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Organization and orchestration of male rat sexual behavior

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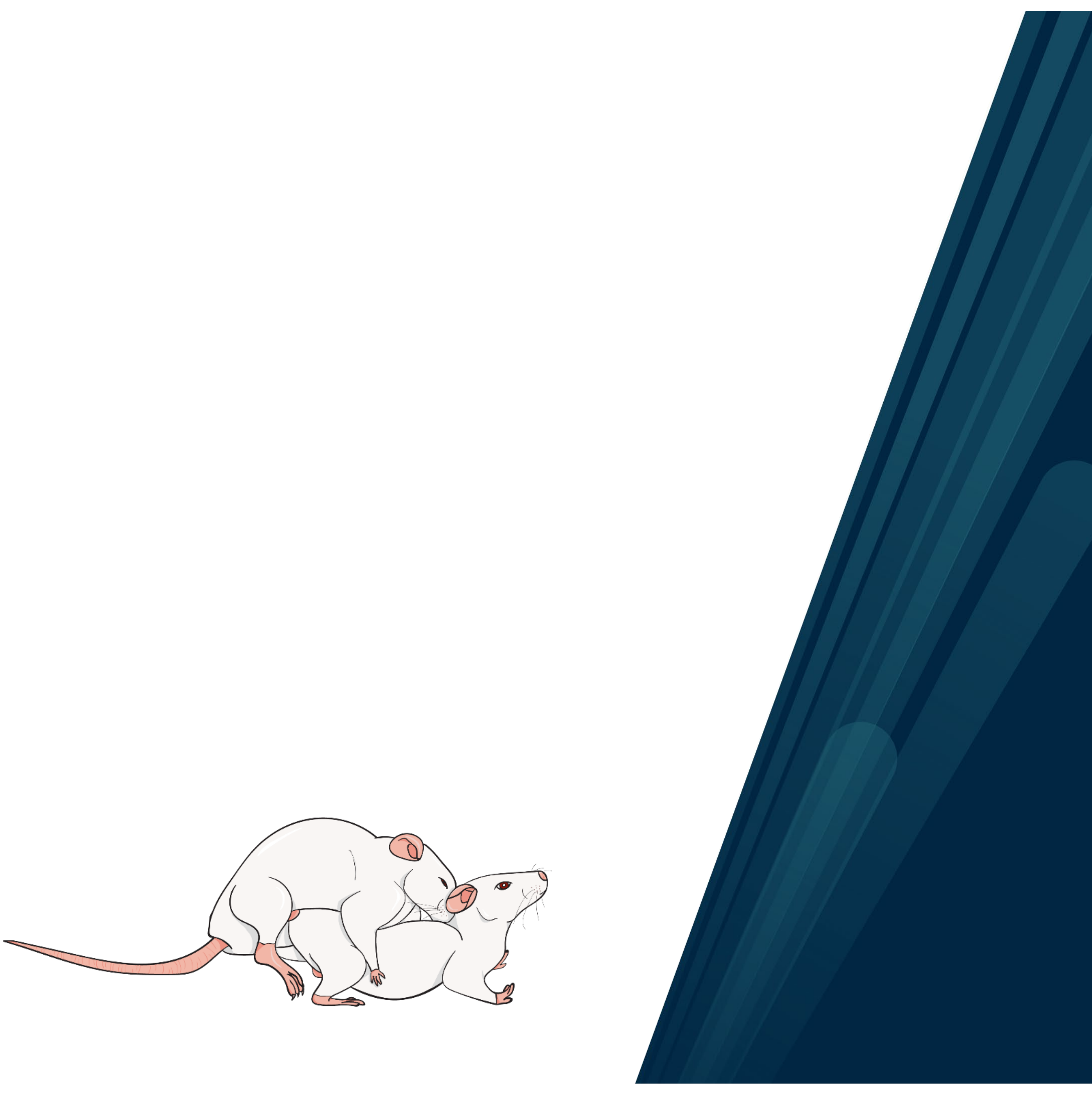

Organization and orchestration of male rat sexual behavior

PhD Thesis

By

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Other

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Abstract of the thesis

Sexual behavior is innately motivated and rewarding. The underlying neurobiological mechanisms of sexual motivation, copulation, and sexual reward have not been completely unraveled. This thesis gives insight in the structure, organization, and neurobiological orchestration of male rat sexual behavior. The research presented in this thesis demonstrates that a more extensive behavioral annotation allows for a more thorough analysis of the behavioral organization of copulation. By utilizing such analyses, we showed that the durations of inter-copulatory intervals are strongly correlated in individual male rats. This advanced understanding is valuable for the formulation of hypotheses about neurobiological underpinnings of sexual behavior. In addition, the more detailed behavioral analysis made it possible to conclude that the medial amygdala influences the latency to ejaculation through the processing of sensory feedback rather than impacting copulatory pace or efficiency, in a study in which we chemogenetically stimulated and silenced the medial amygdala. Neuronal circuitry involved in the orchestration of sexual behavior, including the medial amygdala and the medial preoptic area, is modified by gonadal hormones. It is reported in this thesis that gonadectomy and treatment with dihydrotestosterone affects dendritic spine plasticity in these mentioned brain regions, as well as in the nucleus accumbens, an important node in the mesolimbic reward system. Hormone-induced neuronal plasticity is hypothesized to permit the functional neuronal circuitry to orchestrate sexual behavior, and to be at the basis of long-term sexual reward learning. Together, these findings advance our understanding of the neuronal regulation of sexual behavior, and hopefully stimulate the field to employ more extensive behavioral assessments when studying sexual behavior in male rats.

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Chapter 1

General introduction

1.1 Rationale

Why do we do what we do? This thesis is meant to be a tiny part in the endless pool of research surrounding this ultimate question in neuroscience. The focus of this thesis is on sexual behavior; a behavioral act that is both innately motivated and rewarding, as well as absolutely necessary for reproduction, and thus survival, of all non-human species. These fascinating properties make for sexual behavior to be highly qualified for investigation of neurobiological underpinnings of behavior. A suitable animal model to study sexual behavior is the male rat. Male rats go through several stages of sexual behavior indicative of sexual motivation and execution of copulation, and these stages can be studied separately. In order to advance our understanding of neuronal orchestration of all aspects of sexual behavior, it is important to study both the structure and organization of the behavior in depth, as well as the neurobiological correlates of the behavior. The research presented in this thesis applies and unites both these approaches.

This introduction sets the framework for the experimental research results that are presented in this thesis. The current state of knowledge on endocrine and neuronal control of male rat sexual behavior and the modulatory convergence of hormones and neural circuitry will be discussed. It should be noted that this introduction focuses on outcome measures of all stages of sexual behavior but will mostly gloss over data on physiological parameters such as erections and seminal emissions outside the context of copulation, since these *ex copula* concepts are rather dissociated from the neurobiological control of the same concepts *in copula* (see for a more extensive discussion (Paredes and Ågmo 2004)). In addition, even though research done in other vertebrate species and female rats provides valuable insights for development of hypotheses about neurobiological mechanisms in male rats, this introduction will solely focus on the hormonal and neuronal control of sexual behavior in male rats.

1.2 Rat sexual behavior

Sexual behavior is the result of an interaction between two or more animals. This section briefly introduces the behavioral correlates of such an interaction. Even though rats are rather promiscuous creatures in the wild and in semi-natural conditions (Chu and Ågmo 2015; Schweinfurth 2020), this introduction will focus on interactions between one male and one female as observed in typical laboratory testing conditions.

1.2.1 Male rat sexual behavior

Sexual behavior in male rats can be divided into three stages: approach, copulation, and ejaculation. Approach is dependent on the motivation to engage in sexual behavior, which is typically high in healthy males. Females that are sexually receptive (see female sexual behavior), are identified by odors and pheromones, making them an attractive stimulus for approach. Shortly after a female rat is introduced to a cage with a male, the male will start sniffing and grooming the anogenital region of the female to obtain pheromonal cues. Not long after this initial investigation, the male will attempt to mount the female. During a mount, the male stands on its hind legs and puts its forepaws on the flanks of the female, while thrusting its pelvis. In case the male locates the vagina of the female with its erect penis, an intromission is achieved. Intromissions are characterized by a distinct motor pattern of a backward jump away from the female, and are followed by male autogrooming of the genitals. When no intromission is achieved upon a mount, the male will slowly dismount the female. A sequence of multiple intromissions, and none to multiple mounts is displayed during the copulation phase, interspersed with short rests, (genital) grooming, and chasing and sniffing of the female. When at least some intromissions have taken place, the interaction may proceed into the ejaculation phase. An ejaculation is recognized by the longer duration than an intromission and rhythmic abdominal contractions. After an ejaculation, the male usually raises its front paws and the female will eventually move away from the male. The latency to ejaculation is relatively stable within males across copulation

sessions (Pattij et al. 2005), and is determined by the number of intromissions necessary to reach ejaculation threshold, the efficiency to achieve intromissions during mounts, and the pace of copulation.

Following ejaculation, a refractory period of 3-10 minutes commences. During this refractory period, the male rests and is often observed grooming its genitals or other regions, and pays little to no attention to the female. Towards the end of the refractory period, the male starts sniffing and pursuing the female again, which marks the start of a new ejaculation series. Several ejaculation series can be observed when males are allowed to copulate *ad libitum*. Each following ejaculation series is characterized by an increase in the duration of the refractory period (Rodríguez-Manzo and Fernández-Guasti 1994). Male rats achieve on average seven ejaculations until sexual exhaustion is reached (Rodríguez-Manzo and Fernández-Guasti 1994). After exhaustion, also called sexual satiety, the male needs some days to recover in order for its copulatory parameters to return to baseline.

1.2.2 Female rat sexual behavior

Sexual activity of the female rat is dependent on her hormonal levels, which fluctuate over the estrous cycle (reviewed in (Snoeren 2019)). Only females that are in behavioral estrus are willing to engage in copulation. Females that are not in behavioral estrus, and thus not sexually receptive, will show signs of rejection when males attempt to mount them. It is however very uncommon for male rats to attempt to mount a female that is not in estrous. Sexually receptive females in the presence of a male will show the paracopulatory behaviors hopping (short jumps with all four legs off of the ground) and darting (short and sudden runaway movements, in which she presents her body to the male). These behaviors, only observed in females, are often viewed as a solicitation towards the male. However, it is now known that males and females have an equal part in the initiation of copulatory acts (Bergheim, Chu, and Ågmo 2015). When the male mounts the female, the female assumes the lordosis pose through a spinal reflex induced by the tactile stimulation of the male. The display of

lordosis consists of a marked arching of the back, exposing the vagina and providing access to allow penile insertion.

1.2.3 Rat sexual behavior outcome measures

In order to study rat copulatory behavior, copulatory events (mounts, intromissions, and ejaculations) are manually annotated during a copulation session. From the frequencies and relative time point of these behaviors, outcome measures can be calculated. These outcome measures and their interpretation (as well as the appropriate measures for female rats) are extensively discussed in *paper I*, but a concise overview of definitions in male rat sexual behavior is shown in Table 1 for the purpose of this introduction.

Table 1 Male rat sexual behavior parameters

Outcome measure	**Definition**
Latency to first mount or intromission	Time from the start of the test to the first mount or intromission
Number of mounts	Total number of mounts preceding ejaculation
Number of intromissions	Total number of intromissions preceding ejaculation
Intromission ratio	Number of intromissions in the ejaculation series divided by the total number of copulatory behaviors (mounts + intromissions) in the ejaculation series
Number of mount bouts	Total number of mount bouts preceding ejaculation
Mounts per mount bout	Mean number of mounts per mount bout in an ejaculation series
Intromissions per mount bout	Mean number of intromissions per mount bout in an ejaculation series
Mount bout duration	Time from the first copulatory behavior in a mount bout until the first behavior within the following time out
Time out duration	Time from the end of one mount bout to the start of the next mount bout
Inter-intromission interval	Time between intromissions in an ejaculation series, calculated from the first intromission
Latency to ejaculation	Time from the first mount or intromission to ejaculation
Post-ejaculatory interval	Time from the first ejaculation to the next copulatory behavior (mount or intromission)

In *paper II*, the outcome measures described in Table 1 are further expanded with measures of natural temporal patterning of copulation for the purpose of studying how inter-copulatory intervals relate to each other within male rat copulation. Briefly, copulations are naturally organized

in mount bouts in male rats. These mount bouts consist of one or more copulations that are uninterrupted by non-copulation oriented behavior, and are interspersed by short periods of non-copulation oriented behavior. Mount bouts are the central units of copulation in male rats and determine the temporal pattern of copulation.

1.3 Hormonal control of male rat sexual behavior

Sexual behavior in male rats is dependent on gonadal hormones. This section discusses the facilitating effects of gonadal hormones and their metabolites in the context of both sexual motivation as well as copulation.

1.3.1 Effects of loss and gain of gonadal hormones on sexual behavior

Testosterone, the main gonadal hormone in male rats, is produced in the testes and secreted into the bloodstream. After gonadectomy (removal of the testes), serum testosterone typically quickly declines (<24h) to very low levels in most male rats (Coyotupa, Parlow, and Kovacic 1973; Dessì-Fulgheri et al. 1983; Andò et al. 1986). Following gonadectomy, sexual motivation and copulation gradually decline as well. Some male rats continue to copulate for multiple weeks after gonadectomy and thus loss of gonadal hormones, but in most males copulatory reflexes cease within 3-10 days (Davidson 1966b; Beach and Holz-Tucker 1949). Male rats first lose their ability to ejaculate, then to intromit, and finally they completely stop mounting and have no sexual interest for a receptive female anymore (Davidson 1966b). The unconditioned preference for a receptive female over a non-receptive female is on average absent on day 10-15 after gonadectomy (Ågmo 2003).

The loss of sexual function upon loss of gonadal hormones can be rescued by treating gonadectomized males with testosterone. The preference for a receptive female then returns within about two weeks of daily testosterone treatment (Ågmo 2003), and copulation gradually returns to baseline over the course of 3-10 days, with mounts reinstating before intromissions and ejaculations

(McGinnis et al. 1989; Beach and Holz-Tucker 1949). As mentioned above, testosterone levels fall and rise quickly after gonadectomy and testosterone treatment, respectively, but copulation and sexual incentive motivation take longer to disappear and be reinstated. In addition, prolonged daily exposure to testosterone (>21h/d) is necessary to reinstate the full pattern of sexual behavior (McGinnis et al. 1989). Therefore, hormonal control of male rat sexual behavior seems to mainly be the result of long-term genomic effects of gonadal hormones.

1.3.2 Effects of testosterone metabolites on sexual behavior

While testosterone is the principal hormone secreted by the testes, it is in fact partially converted in target organs, including the brain, into estrogenic and androgenic metabolites. Testosterone can be 5α-reduced into dihydrotestosterone, an androgen with an even higher affinity for the androgen receptor than testosterone, and aromatized by the enzyme aromatase into estradiol, a high-affinity ligand of the estrogen receptor. Testosterone's effects are thus not limited to androgen receptor binding, but may also arise from aromatization into estradiol and subsequent estrogen receptor binding. A longstanding question is whether it is the androgenic effects, the estrogenic effects, or the combination of both that is necessary for the facilitation of sexual behavior by gonadal hormones in male rats. Several observations have given rise to the "aromatization hypothesis", which postulates that aromatization of testosterone into estradiol is vital for sustaining and restoring sexual behavior in male rats. In support of this hypothesis, there are numerous reports that show that estradiol can reinstate most aspects of copulation after gonadectomy (Södersten 1973; Davidson 1969; Pfaff 1970; Larsson, Södersten, and Beyer 1973), as well as sexual motivation (Bakker et al. 1993; Merkx 1984). However, gonadectomized males treated with only estradiol often do not reach ejaculation, and these studies have employed much higher doses of estradiol than physiologically equivalent. Estradiol in the physiological range facilitates mostly mounting and is not sufficient to fully restore copulation (Vagell and McGinnis 1997), and is also not necessary nor sufficient for the preference for a receptive female (Vagell and McGinnis 1997; Attila, Oksala, and Ågmo 2010).

Studies employing drugs that block aromatase or estrogen receptors in testosterone treated gonadectomized males have reported contradictory results, with some studies finding no effect, and others finding large inhibitory effects on sexual behavior reinstatement (Beyer et al. 1976; Luttge 1975). Drug studies should always be interpreted with caution, as the full range of mechanism of action of a drug is often unknown. For example, many anti-estrogens were later shown to actually be partial agonists for the estrogen receptor, and an often used aromatase inhibitor was later demonstrated to also have androgen receptor blocking properties (Kaplan and McGinnis 1989). A series of studies by McGinnis and colleagues have addressed these issues by assessing effects on sexual behavior in the context of the receptor-binding and receptor-blocking properties of the drugs that were studied. They have shown that fadrozole, an aromatase inhibitor without androgen receptor blocking properties, blocks restoration of sexual behavior in testosterone treated gonadectomized males, but not the preference for a receptive female (Vagell and McGinnis 1997). Further, blocking of estrogen receptors in testosterone treated gonadectomized males with an estrogen receptor blocker that reduced nuclear estrogen receptor binding to castrate levels did not affect reinstatement of copulation (Vagell and McGinnis 1998). Taken together, estradiol does seem to contribute to male copulatory behavior, although not to the preference for a receptive female, but its role may be smaller than was long thought, and the effect may be regulated through other mechanisms than nuclear estrogen receptor binding.

If estradiol has only minor effects, it must surely mean that most sexual behavior in males is sustained by the androgenic properties of testosterone. Indeed, blocking of androgen receptors attenuates testosterone reinstatement of copulation and receptive female preference in gonadectomized males (Vagell and McGinnis 1998; McGinnis and Mirth 1986). However, dihydrotestosterone, a “pure” androgen that cannot be aromatized into estradiol, is remarkably ineffective in restoring copulation in gonadectomized males (McDonald et al. 1970; McGinnis and Dreifuss 1989; Beyer et al. 1973; Larsson, Södersten, and Beyer 1973). Dihydrotestosterone alone has some effect on the restoration of sexual motivation, but not to the level of effect of testosterone

(Attila, Oksala, and Ågmo 2010). Studies in which gonadectomized males were treated with either testosterone or a combination of estradiol and dihydrotestosterone showed no difference between these groups in the reinstatement of copulation (Baum and Vreeburg 1973; Larsson, Södersten, and Beyer 1973), as well as sexual incentive motivation (Attila, Oksala, and Ågmo 2010). It thus seems that sustaining a certain minimum level of estradiol is necessary for androgens to facilitate sexual behavior. However, when gonadectomized males are treated with a combination of estradiol and dihydrotestosterone that yields hormone levels and hormone receptor binding in the physiological range, their copulatory behavior is still impaired as compared to males treated with testosterone (McGinnis and Dreifuss 1989). Therefore, testosterone's regulation of sexual behavior in male rats may not just simply be the result of the sum of effects of dihydrotestosterone and estradiol (or: maximal androgen and estrogen receptor binding), but testosterone may have certain regulatory effects that to date remain unidentified.

1.3.3 Concluding remarks on hormonal control of male rat sexual behavior

Overall, these findings suggest that both androgen and estrogen signaling is necessary for the full display of sexual behavior (copulation and sexual motivation) in male rats. Still, the question of androgens' and estrogens' exact roles in sexual behavior in male rats remains largely unresolved. Perhaps local, intra-cellular aromatization of testosterone restricted to certain cell types as opposed to wide-spread estradiol availability is what underlies the full pattern of sexual behavior. Alternatively, testosterone might bind to undiscovered receptors that do not bind dihydrotestosterone, or might have other non-receptor mediated effects. Future research should aim to unravel these mechanisms.

1.4 Neuronal orchestration of male rat sexual behavior

Several brain regions and the circuitry they comprise have been implicated in the neuronal control of male rat sexual behavior. This section will describe some of the most important neural correlates

that have been identified in male rat sexual behavior, with a focus on brain regions that have been investigated in the work presented in the four papers of this thesis

1.4.1 Sensory processing

For sexual arousal and motivation to engage in sexual behavior to arise, it is necessary for a male rat to be able to identify a potential partner. The most important system involved in this process in male rats is the chemosensory system, which includes the processing of olfactory and pheromonal cues. Volatile odors, that can reach the male from a distance, are processed in the **main olfactory bulb** (Lledo, Gheusi, and Vincent 2005). Non-volatile odors, such as pheromones, are sensed by the vomeronasal organ when the male is in close proximity to the female while for example engaging in anogenital sniffing. This information is processed in the **accessory olfactory bulb** (Lledo, Gheusi, and Vincent 2005). Functional olfaction is vital for sexual incentive motivation, as anosmia blocks the preference for a receptive female, and other sensory systems such as vision and audition are not enough to compensate for the loss of olfaction (Ågmo and Snoeren 2017; Bergvall et al. 1991). Anosmic males also show less anogenital sniffing and approach behavior to females, in addition to showing impaired copulation, and these effects are even larger in males without prior sexual experience (Thor and Flannelly 1977; Larsson 1971). Not surprisingly, bulbectomy blocks the preference for a receptive female, and has inhibitory effects on anogenital investigation of a receptive female, initiation of copulation, and ejaculation (Edwards, Walter, and Liang 1996; Meisel, Lumia, and Sachs 1980). Unilateral lesion of the olfactory peduncle causes ipsilateral reduction of *c-fos* expression in the medial amygdala (MeA) upon exposure to a receptive female without intromission and ejaculation (Baum and Everitt 1992), suggesting a pathway of direct chemosensory information relay to the MeA that may be important for the initiation of copulation.

In addition to sensory processing of odors and pheromones, processing of somatosensory information such as penile sensation during intromission and ejaculation is also an important factor in the regulation of sexual behavior. Penile somatosensory feedback is thought to be relayed by the

central tegmental field (CTF), in particular the subparafascicular nucleus (SPFp) and the zona incerta (ZI), as well as the MeA (Baum and Everitt 1992). Unilateral lesion of both these regions reduces copulation-induced *c-fos* expression in the ipsilateral bed nucleus of the stria terminalis (BNST) and medial preoptic area (mPOA), indicating how this information can reach central integrative sites. Within copulatory behavior, ejaculation provides the strongest somatosensory feedback. When focusing on ejaculation specifically, a certain circuit has been delineated through the observation of specific cell clusters expressing *c-fos* solely upon ejaculation. These clusters are found in the BNST, MeA, and SPFp, and the neuronal activity is hypothesized to be mainly the result of the sensory feedback as opposed to control of behavioral output (Coolen et al. 1997). Further, the SPFp receives galanergic input from the spinal ejaculator, and local infusion of galanin into the SPFp severely inhibits copulation, possibly suggesting a role for the SPFp as the central source for copulatory refraction (described in (Coolen et al. 2004)).

1.4.2 The extended amygdala

The amygdala is an agglomeration of several distinct nuclei that have different origin and anatomical connections (Swanson and Petrovich 1998). Of these distinct subregions, the **medial amygdala** (MeA) has the most prominent role in sexual behavior. The MeA is a major hub for sensory processing; as mentioned above, it receives chemosensory information and responds to somatosensory stimuli such as intromissions and ejaculation (Baum and Everitt 1992; Coolen, Peters, and Veening 1997). Despite its role in processing of chemosensory cues, lesions of the MeA do not seem to affect the incentive preference for a receptive female (Beck, Fonberg, and Korczyński 1982; Kondo and Sachs 2002). MeA lesions have been studied extensively in the context of copulation, and it has been repeatedly reported that even though males with MeA lesions still display the full range of copulatory behaviors, the lesions do impair ejaculation as measured by increased ejaculation latency and an increase in mounts and intromissions preceding ejaculation (Giantonio, Lund, and Gerall 1970; Harris and Sachs 1975; de Jonge et al. 1992; McGregor and Herbert 1992; Tsutsui, Shinoda, and Kondo

1994; Kondo, Sachs, and Sakuma 1997; Dominguez et al. 2001; Kondo and Arai 1995). In addition, lesions in sexually inexperienced males are far more detrimental than in experienced males (Kondo 1992), indicating a role for the amygdala in shaping the recognition of and appropriate behavioral response towards receptive females. These previous findings have led to the study described in *paper III*, in which we investigated the role of the MeA in sexual incentive motivation and copulation in more detail, and in *paper IV* in which the MeA was one of the brain regions in which we studied the effects of gonadal hormones on neuronal plasticity.

The MeA has strong connectivity with the **bed nucleus of the stria terminalis** (BNST) through the stria terminalis fiber tract. The BNST is often characterized as a "relay center" for the integration of signals related to fear and anxiety and reward and aversion (Lebow and Chen 2016). Chemosensory investigation as well as copulation (especially ejaculation) induces *c-fos* expression in the BNST, more specifically in the posteromedial division of the BNST (Coolen, Peters, and Veening 1997, 1996). Lesions of the BNST have similar effects as lesions of the MeA; they result in increased ejaculation latency, less ejaculations achieved, and an increased number of mounts and intromissions preceding ejaculation (Emery and Sachs 1976; Claro et al. 1995; Liu, Salamone, and Sachs 1997b; Valcourt and Sachs 1979). Small lesions of the posteromedial BNST additionally delayed initiation of copulation in inexperienced males (Claro et al. 1995), again a similar effect as observed in MeA lesioned males. However, one needs to bear in mind that effects of non-axon sparing lesions of the BNST may be the result of the destruction of the stria terminalis, which contains axonal projections from the MeA to the BNST. Indeed, knife cut lesion of only the stria terminalis results in similar copulatory deficit as MeA and BNST lesions (Kondo and Yamanouchi 1995).

1.4.3 Hypothalamic nuclei

There is overwhelming evidence that the **medial preoptic area** (mPOA) is the most important brain region in the orchestration of sexual behavior in male rats. Electrophysiological recordings *in vivo* have shown that neuronal acitivity in the mPOA is increased upon the introduction of a receptive

female, during copulation, especially responds to copulatory events, and shows prolonged inhibition during the post-ejaculatory interval (Horio et al. 1986; Shimura, Yamamoto, and Shimokochi 1994). Consistent with these *in vivo* measures of neural activity, Fos was found to be significantly increased after several stages of increasing sexual behavior in males (Baum and Everitt 1992; Veening and Coolen 1998). In addition, numerous lesions studies have shown that destruction of the mPOA critically inhibit or even completely abolish male copulation, with large bilateral electrolytic lesions completely and permanently disrupting copulation (Heimer and Larsson 1967; Giantonio, Lund, and Gerall 1970; Hendricks and Scheetz 1973; Chen and Bliss 1974; Ginton and Merari 1977; Ågmo, Soulairac, and Soulairac 1977; Hansen et al. 1982; Klaric and Hendricks 1986; Kondo et al. 1990), and bilateral partial lesions or unilateral lesions having much less severe effects with sometimes regain of the lost copulatory activity over time (Heimer and Larsson 1967; Larsson and Heimer 1964; Chen and Bliss 1974; Ginton and Merari 1977; Arendash and Gorski 1983). Degeneration of cell bodies in the mPOA (while sparing passing axons) by means of the neurotoxin ibotenic acid, or pharmacological suppression of neural activity by microinjection of lidocaine into the mPOA, is just as effective in eliminating copulation as electrolytic lesions, indicating that cell bodies in the mPOA are necessary for copulation in males (Hansen et al. 1982; Hurtazo, Paredes, and Ågmo 2008). In contrast to the effects of lesions, electrical stimulation of the mPOA drastically decreases the duration of the copulatory phase and greatly enhances the speed with which an animal proceeds into the executive phase of ejaculation (Malsbury 1971; Merari and Ginton 1975; Rodríguez-Manzo et al. 2000). These effects are stimulation-bound, meaning they arise immediately and transiently upon stimulation and have no effects outside of episodes of electrical current application. Quite strikingly, continuous stimulation could make a male ejaculate 11 times within 5 minutes, often without any preceding intromissions and with virtually no refractory period (Merari and Ginton 1975). Finally, multiple lines of evidence have shown a role for the release of specific neurotransmitters and neuromodulators into the mPOA during several stages of sexual behavior, such as glutamate, dopamine, nitric oxide, and opioids (Hull, Wood,

and Mckenna 2006; Will, Hull, and Dominguez 2014). These findings strongly implicate the mPOA in the regulation of all aspects of male rat sexual behavior.

In light of the role of the mPOA in sexual motivation, it is reported that males in which copulation was eliminated by mPOA injury still display pursuit of the female, anogenital investigation, and sometimes clasping (Hansen et al. 1982; Everitt et al. 1987). In addition, mPOA lesioned males continue responding in operant tasks to access a receptive female (Everitt et al. 1987). Hence, it has been hypothesized in the past that the mPOA may only be involved in the orchestration of (motor patterns of) copulatory behavior, but perhaps not sexual motivation. However, the frequency and duration of pursuit is dramatically reduced in males with extensive mPOA lesions (which abolished copulation) during interactions with a receptive female (Paredes, Highland, and Karam 1993). Lesion and inactivation of the mPOA with lidocaine also significantly reduce the preference for a receptive female in unconditioned tests, while leaving social motivation unaffected (Edwards and Einhorn 1986; Edwards, Walter, and Liang 1996; Paredes, Tzschentke, and Nakach 1998; Hurtazo, Paredes, and Ågmo 2008). Apparently, conditioned responses for a receptive female are more resilient to mPOA lesions than unconditioned responses. This may be explained by the fact that operant tests actually measure reward memory and reinforcement of copulation instead of incentive sexual motivation, which is more reliant on structures outside of the mPOA such as the reward system (Ventura-Aquino et al. 2017). Moreover, even though a receptive female may not induce strong sexual motivation in a mPOA lesioned male any longer, she is still a social stimulus and thus a rewarding consequence of the conditioned response, especially in a setting where no alternative rewarding stimulus is provided (e.g., compare to the sexual incentive motivation test in which males have a choice in spending time with a sexual stimulus vs. a social stimulus). Thus, it is in my opinion safe to say that the mPOA is critically important for sexual motivation in male rats (see also this review: (Paredes 2003)). Yet, one might ask whether the elimination of copulation through mPOA lesions is a consequence of diminished sexual motivation, or whether the mPOA has an active role in both these stages of sexual behavior. To answer this question is next to impossible, since animals that are not motivated to engage in sexual behavior

obviously have no incentive to show any copulatory behaviors. Still, the *in vivo* neural activity recording studies discussed earlier that showed that each stage of sexual behavior is associated with changes of mPOA neural activity, in addition to the dramatic facilitation of ejaculation upon general mPOA stimulation, provides support for the mPOA being involved in the orchestration of all aspects of sexual behavior in male rats. The exact regulation of the different aspects of sexual behavior by the mPOA remains elusive and highly complex.

The mPOA has reciprocal connections with sensory systems, and sends widespread projections to brain structures that are involved in the regulation of erection, ejaculation, and the general initiation of motor patterns in motivated behavior (Simerly and Swanson 1986, 1988; Coolen, Peters, and Veening 1998). This underscores the role of the mPOA as the major integrative site and "orchestrator" of sexual behavior. For example, whereas unilateral combined lesion of the mPOA and either the MeA or the CTF placed ipsilaterally only result in very minor sexual behavior deficits, contralateral combined lesions severely affect initiation of copulation and abolishes ejaculation and intromission (Kondo and Arai 1995; Brackett and Edwards 1984). Lesion of the MeA also prevents dopamine release in the mPOA during copulation (Dominguez et al. 2001). These findings prompted us to study the role of the MeA as a major mPOA-input area in more detail in *paper III*, which also induced further questions about the role of the specific MeA-mPOA projection. Further, the detrimental effects of posterior lesions to the medial forebrain bundle, through which mPOA efferents (as well as afferents) travel to the midbrain, illustrates the importance of the output of the mPOA (Hendricks and Scheetz 1973; Cagguila, Antelman, and Zigmond 1973; Paxinos and Bindra 1973).

The **paraventricular nucleus of the hypothalamus** (PVN) contains magnocellular neurons that project to the posterior pituitary gland, and parvocellular neurons that release, amongst other neuroactive substances, oxytocin and vasopressin to other central brain regions (Argiolas and Melis 2005). Radiofrequency lesion of the PVN that destroyed both parvocellular as well as magnocellular neurons and passing axons increased ejaculation latency and decreased intromission ratio (Liu, Salamone, and Sachs 1997a). Small electrolytic lesions of the lateral parvocellular division of the PVN

blocked the ejaculation-induced increase in CSF oxytocin concentration and decreased the post-ejaculatory interval (Hughes et al. 1987). A recent study also showed that oxytocin released from PVN neurons into the lumbal spinal cord controls spinal ejaculation generator neurons (Oti et al. 2021). These findings, in combination with a large amount of data on physiological penile effects outside of the context of copulation (reviewed in (Argiolas and Melis 2005)), implicate the PVN as a modulator in the regulation of erection and ejaculation, and possibly the refractory period. The **lateral hypothalamus** (LH) might have a similar role, as the post-ejaculatory interval is associated with serotonin release in the LH, and local administration of a serotonin reuptake inhibitor increased the latencies to first copulatory behavior and ejaculation (Lorrain et al. 1997).

The **ventromedial nucleus of the hypothalamus** (VMN) is strongly implicated in the regulation of female rat sexual behavior, especially lordosis, but very little research has been done on its role in male rat sexual behavior (Flanagan-Cato 2011). Effects of small electrolytic lesions of the VMN in males are contradictory; one study found facilitated ejaculation in testosterone-treated gonadectomized males (Christensen, Nance, and Gorski 1977), while another found no effects on sexual motivation and copulation (Harding and McGinnis 2005). The VMN is sexually dimorphic and less responsive to estrogens in males than in females, which may contribute to the lack of lordosis in males (Flanagan-Cato 2011).

1.4.4 Reward system

Engaging in sexual behavior is rewarding for rats (Martinez and Paredes 2001), suggesting a role for the mesolimbic reward system in sexual behavior. The main pathway involved in reward processing and reward learning of all kinds of behaviors is the dopaminergic projection from the **ventral tegmental area** (VTA) to the **nucleus accumbens (NAc)**(Bromberg-Martin, Matsumoto, and Hikosaka 2010). Expression of *c-fos* is induced in the NAc following sexual behavior, exposure to estrous pheromones, and exposure to conditioned odors paired to copulation in experienced males (Robertson et al. 1991; Kippin, Cain, and Pfaus 2003). Likewise, microdialysis studies have shown that

dopamine release increases in the NAc when sexually experienced males are exposed to odors of receptive females or to an inaccessible receptive female, increases further during copulation, and falls during the post-ejaculatory interval and when the female is removed (Damsma et al. 1992; Mas et al. 1990; Wang et al. 1995; Lorrain et al. 1999; Pleim et al. 1990; Fiorino, Coury, and Phillips 1997). Even sexually naïve animals show a dopamine increase when exposed to chemosensory cues of a receptive female (as well as during copulation) (Louilot et al. 1991; Wenkstern, Pfaus, and Fibiger 1993), indicating that these processes are unconditioned. These findings correspond to the unconditioned preference of naïve males for receptive females and odors of receptive females (Portillo and Paredes 2004). The anticipatory dopamine increases do not occur in non-copulating males (Wang et al. 1995; Pleim et al. 1990), or when sexually active experienced or non-experienced males interact with non-receptive females or another male (Mas et al. 1990; Wenkstern, Pfaus, and Fibiger 1993). A study employing fast scan cyclic voltammetry, which has much better time resolution than microdialysis, showed that dopamine transients during a copulation session are most frequent preceding orientation towards and sniffing of a receptive female during the initial introductory period (Robinson, Heien, and Wightman 2002). These findings imply that dopamine release in the NAc signals sexual reward, and might be involved in the initiation of sexual behavior. Yet, neither radiofrequency lesion of the NAc, nor dopamine depletion by dopaminergic neurotoxin infusion into the NAc resulted in copulatory deficits in sexually experienced males (Liu, Sachs, and Salamone 1998), and dopamine receptor antagonists and agonists do not affect measures of sexual motivation or copulatory performance (Pfaus and Phillips 1991; Moses et al. 1995). However, excitotoxic lesion of the NAc in sexually naïve animals severely impaired intromission and ejaculation and blocked the increase in mounting that occurs over sessions when males gain experience (Kippin et al. 2004). Thus, it seems that the NAc and dopamine release into the NAc is of little relevance to the control of copulation and sexual motivation in experienced males, but these processes may be of importance for the facilitation of copulatory performance after initial experience. We hypothesized that one mechanism through which these processes occur may be hormone-induced spinogenesis, which we investigated in the NAc in *paper IV*.

Since the major dopaminergic input to the NAc is the VTA, this region is of interest as well. Dopaminergic as well as non-dopaminergic neurons of the VTA show increased *c-fos* expression upon first copulatory experience, as well as upon exposure without copulation to an environment in which copulation had previously taken place (Balfour, Yu, and Coolen 2004). A proposed model for the role of the VTA is that endogenous opioids are released upon copulation and copulation-cues in the VTA and inhibit GABAergic interneurons through the mu-opioid receptor, which in turn leads to disinhibition of the dopaminergic projection neurons to the NAc, and thus of increased dopamine release in the NAc. This process induces plasticity of the VTA dopaminergic neurons, which in turn facilitates long-term experience-induced reinforcement of sexual behavior, indicated by shorter latencies to initiate copulation as well as shorter ejaculation latency which can be blocked by a mu-opioid receptor antagonist infused into the VTA during initial gain of sexual experience (Pitchers et al. 2014). The increased *c-fos* expression in the NAc upon anticipation of copulation as well as copulation itself further supports this model (Balfour, Yu, and Coolen 2004). More recently, it was shown that chemogenetic inhibition of dopaminergic VTA neurons did not affect copulatory parameters over short-term multiple sessions in which sexually naïve animals gained experience, nor anogenital investigation, nor copulation-induced *c-fos* expression in the NAc (Beloate et al. 2016). This manipulation did, however, block cross-sensitization to amphetamine, indicating that activation of the VTA during gain of sexual experience is important for long-term reward learning through induction of plasticity.

In summary, the reward system primarily responds to reward-predicting environmental cues and reward experience, signals reward anticipation and mediates reward learning, but does not seem to have a clear role in the orchestration of sexual motivation or copulation per se. Rather, reward learning and reward signaling may serve to facilitate attention shift towards a stimulus (receptive female) in a complex environment, which is not necessary for the eventual initiation of copulation but merely accelerates it.

1.4.5 Motor output centers

The neurobiological mechanisms leading to sexual motivation eventually culminate in the initiation of motor patterns necessary for copulation. Two important brain regions that have a role in this motor output are highlighted in this section. The **periaqueductal gray** (PAG) has long been known to be involved in the regulation of female sexual behavior, especially in the output of the lordosis reflex (Veening, Coolen, and Gerrits 2014). Research in male rats is sparse, but lesions to different PAG subregions result in a facilitation of ejaculation, and sometimes a reduction of the post-ejaculatory interval duration (Heimer and Larsson 1964; Hansen, Köhler, and Ross 1982; Brackett, Iuvone, and Edwards 1986). Specifically, serotonergic cell bodies in the PAG have been implicated to have an inhibitory effect on ejaculation (Normandin and Murphy 2011b). The PAG has extensive reciprocal connections with the mPOA, and mPOA efferents to the PAG may be an important mediator for penile reflex responses (Marson 2004).

The **nucleus paragigantocellularis** (nPGi) is implicated as the major source of tonic inhibition of spinal sexual reflexes, i.e. erection and ejaculation responses, as measured *ex copula* (Marson and McKenna 1990; Marson, List, and McKenna 1992). Lesion studies have resulted in increased ejaculation frequency, decreased ejaculation latency, and less intromissions preceding ejaculation (Yells, Hendricks, and Prendergast 1992; Normandin and Murphy 2011a; Liu and Sachs 1999). It is notable that none of these studies found an effect on intromission ratio, a putative measure of erection during copulation. It might be the case that the nPGi does not inhibit the *likelihood* of erection or ejaculation occurring in the context of copulation, but rather influences the tumescence of occurring erections. If lesions of the nPGi lead to greater tumescence, this might well result in stronger penile sensory feedback – explaining the reduced number of intromissions preceding ejaculation in lesioned animals.

1.5 Convergence of hormones and neural circuitry

The most important mechanism through which gonadal hormones facilitate sexual behavior is through their actions in the central nervous system. Therefore, this section discusses how gonadal hormones modulate the neuronal circuitry involved in sexual behavior and hence aims to integrate hormonal and neuronal control of male rat sexual behavior.

1.5.1 Target brain regions for gonadal hormones

The brain regions described above are all responsive to gonadal hormones, as evidenced by expression of hormonal receptors. The expression of androgen and estrogen receptors is widespread in the brain, but highest in the mPOA, MeA, and BNST, high in other hypothalamic nuclei and the PAG, and somewhat lower but present in the main and accessory olfactory bulbs. The nPGi, CTF and VTA only seem to express androgen receptors (Simerly et al. 1990; Gréco et al. 1998). Androgen receptor expression may be present but very low in the NAc (Tobiansky et al. 2018). The studies in the past have focused on the expression of nuclear receptors, whereas it is now clear that gonadal hormones can exert actions through membrane-bound receptors as well (Meitzen, Meisel, and Mermelstein 2018).

The effect of gonadal hormones on neuronal activity in the context of sexual behavior is evidenced by their requirement for *c-fos* induction that follows exposure to soiled bedding from receptive females (chemosensory cues) in the accessory olfactory bulb, the MeA, the BNST and the POA (Paredes, Lopez, and Baum 1998). Indeed, neurons in these regions, in addition to neurons in the CTF and PAG, that express *c-fos* upon copulation, almost all co-express the androgen receptor (Gréco et al. 1996), and a large amount also express the estrogen receptor (Gréco et al. 1998). The far majority of cells expressing the estrogen receptor in these brain regions co-express the androgen receptor (Gréco et al. 1998), indicating that estrogens mostly act on neurons that are also responsive to androgens, whereas not all androgen-responsive neurons are responsive to estrogens. Furthermore, neurons expressing gonadal hormone receptors in brain regions involved in the regulation of sexual

behavior are interconnected with each other (Gréco et al. 1996; Gréco et al. 1999). These findings suggests that gonadal hormones may function as a "gatekeeper" of neuronal activity, ultimately leading to behavioral output in response to sexual stimuli.

1.5.2 Local effects of gonadal hormones on sexual behavior

Intracerebral implants of gonadal hormones can induce, albeit not completely restore, sexual behavior in gonadectomized males. The most effective brain region for hormonal implants is, not surprisingly, the mPOA. Testosterone implants in the mPOA gradually induce mounts and intromissions in almost all gonadectomized males, and ejaculation in 30-80% of the males (Antonio-Cabrera and Paredes 2014; Christensen and Clemens 1974; Davidson 1966a; Johnston and Davidson 1972; Kierniesky and Gerall 1973). Testosterone in the MeA and VMN also induces mainly mounting and intromission in gonadectomized males, but these effects are smaller and more inconsistent than when testosterone implants are placed in the mPOA (Antonio-Cabrera and Paredes 2014). In non-copulating intact males, testosterone in the mPOA gradually but fully restores complete copulation (Antonio-Cabrera and Paredes 2014). These non-copulators do not have lower hormone serum levels, but may have impaired hormone signaling in the mPOA as suggested by increased expression of aromatase and androgen receptor as compared to normal copulators (Antaramian et al. 2015).

Estradiol implants in the mPOA are also effective in inducing copulation in gonadectomized males and intact non-copulating males (Antonio-Cabrera and Paredes 2014; Christensen and Clemens 1974), but most studies show that estradiol induces mostly mounting and intromissions and ejaculation less so (Antonio-Cabrera and Paredes 2014; Davis and Barfield 1979). Systemic administration of dihydrotestosterone in addition to estradiol in the mPOA resulted in a large increase of animals ejaculating (Davis and Barfield 1979). Likewise, estradiol in the mPOA prevents some of the loss of copulatory behavior as a result of systemic treatment with an aromatase inhibitor (Clancy, Zumpe, and Michael 2000), and administration of an aromatase inhibitor into the mPOA impaired the initiation of copulation and ejaculation (Clancy, Zumpe, and Michael 1995). Estradiol in the MeA and

VMN facilitates some inconsistent mounting and intromission, but not ejaculation, in gonadectomized males (Antonio-Cabrera and Paredes 2014), and, implanted in the MeA, can delay the loss of mounting and intromission when intact males are treated with an aromatase inhibitor systemically (Huddleston et al. 2003).

In agreement with studies utilizing systemic administration, dihydrotestosterone in the mPOA is ineffective in inducing ejaculation in gonadectomized males (Johnston and Davidson 1972). Dihydrotestosterone in the MeA can facilitate copulation in gonadectomized males, provided they are treated with low-dose systemic estradiol (Baum et al. 1982). While dihydrotestosterone alone seems to be relatively ineffective, there are still indications that androgen receptor signaling is of importance in especially the mPOA and VMN. Implants with an androgen receptor blocker in the mPOA attenuate testosterone-induced reinstatement of copulation and preference for a receptive female after gonadectomy (McGinnis, Williams, and Lumia 1996; McGinnis, Montana, and Lumia 2002), whereas VMN implants almost fully block copulation, and MeA implants only slightly affect copulation (McGinnis, Williams, and Lumia 1996). One study also found reduced preference for a receptive female upon VMN androgen receptor bloackage (Harding and McGinnis 2004). However, none of these studies found a complete blockage of preference.

Together, these findings show that the mPOA and VMN are the most important brain sites for gonadal hormone mediated control of sexual behavior in male rats. Even though hormonal manipulations of one brain region may affect sexual behavior, it has never been found to be enough to fully restore it, indicating that hormonal signaling in multiple brain regions or perhaps the periphery is necessary for the full range of sexual behavior. While estradiol is quite effective locally, androgen signaling seems to be necessary for ejaculation. Once again, both estrogen and androgen signaling appears to be necessary in specific brain regions for the full range of sexual behavior. Since most estrogen receptor expressing neurons also express the androgen receptor, the necessity of both estrogen and androgen signaling might already converge on the level of individual neurons in the circuitry.

1.5.3 Hormone-induced neuronal plasticity

It is clear that the central effects of gonadal hormones are most important for the display of sexual behavior. However, the mechanisms of these hormones modulating the neural circuit has not been discussed in this introduction yet. Just like with systemic treatments, the effects of local hormone signaling manipulations typically appear gradually, with effects on ejaculation appearing last. This suggests genomic mechanisms as the main facilitator of hormonal influence on neuronal circuitry, as gene transcription and protein synthesis take time. One study illustrated this by showing that infusion of a protein synthesis inhibitor into the mPOA prevents testosterone-induced reinstatement of copulation after longer term castration, and impairs copulation maintenance when males are immediately treated with testosterone upon castration (McGinnis and Kahn 1997). This demonstrates that protein synthesis is indeed a necessary mediator for hormonal facilitation of sexual behavior. Central protein synthesis under the control of gonadal hormones strongly implicates neuronal plasticity as a key mechanism of neuronal circuit priming for sexual behavior. The fact that electrical stimulation of the mPOA greatly facilitated ejaculation in an intact male, but the same stimulation could only induce mounting in the same male after it had been gonadectomized provides support for this hypothesis (Van Dis and Larsson 1971). Apparently, gonadal hormones act as “gatekeepers” of the functionality of the neural circuitry that underlies the control of sexual behavior.

The importance of this hormone-induced plasticity for the display of sexual behavior has been demonstrated in females (reviewed in (Micevych and Meisel 2017)). Briefly, the rise of estradiol during the estrous cycle induces expression of progesterone receptors in the hypothalamus and initiates a cascade involving multiple neuropeptides and transmitters which leads to inhibition of the mPOA and subsequently the VMN, which ultimately inhibits lordosis. Coincidentally, spine density increases in the arcuate nucleus and the VMN, where dendritic branching is induced as well. This all “primes” the neural circuit for progesterone to be able to activate the stimulatory circuitry that leads to VMN orchestrated lordosis during proestrous, when the inhibition of the mPOA is released due to the fall of estradiol and the rise of progesterone. These processes occur with each estrous cycle, i.e. over the

course of 4-5 days, and rely on hormonal signaling through membrane-bound estrogen receptors. In contrast to genomic effects that arise through nuclear receptors, downstream effects of membrane-bound receptors are very fast, as is illustrated by the neuroplasticity that tracks the estrous cycle and results in rapid and dramatic behavioral effects. Obviously, male rats do not have a hormone cycle, but gonadal hormones have been shown to also impact neuronal plasticity such as spine density in the male brain (Gross et al. 2018). These mechanisms could possibly provide an answer to how gonadal hormones maintain sexual behavior in males. The research presented in *paper IV* further investigates hormone-induced neuroplasticity in brain regions involved in the regulation of sexual behavior in male rats.

1.6 Aims of the thesis

Much work has been done leading to a better understanding of the neurobiological mechanisms underlying male rat sexual behavior. However, sexual motivation was often not studied separately from copulation, and assessment of copulatory behavior has frequently been oversimplified. In addition, the mechanisms of how gonadal hormones regulate sexual behavior in males are still grossly unknown. In light of these considerations, the present thesis aims to formulate more extensive and naturally valid assessments of the structural organization and patterns of male rat sexual behavior, to apply these analyses to unravel the role of the MeA in the control of male rat sexual behavior in more detail, and to unveil the role of gonadal hormones in the regulation of plasticity of the neural circuitry involved in male rat sexual behavior.

One literature review and three experiments were carried out in order to address the aims of this thesis. First, the literature was reviewed to identify valid assessment of copulation and sexual motivation in both male and female rats. Second, behavioral structure and patterns of copulation in male rats were assessed in more detail based on the recommendations that were put forward in the review. Third, these analyses were applied to study the role of the MeA, utilizing chemogenetics to

silence and stimulate the MeA during tests of sexual motivation and copulation in male rats. Finally, DiOlistic labeling was employed to assess the effects of gonadectomy and androgen signaling on spine plasticity in brain regions that are involved in the regulation of sexual behavior in male rats.

The results of these studies will hopefully stimulate the field to employ more extensive behavioral assessments when studying sexual behavior, advance our knowledge of the role of the MeA in sexual behavior, and lay the groundwork for understanding how gonadal hormones influence neuronal plasticity in the neural circuitry of sexual behavior.

Chapter 2

General methods

2.1 Behavioral assessment

Copulation in males has traditionally been tested in a copulation box; a rectangular box in which the male can pace the copulation (**Figure 1**). In the past decades, behavioral assessment has often been limited to the point annotation of copulatory events only (mount, intromission, ejaculation). In the research presented in this thesis, I have employed a more extensive ethogram which entailed annotation of 100% of the time elapsed. This has provided more insight in the natural organization of male rat sexual behavior. A discussion of the copulation test and its outcome measures is found in *paper I*, whereas the more extensive method of assessment is explained in *paper II*.

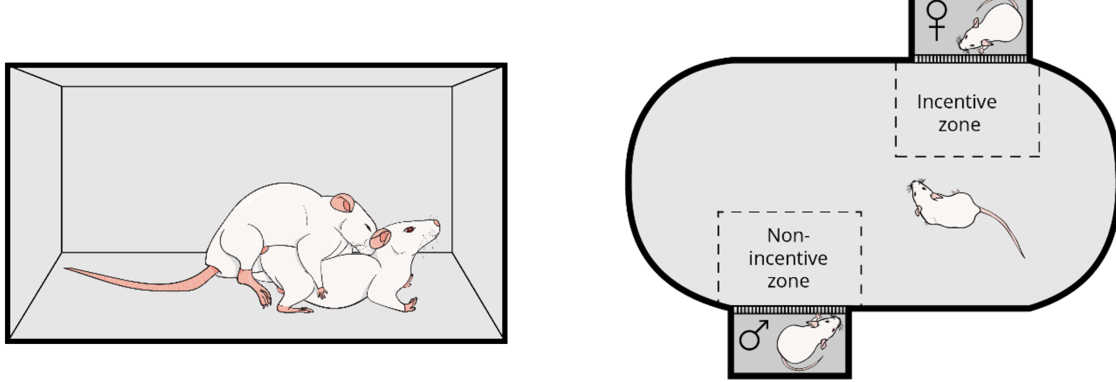

Figure 1 Copulation test (left) and sexual incentive motivation test (right)

The sexual incentive motivation (SIM) test has been characterized and used by my colleagues before (Ågmo 2003; Ågmo and Snoeren 2017). Briefly, the SIM test consists of an open field arena with two small chambers attached on each far end (**Figure 1**). These chambers house a sexual incentive (receptive female) or a social stimulus (male, or non-receptive female), and are separated from the arena by wire mesh. The male subject is placed in the arena and allowed free exploration for 10 minutes, while it can see, smell, and hear the other animals without direct contact being possible. The absolute time spent in the vicinity of each of the stimulus animals, i.e. the total duration the subject male was in the incentive zone (see **Figure** 1) and the total duration the male was in the non-incentive zone is measured. In addition, a preference score is calculated which is the proportion of time the

subject male spends in the sexual incentive zone out of all time spent in either of the zones. An intact male typically has a preference score of around 0.7 (Ågmo 2003). Number of visits to each zone and duration of visits can also be measured. The SIM test is unconditioned, not dependent on sexual experience or memory, and its outcome measure is not determined by speed of movement. In addition, locomotor function can concurrently be determined from the distance traveled and velocity of the subject male. The SIM test, its advantages and disadvantages, and how it relates to other tests of sexual motivation, are further discussed in *paper I*.

2.2 Chemogenetics

Over the recent years, the toolbox for behavioral neuroscience has been greatly expanded. Newly developed techniques have made transient inhibition and stimulation of neuronal populations possible without extensive destruction of tissue necessary. One of these techniques is chemogenetics. Chemogenetics involves a chemical control of cellular mechanisms by an engineered receptor/ligand system (Armbruster et al. 2007; Atasoy and Sternson 2017; Roth 2016). The experimental data presented in *paper III* results from the application of such a chemogenetic system, namely Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), in the MeA. This section explains the mechanisms of chemogenetics and some of its advantages and caveats.

DREADDs are G-protein coupled receptors (GPCRs) that have been engineered through modification of the human muscarinic acetylcholine receptors (hM-receptors). Through random mutagenesis, the ligand binding site of these receptors was altered to selectively bind the ligand clozapine-N-oxide (CNO). The DREADD subtypes hM3Dq and hM4Di have been most widely used in neuroscience. Binding of CNO to hM3Dq has similar downstream effects as the activation of the hM3 receptor upon binding of acetylcholine. As hM3 is a GPCR that interacts with the $G\alpha_q$-protein (hence, the *Dq*-suffix for the DREADD name), ultimate downstream effects of hM3Dq-activation through CNO binding consist of the inhibition of the M-channel, which prevents K^+ efflux and consequently increases

neuronal excitability, as well as the release of Ca^{2+} from internal stores, which can activate the Na^{+}/Ca^{2+}-exchanger and consequently lead to a depolarizing inward current. Similarly, because hM4Di interacts with $G\alpha_i$, ultimate downstream effects of CNO binding consist of induction of hyperpolarization through activation of G-protein inwardly rectifying potassium channels (GIRKs) and consequent K^{+} efflux, as well as a decrease of Ca^{2+} influx through inhibition of voltage-gated Ca^{2+} channels (VGCCs) and subsequent inhibition of presynaptic release of neurotransmitters. Thus, hM3Dq stimulates neuronal firing and hM4Di inhibits neuronal firing, upon CNO binding to these receptors. The DREADDs have low constitutive activity and thus have no behavioral effects without the ligand present.

CNO is a metabolite of the antipsychotic drug clozapine and was chosen as a suitable ligand because of its inertness at endogenous receptors. Recently, doubts have been cast on whether CNO is truly inert, as some behavioral effects have been found in rats treated with CNO that lacked expression of DREADDs (MacLaren et al. 2016). Possibly, these effects, as well as the activation of DREADDs, are mediated by back-metabolizing of CNO into clozapine (Manvich et al. 2018; Gomez et al. 2017). Still, the question is whether compounds that are truly specific without any off-target effects ever exist. As CNO and clozapine have such high affinity for the DREADDs, effective doses are so low that off-target effects are not an issue. Most importantly, a design in which proper controls for the effects of both the DREADD without the ligand as well as the ligand without the DREADD are implemented should always be used, and has been used in the study described in *paper III*.

Delivery of DREADDs to neuronal populations typically is done by means of viral vectors. Several different viruses have been used for this purpose, such as adeno, lenti, and rabies virus strains. Based on their favorable characteristics, recombinant adeno-associated virus (AAV-) vectors are the most broadly used group of viral vectors in neuroscience. Several AAV serotypes exist, which all have specific properties determining their suitability and efficacy regarding infection and DREADD expression of certain neuronal cell types and in specific brain regions (Davidson and Breakefield 2003; Haggerty et al. 2020). For example, AAV5 preferentially infects neurons, and has been shown to induce

high transgene expression in subcortical brain regions (Haggerty et al. 2020). The viral vector carries genetic information necessary for the expression of the DREADD. This expression occurs under a promotor that can be chosen to tailor expression to certain cell types. However, the maximum amount of basepairs a vector can carry, and the relative inactivity of promotors that are very specific have made this approach rather difficult. It has been possible, though, to restrict DREADD expression to neurons with the hsyn-promotor and to glutamatergic neurons in certain brain regions with the CaMKII-promotor. In *paper III*, an AAV5-CaMKII-DREADD viral construct was utilized.

The biggest advantage of using chemogenetics is that activation and inhibition of neurons is transient, and only comes at the cost of very minor brain damage from the small cannula or needle through which the viral construct is delivered (compare to large and non-reversible damage of lesions, and cannula implantations for pharmacological inactivation). In addition, because neurons that express DREADDs can later be identified in the tissue by visualization of their (fluorescent) tag, a very exact target region can be determined (which is more difficult with local infusion of pharmacologically active compounds). Compared to optogenetics, no fibers and lasers are necessary, and whereas optogenetic manipulations lead to strong depolarizations/hyperpolarization, effects of DREADDs are more modest and follow a more "natural" mechanism of inhibition of synaptic neurotransmitter release or increase of excitability of the neuron. Because of these considerations, DREADDs were utilized to study the role of the MeA in sexual behavior in more detail.

2.3 DiOlistic labeling

In *paper IV*, the effect of hormone status on neuroplasticity is analyzed by means of assessment of spine density. Dendritic spines are the thorn-like protrusions on dendrites, primarily found directly opposed to presynaptic boutons (Mancuso et al. 2013). Effects on spine density can be associated with effects on synaptic strength (e.g. more or less connecting spines per presynaptic bouton) and connectivity (e.g. more or less connections with different presynaptic boutons). Spine plasticity can

thus be a functional adaptation of the neural circuitry, and spine density analysis provides a basis for inferences about the circuitry.

The most well-known and broadly applied technique to assess spine density is the Golgi stain and associated adapted protocols such as rapid Golgi staining and the Golgi-Cox method. This technique made it possible to visualize whole neurons with low overlap and low background staining. However, since image acquisition and tracing of dendritic segments occurs by means of widefield microscopy, a limitation is the lack of resolution, especially in the y-axis. Moreover, it is to date unknown why Golgi staining only labels a small percentage of neurons, which introduces an uncertainty as to whether the labeling is random or based on a selection bias. Hence, techniques have been developed that allow for labeling of isolated neurons with fluorescent dyes, making imaging at high resolution possible with laser scanning confocal microscopy. One such technique makes use of the lipophilic dyes DiO, DiI and DiD and is named DiOlistic labeling, after the dyes (Gan et al. 2000). Delivered into a neuron, these dyes can uniformly label the whole neuron through incorporation into membranes. A protocol has been established to deliver the dye into only a small percentage of neurons, resulting in the labeling of relatively isolated fully filled neurons in brain sections, with less uncertainty about selection bias. The technical details of the method are described in *paper IV* and here: (Staffend and Meisel 2011a, 2011b) , but the background rationale for the method is briefly introduced in this section.

As applied in *paper IV*, DiOlistic labeling involves the ballistic delivery of tungsten microparticles coated with DiI to brain tissue sections. Tefzel tubing is coated with the tungsten-DiI microparticles and cut into small pieces (“bullets”) to fit a Helios Gene Gun cartridge. Brains are only lightly fixed to allow for dye transportation within the neurons, and thick sections (300 µm) are cut on a vibratome so that sections do not get destroyed from the gene gun pressure and more whole neurons appear in the tissue. The tungsten-DiI is then shot into the tissue from the bullets to the sections with the gene gun by gas expulsion. Tungsten-DiI particles that hit a neuron soma will spread and label the whole neuron during overnight incubation. Under- and over-labeling can sometimes occur, but can also be contained through picking of the best bullets by an experienced experimenter. Finally, multiple

neurons and dendritic segments are confocally imaged per brain region and animal according to established requirements (e.g. distance from soma, branching- and end points), and reconstructed and analyzed by means of Imaris software.

Multiple non-overlapping dendritic segments can be imaged on a DiI-filled neuron, so that an average spine density per neuron can be calculated. In brain regions that contain many neurons with small soma and thin dendrites (e.g., the mPOA), DiOlistic labeling can be more challenging because the chances are lower to hit a cell body with a microparticle. This also illustrates the limitation of the technique; it is theoretically more likely to label neurons with large soma, which could lead to a certain selection bias in highly heterogenous brain regions. Still, the ease of application, high resolution images, and high-throughput analysis, make DiOlistic labeling a very suitable technique that can be applied to a variety of different tissue or cell cultures.

Chapter 3

Summary of results

Paper I

Assessment of sexual behavior in rats: the potentials and pitfalls

This review article discusses the behavioral tests available to assess copulation and sexual motivation in male and female rats. Considerations for the choice of test, and interpretation of results are discussed, and recommendations on how to optimize this field of study are made. A few important notions are highlighted here.

First, it is vitally important to use the appropriate behavioral test when studying copulation. Since copulation is only rewarding to the rat that paces the interaction, males should be studied in the traditional copulation box set-up, whereas females should be tested in a female-paced mating set-up in which she can escape the male if she chooses to do so. In all cases, it is essential to be critical of the interpretation of results. A few examples are given in which a parameter was interpreted one way in the past, but new knowledge has changed the perspective of interpretation. We also stress that it is important to not take shortcuts and measure all aspects of sexual behavior in order to draw more informed conclusions. We propose that the measured parameters should always be described in the most complete and neutral sense possible, providing better basis for future insights of interpretation of results as well as comparison with results of other studies.

In the case of testing sexual motivation, it is important to distinguish between tests that measure sexual incentive motivation and tests that are actually measuring reward anticipation through a conditioned response. Both these approaches are valuable, but we emphasize that it is important to understand the subtle differences of what these tests are measuring. Some examples are given to show that effects of certain manipulations yield different results in these tests.

Paper II

Male rat sexual behavior: insights from inter-copulatory intervals

With this behavioral study, we analyzed aspects of the organization of male rat copulation based on the natural structure of copulation; mount bouts and inter-copulatory intervals. Mount bouts consist of one or more copulatory events (mount, intromission, ejaculation) that are uninterrupted by behaviors that are not copulation-oriented. Mount bouts are interspersed by time outs, during which the male does not show copulation-oriented behavior. Both time outs and post-ejaculatory intervals are inter-copulatory intervals that follow certain sexual stimulation. We therefore hypothesized that time out and post-ejaculatory interval could be regulated by a similar central mechanism responsible for copulatory inhibition. By studying copulatory behavior in detail, we aimed to determine whether time out duration correlates with post-ejaculatory interval duration, and if any characteristic of a mount bout predicts the duration of the following time out.

The results of this study show that mean time out duration strongly correlates with post-ejaculatory interval duration in individual male rats of different strains, age, background, and laboratory location. Both time out and post-ejaculatory interval increase in the second ejaculation series compared to the first ejaculation series. In addition, even though both these parameters vary over copulation sessions, the correlation is present in each session. When analyzing mount bout characteristics in order to identify predictors for time out duration, this study demonstrates that time out duration is not correlated with the duration of the preceding mount bout, nor is it dependent on the relative time point within the ejaculation series. Instead, time out duration was longer after mount bouts that ended in an intromission. Moreover, this is a secondary effect of the finding that time out duration is at least partially predicted by the sum of sensory stimulation in the preceding mount bout, with more penile stimulation associated with longer time out. Specifically, mount bouts that contain at least 1 intromission are followed by a longer time out than mount bouts that consist of only mounts.

These findings suggest that both time out and post-ejaculatory interval duration may be determined by the magnitude of sensory stimulation, which inhibits copulation. It is therefore hypothesized that the central orchestration of inter-copulatory intervals may share a common mechanism.

Paper III

Silencing and stimulating the medial amygdala impairs ejaculation but not sexual incentive motivation in male rats

As discussed in the general introduction, the MeA is a central hub that is in the position to relay chemosensory information to the mPOA, the major integrative brain region for sexual behavior. This positions the MeA as a candidate for the regulation of sexual motivation. However, the MeA has not been extensively studied in sexual motivation in male rats. The MeA has been repeatedly shown to be involved in the facilitation of ejaculation, but it is unclear through what underlying mechanism. Therefore, we studied the role of the MeA in sexual motivation, as well as in copulation with additional mount bout analysis, by means of chemogenetic stimulation and silencing.

We found no effects of either of the chemogenetic MeA manipulations on sexual incentive motivation. Thus, it seems the MeA is not involved in the regulation of sexual incentive motivation in male rats. In line with earlier reports in which the MeA was lesioned, we found that silencing of the MeA impaired ejaculation. Surprisingly, we found the same effect when stimulating the MeA. Both silencing and stimulating the MeA increased the latency to ejaculation and increased the number of mounts and intromissions preceding ejaculation. Moreover, there was no effect on the duration of time outs and the post-ejaculatory interval, indicating that copulatory pace was unaffected. Therefore, we concluded that the MeA appears to have a role in the processing of sensory feedback from the penis, contributing to the reach of ejaculation threshold. The convergence of the behavioral effects of stimulating as well as inhibiting the MeA may reflect opposing behavioral control of specific neuronal populations within the MeA, or differential effects depending on stimulation protocol.

Paper IV

Effects of gonadectomy and dihydrotestosterone on neuronal plasticity in motivation and reward related brain regions

As mentioned in the general introduction, gonadal hormones can be seen as “gatekeepers” of the functionality of the neural circuitry that underlies the control of sexual behavior. Hormone-induced neuronal plasticity that tracks the estrous cycle has been shown to be vital for sexual receptivity in female rats. Hormone-induced neuronal plasticity in brain regions involved in the regulation of sexual behavior in males has not been extensively studied. In this study, we assessed the effect of gonadectomy and subsequent dihydrotestosterone treatment on spine density and morphology in such brain regions in male rats.

We found that gonadectomy decreased spine density in the mPOA, but not in the MeA, NAc, and caudate putamen. Treatment of gonadectomized males with dihydrotestosterone rescued the loss of spines in the mPOA. Dihydrotestosterone-treated gonadectomized males also had higher spine density than intact animals. In the NAc shell, dihydrotestosterone decreased spine density in gonadectomized males. We found no effects on spine length and spine head diameter in any of the studied brain regions. Overall, gonadectomy and dihydrotestosterone differentially affect spine plasticity in several brain regions that are implicated in the regulation of male sexual behavior.

Because hormone-induced plasticity in females has been shown to be dependent on membrane-bound estrogen receptor signaling, and there are indications a homologous mechanism for membrane-bound androgen receptors may exist in males, we proceeded to study rapid effects of dihydrotestosterone treatment in the NAc. We found that dihydrotestosterone treatment of gonadectomized males rapidly increased the number of cell bodies in the NAc shell that were positive for phosphorylated cAMP response-element binding protein (pCREB), a downstream messenger of the androgen receptor. Thus, androgen signaling plays a role in the regulation of spine plasticity in males

within neurocircuits involved in sexual behavior and motivation, and this may be mediated by rapid signaling through membrane-bound androgen receptors.

Chapter 4

General discussion

4.1 Aims and outcomes of the thesis

This thesis aimed to formulate more extensive and naturally valid assessments of the structural organization and patterns of male rat sexual behavior, to apply these analyses to unravel the role of the MeA in the control of male rat sexual behavior in more detail, and to unveil the role of gonadal hormones in the regulation of plasticity of the neural circuitry involved in male rat sexual behavior. I presented four research papers in this thesis that together have addressed these aims. In this discussion, I will discuss the implications of the key research findings from these papers, how they relate to current literature and behavioral concepts, and what recommendations can be made for future directions of study that build on these findings.

4.2 Towards a more detailed understanding of the organization of male rat sexual behavior

The current standards and directions of behavioral paradigms for the study of sexual motivation and copulation in rats were reviewed in *paper I*. A key notion on the study of sexual motivation is the need to distinguish between measures of sexual incentive motivation, which entails a receptive female being an unconditional incentive independent of sexual experience, and measures of reward anticipation that are dependent on conditioning and sexual experience. Whereas the first is the result of an innate attraction to certain stimulative properties of a receptive female, such as chemosensory cues, the second is the consequence of associating a certain environment with a rewarding event (reward learning), in this case copulation until ejaculation. Possibly, these concepts tune into the theory of liking versus wanting (Berridge and Robinson 2016; Ventura-Aquino et al. 2017). Whereas the unconditioned paradigms could be seen as a measure for how much the male *likes* and perhaps also *wants* to be in the vicinity of a receptive female, the conditioned paradigms lean more towards measuring how much the male *wants* to engage in a previously experienced reward (copulation) and how much effort he is willing to put into receiving that reward. These measures are

of course not completely disentangled, but are regulated differently on the neurobiological level (Berridge and Robinson 2016), as is also explained by example in *paper I*. It would be highly interesting to study the effects of certain manipulations on the measures of several tests of sexual motivation as well as reward anticipation and see how the outcomes relate to each other. This would help advance our understanding of how each of these processes are regulated and possibly related.

As discussed in the method section and in *paper I*, the SIM test has many advantages, but its limitations should also be addressed. One limitation of the SIM test is that it is not possible to distinguish whether a male does not like to be in the vicinity of a sexual incentive more than a social incentive, or whether the manipulation has resulted in a perceptual deficit. If the male has been rendered incapable of recognition of a sexual incentive, a receptive female has become nothing more than a social stimulus. A discussion can be had on whether a perceptual deficit also pertains to disrupted sexual incentive motivation. Without correct identification of an external stimulus, there is no reciprocal interaction with the central motive state (see (Ågmo 1999) for background theory), and hence no perceived incentive to activate approach behavior. These things should be carefully considered when interpreting results of the SIM test.

Regarding copulation, based on some of the discussion on outcome measures of the copulation test in *paper I*, it emerged that a more detailed analysis of copulatory organization is warranted. It became clear that the natural temporal patterning of copulation had over time been lost in shortcuts of simplified analysis over the past decades. We hypothesized that the post-ejaculatory interval and time out may both be the result of a copulatory inhibition mechanism that determines copulatory pace. Exploration of such hypotheses should start at the behavioral level. Therefore, we assessed whether time out duration correlates with post-ejaculatory interval duration. This was indeed the case. In addition, time out duration was at least partially predicted by the total sensory stimulation in the preceding mount bout, with mount bouts that had intromissions inducing longer time outs than mount bouts of only mounts. These findings are important as they increase our understanding of the structure and organization of male sexual behavior, which should be at the basis of studies that test

brain-behavior causality by means of interventions, like in our study investigating the role of the MeA in sexual behavior as described in *paper III*.

Recently, the reductionist approach has been emerging in a growing body of neuroscience research, marginalizing the importance of proper behavioral understanding by employing simplified behavior assessment as an afterthought that is merely slapped on to a bonanza of newly developed neuroscience techniques (also reviewed in (Krakauer et al. 2017)). I want to advocate here that neuroscience really does need more behavior. A great analogy example of this notion is a study that asked whether a neuroscientist could understand a microprocessor (Jonas and Kording 2017). Seeing the microprocessor as a far simplified version of the brain, simulating common neuroscience techniques such as anatomical connectivity assessment, lesioning, and measuring of “neural” activity, did not lead to understanding how the microprocessor orchestrated its “behaviors”, i.e. three different videogames. As the authors state: “many approaches in neuroscience, when used naïvely, fall short of producing a meaningful understanding”. Perhaps playing the videogames, i.e. studying the output of the microprocessor, may have aided in a more guided approach of trying to understand the way the microprocessor worked. Likewise, more detailed understanding of behavior yields more complete insights on which hypothesis-driven brain intervention studies can be based. Importantly, the behavioral work needs to be as refined as the intervention itself.

That said, I propose that a more detailed behavioral understanding is of utmost importance for the generation of hypotheses about neurobiological mechanisms underlying the orchestration of behavior. Our studies of the relation between inter-copulatory intervals and the role of the MeA in sexual behavior are mere examples of how more detailed understanding of structure of behavior can provide a sound basis for hypotheses about causal neurobiological processes. Furthermore, interpretation of outcome measures of behavioral testing paradigms greatly benefits from a more detailed analysis that follows the natural organization of the behavior.

4.3 Correlates of neuronal orchestration of male rat sexual behavior

By applying the more extensive behavioral analysis as discussed in the previous section, the study described in *paper III* provides an example of how utilizing a more extensive behavioral assessment can lead to better informed conclusions of interventionist studies such as ours targeting the MeA. The main finding in this study was that both silencing and stimulating the MeA impaired ejaculation by increasing ejaculation latency in male rats. As mentioned in the general introduction of this thesis, ejaculation latency is determined by the efficiency (intromission ratio; the proportion of mounts that end in intromission), sensitivity (the number of intromissions necessary to reach ejaculation threshold), and the copulatory pace (duration of inter-copulatory intervals). Because of our detailed mount bout-based analysis, we were able to conclude that the increased ejaculation latency was caused by a decrease in sensitivity, implicating the MeA in the processing of somatosensory feedback. I hypothesize that relay of this somatosensory information from the MeA to the mPOA is important, and this should be a topic of future research.

We had expected to find that the MeA is also important for the regulation of sexual incentive motivation, as earlier research has shown the MeA to be responding to chemosensory cues of receptive females, suggesting an integrative role and putatively important input to the mPOA for initiation of copulation. However, we found no indication that the MeA is necessary for sexual incentive motivation. Perhaps there is a role for the MeA in these processes, but other brain regions can compensate for the loss of MeA signaling.

In *paper IV*, we explored how hormone status affects structural plasticity of dendritic spines in several brain regions related to sexual behavior in the male rat. In line with expectations, we found that gonadectomy decreased spine density in the mPOA. I propose that this structural adaptation in the mPOA to loss of gonadal hormones may be one of the mechanisms at the basis of the hormone-induced facilitation of sexual behavior. As already touched upon in the general introduction, gonadal hormones may be seen as the gatekeepers of the functional neural circuitry that ultimately regulates sexual behavior. We showed that treatment with dihydrotestosterone recovered the spine density in

the mPOA to levels found in intact animals within 24 hours. This means that hormone-induced spinogenesis cannot be the complete answer to how gonadal hormones reinstate the full range of sexual behavior, as this process takes days to weeks (reviewed in the introduction). In addition, we have only looked at the effects of dihydrotestosterone, presumably only targeting signaling through the androgen receptor. Studying and comparing effects of estradiol, dihydrotestosterone, and testosterone would be very interesting and perhaps also lead to more insights into the exact roles and convergence of estrogen and androgen signaling in the regulation of male sexual behavior. Furthermore, the differential effects of gonadectomy and dihydrotestosterone treatment on the plasticity of the different brain regions may reflect the gatekeeping of hormones in relation to different aspects of sexual behavior. For example, the NAc is most important for aspects of reward learning, and spine plasticity in this region has also been linked to sexual experience (Pitchers et al. 2010; Staffend et al. 2014).

4.3 A note on studies of sexual behavior in other animal species

Over the years, socio-sexual behavior has been studied in a plethora of animal species. Historically, rats have been the dominating species in studies of sexual behavior, whereas mice seem to be studied more and more in the recent years. A clear reason for this shift in animal model is the much greater availability of transgenic mice that make targeting of very specific neuronal populations and projections possible. This has led to many recent discoveries about population dynamics, neural circuitry, and involvement of very specific neuronal cell types in sexual behavior in mice (see for example: (Karigo et al. 2021; Li et al. 2017; McHenry et al. 2012; Yang et al. 2013; Remedios et al. 2017; Hu et al. 2021)). A discussion of these findings is beyond the scope of this thesis. However, I do think that these developments have led to inevitably asking of the question: “Why don’t we all switch to mice models if they allow for more sophisticated targeting and techniques?” I want to highlight here

why it is important to continue studies of different species, how results can inform each other and ultimately create a better understanding of universal and generalizable concepts.

A longstanding principle in neuroethology is Krogh's principle: "For a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied" (Krogh 1929). Specifically, certain animals are specialized in certain traits, yielding an exaggeration of certain function or behavior, rendering them an excellent model to study the underlying physiology and neurobiology of that trait. This does not mean that results are always generalizable across species, as the same outcome behaviors can be differentially regulated centrally in different animals. Only focusing on one species could lead to tunnel vision while certain mechanisms might in fact be very species specific. A comparative approach in which neurobiology of behavior is studied in different animals can inform us of the neuromechanistic differences but also the communalities. This in turn could help to identify universal principles that could also provide better insights into the function of the human brain, an important goal of basic neuroscience.

Applying these concepts to basic research of sexual behavior may seem obvious, but I will give some examples of what experimental work in different species can add, hopefully illustrating why it is important to perpetuate a broader biological perspective. First of all, mice are not just small rats. For example, rats are generally considered to be more sociable than mice, as they have a higher baseline motivation for social interaction (Netser et al. 2020; Reppucci et al. 2020). This can already be a differentiating factor when studying sexual behavior. Yet, copulation in mice and rats is rather similar, with small differences in for example pattern of intromission. However, the mouse is much slower than the rat in terms of initiation of copulation and ejaculation latency, and most mouse strains only achieve a single ejaculation after which a refractory period is induced that can often last more than 24 hours (McGill 1962). In contrast, rats can achieve multiple ejaculations in a session and only have relatively short post-ejaculatory intervals in between these ejaculation series. Therefore, research on post-ejaculatory intervals and effects over ejaculation series are more suitable in rats. At the same time, comparison of neural correlates of refraction between mice and rats provides excellent

opportunity to try and understand why ejaculation in one species induces much longer refraction than in the other, ultimately helping to understand the overarching neurobiological mechanisms of copulatory inhibition.

A somewhat more obvious example of a specific choice for an animal model for the study of a certain aspect of sexual behavior is found in female hamsters. During copulatory interactions, female hamsters remain immobile in the lordosis posture for up to 10 minutes at a time, also when she is not mounted by the male. When it is necessary for research results to not be confounded by locomotor activity or immobility is required because of methodological considerations, the female hamster is a much better animal model than a female mouse or rat for that purpose (an example: (Moore et al. 2019)). In addition, a comparative approach in this context can teach us a lot about the neural correlates of lordosis, which is apparently maintained in female hamsters without sensory feedback from the male's paws on the flanks, in contrast to female mice and rats. Both the neuromechanistic differences as well as similarities of lordosis control between species can then provide valuable understanding of generalizable concepts.

Even though studying sexual behavior in animals could and should have a purpose on its own within basic research, some research may have the specific goal to be translational to humans. Sexual behavior in animals is necessary for reproduction and species survival, and comes at a cost of energy expenditure. Animals may solely engage in sexual behavior because it is rewarding to them, not being driven by the purpose of reproduction. Similarly, whereas nature ensures that copulation in most animals is confined to the peri-ovulation period through hormonal regulation of female receptivity, this is not the case in humans. Therefore, certain aspects of human sexual behavior are difficult to model in animals. However, sexual dysfunction in humans such as diminished motivation for sexual interaction (decreased libido), erectile dysfunction, or premature ejaculation are aspects that can also be studied in animal models, and the underlying mechanisms may well be conserved across species. Translational research has for example shown similar effects of many drugs on ejaculation latency in rats as in humans (reviewed in (Olivier and Olivier 2019)). This shows the usefulness of research in rats

for screening and characterization of drugs for treatment of sexual dysfunctions. Nonetheless, basic research into neurobiological mechanisms of not directly translatable aspects of animal copulation, such as lordosis and hormonal control of central circuitry underlying sexual behavior provide important building blocks for future comparative and translational research.

In summary, maintaining research in a larger diversity of animals can provide insights in why the brain is organized as it is in different species. Subtle differences in behavior are opportunities for a better understanding of the overarching neuronal basis of the behavioral output. Most importantly, the most suitable animal model should be chosen to answer the question at hand, and it is therefore necessary that advancement of transgenic animals and similar techniques is not just confined to mice but find their way to other species as well.

4.5 Future directions

This thesis advocates for more detailed behavioral analysis in the field of sexual behavior. There is much to be gained from multi-level approaches that pertain to measurements of sexual incentive motivation as well as reward learning. In addition, more detailed analysis of copulation in a wide variety of species, allowing for behaviorally informed hypothesis-driven questions and a comparative approach, is warranted. Furthermore, more variety in the contexts and environments in which we study behavior could greatly advance our understanding of behavior. Animals naturally navigate complex environments and are not merely confined to a simple box in which only a limited amount of possible behaviors can be displayed. Studies in more ecological settings, such as semi-natural environments are for example a valuable addition to the more traditional behavioral tests. Studies that compare outcomes of these approaches are also highly valuable. Applied to further interventional studies, there are many discoveries to be made.

Our study of the role of the MeA induced questions about how it can be that both stimulating and inhibiting the MeA had similar results in the copulation test. A more specific approach in which

certain cell types can be targeted also in rats would make it possible to further investigate these results. More and more transgenic rats are becoming available, for example making viral targeting of GABAergic neurons possible through *cre*-dependent expression (Sharpe et al. 2017). In addition to a more cell-type specific approach, targeting of neuronal projections in addition to brain regions would give more insights in the importance of circuitry. Is the MeA important because it relays sensory information to the mPOA? What exactly does the mPOA do? These questions should also be answered in the context of sexual experience: How does the MeA shape sexual experience? Would our results have been different in sexually naïve rats?

The convergence of hormones and circuitry in the control of sexual behavior is endlessly fascinating. How exactly *do* hormones prime the circuitry for regulation of sexual behavior? Many advances have been made in the study of female sexual behavior, but research in males is lagging behind. The next step is to find out whether the loss and gain of spines in males is indeed a functional process that underlies the orchestration of sexual behavior. In order to find out whether hormone-induced spinogenesis is necessary for sexual motivation, copulation, and reward learning, an approach could for example be taken that prevents local spinogenesis during hormonal treatment of gonadectomized males (see for an example of this approach in females: (Christensen, Dewing, and Micevych 2011)). In addition, the exact roles of testosterone and metabolites of testosterone remain elusive. Many questions remain. How do estrogen and androgen signaling converge? What are unknown mechanisms of testosterone? What specific cell types are responsive to gonadal hormones? How come gonadal hormone action in females is swiftly permissive of sexual motivation and copulation, but it takes much longer in males to induce the full range of sexual behavior? Future research will aim to address these questions.

4.5 Conclusions

Many new questions and interesting topics for further research have arisen from the results described in this thesis. Nevertheless, the research presented in this thesis has 1) provided suggestions and recommendations about the assessment of sexual motivation and copulation in male and female rats; 2) led to a better understanding of the natural organization of male rat sexual behavior, providing a basis for the formulation of new neuromechanistic hypotheses; 3) showed that the MeA is not necessary for sexual incentive motivation in male rats, but has a role in sensory processing which affects ejaculation latency; and 4) showed that loss of gonadal hormones and dihydrotestosterone treatment affects neuroplasticity within brain regions involved in sexual behavior and motivation in male rats.

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Paper I

Assessment of sexual behavior in rats: the potentials and pitfalls

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Invited review

Assessment of sexual behavior in rats: The potentials and pitfalls

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ABSTRACT

In the field of behavioral neuroscience, it is essential to use the appropriate animal models for the topic of investigation. Using the wrong model can result in false interpretation of the results. In this review we will discuss the animal models used to study sexual behavior, with a focus on rats. We will discuss the potentials and pitfalls of the different paradigms and try to make recommendations on how research in this field could be optimized. Both male and female sexual behavior are discussed, in addition to sexual motivation.

1. Introduction

Employing appropriate animal models for research in the field of behavioral neuroscience is essential. The use of the wrong animal model can result in misinterpretation of results and false assumptions about the neurobiological background of these results. In addition, it is possible that these misinterpretations and false assumptions set precedent for future research.

In this review we will explore sexual behavior in both male and female rats, discuss how this behavior should be analyzed and interpreted, and how it fits in behavioral paradigms. Furthermore, we will focus on behavioral paradigms for the investigation of sexual motivation in rats. For both the analyses of the behavioral observations and the paradigms, we will try to show their respective potentials and pitfalls, and argue for a careful approach to the operationalization of notions such as motivation and reward from the given sexual behavioral parameters.

It should be noted that this review is written in the context of the controlled environment of a laboratory. In their natural environment, rats copulate in groups consisting of one or several females and males [1,2]. The sexual behaviors performed by the individuals is similar in nature and in pair-tested tests, just as the complete sexual cycle. There are only some differences in the timing of behaviors, because rats in nature have more space to pursue conspecifics or might get distracted by the environment or fellow rats.

Before we discuss the sexual behavioral parameters, we deem it necessary to first describe the basic observations we can make during sexual encounters between a male and a female.

1.1. General behavioral aspects of the copulatory cycle in rats

The course of sexual interaction between a male and a female rat is to a large degree stereotypical (see Fig. 1) [3–5]. Broadly speaking, a copulation cycle can be divided into three parts, the precopulatory phase, copulatory phase and executive phase [6]. During the precopulatory phase, the male rat and the receptive female (i.e. being in hormonal or behavioral estrus) will engage in anogenital sniffing. The subsequent copulatory phase consists of the female drawing the male's attention with paracopulatory behavior: *hopping* (short jumps with all four legs off of the ground) and *darting* (short and sudden runaway movements, in which she presents her body to the male). In a reaction to these movements, the male rat will try to *mount* the female: he straddles the female from behind, and thrusts his hips in an attempt to locate the vagina with his penis. In the event of penile insertion into the vagina, the male rat continues his thrusting with a sudden deeper thrust. He then dismounts the female, visible as a short jump backwards, away from the female, sometimes raising his forepaws in the process. This behavior is recognized as an *intromission*. The physical stimulation caused by mounts and intromissions can cause the female to arch her back for easier vaginal entry, a receptive phenomenon known as *lordosis*. These behaviors tend to proceed in rapid succession, only to be intermitted by self-grooming, rest, and *pacing* by the female (runaway behavior). Finally, ejaculation constitutes the executive phase for the male, which is followed by a period of male inactivity, usually lasting around 5 min. The beginning of a new cycle of sexual behavior marks the end of the *postejaculatory interval.*

Auditory, olfactory and visual cues play an important role in sexual behavior. Interestingly, a cooperative function seems to exist for the different modalities in the induction of approach behavior of a potential

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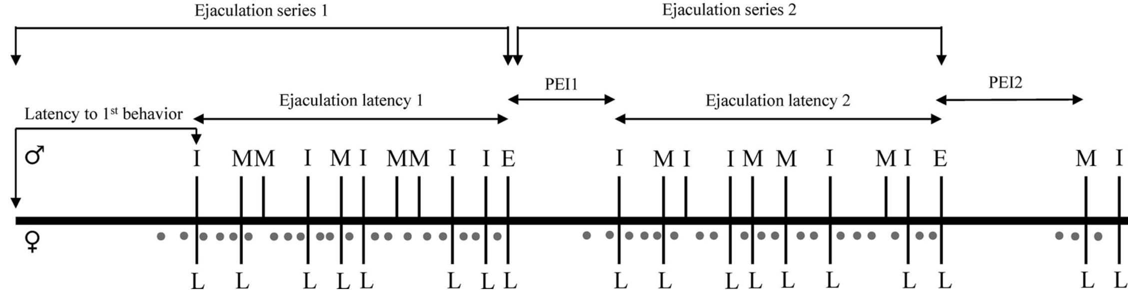

Fig. 1. Sexual behavior cycle. Schematic overview of typical sexual behavior. M = mount, I = intromission, E = ejaculation, L = lordosis, ● = dart/hop, PEI = postejaculatory interval.

mate [7].

2. Male rat sexual behavior

2.1. Parameters

The events described above (mounts, intromissions and ejaculations) are registered at the corresponding time points with a scoring device during sexual behavior assessment, either at the real time test or from video. When trained, an observer can easily recognize mounts, intromissions and ejaculations by looking at the associated behavior as described above. The act of intromission is for example very well correlated with the male rat behavior of a deep thrust and jumping backwards [8]. Analysis of the scoring output yields a set of parameters by which sexual behavior is assessed:

- Mount latency; time from introduction to the female until the first mount
- Intromission latency; time from introduction to the female until the first intromission
- Latency to first behavior; time from introduction to the female until the first behavior − i.e. mount or intromission
- Number of mounts
- Number of intromissions
- Number of ejaculations (if a test is used that allows for observation of multiple ejaculation series)
- Ejaculation latency; time from the first intromission to ejaculation
- Postejaculatory interval; time from ejaculation until next mount or intromission (often time to next intromission is used)

In addition, the following parameters are calculated:

- Intromission ratio; the number of intromissions divided by the sum of the number of intromissions and the number of mounts
- Inter-intromission interval; the total test time divided by the number of intromissions, or the ejaculation latency divided by the number of intromissions
- Copulatory rate; the sum of the number of mounts and the number of intromissions divided by the time from first behavior to ejaculation

Sometimes, sexual behavior is expressed by means of a percentage of ejaculating rats or as a percentage of copulating rats (for example [9]). This makes sense when a treatment is so deteriorating on the sexual behavior of the rats, that there are too few events to score. Analyzing data from too few events can skew the data and augments the problem of how to deal with missing values. If possible, however, we recommend reporting sexual behavior testing results by reporting the abovementioned parameters.

2.2. Interpretation of results

In order to interpret an effect of a certain treatment on any of the mentioned parameters, we first have to more accurately define the key observed behaviors, i.e. mounts and intromissions, and elaborate on the role of those behaviors within the sexual behavior episode and its contribution to the copulatory and executive phase of copulation.

Penile stimulation through intromissions, with a minimum number of two, is essential for a male rat to reach ejaculation [10]. In addition, two or more intromissions are necessary for a female to get into progestational state, necessary to become pregnant [11]. Interestingly, rats that show an innate short ejaculation latency do not necessarily need less intromissions to achieve ejaculation [12]. Moreover, there is a low variability in the temporal pattern of male rat sexual behavior [12,13], meaning that rapid ejaculators need less time to achieve the same amount of intromissions than normal and sluggish copulators. Indeed, normal and sluggish ejaculators show more mounts preceding ejaculation, essentially making rapid ejaculators more "efficient" than their sluggish and normal counterparts [12].

When we look at *mounts* in particular, it is difficult to establish what they really are. Are they failed intromissions? That is, is the "intention" of every mount to end in an intromission? Or, do they represent a behavior independently contributing to the copulation climax and/or do they serve a specific "purpose" within the sexual behavior? We have seen rats only intromitting and not mounting during an ejaculation series, which suggests that mounts are not necessary to reach ejaculation. It is clear, however, that mounts do contribute to the arousal state and facilitate ejaculation: when males mate with a female with a closed vagina for 40 min, less intromissions are necessary to achieve ejaculation during subsequent mating with an intact female. In addition, the ejaculation latency and number of mounts are decreased during this subsequent mating [14]. Mounting is also a self-maintaining behavior. Male rats continue to mount when they are prevented from intromitting through closure of the female vagina, or through local anesthesia of the penis [15,16]. Intriguingly, although intromissions are the essential part of copulatory behavior leading to ejaculation, it is actually the mount bouts that determine the temporal pattern of copulation, independent of intromission behavior. This became evident from a study showing that the inter-mount-bout-interval (the time from the first mount of one mount bout to the first mount of the next mount bout) was highly constant, independent of whether the preceding mount bout ended in a mount or an intromission. In addition, male rats do not keep mounting within a mount bout until they have achieved an intromission, suggesting that the mount bout is not "intromission driven" [17]. This proves that mounts are not just non-essential behaviors for reaching ejaculations, but central behaviors within the sexual behavior pattern of the male rat.

Consequently, interpretation of an effect on the number of mounts and/or intromissions preceding ejaculation is not particularly straightforward. A decrease in the *number of intromissions* preceding ejaculation could be interpreted as an increase of the "arousal state" of the rat, needing less stimulation to achieve ejaculation. It should be

beared in mind though, that the lower need for stimulation in response to any treatment might also be the result of an increase in penile sensitivity. However, this does not mean that penile sensitivity changes are necessarily the mechanism through which rats can become more aroused. For example, male rats require less intromissions to reach ejaculation when the accessibility of the female is limited: single or multiple forced intercopulatory intervals (removing the female for a certain amount of time after intromissions) make the male need less intromissions to reach ejaculation [18,19]. This could not be explained by an increase in penile sensitivity, but it does suggest that males can actually influence their efficiency and arousal state, depending on the circumstances. Another example of this phenomenon is seen in more “natural settings”, in which female rats determine the pace of mating in a multiple choice arena. The non-preferred males in these tests are less often visited by the females, resulting in longer intercopulatory intervals, and become more efficient (more mounts result in intromissions), resulting in shorter ejaculation latencies than when they are tested in a situation where they can pace the mating themselves [20]. The efficiency of the rat is thus reflected in the *intromission ratio*. As mentioned before, the efficiency to reach ejaculation is increased when the rat is more successful at achieving intromission when mounting. Because the occurrence of an intromission is dependent on the occurrence of an erection, effects on the intromission ratio may therefore reflect an effect on erectile function.

The *inter-intromission interval* and *copulatory rate* are parameters that are often interpreted as a measure for temporal patterning of copulation. We question, however, whether these parameters do actually provide any useful information about the temporal pattern of copulation. Previously, we concluded that temporal patterning of copulation in the male rat is entirely determined by the mount bout. Consequently, the inter-intromission interval is actually a function of the intromission ratio and the inter-mount-bout-interval. This means that a decreased inter-intromission interval could be entirely due to a higher efficiency (increased intromission ratio), without any effect on the temporal copulatory pattern (defined by the inter-mount-bout-interval). The copulatory rate in its turn is also very dependent on the efficiency of the rat. For example, interpreting an increased copulatory rate as “increased copulation speed” would be a mistake if there were actually no effects on inter-mount-bout-intervals, but just an increase in the number of mounts within a mount bout, which means the rat is just less efficient − a completely different conclusion! To sum this up, we are inclined to ignore the inter-intromission interval and copulatory rate and instead look at the inter-mount-bout-interval as a measurement for copulation speed. Copulation speed is an interesting measurement in the light of a very basic theory of a “mount generator” within the brain, described by Ågmo [21]. Within this theory, mounts, intromissions and ejaculations all temporarily inhibit this mount generator, in which an intromission has a greater inhibitory effect than a mount. For example, 3–5 mounts (a mount bout) could be necessary to reach the inhibitory threshold already achieved by one intromission. Ejaculation results in the greatest inhibition, reflected by the post-ejaculatory interval (see below for further discussion). In conclusion, measured effects on copulation speed could reflect an influence on the functioning of this mount generator

As for the practical side of scoring *inter-mount-bout-intervals*, it requires either a formula to calculate the parameter from the mount and intromission data points or it needs to be scored separately according to a clear recognizable behavioral definition. Sachs and Barfield defined the mount bout as “a sequence of mounts (one or more), with or without intromission, uninterrupted by any behavior (other than genital autogrooming) that is not oriented toward the female” [17]. This seems to be the only valid way to register mount bouts, since a definition cannot exist in terms of time between behaviors, because time is actually the parameter that is variable here.

Continuing with the interpretation of mounting parameters, *increased mounting* is often interpreted as a measure of motivation. However, a shorter ejaculation latency accompanied by less mounting and intromission behavior does not necessarily mean that the rat is less motivated. It might as well mean that the arousal state of the rat is increased. Another parameter that is usually considered to be a measure of motivation is the *latency to mount*. However, it should be considered that general activity, general arousal and sensory efficiency of the rat also affect this parameter. For example, a treatment that increases tactile sensitivity or sensitivity to smell can affect the ability of the male rat to localize the female and mount faster. Next to that, we cannot be sure in what way the female may affect the mounting latency of the male. Therefore, we need to be very careful when drawing any conclusions from effects on the latency to mount. Finally, there is no reason to believe that the rat has any active choice in starting copulation behavior with a mount or an intromission. Therefore, in contrast to what is common practice, we believe that no different interpretation should be given to whether the first behavior is a mount or an intromission. Consequently, we propose to only report the *latency to first behavior* as a measurement of latency to start copulation.

The interpretation of the *post-ejaculatory interval* is unclear [22]. It is sometimes interpreted as a measure of sexual motivation. However, the post-ejaculatory interval is in general not very variable, as is for example evident from the fact that innate rapid ejaculators do not have a shorter post-ejaculatory interval than other rats [12]. In addition, it is clear that the post-ejaculatory interval can be divided in an absolute and a relative refractory phase [23]. While the rat is absolutely unresponsive to any sexual stimuli, and copulation is completely inhibited during the absolute phase (the first 75% of the post-ejaculatory interval), the rat can be reactivated to start copulating again during the relative refractory phase, by arousing stimuli such as the introduction of a new receptive female, handling or electrical shock [24,25]. Nevertheless, there are examples of treatments that do affect the post-ejaculatory interval, including the absolute refractory phase, sometimes in an extreme fashion (see for instance [23,26]). Furthermore, it is known that the post-ejaculatory interval is not caused by a reduced excitability in the spinal cord control of penile reflexes [27]. Therefore, the post-ejaculatory interval is clearly an effect of some sort of inhibition within the brain. We remind the reader of the mount generator theory, which could explain the refractory period of the post-ejaculatory interval. Small treatment effects on the post-ejaculatory interval could well be effects on general arousal. More extreme effects may suggest an effect on the absolute refractory period. It would be an interesting study to research whether effects on inter-mount-bout-intervals are correlated with effects on the post-ejaculatory interval.

The current standard is to calculate the post-ejaculatory interval as the time from the ejaculation to the next first intromission. Since intromissions require penile erection and coordinated activity of the striated penile muscles, it was seen as a more important sexual behavior than mounts. However, as discussed before, we believe that mounts play an important role in sexual interactions as well, and consider the latency to first behavior a more relevant parameter than the latency to first intromission. For the same reasons, we recommend to calculate the post-ejaculatory interval as the time from the ejaculation to the next first behavior. Only when we calculate the *latency to ejaculation*, the latency to first intromission might become relevant. In comparison to the other parameters, the latency to ejaculation could provide additional information about the efficiency from the first penile sensory stimulation to reach an ejaculation. Mounts do not involve penile insertion and are therefore not considered valid as penile sensory stimulation. Therefore, it could be useful to calculate the latency to ejaculation as the time from the first intromission to the ejaculation. However, with the previously mentioned arguments for that mounts play an important role in sexual interactions as well, it could just as well be interesting to calculate the latency to ejaculation from the first mount, or even the beginning of the test.

A very important point to be made with regard to explanation of results is definition of facilitation and inhibition of sexual behavior in

the literature (see also [28–30]). A decreased ejaculation latency is frequently presented as a facilitation of sexual behavior, whilst it is often accompanied by a decrease in behaviors during the copulatory phase; the rat is more efficient (higher intromission ratio) or has a lower ejaculation threshold (less intromissions preceding ejaculation). On the other hand, decreased ejaculation latency could indeed be accompanied by an increase of behaviors during the copulatory phase, through an increase of the copulatory rate. The fact that the number of pre-ejaculatory intromissions positively influences the amount of sperm reaching the uterus of the female [31] and the chance of pregnancy [11], illustrates that inhibition of the copulatory phase combined with facilitation of the executive phase should not be considered as facilitation of sexual behavior in general, since it can actually have a negative effect on fertility. This makes a case for clearly differentiating between facilitation of the copulatory phase on the one hand and facilitation of the executive phase on the other hand.

2.3. Behavioral paradigms

Excellent protocols have been written on testing paradigms for male rat copulatory behavior assessment [22,32]. Therefore, we will briefly discuss the tests available and considerations that determine the choice of a test without going into too much detail.

Sexual behavior of the male rat is most often assessed by putting the male rat in a transparent test arena together with a receptive female rat. In this set-up, the male has continuous access to the female and can freely copulate at his own chosen pace. It is important to let the test subject pace the copulation, because copulation is only rewarding to the rat that is able to control the mating [33]. This is also illustrated by the fact that the structure of male copulation behavior in a seminatural environment, where females are capable of pacing the copulation, differs from that in a copulation test [5]. Often, the copulation test is conducted for one ejaculation series, ending after the first intromission after the post-ejaculatory interval. Alternatively, the test can be ended after a predefined time period (usually 30 min), independent of the amount of ejaculation series the rat has shown. Sometimes, rats are tested up until exhaustion.

In general, all significant differences among groups can be identified by only looking at the data for the first ejaculation series, except for the number of ejaculations within a defined period of time. Still, the effect of an increase in the number of ejaculations will logically be accompanied by a decreased ejaculation latency and/or a shortened post-ejaculatory interval, and would therefore automatically be reflected in the data from the first ejaculation series. However, although it might not be expected, treatment effects could also only become evident in later ejaculatory series. For example, the ejaculation latency in the first series may remain normal, while it is affected in the following series. Therefore, we recommend to always conduct a 30 min test, if only to rule out this possibility. While the focus of data analysis will lie with the first series, we might come across something unexpected in any of the following series. Additionally, Chan et al. (2010) discussed an interesting argument in favor of the 30-min test: when testing pharmacologically active substances, a 30 min time period will control for individual difference in pharmacokinetics better than a single ejaculation series test [32].

A problem that presents itself when analyzing data from a 30-min test is whether to compare results from the total test time or only from corresponding ejaculation series. In wildtype rat sexual behavior, the number of mounts and intromissions decline during the second to the fourth series, after which the numbers increase again for the series following. Also, the post-ejaculatory interval increases for each ejaculation series after the first [22]. This makes it very difficult to determine how to compare and interpret total test data (except for total ejaculations). Consider the complication in comparing a rat that only ejaculates once, right before the end of the test, with a rat that ejaculated four times. The fast ejaculator will have had four post-ejaculatory intervals, so about 15 min out of 30 min without activity, while the slow ejaculator has been active during the whole duration of the test. Total test number of mounts and intromissions are in this case incomparable between the two situations. The previous example only emphasizes the complexity of drawing conclusions from the data. Therefore, we believe it is most preferable to report raw data as they are, total test and per series, instead of just the interpretations of results. This practice will maintain objectivity in the results as much as possible.

In the end, choosing a suitable test is very dependent on the effect that one is looking for. If the only interest is, for example, an increased or decreased ejaculation latency, a test with one ejaculatory series is obviously sufficient. This is especially applicable in translational research, because humans achieve most often only one ejaculation. For example, in order to assess whether a drug could function as treatment for premature ejaculation, it is sufficient to investigate the effects on the delay in the latency to first ejaculation. However, in case the research is quite fundamental and focuses on mechanisms in rat sexual behavior, it is recommended to assess all effects on behavior which is then tested in a 30-min test. As an example, a treatment might affect the post-ejaculatory interval in such a way that instead of increasing over ejaculatory series in time, it remains the same within each ejaculation series. This effect would not be found in a single ejaculation series test, but will be reflected in data from a 30-min test.

With the use of the 30-min test, it was also discovered that sexual behavior of the male rat is highly variable between rats. A typical population of wild type Wistar rats will show that 10–20% of the animals are so called ‘sluggish copulators’ and 10–20% of the animals are ‘rapid copulators’. Rapid copulators reach double the amount of ejaculations than normal copulators in the same time span, while sluggish copulators will reach less than half of that of normal copulators [12]. Similar endophenotypes can also be found in females, in which about 37%, the male-avoiders, spent significantly less time in the male compartment and showed lower levels of paracopulatory behaviors than the male-approachers. This behavior is also constant over multiple paced-mating tests [34].

3. Female rat sexual behavior

3.1. Parameters

Just as with testing male sexual behavior, the events can be registered by a trained observer at the corresponding time points with a scoring device during sexual behavior assessment. Analysis of the scoring output yields a set of parameters by which sexual behavior is assessed or calculated:

- Number of lordosis responses assessed on a 4-point scale (0–3 with zero as no lordosis and 3 as a full lordosis with a hollow back and lifted head of 45 ° or more [35]), from which can be calculated:
  Lordosis score (the mean of all lordosis intensities)
  Lordosis quotient (the number of lordosis responses divided by the number of received sexual stimulation times 100%)
- Number of paracopulatory behaviors (darts and hops)
- Number of received sexual stimulations (mounts, intromissions and ejaculations)
- Time spent with the male
- Percentage of exits after sexual stimulations (total number of exits after the stimulation within a certain time-frame divided by the total number of the stimulation times 100%). This parameter should be given separately for mounts, intromissions and ejaculations.
- Contact-return-latency (the average time the female needs to enter the male compartment again after an exit). This parameter should be given separately for mounts, intromissions and ejaculations.

Ear wiggling is sometimes also calculated and added to the number

of paracopulatory behaviors. Ear wiggling is a rather fast lateral shaking of the head that is visible as a quiver of the ears, a behavior that is very difficult to score, because it happens very regularly and fast. Therefore, many researchers leave this behavior out of their analysis. In fully receptive females, ear wiggling almost always accompanies the darts and hops, and could therefore (out of practicality) also be considered part of this paracopulatory act of behavior as one event.

3.2. Interpretation of results

Lordosis is the most studied component of female sexual behavior. The *lordosis quotient* (LQ) is considered a measure of sexual receptivity, whereas the *lordosis score* (LS) represents the magnitude of the lordosis response. Lordosis is a reflexive behavior that is very much depending on the hormonal state of the female. The presence of estrogen alone is sufficient to induce receptivity, but progesterone facilitates the estrogen-induced lordosis response [36]. Older studies concluded that lordosis was triggered by sexual stimulations from the male [3,37], but more recent studies have shown that this hormonally regulated response can also be triggered by other forms of tactile stimulations (e.g. upon male sniffing or touching the female or manual stimulations) [38,39]. Surprisingly, researchers keep scoring only the lordosis responses upon mounts, intromissions and ejaculation resulting in a lordosis quotient of maximal 100%. So far, the extra lordosis responses have been measured and reported in only a few publications (e.g. [4,40,41]), which is a missed opportunity. There is a variation between rat strains, but as showed in Snoeren et al. (2011), Wistar rats almost always show an LQ of 100% when the appropriate hormonal treatment is given to ovariectomized rats [40]. Only when females were treated with a low dose of 2 μg of estradiol benzoate alone, an LQ of 40% was (sometimes) found, but the LQ reached 100% in all cases as soon as progesterone was added. Consequently, if the researchers would not have scored the extra lordosis responses to other tactile stimulations, they would not have discovered the positive drug effects on lordosis [40]. The drug-induced increase in LQ is an important finding, because it indicates that the females were extra sensitive to tactile stimulation, which probably is a result of an increased receptivity. This conclusion could never have been drawn if the extra lordosis responses were not measured, and the drug would have been evaluated as having “no results on receptivity”. We therefore suggest that the extra lordosis responses should always be reported in future studies in order to prevent from misinterpretation of results.

It is generally accepted that LQ and LS are the ultimate criterion for female sexual receptivity, but there are some reasons to be careful with the interpretation of the resulting data. For example, sexual behavior tests performed under paced and non-paced mating conditions have resulted in different outcomes on lordosis behavior. POA lesions, for instance, cause an increase in lordosis quotient compared to sham-operated females in a non-paced mating test, while the same lesions disrupt lordosis when the females were allowed to pace their sexual stimulations [42]. Similar conflicting findings were observed on the role of estrogen α receptors in the VMN on lordosis; in a non-paced mating test, females without estrogen α receptors showed impaired lordosis responses [43], while sexual behavior tests performed in a seminatural environment (in which females can escape from the male) indicated normal lordosis capacity in these females [44]. Together, this suggests that the lordosis response might not solely reflect the receptive state of the female, but could also be influenced by her motivational state. In a paced mating set-up, a female can escape from the male when she is not motivated for copulation, while in a non-paced mating paradigm she either overrides her motivation and participates with lordosis responses (in case of the increase in LQ) or she prevents the male from mounting by fighting and/or suppressing the lordosis response (in case of the decrease in LQ). Interestingly, this actually shows that also the reflexive response can be actively suppressed. Therefore, carefulness is needed when analyzing lordosis behavior in a non-paced mating set-up. It actually makes us recommend to always study female sexual behavior in paced mating conditions.

Another measurement for female sexual behavior is the number of paracopulatory behaviors. *Paracopulatory behavior,* also called solicitation or proceptive behavior, is usually described as the species-specific behaviors displayed by an estrus female during sexual interaction in which she encourages the male to mate and regulates the pattern of copulation (also reviewed in [45]). Beach suggested that the darts and hops constitute the female’s assumption of initiative in establishing or maintaining sexual interaction [3], which is then translated in a measurement for female sexual motivation. McClintock and Adler [37] showed that 90% of intromissions were preceded by female approach, while only 3% of intromissions occurred upon approach of a male towards a female [37]. It was, therefore, believed that copulation occurred upon initiation of the female rats. However, a recent study by Bergheim et al. (2015) performed in a seminatural environment showed that the copulatory acts were a consequence of a subtle interaction between the male and female. This indicates that the behavior of both rats are equally important in the initiation of copulation, and thus not controlled solely by the female [46]. Still, there is a linear relationship between the amount of paracopulatory behavior and the amount of copulation: females who dart less, receive less sexual stimulations, while actively darting females receive more sexual stimulations [46]. There is thus an equal proportion of paracopulatory behavior leading to a sexual interaction. Based on the definition that the intensity of execution of a behavior is strictly dependent on the level of motivation (as discussed in [47]), this indicates that paracopulatory behaviors are indeed a parameter for sexual motivation. This idea is strengthened by the observation that the rate of paracopulatory behaviors decreases over time after having received multiple sexual stimulations [48], which attenuates the levels of sexual motivations.

However, some scientists believe that paracopulatory behaviors are not adequate as measure of sexual motivation. They argue that paracopulatory behaviors are very stereotyped, and can be considered entirely reflexive, because hormonally primed females can also show paracopulatory behaviors (just as lordosis responses) upon manually stroking the hind flanks, and thus in a non-sexual context [49]. However, as mentioned before, lordosis is a clear reflexive behavior, that might also be influenced by the motivational state of the female, since lordosis can be actively suppressed when required. In case paracopulatory behaviors are indeed reflexive, it does not prove that this behavior is not a measurement of motivation. Although they can occur upon manually stroking of hind flanks in a non-sexual context, darts and hops performed during copulation can still reflect sexual motivation. An alternative explanation we would like to introduce is that the paracopulatory behaviors might represent the motivational level of keeping participating in the sexual intercourse rather than of the female’s intrinsic sexual motivation. In order to measure the level of intrinsic motivation, a sexual incentive motivation test (as mentioned later in this review) is a better method to use.

Overall, it is important to report the scientific findings as objectively as possible. We could argue that the number of paracopulatory behaviors could be an indicator of the level of sexual motivation, but clear empirical evidence is not available at this moment. Besides, alternative options should not be neglected. We, therefore, strongly support Blaustein and Erskine [50] in using the term *paracopulatory behavior* instead of the older terms (proceptive, solicitation, precopulatory), simply because it obviates the assumptions about the female’s sexual motivation to initiate mating [50].

When a paced mating paradigm is used (as described later), the *time spent with the male* can also be measured. This parameter is thought to reflect the female’s motivation to continue participation in copulation. However, caution should be taken when analyzing this behavior, because this parameter is also affected by a component of social behavior. Male rats do normally not attempt copulating with non-receptive females, defined as females who are not in behavioral estrus. Non-

receptive females, therefore, can safely spend time with the male without the risk of being mounted. The parameter of time spent with the male is probably only a reliable measurement in hormonally primed females who have signs of receptivity. For example, the smell of a receptive female stimulates the male to attempt to mount the female. Now the not-willing female can only reject or escape from the male to be left alone, which is then indicated in less amount of time spent with the male compared to the willing females.

To continue with other components of pacing behavior, it has been shown in the past that the *percentage of exits* increases with the intensity of the received sexual stimulus [51]. In the same line, the *contact-return latency* (CRL) of the female to return to (or to press a lever for) sexually males also changes with the intensity of the previously received sexual stimulus [51–53]; after a mount females return to the male quicker than after an intromission or ejaculation. These parameters are therefore always given per type of stimulation; e.g. percentage of exits after mount or CRL after intromissions. Interestingly, this pacing behavior seems to be a very stable behavior that is innately present in females upon their first sexual contact [54].

Several studies have shown that certain conditions or treatments can have a different effect on the percentage of exits and the CRL [34,40,55,56], suggesting that these measurements of pacing behavior have different read-outs that might be regulated through different brain mechanisms. For example, no differences in percentage of exits were found in ovariectomized females treated with only estradiol or a combination of estradiol and progesterone, while the presence of progesterone decreases the CRL [40]. Furthermore, no change in percentage of exits, but an increase in CRL's after intromissions was found in females receiving more than 15 intromissions [48]. The percentage of exits could, therefore, reflect the female's short-term response to the intensity of the copulatory stimulus (sensory component), while CRL is more a direct measure of the female's motivation to reinitiate mating [57].

However, it is essential to be cautious with the interpretation of the data for a few reasons. First of all, females are more likely to delay their return upon intromissions after they have received multiple intromissions along with ejaculations than after receiving only a few intromissions [45], suggesting that the pacing behavior of the female seen in a copulation test (as described below) is highly dependent on the copulatory activity of the male rat. Since the activity of the male is uncontrollable when studying the sexual behavior of the female, this makes the parameters of pacing behavior very unreliable as indicator of sexual desire or arousal of solely the female. Second, a CRL can only be measured when a female does escape from the male with an exit. As a result, the CRL parameter is biased for the moments that the female escapes from the male and neglects the moments in which the female continues in copulation. At the same time, no clear definition of an exit exists, or an exit is measured with a certain cut-off time, meaning that an escape is scored as exit only if the female runs away from the male within for example 10 or 20 s (but also 120 s has been used). But what does this cut-off point mean and what is it based on? Female rats regularly start running around the cage after a stimulation, in which she might "accidentally" run through her own female compartment before immediately re-entering the male compartment. This would then count as an exit and immediately as a very short CRL, but she might not participate in the sexual interaction straightaway (which is the reason why missing data points for the CRL due to no escape cannot be filled with a zero second count). This kind of situations influence the outcome without explaining the female's short-term response to the stimulation or her motivation to reinitiate mating. One might suggest it is better to calculate a CRL with the time to the next first paracopulatory behavior instead, but since the female often darts in her own compartment, this measurement would also have no significance. In addition, Ellingsen and Ågmo [58] have once calculated the relationship between ambulatory activity and the propensity to escape from the male. By calculating the probability that the female would randomly enter her own compartment, and then compare this to the proportion of escapes after mounts, they discovered that an increase in percentage of escapes (e.g. upon amphetamine treatment) can rather be an effect on ambulatory activity than an increase in sensory responsiveness [58]. Altogether, this supports the idea that the percentages of exits and CRL are useless as indicators for the female's sensory and motivational state. We therefore suggest that if the percentage of exits and CRL are estimated, they should always be evaluated in combination with other parameters of female sexual behavior and never as a measurement of its own.

3.3. Behavioral paradigms

When studying female sexual behavior, different kinds of tests can be used. In many studies, researchers focused solely on investigating lordosis. This was commonly done by allowing females to receive 10 mounts or intromissions and measuring the number of lordosis responses. The lordosis quotient, which is the number of lordosis responses divided by the 10 copulatory stimulations times 100%, was considered a measure of sexual receptivity. This method could be very convenient for the researcher, because it does not take much time to observe 10 mounts, but a disadvantage of this method is that it is always performed in a non-paced mating set-up. As discussed before, female rats seem to be able to suppress the lordosis response to sexual stimulation when no escape possibility is available, which could lead to misinterpretation of the results. But a more important argument for the uselessness of this paradigm is that one only investigates one aspect of the female's sexual behavioral repertoire. Even though, the LQ might provide the information of the receptivity of the female, it does not reflect the willingness of the female to participate in sexual interactions.

A better method to study the full aspects of female sexual behavior would be a complete copulation test in which the female shows its repertoire of copulatory behaviors: ear wiggling, darts, and hops, besides lordosis. A standard copulation test as used for male sexual behavior would be an option. However, this paradigm is also not ideal, because females are not able to pace their sexual interaction. Research has shown that coital stimulations are more effective in inducing pregnancy in a paced mating situation than under non-paced mating conditions [59], suggesting that intromissions become more effective in changing neuroendocrine changes in the female. Besides, copulation only has rewarding properties for a female, when pacing opportunities are available [60]. Thus, a test set-up in which paced mating can be investigated, reflects the *voluntary* participation in sexual behavior better in female rats.

Two standard paced mating set-ups are used for studying female sexual behavior: a bilevel chamber and a two-compartment paced mating set-up in which the chambers are connected with holes (of 4 cm in diameter) through which the female fits, but the male does not (because of his larger size). The bilevel chamber is designed in a such a way that the female can run around and avoid the male by changing levels that are connected by a set of ramps on either side in a narrow cage. This makes it more difficult for the male to mount her during a chase. The disadvantage of this paradigm, however, is the fact that the female needs to keep escaping instead of having a location away from the male to rest. In that perspective, the two-compartment paradigm seems a better way to investigate female sexual behavior. The female can now decide when and for how long she visits the male and receives sexual stimulations, which results in a more direct translational approach.

In the two-compartment paradigm, it is important to mention that the accessibility of multiple holes is essential. If only one hole is available for the female to enter the male compartment, the male can block the hole in his eagerness to get to the female. Practically, this results in less time she spends with the male and less received sexual stimulations, which is then not a measurement of her receptivity, but rather a lack of possibility to visit the male. By making multiple holes accessible, she always has the option to enter the male compartment.

Previously, in the review under male sexual behavior, we discussed the potentials and pitfalls of the 30-min test versus the first ejaculatory series. When studying female sexual behavior in paced mating paradigms, 30-min tests are the standard, although shorter and longer tests have also been used. Just as the lordosis test based on only 10 mounts, a study during only 1 ejaculatory series would not be an appropriate measurement of female sexual behavior. Even though the performance of the male is probably dependent on the accessibility of the female (and thus her sexual motivation and receptivity), it is still better to evaluate the female behavior as independently as possible from the male's performance. A complete 30-min test would minimize the influences from the male, because it would include enough time for a combination of mounts, intromission and ejaculations, whether or not she copulates with a fast or sluggish male. In fact, females spend equal amounts of time and show the same amount of paracopulatory behaviors in the vicinity of a sluggish and a fast male [34], when a sufficient amount of test time is provided. Therefore, we recommend to study the sexual behavior of females in a 30-min paced mating set-up in which all behaviors of the female (lordosis, paracopulatory and pacing behaviors) are evaluated. A two-compartment paradigm seems to be the best option.

4. Behavioral paradigms for sexual motivation

Whereas the paradigms mentioned above describe sexual behavior, they do not investigate sexual incentive motivation. As mentioned before, sexual behavior is divided into three phases, where sexual incentive motivation is part of the first, precopulatory phase. Some of the aforementioned measures of copulation are described (by others) to express motivation. Given the weight motoric responses have in the execution of this behavior, however, we think sexual incentive motivation, as described by the interaction between internal motivational state and incentive stimulus is not a factor in these phases of copulation. If these measures of copulation indicate a kind of motivation, they rather reflect the propensity to continue to participate in copulation.

To investigate sexual incentive motivation, the earlier phase of identification of sexual incentives, and initiation of the efforts to gain physical contact with that incentive, some paradigms have been proposed.

4.1. Runway paradigm

The *straight-arm runway*, as described by Lopez et al. [61], consists of a startbox (25 × 25 × 20 cm), a runway (160 × 10 × 20 cm), and a Plexiglas goalbox (45 cm diameter, 40 cm height; see Fig. 2). A removable, transparent barrier within the goalbox prevents physical contact between subject and stimulus, while retaining access to visual, auditory and olfactory cues. Both the startbox and the goalbox are separated from the runway by removable doors, allowing the entry of the subject to the runway to be controlled. Entry to the runway and subsequent entry to the goalbox are automatically timed by infrared light

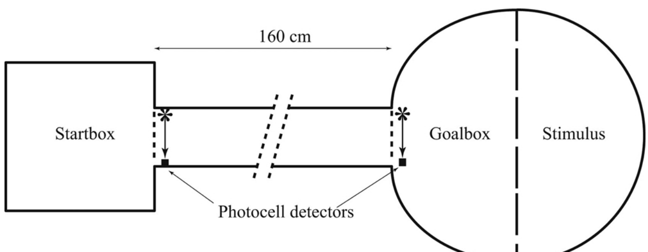

Fig. 2. Runway test. Mechanically removable doors separate the runway from the start and goalbox. Infrared photocell emitter–detector pairs situated at the beginning of the runway and just inside the goalbox allow measurement of the time the rat spends inside the runway.

sensors, which provides a measurement of time needed for the subject to cross the runway and reach the goalbox. Before the subject rat can take a run, they are placed in the goalbox with the target animal first, with the transparent barrier in place. The subject is subsequently placed in the startbox, and the door is opened to start the test and allow the subject to run for the known target stimulus. The runway test has successfully been used with other incentives than sex, e.g. food [62], water [63] and drugs [64].

As shown by Lopez et al. (1999), male rats run faster towards a receptive female than to a non-receptive female or male rat. The previously obtained sexual experience in the goal box did not affect running times. Only after the experience of an ejaculation, the males seem to run faster towards the goal box, but this effect was found for both a receptive female and a non-receptive female as stimulus. Therefore, this confirms previous findings that copulatory experience is not required in order for the male to prefer receptive females over non-receptive females [65–67], or males [68–70]. This indicates that the runway paradigm is indeed suitable to study sexual incentive motivation, and is usable for both sexually naive and experienced rats.

The key benefit of this test for motivation is that it (literally) is straightforward, as its main measurement is the latency to reach the stimulus. If one expresses male sexual motivation as the preparations and actions intended to gain physical contact with a female, the most direct measurement of this approach behavior is the time needed to travel the distance between location A and location B, where the female is. The directness of this test, however, also limits the strength of the measurement: with a relatively short runway, the latency to reach the target is short (in Lopez et al. (1999) a male reaches a receptive female within 25 s), which may limit the possibility to discriminate between subject groups or stimuli. In addition, the short travel time may allow internal states, such as anxiety or stress, and (distracting) extraneous stimuli, such as sound, light, or movement, to possibly prolong or shorten the travel time, and thereby affect the outcome. These effects can be filtered out easier in tests with a longer duration, and indeed, this runway test has been used with runways up to 3 m in length [71]. In any runway paradigm, to reduce this vulnerability to extraneous effects, rats should be habituated to the test set-up in order to reduce exploring and other novelty-associated behavior, and the startbox and runway should be thoroughly cleaned between tests to reduce unwanted olfactory cues.

Compared to procedures where stimulus preference is measured (as in the sexual incentive motivation test, see below), i.e. the subject has the choice between two or more targets with different incentive properties (e.g. receptive female, non-receptive female, male), only one target is present in the runway set-up. Whereas some stimulus preference procedures allow distinction between sexual and social components of the incentive stimuli within one test, the runway test only measures the total incentive value of the stimulus in the goalbox. However, this is a relatively minor objection, since different incentive targets can still be tested with a within-subject design by conducting multiple tests with the different stimuli. In that case, similar conditions should be applied.

4.2. Sexual incentive motivation test

The *sexual incentive motivation (SIM) test* consists of a rectangular arena (100 × 50 cm) of which the short sides are oval shaped (See Fig. 3, based on [72]). On both long sides, but diagonally opposed to each other, a small box (25 × 10 × 25 cm) containing a stimulus can be attached [72]. The arena and stimulus boxes are separated by steel mesh, physically separating the subject from the stimuli, but allowing visual, auditory, and olfactory cues to be perceived by both. Five minutes prior to testing, the stimulus rats are introduced into their respective stimulus boxes. The subject, which is habituated to the arena on three consecutive days before the test, is subsequently placed in the middle of the arena and allowed to move freely during a fixed period of

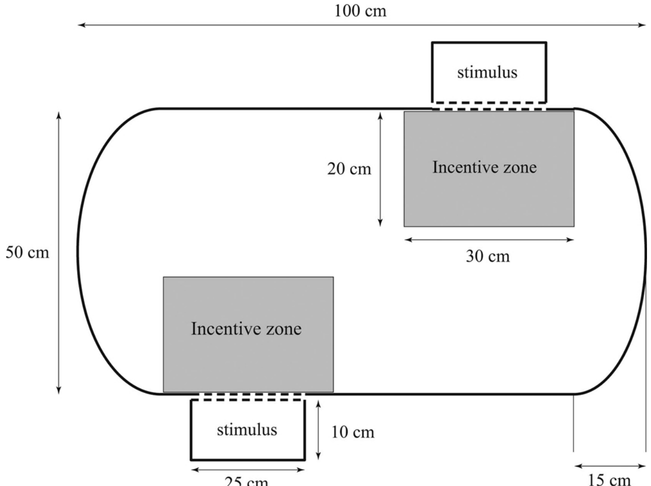

Fig. 3. Design of the sexual incentive motivation test setup.

10 or 20 min, after which the subject is taken out of the arena. Stimulus box A and B can be interchanged to prevent influences of spatial memory. The room in which the SIM test is located is dimly lit, so that a video camera, positioned above the arena, can take recordings, which can be analyzed with tracking software. Using this software, two areas measuring 20 × 30 cm in front of the stimulus boxes are defined, and are called incentive zones. Thus, a host of variables can be measured: time spent in incentive zones, number of visits to the zones, distance moved during the test, and average movement speed. From these variables, the preference score (time spent in incentive zone A/(time spent in incentive zone A + time spent in incentive zone B)) can be calculated. In addition, a number of basic behavioral observations, such as general mobility, self-grooming, freezing, and rearing can be made using the video files.

Several studies performed in this paradigm showed that male rats have a significant preference for a receptive female, when given the choice between this female and a male or non-receptive female [72,73], expressed by a preference score > 0.5. Sexual experience does not affect this outcome. Castration of the male, on the other hand, does lower the preference score by spending more time in the neutral zone instead of in the incentive zones [72]. These effects are reversible with supletion of testosterone propionate.

Similar results have been found with female rats, which spend significantly more time in the incentive zone of an intact male rat than with a castrated male or female rat [58,74]. Interestingly, the sexual incentive motivation test investigates not only the interaction between internal motivational state and a stimulus, but also the relative strength (incentive valence) of specific properties of a stimulus: e.g. a non-castrated male is preferred over a castrated male, while a devocalized male has the same incentive valence as a sham male. The test can also be used to study the incentive value of isolated properties. For example, when only the odor of a receptive and non-receptive females was used in the stimulus boxes, both experienced and in-experienced males prefer the odor of the receptive female. Interestingly, the inexperienced males do not show a preference when the odor of the receptive female was mixed with another odor, e.g. when the bedding was used instead of urine, or when combined with almond odor [72].

Central to the validity of this paradigm of relative choice is the question whether the propensity for a subject to prefer one incentive zone over the other not only depends on the attractiveness (positive incentive value) of the preferred stimulus, but also on the repulsiveness (negative incentive value) of the non-preferred stimulus. This is especially important in a situation where a male stimulus serves as a control for a female stimulus. In a series of tests, Ågmo showed that a male control stimulus does not have a negative incentive value in the SIM test [72]. First of all, male subjects did not show a preference for non-receptive females over male stimuli: no significant differences were found in the preference score, the number of visits, duration of visits, and time spent in incentive zone. Both inexperienced and experienced males showed these results. Second, in a comparison between the first five minutes of the third habituation (empty stimulus boxes) and the first five minutes of the test with either a male stimulus or a non-receptive female stimulus, the experimental rat spent significantly more time in incentive zones when an animal was present. Together, these results rule out the existence of a negative incentive value of either male or non-receptive female stimuli in this sexual incentive motivation test.

Because of the longer and fixed test duration, it seems plausible that the SIM test has a higher discriminative power than the runway test: random, short distractions will have less impact on a ten-minute test than on a 30–60 s test. In addition, because two stimuli are present at the same time, and it is even possible for the subject to withdraw from contact with either of them, it is possible to separate social motivation from sexual motivation. The preference score reflects a measure of stimulus preference relative to the other stimulus (A/(A + B)), thereby taking the potential social motivation out of the equation.

Again, familiarization of the experimental rat to the environment seems to be of specific importance. In a test with male subjects unfamiliar to the environment, the subjects showed no preference for the receptive female compared to a male stimulus. However, when the test was repeated 7 days later, the subject did show a significant preference for the receptive female, suggesting that a previous experience in the test set-up is sufficient to induce the required conditions for the test [72]. Ågmo suggested that one 20-min session in the presence of incentive animals offers sufficient familiarization, but others have confirmed that habituation to the environment without stimuli present for 3 times 10 min offers the same result [73].

4.3. Level searching paradigm

Level searching as a measurement for sexual motivation is a phenomenon first described by Mendelson and Pfaus [75]. It occurs when a sexually experienced rat moves through a familiar behavioral test set-up with different levels, in an apparent search for a sexual partner.

The testing chamber was previously described by Mendelson and Gorzalka (see Fig. 4), who developed the apparatus for easier evaluation of sexual behavior [76]. It consists of a Plexiglas box, with dimensions of approximately 60 × 25 × 15 cm 28 cm above the floor, a platform with the length of 40 cm is mounted. Ramps on either side

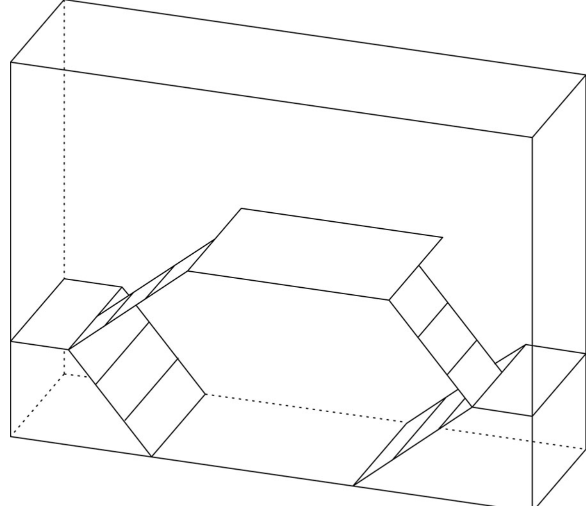

Fig. 4. Bilevel chamber. Schematic impression of the bilevel chamber used in the level searching paradigm (not to scale).

connect this platform to the floor, enabling the rats to move freely. In a typical experiment, a sexually experienced male rat is allowed to explore the chamber for 5 min, after which a female is introduced. A trial lasts until the male rat reached ejaculation or for 15 min, depending on the receptive state of the female.

In a series of experiments, Mendelson and Pfaus showed that male rats that were paired with receptive females had, in the 5-min period before the introduction of the female, increasing level-to-level movements with successive trials, whereas rats that were paired with non-receptive females showed no increase in level changes. Only after these rats had subsequently been paired with receptive females did their level changing rate increase too. Additionally, male rats that had achieved a stable number of level changes (during the 5 min before introduction of the stimulus) were then either paired with a non-receptive female or left alone in the chamber for 15 min. Rats that were left alone showed a decreased number of level changes in trial 4–7 compared to the first trial. Rats that were paired with a non-receptive female did not show a decrease in level changes, a finding that Mendelson and Pfaus explained as a response to a conditional reinforcer, where presence of the non-receptive female was assumed to have an association with previous sexual activity in the chamber.

When the bilevel chamber is used to observe copulation behavior, an obvious advantage of this set-up is the relatively natural aspect of it: all behaviors leading to, and including copulation are possible. In addition, the combination of specific components that make up the total incentive value of both female and male is intact. Visual, olfactory, tactile, and auditory cues can be perceived, and free movement enables female pacing and male pursuit. It is doubtful, however, that this matters when this chamber is used in experiments aimed at *incentive motivation*. After all, the measurement of level changes takes place in the absence of a receptive female, and thus the absence of the sexual incentive. It can therefore be argued that the resulting behavior, in the form of level changes, is not as much attributable to an intrinsic response to a stimulus with a certain positive incentive value, but could rather be explained as a kind of reward anticipation. In the level searching set-up, rats have to be sexually trained in the bilevel chamber in order to obtain a stable number of level changes as measure for 'sexual motivation': they need to know what will happen in this box before they start showing this kind of behavior. As a result, the rewarding aspects of the copulation will get linked to the environment, turning the environment into a conditioned stimulus. Thus, the number of level changes seen by Mendelson and Pfaus could reflect this reward anticipation, which is elicited by the total emotional valence connected to the test environment by previous experience, instead of solely reflecting sexual incentive motivation.

These phenomena of sexual motivation and reward anticipation might have different neuroanatomical substrates. This seems to be supported by the juxtaposition of two papers that investigated the role of the μ-opioid receptor antagonist naloxone on sexual motivation. Using the bilevel chamber, Van Furth and Van Ree found that systemic administration of naloxone to experienced and inexperienced male rats decreases the number of level changes during both the anticipation and the interaction period [77,78]. Ågmo, however, using the SIM test, found no difference between rats that had been injected naloxone, and control rats that had been injected saline: both had an equal preference for a receptive female over a male [79]. This suggests that different neural substrates are activated in different tests, and thus that level changes measure something else than pure sexual incentive motivation (see also Holloway [80]). The level searching paradigm would therefore be unsuitable to study this type of sexual motivation.

The elucidation of these distinct mechanisms is further complicated because naive rats cannot be tested in the level searching paradigm. Sexual experience is a *conditio sine qua non* when level searching and extinction are measured. Sexual experience has been proven to be a modulator for both responses to olfactory stimuli in, and for copulation itself [61,72]. In fact, olfactory cues appear to be the most salient for incentive motivation in experienced males [7], and inexperienced males only seem to react to unambiguous odors [72]. In the bilevel chamber, Van Furth and Van Ree also found odor to be of particular relevance. Rats with a surgically impaired olfactory capacity did not show increased level changes during either the anticipation or the interaction phase, while their copulation behavior was comparable to control animals [77]. These results made them suggest that previously found level changes might have been induced by odors that were still present in the set-up from previous trials. These findings further stress the necessity to remove all odor of receptive females from the chamber in between trials.

4.4. Lever press paradigm

A well-known paradigm to research motivated behavior is the second-order schedule of reinforcement, in which the subject learns to perform work in order to receive a conditioned stimulus (CS), and ultimately the unconditioned stimulus (US). In an elaborate sequence of experiments, Everitt et al. operationalized this paradigm for use in the exploration of male sexual motivation [81].

A Plexiglas box measuring 28 × 26 × 28 cm is fitted with two retractable levers. Between these levers a magazine for the delivery of food pellets is placed. A small light source that functions as the CS is placed on the same wall as the levers. White noise (also CS) can be produced in the chamber. On top of this operant chamber, immediately above a trap door, a second, smaller box is placed, which contains a receptive female (US). Upon reaching of the necessary responses on the lever, the trap door opens and the female enters the center of the operant chamber, making her available for copulation. Prior to testing, rats are allowed to gain sexual experience. The full subsequent second-order schedule can be found in Everitt et al., 1987 and Everitt and Stacey 1987 [81,82]. In short, the main measurement for sexual motivation is expressed as the number of responses in a fixed, 15-min interval.

During the development of this paradigm, Everitt et al. reported some interesting findings, which we will summarize briefly, after which we will discuss the role a second-order paradigm can play in the investigation of sexual behavior: 1) On average, male rats took around 30–36 sessions to reach stable levels of performance. 2) Conditioning with both CS+ and CS- yielded the same results as conditioning with only CS+. 3) Omission of the CS+ during a single session resulted in a significant decrease in responses. 4) Rats that did not have a restricted diet (i.e. food ad libitum the night prior to testing), did not respond to food, if the food was used as the US. Rats' responses to gain access to the female, however did not decrease. 5) During a postejaculatory interval (PEI), the willingness to work for a sexual reward was reduced, but the willingness to work for food remained intact. 6) ejaculation latency is negatively correlated with number of earned CS + 's (i.e. rats that were more willing to work, or more successful to perform the task, had a shorter ejaculation latency). In addition, rats that were more successful with the lever presses, showed less intromissions before ejaculation at the moment they had access to the mate.

An obvious advantage of this paradigm is that both a form of motivation and copulatory behavior can be registered in one test, just as in the level searching paradigm, but not in the runway or SIM test. This way, as shown above, the willingness to work (which serves as a measure for motivation) can be directly linked to the subsequent copulatory parameters. This is a property which makes the test suitable for pharmacological interventions. However, a clear downside of this test paradigm, is that the susceptibility to motor, memory and attentional side effects is high. The paradigm employs learned operant responses as bar pressing for access to a mate. In case pharmacological interventions induce an increase in the number of responses, this could be mistaken for effects of learning, or memory of the procedure. Even more significant, however, is that the rate or speed of responding is an important factor in this operant procedure. A change in the motoric

capacity of the subject could, therefore, severely affect the motivational read-out. The SIM test, on the other hand, employs permanence in a particular area as an index of motivation, minimizing the requirement of motor capacities. The SIM test can, at the same time as investigating sexual motivation, measure the indices of ambulatory behavior (e.g. distance moved and speed of movement) in order to exclude potential effects on motor functions and to diminish the risk of false interpretations. To the contrary, although more relevant in this paradigm, this lever press paradigm alone cannot control for ambulatory behavior. A separate test of motor function can however be added.

More disadvantages can be described to the lever press paradigm, like the lack of relevance for the incentive value of the female as soon as the male had paired the effort to the reward. This lack of relevance is even more present here than in the bilevel chamber, because the male rat will be motivated to work based on previous experiences and the expectation of that happening again, but not because of the inherent attractiveness of the female. This was also evident when the receptive female was substituted by a non-receptive female. Even though it is likely that the male rat had a possibility, however limited, to smell, hear and see the female, it would continue to show the lever press levels as before. Only in session 6 and 7 there were signs of extinction, with the lever press activity decreasing by more than 50%. One explanation of this phenomenon is that the lever press action is decoupled from the incentive properties of the rewarding activity, and that the levers themselves gain reinforcing properties.

Regarding the ease of use, this second-order paradigm would demand involvement of a highly skilled and experienced researcher: planning and execution are intricate and time-consuming, while proper analysis of the data is complex.

4.5. Interpretation of results

In conclusion, the different test paradigms for sexual motivation actually measure different components of motivation or reward anticipation. It is clear that the interpretation of results is complicated and need extra attention. Based on our review, we believe that the level searching and lever press paradigms are not suitable to test sexual incentive motivation. They instead seem to measure reward anticipation more than the interaction between internal motivation state and incentive stimulus. Motivation can be split up in a component of innate sexual incentive motivation, that is activated by a perceived sexual stimulus, and a sexual motivation obtained by previous experiences. The second motivation could, thus, be seen as a strengthened incentive motivational response to the sexual stimuli by an increase in arousal caused by previous rewarding experiences. This complete incentive motivation, however, is still different from reward anticipation, because it is always a response to the presence of a sexual stimulus (which could be a receptive female or just the smell of a receptive female), rather than a reaction towards an associated situation like an environment without the stimulus. In this perspective, only the SIM test and the runway test are suitable to study sexual incentive motivation.

5. Concluding remarks

In summary, after describing all potentials and pitfalls of the different behavioral paradigms to study sexual behavior in rats, a few important lessons can be learned. First, it is absolutely crucial to use the appropriate model for the research. Whereas an incentive sexual motivation test is used to study sexual motivation, a copulation test until the 1st ejaculation can be useful to study e.g. the drug efficiency to treat premature ejaculation. On the other hand, when studying female sexual behavior, the use of a paced mating test allowing the female to control her sexual interactions is important. Second, in all cases, it is essential to be critical of the interpretation of results. We have given some examples in which a parameter was interpreted one way in the past, but where new knowledge has changed the perspective of interpretation. Third, some studies have not always investigated all aspects of the sexual behavioral pattern. Especially in female rat research, a shortcut was often taken by only measuring lordosis behavior and neglecting the paracopulatory behaviors. Therefore, we propose that the measured parameters should always be described in the most complete and neutral sense as possible. When all behaviors are described as they are, it allows for 1) changes in interpretations and 2) comparisons with other studies in the future.

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Paper II

Male rat sexual behavior: insights from inter-copulatory intervals

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Male rat sexual behavior: insights from inter-copulatory intervals

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Abstract

The assessment of sexual behavior in male rats with the aim of unraveling underlying neurobiological mechanisms has in the recent decades been reduced to the annotation of mounts, intromissions and ejaculations. To provide a better understanding of the structure and patterns of copulation, it is necessary to extend and tailor the analysis to the natural organization of male rat copulation. This will lead to better formulation of hypotheses about neurobiological underpinnings of behavior. Mounts and intromissions are naturally organized in mount bouts consisting of one or more copulatory behaviors and are interspersed with time outs. We hypothesized that time outs and the post-ejaculatory interval (inter-copulatory intervals) are related and possibly under the control of a common copulatory inhibition mechanism that is the result of penile sensory stimulation. To test this hypothesis, we analyzed sexual behavior in male rats of three different cohorts from three different laboratories. Results showed that the post-ejaculatory interval and mean time out duration are strongly correlated in all cohorts analyzed. In addition, we showed that individual time out duration is at least partially predicted by the sum of sensory stimulation of copulatory components in the preceding mount bout, with more penile stimulation associated with longer time outs. These findings suggest that both time out and post-ejaculatory interval duration may be determined by the magnitude of sensory stimulation, which inhibits copulation. Whether the same neural pathways are involved in the central orchestration of both time outs and the post-ejaculatory interval should be subject to future studies.

Keywords: mount bout, time out, post-ejaculatory interval, copulation, sexual behavior

1. Introduction

In order to understand the neurobiological mechanisms underlying the orchestration of copulation, it is important to understand the full range and patterning of the behavior in detail. Recently, a critical perspective has warned against a reductionist bias in behavioral neuroscience and called for more detailed behavioral analysis leading to better foundations for hypothesis generation about neurobiological underpinnings of behavior.[1] Male rats are an often-used animal model for sexual behavior in both basic and translational neuroscience research. Yet, behavioral annotation of copulation is often limited to the frequency of mounts, intromissions, and ejaculations, including parameters calculated from the times these behaviors occurred (e.g., latencies, post-ejaculatory interval, intromission ratio). A more detailed analysis of the organization and patterns of male rat copulation has been introduced in the past,[2] but has been underrepresented in studies of the more recent decades.

In the pioneering study by Sachs and Barfield (1970),[2] it was convincingly demonstrated that male rat copulation is temporally organized in mount bouts, which are defined as “a sequence of mounts (one or more), with or without intromission, uninterrupted by any behavior (other than genital autogrooming) that is not oriented towards the female”. Mount bouts are naturally separated by longer periods of no interaction with the female, defined as “time outs”. This mount bout pattern is not driven by intromissions, as males that can only mount still organize copulation in mount bouts of one or multiple mounts interspersed with time outs. Therefore, the mount bout should be considered the basic unit of copulation, and temporal patterning of copulation (copulatory pace) is better reflected in the

time outs between mount bouts than in the more traditionally used inter-intromission interval that disregards mounts.[2] Copulatory pace is an important pillar of male copulation as it determines the latency to ejaculation together with sensitivity (i.e., number of intromissions needed to reach ejaculation) and efficiency (i.e., achieved intromissions per total mounts). Therefore, pursuing a deeper understanding of the temporal organization of male copulation will contribute to the development of better theoretical concepts of the structure of copulatory behavior.

Like the time out, the post-ejaculatory interval (PEI) could also be considered a parameter of copulatory temporal organization. Both the PEI and the time out are inter-copulatory intervals, be it for different durations (e.g., PEI > time out). It is still unclear what neurobiological mechanisms underly the PEI. It has been shown that the PEI is a result of a central, rather than a peripheral (genital), neuronal inhibition,[3] and some brain regions and neurotransmitters have been implicated to be involved in the regulation of the PEI (e.g., galanergic signaling in the medial subparafascicular thalamus, and falling levels of glutamate and dopamine in the medial preoptic area; reviewed by Seizert (2018)[4]). But still, the neurobiological orchestration of this strong and partially absolute central inhibition remains to be elucidated. Likewise, the neurobiological regulation of inter-copulatory-intervals that are observed before ejaculation (i.e., time outs), remain elusive. In view of both the PEI and the time out being the result of a copulatory inhibition, both of these inter-copulatory intervals might be regulated by the same neuronal inhibitory mechanism. Therefore, the investigation of how inter-copulatory intervals relate to each other in the complex structure and pattern of male copulatory behavior is important.

Some evidence for the possible relationship between inter-copulatory intervals is found in several correlational and factor analysis studies of male rat sexual behavior.[5-7] The PEI consistently loads onto the same factor as the inter-intromission interval (III) together with the number of ejaculations and ejaculation latency, referred to as the “copulatory rate factor”. In addition, the PEI, III, and time out are all longer in older compared to younger naive male rats,[8] and both the PEI and III are shortened upon enforced inter-copulatory intervals (making the female unavailable for a short amount of time),[9] suggesting a relationship between these parameters. Conversely, the PEI increases over each subsequent ejaculation series, whereas the mean III duration follows a U-shape over ejaculation series.[10] Following our notion that the time out, and not the III, is the natural inter-copulatory interval before ejaculation, as the mount bout is the basic copulatory unit, we hypothesize that PEI and time out duration are closely related within individual rats, and more strongly correlated than PEI and III.

The PEI is clearly induced by a strong sensory stimulus, namely ejaculation. If the PEI and the time out are related, it is to be expected that time outs are also induced by sensory stimulation in the preceding mount bout. Both mounts and intromissions contribute to achievement of ejaculation, but intromissions provide stronger sensory penile stimulation than mounts.[11] However, it has been found that prevention of intromissions does not change the distribution of time outs,[2,12] and the same lab found that the mean time out duration does not depend on the last behavior (mount or intromission) within the preceding mount bout.[13] Still, intromissions are far more likely to end a mount bout than extravaginal intromissions (motorically identical to intromissions but without penile insertion) or mounts.[13] These results trigger the question of whether the total sensory stimulation of the sum of copulatory components within the mount bout might predict the duration of the following time out. If so, there would be reason to believe that both ejaculation and mount bout induce a similar copulatory inhibition that is determined by the magnitude of sensory stimulation.

We present a detailed description of the mount bout organization of copulation based on behavioral analysis of three different male rat cohorts from three different laboratories. We assessed correlation of PEI and time out within rats, and how these parameters change over ejaculation series as well as across repeated copulation sessions. Moreover, we determined what mount bout characteristics predict the duration of the directly following time out. Our findings lead us to hypothesize that a central inhibitory mechanism might control both the temporal patterning of copulatory behavior within an ejaculation series, as well as the time in between ejaculation series.

2. Materials and Methods

The data presented in this paper consists of three male rat cohorts from three different laboratories in three different locations, from here on referred to as the “Tromsø” (Snoeren lab), “Groningen” (Olivier lab), and “Texas” (Guarraci lab) cohorts.

2.1 Animals

Tromsø. The data from this cohort comes from a previously published experiment [14]. For the purpose of this previous experiment, the 53 male Wistar rats (Charles River, Sulzfeld, Germany) of approximately three months old had undergone brain surgery during which a viral construct coding for Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) was infused bilaterally into the medial amygdala. The data set used in the current paper consists of annotations from a copulation test preceded by an intraperitoneal injection with vehicle (deionized water), 45 minutes before the copulation test. Since DREADDs are inert without the ligand clozapine-N oxide present, no effects are to be expected of these manipulations. The surgery and injections are thus of no significance for the purpose of the current study, for which we were solely interested in behavioral patterns of copulating rats.

Rats were housed in Macrolon IV® cages on a reversed 12h light/dark cycle (lights on between 23:00 and 11:00) in a room with controlled temperature (21 ± 1 °C) and humidity (55 ± 10%), with *ad libitum* access to standard rodent food and tap water. Animals were housed in same-sex pairs with exception of a one-week post-surgery recovery period during which males were single-housed. Males underwent 3 sexual training sessions (once a week) before behavioral testing.

A total of 36 female Wistar rats were ovariectomized as previously described[15] and used as stimulus animals during the copulation sessions. Briefly, a medial dorsal incision of the skin of about 1 cm was made, and the ovaries were located through a small incision in the muscle layer on each side. The ovaries were extirpated and a silastic capsule containing 10% 17β-estradiol (Sigma, St. Louis, USA) in cholesterol (Sigma, St. Louis, USA) was placed subcutaneously through the same incision. The muscle layer was sutured and the skin was closed with a wound clip. One week of recovery was allowed before the females were used in a copulation session. Four hours before behavioral assessment, female rats were subcutaneously injected with 1 mg progesterone (5 mg/mL; Sigma, St. Louis, USA) in peanut oil (Apotekproduksjon, Oslo, Norway)) to induce receptivity.

Groningen. 29 male Wistars Unilever (Envigo, Venray, the Netherlands) Rats (approximately 7-8 months old) were housed under reversed 12h light/dark cycle (lights on between 20:00 and 08:00) with *ad libitum* access to food and water. Males underwent behavioral assessment weekly for 7 weeks.

Forty female rats were tubal ligated in order to prevent pregnancies. To perform tubal ligation surgery, females were anesthetized (Isoflurane) and given pain relief (Fynadine, 0.1 mg/100 g) before surgery, and 24 and 48 h after surgery. Females were at least 12 weeks old when surgery was performed, and 2 weeks of recovery were given before receptivity was induced with estradiol (50 µg in 0.1 ml oil, S.C.) 36–48 h before the copulation test. Females were used not more than once in 2 weeks and not more than two times per experimental day.

Texas. The data from this cohort comes from two different batches of Long-Evans males (Envigo, Indianapolis, IN, USA); 8 males were approximately 7-8 months old, and 4 males were approximately 3-4 months old during the experiment. Rats were pair housed with same-sex cage mates in hanging polycarbonate cages. The animals were kept on a reversed 12h light/dark cycle (lights on between 22:00 and 10:00) in a room with controlled temperature and humidity, with *ad libitum* access to standard rodent food and tap water. The eight older males in this cohort had previously gained sexual experience as stud males in a female paced-mating set-up. The four younger males were trained in the copulation test set up once per week for three weeks prior to observations for the present study.

Ten Long-Evans females (Envigo, Indianapolis, IN, USA) were ovariectomized at least one week before any behavioral testing took place and used as stimulus animals. To induce sexual receptivity, females were subcutaneously administered 10 µg of estradiol benzoate (Sigma, St. Louis, USA) in sesame oil 48 hours prior to the copulation test, and 1 mg of progesterone (Sigma, St. Louis, USA) in sesame oil 4 hours prior to the copulation test.

The males in the Tromsø, Groningen, and Texas cohorts were selected on the basis of the occurrence of at least one post-ejaculatory interval within a standard 30-minute copulation test.

2.2 Copulation test

Tromsø. Male subjects were assessed in the copulation test directly after being tested in the sexual incentive motivation test (as part of a previous study[14]). The sexual incentive motivation test consists of a 10-minute free exploration of an arena and socio-sexual stimulus animals that are not accessible for contact interaction. The male subjects were habituated to the sexual incentive motivation test and so no effects on the copulation tests are to be expected. The copulation test, and focus of the current study, was conducted in

rectangular boxes (40 × 60 × 40 cm) with a Plexiglas front filled with regular wood chips, in a room with lights on. A receptive female was placed in the copulation box, after which the experimental subject was introduced to start the test.

Groningen. The copulation test occurred in wooden rectangular (57 cm × 82 cm × 39 cm; glass wall) boxes with regular wood chips covering the floor, in a room with red light. Rats habituated for 10 min to the testing box right before the test session. After the habituation period, a receptive female was introduced into the box, which started the test.

Texas. The copulation test was conducted in rectangular plexiglass boxes (37 × 50 × 32 cm) with regular bedding material (Aspen wood shavings) covering the floor, in a room with red light. A receptive female was placed in the copulation box, after which the experimental subject was introduced and the test was started.

All copulation tests in all labs were conducted during lights-off time, lasted for 30 minutes, and were recorded on camera. Behavior was later assessed from video.

2.3 Behavioral assessment

Tromsø. Copulation tests were assessed from session 4 (half of the males) and session 5 (half of the males). Males had thus gained sexual experience during 3 or 4 sessions prior to assessment. Behavioral annotation was done for the first ejaculation series (i.e., until the first mount or intromission after the first post-ejaculatory interval).

Groningen. Copulation tests were assessed from session 4 (half of the males) and session 5 (half of the males). Males had thus gained sexual experience during 3 or 4 sessions prior to assessment. In addition, session 7 (i.e., after an additional 2-3 sessions of sexual experience allowance) was assessed for all of the males. Behavioral annotation for all of the sessions was done for the first ejaculation series, as well as for the second ejaculation series if 2 post-ejaculatory intervals occurred during the 30-minute test.

Texas. Eight of the males in the Texas cohort had previously gained extensive sexual experience as stimulus animals during tests of paced mating behavior. Session 2 of the copulation tests as described was used for assessment of these animals. The remaining four animals only gained sexual experience in the copulation test, and behavioral assessment was done from session 5 (these animals had thus gained sexual experience during 4 sessions prior to assessment). Behavioral annotation for all of the males was done for the first ejaculation series, as well as for the second ejaculation series if 2 post-ejaculatory intervals occurred during the 30-minute test.

All cohorts. Behavioral assessment consisted of scoring behavioral events by means of the Observer XT version 12 software (Noldus, Wageningen, the Netherlands). For 1 (Tromsø) or 2 ejaculation series (Groningen and Texas) we behaviorally annotated 100% of the elapsed time according to the following ethogram: the copulatory behaviors mount, intromission, and ejaculation; clasping (mounting the female without pelvic thrusting); genital grooming (grooming of own genital region); other grooming (autogrooming in other regions than genital); chasing (running after the female); anogenital sniffing (sniffing the anogenital region of the female); head towards female (head oriented in the direction of the female while not engaging in other behavior); head not towards female (any behavior that is not oriented towards the female except grooming, such as walking, sniffing the floor, standing still with head direction away from female). For mount bout and time out analysis, the definition as posed by Sachs and Barfield was employed[2]: "A sequence of mounts (one or more), with or without intromission, uninterrupted by any behavior (other than genital autogrooming) that is not oriented towards the female". Mount bouts and time outs during the copulatory tests were identified through review of the events between copulatory behaviors (mounts, intromissions, and ejaculations). If any other behavior other than genital grooming or "head towards female" occurred between copulatory behaviors, this marked the end of one mount bout (i.e., time of the end of the last copulatory behavior) and beginning of the next mount bout (i.e., time of the next copulatory behavior), and the time in between as a time out duration (see Figure 1A for a schematic overview). From these data points the outcome measures as listed in table 1 were determined (see also [16]).

2.4 Data analysis and statistics

Correlation between PEI, III and time outs. The post-ejaculatory interval versus mean time out duration and the inter-intromission interval for the corresponding ejaculation series were analyzed with Pearson correlation coefficients. The mean time out duration was calculated for each subject from all time outs in the corresponding ejaculation series.

Analysis of copulation and mount bout characteristics. The behavioral data used for comparisons between cohorts were not normally

Table 1 Copulation test outcome measure definitions

Outcome measure	Definition
Latency to first mount or intromission	Time from the start of the test to the first mount or intromission
Number of mounts	Total number of mounts preceding ejaculation
Number of intromissions	Total number of intromissions preceding ejaculation
Intromission ratio	Number of intromissions in the ejaculation series divided by the total number of copulatory behaviors (mounts + intromissions) in the ejaculation series
Number of mount bouts	Total number of mount bouts preceding ejaculation
Mounts per mount bout	Mean number of mounts per mount bout in an ejaculation series
Intromissions per mount bout	Mean number of intromissions per mount bout in an ejaculation series
Mount bout duration	Time from the first copulatory behavior in a mount bout until the first behavior within the following time out
Time out duration	Time from the end of one mount bout to the start of the next mount bout
Inter-intromission interval	Time between intromissions in an ejaculation series, calculated from the first intromission
Latency to ejaculation	Time from the first mount or intromission to ejaculation
Post-ejaculatory interval	Time from the first ejaculation to the next copulatory behavior (mount or intromission)

distributed and were therefore analyzed with non-parametric tests. The Kruskal-Wallis test followed by Dunn's multiple comparison posthoc test was employed for comparisons between copulation test outcome parameters of the three different cohorts.

Within-subject consistency within and across copulatory sessions. The Wilcoxon matched-pairs signed rank test was used to analyze the data for ejaculation series 1 compared to ejaculation series 2 in the Groningen and Texas cohorts. Pearson correlation coefficients was employed to analyze the relation of PEI and time out in the different ejaculation series, as well as to analyze the relation of PEI/time out in ejaculation series 1 and PEI/time out in ejaculation series 2.

Time out predictors. The duration of each mount bout versus the duration of its following time out was analyzed with Pearson correlation coefficients. For comparison of data corresponding to individual mount bouts/time outs, data points were z-scored within each rat using the following calculation: z-score = ((data point) – (mean of the data points for the rat))/(standard deviation of the data points for the rat). Z-scores of the different cohorts were then analyzed by means of Mann Whitney U tests in case of 2 groups, or Kruskal-Wallis and Dunn's posthoc tests for 3 or more groups. For the time-binned analysis of time out duration, the first 33% of time outs were defined as time-bin 1, the second 33% as time-bin 2, and the last 33% as time-bin 3. For the time out duration per mount bout stimulation analysis, mount bout types with less than 10 data points were excluded from analysis (e.g. 3 mounts, 2 intromissions).

The behavioral data were extracted from the Observer data files and analyzed using custom Python 3.8 scripts. The scripts are available for sharing upon request. All statistical analyses were performed in GraphPad Prism version 9.0.0 (GraphPad Software, San Diego, CA, USA). In all cases, alpha was set at 0.05 and tests were two-tailed.

3. Results

3.1 Relation of inter-copulatory intervals

Correlation between PEI, III and time outs. Our analysis first focused on how inter-copulatory intervals, i.e. the post-ejaculatory interval (PEI), time outs, and inter-intromission interval (III), relate to one another. Our mount bout-based analysis (Fig. 1A) showed that the PEI was strongly correlated with the mean time out duration in all of the cohorts: Groningen (Figure 1 B; r=0.81, p<0.001), Tromsø (Fig. 1B; r=0.74, p<0.001), and Texas (Fig. 1B; r=0.79, p=0.002). Correlation between the PEI and the III was also strong in the Tromsø cohort (Fig. 1C; r=0.79, p<0.001), but weak in the Groningen cohort (Fig. 1C; r=0.46, p=0.01), and not significant in the Texas cohort (Fig. 1C; r=0.49, NS).

Analysis of copulation and mount bout characteristics. We next examined whether the difference in correlation strength of PEI vs. III between the cohorts could be explained from copulatory parameters. All copulatory parameters and comparisons between the three cohorts can be found in Supplementary Table 1. Only those parameters that are relevant for the current assessment will be discussed in this section. We hypothesized that PEI vs. III correlation is stronger in cohorts in which the III resembles the mean time out duration. If each mount bout consists of only a single intromission, mean time out duration and III are

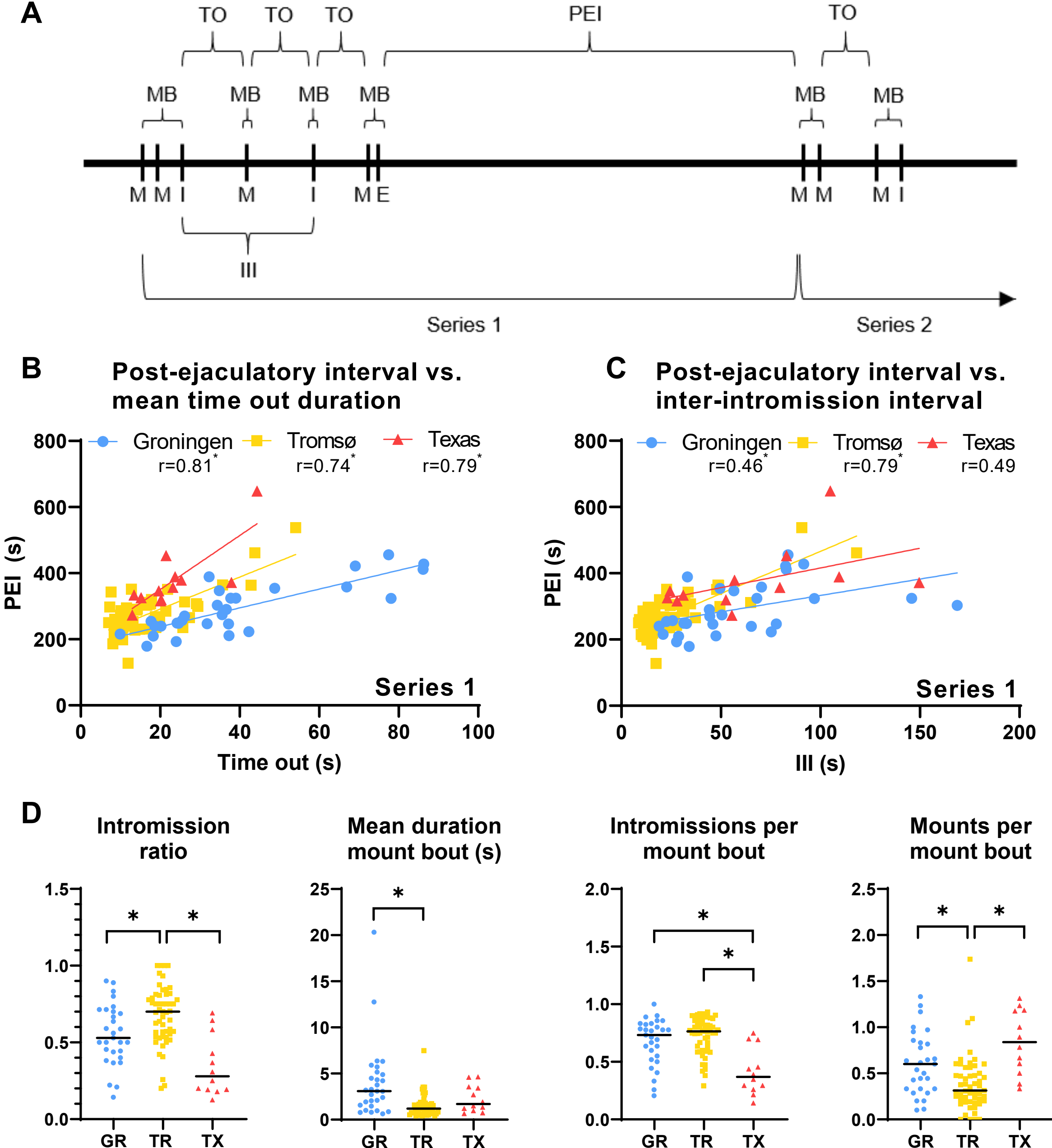

Figure 1 The post-ejaculatory interval correlates with mean time out. **(A)** Schematic overview of male sexual behavior organization. M; mount, I; intromission, MB; mount bout, TO; time out, III; inter-intormission interval, PEI; post-ejaculatory interval. **(B)** Correlation of post-ejaculatory interval and mean time out duration for ejaculation series 1 for Groningen, Tromsø, and Texas cohorts. **(C)** Correlation of post-ejaculatory interval and inter-intromission interval for ejaculation series 1 in Groningen, Tromsø and Texas cohorts. **(D)** Copulation parameters for all cohorts: intromission ratio, mean mount bout duration, mean number of mounts per mount bout, and mean number of intromissions per mount bout. Horizontal lines; median. **All panels:** n=29; 53; 12, *p<0.05

the same. Thus, III more strongly approaches mean time out duration in cohorts with a high number of intromissions, short mount bouts, and more mount bouts with an intromission and relatively few mounts. We found that the copulatory parameters intromission ratio (i.e., number of intromissions divided by total number of copulatory behaviors) (Fig 1D; $H(2)$=21.67, p<0.001), mean duration of mount bout (Fig 1D; $H(2)$=20.30, p<0.001), mean number of mounts per mount bout (Fig 1D; $H(2)$=20.47, p<0.001), and mean number of intromissions per mount bout (Fig 1D; $H(2)$=17.51, p<0.001)in the first ejaculation series differed significantly between the cohorts. The Tromsø cohort had a larger intromission ratio (Fig 1D; p<0.001), more intromissions per mount bout (Fig 1D; p<0.001), and less mounts per mount bout (Fig 1D; p<0.001) than the Texas cohort. The Tromsø cohort also had a larger intromission ratio (Fig. 1D; p=0.023), a shorter mean

mount bout duration (Fig 1D; p<0.001) and less mounts per mount bout (Fig. 1D; p=0.007) than the Groningen cohort. The Groningen cohort had more intromissions per mount bout than the Texas cohort (Fig 1D; p=0.003). These results show that correlation between PEI and III is indeed stronger when time out and III are similar, as is the case in the Tromsø cohort, and explains why the PEI and III correlated stronger in this cohort than in the other cohorts.

Within-subject consistency within a copulatory session. To see whether the mean PEI duration and mean time out duration followed the same pattern over time within the same rats, we looked at how these parameters change from the first ejaculation series to the second ejaculation series within a copulation session, and over different copulation sessions. In the Groningen cohort, the PEI (Fig. 2A; *W*=-251, p<0.001) as well as the mean time out duration (Fig. 2B; *W*=-129, p=0.036) increased in the second ejaculation series compared to the first ejaculation series. We did not find statistically significant effects in the Texas cohort for ejaculation series 1 compared to ejaculation series 2. The PEI of ejaculation series 2 also correlated with the mean time out duration in ejaculation series 2 in the Groningen cohort (Fig. 2C; *r*=0.70, p=0.002) as it did in ejaculation series 1. There was moderate correlation of PEI and mean time out duration in ejaculation series 2 in the Texas cohort, but this was not statistically significant (Fig. 2C; *r*=0.66, NS).

Within-subject consistency across copulatory sessions. Both the PEI and the mean time out duration in the first ejaculation series did not show significant correlation from one copulation session (the 4th or 5th occasion of copulation) to another copulation session (the 7th occasion of copulation) in the Groningen cohort (Fig. 2D-E). However, the correlation of PEI and mean time out duration in the first ejaculation series was persistent over multiple copulation sessions, as the effect was still present and of the same magnitude in the later copulation session of the Groningen cohort (Fig. 2F; r=0.88, p<0.001). Thus, PEI and mean time out duration vary over copulation sessions within rats, but the correlation of the two parameters within each copulation session is consistent.

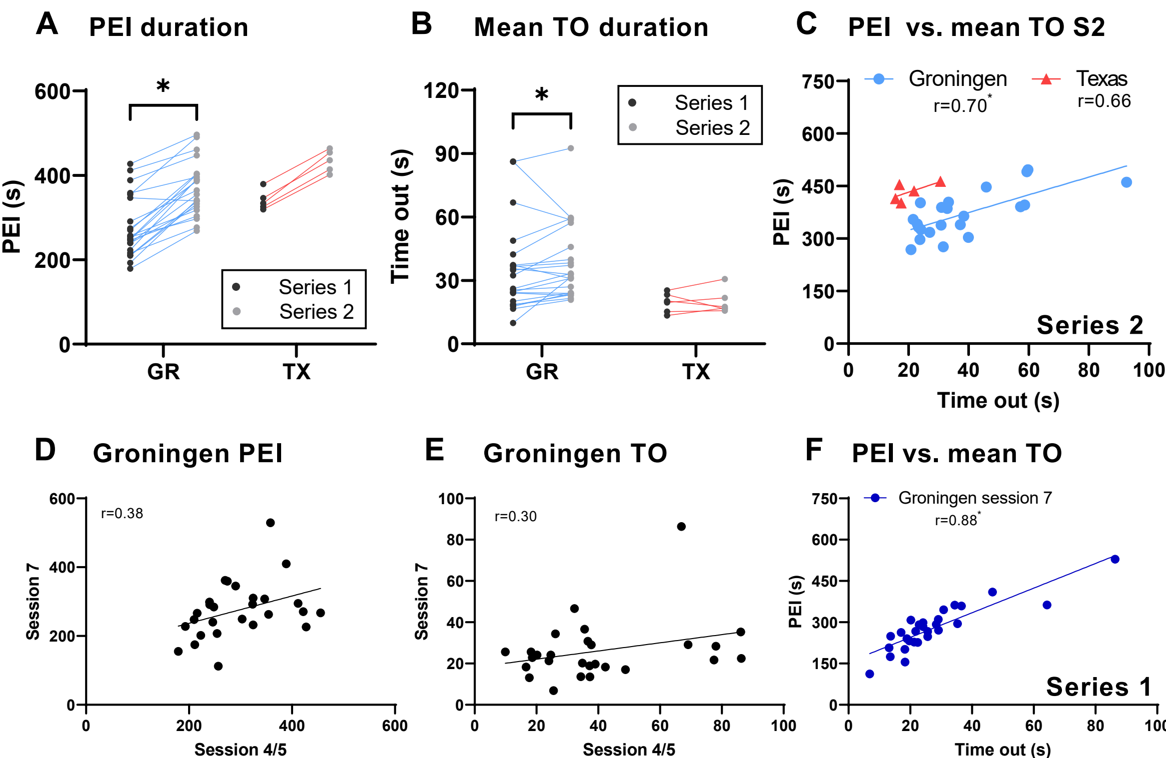

Figure 2 Post-ejaculatory interval and time out both increase over ejaculation series. **(A)** Post-ejaculatory interval duration in ejaculation series 1 compared to ejaculation series 2 within the same animals from the Groningen and Texas cohorts, n=22; 5. **(B)** Mean time out duration in ejaculation series 1 compared to ejaculation series 2 within the same animals from the Groningen and Texas cohorts, n=22; 5. **(C)** Correlation of post-ejaculatory interval and mean time out duration for ejaculation series 2 in the Groningen and Texas cohorts, n=22; 5. **(D)** Correlation of post-ejaculatory interval in copulation session 4/5 with copulation session 7 within the same Groningen animals, n=27. **(E)** Correlation of mean time out duration in copulation session 4/5 with copulation session 7 within the same Groningen animals, n=27. **All panels:** PEI; post-ejaculatory interval, TO; time out, *p<0.05

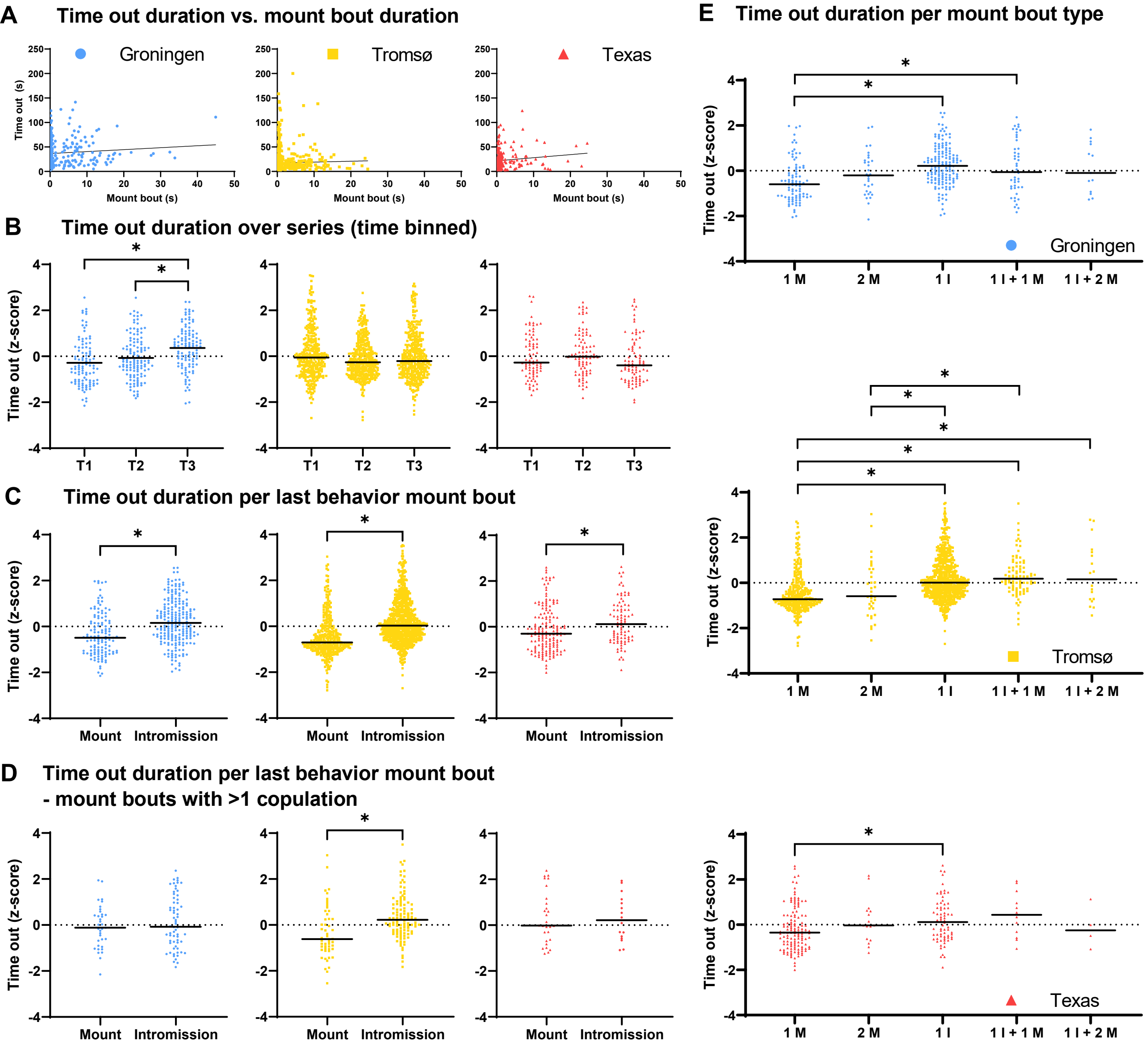

Figure 3 More stimulation within mount bout is associated with longer time out. **(A)** Correlation of individual mount bout duration with subsequent time out duration in Groningen, Tromsø and Texas cohorts, n=341; 1118; 249. **(B)** Z-scores of individual time out durations during the first, second, and third third of the ejaculation series; Groningen (n=103; 123; 114), Tromsø (n=354; 388; 376) and Texas (n=79; 84; 83). **(C)** Z-scores of individual time out duration after mount bouts with mount vs. intromission as last copulation; Groningen (n=124; 216), Tromsø (n=366; 751) and Texas (n=160; 86). **(D)** Z-scores of individual time out duration after mount bouts consisting of multiple copulations with mount vs. intromission as last copulation; Groningen (n=35; 64), Tromsø (n=52; 111) and Texas (n=28; 16). **(E)** Z-scores of individual time out durations after mount bouts with different total copulatory stimulation; Groningen (n=88; 30; 152; 45; 13), Tromsø (n=314; 36; 640; 87; 18) and Texas (n=132; 16; 70; 12; 4), M; mount, I; intromission. **All panels:** Horizontal lines; median, *p<0.05.

3.2 Time out predictors

We next assessed whether any mount bout characteristic predicted the duration of the subsequent time out. First, the duration of individual mount bouts did not correlate with the duration of the subsequent time out (Fig. 3A). Second, we considered that copulatory pace might be faster or slower depending on how close the male is to ejaculation. Therefore, we examined whether individual time out duration is dependent on the relative time point within the ejaculation series. We divided the ejaculation series into three-time bins, each consisting of a third of the total number of time outs within the ejaculation series, and analyzed whether standardized (z-scored within subject) time out duration differs between time bins for each of

the cohorts. We found that standardized time out duration was different over time bins in the Groningen cohort (Fig. 3B; $H(2)$=28.28, p<0.001): the median time out duration was longer in the third time bin compared to both the second (Fig. 3B; p<0.001) and the first time bin (Fig. 3B; p<0.001). We did not find this effect in the Tromsø or Texas cohort (Fig. 3B).

Third, we assessed whether mount bouts that end in an intromission might induce a longer time out than mount bouts that end in a mount. We found that the median duration of time outs that follow a mount bout ending with an intromission was shorter than the duration of time outs that follow a mount bout ending with a mount in the Groningen (Fig. 3C; U=8834, p<0.001), Tromsø (Fig. 3C; U=72940, p<0.001), and Texas (Fig. 3C; U=5496, p=0.009) cohorts. To examine whether this effect of the last behavior within a mount bout is independent of the number of copulations within the mount bout, we ran the same analysis after exclusion of all time outs that followed mount bouts consisting of only a single copulatory behavior. This analysis showed that the effect disappeared in the Groningen and Texas cohorts, but remained in the Tromsø cohort (Fig. 3D; U=1739, p<0.001). We also noted that of the mount bouts with multiple copulations, only 5 out of 117 (4.3%) mount bouts that ended in a mount also contained an intromission (data not shown). This is consistent with our observation that 1057 out of 1068 intromissions (99%) in the full data set ended the mount bout (data not shown). These results indicated that the significant effects of the last behavior within a mount bout on the subsequent time out duration might be a function of the sum of components of the mount bout, which might rather be the true predictor of time out duration.

Fourth, based on the conclusion above, we hypothesized that the total sensory stimulation within the mount bout predicts the following time out duration. We defined mount bout types by the sum of copulatory components of the mount bout and compared the duration of following time outs between mount bout types (bouts with a) 1 mount, b) 2 mounts c) 1 intromission, d) 1 intromission + 1 mount, e) 1 intromission + 2 mounts. Indeed, there was a significant difference between the median standardized time out duration after different mount bout types in all of the cohorts: Groningen (Fig. 3E; $H(4)$=34.30, p<0.001), Tromsø (Fig. 3E; $H(4)$=162.0, p<0.001), and Texas (Fig. 3E; H(4)=11.6, p=0.020). Time outs following mount bouts of 1 mount had a shorter median duration than time outs following mount bouts of 1 intromission in the Groningen (Fig. 3E; p<0.001), Tromsø (Fig. 3E; p<0.001), and Texas (Fig. 3E; p=0.046) cohorts. This was also the case for 1 mount compared to 1 intromission and 1 mount in the Groningen (Fig. 3E; p=0.013) and the Tromsø cohort (Fig. 3E; p<0.001), and compared to 1 intromission and 2 mounts in the Tromsø cohort (Fig. 3E; p=0.009). In addition, time outs following a mount bout of 2 mounts had a shorter mean duration compared to time outs following mount bouts of 1 intromission (Fig. 3E; p=0.008) and compared to 1 intromission and 1 mount (Fig. 3E; p=0.001) in the Tromsø cohort only. Additional mounts in mount bouts with intromissions did not lengthen the subsequent time out any further in any of the data sets (Fig. 3E).

4. Discussion

In behavioral neuroscience, it is important to know as much about the structure and organization of the behavior under investigation as possible, because a detailed understanding of the behavior lends itself to better assessment of causal neurobiological mechanisms underlying the behavior. In light of this, the advancement of research on male rat sexual behavior has been disappointing in the recent decades, as behavioral assessment of copulation is most often reduced to the annotation of mounts, intromissions, and (usually one) ejaculation only. Unfortunately, the pioneering study by Sachs and Barfield on temporal patterning of male rat copulation has not had a lasting impact. The relationship between, and predictors of inter-copulatory intervals in male rat sexual behavior have yet to be elucidated. In the current study, we showed that the PEI is strongly correlated with mean time out duration and that time out duration is at least partially predicted by the total sensory stimulation in the preceding mount bout. These conclusions are remarkable because they were observed in three different cohorts of rats, of two different strains, and in rats of different ages from different origins. In addition, the experiments were carried out in three different laboratories in different geographical locations, with slightly different procedures. This emphasizes the generalizability of our results in the context of male-paced copulation in rats. Our findings advance our understanding of how the PEI and time out are related and possibly regulated by a similar central neuronal inhibition. Our results show how a more detailed analysis of behavioral structure and organization can provide valuable insights for future research.

4.1 Relation of inter-copulatory intervals

That the PEI and the III, the most common measure of temporal patterning in recent literature, are related was already apparent from factor analyses in which these parameters load onto the same factor.[5,6] However, as mount bouts and time outs are a better measure of the natural temporal patterning than III in male rat copulation,[2] our finding that time outs have a stronger correlation with PEI than III is logical. The III disregards mounts even though they are central copulatory behaviors and contribute to the facilitation of ejaculation,[11] and is strongly dependent on the intromission ratio, or efficiency, of the male. Still, in our data set, PEI and III are also strongly correlated in the Tromsø cohort. This can be explained by the notion that this cohort had a high intromission ratio, a short mount bout duration, relatively few mounts per mount bout, and at least 1 intromission in the majority of mount bouts. These copulation characteristics make for the mean time out duration to strongly approximate the III, which would be much larger than the time out when more mounts and less intromissions occur per mount bout. This explains the strong PEI and III correlation in the Tromsø cohort and emphasizes how this correlation is dependent on how closely mean time out duration resembles III. We stress again that mount bout-based analysis should be standard for assessment of copulatory pace of male rat copulation and that III is not sufficient for this goal. As an example for the general utility of mount bout-based analysis, we were recently able to draw more informed conclusions about the cause of increased ejaculation latency upon a manipulation.[14] Because there was no effect on time out duration, and thus on copulatory pace, in this study, we could state that the prolonged ejaculation latency was caused by a decreased sensitivity to reach ejaculation threshold. In order to advance the field of sexual behavior further, it is vital to have a better behavioral understanding in depth and to measure the parameters of the natural organization of copulation in the form of mount bouts and time outs.

It has previously been shown that the PEI increases over each following ejaculation series when males copulate to exhaustion,[10] whereas the III follows a U-shape, and no data to our knowledge has been published on time out. Since PEI and time out strongly correlates with mean time out duration, and not reliably with III, it would follow logically that mean time out would follow a similar pattern over ejaculation series as the PEI. We indeed found that the PEI and mean time out duration in our Groningen cohort was longer in the second ejaculation series than in the first. There was a similar trend in the Texas cohort, but this was not statistically significant due to a much smaller sample size. We did not have the data to investigate the course of the time out over more than two ejaculation series, but it would be interesting if future research could focus on analysis of males copulating to exhaustion, yielding more ejaculation series to study trends over time. The strong correlation between PEI and mean time out, together with the fact that both of these parameters increase over ejaculation series, suggests that the orchestration of both these intervals on the neurobiological level could be related.

4.2 Within-subject consistency of inter-copulatory intervals

We found that both the PEI and the time out are not correlated across copulation sessions within the same cohort of males. Thus, temporal patterning of copulation, as measured by inter-copulatory intervals, varies from session to session. This is consistent with the fact that the PEI does not seem to be a part of sexual behavior endophenotypes in male rats, as rapid ejaculators do not have a shorter PEI than normal ejaculators.[17] Importantly, even though the PEI and time out vary over copulation sessions, the strong correlation between these two parameters was consistent over sessions, indicating that their variation is unidirectional over sessions within a rat. All of the males in our cohorts were allowed sufficient recovery after each copulation session as they were behaviorally tested only once per week, which is enough for all copulation parameters to return to baseline even after exhaustion.[18] We hypothesize that variability over sessions in copulatory pace (as determined by PEI and time out) could simply be caused by daily condition of the male, or is perhaps dependent on the female stimulus. In all of our labs, females are paired with males at random and replaced in case of signs of reduced receptivity or sexual rejection (either of which rarely occur). It has been shown that the III and number of intromissions in the first ejaculation series (as well as a trend for PEI) in a semi-natural environment are different when domesticated males mate with females of the same strain versus females that are caught in the wild.[19] In the same study it was demonstrated that 84% of female paracopulatory behavior episodes are followed by an intromission, whereas only 13% of male-initiated copulations (i.e. not preceded by female paracopulatory behavior) resulted in an intromission. The authors note that differences in paracopulatory behavior frequency seems to account for

the copulation difference of males mating with the same strain versus with a wild female.[19] It needs to be addressed though, that males and females initiate copulation in a semi-natural environment just as often, and that the occurrence of copulatory acts is not mainly controlled by the female.[20] Even though copulatory pacing is thus shared between males and females in a semi-natural environment and seemingly controlled by the male in the standard copulation apparatus, these findings indicate that females are capable of exerting some control over male copulation speed and efficiency. Additional evidence for this notion comes from an experiment in which females were removed after each mount bout and returned to the copulation apparatus upon a bar press.[21] Under these circumstances, the mean time out duration increased, suggesting a stimulatory role of the presence of the female on the reinitiation of mounting by the male. This effect might still be minimal in an *ad libitum* male-paced setting, but possibly relevant for slight session-to-session variability in inter-copulatory intervals if there are individual differences between stimulatory properties of the female in the context of male-paced mating, perhaps found in the number of paracopulatory behaviors displayed by the female. It would be interesting to further investigate the role of the female in male-paced mating protocols.

4.3 Mount bout predictors of following time out duration

Because the III first increases and then decreases over time within the ejaculation series,[22] we examined how the time out duration is distributed within the first ejaculation series. We found that time outs in the third time bin of the ejaculation series in the Groningen cohort were significantly longer than in the first- and second-time bins. We did not find this effect in the other cohorts. A possible explanation for this discrepancy could be that in Groningen, the male subject has a 10-minute habituation to the copulation box (not cleaned) before the female is introduced, whereas in Tromsø and Texas the male is introduced after the female. The habituation in the copulation box, soiled with pheromones and odors, could have increased sexual arousal before introduction of the female, leading to a shortening of time outs in the start of the copulation test and a gradual normalization over the ejaculation series. This is in line with the fact that we did not find effects of time bin on time out duration in the second ejaculation series of the Groningen cohort (Suppl. Fig. 3A). Overall, the time out does not consistently vary over time within the ejaculation series as the III does, but more research into the role of sexual arousal on time out would be interesting

Next, we showed that time outs following mount bouts that ended with an intromission were longer than time outs following mount bouts that ended with a mount. Pollak and Sachs (1976) have reported a similar assessment from two cohorts of males (n=7 and n=5) and found that time out duration after mount bouts that ended with an intromission was increased by 26% and 9% for the two replicates respectively, although not statistically significant.[13] Our data shows a similar magnitude of time out duration increase, but we did find a statistically significant effect in our data set. Because we analyzed on the level of individual time out that was standardized for each rat by z-scoring, instead of analyzing the average for each subject rat, our data set consists of a much larger sample size. The advantage of this approach is that the z-score better reflects the difference in duration between time outs within a rat, while making it possible to still analyze on a group level and compare between different cohorts. This difference in approach compared to Pollak and Sachs, and our much larger number of male subjects, could account for our different statistical outcomes. One other reason that our results reached statistical significance, but not the results from Pollak and Sachs, may be that our data set consisted of a relatively high percentage of mount bouts consisting of only a single copulatory behavior, whereas Pollak and Sachs report a mean number of 1.5 mounts per mount bout in their cohort. This difference in behavioral phenotype may possibly be due to changes in genetic make-up of animals over time. When excluding the mount bouts with a single copulation from analysis, we found a smaller effect of the last event in a mount bout on the following time out. Still, since 99% of intromissions end a mount bout (similar to 90% reported by Pollak and Sachs), mount bouts of multiple copulations ending in a mount are far less likely to include an intromission as well. Therefore, we proceeded with analyzing whether the total stimulation within the mount bout might be the determining factor for the duration of the subsequent time out.

We found that mount bouts consisting of 1 intromission (or 1 intromission and 1 mount in two of the cohorts) induced a longer time out than mount bouts consisting of 1 mount in all of the cohorts. This seems incongruent with earlier reports that show that time out duration distribution is not affected by the prohibition of intromissions.[2,12] However, males that could not intromit tend to have more mounts per mount bout: from 1.5 to 2.6 upon penile lidocaine application[13] and from 2.5 to

3.1 and 9.9 when mating with a female with closed vagina or upon penile tetracaine application, respectively, although not statistically significant.[2] Therefore, if time out duration following mount bouts of 3, 4 or even more mounts is similar to time out duration following mount bouts with at least 1 intromission, it is very possible that no effect would be found of intromission prevention on time out duration distribution. In a data set in which we compiled the data of all three cohorts, we did not find a difference in time out duration following mount bouts of 1 intromission versus mount bouts of three or more mounts (no intromissions), although this data set was small (Suppl. Fig. 4). This underscores that our results are not necessarily in disagreement with the earlier reports and we conclude that time out duration is at least partially under the control of the total stimulation within the preceding mount bout, with a ceiling effect for intromissions.

4.4 Reflections on a hypothesis for a shared central mechanism of inter-copulatory intervals

We showed that PEI strongly correlates with time out, that both of these parameters increase in the first ejaculation series compared to the second ejaculation series, and that even though both of these parameters vary across copulation sessions, their correlation remains present in each copulation session. Moreover, time out is longer after mount bouts with more penile sensory stimulation, whereas the longest inter-copulatory interval (i.e, PEI) follows the strongest sensory stimulation (i.e., ejaculation). Our interpretation of these findings suggests a possibility of PEI and time out being under a similar inhibitory neuronal control. However, alternative hypotheses may be considered. First, one possible cause of increased time out duration after mount bouts that contain intromissions could be that intromissions may induce increased duration of genital grooming. While it is indeed true that mean genital grooming duration is longer after intromissions than after mounts, it was also reported in the same previous study that this effect disappears when only mounts that end a mount bout are considered.[23] Thus, duration of genital grooming after the last behavior within a mount bout is independent of whether that last behavior was an intromission or a mount. In line with this, desensitization of the penis by means of topical application of anesthetic ointment or surgical transection of the penile nerve does not affect genital grooming duration after mounts and intromissions that end a mount bout, suggesting that genital grooming duration is not dependent on the magnitude of sensory feedback within the mount bout.[24] In addition, prevention of genital grooming does not affect ejaculation latency, mounting and intromission frequency, and PEI duration.[25] It is hence postulated that genital grooming might rather be part of a motor program of copulatory behavior.[24] Second, an alternative explanation for the strong correlation of PEI and time out may be that males with a longer PEI simply have more intromissions preceding ejaculation, since mount bouts that contain intromissions induce a longer time out than mount bouts without intromissions. In an extra analysis, we found no correlation whatsoever between PEI duration and the number of intromissions in the first ejaculation series in any of the cohorts (Suppl. Fig. 5). Concluding, our working hypothesis remains that both the PEI and time out are the result of a copulatory inhibition, which is induced by the sum of sensory penile stimulation.

A question that logically arises considering this working hypothesis is whether there is a refractory period after a mount bout like after an ejaculation. The PEI is known to consist of two phases: the absolute refractory period (the first 75% of the PEI duration) and the relative refractory period (the last 25% of the PEI duration). During the relative refractory period, males can be moved to reinitiate copulation faster through non-specific stimulations that presumably increase general arousal, such as handling,[26] electrical shock,[27,28] and removal of the female for short periods of time.[9] These interventions have no effect during the absolute refractory period. If PEI and time out share common mechanisms, one might expect that the time out also consists of an absolute and relative refractory period. There is some evidence for this. Like the PEI, the time to next copulation after an intromission can be decreased by shortly removing the female,[9] handling,[26] or by applying electrical shock after an intromission (as described in Sachs and Barfield (1976)[29]). Interestingly, whereas male rats that have a natural fast copulatory pace (III of less than 30 seconds) are responsive to shocks within 3, 6, 12, or 24 seconds after intromission, naturally slower subjects are unresponsive to shocks within 3 seconds and only marginally responsive to shocks within 6 seconds (as described in Sachs and Barfield (1976)[29]). This is perhaps an indication of an absolute refractory period during inter-copulatory intervals that occur before ejaculation. Future research might provide insight into whether an absolute refraction indeed exists and whether it can be identified as a certain time percentage of a time out.

Another clue about the mechanistic relationship between inter-copulatory intervals and PEI is found in an electrophysiological study. Kurtz and Adler showed that all ejaculations and almost all intromissions are followed by a decrease in hippocampal theta frequency and a desynchronization of hippocampal activity.[30] Mounts, on the other hand, are followed by a theta frequency decrease in 27% of the cases, but by a theta frequency increase in 73% of the cases. Since intromissions almost always end a mount bout and the chance for a mount to end a mount bout is much smaller, it could be hypothesized that the slowing and desynchronization of hippocampal activity might be at the basis of copulatory inhibition, while increased hippocampal theta frequency is indicative of a continuation of copulation (i.e. the mount bout). Studying these oscillations in the context of a mount bout analysis should answer whether the theta frequency increase indeed only happens after the last behavior in a mount bout, and not after copulations within a mount bout, as well as whether similar electrophysiological patterns can be observed throughout the PEI and the time out. Future research should aim to determine whether inter-copulatory intervals indeed share a central mechanism.

4.5 Conclusion

We conclude that PEI and mean time out duration are strongly correlated, and that the total stimulation within a mount bout predicts the length of the following time out. These results were consistent over three different cohorts, despite differences in strain, age, lab, and testing procedure. We hypothesize that both PEI and time out could be regulated by a similar central copulatory inhibition that is at least partially under the control of the magnitude of sensory stimulation. Future research should aim to elucidate the underlying inhibitory mechanisms of both PEI and time out. Moreover, we advocate that the assessment of sexual behavior in male rats should be more extensive and include analysis based on mount bouts, in order to understand measured effects on a more detailed level.

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Data sharing statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supplementary data

Male rat sexual behavior: insights from inter-copulatory intervals

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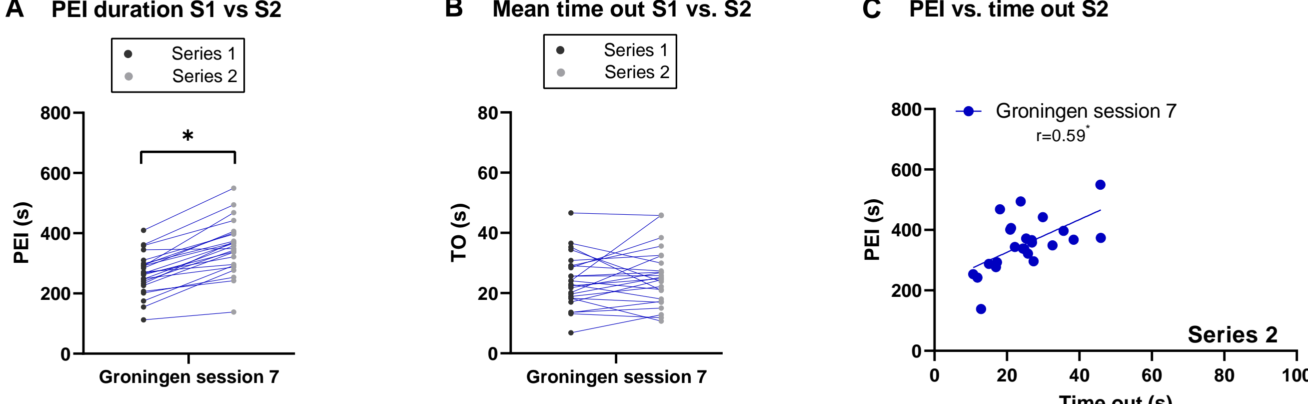
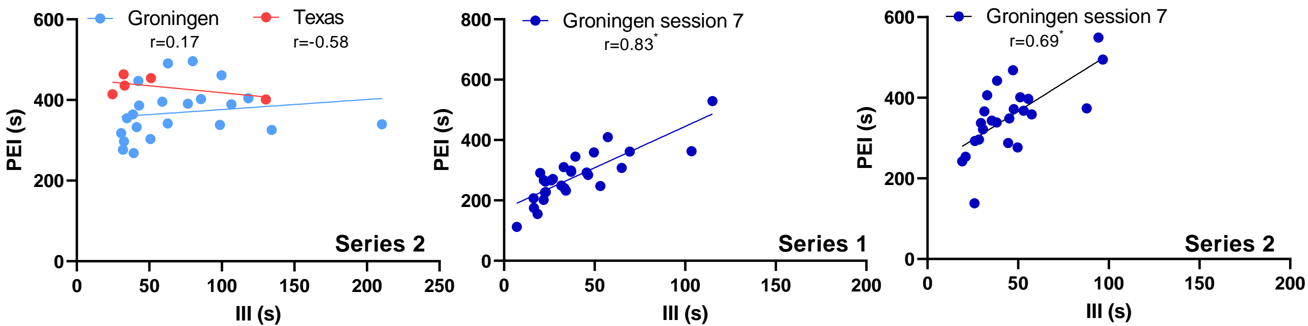
Supplementary Table 1 Copulation test outcome parameters for ejaculation series 1 of all cohorts. Table shows median and (Quartile 1 – Quartile 3), ‡p<0.05 compared to both other cohorts, *p<0.05 compared to the Tromsø cohort.

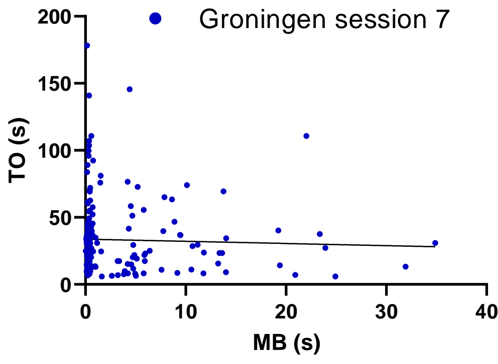
Parameter	**Groningen**	**Tromsø**	**Texas**
Latency to first mount or intromission	15.1 (3.2-8.0)	18.2 (7.1-43.3)‡	3.9 (2.5-12.6)
Number of mounts	6.0 (2.5-10.0)	5.0 (2.5-12.0)	20.0 (6.8-27.3)‡
Number of intromissions	7.0 (5.0-10.5)	13.0 (9.0-16.5)‡	7.0 (6.3-9.0)
Intromission ratio	0.53 (0.42-0.71)	0.70 (0.55-0.81)‡	0.28 (0.19-0.54)
Number of mount bouts	9.0 (6.5-15.0)‡	18.0 (12.0-23.5)	20.5 (13.8-28.0)
Mounts per mount bout	0.66 (0.33-0.85)	0.31 (0.20-0.53)‡	0.84 (0.53-1.18)
Intromissions per mount bout	0.77 (0.53-0.83)	0.76 (0.61-0.87)	0.37 (0.27-0.63)‡
Mount bout duration	3.1 (1.5-5.0)*	1.2 (0.7-1.7)	1.7 (1.0-3.5)
Time out duration	34.8 (24.1-45.6)*	13.14 (10.5-18.9)	20.9 (16.3-24.9)
Inter-intromission interval	49.5 (32.0-80.1)	17.7 (14.6-29.2)‡	56.2 (28.7-99.4)
Latency to ejaculation	411 (208-614)	266 (166-439)	528 (262-728)
Post-ejaculatory interval	270 (240-351)	267 (236-304)	352 (321-386)‡



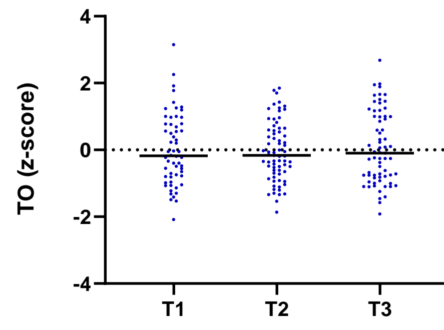


Supplementary figure 1 Post-ejaculatory interval, time out, and inter-intromission interval over series in Groningen copulation session 7. **A** Post-ejaculatory interval duration in ejaculation series 1 compared to ejaculation series 2 within the same animals from the Groningen cohort in copulation session 7 (n=24). **B** Mean time out duration in ejaculation series 1 compared to ejaculation series 2 within the same animals from the Groningen cohort in copulation session 7 (n=24). **C** Correlation of post-ejaculatory interval and mean time out duration for ejaculation series 2 in copulation session 7 of the Groningen cohort (n=24). **D** Correlation of post-ejaculatory interval and inter-intromission interval for ejaculation series 2 in the Groningen (n=25) and Texas (n=6) cohorts, and for ejaculation series 1 (n=28) and 2 (n=24) in copulation session 7 of the Groningen cohort. **All panels:** $^{*}p<0.05$

A **Time out vs. mount bout duration**

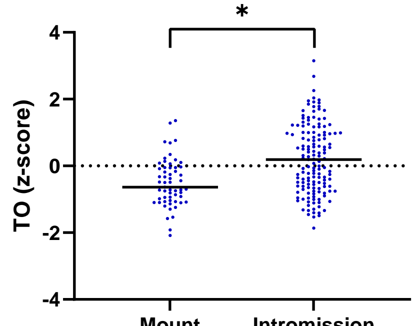


B **Time out duration over series (time binned)**

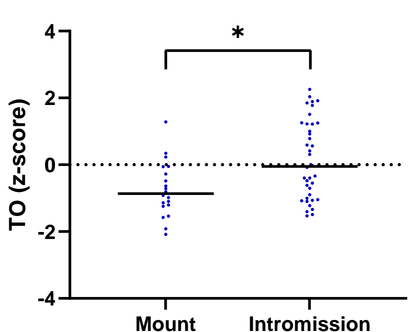


Time out duration per last behavior in mount bout

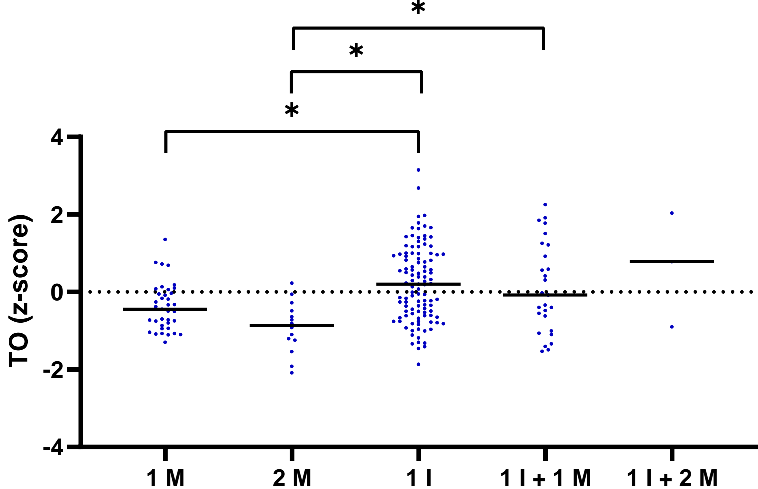
C **All mount bouts**



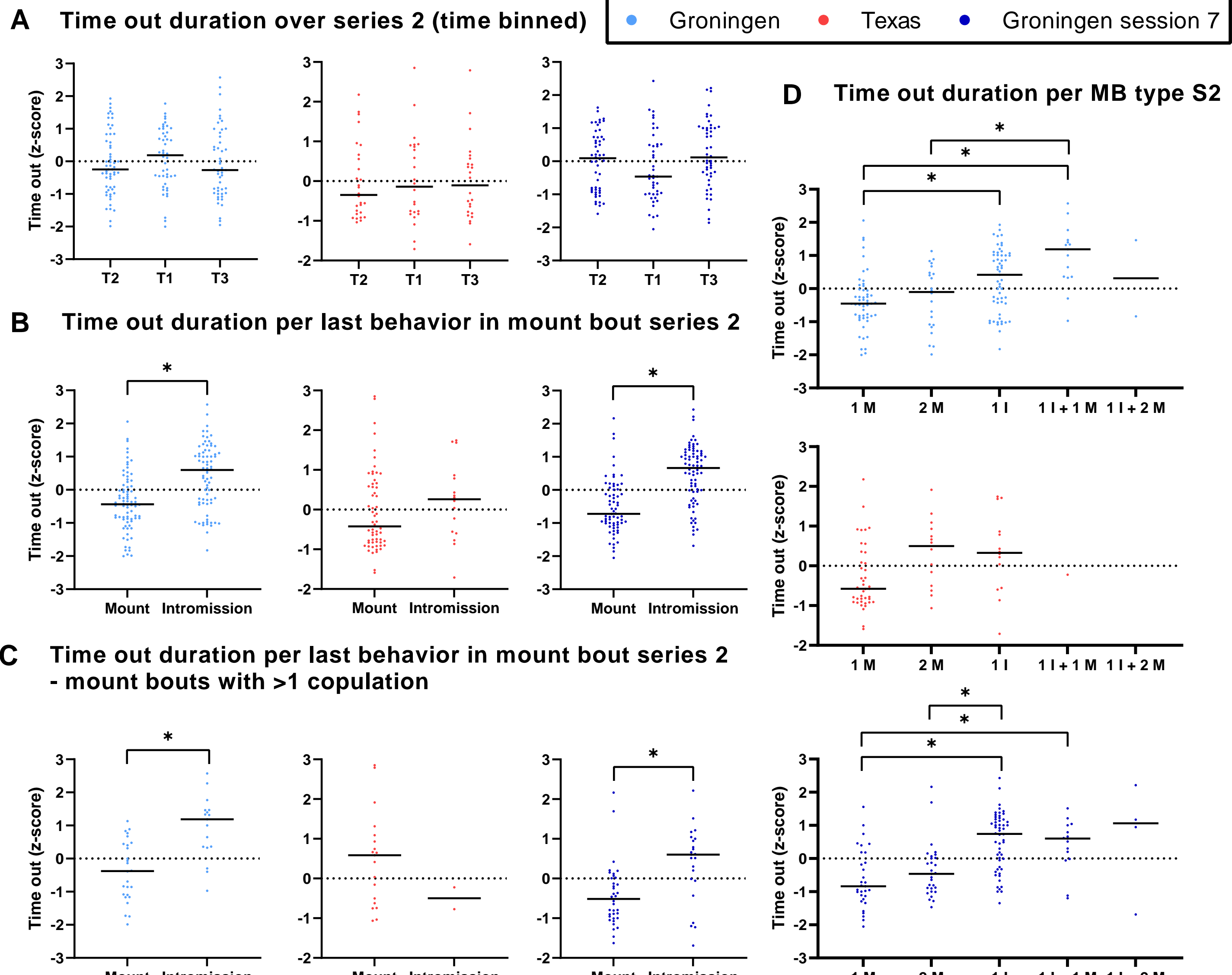
D **Mount bouts with more than 1 copulation**



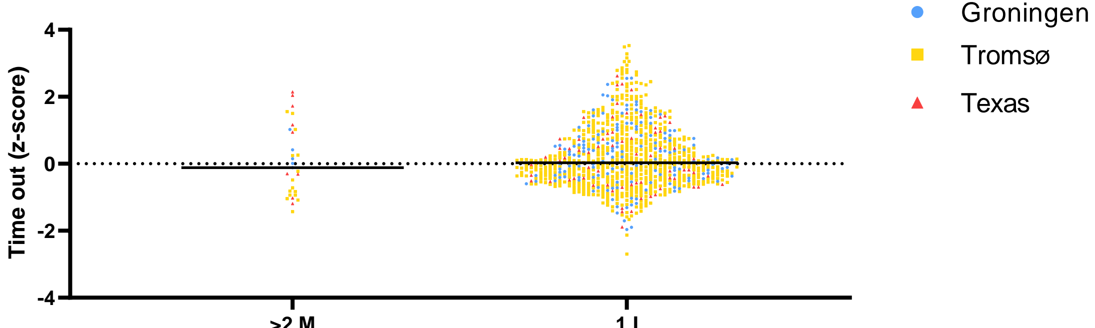
E **Time out duration per mount-bout stimulation**



Supplementary figure 2 Time out duration per mount bout characteristic for Groningen copulation session 7. **A** Correlation of individual mount bout duration with subsequent time out duration (n=202). **B** Z-scores of individual time out durations during the first, second, and third third of the ejaculation series (n=58; 71; 67). **C** Z-scores of individual time out duration after mount bouts with mount vs. intromission as last copulation (n=57; 145). **D** Z-scores of individual time out duration after mount bouts consisting of multiple copulations with mount vs. intromission as last copulation (n=20; 38). **E** Z-scores of individual time out durations after mount bouts with different total copulatory stimulation (n=37; 14; 107; 27; 3), M; mount, I; intromission. **All panels:** TO; time out, horizontal lines; median, $^{*}p<0.05$

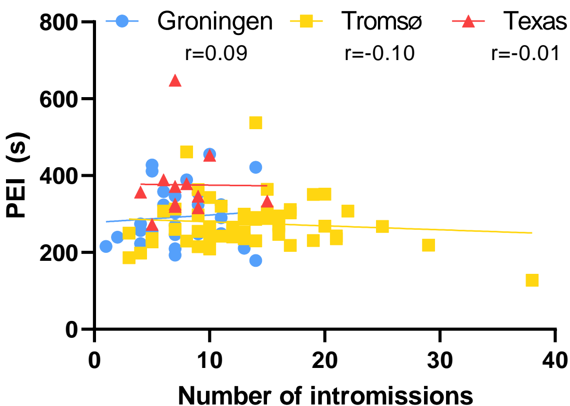


Supplementary figure 3 Time out duration per mount bout characteristic for ejaculation series 2 in the Groningen and Texas cohorts, and Groningen copulation session 7. **A** Z-scores of individual time out durations during the first, second, and third third of the ejaculation series (GR n=46; 57; 50), TX n=24; 28; 24, GR7 n=43; 52; 49)). **B** Z-scores of individual time out duration after mount bouts with mount vs. intromission as last copulation (GR n=79; 74, TX n=60; 16, GR7 n=67; 77). **C** Z-scores of individual time out duration after mount bouts consisting of multiple copulations with mount vs. intromission as last copulation (GR n=26; 17, TX n=19; 2, GR7 n=36; 23). **D** Z-scores of individual time out durations after mount bouts with different total copulatory stimulation (GR n=53; 22; 57; 15; 2, TX n=41; 14; 14; 1, GR7 n=31; 29; 54; 15; 4), M; mount, I; intromission. **All panels:** horizontal lines; median, $^{*}p<0.05$



Supplementary figure 4 Compiled data from Groningen, Tromsø, and Texas cohorts, shows no difference in time out duration following mount bouts of more than 2 mounts vs. mount bouts of 1 intromission, GR n=152; 5, TR n=640; 15, TX n=70, 9, horizontal lines; median.

Post-ejaculatory interval vs. number of intromissions



Supplementary figure 5 Correlation of post-ejaculatory interval and number of intromissions for ejaculation series 1 for Groningen, Tromsø, and Texas cohorts. n=29; 53; 12

Paper III

Silencing and stimulating the medial amygdala impairs ejaculation but not sexual incentive motivation in male rats

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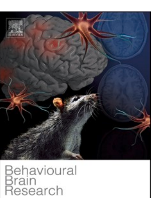

Silencing and stimulating the medial amygdala impairs ejaculation but not sexual incentive motivation in male rats

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ABSTRACT

The medial amygdala (MeA) is a sexually dimorphic brain region that integrates sensory information and hormonal signaling, and is involved in the regulation of social behaviors. Lesion studies have shown a role for the MeA in copulation, most prominently in the promotion of ejaculation. The role of the MeA in sexual motivation, but also in temporal patterning of copulation, has not been extensively studied in rats. Here, we investigated the effect of chemogenetic inhibition and stimulation of the MeA on sexual incentive motivation and copulation in sexually experienced male rats. AAV5-CaMKIIa viral vectors coding for Gi, Gq, or no DREADDs (sham) were bilaterally infused into the MeA. Rats were assessed in the sexual incentive motivation test and copulation test upon systemic clozapine N-oxide (CNO) or vehicle administration. We report that MeA stimulation and inhibition did not affect sexual incentive motivation. Moreover, both stimulation and inhibition of the MeA decreased the number of ejaculations in a 30 min copulation test and increased ejaculation latency and the number of mounts and intromissions preceding ejaculation, while leaving the temporal pattern of copulation intact. These results indicate that the MeA may be involved in the processing of sensory feedback required to reach ejaculation threshold. The convergence of the behavioral effects of stimulating as well as inhibiting the MeA may reflect opposing behavioral control of specific neuronal populations within the MeA.

1. Introduction

Sexual behavior is an innately motivated behavior in the male rat and consists of three phases. During the initial phase, sexual incentive motivation propels a sexually experienced male into approach and investigation of a receptive female. After identification of the receptive female as a potential mate, the second phase of copulation quickly commences. Copulation consists of stereotypical motor output in the form of mounts and intromissions spaced over time in mount bouts, with chasing, genital grooming, and other non-copulation oriented behaviors in between. Multiple mounts and intromissions eventually culminate into ejaculation, the executive phase of sexual behavior. Even though there is no copulation without approach and no ejaculation without copulation, the behavioral output in different phases of sexual behavior might well be independently regulated on the neurobiological level [1]. This is supported by the notion that copulation parameters load onto different factors than anticipatory and approach parameters in factor analysis of male sexual behavior [2]. Studying the different phases of sexual behavior separately will lead to a more precise understanding of temporal and causal relations between neuronal activity and behavior.

The medial amygdala (MeA) is a sexually dimorphic brain region known to be involved in the regulation of a wide array of social behaviors, such as aggression, parental behavior, and sexual behavior, as reviewed in [3,4]. These behaviors require the processing of contextual and sensory information in convergence with the internal state of the animal in order for the animal to display the appropriate behavioral response. Indeed, the high density of estrogen and androgen receptors, together with afferent input containing pheromonal and olfactory information, implicates the MeA as a primary locus for the integration of environmental and sensory information with the internal hormonal milieu of the animal [5,6]. Pheromonal information reaches the MeA directly from the accessory olfactory bulb, and olfactory information reaches the MeA from the main olfactory bulb via the cortical amygdala [7,5]. Major efferent targets of the MeA include the medial preoptic area (mPOA), the bed nucleus of the stria terminalis, and the ventral medial hypothalamic nucleus [8]. These target areas have all been shown to be involved in the regulation of sexual behavior [7,9]. The mPOA specifically is absolutely necessary for the display of sexual motivation and

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copulation in male rats [9]. The MeA regulates dopamine release in the mPOA, and MeA lesion in addition to contralateral mPOA lesion is far more detrimental to copulation than MeA lesion in addition to ipsilateral lesion [10–12]. Considering its involvement in the processing of pheromonal and olfactory cues and its role as a major input area to the mPOA, implicates the MeA as a hub involved in the regulation of motivational, consummatory, and executive phases of sociosexual behaviors.

The role of the MeA in sexual motivation in rats has not been as extensively studied as its role in copulation. The involvement of the MeA in sexual approach would logically follow its integrative role for sensory information and hormonal signaling. Indeed, c-fos is induced in the MeA upon anogenital investigation or exposure to odors of receptive females in sexually experienced males [13]. However, lesions of the MeA do not appear to affect incentive preference for an estrous female in male rats [14], nor do they affect response latencies in a bar-pressing regimen in order to access an estrous female [15]. In male hamsters, both the anterior and posterior MeA seem to be involved in the preference for odors of estrous females [16]. Further investigation of the role of the MeA in sexual incentive motivation is warranted.

The involvement of the MeA in the regulation of the copulatory phase of sexual behavior has been long established [9]. This is apparent from data observing neuronal activity in the MeA during copulation and from studies manipulating the MeA during copulation. Single unit recordings in male rats show a remarkable increase in activity of MeA neurons upon the introduction of a receptive female [17]. This activity remains high during the whole time period the receptive female is present and falls back down to baseline after removal of the female. In addition, neuronal activity spikes in the 20 s after copulation behaviors (a mount, intromission, or ejaculation). No increased neuronal activity was observed when a non-receptive female was introduced [17]. In line with this, c-fos as well as Arc is induced upon copulation in the MeA of sexually experienced male rats [18,19,13]. Even though the MeA seems to clearly respond to copulation, different lesion studies in rats consistently find that the MeA is not essential for any aspect of copulation, including ejaculation [20–22,11,23,24]. However, lesioning of the MeA does increase the ejaculation latency in behavioral tests [20–22,11,23,24]. In addition, whereas the patterns of copulatory behavior look normal in MeA lesioned males, a larger number of mounts and intromissions usually precede ejaculation [10,12,20]. Surprisingly, electrical stimulation of the MeA also dramatically impairs copulation [25]. No further studies have investigated the effect of stimulating the MeA on sexual behavior in male rats. In all, these findings indicate a role for the MeA in copulation with regards to the processing of olfactory and pheromonal cues and somatosensory feedback from the penis, thereby affecting ejaculatory behavior.

Recently, progress has been made in the study of the role of the amygdala in sexual behavior of mice, where methodological advancements enabled a further interrogation of specific neuronal populations of the MeA. So far, studies that make use of more sophisticated techniques in rats are lacking. In addition, analysis of sexual behavior is often reduced to the annotation of only mounts, intromissions, and ejaculations. This prompted us to study the role of the MeA in sexual behavior in male rats by means of chemogenetics, allowing for temporary neuronal inhibition and stimulation with minimal invasiveness. In addition, we employed an extensive behavioral annotation allowing for additional analysis of temporal patterning of copulation through mount bout based assessment [26]. Because so little data exists on stimulation of the MeA in sexual behavior, we looked at the effects of both chemogenetic inhibition and stimulation of the MeA on sexual behavior in male rats. Importantly, with this study we assessed the involvement of the MeA in all stages of sexual behavior; sexual incentive motivation, and copulation (including ejaculation). We found that both stimulation and inhibition of the MeA disrupted ejaculation while increasing the number of copulatory behaviors preceding ejaculation, but did not affect sexual incentive motivation.

2. Materials and methods

2.1. Animals

All rats (Charles River, Sulzfeld, Germany) were housed in Macrolon IV® cages on a reversed 12 h light/dark cycle (lights on between 23:00 and 11:00) in a room with controlled temperature (21 ± 1 °C) and humidity (55 ± 10 %), with *ad libitum* access to standard rodent food pellets (RM1P-E-FG; Special Diets Services, Essex, UK) and tap water. Rats were housed in same-sex pairs, unless otherwise noted (see brain surgery). In this experiment, 54 male Wistar rats were used as subjects. An additional 6 male Wistar rats were used as social incentives in the sexual incentive motivation (SIM) test. A total of 36 female Wistar rats were used as sexual incentives in the SIM test and as stimulus animals in the copulation test.

2.2. Viral constructs and drugs

Three viral constructs (University of North Carolina Vector Core, Chapel Hill, USA) were used in this experiment: AAV5-CaMKIIa-hM4D-mCherry (Gi; inhibitory DREADDs), AAV5-CaMKIIa-hM3D-mCherry (Gq; stimulatory DREADDs) and AAV5-CaMKIIa-EYFP (Sham; no DREADDs). For more information on chemogenetics, see Ref. [27]. Clozapine N-oxide (CNO; synthetic metabolite of clozapine that is the ligand for the DREADDs) (BML-NS105; Enzo Life Sciences, Farmingdale, USA) was dissolved in ddH_2O at a stock concentration of 1 mg/mL (3 mM) and frozen at −20°C in aliquots until further use. For experiments, rats were injected intraperitoneally with 1 mL/kg of the 1 mg/mL CNO solution (a dose that has minimal behavioral effects on its own [28]) or vehicle (ddH_2O).

Silastic capsules (medical grade Silastic tubing, 0.0625 in. inner diameter, 0.125 in. outer diameter, Degania Silicone, Degania Bet, Israel) for females were 5 mm long and contained 10 % 17β-estradiol (Sigma, St. Louis, USA) in cholesterol (Sigma, St. Louis, USA). The silastic tubing was closed off by inserting pieces of toothpick into both ends and sealed off with medical grade adhesive silicone (NuSil Silicone Technology, Carpinteria, USA).

Progesterone (Sigma, St. Louis, USA) was dissolved in peanut oil (Apotekproduksjon, Oslo, Norway) at a concentration of 5 mg/mL. Female rats were subcutaneously injected with 0.2 mL of the solution.

2.3. Surgical procedures

2.3.1. Ovariectomy

Stimulus females were ovariectomized under isoflurane anesthesia as previously described [29]. Briefly, a medial dorsal incision of the skin of about 1 cm was made, and the ovaries were located through a small incision in the muscle layer on each side. The ovaries were extirpated and a silastic capsule containing β-estradiol was placed subcutaneously through the same incision. The muscle layer was sutured and the skin was closed with a wound clip.

2.3.2. Brain surgery

Brain surgery consisted of subsequent bilateral infusions of the viral vector into the MeA. Rats were anesthetized with a mixture of zolazepam/tiletamine/xylazine/fentanyl (73.7 mg/73.7 mg/1.8 mg/10.3 μg per mL; 2 ml/kg) and placed in a stereotaxic apparatus (Stoelting Europe, Ireland). The skull was exposed through incision and small holes were drilled at the appropriate injection sites. A 30 G cannula (Plastics One, Raonoke, USA) was inserted into each brain hemisphere sequentially at the following coordinates: AP -3,1 mm and ML ±3,7 mm from bregma and DV -8,2 mm from the cortical surface [30]. Per infusion site, 750 nl of viral construct solution (Titers; Gi 4.3×10^{12} vg/mL, Gq 1.4×10^{12} vg/mL, Sham 7.4×10^{12} vg/mL) was injected at an infusion rate of 150 nl/min by a Hamilton syringe mounted in a minipump, connected to the infusion cannula by a piece of tubing (Plastics One, Roanoke, USA).

Following infusion, the cannula was left in place for 10 min before withdrawal and closing of the skin with a continuous intradermal suture (Vicryl Rapide 4−0, Ethicon, Cincinnati, USA). After surgery, rats were single-housed for 3–7 days before being rehoused in pairs again. Analgesic treatment consisted of buprenorphine 0.05 mg/kg within 8 h of surgery and every 12 h for 72 h thereafter.

2.4. Behavioral assessment

2.4.1. Sexual incentive motivation

The sexual incentive motivation test is described elsewhere [31]. Briefly, the SIM apparatus consists of a rectangular arena (100 × 50 × 45 cm) with rounded corners placed in a dimly lit (5 lx) room. At each long side, in opposite corners, a closed incentive stimulus cage was attached to the arena and separated from the arena by wire mesh (25 × 25 cm). A social stimulus (intact male rat) was placed in one of the stimulus cages and a sexual stimulus (receptive female rat) was placed in the other stimulus cage. To male subject rats, an intact male and a non-receptive female have the same salience as a social stimulus [31]. The subject rat was placed in the middle of the arena and video-tracked by Ethovision software (Noldus, Wageningen, the Netherlands) for 10 min. In Ethovision, virtual incentive zones (30 × 21 cm) were defined within the arena in front of each stimulus cage. The subject was considered to be within the zone whenever its point of gravity was. The software output consisted of the time the experimental subject spent in each incentive zone, the total distance moved, the time spent moving, and the mean velocity. From this data, the preference score was calculated (time spent in female incentive zones/total time in incentive zones). Subject rats were introduced right after each other, without cleaning the arena in between. The position of the stimulus cages (including the stimulus animal) was randomly changed throughout each experimental session. The SIM arena was cleaned with diluted acetic acid between experimental days.

2.4.2. Copulation

The copulation test was conducted in rectangular boxes (40 × 60 × 40 cm) with a Plexiglas front, in a room with lights on. During behavioral testing, the experimental subject was transferred from the room with the SIM test to the room with the copulation boxes. A receptive female was placed in the copulation box, after which the experimental subject was introduced. The test started upon introduction of the experimental subject male and lasted for 30 min. All test sessions were recorded on camera and behavior was later assessed from video. Behavioral assessment consisted of scoring behavioral events by means of the Observer XT software (Noldus, Wageningen, the Netherlands). For the entire 30 min, the copulatory behaviors mount, intromission and ejaculation were scored. During the first ejaculation series, we also behaviorally annotated 100 % of the elapsed time by expanding the ethogram with clasping (mounting the female without pelvic thrusting), genital grooming (grooming of own genital region), other grooming (autogrooming in other regions than genital), chasing (running after the female), anogenital sniffing (sniffing the anogenital region of the female), head towards female (head oriented in the direction of the female while not engaging in other behavior), head not towards female (any behavior that is not oriented towards the female except grooming, such as walking, sniffing the floor, standing still with head direction away from female). From these data points the outcome measures as listed in Table 1 were determined (see also Ref. [32]). For mount bout based analysis, we employed Sachs' and Barfield's definition of the mount bout: "a sequence of copulatory behaviors (one or more), uninterrupted by any behavior (other than genital autogrooming) that is not oriented towards the female)" [26]. Mount bouts were identified through automated review of the events between each copulatory behavior (i.e. mount or intromission) using a python script (available upon request). Whenever "other grooming" or "head not towards female" occurred in between copulatory behaviors, this marked the end of the previous mount bout (end time was then set on the end of the last copulatory behavior) and the beginning of the next mount bout (start time of the next copulatory behavior), and the time in between these mount bouts as a time out. All behavioral tests were conducted during lights-off time.

Table 1
Copulation test outcome measure definitions.

Outcome measure	Definition
Number of ejaculations	Total number of ejaculations in the 30 min test
Latency to first ejaculation	Time from first copulatory behavior (mount or intromission) to ejaculation (NB: set to 1800 s in case no ejaculation was achieved during the test)
Latency to second ejaculation	Time from the end of the first post-ejaculatory interval to the next ejaculation
Mounts per ejaculation	Number of mounts in the first ejaculation series
Intromissions per ejaculation	Number of intromissions in the first ejaculation series
Intromission ratio	Number of intromissions in the first ejaculation series divided by the total number of copulatory behaviors in the first ejaculation series
Latency to first copulatory behavior	Time from the start of the test to the first copulatory behavior (mount or intromission)
Latency to first intromission	Time from the start of the test to the first intromission
Number of mount bouts per ejaculation	Number of mount bouts (a sequence of copulatory behaviors (one or more), uninterrupted by any behavior (other than genital autogrooming) that is not oriented towards the female) in the first ejaculation series
Mounts per mount bout	Mean number of mounts per mount bout in the first ejaculation series
Intromissions per mount bout	Mean number of intromissions per mount bout in the first ejaculation series
Inter-intromission interval	Time between intromissions in the first ejaculation series, calculated from the first intromission
Mount bout duration	Mean duration of mount bouts in the first ejaculation series
Time out duration	Mean duration of time-out (time from the end of one mount bout to the start of the next mount bout)
Post-ejaculatory interval	Time from the first ejaculation to the next copulatory behavior
Percentage of time spent on [behavior]	Percentage of time spent engaging in each of the annotated behaviors before the first ejaculation
Percentage of time spent in non-copulation oriented behavior	Percentage of time spent engaging in head not towards female + other grooming

2.5. Brain processing, immunostaining and imaging

At the end of the experiment, rats were i.p. injected with a lethal dose of pentobarbital (100 mg/kg; Pentobarbital solution 100 mg/mL, Ås Produksjonslab AS, Ås, Norway) and, when deeply anesthetized, transcardially perfused with 0.1 M phosphate buffered saline (PBS; pH 7.4) followed by 4% formaldehyde in 0.1 M PBS. Brains were quickly removed and post-fixed in 4% formaldehyde in 0.1 M PBS for 48 h. Subsequently, brains were transferred to a 20 % sucrose in 0.1 M PBS solution, followed by a 30 % sucrose in 0.1 M PBS solution until they had sunken. Brains were then either snap frozen by use of isopentane and kept at -80°C until sectioning, or sectioned right away. Brains were sectioned on a cryostat (Leica CM1950, Leica Biosystems, Wetzlar, Germany, and Cryostar NX70, Thermo Fisher Scientific, Waltham, USA) into 30μm thick sections and stored in cryoprotectant solution (30 % sucrose w/v, 30 % ethylene glycol v/v in 0.1 M phosphate buffer, pH 7.4) until further use.

For immunohistochemistry, 1 in every 5th brain section within the area of interest was stained for the corresponding DREADD-conjugated fluorophore. For immunostaining, free-floating sections were washed in

0.1 M Tris-buffered-saline (TBS, pH 7.6), blocked for 30 min in 0.5 % BSA, and incubated on an orbital shaker for 24 h at room temperature +24 h at 4 °C in polyclonal rabbit anti-mCherry (1:30 000, Abcam, cat. ab167453) or polyclonal chicken anti-EYFP (1:200 000, Abcam, cat. ab13970) antibody solution containing 0.1 % Triton-X and 0.1 % BSA in TBS. Sections were then incubated in biotinylated goat anti-rabbit (1:400, Abcam, cat. ab6720) or biotinylated goat anti-chicken (1:400, Abcam, cat. ab6876) antibody solution containing 0.1 % BSA in TBS for 30 min, avidin-biotin-peroxidase complex (VECTASTAIN ABC-HRP kit, Vector laboratories, cat. PK-6100, dilution: 1 drop A + 1 drop B in 10 mL TBS) solution for 30 min, and 3,3′-diaminobenzidine solution (DAB substrate kit (HRP), Vector laboratories, cat. SK-4100, dilution: 1 drop R1 (buffer solution) + 2 drops R2 (3,3′-diaminobenzidine solution) + 1 drop R3 (hydrogen peroxide solution) in 5 mL water) for 5 min, with TBS washes between all steps. Slides were dehydrated, cleared, and cover-slipped using Entellan mounting medium (Sigma, St. Louis, USA).

After drying, the slides were loaded into an Olympus VS120 virtual slide microscope system. High resolution image scans were obtained for each section using a 20x objective (NA 0.75) and automatic focus and exposure settings in single plane. Using OlyVIA online database software (Olympus, Tokyo, Japan), viral spread was determined through assessment of the location and extent of stained cell bodies for separate brain regions. DREADD expression was categorized for each brain region in each animal based on the amount of DREADD + cells per section (1 in 5 throughout the MeA) and the spread of expression throughout the brain region (over sections). We qualified expression using a scoring system per brain region per hemisphere: 0 (no expression in the brain region); 1 (low expression per section and low spread throughout the brain region, i.e. no more than a few positive cells per section), 2 (medium expression throughout the brain region, typically >10 and <30 positive cells per section, or high expression with low spread throughout the brain region); and 3 (high expression throughout the brain region, typically >30 positive cells per section). A second observer validated the qualifications in 5 animals with various expression patterns. We then added the scores for each hemisphere, and excluded animals with a total score (left + right hemisphere) smaller than 3 for the MeA from further analysis.

2.6. Design

Female stimulus animals were ovariectomized and implanted with a silastic capsule with β-estradiol at least one week before use in the SIM and copulation test. The females were injected with 1 mg progesterone 4 h before use in behavioral tests in order to induce sexual receptivity.

Male subjects were first habituated to the SIM arena (10 min per session) and sexually trained immediately after in three sessions over the course of a week. During the copulatory training sessions, that directly followed the SIM habituation, males were allowed to copulate with a receptive female in order to become sexually experienced. Males where then divided into three homogenous experimental groups based on the number of ejaculations in the last 30 min copulation training session. Over the course of the second week, all male rats had brain surgery during which a viral vector carrying Gi(DREADD)-mCherry, Gq (DREADD)-mCherry, or EYFP genetic information, was infused bilaterally into the MeA. A 19–24 day recovery and DREADD expression period was allowed after surgery. Subsequently, rats underwent behavioral testing following an intraperitoneal injection of CNO and vehicle in a latin square within-subject design. Allowing a one week recovery period between copulation testing (enough for copulation parameters to return to baseline even after sexual exhaustion [33]), each male was tested twice, once for each treatment, over the course of two weeks. Rats were first tested in the SIM test 30 min after i.p. injection with either vehicle or CNO. Following the SIM test, rats were tested in the copulation test 5−15 min later. Finally, rats were perfused with formaldehyde and brains were harvested for immunohistochemical analysis of DREADD expression.

The presented data in this manuscript consists of combined data from two separate homologous experiments.

2.7. Data analysis and statistics

Multiple linear mixed models employing virus as between-subject factor and treatment as within-subject factor were tested on the data using SPSS statistical software (IBM, version 26, Armonk, USA). Based on Akaike's Information Criterion, a linear mixed model that included only the factors virus*treatment interaction term and experiment number as a covariate was deemed the best fit for the data. This mixed model was run for each of the separate outcome measures of the SIM test and the copulation test. In case of a significant virus*treatment interaction effect at the alpha 0.05 level, Bonferroni posthoc tests were conducted to identify significant within- and between-group differences. Supplementary analyses on small sample subgroups were done by employing t-tests (alpha 0.05) without multiple comparison correction.

The SIM preference score was compared to chance (0.5) with a one-sample *t*-test for each treatment within each group. Time spent in female zone was compared to time spent in male zone for each treatment within each group with a paired *t*-test. For an effect on sexual incentive motivation, comparisons between both the preference scores and the time spent in female zone needs to be statistically significant, as an increased preference score is irrelevant when the total time spent in incentive zones is relatively small.

3. Results

3.1. DREADD expression

DREADD expression in the MeA was assessed by immunohistochemical staining. Out of 56 animals, 2 animals died before perfusion and were excluded because of a lack of histological data. Another 11 animals (6 Gi, 4 Gq, 1 Sham) were excluded due to insufficient MeA DREADD expression.

Somatic DREADD expression in the remaining 43 animals was observed throughout the anterior and posterior MeA, with higher density posteriorly (Fig. 1). In the majority of animals, DREADD expression extended to amygdaloid structures lateral and posterior from the MeA, namely the intraamygdaloid division of the bed nucleus of the stria terminalis (STIA), the amygdalohippocampal area (Ahi), the posteromedial cortical nucleus (PMCo), and the basomedial amygdaloid nucleus (BM). Most animals also had low-density ventral hippocampal (vHC) DREADD expression. In addition, low-density, mostly unilateral, expression was observed in the peduncular part of the lateral hypothalamus (LH) in 16 animals.

3.2. Sexual incentive motivation

To study the involvement of the MeA in sexual incentive motivation, we compared SIM test (Fig. 2A) parameters in vehicle (VEH) and CNO treated Sham, Gi-DREADD, and Gq-DREADD males. Subject males in each virus group (Sham, Gi, and Gq), and during each treatment, significantly spent more time in the female zone compared to the male zone (Fig. 2C; Sham-CNO $t(15) = 13.7$, Sham-VEH $t(15) = 13.6$, Gi-CNO $t(11) = 15.8$, Gi-VEH $t(11) = 10.8$, Gq-CNO $t(14) = 11.3$, Gq-VEH $t(14) = 9.45$, $p < 0.001$ for all groups). This was also reflected in the preference scores that were significantly larger than 0.5 (Fig. 2D; Sham-CNO $t(15) = 14.6$, Sham-VEH $t(15) = 18.9$, Gi-CNO $t(11) = 19.4$, Gi-VEH $t(11) = 12.6$, Gq-CNO $t(14) = 12.6$, Gq-VEH $t(14) = 11.2$, $p < 0.001$ for all groups). Additionally, subject males visited the female zone more frequently in all but Gi-CNO (Suppl. Fig. 1A; Sham-CNO $t(15) = 3.41$, $p = 0.004$, Sham-VEH $t(15) = 4.25$, $p < 0.001$, Gi-VEH $t(11) = 3.53$, $p = 0.005$, Gq-CNO $t(14) = 2.63$, $p = 0.020$, Gq-VEH $t(14) = 2.86$, $p = 0.013$). There was a shorter latency to visit the female zone than to visit the male zone in Gi-CNO (Suppl. Fig. 1B; $t(11) = 2.27$, $p = 0.044$) and Gi-VEH (Suppl. Fig. 1B; $t(11) = 2.45$, $p = 0.032$). We found no

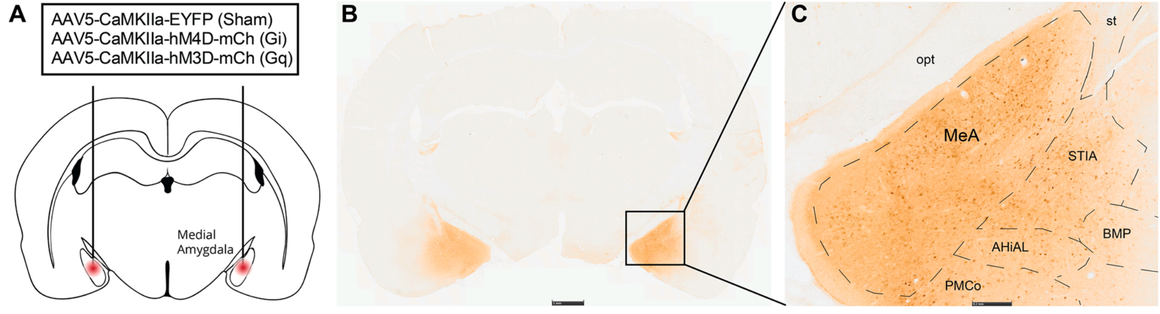

Fig. 1. Medial amygdala DREADD expression. **(A)** Bilateral viral targeting of the MeA. **(B)** Example DREADD expression on whole brain section at approximately AP -3.2 from bregma. **(C)** Magnified inset of (B) showing somatic DREADD expression in the MeA and surrounding structures. MeA = medial amygdala; opt = optic tract; st = stria terminalis; STIA = intraamygdaloid division of the bed nucleus of the stria terminalis; BMP = basomedial amygdaloid nucleus, posterior part; AHiAL = amygdalohippocampal area, anterolateral part; PMCo = posteromedial cortical amygdaloid nucleus.

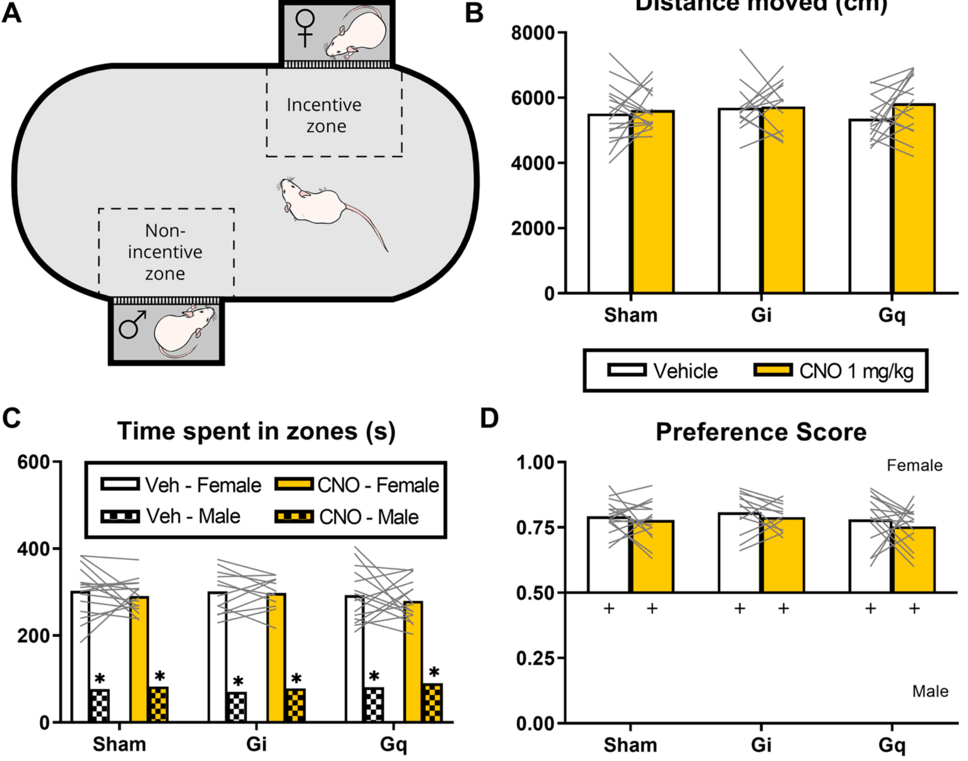

Fig. 2. Silencing or stimulating the MeA does not affect sexual incentive motivation. **(A)** Sexual incentive motivation test (10 min). **(B)** Total distance moved during the 10 min test. **(C)** Total time spent in the incentive zone (female zone) and the non-incentive zone (male zone). *p < 0.05 compared to "female zone" **(D)** Preference score (time spent in female zone/total time spent in female and male zones). $^{+}$p < 0.05 compared to 0.5. **All panels:** n = 16 (sham), 12 (Gi), 15 (Gq); bar represents group mean.

significant interactions of treatment and virus for distance moved (Fig. 2B), time spent in zones (Fig. 2C), preference score (Fig. 2D), frequency of zone entry (Suppl. Fig. 1A), latency to enter zone (Suppl. Fig. 1B), time spent moving (Suppl. Fig. 1C), and mean velocity (Suppl. Fig. 1D).

3.3. Copulation

Immediately after the SIM test, male subjects were tested in the copulation test (Fig. 3A). No effects of MeA silencing or stimulation on latency to first copulatory behavior (Fig. 3B), nor on latency to first intromission were observed (Suppl. Fig. 2). We did find that ejaculation parameters were significantly affected (Fig. 3C). CNO decreased the number of ejaculations during the 30 min test (Fig. 3C; virus x treatment: $F(5,44) = 11.28$, $p < 0.001$) in both the Gi-group (Mean difference (md) = 1.08, $p < 0.001$, $g = 0.83$) and the Gq-group (md = 1.27, $p < 0.001$, $g = 1.267$) compared to vehicle. Although, only in Gi-CNO were the number of ejaculations also significantly decreased (md = 1.22, $p = 0.011$, $g = 1.23$) compared to Sham-CNO. The decrease in number of ejaculations logically followed a significant CNO-induced increase of latency to ejaculation (Fig. 3C; virus x treatment: $F(5,47) = 6.58$, $p < 0.001$) in both the Gi-group (md = 490, $p < 0.001$, $g = 0.91$) and the Gq-group (md = 380, $p = 0.001$, $g = 0.83$) compared to vehicle, and only in Gi-CNO compared to Sham-CNO (md = 481, $p = 0.009$, $g = 1.13$). These effects persisted during the second ejaculation series (Fig. 3C; virus x treatment: $F(5,32) = 4.890$, $p = 0.002$). CNO increased the latency to second ejaculation compared to vehicle in the Gi-group (md = 162, $p = 0.003$, $g = 0.53$), as well as in the Gq-group (md = 162, $p = 0.003$, $g = 1.35$), and compared to Sham-CNO in the Gi-group only (md = 195, $p = 0.009$, $g = 0.98$). Further analysis of the first ejaculation series showed that CNO significantly increased the number of mounts compared to vehicle (Fig. 3D; virus x treatment: $F(5,51) = 2.41$, $p = 0.049$) in both the Gi-group (md = 13.5, $p = 0.012$, $g = 0.86$) and the Gq-group (md = 10.4, $p = 0.029$, $g = 0.76$). The number of intromissions preceding the first ejaculation was also affected by CNO compared to vehicle in the Gi-group (Fig. 3D; virus x treatment: $F(5,48) = 4.63$, $p = 0.002$; Gi md =

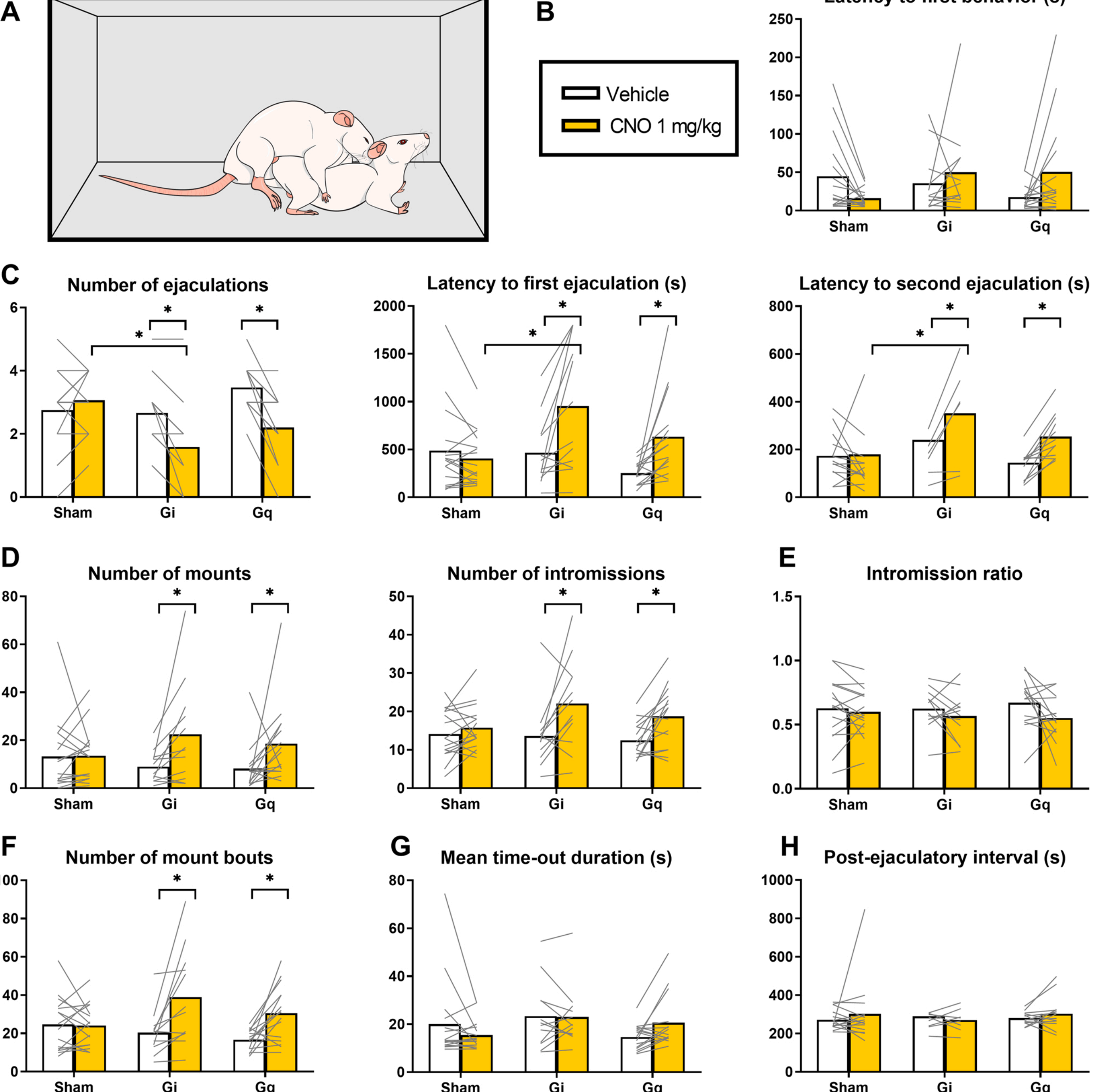

Fig. 3. Silencing and stimulating the MeA affect copulation parameters in the same direction. **(A)** Copulation test (30 min). **(B)** Latency to first copulatory behavior, i.e. mount or intromission. **(C)** Ejaculation parameters: Number of ejaculations, Latency to first ejaculation, and Latency to second ejaculation (n = 13 (sham), 6 (Gi), 12 (Gq)). **(D)** Number of mounts and number of intromissions in the first ejaculation series. **(E)** Intromission ratio (intromissions/(mounts + intromissions)) in the first ejaculation series. **(F)** Number of mount bouts (one or more uninterrupted copulatory behaviors) in the first ejaculation series. **(G)** Mean duration of time-outs (intervals between mount bouts) in the first ejaculation series. **(H)** Post-ejaculatory interval of the first ejaculation series (n = 15 (sham), 8 (Gi), 14 (Gq)). **All panels:** *p < 0.05; n = 16 (sham), 12 (Gi), 15 (Gq) unless otherwise indicated; bar represents group mean.

8.5, p = 0.001, g = 0.83), as well as in the Gq-group (md = 6.27, p = 0.005, g = 0.96). However, no statistical significant effects were observed on the number of mounts and intromissions between Gi-CNO or Gq-CNO compared to Sham-CNO. The numbers of mounts and intromissions were proportionally increased by CNO in the Gi- and Gq-groups compared to vehicle, as intromission ratio remained unaffected by CNO in both these groups (Fig. 3E). The larger number of copulatory behaviors did not lead to an increase in the mean number of mounts and intromissions per mount bout, nor the mean duration of mount bouts (Suppl. Fig. 2). Instead, it was reflected in a CNO-induced increase of the number of mount bouts preceding ejaculation (Fig. 3F; virus x treatment: F(5,49) = 5.55, p < 0.001) in both the Gi-group (md = 18.6, p < 0.001, g = 0.95) and the Gq-group (md = 13.9, p = 0.002, g = 01.25) compared to vehicle. But again, there was no statistical significant effect between Gi-CNO or Gq-CNO compared to Sham-CNO. Finally, no effects were observed on parameters of temporal patterning; mean duration of time-out (Fig. 3G), post-ejaculatory interval (Fig. 3H), and inter-intromission interval (Suppl. Fig. 2).

Analysis of the percentage of time spent on each of the behavioral parameters showed significant effects of CNO on the percentage of time spent on head not towards female (Suppl. Fig. 3; virus x treatment: F (5,45) = 3.37, p = 0.011) compared to vehicle within the Gq-group only (md = 8.01, p = 0.009), but not for Gq-CNO vs. Sham-CNO. Consequently, a statistical significant effect was found for percentage of time

spent on non-copulation oriented behavior (Suppl. Fig. 3; virus x treatment: F(5,44) = 4.08, p = 0.004), which is comprised of percentage of time spent on head not towards female and other grooming, for Gq-CNO compared to Gq-vehicle (md = 9.14, p = 0.004), but not for Gq-CNO compared to Gq-vehicle. No significant interaction of virus and treatment was found in percentage of time spent on other grooming, genital grooming, anogenital sniffing, chasing and clasping (Suppl. Fig. 3).

4. Discussion

The MeA is a sexually dimorphic brain region involved in the regulation of sexual behavior [3,9]. The afferent and efferent connections of the MeA and the expression of hormonal receptors and aromatase in the MeA suggest its involvement in integrating environmental and sensory information with the internal hormonal state of the animal [5–8]. Considering the position of the MeA as an important integration area, and input area of the mPOA, we aimed to shine more light on the role of the MeA during all stages of sexual behavior in male rats. Our main finding here was that both silencing and stimulating the MeA did not impair incentive motivation or alter the structure and patterns of copulatory behavior, but did result in increased ejaculation latency and consequently a decrease in the number of achieved ejaculations during a 30 min test.

Our findings were in line with MeA lesion studies [20–22,11,23,24], as we found that silencing of the MeA impaired ejaculation as shown by an increased latency to ejaculation, and consequently also caused a reduction in the achieved number of ejaculations. Similar to what others found [10,12,20], we also observed that more mounts and intromissions preceded ejaculation, while the intromission ratio was not affected. This indicates that erectile function is not impaired by MeA silencing. In our more extensive behavioral analysis, we annotated 100 % of the time until the second ejaculation series. This allowed the assessment of temporal patterning of copulation by further analysis of mount bouts and time-outs [26]. We showed that the temporal pattern of copulation remained unaffected by silencing of the MeA. Together, these findings lead us to infer that the increased ejaculation latency is not caused by a decreased erectile function or a decreased copulatory pace, but may rather be attributable to a decreased sensitivity to penile stimulation. This is congruent with findings that show that in males, c-fos in the MeA is induced upon penile stimulation (intromissions and ejaculations) [13,34], and in females upon vaginal-cervical stimulation [35], indicating a role for the MeA in the processing of sensory information. Interestingly, c-fos in the MeA upon ejaculation is expressed in a cell cluster more lateral in the MeA, whereas c-fos expression upon copulation and odor exposure is more diffusely located medially in the MeA [19,34]. The activity of the specific subset of lateral neurons associated with ejaculation could mean that these neurons respond to the sensory signal of ejaculation, or it could mean that they are involved in the actual orchestration of ejaculation. Our study shows that chemogenetic manipulation of the MeA impaired ejaculation, showing a role for the MeA in the relay of information that leads to the orchestration of ejaculation. Thus, the processing and accumulation of sensory feedback may occur in the MeA, which ultimately leads to the reach of ejaculation threshold.

Surprisingly, we found the same, attenuated, effects on copulation when stimulating the MeA as when inhibiting the MeA, although only the Gi-group reached statistical significance when comparing ejaculatory parameters to the Sham-group. These findings correspond to a study by Stark et al. [25], who found that electrical stimulation of the MeA in sexually experienced male rats reduced chasing, sniffing, and mounting of an estrous female while it increased these behaviors towards a non-estrous female [25]. The authors hypothesized that the increased mounting of a non-estrous female may actually reflect an increase in aggressive behavior caused by MeA stimulation, which would be suppressed by the sensory cues emitted by an estrous female. Some recent studies in mice might provide an explanation for these findings. It was demonstrated that high laser intensity optogenetic stimulation of all neurons or GABAergic neurons selectively in the MeA leads to aggression towards both male and female intruders, whereas low laser intensity (with same frequency and pulse duration) optogenetic stimulation of GABAergic neurons triggers anogenital sniffing and mounting [36]. A similar scalable behavioral control by laser intensity was found in Esr1+ cells in the mouse ventromedial hypothalamus [37]. It was demonstrated in this latter study that higher laser power both activates more neurons, as well as increases the average activity per neuron. In addition, chemogenetic activation of glutamatergic neurons in the MeA suppressed all social behavior and promoted self-grooming in mice [36]. Next to that, a large proportion of neurons in the MeA respond preferentially to one sex of conspecifics [38], indicating a role for the MeA to identify an appropriate mate and assure the appropriate behavioral response. Thus, a model could be proposed in which different neuronal populations in the MeA, with different activation thresholds, might orchestrate either sexual behavior or aggression or attenuate social behaviors in general, depending on the sensory cues emitted by the conspecific stimulus animal. We observed no aggression or reduced chasing, sniffing, and mounting in any of our subject males towards estrous females upon MeA stimulation, but stimulatory properties of electrical probes, optogenetics and chemogenetics are different in nature. Where effects of electrical and optogenetical stimulation are dependent on the voltage/laser power, and stimulation frequency applied, it is not possible to modulate stimulatory properties of chemogenetic stimulation. If aggressive and copulatory behavioral output in rats is dependent on the intensity of MeA stimulation as it is in mice, the electrical stimulation by Stark et al. and the chemogenetic stimulation in our study, with extensive DREADD expression, could theoretically have been "out of range" for observations of stimulatory effects on ejaculation or copulatory pace. In addition, whereas the CaMKIIa promotor is often used to specifically target glutamatergic neurons based on its absence at GABAergic synapses in the rat cortex and thalamus [39], CaMKIIa activity was shown in GABAergic neurons in several brain regions, such as the commissural and bed nuclei of the stria terminalis [40,41] and cerebellar Purkinje cells [40]. Therefore, we cannot exclude the possibility that our DREADD expression can be found in other than glutamatergic neurons, and so opposing effects of manipulation of GABA-ergic (inter)neurons and glutamatergic neurons in the MeA could have led to a diffuse effect of chemogenetic stimulation as well as inhibition. Whether neuronal subpopulations in the MeA of male rats have similar opposing effects on sexual behavior as in mice remains to be investigated.

In the current study, we employed a more extensive analysis of temporal patterning of copulation. Sachs and Barfield showed that male rats copulate in mount bouts (uninterrupted sequence of mounts and/or intromissions) and that the intervals between these mount bouts (time-outs) are highly constant [26]. Mount bouts are not intromission driven, and copulatory pace is therefore better expressed in the time-out duration than in the inter-intromission interval. Our mount bout analysis here allowed us to conclude that even though males took longer to ejaculate, copulatory behavior patterns remained unaffected, as was reflected in unaffected mount bout structure (mounts and intromissions per mount bout) and interval durations (time outs). Mount bout analysis provides valuable insight in assessment of sexual behavior of male rats and we stress that it should be part of future studies employing behavioral annotation of copulation.

Silencing and stimulation of the MeA did not interfere with the preference for an estrous female over a social stimulus. In a study by Kondo and Sachs [14], small lesions of the posterior MeA also did not affect preference for an estrous female over a non-estrous female in a similar set-up as ours, albeit with the females being behind opaque walls preventing visual cues to the subject male. In the same study it was found that the preference for an estrous female was attenuated in MeA-lesioned males compared to sham-lesioned control males if the stimulus females were anesthetized. In this set-up, the only sensory

modalities available to the subject animal would have been audition and olfaction, which is not sufficient to induce preference over a social stimulus in male rats [42]. These results of these studies imply that olfaction-induced sexual approach is reliant on the MeA, but that the processing of this information is not necessary to maintain sexual incentive motivation and preference when multiple sensory modalities are present. Interestingly, unconditioned pre-exposure to an inaccessible estrous female decreases ejaculation latency in sexually experienced males, but not in naïve males, in a directly following copulation test, and this effect is blocked by lesions of the MeA [21]. In addition, chemogenetic silencing of the MeA attenuated male urine odor preference in sexually naïve female mice [43]. Together with the notion that MeA lesions almost completely block copulation in sexually naïve male rats [44], a far larger effect than in sexually experienced animals, this emphasizes how experience shapes the role of the MeA in different aspects of sexual behavior. Therefore, it could well be that chemogenetic stimulation and inhibition of the MeA of sexually naïve males would result in different findings, even though the fact that we did not find any effects on sexual incentive motivation is in line with the possibility that sexual approach and copulation may rely on different neurobiological mechanisms [1]. Finally, it should be noted that specific neuronal populations in the MeA have been shown to be involved in sexual approach behavior in mice, and that our null-findings could also be a result of non-specific targeting diffusing opposing effects [45,46].

A limitation of our study is that some of the subject males in our study had DREADD expression in the lateral hypothalamus, a brain area known to be involved in sexual behavior, specifically ejaculation, the post-ejaculatory interval, and preference for an estrous female [47–49]. We ran a sub-analysis on our data set excluding all animals with LH expression, and this resulted in similar findings. The expression of DREADD also extended to structures outside of the MeA in this study. The majority of animals expressed DREADD in the STIA, AHi, PMCo, and BM at a similar density as in the MeA, and some animals had low density expression in the vHC as well. Some of the amygdaloid nuclei expressing DREADD have been implicated in the regulation of aspects of sexual behavior [19,44,50–53]. In an additional analysis of a subset of a few animals that solely and substantially expressed DREADD in structures posterior from the MeA (i.e. AHi, PMCo, BM, and vHC), we found no indication of any effects on sexual incentive motivation or copulation. Even though we cannot be completely certain that the DREADD-expressing brain areas outside of the MeA did not contribute to the measured effects in our data set, we conclude that the main effects that we found are attributable to manipulation of the MeA.

Integrating our results on sexual incentive motivation and copulation with the literature suggests that the MeA has a role in the processing of sexually arousing stimuli in male rats before and during copulation. We hypothesize that even though cue processing by the MeA before the start of copulation may not influence the incentive preference for an estrous female in the presence of all sensory modalities, it might rather impact the state of arousal during subsequent copulation, an effect shaped by sexual experience. Our current experimental design did not allow for exploration of this hypothesis, which should be further assessed in future research. Our study showed that the MeA is involved in the regulation of ejaculation. The increased latency to ejaculation is not caused by effects on temporal patterning of copulation or erectile function. Rather, we conclude that the MeA has a role in the processing of sensory feedback necessary to overcome ejaculation threshold during copulation.

CRediT authorship contribution statement

Patty T. Huijgens: Methodology, Formal analysis, Writing - original draft, Conceptualization, Investigation. **Roy Heijkoop:** Methodology, Supervision, Writing - review & editing, Conceptualization. **Eelke M.S. Snoeren:** Methodology, Supervision, Writing - review & editing, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bbr.2021.113206.

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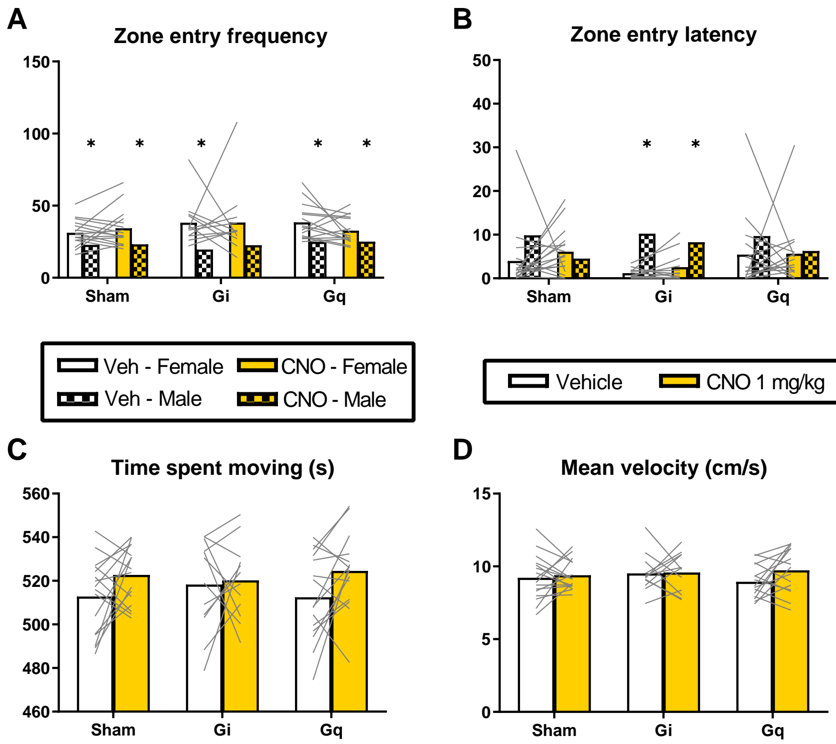
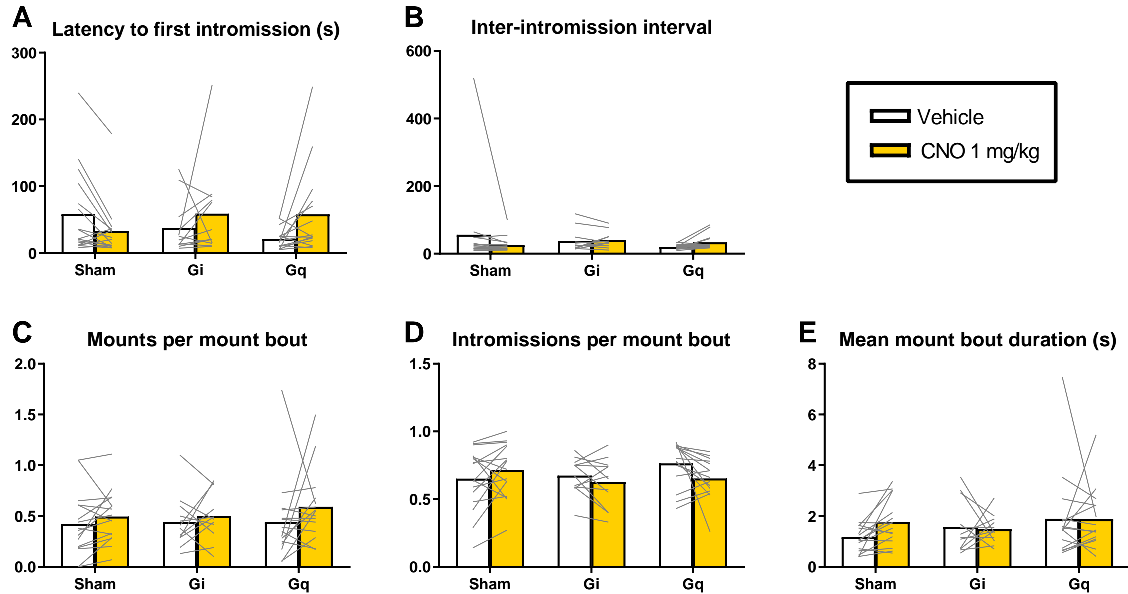
Supplementary figures

Silencing and stimulating the medial amygdala impairs ejaculation but not sexual incentive motivation in male rats

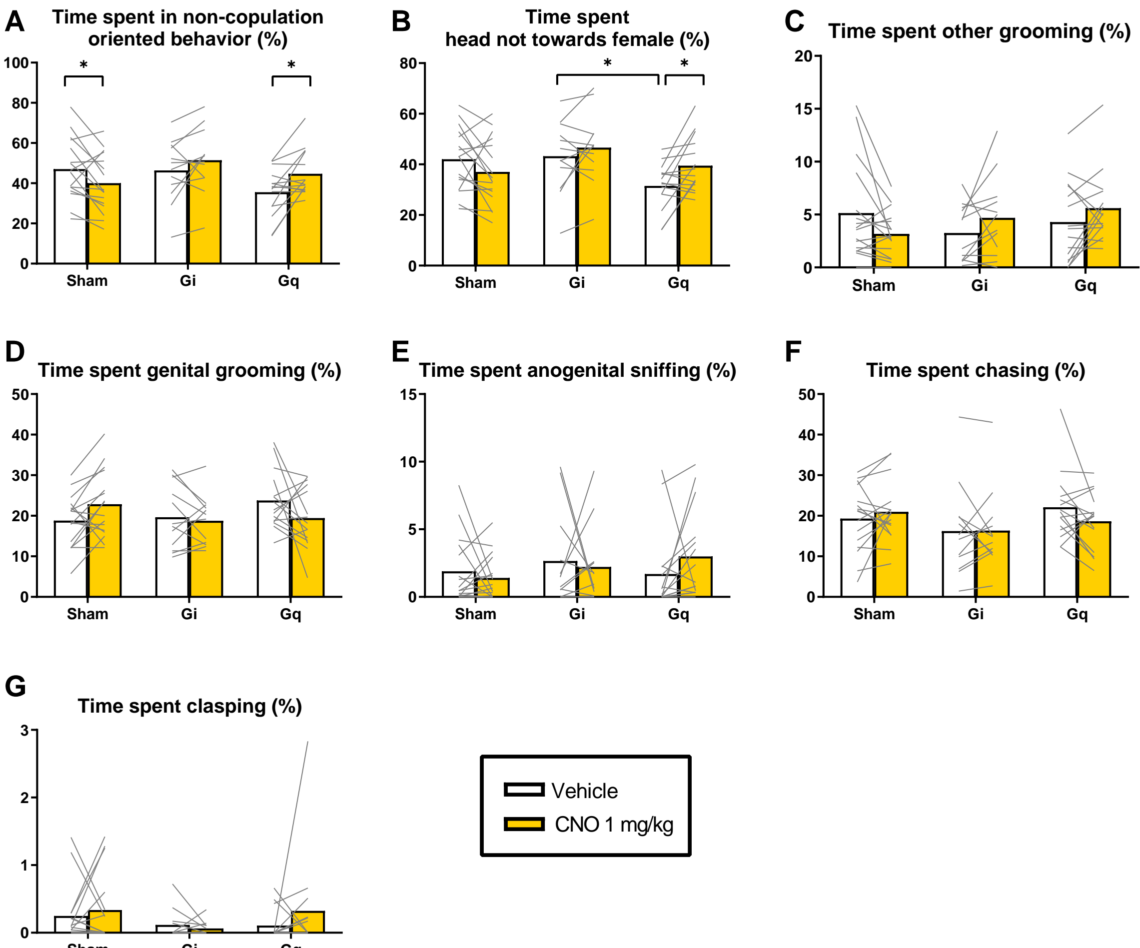
Patty T. Huijgens, Roy Heijkoop, Eelke M.S. Snoeren



Supplementary figure 1 Additional SIM test outcome measures. **(A)** Zone entry frequency. **(B)** Zone entry latency. **(C)** Time spent moving. **(D)** Mean velocity. **All panels:** *p<0.05 compared to "female zone".



Supplementary figure 2 Additional copulation test outcome measures (first ejaculation series). **(A)** Latency to first intromission. **(B)** Inter-intromission interval. **(C)** Mounts per mount bout. **(D)** Intromissions per mount bout **(E)** Mean mount bout duration.



Supplementary figure 3 Percentage of time spent on behaviors during first ejaculation series. **(A)** Non-copulation oriented behavior (other grooming + head not towards female). **(B)** Head not towards female. **(C)** Other grooming. **(D)** Genital grooming. **(E)** Anogenital sniffing. **(F)** Chasing. **(G)** Clasping. **All panels:** $^{*}p<0.05$

Paper IV

Effects of gonadectomy and dihydrotestosterone on neuronal plasticity in motivation and reward related brain regions

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ORIGINAL ARTICLE

Effects of gonadectomy and dihydrotestosterone on neuronal plasticity in motivation and reward related brain regions in the male rat

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Abstract

Gonadal hormones affect neuronal morphology to ultimately regulate behaviour. In female rats, oestradiol mediates spine plasticity in hypothalamic and limbic brain structures, contributing to long-lasting effects on motivated behaviour. Parallel effects of androgens in male rats have not been extensively studied. Here, we investigated the effect of both castration and androgen replacement on spine plasticity in the nucleus accumbens shell and core (NAcSh and NAcC), caudate putamen (CPu), medial amygdala (MeA) and medial preoptic nucleus (MPN). Intact and castrated (gonadectomy [GDX]) male rats were treated with dihydrotestosterone (DHT, 1.5 mg) or vehicle (oil) in three experimental groups: intact-oil, GDX-oil and GDX-DHT. Spine density and morphology, measured 24 hours after injection, were determined through three-dimensional reconstruction of confocal z-stacks of DiI-labelled dendritic segments. We found that GDX decreased spine density in the MPN, which was rescued by DHT treatment. DHT also increased spine density in the MeA in GDX animals compared to intact oil-treated animals. By contrast, DHT decreased spine density in the NAcSh compared to GDX males. No effect on spine density was observed in the NAcC or CPu. Spine length and spine head diameter were unaffected by GDX and DHT in the investigated brain regions. In addition, immunohistochemistry revealed that DHT treatment of GDX animals rapidly increased the number of cell bodies in the NAcSh positive for phosphorylated cAMP response-element binding protein, a downstream messenger of the androgen receptor. These findings indicate that androgen signalling plays a role in the regulation of spine plasticity within neurocircuits involved in motivated behaviours.

KEYWORDS

androgen, gonadectomy, pCREB, plasticity, spine density

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1 | INTRODUCTION

Gonadal hormones are key regulators of rewarding behaviour.[1,2] Oestrogen, progesterone and androgen signalling in the brain is involved in the display of motivated behaviours such as copulation, aggression and physical activity.[3] Moreover, gonadal hormones have been shown to influence the susceptibility to addiction-like behaviour.[4] To understand how hormones affect behaviour, it is important to investigate the underlying neurobiological mechanisms.

One mechanism through which gonadal hormones exert their influence on motivated behaviours is by affecting the structural plasticity of neurones. Previous research has shown that spine density, spine morphology and dendrite length can be impacted by gonadal hormones in multiple brain regions involved in motivation.[5-8] These hormone-induced structural reorganisations are both sexually dimorphic and strikingly different between brain regions, and have been linked to motivated behaviour, learning, memory and addiction.[4,9-11]

Copulation is a naturally occurring motivated behaviour reliant on gonadal hormones. Earlier research has shown that structural neuronal plasticity could be at the basis of hormonal effects on sexual behaviour.[12] For example, within the hypothalamus, oestradiol appears to enhance neuronal connectivity, essential for lordosis.[13-16] Oestrogens impact additional structures in the female limbic system. For example, spine density in the hippocampus fluctuates during the oestrous cycle and oestradiol increases spine density in ovariectomised animals.[17,18] By contrast, oestradiol administration to ovariectomised hamsters or rats produces a decrease in spine density within the nucleus accumbens core (NAcC).[8,19]

Castration gradually ceases all sexual behaviour in male rats and hormonal replacement fully restores copulation.[20] Yet, in males, it remains grossly unknown what neurobiological mechanisms underlie the loss of sexual behaviour following loss of gonadal hormones, and whether hormone effects on structural plasticity could be involved. Although some studies have shown spine plasticity in response to testosterone in males, it remains unclear to what extent this is mediated by oestrogen formed through aromatisation of testosterone.[21-23] It is, however, evident that oestrogens do not simply have the same effects on spine plasticity in males as in females. For example, as mentioned, the hippocampal CA1 region exhibits increased spine density upon oestrogen treatment in females, but is unresponsive to oestrogens in males.[24,25] Instead, CA1 spine density in males is induced by dihydrotestosterone (DHT), a high-affinity ligand of the androgen receptor that is not aromatised into oestradiol.[24] Our laboratory also recently reported similar effects in the nucleus accumbens, where spine plasticity is affected by oestrogens in females and by DHT in males, again indicating that these effects in males are caused by androgens rather than oestrogens.[8,26]

Although the effects of gonadal hormones on spine plasticity are sexually dimorphic, there are indications that the underlying mechanisms through which these effects arise are homologous. Specifically, hormone-induced spine plasticity in the nucleus accumbens is mediated by activation of metabotropic glutamate receptor (mGluR) signalling, via oestradiol in females and DHT in males.[8,26] In females, the oestrogen-induced spine plasticity is reliant on membrane-bound oestrogen receptors that are coupled to mGluRs, which are activated upon oestrogen binding to the oestrogen receptor. The activation of mGluRs can induce a downstream phosphorylation pathway leading to increased phosphorylation of cAMP response-element binding protein (CREB).[27,28] CREB is a transcription factor involved in numerous behavioural outputs and implicated in spine plasticity.[29,30] Because androgen-induced spine plasticity in the nucleus accumbens in males is also mediated by mGluRs, it could be expected that androgen signalling in males has similar effects on CREB phosphorylation as oestradiol in females, perhaps mediated by membrane-associated androgen receptors.[31-33]

In the present study, we investigated the effects of gonadectomy (GDX) and androgen replacement on neuronal plasticity in putatively important brain regions for sexual motivation in male rats. We hypothesised that GDX could lead to alteration of structural plasticity in the medial preoptic nucleus (MPN), medial amygdala (MeA), NAcC and nucleus accumbens shell (NAcSh), possibly indicating a mechanism for GDX-induced loss of sexual behaviour. In addition, we investigated how androgen signalling in GDX males impacts structural plasticity. Finally, we built on the hypothesis that the observed effects could be membrane-bound androgen receptor mediated by looking at rapid induction of phosphorylated CREB (pCREB) in the striatum following DHT treatment in GDX males.

2 | MATERIALS AND METHODS

2.1 | Animals

Intact and castrated Sprague-Dawley rats (200-225 g, 8 weeks old) were purchased from Envigo Laboratories (Indianapolis, IN, USA). Castration took place 48-72 hours before the animals arrived in our facility. Animals were housed two per cage (DiI labelling) or three per cage (pCREB immunohistochemistry) and maintained under a 12:12 hour light/dark photocycle with food and water ad lib. Animals were allowed to habituate to the research facility for at least 1 week prior to the start of any experiment. All animal procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at the University of Minnesota.

2.2 | Treatment, tissue processing and DiI labelling

5α-androstan-17β-ol-3-one (DHT; Steraloids Inc; Newport, RI, USA) was dissolved in cottonseed oil. Ten to 30 days after arrival, the rats were injected s.c. with 1.5 mg DHT or vehicle in a volume of 0.2 mL. The used DHT dose in the present study is based on a recent study from our laboratory showing significant effect on spine density in the NAcSh of castrated males.[26] The experiment was run in batches of two animals (cage mates) at a time. The two animals in each batch were in the same group, and treatment groups were randomised

according to a latin square design, so that average castration duration did not differ between castrated groups. Animals were killed 24 hours after hormone or vehicle treatment.

The tissue was prepared and DiOlistically labelled as described previously.[34] DiOlistic labelling involves the ballistic delivery of tungsten microparticles coated with a lipophilic fluorescent dye (here: DiI) to tissue sections. DiI labels membranes of all neurones in which a tungsten bead is embedded, providing a Golgi-like labelling of neurones, with higher throughput and without bias. Briefly, animals were killed by an Euthasol overdose (0.35 mL i.p; 390 mg mL^{-1} pentobarbital sodium, 50 mg mL^{-1} phenytoin sodium; Virbac AH Inc., Nice, France), injected with 0.25 mL heparin into the left ventricle, and transcardially perfused with 50 mL of 25 mmol L^{-1} phosphate-buffered saline (PBS, pH 7.2) followed by 500 mL of 1.5% paraformaldehyde in PBS. Brains were removed and post-fixed in 1.5% paraformaldehyde for 1 hour. Then, brains were sliced coronally into 300-μm thick sections using a VT1000 S Vibratome (Leica, Buffalo Grove, IL, USA). Sections containing the brain regions of interest (ie the caudate putamen [CPu], NAcC and NAcSh, MPN and MeA) were collected and stored in PBS until ballistic labelling.

DiI bullets were prepared from Tefzel tubing (Bio-Rad, Hercules, CA, USA) pretreated with 15 mg mL^{-1} polyvinylpyrrolidone (PVP) in deionised water. Two milligrams of DiI (Molecular Probes, Carlsbad, CA, USA) was dissolved in 100 μL of dichloromethane and applied to 90 mg of 1.3 μm tungsten microcarrier particles (Bio-Rad) spread out evenly on a glass slide. The coated tungsten particles were suspended in 10 mL PVP solution, and disaggregated by sonication and intermittent vortexing for 12 minutes. The pretreated Tefzel tubing was subsequently coated with the DiI-tungsten particles by allowing the suspension to settle in the tubing for 3 minutes, after which the suspension was quickly expelled. The tubing was dried under 0.4 LPM nitrogen gas flow using a tubing prep station (Bio-Rad) for 30 minutes, after which the tubing was cut into 1.3 cm long 'bullets'. Bullets were loaded into a Helios Gene Gun (modified barrel, 40 mm spacer, 70 μm mesh filter; Bio-Rad) and PBS surrounding brain sections was removed. DiI-tungsten particles were shot into the tissue by shooting one bullet on each section using helium gas expulsion (100 PSI). To allow DiI spreading throughout the labelled neurones, sections were kept overnight in PBS in the dark. The next day, sections were post-fixed in 4% paraformaldehyde for 1 hour, rinsed in PBS, mounted on slides and coverslipped with FluorGlo mounting media for lipophilic dyes (Spectra Services, Ontario, NY, USA) (note that the FluorGlo mounting medium is no longer available).

2.3 | DiI confocal imaging, reconstruction and quantitation

Using a Leica TCS SPE confocal microscope, brain regions of interest were identified and delineated using low magnification brightfield in conjunction with the rat brain atlas of Paxinos & Watson (6th edition) for reference.[35] For each brain region, two or three dendritic segments (70-200 μm away from soma, and more than 10 μm away from dendritic end points and bifurcations) per neurone (completely

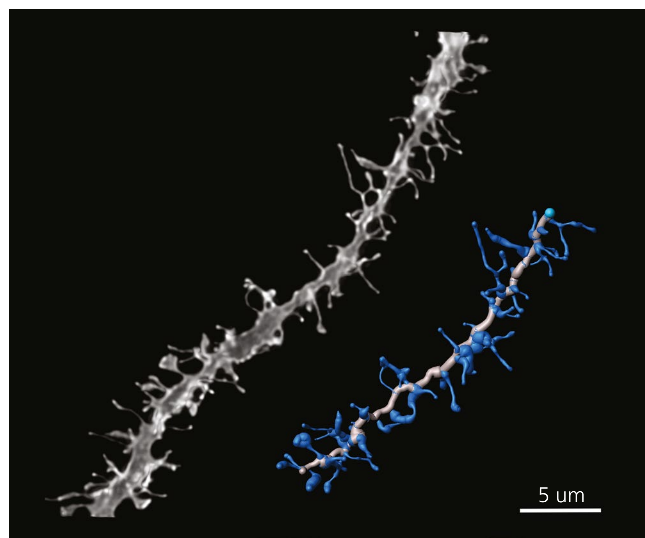

FIGURE 1 Dendritic segment reconstruction. Representative maximum projection image and three-dimensional reconstruction process of a striatal medium spiny neurone dendritic segment labelled with DiI. Image acquired at 63×. Scale bar = 5 μm

and brightly labelled, isolated from other labelled neurones), in two to six neurones (three neurones for the far majority of data points), were imaged and analysed (Figure 1). Our assessment and statistical analyses of dendritic spine densities is based upon a rigorous approach that we and others have previously used.[8,19,34,36-38] With nine or ten animals/group, this equates to approximately 80-90 dendritic segments (and thousands of spines) per group per brain region.

Dendritic segments were imaged using a Leica PLAN APO 63×, 1.4 NA oil immersion objective (11506187; Leica, Mannheim, Germany) and Type LDF immersion oil (Cargille, Cedar Grove, NJ, USA). All images were taken at an *xy* pixel distribution of 512 × 512, a frequency of 400 Hz, a step size of 0.12 μm and optical zoom of 5.6, with the laser power and photomultiplier being adjusted to capture the dendrite in its full dynamic range. Data from nine or ten animals were collected for each treatment group. In case there were less than two neurones in a brain region feasible for imaging, the animal was excluded from further analysis for that region. This predominantly occurred in the MPN and MeA, and explains the smaller sample sizes for these regions.

After imaging, optical sections were processed through three-dimensional (3D) deconvolution using AutoQuant X3 AutoDeblur software (Media Cybernetics, Rockville, MD, USA). Deconvoluted z-stacks were then reconstructed in the Surpass module of Imaris software (Bitplane Inc., Concord, MA, USA), through manual tracing of dendrites and spines using the Filament tool and Autodepth function. A 3D reconstruction of 15-20 μm of dendritic shaft and spines was rendered using the diameter function with a contrast threshold of 0.7, and data on spine density, spine length and head diameter were collected for each segment. Spine densities for each segment (collected as average spine density per 10 μm) were averaged across each neurone and then within each brain region for each animal, providing a region-specific spine density average for each animal that

was then used for statistical analysis. Measurements of spine length and head diameter were pooled for each treatment condition and then plotted in violin plots, as well as binned cumulative probability distributions (bin sizes: spine length, 0.5 μm; head diameter, 0.1 μm).

2.4 | pCREB immunohistochemistry

2-hydroxypropyl-β-cyclodextrin (cyclodextrin; Sigma-Aldrich, St Louis, MO, USA) was dissolved in sterile water to obtain a 45% vehicle solution. DHT was dissolved in cyclodextrin solution and 1.5 mg (high dose) or 0.15 mg (low dose) was injected i.p. in a volume of 0.2 mL. DHT was administered i.p. in this experiment instead of s.c. given the more rapid time course of CREB phosphorylation compared to spine changes measured over 24 hours. After injection, animals were put alone in a cage until lethal i.p. injection with Euthasol (0.35 mL i.p; 390 mg mL^{-1} pentobarbital sodium, 50 mg mL^{-1} phenytoin sodium, Virbac AH Inc.) 15 or 30 minutes later. Then, animals were injected with 0.25 mL of heparin into the left ventricle, and transcardially perfused with 50 mL PBS followed by 500 mL of 4% paraformaldehyde in PBS. Brains were removed and post-fixed in 4% paraformaldehyde for 2 hours, and stored in 10% sucrose in PBS overnight at 4°C. The next day, brains were cut on a freezing microtome into 40-μm sections and every third section throughout the striatum was collected into 0.1% bovine serum albumin (BSA) in 25 mmol L^{-1} PBS (BSA/PBS) for immediate free-floating immunohistochemical processing. After rinsing in BSA/PBS, sections were incubated with a rabbit polyclonal primary antibody directed against serine 133 phosphorylated CREB (dilution 1:2000; cat. 06-519, Merck Millipore, Burlington, MA, USA[39,40]) in 0.3% Triton-X-100 in BSA/PBS for 48 hours at 4°C. Subsequently, sections were incubated in biotinylated goat anti-rabbit (dilution 1:200; VECTASTAIN Elite ABC-HRP rabbit-IgG Kit; Vector Laboratories, Inc., Burlingame, CA, USA) in BSA/PBS for 1 hour, avidin-biotin-peroxidase complex (dilution 1:100; VECTASTAIN Elite Kit) in PBS for 1 hour, and 3,3′-diaminobenzidine (0.8 mg mL^{-1}; Sigma-Aldrich) with 0.3% H_2O_2 in 50 mmol L^{-1} Tris buffer (pH 7.6) for 8 minutes, with repeated buffer washing in between all steps. Sections were then mounted on slides, and coverslipped using DPX mounting medium (Sigma-Aldrich). The experiment was run in batches of three animals at a time, with the same treatment injection timing (15 or 30 minutes) for each animal in the batch, and one animal per treatment group per batch. Administration of different treatments was randomised according to a latin square design so that the order of injection and perfusion would not be a factor.

2.5 | pCREB imaging and quantitation

For each animal, three sections within the central striatum were identified and imaged using a Leica DM 4000 B LED microscope and 10× objective. At the level of the nucleus accumbens, the anterior commissure has a lateral monotonic migration. Consequently, sections were matched on the anterior-posterior axis by selecting those sections in which the distance from the tip of the lateral ventricle to the medial edge of the anterior commissure was 300-350 μm. Images were always taken on the right side of the section, without avoidance of artefacts. The same exposure and white balance settings were used across all images. The images were subsequently loaded in PHOTOSHOP (Adobe Systems Inc., San Jose, CA, USA) and a red box (300 × 500 μm) placed within the brain regions of interest. For the NAc, the box was placed medial to the ventricle for the shell and lateral to the ventricle for the core, and the distance between the boxes was kept at 100 μm for each section imaged. For the medial and lateral CPu, the top corner of the boxes touched the corpus callosum.

For pCREB + cell counting, the cell counter plugin was used in IMAGEJ (NIH, Bethesda, MD, USA). To increase intra-observer reliability, we converted the images to greyscale and used the automated threshold algorithm ‘Otsu’ to acquire a binary image that separated positive cells from background to use as a counting guide. Otsu's method finds a threshold value where foreground and background pixel value variance is at a minimum. Because some batch-to-batch immunohistochemistry variance is to be expected, Otsu's method works well for thresholding here because it uses information from within the image to separate background from staining. Cells were counted if they appeared with at least one pixel in the thresholded image, and standard stereology rules were applied when counting on the box borders. Intra-observer agreement of positive cell count was within the range 97.4%-99.5% for a sample size of 10 duplicate images. Cell counts of three sections were averaged across each brain region within each animal. Out of 384 total boxes, eight images contained very large artefacts in the tissue within the box, and were therefore excluded from analysis.

2.6 | Statistical analysis

All data analysis was conducted in PRISM, version 8 (GraphPad Software Inc., San Diego, CA, USA). For spine density and pCREB expression, groups were compared using a one-way ANOVA, followed by Tukey's multiple comparisons test in case of significant effect, or a Kruskal-Wallis test followed by Dunn's multiple comparisons test when the data did not pass assumptions for parametric analysis. The binned spine morphology probability distributions were compared to each other group using a Kolmogorov-Smirnov test.

3 | RESULTS

3.1 | Dendritic spine plasticity

To investigate the effects of both GDX and androgen replacement on dendritic spine plasticity, we compared intact males treated with oil (vehicle) to GDX males treated with oil, as well as to GDX males treated with DHT. We found that GDX affected spine plasticity in the MPN ($H = 12.16$, $P = 0.0002$, $\eta^2 = 0.59$) (Figure 2A) by decreasing spine density (mean difference vs intact = 2.03, $P = 0.0073$, $g = 2.35$). GDX did not affect spine density in the MeA, NAcSh, NAcC and CPu (Figure 2A). DHT administration to GDX animals rescued the GDX-induced spine loss in the MPN (mean difference vs GDX-oil = 2.15,

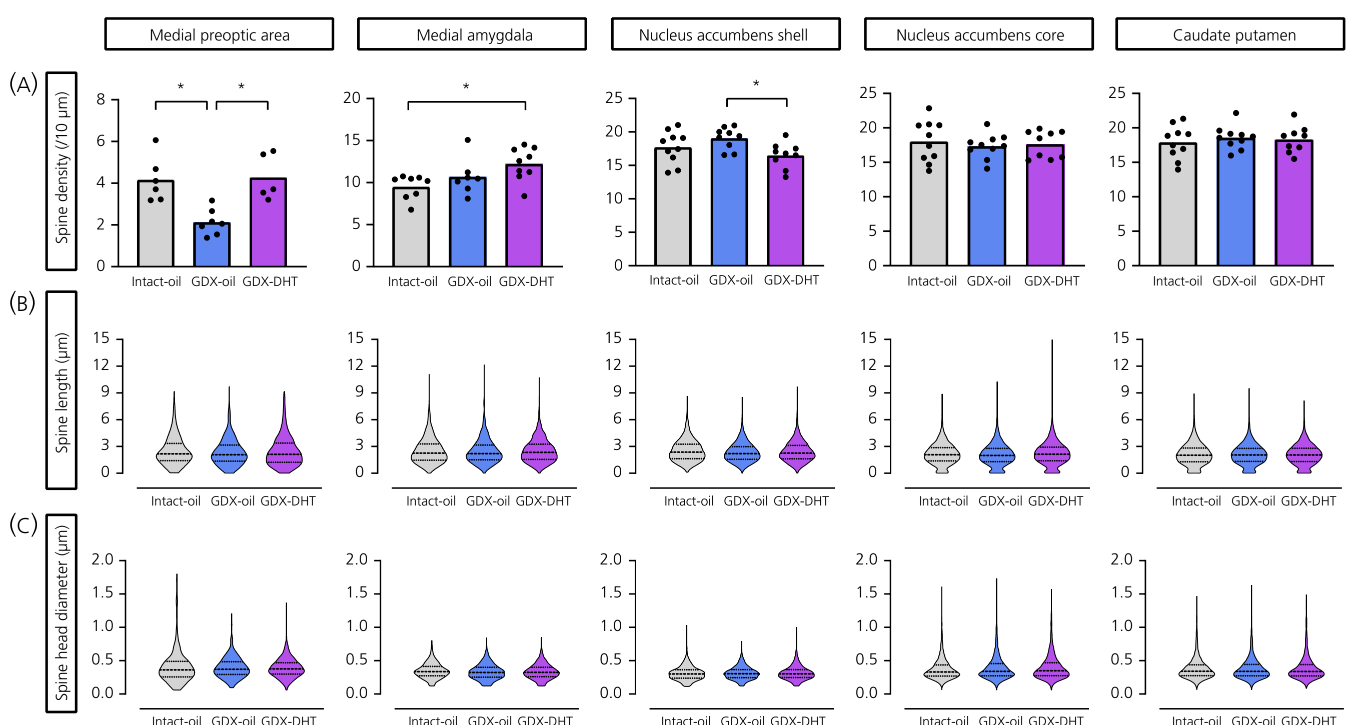

FIGURE 2 Gonadectomy (GDX) and dihydrotestosterone (DHT) affect spine plasticity differentially across several brain regions regulating motivated behaviour. A, Spine density 24 h after treatment with oil or DHT in intact and GDX males in the medial preoptic nucleus (MPN), medial amygdala (MeA), nucleus accumbens shell and core (NAcSh and NAcC), and caudate putamen (CPu). Individual values represent neurone spine density average per animal (= unit of analysis), which is comprised of the average spine density across two or three neurones per animal, calculated from the average spine density from two or three segments per neurone. n = 6, 7, 5 (MPN); 8, 7, 9 (MeA); 10, 10, 9 (NAcC); 10, 9, 9 (NAcSh); and 10, 10, 9 (CPu) animals per group. $^*P < 0.05$. B, Violin plot representation of spine length distribution. For violin plots, all spine data points from all animals within the same group were pooled into one plot. Dashed line, median; dotted lines, quartiles. C, Violin plots of spine head diameter distribution

$P = 0.012$, $g = 2.54$). By contrast, DHT decreased spine density in the NAcSh in GDX animals compared to oil-treated GDX animals ($F_{2,25} = 3.56$, $P = 0.04369$, $\eta^2 = 0.22$) (Figure 2A) (mean difference vs GDX-oil = 2.54, $P = 0.0341$, $g = 1.44$). This effect, however, was not different compared to the intact-oil group. We found effects on spine density in the MeA as well ($F_{2,21} = 4.45$, $P = 0.0245$, $\eta^2 = 0.30$) (Figure 2A). Specifically, although GDX itself did not affect spine density, DHT treated GDX males had a higher spine density than oil-treated intact males (mean difference vs intact = 2.71, $P = 0.0193$, $g = 1.56$). We saw no effects of DHT on spine density in NAcC and CPu (Figure 2A) compared to either the intact-oil or the GDX-oil group.

Spine morphology gives information about spine maturation and function.[41] Because spine morphology is determined by spine length and spine head diameter, we compared the distributions of these two parameters between groups. No effects of castration or DHT treatment were observed on spine length or spine head diameter in any of the brain regions (Figure 2B,C).

3.2 | pCREB expression

To investigate the potential rapid effects of DHT we focused on the striatum, a brain region in which rapid phosphorylation of CREB has been documented.[27] We determined the number of cells expressing pCREB by means of immunohistochemistry (Figure 3A), 15 and 30 minutes after hormone or vehicle treatment of GDX males. DHT treatment had no effect on the amount of pCREB + cells within 15 minutes of hormone administration in any of the investigated subregions (Figure 3B). After 30 minutes, however, a high dose of DHT significantly increased the number of pCREB + cells in the NAcSh ($F_{2,12} = 5.039$, $P = 0.0258$, $\eta^2 = 0.46$) (Figure 3C) compared to vehicle (mean difference 90, $P = 0.0358$, $g = 1.79$). A low dose of DHT also increased the number of pCREB + cells in the medial CPu ($F_{2,12} = 4.350$, $P = 0.038$, $\eta^2 = 0.42$]) (Figure 3C) compared to vehicle (mean difference 82, $P = 0.0321$, $g = 1.77$). No effects of DHT treatment were found in the NAcC and lateral part of the CPu.

4 | DISCUSSION

Gonadal hormones are known to regulate synaptic plasticity.[42,43] Although the literature has so far mostly characterised the effects of oestrogens in females, some evidence exists for effects of gonadal steroids in males as well.[24,31,44] In the present study, we aimed to examine the effects of loss and subsequent replacement of gonadal hormones on spine plasticity in males. We focused on brain regions that are involved in neural circuits of (sexual) motivation: the NAcC, NAcSh and CPu, which are part of dopaminergic reward processing,

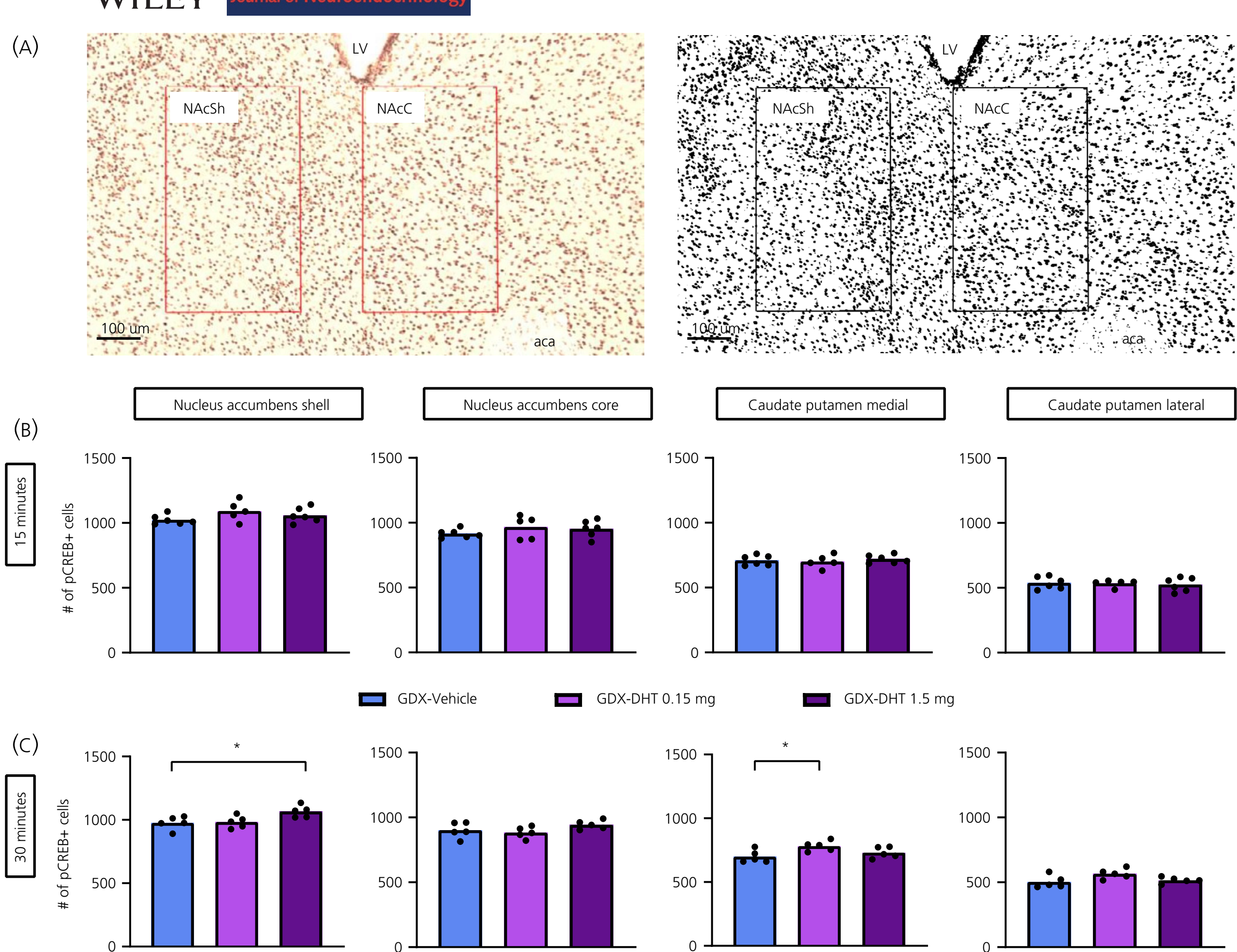

FIGURE 3 Dihydrotestosterone (DHT) rapidly induces cAMP response-element binding protein (CREB) phosphorylation. A, Representative image of phosphorylated CREB (pCREB) staining and counting box (300 μm × 500 μm) delineating in the nucleus accumbens core and shell, and corresponding Otsu thresholded image. Scale bar = 100 μm. LV, lateral ventricle; aca, anterior commissure; NacSh, nucleus accumbens shell; NacC, nucleus accumbens core. B, Number of pCREB positive cells in each brain region 15 min after i.p. treatment with oil or low or high dose DHT in gonadectomised (GDX) males. C, Number of pCREB positive cells 30 min after treatment. *$P < 0.05$

and the MPN and MeA, two regions critically involved in the display of sexual behaviour. The investigated brain regions have all been shown to be androgen target areas in rats.[45] We showed that GDX decreased spine density in the MPN, which was rescued by DHT treatment. In addition, DHT decreased spine density in the NAcSh in GDX animals, whereas it increased spine density in the MeA of GDX animals compared to intact animals. Thus, spine plasticity is differentially affected by gonadal hormones across the studied brain regions.

GDX gradually ceases copulation in male rats as a result of the loss of gonadal hormones. Oestrogen as well as androgen signalling through oestrogen and androgen receptors in the brain is necessary for the full display of male sexual behaviour.[20] Considering the high expression of androgen and oestrogen receptors in the MPN, it is therefore not surprising that the MPN is the most important brain region for regulation of sexual behaviour in males.[20] Disruption of the MPN through lesions causes gonadally intact male rats to stop copulating.[20] Local infusion of an aromatase inhibitor (preventing the formation of oestradiol and thus oestrogen receptor signalling) or an androgen antagonist (preventing androgen receptor signalling) into the MPN suppresses copulation in gonadally intact male rats, showing a vital interaction of gonadal hormones and the MPN in male sexual behaviour.[46,47] Yet, it remains unclear what mechanism underlies the importance of the activity of gonadal hormones in the MPN for copulation in male rats. What has been shown earlier is that GDX reduces dopamine release and c-Fos expression in the male MPN upon exposure to an oestrous female.[48-50] This suggests that a lack of gonadal hormones may reduce afferent sensory information to the MPN. In the present study, we demonstrate a novel effect of GDX in the MPN of male rats. GDX drastically reduces spine density of MPN neurones, an indication of an overall decrease in synapses within the MPN. In line with reduced dopamine release and c-Fos expression in the MPN, this suggests a model in which gonadal hormones act as facilitators contributing to MPN connectivity. This connectivity may then be necessary for sexual behaviour to arise in response to the stimulus of an oestrous female. Our study shows that the GDX-induced spine loss is present in males gonadectomised

for longer than 10 days, a time point at which most male rats would have stopped copulating.[20] Future research should focus on spine plasticity at different time points after GDX aiming to reveal whether loss of spines in the MPN also coincides with the gradual loss of sexual behaviour after GDX in males. That should provide more insight in whether GDX-induced loss of spines in the MPN indeed contributes to loss of sexual behaviour.

Treatment with testosterone given systemically or locally into the MPN facilitates copulation in GDX males.[51-54] In addition, testosterone rescues GDX-induced spine loss in the MPN of male hamsters.[23] Furthermore, functional aromatisation of testosterone into oestrogen is necessary for the display of the full range of sexual behaviour in male rats. Treatment of GDX males with DHT, a high-affinity ligand of the androgen receptor that cannot be aromatised into oestrogen, is ineffective with respect to reinstating sexual behaviour.[52] Furthermore, at the same time that DHT has affinity for oestrogen receptor β (ERβ), our prior work showed that an ERβ agonist did not affect dendritic spine density in the nucleus accumbens.[26] Therefore, we expected to find that treatment with DHT would not be sufficient to rescue the GDX-induced spine loss in the MPN. Nevertheless, in the present study, we show that DHT treatment of GDX males does fully restore spine density on MPN neurones. Even though DHT-induced spinogenesis in the MPN of GDX males does not coincide with restoration of copulation,[55] androgen signalling still contributes to copulatory behaviour. For example, local infusion of an androgen receptor antagonist into the MPN of GDX males prevents the reinstatement of sexual behaviour by systemic testosterone treatment.[56] In line with this, androgen signalling in addition to oestrogen signalling is necessary for the motivational aspects of sexual behaviour, such as preference for an oestrous female, and DHT alone has mild effects on sexual incentive motivation.[57,58] Furthermore, testosterone and oestradiol both show rapid effects on firing rate in MPN neurones, but they rarely affect the same neurones.[59] Therefore, androgenic signalling may perhaps primarily influence and maintain sexual motivation through a distinct neuronal population in the MPN, mediated by spine plasticity. An important research focus in the future will be to unravel the effects of oestrogens on spine plasticity in the MPN of GDX males.

By contrast to our findings in the MPN, we found that GDX did not decrease spine density in the MeA, another important region for copulation.[20] Other studies have reported a decrease in spine density in the posterodorsal MeA, 3 months after GDX, measured on dendrites very proximal to the soma,[59,61] and in males castrated before puberty.[62] Our measures, on the other hand, were taken at least 70 μm away from the soma, not on primary dendrites and within a shorter time frame after GDX. We did find, however, that DHT has spinogenic properties in the MeA, even though we only saw this in comparison with intact males. Possibly, gonadal hormones are not necessary to maintain spine density in the MeA, but do have the ability to affect spine plasticity such as in the MPN. Another study conducted in intact pubertal males showed that a chronic high dose testosterone is transiently spinogenic in the antero- and posterodorsal MeA.[6] Thus, gonadal hormones may affect MeA spine plasticity differentially depending on the distance of a dendritic segment from the soma, the timing of castration within life, the castration duration, and the amount of time that has passed subsequent to hormone treatment.

The present study replicated earlier results obtained from our laboratory, where we showed that, in contrast to its spinogenic effects in the MPN and MeA, DHT decreases spine density after GDX in NAcSh, but not in NAcC and CPu in GDX males.[26] Here, we used an additional control group of intact males to also establish that gonadal hormones are not necessary for maintaining spine density and morphology in the striatum in males because GDX left these variables unaffected. Another group found that, in intact males, a chronic high dose of testosterone decreases spine density in the NAcSh, and has no effect on the NAcC.[63] This suggests that androgens induce loss of spines in the NAcSh regardless of whether the male is gonadectomised or not. The rapid changes in NAcSh dendritic spines following DHT do not appear to underlie the expression of copulation in males because the effects of DHT on spines require mGluR5 signalling[26] and accumbens antagonism of mGluR5 receptors does not disrupt copulation.[64] The medial preoptic area and medial amygdala are better candidates for sites of action of DHT on copulation, and DHT modulated dendritic spines within 24 hours in these regions as well. One study has demonstrated interactions between oestrogen receptor and mGluR signalling in the medial preoptic area[65] with nothing known about similar interactions in the medial amygdala. Because both oestradiol and DHT induce rapid ERK phosphorylation in the medial preoptic area,[66] cooperative signalling through mGluR receptors could be the basis for rapid effects on copulation in males. We propose parallels with the mechanisms through which oestradiol acts to regulate female sexual behaviour. Oestrogens induce rapid membrane-mediated signalling cascades, which are followed by longer lasting transcriptional activation via nuclear receptors.[67] We envision a similar set of actions for male sexual behaviour in which androgens provide both rapid and long-term plasticity.

The small numbers of dendritic spines measured in these studies sometimes raise questions about the functional significance of these spine changes. For striatal medium spiny neurones, Golgi studies suggest that the cumulative dendritic length may be on the order of 2100 μm,[68] whereas cell fills put the number closer to about 3000 μm.[69] With an increase of three spines per 10 μm, as we see in the nucleus accumbens shell, this translates to upwards of 1000 excitatory synapses per medium spiny neurone, producing a substantial impact on the electrotonic potential of these neurones.[69] A limitation of the DiOlistic labelling approach taken in this experiment is that it is not possible to differentiate between specific neuronal cell-types such as D1 vs D2 medium spiny striatal neurones. In the MeA and MPN, neurones were selected based on similar gross morphology, which only partially addresses neuronal heterogeneity. Future research will aim to refine and combine methods in order to distinguish different neuronal populations.

Androgens can exert their action on neurones through multiple signalling pathways.[70] Although the 24 hours after hormone

treatment in the present study comprises sufficient time for genomic effects to occur, previous results from our laboratory show that DHT-induced spine plasticity in the NAcSh is mediated by mGluR5, a G protein-coupled receptor associated with the Gαq protein, suggesting that membrane-initiated signalling pathways are involved.[26] This mechanism is homologous to the mGluR5 mediated oestrogen-induced decrease in spine density in the NAcC of ovariectomised females.[8] The coupling to and regulation of mGluRs by membrane-bound oestrogen receptors has been well characterised and has been shown to mediate spine plasticity and behaviour in females.[15,71,72] Oeestrogens rapidly increase phosphorylation of the transcription factor CREB through its membrane interaction with mGluRs in hippocampal and striatal neurones exclusively in female cultures.[27,28] It is important to note that, although oestrogen receptor/mGluR signalling leads to pCREB across many brain regions, only a subset of these exhibit changes in dendritic spines. Therefore, this signalling pathway may be necessary for structural changes, but it is not sufficient. In male cultures, oestradiol does not induce pCREB, whereas mGluR activation does. In addition, activation of mGluR5 mediates spine plasticity in male NAcC and NAcSh,[73] suggesting that mGluRs possibly couple to membrane-bound androgen receptor in males. The androgen receptor has indeed also been shown to migrate to the membrane,[74] using the same intracellular processes as oestrogen receptors.[75] Here, we show that DHT is capable of inducing striatal pCREB in vivo within 30 minutes of injection. Although the immunohistochemistry method that we used for assessing pCREB expression only allowed for counting of number of positive cells, and not the level of pCREB within a cell, it is possible that DHT *also* induces higher phosphorylation levels of pCREB in each individual positive cell. Still, our results point towards a pathway in which androgen binds to membrane-bound androgen receptors, which activates mGluR5 through coupling in the NAcSh but not in the NAcC. This leads to activation of a downstream signalling cascade culminating into phosphorylation of CREB, thereby enhancing its gene transcription properties. There is a large body of literature on the function of CREB, which, amongst others, is involved in learning and memory and synaptic plasticity.[29] Whether this proposed mechanism of DHT-induced plasticity, through mGluR5 or other mGluRs, can also be applied to the effects we found in the MPN and MeA will be part of future research.

5 | CONCLUSIONS

We conclude that both GDX and androgen differentially affect spine plasticity in the MPN, MeA and NAcSh, whereas NAcC and CPu remain unaffected. In the NAcSh, DHT may exert its effects through pCREB induction mediated by androgen receptor activation of mGluR5.

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CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Patty T. Huijgens: Conceptualisation; data curation; formal analysis; investigation; visualisation; writing – original draft. **Eelke M. S. Snoeren:** Formal analysis; funding acquisition; supervision; writing – review and editing. **Robert L. Meisel:** Conceptualisation; methodology; resources; supervision; writing – review and editing. **Paul G. Mermelstein:** Conceptualisation; funding acquisition; methodology; resources; writing – review and editing.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

The author contributions to the research papers in this thesis were as follows:

Paper I: RH: literature review and writing of the original draft of the sections "Behavioral paradigms for sexual motivation", "Introduction", and "Concluding remarks". PH: literature review and writing of the original draft of the section "Male rat sexual behavior". ES: literature review and writing of the original draft of the section "Female sexual behavior", funding acquisition. All authors took part in reviewing and editing original drafts.

Paper II: PH: conceptualization, investigation, behavioral annotation, methodology, data curation, analysis, writing – original draft. FG – investigation, writing – review and editing. JO – investigation, writing – review and editing. ES - conceptualization, methodology, analysis, supervision, writing – review and editing, funding acquisition.

Paper III: PH: conceptualization, investigation, data curation, methodology, analysis, writing – original draft. RH: conceptualization, methodology, supervision, writing – review and editing. ES: conceptualization, methodology, supervision, writing – review and editing, funding acquisition.

Paper IV: PH: conceptualization, investigation, data curation, analysis, writing – original draft. ES: analysis, funding acquisition, supervision, writing – review and editing. RM: conceptualization, methodology, resources, supervision, writing – review and editing. PM: conceptualization, funding acquisition, methodology, resources, writing – review and editing.