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Differential pup-induced neuronal activation in the maternal medial preoptic area in relation to paternal social status

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1. Introduction

In many animal species, offspring rely on parental interactions in order to survive and develop mentally and physically well. Such behaviour requires sizeable investment of resources and time and ultimately has the goal to increase the likelihood of offspring survival to ensure its maturation to reproductive age (Dulac et al., 2014; Numan, 2014; Wu et al., 2014). Contrary to other social behaviours like aggression or mating, parental care implies a prolonged interaction between parents and infants that may even last years, as well as a non-reciprocal action carried out by adults (Kohl et al., 2017). A multitude of species-specific behaviours towards offspring constitute parental care, such as nest building, grooming, crouching, carrying, nursing and/or feeding as well as defence of young. In mammals, this caregiving behaviour may include alloparental, paternal and maternal behaviour, with laboratory mice displaying the latter (Numan, 2014; Kohl et al., 2017).

1.1 Neural circuitry involved in maternal care behaviour (MCB)

It has been shown that hormonal events associated with pregnancy and parturition play a crucial role in mammals to prepare females for motherhood and to ensure a strong mother-infant relationship. Hormonal priming of the maternal brain is carried out by hormones such as progesterone, estradiol and oxytocin, which enter the brain via the blood stream and cause functional changes in brain regions essential for maternal behaviour through binding to their specific receptors (Numan, 2014; Matsushita et al., 2015) . In conjunction with an initial maternal experience with pups, these hormonal events cause a persistent modification of brain functions, which results in a strong mother-infant bond and is maintained even in the absence of these priming hormone concentrations (Numan, 2014). Extensive research on maternal behaviour and its underlying circuits revealed a core neural circuitry, consisting of the medial preoptic area (MPOA) and the adjoining ventral part of the bed nucleus of the *stria terminalis* (vBNST), both being indispensable for the immediate onset of maternal behaviour after parturition. By forming direct projections to the ventral tegmental area (VTA), which promotes activation of the reward system, maternal behaviour is further regulated (Dulac et al., 2014; Barrière et al., 2021). Projections to the nucleus accumbens (NA) are believed to mediate parental responsiveness with this motivational circuit likely being modulated by projections from the paraventricular

nucleus (PVN) of the hypothalamus (Kohl et al. 2017). Consistent with this, Barrière and colleagues found transient increases in grey matter concentration (GMC) in key regions controlling maternal behaviour (MPOA, BNST and PVN) as well as in other regions involved in motivation and reward (caudate nucleus, orbitofrontal cortex), emotions (amygdala) and mnesic functions (hippocampus). They observed specific increases in GMC in females expressing high levels of maternal behaviour, with these hypertrophies already being significant prior to parturition, thus predicting the quality of maternal care (Barrière et al., 2021).

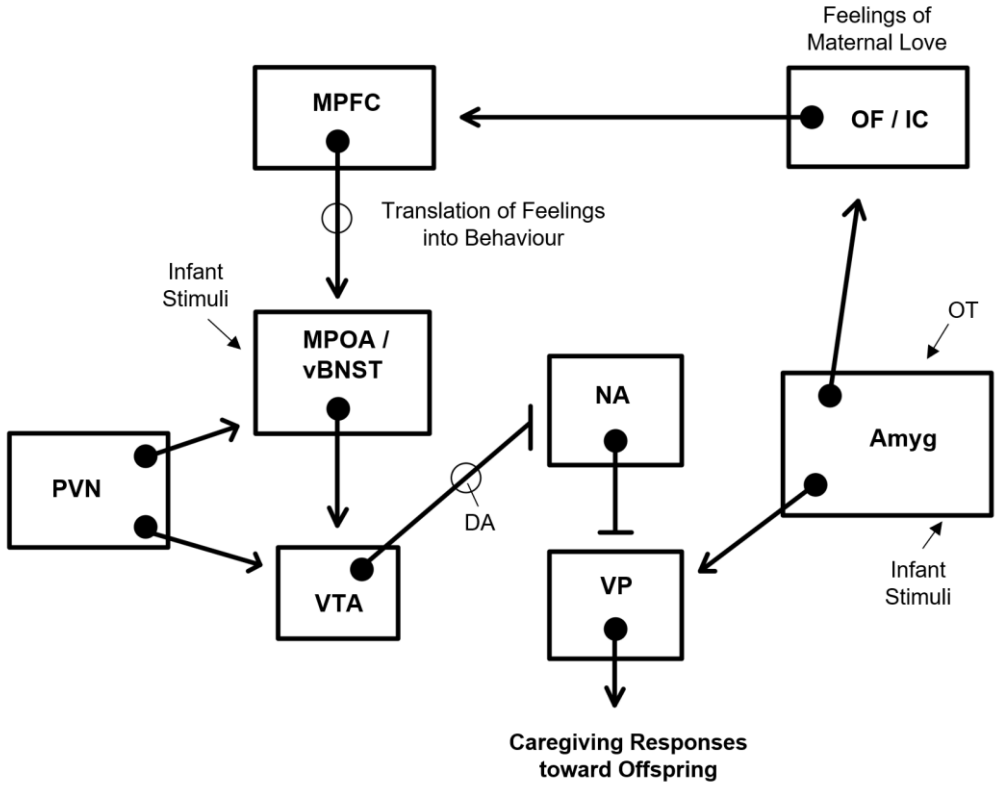

Figure 1: Neural circuitry involved in maternal caregiving responses

The figure shows a model of the brain circuit involved in the expression of maternal care behaviour with the medial preoptic area (MPOA) and ventral part of the bed nucleus of the *stria terminalis* (vBNST) at its centre. Oxytocin (OT) and infant stimuli activate the amygdala, which projects to the ventral pallidum (VP) as well as the orbitofrontal cortex (OF) and insular cortex (IC), which are proposed to give rise to maternal feelings. The OF/IC projects to the medial prefrontal cortex (MPFC), which activates the MPOA. Infant stimuli further activate the MPOA as

well as input from the periventricular nucleus (PVN). MPOA projections activate the mesolimbic dopamine (DA) reward system by projecting to the ventral tegmental area (VTA), which projects to the nucleus accumbens (NA), which in turn projects to the VP. The output from the VP promotes maternal motivation. Effects ending in an arrow indicate stimulation. Inhibitory neurons are depicted ending in a bar. Figure adapted from M. Numan (Numan, 2016)

1.1.1 The medial preoptic area and its role in MCB

The medial preoptic area (MPOA), part of a larger preoptic area (POA), is located in the rostral part of the hypothalamus and has long been considered a key region for the positive control of parental behaviour. The MPOA is further involved in other physiological and behavioural functions such as sexual behaviour, sleep, ovulation, feeding and thermoregulation (Lin et al., 2018; Numan, 2014; Tsuneoka et al., 2013). It is highly heterogenous and receives inputs from, and projects to, multiple brain regions, which impedes the investigation of the exact circuit mechanisms through which this region controls particular aspects of maternal behaviour. Previous studies have shown the specific involvement of certain types of neurons, including galanin-expressing neurons and estrogen receptor α (Esr1) -expressing neurons, in maternal behaviour (Lin et al., 2018; Wu et al., 2014; Wei et al., 2018). Indeed, the importance of Esr1 expressing cells has been further highlighted by Fang et al. (2018), who identified these neurons as key factors for pup approach and retrieval in mice. By comparing projection patterns of MPOA $Esr1^{+}$ and $Esr1^{-}$neurons, Fang and colleagues revealed strong inhibitory projections from MPOA $Esr1^{+}$ cells to VTA non-dopaminergic neurons, suggesting a disinhibition mechanism through which these MPOA $Esr1^{+}$ neurons excite dopamine neurons in the VTA. Considering the involvement of VTA dopamine neurons in motivated behaviours, this pathway might play a central role in transforming sensory cues to motivational drives (Fang et al., 2018; Lin et al., 2018; Matsushita et al., 2015). Interactions of MPOA neurons with the mesolimbic dopaminergic system have been shown to control the appetitive aspects of maternal behaviour, especially the motivational aspects of early postpartum responsiveness to pups through activation of said system. Resulting from continued interactions with offspring, maternal behaviour undergoes considerable plasticity in parallel with the developing pups and along with this, the MPOA becomes differentially engaged throughout the postpartum period. While at first, MPOA activity is crucial to facilitate the onset of maternal behaviour, as the pups grow older and maternal

memory starts to form, the regulation of maternal responsiveness becomes less dependent on MPOA activity and more distributed in the maternal circuit (Numan, 2014; Pereira and Morrell, 2009).

1.2 Social dominance as an indicator of male quality

Mus species typically live in social groups organized into dominance hierarchies. Under natural conditions, high reproductive skew (reproduction is not equally shared among individuals of the same sex (Rubenstein and Lovette, 2009)) with elevated levels of inter-male competition lead to formation of territories with males scent marking it to signal as theirs and to advertise their quality to potential mating partners (Lee et al., 2017). Social status within a group is associated with an increase in resources, food access and territory which can have effects on intra- and transgenerational physiology and fitness and is a trait contributing to the attractiveness of the individual (Cauceglia et al., 2020). Such phenotypic effects serve as multisensory cues for females to detect male quality and are often communicated through behavioural actions or chemosensory cues in urine. Information about the reproductive condition, dominance status and individual identity of the male is conveyed with exposure to these olfactory cues being shown to affect neural activity and behaviour of females. Differential patterns of immediate early gene activation have been observed in females in response to dominant versus subordinate male urinary scents even without previous experience with the urine donors, while in other studies it has been shown that the preference for dominant odors depends on prior sexual experience as wells as hormonal status of the female (Borelli et al., 2009; Lee et al., 2021; Veyrac et al., 2011). MUP20 (darcin), an isoform of major urinary proteins (MUPs), is an involatile protein pheromone in male urine associated with social rank and attractiveness to females (Barabas et al., 2021; Hoffman et al., 2015) with higher levels found after social interaction and in dominant individuals. Since the metabolically costly MUP production is androgen-dependent and testosterone often connected with male dominance, aggressiveness and the ability to defend territory, darcin levels may be indicative of male quality (Hoffman et al., 2015; Lee et al., 2017). These chemosensory cues, additionally with differential physiology and mating behaviour, could elicit molecular and functional changes in the female brain during mating which possibly persist throughout gestation and subsequently may mediate maternal behaviour.

1.3 Reproductive investment when mate quality varies

Decisions on reproductive investment (also referred to as reproductive effort) have great implications on offspring survival and health as well as parents themselves. Reproductive investment refers to the total amount of energy an organism spends on reproduction during a defined time period (Vitt and Caldwell, 2013). These decisions form a crucial part of life-history theory, which predicts an adjustment of current reproductive investment of parents according to both the expected pay-off from the current attempt and estimated future reproductive success (Ratikainen and Kokko, 2010). Life history theory tries to explain how natural selection and other evolutionary events influence an organism to optimize its survival and reproduction (Fabian and Flatt, 2012). Plasticity in reproductive allocation (the proportion of the energy budget an organism allocates to reproduction at any given time (Gilbert, 2012)) is capable to create parental effects on offspring development. For instance, a non-genetic link between offspring fitness and mate attractiveness can be created if mothers invest differentially into offspring based on the attractiveness of their mate. Such a link can have an impact on evolutionary dynamics. Therefore, it is important to know the conditions under which differential investment in relation to mate quality arises as well as the direction in which this effect occurs. In the last decades, two distinct theories have been formed, stating quite opposite predictions. The first, the Differential Allocation Hypothesis (DA), predicting increased maternal investment into offspring when females are mated with high quality males. The latter suggesting an opposite effect, namely increased allocation of resources with low quality males, this body of theory named Reproductive Compensation Theory (RC) (Harris and Uller, 2009).

1.3.1 Differential Allocation Hypothesis

The initial formulation of the DA Hypothesis by Burley based on her research in zebra finches (*Taeniopygia guttata*) postulated an adjustment of parental investment (PI) into the current reproductive attempt according to perceived mate quality (Burley, 1986). Since then, there has been a tremendous amount of experimental work in this field with results demonstrating DA occurring across a wide range of species, parental care patterns and mating systems (Haaland et al., 2017; Sheldon, 2000). The DA Hypothesis predicts increased PI with increasing mate quality under the assumption that investment must be costly in terms of future reproductive

success and that mate quality affects the level of investment, although recently Haaland and colleagues (2017) have shown that this is only true in certain circumstances. According to their models, DA can only be observed when offspring fitness is affected multiplicatively by male quality rather than by purely additive offspring fitness benefits. For this hypothesis to apply, there needs to be a trade-off between current and future reproduction as well as an influence of mate attractiveness on the reproductive value of the breeding attempt. The level of how much mate attractiveness can influence this trade-off is probably dependent on a number of factors: the variance in the effect of mate quality on offspring reproductive value, the estimated future reproductive lifespan of one partner as well as on the expected quality of future mating partners (Sheldon, 2000).

1.3.2 Reproductive Compensation Theory

This theory arose due to experimental findings, which contradicted the predictions of the DA hypothesis. It states that parents increase reproductive investment into offspring in an attempt to compensate for lowered offspring viability when mated to non-preferred partners (Gowaty et al., 2007; Ratikainen and Kokko, 2010). It is important to note the role of mate preference in this context, which is according to Gowaty the driving force for reproductive compensation to occur, rather than mate attractiveness. For this theory to apply, there have to be two assumptions met: (*i*) when individuals have other options than to mate with an unpreferred mate, they resist reproductive efforts with such a mate. But if the individual is forced to mate, then it attempts to compensate for this event by increasing reproductive investment; and (*ii*) offspring viability is negatively affected by constraints on free mate choice. Given that the assumptions are met, the hypothesis predicts increased parental efforts of constrained individuals in an attempt to strengthen offspring fitness and to ensure it is as successful or nearly as successful as offspring from unconstrained competitors (Gowaty et al., 2007). There is no common consensus on what factor reproductive compensation is based on. Some refer to RC when dealing with responses to attractiveness, others strictly exclude attractiveness as a factor when obtaining their results (Ratikainen and Kokko, 2010).

When incorporating theories of differential reproductive investment in empirical studies, results need to be analysed carefully since there are many factors, which may have an impact on

parental investment: according to Harris' and Uller's model, the extent to which females allocate resources differently in relation to male quality is highly dependent on female energetic state and age. In addition to this, another question is what if females do not have complete information about mate quality or are manipulated by mates? Does this change the pattern of reproductive investment and if so, how can this be taken into account? (Harris and Uller, 2009). Furthermore, choosing the characters on which to look at when investigating DA or RC is no trivial task, as a lack of results may root from a wrongly selected trait as a correlate. Moreover, there may be diverse ways in which the level of investment can be measured. Increased investment in one area may be accompanied by a reduction in another by the same parent (Ratikainen and Kokko, 2010).

1.4 Paternal effects on offspring

In rodents, the unproportionally large investment by mothers compared to fathers in the care of offspring has directed much of the research on parental effects to maternal effects. However, there is evidence for paternal effects even amongst species in which paternal investment is limited or absent altogether, which raises questions about the mechanisms driving these effects (Curley et al., 2011). Life-history events of males (*e.g.,* nutritional, social and toxicological exposures) have a lasting epigenetic impact and influence the development of offspring. These observations have led to increased interest in the germline transmission of environmental experiences (Champagne, 2020; Curley et al., 2011). Nevertheless, the role of male-induced maternal effects should not be disregarded. Females are predicted to invest differentially in their young depending on environmental factors, one being mate quality, which can lead to profound variations in offspring development (Harris and Uller, 2009; Ratikainen and Kokko, 2010). Additionally to impacting perceived quality, males may set off post-mating adaptations in the reproductive tract of females. It has been shown that both sperm as well as seminal fluid influence events following mating, such as preimplantation uterine immunological responses, vascular remodelling and cell signalling (Bromfield et al., 2014; Watkins et al., 2018). Since offspring can actively influence maternal care and are not just passive recipients, they too take part in shaping maternal investment, starting *in utero*. The offspring-derived placenta produces hormones which take part in mediating nutrient transfer via the placenta and priming of the maternal brain, thus it is considered a key site for potential indirect genetic effects (Creeth et al.,

2019; Haig, 1996; Potter et al., 2019). Considering the high expression of paternally imprinted genes in the placenta and them having a regulatory effect on postnatal mother-infant interactions, maternal care might be a resource manipulated by the paternal genome (Creeth et al., 2019; Curley et al., 2011). Genomic imprinting is a process whereby genes are monoallelically expressed with the expressed allele being dependent upon which parent it was inherited from. These parent-of-origin effects enable different phenotypes within offspring of the same genotype (Potter et al., 2019). Paternal effects though can also be modulated by the mother. Mashoodh et al. showed that while paternal food restriction in mice had an acceleratory effect on maternal investment when mated under natural conditions, this increase was absent in offspring generated by embryo transfer (Mashoodh et al., 2018). Another study showed transmission of variations in paternal exploratory behaviour being modulated by the time males spent with females such that with increased time, the effect was lessened (Alter et al., 2009). Overall, the direction and degree of impact of these germline effects in offspring is highly dependent on maternal investment pre- and postnatally (Champagne, 2020).

1.5 Behavioural tests to study the social behaviour of mice

Animal models are widely used in behavioural neuroscience to study the biological mechanisms underlying mammalian social behaviour. Particularly rats and mice are leading model organisms in biomedical and behavioural research and are commonly used in behavioural assays (Netser et al., 2020).

1.5.1 Assessment of male social status

The tube test is a commonly used method to determine dominance ranks and the linearity of hierarchies under laboratory conditions (Varholick et al., 2018). The test area consists of a PVC tube in a larger arena. During testing, one male is placed on each end of the tube and enters it. Once the opponents meet, the less dominant will retreat back out of the tube. This procedure is meant to replicate competitive situations without exposure to direct conflict (Barabas et al., 2021). Contrary to home cage observations, this method allows for clear scores of dominance: “lose”, “win” and “tie” (Varholick et al., 2018).

1.5.2 Assessment of maternal care behaviour

In rodents, maternal behaviours are classified in pup-directed and non-pup-directed behaviours, with the former including pup retrieval back to the nest. Pup retrieval is further a component of proactive maternal responses, which require a high level of motivation towards pups to be expressed properly (Salais-López et al., 2021). Pups can influence this behaviour by emitting ultrasonic vocalization (USV). USVs of young mouse pups in response to maternal isolation is a conserved behaviour across mammals and functions as a form of communication to elicit search and retrieval behaviour from dams (Rieger and Dougherty, 2016). They are present from the first postnatal day and can be induced by intense tactile stimulation, loss of body temperature, hunger or maternal separation (Potter et al., 2019).

This innate maternal response (retrieval of displaced pups back to the nest), can be tested under controlled laboratory conditions in the pup retrieval test (PRT), where pups are placed in the opposite corner of the cage than the nest site and retrieval behaviour of the dam is recorded and analysed. Further, neuronal activity in response to pup retrieval can be measured by detecting *c-fos* expression in the maternal brain (Perrin-Terrin et al., 2016).

1.6 *c-fos* expression as a marker for neuronal activity

Analysis of *c-fos* expression in neurons has become a widespread tool in behavioural neuroscience research to detect neuronal activity in response to various stimuli within the brain (Numan, 2014). This proto-oncogene *c-fos*, an immediate early gene, was first described in the early 1980s and subsequently was its product the c-FOS protein, as having gene-activator properties. Changes in neuronal activity induce *c-fos* expression via second messenger signalling cascades, which in turn leads to production of the transcription factor c-FOS. The latter participates in adaptive responses of the nervous system by initiating the expression of late genes. The transcription activation of *c-fos* is 5 to 20 min with a peak of mRNA accumulation between 30 and 45 min after stimulation onset, followed by c-FOS protein synthesis which can be detected 20 to 90 min post stimulation by immunohistochemical techniques (Perrin-Terrin et al., 2016). Since the c-FOS protein is located in the nucleus, this raises the possibility of identifying the phenotype of the activated neuron by double labelling with markers within the cytoplasm of the cell (Hoffman et al., 1993). Its low expression in the absence of stimulation

allows for easier quantification of neuronal activity under a test situation. Compared with electrophysiological approaches for measuring neuronal activity, c-FOS detection permits one to observe activity changes in a larger number of neurons, making it possible to identify active brain areas. Another asset of this method is that test animals do not need to be restrained or anesthetized in order to obtain results. However, when using this technique, one has to consider the delay of c-FOS accumulation in the nucleus, a consequence of this being that there cannot be information obtained about rapid changes in neuronal activity after a specific stimulus. When detecting c-FOS protein, which may have a half-life of 90 to 100 min, it is of importance to account for enough habituation time for the animals before starting any behavioural or other experiments, in order to prevent the measured expression being affected by stress associated with the handling of the animal prior to the procedure (Perrin-Terrin et al., 2016).

2. Aim

Plasticity in reproductive investment has been shown to be associated with a variety of factors, one of which being mate quality. This phenomenon, where a mother exerts different degrees of maternal investment depending on the attractiveness of her mate, is described as the differential allocation hypothesis (DA) and creates an indirect pathway through which paternal effects might be transmitted to offspring. Social status of males is indicative of male quality with dominant males exhibiting biochemical and behavioural differences, which could elicit molecular and functional changes in the female brain during mating and persist throughout gestation and, subsequently may mediate maternal behaviour.

This project explored whether paternal social status can influence specific aspects of a mother's care behaviour in the early postnatal period by examining differential neuronal activation in the maternal brain in response to a specific stimulus eliciting maternal responsiveness (pup retrieval). Observed brain regions include regions of the hypothalamus with particular focus on the medial preoptic area (MPOA) and the amygdala, since these regions are critically involved in parental care behaviour. Using immunofluorescent double-labelling for c-Fos (the protein product of an immediate early gene) and DAPI, the percentage of activated neurons was determined and Spearman correlation analysis between paternal social status and *c-fos* activity as well as to maternal and pup behaviour during the assay was carried out.

Questions this work addresses are:

Is there a relationship between paternal social status and pup-induced neuronal activation in the maternal MPOA, other hypothalamic regions and the amygdala?

How does pup behaviour during the behavioural assay (pup retrieval test) influence maternal neuronal activity?

How is maternal behaviour influenced by pup behaviour during the pup retrieval test?

3. Materials and Methods

3.1 Animal housing and care

All animal experimentation was approved by the national ethical committee on animal care and use (Bundesministerium für Wissenschaft und Forschung) and was conducted in accordance with the EU-directive 2010/63/EU. The registration number for this project is 2021-0.744.367. Both male (20 animals) and female (16 animals) C57Bl6/N mice were obtained from the Charles River Laboratories (Wilmington, MA, USA) at age 7-9 weeks and 5-6 weeks, respectively, and housed in a colony room under controlled conditions (12-hour light/dark cycle; lights on at 8:00 am; 21-22 °C; 25-28 % humidity; and *ad libitum* access to water and food).

3.2 Behaviour assays

All testing was conducted during the light phase of the light/dark cycle and animals were given 1 h habituation time with reduced light intensity and white noise before the start of any experiments. Behavioural assays were conducted by MRes Claire Owen, PhD student at the Department of Neurophysiology and Neuropharmacology at the Medical University of Vienna and are not part of this thesis. However, the obtained data is further used and analysed for this thesis.

3.2.1 Determination of male social dominance using tube test

Males were housed in groups of four and when testing, pairwise trials between cage mates were conducted in an arena composed of a PVC tube in a bigger enclosure. On each end of the tube, one contestant was placed which locomotes to the centre. The less dominant male will retreat once encountering the opponent. Test wins of each male against cage mates were added up and used as a representation of the individuals rank within the dominance hierarchy of the cage.

3.2.2 Ultrasonic vocalizations (USV) of pups

Recording of pup USVs was conducted on post-natal day 4 (PD4). For this, pups were separated from their mother and placed in an incubator at 32-34 °C which was not closed completely to provide sufficient oxygen supply. Pups were sorted by sex and three pups per sex

were used for recordings. Subjects were recorded in random order and identifying marks were noted. For recording a pup, it was moved into an anechoic, sound attenuating chamber and audio was recorded for 3 min using UltraVox XT (Noldus Information Technology, the Netherlands) and the Observer XT (Noldus Information Technology, the Netherlands) using a gain of 95 dB. Before exporting the recordings, a 30 kHz filter was used in order to minimize background noise. For analysis, the EthoVision XT software (Noldus Information Technology, the Netherlands) was used.

3.2.3 Pup retrieval test

Pup retrieval tests were conducted on PD4 immediately after recordings of USVs, therefore the dam has been separated from her litter and placed in a holding cage for 30 min before the start of retrieval. For the pup retrieval test (PRT), four pups which were also used for USV recordings (two of each sex), were placed in the home cage on the opposite end than the nest site and their identification markings (tattooed spots on extremities) were noted. Any remaining pups of the litter were placed back inside the nest. Finally, the experimental female was returned to the nest and her behaviour video-recorded for 3 min using the Media Recorder (Noldus Information Technology, the Netherlands). If the displaced pups were not all retrieved within 3 min, the pups were returned to the nest and the trial was scored as a failure. Recorded videos were analysed for retrieval latencies for each pup (counted as retrieval if mother picked up the pup with its mouth and carried it back to nest). Following retrieval, the home cage was further video-recorded for 15 min in order to observe the amount of time the mother spent in the nest.

3.3 Neuronal activation mapping for c-Fos

Analysis of *c-fos* expression in neurons is a common method in behavioural neuroscience research to measure neuronal activity in the brain in response to a stimulus (Numan, 2014). Using immunofluorescent double-labelling for c-Fos and DAPI, the percentage of c-Fos expressing neurons can easily be determined.

3.3.1 Perfusion and brain extraction

On PD4, 90 min after start of the PRT, maternal brains were fixed by transcardial perfusions to prepare the tissue for immunohistochemistry. For this thesis, I used fixed brains provided by MRes Claire Owen, PhD student at the Department of Neurophysiology and Neuropharmacology at the Medical University of Vienna.

The mice were anesthetized with i.p. injections of a mix of 40 % Ketanest 25 mg/mL (Pfizer Inc., USA), 20 % Rompun 20 mg/mL (Bayer Animal Health GmbH, Germany) and 40 % of 0.9 % NaCl solution. Then, animals were perfused transcardially with 1 x PBS followed by 4 % formaldehyde (PFA) (Electron Microscopy Sciences, USA) in PBS and extracted brains were immersed in 4 % PFA at 4 °C for 24 h before being transferred to 30 % sucrose (Sigma-Aldrich Corp., USA) in PBS and stored for 48 h at 4 °C. To prepare the brains for slicing, they were embedded in a plastic mold containing OCT compound (Scigen Scientific Inc., USA), put on liquid nitrogen for 30 min and stored at -80 °C until further processing.

3.3.2 Cryosectioning

Brains were cut coronally into 30 µm sections with the Leica CM1950 Cryostat (Leica Biosystems GmbH, Germany) at -20 °C and collected free-floating in 24-well plates containing 1 x PBS and stored at 4 °C until immunohistochemical processing. All slices were stored in case more in-depth analysis later on would be necessary.

3.3.3 Immunohistochemistry

Two slices per brain for each of the regions of interest (ROI) were selected using the Allen Mouse Brain Atlas (Figure 2). ROIs are the central amygdala (CeA), medial amygdala (MeA) as well as hypothalamic regions: anterodorsal preoptic nucleus (ADP), anteroventral preoptic nucleus (AVP), anteroventral periventricular nucleus (AVPV), lateral preoptic area (LPOA), medial preoptic nucleus (MPN), medial preoptic area (MPOA), parastrial nucleus (PS) and ventrolateral preoptic nucleus (VLPO).

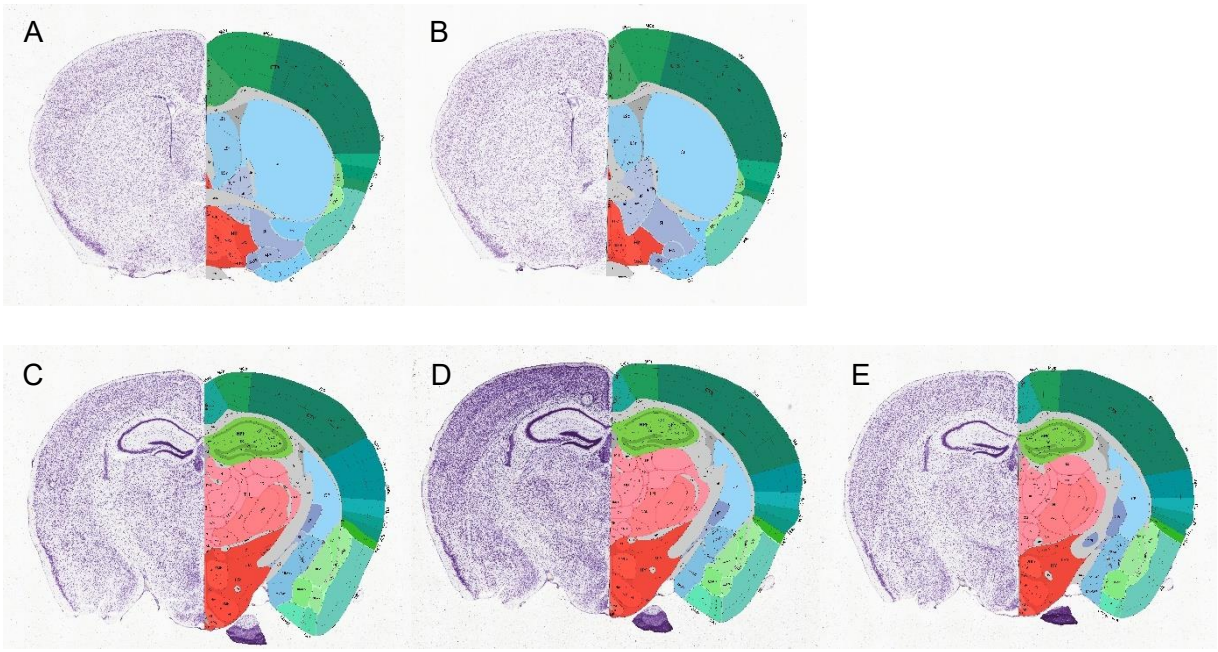

Figure 2: Reference images for ROIs taken from the Allen Mouse Brian Atlas

Coronal sections containing the ROIs of the hypothalamus from rostral to caudal (**A to B;** ROIs in red) and amygdala from rostral to caudal (**C to D to E;** ROIs in dark blue) (©2021. Allen Institute for Brain Science)

All tissue sections were collected in a 24-well plate with each well containing one section. Slices were washed 3 x 10 min in 1 x phosphate buffered saline ((PBS), pH 7.4, 1:10 dilution of 10X PBS with distilled water) at room temperature (RT) (~20 °C) on a shaker before being blocked for 2 h at RT on a shaker with 250 µL/well of blocking solution (10 % NHS (Gibco™, Thermo Fisher Scientific Inc., USA) and 0.5 % TritonX-100 (Triton™X-100, Sigma-Aldrich Corp., USA) in PBS). Following blocking, the plates were incubated for 48 h at 4 °C on a shaker containing 250 µL of primary antibody solution per well. The primary monoclonal antibody rabbit-Phospho-c-Fos (Cell Signaling Technology Inc., USA) was diluted 1:8,000 (stock solution 5 mg/mL) in 2 % NHS and 0.5 % TritonX-100 in PBS. Sections were then washed 3 x 10 min in PBS at RT on a shaker. Then, the polyclonal secondary antibody Alexa Fluor 647 goat anti-rabbit (Invitrogen Corp., USA) was diluted 1:500 (stock solution 2 mg/mL) in 2 % NHS and PBS. 250 µL/well of this antibody solution was added to the sections and incubated for 2 h at RT on a shaker in the dark. The slices were then washed 3 x 5 min in PBS and 250 µL of DAPI (Sigma-

Aldrich Cor., USA) diluted 1:2,000 (stock solution 1 mg/mL) in PBS was added to each well for 5 min at RT on a shaker. Finally, the sections were washed 3 x 5 min in PBS at RT on a shaker and mounted on microscope slides using Dako Fluorescence Mounting Medium (Dako North America Inc., USA), covered with cover slips (VWR® Superfrost® Plus Micro Slide, VWR International, USA) and kept at 4 °C in the dark until imaging.

3.3.4 Image acquisition and analysis

Brain slices were observed under a fluorescence microscope (Zeiss Axio Imager.Z2, Carl Zeiss AG, Germany) and imaged in 20x (numerical aperture (NA) = 0.5) magnification. Images were taken with the tiles modus by applying the stitching method in Zen 2.6 pro (Zeiss®, Carl Zeiss AG, Germany).

Image analysis was performed using ImageJ software. Brain areas were identified using the Allen Mouse Brain Atlas and immunoreactive neurons were counted in the ROI (see figure 3). The number of $cFos^+$ neurons and $DAPI^+$ neurons were automatically counted and the percentage of $cFos^+$ neurons was calculated. Automatically counted $cFos^+$ neurons were further validated by comparing these with manual counts which were carried out blinded.

Table 1: Imaging parameter for cFos and DAPI staining.

	excitation wavelength	emission wavelength	artificial colour	exposure time
Alexa Fluor 647 (cFos)	650 nm	673 nm	red	60 ms
DAPI	353 nm	465 nm	blue	60 ms

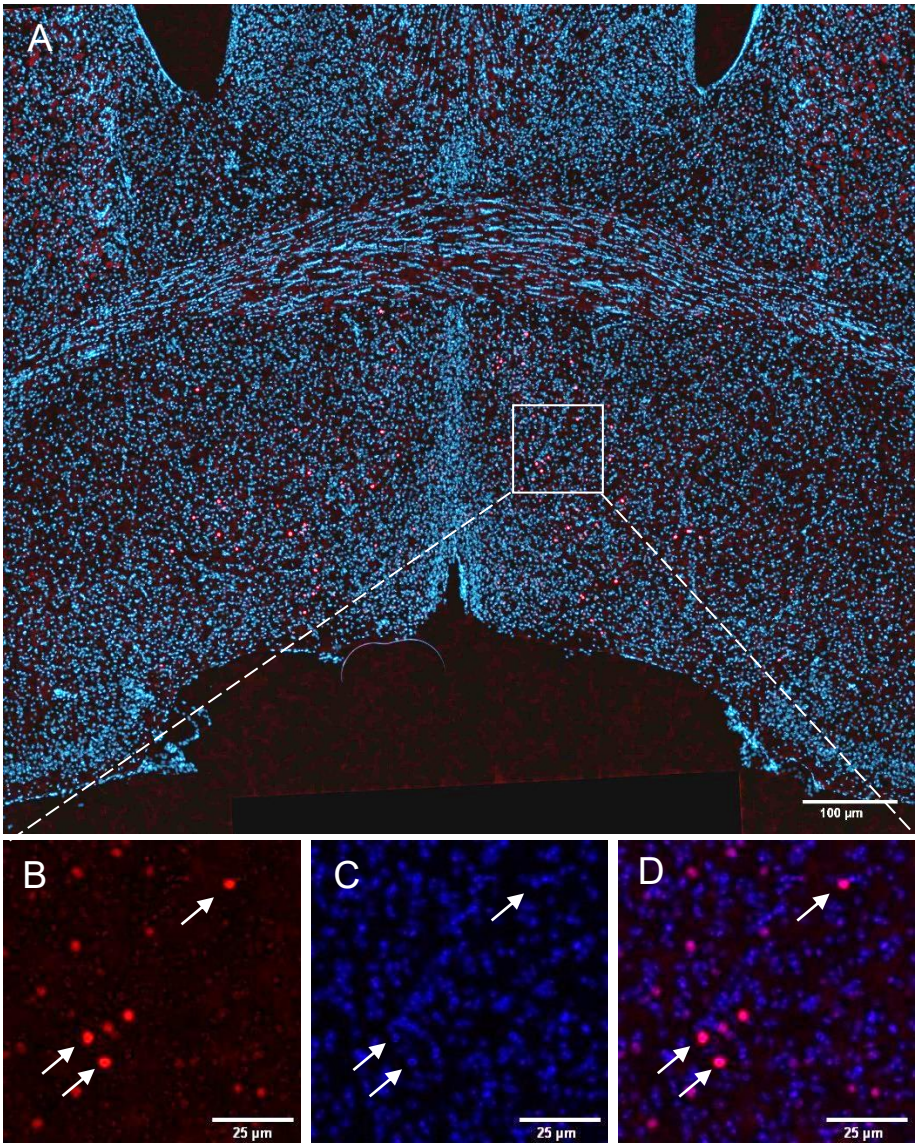

Figure 3: Counting of cFos$^+$ and DAPI$^+$ neurons

The figure demonstrates an example on how cells were counted. **A**: coronal section of the anterior hypothalamus containing the MPOA, brightness adjusted by + 60 %; **B, C and D** are magnifications of A, brightness adjusted by + 40 %; **B**: cFos-positive neurons; **C**: DAPI-positive neurons; **D**: merged image. Images were obtained with a fluorescence microscope with a 20 x (NA = 0.5) objective with the settings stated in table 1.

3.4 Statistical analysis

Before conducting statistical analysis, data was tested for normality with the Levene's test and transformed with either square root or log transformation when necessary. Spearman correlation was performed using SPSS (SPSS Inc., USA) and visualized with GraphPad Prism (GraphPad Software Inc., USA) and Microsoft Office Excel (Microsoft Corp., USA). The outcome results were interpreted according to the degree of association as weak ($r = 0.3$-0.5), moderate ($r = 0.5$-0.7) and strong ($r = 0.7$-1) and considered significant if $p < 0.05$.

4. Results

Nomenclature of mentioned brain regions and their abbreviations are described in part 8: Keywords and abbreviations.

4.1 Relationship between tube test wins and *c-fos* activity in the maternal brain

Descriptive statistics of Spearman's correlation coefficient (r), the level of significance (p) and number of subjects (N) were tabulated (Table 2) and selective correlations between tube test wins and neuronal activity were represented as scatter plots using untransformed data with r and p values of normalized data (Figure 4).

Analysis of $cFos^+$ neurons in hypothalamic regions and the amygdala in response to pup retrieval test and subsequent Spearman correlation analysis did neither show strong nor significant relationships between paternal tube test wins and neuronal activity in selected regions of the maternal brain (Table 2). Contrary to our expectations, paternal dominance did not exert a biologically relevant effect on neuronal activity in the maternal MPOA ($r = -.097$, $p = .721$), rather the CeA seems to be differentially activated ($r = .425$, $p = .168$). Though this effect is not significant and of weak to moderate degree and can be interpreted as a tendency for the CeA being differentially activated in dominant versus subordinate mated mothers. It is apparent that the relationship between tube test wins and neuronal activation differs depending on the brain region, however, based on these correlations, a direct effect of paternal social status represented as tube test wins on maternal neuronal activation in response to pup retrieval cannot be postulated.

Table 2: Correlation between paternal tube test wins and *c-fos* activity in the maternal brain. Spearman's correlation coefficient (r) and its level of significance (p) and number of subjects (N); sorted after the correlation coefficient (r). Amygdala (CeA and MeA); Hypothalamus (ADP, AVP, AVPV, LPOA, MPN, MPOA, PS, VLPO)

Variables between	r	p	N
TTwins & CeA	0.425	0.168	12
TTwins & Amygdala	0.334	0.288	12
TTwins & VLPO	0.231	0.39	16
TTwins & PS	0.202	0.454	16
TTwins & LPOA	0.161	0.552	16
TTwins & Hypothalamus	0.05	0.854	16
TTwins & AVP	-0.055	0.841	16
TTwins & MPOA	-0.097	0.721	16
TTwins & ADP	-0.106	0.695	16
TTwins & AVPV	-0.141	0.602	16
TTwins & MPN	-0.174	0.518	16
TTwins & MeA	-0.251	0.432	12

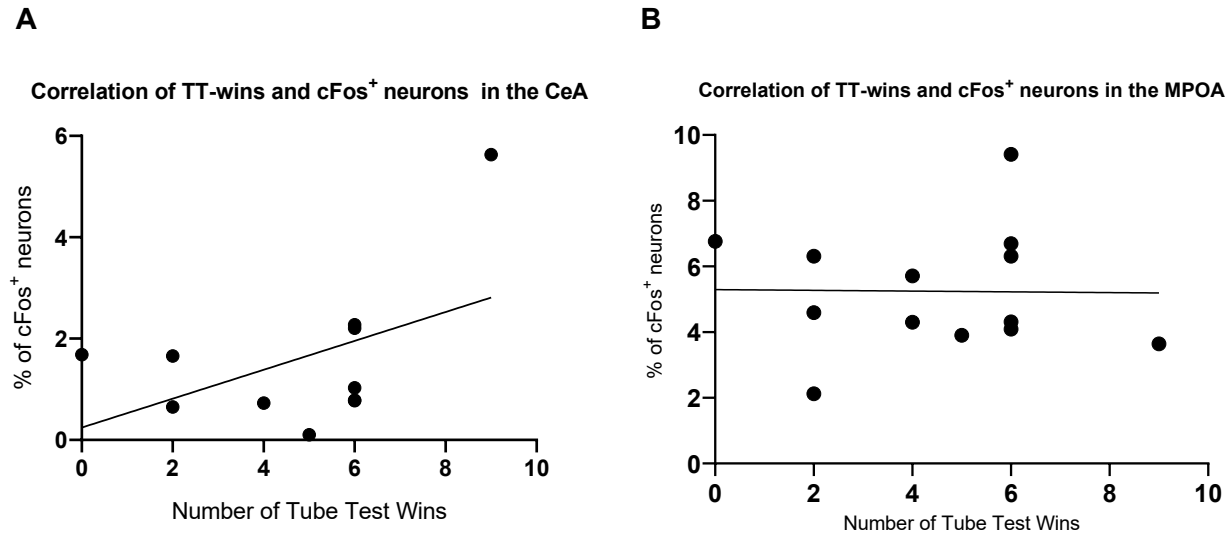

Figure 4: Correlation of tube test wins versus *c-fos* activity in the maternal brain. Scatter plots showing **A**: Spearman positive correlation between paternal tube test wins and cFos$^+$ neurons in the maternal central amygdala (CeA) (r = .425, p= .168). **B**: Spearman negative correlation between paternal tube test wins and cFos$^+$ neurons in the maternal medial preoptic area (MPOA) (r = -.097, p = .721)

4.2 Relationship between pup vocalizations and retrieval behaviour of the dam

Descriptive statistics of Spearman's correlation coefficient (r), the level of significance (p) and number of subjects (N) were tabulated (Table 3 and 4) and selective correlations between pup ultrasonic vocalizations and retrieval behaviour were represented as scatter plots using untransformed data with r and p values of normalized data (Figure 5)

Investigation of pup USVs and retrieval behaviour independently of paternal tube test wins shows a prominent relationship between the percentage of pup USV-complexity and retrieval latencies (Table 3 and 4). USVs are categorized into complex and non-complex vocalizations. Complex USVs are longer with multiple peaks and contain both upward and downward patterns of different frequencies. Non-complex USVs are short and are of one frequency without patterns. It appears the higher the percentage of complex ultrasonic vocalizations pups emit, the faster the dam retrieves them back to the nest site. All retrieval latencies (except latency to retrieve

third pup) are moderately to strongly negatively correlated to the percentage of complex USVs (Table 3), with this relationship being statistically significant. In contrast, the percentage of non-complex USVs has a significantly moderate to strong positive correlation with retrieval latencies (excluding latency to retrieve third pup) (Table 4). These results demonstrate clearly that the proportion of complex to non-complex USVs emitted impacts how quickly pups are retrieved back to the nest. Since there was no noteworthy correlation observed between tube test wins and pup USVs, this effect of pup USVs on retrieval behaviour of the dam is only detected when analysing the data independently of paternal tube test wins and hence, the effect cannot be led back to differential vocalizations of offspring from fathers of different dominance status.

Table 3: Correlations between percentage of complex pup USVs and retrieval latencies. Spearman's correlation coefficient (r) and its level of significance (p) and number of subjects (N); sorted after the correlation coefficient (r). LatToFirst = latency to retrieve the first pup back to the nest; LatToSecond = latency to retrieve the second pup back to the nest; LatToThird = latency to retrieve the third pup back to the nest; LatToFourth = latency to retrieve the fourth pup back to the nest

Variables between	r	p	N
%ComplexUSVs & LatToSecond	-0.73	0.005	13
%ComplexUSVs & LatToFirst	-0.639	0.019	13
%ComplexUSVs & LatToFourth	-0.576	0.039	13
%ComplexUSVs & LatToThird	-0.429	0.144	13

Table 4: Correlations between percentage of none-complex pup USVs and retrieval latencies. Spearman's correlation coefficient (r) and its level of significance (p) and number of subjects (N); sorted after the correlation coefficient (r). LatToFirst = latency to retrieve the first pup back to the nest; LatToSecond = latency to retrieve the second pup back to the nest; LatToThird = latency to retrieve the third pup back to the nest; LatToFourth = latency to retrieve the fourth pup back to the nest

Variables between	r	p	N
%NoneComplexUSVs & LatToSecond	0.766	0.002	13
%NoneComplexUSVs & LatToFirst	0.661	0.014	13
%NoneComplexUSVs & LatToFourth	0.62	0.024	13
%NoneComplexUSVs & LatToThird	0.473	0.103	13

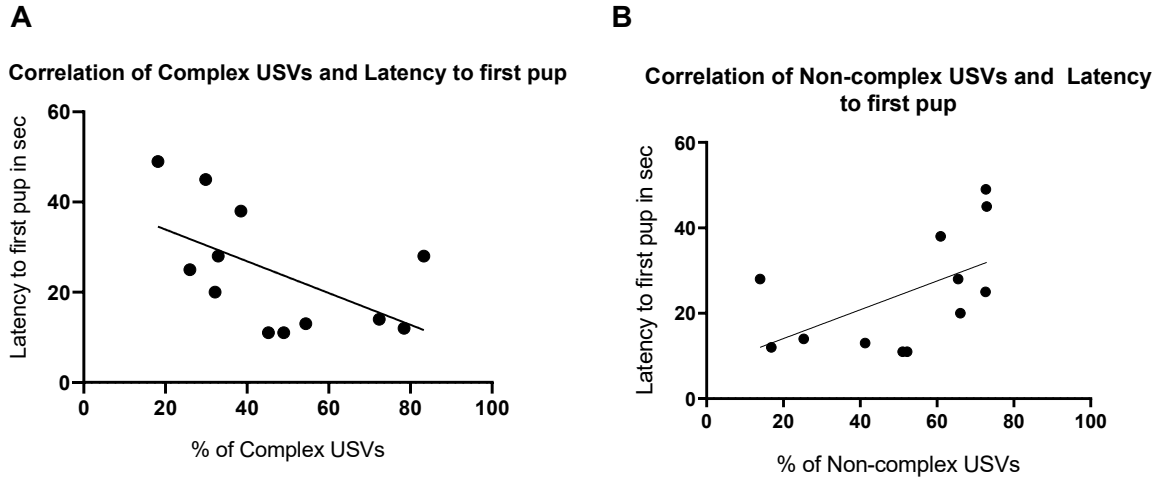

Figure 5: Correlation of pup USVs versus Latency to retrieve first pup. Scatter plots showing **A**: Spearman negative correlation between the percentage of complex USVs and latency to retrieve first pup (r = -.639, p= .019). **B**: Spearman positive correlation between the percentage of non-complex USVs and latency to retrieve first pup (r = .661, p = .014)

4.3 Relationship between pup vocalizations and *c-fos* activity in the maternal brain

Descriptive statistics of Spearman's correlation coefficient (r), the level of significance (p) and number of subjects (N) were tabulated (Table 5 and 6) and selective correlations between pup

ultrasonic vocalizations and neuronal activity were represented as scatter plots using untransformed data with r and p values of normalized data (Figure 6)

Analysis of $cFos^+$ neurons in hypothalamic regions and the amygdala in response to pup retrieval test (independently of paternal tube test wins) and subsequent Spearman correlation analysis showed a significant relationship between pup USVs and neuronal activation in the maternal hypothalamus but not the amygdala (Table 5 and 6). All hypothalamic regions display a moderate to strong negative correlation to the percentage of complex USVs, with the most pronounced effect observed in the anteroventral preoptic nucleus (AVP) ($r = -.824$, $p < .001$). In line with this, neuronal activation in the AVP is strongly positively correlated to the percentage of non-complex USVs emitted from pups ($r = .857$, $p < .001$). Concluding from these results, the AVP, a subregion of the preoptic area (POA), seems to play a crucial part in the assessment of USV-complexity.

Table 5: Correlation between percentage of complex pup USVs and *c-fos* activity in the maternal brain. Spearman's correlation coefficient (r) and its level of significance (p) and number of subjects (N); sorted after the correlation coefficient (r).

Variables between	r	p	N
%ComplexUSVs & AVP	-0.824	<.001	13
%ComplexUSVs & Hypothalamus	-0.747	0.003	13
%ComplexUSVs & MPN	-0.731	0.005	13
%ComplexUSVs & PS	-0.714	0.006	13
%ComplexUSVs & ADP	-0.665	0.013	13
%ComplexUSVs & MPOA	-0.654	0.015	13
%ComplexUSVs & AVPV	-0.637	0.019	13
%ComplexUSVs & VLPO	-0.626	0.022	13
%ComplexUSVs & LPOA	-0.621	0.024	13
%ComplexUSVs & CeA	-0.4	0.223	11
%ComplexUSVs & Amygdala	-0.345	0.298	11
%ComplexUSVs & MeA	-0.218	0.519	11

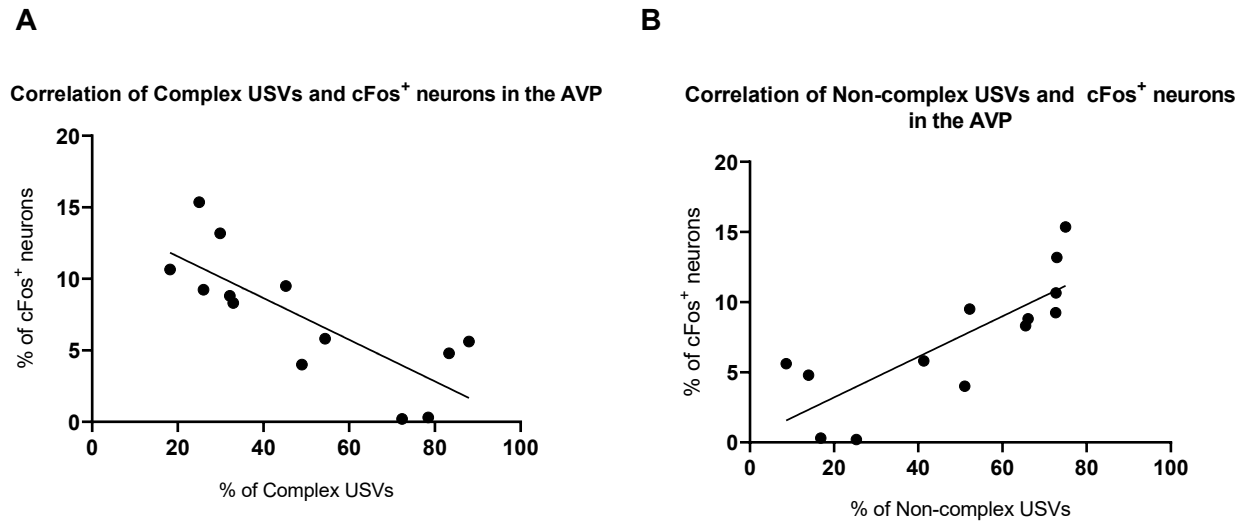

Figure 6: Correlation of pup USVs versus *c-fos* activity in the maternal brain. Scatter plots showing **A**: Spearman negative correlation between the percentage of complex USVs and cFos$^+$ neurons in the maternal AVP (r = -.824, p= <.001). **B**: Spearman positive correlation between the percentage of non-complex USVs and cFos$^+$ neurons in the maternal AVP (r = .857, p = <.001)

Table 6: Correlation between percentage of none-complex pup USVs and *c-fos* activity in the maternal brain. Spearman's correlation coefficient (r) and its level of significance (p) and number of subjects (N); sorted after the correlation coefficient (r).

Variables between	r	p	N
%NoneComplexUSVs & AVP	0.857	<.001	13
%NoneComplexUSVs & Hypothalamus	0.813	<.001	13
%NoneComplexUSVs & MPN	0.808	<.001	13
%NoneComplexUSVs & PS	0.791	0.001	13
%NoneComplexUSVs & ADP	0.747	0.003	13
%NoneComplexUSVs & MPOA	0.742	0.004	13
%NoneComplexUSVs & LPOA	0.709	0.007	13
%NoneComplexUSVs & AVPV	0.692	0.009	13
%NoneComplexUSVs & VLPO	0.654	0.015	13
%NoneComplexUSVs & CeA	0.355	0.285	11
%NoneComplexUSVs & Amygdala	0.355	0.285	11
%NoneComplexUSVs & MeA	0.209	0.537	11

4.4 Relationship between retrieval latencies and *c-fos* activity in the maternal brain

Descriptive statistics of Spearman's correlation coefficient (r), the level of significance (p) and number of subjects (N) were tabulated (Table 7) and the correlation between retrieval latency to first pup and neuronal activity in the AVP was represented as a scatter plot using untransformed data with r and p values of normalized data (Figure 7)

Investigation of retrieval latencies and neuronal activation independently of paternal tube test wins shows a distinct relationship between the start to retrieve pups and *c-fos* activity in the AVP ($r = .625$, $p = .03$) compared to other hypothalamic regions or the amygdala. Seeing as the AVP is also strongly correlated to pup USVs, this leads to the assumption that the AVP may play a critical part in mediating pup retrieval and assessing USVs in mice. To test this hypothesis, more

thorough investigation and classification of cell types in this preoptic subregion needs to be carried out.

Table 7: Correlation between latency to retrieve first pup and *c-fos* activity in the maternal brain. Spearman's correlation coefficient (r) and its level of significance (p) and number of subjects (N); sorted after the correlation coefficient (r). LatToFirst = latency to retrieve the first pup back to the nest

Variable between	r	p	N
LatToFirst & AVP	0.625	0.03	12
LatToFirst & Hypothalamus	0.365	0.243	12
LatToFirst & VLPO	0.351	0.263	12
LatToFirst & LPOA	0.337	0.284	12
LatToFirst & PS	0.337	0.284	12
LatToFirst & MPN	0.27	0.396	12
LatToFirst & MeA	0.231	0.521	10
LatToFirst & MPOA	0.204	0.526	12
LatToFirst & ADP	0.154	0.632	12
LatToFirst & CeA	0.146	0.688	10
LatToFirst & Amygdala	0.109	0.763	10
LatToFirst & AVPV	0.018	0.957	12

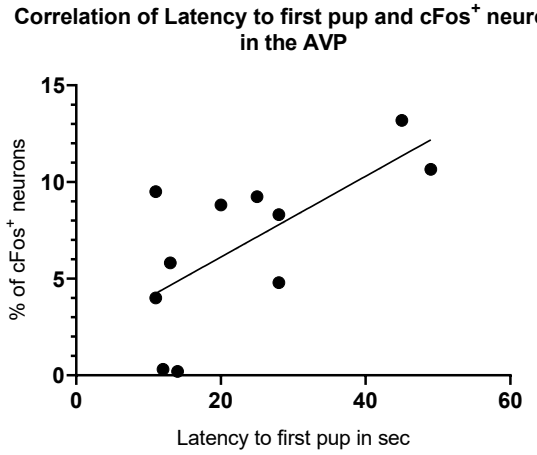

Figure 7: Correlation of Latency to retrieve first pup versus *c-fos* activity in the maternal AVP. Scatter plot showing Spearman positive correlation between Latency to retrieve first pup and cFos$^+$ neurons in the maternal AVP ($r = .625$, $p = .03$).

5. Discussion

The amount and quality of maternal care offspring receive can be predictive of their future physiology, mental state and reproductive success (Dulac et al., 2014). Thus, it is crucial to better understand factors which may exert a modulatory effect on this caregiving behaviour. Plasticity in maternal reproductive investment has been associated with mate quality, which creates an indirect pathway for fathers to influence offspring development (Champagne, 2020). This study investigated the effect of paternal social status on maternal behaviour and neuronal activation in response to a specific stimulus (pup separation and retrieval) eliciting maternal responsiveness.

5.1 Social dominance as an indicator of male quality and mating strategy

One common method to determine social dominance ranks under laboratory conditions is using the tube test (*i.e.,* competitive exclusion task) in which the number of retreats or wins of competitors in a face-to-face encounter within a PVC tube is assessed (Varholick et al., 2018). However, there have been reports of a learned component of tube test outcomes as well as instable hierarchies impacting test results (Barabas et al., 2021; Varholick et al., 2018; Varholick et al., 2019). Further, considering the test being carried out outside the home cage, anxiety may on some occasions be the cause of mice remaining inside the tube and not dominance over an opponent (Barabas et al., 2021). For this reason, additional assessments of male quality might be taken into consideration. Levels of MUP20 (darcin) have been connected to social rank and high levels favoured by potential mating partners (Barabas et al., 2021; Hoffman et al., 2015; Lee et al., 2017; Veyrac et al., 2011). Measuring preputial gland: body length ratio presents an additional method to validate social dominance scores (Barabas et al., 2021) as well as analysis of brain gene expression. Changes in corticotropin-releasing factor (CRF) mRNA expression in MeA, CeA and MPOA and hippocampal brain-derived neurotrophic factor (BDNF) and glucocorticoid receptor (GR) expression have been associated with individual differences in rank (So et al., 2015). As mentioned in the introduction, the absence of clear results may also be the result of a wrongly chosen trait (social dominance) as a correlate. (Ratikainen and Kokko, 2010). Furthermore, the mating strategy has to be taken into consideration when interpreting present results. Past research has shown that the direction of differential allocation (positive/negative) is

highly dependent on mating circumstances. Meaning negative differential allocation (reproductive compensation) is more likely to occur when females are not given free choice in whom they mate with (Gowaty et al., 2007; Sheldon, 2000). When given free choice to mate with preferred males, dams displayed higher maternal investment, and paternally driven maternal effects can be observed (Curley et al., 2011). Thus, the decision to mate randomly in this study may have blurred the direction of DA by introducing additional variables impacting MCB which were not accounted for.

5.2 Neuronal activation of the MPOA in relation to paternal social status

Though the overall percentage of activated neurons in the MPOA was among the highest out of the observed brain regions, correlation analysis between $cFos^{+}$ neurons in the maternal MPOA and paternal social status did not reveal a notable relationship between these two variables ($r = -.097$, $p = .721$). Thus, since all dams displayed appropriate maternal behaviour and retrieved pups, the MPOA seems to be highly active during pup retrieval regardless of paternal social status. This confirms previous findings about the MPOA (and adjacent BNST) being critical for mother-initiated active behaviours like pup retrieval and nest building (Watson et al., 2012). However, since only neuronal activity was observed, no conclusions about possible differential activation of different cell populations can be drawn and their further investigation would present a way to decipher involved cell types. *E.g.,* Fang and colleagues identified estrogen receptor alpha (Esr1)-expressing cells within the MPOA as having a mediating role for pup approach and retrieval. Additionally, a strong inhibitory projection from $MPOA^{Esr1}$ neurons to non-dopaminergic cells in the VTA was observed, which was revealed to be crucial for driving retrieval behaviour (Fang et al., 2018). Not only is Erα expression in the MPOA associated with individual differences in maternal care display and anxiety behaviour, estrogen further enhances oxytocin receptor (OTR) binding in the MPOA. Oxytocin (OT) itself serves diverse functions, including infant reward, maternal care, social attachment and anxiolytic effects. Maternal motivation and infant reward states are potentially facilitated by interactions of OT with the mesolimbic dopamine system (Mchenry et al., 2015). Thus, to understand of which cell types these activated neurons are comprised of, a triple-probe-FISH system can be employed using RNA probes for OTR and Esr1 among other molecular markers relevant in this context like

Galanin (GAL) (Wu et al., 2014) and glutamate decarboxylase 1 (GAD67) (Tsuneoka et al., 2013; Zhang et al., 2021).

5.3 The role of the CeA in mediating stress and fear responses

Spearman correlation analysis of paternal tube test wins and neuronal activation in the maternal brain showed a tendency for the CeA being differentially activated ($r = .425$, $p = .168$) in dominant versus subordinated mated females. Considering the stressful events of pup separation and subsequent retrieval, this observed effect in the CeA might be explained by the crucial role the CeA plays in emotional processing. It was shown to be highly susceptible to stressful events with increased release of neurotransmitters such as glutamate, GABA, serotonin and noradrenaline (Andolina and Borreca, 2017). The CeA expresses a variety of neuropeptides and neuropeptide receptors, including ones for vasopressin and oxytocin. While the former enhances aggressiveness, stress levels and anxiety, the latter leads to decreased stress and anxiety levels and facilitates social encounters and maternal care (Huber et al., 2005). Elevated OT levels in dams are implicated in a number of maternal behaviours such as nursing, pup retrieval, maternal aggression and suppression of freezing when being threatened (Rickenbacher et al., 2017). During the peripartum period, expression of OTR increases within various brain regions, including the MPOA, CeA, ventromedial nucleus of the hypothalamus (VMH), NA, olfactory bulb (OB), PVN, BNST and VTA with these sites being implicated as mediating the behavioural effects of OT during postpartum (Sabihi et al., 2014). A modulatory effect of OT on the processing of olfactory input has been proposed, thereby influencing the amygdaloid function to depress fear-related behaviours (Watson et al., 2012). OT acting in the CeA has been shown to be involved in post-partum reduced anxiety and to underly the suppression of maternal freezing necessary for actively defending pups (Rickenbacher et al., 2017). As this leaves cardiovascular responses unaffected, this specific regulation may preserve the internal, visceral expression of fear but at the same time reduce freezing behaviour, which provides the animal with the ability to react adequately in circumstances where a proactive behavioural response is necessary (Viviani et al., 2011). Considering pup retrieval constitutes a proactive form of maternal behaviour (Salais-López et al., 2021), more in-depth characterization of the detected $cFos^+$ neurons in the CeA may provide better insights into which neurotransmitters and receptors are involved.

5.4 The relationship between pup vocalizations and retrieval latencies

The results of this study show a strong correlation between pup vocalizations and maternal behaviour during pup retrieval test. With rising percentage of complex USVs, retrieval latencies drastically declined, speaking for increased maternal responsiveness. However, non-complex vocalizations do not evoke a similar response, leading to the assumption that the ratio between complex and non-complex USVs is crucial for eliciting fast pup approach and retrieval by the dam. It has been previously shown that dams are able to recognize specific profiles of pup vocalizations, which affirms the observed effect of USV profile on retrieval behaviour. Carrying out the behavioural assay in the home cage might have further reinforced the effect of USVs on maternal behaviour since it has been demonstrated that pup odour in conjunction with USVs synergistically initiate pup approach by the mother (Okabe et al., 2013).

5.5 The relationship between neuronal activity in the AVP, USVs and retrieval behaviour

Correlation analysis shows a considerable link between pup USVs, retrieval behaviour (latencies to retrieve pups) and *c-fos* activity in the maternal anteroventral preoptic nucleus. It appears the AVP is less active the higher the percentage of complex USVs offspring emit, which also correlates with decreased retrieval latencies. Meaning heightened maternal responsiveness is characterized by reduced neuronal activity in the AVP. How this relationship is formed, and underlying mechanisms remain elusive given the lack of literature about the possible involvement of the AVP in maternal behaviours, specifically pup retrieval. The AVP is rather known as the thermointegrative and thermosensitive site of the central nervous system (CNS) and as being involved in mediating fever and regulated hypothermia (Gargaglioni et al., 2006). It has also been proposed that thermoregulatory regions are also involved in ventilatory responses, particularly respiratory frequency, although these functions have been observed mainly in the context of hypoxia (Barros et al., 2006; Kwiatkoski et al., 2014; Ripamonte et al., 2020). Given the strong correlation between retrieval latencies and AVP activity, a hypothetical role of the AVP in respiratory responses might be worth further investigation.

5.6 Concluding remarks

Results from this study suggest only a limited role for paternal social status as a driver for DA and its role in eliciting differential MCB could not be established. Expected differential neuronal activation of the maternal MPOA in response to pup stimuli was not observed, which might be explained by its heterogeneity and broad role in maternal behaviours as well as other physiological and behavioural functions such as sexual behaviour, sleep, ovulation, feeding and thermoregulation (Lin et al., 2018; Numan, 2014). However, an effect of paternal dominance on maternal behaviour was seen in the neuronal activation of the central amygdala (CeA), a region crucial for emotional processing and mediating fear and anxiety responses (Andolina and Borreca, 2017). Considering previously proposed actions of oxytocin in the CeA in the post-partum reduction of anxiety (Rickenbacher et al., 2017), more thorough characterization of involved neurons may shed light on this observed trend. Investigation of pup vocalizations and maternal behaviour independently of paternal social status revealed a strong relationship between pup USVs and retrieval latencies. Specifically, the ratio of complex to non-complex USVs appears to be crucial in evoking fast pup approach and retrieval. Additionally, a considerable correlation between neuronal activation in the AVP and retrieval behaviour as well as to pup USVs was detected. However, a link between these observations and paternal dominance cannot be drawn due to the lack of correlation between paternal tube test wins and these variables. Further, the possibility has to be considered that social dominance might not be the main driver for DA and therefore, other characteristics of males might present a better predictor for DA. Additionally, tube test wins as a sole representative for social status is possibly not accurate enough and additional measures for social dominance should be added to the study design.

The amount and quality of care mothers display towards offspring can be predictive of their physiology, mental state and reproductive success later in life (Dulac et al., 2014). Therefore, it is crucial to better understand factors, like male quality, which may have a modulatory effect on this caregiving behaviour. Especially considering that these adjustments of MCB present an indirect pathway through which fathers can influence offspring development und consequently alter the direction and rate of evolution (Sheldon, 2000).

6. Summary

In many species, offspring depend on parental care in order to survive and develop mentally and physically well. Differences in the degree and quality of this caregiving behaviour can have great implications for offspring physiology and subsequent reproductive success. Such plasticity in reproductive investment has been shown to be associated with a variety of factors, one of which is mate quality. This occurrence, where a mother exerts different degrees of maternal investment depending on mate quality, is described as the differential allocation hypothesis and creates an indirect pathway through which paternal effects might be transmitted to offspring. These variations in maternal care behaviour (MCB) can be observed in expression patterns in brain regions crucial for maternal behaviour such as different regions of the hypothalamus, particularly the medial preoptic area (MPOA), which has long been considered a key region for the positive control of parental behaviour and is involved in both parturition and throughout the postpartum period. A widespread tool to detect such changes in neuronal activity in response to various stimuli is the analysis of *c-fos* expression, an immediate early gene.

This study aimed to uncover differential allocation as a driver for MCB in the mouse, hereby postulating a role for the female in the indirect transmission of paternal effects, by exploring the impact of paternal social status on maternal neuronal activation during a behavioural assay used to evoke MCB. Females were randomly mated with males of different social dominance status, which was used as an indicator of male quality and represented by tube-test wins. On post-natal day 4, *c-fos* expression in hypothalamic regions and the amygdala in response to a brief separation from pups and subsequent pup retrieval test (PRT) was immunohistochemically analysed. Pup retrieval is a behavioural assay used to study maternal care behaviour and subsequent brain activity after a mother is separated from her pups. Potentially ultrasonic vocalisations (USV) emitted by the pups could influence how readily the mother brings them back to the nest. This behaviour is also affected by maternal motivation and phenotype.

Spearman correlation analysis did not show a significant relationship of tube test wins to neither the *c-fos* expression in amygdala, nor the MPOA or other hypothalamic regions. The most pronounced correlation is a weak to moderate observed between tube test wins and the central

amygdala (CeA) ($r = .425$, $p = .168$), although not significant. In general, the relationship between neuronal activity and pup retrieval behaviour varies depending on the hypothalamic region. Investigation of pup behaviour and maternal brain activity independently of paternal tube test wins revealed a particularly noteworthy relationship between *c-fos* activity in the anteroventral preoptic nucleus (AVP) and the complexity of USVs of pups ($r = -.824$, $p < .001$) as well as to the start of retrieving pups ($r = .625$, $p = .03$). Further, pup retrieval behaviours and complexity of pup USVs seem to have a strong association to one another.

The lack of clear results between male social dominance and neuronal activity might stem from a wrongly chosen predictor of social status. Tube test wins may not be fully representative of social status, or social status itself may not be the primary driver for DA to occur. Small sample size may make it difficult to achieve statistical significance. Additionally, natural variations in maternal care, anxiety levels and pup vocalizations may also make it harder to see the main effect of paternal social dominance on MCB in respect to pup retrieval. Another fact to consider is the study design: the lack of choice and forced mating with non-preferred males may have introduced additional variables that impacted MCB. Since the observed *c-fos* expression is in response to the stress induced by pup separation and subsequent retrieval, this neuronal activity is only representative of these specific stimuli and not of other maternal behaviours. Interestingly though is the relationship between paternal social status and activity in the CeA, since this region is critically involved in mediating fear and stress responses. Although there is a sparsity of studies investigating the CeA in relation to MCB, postpartum-associated reduced anxiety has been attributed to oxytocin acting within the CeA, which might be worth further investigation within this context. However, in order to draw more conclusive observations, next steps should include a more thorough determination of male quality as well as a better characterization of the detected c-Fos-ir neurons to the able to decipher cell populations.

7. Zusammenfassung

In vielen Tierspezies sind Nachkommen von elterlicher Fürsorge abhängig, um zu überleben und sich mental und physisch gut zu entwickeln. Unterschiede in Ausmaß und Qualität dieses Pflegeverhaltens können gravierende Auswirkungen auf Physiologie der Nachkommen und deren späteren reproduktiven Erfolg haben und sind assoziiert mit einer Vielzahl von Faktoren, unter anderem die Qualität des Partners. Die differentielle Allokationshypothese beschreibt ebendieses Event, bei welchem eine Mutter, basierend auf der Qualität des Partners, ein unterschiedliches Maß an Fürsorge ihrer Nachkommen gegenüber ausübt. Dies kreiert einen indirekten Weg, durch welchen paternale Effekte auf Nachkommen übertragen werden können. Diese Variationen im Fürsorgeverhalten spiegeln sich in Expressionsmustern bestimmter Gehirnregionen wider, welche in maternalem Verhalten involviert sind. Regionen des Hypothalamus, insbesondere die mediale präoptische Region (MPOA), spielen eine Schlüsselrolle in der positiven Kontrolle elterlicher Fürsorge und sind sowohl bei Geburt als auch postpartal involviert. Eine weitverbreitete Methode, um solche Veränderungen in neuronaler Aktivität in Reaktion auf verschiedene Stimuli zu bestimmen, ist die Analyse der *c-fos*-Expression, eines Immediate Early Gens, welches als Antwort auf extra- oder intrazelluläre Reize exprimiert wird.

Ziel dieser Studie war, differentielle Allokation als treibende Kraft für maternales Verhalten in der Maus aufzuzeigen und postuliert hiermit eine Rolle der Mutter in der indirekten Übertragung paternaler Effekte auf Nachkommen. Der Einfluss des väterlichen sozialen Status in Hinblick auf die maternale neuronale Aktivation während eines Verhaltenstests wird untersucht, wobei der Test dazu dient, mütterliches Verhalten hervorzurufen. Die Weibchen wurden nach dem Zufallsprinzip mit Männchen unterschiedlichem sozialen Status verpaart, welcher als Indikator der Partnerqualität herangezogen wurde und durch Röhrentest-Gewinne repräsentiert wird. Am postnatalen Tag 4 (PD4) wurden Mutter und Jungtiere getrennt und der Pup-Retrieval-Test durchgeführt. Die aus diesem Ereignis resultierende c-fos Expression in hypothalamischen Regionen und Amygdala wurde immunhistochemisch analysiert. Der Pup-Retrieval-Test ist ein Verhaltenstest, in welchem Jungtiere außerhalb des Nests platziert werden und das Muttertier sie zurückholt. Er wird durchgeführt, um mütterliches Verhalten und neuronale Aktivität zu untersuchen. Dieses Verhalten kann potenziell von Vokalisationen der Jungtiere im

Ultraschallbereich (USV) beeinflusst werden, als auch durch mütterliche Motivation und Phänotyp.

Die Spearman-Korrelationsanalyse zeigte keine signifikante Beziehung zwischen Röhrentest-Gewinnen und *c-fos*-Expression weder in Amygdala, noch in MPOA oder anderen hypothalamischen Regionen. Die am stärksten ausgeprägte Korrelation ist eine schwache bis mäßige zwischen Tube Test Gewinnen und zentraler Amygdala ($r = .425$, $p = .168$), ist jedoch nicht signifikant. Im Allgemeinen variiert die Beziehung zwischen neuronaler Aktivität und Pup-Retrieval-Verhalten je nach Region des Hypothalamus. Analyse von Jungtierverhalten und maternaler Gehirnaktivität unabhängig von väterlichen Testsiegen zeigt eine prägnante Beziehung zwischen *c-fos*-Aktivität im anteroventralen präoptischen Nukleus und der Komplexität der USVs ($r = .824$, $p < .001$) als auch zum Beginn der Rückholung des ersten Jungtieres ($r = .625$, $p = .03$). Darüber hinaus scheint eine starke Assoziation zwischen Pup-Retrieval-Verhalten der Mutter und der Komplexität der USVs vorzuliegen.

Das Fehlen eindeutiger Ergebnisse zwischen sozialer Dominanz und neuronaler Aktivität könnte auf einen falsch gewählten Prädiktor für sozialen Status zurückzuführen sein. Tube-Testsiege sind möglicherweise nicht vollständig repräsentativ für sozialen Status, oder letzterer selbst ist potenziell nicht ein primärer Einflussfaktor für DA. Eine zu geringe Zahl an Tieren kann es erschweren, statistische Relevanz zu erreichen. Zusätzlich können natürliche Variationen in maternaler Fürsorge, Angstlevel und Vokalisationen der Jungtiere die Erkennung des Haupteffekts der väterlichen Dominanz im Hinblick auf mütterliches Fürsorgeverhalten erschweren. Weiters zu berücksichtigen ist das Studiendesign: der Mangel an Auswahl und erzwungene Verpaarung mit nicht bevorzugten Partnern hat möglicherweise zusätzliche Variablen geschaffen, welche Auswirkungen auf das Fürsorgeverhalten haben. Da die beobachtete *c-fos*-Expression eine Reaktion auf einen spezifischen Stimulus darstellt (PRT), ist sie nicht repräsentativ für andere mütterliche Verhaltensweisen. Interessant jedoch ist die Beziehung zwischen sozialem Status und Aktivität in der zentralen Amygdala (CeA), da diese Region entscheidend an der Vermittlung von Angst- und Stressreaktionen beteiligt ist. Obwohl es nur wenige Studien über die Beziehung von CeA und Fürsorgeverhalten gibt, wurde die postnatale Reduzierung von Angst auf die Wirkung von Oxytocin in der CeA zurückgeführt, was in diesem Kontext relevant sein könnte. Um jedoch schlüssigere Beobachtungen ziehen zu

können, sollten nächste Schritte eine ausführlichere Bestimmung der Partnerqualität sowie eine bessere Charakterisierung der detektierten c-Fos-positiven Neuronen umfassen.

8. Keywords and Abbreviations

ADP	Anterodorsal preoptic nucleus
AVP	Anteroventral preoptic nucleus
AVPV	Anteroventral periventricular nucleus
BDNF	Brain-derived neurotrophic factor
CeA	Central amygdala
CNS	Central nervous system
CRF	Corticotropin-releasing factor
DA	Differential Allocation
GAD67	Glutamate decarboxylase 1
GAL	Galanin
GMC	Gray matter concentration
GR	Glucocorticoid receptor
IC	Insular cortex
LPOA	Lateral preoptic area
MeA	Medial amygdala
MPN	Medial preoptic nucleus
MPOA	Medial preoptic area
MPFC	Medial prefrontal cortex
MUPs	Major urinary proteins
NA	Nucleus accumbens
NHS	Normal horse serum
OB	Olfactory bulb
OF	Orbitofrontal cortex
OT(R)	Oxytocin(receptor)
PBS	Phosphate buffered saline
PD4	Post-natal day 4
PI	Parental Investment
POA	Preoptic area
PRT	Pup retrieval test

PS	Parastrial nucleus
PVN	Paraventricular nucleus
RC	Reproductive Compensation
USV	Ultrasonic vocalization
vBNST	Ventral part of the bed nucleus of the *stria terminalis*
VLPO	Ventrolateral preoptic nucleus
VMH	Ventromedial nucleus of the hypothalamus
VP	Ventral pallidum

9. List of figures and tables

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