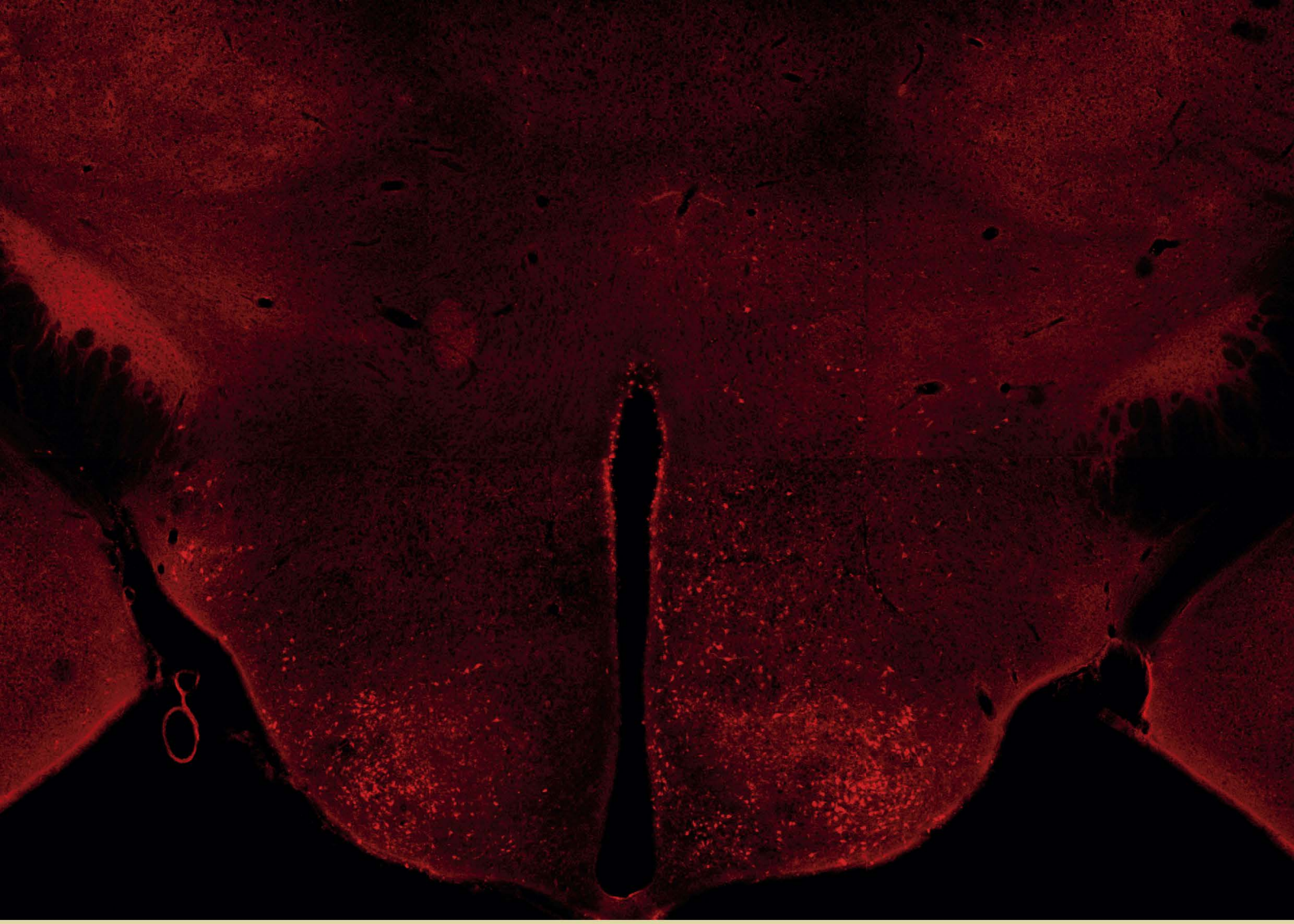

Inês Mendes Preguiça

The Role of PgR+ cells of the Ventromedial Hypothalamus (VMHvl) in Female Sexual Receptivity

Dissertação de mestrado em Biologia Celular e Molecular, orientada pela Doutora Susana Q. Lima
e apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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Universidade de Coimbra

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Inês Mendes Preguiça

August 2017

Dissertation presented to the Life Sciences Department of the Faculty of Sciences and Technologies, University of Coimbra to fulfil the necessary requirements to obtain the Master degree in Cellular and Molecular Biology. The work was performed in the Neuroethology Laboratory at Champalimaud Centre for the Unknown (CCU) under the scientific supervision of Doctor Susana Q. Lima and co-supervision of Doctor Ana Luísa Carvalho (Life Sciences Department of the Faculty of Sciences and Technologies, University of Coimbra).

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LIST OF CONTENTS

ABREVIATIONS

AP	Anterio-posterior
CaM	Calmodulin
CaM-M13	Ca^{2+}/cam-binding M13 peptide
CNO	Clozapine-*N*-oxide
cpGFP	Combination of a permuted green fluorescent protein
DAPI	4,6-Diamidine-2′-phenylindole dihydrochloride
DM	Dorsal medial hypothalamic nucleus
DREADDs	Designer Receptors Exclusively Activated by Designer Drugs
DV	Dorso-ventral
E	Estrogen
ESR1+	Estrogen receptor 1
GIRKs	G protein-coupled inwardly-rectifying potassium channels
GPCR	G protein-coupled receptor
IRES	Internal ribosome entry sequence
l/dmPAG	Lateral and dorsomedial region of periaqueductal gray
mfb	Medial forebrain bundle
ML	Medio-lateral
NSAID	Nonsteroidal anti-inflammatory drug
OVX	Ovariectomy
PAG	Periaqueductal gray
PBS1x	Phosphate-buffered saline
PFA	Paraformaldehyde
Pg	Progesterone
PgR+	Progesterone receptor
PLH	Peduncular lateral part of the hypothalamus
ROI	Region of interest
SBN	Social behaviour network
vlPAG	Ventrolateral region of the periaqueductal gray
VMH	Ventromedial hypothalamus
VMHdm	Dorsomedial VMH
VMHvl	Ventrolateral region of the ventromedial hypothalamus

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Mating is an innate social behaviour exhibited by most animal species, including mice. In females, the reproductive cycle determines the type of interactions they will display upon contact with male conspecifics. Specifically, during proestrous the female is in a receptive state and exhibits lordosis, a receptive posture that aids copulation. The remaining days, the non-receptive phase, is characterized by defensive and aggressive responses towards the male's advances.

The ventrolateral region of the ventromedial hypothalamus (VMHvl) has been described to play a key role in female sexual behaviour: manipulation of this brain region through electrical stimulation or lesion has been shown to alter female sexual behaviour. The VMHvl is composed of different neuronal populations, including neurons that express receptors for the sex hormones progesterone and estrogen (PgR+ and ESR1+, respectively). Consistently, the PgR+ population was suggested to have a critical role for female receptivity: when this population is ablated (by apoptosis), females have a marked decrease in receptivity. However, these experiments were performed in females whose ovaries were removed (OVX) and which were then supplemented with exogenous sex hormones to simulate the reproductive cycle. Therefore, the results from these experiments are difficult to interpret for two main reasons: First, cell ablation, which occurs over days, may lead to changes (structural and activity wise) in the rest of the VMHvl; second, conditions such as ovary removal and fast hormonal replacement are very different from the real physiological scenario where sex hormones are always present and slowly fluctuating. Consequently, the social behaviour is markedly different between naturally cycling and OVX females. Hence, given the fact that so many variables are altered in the previous experiments and given the difficulty to interpret the results, the previous findings still leave us with very little idea regarding the real function of the PgR+ population.

Therefore, in this project, we intended to further understand the role of the PgR+ neurons of the VMHvl in female sexual behaviour. For that, we took advantage of inhibitory Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), specifically activated by Clozapine-*N*-oxide (CNO), to temporarily inhibit PgR+ neurons. Briefly, DREADDs were specifically expressed in PgR+ neurons using a combinatorial strategy with Cre recombinase mouse line and viruses.

The consequent sexual behavioural outcome was recorded and quantified, yet no significant conclusions can be withdrawn for the role of PgR+ neuronal population of the VMHvl in female receptivity. Nevertheless, the observed results in the deviation of sexual behaviour does indicate that this cell population might indeed modulate female receptivity. Overall, the results obtained with this project suggest that further studies need to take place, using more specific tools in order to better identify and distinguish the neurons within this population and, consequently, their function.

Keywords: VMH, progesterone, female, receptivity, DREADD, sexual behaviour

RESUMO

O acto de acasalamento trata-se de um comportamento social inato, exibido pela maioria das espécies de animais, incluindo murganhos. Nas fêmeas, o ciclo reprodutivo determina o tipo de interações que estas demonstram quando em contacto com machos coespecíficos. Mais concretamente, durante a fase de "proestrus" a fêmea encontra-se recetiva e adota a posição de lordose, uma postura que facilita a copulação. Nos restantes dias, a fase não-recetiva, é caracterizada por interações defensivas e, por vezes, agressivas em resposta aos avanços sexuais do macho.

A região ventrolateral do hipotálamo ventromedial (VMHvl) foi descrita como crucial para a manifestação do comportamento sexual feminino. Manipulações desta área cerebral através de estimulações elétricas ou lesões tiveram como consequência alterações do comportamento sexual. O VMHvl é composto por várias populações neuronais, incluindo neurónios que expressam recetores das hormonas sexuais progesterona e estrogénio (PgR+ e ESR1+, respetivamente). A importância da população PgR+ na recetividade sexual feminina foi demonstrada quando a ablação, através de apoptose, desta população celular em fêmeas originou um decréscimo na recetividade. No entanto, estas experiências foram realizadas em fêmeas cujos ovários haviam sido previamente removidos (OVX) e desde então suplementadas com hormonas sexuais exógenas de forma a simular o ciclo reprodutivo. Assim sendo, os resultados consequentes destas experiências tornam-se difíceis de interpretar, principalmente, por duas razões: em primeiro lugar, porque a ablação das células é um processo que decorre ao longo de vários dias, podendo assim levar a mudanças estruturais e de atividade em toda a região do VMHvl; segundo, porque a remoção dos ovários e a rápida substituição hormonal corresponde a uma pobre imitação do que ocorre na realidade, onde as hormonas estão constantemente presentes variando gradualmente a sua concentração. Consequentemente, o comportamento social é bastante diferente entre fêmeas cujo o ciclo reprodutivo decorre naturalmente e OVX. Posto isto, consideramos que os estudos feitos anteriormente apresentam várias variáveis que se distanciam significativamente das condições fisiológicas no que toca a regulação hormonal do ciclo reprodutivo feminino, o que torna os seus resultados difíceis de interpretar. Assim, consideramos que a função da população PgR+ continua sem estar devidamente esclarecida, pelo que novas estratégias devem ser implementadas neste sentido.

Desta forma, este projeto foi desenhado com o compromisso e objetivo de compreender a função dos neurónios PgR+ do VMHvl no comportamento sexual feminino. De forma a atingir esse objetivo, optámos por utilizar uma estratégia que consistiu na inibição temporária dos neurónios PgR+, utilizando *Designer Receptors Exclusively Activated by Designer Drugs* (DREADDs) expressos unicamente em neurónios PgR+ através da combinação de murganhos e vírus que expressam recombinase Cre.

Os resultados obtidos através da análise do comportamento sexual destes animais não nos permitiram tirar conclusões objectivas sobre o papel da população neuronal PgR+ na receptividade sexual feminina. Contudo, de entre as inúmeras observações efectuadas em

relação ao comportamento destes animais, várias sugerem que estas células podem ter de facto um papel relevante na modulação do comportamento sexual feminino. Assim sendo, estamos certos que novas estratégias têm que ser desenhadas, usando ferramentas inovadoras que nos permitam identificar e distinguir os diferentes neurónios desta população e as suas funções.

Palavras-chave: VMH, progesterona, fêmea, recetividade, DREADD, comportamento sexual

Chapter 1 | INTRODUCTION

1.1. INTRODUCTION TO SOCIAL BEHAVIOUR

Many mammals, such as mice, are social species dependent upon the establishment and maintenance of social bonds for survival and perpetuity. Social interactions, which are a consequence of being part of a social group or occasionally encountering conspecifics, are complex behaviours that provide security, reproductive success and social reward[1].

In the wild, *Mus musculus* is a species that establishes group territories initiated by one male with one or two females, and whose area is determined through the density of accessible food. Their social interactions are hierarchically dependent, where the dominant male defends the territory from intruders. However, progenitors and their offspring share the same nest until the pups achieve adulthood and leave to find new territories and a chance to be the dominant male.

But social interactions are not just black and white. The response of an animal to the interaction with a conspecific depends on a variety of factors, such as the outcome of previous interactions[2] or the internal state of the individual: a lactating mother will respond very different to the approach of an unfamiliar male, for example[3]. The richness of these possible outcomes makes social decision making a fascinating topic to study.

Many types of social interactions have been investigated over the last decades, including aggression, juvenile playing, mating and parenting, and currently we already have some information regarding the neuromodulators and the endocrine pathways underlying these behaviours[4–7]. In some instances, we also know some of the neuronal circuits that are involved in social behaviours[7–10]. However, the levels of neuromodulators and hormones are not static: they change in response to many factors, such as behavioural interactions[11] or intrinsic factors[12]. However, very little is known about how the changing levels of neuromodulators and hormones impact on the activity of neuronal circuits, leading to different behavioural outputs depending on their levels. For that we must identify neuronal circuits whose activity can in principle be modulated by the dynamic levels of neuromodulators and hormones to understand how the output of each node changes with varying levels of these ligands.

Sexual interactions are a particular type of social interactions that are observed in species that require sex for reproduction (social and asocial) and which can be displayed without previous training[13]. Sexual behaviour, similarly to other social interactions, in vertebrates is highly dependent on the Social Behaviour Network (SBN)[14,15]. The SBN is composed of a set of reciprocally interconnected brain regions, most of which expresses receptors for sex steroid hormones. Therefore, the SBN has the capacity to be modulated by fluctuating levels of sex hormones and therefore its output can change depending on the internal state of the individuals.

1.1.1 Sexual behavior

Sexual behaviour is a motivated social behaviour exhibited by most species, meaning that it depends on internal and external signals to guarantee its proper execution [16]. In mice, this behaviour is composed of innate and learned actions and can be viewed as a cycle comprised of three phases (Figure 1.1). The first involves the recognition of an individual of the opposite gender

and is known as the *appetitive* phase. It depends upon olfactory cues and is composed of anogenital and nose-to-nose investigation which are known courtship behaviours that promote sexual interaction [16,17]. This is followed by the *consummatory* phase, characterized by a series of mount attempts, where the male chases the female trying to position his front paws on her flank to trigger the lordosis posture (characterized by a dorsi-flexion of the spine to elevate the vagina). When successful, this leads to mounts with intromission, where the female adopts the lordosis posture allowing the male to penetrate her[16,17]. These events repeat themselves until the male ejaculates leading to the *inhibitory/satiety* phase, easily recognizable since the male freezes and falls to the side[16,17].

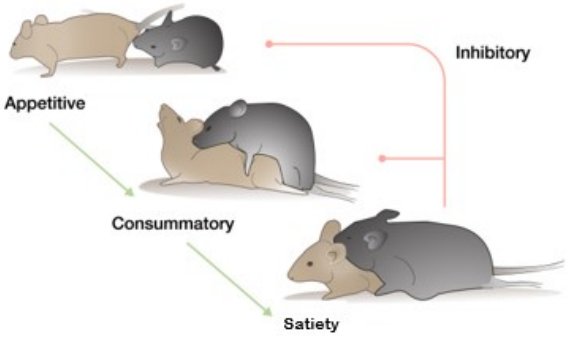

Figure 1.1. Cycle of sexual behaviour in mice. The sexual behaviour of mice depends on internal and external signals and can be subdivided in three main phases: *Appetitive* phase, involving the recognition of an individual of the opposite gender, *Consummatory* phase, involving several mount attempts from the male until the female reaches lordosis, and the *Satiety* phase, characterized by the time point when the male reaches ejaculation.

1.2 FEMALE SEXUAL BEHAVIOUR

In mice, female sexual behaviour is stereotyped, occurring in organized patterns. Initially, it was described to be composed of 3 main components: attractivity or the female's ability to trigger a sexual response in the male through visual and olfactory cues; proceptivity, meaning the female's initiative to establish and maintain sexual interaction; receptivity, where the female would do just enough to allow the male to successfully achieve ejaculation[18].

However, this description of female behaviour is male-centered: it describes the observed behaviour relative to the male, instead of considering it as an intrinsic and independent behaviour of the female[18]. Further on, it was shown that females who had been given the opportunity to control the amount of interaction with the male, therefore pacing the sexual interaction, exhibited a distinguishable behaviour where they solicited intromissions from the male[19]. Additional studies showed that the female did play an active role during sexual encounters[20]. Importantly, it was only when the female could regulate the pace of sexual interactions that brain regions and neuromodulators involved in the display of sexual behaviour accompanied by a rewarding and positive effect were identified [21].

Independently of the perspective adopted to study female sexual behaviour, one key behavioural posture is considered the hallmark of female receptivity, the lordotic posture. In this

posture, the female plants her feet and raises her hindquarters and tail[16] (Figure 1.4); this raised posture helps penetration. Lordosis is observed in several mammalian species and it is usually dependent on the estrous state and the action of ovarian hormones. Therefore, to understand the hallmark of female receptivity in mice, we must understand how the reproductive state and its cycling sex hormones affect female sexual behaviour.

One may ask why should female sexual behaviour and willingness to copulate be coupled to its ability to become pregnant. Wouldn't it be better to copulate all the time and increase the probability of fertilization? Obviously, it is impossible to answer “why” questions. But not everything is positive about sex. In particular, engaging in sexual behaviour increases the probability of predation and exposure to parasites[22]. Therefore, it has been hypothesized that coupling sexual receptivity to moments of high probability of fertilization reduces the risk of predation and infection, by limiting the period where females are available for copulation.

1.2.1. The Estrous cycle

A female will interact differently with a male depending on the phase of the reproductive/estrous cycle that she is in. Receptive behaviour occurs during the proestrus phase, when females ovulate and affiliative behaviours can be triggered and induce the body posture required for copulation, namely lordosis. Non-receptive behaviour extends throughout the remaining phases and is characterized by defensive and aggressive responses towards the male’s attempts at proximity[23]. The estrous cycle of mice can be divided into four phases: proestrus, estrus, metestrus, and diestrus that are consequence of variations in levels of ovarian hormones estradiol and progesterone. Females exhibit either receptive or non-receptive behaviour depending on their hormonal levels and the phase of the cycle they are in[24] (Figure 1.2).

Each stage of the cycle can be identified through a straightforward cytology study, that helps to identify the types and proportions of different cell populations in vaginal smears (Figure 1.3). The proestrus stage, composed of a higher proportion of nucleated epithelial cells than cornified epithelial cells, is when ovulation occurs; this is followed by the estrus phase, which is characterized by the presence of only cornified epithelial cells; after this comes metestrus, where the presence of cornified epithelial cells is higher than nucleated epithelial cells and some leucocytes may appear; and lastly comes diestrus, which is easily identified by an overwhelming presence of leucocytes[24].

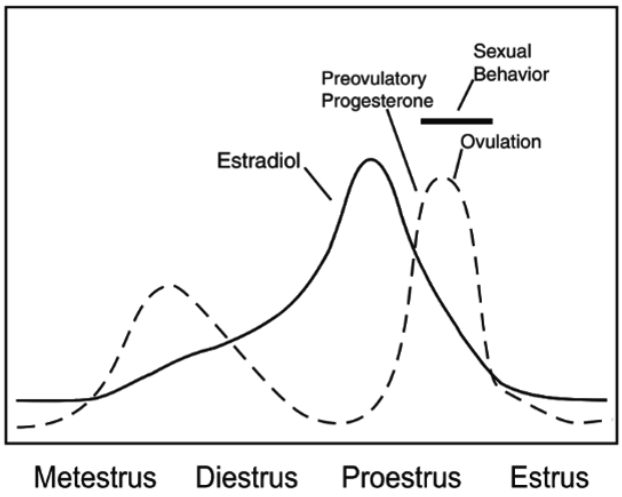

Figure 1.2. Female's receptivity behaviour according to the estrous cycle. Females exhibit a more receptive behaviour during the proestrus phase of the estrous cycle. This period is characterized by a peak of the progesterone hormone and ovulation. During the remaining phases, the female adopts a non-receptive behaviour with defensive and aggressive responses. Adapted from Blaustein, 2008.

Classically, most studies of rodent sexual behaviour are not performed with naturally cycling females, but rather females whose ovaries are removed (ovariectomy, OVX); OVX females are then injected with estrogen and progesterone to induce a fake ovulation[25,26]. Most of the work performed in the host lab has been done with naturally cycling females and, according to unpublished results from our lab (Nomoto et al, unpublished), the behaviour of naturally cycling females is markedly different from the behaviour of OVX females supplemented with hormones (when compared to studies such as Yang et al, 2013). In particular, naturally cycling females reject more the males when in the receptive phase of the cycle and OVX females already accept the male's mount attempts even before hormonal treatment. Therefore, it seems like OVX females are much more permissive to male's attempts to copulate; this is, female's behaviour seems uncoupled from their physiological state.

Consequently, monitoring the estrous cycle is essential when working with naturally cycling females, which according to unpublished results from our lab exhibit sexual behaviour characteristics that differ from ovariectomised (OVX) females.

The estrous cycle itself is not capable of inducing the behaviour on its own. Several brain regions are responsible for reading and interpreting the hormonal signals (circulating hormonal levels) in order to translate them into appropriate behavioural response. The ventromedial hypothalamus has long been connected to female receptivity, among other social behaviours, and in the following topic we will explore the lordosis posture and its connection to this specific structure.

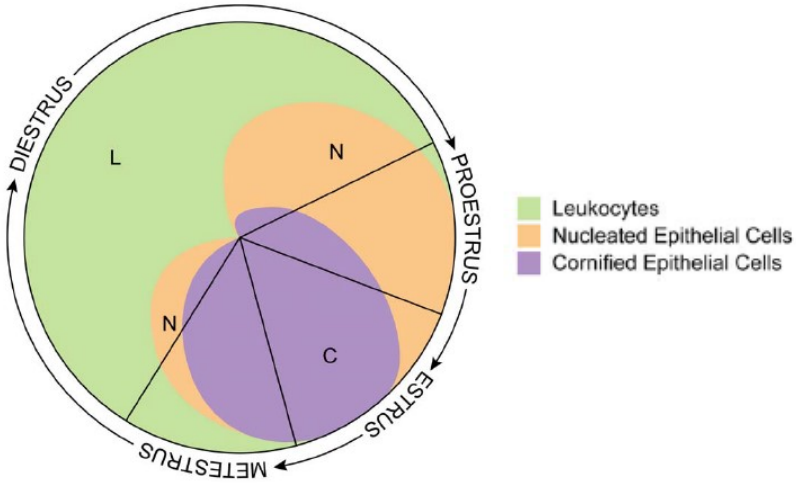

Figure 1.3. Representative scheme of vaginal smears cytology according to the estrous cycle. The proestrus phase is characterized by a higher proportion of nucleated epithelial cells than cornified epithelial cells, followed by the estrus phase which accounts with the presence of only cornified epithelial cells. In the metestrus phase, the presence of cornified epithelial cells is higher than nucleated epithelial cells and some leucocytes may appear. Lastly, the diestrus phase is characterized by an overwhelming presence of leucocytes.

1.2.2. Lordosis

Female sexual receptivity in mice is, as mentioned above, mainly characterized by lordosis, a posture adopted for copulation during the receptive period of their estrous cycle (Figure 1.4). The lordosis reflex is comprised by the arching of the back, an elevation of the pelvis and tail deviation, facilitating penile insertion and ejaculation[27].

Figure 1.4. Representation of the lordosis posture. The lordosis posture is achieved during the receptive period of female's estrous cycle and it is characterized by the arching of the back, an elevation of the pelvis and tail deviation.

This behaviour has been linked to the ventromedial hypothalamus (VMH), more specifically to the ventrolateral region (VMHvl), known for being part of the SBN and its projections to the periaqueductal gray (PAG)[28].

Several studies have aimed to determine the connection between female sexual receptivity and the VMHvl. Initially, lesions of the region were performed, which led to a decrease in receptivity[29,30]. This was followed by stimulation studies, where electrical and hormonal stimulation of the VMHvl, led to a facilitation of lordosis[31,32]. It was later shown through transection that the axonal projections from the VMHvl to the PAG enable the lordosis reflex through modulation of posture-control relays to the reticular formation[33].

1.3. VENTROMEDIAL HYPOTHALAMUS

The Ventromedial Hypothalamus is divided in two hemi-oval structures, the dorsomedial (VMHdm) and the ventrolateral (VMHvl) separated by a central region with few cells [34]

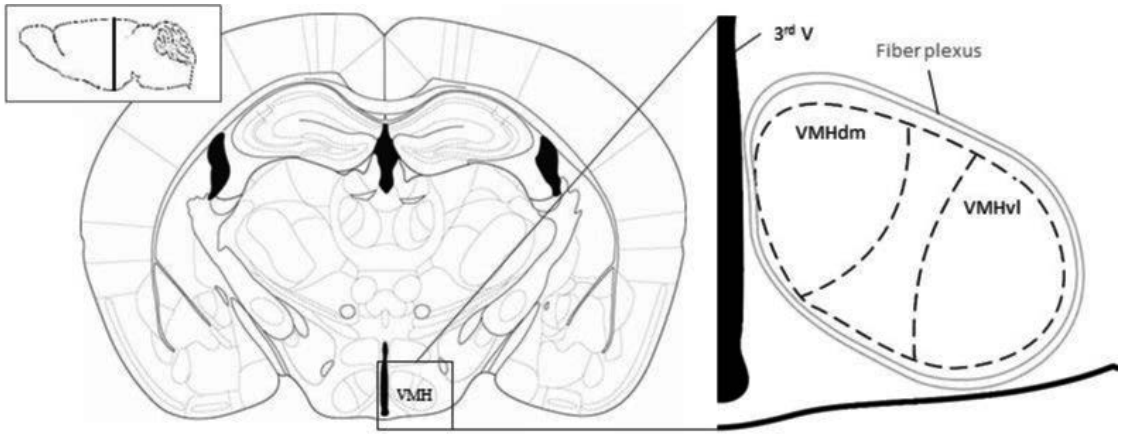

Figure 1.5. Scheme of the VMHvl location and structure details. Schematic illustration of a coronal slice with location of VMH nucleus, the zoomed region shows both partition of the nucleus (dorsomedial-VMHdm and ventrolateral-VMHvl) surrounded by the fiber plexus along the the third ventricle (adapted from Paxinos & Franklin, 2014).

The ventrolateral region of the ventromedial hypothalamus (VMHvl) is located in the caudal hypothalamus along the third ventricle and exhibits quantitative cell and molecular sex differences thought to be important for the exhibition of several dimorphic sexual behaviours [9,35–37].

One of the most important characteristics of this region is the rich expression of sex steroid receptors: estrogen and progesterone receptors; the expression of sex hormone receptors provides a substrate for responsiveness of this region to the fluctuating levels of sex hormones across the reproductive cycle. Sex hormones can affect neuronal activity indirectly by changing gene expression[35] and directly by opening/closing ion channels on the membrane[38,39]. Therefore, the activity of neurons in the VMHvl can in principle change across the cycle, depending on the levels of sex hormones. This was indeed shown to be the case for VMHvl neurons. Our lab has recently shown that the population activity of female VMHvl neurons in response to males changes across the estrous cycle, being enhanced during the proestrous phase[40]. This means that the VMHvl represents a male stimulus in a different manner depending on the internal state of the female.

The number of dendritic spines in the VMHvl is also modulated by the concentration of ovarian hormones[41]; estrogen and progesterone can also rewire VMH dendrites [42]. VMH neurons have a simple dendritic tree with a single long primary dendrite complemented with shorter primary and secondary dendrites, meaning that individual neurons can integrate local signals with inputs from other brain regions, suggesting once again the importance of the high connectivity exhibited by this structure with the nodes of the SBN[43].

Moreover, the VMH is composed of a heterogeneous population of neurons. Importantly, the presence of steroid hormone receptors has been observed in certain cell populations of the

VMHvl. So far, studies have identified the presence of Progesterone Receptor-positive neurons (PR+), and Estrogen Receptor 1-positive neurons (ESR1+). In fact, close to 92% of PR+ neuron population co-labeled with ESR1+ in both genders. Furthermore, Cckar, a G-coupled protein receptor mainly present in females and essential for the execution of female receptive behavior, was co-labelled in roughly 67% of PR+ VMHvl neurons[9].

Recently, the Progesterone Receptor expressing neurons (PR+) population was ablated from the VMHvl through a Cre-LoxP strategy resulting in decreased receptivity and capacity to exhibit the lordosis posture [9]. However, these experiments were performed in OVX females supplemented with sex hormones and therefore their sexual behaviour was already altered from the beginning as it was already mentioned. Also, Yang et al[9] performed an irreversible ablation of the PgR+ cells, and therefore the results could have been the result of long-term changes in response to the insult caused to this brain region.

1.4. FEMALE SEXUAL BEHAVIOUR: BALANCE BETWEEN RECEPTIVITY AND REJECTION

As it was mentioned before, experiments in our lab are primarily performed in naturally cycling females, where females are allowed to follow a natural reproductive cycle and experiments are performed when the appropriate state is reached. Because of this, we have observed that naturally cycling females exhibit higher levels of rejection (and sometimes also aggression) towards males when compared to OVX females, even when in the proestrous phase of the cycle. Importantly, when in proestrous, females initially start by rejecting the male in the beginning of the interaction and sometimes they might never accept him, for example if he is too inexperienced and forceful. As the interaction progresses and females become aroused, the level of rejection decreases and they will primarily exhibit agonistic behaviour. When outside the proestrous phase, a female will vigorously reject a male, sometimes kicking and punching him. In contrast, OVX females will sometimes accept a male even before being treated with hormones and after hormone injection they will immediately accept the male's attempts of copulation.

This raises the idea that sexual behaviour is not only about receptivity per se, but also about inhibiting defense/aggression. To achieve copulation, females can exhibit proceptive behaviour (following the male), receptive behaviour (when approached by the male) and inhibit rejections whenever he approaches them. They should also decrease unsolicited aggressions (go after the male when he is not interacting with them and show aggression). All of these would lead to an increase probability of copulation.

We know that the VMHvl is important for aggression in males[44,45]. Could it be that the VMHvl in females is also important for aggression as it was shown in the male? In particular, ER+ neurons in the male are important for aggression[45] and the majority of these also co-express Progesterone receptor. Therefore, in Yang et al 2013[9], ablation of the PgR+ neurons could only disrupt receptivity (rejection in this study was reported as absence of lordosis and walking away).

We are left without knowing how rejection and defensive behaviour are affected when the PgR+ neurons are ablated in naturally cycling females.

1.5. AIMS AND HYPOTHESIS

Female sexual behaviour is a complex set of actions influenced by the animal's internal state and the activity of certain brain regions, such as the ventromedial hypothalamus (VMH). Yang et al 2013 have shown that in OVX females supplemented with sex hormones, ablation of the progesterone receptor expressing neurons (PgR+ neurons) of the VMHvl lead to reduced female receptivity, this is, less lordosis[9]. However, work from our lab has shown that female sexual behaviour in naturally cycling females is very different from the behaviour of OVX females, in particular, they are more defensive/rejecting than OVX females. Therefore, our main aim was to investigate the effect of disrupting the PgR+ population of the VMHvl (in a reversible manner and in naturally cycling females), on female sexual behaviour. Since sexual behaviour is composed of agonistic and antagonistic behaviours, our hypothesis is that PgR+ neurons could be involved in both aspects of the behaviour: receptive and antagonistic behaviours.

To explore this idea, we decided to disrupt the activity of PgR+ neurons of the VMHvl using Designer Receptors Exclusively Activated by Designer Drugs (DREADDs); these are exogenous receptors that are expressed in cells of choice and that can be manipulated with ligands that do not affect other receptors in the brain.

To do so, we:

- Expressed DREADDs in PgR+ neurons of the VMHvl using a Cre mouse line that expresses Cre recombinase under the control of the progesterone receptor promoter and injected the animals with Cre dependent viruses;
- Tested for inhibition of the PgR+ cells expressing the DREADDs treated with the ligand by measuring neuronal activity with genetically encoded calcium indicators of activity expressed in the same cells;
- Used the DREADD strategy to disrupt female sexual behaviour during the proestrous phase of the cycle in naturally cycling females.

Chapter 2 | MATERIALS AND METHODS

All procedures were in accordance with the Portuguese National Authority for Animal Health (Direcção Geral de Alimentação e Veterinária; DAGV) and the Commission for Experimental and Animal Welfare of the Champalimaud Centre for the Unknown (Órgão para o Bem Estar Animal; ORBEA).

2.1. ANIMALS

All animals used in this study were *Mus musculus domesticus*. For the expression in Progesterone receptor expressing neurons, we used the inbred strain PgR-IRES-Cre (kind gift from Dr. Nirao Shah, UCSF). In this mouse line, Cre recombinase expression is under the control of the progesterone receptor promoter. The gene for Cre was cloned downstream of the progesterone receptor using an internal ribosome entry sequence (IRES). In this case, a single messenger RNA is made after transcription; the IRES is a RNA element that allows for translation initiation in a cap-independent manner, so that the translation of the two genes (progesterone receptor and Cre) is independent[9,46]. Cre dependent viruses are then injected in the region of interest to achieve DREADD expression in PgR+ neurons only. Two C57BL/6J mice, obtained originally from the Jackson Laboratory (stock number 000664), were used as controls to show that expression of DREADDs is dependent on the presence of Cre recombinase.

Animals were kept at a controlled temperature (23 ± 1 °C) in a 12:12 hours reversed light/dark cycle, with light onset at 20:00. Weaning occurred at 21 days of age after which animals were housed in same-sex groups of 2-5 animals per cage (1284L, Techniplast, 365 x 207 x 140 mm). Food chow (Global Diet 2914, Mucedola s.r.l) and water was provided *ad libitum*. Environmental enrichment was provided through cotton (cocoon cylinders, LBS) and paper houses (GLP Des Res Mice Dome Home, LBS). Cages were changed once a week for females and once every two weeks for males; soiled bedding was collected from males at the time of cage changing.

All female PgR-IRES-cre were naturally cycling and were sexually naïve at the time of behavioral testing. However, to ensure that females were cycling, females were exposed to male soiled bedding[47]. Following surgery, females were subjected to daily vaginal smears to determine the phase of the estrous cycle they were in.

After behavioral trials, females who had the viral DREADDs injection were perfused and their brains cryopreserved, and females who had sham surgeries were sacrificed through cervical displacement.

2.2. DRUGS

Clozapine-*N*-oxide (CNO) (4936, TOCRIS) was dissolved at a concentration of 5 $mg.ml^{-1}$ in 0,5% dimethyl sulfoxide (D8418-100ML, Sigma-Aldrich®) in saline solution NaCl-0,9% (Braun). CNO was administered at a dose of 0,2 mL of 5 or 10 $mg.kg^{-1}$ intraperitoneally, and an equally matched volume of vehicle (saline) was used as the control.

2.3. VIRAL CONSTRUCTS

Chemogenetics allows the manipulation of neuronal activity for extended periods of time, even though it does not offer the precise temporal control that accompanies other tools, such as optogenetics. One class of chemogenetic agents that has been used as a tool to manipulate behavior through the inhibition and enhancement of neuron firing rate is the Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). These are modified G protein-coupled receptors (GPCRs) that bind only to certain synthetic ligands and result in either hyperpolarization or depolarization of neurons based on the type of GPCR used. Since we wish to silence neurons, we used the DREADD hM4Di which is a modified Gi-coupled GPCR. hM4Di silences neuronal activity by inducing the neurons into a state of hyperpolarization, specifically by increasing the permeability of G protein-coupled inwardly-rectifying potassium channels (GIRKs) upon specific activation by the ligand CNO[48].

For hM4Di expression in the progesterone receptor positive cell population of the VMHvl, we delivered AAV5.hSyn.DIO.hM4D.mCherry (titer: ≥ 4×10^{12} vg/mL; purchased from the Viral Core at the University of North Carolina, Chapel Hill and Addgene; 44362-AAV5) into the VMHvl of PR-IRES-cre animals With this mouse line, the expression of cre recombinase specifically in PR+ cells would allow the expression of hM4Di only in these cells.

To ensure that DREADDs truly decreased the neuronal activity in the infected PR+ cells of VMHvl (and to determine the minimum amount of CNO needed to achieve that goal), we used a genetically encoded calcium indicator, GCaMP6s[49]. GCaMP6s is a combination of a permuted green fluorescent protein (cpGFP), calmodulin (CaM) and the Ca^{2+}/CaM-binding M13 peptide, resulting in, when expressed, calcium-dependent conformational changes in CaM-M13, including modulation of solvent access and the pK_a of the chromophore, cause increased brightness with calcium binding. Since intracellular calcium levels are increased when neurons fire, GCaMP6s can be used as an indicator of neuronal activity. Thus, to observe neuronal activity of PR+ cells in the VMHvl, we delivered the AAV1.hSyn.FLEX.GCaMP6s.WPRE.SV40 (titer:1.84×10^13 vg/mL ; purchased from Perelman School of Medicine; CS0409) virus into the VMHvl of PR-IRES-cre female mice.

Both the GCaMP6s virus and the DREADD hM4Di virus were injected simultaneously in order to perform neuronal silencing along with activity recordings at the same time. For the behavioral experiments, only the DREADD virus was injected.

2.4. STEREOTAXIC SURGERY FOR VIRAL INJECTION AND/OR FIBER IMPLANT

2.4.1. Viral Injection

Adult females 2-3 months old were weighed and then anesthetized with 0,2 mL of a mixture containing 8% ketamine (Imalgene 1000, 03661103001898, Merial) and 12% xylazine (Rompun, (0104007221017929, Bayer) in saline solution injected intraperitoneally. Once the animal was under anesthesia, verified by pinching its paws with a tweezer, her head was placed in the

stereotaxic apparatus (Model 963 Ultra Precise Small Animal Stereotaxic, KOPF®), with a continuous flow of oxygen and isoflurane (1-2% at 1 L/min), fixating it using ear bars with a 3,7-3,6 equal measure in both sides.

The animals' eyes were covered with ointment (Clorocil, 150626, Edol) to prevent drying, the hair from the top of her head was removed with shears, the skin cleaned with Betadine® (MEDAPharma), and an incision was made with surgical scissors in a sagittal plane direction.

After this, hydrogen peroxide was applied with a swab (300230, Deltalab S.L.) to destroy the structural organization of the periosteum tissue, exposing the surface of the skull. This allows us to observe the three main skull sutures: bregma, sagittal and lambda, which are essential reference points to establish the anterio-posterior (AP), medio-lateral (ML) and dorso-ventral (DV) coordinates of the injection site.

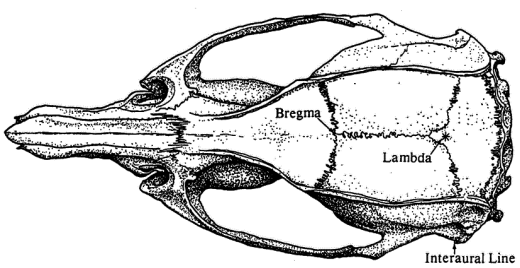

Figure 2.1. Diagram of mice skull. Main points and lines of mice skull, including Bregma, Lambda and Interaural. (Adapted from Paxinos & Franklin, 2014)

First, the bregma and lambda coordinates were noted and the DV coordinates of the two points compared to ensure that the brain was aligned in a horizontal plane (a difference of up to 0,1 mm was allowed in order to proceed to the injection). Subsequently, the following values were added to the bregma coordinates to find the injection site: AP: −1,45, ML: ± 0,7 and DV: −5,8, and two small holes were drilled at these coordinates.

The NANOJECT II (3-000-205/206, Drummond Scientific Co.), assembled to the stereotaxic apparatus with its glass pipette filled with mineral oil (M3516-1L, Sigma-Aldrich®) was then emptied and 1,5 µL of AAV5.hSyn.DIO.hM4D.mCherry (titer: ≥ 4×10^{12} vg/mL) was loaded to the glass pipette.

The pipette was first inserted at ML: −0,70 at a rate of 200 um per 15 seconds, followed by 10 minutes of inactivity until the tissue returned to its original form. Then, the virus was injected into the VMHvl at 0.1 Hz frequency pulses of 4,7 nL injection volumes per pulse for roughly 11 minutes, resulting in a final quantity of 300 nL. The needle was retracted 10 minutes after the full volume of virus was injected, for its proper dissipation into the tissue, at a 200 um per 30 seconds rate. Then the same guidelines were followed when the pipette was inserted on the opposite site, ML: +0,70.

Finally, the incision was sutured with Dafilon® (C0932019, Braun) and the animal remained in isolation until it was fully healed.

2.4.2. Sham surgery

Adult females of 2-3 months old underwent the procedure as described above, however no virus was loaded to pipette that was inserted to the VMHvl.

2.4.3. Fiber Implant

For the fiber implant, after the coordinates for the injection were found and before the holes were drilled, ridges were drilled in the skull to promote the adherence of the first cement: Super-Bond C&B (Sun Medical Co., Ltd); which provides a rough surface to the building cement: Pi-Ku-Plast HP 36 monomer (54000213, Bredent) and polimer (54000215, Bredent) to adhere properly.

Once the Super-Bond solidified, the two holes were drilled. The protocol was then similar to the above described, but this time both AAV5.hSyn.DIO.hM4D.mCherry and AAV1.hSyn.FLEX.GCaMP6s.WPRE.SV40 were injected, 300 nL each per side, resulting in an injection period of 22 minutes on each side.

After both virus were injected bilaterally, the fiber implant (MFC_400/430-0,37_8mm_SM3_FLT, B280-4134-8, Doric lenses) is inserted at ML: −0,70. Once the fiber was in place, several layers of building cement were applied from the surface of the skull to the cannula. Once dry, the incision was sutured and Carprofen, a nonsteroidal anti-inflammatory drug (NSAID) was administered to alleviate pain. Finally, animals were kept in isolation for recovery and until the photometry imaging set of experiments ended, preventing the animals from removing the implants from each other.

2.5. BILATERAL OVARIECTOMY (OVX)

To sexually train the male studs, we used ovariectomized females supplemented with sex hormones to induce receptivity. C57BL/6J female mice were anesthetized with 0,2 mL of 8% ketamine (Imalgene 1000, 03661103001898, Merial) and 12% xylazine (Rompun, (0104007221017929, Bayer) in saline solution injected intraperitoneally and their heads placed in a mask with continuous flow of oxygen and isoflurane (Vetflurane, 575837-4, Virbac). Once the animals were completely unresponsive, their hair was shaved from the lateral flanks and Betadine® was applied to disinfect the skin.

Afterwards, a lateral incision was made in each side to expose the muscle underneath, which was separated from the skin, on the inside, using curved scissors. Then, a small incision was made in the muscle to access the abdominal cavity where tweezers were carefully used to search the ovary, pull it out of the cavity and remove it with a Change-a-tip™ Deluxe High Temperature Cautery kit (Bovie), thus preventing bleeding.

After the same steps were followed on the opposite flank, animals were sutured and administered 0,2 mL Rymadil (5mg/kg, Carprofen, Zoetis).

Each animal remained isolated until the sutures came off, and artificial hormone replacement began one month following surgery. The treatment included the subcutaneous

administration of 5 µl of Estradiol Benzoate in 0,1 mL of Sesame oil and two days later followed by 500 µl of progesterone (Pg) in 0,1 mL sesame oil inducing sexually receptive behavior 4 hours following Pg administration.

2.6. PAP SMEARS

All female PgR-IRES-cre were subjected to pap smears, two weeks following surgery, every day until the day they performed the behavioral trial. At 12:00, they were restrained and using a micropipette, 10 µL of Phosphate Buffered Saline 0.01 M (1X PBS) was introduced in their vaginas through the deposition of a drop, without touching the tip (T-210-Y, Axigen Scientific®) of the micropipette to the skin, thus preventing the induction of pseudopregnancy in the females[25].

The drop of 1X PBS with cells from the lining of their vagina would surface and was collected with the micropipette in the exact same way. After this, the animal was released back to its cage and the 10 uL drop was placed in a microscope slide identified with the proper date.

2.7. STUD TRAINING

Stud is a sexually experienced, highly sexually motivated male mouse presenting a stereotyped behaviour towards females.

C57BL/6J male mice were isolated at 2 months old for two weeks before their training began. Then, a primed OVX C57BL/6J female was placed in their home cage, after the objects for environment enrichment were removed. The hormonally primed OVX female mouse would remain in the cage until the male reached ejaculation or the female started to consecutively reject his advances, which could result in a negative association to sexual behaviour and consequently in a decrease of sexual drive. Each male had three sessions where he would reach ejaculation before being labelled a Stud fit for sexual behaviour experiments.

2.8. SEXUAL BEHAVIOUR EXPERIMENTS

Adult naturally cycling naïve females were placed inside the arena (size 20 x 20 x 20 cm) immediately after the injection of CNO or saline for 45 min – the **habituation period** – providing CNO the time to bind the DREADDs expressed in the neurons and simultaneously monitor its effect on motor function and anxiety, recording it with a PS Eye camera. Afterwards, a male stud is placed inside the arena for:

a) 15 min (after which he is removed) if he does not try to mount (has no drive/motivation), which leads to the placement of another male;

b) 15 min (after which he is removed) if the female is defensive and the male is not able to mount her;

c) until he ejaculates, if the female is receptive and allows copulation.

2.9. SEXUAL BEHAVIOUR ANALYSIS

Mouse behaviour was recorded using a PS Eye camera connected to a computer running BONSAI software and script to acquire the images (30 frames per second). All *habituation periods and trials* for each female were recorded. Analysis of behaviour was performed using BONSAI video annotation package, where a set of behaviours were annotated during the analysis of videos (**Ethogram**). From the annotated behaviours, we defined several measures that gave quantitative aspects of the social and sexual behaviour (**Quantitative measurements of sexual behaviour)**.

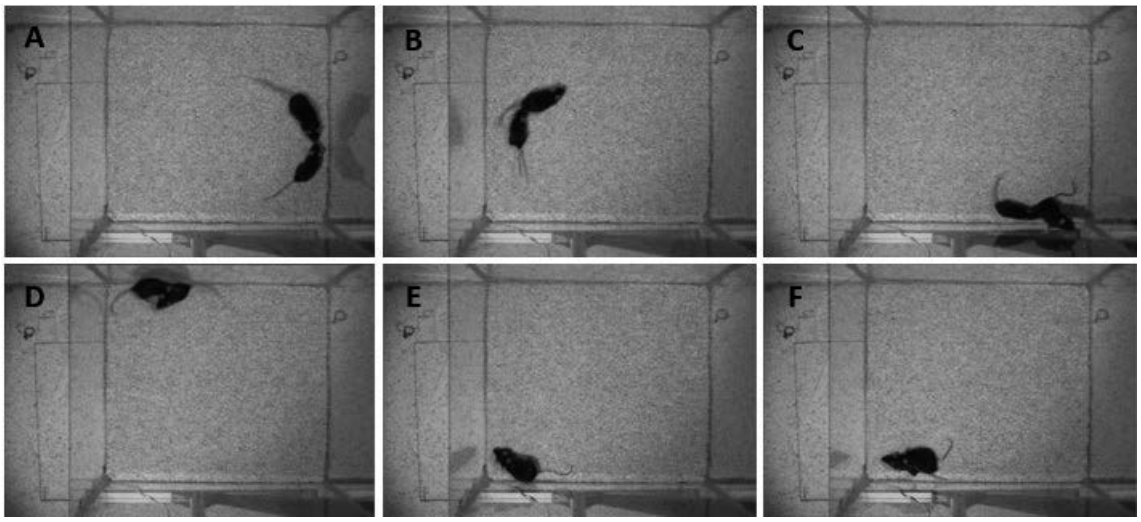

Figure 2.2. Representational images from the sexual behaviour trials. A - Female and male contact nose to nose. B - Female explores the anogenital area of the male. C - Female sniffing the male's body. D - Male attempting to mount the female. E - Male mounting the female successfully with intromissions. F – Male ejaculates and falls to the side.

2.9.1. Ethogram

The following behaviors were manually annotated:

Male in (**M**) – Marks the beginning of behavioural trial, when the male is placed in the open field arena, where the female is already waiting.

Nose-to-nose (N) – Female and male have a nose to nose contact, a socio-investigatory behaviour.

Nose-to-Anogenital (NA) – Female explores the male's anogenital region.

Sniffing Male (S) – Each time the female explores through scent the body of the male, excluding his nose and anogenital region.

Mount Attempt (MA) – Males tries to gain access to the female but due to inexperience or unreceptivity of the female he is not capable to achieve an intromission. Male does unsuccessful pelvic thrust and/or female tries to run away from the male.

Run/Escape (R) – Female escapes and runs away from the male and his thrusts.

Defensive Shoving (DS) – Female pushes or smacks the male when he is in her proximity or trying to mount her.

Defensive Kick (DK) – Female kicks the male upon his attempts to mount her.

Mount with intromission (MI) - Male grabs the female's side and executes a series of pelvic thrusts intromitting the penis in the female vagina for a variable period of time.
Lordosis (L) – When the female achieved the lordosis posture during a mount with intromissions.
Ejaculation (E) – Male is actively mounting the female when he falls to the side carrying the female over with him tightly grasping the female and maintaining genital contact.
Male out (MO) – Represents the end of the trial, when the male is removed from the arena and placed back to his home cage.

2.9.2. Quantitative measurements of sexual behaviour:

Total trial time – The total time of each session, since the male was placed inside the arena until ejaculation or its removal.
Total time of appetitive phase – The total time of the appetitive phase, from the beginning of the trial up to the first mount attempt.
Total time of consummatory phase I – The total time of the consummatory phase I, from the first mount attempt until the first successful mount with intromission.
Total time of consummatory phase II – The total time of the consummatory phase II, from the first mount with intromission until the male ejaculates or is removed from the arena.
Run/Escape time interval (RI) – Represents the time interval in which the female is escaping the grasp of the male attempting to mount
Mount with intromission duration (MID) – Time interval when the male grabs the female's side and executes a series of pelvic thrusts intromitting the penis in the female vagina.
Lordosis Duration (LD) – The time period for which the female, during a mount with intromissions maintained its nose elevated, therefore maintaining the lordosis posture.
Nose to nose events – Total number of times the female's nose contacts with the male's nose.
Anogenital events – Total number of times the female sniffs the anogenital area of the male.
Sniffing male events – Total number of times the females sniffs the whole body of the male, except for the nose and anogenital area.
Total number of Mount Attempts – Total number of times the male attempts to mount the female unsuccessfully.
Mount attempt latency – The period from the begging of the trial until the male first tries to mount the female unsuccessfully.
Total of run away events – Total number of times the female runs away from the male's attempt to mount.
Duration of run away – Sum of the intervals the female spends running away from the male's attempt to mount.

Defensive shoving events – Number of events the female shoves the male away from her.

Defensive kick events – Number of events the female kicks the male away from her.

Mount with intromission latency – The time from the male's placement in the arena until the first mount with intromission successfully.

Total of mounts with intromission – Number of mounts with intromission.

Mean duration of mounts with intromission – The mean of all the time intervals that correspond to a mount with intromission.

Lordosis events – Number of lordosis events the female does during the trial.

Total time of lordosis – Sum of the time intervals where the female is exhibiting lordosis.

Mean duration of Lordosis – The mean of the sum of all time intervals during which the female was in lordosis posture.

Ejaculation Latency – The time comprised between the beginning of the trial until the male ejaculates.

Consecutive mount attempts – The number of times the male tries to consecutively mount the female.

Consecutive run away events – The number of consecutive times the female runs away from the male's advances to mount.

Consecutive defensive events – The number of consecutive times the female defends herself from the male's attempts to mount her.

Consecutive mounts with intromission – The number of consecutive times the male mounts the female with intromissions successfully.

Consecutive lordosis events – The number of consecutive times the female lordosed in mounts with intromissions.

Some measures were not quantified yet, that represent receptive and defensive behaviour, such as Consecutive Mounts with intromissions, temporal order of lordosis events and consecutive defensive and escaping behaviours.

2.10. PHOTOMETRY IMAGING SET UP

The fiber photometry setup is composed of two lasers (473 nm and 556 nm) which are joined to individual patchcords (100 µm core diameter, 0.22 NA) and linked to an individual collimator adapter each (EFL 4.5 mm, NA 0.50) and a neutral density filter, assembled to the main setup unit. Three dichroic mirrors were secure inside the main unit, allowing for 473 nm and 556 nm light emission and consequent GCaMP6f and mCherry fluorescence detection.

The fibers that deliver the 473 nm and 556 nm light were then coupled into a patchcord (200 µm core diameter, 0.48 NA) using a lens (EFL 4.5 mm, NA 0.50) and a rotatory joint. The patchcord was coupled to one of two chronically implanted optical fibers (200 µm core diameter, 0.48 NA). Laser intensities at the patchcord tip, before mating to the chronically implanted fiber, were ~20µW. For GCaMP6f fluorescence detection, light was collected by the lens, transmitted

and reflected by the dichroics before final filtering and focusing into a photodetector. For mCherry fluorescence detection, light was collected by the lens and transmitted through all dichroics before final filtering and focusing into a second photodetector. Photodetector output was digitized at 1 kHz and recorded using custom software in Bonsai[50].

Each animal was submitted to the following protocol, as described in the diagram.

All data analysis will be performed with custom Python software. However, this is ongoing work meaning the protocol which will yield the best results may differ.

2.11. HISTOLOGY

After all behavioural experiments were performed, females with viral injection were anesthetized with 0,3 mL of 8% ketamine (Imalgene 1000, 03661103001898, Merial) and 12% xylazine (Rompun, (0104007221017929, Bayer) in Saline solution and transcardially perfused with 1X PBS followed by 4% paraformaldehyde (PFA) in 1X PBS. Then, their brain was extracted, placed in PFA 4% in PBS1x overnight, after which it was placed in a sucrose (S0389, Sigma-Aldrich®) solution 30% (m/m) with 0,1 % sodium azide (190381000, Acros Organic) for at least two days to cryopreserve.

Following the cryopreservation, a Slinding Microtome SM 2000 R was used to originate three series of coronal brain slices at 40 µm thickness from the extrated brains, after embedding in optimum cutting temperature (OCT) compound (4583, Tissue-Tek), starting at Bregma: -0,82 mm and Interaural: 2,98 mm up to Bregma: -2,30 mm and Interaural: 1,5 mm, so that all the VMH structure would be present. The obtained slices were either stored in 1X PBS-azide 0.1% using 12-Well Plates (712001, NEST® Cell Culture) at 4°C until being assembled in Superfrost® plus microscope slides (4951PLUS4, Thermo Scientific) or directly mounted in Superfrost® plus microscope slides and stored at 4ºC.

2.12. HISTOCHEMISTRY

2.12.1. Vaginal cytology

The samples from vaginal pap smears were stained to identify the cell population ratio and consequently identify the estrous phase each animal was in any given day through brightfield microscopy.The slides with the samples were dried in a hot plate before proceeding with the protocol. Only then, were they placed in container with 96% Ethanol (aga) for 2 minutes, then moved to tap water for 1 minutes, after which they remained in Harris Hematoxilin (05-1211/L, Bio-optic) for 2 minutes, followed by 30 seconds of running tap water and 30 seconds in 96% ethanol. Then, the slides were moved to a hot plate to dry before being placed in Histoclear (HS-202, National Diagnostics) for at least 5 minutes. Last, the slides were covered with DPX Mounting Medium (44581-500ML, Sigma-Aldrich®) and the coverslipped (Mensel-Gläser).

2.12.2. DAPI staining

The first of three series of 40 µm thickness brain slices from each brain obtained from the sliding Microtome was stained with DAPI for nuclei identification through fluorescent microscopy. The samples were previously left to dry in the air, after which the Super PAP pen liquid blocker (Daido Sangyo, Ltd) was used to outline the slices in the slides to prevent liquid to leave its surface. Starting with two 5 minutes wash of 1X PBS to remove the OCT excess, followed by exposure to diluted DAPI (4,6-Diamidine-2′-phenylindole dihydrochloride 1:1000 in 1X PBS) for 20 minutes to slides while protected from direct light preventing bleaching. Then, the solution was removed and the slides washed again 2 times for 5 minutes each. Finally, the slides were rinsed with distilled water and left to air dry before Mowiol mounting medium and coverslips were applied over the tissue sections.

2.13. MICROSCOPY ANALYSIS

All vaginal cytology was observed through brightfield microscopy in Axio Scope.A1 for evaluation and representative images were captured using the Widefield Fluorescence Scanning Microscope Axio Imager.M2.

Axio Scan Z1 slidescaner microscope (Leica) was used to capture semi-automatically representative images of all animals injected, using a 10x magnification objective. In order to have higher resolution images of all animals used for cell quantification, Confocal Laser Point-Scanning Microscope was used, with a 20x magnification objective. All acquired images were analyzed using Zen lite 2.3 software (Zeiss).

2.14. VIDEO ANNOTATION AND DATA ANALYSIS

A tailored python script was created to process the csv files originated from the Bonsai Video annotation script in order to separate, group and count the different behaviors analysed: NoseToNose, Anogenital, Sniffing Male, Mount Attempt, Mount with Intromission, Run Away, Defensive shoving, Defensive kick, Lordosis, Ejaculation; and respective time intervals: MovieStart, Mount with Intromission duration, Run Away Duration, Lordosis Duration, MovieEnd.

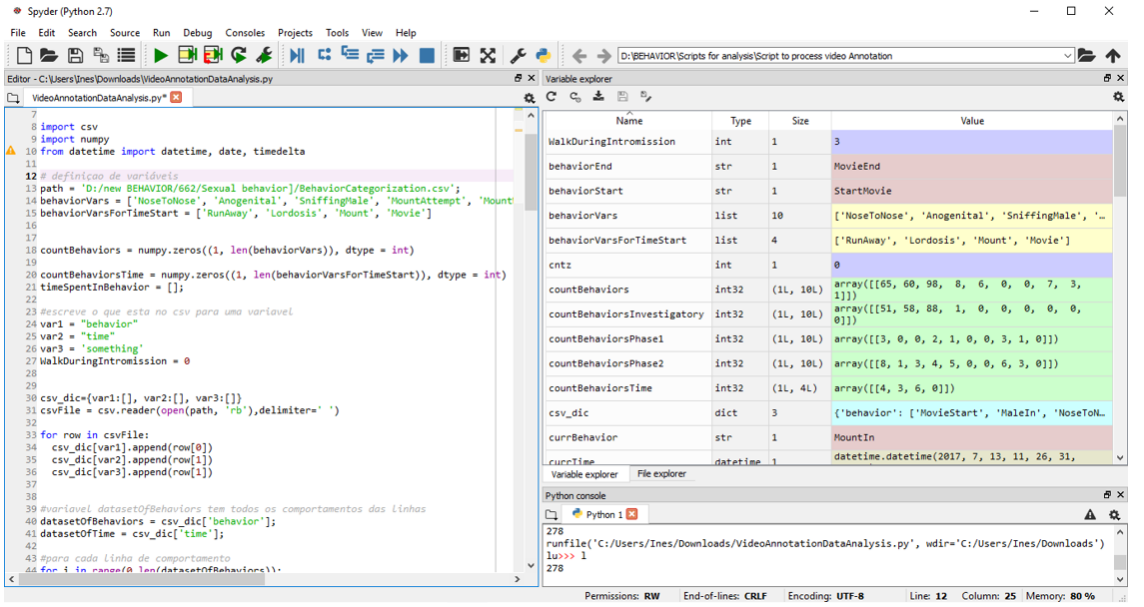

Figure 2.3. Representational image of Spyder python 2.7 software running the tailored script. On the left window part of the script is showing and on the right panel, the console, where the results from the script appear.

2.15. IMAGE QUANTIFICATION

The presence, location and number of PR+ cells infected with DREADD hM4Di was assessed for each successfully injected animal. Subjects with unilateral or non-existent viral expression were excluded from the study.

The number of infected cells was assessed using the ImageJ software, where confocal obtained images where converted to 8-bit files, and an automatic threshold was applied to each image. The region of interest (ROIs) was chosen based on the functional division of the VMH as previously described and a simple ImageJ Macro program was written to automatize this process, yielding the number of cells stained with DAPI and the number of cells infected with mCherry.

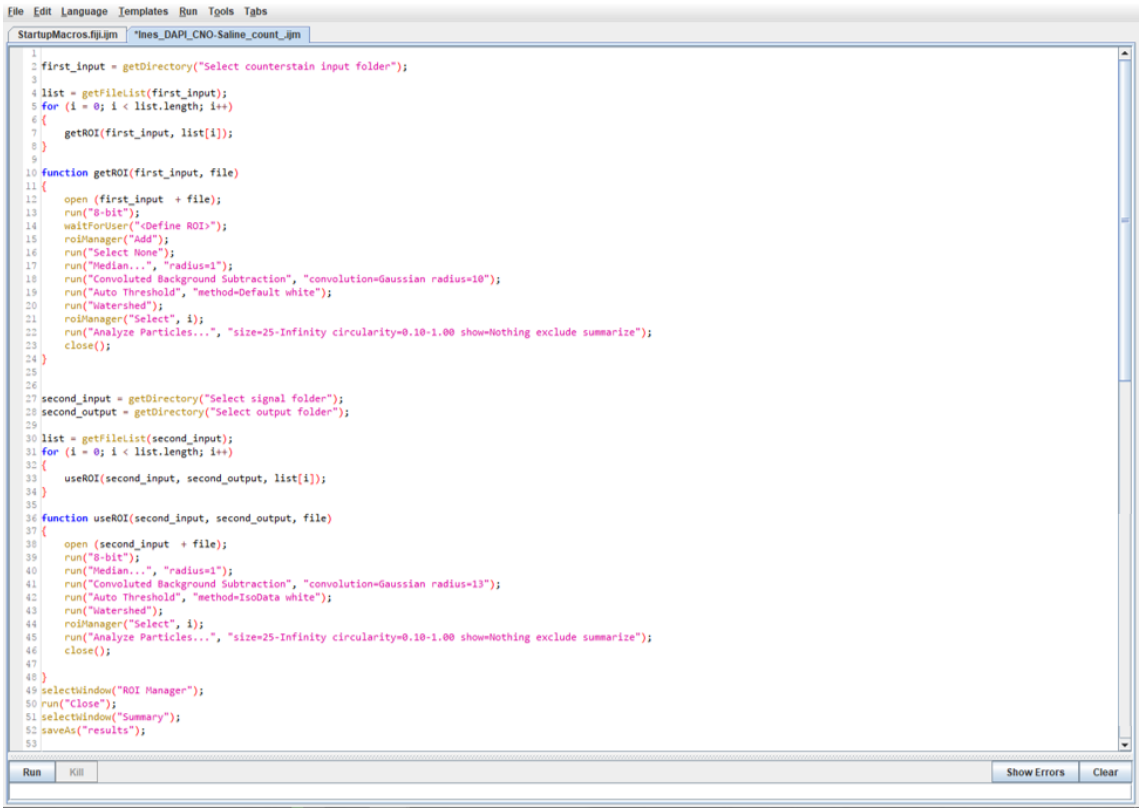

Figure 2.4. ImageJ macro for cell counting. Illustration of the ImageJ macro written to automatize the cell counting process.

2.16. STATISTICS

The values withdrawn from the behaviour annotation were evaluated for their statistical significance between the three groups using the non-parametric Kruskal-Wallis test with a non-Gaussian distribution. Values from each analysis can be consulted in Table 1-4 at the Appendix.

The relation between the number of infected PgR+ cells in the VMHvl and the behavioral output of each animal was obtained through a linear regression, where R^2 values can be view in table 5 at the appendix section.

Chapter 3 | RESULTS

This chapter describes the evaluation of the influence of the Progesterone Receptor positive (PgR+) neuronal population of the ventrolateral portion of the Ventromedial Hypothalamus (VMHvl) in female sexual behaviour. This was possible through the comparison of behavioural parameters between naturally cycling naïve females with an intact VMHvl and females whose VMHvl was silenced. PgR-IRES-cre females were injected with an inhibitory Designer Receptors Exclusively Activated by Designer Drugs (DREADD) cre-dependent virus, silencing the above-mentioned neuronal population. The consequent results of this experiment were examined considering the percentage of cells infected by the virus, allowing us to observe and document a deviation in sexual behaviour from the controls.

To evaluate the effect of disrupting the PgR+ neuronal population of the VMHvl we had three groups (two controls and one experimental) of animals:

- DREADD + CNO: Females subjected to a viral injection through a stereotaxic surgery to express hM4Di DREADD specifically in PgR+ neurons of the VMHvl and subsequently CNO injection.
- SHAM + CNO: Females subjected to a Sham surgery and were injected with the same concentration of CNO as the experimental group. With this group, we tested for the effects of the drug CNO.
- DREADD + Saline: Females subjected to a viral injection through a stereotaxic surgery to express hM4Di DREADD specifically in PgR+ neurons of the VMHvl. These mice were injected with the same volume per weight as the experimental group. With this group we tested for the effects of the viral injection and expression of DREADDs alone.

3.1. ESTROUS CYCLE IS NOT HOMOGENOUS ACROSS INDIVIDUALS

Female sexual behaviour is dependent on the estrous cycle, meaning the fluctuation of Estrogen and Progesterone concentration in the animal's blood flow. Since the experiments were performed with naturally cycling females, it was of extreme importance to monitor their cycle through daily pap smears. The outcome of this procedure, where each sample was stained following the Vaginal Cytology protocol (Figure 3.1.A-D). Each stage of the cycle can be identified by determining the proportion of the different cellular populations in the vaginal smear. Whenever the smear is characterized by a high proportion of nucleated epithelial cells, the female is said to be in proestrous, the most receptive phase of the cycle.

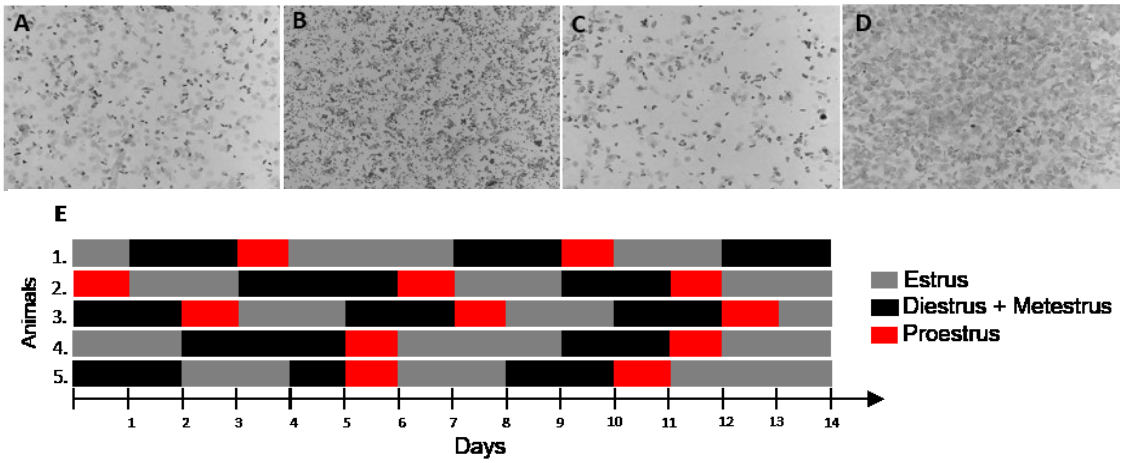

Figure 3.1. Representation of citology of the different phases of the estrous cycle and its transition across time. **A –** Pap smear image of the metestrus phase where the presence of cornified epithelial cells is higher than nucleated epithelial cells and some leucocytes may appear. **B** – Pap smear image of the diestrus phase, which is characterized by an overwhelming presence of leucocytes. **C** – Pap smear image of the proestrus phase which is characterized by a higher proportion of nucleated epithelial cells than cornified epithelial cells. **D** – Pap smear image of the estrus phase which accounts with the presence of only cornified epithelial cells. **E** – Representational diagram of the estrus cycle of five representational animals across 14 days.

Even though the transition between estrous stages is preserved across days (estrous is always followed by diestrous+metestrus and diestrous+metestrus is always followed by proestrous), the length of each phase can vary across individuals (Figure 3.1.E). This means that every day we may, or not, have females in the appropriate phase of the cycle. Therefore, in order to minimize the effect of uncontrollable variables (such as temperature, etc) we made sure to always have a representative proestrous female of each group every experiment day.

3.2. FIBER PHOTOMETRY

Proving the effect of the DREADD inhibition strategy was essential. To do so, in a set of extra females we injected simultaneously the inhibitory virus (AAV5.hSyn.DIO.hM4D.mCherry) and the genetically encoded calcium indicator virus (AAV1.hSyn.FLEX.GCaMP6s) to monitor neuronal activity. The photometry experiments were performed according to the scheme in figure 3.2.

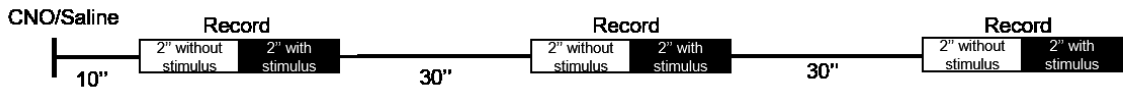

Figure 3.2. Diagram representing the organization of the photometry recordings. After the animal had the patch-cord, which emits the excitatory light, CNO (5mg.Kg^{-1} or 10mg.Kg^{-1}) or Saline is injected and the animal is placed alone in the arena. Following the first 10 minutes, we began recording for 2 minutes and then place the male stimulus inside the arena for the next 2 minutes of recording, after which it is removed. The process is repeated twice following a 30 minutes interval. Then, the male is removed, the patch-cord and the female placed in her home cage.

By chance, and because neurons can be infected by more than one AAV, most neurons should have been infected with AAV-DREADD and AAV-GCaMP. In principle, this experiment should allow us to monitor the activity of PgR+ neurons with GCaMP6 before their inhibition and after CNO was administered. PgR+ neurons are highly active when females interact with male stimuli (Nomoto and Lima, unpublished data). Therefore, before CNO administration we were expecting to observe fluorescence transients when females interact with males. After CNO injection, DREADD expressing neurons should be hyperpolarized and consequently the fluorescence transients should disappear or at least be highly diminished.

No successful recording was obtained in the 6 animals that were co-injected with the two viruses. For that reason, we proceeded to the analysis of the injected brains. Out of the six injected animals, it was possible to assess the histology of four of them. The other two animals tore the fiber from the head of one another (they were being group-caged), making it impossible to analyse the correct placement of the fiber. Consequently, from then on, all animals with fiber implants were in isolation following the surgery until the end of the behavioural tests.

We analysed expression of the virus (mCherry expressing neurons for the DREADD virus and GFP for the GCaMP virus) and fiber placement.

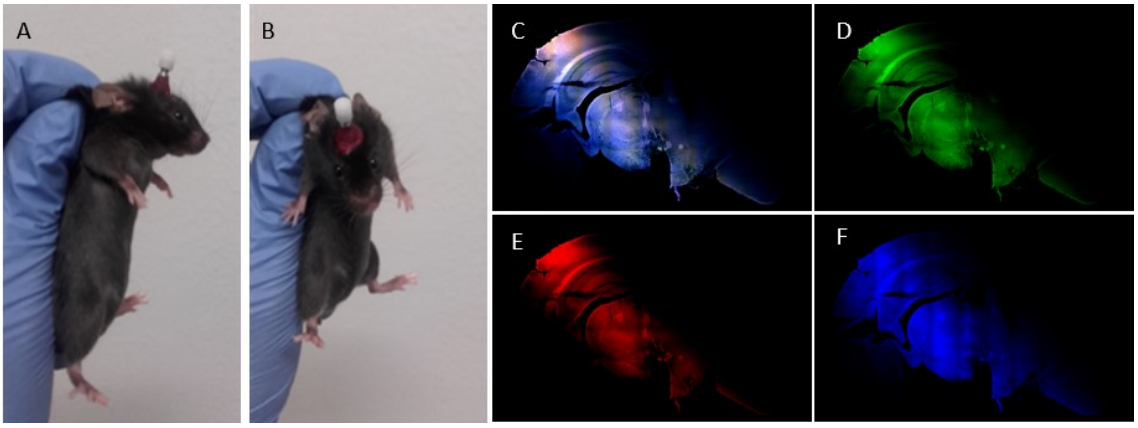

Figure 3.3. Representation of post-surgery mice and histology images after fiber placement for fiber photometry. A-B – frontal and lateral view of PgR+ female following the viral injection and fiber placement. **C-F** – representative image of a fiber placed at the wrong coordinates while the viral injection seems correct at both sites with signal for EGFP, mCherry and DAPI.

In two animals (Figure 3.3.C-F) we observed co-localization of red and green fluorophores in neurons of the VMHvl. However, in these two animals the fiber was placed caudally from the VMHvl, explaining why we could not observe any fluorescence signal in these individuals.

In the remaining two animals (Figure 3.4) we could only detect Green fluorescence and the fiber was placed anterior to the VMHvl. For that reason, in these two animals we could not detect any fluorescence.

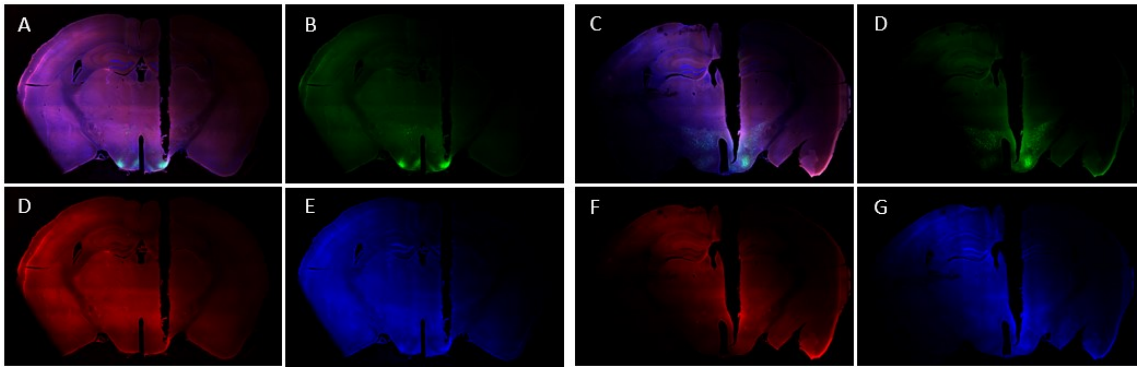

Figure 3.4. Representative images of PgR-IRES-cre animals injected with both DREADD and GCamp6s vírus. A-G – The fiber is placed at the right coordinates and the viral injection seems to be correct at both sites with signal for EGFP and DAPI, while no mCherry signal for the DREADD infection.

3.3. HISTOCHEMISTRY OF ANIMALS FROM THE DREADD EXPERIMENTS

Before analysing the behaviour of the animals infected with DREADDs, we examined the expression of the viral construct in the VMHvl to ensure the expression was performed in the VMHvl and that it was present in both hemispheres (only animals with Red signal on both VMHvl were included).

First of all, it was necessary to ensure that the AAV5.hSyn.DIO.hM4D.mCherry was solely expressed in a Cre dependent manner. To do so, two C57BL/6 animals were injected with the virus, and the results of the viral infection can be seen in the following images in figure 3.5. No Red fluorescence was observed in these two Cre-negative animals, ensuring us that the virus is not leaky (cannot express its transgene before being recombined).

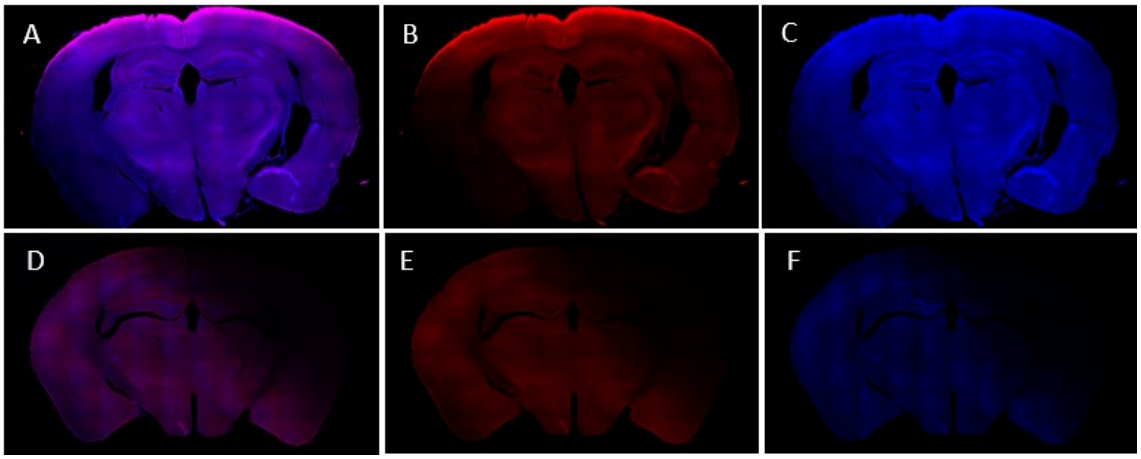

Figure 3.5. Coronal section of the brain of two WT BL6 animals injected with DREADD. A-F – mCherry expression and DAPI staining in a coronal slice of each animal. No red fluorescence (mCherry) is visible in the images.

In total, twenty-one PgR-IRES-cre females were injected with AAV5.hSyn.DIO.hM4D.mCherry and participated in the behavioural experiments; however, only

fourteen of those animals were considered for behaviour and cell quantification. The other seven animals had unilateral expression of Red fluorophore.

Even though, in some of the fourteen animals considered, the injection was spread through the adjacent areas of the VMHvl, all animals had a correct bilateral injection and mCherry-associated DREADD expression in the VMHvl.

The selection process is represented in the following diagram:

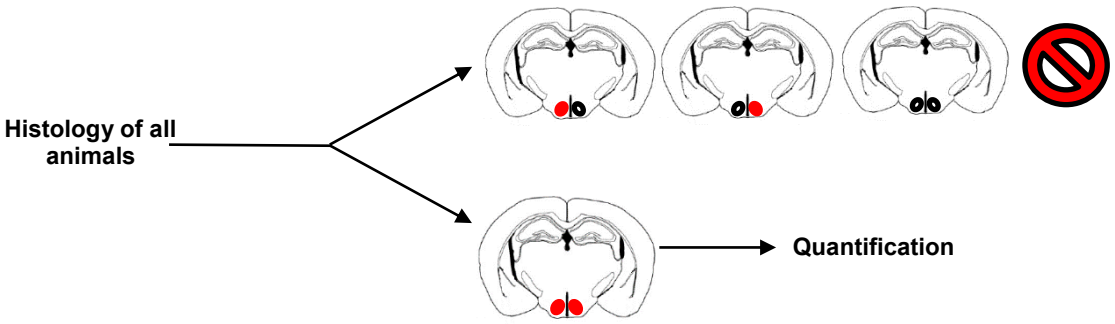

Figure 3.6. Diagram representing the initial screening process. The histology of all animals injected with virus when through a screening before they were quantified, through fluorescence microscope observation to determine whether the animal had a bilateral infection of the VMHvl.

The histology representing the injection site of the final fourteen animals is presented as illustrated in figure 3.7. The DREADD+Saline animals are represented in figure 3.8 while all the DREADD+CNO animals are represented in figure 3.9.

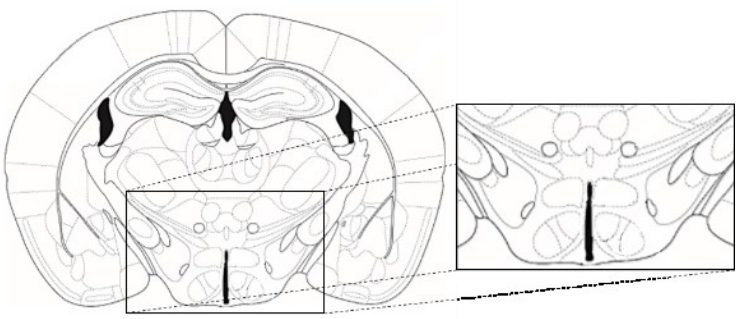

Figure 3.7. Illustration representing the injection site area observed in histology images for all animals. Magnification of hypothalamic region, evidencing the VMHvl and adjacent regions.

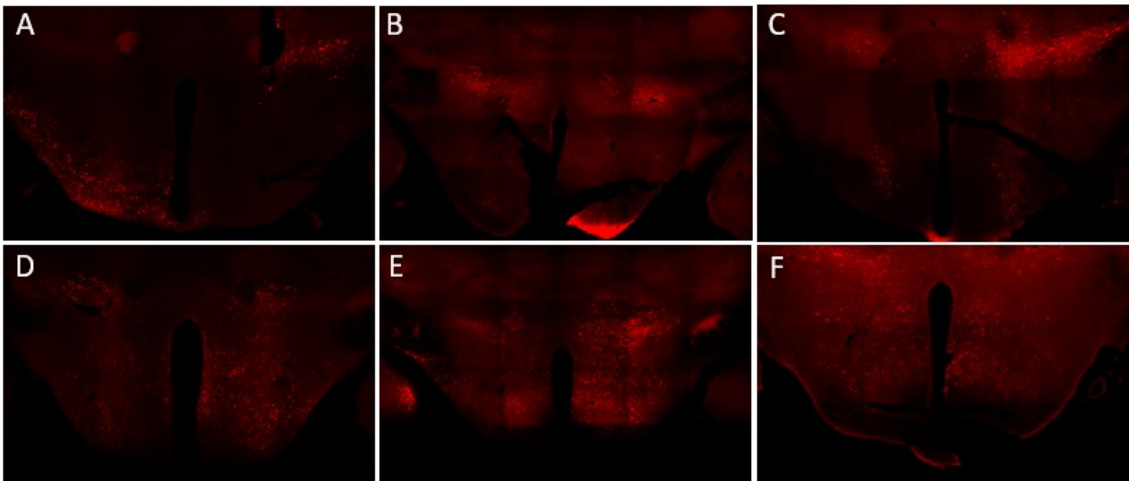

Figure 3.8. Representative images of neuronal mCherry expression in hypothalamic region of all DREADD+Saline animals. A-F – mCherry-expressing neurons in a coronal slice obtained from each one of the animals reflecting the DREADD expression. Red fluorescence can be observed at the injection site in brains slices from all animals.

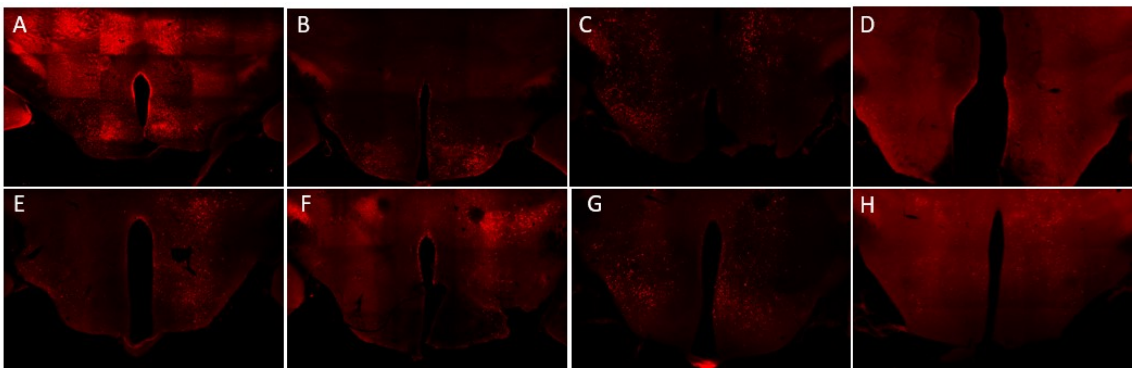

Figure 3.9. Representative images of neuronal mCherry expression in hypothalamic region of all DREADD+CNO animals. A-H – mCherry-expressing neurons in a coronal slice obtained from each one of the animals reflecting the DREADD expression. Red fluorescence can be observed at the injection site in brains slices from all animals.

3.4. QUANTIFICATION OF DREADD INFECTION IN VMHvl PgR+ CELLS

To determine the extent of viral infection, we proceeded to quantify the percentage of cells infected in the animals that presented bilateral mCherry expression.

Fourteen animals had their infected cells quantified through an ImageJ Macro, originating the results illustrated in Table 3.1. Here, it is possible to observe a wide range of infected PgR+ cells in the VMHvl. The percentage of infected cells in DREADD injected animals exposed to CNO ranged from 5,5% to 62,3%, and in animals exposed to only saline from 11,9% to 86,4%.

Table 3. 1. Table containing the values yield from the ImageJ macro for cell quantification. The number of cells contained in each animal, for each side, were originated from the same region of interest (ROI).

Animal	LEFT SIDE			RIGHT SIDE			Average (%)
	mCherry fluorescent cells	DAPI stained nuclei	Infected cells (%)	mCherry fluorescent cells	DAPI stained nuclei	Infected cells (%)	
463	207	605	34,215	17	803	2,12	18,166
587	1286	1622	79,285	642	687	93,45	86,367
596	129	539	23,933	214	686	31,20	27,564
602	189	1537	12,297	254	1806	14,06	13,180
629	151	1256	12,022	128	1094	11,70	11,861
630	595	1863	31,938	350	1662	21,06	26,498
428	65	593	10,961	43	554	7,76	9,361
431	116	461	25,163	373	717	52,02	38,593
588	329	637	51,648	332	586	56,66	54,152
592	101	568	17,782	119	747	15,93	16,856
648	56	1130	4,956	80	1323	6,05	5,501
655	412	668	61,677	455	723	62,93	62,304
658	241	568	42,430	380	839	45,29	43,861
662	197	906	21,744	210	1078	19,48	20,612

3.5. DESCRIPTIVE ANALYSES AND STATISTICS

For a better comprehension of the data collected from the video annotation we will first look at the behaviour as whole and then divide it into the following three parts:

i. the appetitive phase, which starts with the male introduction to the arena until the male does his first mount attempt;
ii. the first part of the consummatory phase, which corresponds to the time between the first mount attempt and the first mount with intromission;
iii. the second part of the consummatory phase which ranges from the first mount with intromission until either the male ejaculates or is removed from the arena.

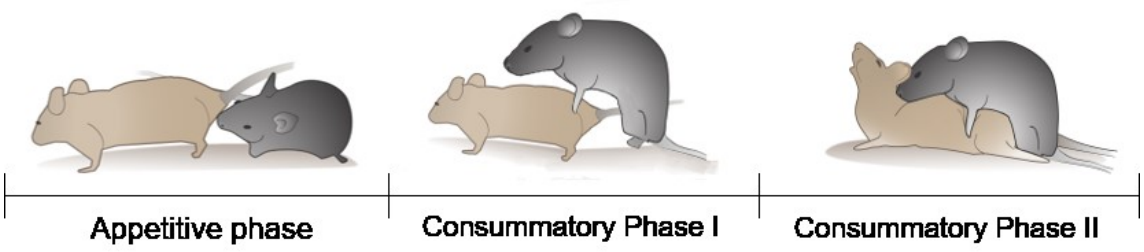

Figure 3.10. Illustration of the different phases that compose sexual behaviour. Sexual behaviour is composed of the appetitive phase, where socio-investigatory behaviours occur; the consummatory phase I, where the male attempts to mount the female; and the consummatory phase II, where the male mounts the female with intromission repeatedly until he reaches ejaculation.

i. General analysis of behaviour

In this section, we describe the general parameters analysed in each session, such as the total duration of trials and the duration of each phase in which the behaviour parameters are examined.

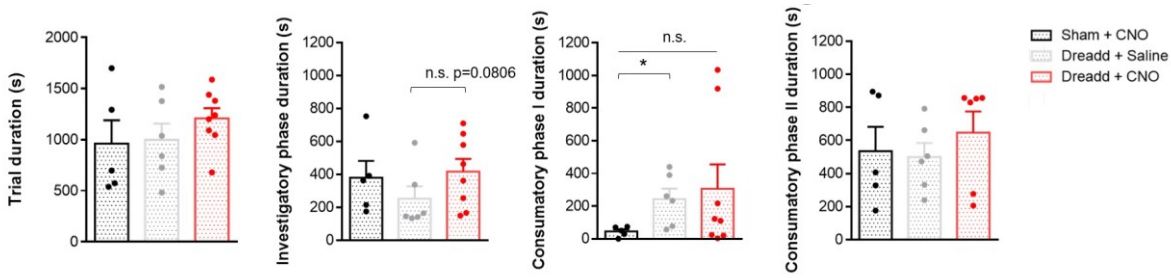

Figure 3.11. Absolute duration of trials and each one of the phases. **A** – Trial duration for each animal group. **B-D** – Time spent by each animal group in the appetitive phase (**B**), the consummatory phase I (**C**) and II (**D**). Data are presented as mean ± sem. Kruskal-Wallis test. Mann-Whitney test, *p<0,05. N=5 for Sham+CNO, N=6 for DREADD+Saline and N=8 for DREADD+CNO.

The total duration of trial for each animal was widely distributed for all conditions (Figure 3.11.A).

We evaluate the time each animal, of each group, spent in the different phases of the trial. In the appetitive phase, no significant differences were observed between the three groups (Figure 3.11.B). In the same graph, it is possible to observe a small cluster of DREADD animals injected with saline, whereas in the other groups, animals have a disperse distribution.

Regarding the time that each animal spent between the first mount attempt and the first successful mount with intromission (Figure 3.11.C), it is possible to observe that, even if not significant, animals subjected to sham surgery who were administered with CNO allowed the males to intromit faster than animals in the remaining groups. Furthermore, it is important to highlight that the two DREADD+CNO animals that spent the longest period in this phase failed to have a successful mount with intromission.

Finally, we quantified the time spent in the second phase of the consummatory behaviour, meaning the time from the first intromission until the male ejaculated or was removed from the arena (Figure 3.11.D). The values of the first two conditions represented in the graph, Sham+CNO and DREADD+Saline, show a scatter distribution, while the values from DREADD+CNO animals seems to cluster around 800 seconds, a consequence of removing the male from the arena, meaning none of those males ejaculated.

The graphs presented in figure 3.12 are still part of the general aspects analysis of the sessions in percentage and in proportion to the complete trial. Here, in A, the two animals which did not reach the final stage (consummatory phase II) were excluded to properly assess the behaviour of the animals who performed all the stages of the behaviour. Observation of this graph indicates that the proportions of time spent in each phase by females DREADD+CNO are similar to Sham+CNO, and in the consummatory phase I the proportion of time spent is smaller than the one by DREADD+Saline females. In B, the two previously excluded animals are included and the

DREADD+CNO behaviour proportion shifts, becoming more similar to the one exhibited by DREADD+Saline females.

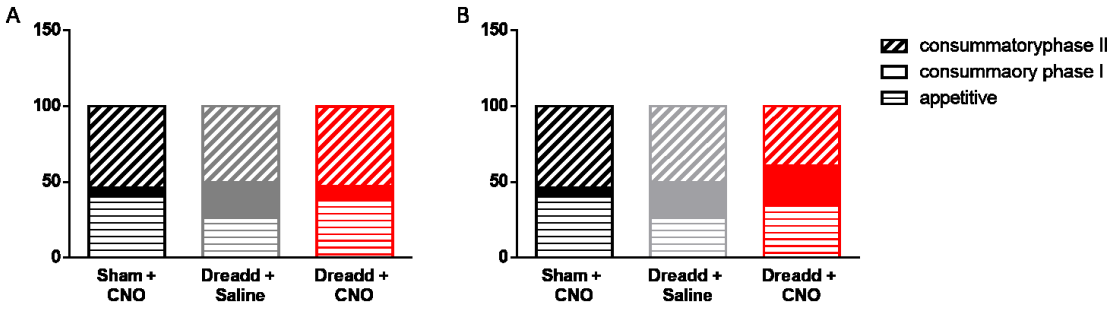

Figure 3.12. Representation of the proportions of time each group spent in each phase of the behaviour. A – Percentage of time the animals spent in each phase of the behavior test without accounting for the two DREADD+CNO animals who did not move to the final stage (consummatory phase II). **B** – Percentage of time all animals spent in each phase of the behavior test during the trial duration. Data are presented as mean ± sd. N=5 for Sham+CNO, N=6 for DREADD+Saline, N=6 for DREADD+CNO (A) and N=8 for DREADD+CNO (B).

Additionally, we performed the following measures:

i. the percentage of animals that were able to intromit female mice (Figure 3.13.A);
ii. the percentage of males who managed to ejaculate without being rejected by the females (Figure 3.13.B).

A comparative observation of these measures show us that 2 of the 8 males (25%) paired with DREADD+CNO females were not able to mount them with intromissions and from the remaining 6, only 2 (33%) managed to reach ejaculation within the trial's time interval. Furthermore, 1 of the 5 males (20%) paired with Sham+CNO and 3 of the 6 males (50%) paired with DREADD+Saline were not able to reach ejaculation either.

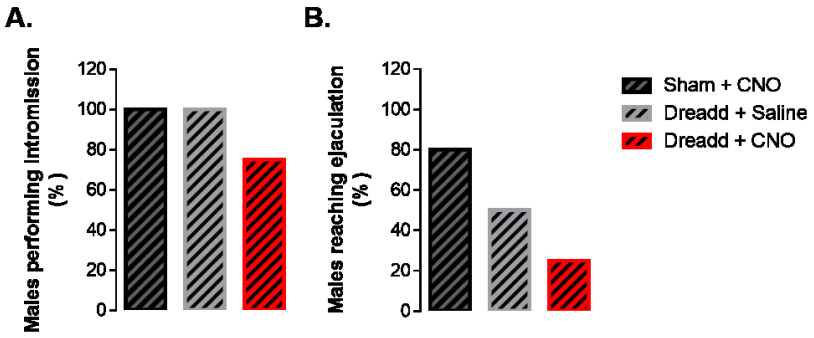

Figure 3.13. Quantification of male related parameters. A - percentage of males who intromitted during each session. **B** - percentage of animals who ejaculated. Data are presented as mean ± sd. N=5 for Sham+CNO, N=6 for DREADD+Saline and N=8 for DREADD+CNO.

ii. Appetitive phase

Here we analysed female behaviour in the investigatory stage, exploring the socio-investigatory component of the behaviour in order to try to perceive any differences that the inhibition of PgR+ cells in the VMHvl might have induced.

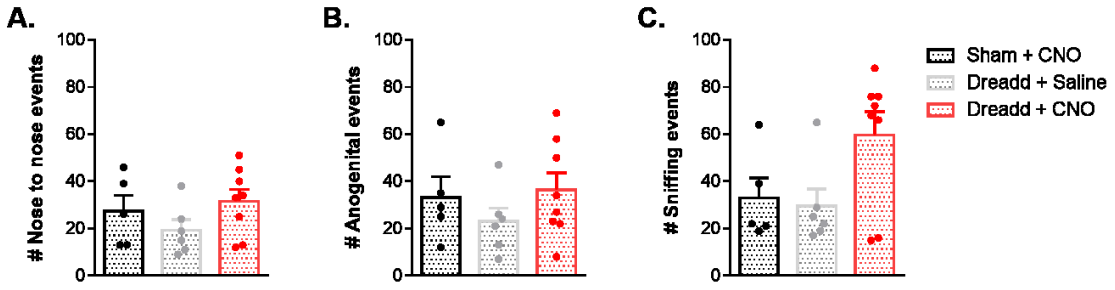

Figure 3.14. Absolute quantification of socio-investigatory events in Appetitive phase. A - Absolute values for nose to nose contacts between the female and the male. **B** - Total number of events where the female explored the anogenital area of the male. **C** – Total number of events where the female explored the male's body. Data are presented as mean ± sem. Kruskal-Wallis test. Mann-Whitney test. N=5 for Sham+CNO, N=6 for DREADD+Saline and N=8 for DREADD+CNO.

Regarding the absolute number of nose to nose interactions between the male and the female, all groups show equivalent values (Figure 3.14.A). Plus, in a general view, no significant differences are visible between groups considering the number of times the female sniffed the anogenital region of the male (Figure 3.14.B). However, it might be of note that DREADD+Saline females seem to be slightly less invested in exploring this anatomic area of the male. Then, by looking at the number of times the female sniffed the male's body, except the nose and anogenital regions, even if not statistically significant, DREADD+CNO animals seem to spend more time exploring the male scent when compared to the other groups (Figure 3.14.C).

Nevertheless, these are absolute values which may not truly represent the interaction displayed by the animals given the fact that the duration of appetitive phase varies between the animals, within all groups. Thus, we analysed the frequency of occurrences for each behaviour (Figure 3.15).

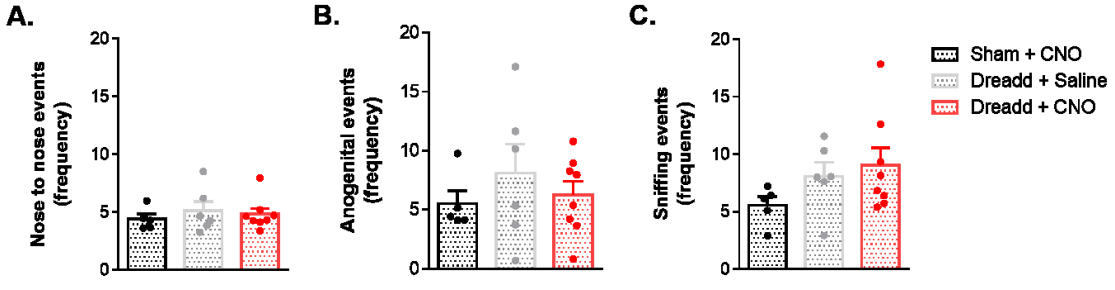

Figure 3.15. Frequency of socio-investigatory events in Appetitive phase. A - Frequency in which the female exhibits nose to nose contact. **B** - Frequency in which the female investigates the anogenital area of the males. **C** - Frequency in which the female explores the male's body. Data are presented for the number of events/min as mean ± sem. Kruskal-Wallis test. Mann-Whitney test. N=5 for Sham+CNO, N=6 for DREADD+Saline and N=8 for DREADD+CNO.

The nose to nose interaction (Figure 3.15.A) seems to be intact, since all considered groups have equal frequency in executing these interaction behaviours with the male. Though, by observing the frequency of anogenital events (Figure 3.15.B), we can see an increase in the distribution of the values in both DREADD+CNO and DREADD+Saline animals, a synonym to variability between animals that contrasts with Sham+CNO animals, which seem to have a consistent frequency of events exploring this portion of the male's body. Finally, when it comes to sniffing the body of the male (Figure 3.15.C), there seems to be a very faded tendency for the increasing of the frequency of this behaviour in DREADD females and even more so if CNO is administered.

iii. **Consummatory phase I**

Here we analysed the behaviours that occurred in the shortest phase of sexual behaviour. Again, we compared the absolute values for the number of occurrences of each group of events with their frequency.

In a first look across the results regarding the total number of investigatory events in this phase (Figure 3.16) we can safely assume that Sham+CNO females, the ones who spent the least amount of time in the first phase of the consummatory behaviour, consequently also have the least number of related events occurring. DREADD+CNO animals present the highest number of events across all behaviors, although the difference is not significant, and overall DREADD+Saline and CNO have similar values.

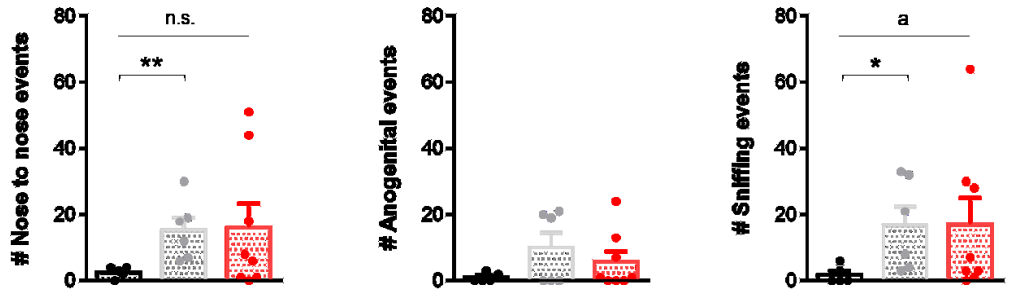

Figure 3.16. Absolute quantification of socio-investigatory events in Consummatory phase I. **A** - Absolute values for nose to nose contacts between the female and the male. **B** - Total number of events where the female explored the anogenital area of the male. **C** – Total number of events where the female explored the male's body. Data are presented as mean ± sem. Kruskal-Wallis test, (a)p<0,05. Mann-Whitney test, *p<0,05, **p<0,01. N=5 for Sham+CNO, N=6 for DREADD+Saline and N=8 for DREADD+CNO.

Regarding the frequency of events (Figure 3.17), it is possible to observe that all the groups seem to share the same tendency as the one presented regarding the total number of occurrences. In fact, besides the fact that DREADD females presented a greater number of events, they also seem to execute them more frequently than Sham+CNO, excepting for the anogenital exploration, where DREADD+CNO values are similar to Sham+CNO.

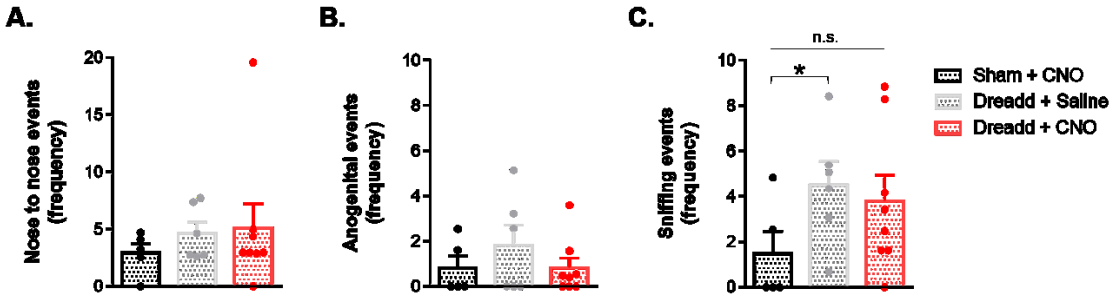

Figure 3.17. Frequency of socio-investigatory events in Consummatory phase I. A - Frequency in which the female exhibits nose to nose contact. **B** - Frequency in which the female investigates the anogenital area of the males. **C** - Frequency in which the female explores the male's body. Data are presented for the number of events/min as mean ± sem. Kruskal-Wallis test. Mann-Whitney test, *p<0,05. N=5 for Sham+CNO, N=6 for DREADD+Saline and N=8 for DREADD+CNO.

Then, we analysed the frequency of mount attempts (Figure 3.18.A) in the present phase, where all groups present similar values with no statistical significant differences. Although, males seem to attempt to mount DREADD+Saline females less than females from the other groups, when considering the percentage of running events and the time the females spent running (Figure 3.18.B-C), all groups have similar values, even if in Sham+CNO animals seem to spend slightly more time running than DREADD+Saline and DREAD+CNO animals. However, an analysis across graphs show that DREADD+CNO females run away less often but for longer time, when compared with the remaining proportions exhibited by Sham+CNO and DREADD+Saline females.

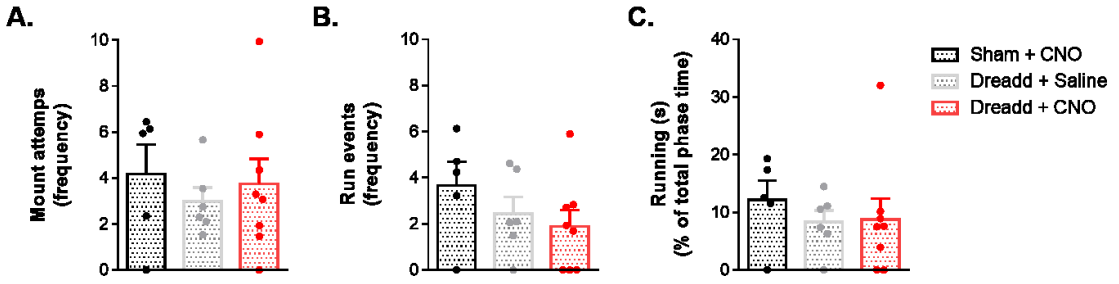

Figure 3.18. Mount attempts and escape events in Consummatory phase I in relation to the time each animal spent in this phase. A - Frequency with which the male attempted to mount the female. **B** - Frequency of events in which the female run away or escaped from the male. **C** – Percentage of time the female spent running away from the male. Data are presented as mean ± sem. Kruskal-Wallis test. Mann-Whitney test. N=5 for Sham+CNO, N=6 for DREADD+Saline and N=8 for DREADD+CNO.

Finally, we analysed the defensive or non-receptive behaviour exhibited by females in the first part of the consummatory phase (Figure 3.19.A). By looking at the results obtained with this analysis, we can clearly see that Sham+CNO females did not produced any defensive behaviour. Contrarily, DREADD+Saline animals presented defensive behaviours, which were displayed in a greater extent in DREADD+CNO animals.

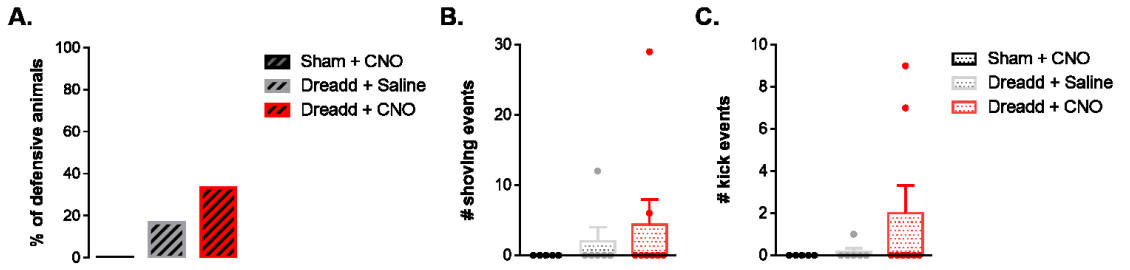

Figure 3.19. Illustration of the defensive behavior exhibited by females during the consummatory phase I of behavior. **A** - Percentage of animals of each group that executed defensive behaviours: shoving and kicking. **B** - Absolute values for the number of shoving events executed by each animal. **C** - Absolute values for the number of kicking events executed by each animal. Data are presented as mean ± sem. Kruskal-Wallis test. Mann-Whitney test. N=5 for Sham+CNO, N=6 for DREADD+Saline and N=8 for DREADD+CNO.

iv. Consummatory phase II

In this section, we present the behavioural parameters measured in the second phase of consummatory behaviour, which began with the male's first intromission up to ejaculation or its removal from the arena.

Even if not statistically significant, is clearly visible that the Sham+CNO animals had a smaller number of investigatory events than the two remaining groups, which present equivalent values between them (Figure 3.20).

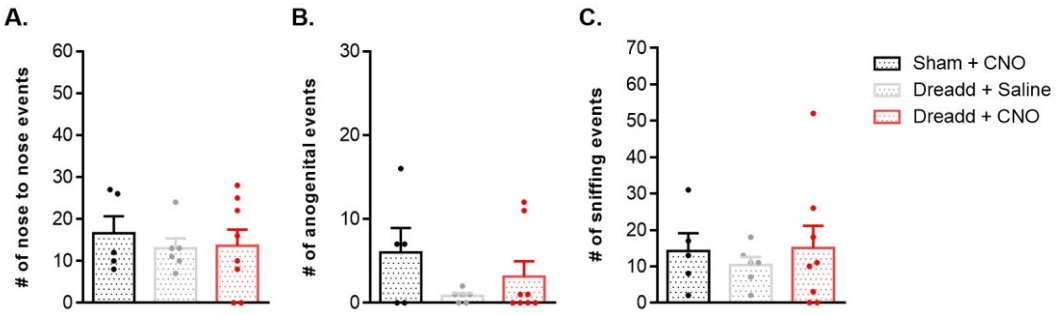

Figure 3.20. Absolute quantification of socio-investigatory events in Consummatory phase II. **A** - Absolute values for nose to nose contacts between the female and the male. **B** - Total number of events where the female explored the anogenital area of the male. **C** – Total number of events where the female explored the male's body. Data are presented as mean ± sem. Kruskal-Wallis test. Mann-Whitney test. N=5 for Sham+CNO, N=6 for DREADD+Saline and N=6 for DREADD+CNO.

However, when comparing the frequency of events (Figure 3.21) in the total duration of phase II, all values between groups are relatively equal. The translation of this results suggests that Sham+CNO animals performed less events due to the fact that they spent fewer time in this phase when compared to animals injected with DREADD.

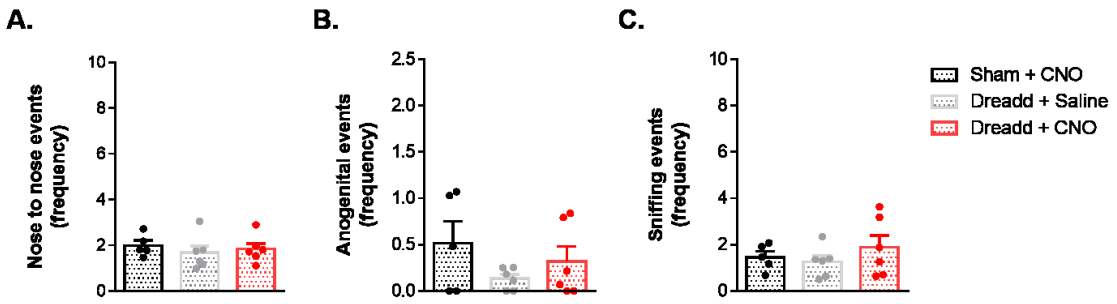

Figure 3.21. Frequency of socio-investigatory events in Consummatory phase II. A - Frequency in which the female exhibits nose to nose contact. **B** - Frequency in which the female investigates the anogenital area of the males. **C** - Frequency in which the female explores the male's body. Data are presented as mean ± sem. Kruskal-Wallis test. Mann-Whitney test. N=5 for Sham+CNO, N=6 for DREADD+Saline and N=6 for DREADD+CNO.

Similarly to what we did in the previous consummatory phase, we evaluated the frequency of mount attempts, run away events and time spent running away from males in consummatory phase II (Figure 3.22). These events are related since most "run away" events occur when the male is trying to mount the female. Therefore, the values for the frequency of both events (Figure 3.22.A-B) seems to be neatly correspondent. However, the DREADD+CNO females have a slight increase in the run away frequency opposing to a slightly diminished frequency of mount attempts compared to SHAM+CNO females. This observation suggests that in this phase, even for a smaller number of mount attempts, DREADD+CNO females tended to run away from males more often, which contrasts with the behaviour displayed by DREADD+Saline mice. Yet, differences between the different groups are not statistically significant. Moreover, the percentage of time spent running in the present phase (Figure 3.22.C) has significantly more variability in all groups, specifically in Sham+CNO animals. For instance, even if the values for most animals of this group are clustered between 4 and 8%, one of these mice spent 22% of their time running from the male. Hence, given this variability it is difficult to speculate whether or not escape behaviours are affected by the CNO-dependent inhibition of VMHvl PgR+ neurons.

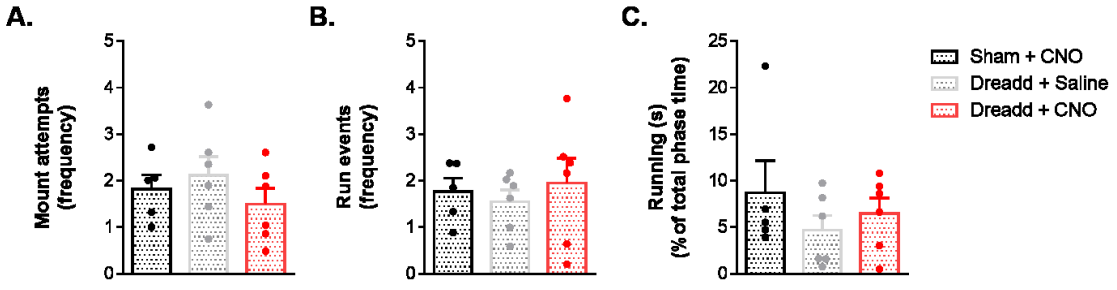

Figure 3.22. Mount attempts and escape events in Consummatory phase II in relation to the time each animal spent in this phase. A - Frequency with which the male attempted to mount the female. **B** - Frequency of events in which the female run away or escaped from the male. **C** – Percentage of time the female spent running away from the male. Data are presented as mean ± sem. Kruskal-Wallis test. Mann-Whitney test. N=5 for Sham+CNO, N=6 for DREADD+Saline and N=6 for DREADD+CNO.

Moving on to the sexual component of this phase, we assessed specific events such as mounts with intromission, female lordosis and the disruption of such.

Regarding the duration of mounts with intromission (Figure 3.23.A), the DREADD animals show a higher variability than the Sham+CNO mice, but no significant differences can be observed in the mean duration of this event between the three groups. Concerning the mean duration of lordosis (Figure 3.23.B), it seems to exist a tendency for an increasing lordosis duration from Sham+CNO to DREADD+CNO. Although, we observed that the highest values for the duration of lordosis per mount with intromission belongs to DREADD+Saline (Figure 3.23.E). Nevertheless, the animals presenting higher values do not necessarily represent the population, since the remaining animals are clustered at lower values, near zero even. In fact, when observing figure 3.23.E, with the exception of one animal, all DREADD+CNO females exhibited the least duration of lordosis per mount with intromission.

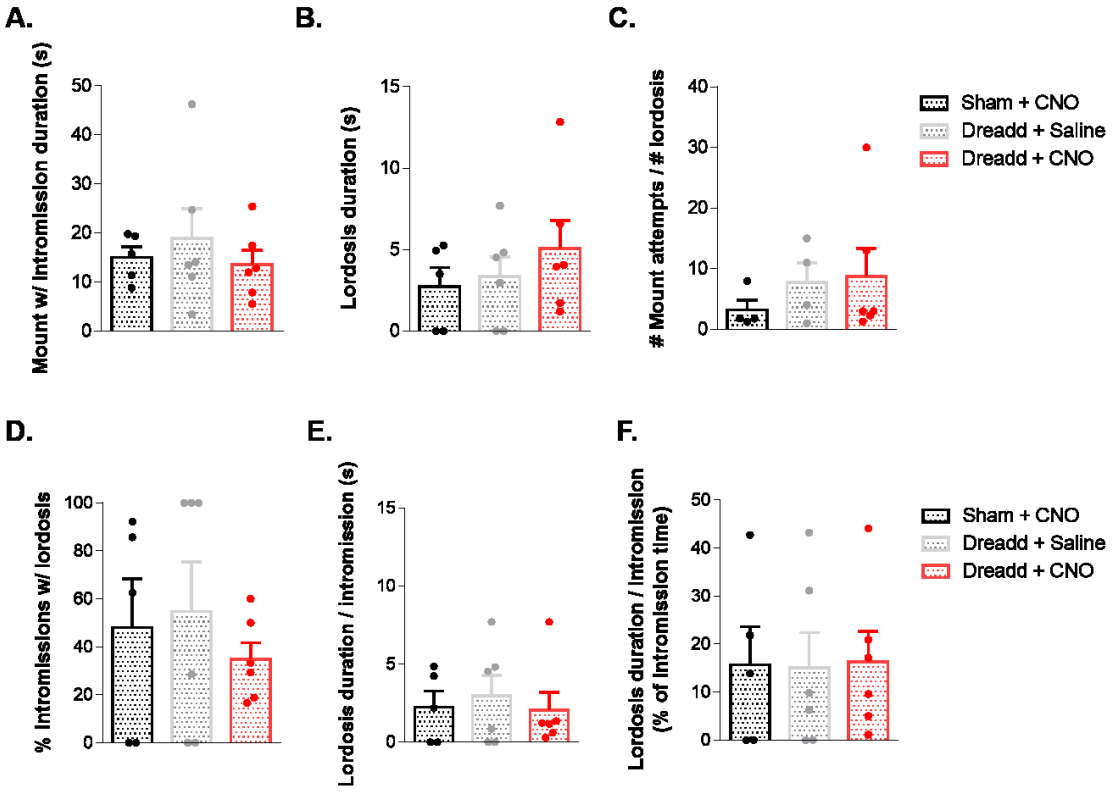

Figure 3.23. Quantification of sexual behavior and female receptivity events in Consummatory phase II. A – Mean duration of each mount with intromission. **B** – Mean for the time each female was in lordosis through all mounts with intromission. **C** – Relation between number of mount attempts and lordosis occurency. **D** – Percentage of intromissions where lordosis occurred. **E** – Mean time each female spent in lordosis position per each mount with intromission event. **F** – Percentage of time the female lordosed per total time of mounts with intromission. Data are presented as mean ± sem. Kruskal-Wallis test. Mann-Whitney test. N=5 for Sham+CNO, N=6 for DREADD+Saline and N=6 for DREADD+CNO.

When observing the lordosis quantification, two results are striking. First, the percentage of mounts with intromission that yielded lordosis (Figure 3.23.D) seems to be smaller in DREADD+CNO females, and, contrary to the other two groups where the behaviour presents itself mostly as present (100%) or absent (0%), in DREADD+CNO the percentage of lordosis per mounts with intromission seems distributed between 20% and 60%. Second, it seems to be a difference regarding the relation between the number of mount attempts and the number of lordosis occurrences, meaning the number of mount attempts that the male had to perform to

successful produce one lordosis event (Figure 3.23.C). The results revealed that for one lordosis event to occur in DREADD females, more mount attempts with no successful intromission are needed. Finally, we measured the proportion of total time the female spent in lordosis position within the total time of mounts with intromission (Figure 3.23.F) and the values appear to be equivalent across the different groups.

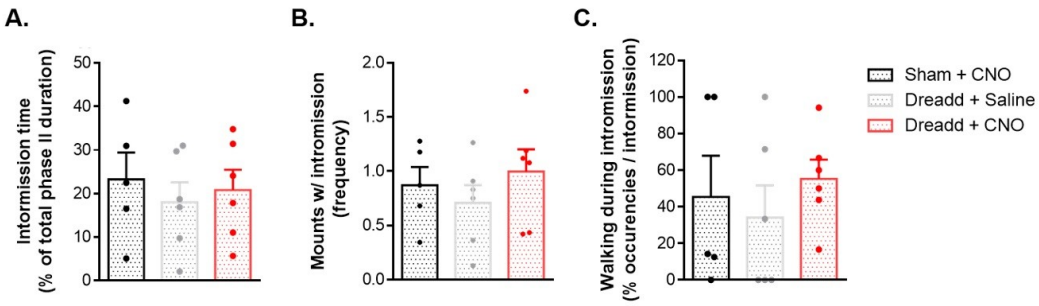

Figure 3.24. Sexual activity and female receptivity events in Consummatory phase II. A – Percentage of time the male spent mounting the female in relation to the total phase time. B – Frequency of mounts with intromission. C – Percentage of mounts with intromission events where the females walked. Data are presented as mean ± sem. Kruskal-Wallis test. Mann-Whitney test. N=5 for Sham+CNO, N=6 for DREADD+Saline and N=6 for DREADD+CNO.

With a careful analysis, considering the results related to the intromissions, it is possible to see that males spent a smaller percentage of time mounting the DREADD+Saline females and with fewer frequency (Figure 3.24.A-B). However, remarkably, it is observable a more distributed percentage of walking during intromission in DREADD+CNO, whereas the other two groups present more absolute values of 0% or 100% (Figure 3.24.C).

Finally, we analysed the mount with intromission and ejaculation latency values, reflecting the time it took the males to perform the first mount and to ejaculate (Figure 3.25.A-B).

First, it is important to mention that for the 8 DREADD+CNO animal's sessions, 2 males were not able to mount the female with intromissions and only 2 males reached ejaculation with these females within the trial time. Similarly, males paired with 1 of the SHAM+CNO and 3 of the DREADD+Saline femles also failed to reach ejaculation.

The comparison of the time that males took to initiate mounts with intromission between groups does not have statistical significance but it appears that males paired with Sham+CNO females took slightly less time. When it comes to the time it took for males to ejaculate, with the exception of one animal, Sham+CNO and DREADD+Saline have similar times, both of slightly lower value than DREADD+CNO.

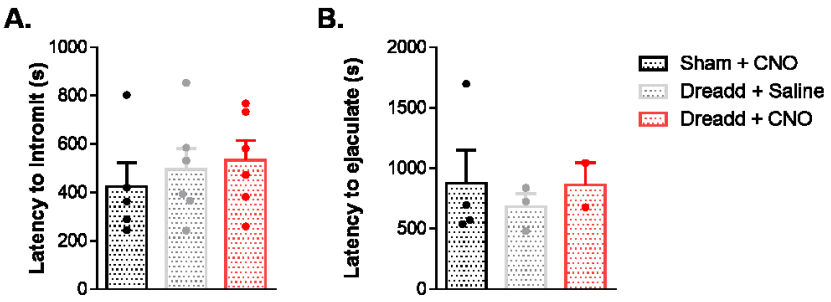

Figure 3.25. Quantification of male related parameters. **A** – Mount intromission latency or the time from the begging of the trial up to the first mount with intromission. N=5 for Sham+CNO, N=6 for DREADD+Saline and N=6 for DREADD+CNO. **B** – Ejaculation latency or the time from the male's entrance in the arena until it ejaculates. N=4 for Sham+CNO, N=3 for DREADD+Saline and N=2 for DREADD+CNO. Data are presented as mean ± sem. Kruskal-Wallis test. Mann-Whitney test.

In this final section of the second part of the consummatory phase, we demonstrate the females performance regarding defensive behaviours (Figure 3.26). Only one Sham+CNO female produced defensive behaviours, whereas the majority of DREADD+Saline and 50% of DREADD+CNO females participated in these (Figure 3.26.A). The number of shoving and kicking events (Figure 3.26.B-C), which reflect the defensive behaviour by the animals and the number of animals who engaged in this behaviour, shows that the Sham+CNO female presents more kicks towards the male than shoving. In contrast, both groups of DREADD females engage in the opposite tendency, with more shoving and less kicking behaviours.

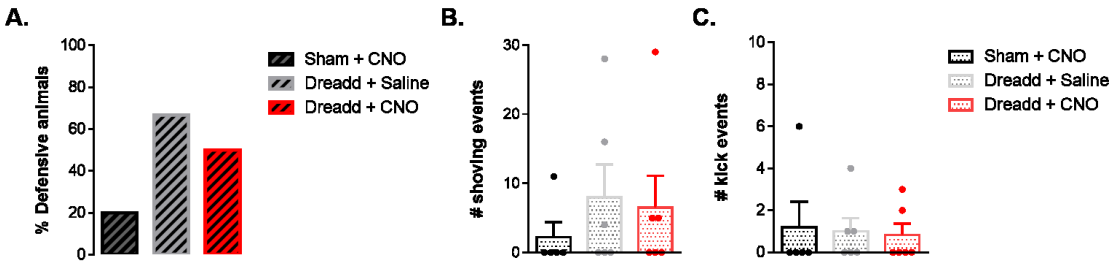

Figure 3.26. Illustration of the defensive behavior exhibited by females during the consummatory phase II of behavior. **A** - Percentage of animals of each group that executed defensive behaviours: shoving and kicking. **B** - Absolute values for the number of shoving events executed by each animal. **C** - Absolute values for the number of kicking events executed by each animal. Data are presented as mean ± sem. Kruskal-Wallis test. Mann-Whitney test. Data are presented as mean ± sem. Kruskal-Wallis test. N=5 for Sham+CNO, N=6 for DREADD+Saline and N=6 for DREADD+CNO.

To have a broader understanding of the relations between behaviours for each animal, the following table 3.2 was assembled.

Here, is possible to see some consequential relation between behaviours, even if the cells infected don't seem to correspond at first sight.

Observing the table is possible to see that all males which had to attempt to mount the female more than 30 times, were not able to reach ejaculation within the trial's time limit and females who presented defensive behaviours in both phases, managed to prevent the male from reaching ejaculation.

Besides, it also appears as if DREADD+CNO females who had the most PgR+ cells silenced were the ones who managed to prevent ejaculation in males, meaning they exhibited the most non-receptive behaviour. In contrast, the DREADD+Saline females which prevented ejaculation in the male were the ones with the lowest level of infection of PgR+ cells.

Table 3. 2. Overall view of defensive and sexual behaviours of each animal. The type of surgery and administered drug followed by the percentage of infected PgR+ cells in the VMHvl and several annotated absolute values for the number of events of several behaviours: Mount Attempts, Defensive Phase I and II, Mounts with Intromission and Ejaculation. Antagonistic behaviours are represented in red and agonistic behaviours in green.

ANIMAL	SURGERY	DRUG	Average number of infected cells (%)	Mount Attempts	Defensive - Phase I	Mount Intromission	Defensive Phase II	Ejaculation
436	SHAM	CNO	0	18	0	8	0	1
452	SHAM	CNO	0	34	0	5	17	0
453	SHAM	CNO	0	19	0	7	0	1
455	SHAM	CNO	0	18	0	13	0	1
457	SHAM	CNO	0	9	0	2	0	1
629	DREADD	Saline	11,861	10	0	7	0	1
602	DREADD	Saline	13,180	34	0	7	1	0
463	DREADD	Saline	18,166	39	13	4	29	0
630	DREADD	Saline	26,498	75	0	12	4	0
596	DREADD	Saline	27,564	8	0	3	0	1
587	DREADD	Saline	86,367	38	0	2	20	1
648	DREADD	CNO	5,501	16	0	6	0	1
428	DREADD	CNO	9,361	34	0	6	32	0
592	DREADD	CNO	16,856	47	36	0	0	0
662	DREADD	CNO	20,612	7	0	5	0	1
431	DREADD	CNO	38,593	57	15	0	0	0
658	DREADD	CNO	43,861	17	0	17	0	0
588	DREADD	CNO	54,152	34	0	6	7	0
655	DREADD	CNO	62,304	8	0	16	5	0

3.6 RELATION BETWEEN THE % OF VMHVL INFECTION TO BEHAVIOUR

Here we analyse how and if the percentage of infected PR+ cells in the VMHvl population affects several parameters of the sexual and defensive behaviour displayed by the females and their interaction with the males.

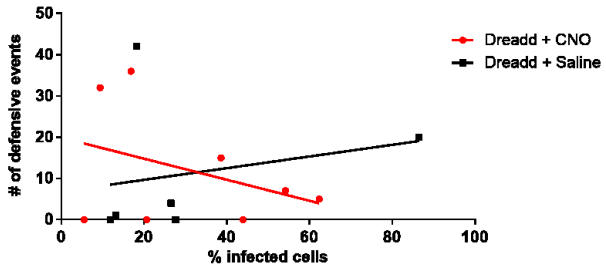

Figure 3.27. Relation between the percentage of infected PgR+ cells in VMHvl with the absolute number of defensive behavior events during the total trial time. Linear regression of the number of kicking and shoving events with the percentage of infected PgR+ cells, showing a positive correlation between both factors in DREAAD+Saline males, in contrast to a negative correlation in DREADD+CNO. N=5 for Sham+CNO, N=6 for DREADD+Saline and N=8 for DREADD+CNO.

When considering the relation between the cell infection percentage and the number of defensive behaviours (Figure 3.27) displayed by the animals injected with CNO versus Saline, it is possible to observe tendency lines with opposite slopes, even if the relation does not present itself as strong since R^2=0,1383 for CNO and R^2=0,05525 for Saline.

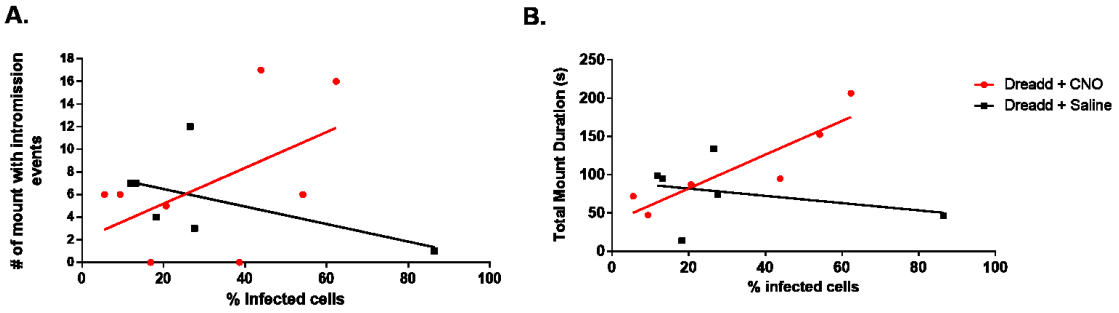

Figure 3.28. Relation between the percentage of infected PgR+ cells in VMHvl and mount with intromission events. A – Linear regression of the number of mounts with intromission with the percentage of infected PgR+ cells. N=5 for Sham+CNO, N=6 for DREADD+Saline and N=8 for DREADD+CNO. **B –** Linear regression of the total duration of mounts with intromission with the percentage of infected PgR+ cells. In both cases, there is a positive correlation between both factors in DREAAD+Saline males, in contrast to a negative correlation in DREADD+CNO. N=5 for Sham+CNO, N=6 for DREADD+Saline and N=6 for DREADD+CNO.

The same observation can be made when the same comparison is applied to the mounts with intromission events (Figure 3.28). Here, the relation between the duration of mounts with intromission and the number of infected PgR+ cells in the VMHvl in DREADD+CNO animals is the strongest of them all, with R^2=0,8139 for CNO and R^2= 0,09915 for Saline, thus, reflecting a more homogeneous behavioural output (Figure 3.28.B). When considering the total number of mounts with intromissions (Figure 3.28..A), even if the slopes are opposite and the same tendency is observed, the relation is not strong for any group (R^2 = 0,2773 for CNO and R^2= 0,3143 for Saline).

In contrast to the previously observed tendencies, the analyses of the relation between percentage of cell infection with the number of times the females performed lordosis during the trials (R^2= 0,0002830 for CNO and R^2= 0,003429 for Saline) and the duration of lordosis (R^2=0,003416 for CNO and R^2=0,001061 for Saline) reveals that there is no slope difference in

the linear regression for these factors, meaning that there is no existing relation between lordosis posture and percentage of infected VMHvl PgR+ cells (Figure 3.29).

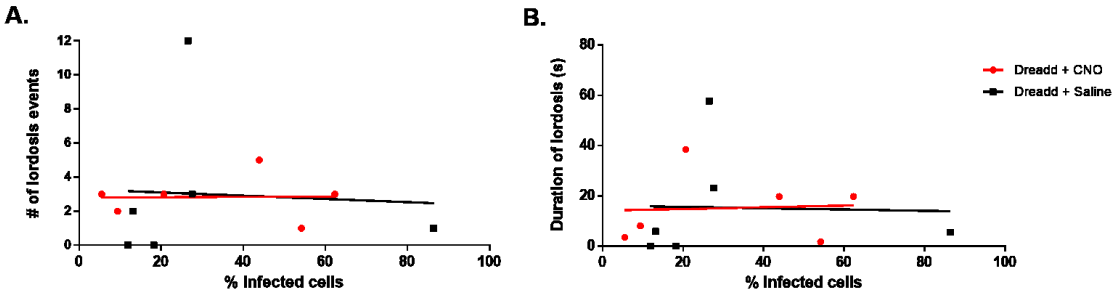

Figure 3. 29. Relation between the percentage of infected PgR+ cells in VMHvl and lordosis occurrences. A. – Linear regression of the percentage of infected PgR+ cells with the absolute values of lordosis events. **B.** – Linear regression of the percentage of infected PgR+ cells with the total duration of lordosis. No correlation is found in both cases. N=5 for Sham+CNO, N=6 for DREADD+Saline and N=6 for DREADD+CNO.

Finally, we decided to evaluate the relation between the percentage of infected PgR+ cells in the VMHvl of females and the incidence of ejaculation in the males they were paired with. Here, we observed that the DREADD+Saline females that allowed males to reach ejaculation, exhibited a higher percentage of PgR+ cells infected in the VMHvl than DREADD+CNO females (Figure 3.30.A). Coherently, the absence of ejaculation occurs in an opposite manner (Figure 3.30.B), where DREADD+CNO females with an increased number of infected cells successfully prevented their paired males from reaching ejaculation, while among DREADD+Saline females, only the ones with a lower percentage of infected cells were successful in the same task.

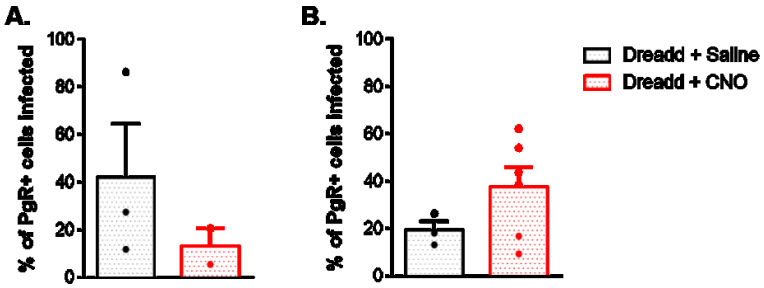

Figure 3. 30. Percentage of PgR+ infected cells in the VMHvl in females paired with males who ejaculated and animals paired with males who did not ejaculate. A - Percentage of infected PgR+ cells in the VMHvl in animals paired with males that reached ejaculation within the trial's time periods. N=3 for DREADD+Saline and N=2 for DREADD+CNO. **B** – Percentage of infected PgR + cells in the VMHvl in animals paired with males that could not reached ejaculation within the trial's time period. N=3 for DREADD+Saline and N=6 for DREADD+CNO.

Chapter 4 | DISCUSSION

Our goal was to investigate the influence of the progesterone receptor expressing (PgR+) neuron population of the VMHvl on the sexual receptivity of female mice. Previous studies have shown, through lesions, electrical stimulation and other perturbations that cause an abrupt interruption of the cellular processes and neuronal firing indiscriminately, that the VMHvl plays a key role in female sexual receptivity. Furthermore, two ovarian hormones that are important regulators of female sexual behaviour - estrogen and progesterone - have their receptors (PgR and ESR1, respectively) expressed in the VMHvl, suggesting the possibility that this region is responsive to circulating levels of these hormones. Interestingly, a recent study showed the ablation of the specific population of PgR+ and ESR1+ cells in the VMHvl disrupted the receptive behaviour displayed by OVX females[9], which, as it has been previously mentioned, is a different display of sexual behaviour, not consequent of the induced estrous cycle and no defensive behaviours are expressed towards males.

Given this, the main aim of the present thesis was to silence the PgR+ cell population of the VMHvl and observe the effects of this on female sexual behaviour of naturally cycling individuals, as this would give us further understanding into the role of this cell population. For this, we used the following tools: (i) To give us exclusive access to PgR+ cells we used PgR cre mice: these mice express cre recombinase in PgR+ cells, allowing the expression of cre-dependent viruses in this population only. (ii) To silence neurons, we employed a chemogenetic strategy: cre-dependent hM4Di DREADD viruses. (iii) To observe the activity of the above-mentioned neurons we used fiber photometry imaging and a cre-dependent GCaMP6s virus: a genetically encoded calcium indicator that fluoresces when bound to calcium ions; together with the inhibitory cre dependent hM4Di DREADD. Below is a discussion of the results we obtained using the above strategies.

4.1 FIBER PHOTOMETRY

The aim of this experiment was to record the activity of PgR+ neurons in the VMHvl using GCaMP6s, while simultaneously silencing the neurons using the hM4Di DREADD. We performed this experiment in 2 batches of animals, both presenting its own set of issues. The first batch of animals injected, due to their tendency to remove the fibers from each other's heads, had to be isolated. In this batch, the injection resulted in a good level of co-localization of both viruses (hM4Di and GCaMP6s), visualized using fluorescence microscopy through the expression of mCherry for DREADD and EGFP for GCaMP6s. However, both the injection site and fiber placement were in coordinates too posterior and lateral (AP: -1.45; ML: ±0.7; DV: -5.8) (Figure 3.3.C-F).

Considering these results, a second batch of animals was injected using corrected coordinates (AP: -1.4; ML: ±0.7; AP: -5.8). Although, in these animals we observed that while GCaMP6s virus infected PgR+ cells, the DREADD virus did not. Since it has already been observed that multiple AAV virus can infect the same cell, we do not know what happened here that prevented this from occurring in these animals. Nonetheless, the injection site was correct.

The fiber placement, however, was not perfect, since it was too dorsal. Consequentially, several layers of non-infected cells remained between the infected PgR+ cell population and the base of the fiber, where light is transmitted and collected, producing a barely discernible, low intensity, signal, rendering any fluorescence recordings impossible (Figure 3.4).

In order to optimize this experiment two features need to be improved. The first is the co-expression of GCaMP6s and DREADD. For this, a batch of animals should be injected with DREADDs from different suppliers simultaneously with GCaMP6s, to discern which one yields optimal co-infection and consequently good co-localization of both signals. The second is the placement of the fiber. To determine the best coordinates, a batch of animals, injected with Evans blue (staining the VMHvl), should be used to position the fiber in several coordinates. Then animals would be immediately perfused to observe and compare for the coordinates which will yield the best imaging recordings. Once all these parameters are optimized, imaging should be possible, yielding accurate recordings of the neuronal activity of the PgR+ population upon administration of CNO and in the presence versus absence of a male stimulus.

4.2 VIRAL INJECTION TO THE VMHVL

The viral infection of the VMHvl through viral injection stereotaxic surgery was observed in the first batch of animals, when the coordinates AP: -1.45; ML: ±0.7 and DV: -5.8 were used. However, it revealed to be too posterior, infecting only the posterior and final part of the VMHvl, if at all. Hence, in the next batch of animals the coordinates were corrected to AP: -1.4; ML: ±0.7 and AP: -5.8, where the injection proved to infect the VMHvl consistently.

When the injection proved successful in infecting the PgR+ cell population of this region, it also infected cells in the surrounding brain areas such as the dorsomedial VMH (VMHdm), the dorsal medial hypothalamic nucleus (DM), peduncular lateral part of the hypothalamus (PLH), the medial forebrain bundle (mfb) and the fornix (f). Regions responsible for behaviours ranging from feeding and drinking to integration of reward and recall memory.

This suggests that our observations may not result only from the inhibition of the PgR+ cells in the VMHvl but rather from the partial inhibition of all or some areas. Although the infection rate in the surrounding areas was smaller than in the VMHvl, we cannot disregard the influence that said areas may have on the behaviour. Perhaps, some of the variability observed in the results is a consequence of such a broad infection. To eliminate this variable, the amount of hM4Di DREADD injected should to be reduced, perhaps to 150 nL, instead of 300 nL, since the aimed area is rather small. Only after the VMHvl is guaranteed to be the sole infected area, can we assure the accuracy of the consequent exhibited behaviours upon the inhibition of the PgR+ neuronal population.

4.3 PGR+ CELLS INFECTED IN THE VMHVL

As discussed above, in some animals the infection area was greater than the VMHvl alone. Nonetheless, for quantification and behaviour analysis purposes we only considered the PgR+ cells within our region of interest (ROI), which encapsulated the VMHvl alone. The consequence of the widespread infection area was the extensive range of viral infection percentage across the VMHvl in all DREADD animals, from 5% up to 86%, which should translate into a wide range of behaviours.

4.4 DESCRIPTIONAL STATISTICS FOR BEHAVIOUR QUANTIFICATION

First, it is important to understand the distinct phases of the sexual behaviour and the reason each one was analysed individually. Sexual behaviour, like all social behaviours, is a complex set of actions and decisions heavily influenced by the arousal state in which the animal finds himself. This state of arousal, in sexual behaviour, is consequent of visual and olfactory cues to which the animal is exposed when in contact with a conspecific[7,18,51].

The division of sexual behaviour into three phases, the appetitive, consummatory phase I and consummatory phase II, is based on the variety of behaviours performed by the animals in each phase. For instance, the appetitive phase is composed only of socio-investigatory behaviours, where the animals explore each other's olfactory cues, which tends to increase their level of arousal. If the increase in arousal occurs, it leads to the next phase, the consummatory phase I, composed of socio-investigatory behaviours and mount attempts. This phase starts at the first mount attempt and lasts until the first mount with intromission. Fluctuations in the duration of this phase reflect the female's receptivity or arousal state, since for the male to be able to intromit she needs to be immobile and adopt a lordosis position to facilitate penile intromission[18]. Hence, when this time interval is longer, it indicates that the female's receptivity is decreased. The last phase of sexual behaviour, the consummatory phase II, is reached when the male mounts the female with intromissions and continues until the male ejaculates. The size of this phase time interval is also a reflection of the female's receptivity, since the time needed for the male to reach ejaculation indicates if the female is facilitating its intromissions. Thus, the more extended this phase seems to be, the lesser is her receptivity.

The goal of this study is to understand female receptivity, a behaviour primarily dependent upon their estrous cycle and the concentration of ovarian hormones in circulation (E and Pg), which vary across days in the cycle[12]. As shown in figure 1.2, it is only when the levels of Pg and E are elevated, in the proestrus phase, that females exhibit affiliative behaviours and receptivity to mate. During the remaining days, they exhibit defensive and even aggressive behaviours towards males. Several studies have shown that the VMHvl, a part of the brain responsible for lordosis, is rich in sex-steroid receptor expressing cells[9,52]. Furthermore, it has been shown that lordosis is mediated through projections from the VMHvl to the periaqueductal grey (PAG)[33]. Consistently, it has been hypothesized that the VMHvl neurons modulate, across the estrous cycle, the behaviour of a female when exposed to a male. In fact, data from our lab shows that male-evoked, but not female-evoked, VMHvl responses are enhanced during proestrus[40]. The

results raised several questions regarding the identity of the cells responsible for this response and whether or not it could be a precursor for consummatory behaviour. Considering this data and studies from ours and other labs, it was expected to observe an increase in the VMHvl PgR+ neurons activity throughout sexual behaviour[51]. Still, fiber photometry analysis in PgR+ neurons of the VMHvl showed that activity in these neurons persisted in socio-investigative behaviours across the hormonal cycle, and it was sustained both when the female lordosed and when she exhibited defensive behaviours. Thus, this data from our lab contradicts the way VMHvl function was perceived in females, leading to the final hypothesis in which, similar to what has it has been shown in the male VMHvl[45], the VMHvl in the female is composed of several sub-populations of neurons, each responsible for a different component of sexual behaviour. Since fiber photometry measures bulk fluorescence, not discriminating individual neurons, the modulation of opposite sub-populations would not be discernible. This hypothesis is further supported by the fact that VMHvl projects to regions of the PAG responsible for opposite behaviours[53], specifically, the ventrolateral region (vlPAG), which is important for lordosis[54,55], and the lateral and dorsomedial PAG (l/dmPAG), which is important for conspecific-triggered aggression and escape[56]. However, the identity of the cells projecting to the different regions is still unknown. Hence, the current hypothesis in our lab is that, since the estrous cycle modulates female sexual behaviour, the varying concentration of E and Pg during the cycle modulates the VMHvl neuron populations differentially as to ensure that the population responsible for mating is dominant during proestrus, while the defensive/rejecting population is dominant through the rest of the cycle.

Therefore, silencing the PgR+ neuron population in the VMHvl would not have a straightforward behaviour inhibition, but rather a disruption of some sexual behaviours and the triggering of a few defensive behaviours. The results obtained seem to reflect a slight imbalance in the neuronal output of the PgR+ cell population correlated with the percentage of infected cells of said population, where the dominant population, as induced through the estrous cycle may no longer have the strongest signal to determine the animal's behavioural output.

The lack of statistical significance observed across the experiment may be a consequence of several factors. First, when studying behaviour, it is necessary to comprehend the inherent variability across naturally behaving animals. Second, to have significant differences, the experiment requires, at least, n≥10 to exclude behavioural outliers from our pool of animals. As can be seen in figure 3.1, the transitions between the phases of the estrous cycle are maintained in all animals, however, the duration of each phase fluctuates from animal to animal and even within the animal during its lifetime. Moreover, working with viral constructs requires the awareness of the cellular death induced by the infection, even if the engineering of these virus eliminates their pathogenicity. Nonetheless, some cell death is bound to take place for as long as the animal is infected. Finally, female sexual behaviour is dependent on the male's performance and motivation. If a male is not as motivated as the remaining males, the outcome of the behaviour changes, increasing its variability. The opposite can also occur, when a male is motivated enough, due to his superior size, he can mount the female even if she is not receptive and eventually reach ejaculation. The use of sexually naïve females in this experiment renders our results difficult to

compare to parallel studies, since most females studied, besides being OVX, are sexually experienced. This leads to their interaction with the male being complemented with a learned factor, the expectation of the male's action. We speculate that one of the reasons our studied females did not present defensive behaviour in the appetitive phase, was due to the fact that they have no expectations regarding the male's behaviour in the arena.

Looking at the general results of the quantification of sexual behaviour, it occurs, in some cases, that the behaviour of the DREADD+Saline group is analogous the one exhibited by DREADD+CNO and in some cases to Sham+CNO. This suggests that DREADD+Saline animals may be suffering from an unbalance in the circuitry output, consequent of DREADD expression or perhaps cellular death, which originates a behavioural exhibition similar to silencing those neurons in DREADD+CNO females. Nonetheless, the opposite also occurs and DREADD+Saline values become comparable to Sham+CNO, which seems to indicate that the cells infected may not responsible for the measured behavioural output. All these hypotheses seem to fit the results, since for some phases of the behaviour and even specific components, DREADD+CNO seems to induce either a variability or a cluster in the values represented. Considering that DREADD+CNO animals would present the same behaviour as DREADD+Saline if the CNO is not administered, the balance in circuitry signal may be restored by the silencing of the neurons disrupting it, thus explaining the similarities between DREADD+CNO and Sham+CNO females. Unfortunately, this can only be verified if the same unbalanced circuit is restored upon silencing of infected PgR+ neurons. To confirm this hypothesis, we would perform behavioural experiments where the same DREADD female was administered saline and CNO in separate sessions, however, this could not be performed in naïve females.

All considered, the interpretation of the following results is complicated due to the primary fact that we do not know if the cells infected in the VMHvl are the ones responsible for sexual receptivity, defensive behaviour or a blend of both. Besides, the fact that the proportion of infected PgR+ cells responsible for the individual behaviours may differ between different animals. Thus, all the following analysis remains speculation until further examination determines the identity and function of these cells.

4.4.1 Global analysis

Here, we discuss the global values of sexual behaviour, the time of each animal's trial and phase of the behaviour. The analysis of the absolute values of behaviour is not a true representation of behaviour, since each female spends different amounts of time with the male in each phase. Thus, all analysis will be done considering the percentages of each phase, consequential of the total time of each trial.

One main result from these experiments, explicit in the global analysis of this behaviour quantification, is the analysis of the males' performance (Figure 3.13.A). When observing all groups of animals concerning the percentage of males which mounted the females with intromission is striking that 2 of the 8 males paired with DREADD+CNO females were not able to

mount them with intromissions, a relevant result considering that, in parallel groups (DREADD+Saline and Sham+CNO females), all males successfully mounted with intromissions. Then, the results concerning the percentage of males which reached ejaculation within the trial's time (Figure 3.13.B) show us that from the remaining 6 males paired with DREADD+CNO (excluding the ones where no intromission was performed), only 2 managed to reach ejaculation within the trial's time interval. Thus, if the males were not removed from the arena, the final stage of behaviour would probably prolong itself until the male reached ejaculation or gave up in consequence of the female's rejection manifestations resulting in an augmented time spent in consummatory phase II. Nonetheless, we must also consider that 1 of the 5 males paired with Sham+CNO and 3 of the 6 males paired with DREADD+Saline were not able to reach ejaculation either. Still, DREADD+CNO females exhibited more efficacy in preventing the progression and conclusion of the sexual behaviour, an indication of the presence of rejecting behaviours which range from: defensive, escape and the refusal to assume a facilitating position for intromissions.

An interesting fact of these results relays on the analysis of the proportion of time each group spends in each phase of sexual behaviour. Initially, the two DREADD+CNO females paired with males who could not reach the final stage of behaviour (consequent of the absence of mounts with intromission) were included in assessment of the proportion of time each animal spends per phase of behaviour. This decision was due to the established time limit for the performance of the behaviour, then, all behavioural output should be considered for analysis (Figure 3.12.B). The overall result reveal that DREADD females spent more time in consummatory phase I than Sham+CNO animals. This result seems to indicate that DREADD females are less receptive, since it takes the males a greater amount of time to intromit with success.

Nevertheless, it is also important, if not more correct, to analyse the proportions of time spent in each phase of sexual behaviour from the perspective of animals who completed the task or reached all the phases of the behaviour (Figure 3.12.A). Especially considering that the comparison is to groups where all the animals completed the passage to all the stages composing sexual behaviour. Interestingly, the proportion of time each group spent in each phase changes, rendering the behavioural output more similar between DREADD+CNO and Sham+CNO. In this context, only DREADD+Saline females seem to difficult the male transition from mount attempts to mounts with intromissions, which alters the interpretation of events. These results seem to support the idea that not only the cells responsible for the female's receptivity positioning are ones being silenced by DREADD. In fact, it suggests that in DREADD+Saline, the unbalance allowed for the cells responsible for defensive behaviours to interfere with the signal from the dominant population (cells responsible for receptivity). Such interference seems to decrease when CNO was administered. Nonetheless, it is important to emphasize that DREADD+CNO females spent an equivalent time in the second part of the consummatory phase consequent of the time limit imposed to the animals. Meaning, if the animals were allowed to stay indefinitely in the arena, the proportion of time relative to final stage of behaviour would have been greater for these females. Thus, these results are not necessarily a true representation of the DREADD+CNO sexual behaviour or of the inherent function of the silenced cells.

4.4.2 Appetitive phase

The following discussion of the appetitive phase, the first stage of sexual behaviour, comprises mostly the analysis of the frequency of socio-investigatory events for each group, since the analysis of the absolute values of behaviour is not a true representation of behaviour.

The most striking result is the slight increase of frequency in both anogenital sniffing and general sniffing of the male's body events in DREADD females (Figure 3.15). DREADD females, seemed to present a stronger urge to investigate the male, while Sham+CNO females presented a more consistent behavioural output. Both ERS1+ and PgR+ neurons in the VMHvl are related to socio-investigation, where it was even demonstrated that photostimulation of ESR1+ neurons in the female VMHvl induced an increased investigatory behaviour[45]. Thus, this result seems to indicate that the distribution of behaviours in DREADD+CNO should correlate negatively with percentage of infected PgR+ cells of the VMHvl, meaning a higher the percentage of silenced infected cells will yield a small frequency of events.

4.4.3 Consummatory phase I

The discussion of the first part of the consummatory phase, the second stage of sexual behaviour, comprises the analysis of the frequency of socio-investigatory, mount attempt and run away events for each group, since, again, the analysis of the absolute values of behaviour is not a true representation.

At this point, we observed certain noteworthy results. First, the same variability in DREADD females is present only in one socio-investigatory parameter: general sniffing of the male. This might indicate that the dysregulation of this type of behaviour decreases as the sexual behaviour advances, meaning the neuronal activity within the PgR+ population shifts to the appropriate cells responsible for the following component of the behaviour.

Next, the fact that DREADD+CNO females appear to run away from the male with less frequency but for longer periods of time (Figure 3.18.B-C), may be related with the exhibition of defensive behaviours as discussed bellow.

Defensive behaviour (Figure 3.19) first appeared in this phase and solely in DREADD females. This is related to the previous result in the way that: DREADD+CNO females may run away less due to fact of using defensive behaviours as a way of intimidating the male and discourage further attempts to mount them. However, a motivated male is not easily discouraged, which leads to the fact that females continues to run away to distance themselves from the male, even if less frequently, but for longer intervals and consequently longer distances. Still, it is not the single observation to be made in consideration of this behaviour. Since it has been established in our lab that wild type naturally cycling females exhibit more defensive behaviours than OVX females, it would have been expected to observe these characteristic defensive behaviours in

Sham+CNO females. Nonetheless, contrary to previous observations, this group demonstrated very little to none antagonistic behaviours towards the males.

Since it was administered CNO to these females, it is important to rule out its influence in this behavioural output. Then, it would be important to study the sexual behaviour on a group of PR-IRES-cre females subjected to Sham surgery to which was administered saline. Besides, behavioural analysis of a group of naturally cycling females with no Sham nor viral injection surgery performed, would help to narrow the variables that may be influencing behaviour besides DREADD inhibition.

4.4.4 Consummatory phase II

The discussion of the second part of the consummatory phase, the last stage of sexual behaviour, includes the analysis of frequency of socio-investigatory, mount attempts, mount with intromission, run away and lordosis events for each group, since the analysis of the absolute values of behaviour is not a true representation of behaviour. Plus, the assessment of the duration of events as lordosis, mounts with intromission and run away.

Finally, we reach the last phase of sexual behaviour, the consummatory phase II. Regarding socio-investigative behaviours, the trends from the previous phases are barely discernible, since females from all groups present an equivalent frequency of interaction with the male (Figure 3.21). The previous observation that DREADD+CNO females run less and for longer periods of time is no longer applicable. However, males in this phase seem to attempt to mount these females less than in the previous phase, which may be a consequence of their display of defensive behaviour as discussed previously and the reason why they no longer run for such long intervals in this phase. These females do seem to run away with the same frequency, perhaps an indication that the behaviour became more preventive than consequential.

In DREADD+Saline females is now observable that their frequency of running events is maintained while the time they spend running decreases (Figure 3.22), which may indicate that they no longer need to distance themselves so far from the male to discourage the intensified mount attempts, especially considering the increase in defensive behaviours which is discussed further along. Still, this does not mean that the males have not been successful in performing mounts with intromissions, simply that DREADD+Saline females do not facilitate the behaviour.

Meanwhile, in Sham+CNO females, the frequency of running away continues to be proportional to the male mount attempts', which indicates a consistency in behaviour not observe in the previous groups.

When observing the parameters of sexual behaviour and receptivity such as mean mount duration, number of mounts, among others we observe no significant differences between groups. However, the small deviations may be enough to indicate what deficits may be occurring in the PgR+ circuitry controlling the behaviour.

Regarding both lordosis and mount with intromissions mean duration (total time interval divided by the event) per trial, we observe opposite consequences. In the mean duration of

mounts with intromissions (Figure 3.23.A), DREADD+Saline female seem to be responsible for inducing the males into performing longer mounts, which is an indication that the female is not exhibiting a correct lordosis position, thus the male needs more time to reach ejaculation. However, the superior average is consequent of one animal, while the other values remain similar the other groups. Still in this graph, DREADD+CNO females present time interval values scattered in a manner that could indicate a relation to the percentage of infected and silenced PgR+ cells in the VMHvl. Regarding lordosis mean duration (Figure 3.23.B), it seems remarkable that both Sham+CNO and DREADD+Saline groups had females which did not exhibited lordosis while DREADD+CNO females exhibited a wide variety of values, which may be consequent of the wide range in the percentage of infected and silenced PgR+ cells in the VMHvl in this group.

Interestingly, this result starts to show how this behaviour display seems to be modulated to either occur in all mounts following the first display of the position or it does not occur at all, a hypothesis which will be discussed further on. Consistently, since this group had the higher values for mount attempts, the results of the relation between the number of mount attempts with the number of consequent lordosis achieved when the male mounts with intromission (Figure 3.23.C) shows that for DREADD females, especially with DREADD+CNO, males require a higher number of mount attempts to induce lordosis, when they do mount, than in the remaining groups.

Even if the lordosis duration remains approximately the same in all groups (Figure 3.23.B) apart from one animal in DREADD+CNO, the percentage of lordosis occurring in mounts with intromission (figure 3.23.D) reveals that DREADD+CNO females exhibit the lowest percentage of lordosis per mount with intromission. More than that, when analysing the results from the other groups is again visible the suggestion that lordosis, as a behaviour display, it either occurs in a session or it does not, as the values in DREADD+Saline and Sham+CNO are clustered in or near 0% or 100%, meaning that every mount yielded one lordosis display or none. A similar observation can be made when observing the mean duration of lordosis per mount with intromission (Figure 3.23.E), where DREADD+CNO females present the lowest duration, a clear indication that their receptive state is not as intense as in the remaining groups. Thus, the silencing of the infected PgR+ cells seems to be decreasing the female's receptivity. However, when viewing the percentage of time females spent in lordosis position in relation to the time of mounts with intromission (Figure 3.23.F), the only difference between groups is that the percentage values in DREADD+CNO are distributed across a range of values, which may correlate negatively with the percentage of infected PgR+ cells in the VMHvl, meaning a higher percentage of infected cells will yield shorter lordosis per the duration of mounts with intromission.

Analysing now the differences in the groups related to mounts with intromission, the most noteworthy result arises in the frequency of mount with intromission (Figure 3.24.B), since the percentage of phase time the females were mounted is equivalent in all groups (Figure 3.24.A). Regarding the frequency of mounts with intromission there is clearly an increase in the frequency with which the males mount DREADD+CNO females when compared to the remaining groups. These results are a consequence of the female decreased receptivity as the male needs to mount

her more often to achieve the same arousal level, when compared with other females that required less mounts.

Considering the parameter of walking during intromission (Figure 3.24.C), the most prevalent observation we made is that in Sham+CNO and DREADD+Saline females this behaviour presents itself mostly as existing or absent, whereas in DREADD+CNO there seems to be a distribution of behaviour, as if the directive to walk was not as imperative as it was for the previous groups, which should probably correlate positively with the percentage of silenced infected PgR+ cells in the VMHvl. This would mean that the highest percentage of silenced infected PgR+ cells yields the most walking with intromission events. All taken together, this parameter seems to indicate that the cells controlling this particular behaviour are not the ones being silenced but the ones responsible for receptive behaviours.

Presently, it is also important to analyse the male related parameters consequent of the female's receptivity (Figure 3.25). Here, is possible to see a clear tendency of increasing latency to intromit the female from Sham+CNO to DREADD+Saline and DREADD+CNO. This suggests a decrease in receptivity as the latency period increases, probably correlated positively with the infection percentage of the PgR+ cells in the VMHvl. However, when analysing the ejaculation latency what needs to be examined is not only what is present in the graph but also what is not, meaning the number of males which were prevented by females from reaching ejaculation. Furthermore, it is important to, once again, consider that the animals did not ejaculated within the time limit, otherwise, the ejaculation latency values would be higher for DREADD+CNO. Thus, these results are not an accurate representation of the female's state, since at first look it appears as if DREADD+CNO has the lowest values, which only means that those were the only males which reached ejaculation before being removed from the arena if the female was in a defensive state rejecting the male's advances.

Interestingly, differences arose in the defensive and rejection behaviours. The percentage of females exhibiting defensive behaviours in this phase is an interesting result. We observed that DREADD+Saline females, which presented less intense defensive behaviours in the previous phase (overall), now exhibit an intense defensive behaviour, mainly characterized by shoving the male. Comparing with DREADD+CNO females, which exhibit a more prevalent defensive state in the consummatory phase I but did not present such an intense behaviour escalation in phase II, leads us to think that perhaps the cells being silenced by CNO are also responsible for defensive behaviours, since the behaviour is initiated but it does not increase as much as in DREADD+Saline females. Nonetheless, it has to be mentioned that, even though only one Sham+CNO female engaged in defensive behaviours, these were more aggressive (more kicks) than the ones demonstrated by several DREADD females (more shoving). Meaning that the defensive behaviour in these females was more pronounced. Thus, the DREADD injection might direct their behaviour towards defensive shoving, but when we silence these cells we seem to decrease the activity of the PgR+ cells responsible for all defensive behaviours, shoving and kicking. Perhaps, the cellular death consequent of DREADD infection diminished the

aggressiveness of defensive behaviours, thus resulting in more shoving than kicks towards the male.

4.5 VMHVL PGR+ CELLS INFECTION AND THE BEHAVIOURAL OUTPUT

In this final part of the discussion we analyse some non-statistical results which originated a table responsible for the following comparison of the percentage infected PgR+ cells in the VMHvl with several behaviours.

Here, we discuss some of the overall aspects of sexual behaviour, in a non-statistical manner, to search relations between the different behaviours. The importance of table 3.1, relays on the fact that, sometimes, when studying variations of specific elements of behaviour, it is difficult to see the whole behavioural dynamics. In an effort to try and find some relations or simultaneity between different behaviours, we compiled the percentage of infected PgR+ cells with the absolute values of behaviours such as mount attempts, defensive behaviours for each phase, mounts with intromission and ejaculation. The definition of agonistic behaviours with the colour red allowed us to observe that when males had to attempt to mount the female for more than 30 times, ejaculation would not occur within the trial's time. Thus, we established a threshold for the values of mount attempt events, which defined that when the number of mount attempt events, which are characteristic and crucial for successful sexual behaviour, were over 30, the behaviour would become agonistic. We also observed that most of the females who had an increased number of mount attempts associated, presented defensive behaviour and successfully prevented the male from reaching ejaculation within the trial's time limit. Finally, the observation that DREADD+CNO female with the highest percentage of silenced PgR+ cells in the VMHvl seemed more successful in preventing males from reaching ejaculation, while DREADD+Saline females with the less percentage yielded the same result, lead to the conclusion that the values of the behaviour events could correlate with the percentage of infected PgR+ cells leading to the following analysis.

In this section, we analyse and discuss the results yield from the effort to find a relation between specific sexual and defensive behaviours and the percentage of PgR+ cells infected in the VMHvl. As mentioned above, we hypothesize that the VMHvl is composed of a heterogeneous PgR+ neuron population modulating opposite behaviours: receptive and non-receptive/rejecting. To determine the influence of the rate of infection on behaviour, different infection percentages were matched with each animal behaviour.

The results yielded were interesting and opened room for more questions. Starting with the defensive behaviours (Figure 3.27), no relation is observed through linear regression analysis. However, observation of the created graph showed two opposite slopes between DREADD+Saline and DREADD+CNO, where the increase in percentage of infected PgR+ cells in the VMHvl resulted in a decreasing number of defensive behaviours for DREADD+CNO females and the opposite for DREADD+Saline females. This suggest that these behaviours are

diminished as the number of silenced PgR+ cells increase, thus, these cells seem to be responsible for this particular behaviour.

Observing the number of mounts with intromission (Figure 3.28.A) we detect that a greater number of PgR+ cells infected results in an increasing number of mounts with intromission in DREADD+CNO animals and a decreasing number in DREADD+Saline animals. Mount duration (Figure 3.28.B) analysis demonstrates an identical tendency, rendering both outcomes congruent with previous results from the statistical analysis of these behaviours, where males mounts with intromission more often and for longer periods of time DREADD+CNO females. Here, these results are complemented by showing that DREADD+CNO females with a high percentage of PgR+ cells infected were mounted more often and for longer periods of time for the male to reach ejaculation. Hinting that DREADD+CNO females with more PgR+ cells silenced were not facilitating intromissions, thus, the silenced neuronal population seems to be responsible for the female's receptive response.

The decrease in both defensive and receptive (facilitating intromission) behaviours with the increase in the percentage of PgR+ cells infected within the PgR+ neuronal population seems to validate our hypothesis of the presence of sub-populations in the PgR+ neurons in the VMHvl which may control opposite behaviours. Since the silencing of the general population yield a decrease in both behaviours, even if not statistically significant, it is reason to continue to the search for the identity of these neuronal populations.

The linear regression of the relation between the percentage of PgR+ cells infected and the number of lordosis events and its duration (Figure 3.29) yielded no relation, statistical or otherwise. Nonetheless, it is important to emphasize that the measure of lordosis, given previous results from the statistical analysis of behaviour, does not seem to be the most accurate, since the disruption of the exhibition of lordosis does not appear to be related to the number of time or duration in which occurs, but the order in which it takes place. Meaning, that during sexual behaviour, when females start to exhibit lordosis during a mount with intromission, if they are receptive, the exhibition will repeat itself in all/almost all following mounts with intromission. Hence, the disruption of lordosis relays in its exhibition in non-consecutive mounts, which was not explored in this data set. So, no conclusions can be withdrawn from these results. However, it may be important to mention that during video annotation we did observe DREADD+CNO females exhibiting lordosis in one mount and then, only repeating it after several mounts with intromission. Thus, this is a result that needs to be properly analysed and quantified to completely understand what is happening and its relation to previous results.

Finally, we compared the percentage of PgR+ cells infected in the VMHvl in the females paired with males that reached ejaculation versus males which did not ejaculate within the trial's time interval (Figure 3.30). The males that reached ejaculation were paired with DREADD+Saline females with a high percentage of PgR+ infected cells, and DREADD+CNO with a low percentage of PgR+ infected cells in the VMHvl. Then, the males which did not ejaculate were paired with DREADD+Saline females with a low percentage of PgR+ infected cells and DREADD+CNO with a wide range of infection percentages, where the majority were increased and higher than the

ones displayed by DREADD+Saline. Once again, we observe opposite behaviours in DREADD+Saline and DREADD+CNO in accordance with the percentage of the PgR+ population which was infected and consequently, silenced upon CNO administration. DREADD+CNO animals with a great percentage of their neurons silenced were successful in preventing the males from reaching ejaculation and DREADD+Saline females with the greater infection were not. However, DREADD+Saline with a lower infection rate were able to do so.

One can only speculate as to why the females DREADD+Saline with a low percentage of infected PgR+ cells demonstrated more non-receptive behaviours than females with a high percentage of infection. Usually, a higher percentage of infection would induce us to think the opposite. Nonetheless, the proposed hypothesis is that lower percentages of infection might be associated with more cellular death, leading to an unbalance in the dominant signal of PgR+ neuronal population. Perhaps, the cells responsible for receptive behaviours are more sensitive to the viral infection, which leads to their cellular death. Nonetheless, none of these hypotheses then explains why the administration of CNO seems to mitigate this effect, only observable in DREADD+Saline.

Then, the best way to tackle this problem would be to perform stereotaxic surgery to inject a simple fluorescent virus (p.e. eGFP) to the VMHvl of a PR-IRES-cre female and perform the same behavioural analysis to determine if the problem is derived from the viral infection of the VMHvl cells and consequent cellular death or from probable self-activation of the expressed DREADDs. Followed by histological analysis of the percentage of infection and the percentage of cellular death on site.

Chapter 5 | CLOSING REMARKS AND FUTURE PERSPECTIVES

The goal of this thesis was to determine the role of PgR+ neurons in the VMHvl in female sexual receptivity, which varies across days consequent of the estrous cycle. Looking back, it is clear that the experimental design was not perfect, its execution presented several challenges along the way and to yield concrete conclusions several improvements are required.

Nonetheless, the yielded preliminary results from this thesis, analysed based on the unproven hypothesis that PgR+ neurons in the VMHvl are a heterogenous population responsible for modulating opposite behaviours across the estrous cycle, seem to corroborate the hypothesis that this population is responsible for both defensive and receptive behaviours. Strikingly, the statistical quantification analysis between values across phases appear to suggest that, among the PgR+ neuronal population, the activation of cells is modulated as the sexual behaviour progresses, so the cells required for behaviours required in the following phase become active while the cells responsible for previous behaviours become less active. This seems to indicate that the dominant neuronal population shifts during the distinct phases of sexual behaviour in a sequential manner. Finally, our effort to establish a relation between the percentage of infected PgR+ neuronal population suggests that PgR+ neurons are indeed a heterogeneous population with opposite functions, since both defensive and receptive behaviours decreased when the silenced percentage of these neurons increased.

However, there is still a lot of work to be done, before reaching any conclusions concerning this subject. Specifically, we need to (i) define the identity of each population; (ii) record their activity during sexual behaviour across the estrous cycle, (iii) define accurate measurements for female sexual behaviour and, eventually, (iv) manipulate the activation/inhibition of the dominant population across the distinct phases of sexual behaviour and the estrous cycle.

We can start by concluding the analysis of the behaviour yielded from this experiment, since lordosis needs to be properly quantified. We believe that the most efficient way to do it, and, simultaneously evaluate the component of sequentially of sexual behaviour, is through the construction of Transitions Maps. In this approach, each behaviour is represented in proportion to its frequency and the probability of transition between the different behaviours is determined and presented in a manner where the arrow that links the different components is, in size, proportional to its probability value. Thus, transition maps present the sequential component of a complex behaviour such as sexual behaviour in an accurate and visually perceptive manner. The use of this method for behaviours occurring within each group will demonstrate in which behaviour transitions the PgR+ neurons are employing their influence.

Then, there are several aspects of our experiment that require a different approach, including the need to minimize the variability inherent to the different infection rate within each animal. To do so, we would have to use sexually experienced females and perform two sessions, one where CNO is administered and another with saline. This is mandatory to observe the influence of DREADD infection versus hyperpolarization – silencing - of the same neurons within the same animal.

Nevertheless, the most important and challenging task is to determine the identity of the neurons composing the heterogenous PgR+ population. Hopefully, a new tactic was developed,

CANE - capturing activated neuronal ensembles – which allows to label, manipulate and trace transsynaptically neuronal circuits with temporal accuracy through the combination of knockin mice and pseudoviruses. This technique would allow us to label and consequently identify neurons activated in each phase of sexual behaviour.

Finally, it would also be interesting to evaluate the learning component and the evolution of sexual behaviour as females become sexually experienced. This could be done using an experimental design similar to the one presented in this project. However, instead of using the animals during one session, it would include multiple sessions per female, where she would start as sexually naïve.

There are almost infinite ways to approach and study sexual behaviour, since most of it is still undetermined, which is a consequence of the complexity and interconnectivity of the regions involved in its modulation. Besides, the regulation of the sexual behaviour by the estrous cycle in females occurs for most of their lives in an everchanging capacity, influencing their decisions and physiology. Hence, understanding how these events occur is essential to comprehend a behaviour essential for the continuity of life.

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APPENDIX

ive phase

ANIMAL	SURGERY	DRUG	Average # of cells infected (%)	Nose-to-nose	Anogenital	Sniffing male	Duration of appetitive phase (s)	Total trial duration (s)	Appetitive phase duration / total trial duration (%)	Frequency of nose to nose (event/min)	Frequency of anogenital (event/min)	Frequency of sniffing male (event/min)
436	SHAM	CNO	0	13	35	22	214,933	696,733	30,84868953	3,629037886	9,770486617	6,141448731
452	SHAM	CNO	0	39	29	19	391,467	1292,934	30,27741555	5,977515346	4,444819104	2,912122861
453	SHAM	CNO	0	13	12	21	174,266	573,466	30,38820087	4,475916128	4,131614888	7,230326053
455	SHAM	CNO	0	46	65	64	752,733	1698,8	44,30968919	3,666638768	5,181119999	5,10141046
457	SHAM	CNO	0	26	25	39	362,866	539,933	67,2057459	4,29910766	4,133757365	6,448661489
629	DREADD	Saline	11,861	24	21	65	337,133	726,333	46,41576247	4,271311322	3,737397407	11,56813483
602	DREADD	Saline	13,180	11	24	19	141,533	1038,133	13,63341691	4,663223418	10,17430564	8,054658631
463	DREADD	Saline	18,166	38	7	29	591,8	1516,133	39,03351487	3,852652923	0,709699223	2,940182494
630	DREADD	Saline	26,498	15	13	25	145,467	1377,867	10,55740503	6,186970241	5,362040875	10,31161707
596	DREADD	Saline	27,564	9	47	22	164,667	482,067	34,15852983	3,279345588	17,12547141	8,016178105
587	DREADD	Saline	86,367	19	26	17	133,8	839,934	15,92982306	8,520179372	11,65919283	7,623318386
648	DREADD	CNO	5,501	34	34	76	255,6	679,533	37,61406731	7,981220657	7,981220657	17,84037559
428	DREADD	CNO	9,361	13	27	16	149,866	1090,2	13,74665199	5,204649487	10,80965663	6,405722445
592	DREADD	CNO	16,856	33	69	72	462,467	1381,467	33,47651446	4,281386564	8,951990088	9,341207048
662	DREADD	CNO	20,612	51	58	88	646,2	1046,533	61,74673899	4,735376045	5,385329619	8,17084494
431	DREADD	CNO	38,593	12	23	15	166,533	1201,267	13,86311286	4,323467421	8,28664589	5,404334276
658	DREADD	CNO	43,861	25	22	76	361,6	1239,8	29,16599452	4,148230088	3,650442478	12,61061947
588	DREADD	CNO	54,152	40	50	68	709,6	1587,6	44,69639708	3,382187148	4,227733935	5,749718151
655	DREADD	CNO	62,304	45	8	66	578,8	1439,133	40,21865943	4,664823773	0,829302004	6,841741534

Table 2.2. Compilation off all data regarding the Consummatory phase I. Compilation off all data regarding the Consummatory phase I.

ANIMAL	SURGERY	DRUG	Average # of cells infected (%)	NoseToNose	Anogenital	Sniffing Male	Mount Attempt	Defensive-shoving	Kick	RunAway	Time spent running away (s)	Duration of consummatory phase I (s)	Total trial duration (s)	Phase I duration/total trial duration (%)
436	SHAM	CNO	0	4	2	6	8	0	0	4	8,6	74,4	696,733	10,67840909
452	SHAM	CNO	0	2	0	0	3	0	0	3	5,666	29,333	1292,934	2,268715959
453	SHAM	CNO	0	3	3	3	7	0	0	5	8,868	70,6	573,466	12,31110476
455	SHAM	CNO	0	4	0	0	2	0	0	4	8,868	50,933	1698,8	2,998175182
457	SHAM	CNO	0	0	0	0	1	0	0	0	0	1	539,933	0,185208165
629	DREADD	Saline	11,861	7	0	8	2	0	0	2	6,001	57	726,333	7,847640132
602	DREADD	Saline	13,180	18	21	33	10	0	0	0	0	390,667	1038,133	37,63169074
463	DREADD	Saline	18,166	12	0	3	12	12	1	9	19,334	261,8	1516,133	17,26761438
630	DREADD	Saline	26,498	19	19	32	26	0	0	11	27,735	440,2	1377,867	31,94793111
596	DREADD	Saline	27,564	6	0	4	3	0	0	6	11,266	77,867	482,067	16,15273396
587	DREADD	Saline	86,367	30	20	21	22	0	0	17	25,933	233,067	839,934	27,74825165
648	DREADD	CNO	5,501	18	13	30	7	0	0	7	8,6	217	679,533	31,93369564
428	DREADD	CNO	9,361	8	1	3	8	0	0	5	8,4	110,334	1090,2	10,12052834
592	DREADD	CNO	16,856	44	24	64	47	29	7	26	69,136	919	1381,467	66,52348554
662	DREADD	CNO	20,612	6	1	7	3	0	0	0	0	122,467	1046,533	11,70216324
431	DREADD	CNO	38,593	51	7	28	57	6	9	49	92,272	1034,734	1201,267	86,13688714
658	DREADD	CNO	43,861	1	0	3	2	0	0	2	2,067	20,334	1239,8	1,640103242
588	DREADD	CNO	54,152	0	0	1	4	0	0	0	7,733	24,133	1587,6	1,520093222
655	DREADD	CNO	62,304	1	0	0	1	0	0	0	0	3,066	1439,133	0,213044937

ANIMAL	SURGERY	DRUG	Average # of cells infected (%)	NoseToNose	Anogenital	Sniffing Male	Mount Attempt	Defensive-shoving	Kick	RunAway	Time spent running away (s)	Duration of consummatory phase I (s)	Total trial duration (s)	Phase I duration/total trial duration (%)
436	SHAM	CNO	0	4	2	6	8	0	0	4	8,6	74,4	696,733	10,67840909
452	SHAM	CNO	0	2	0	0	3	0	0	3	5,666	29,333	1292,934	2,268715959
453	SHAM	CNO	0	3	3	3	7	0	0	5	8,868	70,6	573,466	12,31110476
455	SHAM	CNO	0	4	0	0	2	0	0	4	8,868	50,933	1698,8	2,998175182
457	SHAM	CNO	0	0	0	0	1	0	0	0	0	1	539,933	0,185208165
629	DREADD	Saline	11,861	7	0	8	2	0	0	2	6,001	57	726,333	7,847640132
602	DREADD	Saline	13,180	18	21	33	10	0	0	0	0	390,667	1038,133	37,63169074
463	DREADD	Saline	18,166	12	0	3	12	12	1	9	19,334	261,8	1516,133	17,26761438
630	DREADD	Saline	26,498	19	19	32	26	0	0	11	27,735	440,2	1377,867	31,94793111
596	DREADD	Saline	27,564	6	0	4	3	0	0	6	11,266	77,867	482,067	16,15273396
587	DREADD	Saline	86,367	30	20	21	22	0	0	17	25,933	233,067	839,934	27,74825165
648	DREADD	CNO	5,501	18	13	30	7	0	0	7	8,6	217	679,533	31,93369564
428	DREADD	CNO	9,361	8	1	3	8	0	0	5	8,4	110,334	1090,2	10,12052834
592	DREADD	CNO	16,856	44	24	64	47	29	7	26	69,136	919	1381,467	66,52348554
662	DREADD	CNO	20,612	6	1	7	3	0	0	0	0	122,467	1046,533	11,70216324
431	DREADD	CNO	38,593	51	7	28	57	6	9	49	92,272	1034,734	1201,267	86,13688714
658	DREADD	CNO	43,861	1	0	3	2	0	0	2	2,067	20,334	1239,8	1,640103242
588	DREADD	CNO	54,152	0	0	1	4	0	0	0	7,733	24,133	1587,6	1,520093222
655	DREADD	CNO	62,304	1	0	0	1	0	0	0	0	3,066	1439,133	0,213044937

Table 3.2. Compilation off all data regarding the Consummatory phase II

ll data regarding the Consummatory phase II

ANIMAL	SURGERY	DRUG	Average # of cells infected (%)	Nose-to-nose	Anogenital	Sniffing male	Mount attempt	Mount with intromissions	Defensive-shoving	Kick	Run away	Lordosis	Ejaculation	Walk during intromission	Time spent running away (s)	Duration of phase II (s)	Total trial duration (s)
436	SHAM	CNO	0	10	7	13	9	8	0	0	6	5	1	1	16,198	407,4	696,733
452	SHAM	CNO	0	26	7	17	30	5	11	6	27	0	0	5	41,6	872,134	1292,934
453	SHAM	CNO	0	12	0	8	11	7	0	0	13	6	1	1	23	328,6	573,466
455	SHAM	CNO	0	27	16	31	15	13	0	0	20	12	1		49,599	895,134	1698,8
457	SHAM	CNO	0	8	0	2	8	2	0	0	7	0	1	2	39,401	176,4	539,933
629	DREADD	Saline	11,861	10	1	13	8	7	0	0	9	0	1	5	5,334	332,2	726,333
602	DREADD	Saline	13,180	11	1	11	22	7	0	1	5	2	0	0	8,399	505,933	1038,133
463	DREADD	Saline	18,166	13	0	7	26	4	28	1	24	0	0	4	54,134	662,533	1516,133
630	DREADD	Saline	26,498	13	0	18	48	12	4	0	25	12	0	0	49,334	792,2	1377,867
596	DREADD	Saline	27,564	7	1	2	3	3	0	0	4	3	1	1	1,8	239,533	482,067
587	DREADD	Saline	86,367	24	2	11	15	1	16	4	16	1	1	0	46,133	473,067	839,934
648	DREADD	CNO	5,501	10	0	11	9	6	0	0	13	3	1	4	22,334	206,933	679,533
428	DREADD	CNO	9,361	25	11	26	26	6	29	3	30	2	0	1	55,333	830	1090,2
592	DREADD	CNO	16,856	0	0	0	0	0	0	0	0	0	0	0	0	0	1381,467
662	DREADD	CNO	20,612	8	1	3	4	5	0	0	3	3	1	3	8,399	277,866	1046,533
431	DREADD	CNO	38,593	0	0	0	0	0	0	0	0	0	0	0	0	0	1201,267
658	DREADD	CNO	43,861	28	12	52	15	17	0	0	36	5	0	16	80,8	857,866	1239,8
588	DREADD	CNO	54,152	22	1	10	30	6	5	2	34	1	0	3	73,602	853,867	1587,6
655	DREADD	CNO	62,304	16	0	18	7	16	5	0	3	3	0	7	4,333	857,267	1439,133

ANIMAL	SURGERY	DRUG	Average # of cells infected (%)	Phase II duration/total trial duration (%)	Frequency of nose to nose (event/min)	Frequency of anogenital (event/min)	Frequency of sniffing male (event/min)	Frequency of mount attempts (event/min)	Mount with intromission latency (s)	Frequency of run away (event/min)	Time spent running (%)	Frequency of shoving (event/min)	Frequency of kick (event/min)
436	SHAM	CNO	0	58,47290138	1,47275405	1,030927835	1,914580265	1,325478645	289,333	0,88365243	3,975945017	0	0
452	SHAM	CNO	0	67,45386849	1,788715954	0,481577372	1,169545047	2,063903024	420,8	1,857512722	4,769909211	0,756764442	0,412780605
453	SHAM	CNO	0	57,30069437	2,191113816	0	1,460742544	2,008520998	244,866	2,373706634	6,999391357	0	0
455	SHAM	CNO	0	52,69213563	1,809784904	1,072465128	2,077901186	1,005436058	803,666	1,34058141	5,540958114	0	0
457	SHAM	CNO	0	32,67072026	2,721088435	0	0,680272109	2,721088435	363,533	2,380952381	22,3361678	0	0
629	DREADD	Saline	11,861	45,7365974	1,806140879	0,180614088	2,347983143	1,444912703	394,133	1,625526791	1,605659241	0	0
602	DREADD	Saline	13,180	48,73489235	1,304520559	0,118592778	1,304520559	2,609041118	532,2	0,59296389	1,660101239	0	0,118592778
463	DREADD	Saline	18,166	43,69887075	1,177299848	0	0,633930687	2,354599695	853,6	2,173476642	8,170762815	2,535722749	0,090561527
630	DREADD	Saline	26,498	57,49466385	0,984599849	0	1,363292098	3,635445595	585,667	1,893461247	6,227467811	0,3029538	0
596	DREADD	Saline	27,564	49,68873621	1,753411847	0,250487407	0,500974813	0,75146222	242,534	1,001949627	0,75146222	0	0
587	DREADD	Saline	86,367	56,32192529	3,043966288	0,253663857	1,395151215	1,90247893	366,867	2,029310859	9,751895609	2,029310859	0,507327715
648	DREADD	CNO	5,501	30,45223705	2,899489207	0	3,189438127	2,609540286	472,6	3,769335969	10,79286532	0	0
428	DREADD	CNO	9,361	76,13281967	1,807228916	0,795180723	1,879518072	1,879518072	260,2	2,168674699	6,666626506	2,096385542	0,21686747
592	DREADD	CNO	16,856	0	0	0	0	0	0	0	0	0	0
662	DREADD	CNO	20,612	26,55109777	1,727451361	0,21593142	0,647794261	0,863725681	768,667	0,647794261	3,022679997	0	0
431	DREADD	CNO	38,593	0	0	0	0	0	0	0	0	0	0
658	DREADD	CNO	43,861	69,19390224	1,958347807	0,839291917	3,636931642	1,049114897	381,934	2,517875752	9,418720406	0	0
588	DREADD	CNO	54,152	53,7835097	1,545908204	0,070268555	0,702685547	2,108056641	733,733	2,38913086	8,619843606	0,351342774	0,140537109
655	DREADD	CNO	62,304	59,56829563	1,11983781	0	1,259817536	0,489929042	581,866	0,209969589	0,505443462	0,349949316	0

ANIMAL	SURGERY	DRUG	Average of cells infected (%)	Lordosis duration (s)	Mount duration (s)	Lordosis duration / # intromission (s)	Intromission / phase time (%)	Mounts with intromission frequency (event/min)	Lordosis / intormission (%)	Lordosis time / intromission time (s)	Mount attempt/lordosis	Mean lordosis duration (s)	Mean mount duration (s)
436	SHAM	CNO	0	17,534	126,199	2,19175	30,97668139	1,17820324	62,5	13,89392943	1,8	3,5068	15,77487
452	SHAM	CNO	0	0	44,466	0	5,098528437	0,34398387	0	0	0	-	8,8932
453	SHAM	CNO	0	29,667	135,666	4,238142857	41,28606208	1,27814972	85,71428571	21,86767503	1,833333333	4,9445	19,38085714
455	SHAM	CNO	0	63,199	147,933	4,861461538	16,52635248	0,87137917	92,30769231	42,72136711	1,25	5,266583333	11,37946154
457	SHAM	CNO	0	0	39,666	0	22,48639456	0,68027209	0	0	8	-	19,833
629	DREADD	Saline	11,861	0	98,801	0	29,74142083	1,26429861	0	0	-	-	14,11442857
602	DREADD	Saline	13,180	5,933	94,735	0,847571429	18,72481139	0,83014944	28,57142857	6,262732886	15	2,9665	13,53357143
463	DREADD	Saline	18,166	0	13,866	0	2,092876883	0,36224607	0	0	1	-	3,4665
630	DREADD	Saline	26,498	57,798	133,734	4,8165	16,8813431	0,90886139	100	43,21862802	11	4,8165	11,1445
596	DREADD	Saline	27,564	23,134	74,266	7,711333333	31,00449625	0,7514622	100	31,15018986	-	7,711333333	24,75333333
587	DREADD	Saline	86,367	4,533	46,267	4,533	9,780221406	0,12683192	100	9,797479845	4	4,533	46,267
648	DREADD	CNO	5,501	3,6	72	0,6	34,79387048	1,73969352	50	5	13	1,2	12
428	DREADD	CNO	9,361	8,133	47,335	1,3555	5,703012048	0,43734942	33,33333333	17,18178937	-	4,0665	7,889166667
592	DREADD	CNO	16,856	0	0	0	0	0	0	0	30	-	-
662	DREADD	CNO	20,612	38,533	87,399	7,7066	31,45365032	1,07965710	60	44,08860513	-	12,84433333	17,4798
431	DREADD	CNO	38,593	0	0	0	0	0	0	0	3	-	-
658	DREADD	CNO	43,861	19,799	94,799	1,16467059	11,05056034	1,18896883	29,41176471	20,88524141	2,333333333	3,9598	5,576411765
588	DREADD	CNO	54,152	1,733	152,534	0,288833333	17,86390621	0,42161132	16,66666667	1,13614013	3	1,733	25,42333333
655	DREADD	CNO	62,304	19,799	206,534	1,2374375	24,09214399	1,11983781	18,75	9,586315086	1,333333333	6,599666667	12,90837

Table 1. Provides the results of the statistical analyses (Kruskal-Wallis test). Compilation off all data regarding the Consu

-values resulted from statistical analyses (Kruskal-Wallis test).

	NoseToNose	Anogenital	Sniffing Male	Defensive-shoving	Kick	Phase duration (s)	TotalTimeTrial	Phase duration (%)	Frequency of nose to nose (event/min)	Frequency of anogenital (event/min)	Frequency of sniffing male (event/min)	Frequency of mount attempts (event/min)	Mount Attempt Latency (s)
Appetitive phase	0,2425	0,3438	0,1948	-	-	0,1312	0,4811	0,6022	0,8091	0,741	0,1233	-	-
Consummatory phase I	0,0563	0,6273	0,048*	0,6945	0,6367	0,0913	0,4811	0,1391	0,7987	0,7776	0,1371	0,3637	0,4851
Consummatory phase II	0,8464	0,6977	0,9042	0,6686	0,7019	0,7701	0,4811	0,6309	0,4782	0,6667	0,6762	0,5973	-

	Frequency of run away (event/min)	Time spent running (%)	Lordosis duration (s)	Mount duration (s)	Lordosis duration / #intromission	%intromission / phase time	intromission frequency (event/min)	#lordosis / #intromission (%)	lordosis time / intromission time (s)	lordosis/mount attempt	Mount with Intromission Latency (s)	Ejaculation latency (s)	Walk with intromission / mounts with intromission (s)
Appetitive phase	-	-	-	-	-	-	-	-	-	-	-	-	-
Consummatory phase I	0,2891	0,303	-	-	-	-	-	-	-	-	-	-	-
Consummatory phase II	0,6084	0,6911	0,7263	0,8513	0,9749	0,804	0,5736	0,6426	0,8885	0,4727	0,6309	0,8619	0,5859

Table 5. Relation between VMHvl PgR+ cells infection and behavioural output

Co-relation factors	DREADD+Saline R^2	DREADD+CNO R^2
Mount with intromission events and % of infected cells	0,3143	0,2773
Mount with intromission duration and % of infected cells	0,09915	0,8139
Lordosis events and % of infected cells	0,003429	0,000283
Lordosis duration and % of infected cells	0,001061	0,003416
Defensive events and % of infected cells	0,05525	0,1383