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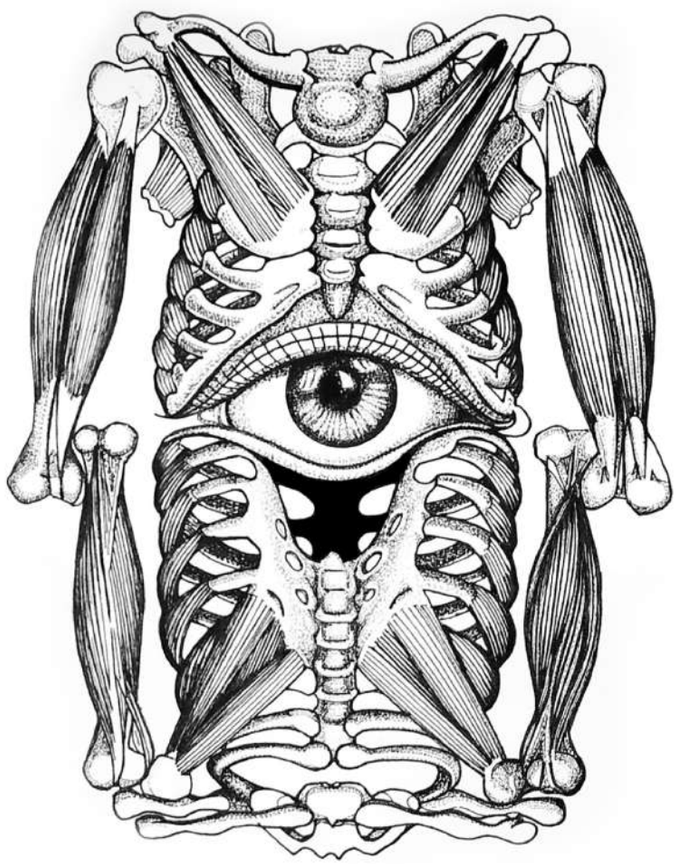

TRANSLATIONAL RESEARCH TOWARD HUMAN HEALTH

From Animal Models to Humans

Translational research toward human health

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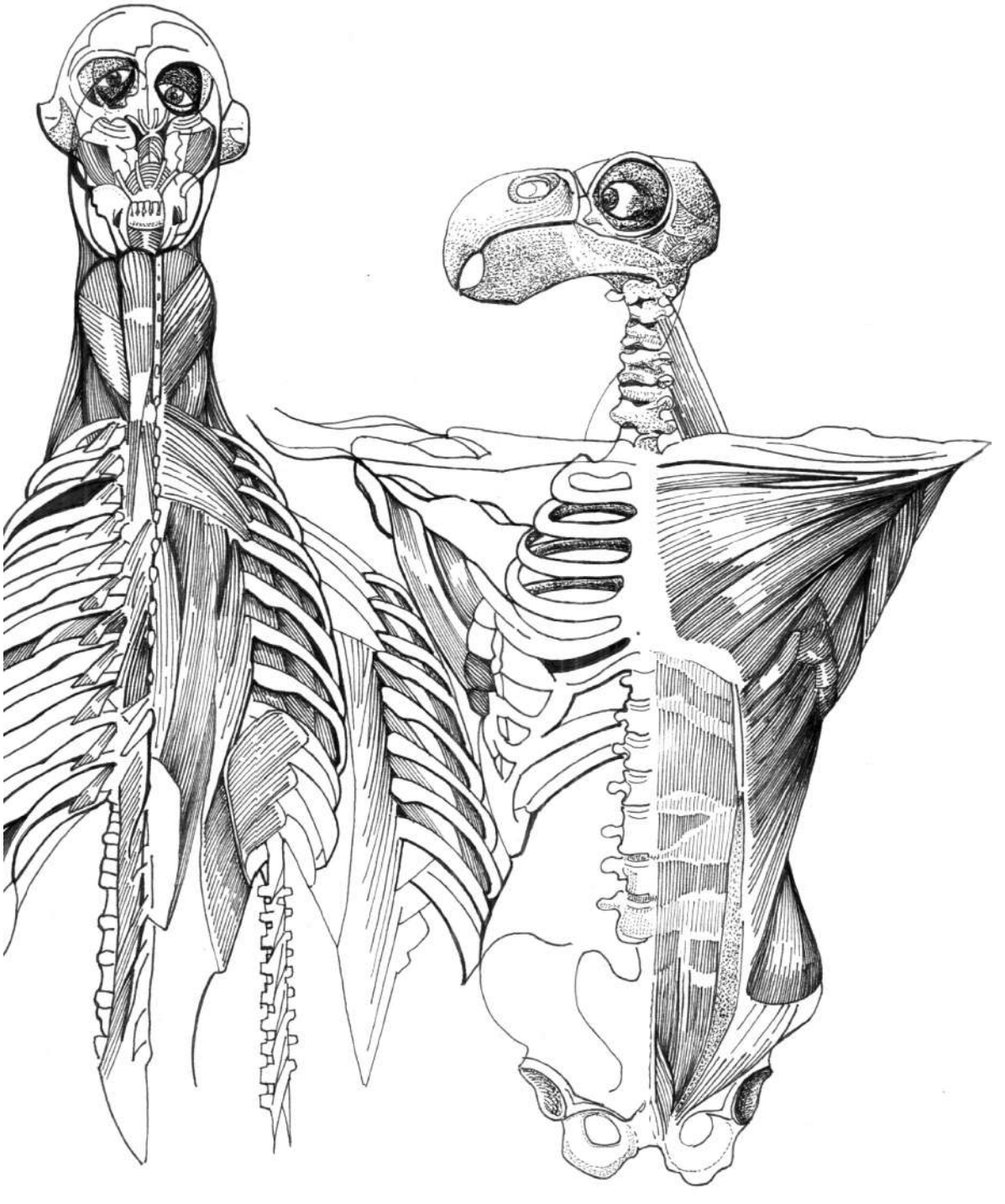

Foreword

Basic scientific research in the biomedical sciences explores the molecular, cellular, genetic, behavioral, and systemic functioning of animals and humans in normal or altered physiological states. Its main objective is to ascertain fundamental biological processes, usually in animal models, that can provide insight into promoting human health and wellbeing. The justification for this approach is based on the evolutionary theory of Charles Darwin and Alfred R. Wallace, that the knowledge of the biological basis of animal models is fundamental to an understanding of humans. In fact, we are members of the zoological class Mammalia, which includes most of laboratory animal models including rodents and primates. However, the path from basic science to applied science for human wellbeing has been more a proposal than a reality. We know that knowledge obtained in the laboratory with animal models (bench) toward application to clinical treatment in humans (bedside) is not easy to attain. One of the reasons is the limited communication between basic research scientists and clinical physicians. The former specialize in biological and behavioral mechanisms in animal models and the latter specialize in practical solutions to pathological conditions. The academic perspective at the university level typically focuses on a reductionist approach rather than construction of an interdisciplinary integration.

Fortunately, toward the end of the 20th century, due to accelerated advances in the biological sciences, a new field was emerging and gaining specialists from the biological and biomedical sciences: translational science and medicine (TM). This emerging scientific field constructs a new paradigm in which students learn basic biological mechanisms, but with an emphasis on their possible application to human wellbeing. To be successful, this new field of TM also requires an active participation of health professionals in the exchange of knowledge with scientists in the basic sciences.

An important caveat is that the mechanisms of a specific physiological or pharmacological process at the organismic level can differ between animal models and humans. Thus, it is imperative to study particular biological processes in both.

Relating basic sciences to clinical application involves three critical steps: 1) observation, 2) experimentation and 3) hypothesis testing. All three can be performed in animal models, but the third must be performed in humans, which may require clinical trials and a complicated process that ultimately may involve the pharmaceutical industry. Also required is feedback from patients about the effects of the treatment. This process can involve myriad barriers at ethical, financial, educational, organizational, and governmental levels, requiring new public health policies.

In México we are still far from this goal. But we are a generation that, after years devoted to basic science, is gradually laying the groundwork for research in humans. This has been an important step, according to Dr. Christopher Austin, of the National Center for Advancing Translational Sciences, NIH, USA. He identifies a key requirement for the advance of translational science: To cross boundaries; to break down the frontiers of our discipline and venture into the domains of other disciplines involved in translational approaches. This book exemplifies the emergence of the "crossing of boundaries" by a group of scientists from México, Spain, Czech Republic, Canada and the USA.

During our university training, most of the knowledge that we gained was based exclusively on the basic sciences. As a consequence, our subsequent professional research involved animal laboratory models, and we became experienced in publishing in specialized journals in our fields. We frequently heard the question: what is the application of your research? What that really means is, what is the relevance of your research *to humans*? Our answer was: basic science is important, is the foundation of all knowledge. This professional position based on our academic training was mainly due to our teachers' also being trained exclusively in basic research. It is now time for our generation to break the boundaries. After becoming expert in animal research, many of us seek new innovative challenges. Thus, it has become acceptable to think: "What about this specific problem in humans? To do research in humans in my research field in animals...perhaps it is not impossible". Three points are critical: the research must involve minimal risk to the persons receiving the intervention, they must give their informed consent to participate, and the proposed research must be approved in advance by an institutional ethics committee. By fulfilling these criteria, we can initiate our translational research in humans. Now

our students have us as their teachers, who have had experience performing research in humans and they can now feel encouraged to engage in such research. As a result, a new generation of scientists is arising. In this book, the reader can learn their advances on several research fronts, and also learn the stories of how they reached this point in their careers. Science it is not just a collection of discoveries, but also a collection of varied personal stories of how those discoveries came to be.

We are fortunate to know the distinguished scientists from the five countries who collaborated on this book, all of them our good friends and colleagues. After a long journey from the basic to the applied sciences, they broke the boundaries before us and advanced to the goal of performing their research-based interventions in humans. We believe that this book will encourage both students and colleagues to look for new frontiers. It is time for a significant translational approach, time to break the boundaries that restrict research to the basic sciences.

This book could not have succeeded without the fundamental support of the Coveicydet (Consejo Veracruzano de Investigación Científica y Desarrollo Tecnológico), which provided the funding for publication. Furthermore, we thank the Sociedad Mexicana de Ciencias Fisiológicas (SMCF), which grants the academic endorsement of this publication; it has the objective of scientific dissemination, and this book was written as a teaching tool for undergraduate and graduate students.

The coordinators and editors of this book appreciate and acknowledge the high level of commitment of the authors who, with generosity and patience, at all times supported the preparation of this book. Thank you very kindly to each. The door is open, and a new path is in front of us for present and future students.

Xalapa, Ver., México, Summer, 2023.

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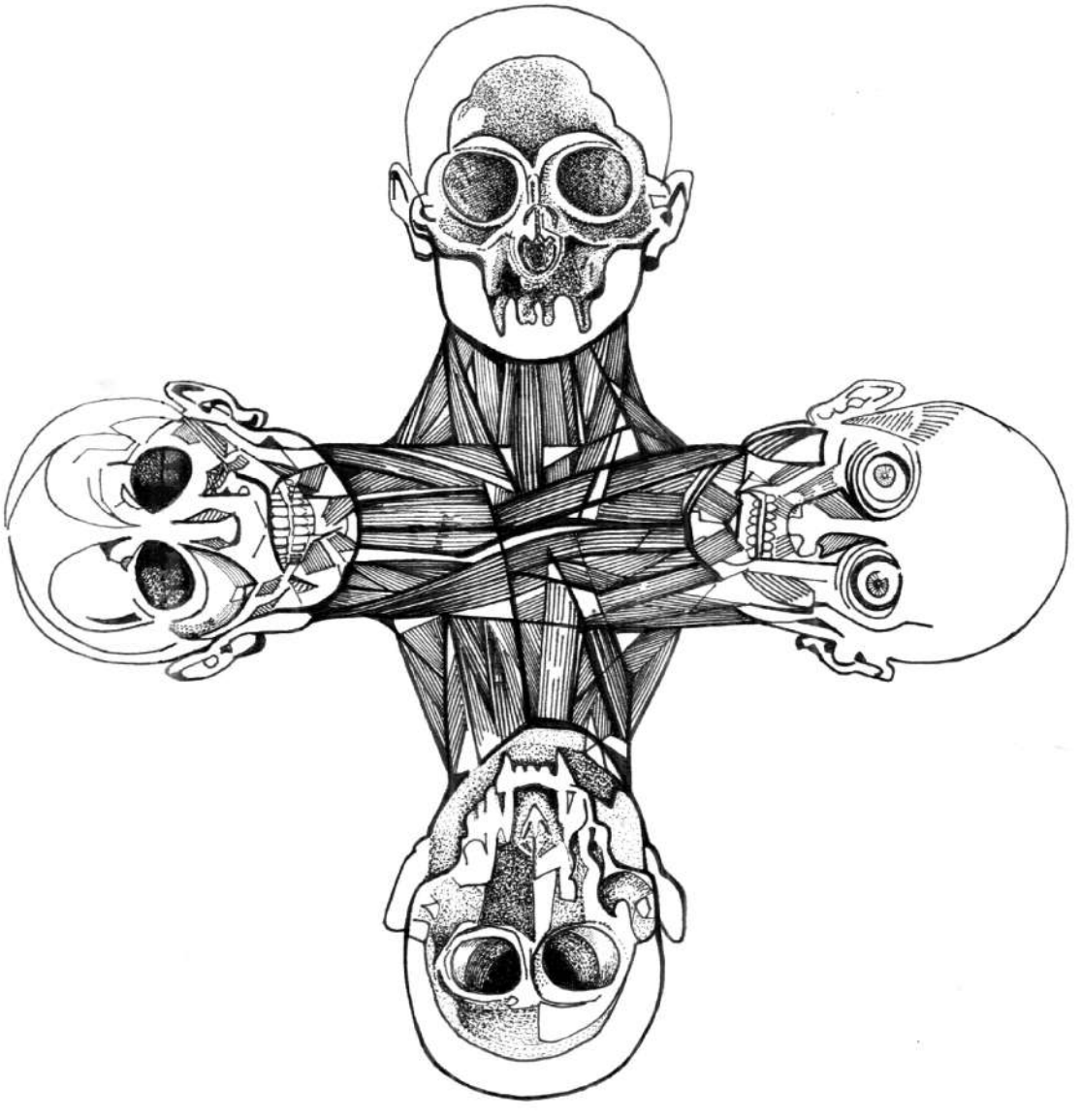

Introductory framework

Translational research is now a growing research emphasis at universities worldwide. Universidad Veracruzana in México is no exception. But the development of this type of research at this university was not initiated by the administration of the institution. Instead, its emergence is the result of a long and slow process by a group of researchers after years of performing basic research in animal models. This book is neither a text on the state of the art of translational research, nor a manual on how to perform translational research. Because our academic training was in basic science, the reader will notice through its 16 chapters that there are multiple references to different species used as animal models. The book also recounts the personal stories of researchers from different universities that led from from basic research in animals to their first studies in humans.

In Chapter 1, Aranda, Hernández, Herrera and Rojas relate their research, using rodent models, to subcellular and molecular mechanisms underlying Alzheimer´s disease. Basic research in this area led to the development of drugs against Alzheimer's disease in humans. The authors provide an analysis of the efficacy of drugs that illustrate how promising findings in animal models do not guarantee efficacy in humans. Why? because animal models have limitations, and more studies are necessary – an example of the problems encountered in the translational approach. In Chapter 2, Professor Noel Kim addresses the path from bench to bedside in sexual medicine, focusing on the quest for an oral therapy for male erectile dysfunction. His chapter provides an excellent example of how drugs developed for different disorders in animal models and humans led to medications to treat sexual dysfunction in humans. It also reveals how financial factors determine and even accelerate research in the pharmaceutical industry. The chapter is a fascinating account of how fortuitous circumstances led to the discovery of medical therapies with enormous economic and social impact.

Herrera, Patraca, Vargas and Fernández in Chapter 3 focus on the ways in which murine models contribute to our understanding of how particular diets can affect our health. We consume a variety of food products with poor nutritious value and high energy content. The result is a growing world tendency to develop obesity and health comorbidities. Unfortunately, the food industry is continually developing food products with an emphasis on financial, rather than nutritional, value. In Chapter 4, Zamora and Cibrián address the translation of research not to the bedside, but rather to the classroom, analyzing the implications of stress and anxiety that result from the study of mathematics, termed "mathphobia". This is an emotional response related to performing calculations with numbers. Studies in animal models reveal how brain structures in the limbic system and prefrontal cortex, and hormones related to stress, are related to mathphobia. Pedagogic and psychological strategies can help to treat this disorder. In Chapter 5, Caba-Flores, Martínez, Camacho and Zurutuza address a critical biomedical problem – infant nutrition, which strongly affects the survival of the newborn. Breast milk is the "gold standard" of infant nutrition, but unfortunately, currently most babies are fed with milk formula. This has led to an increase of health problems for infants that can even extend into adulthood. Unfortunately, infant formula and even breast milk are now contaminated with persistent chemical pollutants, and in recent years also contaminated with microplastics. In contrast to milk formula, breast milk contains myriad immune factors, hormones, and metabolites that contribute to better health in the newborn. Thus it is important to promote nursing in the newborn and infants as the basis for their early and later health. In Chapter 6, Cueto, García, Acosta and Rodríguez, unlike in the other chapters that use mammalian models, these authors address how fish models can help to understand emotional processes in humans and sleep patterns. Fish models are used to test new drugs that are expected to help humans overcome emotional disorders that are common in modern society. Morgado, López, Pérez and Beltrán in Chapter 7 describe how murine models have been useful in understanding the biological basis of spinocerebellar ataxia. They describe the limitations of animal models and the development of new tools, particularly genetic analysis. The translational approach is an opportunity to test genetic and pharmacological therapeutic strategies for this neurological disorder. In Chapter 8, Fleming and Almanza describe their experience and research on the neuroscience of maternal and associated behaviors in rats and their studies about the psychobiology and neuroscience of maternal behavior in humans, especially new mothers. Reciprocally, the work on human maternal behavior

and its emphasis on the importance of psychological factors yields useful directions in animal research, including the examination of variables such as mothers' prior experiences and affective state. It is a chapter by a scientist who, after developing expertise in maternal behavior in rodents, became a leader on this topic in humans. Fleming also is a pioneer in the study of intergenerational effects of how traits in the mother impact their offspring, both in animal models and in humans. Chapter 9 by Pfaus, Coria and Rodriguez is an example of how the use of behavioral and pharmacological approaches in both male and female preclinical animal models led to the emergence of the "third sexual revolution". The authors are world experts in sexual behavior and critically analyze the advantages and limitations of the comparison between rodents and humans. It is a journey through the different components of sexual behavior, the efficacy of current pharmacological approaches, and the development of new drugs for the treatment of sexual dysfunction in humans. Chapter 10 by Aguilar, Álvarez, Alamilla and Góngora focus on how behavioral tests in rodents help to understand the neurobiological and hormonal basis of major depressive disorder in humans. Of particular interest is the chronic mild stress model in rodents to study the pathophysiology of this disorder in humans. Thanks to that knowledge, pharmacological treatment has been developed, but more translational approach is still needed for the treatment of this disorder, an issue of global public health concern. Chapter 11 by Rodriguez del Cerro and Komisaruk addresses the basic question in translational medicine: how knowledge based on research in animal models may apply to humans. When the authors were university students, they were told not to ask how research in animals can be applied to humans, for such questions were considered a heresy. The chapter recounts the significant difficulties they faced to challenge this dogma. The chapter journeys from their studies on neural control of sexual and parental behavior in rodent models to the understanding and treatment of normal and pathological sexual response and affiliative behavior in humans. The culture in science has begun to change, so that now, addressing possible translational implications of one's proposed research is a common requirement for governmental funding decisions on basic research projects. The chapter also incorporates a description of the México-New Jersey student exchange program established by Dr. Komisaruk and Dr. Carlos Beyer, in which multiple authors of this book participated. Their participation facilitated their scientific careers and to a significant extent, led to the creation of this book. Chapter 12 by Manzo, Caycho, Ortega and Velazquez addresses the use of animal models toward the understanding of autism in humans. The comparison exemplifies the

fundamental problem of knowing whether laboratory models truly represent the human disorder. Autism comprises a spectrum of behavioral patterns that lead to developmental problems in children. Despite the obvious limitations, based on their studies in rodents, the authors established a translational line. They developed muscular, visual and auditory stimulation therapies, which increase the motor abilities and also decrease heart rate and systolic pressure in autistic children. Their results demonstrate that it is possible to perform interventions in humans based on knowledge developed from basic science. Chapter 13 by Hernández and Guevara is an example of studies in rats and in humans that explore neural and psychological bases of sexual and maternal behavior. This was possible using non-invasive techniques to study the electroencephalographic brain activity of their experimental subjects. Their studies establish a knowledge base applicable to both rats and humans. Also, the authors identify environmental and social factors essential to a better understanding of these behavior patterns in humans. In Chapter 14 Aguilera, Gómez, García and Orozco address olfactory system research in vertebrates, including humans, and its role in mate sexual selection. They show how specific odors, such as pheromones, play an important role in adaptive sexual behavior patterns, based on research in pigs and humans. They describe how genetic traits, state of women's vaginal cycle and physical activity in men strongly affect physiological responses to odor secretions that affect mate selection. In Chapter 15, Caba, Torres, Viveros and Ramos illustrate how studies in a laboratory animal – the rabbit – led to an extensive line of research on nursing and breast milk in humans. After decades of studying nursing and maternal behavior mainly in rabbits, they came to the point of studying this process in humans. But how to start? The main point was to perform non-invasive studies. Then first came the design of a questionnaire to determine frequency of exclusive nursing and barriers for their establishment just after birth. In parallel they developed electroencephalographic recording and hormonal analyses in relation to maternal attachment and studies of breast milk components. Breast milk composition changes according to the physiological state of the mother and the infant. These studies are an example of successfully implementing an academic program with actual social importance for the general population. In chapter 16 Ángeles, Rojas, Quezada and Arellano describe how, after years of the study of circadian rhythms in the rat, they decided to apply their knowledge to clinical interventions. They controlled light exposure to neonates in intensive care units. Also, they explored the rhythm of the circadian hormone, melatonin, in elderly persons that developed delirium. Based on their analyses they

performed successful interventions that improved the health of children and elderly patients.

In conclusion, we trust that this publication effort can result in an increase of academic networks to reinforce the development of the translational research in our universities. We believe that this book can encourage new generations of scientists to follow in our footsteps to reinforce the development of translational research.

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I

Study models in Alzheimer's disease

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Introduction

Biology is considered a non-linear science; that is, it is not exact like physics or mathematics. The science of life requires models to be studied and statistics to be quantified and validated to a reasonable level.

Every known living being is composed of the fundamental unit of life known as "the cell." Numerous biochemical processes are carried out in the cell, enabling it to reproduce and function properly, thus allowing an organism to sustain its so-called "life."

Biology encompasses both unicellular and multicellular organisms, necessitating laboratory models for their study.

Every biologist in training remembers Antoni Van Leeuwenhoek (1635-1723), the undisputed inventor of the microscope. He practically developed 500 microscopes (van Zuylen, 1981), which opened our eyes to an entirely different world – the world at the cellular level. The development of finely crafted magnifying lenses enabled us to understand the composition of living beings and the various types of cells that exist.

Robert Hooke (1635-1703) is credited with publishing images using a microscope in the book "Micrographia" (1665). He was attributed with pioneering microscopy techniques for observation, along with a great ability to illustrate the cellular nature of life. He was the first to coin the term "cell" (Griffing, 2020).

With the invention of the microscope and cellular description, biology took a significant step forward in the study of microorganisms. This marked the beginning of using cellular models to study life science.

The most effective approach to studying biology is through various cellular models that offer insights into different aspects of the cell, as seen in certain neurodegenerative diseases such as Alzheimer's disease (AD).

Alzheimer's Disease

About 100 years ago, the psychiatrist Alois Alzheimer (1864-1915) described a mental disorder in a patient named Augusta Deter, a 51-year-old woman who presented with memory loss, disorientation, and hallucinations. This set of symptoms was designated as Alzheimer's disease.

Extensive research on this neurodegenerative disease has been conducted since that time, utilizing various biological models such as primary cell cultures, cell lines, transgenic mice, laboratory rats, and the brains of individuals who died from Alzheimer's disease.

To date, more than 30 genes involved in this pathology have been identified, including two important proteins visualized during post-mortem studies: the Tau protein and the beta-amyloid protein. Both proteins accumulate in the brain, leading to the formation of so-called amyloid plaques. As a person ages, these plaques spread across larger portions of the brain, resulting in complete brain atrophy, ultimately culminating in the individual's death.

I have particularly focused on the study of the Tau protein, whose function involves stabilizing microtubules in the axons of neurons.

Tau contributes to shaping the axon, enabling it to synapse with neighboring neurons and facilitating communication between them. This intricate communication network involves around 100 billion neurons, constituting the central nervous system.

Tau mRNA

In the 80s and 90s, the study of the Tau protein commenced within cellular models through molecular biology techniques, which is notably important as it stands as the most linear among all biological sciences.

A question that emerged was how the Tau protein could reach distant sites in the cell body and perform its function. It was already understood that proteins within a cell are synthesized in the cell soma.

However, how did this occur with tau, given that this protein was situated in distant sites of the cell body? Studies conducted by I. Ginzburg in primary cultures of neurons demonstrated, through in situ hybridization, that tau mRNA was localized in the proximal region of the axon (Litman et al., 1993).

The question then arose: how could tau mRNA be localized in the axon of neurons? While it was believed that messenger RNAs, upon leaving the cell nucleus as mature molecules ready for translation by ribosomes, could reach distant regions of the soma, this implied a significant logistical procedure within the cell.

Messenger RNAs are translated into proteins in the cytoplasm rapidly due to their susceptibility to degradation by RNAase enzymes. In other words, these molecules possess a half-life of minutes to a few hours.

The neuron had to resolve the issue of preventing rapid degradation of tau RNA, as it needed to be transported into the axon.

In 1994, the tau mRNA was sequenced (Sadot et al., 1994), revealing its extensive length of 5203 nucleotides and a 3'-UTR region comprising 3848 nitrogenous bases, rendering it atypical in size for a messenger molecule.

The half-life of tau messenger was determined to be around 16 hours in neuronal cytoplasm (Aronov et al., 1999), rendering it highly stable within the cytoplasm.

Studies conducted in neuronal cell lines using molecular biology techniques revealed that the 3'-UTR region of the tau messenger contained a uracil-rich segment where a protein called HuD, a ribonucleoprotein conferring stability to the messenger and preventing its rapid degradation, was attached (Aranda-Abreu et al., 1999). Hence, the cell tackled two challenges: establishing a stability region within its messenger and having a protein providing stability, safeguarding it against RNAases' degradation.

Studies have indicated that the tau messenger localizes in various parts of the neuron axon, necessitating efficient transport to distant soma sites.

In neuronal cultures, it was observed that tau mRNA moved into the axon region alongside ribosomes, enabling on-site translation (Aronov et al., 2001). To achieve this, RNA was anchored through the HuD protein to a kinesin, facilitating anterograde transport of the tau messenger.

The cell effectively resolved another significant issue, transport, by forming a complex of proteins and RNA that, along with ribosomes, were situated at various axon sites. This allowed for translation of the tau messenger on-site when required, considering its 16-hour half-life (Aronov et al., 2002).

These studies marked the first instance of deciphering how a messenger could be translated away from the cell soma, challenging the notion that all RNAs were translated solely in the cell body.

The application of cellular models has extensively been employed to comprehend the subcellular mechanism of Alzheimer's disease. Various

models are utilized today to gain a broader understanding of how this neurodegenerative disease impacts humans.

Other Models for the Study of AD

Several study models exist, ranging from cell cultures to genetically modified organisms. An extensive array of animal models is available for Alzheimer's disease research (https://www.alzforum.org/research-models/alzheimers-disease). Additionally, other models employed for studying the disease include cell cultures, as mentioned earlier. However, it's crucial to note that iPSC (Induced pluripotent stem cell)-derived neurons have also found utility. Investigations into mutations of PSEN1 and PSEN2 genes, APP, and Tau phosphorylation have been conducted (Arber et al., 2017).

Primary cultures of microglia were isolated from human and transgenic mouse brains to examine Tau spreading in the brain (Hopp et al., 2018). Co-cultures of PC12 cells alongside C6 glioma cells serve as a model for sporadic Alzheimer's, assessing APP and b-secretase when insulin receptor expression decreases (Gupta et al., 2018). 3D culture studies and brain organoid cultures have been explored to expedite AD pathology (Choi et al., 2016). Yeast models have been employed to study Tau and b-amyloid aggregation (Moosavi et al., 2015). Other cell lines utilized in AD research encompass HEK 293 cells (Houck et al., 2016), SH-SY5Y (Agholme et al., 2010), P19 (Sberna et al., 1997), and (Aranda-Abreu et al., 1999) (Aronov et al., 2001 & 2002).

The zebrafish model has been implemented for AD studies, as it can function as both a behavioral and pharmacological model (Saleem and Kannan, 2018). Drosophila has also emerged as a promising model for AD research, as it exhibits AD-like genotypes (Tan and Azzam, 2017).

Phase III Drugs Against AD in Humans

Antibodies

Solanezumab is a monoclonal antibody designed to eliminate soluble amyloid peptides that can generate toxic effects in the synapse and precede the deposition of amyloid fibers. However, it showed no significant effect in improving cognitive impairment in a total of 1057 patients with moderate Alzheimer's disease (Honig et al., 2018).

Aducanumab, an antibody capable of reducing Ab plaques in patients with early disease, apparently exhibited mild improvement when studies were conducted using the MMSE (Sevigny et al., 2017).

Gantenerumab is an anti-A monoclonal antibody used in patients with moderate Alzheimer's. It demonstrated a mild decrease in CFS when administered at low doses (Klein et al., 2019).

Crenezumab, a monoclonal antibody designed to remove toxic beta-amyloid oligomers, has not been successful in Alzheimer's patients so far (Avgerinos et al., 2021).

Inhibitory Molecules

Verubecestat (MK-8931) is a BACE1 inhibitor that was administered to patients with the onset of AD at different doses of 12 and 40 mg per day. Results showed that verubecestat did not improve clinical assessments of dementia among patients with prodromal AD, and some measures suggested that cognition and daily function were worse among patients receiving verubecestat than among those receiving placebo (Egan et al., 2019).

Lanabecestat (AZD3293) is a potent BACE1 inhibitor that, at different doses ranging from 50 to 150 mg, demonstrated the ability to reduce CSF and plasma A levels in healthy individuals. This inhibitor is emerging as a promising drug to be studied in Phase III (Sakamoto et al., 2017).

ALZ-OP1 is a combination treatment of cromolyn sodium, a mast cell stabilizer used for asthma treatment, which acts as an anti-inflammatory by inhibiting the release of cytokines, and ibuprofen, a selective COX inhibitor that can reduce Ab1-42 peptide levels by modulating b-secretase activity. Results of this compound are pending in Phase III (Panza et al., 2016).

Azeliragon is a receptor for Advanced Glycation Endproducts (RAGE) inhibitor. Studies have shown its ability to reduce amyloid plaques. In Alzheimer's patients, treatment for 18 months has demonstrated an improvement in mild to moderate cognitive impairment (Burstein et al., 2018).

TRx0237 is a small molecule that inhibits Tau aggregation and is being applied to patients with mild Alzheimer's. The results of this treatment are awaited (Hung and Fu, 2017).

Elenbecestat, an amyloid peptide aggregation inhibitor, is currently in Phase III studies. The results of the application of this inhibitor in CSF are awaited, and the studied doses range from 5 to 50 mg/day (Panza et al., 2018).

Molecules that Reduce the Symptoms of the Disease

Various drugs that can reduce the symptoms of AD have been tested, including escitalopram (serotonin reuptake inhibitor), methylphenidate (central nervous system stimulant), nabilone (cannabinoid agonist), zolpidem

(benzodiazepine analogue), and suvorexant (orexin receptor antagonists) (Cummings et al., 2018).

However, despite the efforts that have been ongoing to find a suitable drug that can at least halt the progression of the disease, such a drug has not been found up to this point.

Another question arises: What are we doing wrong? It has been observed that models provide us with important information about how the disease could originate. However, these models, like any other models in biology, have their limitations.

The drugs that have been developed are undoubtedly well-designed to fulfill their objectives, but why don't they work? Perhaps it's because they are designed for cells that are not damaged, or for neurons that will never suffer from AD, as is the case with the models used today.

Back to Cell Biology

Every cell is surrounded by a phospholipid membrane that maintains cellular contents, such as cytoplasm and organelles, each with its own protective membrane containing molecular content.

Studies have shown that amyloid peptide oligomers can compromise membrane integrity and cellular homeostasis (Bode et al., 2019). It has been determined that the endocytic pathway might be affected, subsequently influencing the trafficking of proteins like APP and BACE1. Proteins integral to the endocytic pathway, such as RAB7a, sorting nexin 3, and sortilin protein-related receptor, among others, could be compromised (Tan and Gleeson, 2019). Additionally, AD could impact the nuclear membrane due to reduced levels of B-type lamin protein expression, attributed to Tau overexpression (Frost, 2016). Amyloid peptides and Tau might also affect the mitochondrial membrane, leading to mitochondrial dysfunction that primarily triggers bioenergetic and respiratory chain failure (Swerdlow, 2018). Mitochondria associated with the endoplasmic reticulum might be affected as well (Area-Gomez and Schon, 2016).

These are instances where membranes suffer damage due to AD. Overexpression of Tau or its hyperphosphorylation, coupled with the presence of amyloid peptides, could contribute to generalized cellular failure. With the endocytic pathway compromised, receptors related to neurotransmission may be damaged, either in their tertiary structure or due to impaired delivery to their intended destinations (Xu et al., 2012). Notably, when cell membranes are affected, neurotransmission becomes inefficient. For instance, the synthesis of the neurotransmitter acetylcholine necessitates acetyl-CoA from mitochondria and choline. In Alzheimer's patients,

the compromised mitochondria impact neurotransmitter synthesis. Additionally, cholinergic receptors might be disrupted, as they might fail to reach their specific membrane sites. Similar issues might occur with glutamate receptors and other neurotransmitters.

Hence, the drugs approved for AD treatment haven't been effective, not because of poor design, but because metabolism and cellular functions are disrupted. These drugs don't address the damage but attempt to maximize neuronal function. They inhibit acetylcholinesterase and exert antagonistic action on glutamate receptors, as seen in the case of memantine.
The challenge lies in AD's multigenic nature, impacting various metabolic pathways and neuronal functions that can't be resolved with a single pill.

So, What Should We Do?

An important aspect is ensuring that both the individual and their family members are cognizant of the warning signs that might manifest. Such awareness could indicate that the disease has already progressed. However, an approach known as "neuronal rehabilitation" could be initiated. This involves attempting to mend cellular damage at the membrane level and mitigate neuroinflammatory processes.

Neural Rehabilitation Process

One of the important aspects that must be addressed first is neuroinflammation, wherein cytotoxicity exists and triggers an inflammatory response involving beta-amyloid protein, cytokines, and prostaglandins that lead to the generation of cyclooxygenase type 2 (COX-2) (McGeer and McGeer, 2001).

An effective strategy to reduce neuroinflammation involves the use of non-steroidal anti-inflammatory drugs, which can decrease COX-2 expression levels. The most commonly used drugs for this purpose have been celecoxib and nimesulide. However, in clinical trials, celecoxib did not adequately decrease COX-2 enzyme levels (Glass et al., 2010). On the other hand, nimesulide has demonstrated effective results by reducing prostaglandin and COX-2 levels (Candelario-Jalil, 2008).

On the other hand, memory loss represents one of the most severe symptoms of AD, as the person begins to forget little by little and loses the details of daily life, to the point of forgetting who he/she is.

In AD, the neuroinflammatory process is of major importance, as neuroinflammation has been determined to be a trigger for the disease (Calsolaro and Edison, 2016). Nonsteroidal anti-inflammatory drugs have a protective effect in rodents where they have been treated with quisqualic acid

injected into the nucleus basalis. Excitotoxin induces cholinergic de-generation and intense glial reaction, as well as production of inflammatory mediators. When rodents are treated with nimesulide (10mg/kg/day, i.m.), it strongly attenuates the microglial reaction, reducing nitric oxide synthase activity and completely inhibits prostaglandin-E2 formation (Scali et al., 2000).

Nimesulide is an NSAID that selectively inhibits COX-2, which may be useful in the treatment of AD (Krause and Müller, 2010) due to the neuroinflammation process characteristic of this pathology, including accumulation of activated microglia and astrocytes, as well as some T-cells, accompanied by characteristic molecular alteration of inflammation such as cytokines, partially triggered by the accumulation and extracellular precipitation of neurofibrillary tangles (McGeer and McGeer, 2015). Some epidemiological studies of individuals using non-steroidal anti-inflammatory drugs (NSAIDs) observed a delay in the onset of AD, for example in identical twins who had received anti-inflammatory therapy, which is associated with damage caused by neuroinflammation (Shaftel et al., 2008). Recent studies indicate that a possible mechanism of action of NSAIDs is by modulating the activity of gamma secretase which is required for proteolytic anchoring of APP and producing ß-amyloid peptide (Medeiros and LaFerla, 2013).

The recommended dose due to the pharmacokinetics of nimesulide is 100 mg per day since plasma levels of up to 4.58 mg/L are reached in about 3 hours (Bernareggi, 1993).

It is recommended only to be ingested for one month due to the risk that could occur due to hepatotoxicity (Donati et al., 2016).

Citalopram is a selective serotonin reuptake inhibitor. Pharmacological evidence suggests that it has a role in the cholinergic modulation of monoamines in AD. Studies related to this drug are mainly aimed at evaluating its efficacy in decreasing agitation in patients with Alzheimer's disease (Peters et al., 2016). In addition, it has been identified that in rat hippocampus it influences Tau hyperphosphorylation. In this experiment, rodents were placed in social isolation, and they reported that the increased level of phosphorylated Tau observed in social isolation can be reversed by citalopram (Ren et al., 2015). Its effect on Tau acetylation or its hyperphosphorylation under ß-amyloid peptide effect is unknown.

The recommended dose in elderly patients is 20 mg per day, which would allow the drug to maintain its effect (Fredricson Overø, 1982).

Resveratrol, which is a polyphenol known for its cardioprotective effect for cardiovascular diseases, cancer and modulates pathological mecha-

nisms of some diseases such as stroke, ischemia and Huntington's disease (Wang et al., 2010). Several studies suggest that it has therapeutic value. It has been reported to reduce the generation of ß-amyloid peptide and may also reduce Tau hyperphosphorylation as well as its abnormal aggregation in animal models (Pasinetti et al., 2015). However, it is still unclear what effect it has on Tau protein. It is proposed that its mechanism of action could be through the activation of SIRT1, which is a deacetylase with antioxidant effects, deacetylates the Tau protein preventing the destabilization of microtubules and decreases the formation of neurofibrillary tangles, inhibits p53 and thus decreases apoptosis, reduces the inflammatory process and neurotoxicity, increases alpha-secretase activity and decreases the production of ß-amyloid peptide (Karagiannis and Ververis, 2012).

It has been determined that a dose of 75 mg daily is sufficient to avoid microvascular dysfunction in people with type II diabetes (Wong et al., 2016) where it has been determined that this condition could be a determining factor for the onset of AD (Vanhanen and Soininen, 1998).

To maintain healthy neurons, the first thing we must do is to maintain plasma membrane integrity and avoid oxidative stress (Aranda-Abreu et al., 2011). Attention has been paid to w-3 acids, which are necessary for the integral maintenance of the plasma membrane (Valentine and Valentine, 2004). It has been determined that w-3 acids are able to maintain brain structure and function and potentially delay dementia (Schwarz et al., 2018).

A dose of 800 mg of omega-3 acids has been recommended to reduce the risk of dementia in older adults (Hooper et al., 2017).
Ginkgo biloba extract has been used for the treatment of memory problems (Field and Vadnal, 1998) (Zhang et al., 2016) obtaining good results on cognitive function. Studies have determined that a dose of 240 mg per day, of ginkgo biloba extract Egb 761 is able to increase prefrontal dopamine (Beck et al., 2016) where this dose can be safely consumed (Herrschaft et al., 2012).

Summary

Biological models (Figure 1) for the study of AD are very important, as they have allowed us to understand how the pathology affects various areas of the brain.

However, the models still do not allow us to know the origin of Alzheimer's disease since more than 30 genes have been involved in the disease.

The best study model to approach this pathology is the human being, since “apparently” it is the only being on earth that suffers from AD. How-

ever, due to ethical principles, this has been a limitation in the study and, of course, it is understandable.

No human being wants to be a research object or laboratory animal to study a disease. Perhaps that is why drug research in AD has been limited.

One drug will not be enough to cure AD. We are of the opinion that neuronal restoration therapy must be carried out first before FDA-approved drugs are used.

Every drug synthesized will not be effective unless the neuronal membranes are functioning properly and cellular function is completely restored.

It is necessary to create cellular models where the different drugs focused on the functioning of the cell can be studied. We must remember that it is the cell that gets sick.

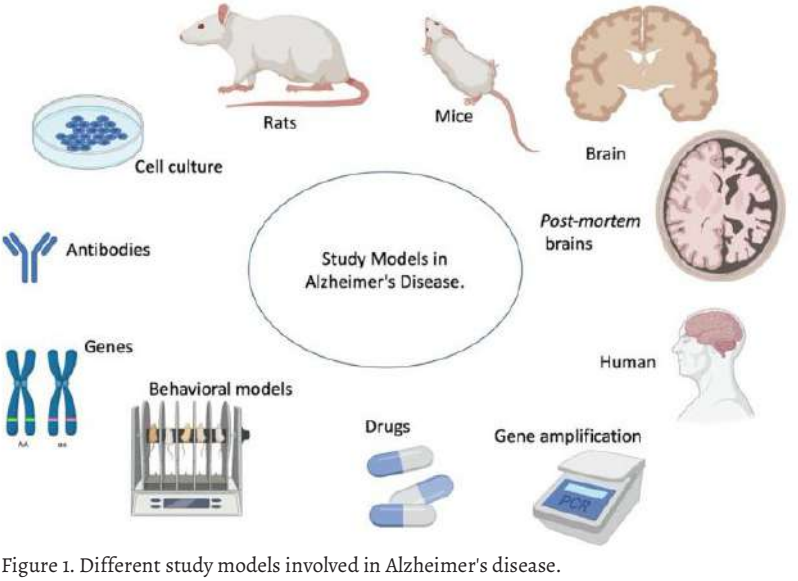

Figure 1. Different study models involved in Alzheimer's disease.

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II

Pathways from bench to bedside in sexual medicine: Lessons from the quest for an oral therapy for male erectile dysfunction

Noel N. Kim

Introduction

Medical treatments for sexual dysfunctions are particularly notable in that very few of the currently approved therapies were specifically developed through a rational process of translational research informed by clinical need. Instead, most of the medications that are currently used to treat sexual dysfunctions were repurposed from development programs focused on other disorders. Unexpected therapeutic responses related to changes in sexual function usually provided insight that the drug could be a viable candidate for a new indication. The role of serendipity in science and medicine is often elevated to mythical status and the degree to which serendipity contributes to the advancement of successfully identifying efficacious therapeutics remains ill defined. Yet, serendipitous circumstances coupled with keen observation and faithful documentation have undoubtedly led to significant clinical advances.

A well-known example is the discovery of penicillin by Alexander Fleming. After returning from holiday in 1928 to his laboratory in London, Fleming observed that fungal colonies had contaminated plates of bacterial cultures and was astute enough to notice clear zones in the periphery of the mold, indicating inhibition of bacterial growth. In 1929, Fleming published his work on penicillin mold extracts, but interest was limited to bacteriologists in the laboratory. The jump to clinical application did not occur until a decade later when Ernst Chain, Howard Florey, and their colleagues at Oxford University performed research on the purification of penicillin and demonstrated its benefits as an antibiotic in animal studies (Gaynes, 2017).

Rather than a comprehensive review of how treatments for sexual dysfunctions were developed, this chapter will focus on key scientific discoveries from different disciplines that progressively provided the momentum toward elucidating a major regulatory pathway in penile erection, set-

ting the stage for one of the most widely used class of drug therapies in sexual medicine.

Treatments for Erectile Dysfunction in Men

Initially, psychotherapeutic approaches were the most commonly used strategies for treating erectile dysfunction (ED). However, in the 1970's, surgical treatment for ED in men (i.e., penile prosthetic implants) became more established for those who were diagnosed as having an organic etiology for their condition (Goldstein et al., 2019). A significant milestone in the appreciation of the physiology of penile erection and medical therapy for ED came in 1982 when Ronald Virag reported that infusing the vasodilator papaverine into the penile circulation of a patient during revascularization surgery caused sustained penile erection (Virag,1982). Despite the importance of this observation, a much more impactful event occurred in 1983 when the British neurophysiologist Giles Brindley famously injected his own penis with phenoxybenzamine, an alpha-adrenergic antagonist. Prior to delivering a lecture at a urological conference, Brindley self-administered the drug by injecting it into the erectile tissue through the side of the penile shaft and then displayed his penile erection to the surprised audience as proof that intracavernosal injection of vasodilators could be effective (Goldstein, 2012).

This remarkable and memorable demonstration provided evidence that vasodilation of the vascular smooth muscle in the erectile tissue of the penis was sufficient to cause an erection. Subsequently, Brindley published a small proof-of-concept clinical study, reporting that low doses of intracavernosal phenoxybenzamine with no systemic effects caused strong and long-lasting erections in 3 of 4 men without ED and in 6 of 11 men with ED (Brindley, 1983). This initiated an era of research and development that quickly led to a series of locally administered vasodilator therapies for the treatment of ED (Brindley, 1986; Gasser, 1987; Padma-Nathan, 1997). One of the most well-known therapeutic classes in sexual medicine is oral phosphodiesterase type 5 inhibitors for the treatment of erectile dysfunction (Goldstein et al., 2019). Even this class of medical treatment owes its existence to a long history of seemingly unrelated discoveries, as delineated in the following sections.

Nitric Oxide (NO)

Gaseous Curiosity to Biological Relevance

Joseph Priestly, an English theologian and chemist, is credited with isolating "nitrous air" (nitric oxide) from chemical reactions with nitric acid and

various metals in 1772 (Smith, 1965; West, 2014). During similar experiments with gases, Priestly also isolated "dephlogisticated air," which was quickly renamed oxygen by Antoine Lavoisier in 1773. From 1872 through the early 1900's, published research on NO in scientific journals documents awareness of the knowledge that NO could be dissolved in the blood and could interact with hemoglobin (Podolinski, 1872; Anson and Mirsky, 1925). It wasn't until the 1960's that researchers began studying the health impact of air pollution and noted the detrimental effects that NO, a component of motor vehicle exhaust, could have due to its ability to interact with hemoglobin in the same way as CO (Waller et al., 1961; Romanovsky et al., 1967). In a separate line of research, the impact of exogenous NO on bacteria and mammalian cells was being studied. It was observed that low concentrations of NO increased the effectiveness of X-rays in killing bacteria (Howard-Flanders, 1957) and that NO also increased the sensitivity of tumor cells to radiation (Gray et al., 1958). Almost 50 years later, endogenous production of NO was postulated to serve as an intrinsic radiosensitizer in a mouse model of fibrosarcoma and lung carcinoma (Jordan et al., 2004).

Aside from synthetic chemical reactions in various contexts and the impact of exogenous NO on physiological processes, it was eventually appreciated that living organisms themselves could synthesize NO by the 1950's (Najjar and Allen, 1954). The enzymatic reduction of nitrite to NO in various species of denitrifying bacteria was characterized, revealing the need for a complex set of cofactors that included multiple nucleotides, and copper and iron as electron carriers (Najjar and Allen, 1954; Chung and Najjar, 1956). In an interesting merging of two lines of research, bacteria in the soil and the oceans were identified as a significant unforeseen source of nitrogen oxides that could rise from the earth's surface to the stratosphere and become photochemically converted by UV radiation from the sun to NO, which then could catalytically destroy ozone (Johnston, 1972). This natural process was of interest due to the advent of commercial supersonic aircraft flights that would add to the burden of NO in the upper atmosphere, possibly accelerating ozone destruction. It was estimated that supersonic aircraft could produce NO in quantities similar to the total amounts produced by terrestrial and aquatic bacteria each year.

The Link to Cardiovascular Physiology

One of the main lines of evidence that NO exerted a physiological effect in the cardiovascular system was research on NO-liberating compounds. In 1849, Playfair identified NO as an important component of nitroprusside and that acid treatment of this compound could liberate NO in solution

(Playfair, 1849). However, nitroprusside was not used clinically until 1928 and its efficacy in lowering blood pressure in severely hypertensive patients was not reported until 1955, eventually gaining US Food and Drug Administration (FDA) approval as an antihypertensive drug in 1974 (Johnson, 1928; Page et al., 1955; Friederich et al, 1995). In a similar but arguably more dramatic vein, when nitroglycerin (glyceryl trinitrate), initially termed pyroglycerine, was discovered in 1847 by Ascanio Sobrero and Théophile Pelouze, they noted that this compound produced a 'violent headache' when placed on the tongue in minute quantities (Marsh and Marsh, 2000). However, they considered their discovery to be too explosive to have any practical use.

In 1849, Constantin Hering performed studies on healthy volunteers, pursuing nitroglycerin as a homeopathic remedy for headache (Marsh and Marsh, 2000). While the early discovery and use of nitroglycerin originated in Paris, it was Lauder Brunton, a British physician, who eventually used nitroglycerin to relieve angina in 1867. In the same year, Alfred Nobel, who had already been manufacturing nitroglycerin for 16 years, recognized its commercial value and applied for and received a patent for his stabilized formulation of nitroglycerin mixed into diatomaceous earth – the initial version of dynamite (Marsh and Marsh, 2000). By 1876, William Murrell was able to formulate a more stable preparation for clinical use to treat angina by mixing 1/100 grain of nitroglycerin (0.6 mg) with chocolate. Ironically, in 1890, Nobel's physicians prescribed nitroglycerin for his angina but Nobel declined, writing to a friend:

> *"My heart trouble will keep me here in Paris for another few days at least, until my doctors are in complete agreement about my immediate treatment. Isn't it the irony of fate that I have been prescribed [nitroglycerin], to be taken internally! They call it Trinitrin, so as not to scare the chemist and the public."* [Nobel Prize website]

Almost a century later in 1977, Ferid Murad and colleagues postulated and confirmed that NO was released from nitroglycerin and activated the intracellular enzyme guanylate cyclase in various tissues, including liver, lung, tracheal smooth muscle, heart, kidney, and brain (Katsuki et al, 1977; Arnold et al., 1977). The measurement of the product cyclic guanosine monophosphate (cGMP) became an indispensable tool in assessing guanylate cyclase activity. Shortly thereafter, Louis Ignarro and colleagues demonstrated the ability of NO-containing compounds to relax vascular smooth muscle (bovine coronary artery) and correlated this relaxation with guanylate cyclase activation (Gruetter et al., 1979).

Through this period that continued studies on the utility of exogenous NO delivered through various NO-containing drugs, the possibility that mammalian systems could synthesize NO had not been explored, relegating NO biosynthesis to a unique capability of bacteria. The turning point came with the publication of Furchgott and Zawadzki's work in 1980, demonstrating that the endothelial lining is necessary for the relaxation of arterial smooth muscle to acetylcholine (Furchgott and Zawadzki, 1980). In essence, this was a methodology paper, examining the reason why acetylcholine did not consistently produce relaxation in the walls of excised blood vessels despite being a potent vasodilator when infused in vivo. The main assay technique was to prepare strips of tissue from the artery, attach the strips to force transducers, and immerse the tissue strips in an isotonic bath of aerated physiological buffer. The conclusion that care must be taken while preparing the excised blood vessel segments to preserve the endothelium had a critical implication – that stimulation of muscarinic cholinergic receptors on endothelial cells caused them to produce a substance or substances that in turn caused relaxation of the underlying vascular smooth muscle.

In a 1983 review article, Furchgott almost casually introduced the term "endothelium-derived relaxing factor (EDRF)," (Furchgott, 1983) which was quickly adopted and widely used by researchers. Furchgott's laboratory and other research groups contributed to the characterization of EDRF as a substance that exhibited: a) short half-life (<60 sec), depending on the conditions; b) rapid inactivation by superoxide anion; c) increased half-life in the presence of superoxide dismutase; d) similar effect on smooth muscle relaxation as acidified nitrite solution; e) inhibition of smooth muscle relaxing effect in the presence of oxyhemoglobin (Furchgott, 1983, & 1987; Cherry et al., 1982; Gryglewzki et al., 1986; Rubanyi and Vanhoutte, 1986). Because these characteristics were consistent with NO, Furchgott postulated that EDRF may be NO (Furchgott, 1987). Furchgott's hypothesis was further verified by the work of Salvador Moncada and colleagues who additionally characterized the similarities between EDRF and NO through various bioassays such as platelet aggregation and chemiluminescent detection of NO after reaction with ozone (Palmer et al, 1987; Radomski et al.,1987). Independently, Louis Ignarro and his research team had been studying the effects of both endogenously produced EDRF and exogenously added NO and NO-containing compounds on guanylate cyclase activation in vascular smooth muscle, eventually arriving at a similar conclusion as Furchgott and Moncada, definitively concluding that EDRF produced in blood vessels is NO (Ignarro et al, 1987).

Characterization of Biosynthetic Pathway and Novel Role as Gaseous Neurotransmitter

With the general acceptance that both bacterial and mammalian species could produce NO, vascular biologists began to study the biosynthesis of NO in endothelial cells while immunologists were engaged in a similar line of investigation in macrophages that were known to produce large quantities of NO that mediated cytotoxic effects during bacterial infection (Palmer et al., 1988; Marletta et al., 1988). Using bioassays and physico-chemical detection methods like mass spectrometry and chemiluminescence, it was determined that L-arginine was oxidized to form NO and citrulline in both endothelial cells and macrophages.

During the same timeframe when NO was becoming firmly established as an endogenously produced vascular signaling molecule, other investigators were characterizing a non-adrenergic, non-cholinergic (NANC) neurotransmitter in rat anococcygeus muscle. This neurotransmitter produced from nerves in a non-vascular smooth muscle was deemed to have properties similar to NO, suggesting that the neurotransmitter was either itself NO or a substance that led to the release of NO (Li and Ram, 1989; Gillespie et al., 1989; Gibson et al., 1989). In a related system, NO was identified as the NANC neurotransmitter in the canine ileocolonic junction (Bult et al., 1990).

Another line of investigation that led to the same juncture was in the brain, where it was shown that glutamate activates NMDA receptors in the cerebellum that results in the release of NO, subsequent activation of guanylate cyclase, and production of cGMP (Garthwaite et al., 1988 &1989). Further examining this phenomenon, David Bredt and Solomon Snyder purified and characterized NO synthase from rat cerebellum, which they found to require NADPH, Ca^{2+}, and calmodulin for its activity (Bredt and Snyder, 1990a). Using the purified NO synthase, Bredt and colleagues developed polyclonal antibodies and demonstrated positive immunostaining outside the brain in the adrenal medulla and neurons of the pituitary, retina, and intestine, firmly establishing the association of NO synthase within neurons (Bredt, et al., 1990b) and demonstrating that NO was a neurotransmitter in addition to being a paracrine factor produced by endothelial cells.

Role in Penile Erection

With the foundation of two centuries of meandering scientific inquiry, an early appreciation for the biological significance of NO in vascular, immunological, and neural physiology was established at a decidedly accelerated

pace during the 1980's and 1990's. Likewise, the 1980's also ushered in a concerted effort at characterizing the regulatory mechanisms involved in penile erection (Andersson et al.,1994). More specifically, the types of nerves and neurotransmitters in the penile corpus cavernosum, the main erectile tissue within the penis, were studied. The discovery of nerves containing vasoactive intestinal polypeptide (VIP) in penile cavernosal tissue and the activity of VIP as vasodilator led to an early interest in VIP as the mediator of penile erection and established the existence of a NANC neurogenic regulatory system in the penis (Andersson et al.,1994; Polak et al., 1981). Despite the identification of VIP as a neurotransmitter, there remained some inconsistencies and debate surrounding whether other NANC neurotransmitters played a more predominant role in penile erection.

Prior to the publications on NO synthesis in the brain, there were few definitive clues about NO being a neurotransmitter. A tenuous link between penile function and EDRF was the characterization of an "inhibitory factor" extracted from bovine retractor penis muscle that caused relaxation of rabbit aorta tissue strips and was indistinguishable from EDRF/NO (Bowman, et al., 1986). Similar studies in the closely related rat anococcygeus muscle (mentioned above) also provided supporting evidence for the involvement of NO in penile function. However, this was in specialized tissues not present in humans and was not directly related to the penile corpus cavernosum. Nevertheless, the stage was set and it was not unreasonable to investigate the possibility that NO may be involved in regulating penile erection. The availability of various inhibitors of NO synthesis, NO scavengers, and guanylate cyclase modulators provided the necessary tools to probe for NO signaling in other tissues.

As a vascular tissue, penile corpus cavernosum is composed of vascular smooth muscle and endothelial cells. In cavernosal tissue strips contracted with an adrenergic agonist such as norepinephrine or phenylephrine, exogenous acetylcholine could reliably produce endothelium-mediated relaxation. Similarly, electrical stimulation of contracted cavernosal tissue strips in the presence of inhibitors of adrenergic neurotransmission (e.g., bretylium) and muscarinic receptor antagonists (e.g., atropine) consistently produced NANC neurogenic relaxation. These endothelium-dependent and neurogenic relaxation responses were found to be inhibited by; a) L-arginine analogs that prevented NO synthase activity; b) the NO scavenger oxyhemoglobin; c) the non-specific guanylate cyclase inhibitor methylene blue; d) hypoxic conditions (Ignarro et al.,1990; Kim et al., 1991). These observations were quickly confirmed by other investigators and further supported by immunohistochemical localization of NO synthase in penile corpus caverno-

sum and penile arteries (Pickard et al., 1991; Holmquist et al., 1991; Knispel et al., 1991; Kimura et al., 1992; Rajfer et al., 1992; Holmquist et al., 1992; Burnett et al., 1992, 1993).

Even though the phenomenon had been previously reported (Johns et al., 1989), the effect of hypoxia on NO synthase activity was underappreciated when examining most tissues, since oxygen is rarely deficient under normal circumstances. However, NO synthase is a cytochrome P-450 hemoprotein that requires molecular oxygen as a co-substrate in addition to L-arginine to produce NO (White and Marletta, 1992). Examining the effects of hypoxia, established by bubbling a mixture of 95% N2/5% CO2 through the tissue baths to sufficiently deplete oxygen, was especially relevant for the penile corpus cavernosum, since the intracavernosal blood is normally in the venous range (25-43 mm Hg) when the penis is flaccid (Kim et al., 1993). During erection, the vascular smooth muscle in the resistance arterioles and cavernosal tissue relax, the flowrate of incoming arterial blood increases to fill the cavernosal sinusoids, and the blood PO2 rapidly rises to arterial levels (~100 mm Hg) (Kim et al., 1993).

The discovery of NO as a key neurotransmitter and paracrine factor mediating penile erection was a major milestone in sexual medicine. However, the various groups of investigators who independently obtained data to support this regulatory pathway in a relatively short period of time (11 publications within 36 months from 5 different nations) was made possible by the establishment of signposts along the path of discovery to guide researchers, pointing them in the right direction.

Discovery of Phosphodiesterase Type 5 (PDE5) Inhibitors as a Treatment for ED

Yet, even with this knowledge, the next phase of developing an effective oral therapy would require additional lines of inquiry that were taking place outside of the field of sexual medicine. After the early work of Virag and Brindley, intracavernosal injection of vasodilator drugs became the principal medical therapy through the 1990's. Injection of a small volume of a single vasodilator or a mixture of multiple vasodilators directly into the erectile tissue was viewed as an ideal method to treat ED, as this form of therapy achieved a high concentration of drug where it was needed while avoiding significant hypotension in the systemic circulation. After the NO/cGMP pathway became well-established as a regulator of penile erection in the early 1990's, researchers proposed that zaprinast, an inhibitor of cGMP phosphodiesterases, could be another treatment for ED when injected intracavernosally (Holmquist et al.,1993). Other investigators pro-

posed the use of different types of vasodilators (e.g., NO donors, potassium channel openers) but the focus was always on administering these drugs by intracavernosal injection to avoid systemic hypotension (Holmquist et al.,1993; Trigo-Rocha et al., 1995). In fact, in 1995, the first FDA-approved treatment for ED was intracavernosal alprostadil (prostaglandin E1), another compound with vasodilator activity (Altman, 1995).

Thus, the development of an oral therapy for ED was seen as problematic because the same signaling pathways regulating blood flow in the penis also existed in the systemic circulation. Further, there was no way to preferentially produce a sufficiently high local concentration of active drug in the penis to reliably cause vascular smooth muscle relaxation without having an effect on the other smooth muscle in the body and thereby adversely impact the patient with ED. Oral medications for ED that had been evaluated were directed towards the central nervous system. Limited data supported the use of the 2-adrenergic antagonist yohimbine and the opioid antagonist naltrexone as oral therapies for ED (Morales et al., 1987, 1988; Fabbri et al., 1989; Brennemann et al., 1993) but their efficacy and safety in treating ED remained unclear. The most novel strategy proposed may have been topical formulations of vasodilators applied directly to the penis, but this still relied upon the paradigm of locally administered drugs (Owen et al., 1989; Cavallini, 1991).

In the mid-1990's, Pfizer's cardiovascular research and development group was engaged in characterizing PDE5 inhibitors for the treatment of angina (Ghofrani et al., 2006). PDE5 specifically breaks down cGMP, thereby terminating NO signaling. Thus, an inhibitor of PDE5 would be expected to elevate intracellular cGMP levels and potentiate the action of substances like NO, facilitating vasodilation. At the time, the main distribution of PDE5 was thought to be in vascular smooth muscle and platelets (Corbin et al., 1999). Eventually, Pfizer's development program became focused on the candidate compound UK-92480 (sildenafil citrate) that was a selective and potent inhibitor for PDE5. Preclinical studies in animals indeed demonstrated that sildenafil caused dilation of coronary arteries and also inhibited thrombus formation (Katzenstien et al., 2001). However, in early clinical studies, sildenafil treatment did not result in a clinically significant effect on hemodynamic outcomes, but some study participants experienced penile erections as an unexpected therapeutic effect (Katzenstien et al., 2001). These erections were increased in frequency or more easily achieved. As sildenafil taken orally reached maximum plasma concentration within 1 hour and had a short half-life of approximately 4 hours, it was proposed that

a drug with pro-erectile activity and rapid onset/clearance could be a good candidate for an on-demand medication to treat ED (Boolell et al., 1996).

For a cardiovascular division at a major pharmaceutical company to even consider developing a medication for ED was remarkable in itself. The convergence between science, medicine, and socio-cultural factors established a point in history when ED was viewed as a valid medical condition with a sufficiently adverse impact on health such that investing in the development of a drug therapy was considered to be worthwhile. The New England Journal of Medicine had published two articles in 1989 on the topic of male erectile dysfunction, a condition termed "impotence" at that time. In 1992, the editor of the highly cited journal Science proclaimed NO the molecule of the year. Then, in 1993, a consensus briefing on impotence was published in the Journal of the American Medical Association, summarizing the recommendations of an expert committee convened by the National Institutes of Health in the US (NIH, 1993). Among the findings from the NIH Consensus Development Conference, the expert panel recommended that the colloquial term "impotence" be replaced by the medical term "erectile dysfunction." This series of publications in high-impact medical and scientific journals within a 5-year period served to validate the condition of ED and changed the perception of this medical condition amongst patients, clinicians, and the general public. Without this momentum and shift in perspective, it is unlikely that a pharmaceutical company would have considered addressing ED or any sexual dysfunction.

This newfound commercial interest motivated researchers to examine the types of phosphodiesterases that were present in penile tissue. Despite previous brief reports at conferences that PDE1 and PDE3 were the most highly expressed, Pfizer investigators using improved purification methods determined that PDE5 was the most prevalent phosphodiesterase isoform in penile cavernosal tissue (Boolell et al., 1996). This fortuitous expression profile of PDE5 in penile erectile tissue and the ability of sildenafil to inhibit PDE5 at low concentrations in the nanomolar range while having minimal impact on systemic blood pressure were the main factors that enabled sildenafil to be an efficacious drug for ED when taken orally and made available systemically. Beyond biochemical and physiological considerations, other challenges included the selection of appropriate outcome measures, and this entailed developing a new validated questionnaire, the International Index of Erectile Function (IIEF) (Rosen et al., 1997). The IIEF has now become among the most widely used validated instruments in ED research and sexual medicine clinical practice, having been translated into 70 different languages, thus far (Goldstein et al.,2019).

As noted in a review publication on the "serendipitous story of sildenafil," Pfizer simultaneously submitted in September 1997 a new drug application for sildenafil to the US FDA and the European Medicines Agency (EMA) "that included 8 years of research, 21 clinical studies in 13 countries, 192 investigators, and almost 4,500 male subjects." (Goldstein et al.,2019). Sildenafil, under the brand name Viagra, was approved by the FDA in March 1998 and by the EMA in September of the same year. In addition to Viagra, multiple drugs and formulations in the PDE5 inhibitor class are commercially available in various countries: vardenafil (Levitra and Staxyn), tadalafil (Cialis), avanafil (Stendra), lodenafil (Helleva), udenafil (Zydena), and mirodenafil (Mvix) (Dhaliwal, 2021).

Summary

The discovery of NO and the inquiry related to this highly reactive gas over two centuries has been carried by and passed between numerous scientific and medical disciplines. Prior to the 1980's, interest in NO was largely relegated to small groups of researchers studying production of NO in denitrifying bacteria, toxicity of NO as a pollutant, and the potential utility of NO in radiation treatment. The most clinically relevant line of work began in the mid-1800's when NO-liberating compounds began to be tested for the treatment of angina. The mechanism of action of these NO-liberating compounds was not elucidated until well into the 1970's, but this knowledge was essential in probing the NO pathway when it became clear through the next decade that NO was synthesized in leukocytes, endothelial cells, the central nervous system, and peripheral nerves.

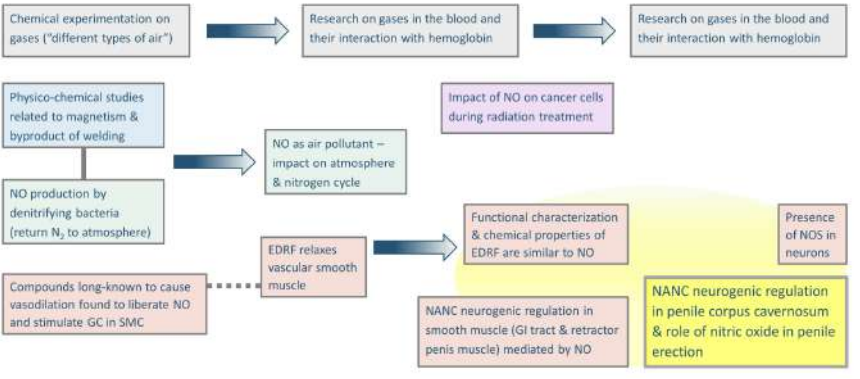
Even with a concerted focus on studying the role of NO in mediating penile erection, therapies were focused on administering drugs locally in the penis. The development of an easily administered, on-demand, oral therapy was not a logical progression from biochemical and physiological data to clinical efficacy. Instead, an unremarkable drug for angina was repurposed into a successful treatment for a sexual dysfunction based upon an unexpected therapeutic effect and insightful decision-making. ED, once the purview of a small community of highly specialized researchers and clinicians, had been brought into general medical practice and into the consciousness of the public with educational efforts through the medical literature and a marketing campaign that seemed omnipresent in newspapers, magazines, billboards, and television advertisements.

As alluded to in the introduction of this chapter, Viagra is not the only drug in the field of sexual medicine to have been originally developed for a different indication and then repurposed to treat a sexual dysfunction. Fli-

banserin, a multifunctional serotonin agonist and antagonist acting on 5-HT1A and 5-HT2A receptors, was originally developed as a novel antidepressant (Clayton et al., 2020). In clinical trials, flibanserin was not consistently superior to placebo or the comparator drug paroxetine in treating major depressive disorder (MDD). However, in a subset of MDD patients who had decreased sexual desire, flibanserin significantly improved their sexual desire that was assessed by a validated instrument measuring sexual function (Clayton et al., 2020). Perhaps in part due to the lesson of Pfizer's experience with sildenafil, the decision was then made to investigate the utility of flibanserin in treating women with hypoactive sexual desire disorder (HSDD). Flibanserin (Addyi), administered orally at bedtime on a daily basis, was eventually approved for the treatment of acquired, generalized HSDD in premenopausal women by the US FDA in 2015 and by Health Canada in 2018.

The second treatment for HSDD, approved in the US in 2019, is bremelanotide (Vyleesi), a non-selective melanocortin receptor agonist, which is administered on-demand by subcutaneous injection. The early history of bremelanotide is the subject of folklore more than documented evidence. Alpha melanocyte stimulating hormone (MSH) had been known to have pro-sexual effects in rats since the 1960s. In a different application, synthetic analogs of -MSH, melanotan-I and melanotan-II, were being developed in the 1980's as sunless tanning agents when a researcher purportedly conducted experiments on himself and injected a higher dose of melanotan-II that resulted in a prolonged erection (~8 hrs) with accompanying nausea and vomiting. After being licensed to a series of private companies, bremelanotide, an active metabolite of melanotan-II, was subsequently developed as a treatment for HSDD. The details of why bremelanotide was repurposed from a potential drug for ED to a treatment for HSDD remain unclear, but the original intent to develop a sunless tanning agent to the unintended therapeutic effect of prolonged erection to a treatment for HSDD is certainly far from a linear progression.

These prominent examples highlight the fact that our knowledge base remains insufficient for many disease states and that trial and error, combined with fortuitous circumstances, plays a larger role in the discovery of medical therapies than we may wish to admit. It remains to be seen whether the push toward personalized medicine using clinico-genomic data and multiple marker diagnostic assays produce more effective therapies and whether these therapies can be cost-effective to the patient and the healthcare system.


Chemical experimentation on gases ("different types of air")
Research on gases in the blood and their interaction with hemoglobin
Research on gases in the blood and their interaction with hemoglobin
Physico-chemical studies related to magnetism & byproduct of welding
Impact of NO on cancer cells during radiation treatment
NO as air pollutant – impact on atmosphere & nitrogen cycle
NO production by denitrifying bacteria (return N_2 to atmosphere)
Functional characterization & chemical properties of EDRF are similar to NO
Presence of NOS in neurons
EDRF relaxes vascular smooth muscle
Compounds long-known to cause vasodilation found to liberate NO and stimulate GC in SMC
NANC neurogenic regulation in smooth muscle (GI tract & retractor penis muscle) mediated by NO
NANC neurogenic regulation in penile corpus cavernosum & role of nitric oxide in penile erection

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III

Modification in feeding: influence in humans and animal models

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Introduction

Food transition and disease

Human nutrition has changed throughout history. Diet was a determining factor that marked the shift for humans to go from nomads to sedentary and to form small groups living together. This situation generated modifications in the eating habits as we pass from being gatherers-hunters consuming fish, roots, berries, wild herbs, vegetables, fruits and having extended periods of famine, to become workers-consumers now collecting in the supermarket (López-Espinoza and Magaña-González, 2014).

The domestication of animals and the cultivation of the land gave rise to agriculture and cattle raising, further accentuating the changes in the eating patterns leaving aside the paleolithic diet. It was undoubtedly during the Industrial Revolution, where a change in the speed and production of food began, leaving the door open to the agro-industries (López-Espinoza and Magaña-González, 2014) that dedicated themselves to producing foods with high contents of sugars, fats, and calories.

Although this entire process took thousands of years, the fact is that today, the large agro-industrial consortia manufacture foods that are not necessarily nutritious for the human being, but fulfill the goal of having larger portions, at exceptionally low costs and attractive to the consumer (López-Espinoza and Magaña-González, 2014). In this sense, because of the marketing strategies used by companies, as well as changes in physical activity and the incorporation of the so-called "Western diet" characterized by its high content in carbohydrates, saturated fats and sodium, a growing epidemic of obesity has been produced (Kopp, 2019).

In this respect, overweight and obesity are risk factors and the main determinants of the Metabolic Syndrome (MS), which is a set of metabolic disorders that includes central obesity, insulin resistance, atherogenic dysli-

pidemia, hypertension, signs that increase the likelihood of developing type II diabetes mellitus, cardiovascular diseases and various types of cancer (Hiriart-Urdanivia et al., 2019; Fahed et al., 2022).

MS currently represents the largest cause of morbidity and mortality in both developed and developing countries, affecting more than one million people worldwide (Saklayen, 2018). Mexico has reported a higher prevalence of MS compared with other countries (Pérez-Galarza et al., 2021), since it affects 39% of male and 38% of female populations (Gutiérrez-Solis et al., 2018).

In addition to the above, the metabolic syndrome is a complex and multifactorial disease related to environmental (nutritional) and genetic factors, where treatments have proved to be complex and ineffective, since the disease must be controlled from several sides. Therefore, the use of animal models within laboratories simulating the symptoms and complications of the disease have been widely used in research, because the environmental conditions in which the animals are found are controllable, reproducible, and stable, Essential characteristics to generate consistent and safe results (Toth, 2015).

In particular, the induction of MS through manipulation or modification in the diet of mouse models is an efficient method that allows to evaluate the metabolism and the energy imbalance in the animal. Although the use of these models has several advantages, they may also have some flaws when used during experimentation. However, proper management will mimic unhealthy eating habits in humans such as increased consumption of carbohydrates, fats, fast food, ultra-processed foods, and sugary drinks, as well as lack of physical activity and exercise (Rodríguez-Correa et al., 2020).

In addition, alterations in animals caused by abrupt changes in feeding allow them to be reflected in a few weeks compared to humans, where it takes several years to observe these changes. Similarly, data collection, mainly serum values and organ damage, can be directly and easily observed (Rodríguez-Correa et al., 2020). For example, standardization of procedures, environmental conditions, and genetic background have been found to reduce variability, and support research reproducibility in pre-clinical research in rodents (Karp, 2018). Highly controlled experiments consider the provision of basic variables including age, body weight, and stage of development of animals, although these patterns are often not reported (Ghasemi et al., 2021).

On the one hand, one of the disadvantages observed in the use of these animal models is that there are no established parameters as in humans, an

example of this is in the definition of obesity. The variability in the composition of the diet per se, is another obstacle that can be presented, because given the diversity in the foods used in the diet, it does not allow an accurate evaluation of the alterations characteristic of the pathology.

On the other hand, scientific research on human beings has brought about great advances in human health. Clinical trials provide a scientific basis for preventing, advising, and treating patients' diseases for the benefit of participants, society, and the medical community. Participants in these types of studies play an active role in their own health care, increasing treatment options when standard therapy does not work, they have access to new treatments before they are available to others and obtain expert medical care during research (Novitzke, 2008).

In this regard, this observational and/or quantitative clinical research begins with the authorization of Research and Ethics Committees and then, they invite volunteers to participate (Novitzke, 2008). As stated in international standards of ethics in research, participants need to sign a document called informed consent and/or assent (Sacristán et al., 2016), which urges respect, autonomy and human dignity, ensuring that the participant understands the benefits and risks associated with the trial and decides whether or not to participate in it (Novitzke, 2008; Constantin, 2018). Overall, to achieve adequate protection of the subjects, researchers must follow certain ethical principles and take due account of the human rights of participants (Dresser, 2009; Constantin, 2018).

Conducting a clinical trial is a complex process, one of the main disadvantages is recruitment. There are often problems with patient or subject retention, which leads to a more complicated and costly process (Sacristán et al., 2016). In addition, there is a risk that the treatment will not be effective for the participant or there are undesired side effects for the subjects (Novitzke, 2008). A controversial point for some researchers is that when the study is completed, patients should be informed about the results of the research and in many cases, this does not happen, and the results should not only be communicated to participants but also, to the general public with appropriate language, in simple and accessible formats for better understanding (Sacristán et al., 2016).

Applicability of experimental models in human diseases

According to Charles Darwin, the human species is an animal. Because of the vestigial reactions we share with some species, animal model experimentation allows us to understand some of the biology and pathophysiological and therapeutic bases in humans (Ghasemi et al., 2021).

Rodents are among the most widely used animals in the laboratory. This species has demonstrated genetic and physiological similarity with humans, allowing for a breakthrough in biomedical research (Rydell-Törmänen and Johnson, 2018). For example, Wistar and Sprague-Dawley rats are used in different medical disciplines such as social stress, toxicology, neurobiology, diabetes, obesity, Among others (Ghasemi et al., 2021). With these models, various aspects can be evaluated from the potential therapeutic effect of some foods on pathologies, to the induction of metabolic alterations similar to those occurring in humans. These dietary models, based on diets or supplements, aim to "imitate" the sedentary lifestyle and unhealthy eating habits that humans have in their daily lives, such as the consumption of fast food, processed foods and/or highly caloric beverages that overall, produce some alterations characteristic of the Metabolic Syndrome (Djalalinia et al., 2015).

To induce the above-mentioned alterations in rats, these models are based on the excessive intake of fats and carbohydrates in diets administered during given periods of time. Generally, in the literature, the use of three types of diet is promoted: 1) high in carbohydrates, where carbohydrates (fructose or sucrose) are added to drinking water or food, 2) high in fat, considered to be one in which the percentage of fat is greater than 10%, and 3) high in carbohydrates and fats, adding a range of commercial foods commonly referred to as a cafeteria diet, to standard meals (Rodríguez-Correa et al., 2020). The cafeteria diet is widely used as a model of obesity, due to its ability to cause metabolic disorders and dysfunction. However, there is no specific consensus on the components of this diet, since we can find various combinations of palatable foods used in the human diet (Feijó et al., 2019), from the use of condensed milk to bacon, among others. To illustrate the characteristics of the diets mentioned above, a compilation of various investigations has been prepared and summarized in Table 1.

Specifically, our research group has sought to induce alterations associated with the Metabolic Syndrome in Wistar male rats, through the modification of the diet. This change is initiated from 21 days of age after weaning, so that we can achieve the desired effects when the subject reaches adulthood. Below, we will describe some of the studies that we have carried out within the laboratory, in which the change in diet played the primary role in the establishment of metabolic alterations. It is worth mentioning that these alterations were always compared with our control groups included in the experimental design of the study:

Guzmán-Gerónimo et al., in 2020 used a diet model consisting of a standard diet plus cholesterol in the croquette (1 g/kg/day) and water intake

with 30% sucrose on demand. After 16 weeks of feeding, we observed the classic alterations present in MS such as increased body weight and abdominal, pericardial, and epididymal adipose tissue. The increase in the weight of organs such as liver, kidney, heart, and spleen became evident. Liver histology showed enlarged hepatocytes due to intracellular accumulation of triglycerides (simple steatosis).

To try a diet high in fat and carbohydrates, we made an adjustment to the diet by adding 1.5% vegetable butter 0.5% cholesterol, 20% sugar to the croquettes and sucrose solution (30%) ad libitum as water in its drink. After 14 weeks, we observed an increase in body weight, abdominal, epididymal and pericardial fat. Similarly, we observed an increase in liver weight and fat accumulation within hepatocytes and sinusoids. In the blood, total cholesterol, triglycerides, insulin and TNF-a levels increased, as well as adiponectin and HDL-cholesterol concentrations decreased (Miñón-Hernández et al., 2021).

In collaboration with the research group of Ramírez-Higuera et al., in 2020, they added 30% of fructose ad libitum as a drinking water for 8 weeks. At the end of the period, an increase in liver weight, visceral and perirenal fat was observed, as well as a significant increase in glucose, total cholesterol, and very low-density lipoprotein (VLDL), triglycerides and insulin. In addition, elevated systolic and diastolic blood pressure levels were obtained.

Summary

As we have seen throughout the writing, all changes in the diet in animal models can generate alterations characteristic of the SM, however, there are still discrepancies about the design of the diet, so to report in detail the composition of the diet, As well as the control diet and caloric intake, it could be useful to standardize a diet and replicate studies appropriately (Rodríguez-Correa et al., 2020).

In conclusion, the modification of the diet in animal models, especially in rodents, has brought with its great advantages for medical research, because these advances have made it possible to understand processes and mechanisms associated with co-morbidities. especially those related to a diet rich in sugars and saturated fats. However, despite the phylogenetic relationship these animal species have with humans, we must consider that they are organisms adapted to different environments, diets and environmental conditions, and care must be taken when replicating and extrapolating the results in another research.

Metabolic syndrome induction studies with experimental animals.

Author	Holemans et al., 2004
Country	Belgium
Experimental Subject	Female Wistar Rats (63 days old)

Model diet	Duration	Effects
Cafeteria-style (CAF), diet contains: 33% ground commercial rat chow, 33% full fat sweetened condensed milk (Nestle), 7% sucrose, and 27% water.	All the experimental period (approximately 8 weeks)	Induces gestational diabetes mellitus (GDM) better than diabetogenic drugs because it causes severe diabetic syndrome by almost complete ablation of pancreatic B cells. This description of GDM in rats provides an attractive model to study the physiological and molecular abnormalities of GDM. Importantly, it also offers the possibility of studying short – and long– term consequences for offspring without the interference of hereditary traits.

Author	Panchal et al., 2011
Country	Australia
Experimental Subject	Male Wistar rats (8–9 weeks old)

Model diet	Duration	Effects
High-carbohydrate, high-fat diet (HCHF) contains: Of 175 g of fructose, 395 g of sweetened dense milk, 200 g of beef tallow, 155 g of powdered rat food, 25 g of Hubble, Mendel and Wakeman salt mixture, and 50 g of water per kilogram of diet.	Total feeding time was 16 weeks.	Metabolic syndrome and cardiovascular remodeling, especially hypertension, hypertrophy, ventricular fibrosis, conduction changes, and endothelial dysfunction, along with nonalcoholic steatohepatitis, degeneration of renal structure, and increased mass of islets of pancreas.

Author	Macedo et al., 2012
Country	Brazil
Experimental Subject	Wistar rats, aged 60 days and weighing 200–250 g

Model diet	Duration	Effects
The cafeteria diet (HD) contains: Increasing the lipid content by 3.33 times and 9.09% to carbohydrates with respect to the standard diet.	6 weeks of treatment	HD increased serum leptin levels, mesenteric, subcutaneous, and visceral addition tissue weight, as well as, weight delta, Lee index, serum triglycerides and total cholesterol levels.

Author	Niu et al., 2016
Country	China
Experimental Subject	Sprague-Dawley rats

Model diet	Duration	Effects
The high-sugar high-fat diet: 70% normal fodder, plus 20% lard, 10% table sugar, 1% cholesterol, and 0.25% cholic acid.	6, 9 or 12 months of treatment.	Found elevated levels of inflammatory factors in blood plasma and brain tissue at all time points, especially insulin resistance. After feeding the diet for a year, rats become obese with histopathological alterations. Chronic inflammation and insulin resistance was evident.

Author	Cheng et al., 2017
Country	Malaysia
Experimental Subject	Male Sprague Dawley rats (Rattus nonverdicts)

Model diet	Duration	Effects
High-fat diet (HFD) contains milk fat that generates an increase in lipids of six times more than the standard diet. High-fat-high-sucrose diet (HFSD) was the previous diet (HFD), and additionally, it was given 30% sucrose water.	8 weeks of treatment	Found central obesity, systemic and diastolic hypertension, impaired fasting glucose, hypertriglyceridemia, and elevated non-HDL cholesterol level. The post-weaning rats was more vulnerable to metabolic dysfunctions.

Author	Oliva et al., 2017
Country	Spain
Experimental Subject	Male and female Wistar rats (Ten-week-old)

Model diet	Duration	Effects
High-fat diet (HF), added with coconut oil and contains: 14.5% of digestible energy derived from protein, 37.0% from lipids, and 48.5% from carbohydrates. Cafeteria diet (K) include plain cookies spread with liver pâte, bacon, water, milk and supplemented with 300 g/L suffocate and 30 g/L of mineral and vitamin.	30 days of treatment	K group showed weight gain in both genders of rats. HF diet elicited significantly higher lactate levels in both males and females, likewise, diets caused triglyceride levels to be increased. Both diets cause the development of disorders associated with metabolic syndrome.

Author	Acosta-Cota et al., 2019
Country	Mexico
Experimental Subject	Male Wistar rats (four weeks old)

Model diet	Duration	Effects
The animals had ad libitum access to water with 30% sucrose for a period of 12, 16 or 20 weeks, and water with 40% and 50% sucrose for 20 weeks.	Period from 12 to 20 weeks	The development of non-alcoholic fatty liver disease (NAFLD) is generated in all concentrations of sucrose and under time cuts, the degree of affection of NAFLD depends on these factors.

Author	Shawky et al., 2019
Country	Egypt
Experimental Subject	Male Sprague-Dawley rats (8-week-old)

Model diet	Duration	Effects
High fructose diet (HFrD) was prepared by mixing 600 g of fructose and 400 g of standard laboratory diet (50% carbohydrates, 23% protein, 3% fat, 4% fiber) plus vitamin and mineral supplements).	60 days of treatment	HFrD caused induced glucose tolerance, also the fastening insulin level increased 2.3-fold, showing elevations in triglycerides (by 1.7 times) and LDL-C (by 2 times). In liver tissue, elevations were found in the levels of MDA (by 1.5 times) and GSH (by 2.8 times), as well as a decrease in SOD activity (by 24%). Likewise, diffuse steatosis was evident in liver samples.

Author	Lasker et al., 2019
Country	Bangladesh
Experimental Subject	Male Wistar rats (10–12 weeks old)

Model diet	Duration	Effects
High-fat (HF) diet was composed of normal food, beef tallow, sugar, and condensed milk. Contained 14% proteins, 37% carbohydrates and 49% fat in terms of the caloric content.	8 weeks of treatment	HF provoked glucose intolerance, dyslipidemia, and oxidative stress in the liver. It also showed increased plasma glucose levels and plasma lipid levels, including cholesterol and triglyceride levels. While in oxidative stress, increased the levels of lipid peroxidase product malondialdehyde, nitric oxide, and advanced protein oxidation products. As well, developed hepatic fibrosis and the intestinal brush border was altered and a disorganized structure of the intestinal tract.

Author	Du Preez et al., 2020
Country	Australia
Experimental Subject	Male Wistar rats (8–9 weeks old)

Model diet	Duration	Effects
H diet contained 175 g of fructose, 395 g of sweetened dense milk, 200 g of beef tallow, 155 g of powdered rat food, 25 g of salt mixture, and 50 g of water per kilogram of diet. Plus, another group was t supplemented with 25% fructose in water.	8 weeks of treatment	Both diets developed obesity, hypertension, dyslipidemia, glucose intolerance, fatty liver and increased left ventricular collagen deposition.

Author	Mbappe et al., 2022
Country	Cameroon
Experimental Subject	Albino Wistar rats (twelve-week-old male)

Model diet	Duration	Effects
High fat/high sucrose (HF/HS) diet consisting of increasing the lipid content 3.5 times plus, and sucrose diet 35.68%.	21 days of treatment	HF/HS caused an increase in body weight, in terms of serum levels it shown an increase in total cholesterol, triglycerides and LDL. Likewise, it caused an increase in cardiac and renal secondary products of lipid peroxidase.

Author	Yue et al., 2022
Country	China
Experimental Subject	Male Sprague Dawley rats (2 months old)

Model diet	Duration	Effects
High-sugar and high-salt diet (HFSSD) is composed of feed 600 g; 130 g lard; 100 g egg yolk; 110 g sugar; 40 g editable salt and 30% of fructose.	4 weeks of treatment	HFSSD induced metabolic syndrome, reflected in increased fasting blood glucose and triglycerides (TG). On the other hand, gut microbes were found to have a strong correlation with serum metabolites, liver genes, obesity, TG, and hyperglycemia.

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IV

From reasoning to fear

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Introduction

Mathematical Anxiety (MA from here on out) is a phenomenon already described in the mid-1970s, when this concept made its debut in the scientific literature, although at that time it was better known as "math phobia" due to the behaviors of fear, panic, impotence, and paralysis caused by the simple fact of interacting with digits or abstract elements, thus adding a different factor to the cognitive one as part of mathematical processing, the emotional factor (Lazarus, 1974). At present, the term MA has not completely changed since it retains the essence that has been used over time, concluding that MA is an emotional response caused by numerical manipulation, this emotional response significantly interrupts the performance during mathematical problem solving (Cipora et al., 2022).

But before describing the term MA in depth, we will give space to explain the importance of mathematics in today's society and how the process to develop it covers aspects beyond the academic requirements of the individual, we fully agree that the use and learning mathematics are an important pillar for cognitive and technological development, but currently, these two factors have been moving away in a modern community that does not find the practical use of mathematics on a day-to-day basis. In general, when the word mathematics is heard, the first thing that comes to mind are numbers, formulas, theorems, and geographical structures, always related to something logical, something full of reason, something that explains a perfect reality.

What is mathematics?

The history of mathematics goes back to antiquity when human beings began to use symbols and numbers to count, measure, and solve practical problems (Molina-González, 2014). The need to measure and control agricultural resources and the construction of buildings and monuments prompted the development of mathematics in ancient civilizations such as the Egyptian, Babylonian, Indian, and Chinese (Sánchez-Gonzales, 2022).

During the Ancient Age, the Greeks made important contributions to the development of mathematics, with characters such as Pythagoras, Euclid, and Archimedes among others. These established the foundations of geometry, algebra, and trigonometry, laying the foundations of what this science is today (Bell, 2021). In the Middle Ages, Arab, Indian, and Chinese mathematicians made major advances in calculus, geometry, algebra, and number theory. In Europe during the Renaissance, mathematicians such as Leonardo da Vinci, Galileo Galilei, and Johannes Kepler used mathematics to describe the motion of celestial bodies and advanced physics and astronomy (Bell, 2021).

In the 17th century, Isaac Newton and Gottfried Wilhelm Leibniz developed calculus, which revolutionized physics and engineering. In the 18th century, Leonhard Euler and Joseph Louis Lagrange made important advances in number theory, differential geometry, and celestial mechanics. In the 19th and 20th centuries, mathematics expanded into new areas such as set theory, topology, algebraic geometry, and the theory of computation. During this time, mathematicians such as Carl Friedrich Gauss, Évariste Galois, David Hilbert, Alan Turing, and John Von Neumann, laid the foundations of modern science and technology (Jean-Luc, 2008; Pla, 2014).

Over time, mathematics could be positioned as a discipline that focuses on the study of the properties and relationships of numbers, geometric figures, functions, sets, and the processes that involve them. It is a formal science that relies on logic and deduction to analyze and solve problems.

Mathematics is used in multiple areas of knowledge and everyday life, from physics and engineering to economics and biology. Its application is fundamental in the development of technologies, the resolution of complex problems, and the understanding of natural and social phenomena (Ashcraft and Ridley, 2005). In it we can find different branches and subdisciplines, such as arithmetic, geometry, algebra, calculus, and statistics, among others. Each of these areas focuses on a specific set of mathematical concepts and tools and has practical applications in different fields of science and technology. To understand in more detail, the practical use of mathematics and what its abstraction implies, the individual must be exposed to formal learning or education (Núñez-Peña and Suárez-Pellicioni, 2014).

Mathematics in education

Teaching mathematics is a crucial task in the educational development of students. Mathematics is a discipline that is used in multiple areas of knowledge and daily life, so its understanding is essential for success in education and the individual's professional life (Pérez-Tyteca et al., 2011).

The teaching of mathematics must be inclusive, promoting equal opportunities for all students, regardless of their socioeconomic origin, gender, race, and ethnicity, among other factors (Sokolowski and Ansari, 2017). Teachers must provide a safe and stimulating learning environment that fosters curiosity, creativity, and problem solving (González-Pienda and Nuñez-Pérez, 1999).

Also, the teaching of mathematics must be meaningful, that is, students must be able to relate mathematical concepts to real-life situations (Cohen and Green, 2002). Teachers must present concepts in a clear and organized way, using concrete examples and practical activities that help students better understand mathematical concepts (Godino et al., 2003).

On the other hand, the teaching of mathematics must be progressive and structured, that is, teachers must carefully plan the learning objectives and design appropriate teaching strategies for each level of teaching. Students must be guided so that they can develop basic mathematical skills, and as they progress in their learning, they must be challenged with more complex concepts (Furner and Berman, 2004; Chavarría-Arroyo and Díaz-Obando, 2013).

As can be seen, mathematics as a formal science is already quite a challenge to be able to master, one, due to the complexity itself to understand and two, due to the entire teaching-learning process that leads to an individual developing them (Dehaene, 2011). But there is also the emotional factor that has a significant impact on the two previous processes. In a very specific way, there is a phenomenon known as Mathematical Anxiety (Meece et al., 1990; Ashcraft et al., 2007).

Math Anxiety

MA is a type of anxiety that is experienced when facing situations related to mathematics, such as solving mathematical problems, taking mathematics tests participating in activities that involve the use of mathematics. MA can have a negative impact on a person's mathematical performance and their attitude towards mathematics in general (Monje-Parrilla et al., 2012). People who experience MA may have negative thoughts about their math skills, feel afraid of failing, or feel overwhelmed when facing math problems (Eccius-Wellmann et al., 2017).

However, MA is treatable. Therapeutic approaches such as Cognitive Behavioral Therapy and gradual exposure can help people manage their anxiety and improve their math performance. In addition, there are practical strategies to reduce MA, such as practicing relaxation and visualization exercises, focusing on the steps needed to solve a problem rather than worr-

ying about the result, and seeking support from teachers, friends, and family (Eysenck and Calvo, 1992; Fernández-Álvarez and Fernández-Álvarez, 2017).

MA is an emotional state that is experienced in response to situations that involve numerical management, when it becomes excessive and persistent, it can greatly affect the performance of the person, and this is due to the over-activation of certain brain regions (Paulus and Steins, 2006). In the last decade, there has been a large amount of research in the neurobiology of MA that has led to important advances in the understanding of the biological bases (Suárez-Pellicioni et al., 2015; Wang et al., 2014).

The amygdala is a brain structure that has been identified as one of the main areas involved in MA. The amygdala processes emotional information, especially related to fear and anxiety, and has been found to be overactive in people with high MA. Neuroimaging studies have shown that the amygdala shows greater activity in response to aversive stimuli in this specific case, to the resolution of mathematical problems (Stein et al., 2007; Young et al., 2012).

Another brain area involved is the ventromedial prefrontal cortex (VMPC). The VMPC is involved in emotional regulation and decision making. Studies have shown that the VMPC is underactive in people with high levels of anxiety, suggesting that there may be a dysfunction in the ability of these people to regulate their emotions (LeDoux and Pine, 2016).

The stress hormone system, especially the hypothalamic-pituitary-adrenal axis, is also involved in MA. Cortisol is a hormone released during the stress response, and chronically elevated cortisol levels have been associated with an increased risk of anxiety disorders and acute aspects such as MA (LeDoux and Pine, 2016; Tovote et al., 2015).

The structures related to emotional processing, in the MA region involved in mathematical processing also participate. Understanding the neurobiology of mathematics has become a growing field of interest, as it may have important implications for the teaching and learning of mathematics (Caballero et al., 2009).

Research has shown that mathematics involves multiple areas of the brain, including the prefrontal cortex, the parietal lobe, and the hippocampus. The prefrontal cortex is involved in abstract reasoning and planning, the parietal lobe is associated with spatial perception and number manipulation, and the hippocampus is involved with memory and information retrieval (Serra-Grabulosa et al., 2010).

Learning mathematics has been found to involve changes in the structure and function of the brain. For example, it has been shown that training

in mathematics can increase the density of gray matter in the parietal lobe, suggesting greater efficiency in mathematical processing (Núñez-Peña and Suárez-Pellicioni, 2014).

Summary

We can say that mathematics has been driven by the need to solve practical problems and by human curiosity to understand the world around us. Mathematics is a fundamental discipline in the development of human knowledge and its application is essential in multiple areas of science and technology.

We must be clear that the teaching of mathematics must be inclusive, meaningful, progressive, and structured. Teachers must be trained and updated on the latest pedagogical and technological trends, and they work in collaboration with other professionals to improve the quality of mathematics teaching and ensure the success of their students.

We can't leave behind the neurophysiological aspects that are behind anxiety itself and the brain areas involved in mathematical processing, considering that it is a constantly evolving field that explores how the human brain processes and uses mathematical information. Understanding this process may have important implications for the teaching and learning of mathematics, as well as for research in the field of cognitive neuroscience.

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V

Human milk contamination and consumption derived from XXI century industrial lifestyle

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Introduction

Breastmilk is essential for infant's nutrition and development

Mother's milk during earliest stages of life is fundamental for a proper growth and maturation of tissues and organs in humans because it provides all nutritional and non-nutritional requirements for the nutrition, health, and development of infants. Besides, milk mitigate and prevent metabolic and immune-related pathologies during infancy and later in life (Mosca and Gianni, 2017). In this regard, breast milk is considered as the gold standard for infant nutrition.

According to the World Health Organization (WHO, 2022) and the United Nations Children's Fund, infants need to receive breast milk for at least the first 6 months and up to two years, or longer, complemented with solid food. However, milk also can have toxic components. In our modern society, high number of industrial compounds, had been released to the environment, they remain for long periods in the food chain and in all tissues in humans, including breast milk (United Nations, 2019 & 2022). Many of them are toxic to laboratory subjects and humans. In this chapter we will review the composition and properties of human milk as well as their toxic compounds, particularly those known as persistent organic pollutants (POPs). Finally, we will compare presence of POPs in human milk with those in cow's milk which is highly consumed by adults and human infants in a wide array of formula milk substitutes.

Breast milk composition

Breast milk is produced by lactocytes which secrete this fluid into the alveoli and duct lumens of the mammary gland. Breast milk is an extraordinarily

complex, highly variable bioactive fluid, with changes in composition depending on the stage of lactation (from colostrum to late lactation), time of day, and physiological/nutritional state of the woman (Mosca and Gianni, 2017). In fact, composition of the human milk as a bioactive fluid has been documented since the twentieth century (Widdows et al., 1930), including their hormonal, immune and nutritional components (Ballard and Morrow, 2013; Field, 2005; Garwolinska et al., 2018; Kim, 2021; Mazzocchi et al., 2019).

Hormonal molecules include adiponectin, leptin, insulin, ghrelin, and obestatin (Mazzocchi et al., 2019), and growth factor's molecules-related such as insulin-like growth factor 1, hepatocyte growth factor, epidermal growth factor, neuronal growth factors and vascular endothelial growth factor (Gila-Diaz et al., 2019). Immune-related components have also been reported in human milk including cytokines (IL-1ß, IL-4), IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, TNF- , INF- , granulocyte-colony stimulating factor, monocytes chemotactic protein 1, and RANTES (Ballard and Marrow, 2013; Camacho-Morales et al., 2021; Field, 2005; Kim, 2021), and immune cell entities such as innate lymphoid cells, natural killer, cytotoxic and T regulatory cells, neutrophils and macrophages (Ballard and Marrow, 2013; Camacho-Morales et al., 2021; Field, 2005; Kim, 2021).

Nutritional components include macronutrients as carbohydrates, proteins and fats and endogenous antioxidants including -carotene, ß-carotene, ß-cryptoxanthin, lycopene, lutein, zeaxanthin, retinol, and -, -tocopherol tocopherol (Hanson et al., 2016). Other important macronutrients are complex oligosaccharides constituted from glucose (Glc), galactose (Gal), fucose (Fuc), N-acetylglucosamine (GlcNAc), and sialic acid with N-acetylneuramic acid (Neu5Ac) (Garwolinska et al., 2018). Proteomic analysis documented more than 1500 different compounds in the human milk, including those modulating immunomodulatory and antimicrobial activities such as lactoferrin, secretory immunoglobulin A, osteopontin, lysozymes, -casein, lactoperoxidases, haptocorrin, a-lactalbumin (Beck et al., 2015; Haschke et al., 2017). Nonprotein molecules containing nitrogen in human milk have also been identified, including amino sugars, creatine, carnitine, nucleic acids, nucleotides, polyamines, urea and uric acid (Garwolinska et al., 2018).

In relation to lipids, they are the major source of energy supply in the breast milk providing 40%–55% of the total energy requirement for the infant (Koletzko et al., 2011), and are the second most abundant macronutrient (Boudry et al., 2021). Initial characterization of lipid composition in human milk determined accumulation of triacylglycerols (TAGs,

98–99%), phospholipids (0.26–0.80%), sterols (0.25–0.34%, mainly cholesterol) and trace amounts of glycerophospholipids such as monoacylglycerols, diacylglycerols and fatty acids (Wei et al., 2019; Yong-Hua et al., 2010).

Recent lipidomic analysis confirmed that sphingolipids (sphingomyelin, ceramide and dihydroceramides) accumulate at higher concentrations in breast milk (Dei Cas et al., 2020), while additional reports documented glycerophospholipids (phosphatidylethanolamine, phosphatidylcholine), glycerolipids (diacylglycerol) or ceramides which changed dramatically according to preterm and term stages of the infant at birth (Xu et al., 2020). As a major component of human milk, triacylglycerols are included into lipid droplets which confine in globules surrounded by cellular membranes in the mammary gland (Michalski et al., 2005). Globules building in the mammary gland depends on de novo lipogenesis provided from dietary components. For instance, fatty acids in milk are derived from the circulating triacylglycerides into the very low-density lipoproteins (VLDL) and chylomicrons or those bound to plasma albumin as non-esterified fatty acids (Shingfield et al., 2010). Also, cholesterol accumulation into the mammary gland is provided by circulating cholesterol bound to the low-density lipoprotein (LDL) (Wong et al., 1993). As happen in the adipose tissue, fatty acids from the triacylglycerides found in the VLDL and chylomicrons are imported into the mammary gland following the action of mammary lipoprotein lipase (Shingfield et al., 2010).

Finally, de novo fatty acid synthesis is secured by the acetyl-CoA carboxylase and fatty acid synthetase enzymes. This evidence confirm that mammary gland integrates an active lipid metabolism by a crosstalk of lipoprotein transporters including LDL, HDL, VLDL, and albumin to provide chemical components of lipogenesis. It is precisely this last lipid component of milk that marks a relevant point for this chapter, since in recent years a series of contaminants known as POPs have been identified, whose chemical characteristics give most of them a high lipid solubility which facilitates their entry, transport, and accumulation in the body, as well as their excretion from the mammary gland and eventual digestion by the newborn (Fig. 1) (Lind and Lind, 2020)

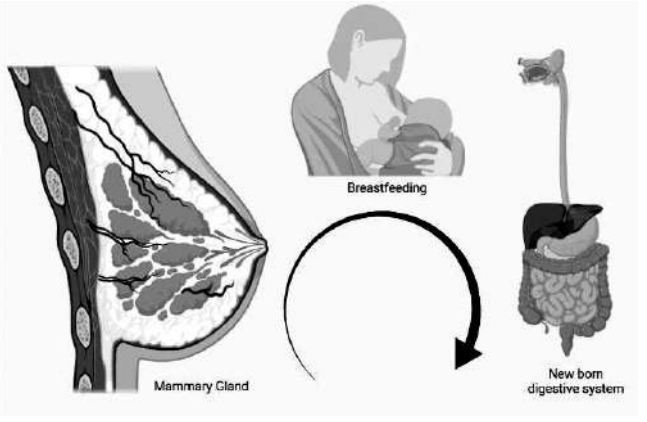

Figure 1. Representation of the cycle of entry of POPs into de body of newborn through breastfeeding. Figure created in BioRender.com.

Persistent Organic Pollutants in milk

The interrelation between the lipid component of milk and POPs has been increasingly investigated in recent years due to their high occurrence in the environment since they were used indiscriminately in low, middle and high-income countries as insecticides for the agricultural industry, as combustion retardants to decrease flammability of several materials and increase their durability, as well as in many plastic products and industrial processes (Lohmann et al., 2007). Also, some are formed from emissions from the combustion of organic matter (Breivik et al., 2004).

In general POPs are easily dispersed and released to the air and water sources and enter the food chain where they bioaccumulate. Chemically they are brominated and chlorinated organic compounds which make them highly resistant to degradation by physical, chemical, or biological means. This rises global concern and for this reason the United Nations Environmental Program launched the Stockholm convention (SC) on POPs to monitor their levels in human tissues in 2001 (United Nations, 2019 & 2022). POPs can be classified in brominated and chlorinated compounds. Among brominated are the diphenyl ether (PBDE) group and hexabromocyclododecane (HBCD) group. Among chlorinated are the dichlorodiphenyltrichloroetane (DDT) metabolites, dioxins, dibenzo-p-furans (PCDFs), hexachlorobenzene (HCB), Aldrin, Endrin, Mirex and chlorinated paraffins. Several other POPs have been included in the SC (Huang et al., 2006).

The risk begins once they have entered the body through the oral, nasal, or dermal route as POPs are transported by lipoproteins to their deposition in tissues with high adipose content. LDL lipids are the main mean of distribution, however, VLDL and HDL lipids can also perform this function in the

body and may be susceptible to changes in POPs function (Lind and Lind, 2020). In addition, POPs have been related to triglycerides and cholesterol molecules in the blood which also transport them (Lind and Lind, 2020). Once in the bloodstream, the lipophilic nature of POPs gives them easy access to the breast since it has a high concentration of fatty tissue. This tissue during the milk production phase requires a large number of lipoproteins, triglycerides, and cholesterol for the generation of its lipidic part. They enter the milk-producing epithelial cells where they are bio-degraded and reused for the de novo synthesis of fat globules that will serve as transport for a large number ofmany fundamental components for the healthy development and growth of the infant. Though, during this process POPs bound to lipids are incorporated in the cycle allowing that such pollutants reach the newborn. This cycle has been schematically represented in figure 2 (Manettje et al., 2012).

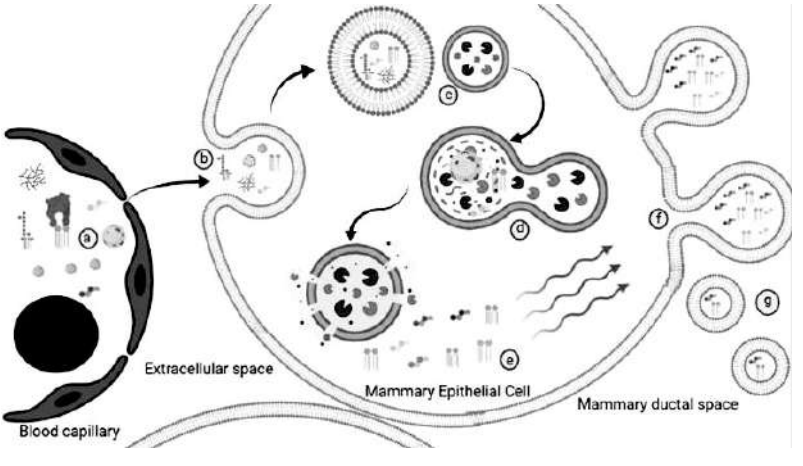

Figure 2. Cycle of lipid-bound POPs from the maternal circulation to the newborn. a) In the bloodstream, a heterogeneous group of molecules can mobilize POPs from the site of entry into the body to milk-producing cells by non-covalent bonds, such as HDL, LDL or VLDL lipoproteins, triglycerides and cholesterol. b) These molecules enter the cell through pinocytosis being identified by receptors on the cell Surface. c) Once inside the cytoplasmic space, the vacuole is identified by lysosomes, which initiate the fusion of their membranes. d) The enzymes within the lysosome are responsible for the degradation of the molecules into their most basic components, the POPs can remain attached to those lipids present in this medium. e) Lipid-bound POPs can be released into the cytoplasmic medium or used for the synthesis of new compounds for milk secretion through new biological synthesis processes. f) The mammary gland performs the release of milk through apocrine secretion for the lipid component, which causes both the intracellular content and part of the membrane where these lipids attached to the POPs that have been reused are immersed. g) Finally, these new lipids linked to POPs enter the baby's digestive system, being used in their biological processes. Figure created in BioRender.com.

Breastmilk to monitor POPs

The milk of mammals had been used to study the levels of POPs, as levels in the mother's tissues indicates the approximate amount of these compounds ingested through their life and correlates with the amount secreted in milk which is ingested by the newborn through breastfeeding (Grešner et al., 2021). As previously mentioned, there is a high concentration of lipids in milk and POPs are highly lipophilic. Owing to this property the Stockholm Convention recommends POPs monitoring in blood and specially in breastmilk as sample collection is easy and less invasive than blood sampling (Grešner et al., 2021). As expected, large number of POPs had been identified in different mammal's milk.

The pollutants chemical composition and concentration vary widely between countries and environments and is modified by human activities such as agriculture as this activity requires pesticides and other contaminant chemical additives. As expected, water can contain more POPs derived from these activities and nearby industries. (Grešner et al., 2021; Manettje et al., 2012; Matovu et al., 2021; Polanco-Rodríguez et al., 2017). Moreover, POPs are present in everyday products as furniture, rugs, paints, and plastic containers (Wong et al., 1993). Consequently, these contaminants are present everywhere. For this reason, to determine their presence and concentration is a global matter of concern to implement strategies for controlling their release.

POPs in cow's milk

Besides human milk, presence of these compounds in cows is of particular importance as there is a high consumption of meat and milk by humans. Moreover, cow's milk is the fundamental basis of most milk formulas which are consumed by millions of infants around the world. Studies in several countries such as Spain, Slovenia, Italy, Greece, China, Colombia and Brazil among many others in the world revealed a wide array of POPs included in the Stockholm Convention list in fresh, pasteurized, and organic bovine milk (Vasconcelos et al., 2019). In México chlorinated compounds as alpha hexachlorocyclohexane, endosulfan, dieldrin, endrin, heptachlor and metabolites of DDT had been found in cow milk in an industrial farm in the state of Hidalgo (Gutiérrez et al., 2013). Also, alpha, beta, gamma and delta hexachlorocyclohexane, heptachlor, aldrin, dieldrin and metabolites of DDT were detected in bovine milk from an organic farm in Chiapas state (Gutiérrez et al., 2012).

As most infant milk formulas are produced from cow milk, then ensues the question whether they can contain POPs. Indeed, POPS are present in

commercial marketed infant milk formulas. Alpha, beta and gamma hexachlorocyclohexane, aldrin, heptachlor and heptachlor epoxide were found in seven dehydrated commercial milk formula brands for infants in México city (Gutiérrez et al., 2014). Although their concentration was below the international permitted limits set by the Codex Alimentarius (Gutiérrez et al., 2014), their presence raise concern about the ingestion of these compounds every day during long periods of months and years, because as mentioned, POPS bioaccumulate in fat tissue, remain in the body and are very difficult to eliminate. Moreover, it is possible that in some cases these toxic compounds reach values above those permitted as their concentration vary widely depending on the sampling area and methods of analysis (Breivik et al., 2004; Hayward et al., 2018; Lohmann et al., 2007).

However as mentioned above as they enter the food chain, they are available everywhere in many of our food products. The interest of monitoring these pollutants changes according to their prevalence in samples of particular environments and their possible toxic effect. It should be considered that for these pollutants the percentage between what is absorbed and what is excreted also varies widely (Amutoya et al., 2021). Despite the variability in concentration of the different POPs among countries it had been estimated that around 18% of the human exposure to Polychlorinated biphenyls and dibenzofuranes is from bovine meat (Driesen et al., 2022). In this study authors report that in contaminated cows' calf in uterus start to accumulate POPs, and after birth they continue to ingest them through milk. Then POPs in calf's blood was 1.5-fold higher compared to that of their mother; they remain in their meat which is consumed by humans. Fish as salmon or canned tuna contain high concentration of some of these contaminants (Saktrakulkla et al., 2020). Then it is clear that POPS are found in all the food chain, as already mentioned. Together this evidence clearly indicates a global contamination of POPs in a wide array of available food. In cattle it is of particular concern because POPs are ingested by humans through meat, milk and dairy products, including milk formulas.

POPS in humans, since utero and in breastmilk

Accumulation of POPS in humans start in the uterus during pregnancy and similar to cows, there is a 2-fold increase in the fetus in comparison to the mother (Driesen et al., 2020; Landrigan et al., 2004). It had been demonstrated that the consumption of contaminated beans and potatoes at least one time per week or of cereals at least two times per week was found to be associated with increased concentration of insecticide metabolites in human milk (Hassan et al., 2022). Also, the ingestion of dairy products and fish was

linked to higher POPs concentration in milk in Poland (Grešner et al., 2021). A study in 53 countries of 5 continents (Asia, Europe, Oceania, Africa and Central/South America) found chlorinated paraffins in all breast milk samples, including in isolated locations such as pacific island countries (Krätschmer et al., 2021). Another multi continent study found higher presence of perfluoroalkane substances in milk in higher income countries such as Europe when compared to lower income countries such as Africa and Latin American Countries (Fiedler and Sadia, 2005).

A recent study in México explored in detail 28 brominated and 75 chlorinated POPS in breast milk from Sinaloa state (Martínez et al., 2022). In relation to brominated compounds, PBDE47 had the highest value and the sum of this and other 5 PBDE was 11.6 ng/g lipid which is considered as a "safe" value. Also, alpha HCB was found but their safety limits had not been established. In relation to chlorinated compounds, metabolites of DDT were in the upper limit in the group of Latin American countries, which have far higher concentrations than in European countries. The reason for the high concentration in México and countries of this group is that DDT was widely used to control malaria. Although the use of this compound was prohibited years ago, it remains in the environment and the food chain. Many other compounds were detected and the concentration of most of them was also in the "safe" limit, except chlorinated dioxin-like compounds. In general, this study is important to compare concentration values in relation to previous and future surveys in México and other countries. As an example, PBDE and DDT compounds are decreasing in comparison to a previous survey in 2011 (Martínez et al., 2022; United Nations, 2019 & 2022).

POPs and human health

Several in vitro or in laboratory animal models studies had explored the concentration and toxic effect of POPS. They showsshow that several of them are neurotoxic, induce developmental and reproductive disturbances, affect the endocrine and immune system and had been associated to the development of diabetes and cancer (United Nations, 2019 & 2022). On this basis the Stockholm Convention include in their list particular POPS. However, their specific effect in humans it is difficult to evaluate due to ethical concerns. Though, some insight had been obtained from unfortunate industrial accidents. In 1976 in Italy there was a large release of dioxin-like compounds in the atmosphere and many pregnant women were exposed to this POP. Thirty years later the analysis revealed hypothyroidism in their now adult offspring's due to an elevation of the thyroid-stimulating hormone (Baccarelli et al., 2008).

Another study in relation to the same industrial accident reported a sex and time related effect as male infants but not adults that exposed to the contaminants showed 22 years later a reduction in sperm counts and motility as well as reduction in the hormone estradiol and increased follicle stimulating-hormone (Mocarelli et al., 2008). These disturbances highlight that the exposure to POP seems to be more harmful to the fetus and during development, than in adults and that males and females are affected differently. However, the degree of immediate and long-lasting detrimental effects is not clear and only can be inferred on basis of in vitro and laboratory animal studies.

Our laboratory contributed to the determination of POPs in breastmilk in a population in México (Martínez-Valenzuela et al., 2022). We are now extending the analysis of several toxics, including microplastic contaminants in breastmilk and milk formulas. Besides we are also exploring immunological compounds, metabolites and hormones in breastmilk in relation to body mass index as the amount of body lipids affect their concentration and concomitantly may have an important impact on health and development of the infant. This is an important issue because as mentioned above, the contaminants in milk are highly lipophilic.

Summary

POPs interaction begins during in utero days and continues after birth through ingestion though cow and human milk. They can accumulate and remain for long periods in the individual and although evidence suggest that they can be associated to an array of health disturbances, their harmful effects are still not completely clear. Despite the probable health outcomes of POP ingestion, the WHO still recommends breastmilk as the only food during at least the first six months of age because the health benefits greatly outcome the risks. As expected, these benefits are not present in cow's milk-based formulas. Strong evidence demonstrates the superior quality of human in comparison to cow's milk for the nutrition, health, and development of the infant with long-lasting benefits (Mosca and Giannì, 2017; WHO, 2022).

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VI

From aquarium to laboratory. The role of fish in human health

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Introduction

Fish are the first group of vertebrates in the phylogenetic scale. They are characterized by a body covered with scales and adaptations to aquatic life as fins and gills to breathe. Locomotion is based on a hydrodynamic shape and lateral movements of the body. Fish were long believed to have a poor behavioral repertoire that was simply regulated by reflex responses. Even Darwin, who studied different aspects of fish, did not mention fish emotions. He only cited S. J. Whitmee, who described that the erection of the dorsal and anal fins protects against being eaten by predators (Darwin, 1890). Due to all their characteristics, fish were considered simple organisms unable to experience complex processes and behaviors such as sleep or emotions. However, in the 1920s, studies of behavior by Konrad Lorenz and Nicolas Tinbergen demonstrated complex behavioral patterns in fish. Tinbergen discovered that in the three-spined stickleback fish (*Gasterosteus gymnurus*, figure 1a), only the red color on the male's sides is the trigger signal of aggression that allows the fish to fight only real competitors for food and females (Tinbergen and Van Lersel, 1948).

Konrad Lorenz studied ritual dances of aggression between male European stickleback fish (*Gasterosteus aculeatus*), Siamese fighting fish (*Betta splendens*), and the Jewelfish cichlid (*Hemichromis bimaculatus*) during mating, spawning, and larva care (Lorenz, 1962). Both Lorenz and Tinbergen are considered the fathers of ethology. Notably, they won the Nobel Prize in Medicine and Physiology for their findings in 1973.

Behavioral analyses of fish are used as an index of health that is associated with water pollution levels where they live. For example, *Betta* fish that were chronically exposed to wastewater from pharmaceutical industries that contains ß-sitosterol (phytosterol), equol (metabolites of isoflavones), and genistein (isoflavone) phytoestrogens exhibited a decrease in aggressi-

ve behavior that was displayed in front of a mirror (Clotfelter and Rodríguez, 2006). Genistein and equol increased dopamine in the prosencephalon in *Betta* fish, whereas ß-sitosterol decreased norepinephrine and dopamine in the prosencephalon and rhombencephalon (Clotfelter et al., 2007). These findings suggest that behavioral alterations are useful indicators of environmental pollution that, although not lethal, modify neuroendocrine and neurochemical systems.

Aggression in *Betta* fish is also decreased by exposure to 5-HT receptor antagonists, such as 8-OH-DPAT (Clotfelter et al., 2007), and antidepressants, such as fluoxetine (Lynn et al., 2007). Aggression displayed toward a mirror by the African cichlid (*Astatotilapia burtoni*) has been related to an anxiety-like state, during which increased neuronal activity in the dorsomedial pallium activating the expression of Fos protein. It is relevant considering that this nucleus in the fish has a similar function to the amygdala in mammals, which has been implicated in regulation of anxiety-like behaviors (Desjardins and Fernald, 2010). Now we know that some fish, such as Discus Fish (Symphysopdon aequifasciatus, Cichlidae; Figure 1b) are even able to recognize "faces" (Satoh et al., 2016). These findings suggest similarities in brain neurochemistry and behavior expression between mammals and fish (Chandroo et al., 2004); but what exactly do we know about fish brain?

Fish Brain

The nervous system of fish is similar to that of other vertebrates. Generally, a fish´s central nervous system (CNS) comprises the brain and the spinal cord. The brain is divided into the telencephalon or prosencephalon (with two olfactory bulbs and the brain), the diencephalon (with epithalamus, thalamus and hypothalamus), mesencephalon or midbrain (with two optical lobes), metencephalon (cerebellum) and brainstem. In most fish, the olfactory bulbs are the most prominent part of the brain, highlighting the importance of olfaction in this group of vertebrates (Wulliman, 1998; Bradbury, 2008). Fish brain is located behind the olfactive lobes and controls voluntary movements. In mammals, it is associated with learning and memory, but in fish, this process has been studied in recent years (Kotrschal, 1998; Wulliman, 1998), although it is known that species such as butterfly fish form mental maps of their territory.

Optical lobes are located behind the cerebellum and connect to eyes trough the optical nerve. Bellow the optical lobes is the pituitary gland, which controls the endocrine system. The cerebellum is the control center of movement that regulates muscular contraction. Brainstem located below the cerebellum connects the brain to the spinal cord; it controls hormone

secretion, heart function, the smooth muscle of internal organs and gill contraction for breathing (Kotrschal, 1998; Bradbury, 2008). The spinal cord contains long nerve fibers that receives information from peripheral nerves to sensorial zones of the brain and transmit action potential to muscles, glands, and other tissues (Wulliman, 1998). Peripheral nervous system is a network of nerves that connect muscles and sensorial organs to the CNS, sensorial nerves transport information from the sensorial organs to the spinal cord and motor nerves send signals from CNS to muscles and glands.

Animal Models in Health Research

Rodents and rabbits are most popular laboratory animals in all areas including neuroscience. Fish were commonly used in biomedical areas as tissue regeneration but excluded from complex processes such as emotions and sleep. Fish CNS could be considered simpler than human, but it is perfectly adapted to the challenges of their environment. Given the similarities, it is a suitable tool in neuroscience to understand emotions, behaviors such as sleep, and disorders such as anxiety and sleep disorders, among others, using validated models. In neuroscience, an animal model is a living organism with specific characteristics that are exploited to explore relationships between the brain and behavior under controlled laboratory conditions to obtain information that is applicable to humans and other species (Van der Staay, 2006; Maximino and Van der Staay, 2019).

Small species are advantageous given their common high fecundity and fast development. For example, in 2000, the first behavioral scores related to sleep under laboratory conditions were made using fruit flyes (*Drosophila melanogaster*). Giulio Tononi and collaborators determinated the activity phase of fruit flies using little cylinders connected to movement detectors that individually scored the leg movement on the cylinder and named this phase as wakefulness. In contrast, the phase of immobility was called "sleep-like" stage. Interestingly, the wakefulness synchronizes with the light phase while sleep-like phase synchronizes with the dark phase of the day, suggesting a circadian rhythm in the cycle of activity-resting of *D. melanogaster.* Tononi's research group tested the effect of the alkaloid caffeine dissolved in water flies drank. Caffeine blocked the adenosine receptors inhibiting sleep; flies that drank several concentrations of caffeine had an evident suppression of the sleep-like stage (Shaw et al., 2000). These results suggest that the sleep-like stage of fruit flies shares similar neurochemical mechanisms of sleep regulation to birds and mammals, despite lacking a complex nervous system. Hence, it seems logical that organisms such as amphibians, reptiles, and fish have a sleep stage.

Studying Sleep in Different Organisms

In general terms, sleep is defined as a behavioral state of immobility characterized by a specific stereotypical posture in each specie. This state is accompanied by changes in the electrical activity of the cerebral cortex and can be reversed by external stimulation (Rattenborg and Amlaner, 2002; Roth et al., 2010; Hartse, 2011). In mammals, specific electrical activity rhythms are associated with a particular behavioral stage of sleep. For example, the stage without rapid eye movements (NREM) is characterized by a cerebral rhythm with waves of 0.5-4 Hz of frequency and amplitude of 200 V. Simultaneously, muscular tone decreases, and ocular movements are absent. On contrary, the phase of rapid eye movements (REM) presents an activity with rapid waves above 16Hz, inhibiting antigravity muscles and rapid eye movements. Both sleep phases are well known in birds and mammals; however, the presence of sleep electrophysiological patterns in other vertebrates as fish is still in debate, and the controversy is more significant in the case of invertebrate organisms.

Not all elements that define sleep can be identified in all species. However, if we define sleep as the quiescent behavioral state with a reduced response to external sensorial stimuli, all vertebrates and invertebrates present sleep. Simpler organisms such as metazoos with a nervous system similar to cnidarians also have a behavioral sleep. In consequence, small laboratory organism as zebrafish (*Danio rerio*), fruit fly (*Drosophilia melanogaster*), and the nematode *Caenorhabditis elegans* show behavioral patterns consistent with sleep behavior.

Sleep in fish

The technical limitations to scan electroencephalogram (EEG) and electromyogram (EMG) parameters in fish and simpler organisms are not evidence of the inexistence of electrophysiological sleep parameters like those seen in birds and mammals. Nowadays, there is a need for more scientific instruments to score brain activity in aquatic environments. In reptiles, amphibians, and fish it is reported a "sleep-like" stage characterized by a decrease in motor activity and a decreased response to external stimulation (Rattenborg et al., 2007; Lesku et al., 2009b; Roth et al., 2010; Volkoff, 2012). EEG studies in reptiles show inconsistent results, and it is hard to establish specific stages of sleep according to the brain´s electrical activity (Ayala-Guerrero, 1991; De Lera, 1994; Siegel, 2008).

In fish, the "sleep-like" stage is characterized by a prolonged inactivity period with a typical posture of resting frequently inside a refuge, accompanied by an increased sensory threshold and a circadian rhythm. In 1976,

Shapiro observed that *Oreochromis mossambicus* spent the night at the bottom of the tank with a low respiratory frequency and without ocular movements in captivity. Additionally, the fish had a decreased response to palatable stimuli at night (Shapiro, 1976). Karmanova and collaborators observed that *Ameiurus nebulosus* fish had a typical posture of resting with the caudal fin extended on the substrate, pectoral fins stretched out, and the body inclined 10 to 30 degrees from the vertical axis; respiratory and heart rates were slow, and fish were less perceptive to sound and tactile stimulation, researchers could slightly touch the head of fish with crystal sticks without interrupting the "sleep-like" stage (Titkov, 1976).

Nowadays, we know about 30 000 species of fish, and approximately only 10 species have been studied under laboratory conditions to explore sleep (Siegel, 2008). Experiments with zebrafish (*Danio rerio*) have shown circadian variations in the responses to external stimuli after a privation of the "sleep-like" period (Yokogawa, 2007; Zhdanova, 2007). It has been explored if "sleep-like" privation during resting time in fish produces a compensation of subsequent sleep-like time as occur in mammals. Results are controverted since some groups find this compensation (Yokogawa, 2007), while other research suggests it does not exist (Zhdanova, 2007). These contradictory observations will have to be reviewed, and the validity of the results will depend on new technologies for scoring.

Interestingly, studies on blind cave fish (*Astyanax mexicanus*, figure 1c) that inhabit the caves of northwest México have shown exciting results on the origin and evolution of sleep along species. *A. mexicanus* has relatively small or no eyes at all and lacks skin pigmentation (Borowsky, 2008; Gross and Wilkens, 2013). It is noteworthy that this fish respond to vibrations, develops hyperphagia and loss of "sleep-like" pattern (Aspiras et al., 2015; Duboué et al., 2011; Kowalko et al., 2013; Yoshizawa et al., 2010). It has been observed that *A. Mexicanus* "sleep-like" time increases during long periods of starvation, which suggest the possibility that cavefish suppress sleep during the rainy season to feed when food is abundant while increasing sleep to preserve energy during the dry season when food is less abundant (Jaggard et al., 2017). Additionally, the ablation of the lateral line of cavefish increases sleep, suggesting that sensory stimulation is necessary to maintain active stages. Thus, cavefish´s sleep loss seems to be an evolutive consequence of the variations in food availability, which offers an opportunity to study the interactions between sleep and metabolism. According to Siegel (2005), food availability and foraging are potent boosters of evolutive change that contribute to variation in sleep duration among species.

The number of neurons positive to orexin (ORX) in the hypothalamus is increased in blind cavefish. On the other hand, pharmacological or genetic inhibition of ORX signaling increases sleep in fish, which suggests that ORX signaling is a conserved element in the evolution of the nervous system and essential to inhibit sleep. Similarly, ablation of the lateral line or low availability of food inhibits ORX expression suggesting that the absence of sleep is a consequence of the high activity of the orexinergic system in blind cavefish (Jaggard et al., 2018). In mammals, administration of ORX induces arousal, increases motor activity, and reduces REM and NREM sleep (Hagan et al., 1999), while mutations in the ORX gene or the loss of orexinergic neurons is associated with an increase in daytime sleepiness (Chemelli et al., 1999; Nishino, 2011). Together with the current scientific literature on fish, these findings suggest the utility of several species as zebrafish as an essential tool in the research of the neurobiological basis of sleep.

Studying Anxiety in Fish

As cold-blood animals, fish were believed to lack emotions. However, as mentioned above, behavioral research has shown that these species can experience pain and emotions and even display stress-related behaviors. In this sense, defensive behavioral responses exhibited by several species as zebrafish (*Danio rerio*), guppies (*Poecilia reticulata*), and goldfish (*Carasius auratus*) to cope with aversive stimuli, are used as indicators of the emotional state of fish (Maximino et al., 2007). Behavioral models of anxiety in zebrafish expose fish to stressful stimuli such as novel environments, alarm substances, illuminated or dark places, and images or dummies of potential natural predators (Cianca et al., 2013); see Table 1.

Table 1. Behavioral models of anxiety in zebrafish

Model	Characteristics	Interpretation	References
Light/Dark Test	Rectangular tank divided into two parts by a central chamber half of the device is white and the other half is black.	Fish are placed in the central chamber for 5 min and then allowed to explore the entire tank for 10 min. The time exploring the light and dark compartments is measured. The latency to leave the central chamber is also measured as an anxiety indicator.	Maximino et al., 2007, 2010, 2011

Black/White maze	Cross-shaped Tank with two arms and a central square area. The long arm is black whereas the short arms are white near to the center, and the rest is gray.	The number of transitions between arms, and the time spent in the white and central compartments is scored. Like rats, zebrafish avoid light, a behavior that decreases with anxiolytic drugs.	Guo, 2004 Sackerman et al., 2010
Novel tank test	The tank is a trapezoid divided into two equal parts by a virtual division. Fish is individually placed into the tank, and variables as the latency and time of exploration of the upper half of the tank, the number of crossings to the upper half, erratic movements, time and frequency of freezing are measured (5-6 min test).	Zebrafish seek protection in novel environments by staying on the bottom of the tank until they feel safe to explore the entire tank. Anxiolytic substances decrease the latency and increase the exploration of the upper parts of the tank.	Egan et al., 2009 Bencan et al., 2009
Predator stimuli	Consists of a rectangular tank with an LCD computer monitor placed at the left and right walls. The monitor allows video presentation of the natural predators of zebrafish, such as gangetic leaf fish (Nandus nandus, figure 1d) and freshwater garfish (Xenentodon cancila). Bird silhouettes can also be displayed by a monitor above the tank. A robot that mimics the shape and movements of gangetic leaf fish can be used in a rectangular tank divided into two separate sections.	The test consists of a habituation period followed by a pre-stimulus period and stimulus period of 5 min each one, during which images are projected eight times, ending with a post-stimulus period (4 min). The time in the upper zone of the tank is considered an indicator of the emotional state. During the test, the fish-shaped robot is placed in the right compartment, and the distance between zebrafish and the robot, and erratic movements are measured as anxiety indicators.	Gerlai et al., 2009 Luca and Gerlai, 2012a,b Cianca et al., 2013
Alarm substances	Test is performed in a rectangular tank visually isolated in all directions except the front. Alarm substances are obtained from superficial cuts on the skin of dead zebrafish. The solution of alarm substances is poured into the water after a habituation period.	Unidentified substances are secreted through skin wounds which are detected by fish and trigger defensive behaviors as erratic movements, freezing, motor activity, and shoal cohesion (when tested in a shoal).	Bass and Gerlai, 2008 Spedie and Gerlai 2008

Table 1. Shows the main behavioral models of anxiety using zebrafish, which have been adapted from validated rodent models.

Throughout the reviewed models, specific behaviors are measured that are sensitive to pharmacological manipulations with anxiolytic drugs used in the therapeutic to treat anxiety disorders in humans. The behaviors evaluated in these models and their interpretations are compared with equivalent hypothetical functions in the natural environment. For example, swim cohesion is considered an anti-predator response since fish schooling is difficult to track by predators because the movement of all fish impairs predator attention. Additionally, schools are useful as zones of feeding and mating (Speedie and Gerlai, 2008). The time on the bottom of the tank (bottom-dwelling) is similar to hiding from aerial predators by avoiding the water surface in wild zebrafish. Staying on the bottom allows fish to mimic substrates in their environment and avoid illuminated areas (Luca and Gerlai, 2012a). Additionally, erratic movements are displayed by fish on the bottom of the tank, slightly oriented downward and moving in a way in which they appear to be trying to penetrate the bottom of the tank. Wild zebrafish (figure 1e) live in small water streams and lakes with steady currents. Erratic movements agitate sediments and vegetation at the bottom to cloud the water, so erratic movements are an adaptive anti-predator response to escape (Speedie and Gerlai, 2008). Finally, erratic movements are often followed by periods of freezing. Under natural conditions, this freezing behavior can be useful for blending in with substratum debris previously disturbed and clouds the water (Speedie and Gerlai, 2008). The models in which these behaviors are scored are validated with substances that have anxiolytic effects at the clinical level, and that reverse the behaviors measured in zebrafish (Van der Stay, 2006).

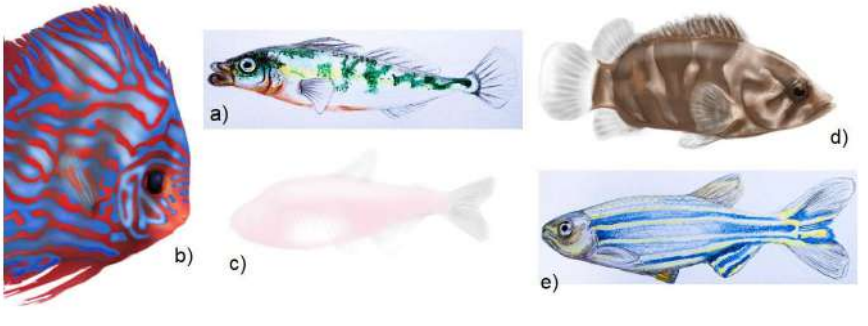

Figure 1. Fish used in scientific research. The figure shows five species of fish used in scientific research among the several species studied in the literature. a) *Gasterosteus gymnurus*, b) *Symphysopdon aequifasciatus*, c) *Astyanax mexicanus*, d) *Nandus nandus*, e) *Danio rerio*. (Original images from authors).

Anxiety models in several species, including fish, have contributed to understanding the neurobiological and neurochemical substrate of anxiety as a natural emotion and as a psychiatric disorder triggered by stress (de Abreu et al., 2021). Nowadays, fish models can be used to explore potential substances with anxiolytic effects, understanding their pharmacological mechanisms and toxic effects, in the same individual or their offspring, given the short lifespan.

Summary

Fish are ideal model species to explore the evolutive processes underlying sleep´s origin. Sleep definition could be amplified to circumscribe beyond electrophysiological elements described in birds and mammals. The ubiquity of sleep among living things suggests that sleep has evolved parallel to nervous system evolution. The absence of technological tools to identify sleep stages slow the study of sleep in these organisms. However, in the future, fish will be used to understand sleep disorders such as narcolepsy and excessive daytime sleepiness, as zebrafish are being used to explore anxiety and emotions. Additionally, the study of anxiety in fish will allow us to explore the evolutive aspects of emotions but also the neurobiological and neurochemical substrate of anxiety and anxiolytic drugs (de Abreu et al., 2020 et al., 2021), including new molecules with potential therapeutic properties (Cueto-Escobedo et al., 2022) that contribute to human well-being.

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VII

Use of animal models for the study of spinocerebellar ataxias

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Introduction

Hereditary dominant spinocerebellar ataxias (SCAs) belong to a group of neurological diseases characterized by the loss of cerebellar gray matter and, therefore, lack of motor control of gait and fine movements. In the first part of this chapter, we review how these neurodegenerative diseases are classified considering the common neurological symptoms that occur in patients and the findings of the loss of cerebellar and cerebral gray matter observed in the several types of SCAs. Currently, with the help of new molecular biology techniques and together with the use of cell and animal models, SCAs have been reclassified and now, depending on the affected gene and the order in which they were described, they have been renamed. In the second part of the chapter, we review how murine models of SCAs have helped to elucidate the transcriptional deregulation of cells that overexpress mutated genes of SCAs, and how this induces neuronal death. In addition, we review how these models have been used to propose and evaluate new genetic and pharmacological therapeutic strategies. Finally, we discuss the advantages and disadvantages of these approaches and the opportunities that new molecular engineering techniques potentially offer to control cerebellar, cerebral, and retinal neurodegeneration in patients with several types of SCAs.

Spinocerebellar ataxias (SCAs, previously known as autosomal dominant cerebellar ataxias, ADCA) represent a clinically and genetically heterogeneous group of neurodegenerative diseases. SCAS were initially classified according to clinical and neuropathological descriptions. Subsequently, a classification depending on the mode of inheritance and clinical signs (ADCA I-III) was suggested. Currently, the numbering of the SCAs corresponds to the order of description of the modified gene that causes the death of cerebellar neurons (SCA1–SCA8, SCA10–SCA23, SCA25). There are

also ataxias named by the gene that encodes the mutation, e.g., the growth factor of fibroblasts 14 (FGF14) or by their anatomy e.g., dentate-rubro-pallidum-Louisiana atrophy (DRPLA) (Verbeek and van de Warrenburg, 2011). This molecular classification is currently the most accepted by the medical and scientific community.

The prevalence of SCAs in the world is between 1 to 4 people per 100,000 inhabitants (Warrenburg et al., 2002). Patients typically present with a slowly progressive cerebellar syndrome with various combinations of oculomotor disorders, dysarthria, dysmetria/kinetic tremor, and/or ataxic gait (Manto et al., 2020). Symptoms can also occur in combination with pigmentary retinopathy, extrapyramidal movement disorders (parkinsonism, dyskinesias, dystonia, chorea), pyramidal signs, cortical symptoms (seizures, cognitive impairment/behavioral symptoms) and, peripheral neuropathy (Manto, 2005).

The most common forms of SCAs are: SCA1, SCA2, SCA3, SCA6 and SCA7. SCA3 could be the most frequent SCA in the world. The average onset of symptoms of SCAs occurs between the ages of 30 and 40 years. However, there is large variability between the different subgroups of SCAs. For example, symptoms of SCA1, SCA2, SCA3, SCA7, SCA8, SCA12, SCA13, SCA17, or SCA25 may begin in the first ten years of life, whereas in SCA6, symptoms may appear around the age 65 (Schöls et al., 2004).

Patients with SCAs present with a cerebellar syndrome that includes a disorganized and uncontrolled gait (the main ataxic sign), postural ataxia, dysmetria and/or kinetic tremor in all four limbs, as well as oculomotor deficits (nystagmus, hypermetria/hypometry of saccades). As a result of a degenerative process, gray matter loss is not limited to the cerebellum, but extends to the retina, optic nerve, brainstem, basal ganglia, cerebral cortex, spinal cord, and nervous system. peripheral nervous system and extracerebellar signs are often associated.

Challenges in diagnosing SCAs

Two main factors difficult clinical diagnosis: 1) That, within a genetic subtype, the clinical signs are highly variable and 2) That there is a surprising phenotypic overlap between the several types of SCA. Magnetic resonance imaging is used to aid in clinical diagnosis and has shown that ataxias have different patterns of atrophy. A pure cerebellar atrophy can be observed in SCA4, SCA5, SCA6, SCA8, SCA9, SCA10, SCA11, SCA14, SCA15, SCA16, SCA18, SCA21 and SCA22; while an olivopontocerebellar atrophy pattern is seen in SCA1, SCA2, SCA3, SCA7 and SCA13, or a pattern of global brain atrophy in SCA12, SCA17, SCA19 and DRPLA (Gandini et al., 2020; Manto et al., 2020;

Manto and Marmolino, 2009; Cloak, 2005). An interesting finding is the presence of cerebellar nuclei calcifications in SCA20 (Knight et al., 2004).

Most SCAs begin with cerebellar atrophy that can later progress to extracerebellar structures. As a result of the marked phenotypic variability of SCAs, even within the same family, diagnosis cannot be based solely on the evaluation of clinical symptoms and a detailed genetic search is required to confirm the disease and the affected gene.

Molecular features

In the last two decades it has become clear that most SCAs are caused by an expansion of trinucleotide repeats. Some SCAs are caused by a CAG expansion encoding a polyglutamine repeat, e.g., SCA1, SCA2, SCA3, SCA6, SCA7, SCA17, DRPLA. In SCA12, the CAG repeat expansion does not encode a polyglutamine stretch, and in SCA8 a CTG repeat is not translated (Knight et al., 2004; Schöls et al., 2004). Furthermore, there is an inverse correlation between the length of repeats and the age of onset. A main characteristic of SCAs is the anticipation phenomenon, which consists in that the descendants of sick parents tend to inherit a more extensive expansion of the CAG trinucleotide, thus presenting the symptoms of the disease in the first decade of life.

SCAs can also be caused by an expansion of triplet repeat sequences (CAGs) in the CACNA1A gene (19p13) encoding an alpha 1 subunit of a P/Q-type voltage-gated calcium channel (Prestori et al., 2019); or by an ATTCT repeat sequence in a non-coding region of the genes associated to SCA8 and SCA10 (Aydin et al., 2018); or by expansion of CAG or CAA repeats in the genes encoding transcription factors associated to SCA12 and SCA17 (Weber et al., 2022).

It is important to note that there are dominant hereditary diseases that are phenotypically remarkably alike SCAs, e.g., episodic ataxias (EA 1-5), hereditary spastic paraplegia, Huntington's disease, essential tremor, hereditary sensory-motor neuropathies (Jen, 2008; Riant et al., 2008; Tabrizi et al., 2019). Autosomal recessive disorders, mitochondrial diseases, X-linked diseases (Zhang et al., 2022), and even sporadic diseases can also mimic the SCA phenotype. Leukodystrophies, mitochondrial cytopathies such as Kearns-Sayre syndrome, MERRF, MELAS, NARP, or Leigh syndrome; as well as Friedreich's ataxia, fragile X syndrome, progressive myoclonus and epilepsies and multiple system atrophy (MSA) must be considered before diagnosing a SCA (Manto et al., 2020).

Even when family history may not indicate a risk for the patient to present a SCA, it is important to consider that there are certain types of SCAs

that show a late onset of ataxia symptoms, as is the case of SCA6 (Solodkin & Gomez, 2012). In addition, the phenotypic variation of pyramidal or extrapyramidal signs that could mask ataxia in any family members should be considered. Lastly, a possible false paternity should be considered.

SCA treatments

Treatments for SCAs are currently a subject of study in the medical and scientific community. Even though some benefits have been reported on the relief of ataxia in SCA6 patients when they are medicated with drugs such as 5-hydroxytryptophan or buspirone and acetazolamide (Takei et al., 2005; Underwood and Rubinsztein, 2008), or a decrease in extracerebellar symptoms of patients with SCA2 and SCA3 when medicated with amantadine, levodopa, and dopaminergic agonists (Park et al., 2015), the benefit with pharmacological treatments is still limited.

The non-pharmacological treatment with thalamic deep brain stimulation suppresses tremor in patients with SCA2 (Pirker et al., 2003). In addition, regular physical therapy in all cases of SCAs is recommended. Clinical reports show a transient improvement in gait control and hand tremor, both in patients following an exercise regimen and in those using orthosis (Freund and Stetts, 2010; Serrao et al., 2017). However, the progression of neurodegeneration persists, forcing patients to use a wheelchair and to depend entirely on the primary caregiver.

Use of animal models in the study of SCAs

To understand the molecular and pathophysiological mechanisms of SCAs, the use of genetically manipulated animals to emulate human diseases is essential. In the field of SCA research, murine models are the most widely used, both to emulate the distinct types of SCAs in order to test the effectiveness of new genetic therapies proposed to inhibit the neurodegeneration induced by the accumulation of mutated SCA proteins and, to test whether cell therapy using stem cell transplants promotes the generation of new neurons to replace the lost ones (Xia et al., 2004). A special emphasis has been placed on preventing the anomalous folding and aggregation of mutated proteins, through the overexpression of chaperonins such as HSP70 or the application of histone deacetylase inhibitors such as divalproex sodium in affected neurons (Cummings, 2001; Wang et al., 2018).

Transgenic mice as animal models for SCAs

Developing transgenic murine models to study SCAs has turned out to be quite a challenge, because as part of the methodology it is necessary the in-

jection of a linear DNA sequence that contains the promoter region of the SCA gene to be modeled, as well as the gene sequence with the expansion of the desired gene. The animals generated with this methodology have two problems. The first is that the levels of protein expression are so high that the animals die in utero. The second problem is the opposite effect, i.e., the mutant protein has an extremely low expression, causing that neither the phenotype nor the signs sought for the SCA of interest can be observed, or alternatively, that the first signs of the disease take months to appear.

To avoid these technical problems, researchers have developed new models using conditional transgenic mice. In these animals it is possible to regulate the expression of the gene of interest either by overexpressing it, lowering the expression levels or suppressing the expression altogether. Regulation is achieved through the presence or absence of an enzyme, drug, or hormone analog, which is supplied to the transgenic animal. This strategy has been used to model SCA3 by using the CamKII gene promoter and the MJD77 gene to emulate the characteristics of the disease seen in humans. This model showed for the first time that the cerebellar expression of the expanded ATXN3 protein is not essential to induce a neurological phenotype, since the neurons of the forebrain are more sensitive to the toxicity of this mutated protein and that sensitivity is enough to generate an ataxic phenotype (Schmidt et al., 2019).

In combination with the use of the drug doxycycline, researchers have developed conditional transgenic mice that display a progressive neurological phenotype, characterized by hyperactivity, impaired motor learning, weak performance on the rotarod test, reduced body weight, and generation of intranuclear inclusions in neurons, all these features found in human patients with SCA3 (Schmidt et al., 2019).

Using in vivo magnetic resonance spectroscopy (MRS) in a conditional mice model of SCA1, neurotransmitters and metabolites concentrations during neurodegeneration have been estimated in a non-invasive way (Öz et al., 2015). Transferring this technology to clinical trials could help to evaluate the effectiveness of new therapies in preclinical and clinical trials.

Knock-in mice (KI mice) have also been used for the study of SCAs. In KI mice, the sequence of the gene of interest is modified by one-for-one substitution with a transgene, or by the addition of a gene sequence not found within the locus (insertion). Models of SCA1, SCA3 and SCA7 have been generated using this technique (Fusco et al., 2021; Haas et al., 2022). However, the main disadvantage of these models is that, by using the endogenous promoters of the genes of interest the expression is weak compared to that of the conditional transgenic mice. An example of this effect was found in

KI Sca1(78Q) mice, where the mice had a mutated allele containing a 78-repeat CAG expansion in the SCA1 gene. However, even though this expansion is considered toxic, the mice did not present a severe ataxic phenotype, only slight coordination problems. Furthermore, when the mice reached an age of 24 months and their brains were analyzed histologically, not significant change from the control group was observed (Lorenzetti et al., 2000). However, more recent studies show that KI mice with the ATXN1(154Q) gene display a typical and progressive ataxic phenotype. These mice also display a change in mood that can be quantified, indicating that mutated expression of the ATXN1 gene contributes directly to the levels of anxiety and depression present in patients with SCA1 (Huang et al., 2022).

Use of lentiviruses in the generation of SCA models

Lentiviruses belong to the Retroviridae family of viruses and are capable of transfecting dividing cells and cells that are not actively dividing, such as neurons. This characteristic distinguishes them from other retroviruses (Maes et al., 2019). Lentiviruses are made up of a reverse transcriptase protein which converts RNA into double-stranded DNA, and of a DNA integrase protein responsible for integrating the double-stranded DNA to the host genome. These components constitute the viral core, which is also surrounded by an envelope generated during the budding process of the host cell membrane. The use of lentiviruses to express transgenes in the central nervous system has increased in the last ten years. In the study of SCAs, lentiviruses have been used to transfect the cerebellum of mice and thus emulate SCA14. In this case, the lentiviruses were used as vector to introduce and overexpressed in Purkinje cells a mutated version of the protein kinase C gamma (mutant PKC). This manipulation changes the membrane translocation kinetics of native PKC . The presence of mutant PKC , interfers with synaptic pruning processes in postnatal day 6 (P6) and with synaptic plasticity and transmission in P21 compared to the control group (Shuvaev et al., 2011). These alterations may be the molecular mechanism for SCA14 pathogenesis.

Lentiviruses have also been used to generate a model of SCA3 in rats, aimed to test whether the expression of a mutated version of ATXN3 in the substantia nigra – a region affected in patients with SCA3, and in cortex and striatum –regions that have not been reported as affected in patients with SCA3, participate in the etiology of the neurological symptoms seen in humans. The results show that the injection of lentiviruses carrying the mutated version of ATXN3 into the three regions cause neurodegeneration, suggesting that neurodegeneration of such regions could be involved in the

etiology of symptoms such as dystonia and chorea occurring in some patients with SCA3 (Boy et al., 2009).

Another example of the use of lentiviruses to generate an ataxia model is that of SCA7. In this case, the researchers constructed and injected a lentivirus with the truncated version of the ATXN7 gene (100Q) into the cerebellum of adult mice. The expression of this protein induced cerebellar histological changes like those found in postmortem analyzes of brains from patients with SCA7. Among the findings are intranuclear aggregates in Purkinje cells (PC), loss of synaptic markers, neuroinflammation and neuronal death. In addition, a correlation between the nuclear inclusions of ATXN7 and the nuclear accumulation of specific proteins that bind to RNA, such as the FUS/TLS and MBNL proteins was found. The accumulation of these proteins in the nucleus suggests that they interfere with alternative splicing processes of multiple mRNAs, causing neuronal death (Alves et al., 2016).

Adeno Associated Viruses (AAV) for generation of SCA models

AAVs are part of the Parvoviridae family and are small, non-enveloped viruses. AAVs establish a latent infection and maintain an extrachromosomal genome, where they can replicate autonomously (Maes et al., 2019). Recently, AAV strains have been created and selected such as that, when injected into the bloodstream, can cross the blood-brain barrier and effectively transfecting both glial cells and neurons (Maes et al., 2019). Using this technology to overexpress the mutated ATXN3 gene (89Q) under the specific control of a neuronal promoter in adult mice, it has been possible the creation of a mouse model of SCA3 disease. In this model, a decreased expression of the orphan retinoid-related receptor protein a (RORa) was observed (Watanave et al., 2019), causing the degeneration of Purkinje neurons, as well as a motor deficit. The importance of RORa is because this protein can regulate the transcription of at least 6 brain transcription factors (Sarachana and Hu, 2013).

AAVs have not only been used to insert mutated ataxin gene sequences. Currently, they are used to knock out the expression of proteins known to change their expression levels in the presence of mutated ataxins and that are critical in activating neuronal death processes during the disease and for the appearance of signs. For example, in adult mice, the injection of AAVs encoding RNA interference (RNAi) that prevents or decreases the expression of the RORa protein, – a protein that changes its expression levels in the presence of the ataxin-3 protein, causes Purkinje neurons death, motor learning deficits, and cerebellar ataxia. Hence, it is confirmed that this

protein plays a critical role in the death of Purkinje neurons, which gives rise to ataxic symptoms in SCA3 (Yasui et al., 2021).

Gene therapy in cellular and animal models of SCAs

Gene therapy provides an important means for repairing mutated genes associated with polyQ expansion diseases. For example, RNAi and antisense oligonucleotides (ASO) are potentially effective therapeutic strategies to treat SCAs, either by inhibiting mRNA expression, eliminating CAG expansions through selective methods, or by silencing mutated ataxin genes (Kotowska-Zimmer et al., 2020; Xia et al., 2004). Recently, a cellular model of differentiated human neurons has been developed. This model is based on the use of fibroblasts from patients with SCA3 to produce human induced pluripotent stem cells (hiPSC) (Fang et al., 2021). By using this cellular model and the CRISP/CAS9 gene technology, it has been possible to correct the abnormal CAG sequence (74 CAG) and replace it with normal repeats (17 CAG) by homologous recombination. With this manipulation, the corrected SCA3-hiPSCs retained the normal pluripotent karyotype (He et al., 2021). In addition, the correction allowed these cells to become neural stem cells, to later differentiate into neurons, which express neuronal markers and electrophysiological characteristics like normal hiPSC-derived neurons (He et al., 2021).

Because the physiological function of many of the ataxins with normal polyQ sequences and the role they play throughout human ontogeny is unknown, the safest therapeutic strategy to treat the overexpression of mutated genes in patients with SCAs should be preferentially to modulate the expression of mutant variants, leaving normal proteins expression intact. To selectively silence mutated alleles, researchers have used the single nucleotide polymorphism (SNPs) linked to repeat expansions. However, this approach has limitations, since the RNAi directed towards these SNPs must be personalized for each patient. A strategy that enables therapy in a broader group of patients requires detection of the PolyQ length of both normal and mutated alleles. Single-stranded oligonucleotides potently and selectively silence the expression of abnormal forms of the alleles of mutant genes (Vázquez-Mojena et al., 2021).

Some other approaches include the use of synthetic molecules such as peptide-nucleic acids, which are polymers like DNA and RNA with the particularity that they have the chemical stability of a protein and can store information like DNA molecules. These molecules are known as ANPs. The interaction between these single-stranded synthetic molecules with the DNA strands is usually very stable. However, given their hydrophobic nature,

they are difficult to introduce into cells. To overcome this disadvantage, RNA analogous molecules have been used, which contain a methylene bridge between the 2'-O and 4'-C positions of ribose and are also known as locked nucleic acid (LNA). This chemical modification allows thermal stability of the oligonucleotides. In contrast to the neutral amide backbone of ANPs, LNAs have a negatively charged phosphodiester in their structure, allowing cationic lipids to introduce these molecules into cells. LNA-like oligomers are currently being tested in clinical trials (Hu et al., 2009). Nanobiotechnology in combination with thermostable LNAs are the future of therapeutics in genetic therapy.

Summary

The clinical characteristics of patients with SCA are highly heterogeneous. To provide neurologists with a guide to identify the type of ataxia that the patient suffers from the clinical symptoms that him/her display, SCAs have been grouped into two major subtypes. The first group is that of pure ataxias, in which ataxia is the first sign displayed in the patient at the onset of the disease, e.g., SCA12, SCA10, SCA31 and SCA6. A second group is that in which multiple signs such as visual loss, parkinsonism, hearing loss, chorea, or dystonia, among other symptoms appear simultaneously, or earlier than the sign of ataxia (Rossi et al., 2014; Schöls et al., 2004). Currently, the diagnosis of which subtype of ataxia a patient suffers is determined by the result of a PCR test, aimed at identifying the SCA gene that is suspected to affect the patient, as well as the estimation of the expansion of the CAG triplet repeat in the alleles. The main reason why genotyping is the only way to determine the type of SCA is due to the wide overlap of clinical symptoms that exists between the different subtypes, as well as the limited clinical information available for each subtype. Currently, molecular analysis is the only alternative for diagnosis (Manto et al., 2020; Rossi et al., 2014; Schöls et al., 2004). To offer more tools for diagnosis, the use of artificial intelligence is warrant. In order to build a clinical diagnostic algorithm that narrows the identification of the type of SCA to be treated, it is very important that clinical reports describe in an extensive and detailed manner the symptoms and signs of patients for each of the non-pure forms of SCA.

Molecular clinical reports and research in SCA animal models have confirmed the importance of knowing the size of the PolyQ expansion, since it is related to the severity of clinical symptoms, the time of onset of the disease and its prognosis. Despite this, currently most laboratory tests only estimate if one of the alleles of the genes suspected of being mutated contains a longer expansion than that of the non-mutated gene, and only a few labora-

tories report the number of repeats of CAGs that contains the mutated allele. This is due to the difficulty for sequencing the nucleotide-rich regions of CAGs. Until now, since SCAs are considered rare diseases, there is little interest in accurately determining the length of polyQ expansions in molecular diagnostics in the clinic. However, knowing this data in the diagnosis of patients is relevant, as can be inferred from scientific reports using models of SCAs in mice in which the effectiveness of potential gene therapies could vary depending on the length of the polyQ expansion.

Most of the genes responsible for the generation of the distinct types of SCAs have been identified in the last two decades. However, in several cases, the physiological role that the proteins encoded by these genes play in the metabolism of neurons and other cell types is unknown. The search for these functions in both health and disease are a matter of current research, but there is still a long way to go in this regard.

Currently, new gene therapies allow us to reduce or silence expression of the mutated genes associated with SCAs in vivo using viral vectors, or to edit the abnormal versions of the genes using CRISPR/CAS9 technology in murine models of different ataxias, and more recently, in humans. However, the secondary effects of the insertion of exogenous genetic material, the stability of the RNAs that suppress the transcription of abnormal genes, the tolerance to their transcription over time (potentially for decades), and other secondary effects in genetically intervened patients are still completely unknown. These questions will only be answered over time and with a wider use of this technology in humans.

The use of murine models for the study of the different SCAs has limitations. For example, in some cases the levels and patterns of expression of ataxin gene mutants in the brains of distinct species vary. This can directly influence the severity and type of the neuropathological manifestation that is induced, distorting the results observed in the SCA model for each specie used. Therefore, it is important that scientific reports always warn of this problem, and interpret the results obtained in animal models with caution.

Lastly, it is important to mention that animal models of SCAs are not limited to the use of vertebrates. In the scientific literature, the use of invertebrate animals has been widely reported. The relative ease for breeding and genetically manipulating these animals with the anomalous versions of the genes of the different SCAs, allows not only to induce motor problems like those observed in humans, but also to evaluate and quantify them objectively. In addition, since the complete genetic sequence of these animals is available, it is possible to accelerate the identification of the proteins that are affected in the presence of the mutated version of the gene associated

with some SCA, and that, potentially, also participate in the neurodegeneration process during the illness.

The identification of potential target proteins for the treatment of SCAs in invertebrate animal models, in combination with new cell models derived directly from patients with distinct types of SCAs, will allow proposing new genetic strategies to correct abnormal polyQ expansions in vivo and discover new drugs that relent the progression of SCAs

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VIII

Rat and human mothering: A translational perspective

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Introduction

This review describes the history, experiences, outlook and research of two scientists who started their research careers working on the neuroscience of maternal and associated behaviors in a female animal model, followed by studies of the psychobiology and neuroscience of maternal behavior in humans, especially new mothers. Alison S. Fleming, now Emerita from the University of Toronto, and retired in México as a painter and a sculptor and Mayra L. Almanza-Sepulveda, a recent postdoctoral fellow, who worked with Alison for many years and is on Psychology faculty at Universidad La Salle Bajío, León, México.

Alison: My training in Behavioral Neuroscience started at the Institute of Animal Behavior (The IAB), an Institute at Rutgers University started by Daniel S Lehrman and overseen for many years by his colleague and my mentor, Jay S Rosenblatt. Being trained in the era of the ethology of Niko Tinbergen and developmental perspectives of Robert Hinde, Zing Yang Kuo, and Gilbert Gotleib, our approach at the IAB was to first understand the components and characteristic of the species-characteristic behaviors, and then to explore the interactive effects on their expression of environmental and physiological influences. At the time of my work at the IAB when I explored the behavioral and physiological mechanisms underlying the expression of maternal behavior in a rat model, I explored the effects of central and peripheral lesions of the olfactory system on maternal behavior, as a function of the parity and experience of the females (virgins vs primiparous vs multiparous mothers). This interest in the additive and interactive role of maternal experience and of hormones on maternal brain and behavior has guided all my work and was initiated in studies of the rat model of mothering. Later in my career, I started my studies of human maternal behavior.

Mayra: My training in Behavioral Neuroscience started at the Institute of Neuroscience of The University of Guadalajara, at the EEG correlation

and behavior lab with Dr Miguel Guevara. We were interested in investigating the physiological bases of cognition and their association with motivated behaviors. While initially we focused on sexual behavior in the rat model, in subsequent studies, I have explored the role of cognitive function and brain in association with human mothering. Maternal behavior and sexual behavior are among the two most highly motivated hormonally affected behaviors that trigger activation and modify brain reward circuitry in both animal models and humans. In our studies with rats, we aimed to determine and established that there occurs greater interhemispheric transfer in the medial prefrontal cortex (mPFC) in sexually motivated male rats who perform better on a working memory task that utilizes sexually-specific rewards. Our findings with animal models showed that the incentive value of sexual interaction, used as a rewarding stimulus, facilitates working memory responses in rats. Asking a similar question, among human mothers, the incentive value of an interaction with the baby has also been associated with enhanced brain activation and increased executive function.

With a focus on determinants of maternal behavior, the basic strategy we adopted in our laboratory has been to first describe in the rat model, the components of maternal behavior and the major psychological changes that are associated with its expression at the time of parturition; then consider how each of the hormones that undergo change during pregnancy, parturition, lactation, and weaning affect those maternal and the associated behaviors. We explore the role of sensory factors, especially olfaction, and of experience in the regulation or effect on mothering. These include experiences of the new mother postpartum that ensures the maintenance of maternal behavior when hormones are no longer effective ('maternal memory') and also the longer-term effects on mothering of mothers' earlier experiences during their neonatal and juvenile periods. Finally, we explore the role of different brain systems and their interconnection in the regulation of both hormonally and non-hormonally mediated maternal behavior. In recent years we have extended this work to consider how the mothers' genotype influences the action of both hormones and experience.

The aim of this chapter is to describe the benefits of translational research where studies of the rat model have furthered our understanding of the regulation of human maternal behavior and where we can describe species' similarities and their differences. We first describe some of the methodological issues in the study of animal and human mothering, followed by examples predominantly from our lab, but also from the wider literature, that illustrate this translational approach.

Methodology

Although research on the physiology of human parenting has been stimulated by animal work, it has depended on very different research strategies than those usually found in animal studies. Whereas animal work usually involves observing behavior in the laboratory under relatively controlled environmental conditions and in 'laboratory' (often inbred) animals with known and controlled prior experiences, human beings have heterogeneous backgrounds and experiences and, in general, are observed under quite varied conditions, as in the home or hospital setting.

With respect to measurement, maternal behavior in animals is usually clearly defined by the species-typical behavioral repertoire exhibited by adults in response to infants. In the rat, for example, this repertoire includes the nest building, pup-retrieval into the nest, licking of pups, – both general body and anogenital– adopting a nursing posture over pups and defending the nest and young from perceived 'intruders' (Lévy et al., 2015; Numan, 2020; Numan et al., 2006).The intensity of these behaviors is established through daily timed observations of mother rats with their own offspring or virgin rats with foster pups (Fleming and Sarker, 1990; Lonstein and Fleming, 2001). Some studies explore the rewarding value of pups by testing animals in a skinner box where the animal is required to bar-press for pups (Lee et al., 1999). In other cases, the speed and willingness for the animal to circumvent a barrier to reach pups is used as a measure of motivation (Morgan et al., 1992).

In human beings, by contrast, 'behavior' is assessed in a wider variety of ways: by assessments of physiological responses to infants and their cues, employing either autonomic measures or endocrine measures (see reviews: Almanza-Sepulveda et al., 2020; Feldman and Bakermans-Kranenburg, 2017; Saltzman and Maestripieri, 2011); by hedonic or recognition responses to specific infant cues (Fleming et al., 1987; Fleming et al.,1997; Saltzman and Maestripieri, 2011); by analysis of mothers' expressed feelings and attitudes measured by their responses in semi-structured interviews, or to attitude items on questionnaires (O'Donnell et al., 2014); or, by analysis of mothers' interactive behavior with their infants, employing either ratings of general attributes across different behaviors, such as maternal sensitivity, or coding of particular maternal behaviors (Ainsworth, 1979; Barrett and Fleming, 2011; Fleming et al., 1988; Jonas et al., 2015; Mileva-Seitz et al., 2011; Pederson and Moran, 1999). Sensitivity has been an ever-increasingly used measure of maternal responsiveness to infants; in most cases, it is rated on the basis of direct observations of mother-infant interaction. It has the apparent advantage of transcending particular motor patterns of maternal

behavior and thus would appear to offer more generality than some measures, but at the same time suffers from the problem of whether rating judgments can be made across different cultural contexts and across different infants.

There are also clear differences in the animal and human work in research design. Whereas the study of the physiology of parenting in animals tends to be invasive and involve surgical and other experimental manipulations, analogous human studies use correlational, non-invasive strategies. The role of a hormone in the regulation of mothering in rats is established first by assessing how behavior changes when the endocrine organ that produces it is removed and then by further changes in behavior with its subsequent replacement. This is how the roles of estrogen, progesterone, and prolactin were first established (Bridges, 1984; Lonstein et al., 2015; Stolzenberg and Champagne, 2016). Similar strategies are adopted when exploring where in the brain and on which receptors the hormone acts, with first a lesion of the site containing the relevant receptors or use of neurochemical or hormonal antagonists and then the intracranial replacement of the hormone into the brain. The role of oxytocin (OT) in the mix was clearly established in this way (Bridges, 1984; Lonstein et al., 2015; Pedersen, 1997; Pedersen et al., 1991; Stolzenberg and Champagne, 2016). Similar strategies are adopted when studying the role of brain neurotransmitters in maternal behavior. Dopamine and serotonin studies for instance, used this approach (Afonso et al., 2008, 2009 & 2011; Mileva-Seitz et al., 2011; Numan and Stolzenberg, 2009; Olazábal et al., 2013). Finally, sometimes in the rat model measurements of hormones and neurotransmitters are done and behavior is measured simultaneously (see review: Lonstein et al., 2015; Numan, 2020).

In contrast, in order to explore the role of hormonal factors in the regulation of human maternal behavior, studies may measure concentrations of biologically active hormones or their metabolites in blood plasma, saliva, or urine and correlate these with ongoing behavior (Fleming et al.,1997; Gonzalez et al., 2012; Gonzalez et al., 2009; Jonas et al., 2018; Worthman and Stallings, 1997). Alternatively, researchers may measure acute changes in hormones in response to the controlled presentation of stimuli or events, as in the presentation of cries or "stressors", or in response to the administration of other hormones or chemicals (for a review: Almanza-Sepulveda et al., 2020). Yet another strategy that has been adopted to determine the role of hormones in human behavior is to evaluate behavioral changes associated with natural changes in the reproductive cycle; for instance, across the menstrual cycle, puberty, the pre- and postpartum periods or menopause.

This strategy has been adopted when comparing pregnant with postpartum women, as in comparisons of cognitive function across the parturitional period (Pawluski et al., 2021). In some instances, as with the contraceptive pill, behavior may also be assessed in relation to the administration of exogenous hormones. (Bridges, 2020; Holtfrerich et al., 2016; Weisman et al., 2014).

Finally, in some cases where endocrine disorders exist, as with the adrenogenital syndrome (involving overproduction of androgens by the fetal adrenal gland) or testicular-feminization syndrome (where certain androgen receptors are absent), it is possible to compare the behavior of people suffering from the endocrine disorder with the behavior of siblings or other matched controls. This has been an approach when exploring issues of sex or gender differences (Hines, 2020). In general, the hormones that have been investigated most thoroughly in the context of parental behavior are the steroid hormones known to be elevated during human pregnancy or parturition and/ or known to play a role in parental behavior in other animals. In the female, these include the steroid hormones, estrogens and progesterone, produced by the placenta and ovaries; cortisol, produced by the fetal and maternal adrenal glands; and the protein hormones, prolactin and OT, produced by the maternal pituitary gland (Lonstein et al., 2015; Numan, 2020).

In addition to correlating endocrine profiles and responses with behavior, physiological states and responses to discrete stimuli, or during actual mother-infant interaction are assessed using indices of autonomic activity, including heart rate, blood pressure, and galvanic skin response (Donovan and Leavitt, 1985). In assessing parental responses, the presented stimuli are in general infant – related cues including – cries, odors, video images, and the infant itself. Different patterns of autonomic responses are assumed to have different meanings.

Finally, relative to the study of neuroanatomy of behavior in animals, which depends on invasive methodologies (including lesioning, stimulating and recording from neural structures deep within the brain), our understanding of functional neuroanatomy associated to maternal behavior in humans is based almost entirely on functional MRI and other non-invasive technologies.

Most of the maternal brain imaging studies focus on neural activation patterns to different infant stimuli, for example, in response to crying, words, videos, or images with emotionally salient infant stimuli generally compared own vs unfamiliar infant (Barba-Müller et al., 2019; Barrett et al., 2012; Bjertrup et al., 2019; Dudin et al., 2019; Noriuchi et al., 2008; Silverman

et al., 2011; Swain, 2008; Wonch et al., 2016). Stimuli are chosen to elicit maternal care-taking responses in mothers compared with non-mothers (Dudin et al., 2019; Gregory et al., 2015; Seifritz et al., 2003), mothers vs fathers (Ranote et al., 2004; Swain, 2008) or depressed versus non-depressed parents (Barrett et al., 2012; Dudin et al., 2019; Wonch et al., 2016) and so on. These studies have allowed us to identify, in humans, brain regions and brain circuitry that are associated with elevated maternal responsiveness and have been shown in animal studies to be important for the exhibition of maternal behavior (Barrett and Fleming, 2011; Noriuchi et al., 2008; Numan, 2020). Included among the sites and systems are the ventral tegmental area, striatum, amygdala (AMY), insula, cingulate cortex, and orbitofrontal cortex. In our lab we have focused on the AMY, as this brain region has been implicated in both the maternal and affective neural circuitry, and is involved in processing socioemotionally salient stimuli (Kim et al., 2016; Swain, 2011) both in human and animal models (Lonstein et al., 2015).

However, fMRI not only allows us to identify the activation of a particular brain region; it also allows for a functional connectivity approach, which examines the temporal synchronization in BOLD signal changes that occur across brain networks (for a review: Eickhoff and Müller, 2015). For instance, a Region of Interest in a connectivity analysis could examine the response of the AMY, anterior cingulate cortex, ventral striatum, hypothalamic medial preoptic area, corresponding to the maternal network (Atzil et al., 2011; Bjertrup et al., 2019; Swain et al., 2014), suggesting that these brain areas are co-activated or that there is an inter-relationship between them associated with particular infant stimuli.

In short, the study of the psychobiology of human parenting depends very heavily on correlational and multivariate strategies, which do not permit definitive causal analysis and which often use quite heterogeneous populations. However, with animal data as a backdrop and through the use of convergent analytic approaches, we may come closer to understanding psychobiological mechanisms of human parenting. Thus, in a heuristic strategy for moving between studies of animal and human mothering, research on rat mothers provides approaches to the analysis of more proximal and mechanistic features of human maternal behavior. Reciprocally, the work on human maternal behavior and its emphasis on the importance of psychological factors yields useful directions in animal research, including the examination of variables such as mothers' prior experiences and affective state.

The next section provides exemplars of how animal work has provided fodder for studies exploring similar questions in humans. Given the focus

of this chapter, most of the cited studies are studies our lab has done which illustrates this translational approach. For these examples we draw liberally from many of our own other previous reviews; and in some cases, text is taken directly from those reviews (Almanza-Sepulveda et al., 2020; Corter and Fleming, 1995; Fleming and Kraemer, 2019; Fleming and Li, 2002; Lomanowska et al., 2017; Lonstein et al., 2015).

The first example describes results in animals relating to the roles of anxiety and cognition and executive function, in mothering-first in the rat model and then in humans. The second set of examples describes studies focusing on the effects of early adversity and experiences early in life on the development of maternal and associated behavior expressed in adulthood. The third example illustrates how one hormone, –and in fact, one that was not for a long time known to be involved– the so-called stress hormone, adrenal glucocorticoid, has been shown to play a role in early maternal and associated behaviors in rat and human studies. The final example is more recent and includes a brief discussion of how some hormone-related genes may be involved in the regulation of mothering.

Due to space constraints, these exemplars are taken primarily from our own studies and hence include a very small proportion of the ever-expanding research on the psychobiology of maternal behavior which illustrates the usefulness of the animal model to guide studies on human mothers. We apologize for these restrictions. Significantly absent from this review are studies that explore the role of brain circuitry, brain neurochemistry, and molecular epigenetics in the regulation of maternal behavior in rat and human maternal behavior (see Numan, 2020). Significantly absent as well from this review is the large body of research on psychobiology of mothering in other mammalian species, including exceptional work on rabbits (for a review: González-Mariscal et al., 2016) and sheep (Lévy et al., 2019; Poindron et al., 2007). Inclusion of outcomes from these species would have further enriched this comparative analysis.

Examples

Anxiety and maternal behavior

Rats

Non-mother rats are often 'neophobic' and display high levels of anxiety-related behaviors, as indicated in anxiety tests, by their cautious approach to and engagement with newly presented stimuli and low exploration of novel environments (Fleming et al., 1980, 1989; Fleming and Luebke, 1981). In contrast, most new mother rats are generally less neophobic, fearful or anxious. This change in emotional regulation in most mothers contributes to the

successful onset and maintenance of caregiving. One of the primary sensory systems that prevents non-mother rats (i.e., adult virgins) from responding maternally, is the olfactory system. Peripheral or central manipulations that prevent olfactory input from reaching emotion-regulating regions of the brain, including the AMY, reduces pup-related avoidance in virgin female rats (Fleming et al., 1980; Fleming and Rosenblatt, 1974; Morgan et al., 1999; Numan et al., 1993).

Most studies find that such a general reduction in neophobia, fear, and anxiety does occur after most females give birth, though and lasts for a few days or up to about one week (Lonstein, 2005 & 2007). Some reduction in neophobia, fear, and anxiety even accompanies maternal state in sensitized nulliparous female rats (Agrati et al., 2008; Ferreira et al., 2002; Pereira et al., 2005). This blunting of maternal emotional reactivity could have implications for many postpartum behaviors. Functionally speaking, it could affect mothering behaviors by allowing dams to ignore relatively benign environmental threats and instead focus on the pups. Reduced anxiety postpartum may also permit the expression of the elevated aggression that dams direct towards intruders to the nest (Lonstein and Gammie, 2002), and promote their foraging for food away from the nest (Woodside et al., 2012).

It has been suggested that a moderate level of anxiety that is neither too high (rendering dams over-reactive to threat) nor too low (rendering them under-reactive) is optimal for maternal ability to focus attention on the needs of the pups despite threats in the environment (Ragan and Lonstein, 2014).

Humans

Sensitive responding to infants requires that mothers are affectively prepared to interact with them. Accordingly, maternal psychological well-being greatly impacts early human mothering. For many women, the first postpartum week is an exciting and positive experience, one that maps on too many changes in a mother's awareness of her new role and the experience of 'falling-in-love' with the baby. Studies of the normal changes in anxiety across the peripartum period and beyond in new mother's report mixed results, with some studies showing a decrease in anxiety (Figueiredo and Conde, 2011; Vesga-López et al., 2008), others showing an increase in anxiety (Britton, 2008), and yet others reporting no change (Leckman et al., 1999).

These discrepancies may be due to different methodologies used for assessing anxiety or the postpartum time when anxiety is evaluated (see Lonstein et al., 2015 for complete analysis). For instance, Figueiredo and Conde

(2011) observed that compared to pregnancy, state anxiety remains high only during the first postpartum days, but then decreases across the first three months postpartum. A variety of hormones, neuro-peptides, and other neurochemicals released or inhibited when human mothers receive suckling or other tactile inputs from their infants are presumed to contribute to a decrease in anxiety, although their mechanisms of action for this purpose in women are not particularly well studied. Some likely candidates are those known through experimental work to act centrally to affect anxiety in postpartum rodents, including OT, prolactin, vasopressin, corticotropin releasing hormone, GABA, norepinephrine and serotonin (Altemus et al., 2004; Carter et al., 2001; Heinrichs et al., 2001; Lonstein, 2007; Pawluski et al., 2017).

As pointed out by Lonstein, Levy et al (2015) regardless of the changes in anxiety that occur for postpartum women as a whole, there is still a subpopulation of mothers who experience a worsening of pre-existing anxiety symptoms or the new onset of an anxiety disorder. It has been suggested that up to 20–30% of women are truly affected by high peripartum anxiety (Britton, 2005 & 2008; Fawcett et al., 2019; Matthey et al., 2003). This high anxiety certainly seems maladaptive because it has a particularly negative effect on maternal caregiving. Anxious mothers show less warmth, more disengagement, are more critical of their infants, and are prone to catastrophizing (Moore et al., 2004; Whaley et al., 1999; Woodruff-Borden et al., 2002). These characteristics of anxious mothers have detrimental consequences for infant cognitive and socioemotional development (Glasheen et al., 2010).

The host of individual, social and contextual variables that contribute to heterogeneity in peripartum anxiety (Agrati and Lonstein, 2016) (include a history of anxiety, socioeconomic status, social support, fatigue, physical exercise (Britton, 2008; McGuinn et al., 2022; Vesga-López et al., 2008), and individual differences in women's genetic profile and physiology (Eley, 2007; Macbeth and Luine, 2010). Furthermore, similar to laboratory rats, physical contact with infants even without suckling transiently reduces anxiety (Heinrichs et al., 2001), so the timing when postpartum women are assessed for anxiety relative to their last interaction with their infant becomes critical.

Finally, womens' early-life experiences and the temperament of their infants modulate postpartum anxiety (Agrati et al., 2015; Agrati and Lonstein, 2016). Moreover, it is also likely that mothers' anxiety itself may affect infant temperament through anxiety-related alterations in maternal behavior. Jonas et al. (2015) found that among mothers who did not breastfeed at

3 months, that mothers' anxiety predicted both reduced maternal sensitivity at 6 months and later elevations in infant negative affectivity over a year later. That is, breastfeeding appeared to protect mothers from anxiety-related effects on later maternal behavior and infant negativity.

Cognition and Executive Function

Rats

Mothering relies to a great extent on good executive function which allows the mother to filter out interference, anticipate the consequences of actions and show adaptation to the environment-processes that ensure the survival and healthy development of the newborn. Mother rats with lesions to the mPFC show impaired attention (reflected in attentional set-shifting performance) and in prepulse inhibition (reflected in a reduction in the amplitude of the acoustic startle responses that affect the smooth temporal sequencing of maternal behaviors as in pup retrieval and pup licking (Afonso et al., 2007). Also, rats raised without mothers show difficulties in their own maternal behavior and executive functions such as attention shifting (Lovic and Fleming, 2004), or impulsivity (Lovic et al., 2011), associated with decreased maternal pup licking and time spent crouching over their own pups, and less time spent in nest (Lovic et al., 2011; Lovic and Fleming, 2004).

Similarly, studies show that maternal separations in early postnatal period leads to changes in the adult's attentional set-shifting (Kuhn et al., 1990; Lehmann et al., 2002; Plotsky et al., 2005; Rhees et al., 2001), associated with alterations in brain development, as in persistent elevations in the number of neurons and supporting astroglia cells in brain areas involved in the circuitry underlying maternal behavior (Chatterjee et al., 2007). Furthermore, mother rats exposed to additional corticosterone express a better maternal memory (the long-term retention of maternal responsiveness as a consequence of rats' prior experiences with pups) (Graham et al., 2006), which in turn facilitates the expression of maternal behavior (Fleming et al., 1989; Li and Fleming, 2003b); moreover, mother rats with lesions on the nucleus accumbens (NA), particularly the shell region, before or directly after pup experience show impediments to forming maternal memory (Li and Fleming, 2003a). Studies in postpartum rats also show that dopamine D1 and D2 receptors on OT receptors in NA shell consolidate maternal memory (Parada et al., 2008). Blocking OT receptors in the NA after a maternal experience postpartum results in a reduced latency to become maternal on retention testing (D'Cunha et al., 2011). Taken together, these studies show that the NA shell plays a role in the consolidation of maternal memory, and

organizes the pattern of behavior exhibited by mother rats during their caregiving.

Humans

Similarly, in human mothers, the quality of maternal behavior has been associated with adequate performance of executive functions. Mothers who exhibit poor sensitivity to their infants show lower performance on cognitive flexibility tasks, attentional set-shifting, difficulty in inhibiting irrelevant stimuli, and lower performance on spatial working memory tasks (Gonzalez et al., 2012; Gonzalez et al., 2009). Moreover, in comparison to adult mothers, teen mothers, are often less sensitive in their interactions with their babies, and tend to show poorer performance on a variety of executive function tasks, including attention and working memory (Almanza-Sepulveda et al., 2018; Chico et al., 2014). During interactions with their babies, they are more easily distracted by their surroundings and participate in fewer contingent activities with their babies (Flanagan et al., 1994; Sadler and Catrone, 1983). Moreover, teen mothers often invest more time than adult mothers in instrumental behaviors and less time in affective behaviors (Krpan et al., 2005).

Compared with adult mothers, adolescent mothers show little change in basal cortisol and heart rate when they listen to their baby's cries (Giardino et al., 2008). Cortisol levels have been associated with quality of maternal behavior, responses to stress, and emotional regulation (Fleming et al., 1987; Fleming et al.,1997; Fleming et al.,1997). See below for additional studies on cortisol and mothering.

Early Experience

Rats

As described by Fleming and Li (2002) the early experiences of being mothered also have long-term effects on the quality of mothering that offspring that will show toward their own offspring when they grow up. In this case, the experience of receiving more somatosensory stimulation and more varied nest experiences may directly affect the animals' physiology and maturational processes; active associative processes may not be involved.

Francis and others from the Meaney lab have demonstrated that young that receive more licking from their mothers grow up to show higher levels of licking toward their own offspring compared with those who received low licking (Francis et al., 1999). This effect was also found if the young from high – and low– licking mothers were cross fostered to mothers showing the opposite pattern of licking than that of their biological mothers. Hence if

offspring from high-licking mothers are cross fostered to low-licking mothers they show a low-licking pattern when they grow up. This cross-generational effect of licking provides a nongenomic mechanism for the transmission of behavioral phenotypes (Gonzalez et al., 2001).

One can also actively manipulate the early environment experienced by the offspring and make marked changes in their adult maternal behavior. Gonzalez et al. (2001) raised rat pups artificially from day 3 to day 18 of life in cups floating in a warm bath. Pups were fed a nutritionally balanced (for rat pups) diet at 1-hr intervals and received either a high level of simulated licking daily (six times daily with a paintbrush) or low levels of licking (two times daily). Sham operated and intact, sibling pups remained with their mothers. When compared with their mother-raised siblings in the quality of their maternal behavior after birth of their own litters, the artificially raised mothers showed clear deficits in maternal licking and crouching, but no deficits in retrieval responses. Interestingly, the artificially raised offspring that received additional licking like stimulation (six daily bouts of stroking) while in the cups showed levels of maternal licking and crouching more similar to those of their mother-raised siblings. Moreover, the effects of early mothering deprivation experienced by one generation are also experienced by subsequent generations that did not experience the maternal deprivation regime. Hence the female offspring of artificially raised mothers who received reduced licking themselves licked their offspring less when they grew up (Gonzalez et al., 2001) These early experience effects can therefore be transmitted from one generation to the next and very likely depend on somatosensory experiences associated with licking stimulation.

In addition to affecting the quality of mothering, early experiences being mothered also affect the development of fear and emotionality which in turn contribute to the quality of mothering (Francis et al., 1999; Gonzalez et al., 2001). Females who are raised artificially (Gonzalez et al., 2001) or who receive reduced licking from their mothers show reduced exploration in the open-field test of emotionality and reduced emission of ultrasonic calls in a novel environment (Francis et al., 1999; Gonzalez et al., 2001); as well as reduced performance on tasks of executive function (Kuhn et al., 1990; Lehmann et al., 2002; Lovic and Fleming, 2004; Plotsky et al., 2005; Rhees et al., 2001).

Humans

Positive early experiences also predict later maternal behaviour (Belsky et al., 2005; Zeng-yin Chen et al., 2008; Zeng-yin Chen and Kaplan, 2001). This is described fully in Lomanowska et al (2017). For instance, mothers who re-

port positive childhood relationships with their parents are more responsive towards their children (Gara et al., 1996). Furthermore, the experience of less authoritarian parents in early childhood, a more positive family 'climate' in middle childhood, and more positive attachment in adolescence, are all predictive of warm, sensitive, and stimulating maternal behaviour in adulthood (Belsky et al., 2005). Not surprisingly, negative early experiences in family of origin increase the risk of negative parenting. Moehler et al, (2007) measured emotional availability during mother-infant interactions and found that mothers with a history of sexual or physical abuse were significantly more intrusive toward their children than were non-abused mothers. Roberts et al (2004) also report that mothers who had experienced sexual abuse in early life are less interested in becoming mothers themselves and when they do, exhibit impaired parenting skills, such as higher levels of child neglect, diminished confidence in their own parenting skills, more negative self-appraisal as a parent, greater use of physical punishment, and a lack of emotional control in parenting situations. Knutson (1995) has reported that a substantial proportion of mothers who were abused during childhood go on to subsequently abuse their own children, when compared to mothers who did not report abuse.

In sum, early life experiences can be viewed as part of a spectrum from very negative to very positive, and both positive and negative parenting behaviours are transmitted intergenerationally. However, the emphasis of this research has been both on the developmental sequelae of inadequate parenting and the outcomes associated with warm and supportive parenting. Initially relations were established by studies in which the early experiences were recalled retrospectively. Recently studies have been prospective, in which families and children have been followed from childhood through adulthood (Belsky et al., 2009; Neppl et al., 2009; Scaramella et al., 2008).

It is important however to indicate here that in spite of these apparent relations between early adversity, including physical abuse (and/or retrospective perception of that adversity), and later emotional and parental problems, it remains that a large proportion of mothers who were abused will not in turn abuse their own children. This suggests that a myriad of factors protect future mothers from the risks accrued through early experiences. Among these, social support to the mother or to the developing child (Jaffee et al., 2007; Wind and Silvern, 1994) as well as forming a relationship with a supportive partner in adulthood (Seeman et al., 2002) appear to play an important role.

Hormones

A general overview: Rats and Humans

There is an abundance of evidence in animal studies that show the effect on the onset of mothering during late pregnancy and the early postpartum period, of changes in the steroids, estrogen and progesterone and in the peptide hormones, prolactin and OT (Bridges, 2020; Carter et al., 2001; Lévy et al., 2015). Studies exploring the role of estrogen rise at the end of rat pregnancy against a backdrop of declining progesterone, suggest that in the rat the shift in the ratio of these two steroids enhance maternal motivation at the time of parturition (Pereira et al., 2015; Numan, 2020). Subsequent studies show that these effects are further enhanced by a subsequent rise in prolactin and OT (Bridges, 2020; Bridges and Feder, 1978; Pedersen, 1997; Pedersen et al., 1994) and have been established primarily through studies showing the effects on maternal behavior to foster pups of gestational ovariectomy and hysterectomy (Rosenblatt and Siegel, 1975; Siegel and Rosenblatt, 1975) along with hormone replacement as well as through a regimen of hormones administered to virgin animals tested with foster pups (Bridges, 1984).

There have also been a number of studies that explore the correlation in human mothers between hormonal changes during pregnancy and feelings of nurturance postpartum. In general, the pattern of 'effects' is similar in humans as in other animals, although too few studies have been undertaken to solidify these relations (Fleming et al., 1997).

Adrenocortical Hormones

What follows is a more in-depth analysis of the potential –and perhaps unlikely– role in mothering of another steroid, the glucocorticoid, corticosterone (in rats) or cortisol (in humans) (Almanza-Sepulveda et al., 2020).

Rats

In both rats and human mothers, circulating plasma concentrations of corticosterone rise across pregnancy, peak at parturition, and fall within the first few weeks postpartum (Fleming et al., 1997; Voogt et al., 1969). Studies had shown a correlation between cortisol concentrations and maternal responsiveness in early postpartum. In rats, adrenalectomy and corticosterone replacement to new mother rats decreased and then enhanced licking of newborn pups, although other behaviors reflecting maternal motivation such as retrieval and nursing responses are unaffected (Rees et al., 2004).

Furthermore, female rats exhibited a better memory or retention of their postpartum experience interacting with pups when exposed to addi-

tional corticosterone, indicating that corticosterone also enhances maternal memory of how to behave with young (Graham et al., 2006). However, in contrast and in comparison, to vehicle-treated controls, Brummelte and Galea (2010) found that rat mothers who were administered very high doses of exogenous corticosterone (over and above their endogenous corticosterone) nursed their pups less and spent more time away from the nest. These results could suggest a biphasic response to corticosterone in rats, with low-to-moderate concentrations facilitating components of maternal responding, at least soon after parturition, whereas high concentrations that might model chronic stress may simply derail maternal capabilities. Finally, in contrast to the effects in new mother rats, the maternal behavior, especially pup-licking in virgin animals induced through continuous contact with foster pups, is enhanced by adrenalectomy and disrupted by corticosterone replacement, indicating that the effects of the adrenal system on mothering depends on hormone dose, the timing of the manipulation and the parity of the animal (Rees et al., 2004).

Humans

Similarly, women with higher salivary cortisol concentrations display longer durations of affective touch with their infants (Fleming et al., 1997). Immediately after parturition, mothers with higher plasma concentrations of baseline cortisol tended to display more contact approach responses towards their nursing infants (Almanza-Sepulveda et al., 2020). More recently, a positive relationship between cortisol concentrations (prepartum and postpartum) across a dyadic interaction and intensity of affectionate behaviors was also found in young primiparous mothers (in their early 20s) but not in multiparous mothers. Cortisol is also positively associated with new mothers' attraction to infant cues (Fleming et al.,1997).

In terms of discriminate one's own versus other infants' odors on a T-shirt, as opposed to attraction to infant cues, the cortisol-parity effects are reversed. Multiparous mothers with higher cortisol show significantly higher accuracy scores, whereas accuracy scores and cortisol concentrations were unrelated in primiparous mothers (Fleming et al.,1997). It may be that for individual recognition, prior experience with infants permits mothers to learn about the specific features of neonates. In that case, cortisol in the early postpartum period enhances maternal attention to the most meaningful features of infant stimuli. In contrast to nearly postpartum, by 6 months postpartum the relation between cortisol and maternal sensitivity is an inverse one. For instance, in one study that explored the interactive effect of mothers' early life experiences and cortisol at 6 months postpartum

on executive function and maternal sensitivity, Gonzalez et al, (2012) report that a more negative early experience was associated with both higher levels of diurnal cortisol at 6 months and with poorer performance on the spatial working memory task. These, in turn, were associated with one another and with lower maternal sensitivity.

A role for altered HPA axis and cortisol function in postpartum anxiety and postpartum disorders has also been proposed, with consequences for mothering (Brummelte and Galea, 2016). Although depressive-like symptoms can be produced in a rat model through exogenous corticosterone, in human mothers the relationship between circulating cortisol and postpartum mental health still remains ambiguous. Some studies measuring cortisol across pregnancy through the early postpartum period report an association between hypocortisolemia and postpartum anxiety and depression, whereas others report an association between hypercortisolemia and postpartum affective disorders; and yet others report no significant association between the two (Brummelte and Galea, 2016). Clearly, these associations suggest that in addition to potentially affecting executive function, other ways elevated cortisol could impact mothering at 6 months and later is through affecting mothers' levels of anxiety and depression. Finally, as indicated above when discussing the changes in executive function and those effects on mothering, cortisol changes may also be implicated.

Oxytocin and genetics

Rats

OT is a neuropeptide hormone involved in parturition and milk let-down across all mammals (Jonas and Woodside, 2016). Estrogen enhances OT receptor (OTR) binding in the medial preoptic area (MPOA), a brain region critical to maternal behaviour across mammals (Pedersen, 1997; Young et al., 1998). Central administration of OT stimulates maternal behaviour, in virgin rats while OTR blockers reduce maternal behaviour (Fahrbach et al., 1985; Pedersen et al., 1994). Moreover, individual differences in specific forms of maternal behaviour, such as pup licking/grooming (LG), correlate with OTR expression in the MPOA; and intracerebral infusion of an OTR antagonist eliminates individual differences in pup LG among lactating rats (Champagne et al., 2001).

Humans

OT is also closely related to human mothering and other affiliative relationships (Feldman and Bakermans-Kranenburg, 2017; Galbally et al., 2011). OT levels in the first trimester predict the expression of maternal behaviour

and coordination with infant state (Feldman et al., 2007). OT seems to be mostly associated with onset of maternal behavior, 'maternal motivation' and warmth, and likely of greatest direct relevance in the early stages of maternal responding when close contact behaviours predominate and nursing may still be occurring (Jonas et al., 2013). OT may also be related to mothers' depression after birth, although the relationship between OT and depression seems to depend on gender and timing of the depression. For instance, low OT pregnancy levels relate to the development of postpartum depression (Jonas et al., 2013). In a different population of non-mothers, plasma OT levels are higher in patients with major depression than in healthy controls (Parker et al., 2010).

Very few studies have explored the association between genetics within the OT system and mothering and they are predominantly human studies (Bakermans-Kranenburg and van IJzendoorn, 2008; Jonas et al., 2013; Mileva-Seitz et al., 2013). Hence, we know more about the role of OT physiology in mothering from the animal work and about OT genetics and mothering from human studies. Among new human mothers, evidence shows that the presence of a C allele on the OT gene is associated with higher stress-induced anxiety and lower emotional well-being (Mileva-Seitz et al., 2013). Variations in OT-related genes modulate both non-postpartum depression and anxiety and possibly, maternal mood and sensitivity in mother-infant interactions (Mileva-Seitz et al., 2013). Genetic variations within the OT system also interact with mothers' own childhood experiences of adverse rearing and current stressors to influence mothering, these effects are often mediated through alterations in mothers' affective state (Mileva-Seitz et al., 2013).

Summary

As we hope these exemplars illustrate, there is a substantial and ever-growing literature on behavioral and physiological mechanisms regulating maternal behavior in both the animal and human research and findings in each domain have informed the questions asked and studies done in the other domain. This is not to say that the outcomes for nonhuman animals (rats in this case) and humans are the same, but their respective analyses fruitfully explore similar questions; albeit using quite different methodologies. Studies of behavior- of anxiety, cognition, perception and their role in the expression of mothering have enhanced our understanding of maternal behavior in rats as well as in humans. Similarly, studies of hormones, the brain and genetics in both the animal models and humans contribute to our understanding of mothering both as well. Finally, intergenerational effects

apply to both, where early and proximal experiences by the mother impact the (F1) offspring and theirs, in turn. While humans are not rats, they share many mammalian characteristics. However, to better understand which are universal mammalian regulators of maternal behavior and which mechanisms are unique to human mothering, we need to explore similar questions in many more mammalian species than we have in this short chapter.

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IX

Translational animal models for sexual health

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Introduction

Preclinical Research in Sexual Medicine

Basic preclinical research uses animal models of human behavior or disease states to generate information that is intended ultimately to benefit patients, inform clinicians, and improve healthcare (Sung et al., 2003; Lean et al., 2008). The process to transfer basic research findings to clinical settings and the community may be divided into different translational phases; i.e. from 1) the laboratory to humans; 2) clinical science to routine practice; and 3) real-life procedures to cost-effective recommendations and solutions (Sung et al., 2003; Lean; 2008). Each of these phases comprises different professional disciplines, methods, study populations, institutions, networks, funding bodies, and regulatory units, that should promote translational medicine based on proper non-biased evidence (Sung et al., 2003).

Although this process generally is considered from "bench to bedside" as it moves forward, translational research in sexual medicine should be considered a bidirectional dynamic process, where findings in clinical practice or health care systems are, in turn, intended to (reversely) excite investigative basic science activities (Homer-Vanniasinkam and Tsui, 2012; Liebman and Marincola, 2012; Marincola, 2003; van der Laan and Boenink, 2012). An important initial decision in addressing a clinical problem in an experimental setting is the selection of a valid model that corresponds to the human condition in etiology, pathophysiology, symptomatology, and response to therapeutic interventions (Varga et al., 2010). Reverse translational research then serves several purposes to feed-back, evaluate, and develop 1) a model's suitability for research, 2) mechanistic evidence for a process or intervention, and 3) advances and restart of forward translation for adjacent discoveries (Homer-Vanniasinkam and Tsui, 2012; Liebman and Marincola, 2012; Marincola, 2003; van der Laan and Boenink, 2012). Thus, translational medical research can be viewed as comprising an interaction

of many disciplines and different procedures that span several levels of analysis.

Homologies, analogies, validities, and practicalities. A practical consideration to any preclinical model is how well it predicts a clinical outcome. Traditionally, it has been perceived that the "closer" a mammal is to humans, e.g. non-human primates, the better the correlation to human functions or disease processes (Denayer et al., 2014; Sams-Dodd, 2006a). This may be true for pathophysiological mechanisms of environmental or spontaneously occurring diseases in humans that similarly occur in other mammals, but probably less so for simulated (artificial) diseases that are caused by various inducers or other interventions (Sams-Dodd, 2006a). However, the notion of an ideal species for translational research that is based on phylogenetic relationships may, if a defined process is well characterized after exploration in several species, be an unnecessary constraint for comparisons to humans (Preuss, 2000). Instead, understanding and further characterization of cross-species commonalities to humans should leave focus to the process studied, and allow for considerations of cost, effectiveness, convenience.

The concept of face validity involves a consideration of how the model is analogous or homologous to the human condition (Denayer et al., 2014; Sams-Dodd, 2006a). In sexual arousal, penile and clitoral erection are examples of a component of sexual response that in many mammals share substantial physiological and biochemical substrates, and for which methods exist that can be used to assess homologous endpoints (Andersson, 2011; Christ and Lue, 2003; Giuliano et al., 2010). Similarly, female precopulatory solicitations are also conserved among different mammalian species, including humans, although they are presented differently between species. Therefore, knowledge of particular species-related behaviors and physiology is crucial for comparisons to be made to humans (Barnett et al., 2006; Giuliano et al., 2010; Pfaus et al., 2003; 2012). Homologies and analogies of human and rat behavior are shown in Figure 1.

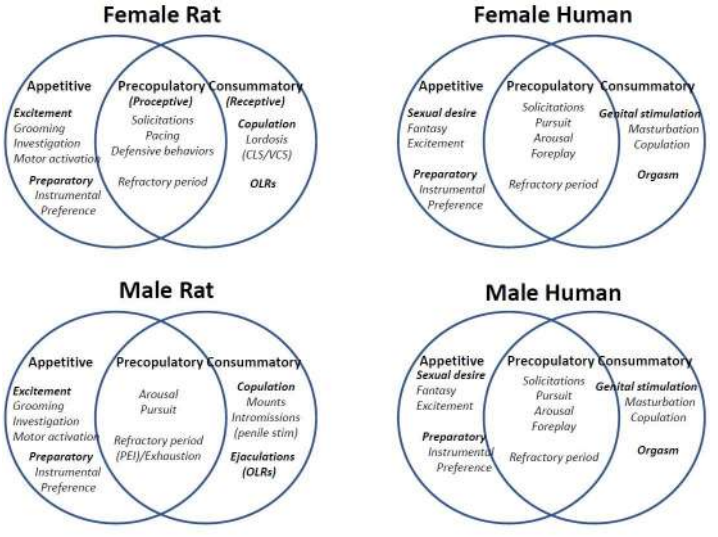


Figure 1: Incentive sequences for human and rat sexual behavior (modified from Pfaus et al., 2003). The behavioral stream moves from left to right, through appetitive, precopulatory, and consummatory phases of behavior. This conforms to the movement of animals from distal to proximal to interactive with respect to sexual incentives. Sexual excitement can manifest itself in learned or unlearned behaviors, whereas preparatory behaviors are learned responses that animals must make in order to acquire the incentive (e.g., operant behaviors, pursuit, preference etc.). Recognizing which responses occupy which parts of the behavioral stream allows researchers to compare behaviors that appear different among different species, but that accomplish a similar motivational endpoint. Such behaviors in one species (e.g., rat) may then serve as models of analogous behaviors in humans. The predictive validity of such models is enhanced when drugs or other treatments modify the behavior of both species the same way, suggesting that a common neural system for those behaviors has been conserved. OLRs = orgasm like responses. CLS = clitoral stimulation. VCS = vaginocervical stimulation.

The relation of disease mechanisms in an animal model versus the corresponding human disease, i.e. construct validity, is important to reflect upon in studies with e.g. pathophysiological or therapeutic endpoint measures (Sams-Dodd, 2006a). The concept of predictive validity concerns whether the analogous or homologous behavior or physiological system can be perturbed in a way that predicts a similar effect in humans, and whether such a perturbed behavior or system can be restored by a particular treatment regimen (e.g., a physiological, pharmacological, and/or behavioral intervention; Denayer et al., 2014; Sams-Dodd, 2006a).

Regarding sexual behavior, all organisms that engage in sexual behavior share a common set of principles and end-points that define the behavior, along with particular excitatory and inhibitory neural mechanisms that make it successful (Pfaus, 2009). First, we must be able to respond to hormonal and neurochemical changes that signal our own sexual desire

and arousal. This ability underlies our moment-to-moment level of sexual arousability (as conceived by Whalen, 1966, see below), and defines a large part of the internal state that is commonly referred to as "sex drive". The rest requires a complex mix of instinct, learning, and feedback; a neural organization that allows us to interact with external sexual stimuli and partners. We must be able to identify external stimuli that predict where potential sex partners can be found, to seek out, solicit, court, or otherwise work to obtain sex partners, distinguish external cues and behavioral patterns of potential sex partners from those that are not sexually receptive, and to pursue sex partners once sexual contact has been made. Neural mechanisms exist that allow sexual responding to become habitual or automated with practice. Similarly, neural mechanisms exist that allow the stimulation received during sexual contact to be perceived as rewarding. Such reward alters subsequent behavior, for example, by contributing to the formation of preferences for salient stimuli associated with positive sexual reinforcement (Pfaus et al., 2012). Many of these aspects of sexual responding go well beyond the traditional focus on copulation or genital reflexes, but they have important implications as models of desire and pleasure. The use of the "right" model (i.e., one that has both face and predictive validity for the sexual function and/or dysfunction in question) is thus a critical consideration in any translation of knowledge from preclinical laboratory models to real humans in a clinical situation. Thus, predictive validity is dependent on similarities between both the disease mechanisms and the biological, pathophysiological, or pharmacological functions in the animal model that pertain to the clinical situation in humans (Denayer et al., 2014; Dothel et al., 2013; Everitt, 2014; Sams-Dodd, 2006b). As mentioned above, the translational process should be considered bi-directional. Reverse translation from clinical findings to animal models not only forms a basis for in-depth mechanistic investigations, but should also be used to refine a model to improve its predictivity (Denayer et al., 2014; Dothel et al., 2013).

Some barriers have been identified for translation of new knowledge from the laboratory to tests and evaluation in human subjects, and for transfer of clinical evidence to national health decision making (Sung et al., 2003). These barriers reflect differences in human resources and practice, discordant methodologies or regulations, and financial limitations or interests. Concerns of a general decline in success rates for drugs during development from preclinical to clinical settings (despite evidence of potential efficacy in experimental models) discourage subsequent preclinical testing, as does complex extrapolation of discoveries from some animal models to humans (Denayer, 2014; Everitt, 2015; Linder, 2007; Morrison et al.,

2015; Perel et al., 2006; Sung et al., 2003). This latter point is especially concerning when the preclinical model examined is not a valid choice for the human clinical endpoint.

Three situations serve as examples for consideration of these ideas. First, despite the fact that female Japanese macaques form stable same-sex partnerships during the breeding season, and that they engage in extensive clitoral contact with one another (Vasey, 2002), this is likely not a model of female homosexuality as humans understand it as a self-concept. This becomes a "level of analysis" issue: do female Japanese macaques consider themselves "gay" or "bi"? And how would we ever know? However, if the clitoral stimulation (CLS) is pleasurable, does it serve to reinforce the particular sexual behavior in both macaques and humans, regardless of the conscious term used to describe it? If so, then CLS as a pleasure-based stimulus can be used as a tool to understand its human counterpart. Second, the lordosis reflex in female rats and other species is affected by brain lesions, hormonal or pharmacological manipulations, and environmental conditions in ways that do not predict effects on "sexual receptivity" in women. Indeed, human females do not show a "lordosis" posture that is driven by estradiol. Solicitations, on the other hand, although they involve motor movements that differ widely between species, are affected by pharmacological manipulations that affect women's sexual desire in a similar manner between species. Thus, lordosis should not be used as an exclusive laboratory model from which "desire" can be extrapolated unambiguously, even if a drug might have inhibitory or excitatory effects on both lordosis and solicitations. Finally, penile erection and the intromission latency in male rats are highly correlated. If a drug or condition diminishes the ability of male rats to gain penile erection, it will necessarily increase the time it takes for male rats to gain vaginal intromission or penetration. Thus, use of the intromission latency as a measure of male sexual "motivation" or "desire" is not necessarily valid because it may well reflect an effect on genital sexual arousal alone. Use of the mount latency, on the other hand, is more a measure of the "desire" to engage in copulation even if the male does not have an erection.

The behavioral models that have been most informative for preclinical translational research in sexual medicine address sexual arousal, desire, orgasm/pleasure, inhibition, and genital pain in rats and mice. Rodent species are cost-efficient, provide high through-put (i.e., multiple animals can be tested at the same time), and require basic maintenance in a laboratory animal colony with environmental enrichment. The sexual behavior of rats and mice conform generally to the 4-phases of the human sexual response cycle (Masters and Johnson, 1966; Moll, 1908), all of which are constrained

by the interplay of excitatory and inhibitory neural systems (Bancroft and Janssen, 2000; Perelman, 2006; Pfaus, 2009). Models of both unconditioned and conditioned sexual behavior have been generated on the basis of more sophisticated testing conditions that utilize pacing chambers that allow the females to initiate and pace the rate of copulation, open fields partner preference tests that allow females or males to choose to copulate with two potential partners, one of which is preferred on the basis of sexual reward learning, and conditioned place preference (CPP) paradigms that allow researchers to determine which aspects of copulation or sensory stimulation induces a sexual reward state. Such chambers are shown in Figure 2.

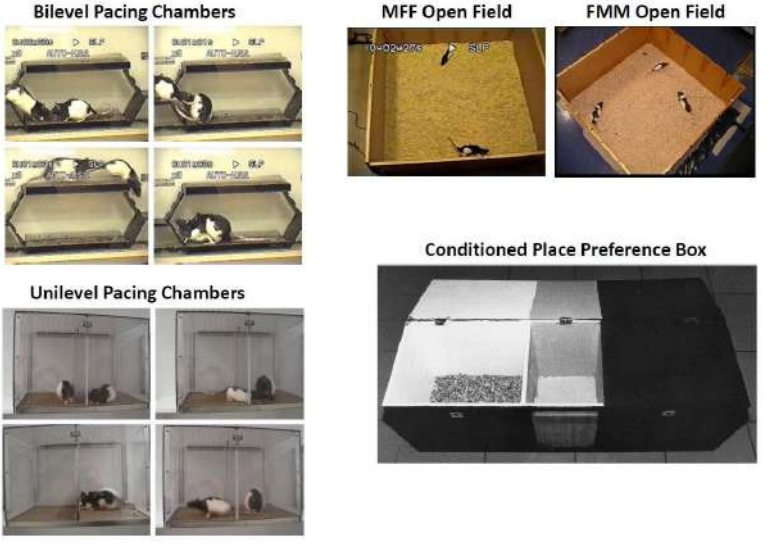


Figure 2. Left: Bilevel and unilevel pacing chambers used for conditioning of partner or place preferences (adapted from Pfaus et al., 2015 with permission). Pacing chambers allow the female to regulate copulatory contact with the male by either running from level to level (top) or by darting through small holes in a divider that are big enough to allow the female to pass through, but too small to allow the male to pass through (bottom). In the bilevel pacing chambers, females make a headwise orientation to the male (upper left panel), then hop over him exposing their anogenital area (top right panel). Females then run away, forcing the male to chase them (bottom left panel), after which they run to the original level and assume a lordosis crouch, allowing the male to mount with intromission (bottom right panel). Similarly, in unilevel pacing chambers females can pass through the hole into the male's side (top left and right panels) and solicit an intromission (bottom left panel), then exit the male's side (bottom right panel). Each of these sequences depict the initiation and termination of a copulatory bout by the female. Right top: Open fields used for testing conditioned partner preferences and CEP (from Pfaus et al., 2015). To test CEP, a male is placed into the open field with two females, one scented or jacketed and the other unscented or unjacketed. The male can then copulate freely with either one. To test partner preferences in females, a female is placed with two tethered males, one scented or jacketed, and the other unscented or unjacketed. Males are tethered to avoid their tendency to huddle together. Females compete, so they can remain untethered. Right bottom: Apparatus used to condition and test

place preferences after sexual reward training (from Paredes and Vazquez, 1999). After a requisite amount of copulatory stimulation, a female or male is placed on one side of the box (usually the light side as this is typically not their naturally preferred side). This is contrasted without stimulation, after which the female or male is placed into the naturally preferred (e.g., dark) side. If the copulatory stimulation induces a reward state, then on a final test, after the animal is placed into the middle compartment, it will move to the side with cues that predict the reward state and spend more time there compared to the other side.

Models of Sexual Arousal

Physiological sexual arousal in humans and animals can be defined as increased autonomic activation that prepares the body for sexual activity. This includes both sympathetic blood flow from the heart to striated and smooth muscle that participate in sexual responses and parasympathetic activation of genital and erectile tissues, in particular the clitoris, labia, vaginal epithelium, and penis, to keep the tissues engorged. Sexual arousal also includes a central component that increases neural "tone" or preparedness to respond to sexual incentives. This latter concept was defined as "arousability" by Whalen (1966), and may form around an intricate interaction of hormone priming and noradrenergic activity in different regions of the brain. Both peripheral and central arousal may be detected as part of the perception of subjective sexual arousal, and both clearly lead to changes in responsiveness in genital tissues and control certain copulatory responses, such as the latency to orgasm or ejaculation (with shorter latencies indicating an increase in arousal).

Male sexual arousal. Among the measures of sexual arousal, erection has been considered as the indicator of autonomic arousal in males, both in men and rats (Sachs, 2007), and a high correlation of this genital response with subjective arousal seems to exist in men (Chivers et al., 2010). There is a considerable degree of similarity between male rats and men in terms of the neuroanatomical, neuroendocrine, and neurochemical regulation of penile erection and ejaculation (Andersson, 2011; Giuliani, 2011; Meisel and Sachs, 1994; Rosen and Sachs, 2000, for comprehensive reviews). Penile erection is the conclusion of integrated neurovascular processes that involve the central nervous system and peripheral nerves, and endothelial and smooth muscle cells of the penile arteries and the erectile tissue (Andersson, 2011).

Fundamentally, neurogenic and endothelial stimuli shift the tonus of penile vascular smooth muscle from contractile to relaxant states and increase arterial inflow and sinusoidal compliance where after the incrementing engorgement activates the veno-occlusive mechanism to achieve erection of the penis (Andersson, 2011; Giuliano, 2011). Extensive research suggests that regulatory mechanisms of penile erection exhibit substantial

physiological, biochemical, and pharmacological homologies among mammalian species, incl. humans (Andersson 2011; Christ and Lue, 2003; Giuliano et al., 2010; Gratzke et al., 2010). As example, human, monkey, rabbit, dog, rat, and mouse corpus cavernosum tissue that is precontracted to simulate penile flaccidity, respond similarly via neuronal and endothelial signals with relevant nitric oxide (NO)-dependent relaxant "erection-simulating" responses (Hedlund et al., 1995; 1999; 2000; Ignarro et al., 1990; Kim et al., 1991; Okamura et al., 1998). Accordingly, a crucial role for the NO-pathway in penile erection has been characterized in vivo during erections in various animals (Ayajiki et al., 2009; Burnett et al.,1996; Holmquist et al., 1991; Mills et al., 1992; Trigo-Rocha et al., 1993). Furthermore, similar to humans, several mammals display in aging or due to various diseases signs of erectile dysfunction and tissue alterations of important regulatory pathways of erection (Giuliano et al., 2010).

Intracavernous pressure (ICP) registrations were initially performed in larger animals such as bulls, goats, dogs, and monkeys, probably mainly due to their larger anatomy (Becket et al., 1972; Lewis et al., 1968; Lue et al., 1983; Purohit and Beckett 1976). Later, in favor of easier handling and faster processing with lower costs, cats, rabbits and rodents were introduced for ICP recordings (Hellstrom et al., 1992; Holmquist et al., 1991; Mills et al., 1992; Sezen et al., 2000; Steers et al., 1988). In agreement to findings in animal models, recordings of ICP in humans have been correlated to tumescence and erections (Lue et al., 1995; Rehman et al., 1999).

Drug-induced penile erection. Studies using compounds that act at various receptors have been performed to clarify the role of certain neurophysiological signals in sexual functions and some of these agents may be mentioned as "standard drugs" in models of erection (Andersson, 2011). One such drug is apomorphine, a non-selective dopamine receptor agonist, that increases the incidence of penile erections in rats (Benassi-Benelli et al., 1979). The compound was later shown to induce erections also in mice, monkeys, and humans (Lal et al., 1984; Pomerantz, 1990; Poncelet et al., 1994; Rampin et al., 2003; Segreaves et al., 1991). Apomorphine was put forward for clinical trials, and although its efficacy was discouraging in relation to peripheral-acting drugs (Moll and Brown, 2011), it remains as an option for treating erectile dysfunction in patients that cannot take phosphodiesterase 5 inhibitors (Burnett et al., 2018). Still, species-similarities in central dopamine-receptor mediated regulatory functions of penile erection and experience with apomorphine in sexual medical research supports its use in models of erectile neurophysiology, pathophysiology, and pharmacology. Apomorphine acts at supraspinal and spi-

nal regions of the CNS to induce erections (Chen et al., 1997; Giuliano et al., 2002; Melis et al., 1987; 2022). In anesthetized rats, apomorphine microinjected into the PVN, or by intravenous or subcutaneous route induces erections depicted as increases in ICP (Chen et al 1999; Giuliano et al., 2002; Matsumoto et al., 2005; Mizusawa et al., 2002). Similarly, apomorphine induces erections recorded as ICP responses in awake rats (Andersson et al., 1999; Bernabe et al., 1999; Hedlund et al., 2005; Ischizuka et al., 2002; Matsumoto et al., 2005). It should be noted that the pathways targeted by apomorphine in the CNS are likely involved in regulation also of other urogenital functions as the drug is reported to induce bladder overactivity and to promote ejaculation and seminal emission in rats, and to reduce post ejaculatory latencies in monkeys (Andersson, 2003; Napoli-Farris et al., 1984; Paglietti et al., 1978; Pehek et al., 1989; Pehrson and Andersson, 2003; Pomerantz, 1992).

Central melanocortin receptor-mediated signals are involved in sexual functions and penile erection in various species, e.g., rats, rabbits, cats, dogs, monkeys, and humans (King et al., 2007). Classically, the endogenous a-melanocyte stimulating hormone (a-MSH) was reported to induce erections and later, proerectile effects of synthetic a-MSH analogues such as melanotan II and its primary metabolite bremelanotide have been evaluated in different animal models and in humans (Diamond et al., 2004; Donovan, 1978; Dorr et al., 1996; Molinoff et al. 2003; Pfaus et al., 2007; Rosen et al., 2004; Wessels et al., 1998). In rats, a-MSH or melanotan II similarly induce erections that have been recorded as ICP responses in anesthetized or awake rats (Giuliano et al., 2005; 2006; Mizusawa et al., 2002).

Oxytocin is a peptide that has been extensively used in models for the study of erection and oxytocin signals have been well characterized in spinal and supraspinal pathways that regulate penile erection (Andersson, 2011). However, even if it is acknowledged that this peptide has an important role for erection in rodents, it is not established if oxytocin has the same relevance in humans (Andersson, 2011; Behnia et al., 2014; Burri et al., 2008). Compared to placebo, intranasal administration of oxytocin was reported not to have any effect on the ability to achieve erections in healthy men (Behnia et al., 2014).

In summary, dopaminergic pathways and MC receptor signals appear to exhibit commonalities in erectile function of various mammals and humans. Compounds such as apomorphine, a-MSH, melanotan II, or bremelanotide, may be considered relevant reference drugs for the study of penile erection.

"Psychogenic" erections

Male rats exposed to inaccessible estrous females show penile erections (Sachs et al., 1994), a phenomenon that is also observed in primates like the chimpanzees and rhesus monkeys (Nadler and Bartlett, 1997; Pomerantz, 1990). These noncontact erections (NCEs) are presumed to reflect sexual arousal (Nadler and Bartlett, 1997; Sachs, 2000) and therefore could serve as a model to study the neurobiology of human male sexual arousal (Nielsen et al., 2011). In support of this idea, rapid effects of androgen receptor activation in the medial amygdala were found to play an important role in the expression of NCE, involving these effects in male rat sexual arousal (Bialy et al., 2011) and the amygdala is among the brain regions consistently activated during male sexual arousal in humans, as determined by fMRI studies. Moreover, concurrent activation of this brain region and the insula by sexual cues and penile turgidity was proposed to reflect the emotional component of sexual arousal (Kuhn and Gallinat, 2011). Penile erections evoked by estrous female odors, in the absence of female rats, have also been considered to reflect male sexual arousal (Nielsen et al., 2011). Using NCE as a model of sexual arousal, it was established that sildenafil (Viagra®), used widely used to treat erectile dysfunction in humans, has not only peripheral actions, but facilitates sexual arousal by acting in the central nervous system, enhancing dopaminergic and serotonergic transmission in the nucleus accumbens (NAc) and medial preoptic area (mPOA) (Kyratsas et al., 2013). These data suggest that the NCE model is useful to study the central component of sexual arousal.

Penile reflex tests

In typical penile reflex tests, male rats are placed supine in a tube with their penile sheaths held back and the penis retracted. The penis then shows a characteristic pattern of tumescence and detumescence accompanied by "quick flips" (quivers following full erection) and "cups" (a flaring of the glans penis thought to be analogous to ejaculation). Latency to erection and the frequency of these different reflexes are counted following an experimental treatment, (e.g., systemic or central drug administration, brain lesions, or lesions of different nerves that innervate the pelvic ganglia). Many treatments that enhance penile erection in men (e.g., sildenafil, apomorphine, prostaglandin E1, oxytocin, a2-adrenergic receptor antagonists like yohimbine, idazoxan and imiloxan, and vasodilators that act through NO substrates, such as nitroglycerine, sodium nitroprusside, and linsidomine) enhance penile erection in rats (reviewed in McKenna, 1999; Meisel and Sachs, 1994). Erectile responses to these drugs are also studied by monito-

ring intracavernosal pressure with electrodes (e.g., Andersson et al., 1999; Bernabe et al., 1999). Conversely, drugs that reduce sexual arousal in men, e.g., dopamine antagonists such as haloperidol (Crenshaw and Goldberg, 1996), also reduce penile reflexes in rats (Pehek et al., 1988). However, selective activation of different dopamine receptors in the mPOA alters autonomic arousal differently, with selective agonist actions on D1 receptors facilitating erections and inhibiting seminal emissions in male rats, and selective actions on D2 receptors having the opposite effect (Hull et al., 1989). This suggests that the erectogenic actions of apomorphine in rats occur through its binding to D2 receptors in this or other brain regions.

Conditioned sexual arousal in the male

Contextual cues that are always associated with sexual pleasure or reward, drug reward, or stress, can induce more vigorous copulatory behavior in male rats. This has been shown in places associated with morphine reward (Mitchell and Stewart, 1990) and in the induction of a somatosensory fetish where a rodent tethering jacket is worn during a male rat's first sexual experiences to ejaculation (Pfaus et al., 2013). In the latter study, males trained to copulate while wearing the jacket showed deficits in copulatory measures of arousal (e.g., increases in intromission and ejaculation latencies, decreased number of ejaculations) with the jacket off relative to males with the jacket on (Figure 3). Penile reflexes are also more likely to occur in chambers previously associated with the ejaculatory reward state in male rats (Sachs and Garinello, 1978).

Female sexual arousal. Increased genital blood flow during normal arousal in women causes clitoral tumescence and a lubrication-swelling response of the lower third of the vagina producing fluid transudations through the vaginal wall (Traish, 1999). The peripheral models of female sexual responses involve monitoring changes in clitoral blood flow, vaginal secretions, temperature, and blood flow, as well as monitoring smooth and striated muscle contractions of the vagina, uterus, anal sphincter and pelvic floor muscles (Giraldi et al., 2013; Munarriz, 2003). The female genital tract is innervated by adrenergic, cholinergic, peptidergic, and NO synthase-containing nerves. Animal studies in rats and rabbits have shown the presence of vasoactive intestinal polypeptide (VIP), nitric oxide synthase (NOS; producing NO), neuropeptide Y (NPY), calcitonin gene-related peptide (CGRP), substance P (SP), as in males (Giraldi et al., 2013; Munarriz, 2003; Giuliano et al., 2010). Nerve branches of the pudendal nerve form the dorsal clitoral nerve have a similar function to the dorsal nerve of the penis and evidence for endothelial and neuronal NO pathways have been documented

(Musicki et al., 2010; Olivier et al., 2014; Tajkarimi and Burnett, 2011). However, in clinical studies, sildenafil did not improve sexual function or significantly increase clitoral engorgement in women with female sexual arousal disorder (Alexander et al., 2011; Leddy et al., 2012). In cell cultures from human and rabbit vaginal smooth muscle, studies of the signal transduction molecules cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) suggest that these signal transduction pathways are of importance for regulation of vaginal smooth muscle tone (Traish, 1999). Furthermore, a recent study showed that cAMP/PDE4 system may be of similar functional importance as the NO/cGMP/PDE5 pathway for neurovascular genital responses of the female rat (Castiglione et al., 2013). Stimulation of the pelvic nerve in anaesthetised female rats (Giuliano et al., 2001a; Vachon et al., 2000) rabbits (Munarriz et al., 2003) and as well in dogs (Angulo et al., 2001) has been shown to increase vaginal blood flow, clitoral ICP, vaginal wall pressure, vaginal length and blood flow, and decreases vaginal luminal pressure.

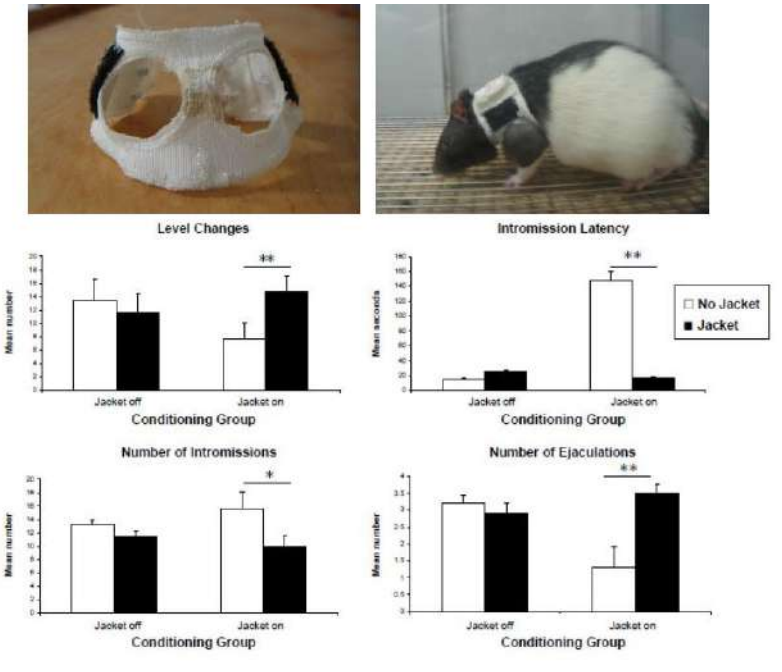


Figure 3. Top: Rodent tethering jacket used to condition sexual arousal in Long-Evans male rats. Bottom: Mean number of conditioned level changes, intromission latency, number of intromissions, and ejaculation latency (+SEM) of male rats trained with or without the jacket (conditioning group, jacket off or jacket on). Data are taken from the final test in which males from both conditioning groups either did not wear the jacket (white bars) or did wear the jacket (black bars). Modified with permission (Pfaus et al., 2013).

Models of Sexual "Desire"

Clinicians and motivational theorists alike view desire as distinct from arousal in both animals and humans. This distinction generally reflects blood flow to the genitals and erectile tissues (which people may or may not be consciously aware of) versus "psychological" sexual interest in which individuals "want" or "crave" sex (with wanting and craving defined here as in Robinson and Berridge, 1993, for drugs of abuse). This has been maintained for decades in subsequent incarnations of the DSM and ICD that categorize sexual arousal disorder (SAD) distinct from hypoactive sexual desire disorder (HSDD). However, in practice desire may well be informed or even confirmed by the presence of autonomic and central responses that define arousal, and people often regard desire and arousal as parts of one another, despite being given distinct definitions (e.g., Basson, 2002; Sarin et al., 2013; Toledano and Pfaus, 2006). This has led to controversy in the current DSM-5 (2013), which views disorders of sexual arousal and interest in women as blended phenomena (called female sexual interest and arousal disorder, or FSIAD), but leave them distinct for men. Disorders of sexual desire can stem from either a lack of sufficient excitation in response to sexual cues, overabundance of inhibition in response to such cues, or a mix of the two (Bancroft and Janssen, 2000; Pfaus, 2009; Sarin et al., 2013).

Sexual desire has been further subdivided theoretically into "spontaneous" versus "receptive" forms (Basson, 2003). The former is reminiscent of older concepts of "drive" (e.g., Hull, 1943), and is thought to occur as an emergent property of internal events (e.g., hormone actions) that suddenly create a conscious realization of being "horny", without any obvious external cue or incentive acting as a trigger for the response. The latter is more obviously related to unconditioned or conditioned cues that are sexually-relevant acting as the trigger(s) (Georgiadis et al., 2012), and to more modern concepts of incentive motivation (e.g., Bindra, 1959; Toates, 1988; 2014). In humans, unconditioned sexual stimuli include genital tactile stimulation and visual/auditory sexual stimuli, although some studies suggest that chemosensory signals like pheromones are involved in human sexual attraction (Lubke and Pause, 2015). In rats the unconditioned sexual stimuli include pheromones, odors, and auditory vocalizations (Pfaus et al., 2012). Animal studies suggest that sexual desire (sexual incentive motivation) is stimulated by dopamine and melanocortins in both mesolimbic and incertohypothalamic circuits, sexual attraction by oxytocin in the hypothalamus and limbic system, and sexual arousal by noradrenaline in hypothalamic, limbic and cortical regions (Pfaus, 2009). In humans, visual sexual stimulation activates different brain regions including the ventral striatum (meso-

limbic circuit), amygdala and anterior cingulate cortex (limbic system) and orbitofrontal cortex (cortical regions) (Childress et al., 2008), and the response to sexual incentive stimuli depends critically on mesolimbic dopamine activity (Georgiadis et al., 2012; Oei et al., 2012), as it does in rodents and virtually all other vertebrates.

Stimulation of sexual desire

Sexual desire requires the coordinated actions of the hypothalamus, limbic system, and cortex, within which dopamine, melanocortins, and oxytocin play key roles in its stimulation in both males and females. Sexual desire in males of a variety of species produces behavioral activation that can be expressed in terms of facilitated motor activity (e.g., nose pokes through a wire-mesh screen, level changes in a bilevel chamber, maze running and partner preference) and operant responses made in anticipation of the arrival of a sexually receptive female or a stimulus (e.g., odor or visual cue) associated with sexual reward. Desire can also be expressed in the willingness to pursue a sexually receptive female and the speed with which the male engages in copulatory activity after it has been solicited by the female. Females show similar and complementary responses that can be taken as measures of desire. At least three important behaviors have emerged in animal models of female desire: approaches, solicitations, and the inhibition of rejection responses. Use of animal models allows researchers to focus on pharmacological and molecular mechanisms of action for these behavioral phenomena.

As mentioned above, there are several compounds and formulations either approved or currently in clinical trials for the treatment of HSDD/FSIAD. Flibanserin was the first drug approved by the US FDA in 2016 for the treatment of HSDD in premenopausal women under the trade name Addyi®. It is a mixed agonist/antagonist at 5-HT1A and 5-HT2A receptors, and its pharmacology and behavioral effects have been examined in female rats. The drug increases solicitations in OVX female rats maintained on estradiol and progesterone or estradiol alone (Figure 4), but only after approximately 21 days of daily administration (Gelez et al., 2013). This time course coincides with a decrease in serotonin in the prefrontal cortex, along with increases in noradrenaline and dopamine in the medial prefrontal cortex, and dopamine in the mPOA (Allers et al., 2010). Chronic treatment with flibanserin also resulted in Fos activation of the NAc, mPOA, arcuate nucleus, ventral tegmental area, locus coeruleus, and lateral paragigantocellularis (Gelez et al., 2013).

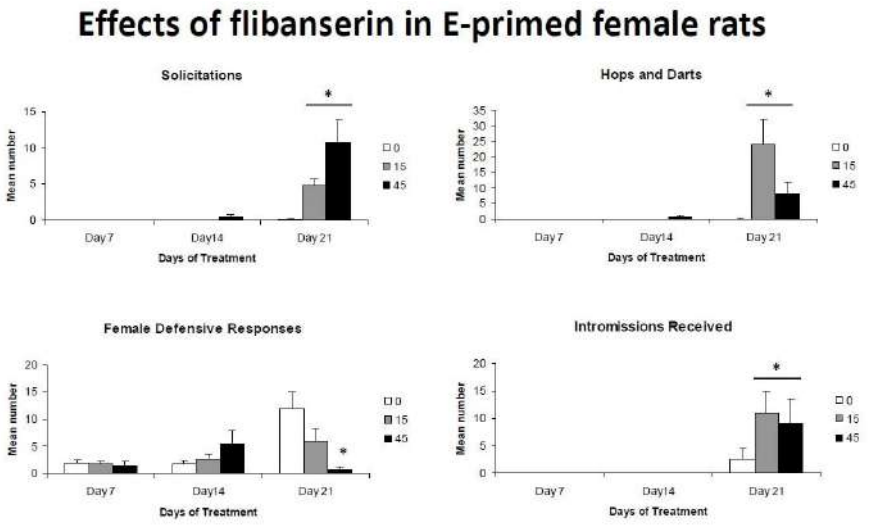


Figure 4. Chronic effects of flibanserin (Addyi®) on solicitations, hops and darts, rejection responses, and the number of intromissions received in OVX Long-Evans rats primed with estradiol benzoate alone. Flibanserin (0. 15, or 45 mg/kg) was administered sc daily and tests conducted at 7-day intervals. Data are means + SEMs. From (Gelez et al., 2013), with permission.

The MC4 receptor agonist bremelanotide was the second drug approved by the US FDA for the treatment of HSDD in premenopausal women in 2019 under the trade name Vyleesi®. It was tested extensively in both female and male rats to establish its behavioral effects and mechanisms of action (Pfaus et al., 2003; 2007). Acute systemic (sc) administration of bremelanotide to OVX female rats produces a dramatic increase in approaches and solicitations, and a decrease in rejection responses, regardless of whether they have been primed with estradiol and progesterone or estradiol alone (Figure 5).

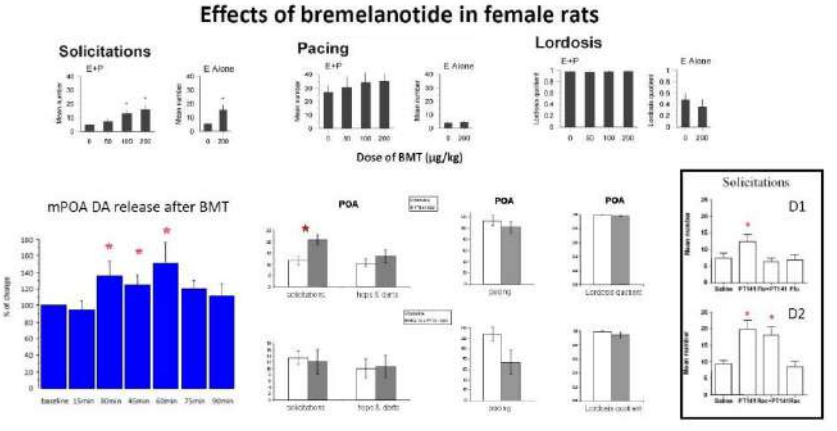


Figure 5. Top: Effects of acute sc injections of bremelanotide on solicitations, pacing, and lordosis in OVX Long-Evans rats primed with estradiol benzoate and progesterone (E+P) or estradiol benzoate alone (E alone). Bottom (left to right): Effects of 200 µg/kg on extracellular concentrations of dopamine in the mPOA. Effects of infusions to the mPOA on solicitations, pacing, and lordosis (facilitation of solicitations was reversed by sc injections of the selective MC4 antagonist HS014). Facilitation of solicitations by bremelanotide reversed by a D1, but not D2, receptor antagonist. Data are means + SEMs. From Pfaus et al., (2007) with permission.

The drug also increases erections (both spontaneous and noncontact) and conditioned level changes in male rats (Figure 6).

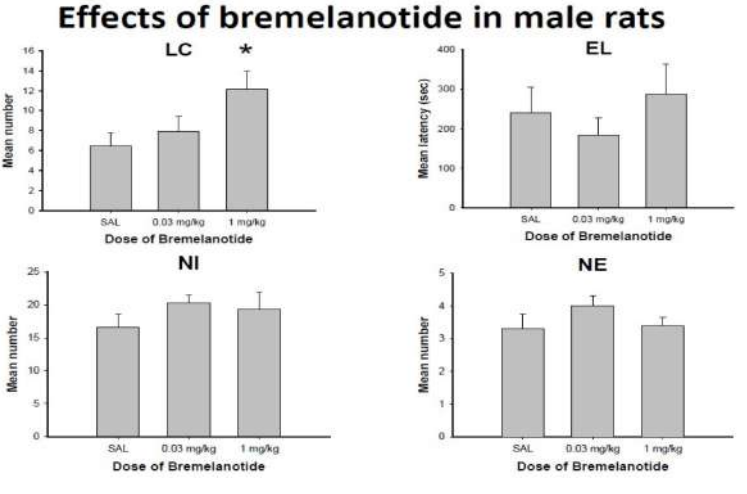
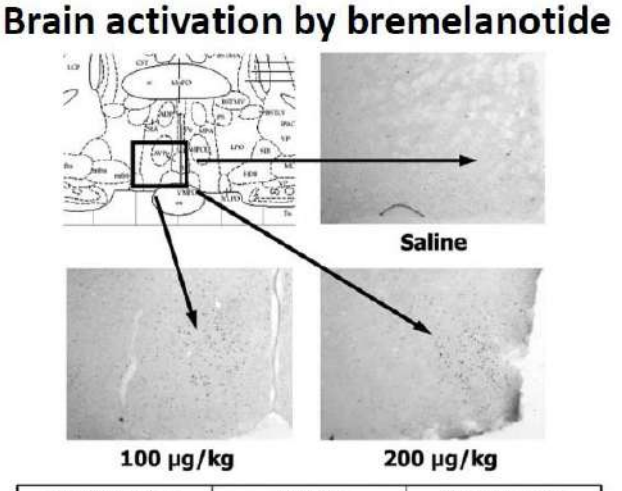


Figure 6. Effects of acute sc injections of bremelanotide on conditioned level changes (LC), ejaculation latencies (EL), number of intromissions (NI), and number of ejaculations (NE) in sexually experienced Long-Evans male rats during a 30-min test. Data are means + SEMs (N=12 males/dose). Unpublished.

It works through actions on MC4 receptors in the mPOA, which leads to a selective release of dopamine, which in turn acts on D1 receptors to activate appetitive sexual responses along with erections. It can induce these effects by direct infusions to the mPOA, and the effects are reversed by co-administration of HS014, an MC4 receptor antagonist, but also by administration of a D1, but not D2, dopamine receptor antagonist (Pfaus et al., 2007). The drug also activates Fos protein (as a marker of neuronal activation) within the nucleus accumbens, mPOA (Figure 7), lateral hypothalamus, basolateral amygdala, and ventral tegmental area (Pfaus et al., 2007).



Brain Region	Activity	Significance
mPOA	+++	Solicitation (Proceptivity)
VTA	+++	Incentive Salience Attention
NAc	+++/++ shell/core	Incentive Salience Attention
PFC	+++	Conditioned Arousal
BLA	+++	Reward-Related Learning
VMH	-	Lordosis

Figure 7. Top: Induction of Fos protein in the mPOA by acute sc injections of saline or bremelanotide. Bottom: Relative induction of Fos protein by acute sc injections of bremelanotide within the mPOA, ventral tegmental area (VTA), nucleus accumbens (NAc), prefrontal cortex (PFC), basolateral amygdala (BLA), and ventromedial hypothalamus (VMH) in OVX female rats primed with E+P. From (Pfaus et al., 2007), with permission.

Although bremelanotide had an excellent safety profile in both rats and humans, a concern of the regulatory agencies was whether its use to treat HSDD in women might lead to hypersexuality and concomitant destabili-

zation of established relationships. Bremelanotide was then tested in female rats using the conditioned partner preference paradigm. Females were trained to associate a scented male with rewarding paced copulation. Then, on a final test, females were injected with bremelanotide or saline and placed into an open field with two tethered males, one scented and the other unscented, and allowed to copulate freely for 30 min. Instead of random copulation with both males, females injected with bremelanotide solicited and copulated significantly more with their preferred (scented) male relative to the saline-injected females (Figure 8). Bremelanotide remains the only compound where the "proof of concept" was established first in rats before human clinical trials commenced.

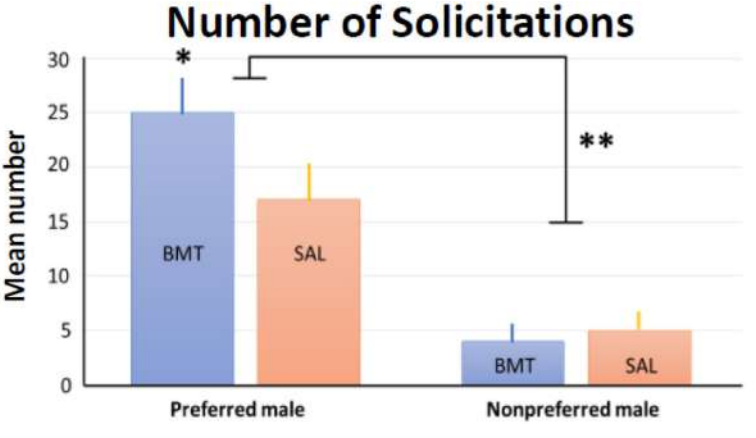


Figure 8. Acute effect of sc injections of saline or bremelanotide (200 µg/kg) on the number of solicitations made by females toward their preferred (scented) versus nonpreferred (unscented) males. Data are means + SEMs. Unpublished.

More compounds are currently in clinical trials for low desire in women. These include Lybrido® (a combination of testosterone and sildenafil) and Lybridos® (a combination of testosterone and buspirone), both of which require a delay of approximately 4 hrs for testosterone to prime neurochemical systems sufficiently for the combination to increase sexual desire and interest in women viewing erotic visual stimuli (Bloemers et al., 2013; Poels et al., 2013; 2014; Tuiten et al., 2001; van Rooij et al., 2013; 2015). Lybrido® is directed to women with a more "bottom-up" problem where arousal may not register sufficiently to influence desire, whereas Lybridos® is directed to women with too much "top-down" inhibition of desire (presumably by increased serotonergic activation of executive function). In OVX rats primed with sub-threshold doses of EB and a normal dose of P (to produce low-to-moderate sexual responding), acute systemic treatment with testos-

terone propionate (TP, 4 hr before tests) and the PDE-5I vardenafil (Snoeren et al., 2011) 1 hr before the test increased precopulatory hops and darts (proximate solicitations) and lordosis quotients significantly relative to TP alone or the drugs alone. Intracerebroventricular (icv) administration of vardenafil had no effect, indicating that an increase in genital blood flow was a critical feature of the drug effect in combination with TP.

Other compounds currently in clinical trials for HSDD include Lorexys® (a mixture of bupropion and trazodone) and a peptide called BP-101. Although trazodone has not been examined to date in female rats, acute bupropion administered sc to hormonally primed females increased the number of ejaculations male rats achieved with them, suggesting a possible increase in attractivity (López et al., 2007). Subsequently, Ventura-Aquino and Fernández-Guasti (2013) found that chronic, 14-day ip administration of bupropion increased proximal solicitations (hops and darts) and ear-wiggling, in OVX rats primed with EB alone, indicating an increase in both attractivity and sexual motivation. Acute intranasal infusions of BP-101 increased both solicitations and lordosis in OVX rats primed with EB alone. Like bremelanotide, this effect was also induced by infusions directly to the mPOA, but not by infusions to the olfactory bulbs, VMH, or VTA (Andreev-Andrievskiy et al., 2017).

In sexually experienced males, treatments such as the presentation of a new female for each ejaculatory series (stimulating the "Coolidge effect") or systemic administration of low-to-moderate doses of sympathomimetic drugs such as cocaine can increase the number of intromissions and ejaculations and decrease the latency to initiation copulation (Pfaus et al., 1990). The dopamine receptor agonist apomorphine also increases conditioned level changes in male rats (Pfaus and Phillips, 1991; Pfaus, 2009). As noted above, increases in conditioned level changes have been observed in male rats following bremelanotide. Taken together with the apomorphine data, it is likely that increased conditioned level changes is induced by augmented dopamine turnover in the mPOA and NAc.

Inhibited sexual desire. As mentioned above, in the DSM-5, HSDD was split into male HSDD for males and FSIAD for females. Male HSDD is defined as "persistently or recurrently deficient (or absent) sexual/ erotic thoughts or fantasies and desire for sexual activity" of which several subtypes are described. The lifelong HSDD subtype implies, by definition, that low or no sexual desire has always been present, a condition resembling the so-called non-copulating (NC) rat. This is a subpopulation of males that can be found in all rat strains and several other mammalian species (Clark and Galef, 2000; Harding et al., 2010). The males are otherwise healthy but will

not copulate despite repeated exposure to sexually receptive females (Anderson, 1936). NC males have intact erectile and ejaculatory functions (Stefanick and Davidson, 1987) and do not show any steroid hormone deficits (Whalen et al.,1961).

There are several ways to transform these sexually-impaired males into sexually competent copulators. One is by the application of unpredicted, low-level footshock (the "Dud-to-Stud" phenomenon; Crowley et al., 1973). Presumably footshock activates both autonomic and central arousal states, which in turn focus the male's attention on salient sexual cues of the female partner. Copulation can also be stimulated in the NC male by pharmacological treatments that either inhibit endogenous opioid systems (e.g., the opioid antagonist naloxone; Pfaus and Wilkins, 1995) or that stimulating the endocannabinoid system with the endogenous ligand anandamide (Canseco-Alba and Rodríguez-Manzo, 2013). Anandamide-induced copulation in NC males can be a long-term effect, when a sexual interaction takes place under the influence of this treatment (Canseco-Alba and Rodríguez-Manzo, 2019a). These results point to the existence of brain mechanisms that inhibit sexual arousal and attention to unconditioned incentive cues. Interestingly, NC males have reduced estrogen receptor density in their mPOA (Clark et al.,1985) and increased enkephalin content in the hypothalamus (Rodríguez-Manzo et al., 2002) relative to males that copulate (Clark et al., 1985). This rat population represents one "natural" model for the study of the neurobiological bases of inhibited sexual desire and as such, potentially useful for the understanding of human male lifelong HSDD. Indeed, a controlled clinical trial recently showed that sexual inhibition is reduced by sexual arousal both in men and women, an effect that might be involved in desire – or drug – driven engagement in sexual risk behavior (Imhoff and Schmidt, 2014; Pfaus et al., 2010).

Sexual refractoriness and inhibited desire. Another important observation is that if sexually competent male rats are allowed to copulate to sexual exhaustion, the majority of the males will not copulate for the next 48 hours (Rodríguez-Manzo and Fernández-Guasti, 1994) and require a 15 day-period of sexual rest to recover their initial sexual capacity (Rodríguez-Manzo et al., 2011). This phenomenon has been used as an animal model to study the neurobiological mechanisms underlying the triggering and maintenance of a sexual inhibition that probably represents an adaptive mechanism developed to protect the individual from potential disadvantages of prolonged engagement in sexual activity (Bancroft, 1999). This inhibition is due to neurochemical events and brain plastic changes triggered by repeated ejaculation within a short period (Rodríguez-Manzo et al., 2011;

Rodríguez-Manzo, 2015) and the mesolimbic dopamine system appears to be one of the main brain regions experiencing these plastic changes (Fiorino et al., 1997; Garduño-Gutiérrez et al., 2013b; 2013c; Rodríguez-Manzo et al., 2021).

Several of the drugs that can partly or fully reverse this inhibitory state, interact directly or indirectly with this system, which appears to be a final common pathway for satiety reversal (Pfaus, 2009; Rodríguez-Manzo, 1999). Particularly, the glutamate receptor antagonists ketamine, MPEP and CNQX (which reduce glutamatergic transmission by blocking NMDA, AMPA and mGluR5 receptors, respectively) (Rodríguez-Manzo, 2015), the opioid receptor antagonists naloxone and naltrexone (which block the binding of endogenous opioids) (Rodríguez-Manzo and Fernández-Guasti, 1995a; Garduño-Gutiérrez et al., 2013a; Garduño-Gutiérrez et al., 2013c), the endocannabinoids anandamide and 2-AG, both CB1 cannabinoid receptor ligands which inhibit GABA and glutamate release (Figure 9; Canseco-Alba and Rodríguez-Manzo, 2014; Canseco-Alba and Rodríguez-Manzo, 2019b), the GABA-A receptor antagonist bicuculline (Rodríguez-Manzo and Canseco-Alba, 2017), the nonspecific dopamine receptor agonist apomorphine and the D1-like dopamine receptor agonist SKF38393 (both activating D1-like dopamine receptors) (Guadarrama-Bazante et al., 2014) have been shown, or are candidates, to act at the mesolimbic system to reverse satiation (Figure 10; Garduño-Gutiérrez et al., 2013b; 2013c; Canseco-Alba and Rodríguez-Manzo, 2016; Rodríguez-Manzo and Canseco-Alba, 2017; Guadarrama- Bazante and Rodríguez-Manzo, 2019).

In addition, the a2-adrenergic receptor antagonist yohimbine (which increases noradrenergic tone) interacts with dopamine to reverse satiation (Rodríguez-Manzo, 1999) and the 5-HT1A receptor agonist 8-OH-DPAT (which decreases serotonin release) requires intact noradrenergic transmission to reverse this inhibitory state (Rodríguez-Manzo and Fernández-Guasti, 1995b).

Besides, in males that received naloxone or the cannabinoid CB1 receptor antagonist AM251 during the copulation to satiety process, the triggering of the sexual inhibitory state was prevented (Garduño-Gutiérrez et al., 2013a; Canseco-Alba and Rodríguez-Manzo, 2014). AM251 also prevented the sexual inhibitory state when infused directly into the VTA (González-Morales and Rodríguez-Manzo, 2020). This suggests that sexual satiety or refractoriness induces a state of inhibited desire to engage in sexual activity and could be studied from the stand point of pharmacological or behavioral treatments that prevent or reverse this inhibition.

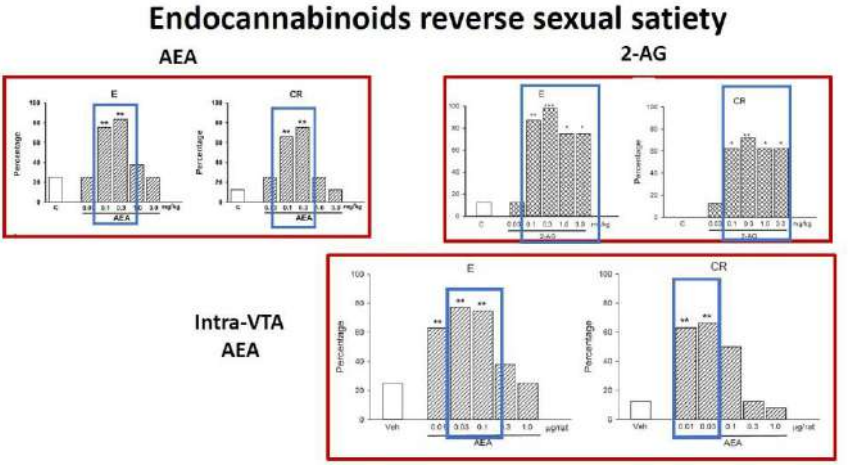


Figure 9. Reversal of sexual satiety by the endocannabinoids anandamide (AEA) and 2-AG. Top: Increase in the proportion of sexually satiated males showing ejaculation (E) and resuming copulation after ejaculation (CR) in response to the acute ip injection of different doses of AEA or 2-AG compared to vehicle injected control animals (C). Bottom: AEA direct infusion into the ventral tegmental area (VTA) also reverses the sexual inhibition of the sexually satiated rats as compared to vehicle. From Canseco-Alba and Rodríguez-Manzo, 2014; 2016; 2019b with permission.

Female rats show a similar kind of refractory state induced by large amounts of vaginocervical stimulation (VCS) during copulation or manual stimulation with a glass rod (Pfaus et al., 2000). Appetitive sexual behaviors like solicitation are the most sensitive to inhibition by those treatments, and as the intensity of the inhibition progresses, females display increases in pacing and defensive behaviors and a decrease in high-intensity lordosis. The inhibition has been traced to the activation of glutamate neurons in the ventrolateral VMH that are inhibited by estradiol and facilitated by VCS (Georgescu and Pfaus, 2006a; 2006b; Georgescu et al., 2012; 2009; 2014; Kow et al., 1985). Interestingly, systemic administration of drugs like bremelanotide, that stimulate sexual desire in female rats, also inhibit an electrophysiological glutamate signature in the ventrolateral VMH (Booth et al., 2010).

Pharmacological reversal of sexual satiety at the mesolimbic system

Brain region	Drug	Pharmacological action	Neurotransmitter system	Effect	Reference
VTA	AEA	CB1R agonist	Endocannabinoid	Reversal	Canseco-Alba & Rodríguez-Manzo 2016
	Bicuculine	GABA-A antagonist	GABAergic	Reversal	Rodríguez-Manzo & Canseco-Alba 2017
	Naltrexone	MOR antagonist	Opioidergic	Reversal	Garduño-Gutiérrez et al 2013c
NAc	Apomorphine	D1R/D2R agonist	Dopaminergic	Reversal	Guadarrama-Bazante & Rodríguez-Manzo 2019
	Quinpirole	D2R agonist		Reversal	

Haloperidol cancels endocannabinoid-mediated reversal of sexual satiety

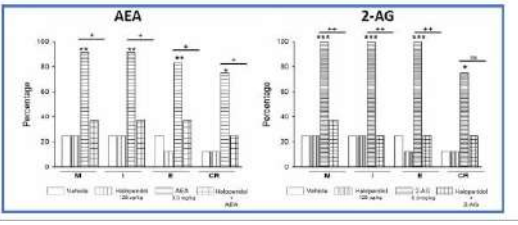


Figure 10. Reversal of sexual satiety at the mesolimbic system. Top: Pharmacological treatments reversing sexual satiety when directly infused into the ventral tegmental area (VTA) or the nucleus accumbens (NAc). Bottom: Reversal of sexual satiety by the systemic injection of an effective dose of AEA or 2-AG (activating CB1 cannabinoid receptors), is blocked by coadministration of a low dose of the dopamine receptor antagonist haloperidol, evidencing the interaction the dopaminergic transmission in their effects. From Canseco-Alba and Rodríguez-Manzo, 2019b.

Conditioned inhibition of sexual desire. Inhibition can also be induced in both female and male rats by stimuli associated with sexual nonreinforcement or nonreward. In one paradigm (Kippin et al., 1998; 2001), sexually nonreceptive female rats were scented with a neutral olfactory cue (almond or lemon odor). Males received 10 sequential trials each with sexually receptive females (unscented) and nonreceptive females (scented) every 48 hrs during the middle third of their dark circadian cycle. Although most males attempted to mount the nonreceptive females on the first trial, their attempts were met with intense rejection responses and fighting, which thwarted their attempts. By the 5th or 6th trials, males were no longer attempting to copulate with the nonreceptive females. A final test was conducted in a large open field with two sexually receptive females, one scented and the other unscented. Despite both females being sexually receptive and displaying solicitations and interceptions, the males avoided the scented females and mounted, intromitted, and ejaculated preferentially with the unscented females. This "frustration"-induced inhibition related to the odor could be disinhibited by a low dose of alcohol or cocaine (Pfaus et al., 2010).

A similar inhibition was induced in males by the opioid receptor antagonist drug naloxone (Ismail et al., 2006). In those studies, males were given access to sexually receptive females scented with almond odor. Prior to access, half the males were injected with naloxone or saline. The males received the opposite order of association with sexually receptive unscented females. These trials were done in sequence for a total of 10 trials in each condition. On the final test, males were placed into an open field with two sexually receptive females, one scented and the other unscented. As with frustration, males that received access to scented females during naloxone, avoided scented females during the final test and copulated preferentially with the unscented females.

Conversely, males that received naloxone during trials with unscented females avoided unscented females during the final test and copulated preferentially with the scented females. Naloxone infused directly into the mPOA of males during copulation with scented females induced this effect, whereas males infused with naloxone to the NAc showed no preference at all (Quintana-Zunino et al., 2019). Thus, frustration or nonreward induced by blocking opioid reward centers (e.g., in the mPOA and/or NAc) leads to an inhibitory state associated with the odor and concomitant avoidance of females bearing the odor despite their solicitations and sexual receptivity.

The same relationship between frustration and nonreward also works for females. Frustration can be induced in female rats by applying clitoral stimulation (CLS) with a paint brush in the presence of an inaccessible male scented with almond behind a wire-mesh screen (Parada et al., 2011). Females actively solicit the scented male following CLS but are not able to make contact or have their solicitations reacted to by active pursuit. Four days later females receive access to an unscented sexually vigorous male in the same chamber but with the wire mesh barrier removed so that she can copulate freely. On a final open field test with two sexually vigorous males, the female avoids the scented male and solicits and takes ejaculations preferentially from the unscented male. As in males, naloxone treatment during conditioning induces a state of sexual nonreward. Females treated with naloxone prior to paced copulation with scented males display few, if any, solicitations, low level lordosis, and active rejection responses by the 6th or 7th conditioning trial, despite being fully primed with estradiol and progesterone.

On the final open field test with two sexually vigorous males, one scented and the other unscented, females previously treated with naloxone solicit and receive ejaculations from the unscented male relative to the scented male. Females treated with saline during paced copulatory conditioning

with scented males solicit and receive ejaculations preferentially from the scented males (Coria-Avila et al., 2008). In fact, naloxone-treated females that copulate with unscented males eventually display sexual rejection responses toward unscented males that appear to be a permanent response to those males when the females are switched to saline and despite their full priming with estradiol and progesterone. Although previous studies have successfully utilized OVX rats primed with low levels of estradiol as a model of low desire, it may well be the case that acquired inhibition through non-reward in fully primed females may constitute a more ecologically valid model of hypoactive sexual desire in premenopausal women that do not have concomitant decreases in circulating hormone levels.

Models of Ejaculation, Orgasm, and Sexual Pleasure

In the 4-stage models of human sexual response, orgasm represents the apex of sexual response and is described as a "release" of sexual tension resulting in a "resolution" of refractoriness. Although in men, orgasm is typically regarded as synonymous with ejaculation, there are cases of ejaculation without orgasm and orgasm without ejaculation (Rowland et al., 2010). As noted above, ejaculation in males of other mammalian species resembles that of human males, and is typically followed by an absolute and relative refractory period. The spinal mechanisms that control sexual climax (which is experienced in the brain as orgasm) is similar in males and females (McKenna, 2001). Orgasms have been more difficult to study in women (Levin, 2004), although there are physiological responses such as vaginal "tenting," and characteristic pelvic floor muscle responses (Bohlen et al., 1982) and urogenital (UG) and cervico-uterine (Shafik) reflexes that accompany orgasm in women (Pfaus et al., 2016; Shafik et al., 2009).

The situation is similar in laboratory animals. Ejaculation occurs in virtually all male mammals and is accompanied by very obvious behavioral patterns that make it easy to distinguish from other copulatory behaviors. In contrast, orgasm-like responses have been described for some female macaque species (Goldfoot et al., 1980; Slob et al., 1986; Troisi and Carosi, 1998), but have yet to be adequately examined in females of other species. This is due, in part, to a general lack of obvious behavioral responses that could be considered analogous to those that occur in women. However, orgasm-like responses occur in female rats and can be distinguished on the basis of ultrasonic vocalization patterns (Gerson et al., 2019a) in response to CLS.

Ejaculatory reflexes

The urethrogenital reflex is a model system that allows the study of sexual reflexes, particularly ejaculation, in anaesthesized, spinally-transected rats (McKenna et al., 1991). In this model, the ejaculatory response is elicited by the mechanical distention of the urethra with saline injection, (mimicking the deposition of seminal secretions into the posterior urethra), while occluding the penile meatus. This stimulus triggers a rhythmic motor pattern in all genital muscles surrounding the male genital tract, controlled by a spinal pattern generator located at the lumbosacral spinal cord (Carro-Juárez et al., 2003). Ejaculatory patterns, similar to those of males have been identified in human and non-human female primates, as well as in female rats (Emery and Sachs, 1975; Marson et al., 2003), which exhibit an ejaculatory rhythmic motor pattern indistinguishable from the one registered in male rats (Carro-Juárez and Rodríguez-Manzo, 2006). This model system has been used to evidence the spinal effects of chronic treatment with different antidepressants on the ejaculatory response (Hueletl-Soto et al., 2010; 2012; 2014).

Models of anorgasmia or delayed ejaculation

In male rats, acute treatment with the selective serotonin reuptake inhibitor (SSRI) fluoxetine (FLU) dose-dependently delays ejaculation and increases the total number of mounts before ejaculation and lengthens the post-ejaculatory refractory period (Vega Matuszcyk et al., 1998b; Yells et al., 1994).

Furthermore, male rats that were chronically administered FLU at 10 or 20 mg/kg showed fewer ejaculations and a decrease in measures of sexual motivation relative to baseline (Cantor et al., 1998; Yells et al., 1994). In female rats, FLU decreased the motivation to interact with a sexual partner, quantified by the number of nose-pokes performed to gain access to a male counterpart (Uphouse et al., 2015). Also, FLU-treated females showed a decrease in the magnitude of the lordosis reflex, number of hops and darts, and solicitations (González Cautela et al., 2021; Vega Matuszcyk et al., 1998a). Because the effect of SSRIs in rats successfully mimics two of the most prominent symptoms of sexual dysfunction observed among humans (anorgasmia and decreased sexual desire), rats have been used as preclinical models to delineate both the basic neuropharmacology of SSRI effects on sexual behavior, and as proof of concept in testing potential treatments (Dulawa et al., 2004). Lesions of the nucleus paragigantocellularis in males (which contains serotonin neurons that project from the brainstem to

spinal cord) reversed the effects of FLU on ejaculation (Yang et al., 2013; Yells et al., 1994).

As with NC rats, some are also sexual sluggish and show a consistent pattern of long ejaculation latencies (Pattij et al., 2005). In this model of naturally occurring delayed ejaculation, a variety of drugs have been reported to facilitate ejaculation. Among them, the dopaminergic drugs L-DOPA and apomorphine (Tagliamonte et al., 1974), SND-919 and sildenafil (Giuliani et al., 2002), the ß-adrenoceptor agonist clenbuterol (Benelli et al., 1990), and the 5-HT1A receptor agonists 8-OH-DPAT and buspirone (Pattij et al., 2005; Chan et al., 2008). The sexually sluggish rat can be a useful model for the study of delayed ejaculation if the selection of this subpopulation includes only animals in which the occurrence of ejaculation, although delayed, is verified. Even though the DSM-5 definition of delayed ejaculation in humans includes both inhibited and retarded ejaculation, the neurobiological bases for inhibited ejaculation might be distinct from those underlying prolonged ejaculation latencies. Off-label pharmacotherapies used for delayed ejaculation include drugs that facilitate ejaculation by stimulating central dopaminergic or oxytocinergic mechanisms, by blocking central serotonergic transmission or by acting at peripheral adrenergic mechanisms. The majority of these drugs have limited efficacy and/or exhibit significant side-effects (McMahon, 2014). Recently, it was reported that acute injection of very low doses of the endocannabinoid anandamide (a retrograde messenger modulating the release of neurotransmitters from nerve endings) specifically restores the ejaculatory threshold of sluggish rats by reducing pre-ejaculatory intromission number (the amount of stimulation) and ejaculation latency (time needed to achieve ejaculation) values to those of normal sexually experienced rats (Rodríguez-Manzo and Canseco-Alba, 2015). This specificity suggests that this compound might be useful to treat lifelong delayed ejaculation in men. Anandamide's effects might involve the direct or indirect suppression of the release of neurotransmitters augmenting the ejaculatory threshold of sluggish rats, as has been observed for its ability to reverse sexual exhaustion.

Models of premature ejaculation

Premature ejaculation (PE) is the most common male sexual dysfunction. PE can be classified as primary (lifelong) or secondary (acquired) when it develops after a period of normal control of ejaculation. Intravaginal ejaculation latency time in men follows a continuum with positively skewed distribution (i.e., the mean is superior to the median) (Waldinger et al., 2005). Accordingly, there are men who have short ejaculation latency time refe-

rred to as PE, others delayed ejaculation, and a last category (the largest one) of men who ejaculate in a range of "normal" ejaculation time. Interestingly, a similar distribution of ejaculation latency has been described in male rats (Pattij et al., 2005), thus giving the opportunity to use a relevant animal model for preclinical researches. In practice, sexually experienced male rats are categorized according to their ejaculatory performance (number and latency of ejaculations) measured during a standardized copulatory test.

Further investigations in rapid ejaculating rats led to the identification of a neurobiological substrate for the variability in ejaculatory performance. It was shown, using the immediate early gene c-Fos level of expression as a marker of neuronal activity, that rapidly ejaculating rats displayed increased activity in the brain network involved in the control of ejaculation (Clement et al., 2012; Pattij et al., 2005). In addition, response of the spinal generator of ejaculation to stimulation was reported accelerated in rapidly ejaculating rats (Borgdorff et al., 2009). However, the causal link between ejaculatory performance and activity in the various components of the brain and spinal pathways of ejaculation remains to be clarified. The predictability of the rat model of premature ejaculation was recently supported by a pharmacological study showing that dapoxetine, the first registered treatment of PE, 'normalized' ejaculatory behavior (most notably ejaculation of the first latency) in rapidly ejaculating rats (Clement et al., 2012).

The ejaculation distribution theory establishes that the intravaginal ejaculatory latency time (IELT) in men consists of a biological continuum, with men with premature ejaculation belonging to the left extreme of the IELT curve (Waldinger, 2002). The biological variability in the ejaculatory behavior of male rats was described and ejaculation latency (EL) was found to follow a Gaussian distribution, with rapid and sluggish ejaculating rats represented at each of its ends (Pattij et al., 2005). This similarity led them to propose that male rats at the extremes of the ejaculatory spectrum might be used as animal models for the study of human premature or delayed ejaculatory disorders (Waldinger and Olivier, 2005).

Behavioral models of sexual pleasure

Sexual stimulation to ejaculation and orgasm induces a pleasurable (rewarding) state that is contingent on particular behaviors that lead to it (operants) and contextual and partner-related cues that predict it through Pavlovian conditioning (Pfaus et al., 2012; Quintana et al., 2022). Rats, for example, learn to run mazes (Whalen, 1961), nose-poke or press bars (Everitt and Stacey, 1987; Everitt et al., 1987; Everitt, 1990; Fitzpatrick et al., 1971;

Jenkins and Becker, 2003; Uphouse et al., 2015; Woodson and Balleine, 2002) for access to copulation with sex partners. Sexual reward can also be assessed using the conditioned place preference (CPP) or conditioned partner preference paradigms. For both operant and Pavlovian paradigms, ejaculation is critical for reward in males, whereas in females, their ability to initiate and control or 'pace" the rate of copulation with is critical for reward (reviewed in Pfaus et al., 2012). Indeed, CLS applied with a fine paintbrush to female rats in a manner that mimics paced copulation is capable of inducing CPP (Parada et al., 2010) and clitoral anesthesia disrupts the pattern of paced copulation (Parada et al., 2014). In males, ejaculation induces the release of endogenous opioids in the brain (Coolen et al., 2004; Szechtman et al.,1981), and treatment with the opioid receptor antagonist naloxone or naltrexone, but not dopamine receptor antagonists, during training blocks the conditioning of both place– and partner–related cues (Burkett et al., 2011; Ismail et al., 2009). Likewise, naloxone treatment during paced copulation trials blocks the conditioning of both place– and partner–related cues (Coria-Avila et al., 2007; Paredes and Martinez, 2001). In fact, naloxone treatment during a female rat's first sexual experiences induces a powerful inhibition of solicitations and lordosis and promotes defensive responses with males, despite hormonal priming that should induce a full complement of sexual responding (Pfaus et al., 2012). This pattern of behavior lasts long after naloxone treatment terminates, and may constitute a more ecologically valid model of inhibited sexual desire in premenopausal women without disrupted levels of circulating ovarian steroids.

Models of Sexual Pain

Although genital pain disorders like vulvodynia, clitorodynia, persistent genital arousal disorder/genitopelvic dysesthesia, and priapism affect millions of people, very few behavioral models of these disorders exist in animals. Genital pain has been induced in female mice by injections of inflammogens like zymosan A or -carrageenan (2%) into genital regions and compared with injections to nongenital areas like the hindpaw, tail, or cheek (Farmer et al., 2014). Sexual behavior was significantly reduced in fully primed female mice experiencing pain (in all combinations). However, male mice similarly treated displayed unimpeded sexual motivation. The pain-induced decrements in female sexual behavior and motivation could be restored by the analgesic drug pregabalin, and the desire-enhancing drugs apomorphine and melanotan-II.

CLS in female rats is generally rewarding when applied manually with a soft, natural fiber paintbrush in a manner that mimics the distributed ti-

ming of CLS that are normally "paced" or regulated during copulation with a male (Parada et al., 2010). However, paintbrushes with synthetic fibers are prickly and can irritate the clitoris. This induces powerful rejection responses and aversive vocalizations, and generalizes to a natural fiber paintbrush or any object that the experimenter uses to attempt to touch the clitoris. These rejection responses occur despite the females being fully primed with estradiol and progesterone (Gerson et al., 2019b). These behaviors could be utilized as an expectancy model of clitorodynia in the rat.

Summary

The advent of sildenafil heralded in a "third sexual revolution" of information about the physiology of sexual behavior. But even prior to this, sophisticated behavioral paradigms were being developed to optimize the recording of sexual responses in rodents and other species and the ability to observe more appetitive aspects of sexual interaction in both females and males (Erskine, 1985; McClintock, 1984; Mendelson and Gorzalka, 1987; Pfaus et al., 1990; Pfaus et al., 1999). The use of the CPP paradigm showed that both male and female rats found particular types of sexual stimulation rewarding (Agmo and Berenfeld, 1990; Martínez and Paredes, 2001; Paredes and Alonso, 1997; Paredes and Vazquez, 1999). The addition of partner preference paradigms helped to reveal the neurochemical substrates of sexual reward and how they are related to the stimulation of sexual desire and arousal. Thus, the innovative use of these behavioral paradigms offers researchers the opportunity to collect preclinical data that have a high degree of predictive validity to human sexual responses, and that translate – literally – from the bench to the bedside.

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Preclinical protocols in rodents for the development of antidepressant drugs

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Introduction

According to Malhi and Mann (2018), major depressive disorder (MDD) is a global public health issue that impacts the lives of millions of individuals, resulting in substantial disability and reduced quality of life. Despite the development of various categories of antidepressant medications, a significant number of patients fail to respond effectively to treatment, underscoring the need for new therapeutic options. In this chapter, we will briefly discuss two animal models used with success to identify potentially helpful antidepressant medications: the forced swim test (FST) and the chronic mild stress (CMS) models.

Forced swim test

Description

The forced swim test (FST), initially developed by Porsolt et al. in 1977, is a behavioral experiment widely used to screen the antidepressant potential of drugs. A rat or mouse is placed inside a Plexiglass® cylindrical tank (40 to 46 cm height, 18 to 20 cm diameter for rats; 24 cm height, 13 cm diameter for mice) from which it cannot escape (Porsolt et al., 1977; Cryan et al., 2005). The tank is filled with water up to a certain height so that animals cannot touch the bottom of the cylinder (originally 15 cm for rats, but later modified to 30 cm; 10 to 15 cm for mice, depending on the animal's size) (Porsolt et al., 2001; Cryan et al., 2005). The water temperature is usually 22 to 25 °C, but some have used 30 °C to prevent hypothermia.

As originally described, the FST for rats consists of two sessions. The first (pretest) lasts for 15 minutes, while the second (the test itself) takes place 24 h after the first and lasts only 5 minutes. During the pretest, the animal

usually shows marked activity by swimming and attempting to climb out of the tank. However, after several minutes it finally stops trying to escape, and remains essentially immobile, only making the necessary movements to stay floating. This immobility has been interpreted as "behavioral despair" or "lowered mood" due to an inescapable situation (Porsolt et al., 1977; Cryan et al., 2005). The rodent strain used strongly influences baseline immobility (Cryan et al., 2005). The first session aims to ensure that the rats will adopt an immobile behavior in the second session, thus facilitating the evaluation of the effects of putative antidepressant drugs (Porsolt et al., 1977; Cryan et al., 2005). The suspected antidepressant drugs are administered sub-acutely (23.5, 5.0, and 1.0 h) before the test session (Porsolt et al., 1977 & 2001; Cryan et al., 2005). However, depending on the particular objectives, some researchers have omitted the pretest session and applied the antidepressants in an acute (<12 h) or chronic (days) schedule before the single (test) session (Bourin et al., 2004; Rodríguez-Landa et al., 2007; D'Aquila et al., 2010; Li et al., 2013). For mice, the FST consists of a single 6-minute session, in which the first 2 minutes are the pretest, while the last 4 min are used to obtain the test measurements (Porsolt et al., 2001). As in rats, mice receive drug treatments before the FST.

Analysis and interpretation of behavioral changes in the FST

Usually, the experiments are videotaped for later scoring by a person blinded to the treatment used in each animal. In the modified version of the FST (water depth = 25 cm), the period of active motion involving escape strategies (swimming, climbing), as well as the immobility (floating) time, are recorded (Cryan et al., 2005). The previous application of antidepressants makes animals subjected to inescapable stress move more than those animals in the control group, thus spending less time immobile. A comparison of times spent swimming, climbing, or being immobile is made between the treated and control animals. Substances that yield a positive response may undergo additional tests before being used clinically.

Drugs that induce a reduction in the immobility time (and thus increase the swimming or climbing time) in the test session are the ones that may eventually turn out to be antidepressants in the clinical setting. Of course, drugs that increase overall mobility may result in false positives in the FST (Cryan et al., 2005). For this reason, the FST should be preceded by the open field test (OFT), which allows the measurement of drug-induced changes in general locomotion. Therefore, the drug of interest is regarded as a potential antidepressant only if it reduces immobility and increases swimming or

climbing of the rodent in the FST while it reduces or does not change locomotion in the OFT (Cryan et al., 2005; Hao et al., 2019).

The FST validity is sustained by the learned helplessness theory, which states that animals develop a maladaptive passiveness when subjected to an uncontrollable stressful situation (Willner, 1986). The critical factor for developing learned helplessness is the lack of control that subjects have on avoiding the aversive condition rather than the stressful stimulus itself (Willner, 1986). This idea is endorsed by the classic triadic experimental design:

1. A group receives the aversive stimulus but can finish this condition by performing a task.
2. A second group (linked to the first group) receives the same aversive stimulus but does not have control over it.
3. A third group does not receive any aversive stimulus.

In this paradigm, about two-thirds of the animals in the second group cease their attempts to escape from the aversive stimulus; that is, they develop learned helplessness, while animals of the first group do not (Willner, 1986). Thus, in the FST, the uncontrollability is given by the incapacity of the animals to escape from the water tank.

Construct validity of the FST

The construct validity of a model depends on the demonstration that it has high homology with the disorder that it intends to represent, providing empirical evidence that the theoretical foundations of the construct are the same or very similar to those of the pathology (Willner, 1986; van der Staay, 2006).

In the case of the FST, the construct validity is the weakest of all criteria because the situation of inescapable stress that triggers the immobility of the animal bears poor resemblance with the stressful situations that usually precede the episodes of major depressive disorder (MDD). Although many processes underlying the etiology of MDD remain unknown, there is strong evidence that the onset of depressive crises is significantly associated with the prior occurrence of life events that produce acute or chronic stress (Hammen et al., 2009; Horesh and Iancu, 2010). For example, a study of twin women (monozygotic and dizygotic) found that two-thirds of major depressive events occurring in one of the pair of monozygotic twins significantly associated (odds ratio 3.58) with having experienced within the same month any of 11 types of personal stressful life events, some of them uncontrollable (e.g., assault, divorce/separation, financial problems, job loss) (Kendler et al., 1999). Thus, although it is impossible to know the cognitive

meaning (e.g., the feelings) that a rodent assigns to its subjective experience during the FST (Willner, 1986; Commons et al., 2017), the increase of immobility during the second session (test) could indeed be conceptualized as a measure of the "despair behavior", resulting from the lack of control over the cause of the acute stress experienced during the pretest, which would be equivalent to the uncontrollable acute stressful events that precede MDD in humans (Kendler et al., 1999).

From the above, it emerges that the inescapable stressful situation experienced by rodents in the FST is not precisely homologous to the acute stressful events (some of them unavoidable) that usually precede the development of depression in humans; however, immobility could represent an element that partially supports the construct validity of the FST as an animal model of MDD, as occurs with other animal models of depression (Willner, 2016).

Face validity of the FST

For an animal model to have high face validity, it must closely resemble the disorder it intends to represent in terms of its etiology and pathophysiological mechanisms, particularly its symptomatology (Willner, 1986; van der Staay, 2006). However, although there are criteria to diagnose MDD in humans based on the presence and duration of specific symptoms (DSM-V, American Psychiatric Association, 2013), knowledge about its causes and the brain mechanisms involved in its development is still limited (Cryan et al., 2005).

Furthermore, many of the symptoms of MDD have a subjective component that can only be expressed verbally (e.g., excessive feelings of sadness, worthlessness, or guilt) (Commons et al., 2017). The above has been the main obstacle for developing an animal model that has high face validity with MDD. In the particular case of the FST, the main drawback is the interpretation of rodent immobility during the test, which was initially conceived as a manifestation of "lowered mood" (Porsolt et al., 1977), and subsequently translated as a "depressive-like state" (de Kloet and Molendijk, 2016). In contrast, other researchers contend that the immobility observed in the FST is merely a passive coping strategy in reaction to an inescapable stressor, which creates uncertainty in the interpretation of this behavior (Kloet and Molendijk, 2016; Commons et al., 2017). However, although seemingly opposed, these postures about what the FST is modeling could be complementary. Thus, there is evidence that individuals facing considerable stress report marked depressive symptoms, and this is significantly associated with a greater propensity to adopt passive stress coping strategies

(give up efforts to achieve goals, despair, or withdraw) than active coping strategies (planning the resolution of a problem, or taking an active behavior to deal with a stressor) (Chou et al., 2011; Orzechowska et al., 2013). Also, passive coping strategies (e.g., helplessness, resignation) for stress management have been significantly associated with greater MDD severity in human subjects (Drapeau et al., 2017; Holubova et al., 2017). Hence, the immobility of rodents in the FST could be regarded as a passive coping strategy (Cryan et al., 2005), which bears some resemblance to "psychomotor retardation (observable by others)" or to "fatigue and loss of energy", which are among the nine symptoms listed in the DSM-V for the diagnosis of MDD (American Psychiatric Association, 2013).

On the other hand, swimming and climbing could be viewed as active coping strategies to fight the stressful situation (Cryan et al., 2005), likely indicating that the despair-like state has subsided or has not emerged yet. Similarly, people who use active coping strategies report less severe depressive symptoms (Chou et al., 2011). Even so, the FST does not fully comply with the face validity criteria since it does not reproduce the sadness and anhedonia experienced by human subjects (Commons et al., 2017), which are the primary symptoms considered for diagnosing MDD (DSM-V). However, as van der Staay (2006) has stated, more than the poor face validity of a model is needed to nullify its usefulness.

Predictive validity of the FST

It is said that a model has predictive validity when it always yields a result consistent with the expected performance in the situation it intends to model (Willner, 1986; van der Staay, 2006). In the case of the FST, predictive validity would refer to the anticipation that the increase in the immobility of the rodents would be triggered not only by the inability to escape from a stressful aversive situation (pretest) but also by experimental manipulations that mimic the neuroendocrine changes that occur during high-stress situations. Thus, in both humans and rodents, exposure to stressful situations produces activation of the hypothalamic-pituitary-adrenal (HPA) axis, which usually returns to its basal state after resolving the stressful contingency (Dudek et al., 2021). However, when this does not occur, HPA hyperactivity is manifested, and could contribute to the development of MDD in susceptible individuals (Dudek et al., 2021). In line with the above, there is evidence that two-thirds of patients with MDD have elevated plasma levels of glucocorticoids (GC) and that there is also a subgroup with lower concentrations and milder depressive symptoms (Dudek et al., 2021).

In rodents exposed to the FST, increased immobility during the second swimming session (test) is linked to HPA hyperactivity since prior adrenalectomy prevents it from occurring (de Kloet et al., 2016). Furthermore, in adrenalectomized animals, the increase in immobility during the test can be restored if they receive treatment with the endogenous GC corticosterone or a synthetic one (dexamethasone) immediately after the pretest. The effect of GCs is specific since it is blocked with an anti-glucocorticoid (de Kloet et al., 2016). Altogether, the above evidence supports the phenomenological prediction validity of the FST.

However, what has contributed most to support the predictive validity of the FST is its ability to correctly identify the therapeutic potential of various medications for MDD, and even of non-pharmacological treatments that are effective in drug-refractory depression, such as electroconvulsive shock (Porsolt et al., 1977) and vagus nerve stimulation (Cunningham et al., 2008; Shin et al., 2019). All these treatments have the common ability to prevent the increase in the immobility of rodents during the FST test session. Nevertheless, false positives can also occur because drugs that increase general motility in animals (e.g., amphetamines) also reduce immobility in the FST (Porsolt et al., 1977), so it is necessary to assess whether candidate antidepressant drugs increase general locomotor activity in the OFT prior to performing the FST.

Antidepressant drugs whose primary mechanism of action is the inhibition of serotonin (5-hydroxytryptamine, 5-HT) and/or norepinephrine (NE) reuptake (including various tricyclic compounds), and that were used to treat patients with MDD for several decades before the advent of the FST, were later shown to reduce the immobility time of rodents in this test. The above laid the foundations to propose the FST as a method with predictive validity to identify new compounds with antidepressant potential (Porsolt et al., 1977; Almeida et al., 2020). Since then, the predictive validity of the FST has been confirmed by the anticipated identification of several drugs capable of reducing immobility in rodents that later showed their efficacy as antidepressants in phase II and III clinical trials, and subsequently led to their approval for therapeutic use in several countries, as described in the next section, which summarizes the effects of these compounds on the FST performed in rats.

Successful cases of the predictive validity of the FST to identify new antidepressant drugs

Antidepressants that act through monoamine reuptake inhibition

Levomilnacipran

Levomilnacipran is the levorotatory enantiomer of milnacipran, which acts as a dual NE and 5-HT reuptake inhibitor (McIntyre, 2017). Before its approval for clinical use, its antidepressant potential was predicted using the FST in rats (Rénéric and Lucki, 1998). Thus, the subacute administration of the racemic mixture milnacipran (3 doses along 24 h) produced a reduction of immobility and an increase in climbing, without changes in swimming, while chronic dosage (16 days) increased climbing and swimming and reduced immobility. Rénéric and Lucki (1998), reported that the decrease in immobility during FST following each treatment regime was not a result of a general increase in motility, as both schemes caused a reduction in locomotion during the open field test. Later, phase III, randomized, double-blind, placebo-controlled studies published in 2013 and 2014 showed that treatment with levomilnacipran for 8-11 weeks was superior to placebo for improving depressive symptoms and functional impairment (McIntyre, 2017). Considering these data, the FDA-USA granted approval of levomilnacipran for the management of MDD in 2013 (Deardorff and Grossberg, 2014). Subsequently, in 2015, Health Canada also approved the drug for this purpose (McIntyre, 2017).

Vilazodone

Vilazodone acts as a selective serotonin reuptake inhibitor (SSRI) and a partial agonist at 5-HT1A receptors (Kalia et al., 2011; McIntyre, 2017). The FST predicted its antidepressant potential in advance of clinical trials. Thus, the rats treated with the dose of 1 mg/kg displayed a decrease in immobility and an increase in swimming, which is a behavioral outcome typical of other SSRIs (Page et al., 2002). Two randomized, double-blind, placebo-controlled trials published in 2009 and 2010 showed the antidepressant efficacy of vilazodone (Kalia et al., 2011), and in 2011 the USA-FDA approved its use for the treatment of MDD (Kalia et al., 2011), while Health Canada approved it in 2015 (McIntyre, 2017).

Vortioxetine

Vortioxetine is a multimodal drug, acting as an SSRI, a 5-HT1B receptor partial agonist, a 5-HT1A receptor agonist, and a 5-HT7 and 5-HT1D receptor antagonist (Li et al., 2013; McIntyre, 2017). Its antidepressant effect was validated in the FST using a model of depressive-like behavior induced by

progesterone withdrawal in female rats. Vortioxetine treatment (10 mg/kg) administered in acute (3 daily doses) or chronic (14 days, either p.o. or i.p.) reduced the immobility of female rats (Li et al., 2013). Several double-blind, placebo-controlled, phase III clinical trials published in 2012 confirmed its effectiveness in major depression, and in 2014 vortioxetine was approved in Canada for the treatment of MDD (McIntyre, 2017).

Antidepressants that do not act through monoamine reuptake inhibition

Agomelatine

The antidepressant action of agomelatine was predicted by the FST in rats, in which acute or chronic doses, administered either in the morning or evening, caused a reduction of immobility (Bourin et al., 2004); this effect was later confirmed and extended by showing that agomelatine also augments swimming (Canpolat et al., 2016). Preclinical studies indicate that the antidepressant action of agomelatine is novel since the synergistic interaction of two mechanisms likely mediates it: stimulation of MT1 and MT2 melatonin receptors and blockage of 5-HT2C serotonergic receptors (Popoli, 2009; de Bodinat et al., 2010). The antidepressant potential of agomelatine was manifested in other rodent models of depression. Also, between 2006 and 2010, several placebo-controlled clinical trials confirmed its effectiveness in major depression, providing evidence that it could also be efficacious for preventing relapses (Carney et al., 2010). Thus, agomelatine was approved in 2009 by the European Medicines Agency for treating subjects with MDD in the European Union (de Bodinat et al., 2010); similarly, in 2010, the Therapeutic Goods Administration of Australia approved it for the treatment of major depression in adults, including prevention of relapse (Australian Government, 2010).

Allopregnanolone

Allopregnenolone, a neurosteroid that exerts a positive allosteric modulation of the GABA-A receptor (Almeida et al., 2020), is another drug whose antidepressant potential was predicted in the FST, reducing immobility and increasing swimming in rats (Rodríguez-Landa et al., 2007; D'Aquila et al., 2010). These observations, along with substantial, additional preclinical evidence in other rodent models of depression (Almeida et al., 2020) led to the clinical evaluation of an intravenous formulation named brexanolone, consisting of a mixture of allopregnenolone plus a solubilizing agent (sulfobutylether-ß-cyclodextrin) (Scott, 2019). The efficacy of brexanolone for the treatment of postpartum depression (PPD) was established in phase II cli-

nical trials (2015-2106) and then entered phase III placebo-controlled trials, during which it received approval from the FDA-USA to treat PPD (Scott, 2019).

Esketamine

Esketamine is the active isomer of ketamine, a non-competitive N-methyl-D-aspartate receptor antagonist (Salahudeen et al., 2020). The antidepressant potential of its racemic mixture, ketamine, was predicted by its ability to reduce the immobility of rats in the FST after receiving a single dose of 25 mg/kg, applied 24 or 1 h earlier (Gigliucci et al., 2013). The efficacy of esketamine for the treatment of MMD resistant to other antidepressants was established in double-blind, placebo-controlled, phase II (2014-2016) and phase III (2015-2021) clinical trials (Salahudeen et al., 2020). In 2019, intranasal esketamine for patients with antidepressant-resistant MDD received approval from the FDA-USA (Salahudeen et al., 2020).

Conclusion about the FST

Despite its limitations in its construct and face validity characteristics, there is broad consensus that the main advantage of the FST is its high validity to predict the therapeutic efficacy of antidepressant treatments (Cryan et al., 2005; de Kloet et al., 2016; Commons et al., 2017; Hao et al., 2019). In addition, when the modified protocol is used, it allows discriminating between drugs whose mechanism is through either serotonergic or noradrenergic transmission (Cryan et al., 2005). Lastly, it is an economical procedure with high reproducibility between laboratories (Cryan et al., 2005; Hao et al., 2019).

The chronic mild stress (CMS) model

The chronic mild stress (CMS; also referred to as chronic unpredictable, variable, or intermittent stress) model is one of the most used experimental approaches in rodents to investigate the pathophysiology of MDD, and possibly the most valid method for the preclinical investigation of antidepressant-like effects (Willner, 2016).

The first experiments, by Katz and colleagues in the early 1980s in rats, were based on clinical and preclinical research regarding the etiology of depression, and involved the rotation of stressors applied over 21 days (Katz, 1981). Willner and colleagues later modified these procedures in the late 1980s by reducing the severity of the deprivation stressors but prolonging their duration and making them unpredictable. The latter resulted in the original

CMS paradigm to assess novel pharmacotherapeutic agents with potential antidepressant efficacy (Strekalova et al., 2011). Thus, the CMS model protocol is an established, relevant model, for inducing behavioral symptoms commonly associated with clinical depression. It involves the repeated exposure of animals to an array of unpredictable mild stressors (most frequent components are summarized in Willner, 2016, Table 4), such as periods of food and water deprivation, wet bedding, inversion of the light/dark cycle, forced swimming in cold water, social isolation, cage tilting, physical restraint in a tube, stroboscopic lighting, changes of cage mates, and other similarly innocuous manipulations over a sustained period (ranging from 10 days to 8 weeks). These mild stressors cause an anhedonic-like behavior (decrease in responsiveness to rewards) in rats or mice.

The most common measure used to track the effects of the CMS model is a decrease in the consumption of a palatable (1–2%) sucrose or saccharin solution, which the chronic administration of antidepressant drugs reverses. Finally, once a hedonic deficit has been established, the administration of chronic treatments or their vehicles begins and continues throughout the stress period (Willner, 2016), as illustrated in Figure 1.

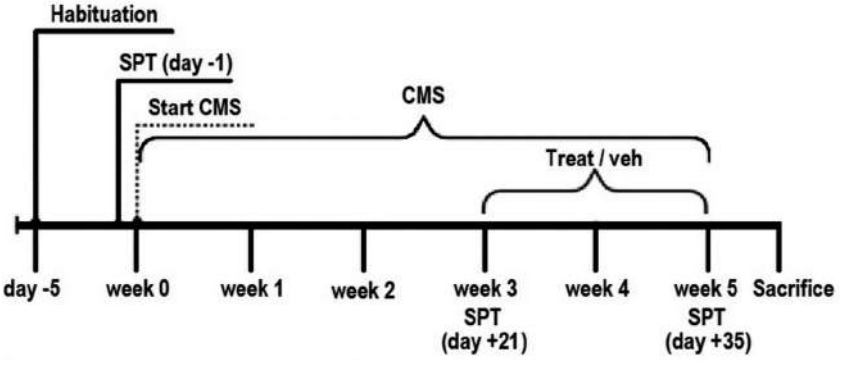

Figure 1. CMS model. Example of a chronic mild stress (CMS) experimental design. Animals are exposed to a series of mild stressors over a period of five weeks. The sucrose preference test (SPT) is conducted on days -1, +21, and +35 to assess anhedonic behavior. During the final 14 days of the model, chronic treatment (Treat) with potential antidepressant drugs is administered, while control animals receive the vehicle (Veh).

Although the endpoint of this model is focused exclusively on sucrose intake and preference, it is worth mentioning that there are complementary in vivo preclinical tests that are not in themselves sufficient to unveil the antidepressant-like effect of a novel drug, but when used in conjunction with the CMS model increase the chances for a successful prediction of their antidepressant efficacy.

These tests refer mainly to the FST and the open field test (OFT). The latter behavioral experiment measures spontaneous locomotor activity in rodents placed inside a transparent acrylic enclosure field for a specific amount of time (Feyissa et al., 2017). In addition to anhedonia-like impairments in tests of rewarded behavior, the effects of CMS include a reduction of locomotion in the OFT during the rat's active period (Gorka et al., 1996), an effect reversed with antidepressant drugs (Willner, 1997). In this respect, psychomotor agitation or retardation symptoms have been reported in humans with MDD, which is a far more complex behavior than a simple decrease in locomotor activity. However, Willner (1997) suggests that: "a rat exposed to CMS could, in principle, legitimately attract a DSM-IV diagnosis of either major depressive disorder or major depressive disorder with melancholic features" (Willner, 1997). Nevertheless, hyperlocomotion has also been reported in the OFT in response to mild threats after rats were subject to a chronic unpredictable stress (CUS) model (Sequeira-Cordero et al., 2019). On the other hand, the FST, as already mentioned in this chapter, is a behavioral experiment used to determine the efficacy of prospective antidepressant drugs. The endpoint is immobility, which is interpreted as a measure of "behavioral despair" or freezing in response to an inescapable stressor, such as forced swimming (Krishnan and Nestler, 2010). In this sense, other behavioral parameters have recently been proposed as more reliable behavioral markers of chronic stress, particularly in juvenile rats, such as hyperlocomotion induced by mild threats (e.g., in the OFT) or high immobility levels in the FST (Brenes et al., 2008; Sequeira-Cordero et al., 2019). Thus, we propose that the implementation of the FST and OFT as complementary tests to the CMS model may contribute to avoiding false positives.

Construct validity

In CMS model trials, rodents are exposed to a variety of unavoidable mild stressors for a prolonged period, which intends to emulate the long-lasting and stressful adverse life events that in humans have been significantly associated with the future development of MDD episodes (Hammen et al., 2009). Hence, the construct validity of the CMS model is based on the possible similarity of psychological processes triggered by uncontrollable stressors that lead to an altered responsiveness to rewarding events (Willner, 2016), and specifically in its capacity to induce anhedonia, a core symptom of the melancholic subtype of MDD (American Psychiatric Association, 2013; Willner, 2016). Anhedonia is defined as a decreased ability to experience pleasure, and is the key characteristic of melancholia; therefore, the

CMS model assumes that sucrose drinking is a valid measure of sensitivity to reward, and that it triggers a generalized decrease of it rather than a specific effect on responses to sweet tastes (Willner, 1997). In rodents, this anhedonic-like behavior is monitored by measuring the relative preference for palatable rewards, such as a dilute sucrose solution. As noted by Krishnan and Nestler (2010), the typical method for determining sucrose preference (%) is calculated as [sucrose solution intake (ml)/total fluid intake (ml)] x100]. Hence, the hedonic deficit might be considered a primary feature to be addressed in preclinical models of depression.

According to prior research conducted by Muscat et al. (1988) and Willner et al. (1987), it has been demonstrated that the CMS does not induce any non-specific alterations in the consumption of plain water, and its impact can only be observed in preference tests involving single-bottle or two-bottle (sucrose-water) conditions. In the case of the latter, when 1% sucrose is added to plain water, the intake of control subjects increases, while that of CMS subjects does not. Besides, this anhedonic-like behavior was later supported by additional research evaluating other measures of reward and hedonic impacts, such as conditioned place preference, brain stimulation reward, and dopaminergic release in response to rewarding stimuli (Hill et al., 2012; Willner, 2005).

Face Validity

As has already been mentioned, the CMS model offers a realistic simulation of the etiology of a major depressive episode, even one with melancholic features, because of its reasonable approximation to the stresses of daily life. In addition to modeling anhedonia, animals subjected to CMS develop other realistic behavioral changes akin to those manifested during major depression episodes in humans (summarized in Willner, 1997, Table 1). For instance, while in humans a major depression episode lasts for at least two weeks, the effects persist for up to 3 months in the CMS model. Also, in humans, it has been established that a markedly diminished interest and pleasure as a core symptom is equivalent to decreases in the performance of motivated behaviors (sexual, aggressive, and investigative), whereas, in animals, this corresponds to decreased responses to rewards (D'Aquila et al., 1994). In addition, significant weight loss in humans and a relative weight loss (a slower rate of weight gain, typically of around 5%) in animals have been reported (Willner, 1997). Moreover, CMS studies have shown psychomotor agitation or retardation and the presence of fatigue or loss of energy in humans, while in animals decreased locomotor activity and "acti-

ve waking" in the EEG during the dark phase have been reported (Cheeta et al., 1997).

Furthermore, animals exposed to the CMS model develop other physiologic changes that correspond to those of major depression, as well as specific characteristics of melancholia. For example, increased activity in the hypothalamus-pituitary-adrenal (HPA) axis, including adrenal hypertrophy (Muscat and Willner, 1992) and corticosterone hypersecretion (Ayensu et al., 1995). Impairments in the immune system have also been detected, including an increase in serum complement (Ayensu et al., 1995), decreases in thymus weight and natural killer cell activity, and altered T-cell reactivity to several mitogens (Kubera et al., 1998). Also, disrupted sleep patterns occur, such as diurnal variations that are worse at the start of the dark (active) phase (D'Aquila et al., 1997), and a phase advance of circadian rhythms. Moreover, many reports demonstrate CMS-induced cognitive impairments and anxiogenesis in various tests, such as spatial learning (Song et al., 2006) and novel object recognition (Orsetti et al., 2007). All these impairments are reversed by chronic antidepressant treatment (Willner, 2016). Likewise, a volume reduction of the hippocampus has been observed in the CMS model and in major depression (Campbell et al., 2004), and this morphological change is also associated with CMS-induced anhedonia (Delgado y Palacios et al., 2011).

Additionally, concerning antidepressant drug treatments, Willner's (1992) four key aspects for the face validity of the CMS model may be pointed out. Firstly, treatment with antidepressant drugs does not commence until anhedonia has been established; thus, the model demonstrates the reversal of this condition by antidepressants rather than their prophylactic effects. Secondly, stress continues during the treatment period, similar to what happens in the clinical situation, since, usually, there would not be an abrupt change in the patient's circumstances associated with the onset of treatment. Thirdly, the time course of recovery (3-5 weeks) corresponds closely to that observed in the clinic. Fourthly, no changes are seen in control animals, corresponding to the failure of antidepressants to elevate mood in non-depressed volunteers (Willner et al., 1992).

Predictive Validity

The tested antidepressant drugs usually require 3-4 weeks of treatment to reverse anhedonia-like behavior in animals exposed to CMS, but do not alter rewarded behavior in non-stressed control animals (Willner, 1997). Nowadays, a wide range of antidepressant agents reverse anhedonia-like behaviors in the CMS model. For example, tricyclics such as imipramine, desipramine, and amitriptyline at low to moderate doses (e.g., 5–10 mg/kg

per day) (Willner et al., 1987), the specific 5-HT reuptake inhibitors (SSRIs) fluoxetine, fluvoxamine and citalopram (Muscat et al., 1992), the specific NA reuptake inhibitor maprotiline (Muscat et al., 1992), the monoamine oxidase inhibitor moclobemide (Moreau et al., 1993), the antimanic agent lithium (Sluzewska and Szczawinska, 1996), and the atypical antidepressant mianserin (Cheeta et al., 1994). Also, electroconvulsive shock restored normal responsiveness to reward in animals exposed to CMS after one week of treatment (Moreau et al., 1995).

Antidepressant drugs whose efficacy was predicted in the CMS model

Historically, in vivo models have been widely used as part of the necessary preclinical tests needed to investigate the efficacy of novel antidepressant drugs intended for MDD treatment. As such, the CMS behavioral model is a valuable asset for pursuing drug discovery research at the preclinical level as an aid to confirm the effectiveness of conventional antidepressants and to identify novel agents for the treatment of MDD. In order to support the predictive validity of the CMS model and highlight the importance of its use for its translation into clinical use, table 1 shows some studies about the most recent antidepressant drugs whose efficacy was predicted in the CMS model before approval for the treatment of MDD.

Table 1. Drugs whose antidepressant potential was predicted by the CMS model.

Antidepressant	Reference	CMS duration (days)	Species	Year of approval
Milnacipran	(Papp et al., 2004)	35	Rat	2009 (FDA)
Agomelatine	(Papp et al., 2003)	63	Rat	2009 (European Union)
Ketamine (i.e., Esketamine)	(Papp et al., 2017)	35	Rat	2019 (FDA)

Milnacipran

The 5-week CMS model caused a decrease in the responsiveness of male Wistar rats to rewarding stimuli with a 1% sucrose solution. The deficit in sucrose consumption in stressed animals was reversed by chronic administration of milnacipran (10 and 30 mg/kg i.p.), a dual 5-HT and NE reuptake inhibitor (SNRI) with proven clinical efficacy in MDD (Papp et al., 2004).

Agomelatine

The 9-week CMS model in male Wistar rats allowed assessing the effects of the melatonin agonist and selective 5-HT2C antagonist agomelatine (S20098) compared with melatonin, imipramine, and fluoxetine. The CMS caused a gradual decrease in 1% sucrose solution consumption, which was reversed by agomelatine (10 and 50 mg/kg i.p.). The administration of agomelatine suggested that its antidepressant-like activity depends on some combination of its melatonin agonist and 5-HT2C antagonist properties (Papp et al., 2003).

Ketamine

The 5-week CMS model in male Wistar rats caused a significant decrease in the consumption of 1% sucrose solution after two weeks of stress exposure, which was reversed by ketamine (5 and 10 mg/kg i.p.) with no drug effects on sucrose intake in control animals. Therefore, this study shows an antidepressant-like effect of ketamine and a procognitive effect in the CMS model (Papp et al., 2017).

Summary

The CMS model is now a valuable tool for investigating the neurobiological, behavioral, and hormonal changes underlying the psychopathological mechanisms associated with stress. The similarity of the neurobiological mechanisms between the CMS model and the major depressive disorder in humans suggests that it has a high translational potential to identify clinically efficacious antidepressants. However, continued research with a complementary and improved battery of behavioral tests evaluating different domains of CMS-induced functional impairment may have greater translational potential as this preclinical area of research expands and matures.

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XI

Breaking an old heresy: Connecting animal and human behavioral neuroscience research

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Introduction

What do we mean by "heresy"? When we were both students, independently we were told that we should NEVER ask how research in animals can be applied to humans, because research in animals is "pure" basic research and should be performed for its own sake. The reason is that if we ask for its relevance to humans, it diminishes the value of basic research, which is important to understand "how things work in the organism in which it is studied". What was offensive to our mentors then was our asking of research on animal behavior, "how does this apply to humans, and what is the practical application of the research?" Probably a preferable way of asking, which we now recognize, would have been "What do you see as the implications of the research?"

While our questioning, as students, of how research in animal behavior could inform human physiology or behavior was then considered "heresy", today the attitude is completely different. In fact, in grant applications to the NIH or NSF we are now required to state the relevance of our proposed research in animals to the "human condition.". When we were discussing formulating our joint chapter, we mentioned to each other the severe criticism that we each received when we were students– the same whether in Spain or in the US –when we asked our mentors whether their research could be relevant to humans.

From our experience with Carlos Beyer and our former students, who are now our colleagues, in Mexico, we thought the attitude in Mexico was always open to considering the link between animal and human research and mechanisms, and that maybe our Mexican colleagues might not have had a similar experience to ours. But we both did have similar experience in being affected by the attitude of professional scientists toward animal and human

research, despite our growing up in two difference cultures, in Spain or the United States.

Two neuroscientists evolved similarly in two different countries and cultures

Cruz: Growing up in Spain: What do you want to be when you grow up?

That was the typical question that an adult asked a child. When I was a little girl, I always answered: "a writer". My answer always sounded like an unusual and even pedantic response for a 7-year-old girl. This response was based on my seventh birthday gift from my father, the book "Gulliver´s travels". I felt so amazed with this reading. I was transported to Lilliput land or the opposite giant land of Brobdingnag. My discovery was that through books, I could travel to a world of fantasy, and any reading thrilled me to create my own tales. Since then, my curiosity grew with every book I read, and my questioning on different doubts arising from them. And, here I am, writing many years later on a topic that is possibly one of the most controversial issues in science: basic vs. applied science. Yes, there has been a radical change in the neuroscience paradigm concept in the last 40-50 years on the extrapolation from animal research to human studies.

As we introduced above, the most widespread convention was that basic animal experimental approach was totally independent of any possible relationship with human research. The most general statement was that basic science was experimental and totally done in animals, and that its aim was the searching of the truth itself with no direct applications, just for searching whatever neurobiological mechanisms in the neuroscience experimental approach underlies the life phenomenon. Thus, this "searching for truth" based on the hypothesis-driven scientific method, involves something very critical, which is the possibility of replication of any results obtained through this scientific method. At the same time, this conceptual key-replication of the results of any experiment done, no matter where or by whom, allowed to the whole scientific community to be able to communicate and be open, thereby making possible the advance in scientific knowledge accessible throughout the world and sharing scientific advances. This was the main principle in the way neuroscience-related research functioned in the last century. But let me follow with my own scientific life experience, since it can be an example of the significant changes that this classical approach has changed throughout the past approximately 50 years.

My earliest experience in basic science

My very first experience as a "neuroscientist" was when as a student, I started to assist in the research line of "Sex differences in Learning" of my dear and never forgotten professor, J.A.I. Carrobles at the Autonomous University of Madrid. He was studying the possible sex differences in the well-established phenomenon of the effects of partial reinforcement on the extinction process (Del Cerro, 1978). This paradigm of animal learning studies referred to the observation that rats have a major resistance to extinction of a learned response after a partial reinforment learning program, in comparison with rats under a continuous reinforcement schedule. In other words, rats continue responding longer on the extinction process-no reward period if the acquisition program was based on a partial reinforcement schedule – random (yes/no…) reward– than those animals with a continuous reinforcement pattern (yes/yes/yes…). The results of these experiments were my own graduation experimental work known as a Tesina (little thesis). The final data demonstrated that there are differences between male and female rats, since female rats show more resistance than males to extinction of a learned task after a partial reinforcement learning program. These results started me on the path of what we are trying to develop in this book and chapter, i.e., to transfer our animal experimental data to human studies: Does it mean that women have a more powerful learning process with a partial reinforcement trial than men? Is it feasible and appropriate to use animal results for addressing human problems?

I took this path on the basis of different situations, seminars, public defense of my Tesina, and talking with colleagues. I always felt trapped to think about the translation of my lab results to understand human behavior, but that it was not acceptable, according to my mentor and orthodox animal learning experts at that time, at least in Spain's schools of psychology and medicine. The same issue arose shortly afterward during my doctoral fellowship, when I tried to apply my research findings to some questions related to human brain and behavior; my mentors took these ideas as completely mistaken. I always received the same advice: … "You should think about your hypothesis and experimental design as the search for the truth". So, the "mantra" that I heard when I tried to interpret beyond my research findings, discussion and conclusions was: "We are doing basic science and we are looking for responding to our working hypothesis, no more. We are following just the truth of what we want to know via the scientific method". These recommendations were based mainly on the Mario Bunge analysis of philosophy of science. During this time, the 1980´s, the work of

Mario Bunge was critical for the new scientific approach in Spanish psychology.

According to Bunge, scientific knowledge describes facts just as they are, producing new facts, and it is possible to explain them by looking for the mechanisms that produce them. Bunge called this concept as "Ciencia Fáctica" (Factual Science; Bunge, 1985). This idea has always been imprinted on my mind and my entire research career. Actually, I think that was good for my scientific training. Possibly human extrapolation, especially in the behavioral neurosciences, needed those "pure" bases in order to create a scientifically rigorous framework in which we can work subsequently using a theoretical approach. Another idea that has been not only in my mind but also imprinted in many of my academic works, belongs to the "father of neuroscience", Santiago Ramón y Cajal, and shows the emotional part of our work. "In science, as in life, the fruit always comes after love." Yes, love and passion for our job in the lab and teaching, even for our way of communicating our results to the scientific community and society, is essential.

This very first experience in the laboratory was for me a difficult way of facing my future dedication to behavioral neuroscience. First of all, I had to read and learn – in English – practically the complete rationale of the current research line. Even though my spoken English wasn't too bad, my reading and written English was far from an acceptable level. This type of requirement in our scientific field is something that English speakers do not have to face. Second, my starting in the daily work in the laboratory and bioterium made me feel like a slave of the experiment´s agenda, because no weekends or holidays exist for our experimental rats. However, very soon I became adjusted and comfortable with the requirements I had to deal with, such as the handling of the animals, or the trials schedule, even during weekends. I must say that I had some advantage in comparison with my lab mates since I was a "country girl", so familiar with animal care, whereas they felt traumatized by the rat management experience. As a result of my first experience in an animal learning lab, I knew that this would be my field in psychology and not any other field more commonly chosen by most of the psychology students, such as clinical, organizational, or social psychology.

The laboratory was located in the medical school of the Autonomous University of Madrid (UAM), and there I met professor Guillamón (Antonio), who had recently arrived from Oxford University after a stay at the Jeffrey Gray laboratory. This meeting showed me the path that I wanted to take, to study the neurobiological basis of learning. Antonio was recruiting students interested in this specific area, and he offered me entry into his

newly-formed laboratory. There were just three students including me, Santiago Segovia and Azucena Valencia, we all continued academic life being good friends and colleagues.

These starting years were intense in work and a huge number of grant applications. Today it seems to me like a dream, that which we were able to accomplish by our young enthusiastic strength and pushing illusions of our psychobiological aims. After a decade we were the well-known "Guillamón group from UAM and UNED", with an important body of data published on the study of the sex differences in a multisynaptic neural system – the vomeronasal system – and we began studying the translation of these sexually dimorphic neural system in rat model behavior (Segovia et al., 1982; Del Cerro et al., 1986)).

From Molecular to Molar research: ions to behavior

During the period of my starting in basic science work during the last decades of the 20th century, molecular research was, in some way, more appreciated than molar or behavioral research. This was one of the reasons that some neuroscientists were very critical of, and underestimated, behavioral studies using animal models. The advances in molecular procedures and techniques in neuroscience, such as molecular or cellular ones, high performance liquid chromatography (HPLC), capillary electrophoresis (CE), or imaging techniques, including fluorescence, functional magnetic resonance imaging (fMRI), positron emission tomography (PET) and mass spectrometry (MS) were, in part, the "switch on" of this neuroscientific pressure. By contrast, the complexities of behavioral patterns and behavioral paradigms were not as clear, reliable, and replicable as the studies performed using the new, different, sophisticated molecular procedures. Consequently, a new interdisciplinary era started in this period, which has been successfully followed today. A good example of this is some of our own recent studies in which geneticists, neuroimmunologists, pharmacologists and others are working on our research projects in laboratories in Spain as well as in the USA.

I want to point out some details related to the way things have changed in academic filters in Spain, such as the defense of the doctoral dissertation. When I defended my dissertation, I had an important body of data that I wanted to publish. However, I was forbidden to do it because at that time –the 1980's – it was not permitted to publish any result prior to the final dissertation defense. Today, it is just the opposite: a doctoral dissertation requires at least two published partial results; otherwise, it is not permissible to defend it.

My doctoral dissertation was my real first contact with a molecular approach to the study of the nervous system. I was fortunate to study with one of the best experimental neuroendocrinologists of Spain in the 20th century, Dr. Francisco Escobar del Rey and his wife, Dra. Gabriela Morreale. They were responsible for introducing a new test protocol in newborn babies for detecting thyroid function, preventing the incidence of hypothyroidism and the introduction of iodide salt in diet in Spain among other important contributions. UNICEF took the Escobar and Morreale thyroid function test in newborns and introduced it all over the world. Furthermore, the WHO followed with the recommendation of increasing the consumption of iodide salt from 20% in 1990 to 70% in developed countries (OMS, 1999).

My topic focused on how early thyroidectomy could differentially affect males or females in terms of neurotransmitter distribution in the brain. The results were important and they showed that neonatal thyroidectomy produces selective changes in receptor density for GABA, and for 5-HT; it increased receptors in cerebellum and striatum and decreased receptors in visual cortex (Del Cerro, 1984). Prior to my doctoral dissertation, the group that I belonged to, Antonio Guillamón laboratory in the School of Medicine of UAM, had discovered some sexually dimorphic patterns in an interesting neural system related to the modulation of reproductive behavior – the vomeronasal system (VNS).

Sexual differentiation of the brain was the framework of our research line

After my doctoral dissertation received the grade of Cum Laude and Extraordinary Award, 1984, I wanted to progress from my molecular studies to behavioral ones. Since the advances in the group research line were very successful, Antonio suggested that I choose a behavior pattern related to the neural control of VNS that may show behavior patterns that differ between males and females. That led to my first encounter with Jay Rosenblatt's excellent research on maternal behavior in rats. Eureka!!! That was my reaction when I saw Jay's well-known Science publication (Rosenblatt, 1967). This study established the nonhormonal basis for maternal behavior in the rat by demonstrating that any condition of a rat, nonpregnant intact, ovariectomized, hypophysectomized, and even intact and castrated males were all able to express the main components of maternal behavior after continuous exposure to young foster pups. These findings provided evidence that all rats have a basic level of maternal responsiveness that is independent of hormonal condition. This publication and others (Rosenblatt, 1980;

1983 &1989) drove me to initiate the parental behavior (PB) research line in our laboratory by studying the possible relation of this complex reproductive behavior to our previously well-established sexually-dimorphic model, the VNS. This research triggered a substantial series of studies and publications that led to the approach about which I am most enthusiastic: the epigenetic basis of PB.

In relation to my first studies, I decided to study how the sexual differences found in the VNS of the rat, by our research group, can be translated into the sex differences found in PB, starting from the most peripheral i.e., the vomeronasal organ, centrally to the accessory olfactory bulbs (AOB), the bed nucleus of the accessory olfactory tract (BAOT) and the medial preoptic area (MPOA) (Del Cerro et al,1991; Izquierdo et al, 1992; Segovia and Guillamón, 1996; Segovia et al., 1996). These studies provided evidence that the greater number and volume of neurons in these vomeronasal structures in males inhibited the female-like maternal patterns, since our experimentally-induced reduction in number and volume facilitated the onset of maternal patterns in males (Izquierdo et al., 1992, Carretero et al., 2003). Studies by others were consistent with our findings, showing an inhibitory role of the vomeronasal input on maternal behavioral patterns (Fleming et al.1979, Malenfant et al.,1991). From these studies, we established the theoretical framework for the PB-inhibitory role of the "supernumerary neurons" in the VNS (Del Cerro, 1998; Segovia and Guillamón, 1993; Segovia et al., 1999). Through this model of VNS-related sexual differentiation we have been able to understand the role and behavioral translation of the organizational action of steroids during the perinatal period in mammals and the related sex differences in PB, sexual behavior, sexually dimorphic, and learning paradigms (Claro et al.,1994; Numan et al.,1999; Segovia et al., 1999).

Neuroanatomical bases of sex differences in parental behavior

At that point, I needed to account for how the sex differences observed in the VNS can underlie the differences in behavior. We became pioneers in the use of a neural system, the VNS, as a model to understand sexual differences in brain and behavior. The best experimental approach was to study how the sexual dimorphism observed in the VNS is reflected in a sexually dimorphic behavior such as parental behavior (paternal vs. maternal) in which this neural system is directly involved. I started studying the implication of sex differences in the BAOT for PB in male and female rats. I must say that this study was my scientific milestone and the reason for my continuing scientific career…but why? Still today, I vividly remember my reaction to the first male that I placed into the experimental cage; it ran rapidly

to retrieve the first pup that was located in the opposite corner to the male's location.

This first retrieval performed by a male, and its short latency, was telling us that the bilateral electrolytic lesions in the BAOT resulted in this experimental male showing a female-like behavioral pattern, a non-expected response in a male cohabiting with a pup. I felt so happy, but I had to react quietly and continue with the experiment, although I really wanted to run out of the experimental room and shout to my colleagues IT WORKED!!! Yes, our hypothesis was supported. Based on the Guillamón and Segovia (1993) concept of the inhibitory role of VNS on female-like behavior, we confirmed this prediction with our finding of an inhibitory role of BAOT on PB in male rats (Izquierdo et al., 1992).

Figure 1. Parental Behavior similarities in mammals. The human father is my son Fran with his own son, Fran jr.

As the only researcher in Spain at that time studying PB (Figure 1), I applied for, and received, an Abroad Mobility grant for full professors from the Ministerio de Educación, Spanish government and a Fulbright Grant, to learn from, and collaborate with, Jay Rosenblatt in the Institute of Animal Behavior (IAB) at Rutgers University in Newark, New Jersey, USA, in 1989. This stay was really fruitful in developing my research skills, and allowed me to see the differences between the Spanish and the American ways of doing research. The major difference was, for then, the easy way of bringing

experimental ideas to the laboratory in the Institute of Animal Behavior versus the Spanish university system, which was much more bureaurocratic and slow. However, currently I see a more common level of filters, some of them necessary, e.g., animal care, but others in Spain still really hyperbureaucratic, e.g., double or triple veterinarian and various committees´ requirements.

My stay at the IAB was not only important in my experimental training, but overall, a full professional experience by which I learnt from excellent scientist-colleagues like Carlos Beyer (Carlos) and Barry R. Komisaruk (Barry). They continued with me as best mentors and friends for the upcoming years. Sadly, Carlos left us as orphans of his enlightening intelligence and erudition too soon. But his imprint is with us forever.

Figure 2. Carlos Beyer and the authors at Barajas Tacos in Todos Santos, Baja California Sur, circa 2010

With Jay and Barry we designed several experiments to understand the neuro-modulatory role of several vomeronasal structures in natural mother and virgin rats, under different hormonal and stimuli conditions. We asked whether the VNS system, with its efferents to the medial preoptic area (MPOA), was activated or not in the maternal behavior (MB) response toward pups. We found that in the MPOA, 14C-2-deoxyglucose (2-DG) levels, measured autoradiographically, were higher during natural parturition with concurrent maternal behavior than in non-pregnant non-maternal controls, whereas levels in the VNS were lower in virgin rats induced to show maternal behavior by cohabitation with young, than in control and parturient rats. Prior studies reported that lesions of MPOA disrupt maternal behavior, whereas lesions of VNS structures elicit it, and that an increase in 2-DG levels is indicative of an increase in firing activity in neuron terminals. These joint results provided evidence that maternal behavior can be

induced by: 1) an increase in parturition-generated sensory stimulatory input to the MPOA in response to the mechano-stimulation of the birth canal that occurs during parturition, and 2) a separate chemosensory vomeronasal pathway whose activity is reduced by cohabitation with young, thereby disinhibiting maternal behavior (Del Cerro et al., 1995). Following these results, we needed to go further, so we took the risk of combining two procedures, the combination of which could provide important neural activity information related to the expression of MB.

On the basis of evidence that 2-DG autoradiography indicates activity (increased or decreased) at axonal terminals, whereas c-fos immunocytochemistry indicates activation (or not) of neuronal cell bodies, we combined these techniques in the same individual rats, evaluated in alternating adjacent histological brain sections, to assess excitatory (increase in 2-DG and c-fos activity in adjacent sections) and disinhibitory (decrease in 2-DG but increase in c-fos activity in adjacent sections) synaptic relations in selected sites in female rats in which maternal behavior was elicited by natural parturition, sensitization (7-10-day cohabitation with foster pups), or hysterectomy. All individuals in these three groups expressed maternal behavior immediately prior to receiving the 2-DG injection. We found elevation (compared with controls and non-maternal virgins) in both 2-DG and c-fos activity in MPOA in the parturient and hysterectomized groups, indicating an increase in its input and output activity, i.e., an excitatory interaction; as mentioned above, the MPOA was previously shown to be critical for maternal behavior. The sensitized (co-habitation) group showed a decrease in 2-DG activity of vomeronasal nuclei (BAOT and medial amygdala), replicating our previous study) plus an elevation in c-fos activity in the alternating sections. This combination indicated disinhibition of these nuclei, which were previously shown to modulate pup-related chemostimulation-induced sensitization. Our findings demonstrated the feasibility of measuring 2-DG and c-fos activity jointly in adjacent sections of the same brain, thereby providing evidence to distinguish between localized excitation and disinhibition in VMS structures modulating MB expression under different hormonal and stimuli conditions (Komisaruk et al., 2000).

While my background was always experimental science using the rat as a research model, I felt the need to jump to human studies. With Barry we started some preliminary human studies on PB based on our animal research and our hypothesis on brain sex differences. The aim of our approach is to analyze the activity of some brain areas related to the response of recent fathers and mothers versus non-mothers or non-fathers. The stimulus was a recording of a baby crying (their own or other) versus white

noise. The response was measured by functional Magnetic Resonance (fMRI). Some of the most relevant preliminary results, not published yet, show differences in activity in the paraventricular nucleus region of the hypothalamus in mothers and their own baby crying in comparison to fathers. We need to continue our studies trying to understand the possible mechanisms related to the oxytocin (Oxy) involvement in these findings based on the sex differences in Oxy levels and distribution (Marazziti et al., 2019).

The most recent publications with Barry (Komisaruk and Del Cerro, 2021 & 2022; Komisaruk et al, 2023) are theoretical essays on the relevant role of pleasure in human well-being. We reviewed the critical function of sensory stimulation, proprioception, and feelings on the way to pleasure, based on the evidence that their disruption leads to pathologies. In Spain, in our laboratory of UNED plus other Spanish academic and health institutions in collaboration with others from Holland and Canada, and my admired colleague and friend, Antonio, we are involved in a recently renewed project (PID2021-127547NB-C21, Ministerio de Ciencia e Innovación, Gobierno de España) on a current and interesting topic: Brain endophenotypes: genetic and epigenetic effects on transgender adolescents, before and after the treatment with puberty blockers. The project is pioneering in this research area, since longitudinal studies before and after the effect of treatment with puberty blocking agents on the brain of transgender adolescents have not been undertaken previously. Once again, this experimental human approach is based on our previously developed animal (rat) models of feminization and masculinization (Gómez et al., 2020). Our animal model was the key to account for the findings we hypothesize to occur in the brain of transgender adolescents under treatment with puberty blocking agents. As we said above, neuroscience advancement is supported by interdisciplinary continuous and fruitful cooperative national and international projects, as in this case of transgender teenagers. Thus, these approaches are a paradigmatic example about the general topic of this book, i.e., the value of animal experimental data toward understanding behavioral phenomena in humans.

From sexual dimorphism in parental behavior to effects of perinatal stress: The role of epigenetic factors in the neurobiological bases of behavior

We published our general findings on sex differences in VNS and its implications in sexual dimorphism in MB controlled by organizational effects of perinatal gonadal steroids "milieu" in several reviews (Del Cerro, 1998; Segovia et al.,1999). Subsequently, I came across some early publications from

a German group (Dörner and Staudt, 1969) that showed a possible prenatal stress effect on the sexually dimorphic nucleus of the hypothalamus. The authors developed a series of epidemiological studies in humans indicating a prevalence of homosexuality in men born during the second world war. Post-mortem neuroanatomical analysis using morphological parameters showed a low volume of the sexually dimorphic nuclei of the hypothalamus of these men, with values similar to those that are characteristic of women (Dörner et al., 1975). These researchers related this finding to the chronic stress that the men's mothers suffered during the bombing at the end of the war in some German cities. This finding inspired me to ask: Is it possible that chronic stress during gestation can alter developmental neuroendocrine patterns in the fetuses, which can affect their subsequent behavioral patterns when adult? I was incorporating three main factors that underlie behavioral neuroscience: brain, hormones and behavior. My research on this topic has been performed in rats. Part of the rationale is based on the evidence that maternal stress hormones during gestation can enter the fetal brain and impair the development of the response of the hypothalamic-pituitary-adrenal (HPA) axis to stress at maturity.

Other studies demonstrated that exposure to stress during pregnancy produces physiological and behavioral alterations in the offspring, disrupting their maternal behavior when adult (Weaver et al, 2004). Some of our most significant findings showed that stress applied to pregnant rats affects not only the postpartum expression of maternal care toward their pups, but also that when those pups develop to adulthood, the maternal care that they express is abnormal and disorganized (Pérez-Laso et al., 2008; Del Cerro et al., 2010). These alterations were not only expressed in maternal behavior, but also in morphological, hormonal and immune-histochemical parameters involving AOB, adrenocorticotrophic hormone (ACTH), testosterone, estradiol (E2) and c-fos. These series of studies in rats provide evidence that stress during the final week of pregnancy exerts long-lasting effects on the expression of induced maternal behavior in the female offspring when they are adult. Moreover, the changes observed in their MB are related to alterations in the development of the AOB, one of the sexually dimorphic brain nuclei involved in the control of MB, and the HPA endocrine axis (Del Cerro et al., 2015). These findings led me to the question: Are these stress effects caused by the neurobiological changes or by the altered maternal behavior per se, or both?

Noteworthy is one of the most cited works by the group of Michael Meany in rats (Francis et al., 1999), demonstrating that variations in maternal care influence the development of behavioral and endocrine responses

to stress in the offspring. In that study, a cross-fostering procedure was used to ascertain whether MB was responsible for the future behavioral alterations of the offspring when adult, versus gestational stress effects. That was precisely my question. Francis et al., (1999) reported not only an influence of maternal behavior on stress reactivity in the offspring, but the subsequent transmission of such individual differences in maternal behavior from one generation of females to the next. In order to study MB as an independent variable in our stress experiments, we had to introduce the "cross-fostering" procedure (i.e., adoption), which consists of the exchange of newborn pups from stressed mothers to non-stressed mothers and vice versa. With this method we could determine whether the effects seen in the pups when they are adult, are related to their prenatal stress or altered MB that they received, or both. We found striking results in our first such study (Del Cerro et al., 2010): The adoption procedure reversed the prenatal stress-induced abnormal MB, without changing morphological and hormonal stress-induced effects.

We found further effects of cross-fostering as follows. We previously found that prenatal stress eliminated sexually dimorphic patterns in brain morphology and MB. Female fetuses whose dams were exposed to stress had an increased BAOT neuronal population similar to the control male values, and they showed a significant reduction in MB. The opposite morphological values were found in males raised by their own stressed mothers.

Moreover, prenatally stressed females showed lower levels of E2 than control female rats, plus high cortisol (Cpd B) and progesterone (P) levels. Remarkable to us was that our cross-fostering procedure counteracted the behavioral stress effects without changing the hormonal and morphological alterations. These results demonstrate how maternal care can act as a non-genomic behavioral factor in the transmission of individual differences in the response to stress across generations. Thus, an environmental manipulation imposed during early development. i.e., stress during gestation, can alter maternal behavior toward the pups when they are born, which, when they are adult, can affect their pattern of maternal care transmission and the MB of subsequent generations. Based on our studies on effects of prenatal stress on hormones, brain and maternal behavior, we demonstrated that environmental factors, such as stress during critical periods of brain development, interact with the neuroendocrine system, inducing permanent morphological alterations in brain structures involved in the neural control of maternal behavior, endocrine dysfunctions and, thereby, changes in MB patterns in female rats. Consequently, prenatal stress exerts long-lasting effects on the ratio of these hormones in a

direction opposite that observed after the early post-partum period in female rats (Del Cerro, 2008, Pérez-Laso et al, 2013, 2015).

In a subsequent study (Del Cerro et al., 2010), using a cross-fostering design, we found that while all the neuro-morphological and hormonal alterations caused by prenatal stress were replicated, the most striking new finding in this study was that positive maternal care provided by non-stressed foster mothers toward prenatally-stressed pups prevented the altered MB, in contrast to the altered MB shown by the stressed pups when adult, that were raised by their own stressed mothers. Thus, we demonstrated the role of maternal care in ameliorating and even preventing possible negative effects of epigenetic factors, such as chronic stress, on the behavior of the pups when they reach adulthood. Based on these findings, we concluded that early learning processes do not only modulate, but they can even overcome, the biological determinants of the individual. In this way we offer an optimistic vision of the old "nature-nurture" controversy, since a behavior early in life, such as maternal care, can counteract the possible biological alterations caused by an epigenetic agent, such as stress, during brain development. Thus, rodents may provide a valid and useful model to understand the effects of the unfortunately all-too-frequent situation of gestational stress occurring during prenatal brain development. It is critical to pay attention to the ways in which prenatal environmental factors can affect lifespan physiology and behavior – especially maternal behavior – that ensures the survival of the species; is there any more crucial behavior?

Toward popular science experience

There is a time in our life when we stop and reflect about the sense of our work and how it could contribute to making a better world. This sounds, perhaps too ambitious, but it is something that happened to me a few years ago in a humble way. After my stress studies and the high impact that they had in the scientific community (Del Cerro et al. 2010) on how MB *per se* can counteract the neurobiological effects of gestational stress…it was at the top 10 in ResearchGate during two consecutive years, 2010-2012…I needed to integrate findings from the rat animal model with human neuroscience research, specifically, human attachment. I felt the necessity of translating my lifetime findings and reflections that could be positive for society. So I wrote El Cerebro Afectivo (Figure 3), a popular science book which now is in its second edition and has been well-received by the readers. Some have given me such enthusiastic feedback that I feel encouraged to continue writing this way. The main theme of the book is shown in a few words in its subtitle: "El afecto recibido durante la gestación y la primera infancia

modela nuestro cerebro y nuestro carácter". This message, "Love received during gestation and childhood shapes our brain and character" underlies the content of the different chapters. I am convinced that this kind of work, making science available for the general public, is necessary to counteract the overwhelming amount of false information and "easy talkers and media gurus" who are ever-present in our daily life.

Figure 3. Dani, my grandson and me at Feria del Libro de Madrid signing autographs.

During the last five years, my main effort has been to make available in any way I could (or I was invited to the media: national Spanish TV channel-TVE, Tele-Madrid, Tele-5, Radio Nacional, Radio 3, El País, El Mundo, etc.), the importance of expressing love to our children during the "in uterus" developmental and postnatal periods. This can be expressed by paying attention not only to the diet, and physical or relaxing exercises pre-partum, but especially to the emotional link from the mother toward her newborn baby. This early period in the life of the individual can be critical in the subsequent way of facing life events, resulting in a healthy adaptive person and, hopefully, ultimately in a more fair and peaceful society. And now, let me tell you something important to think about. While all these ideas are

based on my own research, on the literature, and other's conclusions, my ideas are based on the experience of my own perinatal period's "nesting behavior" toward me by my parents, who gave me a childhood full of love and ethical values.

Now at the beginning of the 21st century, an economic perspective is present in neuroscience, resulting from the effort of transferring progress advancement from animal models to human studies. James J. Heckman is leading this current research in parental care and social well-being. My media message is in complete agreement with the critical message from Heckman, who is the Henry Schultz Distinguished Service Professor of Economics at the University of Chicago, a Nobel Memorial Prize winner in economics (2000), and an expert in the economics of human development. Heckman has conducted valuable work with a consortium of economists, developmental psychologists, sociologists, statisticians and neuroscientists, showing that quality of early childhood development heavily influences health, economic and social outcomes for individuals and society at large. He has shown that there are great economic benefits to be gained by investing in early childhood development (Heckman, 2007). For Heckman, the critical time to shape productivity is from birth to age five, when the brain develops rapidly to build the foundation of cognitive and character skills necessary for success in school, health, career and life. I believe that the critical period actually starts before that – during gestation. Therefore, the investment policies should start in at-risk families or pregnant women even during pregnancy in order to overcome the problem that, according to Heckman, "… society invests too much money in later development when it is often too late to provide great value". Such policies would likely prevent the current problem that children from families that are disadvantaged by low economic conditions may fail to develop cognitive and emotional skills that support their adaptive strategies to environmental changes that thereby create a disparity between poor and wealthy families. That is, affection and love can play a significant role in overcoming some of the deficiencies found in at-risk children despite low economic conditions.

Paying our "scientist social taxes" to society

As scientists, I believe that we have an important responsibility toward society. We should contribute the knowledge that we have gained in our career that could be relevant to the advancement of solutions that could ensure a better understanding and a better life for people. How can we do this? Basic research is necessary to maintain scientific advancement, while application of scientific advances to the human condition should be our ul-

timate purpose. Governmental and other societal policies need our contribution, and we have to be attentive to take opportunities when possible. Equity among people is essential, so applying the best work in our scientific fields can be instrumental toward making possible a better life for all. As scientists we can contribute to the 2030 agenda of the United Nations sustainable development goals (UNSDG), that involve the meaning of our own research and transferring our knowledge to society. Part of this task has to be made through media relationships that must be clear and accessible to the non-specialized public, while also accurate, rigorous and feasible. We need to contribute to society what I term "social taxes". That is, because we have developed our research thanks to public and/or private funds, we must "pay back" with the useful fruit of our labor. This is a task that makes us feel that our life has meaning to human development, even if in a modest way.

During the COVID pandemic, the work of scientists throughout the world was crucial, enabling the most rapid creation ever of a vaccine against a new pandemic disease. This is a good example of how science can contribute to a better life for all. We need to understand that equity is the best way of making life better for all humans. This is the major goal for the advance of science: Equity among All.

Growing up in the United States

Barry: Beginnings: How I got interested in the brain and behavior

As an undergraduate at CCNY (College of the City of New York), I had the opportunity to volunteer as a research assistant in the Department of Animal Behavior on the 5th floor of the American Museum of Natural History (AMNH). My mentor was Prof. Ethel Tobach. The project she assigned to me was to count fecal boluses in the "open field test" as a measure of stress in rats. I once asked Ethel how this research could be applied to humans. This incurred Ethel's wrath! She said we do research in order to understand the mechanisms underlying behavior in nature, not to understand their application to humans. It is pure basic research and not to be confused with applied research. This attitude was shared by others working in the Department. Applied research was considered to be a trivial version of pure basic research. I initially incorporated this attitude as I progressed in my academic career. Ethel advised me to take a course at CCNY with Danny Lehrman, who had done his doctoral research at the same department at the AMNH. So as a senior at CCNY, I took Danny's course in Structure and Function of the Organism. I loved his lectures. They were vivid and intriguing and stuck with me. One day he mentioned recent research by Harris et al (1958) & Lisk (1960), who implanted crystals of estrogen into the brain

of ovariectomized cats and rats, respectively, and thereby induced sexual receptivity. At the end of Danny's lecture, I went up to him and asked, how could a crystal of a hormone in one small region of the brain induce such a complex behavior pattern? He said how would you like to study that question as my doctoral student? It was an offer I couldn't refuse. Danny, who was a visiting professor at CCNY for the course, had just founded the Institute of Animal Behavior (IAB) at Rutgers University in Newark, New Jersey, across the Hudson River from CCNY, which is in Manhattan. Danny had recently found that injection of progesterone in ring doves could induce their egg-incubation behavior, and as a beginning doctoral student, I assisted two advanced doctoral students, Sheli Wortis and Phil Brody, in repeating that study. I observed that progesterone not only induced incubation behavior, but also blocked courtship bow-cooing in the male doves.

So, my doctoral dissertation research, which I eventually published (Komisaruk, 1967), became implanting progesterone crystals in the ring dove brain to see if it would induce incubation and inhibit courtship and if so, where in the brain. I visited Bob Lisk at Princeton, newly appointed after his doctoral dissertation at Harvard, who kindly taught me how to make crystalline hormone brain implants and how to do stereotaxic surgery, and I visited Elizabeth Crosby at University of Michigan, who was in her 90s, who kindly and patiently taught me to identify the brain nuclei in the ring dove based on her pioneering work on bird brain (Kappers et al., 1938). Having convinced myself that hormones can act on the brain to influence reproductive behavior, I wanted to know what the hormones do to the neurons that generate the behavior.

My early discovery that set me on the path to the present

The only person in the USA studying hormonal effects on neuronal activity was Charles Sawyer at the University of California, Los Angeles (UCLA). I got an NIH fellowship to do my postdoctoral training with him. At the time, Sawyer's research was focused on identifying the neurons that produce pseudopregnancy in rats in response to artificial vaginal stimulation, which mimics the effects of sterile mating. And Sawyer was also studying the feedback effects of progesterone, which is produced in pseudopregnancy, on activity of individual neurons in the brain. It was in Sawyer's laboratory that I met Carlos Beyer, who was also in postdoctoral training with Sawyer. In my doctoral dissertation, I had actually cited a then-recent study of Carlos' on limbic system stimulation inducing oxytocin secretion (Beyer et al, 1961), and when I mentioned that to Carlos, we connected; I believe that initial encounter was instrumental in establishing our decades-long brotherly colla-

boration and exchange program, which connected me to this day with his wonderful students and investigators and families of Mexico.

Before I had left Rutgers for my postdoc at UCLA, Danny Lehrman promised me a faculty position at the Institute of Animal Behavior after my postdoc, so again, it was an offer I couldn't refuse. After my return to the Institute, one of Danny's invited seminar speakers was James Olds. Olds said that he had developed a method of recording the activity of individual neurons in awake rats. He commented to me that he was training the rats using operant conditioning to identify neurons involved in memory. He lamented that they were in operant conditioning boxes and nobody was observing their behavior. Would I like to observe his rats and try to see whether there is some correlation between the rats' behavior and neuronal activity in their hippocampus, cortex, hypothalamus or any of the other of his electrode implantation sites? It was another offer I couldn't refuse, so I went to his laboratory at the University of Michigan in the summer of 1967. In Olds laboratory, in the short space of a few weeks, I made two discoveries that changed my career trajectory to the present day.

In order to record the activity of small populations of neurons, Olds was using 62.5 micron-diameter nichrome wire as the electrodes. In order to correlate the activity of the neurons with the rat's behavior, the neuronal signals were amplified to headphones, so I could listen to the neuronal action potential activity while watching the rats. I was struck by hearing rapid rhythmical bursts of neuronal activity in the hippocampal electrodes, something like a train of sounds like "chug–chug–chug–chug...". Timing the trains of bursts of activity, I realized that the bursts were occurring about 7 times per second. Watching the rats, it became clear that the bursts of neuronal activity were precisely synchronized with the rhythmical back and forth movement of the vibrissae (whiskers). Watching the thorax, I realized that the respiration was rhythmical in synchrony with the vibrissa sweeps. Then I realized that 7 per second is the rate of the hippocampal theta rhythm, and the hippocampus is a main component of the rhinencephalic/olfactory system, which must be involved in the rhythmical inhalation sniffs of the rat during its exploratory behavior.

So, I was seeing a precise neuronal correlate of a behavioral movement! Then the rats started drinking. There was a movement artifact, but it showed that the rats were licking the water tube at about 7 licks per second. I gave the rats some chow, and when they nibbled on it, the movement artifact was also at about 7 chews per second. Was there a single pacemaker that drives the exploratory sniffing/vibrissa whisking, licking, and chewing, all at the same, EEG theta rhythm rate? This sudden realization was an exci-

ting moment. I continued analyzing these phenomena for years after this initial discovery (e.g., Komisaruk and Olds, 1968; Komisaruk, 1970; Semba and Komisaruk, 1978 & 1984).

In addition to observing the spontaneous activity of the rats and trying to correlate their behavior with their neuronal activity, I tried as many different sensory stimuli as I could think of, in order to see if I could observe any neuronal activity related to the stimuli or the rats' responses to the stimuli. In addition to milk, water, cookies, chocolate, hamburger, vinegar, and anything else I could find, I also tried vaginal stimulation with a 1cc syringe plunger, as we had used in Sawyer's laboratory at UCLA to activate the pseudopregnancy reflex in anesthetized rats. But now, in the awake rats in Olds' laboratory, when I applied the vaginal stimulation, the rats became immobilized. I applied flank/perineum stimulation to see if lordosis would have an effect on any of the neuron populations, and to my surprise, when I applied the flank/perineum stimulation while the rats were immobilized, they all showed an intense lordosis response. My first thought was that their estrous cycles were synchronized and that they were all in proestrus. So, I took their vaginal smears, but the smears showed all different stages of their estrous cycle; some rats showed intense lordosis to flank-perineum palpation during the vaginal stimulation even if they were in metestrus or diestrus. My next thought was that the multiple implanted electrodes in the brain had damaged the neocortex; Frank Beach (1944) had demonstrated a disinhibition of the lordosis response after cortical damage. When I returned to my own lab at Rutgers, I tried the same vaginal stimulation on intact rats, no electrodes implanted in the brain, at different stages of the estrous cycle, and again, they all showed lordosis in response to the combined vaginal stimulation plus the flank/perineum stimulation, even in ovariectomized rats without estrogen treatment. This observation also led to a series of studies over the next years, starting with Komisaruk and Diakow (1973).

Knut Larsson came to the IAB as a visiting professor and soon after he arrived, I had the opportunity to show him the two effects of vaginal stimulation – induced immobilization and lordosis facilitation. Knut was impressed with both effects. We were discussing the immobilization and its possible significance as we were leaving the building one early evening, and in the elevator, I asked Knut what he thought would happen if during the immobilization, we pinched a foot. Would the immobilization block the leg withdrawal reflex? He said let's see. So, we stayed in the elevator, took it back upstairs, got a rat, did the vaginal stimulation, got the immobilization, pinched the foot, and...no leg withdrawal response! We repeated it in several more rats right then and there. Same effect in all: the leg withdrawal re-

flex was completely blocked. We did some more related studies of the effect, wrote up the results in one day, submitted to Brain Research, and it was published immediately – my fastest paper! (Komisaruk and Larsson, 1971).

My question then was: is it just a motor inhibition – a paralysis, or does the rat not feel the (to humans) painful foot pinch, or both? So, I embarked on several studies to see if the vaginocervical stimulation blocked pain. We recorded single neuron responses in sensory thalamus to noxious and innocuous stimulation. The vaginocervical stimulation attenuated the response to pinching limbs (painful), but not to gently brushing the fur (innocuous), even in the same individual neurons (Komisaruk and Wallman, 1977).

To solve the problem of whether the vaginocervical inhibition of the withdrawal response to foot pinch was due to a true pain blockage or to just paralysis, I recruited a colleague, Prof. Edna Ross, who had then recently completed her doctoral dissertation on operant conditioning in rats. First, we suspended each rat in a sling near a lever, and trained it to press the lever that turned off a continuous mild shock delivered to the skin of the back. Then, instead of turning off the shock when the rat pressed the lever, we kept the shock on for an extended period, and the rats soon extinguished the lever pressing response. Then we left the shock on, but applied vaginocervical stimulation concurrently. The rats soon learned to press the lever again, even though the shock remained on, and continued the vaginocervical stimulation for a few seconds until the shock was turned off concurrently. Thus, during the skin shock, the rats performed the bar press response, which provided them with vaginocervical stimulation, and did so, before the vaginocervical stimulation produced immobilization. Thus, we concluded that vaginocervical stimulation provided a benefit to the rat in the face of inescapable skin shock, i.e., probably provided analgesia. The rats did not press the lever for the vaginocervical stimulation in the absence of the skin shock, indicating that the vaginal stimulation was not in itself "rewarding" (Ross et al, 1979).

During subsequent years, we performed functional neuroanatomical, neuronal recording, behavioral, and pharmacological studies in brain and spinal cord of rats, with Carlos and many faculty and students of Mexico. In 1989, Prof. Maria Cruz Rodriguez del Cerro ("Cruz") first visited the IAB at Rutgers at the invitation of Prof. Jay Rosenblatt, who was the Institute Director. We collaborated on two studies on brain activity during induced maternal behavior in rats, first using 2-DG autoradiography, which shows increases or decreases in axon terminal activity, and subsequently adding c-fos immunohistochemistry that shows only increases in cell body activity

when the relevant neurons are active. Cruz previously reported that lesions of the bed nucleus of the accessory olfactory tract (BAOT) of the vomeronasal system induced maternal behavior in virgin rats (Del Cerro, 1998). In our collaboration, consistent with that finding, 2-DG activity in BAOT was reduced in rats that initiated maternal behavior (Del Cerro et al, 1995).

In our subsequent collaborative study, we found that not only did the 2-DG activity show a reduction in BAOT activity upon initiation of maternal behavior, but there was an increase in c-fos activity in the BAOT in alternate brain sections in the same individual rats (Komisaruk et al, 2000). Thus, we concluded that the BAOT input disinhibits the BAOT output, which is consistent with the role of the vomeronasal system in triggering maternal behavior.

A crucial factor: Setting up the Mexico-New Jersey exchange program

During the same years, Carlos Beyer, who was a visiting professor at Rutgers, and I, with the help of Hugo Aréchiga of CINVESTAV and funding from Norman Samuels, Provost of Rutgers-Newark, set up an exchange program between UNAM in Mexico City and Rutgers University in Newark, NJ. The exchange program eventually came to include Mario Caba (Caba et al, 1998; Beyer et al, 1991), Anna-Maria López-Colomé et al, (1990) and with Beyer et al, 1992; and (Peggy) McCarthy et al, 1991), Rafael Cueva-Rolón, Lizbeth Gómez and Julio Muñoz (1995, 1996; Sansone et al, 1997), Oscar Gonzalez-Flores, (Beyer et al, 1989), Porfirio Gomora, together with Beyer et al (1988) and with Gabriela Gonzalez-Mariscal, Carlos and Oscar González-Flores (Gomora et al, 1994, 2020), Gabriela Moralí (Moralí et al, 1989), Anna-Maria Lopez-Colomé with Mario Caba (Beyer et al, 1992; McCarthy et al, 1989, 1990), Jorge Manzo-Denes with Rosa-Angélica Lucio, Margarita Martinez-Gomez and Pablo Pacheco (Manzo-Denes et al, 1989), Margarita Martinez-Gomez with Pablo Pacheco, Laura Oliva-Zarate, Rosario Chirino, and Jorge Manzo-Denes (Martinez-Gomez, 1988, 1988a,1992), Pablo Pacheco with Margarita Martinez-Gomez, Laura Oliva-Zarate and Beverly Whipple (Pacheco et al, 1989; Whipple, 1989), and Yolanda Sandoval (Sandoval et al, 1988).

Additional studies with Carlos included Beyer and Komisaruk (1971, 1971a, 2009), Beyer et al (1985), Komisaruk and Beyer (1972), Komisaruk et al (2008, 2009), Gonzalez-Diddi et al, 1972), (David) Masters et al (1993), McCarthy et al (1989), and (Lowell) Roberts et al (1985, & 1986). More recently we have continued publishing with Porfirio Gómora-Arrati, Oscar-Gonzales-Flores et al (2019), and Rosa Angélica, Porfirio Gómora-Arrati, and Oscar

Gonzalez-Flores (Aguilar-Perez et al, 2020). Others who participated in the exchange program included Porfirio Carrillo, Ramón Eguibar, Alonso Fernandez, and Maria Elena Hernandez.

Why research on humans became necessary

I realized that the only way I could know for sure whether vaginal stimulation blocked pain was to ask women! So, I decided that I would have to learn how to do research on humans. I figured that I should start with an innocuous project. Based on my prior rhythmicity study in rats, I wondered whether physiological finger tremor in humans, which occurs at around 10-12Hz could be synchronized with the alpha rhythm, which is characterized by the same rate range. So, I proposed that study to the Rutgers IRB. It was approved and, since I was the only person on the Newark Campus of Rutgers (the main campus is in New Brunswick, NJ) to apply to perform human research, I was asked to join the university-wide IRB. I figured that this was a good opportunity to establish a reputation for being ethical and careful, prior to applying for IRB approval to do a project on vaginal self-stimulation and pain thresholds in women. So, I was very diligent as the Newark Campus representative on the Rutgers-wide IRB.

Then several chance events coalesced. I went to the First World Sexology Conference in Jerusalem in 1980. I met and befriended Benjamin Graber there, and Julian Davidson mentioned Beverly Whipple's new idea about the G-spot in his presentation. When I returned home after the Conference, I had a letter from Beverly Whipple asking for my reprints on vaginal stimulation produced pain blockage in the rats, and I saw that she was on the Gloucester County College Nursing faculty in Southern New Jersey. I was teaching a course in Human Sexuality, so I took the opportunity to invite Beverly to give a guest lecture in my course. She accepted and came to Newark…with a bodyguard! After her lecture, Beverly told me that she wanted to get a PhD, so I invited her to apply to Rutgers, and for her dissertation research, she could study whether vaginal self-stimulation would block pain in women. She could study the women whom she studied for her then-recently co-authored book, the G-spot (Ladas et al, 1982). Beverly agreed to apply to our doctoral program in Psychology. There was a lot of hesitancy on the part of the Psychology department faculty, concerned that Beverly would be a publicity magnet because of her new book and would not be a serious student. However, Beverly proved to be serious, dedicated, motivated, highly organized and efficient, earned straight A's in the doctoral courses, and ultimately earned her doctoral degree in record time (2.5 years) for the department.

When she started in our doctoral program, I initiated my IRB application to study the effect of vaginal self-stimulation on pain thresholds. I asked Benjamin Graber for a sample of his IRB applications to do research on humans and he graciously sent me one of his applications, which I used to model my application. To my surprise and delight, our IRB approved my application. Before starting the research, I sent a copy of my IRB application to our Provost to alert him to what I was proposing and to know if there was any problem with it. He forwarded my application to the Rutgers legal department. Their conclusion and his was that academic freedom was the critical factor and hence I was free to perform the research. However, a delegation of Psychology Department faculty members appealed to Jay Rosenblatt (we faculty voted Jay as the Director after Danny Lehrman died unexpectedly and suddenly in 1972). Those faculty complained that research on humans would be inappropriate in the Institute of Animal Behavior and would give it a bad name. Jay dismissed their complaint on the basis that it would interfere with my academic freedom. Then Beverly and I proceeded with the project. We found that, indeed, vaginal self-stimulation significantly and markedly elevated the pain threshold in women (Whipple & Komisaruk, 1985 & 1988).

Why coordinating my research on animals and humans became necessary

I was intrigued by the finding that a "simple" pressure stimulus in the vagina could produce a profound inhibition of pain. Which nerve(s) convey(s) the pain-blocking stimulus? With Tiffany Cunningham, a visiting summer undergraduate who, as the daughter of Winona, who was the IAB Secretary, I had known since she was 7 when she played with my lab ring doves. We studied indicators of pain blockage in rats by surgically transecting the three known pairs of nerves that we had shown previously to innervate the genital tract – the pudendal, pelvic, and hypogastric (Peters et al, 1987). We found that transecting both pelvic nerves almost completely prevented vaginocervical stimulation from blocking two types of response to noxious stimulation – the tail flick response to a hot focused light beam and the vocalization response to tail shock (Cunningham et al, 1991). That finding led to two questions: what is(are) the neurotransmitter(s) in the pelvic nerve that produce the pain blockage, and does pelvic nerve stimulation block pain in women? To address the first question, with behavioral biochemist Alan Gintzler at SUNY Downstate Medical Center in Brooklyn, NY, we performed vaginocervical stimulation in rats while superfusing the spinal cord, and analyzed the content of the superfusate, using immunochemistry

to test for vasoactive intestinal peptide (VIP), which had been reported by deGroat (1987) to be released in response to urinary bladder distention, which is also innervated by the pelvic nerve.

We found that VIP was indeed released in significant amounts by the vaginocervical stimulation (Komisaruk, et al, 1989). With Cindy Banas, who was my research assistant, we then injected VIP into the intrathecal space surrounding the spinal cord in awake rats and found a significant pain blockage (Komisaruk et al, 1989a). My colleague in the Chemistry department, Frank Jordan, suggested that we fragment the VIP enzymatically to ascertain the critical pain-blocking component. So, we incubated homogenates of rat spinal cord with VIP and, using HPLC, we analyzed the amino acid content of the 5 fragments that resulted (Barbato et al, 1987). Then we had the fragments synthesized and tested each of them by injecting each into the spinal cord intrathecal space. We found that a particular fragment, the 11-28 amino acid sequence, was significantly more potent than the parent compound, VIP. So, we applied for, and eventually, 2 years later after back-and-forth with the U.S. patent office, received a patent for the pain-blocking action of the VIP 11-28 peptide fragment (Komisaruk and Jordan, 1995).

The puzzling finding that unexpectedly led me to study the brain activity during orgasm

In order to test whether the pelvic nerve mediates the pain-blocking action of vaginal/cervical self-stimulation in women, a good way to do that was to study the effects of blocking the pelvic or other genital sensory nerves' access to the brain. So, with Beverly and spinal cord physiatrist, MarcaLee Sipski, I obtained IRB approval to study women with complete spinal cord injury at different levels that would selectively block the access of the pudendal, pelvic, and hypogastric nerves' access to the brain. As a means of establishing the effect of blocking all genital sensory input to the brain, I included the condition of a high level of complete spinal cord injury, at or above the thoracic-10 spinal cord level, expecting that the effect of vaginal or cervical self-stimulation would be completely blocked. To my great surprise, and that of my collaborators, the five women in that group actually showed the highest vaginal self-stimulation-induced elevation of the pain threshold, measured at the fingers. In addition, they said that they could feel the stimulation, which was a great surprise to themselves, because their health care providers had told them that after their spinal cord injury, their sex life was over, and since then, they never tried vaginal or cervical stimulation (Whipple et al, 1996 & 1996a; Komisaruk et al, 1997).

In addition, these women said that although they had no sensation below the level of their injury, nevertheless, they did have menstrual discomfort. To check the possibility that the women's spinal cord was not completely severed, we analyzed MRIs of the region of their injury. MarcaLee could not believe our findings and thinking that something must be wrong with our methods, she abandoned the project. My radiologist colleague from the medical school, Andrew Kalnin, confirmed that the spinal cords were, in fact, severed. I thought that the only possible way for the women to be able to respond to the vaginal or cervical self-stimulation was via the vagus nerve, which is the 10th cranial nerve that courses through the neck, thorax, and abdomen, bypassing the spinal cord. The classical concept of the vagus nerve is that it does not project as far as the pelvic region. However, at that time there was one recent study in rats, in which a nerve tracer (horseradish peroxidase) was injected into the cervix and it labeled the sensory (nodose) ganglion of the vagus nerve (Ortega-Villalobos et al, 1990). In order to test the possible genital sensory functionality of the vagus nerve in rats, with my students, we performed a study in which I surgically removed a segment of the spinal cord at the mid-thoracic level and tested for brain-mediated responses to vaginocervical stimulation.

We found that the pupil dilatation response to vaginocervical stimulation, which we previously described (Szechtman et al, 1985), was clearly still present after the spinal cord transection. Then I cut the vagus nerves in these spinal cord-transected rats; that procedure did then abolish the pupil dilatation response to the vaginocervical stimulation. Furthermore, the pupillary light reflex to a light shone in the eye remained intact (Cueva-Rolón et al, 1996), indicating that the pupil was still functional. To test the role of the vagus stimulation further, we electrically stimulated the cut central end of the vagus nerves and found an immediate, marked dilatation of the pupils (Bianca and Komisaruk, 2007).

I then figured that the only way I could determine for sure whether the vagus nerves conveyed vaginal or cervical afferent activity to the brain in women would be to ascertain whether the stimulation would activate the sensory projection site of the vagus nerves, i.e., the nucleus of the solitary tract (NTS: nucleus tractus solitarii) in the medulla oblongata, in the lower brainstem. This would require brain imaging. Fortuitously, I was invited to present a paper at a spinal cord injury conference at Wake Forest University in North Carolina. I presented my findings on the analgesic effect of vaginal or cervical self-stimulation in the women with complete spinal cord injury and mentioned the obvious need to test the possible role of the vagus. The organizer of the conference, John Keyes, invited me to collaborate with him

using the PET (Positron Emission Tomography) facility at the University, of which he was the director, to test the hypothesis… Another offer I couldn't refuse. I obtained a grant to do the study and started by testing an able-bodied woman to develop the methodology (Whipple and Komisaruk, 2002). Before we could proceed with the project, John informed me, sadly and shockingly, that he had developed retinal degeneration, was going blind, and was forced to retire. He was replaced by a woman director of the facility who informed me, through her staff, that there was no way she was going to permit us to proceed with a study of vaginal self-stimulation at what was now "her" Southern Baptist hospital PET facility. So that was the end of our PET study.

But about a year later, at a neuroscience seminar at Rutgers-Newark, attended by some faculty members from the fMRI (functional Magnetic Resonance Imaging) facility at the nearby medical school, UMDNJ (University of Medicine and Dentistry of New Jersey), Kristine Mosier, a member of their Dentistry department mentioned that she was studying swallowing movements in patients after cancer surgery, by analyzing brainstem fMRI activity. I told her about my vagus-brainstem hypothesis, and she offered to collaborate using the fMRI facility. That is how I finally got to study whether vaginal or cervical self-stimulation in women with high-level complete spinal cord injury would show activation of the NTS. Since there was no fMRI atlas of the lower brainstem, we had to perform a study to locate the NTS. I figured the best way was to triangulate on the NTS by functionally locating the surrounding cranial nerve nuclei, and also by activating the NTS by a known sensory modality, i.e., gustation, as Norgren and Leonard (1971) had shown in rats. The NTS is surrounded by the facial, trigeminal, and hypoglossal cranial nerve nuclei that control facial movement, respond to sensory stimulation of the face, and control tongue movement, respectively. So, in order to activate the corresponding cranial nerve nuclei that surround, and thereby delineate the location of the NTS, I asked our able-bodied participants to smile and pucker, tap their face, and press their tongue to the roof of their mouth. Those procedures did, indeed, activate the specific brainstem regions corresponding to the location of the respective cranial nerve nuclei based on their anatomical location. In order to activate the NTS by gustation, I made a sauce of sugar, salt, lemon juice and mustard, which the scan participants drew into their mouth via a long flexible tube. Then they applied vaginal or cervical self-stimulation. The region of the brainstem activated by the tasting sauce was surrounded by the activation of the other three cranial nerve nuclei, and the vaginal/cervical self-stimulation activated the region directly below the region activated by the tasting

sauce. We verified the location of the cranial nerve nuclei in collaboration with Laszlo Zaborszky, an expert in human neuroanatomy, in our Rutgers Center for Molecular and Behavioral Neuroscience. This provided the location of the vaginal/cervical sensory input, which we then used to ascertain that all five of the women with the complete spinal cord injury did, in fact, show activation of the NTS in response to their vaginal and cervical self-stimulation (Komisaruk et al, 2002). In the course of their self-stimulation, 3 of the 5 women reported that they experienced orgasms. Thus, we were able to identify brain regions activated by orgasm in women – the first evidence of this phenomenon (Komisaruk et al, 2004).

With doctoral students Nan Wise and Eleni Frangos, we subsequently performed an extensive study in able-bodied women on the brain regions activated by orgasm, and mapping the brain regions activated by clitoral, vaginal or cervical self-stimulation. We found, to my surprise, that nipple self-stimulation also activated the same brain region, i.e., the paracentral lobule, as did the genital self-stimulation (Komisaruk et al, 2011). We have subsequently identified the brain regions activated during orgasm in able-bodied men (Allen et al, 2020). The similarities in brain regions activated during orgasm between women and men, studies performed in collaboration with Kachina Allen, who was doing a postdoctorate with us, seem to be greater than the differences. In men, we are now seeing the onset of inhibition occurring during, and continuing after, orgasm most likely related to the refractory period.

My unexpected entry into studying sexual pathologies

In 2009, Sandra Leiblum of the Rutgers University Medical School contacted me, having learned of my fMRI genital system mapping. She had characterized a condition that she termed, Persistent Sexual Arousal Syndrome (PSAS), in which women experienced intense, unwanted genital sensations, which some described as an intense itch that they couldn't scratch, or being on the verge of orgasm for prolonged periods without relief by sexual stimulation, which could even make the feelings more disturbingly intense (Leiblum and Nathan, 2001). Sandra decided to change the name of the syndrome to Persistent Genital Arousal Disorder (PGAD) in recognition that the women did not feel that the distressing symptoms were "sexual" (Leiblum, 2006). She asked me to test whether we could detect brain activity correlates of the condition. Sandra and I started to do some recordings, but tragically, she had a terrible fall while bicycle riding, hit her head, fell into a coma, and died soon afterward. I didn't have the heart to continue our study. Shortly afterwards, Walter Fanburg, a psychiatrist,

phoned me, having heard of our work with PGAD, and told me that his wife had the condition, which he believed was caused by a Tarlov cyst producing pelvic vasocongestion. I had never heard of a Tarlov cyst, so I looked it up, and found that Tarlov cysts typically form as a "blister" on the Sacral 2-4 nerve roots in the spinal canal (Tarlov, 1932). I realized that of course, these are the nerve roots of the pudendal and pelvic nerves, which convey genital sensation. The cyst could irritate the nerves, thereby generating the PGAD symptoms. To test this hypothesis, I got IRB approval to contact women in a PGAD support group to ask if they had MRIs of their sacral region that they would be willing to send to me on CDs. I asked Huey-Jen Lee, the Chief of Neuroradiology in our medical school, if he would be willing to review the MRIs with me, and he agreed. By chance, he had already published a paper claiming that he found Tarlov cysts in about 1% of 700 patients who had come to his facility for complaints of back and leg pain. As I started to receive MRI CDs from the women in the PGAD support group, and brought them to Huey-Jen, one after another had at least one Tarlov cyst. After about 8 such MRIs in succession, he said he had never seen so many Tarlov cysts in his entire career! More MRI CDs came in and we saw more Tarlov cysts, until we tallied 12 cases of Tarlov cysts in the first 18 women. At that point, we decided to publish the findings (Komisaruk and Lee, 2012) in the Journal of Sexual Medicine (JSM)). About a year later, Irwin Goldstein, the Editor-in-Chief of JSM, phoned me and said a young male patient of his has symptoms of intense pain upon erection, and could it possibly be related to a Tarlov cyst? I said possibly, so he arranged an MRI. When we reviewed those MRI images, we observed 4 large Tarlov cysts (Goldstein et al, 2017). The young man had the Tarlov cysts treated surgically by Frank Feigenbaum, who specialized in the surgery mainly for back problems, and his painful erections subsided gradually over the next 13 months until they were completely pain-free. Subsequent to our 2012 paper, Feigenbaum and Boone (2015) published their results of performing surgery on Tarlov cysts in 11 women with PGAD, in whom they reported that 8 were entirely free of symptoms post-surgically and another 2 had significant relief.

These experiences induced Irwin Goldstein to obtain MRIs in the women and men who were PGAD patients in his Sexual Medicine facility at Alvarado Hospital in San Diego, CA. He contacted me to review the MRIs. Irwin also enlisted the assistance of Choll Kim, an orthopedic spine surgeon at the Spine Center affiliated with the hospital. Together, via weekly video conferences, we analyzed the MRIs jointly. We found many cases of Tarlov cysts in the patients, both women and men. Choll started to perform surgery on the Tarlov cysts in some of the cases, with great success at allevia-

ting the PGAD symptoms. However, we were also seeing a substantial number of patients who complained of the symptoms of PGAD but we found no evidence of Tarlov cysts. I mentioned that it seemed to me that their MRIs showed what looked like one or two bulging intervertebral discs that could be impacting the nerve roots in the cauda equina. Choll was skeptical, but was willing to try an epidural injection of anesthetic at the site of the bulging disc(s) to test whether it would alleviate the symptoms temporarily for the duration of the anesthetic.

The first woman on whom he performed that procedure had immediate relief of her PGAD symptoms. Choll's specialty is minimally-invasive intervertebral disc surgery using laser and suction under CT-X-ray guidance to remove the bulging disc tissue. In cases of a bulging disc, the gel-like core (pulposus) of the disc leaks out through the torn tough outer ring (the annulus) of the disc, a condition termed "annular tear". As it is a "foreign" protein, it produces inflammation of the dura mater and diffuses onto some of the nerve roots that comprise the cauda equina containing the genital sensory nerve roots (sacral roots 2-4). We have now seen more than 20 such cases, with which Choll has about a 70% rate of clinically significant alleviation of the PGAD symptoms as a result of his minimally invasive endoscopic surgery. Before such decision to perform the surgery, we use a specific "algorithm" that we developed that entails extensive sequential testing to ascertain whether the PGAD symptoms could be due to local genital pathology per se (e.g., vaginal infection) or some other factor (e.g., compression of the pudendal nerve in the perineum). In the algorithm, standard gynecological examination, local surface anesthetic administration on the genitals, neurogenital reflex testing, and pudendal nerve block if indicated, are first performed to assess for possible peripheral genital pathology. If the pain and discomfort of the PGAD symptoms persist after all those procedures, then Choll performs the epidural anesthetic injection. If that procedure alleviates the symptoms temporarily for the duration of the anesthetic effect, then he proceeds with the disc surgery (Goldstein et al, 2021).

This brings us to the present. We are seeing a combination of symptoms that accompany the PGAD symptoms; they include vaginal and cervical pain, bladder and rectal discomfort and pain, even umbilicus (belly-button) pain, and back and leg pain. The combination of pudendal, pelvic, and sciatic nerves convey sensation from these body regions, and all three nerves enter the sacrum at levels 2 and 3 and converge in the sacral roots that are included in the cauda equina. The nerve roots formed by these nerves can all be irritated by the bulging intervertebral discs with annular tear, and the symptoms are alleviated by the disc surgery. Many of these women and men

have for years been treated unsuccessfully by their health care providers, who have focused on the peripheral organs, finding no pathology there. It is clear that the sensory nerve roots conveying the sensations from the peripheral organs can be irritated where they pass up in the cauda equina toward the spinal cord in the spinal canal. Many health care providers – gynecologists and urologists among them – tell us that it never occurred to them that genital pain could be due to non-genital pathology at sites that are remote from the genitals…they were never required to study neurology! We tell them that it is a kind of genital "sciatica". That, they understand – that a pinched nerve at its entrance to the spine can produce a sensation perceived as originating in the foot or leg. Little by little, our understanding is increasing of the role of the nerves, spinal cord, and brain in genital processes and pathologies.

Most recently, Cruz and I have discussed the possible neural bases of cognitive aspects of sexual feelings, and how the neurons of the brain could generate such complex effects, rather than just the regions of the brain activated during sexual response and orgasm; we collaborated on writing two theoretical articles, speculating on possible underlying mechanisms including orgasm and pain (Komisaruk and del Cerro, 2021 & 2022).

My main lesson learned: follow my findings, not my hypothesis

So, my first experience of being intimidated for asking Ethel Tobach how our basic research on animals could be applicable to the human condition has been redeemed after my decades of research. What started out as basic research on how a hormone crystal in one part of the brain could "turn on" a complex behavior pattern, led to my pain research, that led to my brain imaging research in humans, that led to my current collaborative work that is eliminating genital pain in women and men. As Noel Kim points out in his chapter in this book, you really never know how "basic" research may lead to practice that is of immediate and direct benefit to humankind. I had no idea where possible answers to each question that I asked would lead. One really never knows. But I have found in my experience that if something looks incongruent, strange, unexpected, puzzling…and keeps cropping up in my study, it is probably more important to try to understand that – it is reality – than to stick with my initial hypothesis. Understanding the bases for reality is the goal and power of science; biological reality is a miracle. If through our research, we are able to decipher some little bit of reality, we feel like we have a handle on the universe. If through our research, we can figure out something, we understand it! What a gratifying feeling…all the better if in some way it actually helps people!

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XII

The precious animal models to understand human autism

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Introduction

Autism spectrum disorder (ASD) represents a set of behavioral patterns appearing in childhood that do not correspond with the known typical development. Such behaviors appear after modifications in the growth and maturation of several structures in the brain. The worldwide elevated number of children in the spectrum made ASD a challenging concern for science and society because it remains for life and the neurodevelopmental complexity of the disorder slows down the innovation of new therapeutic approaches. Although research in humans gives appropriate knowledge, the advance to date also depends on several research designs using non-human models of autism.

Animal models are relevant to comprehend human disorders like autism. Although human-animal differences exist, it is essential to notice that similarities exist, allowing proper translational efforts ranging from behavior to neurobiology. In our experience, animal models strengthen any approach we can design to benefit people with ASD. Thus, our vision is that translational work is a must when studying disorders with the confidence that its proper practice will result in more robust knowledge.

Autism Spectrum Disorder in Humans

There is only one way to diagnose ASD, i.e., behavioral observation. Neither laboratory test nor brain EEG, or any other lab test, is helpful to determine whether a child is in the spectrum. Thus, when asking about what autism is, we must consider the set of behavioral parameters that exhibit eye-catching differences. The word autism originated from the Greek autos, meaning self, introduced by Eugen Bleuler in 1912 (Kuhn and Cahn, 2004) to indicate the self-isolation from social contact observed in ASD children. To date, this is still one of the critical behaviors to recognize autism, but now we know

that the core of ASD is a triad of behaviors. In addition to social isolation, children display alterations in language and movement, including stereotyped movements (Lai et al., 2014). However, the spectrum refers to other behaviors expressed by children, including hyperactivity, sleep problems, gastrointestinal alterations, or abnormal facial expressions (Coleman and Gillberg, 2012; Manzo, 2019).

Data on autism prevalence show that 1 in 44 children (Center for Disease Control and Prevention) or 1 in 100 children (World Health Organization) display autistic behaviors, which indicates a severe problem worldwide because it occurs in every society. One hundred years after Bleuler, the detailed analysis finally agreed to name the set of behaviors as Autism Spectrum Disorder (ASD) published in the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 2013). ASD behaviors result from brain alterations characterized by modifications in neurons, neurotransmission, and circuits in the central nervous system. The etiology of such changes came from genetic, epigenetic, or environmental factors (Yoon et al., 2020). Mutations in different genes are responsible for the appearance of autistic behaviors (Muhle et al., 2004), with many genes found in chromosome 7 (Ashley-Koch et al., 1999). Epigenetic mechanisms, which regulate gene expression and chromatin, also play an essential role in ASD (Waye and Cheng, 2018). Furthermore, environmental factors such as viral infections, zinc deficiency, valproate exposure, or pollution also play an essential role in triggering autism (Waye and Cheng, 2018; Yoon et al., 2020).

Neuroimaging studies show that modifications in the brain of autistic subjects can comprise several regions, including the amygdala, the hippocampus, the basal ganglia, and the whole cortex (Manzo, 2019). Thus, ASD behaviors seem to depend on the altered region. The size of the amygdala increases, which is related to anxiety and aggressiveness. The hippocampus shows reduced connectivity with different cortex areas, which affects the memory process (Cooper et al., 2017). The caudate and putamen nuclei at the basal ganglia are enlarged; the nucleus accumbens shows hyporeactivity to pleasant stimuli, the globus pallidus shows an increase in its curvature, and the substantia nigra show failures in the expression of the Auts2 gene (Manzo, 2019).

The frontal cortex is involved in functions of attention, emotions, and social interaction, and such behaviors are modified when their convolution folding is defective in ASD (Nordahl et al., 2007); the left temporal cortex shows reduced activity in ASD, which explains the alteration of language (Eyler et al., 2012); and the cortex of the parietal and occipital regions are

thinner in ASD; hence it seems that it underlie changes in the vision processes to identify scenes or to track objects in movement (Robertson et al., 2014). Furthermore, another structure, the cerebellum, has a unique role underlying ASD because it is one of the regions with the most significant and expected changes in the autistic brain. Cerebellar modifications can range from cytoarchitecture, as a decreased number of Purkinje neurons, to hypoplasia and alterations in the connectivity of higher structures such as the prefrontal cortex (Belmonte et al., 2004). Also, along with the oliva complex, the cerebellum seems to underlie sound sensitivity because its function is also related to auditory processing (Monje-Reyna et al., 2019; Ida-Eto et al., 2017).

In addition to the central nervous system, the complexity of the gastrointestinal tract is now considered another area underlying ASD behaviors. The microbiome and the enteric nervous system are two critical regions under intense research.

Some authors regard the microbiome as a human organ. However, its composition depends on a wide variety of microorganisms living in our gut (Baquero and Nombela, 2012), some of them with the peculiarity of being transferred from mother to offspring (Schei et al., 2017). This complex organization of the microbiome exists in all mammals, significantly impacting the subject's physiology, including neural development (Barko et al., 2018). Because ASD is a neurodevelopmental disorder, the role of the microbiome is under intensive research. Children with autism frequently suffer gastrointestinal discomfort, including constipation, diarrhea, and acid reflux. All of them seem to be directly related to the disorder (Buie, 2015), which has stimulated the innovation of therapies for ASD focused on the microbiome as probiotics administration or fecal microbiota transplant (Yang et al., 2018).

The enteric nervous system is the distinctive set of more than 100 million neurons and glia found in the gastrointestinal tract serving just for its function (Rao and Gershon, 2018). These nerve cells have close interactions with the microbiome and show bidirectional communication with the brain (Mayer et al., 2015). They can regulate the physiology of the gut without inputs from the spinal cord or the brain, and its alterations underlie different neurological disorders, including ASD (Rao and Gershon, 2016). The knowledge about the enteric nervous system is scarce, but to date, research indicates that we should view the role of enteric neurons in the development and therapy for autism.

Autism in Animal Models

Does autism occur in animals? The answer is uncertain because we ignore if autism does not exist in animals or are not aware of this, perhaps because they do not survive in the wild. Whatever the explanation, science found a way to develop animal models for ASD and several other disorders in laboratories. The fact is that scientific knowledge of alterations underlying several human disorders and diseases is strengthened by using animal models. Although models lack to represent the whole set of alterations observed in humans, they are helpful because they exhibit some worthy features of the disease that undoubtedly increase our comprehension. For example, in studying the neural basis of ASD, animal models are of great value. Thus as far as scientific information on this topic is coming into view, the number of animal models is constantly increasing.

The effect of hereditary challenges is of great concern in the etiology of autism. Studies in human twins show a significant impact of genetics on ASD (Tick et al., 2016), and it is known that mutations in several genes at different chromosomes underlie the expression of ASD behaviors (Muhle et al., 2004); therefore, it is highlighted the importance of understanding autism genetics for clinical practice (Vorstman et al., 2017). Accordingly, many models of mutations in animals have been developed to help increase knowledge in this field. For example, a remarkable group of proteins known as neuroligins is attached to the postsynaptic neuron membrane and interact with presynaptic proteins to allow a proper synaptic transmission (Chanda et al., 2017). The family of neuroligins is coded in five different genes, and each protein could be related to an excitatory (Dang et al., 2018) or inhibitory synapse (Varoqueaux et al., 2004). Some mutations in the sequence of neuroligins link to ASD (Jaco et al., 2008); hence several mice models of neuroligins mutations have been developed for studying ASD (Trobiani et al., 2020). To date, such models help understand the role of these proteins in the expression of autistic behaviors (Jaramillo et al., 2014). Like neuroligins, other rodent models are used to study genes involved in neurogenesis, migration, morphogenesis, and synaptogenesis in the different layers of the cerebral cortex (Garcia-Forn et al., 2020).

Other approaches are modeling ASD in mammals using teratogens such as valproic acid. They have been useful in determining the hyperconnectivity of the rat brain cortex (Rinaldi et al., 2007 & 2008), which increases the local connection but decreases the strength in excitability resulting in hyper reactions. A similar effect of ASD has been described in the amygdala and the entire limbic system (Markram et al., 2008). Another key region analyzed in murine models of autism is the cerebellum because it is one of

the most affected areas in human ASD (Courchesne, 1997). Animal models have shown abnormal architecture and loss of Purkinje neurons (Hampson and Blatt, 2015) and alteration in androgen receptors (Perez-Pouchoulen et al., 2016).

In the zebrafish, a non-mammalian vertebrate ASD model, mutations in the chd8 gene are associated with enlarged prosencephalon and metencephalon and gastrointestinal alterations, as observed in humans (Bernier et al., 2014). Furthermore, the macro anatomical characteristics in autistic humans, such as the increase of the brain volume in the first year of life and the concurrent molecular alterations, produce abnormal social behavior that is also exhibited by the zebrafish ASD model (Zimmermann et al., 2015).

Invertebrates are also helpful animal models of ASD. For example, studies in nematodes have contributed to understanding the role of neuroligins (Rawsthorne et al., 2020), androgens (Gámez-Del-Estal et al., 2014), and alterations in the GABAergic system (Schmeisser and Parker, 2018) and serotonergic and dopaminergic systems (Izquierdo et al., 2013). Furthermore, nematodes are of great value in understanding the role of genes in the altered social behavior in autism (Rawsthorne et al., 2021).

Animals to humans: the translational challenge

In any field of biomedical research, a fundamental purpose is to translate new knowledge obtained from animal research for proper use in human health (Fontanarosa and DeAngelis, 2002). This effort is subdivided into T1 and T2 tasks, the former described as "the transfer of new understandings of disease mechanisms gained in the laboratory into the development of new methods for diagnosis, therapy, and prevention and their first testing in humans" and the latter described as "the translation of results from clinical studies into everyday clinical practice and health decision making" (Woolf, 2008). These two steps are essential to link basic research in animal models to a massive use in the human health system.

The pathway to procure animal models of autism goes from manipulating genes to the use of teratogens, and all these models are precious paradigms for understanding the disorder in humans. Notwithstanding, translational evidence is an overcomplex challenge. However, the known cases of autism in children after exposure to teratogens in-utero (valproic acid, ethanol, thalidomide, and misoprostol) allowed us to understand the genetics of autism (Dufour-Rainfray et al., 2011) and also allowed to develop animal models because it is possible to find parallelisms between humans and animals (Arndt et al., 2005). This parallelism is represented in the complex

known as mirror neurons in behavioral neurophysiology. The group of Giacomo Rizzolatti found that in monkeys some neurons at the premotor cortex discharge during movements of grasping, holding, and tearing with the hand and that those neurons also discharge when the monkey just observes the movements executed even by a human hand (Pellegrino et al., 1992). The same kind of neurons was also reported in humans and named mirror neurons as they trigger imitation behavior (Iacoboni and Dapretto, 2006). The whole story of mirror neurons can be read in a fascinating paper from the same group, where they indicate how the impairment of mirror neurons could result in autistic behaviors (Rizzolatti and Fabbri-Destro, 2010). To date, it is accepted that the mirror neurons system is crucial in understanding the neurobiology of autism.

The translational efforts are not focused just on genetic and teratogenic models. They also include studies in groups of animals that exhibit specific behaviors, such as those that by nature are solitary in the wild. A list of several species of solitary mammals is starting now being used as models to analyze the neural basis of reduced social behavior (Reser, 2014) because it represents one of the critical autistic behaviors. Thus, in studying ASD, we still need more research in basic science and translational efforts. Notwithstanding, what we have learned in autism is that translation of knowledge has been a bidirectional task, i.e., human experiences allow the development of animal models, which in turn allow a better understanding and innovation of new therapies for humans in the spectrum. Where are we now? to respond, we can paraphrase the sentence that John Lennon sang to his beautiful boy, "but I guess we'll both just have to be patient, 'cause it's a long way to go, a hard row to hoe."

Summary

For several years our laboratory was focused on basic science for studying the neurobiology of sexual behavior in rats. Still, ten years ago, our goal turned to focus on autistic behaviors. The detour followed our awareness of the high autism prevalence in children, in the world and our community, and we decided to develop research for the benefit of ASD children. The literature taught us one characteristic of the autistic brain, as it is enlarged compared to typical developing brains, with some regions showing an increased number of neurons (Amaral et al., 2008); hence, it seems that, at least for autism, nothing is missing in the brain. Then, we launched our research line by asking what we could do to change the functions of those neurons, and the core of our work considered two concepts, brain plasticity and enriched environment.

The knowledge from behavioral neuroscience studies has shown for a long time the potency of the brain to modify its structure, function, neurochemistry, and synaptic organization in response to experience, all of them to evoke more effective or adapted behaviors. Brain plasticity is the concept that encircles the experience-induced brain changes (Kolb and Whishaw, 1998). On the other hand, one of the best stimuli to induce brain plasticity is an enriched environment, defined as an environment that stimulates sense organs and promotes subjects' physical activity (Sale et al., 2009). Therefore, in responding to the "what we can do" question, we first decided to increase the body movements of children with autism not to see brain plasticity but to see instead if this enriched stimulation impact cognition and behavior.

A series of experiments stimulated body movements in children with autism. First, we took advantage of the high interest that autistic children show for electronic screens and used a video console to increase movements with virtual sports (Crespo et al., 2016). After a year of a controlled program of virtual sports, we found that children showed more motor abilities and increased language, social interactions, and better expression of emotions. An important finding in this study is that girls acquired abilities faster than boys. Thus, further work confirmed that both sexes have similar potencies to increase capabilities. However, girls always learn more quickly than boys, which must be considered in treating autism.

In another set of experiments, we used auditory stimulation and analysis at different levels in a rat model of autism and ASD children (Monje-Reyna et al., 2019; Nuñez-Arcos et al., 2020). In both species, we used Mozart's Sonata K448 at a volume of 70 decibels. In rats, we found that musical stimulation activated cannabinoid receptors in the cerebellum, while in children we observed stability in heart rate and systolic pressure. Although it is not possible to explain both findings, it is clear that a translational effort is needed to decipher how they are connected. However, a key topic is that using the same methodology in basic and applied science is a must translational task.

Our experience tells us that it is possible to carry out both basic and applied research that helps to understand autism and to develop new therapeutic approaches, no matter that we do not have access to the latest technologies, that is desirable. Also, limited budgets decelerate the potency to design new strategies and do not stimulate more translational procedures. However, all of us must go on; the cause is worthy.

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XIII

Sexual and maternal motivation: the challenge of their study in animals and humans

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Introduction

In our quest to identify and understand our behavior, biology, and health, humans have employed animal models in research for over two millennia. It is precisely the question "why do we behave in a certain way?" that has promoted the development of the psychological aspects of animal research. Numerous discoveries in neurophysiology based on animal models have formed an integral part of the development of human knowledge with great translational relevance. However, the continued advancement of non-invasive technologies and methodologies has led many researchers to question whether animal experiments still have a place in behavioral psychology and neuroscience. If this is debatable in relation to behavior, it is even more so in terms of neurophysiological processes, such as motivation, that are difficult to assess in animals and then compare to humans.

Without doubt, the anatomical insights gained through studies using animal models have provided the basis for a better understanding of the neural substrates of motivational processes. Indeed, the complex anatomical organization of the reward system and the topographical ordering of its cortical and subcortical connections could not have been observed in such detail without the technique of neuroanatomical pathway tracing in animals. However, there is justification for the reluctance to accept this approach when it comes to comparisons with humans because environmental, social, cultural and educational factors all play major roles in the molding of behavior, and they are difficult or almost impossible to replicate in animal models.

Largely for this reason the study of motivation in animal models has consisted in applying behavioral evaluation paradigms involving aspects of positive reinforcement using various types of rewards (food, drink, or hedonic rewards like sexual or maternal interaction). Sexual and maternal

behaviors are motivated and rewarding and contain an implicit hedonic component. They occur in response to the detection and processing of stimuli with sexual or maternal content, in which the prefrontal cortex (PFC) and its connections to the amygdala (Amy) and other structures of the mesocorticolimbic dopaminergic system (MCDS) play important roles in assigning the incentive value to stimuli that makes it possible to induce and maintain motivational states.

Non-invasive neurophysiological methodologies like functional magnetic resonance imaging (fMRI) and electroencephalography (EEG) have allowed researchers to study degrees of localization and functional organization at the brain level and the cortical activity associated with motivated states in humans. But the finer details, such as identifying the anatomical structures of specific pathways, would not have been possible without the use of animal models based, for example, on rats.

Based on the knowledge obtained from EEG and behavioral studies performed with rats, we have been able to carry out a translational approach by applying non-invasive techniques and neuropsychological questionnaires to humans to elucidate the brain functioning associated with sexual and maternal motivation. This chapter presents a compilation of EEG results that support the participation of some structures of the MCDS in the motivational processes of sexual and maternal behavior in rats, complemented with descriptions of studies carried out with humans. It also elucidates the series of challenges and difficulties that we faced as we sought to implement, execute, and publish the cited works.

Sexual and maternal motivation, definitions and how they are measured

The onset of sexual and maternal interactions between individuals requires several phases, including the detection and perception of the stimuli, assigning incentive value to each one, and inducing motivation. Ågmo (1999) defined sexual motivation as "the process that makes an animal search for sexual contact with another animal". In a more operational form, Ågmo and Laan (2022), conceptualized this as "responsivity to sexually-relevant stimuli". In this order of ideas, maternal motivation can be defined as the process that makes a mother search for maternal contact with her offspring (her own or an alien one); thus, it promotes nurturing behaviors (Hernández-González and Guevara, 2009). Sexual motivation in male rats is induced when a suitable stimulus (e.g., a receptive female) is perceived as being sexually-relevant. Similarly, maternal motivation is induced when a female perceives an adequate stimulus; in the case of rats, a pup. Because these sti-

muli tend to induce approach behaviors, they are scored as positive incentives.

Research on motivated behaviors has divided them into two components: appetitive and consummatory (Everitt, 1990). The former includes behaviors that bring one organism into contact with an attractive or desired stimulus, object, or goal, while the latter involves behaviors performed after contact has been made and that often result in achieving a set goal. Hence, the motivated states that characterize sexual and maternal motivation impel individuals to perform sexual or maternal acts. In general, manifestations of the appetitive component have been taken as evidence of increased motivation. Taking this into consideration, the following section describes the appetitive components of sexual and maternal behaviors in rats and humans.

Sexual and maternal motivation in rats

Sexual motivation in the male rat has been evaluated both *in-copula* and *ex-copula*. The parameters of sexual motivation *in-copula* include attention to, and the pursuit of, the female, as well as mounting and intromission latencies (for a review, see Meisel and Sachs, 1994). For *ex-copula* evaluations, paradigms like mazes, Skinner boxes (Jowaisas et al., 1971; Everitt and Stacey, 1987; Hernández-González et al., 2014), and conditioned place preference (Paredes et al., 1998; Ramírez-Rodríguez et al., 2017) have been used to evaluate sexual motivation without displays of direct sexual behavior. Paradigms like the box used to measure sexual incentive motivation have also been very useful in measuring *ex-copula* motivation, since the time that a male rat remains in the area near an inaccessible receptive female (a sexual incentive stimulus) in contrast to other stimuli (such as a non-receptive female or a male) is a parameter of sexual motivation. Because this parameter can be obtained without the interference of other variables, such as learning and memory processes, some researchers have proposed that it may be isomorphic and homologous to the human condition (Ågmo, 1999; Ågmo et al., 2004).

Sachs (2007) sustained that there is a clear distinction between sexual motivation and sexual arousal and posited that penile erection is the gold index of the latter condition, but not all researchers in the field agree (Ågmo 2011). This phenomenon is easily observed in non-copulatory male rats that present non-contact erections in the presence of a receptive female but make no attempt to copulate (Portillo and Paredes, 2019). Similarly, males may be sexually motivated and engage in behavior directed toward recep-

tive females, but are incapable of achieving penile erection and, there- fore, fail to copulate.

Turning to maternal motivation in rats, parameters such as nest-building, pup retrieval, and pup licking have been considered indicators of this type of motivation (Stern, 1996). As in the case of sexual motivation, paradigms such as Skinner boxes (Lee et al., 2000), open-field or elevated-plus mazes (Stern and Mackinnon., 1976; Fleming et al., 1994), and the box designed to measure maternal incentive motivation have been used to study the motivation of mother rats to search for, and then stay close to, their pups.

Sexual and maternal motivation in humans

As in research on motivation using animal models, experimental reinforcement paradigms have been applied in humans, though with much greater difficulty and significant ethical restrictions. Those paradigms use measures like monetary gain (Wilson and Daly, 2004) or the observation of pleasant images or videos (Amezcua-Gutiérrez et al., 2020; Hernández-González et al., 2012, 2013). Unlike animal models, using sexual or maternal rewards with humans is rarely feasible, so only a few approaches using classic and operant conditioning have been carried out (for reviews, see Pfaus et al., 2001; Gewirtz, 1976; Leuner and Shors, 2006).

As a result, studies of sexual and maternal motivation in humans have applied scales, questionnaires, and measurements of objective parameters (e.g., indicators of general arousal) like respiration, pupil dilatation, galvanic skin responses, or heart rate in response to stimuli with sexual (Cohen et al., 1985) or maternal content (Casanova et al., 1994; Brewster et al., 1998). In fact, most studies of sexual motivation or activation in humans have used visual and auditory stimuli with sexual meaning (Stolerú et al., 1999; Hernández-González et al., 2013; Ruíz-Díaz et al., 2012). The most objective works have measured penile tumescence scores (strain gauge measurements) (Chivers et al., 2010; Cohen et al., 1985; Arnow et al., 2002) that include changes in penile volume or circumference in men (for a review, see Barker and Howell, 1992) and vaginal self-stimulation (Komisaruk et al., 2004) or vaginal lubrication in women in response to observing stimuli with sexual meaning (Handy and Meston, 2020).

In the case of maternal motivation, experiments are often made during direct interaction of mothers with their babies (Cervantes et al., 1992; Fleming et al., 1997; Giardino et al., 2008) by evaluating their behavior and answering various scales, or using visual or auditory stimuli with maternal-related content (Casanova et al., 1994; Brewster et al., 1998; Pérez-Hernández et al., 2017; Hidalgo-Aguirre et al., 2020; Sandoval-Carrillo et al., 2020).

The neural substrate of motivation

It is well known that motivated behaviors occur spontaneously in response to specific external sensory stimuli. In order for a subject to detect a stimulus as sexually or maternally attractive, an appropriate internal state is required (*i.e.*, adequate neural functionality and hormonal and neurotransmitter levels), one that promotes an awareness of incentive stimuli. This also requires adequate processing of stimuli with sexual or maternal meaning that will allow the individual to assign incentive value and perceive a stimulus as rewarding. The role of the medial preoptic area in assigning incentive value to stimuli and performing sexual behaviors has been broadly described in rats (Liu et al., 1997; Hurtazo and Paredes, 2005; Hurtazo et al., 2008). Other studies, including some performed in our laboratory, have demonstrated the participation of limbic-prefrontal circuits including, mainly, the reciprocal projections between the PFC and Amy, in processing that entails acquiring the incentive value of stimuli. The PFC and Amy are two cerebral structures that form part of the mesocorticolimbic dopaminergic system (MCDS) and hence, the reward system, which is considered the pivotal substrate for rewarding and motivational states (Berridge and Kringelbach, 2015; for a more detailed description of the neural networks involved in sexual behavior, see the review by LeMöene and Ågmo, 2019). Based on those approaches, our first experiments focused on characterizing the electroencephalographic functioning of the PFC during the motivational and consummatory phases of copulatory interaction in rats. Subsequently, we studied other areas of the MCDS during more specific tasks of sexual and maternal incentive motivation.

A brief review of EEG activity

An electroencephalogram (EEG) is defined as a mixture of rhythmic sinusoidal-like fluctuations in voltage generated by the brain that, it has been suggested, represent the global activity of neurons located in the cortex and subcortical structures. The quantitative analysis of EEG recordings is a useful tool that has allowed researchers to relate changes in brain electrical activity to specific behavioral situations and/or experimental manipulations. By applying Fast Fourier Transformation (FFT), EEG signals can be divided into frequency bands. In the case of rats, EEG recordings are divided into three principal bands: delta (0.5-3.5 Hz), slow theta (4-7.5 Hz) and fast theta (8-12 Hz), and fast frequencies (above 12 Hz), while in humans five main bands have been described: delta (0.5-3.5 Hz), theta (4-7.5 Hz), alpha (8-12.5 Hz), beta (13-25 Hz), and gamma (25-50 Hz). These bands have been associated with certain physiological or behavioral states (Berger, 1929 cited in

Andreassi, 2000). Specifically, the theta band is a frequency range that, as several works on both rats and humans have reported, shows a prevalence during relaxed and motivated states, as well as during processes of selective attention (Cervantes et al., 1992; Kurtz and Adler, 1973). Three important parameters can be calculated from EEG recordings: Absolute Power (AP), which is an index of the energy of the signal measured in μV; Relative Power (RP), defined as the proportional contribution of each band expressed as a percentage of total power; and electroencephalographic correlation (rEEG), an index of the degree of similarity between the EEG activity of two brain structures (López da Silva, 1991; Shaw, 1984). Low correlation reflects higher functional differences and *vice versa*, while high correlation indicates a more homogeneous functioning between structures. Interhemispheric correlation is calculated between structures in the right and left hemisphere, while intrahemispheric correlation is calculated between structures in the same hemisphere. rEEG is calculated by applying Pearson product-moment correlation coefficients between successive amplitude values of EEG segments from two cerebral regions.

EEG studies in rats

Sexual motivation

In a first experiment with male rats, we recorded EEG activity in the prefrontal cortex simultaneously with accelerometric recording of the pelvic thrusting that the rats perform as part of the copulatory responses of mounting, intromission, and ejaculation. During approach behaviors (orientation to, and persecution of, the female rat, two indices of sexual motivation), the PFC showed a lower AP of the fast frequencies but a higher AP of the theta frequencies. While the rats performed pelvic thrusting, in contrast, the EEG bands analyzed (4-16, 18-24, and 26-32 Hz) showed a distinct pattern. Those findings suggest that the PFC manifests characteristic functionalities in relation to the motivational and motor components of sexual behavior, respectively (Hernández-González et al., 1998).

In another study, we determined the characteristic EEG patterns of the PFC during the approach behavior to a receptive female in a T-maze. After a male rat had performed 1 or 2 intromissions with a receptive female (to generate a sexually-motivated state), it was placed in the start box of the T-maze and two female rats, one receptive, the other one non-receptive, were placed in the goal boxes of each arm. Male rats without previous intromissions were used for the non-motivated condition. In that experiment, the sexually-motivated male rats showed a higher motivation to engage in sexual behavior indicated by lower latencies and times to reach the goal box

where the receptive female had been placed, compared to the non-motivated males. This higher sexual motivation was associated with a greater prevalence of slow frequencies (6-7 Hz) in the PFC and a higher interprefrontal correlation in that band while they remained near the goal box with the receptive female. These EEG changes in the PFC could be associated with various processes: the "motivated state" that resulted from the intromissions with the female before entering the maze; spatial orientation and selection of the goal; and processing of the odors and sounds emitted by the female (Hernández-González et al., 2007).

In more recent work, we characterized the EEG activity of other brain structures of the MCDS during sexual incentive motivation in male rats. In addition to the PFC, we have recorded activation and degrees of coupling (or rEEG) among the PFC, nucleus accumbens (NAcc), Amy, and other cortical regions, such as the parietal cortex, that participate in the attention and processing of incentive stimuli. These studies have consistently found a prevalence of slow frequencies in the theta range during sexually-motivated states. For example, work by Domínguez-Estrada (2019) showed that while a sexually-motivated male rat remained near a receptive female, an increased AP of the slow frequencies (7-12 Hz) in the left and right prefrontal cortex was observed. The prevalence of slow frequencies during sexually-motivated states in male rats has been observed not only in the prefrontal and parietal cortices but also in the Nacc, Amy, and vental tegmental area (VTA) (Guevara et al., 2008). Interestingly, there are reports that the electrical functioning of these areas can be altered by several factors, including alcohol (Hernández-González et al., 2009, 2014), stress (Hernández-González et al., 2017, 2020, 2021; Hernández-Arteaga et al., 2020), and suppression of dopaminergic innervation from the VTA (Cortes et al., 2019). Similarly, distinct degrees of coupling, or rEEG, between structures in the MCDS has been observed in relation to motivational states in both male (Guevara et al., 2008; Domínguez-Estrada, 2019; Hernández-Arteaga et al., 2020) and female rats (Hernández-González et al., 2009, 2016).

Maternal motivation

After several years of work on the functioning of structures in the MCDS associated with sexual motivation in rats, we decided to analyze the functioning of this system during the motivational components of the female rat's maternal behavior, since this system is also involved in the rewarding aspects of that type of behavior. In a first study, we compared the functioning of the PFC and VTA during motivational and motorically active (pup retrieval, pup licking) behaviors of rat dams (Hernández-González et al., 2005A).

A significant increase was obtained in the AP of the 8-11-Hz band during pup retrieval compared to basal walking behavior, and during pup licking compared to forepaw licking, the latter a non-maternal behavior that has equivalent postural adjustments and motor elements to those of pup licking. Interestingly, when the mother rats performed consummatory inactive maternal behaviors (*i.e.*, low and high crouching on the pups) no EEG changes were detected compared to the awake-quiet basal state. This indicated the participation of the PFC and VTA mainly in manifestations of the appetitive maternal behaviors of the female rat.

Considering that the detection and processing of odors changes in relation to the estrous cycle (Pietras and Moulton, 1974; Fleming et al., 1989), in another study we reported that during the smelling of nest bedding, lactating rats presented a higher RP of the 8-11 Hz band in the PFC and VTA compared to proestrus-estrus and diestrus rats, while during the smelling of female bedding, no significant differences were observed between these reproductive states (Hernández-González et al., 2005b). Those results led us to propose that the slow frequencies observed during the smelling of the nest bedding could be associated with the adequate integration of olfactory stimuli to modulate the motivational processes involved in expressing maternal behavior in lactating rats.

The transition to studying sexual and maternal motivation in humans

The transition to studying the neurophysiological processes involved in sexual and maternal motivation in humans presented numerous challenges. Although one of the authors had training and experience in recording and analyzing EEG activity in humans, it was not until a colleague psychologist was brought to our laboratory that we ventured to design an experimental protocol for humans. The objective of our first study was to determine the EEG functioning of the PFC in young men while observing erotic stimuli. One of our first concerns was to familiarize ourselves with international ethical protocols for performing experimental research with humans, and determining if it was feasible to follow and apply those ethical specifications in our proposed protocol. As a first step, we submitted our research protocol for evaluation by our institutional ethics committee. That led to the emergence of other concerns, such as where to recruit participants for the experiment? This was never a problem in work with rats because we simply had our institute's animal care facility breed the rats we required. For obvious reasons, this procedure could not be followed for research with humans, so we had to deal with the reality of needing to "convince" potential partici-

pants to agree to collaborate in experiments even though they really had nothing to gain by doing so. Other concerns we had to resolve were of a more technical nature: we needed a specific type of polygraph, a suitable locale for EEG recording, and had to purchase the required instruments and materials, including neuropsychological scales, tests, and stimuli for use in the experiment.

We managed to resolve all those problems and concerns and concluded our first experiment in the study of sexual motivation in humans. But then we faced the even more difficult challenge of completing the steps required to publish the study. During the publication process we realized that several aspects required an approach distinct from the one followed for studies using animal models. 1) We had to obtain samples large enough to satisfy all our experimental controls. That was not an easy task, but required extensive invitations offered on social media, personal inquiries, and even classroom recruitment, especially since we did not have the resources to induce participation by offering a monetary incentive. 2) The ethical aspects are quite distinct and must be observed strictly in all interactions with participants, from the first interview through to the publication of the experiment data. 3) A more specific point that derived from the nature of our research was the need to highlight the advantages of EEG recording for the study of brain functions during motivated states. Our early studies were often challenged –even rejected– by reviewers on the grounds that more sophisticated techniques existed to study brain functioning, such as fMRI and positron emission tomography (PET). While the advantages of those techniques cannot be denied, EEG recordings allow a more flexible environment for recording and offer outstanding temporal resolution. It can be argued, in fact, that the most significant advantage of the EEG approach is that it facilitates frequency analysis since these recordings allow researchers to decompose signals into the distinct frequencies that have been associated with specific psychological processes (see above), and make broader analyses than other recording techniques.

EEG studies in humans

The results of several studies in rats provided us with a general view of the pivotal role that the PFC and other regions of the MCDS play in the processing of the incentive value of stimuli with sexual or maternal meaning, so an obvious question arose: how similar is cortical functioning during sexual and maternal motivation in rats to that of humans? Our attempt to answer this question began with an extensive review of the literature, which revealed that the majority of EEG studies had been conducted many years earlier.

Those works had focused mainly on evaluating cerebral responses associated with orgasm or masturbation, in the case of sexual behavior, or with direct interaction of mothers with their babies, complemented by interviews and personal reports. With the arrival of imaging techniques, several studies reported functional changes in response to sexual and maternal stimuli, but very few used an approach based on EEG recording and frequency analysis. The scarcity of studies of this nature in the existing literature motivated us to develop the works we describe in the following section.

Sexual motivation

Before describing some of the works we have published on humans, it is important to explain that measuring sexual motivation based on a strict definition –"that which drives individuals to seek and approach a goal"– proved to be very difficult. For this reason, various studies, including ours, have opted to measure sexual arousal as an indirect index of sexual motivation. In fact, the excellent review by Ågmo and Laan (2022) states that "effective sexual motivation" is always a combination of sexual arousal and general arousal. Thus, the objective of our first study (Hernández-González et al., 2013) was to determine whether sexual arousal in young men is associated with specific rEEG patterns involving the prefrontal, parietal, and temporal cortices. In that experiment we used a battery of photographs that had been tested in a pilot group of young men who answered two scales: the Self-Assessment Manikin Scale (SAM) (Bradley and Lang, 1994), and the Sexual Arousal Scale (SAS), a Likert-type scale (length from 0-10 cm) which shows images that represent non-erect to fully erect penises with values that range from 0 (erection totally absent) to 10 (maximum erection). Participants in that pilot study (men aged 18-26) used the first scale to indicate valence (pleasant or unpleasant) and general arousal (higher or lower), and the second to indicate the degree of sexual arousal they experienced while observing images with erotic *vs.* neutral content while EEGs were being recorded from the prefrontal and parietal cortices. As expected, when exposed to sexual stimuli, participants reported an overall activation, pleasurable experience and a penile erection. These changes where associated with a decrease in brain connectivity (rEEG) between frontal and posterior cortices in fast frequencies (alpha and beta bands). These results suggest a dissociation between the sensory-processing areas (parietal cortex) and the executive control systems that allows the cold, logical responses (dorsolateral prefrontal cortex).

In another study, one similar to our previous work with rats, we found that alcohol affects cortical functionality (Hernández-González et al., 2012).

In fact, alcohol suppressed the prefrontal EEG changes associated with watching erotic visual stimuli without affecting sexual arousal. This could be associated with interference in the adequate processing of visual erotic stimuli.

It is well known that in addition to the important role that the prefrontal cortex plays in the processing of stimuli with emotional meaning, this cortical area is also involved in various cognitive processes, including planning, decision-making, goal-setting, inhibition, and memory, among others (Godefroy et al., 1999; Fuster, 2002). Taking this aspect into account and considering that stimuli with emotional meaning exert a distractor effect during execution of cognitive tasks, we evaluated the cortical functioning of sexually-aroused young men while solving the Tower of Hanoi (TH) task. We found that those subjects presented a decreased correlation between prefrontal areas with an increased correlation between parietal and prefrontotemporal areas (Amezcua-Gutiérrez et al., 2017). In a similar study, we compared the effect of observing three kinds of stimuli with emotional meaning (erotic, aggressive, neutral) on cortical responses during execution of two cognitive tests, the Wisconsin Card-Sorting Test (WCST) and the TH. Only after observation of stimuli with sexual meaning was a lower rEEG coupling between prefrontal areas observed during execution of the WCST, while a higher rEEG was observed during execution of the TH. Since participants reported a higher subjective degree of penile erection after watching erotic videos, it is likely that enhanced sexual arousal was the main cause of this change. This suggest that such correlation patterns could be associated with cognitive strategies or functional adaptations while participants are being sexually aroused.

Visual stimuli with sexual content have been widely used in laboratory conditions to induce states of sexual arousal in humans, but other types of stimuli have also been employed; for example, reading erotic literature (e.g., Osborn and Pollack, 1977). Due to reports that women perform this activity with greater frequency than men, we explored the valence and degree of sexual arousal that young women reported together with the cortical functionality related to the reading of a sexually-explicit text (Guevara et al., 2018). Participants rated the sexually-explicit text as more pleasurable and, using a modified version of the SAS scale, reported higher sexual arousal compared to reading an emotionally-neutral text. The reading of the erotic text was associated with higher activation in temporal and parietal areas and to a lower rEEG between prefrontal and temporal areas. Those results indicated that while both hemispheres were activated during reading of the erotic texts, the functionality of the prefrontal and parietal cortices was in-

dependent, so this may be a requirement for generating and maintaining sexual arousal while women read texts with sexual content.

It has been proposed that the assigning of incentive value to stimuli with sexual meaning varies depending on several factors, including sexual orientation. Studies have reported, for example, that heterosexual men prefer films with heterosexual content, but homosexual men rate heterosexual stimuli as only mildly pleasant (Mavissakalian et al., 1975). Results suggested the participation of cognitive processes that induce pleasant or unpleasant sensations (Hu et al., 2011) Thus, in a study published in 2020, we reported that while watching an erotic heterosexual video, only heterosexual participants reported a higher degree of sexual arousal (due to greater penile erection). Both groups showed a decreased amplitude of the alpha band in the prefrontal and parietal cortices during observation of both the erotic and neutral videos, indicating increased attention, while only during observation of the erotic heterosexual video did the homosexual men show an increased amplitude of the theta and fast bands in the prefrontal cortex, possibly related to the cognitive processing of the erotic stimulus (Amezcua-Gutiérrez et al., 2020).

Maternal motivation

As mentioned above, the detection and assigning of incentive value to sexual and maternal stimuli varies with distinct physiological states (nulliparity, gestation, lactation, postpartum period), as well as the life history of participants. In the case of responsivity to stimuli emitted by a baby, a higher response has been observed in primiparous lactating mothers compared to multiparous or virgin women. In this regard, some reports indicate that responsivity to stimuli emitted by a baby is similar in biological (BM) and adoptive mothers (AM). Taking these aspects into account, we conducted an experiment to determine the EEG synchronization among prefrontal, temporal, and parietal cortices in non-mothers, BM, and AM while listening to recordings of babies' cries. The BM had a lower rEEG between the prefrontal and temporal cortices while listening that could be associated with lower control of the prefrontal cortex (cognitive process) in the temporal areas (emotional process). The AM had a greater EEG correlation between cortical areas in both hemispheres, probably indicative of a greater modulation of the affective information of the crying baby (Pérez-Hernández et al., 2017).

Mothers change their reactivity to different sensory stimuli, as during the postpartum period (PP) they become more sensible to baby related signal and other type of stimuli (such as sexual) gets relegated, this becomes

evident not only by the change on behavior but also in brain activity. As postpartum period progresses, reactivity to baby signal is maintained but reactivity to sexual stimuli is reestablished. These changes are accompanied by a reconfiguration in brain connectivity, as correlation is increased albeit among fewer cortical areas. This could be related to maternal experience establishment, and as such, optimal response to infant signals could be perform albeit with fewer brain resources and thus, responses to sexual stimuli gets heightened (Hidalgo-Aguirre et al., 2020).

Although a baby's cries constitute one of the most salient stimuli that motivate sensitive maternal behaviors, such as soothing or feeding, human mothers can perceive those cries in different ways and they can evoke diverse reactions, ranging from feelings of love for the baby to infant abuse or neglect. Attachment theory postulates that individuals develop different ways of responding to emotional and challenging situations of attachment depending on the degree to which they trust their parents or caregivers in stressful situations. Women with secure attachment (SA) are better able to regulate emotional demands and more capable of responding contingently to attachment demands than those who developed insecure attachment (IA) (Bowlby, 1969). Against this background, the aim of another of our studies was to compare the degree of activation and synchronization of the different EEG frequency bands between the prefrontal and parietal cortices in mothers with SA and IA while watching an audio-visual stimulus of a baby crying. The mothers' degrees of attachment were measured by the Adult Attachment Interview (AAI). While watching the crying video, SA presented a characteristic EEG activation, mainly in the fast bands (alpha and beta) for the frontal areas and a mixture of slow and fast frequencies (delta and gamma) in the parietal areas. As the SA can adequately response to the baby´s signals, this changes in brain activity could be an indicator of higher emotional regulation and attention when compared to the IA group (Sandoval-Carrillo et al., 2020).

Pregnancy is one of the most important phases of a woman's reproductive cycle. It is characterized by neurophysiological, emotional, and cognitive changes. Numerous reports indicate that cognitive changes occur during pregnancy and the postpartum period, with memory deficits being the most common complaint (Brett and Baxendale, 2001). Thus, in a study developed by Almanza and cols. we characterized the EEG correlations during performance of two working memory tasks (WM), one verbal the other visuospatial, in each trimester of pregnancy (Almanza-Sepúlveda et al., 2018). Distinct patterns of cortical synchronization in each trimester were observed depending on the WM tasks that the women were asked to solve. Thus,

as pregnant women need to focus their attention, the results could hint at some adaptative mechanism in the brain to adequately adapt behavior, such as when solving the WM tasks.

The response women have toward infants 'cues, as well as their cognitive processing, has been associated to reproductive experience (parity) as it can modulate cerebral functioning (Bunderson et al., 2020). In fact, multiparous mothers recognize and interpret a baby's cries faster and more effectively than first-time mothers. Pérez-Hernández et al. (2021) evaluated working memory (WM) in primiparous and multiparous mothers and the cortical functionality associated with this cognitive process while they were listening to a distractor stimulus. During performance of a WM task while listening to babies' cries or a neutral sound, the multiparous mothers solved the cognitive task faster and presented a lower rEEG between left prefrontal and parietal areas, as well as a higher rEEG between parietal areas, compared to first-time mothers. It is probable that these different degrees of correlation between prefrontal and parietal cortices in multiparous mothers could reflect a cerebral mechanism that is required to maintain better memory and suppress the effect of the distractor stimuli, thus allowing them to solve the WM task more efficiently.

Summary

The experiments described above, despite their limitations, have allowed us to reach two main conclusions that could be applicable to both rats and humans. During states of sexual and maternal motivation: 1) some MCDS structures present a prevalence of slow frequencies; and 2) the correlation between the prefrontal and posterior cortices varies depending on physiological states or cognitive processing. It is likely that these EEG changes could be associated with greater attention to the incentive stimuli, modulation of the affective information, or cerebral strategies during cognitive processing. To date, several works have sought to emulate, or make equivalencies between, the behaviors and brain processes involved in sexual and maternal motivation. They have reached important conclusions and revealed many similarities between animals and humans (see Ågmo and Laan, 2022; Georgiadis et al., 2012; Natan et al., 2013). There is a clear need for additional studies that incorporate multiple factors –environmental, social, and learning-related– to help elucidate how they act in conjunction to influence the induction and maintenance of sexual and maternal motivation in humans. Integrative studies of this kind may well reveal novel mechanisms that have important translational relevance to human beings.

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XIV

The importance of smell in mate selection in animals and humans

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Introduction

In recent years, the olfactory system has been the object of study due to its importance in various physiological processes. Smell is the sensory system that most easily carries information to the brain, particularly to the limbic system. Its stimulation can produce vegetative, motor and psychological responses (Fuentes et al., 2011). The response that is produced depends on the type of odor and its intensity. Some of its main functions are: the choice and intake of nutrients, establishment of social relationships, detection of potentially toxic and harmful substances, identification of individuals, etc. However, even when it has important functions, it continues to be one of the least known and studied (Drucker, 2005).

From an anatomical and functional point of view, the olfactory system has several subdivisions: the main olfactory system, the accessory or vomeronasal olfactory system (VNO), the trigeminal system, the terminal nerve ("cranial nerve 0"), and the septal organ of masera. Thus, it can be said that there is an archaic olfactory system, which is in charge of the basic olfactory reflexes; then an ancient olfactory system, which provides automatic control for partial learning of food intake, as well as the rejection of toxic or unhealthy foods; Finally, there is a third way, a recently identified system that is responsible for the conscious perception of smell (Fuentes et al., 2011; Collado et al., 2014; Parra et al., 2016).

One of the most accepted proposals is that stimulation of the main olfactory system produces conscious responses because the information projects primarily to the cerebral cortex, while stimulation of the accessory olfactory system (vomeronasal organ or Jacobson's organ) produces unconscious responses, since the information projects to subcortical areas, primarily limbic (Fuentes et al., 2011) From a physiological point of view, the sense of smell and taste are related to each other and are part of a chemosensory

system. Almost everything we consider taste (95%) we detect with smell (Saavedra et al, 2012).

Accessory or vomeronasal olfactory system

The vomeronasal or accessory olfactory system (VNO) was described by Ludvig Levis Jacobson, a Danish anatomist, who, in 1813, proposed that this organ fulfills sensory functions (Zeller, 2007).

The presence of this organ is well known in reptiles, however, Jacobson also identified it in mammals. Anatomically, the vomeronasal organ is located in the anterior third of the epithelium of the nasal septum at the base of the nasal cavity (Fig.1) (Zeller, 2007).

The VNO has the appearance of a tubular structure, which is divided by the nasal septum. It is a chemosensory organ clearly separate from the nasal cavity in most amphibians, reptiles, and non-primate mammals. The lumen is crescent-shaped and is lined with receptor neurons, in addition to being covered by fluid from the vomeronasal glands, lateral to the lumen, there are large blood vessels innervated by the autonomic nervous system, these perform vasodilation and vasoconstriction, to stimulate to the lumen (Keverne, 1999).

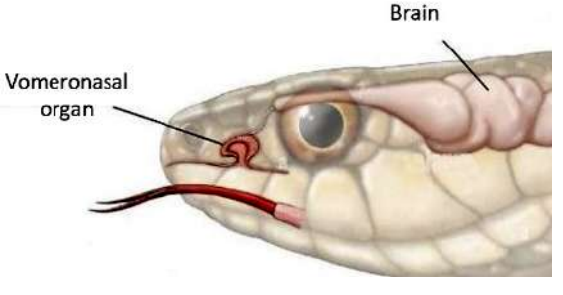

Fig 1. Schematic representation of the anatomical position of the VNO in snakes

The organ is covered by a bony or cartilaginous capsule and goes through a duct at the base of the nose. In carnivores and ungulates there is a connection with the oral cavity through the nasopalatine duct. Throughout the base you can find stem cells. The sensory epithelium layer can also be seen, which increases in volume from birth to puberty. Receptor neurons have apical microvilli, unlike the cilia found in the main olfactory receptors, and their axons unite to form vomeronasal nerves (Fig. 1) (Keverne, 1999; Jungblut, 2012).

The VNO is a chemosensory subsystem found in the nose of most mammals. That is primarily responsible for detecting pheromones and other chemical signals that initiate innate behavioral responses. Vomeronasal

neurons are very similar to the sensory neurons of the main olfactory epithelium, they are also bipolar and exhibit cilia at one end, in which the so-called vomeronasal receptors are expressed (Dulac et al., 1995; Loconto et al., 2003); Ferrando & Gallus, 2013; Ibarra-Soria et al., 2014).

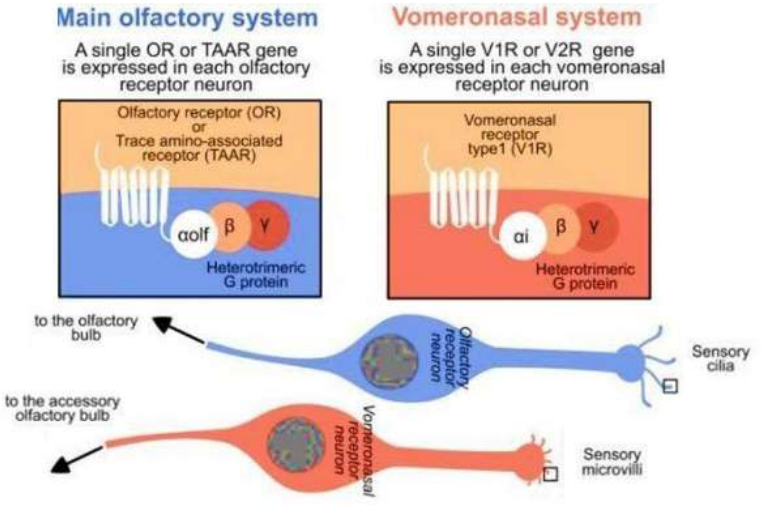

Figure 2. Schematic representation of the main differences and similarities between SOP and SOA neurons and receptors. VNO neurons are also hair cells and display their receptors on dendrites. Both types of receptors, both those of the VNO and those of the SOP, are metabotropic receptors, one of the main differences being that the VNO cells mainly send sensory information to the accessory olfactory bulb (Ferrando and Gallus, 2013).

Even though the data is controversial, it is proposed that in tetrapods the vomeronasal organ specializes in detecting pheromones (Jungblut, 2012; Ibarra-Soria et al., 2014). Pheromones were described in the 1950s as chemical signaling agents used for intraspecific communication in insects, some four decades later vomeronasal receptors type 1 (V1R) and type 2 (V2R), associated with the VNO, were identified as pheromone receptor molecules. VRs are present in amphibians, reptiles and mammals, while birds and fish lack them. The VR1 and VR2 receptors are metabotropic receptors that activate Gs and Go proteins, signal transduction is activated through phospholipase C, while most olfactory receptors activate signal transduction through adenylate. cyclase-cAMP (Figs. 2 and 3) (Silva and Antunes, 2017). The VNO expresses subfamilies of vomeronasal receptors (VRs) in a cell-specific manner as each neuron can express different numbers of VR receptors ranging from just one or two to hundreds of these receptors, for example, in rodents (Loconto et al,. 2003; Ibarra-Soria et al., 2014).

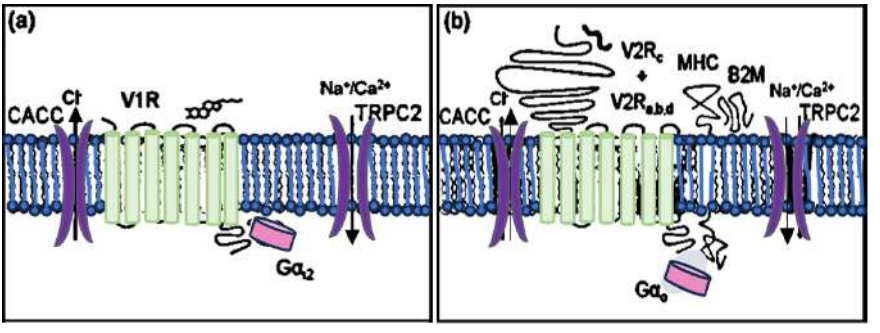

Fig 3. Schematic representation of the VR1 and VR2 receptors. On the left (a) a VR1 receptor associated with a Gai2 protein. The G protein subunit is activated by a volatile chemical ligand. On the right (b), a VR2 receptor from subfamily C is co-expressed with one from subfamily A, B or D. These are associated with Gao proteins and are individually or collectively activated by a peptide or protein ligand. One or more of the nine major histocompatibility complex (MHC) class 1b and b2-microglobulin (B2M) proteins are also expressed in a subset of these neurons. Both types of neurons additionally express a transient receptor potential ion channel (TRPC2) and calcium-activated chloride channels (CACC), which together depolarize the cell (Taken from Ibarra-Soria et al., 2014)Axonal projections

The vomeronasal axons, unlike the axons from the main olfactory system (SOP), project to the accessory olfactory bulb (SOA), from there they send projections to the middle amygdala (MeA), in turn these projects massively to the hypothalamus (Loconto et al., 2003; Moreno et al., 2005).

It is quite clear that the projections of the axons from the SOP and the SOA have different projections towards the amygdaloid complex, these differences are observed in amphibians, reptiles and mammals, which suggests clear differences in the physiological and behavioral influences of both systems (Scalia et al., 1991; Halpern and Martínez-Marcos, 2003; Halpern and Zuri, 2005; Moreno and González, 2006; Moreno and Gonzalez, 2007).

Some studies have shown that the VNO mediates a range of behaviors such as identification of individuals, establishment of hierarchies in social organizations, identification of kinship, location of prey in reptiles, amphibians and urodels, food preferences, generating defensive responses triggered by detection of interspecific alarm signals, among others (Graves and Halpern, 1990; Alving and Kardong, 1996; Placyk and Graves, 2002; Papes et al., 2010).

The VNO also mediates sexual behaviors including masculine and maternal aggressive behaviors, sexual attraction, lordosis, selective termination of pregnancy, as well as avoidance and defensive behaviors, this is attributed to the fact that sex pheromones can be detected in mammals, reptiles and amphibians through the VNO. (Shine, 2003; Keverne, 2004; Kikuyama et al., 2002; Wirsig-Wiechmann et al., 2002).

The above is clear evidence of the link between the VNO and the sexual and reproductive behavior of various organisms. Some of the studies that support this hypothesis are described below.

Michael et al., (1968) documented that oophorectomized or ovariohysterectomized *Rhesus* monkeys lost their ability to sexually attract males, since they lost the ability to emit the characteristic body odor that they normally emit when estrogen levels are higher. When oophorectomized females were smeared with vaginal fluids from fertile females, the ability to attract the opposite sex was restored. These substances with pheromone effects were called copulins. When these monkey pheromones were tested on the breasts of mature women, they did not have the same effect, meaning that the pheromones only work on individuals of the same species (Michael et al., 1975; Cerda et al., 2000).

The saliva of sexually mature male pigs contains two sex attractants, which are produced in the testes and transported through the bloodstream to the submandibular salivary glands, where they bind to a specific protein called pheromaxein. The sex attractants are the 5a-androgenic steroids, androstenone (5a-androst-16-en-3-one) and 5a-androstenol (5a-androst16-en-3a-ol), another example of a well-known "pheromonal" chemical signal is the amino acid felinine (2-amino- 7-hydroxy-5,5-dimethyl-4-thioheptanoic acid) in the urine of domestic cats (Castañeda et al, 2007).

Human accessory olfactory system

The existence of a human vomeronasal organ has been questioned, currently, it is known, through the data accumulated in different investigations, that said organ is present in adult subjects, is fully functional, actively participating in the modulation of the neuroendocrine axis and which has peripheral cells, located in the VNO, capable of acting as pheromone receptors, generating a connection with the hypothalamus and the limbic system, with the capacity to generate behavioral changes in humans (Liliam et al., 2012).

Sarría-Echegaray et al., (2014), documented the presence of VNO in human patients explored by rigid sinonasal endoscopy, these subjects underwent extraction of the nasal septum, later it was determined that these patients presented decreased libido.

Borgarelli, (2007) mention that the human vomeronasal organ shows some differences in its structure when compared to that of other mammals. Some scientists have postulated that the human vomeronasal organ could be in a small group of nerve cells located in the dividing septum of the nos-

trils. Although it does not have a thick epithelium like other species, it does have some cells that have been described as bipolar receptor neurons.

Besli et al., (2004) identified the presence of VNO in 112 live subjects and in eight cadavers. Other studies have documented the presence of VNO in 100% of study subjects. The VNO has been identified in human embryos, an interesting fact is that its nerve fibers constitute a substrate for the migration of GnRH-secreting cells from the olfactory placode to the hypothalamus (Zancanaro, 2014).

Two human genes have been described that are very similar to those responsible for the synthesis of receptor proteins in the vomeronasal organ of rats, the place where pheromones are attached. In the mouse 364 genes code for VR receptors, of which 190 have been identified as functional, in the rat 220 code for VR proteins, of which 120 have functions, in the dog 65 and 7 function, in humans they have been identified 117 and at least two are known to be functional (Miras-Portugal, 2006).

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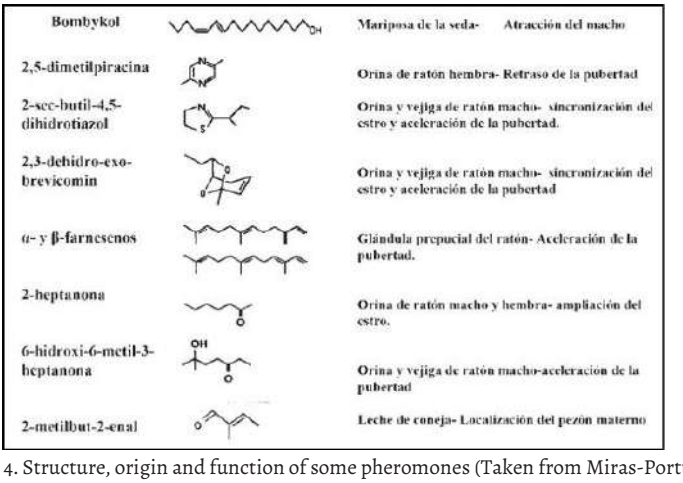

Figure 4. Structure, origin and function of some pheromones (Taken from Miras-Portugal, 2006).

In studies with secretions from humans, the presence of some pheromones that seem to influence social and sexual behavior has been described. These have been identified at least in vaginal and axillary secretions. Studies of the fatty acid composition of human vaginal fluids during the

menstrual cycle show the existence of estrogen-derived chemicals; They are five short-chain volatile fatty acids: propanoic acid, butanoic acid, methylpropanoic acid, methylbutyric acid, and methylpentanoic acid (Curtis, 1971). As the vaginal wall is devoid of glands, it is likely that these acids come from bacteria that metabolize glycogen to form lactic acid and all other aliphatic acids (Junqueira et al., 1977), which could be responsible for the synthesis of these pheromones. They have been called copulins and they are found in different proportions, depending on the phase of the menstrual cycle, increasing the concentration around the time of ovulation (Doty et al., 1975; Preti, 1987; Muller-Schwarze, 2012).

The invisible cloud of pheromones that wraps around the body "like a second garment" is a powerful factor in subliminal attraction that increases libido and activates sexual arousal. In women, a reduction in the production of chemically attractive substances in areas such as the vagina, whose pheromone load is very high, contributing to the typical "woman's aroma" of childbearing age, can cause a reduction in the self-perception as object of desire and less attraction of men. Therefore, hormone-dependent olfactory modifications can contribute biologically and functionally in an important way to the variation of libido in the different phases of a woman's life (Graziottin, 1998).

The aroma of copulins is perceived at the level of the limbic system, and appears to have an effect that causes increased dopamine release in the brain (Vosshall, 2001; Wysocki et al., 2009).

There are various research papers in which attempts have been made to study the intensity and attractiveness of odors from vaginal secretion during the phases of the menstrual cycle; in some, questionnaires have been used to assess these characteristics. The men were asked to rate both qualities of odors from tampons used at various phases of the menstrual cycle. The results of some studies indicated that in the preovulatory and ovulatory stages the odor was slightly weaker than in the menstrual, early luteal, and late luteal stages. The odor released by the woman is increased in the fertile time. All this indicates that the phases of the menstrual cycle involve secretions of quantitative and perhaps qualitative composition of odorous chemical substances, perhaps with characteristics of pheromones.

Women, especially if they are in a state of ovulation, secrete sex pheromones that release certain hormones in the brain of men such as dopamine and serotonin that have an impact on the nervous and circulatory systems. Pheromones frequently cause an unconscious arousal state in young men and adolescents (Doty et al., 1975).

In 1999, the vaginal fluids of the menstrual and ovulatory phase were studied, which, as previously mentioned, had already been shown to vary in copulin composition, and the influence of these substances on male behavior was investigated. The synthetic fluids and a placebo fluid composed exclusively of water were presented to a sample of 60 men, applying the double-blind method. The men were required to refrain from smoking for 25 minutes prior to the experiment. In addition, the study was carried out in duplicate. To control the hormonal changes induced by copulins, saliva samples were taken before and after the experiment. Men were required to inhale the submitted substance and rate whether they found the women in the submitted photographs attractive. The typical fluid of the ovulatory phase induced an increase in androgen secretion and altered the cognitive discriminatory capacity regarding female attractiveness. Men became less demanding. This study demonstrates the pheromonal nature of copulins (Filsinger, 1990; Borgarelli, 2007).

On the other hand, Osvaldo N. Mazza carried out an attractive experimentation with copulins. Cotton swabs were soaked, first in vaginal secretions for the impregnation of said copulins, and then in alcohol; other cottons were impregnated only with alcohol. A group of volunteers were given to smell one or the other; the perceived odor was the same, of alcohol, for the swabs with or without copulin. The results showed that, in the men who had smelled the pheromone swab, testosterone levels increased between 100 and 150 percent, compared to previous information, Mazza clarified that this was true only for those volunteers who had in the vomeronasal organ, which detects pheromones, in good condition, as some studies mention that it is atrophied in many people (Stoddart, 2006).

Regarding men, several studies have addressed the actions of the volatile odorous steroid 4,16-androstadien3-one (androstadienone), which is excreted by sweating in the axillary region of men, to a lesser extent in women, the application of androstadienone in the upper lip exerted a subtle positive effect on mood in female subjects, enhanced perceived Masculinity Index of male faces in male subjects, and increased cortisol secretion in females (Baum and Kellier, 2009).

In studies such as that of Rantala et al., (2006) they mention that there is a positive correlation between the level of salivary cortisol and attractiveness ratings of men's T-shirts in all groups of women, or in another study such as that of Thornhill et al. al., (2003) says that the scent of relatively symmetrical men is more attractive to women who have a regular ovulation period.

The vomeronasal organ and the major histocompatibility complex

The major histocompatibility complex (MHC) plays important roles in intercellular recognition and differentiation between self and foreign. It is involved in the development of both humoral and cell-mediated immune responses (Goldsby et al, 2004). The major histocompatibility complex is located on chromosome six in humans, it is made up of at least 4x106 base pairs. In humans these genes are called human leukocyte antigen or HLA genes because they were first discovered by antigenic differences between leukocytes from different individuals (Murphy et al, 2009).

The MHC has a non-classical function, related to activation signals of the sensory neurons of the olfactory system, therefore, it is proposed that it has an important participation in the regulation of animal and human behavior (Milinski et al, 2013). Some of the works that support the above are those carried out by Dulac et al., (2003) who, after studying a subpopulation of neurons in the VNO, identified the expression of two gene families (M1 and M10) of the MHC. The M10 proteins found in the VNO were different in structure and function from similar molecules expressed elsewhere in the body, which commonly function on the surface of immune cells to present foreign proteins to the immune system in order to activate the destruction of invading pathogens. Those found in the VNO neurons seem to have specific functions related to pheromone recognition; apparently, there is a population of neurons in which each one only expresses the gene for one type of pheromone receptor. Within the VNO, the identified genes are expressed only in V2R- positive VNO neurons. Dulac et al., (2003), also mentioned that the M10 gene was only activated after birth, which suggested that M10 only functions in the detection of pheromones in the adult individual.

According to Dulac et al., (2003), the M10 protein and its accessory molecule are necessary for the pheromone receptor to reach the surface of the neuron, which is why they described them with "haperone" functions. Dulac said the recently discovered MHC molecule could have important implications for understanding the pheromone system. This association opens up all kinds of possibilities about the understanding of the pheromone detection mechanism, and how some body secretions of organisms can modulate the behavior of another organism, according to sex, genetic origin, social status, etc.

Based on the above information, in the area of Psychobiology of the Autonomous University of the State of Mexico, we set out to study the influence of body odor of people and non-human organisms (pigs) on the choice of a partner.

The studies are focused on contributing information related to the functionality of the human accessory olfactory system. We did three experimental works, which are described below.

Sow (*sus scrofa*) preference for males through preputial and salivary secretions

In this work, our main objective was to determine if in captivity conditions there is a preference of the sows towards a particular male, we collected foreskin and salivary secretions from four males of different breeds and exposed them to 11 females of the same breeds to evaluate if the female skews in terms of time and frequency her preference towards any of them.

The most relevant results were that the females of the "Landrace", "Duroc" and "Pietrain" breeds (of possible European origin) preferred to sniff the secretions of the male of the "Yorkshire" breed (of Asian origin) and the females of the "Yorkshire" breed preferred the male of the "Duroc" breed.

The main results of this first study showed that females spent more time sniffing secretions from the male of the breed to which they are less genetically related, as predicted by MHC theory, where related individuals exhibit similar odors and females tend to reject them.

Another possible explanation for these results is that females learn the scent of their breed during the suckling stage and therefore avoid said scent in adulthood. It is also possible that we are observing a combined effect, where the female has not only learned to recognize individuals of its own race through imprinting, but it is also likely that it is also recognizing genetically more distant individuals through the products of metabolism of the major histocompatibility complex (Carrillo-Castilla et al., 2020).

Male neurovegetative response to olfactory stimuli from vaginal secretions at different stages of the menstrual cycle

Twelve female students from the Faculty of Sciences of the Autonomous University of the State of Mexico were selected to participate in the study. The inclusion criteria were: age between 18-25 years, regular menstrual cycle, not being under hormonal contraceptive treatment, not being breastfeeding, having correctly filled out the informed consent questionnaire and having given their authorization to participate in the study. With the data obtained about the duration and dates of their menstrual cycle, a calendar was made to be able to have an exact follow-up of the menstrual cycle of each woman and in this way, to be able to group them according to their dates in those that at the time of the experimentation presented its follicular, perio-

vulatory or luteal stage. Each of the women was asked to donate vaginal secretions.

Three samples of vaginal secretion were taken from each woman (one per week), where initially they were in their follicular stage, then in their periovulatory stage and finally in the luteal phase. Two repetitions of each stage were carried out, that is, each woman's donation was carried out for approximately two months.

An informative session was held in which they were explained how to take the vaginal secretions, a light rub should be done with a sterilized swab in the vaginal canal. Each of the samples was stored instantly in 1.5mL vial tubes containing isopropyl alcohol. Each of the fresh samples was stored and labeled with the donor's initial and stage at the time of donation to facilitate subsequent identification.

Experimentation with men

Here 33 men participated, they were individually numbered from 1 to 33 and later organized in groups of 5 or 6 to carry out the experimentation.

The test was carried out in a laboratory completely isolated from external noise, in which the subjects were placed comfortably seated with their legs resting on the floor and at the same time, they were given clear instructions to remain silent during the entire procedure. Each of the men was connected to the Biofeedback Center® brand Biofeedback computer, model I-330C2+6ch with USE3 software, to measure neurovegetative responses (temperature, heart rate, respiratory rate, skin conductance, and electromyogram). The electrodes were installed in specific areas such as the index, middle, and ring fingers, wrists, thorax, and facial muscles approximately in the zygomaticus major and minor. In addition, they were blindfolded in order to minimize alterations in the responses emitted to the moment of stimulation. The first step for the experimentation was to record a baseline with an approximate duration of 1 minute; this with the purpose of making pre-stimulation comparisons, observing the responses emitted by each subject before subjecting him to the stimulus. Once the baseline was obtained, the swabs were given to smell with fresh samples of vaginal secretions. It should be mentioned that a swab with water was also used as a control. Therefore, each male was randomly subjected to the stimulation of 3 or 4 secretions from different women who were in the follicular stage, 3 or 4 in the ovulatory stage and 3 or 4 in the luteal stage, as well as the control swabs. Recordings were made in sessions of approximately 15 minutes (Quintos-Meneses, H.A., 2010).

The main results of this study showed that the values of some of the vital signs changed when men were exposed to odors coming from women in the periovulatory stage.

The electromyogram and body temperature showed a significant increase in heart rate. These results are related with the results obtained from similar studies with human pheromones (Grosser et al., 2000 and Monti-Bloch et al., 1998), but differing on a par with what these authors report about the responses of respiratory frequency and electrodermal activity or skin conductance, since in their studies they report a change and/or increase of both responses; meanwhile in our study, we found for those vital signs not significance.

The electromyographic response measures the electrical potentials that arise from the concentration or relaxation of muscle fibers, that is, it provides information on the activity of the muscle or muscle group on which the electrodes are placed (Conde and Menéndez, 2002). In men, the aspect of the motor apparatus that has to express emotions before different stimuli are the dermal muscles of the face called facial muscles. Although the movements and gestures of the whole body are important in the expression of many emotions, the facial muscles represent a special apparatus for this function; therefore, this motor pathway is added to the limbic system (Graziottin, 1998). Given the above, this was the reason why the electrodes that measured the electromyographic response in the participants were placed close to the facial muscle area of the zygomaticus minor. The electromyographic response is considered a phenomenon closely linked to the emotional state of the individual. In this study, obtaining greater muscle tension during olfactory stimulation, with the vaginal secretions presented from the three stages of the women's menstrual cycle, it means that a positive reaction of the autonomic nervous system was achieved, due to the generation of high values in the results. This result coincides with previously published research where a relationship with the different levels of muscle activation and tension is affirmed, being that the greater the activation of the nervous system, the greater the muscular tension (Contreras and Juárez, 2003).

Cardiac activity is the record that presents the most variations in any situation, whether it is highly emotionally charged, such as stressful or anxious situations, or in those in which the subject is relaxed (Conde and Menéndez, 2002). Indeed, the records in the study show that this neurovegetative response was the one that presented the highest values with olfactory stimulation when presenting them with the swab containing vaginal secretions, this in comparison with the other responses measured, which could

also indicate that the men felt, unconsciously, an attraction to the aroma that was presented to them, proving once again the existence of a relationship that indicates that the greater the activation of the autonomic nervous system, the higher the heart rate. The temperature was measured by sensors placed on the fingers. It indicates the contraction or relaxation of the smooth muscles that surround the blood vessels, which determine the amount of blood that flows to the fingertips. When these muscles contract, the temperature is lower because less blood flows to the fingers. The increase in peripheral temperature correlates with the decrease in activation, and therefore can serve as an index of the level of general relaxation of the subject (Dosil and González, 2003). The study presents records of a lower temperature when the control swab was used, contrary to the records that mark an increase in temperature with the swab containing samples of vaginal secretions, which could indicate that the stimulus with the latter produced in males a state of relaxation that generated an increase in the values of this neurovegetative response.

The three stages of the female menstrual cycle (follicular stage, ovulatory stage and luteal stage) that were used in the study undoubtedly produced an activation of the autonomic nervous system, generating significant responses in the three aforementioned neurovegetative responses with an increase in their recorded values; however, there was a stage that stood out above the other two: the ovulatory stage or, as recorded in the study, stage 2 of the cycle. As well reported, female vaginal secretions contain substances called copulins (Michael et al., 1968) with a quantitative and qualitative composition of odorous chemical substances that play the role of pheromones, and are found in different proportions, depending on the phase or stage of the menstrual cycle (Doty et al., 1975).

When a woman's cycle is in her ovulatory stage or close to it (Stoddart, 2006), there is a higher concentration of copulins or, put differently, of sexual pheromones, capable of achieving a perception of an aroma at a level of the limbic system (Vosshall, 2001; Wysocki et al., 2009) which releases certain hormones in the male brain such as dopamine and serotonin, consequently having a high impact on the nervous and circulatory systems (Doty et al., 1975).

Based on this work, it is suggested to implement or add to the method the use of isopropyl alcohol for the conservation of pheromones found in vaginal secretion, while at the same time disguising the direct aroma of the secretion as reported by Stoddart (2006) in the experimentation carried out by Osvaldo N. Mazza. Likewise, it is also suggested to establish a standard time for when women perform vaginal rubbing with the sterilized swab,

since perhaps the amount that was given influenced in some way that not all of the neurovegetative responses that were expected were not generated in the end.

Changes in the vegetative response of young university students to exposure to axillary odoriferous stimuli from male athletes and non-athletes

In this study, Osorio-Mercado (2020) found the response of the autonomic nervous system (heart rate, mean arterial pressure, respiratory rate, and temperature) of a group of young university students when exposed to axillary odoriferous stimuli from male athletes (Type A) and non-athlete males (Type B) was evaluated. which were exposed to cotton shirts that the men used for a certain period of time. The results showed significant differences in odor preference. These data lead us to speculate that the odor preference found in this study is that women feel more attracted to the odor produced by men who exercise as part of their daily routine; according to Alcázar (2011) hormonal concentrations are higher in athletes than in sedentary subjects, which may be due to physical activity; According to this and with the results we can say that the women chose the male athletes because of the greater hormonal load that they present and given this the smell they perceived from them is more pleasant according to Rantala (2006) women prefer the body odor from symmetrical men and odor from men with immunocompetent genotypes.

A significant difference in body temperature was found when women were given the Type B odorant stimulus to smell compared to when women were at rest, Grosser (2000) show a significant increase in temperature when humans are exposed to the Androstadienone pheromone. We found a significant difference in the results of heart rate, they tell us that there is a significant variation in heart rate when women are exposed to Type A odoriferous stimuli, according to Guzmán (2004) pheromones, when activating the VNO, effects appear autonomic increase in heart rate and increase in alpha waves. For the values found for respiratory rate and mean arterial pressure, no significant differences were found, but in the values observed in Table 1, we can see that if there are changes in the values when the women are in the basal state and when they are stimulated by odors, observing the mean arterial pressure data, we can get an idea that when women sniff the shirt with the type A odor they suffer observable changes, in the same way when the respiratory rate was measured, changes were observed in this when women aspire the odoriferous stimuli of both men.

Summary

With the results obtained in this study, we can come to the idea that the olfactory system, and in particular the vomeronasal organ in humans, have an important role in sexual selection in humans, and that not only sexual selection in humans is determined by factors such as: physical attractiveness, where tall, athletic men are generally preferred, with a waist-hip ratio of 0.9 and a symmetrical body, with facial characteristics such as above- average eye size, prominent jaws, bone and facial symmetry (Hernández-López and Cerda-Molina, 2012).

On the other hand, Grammer (2005) says pheromones have the potential to influence human behavior and physiology; and that olfactory/pheromonal cues profoundly influence neuroendocrine functioning and regulate a wide variety of social behaviors including sexual interactions, parent-child relationships among others (Keller et al. 2009); it can be said that body odor can provide significant signals about the genetic quality of a potential sexual partner, reproductive status and health (Havlicek et al, 2005).

Physiologically, it is possible that cortisol has a direct or indirect link via adrenaline to apocrine sweat gland activity that may have effects on the attractiveness of body odor. In addition, sweat metabolism by commensal microbes may cause odor of the individual that is more pleasant depending on the rate of sweating (Rantala et al, 2006) and evolutionarily, immunosuppressive hormones are said to be essential for the production of good quality sperm and thus spermatozoa may be less susceptible to autoimmune attacks (Skau and Folstad, 2004).

It was found a significant difference in the woman´s preferences with the male athletes; the women felt more attracted to them. It is assumed that these males have higher immune quality. The qualitative evidence indicate it is because of the amount of hormones, therefore, we can say the women select that odors with a different MHC. As mentioned by Guzmán (2004) there is a certain degree of certainty that steroid pheromones are modulating the activity of the hypothalamic-pituitary axis through the VNO.

This would explain the sensation that is experienced during the attraction process, and which colloquially translates into expressions such as “my heart jumped” or “even my breath stopped”, which would speak of the participation of the hypothalamic pituitary axis and the system autonomic, with consequent changes in heart and respiratory rates.

Due to the lack of information, this experimentation allows us to add as a new report, the results obtained for the neurovegetative responses of heart rate and body temperature, registering changes and even an increase considered successful during olfactory stimulation.

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XV

Lactation in humans: the long path from animal studies to multidisciplinary translational research

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Introduction

The most important characteristic of class Mammalia is the production of milk from mammary glands in the mother to nourish their young. We, as mammals, are very familiar with this important reproductive process in nature and consider that this is the proper, natural way to feed newborns after birth. However, at the same time, many from our species consider this is right "for animals", as we were not mammals! As we will see in this chapter this is due to a general lack of education on breastfeeding benefits for the offspring and the mother, in the general population and even in health professionals. Besides there are several social and governmental barriers that collectively impact on the establishment of a successful breastfeeding. Due to the importance of breastfeeding for the nourishment, health, and better development of the young after years of studying maternal behavior and lactation in laboratory animal models we decided to focus on maternal lactation in our species. It had been a fascinating journey full of new research opportunities, with an additional advantage to basic research: it is in humans. Unfortunately, frequency of breastfeeding in México is low and different feeding practices for newborns and infants, different than breastmilk, are increasing dramatically with important negative consequences. Lack of breastfeeding is associated to several health problems including infections and higher probability to develop several comorbidities. In fact, in parallel to the low frequency of breastfeeding México is one of the world champions in overweight, obesity and diabetes. As we will see, this line of research has a great social impact because breastfeeding is the basis of a

better health for the infant with long lasting effects in adults. But the journey of this translational research begun around 30 years ago.

Everything starts with the rabbit

Rabbits are unusual among mammals. Unlike rodent and human newborns which suck milk several times during the day and night, rabbit does nurse their pups only one time every 24 hours. Either in the wild and in laboratory conditions after parturition the mother leaves pups in the nest and return around 24 hours later for a few minutes. During this short time, she only nurses their pups and does not engage in maternal behaviors as licking and retrieving as in other mammals. During this moment pups engage in a competition for the nipples to suck milk. Afterwards she leaves the nest and pups remains mostly quiescent most part of the time. However around 21 hours later, three hours before the next visit of their mother they steadily increase their activity and at mother arrival they show strong locomotor behavior and particular vocalizations. This increase in alertness is named food anticipatory activity (FAA), which is a phenomenon also studied in adult rodents. But as rodents eat several times during their active period, they need to be food deprived and then fed one time in 24 hours. This involves several manipulations and stress for the subjects. In sharp contrast rabbit pups naturally eat just one time in 24 hours. For this reason, they are considered a more natural model to study FAA phenomenon (Caba et al., 2008). Once subjects learn that food is available every day at the same hour for just a short period of time, they develop FAA even whether food it is not available for at least two days. This is a very interested phenomenon. It had been proposed that FAA is controlled by a putative oscillator controlled by food. But to date the locus of the possible oscillator had not been identified either in the nervous system or in peripheral organs (Caba 2021; Mistlberger 2020). Another proposal is that there is an interplay of oscillators between the nervous system and peripheral organs. On this sense recent research from our laboratory proposed a possible important link between the brain and organs innervated by the autonomic system. A particular group of oxytocin producing cells in a subregion of the caudal part of the hypothalamic paraventricular nucleus are activated at the time of FAA. These cells are in a region of preganglionic cells of the autonomic nervous system that project to peripheral organs as the liver and adrenal glands. This finding suggests that these cells could be part of the oscillatory network between brain and the periphery that sustain FAA (Caba et al., 2021a; Caba et al., 2021b). This proposal reinforces the idea that FAA is controlled by an oscillatory network. Moreover, the mother also seems to have a similar oscillatory mechanism

with circadian periodicity. Before their return to nursing every 24 h the mother also has an increase in locomotor behavior and there is an increase in activation of some brain regions. Of note we found that there is an activation of the mesolimbic system and other areas related to motivation and reward before nursing (Aguirre et al., 2017; Huerta et al., 2023). This suggests that she is motivated around every 24 h to visit their pups for nursing. Specifically, it had been proposed that during this time suckling of the pups plays a main role (González-Mariscal et al., 2013; Huerta et al., 2023). A fascinating story of clocks synchronization, by food in the pups and suckling stimulus in the mother, with circadian periodicity. Milk is at the center of this phenomenon.

The research on this dyad model on maternal behavior and lactation continues. But suddenly a question arose, which is the state of the art of maternal behavior and lactation studies in humans? How to study these topics in considering that academic training in neurosciences usually focus on basic science in laboratory animal models. Besides there are ethical important issues to do research in humans. But what about to start with non-invasive methods of research? As for example a census about maternal lactation practices and barriers in our population? Or perhaps milk analysis? With these ideas in mind, we decided to enter to this new fascinating line of research. First, we will focus in one of the most marvelous biofluids in nature: milk. We will explore the composition of this complex fluid and their "flexibility" across time and physiological condition of the mother and child. After we will focus on a census about maternal lactation practices in Xalapa city in México. Then we will describe some of our ongoing research lines on these topics as well as in educational programs about the importance and benefits of breastfeeding for the young and the mother.

First was the egg

Although we are familiar with lactation in mammals it is considered that mammary gland secretions evolved in a group of synapsid reptiles, considered mammalian ancestors, around 250 million years ago. These animals lay parchment-shelled eggs which dehydrate faster than rigid-shelled eggs of birds. Then the secretion from their glands provides them water which was absorbed through the shell to prevent desiccation, and it is possible that some ions and nutrients were also secreted and absorbed (Oftedal, 2002). Mammalian ancestors laying eggs? Yes, indeed this is not surprising. Modern monotremes as platypus and echidna, both mammals, lay and incubate eggs and after hatching, they nurse their immature young, not from nipples but through openings in their skin. But unlike synapsids that secre-

te mostly water from their glands, monotremes as well as all mammals secrete, besides water, a complex array of molecules and cells, a biofluid known as milk, which was developed during around 250 million years of evolution. This evolutionary process began at the end of the Triassic period and several morphological, biochemical, physiological and behavioral adaptations both in the mother and in the young were developed. As a result, a complex fluid known as milk was produced which is considered the gold standard for infant nutrition.

Milk, a complex biofluid

World Health Organization recommends that the newborn must be fed only with milk during at least the first six months of age; nothing else, like water, tea, etc. This is because milk it contains all nutritive and nonnutritive necessary elements for the proper growth and development of the newborn (Camacho-Morales et al., 2021). At birth, the human infant it is immature, and their priority is survival. Unlike the general belief that milk contain only nutrients like proteins, fats and carbohydrates, it is well known that milk contains a myriad of immunological factors, both molecules and cells to help infant survive during this critical period after birth where he is exposed to a myriad of pathogens. These factors belong to either category of the macronutrients: Fats: long chain-fatty acids; Proteins: lactoferrin, lysozyme, Secretory IgA, -Lactalbumin, osteopontin, amylase and carbohidrates: milk oligosaccharides, just to name a few molecules which confer passive and active immune protection of the neonate. Besides, milk also contains growth factors and biologically active cells as leukocytes, epithelial and stem cells. Also, in contrast to the old belief that milk is sterile, a complex balance of probiotic bacteria and other microorganisms, are secreted by the breast, which colonize the infant's gut. In recent years non-codificant micro Ribonucleic acids (miRNAs) were discovered in milk and are currently studied. It had been postulated that they participate in regulation of many immunological and developmental processes (Witkowska-Zimny and Kaminska-El-Hassan, 2017; Fig. 1). Besides their rich and complex composition milk also have some remarkable properties. Their composition changes according to the diet and physiological condition of the mother and infant health status (Camacho-Morales et al., 2021). These adaptations are very impressive because implicates that milk can be "personalized" according to infant's requirement. But even more, milk composition also changes across time, not only from colostrum upon parturition to mature milk weeks and months later, but even more, it changes according to the time of day and night.

Why newborn babies ask for milk during the night?

Rabbit pups eat, suck milk, just one time every 24 hours. That is enough for around the first 15 days of age. Then their eyes are open, and besides milk, they start to consume solid food mostly during their activity period. But the human baby after birth begs for food constantly during day and night. Why? Why the system is not as effective as that of rabbit pups? This is an important issue which had received scarce attention and the answer it is clearly on sight. During their development in the womb, the fetus was exposed to daily changes in the mother. All the metabolites, hormones, temperature, behavior in the mother and in our entire species have cyclic changes every day and night, known as circadian rhythms. Then the development of the baby ensued under a rhythm milleu during around nine months. Suddenly this rhythmic environment is interrupted by parturition. But nature creates the perfect substitute: rhythmic breastmilk.

All we know that milk has nutritive compounds, i.e., it is food. Few know that milk also have immunological components, as mentioned above, which are crucial for their survival particularly during the first months of life when their immune system it is immature. But in general, the population does not know that milk components also have rhythmic changes. Indeed, it has circadian rhythms (Caba-Flores et al., 2022b). These rhythms are daily changes imposed by our diurnal activity pattern which is synchronized by the sun's light every day. Then after delivery the baby ask for food, in this case milk, day and night, yes, but ask for "circadian milk". Recently there is great interest in chrononutrition, the science of how temporal eating patterns, the metabolism and the circadian clock interacts and affect the human health (Flanagan et al., 2021). Recently this topic is receiving attention in relation to breastmilk because it had been demonstrated that has important benefits and clinical applications for infant's health.

Several hormones, as glucocorticoids, melatonin (MEL), and leptin, among others, had been discovered in milk and they have circadian variations. For example, glucocorticoids, as cortisol (CORT), increase in the early morning and decrease later in the day. In sharp contrast the hormone MEL is secreted only during the night. In adults it is considered that the cyclic pattern of these two hormones communicate "time of day" to the body. Specifically, they are related to activity during the day and rest during the night. Several other molecules also had been explored. The hormone leptin reaches highest levels during the night whereas lipids are higher during the day and lower during the night. These compounds circulate in the blood plasma then are transferred to milk in the mother's breast, and are ingested by the newborn. On this way the neonate continues to receive time cues

from the mother through milk. For this reason, the infant awakes during the night, ingest milk and continue sleeping. Many other components as aminoacids and nucleotides had circadian variations and their changes had been tested on infants. Levels of the aminoacidic tryptophan increase in the night and are higher at around 04:00, h then levels decrease during the last part of the night and during the day, reaching lowest levels in the evening, to then increase again in the night. This pattern is maintained during several months in colostrum, transitional and mature milk. In relation to nucleotides, adenosine 5'monophosphate (5'AMP), guanosine 5'phosphate and uridine 5'phosphate (5'UMP) had higher levels at night. In considering the higher increase of tryptophan and nucleotides during the night they were added to milk formulas and cereals fed by infants at nighttime, and they significantly improved sleep quality (Cubero et al., 2007, 2009). Among the many other nutritional and non-nutritional compounds with circadian rhythms in milk some are very intriguing. 2-arachidonoyl glycerol (2-AG) concentrations is higher during the transition between day and night. This lipid is a cannabinoid, an endogenous substance similar to products from the *Cannabis sativa* plant, commonly known as marihuana. In considering the role of these compounds in hunger and hedonic states in adults it is possible that they could play similar actions in the baby. Experiments in laboratory animals demonstrate that 2-AG it is essential for the normal development of the mouth muscles as the administration of a 2-AG antagonist on postnatal day one in mice, disrupt development of those muscles and the pups are unable to suck milk and die of starvation (Fride, 2004). To date nothing it is known about the physiological action of 2-AG in the human neonate but in considering the above evidence it is possible that 2-AG may play an important role. Another substance also found in human milk is -endorphin (-EP), and endogenous opioid, again, similar to opioid alkaloids found in the poppy plant *Papaver somniferum*. -EP it is present in colostrum in higher concentration than in transitional and mature milk. Moreover, their concentration in colostrum is twofold higher than in mother's plasma. It had been proposed that this high concentration helps the newborn to overcome the stress of parturition and perhaps contribute to their well-being throughout the lactation period (Zanardo et al., 2001). Besides, it is possible a role in pain and sleep modulation in babies, which had not been explored. Also, it not known whether -EP secretion in the milk has a circadian rhythm. As can be appreciated the call of the babies during the night goes beyond the nutritional value of milk. It also requests the different concentration of many substances, besides that of nutrients, but very little had been explored about their physiological importance on the neonate.

Other milk components also have a pattern of circadian secretion, but they are mainly found during the day. There is evidence that some immunological factors as immunoglobulins IgA, IgM and IgG are secreted in colostrum, transitional and mature milk mainly during the day. C3 and C4, complement proteins that destroy pathogens are also found during the day in colostrum. A more complex pattern had been reported in relation to cytokines. In colostrum, some as IL-6 and TNF, have higher concentrations during the day whereas IL-2 and IL-10 are higher during the night. This tendency changes in relation to mature milk on these and other cytokines (Morais et al., 2015). Then, there is a complex dynamic pattern on the concentration of these immunological factors trough time.

Besides it had been reported an interaction with MEL, which is secreted only during the night as mentioned above, and lymphocytes. This blood cells contain MEL receptors in their plasmatic membrane and in vitro experiments demonstrates that MEL increases the phagocytic activity of some lymphocytes from colostrum against pathogens. Besides MEL also regulates action of pro-inflammatory cytokines (Rev. in Caba-Flores et al., 2022b). Together these evidence points to the importance of the compounds of milk during the day and night and their benefits for the infant.

Importance of circadian rhythms for the infant

The plethora of rhythmic variations of nutritive and non-nutritive milk components through the day and night must be of important physiological relevance for the infant. As mentioned above, tryptophan and some nucleotides were tested in infants. This aminoacid and nucleotides 5-AMP and 5'UMP were added to formula milk to children age 1-5 months to test their effect on sleep. Children reported a general improvement in sleep parameters. The same group of researchers in a different experiment enriched cereal with the same aminoacid and nucleotides, in children of similar age, and also sleep parameters were improved. This evidence is important as 5-AMP induce sleep in adults and tryptophan is a MEL precursor. It is well known that MEL facilitate sleep in adults. Other researchers had reported that breastfeed at will, during the day and the night improve sleep parameters and decrease colic and irritability episodes, in contrast to infants fed with milk formulas (Cohen Engler et al., 2012) In general the evidence supports the assumption that breastmilk contain chronobiotic substances that improve sleep and the well-being of babies (see revs. Caba-Flores et al., 2022; Hahn-Holbrook et al., 2019; Fig. 1; Flanagan et al., 2021).

Besides the importance of the substances in milk for the welfare of infants the control of lighting also dramatically increases not only health but

even survival of newborns. Premature babies usually are maintained in strong constant light through 24 h at the neonatal intensive care facilities. When they were exposed only for 12 h to light of the room and 12 h to dim light, they attained higher food intake, body weight and oxygen consumption in less days than newborn exposed to constant strong light. Therefore, they were discharged early from the neonatal intensive care unit than babies that were maintained in constant light during 24 h (Vásquez-Ruiz et al., 2014). The overall evidence confirms the great importance of breastfed the infant at will, during the day and the night and in general the important benefits of circadian rhythms for the proper development and health of the infant. Unfortunately, very few studies had explored this issue.

Long term benefits of breastfeeding

Upon birth breastmilk protect against gastrointestinal, lung and ear infections decreasing the risk of pneumonia and chronic diarrhea and improve significantly a "healthy" microbiota in the gut (Victora et al., 2016). But also, there are long term benefits. A plethora of studies indicates that breastmilk had benefits to prevent dietary disorders and several comorbidities. Breastmilk decreases the risk of overweight and obesity, diabetes and metabolic disorders in infants with long lasting effects until adulthood in sharp contrast to bottle-fed infants. It is known that milk contains several hormones as leptin, ghrelin, adiponectin and obestatin which are related to the control of metabolism and they seem to have a main role in the epigenetic metabolic programming of the infant (Badillo-Suárez et al., 2017). As an example, several risk factors lead to overweight and obesity, genes, among them. Individuals with the gene FTO have a higher predisposition to develop overweight and obesity among other comorbidities as type 2 diabetes. In a longitudinal study of 5590 children, it was demonstrated that breastfeed infants for at least 5 months of age have a decreased risk to develop overweight and obesity despite they were FTO gene carriers, in contrast to milk formula fed infants (Wu et al., 2017).

It is evident that nothing can replace the complexity and benefits of breastmilk. It is interesting to compare that the huge increase in profits and production of milk formulas grows in parallel to overweigh, obesity and other comorbidities in children and in adults in the world. Besides, the lack of proper breastfeeding induces more than 500,000 child deaths/year (Walters et al., 2019). Finally breastfeeding promotes attachment between mother and child, promotes a better neurological and psychological development of the infant and improves their performance in intelligence tests (Horta et al., 2015; Victora et al., 2016).

Breastfeeding is excellent for nutrition and health, then why their prevalence is low?

In considering the multiple benefits of breastfeeding, then why their prevalence is low? We consider that there are two main factors. One is the absence of interest in the Universities as none in México have in their academic programs a formal class in lactation and their benefits for a better health. The second is a lack of effective governmental support to an effective program to increase breastfeeding. In considering our social responsibility with the society we decided to implement a broad program covering two main points: research and education. In the following sections we will describe some of our ongoing research projects.

Immunological compounds in milk

Milk' analysis is a fascinating field of work, but how start their analysis in our population? How milk can be collected and stored, and then which methodology needs to be used? As a first approach we aimed to use flow cytometry to explore leukocytes in mature breastmilk samples collected during an interval of 60 days, from 8-9.5 months of lactation. After reviewing several reports, and in considering our experience on this technique, we implemented a methodology to analyze some leucocyte T cells populations. In parallel of breastmilk samples, we gather clinical information of the mother and the infant. We found a mean of 30.3% of CD3+, 19.2% of CD4+, 10.5% of CD8 and 2.9 of , lymphocyte T cells. No difference was observed in most lymphocyte cells throughout the study, except in a subpopulation of T cells. At day sixteen of sampling the clinical report indicates that the baby had a process of viral infection and the number of a subpopulation of T cells showed a 9.9 fold increase. Then at day 40 of sampling the clinical report also indicates that the mother had a period of asthenia, and the same population of T cells had a significant decrease in comparison to mean basal levels. The implications of these results are amazing as they suggest that there is a bidirectional communication between the mother and the baby as the breastmilk content of T cells increases in response to the infant needs to help control the viral infection. On the other hand, the results also show that the psychological state of the mother also can affect the composition of the milk, on this case that population of T cells, which can have important consequences for the health of the infant (Caba-Flores et al., 2022a). Our results are important in considering that breastfeeding it is very important for the first 1,000 days of life. During this period milk components contribute to the to the metabolic and physiological programming of the newborn which has consequences for the rest of their life. Besides that, we recently pro-

posed how breastfeeding trough milk components, including their microbiome, contribute strongly programming of the immunological system of the infant (Camacho-Morales et al., 2021). During this time frame after birth the newborn also ingest toxic compounds present in the mother's milk. Recently we participated in a global effort of the United Nations through the Stockholm convention to monitor toxic compounds in breastmilk in the world. Our research is the reference for México which will be used for public health policies to control the release of toxic compounds in the environment (Martínez-Valenzuela et al., 2022).

Maternal attachment

During motherhood there are several changes in the physiology and the brain as the mother faces new challenges. The most important is to feed and protect her newborn. Several changes in the brain had been identified in neural structures as the hippocampus, hypothalamus and the cerebral cortex and collectively they had been named "maternal brain". These studies had been performed mainly in laboratory subjects. In humans some evidence about changes in the brain had been obtained on basis of electroencephalographic recordings and resonance imaging studies, peripheral release of hormones and psychological studies. After parturition in the brain there is an activation in the amygdala, the striatum and the superior frontal gyrus, among other areas, which had been associated to a higher sensitivity and response to her infant (Kim et al., 2011). This evidence suggests that the mother is in a state of high arousal in comparison to no-mothers. In considering the studies in laboratory animals, as mentioned previously about an increase in glucocorticoids during high arousal states, we hypothesized a higher concentration of glucocorticoids in lactating mothers in comparison to control, no-lactating, no-pregnant women. Then we performed an experiment in which we tested the reactivity of lactating women to an image of a baby or to an adult woman in comparison to controls. Before and after image presentations we recorded their electrical activity and collected saliva samples to determine the hormone cortisol. To date we already completed the processing and analysis of saliva samples. To our surprise, in contrast to our hypothesis lactating women had significantly lower concentration of cortisol in the basal state, before image presentations, than controls. Interestingly levels further decrease after an image of an adult woman but increase again after the image of a baby. It is considered that glucocorticoids are "stress hormones" which increase their levels during stressful situations to help face an imminent threat. But high levels of these hormones are harmful and lead to several health physical and psychological problems

as high blood pressure, weight gain, anxiety, and depression. In fact, it had been reported that lactation is associated to a decreased activity of the hypothalamic-pituitary-adrenal axis that control the release of circulating glucocorticoids (Heinrichs et al., 2001). Low levels of glucocorticoids during lactation are important as these hormones are transferred from the blood to the milk and then to the baby, and they can have negative consequences on the infant development (Manuela et al., 2021). Then our results agree with the proposal that low levels of glucocorticoids during lactation are adaptive to protect the proper development of the infant despite the mother is under a high state of physical and psychological pressure (rev. Almanza-Sepulveda et al., 2020). This can lead to psychological disturbances in the mother which can also influence the infant.

Prevalence of breastfeeding and psychological state of mothers in Veracruz

There are no data about breastfeeding prevalence in the state of Veracruz. The only data available for the country of México had been obtained from a national survey about nutrition and health status named ENSANUT. World Health Organization (WHO) recommends that newborns need to be fed exclusively with breastmilk for at least the first six months of age, which is named exclusive breastfeeding (EBF). EBF in ENSANUT 2012 survey was 14.4%, one of the lowest ratios in Latin America. In the survey of 2018, the prevalence increased to 28.6% (ENSANUT, 2018). Although still low this data indicates a significant EBF improvement. No data were reported from Veracruz and also for any other state of the country. The number of women in the survey were only 350, which is roughly 11 mothers per every state of the country! In considering this we decided to gather a census in some regions of the state of Veracruz, but the first question was, which questionary should be used? Our idea was to know prevalence of EBF in comparison to other feeding practices and also explore about barriers for establishment and prevalence of EBF. Besides we also wanted to explore prevalence of mood disorders in relation to breastfeeding in comparison to other feeding practices. Soon we realized that the only questionary available in México was that of ENSANUT, but it is very long with broader scope than our objectives. Then we designed a questionary with 20 questions which was validated by two Physicians with postgraduate degrees in epidemiology. This was a long process with three main steps. The first one was to design a first set of questions with different options. They were sent to 7 health professionals in medicine, nursery, psychology, nutrition, and international certification in lactation (IBCLC). They sent us comments and corrections to the questions

and suggested additional questions. With this information we organized a second set of questions, and we obtained a confident statistical significance ($p<0.05$; Torres-Morales et al., 2021) about the reliability of the questionary. Then it was applied to a group of 20 mothers with babies less than two years old. The same questionary was applied to the same mothers one month later. Again, we obtained an index of reliability about their answers and the epidemiologists consider that the questionary was ready to apply to a broader population. So far, we had applied the survey to 347 women older than 18 years old. To our surprise the prevalence of EBF was 61%. 29% of the mothers breastfeed in combination with milk formulas and only 10% feed their babies exclusively with milk formulas. 17% of the mother's report that a physician recommends feed the babies with milk formulas. Depression index in mothers with EBF was 8.4%, 12.5% in mothers that feed their baby with milk formulas and 18% in mothers that breastfed and use milk formulas.

Our results are important as they are the first reliably data about breastfeeding practices in the state of Veracruz. The survey was conducted mainly in Xalapa city and a few data were obtained from other small towns in the state. The survey is demonstrating a much higher prevalence of EBF than those reported by ENSANUT. Also indicates that the mothers that use milk formulas were influenced by a health professional. Another important result is that breastfeeding is associated to a lower prevalence of depression in mothers. This last point is interesting because during breastfeeding several neurochemicals are released in the mother which had been associated to a state of well-being.

Summary

Besides the research work an educational effort was also implemented, a "Maternal lactation" course for bachelor's degree students and a postgraduate Diploma degree in "Maternal lactation and health". We also published a manual "Alimentación durante los dos primeros años de vida", (Feeding during the first two years of life). Interestingly, this manual was also traduced to zapoteco and mazateco, two prehispanic languages, natives from México. These manuals along with other bibliographic resources about maternal lactation are available in our web page (https://www.uv.mx/cib/lactancia-materna/libros/).

We are convinced that education about the importance and benefits of lactation for the infant and the mother can contribute significantly to a healthier population. As stated, it is well known that breastmilk, in contrast to milk formulas, contribute to a physiological and immunological programming in the newborn which decrease the risk of developing several co-

morbidities in adult life. Unfortunately, very few people know about these benefits. For this reason, we consider that educational programs are at the core of a desired change in the society about appreciation of breastmilk benefits.

It had been a long journey, more than 30 years of research in basic science, full of academic production, formation of human resources and creation of new infrastructure. This experience was the basis for this translational research about breastfeeding and milk benefits. It was demonstrated that it is possible to successfully implement an academic program with true social importance. However, the future of this effort is uncertain due to several administrative and institutional drawbacks at our research center. Despite that, the door was already open for novel translational research with important potential impact to the general population. This represents new academic roads for present and future generations.

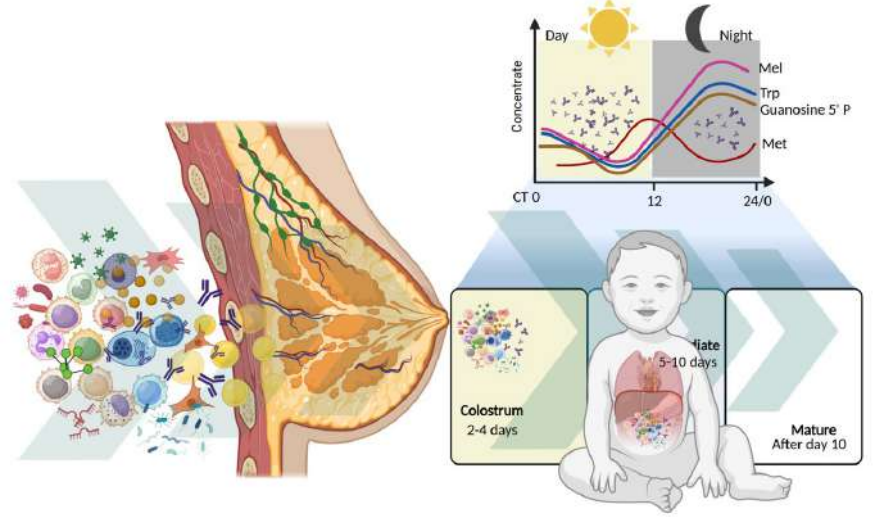

Fig. 1. Breast milk a complex and dynamic biofluid. Human milk has an array of nutritional and non-nutritional components which are necessary for the optimal nutrition and development of the newborn. Among them it has a plethora of immunological components, in sharp contrast to milk formulas. Milk composition is dynamic, it changes according to the physiological and pathological state of the infant, throughout the lactation period and during day and night. Graph represents some day/night changes in Melatonin (Mel), Tryptophan (Trp), the nucleotide Guanosine 5'P and the aminoacid methionine (Met) and immunological factors. Modified from Caba-Flores et al., 2022b.

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XVI

Clinical application of chronobiology

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"Measure over time what is measurable,
and make measurable what is not yet measurable".
Franz Halberg (1919-2013)

Introduction

Chronobiology is considered the scientific discipline that deals with the study of biological rhythms, which occur in living organisms at different levels of organization. The biological rhythms of greatest interest from the biomedical point of view are those of circadian period (period of 22 to 28h), these rhythms are found in all species of animals, including humans (Aschoff, 1981).

However, the greatest development of chronobiology has been in basic research laboratories, especially where researchers are attracted by the study of biological time, most of these observations have been made experimentally at different levels, especially in rodents, from genetics to cell biology these studies have been limited especially to the characterization of circadian rhythms, searching for the components of the circadian system both structurally and functionally. These studies have been of great importance to understand the molecular, cellular, neurobiological, and behavioral mechanisms, among others, that underlie circadian rhythms.

On the other hand, it is known that up to 30% of the human genome is related to circadian rhythms, which is reflected in most of the physiological, behavioral, and biochemical variables, for example: temperature, sleep-wake cycle, feeding, plasma cortisol, growth hormone, leptin, and melatonin levels, etc., which has important effects on homeostatic level, health, and susceptibility to diseases (Rijo-Ferreira et al., 2019).

Clinical Chronobiology (Chronomedicine)

Clinical chronobiology is the result of the need to bring laboratory findings to clinical application, as there is a large amount of basic biomedical knowledge not applied clinically. Major scientific advances, as well as very specific lines of research, have moved the basic biomedical researcher away from the patient's bedside (Lenfant, 2003).

Based on temporal fluctuations as a determinant factor of our physiology Chronomedicine tries to address the prevention, etiology, diagnosis, and treatment of diseases in humans (Cortelli, 2015). It is important to note that chronobiology in the last two decades has generated a great deal of knowledge from experiments in animal models to understand human health and disease, as well as strategies in chronotherapy, unfortunately very few clinicians take interest or apply them.

Chronodisruption (Circadian Desynchronization)

Desynchronization refers to the temporal disorganization of the circadian system, which has effects on health, as it significantly disturbs the temporal organization of physiology (Figure 1). The stability of circadian rhythms such as the sleep-wake cycle, body temperature, as well as the synthesis of hormones such as melatonin and cortisol, for example, are indispensable to ensure an appropriate physiological response and to anticipate events in the external environment (Erren et al., 2003).

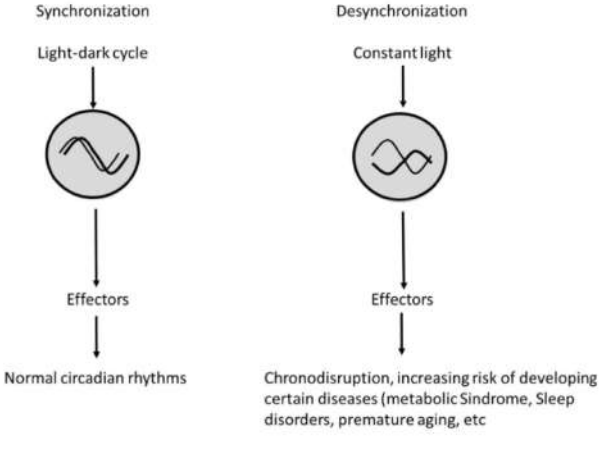

Figure 1. Circadian disruption can be induced by different factors: continuous light, nocturnal feeding, nocturnal physical exercise or by frequent shifts in the time provided by zeitgebers (i.e., jet lag, shift work).

In our modern society, environmental conditions have been recognized and studied that generate inadequate temporal signals, which can trigger

desynchronization events, for example: jet lag (Jet-Lag Syndrome), night work (Nightwork or Shiftwork), light pollution associated with social jet lag, all the above examples can evolve into a disease in cases of chronic or permanent dysfunction, or to the trigger for more complex alterations. The main consequences of desynchronization are premature aging, insomnia, increased propensity to develop depression, cognitive impairment, dementia, and other mood conditions. Chronodisruption also affects metabolic status by promoting inflammation, obesity, metabolic syndrome, cardiovascular disease, and premature mortality (Tapia-Osorio et al., 2013).

In such a way that the chronic light stimulus at the beginning of the dark phase (night) promotes the extension of the waking state, delaying the onset of sleep and consequently shortening the hours of rest and causing chronic sleep deprivation, in addition to altering the synchrony of hormonal rhythms that have their peaks of maximum release at night, for example growth hormone or melatonin, whose synthesis and release are suppressed by exposure to light during the night, melatonin is a hormone with many physiological properties, mainly is a potent antioxidant, besides being considered as a signal of darkness that has the ability to synchronize and couple to the circadian system (Angeles-Castellanos et al., 2010).

As we can observe the time of day is a critical factor for most biological functions, but the concepts of the field of chronobiology have not been yet fully translated into clinical practice (Lenfant, 2003). However, in many clinical situations the patients are subjected to a phenomenon of chronic desynchronization due to diseases whose symptomatology is exacerbated during the night, our group has had the opportunity to study one of these diseases.

Chronobiology and chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is a chronic degenerative disease and a public health problem associated with aging and continued exposure to risk factors such as smoking, pollution, and mainly chronic exposure to biomass combustion. This disease is characterized by respiratory symptoms such as cough, dyspnea, among other symptoms generated by airway obstruction secondary to chronic exposure to harmful particles or gases that distort the architecture of the bronchi (Hattab, 2016).

It is known that COPD patients have sleep-wake cycle disturbances, characterized by poor sleep quality, difficulty initiating sleep, frequent awakenings, and consequently increased daytime sleepiness. Approximately 60 to 70% of these patients have poor sleep quality. The pathogenesis of sleep disorders is very complex and multifactorial, involving physiological

changes associated with sleep, hypoxemia, hypercapnia, inflammation, COPD medications, all of which could potentially contribute to the development of sleep disturbances in these patients. Generally, in the explanation of the pathogenesis of sleep disorders, biological rhythms are left aside, which we consider to be an important physiological process that should be considered in the diagnosis and treatment of some diseases that occur with chronodisruption such as COPD (Akinci et al., 2018).

If we consider that sleep is regulated by the circadian system and modulated by the hormone melatonin, which is synthesized in the pineal gland and is a nocturnally released hormone, its maximum levels of secretion occur during the night approximately between 01:00 and 03:00 h, with concentrations that can vary from 29.5 ± 2.2 to 37.6 ± 1 pg/ml, in older patients. We found that the group of healthy patients had melatonin values considered adequate for their age range (69 ± 9 years). The melatonin levels found could indicate that poor sleep quality in these patients is influenced by low values and altered melatonin release pattern. In the group of patients with COPD, they show a loss of nocturnal melatonin release, which may be related to the chronicity of the disease and especially with exposure to light during the night when they present symptoms, which then becomes negative feedback on sleep quality, since melatonin is a hormone that is synthesized through a phototransduction process that is stimulated in the dark and inhibited by exposure to artificial light during the night, in addition to suppressing its secretion, decreases its release time (Martínez-Luna et al., 2021).

On the other hand, different studies have shown that the administration of exogenous melatonin improves sleep quality in COPD patients. However, the mechanisms have not been fully described, although two could be proposed. The first is due to the action melatonin has shown to suppress inflammatory cell infiltration in lung tissue, reduce lung destruction and necroptosis, and decrease oxidative stress and dyspnea. So far, most of the studies supporting these results have been performed in animal models, but they suggest a limitation in the progression of the disease and the reduction of the symptomatology in COPD patients, due to the reduction of the inflammation of the airways, favoring a reduction of the symptomatology and a better quality of sleep (Halvani et al., 2013). Another mechanism would be regulated by the chronobiotic actions of melatonin on the circadian sleep-wake rhythm; if patients have low melatonin levels, they could recover adequate levels with exogenous administration of melatonin, which are required during the night to promote sleepiness and maintain sleep.

From the functional point of view, low values of this ratio FEV1/FVC were observed in patients with COPD; where FEV1 is the air capacity that a person can expire in the first second of forced expiration and FVC is the complete forced vital capacity; low values were observed indicating a greater obstruction of the patient's airways. This variable was correlated with low nocturnal melatonin values, together with sleep quality and sleepiness. This establishes a relation among a greater airway obstruction, lower nocturnal melatonin, the worse the sleep quality and the greater the daytime sleepiness.

Therefore, we can recommend that in COPD patients their treatment should include melatonin supplementation to ensure that they have a good quality of sleep and not only the treatment of respiratory symptoms, for example, oral administration of 3 mg of exogenous melatonin one hour before bedtime for twenty-one days has been shown to be an alternative for the treatment of poor sleep quality in COPD patients. The evidence obtained with evaluations of the Pittsburgh Sleep Quality Index shows an improvement in the group treated with melatonin, compared to the group that received a placebo, suggesting that melatonin has an important role in improving the quality of sleep of these patients (Martínez-Luna et al., 2021)

Chronobiology in the hospital

Most studies of chronobiology put special emphasis on light as the main environmental signal for the regulation and proper functioning of the circadian system, however, we should not overlook the importance of darkness as a temporal signal with the same importance as light. In such a way that it should be considered that the most important for the circadian system is the stable alternation of these two signals, to maintain a circadian temporal stability in all body functions.

Therefore, we consider that the hospital environment is the most appropriate space to characterize the harmful effects of the loss of the alternation of the light-dark cycle, since almost all hospital units are kept 24 hours a day with the light on to allow health care staff, adequate supervision of hospitalized patients, in both patients and medical staff is in constant lighting conditions. However, for the moment we have only been concerned about the patients, leaving aside the personnel who work in these conditions, which should be considered in the modality of circadian alterations generated by rotational work or night work (Table 1).

Our group has carried out an analysis of the conditions and intensities of nocturnal lighting in three different hospitals, in different areas or hospital floors, using a luxmeter to measure the intensity of illumination and

we could observe that during the night the different hospital spaces are illuminated above 100 lx (Figure 2). These conditions of constant illumination and high intensity of direct light in patients can generate circadian alterations of different physiological variables including melatonin and alterations of the sleep-wake cycle, predisposing hospitalized patients to trigger alterations or clinical manifestations that complicate the base diagnosis, consequently their treatment and evolution. One of these disorders that our group has studied is the development of in-hospital delirium and its association with circadian disturbances.

Figure 2. Photograph showing the lighting conditions, two newborns of the experimental group were under a light-dark cycle of 12:12, (275.8 ± 10 lux) of 7:00 am at 07:00 p.m. and darkness (25.23 ± 7.8 lux) of 7:00 p.m. at 7:00 a.m., with the infant's head covered by a cloth.

Chronodisruption and delirium

Delirium is a common and complex hospital complication in older adults, which can affect independence, increase morbidity and mortality. This syndrome is characterized as an acute and fluctuating disorder of attention and cognition. It usually occurs in a vulnerable adult patient and is usually secondary to acute medical pathology, drug use and/or an elective or emergency surgical-anesthetic event.

Delirium manifests itself through various symptoms, among which attention disorder stands out. Interestingly, its presentation is fluctuating throughout the day. Among its symptoms are variable degrees of changes in the level of consciousness with modification of the level of alertness, memory alterations, difficulties in the organization of thought, disorientation in space and time, the appearance of symptoms such as hallucinations, delusions, increased motor activity or physical agitation and mainly disorders of the rhythm of sleep and wakefulness (Al-Aama et al., 2011).

Delirium is most prevalent among older patients, with a proportion of 11-42%. Interestingly, the symptoms of delirium are closely related to circadian rhythm. In patients who develop delirium, the sleep/wake cycle is re-

versed, and patients are drowsy and take naps during the day, while nighttime sleep is brief and fragmented. Up to 98% of older patients with delirium are known to have at least moderate sleep/wake cycle disturbances.

As previously discussed, the integrity of the sleep/wake cycle and the temporal order of all physiological and behavioral functions, including melatonin synthesis and release, depends on proper internal synchronization between the suprachiasmatic nucleus and the pineal gland, and it has also been suggested that delirium is accompanied by a loss of temporal variation of plasma melatonin (Reiter, 2003). On the other hand, it has been documented that patient undergoing major abdominal surgery developed postoperative delirium and have reduced plasma melatonin levels and sleep disturbances, including sleep phase delay syndrome. Another study reported cases of in-hospital delirium in the Intensive Care Unit associated with loss of the circadian rhythm of melatonin (Shilo et al., 2000).

To determine whether the loss of melatonin rhythm may be an indicator of the onset of delirium, our group conducted a study in which salivary melatonin levels were evaluated in older adult patients from the first day of hospital admission until the onset of delirium or until hospital discharge. The patients were distributed in two groups, patients who did not develop delirium (ND) and those who did develop delirium (D). The mean age of the patients studied was 78.5±5.8; in the ND patients salivary melatonin showed low levels during the day and high levels at night, resulting in a daily rhythm of melatonin concentration in the 3 days prior to hospital discharge. In the group that developed delirium, melatonin levels were analyzed for seven consecutive days, that is, three days before and four days after the development of delirium, in which melatonin levels remained low both during the day and at night, they did not show a circadian rhythm (Angeles-Castellanos et al., 2016).

As we have already reviewed, delirium is a common syndrome that increases morbidity and mortality in the hospitalized population, which can be a frightening and distressing experience for patients and their families. Therefore, the need for early diagnosis is crucial to improve the patient's prognosis, so the results obtained in this study suggest that there is a close relationship between circadian system disturbance and the development of delirium. These findings indicate a strong association between daily melatonin rhythms and the onset of delirium, so that determining the daily rhythm of salivary melatonin, prior to the development of delirium may be a useful predictive strategy, in addition to the fact that this strategy is not invasive and does not interfere with the patient's general condition.

On the other hand, the relationship between delirium and the loss of the daily rhythm of melatonin synthesis and release has been previously demonstrated in other studies. Therefore, we propose, melatonin administration of exogenous melatonin as a preventive treatment for the development of delirium in the hospital environment, thus reducing the severity and most likely the length of hospital stay.

Neonatal intensive care unit and constant light

Fetal circadian rhythms can be observed in utero from 30 weeks gestation, coupled to the maternal rhythm, but after birth newborns synchronize with the external environment. Important cues for synchronization include the light/dark cycle, the timing of feeding, and exposure to hormonal circadian cues in breast milk. Disruption or lack of these signals may occur during admission to the neonatal intensive care unit (NICU). This may affect the development of circadian rhythms and influence neonatal survival and development, with the potential to affect health and well-being throughout adulthood (Boo et al., 2002).

We describe below the rationale and evidence to support a chronobiological approach to inpatient neonatal care. The NICU is one of the most stressful hospital spaces, due to the medical condition of the patients who are admitted there, the reason there is a need for constant attention and vigilance for premature newborns (PNB) hospitalized in these spaces. In such a way that a large number of hospitals internationally, NICUs are kept in constant lighting conditions (LL), 24 hours a day, without significant variations in light intensity (Escobar et al., 2021).

Our group had the opportunity to determine the lighting conditions in the NICU in three Mexican hospitals, where we measured the intensity of illumination with a lux meter, at 6 different time points of the day, the intensity of illumination of the NICU at the level of the incubators was 275.82±14 lux during the day and 145.28±14 lux during the night, the illumination is light provided by white fluorescent lamps placed on the ceiling of the hospitalization area and we can observe that these are very high intensities, since at least during the night an intensity of less than 5 lux is recommended, above this level they are considered negative for circadian system. Therefore, artificial light during long intervals in the dark or night phase promotes the alteration of activity schedules and the reduction of sleeping hours, on the other hand, light during the night generates contradictory time signals for the biological clock and can lead to the deregulation of circadian rhythms (Sánchez-Sánchez et al., 2022).

It is known that during the fetal stage circadian function depends on maternal signals, especially melatonin, which is one of the few hormones able to cross the placental barrier. Experimentally it has been observed that exposure of pregnant rats to light during the night suppresses their own melatonin production and affects the circadian rhythms exhibited by their offspring, which show low intrauterine growth and deficiency of corticosterone secretion in postnatal stages. It has also been demonstrated that LL during development permanently damages the biological clock, resulting in a lower number of neurons and glial cells in the suprachiasmatic nucleus considered the main biological clock. Mouse pups exposed to light at night during infancy are unable to exhibit coordinated circadian rhythms throughout their lives. It has also been demonstrated that rat pups exposed to irregular light-dark cycles during lactation exhibit anxiety behaviors, alterations in social interaction and poor performance in learning tests in adulthood. Therefore, it has been proposed that during development the excess of light at night alters brain organization and may be a risk factor for developing depression, anxiety, and other mood disorders.

Thus, preterm infants kept in the NICU under LL conditions are exposed to chaotic environmental time cues with no cyclical variations, rather than predictable time cues such as a daily light-dark cycle. Surprisingly, the light levels to which babies are exposed are one of the few environmental variables that are not controlled and have not been considered in the protocols of care of these patients. In these hospitalization conditions, the PNB are kept for prolonged periods of time, generating negative effects on their growth and development. As we have previously mentioned, exposure to constant light can generate potential damage to the eyes of newborns. We have experimentally observed damage to the cellular organization of the retina in newborn rats exposed to a constant lighting environment from the first day of birth. On the other hand, we have shown that establishing a light-dark cycle (LD) 12:12 hours, in PNB promotes an increase in sleep time decreasing the time devoted to feeding and weight gain, compared to PNB that remain in constant light or darkness. It has also been observed that PNB who were exposed to a cycle of LD from birth, acquired circadian rhythmicity in activity/rest, from the 34th week of life (Mann et al., 1989; Vásquez-Ruiz et al., 2014).

One of the recent objectives has been to transfer the chronobiological knowledge acquired in laboratories to hospital application in the care and treatment of patients. One of the strategies in which we have tried to apply chronobiology is to intervene directly by modifying the environmental lighting conditions in hospitalization areas, for example: our group has carried

out a series of clinical-experimental studies with healthy preterm newborn babies, but with low body weight at birth, as mentioned above, the light/dark conditions have been modified individually, to verify the direct benefits of the light/dark cycle, we had a control group, which was kept in constant lighting conditions in the NICU with an average of 250±21 lux illumination, 24 hours a day. The infants in the experimental group remained in the same room, but under a light-dark cycle, which was achieved by placing an acrylic helmet covered with folded blue or green surgical drapes over the infant's head, leaving the front part open to allow good air flow. This helmet was placed individually over the head and part of the upper thorax of each PNB, which resulted in reduced illumination, with a light intensity of 25.23 ± 7.8 lux, at eye level during the night period from 19:00 to 07:00. During the day, from 07:00 to 19:00 the cloth drape was removed allowing the PNB to receive the illumination intensity corresponding to the day, 275.82±10 lux (Figure 3). When the light/dark cycle was established, the infants maintained under this condition showed greater tolerance to milk formula, compared to the infants in the control group who were in constant light conditions. This positive change in feeding results directly in faster and more constant body weight gain, therefore, once these babies reached the required body weight (2 kg), they were discharged from the hospital much earlier, on average 20 days earlier than the babies who were in constant light. We consider this result to be very positive, first of all, because it significantly reduces the number of days of hospitalization, considerably reducing the exposure of babies to nosocomial diseases, and also the newborn is integrated more quickly to the family bed and maternal care, which is important for their perinatal development. On the other hand, it is evident that there is an economic saving for the family as well as for the hospital services and a better use of supplies and personnel for neonatal medical care. With these results we have been able to prove the advantages offered by chronomedicine, at implementing a light/dark cycle during the first days after birth, so we propose that all NICUs should reevaluate the internal rules that suggest keeping babies in constant lighting conditions and assess the advantages and disadvantages on health (Sánchez-Sánchez et al., 2022).

Perspectives of medical chronobiology

As we have been able to verify, the chronobiological management of patients still presents various logistical and methodological problems, especially the fact that medical care units do not have adequate devices for the continuous monitoring of physiological variables; however, the chronobiological care of patients implies a continuous follow-up and, in many cases,

ambulatory accompanied by high costs. Unfortunately, the results offered by medical chronobiology are not immediate or spectacular as we are accustomed to observing with some medical-surgical treatments. However, chronobiology proposes a better understanding and manipulation of the physiology, understanding its circadian variations and using them as part of the treatment.

Chronobiology has established itself as a scientific discipline that explains physiopathological phenomena from the point of view of the temporal order that had only been approached in a descriptive and anecdotal way, giving support not only to therapeutic schemes but also to diagnostic ones. From the clinical point of view, there are some specialties that already identify a chronobiological component, both in the diagnosis and in the treatment of some pathologies (Smolensky and D'Alonzo, 1991). Chronobiology thus provides new tools for the analysis and treatment of sleep disorders and makes a special contribution to the understanding of psychiatric disorders, such as seasonal depression. Currently in several parts of the world including Mexico, chronobiological studies of chronic degenerative diseases and internal desynchronization is studied as part of the pathogenesis of some diseases such as obesity and metabolic syndrome.

As we have been able to review, circadian rhythmic modifications in developmental biology, for example, the changes that occur from infancy to adulthood, and the rhythmicity disorders that occur with old age, are of great interest. The pharmaceutical industry is beginning to design drugs based on chronobiology to be released at specific times of the circadian cycle; thus, we see the multilayer tablets, which allow its modulated release, for example: fast in the proximal portion of the small intestine, and slow in its distal portion, combining these techniques is intended to design a system of programmed release by pulses. Infusion pumps are currently the most effective method for the release of one or more drugs. An alternative method of achieving a pulsatile release of a drug involves the use of nanotechnology. In the future, microchips could be the necessary tool to obtain efficient chrono-pharmacokinetic systems (Ohdo, 2007).

We can also observe that the "complexity" inherent to basic research depends on the type of manipulation, as well as on the experimental models used. The present paper allows us to realize that the knowledge obtained in basic chronobiology can be transferred to its clinical application (chronomedicine), logically with the reservations in its limitations and pending challenges of preclinical studies. On the other hand, we believe that the official incorporation of translational research should become a priority in medical education. Such inclusion in academic and health systems programs

would allow to initiate a positive discussion around an important issue in many developed and developing countries: what should be researched, who should finance it, and how much money should be allocated for a sustainable and balanced development of research, in favor of the health of the population. Currently, only 14% of scientific advances related to health are applied in the clinical field and the process of transforming basic knowledge into clinical knowledge takes up to 17 years on average. For example, the UK Centre for Advancing Translational Science recommends that all projects undergo a full evaluation 3 to 5 years after initiation, which has resulted in a 30% increase in translational research projects. It is also known that the time taken by researchers to write up and publish their results is too long and it is recommended that this should be a period of 3 to 10 months after results are obtained. This would bring enormous advantages for the translational application of not only chronobiological knowledge but also of many advances that are being made today in biomedical laboratories around the world (Straif et al., 2007).

Summary

We believe that chronomedicine proposes a better understanding and manipulation of physiology, using its circadian variations as part of the treatment. One way to achieve better progress in this branch will be to include a change in the teaching and training of future doctors, including concepts of biological rhythms and chronotherapy, which will undoubtedly have an impact on a better understanding of physiology and greater effectiveness in the prevention of diseases and treatment of pathologies. Nowadays, the teaching of medicine has its back turned to chronobiology, since most doctors, even those who claim to know something about chronobiology, do not know that some common diseases become more acute at certain times of the day, even they do not know that blood pressure varies significantly during the day and believe that diagnostic tests always give the same results regardless of the time they are performed, also that drugs work the same and their side effects are the same regardless of the time of administration. Also, we are convinced of the usefulness that chronobiology can provide to improve people's health and quality of life, so we cannot leave aside the ethical commitment that certain chronobiological principles and applications can help to improve the effectiveness of conventional pharmacological treatments and to prevent disease. If we show future doctors and biomedical researchers that drugs used in cancer therapy have a cytotoxic effect on cells during the proliferative phase of the cell cycle and that the number of cells in the DNA synthesis phase and mitoses varies throughout the 24

hours, so that the mechanisms responsible for this circadian regulation act on growth factors, membrane receptors and/or enzyme systems, so it should be given in low doses at night and high doses during the day, without forgetting that it should ideally be given at the time of least mitosis (nadir) of the circadian rhythm of healthy tissue or during the acrophase or maximum mitotic peak of the cancer in question. However, for this it is not enough to present the results of the many existing works on cancer chronotherapy, it is necessary to teach solidly the foundations on which these treatments rest. Finally, we believe that no single technology is sufficient to contribute to the advancement of the big questions of biomedical research. Success depends on asking questions that make biological and medical sense, and these questions will arise from close collaboration between basic and clinical researchers.

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