UNIVERSIDADE D
COIMBRA

João Daniel Ribeiro Neves

The role of kisspeptin in the sexual differentiation of the developing brain

Dissertação no âmbito do Mestrado em Neurociências Molecular e de Translação, orientada pela Doutora Joana Teresa Ferreira Gonçalves e coorientada pelo Professor Doutor Miguel Castelo Branco e apresentada à Faculdade de Medicina da Universidade de Coimbra.

setembro 2023

UNIVERSIDADE D
COIMBRA

THE ROLE OF KISSPEPTIN IN THE SEXUAL DIFFERENTIATION OF THE DEVELOPING BRAIN

JOÃO DANIEL RIBEIRO NEVES

This work was supported by FCT Exploratory project 2022.01066.PTDC and FCT Strategic plan UIDP/04950/2021 (CIBIT).

Ao professor Miguel Castelo-Branco e à Dra. Joana Gonçalves, por toda a confiança, pelo know-how científico e por todos os conselhos que transcenderam o âmbito académico.

Aos investigadores do iCBR, nomeadamente do grupo do Doutor Francisco Ambrósio, pela transferência de conhecimento constante.

Aos meus colegas de laboratório, Doutor João Martins, Beatriz Martins, Marina Pais e Helena Ferreira pela ajuda incansável na concretização deste trabalho.

À Carolina Fortunato, por ser a companheira desde o primeiro dia nesta aventura.

Aos meus parceiros de mestrado, pelos desabafos, favores, discussões científicas e lazer.

À amiga de longa data Rafaela Clamote, pelos favores, cafés, intervalos e companheirismo fiel.

Ao Bruno, por entender os silêncios, as ausências, os cansaços e acima de tudo por ser o meu porto seguro.

Aos meus familiares, em especial aos meus pais, que são e serão exemplo, pelo apoio incondicional e pelo amor imensurável. A quem dedico esta tese.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AKT - Protein Kinase B (also known as Akt)

AMY - Amygdala

ARC - Arcuate Nucleus

AR - Androgen Receptor

AVPV - Anteroventral Periventricular Nucleus

BCA - Bicinchoninic Acid

BDNF - Brain-Derived Neurotrophic Factor

BNST - Bed Nucleus of the Stria Terminalis

BMI - Body Mass Index

CNS - Central Nervous System

DAG - Diacylglycerol

DTT - Dithiothreitol

EDTA - Ethylenediaminetetraacetic Acid

ELISA - Enzyme-Linked Immunosorbent Assay

ER - Estrogen Receptor

ERK - Extracellular Signal-Regulated Kinase

FSH - Follicle-Stimulating Hormone

GABA - Gamma-Aminobutyric Acid

GMV - Grey Matter Volume

GPCRs - G-Protein Coupled Receptors

GnRH - Gonadotropin-Releasing Hormone

HPG - Hypothalamic-Pituitary-Gonadal

ICV - Intracerebroventricular

IP - Intraperitoneal

IP3 - Inositol Triphosphate

JAK - Janus Kinase

KO - Knockout

KP - Kisspeptin

KISS1R - Kisspeptin Receptor

Kp234 - Kisspeptin-234

LH - Luteinizing Hormone

MAPK - Mitogen-Activated Protein Kinase

mPOA - Medial Preoptic Area

MRI - Magnetic Resonance Imaging

mTOR - Mammalian Target of Rapamycin

NTS - Neonatal Testosterone Surge

PAGE - Polyacrylamide Gel Electrophoresis

PBS - Phosphate-Buffered Saline

PCA - Principal Component Analysis

PCs - Principal Components

PCR - Polymerase Chain Reaction

PIP2 - Phosphatidylinositol 4,5-Bisphosphate

PKA - Protein Kinase A

PKC - Protein Kinase C

POA - Preoptic Area

POMC- proopiomelanocortin

PND - Postnatal Day

Ptf1a - Pancreas Transcription Factor 1a

RIPA - RadioImmunoPrecipitation Lysis Buffer

SDS - Sodium Dodecyl Sulfate

SEM - Standard Error of the Mean

SNc - Substantia Nigra

SRY - Sex Determining Region

STAT - Signal Transducer and Activator of Transcription

TBST - Tris-Buffered Saline with Tween 20

TRPC - Transient Receptor Potential Canonical

USV - Ultrasonic Vocalization

Veh - Vehicle

VTA - Ventral Tegmental Area

LIST OF TABLES

LIST OF FIGURES

RESUMO

Ao longo de décadas, muito se tem estudado sobre diferenciação sexual do cérebro. No entanto, apesar de se ter explorado abordagens genéticas, o impacto das hormonas sexuais e as diferenças anatómicas, as razões fundamentais por trás das distinções entre homens e mulheres, permanecem pouco elucidativas. O dimorfismo sexual, influenciado principalmente pelos cromossomas sexuais X e Y que contêm genes específicos para a masculinização/feminização, particularmente no cérebro, continua a ser enigmática. Embora os recetores de hormonas sexuais estejam distribuídos por todo o cérebro, eles não fornecem uma explicação abrangente para a diferenciação sexual. A evidência anatómica, embora importante, muitas vezes não apresenta correlações com o comportamento. Assim, a pergunta persiste: o que une todos estes aspetos? O que impulsiona o dimorfismo sexual desde a fase embrionária até à fase adulta?

Os kisspeptinas, uma família de péptidos expressos principalmente no hipotálamo, desempenham um papel vital na regulação e libertação da hormona libertadora de gonadotrofinas (GnRH). Os neurónios de kisspeptina contribuem significativamente para os mecanismos de feedback das hormonas sexuais. Curiosamente, os recetores e os kisspeptinas apresentam padrões de expressão sexualmente dependentes em regiões críticas que governam comportamentos típicos de cada género. Por exemplo, no comportamento de jogo, a amígdala medial está altamente envolvida. Uma outra descoberta cativante é o pico neonatal de kisspeptina, que se alinha com o aumento de testosterona observado em machos e homens durante esta fase de diferenciação sexual crucial. Esta sincronização incita-nos a formular a hipótese de que o kisspeptina influencia as diferenças sexuais e a diferenciação sexual do cérebro.

Para investigar esta hipótese, ratos Wistar foram injetados intracerebroventricularmente com um antagonista do kisspeptina, Kp234, a PND0. A bateria de testes comportamentais incluiu marcos de neurodesenvolvimento, vocalizações ultrassónicas induzidas pelo isolamento de crias, teste de “open field”, avaliação do comportamento de jogo e análise do comportamento sexual. Adicionalmente, avaliámos a expressão do recetor de kisspeptina 1 através de western blotting e avaliámos a morfologia da microglia através de imunomarcação.

As nossas descobertas demonstram um bloqueio bem-sucedido das vias de sinalização do kisspeptina, uma vez que, houve uma redução dos níveis plasmáticos de testosterona após injeção com o antagonista nos machos. O bloqueio do kisspeptina tem também um impacto no peso corporal, no desenvolvimento dos testículos e no peso total do cérebro nos ratos machos adultos. Além disso, observamos que o kisspeptina modula a comunicação dos ratos, tanto na infância como em fase juvenil. Apesar de explorarmos uma variedade de resultados, não observámos alterações no neurodesenvolvimento dos animais. No entanto, os ratos injetados com Kp234 exibiram comportamento hiper-locomotor e um aumento do comportamento exploratório.

Este estudo destaca influências distintas do bloqueio neonatal do kisspeptina no comportamento de jogo, revelando tendências de brincadeira reduzida em ambos os sexos, com um efeito mais pronunciado nos machos. Embora o comportamento sexual permaneça em grande parte inalterado, observa-se uma redução inesperada na latência para se envolver em comportamentos sexuais nos machos injetados com Kp234, sugerindo fatores motivacionais complexos.

O estudo culmina numa análise de componentes principais, que agrupa eficazmente populações distintas em ambos os sexos. Isso fornece evidência convincente para o impacto duradouro da modulação neonatal da kisspeptina nos perfis de comportamento típicos de cada género.

Em resumo, este estudo explora a interação intricada entre o aumento neonatal de kisspeptina e os comportamentos típicos de género ao longo de várias fases da vida, oferecendo novas perspetivas sobre os mecanismos moleculares e celulares subjacentes.

Palavras-chave: kisspeptina; diferenciação sexual; cérebro em desenvolvimento; in vivo

ABSTRACT

For decades, the study of brain sexual differentiation has been extensive. However, despite exploring genetic approaches, the impact of sex hormones, and anatomical differences, the fundamental reasons behind male-female distinctions remain elusive. Sexual dimorphism, influenced mainly by the X and Y sexual chromosomes housing specific genes for masculinization/feminization, particularly in the brain, remains enigmatic. While sex hormone receptors are distributed throughout the brain, they fall short of providing a comprehensive explanation for sexual dimorphism. Anatomical evidence, though necessary, often lacks correlations with behavior. Thus, the question remains: What bonds all these aspects? What drives sexual dimorphism from its inception through life?

Kisspeptins, a family of peptides mainly expressed in the hypothalamus, play a vital role in regulating and releasing gonadotrophin-releasing hormone (GnRH). Kisspeptin neurons contribute significantly to the feedback loop of the sexual hormone cycle. Intriguingly, kisspeptin receptors and peptides exhibit sexually dependent expression patterns in critical regions that govern gender-typical behaviors. For example, in play behavior, the medial amygdala is involved. Another compelling discovery is the neonatal peak of kisspeptin, which aligns with the testosterone surge observed in males and men during this pivotal sexual differentiation phase. This synchronization prompts us to hypothesize that kisspeptin substantially influences sexual dimorphism and differentiation.

To investigate this hypothesis, Wistar rats were intracerebroventricularly (ICV) injected with a kisspeptin antagonist, Kp234, on PND0. The battery of behavioral tests encompassed developmental milestones, pup isolation-induced ultrasonic vocalizations (USVs), open field tests, play behavior assessments, and sexual behavior analyses. Additionally, we evaluated the expression of the kisspeptin 1 receptor through western blotting and assessed microglia morphology via immunolabeling.

Our findings demonstrated the successful blockade of the testosterone surge through ICV injection. Kisspeptin blockade exerted impacts on body weight, testis development,

and total brain weight during rat adulthood. Furthermore, we unveil the modulation of communication, both in pup and juvenile rats. Despite exploring a range of behavioral outcomes, we did not observe alterations in kisspeptin-induced neurodevelopment milestones. However, rats injected with Kp234 exhibited hyperlocomotive behavior and increased exploratory tendencies.

This study highlighted the nuanced influences of neonatal kisspeptin blockade on play behavior, revealing reduced play tendencies in both sexes, with a more pronounced effect in males. While sexual behavior remains largely unchanged, an unexpected reduction in latency to engage in sexual behavior was evident in male Kp234 animals, hinting at intricate motivational factors.

The study's culmination involves a Principal Component Analysis, effectively clustering distinct populations in both sexes. This provides compelling evidence for the lasting impact of neonatal kisspeptin modulation on gender-typical behavior profiles.

In summary, this study delves into the intricate interplay between neonatal kisspeptin surge and gender-typical behaviors across various life stages, offering novel insights into the underlying molecular and cellular mechanisms.

Keywords: kisspeptin; sexual differentiation; developing brain; in vivo

1. INTRODUCTION

1.1. An extended overview of the sex differentiation and sexual differences in the brain: from genome to behavior

Over the past years, grew in the scientific community the interest in studying the biology of sex (1). Rooted in the intricacies of genetic, hormonal, and neural interactions, the biology of sex unveils an elaborate arrangement of molecular orchestrations that shape sexual dimorphism. Today, evidence demonstrates that sex influences, not only physiological processes (1) but also disease onset, progression, severity, symptomatology, and treatment response (2). Thence it is increasingly apparent that sex occupies a pivotal role since the genomic level, passing through the whole organism, and extends its impact even to the dynamics of interpersonal interactions (Figure 1) (3,4).

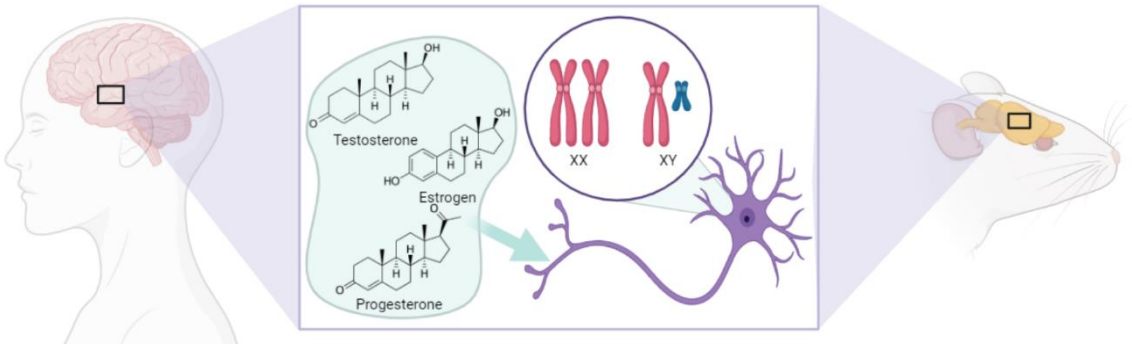


FIGURE 1 THE DIFFERENT LEVELS OF SEX DIMORPHISM
Sex influences the mechanisms both in humans and animals. From the genetic background to the behavioral differences, passing through molecular changes, cellular differences, and anatomic variations. Original image created with BioRender.

Building upon this genetic and hormonal framework, in mammals, the beginning of life starts with the fusion of an ovum with a sperm cell (1,2). It is at this point that sex differentiation starts. Depending on whether the sperm that carries half of the genetic information of the male progenitor has a sexual chromosome X or Y, meaning that if it carries an X chromosome it will develop a female descendent and if it is a Y it develops a male offspring (5).

A pivotal gene, sex-determining region Y (SRY), found on the Y chromosome, initially thought to dictate male sexual development, now faces challenges due to complexities in its action and regulation (6,7). Since only males have the Y chromosome and therefore SRY, the prediction that this gene mediated the formation of the gonads and other secondary sexual male characteristics gained force in the scientific community

(7). SRY acts as an activator of the transcription of genes that mediate testicular development and also as an inhibitor of ovarian-promoting genes (7). Studies demonstrated that this gene exerts effects in the brain directly, being expressed in the hypothalamus and mid-brain in mice, in the hypothalamus and temporal cortex in adult man, and tyrosine hydroxylase (TH)-positive neurons in the substantia nigra (SN), acting as a positive regulator of dopamine biosynthesis and function (8).

However, SRY only functions as a testis-determining factor if during a specific time point in development, expression reaches a threshold (7). Also, this gene has a very frail transactivation potential and is expressed either very transiently or very badly in almost all mammals (9). Hence, researchers focused their attention on SRY box 9 (SOX9), since it is regulated by SRY, but only during the high expression period, after that SOX9 is upregulated by either a feed-forward mechanism maintaining its expression or silenced by an opposite set of genes that are responsible for the ovarian differentiation (10). This finding placed SRY in a less pivotal place, meaning that despite its importance, it is insufficient to explain how sexual differentiation perpetuates throughout life (7).

Studying sex differentiation and sexual differences is crucial since it significantly impacts the neuronal phenotype, and therefore, when neglected, difficult translation. A traditional view of how sex influences the brain is very simplistic, genes impact different gonad formation and produce different sexual hormones that will impact distinctly the brain circuitry and function (11). This classic perspective attributes sexual hormones as the core factor of sexual differentiation (12).

The conventional perspective of brain sexual differentiation asserts that sexual hormones manifest their influence through two distinct mechanisms: testosterone released by embryonic testes act in the whole organism to masculinize the genitalia, sperm ducts, and other organs like the brain, having an organizational effect (11,13). On the other hand, they can have a reversible activational effect, where at any time in life, mainly in adulthood, the exposure to sexual hormones leads to an increased sexual differentiation that may be reversed by gonadectomy (Figure 2) (11).

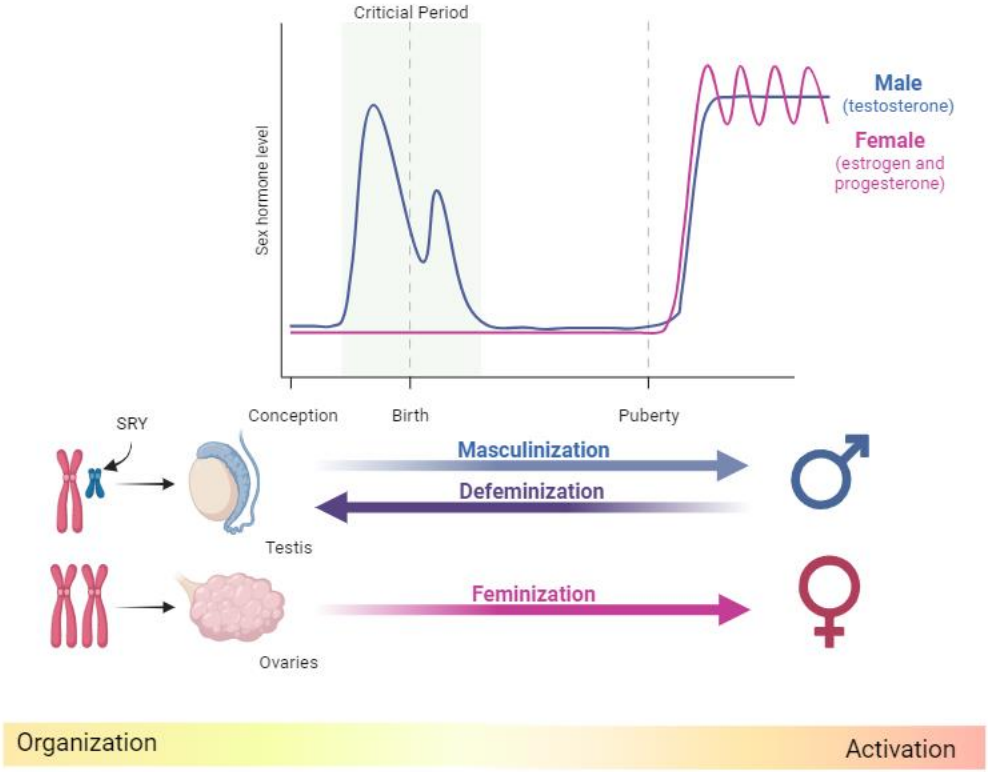


FIGURE 2 CLASSIC PERSPECTIVE OF SEXUAL DIFFERENTIATION.
Beginning in the genome, being SRY gene is pivotal in starting and maintaining the masculinization of the organism, by triggering male gonad formation and therefore, testosterone production. Alongside, masculinization and the process of defeminization take place, being responsible for blocking female-specific behaviors during the lifespan. On the female side, since there is no testosterone triggering, feminization takes place. Masculinization triggering is seen as organizational and essential for activational effects that occur during life, mainly in puberty and during reproduction (14). Original image created with BioRender.

Supporting this theory was the finding that during the perinatal period in many mammalian species, including rodents, non-human primates, and humans, occurs an abrupt and short-lived increase in plasma testosterone (15). This testosterone peak is so relevant for male/female-like sexual differentiation that studies have shown that castrated rats at birth present a female-like development, and a single dose of testosterone right after the castration rescues the male-like phenotype (16). Also, if testosterone is administrated to female rats after birth, they present masculinized behaviors (16,17). Nevertheless, the mechanisms underlying the generation of this perinatal testosterone peak and its enduring impact on long-term behavior remain largely uncharted.

Also, in the central nervous system (CNS) steroid hormones have important roles in neurogenesis and neuroprotection (13). To reach the brain, they are transported through the circulatory system bound to transport proteins, easily crossing the blood-brain barrier

(BBB). Additionally, these hormones can be produced directly on the CNS from the conversion of cholesterol or other steroid molecules, they, as well, were found to masculinize/feminize the brain (13). Testosterone, during development, mediates the wiring of a male-typical circuitry and suppresses the female-typical one. The mechanisms underlying these changes are still largely unknown (13). It is still unspecified any mechanism in females similar to the males, the theory is that in the absence of this activational system, the female organs, and secondary mechanism are triggered (1).

Steroid hormones, and also steroids produced in the brain, action could be done in 2 different ways: a more straightforward approach, where the molecules pass the cellular membrane and bind to intracellular steroid receptors that will promote or inhibit the transcription of specific genes involved on neuronal differentiation or neuronal functions (18,19), or act by binding to membrane-associated or transmembrane receptors, triggering a plethora of cellular mechanisms, like membrane depolarization, gene expression, and neuroprotection (Figure 3) (20).

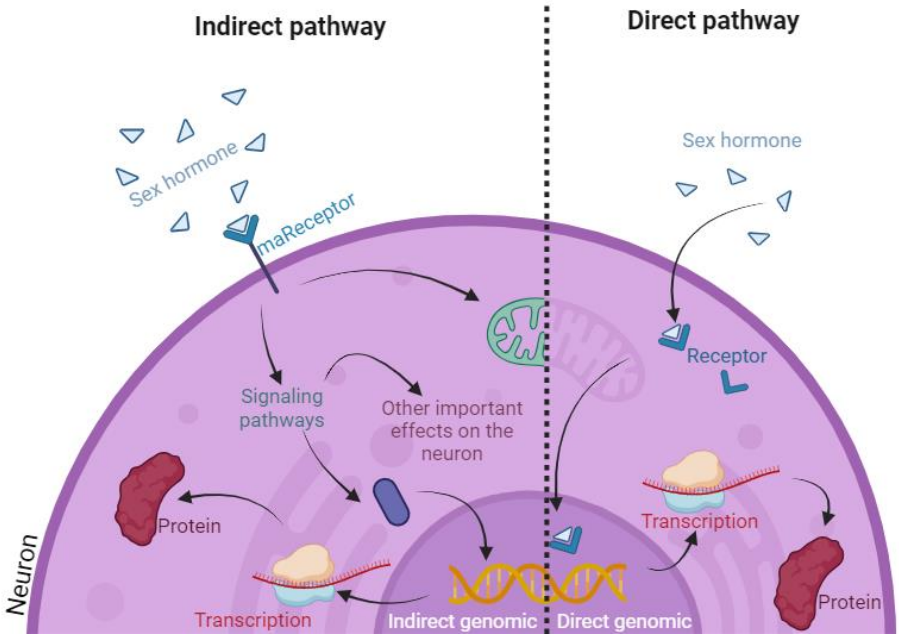


FIGURE 3 THE TWO PATHWAYS IN WHICH SEX HORMONES ACT

Sex hormones could act in the cell in two different pathways. Either by binding to intracellular receptors that when activated enter the nucleus and exert genomic functions. Or either by an indirect pathway binding to membrane-associated receptors (maReceptors), where a signaling pathway is activated and therefore, secondary messengers are triggered, which not only will activate or block transcription but also will initiate other important mechanisms in the neuron. Also, some receptors can play a role in mitochondrial transcription (21). Original image created with BioRender.

Sexual hormones are steroids and can be divided into 3 categories: androgens, such as testosterone; estrogens, like estradiol and estrone; and progestins, namely progesterone. Importantly, testosterone could be converted into dihydrotestosterone (DHT) or estradiol, and these forms are normally the ones that act more prominently in the brain (21). Notably, steroids could directly influence the transcription of mitochondrial genes since it was found a receptor responsive to steroids in this organelle (22). This action is found to have a neuroprotective function (23).

Estrogens have been extensively studied regarding sexual differentiation of the brain. They play critical roles in gamma-aminobutyric acid (GABA)ergic neurons, hypothalamic development, neuron migration during development, and microglia reactivity (24–26). They act through estrogen receptors (ER), expressed mainly in the amygdala (AMY) (27). Nonetheless, other brain regions respond to these hormones, namely the hippocampus, the prefrontal cortex (PFC), the cerebellum, the ventral tegmental area (VTA), the substantia nigra pars compacta (SNc), the striatum, and thalamus (27). Despite, being present all over the mammalian brain, ER have different distributions depending on the time of development and sex (28). This receptor has two isoforms, Erα, more associated with the masculinization effect of estrogens, and Erβ more associated with the defeminization process (26). ER receptors are membrane-bound, and when activated initiate signal cascades involving protein kinases and phosphatases (29). Estradiol, also, promotes presynaptic glutamate release, through phosphoinositide 3-kinase (PIP3-k) activation, consequently affecting N-methyl D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (30,31).

Androgens, on the other hand, act through androgen receptors (AR) members of the steroid receptors (7). They can increase spine formation in hippocampal neurons, through signaling pathways, like mitogen-activated protein kinase/ extracellular signal-regulated kinase (MAPK/ERK), cyclic adenosine mono-phosphate/ protein kinase A (cAMP/PKA), and phosphatidylinositol-3-kinase (PIP3K)/ AKT/ mammalian target of rapamycin (mTOR), contributing also, for synaptic modulation, structural protein expression, and dendritic spine density (32). Ultimately androgens could regulate brain-derived neurotrophic factor (BDNF) levels (32). A factor important in neuronal survival and growth, as a neurotransmitter modulator, and participant in neuronal plasticity (33). Finally, progesterone activates progesterone receptors and is highly involved in brain

development as well. They could modulate GABAA receptors, and they activate a wide range of cascades, such as GPRCs, anus kinase/signal transducer and activator of transcription (JAK/STAT), MAPK, and Src pathways, thus intervening in transcriptional processes (34). Altogether, steroid hormones are crucial for brain development (27).

Nonetheless, many cells are influenced by steroids without expressing a steroid receptor. It seems that steroid action in a cell triggers an alteration in the releasable factors and the cell-to-cell communication that will ultimately change the microenvironment (11). As an example, despite no evidence of ER in astrocytes, the effect of steroid hormones in GABA and glutamate release indirectly influences their morphology (11). Additionally, these changes in a specific brain region will project different outcomes to other brain regions, initiating a domino reaction (11). These findings illuminate a novel yet logical concept: within the brain, cells are impacted not only by direct pathways but also by alterations in the surrounding microenvironment (11).

Despite all this evidence, the classic approach of sex hormones as the single players in sexual differentiation is outdated. New studies have shown this phenomenon is a more complex case (8). It is now hypothesized, that various sex-specific factors, including not only hormonal and genetic but also epigenetic, have a region-specific action in the brain and act heterogeneously on intracellular mechanisms and on cell-to-cell communication to perpetuate or eliminate sexual differences (27,35,36).

As we delve into specific brain regions and their sexual dimorphism, it becomes evident that these differences are intertwined throughout the entire brain (27). This convergence of structural disparities and behavioral correlations is underscored in regions like the PFC, AMY, hippocampus, and hypothalamus. (27)

Starting with the PFC, the response to stressors varies significantly. In male rats subjected to chronic stress, dendritic debranching and shrinkage occur, whereas females show no such changes (37). However, neurons projecting from the medial PFC to the AMY in females exhibit dendritic expansion, a process influenced by estrogen (27). Ovariectomized females display increased spine density, while the opposite is observed in the contralateral prefrontal projection to the AMY (37). A recent study demonstrated, also in the PFC, microglial changes between males and females mainly in adulthood (17). Being the male microglia more ramified than females in adolescence. Interestingly in

adulthood, there is a shift in the microglia morphology, with females having a more complex morphology. Also, females injected with testosterone after birth showed a phenotype more similar to control males than the control females (17). Remarkably, these morphological changes in adulthood demonstrate the lasting impact of early-life sex hormone manipulation on cellular morphology and brain plasticity in this region (17).

Moving to the AMY, a very important region for social behaviors (38), differences largely stem from the endocannabinoid system's influence on cell proliferation, neurogenesis, differentiation, and synaptogenesis (39–42). A study reported a female increase in cell proliferation on the medial AMY, mediated by the cannabinoid 2 receptor (CB2) (38). Interestingly, further studies demonstrated, that the phagocytic capacity of microglia in the medial AMY is increased in males. The proposed model links the perinatal surge of testosterone with elevated endocannabinoid (eCB) metabolites in the medial AMY (43). Consequently, an increase in eCB metabolites recruits more phagocytic microglia, that control the proliferation of astrocytes. Hence, the male medial AMY develops with fewer astrocytes, increasing excitability (44).

The AMY was found to be extremely important in modulating play behavior (44). This behavior in rodents is evaluated during the juvenile period and is easily observed in rats since they are very social animals (44). Within play-behavior pin, pounce, and box are shown to present sexual dimorphism (45). It is hypothesized that these changes are testosterone-dependent (38). Studies, where females were injected with testosterone directly on the AMY during the perinatal period, showed an increase in play behavior (38). Also, endocannabinoid agonist injection in females, during the perinatal period was shown to increase the play behavior to the levels of males. Showcasing the important role of this system on the sexual differentiation of the AMY and therefore in play behaviors (44). On the other hand, changes in microglia structure, mainly in their phagocytic appearance in the AMY strongly correlated with an increased play behavior (46).

In the hippocampus, males under stress conditions, present a retraction and loss of CA3 neurons. Acute stress reduces estrogen-dependent spine formation in females, whereas it enhances such formation in males (27). Injecting females with testosterone after birth approximates them from the males. Corticotropin-releasing factor receptor (CRF) might underlie these sex-specific hippocampal changes, given Gs protein and beta-arrestin 2 dimorphism, rendering females less responsive to acute stress but more

susceptible to chronic stress (27). Regarding memory, the changes observed in the hippocampus, under stress conditions, females show enhancement and males an impairment (27).

Lastly, the hypothalamus is an integrator of the sensory stimuli, controlling endocrine, autonomic, and somatic behavior, and is divided into 11 nuclei (47). Importantly, the BBB in this region is particularly more permeable, allowing easier transport of hormones and other molecules crucial for homeostasis (47). This region plays a role in nociception, limbic sensory integration, and processing of light signals through the retinohypothalamic tract. Integration in the hypothalamus yields proper homeostatic and behavioral responses (47). To highlight the findings regarding sexual dimorphism in this brain region, Table 1 elucidates the principal effects of each nucleus and Figure 4 gives an anatomical visualization of their location.

TABLE 1 NAME AND FUNCTION OF THE ELEVEN NUCLEI OF THE HYPOTHALAMUS.

Nuclei	*Function*
Paraventricular and supraoptic	Production of oxytocin (controls uterine contraction during labor and milk disposal in breastfeeding) and antidiuretic hormone (controls the translocation of aquaporin receptor to the membrane of the renal distal convoluted tubules) (48)
Preoptic, anterior, and posterior	Regulation of the body temperature. Dimorphic levels of ER on the preoptic nuclei modulate sexual and maternal behavior (47,49)
Suprachiasmatic	Controls hormone secretion and behavior during the day since it integrates light input. Role on the circadian rhythms (50)
Ventromedial	Regulates feeding behavior (51)
Lateral	Related to hunger perception and increases eating (51)
Dorsomedial	Controls rage behavior (51)
Arcuate	Releases hormones to the pituitary glands, that will consequently influence the production of hormones in the gonads, such as cortisol in stress, thyroid hormone in metabolism, and sex hormones in sexual regulation. (52)
Mamillary	Contribution to the limbic system. Important in memory formation, and control of exploratory behavior. (53)

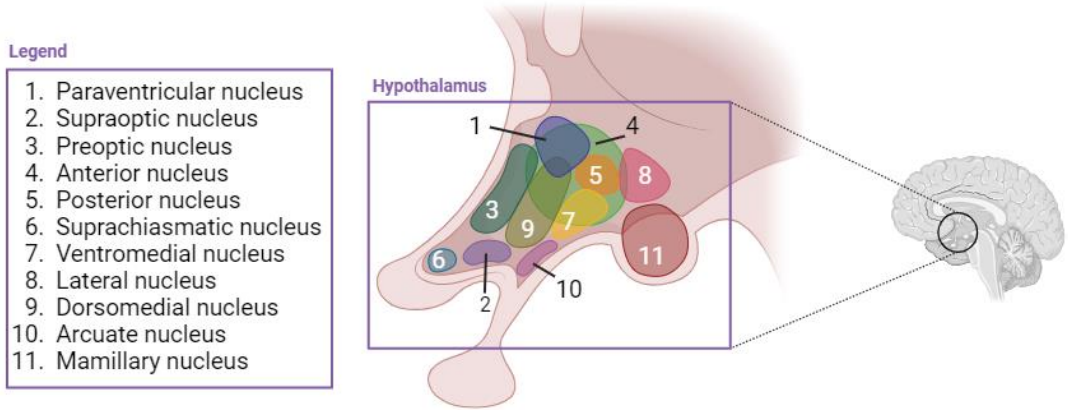


FIGURE 4 THE LOCATION OF THE HYPOTHALAMUS IN THE BRAIN AND THE APPROXIMATE LOCATION OF ALL THE NUCLEI OF THE HYPOTHALAMUS

Original image created with BioRender.

The preoptic area (POA), extensively studied and recognized as the most prominent dimorphic nucleus of the hypothalamus (14), is characterized by varying levels of steroid receptors. This region holds a critical role in regulating parental care, mating behaviors, aggression, as well as body temperature, and sleep-wake cycles (54–61). Also, POA receives input and sends outputs to other nuclei of the hypothalamus, AMY, bed nucleus of the *stria terminalis* (BNST), septum, and brainstem (62). Hence, this nucleus is very complex in its composition and function. Notably, the POA stands out as the brain's most densely populated region with AR and ER (14).

Studies have shown that the key players in sexual differences in POA are mast cells and microglia (63). This is particularly intriguing due to the infrequent presence of mast cells in the brain, except in the crucial period of sexual dimorphism (63). During this window, mainly in males, mast cells populate this region and are constantly realizing histamine, upon degranulation (64). In response microglia, since are very sensitive to histamine, are attracted to this region, beginning the high production of prostaglandins, which will ultimately activate signaling cascades and promote the formation and stabilization of excitatory dendritic spines (64,65). This phenomenon is highly correlated with male-typical sexual behavior, for example (66). Also, the increase in glutamatergic synapses and therefore, the increase in excitation, enhances mating, by increasing the responsiveness to the cues of receptive females. This important role of microglia is more evident when its temporary depletion inhibits completely male sexual behavior but maintains female sexual behavior (67).

Evidence of a sexually dimorphic brain is also found in neuroanatomic studies, both in rodents and humans (33). Already in development, in humans, boys brains are 8% larger than those of girls (68). In adult humans, studies have demonstrated an overall 11% larger brain size in men compared to women (69). Correspondingly, parallel observations regarding brain volume have emerged from studies on rodents (68). Notably, women exhibit greater grey matter volume (GMV) in areas like the superior parietal, dorsolateral prefrontal, and anterior cingulate cortices, while men showcase larger GMV in the occipital cortex, fusiform, parahippocampal gyrus, AMY, and putamen (68). Histological examinations of mice confirm these trends: males exhibit an enlarged POA of the hypothalamus, BNST, and medial nucleus of the AMY, whereas females possess a larger anteroventral periventricular nucleus (AVPV) of the hypothalamus (11,68)

These differences in size could be explained by the molecular effect sex hormones have in these regions. Firstly, in POA, during the perinatal period of hormonal sensitivity, male neurons survive more in the environment opposing the increased cellular death observed in females (70,71). Secondly, in AVPV, estradiol activates mechanisms of cellular death in males, a process directly modulated by ER (72). Ultimately, these alterations might be attributed to an augmented cell count within the specific cell phenotype associated with each brain region. For instance, the more significant number of vasopressin neurons in the BNST in males contributes to an expanded region size (73). Nevertheless, it is crucial to acknowledge that these alterations merely establish a comparative correlation. Females, still have, although smaller than males, a POA. Also, the increased number of vasopressin neurons in males does not exclude females from having them. Indicating that despite existing structural differences, both sexes likely share the same neural networks (11).

Finally other behavioral differences between sexes are being constantly reported in health and disease. Taken together, despite the significant advances in recent years regarding sexual differentiation and sexual differences in the brain, there still exists a long road to take. Little is still known about the exact mechanism by which sex hormones exert morphological changes, how they are maintained throughout life, and how these cellular changes impact behavioral alterations. Thus, establishing correlations between these sex-based structural alterations and behavior proves challenging (68).

Amid these discoveries, a crucial element remains conspicuously absent- a connecting factor capable of bridging the genome-hormonal gap, explaining cellular influences, and elucidating microenvironmental manipulations that impact structural and behavioral outcomes. As we advance in understanding sexual differentiation's intricacies, this missing link gains importance, hinting at a more comprehensive perspective awaiting exploration.

1.2. Kisspeptin a possible mediator of sexual differentiation

At the end of the previous century, a new gene was discovered to have cancer-suppressing properties, mainly in melanoma (74). This peptide was discovered in Hershey, Pennsylvania, and was named after a famous local chocolate, Hershey Kisses (74). In 2003, two studies underscored the significance of the Kiss1 receptor (KISS1R) in the process of pubertal maturation. Moreover, investigations involving mice with KISS1R mutations revealed their inability to undergo regular pubertal maturation, while in humans, mutations in this receptor led to precocious puberty (75,76).

The *Kiss1* gene localized in chromosome 1 (1q32), encodes for a protein with 145 amino acids (77), subsequently cleaved into a 54-amino-acid segment. These segments, along with further cleavages produce 10, 13, or 14 amino acid peptides, forming the Kisspeptin (KP) family (Figure 5) (78). Interestingly, while the larger peptide shows variability across species, the smaller peptides remain highly conserved. Additionally, the binding site on the C-terminal is consistently preserved and always activates KISS1R (79).

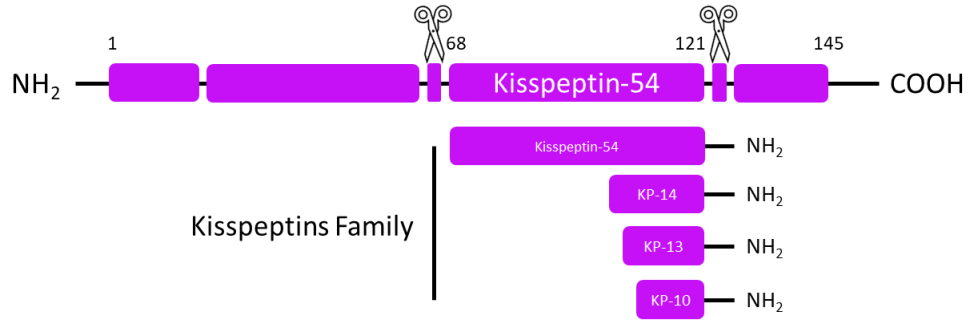


FIGURE 5 KISSPEPTIN FAMILY

Kisspeptins are encoded from the same gene, the peptides are then formed by cleavage in different sites, forming peptides of 54, 14, 13, and 10 amino acids. Original image created with BioRender.

Importantly, the literature concerning this new peptide family and receptor is ambiguous, due to the multiple nomenclatures. For the receptor alone, various terms are employed, including AXOR12, hOT7T175, GPR54, KISS1R, KiSS1, and the metastin receptor (79). The complexity escalates when addressing mRNA and protein nomenclature, with differences notable across species (79). The more consensual terms (79), and the ones used in this thesis will be, as mentioned before KISS1R for the receptor, and KP for the peptides in all the species mentioned.

KP molecular signaling pathway follows a typical receptor coupled to a G-protein (GPCRs) (80). Briefly, after binding, G-protein subunit α activates phospholipase C (PLC), which will break the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into the secondary messenger's inositol triphosphate (IP_3) and diacylglycerol (DAG), consequently calcium levels increase inside the cell and protein kinase C (PKC) is activated. These events will stimulate transient receptor potential canonical (TRPC)-like channels and inhibit inwardly rectifying potassium channels (80). Other pathways include activation of ERK 1/2, p38, and Rho, important transcriptional factors (Figure 6).

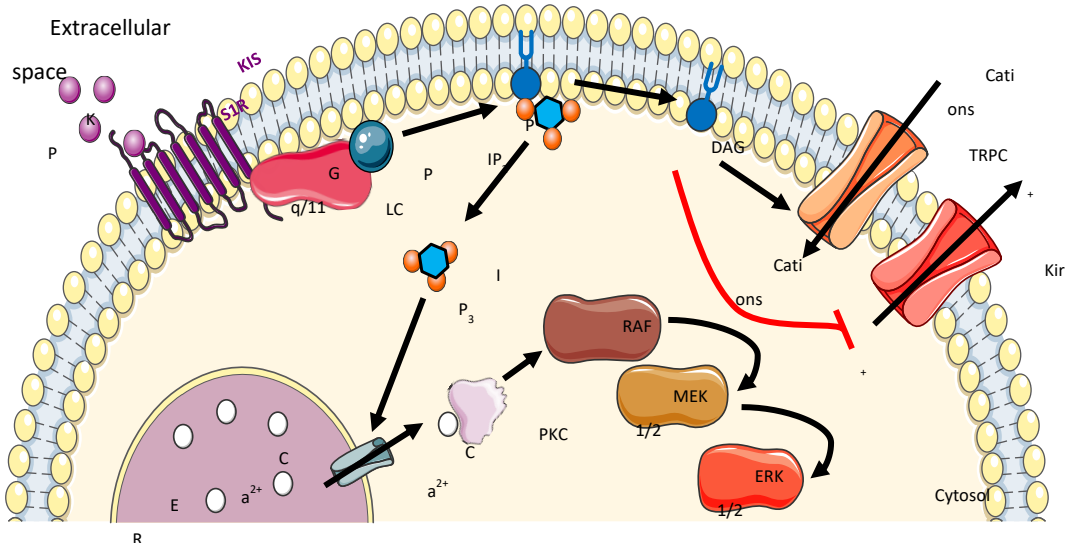


FIGURE 6 THE MAIN SIGNALING PATHWAY OF KISSPEPTIN

The receptor is coupled to a g-protein that activates PLC. PLC will brake PIP_2 in IP_3 and DAG. DAG will open the TRPC channels promoting the entry of cations and close the channel Kir inhibiting the outflux of potassium. IP3 will open a calcium channel of the endoplasmic reticulum promoting the increase of calcium in cytosol. Calcium, in turn, will activate PKC, which will consequently activate RAF, then it activates MEK ½, and finally, ERK 1/2 is activated. Erk enters the nuclei and promotes the transcription of specific genes (not illustrated). G-protein q/11 (Gq/11), Phospholipase C (PLC), phosphatidylinositol 4,5-biphosphate (PIP_2), inositol phosphate (IP_3), diacylglycerol (DAG), transient receptor potential canonic-like channel (TRPC), inwardly rectifying potassium channel (Kir), potassium ions (k^+), endoplasmatic reticulum (ER), calcium ions (ca^{2+}) and protein kinase c (PKC). Original image created with smart.servier.com.

KISS1R could be also found outside the brain, namely in the liver, pancreas, and adipose tissues mediating metabolism, namely, modulating energy expenditure, reducing food intake, regulating fat mass, and increasing glucose-stimulated insulin secretion (81,82). Also, in humans and rodents uterus, the receptor is important for embryo implantation, and in the placenta, which contributes to fetal growth among other functions (83,84). At last, other main KP locations are in the testes (85) and ovaries (86), by improving male gametogenesis and sperm motility and promoting oocyte survival and maturation, respectively. Additionally, in the brain KISS1R is present in the AMY,

nucleus acumbens, hippocampus, cingulate gyrus, cerebral cortex, thalamus, pons-medulla, and cerebellum in humans (87). In rodents KISS1R is widespread in the CNS, mainly in the hypothalamus, the dentate gyrus of the hippocampus, pons, midbrain, thalamus, hippocampus, AMY, cortex, PFC, and striatum (88,89). Also, KP was found in the infundibular stalk and the lamina terminalis in humans. In rodents in the AVPV, arcuate nucleus (ARC), periventral, ventromedial, paraventricular, and dorsomedial nucleus of the hypothalamus, BNST, and AMY. This widespread location of the KP-KISS1R signaling is crucial in multiple brain functions (89).

The most studied function of KP-KISS1R signaling is in the hypothalamus and the hypothalamic-pituitary-gonadal axis (HPG). Gonadotropin-releasing hormone (GnRH) neurons have KiSS1R and receive direct input from KP neurons in the hypothalamus (90). Consequently, hypothalamic GnRH regulates the release of gonadotropins (GPNs), such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the anterior pituitary gland. GNPs are released into the bloodstream reaching ovaries or testes controlling gonad steroid hormone levels. Finally, and closing the feedback cycle KP neurons have ER and AR that sense these molecules (Figure 7) (91). The KP neurons in different hypothalamic nuclei have different outcomes (92). Indeed, KP neurons in AVPV/PeN promote increased levels of GnRH in preovulation, and GNPs increase in response to high levels of estrogen (92).

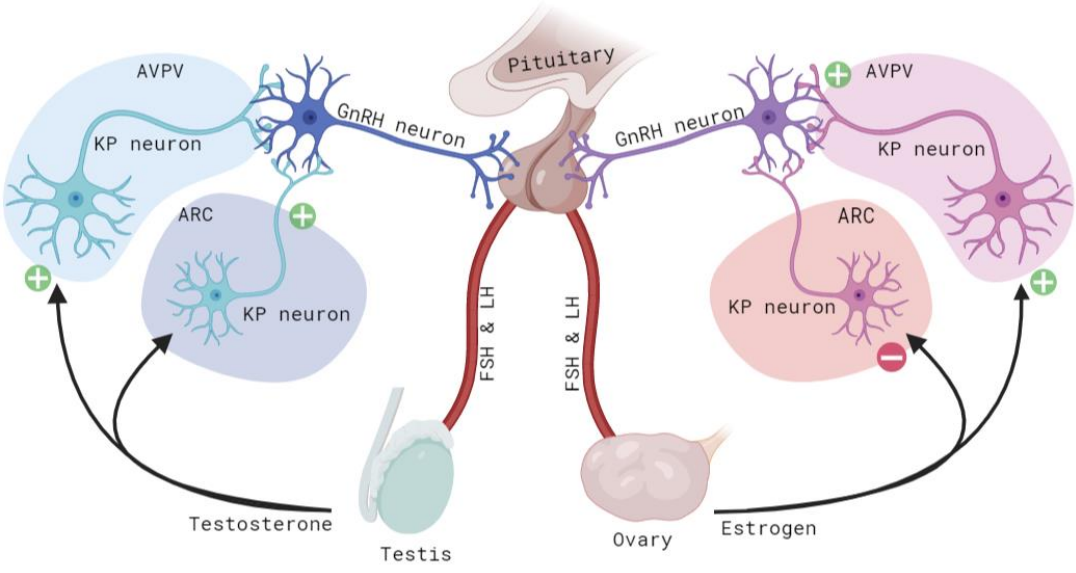


FIGURE 7 FEEDBACK MECHANISMS OF THE HPG AXIS AND THE DIMORPHISM PRESENT.
KP neurons release KP that is sensed by the GnRH neurons triggering the release of GnRH that will stimulate the pituitary glands, promoting the release of LH and FSH to the bloodstream. These hormones stimulate the gonads to release their sexual hormones. On one hand, testosterone positively inputs the KP neurons in the AVPV and the ARC. On the other hand, estrogen positively inputs KP neurons in the AVPV but negatively inputs ARC KP neurons. Original image created with BioRender.

Importantly, the GABA B receptor can also regulate KP expression. Studies demonstrated that the administration of a selective antagonist of GABA B reduces FSH levels, delays the onset of puberty in males, and decreases the number of ovulatory follicles (92). Many studies report epigenetic mechanisms regulating KP expression (92). Importantly, it is still very unclear the role of KP neurons in extra hypothalamic functions.

The convergence of several factors underscores the significant role of this peptide in the sexual differentiation of the brain. The prominent presence of both KP and KISS1R in dimorphic brain regions, coupled with the existence of sexual hormonal receptors on these neurons, positions this peptide as a compelling contender for mediating the intricate process of sexual brain differentiation. Furthermore, the correlation between KP levels and hormonal fluctuations during crucial periods for sexual differentiation, such as the neonatal testosterone surge (NTS) in males, further solidifies its role in shaping these developmental pathways (93).

NTS has a vital role in the initiation of the brain's sexual dimorphism that consequently mediates male reproductive physiology and function and the development of reproductive organs (94,95). This peak occurs within hours after birth as described in most mammals (96), however, this surge in non-human primates and humans could extend to weeks (96–98) and agrees with an increase in LH, GnRH, and KP levels (Figure 8) (91). Interestingly, this surge has no equivalent in females (99). Knockouts (KO) of the GnRH receptor and depletion of KISS1R in GnRH neurons lead to a demasculinization of rodent male brains (100,101)

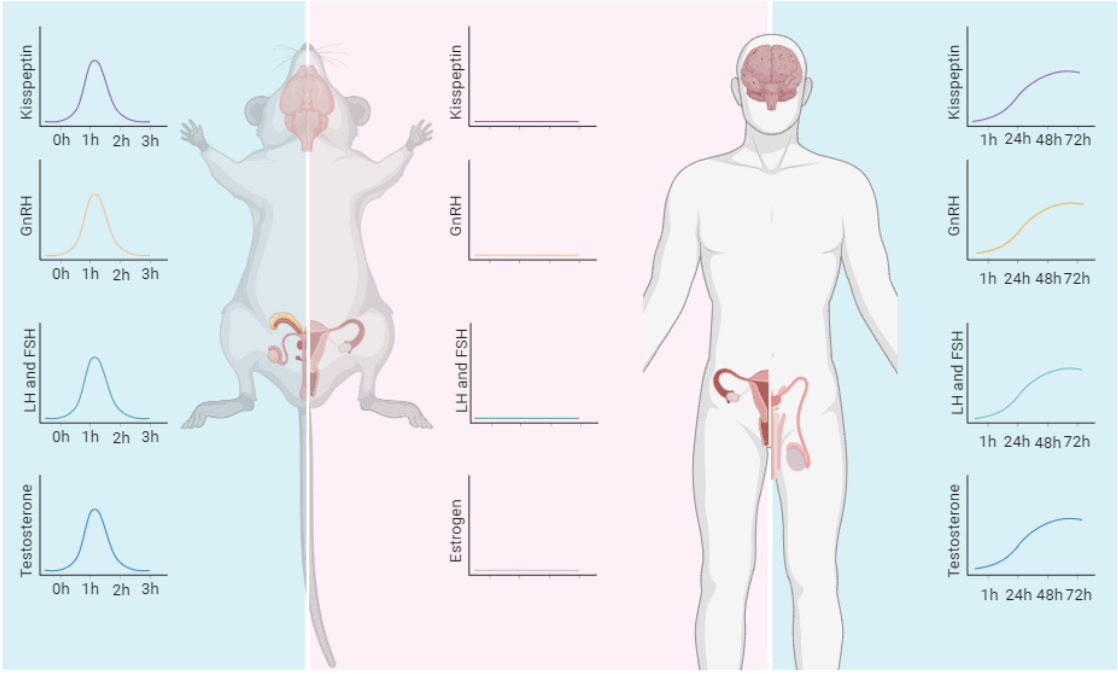


FIGURE 8 REPRESENTATIVE IMAGE OF THE NEONATAL TESTOSTERONE SURGE (NTS) AND THE FEEDBACK MECHANISMS UNDERLYING IT.

In male rodents, this NTS occurs within the first 2 hours after birth. While in humans it could take weeks. In females and women, no such mechanism is documented. Original image created with BioRender.

Importantly, this NTS in rodents is proven to be responsible for physiological and behavioral dimorphisms (94). In humans, this correlation is not so direct. The majority of the sexual differentiation of the brain occurs in the prenatal period, the effect of postnatal testosterone surge is still understudied (102). Recent studies correlated this surge in humans with gender-linked social development (103).

During postnatal development, KP is expressed differently in males and females (104). Females express a slightly higher number of KP neurons in the ARC than males (105). However, this dimorphism disappears during puberty and adulthood(106). In the AVPV/PeN, the expression of KP increases significantly more in females than in males mainly during puberty and adulthood (91). An important regulator of the KISS1R-KP in the sexual dimorphism of the brain is the pancreas transcription factor 1a (Ptf1a) (107). In conditional KO of Ptf1a, there is a disruption of the KP neuron development similar to KISS1R-KP deficient mice (108).

The study of KP function, despite differential importance over the lifespan, is conducted in KO animals. These studies revealed an overall demasculinization of males,

and a masculinization of females, mainly in sexual behavior (109,110). A study reported anxiogenic properties of KP since KO rescue male animals spent twice as much time in the open arms of the elevated plus maze test compared with intact control (111).

To sum up, many studies have shown that KP is crucial for sex differentiation throughout development. In addition, KP has a time-dependent function, being more expressed and more relevant during the neonatal period. Still, there is a big gap in knowledge on the overall effects of KP on the dimorphism of the brain. How KP mediates behavioral alteration and a full characterization of the real effects of this peptide throughout life is needed. More importantly, lacks literature concerning the effects of the neonatal KP surge, which carries the big translational potential to explain the correlation found in humans concerning gendered behavior.

2. OBJECTIVES OF THE MASTER THESIS

OBJECTIVES OF THE MASTER THESIS

The scope of this thesis is to shed light on the role of KP in the sexual differentiation of the brain. It is important to understand if the time-specific blockade of KP signaling during neonatal testosterone surge is sufficient to determine male/female-like behavior throughout life.

The primary anticipated outcome revolves around discerning behavioral disparities between control animals and those in which the KP signaling pathway has been blocked. Additionally, we seek to gain a deeper understanding of several key aspects:

- The interplay between KP and sex-related factors
- Sexual variations in microglia morphology, particularly in adulthood, as previously reported, and offer initial insights into the impact of KP on microglia

3. Material and Methods

3.1. Animals

For this study, nine Wistar Rats (six females and three males) were initially purchased at Charles River Laboratories (Germany). Upon arrival animals were kept at standard conditions at the animal facility of Institute of Nuclear Sciences Applied to Health (ICNAS), University of Coimbra: 2-3 animals per polycarbonate cages (395 x 346 x 213 cm) (GR900 for Rats, TechniPlast, Milan, Italy), at 21 ± 2 °C, humidity controlled and 12:12-h light/dark cycles with *ad libitum* water and food, enriched with nesting material and cardboard tubes. The cage was changed once a week or whenever it was justifiable to do so.

After acclimatization, the breeding process was initiated. Animals were tattooed with a small ink injection in their paws and were mated in trios (two females and one male), males were allowed to spend ten to fourteen days with the females. When they showed signs of pregnancy (rounded belly and weight increase) males were removed and two to three days before the expected delivery day females were separated. Females were allowed to deliver naturally, with the day of birth designated postnatal day 0 (PND0). Between the first 24 hours of life, pups underwent intracerebroventricular (ICV) injections (the procedure will be detailed later on), were tattooed in the paws and were returned to their mothers.

Six experimental groups were established based on the sex and procedure: males injected with vehicle or with Kp234, females injected with vehicle or Kp234, and a male and female control group that did not undergo any injection. Overall, eighty-nine animals were born, from eleven litters (ranging from 4-12 pups). Group formation was blind, and the litters were equally divided between the groups, to reduce bias. All pups were housed together with the mother until PND21. After, each litter was segregated by sex and treatment. All procedures and personnel were authorized and complied with the European Union Council Directive (2010/63/EU), National Regulations, DGAV, and ORBEA board of ICNAS (5/2021).

3.2. Intracerebroventricular Injection

In the first 24 hours after birth, animals underwent ICV injection. One by one, animals were placed on ice with an aluminum foil between to avoid direct contact with ice (Figure 9A). Animals were placed on ice for around 15-30min, until their paw reflex disappeared, and their coloration became blueish. While the pups were being crio-anesthetized a 10μL syringe (1700 series, Hamilton, USA) with a 32G needle (17762-05, Hamilton, USA) was loaded with either Kp234 (Tocris Bioscience, USA) solution (10nmol in 0.9% NaCl solution) or vehicle (0.9% NaCl) and was placed in the stereotaxic arm (Figure 9B). Anesthetized animals were placed with the head between the ear bars (reversed), parallel to the x-axis, and the bregma-lambda line was placed parallel to the y-axis (Figure 9C). The head was sterilized with cotton embedded in 70% ethanol. The syringe was placed above lambda and X and Y coordinates were put on zero (Figure 9D), the arm was first moved (0.90,4.20) mm. The needle was lowered until entering the skull and the z-axis was put on zero. The needle was lowered until z=1.90mm and then retracted until 1.70mm. At this point the syringe was inside of one of the lateral ventricles (coordinates were previously tested on 3 pups injected with ink, the brain was removed and sliced to test if the lateral ventricles were hit see Supplementary Data- Annex I). 1μL of the solution was slowly injected and the needle was kept in place for around one minute, after that, the syringe was retracted and the same procedure was performed but to the other lateral ventricle (x coordinate was -0,90 mm). After the two injections, and while the animals were still anesthetized, they were tattooed. Animals returned to the warming pad until fully recovered. After all the animals were injected they were returned to their mothers and were observed until guaranteed proper mother nursing. In all litters, vehicle was injected first and only then Kp234 solution. During this procedure, all the mothers nursed their pups and there was no mortality registered. After one hour of injection (this time point was chosen based on pharmacokinetics studies of Kp234 (112)), five males and six females injected with vehicle and six males and five females injected with Kp234 solution were sacrificed by decapitation, tail cut, blood collected, and their whole brains removed.

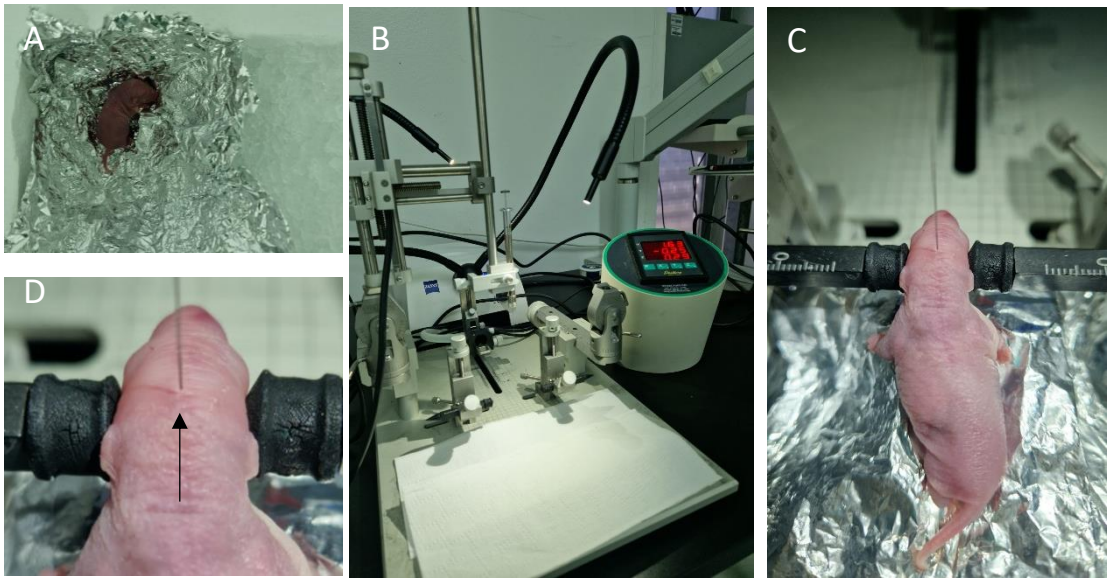


FIGURE 9 INTRACEROBROVENTRICULAR INJECTION

A- Crio-anesthesia, the animal is placed in contact with ice until loss of reflection. **B-** Stereotaxic apparatus, digital coordinates equipment installed and lights in position to start ICV procedure. **C-** Animal placed in the stereotaxic apparatus ready to be injected. **D-** Bregma location, the black arrow points to the exact location.

3.3. Genotyping

Tail (5mm maximal length) collected upon sacrifice underwent digestion using a lysis solution [containing 10mM Tris, 5mM EDTA, 200mM NaCl, 0.3% sodium dodecyl sulfate (SDS), and 2.5% proteinase K (Invitrogen, Waltham, MA, USA)] for 4 hours at 55ºC. Subsequently, the resultant solution underwent centrifugation (1730R, Gyrozen, Gimpo, South Korea) at 12000 rpm for 15 minutes at 4ºC. The supernatant was collected, and DNA was precipitated through two sequential centrifugations at 4000 rpm for 15 minutes at 4ºC, using 100% ethanol and 75% ethanol (Sigma-Aldrich, St. Louis, MO, USA). The resulting pellet was air-dried overnight, then reconstituted in TE buffer (Tris 10mM, EDTA 1mM, pH 8.0). The DNA strands were uncoiled with a 10-minute heating at 70ºC. DNA quantification was performed using Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA), and the polymerase chain reaction (PCR) mix was prepared with the following components: 50% Taq Master Mix (Bioron Diagnostics GmbH, Römerberg, Germany), 10% SRY primers (Forward primer: AAGCCTTACAGAAGCCGAAAAA; Reverse primer: TGTGGCACTTTAACCCTTCGA (113)) (Metabion, Planegg, Germany), PCR Water

(Bioron Diagnostics GmbH, Römerberg, Germany) to complete the volume, and DNA (200ng/uL). PCR amplification was carried out (T100 Thermal Cycler, Bio-Rad Laboratories, Hercules, CA, USA) with the following temperature profile: 2 minutes at 72ºC; 30 seconds at 94ºC, 30 seconds at 58ºC, 20 seconds at 72ºC for 35 cycles; followed by 10 minutes at 72ºC; and an infinite hold at 4ºC. Each PCR product was mixed with 6x loading buffer (Thermo Fisher Scientific, Waltham, MA, USA) and loaded onto a 2% agarose gel (NZYTech, Lisboa, Portugal) in TAE buffer (Tris 40mM, EDTA 5mM, acetic acid 5.71%, pH 8.0) containing GreenSafe Premium (NZYTech, Lisboa, Portugal) for band visualization. Electrophoresis was conducted in TAE buffer at 90V for 40 minutes (PowerPac, Bio-Rad Laboratories, Hercules, CA, USA). The resulting outcomes were observed under UV light. An example of the gel can be seen in Supplementary Data-Annex II.

3.4. ELISA

Blood samples were collected in EDTA-coated tubes (BD Vacutainer, Franklin Lakes, NJ, USA) and centrifuged at 1600 x g, for 15 min, at 4ºC, right after sacrificed, and plasma was collected and stored at -80ºC until being used. The whole brain was stored at -80ºC until analysis.

3.4.1. Testosterone ELISA

Plasma samples were used on this ELISA kit and the manufacturer protocol was followed (ELISA Kit for Testosterone, Cloud-clone Corp., Houston, USA). The samples were diluted in miliQ water until the minimum volume necessary was reached. In summary, the samples and testosterone standards were added to a plate coated with testosterone antibody and exposed to the biotin-labeled antigen (detection reagent A), undergoing a 2-hour incubation at 37ºC. Following this, the wells were washed three times with wash buffer and subsequently treated with Horseradish Peroxidase (HRP)conjugated by avidin (detection reagent B), going through 2 hours of incubation also at 37ºC. After being washed 3 times substrate solution TMB was added, and the plate was incubated 30min. The addition of the stop solution was followed by a reading of the plate at 450 nm. As the concentration of the tested sample increases, there is a greater inhibition of the binding between labeled antigen and antibody, resulting in reduced color intensity. This color intensity is directly related to the enzyme quantity but inversely proportional to the concentration of the target molecule in the tested sample. The results

were adjusted based on the mean absorbance of blank wells, and the testosterone concentration was determined by extrapolating the log of the standard concentration with the Abs. Finally, the concentrations were corrected for the dilution of the sample.

3.4.2. GnRH ELISA

Brain samples were digested in lysis solution [1 pill of Mini Protease Inhibitor Cocktail (Roche, Basel, Switzerland) for each 10mL of phosphate-buffered saline (PBS) [137 mM NaCl; 2.7 mM KCl; 1.8 mM KH2PO4; 10 mM Na2HPO4·2H2O; pH 7.4], mechanically macerated and sonicated. The protocol of this GnRH Elisa Kit (Cloud-clone Corp., Houston, USA) was the same as the testosterone ELISA Kit previously detailed.

3.5. Behavioral tests

Behavioral tests were performed during 3 stages of life, infancy (between PND4 and PND14), juvenile (PND24 until PND30), and adulthood (after PND60). Figure 10 represents all the behavioral tests performed in this thesis. After each test animals were weighed, measured in total length and anogenital distance.

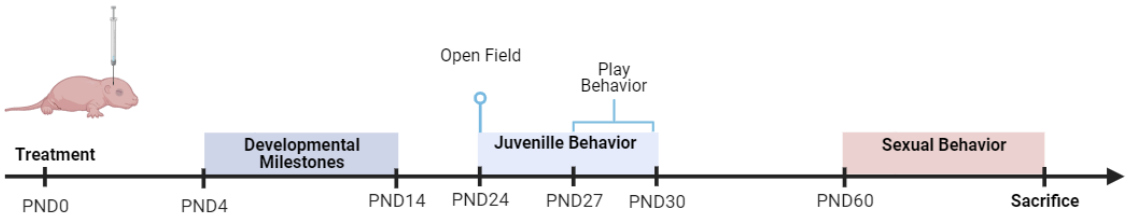


FIGURE 10 TIMELINE OF THE BATTERY OF BEHAVIORAL TESTS

Original image created with BioRender.

3.5.1. Developmental milestones tests

Developmental milestones were conducted at PND4, 6, 8, 10, 11, 12 and 14. Always between the afternoon period (12 am and 6 pm), in a quiet room, under dim light with temperature, and humidity controlled, and a 30 second interval was taken between each test.

3.5.2. Surface righting reflex

Each pup was placed on its back on a flat surface, and the time to return to its 4 paws was recorded. The cutoff time was 30 seconds (Figure 11A) (67,114).

3.5.3. Negative geotaxis reflex

Pups were placed face down on a ramp with a 30-degree inclination, covered with a metal grid to enable traction. The time the pup took to turn 180 degrees was scored with a cutoff time of 30 seconds and pups had 3 trials. If the animal didn't complete the test the sum of the 3 trials was considered (Figure 11B) (67,114).

3.5.4. Locomotion

A flat surface had a circle with a 13 cm diameter. Pups were placed in the center and the time the 4 paws were out of the circle was recorded until a maximum time of 30 seconds (Figure 11C) (67,114).

3.5.5. Cliff aversion

Animals were placed on an elevated surface with their front paws and snouts hanging over the edge. The time the animals took to retract completely from the edge was recorded, with a cutoff time of 10 seconds. If the animal did not move or fell they were given 2 more trials (Figure 11D) (67,114).

3.5.6. Wire Suspension

The pup's forepaws were placed against a horizontal wire rod suspended above a padded recipient. The time each pup hung in the wire was recorded for a maximum of 10 seconds, if the animals unsuccessfully hung the wire 2 more trials were performed (Figure 11E) (67,114). If they unsuccessfully hung the wire the sum of the 3 trials was considered.

3.5.7. Nest seeking

A rectangular recipient (25cm x 10cm) was sectioned into three divisions by delineating a target line on each of its sides, placed 6.5 cm away from the central area. On one of the divisions, used home bedding material was placed, while a similar quantity of fresh bedding material was positioned in the opposite division. The pups were placed in the central part of the recipient, positioned at a 90° angle with the bedding sections, and allowed to explore the enclosure. The experiment consisted of two trials, each lasting 120 seconds, with a 30-second break between them. To counteract any potential preferences for head orientation, during each trial, the animal's initial facing direction was adjusted. The recorded metrics were the first orientation of the body (1 point if it was towards the home bedding and -1 if it was facing the fresh bedding), the final bedding choice (1 point if it was the home bedding and -1 if it was the fresh bedding) and the time taken for the animal to cross the designated goal line on the bedding material using both four paws, with a maximum duration of 120 seconds for each trial. If the animal crossed the goal line on the fresh bedding material, the 120-second limit was considered. The final score was calculated by the sum of the scores from both trials (Figure 11F) (67,114).

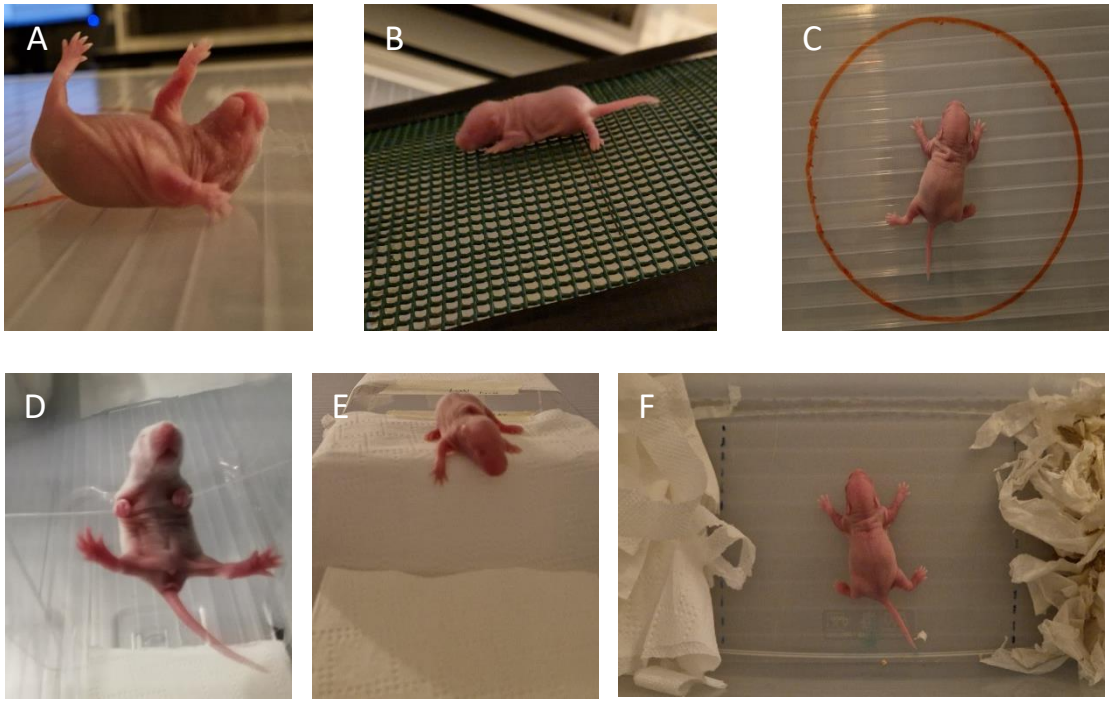


FIGURE 11 REPRESENTATIVE IMAGES OF THE DEVELOPMENTAL MILESTONES
A- Rightening reflex. **B-** Negative geotaxis reflex. **C-** Locomotion **D-** Wire suspension **E-** Cliff aversion **F-** Nest seeking.

3.6. Pup isolation-induced USVs

An anechoic chamber measuring 55 cm x 50 cm x 70 cm (H x D x W) was constructed by assembling acrylic sheets 1.5 cm thick. The interior of the chamber was entirely covered with sound-absorbing foam to eliminate external noises (Figures 12A and B). Recordings of pup USVs were conducted on Postnatal Days 4, 6, and 8, after the developmental milestones, all within the light period (12:00 AM - 6:00 PM). To ensure the pup's comfort and maintenance of body temperature, the pup was positioned within a plastic container (15 cm x 10 cm x 8 cm) cushioned with tissue paper. This container was then placed inside the anechoic chamber. A recording lasting 5 minutes commenced immediately after the chamber's door was closed. USVs were captured using an ultrasound recording system that incorporated an Avisoft CM16/CMPA condenser microphone, positioned 28 cm above the base of the test container. This setup included an UltrasoundGate 416H amplifier and Avisoft Recorder software (Avisoft Bioacoustics, Glienicke/Nordbahn, Germany).

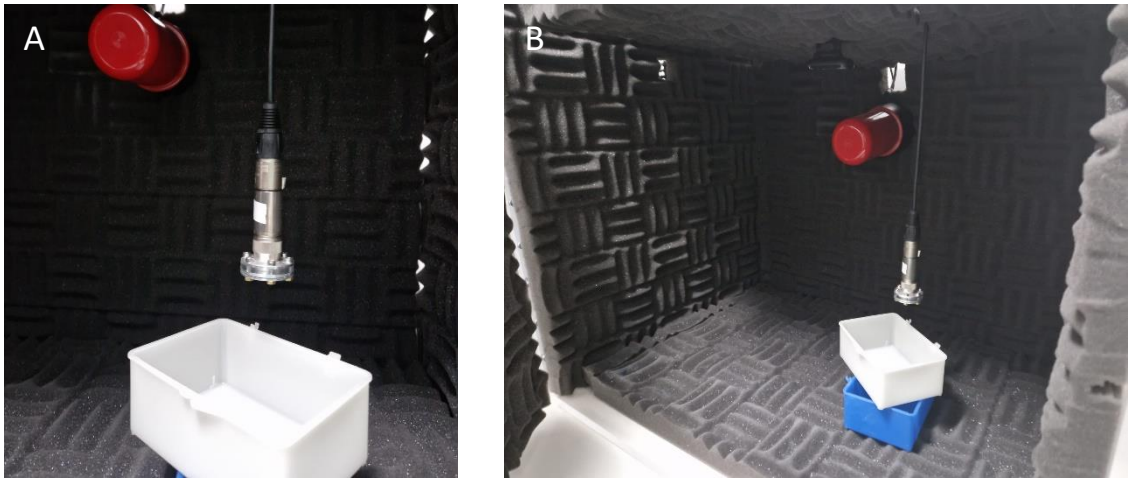


FIGURE 12 MILESTONES SETUP FOR THE ISOLATION INDUCED USVS RECORDED AT PND4, 6 AND 8. **A-** The anechoic chamber was completely covered with sound-absorbing foam to prevent external noise. **B-** An ultrasonic condenser microphone was positioned over the test container.

After the recording of USVs, spectrograms were produced using an FFT length of 512 points, a 16-bit format, a sampling frequency of 250 kHz, a time resolution of 1 ms, a frequency resolution of 488 Hz, and an overlap of 50%. The USVs were further assessed utilizing the MATLAB toolbox DeepSqueak version 3.1.0. This facilitated the extraction of individual rat USV fragments through the application of the Rat Call_Detector_Neural network. The analysis utilized a chunk length of 6 seconds, an overlap of 0.1 seconds, a high-frequency cut-off set at 125 kHz, and no score threshold. Key parameters such as

call length, principal frequency (median), lowest frequency, highest frequency, delta frequency, peak frequency (frequency at the highest power), tonality (1 minus the geometric mean of the spectrum divided by its arithmetic mean), sinuosity (length of the initial and last point of the call divided by the Euclidean distance between these 2 points), and power of each USV were subsequently extracted. Also, calls were classified by the number of fragments, singles with one fragment, doubles with two fragments, triples with three, and multiples with more than three fragments. The metrics for the calls were then manually calculated in Excel.

3.7. Juvenile behavior tests

All juvenile behavior tests were conducted within the light period (12:00 AM – 6:00 PM) in a quiet room where animals were placed at least 30 minutes before the test for habituation. These tests were recorded for later analysis using a video camera (LifeCam HD-3000, Microsoft, Redmond, WA, USA) positioned on the room's ceiling and a telephone to have a lateral perspective. Simultaneously, USVs were recorded during these behavioral tests. For this purpose, a 60 cm x 100 cm x 60 cm (H x D x W) box was constructed and lined with absorbing foam on the bottom and walls to prevent external noise interference. an open polycarbonate arena (50x50 cm, 40 cm high) was placed inside this foam-lined box (Figure 13). An ultrasound recording system consisting of an Avisoft CM16/CMPA condenser microphone located 20 cm above the bottom of the foam-lined box, an UltrasoundGate 416H amplifier, and Avisoft Recorder software (Avisoft Bioacoustics, Glienicke/Nordbahn, Germany) was employed to capture the USVs.

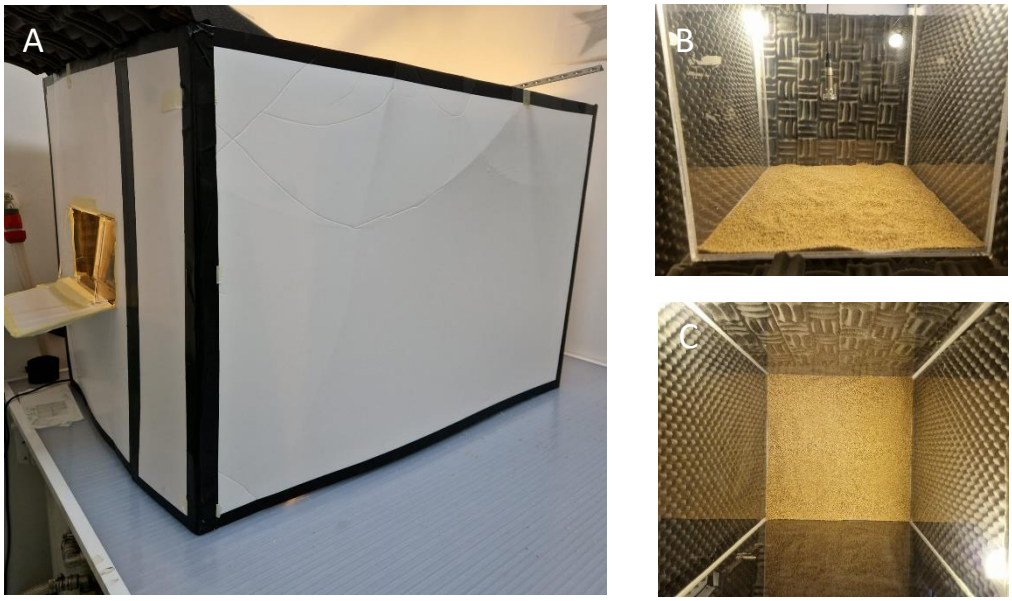


FIGURE 13 TEST SETUP FOR THE JUVENILE TESTS.
A- Anechoic box with side window for telephone positioning **B-** side-view from the telephone window **C-** ceiling perspective of the arena.

3.7.1. Open Field Test

At PND24, rats were placed in the open arena and let to explore freely for 10min (67). The floor had bedding to make the ambiance less stressful and allow proper recording, the light was set to the minimum possible (less than 50 lux), and the test was performed always between 12 am and 6 pm. The time spent in each zone, the number of zone crosses, resting time, mean speed, and total distance in each zone were automatically evaluated using Smart Video Tracking Software version 3.0.06 (Panlab, Barcelona, Spain). Also, lateral recordings were used to evaluate the number of poops, supported and unsupported rearing and grooming number, total time, and inter-interval mean time using Behavioral Observation Research Interactive Software (BORIS)-App v. 0.9 (115). USVs were recorded but for the scope of this thesis, they were not analyzed. Between each animal, only the poops were collected.

3.7.2. Social Juvenile test

Between PND24 and PND27, animals were isolated to enhance social behavior. The juvenile behavior was accessed on 4 consecutive days between PND27 and 30. Pairs of rats (sex- treatment- and age-matched) were placed in the open arena and let to explore freely for 12min (2 min of habituation and 10 min of effective test). The floor had bedding to make the ambiance less stressful and allow proper recording, a red light was set to the

minimum possible (less than 50 lux), and the test was performed always between 12 am and 6 pm. Video recordings were taken from the ceiling and the side to ensure proper behavior scoring. It was scored manually 3 types of behaviors:

- Box- scored when both animals rise on their hind legs and use their front paws to paw at each other. Both animals involved in boxing receive a score
- Pounce- scored when one animal launches itself towards the nape or back of the other. It typically involves vigorous movement, and the score is attributed to the animal performing the pounce.
- Pin- scored when a pouncing animal successfully flips the other onto its back, with all four limbs off the ground. The score for pinning is given to the animal that executed the pounce. Often, pinning occurs shortly after a pounce (45).

Figure 14 shows a representation of these particular behaviors. USVs were recorded in all 4 days but only PND29 was analyzed. Between each pair only the poops were collected. The bedding was replaced each day.

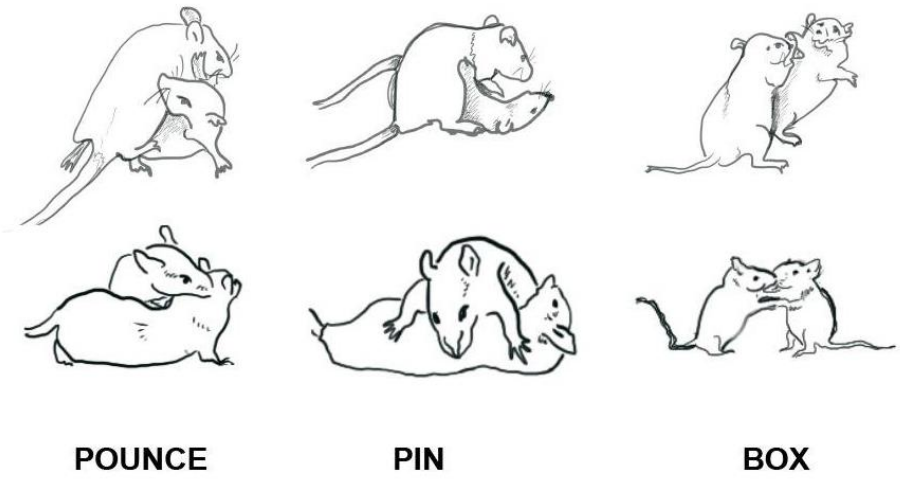


FIGURE 14 ILLUSTRATIONS EXEMPLIFYING RATS ENGAGING IN THE THREE MAIN PLAY BEHAVIORS– POUNCING, PINNING, AND BOXING.

Directly extracted from VanRyzin et al, Assessing Rough-and-tumble Play Behavior in Juvenile Rats (2020).

3.8. Sexual behavior test

Sexual behavior tests were conducted within the dark period, one hour after the beginning of the dark phase in a quiet room where animals were placed at least 2 hours before the test for habituation. These tests were recorded for later analysis using a video camera (LifeCam HD-3000, Microsoft, Redmond, WA, USA) positioned on the room's ceiling and a telephone to have a lateral perspective. In this test, the same apparatus shown in Figure 13 was used.

3.8.1. Male sexual behavior

Intact male rats were assessed for male copulatory behaviors in response to natural cycling and experienced female rats, whenever they were receptive (previously tested with a 2-minute interaction with an experienced male). Animals underwent a 20-minute behavioral test after PND60. The behaviors recorded included the count of mounts and intromission-like behaviors. Additionally, the latency to the first occurrence of each behavior was documented. When none of these behaviors was observed, the latency was assigned the duration of the test (1200 seconds). A mount was scored when the male mounted the female from behind, placing both front paws on the female's flanks and thrusting against her. Intromission-like behaviors were tallied when the male performed a mount but, during thrusts, swiftly shifted his weight, engaged in significant flexion of his haunches, and involuntarily disengaged (67).

3.8.2. Female sexual behavior

Female rats with their natural reproductive cycles intact were subjected to evaluations of female proceptive behaviors and lordosis in the presence of experienced males. These females were tested daily across their estrus cycle (lasting 4.5 to 5 days), with each test lasting for 20 minutes. The behaviors were assessed whenever the female presented receptiveness in the first 5 minutes of the test. Only 3 females injected with Kp234 were not receptive on 5 consecutive days. The behaviors examined included proceptive behaviors (hops, darts, and solicitations) as well as lordosis, which is a posture indicating sexual receptivity, in response to a male rat actively mounting them. In this context, hops were defined as brief jumps wherein the female landed on all four paws in a crouched stance. Darts were characterized as short sprints where the female suddenly halted in a crouched position. Solicitations were identified by the female facing the male and then promptly moving away before adopting a presenting posture.

3.9. Cellular and molecular analysis

All the procedures and molecular analyses therefore mentioned were conducted at the Coimbra Institute for Clinical and Biomedical Research (iCBR), at the Retinal Dysfunction & Neuroinflammation Group facilities. After sexual behavior animals were sacrificed by decapitation, and their weight, length, and anogenital distance were measured. Also, the brain and testicles were extracted and weighed. Blood samples were taken by cardiac puncture and collected in EDTA-coated tubes, which were then centrifuged at 1600 x g, for 15 min, at 4ºC, being plasma collected and stored at -80ºC. Brains were either dissected or fixed, depending on their purpose.

3.9.1. Immunohistochemistry for microglia morphology

Animals were deeply anesthetized using an intraperitoneal (IP) injection of ketamine (90 mg/kg; Nimatek) and xylazine (10 mg/kg; Ronpum 2%), after checking for paw and tail reflexes animals were transcardially perfused with around 200mL of PBS1x and after with 150mL of PFA 4% [4% paraformaldehyde in 0.01 M PBS, pH 7.4]. Animals were then decapitated, and their brains extracted. Brains remained in PFA 4% for 24 hours at 4ºC and were immersed in 20% sucrose in 0.01 M PBS until reached the bottom of the falcon. Brains were dried, wrapped in parafilm and aluminum foil, and stored at -80ºC until further use.

Afterward, the brains were cut into 50-µm coronal sections on a cryostat (LEICA CM3050 S, Germany) and slices were collected in cryoprotection solution (30% Sucrose; 30% Ethylene glycol; 10 mM phosphate buffer, pH 7.2). Slices containing the medial preoptic area (mPOA), as determined by the "The rat brain in stereotaxic coordinates" atlas (Paxinos, George, and Charles Watson, 2006), were carefully chosen. Following this, they were incubated with the primary antibody (rabbit anti-Iba-1, 1:1000, WAKO, Osaka, Japan) for a period of 48 hours at 4 °C. After thorough washing, the sections were treated with the secondary antibody (donkey anti-rabbit, 1:1000, Invitrogen, Waltham, MA, USA) for 2 hours at RT. Additionally, 4',6-diamidino-2-phenylindole (DAPI) was applied at a concentration of 1:5000 for 10 minutes at RT to label cell nuclei. Following this process, the sections were mounted onto the slides using a DAKO glycergel mounting medium. Utilizing a laser scanning confocal microscope LSM 710 META connected to

ZEN software (Zeiss, Oberkochen, Germany) with a × 63 objective, images of 10 randomly chosen microglial cells per animal were captured.

3.9.2. Western Blot of KISS1R

Rat brains were extracted, and hypothalamus was carefully dissected on ice and subsequently stored at -80ºC. The hypothalamus was homogenized using a sonicator in a radioimmunoprecipitation lysis buffer (RIPA) [150mM NaCl, 50nM tris-base, 5mM egtazic acid (EGTA), 1% triton X-100, 0.5% sodium deoxycholate (DOC), 0.1% sodium dodecyl sulfate (SDS), and a pH of 7.5]. This mixture was supplemented with protease inhibitors (Roche Diagnostics GmbH, Germany) and 0.1% dithiothreitol (DTT). The samples were then subjected to centrifugation at 13,000g for 15 minutes at 4ºC, and the resulting supernatant was collected.

To quantify the sample concentrations, a portion of each sample was subjected to a bicinchoninic acid (BCA) assay (Thermo Fisher) for 30 minutes at 37ºC. Subsequently, all samples were standardized to a concentration of 50µg of protein per well using sample buffer [0.5M Tris-HCl, 10.28% SDS, 30% glycerol, 600 mM DTT, 0.01% bromophenol blue, and a pH of 6.8] along with 5% SDS. The samples were denatured at 95ºC for 5 minutes and then loaded onto sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels, where proteins were separated by electrophoresis. The separated proteins were subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane (Merk Millipore, Ireland) using a Mini Trans-Blot Cell (BioRad) and PowerPac HC (BioRad). Afterward, the membranes were blocked using a solution of 5% skimmed milk (Nestlé Portugal) in tris-buffered saline containing 0.1% Tween 20 (TBST; pH = 7.6). The membranes were then incubated overnight at 4ºC with the primary antibody rabbit Anti-Kiss1 (GRPR54) (Alomone Labs, Israel) at a dilution of 1:500. After being washed with TBST, the membranes were exposed to an alkaline phosphatase-conjugated secondary antibody (anti-rabbit 1:10,000; Thermofisher, Massachusetts, U.S.A) and visualized using ECF reagent (RPN5785, GE Healthcare, U.S.A.) on a ChemiDoc MP (BioRad). Densitometry analysis of the resulting bands was conducted using BioRad's ImageLab software (version 6.1.0). Immunoblots were reprobed with anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) antibody. Protein expression levels were normalized with GAPDH.

3.10. Statistical Analysis

The statistical analysis was performed using IBM SPSS Statistics (version 27) with a significance level of $p < 0.05$. Before analysis, outliers were removed, based on the number of correlated parameters that fell outside the interval (mean $\pm$ 3*standard deviation), for a given test (Supplementary Data- Annex III, shows each test the criteria and the number of animals excluded). Data distribution was assessed using the Shapiro-Wilk test, while Levene's test was used to test for homogeneity of variance. Generally, normal distribution was assumed, parametric tests were employed, and the results were reported as $\bar{x}$ = mean $\pm$ standard error of the mean (SEM). When comparing two groups, we used a two-tailed independent samples t-test (a Welch's correction was applied if homoscedasticity was not met). Graphs were constructed on GraphPad Prism version 8.0.1 (GraphPad Software, San Diego, CA, USA).

Principal component analysis (PCA) was conducted at the end of the behavioral tests to have a spatial representation of group clustering. For this analysis, all the parameters regarding behavioral tests were used. Briefly, PCA is a technique that transforms a dataset with interrelated or correlated variables into a new dataset with uncorrelated variables known as principal components (PCs). These PCs are linear combinations of the original variables and are ordered based on their explained variances, with PC1 having the highest explained variance. The eigenvalues, and the coefficients are eigenvectors that allow to understand the directions of the spread of the data derived from the covariance matrix. The primary objective is to retain most of the information in a reduced number of PCs, which helps in describing multivariate patterns and variation in behavioral outcomes across groups. The eigenvectors, when multiplied by the square root of eigenvalues, form loadings that represent the significance of each original variable for a particular PC. Additionally, the sum of the product of loadings and original data values generates scores, which indicate values of new variables that display spatial patterns. Each animal's p metrics are transformed into p scores through this process.

4. RESULTS

This thesis aimed to provide initial insights into the lifelong impact of neonatal KP, focusing on behavioral changes in both males and females. Additionally, we sought to unravel the molecular mechanisms and key factors involved in these alterations. To achieve this, we designed four experimental groups: males injected with a vehicle (referred to as "Males Vehicle"), males injected with the KP antagonist kisspeptin-234 ("Males Kp234"), and two female groups ("Females Vehicle" and "Females Kp234"). These animals underwent a comprehensive series of behavioral tests, spanning from developmental stages to adult sexual behavior, to assess the potential influence of neonatal KP on gender-specific behaviors. Notably, a non-injected control group also underwent the same battery of behavioral tests to ensure that the ICV injection had no impact on the outcomes, and no significance was observed in any parameter between the sex-paired vehicle group.

4.1. Intracerebroventricular injection successfully blocks neonatal testosterone surge

To test the influence of neonatal KP surge on sex-typical behavior throughout life, it was important to check if the ICV injection of KP-234 successfully blocked the KP peak and the downregulated testosterone surge. For that, brain levels of GnRH and plasma levels of testosterone were checked by ELISA (Figure 15).

Among the treatment groups, testosterone levels were the only ones that displayed statistical significance in both sexes. Interestingly, males exhibited the anticipated reduction in plasma testosterone levels one hour after injection, while females experienced an unpredictable increase in testosterone plasma levels due to the blockade of the KP signaling pathway (Figure 15A). On the other hand, GnRH levels did not exhibit statistical significance. However, among females, a trend towards decreased GnRH levels between treatments was observed (Figure 15B). It is noteworthy that GnRH ELISA was conducted on the whole brain, potentially masking the blockade's effect specifically in the hypothalamus. In conclusion, the ICV injection yielded successful outcomes, illustrating that the KP antagonist effectively curtailed, at least partially, the testosterone surge.

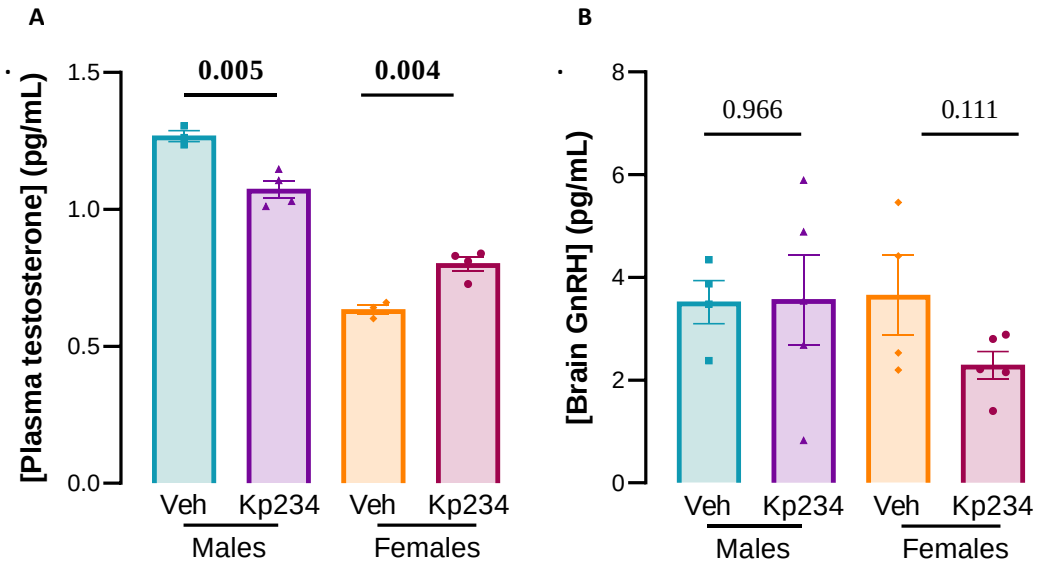


FIGURE 15 GNRH AND TESTOSTERONE LEVELS AFTER 1H OF INJECTION

A- Levels of plasma testosterone in males and females injected with vehicle solution and the kisspeptin antagonist. Two-tailed independent samples t-test; p<0.050; numbers above the bar indicate p-values: in bolt<0.050; n (males Veh) = 3; n (males Kp234) = 4; n (females Veh) = 3; n (females Kp234) = 4. **B-** Levels of whole brain GnRH in males and females injected with vehicle solution and the kisspeptin antagonist. Two-tailed independent samples t-test; p<0.050; numbers above the bar indicate p-values: no significant difference between treatment groups; n (males Veh) = 4; n (males Kp234) = 5; n (females Veh) = 4; n (females Kp234) = 5. Data represented as mean ± SEM.

4.2. Neonatal kisspeptin disruption affects body weight and total mass of the gonads and brain in adult males

After every behavioral test (PND 4, 6, 8, 12, 14, 24, 27, and 30), the weight, length, and anogenital distance were measured, and the body mass index (BMI) was calculated. No significance between treatment groups was found during this period in any of the parameters (Supplementary Data - Annex IV). However, upon sacrifice (PND60+) males Kp234 presented increased weight and consequently BMI compared with males vehicle (Figure 16A and C). Additionally, males Kp234 have lighter brains and testis (Figure 16E and F). Concerning length and anogenital distance no differences were found (Figure 16B and D), nor even any difference between females (Figures 16A-F). These results provide evidence for the hypothesis that KP exerts distinct effects on females and males, highlighting the significance of neonatal KP in shaping metabolic mechanisms, gonadal development, and overall brain formation in males.

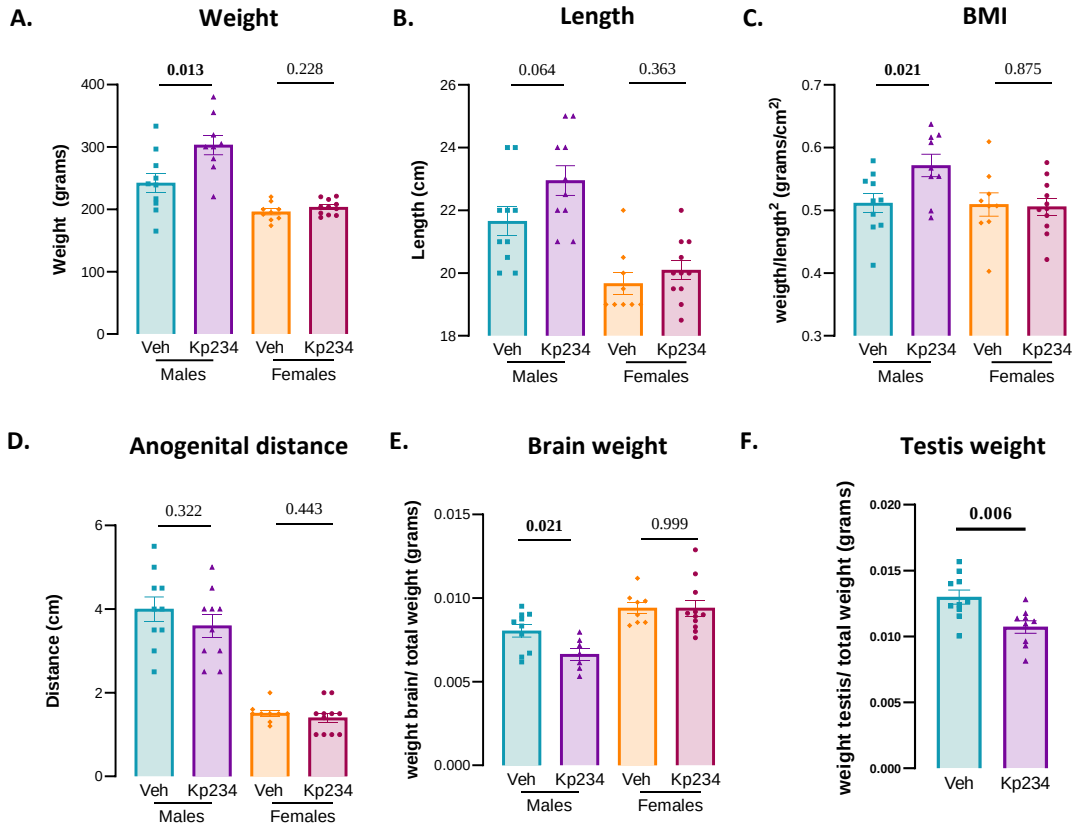


FIGURE 16 ANATOMICAL MEASUREMENTS UPON SACRIFICE

A- Weight. **B-** Length **C-** Body Mass Index (BMI, calculated as the division of the weight by the square of the length). **D-** Anogenital distance, **E-** Brain weight normalized by the total weight **F-** Testis weight normalized by the total weight of male rats. Two-tailed independent samples t-test; numbers above the bar indicate p-values: in bolt<0.050. n (males Veh) = 10; n (males Kp234) = 10; n (females Veh) = 9; n (females Kp234) = 11. Data represented as mean ± SEM.

4.3. Kisspeptin is not required for development of sensorimotor skills

Between PND4 and PND14, a battery of milestones tests was conducted to evaluate the development of sensorimotor skills. These tests included assessments for surface righting reflex, negative geotaxis reflex, cliff aversion, locomotion, wire suspension, and nest seeking in females (Figure 17) and males (Figure 18). Supplementary Data - Annex V shows the other two metrics of the nest-seeking test, first orientation, and final bedding choice.

Major differences and tendencies are found in locomotion activities. Females Kp234 showed a delay in exiting the circle at PND14 (Figure 17C). In any other developmental test, no significant differences were detected between groups. These results indicate that KP does not affect motor and vestibular development.

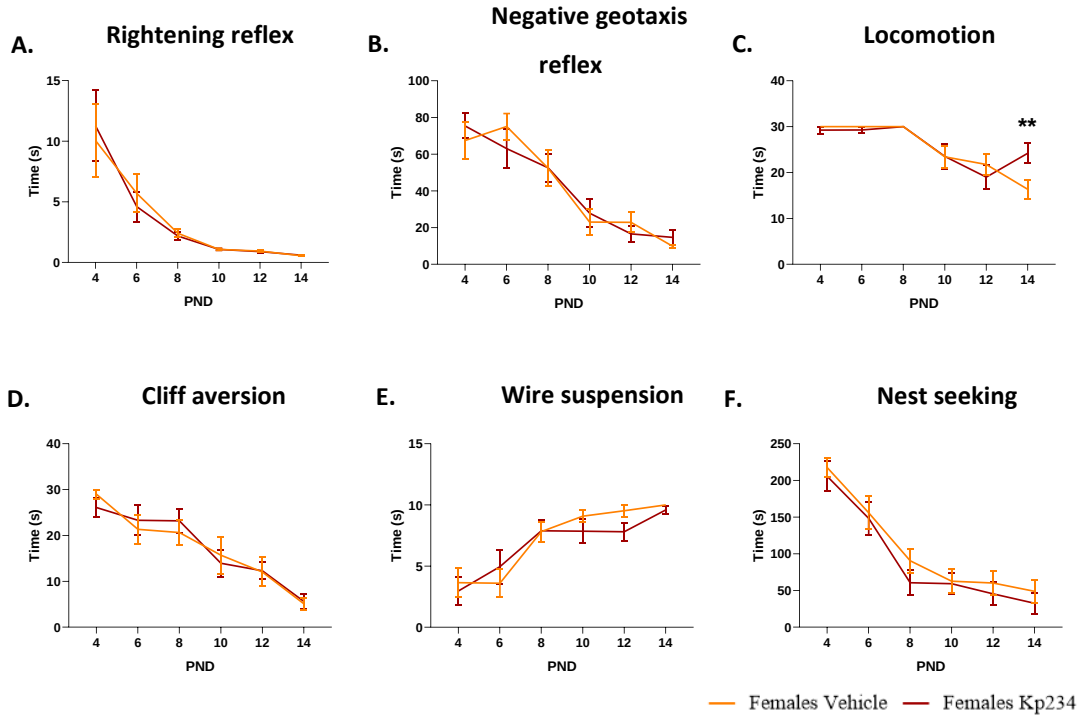


FIGURE 17 FEMALE DEVELOPMENTAL MILESTONES

A- Surface righting reflex, **B-** negative geotaxis reflex**, C-** locomotion, **D-** cliff aversion, **E-** wire suspension**,** and **F-** nest-seeking tests, performed on PND4, 6, 8, 10, 12, and 14. Two-tailed independent samples t-test, for each PND; **: p-value<0.010. n (females Veh) = 11; n (females Kp234) = 11. Data represented as mean ± SEM.

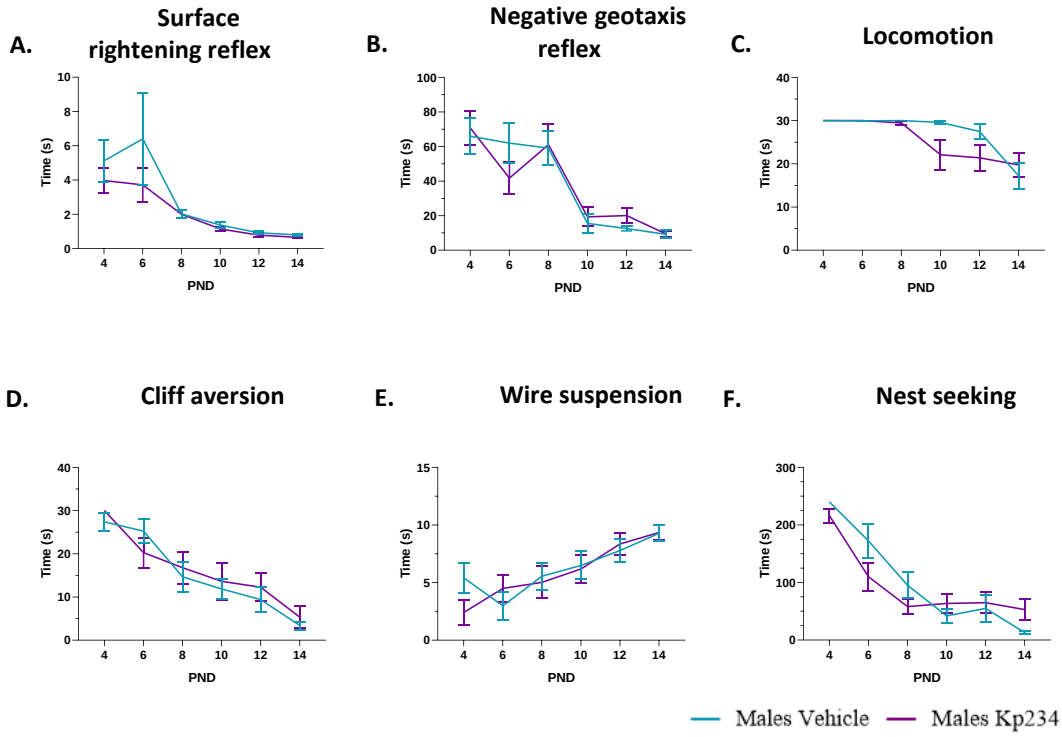


FIGURE 18 MALE DEVELOPMENTAL MILESTONES

A- Surface righting reflex, **B-** negative geotaxis reflex**, C-** locomotion, **D-** cliff aversion, **E-** wire suspension**,** and **F-** nest-seeking tests, performed on PND4, 6, 8, 10, 12, and 14. Two-tailed independent samples t-test, for each PND: no differences between groups found. n (males Veh) = 11; n (males Kp234) = 10. Data represented as mean ± SEM.

4.4. Kisspeptin is relevant in neonatal communication

Isolation-induced USVs were recorded on PND4, 6 and 8. Initially, USVs were analyzed based on the general characteristics of their fragments (Supplementary Data - Annex VI). Subsequently, calls were categorized by the number of fragments they contained, including single, double, triple, and multiple fragments. The overall number of calls for each PND was quantified (Figure 19), as well as the specific call counts per PND (Figure 20). Once again, the inhibition of KP during the neonatal period yields divergent effects on males and females. In general, the blockade of KP signaling tends to decrease the number of USVs in males while in females statistical significance was not observed (Figure 19). Remarkably, PND6 appears to be a particularly sensitive period for males, as those treated with Kp234 display an overall reduction in the number of calls (Figure 19B), driven by a significant decrease in single calls (Figures 20B). Notably, among all PNDs, the double call is the most prominent (Figures 20D-F).

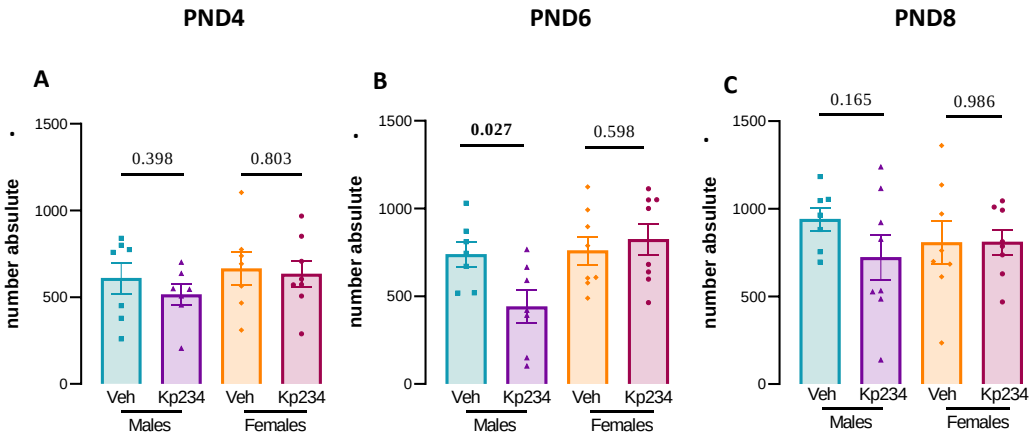


FIGURE 19 TOTAL NUMBER OF CALLS PER PND

A- Total number of calls at PND4. **B-** Total number of calls at PND6. **C-** Total number of calls at PND8. Two-tailed independent samples t-test: numbers above the bar indicate p-values: in bolt<0.050. n (males Veh) = 7; n (males Kp234) = 7; n (females Veh) = 7; n (females Kp234) = 8. Data represented as mean ± SEM.

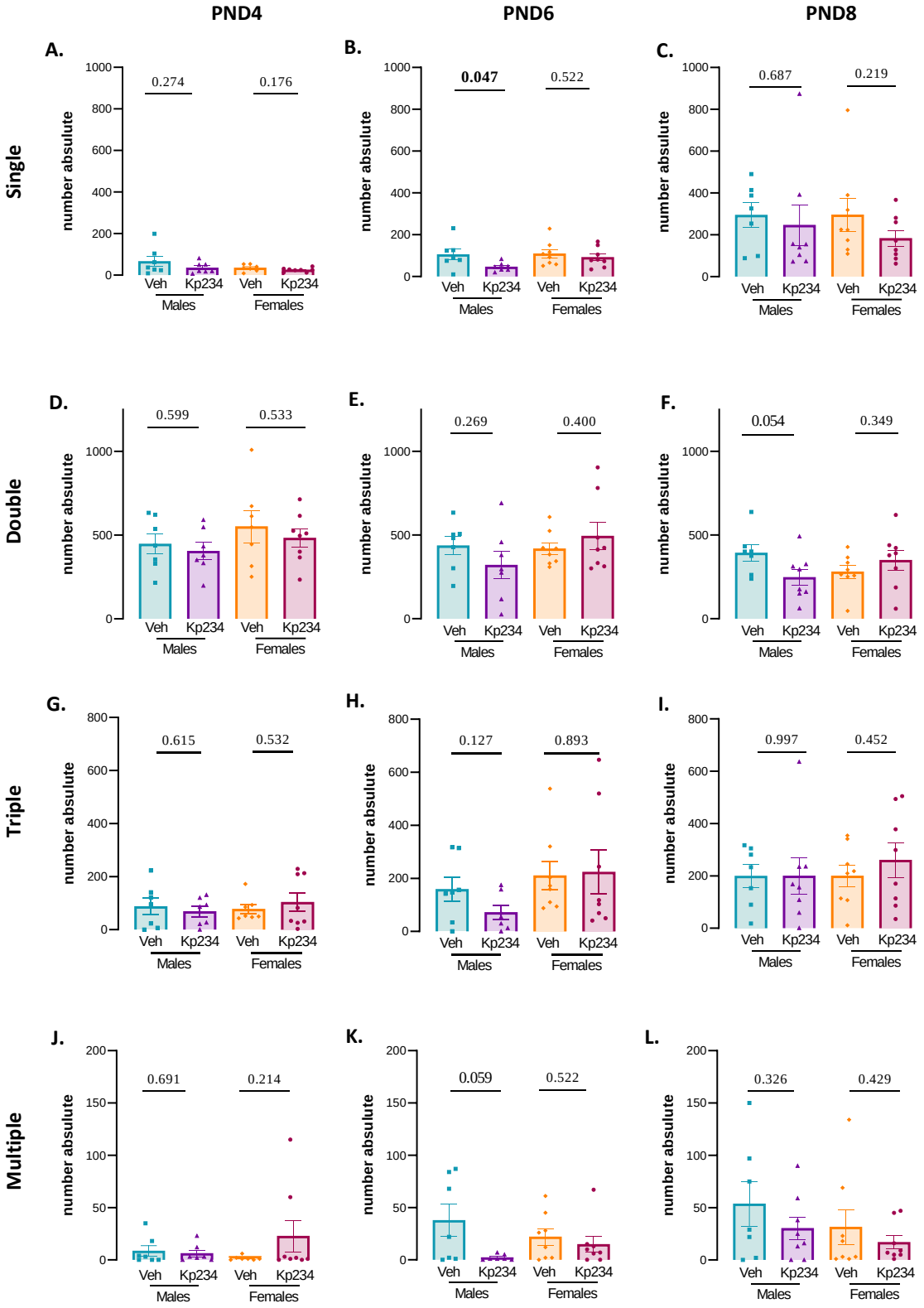


FIGURE 20 TOTAL NUMBER OF CALLS PER CALL TYPE AND PND

A- Number of single calls at PND4. **B-** Number of single calls at PND6. **C-** Number of single calls at PND8. **D-** Number of double calls at PND4. **E-** Number of double calls at PND6. **F-** Number of double calls at PND8. **G-** Number of triple calls at PND4. **H-** Number of triple calls at PND6. **I-** Number of triple calls at PND8. **J-** Number of multiple calls at PND4. **K-** Number of multiple calls at PND6. **L-** Number of multiple calls at PND8. Two-tailed independent samples t-test: numbers above the bar indicate p-values: in bolt<0.050. n (males Veh) = 7; n (males Kp234) = 7; n (females Veh) = 7; n (females Kp234) = 8. Data represented as mean ± SEM.

To delve deeper into the analysis of these USVs, we extracted and calculated the metrics obtained from DeepSqueak. This included evaluating call length, inter-interval, low, high, delta, peak, and principal frequencies. Additionally, mean power, tonality, sinuosity, and the slope of the calls were examined. For this discussion, we will concentrate on the characteristics of single calls, as they exhibit more pronounced differences between groups and are prevalent throughout the lifespan (Figure 21 and 22). However, characteristics of double and triple calls can be found in the Supplementary Data- Annexes VII and Annex VIII (respectively).

In terms of single calls, males treated with Kp234 exhibited a reduced low, high, delta, peak, and principal frequencies at PND6 compared to males in the vehicle group (Figure 21D-F, H, I). These observations suggest that the influence of KP signaling, particularly at PND6, has a significant impact on the communicative abilities of males, with noticeable changes evident in the overall reduction of call frequencies. Conversely, although not accompanied by significant differences in call numbers, females Kp234 also exhibited lower low, high, delta, principal, and peak frequencies (Figure 22D-F, H, I), sinuosity (Figure 22J), and slope (Figure 22C) at PND 8, in comparison to female vehicle. Furthermore, at PND4, females Kp234 showed reduced tonality relative to females in the vehicle group (Figure 22K).

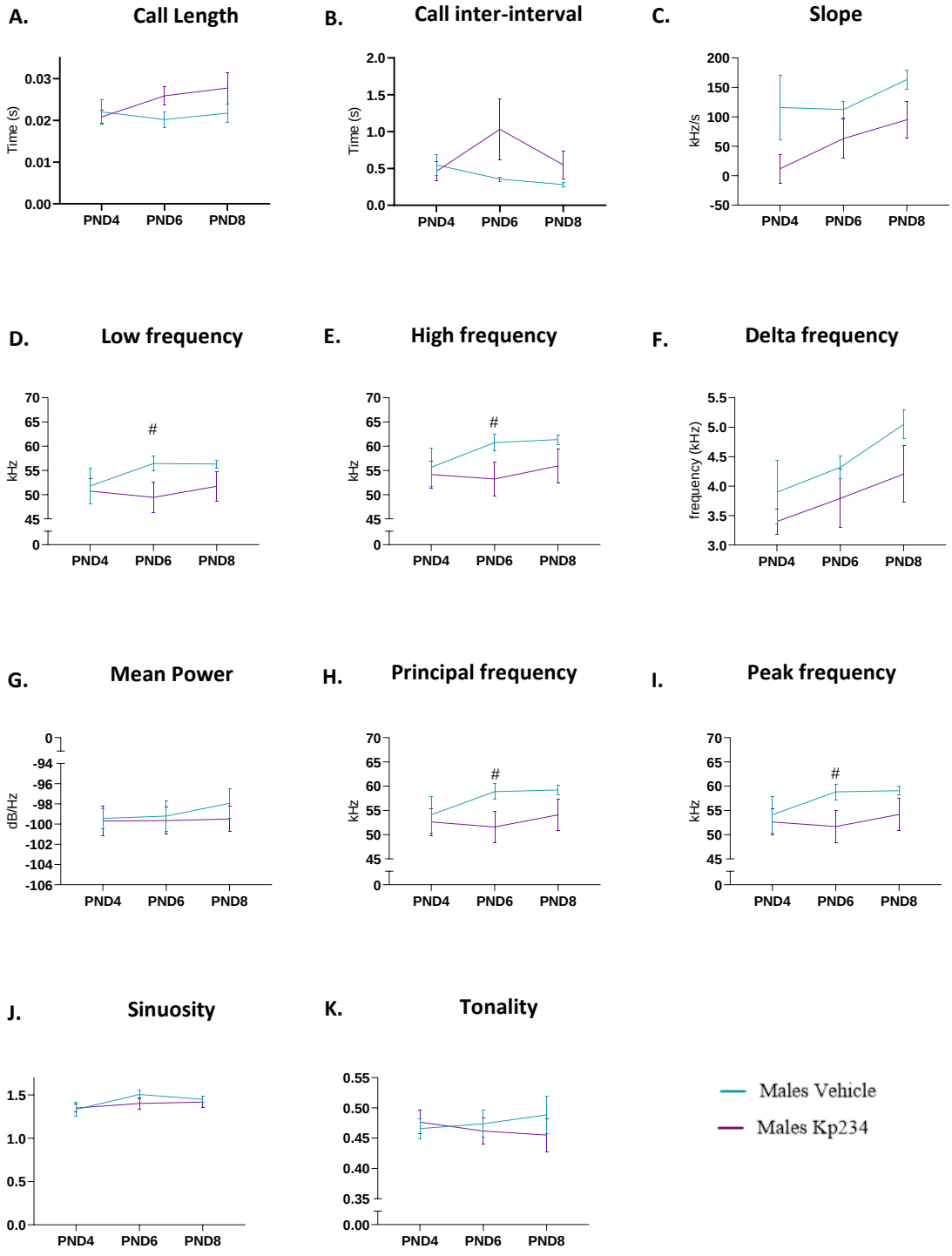


FIGURE 21 MALE SINGLE CALL CHARACTERISTICS

A- Call length. **B-** Call inter-interval. **C-** Slope. **D-** Low frequency. **E-** High frequency. **F-** Delta frequency. **G-** Mean power. **H-** Principal Frequency. **I-** Peak frequency. **J-** Sinuosity. **K-** Tonality performed on PND4, 6, and 8. Two-tailed independent samples t-test, for each PND, #: p-value<0.050. n (males Veh) = 7; n (males Kp234) = 7. Data represented as mean ± SEM.

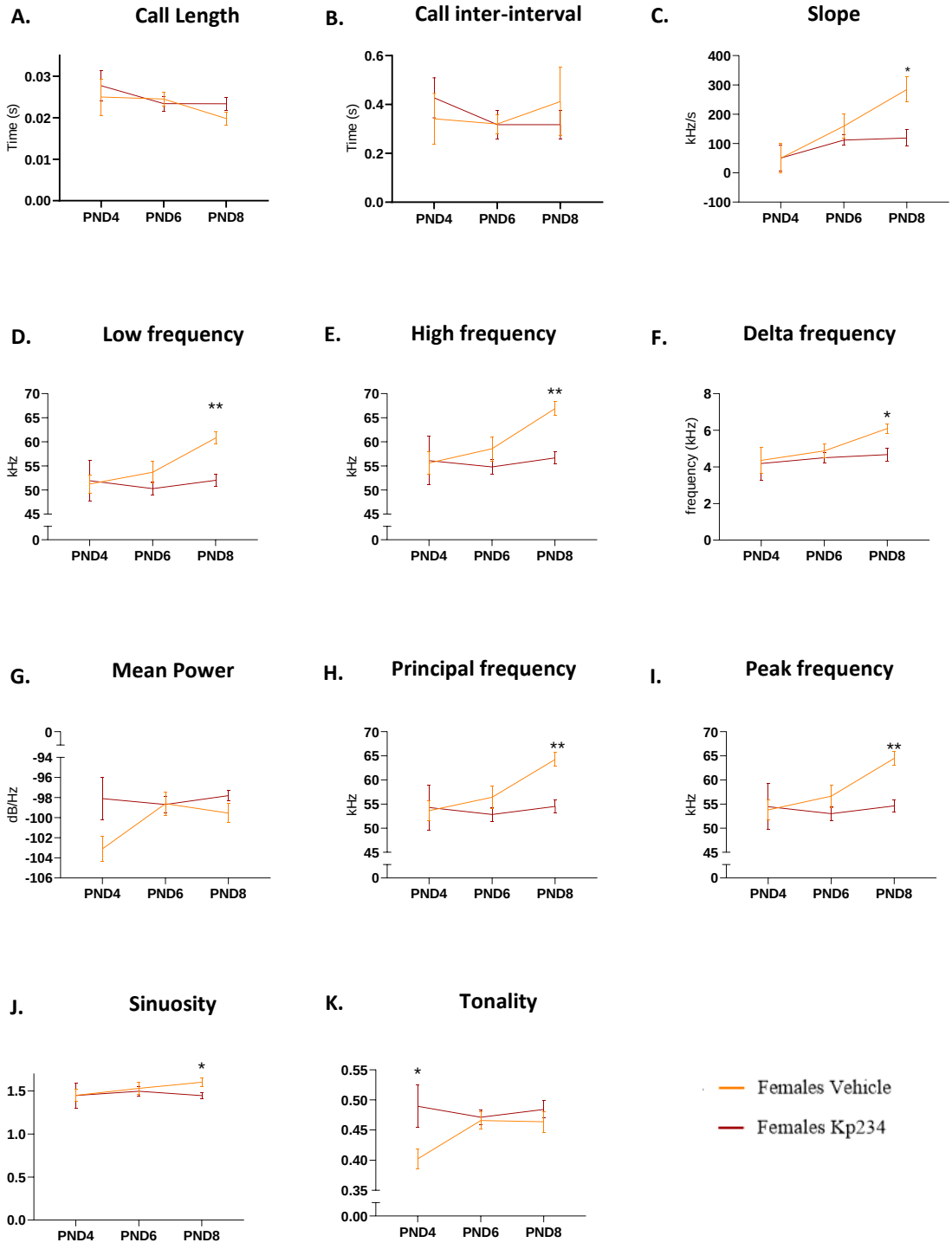


FIGURE 22 FEMALES SINGLE CALL CHARACTERISTICS

A- Call length. **B-** Call inter-interval. **C-** Slope. **D-** Low frequency. **E-** High frequency. **F-** Delta frequency. **G-** Mean power. **H-** Principal Frequency. **I-** Peak frequency. **J-** Sinuosity. **K-** Tonality performed on PND4, 6, and 8. Two-tailed independent samples t-test, for each PND: *: p-value<0.050; **: p-value<0.010. n (females Veh) = 6; n (females Kp234) = 8. Data represented as mean ± SEM.

Interestingly, double calls of males Kp234 displayed an intriguing pattern, with frequencies experiencing a substantial drop between PND6 and PND8 (not statistically significant). However, the trends observed were opposite in females Kp234 when compared to female vehicle, indicating an overall increase (Supplementary Data - Annex VII). As for triple calls, it is noteworthy that over time, females Kp234 tended to align more closely with the metrics of males, whereas this tendency was not observed among males themselves (Supplementary Data- Annex VIII).

Overall, the isolation-induced USVs underscore the significance of KP in social communication, with more pronounced effects observed in simple calls and noteworthy trends in more complex calls. Interestingly, in females, these alterations exhibited a delayed onset. The pivotal observation lies in the frequency modulation of neonatal KP.

4.5. Kisspeptin blockade at birth induces hyperlocomotive and increased exploratory behavior

To test locomotor activity and stress-like behavior, open field test was performed at PND24. Animals were allowed to explore an arena with fresh bedding, under low light, for 10min. Ceiling perspective recordings were used to analyze the total distance and distance travel in each zone (center and periphery, marked using the zone function on Smart software) (Figure 23). Also, latency for the first entrance on the periphery, time in the center, mean speed, resting time in each zone (Figure 24), entries in each zone, and number of poops (Supplementary Data- Annex IX) were accessed.

Overall, animals injected with Kp234 traveled bigger distances throughout the test (Figure 23A). Since they spent more time in the periphery (Figure 24A) they traveled longer distances in the periphery (Figure 23B and C). Additionally, they traveled faster in both regions (except females in the center) (Figures 24C and D) and consequently males spent less time resting in the periphery (since they spent almost no time in the center) (Figures24E and F). This could indicate a more stressful behavior, however, looking for the time spent in the center, a common measure of stress-like behavior (less time in the center, means increased stress), no differences were found (Figure 24A). Also, the number of entries in each zone and the number of poops had no significant differences between groups (Supplementary Data- Annex IX).

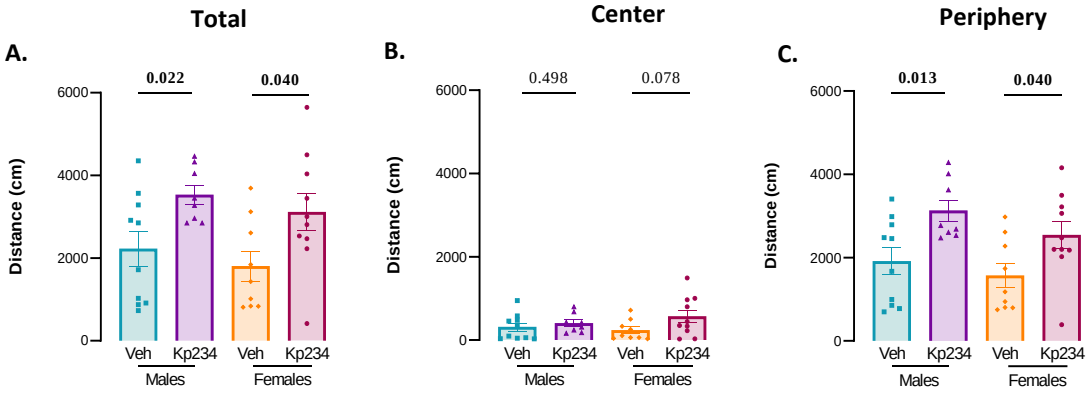


FIGURE 23 DISTANCE TRAVELED ON OPEN FIELD TEST

A- Total distance. **B-** Distance traveled in the center. **C-** Distance traveled in the periphery. Two-tailed independent samples t-test: numbers above the bar indicate p-values: in bolt<0.050. n (males Veh) = 10; n (males Kp234) = 8; n (females Veh) = 9; n (females Kp234) = 10. Data represented as mean ± SEM.

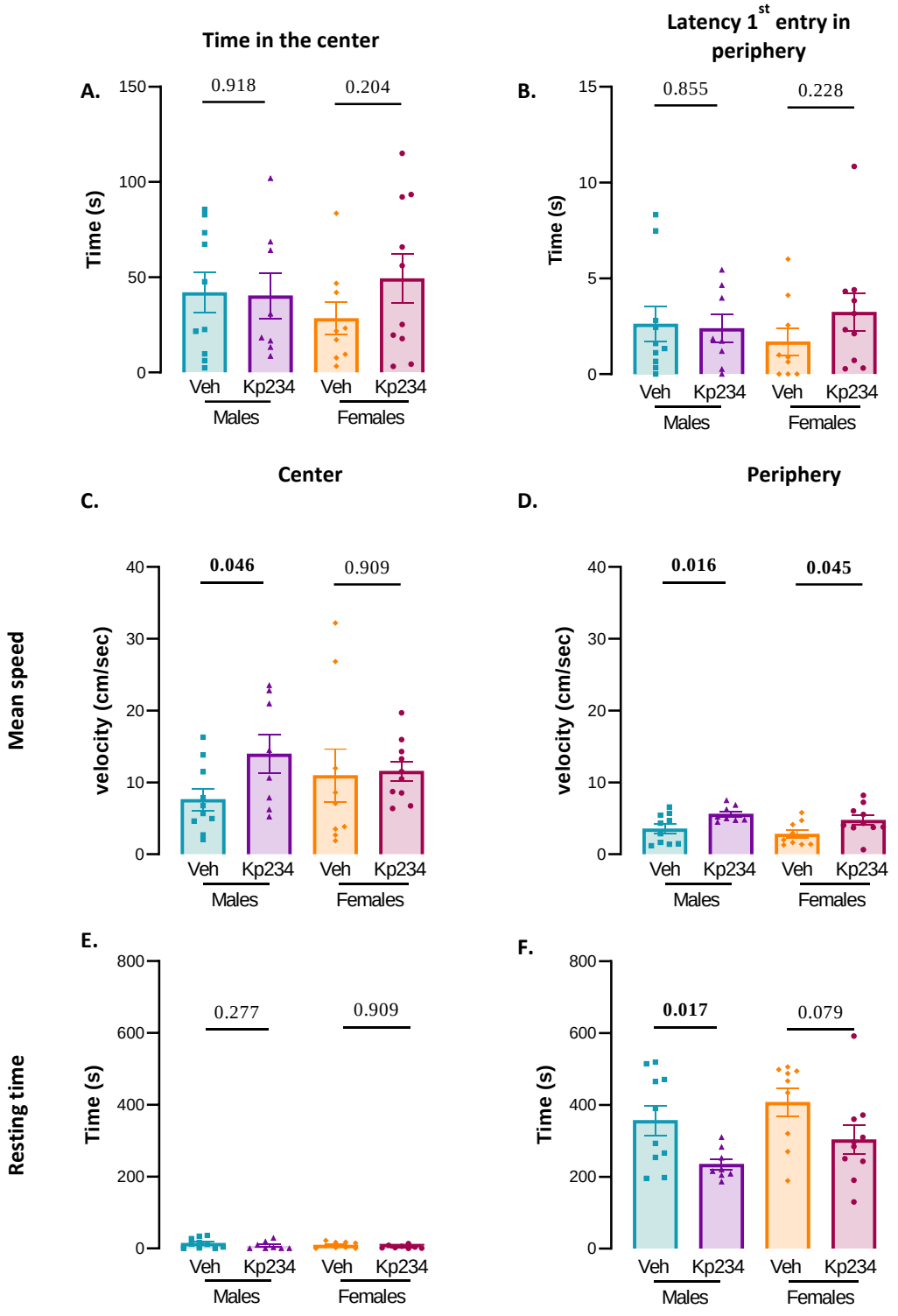


FIGURE 24 OPEN FIELD TEST METRICS

A- Time spent in the center**. B-**Latency for first entrance in the periphery**. C-** Mean speed in the center. **D-** Mean speed in the periphery. **E-** Resting time in the center. **F-** Resting time in the periphery. Two-tailed independent samples t-test: numbers above the bar indicate p-values: in bolt<0.050. n (males Veh) = 10; n (males Kp234) = 8; n (females Veh) = 9; n (females Kp234) = 10. Data represented as mean ± SEM.

To further study the possibility of increased stress in animals injected with Kp234, grooming (Supplementary Data- Annex X), supported rearing (Figure 25 A, C, and E), and unsupported rearing (Figures 25B, D and F) were analyzed using BORIS. From each behavior, the total number and duration, and the mean duration were accessed.

Overall, no significant differences were found between groups. However, males Kp234 tended to spent more time performing supported rearing, a result of the statistical significant increased duration of this behavior (Figure 25 F and E). This type of rearing is more related to locomotive behaviors and exploration, while unsupported rearing is more associated with stress, mainly acute stress (more unsupported rearing means increased stress) (116). No differences were found in grooming behavior (Supplementary Data- Annex X), showing that animals were unlikely more stressed compared with vehicles.

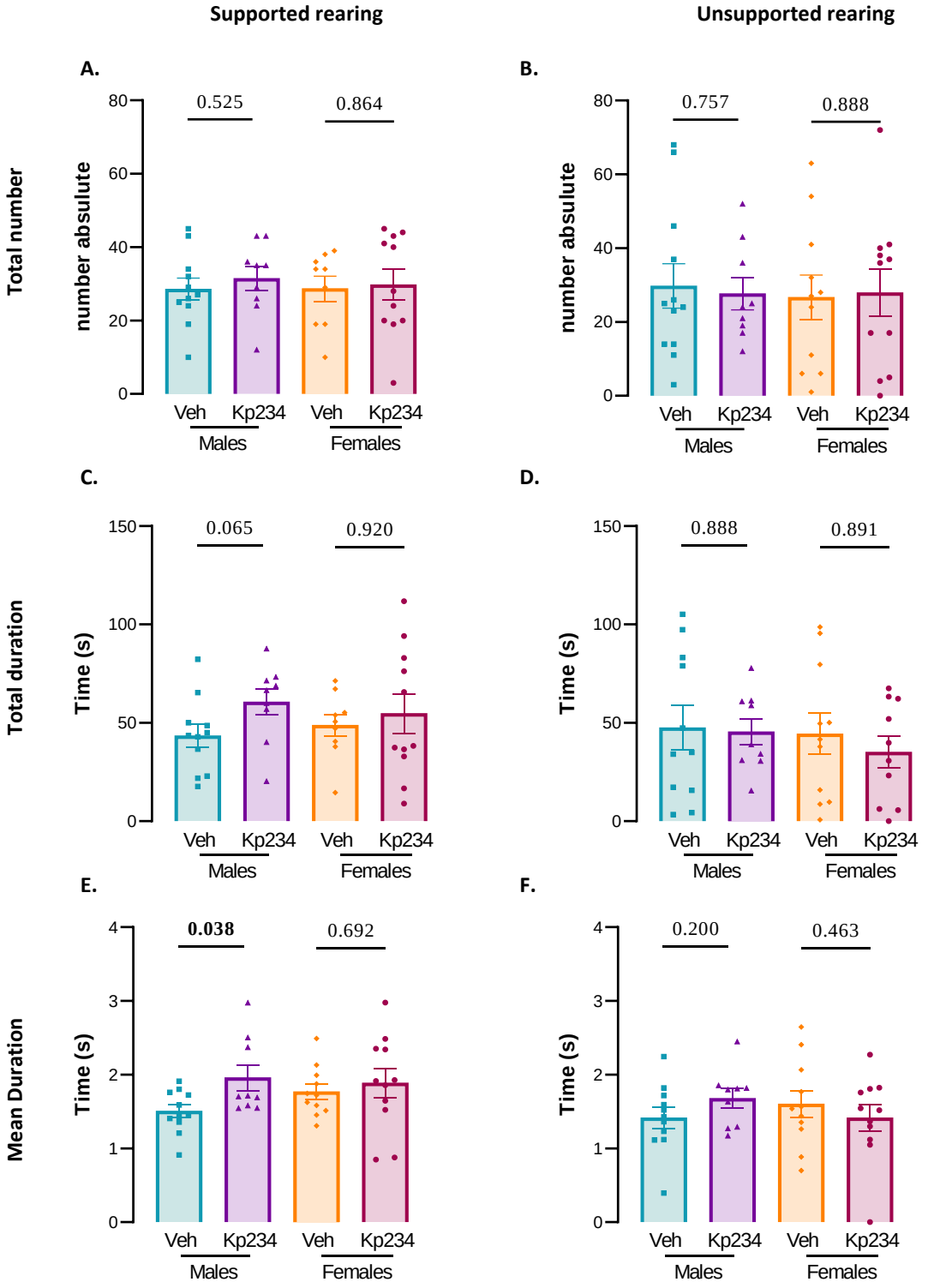


FIGURE 25 SUPPORTED AND UNSUPPORTED REARING

A- Total number of supported rearing**. B-**Total number of unsupported rearing**. C-** Total duration of supported rearing. **D-** Total duration of unsupported rearing. **E-** Mean duration of supported rearing. **F-** Mean duration of supported rearing. Two-tailed independent samples t-test: numbers above the bar indicate p-values: in bolt<0.050. n (males Veh) = 11; n (males Kp234) = 9; n (females Veh) = 10; n (females Kp234) = 10. Data represented as mean ± SEM.

In summary, the open-field test results indicate that KP does not appear to influence stress-related behaviors. Instead, it appears to amplify hyperactive locomotion and heightened exploratory behaviors, particularly in males.

4.6. Kisspeptin blockade at birth impairs play behavior

Between PND24 and PND27 animals were isolated to enhance social behavior. The test was performed on 4 consecutive days and the pair was maintained. An average of the three behaviors was totaled (total play is the sum of pin, pounce, and box) (Figure 26) and each behavior was scored on each test day, manually (Figure 27).

Males treated with Kp234 exhibit a tendency to play less compared to male Vehicle (Figure 26A), with a more pronounced impact observed on the pouncing behavior (Figure 27A). The involvement of KP in male play behavior is noteworthy, especially evident in PND29 (Figure 26B). Pouncing, often linked to play initiation, has a tendency to be less frequent in males treated with Kp234, suggesting a reduced tendency to initiate social interactions. Notably, both males and females display an overall decrease in play behavior across all testing days (Figure 26B), encompassing all behavioral aspects (Figure 27). Moreover, as documented, female Vehicle, although not significantly, exhibit less play behavior compared to males in the vehicle group. This pattern is disrupted when animals are subjected to antagonist treatment (Figure 27B).

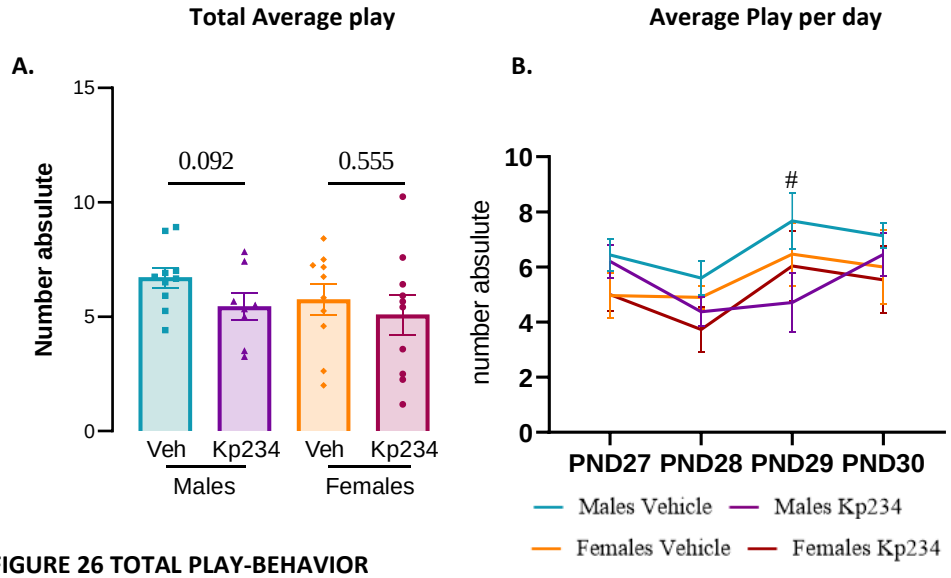


FIGURE 26 TOTAL PLAY-BEHAVIOR

A- Average total play of each day. **B-** Total play per day. Two-tailed independent samples t-test: numbers above the bar indicate p-values: # p-value<0.05 between males. n (males Veh) = 10; n (males Kp234) = 8; n (females Veh) = 10; n (females Kp234) = 10. Data represented as mean ± SEM.

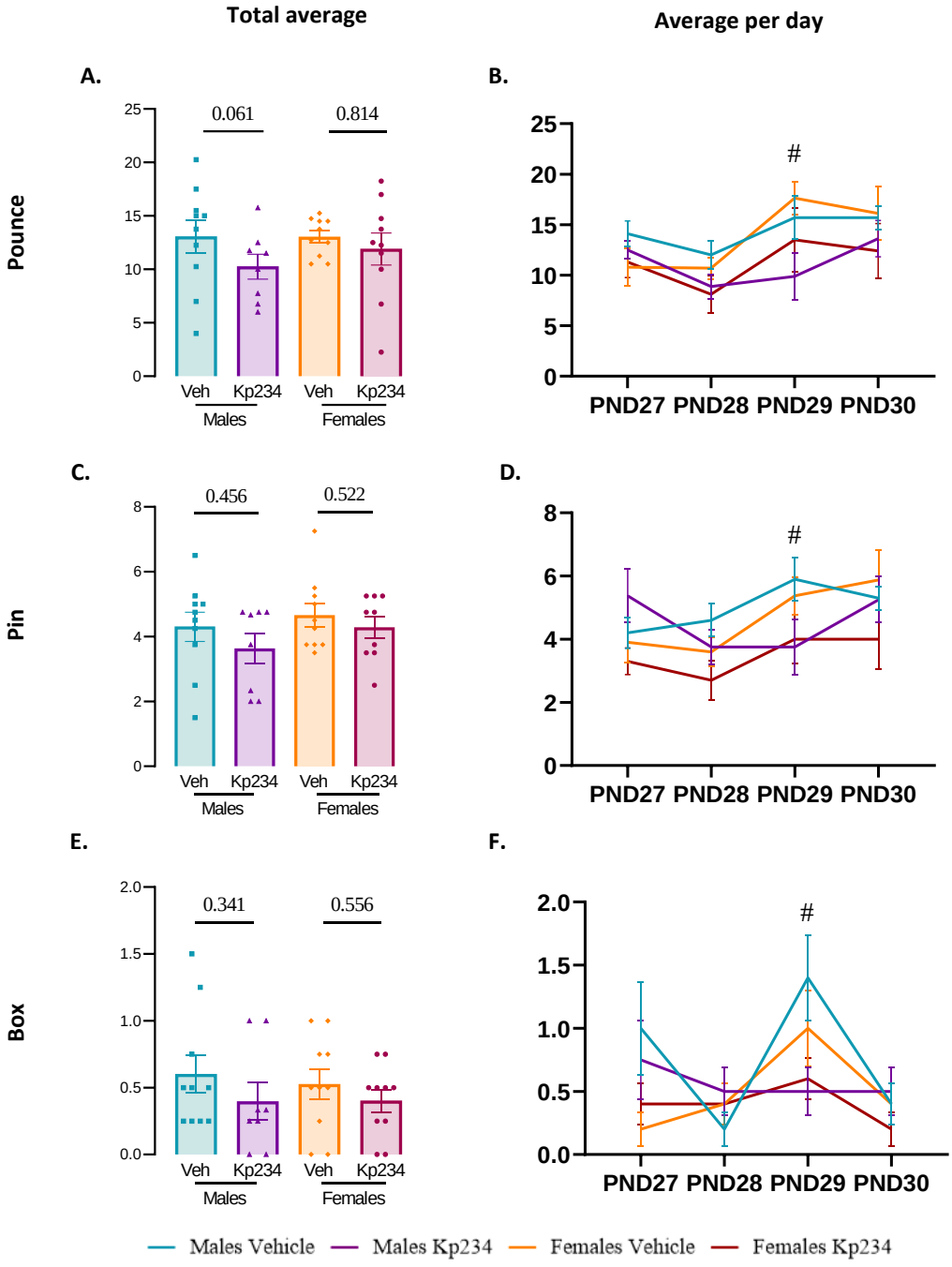


FIGURE 27 PIN, POUNCE, AND BOX BEHAVIORS

A- Average of pounce. B- Pounce per day**. C-** Average of pin. **D-** Pin per day. **E-** Average of box. **F-** Box per day. Two-tailed independent samples t-test: numbers above the bar indicate p-values: # p-value<0.05 between males. n (males Veh) = 10; n (males Kp234) = 8; n (females Veh) = 10; n (females Kp234) = 10. Data represented as mean ± SEM.

USVs were recorded in all test days, but since PND29 seems more promising to show differences between treatments it was the only one analyzed. Like the isolation-induced USVs, the number and their low, high, delta, peak, and principal frequencies were accessed (Figure 28). Additionally, call length, inter-interval, mean power, tonality, sinuosity, and slope were studied (Supplementary Data- Annex XI).

Due to the use of a single microphone and the presence of two animals within the arena during recording, the sample size in this context corresponds to a pair of animals rather than an individual animal. Consequently, the statistical power of the analysis is reduced due to the limited sample size. Despite this limitation, like pup USVs, frequency parameters displayed the most noticeable variation. However, contrary to what was observed in pups, males injected with Kp234 exhibited increased frequencies, particularly higher frequency, in comparison to males vehicle (Figure 28E). Conversely, females displayed an opposing pattern, as those injected with Kp234 showed an overall decrease in frequency, predominantly pronounced in high frequencies (Figure 28E). As for the remaining metrics, no significant distinctions emerged between the treatment groups (Supplementary Data- Annex XI).

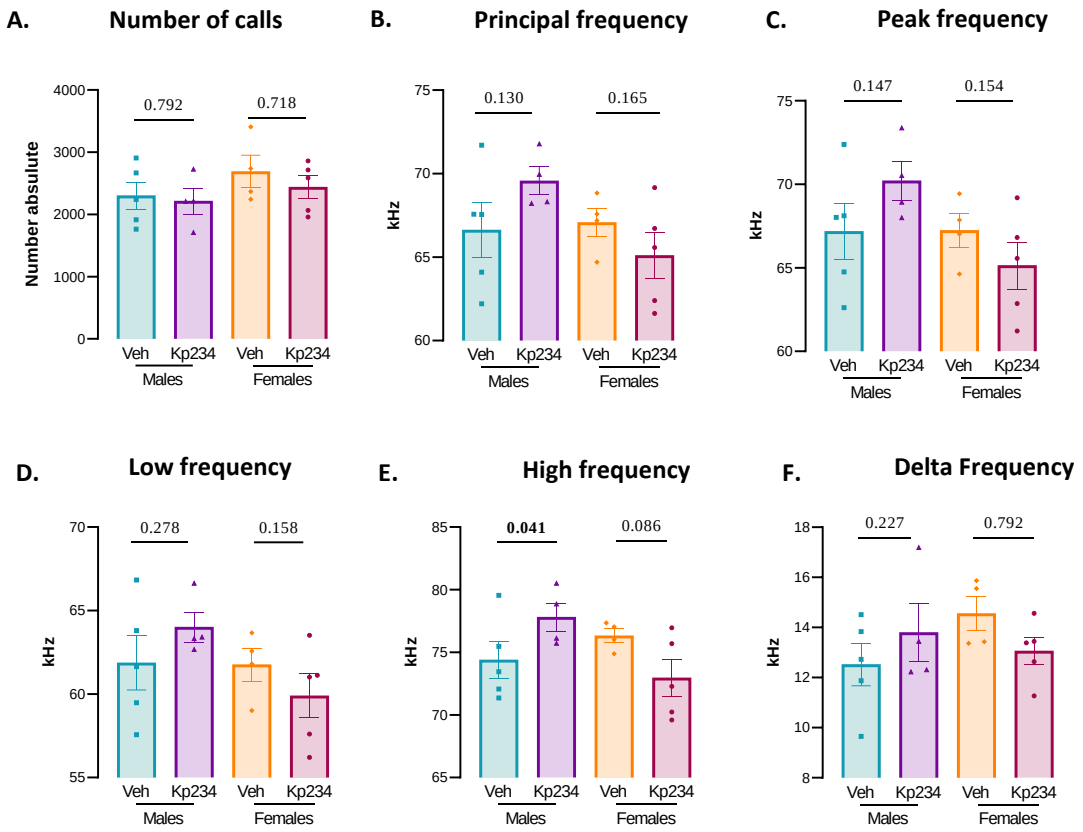
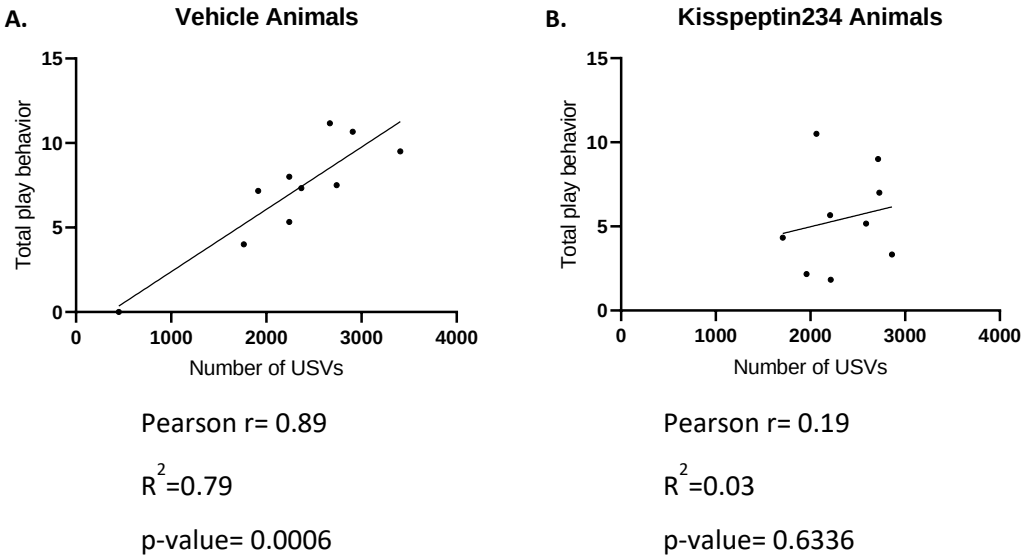


FIGURE 28 PND29 USV CHARACTERISTICS

A- Number of calls. **B-** Principal frequency. **C-** Peak frequency. **D-** Low frequency. **E-** High frequency. **F-** Delta frequency. Two-tailed independent samples t-test, bolt: p-value<0.050. n (pair of males Veh) = 5; n (pair of males Kp234) = 4; n (pair of females Veh) = 4; n (pair of females Kp234) = 5. Data represented as mean ± SEM.

Crucially, the number of calls remained unchanged in this test (Figure 28A). However, this raised the question whether the call numbers correlated with the average play behavior on that specific test day. A Pearson's correlation analysis was undertaken, with sex not being segregated, utilizing the mean of average play at PND29 for each pair (Figure 29).

The number of USVs and the average of play highly correlated in animals vehicle (Figure29A). However, no correlation was found in animals injected with Kp234 (Figure 29B). These results show that despite no difference in USV number their context or purpose was not the same.



Pearson r= 0.89

R^2=0.79

p-value= 0.0006

Pearson r= 0.19

R^2=0.03

p-value= 0.6336

FIGURE 29 PEARSON'S CORRELATION BETWEEN AVERAGE PLAY AND USV NUMBER AT PND29

A- Pearson's correlation of animals injected with vehicle. **B-** Pearson's correlation of animals injected with Kp234. n (Vehicle) = 9; n (Kp234) = 9.

Overall, these results show that KP is important in shaping play behavior, mainly in males, which is in agreement with the fact that this behavior is more male-typical. Also, despite presenting the same USV number animals injected with KP antagonist poorly correlate with play behavior, predicting the importance of KP in shaping social communication in the juvenile period.

4.7. Kisspeptin disruption at birth induces a faster sexual approach in males

After PND60, sexual behavior was recorded. Male sexual behavior comprehended mounts and intromission-like behaviors number and latency (Figure 30).

Regarding the number of mounts and intromissions, there was no difference reported (Figures 30A and C). However, the latency of both behaviors was significantly decreased in males Kp234 compared to male Vehicle (Figures 30B and D). Importantly, males were inexperienced and therefore, the day of the test was the first time a male was presented to a female. Still, males injected with the antagonist engaged faster in sexual behavior.

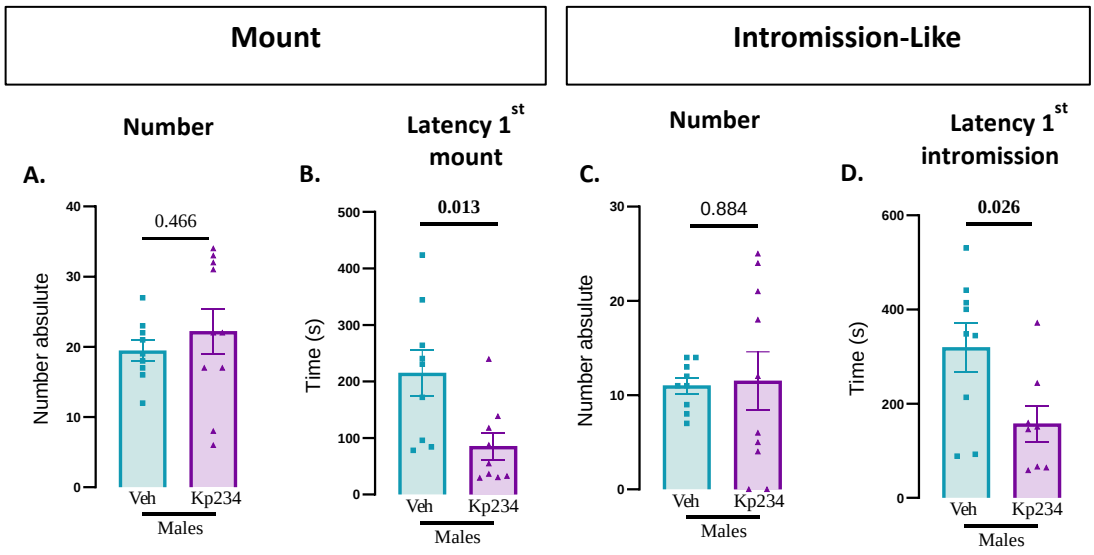


FIGURE 31 MALE SEXUAL BEHAVIOR

A- Number of mounts. **B-** Latency of the first mount. **C-** Number of intromissions. **D-** Latency first intromission. Two-tailed independent samples t-test: numbers above the bar indicate p-values: in bolt<0.050. n (males Veh) = 8; n (males Kp234) = 9. Data represented as mean ± SEM.

Female behavior was also analyzed after PND60, the number of solicitations (Figure 31A), hops and darts (Figure 31B), and lordosis (Figure 31C) were accessed. No significant difference was found regarding these behaviors. Indicating KP does not affect female sexual behavior.

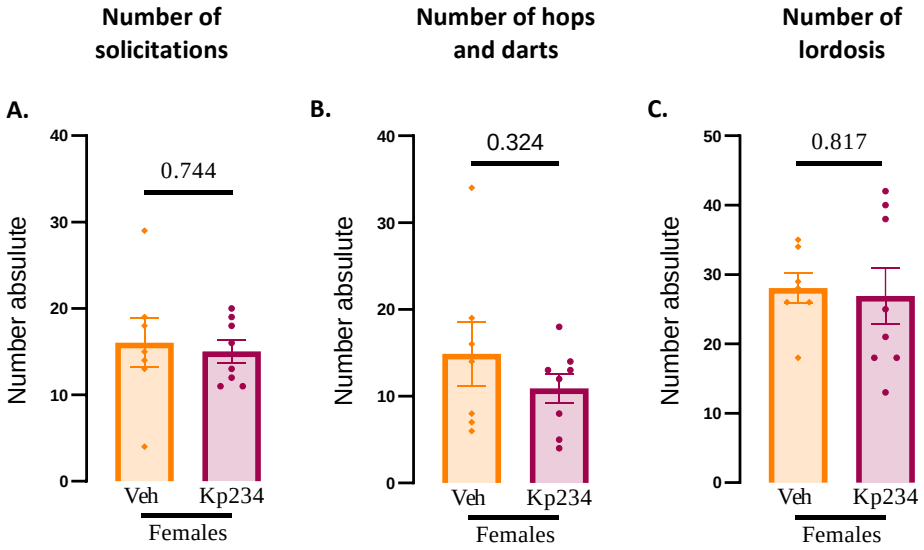


FIGURE 30 FEMALE SEXUAL BEHAVIOR

A- Number of solicitations. **B-** Number of hops and darts. **C-** Number of lordosis. Two-tailed independent samples t-test: numbers above the bar indicate p-values: no significance between groups. n (females Veh) = 7; n (females Kp234) = 8. Data represented as mean ± SEM.

Overall, male sexual behavior seemed to have little modulation by KP. Latency in sexual behavior could predict more sexual drive rather than better sexual performance. Female behavior is not affected by neonatal KP disruption.

4.8. Neonatal kisspeptin blockade is sufficient to build distinct behavioral populations within the same sex

To have a better insight into the overall behavioral effect of neonatal KP along life, a PCA analysis was performed with all behavioral parameters from all tests. The loadings of the variables and their relative importance for each PC were extrapolated when comparing male Vehicle vs. male Kp234 and female Vehicle Vs. female Kp234 (Supplementary Data- Annex XII). Also, the individuals were placed on an XY plan to see how they were distributed according to the PC (males: Figure 32A; females: Figure 32B).

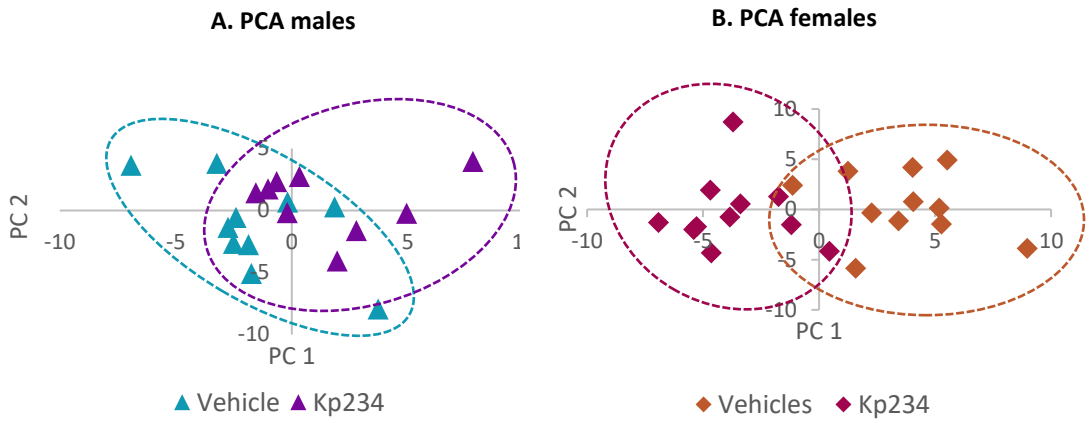


FIGURE 32 PRINCIPAL COMPONENT ANALYSIS
A- Males PCA. **B-** Females PCA. n (males Veh) = 10; n (males Kp234) = 9; n (females Veh) = 10; n (females Kp234) = 11.

The results of the PCA reveal that this comprehensive behavioral battery differentiated distinct populations within each sex. This underscores the critical role of neonatal KP signaling in shaping gender-typical behavior throughout the lifespan. Across both sexes, the variables that primarily contributed to explaining individual variance were associated with play behavior and open field test (OFT) outcomes (Supplementary Data- Annex XII). These findings seem to underscore that KP exerts a more pronounced influence on juvenile social interactions, as well as locomotion and exploratory activities.

4.9. Hypothalamic Kisspeptin 1 receptors levels are not altered in adulthood in both sexes

After sacrifice, at PND60+, the hypothalamus was dissected and levels of KISS1R were evaluated by western blot. Receptor levels could predict the mechanistic pathways by which neonatal KP blockade affected the behavioral outcomes observed throughout life.

However, there were no significant differences observed between treatment groups (Figure 33). It is noteworthy that this analysis encompassed the entire hypothalamus and all cell types, which might have obscured some outcomes. For instance, the blockade of KP could have caused a reduction in receptor quantity within one hypothalamic nucleus while elevating it in another. Our understanding of KISS1R-positive cell types remains limited. Additionally, the diminished sample size impacted the statistical power of the analysis. A crucial observation arises: comparison of the number of KISS1R between females and males showed a significant reduction in the former, revealing the high degree of sexual dimorphism in this region.

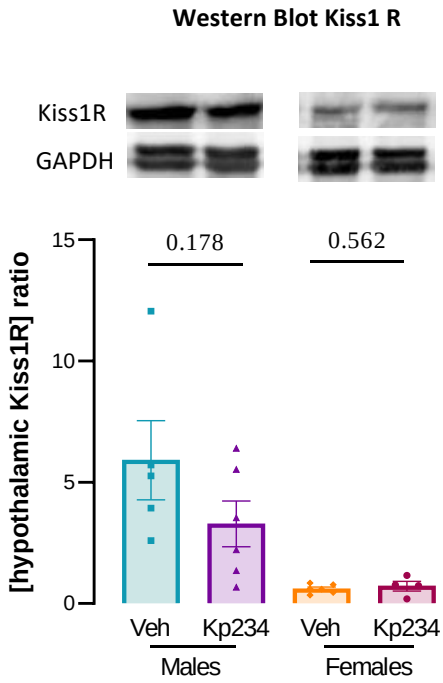


FIGURE 33 HYPOTHALAMIC LEVELS OF KISS1R IN ADULTHOOD

Two-tailed independent samples t-test: numbers above the bar indicate p-values: no difference between treatment. n (males Veh) = 5; n (males Kp234) = 6; n (females Veh) = 5; n (females Kp234) = 5. Data represented as mean ± SEM.

4.10. Kisspeptin neonatal blockade appears to induce morphological microglia changes on the media preoptic area of the hypothalamus

Microglia emerges as a pivotal contributor to sexual dimorphism in the mPOA. Consequently, our objective was to illuminate the impact of KP on microglia morphology. In this study, we limited our examination to immunolabeling and qualitative remarks on microglial structure. Moving forward, we plan to undertake a Sholl analysis to validate our observations. Upon reviewing the cell examples from each group, a noticeable contrast arises in males, microglia appears to exhibit reduced complexity in those injected with Kp234 compared to Vehicle (Figures 34A and B), whereas in females, no evident differences manifest (Figures 34C and D). We postulated that neonatal KP orchestrates enduring alterations in microglia morphology, with these changes being sex dependent.

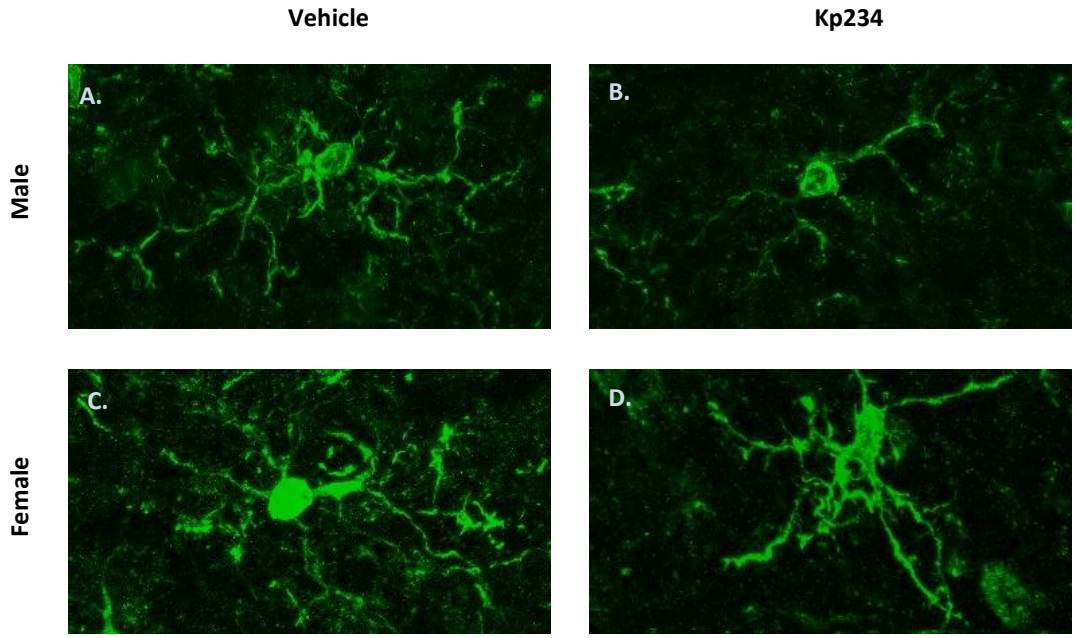


FIGURE 34 MICROGLIA MORPHOLOGY

A- Representative microglia of males vehicle. **B-** Representative microglia of males Kp234. **C-** Representative microglia of females vehicle. **D-** Representative microglia of females Kp234.

5. DISCUSSION

The present study has made noteworthy contributions in decoding the impact of neonatal KP surge on gender-typical behaviors across all life stages. Additionally, it has provided initial insights into novel and promising molecular and cellular mechanisms underlying these changes. While this research represents some leap forward, it also unveils a new realm of questions that necessitate further exploration and investigation.

The initial phase involved confirming the effectiveness of ICV injection in blocking the KP signaling cascade. Unfortunately, the assessment of GnRH ELISA yielded less promising outcomes. We attribute these outcomes more to specific protocol choices than to an indication of the ineffectiveness of the ICV approach. This is primarily underscored by the fact that plasma testosterone levels, regulated by GnRH, showed alterations. It is worth noting that the analysis covered the entire brain, whereas our primary interest lay in hypothalamic GnRH levels. Dissecting brain regions at PND0 is challenging due to the small and delicate nature of the neonatal brain. However, future studies utilizing ICV injection should ideally focus on optimally isolating the hypothalamus.

Our findings from testosterone ELISA in animals sacrificed one-hour post-injection underscore this observation. Specifically, in males, the anticipated outcome was observed: plasma testosterone levels were significantly reduced after Kp234 injection. Interestingly, a novel and unreported effect emerged in females-Kp234 injection led to elevated plasma testosterone levels. To delve deeper into the mechanistic underpinnings of this phenomenon, further investigations are warranted. It is plausible that females exhibit distinct mechanisms from males, providing an intriguing avenue for exploration.

The validation of hormonal effects through ICV injection confirms all the group differences found on the further behavioral tests. Firstly, we observed that males injected with Kp234 exhibited an increase in body mass, which became significant in adulthood. Several factors could account for this outcome. For instance, KP neurons are directly connect to proopiomelanocortin (POMC) and neuropeptide Y (NPY) neurons in the ARC. Previous studies have indicated that antagonist KP injections inhibit POMC neurons (117). NPY neurons stimulate appetite, while POMC activation suppresses it. While manipulating appetite to address food disorders remains of great interest, rodent studies have yielded conflicting results due to inconsistent protocols and the time-dependent

effects of KP (82). Nevertheless, our findings suggest that neonatal KP blockade influences appetitive behavior across the lifespan. We posit that the KP antagonist disrupts the usual wiring to POMC and NPY neurons, favoring increased activity in the NPY neurons. Further investigation is necessary; for instance, food intake was not controlled in our study, and we did not examine the count of NPY neurons or KP neurons in the ARC. Notably, our results indicate that these weight differences are sex-dependent, with males relying more on KP for proper wiring of the body's regulation neural circuit.

In terms of body measurements, we also observed a decrease in testis weight, underscoring the importance of KP in gonadal development. Interestingly, studies have shown that hypogonadism is correlated with increased body weight (82). KP appears to interconnect these two findings by modulating different nuclei of the hypothalamus.

Lastly, neonatal KP blockade led to a reduction in brain mass. During the neonatal period, KP may play a pivotal role in shaping neural connections. The brain undergoes dynamic growth and structural changes during early life, influenced by factors such as hormonal fluctuations and genetic predispositions. If KP signaling is temporarily disrupted during this critical period, it could potentially affect the delicate balance of neural connections guiding brain development. For instance, a study demonstrated that KP signaling mediates growth hormone release (118). This, in turn, might contribute to the observed differences in brain mass between treatments in males, particularly if the alterations disproportionately affect brain regions contributing to overall brain size. Nonetheless, the role of KP in circuitry wiring and neurodevelopment remains understudied, necessitating further investigations into the mechanisms influenced by KP and their potential contribution to an overall effect on brain size.

A significant achievement of this study is the revelation of the significance of neonatal KP modulation in shaping vocalizations in pups and juveniles. Interestingly, even in infancy, animals injected with Kp234 exhibited a reduced number of vocalizations on PND6, along with an overall reduction in call frequency. Interpreting these outcomes remains challenging due to mixed literature on rat call classification and a limited understanding of the nuanced implications of various USV characteristics in pups. Our research offers a novel perspective on how USVs could serve as robust indicators of KP's influence. However, further investigations are needed to delve into how KP precisely modulates USVs during infancy. For instance, the production of isolation-induced USVs

could potentially result from temperature fluctuations rather than isolation itself (117). The hypothalamus, a hub for body temperature regulation, houses a substantial concentration of KP neurons, which are highly implicated in this regulation (118). It is conceivable that Kp234-injected pups might exhibit heightened sensitivity to temperature changes, leading to reduced vocalizations. This suggests an experimental control of pups' body temperature to test its role in isolation-induced USVs.

Furthermore, KP's role in communication modulation is paramount. The influence of sexual hormones on communication is well-documented. A study involving mice demonstrated that testosterone injections in females increased the number of USVs during sexual behavior (119). Another study highlighted substantial correlations between KP neuron activation in the ARC and female sexual approach, whether in the presence of male USVs or not. USVs play a pivotal role in mediating social interactions (120). Our study demonstrated a sex-dependent effect on frequency modulation (increase in males and decrease in females) between treatments. Importantly, we established that USV number and play behavior only correlate in vehicle-injected animals, suggesting a different purpose for USVs in Kp234 animals. These findings underscore the impact of KP on modulating social communication that ultimately impairs behavior. No literature was found regarding USVs in the juvenile period nor even their importance in play behavior. USVs are important in modulating behavior and should be more frequently analyzed in various contexts and behavioral tests to have a better insight into how rodents use oral communication and therefore improve translational outcomes.

The primary aim of this study was to comprehend the importance of KP in shaping gender-typical behavior. To achieve this, we designed a battery of behavioral tests that encompassed neurodevelopmental tests, juvenile tests, and adult behavior tests.

In terms of neurodevelopmental tests, as anticipated, no significant differences were found, indicating that KP likely does not affect motor and vestibular development. Despite significant differences in the locomotion test at PND14 in females, these differences are likely related to orientation skills. In this test, the threshold for success is exiting the circle, and animals frequently spent the test exploring and moving without exiting the perimeter. However, as no other differences were consistent throughout subsequent days, this locomotor difference likely had no lasting impact.

Supporting the notion that motor performance remains unaffected, the outcomes of the OFT indicate a significant increase in the distance traveled by animals injected with Kp234 compared to the vehicle group. Additionally, their mean speed exhibited an increase, leading to reduced resting time. These findings might suggest a heightened sense of stress, as animals spend considerable time circling. However, metrics directly linked to stress-like behavior, such as time spent in the center and grooming behavior, showed no changes. Notably, whether greater distances traversed inherently denotes increased stress remains debatable, as animals may simply be more curious.

A study conducted by Sturman et al. in 2018 (116) proposed two metrics aimed at distinguishing exploratory and stress behaviors. They demonstrated that supported and unsupported rearing correlated distinctly with stress metrics. Unsupported rearing strongly correlated with center time, while supported rearing showed a closer association with distance covered and exploratory actions. Given the absence of differences in unsupported rearing, these findings align with the hypothesis that Kp234 animals did not experience more stress. Furthermore, among males, an increased time in supported rearing was observed, suggesting that males injected with Kp234 are more inclined to explore. This aligns with results from a study by Vanryzin, which involved temporary microglia depletion at birth and showcased heightened hyperlocomotive behavior and increased disinhibition behaviors. Therefore, we posit that KP neonatal blockade induces hyperlocomotive behavior as well, hinting at the possibility that the blockade of KP and microglia in the same period elicits similar outcomes.

The findings related to play behavior shed light on the nuanced influence of neonatal KP blockade. Notably, our results indicate that male Kp234 subjects exhibited reduced play behavior compared to their vehicle-treated counterparts, particularly affecting pouncing behavior. Moreover, this influence was notably prominent around PND29, a crucial developmental stage. This effect could be rooted in altered neural circuits and signaling mechanisms that govern such behaviors.

Interestingly, our study also unveiled a broader trend—a decrease in play behavior across all test days, observed in both male and female animals. This trend raises questions about the interplay of various factors contributing to the modulation of play behavior. Additionally, the disturbance in play behavior following treatment with the antagonist implies a multifaceted interrelationship between KP and the intricate neural networks

underpinning play behavior. This points to the role of KP in shaping the overall neural landscape that orchestrates social interactions.

Furthermore, our results align with a study by Vanryzin in 2019 (42), which explored the impact of testosterone in females at birth. Their study revealed that masculinized females presented male-like play behavior mediated by microglia morphology in the AMY. These behavioral outcomes strikingly mirror the effects of KP blockade within the same developmental timeframe, highlighting the interconnected nature of KP and microglia in influencing play behavior.

In essence, our exploration of play behavior provides a comprehensive view of how neonatal KP mainly influences male social interactions during playful activities. The subtle interplay between KP and microglia underscores the complex nature of play behavior and points towards avenues for deeper investigation into their interplay in shaping social behavior.

However, sexual behavior did not present significant alterations. Importantly, we did not strictly follow the normal protocol for testing male sexual behavior. Most studies employ receptive primed females, while we used naturally cycling females. Additionally, the literature advises conducting two trials in two consecutive weeks to familiarize males and enhance sexual behavior. However, due to time limitations, we only performed one trial, potentially missing important insights into male sexual performance. Nonetheless, significant differences were found in the latency to engage in sexual behavior in males. Male Kp234 engaged in these behaviors much faster than male Vehicle. Latency is more associated with the motivation to engage in the behavior rather than better performance in sexual behavior (121). These findings were unexpected since a recent study reported that KP IP injection enhances sexual motivation in males (122). Consequently, further studies are imperative to elucidate the true impact of neonatal KP on male sexual behavior. If these findings prove reproducible, it may suggest that KP serves distinct functions during different developmental stages in modulating male sexual behavior. Moreover, we can speculate that the heightened motivation observed in males injected with Kp234 could be associated with the increased exploratory tendencies noticed in these animals.

Ultimately, our goal was to create a comprehensive picture to understand how these behavioral differences impact gendered behavior. To achieve this, we conducted a PCA with all behavioral parameters from all tests. The results are self-explanatory: in both males and females, we were surprisingly able to cluster two distinct populations in our sample. This result serves as proof of concept that neonatal KP signaling triggers essential mechanisms that persist throughout life to maintain a gender-typical behavior profile.

As we endeavor to shed light on the underlying mechanisms of our behavioral discoveries, we aimed to investigate the potential factors at play. However, our examination of KP receptor levels in the hypothalamus did not yield any significant findings. It is important to note that these results offer a broad overview. The diversity of hypothalamic nuclei could potentially lead to varying receptor levels between groups in different nuclei. Additionally, given the multitude of neuronal cell types populating the hypothalamus, the impact of KP blockade on KISS1R might manifest differently.

However, there seems to exist an influence of KP on microglia morphology and therefore function in the mPOA, mainly in males. However confirmatory quantitative studies are needed. To gain a more comprehensive understanding, more refined studies are imperative. Conducting immunolabeling of KISS1R and assessing receptor levels across each hypothalamic nucleus and different brain regions such as the AMY would provide valuable insights. Performing colocalization studies with various cell types within the hypothalamus would elucidate whether KP exerts a direct influence on these specific cells or not.

6. CONCLUSION AND FUTURE RESEARCH

This study comprehended sustained clear evidence on the role of KP in the sexual differentiation of the brain. For instance, a neonatal short-lived blockade of the signaling KP pathway was sufficient to shift gender-typical behavior.

Beginning in infancy with communication alterations, passing through the intricacies of juvenile behavior, including a hyperlocomotive profile, an increased tendency to explore, and also, impaired social interaction and social communication. Ending in an increased male sexual motivation. Males and females were differently affected by KP blockade, but overall, we were able to demonstrate that KP signaling affects both sexes.

In the end, there is a hunger to uncover so much more after what we have discovered in this study. The results have opened up new pathways for research that hold a lot of promise. Now that we have a sense of the big picture, it is time to zoom in and understand the nitty-gritty details of how neonatal KP affects our behaviors. Techniques like immunolabeling of KISS1R could give us more insights into the specific brain regions and neural pathways that are influenced.

Our study has shown that the effects of neonatal KP modulation last into adulthood. But what happens in the long run? How do these changes impact our development and overall behavior over time? This is a crucial area for further investigation. Additionally, it is fascinating how KP seems to impact males and females differently. Delving into the molecular and neural drivers behind these gender-specific outcomes will deepen our understanding of how KP contributes to shaping gender-typical behaviors.

A key point that triggers great interest is to find the connection between KP and the role of the microglia. Future research efforts should aim to unravel the intricate signaling pathways and molecular interplays that underlie this intriguing relationship.

As we learn more, it might be worth thinking about how our findings could be applied in real-world scenarios. Could this knowledge lead to new treatments for certain conditions? It could help provide new approaches to treating sex-dependent diseases? Exploring practical applications of our research could be exciting. Advanced imaging techniques like optogenetics and functional magnetic resonance imaging (MRI) could give us a closer look at what is happening in the brain. This could reveal the specific brain

regions that are influenced by KP shed light on the intricate neural circuits in numerous behavioral outcomes and help translate our findings.

All in all, we have laid a strong foundation with this study, but there is so much more to uncover. The journey ahead promises to be full of exciting discoveries that will deepen our understanding of how neonatal KP shapes our behaviors.

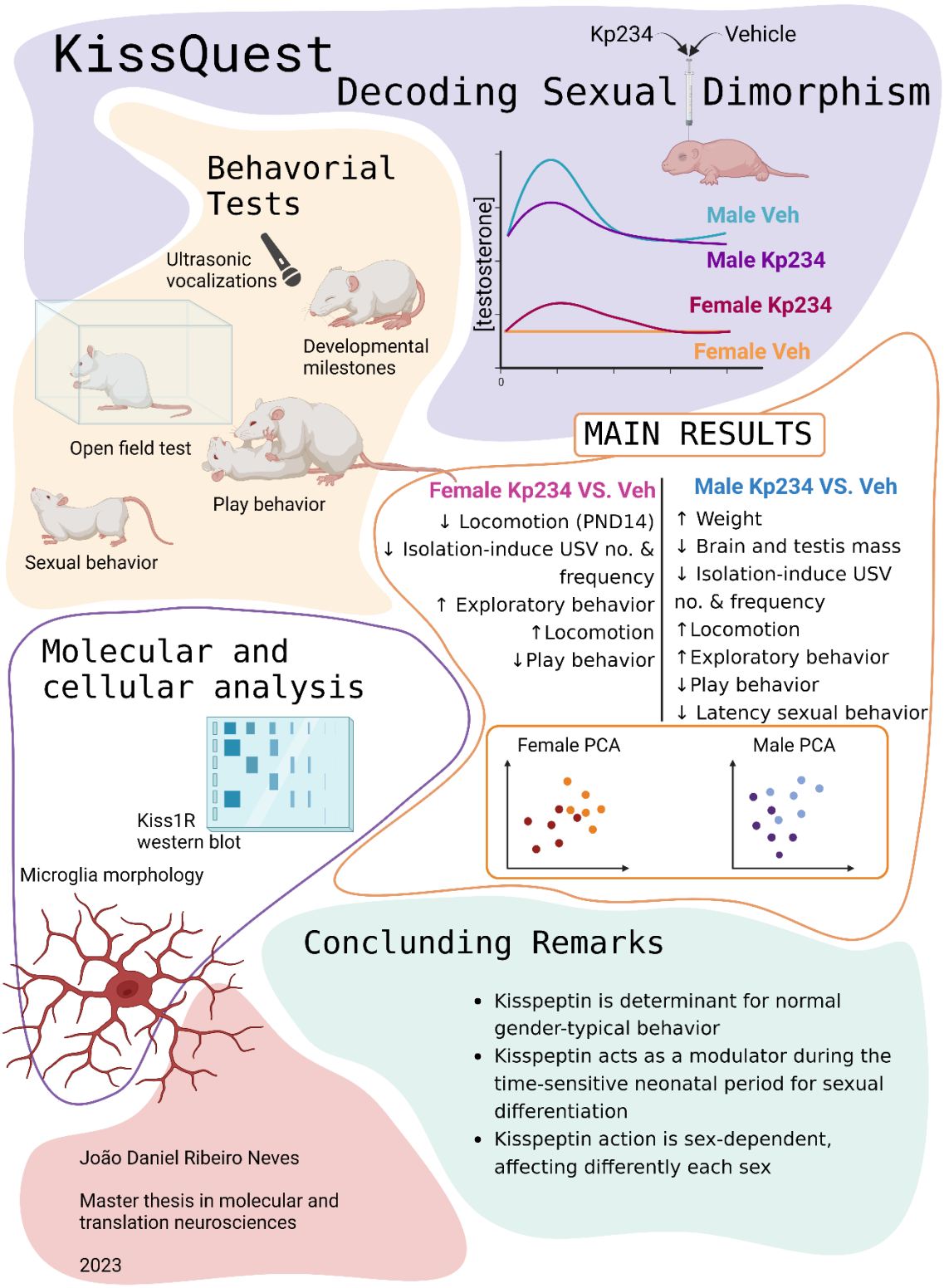


FIGURE 35 GRAPHICAL ABSTRACT

Original image created with BioRender.

7. REFERENCES

1. Terrin F, Tesoriere A, Plotegher N, Dalla Valle L. Sex and Brain: The Role of Sex Chromosomes and Hormones in Brain Development and Parkinson's Disease. Cells 2023, Vol 12, Page 1486 [Internet]. 2023 May 27 [cited 2023 Aug 10];12(11):1486. Available from: https://www.mdpi.com/2073-4409/12/11/1486/htm

2. Mauvais-Jarvis F, Merz NB, Barnes PJ, Brinton RD, Carrero JJ, Demeo DL, et al. Sex and gender: modifiers of health, disease, and medicine [Internet]. Vol. 396, www.thelancet.com. 2020. Available from: www.thelancet.com

3. Cahill L. Why sex matters for neuroscience. Vol. 7, Nature Reviews Neuroscience. 2006. p. 477–84.

4. McCarthy MM, Arnold AP, Ball GF, Blaustein JD, de Vries GJ. Sex Differences in the Brain: The Not So Inconvenient Truth. The Journal of Neuroscience [Internet]. 2012 Feb 2 [cited 2022 Oct 22];32(7):2241. Available from: /pmc/articles/PMC3295598/

5. He Q, Huang M, Cao X, Zhang K, Liu J, Quan F. Advancements in mammalian X and Y sperm differences and sex control technology. Zygote [Internet]. 2022 [cited 2023 Aug 11];30(4). Available from: https://pubmed.ncbi.nlm.nih.gov/35220992/

6. Hiramatsu R, Matoba S, Kanai-Azuma M, Tsunekawa N, Katoh-Fukui Y, Kurohmaru M, et al. A critical time window of Sry action in gonadal sex determination in mice. Development [Internet]. 2009 [cited 2023 Aug 11];136(1):129–38. Available from: https://pubmed.ncbi.nlm.nih.gov/19036799/

7. Sinisi AA, Pasquali D, Notaro A, Bellastella A. Sexual differentiation. J Endocrinol Invest. 2003;26(3 Suppl):23–8.

8. McCarthy MM, Arnold AP. Reframing sexual differentiation of the brain. Nat Neurosci [Internet]. 2011 Jun [cited 2023 Aug 12];14(6):677. Available from: /pmc/articles/PMC3165173/

9. Sekido R, Lovell-Badge R. Genetic control of testis development. Sex Dev [Internet]. 2013 [cited 2023 Aug 12];7(1–3):21–32. Available from: https://pubmed.ncbi.nlm.nih.gov/22964823/

10. Sekido R, Lovell-Badge R. Genetic control of testis development. Sex Dev [Internet]. 2013 [cited 2023 Aug 11];7(1–3):21–32. Available from: https://pubmed.ncbi.nlm.nih.gov/22964823/

11. Mccarthy MM, Arnold AP. Reframing sexual differentiation of the brain. Nat Neurosci. 2011;14(6):677–83.

12. Joel D, Garcia-Falgueras A, Swaab D. The Complex Relationships between Sex and the Brain. https://doi.org/101177/1073858419867298 [Internet]. 2019 Sep 11 [cited 2023 Aug 12];26(2):156–69. Available from: https://journals.sagepub.com/doi/10.1177/1073858419867298?url_ver=Z39.88-2003&rfr_id=ori%3Arid%3Acrossref.org&rfr_dat=cr_pub++0pubmed

13. Sánchez FJ, Vilain E. Genes and brain sex differences. Prog Brain Res [Internet]. 2010 [cited 2022 Oct 22];186(C):64–76. Available from: https://pubmed.ncbi.nlm.nih.gov/21094886/

14. McCarthy MM. A new view of sexual differentiation of mammalian brain. J Comp Physiol A Neuroethol Sens Neural Behav Physiol [Internet]. 2020 May 1 [cited 2023 Aug 12];206(3):369–78. Available from: https://link.springer.com/article/10.1007/s00359-019-01376-8

15. Chen J, Minabe S, Munetomo A, Magata F, Sato M, Nakamura S, et al. Kiss1-dependent and independent release of luteinizing hormone and testosterone in perinatal male rats. Endocr J [Internet]. 2022 [cited 2022 Oct 22];69(7):797–807. Available from: https://pubmed.ncbi.nlm.nih.gov/35125377/

16. Corbier P, Roffi J, Rhoda J. Female Sexual Behavior in Male Rats: Effect of Hour of Castration at Birth. Vol. 30, Physiology & Behavior. Pergamon Press Ltd; 1983.

17. Simões-Henriques CF, Rodrigues-Neves AC, Sousa FJ, Gaspar R, Almeida I, Baptista FI, et al. Neonatal testosterone voids sexually differentiated microglia morphology and behavior. Front Endocrinol (Lausanne) [Internet]. 2023 [cited 2023 Aug 11];14. Available from: https://pubmed.ncbi.nlm.nih.gov/36926023/

18. Panzica GC, Melcangi RC. The endocrine nervous system: source and target for neuroactive steroids. Brain Res Rev [Internet]. 2008 Mar 14 [cited 2023 Aug 12];57(2):271–6. Available from: https://pubmed.ncbi.nlm.nih.gov/18355582/

19. Kawata M. Roles of steroid hormones and their receptors in structural organization in the nervous system. Neurosci Res [Internet]. 1995 [cited 2023 Aug 12];24(1):1–46. Available from: https://pubmed.ncbi.nlm.nih.gov/8848287/

20. Sakamoto H, Takahashi H, Matsuda KI, Nishi M, Takanami K, Ogoshi M, et al. Rapid signaling of steroid hormones in the vertebrate nervous system. Front Biosci (Landmark Ed) [Internet]. 2012 Jan 1 [cited 2023 Aug 12];17(3):996–1019. Available from: https://pubmed.ncbi.nlm.nih.gov/22201787/

21. Marrocco J, McEwen BS. Sex in the brain: hormones and sex differences. Dialogues Clin Neurosci [Internet]. 2016 [cited 2022 Oct 22];18(4):373. Available from: /pmc/articles/PMC5286723/

22. Psarra AMG, Solakidi S, Sekeris CE. The mitochondrion as a primary site of action of steroid and thyroid hormones: presence and action of steroid and thyroid hormone receptors in mitochondria of animal cells. Mol Cell Endocrinol [Internet]. 2006 Feb 26 [cited 2023 Aug 12];246(1–2):21–33. Available from: https://pubmed.ncbi.nlm.nih.gov/16388892/

23. Gaignard P, Liere P, Thérond P, Schumacher M, Slama A, Guennoun R. Role of sex hormones on brain mitochondrial function, with special reference to aging and neurodegenerative diseases. Front Aging Neurosci. 2017 Dec 7;9(DEC):318013.

24. Loiola RA, Wickstead ES, Solito E, McArthur S. Estrogen Promotes Pro-resolving Microglial Behavior and Phagocytic Cell Clearance through the Actions of Annexin A1. Front Endocrinol (Lausanne) [Internet]. 2019 [cited 2023 Aug 12];10(JUN):420. Available from: /pmc/articles/PMC6607409/

25. Mong JA, Nuñez JL, McCarthy MM. GABA Mediates Steroid-Induced Astrocyte Differentiation in the Neonatal Rat Hypothalamus. J Neuroendocrinol [Internet]. 2002 Jan 1 [cited 2023 Aug 12];14(1):45–55. Available from: https://onlinelibrary.wiley.com/doi/full/10.1046/j.1365-2826.2002.00737.x

26. Tobet S, Knoll JG, Hartshorn C, Aurand E, Stratton M, Kumar P, et al. Brain sex differences and hormone influences: a moving experience? J Neuroendocrinol [Internet]. 2009 [cited 2023 Aug 12];21(4):387–92. Available from: https://pubmed.ncbi.nlm.nih.gov/19207813/

27. McEwen BS, Milner TA. Understanding the broad influence of sex hormones and sex differences in the brain. J Neurosci Res [Internet]. 2017 Jan 1 [cited 2022 Oct 22];95(1–2):24–39. Available from: https://pubmed.ncbi.nlm.nih.gov/27870427/

28. Pérez SE, Chen EY, Mufson EJ. Distribution of estrogen receptor alpha and beta immunoreactive profiles in the postnatal rat brain. Developmental Brain Research. 2003 Oct 10;145(1):117–39.

29. Hammes SR, Levin ER. Extranuclear Steroid Receptors: Nature and Actions. Endocr Rev [Internet]. 2007 Dec 1 [cited 2023 Aug 12];28(7):726–41. Available from: https://dx.doi.org/10.1210/er.2007-0022

30. Schwarz JM, McCarthy MM. The role of neonatal NMDA receptor activation in defeminization and masculinization of sex behavior in the rat. Horm Behav. 2008 Nov 1;54(5):662–8.

31. Schwarz JM, Liang SL, Thompson SM, McCarthy MM. Estradiol Induces Hypothalamic Dendritic Spines by Enhancing Glutamate Release: A Mechanism for Organizational Sex Differences. Neuron [Internet]. 2008 May 22 [cited 2023 Aug 12];58(4):584–98. Available from: http://www.cell.com/article/S0896627308002225/fulltext

32. Kuwahara N, Nicholson K, Isaacs L, MacLusky NJ. Androgen Effects on Neural Plasticity. Androgens [Internet]. 2021 Dec 1 [cited 2023 Aug 13];2(1):216. Available from: /pmc/articles/PMC8744448/

33. Bathina S, Das UN. Brain-derived neurotrophic factor and its clinical implications. Arch Med Sci [Internet]. 2015 Dec 12 [cited 2023 Aug 23];11(6):1164. Available from: /pmc/articles/PMC4697050/

34. Guennoun R. Progesterone in the Brain: Hormone, Neurosteroid and Neuroprotectant. International Journal of Molecular Sciences 2020, Vol 21, Page 5271 [Internet]. 2020 Jul 24 [cited 2023 Aug 12];21(15):5271. Available from: https://www.mdpi.com/1422-0067/21/15/5271/htm

35. McCarthy MM, Herold K, Stockman SL. Fast, furious and enduring: Sensitive versus critical periods in sexual differentiation of the brain. Physiol Behav [Internet]. 2018 Apr 1 [cited 2023 Aug 12];187:13–9. Available from: https://pubmed.ncbi.nlm.nih.gov/29101011/

36. Grgurevic N, Majdic G. Sex differences in the brain–an interplay of sex steroid hormones and sex chromosomes. Clin Sci [Internet]. 2016 Sep 1 [cited 2023 Aug 12];130(17):1481–97. Available from: /clinsci/article/130/17/1481/71348/Sex-differences-in-the-brain-an-interplay-of-sex

37. McEwen BS, Morrison JH. The brain on stress: vulnerability and plasticity of the prefrontal cortex over the life course. Neuron [Internet]. 2013 Jul 10 [cited 2023 Aug 14];79(1):16–29. Available from: https://pubmed.ncbi.nlm.nih.gov/23849196/

38. Krebs-Kraft DL, Hill MN, Hillard CJ, McCarthy MM. Sex difference in cell proliferation in developing rat amygdala mediated by endocannabinoids has implications for social behavior. Proc Natl Acad Sci U S A [Internet]. 2010 Nov 23 [cited 2023 Aug 14];107(47):20535–40. Available from: https://pubmed.ncbi.nlm.nih.gov/21059913/

39. Kim D, Thayer SA. Cannabinoids Inhibit the Formation of New Synapses between Hippocampal Neurons in Culture. Journal of Neuroscience [Internet]. 2001 May 15 [cited 2023 Aug 14];21(10):RC146–RC146. Available from: https://www.jneurosci.org/content/21/10/RC146

40. Rueda D, Navarro B, Martínez-Serrano A, Guzmán M, Galve-Roperh I. The endocannabinoid anandamide inhibits neuronal progenitor cell differentiation through attenuation of the Rap1/B-Raf/ERK pathway. Journal of Biological Chemistry [Internet]. 2002 Nov 29 [cited 2023 Aug 14];277(48):46645–50. Available from: http://www.jbc.org/article/S0021925819332818/fulltext

41. Galve-Roperh I, Aguado T, Rueda D, Velasco G, Guzman M. Endocannabinoids: A New Family of Lipid Mediators Involved in the Regulation of Neural Cell Development. Curr Pharm Des. 2006 Jun 10;12(18):2319–25.

42. Aguado T, Palazuelos J, Monory K, Stella N, Cravatt B, Lutz B, et al. The Endocannabinoid System Promotes Astroglial Differentiation by Acting on Neural Progenitor Cells. Journal of Neuroscience [Internet]. 2006 Feb 1 [cited 2023 Aug 14];26(5):1551–61. Available from: https://www.jneurosci.org/content/26/5/1551

43. VanRyzin JW, Marquardt AE, Argue KJ, Vecchiarelli HA, Ashton SE, Arambula SE, et al. Microglial Phagocytosis of Newborn Cells Is Induced by Endocannabinoids and Sculpts Sex Differences in Juvenile Rat Social Play. Neuron [Internet]. 2019 Apr 17 [cited 2023 Aug 14];102(2):435-449.e6. Available from: https://pubmed.ncbi.nlm.nih.gov/30827729/

44. Meeh KL, Rickel CT, Sansano AJ, Shirangi TR. The development of sex differences in the nervous system and behavior of flies, worms, and rodents. Dev Biol. 2021 Apr 1;472:75–84.

45. VanRyzin J, Marquardt A, McCarthy M. Assessing Rough-and-tumble Play Behavior in Juvenile Rats. Bio Protoc. 2020;10(1).

46. VanRyzin JW, Marquardt AE, Pickett LA, McCarthy MM. Microglia and sexual differentiation of the developing brain: A focus on extrinsic factors. Glia [Internet]. 2020 Jun 1 [cited 2022 Oct 22];68(6):1100–13. Available from: https://onlinelibrary.wiley.com/doi/full/10.1002/glia.23740

47. Bear MH, Reddy V, Bollu PC. Neuroanatomy, Hypothalamus. StatPearls [Internet]. 2022 Oct 10 [cited 2023 Aug 14]; Available from: https://www.ncbi.nlm.nih.gov/books/NBK525993/

48. Qin C, Li J, Tang K. The Paraventricular Nucleus of the Hypothalamus: Development, Function, and Human Diseases. Endocrinology [Internet]. 2018 [cited 2023 Aug 14];159(9):3458–72. Available from: https://pubmed.ncbi.nlm.nih.gov/30052854/

49. Wei YC, Wang SR, Jiao ZL, Zhang W, Lin JK, Li XY, et al. Medial preoptic area in mice is capable of mediating sexually dimorphic behaviors regardless of gender. Nat Commun [Internet]. 2018 Dec 1 [cited 2023 Aug 14];9(1). Available from: https://pubmed.ncbi.nlm.nih.gov/29348568/

50. Patton AP, Hastings MH. The suprachiasmatic nucleus. Curr Biol [Internet]. 2018 Aug 6 [cited 2023 Aug 14];28(15):R816–22. Available from: https://pubmed.ncbi.nlm.nih.gov/30086310/

51. Whiting AC, Oh MY, Whiting DM. Deep brain stimulation for appetite disorders: a review. Neurosurg Focus [Internet]. 2018 Aug 1 [cited 2023 Aug 14];45(2). Available from: https://pubmed.ncbi.nlm.nih.gov/30064311/

52. Harter CJL, Kavanagh GS, Smith JT. The role of kisspeptin neurons in reproduction and metabolism. J Endocrinol [Internet]. 2018 Sep 1 [cited 2023 Aug 14];238(3):R173–83. Available from: https://pubmed.ncbi.nlm.nih.gov/30042117/

53. Dillingham CM, Frizzati A, Nelson AJD, Vann SD. How do mammillary body inputs contribute to anterior thalamic function? Neurosci Biobehav Rev [Internet]. 2015 Jul 1 [cited 2023 Aug 14];54:108–19. Available from: https://pubmed.ncbi.nlm.nih.gov/25107491/

54. Park SG, Jeong YC, Kim DG, Lee MH, Shin A, Park G, et al. Medial preoptic circuit induces hunting-like actions to target objects and prey. Nature Neuroscience 2018 21:3 [Internet]. 2018 Jan 29 [cited 2023 Aug 14];21(3):364–72. Available from: https://www.nature.com/articles/s41593-018-0072-x

55. Kohl J, Babayan BM, Rubinstein ND, Autry AE, Marin-Rodriguez B, Kapoor V, et al. Functional circuit architecture underlying parental behaviour. Nature 2018 556:7701 [Internet]. 2018 Apr 11 [cited 2023 Aug 14];556(7701):326–31. Available from: https://www.nature.com/articles/s41586-018-0027-0

56. Ishii KK, Osakada T, Mori H, Miyasaka N, Yoshihara Y, Miyamichi K, et al. A Labeled-Line Neural Circuit for Pheromone-Mediated Sexual Behaviors in Mice. Neuron [Internet]. 2017 Jul 5 [cited 2023 Aug 14];95(1):123-137.e8. Available from: https://pubmed.ncbi.nlm.nih.gov/28648498/

57. Chung S, Weber F, Zhong P, Tan CL, Nguyen TN, Beier KT, et al. Identification of preoptic sleep neurons using retrograde labelling and gene profiling. Nature [Internet]. 2017 May 25 [cited 2023 Aug 14];545(7655):477–81. Available from: https://pubmed.ncbi.nlm.nih.gov/28514446/

58. Hashikawa K, Hashikawa Y, Falkner A, Lin D. The neural circuits of mating and fighting in male mice. Curr Opin Neurobiol [Internet]. 2016 Jun 1 [cited 2023 Aug 14];38:27–37. Available from: https://pubmed.ncbi.nlm.nih.gov/26849838/

59. Hull EM, Dominguez JM. Sexual behavior in male rodents. Horm Behav [Internet]. 2007 Jun [cited 2023 Aug 14];52(1):45–55. Available from: https://pubmed.ncbi.nlm.nih.gov/17499249/

60. Tsuneoka Y, Maruyama T, Yoshida S, Nishimori K, Kato T, Numan M, et al. Functional, anatomical, and neurochemical differentiation of medial preoptic area subregions in relation to maternal behavior in the mouse. J Comp Neurol [Internet]. 2013 May 1 [cited 2023 Aug 14];521(7):1633–63. Available from: https://pubmed.ncbi.nlm.nih.gov/23124836/

61. Nakamura K. Central circuitries for body temperature regulation and fever. Am J Physiol Regul Integr Comp Physiol [Internet]. 2011 Nov [cited 2023 Aug 14];301(5). Available from: https://pubmed.ncbi.nlm.nih.gov/21900642/

62. Tsuneoka Y. Molecular neuroanatomy of the mouse medial preoptic area with reference to parental behavior. Anat Sci Int [Internet]. 2019 Jan 25 [cited 2023 Aug 14];94(1):39–52. Available from: https://pubmed.ncbi.nlm.nih.gov/30392107/

63. Lenz KM, Pickett LA, Wright CL, Davis KT, Joshi A, McCarthy MM. Mast Cells in the Developing Brain Determine Adult Sexual Behavior. J Neurosci [Internet]. 2018 Sep 12 [cited 2023 Aug 14];38(37):8044–59. Available from: https://pubmed.ncbi.nlm.nih.gov/30093566/

64. Lazarus M, Yoshida K, Coppari R, Bass CE, Mochizuki T, Lowell BB, et al. EP3 prostaglandin receptors in the median preoptic nucleus are critical for fever responses. Nat Neurosci [Internet]. 2007 Sep [cited 2023 Aug 14];10(9):1131–3. Available from: https://pubmed.ncbi.nlm.nih.gov/17676060/

65. Lenz KM, Nugent BM, Haliyur R, McCarthy MM. Microglia are essential to masculinization of brain and behavior. J Neurosci [Internet]. 2013 Feb 13 [cited 2023 Aug 14];33(7):2761–72. Available from: https://pubmed.ncbi.nlm.nih.gov/23407936/

66. Wright CL, Burks SR, McCarthy MM. Identification of prostaglandin E2 receptors mediating perinatal masculinization of adult sex behavior and neuroanatomical correlates. Dev Neurobiol [Internet]. 2008 Oct [cited 2023 Aug 14];68(12):1406–19. Available from: https://pubmed.ncbi.nlm.nih.gov/18726914/

67. Vanryzin JW, Yu SJ, Perez-Pouchoulen M, McCarthy MM. Temporary depletion of microglia during the early postnatal period induces lasting sex-dependent and sex-independent effects on behavior in rats. eNeuro. 2016 Nov 29;3(6).

68. Raznahan A, Disteche CM. X-chromosome regulation and sex differences in brain anatomy. Neurosci Biobehav Rev [Internet]. 2021 Jan 1 [cited 2022 Oct 22];120:28–47. Available from: https://pubmed.ncbi.nlm.nih.gov/33171144/

69. Bakker JU de LUl> G> GNN. The role of steroid hormones in the sexual differentiation of the human brain. J Neuroendocrinol [Internet]. 2022 Feb 1 [cited 2023 Aug 13];34(2). Available from: https://orbi.uliege.be/handle/2268/289284

70. Krishnan S, Intlekofer KA, Aggison LK, Petersen SL. Central role of TRAF-interacting protein in a new model of brain sexual differentiation. Proc Natl Acad Sci U S A [Internet]. 2009 Sep 29 [cited 2023 Aug 13];106(39):16692–7. Available from: https://pubmed.ncbi.nlm.nih.gov/19805359/

71. Waters EM, Simerly RB. Estrogen induces caspase-dependent cell death during hypothalamic development. J Neurosci [Internet]. 2009 Aug 5 [cited 2023 Aug 13];29(31):9714–8. Available from: https://pubmed.ncbi.nlm.nih.gov/19657024/

72. Simerly RB. Wired for reproduction: organization and development of sexually dimorphic circuits in the mammalian forebrain. Annu Rev Neurosci [Internet]. 2002 [cited 2023 Aug 13];25:507–36. Available from: https://pubmed.ncbi.nlm.nih.gov/12052919/

73. De Vries GJ, Jardon M, Reza M, Rosen GJ, Immerman E, Forger NG. Sexual Differentiation of Vasopressin Innervation of the Brain: Cell Death Versus Phenotypic Differentiation. Endocrinology [Internet]. 2008 Sep [cited 2023 Aug 13];149(9):4632. Available from: /pmc/articles/PMC2553370/

74. Lee JH, Miele ME, Hicks DJ, Phillips KK, Trent JM, Weissman BE, et al. KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. J Natl Cancer Inst [Internet]. 1996 [cited 2023 Aug 16];88(23):1731–7. Available from: https://pubmed.ncbi.nlm.nih.gov/8944003/

75. De Tassigny XDA, Fagg LA, Dixon JPC, Day K, Leitch HG, Hendrick AG, et al. Hypogonadotropic hypogonadism in mice lacking a functional Kiss1 gene. Proc Natl Acad Sci U S A [Internet]. 2007 Jun 19 [cited 2023 Aug 16];104(25):10714–9. Available from: https://pubmed.ncbi.nlm.nih.gov/17563351/

76. Teles MG, Bianco SDC, Brito VN, Trarbach EB, Kuohung W, Xu S, et al. A GPR54-activating mutation in a patient with central precocious puberty. N Engl J Med [Internet]. 2008 Feb 14 [cited 2023 Aug 16];358(7):709–15. Available from: https://pubmed.ncbi.nlm.nih.gov/18272894/

77. West A, Vojta PJ, Welch DR, Weissman BE. Chromosome localization and genomic structure of the KiSS-1 metastasis suppressor gene (KISS1). Genomics [Internet]. 1998 Nov 15 [cited 2022 Oct 28];54(1):145–8. Available from: https://pubmed.ncbi.nlm.nih.gov/9806840/

78. Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, et al. The Metastasis Suppressor Gene KiSS-1 Encodes Kisspeptins, the Natural Ligands of the Orphan G Protein-coupled Receptor GPR54. Journal of Biological Chemistry. 2001 Sep 14;276(37):34631–6.

79. Gottsch ML, Clifton DK, Steiner RA. From KISS1 to Kisspeptins: An Historical Perspective and Suggested Nomenclature. Peptides (NY) [Internet]. 2009 Jan [cited 2023 Aug 16];30(1):4. Available from: /pmc/articles/PMC2683679/

80. Oakley AE, Clifton DK, Steiner RA. Kisspeptin signaling in the brain. Vol. 30, Endocrine Reviews. 2009. p. 713–43.

81. Silvestre RA, Egido EM, Hernández R, Marco J. Kisspeptin-13 inhibits insulin secretion without affecting glucagon or somatostatin release: Study in the perfused rat pancreas. Journal of Endocrinology. 2008 Feb;196(2):283–90.

82. Izzi-Engbeaya C, Dhillo WS. Emerging roles for kisspeptin in metabolism. J Physiol [Internet]. 2022 Mar 1 [cited 2023 Aug 16];600(5):1079–88. Available from: https://pubmed.ncbi.nlm.nih.gov/33977536/

83. Zhang P, Tang M, Zhong T, Lin Y, Zong T, Zhong C, et al. Expression and function of kisspeptin during mouse decidualization. PLoS One. 2014 May 15;9(5).

84. Cejudo Roman A, Pinto FM, Dorta I, Almeida TA, Hernández M, Illanes M, et al. Analysis of the expression of neurokinin B, kisspeptin, and their cognate receptors NK3R and KISS1R in the human female genital tract. Fertil Steril. 2012 May;97(5):1213–9.

85. Mei H, Doran J, Kyle V, Yeo SH, Colledge WH. Does kisspeptin signaling have a role in the testes? Front Endocrinol (Lausanne). 2013;4(DEC).

86. Hu KL, Zhao H, Chang HM, Yu Y, Qiao J. Kisspeptin/kisspeptin receptor system in the ovary. Vol. 8, Frontiers in Endocrinology. Frontiers Media S.A.; 2018.

87. Muir AI, Chamberlain L, Elshourbagy NA, Michalovich D, Moore DJ, Calamari A, et al. AXOR12, a Novel Human G Protein-coupled Receptor, Activated by the Peptide KiSS-1. Journal of Biological Chemistry. 2001 Aug 1;276(31):28969–75.

88. Lee DK, Nguyen T, O'Neill GP, Cheng R, Liu Y, Howard AD, et al. Discovery of a receptor related to the galanin receptors. FEBS Lett. 1999 Mar 5;446(1):103–7.

89. Herbison AE, De Tassigny XDA, Doran J, Colledge WH. Distribution and Postnatal Development of Gpr54 Gene Expression in Mouse Brain and Gonadotropin-Releasing Hormone Neurons. Endocrinology [Internet]. 2010 Jan 1 [cited 2023 Aug 16];151(1):312–21. Available from: https://dx.doi.org/10.1210/en.2009-0552

90. Zhang C, Roepke TA, Kelly MJ, Rønnekleiv OK. Kisspeptin depolarizes gonadotropin-releasing hormone neurons through activation of TRPC-like cationic channels. Journal of Neuroscience. 2008 Apr 23;28(17):4423–34.

91. Babiker A, Shaikh A Al. The role of kisspeptin signalling in control of reproduction in genetically similar species. Sudan J Paediatr [Internet]. 2016 [cited 2022 Oct 28];16(1):9. Available from: /pmc/articles/PMC5025939/

92. Ruohonen ST, Poutanen M, Tena-Sempere M. Role of kisspeptins in the control of the hypothalamic-pituitary-ovarian axis: old dogmas and new challenges. Vol. 114, Fertility and Sterility. Elsevier Inc.; 2020. p. 465–74.

93. Jayasena CN, Nijher GMK, Narayanaswamy S, De Silva A, Abbara A, Ghatei MA, et al. Age-dependent elevations in plasma kisspeptin are observed in boys and girls when compared with adults. Ann Clin Biochem. 2014 Jan 1;51(1):89–96.

94. Lenz KM, McCarthy MM. Organized for sex – steroid hormones and the developing hypothalamus. Eur J Neurosci [Internet]. 2010 Dec [cited 2022 Oct 28];32(12):2096. Available from: /pmc/articles/PMC5350613/

95. Kuiri-Hänninen T, Seuri R, Tyrväinen E, Turpeinen U, Hämäläinen E, Stenman UH, et al. Increased activity of the hypothalamic-pituitary-testicular axis in infancy results in increased androgen action in premature boys. J Clin Endocrinol Metab [Internet]. 2011 [cited 2022 Oct 28];96(1):98–105. Available from: https://pubmed.ncbi.nlm.nih.gov/20881260/

96. Corbier P, Edwards DA, Roffi J. The neonatal testosterone surge: a comparative study. Arch Int Physiol Biochim Biophys [Internet]. 1992 [cited 2023 Aug 16];100(2):127–31. Available from: https://pubmed.ncbi.nlm.nih.gov/1379488/

97. Lunn SF, Recio R, Morris K, Fraser HM. Blockade of the neonatal rise in testosterone by a gonadotrophin-releasing hormone antagonist: effects on timing of puberty and sexual behaviour in the male marmoset monkey. J Endocrinol [Internet]. 1994 [cited 2023 Aug 16];141(3):439–47. Available from: https://pubmed.ncbi.nlm.nih.gov/8071642/

98. De Zegher F, Devlieger H, Veldhuis JD. Pulsatile and sexually dimorphic secretion of luteinizing hormone in the human infant on the day of birth. Pediatr Res [Internet]. 1992 [cited 2023 Aug 16];32(5):605–7. Available from: https://pubmed.ncbi.nlm.nih.gov/1480465/

99. Ghosh MK, Chen K hui E, Dill-Garlow R, Ma LJ, Yonezawa T, Itoh Y, et al. Sex Differences in the Immune System Become Evident in the Perinatal Period in the Four Core Genotypes Mouse. Front Endocrinol (Lausanne) [Internet]. 2021 May 27 [cited 2022 Oct 28];12. Available from: https://pubmed.ncbi.nlm.nih.gov/34122327/

100. George JT, Seminara SB. Kisspeptin and the hypothalamic control of reproduction: lessons from the human. Endocrinology [Internet]. 2012 Nov 1 [cited 2022 Oct 28];153(11):5130–6. Available from: https://pubmed.ncbi.nlm.nih.gov/23015291/

101. Homma T, Sakakibara M, Yamada S, Kinoshita M, Iwata K, Tomikawa J, et al. Significance of neonatal testicular sex steroids to defeminize anteroventral periventricular kisspeptin neurons and the GnRH/LH surge system in male rats. Biol Reprod. 2009;81(6):1216–25.

102. Swaab DF, Hofman MA. Sexual differentiation of the human hypothalamus: ontogeny of the sexually dimorphic nucleus of the preoptic area. Brain Res Dev Brain Res [Internet]. 1988 Dec 1 [cited 2023 Aug 16];44(2):314–8. Available from: https://pubmed.ncbi.nlm.nih.gov/3224432/

103. Alexander GM. Postnatal testosterone concentrations and male social development. Front Endocrinol (Lausanne) [Internet]. 2014 [cited 2023 Aug 16];5(FEB). Available from: https://pubmed.ncbi.nlm.nih.gov/24600437/

104. Padilla SL, Carmody JS, Zeltser LM. Pomc-expressing progenitors give rise to antagonistic neuronal populations in hypothalamic feeding circuits. Nat Med. 2010 Apr;16(4):403–5.

105. Lee EB, Dilower I, Marsh CA, Wolfe MW, Masumi S, Upadhyaya S, et al. Sexual Dimorphism in Kisspeptin Signaling. Cells [Internet]. 2022 Apr 1 [cited 2022 Oct 22];11(7). Available from: https://pubmed.ncbi.nlm.nih.gov/35406710/

106. Takumi K, Iijima N, Ozawa H. Developmental changes in the expression of kisspeptin mRNA in rat hypothalamus. J Mol Neurosci [Internet]. 2011 Feb [cited 2022 Oct 28];43(2):138–45. Available from: https://pubmed.ncbi.nlm.nih.gov/20665248/

107. Sivalingam M, Parhar IS. Hypothalamic kisspeptin and kisspeptin receptors: Species variation in reproduction and reproductive behaviours. Front Neuroendocrinol. 2022 Jan 1;64:100951.

108. Fujiyama T, Miyashita S, Tsuneoka Y, Kanemaru K, Kakizaki M, Kanno S, et al. Forebrain Ptf1a Is Required for Sexual Differentiation of the Brain. Cell Rep. 2018 Jul 3;24(1):79–94.

109. Hellier V, Brock O, Candlish M, Desroziers E, Aoki M, Mayer C, et al. Female sexual behavior in mice is controlled by kisspeptin neurons. Nat Commun [Internet]. 2018 Dec 1 [cited 2023 Aug 16];9(1). Available from: https://pubmed.ncbi.nlm.nih.gov/29374161/

110. Gresham R, Li S, Adekunbi DA, Hu M, Li XF, TO'Byrne K. Kisspeptin in the medial amygdala and sexual behavior in male rats. Neurosci Lett [Internet]. 2016 Aug 3 [cited 2023 Aug 16];627:13–7. Available from: https://pubmed.ncbi.nlm.nih.gov/27233219/

111. Delmas S, Porteous R, Bergin DH, Herbison AE. Altered aspects of anxiety-related behavior in kisspeptin receptor-deleted male mice. Sci Rep [Internet]. 2018 Dec 1 [cited 2023 Aug 16];8(1):2794. Available from: /pmc/articles/PMC5809376/

112. Roseweir AK, Kauffman AS, Smith JT, Guerriero KA, Morgan K, Pielecka-Fortuna J, et al. Discovery of potent kisspeptin antagonists delineate physiological mechanisms of gonadotropin regulation. J Neurosci [Internet]. 2009 Mar 25 [cited 2023 Aug 17];29(12):3920–9. Available from: https://pubmed.ncbi.nlm.nih.gov/19321788/

113. Xiang J, Li Z, Wan Q, Chen Q, Liu M, Jiang X, et al. A qPCR method to characterize the sex type of the cell strains from rats. OUP [Internet]. 2016 [cited 2023 Aug 16];80(10):1917–24. Available from: https://www.tandfonline.com/doi/abs/10.1080/09168451.2016.1194180

114. Feather-Schussler DN, Ferguson TS. A Battery of Motor Tests in a Neonatal Mouse Model of Cerebral Palsy. J Vis Exp [Internet]. 2016 Nov 3 [cited 2023 Aug 17];2016(117):53569. Available from: /pmc/articles/PMC5226120/

115. Friard O, Gamba M. BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. Methods Ecol Evol [Internet]. 2016 Nov 1 [cited 2023 Aug 16];7(11):1325–30. Available from: https://onlinelibrary.wiley.com/doi/full/10.1111/2041-210X.12584

116. Sturman O, Germain PL, Bohacek J. Exploratory rearing: a context- and stress-sensitive behavior recorded in the open-field test. Stress [Internet]. 2018 Sep 3 [cited 2023 Aug 20];21(5):443–52. Available from: https://www.tandfonline.com/doi/abs/10.1080/10253890.2018.1438405

117. Boulanger-Bertolus J, Mouly AM. Ultrasonic Vocalizations Emission across Development in Rats: Coordination with Respiration and Impact on Brain Neural Dynamics. Brain Sci [Internet]. 2021 [cited 2023 Aug 23];11(5). Available from: /pmc/articles/PMC8150956/

118. Rønnekleiv OK, Qiu J, Kelly MJ. Hypothalamic Kisspeptin Neurons and the Control of Homeostasis. Endocrinology [Internet]. 2022 Feb 1 [cited 2023 Aug 23];163(2). Available from: https://pubmed.ncbi.nlm.nih.gov/34953135/

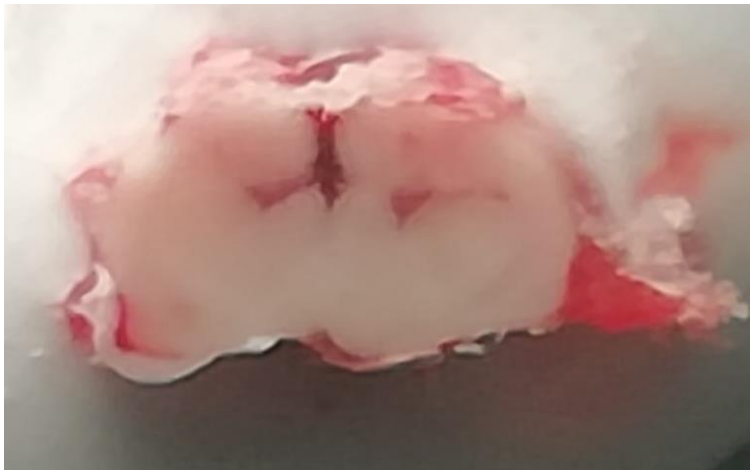
119. Geyer LA, Barfield RJ. Influence of gonadal hormones and sexual behavior on ultrasonic vocalization in rats: I. Treatment of females. J Comp Physiol Psychol [Internet]. 1978 Jun [cited 2023 Aug 23];92(3):438–46. Available from: https://pubmed.ncbi.nlm.nih.gov/681563/

120. Asaba A, Osakada T, Touhara K, Kato M, Mogi K, Kikusui T. Male mice ultrasonic vocalizations enhance female sexual approach and hypothalamic kisspeptin neuron activity. Horm Behav. 2017 Aug 1;94:53–60.

121. Ågmo A. Male rat sexual behavior. Brain Research Protocols. 1997 May 1;1(2):203–9.

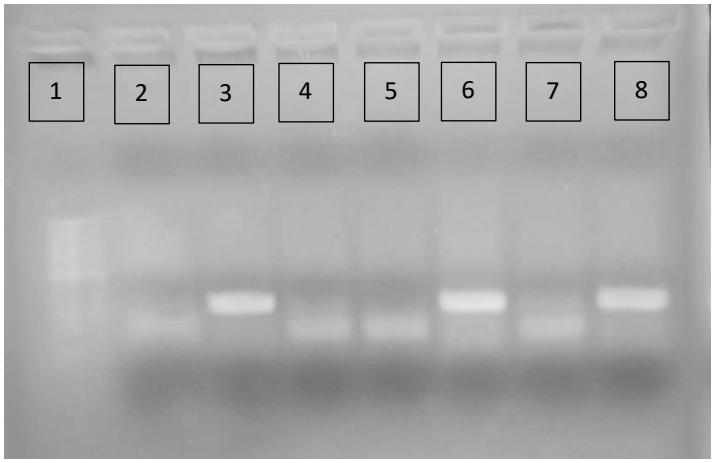
122. Magarramova LA, Tissen IY, Blazhenko AA, Lebedev AA, Loskutov SI, Proshin SN. Kisspeptin is Testosterone independent regulator of Sexual Motivation in Male Rats. Journal of Experimental Biology and Agricultural Sciences [Internet]. 2022 Feb 28 [cited 2023 Aug 22];10(1):131–4. Available from: https://jebas.org/ojs/index.php/jebas/article/view/256

8. SUPPLEMENTARY DATA



ANNEX I REPRESENTATIVE IMAGE OF THE INJECTION SITE WITH THE COORDINATES (0.8,1.2,1.7) MM

Three animals were injected with ink and were sacrificed with their brain removed. The brain was sliced in a cryostat to check macroscopically the location of the injection. In this case, it reaches the ventricles. Importantly a bigger needle was used in this test.



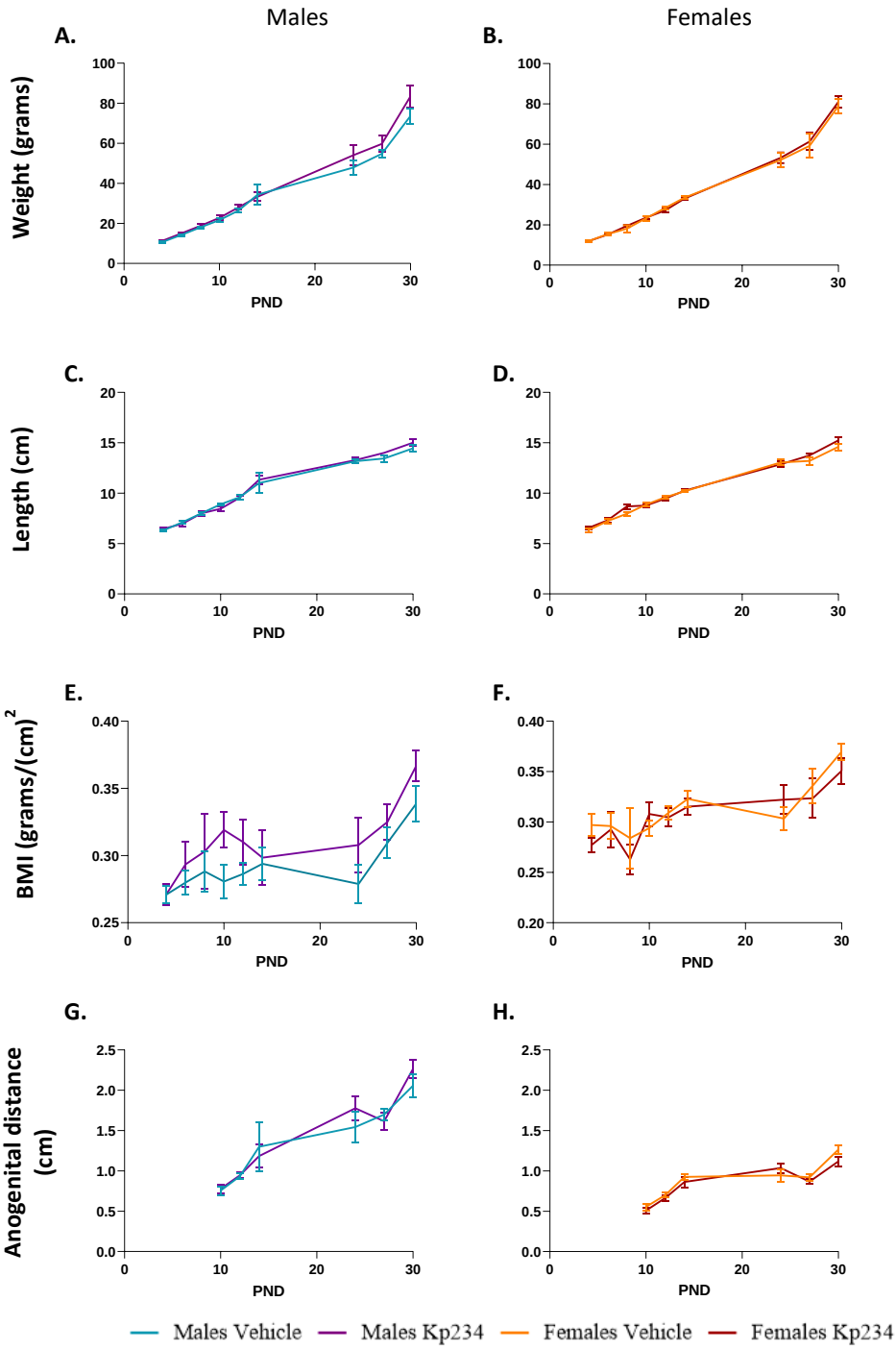
ANNEX II REPRESENTATIVE AGAROSE GEL TO DETERMINE THE SEX OF THE PUPS

An agarose gel was utilized to display the results of genotyping the animals to determine their sex. The gel contained several lanes, each representing a specific sample. The first lane (1) served as a marker for reference. Lane 2 contained DNA from a female adult rat, acting as a positive control for females. Lane 3 contained DNA from a male adult rat, functioning as a positive control for males. Lanes 4, 5, and 7 corresponded to samples that tested negative for SRY, indicating that these pups were females. In contrast, lanes 6 and 8 contained samples that were positive for SRY, confirming that these pups were males.

ANNEX III OUTLIER CRITERIA FOR EACH TEST OR ANALYSIS AND NUMBER OF ANIMALS EXCLUDED PER TEST

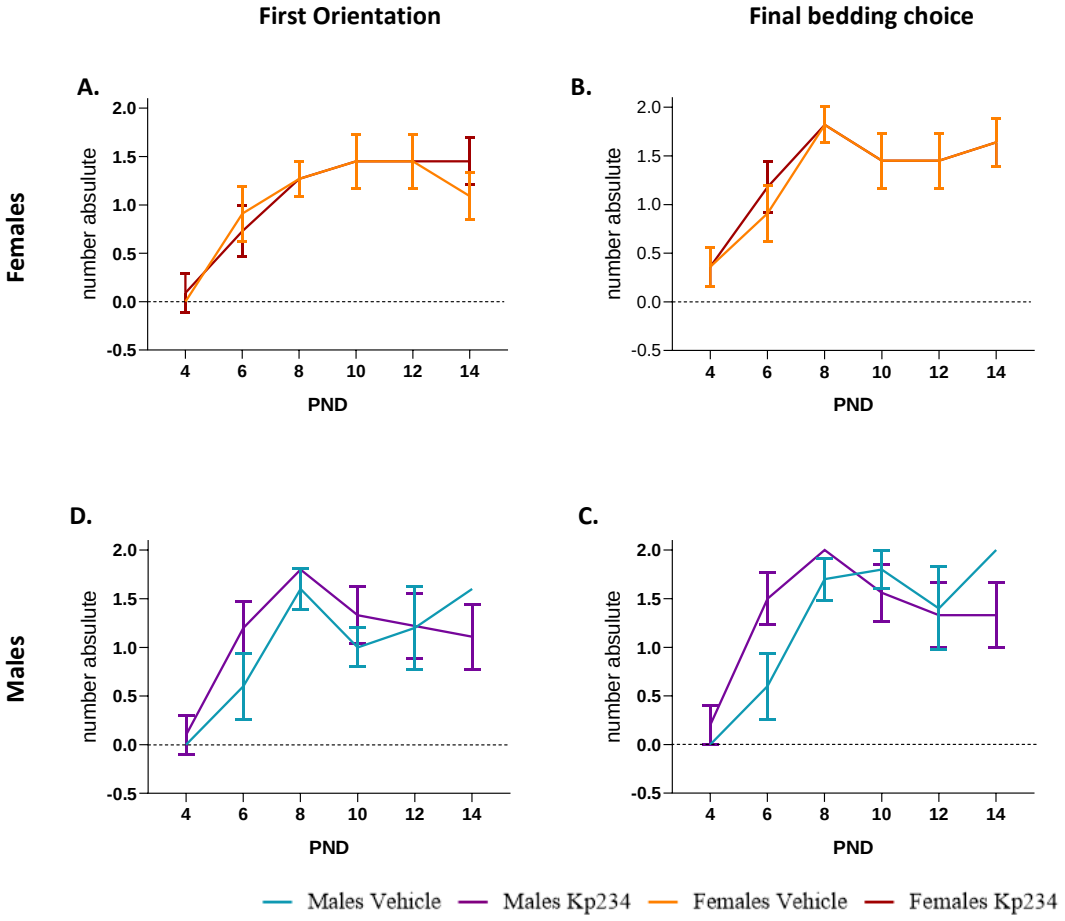
In multiparametric tests, several days or parameters were selected. ROUT was performed with aggressivity of 10%.

Tests	*Criteria*	*No. of exclusion*
Daily records	Outlier in one of the parameters independently of the day	none
Developmental milestones	Outlier in more than 2 PNDs for each test	none
Pup USVs		
Fragments		none
Single		One female vehicle
Double	More than 4 parameters within the same PND as the outlier	One male Kp234, one male vehicle, and one female Kp234
Triple		none
Open Field test		
Metrics from Smart	More than 3 parameters as an outlier	none
Manually scored behaviors	Outlier in the no. of one of the behaviors	none
Juvenile Behavior	Outliers in 2 days of the average play	none
Sexual Behavior	Outlier in the total number of one of the behaviors	none
Testosterone ELISA	Rout test of GraphPad	One male vehicle, one male Kp234, one female Vehicle, and one female Kp234
GnRH ELISA	Rout test of GraphPad	none
Western Blot	Rout test of GraphPad	none



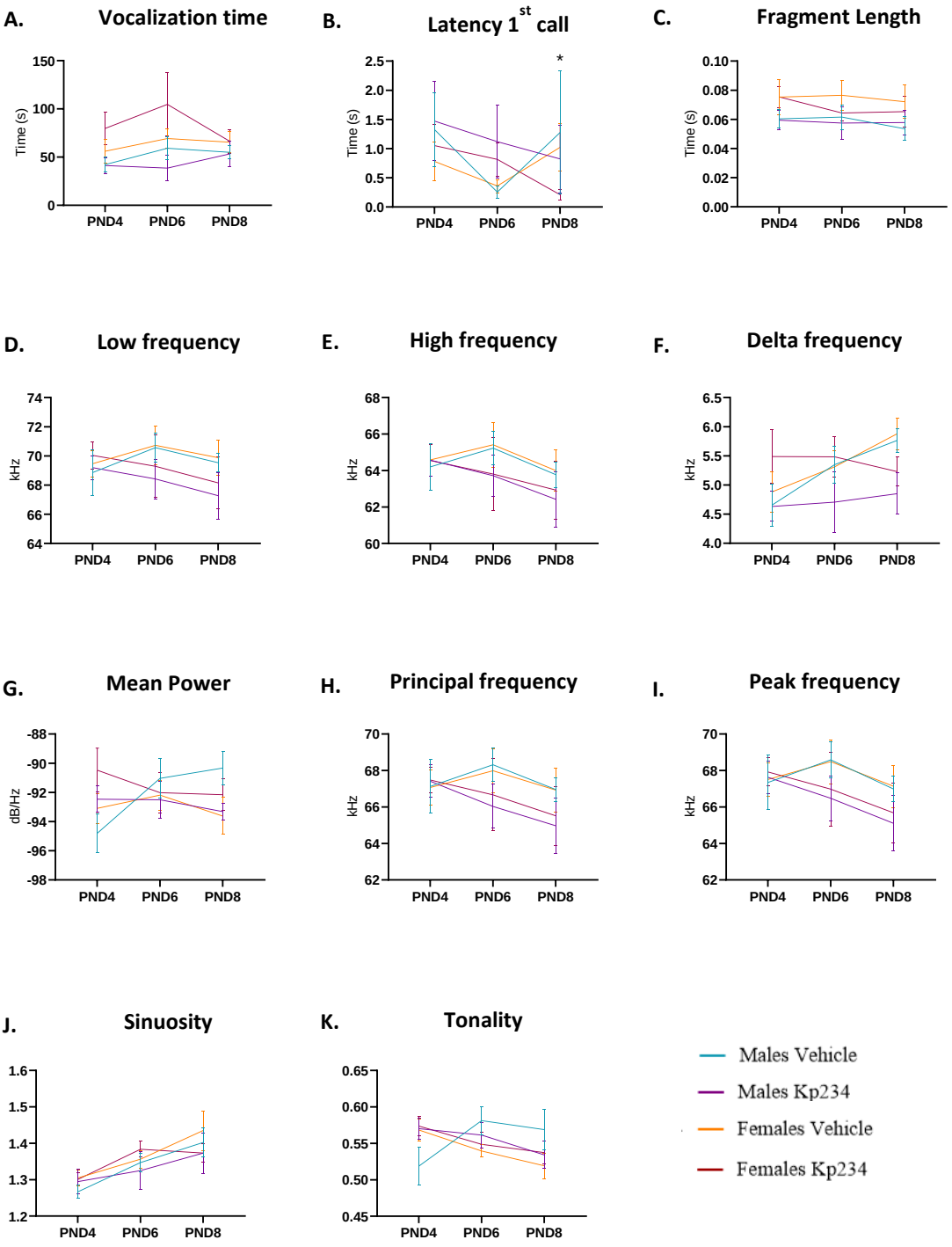
ANNEX IV ANATOMICAL MEASUREMENTS DURING DEVELOPMENT

A- Weight of males. **B-** Weight of females. **C-** Length of males. **D-** Length of females **E-** BMI (calculated as the division of the weight by the square of the length) of males **F-** BMI of females. **G-** Anogenital distance of males. **H-** Anogenital distance of females. In all graphs, performed two-tailed independent samples t-test for each PND; no significant differences between groups; n (males Veh) = 11; n (males Kp234) = 10; n (females Veh) = 11; n (females Kp234) = 11. Data represented as mean ± SEM.



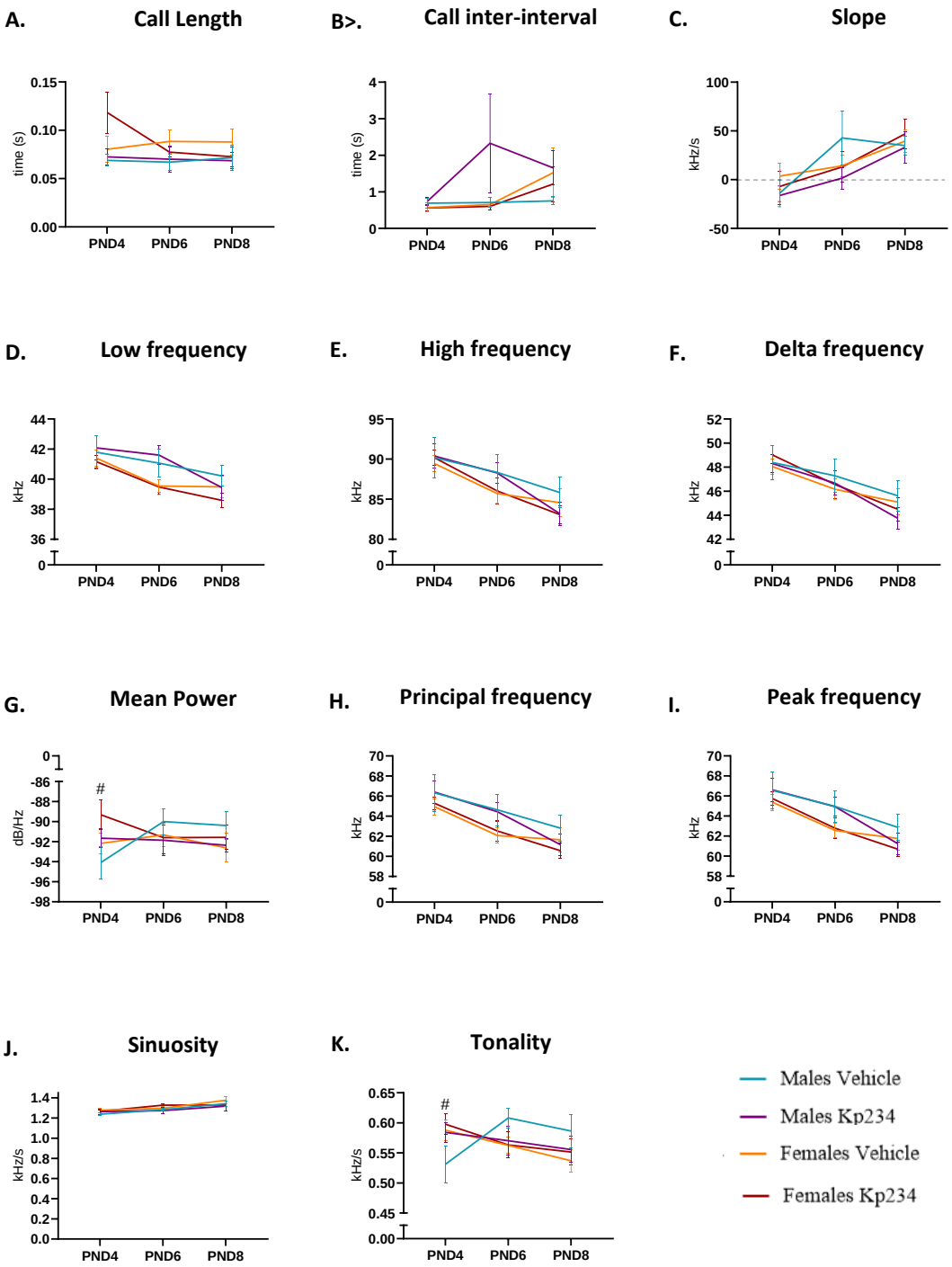
ANNEX VI NEST SEEKING ADDITIONAL MEASUREMENTS

A- Females' first orientation. **B-** Females' final bedding choice. **C-** Males' first orientation. **D** Males' final bedding choice In all graphs, performed two-tailed independent samples t-test for each PND; #: p-value <0.050 between males vehicle and males Kp234; n (males Veh) = 11; n (males Kp234) = 10; n (females Veh) = 11; n (females Kp234) = 11. Data represented as mean ± SEM.



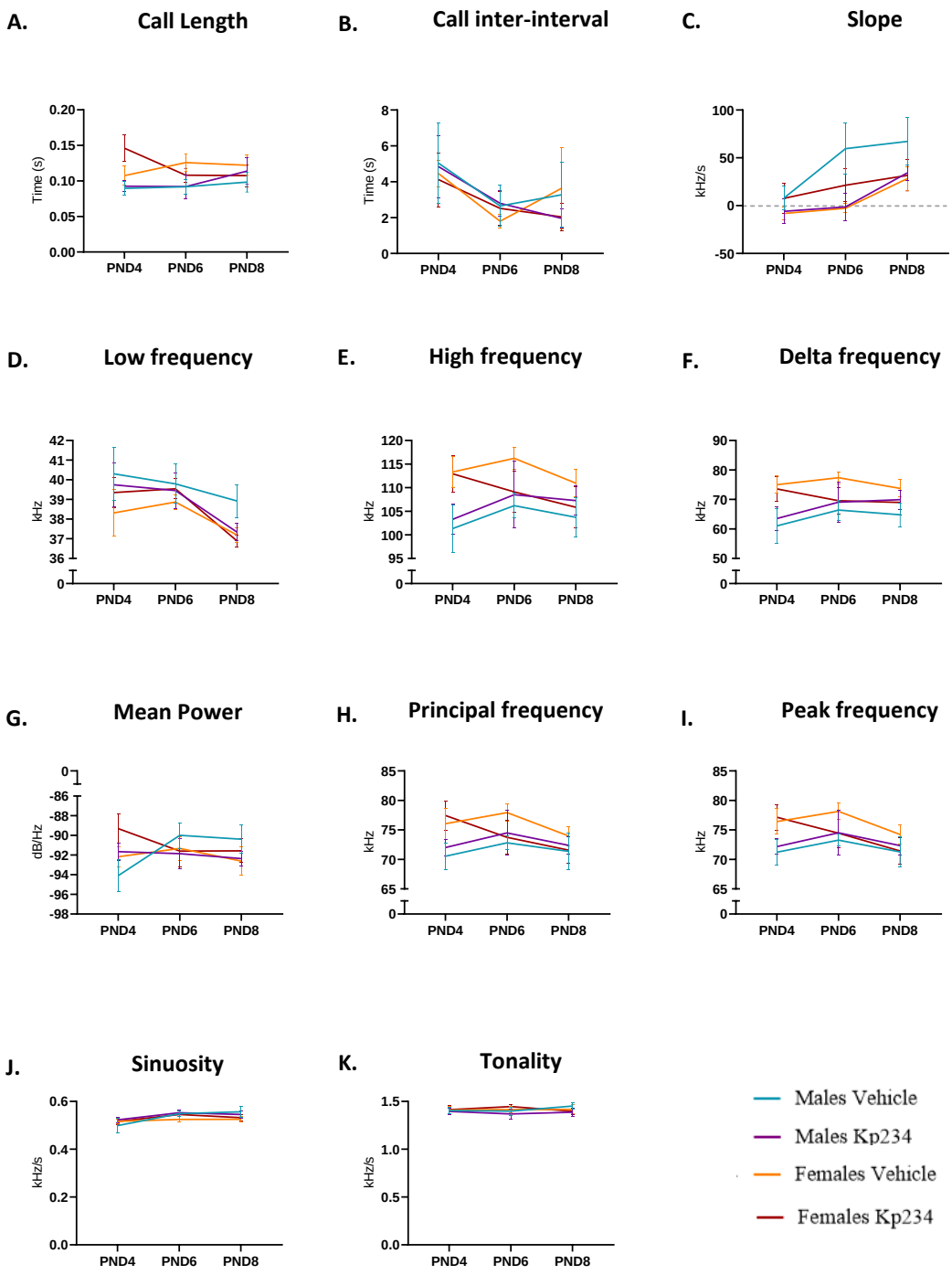
ANNEX VII FRAGMENT CHARACTERISTICS

A- Total vocalization time **B-** Latency first call **C-** fragment length **D-** Low frequency. **E-** High frequency. **F-** Delta frequency. **G-** Mean power. **H-** Principal Frequency. **I-** Peak frequency. **J-** Sinuosity. **K-** Tonality performed on PND4, 6, and 8. two-tailed independent samples t-test, for each PND, between females: *: p-value<0.050; between males: #: p-value<0.050. n (males Veh) = 7; n (males Kp234) = 7; n (females Veh) = 7; n (females Kp234) = 8. Data represented as mean ± SEM.



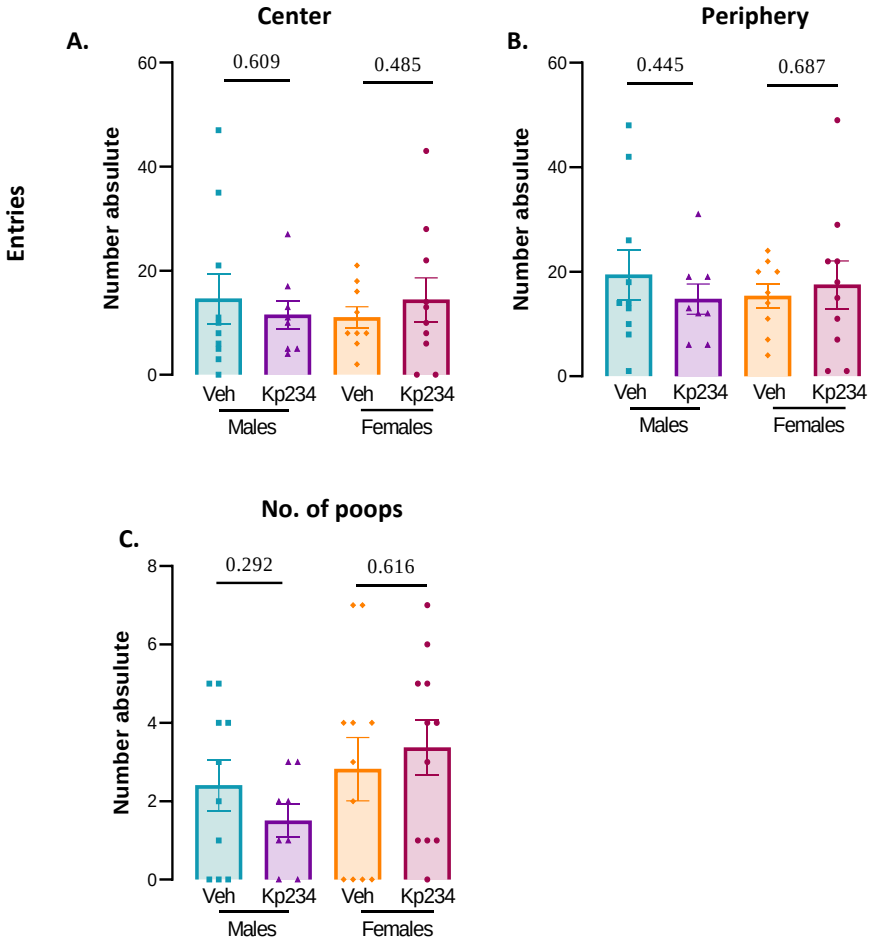
ANNEX VIII DOUBLE CALL CHARACTERISTICS

A- Call length. **B-** Call inter-interval. **C-** Slope. **D-** Low frequency. **E-** High frequency. **F-** Delta frequency. **G-** Mean power. **H-** Principal Frequency. **I-** Peak frequency. **J-** Sinuosity. **K-** Tonality performed on PND4, 6, and 8. two-tailed independent samples t-test, for each PND, between males: #: p-value<0.050. n (males Veh) = 6; n (males Kp234) = 6; n (females Veh) = 6; n (females Kp234) = 7. Data represented as mean ± SEM.



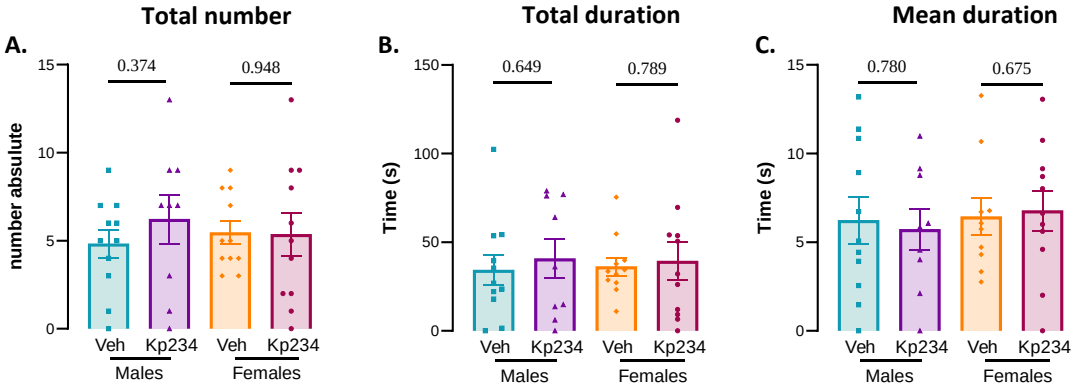
ANNEX IX TRIPLE CALL CHARACTERISTICS

A- Call length. **B-** Call inter-interval. **C-** Slope. **D-** Low frequency. **E-** High frequency. **F-** Delta frequency. **G-** Mean power. **H-** Principal Frequency. **I-** Peak frequency. **J-** Sinuosity. **K-** Tonality performed on PND4, 6, and 8. two-tailed independent samples t-test, for each PND, no differences found between groups. n (males Veh) = 7; n (males Kp234) = 7; n (females Veh) = 6; n (females Kp234) = 8. Data represented as mean ± SEM.



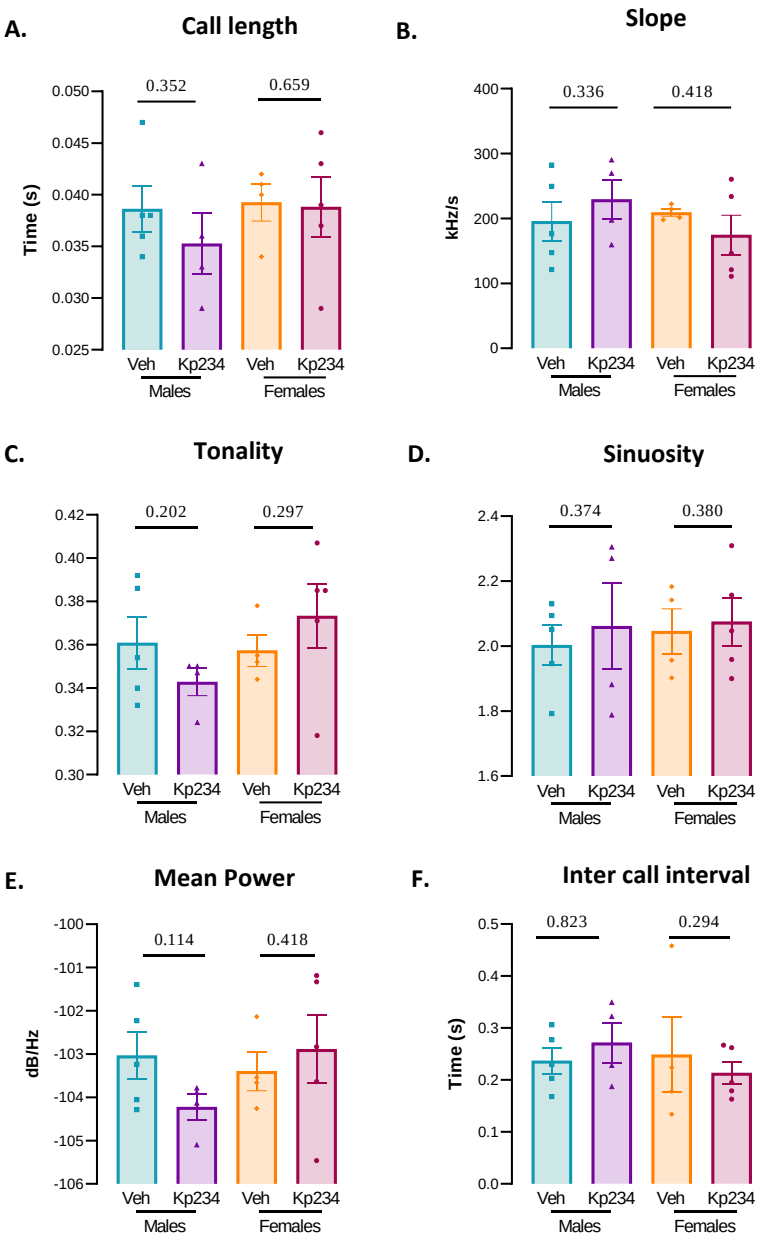
ANNEX XI ADDITONAL OPEN FIELD TEST METRICS

A- Entries in the center. **B-** Entries in the periphery. **C-** Total number of poops. Two-tailed independent samples t-test: numbers above the bar indicate p-values: in bolt<0.050. N (males Veh) = 10; n (males Kp234) = 8; n (females Veh) = 9; n (females Kp234) = 10. Data represented as mean ± SEM.



ANNEX XII GROOMING BEHAVIOR

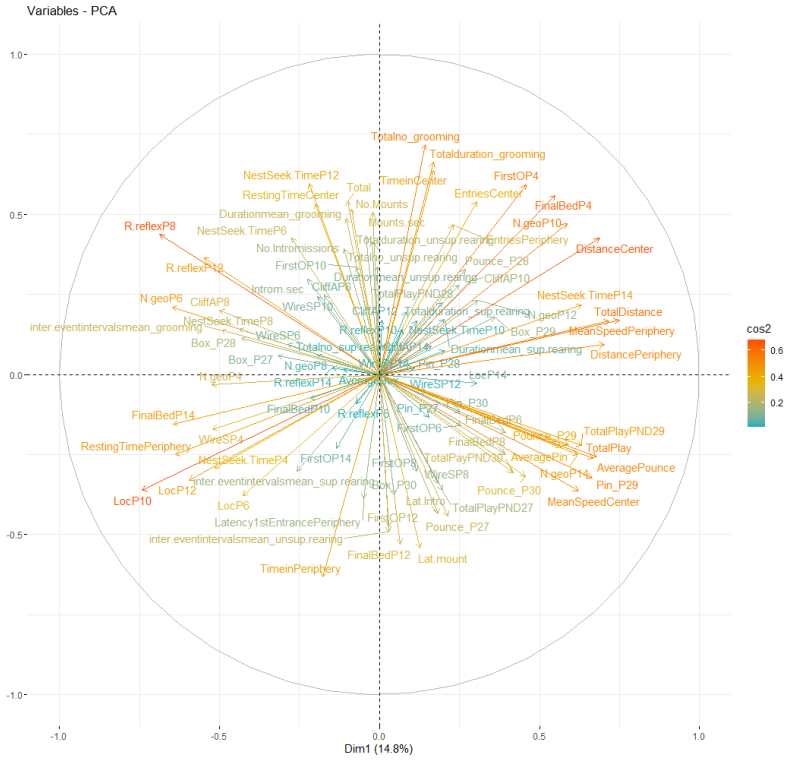
A- Total number of grooming**. B-** Total duration of grooming**. C-** Mean duration of grooming. Two-tailed independent samples t-test: numbers above the bar indicate p-values: in bolt<0.050. n (males Veh) = 11; n (males Kp234) = 9; n (females Veh) = 10; n (females Kp234) = 10. Data represented as mean ± SEM.



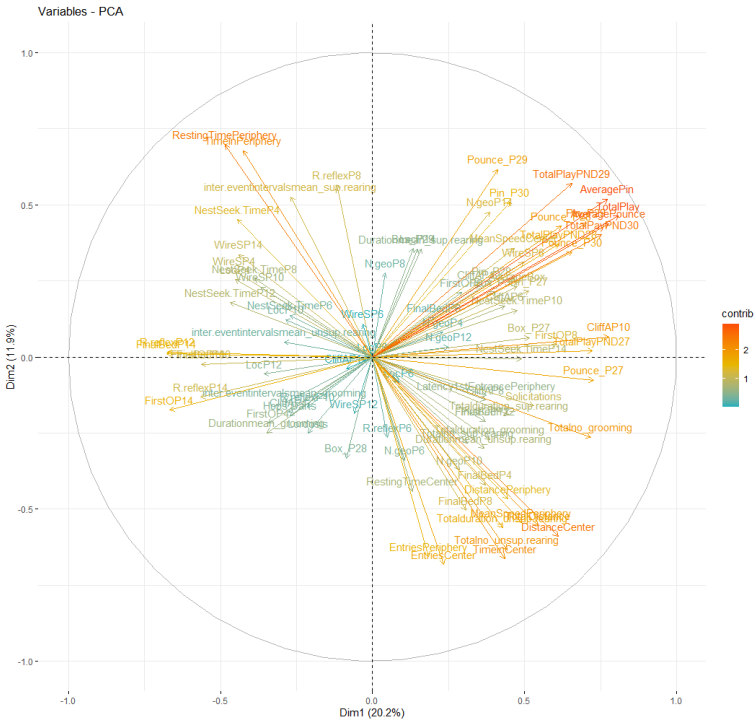
Annex XIII PND29 USV characteristics

A- Call length. **B-** Slope. **C-** Tonality **D-** Sinuosity. **E-** Mean power. **F-** Call inter-interval. Two-tailed independent samples t-test, bolt: p-value<0.050. n (pair of males Veh) = 5; n (pair of males Kp234) = 4; n (pair of females Veh) = 4; n (pair of females Kp234) = 5. Data represented as mean ± SEM.

A.



B.



ANNEX XIV LOADINGS OF THE VARIABLES ON PCA

A- Distribution of the variables that explain the variance of each Dimension (PC) in males. **B-** Distribution of the variables that explain the variance of each Dimension (PC) in females.