KU LEUVEN

FACULTEIT PSYCHOLOGIE EN
PEDAGOGISCHE WETENSCHAPPEN

The effects of chronic oxytocin treatment in a mouse model of delayed brain development

Masterproef aangeboden tot het
verkrijgen van de graad van Master of
Science in de pedagogische
wetenschappen
Door
Carmen Winters

promotor: Prof. Dr. Rudi D'Hooge
copromotor: Prof. Zsuzsanna Vegh

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Summary

Carmen Winters, The effects of chronic oxytocin treatment in a mouse model of delayed brain development
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Promotor: Prof. Dr. Rudi D'Hooge
Co-promotor: Dr. Zsuzsanna Vegh

For centuries, farmers have used oxytocin (OXT) via vaginal stimulation to trigger maternal behaviour in foster animals without having any knowledge about the existence of this hormone. Thus while its modulating effects on behaviour were generally known, it was not until the 1970s that OXT was identified as activator/modulator of various social behaviours. Above all, it was primarily this hormone's role in parental bonding and pair bonding ("love") that made it both commercially and clinically an appealing and relevant subject. However, it was the finding that OXT is involved in trust and empathy that has put it on the map as potential treatment for various psychiatric disorders. From then on, studies have reported beneficial, neutral, and detrimental effects of OXT in both neurotypical and patient populations. Gradually, it has become clear that OXT's nature is more complex than it was originally perceived.

In this project, the overall hypothesis was that OXT has beneficial effects in developmental disorders with social impairment. Because of the sexually dimorphic nature of neurodevelopmental disorders, it was hypothesized that OXT treatment would show different effects in males and females. In an effort to answer these main hypotheses, a mouse model of neurodevelopmental delay (Sey/+) was used. At the age of 8-14 weeks, mice of this strain were compared to controls (WT) in a testing battery for social behaviour. All animals were subjected twice to this testing battery: first to evaluate a baseline and subsequently to assess the effects of OXT treatment. OXT (3 IU) was administered intranasally and in a 25-day chronic regime. Finally, after this treatment period, an additional paradigm to investigate cognitive impairments was performed.

The results showed a consistent pattern of differential social behaviours when comparing SEYs to WTs at baseline. Dependent on the behavioural test, OXT appeared to alleviate social deficits in SEY but also in WTs. It is assumed that the observed prosocial effects are due to OXT's anxiolytic effect. In this context, data provided evidence for a sex-specific mechanism of OXT underlying different social behaviours. Against all expectations, the chronic OXT treatment appeared to have detrimental effects on cognitive functioning of SEYs and WTs of both genders.

In general it can be concluded that mice with the small eye mutation provide an adequate model for neurodevelopmental disorders with social deficits at their core (e.g. ASD). Moreover, based on this project, the belief in OXT's potential as psychiatric anxiolytic is justified. However, the putative cognitive impairments show that there are more dots which need to be connected before establishing its clinical use.

Woord van dank

Zoals Atlas volgens de Griekse mythologie de wereld droeg, heb ik een aantal personen te danken die mij geholpen hebben deze thesis te dragen.

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Eludication on approach and own input

I presented the subject of my thesis to professor Rudi D'Hooge and in collaboration with professor Zsuzsanna Vegh this concept was operationalized.

In February Zsuzsa has taken me under her wings by providing me basic literature and introducing me in both the handling of the animals and apparatuses/setups. On this basis I started working every day autonomously on tasks such as testing, taking care of the animals during the week, maintaining the apparatuses,… I performed the statistical analyses myself, though they were thightened up by Zsuzsa now and then.

List of abbreviations in alphabetical order

ASD = Autism Spectrum Disorders

AVP = arginine vasopressin

AVPR = arginine vasopressin receptor

BBB = blood-brain barrier

CNS = central nervous system

CSF = cerebrospinal fluid

OXT = oxytocin

OXTR = oxytocin receptor

PVN = paraventricular nucleus

SEY = small eye

SON = supraoptic nucleus

WT = wild type

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1 Introduction

For centuries, farmers have used oxytocin (OXT) via vaginal stimulation to trigger maternal behaviour in foster animals without having any knowledge about the existence of this hormone (Swaab, 2011). Thus while its modulating effects on behaviour were generally known, it was not until the 1970s that OXT was identified as activator/modulator of different social behaviours (Shen, 2015). Above all, it was this hormone's role in parental and pair bonding ("love") among many other social behaviours that made it a socially appealing and relevant subject (Huang et al., 2014; Dölen, 2015b).

Commercially, these initial findings opened a new market for products such as cosmetics and perfumes containing OXT with promising effects on attractiveness, relationships, intimacy and self-confidence (Attrakt for Him Oxytocin Spray, n.d). Scientifically this has no solid ground, since both the chemical and physical characteristics of the OXT molecule prevent it from fulfilling these promises.

In the clinic, OXT has been postulated to be effective in treating disorders with social deficits at their core ranging from autism spectrum disorders (ASDs; Li, Nakajima, Ibañez-Tallon, & Heintz, 2016; Huang et al., 2014) to addiction (Striepens, Kendrick, Maier, & Hurlemann, 2011). However, the preliminary positive findings of early studies might be distorted since they focused on a single dose and had rather small subject samples (Shen, 2015).

To date, different studies have shed a light on OXT's complex nature, contesting the primarily belief in OXT as *the* love/hug/trust hormone par excellence. For example, while Guastella and colleagues found that a single OXT dose in male ASD participants improved the ability to communicate non-verbally, they could not duplicate this effect as OXT was administered twice a day (Guastella 2010; 2015). As Sue Carter argues (Shen, 2015): "*Oxytocin is part of a system and it's not the only molecule that matters, but it's one that in some way is regulatory over a large number of systems.*"

2 Oxytocin and social behaviour: state of affairs

2.1 Short history: so close, yet so far

In 1906, sir Henry Dale injected human pituitary extracts into a pregnant cat, which led to the observation of uterine contractions (Dölen, 2015b). In light of this function, Dale named this pituitary extract 'oxytocin' after the Greek for 'rapid birth'. However, it didn't take long for its line of functions to expand. In less than a decade, an oxytonergic injection was found to induce milk ejection in a goat, to lower blood-pressure in birds, and to inhibit urine secretion in a human (Magon & Kalra, 2011).

Despite these findings, it was not until 1953 that du Vigneaud and colleagues were able to isolate the responsible substance for the uterine contractions and the milk-ejection (Magon & Kalra, 2011). Du Vigneaud also determined OXT's chemical composition (Figure 1A). This was the stepping stone to the observation that OXT is very similar to another posterior pituitary hormone, arginine vasopressin (AVP). Both hormones differ in only two of their nine amino acids (Figure 1B) which determines their differential biological activity (van Kesteren et al., 1995). Also with regard to genetics, both neuropeptides are very similar as their genes are located on the same chromosomes within a species (e.g. chromosome 2 in mice and 20 in humans) (Choleris, Pfaff, & Kavaliers, 2013). The similarity between these hormones is a consequence of a common ancestor, vasotocin, which underwent a gene duplication millions of years ago (Choleris et al., 2013).

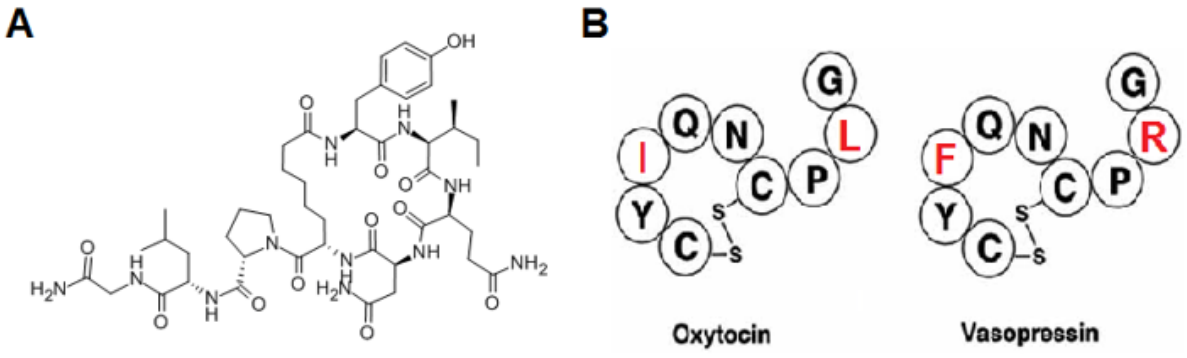

Figure 1A-B| Structural formula of oxytocin (A) and amino acid sequences of oxytocin and vasopressin (B). *In B letters denote single-letter data-base codes with C = cysteine; Y = tyrosine; I = isoleucine; F = phenylalanine ; Q = glutamine; N = asparagine; P = proline; Leu = leucine; R = arginine; G = glycine; S-S = disulfide bridge.*

OXT and AVP fall into the nonapeptide superfamily, a number of other structurally related

peptides which in general are widely conserved in phyla. However, AVP/OXT-like peptides are also identified in some invertebrate species where they play similar roles as in vertebrates (Gruber, 2014). In snails, for example, OXT is known to modulate ejaculation and egg deposition, whereas it is involved in sex-specific mating behaviours in roundworms (Caenorhabditis elegans) (Beery, 2015). Moreover, there is not 'one universal OXT' and even in some mammalian species such as new world primates, specific oxytonergic mutations are identified (Lee et al., 2011). These findings all indicate that OXT/AVP-like peptides are highly conserved both in function and structure and thus reflect the evolutionary importance of both hormones.

2.2 Oxytocin: what's in the *brain*?

In vertebrates, synthesis of OXT (Figure 2) and AVP primarily takes place in two nuclei of the hypothalamus: the supraoptic nucleus (SON) and paraventricular nucleus (PVN). These nuclei, however, differ histologically with regard to the cell population and the associated mode of transmission (Insel, 2010; Dölen, 2015b).

The **SON** is composed of *magnocellular* neurons, a type of neurons that secrete OXT from large dense core vesicles at the soma, dendrites and axons. Via the somatodendric mechanism OXT and AVP are released directly into to cerebrospinal fluid (CSF) thus being able to activate multiple extrasynaptic receptors within the central nervous system (CNS; *paracrine or volume transmission).* The axonal terminals, on the other hand, are situated within the posterior pituitary outside the blood-brain barrier (BBB). Here, released hormonal secretes come directly into the systemic bloodstream which enables them to activate distal receptors in for example uterus or breasts (*endocrine transmission*) (Dölen, 2015b).

Secondly, the **PVN** contains two building blocks: the previously mentioned magnocellular neurons and *parvocellular* neurons. Again, this type of neurons uses dense core vesicles for secretion, although these are much smaller than those used in magnocellular secretion. Parvocellular axons form synapses with the local neurons of certain brain regions (e.g. nucleus accumbens), where the released OXT/AVP resembles a neurotransmitter (e.g. rapid release, rather small quantities and spatially restricted) (Dölen, 2015b).

For both nonapeptides the principle holds that dependent on factors such as species and sex, different brain areas cumulate OXT/AVP production. However, unlike OXT, AVP is produced in multiple additional areas with consistenty over all mammalian species (Choleris et al., 2013). That is, within the present state of affairs, AVP producing neurons have been identified in the

preoptic area, the anterior hypothalamus, and suprachiasmatic nucleus (Choleris et al., 2013; Vandesande, Dierickx, & De Mey, 1975; Hofman & Swaab, 1995; Mammen & Jagota, 2011). In case of OXT, murine neurons in the bed nucleus of the stria terminalis, medial preoptic area and amygdala also accumulate OXT productio. However, these additional hormone producing areas deliver mostly smaller quantities and thus SON and PVN remain the main production sites (Choleris et al., 2013).

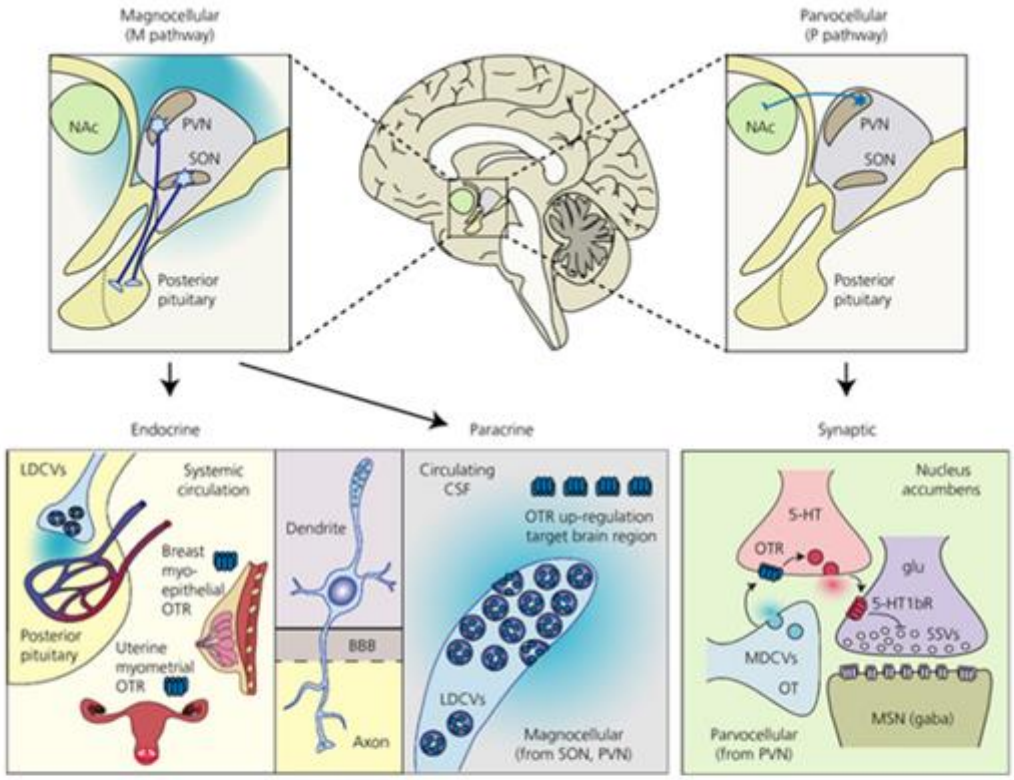

Figure 2| OXT pathways in the social brain. *In the upper panels, the magnocellular and parvocellular pathways are located in the brain. The lower pannels display the transmission mechanisms associated with the pathways. Nac = nucleus accumbens; LDCV = large dense core vesicle; OTR = oxytocin receptor; BBB = blood-brain barrier; CSF = cerebrospinal fluid; 5-HT = serotonin; MDCV = medium dense core vesicle; OT = oxytocin; MSN = medium spiny neurone; SSV = small synaptic vesicle; 5-HT1bR = serotonin receptor subtype 1b; glu =glutamate. Adapted from "Oxytocin: Parallel Processing in the Social Brain?," by G. Dölen, 2015, Journal of neuroendocrinology, 27(6), 516-535.*

Adding to the complexity, production of both hormones is not limited to the CNS and both OXT and AVP can originate from peripheral tissues. Among many peripheral tissues, OXT can originate from retina, adrenal medulla, thymus, pancreas, placenta, and heart (Yang, Wang, Han, & Wang, 2013; Mouilac et al., 2015). Also AVP is secreted in various peripheral tissues such as thymus, testes, and adrenal gland (Mouilac et al., 2015).

Both OXT and AVP function through the activation of specific receptors. To date, four nonapeptide receptors are identified in mammals (Magon & Kalra, 2011). As previously mentioned, the structural differences between both nonapeptides result in differential affinity to bind specific receptors. Based on this criterium, OXT has one specific receptor whereas AVP has three distinct receptor types (V1a, V1b and V2) (Holmes, Landry, & Granton, 2003).

The effect OXT or AVP will have by activating their receptor is highly dependent on the tissue in which the receptor is situated (Mouilac et al., 2015; Bell, Erickson, & Carter, 2014). For example, the V1 receptors are primarily expressed in hepatic/vascular tissues in peripheral and central nervous system and thus will function accordingly (Mouilac et al., 2015). The OXT receptor (OXTR), on the other hand, is widely distributed within vertebrates, with locally high concentrations in for example heart, intestines, uterus, and breasts (Bell, Erickson, & Carter, 2014).

Before immersing in both hormones' wide range of functions, it is important to note that receptor distribution – and thus the effects – is remarkably plastic and dependent on factors such as species, social lifestyle, sex, reproductive state and social rank (Wöhr & Krach, 2017). In addition, the close similarity of OXT and AVP complicates the understanding of the functionality of both hormones. OXT is able to bind an AVPR and vica versa thus resulting in either agonistic or antagonistic effects (Bell et al., 2014; Mouilac et al., 2015).

2.2.1 *Activation of peripheral receptors*

Primarily, peripheral binding of OXT and AVP to their receptors is best known for its **myoactive properties** to stimulate contractions in a variety of tissues. Hemodynamic stress (e.g. hypotension and hypovolemia) evokes AVP to cause vasoconstriction (Mouilac et al., 2015). After adequate stimulation, OXT targets its receptors in smooth muscle cells in order to induce contractions in for example orgasms, breast for milk ejection or uterus for parturition (Yang et al., 2013; Swaab, 2011; Borrow & Cameron, 2012).

On the other hand, both OXT and AVP are identified as regulators of other homeostatic processes. As the name Antidiuretic hormorne suggests, are AVP-like peptides associated mainly with **fluid homeostasis** (Mouilac et al., 2015). By binding its renal V2 receptors, systemic AVP initiates water reabsorbtion in the renal collecting ducts (McCormick & Bradshaw, 2006). However, because the major similarities between OXT and AVP, OXT can also slightly perform this antidiuretic function (Li et al., 2008).

Other homeostatic processes which are regulated by AVP/OXT are **glucose metabolism**, **feeding**, **circadian rhytms**, **body temperature**, **control of stress responses**, **analgesia**, and **wound healing** (Ramos et al., 2014; Goodson & Bass, 2001; Swaab, 2011)

2.2.2 *Activation of central receptors*

To date, more and more interest is directed at the central role of OXT and AVP. Here, both hormones are involved in the regulation and mediation of **complex social behaviours** and **cognition**. Accordingly, social behaviour in se is a complex subject since its a careful consideration of both sensory information of the environment and internal stimuli (e.g. mental state and experiences). In addition, similar hormonal processes underly the following behaviours indicating that they show more overlap than meets the eye (Feldman, 2016). As a final remark, the neural basis of social behaviors is rather plastic and highly dependent on factors such as sex, species, social lifestyle, reproductive state, and social rank (Goodson & Bass, 2001; Wöhr & Krach, 2017; Choleris et al., 2013). Moreover, it is a crosstalk of different hormones and neurotransmitters in which OXT and AVP are only a piece of the puzzle (Feldman, 2016).

A first important domain within the influence of OXT and AVP are the affiliative behaviors such as **parental behaviour**. Pedersen and Prange (1979) found that, after centrally injecting OXT, – and to a smaller extent AVP – adult nulliparous female rats exhibited maternal behaviours such as nest building. In addition, OXT prevented such rats from attacking or avoiding pups as they usual do. Rather, these adult nulliparous rats took maternally care of these pups (Pedersen, Ascher, Monroe, & Prange, 1982). In contrast to rats, mice do not need steroid induction, nor parturition to perform maternal caring (Insel, 2010). While previous studies have focused only on maternal care, evidence suggests that similar hormonal processes underly paternal care. Studies found differential patterns of OXTR and AVPR in the brain of parental and non-parental voles (Microtus). More in particular, paternal behaviour is associated with a decreased AVPR binding in the lateral septum, a major player in aggressive behaviour. On the other hand, a higher OXTR binding was reported in areas of the extended amygdala which are associated with maternal care (Parker, Kinney, Phillips, & Lee, 2001). Also in humans an association between OXT levels and typical parenting behaviours in both new fathers and new mothers was established (Gordon, Zagoory-Sharon, Leckman, & Feldman, 2010).

Closely related to this parental care is, **pair bonding** with a sexual partner. Because of their pronounced monogamous social structure, studies have mainly focused on prairie voles to investigate this subject (Parker et al., 2001). A study of Insel and Shapiro (1992) compared the monogamous prairie vole to the polygamous montane vole. They found that despite their

descendance of the same genus (Microtus or Voles), OXTR expression patterns in the brain were rather different. Furthermore, manipulation studies show that when OXTR or AVP V1a antagonists are administered no pair bonding takes place (Cho, DeVries, Williams, & Carter, 1999). Also, in regard partner preference, OXT is the main facilitator in females, whereas AVP performs this role in males (Winslow, Hastings, Carter, Harbaugh, & Insel, 1993; Parker et al., 2001). In humans this is more difficult to investigate, though intranasal OXT seems to increase some aspects of human bonding such as trust, prosocial behaviour and trustworthiness (Parker et al., 2001).

A third affiliative behaviour under regulation of OXT and AVP is **within-group bonding.** De Dreu et al. (2011) have shown OXT's involvement in human ethnocentrism. OXT administration in males, resulted in increased in-group favoritism and to a lesser extent out-group derogation. In animal studies, this is mostly studied by blocking OXT/AVP-like peptide receptors of zebra finches which decreases their flocking behaviour (Kelly et al., 2011; Goodson, Schrock, Klatt, Kabelik, & Kingsbury, 2009).

As opposed to what names such as "love hormone", "cuddle hormone" or "trust hormone" suggest, OXT is not limited to prosocial behaviour and neither is AVP. **Aggressive behaviour** has been linked to higher OXT levels in male stickleback fish trying to aggressively defend their eggs or climb the social ladder (Parker et al., 2001). Contesting this finding, aggression seems to be increased in male OXT knock-out mice, suggesting a compensating mechanism by AVP and/or decreased fearfulness in these mice (Parker et al., 2001; Pobbe et al., 2012). Furthermore, experimentally activating V1a receptors has shown the excitatory nature of AVP on aggression, whereas blocking of the receptors resulted in a decrease in aggression (Parker et al., 2001; Goodson & Bass, 2001).

In females, AVP has the opposite effect. It has a inhibitory role on aggression of both maternal as non-maternal animals (Parker et al., 2001). Studies reporting OXT's role in aggression are inconclusive (Parker et al., 2001). However, it might be interesting to refer to studies investigating social hierarchy in this matter. That is, because an individual's competitive ability will determine mainly his/her social rank, it is intriguing that OXT seems to play a role in this. Michopoulos and colleagues (2011) showed that in female rhesus monkeys, OXT levels in the serum were higher in dominant individuals as compared to subordinate individuals.

In humans, offensive aggression is more difficult to test (Parker et al., 2001), though OXT administration seems to influence negatively valenced social behaviours such as envy and group

identity, and emotions such as stress (Beery, 2015). This should be nuanced by mentioning the dependency on context (present social information, prior contact), individual (tendency to physical aggression, relationship status, attachment), gender, and mental illness (borderline personality disorder, psychopathic characteristics, schizophrenia).

Both nonapeptides also play an important role in functions related to **learning and memory**.

Social behaviours based on social attachment require the **recognition** of specific individuals which is based on memorizing cues of primarily olfactory, visual, and auditory nature (Campbell, 2008). In these social cognitive processes again OXT and AVP have a role to play. Male mice lacking the OXT gene, do not show the normally observed decrease in olfactory investigation of a conspecific which is a read-out for social recognition. However, treatment with OXT could restore this social memory in knock-outs (Ferguson et al., 2000) and a similar effect was observed in female rats (Engelmann, Ebner, Wotjak, & Landgraf, 1998). After intranasal administration in human male participants, OXT specifically improves the ability to recognize familiar faces, which was not the case for nonsocial stimuli (Rimmle, Hediger, Heinrichs, & Klaver, 2009). The role of AVP in animal studies was less straightforward whereas of V1a and V1b knockouts provided mixed results (Parker et al., 2001).

Also **learning and memory in a non-social context** are subject to OXT/AVP influences. That is, AVP knock-out rats do not acquire the ability to learn avoidance responses in active/passive avoidance tasks (de Wied, Diamant, & Fodor, 1993). Despite the inconclusive nature, AVP roughly seems to have facilitating effects whereas OXT has an inhibitory effect when considering cognitive performances (de Wied et al., 1993).

To date, the regulatory mechanisms that underlie OXT's function in distinct social behaviours are unknown. But both the mode of transmission and the targeted brain areas seem to play a key role in it. For example, the magnocellular pathway is believed to play a role in parental and conjugal attachment, buffering of fear and anxiety, and emotional empathy. The parvocellular pathway on the other hand, is believed to play a role in consociate attachment, social recognition memory and cognitive empathy (Dölen, 2015b).

To understand the role of both OXT and AVP on social behaviour in adults it is important to stress their steroidal dependency. That is, to induce behavioural effects of both neuropeptides, increased steroid levels are either enhanced or necessitated. It logically follows that the affected behaviours are directly or indirectly associated with reproductive effort (Choleris et al., 2013).

2.3 Clinical relevance of oxytocin

To date, synthetic forms of OXT such as Pitocin or Synotocinin are used in clinical settings to induce labor in several indicative situations (Bell et al., 2014). In addition, OXT is also used to augment contractions during labor, to control uterine bleeding postpartum, accelerate abortion induction, and facilitation of milk ejection (American Society of Health-System Pharmacists, 2017).

In 2005, a ground-breaking paper (Kosfeld, Heinrichs, Zak, Fischbacher, & Fehr, 2005) laid the foundation for a wider clinical relevance of OXT. The authors reported that after OXT administration in an experimental economic game, human participants were willing to take increased social risks. Alongside this trust effect, Barraza and Zak (2009) demonstrated that higher OXT levels are correlated with a higher sense of empathy in a person. Altogether, these findings raised an important question: could OXT be used as a therapy for individuals who are missing these characteristics?

In the first place, the focus shifted towards **autism spectrum disorders** (ASDs; autism), a set of neurodevelopmental disorders. To date, no cure has been identified for ASDs and gradually the focus of potential treatments has shifted to managing the symptoms (Dölen, 2015b). Individuals with ASD show deficits on two major behavioural levels. The first level concerns deficits in social communication and social interaction, whereas the second deals with the stereotyped actions and interests of individuals with ASD (5th ed.; DSM–5; American Psychiatric Association, 2013). Genetic research has indicated the oxytonergic system as an important component within the neurobiology of ASDs. Most attention goes to the OXTR, in which certain genetic mutations have been associated with empathy, maternal sensitivity, attachment behaviour, positive affect, and reduced amygdala responses to emotional faces (Striepens, et al., 2011; Wermter et al., 2010). In both human and animal studies, evidence has supported the assumption that OXT can – at least partially – ameliorate core characteristics of ASDs such as repetitive behaviour (Hollander et al., 2003), emotion recognition (Guastella et al., 2015), and non-verbal communication (Guastella, 2010). However, it should be noted that not studies point out the beneficial effects of OXT's on ASD-related characteristics. For example, after a 4-day OXT treatment, Dadds and colleagues (2014) did not find improvements in emotion recognitions, social interaction skills, or general behavioural adjustments.

Another clinical field that was drawn to the indicative behavioural effects of OXT are the **social anxiety disorders** (SADs). These disorders are defined as "clinically significant anxious

reactions and extreme discomfort in anticipation – or following – exposure to social settings, including performance and test situations" (Meyer-Lindenberg et al., 2011). Within this subject, it has been suggested that OXT influences fear-related amygdala activity in particular when the amygdala is hyperactive (Meyer-Lindenberg et al., 2011). Moreover, research indicates that systemic OXT can have an anxiolytic function since it has the potential to induce a decrease in peripheral cortisol levels (Dölen, 2015a).

Furthermore, OXT is considered as a potential treatment for **borderline personality disorders.** These disorders can be understood as a pervasive pattern of impulsivity combined with instability of interpersonal relations, self-image, and affects (Sienaerts, 2015). Mostly, these behaviours are provoked by events such as perceived rejection and loss. This indicates the attachment and affiliative system as a candidate target (Meyer-Lindenberg et al., 2011) and it was previously mentioned that this is the playground of OXT. However, the clinical use of OXT within this domain of disorders has been scarcely studied. The limited studies that have explored OXT's potential in borderline personality disorder, have highlighted potential negative effects. That is, in patients with this disorder, OXT seemed to reduce trust and cooperative behaviour, suggesting a mediating role of the attachment style (Bales et al., 2014; Rilling et al., 2014).

Next, evidence has suggested that oxytonergic abnormalities might play a role in social deficits of **schizophrenia** (Striepens et al., 2011). Despite several attemps to associate either central or peripheral OXT levels to this disorder, results are still open to doubt. Overall, initial studies have suggested beneficial effects of OXT treatment on negative symptoms (e.g. social withdrawal or anhedonia), and acute psychosis, but to a lesser extent on the positive symptoms (e.g. delusions or hallucinations) (Striepens et al., 2011; Feifel et al., 2010).

Finally, OXT also has been put forward as a potential treatment for **depression**, **obsessive compulsive disorder**, **post-traumatic stress disorder**, **addiction** and **eating disorders**. As mentioned in all previous disorders, clinical use of OXT in these psychiatric disorders is still a work-in-progress and not a well-established treatment. More in particular, scientifically, there is no agreement on what are the best administration routes, dosages, and treatment durations. Additionally, available evidence stems from rather small subject sizes. On the bright side, the technical revolution in neuroimaging will increasingly help to unravel the action domains of OXT (Meyer-Lindenberg et al., 2011).

2.4 Finding the proper dosage regime

Pharmacological studies on the effects of OXT require an in-depth consideration of how the hormone will be administered. When targetting features of psychiatric disorders, the obvious routes of administration are oral, intravenous, and intranasal (Lee, Lee, Hwangbo, Han, Hong, & Bahn, 2015). However, OXT is extensively metabolized by the liver and the gastrointestinal tract, which obviously is a large disadvantage of oral administration. Furthermore, OXT is a relatively large molecule so when injected in an artery, it does not effectively cross the blood-brain barrier (BBB). For that reason, the remaining options are either peripheral administered BBB-permeable agonists/antagonists or central administration. However, in case of peripheral administration it is unclear where, when and which amount of OXT is able to act centrally. Moreover, there might be a possibility that the OXT acts peripherally on the sensory or autonomic system (f.e. stress system) and thus affects behaviour indirectly (Dölen, 2015b).

The olfactory epithelium, however, is suggested to have a loophole for the non-permeable BBB. Every 3-4 weeks the olfactory receptor neurons regenerate. This results in a certain degree of porosity of the BBB at this epithelium. Accordingly, via intranasal administration, OXT is able to bypass the bloodstream and actually access the CNS. Evidence indicates that its central access is confirmed because changes in brain functioning, perception, and behaviour are replicable (Bakermans-Kranenburg & Van Ijzendoorn, 2013). Intranasally administerd OXT finds its way into the CSF and accordingly will be distributed through its flow (Dölen, 2015b). Therefore, it has the potential to activate large volumes of the brain, which is a necessary condition for potential treatment of social deficits. Another major advantage of intranasal administration is that no systemic hormone-like side effects (e.g. milk ejection) are elicited (Huang et al., 2014). Furhtermore, one spray of OXT can have effects which can be observed after 7h (Bakermans-Kranenburg & Van Ijzendoorn, 2013). Presumably, the exogenous OXT activates a feed-forward mechanism of the oxytonergic system which will respond with endogenous secretion of the hormone. A disadvantage of intranasal administration is that it is not clear where, when and in what amount OXT reaches targeted areas. More in particular, because it is transmitted via CSF concentration gradients are induced, in which concentration is higher in the rostral regions compared to caudal regions. In this regard, it is important to consider which area one wants to affect because distal regions (e.g. nucleus accumbens) call for higher concentrations.

In a meta-analysis, 19 clinical trials which used OXT to target various psychiatric disorders were analysed. In a neurotypical population, smaller doses markedly increased salivary OXT levels, whereas excessively high IUs (7000 IU) were not successful in clinical trials. Resorting

to OXT's similarity to AVP, an oxytonergic excess might occupy AVPR and thus shift their balance in the brain (Bakermans-Kranenburg & Van Ijzendoorn, 2013). This could result in unwanted side effects.

Most of the previously mentioned psychiatric disorders are life-long conditions. This raises questions regarding the relevance of studies using acute, single-dose administrations. Huang and colleagues (2014) found that acute intranasal administration facilitates social behaviour, whereas chronic (7-21 days) administration decreases specific forms of social behaviour. Moreover, chronic administration reduced OXTR throughout the brain. Recently, another administration regime (i.e., sub-chronic or intermittent administration) was used and seemed to ameliorate social deficits (Teng et al., 2013). Herein, four doses OXT were administered at 48h intervals, which might better resemble pulses of endogenous OXT release (Bales et al., 2014).

2.5 Pax-6 heterozygous mutants: a mouse model of delayed brain development

Pax-6 gene is paired-box containing genes, a gene family which plays a key regulatory role in both the formation of tissues and organs during embryogenesis (Walther & Gruss, 1991; Robertson, 2012). In vertebrates, Pax-6 genes are expressed in a similar pattern, predominantly in central nervous system, eyes, nose, pituitary and pancreas (Schmahl, Knoedlseder, Favor, & Davidson, 1993; Callaerts, Halder, & Gehring, 1997).

A mutation in the Pax-6 gene, Small eye (Sey), is due to either a small deletion or point mutation and results in loss of the functional gene product (Umeda et al., 2010). The homozygous (Sey/Sey) condition is associated with ophthalmic and gross cerebral abnormalities and thus is a lethal genotype in mice (Thompson et al., 2004) and humans (Heyman et al., 1999). In contrast, the heterozygous (Sey/+) mutants are viable (Kaufman, Chang, & Shaw, 1995) and compared to Sey/Sey, the murine Sey/+ mutants display milder ophthalmic abnormalities and less severe cortical abnormalities (Thompson et al., 2004). Human Sey/+ mutations, on the other hand, show more severe cerebral abnormalities than murine models do (Thompson, et al., 2004).

In mice, Pax-6 has been identified to play a crucial role in various cortical developmental processes such as regional differentiation, neuronal migration and axonal guidance (Mitchell et al., 2003; Bamiou et al., 2007). In this regard, it should not come as a surprise that besides eye phenotypes, Pax-6 mutations also play part in behavioural and neurodevelopmental phenotypes.

The human Pax-6 gene is associated with a rare genetic disorder: WAGR syndrome, an acronym for Wilms tumor, Aniridia, Genitourinary malformation, and mental Retardation (Yamato et al.,

2013). The majority of WAGR patients display mental retardation and behavioural problems. More than 1/5 also show autistic features (Umeda et al., 2010). Umeda and colleagues (2010) have investigated these mice in light of neurodevelopmental disorders such as ASDs. They found that the heterozygous Sey rats indeed showed autism-related abnormalities in behaviour and deficits in the 5-HT system. In conclusion, taking into account the face validity (symptom replication), predictive validity (treatment effectiveness), construct validity (conceptual analogy to human cause) and other conceptual analogy with human disorder (D'Hooge, 2016), Pax-6 appears to be a valid mouse model.

However, the Sey/+ mutants show severe abnormalities on the previously mentioned domains and provide both genetically and phenotypically a rather good model for the human disorder aniridia (Van Heyningen & Williamson, 2002).

2.6 Hypotheses and methodological rationale

The overall central hypothesis of the present project related to the putative beneficial effect of OXT in developmental disorders with social impairment. In addition, it was hypothesized that males and females would display different effects after OXT treatment. These hypotheses were investigated by comparing wildtype C57BL6 to Pax-6 heterozygous Sey mutants. This strain is a mouse model for neurodevelopmental delay. Moreover, the neurodevelopmental abnormalities in this murine strain ressemble abnormalities which characterize ASD and other related neurodevelopmental disorders (Yoshizaki et al., 2016).To test these hypotheses, a murine test battery was used to assess deficits in various domains of social interaction. The first domain focuses on deficits in social interactions and contains three paradigms.

The first paradigm was the **Social Proximity Test**. In this test, different same-sex pairs (WT vs. WT; SEY vs. SEY; WT vs. SEY) were placed into an arena with limited space. Therefore, an animal's tendency to avoid specific contact could be observed. The rationale for this test was that SEYs would display avoidance behaviours and thus higher levels of social anxiety.

Using a **SPSN** protocol in a three-chamber setup, mice were confronted with unfamiliar stranger conspecifics and their behaviour towards these animals is recorded. To test an animal's sociability, the animal is presented the option to explore either an unknown mouse or an empty cage. Being very social animals, mice tend to be attracted to other mice and will preferable approach the stranger mouse. Since this behaviour is altered in mouse models for ASDs (Silverman, Yang, Lord, & Crawley, 2010), the hypothesis in this test was that SEYs display decreased approach behaviours towards the stimulus mouse.

Consequently, the same **SPSN** protocol enabled to assess a mouse's preference for social novelty. To do so, a novel animal is placed in the previously empty cage thus presenting the option to approach either a novel or previously encountered animal. Under normal circumstances, the subject mouse will show an increased interest towards the novel mouse. This ability of a mouse to recognize other previously encountered mice is altered in mouse models of ASDs (Silverman, Yang, Lord, & Crawley, 2010). Therefore, the rationale was that SEYs would potentially show deficits in this behaviour when compared to WTs.

The last test to assess deficits in social interaction was the **Automated Tube Test**. In this setup an animal's response to frontal confrontation with another mouse was physically limited. That is, a mouse could either chose to force the other animal to retreat (dominance) or to retreat itself (subordinance). Since it was reported that a mouse model of ASD always retreated (Irie, Badie-Mahdavi, & Yamaguchi, 2012), the hypothesis for this test was that SEY mice would show a similar tendency.

The second domain assessed communication deficits in mice. Using a **Olfactory Habituation/Dishabituation paradigm**, a mouse's ability to distinguish among different non-social and social odors was tested. In line with various mouse models of ASD, the hypothesis for this test was that SEYs would display impairments in social recognition (Silverman, Yang, Lord, & Crawley, 2010).

Finally, in addition to this testing battery for social deficits, a cognitive paradigm was used to evaluate the mice's ability to learn to discriminate a defined context. In this context, the mouse is presented to an electroshock which results in contextual fear conditioning. As the animal is able to recognize this defined context it wil display freezing behaviour. However, in case it is unable to discriminate other contexts, these will also elicit freezing behaviour. In this **Contextual Fear Conditioning test**, it was hypothesized that this fear-cued memory was impaired in Pax-6 heterozygous mutants.

3 Methods

3.1 Experimental design

The current study adopted a pre-post design to test the effect of oxytocin (OXT) on social behaviour in two mice genotypes with the same C57BL6 background. To do so, the non-intervened behaviour of all mice was mapped using a social behavioural test battery. Hereafter, all mice performed the exact same test battery, only now an OXT-treatment was provided. After this chronic treatment period, a Contextual Fear Response test was performed without OXT administration.

OXT was dissolved in saline (1mg/ml) and applied intranasally daily for 25 days (weekends not included). A small drop of 5µl was placed between the two nostrils with a 0.5-10 µl Eppendorf pipette. This dosage is based on Teng et al. (2017) and was administered once a day, 15 min prior testing. After administration, mice were kept individually in holding cages until testing. A maximum administration limit was set on once a day, with the result that some tests such as the Automated Tube Test took longer than in the baseline-condition.

3.2 Animals

Subjects were 8-14-week-old wildtype C57BL/6 mice (WT; 6 males; 8 females) and 11 Pax-6 heterozygous mice (SEY; 7 males; 4 females) of a C57BL/6 background. All mice were group housed and kept under standard laboratory conditions (20-22 °C; 12-h light/dark cycle, lights on at 8.00 a.m.). Food and water were available ad libitum. Wood shavings were used as bedding and cages were enriched with paper snippets and toilet roles. Because the OXT treatment seemed to trigger more aggression in males, extra enrichment (more paper snippets and cardbord boxes) was provided as shelter. Before starting the OXT treatment one female WT died without a clear cause.

All procedures were reviewed and approved by the KU Leuven animal ethics commission and were in compliance with the European Community Council Directive.

3.3 Testing battery

3.3.1 *Social Proximity Test*

Within a transparent plexiglass cylinder social distancing of the two subject mice was restricted (Ø: 20 cm; Figure 3). This restriction enabled assessment of behaviour in forced contact since social contact could not be directly avoided. To limit environmental distractions, the setup was placed into an enclosure. Illumination was indirect from underneath the setup. Trials were

recorded by two cameras (one top-mounted and one front-mounted) and a videocamera (front-mounted).

After starting all cameras, 2 non-cagemate animals were placed into the setup where they could freely interact with eachother for 10 min. To avoid (social) odor transmission, animals were placed in different holding cages to separate them from naïve cagemates. The setup was cleaned with water (70% ethanol when testing other gender). Testing was always performed for gender-matched mice. First, pairs of same genotype (WT-WT; SEY-SEY) were tested, whereas subsequently pairs of different genotypes (WT-SEY) were tested.

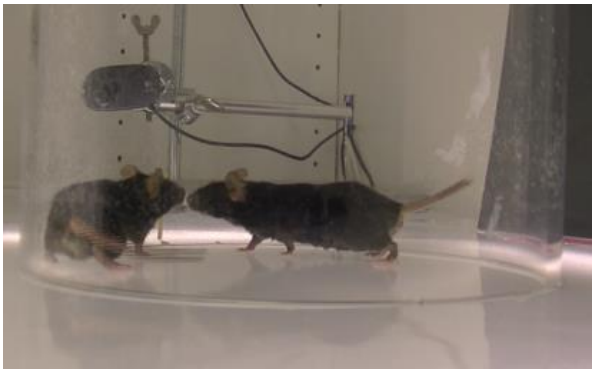

FIGURE 3| Test setup of the Social Proximity Test. *A transparant plexiglass cylinder with a limited (Ø: 20 cm) diameter was used to force interaction of the individuals.*

Because of image quality reasons scoring was performed using the recordings of the camera and were decelerated with .33 using VLC media player. The behavioural quantification method was based on Defensor et al. (2011) and included the following behaviours: nose tip-to-nose tip (NN), nose-to-head (NH), nose-to-anogenital (NA), crawl over (CO), crawl under (CU), upright (U) and jump escape (JE). For observational reasons this was extended with the behaviours autogrooming, allogrooming and sniffing urine or feces. To limit interpretation, a visual manual was drawn up to provide a more objective scoring method (Appendix A).

3.3.2 *Sociability/preference for social novelty (three chamber test)*

Social approach was tested in a rather simple three chambered setup out of transparent plexiglass box (w x d x h: 94 x 28 x 30 cm). The two outer chambers (w x d x h: 29 x 28 x 30 cm) are accessible via plexiglass sliding doors (w x h: 6 x 8 cm) in the division walls between the chambers. Each chamber contained a cylindrical wire cup (h x Ø: 11 x 12 cm) in the center in which a mouse could be placed. The setup was illuminated indirectly from underneath an opaque floor and placed into an enclosure to limit environmental distractions.

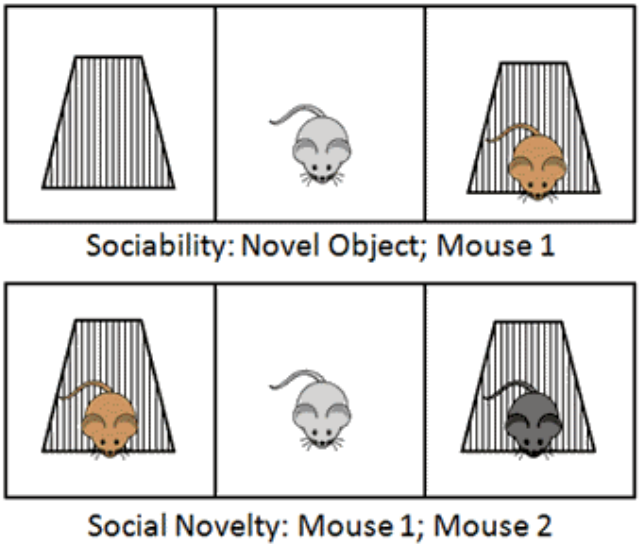

Figure 4| Schematic representation of the Sociability and Preference for Social Novelty Test. *During the Sociability phase (upper panel), the test animal can choose to approach either a unknown mouse or an empty cage. During the Preference for Social Novelty phase, the choice is increased by placing a second novel mouse in the previously empty cage. Adapted from "Social Interaction Tests", from Stanford Medicine, 2017, Retrieved from http://med.stanford.edu/sbfnl/services/bm/si.html*

Mouse movements were registered by two top-mounted cameras positioned 60 cm above the setup and linked to the ANY-maze^{TM} Video Tracking System software (Stoelting Co., IL, USA) for automatical recording and analysis. Stranger mice were gender-matched C57BL/6J mice specifically used for SPSN testing and were randomly assigned to one of the wire cups in which they remained during consecutive phases of the same mouse. Between animals, the setup was cleaned with water, soap and paper towels, whereas 70% ethanol was used in between genders.

SPSN testing started with a 5-min **acclimatization phase** in which mice could freely explore the central chamber of the setup. The distance a mouse travelled (m) was used as a read-out for exploration behaviour and was measured in all three phases.

Subsequently, a 10 minute lasting **sociability phase** (Figure 4) followed, in which the testing animal could choose to approach either an unknown mouse (STR1) in a wire cup or an empty wire cup. 5 s after ANY-maze was started, the sliding doors were manually opened enabling the mouse to enter the side chambers.

Since mice are very social animals, other conspecifics will generally attract them and thus it can be assumed they will spent more time with the unknown mouse. This preference for social stimuli was operationalized by time the head of testing mouse was within a small (2 cm) periphery of both the wire cage. More in particular, the ratio of time spent in the periphery of the social wire cage over the total time spent in the 2 cm periphery of the wirecages [$Time_{social}/(Time_{social} + Time_{empty})$] is taken.

During the **preference for social novelty phase** (Figure 4), STR1 stays in the same cage but a second stranger mouse (STR2) was placed in the empty wire cup. The mouse started again in the central chamber and the same procedure as in the sociability was repeated. Similar to the sociability ratio, a preference for social novelty ratio was calculated as $Time_{new}/(Time_{old} + Time_{new})$.

3.3.3 *Olfactory Habituation/Dishabituation Test*

To assess a mouse's response to olfactory cues, the Olfactory Habituation/Dishabituation Test was performed (Figure 5).

FIGURE 5| Test setup of the Olfactory Habituation/Dishabituation Test. *Sniffing was operationalized as the time the nose was within the red rectangle.*

Testing was performed in the same enclosure as that used in social proximity and was recorded using a video camera. Mice were first acclimatized in a clean cage containing only a thin layer of clean bedding for 30 min. In 2 min trials, saturated cotton swabs with different odors were fixed on top of the cage with a binder clip. Each odor was presented 3 times in a row and the sequence of odor presentation was fixed: *H_2O* – *Banana* (1:100 dilution; 2-methylbutyl-acetate) – *Grape* (1:100 dilution; methylanthranilat) – *Social 1* – *Social 2*. These social odors

were obtained by sweeping the cotton swabs through the dirty bedding – in particular in the urinary corners – of unfamiliar mice.

The OXT phase was slightly different than previously described. First, after the acclimatization period, OXT was administred and a 15-min infiltration period followed. Secondly, during this phase, also social odors of the opposite sex were presented, again using a fixed sequence over different genders (female-male-female-male).

After each odor, both the binder clip and grid were cleaned with 70% ethanol and gloves were continuously changed to prevent odor interference.

Based on the videos, the amount of time the nose of the mouse was within a defined periphery (w x h: 8 x 5 cm) around the cotton swab was scored. Both chewing and direct manipulation of the cotton swab were included in the scoring. This was operationalized in two variables: total sniffing time (%) and mean sniffing duration (s). For these variables, habituation was operationalized as a decrease in investigation as an odor is repeatedly presentated, whereas dishabituation was the reinstatement of investigation behaviour as a novel odor was presented.

3.3.4 *Automated Tube Test*

The testing apparatus (Benedictus Systems, Rotterdam, the Netherlands) was a transparent fiberglass setup consisting of a tube (w x d x h: 47 x 2.5 x 2.5 cm) which connects two boxes (w x d: 12 x 8 cm). This setup was sectioned by three doors: two transparent doors to manipulate the accessibility of the tube and one opaque (black) door halfway the tube to avoid prior awareness of another mouse during the tournament phase. On the opposite wall of the tube entrance each box has some valves (1 cm above bottom) to provide puffs of pressurized air. The position of the doors and the activity of the air valves were fully automated using real-time mouse tracking with infrared photo-detectors on the bottom of the apparatus.

Initially, the protocol started with a 5-day non-social training phase to habituate the mice to enter the tube and reach the goal box (Automated Tube Test ©, n.d.). That is, after a mouse was placed in one of the boxes, the start of a trial was announced by the opening of the tube entrance door. After this cue the mouse should enter the tube and run towards the closed opaque door which automatically opens whenever the mouse reaches within 4 cm with a random delay of 1-3 s. Then, the mouse proceeds and enters the goals box which triggered the third door to close. For fluidity reasons, a maximum trial limit of 180 s after opening of the entrance door was set. Whenever this limit was exceeded, the animal was gently assisted

towards the goal box with a knotted wire. After a trial, the mouse returned to the homecage and the setup was cleaned with 70% ethanol. On day 1, mice performed two habituation trials to explore the tube freely (left-right and right-left). On day 2-5, mice performed 3 sets of 2 trials (left-right and right-left) with at least 45 minutes between each set. If the animal did not enter the tube 5 s after the entrance door opened, the air valve was activated until the animal entered the tube.

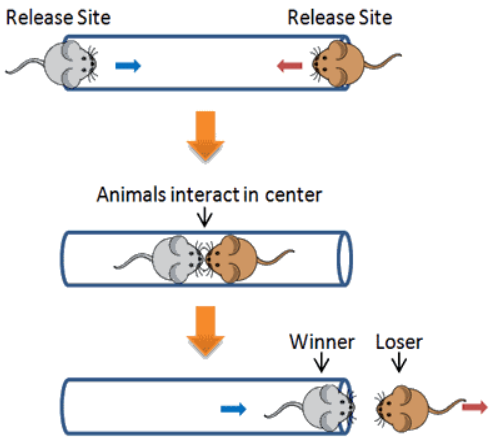

FIGURE 6| Schematic representation of the Automated Tube Test. *A tube connects two chambers in which a mouse can be placed. The aim is that mice run towards the central, black door and start pushing one another out of the tube to reach the opposite chamber as the one they started in. This is used as a read-out for dominance/subordinance which can be linked to aggressiveness. Adapted from "Social Interaction Tests", from Stanford Medicine, 2017, Retrieved from http://med.stanford.edu/sbfnl/services/bm/si.html*

Next, a 2-day resting phase followed this training week to enhance the behaviour during the tournament week.

To explore changes in social interaction of SEY mice, a group-vs-group design was used during the tournament phase. That is, within each gender, all SEY mice competed against all non-cagemate WT mice. The number of matches a mouse had to play was limited to max. 2 matches per day in the pre-phase and max. 1 match per day during the OXT-phase. A tournament day started with randomly assigning starting boxes to mice per match and two non-social training trails (*Pre*: min. 45 min prior to match; *OXT*: min. 60 min prior to match). A schematic representation of this this is shown in Figure 6. After mice were placed in their box, entrance doors opened leaving the mice 5 s to enter the tube before the air valves were activated. When both mice were positioned within 4 cm of the door, the opaque door opened as

starting sign of the match. An animal could lose if the opponent mouse forces it to retreat to the starting box (even if the door not closes). After every match the apparatus was cleaned with 70% ethanol.

Social dominance was operationalized as a dominance ratio [Wins/(Wins + Losses)] because the number of matches played was dependent on both the genotype and homecage of the mouse.

3.3.5 *Contextual Fear Conditioning*

The test box (w x d x h: 25 x 25 x 25 cm) had a stainless steel grid floor which could deliver shocks. Underneath the grid floor was a motion sensitive floor connected to a computer equipped with Panlab Freezing v1.2.0 software to register movements during the test. Movements could range from 0 to 100, whereas freezing was operated as movement remained below a threshold of 2.5 for at least 1 second. This apparatus was located in a sound attenuating cubicle. To perform the experiment Panlab Startle & Fear Combined system (Panlab, S.L., Cornellà, Spain) was used. Testing started daily at 9.00 AM and ended before 1.00 PM (dependent on phase).

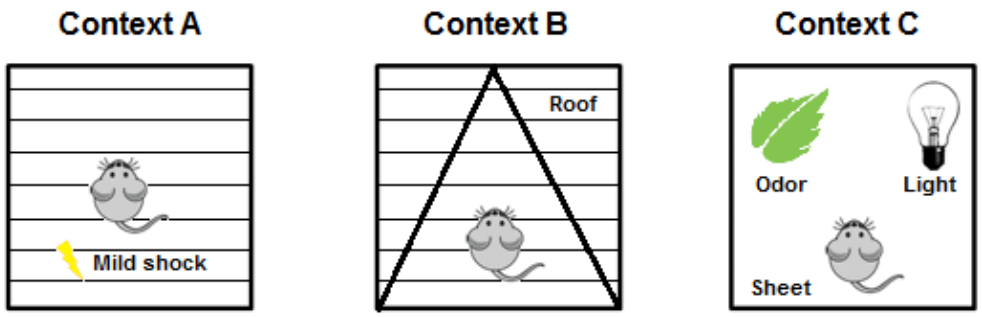

FIGURE 7| Schematic representation of the three different contexts for the Contextual Fear Conditioning Test. *Context A (left) was the defined context in which an electroshock was presented. Context B (middle) was very similar to Context A, though without an electroshock, and a roof was added. Context C (right) was visually, tactile, and olfactory different from Context A.*

The test was build out of 4 phases, and for each of the phases the read-out was the mean % of freezing during the first three minutes of testing (i.e. before the shock), whereas freezing was defined as a movement score below 2.5 for at least 1 s.

On **day 1-3** (contextual fear conditioning phase) mice were once a day conditioned in Context A, where they were placed on a grid floor (tactile), without light (visual) and an ethanol odor (olfactory). A trial started with a 3-min exploration period, which was followed with a 2 s lasting foot shock (0.5 mA) to induce fear. After the shock, the mouse remained in the testing box for 1 min and was then placed back into its homecage. If fear conditioning is succesful, freezing % should increase after day 1.

During the contextual testing phase (**day 4-5**), every testing day each mouse was tested in 3 different contexts (Figure 7) to test the contextual memory of the mouse. However, in this testing phase no shocks were given in Context A and two new contexts were introduced. Context B was very similar to Context A, but here a V-shaped roof was added in the box, whereas Context C did not resemble Context A. In Context C a white plastic covered the grid floor (tactile), the box was illuminated (visual) and a peppermint odor was used (olfactory). The procedure and length of the trials remained the same as during the previous phase. During this phase the % of freezing per context would give an indication of the specificity of the contextual memory (theoretically: A > B > C).

On **day 6-15**, the contextual discrimination learning phase started, in which mice were presented each day to both context A with shocks and context B with a roof and no shocks. The order in which the contexts were presented was random to avoid sequence learning. If successful, a reduction of freezing percentage in Context B would be visible.

Hereafter (**day 16-17**), again a contextual testing phase (identical to day 4-5) was used to test the discriminative ability of the mice. During this phase, one could theoretically assume that while freezing is high in Context A, the mice can discriminate Context B and C and thus show less freezing behaviour in these contexts.

Because of technical errors some data were removed manually.

3.3 Statistical Analysis

All statistics were performed with Statistica 13.1 (StatSoft. Inc. Tulsa, USA) and for all analyses a significance criterion of $p < 0.05$ was used. Main effects, interaction effects (and contrasts if possible) were performed using the General Linear Model (GLM).

4 Results

4.1 Social Proximity Test

In an ANOVA both SEY-SEY and WT-SEY pairings were compared to WT-WT interactions (see Appendix B1 for an overview). In the **baseline condition** no general differences were found between SEY-SEY and WT-WT interactions, though the large effect sizes draw the attention on nose-nose interaction ($F_{1, 5} = 5.99$, $p = 0.06$, $\eta_p^2 = 0.55$) and feces/urine investigation ($F_{1, 5} = 4.66$, $p = 0.08$, $\eta_p^2 = 0.48$). In both behaviours, frequencies were higher in interactions of two WT mice than they were in interactions of SEY pairs.

In comparison of the mixed and WT pairs, a significant difference in escape jumping showed, whereas this behaviour was more observed in mixed pairs ($F_{1, 6} = 6.942$, $p = 0.04$, $\eta_p^2 = 0.54$). Furthermore, a large effect size draw attention on the higher frequencies of upright interactions ($F_{1, 6} = 5.79$, $p = 0.05$, $\eta_p^2 = 0.49$) in mixed pairs when compared to WT-WT pairs.

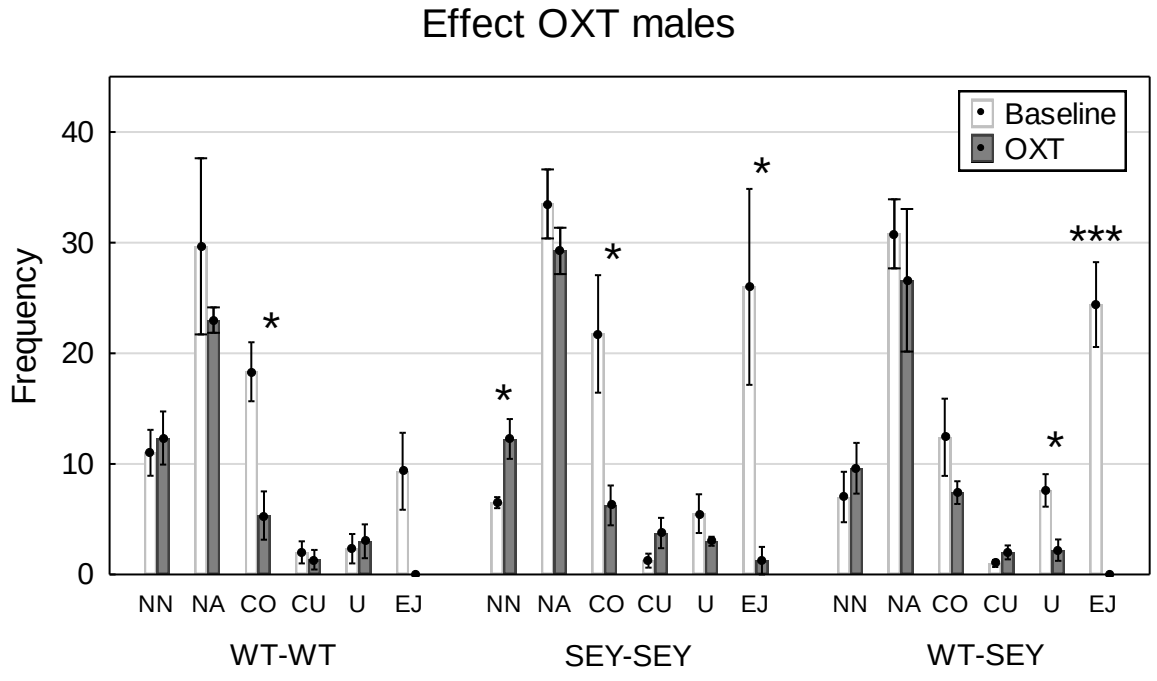

FIGURE 8| Occurance of interaction behaviours in different pairs. *Data is expressed as mean ± SEM. Significant effects of OXT administration are shown with an asterisk (*, p < 0.05; **, p < 0.01; ***, p < 0.001). NN = nose-nose; NA = nose-anogenital; CO = crawl over; CU = crawl under; U = upright; EJ = escape jumping.*

The effects of **OXT Treatment** within different pair types are displayed in Figure 8 and the underlying statistics can be found in Appendix B2. The GLM (for overview of all results see

Appendix B3) showed large effect sizes for all behaviours except allogrooming. OXT treatment significantly decreased crawl over behaviour ($F_{1, 9} = 24.10$, $p = 0.00$, $\eta_p^2 = 0.73$), escape jumping ($F_{1, 9} = 32.79$, $p = 0.00$, $\eta_p^2 = 0.78$), and upright contact ($F_{1, 9} = 12.83$, $p = 0.01$, $\eta_p^2 = 0.59$). The latter should be nuanced because an interaction with Treatment and Pair type was shown ($F_{2, 9} = 6.973$, $p = 0.02$, $\eta_p^2 = 0.60$).

Due to time limitations, female data were not yet fully analysed and thus could not be shown in this thesis.

4.2 Sociability/preference for social novelty

4.2.1 *Acclimatization trials*

During the acclimatization trials animals could freely explore the central box of the setup. Distance travelled was measured as a read-out for exploratory behaviour. During the **baseline condition**, the GLM did not indicate differences in Genotype with regard to general exploratory behaviour in males ($F_{1, 11} = 0.03$, $p = 0.88$, $\eta_p^2 = 0.00$), nor females ($F_{1, 10} = 0.54$, $p = 0.48$, $\eta_p^2 = 0.05$). On the other hand, the GLM did not show a difference between both Genders ($F_{1, 23} = 0.55$, $p = 0.47$, $\eta_p^2 = 0.02$). After **OXT administration**, again, no differences were found for Genotye in males ($F_{1, 11} = 0.00$, $p = 0.97$, $\eta_p^2 = 0.00$), nor females ($F_{1, 9} = 0.00$, $p = 0.99$, $\eta_p^2 = 0.00$). However, a significant interaction effect of Gender x Treatment suggested that distance travelled by males increased significantly ($F_{1, 11} = 8.89$, $p = 0.01$, $\eta_p^2 = 0.45$), but no effect was found for females ($F_{1, 9} = 0.10$, $p = 0.80$, $\eta_p^2 = 0.011$).

4.2.2 *Sociability trials*

Next, the test animal's sociability was tested by giving the animal the choice to approach either an unknown mouse or an empty cage. This preference was operationalized in time the head of the subject mouse was in a 2 cm periphery of the other animal relative to the time the head was in either the social or non-social periphery.

During the **baseline condition**, preference to approach another conspecific over an empty cage (Figure 9A). was significant for males ($F_{1, 11} = 6.63$, $p = .03$, $\eta_p^2 = .38$), but not for females ($F_{1, 9} = 2.87$, $p = 0.13$, $\eta_p^2 = 0.24$). No differences were observed between WTs and SEYs (Males: $F_{1, 11} = 0.36$, $p = 0.56$, $\eta_p^2 = 0.03$; Females: $F_{1, 9} = 0.18$, $p = 0.68$, $\eta_p^2 = 0.02$), and between males and females in general ($F_{1, 23} = 0.00$, $p = 0.99$, $\eta_p^2 = 0.00$). Administration of intranasal OXT did not affect sociability. No significance was found for either the main effect of Treatment (Males: $F_{1, 11} = 0.03$, $p = 0.90$, $\eta_p^2 = 0.00$; Females: $F_{1, 9} = 0.18$, $p = 0.68$, $\eta_p^2 = 0.02$), nor the Treatment

Genotype interaction (Males: $F_{1, 11} = 0.01$, $p = 0.93$, $\eta_p^2 = 0.00$; Females: $F_{1, 9} = 0.53$, $p = 0.49$, $\eta_p^2 = 0.06$).

For the variable distance travelled (Figure 9B) at **baseline**, the GLM did show differences in Genotypes (Males: $F_{1, 11} = 0.03$, $p = 0.88$, $\eta_p^2 = 0.00$; Females: $F_{1, 9} = 0.54$, $p = 0.48$, $\eta_p^2 = 0.05$) or Gender in general ($F_{1, 23} = 0.55$, $p = 0.47$, $\eta_p^2 = 0.02$). However, the OXT appeared to have differential effects on males and females ($F_{1, 22} = 28.41$, $p = 0.00$, $\eta_p^2 = 0.56$). More in particular, **males** showed an increase in exploration ($F_{1, 11} = 44.41$, $p = 0.00$, $\eta_p^2 = 0.80$), whereas treatment had no significant effect on females ($F_{1, 9} = 1.83$, $p = 0.21$, $\eta_p^2 = 0.03$). Both tendencies were similar in WTs and SEYs (Males: $F_{1, 11} = 3.12$, $p = 0.10$, $\eta_p^2 = 0.23$; Females: $F_{1, 9} = 0.26$, $p = 0.62$, $\eta_p^2 = 0.03$).

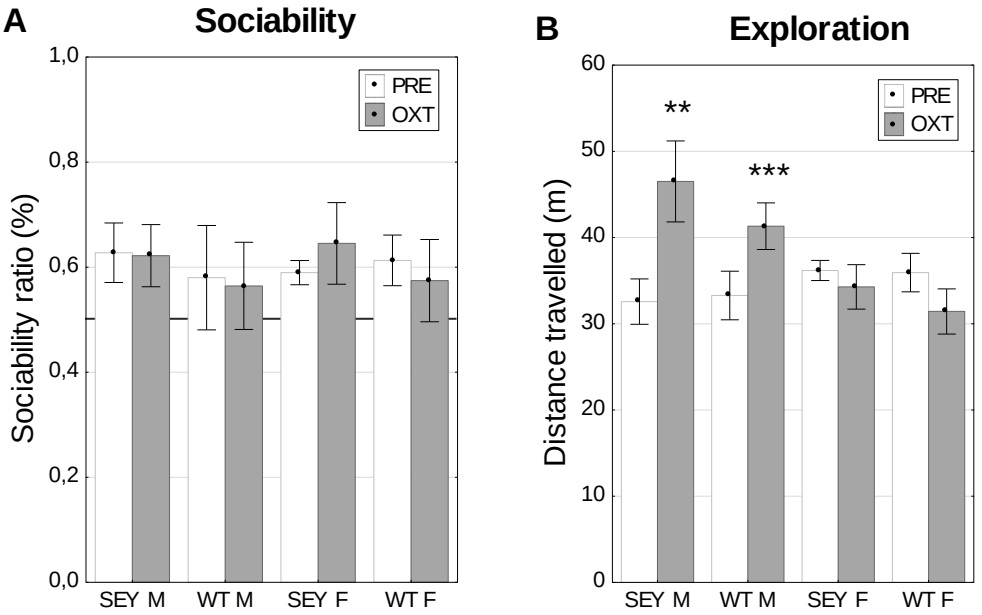

FIGURE 9| Sociability ratio (A) and distance travelled (B) during the sociability trials in the SPSN test in baseline and Treatment condition. *SEY M = male small eyes; WT M = male wildtypes; SEY F = female small eyes; WT F = female wildtypes. Data is expressed as mean ± SEM. Significant differences after OXT administration compared to pre are shown with an asterisk (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).*

4.2.3 *Preference for social novelty trials.*

When mice were presented the choice to approach either a known mouse or a novel mouse, at **baseline** significant differences were shown for either of the testing groups (Table 1; Figure 10).

Furthermore, no differences in Genotype showed (Males: $F_{1, 11} = 0.01$, $p = 0.91$, $\eta_p^2 = 0.00$; Females: $F_{1, 9} = 0.45$, $p = 0.52$, $\eta_p^2 = 0.05$) nor for Gender in general ($F_{1, 23} = 1.66$, $p = 0.21$, $\eta_p^2 = 0.07$). In the Treatment phase, both males and females did seem to prefer the previously encountered animal over the novel (Table 1). Data did not show an interaction effect of Treatment and Genotype in males ($F_{1, 11} = 0.22$, $p = 0.65$, $\eta_p^2 = 0.02$), nor females ($F_{1, 9} = 0.45$, $p = 0.52$, $\eta_p^2 = 0.05$).

	Mean	Std.Dv.	N	Std.Err.	Reference	t-value	df	p
Baseline								
SEY M	0,50	0,24	7	0,09	0,50	0,01	6	0,99
WT M	0,47	0,12	6	0,05	0,50	-0,62	5	0,57
SEY F	0,48	0,19	4	0,09	0,50	-0,24	3	0,82
WT F	0,64	0,17	8	0,06	0,50	2,22	7	0,06
Treatment								
SEY M	**0,34**	**0,14**	**7**	**0,05**	**0,50**	**-3,00**	**6**	**0,02**
WT M	**0,38**	**0,10**	**6**	**0,04**	**0,50**	**-2,97**	**5**	**0,03**
SEY F	0,48	0,19	4	0,09	0,50	-0,24	3	0,82
WT F	0,42	0,22	7	0,08	0,50	-0,90	6	0,40

Table 1| *T-statistic of the Preference for Social Novelty ratio to a 50% reference.*

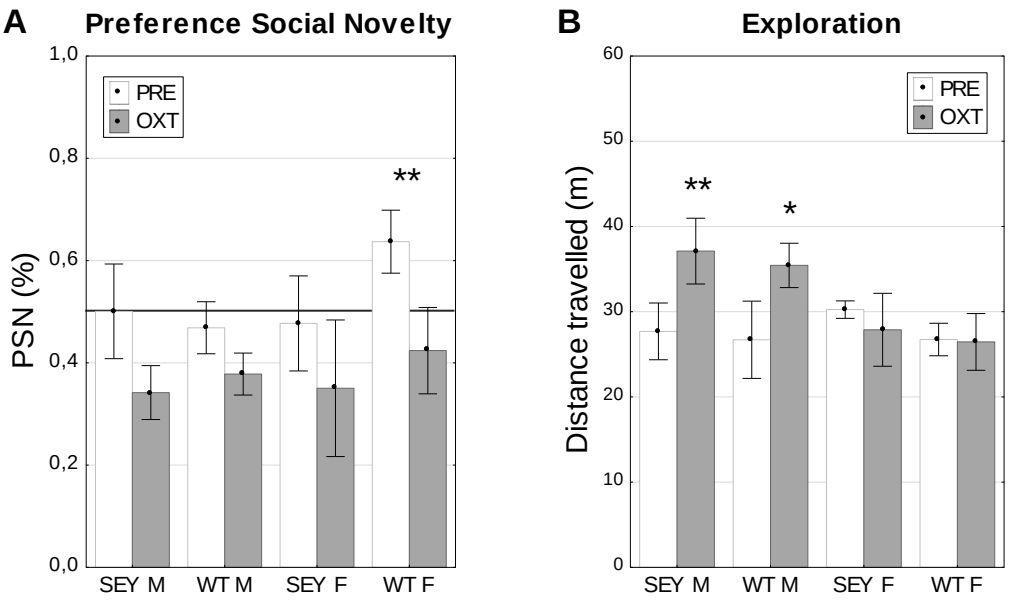

FIGURE 10| Preference for Social Novelty ratio (A) and distance travelled (B) during the sociability trials in the SPSN test in pre and OXT condition. *SEY M = male small eyes; WT M = male wildtypes; SEY F = female small eyes; WT F = female wildtypes. Data is expressed as mean ± SEM. Significant differences after OXT administration compared to pre are shown with an asterisk (*, p < 0.05; **, p < 0.01; ***, p < 0.001).*

With regard to the variable distance, no difference showed **at baseline** between male and female mice ($F_{1, 23} = 0.05$, $p = 0.83$, $\eta_p^2 = 0.00$). During this phase of the project, WT and SEY mice travelled similar distances (Males: $F_{1, 11} = 0.07$, $p = 0.79$, $\eta_p^2 = 0.01$; Females: $F_{1, 9} = 0.49$, $p = 0.50$, $\eta_p^2 = 0.05$). OXT administration affected the distance travelled on a gender-specific way: males tended to explore more ($F_{1, 11} = 31.93$, $p = 0.00$, $\eta_p^2 = 0.74$), whereas no effect was found in females ($F_{1, 9} = 0.25$, $p = 0.63$, $\eta_p^2 = 0.03$). Treatment and Genotype did not interact (Males: $F_{1, 11} = 0.05$, $p = 0.83$, $\eta_p^2 = 0.00$; Females: $F_{1, 9} = 0.27$, $p = 0.62$, $\eta_p^2 = 0.03$).

4.3 Olfactory Habituation/Dishabituation Test (only male data)

Statistics for Habituation and Dishabituation are shown in Appendix C. In the **Baseline condition**, no differences in *total sniffing time* (Figure 8) showed when comparing both genotypes ($F_{1, 11} = 0.06$, $p = 0.82$, $\eta_p^2 = 0.01$). Furthermore, mice showed a significantly larger attraction towards social odors when compared to water ($F_{1, 11} = 4.87$, $p = 0.05$, $\eta_p^2 = 0.31$) and non-social odors ($F_{1, 11} = 17.71$, $p = 0.00$, $\eta_p^2 = 0.62$). These effects did not interact with Genotype (Water: $F_{1, 11} = 0.47$, $p = 0.51$, $\eta_p^2 = 0.04$; Non-social: $F_{1, 11} = 0.43$, $p = 0.53$, $\eta_p^2 = 0.04$).

Similarly, no significant effect of Genotype was observed for the variable *mean duration* of the sniffing ($F_{1, 11} = 0.99$, $p = 0.34$, $\eta_p^2 = 0.08$).

On average WT males seemed to sniff longer than SEY's in general, but this was not significant ($F_{1, 11} = 1.05$, $p = 0.33$, $\eta_p^2 = 0.09$) nor was the Odor x Genotype interaction ($F_{1, 11} = 1.17$, $p = 0.34$, $\eta_p^2 = 0.10$). In general, mice tended to sniff non-significantly longer to the initial presentation of social odors when compared to non-social odors ($F_{1, 11} = 1.73$, $p = 0.22$, $\eta_p^2 = 0.14$) and water ($F_{1, 11} = 1.61$, $p = 0.23$, $\eta_p^2 = 0.13$). For none of these effects a significant interaction with Genotype (non-social: $F_{1, 11} = 1.09$, $p = 0.32$, $\eta_p^2 = 0.09$; water: $F_{1, 11} = 1.07$, $p = 0.32$, $\eta_p^2 = 0.09$), though it appeared that WT's had on average longer sniffing times for social odors whereas SEY remained approximately the same.

In general **OXT administration** did not have a significant effect on total sniffing time for males ($F_{1, 23} = 0.50$, $p = 0.49$, $\eta_p^2 = 0.02$). However, this effect interacted significantly with Type of olfactory stimulus ($F_{4, 92} = 3.92$, $p = 0.01$, $\eta_p^2 = 0.15$), suggesting an increase in total sniffing behaviour for the social stimuli when compared to non-social stimuli. The GLM did not show differences between both genotypes in general ($F_{1, 23} = 0.38$, $p = 0.54$, $\eta_p^2 = 0.00$).

At the initial presentation of an odor, *total sniffing* time (Figure 11A-B) did not differ between social odors when compared to water ($F_{1, 11} = 0.42$, $p = 0.53$, $\eta_p^2 = 0.04$) and no Stimulus-type x

Genotype effect was observed ($F_{1, 11}$ = 0.02, p = 0.89, η_p^2= 0.00). However, social odors elicited more sniffing when compared to nonsocial odors ($F_{1, 11}$ = 7.41, p = 0.02, η_p^2= 0.40) which did not differ between Genotypes ($F_{1, 11}$ = 0.27, p = 0.61, η_p^2= 0.02). More in particular, mice tended to sniff more when presented male odors compared to female odors ($F_{1, 11}$ = 5.41, p = 0.04, η_p^2= 0.33). The interaction effect of Stimulus-gender x Genotype showed a decrease for WT mice and no effect in SEY ($F_{1, 11}$ = 2.64, p = 0.13, η_p^2= 0.19), but was not significant.

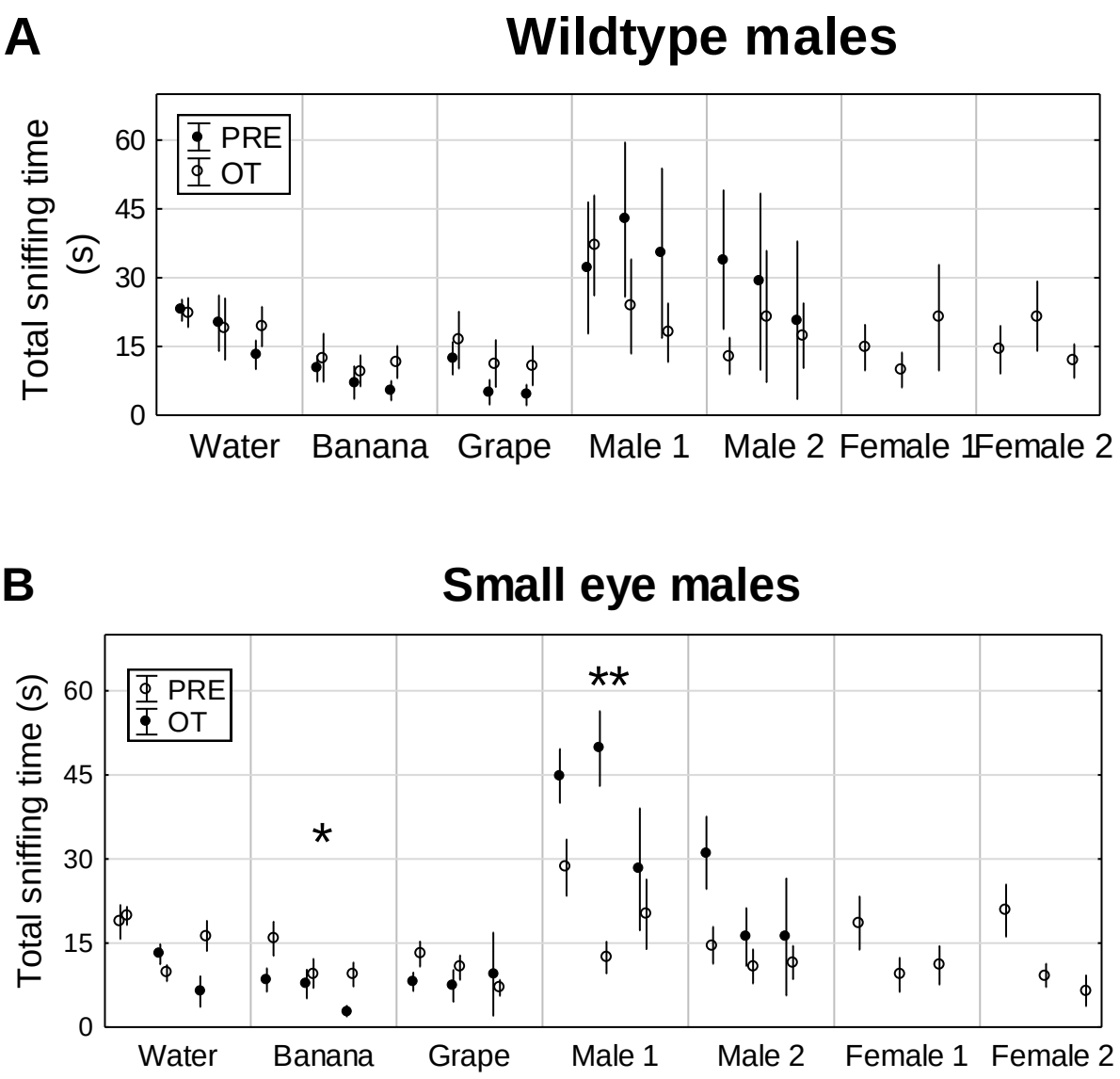

FIGURE 11| Total sniffing time in seconds for male WT (A) and SEY mice (B) during the olfactory habituation/dishabituation test in baseline and OXT condition. Data is expressed as mean ± SEM. Significant differences after OXT administration compared to pre are shown with an asterisk (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

In general *sniffing duration* at the initial presentation of an odor was not different for Genotypes ($F_{1, 11} = 0.25$, $p = 0.63$, $\eta_p^2 = 0.02$). Sniffing was significantly longer for social odors than non-social odor ($F_{1, 11} = 5.52$, $p = 0.04$, $\eta_p^2 = 0.33$) and this was more pronounced in WT's than SEY's ($F_{1, 11} = 1.16$, $p = 0.24$, $\eta_p^2 = 0.13$). A similar difference showed when social odors were compared to water (Main effect: $F_{1, 11} = 5.66$, $p = 0.04$, $\eta_p^2 = 0.34$; Interaction effect: ($F_{1, 11} = 0.97$, $p = 0.35$, $\eta_p^2 = 0.08$). No differences were found for male and female social odors ($F_{1, 11} = 2.32$, $p = 0.16$, $\eta_p^2 = 0.17$), with a preference for male odors in WT's and no observed preference in SEY's ($F_{1, 11} = 3.28$, $p = 0.99$, $\eta_p^2 = 0.23$).

Due to time limitations, female data were not yet fully analysed and thus could not be shown in this thesis.

4.1 Automated Tube Test

A social dominance ratio was calculated as a read-out for social dominance and Figures 12A-D show the cumulative wins/losses per individual mouse. Herein, a win is attributed with +1 whereas losses are attributed with -1.

For the **baseline** condition, genotypical differences were just over significance level in males ($F_{1, 11} = 4.70$, $p = 0.05$, $\eta_p^2 = 0.30$; Figure 12A), and were significant in females ($F_{1, 10} = 20.06$, $p = 0.00$, $\eta_p^2 = 0.67$; Figure 12C). After **administering OXT**, the main effect of Genotype was significant for males ($F_{1, 11} = 98.90$, $p = 0.00$, $\eta_p^2 = 0.90$; see Figure 12B), but not for females ($F_{1, 9} = 2.07$, $p = 0.18$, $\eta_p^2 = 0.19$; see Figure 12D). Moreover, the Treatment x Genotype interaction showed significant in males ($F_{1, 11} = 5.45$, $p = 0.04$, $\eta_p^2 = 0.33$), but not females ($F_{1, 9} = 4.16$, $p = 0.07$, $\eta_p^2 = 0.32$). In WTs, there appeared to be a Gender x Treatment interaction, albeit non-significant ($F_{1, 11} = 4.18$, $p = 0.07$, $\eta_p^2 = 0.28$). This interaction was significant in SEY mice ($F_{1, 9} = 8.17$, $p = 0.02$, $\eta_p^2 = 0.48$).

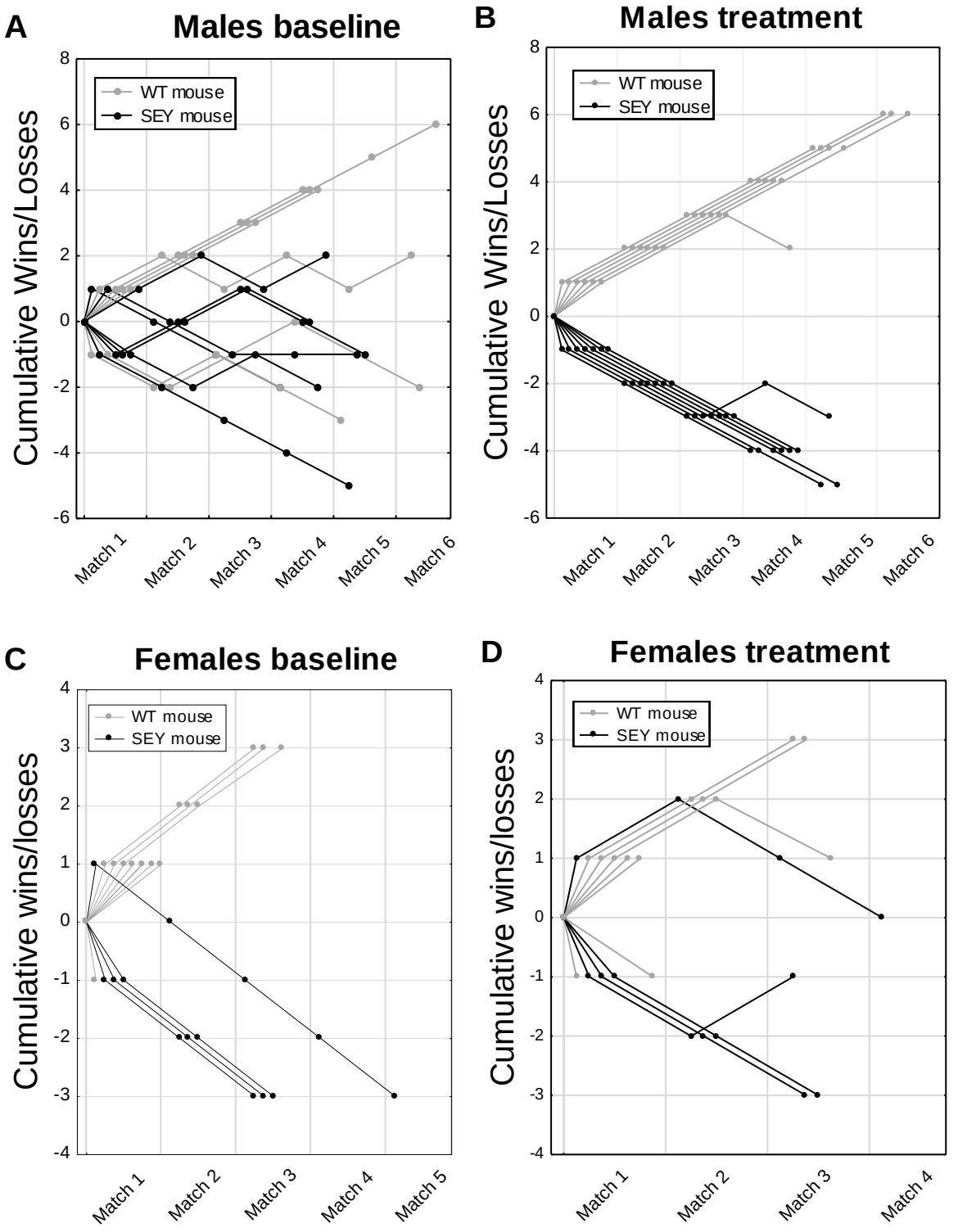

Figure 12| Representative plots of cumulative win/loss scores in the Automated Tube Test for individual mice per gender and per experimental condition. *The upper panels represent the male tournaments in baseline (A) and OXT condition (B), while the lower panels represent the female tournaments in baseline (C) and OXT condition (D). Line patterns represent individual mice for which a won match is registered as +1 and lost match is registered as -1.*

4.2 Contextual Fear Conditioning

4.5.1 *Conditioning phase*

Briefly, during this phase mice were placed only in Context A for three consecutive days, where they received a shock. In all genotypes this resulted in an increased freezing significantly during the first three minutes per day ($F_{2, 38}$ = 87.26, p = 0.00, η_p^2= 0.82) (Figure 13).

In both genders a main effect of Day indicated an effective fear conditioning (Males: $F_{2, 22}$ = 35.62, p = 0.00, η_p^2= 0.76; Females: $F_{2, 16}$ =53.66, p = 0.00, η_p^2= 0.87) which did not differ between SEYs and WTs (Males: $F_{1, 11}$ = 0.00, p = 0.96, η_p^2= 0.00; Females: $F_{1, 8}$ =0.93, p = .36, η_p^2= 0.10).

When controlling for genotype and modelling differences in gender, the GLM of **WT** mice shows a significant main effect of Gender ($F_{1, 10}$ = 6.48, p = 0.03, η_p^2= 0.39) which interacted with Day, suggesting a steeper increase for males when compared to females ($F_{2, 20}$ = 5.06, p = 0.02, η_p^2= 0.34). For **SEY** mice, no differences showed between males and females ($F_{1, 9}$ = 0.47, p = 0.51, η_p^2= 0.05), nor was the Gender x Day effect significant ($F_{2, 18}$ = 0.95, p = 0.41, η_p^2= 0.10).

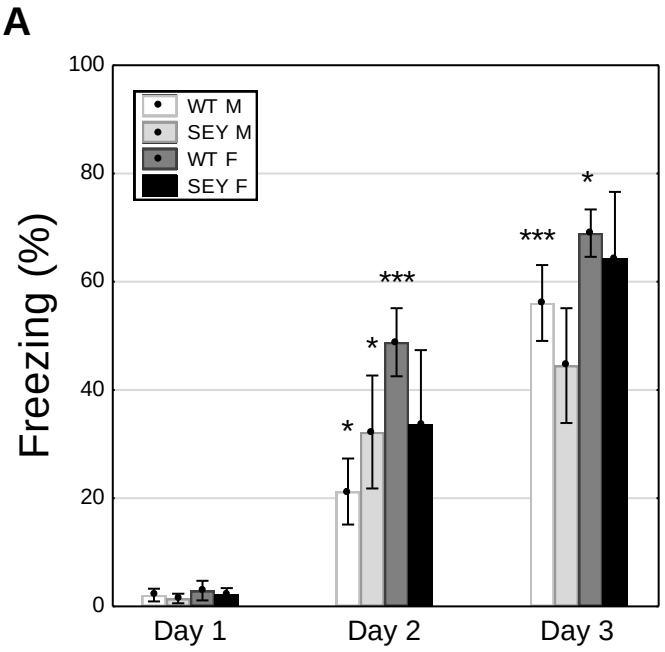

Figure 13| Mean percentage of freezing during a three-day Fear Conditioning phase.
Data is expressed as mean ± SEM. Significant differences in day are shown with an asterisk (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

4.5.2 *Contextual Memory*

During this phase a mouse's specifity of contextual memory was tested by introducing two novel contexts which differed in their similarity to Context A. Figure 14A-B shows the mean freezing times per day in each context, separately for males (A) and females (B).

Males of both genotypes were unable to distinguish Context A and B (WT: $F_{1, 5} = 0.04$, $p = 0.85$, $\eta_p^2 = 0.01$; SEY: $F_{1, 5} = 0.00$, $p = 0.95$, $\eta_p^2 = 0.00$) and did not differ from each other ($F_{1, 10} = 1.65$, $p = 0.23$, $\eta_p^2 = 0.14$). On the other hand, both WTs ($F_{1, 5} = 50.58$, $p = 0.00$, $\eta_p^2 = 0.91$) and SEYs ($F_{1, 5} = 15.36$, $p = 0.01$, $\eta_p^2 = 0.75$) were able to differentiate Context A and Context C, whereas both Genotypes were not significantly different ($F_{1, 10} = 0.43$, $p = 0.53$, $\eta_p^2 = 0.04$).

A similar tendency was found in **females**, whereas both WTs ($F_{1, 6} = 0.06$, $p = 0.82$, $\eta_p^2 = 0.01$) and SEYs ($F_{1, 4} = 1.92$, $p = 0.26$, $\eta_p^2 = 0.39$) were unable to differentiate Context A from Context B without a main effect of Genotype ($F_{1, 9} = 2.96$, $p = 0.12$, $\eta_p^2 = 0.25$). Despite no main effect of Genotype was shown ($F_{1, 9} = 2.39$, $p = 0.16$, $\eta_p^2 = 0.21$), it appeared that SEYs ($F_{1, 3} = 16.55$, $p = 0.03$, $\eta_p^2 = 0.85$) but not WTs ($F_{1, 6} = 5.52$, $p = 0.06$, $\eta_p^2 = 0.48$) were able to distinguish Context A and C.

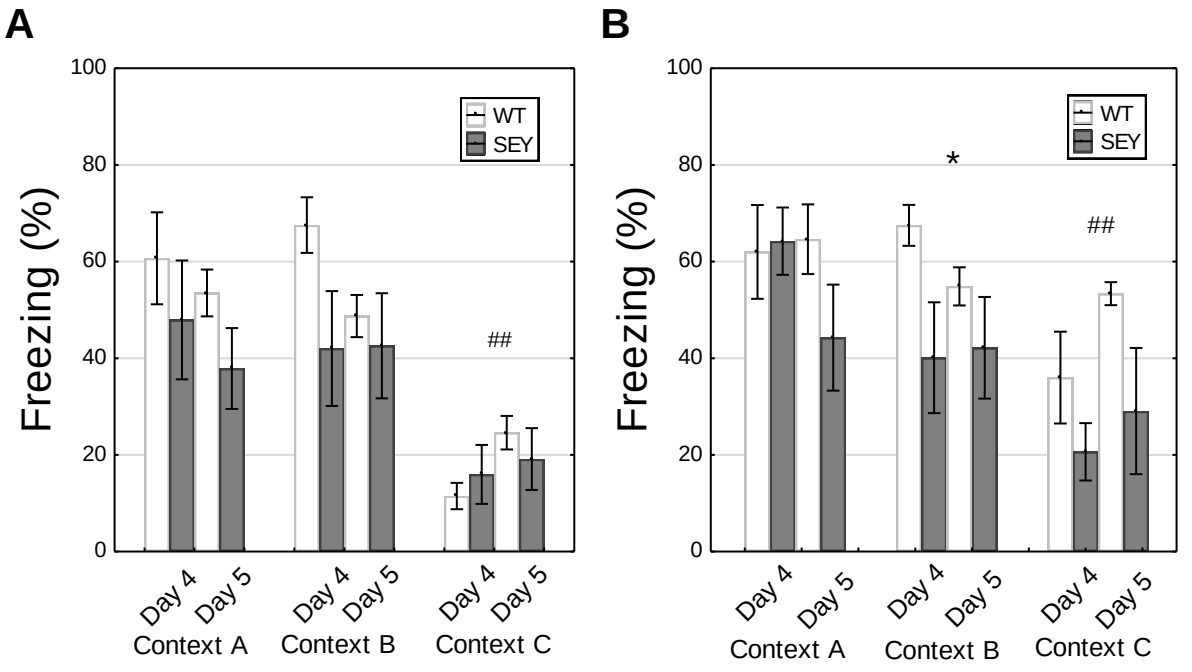

Figure 14| Mean percentage of freezing during the Contextual Fear Learning test phase for males (A) and females (B). Data is expressed as mean ± SEM. Significant main effects of Genotype are shown with an asterisk (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Significant differences between Context B/C with Context A are shown with a hashtag (#, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$).

Briefly, no gender differences were found between **WT** males and females considering Context A-B discrimination ($F_{1, 11}$ = 0.65, p = 0.44, η_p^2= 0.06), but was found for Context A-C discrimination ($F_{1, 11}$ = 6.19, p = 0.03, η_p^2= 0.36). That is, data suggested that females showed increased fear responses. Furthermore, no differences were found for both SEY genders (Context A-B: $F_{1, 8}$ = 0.08, p = 0.79, η_p^2= 0.01; Context A-C: $F_{1, 8}$ = 0.41, p = 0.54, η_p^2= 0.05).

4.5.3 *Discrimination learning phase*

Next, a 9-day discrimination learning phase was performed, in which the animals were subjected randomly to Context A (with shock) and Context B (no shock). Data for the individual genotypes are shown in Figure 15A-D.

For the SEY males the effect for Day was significant ($F_{8, 96}$ =2.81, p = 0.01, η_p^2= 0.19). However, the two context seemed to trigger same amounts of freezing behaviour ($F_{1, 12}$ =0.03, p = 0.86, η_p^2= 0.00), with no interaction of Day x Context ($F_{8, 96}$ =0.84, p = 0.57, η_p^2= 0.07). A similar pattern was found for WT males, with a significant main effect for Day ($F_{8, 80}$ =2.57, p = 0.02, η_p^2= 0.21), but not for Context ($F_{1, 10}$ =0.47, p = 0.51, η_p^2= 0.05). No interaction was shown between both effects ($F_{8, 80}$ =.78, p = 0.63, η_p^2= 0.07).

The effect of Day was not significant for SEY females ($F_{8, 48}$ =1.88, p = 0.09, η_p^2= 0.24), nor was the effect of Context ($F_{1, 6}$ =0.41, p = 0.54, η_p^2= 0.07). The GLM did not show an interaction-effect between both effects ($F_{8, 48}$ =1.49, p = 0.19, η_p^2=0.20). For female WTs the GLM did show a significant effect of Day ($F_{8, 88}$ =5.03, p = 0.00, η_p^2= 0.31), but not of Context ($F_{1, 11}$ =0.03, p = 0.87, η_p^2= 0.00), nor an interaction between Day x Context ($F_{8, 88}$ =1.98, p = 0.06, η_p^2= 0.15).

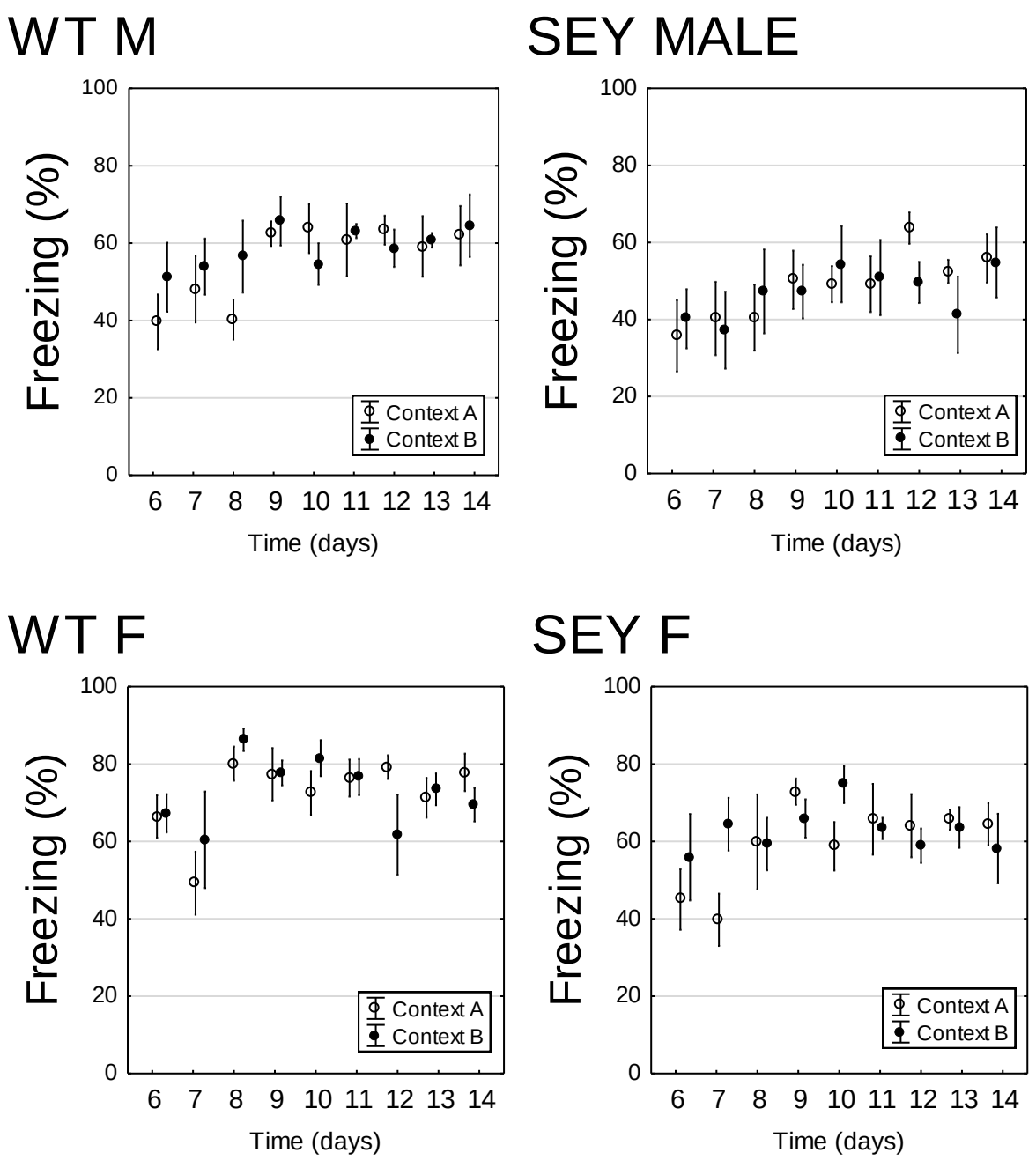

Figure 15| Mean percentage of freezing per genotype and gender during a 9-day Discrimination Learning phase. The upper panels display male data with WTs on the left (A) and SEYs on the right (B). The lower panels display data for female WTs (C) and female SEYs (D). Data is expressed as mean ± SEM.

4.5.4 *Contextual Memory Test*

After the contextual discrimination phase, the contextual memory of an animal was tested again by using the previously mentioned three contexts.

For **males**, the induced amount of freezing differed significantly among contexts ($F_{2, 22}$ = 33.77, p = 0.00, η_p^2= 0.75), with no observed difference between Context A and B ($F_{1, 11}$ =0.04, p = 0.85, η_p^2=0.00), but significant less freezing behaviour in Context C compared to Context A ($F_{1, 11}$ =54.20, p = 0.00, η_p^2= 0.83). In general, WT mice showed nonsignificantly more freezing behaviour than SEY mice did ($F_{1, 11}$ =2.60, p = 0.14, η_p^2= 0.19).

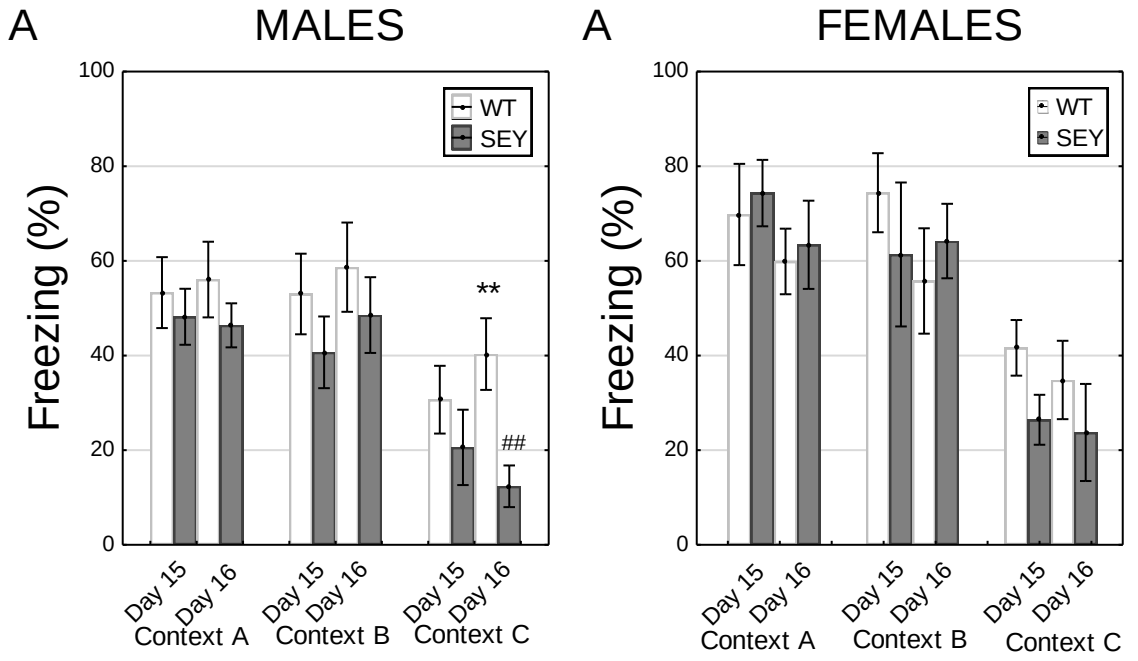

Figure 16| Mean percentage of freezing during the Contextual Discrimination test phase for males (A) and females (B). Data is expressed as mean ± SEM. Significant main effects of Genotype are shown with an asterisk (*, p < 0.05; **, p < 0.01; ***, p < 0.001). Significant differences of Context B-C with Context A are shown with a hashtag (#, p < 0.05; ##, p < 0.01; ###, p < 0.001).

Also for females in general, the GLM did show a significant main effect of Context ($F_{2, 18}$ =20.93, p = 0.00, η_p^2= 0.70), whereas Context A and B did not differ significantly ($F_{1, 9}$ =1.16, p = 0.31, η_p^2= 0.11) freezing was significantly lower in Context C compared to Context A ($F_{1, 9}$ =30.89, p = 0.00, η_p^2= 0.77). Over contexts, SEY non-significantly had lower induced fear when compared to WT mice ($F_{1, 9}$ =.14, p = 0.71, η_p^2= 0.02), which did not interact with the main effect of Context ($F_{2, 18}$ =1.04, p =0.38, η_p^2= 0.10). Briefly, males in general appeared to freeze nonsignificantly less than females did ($F_{1, 22}$ =4.24, p = 0.05, η_p^2= 0.16). Differences between Contextual Memory test on day 4-5 and day 15-16 are shown in Table 2.

	WT M	WT F	SEY M	SEY F
TEST 1 (Day 4-5)				
Context A	57.101 (8.503)	63.318 (7.872)	43.365 (8.503)	54.256 (10.414)
Context B	58.144 (7.496)	61.194 (6.940)	42.306 (6.940)	41.136 (9.180)
Context C	18.040 (5.532)	44.678 (5.122)	17.561 (5.122)	24.861 (6.776)
TEST 2 (Day 15-16)				
Context A	54.684 (7.001)	64.842 (6.490)	47.290 (6.490)	68.871 (8.585)
Context B	55.843 (8.042)	65.086 (7.445)	44.624 (7.445)	62.778 (9.849)
Context C	35.495 (6.336)*	38.227 (5.866)	16.480 (5.866)	25.078 (7.760)

Table 2| *Means with their standard errors in parentheses. Significant differences between Test 1 and 2 for the same Context are shown with asterisk (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).*

5 Discussion

The aim of this thesis was to evaluate the influence of chronic OXT treatment in male and female heterozygous Pax-6 mutants (SEY), a mouse model of delayed brain development. First, a testing battery was used to map several social behaviours in both Pax-6 mutant (SEY) and C57BL6 (WT) mice. Subsequently, mice were subjected to the same testing battery to point out effects induced by chronic OXT administration. The behavioural testing battery used to do so, assesses two major paradigms of social behaviour, i.e. social interaction and communication.

Within the social interaction paradigm, behaviours such as reciprocal social interactions (Social Proximity Test), sociability and social recognition (SPSN), and social dominance behaviour (Automated Tube Test) were measured. Communicative abilities of mice were mapped using the Odor Habituation/Dishabituation Test. After the chronic treatment regime, mice's ability to discriminate a defined shock-related context was evaluated using a Contextual Fear Response paradigm.

In the following section, the behavioural differences between WT and SEY genotype will be discussed. More in particular, the relevance of SEY mutants as a mouse model for neurodevelopmental delays with regard to social deficits is evaluated (5.1). In addition, the effect of OXT and its clinical implications will be debated (5.2), followed by its gender-specific differences (5.3). Furthermore, the effects of chronic OXT administration on the cognitive phenotype of mice will be discussed in section 5.4.

5.1 Social differences between SEYs and WTs

In this project a mouse's approach behaviour was tested in three tests with differences with regard to context. In the first place, a three chambered apparatus was used in which the mouse could *freely* explore either an encaged conspecific or an empty cage. The results of this test showed that both SEYs and WTs prefer to investigate a social over a non-social stimulus (empty cage) (Figure 9A). This same tendency to approach a social over a non-social stimulus was found both in C57BL6 (WT) and a mouse model for ASD (Bales et al., 2014). However, this article reports that the sociability in an ASD model is a contra-indication of previously reported findings. The authors attribute this counterintuitive finding to long-term handling stress. However, in the current study, this might not be the case since this effect was found previous to the long-term handling. A possible explanation could be that a C57BL6 background is characterized as a highly social genetic strain (Bales et al., 2014). However, social behaviour has a very complex genetic background thus a small genetic alteration such as Sey mutation will

presumably not fully alter its social behaviour. On the other hand, this might reflect the tendency of individuals with ASD to be interested in social events despite their reluctancy to participate in such social events. As Dölen (2015b) suggests, individuals with ASD might not experience the same rewarding effect of social interaction as neurotypical individuals do.

Next, the Social Proximity Test was used to assess a mouse's approach/avoid tendencies in a *forced context*. Because the spatial restriction of this test, the assumption can be made that this context is more stressful than the previously discussed free exploration. That is, this restricted context introduces a sort of 'butterfly effect', since even small movements of one animal affects the other animal thus forcing interactions. This test confirmed the hypothesis that SEYs are displaying different interaction strategies than WTs. First, SEYs showed a lower occurance frequency of direct frontal interactions (Figure 8). A sequel study which tried to replicate the findings of this thesis on a bigger scale, confirmed this finding (A. Murueta-Goyena, personal communication, July 5, 2017). On the other hand, for SEYs in particular, the aversive/stressful nature of this context is confirmed by displaying significantly more escape jumping and upright (defensive) posture (Figure 8). In literature these two behaviours could be decreased by administering Diazepam, an anxiolytic drug, indicating their link to stress and/or anxiety (Defensor et al., 2011). Additionally, the increase in defensive posture in SEY mice might be in line with the observation of Umeda and colleagues (2010) that SEY rats show more aggression-related behaviours than WTs did. Overall, these results suggest a higher stress sensitivity in SEY mice when compared to WTs as a result of this particular social environment. Consequently, SEY might model the higher stress levels due to social cues which have been reported in humans with ASD (Dölen, 2015b).

Finally, the approach/avoidance tendency of mice was tested in a third context, the Automated Tube Test. This context was even more restrictive, since mice could only move towards the other mouse or away from the other mouse. The results showed that SEYs of both genders rather than WTs showed a tendency to retrieve to their starting box which was used as a read-out for subordinance to a more dominant WT. This tendency was confirmed in the sequel study (A. Murueta-Goyena, personal communication, July 5, 2017). In line with the findings of the Social Proximity test, it can be expected that SEYs had a lower social status. Timmer and colleagues (2011) reported that male rats which were stressed did obtaine a subordinate position in the social hierarchy. This makes sense since social hierarchy is established by direct confrontations between animals and SEYs appear to avoid these behaviours because of anxiety/stress. On the other hand, this finding is consistent with the previously mentioned findings in the Social Proximity Test. Altogether, it appears that SEY mice are not less interested in unfamiliar

conspecifics. Rather they appear to be reluctant to participate in social interactions. Similar findings were reported in other mouse models of ASD, with exhibition of more withdrawal/ avoidance behaviours (Defensor et al., 2011) and less approach behaviours (Bales et al., 2014).

Another aim of this project was to map deficits in **social recognition**. In contrast with the general expectation, none of the testing groups showed a preference for a novel social stimulus over a previously encountered animal. Accordingly, neither of the male genotypes appeared to recognize and distinguish social odors properly through demonstration of dishabituation during the Olfactory Habituation/Dishabituation paradigm. A similar pattern was reported by Pearson and colleagues (2010) whereas they suggest that the preference might not be due to the social characteristics but rather to the environmental changes created by the addition of a novel animal. An explanation for the observed results might be that in both the SPSN and Olfactory Habituation/Dishabituation same sex and strain (C57BL6) conspecifics are used as stimulus animals. Another study using C57BL6 as stimulus mouse was unable to report the social recognition in the subject mice (Martin, Sample, Gregg, & Wood, 2014). It appears that because of these similarities the subject mice are unable to differentiate among stimulus mice. This might explain why Bales et al. (2014) reported a preference for social novelty in C57BL6 mice when 129Sv/ImJ mice were used as stimulus mice. Thus the different genetic backgrounds of stimulus mice appears to allow subject mice to differentiate among them.

5.2 Effects of OXT on social behaviour

Following the rationale of evaluation approach behaviour in different contexts, here, the effects of OXT are discussed. In a non-stressful situation (SPSN), OXT treatment did not seem to have an effect. However, in this treatment condition, mice appeared to travel significantly further than they did during the baseline condition. This was in line with the observation of higher approach behaviours towards both the animal as the empty cage, though the ratio was stable. A possible interpretation is that OXT does not influence social interest itself, but rather the inhibitory exploration mechanisms. While it is tempting to attribute the observed effect to an anxyiolytic effect of OXT, it should be noted that at this point of testing, mice were no longer naïve. That is, during previous tests they have encountered various ‘novel’ environments in which they were confronted with social stimuli. More importantly, they have already encountered this specific environment which might result in a reduction of stress and anxiety due to habituation.

Interestingly, in the Social Proximity Test, OXT treatment was able to restore the deficit in nose-nose interactions in SEYs. In addition, the treatment appeared to significantly decrease upright behaviours in the mixed pairs, which ressembles the tendency in SEYs. This suggests

that OXT has a comparable anxiolytic effect in SEYs, but to a lesser extent in WTs since they displayed minimal upright behaviors under any condition. In addition, the steep decrease in escape jumping in all three pair types (see Figure 8) appears to confirm this anxiolytic effect of OXT. As previously mentioned, behavioural reduction in these behaviors is associated with less anxiety/stress (Defensor et al., 2011).

In the context with the highest degree of imposed physical limitation, the Automated Tube Test, data showed apparent genotypical differences after OXT administration. More in particular, in both genders, SEYs retrieved the majority of times to their starting box after OXT administration. The sequel study, which tested only males, was able to replicate this finding (Murueta-Goyena, personal communication, July 5, 2017). This might learn something about the mechanism underlying the anxiolytic effect. More in particular, it appears that OXT has a gender-specific way of reducing stress/anxiety in mice (discussed in 5.3). The data thus indicate the existence of an association between OXT and social dominance. However, the earlier research has already linked OXT and social dominance in female rhesus macaque monkeys (Michopoulos, Checchi, Sharpe, & Wilson, 2011) and in rats (Timmer, Cordero, Sevelinges, & Sandi, 2011).

Clinically, these findings weigh in favour of a beneficial effect of OXT. However, in line with both human (Wöhr & Krach, 2017) and animal (Lawson, Gray, & Woehrle, 2016) studies, this project confirms that OXT's prosocial effects are at least partially due to its potential to alleviate anxiety. More in particular, it might be an interesting lead to further explore this effect with an eye on disorders such as ASDs. Despite findings suggest that intranasal OXT is correlated with changes in at least some brain activation patters, it always should be taken into account that a minimum of the administered OXT will in the systemic circulation. Here, studies have provided evidence that OXT can mediate the stress response through attenuation (Dölen, 2015a). Alltogether, there exists common ground in these findings and the finding that intranasal OXT has beneficial effects in individuals with ASD by decreasing anxiety about social cues might be a hopefull lead to use OXT as a potential treatment in the future (Dölen, 2015a).

Furthermore, it is reported that OXT knock-out mice in contrast to WTs do not habituate to social stimuli (Modi & Young, 2012). Following this rationale, the expectation was that OXT administration would enhance a mouse's capability to both recognize and habituate to social odors. In contrast to the results at baseline, after OXT administration, males of both genotypes appeared to be able to distinguish among a previously encountered and a novel mouse.

5.3 Gender-specific mechanisms of OXT

The way OXT affects social behaviour is not only influenced by genotypical differences. Differences in gender play another important role. In a low-stress environment (SPSN), males and females showed a similar tendency to approach another mouse over an empty cage after OXT administration. Both in the acclimatization and sociability phase of this test, baseline measurements were similar in males and females. However, after administering OXT, males tended to explore significantly more while females even tended to explore less, albeit non-significant (Figure 8). Similar findings were found during the Preference for Social Novelty phase.

At first sight, the high-stress environment (Automated Tube Test) shows a similar pattern for males and females: WTs show high social dominance and SEYs are rather subordinate. However, based on Van Den Berg and colleagues' (2015) theory of gender-specificity in the formation of social hierarchy, one caveat is visible. This theory suggests that social hierarchy in females is primarily based on stable intrinsic attributes. In males, on the other hand, the foundation of social hierarchy is formed by prior experience. The results of the Automated Tube Test suggested that in males OXT administration has a rather strong effect, but this was not the case in females. When looking at Figure 12A-B, OXT appears to play (at least partially) a role in the genotypical dichotomy of social dominance behaviour. On the contrary, this was not the case in females and Figure 12C-D both display a stable pattern of hierarchy. For example, both figures show that there is one (and the same) SEY mouse which wins in the baseline and in the treatment condition. Overall, it appears that OXT is able to increase the effects of prior experience, but not the stable intrinsic attributes. A possible explanation can be found in the study of Timmer and colleagues (2011). This study showed that a stressed rat will become subordinate after a hierarchical encounter with a non-stressed rat. Moreover, they reported that even 3 hours after this encounter, status- and region-specific changes were found in the OXTR on the level of mRNA. In this regard, it appears that OXT has more binding sites in the brain of a dominant animal and thus will be differentially expressed.

Clinically, these findings highlight the importance to consider gender-specific effects of OXT treatment. Based on the available results, OXT's anxiolytic effects appear to have a more pronounced basis in males than in females. Specifically in the context of disorders such as schizophrenia and ASD which have a relative high male:female ratio, it is important to keep this gender-specific mechanism in mind.

5.4 Cognition after chronic OXT treatment

The Contextual Fear Conditioning showed that after chronic OXT treatment, mice were able to condition to an aversive stimulus. Mice were able to differentiate between the context of the aversive stimulus and a non-ressembling context. However, they were not able to do this with a context wich ressembled the aversive stimulus context. Even after a 9-day discrimination learning phase this seemed to worsen. With regard to the prior beliefs, the inability of SEY to discriminate among contexts was expected. That is, Pax-6 knock-out mice display deficits in contextual fear-conditioning tasks due to hippocampal dysfunction (Tuoc et al., 2009). However in humans, some studies report associations between Pax-6 mutation and severe cognitive or behavioural abnormalities (Davis et al., 2008), while others did not (Thompson et al., 2004). More interestingly is the observed inability of WT mice to discriminate the defined 'aversive' context. OXT plays a role in the learning of fearful stimuli (Bales et al., 2014). This finding thus might ressemble the previously reported negative findings of chronic OXT administration in the young brain (Bales et al., 2014). Further studies are needed to assess the validity of this finding.

6 Conclusion

In this study a mouse model for delayed brain development, small eye, was tested at the age of 8-14 weeks to evaluate whether OXT had beneficial effects on its social behavior and cognition. Because of reported gender-specific mechanisms underlying the effect of OXT both males and females were tested. In a baseline condition, the evaluation of SEY as a mouse model of neurodevelopmental disorders confirmed the validity for this model. Results showed a consistent pattern of avoidance/withdrawal which were associated with higher amounts of stress. The paradigms assessing social recognition of the animals were not able to distinguish WTs and SEYs. However, this appeared to be caused by an inability to distinguish two animals of the same genotype. Therefore, it might be useful for future studies to use stimulus animals which substantially differ in genetic background from the subject animals.

During a chronic OXT treatment regime, benificial effects were found both in WTs and SEYs. More in particular, OXT was able to restore at least some of the social deficits observed in SEYs. In general, the results confirmed that OXT's prosocial effects are the consequence of an anxiolytic effect. Further research will be needed to confirm whether this is because of a systemic effect or an effect at the level of the brain. On the other hand, this project higlights that OXT to some extent has detrimental effects on the cognition of SEYs and WTs of both genders. Based on the available results, it appears that OXT has a larger potential to intervene as an anxiolytic in males than in females. This should be taken into account when translating OXT into the clinical context of sexual dimorphic disorders such as ASD.

Finally, using a rather small sample size, the findings in this study are indicative and useful, though they will need further investigation on a larger scale to be confirmed. To conclude: this is a small step forward in OXT's clinical relevance, but to translate into an effective drug for mankind, a whole lot of work still needs to be done.

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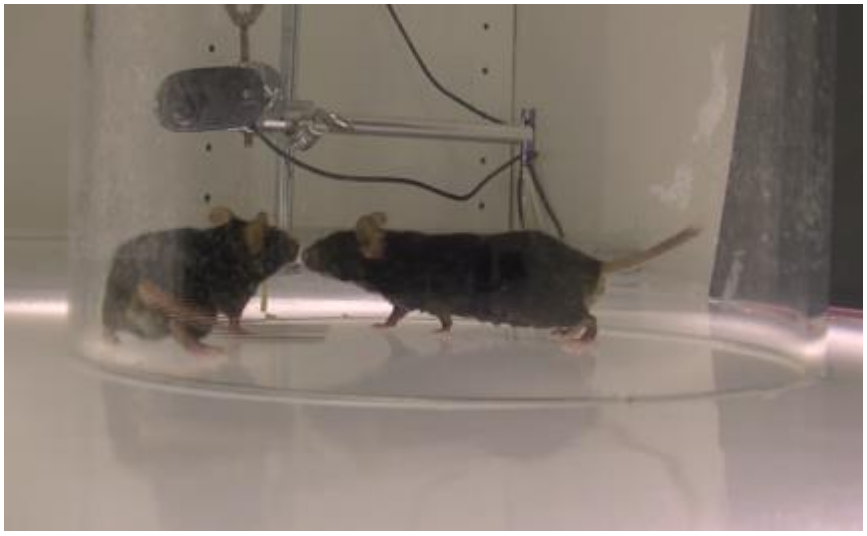
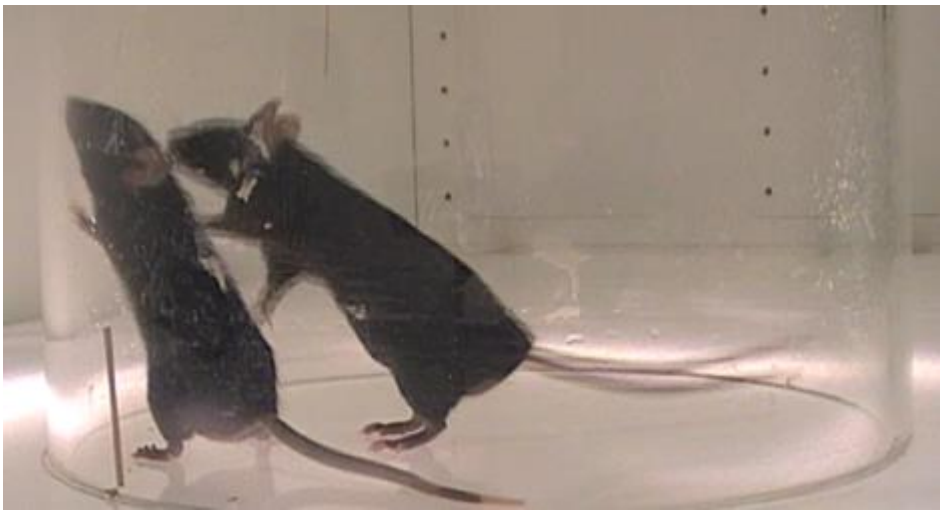
8 Appendix

A. Social proximity: mouse behaviour scoring manual

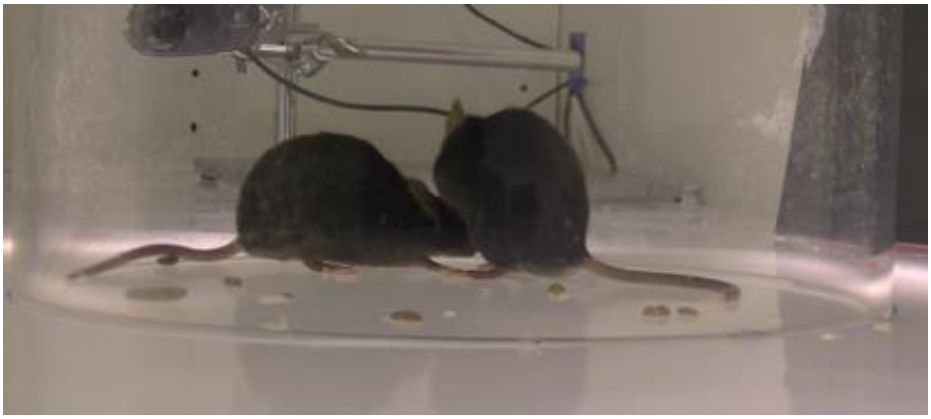
Nose tip nose tip

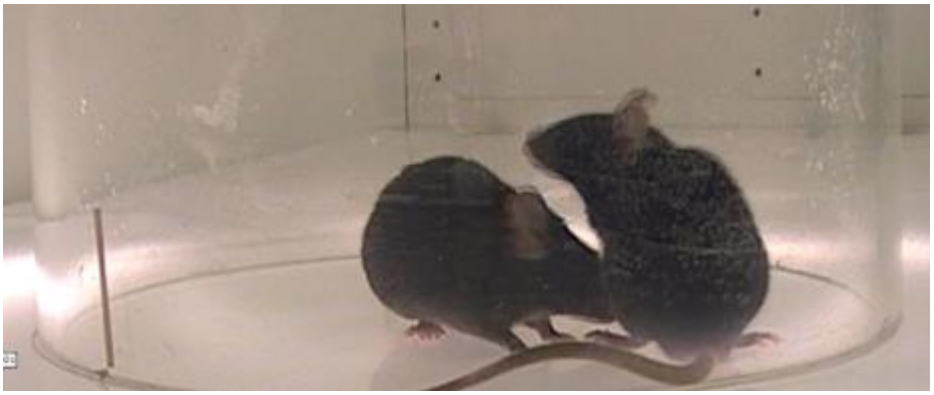


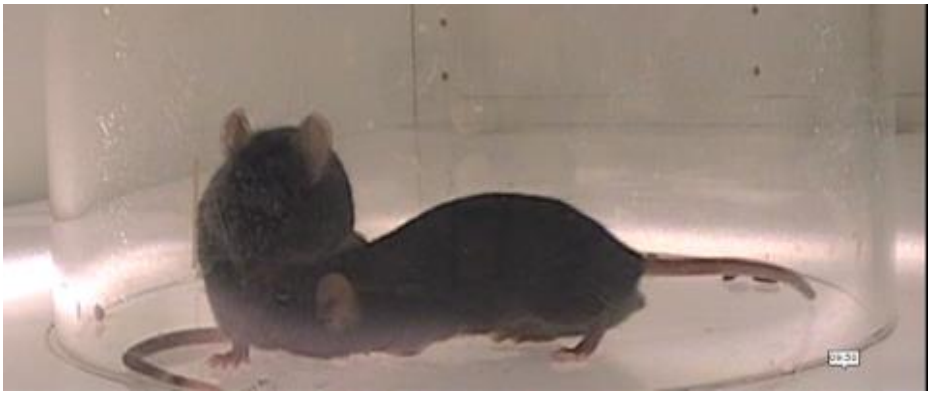
Crawl over (= unidirectional interaction)



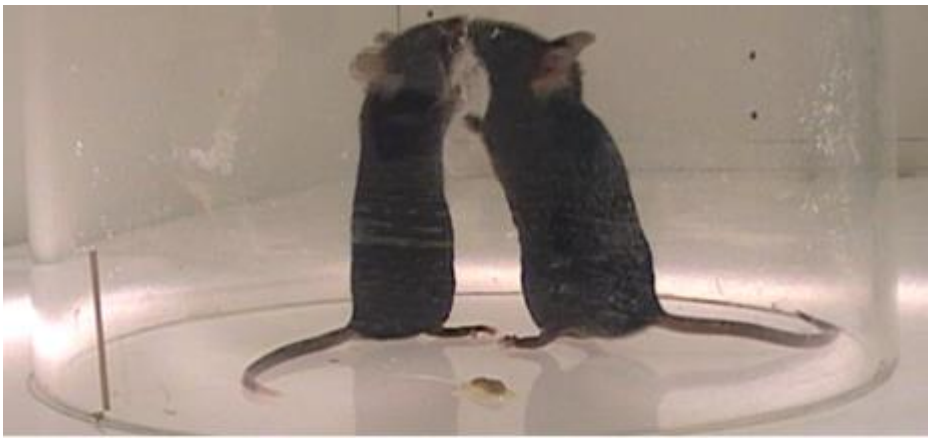
Crawl under

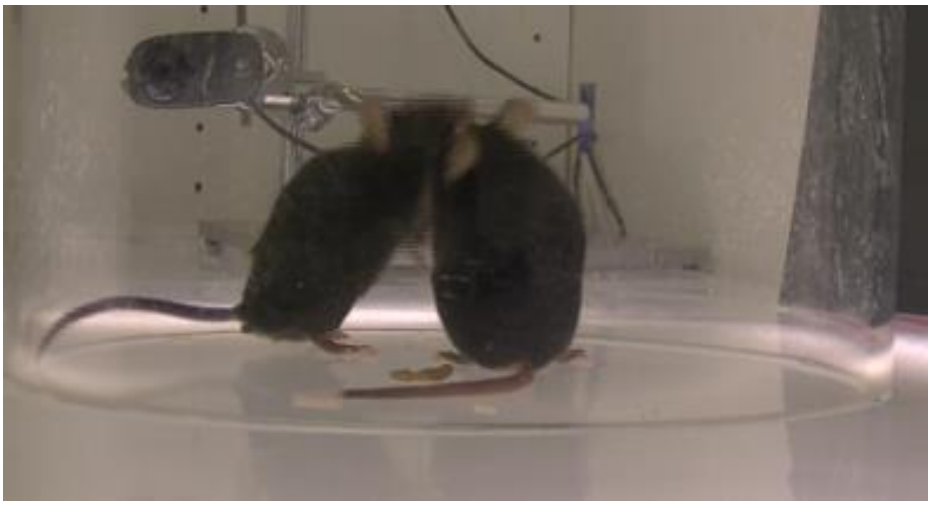




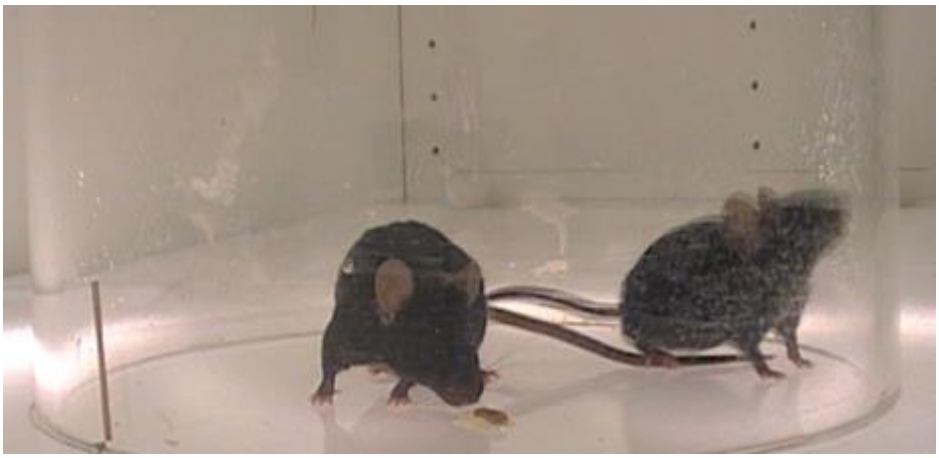


Upright (= Bidirectional interaction)

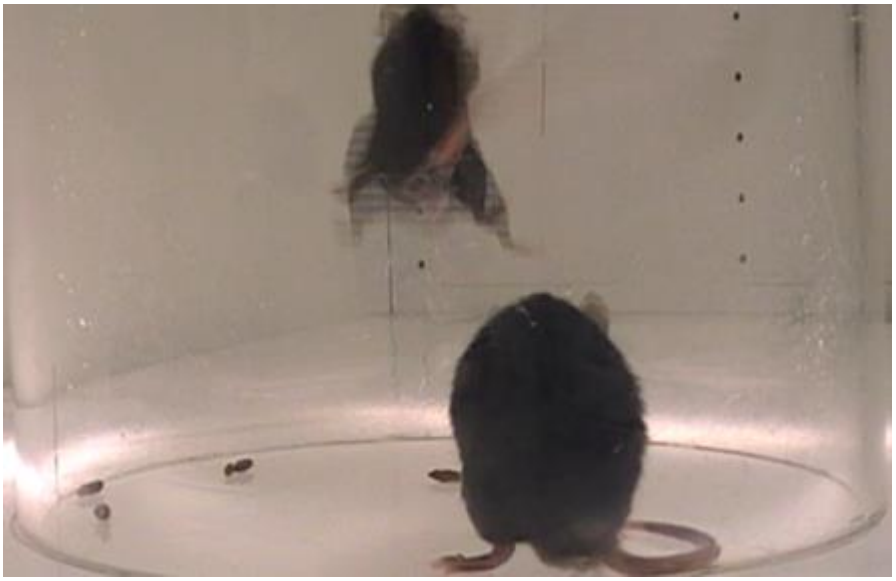




Sniffing faeces/urine



Escape jumping



Allogrooming

B. Social proximity: separate results

B.1. Comparison between SEY or mixed pairing to WT-WT interactions during baseline and OXT treatment.

	Baseline					OXT treatment				
	df Effect	df Error	F	p	η_p^2	df Effect	df Error	F	p	η_p^2
SEY-SEY										
NN	*1*	5	5,985	0,058	.545	1	5	0,001	0,978	.000
NA	*1*	5	0,253	0,636	.048	1	5	5,511	0,066	.524
CO	*1*	5	0,262	0,630	.050	1	5	0,107	0,757	.021
CU	*1*	5	0,449	0,533	.082	1	5	1,826	0,235	.267
U	*1*	5	1,803	0,237	.265	1	5	0,000	1,000	.000
EJ	*1*	5	2,347	0,186	.319	1	5	0,714	0,437	.125
AUG	*1*	5	3,145	0,136	.386	1	5	5,320	0,069	.516
ALG	*1*	5	0,042	0,846	.008	1	5	2,143	0,203	.300
FU	*1*	5	4,658	0,083	.482	1	5	2,545	0,172	.337
WT-SEY										
NN	*1*	6	1,385	0,284	.188	1	6	0,601	0,468	.091
NA	*1*	6	0,025	0,879	.004	1	6	0,174	0,691	.028
CO	*1*	6	1,385	0,284	.188	1	6	0,964	0,364	.138
CU	*1*	6	1,406	0,281	.190	1	6	0,395	0,553	.062
U	*1*	6	5,793	0,053	.491	1	6	0,220	0,656	.035
EJ	***1***	**6**	**6,942**	**0,039**	**.536**	1	6			
AUG	*1*	6	2,465	0,167	.291	1	6	0,479	0,515	.074
ALG	*1*	6	0,207	0,665	.033	1	6	0,563	0,482	.086
FU	*1*	6	0,607	0,466	.092	1	6	2,951	0,137	.330

B.2. Within effect of OXT

	WT-WT				SEY-SEY				WT-SEY			
	df Effect	df Error	F	p	df Effect	df Error	F	p	df Effect	df Error	F	p
NN	*1*	4	0,176	0,697	***1***	**6**	**9,503**	**0,022**	*1*	8	0,646	0,445
NA	*1*	4	0,686	0,454	*1*	6	1,277	0,302	*1*	8	0,344	0,574
CO	***1***	**4**	**14,215**	**0,020**	***1***	**6**	**7,637**	**0,033**	*1*	8	1,891	0,206
CU	*1*	4	0,250	0,643	*1*	6	2,727	0,150	*1*	8	2,000	0,195
U	*1*	4	0,108	0,759	*1*	6	1,923	0,215	***1***	**8**	**9,406**	**0,015**
EJ	*1*	4	7,193	0,055	***1***	**6**	**7,651**	**0,033**	***1***	**8**	**40,336**	**0,000**
AUG	*1*	4	0,450	0,539	*1*	6	0,752	0,419	*1*	8	4,439	0,068
ALG	*1*	4	1,000	0,374	*1*	6	0,429	0,537	*1*	8	0,800	0,397
FU	*1*	4	1,433	0,297	*1*	6	1,900	0,217	*1*	8	0,007	0,934

B.3. Between pair effect of OXT

	Effect Genotype			Effect Treatment			Interaction		
	$F_{2,9}$	p	η_p^2	$F_{1,9}$	p	η_p^2	$F_{2,9}$	p	η_p^2
NN	1,56	0,26	0,26	2,86	0,13	0,24	0,45	0,65	0,09
NA	0,36	0,71	0,07	2,74	0,13	0,23	0,06	0,94	0,01
CO	0,75	0,50	0,14	**24,10**	**0,00**	**0,73**	2,27	0,16	0,34
CU	0,67	0,54	0,13	2,91	0,12	0,24	2,45	0,14	0,35
U	0,83	0,47	0,16	**12,83**	**0,01**	**0,59**	**6,73**	**0,02**	**0,60**
EJ	1,91	0,20	0,30	**32,79**	**0,00**	**0,78**	1,96	0,20	0,30
AuG	3,74	0,07	0,45	3,13	0,11	0,26	0,82	0,47	0,15
AlG	0,25	0,79	0,05	0,58	0,47	0,06	1,02	0,40	0,19
FU	3,01	0,10	0,40	2,97	0,12	0,25	0,86	0,45	0,16

C. Statistical results for Habituation and Dishabituation in male SEYs and WTs

C.1. Habituation

	Baseline					OXT treatment				
	df Effect	df Error	F	p	η_p^2	df Effect	df Error	F	p	η_p^2
SEY										
Water	**2**	**12**	**7,51**	**0,01**	**0,56**	**2**	**12**	**4,67**	**0,03**	**0,44**
Banana	*2*	12	2,01	0,18	0,25	2	12	3,38	0,07	0,36
Grape	*2*	12	0,08	0,92	0,01	2	12	3,46	0,07	0,37
Social 1	*2*	12	2,41	0,13	0,29	**2**	**12**	**3,97**	**0,05**	**0,40**
Social 2	*2*	12	2,07	0,17	0,26	2	12	0,55	0,59	0,08
Social 3						**2**	**12**	**10,00**	**0,00**	**0,63**
Social 4						**2**	**12**	**8,25**	**0,01**	**0,58**
WT										
Water	2	10	3,71	0,06	0,43	2	10	0,47	0,64	0,09
Banana	*2*	10	1,05	0,39	0,17	*2*	10	0,36	0,71	0,07
Grape	**2**	**10**	**7,04**	**0,01**	**0,58**	*2*	10	0,68	0,53	0,12
Social 1	*2*	10	1,40	0,29	0,22	*2*	10	2,72	0,11	0,35
Social 2	2	10	2,60	0,12	0,34	2	10	0,45	0,65	0,08
Social 3						2	10	1,24	0,33	0,20
Social 4						*2*	10	1,19	0,34	0,19

C.2. Dishabituation

	Baseline					OXT treatment				
	df Effect	df Error	F	p	η_p^2	df Effect	df Error	F	p	η_p^2
SEY										
Water-Banana	1	6	0,24	0,64	0,04	1	6	0,01	0,92	0,00
Banana-Grape	**1**	**6**	**10,57**	**0,02**	**0,64**	1	6	1,55	0,26	0,21
Grape-Social 1	**1**	**6**	**9,66**	**0,02**	**0,62**	**1**	**6**	**21,40**	**0,00**	**0,78**
Social 1-Social 2	1	6	0,18	0,68	0,03	1	6	0,73	0,43	0,11
Social 2-Social 3	1	6	0,24	0,64	0,04	1	6	2,77	0,15	0,32
Social 3-Social 4						1	6	4,00	0,09	0,40
WT										
Water-Banana	1	5	3,41	0,12	0,41	1	5	4,18	0,10	0,46
Banana-Grape	1	5	3,16	0,14	0,39	1	5	1,70	0,25	0,25
Grape-Social 1	1	5	4,77	0,08	0,49	**1**	**5**	**13,53**	**0,01**	**0,73**
Social 1-Social 2	1	5	0,10	0,77	0,02	1	5	1,02	0,36	0,17
Social 2-Social 3						1	5	0,19	0,68	0,04
Social 3-Social 4						1	5	0,75	0,43	0,13