Exploring sex differences in the response to tickling in juvenile Wistar rats

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Declaration

I declare that this thesis has been composed by myself, the work contained within it is my own and the assistance and help received during the course of the research has been fully acknowledged. This work has not been submitted for any other degree or professional qualification.

E. K. L. Tivey

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Abstract

Positive welfare is considered not simply the absence of suffering, but also the presence of positive experiences. ‘Tickling’ induces positive affective states in laboratory rats as evidenced by the production of 50 kHz ultrasonic vocalisations (USVs). Few studies have investigated the effect of tickling on females, and whether there is a sex difference in response to tickling and the involvement of the neuroendocrine system. Here I investigated whether there are sex differences in behavioural responses to tickling in juvenile Wistar rats due to sex-specific neural regulation of positive affective states. I tested the following hypotheses that: (i) specific elements of the behavioural response to tickling (including USVs) differ between sexes; (ii) 50 kHz USV subtypes are produced in a temporally specific manner during tickling, and they may be paired with different behaviours observed during tickling; (iii) the behavioural response to tickling is regulated by brain regions and neural circuits that are associated with social play in rats, and this is sex specific; (iv) oxytocin and vasopressin neurons in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) are involved in the behavioural response to tickling in a sex specific manner; and (v) 50 kHz USV production in response to tickling is related to measures of motivation for the reward of tickling, and this is greater in females.

In experiment 1, rats were tickled or had no hand contact during two minutes of testing per day for 10 days. Play-related behaviours (hand approaches and solitary play) and 50 kHz USVs were quantified. Female rats made more 50 kHz USVs after 10, but not five, days of tickling. Tickled rats made more trill 50 kHz USVs, regardless of sex, while female rats emitted more flat 50 kHz USVs than males after 10 days of testing. Both treatment and sex affected flat 50 kHz USVs, with females producing more flat 50 kHz USVs and regardless of sex, tickled rats producing more trill 50 kHz USVs than controls. Females paired more flat 50 kHz USVs with scampering and approaches, while tickled rats of both sexes paired more trills with those behaviours. Tickled rats elicited more solitary play scampering than control

rats, and this was greatest in females. Tickling did not increase hand approaches, and control females approached the hand more than tickled rats.

Double-labelled immunohistochemistry was used to quantify c-*fos* expression (a neuronal activity marker) in oxytocinergic and vasopressinergic neurons that are known to be involved in social behaviours (e.g. social conspecific play) and reward pathways. Tickling did not increase Fos immunoreactivity in the social behaviour, reward or mid- or hind-brain brain regions. Control rats had more correlated Fos immunoreactivity between regions of the social behaviour network and reward pathway. In the PVN, tickled rats, regardless of sex, had lower numbers of Fos positive parvocellular, but not magnocellular, oxytocin and vasopressin neurons. There was no effect of tickling on Fos immunoreactivity in oxytocin and vasopressin SON neurons.

In experiment 2, rats were tickled or had no hand contact during two minutes of testing per day for five days, followed by testing in a modified runway test for two days to measure motivation for tickling, and a modified preference test to measure preference for the hand over a novel object. Tickling did not affect the time taken to traverse the runway, but tickled rats made more total and trill 50 kHz USVs while traversing the runway. Tickling did not change preference for a novel object or the hand, but control rats made more visits to the novel object.

In summary, there are sex differences in the behavioural response to tickling, but this appears to be dependent on the number of days of tickling and specific to individuals. Tickling did not increase neuronal activity in key regions associated with social behaviours and reward and this may be a consequence of individual responses to tickling. Alternatively, tickling, while rewarding, was not perceived as a social stimulus. This is an important consideration for future tickling studies as tickling should not necessarily be construed as mimicking rat social conspecific play and responses to tickling by both sexes should be considered.

Lay Summary

Play is generally understood as being enjoyable in many species. Rats are known to play together as pups and adolescents, and play is commonly seen up until adulthood. Tickling is a type of play that humans can perform, which is carried out by making rapid movements with fingers on certain parts of the body. In 2000, scientists tried tickling rats, using their knowledge of how rats play with each other to design a method of tickling, using the same rapid finger movements as when tickling another human, on the areas of the rat's body that are contacted during play in young rats. The scientists discovered that rats make high pitched (ultrasonic) calls during tickling. These specific calls were identified as being the same calls that are also made during rewarding behaviours including feeding, play and mating, suggesting that rats also enjoy tickling. Many experiments since have used tickling as a way of creating a positive emotional state (e.g. pleasure) in rats. However, many of these experiments have only used male rats, and so whether tickling creates a similar sense of pleasure in both female and male rats is not well understood. Scientists have also been interested in the areas in the brain that might control tickling in rats. Experiments have shown that brain areas that control gratifying, rewarding experiences, such as sweet, sugary foods and sex, may also control tickling. However, it is not yet known whether any areas of the brain that control social interactions between people or animals are also involved with controlling tickling. Social behaviour can be different in females and males, therefore brain regions that control social interactions can also be different between sexes. I set out to understand whether female and male rats enjoyed tickling to the same extent, and if the brain regions controlling tickling were also the same in female and male rats, focussing on areas that are known to be involved in social interactions.

I tickled female and male rats and recorded the rats' behaviours while I was tickling them using a video camera and specialised microphone to record the ultrasonic calls. I studied the brain areas I was interested in by using antibodies that bind to a specific protein in neurons. The protein that the

antibody attached to indicates which neurons were activated in response to tickling allowing me to count the activated cells in different brain regions. I found that both female and male rats seemed to enjoy tickling based on the ultrasonic calls they produced. I also found that one particular sub-type of ultrasonic call (in rats, there are different types of ultrasonic calls) was used more by female rats than males. In addition, I found that rats pair certain types of ultrasonic calls with certain play-related behaviours and this is different between females and males. This may show how female rats are sharing information with other rats during playful interactions. In the brains of the rats, I found that the majority of the areas that I looked at did not appear to be associated with tickling as the number of cells that had been activated was not different between rats that had been tickled compared to rats that hadn't. However, I did find that in the area of the brain that produces the hormones oxytocin and vasopressin (which have important roles in social interactions), more cells that produce these hormones were activated in rats that had not been tickled compared to rats that had been tickled. This suggests that tickling had caused less activity in these cells, and that therefore, tickling might not be perceived as a social interaction by rats. I tested this further by investigating whether rats preferred to approach the hand that had been tickling them or a new object that they hadn't experienced before. Adolescent rats have a curious nature and are attracted to novel objects over familiar objects. Rats are also highly social animals, and tend to prefer spending time with another rat over a novel object. Measuring the time that a rat spends with a novel object compared to having social contact can show how highly they value the social contact. I found that rats that had been tickled did not prefer to visit the hand that had been tickling them over the novel object, whereas rats that had not been tickled made more visits to the novel object over the hand compared to tickled rats. This suggests that rats did not show a preference for tickling over investigating a novel object.

Overall, this may suggest that tickling is not perceived as a social interaction for rats. Another explanation may be that individual rats vary in how much they enjoy being tickled, with some rats finding tickling to be highly

pleasurable, but some rats did not find it pleasurable or were ambivalent towards being tickled. Investigating individual differences in future studies is warranted. In addition, my findings have provided preliminary evidence that can be used to inform future experiments to understand in greater depth the areas and cellular networks in the brain that are associated with the response to tickling in both female and male rats. I also found that there may be sex differences in the pairing of ultrasonic calls with play-related behaviours. This novel finding suggests that female and male rats may communicate using different patterns of calls, and shows the importance of using female animals in scientific studies.

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Finally, to the rats that were used in this study. I truly hope that I have done them justice through this body of work. Most of all, I hope that their short lives with me were somewhat enjoyable and that they had many 'positive experiences'.

Contents

Chapter 1

Literature review

The main aims of this thesis are to investigate the positive affective state and neural and neuroendocrine correlates induced by tickling, and to elucidate whether these correlates are sexually dimorphic. Given these aims, this literature review has two parts; the first discusses welfare and affect, social behaviours and the rat tickling model. The second part discusses the neurobiological basis of social behaviours, particularly social conspecific play, and of tickling.

1.1 Animal welfare and affective states

There has been increasing interest and scientific study of animal welfare in the past approximately 60 years (Broom, 2011). In the UK, Harrison's book on the welfare concerns of intensive farming practices (Harrison, 1964) led to an investigation into intensive farming and the subsequent Brambell report (1965). Following this, the Five Freedoms were conceptualized by the Farm Animal Welfare Council (FAWC) in 1979 and have since been used as one of the main frameworks used in animal welfare legislation (Vapnek and Chapman, 2010; OIE, 2019).

Animal welfare is a multi-dimensional concept, with several aspects needing to be fulfilled to ensure good welfare, such as health, expression of normal behaviours and a comfortable environment (Botreau et al., 2007; Cornish et al., 2016). Fraser et al. (1995) described that key concepts of animal welfare are that animals should live natural lives, animals should feel well and animals should function well. A key concept of animal welfare is that non-human animals are sentient beings and can experience emotions (Boissy et al., 2007; Mendl et al., 2009; Cornish et al., 2016). Animal sentience, in part, refers to the capacity of an animal to experience states of emotion such as pleasure and aversion (Dawkins, 2006; Broom, 2007). By scientifically

demonstrating that animals are sentient may help to promote the importance of safeguarding animals' welfare (Proctor, 2012). Currently, the difficulty lies in measuring and proving sentience because, without being able to communicate them verbally, it is extremely challenging to directly provide proof of emotions (e.g. Mendl et al., 2009; Proctor, 2012). Understanding fully what animals experience is not certain, but accumulated evidence supports that animals have a level of sentience (e.g. Darwin, 1872, Dawkins, 2006, Low et al., 2012, Proctor et al., 2013). This would include the precautionary principle which should be applied when evidence of sentience is inconclusive (Birch, 2017), and this is reflected in law, for example, the Animal Sentience Bill which will underpin the Government's 'Action Plan for Animal Welfare' (UK Government, 2021; Lawrence and Vigors, 2020).

Definitions of key terms

The following section is to clarify the meaning of key terms that will be used in the rest of this review.

'Positive welfare' has recently been discussed as referring to either a welfare state arising from the outcome of likes and wants, or an overall state of welfare that results from the balance between positive and negative experiences (Rault et al., 2020). In this thesis, positive welfare will refer to the latter, that when positive experiences outweigh negative experiences, the overall result is a positive welfare state (Rault et al., 2020).

'Emotion' is a term used for processes (e.g. behavioural, physiological and neural) that evolved to allow the animal to avoid harm and seek out valuable recourses for survival (Paul et al., 2005). Emotions in human psychology have a conscious, subjective component, where we can be aware of the emotion, i.e. how it 'feels' (Paul et al., 2005). Emotions are hypothesised to serve to guide an animal's behaviours in order to survive, such as fear and fleeing behaviours (Mendl et al., 2009).

‘Emotional experiences’ may be defined as subjective states of emotion that are valenced, so they can be positive or negative (Mendl et al., 2010).

For years it was argued that there are ‘basic categories of emotion’ which suggested that one can feel an emotion, such as feel fear or feel happiness, but they are separate from each other and to some extent are binary (Russell and Barrett, 1999). Discrete emotions are event- or object-focused, arising in response to a stimulus and possibly serve to mediate a behavioural response that will secure the animal’s immediate survival (Paul et al., 2005). However, there is an alternative theory that suggests that emotions vary along dimensions, for example, the intensity of an emotion is not always the same; one can feel happy and excited, or happy and content, and these two states would differ in the level of arousal (Fig.1; Russell and Barrett, 1999). This theory relates to ‘affective states’. ‘Affect’ is considered to refer to the valence (e.g. pleasantness or unpleasantness) and intensity (arousal) of an emotional state (Fig. 1; Paul et al., 2005).

Emotions or emotional states tend to reference states that ‘are attached in some way to an object’ and are temporally short-lived, whereas affect or affective states can be viewed as ‘moods’, they are longer-lasting and not attached to an object (Paul et al., 2005). The clear distinction between emotional and affective states is often debated, therefore for the purposes of this review, the terms ‘emotion/ emotional states’ and ‘affect/ affective states’ will be used synonymously (de Vere and Kuczaj, 2016; Kremer et al., 2020).

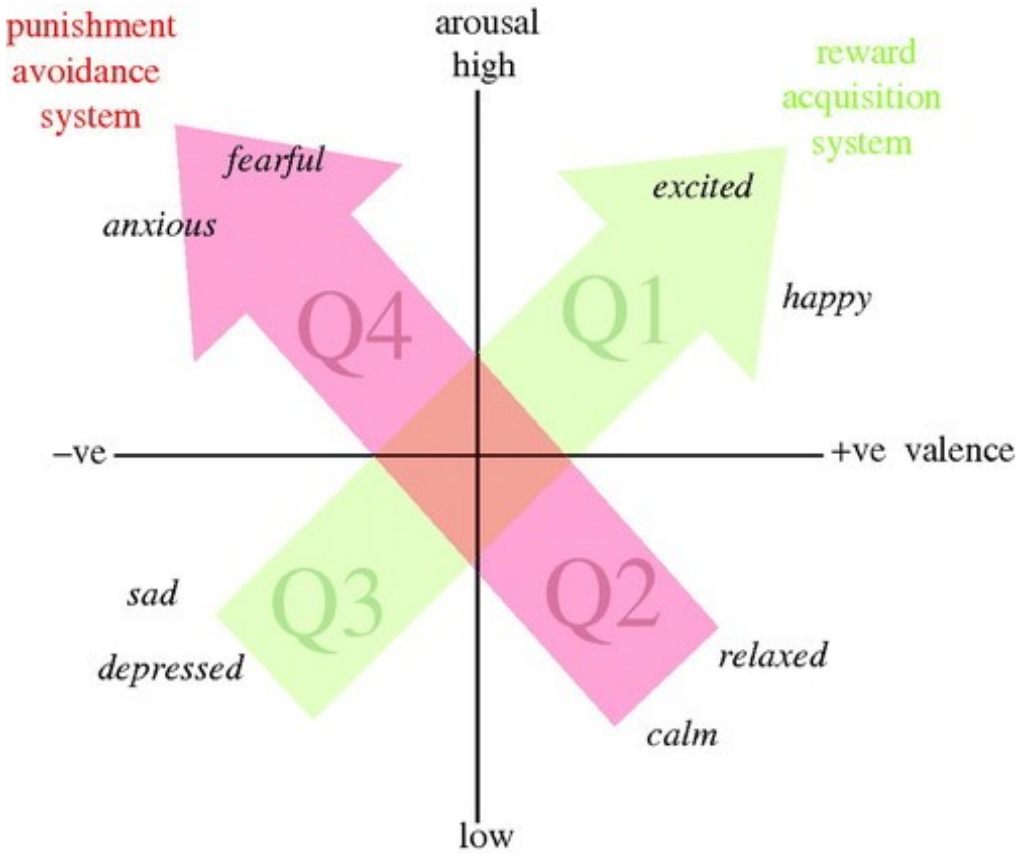

Figure 1. Core affect presented on a two-dimensional axis of arousal (low to high) and valence (positive to negative).
In italics are possible affective states and where they would lie on this axis given their valence and arousal. Image taken from Mendl et al., 2010.

1.1.1 Positive welfare

Positive welfare is thought to have arisen as a reaction to the focus on negative states in animal welfare science for many years (Lawrence et al., 2019). An example of focusing on negative states may be the Five Freedoms, four of which specify "freedom from" negative stimuli such as pain, thirst/hunger, discomfort, fear, disease (Brambell, 1965; FAWC, 2013; McCulloch, 2013). Even the "Freedom to express normal behaviour", which may have the potential to provide an animal with the opportunity to have positive experiences (Phillips, 2008), is underpinned by the objective to avoid a negative (i.e. frustration; Lawrence et al., 2019). While the Five Freedoms have had an important impact on animal welfare from the legislation and welfare standards which are based upon them (McCulloch, 2013), it is argued that the absence of negative states does not automatically result in the animal having positive welfare (Phillips, 2008; Yeates and Main, 2007). Therefore,

inclusion of indicators of positive states in welfare standards have been called for (Yeates and Main, 2007). Yeates and Main (2007) give different positive outcomes which may imply or have the potential to induce positive affect: for example, play, sleep, thermal comfort, companions, space, opportunity. By including positive states in welfare standards, it allows for not just satisfying some essential needs (e.g. feeding), but it also allows for positive experiences which are species (and perhaps individual), time and context specific and so add something extra to the life of the animal rather than simply alleviating negatives (Yeates and Main, 2007).

Edgar et al. (2013) argue a similar point, that a ‘good life’ is when the quality of life exceeds the minimum legal requirements which usually rest on the Five Freedoms. The authors tested a framework with which to evaluate positive welfare on laying hen farms (Edgar et al., 2013). Each farm was scored on the resources available for the hens to have a ’good life’, which included, comfort within their environment, pleasure (e.g. food enrichment and play), positive social interactions, environmental and cognitive enrichment and positive health based on the FAWC report (2009) (Edgar et al., 2013). This study showed that it was possible to evaluate positive welfare criteria via a resource input approach in different laying hen systems, and to give farms a tier system through which they could progress towards the best welfare for their animals (Edgar et al., 2013).

Vigors and Lawrence (2019) assessed the perspective of livestock farmers on positive welfare in a qualitative interview study. It was found that the farmers in this study had welfare indictors that they tried to provide directly through their management practices (linked to the needs of their animals), and others (such as play) which happened indirectly of management practices (Vigors and Lawrence, 2019). Farmers would use play as a welfare indicator, and that evidence of play signified overall animal wellbeing and good welfare (Vigors and Lawrence, 2019). Contentment was the most frequently mentioned positive affective state (Vigors and Lawrence, 2019). Many of the farmers considered their job was to minimize negative experiences, and

positive ones would occur indirectly of management practices or by the animal themselves (Vigors and Lawrence, 2019). The relationship between positive and negative affective states is complex; affective states have been argued as being mutually exclusive if they are differently valenced, but emotions of the same valence can co-occur (Vansteelandt et al., 2005). However, independence of positive and negative affect has been shown in humans in some instances (e.g. Diener and Emmons, 1984; Goldstein and Strube, 1994), while they can co-occur in others (e.g. Larsen et al., 2017, for review, see Russell and Carroll, 1999 and Larsen and McGraw, 2014). In animals, measuring the relationship between positive and negative affect is largely unknown (Clarkson et al., 2020), likely due to the challenge of developing and validating measures, such as ear and tail positions (e.g. in sheep Reefman et al., 2009, and in pigs, Reimert et al., 2013) and vocalisations (e.g. in elephants, Soltis et al., 2011, in goats, Baciadonna et al., 2019, and in rats, Brudzynski, 2007) to directly compare between valenced states. The next section will explore affective states in animals in more detail.

1.1.2 Affective states

The majority of research carried out into emotion and animal welfare has tended to focus on negative affective states (e.g. fear, pain, stress; Paul et al., 2005; Boissy et al., 2007). However, positive affective states are beginning to receive more attention in both animal welfare research and in behavioural neuroscience (Burgdorf and Panksepp, 2006; Boissy et al., 2007; Mendl et al., 2009; Mellor, 2015; Webb et al., 2018). There are four main features defining the literature on positive animal welfare: positive emotions, positive affective engagement (the rewarding nature of engaging in motivated behaviours), quality of life and happiness (Lawrence et al., 2019). In particular, positive emotions have played a central role in the development of positive animal welfare research to date, possibly because evidence for positive emotions in animals is necessary to vindicate positive animal welfare (Lawrence et al., 2019). Expression of behaviours in which the animal is genetically pre-programmed and highly motivated to engage, such as nest building in pregnant sows (Jensen, 1993; Spoolder et al., 1995), may result in

inducing positive affective states in the animal as they are responding to the motivation to partake in a rewarding behaviour (i.e. positive affective engagement; Mellor, 2015). By promoting positive affective states, it may be possible to enhance the welfare of the animal (Mellor, 2015), helping them to have lives worth living (FAWC, 2009) rather than predominantly focusing on reducing negative affective states (Mellor, 2016).

Affective states are difficult to assess and measure in animals because of an absence of verbal communication (Boissy et al., 2007). By developing tests to induce affective states, it may be possible to measure affective states, for example, by taking behavioural and neurobiological indictors (Boissy et al., 2007; Mendl et al., 2010). In the next two sections I will discuss some behavioural and neurobiological indicators.

Behavioural measures of affective state

Rodent behavioural measures of affect often allow for the study of both positive and negative states, with a simple example being approach and avoidance behaviours conveying the valence of a stimulus (Paul et al., 2005; for review, see Kremer et al., 2020). For negative affective states, relevant rat behaviours include freezing, aggression, fleeing (Paul et al., 2005). A recent study in mice showed that an aversive handling method, being lifted by the tail, increased negative affective states shown through behavioural measures in the elevated plus maze and open field test (Clarkson et al., 2020). They also showed that tail-handling impacted reward by making them less resilient to the loss of a reward of sucrose, but it did not impact their reaction to reward gain, suggesting tail-handling did not influence positive affect (Clarkson et al., 2020).

Grimace scales in rodents and other animals use changes in facial expression to determine the level of pain being experienced in response to aversive stimuli (Mogil et al., 2020). The use of facial expressions as a measure of pain started out in human neonates (Grunau and Craig, 1987) and since then grimace scales have been developed for 10 mammalian species, the first of which was the mouse (Langford et al., 2010). The Rat Grimace

Scale followed in 2011 which featured four action units within the scale: Orbital tightening (closure of the eye), nose/cheek flattening (bulging of the nose and cheek), ear changes (the fold, curl and angle of the ears), and whisker change (position of the whiskers; Sotocina et al., 2011). These grimace scales are now widely used as pain assessment tools, although a limitation of these scales is that multiple negative affective states can influence facial expressions in similar ways. For example, aggression and fear both result in orbital tightening and flattened ears in mice (Defensor et al., 2012; Mogil et al., 2020).

For positive affective states, a good example of a behavioural measure lies with rats and the production of ultrasonic vocalisations (Knutson et al., 2002). Rats spontaneously emit vocalisations in a number of different contexts, for example, during play (Knutson et al., 1998), and sexual behaviours (McIntosh et al., 1978). Ultrasonic vocalisations made at specific frequencies are thought to be indicative of affective states, for example vocalisations produced in response to fearful stimuli, or to rewarding stimuli (Knutson et al., 2002). Other behaviours that are used as indicators of positive affective states include play behaviours (Held and Špinka, 2011), exploration and bond-formation (Mellor, 2012; Mellor, 2015b). Finlayson et al. (2016) identified facial indicators of positive emotional states following a heterospecific play paradigm. Similarly to the Rat Grimace Scale (Sotocina et al., 2011), orbital tightening, nose/ cheek flattening, ear position and whisker position were used as units, and also included with the addition of ear colour (pinker/ more flushed), nictitating membrane (visibility of the membrane at the front of the eye), ear angle, and eyebrow measurements (Finlayson et al., 2016). Out of these measures, Finlayson et al. (2016) found that ear colour and ear angle changed significantly in response to the positive treatment, while the other measurements did not differ between treatments. These findings suggest that facial expression may indicate positive affective states in rats, although this requires validation (Finlayson et al., 2016).

Cognitive bias tests (Kremer et al., 2020), conditioning and operant tasks (Mendl et al., 2009) allow for the underlying neurobiology and

neurochemical substrates of affective states to be studied (Mendl, Burman and Paul, 2010). Affective states may also be measured using preference testing (Dawkins, 1980), through which the choices of animals and their motivation to access certain rewards are used to indicate the affective state of the animal. Qualitative behavioural assessments can also be used, by studying the behaviour of an animal in a qualitative manner and relating these behaviours to the affective state of the animal (Wemelsfelder, 2008).

Neurobiological measures of affective state

Neurobiological measures are used to assess emotional states. For example, in the study of the effect of stress on emotional states and the effect on the neuroendocrine system, the hypothalamic-pituitary-adrenal (HPA) axis, by measuring corticotrophin releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and cortisol (or corticosterone) levels (e.g. Weiss et al., 2004; Paul et al., 2005, Boissy et al., 2007). However, many neurobiological measures tend to reflect negative rather than positive emotional states (Paul et al., 2005). The autonomic nervous system, receiving information from limbic structures in the brain, influences aspects of an animal's physiological state, including homeostasis, in response to affective states, for example heart rate as an index of the response to a stressor (Boissy et al., 2007). Such physiological measures of positive affective states in animal studies are less numerous than with negative affective states and therefore more research is needed on potential physiological measures of positive affect (Boissy et al., 2007).

Neurobiological techniques, including imaging (e.g. Knutson et al., 2014), electrical stimulation (e.g. Burgdorf et al., 2000), pharmacological manipulations (e.g. Burgdorf et al., 2007), chemogenetics (e.g. Massaly et al., 2019) and optogenetics (e.g. Wahis et al., 2021), allow us to study which brain regions and circuits produce different emotional responses to specific stimuli, elucidating the neural correlates underpinning emotional processes (Mendl et al., 2009). For many years it was argued that there are discrete emotional systems that are embedded in specific regions of the brain (Paul et al., 2005),

for example the fear system and the amygdala and periaqueductal grey (Panksepp, 1998a). However, there is growing evidence that neural networks spanning multiple brain regions have a coordinated role in generating emotional states, for example the limbic system (for review see Boissy et al., 2007, Paul et al., 2020 and Zych and Gogolla, 2021). Positive emotional states, like reward and appetitive motivation, are considered to arise from the mesolimbic reward system which comprises the nucleus accumbens, ventral tegmental area and medial prefrontal cortex (e.g. Boissy et al., 2007). There is also evidence that positive and negative affective states may arise from specific neuronal populations within a discrete brain region (e.g. in song birds, Goodson and Wang, 2006; in humans, Sieger et al., 2015; in mice, Beyeler et al., 2018). A recent study found that there were positive-valence and negative-valence neuronal sub-populations in the basolateral amygdala (Zhang et al., 2021). These distinct neuronal types regulate positive and negative reinforcement in the response to appetitive and aversive stimuli in mice (Zhang et al., 2021). They also found that these neuronal types send the reward and punishment information through projections to the ventral striatum (the olfactory tubercle and nucleus accumbens, respectively; Zhang et al., 2021).

Affective states tend to be studied by using discrete emotions that are induced by a specific stimulus, and behavioural, neurobiological and physiological responses measured as indicators of affect (Paul et al., 2005; Webb et al., 2018). I discuss the neurological basis of affect further in Section 1.4.

1.2 Social behaviours

Social behaviours are thought to have arisen from the benefit to the survival if the individual of living in social groups as opposed to living alone (Hamilton, 1971; Ward and Webster, 2016). Pro-social behaviour can be defined as an action performed by an animal to the benefit of others, such as parental care, group affiliation, bond-formation and play (Rault, 2019). Social animals that perform these behaviours naturally derive benefit from such behaviours (Rault, 2019). For example, social species have been known to

show better recovery from stressful experiences when they are with other conspecifics (social buffering), found in rodents, birds and primates (Kikusui et al., 2006). On exposure to a stressor, the presence of a conspecific has been shown to decrease the activity of the HPA axis (Hennessy et al., 2009).

The absence or presence of prosocial behaviours may be important in welfare assessments as they may reflect the conditions in which the assessments are occurring, where an absence of prosocial behaviours may reflect a stress response or presence of prosocial behaviours can be used as positive welfare indicators (Rault, 2019). Prosocial behaviours may also help to improve welfare; a number of studies found improvements in physiological measures when the animal could partake in a social behaviour, for example, allogrooming in dairy cattle resulted in lower heart rate (Laister et al., 2011); pigs that received social nosing had a higher growth rate than pigs that received no social nosing (Camerlink et al., 2012); and female rats that affiliated with conspecifics in a more reciprocal manner had a lower circulating corticosterone peak in response to an acute stressor and lived for longer in a lifespan study (Yee et al., 2008).

Play is one of the earliest prosocial behaviours to emerge after mother-directed social behaviour (Vanderschuren et al., 1997) and in many mammalian species is easily recognizable and quantifiable (Vanderschuren and Trezza, 2013) which makes it a useful tool for studying prosocial behaviours and positive welfare.

1.2.1 Play

Play is seen in a wide variety of mammalian species, mainly occurring in juvenile animals (Northcutt and Nwanko, 2018; Burgdorf and Panksepp, 2001). Play consists of exaggerated, incomplete movements (often in patterns) which are repeated in a non-stereotyped way (Poole and Fish, 1976; Vanderschuren and Trezza, 2013). Burghardt (2010) described play by five categorical criteria: it is does not completely contribute to the current survival of the individual; it occurs spontaneously and the individual engages in play

voluntarily; it differs from other behaviours in that it can be exaggerated, incomplete and involved specific behavioural patterns; play behaviours are performed repeatedly but not in a stereotypical way; and it is initiated in the absence of negative states such as stress or pain.

It has long been suggested that there is a link between welfare and play (Held and Špinka, 2011). Play is engaged in voluntarily and tends to occur in non-life threatening conditions, for example in the absence of pain, so it is thought to indicate a positive affective state (Held and Špinka, 2011; Lampe et al., 2017; Burghardt, 2005; Pellis and Pellis, 2007). Fraser and Duncan (1998) argued that play is a behaviour that is considered to be low cost to the animal; it is observed when the other needs of the animal have been met and is often suppressed in poor conditions, such as food deprivation (Held and Špinka, 2011; Siviy and Panksepp, 1985).Thus when play is observed may indicate a high level of well-being in the animal (Lawrence, 1987). There is a motivation to play likely as it has a low fitness cost to the individual, which results in promoting a positive motivational affective state (Fraser and Duncan, 1998). Play may not only be as a result of good welfare, but it may also actively lead to an improvement in welfare due to its 'contagious' nature, where play in one individual can lead to more play within the group (Held and Špinka, 2011). Therefore, better understanding play may have applications for welfare assessments and improvements in captive animals (Held and Špinka, 2011).

There are three main subcategories of play: solitary play, object-directed play and social play (Vanderschuren and Trezza, 2013). Object-directed play is not of particular relevance to this review and so will not be included. While play has been studied across a number of different species, this review will focus on play in the laboratory rat, relating to the welfare of rats used for scientific experimentation.

Solitary Play in rats

Solitary play in rats is also termed locomotor or locomotor-rotational play and refers to spontaneous movements (Pellis and Pellis, 1983), such as

running, leaping, pirouetting, that are exaggerated forms of normal locomotor movements and are made with no obvious goal (Wilson and Kleiman, 1974). Pellis and Pellis (1983) describe rat locomotor-rotational play to be made up of 5 main types of movement: ‘running’ (galloping and changing directions frequently); ‘forward jumps’ or ‘galloping hops’ (hops made either in isolation or as part of the galloping/running);‘up jumps’ (upon landing, all four paws contact the ground at the same time); ‘jerk (jump/rotation)’ (‘a jerky, jumping motion’); ‘anterior jerky rotation’ (movements of the head, neck and upper thorax will sniffing the environment;Pellis and Pellis, 1983). These different solitary play movements can be combined and occur simultaneously (Wilson and Kleiman, 1974), such as scampering behaviours which comprise running rapidly and performing hops (Melotti et al 2014; Lampe et al., 2017). These locomotory play elements can also occur in different frequencies and contexts (Pellis and Pellis, 1983). Animals may repeatedly come back to the stimulus which initiated the play, distinguishing these play behaviours from behaviours with which the animal moves away from negative stimuli (Wilson and Kleiman, 1974). While this form of play, as the name may suggest, may occur when the animal is in the absence of conspecifics, this is not always the case as olfactory and visual cues from conspecifics can result in solitary play behaviours being performed (Wilson and Kleiman, 1974). Solitary play can also occur between social play bouts (Pellis and Pellis, 1983) but these two play types seem to be unrelated (Melotti et al., 2014). Melotti et al (2014) used a play-in-pairs test with juvenile male rats, finding that solitary play was unrelated to the duration of social play bouts or the quantity of social play in which the rat engaged. The authors compared the frequency of scampering behaviours and the number of pinned-and-being-pinned behaviours (pinning is where one rat rolls onto its back in defense and is held down by its playmate who attempts to make contacts with the nape of the neck of its playmate), finding that social play in the home cage occurred more often than solitary play (Melotti et al., 2014). Lampe et al. (2017) similarly found that in a play-in-pairs test pinning (a social play behaviour) was not correlated with scampering. This may present supporting evidence for different types of play stemming from independent

evolutionary origins and serving different functions in the development of the animal (Burghardt, 2005; Melotti et al., 2014; Lampe et al., 2017).

Social play in rats

Social play (play directed towards others;Pellis and Pellis, 2007) is one of the most extensively studied forms of play in animals (Burghardt, 2005). Social play is observed in rats (Small, 1899; Northcutt and Nwanko, 2018) and it mainly takes the form of rough-and-tumble play (Pellis and Pellis, 2007) which has a complex structure, made up of multiple behavioural elements (Panksepp, 1979; Panksepp and Beatty, 1980). In rough-and-tumble play, one rat performs initiation contacts to the back of the neck of the play mate (Vanderschuren and Trezza, 2013). The solicited rat can respond to this by rolling over onto its dorsal aspect and is pinned by the other rat (Vanderschuren and Trezza, 2013). The supine rat can then attempt to make a contact with the nape of the neck of the playmate and initiate another bout of play, and so the play partners alternate between pinning their partner and being pinned (Vanderschuren and Trezza, 2013; Vanderschuren, Niessink and Van Ree, 1997). It is thought that pinning is a good indicator of social play in rats (Panksepp, 1979; Panksepp and Beatty, 1980) and that the initiation ‘pouncing’ behaviour represents a solicitation to play (Vanderschuren and Trezza, 2013). Both pinning and pouncing are discrete behaviours that are possible to count (Fig. 2) and they occur repeatedly when young rats are playing; therefore, these characteristic behaviours represent a reliable measure of when play is occurring (Panksepp and Beatty, 1980).

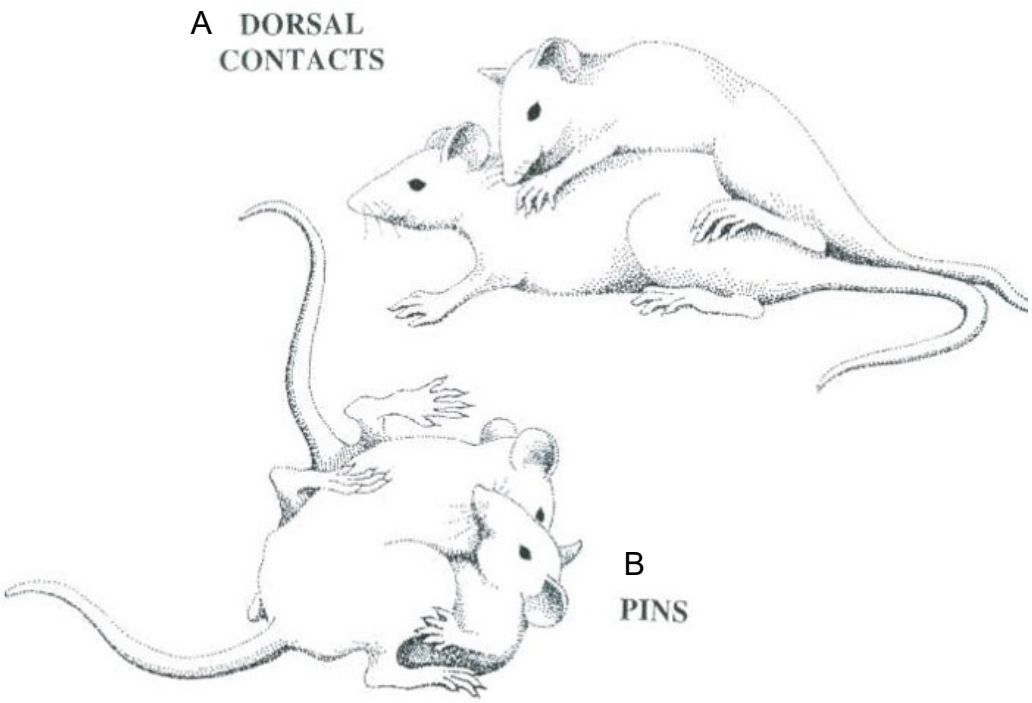

Figure 2. Rat rough-and-tumble play behaviour.
(**A**) Dorsal contact; (**B**) Pin (Reproduced from Panksepp, 1998b)

In rats, rough-and-tumble play begins to emerge pre-weaning (postnatal day 17-19) andrats increasingly engage in social play up to a peak at around postnatal day 28-40 where it declines as animals approach sexual maturity (Vanderschuren and Trezza, 2013). In juvenile rats, the structure of social play most commonly includes pouncing, standing defense, boxing with paws, initiation contacts to the nape of the neck, supine lying and pinning (Vanderschuren and Trezza, 2013). At the onset of play, standing defense is commonly observed, which around the time of weaning, shifts to supine lying, resulting in pinning, becoming the most frequent response to play initiation (Vanderschuren and Trezza, 2013).

The immediate and long-term benefits of play

Solitary (locomotor) play may serve to develop motor skills required in adulthood as it mimics behaviours such as escaping from predators (Byers and Walker, 1995). Whereas, social play in rats is thought to be important in

cognitive development, for example, task-learning and decision-making (Baarendse et al., 2013), emotional development, for example, anxiety (Einon and Morgan, 1977), and social development, for example, preparing animals for adult behaviour such as aggressive or sexual behaviours (Auger and Olesen, 2009). In studies where play is suppressed during development, rats go on to show abnormal patterns of social, sexual and aggressive behaviours (Vanderschuren et al., 1997), so play may function to 'train' adolescent rats in adaptive social behaviours observed in adult animals (Auger and Olesen, 2009). A number of studies have investigated the effect of rearing in social isolation, isolating rats between postnatal day 20 up to adulthood (Vanderschuren and Trezza, 2013). A number of negative effects on social development have been reported, including rats showing lower levels of social approach (Hol et al., 1999) and social exploration (Van den Berg et al, 1999), poor coping with territorial aggression as adults (Von Frijtag et al., 2002) and a general increase in aggressive behaviours (Meng et al., 2010). A similar detrimental effect of social rearing in juvenile rats (between postnatal days 21-25) upon aspects of cognitive development are found. For example, Einon et al. (1978) found that rats were slower to investigate novel objects in an open field test. Similarly, Baarendse et al. (2013) observed abnormal responses in a multiple choice serial reaction time task. Social isolation during adolescence may effect emotional development in rats, resulting in increased anxiety-like behaviours in elevated plus maze tests and open field tests (Wright et al., 1991; Da Silva et al., 1996; Leussis and Andersen, 2008; Lukkas et al, 2009).

Restriction of social contact with conspecifics during a period when social play is most abundantly observed in juvenile rats may have a negative impact on social, cognitive and emotional development (Vanderschuren and Trezza, 2013). Less is understood about potential short-term benefits of play, but it may include helping an animal to glean information about its environment or group members, or decreasing social stress (Held and Špinka, 2011). For captive animals, play may serve to improve their welfare by honing skills such as stress resilience and emotional flexibility (Held and Špinka, 2011).

Differences in play

Play is observed in both female and male rats (Pellis et al., 1997). However, given the putative role of play in behavioural and neural development, it may be expected that play would differ between sexes given their different functional behavioural repertoire (Pellis et al., 1997; Vanderschruchen and Trezza, 2013). There are indeed known sex differences in rough-and-tumble play in rats (Pellis et al, 1997). Females exhibit play, although this differs in structure, duration and frequency compared to males (Poole and Fish, 1976; Schwarting, 2018). Male rats play more than females, which may be the result of higher levels of play initiation in males (Auger and Olesen, 2009). Male and female rats also play together, although both males and females may play more with males so females are likely to be less involved in social play (Poole and Fish, 1976). The structure of play is sexually dimorphic, with males tending to make more pins than females and females being more likely to be pinned (Auger and Olesen, 2009; Pellis et al., 1997). Pellis and Pellis (1983) report that male and female rats aged between 20-25 days old exhibited similar solitary play trends, suggesting there was no effect of sex on solitary play. The neural basis for the sexual dimorphism in play in rats remains to be fully elucidated.

Play can differ between strains of rat (Himmler et al., 2014). Wistar, Long-Evans and Brown Norway strains have been found to engage in high levels of play behaviour. However, the structure of play, mainly the tactics to promote body contact in play, differed between strains (Himmler et al., 2014). These strain differences suggest there is a genetic component to the phenotype of play (Siviy, 2020), as with other behaviours such as maternal care (Pan et al., 2018). Similarly, differences in play are often observed between individuals, with studies describing ‘high play’ and ‘low play’ individuals (Pellis and McKenna, 1992). Highly playful individuals may play more consistently in different contexts and at different ages, they may have different intrinsic motivations to play (Pellis and McKenna, 1992; Lampe et al., 2017).

Rough and tumble play is found to be age-dependent; it is more abundant in juveniles, increasing from 18 (pre-weaning) - 28 days old, peaking between 32 - 40 days old and declining after 40 days of age (post-puberty) (Panksepp, 1981; Pellis and Pellis, 1990; Auger and Olesen, 2009).

Play in rats is a highly complex behaviour that is sensitive to several variables, such as sex (Pellis et al, 1997), age (Panksepp, 1981) and environmental conditions, for example, food availability (Siviy and Panksepp, 1985). This needs to be taken into account when using play as a measure of affective state. There is a growing interest in when and how animals may experience positive affect, such as pleasure (Boissy et al., 2007). Following several years of studying play and reward-induced 'chirping', the 'tickling' paradigm was developed by Panksepp and his lab in approximately 2000 and has become a technique that is used to study positive affect in rats (Panksepp and Burgdorf, 2003).

1.3 The tickling rat model

'Tickling' is a complex behavioural phenomenon; in humans the behavioural response has stereotypic, reflex-like properties (Harris, 2012). Notably, in humans the tickled individual usually exhibits laughter as well as struggling to escape or fight off the person doing the tickling (Harris, 2012). There is also the interesting element that we cannot tickle ourselves, and therefore tickling may require a social stimulus (Provine, 2004; Harris, 2012). There are two forms of tickling that have been described: knismesis and gargalesis (Harris, 2012). Knismesis refers to a light tickling sensation which can be elicited anywhere on the body and is unlikely to result in laughter (Harris, 2012). Gargalesis, on the other hand, refers to a repeated, heavier movement directed at certain parts of the body, such as the ribcage or armpits, and causes laughter (Harris, 2012). Along with the ability to evoke laughter, another fascinating distinction between these two forms of tickling is that humans can induce knismesis in themselves, while gargalesis (resulting in laughter) is almost impossible to carry out to yourself (Harris, 2012).

In animals, knismesis is observed across mammalian species, with a typical behavioural output being scratching in response to very light touch (Harris, 2012). Gargalesis is less common; it is observed in some great apes (Ross et al., 2010), for example chimpanzees tickle a playmate during rough and tumble play and this results in human-like laughter (van Lawick-Goodall, 1968). Tickling in rats refers to gargalesis, whereby a human hand serves to mimic the contacts made in conspecific rough-and-tumble play in rats, while also making rapid, heavy finger movements on the nape of the neck and ventral side of the rat, eliciting a specific call profile that has been equated to laughter (Panksepp and Burgdorf, 1999 and 2000). This can also be referred to as heterospecific play (Panksepp and Burgdorf, 2000). Panksepp and Burgdorf developed the technique of tickling rats and set out to establish if this heterospecific playful social contact was rewarding to juvenile rats. Their original tickling protocol was to expose rats to two minutes of a tickle test, consisting of four cycles of 15 seconds of release where the rats received no stimulation, followed by 15 seconds of tickling. Tickling was carried out by making rapid finger movements across the dorsal aspect of the rat, with particular focus on the nape of the neck, and turning the rats onto their backs, pinning them and tickling their ventral surface for a few seconds before releasing them again. This was repeated throughout the two minute tickling test. (Panksepp and Burgdorf, 2000)

Since the initial study by Panksepp and Burgdorf (2000), numerous other studies have utilized tickling, and a variety of methods for tickling are now reported in the literature (LaFollette et al., 2017). Most commonly used is the original method described above by Panksepp and Burgdorf (2000), but there are variations in the session duration (from one minute; Cloutier et al., 2008) to 10 minutes (Schwarting et al., 2007) or number of days of tickling (e.g. one, three and five days, LaFollette et al., 2018; 10 days, Hammond et al., 2019). Rats are mostly tickled in a test arena, although some studies do tickle rats in their home cage (e.g. Schwarting et al., 2007), and tickling usually is carried out individually rather than in groups (LaFollette et al., 2017).

In terms of the strains of rats used in tickling studies, the most commonly used are outbred Long-Evans Hooded, Sprague- Dawley and Wistar, possibly because these strains abundantly produce ultrasonic vocalisations in response to tickling (LaFollette et al., 2017; Wright et al., 2010; Schwarting, 2018). Tickling is mostly studied in juvenile rather than adult animals, usually between weaning at around 21 - 23 days old up to puberty at around 45 days old (Burgdorf and Panksepp, 2001; LaFollette et al., 2017), tickling is found to be robustly rewarding in rats at this age (Cloutier et al., 2018; Cloutier et al., 2012). Some studies have investigated tickling in adult animals (e.g. Panksepp and Burgdorf, 2000) finding adult rats do respond to the tickling stimulus but this declines as a function of age and is substantially reduced when animals are well into adulthood (Panksepp and Burgdorf, 2003).

Male rats have predominantly been used in tickling experiments, which is similar to other biomedical research where there has long been a bias to using males in research (Beery and Zucker, 2011). In a review by Lafollette et al. (2017), the authors highlight that out of 32 papers that evaluate outcomes of tickling rats, 25% used both males and females in their experiments, and only one article used just female animals. Therefore, female rats have thus far been under-represented compared to males in tickling studies.

There is growing evidence that tickling is a useful paradigm for investigating and measuring positive affective states in rats (LaFollette et al., 2017). As with conspecific play, anxiogenic conditions suppress a rat's tickling responses (Panksepp and Burgdorf, 2003). Studies investigating the relationship between tickling and negative affective stimuli, such as foot shocks, bright light, the scent of a predator (e.g. cat), have found this to reduce the tickling response (Panksepp and Burgdorf, 1999). Mällo et al. (2007) found that tickling had an anxiolytic effect in both female and male rats, tickling lead to an increase in activity in an elevated plus maze and exploration box test. Similarly, it has been found that juvenile rats find tickling rewarding (Panksepp and Burgdorf, 2003). Burgdorf and Panksepp (2001) demonstrated that tickling of adolescent rats was reinforcing: tickling established a robust place

preference in the rats and was found to serve as a reward for the acquisition of an operant lever-pressing task. This suggests that tickling is a positive experience for juvenile rats (Burgdorf and Panksepp, 2001).

The response to tickling follows a similar age-related trajectory as social play: tickling declines when the animals are well into adulthood (at least 70 days of age; Panksepp and Burgdorf, 2003) and rough-and-tumble play peaks between 30 - 40 days old (Lampe et al., 2017) before declining into adulthood (Vanderschuren and Trezza, 2013). This suggests that if tickling can be useful for inducing positive states in rats, it may be most important during adolescence, however, this is yet to be fully established (e.g. Panksepp and Burgdorf, 2003).

1.3.1 Measuring the response to tickling

Behavioural measures can be used to assess the affective response to tickling including approach behaviours, or hand-following, where the rat makes forward motions, seeking out the hand that has been tickling it (Fig. 3; Burgdorf and Panksepp, 2001). Hammond et al. (2019) report that tickled male juvenile Wistar rats had a significantly longer duration of hand-following by the end of the 10 day testing period than control rats and cage mates of tickled rats also exhibited more hand-following than the cage mates of non-tickled control rats. This suggests that not only was tickling rewarding for the rats as they expressed a motivation to be tickled, but also that this may have had a contagious effect on their cage mates, although the authors question whether approach tests are sensitive enough to discriminate between whether the rats found tickling rewarding (i.e. pleasurable) or whether they increased arousal (i.e. curiosity; Hammond et al., 2019).

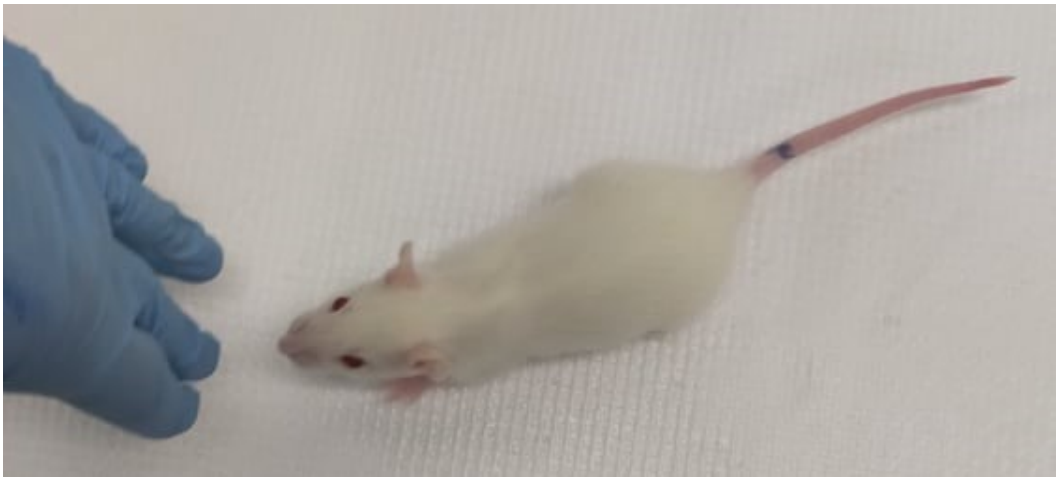

Figure 3. An example of a rat approaching the hand that has been tickling it.

The time taken to first make an approach towards the hand, otherwise called approach latency, can be used as a measure of whether tickling was positively reinforcing for the rat (Burgdorf and Panksepp, 2001). Burgdorf and Panksepp (2001) measured approach latency by placing the rat in a corner of the testing box, facing the experimenter's hand, which was across the diagonal to the rat. The time taken for the rat to touch the experimenter's hand with its head or front paws was recorded, with a maximum latency set to 30 seconds (Burgdorf and Panksepp, 2001). After an approach was made or the maximum latency reached, the rat received a 15 second bout of tickling, followed by 15 seconds of no stimulation, after which approach latency was measured again and this was repeated three times (Burgdorf and Panksepp, 2001). Burgdorf and Panksepp (2001) found that, when comparing tickling stimulation to a light touch (repeated, gentle stroking of the animal's dorsal surface) control group, tickled animals were significantly faster to approach the hand, suggesting that tickling had been more reinforcing than just a light tactile stimulus.

Solitary play, as previously discussed, consists of spontaneous locomotor movements, such as running, leaping and pirouetting (Vanderschuren and Trezza, 2013). Scampering (a solitary play behaviour consisting of rapidly running alone and performing hops) has been used as an outcome of social play and tickling (Lampe et al., 2017; Melotti et al., 2014; Hammond et al., 2019). Hammond et al. (2019) found that tickling increased

the amount of scampering performed before handling in the home cage (either tickling or control handling where the experimenter's hand was motionless in the centre of the arena) was carried out, while tickling did not affect the amount of social play. This may suggest that solitary play could be an anticipatory behaviour for being tickling; Hammond et al. (2019) postulate that the amount of solitary play may reflect the reward of expecting to be tickled.

1.3.2 Ultrasonic vocalisations

Rats emit different types of vocalisation, both audible and ultrasonic, that are distinct and are produced during various social situations (Burgdorf and Panksepp, 2001). Of these, two types of ultrasonic vocalisations (USVs) – 22 kHz and 50 kHz - are thought to reflect certain emotional states in juvenile and adult rats (Simola and Brudynski, 2018; Burgdorf and Panksepp, 2001).

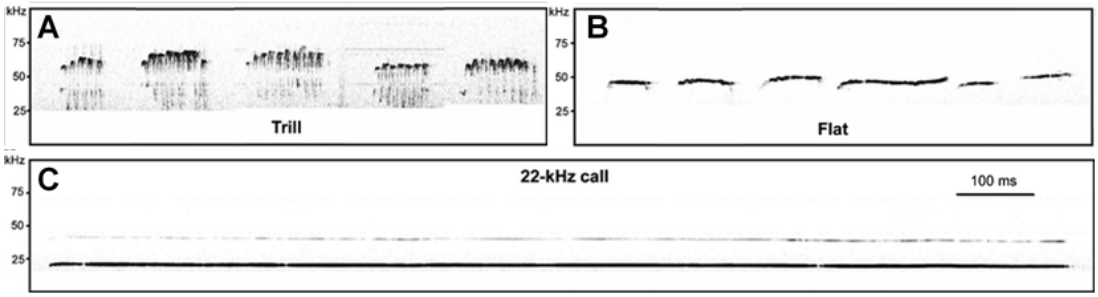

Figure 4. An example of ultrasonic vocalisations in the rat.
Trill 50 kHz USVs (A); flat 50 kHz USVs (B); and 22 kHz USVs (C). (Adapted from Wright et al., 2010)

22 kHz USVs

USVs in the narrow range of 20 - 23 kHz, known as 22 kHz USVs, are produced during painful or aversive stimuli, such as foot shock and social isolation, and are thought to indicate negative states (Brudzynski, 2009; Simola and Brudynski, 2018). They are characteristically flat calls with a long duration ranging from 100ms - over 3000ms (Fig. 4; Brudzynski, 2009).

22 kHz USVs are strongly associated with aggressive social interactions, such as an intruder male rat introduced to the home cage of another male rat resulting in the emission of 22 kHz USVs (Panksepp et al., 2004). Similarly, exposure to a predator resulted in rats showing high levels of

22-kHz calling (Blanchard et al., 1991). Studies have shown that rats produced more 22 kHz USVs during and for 30 minutes following the presence of a cat when they were in a group with other conspecifics than if they were exposed individually to the cat; individual rats showed low or no 22 kHz calling (Blanchard et al., 1991). This suggests that these calls are facilitated by the presence of conspecifics and may have the function of being alarm calls (Blanchard et al., 1991).

50 kHz USVs

USVs in the 30 - 90 kHz range are known as 50 kHz USVs; they tend to be much shorter than 22 kHz USVs, on average 20 - 40ms in duration (Wright et al., 2010; Brudzynski, 2009). 50 kHz USVs are produced by rats in response to hedonic stimuli, for example, during conspecific rough-and-tumble play, sexual behaviours and anticipation of a food reward, and are suppressed by aversive stimuli, such as foot shocks or social defeat (Burgdorf et al., 2011). This suggests they reflect positive affective states in rats (Burgdorf and Panksepp, 2001; Mällo et al., 2007) by conveying the valence of a situation, and even function to communicate this information to conspecifics (Simola and Brudynski, 2018).

There are a number of different categories of 50 kHz calls, such as ‘flat’ (constant frequency) or ‘trill’ (frequency modulated) calls (Fig. 4), and individuals can produce a diverse range of 50 kHz call subtypes (Wright et al., 2010; Simola and Brudynski, 2018). Flat 50 kHz USVs may act as social-exploration signals (Burgdorf et al., 2011), or as social coordination signals (Burke et al., 2017a), for example, they are produced in response to separation from the cage mate (Wöhr et al., 2008) and by singly-housed rats more prevalently than pair-housed rats (Wright et al., 2010). It is not clear if flat 50 kHz calls are produced in response to pleasurable stimuli (Wöhr et al., 2008; Burgdorf et al., 2008).

Trill 50 kHz calls, on the other hand, have been associated with positive emotional states in rats (Burgdorf et al., 2011), for example, rewarding social

interactions like mating, conspecific rough-and-tumble play and tickling (Burgdorf et al., 2008). Trill USVs are also associated with the reward pathway of the brain, for example, injection of the euphorigenic drug, amphetamine, into the nucleus accumbens (a region of the forebrain which is part of the dopaminergic reward pathway) of rats increased the number of 50 kHz USVs emitted (Burgdorf et al., 2001).The distinction between the different 50 kHz USV subtypes is challenging, with the easiest distinction being made between flat and trill calls (Burke et al., 2017a). Wright et al. (2010) identified 14 categories of 50 kHz USVs based on the acoustic parameters of the calls, including duration and bandwidth. The authors found that three subtypes were prevalently emitted: trills, flat/ trill combination, and flat calls, with these three subtypes together comprising ~50% of the total observed calls. Systemic amphetamine administration increased the proportion of trill calls produced and suppressed the number of flat calls produced in a dose-dependent manner, in both singly-house and pair-housed rats, suggesting that trill calls may be associated with reward (Wright et al., 2010). Pair-housed rats produced more trill 50 kHz calls than singly-housed rats, suggesting that the profile of calls produced by these rats was affected by social context (Wright et al., 2010). This study demonstrated that the subtypes of 50 kHz USVs produced may be affected by context, and that trill and flat 50 kHz USVs may carry different behavioural significance (Wright et al., 2010).

To address the question of the behavioural significance of each 50 kHz USV subtype, Burke et al. (2017a) examined the correlation between the behaviours and vocalisations of juvenile male rats while the rats were in anticipation of the arrival of a play-partner (which normally would elicit 50 kHz USVs (Knutson et al., 1998). The authors found that when anticipating the arrival of a play partner, juvenile rats emit predominantly trill 50 kHz USVs; 77% of the total number of calls were trills (Burke et al., 2017a). The total number of 50 kHz calls increased significantly over the seven days of testing, along with the total number of jumps and runs (Burke et al., 2017a). The authors summarised that slower locomotive movements (turning and walking), were associated with calls that had some frequency modulation, and during

energetic locomotion (running and jumping), vocalisations were more complex and composite (calls comprising two or more subtypes; Wright et al., 2010) and had frequency modulation (Burke et al., 2017a). These results suggest that subtypes of 50 kHz USVs are strongly linked to specific behaviours made in response to play anticipation (Burke et al., 2017a). This study also provides evidence for positive affect being conveyed by 50 kHz calls as rats increased the production of these calls over the seven day testing period as the rats' prior experience led them to expect the arrival of a play partner (Burke et al., 2017a). The amount of running and jumping similarly increased over time, and this may suggest that both 50 kHz calls and these movements could indicate positive affective states (Burke et al., 2017a). This is consistent with the findings that male Long-Evans rats selectively bred for high 50 kHz calling rates also showed high levels of spontaneous locomotor activity (Brudzynski et al., 2011), and the mesolimbic dopamine reward system has been implicated both in 50 kHz USV production (Burgdorf et al., 2011) and in the regulation of movement and motivated behaviours (Alcaro et al., 2007).

Together the current literature suggests that it is plausible that different subtypes of ultrasonic vocalisations may have specific meanings, and that these call subtypes can be temporally coupled to behavioural movements (also reported by Laplagne and Costa (2016) and Takashi et al. (2010)), possibly acting as a method of moment-by-moment social communication (Burke et al., 2017). Burke et al. (2017a) posit that if these behaviour-vocalisation associations can be further validated, they could be useful in more thorough investigations of the neurophysiological mechanisms underlying positive affective states, which may have implications for models of human mental health disorders and of animal welfare.

50 kHz USVs as a response to tickling

As has been discussed, 50 kHz calls can be recorded and used as a measure of affect during behaviour (Panksepp and Burgdorf, 2000; Garcia et al., 2015). 50 kHz USVs are produced in positive social situations, for example, during or in anticipation of direct social contact (Brudzynski and Pniak, 2002)

and they are abundantly produced during tickling (Panksepp and Burgdorf, 2001; Panksepp and Burgdorf, 2003; Lampe et al., 2017). Rats have been observed to emit more 50 kHz USVs in response to tickling than in response to light touch (Ishiyama and Brecht, 2016) and trill 50 kHz USVs have been shown to increase in response to tickling (Garcia et al., 2015). This suggests that 50 kHz USVs that are evoked in response to being tickled could be a useful behavioural marker for the positive affective state induced by tickling in rats (Panksepp and Burgdorf, 2000).

To measure USV production during tickling, specialised equipment, such as an ultrasonic microphone or a bat detector, is used (Panskepp and Burgdorf, 1999). Spectograms are generated from these recordings, from which the calls can then be quantified (LaFollette et al., 2017). While both male and female rats produce USVs in response to tickling (Schwarting et al., 2018; Himmler et al., 2014), there are only a few studies that have investigated the effect of tickling on USV production in females (LaFollette et al., 2017). Those studies that have compared tickling in male and female rats have presented varying results, reporting both male and female rats abundantly producing USVs (Mällo et al. 2007; Schwarting et al., 2018), or male rats calling more than female rats (Burgdorf and Panksepp, 1999). If 50 kHz USVs are to be used as behavioural markers of positive affect induced by tickling, it is important to consider their generalisability, such as whether the production of 50 kHz USVs, including specific subtypes, is affected by the sex of the animal (Knutson et al., 1999).

1.4 The neural systems and substrates of social behaviours and tickling

There is a known association between observed affective states and the neurological basis of the behavioural output of these affective states (Panksepp, 1998a, b). This is true for many mammalian species and is thought to originate in conserved limbic structures (Panksepp, 1998a, b). The processing of stimuli, which induce affective states, can be carried out by particular brain regions (for example, fear-processing in the amygdala; Fossati, 2012), or brain networks (for example, the reward pathway; Panksepp, 1998b). Likewise, positive affective states may also be linked to particular neural correlates, such as the NMDA receptor subunit NR2B which has a regulatory role in the positive affect of social conspecific play in rats (Burgdorf et al., 2011).

The specific neural signalling pathways underpinning the positive affective states induced by tickling have yet to be fully elucidated (Paredes-Ramos et al., 2012). This section will discuss some of the evidence for the neurobiology of social behaviour, particularly social play, and tickling, in line with the aims of this thesis to further investigate the neural and neuroendocrine signalling pathways that are involved in regulating the behavioural response to tickling.

1.4.1 The neural systems of social behaviour

The neurobiological basis of prosocial behaviour is highly complex and is not yet completely understood. A number of studies have implicated the involvement of the neuropeptide, oxytocin, in many aspects of prosocial behaviours (e.g. Insel and Young, 2001; Ferguson et al., 2002; Young and Wang, 2004). Oxytocin is released centrally in response to social stimuli (Uvnas-Moberg, 1998), it is found to facilitate bond-formation and plays a vital role in maternal behaviour and parturition (Neumann, 2008). The related neuropeptide, vasopressin, is similarly associated with social behaviours, such as partner preference formation in prairie voles, however vasopressin also underlies more negative social behaviours such as aggression and anxiety in

social contexts (Goodson and Thompson, 2010). The mesolimbic dopamine, or reward, system and the endogenous opioid system are also thought to be involved in certain prosocial behaviours (Beery and Kaufer, 2015). For example, transmission of the neurotransmitter, dopamine, within the shell of the nucleus accumbens of the reward system facilitates pair-bond formation in prairie voles (Aragona et al., 2005), and kappa-opioid receptors within the shell of the nucleus accumbens are important in pair bond maintenance in prairie voles (Resendez et al., 2012).

1.4.1.1 The social behaviour network

The Social Behaviour Network (SBN) is a network of key brain regions thought to regulate social behaviours in all vertebrates (e.g. Newman, 1999; Goodson, 2005). This network is comprised of at least six core nodes which are essential to basic social behaviours, including maternal behaviours, aggression, forms of social communication, social recognition and bonding (Goodson, 2005; Goodson and Kingsbury, 2013). They are reciprocally connected (Dong and Swanson, 2004; Coolen and Wood, 1998; Coolen et al., 1997) and contain sex steroid receptors which enables sexual differentiation of social behaviour (Simerly et al, 1990; Goodson, 2005). These six nodes are: the extended amygdala which encompasses the medial amygdala (meA) and medial bed nucleus of the stria terminalis (BNST); the lateral septum (LS); the preoptic area (POA); the anterior hypothalamus (AH); the ventromedial hypothalamus (VMH); areas of the midbrain, including the periaqueductal gray (PAG) and adjacent tegmentum (Newman, 1999; Goodson and Kingsbury, 2013; Fig. 5). Within this closely connected network, a single node can mediate multiple forms of social behaviours (e.g. BNST and meA with copulatory behaviours, Coolen et al., 1997; meA, BNST, POA and VMH with pair bonding, Cushing et al., 2003; BNST, meA, POA and the paraventricular nucleus (PVN) of the hypothalamus with maternal aggression, Gammie and Nelson, 2001; BNST, meA and POA with maternal behaviours, Kalinichev et al., 2000; Goodson, 2005).

In mammals, other brains regions are also considered to act as part of the SBN, such as areas of the mesolimbic dopamine system (Fig. 4) and PVN (Goodson and Kingsbury, 2013). The PVN is the site of synthesis of neuropeptides (oxytocin, vasopressin and corticotrophin releasing hormone (CRH)) that play a major role in social behaviour (Cunningham and Sawchenko, 1991) that sends projections to the nodes of the SBN (Goodson and Thompson, 2010). There is evidence that the mesolimbic dopamine system and the SBN are functionally linked and share common regions, suggesting that information can be passed between the two networks (O'Connell and Hofmann, 2011). The primary regions involved in both systems are the LS, BNST and the meA (Fig. 5; O'Connell and Hofmann, 2011). These regions are implicated in processing the rewarding nature of social stimuli and generating a behavioural output in response; for example, showing sexual behaviours towards a receptive, prospective mate (O'Connell and Hofmann, 2011).

In a study by van Kerkhof et al. (2014), the authors identified the brain circuitry that was activated following social play behaviours in male rats by using the expression of the c-*fos* gene as a marker of cellular activity. The brain regions that were identified in the study as having increased expression of *c-fos* following play included regions of the reward circuit and SBN: medial prefrontal cortex (mPFC), striatum, nucleus accumbens (NA), amygdala, ventral tegmental area (VTA) (van Kerkhof et al., 2014). It was also found that these increases in neural activity were organized in topographical manner, suggesting that there were correlations in c-*fos* between areas that are anatomically connected, such as between the basolateral amygdala (BLA) and striatum, and VTA and NA (van Kerkhof et al., 2014). The findings of this study may provide evidence for the reward circuitry and nodes of the SBN having a role in social play behaviours in male rats. Any role in the response to tickling, and whether this network governing play behaviours is different in the male versus the female brain (van Kerkhof et al., 2014), is yet to be explored.

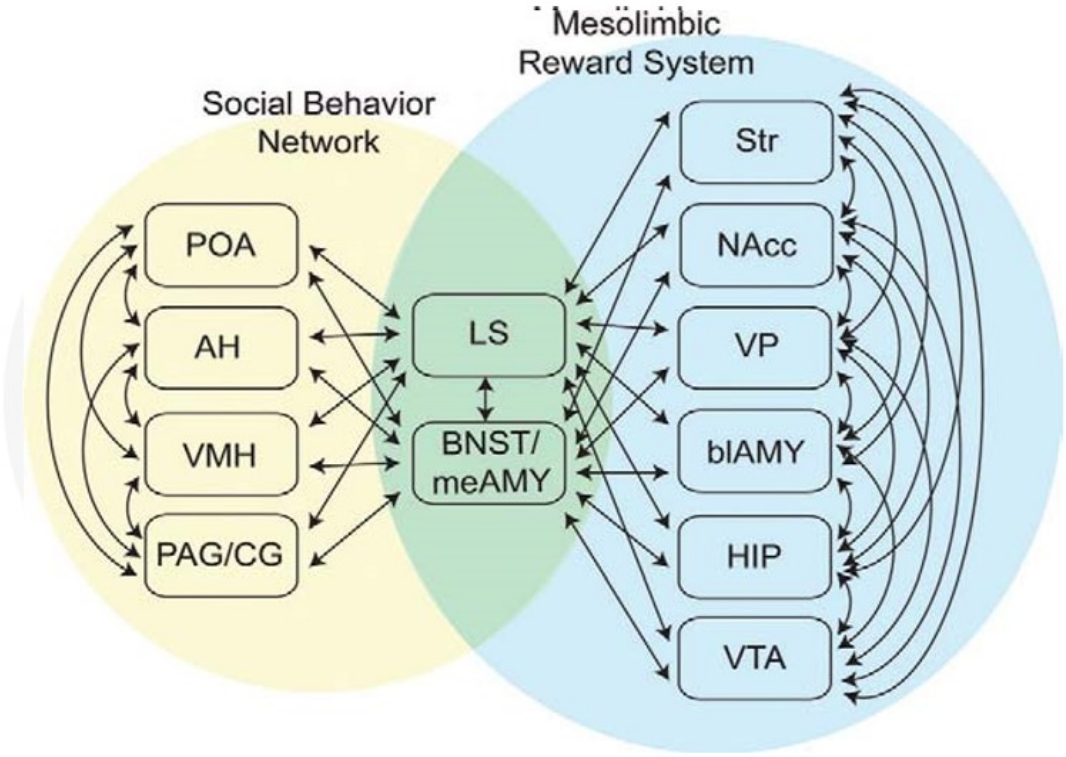

Figure 5. Connections of the social behaviour network.
Nodes of the SBN (yellow), regions of the reward circuit (blue) and overlap between both systems (green). Arrows denote reciprocal anatomical connections between regions in mammals.
Abbreviations: POA, preoptic area; AH, anterior hypothalamus; VMH, ventromedial hypothalamus; PAG/ CG, periaqueductal gray/ central gray; LS, lateral septum; BNST/meAMY, bed nucleus of the stria terminalis/ medial amygdala; Str, striatum; NAcc, nucleus accumbens; VP, ventral pallidum; blAMY, basolateral amygdala; HIP, hippocampus; VTA, ventral tegmental area. (From O'Connell and Hofmann, 2011)

Mapping neural systems using immediate early gene expression: c-fos

The proto-oncogene, c-*fos,* has been extensively studied for decades (Greenberg and Ziff, 1984; Herrera and Robertson, 1996). It is an immediate early gene that is stimulated by external signals to modify gene expression in neurons (Herrera and Robertson, 1996). It is normally expressed in tissues and encodes the protein, Fos, and is a useful marker of cellular activation in the nervous system (Herrera and Robertson, 1996).

The induction of c-*fos* mRNA is transient, with transcription occurring within minutes of extracellular stimulation (Greenberg and Ziff, 1984), for example addition of growth factor (Muller et al., 1984), and peaks between 30-

60 minutes, for example, following acute stress (a forced swim test) in male Sprague-Dawley rats, c-*fos* mRNA (visualized via *in situ* hybridization) throughout the brain is found to peak at 30 minutes post-stimulus and the levels are significantly reduced 120 minutes post-stimulus (Cullinan et al., 1995). c-*fos* mRNA has a short half-life (approximately 10-15 minutes) (Sheng and Greenberg, 1990). Therefore, the expression of c-*fos* is tightly controlled, suggesting that the protein product may have a role in the regulation of the cellular response to external stimuli (Sheng and Greenberg, 1990). The protein product, Fos, typically reaches maximal levels between 60 - 90 minutes post stimulus (for review, see Morgan and Curran, 1991). For example, this peak in Fos has been shown in the amygdala and hippocampus of mice following a foot shock (Milanovic et al., 1998; Radulovic et al., 1998; Stanciu et al., 2001). Also, this time course has been observed in male Sprague-Dawley rats in response to food intake in the nucleus of the solitary tract (NTS) (Zittel et al., 1999). Similarly, following systemic administration of nicotine throughout the brain, Fos immunoreactivity in the superior colliculus and several area of the visual pathway was detectable after 60 minutes post- nicotine administration, reaching a peak at 90 minutes and declined between 180 - 240 minutes (Ren and Sagar, 1992).

c-*fos* has been widely used as a functional marker of neuronal activation patterns to certain stimuli (Sagar et al., 1988; Dragunow and Faull, 1989). It was first reported in the adult rat brain by Dragunow and Robertson (1987) who used immunohistochemistry with polyclonal antibodies raised against the Fos protein to visualise neuronal cells in which c-*fos* had been induced (Dragunow and Robertson, 1987).

Quantifying the levels of c-*fos* gene mRNA or Fos protein expression in brain regions is a useful tool for exploring which brain regions have been active during a behaviour of interest, such as during rough-and-tumble play in rats (e.g. Reppucci et al., 2018; Gordon et al., 2002).

By using the expression of the immediate-early gene, c-*fos*, as a marker of cellular activity, in conjunction with discrete behaviours, it is possible to build

a map of the brain regions that were engaged in that behaviour (Siviy, 2016; Gordon et al., 2002). Enhanced c-*fos* gene expression was found following 15 minutes of rough-and-tumble play in juvenile male Wistar rats in subregions of the prefrontal cortex, the orbital frontal cortex, the nucleus accumbens, the amygdala and the bed nucleus of the stria terminalis (van Kerkhof et al., 2014). This study highlights key brain regions that are active during social play and in particular, demonstrating the role of subregions in regulating play behaviours (van Kerkhof et al., 2014). Importantly, the c-*fos* activation patterns after play were correlated between regions with known direct connections, such as projections between the mPFC to the striatum. This suggests a neural network is involved in mediating social play behaviour in rats (van Kerkhof et al., 2014).

1.4.1.2 The oxytocin and vasopressin system

Oxytocin and arginine vasopressin are neuropeptides synthesised in the PVN and supraoptic nucleus (SON) of the hypothalamus of the rat brain (Cunningham and Sawchenko, 1991). The PVN is made up of two divisions: the magnocellular (consisting of three distinct regions) and the parvocellular division (consisting of five distinct regions; Swanson and Sawchenko, 1980). The neurons are typically divided into magnocellular and parvocellular neurons based on the morphology, projections, electrophysiological activity and the function of the cells (Althammer and Grinevich, 2018). The neurons of the magnocellular division contain mainly oxytocin or vasopressin, and these neurons project mainly to the posterior pituitary gland, the site at which oxytocin and vasopressin are released into the bloodstream (Swanson and Kuypers 1980; Swanson and Sawchenko, 1980). Similarly, in the SON, magnocellular neurons also contain oxytocin and vasopressin (Pow and Morris, 1989) and these neurons project to the pituitary gland (Sherlock et al., 1975; Poulain and Wakerley, 1982). Oxytocin and vasopressin are also released from the somas and dendrites of magnocellular neurons in the PVN and SON (Ludwig, 1998; Ludwig et al., 2002). Parvocellular neurons in the PVN project to multiple hindbrain structure, such as the brain stem and spinal cord, as well as the dorsal vagus complex and nucleus of the solitary tract (NTS) (Swanson and Sawchenko, 1980; Swanson and Kuypers, 1980;

Althammer and Grinevich, 2018). These parvocellular projections modulate different autonomic functions, such as breathing (Mack et al., 2002) and cardiovascular responses (Petersson, 2002; Althammer and Grinevich, 2018).

The next two sections will discuss the two neuropeptides oxytocin and vasopressin in more detail.

Oxytocin

Oxytocin is a nonapeptide (nine-amino acid protein) that is highly conserved across taxa; it is not just found in mammals but is also found as mesotocin in birds and isotocin in fish (Rault, 2019). Oxytocin is synthesised in the PVN and SON in rats (Cunningham and Sawchenko, 1991). The majority of oxytocinergic neurons project to the posterior pituitary gland, and from here, oxytocin is released into the circulation where it has a number of physiological roles (Leng, 2005), for example regulating uterine contraction during parturition, the milk ejection reflex and ejaculation (Russell et al., 2003). Peripheral stimuli, such as suckling, can stimulate the release of oxytocin from the pituitary gland to act on target organs (Cunningham and Sawchenko, 1991). Oxytocin is also centrally-released from neurosecretory neurons and has a widespread action throughout the brain, modulating activity in various regions via its receptor (Cunningham and Sawchenko, 1991; Ludwig and Leng, 2006). Magnocellular oxytocin neurons can send widespread axonal projections throughout the brain, where oxytocin is locally released, and control behaviours, for example fear responses in female Wistar rats (Knobloch et al., 2012; Althammer and Grinevich, 2017). There is building evidence for a population of parvocellular oxytocin cells that make direct synaptic contact with magnocellular oxytocin cells and this allows the parvocellular neurons to drive activity in the magnocellular neurons (Eliava et al., 2016; Tang et al., 2020). Oxytocin plays an important role in the regulation of several social behaviours, for example, aggression, social recognition, pair bonding and mother-offspring bonding (Insel, 1992) and in some cases in a sex-specific manner (Bredewold et al., 2014; Dumais and Veenema, 2016).

Play in rats is one of a few behaviours that differs between males and females, like sexual and aggressive behaviours (Pellis et al., 1997). Given oxytocin's role in these behaviours, and as play behaviour is thought to function in preparing animals for these social encounters as adults, oxytocin is a good candidate for being involved in regulating social play in rats (Baskerville and Douglas et al., 2010). Reppucci et al. (2018) investigated the role of vasopressin and oxytocin systems in regulating social play in a sex-specific manner. Vasopressin is a highly conserved neuropeptide that differs to oxytocin by two amino acid positions and is implicated in the regulation of social behaviours, including social play, often in sex-specific ways (see below and Dumais and Veenema, 2016). Recruitment of vasopressin or oxytocin neurons was not increased in the SON or PVN in either males or females, but there was an increase in Fos protein in the posterior BNST and posterodorsal medial amygdala (MEApd) following 10 minutes of play in females. This suggests a potential sex difference in control of social play behaviours in the amygdala and extended amygdala (Reppucci et al., 2018).

Any role of oxytocin in tickling has yet to be explored, however, Okabe et al. (2015) found that in rats, gentle stroking stimuli increased the number of active oxytocin cells, as indicated by the expression of Fos protein, in the medial parvocellular PVN and also increased the number of 50 kHz USVs emitted during the stroking stimulus. These findings suggest that a pleasant sensory stimulus may activate oxytocin neurons in a sub region of the PVN (Okabe et al., 2015).

In a recent study by Tang et al. (2020), the neuronal activity of oxytocin neurons in the PVN was recorded during a social interaction paradigm using female rats to identify whether components of social behaviour affected the firing rate of oxytocin neurons. The authors found that social interactions that were made up of physical touch (being crawled on by or crawling over a conspecific) resulted in increased neuronal activity of PVN oxytocin neurons, likely magnocellular oxytocin neurons (Tang et al., 2020). It was also found that targeted excitation of small population of parvocellular neurons increased

the amount of social interaction that a rat made with a conspecific, and inhibition of these parvocellular oxytocin cells decreased this social interaction (Tang et al., 2020). The authors discovered synaptic-like contacts between parvocellular and magnocellular neurons, suggesting that parvocellular neurons are anatomically connected with magnocellular neurons (Tang et al., 2020). Therefore, parvocellular neurons that respond to somatosensory stimuli may control neuronal activity in a population of magnocellular neurons which project to brain regions involved in social behaviours by synapsing onto them, and therefore regulate aspects of social interactions between female rats (Tang et al., 2020).

Oxytocin acts in the meA and LS to regulate social memory, and these effects may be sex-specific (Lukas et al., 2013). Dumais and Veenema (2016) investigated the role of oxytocin via its receptor in the central (ceA) and medial amygdala on social interest (reflected by the time spent investigating a novel conspecific) in males and females. Social interest is thought to be the initiator that leads to other social behaviours, such as mating and play (Dumais and Veenema, 2016). The authors found that infusion of an oxytocin receptor antagonist in the ceA decreased social interest in a sex-specific way; males decreased social interest whereas females did not. There was a correlation between oxytocin release in the ceA and time spent in social investigation in females (Dumais and Veenema, 2016) suggesting that there may be sex differences in the role of oxytocin in the amygdala to regulate social behaviour.

Sexually dimorphic central oxytocin systems are thought to be present in the juvenile brain (Bredewold et al., 2014). Exogenous oxytocin delivered into the LS in juvenile female rats increased social play behaviours in a novel cage (Bredewold et al., 2014).Also, an oxytocin receptor antagonist in the lateral septum reduced social play in a novel environment as opposed to the home cage; these differences were not observed in juvenile males (Bredewold et al., 2014). In a related study, Veenema et al. (2013) found that intracerebroventricular injection of an oxytocin receptor antagonist did not alter social play with a conspecific in juvenile of either sex. These findings suggest

that oxytocin may have a role in governing social play behaviour in sex- and context-specific ways, although it is likely to be complex.

Oxytocin and dopamine pathways interact to regulate aspects of social behaviour (Baskerville and Douglas, 2010; Young and Wang, 2004). Liu and Wang (2003) demonstrated that blocking both oxytocin receptors and dopamine receptors (D2 subtype) prevented the formation of partner preferences in monogamous female prairie voles, and this was specific to the NA, suggesting that parallel activation of oxytocin and dopamine D2 receptors in the NA was critical for social bonding. Similarly, Dölen et al. (2013) showed that, in male mice, oxytocin acting in the NA was required for the formation of place preference for a socially rewarding context. There is evidence that oxytocin neurons in the medial POA, SON and PVN co-express dopamine D2 receptors which allows for direct regulation of oxytocinergic cells by dopamine, and this could mediate male sexual behaviours (Baskerville et al., 2009).

There is growing evidence that oxytocinergic innervation onto the VTA from the PVN of the hypothalamus is important for social reward (Song et al., 2016; Hung et al. 2017). Oxytocin administered into the VTA of male hamsters resulted in the animals choosing to spend an increased amount of time in a chamber where they have received social interaction with a conspecific (Song et al., 2016). The authors found that this preference for social interaction was dependent on the activation of oxytocin receptors in the VTA (Song et al., 2016). Hung et al. (2017) built on these findings, and reported that during social interactions the activity of oxytocinergic projections from the PVN to the VTA increased. Inhibition of these neurons and blocking oxytocin receptors in the VTA significantly reduced socially conditioned place preference in mice (Hung et al., 2017). Furthermore, oxytocin receptor activation in the VTA was found to increase the excitability of nucleus accumbens-projecting dopaminergic neurons (Hung et al., 2017). This suggests that oxytocin release in the VTA influences the activity of a

subset of dopaminergic neurons that project to the NA, which promotes the rewarding aspect of social interactions (Hung et al., 2017).

Vasopressin

Vasopressin (or arginine vasopressin in rats) is a nonapeptide found in the mammalian system, and as a nonmammalian homologue, vasotocin in birds (Goodson and Thompson, 2010). Vasopressin is related to oxytocin, differing only at two amino acid positions (for review see Goodson and Thompson, 2010), and it is also synthesised in magnocellular neurons in the PVN and SON, with both central and peripheral modes of release (Cunningham and Sawchenko, 1991; Ludwig, 1998). Vasopressin modulates a number of social behaviours in mammals (Goodson and Bass, 2001), for example, social memory (Dantzer et al., 1988), aggression (Wersinger et al., 2002), partner preference (Cho et al., 1999) and sexual behaviours (Pedersen and Bocia, 2006). Vasopressin neurons in the olfactory bulb of female and male rats are important for social recognition; inhibiting vasopressin V1a receptor transmission resulted in no discrimination between familiar and unfamiliar conspecifics, suggesting there was impaired social recognition in juvenile female and male rats (Tobin et al., 2010).

Vasopressin also has an important regulatory role in social play behaviours; blocking vasopressin with a vasopressin V1a receptor antagonist reduces social play in male rats as well as reducing the number of 50 kHz USVs produced in response to social play (Lukas and Wohr, 2015). Veenema et al. (2013) showed that central vasopressin regulates rough and tumble play in juvenile rats in a sex-specific way. An intracerebral injection of a vasopressin V1a receptor antagonist decreased the amount of social play in male rats, having the opposite effect in females (Veenema et al., 2013). Blockade of the V1a receptor in the LS increased social play in males, but decreased it in females, which suggests different brain regions may be involved in any sex-specific regulation of social play behaviours in juvenile rats (Veenema et al., 2013). Bredewold et al. (2014) confirmed this finding, and also showed that vasopressin increased anxiety-like behaviours in an elevated plus maze in

male but not female rats. Paul et al. (2014) reported that vasopressin may also regulate the onset of play in weanling rats (aged 18 - 21 PND) in a sex-specific way. The development of vasopressin mRNA correlated with playful behaviours in male, not female, Wistar rats (Paul et al., 2014). An increase in play behaviours negatively correlated with vasopressin gene expression in the BNST of male rats, whereas vasopressin mRNA in the PVN correlated positively with the amount of play (Paul et al., 2014). This may suggest that vasopressin acts on multiple regions to have both a stimulatory and inhibitory effect on social play behaviours in rats (Paul et al., 2014).

Similarly to oxytocin, there is evidence to suggest that vasopressin interacts with the dopaminergic system to regulate social play in rats (Bredewold et al., 2018). Social play was found to increase the release of dopamine in the LS in female but not male juvenile rats (Bredewold et al., 2018). Injecting vasopressin into the LS increased dopamine release in LS in female but not male rats (Bredewold et al., 2018). A vasopressin V1a receptor antagonist infusion into the LS reduced social play in females and also prevented the social play-induced dopamine release in the lateral septum in females (Bredewold et al., 2018). This suggests that dopamine transmission in LS may interact with vasopressin to moderate social play in a sex-specific manner (Bredewold et al., 2018).

1.4.2 The neural correlates of tickling

To the best of my knowledge, the extent of our understanding of the neural underpinnings of tickling is limited to the somatosensory cortex and the mesolimbic reward pathway, which I will now discuss.

1.4.2.1 The somatosensory cortex

Somatosensory stimulation may be necessary for social rough-and-tumble play in rats; Siviy and Panksepp (1987) found that anesthetisation of the dorsal surface of the body decreased pinning behaviours during play by up to 70%. This treatment did not reduce the number of dorsal contacts to the nape of the neck, which are indicators of play solicitation, suggesting that the motivation to play was still intact (Siviy and Panksepp, 1987).

Tickling is a tactile stimulus, and the involvement of the somatosensory signalling pathway has been investigated. In humans, it has been reported that you cannot tickle yourself and this phenomenon is due to the attenuation of the sensory effects of tactile stimuli that are recognized as being self-produced (Blakemore et al., 2000). Using functional MRI (fMRI) to image the brains of human volunteers, it was found that a self-produced tactile stimulus resulted in less activity in the somatosensory cortex than an external tactile stimulus, possibly due to these self-produced stimuli matching predicted sensory feedback coming from the cerebellum and the author conclude that tickling is associated with neuronal activity in the somatosensory cortex (Blakemore et al., 1998).

Using the tickling rat model, Ishiyama and Brecht (2016) report that single unit neuronal recordings in the trunk region of the somatosensory cortex showed neuronal firing increased during tickling, which was concomitant with 50 kHz USV production and microstimulation of deep layers of the somatosensory cortex evoked 50 kHz USVs (Ishiyama and Brecht, 2016). This suggests that the trunk of the somatosensory cortex, an area largely represented in mammals for tactile stimulation, is involved in responses to tickling in rats and it is the deep cortical layers, rather than superficial, that triggers 50 kHz vocalisations (Ishiyama and Brecht, 2016). The authors suggest a neural link between tickling, play and the somatosensory cortex (Ishiyama and Brecht, 2016).

1.4.2.2 The mesolimbic dopamine (reward) system

Both social play and tickling are found to be rewarding forms of social interaction (Vanderschuren et al., 1997; Burgdorf and Panksepp, 2001) and brain regions involved in reward present regions of interest in the regulation of positive affect (Mällo et al., 2009).Tickling is thought to be associated with the ascending mesolimbic dopamine system, a neural network which has an important role in reward, motivation, the reinforcement of different stimuli (Burgdorf et al., 2011; Alcaro et al., 2007). The network includes the VTA, NA and mPFC (Fig. 6; Burgdorf and Panksepp, 2006; Young and Wang, 2004).

Firing in the dopaminergic projections that originate from the VTA can result in dopamine release in the NA and mPFC, encoding the rewarding value of the stimulus (Ikemoto, 2007)

Dopamine release is increased in the NA of adolescent rats during tickling (Hori et al., 2013) and dopamine antagonists injected into the VTAdecreased rates of 50 kHz USVs in rats (Burgdorf et al., 2007). Burgdorf et al. (2001) found that using a dopamine agonist, amphetamine, in the NA of adult rats robustly increased the production of 50 kHz USVs in a dose-dependent manner. Similarly, electrical brain stimulation of the mesolimbic dopamine system also increased the amount of 50 kHz calls emitted by rats (Burgdorf et al., 2007). The importance of dopamine for social play has also been shown through studies that show that dopamine receptors in the NA are required. Increasing extracellular dopamine in the NA using amphetamine increased social play behaviour in male juvenile rats and this was dependent on activation of both dopamine D1 and D2 receptors (Manduca et al., 2016). Blockade of dopamine receptors in the NA inhibits the production of tickling-induced 50 kHz USVs (Hori et al., 2013). Therefore, this increase in dopamine in the NA during tickling and subsequent neural activation is likely linked to the emission of 50 kHz USVs during tickling and explains why tickling is rewarding for rats (Hori et al., 2013).

The dopaminergic system is also important in the sexual differentiation of social play behaviour, with dopamine D1-like receptor agonists masculinising female juvenile social play (Auger and Olesen, 2009). In a study by Burgdorf et al. (2007), electrical brain stimulation was used to examine the brain substrates mediating reward in juvenile female rats. By stimulating different brain regions and recording 50 kHz USVs, they identified the PFC, NA, ventral pallidum, lateral POA, lateral hypothalamus (LH) and VTA as initiating USVs in these rats. Dopamine antagonist injection in the VTA reduced 50 kHz USVs. This suggests that similarly to males, females emit 50 kHz USVs in response to rewarding stimuli, and the neural circuitry regulating these behaviours are linked to the mesolimbic dopamine system (Burgdorf et

al., 2007). This suggests that key neurotransmitters, such as dopamine, acting in key brain regions, regulate social play behaviour in a network in the male and female rat brain.

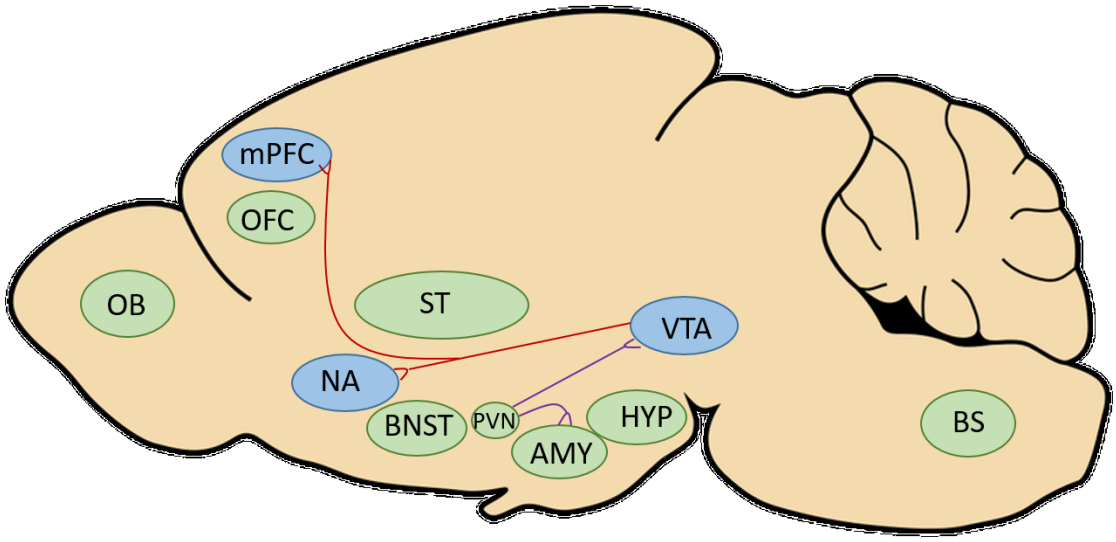

Figure 6. Brain regions thought to be involved in the response to play and tickling in rats.
The major regions of the mesolimbic dopamine pathway are the VTA, NA and mPFC (shown in blue) with red lines denoting the dopaminergic projections. Purple lines, projecting from the PVN to the AMY and the VTA, represent oxytocinergic projections. Other regions involved are the OFC, ST, BNST. VTA, ventral tegmental area; NA, nucleus accumbens; mPFC, medial prefrontal cortex; PVN, paraventricular nucleus; AMY, amygdala; OFC, orbital frontal cortex; ST, striatum; BNST, bed nucleus of the stria terminalis; HPY, hypothalamus; OB, olfactory bulb; BS, brainstem. (Modified from Baskerville and Douglas (2010).

1.5 Conclusions

Tickling provides a robust model of positive affect in rats as evidenced by the production of appetitive 50 kHz USVs and activation of the mesolimbic reward circuitry during tickling in both sexes (Burgdorf and Panksepp, 2001; Hori et al., 2013; Burgdorf et al., 2007). There is some evidence of a sexually dimorphic response to being tickled, but the results are varied and further work needs to be carried out to investigate the neural correlates underlying this sex difference. Potential candidates may be the neuropeptides, oxytocin and vasopressin, as they have recently been implicated in the sex-specific regulation of social play in juvenile rats (Bredewold et al., 2014; Paul et al., 2014).

1.6 Overarching thesis aims

The aims of the thesis are to:

1. Develop a model of positive affective state to investigate the neural and neuroendocrine signalling pathways that are involved in regulating the behavioural and physiological response to positive affective states.
2. Elucidate whether the behavioural response to and the neurobiological regulation of positive affective states are sexually dimorphic.

This has relevance to laboratory animal welfare as well as to farm animal welfare.

Chapter 2

Behavioural and physiological responses to tickling in female and male juvenile Wistar rats.

2.1 Introduction

Sex differences in social behaviours are widespread across mammalian species. Classically this has been well described in relation to sexual behaviours. Courtship and mating behaviours vary dramatically between sexes and it is usually the male that courts the female (Mitoyen et al., 2019). There are well defined, sex-specific mating behaviours in rodents. For example in rats prior to copulation, the female engages in paracopulatory behaviours, including hopping (small jumps where all four paws leave the ground) and darting (rapid running movements), to invite the male to mount (see the recent review, Heijkoop et al., 2018). Intromission involves penile insertion and thrusting during mounting in the male and the receptive female exhibits lordosis behaviour (Heijkoop et al., 2018).

There are several social behaviours which are observed in one sex more than the other. Parental behaviours are often complex and the maternal and paternal roles differ significantly between species (Lonstein and De Vries, 2000). In mammals, the female gives birth and provides nourishment, warmth and protection, so the maternal role is more prominent. However, there are examples of where the male provides the majority of care (e.g. in *Callicebus* primates; Mendoza and Mason, 1986; Lonstein and De Vries, 2000). Rats are uniparental; the mother provides the care for her pups, which includes lactation, retrieval, licking and grooming behaviours towards her offspring (e.g. Lonstein and De Vries, 2000). Males are commonly found to be more aggressive than females in many species (Gray, 1971). Offensive aggression (e.g. attacking an intruder) is mostly seen in post-pubescent males (Blanchard et al. 2003), while females generally only exhibit maternal aggression

postpartum when protecting offspring (e.g. Lonstein and Gammie, 2002; Brunton and Russell, 2010).

There is evidence that social play is a sexually dimorphic behaviour (Chau et al. 2008), including in dogs (Ward et al., 2008) and primate species (Meaney et al., 1985). In rats, numerous studies have found that juvenile males tend to engage in more rough-and-tumble play than females (e.g. Poole and Fish, 1976; Olioff and Stewart, 1978; Meaney et al., 1985; Pellis et al., 1997). Importantly, the structure of rough-and-tumble play and frequency of play bouts differs between female and male rats (Pellis et al., 1997, Auger and Olesen, 2009). Male rats have been found to solicit play by making more pounces than females (Meaney and Stewart, 1981). Males also make more pins (where the rat rolls onto its back in defence and is held down by its playmate which attempts to make contacts with the nape of the supine rat's neck; Pellis et al., 1997), playful attacks and playful supine defences (when the rats allows itself to be pinned by its playmate) than females (Pellis and Pellis, 1990). In fact, it is possible to masculinise social play in juvenile female rats by administering androgens into the amygdala (part of the brain's limbic system), causing an increase in rough and tumble play (Meaney and McEwen, 1986) and play solicitation (Thor and Holloway, 1986) in females to levels seen in juvenile male rats.

Play behaviour is generally accepted to be associated with positive emotional states (Held and Špinka, 2011), although this is disputed by some (see Ahloy-Dallaire et al., 2018). The expression of play behaviours is sensitive to environmental conditions and it is believed that play is observed when the animal's other needs have been met and its welfare is not compromised (Lawrence, 1987; Held and Špinka, 2011). Therefore play may present when an animal is in a more positive emotional state (Held and Špinka, 2011). However, studies have not yet investigated whether the expression of positive emotional state is different between sexes.

Heterospecific play, or tickling, has been used for over 20 years to model positive affective states in rats (Panksepp and Burgdorf, 2000). 50 kHz

ultrasonic vocalisations (USVs) are associated with positive affective states in rats (e.g Burgdorf et al., 2011). Rewarding stimuli, such as rough and tumble play (Knutson et al., 1998), anticipation of food (Burgdorf et al., 2000), euphorigenic drugs (Burgdorf et al., 2001), and alcohol intake (Buck et al., 2014), all increase the number of 50 kHz USVs produced by rats (Burgdorf et al., 2011). 50 kHz USVs are also abundantly produced by rats during tickling (e.g. Burgdorf and Panksepp, 2001). Only a small number of studies to date have investigated the effect of tickling on female rats (Lafollette et al., 2017) and those studies that have presented varying results. For example, where 50 kHz USV production has been used as a response measure, it has been shown that both sexes produce 50 kHz USVs during tickling (Burgdorf et al., 2009; Mällo et al., 2007), whilst another study reported no such differences in 50 kHz USVs between sexes (Burgdorf and Panksepp, 2001). Similarly, during tickling, males were reported to call more than females (Panksepp and Burgdorf, 1999; Panksepp and Burgdorf, 2003), but in another study the opposite was reported with females producing more 50 kHz USVs than males (LaFollette et al., 2018). Therefore it is unclear whether female and male rats respond differently to being tickled, and whether tickling induces positive affect to the same extent in female and male rats.

The overall aim of the study in this chapter was to investigate if there are sex differences in the behavioural response to positive affective states induced by tickling in juvenile Wistar rats, to test the hypothesis that specific elements of the behavioural response to tickling differ between female and male juvenile rats. I predicted that the tickling protocol would successfully induce positive affective states in juvenile rats of both sexes, but that the behavioural phenotype would be different between female and male rats as conspecific social play elements show sexual dimorphisms. To address this, solitary play (scampering), approach behaviours and ultrasonic vocalisations were used as behavioural measures of affect as they are commonly used in tickling studies (e.g. Burgdorf and Panksepp, 2001; LaFollette et al., 2018; Hammond et al., 2019) and directly compared between tickled and control rats of both sexes.

2.2 Materials and Methods

2.2.1 Subjects

Across two replicates, 64 juvenile Wistar rats (Replicate 1: Females 41.0-69.1g, Males 48.6-75.5g; Replicate 2: Females 39.1-62.4g, Males 42.7-64.3g) were sampled (32 per replicate; Charles River, Kent, UK). Each replicate was split evenly between males and females. Rats arrived at the Roslin Institute Biological Research facility at 23-24 days of age. The rats were derived from four different litters: four female and four male rats from the same litter were used (four litters in total). Treatment (Control or Tickled) was randomly assigned to each rat, balancing for body weight and littermates so that average weights for each treatment group and for each sex were as balanced as possible. There was an equal number of animals from each litter in both treatment groups.

Following arrival rat body weight (g) was taken daily and rats were acclimatised to their new surroundings for five days before they were habituated to the tickling test arena (a Perspex open box, 60 (length) x 60 (width) x 25 (height) that was lined with LabMat; LabLogic Systems Ltd., England). Rats were housed in same-sex pairs, with each cage containing a tickled and control rat (the tickled rats were marked with a black mark on their tail in marker pen, treatment was pseudo-randomly assigned balancing for body weight (Matsuzaki et al., 2018) and litter (no littermates were housed in the same cage)), in clear plastic cages (46 x 25 x 21 cm) with a wire lid. Each cage contained aspen chip bedding, one shredded paper nest, one aspen chew stick (Nepco, Warrensburgh, USA). Food (14% protein rodent maintenance diet, Envigo, UK) and water were available *ad libitum* and the room temperature, humidity and light intensity was held stable at 18-23 ^{o}C, 40-60% and 25 lux respectively. The cages were randomly arranged in a cage rack to account for differing lux levels through the height of the rack to balance for sex and litter. The rats were held on a reversed 12-hour light/12-hour light dark cycle (lights on: 00:00, lights off: 12:00) and were tested on the EPM and in the tickling test arena in the dark phase. Body weight (g) was recorded daily following testing between 16:00-18:00 in the dark phase. The rats were

checked daily (by laboratory personel at 08:00, during the light phase) and nitrile gloves were worn when handling the animals. To minimise handling stress rats were picked up gently by holding them behind their forelegs and then cupping them with both hands.

2.2.2 Experimental design

Rat sample size was determined using a power equation using variance and mean values from previous data (Hammond et al., 2019). The order in which the cages and cage mates were tested each day was pseudo-randomised to account for time of day, sex, treatment and lux levels of the cages in the cage rack.

All testing (weighing, habituation, EPM and tickling) was carried out in the home room. The area used for all testing was enclosed by a thick, plastic curtain, at the opposite end of the room to the cage rack. Testing was carried out in the dark phase under red light for the experimenter (myself, Emma Tivey) to see. The only personel present in the room for testing and carried out all testing was myself. The rats were brought to a bench in the enclosed area used for testing in their home cage. One rat was tested while the second cage mate remained in the home cage. The home cage was placed away from the testing apparatus so that no USVs were detected by the rat in the home cage. Each cage mate was identified by a mark on the tail made at the time of assigning treatment: tickled rats had a black mark, control rats had no black mark.

Animals were given a five day habituation period during which they were placed in the centre of the testing arena for a total of five minutes. An immobile right hand (wearing a nitrile glove covered with a white cotton glove) was placed in the arena to habituate the rats to the glove and the researcher.

On the final day of habituation, after all rats had experienced the tickle test arena, the rats were then tested on an elevated plus maze (EPM) to quantify anxiety (Pellow et al., 1985). The order in which the rats were put into the tickle test arena for habituation was used for the EPM so that each rat had had an equal amount of time in between the habituation and the EPM. The

rats were individually run on the EPM (each arm 100 x 10 cm; centre area 10 cm^2; walls of closed arms 34 cm high; maze elevation off floor 60 cm; Tracksys Ltd UK) for five minutes. Data was collected using a digital infrared camera (VCC-6594 1/3-Inch IR CCD, SANYO, Japan) suspended above the maze and later analysed using automated behaviour tracking software (Ethovision XT 11.5; Noldus Information Technology, Wageningen, Netherlands). The EPM was thoroughly cleaned between each test using 70% ethanol and testing was carried out during the dark phase. After the habituation phase, on day six, the rats began ten days of behavioural testing. Each animal was tested in the arena for two minutes per day for ten days. One cage mate (randomised order) was taken from the home cage and placed in the test arena. The rat was tested for two minutes, timed with a stopwatch and both video and sound recordings of the behaviour were recorded digitally using a video camera (Panasonic HD HC-V10) and ultrasound microphone (Pettersson M500-384 USB Ultrasound microphone, PetterssonElectronik, Sweden). Audacity software (Version 2.1.3, Pennsylvania, United States of America) was used to record the spectrograms. The microphone was suspended about 30 cm above the testing arena. A video camera (Panasonic HD HC-V10) was also placed above the testing arena to record behaviour during testing. Spectrograms of USVs were collected and the total number of 50 kHz calls counted manually in Ultravox 14 (Noldus Information Technology, Wageningen, Netherlands) for each animal over the 10 days of testing. Test arena behaviour was analysed in Observer 15 (Noldus Information Technology, Wageningen, Netherlands) by the experimenter who was blinded to sex but not to treatment due to being able to see the animal being tickled or not in the video. The arena was cleaned with 70% ethanol gel and allowed to dry between the testing of each rat and testing was carried out in the first three hours of the dark phase.

For the tickled group, rats were placed in the arena, and a hand (wearing a white cotton glove) was placed motionless on one wall of the arena (the wall position and placement of the hand was randomised each time) for the first 15 seconds of testing. Following these 15 seconds of release (i.e. where the experimenter's hand was motionless on the side of the arena and

the rat received no contact with the hand), the rat was tickled for 15 seconds by making rapid finger movements on the nape of the neck. If the rat reared

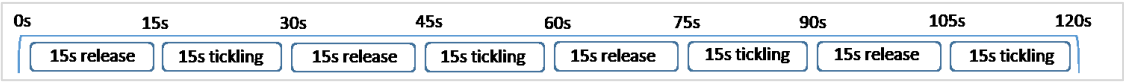

Figure 1. Representation of the release and tickling two minute protocol.

up at the hand it was tickled on its ventral side. The 15-second bouts of ticking and release (Tickle and Release Sessions) were alternated during the two minutes of testing (Fig. 1). For the control group, rats were placed in the arena for two minutes, with a hand (wearing a white cotton glove) resting motionless on one wall of the arena (the wall position and placement of the hand was randomised each time). If the rat reared at the hand, the hand was gently moved away to one side and then replaced back in position. Following testing, the rat was gently picked up (as described previously) and body weight was measured before it was returned to its home cage. The other cage mate was then immediately removed and placed in the arena for testing and the behavioural testing was repeated. The cotton gloves were only worn by the hand in the testing arena during testing and each rat was exposed to its own individual cotton glove to avoid any potential olfactory bias.

On the final day of testing (day 10) rats were removed from their home cage between 60-90 minutes after they were tested in the tickle arena (time specified for neural analysis using c-*fos* gene expression, please see Chapters 4 and 5). For those 60-90 minutes between the final testing being carried out and being euthanised, the rats were returned in their home cage to the cage rack and remained in the home room. Rats were then weighed and euthanized with an intraperitoneal injection of sodium pentobarbitone (Pentoject; 20% w/v 200 mg/ml) carried out by experienced laboratory personnel. The order in which the rats were euthanised was pseudo-randomised and balanced for treatment group and sex. Following confirmation of death, the rats were decapitated and a trunk blood sample and faecal bolus were collected. The blood was collected into an Eppendorf tube containing EDTA and allowed to clot for 10-20 minutes at room temperature. Blood was then centrifuged at

14,000g for 20 minutes and the serum pipetted off (carried out by Dr Sarah Brown). Plasma and faecal samples were frozen in labelled Eppendorp tubes and stored at -20^{o}C until analysis. The brains were removed from the skull and prepared for paraformaldehyde fixation (carried out by Mrs Valerie Bishop).

All animal work was carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 following ethical approval by the Roslin Institute's Animal Welfare and Ethical Review Body, and carried out in the Roslin Institute's Biological Research Facility.

2.2.3 Measurements

All data were collected and analysed by myself (Emma Tivey).

Ultrasonic vocalisation analysis

Firstly, to establish changes in calling frequency over time, the total number of 50 kHz USVs emitted during testing on the first, middle and final day (days 1, 5 and 10) were manually counted on day 10 in Ultravox 14 (Noldus Information Technology, Wageningen, Netherlands) (see Table 1 for call definitions).

Secondly, flat and trill 50 kHz USV subtypes, and 22 kHz USVs were manually counted on day 10 in Ultravox 14 (see Table 1 for call definitions). This was done because the animals were also being used to investigate the underlying neural correlates of the behavioural phenotype, and so were culled 90 minutes after testing on day 10 (refer to chapters 4 and 5). Therefore, a more in depth analysis of the behaviours on day 10 was carried out to relate to the neural correlates. Trill and flat 50 kHz calls subtypes are abundantly produced during tickling (Burgdorf et al., 2011) and may serve different functions, so these two subtypes were counted along with total number of all 50 kHz USVs.

Thirdly, the number of flat and trill 50 kHz USVs during the 15 second bouts of tickling and release through the two minutes of testing on day 10 were counted for comparison in the analysis. Trill 50 kHz USVs were counted in each 15 second segment of the 2 minutes of testing on day 10. The trill 50 kHz USVs

produced in the four 15 second segments that equated to when the rat was in a ‘release’ session (i.e. not being tickled) were added together to give a total number of trill 50 kHz USVs emitted when the rat was not being tickled. The trill 50 kHz USVs produced in the four 15 second segments that equated to when the rat was in a ‘tickle’ session (i.e. being tickled) were added together to give a total number of trill 50 kHz USVs emitted during active tickling. This was done for each rat; even though the control rats were not tickled, the same 15 second segments were used to split up the 2 minutes of testing that the control rats received in order to compare 50 kHz USVs in the same segments between treatment groups. This same procedure was repeated for flat 50 kHz USVs.

Test arena behavioural analysis

In order to analyse the effect of tickling on scampering, hand approaches and approach latency over the course of the 10 day experiment, behaviours were analysed on days 1, 5 and 10 of testing. Using video footage the total number of scampers and approaches made towards the hand, and the latency to first approach were manually counted on days 1, 5 and 10. On day 10 of testing a greater in depth analysis of scampering behaviours was carried out. This was done to relate the specific behavioural elements to the neural correlates (refer to chapters 4 and 5). Scampering was broken down into its constituents of hopping and darting (see Table 1 for definitions of behaviours). The total number of hops and darts were counted manually using video footage from day 10.

Table 1. Ethogram used to carry out the behavioural analysis of the rats response to tickling.

Behaviour	Definition	Reference
Still	Motionless, including resting, sitting or lying still, each bout was measured – a bout was determined as >1 second participating in the behaviour.	LaFollette et al., 2018.
Exploratory	Sniffing directed at the environment, including during rearing. Each bout of sniffing (with or without rearing) was measured- a bout was determined as >1 second participating in the behaviour.	Mällo et al., 2007.
Scampers	Comprised of hops followed directly by rapid darting movements, not as a result of being pursued. Also referred to as solitary play: spontaneous locomotor movements, such a leaping, pirouetting and running in a sequence. One scamper consisted of >2 hops and >1 dart.	Richter et al., 2016; Ahloy-Dallaire et al., 2018; Vanderschuren and Trezza, 2013.
Hop	'Joy-leaps'/ 'jerk-jumps'. These hops or jumps are an element of scampering when combined with darts. One hop was determined as when all four feet left the floor.	Richter et al., 2016; Melotti et al., 2014.
Dart	Locomotor play, a rapid darting movement/ running alone. Darts are an element of scampering when combined with hops. One dart was determined as a rapid locomotory movement in the absence of sniffing, lasting >1 second, usually in a circular motion returning to the hand.	Vanderschuren and Trezza, 2013; Melotti et al 2014; Pellis and Pellis, 1983.
Approach	Forward motion, directed movements including rears directed at the hand of the experimenter. One approach was determined as a forward locomotor movement directed towards the hand ending in the rat touching the hand with the nose.	Ishiyama and Brecht, 2016.

Latency to approach	The time taken to first approach and touch the hand of the experimenter. Touching of the hand with the nose following forward movement directed towards the hand.	Burgdorf and Panksepp, 2001.
50 kHz USV	Broad range of calls in the ultrasonic frequency range, from 30kHz up to 90kHz. Anything below 30 kHz was not coded as a 50kHz 'positive' call.	Wöhr, 2018.
Flat 50 kHz USV	50 kHz calls with a near-constant frequency greater than 30 kHz. Flat-trill combination calls were not distinguished.	Wright, Gourdon and Clarke, 2010.
Trill 50 kHz USV	50 kHz calls with rapid frequency oscillations greater than 30 kHz. Flat-trill combination calls were not distinguished.	Wright, Gourdon and Clarke, 2010.
22kHz USV	Low level of modulation, long calls, often occurring in bouts of between 2-8 calls in the 18-24kHz frequency range.	Wöhr, 2018.

Elevated plus maze

The rats were run on the EPM in a randomised order, taking into account treatment, sex and where the home cage was located on the cage rack. The home cage was taken off the rack and placed on a bench surrounded by a plastic curtain. For each cage, the rat which was to be tested first was randomly selected. The rat was removed from its home cage and placed in the centre of the elevated plus maze facing one of the closed arms. A digital infrared camera and Ethovision software was used to record testing. The rat was allowed to explore the maze for five minutes. At the end of the testing period, the rat was removed from the elevated plus maze and returned to its home cage. The maze was cleaned with 70% ethanol gel, and the second cage mate was removed from the cage and underwent the same procedure as the first cage mate. After both cage mates were tested, the home cage was returned to the rack and this was done for all of the cages. In Ethovision software, data profiles were built to extract measures of frequencies of

open/closed arm entries, time spent in open/closed arms, and latency to first enter open/closed arms. The data was exported as an Excel spreadsheet for later statistical analysis.

Body weight

Rat body weight (g) was measured every afternoon (after testing) and an average daily live bodyweight gain calculated using the following formula:

$$\text{Average daily live body weight gain (g)} = \frac{\text{(Weight on final day- weight on first day)}}{\text{(Date of cull – Date of start)}}$$

2.2.4 Statistical analysis

Calls and behaviours over time (days 1, 5 and 10) were analysed in Minitab 17 (Minitab Ltd., Coventry, UK) using a generalised linear model to account for multiple groups being tested across time (sex, treatment, day, and the interactions) followed by Tukey pairwise comparisons. 50 kHz USVs, flat 50 kHz USVs, trill 50 kHz USVs, approach behaviour, latency to approach, scampering, darting and hopping behaviours from day 10 were analysed in Minitab 17 using a generalised linear model to account for multiple factors (sex, treatment, sex x treatment) followed by Tukey pairwise comparisons. 50 kHz USVs in 'release' or 'tickle' sessions were analysed in Minitab 17 using a generalised linear model to account for multiple factors (sex, treatment, sex x treatment). Elevated plus maze data were analysed in Minitab 17 using a two-sample t-test. Average daily live body weight gain was analysed across time in Minitab 17 using a generalised linear model. A regression analysis was carried out: daily live body weight gain was regressed against behavioural responses (50 kHz USVs, trill 50 kHz USVs, flat 50 kHz USVs, scampers, hops, darts, hand approaches and latency to approach on day 10 in Minitab 17. Average daily live body weight gain was included as a continuous predictor, while treatment and sex were included as categorical predictors. All graphs were generated in GraphPad Prism (version 9 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com). The data in the graphs are presented as the means of the raw data ± standard error of the mean (SEM).

2.3 Results

2.3.1 The effect of tickling on behaviour across 10 days of testing

50 kHz USV production

There was no significant overall interaction between sex x treatment ($F_{1,60}$ = 0.12, p = 0.727), although the raw data indicated that there might be pairwise differences between groups and this warranted exploring (Fig. 2). Indeed, a Tukey pairwise comparison showed that tickled females (mean = 469.1 ± 34.1) called more than tickled males (343.1 ± 34.1), control females (mean = 230.3 ± 34.1) and control males, with this latter group calling the least (mean = 120.6 ± 34.1). This result should be taken into consideration in the context of the non-significant interaction effect. There was a significant interaction between sex x day ($F_{2,60}$ = 3.34, p = 0.038) with female rats on day 10 calling significantly more (mean = 503.4 ± 40.7) than the other groups. There was also a significant interaction between treatment x day ($F_{2,60}$ = 3.83, p = 0.024), as tickled animals on day 10 called the most (mean = 556.1 ± 40.7). While there was no interaction between sex x treatment x day ($F_{2,60}$= 1.77, p = 0.174), post-hoc analysis revealed that tickled female rats on day 10 called more than any other group (mean = 675.6 ± 57.3). All groups (tickled females, tickled males, control females, control males) were found to increase calling across the 10 days ($F_{2,60}$ = 31.78, p < 0.001). There was a sex difference in the total number of 50 kHz USVs produced in response to tickling ($F_{1,60}$ = 25.40, p<0.001; Fig. 2) with females (mean = 349.7 ± 24.1) producing more 50 kHz USVs than males (mean = 231.9 ± 24.1). Treatment also affected the number of 50 kHz USVs that were produced ($F_{1,60}$ = 97.41, p < 0.001) with tickled animals (mean = 406.1 ± 24.1) calling more than control animals (mean = 175.4 ± 24.1). On day 10, rats called more (mean = 402.3 ± 29.5) than on day 5 (mean = 295.9 ± 29.5) and day 1 (mean = 174.2 ± 29.5).

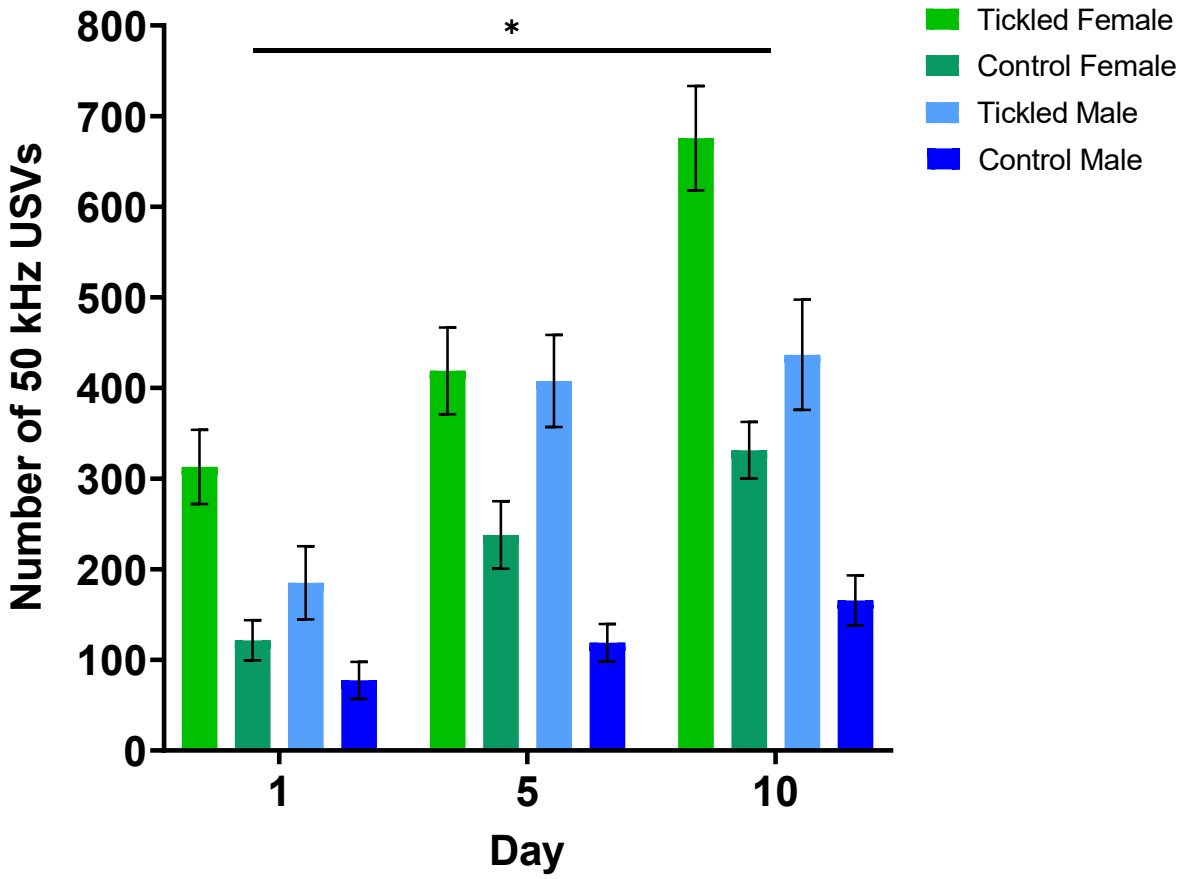

Figure 2. The number of 50 kHz USVs emitted increased across the 10 days and was greatest in tickled rats.

Total number of 50 kHz USVs produced during the 2 minutes testing period on day 1, day 5 and day 10. Female rats (green) and male rats (blue). Mean ± SEM; n = 16 / group. * denotes $p < 0.05$ for significant interaction between treatment x day.

Scampering behaviour

Similarly to total 50 kHz USVs, there was no significant interaction of sex x treatment ($F_{1,60}$ = 0.02, p = 0.886), but exploring potential pairwise differences between groups was warranted after visualising the raw data (Fig. 3). A post-hoc Tukey pairwise comparison analysis showed that tickled females scampered more than any other group (mean = 9.04 ± 0.72). There was no significant interaction of sex x day ($F_{2,60}$ = 0.90, p = 0.408). There was a significant interaction of treatment x day ($F_{2,60}$ = 5.56, p = 0.005) with scampering increasing across testing in tickled animals, with tickled rats on day 5 (mean = 8.72 ± 0.88) and 10 (mean = 8.97 ± 0.88) scampering more than other groups. Rat sex ($F_{1,60}$ = 28.30, p < 0.001), treatment ($F_{2,60}$ = 88.38, p < 0.001) and the day of testing ($F_{2,60}$ = 5.61, p = 0.004) each had an independent effect on the total number of scampers observed during the 2 minute tickling test (Fig. 3).

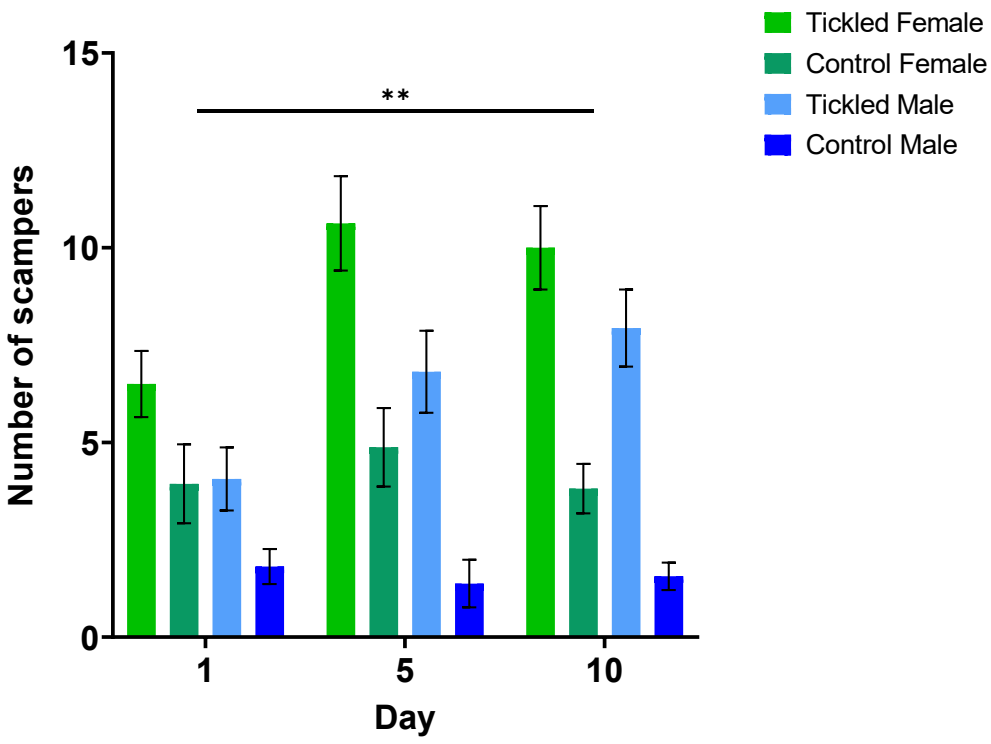

Figure 3. Solitary play behaviour in rats.
Total number of scampers made during 2 minutes of testing on day 1, day 5 and day 10. Female rats (green) and male rats (blue). Mean ± SEM; n =16 / group. ** denotes p ≤ 0.01 for significance of the interaction of treatment x day.

Approach to the hand

There was a significant interaction of treatment x day ($F_{2,60} = 4.27$, $p = 0.015$) as the number of approaches decreased across testing days except in the control female group (Fig. 4). There was a significant effect of sex on the number of approaches towards the hand ($F_{1,60} = 16.86$, $p < 0.001$), with females (mean = 9.58 ± 0.49) approaching the hand significantly more than males (mean = 7.57 ± 0.49), but there was no effect of treatment ($F_{1,60} = 0.62$, $p = 0.432$; Fig. 4).

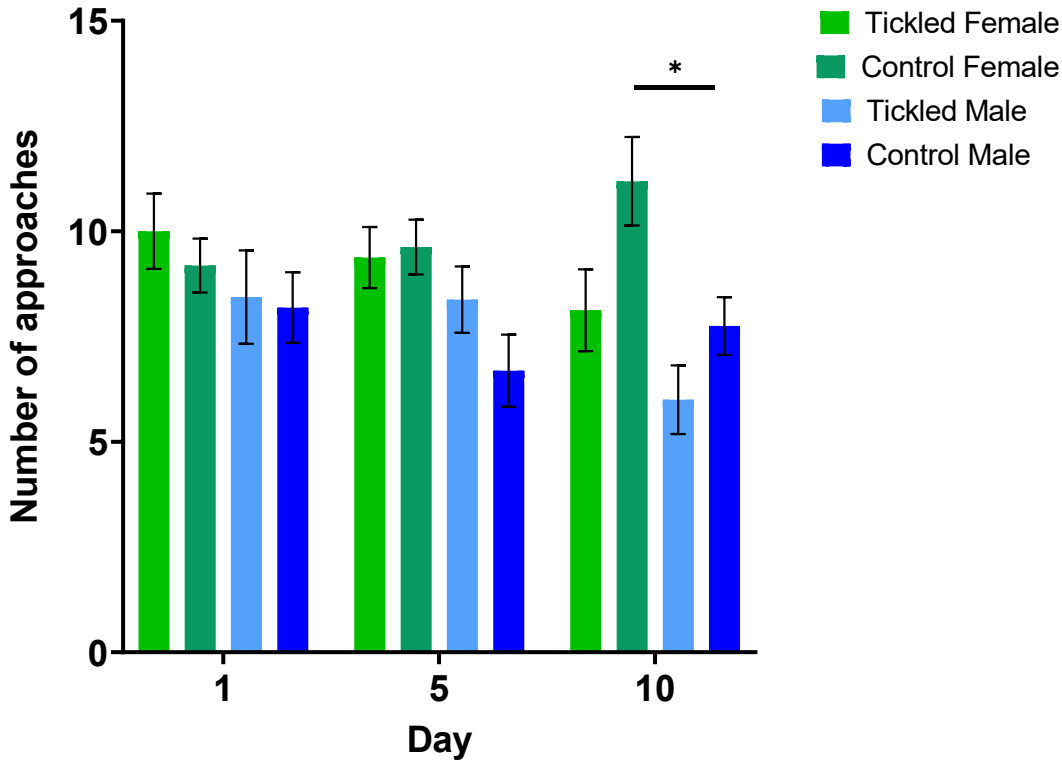

Figure 4. Approach made towards the hand.
Total number of approaches towards the hand made during 2 minutes of testing on day 1, day 5 and day 10. Female rats (green) and male rats (blue). Mean ± SEM; n = 16 / group. * denotes $p < 0.05$ for significant interaction of treatment x day.

Latency to Approach the hand

There was no significant effect of sex ($F_{1,60} = 2.80$, $p = 0.096$), treatment ($F_{1,60} = 2.25$, $p = 0.135$) or day ($F_{2,60} = 0.70$, $p = 0.469$) or the interaction of sex x treatment x day ($F_{2,60} = 0.00$, $p = 0.997$) on the time taken to first approach the tickling hand (Fig. 5). On day 10, control rats took longer to approach the hand compared to tickled rats ($F_{2, 60} = 3.68$, $p = 0.027$, mean = 22.2 ± 4.38 s). A Tukey pairwise comparison revealed that this effect was due to male control rats (mean = 28.7 ± 6.19 s).

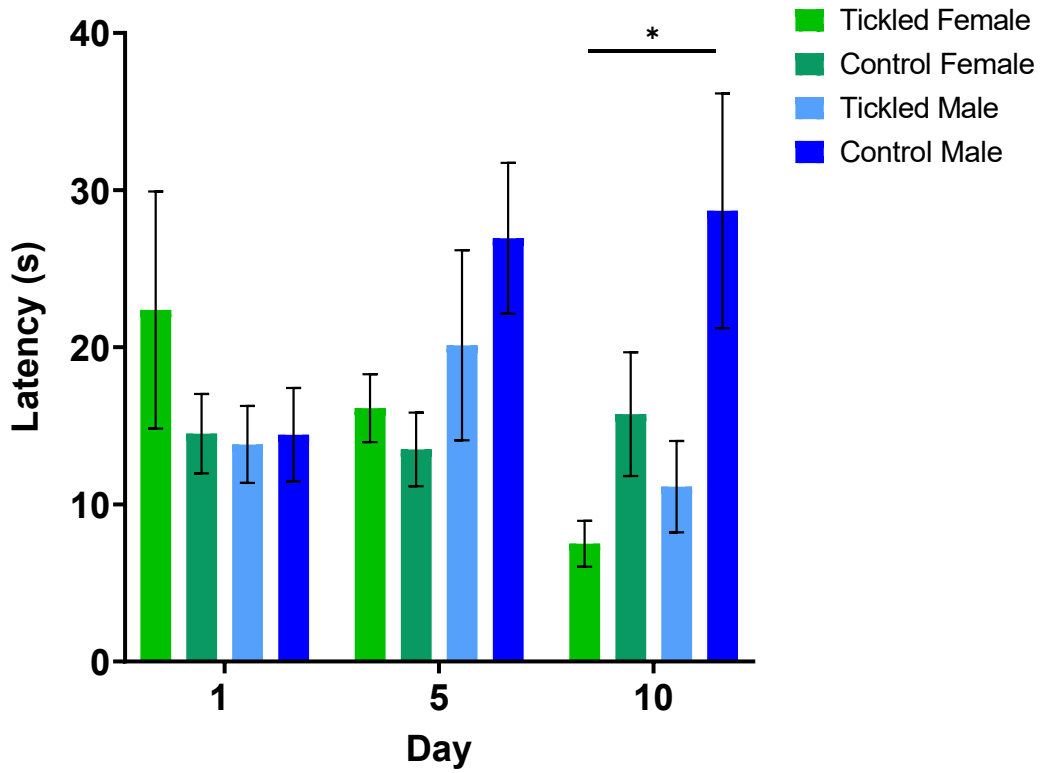

Figure 5. Latency to approach the hand.
Time taken (seconds) for the rat to make its first approach towards the hand on day 1, day 5 and day 10. Female rats (green) and male rats (blue). Mean ± SEM; n = 16 / group. * denotes $p < 0.05$ for significant interaction of treatment x day.

2.3.2 The effect of tickling on behaviour on day 10

On day 10 of testing, animals were culled and whole brains taken for neural analysis. To relate the behavioural phenotype to underlying neural correlates, behavioural measures from day 10 were analysed separately to then be regressed against the neural data (see Chapters 4 and 5, for neural data relating to the behavioural data in this section). Scampering was split up into its elements of hops and darts, and flat and trill 50 kHz USV subtypes were differentiated from other subtypes and counted separately.

50 kHz USV production (day 10)

By day 10, there was an effect of sex ($F_{1,60} = 18.70$, $p < 0.001$) and treatment ($F_{1,60} = 43.23$, $p < 0.001$) on the total number of 50 kHz USVs produced (Fig. 2). The call profiles were predominantly made up of frequency-modulated/trill and flat 50 kHz calls and these subtypes were analysed.

Frequency modulated (FM) or Trill 50 kHz USVs (day 10)

There was no significant effect of the interaction of sex x treatment on the number of trill 50 kHz USVs ($F_{1,60} = 2.19$, $p = 0.144$; Fig. 6A). There was no significant difference between the number of trill 50 kHz USVs produced by female and male rats ($F_{1,60} = 2.73$, $p = 0.104$). Treatment had an effect, with tickled rats emitting more trill 50 kHz USVs than control rats ($F_{1,60} = 36.55$, $p < 0.001$).

Non-modulated or Flat 50 kHz USVs (day 10)

There was no significant effect of sex x treatment on the number of flat 50 kHz USVs ($F_{1,60} = 0.83$, $p = 0.367$; Fig. 6B). There was an effect of sex on the number of flat 50 kHz USVs produced ($F_{1,60} = 9.85$, $p = 0.003$), with female rats producing more flat 50 kHz USVs than males. Tickled rats produced more flat calls than controls ($F_{1,60} = 21.55$, $p < 0.001$).

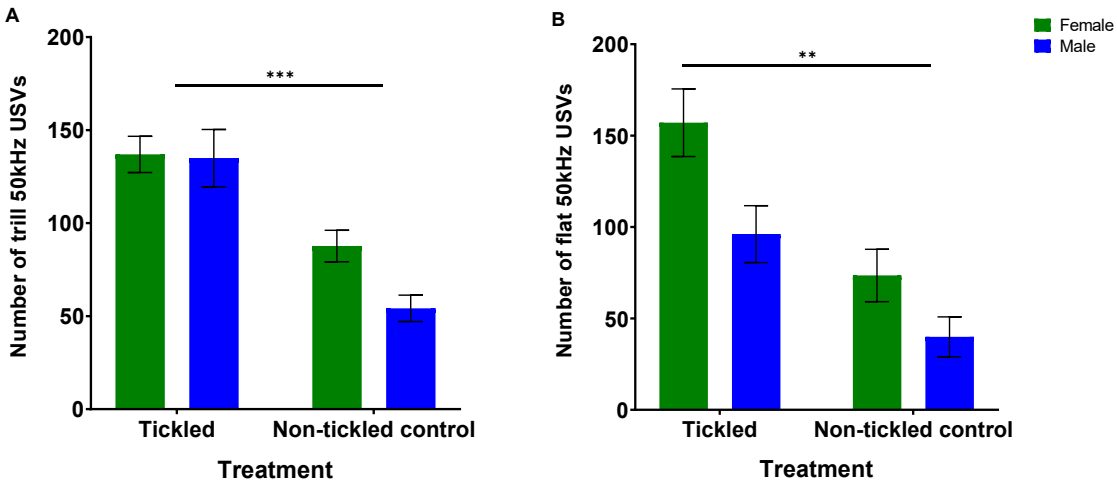

Figure 6. 50 kHz USV subtypes emitted by rats on day 10.

Total number of trill 50 kHz USVs were affected by treatment (A) and total number of flat 50 kHz USVs were affected by sex (B). Female rats (green) and male rats (blue). Mean ± SEM; n = 16 / group. ** denotes $p \leq 0.01$ for significant effect of treatment; *** denotes $p \leq 0.001$ for significant effect of treatment.

22 kHz USV production (day 10)

The number of aversive 22 kHz USVs produced was low throughout the testing period (Fig.7). There was no significant interaction between sex x treatment ($F_{1,180}$ = 0.01, p = 0.932), sex x day ($F_{2,180}$ = 0.75, p = 0.474), treatment x day ($F_{2,180}$= 0.871, p = 0.871), and sex x treatment x day ($F_{2,180}$ = 1.27, p = 0.282) on 22 kHz USVs. There was no significant effect of sex ($F_{1,180}$ = 0.01, p = 0.932), treatment ($F_{1,180}$ = 0.01, p = 0.932) or day ($F_{2,180}$ = 2.38, p = 0.095). This suggests that tickling was not more aversive to the rats than spending time in the test arena; this did not change across time.

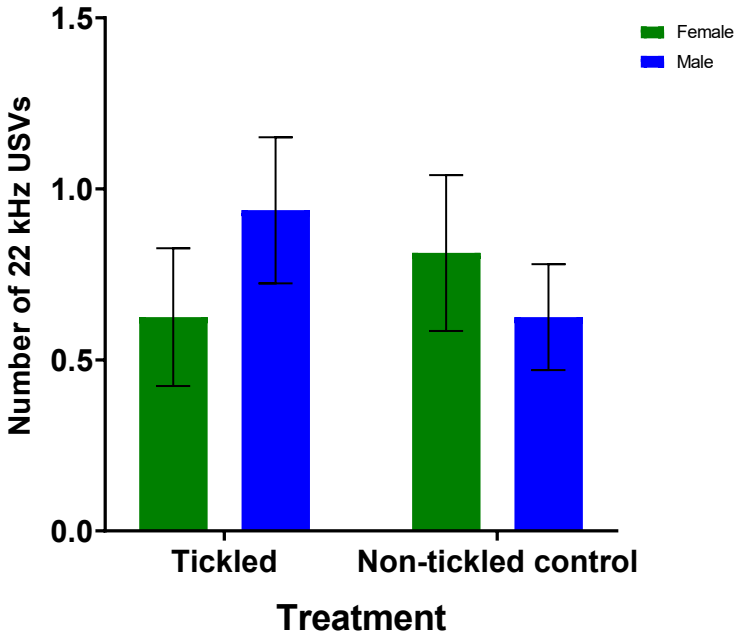

Figure 7. 22 kHz USVs did not differ between tickled and control rats.
Total number of aversive 22 kHz USVs emitted during the 2 minute testing phase on day 10 in female and male rats. Female rats (green) and male rats (blue). Mean ± SEM; n = 16 / group. [n.s].

Scampering, hops and darts (day 10)

Scampers are comprised of hopping and darting. These separate components of scampering were analysed on day 10 to investigate whether these elements of solitary play behaviour were affected by treatment, or by the sex. Treatment had a significant effect on the number of scampers ($F_{1,60}$ = 51.9, p <0.001), with both sexes in each treatment group making similar numbers of scampers ($F_{1, 60}$= 1.16, p = 0.286; Fig. 3). There was an effect of treatment on hopping ($F_{1,60}$ = 5.37, p = 0.024; Fig. 8A); tickled rats made more hops compared to control rats (mean $_{tickled}$ = 9.5 ± 1.31, mean $_{control}$ = 6.4 ± 1.31). There was no effect of sex on the number of hops performed in the 2 minutes of testing on day 10 ($F_{1,60}$ = 1.16, p = 0.287). Tickled rats darted significantly more than control rats ($F_{1,60}$ = 110.9, p < 0.001, mean $_{tickled}$ = 9.03 ± 0.78, mean $_{control}$ = 0.84 ± 0.78). Male and female rats performed a similar numbers of darts during testing on day 10 and there was no significant difference between the sexes in the number of darts made ($F_{1,60}$ = 2.33, p = 0.132; Fig. 8B).

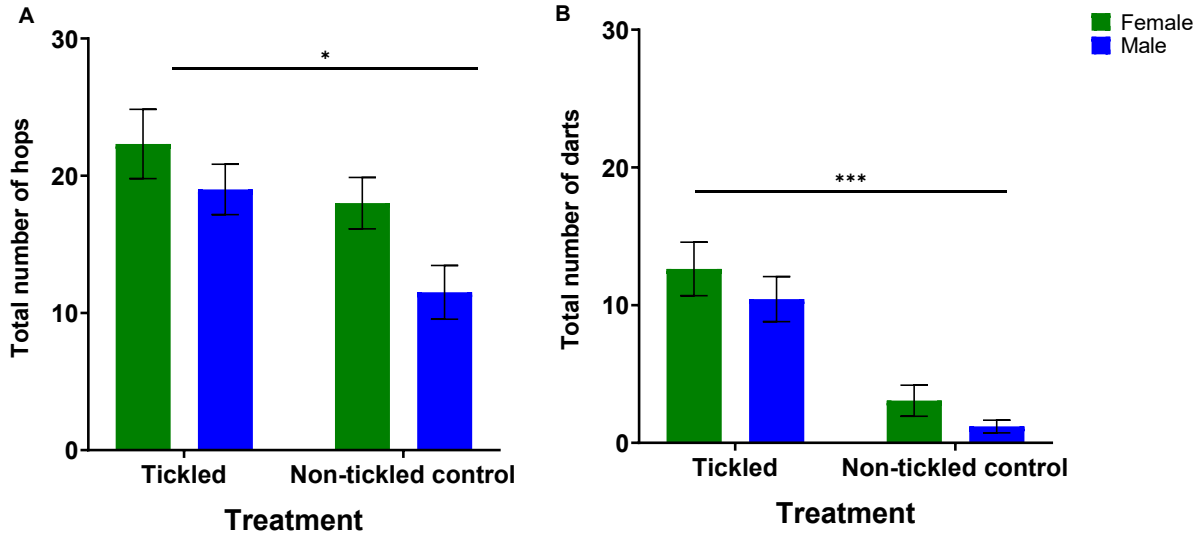

Figure 8. Solitary play behaviour in rats was affected by treatment.

The total number of hops made (A), and the total number of darts made (B), during 2 minutes of testing on the final day (day 10) in female and male rats. Female rats (green) and male rats (blue). Mean ± SEM; n = 16 / group. * denotes $p < 0.05$; *** denotes $p \leq 0.001$ for significant effect of treatment.

Approach to the hand (day 10)

There was no significant interaction between sex x treatment on approaches to the hand ($F_{1, 60} = 0.61$, $p = 0.437$; Fig. 9A). Sex ($F_{1, 60} = 17.76$, $p < 0.001$) and treatment ($F_{1, 60} = 9.58$, $p = 0.003$) both affected approaches towards the hand (Fig. 9A). There was no significant interaction between sex x treatment on latency to approach the hand ($F_{1, 60} = 0.34$, $p = 0.562$), although pairwise comparisons suggested a trend for tickled females to take the least amount of time to approach the hand (mean $_{\text{tickled female}} = 5.63 \pm 6.60$) and control males look the longest amount of time (mean $_{\text{control male}} = 26.25 \pm 6.60$; Fig. 9B). Latency to first approach the hand was affected by sex ($F_{1,60} = 4.91$, $p = 0.03$) and treatment ($F_{1,60} = 4.85$, $p = 0.031$; Fig. 9B).

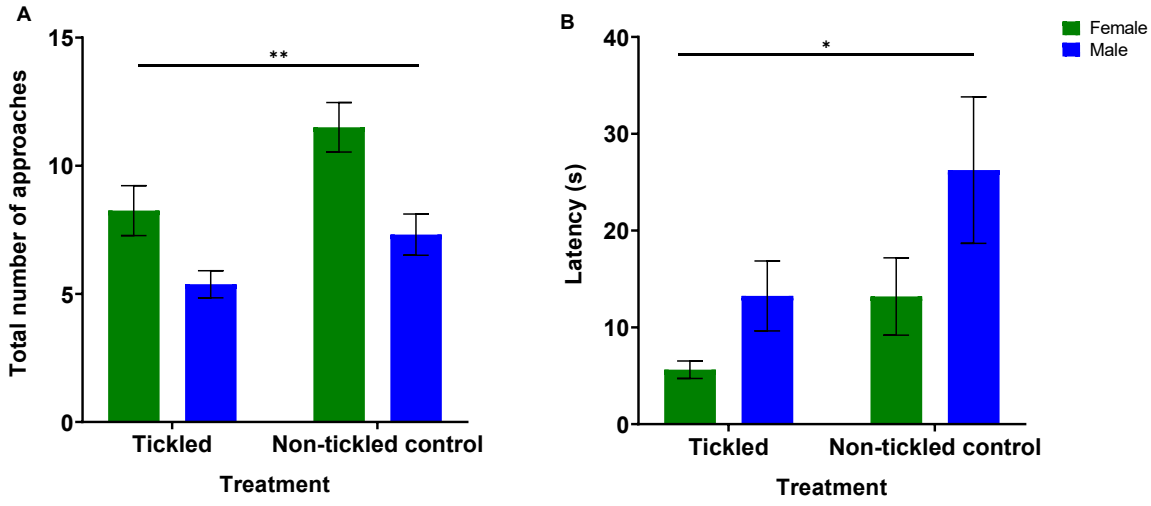

Figure 9. Approach to the hand by rats on day 10.
Total number of approaches made towards the hand (A), and the latency in seconds to first approach the hand (B), during 2 minutes of testing on the final day (day 10) in female and male rats. Female rats (green) and male rats (blue). Mean ± SEM; n = 16 / group. * denotes $p < 0.05$; ** denotes $p \leq 0.01$ for significant effect of treatment.

2.3.3 The distribution of 50 kHz USVs during 'tickle' versus 'release' sessions on day 10

Flat 50 kHz USV calls (day 10)

There was no significant interaction between sex x treatment on the number of flat 50 kHz USVs produced during the release sessions ($F_{1,60} = 1.13$, $p = 0.292$; Fig. 10A). While this should be interpreted cautiously, in a pairwise comparison, there was a trend for tickled females to produce more flat USVs during release sessions compared to the other three groups (mean $_{femalecontrol} = 37.43 \pm 9.39$, mean $_{femaletickled} = 67.2 \pm 9.39$, mean $_{malecontrol} = 19.6 \pm 9.39$, mean $_{maletickled} = 35.3 \pm 9.39$). There was an overall effect of sex ($F_{1,60} = 13.97$, $p < 0.001$) and treatment ($F_{1,60} = 11.68$, $p = 0.001$) on the number of flat 50 kHz USVs produced during the release sessions (Fig. 10A). There was no significant interaction between sex x treatment on the number of flat 50 kHz USVs produced during the tickle sessions ($F_{1,60} = 0.53$, $p = 0.468$; Fig. 10B). Similarly, in a pairwise comparison, there was a trend for tickled females to produce more flat USVs than the other groups (mean $_{femalecontrol} = 36.1 \pm 12.8$, mean $_{femaletickled} = 89.9 \pm 12.8$, mean $_{malecontrol} = 20.3 \pm 12.8$, mean $_{maletickled} = 60.8 \pm 12.8$). There was an overall effect of sex ($F_{1,60} = 6.11$, $p = 0.016$) and

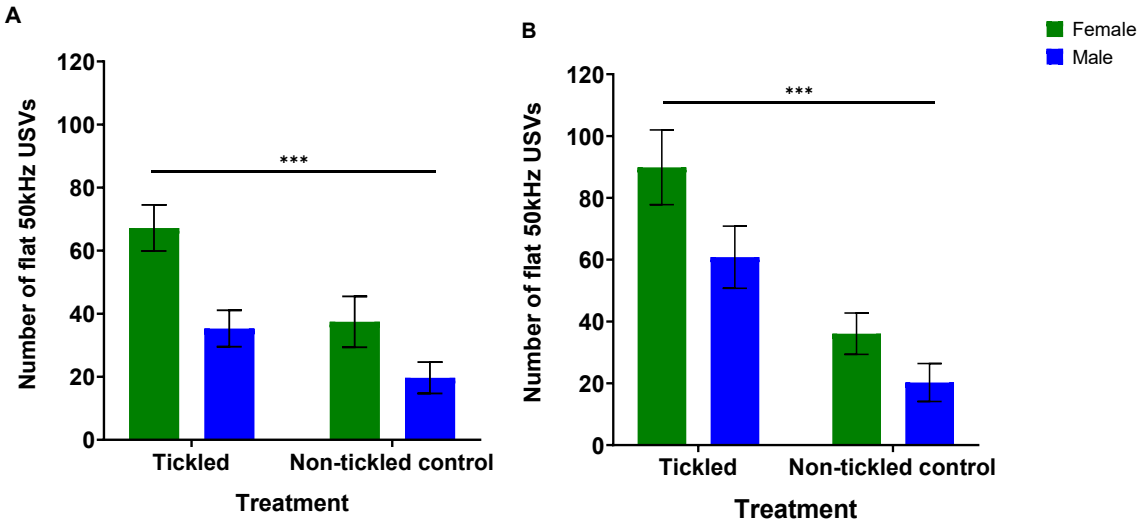

Figure 10. Flat 50 kHz USVs emitted during release and tickle sessions were affected by treatment and sex.

The number of flat 50 kHz USVs produced during the release sessions (A), and the number of flat 50 kHz USVs produced during the tickle sessions (B). Female rats (green) and male rats (blue). Mean ± SEM; n = 16 / group. *** denotes $p \leq 0.001$ for significant effect of treatment.

treatment ($F_{1,60}$ = 27.02, p < 0.001) on the number of flat 50 kHz USVs produced during the tickle sessions (Fig. 10B).

Trill 50 kHz USVs calls (day 10)

There was no significant interaction between sex x treatment on the number of trill 50 kHz USVs produced during release sessions ($F_{1,60}$ = 2.19, p = 0.1.45; Fig. 11A). There was an overall effect of treatment ($F_{1,60}$ = 14.52, p < 0.001) but no effect of sex ($F_{1,60}$ = 2.67, p = 0.107) on the number of trill 50 kHz USVs produced during release sessions (Fig. 11A). There was a significant effect of treatment ($F_{1,60}$ = 55.87, p < 0.001) on the number of trill 50 kHz USVs produced during tickled sessions (Fig. 11B). Similar to the number of trills produced during periods of release, there was no effect of sex ($F_{1,60}$ = 2.16, p = 0.146) or the interaction between sex x treatment ($F_{1,60}$ = 1.64, p = 0.206), likely due to tickled females and tickled males producing very similar numbers of trills (mean $_{femalecontrol}$ = 41.3 ± 8.26, mean $_{femaletickled}$ = 77.5 ± 8.26, mean $_{malecontrol}$ = 25.3 ± 8.26, mean $_{maletickled}$ = 76.4 ± 8.26).

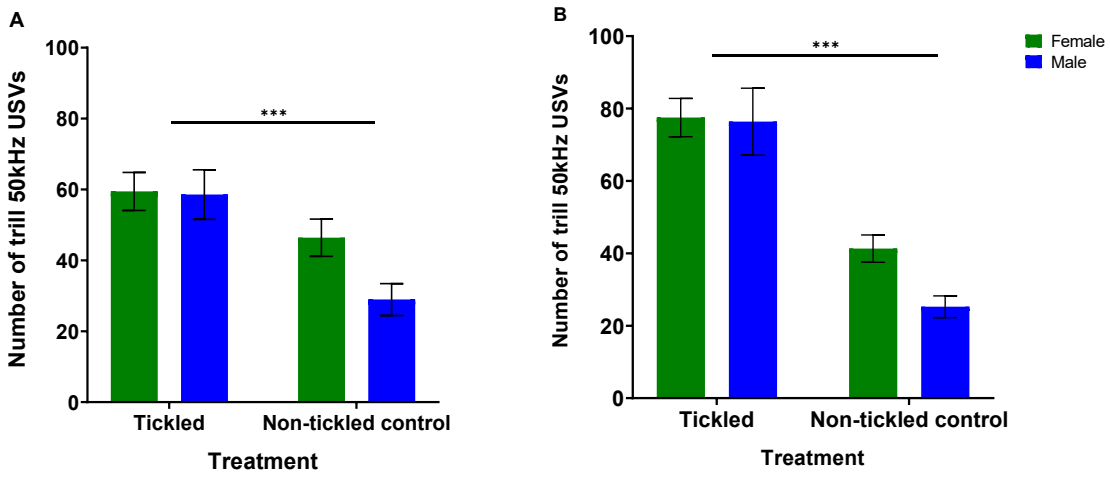

Figure 11. Trill 50 kHz USVs emitted during release and tickle sessions were affected by treatment, but not sex.

The number of trill 50 kHz USVs produced during the release sessions (A), and the number of trill 50 kHz USVs produced during the tickle sessions (B). Female rats (green) and male rats (blue). Mean ± SEM n = 16 / group. *** denotes p ≤ 0.001 for significant effect of treatment.

2.3.4 Anxiety levels measured on the elevated plus maze

Male and female rats did not differ in their anxiety levels at the start of the study (Table 2). There was no significant difference between male and female rats in the total time spent in the closed arms of the EPM ($t = -1.81$, $p = 0.076$), the number of entries made into the open arms of the maze ($t = 1.44$, $p = 0.155$), the total time spent in the open arms of the maze ($t = 1.74$; $p = 0.088$) or the latency to first enter one of the open arms ($t = -0.97$, $p = 0.336$).

Table 2. Behavioural measures in the EPM before testing began. Sex had no significant effect on baseline anxiety levels before testing commenced.

Measure	Mean ± SEM	
	Male	Female
Total time spent in the closed arms (s)	98.4 ± 4.8	85.9 ± 4.9
Number of entries made into the closed arms	12.6 ± 0.86	12.3 ± 0.54
Number of entries made into the open arms	13.3 ± 0.76	14.8 ± 0.72
Total time spent in the open arms (s)	137.5 ± 5.6	151.7 ± 6.02
Latency to first enter one of the open arms (s)	48.7 ± 4.8	41.6 ± 5.5

2.3.5 Body weight

Male rats had a significantly greater average daily live body weight gain compared to female rats ($F_{1, 60} = 121.1$, $p < 0.001$, mean $_{males} = 7.16$g, mean $_{females} = 5.07$g), but there was no significant effect of treatment on weight gain ($F_{1, 60} = 0.05$, $p = 0.827$, mean $_{tickled} = 6.13$g, mean $_{control} = 6.09$g), or interaction of sex x treatment ($F_{1, 60} = 0.34$, $p = 0.563$; Fig. 12). In a regression analysis, there was a negative relationship between average daily live weight gain and 50 kHz USV production ($F_{1, 61} = 23.14$, $p < 0.001$), flat 50 kHz USVs ($F_{1, 61} = 12.24$, $p = 0.001$) and trill 50 kHz USVs ($F_{1, 61} = 5.31$, $p = 0.025$).

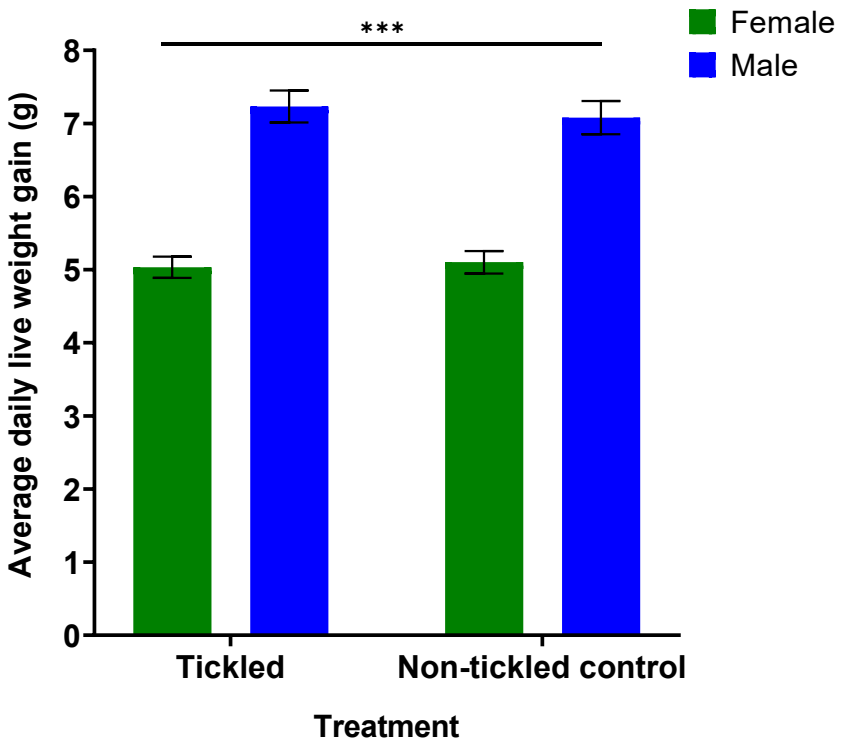

Figure 12. Average daily live weight gain was higher in male compared to female rats.

Average daily live weight gain of rats across the three week testing period. Female rats (green) and male rats (blue). Mean ± SEM; n = 16 / group. *** denotes $p \leq 0.001$ significance.

2.4 Discussion

Tickling elicited behavioural responses in both sexes, and tickled rats produced more 50 kHz ultrasonic vocalisations than controls which is in line with previously reported studies (e.g. Burgdorf and Panksepp 2001, LaFollette et al. 2018). In a related study in juvenile Wistar rats, 50 kHz USV production increased over time in response to tickling in both sexes (Mällo et al. 2007). In the current study there are sex differences in the rates of calling with female rats producing significantly more total 50 kHz USVs and flat 50 kHz USVs. There was no significant interaction of treatment and sex, however, the data suggested a trend towards tickled females producing the most 50 kHz calls compared to any other group, although this needs to be interpreted within the context of the non-significant interaction. Other studies have showed contrasting results in terms of sex differences in the response to tickling: adult male Long-Evans hooded rats were found to produce more trill 50 kHz USVs during tickling compared to females (Panksepp and Burgdorf, 1999). In another study, female Long-Evans hooded rats produced more 50 kHz USVs than male rats and the author's postulate that this may be due to the sex of the experimenter (LaFollette et al., 2018). Together these findings indicate that there may be sex differences in the rate of 50 kHz USVs produced during tickling; these sex differences may be affected by strain, and strain differences have been previously reported in social play behaviours and the number of 50 kHz USVs produced during play (Manduca et al., 2014).

On the final day of testing, there was both a sex and treatment effect on the total number of 50 kHz USVs. However, on examination of the 50 kHz USV subtypes, flat 50 kHz USVs were affected by sex and treatment, but trill 50 kHz USVs were only affected by the treatment and not sex. Flat 50 kHz USVs are thought to act as social communication signals (Burgdorf et al., 2011; Burke et al., 2017; Wöhr et al., 2008) and it is not altogether clear if they are always produced in positive affective states (Wöhr et al., 2008; Burgdorf et al., 2008). There are a growing number of studies which support the hypothesis that trill 50 kHz USVs are related to reward and positive affect (e.g. Burgdorf et al., 2001; Burgdorf et al., 2008; Burgdorf et al., 2011, Brudzynski, 2013).

Therefore, the findings from this study suggest that both female and male rats emit trill 50 kHz USVs in response to tickling, indicating that tickling is a positive, rewarding stimuli for females as it is for males. The role of flat 50 kHz USVs, which were found to be produced by tickled rats significantly more than controls, during tickling is intriguing and at present, unknown. To investigate this further, flat and trill USV subtypes were analysed with the temporal aspect of whether the tickled rats were in a period of release (not being touched) or a period of being tickled. There was again evidence of flat and trill 50 kHz USVs being affected by different factors: flat 50 kHz USVs were likewise affected by treatment and sex in both release and tickle sessions, but trill production was independent of sex, with female and male tickled rats producing more trills during both release and tickle session than control rats again supporting that trills reflect a positive affective response. This again provides evidence for trill 50 kHz USV production during tickling being independent of sex and that trills are emitted at a similar rate between release and tickle sessions in females and males. Further investigation is required to ascertain whether flat 50 kHz USVs serve different roles for each sex.

Taken together, the USV results suggest that trill USVs are produced independently of sex in response to tickling, but that flat 50 kHz are emitted in a sex-specific way. There are other subtypes of 50 kHz USVs not studied here (Wright et al., 2010) that also could be affected by sex. As with previous studies (Panskepp and Burgdorf, 2003; Burgdorf et al., 2008; Cloutier, Panksepp and Newberry, 2012; LaFollette et al., 2018), 22 kHz USVs remained low throughout testing and we found no effect of sex or treatment. Thus, the tickling protocol used in this study was not found to be aversive and this data provides further evidence for tickling being a seemingly positive experience for juvenile female and male rats.

Approach behaviours to the hand decreased across the 10 days of testing and tickling did not affect the number of approaches made or the latency to approach. This finding is interesting because hand approach behaviour is often used as an indicator of whether tickling is a rewarding experience (Burgdorf et al., 2011). A number of studies have found a positive

outcome of tickling on reducing approach latency and increasing the number of contacts with the human hand (e.g. Burgdorf and Panksepp, 2001, Cloutier, Panksepp and Newberry, 2012). Therefore, it is surprising that there was no effect of tickling on approach to the hand over the 10 days of testing in the present study, although Cloutier and Newberry (2008) report that there was no effect of tickling in a Human Approach test, which assessed rat motivation for human contact. Similarly, Hinchcliffe et al. (2020) found no relationship between approach latency and either 50 kHz calling during tickling or the affective bias test, showing that in their study hand approach did not reflect tickle-induced positive affect. LaFollette et al. (2018) measured approach behaviours before and after tickling in male and female rats, finding that male rats that had been tickled for 5 days had longer latency to approach the hand than male rats that had been tickled for 1 day, and they report that tickling seemed to have little positive effect on approach behaviours. Different methods for evaluating approach have been used, such as evaluating approach directly after tickling (LaFollette et al., 2017). In the present study, approach to the hand was assessed during the testing phase and so may not be a reliable measure of the responses to testing; this was revised for future experiments. However, by the end of testing, sex and treatment did have an effect on approach behaviours, but rather surprisingly, female control rats were found to approach the hand the most, and tickled males the least. The latency to approach was affected by sex and treatment, with tickled females taking the least amount of time to approach the hand and control males the most. Such inconsistent effects of tickling on measures of approach have been reported in previous studies (LaFollette et al., 2018; Hammond et al., 2019; Hinchcliffe et al., 2020), suggesting that measuring approach behaviours is an inconsistent measure of the response to tickling compared to measuring USVs.

Scampering, a solitary play behaviour where rats dart and hop, was increased in tickled animals in the test arena, and tickled rats significantly increased their scampering across the testing phase. By day 10 of the present study, treatment had an effect on scampering, as well as on hopping and darting components of solitary play behaviours. As scampering was increased

in tickled rats, it was also expected that hopping and darting would be increased. These darts and hops have been described by Panksepp and Burgdorf (1999) during tickling and they are hypothesised as representing the playful nature of tickling. Hammond et al. (2019) also found tickling increased solitary play events in young male Wistar rats in the home cage and prior to release into the test arena. A sex effect was detected across the 10 days of testing, although this was likely due to the tickled females, and by day 10 there was no sex effect detected. This finding is corroborated by previous studies that have found no sex differences in solitary play in juvenile rats (e.g. Pellis and Pellis, 1983). Therefore, tickling appears to increase the frequency of these solitary play behaviours in young rats, but this is likely not dependent on sex.

There was no detectable sex difference in baseline anxiety in the juvenile rats during testing on an elevated plus maze. Both male and female juvenile rats entered the open arms of the plus maze and spent time in those open arms. This is consistent with the findings of Imhof et al. (1993) who reported no sex differences in Wistar rats up to 60 days of age. This suggests that in the present study young rats showed low levels of anxiety and this is independent of sex (Imhof et al., 1993).

Tickling had no effect on body weight gain over the 10 days and this finding is similar to that published by Cloutier et al. (2012) in which they tickled male Sprague-Dawley rats. There was a negative relationship between 50 kHz USVs and trill and flat subtypes, and daily weight gain. Hammond et al. (2019) report a significant positive correlation between the weight at the start of 10 days of tickling and 50 kHz USVs produced in adolescent male Wistar rats, although by the end of testing there was no relationship between final weight and 50 kHz USVs. Brown et al. (2015) found that in piglets, an increase in body weight gain was positively associated with the amount and type of play in which the piglets engaged. However, in the present study, the negative relationship was likely due to the effect of sex on body weight, with males being significantly heavier than females, and the sex differences in 50 kHz USV types.

Conclusions

Tickling is associated with positive affective states in both female and male juvenile Wistar rats, as shown by higher levels of 50 kHz USV and in particular trill 50 kHz USV production. Flat 50 kHz USVs were emitted differently by female and male rats regardless of treatment, and may therefore have a sexually dimorphic role in rats, for example related to communication. Trill 50 kHz USV emission was not different between sexes, and is therefore likely to be a good indicator of positive affective states in both female and male rats. Similarly, scampering behaviour during tickling was also increased in tickled rats, suggesting that it is associated with positive affect. Scampering may be useful as an indicator of positive states that does not require recording equipment, if tickling is being implemented as an intervention to improve laboratory rat welfare. Conversely, hand approach behaviours did not differ between sex or treatment which supports previous evidence that hand approaches or latency to approach may not be a sufficiently sensitive measure of affect.

Chapter 3

Coupling 50 kHz ultrasonic vocalisations (USVs) subtypes with play and non-play related behaviours.

3.1 Introduction

During social situations, rats emit different types of vocalisation, both audible and ultrasonic, that are distinct in their frequency and modulation (Burgdorf and Panksepp, 2001). Ultrasonic vocalisations (USVs) in the 40 kHz frequency range are emitted by rat pups when they are isolated from their mother (Noirot, 1968) and these trigger pup retrieval behaviours in the dam (Allin and Banks, 1972). 22 kHz and 50 kHz USVs are thought to reflect certain emotional states in both juvenile and adult rats (Simola and Brudynski, 2018; Burgdorf and Panksepp, 2001). 22 kHz USVs are associated with aggression (Panksepp et al., 2004) and exposure to a predator (Blanchard et al., 1991) and are thought to be used as alarm cries (Litvin et al., 2007). On the other hand, 50 kHz USVs are produced by rats in response to hedonic stimuli, for example, during conspecific rough-and-tumble play, sexual behaviours and anticipation of a food reward (Burgdorf et al., 2011). 50 kHz USVs are suppressed by aversive stimuli, such as foot shocks or social defeat (Burgdorf et al., 2011).

Juvenile rats emit 50 kHz USVs during tickling, a heterospecific playful handling technique (e.g. Panksepp and Burgdorf, 2000 and 2003, Cloutier et al., 2013, Hammond et al., 2019). The 50 kHz USVs can be classified into a number of distinct subtypes of calls; Wright et al. (2010) described 14 categories of 50 kHz calls, with 13 categories having some degree of frequency modulation. Whilst the role of different frequency-modulated 50 kHz call subtypes has yet to be explained, it is widely accepted that frequency-modulated, or ‘trill’, 50 kHz USVs have a different meaning to non-frequency-modulated, or ‘flat’, 50 kHz USVs (Burgdorf et al., 2008; Burke et al., 2020). Trill 50 kHz USVs have been associated with positive affect in young rats

(Burgdorf et al., 2008) and are emitted during rewarding social interactions like mating, conspecific rough-and-tumble play and tickling (Burgdorf et al., 2008). Flat 50 kHz USVs are thought to have a social communicatory role (Wöhr et al., 2008; Wright et al., 2010; Burke et al., 2017a; Burgdorf et al., 2011). Different USV subtypes are produced in different contexts, so it is likely that trill and flat 50 kHz USVs may have a different communicative significance (Wright et al., 2010), for example, sharing specific information to conspecifics (Burke et al., 2018).

To address the question of which 50 kHz USV subtypes are important for different behavioural functions, several studies have investigated the co-occurrence of calls with behaviours (Burke et al., 2020). A fascinating example of when rats emit calls in relation to behaviour came from Reinhold et al. (2019) who found that during a hide and seek paradigm, adult male Long-Evans hooded rats emitted 50 kHz USVs differently depending on whether the rat was in the 'hide' or the 'seek' role. The authors describe complexity in the call profiles as well as flat and trill calls being the most frequent (Reinhold et al., 2019). In the 'hide trials', rats produced low numbers of 50 kHz USVs (irrespective of call type) compared to 'seek trials' where the rats generally called more (Reinhold et al., 2019) suggesting a role for calls in coordinating play, however, flat and trill subtypes were not explored in detail. Different call subtypes were investigated by Burke et al. (2017a), and different subtypes were produced in relation to specific social behaviour during anticipation of play in juvenile male Long-Evans rats. Calls involving trills were found to be correlated with slow movements, such as walking, although there did not appear to be a binary relationship between any one movement and call subtype (Burke et al., 2017a). The pairing of trill calls with walking was later replicated in male Wistar rats (Burke et al., 2021). Similarly, during social play, different calls may be correlated with different play behaviours, such as trill USVs being associated with play-initiating nape contacts and approaching the play-mate (Burke et al., 2018). Together this evidence suggests that male rats couple certain call subtypes with behaviour which may serve to communicate to another rat during play (Burke et al., 2020). So far, the relationship between

call subtypes and behaviours has mostly been explored in males, with few studies investigating calls in relation to behaviour in female rats. Himmler et al. (2014) found that in both sexes, pairs of rats vocalized more before a playful contact than after a contact. These vocalisations were found to be mostly made up of trill 50 kHz USVs, although there was no direct evidence that these calls were being used strategically by the rats (Himmler et al., 2014).

One limitation with using conspecific play between two rats to investigate USV-behaviour pairings is that it is very difficult to determine which rat is calling (Himmler et al., 2014; Burke et al., 2020) unless one rat has been devocalized (Burke et al., 2018). The tickling paradigm may be a useful tool with which to investigate call-behaviour associations as tickling can be carried out with an individual rat (e.g. LaFollette et al., 2017). There are discreet behaviours that are associated with tickling such as approaches and scampers (Hammond et al., 2019; Chapter 2). Approach behaviours are often used in tickling studies as a measure of the rewarding aspect of tickling (Burgdorf and Panksepp, 2001; Hammond et al., 2019), while scampering is a solitary play behaviour, comprised of hopping and darting (Lampe et al., 2017; Melotti et al., 2014; Hammond et al., 2019). It has yet to be investigated whether rats also pair their 50 kHz USVs with behaviours seen during tickling in a similar way to that recorded during conspecific play.

The data presented in Chapter 2 showed that female rats produced significantly more total 50 kHz USVs in response to tickling than male rats, in particular flat 50 kHz USVs, whereas tickled rats, regardless of sex, produced significantly more trill 50 kHz USVs. The aim of this chapter was to investigate whether the two call subtypes, flat and trill 50 kHz USVs, were associated with certain play-related behaviours in a sex specific way. I tested the hypothesis that trill 50 kHz USVs are associated with tickled-induced play behaviours in both female and male rats, and that flat 50 kHz USVs accompany play-related behaviours in a sex-specific way. I expected females to pair flat USVs more with play-related behaviours as there was a trend in Chapter 2 for females to produce higher rates of flat 50 kHz USVs. I used exploration and running as

two non-play related behaviours to compare the number of 50 kHz USV subtypes produced during behaviours that are not associated with play.

3.2 Materials and Methods

Please refer to Chapter 2: Materials and Methods for detailed Animal and Husbandry, and Experimental Testing.

3.2.1 Subjects

In brief, 64 juvenile Wistar rats (23-24 days of age; Replicate 1: Females 41.0-69.1g, Males 48.6-75.5g body weight; Replicate 2: Females 39.1-62.4g, Males 42.7-64.3g body weight) were sampled (32 per replicate; Charles River, Kent, UK). Each replicate was split evenly between males and females. Treatment (Control or Tickled) was randomly assigned to each rat, balancing for body weight and littermates so that average body weights for each treatment group for each sex were comparable. Rats were pseudo-randomly assigned to groups, balanced for body weight (Matsuzaki et al., 2018) and litter, in same-sex pairs with each cage containing a tickled and a control rat.

3.2.2 Experimental design

In brief, rats were acclimatised for a period of five days before being habituated for a further five days in the testing arena. After the habituation in the testing arena phase, the animals began ten days of testing with each rat being tested for two minutes. Each test session was recorded using a video camera and ultrasonic microphone for later quantification of behaviour and ultrasonic vocalisations respectively. One cage mate was taken from the home cage and placed in the testing arena: for tickled animals, the researcher's hand (wearing a white cotton glove) was placed motionless on one wall of the testing arena for the first 15 seconds of testing, followed by 15 seconds of tickling where the researcher made rapid finger movements on the nape of the neck and if the rat reared at the hand, on the ventral side. These 15 second bouts of tickling and release were alternated for the two minutes of testing. Control animals were placed in the testing arena for two minutes, with the hand of the researcher (wearing a white cotton glove) resting motionless on one wall of the testing arena. Following the two minutes in the testing arena, the rat was picked up (without the cotton glove but wearing nitrile gloves) and weighed before being returned to the home cage. The other cage mate was then

removed and placed in the arena for testing. The order in which the cage mates were tested was alternated each day. Testing was carried out in the first three hours of the dark phase. This was carried out every day for the ten days of testing.

3.2.3 Measurements

USVs

An ultrasound microphone (Pettersson M500-384 USB Ultrasound microphone, Pettersson Electronik, Sweden) and Audacity software (Version 2.1.3, Pennsylvania, United States of America) were used to record USVs. Spectrograms of USVs were collected and the number of trill and flat 50 kHz USVs emitted on the final day of testing were counted manually in Ultravox 14 (Noldus Information Technology, Wageningen, Netherlands) using call parameters outlined by Wright et al. (2010) (see Table 1).

Testing arena behaviour

Video camera (Panasonic HD HC-V10) footage was obtained during testing for all animals across all days. Behaviour was analysed in Observer 15 (Noldus Information Technology, Wageningen, Netherlands) by the experimenter who was blinded to sex but not to treatment due to being able to see whether individual rats were tickled or not in the digital recording. The total number of hops, darts, approaches, exploration and runs were scored in Observer 15 using the ethogram shown in Table 1. Hops, darts and approaches were assigned as being ‘play related’ behaviours because they are observed during conspecific and heterospecific play in rats (Richter et al., 2016; Melotti et al., 2014; Hlinak and Madlafousek, 1977; Vanderschuren and Trezza, 2013; Pellis and Pellis, 1983; Ishiyama and Brecht, 2016; Hammond et al., 2019). Exploration and runs were assigned as ‘non-play related’ behaviours because they are described as locomotion which is not associated with playful behaviours such as scampers or jumps (e.g. Hughes, 1968 and Pellis and Pellis, 1983). These behaviours were used to compare the number of USVs produced during or before non-play related locomotion and during or one second before locomotor play behaviours. A one second duration before

a behaviour was selected to allow for human error in coding behaviour times (Burke et al., 2018) in addition to whether there was emission of USVs in the anticipation of play-related behaviours (Knutson et al., 1998) given that there may be association of calls with a behaviour up to 600 milliseconds before the call is emitted (Burke et al., 2020).

Synchronising USVs and observed behaviours

USV data were imported into Observer 15 from Ultravox 14. The video footage and sound files from Ultravox were then played concurrently and the behaviours scored using the ethogram (Table 1) which was written in Observer 15. This generated a file for each animal where the behaviours and USVs can be temporally compared: the USVs produced by the rat during or in the one second leading up to a behaviour of interest were counted. Using Observer 15, data profiles were built for each group (tickled female, tickled male, control female, control male) and the number of flat or trill 50 kHz USVs made during, and one second before any of the scored behaviours were counted.

Table 1. Ethogram used to carry out the behavioural analysis of the rats response to tickling.

Behaviour	Definition	Reference
Exploration	Sniffing directed at the environment, either when still or during slow walking, including rearing behaviours. Each bout of sniffing (with or without rearing) was measured- a bout was determined as >1 second participating in the behaviour.	Hughes, 1968; Mällo et al., 2007.
Run	Locomotion which is not locomotor play, so does not include scampering, hopping or darting. Slower locomotion than darting, at least one paw is on the floor at any given time, not directed at or in response to the hand, usually in one direction. A bout was determined as >1 second participating in the behaviour.	Pellis and Pellis, 1983.
Hop	'Joy-leaps'/ 'jerk-jumps'. One hop was determined as when all four feet left the floor.	Richter et al., 2016; Melotti et al., 2014; Hlinak and Madlafousek, 1977.

Dart	Rapid darting movements, locomotion with frequent changes in direction. One dart was determined as a rapid locomotory movement in the absence of sniffing, lasting >1 second, usually in a circular motion returning to the hand.	Vanderschuren and Trezza, 2013; Melotti et al 2014; Pellis and Pellis, 1983; Hlinak and Madlafousek, 1977
Approach	Forward motion, directed movements including rears directed at the hand of the experimenter. One approach was determined as a forward locomotor movement directed towards the hand ending in the rat touching the hand with the nose.	Ishiyama and Brecht, 2016.
Flat 50 kHz USV	50 kHz calls with a near-constant frequency greater than 30 kHz. Flat-trill combination calls were not distinguished.	Wright, Gourdon and Clarke, 2010.
Trill 50 kHz USV	50 kHz calls with rapid frequency oscillations. Flat-trill combination calls were not distinguished.	Wright, Gourdon and Clarke, 2010.

3.2.4 Statistical analysis

Statistical analysis was carried out in R Studio and R (v 4.0.3, The R Foundation for Statistical Computing Platform (2020)). Model adequacy was verified by examination of residuals (McCullagh and Nelder, 2019) via the DHARMa package (Hartig, 2020). Generalised linear mixed models (GLMMs) using the glmmTMB package (Brooks et al., 2017) were used to compare frequencies of flat and trill 50 kHz USVs emitted before or during observed locomotory behaviours within allocated treatments. Dependent on model fitting and overdispersion, family links were set to either poisson or negative binomial distributed errors ('nbinom2' in the MASS package; Venables and Ripley, 2002) with default transformations. All models included batch and cage as random effects and were nested (batch followed by cage). This was done to account for the variation from the non-independence of rats from the same cage and potential batch effects. All models included both sex and treatment as fixed effects, as well as the interaction between them, with effects reported through ANOVA comparisons (Fox and Weisberg, 2019) to compare the differences between group means rather than the linear relationships between variables. Pairwise comparisons were identified and reported using the

emmeans package (Lenth, 2020), with statistical significance based on $p < 0.05$ threshold level and adjusted for multiple comparisons using the Tukey method (Lenth, 2020). All data in the tables are presented as ± standard error of the estimated marginal mean (SEM) and 95% confidence intervals (CI). All graphs were generated in GraphPad Prism (version 9 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com). The data in the graphs are presented as the means of the raw data ± standard error of the mean (SEM).

3.3 Results

3.3.1 USV production associated with play-related behaviours

Hopping

Within the control group, female rats had a higher mean frequency of flat 50 kHz USVs emitted in the one second before hopping compared to males (t_{ratio} = 4.254, p = 0.0004) and the same was seen within the tickled group (t_{ratio} = 2.729, p = 0.0407) (Table 2, Fig. 1). During hopping, there was no interaction of treatment and sex ($X^2_{(1,63)}$ = 0.31, p = 0.5768), as control female and male rats emitted similar numbers of flat USVs and the same was true for USV production during hopping in tickled female and male rats (Table 2). There was a sex difference in flat 50 kHz USVs when paired with hopping (Table 2). Female rats emitted more flat 50 kHz USVs before ($X^2_{(1,63)}$ = 11.16, p = 0.0008), and during ($X^2_{(1,63)}$ = 7.78, p = 0.0053) hopping compared to male rats. Overall tickled rats produced more flat USVs in the one second before ($X^2_{(1,63)}$ = 86.02, p < 0.0001) and during ($X^2_{(1,63)}$ = 15.35, p < 0.0001) hopping compared to control rats, this was highest in tickled females before hopping ($X^2_{(1,63)}$ = 7.22, p = 0.0072; Table 2).

The interaction between treatment and sex had an overall effect on the frequency of trill USVs emitted in the one second before hopping ($X^2_{(1,63)}$ = 7.16, p = 0.0074) which was likely due to treatment as tickled female and male rats made more trills before hopping than control female or male rats (Table 3, Fig. 2). There was no significant interaction between treatment and sex on the frequency of trill USVs emitted during hopping ($X^2_{(1,63)}$ = 2.20, p = 0.1383). Treatment, but not sex, affected the pairing of trill 50 kHz USVs with hopping (Table 3). Tickled rats, regardless of sex, made more trill USVs in the one second before ($X^2_{(1,63)}$ = 121.10, p < 0.0001) and during ($X^2_{(1,63)}$ = 98.71, p < 0.0001) hopping compared to control rats (Table 3).

Darting

The interaction between treatment and sex did not have an overall effect on the frequency of flat USVs emitted in the one second before darting

($X^2_{(1,63)}$ = 3.47, p = 0.0624) and this is likely due to no differences between sexes in the control group (Table 2, Fig. 1). During darting, there was no interaction between treatment and sex ($X^2_{(1,63)}$ = 3.22, p = 0.0730). Female rats paired more flat USVs with darting than males (Table 2). Female rats had higher rates of flat USVs before ($X^2_{(1,63)}$ = 7.73, p = 0.0054) and during ($X^2_{(1,63)}$ = 6.62, p = 0.0101) darting compared to males (Table 2). Tickled rats made more flat USVs before ($X^2_{(1,63)}$ = 182.04, p < 0.0001) and during darting ($X^2_{(1,63)}$ = 339.12, p < 0.0001) compared to control rats (Table 2).

There was no effect of the interaction between treatment and sex on trill USVs before ($X^2_{(1,63)}$ = 0.06, p = 0.7938) or during darting ($X^2_{(1,63)}$ = 1.99, p = 0.1585; Table 3, Fig. 2). As with hopping, tickled rats, regardless of sex made more trill USVs in the one second before ($X^2_{(1,63)}$ = 103.76, p < 0.0001) and during ($X^2_{(1,63)}$ = 284.87, p < 0.0001) darting compared to control rats and there was no overall effect of sex (Table 3, Fig. 2).

Approaches to the hand

There was no effect of the interaction between treatment and sex before ($X^2_{(1,63)}$ = 0.33, p = 0.5643) or during approaches ($X^2_{(1,63)}$ = 1.36, p = 0.2434). There was a sex difference in flat 50 kHz USVs being paired with hand approaches (Table 2, Fig. 1). Female rats emitted higher rates of flat USVs in the one second before ($X^2_{(1,63)}$ = 9.10, p = 0.0026) and during ($X^2_{(1,63)}$ = 7.92, p = 0.0049) approaches to the hand compared to males (Table 2). Tickled rats made more flat USVs during approaches to the hand compared to control rats ($X^2_{(1,63)}$ = 5.27, p = 0.0217), but not before approaches ($X^2_{(1,63)}$ = 2.81, p = 0.0936; Table 2).

The interaction between treatment and sex had an overall effect on the frequency of trill USVs emitted in the one second before approaching the hand ($X^2_{(1,63)}$ = 16.74, p < 0.0001; Table 3, Fig. 2). Pairwise comparisons revealed that control male rats emitted lower mean frequencies of trill 50 kHz USVs in the one second before approaching the hand compared to tickled males (t_{ratio} = -5.37, p <0.0001) and control females (t_{ratio} = 2.97, p = 0.0219; Table 3). Similarly, the interaction between treatment and sex had an overall effect on

the frequency of trill USVs emitted during hand approaches ($X^2_{(1,63)} = 23.25$, $p < 0.0001$), with control males making fewer trill 50 kHz USVs during approaches than control females ($t_{ratio} = 2.94$, $p = 0.0236$), tickled females ($t_{ratio} = 3.11$, $p = 0.0149$) and tickled males ($t_{ratio} = -4.08$, $p = 0.0008$; Table 3). Control male rats did not pair trill 50 kHz USVs with hand approaches (Table 3). Tickled rats, regardless of sex, made more trill USVs in the one second before ($X^2_{(1,63)} = 12.13$, $p = 0.0005$) but not during ($X^2_{(1,63)} = 0.22$, $p = 0.6405$) hand approaches as compared to control rats (Table 3).

Table 2. Statistical output of generalised linear mixed model of flat 50 kHz USVs emitted before and during play-related behaviours. Significant effects ($p < 0.05$) shown in bold.

Behaviour	Before/ During	Fixed Effects	Levels	Estimated marginal mean	±SE	95% CI	Chi-Square	p-value
Hopping	Before	Treatment	Control	3.38	0.90	1.99-5.75	86.02	**<0.0001**
			Tickled	9.13	2.33	5.48-15.22		
		Sex	Female	9.09	2.53	5.21-15.87	11.16	**0.0008**
			Male	3.40	1.00	1.89-6.11		
		Treatment:Sex	Control-Female	6.36	1.83	3.58-11.32	7.22	**0.0072**
			Tickled-Female	13.00	3.64	7.42-22.77		
			Control-Male	1.80	0.58	0.95-3.41		
			Tickled-Male	6.42	1.87	3.58-11.51		
Hopping	During	Treatment	Control	3.66	0.77	2.40-5.58	15.35	**< 0.0001**
			Tickled	11.33	2.20	7.68-16.73		
		Sex	Female	9.65	1.89	6.52-14.30	7.78	**0.00053**
			Male	4.29	0.90	2.82-6.52		
		Treatment:Sex	Control-Female	5.94	1.68	3.37-10.47	0.31	0.5768
			Tickled-Female	15.69	4.26	9.11-27.02		
			Control-Male	2.25	0.70	1.20-4.21		
			Tickled-Male	8.19	2.28	4.69-14.29		
Darting	Before	Treatment	Control	0.65	0.16	0.40-1.07	182.04	**<0.0001**
			Tickled	8.61	1.50	6.07-12.21		
		Sex	Female	3.26	0.85	1.93-5.51	7.73	**0.0054**
			Male	1.72	0.48	0.99-3.00		
		Treatment:Sex	Control-Female	0.75	0.26	0.39-1.49	3.47	0.0624
			Tickled-Female	14.20	3.31	8.90-22.63		
			Control-Male	0.57	0.20	0.28-1.16		
			Tickled-Male	5.22	1.33	3.14-8.70		
Darting	During	Treatment	Control	0.71	0.19	0.42-1.2	339.12	**< 0.0001**
			Tickled	15.65	3.26	10.31-23.7		
		Sex	Female	4.91	1.50	2.66-9.04	6.62	**0.0101**
			Male	2.25	0.72	1.19-4.27		
		Treatment:Sex	Control-Female	0.89	0.32	0.43-1.84	3.22	0.0730
			Tickled-Female	26.96	7.72	15.19-47.83		
			Control-Male	0.58	0.21	0.26-1.20		
			Tickled-Male	9.08	2.72	4.99-16.54		
Approach	Before	Treatment	Control	3.15	0.49	2.32-4.30	2.81	0.0936
			Tickled	3.94	0.58	2.93-5.30		
		Sex	Female	5.30	0.96	3.69-7.61	9.10	**0.0026**
			Male	2.34	0.48	1.56-3.52		
		Treatment:Sex	Control-Female	4.92	0.96	3.32-7.28	0.33	0.5643
			Tickled-Female	5.71	1.10	3.89-8.39		
			Control-Male	2.02	0.48	1.26-3.24		
			Tickled-Male	2.71	0.60	1.74-4.22		
Approach	During	Treatment	Control	4.77	0.72	3.53-6.45	5.27	**0.0217**
			Tickled	6.16	0.90	4.60-8.26		
		Sex	Female	8.05	1.52	5.52-11.26	7.92	**0.0049**
			Male	3.65	0.74	2.43-5.48		
		Treatment:Sex	Control-Female	7.51	1.49	5.05-11.17	1.36	0.2434
			Tickled-Female	8.63	1.69	5.83-12.79		
			Control-Male	3.03	0.68	1.93-4.75		
			Tickled-Male	4.40	0.94	2.87-6.75		

Table 3. Statistical output of generalised linear mixed model of trill 50 kHz USVs emitted before and during play-related behaviours. Significant effects ($p < 0.05$) shown in bold.

Behaviour	Before/ During	Fixed Effects	Levels	Estimated marginal means	±SE	95% CI	Chi-Square	P-value
Hopping	Before	Treatment	Control	4.16	1.06	2.50-6.91	121.10	**<0.0001**
			Tickled	12.00	2.94	7.35-19.60		
		Sex	Female	8.40	2.19	4.98-14.2	1.35	0.2450
			Male	5.94	1.59	3.49-10.1		
		Treatment:Sex	Control-Female	5.60	1.53	3.24-9.68	7.16	**0.0074**
			Tickled-Female	12.58	3.31	7.43-21.29		
			Control-Male	3.09	0.89	1.74-5.48		
			Tickled-Male	11.45	3.04	6.73-19.47		
Hopping	During	Treatment	Control	4.44	0.78	3.12-6.31	98.71	**< 0.0001**
			Tickled	11.19	1.84	8.05-15.55		
		Sex	Female	9.05	1.77	6.12-13.39	3.83	0.0505
			Male	5.49	1.12	3.65-8.25		
		Treatment:Sex	Control-Female	6.11	1.28	4.01-9.29	2.20	0.1383
			Tickled-Female	13.42	2.65	9.04-19.93		
			Control-Male	3.23	0.74	2.04-5.09		
			Tickled-Male	9.33	1.91	6.20-14.04		
Darting	Before	Treatment	Control	0.57	0.16	0.33-1.01	103.76	**< 0.0001**
			Tickled	12.85	1.74	9.80-16.85		
		Sex	Female	3.07	0.63	2.03-4.62	0.65	0.4189
			Male	2.40	0.56	1.50-3.83		
		Treatment:Sex	Control-Female	0.67	0.24	0.33-1.39	0.06	0.7938
			Tickled-Female	13.97	2.64	9.57-20.40		
			Control-Male	0.49	0.20	0.22-1.11		
			Tickled-Male	11.82	2.31	7.99-17.49		
Darting	During	Treatment	Control	0.40	0.13	0.21-0.77	284.87	**< 0.0001**
			Tickled	20.09	4.17	13.26-30.44		
		Sex	Female	4.36	1.29	2.41-7.89	2.03	0.1541
			Male	1.84	0.65	0.91-3.73		
		Treatment:Sex	Control-Female	0.74	0.27	0.35-1.54	1.99	0.1585
			Tickled-Female	25.75	6.99	14.69-44.33		
			Control-Male	0.22	0.11	0.08-0.62		
			Tickled-Male	15.68	4.33	27.23-9.96		
Approach	Before	Treatment	Control	3.23	0.49	2.39-4.37	12.13	**0.0005**
			Tickled	5.42	0.74	4.12-7.13		
		Sex	Female	5.13	0.92	3.58-7.34	1.07	0.3015
			Male	3.42	0.65	2.34-5.00		
		Treatment:Sex	Control-Female	5.03	0.97	3.42-7.40	16.74	**< 0.0001**
			Tickled-Female	5.23	1.01	3.56-7.69		
			Control-Male	2.08	0.48	1.31-3.29		
			Tickled-Male	5.61	1.08	3.82-8.24		
Approach	During	Treatment	Control	5.77	0.82	4.34-7.68	0.22	0.6405
			Tickled	6.70	0.93	5.08-8.84		
		Sex	Female	7.51	1.38	5.20-10.84	1.52	0.2169
			Male	5.15	0.97	3.53-7.51		
		Treatment:Sex	Control-Female	8.73	1.66	5.97-12.76	23.25	**< 0.0001**
			Tickled-Female	6.46	1.26	4.37-9.55		
			Control-Male	3.82	0.81	2.51-5.81		
			Tickled-Male	6.95	1.35	4.71-10.25		

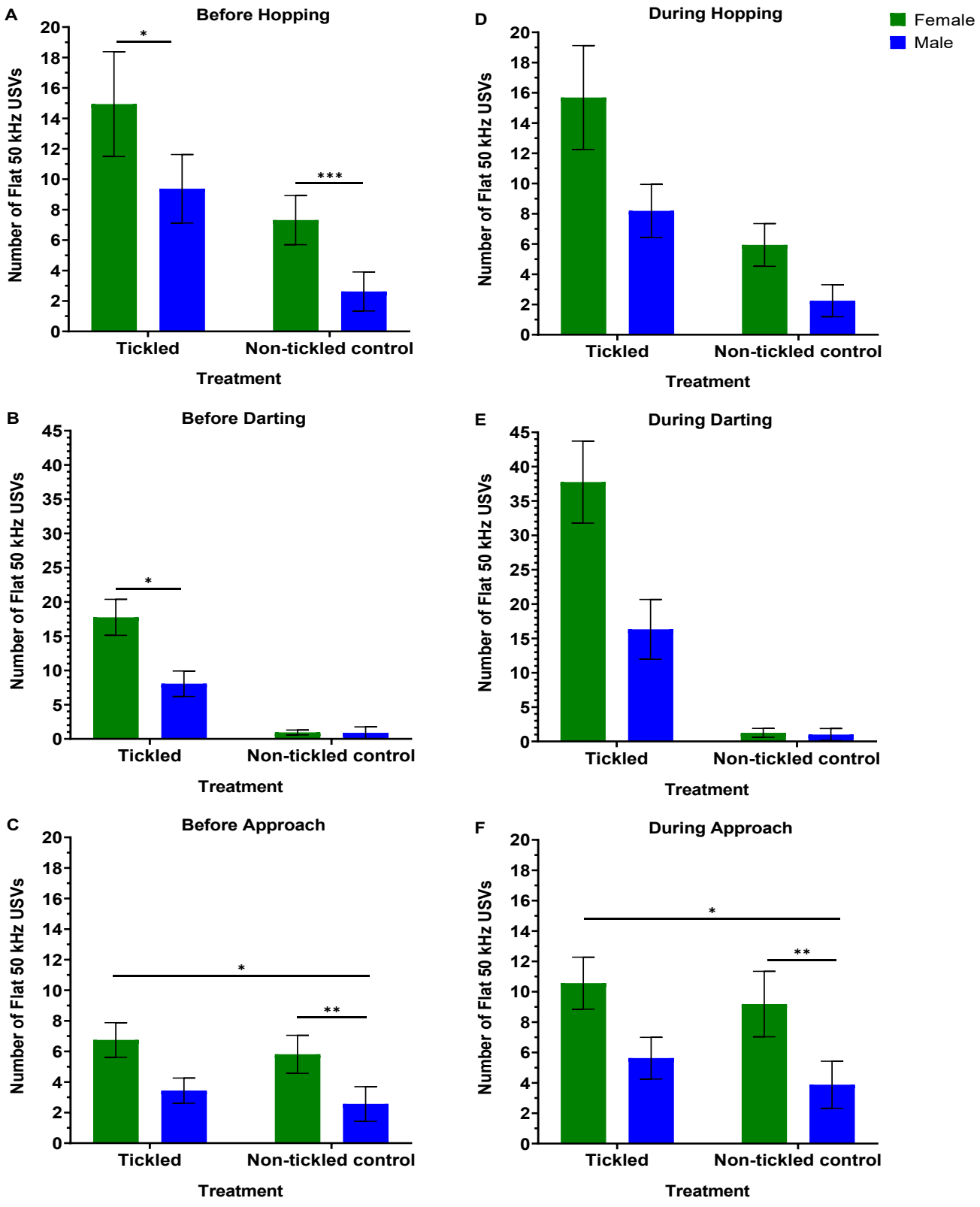

Figure 1. Flat USVs paired with play-related behaviours in tickled compared to control rats. Number of flat 50 kHz USVs produced in the one second before hopping (A), darting (B) and approach to the hand (C), and flat 50 kHz USVs produced during hopping (D), darting (E) and approach to the hand (F) 'play-related' behaviours. Females (green), males (blue). Mean ± SEM; n = 16 / group. * denotes $p \leq 0.05$; ** denotes $p \leq 0.01$; *** denotes $p \leq 0.001$; **** denotes $p \leq 0.0001$ for significant pairwise interactions.

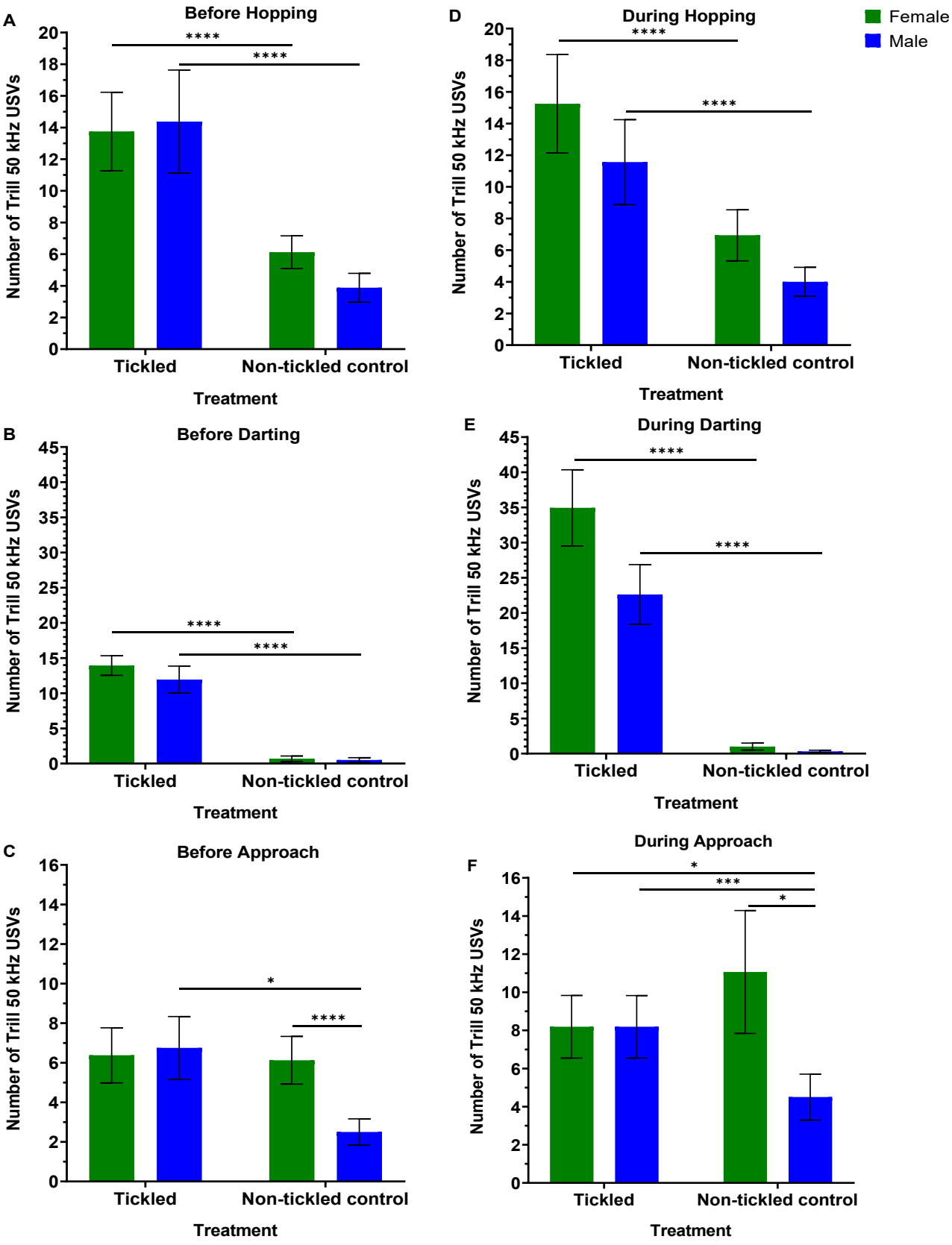

Figure 2. Trill USVs paired with play-related behaviours in tickled compared to control rats. Number of trill 50 kHz USVs produced in the one second before hopping (A), darting (B) and approach to the hand (C) 'play-related' behaviours, and trill 50 kHz USVs produced during hopping (D), darting (E) and approach to the hand (F) 'play-related' behaviours. Females (green), males (blue). Mean ± SEM; n = 16 / group. * denotes $p \leq 0.05$; ** denotes $p \leq 0.01$; *** denotes $p \leq 0.001$; **** denotes $p \leq 0.0001$ for significant pairwise interactions.

3.3.2 USV production associated with non-play-related behaviours

Exploring

The interaction between treatment and sex had an overall effect on the frequency of flat USVs emitted in the one second before ($X^2_{(1,63)}$ = 10.86, p = 0.0010) and during ($X^2_{(1,63)}$ = 22.41, p < 0.0001) exploring (Table 4, Fig. 3). Pairwise comparisons revealed differences between control and tickled males, with tickled males having a higher mean frequency of flat 50 kHz USVs emitted before (t_{ratio} = -4.35, p =0.0003) and during (t_{ratio} = -7.50, p < 0.0001) exploring than control males (Table 4). In contrast to play-related behaviours, there was a treatment, but not a sex, effect on flat USVs being paired with exploration (Table 4, Fig. 3). Irrespective of sex, tickled rats made more flat USVs before ($X^2_{(1,63)}$ = 8.062, p = 0.0045) and during exploration compared to control rats ($X^2_{(1,63)}$ = 34.98, p < 0.0001; Table 4).

The interaction between treatment and sex had an overall effect on the frequency of trill USVs emitted in the one second before ($X^2_{(1,63)}$ = 29.99, p < 0.0001) and during ($X^2_{(1,63)}$ = 116.11, p < 0.0001) exploration (Table 5, Fig. 4). Control females made more trill USVs before exploration than tickled females (t_{ratio} = 2.74, p = 0.0400), while tickled males had higher rates of trill USVs before exploration than control males (t_{ratio} = -5.10, p < 0.0001; Table 5). This was also observed during exploratory behaviour with control females making more trill 50 kHz USVs during exploration than tickled females (t_{ratio} = 7.15, p < 0.0001), and tickled males made more trills than control males (t_{ratio} = -8.14, p < 0.0001; Table 5). Within the tickled groups, males had a higher mean frequency of trill 50 kHz USVs during exploration compared to females (t_{ratio} = -4.09, p = 0.0008; Table 5). In contrast to play-related behaviours, tickling did not affect trill USVs when paired with exploration. There was no main effect of treatment (before: $X^2_{(1,63)}$ = 3.47, p = 0.0624; during: $X^2_{(1,63)}$ = 1.36, p = 0.2434) or sex (before: $X^2_{(1,63)}$ = 0.55, p = 0.4589; during: $X^2_{(1,63)}$ = 1.44, p = 0.2295) on the frequency of trill 50 kHz USVs paired with exploration (Table 5).

Running

The interaction between treatment and sex had no effect on flat 50 kHz USVs when paired with running (Before: $X^2_{(1,63)} = 3.33$, $p = 0.0679$; During: $X^2_{(1,63)} = 2.93$, $p = 0.0869$; Table 4, Fig. 3). Also, there was no effect of treatment (before: $X^2_{(1,63)} = 2.62$, $p = 0.1057$; during: $X^2_{(1,63)} = 3.31$, $p = 0.0689$) or sex (before: $X^2_{(1,63)} = 0.21$, $p = 0.6434$; during: $X^2_{(1,63)} = 2.73$, $p = 0.0986$; Table 4, Fig. 3).

Tickled females had the lowest trill USV rate before and during running (Table 5, Fig. 4). The interaction between treatment and sex had an overall effect on the frequency of trill USVs emitted in the one second before ($X^2_{(1,63)} = 32.66$, $p < 0.0001$) and during ($X^2_{(1,63)} = 47.02$, $p < 0.0001$) running and this may be explained by the low call rates of tickled females (Table 5, Fig. 4). Control females (before: $t_{ratio} = 7.34$, $p < 0.0001$; during: $t_{ratio} = 8.66$, $p < 0.0001$), tickled males (before: $t_{ratio} = -4.59$, $p = 0.0001$; during: $t_{ratio} = -4.56$, $p = 0.0002$) and control males (before: $t_{ratio} = -5.03$, $p < 0.0001$); during: $t_{ratio} = -4.94$, $p < 0.0001$) had a higher mean frequency of trill 50 kHz USVs before and during running compared to tickled females. Control rats made more trill USVs in the one second before ($X^2_{(1,63)} = 21.97$, $p < 0.0001$) and during running compared to tickled rats ($X^2_{(1,63)} = 29.14$, $p < 0.0001$), regardless of sex (Table 5).

Table 4. Statistical output of generalised linear mixed model of flat 50 kHz USVs emitted before and during non-play related behaviours. Significant effects ($p < 0.05$) shown in bold.

Behaviour	Before/ During	Fixed Effects	Levels	Estimated marginal means	±SE	95% CI	Chi-Square	P-value
Exploration	Before	Treatment	Control	6.35	1.49	3.97-10.2	8.06	**0.0045**
			Tickled	8.14	1.89	5.11-13.0		
		Sex	Female	8.51	2.28	4.97-14.6	1.13	0.2883
			Male	6.08	1.66	3.52-10.5		
		Treatment:Sex	Control-Female	8.58	2.35	4.96-14.84	10.86	**0.0010**
			Tickled-Female	8.43	2.31	4.87-14.58		
			Control-Male	4.70	1.33	2.67-8.28		
			Tickled-Male	7.85	2.17	4.52-13.64		
Exploration	During	Treatment	Control	20.8	6.39	11.2-38.4	34.98	**< 0.0001**
			Tickled	27.5	8.44	14.8-50.8		
		Sex	Female	26.4	8.65	13.7-50.8	0.52	0.4745
			Male	21.6	7.11	11.2-41.8		
		Treatment:Sex	Control-Female	25.5	8.40	132.-49.3	22.41	**< 0.0001**
			Tickled-Female	27.3	8.98	14.1-52.7		
			Control-Male	16.9	5.59	8.7-32.8		
			Tickled-Male	27.7	9.12	14.3-53.5		
Running	Before	Treatment	Control	4.72	1.63	2.36-9.43	2.62	0.1057
			Tickled	2.67	0.95	1.31-5.43		
		Sex	Female	3.25	1.14	1.61-6.57	0.21	0.6434
			Male	3.89	1.35	1.94-7.80		
		Treatment:Sex	Control-Female	6.11	2.56	2.64-14.14	3.33	0.0679
			Tickled-Female	1.73	0.78	0.70-4.28		
			Control-Male	3.65	1.58	1.54-8.66		
			Tickled-Male	4.14	1.75	1.78-9.65		
Running	During	Treatment	Control	7.08	1.97	4.06-12.35	3.31	0.0689
			Tickled	3.88	1.10	2.20-6.84		
		Sex	Female	3.94	1.12	2.23-6.95	2.73	0.0986
			Male	6.98	1.92	4.02-12.11		
		Treatment:Sex	Control-Female	7.28	2.61	3.55-14.92	2.93	0.0869
			Tickled-Female	2.13	0.84	0.97-4.68		
			Control-Male	6.88	2.60	3.23-14.67		
			Tickled-Male	7.08	2.58	3.41-14.70		

Table 5. Statistical output of generalised linear mixed model of trill 50 kHz USVs emitted before and during non-play related behaviours. Significant effects ($p < 0.05$) shown in bold.

Behaviour	Before/ During	Fixed Effects	Levels	Estimated marginal means	±SE	95% CI	Chi-Square	P-value
Exploration	Before	Treatment	Control	9.38	2.33	5.71-15.4	3.47	0.0624
			Tickled	10.47	2.59	6.38-17.2		
		Sex	Female	9.30	2.45	5.49-15.8	0.55	0.4589
			Male	10.6	2.77	6.24-17.9		
		Treatment:Sex	Control-Female	10.80	2.88	6.33-18.4	29.99	**<0.0001**
			Tickled-Female	8.02	2.17	4.67-13.8		
			Control-Male	8.15	2.20	4.75-14.0		
			Tickled-Male	13.67	3.63	8.04-23.2		
Exploration	During	Treatment	Control	32.5	8.46	19.3-54.8	1.36	0.2434
			Tickled	32.9	8.54	19.5-55.3		
		Sex	Female	29.8	8.04	17.4-51.2	1.44	0.2295
			Male	35.9	9.67	20.9-61.5		
		Treatment:Sex	Control-Female	37.1	10.05	21.6-63.8	116.11	**< 0.0001**
			Tickled-Female	23.9	6.52	13.9-41.3		
			Control-Male	28.5	7.75	16.6-49.2		
			Tickled-Male	45.1	12.19	26.3-77.5		
Running	Before	Treatment	Control	5.59	1.68	3.06-10.22	21.97	**< 0.0001**
			Tickled	2.28	0.72	1.21-4.29		
		Sex	Female	2.52	0.85	1.29-4.94	1.54	0.2141
			Male	5.05	1.61	2.67-9.55		
		Treatment:Sex	Control-Female	5.81	1.91	3.01-11.22	32.66	**< 0.0001**
			Tickled-Female	1.10	0.42	0.51-2.34		
			Control-Male	5.38	1.75	2.80-10.32		
			Tickled-Male	4.74	1.56	2.46-9.13		
Running	During	Treatment	Control	8.20	1.81	5.28-12.74	29.14	**< 0.0001**
			Tickled	3.46	0.81	2.17-5.52		
		Sex	Female	3.56	0.98	2.05-6.17	2.11	0.1459
			Male	7.98	2.07	4.75-13.40		
		Treatment:Sex	Control-Female	7.93	2.14	4.62-13.62	47.02	**< 0.0001**
			Tickled-Female	1.59	0.49	0.86-2.96		
			Control-Male	8.48	2.24	4.99-14.40		
			Tickled-Male	7.50	1.99	4.41-12.77		

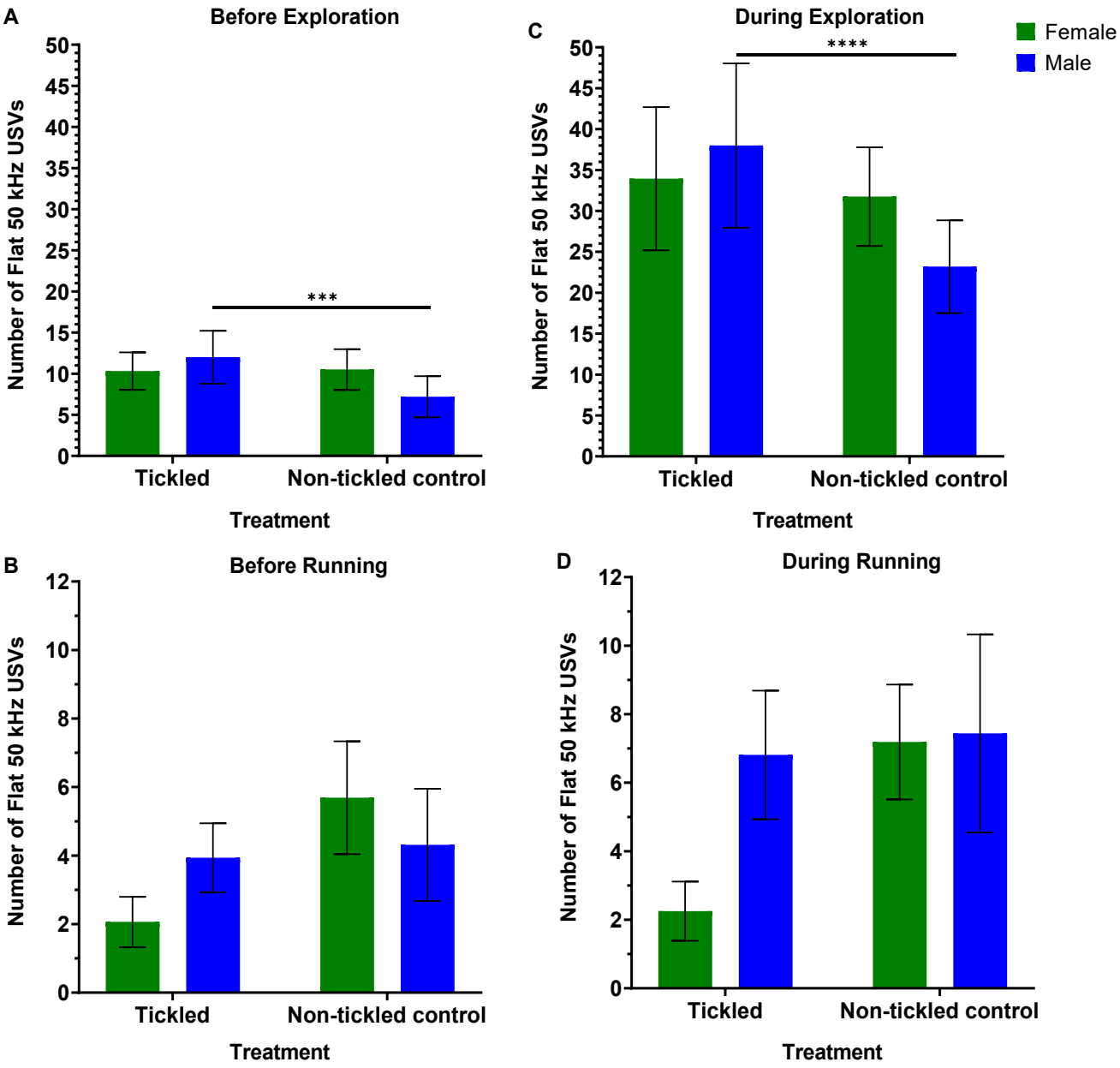

Figure 3. Flat USVs paired with explorations, but not running, in tickled compared to control rats.

Number of flat 50 kHz USVs produced one second before exploration (A) and running (B), and flat 50 kHz USVs produced during exploration (C) and running (D) 'non-play-related' behaviours. Females (green), males (blue). Mean ± SEM; n = 16 / group. * denotes $p \leq 0.05$; ** denotes $p \leq 0.01$; *** denotes $p \leq 0.001$; **** denotes $p \leq 0.0001$ for significant pairwise interactions.

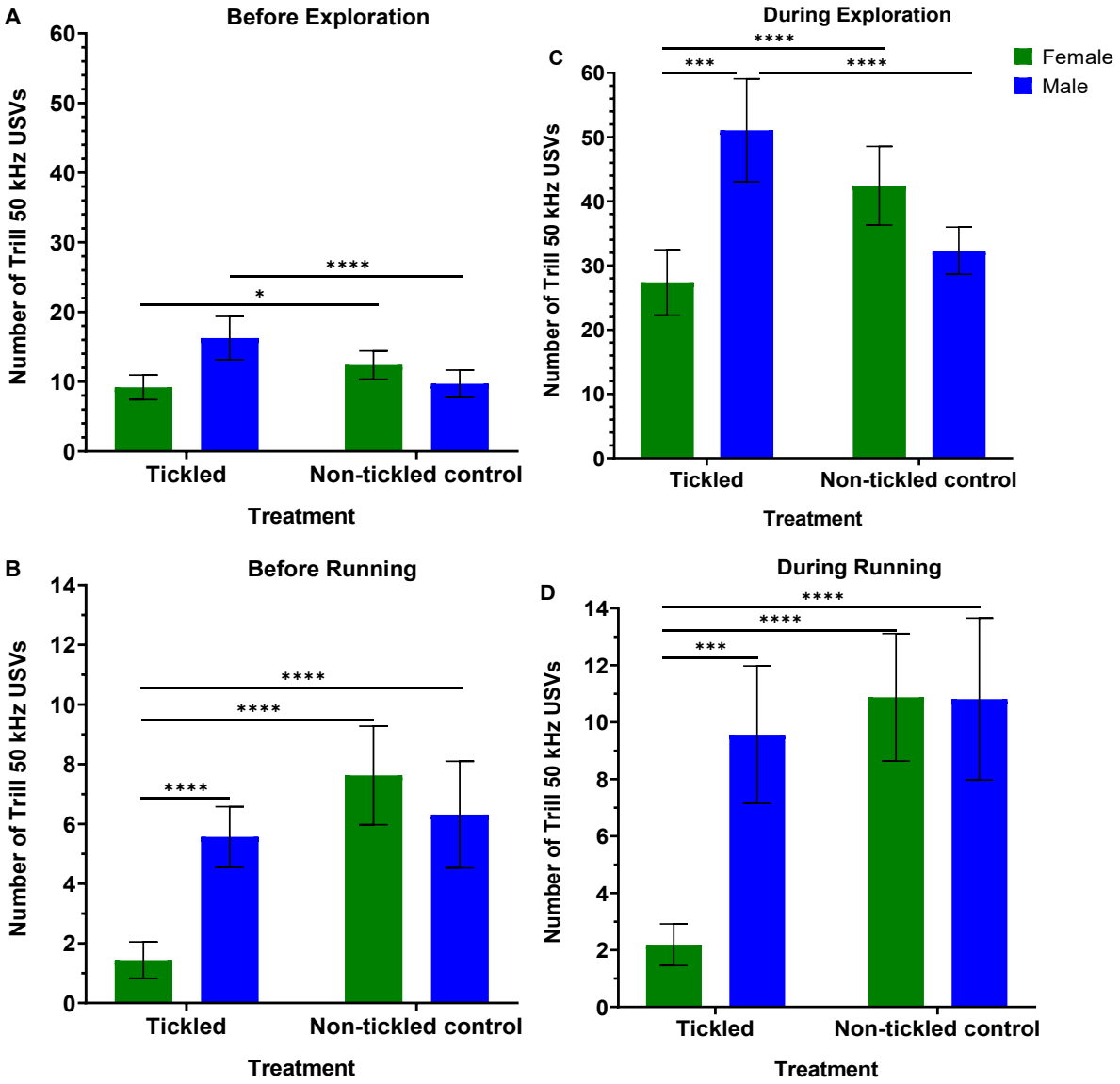

Figure 4. Trill USVs paired with explorations and running in tickled compared to control rats.

Number of trill 50 kHz USVs produced 1 second before exploration (**A**) and running (**B**) 'non-play-related' behaviours, and trill 50 kHz USVs produced during exploration (**C**) and running (**D**) 'non-play-related' behaviours. Females (green), males (blue). Mean ± SEM; n = 16 / group. * denotes $p \leq 0.05$; ** denotes $p \leq 0.01$; *** denotes $p \leq 0.001$; **** denotes $p \leq 0.0001$ for significant pairwise interactions.

3.4 Discussion

To my knowledge, this is the first study to show that there is a sex difference in 50 kHz USV subtypes being emitted in relation to certain tickle-induced behaviours. Female rats paired more flat 50 kHz USVs than males with hopping and darting solitary play behaviours. While there were no significant interactions between treatment and sex, the estimated marginal means suggest a trend for tickled female rats to pair more flat USVs with hopping and darting than tickled males and control rats. Females, irrespective of treatment, paired flat USVs with approaches to the hand significantly more than males. In contrast, tickled rats of both sexes paired trill 50 kHz USVs with hopping, darting and approach behaviours significantly more than control rats. This suggests that flat USVs are being used during these behaviours differently in female rats, while tickling induces higher trill USV call rates with hopping, darting and approaches, irrespective of sex.

Female and male juvenile Wistar rats produced trill and flat 50 kHz USVs in relation to the playful stimulus of tickling, which is consistent with those expressed during conspecific play in males. Burke et al. (2018) found that the most common types of calls produced by pairs of playing male rats were trills, trills with jumps, flat/trill combinations and flats. Also, Burke et al. (2021) reported that male juvenile Wistar rats produce similar numbers of trill and flat 50 kHz USVs during conspecific play and these two call subtypes are more common than other more complex subtypes, such as composite, split and down ramp 50 kHz USVs. To date most tickling studies do not subcategorise 50 kHz USVs (e.g. LaFollette et al., 2018; Hammond et al., 2019) and other studies focus on the frequency-modulated trill (e.g. Cloutier et al., 2018) subtype, which are also referred to as ‘chirps’ (e.g. Panksepp and Burgdorf, 2000; Mällo et al., 2007; Panksepp and Burgdorf, 2003). Therefore, there is not a lot of evidence in the literature for 50 kHz USVs subtypes including trills and flats being expressed differently by rats during tickling, and even less comparing this between sexes.

It is clear from these data that there are patterns in the association of USV subtypes and behaviours in juvenile Wistar rats, which is similar to the findings from Burke et al. (2018) in male Long-Evans rats, where they found that calls are linked to certain play behaviours. Takahashi et al. (2010) similarly found that USV subtypes correspond with fighting, feeding and locomotive behaviours. In other species, ultrasonic vocalisations play a role in coordinating behaviours. For example, USVs are thought to maintain social cohesion in slow loris' (*Nycticebus javanicus*; Geerah et al., 2019), kin recognition in grey mouse lemurs (*Microcebus murinus*; Kessler et al., 2012) and social communication in the common (*Microtus arvalis*), bank (*Myodes glareolus*) and field (*Microtus agrestis*) vole species (Kapusta and Sales, 2008). Similarly, it has been suggested that USVs function to coordinate playful actions between rats (e.g. Kisko et al., 2015a; Burke et al., 2018). I found that USVs are linked to play-related behaviours during tickling, which may therefore suggest that rats are using these calls for a function during tickling. This current study adds to the findings of other studies which have mainly focussed on male Long-Evans rats (Burke et al., 2018) by showing coupling of UVSs to behaviours in juvenile female and male Wistar rats.

Calls paired with play-related behaviours

Control rats appear to call infrequently both before and during play-related hopping and darting behaviours, while tickling induced higher call rates both before and during play-related behaviours in both female and male juvenile Wistar rats, suggesting that USVs are linked to play behaviours occurring during tickling. To add to this, there was an association of trills both before and during play-related behaviours which may suggest that trills are used by both sexes as signals of affective state (induced by both conspecific and heterospecific playful interactions). This provides evidence that trills may act as an indicator of positive affect, or even to enhance positive affect (Burke et al., 2020; Knutson et al., 2002). To investigate whether these behaviours were qualitatively different in tickled compared to non-tickled control rats a Qualitative Behavioural Assessment (Wemelsfelder et al., 2001; Rutherford et al., 2012) could be carried out on the play-related behaviours in the future. This

may help to support the existing evidence for trill 50 kHz USVs indicating positive affect.

Tickling resulted in higher numbers of flat 50 kHz USVs produced one second before hopping and darting behaviours, and higher numbers of trill USVs before hops, darts and approaches compared to controls. Female rats emitted more flat, but not trill, 50 kHz calls than males before hopping, darting and approaching. Tickled rats of both sexes made more trill calls before play-related, versus non-play-related, behaviours. This supports previous findings for 50 kHz USVs, particularly of the trill subtype, being a play signal. Trills have been found to be important for playful encounters and they may facilitate playful contact (Burke et al., 2020). Similarly, rats emit calls immediately before making playful contact in conspecific social play (Himmler et al, 2014), and pre-contact calls are emitted by both playmates during conspecific social play (Kisko et al., 2015b). Male rats emit trill 50 kHz USVs (Bialy et al., 2000) and female rats emit both trill and flat 50 kHz USVs (Börner et al., 2016) before sexual encounters, suggesting that these calls have a function in sociosexual communication. Also, male rats produce both flat and trill USVs during the ejaculatory phase of copulation (White et al., 1990) and these may affect female sexual receptive behaviours (White and Barfield, 1990). The findings from the present study suggest that female and male juvenile Wistar rats may differentially use trill and flat calls as an affective signal during tickling.

Female rats emitted more flat 50 kHz USVs in relation to hopping, darting and hand approaches than males, and there was a trend towards tickled female rats pairing more flat USVs with hopping and darting compared to other groups. Based on the few studies to use both sexes in tickle experiments, there are inconsistent findings on whether female or male rats vocalise more in response to tickling (e.g. Panksepp and Burgdorf, 1999; Panksepp and Burgdorf, 2003; Mällo et al., 2007; Wöhr and Schwarting, 2013). There is evidence that female rats from three outbred stocks (Wistar, Long-Evans and Sprague-Dawley) produce multiple types of 50 kHz USVs in response to being tickled (Schwarting, 2018). While there may be different factors affecting whether each sex produces more calls in response to tickling

when directly compared (for example, the sex of the experimenter; LaFollette et al., 2018), there has been a historical tendency to focus on trill 50 kHz USVs (e.g. Panksepp and Burgdorf, 2000, Burgdorf et al., 2008). The present study suggested that tickled female rats produce more flat, rather than trill, 50 kHz USVs in response to tickling. This is the first time this trend has been shown and therefore warrants further investigation as the interaction between treatment and sex may have been affected by the power of the model due to the sample size used in the present study. Female rats have been found to produce flat USVs abundantly during mating (Thomas and Barfield, 1985) which may suggest that flat 50 kHz USVs have a sex-specific communicatory role for female rats. Future studies that further investigate sex differences in vocalisations will be important to interpret USV call profiles in rats during tickling.

Tickled rats made more flat calls during play-related behaviours than control rats. Burke et al. (2018) also found that flat calls were associated with conspecific play behaviours such as active wrestling between two male rats, and flats were not associated with passive contact. Flat calls have been postulated to be used as cues of dominance and submission between playing male rats (Burke et al., 2018) and this communication may be used to avoid escalation of play fighting to aggression (Burke et al., 2017b). It is plausible that flat USVs may be used by each sex differently. For example in males, flat USVs may be used to establish dominance (Burke et al., 2018). My results suggest that flat USVs may serve a particular communicatory purpose during a playful interaction, and there was a trend that this was specifically in female rats.

Female and male tickled rats made more trills during the play related behaviours such as hopping and darting. USVs have been previously linked to hopping and darting in the context of sexual behaviours (Blumberg, 1992), particularly for females. Trill USVs have been positively correlated with conspecific social play and heterospecific play in female and male Long-Evans rats (Burgdorf et al., 2008), and calls that had trill elements have been associated with play behaviours, such as nape contacts, chasing and wrestling

(Burke et al., 2018). However, Burke et al. (2018) found that trills were also associated with approaching the hand, unlike in the present study, where trills seem to be made just before but not during approaching the hand. This may be as a result of the control males having a low call rate during approach behaviours in the present study.

Calls paired with non-play related behaviours

Flat and trill USVs appeared to be used differently by tickled and control rats during non-play related behaviours. Flat calls were paired with explorations in tickled rats compared to controls, while there was no main effect of sex and treatment in the number of trills paired with exploration. Whereas flat USVs were not paired with running in any group, control rats paired more trills with running than tickled rats, with tickled females in particular producing low numbers of trills before and during running. Overall, this may suggest that tickled rats were pairing flat USVs with explorations, while control rats paired trill USVs with running. This argues against the *movement by product hypothesis* that was proposed by Thiessen and Kittrell, (1979) in gerbils in which they concluded that USVs are a result of the compression of the thorax during locomotion. LaPlagne and Costa (2016) also found that 50 kHz USVs do not appear to be just a by-product of vigorous movement, although USVs were associated with locomotion. My results suggest that while USVs are paired with locomotive behaviours, such as running, specific subtypes of USV are paired with particular behaviours, and this was different in rats that were tickled compared to those who were not. Other studies have found that rats emit USVs during exploration and running. Male Wistar and Sprague-Dawley rats were found to emit USVs during cage exploration (Manduca et al., 2014), and Burke et al. (2021) found that running was associated with trill calls in juvenile male Wistar rats and flat calls had less of an association, which is consistent with the present findings. In the same study trill USVs did not have a strong association with exploration behaviours, which is also consistent with my data. While there is evidence that USVs are associated more strongly with playful behaviours (during social conspecific play) than non-social behaviours in male rats (Burke et al., 2018), my findings

suggest that rats pair 50 kHz USVs with both playful and non-play related behaviours, and that tickling may affect which USV subtypes are paired with specific behaviours.

Limitations

There are several limitations to this study that should be addressed. Firstly, using the Observer and Ultravox software in the current study allowed for a unique analysis by synchronising the audio and video recordings. However, with this method there is a possibility that trill and flat 50 kHz USVs (both commonly occurring calls) may overlap by chance with the different behaviours in this analysis (Burke et al., 2020). An alternative technique would have been to use the Monte Carlo shuffling technique used by Burke et al. (2017a, 2018).

Secondly, only calls containing trill elements and flat 50 kHz USVs were incorporated into this analysis whilst other subtypes were not. Using the more detailed subcategories of Wright et al. (2010) may be more accurate in establishing which calls, including more rarely produced USVs, are related to which behaviours observed during tickling (Burke et al., 2020), although trill and flat calls are the predominant subtypes emitted during playful interactions (Burke et al., 2018; Burke et al., 2021).

Thirdly, one second before the start of a behaviour was used in this study for the analysis of calls produced in anticipation of a behaviour. This was done to account for human error in the coding of the behaviours of several hundred milliseconds (Burke et al., 2017a, 2018) and also because a previous study found that there was association of calls with behaviours up to 600 milliseconds after the call is emitted (Burke et al., 2020). Rats may synchronise USVs and behaviours on a much shorter time scale, for example < 200 milliseconds (LaPlagne and Costa, 2016) and so any association between the call and the ensuing behaviour may be lost in a one second time frame. However, as our findings show robust patterns of USV subtypes produced in the lead up to specific play behaviours, there is evidence that the one second frame used did not lose this association.

Lastly, the analysis of call-behaviour association spanned the entire two minute testing period, during which the tickled animals had alternating fifteen second bouts of tickling while the control rats received no contact. This could be refined by analysing the fifteen second segments when the tickled rats were being tickled and compare to the same fifteen seconds in the control rat trials. This might better capture vocalisations when the rat is undergoing tickling, rather than in the present study which represents the general effect of being tickled or not. However, my results in Chapter 2 show that tickled and control rats call in similar frequencies in the release and tickle sessions so this is unlikely to have affected the present findings.

Conclusions

This study has shown for the first time that juvenile Wistar rats couple reward-associated trill 50 kHz USVs with play-related behaviours of hopping, darting and approaching the hand, and importantly that this finding is consistent between females and males. The results indicate that tickling has a substantial effect on USVs being paired with play-related behaviours. Sex differences appear in emission of flat 50 kHz USVs, with female rats producing more flats in association with play-related behaviours than males. As flat and trill USVs are thought to act in a communication role during play, this indicates that female rats use different USV subtypes to communicate compared to males. Further investigations are required to clarify the meaning of these USV subtypes.

Chapter 4

Fos immunoreactivity in the Wistar rat brain following tickling: a sex comparison.

4.1 Introduction

Tickling, or playful handling, is a form of heterospecific play (Cloutier et al., 2013) where the human hand mimics some aspects of social play in rats (Panksepp and Burgdorf, 2000). Tickling is found to be rewarding in juvenile rats (Burgdorf and Panksepp, 2001) and has been proposed as a technique to induce positive affective states in laboratory rats and attenuate the stress associated with routine handling and procedures (Cloutier et al., 2012; Cloutier et al., 2014; Cloutier et al., 2018). Tickling is often seen as a social stimulus (e.g. Burgdorf et al., 2020) and tickling and social play have similar aspects, with tickling being originally developed by mimicking elements of social conspecific play using the human hand, such as making contact with the nape of the neck (Panksepp and Burgdorf, 2000). Also, some behaviours observed during social conspecific play are seen during tickling, mainly 50 kHz ultrasonic vocalisations (USVs) which are abundantly produced during both tickling (Panksepp and Burgdorf, 2000) and social play (Knutson et al., 1998), as well as locomotor play (or scampering; Pellis and Pellis, 1983; Hammond et al., 2019). However, individual rats vary in their response to tickling (Hinchcliffe et al., 2020), as with humans where tickling is both pleasant and unpleasant (Harris and Alvarado, 2005), and many elements of tickling are very different from social conspecific play (Bombail et al., 2021). There have been no published studies to date that have investigated whether the neural pathways that underpin social conspecific play also underpin tickling; studies that have investigated the neurobiology of tickling have shown that the reward system (Burgdorf et al., 2001; Burgdorf et al., 2007; Hori et al., 2013; Burgdorf et al., 2020) and the deep layers of the somatosensory cortex (Ishiyama and Brecht, 2016; Ishiyama, Kaufmann and Brecht, 2019) are activated in response to

tickling, and that tickling induces neurogenesis in the hippocampus (Wohr et al., 2009; Yamamuro et al., 2010). There is sparse evidence that hypothalamic nuclei may be involved in the response to tickling in male rats (lesioning the lateral hypothalamix parvafox nucleus decreased tickle-induced behaviours, Roccano-Waldmeyer et al., 2016; and tickling increased gene expression in the hypothalamus, Hori et al., 2009).

Elucidating the neurobiological substrates of tickling would allow better understanding of how the brain processes the stimulus of tickling, and may provide greater insight into how tickling is perceived by the rat. This is important as tickling is being advised as an intervention to improve the welfare of laboratory rats (NC3Rs, 2017).

4.1.1 Brain regions that regulate social conspecific play

As tickling is widely considered to be a form of play (heterospecific play; Panksepp and Burgdorf, 2000), brain regions that have known involvement in social conspecific behaviours may be good candidates for being involved in the behavioural response to tickling. I will provide a brief introduction to several key brain regions implicated in social conspecific play in rats.

4.1.1.1 Forebrain regions

Medial prefrontal cortex

The prefrontal cortex (PFC) is a neocortical area, made up of interconnected regions such as the orbitofrontal cortex (OFC) and medial prefrontal cortex (mPFC) (Miller and Cohen, 2001). The PFC sends and receives connections throughout the brain and plays an important role in higher cognitive functions such as memory, decision making, reward and affect (Miller and Cohen, 2001). In particular, the mPFC has direct connections to limbic structures, such as the amygdala, and has an important role in emotional processing (Cassell and Wright, 1986). The mPFC has four divisions: the frontal agranular, anterior cingulate, prelimbic and infralimbic cortices (Hoover and Vertes, 2007). The prelimbic (PL) and infralimbic (IL) cortices receive input from the hypothalamus, amygdala, thalamus, and limbic cortex (including the orbital cortex, anterior cingulate cortex, hippocampus,

perirhinal cortex, entorhinal cortex; Hoover and Vertes, 2007). Given these inputs, the IL and PL have important functions in emotional behaviours, such as anxiety (e.g. in male mice; Suzuki et al., 2016), and fear (e.g. in male Sprague-Dawley rats; Milad and Quirk, 2002). The mPFC is also thought to be instrumental for affective states (Burgdorf et al., 2020), for example, in humans, deep brain stimulation of the mPFC induced positive affective states (Choi et al., 2015). In rats, electrical brain stimulation of the mPFC cause rats to elicit 50 kHz USVs which are associated with positive affect in rats (Burgdorf et al., 2007).

The mPFC is considered to be part of the reward circuitry in the brain (Tzschentke, 1999). mPFC glutamatergic neurons project to the ventral vegmental area (VTA) and nucleus accumbens (NA) regions of the reward circuit (Fig. 1; Gorelova and Yang, 1996, Han et al., 2017) and electrical (Gariano and Groves, 1988) and pharmacological (via *in vivo* microdialysis; Taber et al., 1995) stimulation of the mPFC results in an activation of dopaminergic VTA neurons and an increase of dopamine release in the NA. Directly stimulating the mPFC with electrodes induces the reward-associated behaviour of self-stimulation in male albino rats (Routtenberg and Sloan, 1972).

The PFC appears to have an important role in social conspecific play. Lesioning of the PFC was found to alter the structure of play in Long-Evans rats (unspecified sex), particularly, the duration of pinning was affected (Panksepp et al., 1994), and lead to less complex defensive tactics during play bouts in female and male Long-Evans rats (Bell et al. 2009). Pharmacological inhibition of the IL and PL mPFC is also found to reduce the frequency and duration of social play in male Wistar rats (van Kerkhof et al., 2013). Similarly, inhibition of the IL decreased pinning and pouncing elements of social play, but not social exploratory behaviours or locomotor activity in male Wistar rats (Achterberg et al., 2015). van Kerkhof et al., (2014) used c-*fos* gene expression as a marker of neuronal activity and showed that following 15 minutes of social play in male Wistar rats, there was an increase in Fos immunoreactivity (Fos-ir) in the PL mPFC but not the IL mPFC. Also, Fos-ir in

the PL and IL was correlated with Fos-ir in regions with which they are connected, including the basolateral nucleus of the amygdala (BLA), medial amygdala (meA) and VTA in rats exposed to social play, suggesting these connections may be important in the expression of social play (van Kerkhof et al., 2014). In addition, social play induced changes in neural plasticity in the mPFC of female Long-Evans hooded rats, and this may function to ready the mPFC in juvenile rats to be more responsive to plasticity-inducing experiences later in life (Himmler et al., 2013).

Nucleus accumbens

The NA is part of the ventral striatum, and is made up of two main subregions, the core and shell regions (Zaborszky et al., 1985). The core and shell subregions send projections to different brain regions, for example the core subregion projects to the globus pallidus (GP), and the dorsolateral ventral pallidum (dlVP) which are involved in motor outputs, whereas the shell projects to the VTA and ventral pallidum (VP) (Zahm and Heimer, 1993).

The NA receives glutamatergic inputs from the amygdala (Fuller et al., 1987; Mcdonald, 1991), hippocampus (Yang et al., 1984), thalamus (Phillipson and Griffiths, 1985) and PFC (Montaron et al., 1996; Fig. 1). The discovery that injections of dopamine into the NA increased locomotor activity in rats (Pijnenburg et al., 1973; Costall and Naylor, 1975) lead to interest in dopamine within the NA, which was found to be a key region of the mesolimbic reward pathway of the brain. Self-administration of drugs (e.g. D-amphetamine) stimulates dopamine release within the NA (Hoebel et al., 1983; Phillips et al., 1994). Rats also maintain self-stimulation of dopamine receptor agonists into the NA (Ikemoto et al., 1997) suggesting a role for dopamine in the NA in reward (Ikemoto and Panksepp, 1999). The VTA also sends dopaminergic projections to the shell region of the NA (Gerfen et al., 1987; Berlanga et al., 2003) and firing of these projections results in dopamine release in the NA, encoding the rewarding nature of the stimulus (Ikemoto, 2007).

The NA has an important role in the expression of social play in rats. For example, inactivating the NA core region increased the duration, but not

the frequency, of social play in male Wistar rats while inactivating the NA shell did not impact social play behaviour (van Kerkhof et al 2013). Social play may be dependent specifically on dopamine transmission within the NA, given that activation of dopamine D1 and D2 receptors in the NA increased social play in male Wistar rats (Manduca et al., 2016). Blockade of dopamine receptors reduced social play in socially isolated rats that should have been highly motivated to engage in play (Manduca et al., 2016). In addition to dopamine transmission, opioid transmission in the NA also appears to affect social play as mu-opioid receptor agonists in the NA increase social play in male Wistar rats (Trezza et al., 2011). Levels c-*fos* mRNA were increased in Long-Evans hooded male rats in the dorsal and ventral striatum after 30 minutes of social play (Gordon et al., 2002), and Fos-ir in the NA core and shell increased following 15 minutes of social play in male Wistar rats (van Kerkhof et al., 2014).

Bed nucleus of the stria terminalis

The bed nucleus of the stria terminalis (BNST) is located in the basal forebrain and is considered to be an extension of the centromedial amygdaloid complex as there are interconnecting cell columns bridging these two structures (Johnston, 1923; de Olmos and Ingram, 1972; Alheid et al., 1998). The BNST can be divided into lateral and medial subdivisions based on connections with the amygdala; the central amygdala (ceA) projects primarily to the lateral BNST (lBNST), and the meA projects primarily to the medial BNST (mBNST) (Alheid et al., 1998). The lBNST also receives major projections from the BLA (Dong et al., 2001). These connections to the amygdala are thought to facilitate the role of the BNST in fear responses (Davis et al., 2010), while Kim et al. (2013) showed that projections from the BNST to the lateral hypothalamus (LH), parabrachial nucleus (PB) and VTA were involved in anxiolytic responses: risk avoidance, respiratory rate and positive valence, respectively. The BNST also has a role in stress through projections from the frontal cortex; GABAergic projections from the PL mPFC to the BNST (anterior portion) mediate stress responses by activating the hypothalamic-pituitary-adrenal stress axis (Radley et al., 2009). The BNST

projects to the lateral septum (LS), the periaqueductal gray (PAG) and to the LH and anterior hypothalamus (AH) which is thought to modulate reproductive and visceromotor responses (Dong and Swanson, 2004).

The BNST, particularly the medial division, is part of a brain network that regulates social behaviours, such as maternal pup retrieval in rats (Numan and Numan, 1996) and copulatory behaviours (e.g. lesioning of the BNST increased the number of intromissions, Emery and Sachs, 1976; longer post-ejaculatory periods, Valcourt and Sachs, 1979; and deficits in non-contact erections, Lui et al., 1997, in male Long-Evans rats). Also, the BNST has been shown to have a role in social play in rats. Fos-ir in the BNST was increased in male Wistar rats following 15 minutes of social play (van Kerkhof et al., 2014) and Reppucci et al. (2018) found that 10 minutes of social play increased Fos-ir in the posterior BNST, and this was higher in female Wistar rats than males. Whereas Paul et al. (2014) found that vasopressin mRNA expression in the BNST was negatively correlated with social play (after a 30-minute play session) and this was only in male Wistar rats, not females. This suggests that the BNST exerts sexually dimorphic regulation of social play in rats, which is seen with social interactions in Fischer 344 rats where Fos-ir was higher in females following 10 minutes of social investigation of a conspecific (Perkins et al., 2017).

Lateral septum

The LS is located in the forebrain, within the rostrodorsal septal region (Rishold and Swanson, 1997). The LS was shown to project to the periventricular hypothalamus, ventral pallidum and thalamus using fluorogold for anterograde tracing in male Sprague-Dawley rats (Rishold and Swanson, 1997). The authors also found that the LS was innervated by the hippocampus (Rishold and Swanson, 1997). There is also evidence that the LS is interconnected to the ceA (Thomas et al., 2012), meA (Caffe et al., 1987), BNST (de Vries and Buijs, 1983), mPFC (Beckstead, 1979), entorhinal cortex (Alonso and Kohler, 1984) and VTA (Deniau et al., 1980; Swanson, 1982) and is part of the social behaviour network of the brain (Newman, 1999). It also

receives direct dopamine projections from the VTA (Lindvall and Stenevi, 1978; Gaspar et al., 1985).

The LS is found to play a role in social behaviours, including aggression, anxiety and social play. Lesion studies have shown the role of LS, for example, lesions to the LS reduced sibling preference in female and male Long-Evans rat pups (Clemens et al., 2020). Lesions to the LS had an anxiogenic effect on the response to electrical shock in male Sprague-Dawley rats (Yadin et al., 1993) and the hippocampal-LS circuit may regulate social aggression as activation of the LS by the CA2 region of the hippocampus and triggered attack behaviour in mice (Leroy et al., 2018). Also, aggressive behaviours in male rats is associated with vasopressin in the lateral septum (Beiderbeck et al., 2007). The LS has been implicated in social play behaviour in rats. Blocking GABA-A receptors in the LS decreased the duration of social play in both females and males Wistar rats, and this was dose-dependent (Bredewold et al., 2015). In addition, blocking glutamate receptors in the LS decreased the duration of social play in female, but not male Wistar rats in a dose-dependent manner (Bredewold et al., 2015). Vasopressin receptor transmission in the lateral septum has been shown to regulate social play in a sexually dimorphic manner. The blocking of vasopressin V1aR receptors increased the duration of social play, the frequency of nape attacks and pins (i.e. social behaviours) in male Wistar rats, but decreased the duration of social play and the number of pins in female Wistar rats (Veenema et al., 2013). Septal lesions increased the frequency of social play and of play initiation in female and male rats (unspecified strain; Beatty et al., 1982). Bredewold et al. (2014) found that oxytocin and vasopressin injected into the LS affected social play; oxytocin receptor blockade in the LS decreased nape attacks and pins in female Wistar rats, but not males, while vasopressin receptor blockade increased the duration of social play and the number of pins in males compared to females.

4.1.1.2 Midbrain regions

Amygdala

The amygdala is located in the midtemporal lobe of the brain and was identified in the early 19th century (Burdach, 1822). It is a heterogeneous region that is formed of around 13 nuclei that can be distinguished based on their cytoarchitecture and connections throughout the brain (Sah et al., 2003; Swanson and Petrovich, 1998). The BLA is part of the basolateral complex, along with the lateral nucleus and the accessory basal nucleus (or basomedial nucleus; Sah et al., 2003). The ceA and meA form the centromedial nuclear group in the dorsomedial amygdaloid complex (Sah et al., 2003). The BLA projects to the hippocampus and perirhinal cortex (regions associated with memory; Petrovich et al., 2001; Sah et al., 2003) and also to the NA as shown by MacDonald (1991) using retrograde tracing in Sprague-Dawley rats (sex not specified). The meA sends projections to the hypothalamus, BNST (Dong et al., 2001), and three main areas of the brainstem: the PAG (which has a role in vocalisations, analgesia and cardiovascular responses; Behbehani, 1995), the PB (which is involved in pain processing; Gauriau and Bernard, 2002) and the nucleus of the solitary tract (NTS; which is involved with somatosensory processing and autonomic processes; van der Kooy et al., 1984; Sah et al., 2003). The ceA has projections to the hypothalamus, mainly the dorsolateral and caudolateral regions (Petrovich et al., 2001), the BNST (Gungor et al., 2015) as well as the NTS (Saha et al., 2000).

The amygdala is involved in emotional responses, originally identified in rhesus monkeys (*Macaca mulatta*) with temporal lobe lesions that had attenuated fear and anger, and loss of social interactions (Kulver and Bucy, 1937), which was shown later by Zola-Morgan et al. (1991) where lesions to the amygdala in long tailed macaques (*Macaca fascicularis*) affected emotional behaviours but not memory. The amygdala has a well-established role in fear responses (for review, see LeDoux, 2003 and Fendt and Fanselow, 1999). Early studies showed that lesions to the amygdala impair fear responses in fear conditioning paradigms, for example, electrolytic or ibotenic acid lesions of the ceA and meA reduced the fear-induced startle response in male Sprague-Dawley rats (Campeau and Davis, 1995), and injection of lidocaine HCl into the amygdala before presentation of a conditioned stimulus

paired to a foot shock reduced the amount of time that rats spent expressing defensive freezing behaviour (Helmstetter, 1994). The ceA and BLA are key areas in fear responses (e.g. Duvarci et al., 2011, Li et al., 2013, Campeau and Davis, 1995; Koo et al., 2004). More recently, it has been shown that the amygdala is necessary for conditioned fear memories (Krabbe et al., 2018) and the extinction of fear following conditioned fear learning (Lee et al., 2021; Lingawi et al., 2021).

In addition to its known role in emotion processing, divisions of the amygdala have been implicated in social play in rats. van Kerkhof et al. (2014) found that Fos-ir was increased in the lateral amygdala but not the ceA, meA, or BLA in male Wistar rats. However, meA and BLA Fos-ir was correlated with Fos-ir in the mPFC, therefore the amygdala may be involved in the expression of social play via projections to other regions, such as the mPFC (van Kerkhof et al., 2014). Reppucci et al (2018) found that social play increased Fos-ir in the meA, and this was higher in female Wistar rats than males. Similarly, electrolytic lesions of the whole amygdala reduced social play in male but not female albino rats (Meaney et al., 1981). These studies may suggest that the amygdala is an important area for sex differences of social play in rats.

Periaqueductal gray

The PAG is a midbrain structure that is considered to act as an interface between forebrain and brainstem structures, processing sensory stimuli into behavioural responses (Benarroch, 2012). The PAG has reciprocal connections to a number of brain regions, including the mPFC (An et al., 1998), anterior cingulate cortex (ACC) (An et al., 1998), amygdala (An et al., 1998), hypothalamus (Behbehani et al., 1988) and the VTA (Ntamati et al., 2018). Mantyh (1983) showed that the PAG sends descending efferent projections to brainstem structures (the superior colliculus (SC), locus coeruleus (LC), and reticular formation) and spinal cord in squirrel monkeys (*Simia sciurea*).

The PAG can be subdivided into longitudinal columns based on morphological characteristics of the neurons in each division (Beitz, 1985): the dorsal (dPAG) (which includes the dorsomedial and dorsolateral PAG), lateral

(lPAG) and lateroventral (lvPAG) PAG. These divisions have differing connections throughout the brain. For example, using retrograde tracing of horseradish peroxidase in male Sprague-Dawley rats, Beitz (1982) showed that the dlPAG sends projections to the LH, SC and the pons, the dPAG projects to the dorsomedial hypothalamus (DMH) and the PB, and the lvPAG projects to the ventrolateral hypothalamus (VLH), the PFC, medial preoptic area (mPOA), the VTA, amygdala, as well as hindbrain structures, the medulla, pons and cerebellum.

The PAG is involved in a number of behaviours; it was first identified as a critical region for pain processing, for example, the PAG was found to be responsive to analgesic morphine of electrical stimulation following painful stimuli in male Holtzman rats (Yeung et al., 1978). Similarly, Fardin et al. (1984) found that electrical stimulation of the PAG also had an analgesic effect on electrical tail shocks in male Sprague-Dawley rats. Heinricher et al. (1987) showed that the PAG has distinct classes of neurons which modulate nociception, by measuring cellular activity during a tail-flick behavioural response to noxious heat stimulus in male Sprague-Dawley rats. The PAG is also involved in fear and anxiety responses (e.g. Borelli et al., 2013; Broiz et al., 2008; see for review: Brandao et al., 2008). For example, a GABA-A receptor agonist injected in the dPAG caused a reduction in fear-potentiated startle response and conditioned freezing in male Wistar rats (Reimer et al., 2008). The PAG is also important for sexual behaviours. Electrical stimulation of the PAG induced lordosis in female Sprague-Dawley rats (Sakuma and Pfaff, 1979) and lesions of the PAG resulted in an absence of lordosis, an increased in maternal attacks towards a male intruder and increased anxiety in an elevated plus maze in female Long-Evans rats (Lonstein et al., 1998). Also, lesions to the PAG that resulted in a decrease of serotoninergic neurons in this region disrupted male sexual behaviours, there was an increase in number of ejaculations and a decrease in ejaculatory latency in male Sprague-Dawley rats (Normandin et al., 2011).

There is growing evidence that each subregion of the PAG has a role in distinct behaviours, for example, stimulating the dPAG increased the

expression of defensive behaviours in male Wistar rats (Bittencourt et al., 2004). Lesions to the lPAG affected prey hunting in male Wistar rats, with decreased chasing and attacking behaviours observed (Mota-Ortiz et al., 2012). Optogenetic inhibition of the lPAG and lvPAG improves the acquisition of contextual fear conditioning, and increases defensive responses to a non-noxious stimulus in male Sprague-Dawley rats (Assareh et al., 2017). Fos-ir in the lvPAG was greater than that in the lPAG following fear conditioning in male Wistar rats (Carrive et al., 1997). Chemogenetic excitation of the lvPAG impairs fear learning in a Pavlovian fear conditioning paradigm with male Sprague-Dawley rats (Arico et al., 2017).

The PAG is associated with the production of vocalisations in several mammalian species (Jurgens, 1994; Gruber-Dujardin, 2010), from cats (Adamentz and O'Leary, 1959) to bats (Suga and Yajima, 1988; Schuller and Radtke Schuller, 1990), with even some evidence in humans where lesions to the PAG result in complete mutism (e.g. Esposito et al., 1999) and laughter activated the PAG in healthy human patients (Wattendorf et al., 2013). In monkeys, activity in PAG neurons corresponds with vocalisations in macaques (Larson, 1991) and glutamate agonists injected into the PAG elicited vocalisations in squirrel monkeys (Lu and Jurgens, 1993). Electrical stimulation of the PAG elicited several types of vocalisations (clucking, squealing, and purring) in male and female guinea pigs (*Cavia porcellus*; Martin, 1976). It was later found that separation vocalisations (low whistle) and mating calls (purr) in male guinea pigs were evoked differently based on the division of the PAG that was being stimulated (rostral and caudal PAG, respectively; Kyuhou and Gemba, 1998). Similarly, ultrasonic vocalisations (that were uncategorized) in male Wistar rats (Yajima et al., 1980) and audible vocalisations (defined as ranging from "a faint high-pitched squeak to a deep-throated squeal") male Long-Evans rats (Waldbillig, 1975) were induced by electrical stimulation of the PAG.

Due to the role of the PAG, specifically the lPAG and lvPAG (Jurgens, 2009), in vocalisations in several mammalian species it is warranted to investigate whether PAG neuronal populations in these regions are active

during tickling where ultrasonic vocalisations are abundantly produced (Panskepp and Burgdorf, 2000).

Ventral tegmental area

The VTA is a midbrain structure that is known for being a part of the mesolimbic dopamine system (Ikemoto, 2007), a neural network that encodes the rewarding nature of different stimuli (Wise, 1977; Wise and Rompre, 1989). Andén et al. (1964) first identified a large population of dopaminergic neurons in the VTA. Following this discovery, interest in the VTA grew, and a number of subsequent tracing studies showed the efferent and afferent projections of the VTA throughout the brain. The VTA has connections to the PFC, LS, BNST, NA, thalamus, ACC, entorhinal cortex, central, medial and lateral nuclei of the amygdala, hypothalamus, dorsal raphe and the LC (Lindvall et al., 1974; Fallon and Moore, 1978; Simon et al., 1979; Beckstead et al., 1979; Swanson, 1982; Fig. 1). Swanson used a fluorescent tracing technique in male albino rats to show that a bundle of VTA neurons project to the PAG, PB, LC and raphe nucleus of the hindbrain (Swanson, 1982).

While the VTA contains other neuronal types (GABAergic (Steffensen et al., 1998) and glutamatergic (Hnasko et al., 2012) neurons), VTA dopaminergic neurons have been extensively implicated in reward (for review see Ranaldi, 2014; Wise, 2004). Pharmacological stimuli, such as amphetamine, cocaine, nicotine, become rewarding because they elevate brain dopamine. Dopamine neurons are commonly identified by labelling neurons that express tyrosine hydroxylase (TH), an enzyme that is involved in the synthesis of dopamine (tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA); Pickel et al., 1976; Verney et al., 1982; Bayer and Pickel, 1990; Molinoff and Axelrod, 1971; Daubner et al., 2011). Dopaminergic neurons of the VTA project to the NA and mPFC; activation of these neurons results in dopamine release in these regions which encodes reward (Ikemoto, 2007; Ranaldi, 2014). For example, optogenetic studies have showed that male mice will learn to nose poke to gain VTA dopamine neuronal excitation (Kim et al.,

2012) and optogenetic excitation of VTA dopamine neurons induces conditioned place preference in mice (Tsai et al., 2009).

Sex differences in dopaminergic neurons of the VTA have been shown in several studies, for example, retrograde labelling to identify VTA dopaminergic neurons projecting to the PFC and motor cortices showed female Sprague-Dawley rats had more TH immunoreactive (TH-ir) neurons than males (Kritzer and Creutz, 2008). McArthur et al., (2007) found that female Sprague-Dawley rats had significantly more TH-ir cells in the VTA compared to male rats. Northcutt and Nguyen (2014) found that overall female Long-Evans rats had more Fos-ir TH cells in the VTA than males, and social play increased the number of Fos-ir TH cells in females.

The reinforcing effects of natural hedonic stimuli such as food and mating are mediated by the mesolimbic dopamine reward system (Wise, 2006; Young and Wang, 2004). Social play is also a rewarding behaviour (Vanderschuren et al., 1997); it has been used as a reward in conditioned place preference in male Sprague-Dawley rats (Crowder et al., 1992) and it has been shown that rats learn a task in order to play (Humphreys and Einon, 1981; Normansell and Panksepp, 1990). Dopamine transmission in the brain is involved in social play. A catecholamine synthesis inhibitor and pharmacological blockers of dopamine transmission decreased social play in male albino rats (Beatty et al., 1982). Blocking dopamine transmission reduced pinning and nape contacts in male Sprague-Dawley rats (Siviy et al., 1996) and similarly decreased pinning during social play in male Wistar rats (Niesink and van Ree, 1989). Also, Fos-ir in dopaminergic neurons of the VTA (TH-positive neurons) was significantly increased in female, but not male, Long-Evans rats (rats were observed for a one-hour session during which play could occur; Northcutt and Nguyen, 2014). Given that Fos-ir was not increased in VTA neurons following social play in male Wistar rats (van Kerkhof et al., 2014), there may be a sex-specific role of the VTA in the expression of social play in rats. Dopamine transmission in the reward circuit has been associated with tickle-induced 50 kHz USVs in female and male rats (Burgdorf et al., 2007; Hori et al., 2013). Burgdorf et al. (2007) found that lesions of the VTA and

dopamine receptor D1 and D2 antagonists reduced the number of 50 kHz USVs emitted during tickling in female Long-Evans rats. Similarly, Hori et al. (2013) found that dopamine D1, D2 and D3 receptor antagonist administration decreased 50 kHz USV frequency in male rats. However, to date, no studies have investigated any role of VTA dopamine transmission in the behavioural response to tickling in rats.

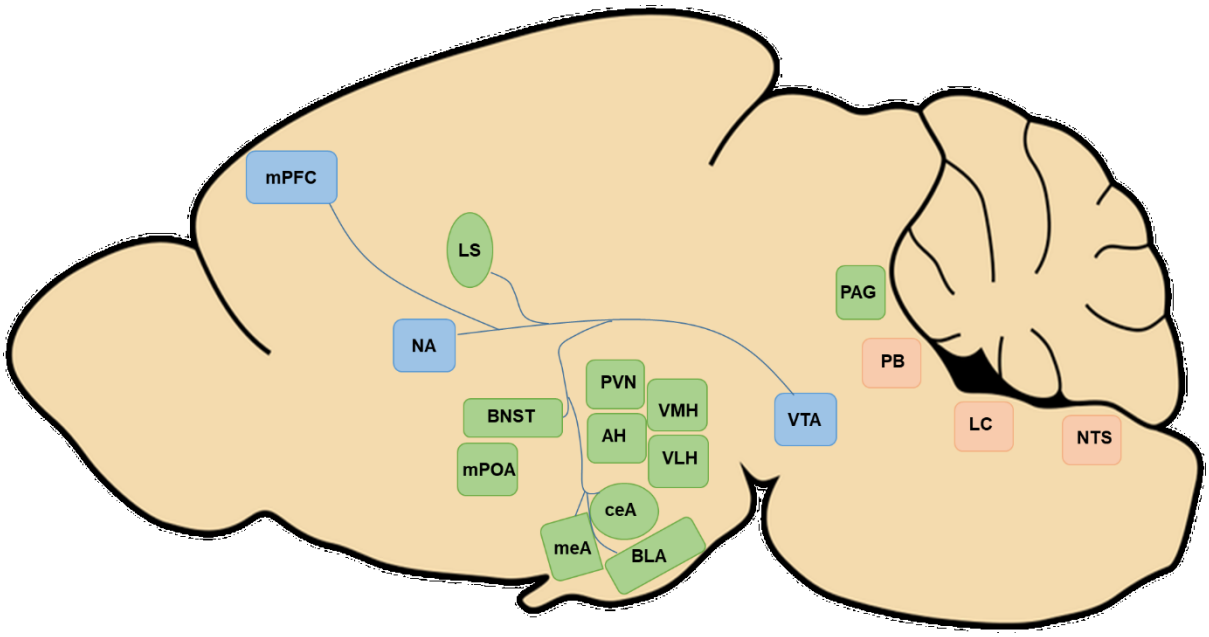

Figure 1. Main projections between brain regions of the mesolimbic dopaminergic reward pathway.
The major regions of the reward pathway are the VTA, NA and mPFC (shown in blue) with blue lines denoting the dopaminergic projections. Other regions involved are the LS, BNST and ceA, meA and BLA. Regions of the social decision making network shown in green; hindbrain regions shown in orange.
mPFC, medial prefrontal cortex; NA, nucleus accumbens; LS, lateral septum; BNST, bed nucleus of the stria terminalis; mPOA, medial preoptic area; ceA, central amygdala; meA, medial amygdala; BLA, basolateral amygdala; PVN, paraventricular nucleus of the hypothalamus; AH, anterior hypothalamus; VMH, ventromedial hypothalamus; VLM, ventrolateral hypothalamus; VTA, ventral tegmental area; PAG, periaqueductal gray; PB, parabrachial nucleus; LC, locus coeruleus; NTS, nucleus of the solitary tract. (Modified from Alcaro et al., 2007).

4.1.1.3 The social behaviour network (SBN)

Some of the regions that I have discussed have roles in multiple social behaviours in both sexes, and form part of an integrated network referred to as the social behaviour network (SBN; Newman, 1999; Fig. 2). The SBN is comprised of the LS, meA, mBNST, mPOA, AH, VLH and ventromedial hypothalamus (VMH), PAG and tegmentum, which are reciprocally interconnected (Newman, 1999). Sequences of social behaviours, such as sniffing, mounting, ejaculation in male sexual behaviour in rats, are controlled by not just one node, but patterns of activity across the network (Newman, 1999). Therefore, the nodes in this closely connected network can mediate multiple forms of social behaviours. For example, Fos-ir increased in the BNST and meA following male sexual behaviours, copulation and anogenital investigations, in Wistar rats (Coolen et al., 1997), and Fos-ir increased in the meA, BNST, POA and VMH after pair bonding between pairs of female and male prairie voles (*Microtus ochrogaster*; Cushing et al., 2003). Fos-ir increased in the BNST, meA, POA and paraventricular nucleus of the hypothalamus (PVN) in association with maternal aggression in female mice in response to a male intruder (Gammie and Nelson, 2001). Similarly in adult rats, maternal behaviours towards foster pups were associated with increased Fos-ir in the dorsal BNST, meA and cortical amygdala (Kalinichev et al., 2000). There were also correlations in Fos-ir between the mPFC and striatum, and the amygdala, VTA, NA and mPFC on male Wistar rats that were exposed to social conspecific play (van Kerkhof, et al., 2014). The nodes of the SBN contain sex hormone receptors enabling sexual differentiation of social behaviours, such as aggression and sexual behaviours (Simerly et al, 1990; Newman, 1999; O'Connell and Hofmann, 2011). Other brain regions, such as the PVN, that send and receive projections from the aforementioned nodes are also involved in the SBN as it is an important region for neuropeptide secretion and projections, and has involvement in a number of social behaviours (Goodson and Kingsbury, 2013; de Vries, 2008; Goodson and Thompson, 2010; Fig. 2).

Regions of the reward circuit, such as the VTA and NA, are anatomically connected with the SBN, mainly via the LS and BNST nodes (O'Connell and Hofmann, 2011). This is argued to function as a social decision-making brain network which is thought to code the rewarding nature of salient stimuli, including social and non-social stimuli (O'Connell and Hofmann, 2011). Given how important social behaviours, like reproductive behaviours, aggression, parental behaviour and gregariousness, are to the survival of the individual and evolution of the species, encoding the rewarding nature of these social behaviours likely contributes to the behaviour becoming adaptive (O'Connell and Hofmann, 2011). Evidence for homologues of the brain nodes and connections between nodes identified in mammals across birds, reptiles, amphibians and teleosts supports the concept of the social decision-making network and its role in modulating social behaviours (O'Connell and Hofmann, 2011).

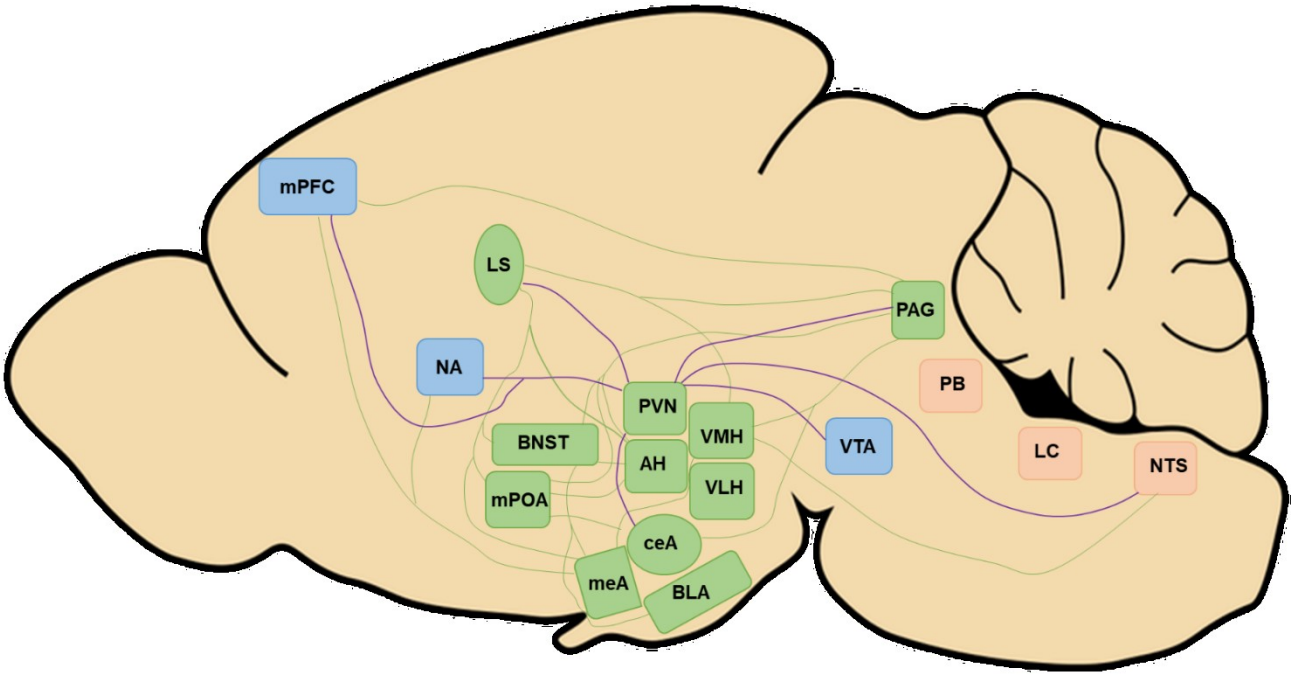

Figure 2. Main projections between brain regions of the social behaviour network. The major regions of the social behaviour network (shown in green) with green lines denoting connections between them, and also to regions of the reward pathway (shown in blue) and the hindbrain (shown in orange). Purple lines denote oxytocinergic projections. Together, this integrated neural network comprises the social decision making network. mPFC, medial prefrontal cortex; NA, nucleus accumbens; LS, lateral septum; BNST, bed nucleus of the stria terminalis; mPOA, medial preoptic area; ceA, central amygdala; meA, medial amygdala; BLA, basolateral amygdala; PVN, paraventricular nucleus of the hypothalamus; AH, anterior hypothalamus; VMH, ventromedial hypothalamus; VLM, ventrolateral hypothalamus; VTA, ventral tegmental area; PAG, periaqueductal gray; PB, parabrachial nucleus; LC, locus coeruleus; NTS, nucleus of the solitary tract. (Modified from O'Connell and Hofmann, 2011, Grinevich and Neumann 2021 and Ike et al., 2020)

4.1.1.4 Hindbrain regions

Tickling provides a somatosensory stimulus as evidenced by the activation of the deep layers of that the somatosensory cortex following tickling in male Long-Evans rats (Ishiyama and Brecht, 2016). The somatosensory system in rodents spans from sensory receptors (which detect thermal, mechanical and noxious stimuli) in the periphery (skin, muscles and joints) that transmit somatosensory information via dorsal root ganglia and trigeminal ganglia to the spinal cord (Watson, 2012). The spinal cord sends projections to regions in the hindbrain (e.g. the NTS, cerebellum, LC, raphe nuclei and PB), which in turn project to the thalamus (Watson, 2012). From the thalamus, somatosensory information is relayed to the somatosensory cortex (Watson, 2012). Any involvement of hindbrain structures in tickling has yet to be elucidated.

The NTS may be of interest in the response to tickling because it provides a gateway to the rest of the brain conveying sensory cues relating to social behaviours, and sending catecholaminergic projections to regions of the reward pathway. Therefore, investigation is warranted as to whether the NTS serves as an intermediary between the somatosensory input (Ishiyama and Brecht, 2016) and the rewarding aspect of tickling (Burgdorf and Panksepp, 2001).

The nucleus of the solitary tract

The NTS is a hindbrain structure located in the dorsomedial medulla, and is made up of multiple subnuclei based on cytoarchitecture and the projections of afferent nerve endings from the periphery (Jean, 1991). The NTS relays signals coming in from the periphery via ascending spinal tracts, for example, vagal and glossopharyngeal afferent projections and facial and trigeminal nerve endings (Jean, 1991), and transmits this somatosensory information via ascending projections to forebrain and limbic structures through the PB and LC (Porges, 2003; Fig. 3). These target forebrain structures include the hypothalamus, the amygdala, and the thalamus, which are key regions in the modulation of social behaviours and emotional

processing (Porges, 2003). The NTS also receives descending projections from brain structures that are, again, involved in emotion, social behaviours and higher cognitive functions such as the PVN, BNST, ceA and mPFC, mPOA (Jean, 1991; Norgren, 1978; Bailey and Wakerley, 1997; Ricardo and Koh, 1978; Terreberry and Neafsey, 1983; Fig. 3). Therefore, these extensive connections throughout the brain, many of which are reciprocal, make the NTS a key structure for autonomic, neuroendocrine, sensory and behavioural functions (Jean, 1991).

The NTS has been shown to be involved in a number of social behaviours, such as social stress in male Lister rats (Martinez et al., 1998), male sexual behaviours in mice (Vestlund and Jerlhag, 2020) and also male sexual behaviours in prairie voles (*Microtus ochrogaster*; Curtis et al., 2003). During parturition, the activity of NTS neurons that project to the supraoptic nucleus of the hypothalamus (SON) increases in female Sprague-Dawley rats (Meddle et al., 2007). In addition, oxytocin mRNA was found to increase in the NTS at parturition (Meddle et al., 2007). This suggests that the NTS plays a key role in relaying information from the uterus to the SON, activating oxytocin and vasopressin neurons during labour to drive maternal behaviours (Meddle et al., 2007). The NTS is involved in reward processing. The NTS contains catecholamine neurons; catecholamine neurons containing tyrosine hydroxylase project to the nucleus accumbens of the reward pathway in rats (Wang et al., 1992). Also, NTS catecholamine neurons projecting to the NA and the BNST are critical for the rewarding aspect of opiates in mice (Olson et al., 2006). This indicates that this pathway from the NTS has a role in altered reward processing that is associated with addiction (Smith and Aston-Jones, 2008). The NTS is found to have a role in the rewarding aspect of food. Glucagon positive neurons in the NTS project directly to the VTA and NA core and shell regions in male Sprague- Dawley rats, and blocking this transmission of glucagon from NTS neurons in the VTA and NA increases food intake (Alhadeff et al., 2012). Richard et al. (2015) found that glucagon receptors in the NTS of male Sprague-Dawley rats inhibits food reward behaviours, intake

of palatable peanut butter, and food motivated behaviour, lever-pressing to receive a sucrose reward.

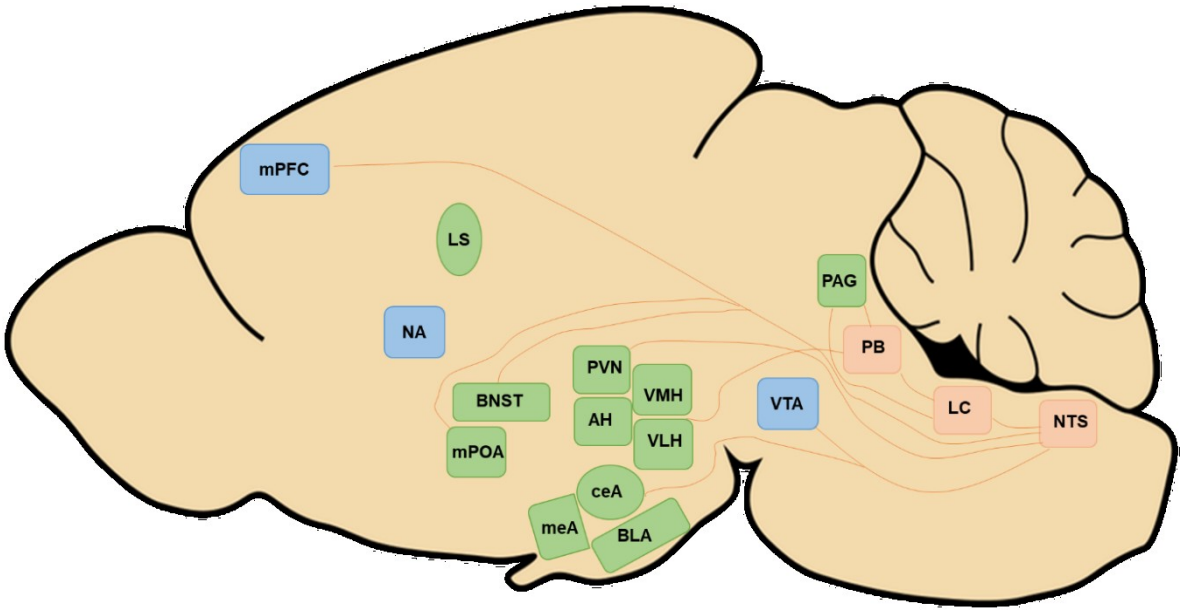

Figure 3. Main projections from the NTS to midbrain and forebrain regions.
The major projections from the NTS and other hindbrain regions, parabrachial nucleus and locus coeruleus, (shown in orange) with orange lines denoting the major projections to regions of the social decision making network (shown in green) and reward pathway (shown in blue).
mPFC, medial prefrontal cortex; NA, nucleus accumbens; LS, lateral septum; BNST, bed nucleus of the stria terminalis; mPOA, medial preoptic area; ceA, central amygdala; meA, medial amygdala; BLA, basolateral amygdala; PVN, paraventricular nucleus of the hypothalamus; AH, anterior hypothalamus; VMH, ventromedial hypothalamus; VLM, ventrolateral hypothalamus; VTA, ventral tegmental area; PAG, periaqueductal gray; PB, parabrachial nucleus; LC, locus coeruleus; NTS, nucleus of the solitary tract. (Porges, 2003; Jean, 1991; Norgren, 1978; Bailey and Wakerley, 1997; Ricardo and Koh, 1978; Terreberry and Neafsey, 1983)

4.1.2 Experimental aims

I set out to conduct a study to map Fos-ir throughout the brains of tickled rats, and compare this to non-tickled control rats, with the overall aim to guide future hypotheses as to areas of interest for the control of the behavioural response to tickling. The immediate early gene, c-*fos*, can be used as a functional marker of neural activity in response to certain stimuli (Sagar and Curran, 1988; Dragunow and Faull, 1989). Quantifying the levels of c-*fos* gene expression in brain regions or functional circuitry of interest is a useful tool for exploring which brain regions have been active during social behaviours (e.g. Baum and Everitt, 1992; Gordon et al., 2002; Veening et al., 2005; Varlinskaya et al., 2013). I analysed the effect of treatment, sex and their interaction on Fos-ir in the BLA, meA, ceA mBNST, lBNST, PLmPFC, lLmPFC, VTA, NA, LS, lPAG, lvPAG, dPAG and NTS. I also regressed behaviours that are expressed during tickling against Fos-ir in order to investigate associations between specific behaviours and neuronal activation in these regions.

To investigate whether tickling affected the activity across neural networks rather than in isolated regions, I investigated whether there were correlations in Fos-ir between connected regions of the SBN in tickled versus control rats: BLA, meA, mBNST, lBNST, PLmPFC, lLmPFC, VTA, NA, LS, lPAG, lvPAG, dPAG. Fos-ir was also quantified in the reward circuit: VTA, NA, PL mPFC and lL mPFC.

As the NTS receives and sends projections to limbic structures (Jean, 1991; Ricardo and Koh, 1978), including the medial prefrontal cortex (Terreberry and Neafsey, 1986), I also investigated whether tickling had an effect on neural activity patterns between the NTS and the PL mPFC and lL mPFC, mBNST and lBNST and ceA.

Aims

Aim 1. To quantify Fos-ir in discrete brain regions following tickling:

1.1. To identify brain regions which are active during tickling based on regions with known involvement in social conspecific play in female and male juvenile

Wistar rats. I predicted that there would be an increase in Fos-ir in areas that are involved in social play, because tickling was developed by mimicking elements of social play.

1.2. To compare Fos-ir throughout the brain between females and males in order to address whether there are any sex differences in brain regions activated in the response to tickling. I predicted that there would be sex differences in the regions involved in the response to tickling, as there were sex differences in some of the behavioural responses to tickling, and we know that there are sex differences in the neural networks of a number of different behaviours, and sex differences in the brain in general.

1.3. To investigate whether Fos-ir in individual brain regions was associated with specific play-related behavioural measures (total 50 kHz USVs, flat 50 kHz USVS, trill 50 kHz USVs, scampering, hopping, darting, approach and latency to approach). I predicted that there would be a difference in Fos-ir that parallels the differences in behaviours found in Chapter 2, and these behaviours would predict Fos-ir in regions that are known to control similar behaviours.

1.4. To build on previous findings where the reward pathway has previously been shown to be activated in response to tickling (Burgdorf et al., 2001; Burgdorf et al., 2007; Hori et al., 2013) by directly comparing activation of dopaminergic VTA neurons (visualised by tyrosine hydroxylase) between female and male juvenile Wistar rats.

1.5. To investigate whether neuronal populations in the NTS were active during tickling in female and male juvenile Wistar rats. Given its involvement in relaying somatosensory stimuli to forebrain regions, and its connections to the reward pathway, I predicted that there would be an increase in Fos-ir in the NTS because tickling is a sensory stimulus.

Aim 2. To quantify Fos-ir correlations between connected brain regions:

2.1 To investigate which neural networks are activated in response to tickling in Wistar rats using correlations of Fos-ir between anatomically related brain regions that regulate social behaviours.

2.2 To build on previous findings that the reward pathway is activated in response to tickling using correlations of Fos-ir between anatomically related brain regions of the reward pathway.

2.3 To investigate whether there were correlations in Fos-ir between the NTS and anatomically connected regions of the limbic forebrain and midbrain.

I predicted that Fos-ir would be correlated between anatomically related areas if that particular neural network was involved in the response to tickling.

Tested hypotheses

1. That the behavioural response to tickling is associated with brain regions and neural circuits that are involved in social play.
2. That there are sex differences in the neural correlates of tickling.
3. That tickling activates the reward pathway in both female and male rats given the emission of trill 50 kHz USVs during tickling in both sexes.
4. That NTS neurons are recruited in response to tickling, as this region has been reported to transmit somatosensory signals from the spinal cord to limbic and reward structures in the brain.

4.2 Materials and Methods

Please refer to Chapter 2: Materials and Methods for Animal and Husbandry, and Experimental Testing of the rats used here.

4.2.1 Immunohistochemistry for Fos immunoreactivity

Immediately following brain dissection each brain was immersed in 4% PFA in 1x PBS at 4°C for five days in a labelled plastic 50 ml tube. The brains were then transferred into 15% sucrose in 4% PFA for a further 24 - 48 hours. When the brains had sunk to the bottom of the tube, they were then transferred into 30% sucrose in 1x PBS at 4°C for 24 - 48 hours or until the tissue had sunk to the bottom. Preparation of all solutions was supervised by Mrs Valerie Bishop.

Post fixation whole brains were dissected into three sections: 1. the cerebellum, brainstem and spinal cord; 2. the olfactory bulb and 3. The main brain (including cerebral cortex, diencephalon and mesencephalon) before freezing. Any excess solution was blotted from the brain before dissection. The three samples were snap frozen on dry ice, wrapped in foil and stored in small labelled bags at -70^{o}C until they were sectioned using a freezing microtome. This part of the experiment was carried out by myself and Prof. Simone Meddle.

The main brain was sectioned coronally at 50 µm using a freezing microtome (8000 sledge microtome, Bright Instruments Ltd. Cambridgeshire, UK). Free floating sections were collected into three sets to allow for three immunohistochemical staining runs to be carried out. One set was used for double labelling with oxytocin, one set for double labelling with vasopressin (see Chapter 5 for oxytocin and vasopressin double labelling) and one set for double labelling with tyrosine hydroxylase. During sectioning, brain sections were collected into 1 x PBS, before being transferred into ethylene glycol cryoprotectant and stored at -20^{o}C.

Fos-ir and tyrosine hydroxylase double immunohistochemistry

Double labelling was performed using the free floating sections. To visualise active neural populations, rabbit anti-Fos antibody (K-25 Santa Cruz sc253) was used to stain c-*fos* expressing neurons and mouse anti-tyrosine hydroxylase monoclonal antibody (MAB318, Sigma-Aldrich) was used to stain TH-positive cell bodies. On a shaking platform set to 95 rpm, sections were washed four times 15 minutes in 0.2% PBS-T, rinsed for 5 minutes in 1x PBS, endogenous peroxidases were quenched in 0.3% hydrogen peroxide for 20 minutes, rinsed for 5 minutes in 1x PBS, incubated with 0.3% hydrogen peroxide for a further 20 minutes, washed three times 10 minutes in 0.2% PBS-T, immersed in 0.2% PBS-T and 5% normal goat serum (NGS) for 60 minutes to block non-specific binding, and incubated in rabbit anti-Fos antibody (K-25 Santa Cruz sc253) at 1:1000 diluted with 0.2% PBS-T and 3% NGS for 3.5 days at 4°C.

Sections were then washed four times 10 minutes in 0.2% PBS-T to remove excess, unbound antibody. Sections were incubated in biotinylated anti-rabbit secondary antibody (Vectastain elite ABC rabbit kit; Vector; PK6101) to amplify antibody-antigen complexes, using 10 μl biotinylated anti-rabbit secondary antibody and 30 μl NGS to every 1 ml of 0.2% PBS-T. 3 ml per sample was allowed, and sections were incubated for 60 minutes on a shaking platform (set to 65 rpm) at room temperature. Sections were washed three times 10 minutes in 0.2% PBS-T. The sections were then incubated in Avidin / Biotinylated horseradish peroxidase conjugate (Vectastain elite ABC rabbit kit; Vector; PK6101) to amplify the signal using 20 μl Avidin DH and 20 μl biotinylated horseradish peroxidase to every 1 ml of 0.2% PBS-T (made up at least 30 minutes before use). Sections were incubated for 60 minutes on a shaking platform (set to 65 rpm) at room temperature. Sections were washed three times 10 minutes in 0.2% PBS-T, rinsed in 1x PBS for 5 minutes, rinsed in 0.2M Sodium acetate buffer, antibody-antigen complexes visualised using nickel II sulphate, ammonium chloride and 0.2M sodium acetate buffer solution mixed with 25 mg/ml DAB solution and 30% hydrogen peroxide. Sections were monitored under a light microscope after 2 minutes of adding the colour substrate solution and the colour reaction halted once positive staining was

seen by immersing sections in 0.2M sodium acetate buffer for 5 minutes, sections were rinsed five times 5 minutes in 1x PBS. Endogenous peroxidases were quenched by incubating section for 20 minutes in 0.3% hydrogen peroxide. Sections were washed two times 10 minutes in 1x PBS-T before being incubated overnight at 4°C in mouse anti-TH monoclonal IgG (MAB318, Sigma-Aldrich) at [1:4000] diluted in 0.2% PBS-T and 5% NGS.

Sections were then washed three times 10 minutes in 0.2% PBS-T to remove excess, unbound antibody. Sections were incubated in biotinylated anti-mouse secondary antibody (Vectastain elite ABC mouse kit; 2B Scientific; PK6102) to amplify antibody-antigen complexes, using 10μl biotinylated anti-mouse secondary antibody and 30μl NGS to every 1 ml of 0.2% PBS-T. 3 ml per sample was allowed, and sections were incubated for 60 minutes on shaking platform (set to 65rpm) at room temperature. Sections were washed three times 10 minutes in 0.2% PBS-T. The sections were then incubated in Avidin / Biotinylated horseradish peroxidase conjugate (Vectastain elite ABC mouse kit (2B Scientific; PK6102)) to amplify the signal from immuno-positive cells using 20μl Avidin DH and 20μl Biotinylated horseradish peroxidase to every 1ml of 0.2% PBS-T (made up at least 30 minutes before use). 3 ml per sample was allowed, and sections were incubated for 60 minutes on shaking platform (set to 65rpm) at room temperature. Sections were washed three times 10 minutes in 0.2% PBS-T, rinsed in 1 x PBS for 5 minutes, TH-positive cells were visualised using DAB in 1 x PBS and 30% hydrogen peroxide (without nickel enhancement). Sections were monitored under a light microscope after 2 minutes of adding the DAB solution and the colour reaction halted once positive staining was seen by immersing sections in 1 x PBS for 5 minutes. Sections were washed five times 5 minutes in 1 x PBS before the immuno-stained sections were stored in 1 x PBS at 4°C until being mounted onto chrome-alum subbed slides.

Following all immunostaining sections were float-mounted from 1 x PBS onto gelatinised slides using a paintbrush. Slides were allowed to dry from 2 to 48 hours before dehydrating and coverslipping. Following float mounting of

sections onto the slides, the slides were dehydrated through an increasing series of ethanol as follows: 70% 5 minutes, 95% 5 minutes, two times 99% 5 minutes, xylene/99% 5 minutes, three times xylene 5 minutes. Slides were finally cover slipped straight from the last xylene using xylene-based mounting medium (Pirtex) and left on downdraft table overnight to dry. Slides were then cleaned using a razor blade and ethanol before being analysed.

4.2.2 Image acquisition and analysis

Brain regions of interest (ROI) were analysed separately in the following order: basolateral amygdala, medial amygdala, central amygdala, lateral bed nucleus of the stria terminalis, medial bed nucleus of the stria terminalis, prelimbic prefrontal cortex, infralimbic prefrontal cortex, nucleus accumbens, lateral septum, dorsal periaqueductal gray, lateral periaqueductal gray, lateroventral periaqueductal gray, ventral tegmental area, and nucleus of the solitary tract. Boundaries were defined using Paxinos and Watson's stereotaxic rat brain atlas (Paxinos and Watson, 1998) (Fig. 4 - 6). A light microscope (Nikon E600 Brightfield Microscope) was used and the ROI was identified with a 4 x objective. An image was taken at the centre of the ROI at 10 x magnification using Zen 2 (ZEISS Microscopy, Germany) software for image capture (Fig. 7-10). This was performed for both left and right hemispheres across four representative sections per animal. Sections were selected to contain the same coronal plane in order to maintain consistency across individual rats.

Fos-ir positive cells were identified as having a dark grey/ black nucleus (Fig. 7, 8, 10). Cell counting was performed blinded to sex and treatment by one observer using ImageJ 1.48v software (W. Rashband, National Institutes of Health, USA). Each image was opened in ImageJ and the *Threshold* function was used to saturate the Fos-ir positive nuclei as black and the absence of Fos-ir as white. The *Threshold* level was adjusted for each region because there was variation in background staining between regions, but kept consistent for all samples in the same region (Table 1). After the threshold levels function, the *Iterations* and *Open* functions in the binary submenu were

used to remove isolated pixels and smooth the outlines of the nuclei (https://imagej.nih.gov/ij/docs/menus/process.html; Table 1). The number of highlighted Fos-ir positive nuclei were automatically counted using the *Analyse Particles* function in ImageJ. A range of pixel units for nuclei were counted and the circularity of the outlines was assigned for each region to ensure that whole nuclei were counted (Table 1). A series of randomly selected images were used to decide on the size and intensity of Fos-ir. A random selection of images were counted manually to compare to the count data obtained from the automated counting to ensure consistency between counting methods. The reliability values between manual counting and automated counting were > 90% for each ROI (for example, for the meA, manual and automated counts had 94.4-97.3% reliability; for the lvPAG, manual and automated counts had 96.5-99.4% reliability). The number of Fos-ir positive nuclei in each section per rat were summed and then averaged to give a single average of Fos-ir positive cells per ROI per rat.

Table 1. Criteria used for quantifying Fos immunoreactivity for in each brain region of interest.

Brain Region	Threshold units	Iterations	Pixel Units	Circularity
BLA	0-100	3	70-Infinity	0.20-1.00
Medial amygdala	0-100	3	70-Infinity	0.20-1.00
Central amygdala	0-100	3	70-Infinity	0.20-1.00
Lateral BNST	0-75	4	80-Infinity	0.40-1.00
Medial BNST	0-75	4	80-Infinity	0.40-1.00
PL medial PFC	0-75	4	60-400	0.30-1.00
IL medial PFC	0-75	4	60-400	0.30-1.00
NA	0-75	4	80-Infinity	0.40-1.00
LS	0-75	4	80-Infinity	0.40-1.00
Dorsal PAG	0-90	4	50-400	0.30-1.00
Lateral PAG	0-90	4	50-400	0.30-1.00
Lateroventral PAG	0-90	4	50-400	0.30-1.00
VTA	0-70	4	70-Infinity	0.30-1.00
NTS	0-70	4	70-Infinity	0.30-1.00

Fos-ir / TH double-labelled neurons were quantified in the VTA (Fig. 9). TH-positive cells were identified as having a light brown/ tan coloured cell body. Fos-ir / TH double labelled cells had both a black nucleus and brown cell

body. The total number of Fos-ir nuclei, the number of Fos-ir /TH double-labelled cells, and TH positive cells were quantified separately in the VTA. Each image was opened in ImageJ and the Brightness and Contrast function was set to auto levels. A 10cm^2 square was used to define the counting area: the square was placed on the bottom left hand corner (randomly assigned) of the image and cells in the area alone were counted. The multi-point tool was used to manually count Fos-ir cells which were identified as having a dark grey/ black nucleus, and double-labelled cells which had a black nucleus and brown cell body. Any ambiguity in whether cells were positive or not (e.g. faint staining, or dark staining where it was not possible to distinguish between a black nucleus and brown cell body), the cells were not counted as positive. The number of Fos-ir nuclei in each section per rat were summed and then averaged to give a single average of Fos-ir cells. This was also carried out for TH positive, and Fos-ir /TH double-labelled cells.

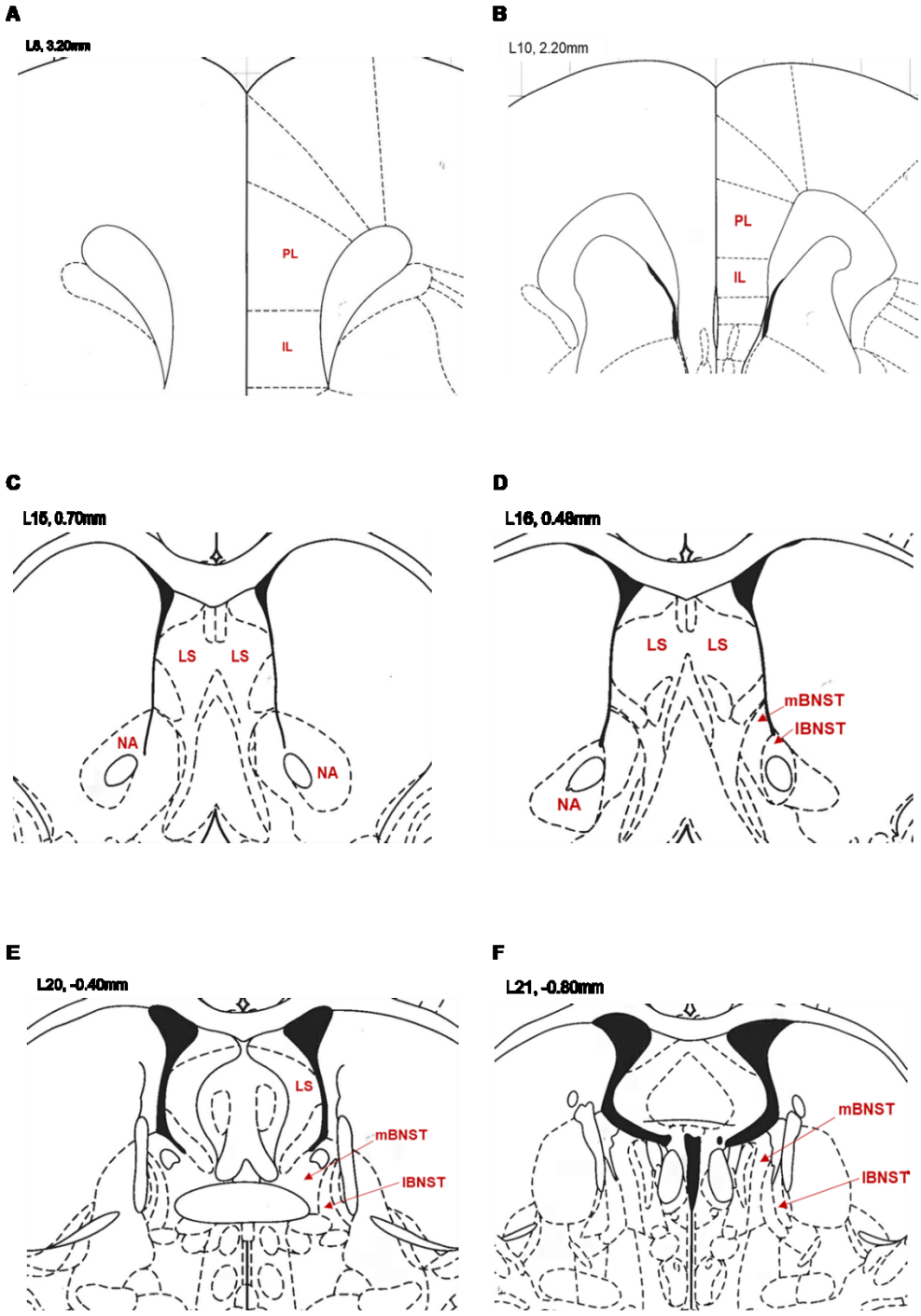

Figure 4. Representation of sampling locations in the forebrain on modified rat brain atlas templates (Paxinos and Watson, 1998).
Locations of the IL mPFC (A, B), PL mPFC (A, B), NA (C, D), LS (C, D, E), lBNST (D, E, F) and mBNST (D, E, F) moving caudally through the brain on coronal sections. Regions defined by black dashed lines. Headings refer to atlas level and distance in mm from bregma. IL mPFC, infralimbic prefrontal cortex; PL mPFC, prelimbic prefrontal cortex; NA, nucleus accumbens; LS, lateral septum; lBNST, lateral bed nucleus of the stria terminalis; mBNST, medial bed nucleus of the stria terminalis.

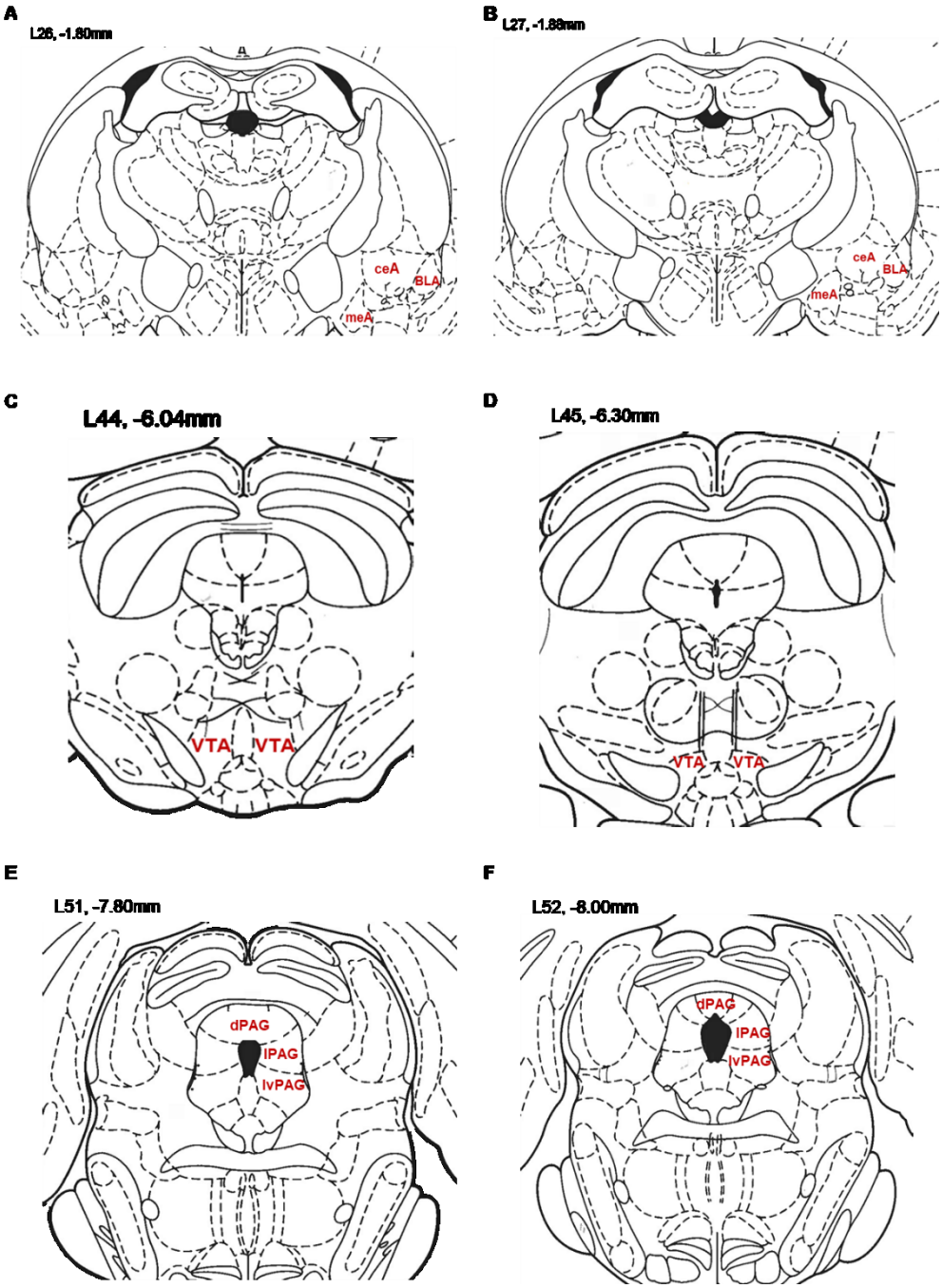

Figure 5. Representation of sampling locations in the midbrain on modified rat brain atlas templates (Paxinos and Watson, 1998).
Locations of the BLA (A, B), ceA (A, B), meA (A, B), VTA (C, D), dPAG (E, F), lPAG (E, F), and lvPAG (E, F) moving caudally through the brain on coronal sections. Regions defined by black dashed lines. Headings refer to atlas level and distance in mm from bregma. BLA, basolateral amygdala; ceA, central amygdala; meA, medial amygdala; VTA, ventral tegmental area; dPAG, dorsal periaqueductal gray; lPAG, lateral periaqueductal gray; lvPAG, lateroventral periaqueductal gray.

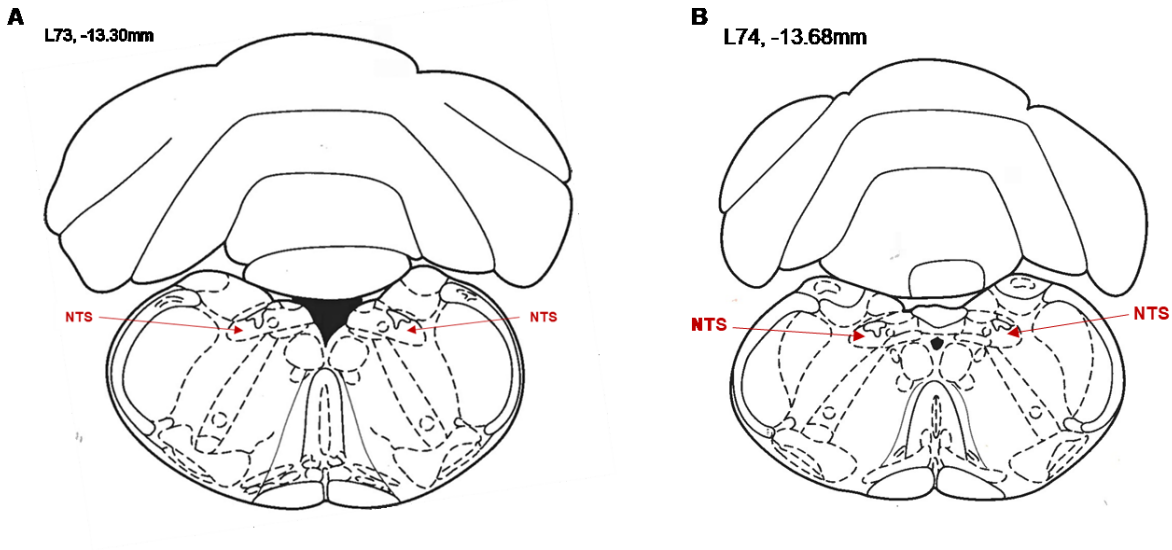

Figure 6. Representation of sampling locations for the NTS on modified rat brain atlas templates (Paxinos and Watson, 1998).
Locations of the NTS (A, B) moving caudally through the brain on coronal sections. Regions defined by black dashed lines. Headings refer to atlas level and distance in mm from bregma. NTS, nucleus of the solitary tract.

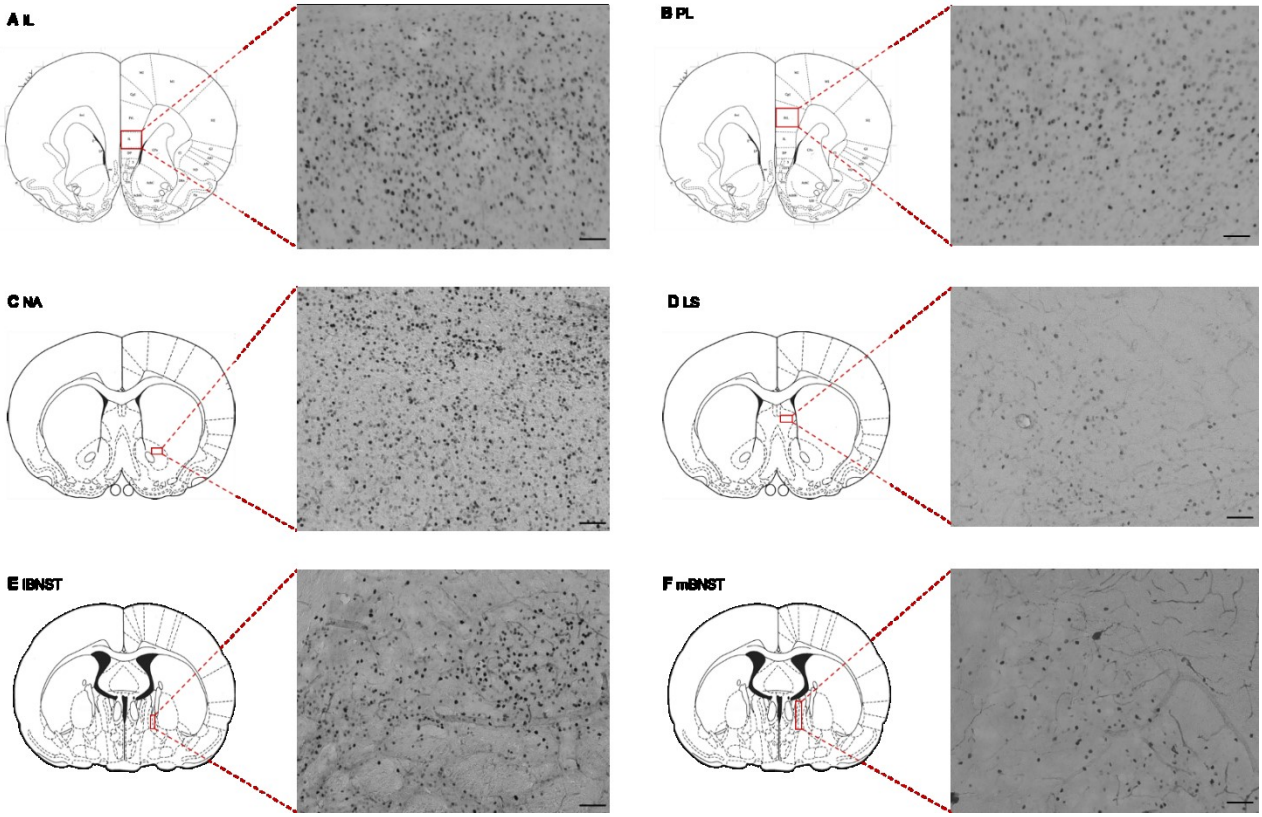

Figure 7. Representative image of immunohistochemical staining for Fos-ir and sampling locations for each forebrain region on modified rat brain atlas templates (Paxinos and Watson, 1998).
Examples of Fos nuclear staining in the IL mPFC (A), PL mPFC (B), NA (C), LS (D), lBNST (E), mBNST (F) at x10 magnification. Red rectangles refer to the location used to carry out cell counting in each subregion. Scale bar = 50μm. IL mPFC, infralimbic prefrontal cortex; PL mPFC, prelimbic prefrontal cortex; NA, nucleus accumbens; LS, lateral septum; lBNST, lateral bed nucleus of the stria terminalis; mBNST, medial bed nucleus of the stria terminalis.

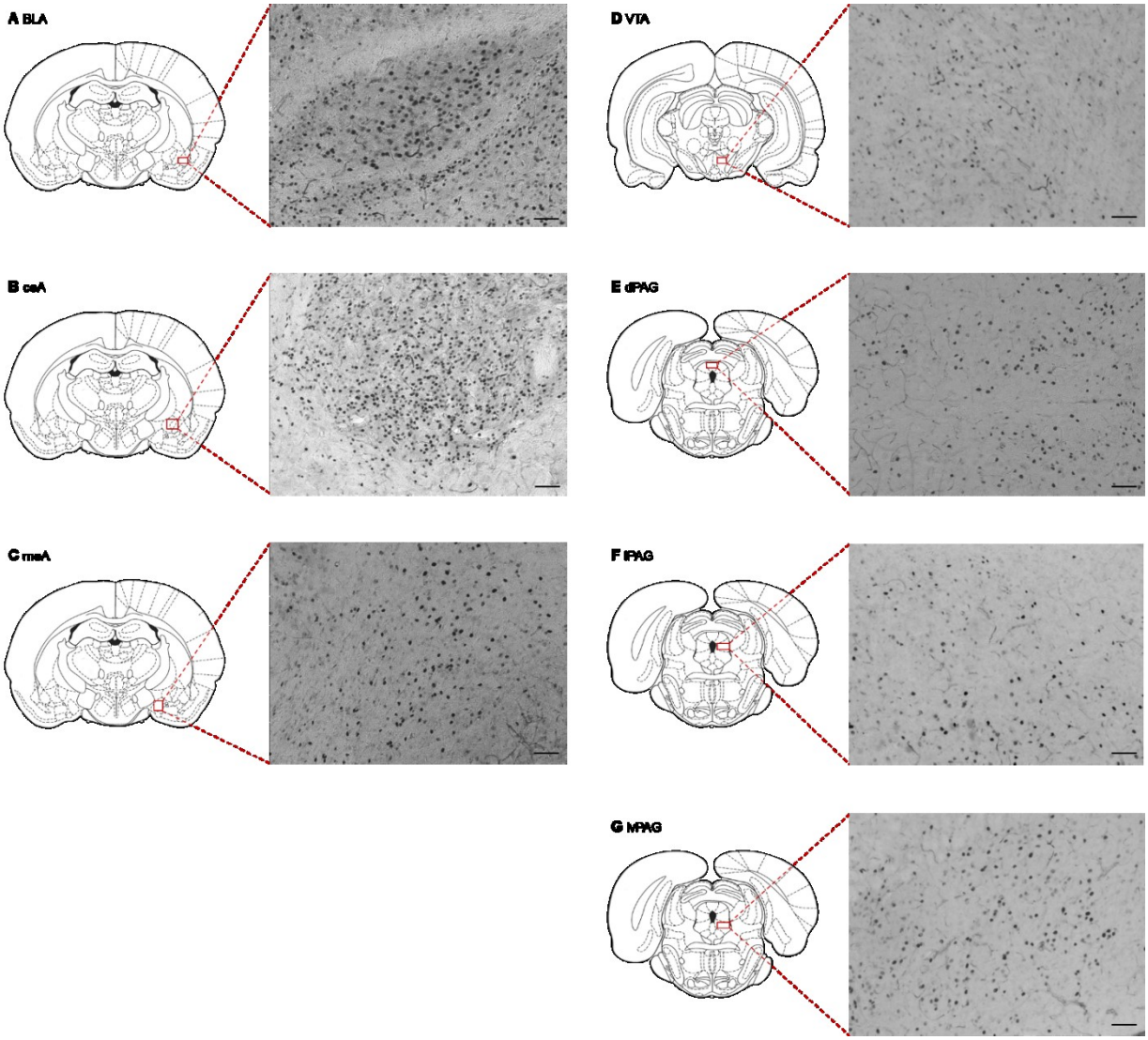

Figure 8. Representative image of immunohistochemical staining for Fos-ir and sampling locations for each midbrain region on modified rat brain atlas templates (Paxinos and Watson, 1998).

Examples of Fos nuclear staining in the BLA (A), ceA (B), meA (C), VTA (D), dPAG (E), lPAG (F) and lvPAG (G) at x10 magnification. Red rectangles refer to the location used to carry out cell counting in each subregion. Scale bar = 50μm. BLA, basolateral amygdala; ceA, central amygdala; meA, medial amygdala; VTA, ventral tegmental area; dPAG, dorsal periaqueductal gray; lPAG, lateral periaqueductal gray; lvPAG, lateroventral periaqueductal gray.

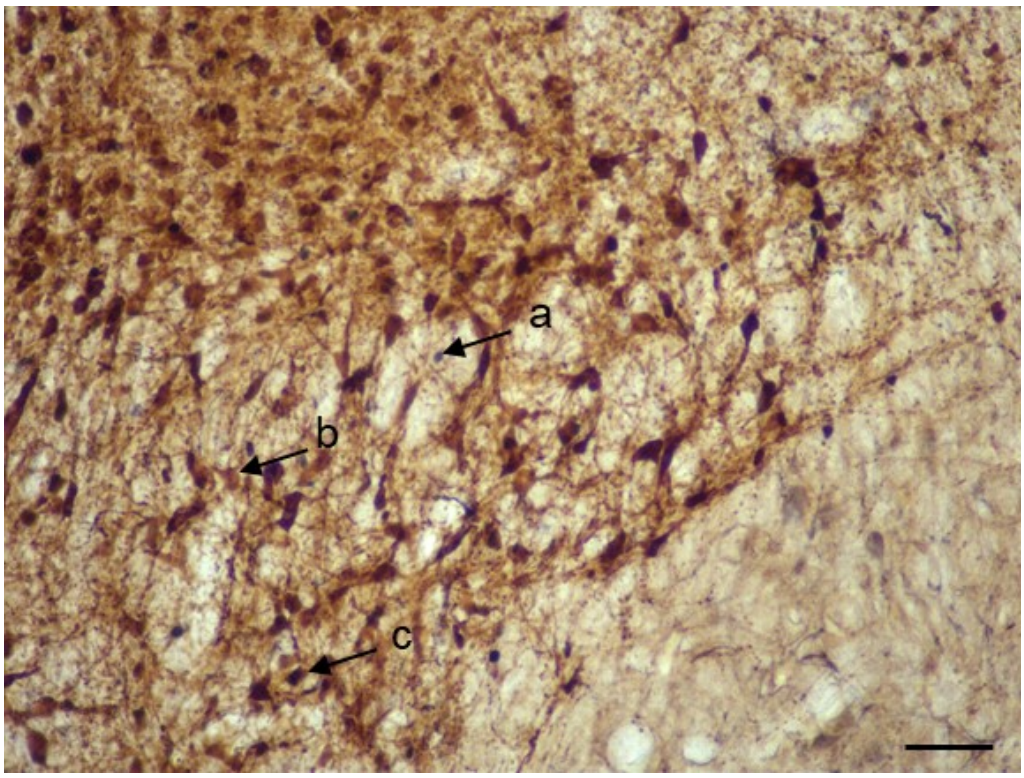

Figure 9. Representative image of immunohistochemical staining in the VTA.
Fos-ir nuclear staining and tyrosine hydroxylase cytoplasmic staining in the VTA at x10 magnification. a, a Fos-ir nucleus; b, a TH positive cell body; c, a double-labelled Fos-ir TH neuron. Micrograph of coronal section approximately level 44, distance from the interaural line 2.96mm, distance from bregma -6.04mm (Swanson and Kuypers, 1980). Scale bar = 50μm.

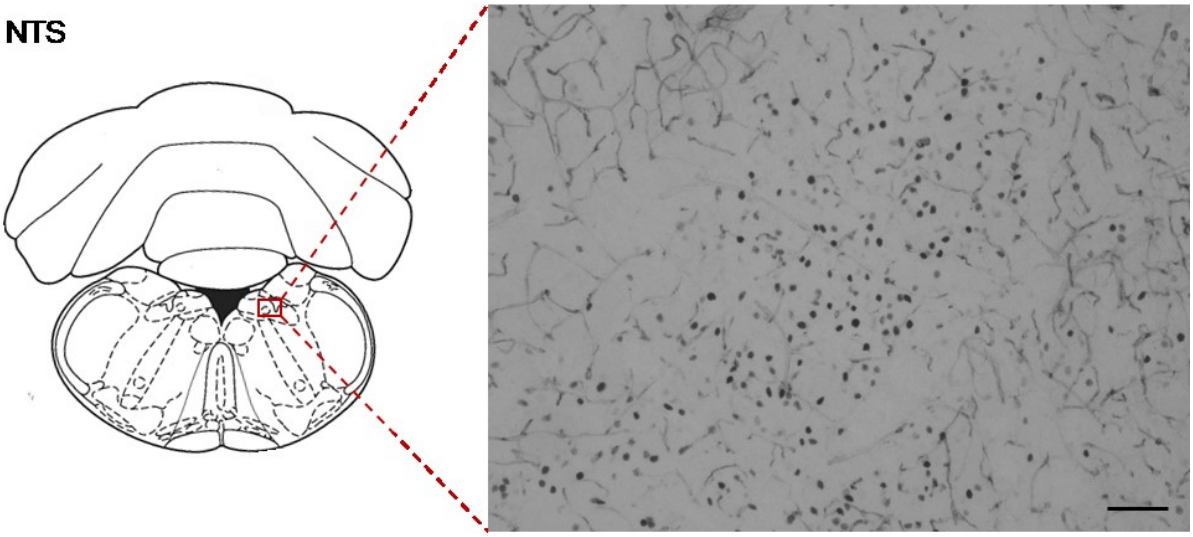

Figure 10. Representative image of immunohistochemical staining for Fos-ir and sampling locations for the NTS of the hindbrain.
Examples of Fos-ir nuclear staining in the NTS (A, B) at x10 magnification. Red rectangles refer to the location used to carry out cell counting in each subregion. Scale bar = 50μm. NTS, nucleus of the solitary tract.

4.2.3 Statistical analysis

Statistical analysis was carried out in R Studio and R (v 4.0.3, The R Foundation for Statistical Computing Platform (2020)). Model adequacy was verified by examination of residuals (McCullagh and Nelder, 2019) via the DHARMa package (Hartig, 2020). Generalised linear mixed models (GLMMs) in the glmmTMB package (Brooks et al., 2017) were used to analyse the effect of treatment (tickled or non-tickled control), sex and the interaction of treatment and sex on Fos-ir in the BLA, meA, ceA, lBNST, mBNST, PL mPFC, IL mPFC, NA, LS, dPAG, lPAG, lvPAG, VTA and NTS (each area was analysed separately). Dependent on model fitting and overdispersion, family links were set to either poisson or negative binomial distributed errors ('nbinom2' in the MASS package; Venables and Ripley, 2002) with default transformations. All models included batch and cage as random effects and were nested (batch followed by cage). This was done to account for the variation from the non-independence of rats from the same cage and potential batch effects. For all models, sex and treatment were included as fixed effects, as well as the interaction between them. A regression was carried out to test the linear relationships between Fos-ir and the fixed effects. Statistical significance based on $p<0.05$ threshold level and adjusted for multiple comparisons using the Tukey method (Lenth, 2020).

Likewise, GLMMs in the glmmTMB package (Brooks et al., 2017) were used to analyse the effect of treatment (tickled or non-tickled control), sex and the interaction of treatment and sex on Fos-ir immunoreactivity in TH-positive neural populations in the VTA. Due to model fitting and overdispersion, family links were set to negative binomial distributed errors ('nbinom2' in the MASS package; Venables and Ripley, 2002) with default transformations. Batch and cage as random effects and were nested (batch followed by cage). Sex, treatment and their interaction were included as fixed effects in the final models. A regression was carried out to test the linear relationships between Fos-ir in TH- neurons and the fixed effects. Statistical significance based on $p<0.05$ threshold level and adjusted for multiple comparisons using the Tukey method (Lenth, 2020). The model included an offset of the total number of Fos

positive nuclei, to account for this uncontrolled variable in each sampling event, effectively calculating proportions of double-labelled cells/ total Fos-ir positive cells.

A bottom-up, stepwise regression analysis was used to investigate whether the measured behaviours could explain differences in Fos-ir in TH-positive VTA neurons. Each behaviour was added into the GLMM as a covariate in a bottom-up, stepwise regression. Covariate effects were established by interpretation on estimated coefficients, associated z-values and p-values (Bates et al., 2015), with statistical significance based on $p < 0.05$ threshold level. Behaviours were sequentially included in the model based on estimated coefficients, associated z-values and p-values (Bates et al., 2015), with non-significant co-variates dropped, producing final minimal models with maximised predictive parameters. All final models included as a minimum treatment, sex and their interaction. All data in the tables are presented as ± standard error of beta coefficient estimates (SE) and 95% confidence intervals (CI). The significance level was set at $p < 0.05$.

A top-down, stepwise regression analysis was used to investigate whether the measured behaviours could explain differences in Fos-ir in the BLA, meA, ceA, lBNST, mBNST, PL mPFC, IL mPFC, NA, LS, dPAG, lPAG, lvPAG, VTA and NTS (each area was analysed separately). Every behaviour was included as a co-variate in the initial GLMM. Covariate effects were established by interpretation on estimated coefficients, associated z-values and p-values (Bates et al., 2015), with statistical significance based on $p < 0.05$ threshold level. Behaviours were sequentially removed from the model based on estimated coefficients, associated z-values and p-values (Bates et al., 2015), with non-significant co-variates dropped, producing final minimal models with maximised predictive parameters. All final models included as a minimum treatment, sex and their interaction. All data in the tables are presented ± standard error of beta coefficient estimates (SE) and 95% confidence intervals (CI). The significance level was set at $p < 0.05$.

Correlations of average number of Fos-ir nuclei between the different regions were carried out using the average number of Fos-ir positive nuclei for each rat. Raw data were tested for normality using the Shapiro test (the data were deemed not normal if $p < 0.05$) and then analysed with either a Pearson (if tested normal) or Spearman rank (if not normal) correlation. This was carried out between treatment conditions and not sex to increase power in the analysis as sex did not affect Fos-ir in isolated regions (Tables 2, 3 and 5). The significance level was set at $p < 0.05$.

All bar graphs were generated in GraphPad Prism (version 9 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com). The data in the graphs are presented as the means of the raw data ± standard error of the mean (SEM). Scatter plots were generated in R Studio and R (v 4.0.3, The R Foundation for Statistical Computing Platform (2020)) using the ggplot2 package (Wickham, 2011).

4.3 Results

4.3.1 The effect of tickling on Fos-ir expression in the forebrain

Medial prefrontal cortex

There was no difference in Fos-ir expression in the mPFC of tickled and control rats. Treatment, sex or their interaction had no main effect on the number of Fos-ir cells in the two divisions of the mPFC: the infralimbic mPFC (Treatment: Z = -1.08, p = 0.279; sex: Z = 0.246, p = 0.805; Treatment x Sex: Z = 0.127, p = 0.899), and the prelimbic mPFC (Treatment: Z = 1.363, p = 0.173; sex: Z =-0.429, p = 0.668; Treatment x Sex: Z = -0.200, p = 0.842; Table 2; Fig. 11). There was no significant involvement of any tickle-associated behaviours on neural activity in this region.

Nucleus accumbens

There was no difference in Fos-ir in the NA of tickled and control rats. There was no effect of the interaction of treatment and sex (Z = 0.44, p = 0.660) and overall effect of treatment (Z = -0.57, p = 0.568), sex (Z = -1.07, p = 0.286) on the number of Fos-ir cells in the NA (Table 2; Fig 11). There was no significant involvement of any tickle-associated behaviours on neural activity in the NA.

Bed nucleus of the stria terminalis

There was no effect of the interaction of treatment and sex on the number of Fos-ir in the lBNST (Treatment x Sex: Z = 1.208, p = 0.227) or mBNST (Treatment x Sex: Z = 1.584, p = 0.1132; Table 2; Fig. 11). There was no difference in Fos-ir in the BNST of tickled and control rats (lBNST: Z = -0.471, p = 0.638; mBNST: Z = 0.180, p = 0.857; Table 2; Fig. 11). There was a main effect of sex on Fos-ir in the mBNST (sex: Z = -2.208, p = 0.027), although not in the lBNST (sex: Z = 0.375, p = 0.708; Table 2; Fig. 11).

Three behaviours were associated with tickling: flat 50 kHz USVs, scampering and darting predicted Fos-ir in the BNST. Fos-ir in the lBNST was significantly explained by both flat 50 kHz USVs (Z = 2.024, p = 0.043) and

darting behaviours (Z = -2.001, p = 0.045). Flat 50 kHz USVs predicted an increase in Fos-ir in the lBNST (Fig. 12), while darting predicted the opposite, with a higher frequency of darting being associated with lower Fos-ir (Fig. 12). Scampering behaviours explained Fos-ir in the mBNST (Z = -2.430, p = 0.0151). The association was negative, with a higher rate of scampering being related to lower Fos-ir (Fig. 12).

Lateral septum

There was no difference in Fos-ir in the LS of tickled and control rats. There was no effect of the interaction between treatment and sex (Z = -0.359, p = 0.719) and no overall effect of treatment (Z = -0.274, p = 0.784), sex (Z = -0.536, p = 0.592) on Fos-ir in the LS (Table 2; Fig. 11). There was no significant involvement of any tickle-associated behaviours on neural activity in this brain region.

Table 2. Statistical output of final predictive model for Fos-ir in the forebrain.
Significant effects ($p < 0.05$) shown in bold.

Region	Fixed Effects	β-coefficient	±SE	95% CI	Z value	P-value
IL PFC	Treatment	-0.473	0.4364	-1.328- 0.383	-1.08	0.279
	Sex	0.059	0.239	-0.411- 0.529	0.246	0.805
	Treatment:Sex	0.043	0.336	-0.616- 0.701	0.127	0.899
PL PFC	Treatment	0.457	0.335	-0.200-1.113	1.363	0.173
	Sex	-0.136	0.318	-0.760- 0.487	-0.429	0.668
	Treatment:Sex	-0.088	0.439	-0.949- 0.773	-0.200	0.842
NA	Treatment	-0.097	0.170	-0.430- 0.236	-0.57	0.568
	Sex	-0.177	0.166	-0.503- 0.148	-1.07	0.286
	Treatment:Sex	0.103	0.234	-0.356- 0.562	0.44	0.660
	Sex	-0.086	0.166	-0.412- 0.239	-0.52	0.602
	Treatment:Sex	0.037	0.231	-0.416- 0.491	0.16	0.871
Lateral BNST	Treatment	-0.201	0.427	-0.001-0.636	-0.471	0.638
	Sex	0.092	0.244	-0.387- 0.571	0.375	0.708
	Treatment:Sex	0.428	0.354	-0.266- 1.122	1.208	0.227
	Flat	0.003	0.002	0.0001- 0.006	2.024	**0.043**
	Dart	-0.060	0.030	-0.119- -0.001	-2.001	**0.045**
Medial BNST	Treatment	0.047	0.263	-0.467- 0.562	0.180	0.857
	Sex	-0.490	0.222	-0.926- -0.055	-2.208	**0.027**
	Treatment:Sex	0.473	0.299	-0.112- 1.059	1.584	0.1132
	Scamper	-0.055	0.023	-0.099- -0.011	-2.430	**0.0151**
LS	Treatment	-0.069	0.252	-0.564- 0.426	-0.274	0.784
	Sex	-0.138	0.258	-0.644- 0.367	-0.536	0.592
	Treatment:Sex	-0.128	0.357	-0.828- 0.572	-0.359	0.719

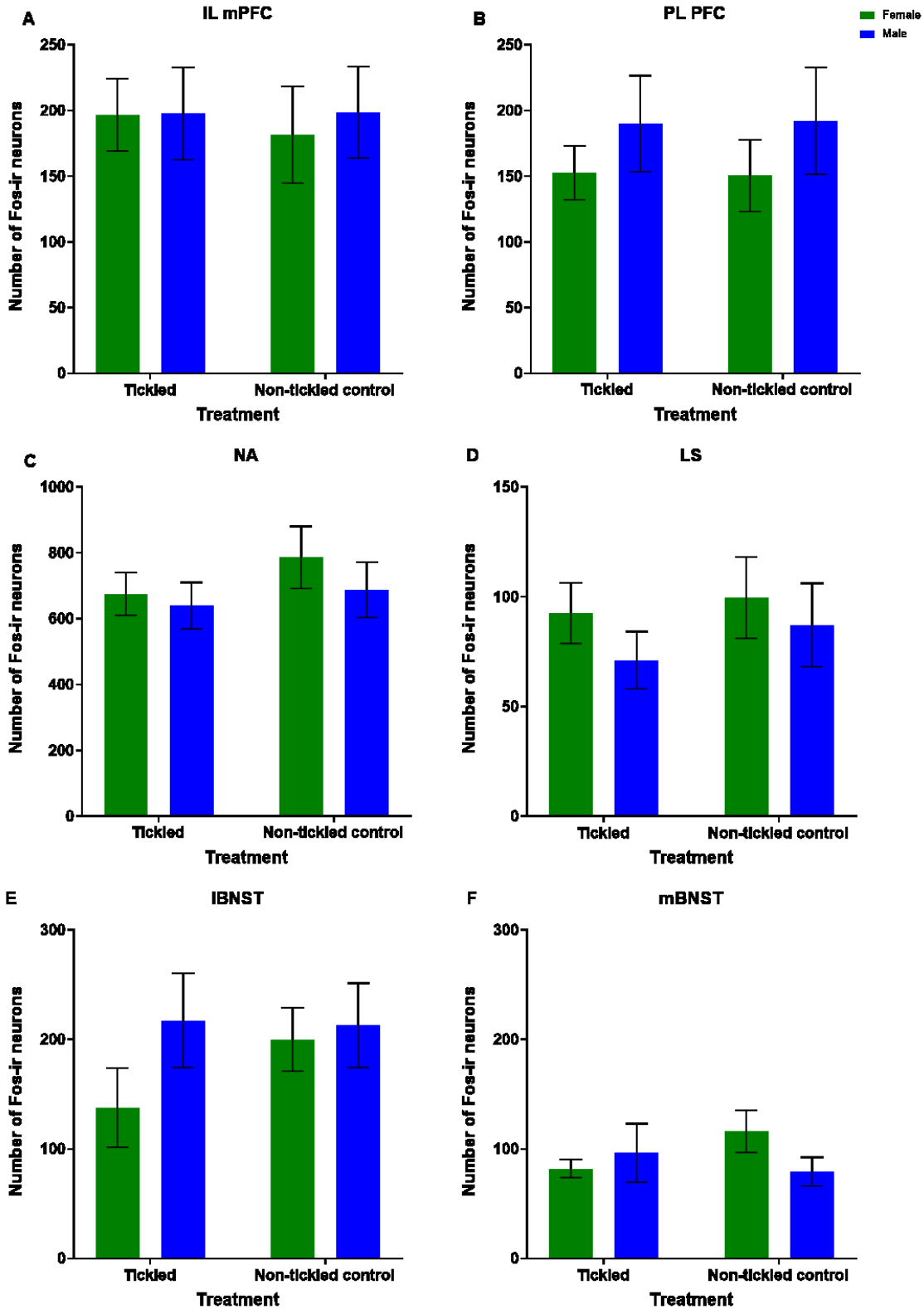

Figure 11. Tickling did not affect Fos-ir in regions of the forebrain of female and male rats.

Number of Fos-ir cells in the infralimbic medial prefrontal cortex (A), prelimbic medial prefrontal cortex (B), nucleus accumbens (C), lateral septum (D), lateral bed nucleus of the stria terminalis (E), and medial bed nucleus of the stria terminalis (F). Females (green), males (blue). Mean ± SEM; n = 16 / group. [n.s.]

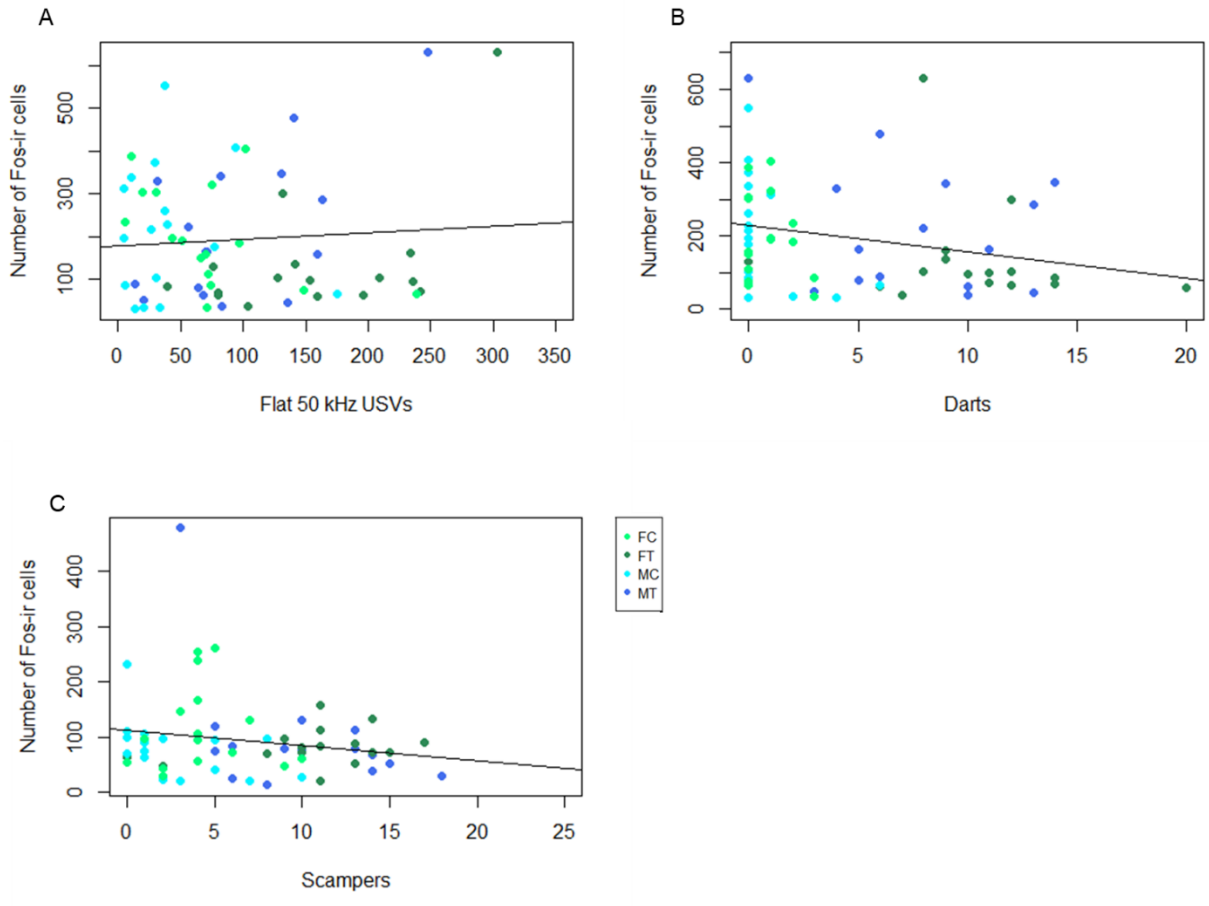

Figure 12. Flat 50 kHz USVs, darts and scampers were found to predict Fos-ir in the BNST.
Correlations between flat 50 kHz USVs and Fos-ir in the lBNST (A), darts and Fos-ir in the lBNST (B), and scampers and Fos-ir in the mBNST (C). FT, female tickled (light green); FC, female control (dark green); MT, male tickled (light blue); MC, male control (dark blue). Black line, linear regression line.

4.3.2 The effect of tickling on Fos-ir in the midbrain

Amygdala

Three regions of the amygdala were analysed as part of this study: the basolateral, central and medial subdivisions. Treatment, sex or their interaction had no effect on the number of Fos-ir nuclei in the BLA (Treatment: Z = 1.06, p = 0.290; sex: Z = 0.51, p = 0.610; Treatment x Sex: Z = -0.81, p = 0.853), the ceA (Treatment: Z = 0.89, p = 0.375; sex: Z = 0.24, p = 0.813; Treatment x Sex: Z = -0.86, p = 0.390), or the meA (Treatment: Z =0.50, p = 0.619; sex: Z = -0.52, p = 0.602; Treatment x Sex: Z = 0.16, p = 0.871; Table 3; Fig. 13). No behaviours were significantly correlated with Fos-ir in the basolateral or medial divisions of the amygdala, however scampering was correlated with Fos-ir in the ceA (Z = -2.02, p = 0.043) and a lower frequency of scampering was associated with more Fos-ir in the ceA (Fig. 14).

Periaqueductal gray

Fos-ir did not differ between treatments in the PAG, however, tickle-associated behaviours were correlated with Fos-ir in the lvPAG. Treatment, sex or their interaction had no effect on the number of Fos-ir cells in the three divisions of the PAG: dPAG (Treatment: Z = -0.438, p = 0.661; sex: Z = -0.846, p = 0.398; Treatment x Sex: Z = 0.748, p = 0.454), lPAG (Treatment: Z = 0.871, p = 0.384; sex: Z = -0.842, p = 0.400; Treatment x Sex: Z = 0.106, p = 0.916) and lv PAG (Treatment: Z = -0.008, p = 0.994; sex: Z = -1.509, p = 0.131; Treatment x Sex: Z = -0.332, p = 0.739; see Table 3; Fig. 13).

There was no involvement of any tickle-associated behaviours on Fos-ir in the dPAG and lPAG, however, there was an association between trill 50 kHz USVs and Fos-ir in the lvPAG (Z = -2.029, p = 0.043; Fig. 15). Higher frequency trill USVs predicted a decrease in the number of Fos-ir cells in the lvPAG. Conversely, as flat 50 kHz USVs increased, the number of Fos-ir cells also increased in the lvPAG (Z = 2.090, p = 0.036; Fig. 15).

Ventral tegmental area

There was no effect of the interaction of treatment and sex on Fos-ir in the VTA ($Z = 0.503$, $p = 0.615$). There was a significant overall effect of sex on Fos-ir in the VTA ($Z = -2.23$, $p = 0.026$), with females having more Fos-ir cells in the VTA than males (Table 3, Fig. 13). Tickling did not affect Fos-ir in the VTA ($Z = 0.329$, $p = 0.742$). There was no significant involvement of tickled-related behaviours on neural activity in this region.

Tyrosine Hydroxylase in the VTA

There was no significant relationship between Fos-ir TH neurons in the VTA and the interaction of treatment and sex ($Z = 0.726$, $p = 0.468$; Table 4, Fig. 16), treatment ($Z = -0.115$, $p = 0.908$), and sex ($Z = -0.708$, $p = 0.479$). No quantified behaviours were correlated with the number of Fos-ir TH neurons.

Table 3. Statistical output of final predictive model for Fos-ir in regions of the midbrain. Significant effects ($p < 0.05$) shown in bold.

Region	Fixed Effects	β-coefficient	±SE	95% CI	Z value	P-value
BLA	Treatment	0.241	0.227	-0.205- 0.686	1.06	0.290
	Sex	0.074	0.146	-0.212- 0.360	0.51	0.610
	Treatment:Sex	-0.039	0.212	-0.454- 0.376	-0.81	0.853
Central Amygdala	Treatment	0.244	0.275	-0.296- 0.784	0.89	0.375
	Sex	0.042	0.178	-0.308- 0.392	0.24	0.813
	Treatment:Sex	-0.244	0.283	-0.799- 0.312	-0.86	0.390
	Scamper	-0.064	0.032	-0.127- -0.002	-2.02	**0.043**
Medial Amygdala	Treatment	0.083	0.167	-0.244- 0.410	0.50	0.619
	Sex	-0.086	0.166	-0.412- 0.239	-0.52	0.602
	Treatment:Sex	0.037	0.231	-0.416- 0.491	0.16	0.871
Dorsal PAG	Treatment	-0.2198	0.5016	-1.203- 0.763	-0.438	0.661
	Sex	-0.2832	0.3348	-0.939- 0.373	-0.846	0.398
	Treatment:Sex	0.3047	0.4070	-0.493- 1.103	0.748	0.454
Lateral PAG	Treatment	0.199	0.229	-0.249- 0.648	0.871	0.384
	Sex	-0.181	0.215	-0.601- 0.240	-0.842	0.400
	Treatment:Sex	0.032	0.298	-0.553- 0.616	0.106	0.916
Lateroventral PAG	Treatment	-0.004	0.5411	-1.065- 1.056	-0.008	0.994
	Sex	-0.510	0.338	-1.174- 0.153	-1.509	0.131
	Treatment:Sex	-0.137	0.413	-0.947- 0.672	-0.332	0.739
	Trill	-0.007	0.004	-0.014- -0.0002	-2.029	**0.043**
	Flat	0.003	0.001	0.0002- 0.005	2.090	**0.036**
VTA	Treatment	0.155	0.472	-0.769- 1.080	0.329	0.742
	Sex	-0.760	0.341	-1.428- -0.091	-2.23	**0.026**
	Treatment:Sex	0.247	0.491	-0.716- 1.210	0.503	0.615

Table 4. Statistical output of final predictive model for Fos-ir and tyrosine hydroxylase neurons in the VTA.

Region	Neuropeptide	Fixed Effects	β-coefficient	±SE	95% CI	Z value	P-value
VTA	Tyrosine Hydroxylase	Treatment	-0.0043	0.037	-0.077- 0.068	-0.115	0.908
		Sex	-0.0269	0.038	-0.101- 0.048	-0.708	0.479
		Treatment: Sex	0.0384	0.053	-0.065- 0.142	0.726	0.468

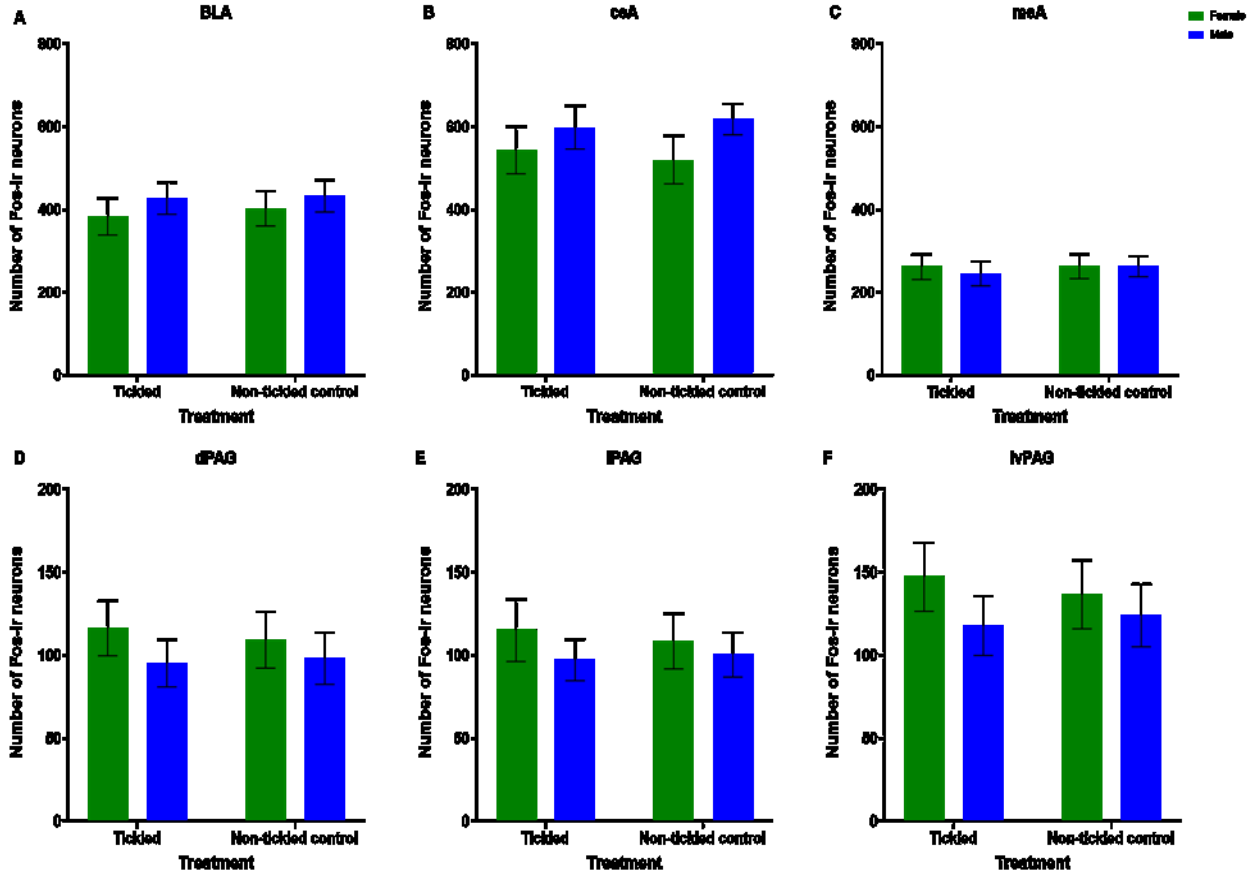

Figure 13. Tickling did not affect Fos-ir in regions of the midbrain of female and male rats.

Number of Fos-ir cells in the basolateral amygdala (A), central amygdala (B), medial amygdala (C), dorsal PAG (D) lateral PAG (E), and lateroventral PAG (F). Female (blue) and male rats (green). Mean ± SEM; n = 16 / group.

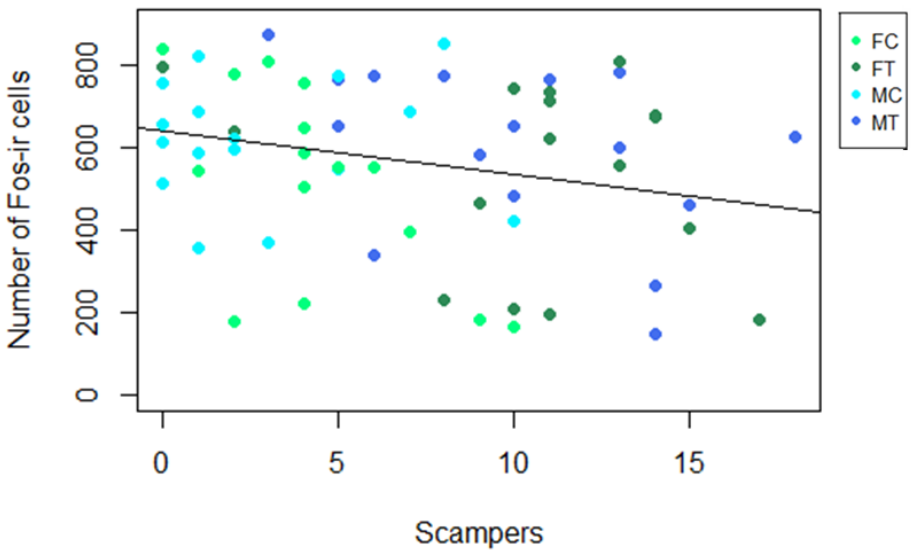

Figure 14. Scampering was negatively associated with Fos-ir in the central amygdala.
Correlations between the numbers of scampers and Fos-ir in the central amygdala. FT, female tickled (light green); FC, female control (dark green); MT, male tickled (light blue); MC, male control (dark blue). Black line, linear regression line.

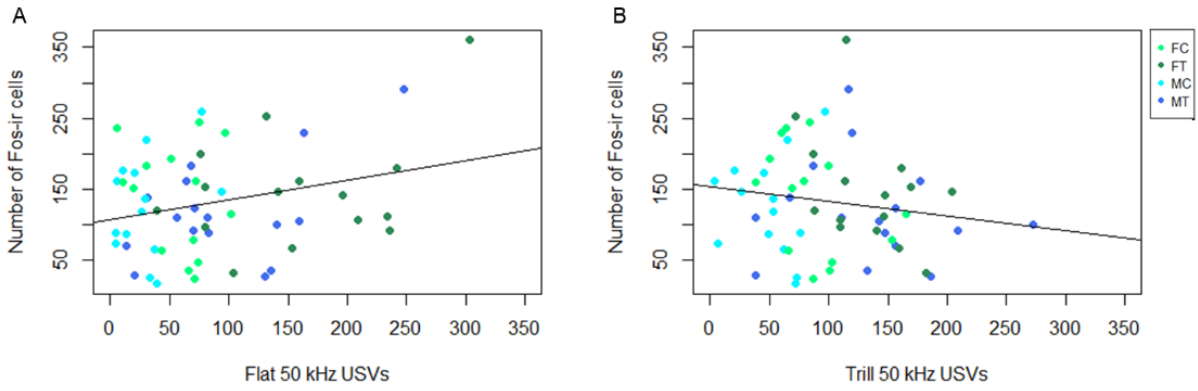

Figure 15. Sub types of 50 kHz USVs were found to predict Fos-ir in the lateroventral PAG.
Flat 50 kHz USVs and Fos-ir in the lateroventral periaqueductal gray were positively associated (A), while trill 50 kHz USVs and Fos-ir in the lateroventral periaqueductal gray were negatively associated (B). FT, female tickled (light green); FC, female control (dark green); MT, male tickled (light blue); MC, male control (dark blue). Black line, linear regression line.

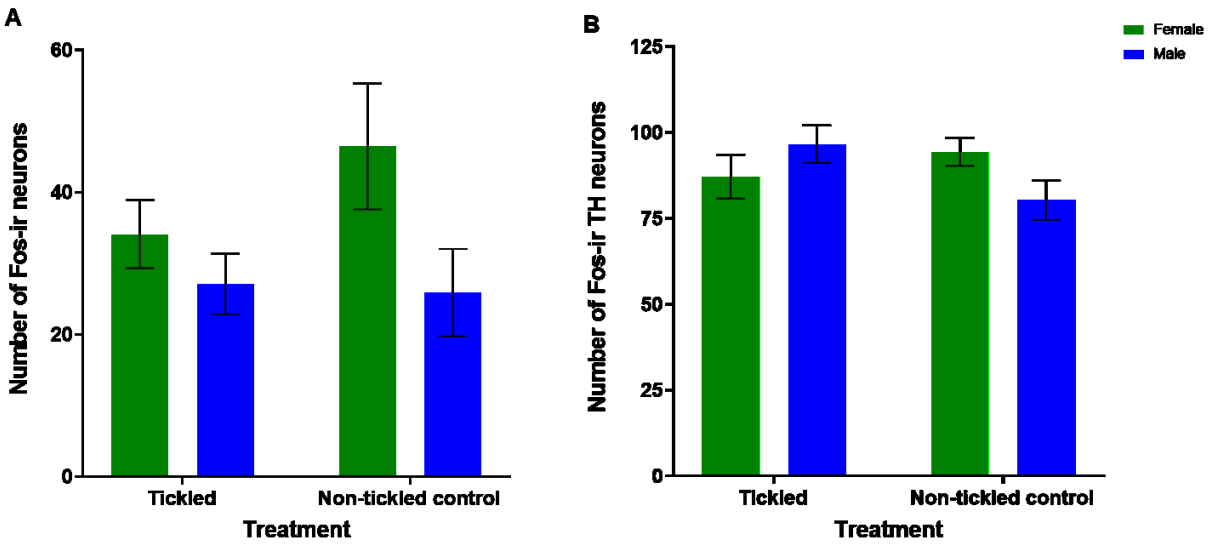

Figure 16. Tickling did not affect Fos-ir in TH neurons of the VTA in female and male rats. The number of Fos-ir non-TH neurons (A) and Fos-ir TH neurons in the VTA (B). Females (green), males (blue). Mean ± SEM; n = 16 / group. [n.s.]

4.3.3 The effect of tickling on Fos-ir in the hindbrain

Nucleus of the solitary tract

While there was no overall effect of the interaction of treatment and sex (Z = 0.870, p = 0.384), treatment (Z = 0.427, p = 0.669) and sex (Z = 0.397, p = 0.691) on the number of Fos-r cells in the NTS (Table 5, Fig. 17), darting and scampering behaviours were associated with Fos-ir in the NTS (Fig. 18).

Darting behaviours were significantly related to Fos-ir (Z = 2.075, p = 0.0379); a higher frequency of darting was correlated with greater Fos-ir. This is in contrast to scampering, where higher rates predicted lower Fos-ir in the NTS (Z = -3.361, p = 0.0008; Fig. 18).

Table 5. Statistical output of final predictive model for Fos-irin the NTS of the hindbrain. Significant effects (p < 0.05) shown in bold.

Region	Fixed Effects	β-coefficient	±SE	95% CI	Z value	P-value
NTS	Treatment	0.183	0.424	-0.657- 1.024	0.427	0.669
	Sex	0.129	0.325	-0.507- 0.765	0.397	0.691
	Treatment:Sex	0.355	0.408	-0.444- 1.154	0.870	0.384
	Scamper	-0.160	0.048	-0.254- -0.067	-3.361	**0.0008**
	Dart	0.106	0.051	0.005- 0.207	2.075	**0.0379**

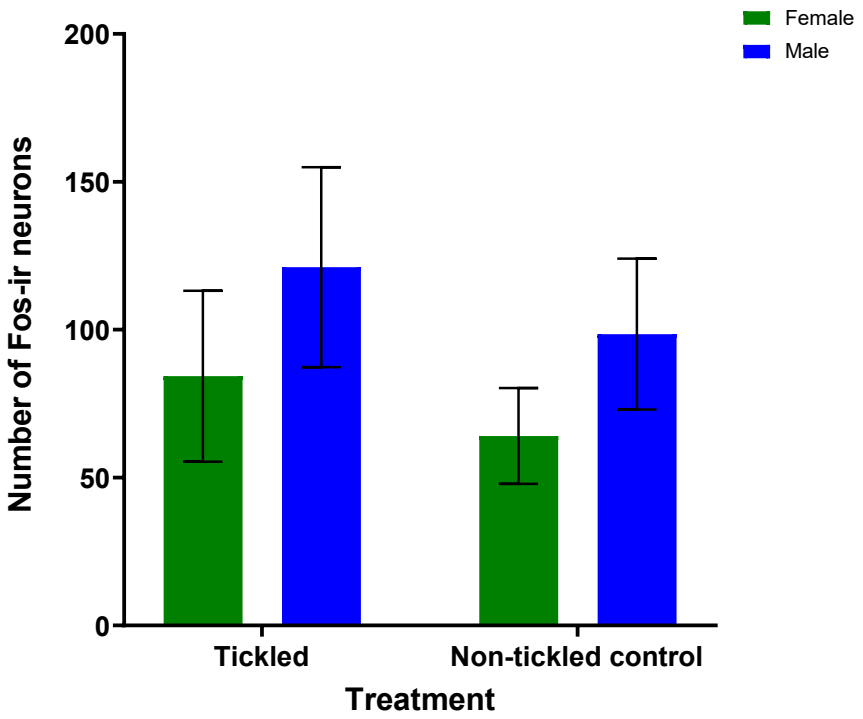

Figure 17. Tickling did not affect Fos-ir in the NTS of the hindbrain in female and male rats.
Number of Fos-ir cells in the NTS. Females (green), males (blue). Mean ± SEM; n = 16 / group. [n.s.]

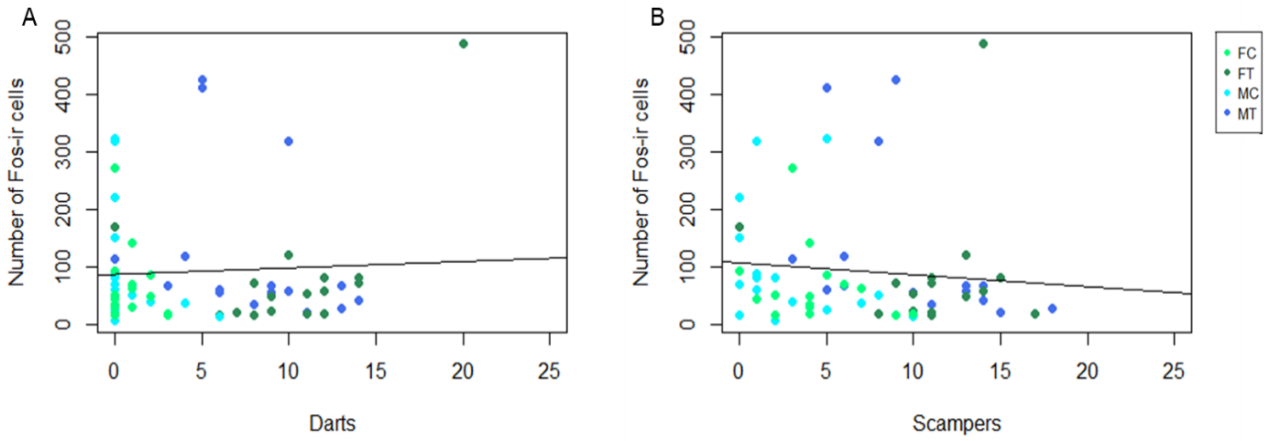

Figure 18. The number of darts was found to positively predict Fos-ir in the NTS, while scampers negatively predicted Fos-ir in the NTS.
Correlations between darts and Fos-ir in the NTS (A) and scampers and Fos-ir in the NTS (B). FT, female tickled (light green); FC, female control (dark green); MT, male tickled (light blue); MC, male control (dark blue). Black line, linear regression line.

4.3.3 The effect of tickling on neural activation patterns between anatomically related regions

Connected regions underpinning social play

Fos-ir in the NA and the mBNST, lBNST and LS were not correlated in tickled compared to control rats (Table 6) while activity between NA and the BLA and meA was maintained in tickled rats similarly to control rats (Table 6). Therefore, Fos-ir patterns in the NA and its projections to the amygdala, BNST and LS were correlated in control rats. Tickled rats also had similar activity patterns between the NA and the amygdala.

Fos-ir patterns between the prefrontal cortex and the amygdala were similar in tickled and control rats. In the prefrontal cortex, both tickled and control rats had significant correlations between the Fos-ir in the IL and BLA, and between the PL and BLA, while between the IL and meA, neither group had a significant correlation (Table 6).

Fos-ir in the basolateral and medial amygdala was correlated with that in the VTA in control but not tickled rats (Table 6). Similarly, Fos-ir in the lBNST was correlated with expression in the BLA and meA in control but not tickled rats (Table 6). In both tickled and control rats, there was no correlation in activity between the basolateral and medial amygdala and the mBNST (Table 6). Fos-ir was correlated between the BLA and meA in both tickled and control rats (Table 6). There was no correlation in Fos-ir between the VTA and the mBNST and lBNST in tickled or control rats (Table 6).

Fos-ir in the LS was significantly correlated with expression in the lBNST and mBNST, and lvPAG in both tickled and control rats (Table 6). There was no correlation between Fos-ir in the LS and VTA in either tickled or control rats (Table 6). Between the LS and BLA, there was a correlation in control but not tickled rats (Table 6). Therefore, tickling appears to affect correlations in Fos-ir between the LS and BLA compared to correlations between LS and other regions.

Between the dPAG and mBNST and lBNST, Fos-ir was correlated in control but not tickled rats (Table 6). There was a significant correlation in

activity in the dPAG and meA in both tickled and control rats, but only control rats had a significant correlation between dPAG and the LS (Table 6). Tickling may affect correlations in Fos-ir between dPAG and the BNST and LS.

In the lPAG, tickled and control rats had similar activity patterns. There was a significant correlation between Fos-ir in the lPAG and the mBNST and meA in both groups, and no correlation between lPAG and lBNST (Table 6). This suggests that treatment did not alter correlations in Fos-ir between the lPAG and other structurally connected regions.

There was no correlation between Fos-ir in the lvPAG and the lBNST in tickled or control rats (Table 6). There was a significant correlation in Fos-ir between the lvPAG and meA in both tickled and control rats (Table 6). Only tickled rats showed a correlation between lvPAG and mBNST (Table 6).

Table 6. Correlations between structurally related regions of the brain that have been associated with the regulation of social behaviours in rats.
Significant effects ($p < 0.05$) shown in bold.

SBN		Control			Tickled		
Brain region 1	Brain Region 2	Correlation Co-efficient	t/S value	p value	Correlation Co-efficient	t/S value	p value
NA	**BLA**	0.404	3250	**0.022**	0.677	5.05	**<0.001**
NA	**Lateral BNST**	0.471	2.92	**0.006**	0.095	4939.9	0.607
NA	**LS**	0.582	2276	**<0.001**	0.275	1.566	0.128
NA	**Medial BNST**	0.383	3364.6	**0.03**	0.204	4342	0.261
NA	**Medial amygdala**	0.41	2.46	**0.01**	0.523	3.365	**0.002**
IL	**BLA**	0.545	700	**0.011**	0.574	3.21	**0.004**
IL	**Medial amygdala**	0.417	2.00	0.061	0.394	1.96	0.063
PL	**BLA**	0.542	704	**0.012**	0.576	858	**0.004**
PL	**Medial amygdala**	0.392	1.85	0.075	0.446	1120	**0.033**
BLA	**Lateral BNST**	0.531	2558.5	**0.001**	0.277	3942.7	0.124
BLA	**Medial BNST**	0.284	3902.7	0.114	0.305	3794	0.09
BLA	**VTA**	0.371	3431	**0.03**	0.07	0.406	0.688
BLA	**Medial amygdala**	0.743	1402	**<0.001**	0.826	8.04	**<0.001**
Medial amygdala	**Lateral BNST**	0.491	3.09	**0.004**	0.155	4607.8	0.39
Medial amygdala	**Medial BNST**	0.303	3802.7	0.09	0.273	3966	0.13
Medial amygdala	**VTA**	0.417	3175.6	**0.01**	0.16	0.88	0.38
Medial BNST	**VTA**	0.166	4547.2	0.362	0.117	4812.9	0.52
Lateral BNST	**VTA**	0.111	4852.3	0.54	0.116	4820	0.53
LS	**BLA**	0.496	2746	**0.004**	0.211	1.187	0.245
LS	**Lateral BNST**	0.651	1906.3	**<0.001**	0.477	2848.3	**0.005**
LS	**Lateral PAG**	0.423	3154	**0.001**	0.084	4999	0.64
LS	**Lateroventral PAG**	0.413	2144	**0.02**	0.377	3085	**0.03**
LS	**Medial BNST**	0.719	1533.3	**<0.001**	0.544	2482.7	**0.001**
LS	**VTA**	0.085	4989.9	0.642	0.044	0.24	0.81
dPAG	**Lateral BNST**	0.405	2.43	**0.021**	0.121	4790.9	0.506
dPAG	**Medial BNST**	0.454	2975.5	**0.008**	0.23	4200	0.204
dPAG	**Medial amygdala**	0.602	4.13	**<0.001**	0.413	2.487	**0.01**
dPAG	**LS**	0.489	2786	**0.004**	0.112	0.62	0.54
Lateral PAG	**Lateral BNST**	0.307	1.77	0.08	0.175	4498.8	0.336
Lateral PAG	**Medial amygdala**	0.539	3.51	**0.001**	0.49	2782	**0.005**

Lateral PAG	**Medial BNST**	0.423	3149.6	**0.01**	0.394	3304	**0.02**
lvPAG	**Lateral BNST**	0.146	0.75	0.45	0.323	3354.8	0.075
lvPAG	**Medial amygdala**	0.509	3.01	**0.005**	0.41	2924	**0.023**
lvPAG	**Medial BNST**	0.322	2474.7	0.09	0.578	2090	**<0.001**

Connected regions of the reward circuitry

In control rats there was a correlation in Fos-ir between the NA and PL mPFC but this was not observed in tickled rats (Table 7). Both tickled and control rats had a significant correlation between the NA and IL mPFC.

Tickling seemed to affect correlations between the VTA and the PL and IL mPFC (Table 7). Neither control nor tickled rats had a significant correlation of Fos-ir in the VTA and NA.

Table 7. Correlations between structurally related regions of the reward circuitry. Significant effects ($p < 0.05$) shown in bold.

Reward		Control			Tickled		
Brain region 1	Brain Region 2	Correlation Co-efficient	t/S value	p value	Correlation Co-efficient	t/S value	p value
NA	**PL**	0.538	2.78	**0.011**	0.406	1202	0.056
NA	**IL**	0.561	2.95	**0.008**	0.629	3.71	**0.001**
VTA	**PL**	0.658	526.3	**0.001**	0.273	1470.9	0.207
VTA	**IL**	0.628	573.4	**0.002**	0.373	1.84	0.079
VTA	**NA**	0.269	3988.7	0.134	0.191	1.07	0.290

Connected regions of the NTS

In tickled compared to control rats, correlations between the NTS and IL and PL mPFC, and the mBNST and lBNST were disrupted (Table 8). There was a significant correlation between the NTS and ceA in both tickled and control rats. There was no correlation in Fos-ir between the NTS and NA in tickled and control rats.

Table 8. Correlations between the NTS and midbrain and forebrain regions to which it sends projections. Significant effects ($p < 0.05$) shown in bold.

Hindbrain		Control			Tickled		
Brain region1	Brain Region 2	Correlation Co-efficient	t/S value	p value	Correlation Co-efficient	t/S value	p value
NTS	**ceA**	**0.665**	**1822.7**	**<0.001**	**0.444**	**3030**	**0.01**
NTS	**IL mPFC**	**0.541**	**706**	**0.012**	0.164	1692	0.453
NTS	**PL mPFC**	**0.603**	**610**	**0.004**	0.011	2002	0.9622
NTS	**lBNST**	**0.577**	**2305.4**	**<0.001**	-0.025	5592	0.8923
NTS	**mBNST**	**0.376**	**3401.6**	**0.034**	0.242	4134	0.181
NTS	**NA**	0.262	4026	0.147	0.266	4000	0.139

4.4 Discussion

In the present study, tickle-induced Fos-ir was measured in regions of the social behaviour network, reward pathway and hindbrain to investigate the neural substrates underpinning responses to tickling. Overall, there was no difference between Fos-ir in control and tickled female and male rats in individual forebrain, midbrain and hindbrain regions (Tables 2-5). Unexpectedly, control rats had more correlated activity in known neural circuits compared to tickled rats. While several behaviours that occurred during tickling were associated with Fos-ir in specific brain regions and Fos-ir was correlated between certain regions with known anatomical connections, these data represent correlational and not functional associations. Overall, this study should provide preliminary evidence that may inform future studies to understand in greater depth the neural correlates of tickling in both female and male rats.

4.4.1 Regions of the SBN

The mPFC, NA, BNST, LS, amygdala, PAG and VTA are part of the SBN that extends throughout the brain and mediates numerous social behaviours (Newman, 1999; O'Connell and Hofmann, 2011). Given that tickling did not appear to significantly increase neuronal activity in any of these regions, this may suggest that tickling does not activate the same brain regions as social behaviours.

Lateral and medial BNST

The BNST is an important region for social behaviours ranging from maternal pup retrieval in rats (Numan and Numan, 1996), intromission and ejaculation copulatory behaviours in male rats (Emery and Sachs. 1976; Valcourt and Sachs, 1979) to social play. Social play was found to increase Fos-ir in the BNST of male Wistar rats (van Kerkhof et al., 2014). Reppucci et al. (2018) found that social play increased Fos-ir in the BNST of rats, and this was greater in female compared to male rats. Therefore, the lack of effect of tickling on neuronal activation in the lateral and medial BNST may suggest that this region is not involved in the tickling response. The BNST has extensive

projections to the amygdala (Alheid et al., 1998; Dong et al., 2001), and is considered part of the extended amygdala due to the connections between the central amygdala and the BNST (Johnston, 1923; de Olmos, 1972; Alheid et al., 1998). Through these projections, the BNST modulates fear (Davis et al., 2010) and stress responses (Radley et al., 2009). Therefore, one interpretation of my results may be that by the stimulus of tickling was not a fearful or stressful stimulus, which is consistent with the lack of increased Fos-ir also found in the amygdala. This is supported by the behavioural data, which show low levels of 22 kHz USVs being emitted (Chapter 2, Fig. 8) and tickled rats produced abundant numbers of appetitive 50 kHz USVs (Chapter 2, Fig. 6) which are suppressed in response to aversive stimuli (Burgdorf et al., 2011).

Lateral septum

The LS regulates social behaviours such as bonding between rat pups (Clemens et al., 2020) and social aggression (Leroy et al., 2018; Beiderbeck et al., 2007). The LS is important in the expression of social play in female and male rats (Beatty et al., 1982; Bredewold et al., 2015). Veenema et al. (2013) showed that vasopressin receptor activation in the LS regulated social play in female and male rats, while Bredewold et al. (2014) showed that oxytocin and vasopressin affected nape attack and pin elements of play, as well as the duration of play bouts. Therefore, while Fos-ir was not affected by tickling, another avenue to explore may be whether oxytocin and vasopressin receptor activation in the LS is involved in the response to tickling. This could be carried out by using receptor antagonists injected into the LS and quantifying any behavioural changes during tickling.

Basolateral, central and medial amygdala

Meaney, Dodge and Beatty (1981) found that lesions to the amygdala decreased the frequency of social play in male Holtzman rats, but did not affect social play in females. Reppucci et al. (2018) found that social play increased Fos expression in the meA of female and male Wistar rats, and they reported this effect to be greater in females. In contrast, van Kerkhof et al. (2014) showed no increase in Fos-ir in the ceA, meA or BLA as a result of conspecific

play. This may suggest that the role of the amygdala in play is not binary, and measuring neural activation in this region may not be a sensitive enough technique to determine the amygdala's role in play and tickling. Alternatively, transmission of different neurotransmitters, such as endocannabinoids (Argue et al., 2017) and oxytocin (Bertelsen et al., 2017) may be responsible for the role of the amygdala in play. Due to the amygdala's established role in fear responses (LeDoux, 2003; Fendt and Fanselow, 1999), that tickling did not significantly increase neuronal activation in this region may suggest that tickling was not perceived as an aversive, fearful stimulus for the rats.

Periaqueductal gray

The PAG has been implicated in a number of behaviours, for example, in fear responses (Borelli et al., 2013; Broiz et al., 2008) and sexual behaviours in female (lordosis) and male (ejaculation) rats (Lonstein et al., 1998; Normandin et al., 2011). Also, the dPAG is involved in defensive behaviours in male rats (Bittencourt et al., 2004), the lPAG is necessary for prey hunting, chasing and attacking behaviours (Mota-Oritz et al., 2012), and the lvPAG is important in fear learning (Assareh et al., 2017; Carrive et al., 1997; Arico et al., 2017). There is not a lot of evidence for the role of PAG in social play, although Gordon et al. (2002) found that elements of social play, for example dorsal contacts, were associated with increased Fos expression in the dPAG in juvenile male Long-Evans rats. Social play in rats is thought to be important for the development of social behaviours in adulthood, like sexual behaviours, fighting and aggression (Auger and Olesen, 2009) and therefore the role of the PAG is likely for those adult behaviours such as mating (Lonstein et al., 1998; Normandin et al., 2011) and aggression (Mota-Oritz et al., 2012). In tickling, the PAG may be more important in the control of vocalisations (see section '*Associations between behaviour and Fos-ir throughout the brain*').

4.4.2 Correlations in Fos-ir between anatomically related regions

When correlations in Fos-ir between structurally related brain regions implicated in social conspecific play were analysed (Table 6), I found that in

24 of the 34 correlations there were significant correlations between brain regions in control rats (correlation rate of 70.6%) while tickled rats had significant correlations in 14 of the 34 correlations (correlation rate of 41.2%). This was different to my predications as I hypothesised that tickled rats would have increased Fos-ir in structurally related regions compared to control rats. Instead, these data suggest that tickling is affecting neuronal activation in the SBN in a different pattern across regions compared to control rats.

Except for the PL to meA projection, there were significant correlations in Fos-ir between the PL and BLA, and the IL and BLA in both tickled and control rats. Projections between the amygdala and PFC are associated with emotional processing, such as fear responses in rats (Likhtik et al., 2005; Quirk et al., 2003), reward learning in rats (Keefer and Petrovich, 2017) and inducing affective states in humans (Banks et al., 2007). Specifically, the mPFC-BLA pathway has been implicated in reducing fear in response to non-aversive stimuli, by firing of neurons in the mPFC entraining BLA neuronal firing, resulting in a reduced fear response in both rats and mice (Likhtik et al., 2014). The correlated activity across this pathway in both tickled and control rats in this study indicates that the testing paradigm for both control and tickled rats was not perceived as an aversive stimuli, which is consistent with the low number of 22 kHz USV emitted by these rats (Chapter 2, Fig. 8).

There was correlated Fos-ir activity in the meA to the lBNST and to the VTA in control but not tickled rats, and also between the BLA to the lBNST and VTA. Neither tickled nor control rats had correlations in Fos-ir between the BLA and meA to the mBNST. van Kerkhof et al. (2014) found that Fos-ir in the BLA and BNST was increased following social play, but not in the ceA and meA. Also, Cheng et al. (2008) found that following play in male hamsters, there was increased expression in the meA and BNST. This suggests that these regions were not activated in a similar way by tickling, as they are by social conspecific play.

The LS has a role in multiple social behaviours, such as maternal behaviour (Menon et al., 2018), pair bonding (Liu et al., 2001), social

aggression (Leroy et al., 2018; Wong et al., 2016) and social memory (Lukas et al., 2013). It is connected to the hypothalamus, PAG and meA and all regions are thought to play an important role in regulating social behaviours (Risold and Swanson, 1997; Clemens et al., 2020). In the present study, I found that there were significant correlations between the LS and the amygdala, extended amygdala, and PAG in control rats, while Fos-ir was not correlated in tickled rats. This may suggest that with tickling there was less coordinated activity between regions that are activated by social behaviours.

fMRI studies have reported that the PAG is activated during tickling and laughter in humans (Wattendorf et al., 2013) and the PAG plays a role in vocalisations in multiple species such as monkeys, rats and guinea pigs (Yajima et al., 1980; Jurgens, 1994; Kyuhou and Gemba, 1998). Due to connections from the PAG to brain regions of the social behaviour network, such as the LS (Risold and Swanson, 1997; Clemens et al., 2020) and amygdala, it is proposed that the PAG has a role in conveying emotional behaviours (Paredes et al., 2000). In the current study I found that there were positive correlations in Fos-ir between the dPAG and the BNST, meA and LS in control rats, but this was not the case in tickled rats. Therefore, this may again suggest that in tickled rats there is less coordinated activity between regions that are related to social behaviours.

Overall, these correlational data suggest that tickled rats have less synchronised activity between regions with known involvement in social behaviours, including social play. A possible explanation for this may be that the rats were pair-housed and so had access to social interactions, including social play, in the home cage. Therefore, the correlations between regions in the control rats in the present study may represent neuronal activation due to the social interaction in the home cage. The stimulus of tickling may affect neuronal activity between regions that modulate social behaviours because tickling is not perceived as a social stimulus to the rat. An alternative explanation may be related to differences in experimental design between tickling and social play studies. In the present study, the rats were tickled for two minutes per day for 10 days. Two minutes of testing is consistent with the

method developed by Burgdorf and Panksepp (2001) and is widely used, while longer durations of tickling are less common (LaFollette et al., 2017; e.g. 10 minutes; Schwarting et al., 2007; Wohr et al., 2009). In contrast, many studies on social play use longer testing periods during which play can occur (e.g. 10 minutes, Reppucci et al., 2018 and Perkins et al., 2017; 15 minutes, van Kerkhof et al., 2014; 30 minutes, Paul et al., 2014 and Gordon et al., 2002; 60 minutes, Northcutt and Nguyen, 2014). Therefore, the two minutes of testing in the present study may not have been a strong enough stimulus to result in changes in Fos-ir in the present study, compared to those changes reported following social conspecific play.

4.4.3 Regions of the reward pathway

I found that there was no effect of tickling on Fos-ir in the PL and IL mPFC, or in the NA. There was also no effect of treatment on Fos-ir in the VTA, nor on Fos-ir in VTA TH neurons. There were significant correlations in Fos-ir between the IL mPFC and the NA in both tickled and control rats, however control rats had significant correlations in Fos-ir between the NA and PL mPFC, and between the VTA and mPFC, while tickled rats did not. Taken together, these data suggest that tickling did not increase neuronal activity across the reward circuitry compared to control conditions. This is surprising as tickling has been linked to the reward pathway, as indicated by trill 50 kHz USVs (Burgdorf and Panksepp, 2001; Burgdorf et al., 2011; Hori et al., 2013), which were produced significantly more by tickled than control rats in the present study (Chapter 2, Fig. 6). However, while tickled rats produced more trill 50 kHz USVs, control rats were also abundantly producing trill 50 kHz USVs and there was individual variation within treatment groups (Chapter 2). It is possible that some control rats were finding entering the tickling arena to be rewarding. Therefore, the treatment difference in the behaviours may not have been large enough to be detected by quantifying Fos-ir in the regions analysed. An alternative control group, where the rats do not enter the test arena but remain in their home cage and are not touched, similar to the control used by van Kerkhof et al. (2014) when investigating the neural substrates of social conspecific play in male rats, could be used to investigate whether the

environment of the test arena resulted in trill 50 kHz USVs and activation of the reward pathway in control rats.

Studies have shown the involvement of the reward pathway in tickling; lesions of the VTA significantly decreased trill 50 kHz USVs produced during tickling (Burgdorf et al., 2007). Burgdorf et al. (2007) also showed that electrical brain stimulation of the mesolimbic dopamine system via the VTA increased the amount of 50 kHz calls emitted by rats, although these rats were not tickled. Tickling was found to increase dopamine release in the NA of adolescent rats (Hori et al., 2013). Similarly, Burgdorf et al. (2001) found that a dopamine agonist, amphetamine, applied to the NA of adult rats that had not been tickled increased the number of 50 kHz USVs in a dose-dependent manner. A possible explanation for the differing findings of the present study may be due to the difference in experimental design between studies. In the present study, the rats were tickled for 10 days and then the brains were quantified for Fos-ir. Alternatively, Burgdorf et al. (2007) carried out three days of tickling before lesioning or electrically stimulating the VTA, while Hori et al. (2013) tickled the rats for five minutes before measuring dopamine release in the NA. The rewarding nature of tickling may be greatest at a particular time point of the tickling protocol, and after 10 days of tickling extinction of the reward may be occurring (extinction is a behavioural event that occurs when a learned behaviour decreases in strength if the reinforcing stimulus is removed;Todd, Vurbic and Bouton, 2014). This could be investigated by quantifying Fos-ir in the reward pathway in rats that had undergone different lengths of tickling, for example, one, three, five and 10 days of tickling.

The reward pathway plays a vital role in the rewarding aspect of a number of non-social stimuli, such as feeding (e.g. Nalied et al., 2005), and social stimuli (see Trezza et al., 2011 for review), such as sexual behaviours in male rats (e.g. Pfaus et al., 1990) and social play in rats (Vanderschuren et al., 1997, 2016). Specifically in terms of social play, functioning of the mPFC is vital for normal expression of different elements of social play, such as pinning (Panksepp et al., 1994) and defensive tactics (Bell et al. 2009), and the duration and frequency of play bouts (van Kerkhof et al., 2013). Achterberg

et al. (2015) showed that pharmacological inhibition of the IL mPFC reduced pinning and pouncing elements of social play in male Wistar rats. van Kerkhof et al. (2014) showed that male Wistar rats that had been exposed to social conspecific play had increased Fos-ir in the PL mPFC, but not the IL mPFC, and in the nucleus accumbens (core and shell regions) in male rats that had engaged in social play. The authors also showed that there were correlations between the VTA and the mPFC and the NA and the mPFC (van Kerkhof et al., 2014). van Kerkhof et al. (2014) found no association between Fos-ir in the VTA and social play in male juvenile Wistar rats which is consistent with the findings of the present study. However, the VTA is involved in other social behaviours, such as pair-bonding in male prairie voles (Curtis and Wang, 2005), establishing a conditioned place preference for a parent in mice pups (Wang et al., 2017), licking and grooming maternal behaviours impacting TH-ir in the VTA (Pena et al., 2014), sexual pacing behaviours in female rats (Coria-Avila and Pfaus, 2007) and promoting prosocial behaviours in mice (Hung et al., 2017). Taken together, this may suggest that tickling did not activate the reward pathway as with other social behaviours, such as social play, pair-bonding and sexual behaviours.

4.4.4 The nucleus of the solitary tract

To my knowledge, no studies have investigated the role of the NTS in relation to play behaviours and tickling. The NTS has known involvement in numerous autonomic functions, such as cardiovascular effects (de Oliveira et al., 2003), breathing (Huda et al., 2018), sodium appetite (Jarvie and Palmiter, 2017) and feeding (Eckel and Geary et al., 2001; Lira et al., 2014). The NTS is also involved social behaviours, such as social stress in male rats (Martinez et al., 1998), male sexual behaviours (Vestlund and Jerlhag, 2020) and parturition in female rats (Meddle et al., 2007). In the present study, there was no overall effect of treatment on Fos-ir in the NTS. Also, there were significant correlations between the NTS and the mPFC, as well as the BNST in control but not tickled rats. These results suggest that the NTS does not have a key role in the response to tickling in female and male rats. Projections from the NTS to regions such as the mPFC and BNST are thought to be the link

between the limbic and visceral systems (Terreberry and Neafsey, 1983; Schwaber et al., 1980) and the projection from the BNST to the NTS is involved in attenuating the stress response (Scheuer et al., 2018). Therefore, these projections appear to have a role in emotional processing (Jean, 1991). Correlations in neuronal activity between these regions appears to be different in tickled rats compared to control rats. As the NTS is also involved in social behaviours, and tickling did not increased Fos-ir in or between other regions involved in regulating social behaviours as already discussed, this may again suggest that tickling is not being perceived as a social stimulus by the rats.

4.4.5 The effect of sex on Fos-ir throughout the brain

There were no sex differences in Fos-ir in any region other than in the mBNST and VTA, and this was independent of treatment. This result was surprising given the sex differences found in total 50 kHz USVs, flat 50 kHz USVs, and approaches to the hand on day 10 (Chapter 2), and sex differences are widespread throughout the brain which mediate sex differences in social behaviours. For example, the mPOA is essential for male sexual behaviour in rats (Hansen, 1982), while oestradiol signalling in the VMH is crucial for lordosis in female rats (Rubin and Barfield, 1980). Oestrogens and androgens mediate numerous social behaviours in rats, including social recognition and aggression (e.g. O'Connell et al., 2013; for review see Choleris et al., 2018), and sex differences in vasopressin signalling mediate sex differences in aggression (Terranova et al., 2017). A possible explanation may be that there were sex differences in some, but not all, behaviours expressed during tickling, such as trill 50 kHz USVs and scampering behaviours (Chapter 2). Fos-ir may not be not sensitive enough to detect sex differences in behaviours that are happening simultaneously with behaviours that are not different between sexes, as with the present study (Chapter 3). Additionally, sex differences in the regulation of the behavioural response to tickling may lie in other brain regions or specific neuronal subtypes not included in the present study. For example, vasopressin in the lateral septum regulates social play behaviours differently between female and male rats, with vasopressin receptor

antagonists increasing social play behaviours in male rats, but decreasing them in female rats (Veenema et al., 2013).

Female rats had higher Fos-ir in the mBNST compared to male rats. Several studies show sex differences in the BNST, for example, following social investigation (Perkins et al., 2017) and social play (Reppucci et al., 2018) with Fos-ir being higher in female than male rats, whereas Paul et al. (2014) found that male rats had greater vasopressin mRNA expression in the BNST following social play compared to females. The BNST is implicated in behaviours which are sexually dimorphic, such as maternal behaviours (Numan and Numan, 1996) and copulatory behaviours (Emery and Sachs, 1976; Liu et al., 1997). This may account for the sex differences in Fos-ir that were independent of treatment in the present study.

Females had higher Fos-ir in the VTA compared to male rats, but this was independent of tickling. A possible explanation for this may be that another stimulus was activating VTA neurons in the brains of the female, but not male, rats, for example, female rats, regardless of treatment made more flat 50 kHz USVs than males (Chapter 2, Fig. 7). There was no difference between females and males in double labelled Fos-ir TH neurons in the VTA. This is in contrast to Northcutt and Nguyen (2014) who found that overall female Long-Evans rats had more Fos-ir TH cells in the VTA than males. The authors also found that female rats that had been exposed to social play had significantly more For-ir TH cells than males that had played and females and males that had not played (Northcutt and Nguyen, 2014). This may suggest that tickling was not activating dopaminergic neurons in the VTA differently between female and male rats.

4.4.6 Associations between behaviour and Fos-ir throughout the brain

Solitary play behaviours (scampering and darting) were found to be associated with a decrease in Fos-ir in the lateral and medial BNST and ceA. These regions comprise the extended amygdala (Johnston, 1923; de Olmos, 1972; Alheid et al., 1998). The extended amygdala is involved in male sexual

behaviours (Newman, 1999) and social interaction (Maruska et al., 2012) and emotional processing (Regev et al., 2010; Toufexis, 2007). The ceA has a role in stress responses (Roozendaal et al., 1990; Hernandez et al., 2016) and maternal aggression (Bosch and Neumann, 2010), while the BNST is involved in anxiety and fear responses (Duvarci et al., 2009) as well as reward (Park et al., 2012). Therefore, the role of the extended amygdala is complex with regard to social behaviours and emotional processing. The BNST has a role in social play (Reppucci et al., 2018; Veenema et al., 2013; Cheng et al., 2008), therefore a possible explanation for the negative associated found is that neuronal activation decreased with solitary play.

Flat 50 kHz USVs were positively associated with increased Fos-ir in the lBNST and also the lvPAG. There is evidence that the BNST projects to the PAG (Dong and Swanson, 2004). The BNST is part of the social behaviour network and plays an important role in regulating social behaviours in rats, such as maternal pup retrieval (Numan and Numan, 1996), copulatory behaviours (Valcourt and Sachs, 1979; Liu et al., 1997) and social play (van Kerkhof et al., 2014; Reppucci et al., 2018; Perkins et al., 2017). Whereas the PAG has been shown to be important in the production of vocalisations in many different species, for example, the PAG was activated during voluntary laughter, and involuntary laughter following tickling in healthy human participants (Wattendorf et al., 2013). In rats, electrical stimulation of the PAG evoked USVs in male Wistar rats (Yajima et al., 1980). In squirrel monkeys, stimulation of the PAG by an electrode resulted in vocalisations which were found to be negatively reinforcing (Jurgens, 1976). Also, there is evidence that PAG subregions control different call types in squirrel monkeys, and this depends on the origin of the inputs to the PAG sub region, for example, PAG subregions that control aversive, shriek calls have limited input from the nucleus accumbens (Dujardin and Jurgens, 2006). Similarly in guinea pigs, electrical stimulation of the rostral PAG (comprising the dorsal and lateral PAG) resulted in low whistle calls (produced during separation), while electrical stimulation of the caudal PAG (lateral and ventrolateral PAG), which has input from the mPOAunlike the rostral PAG, induced purr calls which are produced

by male guinea pigs during sexual behaviour (Kyuhou and Gemba, 1998). This may suggest that different PAG subregions generate calls depending on their valence or communicatory function to the animal and this seems to be determined by the areas projecting to each PAG sub region (Dujardin and Jurgens, 2006). The lvPAG projects to other areas of the social behaviour network, the VLH, mPOA, PFC, amygdala and VTA (Beitz, 1982). Therefore, flat 50 kHz USVs may be associated with Fos-ir in the lBNST and the lvPAG because of flat 50 kHz USVs' social communicatory role as social exploration (Burgdorf et al., 2011) and social coordination signals (Burke et al., 2017). The positive association between flat 50 kHz USVs and both the lBNST and lvPAG may guide a future study to test the hypothesis that there is a pathway encompassing these two brain regions that control flat 50 kHz USV emission in rats.

I found that trill USVs were negatively associated with Fos-ir in the lvPAG. The lvPAG projects to regions of the reward pathway (PFC and VTA) (Beitz, 1982) and tickle-induced trill 50 kHz USVs have been shown to be dependent on dopamine transmission and activity in the mesolimbic reward pathway (Burgdorf et al., 2007). While the correlation between trill 50 kHz USVs and Fos-ir in the lvPAG was negative, is it a weak correlation (Fig. 15) and should be interpreted cautiously. However, it may provide preliminary evidence that the lvPAG is involved in the emission of trill 50 kHz USVs.

Fos-ir in the NTS was negatively associated with scampering, in contrast darting was positively associated with increased Fos immunoreactivity. However, these correlations were weak (Fig. 18) and caution should be employed in their interpretation. Scampering, hopping and darting behaviours are exhibited during solitary play (Melotti et al., 2014) and during sexual behaviours where hopping and darting are exhibited before lordosis in female rats (Beach, 1976). The NTS has not been shown to have a role in either solitary play nor female sexual behaviours, but the NTS receives projections from multiple regions involved in regulating social behaviours, such as the ceA, BNST, hypothalamisc nuclei, mPOA, the thalamus, IL mPFC and the PAG (Schwaber et al., 1980; Ricardo and Koh, 1978; Gasparini et al.,

2020). Therefore, one possible explanation for the association between scampering and darting behaviours may be the projections of the NTS.

Conclusions

Tickled and control rats did not differ in neuronal activation in several regions that comprise the social behaviour network and are implicated in a number of social behaviours, and importantly social conspecific play in rats. This would suggest that tickling may have different neural correlates to social conspecific play. While tickling was modelled on social conspecific play (Panksepp and Burgdorf, 2000), the structure and elements of social play and tickling are not identical: the tickling protocol used in the present study used vigorous tickling movements on the dorsal and ventral aspect of the rat in distinct 15 second segments (Panksepp and Burgdorf, 1999), while social play also contains pouncing, wrestling and reciprocal attacks to the nape of the neck (Pellis and Pellis, 2007) which cannot be replicated by the human hand and does not completely allow for the rat to choose when to be tickled or not (Bombail et al., 2021). Therefore, tickling may not activate the same neural circuitry as social play, despite it originally being based on this behaviour.

It is unclear whether tickling resulted in activation of reward circuitry in this study. This may differ from other studies due to differences in experimental technique, for example, between Fos-ir quantification, lesioning and electrophysiological manipulations, and control rats may be finding the environment of the test arena to be rewarding which may explain the lack of difference in Fos-ir in the regions of the neural reward pathway.

The NTS plays a key role in processing sensory information from the periphery. The lack of difference in neuronal activation between tickled and control rats, and its known role in social behaviours may again suggest that tickling may not be perceived as a social stimulus by rats.

Chapter 5

Oxytocin and vasopressin neuronal activity in response to tickling differs from that observed following social play in female and male Wistar rats.

5.1 Introduction

Oxytocin and arginine vasopressin are neuropeptides synthesised in the paraventricular (PVN) and supraoptic (SON) hypothalamic nuclei of the rat brain (Cunningham and Sawchenko, 1991). These two hormones are closely related, both being made up of nine amino acids that differ at the third and eighth position (see Stoop, 2012 for review). They have been conserved across evolution: they are found in most mammalian species, and homologues are found in species of fish, amphibians, reptiles and birds (see Knobloch and Grinevich, 2014 for review).

Both nonapeptides are found within the magnocellular and parvocellular neurons of the PVN, and within the magnocellular neurons of the SON (Swanson and Sawchenko, 1983; Althammer and Grinevich, 2018). Magnocellular and parvocellular neurons are distinguished mainly by size; magnocellular neurons have larger somas compared to parvocellular neurons (Swanson and Kuypers, 1980). The PVN is divided into distinct divisions based on the distribution of magnocellular and parvocellular neurons (Swanson and Kuypers 1980). For example, the medial parvocellular PVN is made up of small, densely packed cells and lies medial to the posterior magnocellular PVN (Swanson and Kuypers, 1980). The dorsal parvocellular region has a horizontal orientation and is comprised of medium sized cells (Swanson and Kuypers, 198; Fig. 1). Each division of the PVN is comprised of oxytocin- , vasopressin- and corticotrophin releasing hormone (CRH) - synthesising neurons (Swanson and Kuypers, 1980).

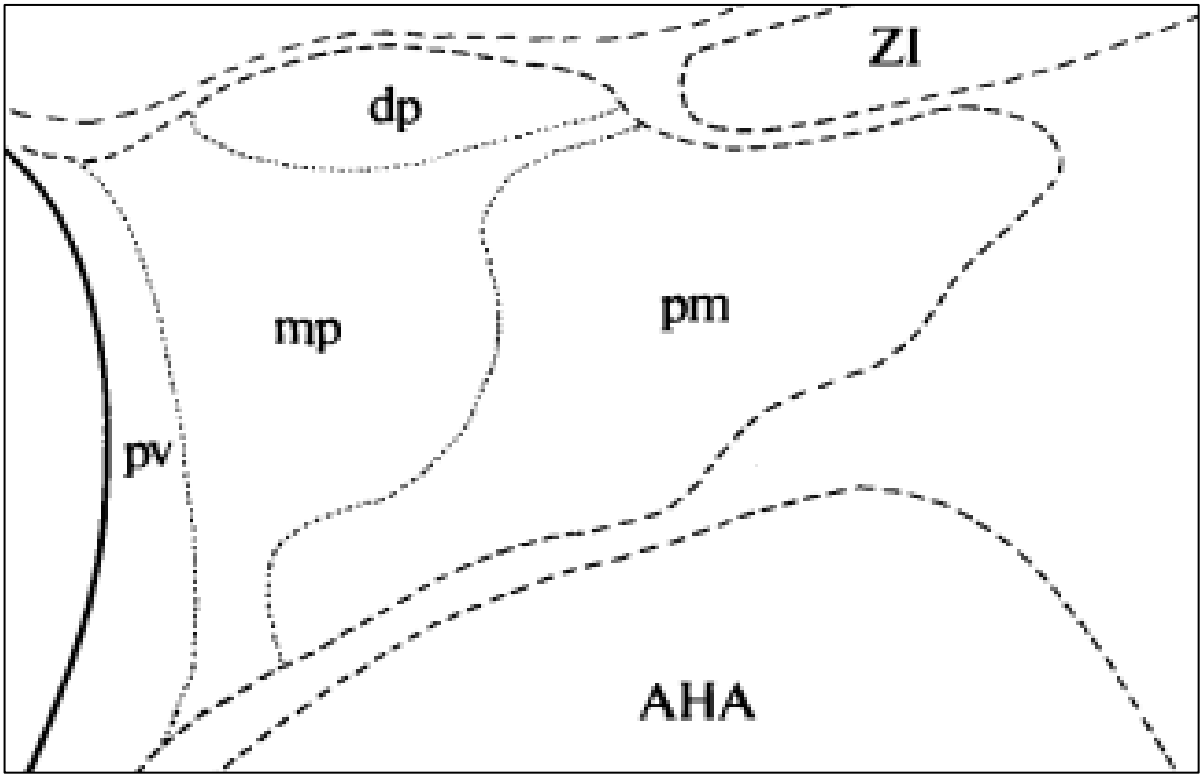

Figure 1. Representation of the boundaries of divisions in the PVN.
The pvPVN lies most medial to the third ventricle. The mpPVN is medial to the pmPVN, and the dpPVN has a more horizontal orientation and lies dorsal to the mpPVN (from Swanson and Kuypers, 1980). Approximate bregma -1.80mm, interaural 7.20mm. dp, dorsal parvocellular PVN; mp, medial parvocellular PVN; pm, posterior magnocellular PVN; pv, periventricular PVN; AHA, anterior hypothalamic area; ZI, zona incerta (adapted from Swanson and Kuypers, 1980).

The magnocellular oxytocin and vasopressin neurons project down to the posterior pituitary gland and release oxytocin and vasopressin into the peripheral circulation where these hormones have widespread physiological effects (Baribeau and Anagnostou, 2015; Swanson and Kuypers, 1980), regulating target organs and many physiological processes (Leng et al., 2005). The physiological functions that oxytocin facilitates include parturition and lactation (e.g. Higuchi et al., 1986; Russell et al., 2003), food intake (e.g. Olson et al., 1991, Sabatier et al., 2013), stress responses (e.g. Torner et al., 2017) and sexual behaviours (e.g. Melis et al., 1986, Pedersen and Boccia, 2002, Melin and Kihlstrom, 1963, Hillegaart et al., 1998). Vasopressin similarly has an important role in a number of physiological processes, such as cardiovascular functions (Share, 1988), glucose homeostasis (Aoyagi et al., 2007), stress responses via the HPA axis (Scaccianoce et al., 1991; Scott and Dinan, 1998), and body water homeostasis through its action on the kidney via the vasopressin V2 receptor (Bankir et al., 2017).

Using injections of retrogradely labelled virus injected into the pituitary gland and the spinal cord, Swanson and Kuypers (1980) showed that there are parvocellular neuronal populations that project to the brain stem and spinal cord. A small number of parvocellular neurons also project to the dorsal vagal complex (Swanson and Kuypers, 1980). The dorsal vagal complex is made up of the NTS and the dorsal motor nucleus of the vagus, playing an important role in the control of autonomic functions (Tolchard and Ingram, 1993). Hindbrain-projecting parvocellular neurons are thought to be involved in food intake (Perello et al., 2013; Sarkar et al., 2003), as well as autonomic functions such as breathing (Mack et al., 2002) and cardiovascular effects (Pettersson, 2002; Althammer and Grinevich, 2018).

Oxytocin and vasopressin magnocellular neurons project to numerous different brain regions, including the hippocampus, amgydala, SON, LS and BNST (Grinevich and Neumann, 2020; Caldwell, 2017). Also, both oxytocin and vasopressin are released centrally from dendrites of magnocellular neurons, and this is regulated independently of axonal release (Ludwig, 1998; Leng et al., 2008). Release from neuronal dendrites means that a

neuropeptide can act back onto the dendrites, and stimulate further release of neuropeptide (Ludwig et al., 2002; Ludwig and Leng, 2006). Centrally released neuropeptides can also diffuse throughout the brain and target sites where their receptors are expressed, which may be distant from the site of release (Ludwig and Leng, 2006). As the oxytocin and vasopressin receptors are widespread throughout the brain, oxytocin and vasopressin can act in many brain regions, which may facilitate their role in numerous physiological and behavioural responses (Ludwig and Leng, 2006).

In addition to their physiological roles, oxytocin and vasopressin have widespread effects on social behaviorsOxytocin is involved in the regulation of a number of key prosocial behaviours such as social recognition in female and male mice (Ferguson et al., 2001; Choleris et al., 2007), partner preferences in male and female prairie voles (*Microtus ochrogaster*; Cho et al., 1999), social preference in male and female Wistar rats (Lukas et al., 2011; Lukas and Neumann, 2014), social memory in male mice (Ferguson et al., 2000), social interaction in male Listar-hooded rats (Kohli et al., 2019), social investigation in male mice (Resendez et al., 2020), social defeat in female mandarin voles (*Microtus mandarinus*; Wang et al., 2018), and maternal behaviours in female Wistar rats (Bosch et al., 2005; Leng et al., 2008). Vasopressin also has an important role in modulating social behaviours in mammals (see Goodson and Bass, 2001 for review), such as aggression in male mice (Wersinger et al., 2002) and partner preferences in male and female prairie voles (*Microtus ochrogaster*; Cho et al., 1999), and social memory in male Wistar rats (Le Moal et al., 1987). Both oxytocin and vasopressin are implicated in the regulation of juvenile social play in rats (e.g. Veenema et al., 2013; Paul et al, 2014; Bredewold et al., 2014; Lukas and Wohr, 2015) and this may be sex-specific (e.g. Veenema et al., 2013; Bredewold et al., 2014; Bredewold and Veenema, 2018).

While there is evidence showing that rats find tickling to be rewarding (e.g. Panksepp and Burgdorf, 2001) and the behavioural response to tickling is thought to be associated with the ascending mesolimbic dopamine system (Burgdorf, et al., 2011; Alcaro et al., 2007), there have been no published

studies to date that have explored the neuroendocrine response to tickling in rats. Given that tickling was modelled on rat social rough and tumble play (Panksepp and Burgdorf, 2000), it may be predicted that oxytocin and vasopressin are involved in tickling, just as they are in conspecific play (Bredewold et al., 2014). Furthermore, in rats, gentle stroking stimuli increased the number of 50 kHz ultrasonic vocalisations emitted during the stroking stimulus but also increased the number of Fos positive oxytocin neurons in the medial parvocellular PVN (Okabe et al 2015). The ventral tegmental area (part of the reward network) receives input from PVN oxytocinergic neurons; firing of these oxytocinergic neurons increases dopamine activity in the VTA (Xiao et al., 2017). This innervation from the PVN onto dopaminergic neurons is important for social reward (Song et al., 2016; Hung et al., 2017). Similarly, there is evidence that vasopressin interacts with the dopaminergic system in the lateral septum (Newman, 1999; Goodson and Kingsbury, 2013) to moderate social play in a sex-specific manner (Bredewold and Veenema, 2018). The lateral septum has a role in a number of social behaviours such as pair bonding in male prairie voles (Liu et al., 2001), social memory in rats (Everts et al., 1999), and social aggression in male mice (Wong et al., 2016).

As the oxytocinergic and vasopressinergic systems form part of the neural circuitry underlying social conspecific play and they interact with the dopaminergic reward system which is associated with the response to tickling (Burgdorf et al., 2011; Hori et al., 2013), the aim of this study was to investigate the involvement of these two neuropeptides in the response to the playful stimulus of tickling. I predicted that the magnocellular and parvocellular oxytocin and vasopressin neurons in the PVN would be recruited differently due to their differing projections to the pituitary or the hindbrain and spinal cord, respectively. I also aimed to investigate if the sex differences in the behavioural response to tickling as described in Chapter 2 would be reflected in the involvement of the oxytocin and vasopressin system as oxytocin and vasopressin have a sexually dimorphic role in a number of behaviours. I tested the hypothesis that tickling increases Fos-ir in oxytocin and vasopressin neurons of the PVN, predicting that the number of Fos-ir neuropeptidergic

neurons would correlate with tickle-induced play behaviours and that sex differences in the activation of the oxytocin and vasopressin systems would be observed that match the behavioural response. I used immunohistochemistry to quantify Fos protein (a marker of cellular activity) expression in oxytocinergic and vasopressinergic neurons of PVN and SON of the hypothalamus. I predicted that Fos-ir in SON oxytocin and vasopressin neurons may also differ between tickled and control rats as gentle stroking has been shown to increase Fos-ir oxytocin neurons in the SON of male rats (Okabe et al., 2015). Also, male golden hamsters (*Mesocricetus auratus*) had increased neural activity in vasopressin neurons in the SON following play fighting (comparable to rough and tumble play in rats; Cheng et al., 2008).

5.2 Materials and Methods

Please refer to Results Chapter 1: Materials and Methods for Animal and Husbandry, and Experimental Testing of the rats used in this neural analysis.

5.2.1 Enzyme-linked immunosorbent assay (ELISA)

An enzyme-linked immunosorbent assay (ELISA) was used to quantify serum concentrations of oxytocin and vasopressin using rat ELISA kits (Oxytocin: E1216Ra; Vasopressin: E038Ra, Bioassay Technology Laboratory) following the manufacturer's instructions. All samples were run in a single assay and the sensitivity of each assay was 1.08ng/l for oxytocin and 0.11ng/l for vasopressin. The intra assay variation was measured by the coefficient of variation (CV) with a CV < 8% for oxytocin and a CV < 8% for vasopressin

Oxytocin

For reagent preparation, 120µl of the standard (640 ng/l) was reconstituted with 120 µl of standard diluent to generate a 320ng/l standard stock solution. The standard was allowed to sit for 15 minutes with gentle agitation (shaker; Janke & Kunkel IKA-Schüttler MTS 4 Laborschüttler Schüttler Kreisschüttler) prior to dilutions. Duplicate standard points were prepared by serially diluting the standard stock solution (320 ng/l) 1:2 with standard diluent to produce 160 ng/l, 80 ng/l, 40 ng/l and 20 ng/l solutions. Standard diluent served as the zero standard (0 ng/l). Dilution of standard solutions were as follows:

320 ng/l	Standard No.5	120 µl Original Standard + 120 µl Standard
160 ng/l	Standard No.4	120 µl Standard No.5 + 120 µl Standard
80 ng/l	Standard No.3	120 µl Standard No.4 + 120 µl Standard
40 ng/l	Standard No.2	120 µl Standard No.3 + 120 µl Standard
20 ng/l	Standard No.1	120 µl Standard No.2 + 120 µl Standard

The wash buffer was prepared by diluting 20 ml of wash buffer concentrate into distilled water to yield 500 ml of 1x Wash Buffer. The assay

was performed at room temperature. 50 µl of each standard was added to the standard wells of the ELISA plate, 40 µl of each sample was added to the sample wells and 10µl anti-oxytocin antibody to sample wells and 50 µl streptavidin-HRP to the sample wells and standard wells (except blank control well). The plate was sealed with a plastic cover provided in the ELISA test kit and incubated (SANYO CFC FREE MIR-253 Laboratory Incubator) for 60 minutes at 37°C. The plate was washed five times with wash buffer using automated washing (Bio-Rad Bio-Plex Pro II wash station), aspirating all wells, overfilling wells with wash buffer. 50 µl of substrate solution A was added to each well and followed by 50 µl substrate solution B to each well. The plate was incubated covered with a new sealer for 10 minutes at 37°C in the dark. 50 µl of stop solution was added to each well, changing the colour from blue into yellow immediately. The optical density (OD value) of each well was determined immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Vasopressin

For reagent preparation, 120 µl of the standard (80 ng/l) was reconstituted with 120 µl of standard diluent to generate a 40 ng/l standard stock solution. The standard was allowed to sit for 15 minutes with gentle agitation (shaker; Janke & Kunkel IKA-Schüttler MTS 4 Laborschüttler Schüttler Kreisschüttler) prior to making dilutions. Duplicate standard points were prepared by serially diluting the standard stock solution (40 ng/l) 1:2 with standard diluent to produce 20 ng/l, 10 ng/l, 5 ng/l and 2.5 ng/l solutions. Standard diluent served as the zero standard (0 ng/l). Dilution of standard solutions were as follows:

40 ng/l	Standard No.5	120 µl Original Standard + 120 µl Standard
20 ng/l	Standard No.4	120 µl Standard No.5 + 120 µl Standard
10 ng/l	Standard No.3	120 µl Standard No.4 + 120 µl Standard
5 ng/l	Standard No.2	120 µl Standard No.3 + 120 µl Standard
2.5 ng/l	Standard No.1	120 µl Standard No.2 + 120 µl Standard

The wash buffer was prepared by diluting 20 ml of Wash Buffer Concentrate distilled water to yield 500 ml of 1x Wash Buffer. The assay was performed at room temperature. 50 µl of each standard was added to the standard wells, 40 µl of each sample was added to the sample wells and 10 µl anti-ADH/VP/AVP antibody to sample wells and 50 µl streptavidin-HRP to the sample wells and standard wells (except blank control well). The plate was sealed and incubated (SANYO CFC FREE MIR-253 Laboratory Incubator) for 60 minutes at 37°C. The plate was washed five times with wash buffer using automated washing (Bio-Rad Bio-Plex Pro II wash station), aspirating all wells, overfilling wells with wash buffer. 50 µl of substrate solution A was added to each well and followed by 50 µl substrate solution B to each well. The plate was incubated covered with a new plastic sealer for 10 minutes at 37°C in the dark. 50 µl of stop solution was added to each well, changing the blue colour into yellow immediately. The optical density (OD value) of each well was determined immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

5.2.2 Immunohistochemical procedures

Following dissection, brains were immediately immersed in 4% PFA in 1 x PBS at 4^{o}C for five days. The brains were then transferred into 15% sucrose in 4% PFA for a further 24 - 48 hours at 4^{o}C. When the brain had sunk to the bottom of each tube, they were transferred into 30% sucrose in 1 x PBS for 24 - 48 hours incubation at 4^{o}C or until the tissue had sunk to the bottom. Preparation of all solutions was supervised by Mrs Valerie Bishop.

The brains were dissected into three sections: the cerebellum, brainstem and spinal cord; the olfactory bulb; the main brain (including cerebral cortex, diencephalon and mesencephalon). Any excess solution was blotted from the brain before dissection. The three samples were snap frozen on dry ice, wrapped in foil and stored in a labelled small plastic bag at -70 ^{o}C until they were sectioned using a freezing microtome. This was carried out by myself and Prof. Simone Meddle.

The main brain was sectioned coronally into 1 x PBS in a petridish at 50 µm on a freezing microtome. The sections were collected into three alternative sets to allow for multiple immunohistochemical staining to be carried out. One set of sections was stained for Fos and oxytocin, one set was immunolabelled for Fos and vasopressin and the third set for Fos and tyrosine hydroxylase (see Chapter 4) staining. Following sectioning, the brain sections were transferred into cryoprotectant and stored at -20 ^{o}C.

Fos and oxytocin or arginine vasopressin double immunohistochemistry

Double labelling was performed using the free floating sections. To visualise active neural populations, rabbit anti-Fos antibody (K-25 Santa Cruz sc253) was used to stain *c-fos* expressing neurons and rabbit anti-oxytocin polyclonal IgG (Immunostar; 20068) was used to stain oxytocinergic cell bodies. Rabbit anti-vasopressin polyclonal IgG (Chemicon; AB1565) was used to stain oxytocinergic cell bodies. On a shaking platform set to 95 rpm, sections were washed four times 15 minutes in 0.2% PBS-T, rinsed for 5 minutes in 1x PBS, endogenous peroxidases were quenched in 0.3% hydrogen peroxide for 20 minutes, rinsed for 5 minutes in 1x PBS, incubated with 0.3% hydrogen peroxide for a further 20 minutes, washed three times 10 minutes in 0.2% PBS-T, immersed in 0.2% PBS-T and 5% normal goat serum (NGS) for 60 minutes to block non-specific binding, and incubated in rabbit anti-Fos antibody (K-25 Santa Cruz sc253) at 1:1000 diluted with 0.2% PBS-T and 3% NGS for 3.5 days at 4°C.

Sections were then washed four times 10 minutes in 0.2% PBS-T to remove excess, unbound antibody. Sections were incubated in biotinylated anti-rabbit secondary antibody (Vectastain elite ABC rabbit kit; Vector; PK6101) to amplify antibody-antigen complexes, using 10 µl biotinylated anti-rabbit secondary antibody and 30 µl NGS to every 1 ml of 0.2% PBS-T. 3 ml per sample was allowed, and sections were incubated for 60 minutes on a shaking platform (set to 65 rpm) at room temperature. Sections were washed three times 10 minutes in 0.2% PBS-T. The sections were then incubated in Avidin / Biotinylated horseradish peroxidase conjugate (Vectastain elite ABC

rabbit kit; Vector; PK6101) to amplify the signal using 20 μl Avidin DH and 20 μl biotinylated horseradish peroxidase to every 1 ml of 0.2% PBS-T (made up at least 30 minutes before use). Sections were incubated for 60 minutes on a shaking platform (set to 65 rpm) at room temperature. Sections were washed three times 10 minutes in 0.2% PBS-T, rinsed in 1 x PBS for 5 minutes, rinsed in 0.2M Sodium acetate buffer, antibody-antigen complexes visualised using nickel II sulphate, ammonium chloride and 0.2M sodium acetate buffer solution mixed with 25 mg/ml DAB solution and 30% hydrogen peroxide. Sections were monitored under a light microscope after 2 minutes of adding the colour substrate solution and the colour reaction halted once positive staining was seen by immersing sections in 0.2M sodium acetate buffer for 5 minutes, sections were rinsed five times 5 minutes in 1x PBS. Endogenous peroxidases were quenched by incubating sections for 20 minutes in 0.3% hydrogen peroxide. Sections were washed two times 10 minutes in 1x PBS-T before being incubated overnight at 4°C in rabbit anti-oxytocin polyclonal IgG (Immunostar; 20068) at 1:10,000 diluted with 0.2% PBS-T and 5% NGS. The second set of sections for visualising vasopressin were incubated overnight at 4°C in rabbit anti-vasopressin polyclonal IgG (Chemicon; AB1565) at [1:4,000] diluted in 0.2% PBS-T and 5% NGS.

Sections were then washed three times 10 minutes in 0.2% PBS-T to remove excess, unbound antibody. Sections were incubated in biotinylated anti-rabbit secondary antibody (Vectastain elite ABC rabbit kit; Vector; PK6101) to amplify antibody-antigen complexes, using 10 μl biotinylated anti-rabbit secondary antibody and 30 μl NGS to every 1 ml of 0.2% PBS-T. Sections were incubated for 60 minutes on a shaking platform (set to 65 rpm) at room temperature. Sections were washed three times 10 minutes in 0.2% PBS-T. The sections were then incubated in Avidin / Biotinylated horseradish peroxidase conjugate (Vectastain elite ABC rabbit kit; Vector; PK6101) to amplify the signal using 20 μl Avidin DH and 20 μl biotinylated horseradish peroxidase to every 1 ml of 0.2% PBS-T (made up at least 30 minutes before use). Sections were then incubated for 60 minutes on shaking platform (set to 65 rpm) at room temperature. Sections were washed three times 10 minutes

in 0.2% PBS-T, rinsed in 1 x PBS for 5 minutes, oxytocin positive cells were visualised using DAB in 1x PBS and 30% hydrogen peroxide (without nickel enhancement). Sections were monitored under a light microscope after 2 minutes of adding the DAB solution and the colour reaction halted once positive staining was seen by immersing sections in 1 x PBS for 5 minutes. Sections were washed five times 5 minutes in 1 x PBS before the immuno-stained sections were stored in 1 x PBS at 4°C until mounted onto chrome-alum subbed slides.

Sections were float-mounted from 1 x PBS onto gelatinised slides using a paintbrush. Slides were allowed to dry for at least 2 hours before they were dehydrated and coverslipped. Following mounting onto slides, sections were dehydrated through an increasing series of alcohol as follows: 70% IMS 5 minutes, 95% IMS 5 minutes, two times 99% IMS 5 minutes, xylene/99% IMS 5 minutes, three times Xylene 5 minutes. Slides were coverslipped straight from the last xylene using xylene-based mounting medium (Pirtex) and left on a downdraft table to dry overnight.

5.2.3 Image acquisition and analysis

Brain regions of interest (ROI) were analysed in the following order: medial parvocellular PVN, posterior magnocellular PVN, dorsal parvocellular PVN, and SON. The ROI boundaries were defined using Paxinos and Watson's stereotaxic rat brain atlas (Paxinos and Watson, 1998) and referring to Swanson and Kuypers (1980). A light microscope (Nikon E600 Brightfield Microscope) was used; the region was identified with a 4 x objective, an image was taken at the centre of the region at 10x magnification using Zen 2 software for image capture. This was done for each ROI (see Table 2) for both left and right hemispheres and across four representative sections per rat.

Cell counting was performed blinded to sex and treatment by one observer using ImageJ 1.48v software (W. Rashband, National Institutes of Health, USA). Each image was opened in ImageJ and the Brightness and Contrast function was set to auto levels. The multi-point tool was used to manually count Fos-ir positive cells which were identified as having a dark

grey/ black nucleus, and double-labelled cells which had a black nucleus and brown cell body. Any ambiguity in whether cells were positive or not (e.g. faint staining, or dark staining where it was not possible to distinguish between a black nucleus and brown cell body), led to the cells not being counted as positive.

The total number of Fos-ir positive nuclei and total number of Fos-ir and oxytocin positive double-labelled cells were quantified separately. Counts were made in the posterior magnocellular, medial parvocellular and dorso-parvocellular subregions of the PVN (Swanson and Kuypers, 1980; Sawchenco and Swanson, 1982; Fig. 1) and the SON (Fig. 2). Total number of Fos-ir positive nuclei and total number of Fos-ir and vasopressin positive double-labelled cells were quantified separately. Counts were made in the posterior magnocellular and medial parvocellular subregions of the PVN (Swanson and Kuypers, 1980; Sawchenco and Swanson, 1982) and the SON.

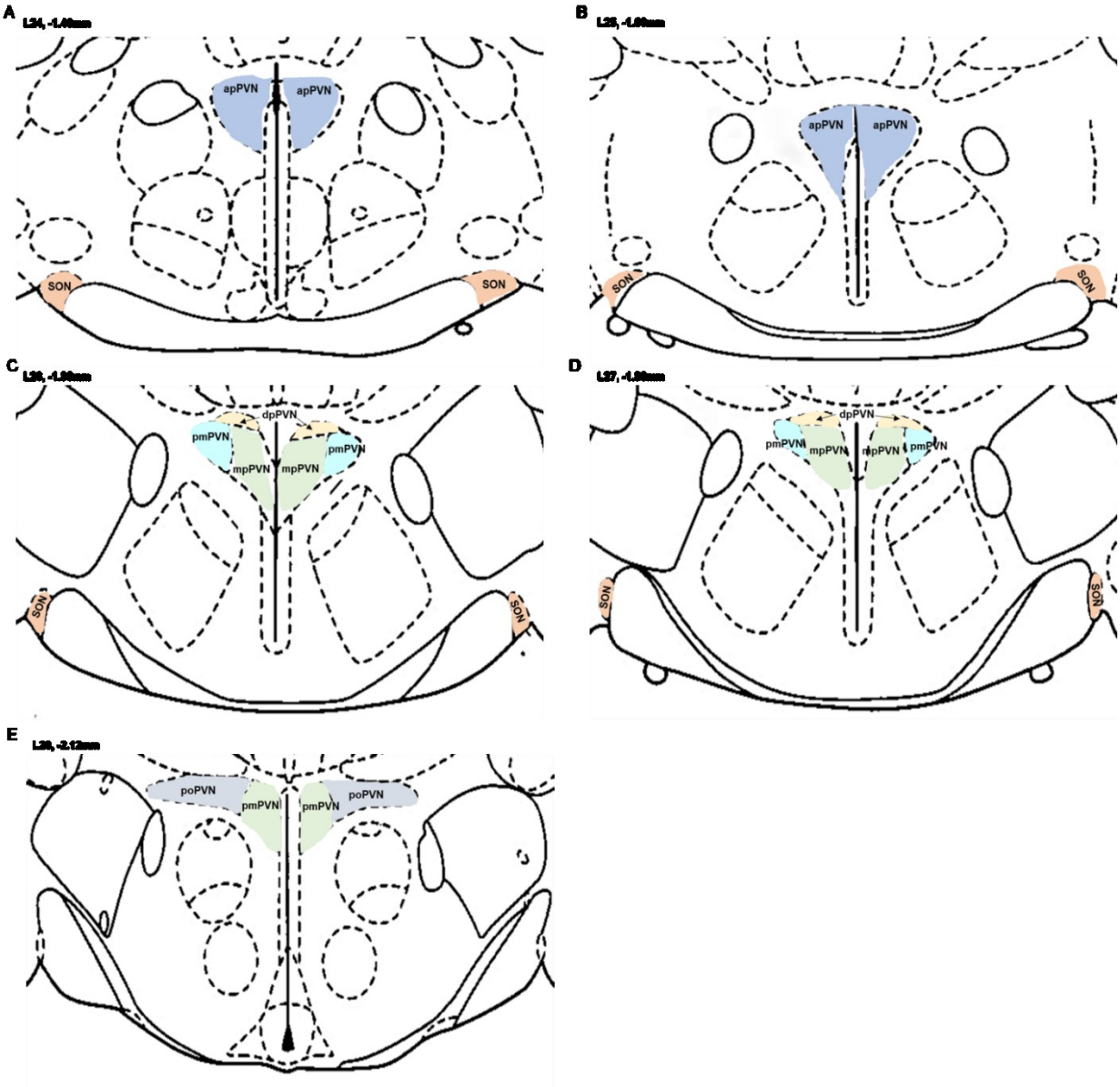

Figure 2. Representation of sampling locations on modified rat brain atlas templates (Paxinos and Watson, 1998).
Locations of the PVN and SON moving caudally through the brain on coronal sections. Filled areas refer to the analysed locations. apPVN (dark blue) and SON (orange) (A-B); dpPVN (yellow), pmPVN (light blue), mpPVN (light green) and SON (orange) (C-D); poPVN (grey) and pmPVN (light green) (E). Headings refer to atlas level and distance in mm from bregma. PVN, paraventricular nucleus of the hypothalamus; SON, supraoptic nucleus of the hypothalamus; apPVN, anterior PVN; dp, dorsal parvocellular PVN; mp, medial parvocellular PVN; pm, posterior magnocellular PVN; poPVN, posterior PVN (Swanson and Kuypers, 1980).

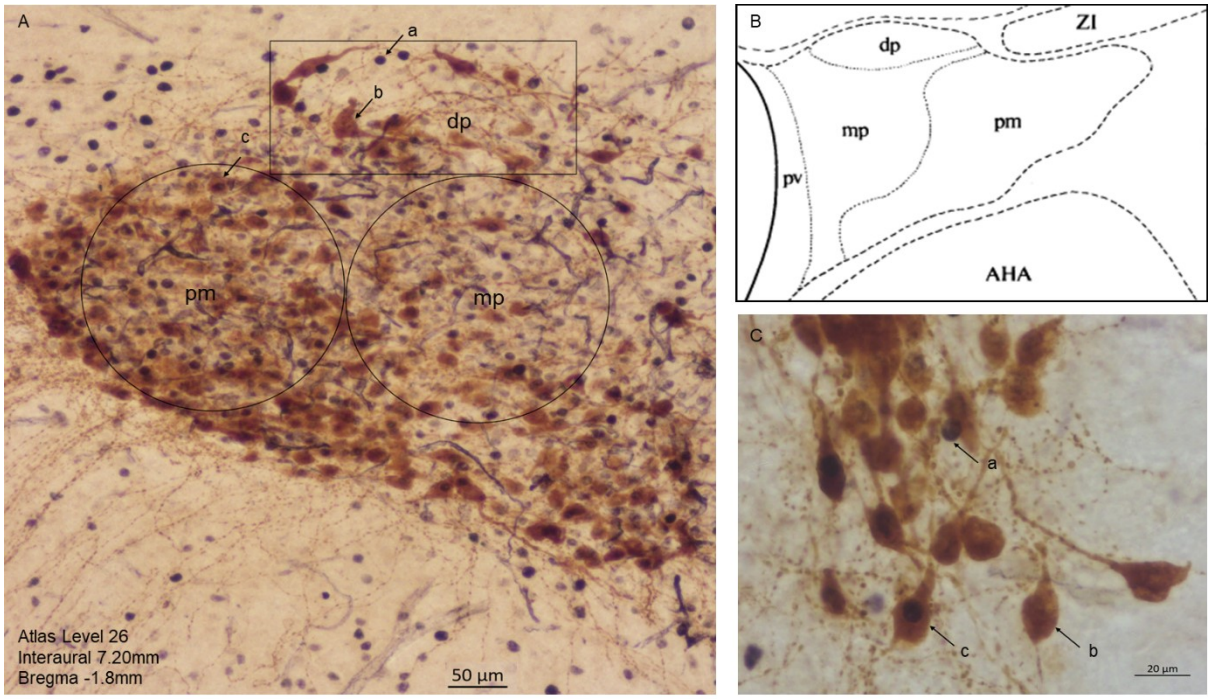

Figure 3. Representative image of immunohistochemical staining and sampling locations in the PVN.
Fos-ir nuclear staining and oxytocin cytoplasmic staining in the PVN of a tickled male rat at x10 magnification. Black rectangle and circles refer to the location used to carry out cell counting in each subregion of the PVN for coronal sections at atlas level 26 and distance in mm from the interaural line and bregma. Scale bar = 50µm (A); Boundaries of cell groups in the PVN, adapted from Swanson and Kuypers, 1980 (B); 40x magnification of oxytocin Fos-ir cells in the PVN. Scale bar = 20µm (C). **a**, a Fos-ir nucleus; **b**, an oxytocin positive cell body; **c**, a double-labelled Fos-ir oxytocin neuron. dp, dorsal parvocellular PVN; mp, medial parvocellular PVN; pm, posterior magnocellular PVN; pv, periventricular PVN; AHA, anterior hypothalamic area; ZI, zona incerta (Swanson and Kuypers, 1980).

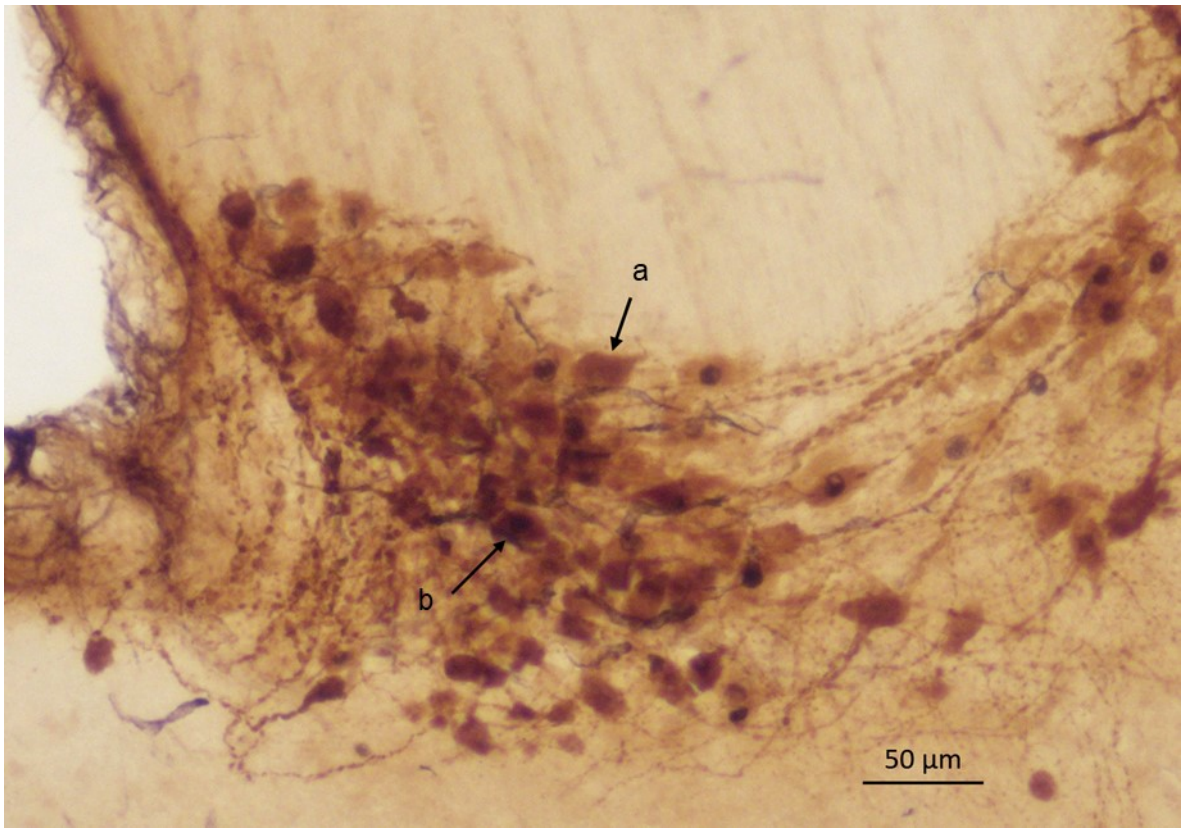

Figure 4. Representative image of immunohistochemical staining in the SON. Fos-ir nuclear staining and vasopressin cytoplasmic staining in the supraoptic nucleus of a female control animal. **a**, an vasopressin positive cell body; **b**, a double-labelled Fos-ir vasopressin neuron. Scale bar = 50µm.

Table 1. Brain regions analysed for Fos-ir positive cells and double-labelled cells.

'+' indicates the immunohistochemical staining was quantified in that region or subregion. '-' indicates that Fos-ir / vasopressin positive neurons were not quantified in the dpPVN because this area is comprised almost entirely of oxytocin neurons (Sawchenko and Swanson, 1982).

Brain region	Subregion	Fos-ir	Fos-ir/ Oxytocin	Fos-ir / Vasopressin
Supraoptic Nucleus		+	+	+
Paraventricular Nucleus	Dorsal parvocellular PVN	+	+	-
	Medial parvocellular PVN	+	+	+
	Posterior magnocellular PVN	+	+	+

5.2.4 Statistical analysis

ELISA data were analysed in GraphPad Prism 5 (Graphpad Software, California, USA) to generate a standard curve and interpolated results which were then analysed in Minitab 17 using a two-way balanced ANOVA. A standard curve for each ELISA was calculated using the average optical density for each standard, calculating the concentration of the samples by interpolation of the standard curve and determining a best fit curve by regression analysis using Graphpad Prism 5 software. For oxytocin, the standards were logged to normalise the data and standard 0 was removed for analysis due to an error.

Statistical analysis was carried out in R Studio and R (v 4.0.3, The R Foundation for Statistical Computing Platform (2020)). Model adequacy was verified by examination of residuals (McCullagh and Nelder, 2019) via the DHARMa package (Hartig, 2020). Generalised linear mixed models (GLMMs) in the glmmTMB package (Brooks et al., 2017) were used to analyse the effect of treatment (tickled or non-tickled control), sex and the interaction of treatment and sex on Fos-ir immunoreactivity in oxytocinergic and vasopressinergic neural populations in the PVN (each region of the PVN was analysed separately). Dependent on model fitting and overdispersion, family links were set to either poisson or negative binomial distributed errors ('nbinom2' in the MASS package; Venables and Ripley, 2002) with default transformations. All models included batch and cage as random effects and were nested (batch followed by cage). This was done to account for the variation from the non-independence of rats from the same cage and potential batch effects. For all models, both sex and treatment were included as fixed effects, as well as the interaction between them. A regression was carried out to test the linear relationships between Fos-ir and the fixed effects. Statistical significance was based on $p < 0.05$ threshold level and adjusted for multiple comparisons using the Tukey method (Lenth, 2020). All models included an offset of the total number of Fos-ir positive nuclei, to account for this uncontrolled variable in each sampling event, effectively calculating proportions of double-labelled cells/ total Fos-ir positive cells.

A bottom-up, stepwise regression analysis was used to investigate whether the measured behaviours could explain differences in Fos-ir in the oxytocinergic and vasopressinergic neural populations of the PVN. Each behaviour was added into the GLMM as a covariate in a bottom-up, stepwise regression. Covariate effects were established by interpretation of estimated coefficients, associated z-values and p-values (Bates et al., 2015), with statistical significance based on $p < 0.05$ threshold level. Behaviours were sequentially included in the model based on estimated coefficients, associated z-values and p-values (Bates et al., 2015), with non-significant co-variates dropped, producing final minimal models with maximised predictive parameters. All final models included as a minimum treatment, sex and their interaction. All data in the tables are presented ± standard error of beta coefficient estimates (SEM) and 95% confidence intervals (CI). The significance level was set at $p < 0.05$.

All bar graphs were generated in GraphPad Prism (version 9 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com). The data in the graphs are presented as the means of the raw data ± standard error of the mean (SEM). Scatter plots were generated in R Studio and R (v 4.0.3, The R Foundation for Statistical Computing Platform (2020)) using the ggplot2 package (Wickham, 2011).

5.3 Results

5.3.1 The effect of tickling on Fos-ir expression in oxytocin and vasopressin neurons in the PVN

Parvocellular divisions of the PVN

There was a significant interaction between treatment and sex on the number of Fos-ir oxytocin cells in the mpPVN ($Z = 3.089$, $p = 0.002$; Table 2, Fig. 5). Tickled male, control female and control male groups had more double labelled Fos-ir oxytocin cells than tickled female rats. There was a significant relationship between treatment and the number of Fos-ir positive parvocellular oxytocin neurons in the dpPVN ($Z = -2.169$, $p = 0.0301$) and the mpPVN ($Z = -3.101$, $p = 0.0019$). Specifically, tickled rats had lower double labelled Fos-ir oxytocin neurons in dpPVN and mpPVN than corresponding control rats. There was no interaction of sex and treatment on Fos-ir oxytocin neurons in the dpPVN ($Z = 0.668$, $p = 0.4360$). There was no relationship between Fos-ir oxytocin cells in the dpPVN and sex ($Z = 1.590$, $p = 0.112$). There was also no effect of sex on Fos-ir in the mpPVN ($Z = 0.378$, $p = 0.7054$).

There was no interaction of treatment and sex ($Z = 1.080$, $p = 0.280$) on the number of Fos-ir vasopressin cells in the mpPVN (Table 2, Fig. 5). There was a significant relationship between the number of Fos-ir vasopressin cells in the mpPVN and treatment ($Z = -2.169$, $p = 0.030$). As with parvocellular oxytocin neurons, tickled rats had fewer Fos-ir vasopressin neurons in mpPVN than corresponding control rats. There was no significant relationship between Fos-ir vasopressin cells and sex ($Z = -0.275$, $p = 0.783$) on the number of Fos-ir vasopressin cells in the mpPVN.

The number of 50 kHz USVs emitted was correlated with the number of Fos-ir parvocellular oxytocin neurons in the dpPVN ($Z = 3.158$, $p = 0.0016$) although there was no interaction between 50 kHz USVs and treatment ($Z = -0.549$, $p = 0.583$) or between 50 kHz USVs and sex ($Z = -0.259$, $p = 0.796$) on the number of Fos-ir dpPVN oxytocin neurons (Table 2, Fig. 6). Similarly in the mpPVN, the number of 50 kHz USVs emitted was correlated with the number of Fos-ir parvocellular oxytocin neurons ($Z = 3.001$, $p = 0.0027$), but there was

no interaction between 50 kHz USVs and treatment ($Z = 1.55$, $p = 0.121$) or between 50 kHz USVs and sex ($Z = 1.316$, $p = 0.188$) on the number of Fos-ir dpPVN oxytocin neurons. As 50 kHz USV emission increased, so did the number of Fos-ir oxytocin cells in the dpPVN and mpPVN (Fig. 6). No behaviours were significantly correlated with Fos-ir vasopressin cells in the mpPVN (data not shown).

Table 2. Statistical output of final predictive model for parvocellular double labelled Fos-ir oxytocin and vasopressin neurons in the PVN. Significant effects ($p < 0.05$) shown in bold.

Region	Neuropeptide	Fixed Effects	Estimates	±SE	95% CI	Z value	P-value
dpPVN	Oxytocin	Treatment	-0.3843	0.1772	-0.7316- -0.0371	-2.169	**0.0301**
		Sex	0.1143	0.1468	-0.1734- 0.4021	0.779	0.4360
		Treatment: Sex	0.1358	0.2031	-0.2623- 0.5339	0.668	0.5039
		50 kHz USVs	0.0008	0.0002	0.0003- 0.0013	3.158	**0.0016**
mpPVN	Oxytocin	Treatment	-0.3738	0.1205	-0.6101- -0.1376	-3.101	**0.0019**
		Sex	0.0431	0.1140	-0.1804- 0.2666	0.378	0.7054
		Treatment: Sex	0.3818	0.1236	0.1395- 0.6240	3.089	**0.0020**
		50 kHz USVs	0.0006	0.0002	0.0002- 0.0011	3.001	**0.0027**
	Vasopressin	Treatment	-0.2355	0.1086	-0.4483- -0.0227	-2.169	**0.0300**
		Sex	-0.0306	0.1114	-0.2489- 0.1876	-0.275	0.7830
		Treatment: Sex	0.1620	0.1500	-0.1320- 0.4560	1.080	0.2800

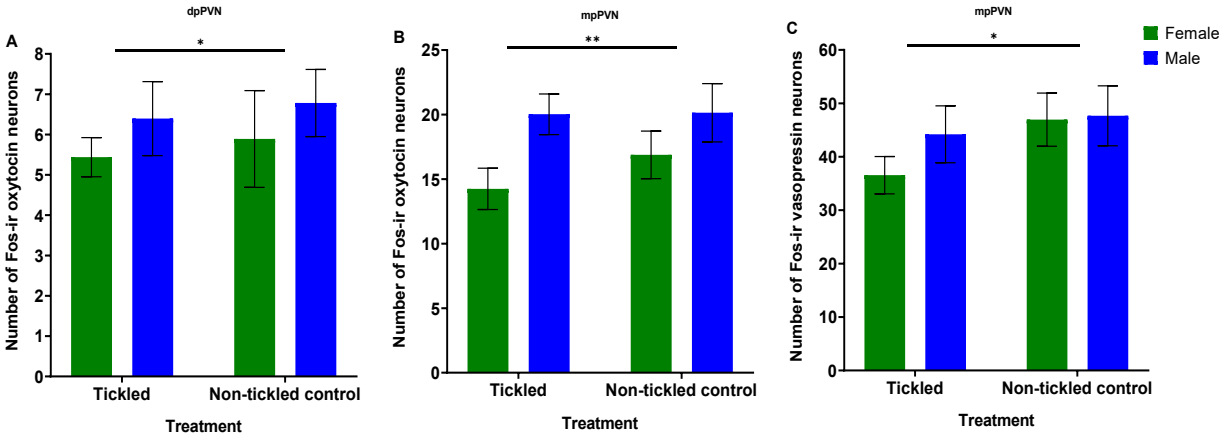

Figure 5. Tickled rats had lower numbers of Fos-ir oxytocin and vasopressin parvocellular neurons than control rats.
Number of Fos-ir parvocellular neurons in the PVN: dpPVN oxytocin (A); mpPVN oxytocin (B); mpPVN vasopressin (C). Females (green), males (blue). Individual axes were adjusted for better visualization of the graphs as there was a wide range in the number of cells in each division. Mean ± SEM; n = 16 / group. * denotes $p \leq 0.05$; ** denotes $p \leq 0.01$; *** denotes $p \leq 0.001$; **** denotes $p \leq 0.0001$ for significance.

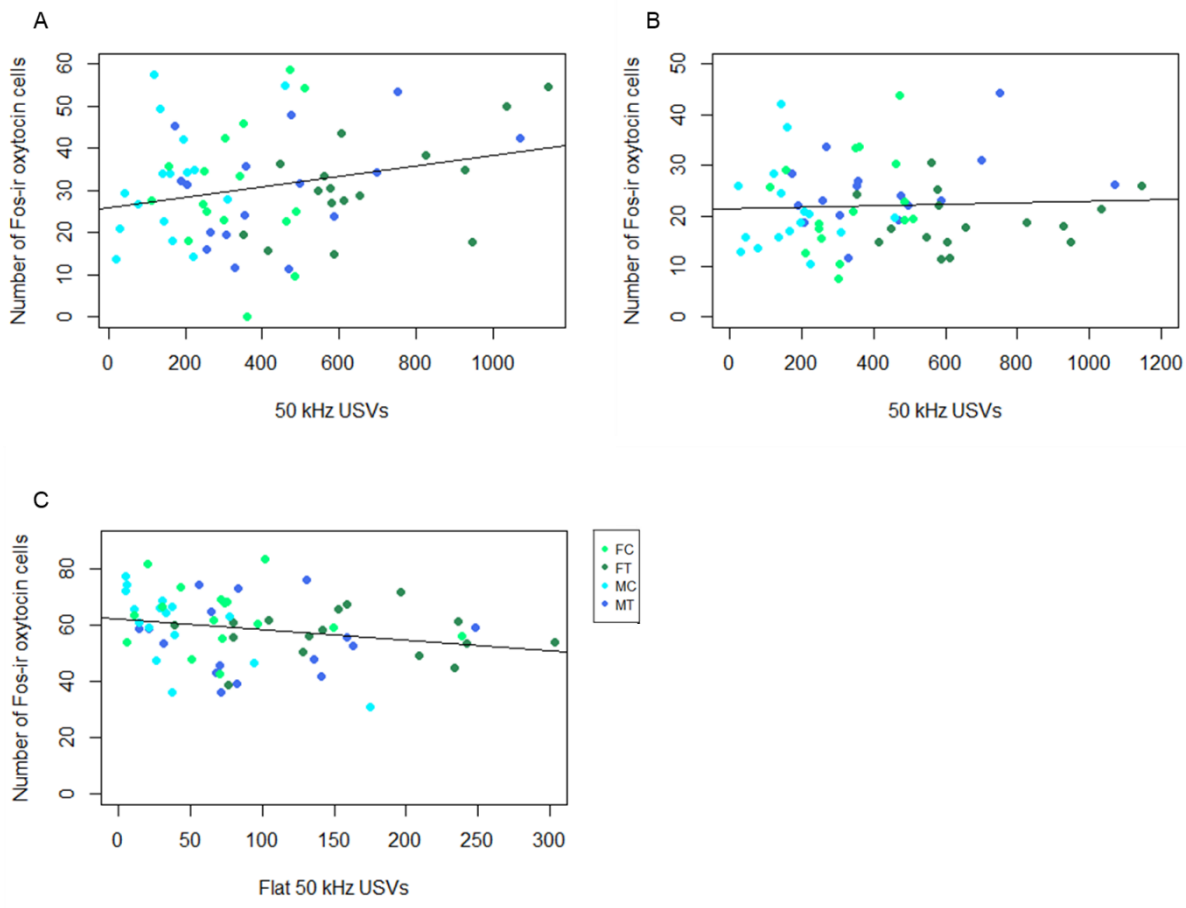

Figure 6. 50 kHz USVs were found to positively predict the number of Fos-ir parvocellular oxytocin neurons in the PVN, while flat 50 kHz USV negatively predicted the number of Fos-ir oxytocin neurons in the SON.
Correlations between 50 kHz USVs and Fos-ir positive oxytocin neurons in the dpPVN (A) and mpPVN (B), and flat 50 kHz USVs and Fos-ir positive oxytocin neurons in the SON (C). FT, female tickled (light green); FC, female control (dark green); MT, male tickled (light blue); MC, male control (dark blue). Black line, linear regression line.

Magnocellular division of the PVN

There was no interaction of treatment and sex (Z = 1.191, p = 0.233) and no significant relationship between double labelled Fos-ir and oxytocin cells in the pmPVN and treatment (Z = -1.517, p = 0.129), sex (Z = -0.011, p = 0.991) (Table 3, Fig. 7). For double labelled vasopressin magnocellular neurons, there was no interaction of treatment and sex (Z = -0.233, p = 0.816; Table 3, Fig. 7) and no correlation with treatment (Z = -0.074, p = 0.941), sex (Z = 0.201, p = 0.840). No behaviours were correlated with double labelled Fos-ir oxytocin or vasopressin cells in the pmPVN (data not shown).

Table 3. Statistical output of final predictive model for double labelled magnocellular oxytocin and vasopressin neurons in the PVN. Significant effects (p < 0.05) shown in bold.

Region	Neuropeptide	Fixed Effects	Estimates	±SE	95% CI	Z value	P-value
pmPVN	Oxytocin	Treatment	-0.1312	0.0865	-0.3008-0.0383	-1.517	0.129
		Sex	-0.0009	0.0848	-0.1671-0.1652	-0.011	0.991
		Treatment: Sex	0.1436	0.1206	-0.0926-0.3799	1.191	0.233
	Vasopressin	Treatment	-0.0054	0.0731	-0.1487-0.1379	-0.074	0.941
		Sex	0.0168	0.0833	-0.1465-0.1801	0.201	0.840
		Treatment: Sex	-0.0237	0.1021	-0.2239-0.1764	-0.233	0.816

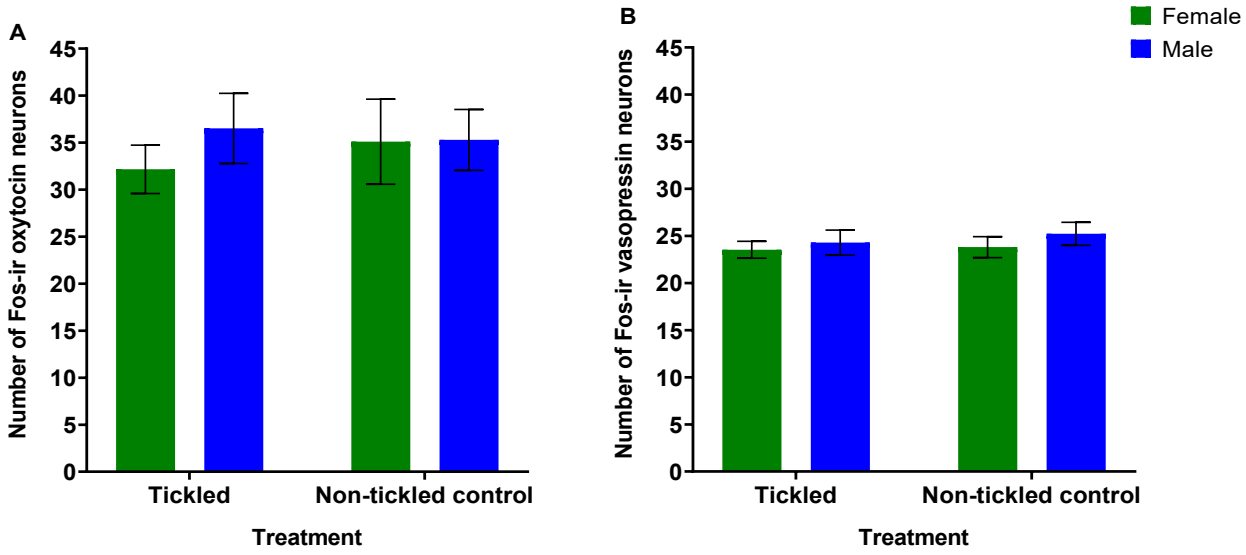

Figure 7. Tickling did not affect Fos-ir in oxytocin or vasopressin magnocellular neurons in the PVN.
Number of Fos-ir oxytocin (A) and vasopressin (B) magnocellular neurons in the pmPVN. Females (green), males (blue). Mean ± SEM; n = 16 / group. [n.s.].

5.3.2 The effect of tickling on Fos-ir in oxytocin and vasopressin neurons in the SON

There was no interaction of treatment and sex (Z = -0.323, p = 0.7467) and no significant relationship between Fos-ir oxytocin in the SON and treatment (Z = -0.079, p = 0.9368), sex (Z = -1.044, p = 0.2966; Table 4, Fig. 8). This was the same with Fos-ir vasopressin cells (treatment: Z = -0.361, p = 0.718; sex: Z = -0.556, p = 0.578; treatment x sex: Z = 0.609, p = 0.542).

No behaviours were significantly correlated with Fos-ir vasopressin cells in the SON, but there was a significant relationship between the number of Fos-ir oxytocin cells in the SON and the number of flat 50 kHz USVs emitted (Z = -2.256, p = 0.0241; Table 4). There was a significant interaction between flat 50 kHz USVs and treatment (Z = 3.01, p = 0.0027), and also between flat 50 kHz USVs and sex (Z = -2.19, p = 0.0285) on the number of Fos-ir SON oxytocin neurons. Flat 50 kHz USV emission predicted a decrease in number of Fos-ir oxytocin cells in the SON (Fig. 6).

Table 4. Statistical output of final predictive model for oxytocin and vasopressin neurons in the SON. Significant effects ($p < 0.05$) shown in bold.

Region	Neuropeptide	Fixed Effects	Estimates	±SE	95% CI	Z value	P-value
SON	Oxytocin	Treatment	-0.0052	0.0651	-0.1327-0.1224	-0.079	0.9368
		Sex	-0.0650	0.0623	-0.1872-0.0571	-1.044	0.2966
		Treatment: Sex	-0.0256	0.0794	-0.1813-0.1300	-0.323	0.7467
		Flat 50 kHz USVs	-0.0008	0.0004	-0.0016- -0.0001	-2.256	**0.0241**
	Vasopressin	Treatment	-0.0195	0.0540	-0.1254-0.0864	-0.361	0.718
		Sex	-0.0304	0.0546	-0.1374-0.0767	-0.556	0.578
		Treatment: Sex	0.0459	0.0753	-0.1017-0.1935	0.609	0.542

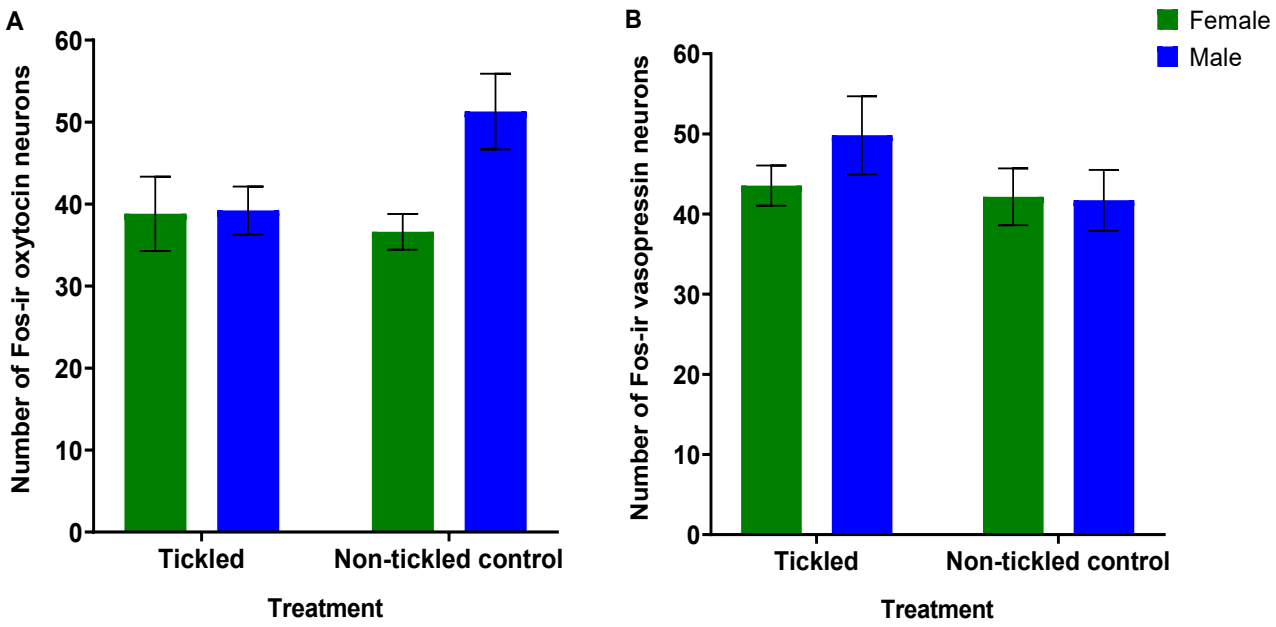

Figure 8. Tickling did not affect Fos-ir in oxytocin or vasopressin magnocellular neurons in the SON.
Number of Fos-ir oxytocin (A) and vasopressin (B) neurons in the SON. Females (green), males (blue). Mean ± SEM; n = 16 / group. [n.s.].

5.3.3 The effect of tickling on circulating hormone levels

There was no significant difference between control and tickled rats in serum concentrations of oxytocin ($F_{(3,60)} = 1.21$, $p = 0.313$; Fig. 9A) or vasopressin ($F_{(3,60)} = 1.80$; $p = 0.157$; Fig. 9B). Male rats, independent of treatment, had a higher concentration of circulating vasopressin compared to females ($F_{(1,62)} = 4.37$; $p = 0.041$), but there was no difference between sexes in circulating oxytocin levels ($F_{(1,62)} = 3.23$; $p = 0.077$).

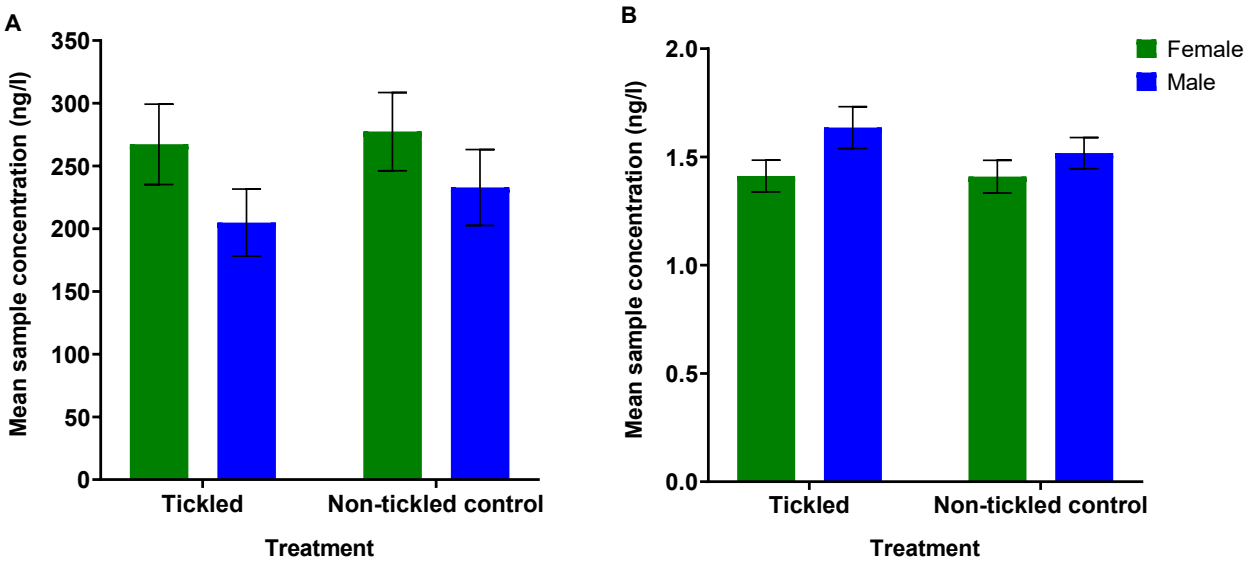

Figure 9. Circulating oxytocin and vasopressin levels were not different between tickled and control rats.
Tickling had no significant effect on plasma levels of oxytocin (A) or vasopressin (B) in male and female rats compared to non-tickled controls. Female rats (green) and male rats (blue). Mean ± SEM. n = 16 / group.

5.4 Discussion

To my knowledge, this is the first study to investigate if the hypothalamic oxytocin and vasopressin systems are active during a playful tickling stimulus in female and male juvenile Wistar rats by quantifying immediate early gene expression in oxytocin and vasopressin neurons via immunohistochemical double labelling. Tickling resulted in less Fos-ir in oxytocin neurons in the dorsal parvocellular PVN and oxytocin and vasopressin neurons in the medial parvocellular PVN compared to untickled control rats, and this finding was consistent for both sexes. Although, there was a significant interaction between treatment and sex in oxytocinergic mpPVN neurons. There was no association between tickling and Fos-ir in the posterior magnocellular PVN, nor in the SON, in either oxytocinergic or vasopressinergic neurons; again the finding was the same for females and males.

The effect of tickling on Fos-ir expression in oxytocin and vasopressin neurons in the PVN and the SON

In the present study, neuronal activation in magnocellular oxytocin and vasopressin neurons was not different in tickled compared to control rats, and there was less neuronal activation in parvocellular oxytocin and vasopressin neurons in tickled compared to control rats. Similarly, there was no effect of treatment on Fos-ir in the SON. A possible interpretation for these data is that the oxytocin and vasopressin systems are not involved in the behavioural response to tickling, or that there is even an inhibitory effect of tickling on parvocellular oxytocinergic and vasopressinergic neurons. This is in contrast to studies that have shown a key role of these two neuropeptides in response to conspecific social play (Veenema et al., 2013; Paul et al., 2014; Bredewold et al., 2014; Bredewold and Veenema, 2018; Lee et al., 2021), upon which the tickling paradigm was modelled (Panksepp and Burgdorf, 2000). Intracerebroventricular administration of a vasopressin receptor antagonist led to a reduction in social play in male Wistar rats, but an increase in social play in females, while blocking vasopressin receptors in the lateral septum increased social play in males but decreased play in females (Veenema et al., 2013). Similarly, oxytocin and vasopressin receptor antagonists administered

centrally altered social play in a sex- and context- specific manner in female and male Wistar rats; for example, a vasopressin antagonist enhanced play in males, but this effect was observed in the home cage and not in a novel cage (Bredewold et al., 2014). A recent study has showed that exposure to social play increased Fos-ir in vasopressin-receptor (V1aR) expressing neurons in the ventral pallidum (a region associated with regulating rewarding social behaviours, including partner preference in prairie voles (*Microtus ochrogaster*; Lim et al., 2004; Lee et al., 2021). The authors also found that a V1aR antagonist administered into the ventral pallidum increased the duration of social play bouts in male Wistar rats, but this decreased play duration in female Wistar rats (Lee et al., 2021). Reppucci et al. (2018) found that there was no difference in Fos-ir in vasopressin and oxytocin neurons in the PVN between female and male Wistar rats that had been engaged in 10 minutes of social play, and those that had not, which is consistent with the findings of the present study. However, the authors found that SON oxytocin neurons were positively correlated with the amount of time female and male rats engaged in social play (Reppucci et al. 2018), while there was no effect of tickling on the activity of oxytocin and vasopressin neurons in the SON in the current study. Indeed, oxytocin and vasopressin are also known to facilitate numerous other social behaviours in various species, such as bonding between individuals (for example, vasopressin receptor (V1aR) expression facilitates pair bond formation in prairie (*Microtus ochrogaster*) and meadow (*Microtus pennsylanicus*) voles; Lim et al., 2004) and maternal behaviours (for example, oxytocin enables pup retrieval in female mice by enhancing the mother's recognition of pup distress calls; Marlin et al., 2015). Oxytocin neurons in the SON are implicated in several social behaviours, such as social recognition (Takayanagi et al., 2017) and maternal pup retrieving behaviour (Okabe et al., 2017). Therefore, one interpretation of the current study is that tickling is not perceived as a social behaviour by female and male Wistar rats, hence the lack of involvement in oxytocin and vasopressin neurons in the PVN and SON or changes in circulating levels of oxytocin or vasopressin.

Tickling was originally modelled on social conspecific play in rats (Panksepp and Burgdorf, 2000), however, many of the behavioural elements that exist in conspecific play, such as pouncing, wrestling and reciprocal attacks to the nape of the neck (Pellis and Pellis, 2007) cannot be replicated by the human hand (Bombail et al., 2021). Similarly, the rat may not have a choice in being tickled when following the alternating 15 seconds of tickling and 15 seconds of no tickling (Panksepp and Burgdorf, 2000) as this prescriptive protocol does not necessarily allow for the rats to choose when it does or does not want to be tickled (Bombail et al., 2021). Therefore, current findings suggest that the neural correlates of tickling differ from those of conspecific social play, and this is may be due to tickling not fully resembling social play, despite it originally being based on this behaviour.

This interpretation is further supported by findings that gentle touch or stroking activates oxytocin hypothalamic neurons (Okabe et al., 2015, 2020, 2021; Tang et al., 2020). Touch is a sensory stimulus found in several social behaviours, for example parental care (e.g. maternal care in Macaque monkeys; Harlow and Zimmerman, 1959) and social bonding (e.g. social cohesion in a number of primate species; Dunbar, 2010), and is thought to play a role in communication, emotion and bond-formation (Hertenstein et al., 2006). Löken and Olausson (2010) describe touch as “a channel for social information”. Studies have shown that gentle stroking stimuli facilitate affiliative relationships between individuals (for example, between piglets and humans, Tallet et al., 2014; between mice and humans, Cho et al., 2021; between cattle and humans, Schmied et al., 2008). Tang et al. (2020) showed that during a social interaction paradigm with adolescent female Wistar rats, physical touch by a conspecific increased the number of Fos-ir oxytocin neurons in the PVN and the SON. Similarly, Okabe et al. (2015) found that adolescent male Long-Evans rats that received gentle stroking for five minutes (the rat was placed on the experimenter’s lap and stroked on its back by the experimenter, wearing a cotton glove at a defined speed and frequency) increased the number of Fos-ir oxytocin cells in the medial parvocellular PVN. More recently, it has been shown that five minutes of gentle stroking activated oxytocin neurons in the

PVN, in both female (Okabe et al., 2021) and male (Okabe et al., 2020) Lewis rats, suggesting that this type of gentle stroking incudes an affiliative relationship between the experimenter and the rats (Okabe et al., 2020, 2021). Therefore, tickling may not activate oxytocin neuronal populations as gentle stroking does, because gentle stroking has more of a social, affiliative role for rats (Okabe et al., 2020, 2021) which has thus far not been explored with tickling. Tickling differs from gentle touch in other neural correlates, for example, tickling was found to evoke increased neuronal firing in the trunk region of the somatosensory cortex compared to gentle touch in rats (Ishiyama and Brecht, 2016). The behavioural response to tickling and light touch (repeated gentle stroking along the rats' dorsal side) differs too as rats are found to emit more 50 kHz USVs in response to tickling than gentle touch (female and male Long-Evans rats in Burgdorf and Panksepp, 2001; unspecified sex and strain in Ishiyama and Brecht, 2016), although rats did produce 50 kHz USVs in response to gentle stroking (Burgdorf and Panksepp, 2001; Ishiyama and Brecht, 2016; and also reported by Okabe et al., 2015, 2020, 2021). Therefore, tickling and gentle touch may have differing neural correlates, with gentle touch being more of a social stimulus than tickling. It is worth noting that Okabe et al. (2015, 2020, 2021) carried out gentle stroking for five minutes, compared to the two minutes of tickling in the present study. Therefore, it is possible that Okabe at al. (2015, 2020, 2021) found differences in Fos-ir due to a longer duration of stimulus.

The effect of tickling on Fos-ir in parvocellular versus magnocellular neural populations of the PVN

Oxytocin and vasopressin neurons in the PVN can be categorised into two types, magnocellular and parvocellular neurons based on several criteria including shape, size, function, projection sites and modes of release (Althammer and Grinevich, 2017; Swanson and Sawchenko, 1980, 1983; Stern and Armstrong, 1995). Parvocellular oxytocin neurons project to structures in the brainstem and spinal cord (Swanson and Kuypers, 1980), and have varying roles in autonomic functions. For example, an increase in Fos-ir expression in parvocellular oxytocin neurons occurred following acute

myocardial infarction in rats (Roy et al., 2018) and activation of parvocellular oxytocin neurons lead to attenuated nociceptive signalling (Eliava et al., 2016). A small subset of parvocellular oxytocin neurons are also found to synapse onto magnocellular oxytocin neurons in the PVN, and this is thought to control the activity of those oxytocin magnocellular neurons (Eliava et al., 2016). Much less is known about the projections of parvocellular vasopressin neurons, and the interaction of parvocellular and magnocellular neurons maybe unique to the oxytocin system (Grinevich and Ludwig, 2021). Magnocellular oxytocin and vasopressin neurons project to the posterior pituitary gland as well as to midbrain regions, for example the hippocampus, amygdala, and locus coeruleus (Zhang and Hernandez, 2013; Hernandez-Perez et al., 2019; Knobloch et al., 2012) and mediate social and emotional responses, for example, female aggression (de Moura et al., 2021), and social fear responses (Menon et al., 2018; Grinevich and Ludwig, 2021; Grinevich and Stoop, 2018). The current study suggests that tickling may lead to lower parvocellular oxytocin and vasopressin neuronal activity, but there was no effect of tickling on magnocellular neuron activity. The projections of the parvocellular neurons cannot be discerned in the current study because Fos immunohistochemistry does not visualise neuronal projections. However, it is interesting to note that there was a decrease in Fos-ir in parvocellular, but not magnocellular oxytocin neurons. There is evidence that somatosensory information is received by parvocellular oxytocin neurons, which then activate magnocellular oxytocin neurons, and this is found to occur during social interactions in female Wistar rats (Tang et al., 2020). Tang et al. (2020) also found that a non-nociceptive air puff applied to the skin of the abdomen led to an increase in Fos-ir in parvocellular oxytocin neurons. While it is not clear why a puff of air increased Fos-ir in these cells while tickling decreased Fos-ir in parvocellular oxytocin cells in the present study, this may indicate that the somatosensory information of tickling, as it is a somatosensory stimulus (Ishiyama and Brecht, 2016), may be received by parvocellular oxytocin neurons initially. This would need to be explored in a future study, possibly via a similar technique as Tang et al. (2020), using designer receptors exclusively activated by designer drugs

(DREADDs; Armbuster et al., 2007) to explore whether chemogenetic manipulation of these parvocellular oxytocin neurons altered these neurons' response to tickling.

The effect of sex on Fos immunoreactivity in the PVN and SON

There were no sex differences in the number of Fos-ir double labelled oxytocin and vasopressin neurons in either tickled or control rats. There are known sex differences in the oxytocin and vasopressin systems, for example, there are higher numbers of vasopressin-ir neurons in males compared to females in the BNST in Wistar rats (Van Leeuwen et al., 1985), more vasopressin-ir fibres in the lateral septum of male versus female Wistar rats (De Vries et al., 1981) and more vasopressin-ir fibres in the BNST and medial AMG in male versus female mice (De Vries et al., 2002; Bakker et al., 2006). There are also sex differences in vasopressin V1aR receptor binding in Wistar rats, with males having higher V1aR binding densities in the BNST, hippocampus and somatosensory cortex (Dumais and Veenema, 2016). Female Wistar rats were shown to have lower oxytocin receptor binding densities in the NA, caudate putamen, LS, BNST, amygdala and VMH than male rats (Dumais et al., 2013). However, there are few sex differences in vasopressin-ir neurons or fibres specifically in the PVN and SON in multiple species, including Wistar rats (e.g. Tayla et al., 2012), mice (e.g. Joca et al., 2013), vole species (e.g. Wang et al., 1996), hamster species (e.g. Wang et al., 2013), humans (e.g. Fliers et al., 1986) and macaques *(Macaca fascicularis;* e.g. Caffe et al., 1989; see Dumais and Veenema et al. (2016) for complete review). No differences have been reported in the expression of vasopressin mRNA in the PVN and SON of adult Wistar rats (Dumais and Veenema, 2016). In the oxytocin system, numbers of oxytocin-ir neurons in the PVN and SON have been found to be higher in female Brandt's voles *(Lasiopodomys brandtii*; Xu et al., 2010), mandarin voles (*Lasiopodomys mandarinus*; Qiao et al., 2014) and mice (Haussler et al. 1990). However, there was no difference in oxytocin mRNA optical density in the PVN and SON of female and male Wistar rats (Dumais et al., 2013), nor in the number of oxytocin-ir neurons in the PVN and SON of prairie *(Microtus ochrogaster*), pine

(*Microtus pinetorum*), meadow (*Microtus pennsylvanicus*) and montane (*Microtus montanus*) voles (Wang et al., 1996), naked mole rats (*Heterocephalus glaber*; Rosen et al., 2008) and macaques *(Macaca fascicularis*; Caffe et al., 1989). Contrary to this, Reppucci et al. (2018) found that male Wistar rats had double the number of Fos-ir oxytocin and vasopressin neurons in the SON than female Wistar rats following social conspecific play, which was not replicated in the present study. While sex differences in the oxytocin and vasopressin system do exist in multiple mammalian species, there are only a few reported sex differences in the PVN and SON of Wistar rats. This may help to explain the finding of the present study as overall the literature suggests that any sex differences in the role of the oxytocin and vasopressin systems in tickling may not lie in the PVN and SON.

Along with the sex differences in the oxytocin and vasopressin neural systems, there are known sex differences in oxytocin and vasopressin's roles in various behaviours (e.g. Stack et al. 2010; Lukas and Neumann, 2014; Dumais and Veenema, 2016; DiBenedictis et al., 2017). These include social interest in Wistar rats (Dumais et al., 2016), aggression in Syrian hamsters (*Mesocricetus auratus*; Gutzler et al., 2010), partner preference formation in prairie voles *(Microtus ochrogaster*; Winslow et al., 1993; Insel and Hulihan, 1995), and in social play (Veenema et al., 2013; Bredewold et al., 2014). Inhibiting vasopressin V1aR receptors reduces social play in male Wistar rats, but increases social play in females, while an oxytocin receptor antagonist had no effect on play in either sex (Veenema et al., 2013; Bredewold et al., 2014). In the present study there were sex differences in some, but not all, behaviours induced by tickling in these rats. For example, female tickled rats emitted more total 50 kHz USVs than tickled male rats or control rats (Chapter 2, Fig. 6), and there was no sex difference in the number of trill 50 kHz USVs emitted, while female rats made more flat 50 kHz USVs (Chapter 2, Fig. 7). Also, in solitary play behaviour, there was no difference between sexes in the number of scampers, or the hopping and darting elements of this behaviour (Chapter 2, Fig. 9). Given the lack of sex differences in oxytocin-ir and vasopressin-ir

neurons in the PVN, this may suggest that any sex differences in the regulation of the behavioural response to tickling may lie in other brain regions or circuitry and not in the oxytocin and vasopressin PVN systems. For example, Reppucci et al. (2018) found robust sex differences in Fos-ir in the posterior bed nucleus of the stria terminalis and the posterodorsal medial amygdala nucleus, but not in oxytocin and vasopressin neurons in the PVN in rats that had engaged in social conspecific play over a 10 minute period. Studying the neural correlates of each individual play behaviour (e.g. scampers or 50 kHz USVs separately) may deduce whether there are sex differences in the neural pathways underlying these behaviours. This wasn't possible when using immunohistochemistry against Fos because this technique is carried out post-mortem and not in awake, behaving animals. Therefore, which particular behaviour activated each neuron or population of neurons in the PVN cannot be deduced. An alternative may be to use optogenetics (Boyden et al., 2005; Deisseroth, 2010); with this technique, it is possible to switch specific cell types on or off during a certain behaviour, and could be used, for example, to target parvocellular oxytocin neurons during tickling and compare between female and males rats which, if any, behaviours were affected.

Behaviours regressed against Fos-ir

While there were significant correlations between 50 kHz USVs and Fos-ir parvocellular oxytocin neurons in the PVN, and between flat 50 kHz USVs and Fos-ir oxytocin neurons in the SON, the correlations are weak and there is a lot of individual variation. Therefore, caution should be used with their interpretation. A tentative explanation for the association between 50 kHz USVs and Fos-ir parvocellular oxytocin neurons in the PVN may be that 50 kHz USVs have been related to the reward circuitry (Burgdorf et al., 2007) and dopamine release in the nucleus accumbens (Willuhn et al., 2014) and it has been shown that there are both magnocellular and parvocellular oxytocinergic projections from the PVN to the VTA (Hung et al., 2017). Blocking oxytocin neurons projecting to the VTA reduces socially conditioned place preference, and oxytocin receptor activation in the VTA results in increased activity of nucleus accumbens- projecting dopaminergic neurons in mice (Hung et al.,

2017). Given that this correlation between 50 kHz USVs and neural activity of parvocellular oxytocin neurons was independent of treatment, this weak correlation may be due to some high-calling individuals in the control groups. It is possible that this result may represent the reward associated with going into the tickling arena (but not necessarily from being tickled), as some individuals in the control group, particularly control females, were producing abundant numbers of 50 kHz USVs during the two minutes of testing (Fig. 4). A tracing study (for example with viral vectors (Oztas, 2003), or a fluorescent dye (Lu, 2011) could be used to investigate this putative circuit and compare between tickled and control rats.

Circulating hormone levels

Tickling did not affect plasma oxytocin and vasopressin concentrations, although male rats in general had higher levels of circulating vasopressin than females, which is consistent with prior published findings (Crofton et al., 1986). To my knowledge, no previous studies have analysed the effect of ticking on plasma oxytocin and vasopressin concentrations. Tickling has been found at alter plasma concentrations of adrenaline and noradrenaline, but it did not affect plasma corticosterone concentrations (Hori et al., 2013). The stimulus of tickling may induce changes in some, but not all, endogenous hormones and this likely depends on the role of the hormone.

Oxytocin and vasopressin are synthesised in the PVN and SON, transported along axons to the posterior pituitary gland where these hormones are released into the blood stream to act on distant targets in the periphery (Leng, et al., 2012). As there was little effect of tickling on the activity of oxytocin and vasopressin neurons in either the PVN or SON, it is unsurprising that there was no main effect of tickling on circulating oxytocin and vasopressin concentration. However, there is evidence in other species that interaction with a human may alter plasma oxytocin and vasopressin concentrations: in dogs, human interaction increased plasma oxytocin concentration compared to control dogs who did not receive any human interaction, and plasma vasopressin was found to decrease following human interaction (MacLean et

al., 2017). Massage has been found to increase plasma oxytocin in humans (Morhenn et al., 2012). There is some evidence that stroking in rats also alters plasma oxytocin concentrations (Lund et al., 2002; Stock and Uvnas-Moberg, 1988). As with PVN and SON neuronal activity, a tickling stimulus did not appear to activate the oxytocin and vasopressin systems in the present study. However, the results of this ELISA should be interpreted cautiously as there are two important limitations to this analysis: sample extraction was not carried out and this may have resulted in nonspecific binding which may result in falsely measuring more hormone than was in the sample (for review, see Leng and Sabatier, 2016). Also, the half-life of oxytocin and vasopressin in plasma is found to be on a minute scale (bioreactivity is found to disappear in less than 10 minutes; for review, see Leng and Sabatier, 2016). Therefore, the 60 - 90 minutes between testing and euthanasia is likely too long to be able to detect changes in plasma concentrations of these neuropeptides due to tickling.

Conclusions

In conclusion, the findings suggest that tickling may not involve the oxytocin and vasopressin systems providing the first evidence that tickling does not replicate the involvement of the central oxytocin and vasopressin systems found for social play in juvenile rats. In contrast to social play, rats can’t choose if they want to be tickled, and many elements of social play cannot be replicated by the human hand. This is an important consideration for future interpretation of tickling. Other systems, such as the somatosensory cortex (Ishiyama and Brecht, 2016) and the dopaminergic reward circuit (Burgdorf and Panksepp, 2001; Hori et al., 2013), may instead play a more prominent role in the response to tickling than regions involved in social conspecific play.

Chapter 6

Measuring the motivation and preference of female and male Wistar rats for tickling using modified runway and social preference tests.

6.1 Introduction

Tickling by a human hand is thought to be a rewarding experience for juvenile rats; 50 kHz ultrasonic vocalisations (USVs), in particular the trill subtype, produced during tickling have been linked to hedonic behaviours such as mating, conspecific rough-and-tumble play and feeding (Burgdorf et al., 2011). While both female and male rats produce 50 kHz USVs in response to tickling (Schwarting et al., 2018; Himmler et al., 2014), there are only a few studies that have investigated USV production during tickling in females (LaFollette et al., 2017). Several studies that have compared tickling in male and female rats reported abundant USV production by both sexes and have presented varying results (e.g. Mällo et al. 2007; Schwarting et al., 2018), or found that male rats called more than females (Burgdorf and Panksepp, 1999). However, there are known sex differences in USV production in rats (Lenell et al., 2021), in particular during rough and tumble play (Kisko et al., 2020) and mating behaviours (Thomas and Barfield, 1985; White et al., 1990). In Chapter 2, I found that female rats emitted significantly more total 50 kHz and flat 50 kHz USVs than male rats. While there was no interaction between treatment and sex, a post-hoc analysis suggested that tickled females produced more total 50 kHz USVs than tickled males or controls. In contrast, I found that there was no sex difference in the number of reward-associated (Burgdorf and Panksepp, 2001, 2003) trill 50 kHz USVs produced in response to tickling. I also found that female rats, regardless of treatment, made more scampers (solitary play expressed during tickling; Panksepp and Burgdorf, 1999; Hammond et al., 2019; Chapter 2, Fig. 3) and approaches towards the hand (a measure of positive reinforcement of tickling; Burgdorf and Panksepp, 2001;

Chapter 2, Fig. 4) compared to male rats. Therefore, it is not clear whether the sex differences found in these behavioural outputs were due to the rewarding nature of tickling or due to female rats responding to a stimulus other than tickling.

Runway tests, where a rat is placed at one end of a straight platform and runs along a runway to reach the goal at the opposite end, can be used to measure motivation for incentive stimuli (Ettenberg, 2008), such as food (White et al., 1977) and drug reinforcers (e.g. caffeine, Miller and Milles 1935; cocaine, Ben-Shahar et al., 2008; and heroin, Ettenberg et al., 1996). The speed, or time taken, to traverse the straight runway is used as an indicator of the motivation to seek out the goal stimuli, and is referred to as 'goal-seeking motivated' behaviour (Ettenberg, 2008). For example, White et al. (1977) found that rats ran faster along a runway to receive an injection of morphine in comparison to food delivered into the goal-box, suggesting that the rats were more motivated to reach the morphine than the food. The runway test has been successfully used in a number of studies. For example, it has been used to measure motivation for a food reward following gastric bypass in rats (Zheng et al., 2010), to investigate the role of dopaminergic transmission in male sexual behaviours, where male rats traversed the runway to reach an oestrous female (Lopez and Ettenberg, 2001), and to compare the reinforcement properties of drugs (e.g. morphine and ecstasy) versus food (Crespo et al., 2008; Wakonigg et al., 2003). Given that tickling activates similar neural pathways as hedonic drugs (Burgdorf et al., 2011; Alcaro et al., 2007; Ikemoto et al., 2020) and evokes 50 kHz USVs that are also produced in response to drugs of abuse (Burgdorf et al., 2001), the runway test may be a useful paradigm with which to measure the motivation of rats to be tickled, and in particular, to compare between females and males.

In Chapter 5, I reported a decrease in active oxytocin neurons in the paraventricular nucleus of the hypothalamus in tickled rats, and postulated that this may be due to tickling not necessarily being perceived as a social stimulus. Previous studies have shown that oxytocin administration can induce social preference (e.g. in female mice, Kent et al., 2013; in male rats, Lukas et al.,

2011), although Lukas and Neumann (2014) found that oxytocin and vasopressin systems were not involved in naturally occurring social preference behaviour in female rats. The question of whether tickled rats form a social preference for the human hand has yet to be explored. Social preference tests can be used to establish preference for a social cue, for example, a conspecific (e.g. Lukas et al., 2011, 2014) over a non-social cue, usually an inanimate, novel object (e.g. Lukas et al., 2011, 2014). A recent study has shown that female Long-Evans rats expressed a preference for social (another conspecific) over non-social (an empty chamber) options (Hackenberg et al., 2021). Okabe et al. (2020) used a social preference test to measure male rats' preference for the hand that had been applying a gentle stroking stimulus, compared to a novel object. They found that oxytocin neurons in the paraventricular nucleus of the rats were activated in response to stroking, and the rats showed a preference for the hand of the experimenter in the social preference test (Okabe et al., 2020). Therefore, a social preference test may be used to investigate whether rats perceive tickling as a social stimulus.

To further investigate the sex differences found in Chapter 2, I measured 50 kHz USVs, including trill and flat subtypes, to test whether female rats made more 50 kHz USVs in general, or whether tickled females emitted the most 50 kHz USVs. I also used a modified runway test (adapted from Lopez and Ettenberg, 2001, Crespo et al., 2008, and Ettenberg, 2009) to address whether female rats have a higher motivation to seek out tickling than males as they find tickling to be rewarding. In addition, I used a preference test to further investigate whether tickling could induce a preference for the hand that was carrying out the tickling. In the literature, a 'social preference test' is the term used to describe a behavioural test that distinguishes between a rat's preference for a social cue over a non-social cue (e.g. Hackenberg et al., 2021). Because it is not clear from my previous data (Chapters 4 and 5) whether tickling is perceived by rats as a social stimulus, I will refer to the modified 'social preference test' as a 'preference test' in this study.

I sought to compare the outcomes of the runway test and preference test to the 50 kHz USVs produced during tickling to understand if 50 kHz USV

production matched the motivation and preference for tickling. I tested the hypothesis that the number of 50 kHz USVs produced by female rats in response to tickling correlates with measures of motivation for the reward of tickling. I expected to find that if rats emit higher numbers of 50 kHz USVs, and in particular reward associated-trill 50 kHz USVs (Burgdorf et al., 2011) during tickling, that they will also express higher levels of motivation to be tickled (Panksepp and Burgdorf, 2000). Hence, in the runway test, I predicted that tickled rats should be more motivated to reach the tickling hand, and should traverse the runway more quickly than the control rats, and this would also be greater in female compared to male tickled rats. The control rats should not associate the hand with reward and so should take longer to approach the hand than the tickled rats (Ettenberg, 2008). I also hypothesised that tickled rats do not perceive the tickling stimulus as a social interaction (based on my own findings in Chapters 4 and 5). Therefore, in the preference test, I predicted that there would be no difference between tickled and control rats in their preference for the hand over the novel object, and that both tickled and control rats should find a novel object more interesting to approach and to explore than the hand because of the juvenile rat's natural propensity for novelty (Stansfield et al., 2006; Berlyne, 1950).

6.2 Materials and Methods

6.2.1 Subjects

Forty-eight juvenile Wistar rats (24 females 35.6 - 55.2g; 24 males 39.4 - 70.9g) were used in the current study (Charles River, Kent, UK). Rats arrived at the Roslin Institute Biological Research facility at 21 - 23 days of age. Following arrival, the body weight of each rat was recorded daily. Rats were acclimatised to their new surroundings for four days before they were habituated to the tickling test arena (Perspex open box, 60 (length) x 60 (width) x 25 (height) that was lined with LabMat; LabLogic Systems Ltd., England) and plus maze (each arm 100 x 10 cm; centre area 10 cm^2; walls of closed arms 34 cm high; maze elevation off floor 60 cm; Tracksys Ltd UK).

The rats were derived from four different litters; they were sourced from as many litters as possible to account for the influence of genetics and early life experience. Rats were housed in same-sex pairs, with each cage containing two rats in the same treatment group (Control / Tickled, pseudo-randomly assigned, balanced for body weight (Matsuzaki et al., 2018) and litter (no littermates were housed in the same cage)), in clear plastic cages (46 x 25 x 21cm) with a wire lid. There was an equal number of animals from each litter in both treatment groups. Each cage contained aspen chip bedding, one shredded paper nest, one aspen chew stick (Nepco, Warrensburgh, USA). Food (14% protein rodent maintenance diet, Envigo, UK) and water were available *ad libitum* and the room temperature, humidity and light intensity was held stable at 18 - 23^{o}C, 40-60% and 25 lux respectively. The cages were randomly positioned in a cage rack to account for differing lux levels through the height of the rack to balance for sex and litter. The rats were tested on the EPM and in the tickling test arena in the dark phase and were held on a reversed 12-hour light/12-hour light dark cycle (lights on: 19:00, lights off: 07:00). Body weight (g) was recorded daily following testing between 16:00 - 18:00 in the dark phase. The rats were checked daily (by laboratory personnel at 08:00, during the light phase) and nitrile gloves were worn when handling the animals. To minimise handling stress rats were picked up gently by holding them behind their forelegs and then cupping them with both hands.

6.2.2 Experimental design

Rat sample size was determined using a power equation using variance and mean values from a previous data (Chapter 2). The difference to detect was set at 0.60, the standard deviation 0.50, power 80%. The order in which the cages and cage mates were tested each day was pseudo-randomised to account for time of day, sex, treatment and lux levels of the cages in the cage rack.

All testing (weighing, habituation, tickling, runway and preference testing) was carried out in the home room. The area used for all testing was enclosed by a thick, plastic curtain, at the opposite end of the room to the cage rack. Testing was carried out in the dark phase under red light for the experimenter (myself, Emma Tivey) to see. The only personnel present in the room for testing and carried out all testing was myself. The rats were brought to a bench in the enclosed area used for testing in their home cage. One rat was tested while the second cage mate remained in the home cage. The home cage was placed away from the testing apparatus so that no USVs were detected by the rat in the home cage. Each cage mate was identified by a mark on the tail made at the time of assigning treatment: tickled rats had a black mark, control rats had no black mark.

Habituation phase

Rats were subjected to four days of habituation to the experimenter and the arenas in which the testing was carried out. The rats were habituated as individuals to handling, the testing arena for two days, and the elevated plus maze (each arm 100 x 10 cm; centre area 10 cm^2; walls of closed arms 34 cm high; maze elevation off floor 60 cm; Tracksys Ltd UK) for two days (Fig. 1).

Figure 1. Representation of the order of the behavioural testing.
All rats (n = 48) went through a 4 day habituation phase, habituating them to the testing arena where the tickling protocol took place for 2 days, and the elevated plus maze where the runway test and preference test took place for 2 days. The tickling phase lasted 5 days, followed by the runway test for 2 days and the preference day on 1 day.

The elevated plus maze had the two open arms closed off, allowing the rat to explore the start box and the two closed arms that would be used during testing. During the habituation phase, the rats were placed in the testing arena or maze for a total of two minutes per day. The researcher placed an immobile hand (wearing a nitrile glove covered with a white cotton glove) on the wall of the arena or maze to habituate the rats to the glove and the researcher. The right hand was used with each rat as this was the dominant hand of the researcher. After the habituation phase, the rats began eight days of behavioural testing. Each animal was tested for two minutes per day for five days in the tickling paradigm. Each animal had a total of five trials per day for two days in the runway test. Each animal was tested for five minutes on one day for the preference test.

Tickling

Tickled rats underwent five days of the tickling procedure (Fig. 1). The number of days for which rats are tickled varies greatly in the literature, ranging from one to 46 days (LaFollette et al., 2017). Five days of tickling was chosen based on the study by LaFollette et al. (2018) which showed that between three to five days of tickling is an effective dosage to induce positive affective states in young rats. Control rats were placed in the tickling arena but received no tickling, with the hand (cotton glove) present but immobile on the side wall of the cage (Fig. 2). One cage mate (in a randomised order) was taken from the home cage and placed in the testing arena. Each rat was tested for two minutes, timed with a stopwatch and both video and sound recordings of the behaviour were recorded digitally using a video camera (Panasonic HD HC-V10) and an ultrasound microphone (Pettersson M500-384 USB Ultrasound microphone, PetterssonElectronik, Sweden). Audacity software (Version 2.1.3, Pennsylvania, United States of America) was used to record the spectrograms. The microphone was suspended about 30 cm above the testing arena. A video camera (Panasonic HD HC-V10) was also placed above the testing arena to record behaviour during testing. Spectrograms of USVs were collected and the total number of 50 kHz calls, flat 50 kHz USVs, trill 50 kHz USVs and 22 kHz USVs were counted manually for the testing period in

Ultravox 14 (Noldus Information Technology, Wageningen, Netherlands) for each animal over the five days of testing. The arena was cleaned with 70% ethanol gel and allowed to dry between the testing of each rat and testing was carried out in the first three hours of the dark phase.

For the tickled group, rats were placed in the arena, and a human hand (wearing a white cotton glove over a nitrile glove) was placed motionless on one wall of the arena (the wall position and placement of the hand was randomised each time) for the first 15 seconds of testing. Following these 15 seconds of release (i.e. where the experimenter's hand is motionless on the side of the arena and the rat receives no contact with the hand), the rat was tickled for 15 seconds by making rapid finger movements on the nape of the neck. If the rat reared up at the hand it was tickled on its ventral side. The 15-second bouts of tickling and release were alternated during the two minutes of testing. For the control group, rats were placed in the arena for two minutes, with the hand (wearing a white cotton glove over a nitrile glove) resting motionless on one wall of the arena (the wall position and placement of the hand was randomised each time). If the rat reared at the hand, the hand was gently moved away to one side and then replaced back in position. Following testing, the rat was gently picked up (as described previously). The other cage mate was then immediately removed and placed in the arena for testing and the behavioural testing was repeated. The cotton gloves were only worn by the hand in the testing arena during testing and each rat was exposed to its own individual cotton glove to avoid any potential olfactory bias.

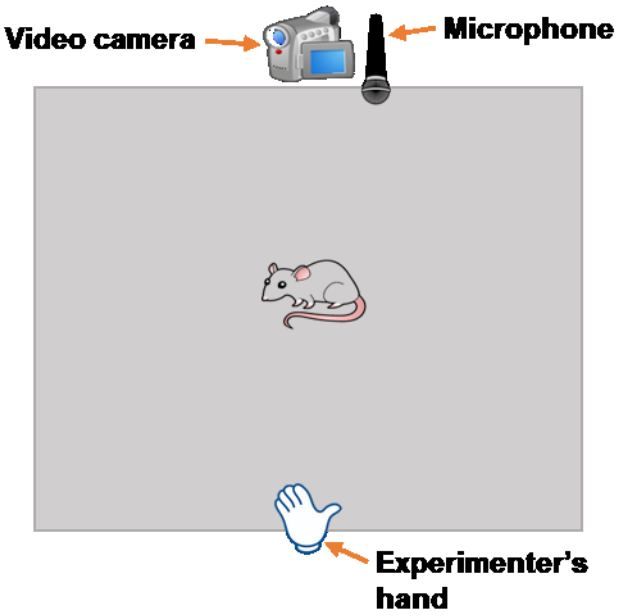

Figure 2. Representation of the testing arena used in the tickling protocol. The grey square represents the testing area. The experimenter's hand was placed on the wall of the arena during the 15 seconds of release during tickling, and for the two minutes of testing for the control rats. A video camera and microphone were suspended above the arena to record behaviours and USVs during testing.

Runway test

The runway test was carried out after the end of the five days of tickling (Fig. 1) over two days: on the first day, the rats had five training trials to learn to run along the runway, followed by five test trials the following day. The runway was created using an elevated plus maze with the two open arms blocked off with a rectangular piece of black Perspex and the closed arms forming the 1m long runway (Fig. 3). Each rat was lifted out of its cage and placed in the start box at one end of the runway (which was selected at random). The test involved the rat running towards the human hand of the experimenter which was resting on the wall of the maze at the opposite end of the maze to the start box (the hand was wearing a white cotton glove as in the tickling test). Each trial was recorded using a digital infrared camera (VCC-6594 1/3-Inch IR CCD, SANYO, Japan) suspended above the maze and Noldus Media Recorder software (Noldus Information Technology, Wageningen, Netherlands). This generated media files that were imported into Ethovision XT 11.5 software (Noldus Information Technology, Wageningen,

Netherlands). This allowed the rat to be tracked, with the time taken and speed for the rat to traverse the runway during each trial of the runway task being automatically calculated. USVs were also recorded using an ultrasonic microphone (Pettersson M500-384 USB Ultrasound microphone, Pettersson Electronik, Sweden) and Audacity software (Version 2.1.3, Pennsylvania, United States of America) was used to record the spectrograms. The microphone was attached to a wall at the centre of the runway so as not to obscure any of the maze and to allow for video tracking to be used in the behavioural analysis. USVs were detected throughout the maze. During habituation to the maze, video tracking and the microphone were tested to ensure that the video tracking worked and USVs were being detected.

The protocol was based on previous studies using a runway test to measure the motivation for rewarding stimuli. Experimental designs for runway tests vary widely in the number of trials and days (e.g. between five trials (Wakonigg et al., 2003) to 30 trials (Esumi et al., 2003), between one day (Crespo 2005) to 11 days (Shin et al., 2011)). Five trials per day for two days was determined to provide sufficient time for the rats to learn to traverse the runway and for their runtimes to stabilise. Wakonigg et al. (2003) showed that run times stabilised over three to four runs (i.e. trials) on a single day. Crespo et al. (2005, 2006) showed that five consecutive trials on one day was sufficient to measure the reinforcing strength of opioids and psychostimulants. Testing the rats over two days (five trials a day) was based on Crespo et al. (2008) who showed that across two days, runtimes were more stable by the second day showing that acquisition of the reward may be improved by the second day. Zernig et al. (2002) also reported that rats learned to run along a maze to reach a food reward within five consecutive trials. This study gave rats a total time of 60 seconds to reach the end of the runway, and if the rat exceeded this time they were gently moved towards the end of the maze to enable them to find the food that acts as a reinforcer. 15 seconds of tickling was established as the reward based on Reinhold et al. (2019) who used a short bout of tickling as a reward in a hide and seek paradigm with adult Long-Evans rats.

The rat had 60 seconds to complete each trial. If the rat did not approach the hand (i.e. if they didn't get closer than one rat's length to the hand) within 60 seconds then the rat was encouraged gently to the end of the apparatus. This was done by gently moving the start box along the runway and encouraging the rat to move to give them experience of the hand at the end of the runway. The trial was completed when the rat was a minimum of one rat's length from the hand. The rats in the tickled group received 15 seconds of brief tickling as a reward (as in Reinhold et al., 2019). The control rats were not handled in any way other than lifting them in and out of the arena with a nitrile glove, as in the tickle test. After each trial was completed, the rat was placed into the plastic pot used for weighing (as this was a familiar environment for them) for 30s while the runway was cleaned with 70% ethanol and the recording equipment reset. The rat was then placed back into the start box for the next trial. At the end of the five trials, the rat was returned to the home cage. All rats learned to run along the runway to the end. Testing as carried out in the first half of the dark phase between the hours of 07:00 and 13:00. Each rat was weighed once all the rats had been tested.

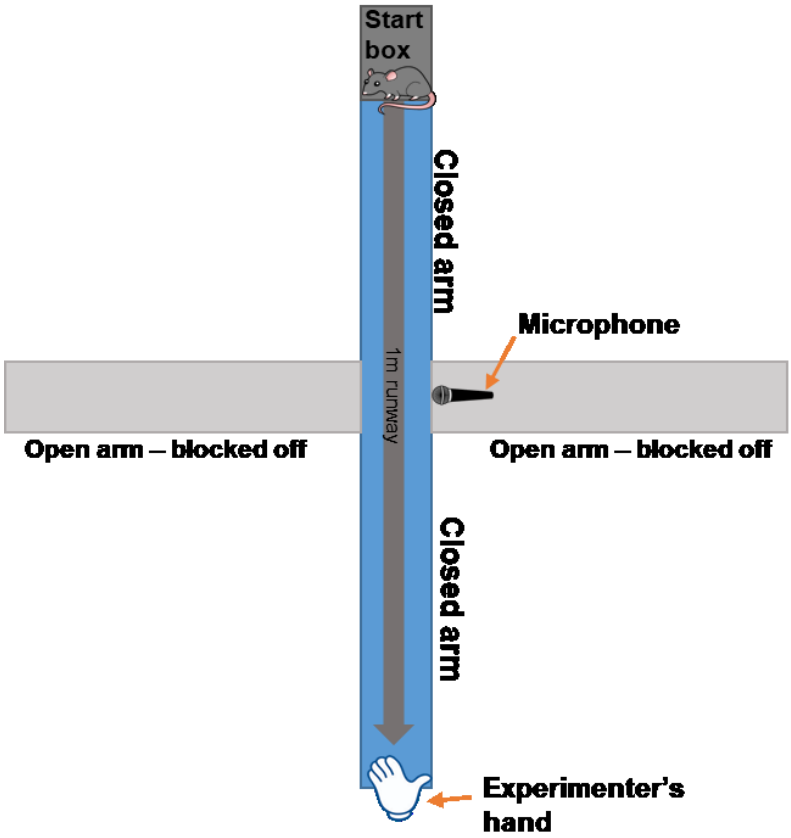

Figure 3. Representation of the apparatus used in the runway test.
The 1m long runway was built using the two closed arms of an elevated plus maze. The open arms were blocked off using rectangular pieces of black Perspex. The start box was placed at one end of the runway, rats were placed in it and once the door to the start box was lifted, they could traverse the runway to reach the experimenters hand located at the other end. A microphone was placed halfway along the runway to record USVs emitted while the rat was traversing the runway. A camera was suspended above the maze and used to track the rat moving along the runway. Light grey denotes areas which are not accessible to the rat. Blue represents the area which was accessible during testing. The start box is shaded dark grey.

Preference test

The protocol for the preference test was based on Lukas et al. (2011 and 2014) who placed the rat into an area for four minutes, and the stimulus (non-social: empty wire-mesh cage; or social: identical cage containing a same-sex Wistar rat) was placed at one wall of the arena. Therefore, four minutes in the testing arena was used in the present study. This decision was also based on Okabe et al. (2020) who tested the preference of rats for a novel object or the hand of the experimenter that was associated with gentle

stroking. The hand and the object were placed in diagonally opposite corners of an arena. Therefore, a modified plus maze was used in the present study, with the hand and a novel object at opposite ends of the maze (Fig. 4).

The preference test occurred the day after the runway test was complete (Fig. 1). The elevated plus maze was again used with one open arm blocked off and the other open arm used as the start arm (Fig. 4a). The two closed arms of the maze were used as the goal arms (each containing the novel object or the hand of the experimenter) and the start box was fixed into place on the open arm being used as the start arm, half a rat's length from the centre of the plus maze. This created a modified T maze (Fig. 4a). The rat was placed into the start box and when the door to the box was lifted, the rat was able to exit the start box and to make a turn either right or left. At the end of one arm was a novel object (a circular plastic object was selected to be of interest to a rat; Fig. 4b, 4c), at the end of the other arm was the right hand of the experimenter wearing a glove. Each rat was given four minutes to explore the arms of the maze. Each trial was recorded using a digital infrared camera (VCC-6594 1/3-Inch IR CCD, SANYO, Japan) suspended above the maze and Media recorder software (Noldus Information Technology, Wageningen, Netherlands). This generated media files that were imported into Ethovision XT 11.5 software (Noldus Information Technology, Wageningen, Netherlands). This allowed the rat to be tracked and the time spent with either the human hand or the object to be calculated. Ultrasonic vocalisations were also recorded using an ultrasonic microphone (Pettersson M500-384 USB Ultrasound microphone, Pettersson Electronik, Sweden) and Audacity software (Version 2.1.3, Pennsylvania, United States of America) was used to record the spectrograms. The microphone was attached to a wall at the centre of the T-maze so as not to obscure any of the maze to allow for video tracking to be used in the behavioural analysis. USVs were detected throughout the maze (which was verifying during the habituation phase as described above). At the end of the four minutes, the rat was returned to the home cage. The maze was cleaned with 70% ethanol gel between each animal. Testing was

carried out in the first half of the dark phase. Each rat was weighed once all the rats had been tested.

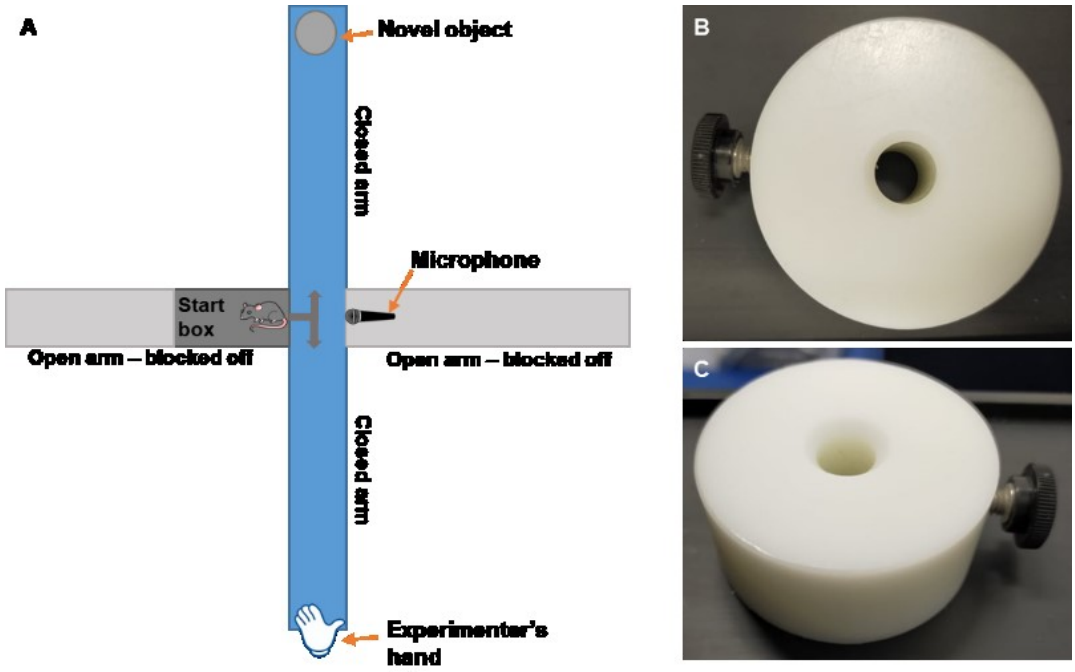

Figure 4. Representation of the apparatus used in the preference test.
Diagram of the modified T-maze used for the preference test (A). Photograph of the item used as a novel object (B, C).
The T-maze was built using the two closed arms of an elevated plus maze. One of the open arms was blocked off using a rectangular piece of Perspex. The start box was placed in the other open arm, rats were placed in it and once the door to the start box was lifted, they could exit the start box and chose to turn right towards the experimenter's hand, or left to the novel object. The rat had four minutes to explore the maze. A microphone was placed in the middle of the T-maze to record USVs emitted while the rat was moving through the maze. A camera was suspended above the maze and used to track the rats as they moved through the maze. Light grey denotes areas which are not accessible to the rat. Blue represents the area that was accessible during testing. The start box is shaded dark grey.

The day after testing had been completed, the rats were euthanized using an intraperitoneal injection of Pentoject (Pentobarbitone Sodium 20% w/v i.e. 200mg/ml) carried out by an experienced member of laboratory personnel. Between the final testing being carried out and being euthanised, the rats were returned in their home cage to the cage rack and remained in the home room. The order in which the rats were euthanised was pseudo-randomised and balanced for treatment group and sex. Following confirmation of death (cessation of heart beat) the rats were decapitated; the brains were

removed from the skull and prepared for fixation. This was carried out by myself and Dr Sarah Brown. Following fixation with paraformaldehyde (as described in Chapter 4) brains were frozen and stored at -80^{o}C for future studies.

All animal work was carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 following ethical approval by the Roslin Institute's Animal Welfare and Ethical Review Body, and carried out in the Roslin Institute's Biological Research Facility.

6.2.3 Measurements

All data were collected and analysed by myself (Emma Tivey).

Ultrasonic vocalisations

To establish changes in calling frequency over days, the total number of 50 kHz USVs and flat and trill 50 kHz USV subtypes, and 22 kHz USVs (see Table 1. for definitions) emitted on day 1, 3 and 5 of the tickle testing were analysed in Ultravox 14 (Noldus Information Technology, Wageningen, Netherlands). The total number of 50 kHz calls, trill 50 kHz USVs, flat 50 kHz USVs and 22 kHz USVs were also counted manually in Ultravox 14 on day 1 and 2 of the runway test (not adjusted for differing lengths of trial), and in the preference test.

Testing arena behavioural analysis

For the runway test, the runtime (the time taken for the rat to move from the start box to the experimenter's hand) and speed (calculated as the number of centimetres moved per second; cm/s) for the rat to traverse the runway during each trial on the training and testing days (days one and two) of the runway test, were automatically calculated using Ethovision XT 11.5 software (Noldus Information Technology, Wageningen, Netherlands). Speed was included to take into account that the trial was completed when the rat was a minimum of one rat's length from the hand so the distance.

For the preference test, the time spent with the novel object and the experimenter's hand (the total time spent within one rat's length of the novel object or hand), and the number of visits made to the novel object and hand (the number of times that the rat approached the novel object or hand to within one rat's length) were calculated using Ethovision XT 11.5 software (Noldus Information Technology, Wageningen, Netherlands).

Table 1. Definition of USV types.

Behaviour	Definition	Reference
50 kHz USV	Broad range calls in the ultrasonic frequency range, from 30kHz up to 90kHz. Anything below 30 kHz was not coded as a 50kHz 'positive' call.	Wohr, 2018.
Flat 50 kHz USV	50 kHz calls with a near-constant frequency greater than 30 kHz. Flat-trill combination calls were not distinguished.	Wright, Gourdon and Clarke, 2010.
Trill 50 kHz USV	50 kHz calls with rapid frequency oscillations greater than 30 kHz. Flat-trill combination calls were not distinguished.	Wright, Gourdon and Clarke, 2010.
22kHz USV	Low level of modulation, long calls, often occurring in bouts of between 2-8 calls in the 18-24kHz frequency range.	Wohr, 2018.

Body weight

Rat body weight (g) was measured every evening (after testing) and an average daily live weight gain calculated using the following formula:

$$\text{Average daily live weight gain (g)} = \frac{\text{(Weight on final day- weight on first day)}}{\text{(Date of cull – Date of start)}}$$

6.2.4 Statistical analysis

Statistical analysis for the behavioural data was carried out in R Studio and R (v 4.0.3, The R Foundation for Statistical Computing Platform (2020)). Model adequacy was verified by examination of residuals (McCullagh and Nelder, 2019) via the DHARMa package (Hartig, 2020). Generalised linear mixed models (GLMMs) in the glmmTMB package (Brooks et al., 2017) were used to analyse the effect of treatment (tickled or non-tickled control), sex and

the interaction of treatment and sex on total 50 kHz USVs, trill 50 kHz USVs, flat 50 kHz USVs and 22 kHz USVs during tickling, the runway test and the preference test. GLMMs (glmmTMB package; Brooks et al., 2017) were used to analyse the effect of treatment (tickled or non-tickled control), sex and the interaction of treatment and sex on the runtime and speed on each trial on day 1 and 2 of the runway test. Likewise, GLMMs in the glmmTMB package (Brooks et al., 2017) were used to analyse the effect of treatment (tickled or non-tickled control), sex and the interaction of treatment and sex on the time spent with the novel object / hand and the number of visits to the novel object / hand in the preference test.

Dependent on model fitting and over dispersion, family links were set to either poisson or negative binomial distributed errors ('nbinom2' in the MASS package; Venables and Ripley, 2002) with default transformations. All models included litter and cage as random effects and were nested (litter followed by cage) to account for the variation from the non-independence of rats from the same cage and potential litter effects. All models included sex and treatment, as well as the interaction between them, with effects reported through ANOVA comparisons (Fox and Weisberg, 2019) to compare the differences between group means rather than the linear relationships between variables. Pairwise comparisons were identified and reported using the emmeans package (Lenth, 2020), with statistical significance based on $p < 0.05$ threshold level adjusted for multiple comparisons using the Tukey method (Lenth, 2020).

Average daily live weight gain was analysed across days in Graphpad Prism (version 9 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com) using a 2-way ANOVA. Treatment, sex and their interaction were included as fixed effects. Statistical significance was set at 0.05.

All data in the tables are presented ± standard error of the estimated marginal mean (SEM) and 95% confidence intervals (CI). The significance level was set at $p < 0.05$. Graphs were generated in GraphPad Prism (version 9 for Windows, GraphPad Software, San Diego, California USA,

www.graphpad.com). The data in the graphs are presented as the means of the raw data ± standard error of the mean (SEM).

6.3 Results

6.3.1 The effect of tickling on USV production

On the first day of tickling, there was no effect of treatment and sex, or their interaction, on total 50 kHz USVs (treatment: p = 0.581; sex: p = 0.606; treatment x sex: p = 0.544), or trill 50 kHz USVs (treatment: p = 0.862; sex: p = 0.304; treatment x sex: p = 0.292; Fig. 5, Table 2). Tickled rats, regardless of sex, made more flat 50 kHz USVs ($X^2_{(1,47)}$ = 5.44, p = 0.0197). In contrast, by day 3 (the middle day of testing) tickled rats produced significantly more total 50 kHz USVs (treatment: p = 0.0102) and trill 50 kHz USVs (treatment: p = 0.0126) than control rats (Table 3). There was no effect of treatment and sex, or their interaction, on flat 50 kHz USVs (treatment: p = 0.090; sex: p = 0.131; treatment x sex: p = 0.997). Similarly, on day 5 (the final day of testing) tickled rats produced significantly more total 50 kHz USVs (treatment: p = 0.002) and trill 50 kHz USVs (treatment: p = 0.003) than control rats (Table 4). There was again no effect of treatment and sex, or their interaction, on flat 50 kHz USVs (treatment: p = 0.182; sex: p = 0.752; treatment x sex: p = 0.957). 22 kHz USVs were low throughout testing on average although there was individual variation within groups (Fig. 2). There was no main effect of treatment or sex on any day (Tables 2 - 4).

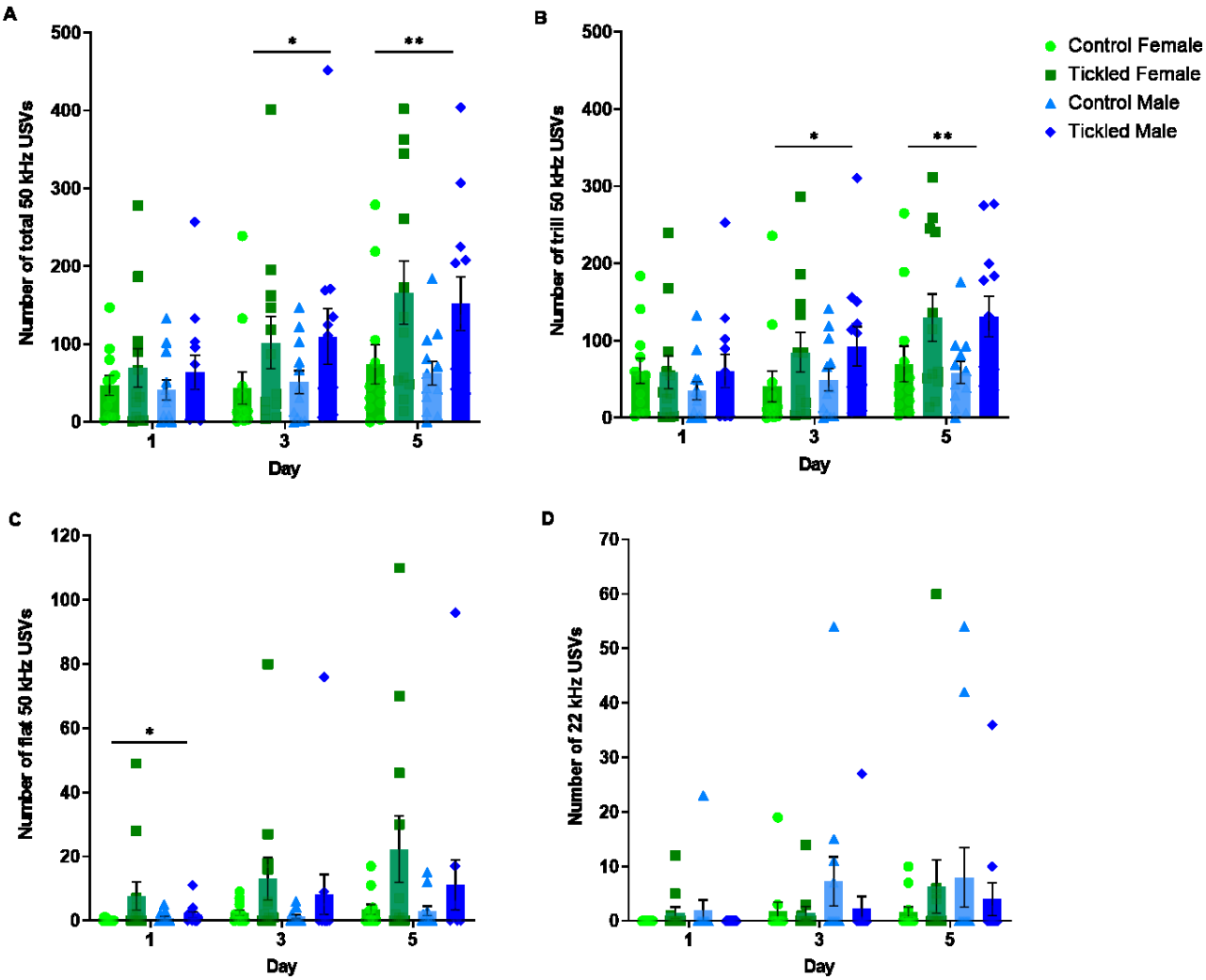

Figure 5. Call profiles of tickled and control rats across five days of testing in the tickling protocol.

Total 50 kHz USVs (A), trill 50 kHz USVs (B), flat 50 kHz USVs (C) and 22 kHz USVs (D) produced over five days of tickling. Control non-tickled females (light green circles), tickled females (dark green squares), control non-tickled males (light blue triangles), tickled males (dark blue diamonds). Axes ranges were modified to allow for better visualization of the data points. Mean ± SEM; n =12 / group. * denotes $p \leq 0.05$; ** denotes $p \leq 0.01$; *** denotes $p \leq 0.001$; **** denotes $p \leq 0.0001$ for significant pairwise interactions.

Table 2. Statistical output of generalised linear mixed model of the number of total, flat and trill 50 kHz USVs, and 22 kHz USVs on day 1 of the tickling procedure. Significant effects ($p < 0.05$) shown in bold.

Day	Behaviour	Fixed Effects	Levels	Estimated marginal mean	±SE	95% CI	Chi squared	P-value
1	Total 50 kHz USVs	Treatment	Control	19.7	6.64	9.96-38.9	0.305	0.581
			Tickled	25.6	8.58	13.06-50.4		
		Sex	Female	25.4	8.48	13.0-49.9	0.266	0.606
			Male	19.9	6.72	10.0-39.3		
		Treatment:Sex	Control-Female	25.7	12.07	9.97-66.3	0.369	0.544
			Tickled-Female	25.1	11.91	9.65-65.4		
			Control-Male	15.1	7.29	5.67-40.0		
			Tickled-Male	26.2	12.33	10.12-67.7		
	Trill 50 kHz USVs	Treatment	Control	19.7	7.00	9.59-40.3	0.03	0.862
			Tickled	21.7	7.64	10.65-44.1		
		Sex	Female	26.8	9.37	13.20-54.2	1.06	0.304
			Male	15.9	5.71	7.73-32.8		
		Treatment:Sex	Control-Female	33.2	16.30	12.31-89.4	1.11	0.292
			Tickled-Female	21.6	10.76	7.89-59.0		
			Control-Male	11.7	5.99	4.13-32.9		
			Tickled-Male	21.8	10.81	7.99-59.3		
	Flat 50 kHz USVs	Treatment	Control	0.118	0.086	0.03-0.51	5.44	**0.0197**
			Tickled	0.911	0.489	2.69- 2.69		
		Sex	Female	0.269	0.182	0.07- 1.05	0.022	0.882
			Male	0.398	0.240	0.12- 1.34		
		Treatment:Sex	Control-Female	0.056	0.06	0.01-0.48	1.829	0.176
			Tickled-Female	1.290	0.93	0.30-5.53		
			Control-Male	0.247	0.214	0.04-1.47		
			Tickled-Male	0.644	0.474	0.14-2.84		
	22 kHz USVs	Treatment	Control	1.13x10-5	0.0008	2.2x10x-11-8.6	0.004	0.951
			Tickled	5.46x10-7	0.001	2.2x10-16-Inf		
		Sex	Female	2.02x10-5	0.001	2.2x16x-11-8.8	0.003	0.957
			Male	3.07x10-7	0.0008	2.2x10-16-Inf		
		Treatment:Sex	Control-Female	1.71x10-7	2.6x10-5	0.0-5.92	<0.0001	0.996
			Tickled-Female	2.37x10-3	7.9x10-3	2.8x10-5-2.0		
			Control-Male	7.52x10-4	2.9x10-7	2.9x10-7-2.0		
			Tickled-Male	1.00x10-10	6.4x10-7	0-Inf		

Table 3. Statistical output of generalised linear mixed model of the number of total, flat and trill 50 kHz USVs, and 22 kHz USVs on day 3 of the tickling procedure.
Significant effects ($p < 0.05$) shown in bold.

Day	Behaviour	Fixed Effects	Levels	Estimated marginal mean	±SE	95% CI	Chi squared	P-value
3	Total 50 kHz USVs	Treatment	Control	17.2	5.42	9.08-32.5	6.597	**0.0102**
			Tickled	53.2	16.32	28.62-98.8		
		Sex	Female	26.8	8.33	14.3-50.2	0.296	0.5861
			Male	34.1	10.61	18.2-63.9		
		Treatment:Sex	Control-Female	14.8	6.56	6.03-36.2	0.019	0.8891
			Tickled-Female	48.6	21.12	20.22-116.8		
			Control-Male	20.0	8.92	8.11-49.2		
			Tickled-Male	58.2	25.21	24.25-139.5		
	Trill 50 kHz USVs	Treatment	Control	15.2	4.94	7.87-29.3	6.229	**0.0126**
			Tickled	46.9	14.76	24.88-88.5		
		Sex	Female	21.8	6.99	11.4-41.6	0.804	0.3699
			Male	32.7	10.41	17.2-62.2		
		Treatment:Sex	Control-Female	11.5	5.32	4.54-29.3	0.098	0.7548
			Tickled-Female	41.1	18.30	16.70-100.9		
			Control-Male	20.0	9.09	7.96-50.1		
			Tickled-Male	53.6	23.80	21.91-131.3		
	Flat 50 kHz USVs	Treatment	Control	0.547	0.302	0.179-1.67	2.871	0.090
			Tickled	1.764	0.882	0.644-4.84		
		Sex	Female	1.669	0.814	0.624-4.47	2.279	0.131
			Male	0.578	0.329	0.183-1.82		
		Treatment:Sex	Control-Female	0.928	0.644	0.229-3.76	<0.0001	0.997
			Tickled-Female	3.002	1.973	0.796-11.31		
			Control-Male	0.322	0.260	0.063-1.65		
			Tickled-Male	1.037	0.749	0.241-4.46		
	22 kHz USVs	Treatment	Control	0.002	0.005	7.4x10-5-0.11	0.119	0.729
			Tickled	0.001	0.002	3.1x10-5-0.05		
		Sex	Female	0.002	0.003	5.4x10-05-0.06	0.019	0.888
			Male	0.002	0.003	4.1x10-05-0.09		
		Treatment:Sex	Control-Female	0.001	0.003	1.8x10-5-0.13	0.411	0.521
			Tickled-Female	0.002	0.004	3.5x10-5-0.16		
			Control-Male	0.005	0.011	7.2x10-5-0.41		
			Tickled-Male	0.0007	0.002	4.7x10-6-0.13		

Table 4. Statistical output of generalised linear mixed model of the number of total, flat and trill 50 kHz USVs, and 22 kHz USVs on day 5 of the tickling procedure.
Significant effects ($p < 0.05$) shown in bold.

Day	Behaviour	Fixed Effects	Levels	Estimated marginal mean	±SE	95% CI	Chi squared	P-value
5	Total 50 kHz USVs	Treatment	Control	36.5	9.39	21.8-61.4	8.966	**0.002**
			Tickled	107.2	27.01	64.6-178.3		
		Sex	Female	61.3	15.6	36.7-103.0	0.013	0.910
			Male	63.9	16.2	38.3-107.0		
		Treatment:Sex	Control-Female	35.2	12.8	16.9-73.5	0.008	0.930
			Tickled-Female	106.8	38.0	52.0-219.1		
			Control-Male	37.9	13.7	18.3-78.7		
			Tickled-Male	107.8	38.4	52.6-221.1		
	Trill 50 kHz USVs	Treatment	Control	37.4	8.32	23.9-58.6	8.406	**0.003**
			Tickled	92.3	20.17	59.4-143.5		
		Sex	Female	58.2	12.8	37.3-90.7	0.005	0.943
			Male	59.4	13.1	38.0-92.7		
		Treatment:Sex	Control-Female	38.8	12.2	20.6-73.0	0.090	0.7636
			Tickled-Female	87.2	27.0	46.7-162.8		
			Control-Male	36.1	11.4	19.1-68.1		
			Tickled-Male	97.8	30.2	52.4-182.3		
	Flat 50 kHz USVs	Treatment	Control	0.967	2.87	0.325-2.87	1.786	0.182
			Tickled	2.545	1.32	0.896-7.23		
		Sex	Female	1.76	0.95	0.593-5.22	0.100	0.752
			Male	1.40	0.731	0.487-4.02		
		Treatment:Sex	Control-Female	1.06	0.80	0.233-4.85	0.003	0.957
			Tickled-Female	2.91	2.15	0.657-12.90		
			Control-Male	0.879	0.66	0.193-4.00		
			Tickled-Male	2.225	1.58	0.531-9.32		
	22 kHz USVs	Treatment	Control	0.005	0.017	9.6x10-6-3.11	0.229	0.632
			Tickled	0.013	0.049	8.1x10-6-22.5		
		Sex	Female	0.013	0.053	3.7x10-6-45.8	0.124	0.725
			Male	0.005	0.016	2.0x10-5-1.59		
		Treatment:Sex	Control-Female	0.006	0.025	2.8x10-6-14.8	0.089	0.765
			Tickled-Female	0.026	0.121	2.4x10-6-294.2		
			Control-Male	0.004	0.013	1.4x10-5-1.58		
			Tickled-Male	0.007	0.022	1.2x10-5-3.9		

6.3.2 Runway test

There was a main effect of trial on days 1 and 2 on all behavioural measures (Tables 5 and 6). Runtimes increased over the five trials on day 1 and 2 of the runway test (Fig. 6 and 7). There was no significant difference in runtime between tickled and control rats on either day 1 ($p = 0.268$) or day 2 ($p = 0.354$). This was similar with speed (day 1: $p = 0.649$; day 2: $p = 0.097$), although female rats were found to run significantly faster than males on day 1 ($p = 0.044$). Total 50 kHz and trill USVs increased over the five trials on day 1 (Fig. 6) and two (Fig. 7), and tickled rats made significantly more of these vocalisations than control rats (Tables 5 and 6). Treatment and sex did not have a significant effect on flat 50 kHz USVs, although there was an increase in flat USVs across trials on both day 1 and day 2 (Fig. 6 and 7, Table 5 and 6).

22 kHz USVs were low (mean < 10) throughout day 1 and 2, although the number of 22 kHz USVs did vary between individuals (Fig. 6 and 7) and there was no difference between trial ($p = 0.228$), sex ($p = 0.104$), or treatment ($p = 0.256$) on day 1. On day 2, there was a main effect of trial on 22 kHz USVs ($p = 0.0145$), in a pairwise comparison there was a small but significant increase between trial 1 to trial 2 ($p = 0.042$) and between trial 1 to 3 ($p = 0.015$). There was no main effect of treatment on 22 kHz USVs ($p = 0.313$) or sex ($p = 0.750$).

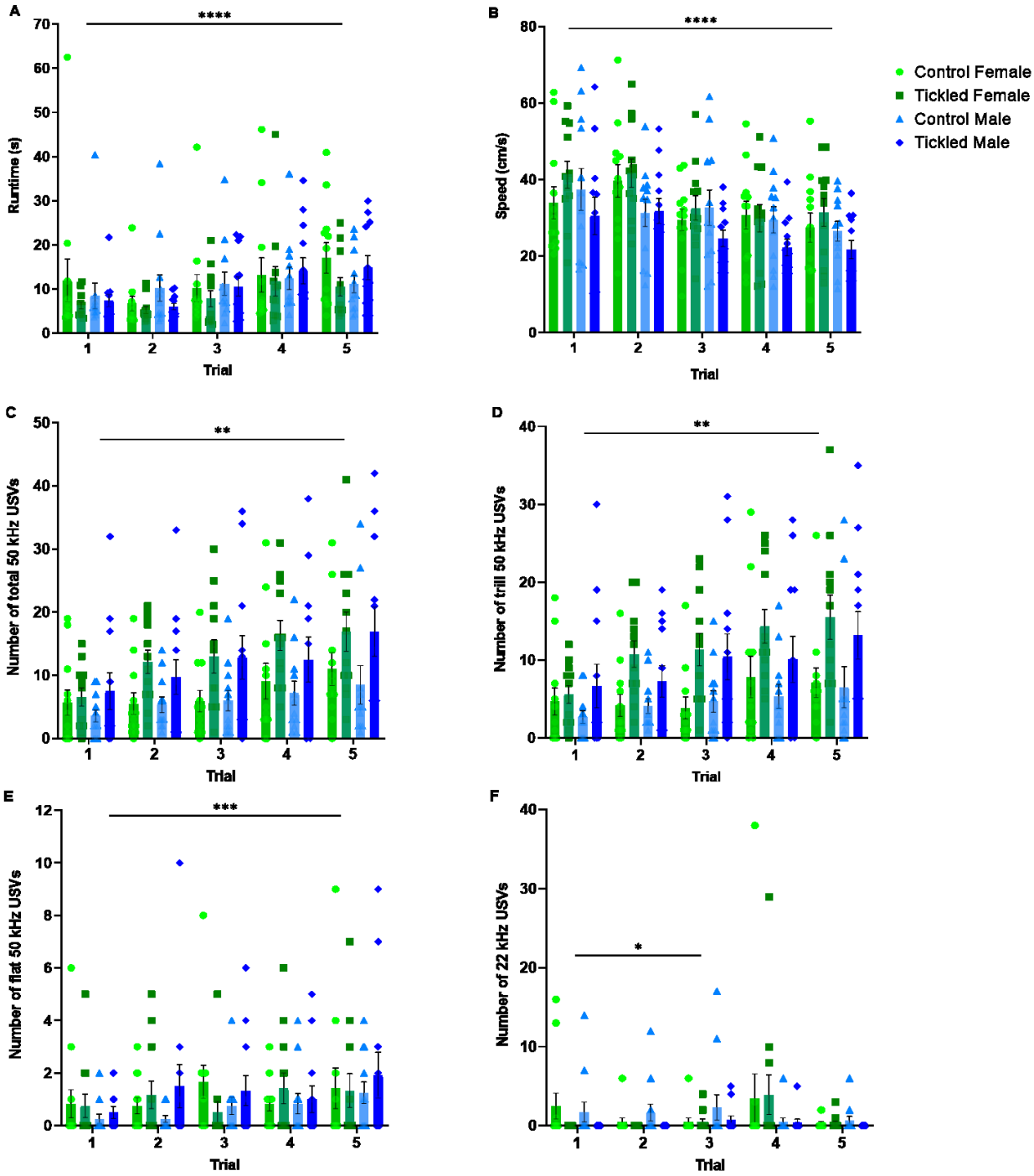

Figure 6. Tickling had no effect on runtime or speed on day one in the runway test. Tickled rats made more 50 kHz and trill 50 kHz USVs than control rats.
Runtime (s) (A), speed (cm/s) (B), total 50 kHz USVs (C), trill 50 kHz USVs (D), flat 50 kHz USVs (E) and 22 kHz USVs (F) produced over five trials on day one of the runway test. Control non-tickled females (light green circles), tickled females (dark green squares), control non-tickled males (light blue triangles), tickled males (dark blue diamonds). Axes ranges were modified to allow for better visualization of the data points. Mean ± SEM; n =12 / group. * denotes $p \leq 0.05$; ** denotes $p \leq 0.01$; *** denotes $p \leq 0.001$; **** denotes $p \leq 0.0001$ for significant pairwise interactions.

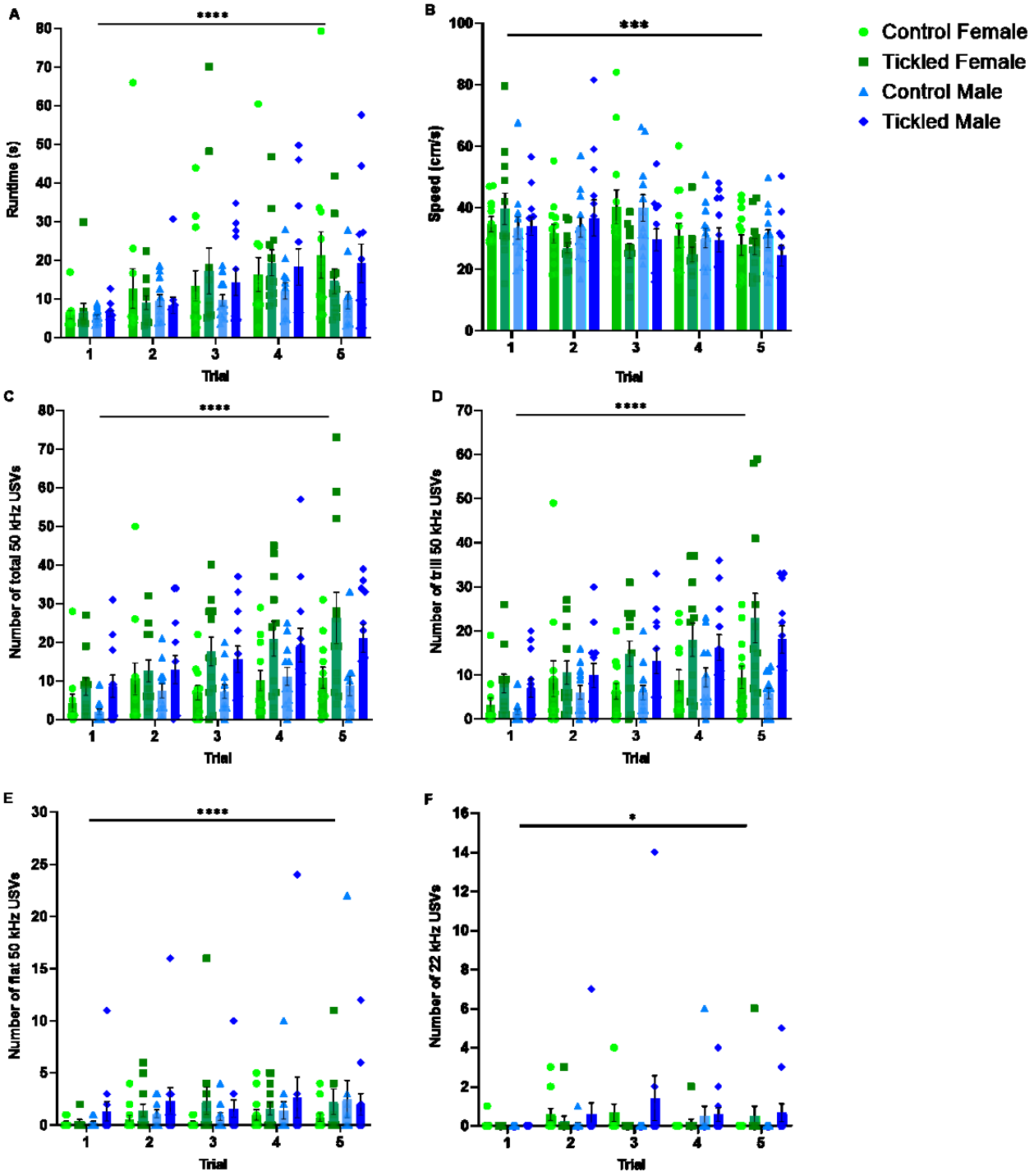

Figure 7. Tickling had no effect on runtime or speed on day two in the runway test. Tickled rats made more 50 kHz and trill 50 kHz USVs than control rats.
Runtime (s) (A), speed (cm/s) (B), total 50 kHz USVs (C), trill 50 kHz USVs (D), flat 50 kHz USVs (E) and 22 kHz USVs (F) produced over five trials on day two of the runway test. Control non-tickled females (light green circles), tickled females (dark green squares), control non-tickled males (light blue triangles), tickled males (dark blue diamonds). Axes ranges were modified to allow for better visualization of the data points. Mean ± SEM; n =12 / group. * denotes $p \leq 0.05$; ** denotes $p \leq 0.01$; *** denotes $p \leq 0.001$; **** denotes $p \leq 0.0001$ for significant pairwise interactions.

Table 5. Statistical output of generalised linear mixed model of the number of total, flat and trill 50 kHz USVs, runtime and speed on day 1 of the runway test. Significant effects ($p < 0.05$) shown in bold.

Day	Behaviour	Fixed Effects	Level	Estimated marginal mean	±SE	95% CI	Chi squared	P-value
1	Total 50 kHz USVs	Treatment	Control	5.08	0.826	3.69-7.0	7.17	**0.0074**
			Tickled	9.34	1.492	6.82-12.8		
		Sex	Female	7.91	1.268	5.76-10.85	1.48	0.223
			Male	6.00	0.972	4.36-8.26		
		Treatment:Sex	Control-Female	5.61	1.29	3.57-8.81	0.11	0.734
			Tickled-Female	11.14	2.50	7.16-17.34		
			Control-Male	4.60	1.06	2.92-7.24		
			Tickled-Male	7.83	1.78	5.00-12.25		
		Trial	1	4.24	0.612	3.19-5.63	57.47	**<0.0001**
			2	6.09	0.848	4.62-8.02		
			3	6.90	0.951	5.26-9.05		
			4	8.32	1.131	6.36-10.87		
			5	10.47	1.41	8.03-13.65		
	Trill 50 kHz USVs	Treatment	Control	3.56	0.614	2.53-5.0	10.12	**0.001**
			Tickled	7.64	1.288	5.48-10.7		
		Sex	Female	6.29	1.064	4.50-8.77	2.45	0.117
			Male	4.32	0.744	3.08-6.07		
		Treatment:Sex	Control-Female	4.03	0.978	2.50-6.50	0.258	0.611
			Tickled-Female	9.79	2.311	6.15-15.59		
			Control-Male	3.14	0.77	1.93-5.09		
			Tickled-Male	5.96	1.4343	3.71-9.57		
		Trial	1	3.39	0.459	2.6-4.43	120.86	**<0.0001**
			2	4.55	0.597	3.5-5.89		
			3	5.24	0.679	4.06-6.79		
			4	6.51	0.83	5.06-8.37		
			5	7.32	0.927	5.7-9.39		
	Flat 50 kHz USVs	Treatment	Control	0.420	0.148	0.21-0.84	0.024	0.876
			Tickled	0.454	0.162	0.23-0.92		
		Sex	Female	0.477	0.168	0.23-0.956	0.134	0.714
			Male	0.400	0.143	0.19-0.81		
		Treatment:Sex	Control-Female	0.526	0.253	0.20-1.36	0.319	0.572
			Tickled-Female	0.433	0.215	0.16-1.15		
			Control-Male	0.335	0.167	0.13-0.89		
			Tickled-Male	0.477	0.235	0.18-1.25		
		Trial	1	0.263	0.083	0.14-0.49	18.86	**0.0008**
			2	0.413	0.121	0.23-0.74		
			3	0.478	0.138	0.27-0.84		
			4	0.460	0.133	0.26-0.81		
			5	0.666	0.185	0.39-1.15		
	Runtime (s)	Treatment	Control	10.33	1.084	8.4-12.7	1.23	0.268
			Tickled	8.76	0.923	7.11-10.8		
		Sex	Female	9.07	0.956	7.37-11.2	0.405	0.525
			Male	9.98	1.046	8.11-12.3		
		Treatment:Sex	Control-Female	10.73	1.59	8.01-14.4	1.312	0.252
			Tickled-Female	7.67	1.15	5.71-10.3		
			Control-Male	9.95	1.48	7.43-13.3		
			Tickled-Male	10.0	1.48	7.47-13.4		
		Trial	1	7.98	0.907	6.38-9.99	36.11	**<0.0001**
			2	6.61	0.765	5.27-8.31		
			3	9.35	1.044	7.51-11.65		
			4	12.15	1.33	7.79-15.07		
			5	12.98	1.411	10.47-16.08		
	Speed (cm/s)	Treatment	Control	31.0	1.97	27.4-35.2	0.206	0.649
			Tickled	29.8	1.90	26.3-33.8		
		Sex	Female	33.3	2.11	29.4-37.7	4.042	**0.044**
			Male	27.8	1.77	24.5-31.5		
		Treatment:Sex	Control-Female	32.0	2.87	26.8-38.1	1.868	0.172
			Tickled-Female	34.7	3.10	29.1-41.3		
			Control-Male	30.2	2.72	25.3-36.0		
			Tickled-Male	25.6	2.31	21.4-30.6		
		Trial	1	35.0	2.21	30.9-39.7	30.59	**<0.0001**

			2	35.5	2.24	31.3-40.2		
			3	29.2	1.87	25.7-33.1		
			4	27.4	1.76	24.1-31.1		
			5	26.2	1.70	23.1-29.8		

Table 6. Statistical output of generalised linear mixed model of the number of total, flat and trill 50 kHz USVs, runtime and speed on day 2 of the runway test. Significant effects ($p < 0.05$) shown in bold.

Day	Behaviour	Fixed Effects	Level	Estimated marginal mean	±SE	95% CI	Z value	P-value
2	Total 50 kHz USVs	Treatment	Control	5.35	0.928	3.80-7.53	11.57	**0.0006**
			Tickled	12.19	2.067	8.73-17.03		
		Sex	Female	8.30	1.42	5.92-11.6	0.05	0.818
			Male	7.86	1.35	5.61-11.0		
		Treatment:Sex	Control-Female	5.47	1.34	3.37-8.86	0.002	0.960
			Tickled-Female	12.61	3.02	7.86-20.22		
			Control-Male	5.24	1.28	3.23-8.48		
			Tickled-Male	11.79	2.82	7.36-18.9		
		Trial	1	3.68	0.565	2.72-4.98	101.48	**<0.0001**
			2	7.59	1.105	5.69-10.11		
			3	8.60	1.242	6.47-11.43		
			4	11.83	1.685	8.94-15.67		
			5	12.11	1.721	9.15-16.02		
	Trill 50 kHz USVs	Treatment	Control	4.43	0.751	3.17-6.18	13.72	**0.0002**
			Tickled	10.61	1.747	7.67-14.67		
		Sex	Female	7.11	1.19	5.12-9.89	0.101	0.751
			Male	6.60	1.10	4.75-9.17		
		Treatment:Sex	Control-Female	4.56	1.09	2.85-7.31	0.003	0.955
			Tickled-Female	11.08	2.58	7.01-17.53		
			Control-Male	4.29	1.03	2.68-6.88		
			Tickled-Male	10.15	2.36	6.42-16.06		
		Trial	1	3.56	0.474	2.74-4.63	230.91	**<0.0001**
			2	6.34	0.800	4.95-8.13		
			3	7.17	0.896	5.61-9.17		
			4	9.33	1.148	7.32-11.89		
			5	9.99	1.226	7.85-12.73		
	Flat 50 kHz USVs	Treatment	Control	0.315	0.112	0.157-0.634	1.689	0.194
			Tickled	0.591	0.196	0.307-1.137		
		Sex	Female	0.366	0.128	0.183-0.73	0.433	0.511
			Male	0.509	0.171	0.263-0.988		
		Treatment:Sex	Control-Female	0.222	0.114	0.08-0.611	0.607	0.436
			Tickled-Female	0.602	0.279	0.241-1.502		
			Control-Male	0.448	0.212	0.176-1.138		
			Tickled-Male	0.580	0.270	0.232-1.453		
		Trial	1	0.189	0.059	0.103-0.349	34.26	**<0.0001**
			2	0.474	0.128	0.278-0.808		
			3	0.437	0.119	0.255-0.749		
			4	0.583	0.155	0.345-0.984		
			5	0.656	0.173	0.390-1.101		
	Runtime (s)	Treatment	Control	10.4	1.03	8.59-12.7	0.861	0.354
			Tickled	11.9	1.17	9.79-14.4		
		Sex	Female	12.1	1.18	9.94-14.6	1.302	0.254
			Male	10.3	1.01	8.46-12.5		
		Treatment:Sex	Control-Female	12.17	1.69	9.26-16.0	1.149	0.284
			Tickled-Female	11.94	1.65	9.09-15.7		

			Control-Male	8.94	1.25	6.78-11.8		
			Tickled-Male	11.82	1.64	8.99-15.5		
		Trial	1	5.91	0.706	4.67-7.47	62.25	**<0.0001**
			2	9.46	1.081	7.56-11.85		
			3	12.84	1.427	10.31-15.98		
			4	15.71	1.726	12.65-19.50		
			5	15.16	1.671	12.2-18.84		
	Speed (cm/s)	Treatment	Control	32.7	1.58	29.8-36.0	2.741	0.097
			Tickled	29.2	1.42	26.6-32.1		
		Sex	Female	30.3	1.47	27.6-33.4	0.303	0.582
			Male	31.5	1.52	28.6-34.6		
		Treatment:Sex	Control-Female	32.2	2.20	28.2-36.8	0.0073	0.932
			Tickled-Female	28.6	1.96	25.0-32.7		
			Control-Male	33.2	2.26	29.1-38.0		
			Tickled-Male	29.9	2.05	26.1-34.2		
		Trial	1	35.3	1.92	31.8-39.3	21.16	**0.0003**
			2	31.7	1.74	28.4-35.3		
			3	33.1	1.81	29.7-36.8		
			4	38.3	1.57	25.3-31.6		
			5	27.0	1.51	24.2-30.1		

6.3.3 Preference Test

Treatment and the interaction between treatment and sex had no significant effect on the emission of any USV type (Table 7). There was a main effect of sex on the number of total 50 kHz USVs ($p = 0.007$) and trill 50 kHz USVs ($p = 0.008$) produced during the preference test with female rats calling significantly more than males (Fig. 8, Table 7).

The time spent with the novel object did not differ between tickled or control rats (treatment: $p = 0.810$; sex: $p = 0.792$; treatment x sex: $p = 0.065$; Fig. 9, Table 7). However, control rats, irrespective of sex, made more visits to the novel object compared to tickled rats ($p = 0.0231$; Table 7). In contrast, treatment had no main effect on the time spent with the hand ($p = 0.813$), but male rats spent more time with the hand than female rats ($p = 0.048$). There was no difference between groups in the number of visits made to the hand (treatment: $p = 0.897$; sex: $p = 0.137$; treatment x sex: $p = 0.692$).

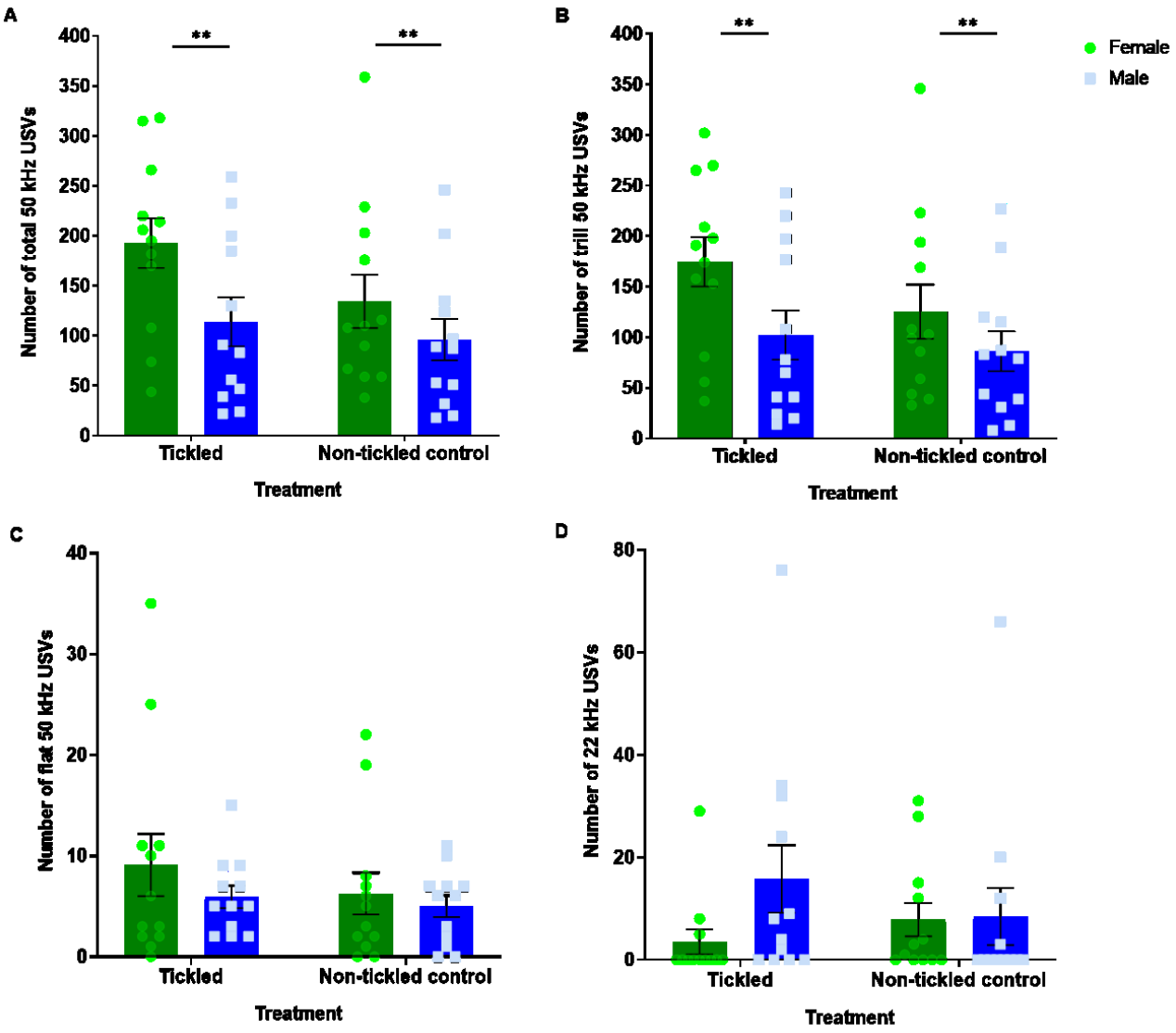

Figure 8. Female rats, regardless of treatment, produced more 50 kHz and trill 50 kHz USVs during the preference test than males.
Total 50 kHz USVs (A), trill 50 kHz USVs (B), flat 50 kHz USVs (C) and 22 kHz USVs (D) produced during social preference test. Females (light green circles), males (light blue triangles). Axes ranges were modified to allow for better visualization of the data points. Mean ± SEM; n =12 / group. * denotes $p \leq 0.05$; ** denotes $p \leq 0.01$; *** denotes $p \leq 0.001$; **** denotes $p \leq 0.0001$ for significant pairwise interactions.

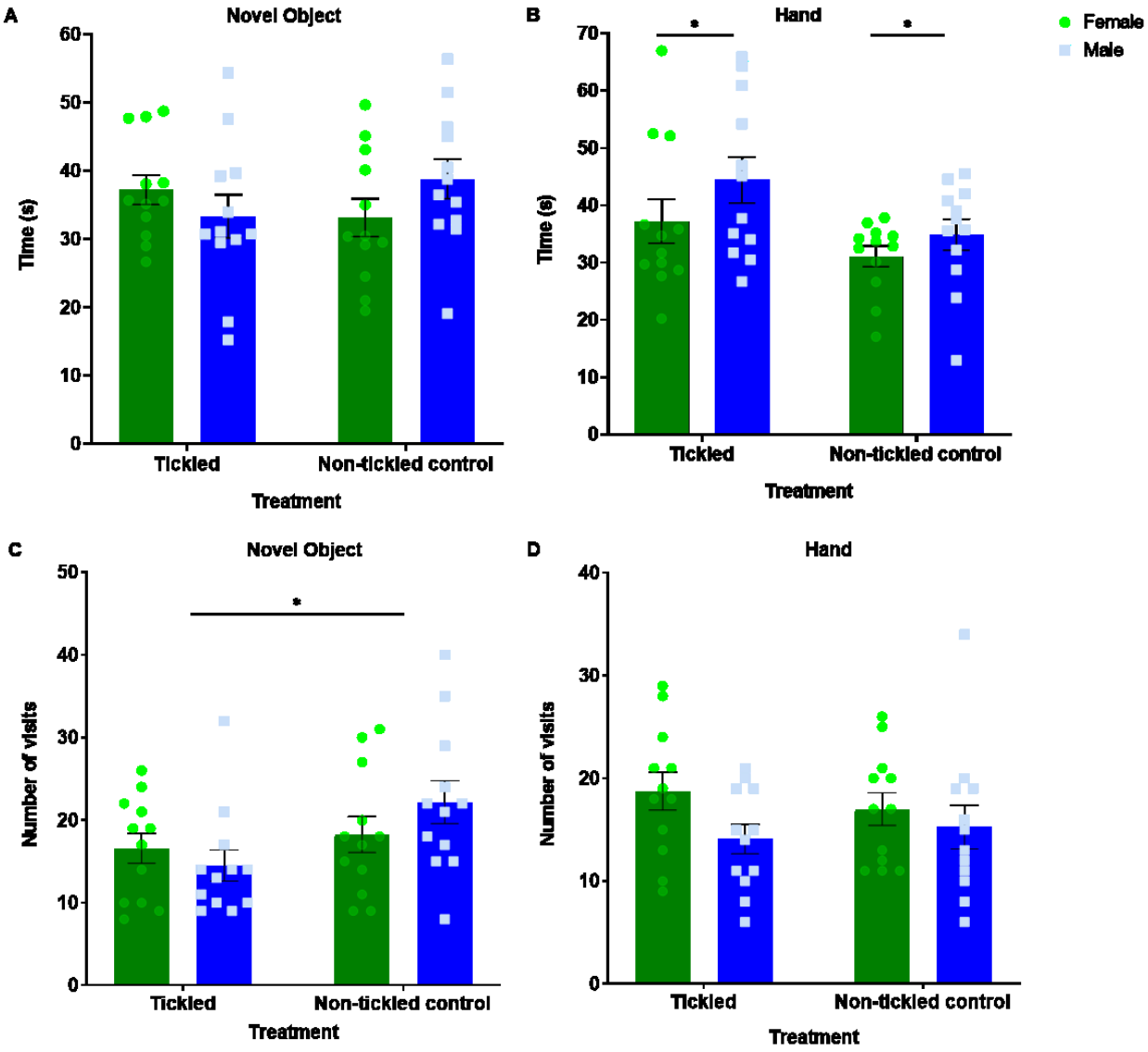

Figure 9. Tickling did not affect preference for the hand in female and male rats. Time spent with the Novel Object (A), Time spent with the Hand (B), Number of visits to the Novel Object (C) and Number of visits to the Hand (D) produced during social preference test. Females (light green circles), males (light blue triangles). Axes ranges were modified to allow for better visualization of the data points. Mean ± SEM. n =12 / group. * denotes $p \leq 0.05$; ** denotes $p \leq 0.01$; *** denotes $p \leq 0.001$; **** denotes $p \leq 0.0001$ for significant pairwise interactions.

Table 7. Statistical output of generalised linear mixed model of the number of total, flat and trill 50 kHz USVs, and 22kHz USVs, time spent with the novel object and hand, and number of visits to the novel object and hand in the social preference test. Significant effects ($p < 0.05$) shown in bold.

Behaviour	Fixed Effects	Levels	Estimated marginal mean	±SE	95% CI	Chi squared	P-value
Total 50 kHz USVs	Treatment	Control	89.8	13.1	66.9-121.0	1.979	0.159
		Tickled	119.9	17.4	89.4-161.0		
	Sex	Female	136.9	19.8	102.2-183.0	7.243	**0.007**
		Male	78.7	11.5	58.6-106.0		
	Treatment:Sex	Control-Female	110.5	22.7	73.0-167.0	0.458	0.498
		Tickled-Female	169.5	34.7	112.2-56.0		
		Control-Male	73.0	15.1	48.1-111.0		
		Tickled-Male	84.8	17.5	55.9-129.0		
Trill 50 kHz USVs	Treatment	Control	77.4	12.9	55.2-108.0	1.415	0.234
		Tickled	102.3	17.0	73.1-143.0		
	Sex	Female	121.2	20.1	86.8-169.4	6.917	**0.008**
		Male	65.3	10.9	46.5-91.5		
	Treatment:Sex	Control-Female	98.4	23.1	61.2-158.1	0.346	0.556
		Tickled-Female	149.4	35.0	93.1-239.6		
		Control-Male	60.8	14.4	37.7-98.2		
		Tickled-Male	70.0	16.6	43.4-112.8		
Flat 50 kHz USVs	Treatment	Control	3.83	1.00	2.26-6.49	0.607	0.436
		Tickled	5.07	1.28	3.04-8.45		
	Sex	Female	4.53	1.18	2.68-7.67	0.025	0.874
		Male	4.28	1.09	2.56-7.16		
	Treatment:Sex	Control-Female	3.90	1.44	1.85-8.20	0.004	0.951
		Tickled-Female	5.27	1.90	2.55-10.92		
		Control-Male	3.77	1.37	1.81-7.85		
		Tickled-Male	4.87	1.72	2.39-9.95		
22 kHz USVs	Treatment	Control	0.523	0.479	0.08-3.32	0.026	0.872
		Tickled	0.602	0.541	0.09-3.70		
	Sex	Female	0.376	0.352	0.057-2.49	0.469	0.493
		Male	0.837	0.735	0.142-4.93		
	Treatment:Sex	Control-Female	1.009	1.147	0.102-10.0	3.228	0.072
		Tickled-Female	0.140	0.189	0.009-2.12		
		Control-Male	0.272	0.353	0.019-3.74		
		Tickled-Male	2.579	2.768	0.296-22.50		
Time spent with the Novel Object (s)	Treatment	Control	35.1	1.89	31.5-39.2	0.058	0.810
		Tickled	34.5	1.87	31.0-38.5		
	Sex	Female	34.5	1.86	30.9-38.5	0.069	0.792
		Male	35.2	1.90	31.5-39.2		
	Treatment:Sex	Control-Female	32.4	2.51	27.8-37.9	3.414	0.065
		Tickled-Female	36.7	2.76	31.5-42.7		
		Control-Male	38.1	2.85	32.7-44.3		
		Tickled-Male	32.5	2.52	27.8-38.0		
Number of visits to the Novel Object	Treatment	Control	19.2	1.51	16.3-22.5	5.159	**0.0231**
		Tickled	14.8	1.22	12.5-17.5		
	Sex	Female	16.6	1.34	14.1-19.6	0.097	0.755
		Male	17.1	1.38	14.5-20.1		
	Treatment:Sex	Control-Female	17.4	1.96	13.8-21.8	2.172	0.141
		Tickled-Female	15.9	1.82	12.6-20.0		
		Control-Male	21.1	2.30	16.9-26.3		
		Tickled-Male	13.8	1.63	10.9-17.5		
Time spent with the Hand (s)	Treatment	Control	32.0	2.38	27.5-37.2	1.770	0.813
		Tickled	36.7	2.71	31.6-42.6		
	Sex	Female	30.9	2.32	26.6-36.0	3.912	**0.048**
		Male	38.0	2.77	32.8-44.0		
	Treatment:Sex	Control-Female	30.4	3.21	24.5-37.6	0.927	0.335
		Tickled-Female	31.5	3.35	25.4-39.0		
		Control-Male	33.7	3.52	27.3-41.6		
		Tickled-Male	42.7	4.36	34.8-52.5		
	Treatment	Control	15.4	1.28	13.0-18.2	0.017	0.897
		Tickled	15.1	1.27	12.7-17.9		

Number of visits to the Hand	Sex	Female	16.6	1.37	14.1-19.6	2.205	0.137
		Male	14.0	1.19	11.8-16.6		
	Treatment:Sex	Control-Female	16.4	1.90	13.0-20.7	0.157	0.692
		Tickled-Female	16.9	1.96	13.3-21.3		
		Control-Male	14.4	1.72	11.3-18.3		
		Tickled-Male	13.5	1.63	10.6-17.3		

6.3.4 Average daily live body weight gain

There was no significant interaction between treatment and sex ($F_{1, 44}$ = 3.09e-29, p > 0.9999) on daily live weight gain (Fig. 10). There was no main effect of treatment ($F_{1, 44}$ = 0.013, p = 0.9096). There was a significant difference between sexes ($F_{1, 44}$ = 65.59, p < 0.0001), with male rats having a higher daily live body weight gain than females.

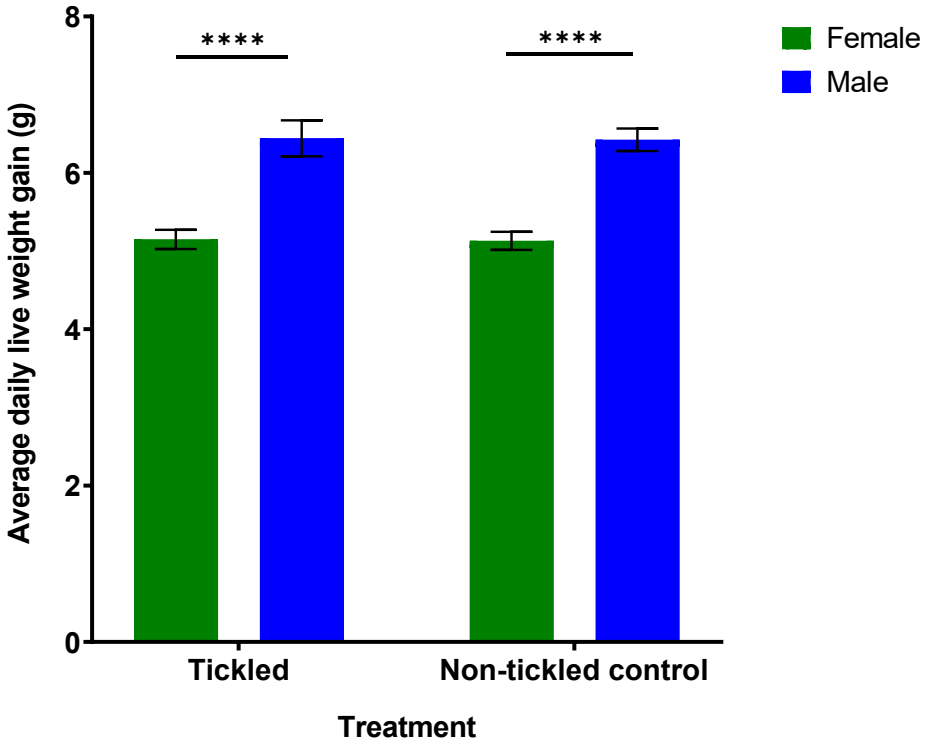

Figure 10. Average daily live body weight (g) gain taken across the three-week whole testing phase. Females (green), males (blue). Mean ± SEM. n =12 / group. **** denotes p ≤ 0.0001 for significant pairwise interactions.

6.4 Discussion

The present study aimed to investigate whether female rats were more motivated to be tickled than male rats, and if they showed higher levels of motivation whether this would correlate with increased 50 kHz USVs during tickling. I also investigated whether tickled rats have a preference for tickling over a novel object in a preference test. Tickled rats made more 50 kHz USVs in total and more of the trill USV sub-type than control rats on days 3 and 5 of the tickling protocol, and this was not different between sexes. While there was no main effect of treatment or sex on the number of 22 kHz USVs produced during tickling, there were individual differences, suggesting there was individual variation in the response to tickling. In the runway test, tickling did not affect the time taken to traverse the runway, while tickled rats made more 50 kHz and trill 50 kHz USVs than controls when traversing the runway. In contrast, in the preference test, there was no difference in 50 kHz, trill 50 kHz or flat 50 kHz between tickled and control animals, but there was a sex difference, with female rats emitting more 50 kHz and trill 50 kHz USVs than males. Tickled and control rats spent a similar amount of time with the novel object and the hand, and made a similar number of visits to the hand. However, control rats made more visits to the novel object compared to tickled rats.

Tickling

Tickling was successful in evoking 50 kHz USVs, including the trill subtype, in both female and male rats. This is consistent with previously published studies and in Chapters 2 and 3 that have shown tickling to induce positive affective states in young rats as reflected by emissions of 50 kHz USVs (e.g. Panksepp and Burgdorf, 2000; Burgdorf and Panksep, 2001; Cloutier et al., 2018; Hinchcliffe et al., 2020). Tickling resulted in significantly more trill and total 50 kHz USVs than control rats on days 3 and 5 of tickling, which is consistent with the study by Lafollette et al. (2018) where three days of tickling was sufficient to cause a significant increase in 50 kHz USV production compared to one day of tickling.

While there was an effect of tickling, irrespective of sex, on flat 50 kHz USVs on day 1 of the tickling procedure, on days 3 and 5, there was no effect of tickling or sex on the number of flat 50 kHz USVs emitted. This is in contrast to my results in Chapter 2, where I found that after 10 days of testing, treatment and sex had main effects on the number of flat 50 kHz USVs made. Most tickling studies focus on total 50 kHz USVs (e.g. Cloutier et al., 2017; LaFollette et al., 2018; Hammond et al., 2019), or trill 50 kHz USVs (e.g. Panksepp and Burgdorf, 200; Burgdorf et al., 2008) as indicators of positive affect induced by tickling. Thus, there is little evidence for the role of flat 50 kHz USVs in tickling. Burgdorf et al. (2008) showed that flat 50 kHz USVs were associated more with aggression than conspecific play or tickling in female and male Long-Evans rats. Whereas, in Chapter 3, I showed that flat 50 kHz USVs were paired with specific tickle-induced behaviours, hopping, darting and hand approaches, but they were also paired with exploration in tickled rats and running in control rats. Other studies that have investigated the role of flat 50 kHz USVs suggest they have a social communicatory role (Wöhr et al., 2008), and flat 50 kHz USVs are produced during rough and tumble play (Burke et al., 2021, 2018) and mating by female (Thomas and Barfield, 1985; Burgdorf et al., 2008) and male rats (White et al., 1990). Flat 50 kHz USVs may be used as cues of dominance and submission between playing male rats (Burke et al., 2018) and this communication may be used to avoid escalation of play fighting to aggression (Burke et al., 2017b). Therefore, while the relationship between flat 50 kHz USVs and tickling is not yet clear, these calls may be used by rats to communicate complex signals during different social interactions.

The majority of tickling studies have used males rats (LaFollette et al., 2017), and this study was intended to directly compare the response of female and male juvenile Wistar rats to tickling, adding to the relatively limited literature where both sexes have been used (e.g. Panksepp and Burgdorf, 2000; Burgdorf and Panksep, 2001; Mällo et al., 2007, 2009; Chapters 2 and 3). In the present study, there were no sex differences in 50 kHz USV call rates on any of the days studied. This result argues against the trend for tickled females to produce the most 50 kHz USVs after 10 days of tickling in Chapter

2. Rats were tickled for five days in the present study based on LaFollette et al. (2018) who showed that three and five days of tickling were highly effective in producing higher rates of 50 kHz USVs, more play behaviours and less inactivity in the home cage compared to one day of tickling. In Chapter 2, there was no sex difference in total 50 kHz USVs by day 5, which is consistent with the present study, and a sex difference in total 50 kHz USVs and flat 50 kHz USVs was only found by day 10 (Chapter 2, Fig. 1). Mällo et al. (2007) report that stabilisation in the number of 50 kHz USVs produced by both female and male Wistar rats occurred after the first week of tickling, although in that study they did not directly compare call rates between sexes. Therefore, together with the present findings, this may suggest that the length of time rats are tickled for may alter their call rates, and this may differ between sexes. A longitudinal study spanning > 2 weeks (to build on the findings in Chapter 2 where rats were tickled for 10 days) to compare the change in USVs in response to tickling over time between female and male rats, similar to Mällo et al. (2007), would be needed to verify this.

Numbers of 22 kHz USVs were low throughout the whole tickling test period, which shows that tickling was not overly aversive. This is consistent with the findings of other tickling studies in which the number of 22 kHz USVs remained low throughout testing (e.g. Panskepp and Burgdorf, 2003; Burgdorf et al., 2008; Cloutier et al., 2012; LaFollette et al., 2018) and my own results in Chapter 2. However, there was individual variation, with a small number of rats emitting 22 kHz USVs at much higher rates than other individuals (Fig. 5d). There appears to be individual variation in the response to tickling, with some rats finding tickling less rewarding than others (Lafollette et al., 2017; Hinchcliffe et al., 2020; Bombail et al., 2021). Therefore, individual preference for tickling may account for the small number of rats producing 22 kHz USVs in the present study.

Runway Test

In the runway test, rats took longer to traverse the runway over the five trials on both day 1 and 2 and there was no difference between sexes. The

speed at which the rats traversed the runway also decreased over the trials. Runtimes are inversely proportional to motivation for the reward at the end of the runway (Ettenberg, 2008). This suggests that both tickled and control rats were decreasing in motivation to reach hand of the experimenter across the trials. The runway test has been used to quantify the motivation of rats to seek out other rewarding stimuli, such as reinforcing drugs in male rats (Crespi, 1942; White et al., 1977; Crespo et al., 2008; Wakonigg et al., 2003; Geist and Ettenberg, 1990, 1997; Zernig et al., 2002), a sexual partner in male Long-Evans rats (Lopez and Ettenberg, 2001), palatable foods in male Sprague-Dawley rats (Shin et al., 2010), and a dopamine uptake inhibitor in male Wistar rats (Esumi et al., 2013). Rats in this current study were not more motivated to receive 15 seconds of tickling at the end of the runway compared control rats who traversed the runway and received no tickling. However, trill 50 kHz USVs were found to increase on both days over the five trials, and tickled rats, regardless of sex, produced significantly more appetitive 50 kHz USVs than control rats. This mirrors the increase in these USVs over the five days of the tickling protocol. This may suggest that even though tickled rats were not more motivated than controls to receive the short bout of tickling when they reached the hand, this tickling was perceived as rewarding as reflected by the increasing trill vocalisations. This is similar to the findings of Hinchcliffe et al. (2020), where male Lister Hooded rats increased their production of 50 kHz USVs in response to tickling, which was associated with positive affect in an affective bias test, but was not associated with approach latency to the experimenter's hand. There is conflicting evidence that tickling induces approach behaviours; some studies report that tickled female and male rats show more approach behaviours to the tickling hand, take less time to approach the hand and interact more with the hand than non-tickled control rats (Cloutier et al., 2012; LaFollette et al., 2018; Hammond et al., 2019; Burgdorf and Panksepp, 2001). Whereas other studies show that approach behaviours are not different between tickled and control rats (Cloutier and Newberry, 2008), or even that tickled males took longer to approach the hand than control rats (LaFollette et al., 2018; Hammond et al., 2019). Arguably, the

latency to approach the hand and the runway test used in the present study are both measuring the time taken to reach the hand that is associated with tickling and represent the motivation to be tickled (Burgdorf and Panksepp, 2001). Together, this may suggest that approaches towards the hand (in a hand approach test or similarly in a runway test) are not associated with the reward of tickling or are not sensitive enough measures to detect the reward of tickling.

Preference Test

In the preference test, female rats, irrespective of treatment, made more 50 kHz USVs, and in particular trill 50 kHz USVs, compared to male rats. As the rats were not touched or tickled during the preference test and there was no effect of treatment on the number of 50 kHz, trill or flat 50 kHz vocalisations, this result shows that female rats calling during the preference test is unrelated to their tickling experience. As trill USVs are linked to the rewarding nature of stimuli (Panksepp and Burgdorf, 2000; Burgdorf and Panksep, 2001; Brudzynski, 2013), this may indicate that female rats are finding spending time in the testing arena rewarding. This is similar to the finding in Chapter 2, that female rats were producing more 50 kHz USVs that males, within the same treatment group. There is some evidence that female rats respond differently to reward than males: female Sprague-Dawley rats showed better learning of a cue-reward association between an audio cue and a sucrose reward, and were less affected by tests to reduce the motivation for the sucrose reward than males (Hammerslag and Gulley, 2013). Female Fischer rats were found to develop a condition place preference for cocaine twice as quickly compared to males, and at a lower dose to males (Russo et al., 2003). Similarly, female Sprague-Dawley rats showed a more robust conditioned place preference for a palatable food reward compared to males, and this was linked to increased Fos expression in the nucleus accumbens and infralimbic medial prefrontal cortex of the reward pathway in female compared to male rats (Sinclair et al., 2017). Therefore, it is plausible that juvenile female rats may find certain stimuli more rewarding than male rats, which may include entering a different environment to their home cage. Although this cannot be verified in the present

study, this could be important for future considerations for promoting positive affective states in rats. It also highlights the importance to include both sexes in future studies.

The time spent with the novel object and the hand of the experimenter did not differ between treatment groups. Control rats made more visits to the novel object over the hand than tickled rats. This indicates that, as predicted, control rats had a preference for the novel object over the hand. This is likely due to the natural propensity for novelty displayed by young rats (Stansfield et al., 2006; Berlyne, 1950), such that they prefer to visit a novel object over the hand that they have become familiar with over the course of the tickling protocol (the control rats received no tickling). The results also indicate that tickled rats did not have a preference for either the tickling hand or the novel object. This is in contrast to Okabe et al. (2020) who report that five minutes of gentle stroking, as opposed to tickling, resulted in male Lewis rats spending more time with the hand over a novel object. The authors used a tactile stimulus that has been shown to evoke 50 kHz USVs in male rats (Okabe et al., 2015), although not as effectively as tickling (Burgdorf and Panksepp, 2001). Tickling differs from light stroking in its components, with tickling mimicking some aspects of social rough and tumble play, whereas gentle stroking aims to simulate social grooming (Burgdorf and Panksepp, 2001), and stroking has been used as a control for tickling (e.g. Cloutier and Newberry, 2008). These differences between tickling and stroking may account for the different outcomes between the present study and Okabe et al. (2020). Also, Okabe et al. (2020) used male rats in their study, and I found that males spent more time with the hand than females, so their findings may be specific to male, not female, rats. Finally, Okabe et al. (2020) tested the rats in the preference test for 10 minutes, as opposed to five minutes in the current study, which may also help to explain the differences in results of the present study. As with other studies (e.g. Lukas and Neumann, 2014; Kent et al., 2013; Lukas et al., 2011), Okabe et al. (2015) found that oxytocin facilitates social preferences in male and female rodents. My previous findings (Chapter 5) showed that fewer oxytocin neurons in the paraventricular nucleus of the

hypothalamus are active in tickled female and male rats suggesting that tickling is not perceived as a form of social interaction and may help to explain why social preference for the hand was not formed in the present study. While tickling is found to induce place preferences (Burgdorf and Panksepp, 2001; Paredes-Ramos et al., 2010), Parades-Ramos et al. (2021) found that tickling did not induce a partner preference for a rat that bore an odour associated with tickling suggesting that preferences for tickling are complex and may differ between individuals (Hinchcliffe et al., 2020).

Conclusions

Together, these results suggest that both female and male rats produce 50 kHz USVs in response to tickling, and the prevalence of trill USVs in tickled rats suggests that tickling was rewarding. Unlike previous findings in Chapter 2, there was no sex difference in the numbers of vocalisations produced in response to tickling. This may suggest that female rats were not producing more 50 kHz USVs in response to tickling as was suggested by the post-hoc analysis in Chapter 2 (Fig. 2). Tickling did not affect the time taken for the rat to traverse the runway to reach the hand, where they received a short bout of tickling. Runtime is described as being inversely proportional to the motivation to reach the reinforcing stimulus, indicating that being tickled at the end of the maze in one trial did not increase the motivation to be tickled in the ensuing trial. In contrast, the number of reward-associated trill 50 kHz USVs did increase across trials, which may suggest that the rewarding nature of tickling was increasing, based on the findings of Hinchcliffe et al. (2020) that 50 kHz USVs are a graded measure of positive affect induced by tickling. Measures of approaching the hand of the experimenter, either in a runway paradigm or other versions of approach test, may therefore not reliably represent the rewarding nature of tickling. Finally, the current study adds to the working hypothesis that rats do not perceive tickling as a social interaction as no preference for the hand of the experimenter was formed in tickled rats of either sex in a modified preference test. This is supported by the finding that control rats formed a preference for the novel object, making more visits to the novel

object than tickled rats. This finding is important as it may affect how tickle studies are interpreted in the future.

Chapter 7

Discussion

7.1 Overall discussion

In this thesis, I set out to establish whether there are sex differences in the behavioural phenotype of tickling in juvenile Wistar rats. I measured the effect of tickling and sex on solitary play behaviours, hand approach behaviours and subtypes of 50 kHz USVs. In particular, I was interested as to whether females and males synchronised 50 kHz USV subtypes with other play behaviours during tickling. I also set out to investigate the neural correlates of the behavioural phenotype of tickling, in particular if there was any involvement of the neuroendocrine system. I was interested in brain regions that have been shown to regulate social conspecific play as tickling was originally developed by mimicking aspects of social play (Panksepp and Burdorf, 2000). I analysed changes in neuronal activity by quantifying Fos immunoreactivity in multiple regions of the social behaviour network and in oxytocin and vasopressin neurons of the PVN and SON.

Within the tickling literature, there is large variation in tickling methods. For example, the number of days of tickling ranges between one to twenty eight days (with an average of 9.4 days), and duration of tickling bouts ranges between thirty seconds to ten minutes (with an average of 3.3 minutes; Lafollette et al., 2017). Methods of tickling also differ between studies, with some studies using structured, repeated pinning (e.g. Burgdorf and Panksepp, 2001; Cloutier et al., 2018; Lafollette et al., 2018) whereas other researchers pin if the rat exposes its ventral side (e.g. Bombail et al., 2019; Hammond et al., 2019) which may adjust the tickling procedure to the response of the individual rat (Bombail et al., 2021). While methods vary, 50 kHz USVs were found to increase as a result of tickling in 94% of studies (LaFollette et al., 2017). This suggests that different methods still induce a positive affective state in these rats. However, Hinchcliffe et al. (2020) recently showed that the

number of 50 kHz USVs produced may present a graded measure of positive affect induced by tickling, which may indicate individual preference for tickling. It is therefore possible that if there are individual preferences to being tickled, there may also be individual preferences to the type of tickling method, as argued by Bombail et al. (2021).

An important consideration for variation in preference for tickling, and the first main research question of this thesis, was 'do rats of different sexes respond to tickling in the same way'. Many studies have not included female rats, for example, Lafollette et al. (2017) cited eight studies out of thirty two studies which used both sexes. Since this paper was published, other tickling publications have only used male rats (e.g. Bombail et al., 2019; Hammond et al., 2019; Hinchcliffe et al., 2021). Studies using both sexes have shown mixed results on whether sex differences in the response to tickling exist (e.g. Burgdorf and Panksepp, 1999; Mällo et al. 2007; Schwarting et al., 2018). My own data have showed inconsistent sex differences. After 10 days of tickling, I found that, although there was no interaction of treatment and sex, a post-hoc analysis revealed that tickled females produced more 50 kHz USVs than tickled males and control rats. In contrast, after five days of tickling in both experiments, there was no difference in 50 kHz USVs between female and male tickled rats. Together, this suggests that female rats do not produce more 50 kHz USVs than males in response to tickling. However, this differs from Lafollette et al. (2018) who found that female rats emitted more 50 kHz USVs in response to tickling than male rats after three days of tickling. One interpretation of these conflicting results could be that, given the papers that have cited sex differences in the phenotype of tickling, there may be effects of experimental design and method. For example, Lafollette et al. (2018), Panksepp and Burgdorf (2000), Burgdorf and Panksep (2001), and Mällo et al. (2007 and 2009) all used structured pinning in their tickling method, while I made tickling movements on the nape of the rat's neck, and if the rat exposed its ventral side by rolling over or crawling onto my hand, I made the same tickling movements on the stomach without pinning the rat. Other studies have not always analysed USV subtypes, quantifying total 50 kHz USVs (e.g.

LaFollette et al., 2018; Hammond et al., 2019), or focussing on trill USVs (e.g. Panksepp and Burgdorf, 2000; Mällo et al., 2007; Panksepp and Burgdorf, 2003; Cloutier et al., 2018). I found sex differences in flat, but not trill, 50 kHz USVs, suggesting that females may use subtypes differently to males during tickling. Further to this, female rats paired flat 50 kHz USVs with hops, darts and approaches to the hand, while both females and males that had been tickled paired trill 50 kHz USVs with hops, darts and approaches to the hand, suggesting there is a sex difference in how specific USV subtypes are associated with play-related behaviours. This highlights the impact that nuances in tickling method and experimental design, for example sex, number of days of tickling, and the behavioural measures taken, have on the behavioural output of tickling and this is an important consideration for future tickling studies. This is also important in terms of tickling being recommended as a welfare intervention for rats kept for scientific experimentation (NC3Rs, 2017): not all tickling methods may have the same effect on all rats, possibly due to factors such as sex, individual variability or prior experience of tickling, and therefore where possible tickling should be adapted based on the responses of the rat to the tickling stimulus (Bombail et al., 2021).

My second research question was whether there was any neuroendocrine regulation of tickling, on the premise that oxytocin and vasopressin are both implicated in social conspecific play, and there is continuing interest in potential sex differences as these neuropeptides have well established sexually dimorphic roles in a number of behaviours, including social play in rats. The neural analysis grew from an initial interest in the hypothalamus, to investigating changes in neuronal activity in several regions that regulate social behaviours (i.e. the social behaviour network; Newman, 1999). I predicted that tickling was a rewarding, social interaction for rats, and so would lead to increased neuronal activity in these regions of interest. Surprisingly, in multiple brain regions there was no change in neuronal activity as a result of tickling. For example, in the magnocellular oxytocin and vasopressin neurons of the PVN, tickling did not affect Fos-ir. However I found a decrease in the double labelled cells for Fos-ir in oxytocin and vasopressin

neurons in the parvocellular division of the PVN. When I correlated Fos-ir between structurally related regions, tickled rats had fewer significant correlations than control rats. An equally surprising result was that there were very few sex differences found. Fos expression is widely and reliably used to study changes in neuronal activity during certain behaviours, for example, nest building in zebra finches (Hall et al., 2014; Edwards et al., 2020), social conspecific play in rats (e.g. van Kerkhof et al., 2014; Reppucci et al., 2018), sexual behaviours in rats (e.g. Robertson et al., 1991; Veening and Coolen, 1998), maternal behaviours in rats (e.g. Lonstein et al., 1997) and gentle stroking in rats (e.g. Okabe et al., 2015). As I have discussed in other chapters, one possible explanation for the surprising results in the present study may be that tickling is not perceived as a social interaction by rats. This is supported by the findings in Chapter 6, where tickled rats did not show increased preference for the hand that had been tickling them over a novel object compared to control rats. Contrary to my results is the study by Okabe et al. (2020) who showed that five minutes of gentle stroking induced affiliative behaviours towards the experimenter and increased Fos-ir in PVN oxytocin neurons, while I found that two minutes of tickling decreased Fos-ir in both oxytocin and vasopressin parvocellular neurons in the PVN. While still a behaviour that is being mimicked by a human (e.g. gentle stroking mimicking social grooming behaviours in primates and humans lead to a development in an affiliative relationship; Lehmann et al., 2006; Dunbar, 2010), the response to gentle stroking may be less polarised than to tickling. Tickling is a complex behavioural phenomenon. In humans, the tickled person usually exhibits laughter as well as struggling to escape or fight off the person doing the tickling (Provine, 2004) and facial expressions during tickling included Duchenne smiling as well as expressions associated with displeasure (Harris and Alvarado, 2010).

Arguably, tickling is not an ethologically relevant behaviour in the same way as other social behaviours that have evolved to contribute to the survival of the animal and propagation of its genes, such as reproductive behaviours, parental behaviours, aggression, and social cohesion for group living (e.g. for

review see Debarre et al.,, 2014). Indeed, the relationship between rats and humans is complex; throughout history, humans and wild rats have lived in close proximity, with human activity (e.g. agriculture) often acting as a food source for rats (Meerburg et al., 2009) whereas humans use pest-control methods to cope with rat populations around the world (Barnett, 2001). Rats are commonly kept as pets (PFMA, 2020) and are one of the most used research animals for scientific experimentation (Baumans, 2004). Therefore, it is unclear whether human interaction would be perceived as aiding or hindering a rat's survival. While tickling is evidently pleasurable for young rats (Burgdorf and Panksepp, 2001), it may not be necessarily perceived as contributing to their survival because it is not a natural reward, such as food and sex (Kelley and Berridge, 2002). Unlike social conspecific play which is considered to serve a role in developing behaviours later in life, such as mating and aggression (Auger and Olesen, 2009), tickling may be a less salient stimulus, having been developed only ~20 years ago (Panksepp and Burgdorf, 2000), that may not activate neuronal populations in regions that are active during evolved social behaviours.

An alternative explanation for my findings may be that methodology for quantifying Fos-ir was not sensitive enough to detect neuronal changes in the regions on the social brain network. While tickled animals made more total 50 kHz USVs, trill 50 kHz USVs and more scampers, the control rats also expressed these behaviours, just not at the same frequency. The control group used in both studies (see Material and Methods sections in Chapters 2 and 6) was designed to control for all other stimuli except for the stimulus of tickling. The control rats were removed from the home cage and entered the tickling arena, but were not touched by the experimenter during the testing period. While tickled rats produced significantly more 50 kHz USVs, in particular trill 50 kHz USVs, than control rats (see Chapters 2 and 6), control rats were also found to increase their 50 kHz USV call rates across the testing phase (see Chapters 2 and 6). This may suggest that the control rats were finding leaving the home cage, and/ or spending time in the arena, to be rewarding (although not as rewarding as tickling based on the number of 50 kHz USVs emitted;

Hinchcliffe et al., 2020). It is unlikely that control rats were engaging in a different activity than tickled rats because apart from the alternating 15 seconds of tickling (totalling one minute) in the arena, the rats in each group were treated the same. By alternating 15 seconds of no tickling, tickled rats also had the opportunity to explore the arena as the control rats did (although only for one minute in total for tickled rats as opposed to two minutes for control rats), and rats of both groups engaged in non-play associated locomotion and exploration which included sniffing and rearing at the walls of the arena (see Chapter 3). Therefore, this control group may have lacked a large enough difference from the tickled group for any difference in Fos-ir to be detected and could explain the results presented in Chapters 4 and 5. An additional control group that did not leave the home cage, and therefore would control for any rewarding effect of changing environments could be used in future studies to address this. As there was no absence of these behaviours in the control rats, there may not have been a large enough difference in neuronal activity to be detected by Fos-ir, given that c-*fos* is an IEG that is generic, being transcribed in response to a wide range of stimuli.

Additionally, the length of the tickling stimulus in the present study was shorter than studies carrying out gentle stroking (Okabe et al. (2015, 2020, 2021) carried out five minutes of gentle stroking) and most studies quantifying Fos-ir or c-*fos* mRNA following social play in rats. For example, sessions range from 10 minutes (quantifying levels of Fos protein, Reppucci et al., 2018; quantifying levels of c-*fos* mRNA, Perkins et al., 2017) to one hour of observation during which play can occur (quantifying levels of Fos protein, Northcutt and Nguyen, 2014). It is also important to consider that quantifying c-*fos* mRNA (for which rats are typically culled 30 - 45 minutes after final testing, e.g. Perkins et al., 2017; Gordon et al., 2002; van Kerkhof et al., 2014) and quantifying Fos protein (for which animals are typically culled between 60 - 90 minutes after final testing e.g. Chapter 4 and 5 of the present study; Northcutt and Nguyen, 2014; Reppucci et al., 2018; Okabe et al., 2015, 2020, 2021) are two different measures of changes in the expression of the c-*fos* gene. To my knowledge, this is the first study to quantify Fos-ir following

tickling and therefore is the first evidence for the effect (or lack of) of tickling on Fos-ir in the brain of juvenile rats. The stimulus of tickling for two minutes might have been too brief a stimulus to induce a change in Fos protein levels that is found in those studies investigating social play where the rats had more opportunities to engage in play over 10 to 60 minutes, or five minutes of gentle stroking, compared to the two minutes of tickling in the present study.

Conclusions

The tickling protocol employed in this study successfully induced a positive affective state in female and male juvenile Wistar rats, as shown by an increase in total and trill 50 kHz USVs. Trill 50 kHz USVs were not found to be emitted differently by female and male rats, whereas flat 50 kHz USVs were, but only after 10 days of tickling. This sex difference in 50 kHz USVs became apparent when synchronising USVs with behaviours; I found that subtypes of 50 kHz USVs may be used differently during tickling in a sexually dimorphic manner by being paired with play-related behaviours. For example, female rats associated flat 50 kHz USVs with solitary play behaviours, while tickled rats, irrespective of sex, paired trill 50 kHz USVs with hopping, darting and approach behaviours significantly more than control rats. This highlights the importance of using both sexes in tickling studies as they may differ in their behavioural response to tickling. Tickling did not increase neuronal activity in key brain regions associated with social behaviours and reward. This may be due to individual responses to tickling, experimental design such as the control group used and length of stimulus, or that tickling, while rewarding, was not perceived as a social stimulus. This study provides preliminary evidence that may be used to inform future studies to understand in greater depth the neural correlates of tickling in both female and male rats. This study has also highlighted the effect that subtle differences in tickling method and experimental design have on the outcomes of tickling studies.

7.2 Key limitations

Total 50 kHz USVs were quantified in this study, and the number produced was analysed across the testing phase in both animal experiments carried out. However, only trill and flat 50 kHz USV subtypes were incorporated into this analysis, because trill and flat calls are the predominant subtypes emitted during social conspecific playful interactions in rats (Burke et al., 2018; Burke et al., 2021). During tickling, most studies have focussed on total 50 kHz USVs and trill 50 kHz USVs (e.g. Panksepp and Burgdorf, 2000 and 2003; Cloutier et al., 2013; Hammond et al., 2019), while Burgdorf et al. (2008) analysed flat and trill 50 kHz USVs only, due to the complex nature of call profiles emitted by rats. However, Wright et al. (2010) detailed 14 subcategories of 50 kHz USVs, and analysing a wide variety of these subtypes is warranted to establish which calls, including more rarely produced USVs, may be related to which behaviours observed during tickling, and particularly whether any subtypes are produced differently by female and male rats. Another limitation of the behavioural analysis was that there was only one experimenter carrying out the testing and scoring of the behavioural measures. There was no comparison between observers for the behavioural measures which may affect repeatability and accuracy of the results presented in this thesis. Factors such as sex of the tickler or previous handling experience may affect the response of the rat to tickling, however there is a lack of published evidence showing how having a single experimenter may affect the response (Cloutier et al., 2018).

There are also general limitations to using Fos-ir as a neuronal marker of activity. The Fos protein product is produced in a time window of 60 to 90 minutes after the acute stimuli, therefore the animal must be culled in that time frame (Kovacs, 1998). This time frame can be variable; in some cases, the maximal Fos expression can be delayed or prolonged beyond the standard 60 - 90 minute range, and so in each brain or indeed each brain region, the Fos protein product may vary (Kovacs, 2008). Similarly, neuronal activation can occur in the absence of the c-*fos* gene being transcribed, and in chronically activated neurons, Fos-ir may not be detected (van Kerkhof et al., 2014;

Kovacs, 2008). Also, Fos-related antigens may also be detected and this is determined by the specificity of the antibody. Quantifying Fos-ir is correlational and not causal, other techniques such as designer receptors exclusively activated by designer drugs (DREADDs) or optogenetics are required to determine functionality. However, using immunohistochemistry to visualise Fos protein expression is a widely used technique to visualise changes in neural activity during behaviours (Kovacs, 2008), for example, nest building in zebra finches (Hall et al., 2014; Edwards et al., 2020), social conspecific play in rats (van Kerkhof et al., 2014; Reppucci et al., 2018), sexual behaviours (e.g. Robertson et al., 1991; Veening and Coolen, 1998), maternal behaviours (e.g. Lonstein et al., 1997) and gentle stroking in rats (Okabe et al., 2015). While the aforementioned limitations are important and should be taken into account, it is unlikely that they significantly affected the results of the present study because protocols for Fos-ir immunohistochemistry have been well established and anti-Fos antibodies in laboratory rats are optimised and reliable.

This study aimed to map brain regions that may have involvement in the response to tickling, and to compare this between female and male rats. Previous studies investigating the response to tickling have implicated the somatosensory cortex (using *in vivo* electrophysiology; Ishiyama and Brecht, 2019; Ishiyama et al., 2019) and regions of the reward pathway (via electrical stimulation and pharmacological manipulation, Burgdorf et al., 2007; via dopamine release, Hori et al., 2013). The present study has indicated certain areas of interest, such as tickling affecting Fos-ir in parvocellular neurons of the PVN and Fos-ir in the PAG being associated with 50 kHz USV production. However, this exploratory design meant that repeated sampling across multiple brain regions was carried out. This was corrected for in the statistical models using the Tukey method, through which the combined confidence interval across all of the comparisons was 95% (Lenth, 2020). This method has an impact on the power in the analysis in Chapters 4 and 5, as for each individual comparison, the confidence interval is greater than 95% to account

for repeated testing. Further, more specified studies are now warranted to investigate these initial findings.

Along with immunohistochemical double staining for Fos protein and oxytocin and vasopressin in the PVN, this study originally aimed to double stain for Fos-ir in corticotrophin releasing hormone (CRH) positive neurons in the PVN. Oxytocin, vasopressin and CRH-releasing neurons in the PVN are distributed throughout the magnocellular and parvocellular subdivisions (Sawchenko et al., 1983). CRH released from these neurons activates the hypothalamic-pituitary-adrenal (HPA) axis, eliciting a stress response (for review see Dunn and Berridge, 1990 and Smagin et al., 2001). CRH-releasing neurons project from the PVN to the median eminence where they release CRH which stimulates the secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland corticotrophs which in turn stimulates secretion of glucocorticoids (corticosterone in rats) from the adrenal cortex (Vale et al., 1981; Rivier and Plotsky, 1986). Visualising neuronal activity (via Fos-ir) of CRH parvocellular neurons can be used as a marker of the stress axis (for example, Pezzone et al., 1992, Helfferich and Palkovits, 2003 and Yanagita et al., 2007). Tickling has been found to significantly reduce the sympathoadrenal stress response to fear conditioning in rats, as tickled male rats had reduced levels of plasma adrenaline and noradrenaline in response to an aversive foot-shock (Hori et al., 2013). Tickling is also found to reduce audible vocalisations which are indicative of pain in response to injections (Cloutier et al., 2014). However, there was no effect of tickling on plasma corticosterone levels (Hori et al., 2013) or faecal corticosterone (Cloutier et al., 2014; Lafollette et al., 2018). Therefore, the mechanisms by which tickling affects the stress response to aversive stimuli is not yet clear. A recent study found that CRH PVN neurons, shown to be activated in response to a stressful stimulus of a foot shock, were inhibited by a reward of sucrose solution in mice (Yuan et al., 2019). As tickling is considered to be a rewarding stimulus (Burgdorf and Panksepp, 2001), I wanted to investigate whether activity in CRH neurons in the PVN was different between tickled and control rats. Unfortunately, despite several anti-CRH antibodies being tested on the neural tissue obtained from

the animals used in the first animal study, no positive staining was confirmed. Different techniques, such as antigen retrieval or fluorescent tags, were needed. However, due to time constraints resulting from COVID-19 restrictions at that time, an alternative research question was addressed instead to visualise dopaminergic neurons in the VTA by double labelling for tyrosine hydroxylase. Nevertheless, investigating changes in activity of CRH-releasing neurons would have yielded interesting and important information alongside the oxytocin and vasopressin immunohistochemistry data and is still warranted (please see Section 7.3 Future directions).

7.3 Future directions

Exploring the effect of tickling on parvocellular neurons in the PVN

Aim: To build on the Fos-ir results in Chapter 5 where Fos-ir was found to be lower in parvocellular oxytocin and vasopressin neurons in tickled compared to control rats.

I would use *in vivo* electrophysiological recordings via tetrode recordings of single neurons (as in Ishiyama and Brecht, 2016 and Reinhold et al., 2019) to selectively target populations of parvocellular oxytocin and vasopressin neurons during tickling, as well as CRH neurons in the PVN. Differentiating between specific neuronal types can now be achieved using transgenic rat lines that express fluorescent proteins in the cell type of interest (for review see Ohbuchi and Ueta, 2014; e.g. Tang et al., 2020). I would use these techniques to test the hypothesis that tickling changes neuronal firing in neuroendocrine neurons of the PVN in juvenile rats. For this study, I would have two control groups, with one group being removed from the home cage and entering the testing arena as in the present study, and a control group where the rats do not leave the home cage, thus controlling for the change in environment. There appears to be individual variation in the response to tickling, for example, 50 kHz USVs may provide a graded measure of positive affective state, representing individual differences in the response to being tickled (Hinchcliffe et al., 2020). Burgdorf et al. (2008) selectively bred two populations of rats that produce high or low rates of 50 kHz USVs and show differing responses to tickling. Given the variation in the neural data presented in this study, using rats that have been identified as high callers and low callers may provide more polarised data from *in vivo* electrophysiological recordings (e.g. in Wohr et al., 2009).

If the firing of parvocellular neurons (oxytocin, vasopressin and CRH-expressing) was affected by tickling as in the present study, I would use an anterograde tracer (for example by using an adeno-associated virus and fluorescent protein expression; e.g. see Dabrowska et al., 2016) to investigate where these parvocellular neurons project to, such as centrally or

oxytocinergic parvocellular neurons synapsing onto magnocellular neurons (Tang et al., 2020), in order to explore the circuitry that is being affected by tickling. To then test the functional relationship of parvocellular oxytocin and vasopressin PVN neurons and tickling, optogenetic techniques (for review see Fenno, Yizhar and Deisseroth, 2011; e.g. Gunaydin et al., 2014; Balazsfi et al., 2018) or DREADDs (e.g. Grund et al., 2019, Tang et al., 2020) could be used to switch on these parvocellular neuronal populations in order to measure the change in the response to tickling and assess in real time the function of these neurons on behaviours associated with the response to tickling.

Exploring the emission of flat 50 kHz USVs during playful behaviours in female rats

Aim: To investigate the use of flat 50 kHz USVs during tickling and conspecific play in female rats.

In Chapter 2, I found that there was a significant effect of sex on the number of flat 50 kHz USVs made, with female rats in general making more flat USVs during testing, in particular during 'release' sessions, than male rats. In Chapter 3, female rats, irrespective of treatment, paired flat USVs with play-related behaviours significantly more than males. Flat USVs may have a role in social communication (Wöhr et al., 2008; Wright et al., 2010; Burke et al., 2017a; Burgdorf et al., 2011), for example to communicate dominance during play fighting in male rats (Burke et al., 2017b). Female rats produce flat USVs during mating (Thomas and Barfield, 1985), however, their role during play in females is not fully understood. My results suggested that female rats may use flat USVs for a communicatory purpose during playful behaviours. Therefore, I would investigate whether females are producing flat USVs in relation to play-related behaviours during social conspecific play in the home cage, as well as in relation to play-related behaviours during tickling, testing the hypothesis that female rats use flat 50 kHz USVs to communicate during playful interactions. I would record USVs produced in the home cage during conspecific play between female rats and during ticking, comparing the rate of flat 50 kHz USVs produced during conspecific play and during tickling and whether flat USVs

were paired with play-related behaviours during both conspecific play and tickling. As I did not find a sex difference in total 50 kHz USVs, or in trill or flat subtypes after five days of testing in Chapter 6, this may indicate that the number of days of testing may influence the response of female rats. Therefore, the study would span 20 days to also measure whether the emission of flat 50 kHz USVs during both social play and tickling changes over time.

Effect of tickling female rats on maternal and offspring behaviours

Aim: To investigate whether tickling induces a positive affective state that affects maternal behaviours and the development of the offspring.

In the present study, I showed that tickling robustly induced a positive affective state in female rats, as evidenced by the number of trill 50 kHz USVs produced. Many studies have shown the negative effects of stress on maternal behaviours and subsequently in the offspring. However, the effect of enriching the maternal environment and the effect of positive stimuli on the mother and her offspring is yet to be fully explored. I designed a study that aimed to investigate whether the positive heterospecific stimulus of tickling during adolescence, through puberty and in early gestation would have a positive effect on puberty, sexual behaviours, pregnancy and maternal behaviours, as well as social and cognitive behaviours in the offspring. This study was planned to be carried out in April-June 2020, however, due to the UK lockdown at this time, the study could not go ahead. Due to subsequent COVID-19 restrictions in the Roslin Biological Research Facility and the length of time needed for the study and analysis of the data, the decision was made that this study was not suitable to be carried out in the time remaining on my PhD. The findings of this experiment could have had important implications for the welfare of breeding dams and their offspring, and thus I believe such studies are warranted. An important aspect of this study would be to use females that showed high call rates in response to tickling (Hinchcliffe et al., 2020) in order to use females that had a positive affective state induced by tickling. This is to take into account the individual variability to tickling exhibited by rats.

I would test the hypothesis that the positive enrichment of tickling in adolescent female rats improves mating behaviours, maternal care and postnatal cognitive and social behaviours in the offspring.

Objectives

1. To measure the physiological effect of tickling on the onset of female puberty, sexual behaviours, duration of pregnancy and litter size.
2. To measure the effect of tickling on maternal care (nest building and licking and grooming behaviours) and lactation in female rats.
3. To measure the effect of tickling on offspring cognitive function, hippocampal development, onset of play behaviours, anxiety levels and the reaction to the positive stimulus of tickling as adolescents themselves.
4. To investigate any gene up-regulation due to tickling in both the tickled females and their offspring.

Predictions

1. Tickling before puberty and in early pregnancy may lead to: earlier onset of puberty in female rats; more solicitation behaviours/ successful copulation and conception rates; females will be better mothers i.e. they will show more licking/ grooming behaviours and pup retrieval.
2. The offspring of tickled mothers will be more resilient to anxiety tests in an open field and elevated plus maze and show better cognitive abilities.
3. The offspring of tickled mothers will exhibit altered neural responses to positive stimuli.
4. There may be sex differences in these effects in the offspring.

Measures used

Puberty would be confirmed by vaginal opening. Copulatory and solicitation behaviours would be recorded and mating confirmed by identification of the remnants of a vaginal plug found in the cage. The length of pregnancy would be measured. Home cage behaviours would be video-recorded to measure maternal behaviours (nest building and licking and grooming behaviours) and onset of play behaviours in the pups.

The offspring would be tested in an elevated plus maze, open field maze and cognitive judgement bias test.

Dopamine levels would be quantified by using immunohistochemistry to compare the number of active dopaminergic neurons in the VTA between groups. Alternatively, to determine the concentration of dopamine in the nucleus accumbens, high-pressure liquid chromatography could be employed. Serotonin may also be measured in this way. RNA-Seq (next-generation sequencing technique) would be used to analyse gene expression in the paraventricular nucleus and hippocampus.

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