Sex-dependent implications of Melanotan II treatment and inflammatory responses following partner loss in the prairie vole (*Microtus ochrogaster*)

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Abstract

Abstract

In mammals, social relationships are important for the well-being and the survival of individuals. Social relationships can be exemplified as parent-infant bonds, relationships between conspecifics, and partner bonds. A key regulator which is involved in the formation of social relationships is the neuropeptide oxytocin (OT). Positive social relationships affect both mental and physical health, buffer against stressful experiences, and exert beneficial effects on immune system functions. In contrast, impaired social relationships, social isolation or the loss of the bonded partner are accompanied by an aggravation of physical and mental health. The loss of a partner is associated with a disruption of the immune system functioning in humans and can lead to a higher susceptibility for the development of post-traumatic stress disorders, anxiety disorders, and major depressive disorders (MDD). Interestingly, patients with MDD can show a dysregulation of the OT system and have a higher prevalence of an activated neuroinflammatory response.

The prairie vole (*Microtus ochrogaster*) has emerged as an excellent model for elucidating the effects of partner loss and the involvement of neuropeptide systems such as the OT and the corticotropin-releasing factor (CRF) system. A dysregulated CRF system following the loss of a partner leads to a higher basal activity of the hypothalamic-pituitary-adrenal (HPA) axis. This increases the release of glucocorticoids and passive stress-coping behavior. Altered stress-coping can be reversed by enhancing the OT release within the nucleus accumbens (NAcc) shell through invasive manipulations of both CRF and OT systems. Therefore, a translational approach would be beneficial for potential therapeutic implications. Furthermore, dysregulations of neuropeptide systems can alter the homeostasis of microglia, which represent a fundamental pillar of the neuroimmune response. Changes of microglia homeostasis can cause pro- or anti-inflammatory reactions of the neuroimmune system. These are linked to the development of psychiatric disorders, commonly caused by chronic stress. To date, studies investigating the neuroinflammatory response in patients with complicated grief following the loss of a partner are lacking. However, chronic activation of the neuroimmune system is common in patients suffering from MDD, which in turn is comorbid with complicated grief. Furthermore, the peripheral immune system is altered during grief and MDD and acute stress evokes an exaggerated response of this system in patients suffering from MDD.

Therefore, in the first study, I aimed to rescue partner loss-induced altered stress-coping and anxiety-like behaviors by potentiating the release of endogenous OT in the NAcc shell. To achieve this, after the formation of a pair bond, male and female prairie voles were either separated from their partner or remained pair housed for 9 days. Separated male and female prairie voles were allowed to briefly socially interact with an opposite sex-stranger and received daily intraperitoneal

(*i.p.*) injections of Melanotan II (MTII). MTII is a melanocortin receptor 4 (MC4R) agonist, capable to potentiate the release of endogenous OT during social interactions. The social behavioral response was assessed following the treatment with MTII. Interestingly, MTII treatment did not alter passive stress-coping and anxiety-like behaviors of separated male and female prairie voles. Furthermore, no alterations of basal physiological parameters of the stress response were observed. MTII treatment did not alter social behaviors but increased autogrooming which was coupled to a decrease of explorative behaviors.

In the second study, I aimed to evaluate the effect of separation from a partner on the activation of the neuro- and the peripheral immune system and a potentially exaggerated immune response following acute stress in separated partners. To achieve this, using the same model of partner loss for 4 days, I assessed stress-coping in the forced swim test (FST) on the last day of separation. For analyzing the neuroinflammatory status, a novel approach was developed to classify morphological microglia activation states (ramified, primed, reactive, or amoeboid). This was performed for the prelimbic cortex (PrL), NAcc shell, and the paraventricular nucleus of the hypothalamus (PVN) combined with other morphological analyses of microglia, gene expression analysis of microglia- and stress-associated genes, and the assessment of neuronal activation. Following the loss of a partner, only male prairie voles showed an increase in passive stress-coping but without altered basal parameters of the stress response. Partner loss led to an increase of microglia percentages only within the PVN of female prairie voles. In separated male prairie voles, the morphological activation of microglia remained unaltered. In females, partner loss led to lower morphological microglia activation in the PrL and the PVN, indicated by an increase of ramified and a decrease of primed microglia. Acute stress resulted in a shift towards a more activated morphology only in the NAcc shell of paired male prairie voles, indicated by a reduction of ramified microglia. Expression of the selected genes (*CX3CR1*, *CX3CL1*, *LGALS3*, *FKBP51*) remained unaltered following partner loss. Only male prairie voles showed a partner loss-induced increase of neuronal activation within the PrL under basal conditions, whereas acute stress increased neuronal activation within the PVN independent of sex and separation. None of the peripheral immune system parameters were altered by separation from a partner or acute stress.

In conclusion, no clear effects of MTII could be observed since the route of administration might have interfered with the observed emotional and social behaviors. In the second study, separation from a partner increased passive stress-coping only in male prairie voles. Furthermore, separation and acute stress are having a diverse influence on the neuroimmune system homeostasis and neuronal activity in male and female prairie voles. The observed microglia and neuronal activation responses towards either separation from a partner or acute stress are sex- and brain region-dependent. This study is advancing our understanding of the impact of partner loss on the

neuroinflammatory response. Furthermore, it is highlighting the importance of sex differences in the light of the susceptibility for emotional dysfunctions.

Zusammenfassung

Zusammenfassung

Soziale Beziehungen sind wichtig für das Wohlbefinden und das Überleben von Säugetieren. Als Beispiel für soziale Beziehungen dienen die Bindung zwischen Eltern und Kind, Artgenossen und Partnern. Das Neuropeptid Oxytocin (OT) ist fundamental für die Bildung von Bindungen zwischen sozialen Individuen. Positive soziale Beziehungen beeinflussen die mentale und physische Gesundheit, sind bedeutend in der Dämpfung von Stress und haben einen vorteilhaften Einfluss auf die Funktion des Immunsystems. Gestörte soziale Beziehungen, soziale Isolation oder der Verlust eines Partners, im Gegenzug, führen zu einer Verschlechterung der physischen und mentalen Gesundheit. Im Menschen geht der Verlust eines Partners mit einer Störung des Immunsystems einher und kann zu einer höheren Anfälligkeit für die Ausprägung von posttraumatischen Belastungsstörungen, Angsterkrankungen und Depressionen führen. Eine Dysregulation des OT Systems kann interessanterweise auch in depressiven Patienten gefunden werden, welche ebenfalls eine höhere Aktivierung des Neuroimmunsystems aufzeigen können.
Die Präriewühlmaus (*Microtus ochrogaster*) ist ein exzellentes Tiermodell, um den Einfluss vom Verlust des Partners auf Dysregulationen von verschiedenen Neurotransmittersystemen, wie zum Beispiel dem OT oder Corticotropin-Releasing Factor (CRF) System, zu untersuchen. Eine Dysregulation des CRF Systems nach dem Verlust des Partners führt zu einer erhöhten basalen Aktivität der Hypothalamus-Hypophysen-Nebennierenrinden (HPA) Achse. Dadurch wird eine erhöhte Freisetzung von Glucocorticoiden und vermehrte passive Stressbewältigung bedingt. Die Änderung der Stressbewältigung kann durch eine erhöhte Freisetzung von OT im Nucleus accumbens (NAcc) shell mittels invasiver Manipulationen der OT und CRF Systeme umgekehrt werden. Ein translationaler Ansatz wäre daher für therapeutische Anwendungen wünschenswert. Weiterhin können Dysregulationen von Neurotransmittersystemen auch zur Änderung der Homöostase von Microglia, welche fundamental für das Neuroimmunsystem sind, führen. Änderungen der Homöostase von Microglia können in pro- oder anti-inflammatorischen Reaktionen des Neuroimmunsystems resultieren. Diese stehen in Verbindung mit der Entstehung von psychiatrischen Störungen, welche häufig durch chronischen Stress ausgelöst werden. Studien, welche die neuroinflammatorische Reaktion in Verbindung mit komplizierter Trauer nach dem Verlust des Partners untersuchen, fehlen. Depressionen stehen jedoch im Zusammenhang mit einer chronischen Aktivierung des Neuroimmunsystems und sind komorbid mit komplizierter Trauer. Des Weiteren zeigt das periphere Immunsystem Änderungen während Trauer und Depressionen. Auch zeigen depressive Patienten eine verstärkte Reaktion des peripheren Immunsystems nach akutem Stress.

In der ersten Studie war es das Ziel, den negativen Einfluss der Separation von einem Partner auf die Stressbewältigung und auf angstähnliches Verhalten durch eine erhöhte Freisetzung von endogenem OT im NAcc shell umzukehren. Dafür wurden männliche und weibliche Präriewühlmäuse nach der Bildung einer Paarbindung entweder für 9 Tage separiert oder verblieben bei ihrem Partner. Separierte Männchen und Weibchen konnten kurzzeitig frei mit einem Individuum des anderen Geschlechts interagieren und erhielten tägliche intraperitoneale (*i.p.*) Melanotan II (MTII) Injektionen. MTII ist ein Agonist für den Melanocortin Rezeptor 4 (MC4R), welcher die Freisetzung von OT während der sozialen Interaktion potenziert. Zusätzlich wurde das Sozialverhalten nach der Behandlung mit MTII analysiert. Interessanterweise hatte die Behandlung mit MTII keinen Effekt auf die Stressbewältigung und das angstähnliche Verhalten von separierten männlichen und weiblichen Präriewühlmäusen. Auch wurden keine Änderungen von basalen physiologischen Parametern der Stressreaktion observiert. Das Sozialverhalten blieb nach der Behandlung mit MTII unverändert, führte jedoch zu vermehrter Fellpflege und reduziertem Explorationsverhalten.

In der zweiten Studie war es das Ziel, die Aktivierung des neuronalen und peripheren Immunsystems nach der Separation von einem Partner und die wohlmöglichen übersteigerten Reaktionen dieser Systeme nach akutem Stress in separierten Partnern zu untersuchen.

Hierfür wurde das gleiche Modell der Separation von einem Partner genutzt, jedoch betrug die Dauer der Separation 4 Tage. Am 4. und letzten Tag der Separation wurde die passive Stressbewältigung im forced swim test (FST) analysiert. Im Rahmen der Analyse der Neuroinflammation wurde eine neuartige Herangehensweise zur morphologischen Klassifizierung der Aktivierungsstadien von Microglia (ramified, primed, reactive, amoeboid) entwickelt. Diese wurde für den prälimbischen Cortex (PrL), NAcc shell und den Nucleus paraventricularis (PVN) angewandt und mit anderen morphologischen Analysen, Genexpressionsanalysen für Microglia- und Stress-assoziierte Gene und der Analyse der neuronalen Aktivierung kombiniert.

Nur männliche Präriewühlmäuse zeigten eine Steigerung der passiven Stressbewältigung nach der Separation von einem Partner, ohne dass diese einen Einfluss auf basale Parameter der Stressreaktion hatte. Nur im PVN von weiblichen Präriewühlmäusen führte der Verlust des Partners zu einer prozentuellen Zunahme von Microglia. Die morphologische Aktivierung der Microglia von männlichen Präriewühlmäusen blieb nach dem Verlust des Partners unverändert. Separierte Weibchen zeigten eine niedrigere morphologischen Aktivierung von Microglia im PrL und PVN, was durch eine Reduzierung von primed Microglia und einen Anstieg von ramified Microglia verdeutlicht wurde. Akuter Stress führte im NAcc shell von gepaarten Männchen zu einer Verschiebung in Richtung einer pro-inflammatorischen Morphologie, was durch eine Reduzierung von ramified Microglia verdeutlicht wurde. Die Expression der Kandidatengene (*CX3CR1*,

CX3CL1, *LGALS3*, *FKBP51*) blieb nach der Separation generell unverändert. Interessanterweise bewirkte der Verlust des Partners unter basalen Bedingungen nur im PrL von männlichen Präriewühlmäusen eine erhöhte neuronale Aktivität. In männlichen und weiblichen Präriewühlmäusen führte akuter Stress zu einer erhöhten neuronalen Aktivität im PVN, unabhängig von der Separation. Der Verlust eines Partners und akuter Stress hatten keinen Einfluss auf die periphere Immunantwort.

Aus den Ergebnissen lässt sich daher schlussfolgern, dass kein klarer Effekt der Behandlung mit MTII zu sehen war, da der Verabreichungsweg der Behandlung wohlmöglich die emotionalen und sozialen Verhaltensweisen beeinträchtigt hat. In der zweiten Studie zeigten nur männliche Präriewühlmäuse eine verstärkte passive Stressbewältigung nach der Separation von einem Partner. Zugleich führten der Verlust des Partners und akuter Stress zu Unterschieden in der Homöostase des Neuroimmunsystems und neuronalen Aktivität in männlichen und weiblichen Präriewühlmäusen. Die beobachteten Effekte von Partnerverlust und akutem Stress ihm Rahmen der Aktivierung von Microglia und Neuronen sind abhängig vom Geschlecht und der Hirnregion. Diese Studie trägt zu einem besseren Verständnis der Auswirkungen vom Verlust des Partners auf die neuroinflammatorische Antwort bei und hebt ferner die Bedeutsamkeit von Geschlechtsunterschieden im Rahmen der Anfälligkeit für emotionale Dysfunktionen hervor.

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Abbreviations

Abbreviations

-RT	minus reverse transcriptase
°C	degree Celsius
+RT	plus reverse transcriptase
ACC	anterior cingulate cortex
ACTH	adrenocorticotropic hormone
AF	Alexa Fluor
AgRP	Agouti-related protein
Amyg	amygdala
ANOVA	analysis of variance
ANS	autonomic nervous system
AON	anterior olfactory nucleus
Arg-1	arginase 1
ASD	autism spectrum disorder
Atp5f1	adenosine triphosphate synthase subunit b, mitochondrial
AU	airy unit
AVP	arginine vasopressin
AVPR	arginine vasopressin receptor
BBB	blood-brain barrier
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
BPD	bipolar disorder
c-Fos	AP-1 transcription factor subunit
CD14	cluster of differentiation 14
CD200R	cell surface transmembrane glycoprotein CD200 receptor
CD68	cluster of differentiation 68
CD86	cluster of differentiation 86
cDNA	complementary DNA
CeA	central amygdala
CFU	colony forming units
cm	centimeter
CNS	central nervous system
CO2	carbon dioxide
CORT	corticosterone
CRF	corticotropin-releasing factor
CRFR	corticotropin-releasing factor receptor
CSC	chronic subordinate colony
CX3CL1	fractalkine
CX3CR1	fractalkine receptor
d	day
DA	dopamine
DAB	3,3'-diaminobenzidine

DAMP	damage-associated molecular pattern
DAPI	4',6-diamidin-2-phenylindol
ddH_2O	double-distilled water
ΔΔCT	delta delta cycle threshold
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELA	early life adversity
ELISA	enzyme-linked immunosorbent assay
EPM	elevated plus maze
EtOH	ethanol
FKBP51	FK506-binding protein 5
FST	forced swim test
g	gram
GABA	γ-aminobutyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	glucocorticoid
GILZ	glucocorticoid-induced leucine zipper
GR	glucocorticoid receptor
h	hour
H_2O_2	hydrogen peroxide
Hipp	hippocampus
HPA	hypothalamic–pituitary–adrenal
HSP90	heat shock protein 90
HyD	hybrid detector
i.p.	intraperitoneal
IBA1	ionized calcium-binding adapter molecule 1
IFNγ	interferon gamma
IgG	immunoglobulin G
IL	infralimbic cortex
iNOS	inducible nitric oxide synthase
L	liter
LGALS3	galectin-3
LS	lateral septum
M	molar concentration
MAC	membrane attack complex
MC1R	melanocortin receptor 1
MC2R	melanocortin receptor 2
MC3R	melanocortin receptor 3
MC4R	melanocortin receptor 4
MC5R	melanocortin receptor 5
MCP-1	monocyte chemoattractant protein 1
MDD	major depressive disorder
MeA	medial amygdala

MeOH	methanol
mg	milligram
MHC-II	major histocompatibility complex class II
min	minute
miRNA	micro RNA
ml	milliliter
mm	millimeter
mPFC	medial prefrontal cortex
MPOA	medial preoptic area
MR	mineralocorticoid receptor
mRNA	messenger RNA
MSH	melanocyte-stimulating hormone
MTII	Melanotan II
NAcc	nucleus accumbens
nGRE	negative glucocorticoid response element
NGS	normal goat serum
ni	no interaction
NOS2	inducible nitric oxide synthase
OB	olfactory bulb
OFT	open field test
OT	oxytocin
OTR	oxytocin receptor
OXTR	oxytocin receptor gene
PAMP	pathogen-associated molecular pattern
pbp	pair bond paired
pbs	pair bond separated
PBS	phosphate-buffered saline
PFA	Paraformaldehyde
PFC	prefrontal cortex
pH	power of hydrogen
PMT	photomultiplier tube
PND	postnatal day
POMC	pro-opiomelanocortin
PrL	prelimbic cortex
PRR	pattern recognition receptors
PTSD	posttraumatic stress disorder
PVN	paraventricular nucleus
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
ROI	region of interest
RT	room temperature
s	second
sACC	subgenual anterior cingulate cortex

SD	standard deviation
SEM	Standard error of the mean
si	social interaction
SNS	sympathetic nervous system
SON	supraoptic nucleus
SpO2	Oxygen saturation
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha
TSST	Trier social stress test
UCN	urocortin
VEH	vehicle
VP	ventral pallidum
vsx	vasectomized
VTA	ventral tegmental area
x g	times G-force
µl	microliter
µM	micromolar concentration
µs	microsecond

General Introduction

As a reference for more in-depth reading; this introduction is referencing to the following publications:

Lost connections: Oxytocin and the neural, physiological, and behavioral consequences of disrupted relationships. (2019)
Int J Psychophysiol, 136, 54-63. https://doi.org/10.1016/j.ijpsycho.2017.12.011

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TP wrote first draft of manuscript and final version.

Abandoned prairie vole mothers show normal maternal care but altered emotionality: Potential influence of the brain corticotropin-releasing factor system. (2018)
Behav Brain Res, 341, 114-121. https://doi.org/10.1016/j.bbr.2017.12.034

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TP analyzed brain regions and contributed to writing the manuscript.

1 General Introduction

1.1 Social relationships

Social relationships are important for survival within a social group. Humans have the natural need to interact with others and to gain acceptance within their social group (Ryan & Deci, 2000). One of the earliest social relationships in mammals is formed through the contact between the mother and its offspring (Numan & Young, 2016; Porges, 2003). This relationship is securing the survival of the young and the transmission of the parental genes. Apart from parent-offspring interactions, humans are engaging in numerous social relationships throughout their lives, and they are formed through repeated contact a conspecific (Carter, 2007; Cheney, Seyfarth, & Smuts, 1986). The possession, as well as the perception, of social relationships during early and later life, have a beneficial impact on the physical and mental health, including a higher life expectancy and lower susceptibility for the development of depression and distress (Beach, Martin, Blum, & Roman, 1993; Cruwys, Haslam, Dingle, Haslam, & Jetten, 2014; Henderson, 1981; Holt-Lunstad, Robles, & Sbarra, 2017; Holt-Lunstad, Smith, & Layton, 2010; House, Landis, & Umberson, 1988; Kenny, Dooley, & Fitzgerald, 2013; Lepore, 1992; Saeri, Cruwys, Barlow, Stronge, & Sibley, 2018; Uchino, 2006; Yang et al., 2016). Partner and family relationships are having a major beneficial impact on health by buffering against adverse life experiences (Glenn & Weaver, 1981; Uchino, Cacioppo, & Kiecolt-Glaser, 1996). The buffering against adverse events by social interactions is termed social buffering (Grewen, Girdler, Amico, & Light, 2005). Perceived social connectedness is protective against feelings of loneliness, which in turn is related to negative thoughts and behavior (Cacioppo & Patrick, 2008). Furthermore, engaging in a marital relationship positively correlates with better immune system function and cardiovascular health (Kiecolt-Glaser, Gouin, & Hantsoo, 2010; Kiecolt-Glaser & Newton, 2001).

In animal research, studies are mainly focused on early life social relationships by investigating the role of parental care towards the offspring (Bendesky et al., 2017). An important aspect regulating the physical wellbeing of the offspring is provided by the mother in the form of maternal care (Bosch & Neumann, 2012). Ample parental care during early life can positively affect the offspring's social behavior, rank, and the response towards fearful stimuli in adolescence and adulthood (Caldji, Diorio, Anisman, & Meaney, 2004; Parent, Del Corpo, Cameron, & Meaney, 2013; Perkeybile, Griffin, & Bales, 2013). In the past decade, further animal studies demonstrated that social interactions are an important mediator for general health as well. Among others, this is mediated by attenuating the stress response (Babygirija, Yoshimoto, et al., 2012), which is also observed in group-housed animals (Neumann, 2009). An important regulator of social behavior and the

beneficial effects arising from social relationships is the neuropeptide oxytocin (OT) (Marlin & Froemke, 2017).

1.2 OT and the modulation of the social salience network

The nonapeptide OT is known for its modulation of social behaviors, including parental care (Rilling & Young, 2014), social recognition (Bielsky & Young, 2004), empathy and social buffering (Burkett et al., 2016; Domes et al., 2019; Smith & Wang, 2014), as well as pair bonding (Walum et al., 2012; Young & Wang, 2004). It is synthetized within hypothalamic magnocellular neurons of the paraventricular nucleus (PVN), the supraoptic nucleus of the hypothalamus (SON), and the accessory nuclei. These hypothalamic magnocellular OT neurons are projecting into the posterior pituitary and the forebrain including the nucleus accumbens (NAcc) and central amygdala (CeA) (Dölen, Darvishzadeh, Huang, & Malenka, 2013; Knobloch et al., 2012; Ross et al., 2009). Parvocellular OT neurons are projecting to and terminating in hindbrain structures and the spinal cord and are involved in the control of autonomic functions and the perception of pain (Althammer & Grinevich, 2017; Sawchenko & Swanson, 1982; Swanson & Sawchenko, 1983). Within the posterior pituitary, OT is stored and released into the peripheral circulation and can induce its effects as a hormone (Ludwig & Leng, 2006). In the periphery, OT affects the induction of uterine contractions during child labor, milk ejection, and reproductive processes (Douglas, Leng, & Russell, 2002; Hatton & Wang, 2008; Jurek & Neumann, 2018). Centrally, OT is important for the onset of maternal behavior following birth (Bosch & Neumann, 2012).

Human studies describe the connection between OT and the facilitation of face recognition and emotions (Bethlehem, Baron-Cohen, van Honk, Auyeung, & Bos, 2014; Shahrestani, Kemp, & Guastella, 2013; Van & Bakermans-Kranenburg, 2012). Furthermore, OT affects social behavior by enhancing the salience of social cues (Bartz, Zaki, Bolger, & Ochsner, 2011; Shamay-Tsoory & Abu-Akel, 2016; Shamay-Tsoory et al., 2009). OT is enhancing the perception of emotional faces, which is in line with research suggesting an impact of OT on the salience of social cues (Tillman et al., 2019). fMRI studies show that OT enhances the neuronal response of the NAcc when viewing the face of a partner, implying a higher perception of reward (Scheele et al., 2013). When viewing emotional faces, the amygdala shows decreased activation, which is dependent on the facial expression, the gender of the subject, sociality and agreeableness, and the subregions of the amygdala (Domes et al., 2010; Gamer, Zurowski, & Buchel, 2010; Groppe et al., 2013). The wide range of involvement of different regions within the brain during social interactions is hinting at circuit-mediated processing of social cues (Bielsky & Young, 2004). Animal research revealed that hypothalamic magnocellular OT neurons are projecting throughout the central nervous system (CNS), to several regions that are rich in OT receptor (OTR) expression, through which OT can

exert its neuromodulatory effects (Jurek & Neumann, 2018). Originating from the PVN, OT neurons are projecting into the prefrontal cortex (PFC) comprising the anterior cingulate cortex (ACC), the infralimbic cortex (IL) and the prelimbic cortex (PrL). Furthermore, projections from the PVN can be found in the anterior olfactory nucleus (AON), the hippocampus (Hipp), the lateral septum (LS), the bed nucleus of the stria terminalis (BNST), the medial preoptic area (MPOA), the nucleus accumbens (NAcc), the amygdala including the CeA, medial- (MeA) and basolateral amygdala (BLA), the posterior pituitary gland, and the ventral tegmental area (VTA). Those numerous projection sites might explain the broad range of effects of OT (**Figure 1**). Through stimulus-dependent activation and release of OT during social interactions, OT exerts circuit-specific effects on the modulation of social behaviors and cognition (Hammock, 2017; Ross & Young, 2009).

The recognition of other social individuals is of importance when navigating through a social world (Bielsky & Young, 2004). OT acts as an enhancer in the processing of social information by increasing the signal-to-noise ratio in the olfactory bulb (OB) through the release of OT in the AON and thereby aiding social recognition (Marlin & Froemke, 2017; Oettl et al., 2016; Walum & Young, 2018), which is of importance in the formation of a pair bond. Furthermore, the MeA, which plays an important role in social recognition is innervated by OT neurons and receives olfactory sensory information from the AON during social interaction and mating (Bielsky & Young, 2004). Studies show that OT release in the MeA is necessary for social recognition in mice and an intact MeA is fundamental for partner discrimination and affiliative behaviors in prairie voles (Ferguson, Aldag, Insel, & Young, 2001; Kirkpatrick, Carter, Newman, & Insel, 1994). Emotional salience of social sensory information is thereby relayed through the MeA to the BLA which is important for associative learning and valence processing (Walum & Young, 2018; Zhang & Li, 2018). Not only the recognition of other social individuals is of relevance, but it is also vital to perceive emotional states to adopt adequate behavioral responses (Phillips, Drevets, Rauch, & Lane, 2003). This is termed emotion recognition and is also facilitated by OT within the CeA of mice (Ferretti et al., 2019). Regarding empathy-related behaviors, studies in rats show the presence of emotional mirror neurons in the ACC (Carrillo et al., 2019) and inhibition of this region blocks empathy-related behaviors (Hernandez-Lallement et al., 2020). Those effects are potentially modulated by the OT system in the ACC since it is facilitating partner-directed prosocial behaviors (Burkett et al., 2016). One positive outcome is the reduction of the stress response of a pair bonded partner (Burkett et al., 2016; Smith & Wang, 2014).

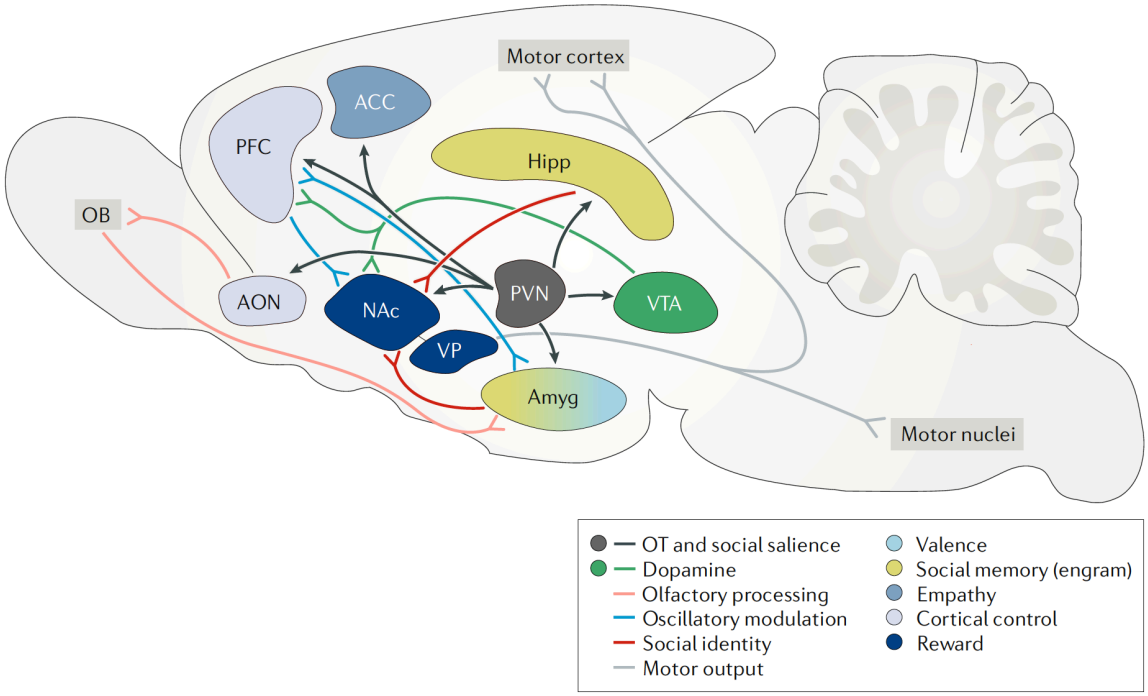

Figure 1 | Projections within the neuronal model of pair bond formation.
Regions in the neuronal model for pair bond formation highly overlap with brain areas associated with social recognition (OB, AON, Amyg, Hipp), affiliative behaviors and emotional processing (PFC, ACC), and reward (VTA, NAcc, VP). Those regions are also heavily innervated by OT projections stemming from the PVN. Figure adapted from (Walum & Young, 2018).

1.3 Pair bonding

Socially monogamous species form long-lasting partner preferences, while not excluding the prevalence of extra-pair mating (Ophir, 2017). The preference for a specific individual is termed pair bonding and is observed relatively rarely among animals. Only 5% of mammals and 29% of primates are living socially monogamous (Cockburn, 2006; Kleiman, 1977; Lukas & Clutton-Brock, 2013). The evolutionary development of social monogamy is hypothesized to be driven by the availability of resources (e.g. mate availability, habitat limitation, breeding synchrony). Under circumstances of low natural resources availability and high risk of predation, adopting social monogamy is thought to be beneficial for the individual's fitness by increasing the probability of reproduction (Kvarnemo, 2018; Lukas & Clutton-Brock, 2013). From the developmental perspective of the neurobiological foundation of a pair bond, it is probably derived from the bond between a mother and its offspring (Numan & Young, 2016). In both types of relationships, similar neurotransmitter systems and brain areas are involved in the formation of a bond (Numan & Young, 2016; Young, 2009; Young & Alexander, 2012).

Besides increasing the probability of breeding success, pair bonding was shown to be advantageous for the physiological and psychological well-being (Young, Gobrogge, Liu, & Wang, 2011).

In humans, being bonded to a partner is linked to better physical health, lower susceptibility for the development of long-term illnesses or work disability, and increased life expectancy (House et al., 1988; Murphy, Glaser, & Grundy, 1997; Waite & Lehrer, 2003). Also, cohabiting with a partner increases the general satisfaction with life in humans (Zimmermann & Easterlin, 2006). Formation of a bond between partners is governed by social interactions. To study the formation of a pair bond, the prairie vole (*Microtus ochrogaster*) has been proven to be an excellent organism for studies unraveling the neurobiological underpinnings of partner preference (McGraw & Young, 2010).

Prairie voles are characterized by the formation of a partner preference following mating, bi-parental care, and selective aggression towards stranger conspecifics in the light of territorial, as well as mate and nest defense (Clutton-Brock, 1991; Kleiman, 1977). Partner preference was described in field studies (by e.g. repeated capture of the same male-female pairs) (Getz, Carter, & Gavish, 1981; Getz & Hofmann, 1986), but also in the laboratory. Upon the formation of a pair bond, prairie voles prefer to stay in close contact with their respective partner over a stranger which can be assessed in experimental settings in the partner preference test (Williams, Insel, Harbaugh, & Carter, 1994). This preference for a partner is governed by several regions within the brain, linking the partner's sociosensory information with reward, thereby intensifying the perceived value of a partner compared to other social subjects (Walum & Young, 2018). In prairie voles, the interplay between OT, arginine vasopressin (AVP), and the dopamine (DA) reward system within mesolimbic brain structures is proposed to be central for regulating the formation of a partner preference (Walum & Young, 2018; Young & Wang, 2004). Research focusing on the OT system of prairie voles has given a multitude of insights about its involvement in the regulation of a partner preference (Walum & Young, 2018). The production and release of OT is relatively conserved between and within species, however, the distribution and abundance of the OTR within the CNS seems to be decisive for observed inter- and intra-species differences in social behavior and partner preference formation (Insel & Shapiro, 1992; King, Walum, Inoue, Eyrich, & Young, 2016).

Inter-species differences in OTR densities within the NAcc are associated with different mating strategies (Young & Wang, 2004). Promiscuity as observed in the meadow (*Microtus pennsylvanicus*) and the montane (*Microtus montanus*) vole is closely linked to lower OTR abundance in the NAcc shell. Higher abundance of OTR within the NAcc shell of prairie voles, however, has been linked to the ability of partner preference formation (Ophir, Gessel, Zheng, & Phelps, 2012). Interestingly, in prairie voles, genotype differences in the *OTR* gene result in altered expression patterns of OTR within the NAcc, leading to variation in the facilitation of a partner preference (King et al., 2016). Partner preference formation in the female prairie vole can be blocked by infusion of an OTR antagonist locally into the PrL and the NAcc shell (Young, Lim, Gingrich, & Insel, 2001). Additionally, the OT receptor (OTR) density determines partner preference formation

in male prairie voles (Ophir et al., 2012). Mating stimulates the release of OT within the NAcc shell of prairie voles (Ross et al., 2009). While cohabitation for 6 hours without mating is usually not sufficient for the establishment of a pair bond, partner preferences can be observed following 24 h with mating (Insel, Preston, & Winslow, 1995). Cohabitation with an opposite sex stranger for 6 h and centrally administered OT, however, facilitates partner preference formation in female prairie voles (Williams et al., 1994). Furthermore, partner preference formation and the display of affiliative behaviors is dependent on local field potential connectivity and cross-frequency coupling between the mPFC and the NAcc, which is hypothesized to be modulated by the expression of OTR within the NAcc (Amadei et al., 2017).

Another system that is involved in the modulation of partner preference is the corticotropin-releasing factor (CRF) system (DeVries, Guptaa, Cardillo, Cho, & Carter, 2002). Inter-species variability of CRF receptor (CRFR) distribution and abundance are observed between promiscuous meadow and montane and socially monogamous prairie and pine voles (*Microtus pinetorum*) (Lim, Nair, & Young, 2005). CRFR1 are more abundant within the NAcc shell of meadow and montane voles when compared to prairie and pine voles, respectively. The abundance of CRFR2 in the pole of the NAcc is higher in both monogamous species than in both promiscuous species (Lim et al., 2005). Infusion of CRF into the NAcc facilitates partner preference formation, while co-injection with either CRFR1 or CRFR2 antagonists blocked this effect (Lim et al., 2007). Furthermore, CRF mRNA is increased within the BNST of male voles following pairing with a female (Bosch, Nair, Ahern, Neumann, & Young, 2009).

The release of CRF is also associated with the response towards a stressor and the release of corticosterone (CORT). CORT was shown to have sex-specific effects on the formation of a partner preference in male and female prairie voles. Cohabitating with a paired male reduces plasma CORT levels in females (DeVries, DeVries, Taymans, & Carter, 1995) and experimentally reducing the levels of circulating CORT by adrenalectomy or antagonizing glucocorticoid receptors (GRs) facilitates partner preference formation (Curtis & Wang, 2005; DeVries et al., 1995). In male prairie voles, however, adrenalectomy prevented the formation of a partner preference which could be reversed by treatment with CORT (DeVries, DeVries, Taymans, & Carter, 1996). Interestingly, both described systems are involved in the response to partner loss (Bosch et al., 2016; Bosch et al., 2009; Pohl et al., 2019).

1.3.1 Dysregulation following the separation from a partner

Alongside the development of anxiety or depressive disorders, disruptions of the immune system or cardiovascular problems are frequently observed following separation from a partner in humans (Barger, 2013; Cacioppo & Hawkley, 2003; Carey et al., 2014; Gerra et al., 2003; Goforth et al., 2009; Holt-Lunstad & Smith, 2016; Shear & Shair, 2005; Uchino, 2006). Bereavement or the loss of a partner can cause grief and is often referred to as one of the most traumatic experiences (Keyes et al., 2014). Episodes of grief of up to 6 months are not regarded as maladaptive. However, grieving episodes that exceed the duration of 6 months are termed complicated grief with a prevalence between 10-20% of bereaved individuals (Prigerson et al., 2009; Shear, 2015; Shear et al., 2011). Characteristic features of complicated grief include but are not limited to the inability to function in everyday life, problems with social relationships and work (Boelen & Prigerson, 2007; Monk, Houck, & Shear, 2006; Simon et al., 2007), as well as an increase in the consumption of alcohol and nicotine (Zisook, Shuchter, & Mulvihill, 1990). Furthermore, patients experiencing complicated grief show a higher incidence of cardiovascular diseases, sleep disturbances, psychosomatic and psychiatric disorders, and higher prevalence of suicidal tendencies (Buckley et al., 2012; Latham & Prigerson, 2004; Shear, 2015). Increased risk for the development of psychiatric disorders includes disorders such as major depressive disorder (MDD), post-traumatic stress disorder (PTSD), and anxiety disorders (Keyes et al., 2014; Monroe, Rohde, Seeley, & Lewinsohn, 1999; Zisook, Chentsova-Dutton, & Shuchter, 1998). Interestingly, those disorders are also known for being modulated by the OT and CRF system (Bangasser & Kawasumi, 2015; Binder & Nemeroff, 2010; Dodhia et al., 2014; Heim et al., 2009; Labuschagne et al., 2010; Ozsoy, Esel, & Kula, 2009; Parker et al., 2010). Human research is usually limited to non-invasive techniques and self-reporting. A brain region that is associated with complicated grief is the NAcc, which is important in the perception of social reward (Walum & Young, 2018). Importantly, self-reported yearning of the deceased correlates with higher reward-related activity in the NAcc when viewing pictures of the deceased partner (O'Connor et al., 2008).

To gain a deeper understanding of the interplay between dysregulations of neuropeptide systems and emotional regulation, research has turned to the socially monogamous prairie vole. Previous studies demonstrate that loss of a partner induces fundamental changes to their emotionality and neuronal and peripheral homeostasis (Bosch et al., 2016; Bosch et al., 2009; Bosch, Pohl, Neumann, & Young, 2018; McNeal et al., 2014; Sun, Smith, Lei, Liu, & Wang, 2014). Firstly, separation from a bonded partner alters the activity and functionality of the OT system. Reduced excitatory input from glutamatergic neurons which are making synapses onto OT^{+} neurons within the PVN results in a decreased expression of OT mRNA. This, in turn, leads to reduced OT release within the NAcc shell and a lower OTR density within the NAcc shell (Bosch et al., 2016). Further evidence for a

decrease of OT release following separation is the increase of OT^+ neurons within the PVN following four weeks of separation from a bonded partner (Sun et al., 2014). A functional study describes the direct connection between OTR expression in the NAcc shell and passive stress-coping, indicative of depressive-like behavior (Bosch et al., 2016). Knockdown of OTR within the NAcc shell results in increased passive stress-coping even in non-separated prairie voles. Furthermore, separation from a partner is leading to enhanced CRF synthesis and activation of downstream processes through the HPA axis (Bosch et al., 2009). Interestingly, central infusions of CRFR2 agonists reduce the release of OT within the NAcc shell while antagonists increase the release of OT at baseline (Bosch et al., 2016), suggesting a connection between CRF system activation and OT release. Indeed, CRFR2 are also located on OT neurons projecting from the PVN to the NAcc (Bosch et al., 2016), Subsequent to the loss of a partner, it is thought that CRF or UCN are increasingly binding to the CRFR2, thereby inhibiting the release of OT within the NAcc shell and negatively affecting the well-being (Bosch et al., 2016). However, CRF and other urocortins (UCN) are also able to bind directly to their respective receptors located throughout the brain, affecting signaling of multiple systems (Deussing & Chen, 2018). It is, therefore, possible that other regions besides the PVN and NAcc shell are also affected by changed CRF signaling following the loss of a partner. **Figure 2** summarizes the effects of partner loss in the monogamous prairie vole on the OT and CRF system.

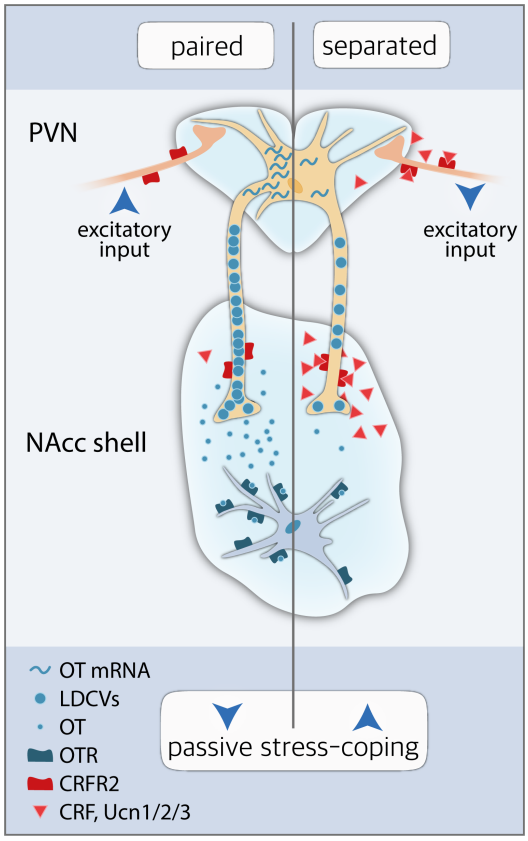

Figure 2 | Schematic depiction of the influence of CRFR2 binding on OT signaling in the NAcc shell following partner loss in prairie voles.
Partner loss induces a reduction in the synthesis of OT in the PVN and a reduction of OTR within the NAcc shell. Separation-induced release of CRF or urocortins (UCN1, UCN2, or UCN3) and binding to CRFR2, further dampens the release of OT within the NAcc shell. Adapted from (Pohl, Young, & Bosch, 2019).

1.4 Dysregulation of the OT system

As described above, separation from a partner causes imbalances and dysregulation of the stress and the OT system. Dysregulations of the OT system can be caused by environmental and genetic factors are also observed in humans (Alves, Fielder, Ghabriel, Sawyer, & Buisman-Pijlman, 2015; Kumsta & Heinrichs, 2013). Numerous studies describe the connection between parent-child interactions or the absence of those and peripheral OT release. Urinary OT levels are decreased in children that were placed into orphanages after birth (Wismer Fries, Ziegler, Kurian, Jacoris, & Pollak, 2005). Childhood maltreatment is also associated with lower CSF OT levels in adult women and men (Heim et al., 2009; Opacka-Juffry & Mohiyeddini, 2012). Moreover, parental separation alters the cortisol response to administration of intranasal OT, resulting in an attenuated decrease following parental separation in adult men (Meinlschmidt & Heim, 2007). In autism spectrum disorder (ASD), lower levels of plasma OT are observed (Modahl et al., 1998) and intranasal OT studies suggest a beneficial role of the OT system on social behavior (Andari et al., 2010; Guastella

et al., 2010). Besides being affected by early life experiences, dysregulations of the OT system are also frequently observed in psychiatric disorders such as major depressive disorder (MDD), where plasma OT levels are increased in MDD patients (Parker et al., 2010). Furthermore, OTR mRNA is increased in the dorsolateral PFC in patients with a history of MDD and bipolar disorder (BPD) (Lee et al., 2018). A higher abundance of OT neurons within the PVN is observed in patients with MDD or BPD, indicating neuronal remodeling of the OT system (Dai et al., 2017). Dysregulations of the OT system can, moreover, affect the modulation and regulation of homeostasis of cortisol levels following stress in humans and animal models (Amico, Mantella, Vollmer, & Li, 2004; Gulpinar & Yegen, 2004; Heinrichs, Baumgartner, Kirschbaum, & Ehlert, 2003). The stress-attenuating effect of intranasal OT, however, should be considered with caution (Cardoso, Kingdon, & Ellenbogen, 2014). Although peripheral administration of OT has been described as not being feasible due to the low blood-brain-barrier (BBB) penetration of OT (Ermisch et al., 1985), recent studies demonstrate that treatment with intranasal OT can reach the brain via different delivery routes (Quintana, Alvares, Hickie, & Guastella, 2015; Yamamoto & Higashida, 2020).

In animal models, male OT knockout mice show social deficits by reduced social recognition (Ferguson et al., 2000; Modi & Young, 2012) and enhanced anxiety- and stress responses during stress exposure in female mice (Amico et al., 2004; Mantella, Vollmer, Li, & Amico, 2003). Furthermore, alterations of the release of OT can also lead to the development of social behavior deficits in mice (Jin et al., 2007). This is further exemplified by OTR knockout, which causes a decrease of ultrasonic vocalizations, higher levels of aggression, and an impairment in social memory in mice (Crawley et al., 2007; Macbeth, Lee, Edds, & Young, 2009; Takayanagi et al., 2005). Interestingly, the effects on aggression and social memory impairments could be rescued by i.c.v. injection of OT acting on the AVP receptor 1A (AVPR1A) (Sala et al., 2011).

Recently, Melanotan II (MTII), an agonist for melanocortin receptors 1, 3, 4, and 5 (MC1R, MC3R, MC4R, and MC5R, respectively), was proposed to influence the endogenous release of OT (Modi et al., 2015). Neonatal chronic administration of MTII enhances social behaviors in adult prairie voles (Barrett et al., 2014). This is most likely caused by the OT system, as MTII treatment increases the activity of magnocellular OT neurons in rats (Paiva, Sabatier, Leng, & Ludwig, 2017) and facilitates the partner preference formation in prairie voles by enhancing the release of endogenous OT within the NAcc during social interaction (Modi et al., 2015). Since MTII is able to penetrate the BBB and is relatively stable, it has a high potential for therapeutic administration (Zhou & Cai, 2017).

1.5 The melanocortin system

The melanocortin system is comprised of 5 G protein-coupled receptors, which are expressed throughout the CNS and the periphery, and have different affinities for specific peptide hormones (Moscowitz et al., 2019). Melanocortin neuropeptides are derived from the preprohormone proopiomelanocortin (POMC), which is mainly synthesized in the anterior pituitary gland. Upon stress exposure, POMC is cleaved into melanocyte-stimulating hormones (MSH), ACTH, and β-Endorphin and Metenkephalin.

The melanocortin receptor MC1R can be found on the plasma membrane of melanocytes and binds melanocortins such as ACTH and MSH. This receptor is involved in the cutaneous pigmentation of the skin (Herraiz, Garcia-Borron, Jimenez-Cervantes, & Olivares, 2017). MC2Rs are located in the *zona fasciculata* of the adrenal cortex and specifically bind ACTH, which facilitates the release of glucocorticoids (GCs) such as CORT and, therefore, are involved in the stress response (Russell & Lightman, 2019). MC3Rs are expressed within the CNS and are thought to be involved in energy homeostasis and food intake (Sutton et al., 2008). Endogenous ligands for the MC3R are α- and γ-MSH, agouti-related protein (AgRP), and ACTH (Getting, 2006; Nasrallah & Horvath, 2014). MC4Rs are expressed within the CNS, specifically within the hypothalamus on magnocellular OT neurons (Sabatier, 2006), and in the intestine (Panaro et al., 2014). The endogenous ligands are α- and β-MSH (Lotta et al., 2019) and MC4Rs are involved in energy homeostasis and food intake (Getting, 2006; Nasrallah & Horvath, 2014). MC5Rs are expressed in the CNS and the periphery and are involved in the regulation of immune system function and inflammatory response, thermoregulation, exocrine secretion, and sexual behavior (Xu, Guan, Zhou, & Gong, 2020). Ligands for the MC5R are α- and β-MSH and ACTH (Getting, 2006).

1.6 The stress response

The terminology of stress is often linked to western lifestyle and commonly used for the description of the stressor, the system that is processing the stress, and the resulting physiological or behavioral responses. To avoid confusion in the terminology of the definition of stress, Selye defined this term in a more specific way as stress being the nonspecific response of the body to any form of demand and stressor as the stimulus to cause the stress response (Selye, 1976). Interestingly, even in the absence of a stressor, the anticipation of a stressor itself can already activate the stress response (Engert et al., 2013). Stressors can be of physical nature, e.g. through heat, cold, pain, or of emotional nature such as psychological loss or uncertainty (Chrousos, Loriaux, & Gold, 1988). Two models of the response to stress have been proposed. On the one hand, it is described as being a system in homeostasis. This describes a stressor-induced response (physiologically or behaviorally) to return to equilibrium (Chrousos et al., 1988). However, since the stress system by nature is fluctuating in

its basal activity (Russell & Lightman, 2019), the other definition is describing it as a system in allostasis, meaning that stability is achieved by change (Sterling & Eyer, 1988). Natural or stressor-induced fluctuations within this system certainly are in need of regulation. Regulation of the stress system occurs through either direct interactions with other hormonal systems or by self-regulation (de Kloet, Joëls, & Holsboer, 2005). This way, the stress system can prevent a constant activation of the stress response. However, while the response to a stressor is beneficial for acute phases of stress, prolonged or chronic stress, in turn, might cause the system to become insensitive towards other stressors, rendering the organism incapable of adequately responding. This is defined as allostatic load (de Kloet et al., 2005).

The two neuropeptides CRF and AVP are essential for regulating the physiological response to stress (Papadimitriou & Priftis, 2009). CRF is mostly synthesized in parvocellular glutamatergic neurons within the PVN and stored in large dense-core vesicles (LDCVs) at axon terminals within the external zone of the median eminence (Deussing & Chen, 2018). Upon exposure to a stressor but also in circadian rhythmicity, CRF is released into the portal vasculature and from there transported to the adenohypophysis, which is the anterior part of the pituitary gland and made up by the *pars distalis*, *pars tuberalis*, and the *pars intermedia* (Deussing & Chen, 2018). Upon binding of CRF to CRFR1 on corticotrope cells of the anterior pituitary, POMC is cleaved to ACTH which is then released into the peripheral circulation via the hypophyseal vein (Cawley, Li, & Loh, 2016). ACTH then binds to MC2R located in the adrenal cortex, stimulating the release of GCs from the *zona fasciculata* into the peripheral circulation (Clark & Chan, 2019). In humans, cortisol is released, whereas in rodents the main GC is CORT (Häusl et al., 2019). Because of their lipophilic properties, GCs are then crossing the BBB and bind to nuclear mineralocorticoid (MR) and glucocorticoid receptors (GR), which both are widely distributed throughout the CNS (Gray, Kogan, Marrocco, & McEwen, 2017). Because MRs have a higher affinity for GCs, GR activation is mainly linked to the exposure to a stressor (de Kloet et al., 2005). Binding to membrane-bound MR or GR modulates glutamate release and dendritic spine dynamics (Gray et al., 2017). Binding of GCs to cytosolic GRs promotes the detachment of the inhibitory complexes heat shock protein 90 (HSP90) and FK506 binding protein 5 (FKBP51) and the translocation of the GR to the nucleus (Häusl et al., 2019). Binding of dimerized GRs to a negative glucocorticoid response element (nGRE) attenuates the expression of ACTH in the pituitary gland, whereas binding to transcription factors can inhibit transactivation of the CRF gene expression in the PVN (Holsboer, 1999). Those mechanisms are essential for the negative feedback loop of the HPA axis and attenuation of the stress response (**Figure 3**).

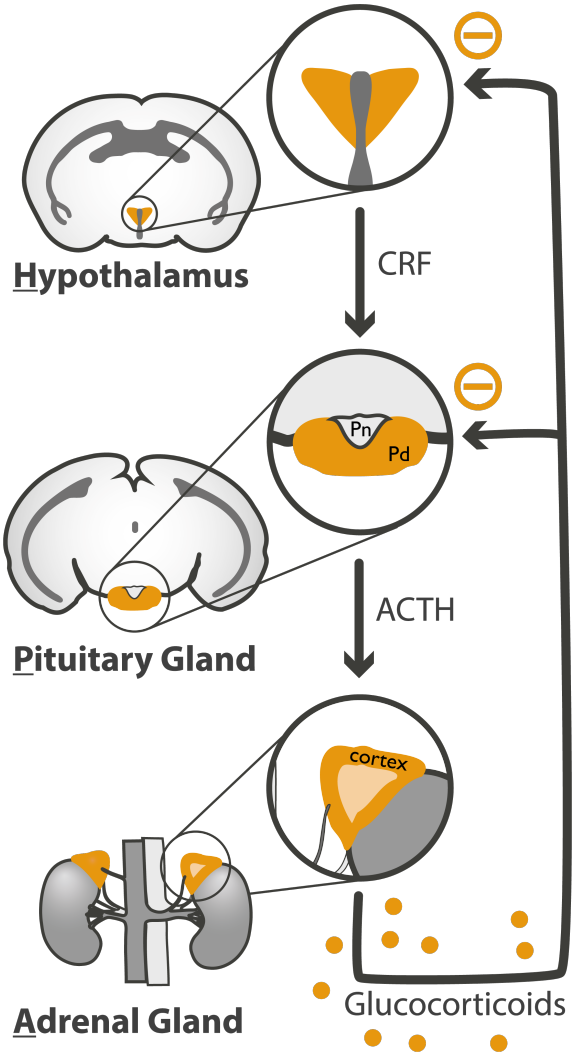

Figure 3 | Overview of the hypothalamic-pituitary-adrenal (HPA) axis.
Upon stress exposure, CRF is released into the median eminence and transported by the vasculature system to the anterior pituitary. Within the *pars distalis*, CRF binding to CRFR1 on corticotropes, ACTH is released into the systemic circulation and binds to MC2R within the cortex of the adrenal glands. From there, glucocorticoids are released into the circulation and exert negative feedback on HPA axis activity within the pituitary and the hypothalamus. CRF: Corticotropin-releasing factor, ACTH: Adreno-corticotropic hormone, Pd: *Pars distalis*, Pn: *Pars nervosa*.

1.6.1 Stress-induced pathophysiology

Under healthy conditions, the stress response can return to homeostasis by interactions between the self-regulating mechanisms of the HPA axis, autonomous nervous system (ANS), and immune system (Russell & Lightman, 2019). Extensive or chronic stress can lead to alterations of HPA axis activity, which can promote the manifestation of psychiatric disorders such as MDD, PTSD, and generalized anxiety disorder (McEwen, 2004; Patriquin & Mathew, 2017). The cumulative exposure to stress or the dysregulation of maintaining allostasis is described as allostatic load (McEwen et al., 2015). Chronic stress is linked to a continuing upregulation of basal cortisol levels, indicative of allostatic load and GC resistance (Cohen et al., 2012; Vyas et al., 2016). In humans,

hypercortisolemia was observed in 40 – 60% of depressed patients (Murphy, 1991). Furthermore, high levels of basal cortisol and depression can cause a decline of cognitive abilities (Lara et al., 2013; Rock, Roiser, Riedel, & Blackwell, 2014).

In mice and rats, chronic stress induces long-lasting effects on the expression of specific genes, altered neuron structures, and changes of neuronal activity (Joëls & Baram, 2009). Due to the effects on synapse plasticity, transcription factors, and on genomic modulation, GCs are involved in the regulation of the negative effects following chronic stress (Joëls & Baram, 2009; Timmermans, Souffriau, & Libert, 2019). Repeated treatment with GCs simulates the effects of chronic stress (Mitra & Sapolsky, 2008). Behaviorally, chronic stress negatively affects social behavior; Chronic restraint impairs sociability indicated by lower social motivation, social interactions, and an increased display of aggressive behavior in rats (van der Kooij, Fantin, Kraev, et al., 2014; van der Kooij, Fantin, Rejmak, et al., 2014). Furthermore, chronic stress or an altered stress response can also induce cognitive deficits; While acute exposure to CORT has beneficial outcomes for the cognitive processes, chronic stress and alterations of the HPA axis reactivity can have detrimental effects on cognition (McEwen, 2017; McIlwrick, Pohl, Chen, & Touma, 2017). Besides its regulating role in behavior and cognition, a dysregulation of the HPA axis is also linked to alterations of the immune system (Hodes, Kana, Menard, Merad, & Russo, 2015).

1.7 Immune system

1.7.1 Basics of immunity

The response of the immune system towards pathogens can be divided into the innate and the adaptive immune system. The innate immune system's response is non-specific, composed of leukocytes, shows an immediate maximal response upon exposure to pathogens, and is found in nearly all life forms (Wilson, 2018). It is already functional immediately after birth and does not require previous exposure to a particular pathogen (J. Stewart, 2012). Besides being differentially regulated by the age of an individual, the response of the innate and adaptive immunity is sex-dependent (Klein & Flanagan, 2016). This variability between the sexes emerges from differences between the sex chromosomes and hormones (e.g. estradiol, progesterone, and androgens), highlighting the importance of differential immune system reactivity in health and disease (Klein & Flanagan, 2016).

The innate immune system

The first lines of defense of the innate immune system are the mechanical barriers formed by the skin and mucous membranes (Wilson, 2018). On the cellular level, the innate immune system is regulated by pattern-recognition receptors (PRRs) which are mainly expressed by leukocytes and can be found in the cell membrane or the cytoplasm. PRR such as toll-like receptors (TLRs) can

detect pathogen-associated molecular patterns (PAMPs) which are found in the cell walls of bacteria and viral nucleic acids (Lee, Chathuranga, & Lee, 2019; Medzhitov, 2001) or damage-associated molecular patterns (DAMPs) which are host cell molecules released following cellular stress or damage (Gong, Liu, Jiang, & Zhou, 2020).

Cell-mediated innate immunity is facilitated by leukocytes (eosinophils, basophils, natural killer cells, phagocytes including macrophages, neutrophils and dendritic cells) (Chaplin, 2010). The recognition of PAMPs and DAMPs by PRRs is leading to the activation of those cells, inducing phagocytosis, antigen presentation and the release of cytokines, which in turn can have regulatory effects on the immune system (Chaplin, 2010). Antigen presentation is accomplished by phagocytes, which circulate through the lymphatic vessels to the lymph nodes where they present antigens to B and T cells of the adaptive immune system, leading to specialization of those cells (Chaplin, 2010).

The humoral (non-cell-mediated) response of the innate immune system is facilitated by acute-phase proteins, interferons, lysozymes, and the complement system (Riera Romo, Perez-Martinez, & Castillo Ferrer, 2016). One mechanism by which the innate immune system can detect and neutralize invading Gram-negative bacteria (e.g. *E. coli*) is the complement system. The complement system is made up of approximately 30 different proteins. They are present in the blood serum and, upon recognition of a pathogen, form a membrane attack complex (MAC) for the lysis of the invading microorganisms (Bayly-Jones, Bubeck, & Dunstone, 2017).

The adaptive immune system

The adaptive/acquired immunity, in contrast, is highly specific through humoral responses facilitated by antibodies recognizing specific antigens (Medzhitov & Janeway, 1998). The adaptive immune system shows a timely lag between exposure and maximal response, is responsible for immunological memory, and is only found in jawed vertebrates (Cooper & Alder, 2006). It comprises B and T cells that derive from hematopoietic stem cells, originating from the bone marrow (Kondo, 2010). T cells are matured in the thymus while B cell maturation occurs within the lymph nodes and spleen (LeBien & Tedder, 2008). T lymphocytes (such as killer T cells, helper T cells, and γδ T cells) are regulating the cell-mediated response of the acquired immunity (Gao & Williams, 2015). B cells are important for the production of antibodies which is a fundamental part of the non-cell response of the adaptive immunity (Bonilla & Oettgen, 2010).

1.7.2 Inflammatory response following stress exposure

Influence of GCs

Immune responses are playing a vital role in the response to stress and the immune system is connected to the HPA axis, the ANS, as well as the CNS by either giving feedback to those systems

or by initiating adequate responses and adaptations following exposure to a stressor (Black, 1994). Chronic stress and high levels of GCs can lead to GC resistance (Cohen et al., 2012; Miller, Cohen, & Ritchey, 2002) and impair the immune system. This is resulting in increased susceptibility to infection and inflammatory processes (Cohen et al., 2012; Elenkov & Chrousos, 2006). GCs can either induce a pro-inflammatory or an anti-inflammatory effect on the immune system by GR-mediated transcriptional regulation (Busillo & Cidlowski, 2013; Ronchetti, Migliorati, Bruscoli, & Riccardi, 2018). Anti-inflammatory effects are evoked by a downregulation of the expression of pro-inflammatory genes (e.g. PPRs, MHC-II, pro-inflammatory cytokines). However, the pro-inflammatory effects of GCs are not completely understood (Cruz-Topete & Cidlowski, 2015). It seems that GCs promote both the expression of pro- and anti-inflammatory genes simultaneously and by this priming the organism for responding to harmful stimuli (Frank, Watkins, & Maier, 2015). Chronic exposure to GCs can, however, suppress the immune function (Sorrells & Sapolsky, 2007). Therefore, the actions of GCs on the immune system depend on several factors such as the duration of the stimulus and the physiological state of the immune system (Cruz-Topete & Cidlowski, 2015).

Stress and the immune system

Stressors can increase the inflammatory activity that might promote depressive symptoms (Slavich & Irwin, 2014). There is a body of evidence for the impact of psychological stress on the immune system, ranging from stress during development to stress during later life. Early life adversity (ELA) affects the reactivity of the immune system to acute stress with a heightened response of proinflammatory cytokines (Pace et al., 2006), and increased inflammation in adulthood (Coelho, Viola, Walss-Bass, Brietzke, & Grassi-Oliveira, 2014; Danese et al., 2008; Kiecolt-Glaser et al., 2011). It also exerts negative effects on psychological and physiological well-being, including a higher risk for the development of depression (Hostinar, Nusslock, & Miller, 2018). Interestingly, individuals with psychiatric diseases such as depression have higher levels of pro-inflammatory cytokines (Danese et al., 2008; Kim et al., 2008; Morris et al., 2011; Shelton et al., 2011; Vogelzangs et al., 2012). A study on the effects of pro-inflammatory cytokines interleukin 2 (IL-2) and IFNγ treatment found that these evoke a higher probability for the development of somatic symptoms during the early stages of treatment (Capuron & Miller, 2004) and prophylactic antidepressant treatment is sufficient to prevent the development of those symptoms (Musselman et al., 2001). This is further strengthening the link between inflammation and mood disorders. Individual differences in the response to antidepressant treatments are also linked to differences in immune system activation (Maes et al., 1997). Heightened proinflammatory profiles, as observed during depression, remain heightened after antidepressant treatment in non-responders and elevations of proinflammatory cytokines positively correlate with symptom severity and episode duration (Thomas et al., 2005).

Another source of stress emerges from social interactions. In humans, exposure to acute social stress during the Trier Social Stress Test (TSST) (Kirschbaum, Pirke, & Hellhammer, 1993) increases levels of proinflammatory cytokines such as IL-1β (Yamakawa et al., 2009), IL-1RA, IL-6, and TNF-α (Goebel, Mills, Irwin, & Ziegler, 2000; Hackett, Hamer, Endrighi, Brydon, & Steptoe, 2012; O'Donnell, Brydon, Wright, & Steptoe, 2008). Interestingly, the release of IL-1β during the TSST is positively associated with the susceptibility to develop depressive symptoms one year after the TSST was conducted (Aschbacher et al., 2012). Interestingly, a study investigating the levels of pro-inflammatory cytokines following bereavement found upregulated levels of pro-inflammatory cytokines within the blood plasma (Schultze-Florey et al., 2012), further strengthening the link between bereavement as a stressful experience that can influence immune system activation (Mason & Duffy, 2019).

Animal studies that investigate the link between dysregulations of the stress response and the immune system are describing similar findings. Rodent models of depression are also showing an increased peripheral and CNS inflammation (Goshen et al., 2008; Grippo, Francis, Beltz, Felder, & Johnson, 2005; You et al., 2011). Furthermore, the absence or even the loss of social interactions can lead to increased levels of peripheral immune system activation in female prairie voles (Scotti, Carlton, Demas, & Grippo, 2015). The increased immune system activation can be evidenced by a higher abundance of pro-inflammatory cytokines which is associated with lower food and water consumption, changes of social behaviors, and sleeping patterns, forming a direct link between the inflammatory status and behavior (Crestani, Seguy, & Dantzer, 1991; Hart, 1987; O'Reilly, Vander, & Kluger, 1988; Yirmiya, 1996). While inter-individual conflict, social isolation, and non-social stress have been extensively studied for its impacts on the immune system, no study to date is describing the impact of losing a bonded partner on the immune functioning.

1.8 Microglia

Different from the peripheral immune system, microglia are the CNS' resident immune cells and were first discovered and described in 1919 in a series of publications by del Río-Hortega, a student of Santiago Ramón y Cajal (del Río-Hortega, 1919a, 1919b, 1919c, 1919d; Sierra et al., 2016). These early descriptions were later put into context del Río-Hortega's 1932 publication (del Río-Hortega, 1932; Kettenmann, Hanisch, Noda, & Verkhratsky, 2011) where he postulated that:

a) microglia enter the brain early during development,

b) those cells are of mesodermal origin and have amoeboid morphology,

c) they utilize vessels and white matter tracts for the guidance within the brain,

d) they have a more ramified, branched morphology in the adult brain,

e) they have their own territory,

f) following stimulation through e.g. a pathological event, they transform morphologically,
g) following transformation, they acquire amoeboid morphology,
h) once they show amoeboid morphology, they can migrate, proliferate, and phagocytose.

Remarkably, these early postulates remain to be disproven until the present day. The original terming of microglia was microgliocyte, which was changed to microglia soon after (Kettenmann et al., 2011). However, del Río-Hortega wasn't first to recognize microglia. Previously, microglial cells were already visualized by Nissl, Alzheimer, and Merzbacher (Alzheimer, 1910; Merzbacher, 1907). However, those descriptions lacked clarifications of the origin of microglia and they were termed rod cells, grid cells, and clearance cells.

Microglia are infiltrating the CNS early during development and are of mesodermal origin, descending from hematopoietic stem cells (Ginhoux & Garel, 2018). This distinctive origin separates microglia from other types of glial cells like astrocytes and oligodendrocytes which share the same precursor derived from neuroepithelial cells (Rowitch & Kriegstein, 2010). Interestingly, other glia cells only start to emerge towards the end of neurogenesis, which further highlights the importance of the microglia's regulatory processes in shaping the functioning of the brain (Reemst, Noctor, Lucassen, & Hol, 2016). Apart from being vital to the development of the CNS, they also provide crucial support and mediation to neuronal processes during adulthood (Reemst et al., 2016).

Microglia make up between 10 – 15% of the cells of the CNS (Kettenmann et al., 2011) and are actively regulating the homeostasis and functioning of the brain, not only in health, but also during pathological conditions (Greenhalgh, David, & Bennett, 2020). They potentially influence axonal outgrowth and guidance (Reemst et al., 2016) as they are found in close proximity to axonal tracks (Pont-Lezica et al., 2014; Verney, Monier, Fallet-Bianco, & Gressens, 2010) and can inhibit axonal outgrowth (Kitayama, Ueno, Itakura, & Yamashita, 2011). Microglia are also involved in synaptic pruning and neuronal remodeling through e.g. fractalkine-fractalkine receptor (CX3CL1-CX3CR1) signaling (Bialas & Stevens, 2012; Limatola & Ransohoff, 2014) and can stimulate synaptogenesis during brain development (Kettenmann, Kirchhoff, & Verkhratsky, 2013). Furthermore, they are also directly involved in programmed neuronal cell death (Marin-Teva et al., 2004; Wakselman et al., 2008), but can also promote neuronal survival (Ueno et al., 2013).

Microglia express a wide range of receptors, not only limited to inflammatory signaling through cytokines but also receptors for most neurotransmitters (Kettenmann et al., 2011). Since they are a regulatory part of the CNS, they need to monitor the brain parenchyma for changes of the homeostasis in order to, once dysregulated, re-establish the homeostasis (Li & Barres, 2018). This can be modulated by the sex of the individual as microglia also show differences in functionality and morphology between both sexes (Guneykaya et al., 2018); they differ in their membrane

potential properties, show different transcriptome- and proteome profiles as well as region-dependent differences in microglia soma size, with male microglia covering a larger area within the cortex, hippocampus, and amygdala (Guneykaya et al., 2018). Furthermore, also the density of microglia and their function is different between males and females (Bilbo, Smith, & Schwarz, 2012). Those differences might explain why the response of microglia towards exogenic stressors differs between sexes (Bollinger, Bergeon Burns, & Wellman, 2016; Diz-Chaves, Pernia, Carrero, & Garcia-Segura, 2012).

Activation of microglia

Microglia activation is linked to an increase of proliferation (Kettenmann et al., 2011) which can be categorized into four different states of activation (Torres-Platas, Comeau, et al., 2014) (**Figure 4)**. The first state comprises ramified microglia, which are morphologically characterized by the possession of a small, round soma with numerous highly ramified processes (Torres-Platas, Comeau, et al., 2014). They are actively surveilling their surrounding brain parenchyma that might be altered by injury, neuronal activity, and cytokines (Deverman & Patterson, 2009; Liu et al., 2019). Upon detection of a molecular change or PAMPs or DAMPS and depending on the nature of the stimulus, microglia can transform into an activated state and actively modulate inflammatory processes or phagocytose cell debris.

The second state of activation is encompassing primed microglia (Walker, Nilsson, & Jones, 2013). Morphologically, the soma of those microglia is elongated and increased in size, and they possess highly ramified processes (Torres-Platas, Comeau, et al., 2014). Microglial priming is linked to an exaggerated inflammatory response following immune challenge or stress (Niraula, Sheridan, & Godbout, 2017; Wohleb et al., 2011). Inflammatory processes are not necessarily increased following priming, however an increase in the expression of IL-1β, major histocompatibility complex class II (MHCII), LGALS3, and TLRs can be observed (Holtman et al., 2015; Niraula et al., 2017). Together with a reduction of fractalkine receptor (CX3CR1), microglial priming is linked to a pro-inflammatory phenotype (Frank, Fonken, Annis, Watkins, & Maier, 2018; Niraula et al., 2017). Furthermore, microglia can further transform into a reactive phenotype, the third state of microglia activation. Reactive microglia can either possess a pro-inflammatory (M1) or anti-inflammatory (M2) polarization state (Prinz & Priller, 2014). Morphologically, reactive microglia have large soma and a reduced number and ramification of processes (Torres-Platas, Comeau, et al., 2014). Both polarization states can actively phagocytize cell debris. The M1 polarization state is more commonly observed during the onset of microglia activation, while the M2 state usually is linked to receding inflammation, neuronal repair and wound healing, and return to homeostasis (Tang & Le, 2016). Molecularly, microglia in the M1 polarization state are characterized by the synthesis and release of pro-inflammatory cytokines such as IL-1β, IL-6, or TNF-α, thereby aiding

the further activation and upholding of the inflammatory response (Krady et al., 2005). Microglia in the M2 state release anti-inflammatory cytokines such as IL-10 and IL-4 and are involved in the repair mechanisms and returning to an anti-inflammatory state (Bolós, Perea, & Avila, 2017; Cherry, Olschowka, & O'Banion, 2014). The fourth state of microglial activation is the amoeboid state. It can be morphologically distinguished by the possession of large soma with few unramified processes (Torres-Platas, Comeau, et al., 2014). Microglia that fall into this category are mainly phagocytotic and highly mobile (Torres-Platas, Comeau, et al., 2014). If the inflammatory response following activation of microglia remains unresolved, microglia can become chronically activated, which can be observed in patients suffering from neurodegenerative diseases and MDD. (Pasqualetti, Brooks, & Edison, 2015; Perry, 2012; Sarlus & Heneka, 2017; Torres-Platas, Cruceanu, Chen, Turecki, & Mechawar, 2014).

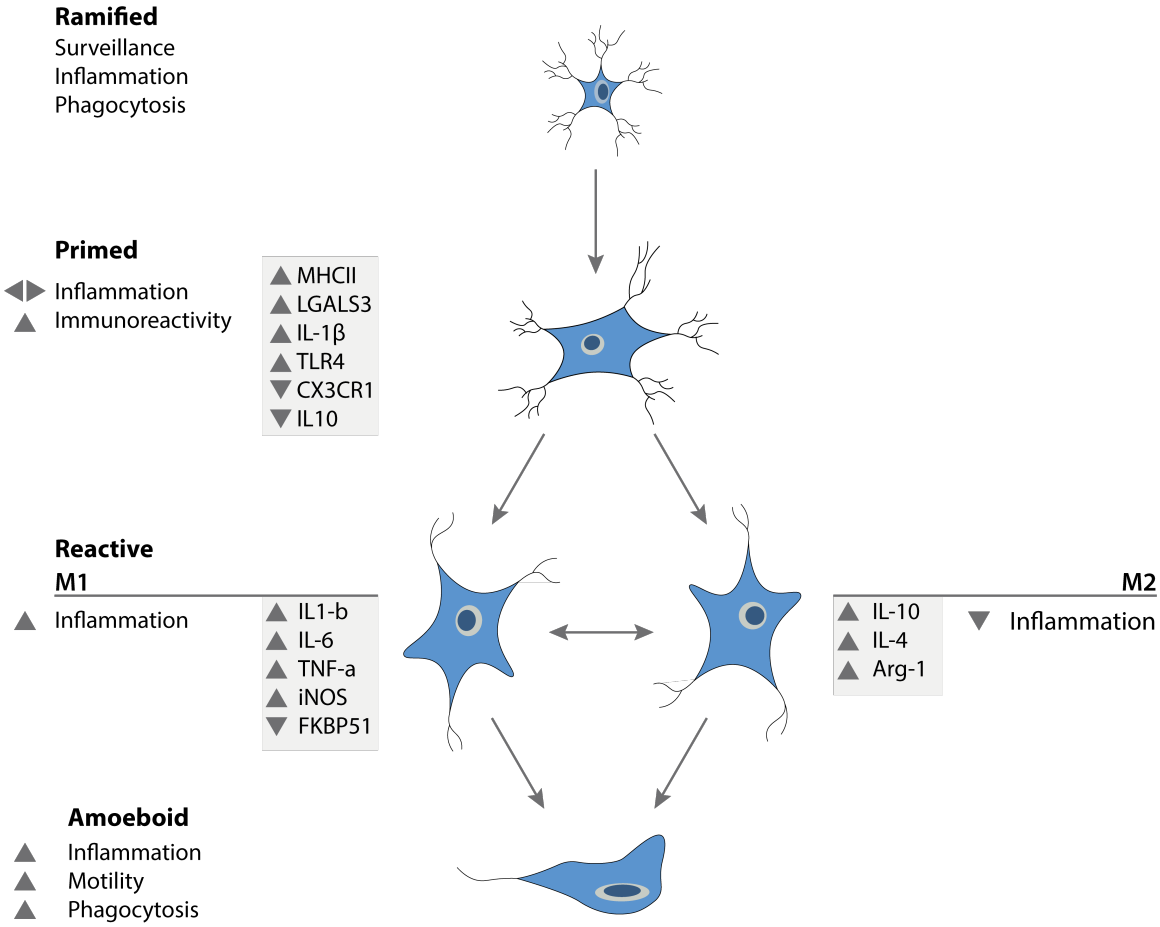

Figure 4 | Microglia activation states.
Under homeostasis, microglia are in the ramified state. Ramified microglia can be distinguished by the possession of a small soma and several highly ramified processes. Upon stimulation, microglia transform to either the primed state, reactive state, or amoeboid state. Primed microglia are morphologically characterized by an increased soma size and highly ramified processes. They have a pro-inflammatory phenotype while inflammation is not necessarily changed compared to ramified cells. The expression of MHCII, galectin 3 (LGALS3), TLR4 and cytokines IL-1β is increased while a decrease in CX3CR1 and IL-10 can be observed. Reactive microglia have an increased soma size and reduced number of processes and ramifications. Both the M1 and M2

Figure 4 | Continued.
polarization state are mobile and perform phagocytosis. The M1 polarization state is marked by an increase in the expression of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α, paired with increased MHCII and iNOS expression. The M2 polarization state is marked by an increase in the expression of anti-inflammatory cytokines IL-10, IL-4, and Arg-1. While reactive microglia in the M1 polarization state are linked to inflammatory responses, M2 microglia are linked to receding inflammation. Amoeboid microglia have an amoeboid-like shape with few unramified processes and are mainly phagocytotic and highly mobile.

1.8.1 Microglia response following stress exposure

Due to the expression of receptors for e.g. glutamate, norepinephrine, serotonin, and GCs on microglial cells, microglia can become activated due to changes of neuronal activity following exposure to a stressor (Pocock & Kettenmann, 2007). Chronic stress can lead to the activation of the HPA axis and the sympathetic nervous system, and the release of GCs has been implied to affect the neuroimmune system (Frank, Fonken, Watkins, & Maier, 2019). Interestingly, the microglial expression of GR is higher than of MR, suggesting that GC-GR interactions are more prevalent within those cells during stressor exposure (Sierra, Gottfried-Blackmore, Milner, McEwen, & Bulloch, 2008). *In vitro* studies show that GCs preferentially bind to MRs at low doses of GC, activating NF-κB (Chantong, Kratschmar, Nashev, Balazs, & Odermatt, 2012). Higher levels of GCs, on the other hand, lead to increased binding to GRs which, in turn, suppresses NF-κB. Thus, GCs can cause differential effects on microglia gene regulation which is dependent on the levels of GC release (Chantong et al., 2012). Acutely, GCs can prime microglia towards an inflammatory response to a following inflammatory stimulus (Frank, Miguel, Watkins, & Maier, 2010; Frank, Thompson, Watkins, & Maier, 2012). Chronic stress, however, can lead to GC resistance and a reduction of GR expression of microglia cells, which, on the one hand, might have protective effects against the overactivation of microglia cells, but also can cause a dysregulation of the neuroimmune system regulation of inflammatory responses (Watters & Pocock, 2014). Furthermore, microglia are sensitive to CRF through the expression of CRFR1 and CRFR2 (Stevens et al., 2003). Following the exposure to stress, the release of CRF is increased which initiates the stress response but also induces pro-inflammatory responses of microglia. Those responses include an elevated secretion of pro-inflammatory cytokines, which in turn can further induce the release of CRF, generating a positive feedback loop (Y. Jiang et al., 2019). Besides enhancing the release of CRF, exposure to a stressor (either acute or chronic) can also alter the release of other neurotransmitters such as glutamate and GABA (McEwen, 2000). While glutamate has a pro-inflammatory effect on microglia, GABA induces anti-inflammatory responses (Pocock & Kettenmann, 2007). Therefore, microglia functioning can be altered through multiple systems, which can get dysregulated during acute or chronic stress. A dysregulation of the neuroimmune system, through for example early life adversity, can contribute to the development of psychiatric disorders such as MDD, as they were

shown to alter microglia activity (Tay et al., 2017). Interestingly, depressive symptoms as observed during MDD can be directly caused by the activation of the immune system through lipopolysaccharide (LPS) administration, potentially linking observed behaviors to inflammatory processes (Yirmiya et al., 2000). Besides early life adversity, chronic stress is associated with the development of MDD, which is positively correlating with neuroinflammation, marked by increased microglial activation (Torres-Platas, Cruceanu, et al., 2014).

Studies in rodents reveal that chronic stress is enhancing the activation of microglia through GRs and the release of cytokines can be diminished by blocking GRs (Herrera et al., 2015), which is suggesting Interestingly, following chronic stress exposure, a reduction of the expression of glucocorticoid-induced leucin zipper (GILZ) and FKBP51, which are negatively regulating GR activity (Ratajczak, 2015), was observed.

Sex-specific effects of stress on microglia morphology

Sex differences in microglia reactivity towards stressors were recently described (Bollinger et al., 2016; Bollinger, Collins, Patel, & Wellman, 2017). Microglia within the mPFC of male rats show a more pro-inflammatory morphology following acute stress and no changes following chronic stress, while in female rats microglia resemble a more anti-inflammatory state following acute and chronic stress (Bollinger et al., 2016). Furthermore, acute stress affects microglia priming in a sex-dependent manner, which in turn influences the response towards acute immune challenges (Fonken et al., 2018).

The cause for the difference in the function of microglia still needs to be clarified. One driving factor of sex-differences in the response of microglia to stress might be the difference in the expression of sex steroids. Estrogens are known to play a major role in the regulation of microglia activity (Villa, Vegeto, Poletti, & Maggi, 2016) as microglia express receptors for both estrogen and progesterone (Sierra et al., 2008). In detail, estrogen and progesterone reduce the expression of pro-inflammatory mediators of the inflammatory response and by this exert anti-inflammatory effects on the neuroimmune system (Sierra et al., 2008). Therefore, sex-dependent differences in the expression of both steroids during or following stressor-exposure might modulate the pro- or anti-inflammatory response of the neuroimmune system.

Behavioral and neuroinflammatory changes following stress exposure

In animal models, stressors induce behavioral alterations such as anxiety-like and depressive-like behaviors also affect microglia in a multitude of brain regions important for the regulation of emotionality, sociality, and memory (Bollinger et al., 2016; Wang et al., 2018). In male rats, repeated restraint stress causes a reduction in body weight, anhedonia, and less time spent struggling during the restraint (Tynan et al., 2010). Those findings were correlated with an increase in ionized

calcium-binding adapter molecule 1 (IBA1) signal in the NAcc, IL, and PrL, while no changes were observed in the PVN (Tynan et al., 2010). An increase in $IBA1^+$ immunofluorescence is linked to an elevation in microglia activation. Chronic unpredictable stress is also altering cognitive capabilities in male mice by negatively affecting spatial learning and memory which is coupled to an increase in $IBA1^+$ cells in the CA3 region of the hippocampus and the PrL (Bian et al., 2012). Social stressors such as chronic social defeat can cause social deficits such as a decrease in scent marking and social interaction in mice (Lehmann, Cooper, Maric, & Herkenham, 2016). Interestingly, this is correlated with an increase in CD68 expression of microglia within the PVN, indicative of an activated microglia profile (Lehmann et al., 2016). Interestingly, those differences in microglia responses are attributed to differences in the ability to cope with named stressors (Lehmann et al., 2018). Acute stress does not alter those parameters but increases microglia proliferation (Lehmann et al., 2016). This is hinting at dynamic alterations of microglia activation based on the temporal duration of the stressor exposure as it has been described elsewhere (Kreisel et al., 2014). Furthermore, repeated social defeat in mice evokes an increase in $CD14^+$ and $CD86^+$ microglia cells, and an increased proportional area covered, indicating an increase of activated microglia morphology and molecular profile of microglia in the MeA, the PFC, and the Hipp (Wohleb et al., 2011). The same study also reports an increase in the pro-inflammatory cytokine IL-1β and decreased GILZ and FKBP51 mRNA levels in microglia cell cultures derived from repeatedly socially defeated animals. Also, microglia of defeated animals are more sensitive to treatment with LPS, marked by a release of pro-inflammatory cytokines IL-6, TNF-α, and MCP-1 (Wohleb et al., 2011). This is indicative of microglial priming following stressor exposure as discussed previously. Besides social defeat, also the absence of social interactions causes microglia activation (Schiavone et al., 2009). Social isolation causes an increase in oxidative stress and increased immunoreactivity for IBA1 in the PFC and NAcc of male rats (Schiavone et al., 2009). Prolonged stress exposure, however, can cause a decrease in inflammatory profiles of microglia (Kreisel et al., 2014), which might be linked to negative feedback mechanisms of the HPA axis via GR activation (Adzic et al., 2009; Calcia et al., 2016). Animals that are resilient to chronic social defeat showed lower phagocytosis and oxidative stress as well as intact BBB function, which was disrupted in susceptible animals and might further lead to microglial activation (Lehmann et al., 2018). Therefore, the modulation of the neuroinflammatory response and its impact on cognition and behavior represents a promising target to elucidate the effects of stressors on the on the individual.

1.9 Aims

The loss of a partner is a disruptive event that can severely impact the physiology and emotionality by altering the homeostasis of the stress system, the ability to cope with stressors as well as the central release of neuropeptides. One neuropeptide system which is dysregulated following the loss of a partner is the OT system. It plays a central role in the formation of a partner preference, is also involved in the negative effects on emotionality and behavior following the loss of a partner. Reversal of the attenuated OT release within the NAcc shell can counter altered stress-coping following separation from a partner. As mentioned earlier, besides the OT system, other systems such as the CRF system are dysregulated following the loss of a partner. Dysregulations of neuropeptide systems can, in turn, affect both the neuro- and peripheral immune system. Prolonged immune system alterations are furthermore linked to either complicated grief following the loss of a partner or other psychiatric disorders, in which both sexes show differences in the susceptibility for their development.

While the peripheral immune system of grieving individuals has received some attention in the literature, insights into the neuroinflammatory response following the loss of a partner are scarce. To date, there are no studies in rodent models investigated the impact of partner loss in conjunction with acute stress on the neuro- and peripheral immune system or even its differences between both sexes.

Therefore, this thesis aimed to:

1. Reverse the negative effects of partner loss on the emotio-behavioral response using a pharmacological approach by increasing the endogenous release of OT in male and female prairie voles.
2. Investigate the effects of separation from a partner in conjunction with acute stress on the neuro- and peripheral immune system in male and female prairie voles.

Material and Methods

2 Material and Methods

2.1 Animals

All animal experiments were conducted at the Yerkes National Primate Research Center and performed on sexually naïve female and male adult prairie voles of a laboratory breeding colony originally derived from field-captured voles in Illinois. Prairie voles for the first study were between 90-160 days of age with a body weight between 24-60 g and group sizes of *n*=8-12. The prairie voles used for the second study were between 60 to 124 days of age for analysis of neuro- and peripheral immune system parameters and 139 to 212 days of age for analysis of gene expression. The body weight of those prairie voles was between 26-58 g and the groups sizes were *n*=10. Prior to the experiment, male and female voles were housed in groups of 2-3 voles. The prairie voles were housed in pairs or alone under a 14/10 h light/dark cycle at 20 °C and 60% humidity with food (LabDiet rabbit chow) and water *ad libitum*. All experiments were performed between 0800 h and 1600 h in accordance with the guidelines set by the National Institutes of Health and were approved by Emory University's Institutional Animal Care and Use Committee.

2.2 Experimental design

To avoid pregnancies in females, all male voles including the stimulus animals were sterilized by electrocauterization of the *vas deferens*. Following of 14 d of recovery post-surgery, voles were paired with an unfamiliar opposite sex-stranger. Voles were then allowed to cohabitate for 5 days which is sufficient for partner preference formation (Bosch et al., 2009). Subsequently, voles were assigned to groups of voles that continued to stay with their partner (pair bonded with partner: *pbp*) or voles that were separated from their partner (pair bonded separated: *pbs*).

For the first study, treatment with either MTII or vehicle commenced on the first day of separation and was administered once per day for 8 consecutive days. 30 min after the daily treatment, separated voles were either subjected to 30 min social interaction or remained alone. Social interaction was recorded on post-cohabitation day 5 and 12. Behavioral testing was conducted on post cohabitation day 9 (elevated plus maze: EPM) and 11 (forced swim test: FST). The prairie voles were sacrificed in the morning of post-cohabitation day 13. The experimental timeline and group compositions are shown in **Figure 5** and **Figure 6.**

For the second study, subsequent to the 5 d cohabitation period, the voles were either separated or stayed in pairs until euthanasia in the morning of post-pairing day 8 (**Figure 7**). Group composition and treatment is shown in more detail in **Figure 8 A** for the experiment investigating microglia morphology and **Figure 8 B** for the depiction of group composition of the gene expression experiment.

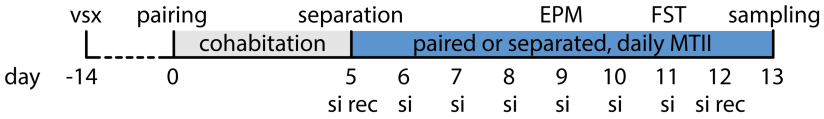

Figure 5 | Timeline of the investigation of MTII treatment and social interaction on stress coping, anxiety, and social interaction.
Animals were vasectomized (vsx) 14 days prior to pairing with an opposite sex stranger vole. Following 5 days of cohabitation, voles remained paired or were separated. Starting on the separation day, the voles received either VEH or MTII daily for 8 consecutive days. After this treatment they were either subjected to 30 min social interaction (si) or no social interaction. Si was recorded (si rec) on the first and the last day of treatment. Behavior was assessed on post-pairing day 9 (EPM) and 11 (FST). Animal tissue was sampled on experimental day 13.

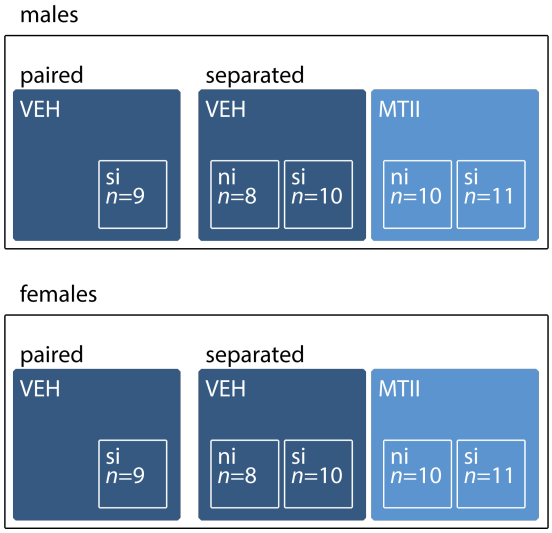

Figure 6 | Experimental groups of the investigation of MTII treatment and social interaction on stress coping, anxiety, and social interaction.
Individual group sizes were *n*=8-11. In detail, male (*n*=48) and female (*n*=48) voles were split into groups which remained either paired (*n*=9) or were separated (*n*=39) from their partner. Separated voles were split into groups which received either VEH (*n*=18) or MTII (*n*=21) treatment. Separated voles receiving VEH were further split into groups of voles that received daily social interaction (si, *n*=10) or no social interaction (ni, *n*=8). MTII-treated voles were split into groups receiving si (*n*=11) or ni (*n*=10). Voles that remained paired received VEH treatment and remained with their partner for the analysis of si.

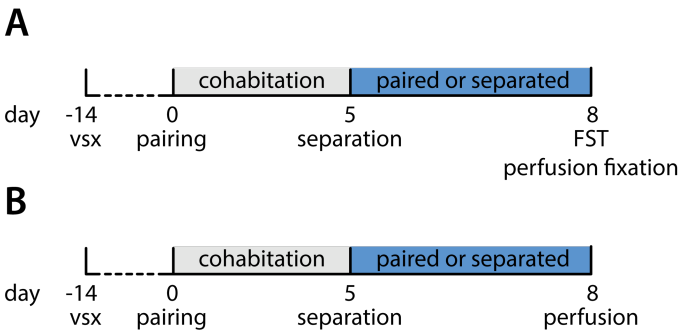

Figure 7 | Timelines of experiments investigating the immune system response following partner loss.
(A) Males were vasectomized (vsx) 14 days prior to cohabitation. After a cohabitation period of 5 days, voles were either separated or remained with their partner. On the 8th day after pairing, voles were subjected to the FST and were euthanized and fixated using 4% PFA. **(B)** Male voles were vasectomized 14 days prior to cohabitation. After a cohabitation period of 5 days, voles were either separated or remained with their partner. On the 8th day after pairing, voles were euthanized, and samples were collected following perfusion.

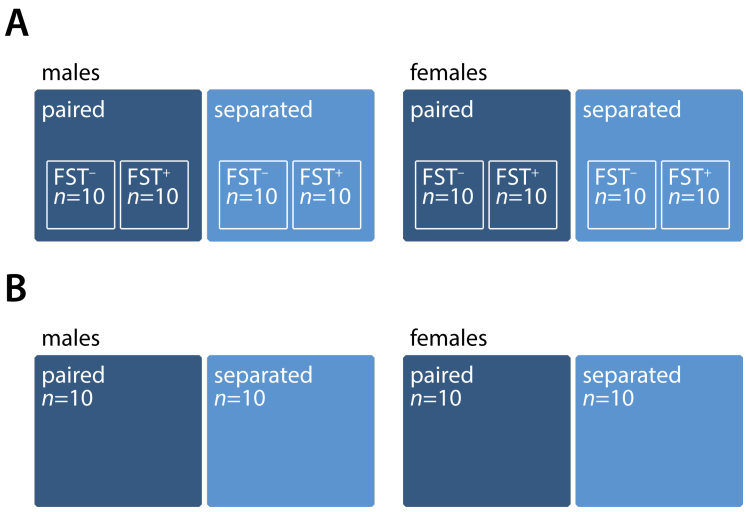

Figure 8 | Group compositions of experiments investigating the immune system response following partner loss.
(A) To assess morphological changes of microglia morphology, the voles were assigned to 8 different groups. In detail, male (n=40) and female (n=40) voles were split into groups which remained either paired (n=20) or were separated (n=20) from their partner. Paired and separated voles were then split into groups of FST$^-$ (n=10) or FST$^+$ (n=10), resulting in a total of 8 groups with n=10 for each group. Animals were split into 8 groups based on sex, pairing status and stress exposure by the means of the FST. **(B)** To study gene expression following partner loss, male (n=20) and female (n=20) voles were either kept paired (n=10) or separated (n=10) from their partner resulting in four groups of n=10 each.

2.3 Generation of sterile male voles

For the generation of sterile voles, males were vasectomized. Males were briefly anaesthetized with 1.5% - 2.5% Isoflurane (Piramal Critical Care, Inc., Bethlehem, PA, USA), procedure was carried out under continuous anesthesia with isoflurane through a nose cone, covering the nose and mouth. First, the abdomen was shaved, the voles were weighed and placed onto the surgery site. Voles were injected with 2 mg / kg Meloxicam (Alloxate, Patterson Veterinary, Devens, MA, USA) at 0.5 mg / ml in sterile saline (Bacteriostatic Sodium Chloride 0.9%, #63323092410, Fresenius Medical Care, Waltham, Massachusetts, USA) using a 25 G syringe (Catalog no. 329651, Becton, Dickinson and Company, Franklin Lakes, USA). The incision area was cleaned with 3 swabs of alcohol and iodine. Vital signs (breathing, heart rate, SpO_2, and body temperature) were continuously monitored, and temperature of the warming pad was automatically controlled using a PhysioSuite™ (Kent Scientific Corporation, Torrington, USA). After assessment of anesthesia by toe pinching, a ventral midline incision with the size of 1 cm was made on the abdomen of the animals to open the skin. Another 1 cm incision was then made in the body wall to expose the body cavity. Following, the *vas deferens* was located by externalizing the fat pads located in the abdominal pelvic cavity attached to the testes and held firmly using forceps. Using electrocautery, the *vas deferens* was severed at two points and the intervening section (±5 mm) was removed. The remaining parts of the *vas deferens* and fat tissue were returned into the abdominal cavity and the same steps were repeated on the opposite *vas deferens*. Finally, the abdominal cavity and skin were closed with 3 to 4 stitches using non-absorbable braided suture for the body wall and absorbable monofilament suture for the skin (Ethicon, Inc., NJ, USA). The voles were placed in a fresh cage with bedding added from their previous home cage. All voles were monitored until they were in sternal position. Continuous warming of the cage was provided for at least 1 h following the surgery to prevent the voles from cooling.

2.4 Treatment administration

Drug administration commenced on the first day of separation between 1200 and 1600 h. Voles were injected *i.p.* using a 1 ml syringe attached to a 25 G needle (Catalog no. 329651, Becton, Dickinson and Company, Franklin Lakes, USA) with either the melanocortin receptor 4 agonist MTII (10 mg / kg in 0.9% saline; Toronto Research Chemicals, North York, Canada) or vehicle (0.2 ml 0.9% saline). Following the injections, voles were placed back into their homecages for 30 min until social interaction with a stimulus animal. Stimulus animals were opposite-sex strangers, whereas male stimulus voles were vasectomized at least 2 weeks prior to the first social interaction.

2.5 Behavioral testing

2.5.1 Social Interaction

Since MTII is affecting the OTergic and other systems involved in social behavior, voles were either subjected to social interaction (si) or no social interaction (ni) on the first day of separation 30 min following injection of either MTII or vehicle. In the case of separated voles, an opposite-sex stranger was introduced the homecage. Each social interaction lasted for 30 min and was repeated daily following the injection for 8 consecutive days. Non-separated voles did not receive a stranger animal. A camera was placed in an overhead view approximately 40 cm from the cage. Social interaction between voles was recorded and analyzed on the first and the last day of treatment, and conducted in the afternoon between 1200 and 1600 h. The time and frequency of behaviors was analyzed using JWatcher (Version 1.0, downloaded from http://www.jwatcher.ucla.edu, (Blumstein & Daniel, 2007)). Analyzed behaviors included: immobile, mating, attack, chasing, being sniffed, autogrooming, grooming, being groomed, other social non-aggressive, huddling, offensive upright, investigating, sniffing, being chased, lateral threat, being investigated, and exploration. Some behaviors were grouped into active aggressive (attack, chase, lateral threat, offensive upright), passive aggressive (being attacked, being chased), active social (grooming, sniffing, investigating, huddling, mating), passive social (being groomed, being sniffed, being investigated).

2.5.2 Forced Swim Test

To assess passive stress coping following the separation from a partner the FST was performed as previously described (Bosch et al., 2009). For the first study, prairie voles were tested in the FST on the day before euthanasia, in the second study, the FST was performed on post-cohabitation day 8. Briefly, animals were placed in a 5 L water beaker filled with 3 liters of tap water (24±1 °C) for 5 min. Behavior was recorded from the side with a video camera placed approximately 30 cm from the beaker. After 5 min of testing, the animals were removed from the water, briefly dried with tissue paper and placed back into their home cage. Paired animals were tested simultaneously by placing each in a separate beaker. The order by which paired male and female were taken from their cage was altered to balance out the probability of confounding the test by removing one partner first. Behaviors scored were struggling (animal breaking the water surface with their front paws), swimming (directed movement within the beaker without the front paws breaking the water surface), and floating (animals not actively swimming, non-directional, only with some movements of front and rear paws to keep equilibrium). Assessed were the time of the animals behaving, the frequency, and the time until first float.

2.5.3 Elevated Plus Maze

The elevated plus maze was used to assess anxiety-related behavior and is creating a conflict between the animal's exploratory curiosity and their innate fear of open and exposed areas (Handley & Mithani, 1984). The plus maze consisted of an elevated plus shaped maze with two open and two closed arms and a center compartment (dimensions per arm: 60 cm × 10 cm, center: 10 cm × 10 cm). The closed arms were surrounded by a 40 cm high dark-colored PVC wall and an illuminance of 21.7 ±5.4 lux. Open arms were illuminated with 35.7 ±2.3 lux. Two cages with nesting material were placed underneath the EPM to protect the voles that fall and prevent them from escaping. Testing was conducted between 0900 and 1400 h. At the beginning of each trial, the maze was cleaned using soapy water and quatricide and dried prior to placing the voles in the center, facing the open arm. The voles were allowed to freely explore the maze for 5 min. A video camera placed above the center of the elevated plus maze recorded the prairie voles' behavior. Videos were analyzed using JWatcher. For the analysis, the EPM was split into three regions (closed arm, center, open arm). Additional to time until the first entry to an open arm, the number of entries into each arm and the time spent in each arm was recorded, whereas an entry was defined by the center of mass of the vole entering an arm.

2.6 Tissue sampling

Prairie voles were killed in the morning of post cohabitation day 13 between 1000 and 1400 h. Briefly, the voles were removed from their home cages, anaesthetized by CO_2 and quickly decapitated using scissors. Trunk blood was taken, brains were removed and snap frozen in ice cold 2-methylbutane (Sigma Aldrich, St. Louis, USA) on dry ice, wrapped in aluminum foil, and stored at -80 °C until further processing. Adrenal glands were removed and stored on ice in 1× phosphate-buffered saline (PBS, pH 7.4) until weighing. Trunk blood collection was centrifuged for 5 min at 4000 × g, and the supernatant containing the plasma was then transferred to 1.5 ml micro tubes and stored at -80 °C.

2.7 Perfusion

Subjects were removed from their home cage and within 3 min killed using CO_2. The abdominal and thoracic cavity were opened, exposing the heart. To ensure the sterility of blood collection, blood was collected directly from the heart by punctuation of the right ventricle using a sterile 25 G syringe with the needle pointing in distal direction towards the right atrium to avoid laceration of the septum.

The blood was then transferred into a K3 EDTA tube (VACUETTE®, Greiner Bio-One, Kremsmünster, Austria), and shaken to allow even EDTA distribution within the blood samples

and stored on ice until centrifugation. Subsequently, the voles were perfused transcardially through the left ventricle with 30 ml ice cold 1× PBS (pH 7.4) for 5 min, followed by 30 ml of ice cold 4% PFA for 5 min. To ensure correct perfusion, the right atrium was opened using scissors directly after the onset of the perfusion. After the perfusion, the voles were decapitated, brains were removed and placed into a falcon tube filled with 20 ml of 4% PFA for overnight post fixation at 4 °C. Adrenal glands were removed and stored on ice in 1× PBS (pH 7.4) until weighing. The blood samples were centrifuged for 5 min at 4000 × g and the supernatant containing the blood plasma was then transferred to 1.5 ml micro tubes and stored at -80 °C. After overnight fixation of the brains, they were placed into a falcon tube containing 20 ml of 30% sucrose solution and stored at 4 °C for three consecutive days. The brains were then briefly dried and snap frozen in ice cold 2-methylbutane (Sigma Aldrich, St. Louis, USA) on dry ice, wrapped in aluminum foil, and stored at -80 °C until sectioning.

Similarly, in voles which were part of the experiment to measure gene expression, blood was collected by heart puncture and processed as described previously. Perfusion was performed through the left heart ventricle with for 5 min with 30 ml ice cold 1× PBS (pH 7.4) for clearance of brain vasculature of blood and immune system molecules deriving from the periphery. After the perfusion, the voles were decapitated and the brains were removed and snap frozen in ice cold 2-methylbutane (Sigma Aldrich, St. Louis, USA) on dry ice, wrapped in aluminum foil, and stored at -80 °C until sectioning. Adrenal glands and spleen were collected and stored in 1× PBS (pH 7.4) until weighing.

2.8 Measurement of basal CORT levels

Blood plasma was diluted 1:250 by diluting 10 µl plasma in 90 µl of 1× PBS (pH 7.4). Subsequently, 10 µl of the 1:10 dilution was further diluted to 1:200 by adding 190 µl 1× PBS. Afterwards, 8 µl of 1:200 diluted plasma was added to 92 µl of PBS to reach the final concentration of 1:250. All steps were performed on ice. The diluted plasma was then added to the ELISA plates (Corticosterone (Human, Rat, Mouse) ELISA, IBL International GmbH, Hamburg, Germany) and the measurement was performed following the manufacturers' protocol. All plasma samples were measured in duplicates. For the analysis, only measurements in which the intra assay coefficient of variation was ≤ 10% were included.

2.9 Morphological characterization of microglia

2.9.1 Immunohistochemical staining of microglia

To measure microglia morphology and to assess their morphological activation, brains were cut in series of 5 using a cryostat (Leica CM3050 S, Leica Biosystems, Wetzlar, Germany). In detail, 30 µm

thick slices of previously selected regions (PrL, NAcc shell, and PVN) were collected in 2 ml freezing solution at -20 °C. Until further utilization, the brain slices were stored at -20 °C. Brain slices were selected and placed into a 24 well plate, each well filled with 1 ml 1× PBS (pH 7.4). The slices were then washed 3× 15 min in 1× PBS to wash out residual cryoprotectant. Following, endogenous peroxidases were blocked by adding 500 µl of a H_2O_2 solution (3% H_2O_2, 10% MeOH in 1× PBS (pH 7.4)) to each well and placing the well plate on an orbital shaker for 15 min. Subsequently, slices were washed in 1× PBS for 3× 15 min. Afterwards, slices were blocked for 1 h in 250 µl blocking solution (1% BSA, 0.3% Triton X-100 in 1× PBS (pH 7.4)). Following, the slices were incubated in 200 µl blocking solution containing 1:1000 rabbit anti-IBA1 (Lot # WDE1198; FUJIFILM Wako Chemicals, Osaka, Japan) and 1:500 goat anti-NOS2 (sc-7271, Lot # D0317; Santa Cruz Biotechnology, Dallas, TX, USA) primary antibodies overnight at 4 °C on an orbital shaker. After overnight incubation, the primary antibody solution was removed, and the slices were washed 3× 10 min in 1 ml 1× PBS. For the following secondary antibody incubation, 200 µl of blocking solution (1% BSA in 1× PBS (pH 7.4)) with 1:1000 Alexa Fluor 488 goat anti-rabbit (Lot # 1910795D; Life Technologies, Invitrogen, Carlsbad, CA, USA) and 1:1000 Alexa Fluor 594 donkey anti-mouse (Lot # 1918277; Life Technologies, Invitrogen, Carlsbad, CA, USA) secondary antibodies were added and incubated in the dark for 2 h at RT on an orbital shaker. Following the incubation, the slices were washed 3× 10 min in 1× PBS. Finally, slices were mounted on microscope slides (SUPERFROST® Menzel-Gläser, Thermo Scientific, Waltham, MA, USA), and coverslipped with mounting medium containing DAPI for the visualization of cell nuclei (Lot # 1972784; ProLong™ Gold antifade reagent with DAPI, Life Technologies, Invitrogen, CA, USA) and cover glasses (High Precision cover glasses thickness No. 1.5H, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). The slides were then stored in the dark at 4 °C until acquisition of confocal pictures. More detail about used antibodies can be found in **Table 1.**

Table 1 | Antibodies used for the visualization of microglia.

Antibody	Immunogen	Source (Cat. No.), species	RRID	Dilution
PRIMARY				
IBA1	Ionized calcium-binding adapter molecule 1	FUJIFILM Wako Chemicals (019-19741), Rabbit (polyclonal)	AB_839504	1:1000
NOS2	Nitric oxide synthase 2 (inducible)	Santa Cruz Biotechnology (sc-7271), Mouse (monoclonal)	AB_627810	1:500
SECONDARY				
AF488	Rabbit IgG	Invitrogen (A-11034), Goat (polyclonal)	AB_2576217	1:1000
AF594	Mouse IgG	Life Technologies (A-21203), Donkey (polyclonal)	AB_2535789	1:1000

2.9.2 Confocal microscopy

For the evaluation of different activation states of microglia, confocal images were acquired on a Leica TCS SP8 (Leica Microsystems, Wetzlar, Germany) for the PrL and NAcc shell, and a Zeiss LSM 880 (Carl Zeiss Microscopy GmbH, Jena, Germany) for the PVN. Adjusting digital images and image capture were performed using LASX software (Leica Microsystems, Wetzlar, Germany) on the Leica system and ZEN (ZEN 2.3 SP1, Carl Zeiss Microscopy GmbH, Jena, Germany) in conjunction with the Zeiss system. Images were captured using 40× magnification and 1× digital zoom. To optimize the image quality, an immersion objective was used in combination with water-based glycerin to adjust to the refraction index of the cover glass and mounting medium. Imaging settings were adjusted for optimal acquisition quality and were kept constant throughout the acquisition of all stacks for the experiment. Bit depth was set to 16 Bit, pixel dwell time to 4.12 µs and the scan mode was set to sequential line scanning. 41 pictures with the resolution of 512×512 pixels were acquired with 0.5 µm z-step size, resulting in a Z-Stack with 20 µm thickness. More detailed information about the setup of the confocal systems and acquisition parameters can be found in **Table 2**. For each prairie vole, three Z-Stacks were captured per brain region and chosen randomly. The Stacks were acquired between Bregma 1.98 mm to 1.54 mm for the PrL, between Bregma 1.94 mm to 1.54 mm for the NAcc shell, and between Bregma -0.70 mm to -0.94 for the PVN. Image acquisition included both hemispheres and were pooled in the subsequent analysis.

Table 2 | Confocal imaging settings for the acquisition of microglia Z-Stacks.

	Zeiss LSM 880				**Leica TCS SP8**		
Z-Stack	41 slices (20 µm)			Z-Stack	41 slices (20 µm)		
Bit depth	16 Bit			Bit depth	16 Bit		
Image size (pixels)	512×512			Image size (pixels)	512×512		
Image size (scaled)	212.55 µm × 212.55 µm			Image size (scaled)	291.19 µm × 291.19 µm		
Objective	Plan-Apochromat 40x/1.4 Oil DIC M27			Objective	HC PL APO CS2 40x/1.3 Oil		
Channel	DAPI	AF488	AF594	**Channel**	DAPI	AF488	AF594
Pinhole	0.76 AU	0.65 AU	0.76 AU	Pinhole	1 AU		
Scan mode	Line Sequential			Scan mode	Line Sequential		
Scan zoom	1			Scan zoom	1		
Pixel dwell time	4.12 µs			Pixel dwell time	4.12 µs		
Averaging	4			Averaging	2		
NA	1.4			NA	1.3		
Wavelength	415-477	499-553	604-650	Wavelength	415-460	498-550	604-650
Gain	750	800	750	Gain	750	100	750
Digital gain	1.2	1	1.2	Intensity	6.60%	7.40%	30%
Detector type	PMT	HyD	PMT	Detector type	PMT	HyD	PMT

2.9.3 Distinction between different activation states

Microglia activation states were determined using Fiji for ImageJ (ver. 1.52p, (Schindelin et al., 2012; Schneider, Rasband, & Eliceiri, 2012)). Image processing was kept uniformly throughout the analysis. To prepare the images for the morphological analysis, pseudo-colors were assigned to the blue, green, and red channel and the channels were merged. Following, a maximum intensity projection was applied on the merged image as well as on the green channel separately. To facilitate easier visual detection of microglia soma, gray scale attribute filtering was applied on the green channel with the following settings: operation=Opening, attribute=Area, minimum=25, connectivity=8. Using the "Polygon selections" tool, ROIs were created at the border of the soma of each microglial cell. Microglia whose soma areas extended past the borders on the x, y, or z axis were excluded from further analyses since no precise measurement of microglial soma area, shape, and counting of primary processes and endpoints could be ensured. Microglia processes and end points were counted with the aid of the counter function of the multi-point tool. Total numbers of primary processes and end points were noted. For the evaluation of the microglial reactivity states, all four parameters (soma area, soma roundness, number of primary processes, and number of endpoints) were measured and analyzed. In the case of soma with an area of more than 35 µm^2, a roundness of >0.7, and >5 primary processes, end points were not counted since the microglia could

already be characterized as belonging to the ramified state. The complete workflow of this characterization is described in **Figure 9**.

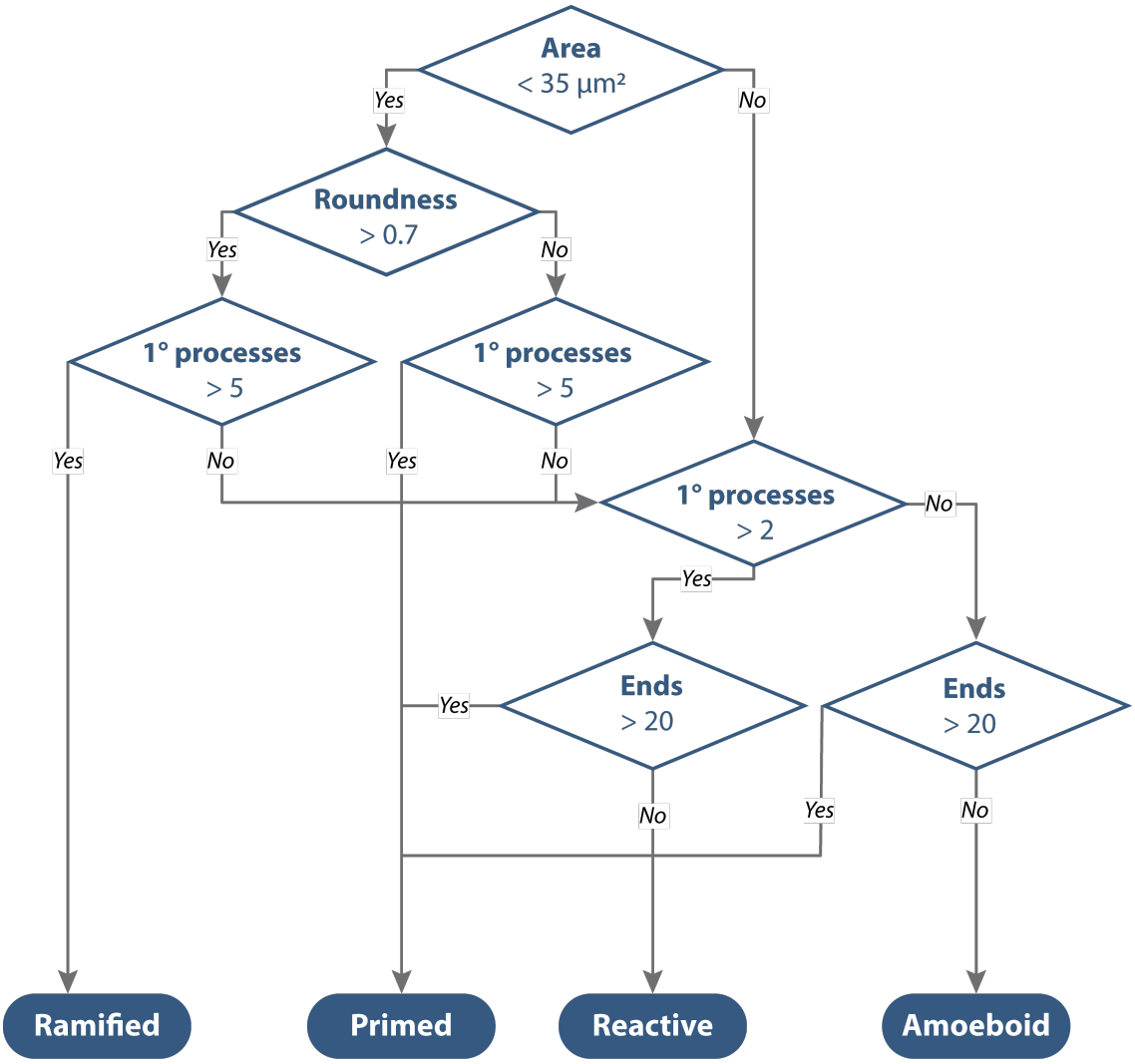

Figure 9 | Characterization of microglial activation.
The workflow for the assignment of individual microglia cells to the respective activation states. Briefly, cells were assessed for the area the microglia soma covered, the roundness was assessed, followed by the number of primary processes. Following, the number of endpoints was counted and in combination with previously assessed cell properties, microglia cells were assigned to their respective activation states.

2.9.4 Other morphological analyses

2.9.4.1 Skeleton Analysis

To measure the general complexity of the microglia, a skeleton analysis was performed. For this, multichannel confocal pictures were imported to Fiji for ImageJ (ver. 1.52p, (Schindelin et al., 2012; Schneider et al., 2012)) and split into three separate channels. The channel containing microglia staining was selected, and a maximum intensity projection was applied, and the Brightness/Contrast was adjusted. After that, gray scale attribute filtering was applied with the

settings set to operation=Opening, attribute=Area, minimum=25, and connectivity=8. Afterwards, the image was smoothened, and noise reduced by applying "Despeckle". Following, the threshold was automatically adjusted with "dark background" selected. The image was again despeckled and outliers were removed with the settings Radius=1, Threshold=50, and Which outliers=Bright. Following, the image was skeletonized with the function skeletonize. Subsequently, the skeleton was analyzed using the function Analyze skeleton. Finally, branch length and endpoint voxels were summed for each image and divided by the number of microglia cells for each image.

2.9.4.2 Sholl Analysis

The microglia complexity was measured by sholl analysis. Confocal images were imported to Fiji for ImageJ (ver. 1.52p, (Schindelin et al., 2012; Schneider et al., 2012)), the channels were split and a maximum intensity z projection was created for the channel containing IBA1 staining. The image LUT color was converted to gray scale. Following, the image color was inverted. Subsequently, the brightness and contrast were adjusted by setting the minimum and maximum limits of the histogram to the borders of the intensity curve. Following, D was determined by using the function "Set scale" and using the value of the correspondence between pixels and µm. Per region, two microglial cells were chosen randomly, resulting in a total of 6 analyzed cells. For the creation of concentric circles, the cell center of a microglia cell was picked by hovering with the mouse over the center and noting its position with the help of the x and y coordinates. The plugin Concentric Circles was executed, and the x and y center values were set by multiplying the respective x and y values by D. The inner center value was set to 10 µm. The outer radius value was calculated by 2×D+(N×S×D), whereas N corresponds to the number of concentric circles additionally to the inner circle and S corresponds to the spacing value between two circles in µm. In the case of this study, 4 µm of between-circle distance was selected. The circle value was then set to N+1 resulting in a total of 9 circles and the number of intersections of the processes with the circles was noted.

2.10 Analysis of neuronal activation

2.10.1 Immunohistochemical staining of c-Fos$^+$ cells

To determine whether microglial changes could be due to changes of neuronal activity, brain slides were stained for the immediate early gene *c-Fos*. In advance to the staining procedure, the blocking solution for the first day of the staining was prepared. The blocking solution contained 5% normal goat serum (NGS) (S-1000, Vector laboratories, Burlingame, CA, USA), 0.5% Triton X-100 (Lot # SLBV4122; Sigma-Aldrich, St. Louis, MO, USA) in 1× PBS (pH 7.4). To block endogenous peroxidases, the H_2O_2 solution was prepared prior to the staining, which comprised 3% H_2O_2, 10% MeOH (AnalaR NORMAPUR Methanol, VWR Chemicals, Radnor, PA, USA) in 1× PBS (pH 7.4).

Brain slices were selected with a fine brush and 6-8 slices were transferred from the cryoprotectant into a well containing 1 ml 1× PBS (pH 7.4). Brain slices were then washed 3× 15 min at RT on an orbital shaker. Subsequent to the washes, the slices were incubated with the H_2O_2 solution for 15 min at RT on an orbital shaker. The blocking of endogenous peroxidases was then followed by 3× 15 min washes in 1 ml 1× PBS. Subsequently, the slices were blocked in 250 µl blocking solution for 1 h at RT while being placed on an orbital shaker. Primary AB incubation was conducted using 1:20,000 rabbit anti-c-Fos (sc-52, Lot # E2903; Santa Cruz Biotechnology, Dallas, TX, USA) in blocking solution. After 1 h at RT on an orbital shaker, the well plate was sealed using Parafilm to avoid evaporation of fluids and the slices were placed on an orbital shaker at 4 °C for two successive nights. After two nights, the primary AB was removed, and slices were washed for 3× 10 min in 1× PBS. Following, secondary AB was performed for 2 h at RT using 1:500 biotinylated goat anti-rabbit IgG (BA-1000, Vector Laboratories, Burlingame, CA, USA) in 2% NGS (S-1000, Vector laboratories), 0.5% Triton X-100 in 1× PBS (pH 7.4). During incubation of the secondary AB, the ABC kit solution was prepared (PK-4000, Vector laboratories, Burlingame, CA, USA). For 10 ml solution, 90 µl of Reagent A, 90 µl of Reagent B were mixed with 9.82 ml of 1× PBS. Secondary AB incubation was followed by 3× 10 min washes in 1× PBS at RT while being placed on an orbital shaker. Following, 200 µl of ABC kit solution were transferred into each well and the well and the slices were incubated for 1 h at RT while being placed on an orbital shaker. After removal of the ABC kit solution, slices were washed 3× 10 min in 1× PBS at RT on an orbital shaker. After washing, DAB staining was performed. The DAB solution was prepared as follows: 84 µl of buffer stock solution, 100 µl of DAB stock solution, 80 µl of H_2O_2 stock solution, and 80 µl Nickel solution were added to 5 ml of distilled water. 200 µl of the prepared DAB solution were added to each well. Four wells were simultaneously incubated, and the plate was gently shaken by hand to facilitate even incubation. After 1-2 min, the reaction was stopped by removing the DAB solution and washing with 1× PBS as soon as background staining became visible. The slices were then washed 6× 10 min in 1× PBS. Using a fine brush, the slices were then mounted on to microscope slides (SUPERFROST® Menzel-Gläser) and allowed to dry overnight. On the next day, the slides were covered with mounting medium (Roti-Histokitt, Carl Roth GmbH & Co. KG) and covered with coverslips (Art. Nr. H 878, Carl Roth GmbH & Co. KG). For subsequent image analysis, a minimum of six pictures were taken per region on a brightfield microscope at 20× magnification (DM5000 B, Leica Microsystems, Wetzlar, Germany). More detail about antibodies used can be found in **Table 3.**

Table 3 | Antibodies used for the analysis of neuronal activation.

Antibody	Immunogen	Source (Cat. No.), species	RRID	Dilution
PRIMARY				
c-Fos	FOS human, mouse, rat	Santa Cruz Biotechnology (sc-52), Rabbit (polyclonal)	AB_2106783	1:20,000
SECONDARY				
Biotinylated Goat Anti-Rabbit IgG	Rabbit IgG	Vector Laboratories (BA-1000), Goat (polyclonal)	AB_2313606	1:500

2.10.2 Cell counting

Total cell number was counted by using the Cell counter plugin for Fiji for ImageJ (ver. 1.52p, (Schindelin et al., 2012; Schneider et al., 2012)). For the NAcc shell and the PVN, I determined the borders of the selected regions referring to a mouse brain atlas. The regions were then outlined and the number of c-Fos$^+$ cells was determined. In the PVN, we discriminated between the magnocellular and the parvocellular region. Since the PrL covered the whole area of the image, no further determination of the region was needed.

2.11 Assays for the assessment of peripheral immune system activation

2.11.1 Luminex Multiplex Cytokine Detection

Plasma cytokine levels were measured commercially by Microbiomix (Regensburg, Germany). Using a homebrew cytokine multiplex bead immunoassay, IL-10, IL-12p40, IL-6, interferon gamma-induced protein 10 (IP-10), and TNF-α were quantified in the plasma of voles.

2.11.2 Bacterial Killing Test

Bacterial killing was performed utilizing an *ex vivo* assay, which is measuring the functional ability of the innate immune system to clear the blood from pathogens. This assay is measuring the relative number *Escherichia coli* (*E. coli*) colony forming units (CFU) growing on agar following the incubation with blood plasma. We used *E. coli* from the strain ATCC #8739 which is complement dependent, i.e. bacterial killing relies on the complement system (Scotti et al., 2015; Demas et al., 2011). Agar plates were prepared by adding 50 g of tryptic soy agar (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) to 1,250 ml ddH_2O. The solution was autoclaved at the liquids setting and cooled down to 80 °C. Following, agar plates were prepared under a sterile laminar flow hood by adding 20 ml of agar to each plate. Following cooling down, lids were added to the plates and they were stored in the refrigerator at 4 °C until further use. To assess killing activity, one pellet (10^7) of lyophilized *E. coli* (Lot # 483-809-2, Microbiologics® Epower™ *Escherichia coli*, ATCC™ 8739™, St. Cloud, MN, USA) were dissolved in 40 ml 37 °C warm 1 M PBS (sterile, 900 ml dH_2O, 74 g NaCl, 18.775 g Na_2HPO_4-7H_2O, 4.15 g NaH_2PO_4-H_2O, pH 7.4, filled up to final volume of 1 L) using sterile forceps and vortexed. The solution was then activated by incubating it at 37 °C for 30 min. Following incubation, the solution was vortexed again and stored at 4 °C. For the bacterial killing,

blood plasma was diluted 1:20 in glutamine enriched CO_2-independent medium (Gibco, 18045054, Thermo Fisher Scientific, Waltham, USA) with 2.34 mg L-glutamine (G8540-25G, Merck, Darmstadt, Germany). Following, 10 µl of blood plasma were added to 190 µl of medium and 1 ml of the bacterial stock solution was diluted in 9 ml sterile 1 M PBS, resulting in a working solution with 50,000 bacteria/ml. The working solution was prepared fresh every day. For the positive control, 20 µl of the working solution were added to 200 µl of media. The negative control was prepared by using 220 µl of media. 20 µl of working solution were added to each 200 ml of diluted (1:20) plasma sample. All steps were performed on ice. The samples were then vortexed and incubated for 30 min at 37 °C. After the incubation, 50 µl of each sample was plated in duplicates on the previously prepared agar plates by placing the 50 µl in the middle and spreading it with a bacterial spreader. Following, plates were covered with the cover slips and incubated upside down over night at 37 °C. On the following day, the plates were photographed, and colonies were counted using the cell counter plugin in Fiji for ImageJ (ver. 1.52p, (Schindelin et al., 2012; Schneider et al., 2012)). Killing capacity was determined by dividing the mean of the replicates by the mean of the positive controls.

2.12 Molecular profile of microglia following separation

Changes to microglial morphology and therefore functionality and homeostasis are robust indicators for measuring the activation of microglia following given manipulation. To my knowledge, the morphology of microglia has not been described in prairie voles. and the employed model of morphological classification has also been developed using samples from the prairie vole, we aimed to link molecular changes with morphological changes observed. For this, I performed quantitative polymerase chain reactions (qPCRs) for selected target genes that might be affected by changes of microglia reactivity and genes that might be involved in the activation pathways stemming from changed HPA axis (re)-activity.

2.12.1 Preparation of punches from brain tissue

One day prior to sectioning, the brains were moved from -80 °C to -20 °C to adapt to the cutting temperature. The brains were cut into 200 µm thick slices using a cryostat and the slices were mounted on microscope slides (SUPERFROST® Menzel-Gläser). Slices from the PrL, NAcc shell, and the PVN were obtained. The brain slices were then stored at -20 °C until further processing. Areas of interest were identified using a brain atlas (Paxinos & Franklin, 2001). Micropunches were obtained from the selected regions using a sample corer (Fine Science Tools GmbH, Heidelberg, Germany) with a diameter of 1 mm. With reference to the brain atlas, 6-8 punches were collected between Bregma 2.10 mm and 1.54 mm (PrL), Bregma 1.70 mm and 1.18 mm (NAcc shell), and Bregma -0.58 mm and -1.06 mm (PVN). The extracted tissue punches where stored in RNase free

1.5 ml DNA LowBind micro tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany) at -20 °C until further processing.

2.12.2 RNA and protein isolation

Total RNA and protein were isolated using a NucleoSpin® miRNA Kit (Lot # 1907/002; MACHEREY-NAGEL GmbH, Düren, Germany). Preparation of the samples was conducted following the manufacturers protocol with minor modifications. In brief, 150 µl Buffer ML was added to the previously extracted tissue punches and homogenized using a pestle. Following homogenization of the samples, further 150 µl of Buffer ML were added to the micro tubes. The samples were vortexed for 5 s and incubated at RT for 5 min. Afterwards, the lysate was filtered through a NucleoSpin® filter column by centrifugation at 11,000 × g for 1 min. Afterwards, 150 µl of 99% EtOH were added to the flow through and the samples were vortexed for 5 s before incubating at RT for 5 min. Following the incubation, the sample was transferred to a NucleoSpin® RNA Column and centrifuged at 14,000 × g for 1 min. The flow-through was then stored on ice until further processing. The NucleoSpin® RNA Column was placed in a collection tube and 350 µl membrane desalting buffer was added to the column. The column was then centrifuged at 11,000 × g for 1 min and the flow-through discarded. Following, 100 µl of rDNase was added directly onto the membrane to digest contaminating DNA and incubated for at least 15 min at RT. 300 µl Buffer MP was added to the previously stored flow-through and the samples were vortexed for 5 s before pelleting the protein by centrifuging for 3 min at 11,000 × g. The supernatant was then transferred to a NucleoSpin® Protein Removal Column and centrifuged for 1 min at 11,000 × g. The column was then discarded and 800 µl Buffer MX was added. The sample was then vortexed for 5 s. Following, the sample was transferred to the NucleoSpin® RNA Column and centrifuged for 30 s at 11,000 × g. The flow-through was discarded and 600 µl washing buffer MW1 was added to the column before centrifuging for 30 s at 11,000 × g. Afterwards, the membrane was washed with 700 µl and 250 µl washing buffer MW2 and spun down for 30 s and 2 min, respectively. To elute the RNA, 30 µl of RNase free water was added directly on the membrane and after 1 min of incubation at RT was spun down for 30 s at 11,000 × g. The absorbance and concentration of the eluted RNA was then measured using a NanoDrop spectrophotometer and stored at -20 °C until further processing.

2.12.3 Polymerase chain reaction

Transcription of mRNA was performed using the UltraScript 2.0 following the manufacturer's protocol with minor adaptations. Briefly, a total of 350 ng was used for the transcription. RNA was added to an 8 well strip tube and RNase free ddH_2O was pipetted to a total volume of 15 µl.

Following, 4 µl 5× UltraScript Buffer and 1 µl Random Hexamers (100 µM) was added to the reaction. The sample was then incubated for 2 min at 70 °C and placed on ice immediately after the incubation was finished. For the -RT samples, 1 µl of the sample were placed in new 8 well strip tubes. For the +RT samples, 1 µl of UltraScript 2.0 was added before both, the -RT and +RT samples were incubated. The cycler settings for the incubations were 10 min at 25 °C, 10 min at 50 °C, 10 min at 80 °C, and 10 min at 95 °C. Following the incubation, the transcribed cDNA was removed from the thermal cycler and stored at -20 °C until utilization.

2.12.4 Quantitative polymerase chain reaction

Genes were selected based on their involvement in the activation process of microglial cells which also happens during morphological activation of microglia. Furthermore, I assessed *FKBP51* for its implication in the involvement of the stress response and also in microglial activation. The genes *ATP5F1* and *GAPDH* were used as housekeeping genes (Panina, Germond, Masui, & Watanabe, 2018). Primer efficiency for all targets was calculated using test RNA prior to the experiment. To evaluate specificity of the primer, -RT samples and ddH_2O as template were used. To verify the qPCR products, the sample product length was verified using northern blotting. For the qPCR, each well contained 5 µl SYBR Green, 1 µl 1 µM forward primer, 1 µl 1 µM reverse primer, 1 µl H_2O, and 2 µl cDNA template. The cycling conditions were 2 min at 50 °C, 2 min denaturation at 95 °C during the hold stage. For the amplification, 50 cycles were performed, each cycle consisting of 3 s at 95 °C for denaturation and 30 s at 60 °C for annealing / extension. After completion of the 50 cycles, a melt curve was created by heating the sample to 95 °C for 1 s. All samples were measured in triplicates. Housekeeping genes, standard curves, and -RT samples were measured in duplicates on 384 well plates. No template control samples were measured in single reactions. A list of assessed candidate genes including the oligonucleotide sequences of the primers are presented in **Table 4**. The relative fold expression of each gene was determined using the ΔΔCT method (Livak & Schmittgen, 2001) and by normalization of the two housekeeping genes.

Table 4 | List of candidate genes.

Candidate gene	Designation	Direction	Sequence	T_M	Amplicon length [bp]
ATP5F1	ATP synthase subunit b, mitochondrial	forward reverse	CGTCTTGGGCTGATTCCTGA TAGGGTCCTGTTACGCCAGT	59.75 60.25	68
GAPDH	Glycerinaldehyde 3-phosphate dehydrogenase	forward reverse	TGCTCCTCCCTGTTTTGGAG TACGGCCAAATCCGTTCACT	59.6 59.68	100
LGALS3	Galectin-3	forward reverse	TTCCACTTTAACCCCCGCTT TTCCCCCAGTTGTTAGCCTG	59.52 59.6	80
CX3CR1	CX3C chemokine receptor 1	forward reverse	AGATCCAAGACAGGAGCACAAG GGCCAGCAGAACACAAGTCA	60.03 60.82	198
CX3CL1	chemokine (C-X3-C motif) ligand 1	forward reverse	TTCCACTTTAACCCCCGCTT TTCCCCCAGTTGTTAGCCTG	59.52 59.6	80
FKBP51	FK506 binding protein 5	forward reverse	CGGCGGCGACAGGTTTTC AATCATCGGGGCCTCATCAC	62.08 59.89	177

2.13 Statistics

All statistical data analysis was performed in SPSS Statistics (Version 23.0.0.2, IBM Corp. Armonk, NY, USA) or in Prism 8 (Version 8.2.1 (279), GraphPad Software, Inc., San Diego, CA, USA). Data distribution was tested for normality using the Shapiro-Wilk test. If normality was given, Student's t-tests were performed to calculate planned comparisons. For independent t-tests, effect sizes were calculated using Cohen's *d* (same sample size) or Hedges' *g* (different sample sizes). If variances differed significantly as determined by the F-test, Welch's independent t-test was used, and Glass's *delta* was calculated for the reporting of the effect size. If normality of the data could not be assumed, planned comparisons were performed using the non-parametric Mann-Whitney-U test and effect sizes were calculated through Pearson's correlation coefficient. For analyses of multiple independent factors and groups, one-way and two-way ANOVAs were performed with Bonferroni-corrected *post hoc* tests to correct for multiple comparisons, if adequate. For microglia morphology analyses, a mixed model ANOVA was performed to account for correlation between the individual activation states. Microglia states were defined as repeated (connected) values and the factor "housing" was used as the independent variable. If sphericity could not be assumed by Mauchly's *W*, a Greenhouse-Geisser-corrected mixed model ANOVA was calculated. For all ANOVAs, effect size was calculated by determination of η^2. Statistical significance was accepted for $p \leq 0.05$.

Results

3 Results

3.1 Reversal of partner loss-induced altered emotionality using MTII

3.1.1 Validation of the stress response following separation from a partner

3.1.1.1 Elevated Plus Maze

To analyze the impact of separation with or without treatment of MTII and either ni or si on anxiety-like behavior, the EPM was conducted. Detailed statistical descriptions are presented in **Table 5**. In both male (**Figure 10 A,C**) and female (**Figure 10 B,D**) prairie voles, treatment with MTII and si following separation from a partner did not affect the percentage of time spent in the open arm and the percentage of entries to the open arms compared with all control groups.

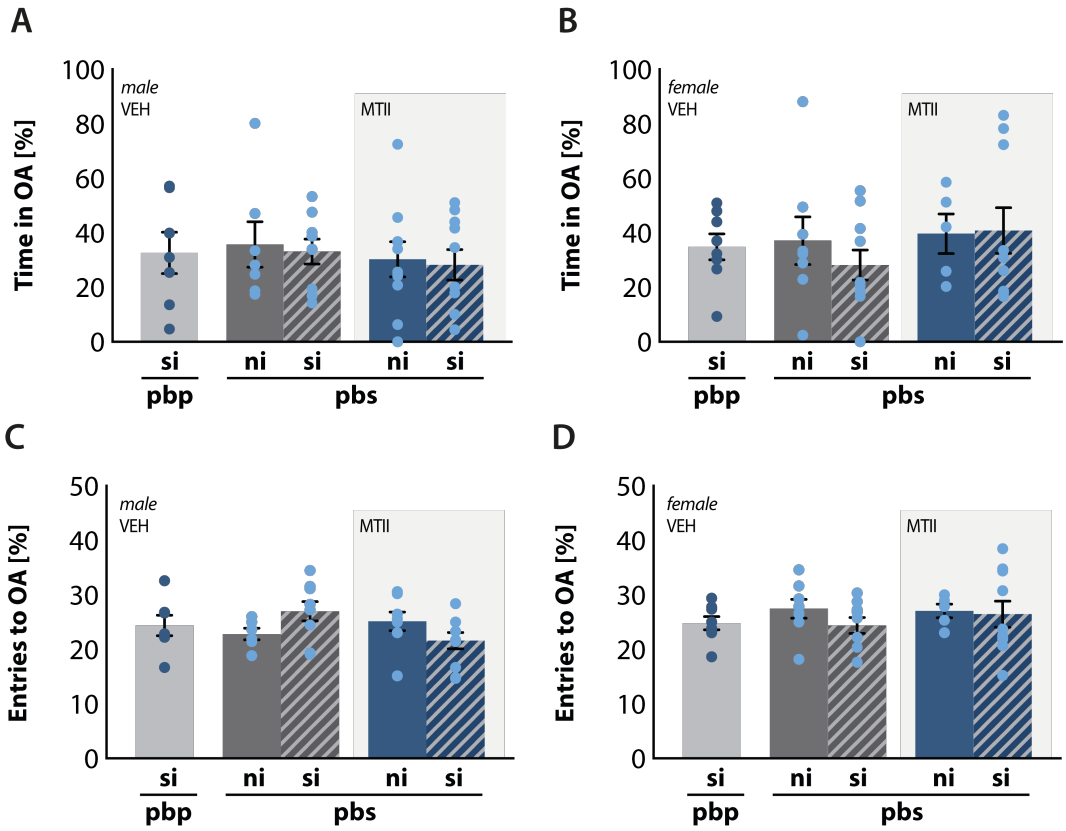

Figure 10 | Results from the EPM following partner loss and treatment with MTII.
Shown are the percentages of time spent in the open arms of (**A**) male and (**B**) female voles and the percentages of entries into the open arms (OA) of (**C**) male and (**D**) female voles. (**A**) No difference between *pbp* or *pbs* groups treated with either VEH or MTII with ni or si was observed. (**B**) In females, no difference was observed in the percentage of time spent in the OA between *pbp* and *pbs* groups with MTII or VEH treatment and ni or si. (**C**) Neither separation nor treatment with either MTII or social interaction affected the percentage of entries to the OA in male voles. (**D**) Regarding the percentage of OA entries of female voles, no difference was observed between *pbp* or *pbs* groups treated with either MTII or VEH and ni or si. The data is presented as bar charts with aligned dot plots with mean ± SEM. All groups n=5-10. Main effects of experimental groups are indicated above the horizontal line with (*) p<0.07, * p<0.05, ** p<0.01, *** p<0.001, the respective *post hoc* statistics are indicated underneath the horizontal line as ≈ p≥0.7, ≤/≥ p<0.07, </> p<0.05.

Table 5 | One-way ANOVA for the assessment of anxiety-like behavior in the EPM.

Parameter	Sex	*N*	Main effect: Experimental Group
Percent of time in OA	M	42	$F(4,37)=0.200$, $p=0.937$, $\eta^2=0.021$
	F	41	$F(4,36)=0.535$, $p=0.711$, $\eta^2=0.056$
Percent of entries to OA	M	42	$F(4,37)=1.937$, $p=0.125$, $\eta^2=0.173$
	F	42	$F(4,16.974)=806$, $p=0.538$

3.1.1.2 Forced Swim Test

The FST was conducted to assess separation-induced passive stress-coping behavior. Previous studies showed that pair bond separation results in increased passive stress-coping in both male and female prairie voles, which can be rescued by increasing OT signaling in the NAcc shell. Here, the aim was to increase endogenous release of OT within the brain via *i.p.* infusions of MTII in combination with social contact to an opposite sex stranger vole, which might result in reduced passive stress coping. Detailed statistics can be found in **Table 6**. Neither in male (**Figure 11 A,C**) nor in female (**Figure 11 B,D**) prairie voles, separation from a partner increased passive stress-coping, and treatment with MTII and social interaction had no further behavioral effect compared to their controls.

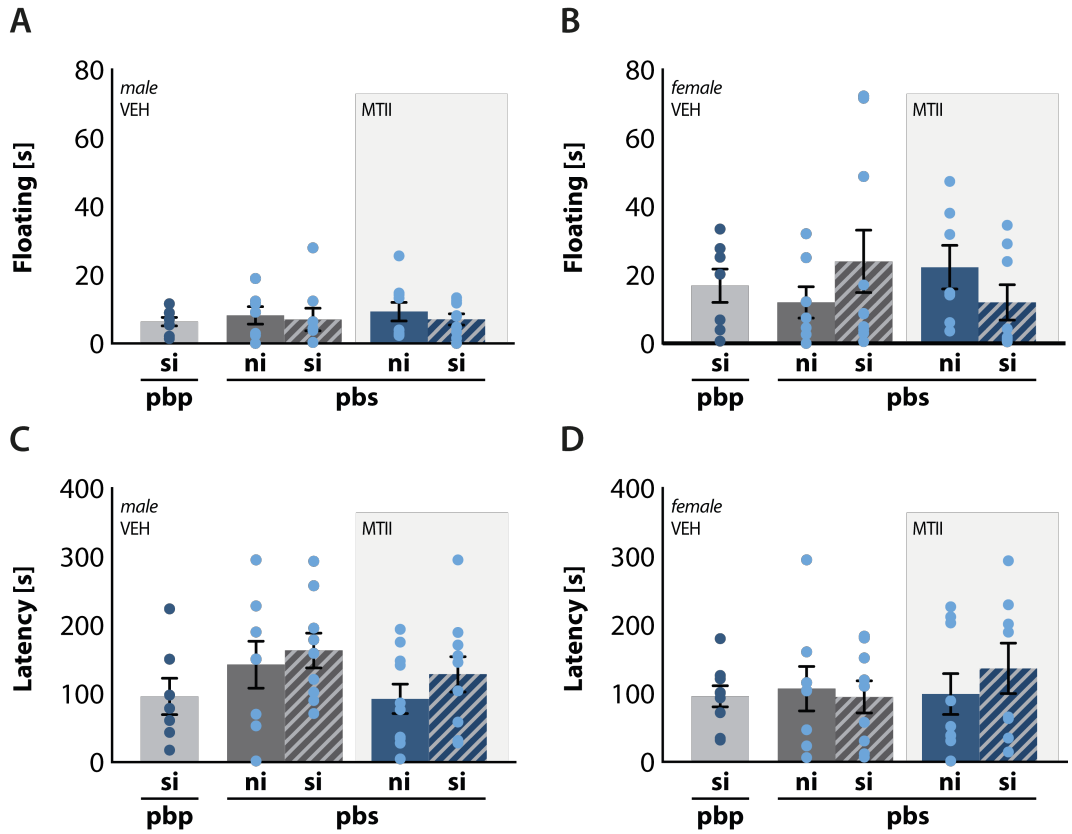

Figure 11 | Results from the FST and MTII treatment following separation from a partner. Depicted are the time (**A**) male and (**B**) female voles spent floating and the latency until the first floating episode in (**C**) male and (**D**) female voles. (**A**) No difference between *pbp* or *pbs* groups treated with either vehicle (VEH) or MTII and no social interaction (ni) or with social interaction (si) was observed. (**B**) In females, no difference was observed in the time spent floating between *pbp* and *pbs* groups with MTII or VEH treatment and ni or si. (**C**) No effect on the latency until the first floating episode was observed following treatment with either MTII and VEH and ni or si between *pbp* and *pbs* groups of male voles. **(D)** No difference was observed between female *pbp* or *pbs* groups treated with either MTII or VEH and ni or si. The data is presented as bar charts with aligned dot plots with mean ± SEM. All groups n=7-10. Main effects of experimental group are indicated above the horizontal line with (*) $p<0.07$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, the respective *post hoc* statistics are indicated underneath the horizontal line as ≈ $p\geq0.7$, ≤/≥ $p<0.07$, </> $p<0.05$

Table 6 | Results from the FST following separation from a partner and treatment with MTII.

Parameter	Sex	*N*	Main effect: Experimental Group
Floating (time)	M	41	$H(4)=1.266$, $p=0.867$
	F	39	$H(4)=3.293$, $p=0.510$
Latency until first float	M	44	$F(4,39)=1.284$, $p=0.293$, $\eta^2=0.116$
	F	43	$H(4)=1.011$ $p=0.908$

3.1.1.3 Measurement of the physiological stress response

Separation from a partner was previously shown to affect physiological parameters such as adrenal gland weight and basal plasma CORT levels (Bosch et al., 2009). Since chronic stress is reported to affect body weight, the voles' body weight was additionally monitored throughout the experiment. Detailed results from the statistical analyses can be found in **Table 7** (body weight development),

Table 8 (relative adrenal gland weight), **Table 9** (basal plasma CORT levels). Furthermore, *post hoc* analyses for comparisons within experimental day and between experimental days can be found in appendix **Table 33** and **Table 34**. Body weight was assessed on experimental days 0, 5, 8, and 12.

A decrease in body weight was observed during the first 5 days of cohabitation in male prairie voles (**Figure 12 A**). MTII treatment led to a significant reduction of body weight compared to the control groups between experimental day 5 and 8. Repeated measures ANOVA with Greenhouse-Geisser correction revealed a main effect of experimental day ($F(2.234,80.422)=73.764$, $p<0.001$, $\eta^2=0.672$), a between-subjects effect of experimental group ($F(4,36)=3.356$, $p=0.020$, $\eta^2=0.272$), and an interaction between experimental day and experimental group ($F(8.936,80.422)=3.538$, $p=0.001$, $\eta^2=0.282$). Bonferroni-corrected *post hoc* tests revealed that MTII treatment significantly reduced body weight on experimental day 8 in *pbs MTII ni* (vs. *pbp VEH si*: $p=0.001$, vs. *pbs VEH ni*: $p<0.001$, vs. *pbs VEH si*: $p=0.013$) and *pbs MTII si* males (vs. *pbp VEH si*: $p<0.001$, vs. *pbs VEH ni*: $p<0.001$, vs. *pbs VEH si*: $p=0.005$).

In female voles, no weight changes were observed during the first 5 days of cohabitation (**Figure 12 B**). While *pbp VEH si* female prairie showed an increase in body weight on experimental day 8 and 12, females that received MTII treatment showed no weight gain and had significantly lower body weight compared to the control females on experimental day 8 and 12. Greenhouse-Geisser-corrected repeated measures ANOVA revealed a main effect of experimental day ($F(2.078,70.652)=12.573$, $p<0.001$, $\eta^2=0.270$), a between-subjects effect of experimental group ($F(4,34)=5.080$, $p=0.003$, $\eta^2=0.374$), and an interaction between experimental day and experimental group ($F(8.312,70.652)=4.262$, $p<0.001$, $\eta^2=0.334$). Both MTII-treated groups had significantly lower weight compared to the *pbp VEH si* group ($p=0.007$ and $p=0.005$). On experimental day 8, *pbs MTII ni* had significantly lower body weight than the *pbp VEH si* group ($p=0.002$). *Pbs MTII si* had significantly or trended towards less body weight than all other VEH-treated groups (vs. *pbp VEH si* $p=0.001$, vs. *pbs VEH ni* $p=0.068$, vs. *pbs VEH si* $p=0.028$). On experimental day 12, both *pbs MTII ni* and *pbs MTII si* had lower body weight compared to *pbp VEH si* ($p=0.028$ and $p=0.009$).

In males *pbs MTII ni*, the treatment increased the adrenal gland weight (**Figure 12 C**). One-way ANOVA revealed a main effect of experimental group ($F(4,40)=3.875$, $p=0.009$, $\eta^2=0.279$). Bonferroni-corrected *post hoc* tests revealed that *pbs MTII ni* had significantly higher relative adrenal gland weight than *pbp VEH si* males ($p=0.017$). All other groups did not differ from the control *pbp* group. In female voles, adrenal gland weight was not affected by separation and treatment with either MTII or VEH in conjunction with ni or si (**Figure 12 D**). Separation from a partner, MTII treatment, and si did not significantly affect plasma CORT levels in male voles (**Figure 12 E**). In females, no difference in plasma CORT was observed between *pbp VEH si* and all

other experimental groups (**Figure 12 F**). A Welch-corrected one-way ANOVA revealed a main effect of experimental group ($F(4,10.024)=6.116$, $p=0.009$). Games-Howell-corrected *post hoc* tests revealed that *pbs MTII ni* females had significantly higher basal CORT levels than females from the *pbs VEH ni* group ($p=0.017$).

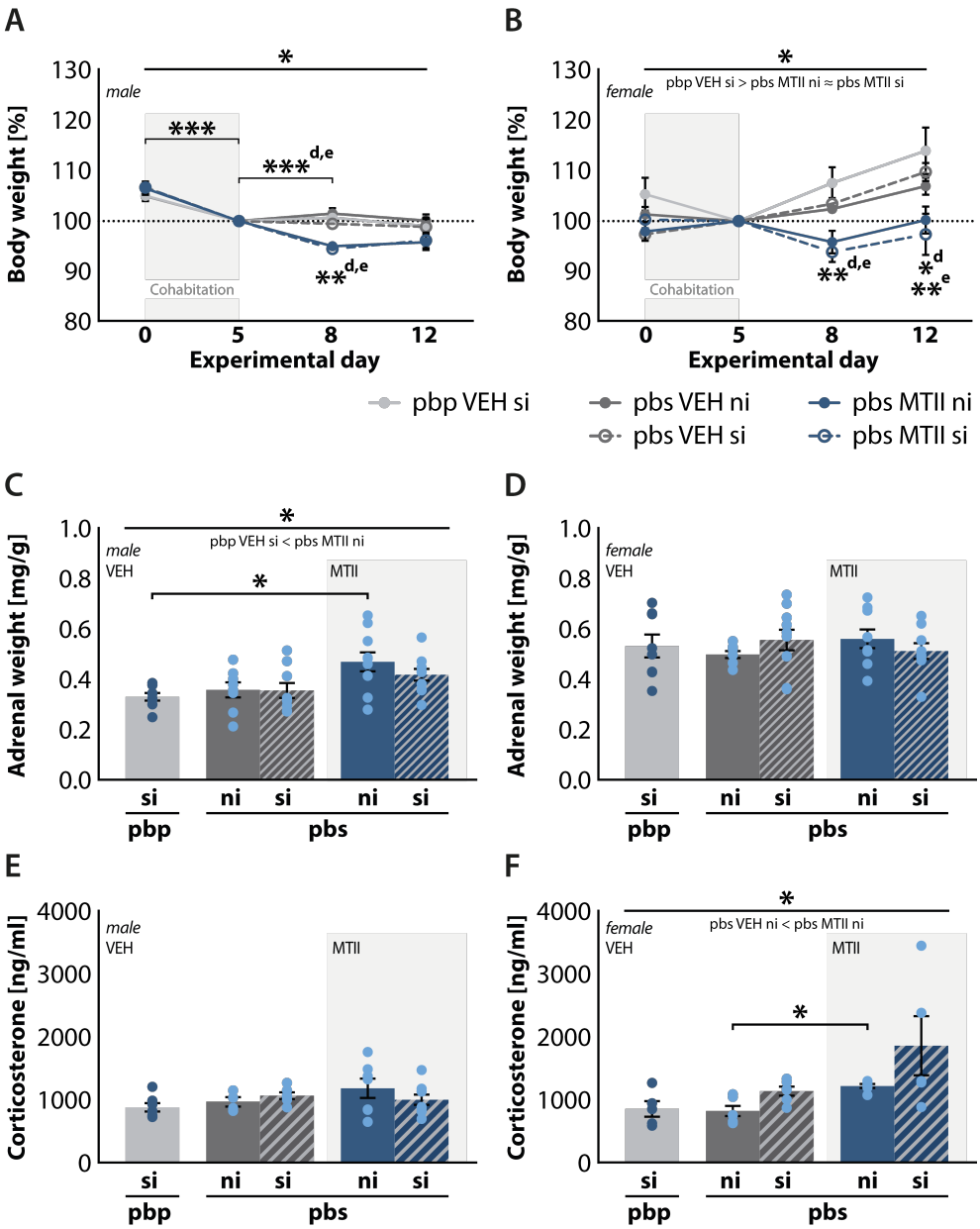

Figure 12 | Assessment of the physiological stress response.
Presented are the development of body weight throughout the experiment in **(A)** male and **(B)** female voles. Adrenal gland weight and basal CORT levels of male and female voles are presented in **(C-D)** and **(E-F)**, respectively. **(A)** Body weight was significantly altered during the course of experiment in male voles, all experimental groups showed a reduction of body weight between experimental day 0 and 5. MTII treatment led to a significant reduction in body weight on experimental day 8 (vs. experimental day 5 vs. experimental day 8 or vs. VEH control on experimental day 8; d, e). **(B)** In female voles, body weight significantly differed between time points of measurement throughout the experiment. MTII treatment led to a reduction of body weight compared to *pbp VEH si* voles on experimental day 5 and 8 (d, e).

Figure 12 | Continued.
(C) MTII treatment with ni did significantly affect relative adrenal gland weight following separation in male voles. **(D)** In female voles, MTII and si treatment following separation did not affect relative adrenal gland weight. **(E)** Following separation from a partner, MTII treatment and si did not significantly alter basal CORT levels. **(F)** In females that were separated from a partner, MTII treatment led to an increase of basal CORT levels in *pbs MTII ni* females. The data is presented as line plots or bar charts with aligned dot plots with mean ± SEM. All groups n=5-10. Main effects of experimental group are indicated above the horizontal line with (*) p<0.07, * p<0.05, ** p<0.01, *** p<0.001 and the respective *post hoc* statistics are indicated underneath the horizontal line as ≈ p≥0.7, (*) p<0.07, * p<0.05, ** p<0.01, *** p<0.001). Statistical significance for the *post hoc* tests for housing condition and interaction are indicated above the respective bars with (*) p<0.07, * p<0.05, ** p<0.01, *** p<0.001. Statistical significance for planned comparisons is indicated above the respective bars with (#) p<0.07, # p<0.05, ## p<0.01, ### p<0.001.

Table 7 | Repeated measures one-way ANOVA for body weight development following MTII treatment and social interaction.

Sex	*N*	Main Effect: Experimental Day	Between-subjects: Experimental Group	Interaction
M	41	F(2.234,80.422)=73.764, p<0.001, η^2=0.672	F(4,36)=3.356, p=0.020, η^2=0.272	F(8.936,80.422)=3.538, p=0.001, η^2=0.282
F	39	F(2.078,70.652)=12.573, p<0.001, η^2=0.270	F(4,34)=5.080, p=0.003, η^2=0.374	F(8.312,70.652)=4.262, p<0.001, η^2=0.334

Table 8 | One-way ANOVA of adrenal gland weight following MTII treatment and social interaction.

Sex	*N*	Main effect: Experimental Group	*Post hoc* tests				
			Exp. Group	pbp VEH si	pbs VEH ni	pbs VEH si	pbs MTII ni	pbs MTII si
M	45	F(4,40)=3.875, p=0.009, η^2=0.279	pbp VEH si	1.000	1.000	1.000	0.017	0.413
			pbs VEH ni		1.000	1.000	0.104	1.000
			pbs VEH si			1.000	0.071	1.000
			pbs MTII ni				1.000	1.000
			pbs MTII si					1.000
F	44	F(4,39)=0.563, p=0.691, η^2=0.055						

Table 9 | One-way ANOVA for basal plasma CORT levels following MTII treatment and social interaction.

Sex	*N*	Main effect:	*Post hoc* tests

		Experimental Group
M	33	$F(4,12.584)=1.364$, $p=0.301$

			Exp. Group	pbp VEH si	pbs VEH ni	pbs VEH si	pbs MTII ni	pbs MTII si
F	27	$F(4,10.024)=6.116$, $p=0.009$	pbp VEH si	1	0.999	0.364	0.166	0.364
			pbs VEH ni		1	0.086	0.017	0.335
			pbs VEH si			1	0.819	0.600
			pbs MTII ni				1	0.679
			pbs MTII si					1

3.1.2 Behavioral responses following MTII treatment

3.1.2.1 Social Behavior

Prosocial behavior

In order to assess the changes by the MTII-evoked increase of endogenous OT release during social interaction, prosocial behavior was assessed by measuring the time spent in grooming, mating, sniffing, side by side contact, and huddling. Those behaviors were then grouped together as active prosocial behavior. Detailed statistical descriptions are presented in **Table 10.**

In male prairie voles, MTII treatment did not affect the display of active prosocial behaviors (**Figure 13 A**). In general, active prosocial behavior remained unaltered between the first (experimental day 5) and the second assessment (experimental day 12). However, *pbp VEH* showed a change in active prosocial behavior. A repeated measures ANOVA revealed no main effect of experimental day, a main effect of experimental group ($F(2,22)=5.061$, $p=0.016$, $\eta^2=0.315$) and an interaction between experimental day and experimental group ($F(2,22)=3.732$, $p=0.040$, $\eta^2=0.253$). Bonferroni-corrected *post hoc* tests revealed that *pbs MTII*-treated males showed a trend towards less active positive social behavior compared to *pbp VEH* voles ($p=0.057$). Interestingly, *pbp VEH* voles showed a decrease in active prosocial behavior following 8 days of treatment ($p=0.011$). In female prairie voles (**Figure 13 B**), separation and MTII treatment did not significantly affect active prosocial behavior. While a repeated measures ANOVA revealed no main effects of experimental day and between-subjects comparisons did not reveal an effect of experimental group, an interaction between time point and treatment was observed ($F(2,23)=6.083$, $p=0.008$, $\eta^2=0.346$). *Pbp VEH*-treated females showed a significant decrease of active prosocial behaviors ($p=0.002$).

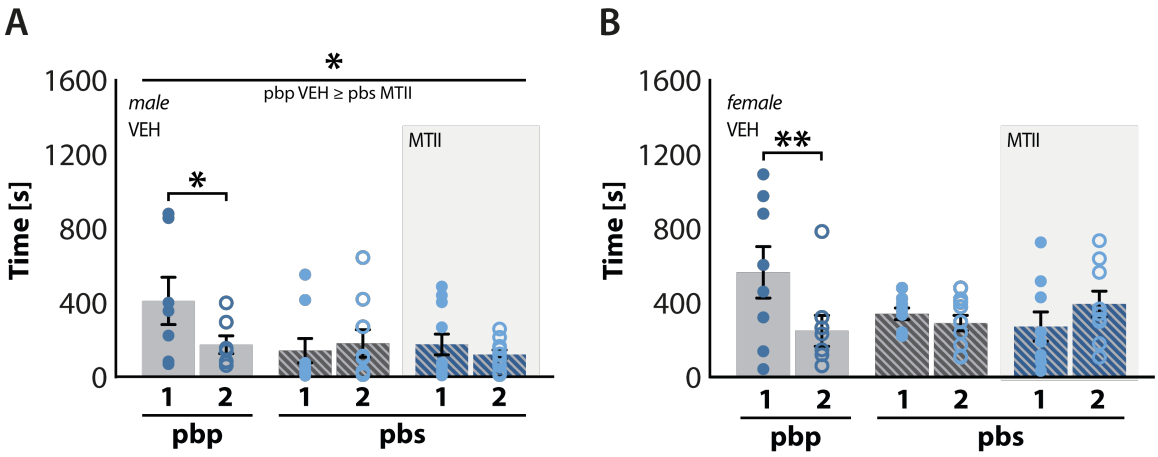

Figure 13 | Effects of MTII treatment on prosocial behaviors.
Active prosocial behavior in paired (*pbp*, grey bars) and separated (*pbs*, dashed bars) prairie voles on both experimental day 5 and 12, indicated as 1 and 2, respectively. **(A)** MTII treatment did not affect active prosocial behaviors in separated males. **(B)** MTII treatment did not affect active prosocial behaviors in female voles. The data is presented as bar charts with aligned dot plots with mean ± SEM. All groups n=7-11. Main effects of experimental group are indicated above the horizontal line with (*) p<0.07, * p<0.05, ** p<0.01, *** p<0.001, the respective *post hoc* statistics are indicated underneath the horizontal line as ≈ p≥0.7, ≤/≥ p<0.07, </> p<0.05. Statistical significance for the *post hoc* tests for the interaction is indicated above the respective bars with (*) p<0.07, * p<0.05, ** p<0.01, *** p<0.001.

Table 10 | Repeated measures ANOVA for prosocial behavior following MTII treatment.

Sex	*N*	Main effect: Time point	Between-subjects: Treatment	*Post hoc* tests		Interaction	*Post hoc* tests	
M	25	$F(1,22)=1.805$, $p=0.193$, $\eta^2=0.076$	$F(2,22)=5.061$, $p=0.016$, $\eta^2=0.315$	pbp VEH vs. pbs VEH	$p=0.112$	$F(2,22)=3.732$, $p=0.040$, $\eta^2=0.253$	pbp VEH	$p=0.011$
				pbp VEH vs. pbs MTII	$p=0.057$		pbs VEH	$p=0.356$
				pbs VEH vs. pbs MTII	$p=0.995$		pbs MTII	$p=0.766$
F	26	$F(1,23)=2.763$, $p=0.110$, $\eta^2=0.107$	$F(2,23)=0.509$, $p=0.608$, $\eta^2=0.042$			$F(2,23)=6.083$, $p=0.008$, $\eta^2=0.346$	pbp VEH	$p=0.002$
							pbs VEH	$p=0.447$
							pbs MTII	$p=0.158$

Affiliative behavior

Affiliative behaviors were assessed to identify the effect of MTII treatment. Huddling and side-by-side contacts were counted as affiliative behaviors and the total duration of both behaviors was measured during 30 min social interaction following treatment with either VEH or MTII. Detailed statistics are presented in **Table 11** and **Table 12**.

In male voles, MTII treatment following separation from a partner had no impact on affiliative behaviors both on experimental day 5 and 12 (**Figure 14 A**). Repeated measures ANOVA revealed a trend towards a main effect of experimental day ($F(1,16)=4.499$, $p=0.050$, $\eta^2=0.219$), a between-subjects effect of experimental group ($F(2,16)=4.724$, $p=0.024$, $\eta^2=0.371$), and an interaction between experimental day and experimental group ($F(2,16)=4.708$, $p=0.025$, $\eta^2=0.370$). On

experimental day 5, Bonferroni-corrected *post hoc* tests revealed that *pbs VEH*-treated voles trended towards a reduction in affiliative behaviors (p=0.052) and *pbs MTII* voles showed a significant reduction compared to *pbp VEH* (p=0.042). Separation and MTII treatment trended to reduce affiliative behaviors towards a stranger on experimental day 12 compared to affiliative behaviors displayed by the *pbp VEH* group (p=0.056). Interestingly, affiliative behaviors of *pbp VEH* males were significantly reduced on experimental day 12 compared to experimental day 5 (p=0.002). No effect of MTII treatment following separation from a partner was observed between both separated groups (**Figure 14 B**). A repeated measures ANOVA revealed a main effect of experimental day (F(1,16)=3.781, p=0.070, η^2=0.191), a between-subjects effect of experimental group (F(2,16)=7.484, p=0.005, η^2=0.483), and an interaction between experimental day and experimental group (F(2,16)=5.831, p=0.013, η^2=0.422). Games-Howell-corrected *post hoc* tests revealed that separation with MTII treatment tended to reduce affiliative behaviors (p=0.066) while separation and VEH treatment significantly reduced huddling and side-by-side contact (p=0.049). On the first day of treatment, both *pbs VEH* and *pbs MTII* females exhibited significantly less affiliative behaviors than *pbp VEH*-treated voles (p=0.014 and p=0.027, respectively). However, on the last day of treatment, no differences were observed between the groups. This was largely due to the reduction of affiliative behaviors in *pbp* females on experimental day 12 (p=0.001).

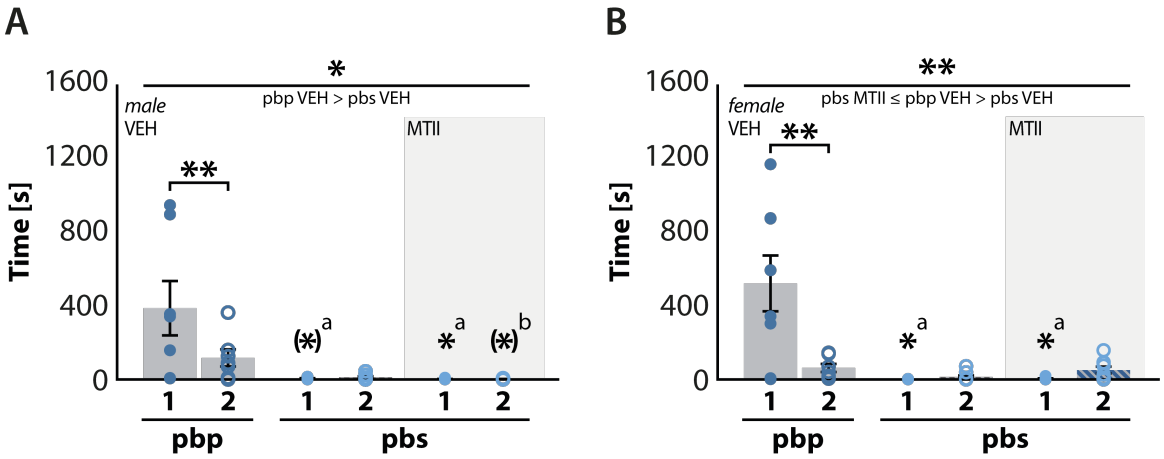

Figure 14 | Affiliative behaviors during social interaction following MTII treatment.
Affiliative behaviors were assessed in male and female prairie voles during social interaction on both experimental day 5 and 12. Affiliative behaviors comprised huddling and side-by-side contact. **(A)** MTII treatment following separation had no impact on affiliative behaviors on experimental day 5 and 12 compared to the *pbs VEH* group. Affiliative behaviors significantly decreased over the time course of the experiment in *pbp VEH*-treated males. **(B)** In female voles, separation from a partner and VEH treatment caused a decrease in affiliative behaviors towards stranger males when compared to *pbp VEH* voles and social interaction with their partner. Also, *pbs MTII* treatment caused a trend towards lower affiliative behaviors when compared to *pbp VEH* voles. No difference was observed between both separated groups. Main effects of experimental group are indicated above the horizontal line with (*) p<0.07, * p<0.05, ** p<0.01, *** p<0.001, the respective *post hoc* statistics are indicated underneath the horizontal line as ≈ p≥0.7, ≤/≥ p<0.07, </> p<0.05. Statistical significance for the *post hoc* tests for the interaction is indicated above the respective bars with (*) p<0.07, * p<0.05, ** p<0.01, *** p<0.001.

Table 11 | Repeated measures ANOVA for affiliative behavior following MTII treatment.

Sex	*N*	Main effect: Time point	Between-subjects: Treatment	Interaction
M	22	$F(1,19)=5.334$, $p=0.032$, $\eta^2=0.219$	$F(2,19)=6.085$, $p=0.009$, $\eta^2=0.390$	$F(2,19)=5.354$, $p=0.014$, $\eta^2=0.360$
F	21	$F(1,18)=4.102$, $p=0.058$, $\eta^2=0.186$	$F(2,21)=4.270$, $p=0.030$, $\eta^2=0.322$	$F(2,21)=5.048$, $p=0.018$, $\eta^2=0.359$

Table 12 | *Post hoc* tests for affiliative behavior following MTII treatment.

Sex	*N*	Treatment		Interaction				
M	22	pbp VEH vs. pbs VEH	$p=0.120$	1st assessment	pbp VEH vs. pbs VEH	$p=0.034$	pbp VEH	$p=0.001$
					pbp VEH vs. pbs MTII	$p=0.018$		
		pbp VEH vs. pbs MTII	$p=0.120$		pbs VEH vs. pbs MTII	$p=1.000$	pbs VEH	$p=1.000$
					pbp VEH vs. pbs VEH	$p=0.357$		
		pbs VEH vs. pbs MTII	$p=1.000$	2nd assessment	pbp VEH vs. pbs MTII	$p=0.262$	pbs MTII	$p=1.000$
					pbs VEH vs. pbs MTII	$p=1.000$		
F	21	pbp VEH vs. pbs VEH	$p=0.229$	1st assessment	pbp VEH vs. pbs VEH	$p=0.040$	pbp VEH	$p=0.002$
					pbp VEH vs. pbs MTII	$p=0.047$		
		pbp VEH vs. pbs MTII	$p=0.281$		pbs VEH vs. pbs MTII	$p=1.000$	pbs VEH	$p=1.000$
					pbp VEH vs. pbs VEH	$p=1.000$		
		pbs VEH vs. pbs MTII	$p=0.387$	2nd assessment	pbp VEH vs. pbs MTII	$p=0.375$	pbs MTII	$p=0.729$
					pbs VEH vs. pbs MTII	$p=0.265$		

Aggressive behavior

Aggressive behaviors were analyzed to assess potential changes in aggression following treatment with MTII. Aggressive behaviors that were analyzed comprised the durations of attacks, lateral threat, offensive upright and chases during the 30 min social interaction following treatment with either VEH or MTII. Detailed statistics are presented in **Table 13**.

In male prairie voles, treatment with MTII caused an increase in the total duration of aggressive behaviors (**Figure 15 A**). A repeated measures ANOVA revealed no main effect of experimental day, a trend towards a between-subjects effect of experimental group ($F(2,17)=3.207$, $p=0.066$, $\eta^2=0.274$), but no interaction between experimental day and experimental group. Games-Howell-corrected *post hoc* tests revealed a significant increase in aggressive behaviors in *pbs MTII*-treated voles compared to *pbp VEH* ($p=0.015$), but not between *pbs VEH* and *pbs MTII*.

In female prairie voles, both *pbs VEH* and *pbs MTII* led to an increase in aggressive behaviors which were more pronounced on experimental day 12 (**Figure 15 B**). Repeated measures ANOVA revealed a main effect of experimental day ($F(1,17)=6.576$, $p=0.020$, $\eta^2=0.279$), a between-subjects

effect of experimental group ($F(2,17)=10.178$, $p=0.001$, $\eta^2=0.545$), but no interaction between experimental day and experimental group. Games-Howell-corrected *post hoc* tests revealed an increase in aggressive behaviors in *pbs VEH* and *pbs MTII* voles compared to *pbp VEH* ($p=0.017$ and $p=0.004$, respectively).

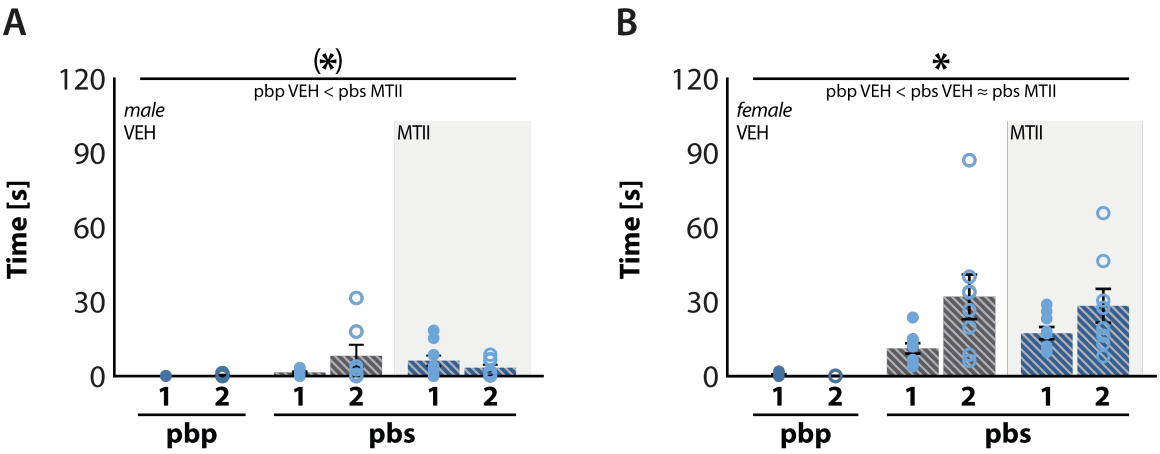

Figure 15 | Aggressive behaviors during social interaction following MTII treatment.
Aggressive behaviors were assessed in **(A)** male and **(B)** female prairie voles during social interaction on both experimental day 5 and 12. **(A)** MTII treatment led to an increase in aggressive behaviors in *pbs MTII* voles compared to *pbp VEH*. **(B)** In females, both *pbs VEH* and *pbs MTII* voles showed significantly more aggressive behaviors than *pbp VEH*. All data is presented as mean ± SEM. The data is presented as bar charts with aligned dot plots with mean ± SEM. All groups n=6-10. Main effects of experimental group are indicated above the horizontal line with (*) $p<0.07$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, the respective *post hoc* statistics are indicated underneath the horizontal line as ≈ $p\geq0.7$, ≤/≥ $p<0.07$, </> $p<0.05$. Statistical significances for the *post hoc* tests for the interaction are indicated above the respective bars with (*) $p<0.07$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Table 13 | Repeated measures ANOVA for aggressive behaviors following MTII treatment.

Sex	*N*	Main effect: Time point	Between-subjects: Treatment	*Post hoc* tests		Interaction
M	20	$F(1,17)=1.261$, $p=0.277$, $\eta^2=0.069$	$F(2,17)=3.207$, $p=0.066$, $\eta^2=0.274$	pbp VEH vs. pbs VEH	$p=0.239$	$F(2,17)=1.943$, $p=0.174$, $\eta^2=0.186$
				pbp VEH vs. pbs MTII	$p=0.015$	
				pbs VEH vs. pbs MTII	$p=0.957$	
F	20	$F(1,17)=6.576$, $p=0.020$, $\eta^2=0.279$	$F(2,17)=10.178$, $p=0.001$, $\eta^2=0.545$	pbp VEH vs. pbs VEH	$p=0.017$	$F(2,17)=2.387$, $p=0.122$, $\eta^2=0.219$
				pbp VEH vs. pbs MTII	$p=0.004$	
				pbs VEH vs. pbs MTII	$p=0.952$	

3.1.2.2 Nonsocial behavior

Explorative behavior

Explorative behaviors were assessed to quantify the impact of MTII treatment on general activity in the presence of another social individual. Explorative behaviors included exploring of walls, digging, and running. Detailed statistics are presented in **Table 14** and **Table 15**. In male prairie voles, *pbs MTII* treatment caused a significant decrease of exploration (**Figure 16 A**). A repeated measures ANOVA revealed no main effect of experimental day, a between-subjects effect of experimental group ($F(2,23)=6.269$, $p=0.007$, $\eta^2=0.353$), and no interaction between experimental day and experimental group. Bonferroni-corrected *post hoc* tests revealed that *pbs MTII* voles spent significantly less time exploring compared to *pbp VEH* ($p=0.018$) and *pbs VEH* ($p=0.043$). The same effect of MTII treatment was also observed in female voles (**Figure 16 B**); *Pbs MTII* voles showed a decrease in the time spent in explorative behaviors. Repeated measures ANOVA revealed no main effect of experimental day, a between-subjects effect of experimental group ($F(2,18)=6.540$, $p=0.007$, $\eta^2=0.421$), and an interaction between experimental day and experimental group ($F(2,18)=4.153$, $p=0.033$, $\eta^2=0.316$). Games-Howell-corrected *post hoc* tests revealed that females from the *pbs MTII* group explored significantly less compared to *pbp VEH* ($p=0.029$) and *pbs VEH* ($p=0.004$). Also, female *pbp VEH* voles explored significantly more on experimental day 12 compared to experimental day 5 ($p=0.039$).

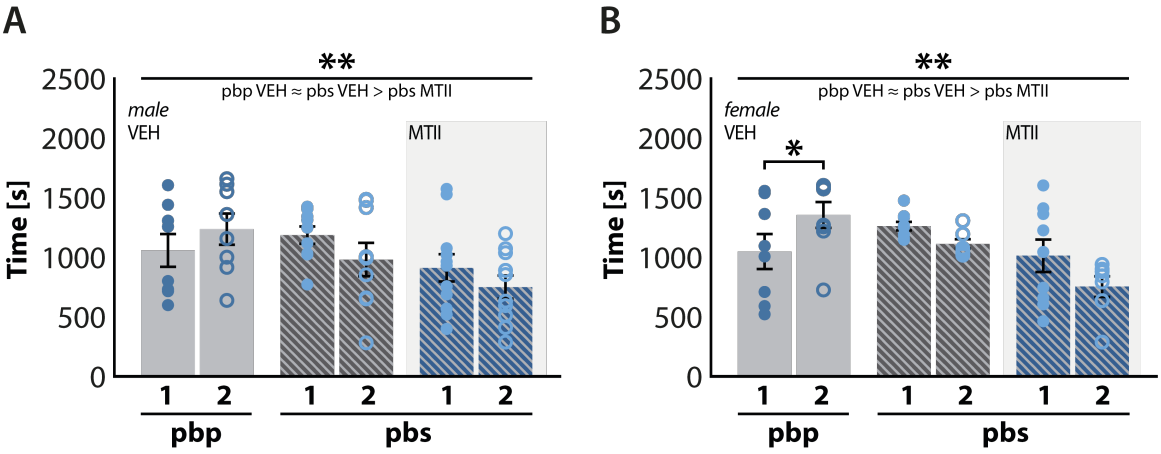

Figure 16 | Explorative behaviors in social context and MTII treatment.
Exploration was assessed in **(A)** male and **(B)** female voles in the presence of another social stimulus on experimental day 5 and 12. **(A)** MTII treatment led to a decrease in exploration in *pbs MTII* voles compared to *pbp VEH*. **(B)** Female *pbs MTII* voles showed a significant reduction in explorative behaviors. The data is presented as bar charts with aligned dot plots with mean ± SEM. All groups n=7-11. Main effects of experimental groups are indicated above the horizontal line with (*) $p<0.07$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, the respective *post hoc* statistics are indicated underneath the horizontal line as ≈ $p\geq0.7$, ≤/≥ $p<0.07$, </> $p<0.05$. Statistical significance for the *post hoc* tests for the interaction is indicated above the respective bars with (*) $p<0.07$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Table 14 | Repeated measures ANOVA for explorative behavior following MTII treatment.

Sex	*N*	Main effect: Time point	Between-subjects: Treatment	Interaction
M	26	$F(1,23)=0.112$, $p=0.740$, $\eta^2=0.005$	$F(2,23)=6.269$, $p=0.007$, $\eta^2=0.353$	$F(1,23)=1.005$, $p=0.382$, $\eta^2=0.080$
F	21	$F(1,18)=0.125$, $p=0.728$, $\eta^2=0.007$	$F(2,18)=6.540$, $p=0.007$, $\eta^2=0.421$	$F(2,18)=4.153$, $p=0.033$, $\eta^2=0.316$

Table 15 | *Post hoc* tests for explorative behavior following MTII treatment.

Sex	*N*	Treatment		Interaction		
M	26	pbp VEH vs. pbs VEH	$p=1.000$	1st assessment		
		pbp VEH vs. pbs MTII	$p=0.018$			
		pbs VEH vs. pbs MTII	$p=0.043$	2nd assessment		
F	21	pbp VEH vs. pbs VEH	$p=0.929$	1st assessment	pbp VEH vs. pbs VEH	$p=0.866$
					pbp VEH vs. pbs MTII	$p=0.813$
					pbs VEH vs. pbs MTII	$p=0.145$
		pbp VEH vs. pbs MTII	$p=0.029$	2nd assessment	pbp VEH vs. pbs VEH	$p=0.129$
					pbp VEH vs. pbs MTII	$p<0.001$
		pbs VEH vs. pbs MTII	$p=0.004$		pbs VEH vs. pbs MTII	$p=0.069$

Autogrooming

Central treatment with OT has previously been linked to an increase in autogrooming (Calcagnoli, de Boer, Althaus, den Boer, & Koolhaas, 2013) and is also an indicator for the measurement of anxiety. Since MTII treatment is increasing the release of endogenous OT in social context, autogrooming was assessed. Detailed statistics can be found in **Table 16**. In *pbs MTII* males, a significant increase in autogrooming was observed (**Figure 17 A**). A repeated measures ANOVA revealed no main effect of experimental day, a between-subjects effect of experimental group ($F(2,23)=23.910$, $p<0.001$, $\eta^2=0.675$), and no interaction between experimental day and experimental group. Games-Howell-corrected *post hoc* tests verified that *pbs MTII*-treated voles spent more time autogrooming than *pbp VEH* or *pbs VEH*-treated voles (both $p<0.001$). The same observation was made in female *pbs MTII*-treated voles (**Figure 17 B**). Repeated measures ANOVA revealed no main effect of experimental day, a between-subjects effect of experimental group ($F(2,19)=12.316$, $p<0.001$, $\eta^2=0.565$), and no interaction between experimental day and experimental group. Games-Howell-corrected *post hoc* tests revealed that *pbs MTII* voles spent significantly more time autogrooming compared to *pbp VEH* or *pbs VEH* females (both $p<0.001$).

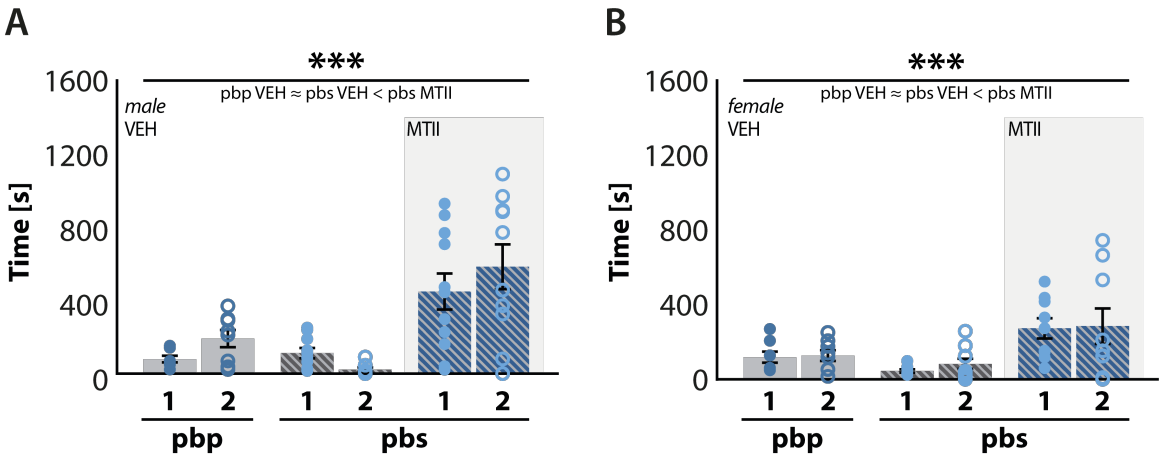

Figure 17 | Autogrooming following treatment with MTII.
Autogrooming was assessed in **(A)** male and **(B)** female voles in the presence of another social stimulus on experimental day 5 and 12. **(A)** MTII treatment led to increased autogrooming observed during 30 min of social interaction. **(B)** Female *pbs MTII* voles showed a significant increase in autogrooming. The data is presented as bar charts with aligned dot plots with mean ± SEM. All groups n=7-10. Main effects of experimental group are indicated above the horizontal line with (*) p<0.07, * p<0.05, ** p<0.01, *** p<0.001, the respective *post hoc* statistics are indicated underneath the horizontal line as ≈ p≥0.7, ≤/≥ p<0.07, </> p<0.05. Statistical significance for the *post hoc* tests for the interaction is indicated above the respective bars with (*) p<0.07, * p<0.05, ** p<0.01, *** p<0.001.

Table 16 | Repeated measures ANOVA for autogrooming following MTII treatment.

Sex	*N*	Main effect: Time point	Between-subjects: Treatment	*Post hoc* tests		Interaction
M	26	$F(1,23)=0.383$, $p=0.542$, $\eta^2=0.016$	$F(2,23)=23.910$, $p<0.001$, $\eta^2=0.675$	pbp VEH vs. pbs VEH	$p=0.085$	$F(2,23)=0.886$, $p=0.426$, $\eta^2=0.072$
				pbp VEH vs. pbs MTII	$p=0.001$	
				pbs VEH vs. pbs MTII	$p<0.001$	
F	25	$F(1,19)=0.225$, $p=0.641$, $\eta^2=0.012$	$F(2,19)=12.316$, $p<0.001$, $\eta^2=0.565$	pbp VEH vs. pbs VEH	$p=0.396$	$F(2,19)=0.022$, $p=0.978$, $\eta^2=0.002$
				pbp VEH vs. pbs MTII	$p=0.047$	
				pbs VEH vs. pbs MTII	$p=0.017$	

3.2 The impact of partner loss and acute stress on the neuro- and peripheral immune system

3.2.1 Validation of the stress response following separation

3.2.1.1 Forced Swim Test

To validate the effect of separation from a partner on emotionality, the FST was performed. Passive stress-coping was assessed in both male and female voles on the 4th day after separation from an opposite sex partner vs. still being with the partner. Detailed statistical descriptions can be found in **Table 17**. Male and female prairie voles displayed differential stress-coping behavior following separation from a partner. In male voles, following separation from a partner, a significant increase in the passive stress-coping behavior, marked by an increase in the time spent floating, was observed compared to paired males ($t(10.25)=2.67$, $p=0.020$, *delta*=0.871, **Figure 18 A**). Female voles did not show an increase in the time spent floating following separation from a male (**Figure 18 B**). Separation from a partner did not affect the time until the first floating episode in male and female prairie voles (**Figure 18 C,D** respectively).

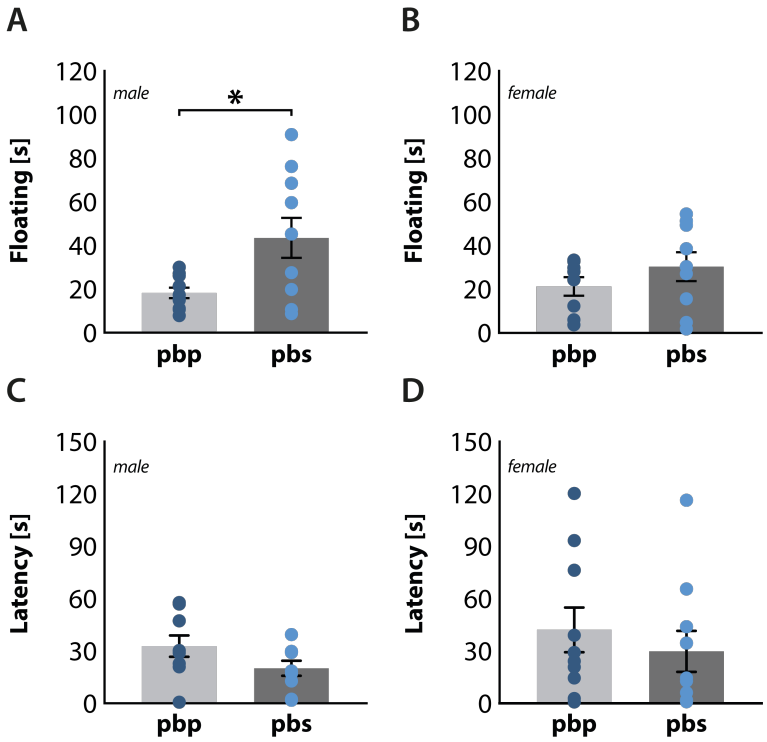

Figure 18 | Results of the FST following the separation from a partner.
To assess passive stress-coping behavior following separation, the FST was performed. **(A,B)** The total time spent floating and **(C,D)** the latency until the first floating episode was assessed in **(A,C)** male and **(B,D)** female prairie voles**. (A)** Separation from a partner induced an increase in the time spent floating in male prairie voles. **(B)** Separation did not affect the time spent floating in female prairie voles. **(C)** No alterations in the time until the first floating episode were observed following separation from a partner in male prairie voles. **(D)** Separation did not affect the time until the first floating episode in female voles. The data is presented as bar charts with aligned dot plot and mean ± SEM. All groups n=8-10, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, (*) $p \leq 0.7$.

Table 17 | Planned comparisons of passive stress-coping behavior following partner loss.

Parameter	Sex	Treatment	*n*	*M*	*Mdn*	*SD*	*df*	*t*	*U*	*p*	Effect Size
Floating (time)	M	pbp	10	18.24	16.50	7.66	10.25	2.670		0.023	0.871[c]
		pbs	10	43.49	36.43	28.98					
	F	pbp	8	21.31	26.22	12.09	15	1.120		0.279	0.546[b]
		pbs	9	30.39	30.35	19.78					
Swimming (time)	M	pbp	9		135.20	95.75			48	0.912	-0.025[d]
		pbs	9		75.89	108.60					
	F	pbp	10	147.10	130.00	115.30	18	0.061		0.952	0.027[a]
		pbs	10	144.10	158.70	103.70					
Struggling (time)	M	pbp	10		114.60	97.78			37	0.352	0.211[d]
		pbs	10		3.45	107.90					
	F	pbp	10		60.94	124.20			48	0.910	0.025[d]
		pbs	10		95.60	103.90					
Time until floating	M	pbp	9	32.84	26.72	18.45	16	1.693		0.343	0.798[a]
		pbs	9	20.10	13.66	13.01					
	F	pbp	10		26.72	40.63			38	0.393	0.194[d]
		pbs	10		13.66	37.31					

[a] = Cohen's *d*, [b] = Hedge's *g*, [c] = Glass's *delta*, [d] = Pearson's correlation

3.2.1.2 Measurement of the physiological stress response

Body weight of male and female voles was assessed at the onset of cohabitation on experimental day 0, on experimental day 5 (day of separation), and on experimental day 8 (4th day after separation). Male voles showed a decrease of body weight during the cohabitation period between experimental day 0 and 5, no body weight changes were observed following separation between experimental day 5 and 8 (**Figure 19 A**). A repeated measures ANOVA revealed a main effect of experimental day (F(1.606,54.607)=32.728, p<0.001, η^2=0.490), no between-subjects effect of housing condition, and no interaction between housing condition and experimental day. Bonferroni-corrected *post hoc* tests revealed that both *pbp* and *pbs* showed a significant reduction in body weight during the cohabitation period (both p<0.001). Separation had no effect on female body weight and the body weight did not differ between experimental days (**Figure 19 B**). Adrenal gland weight was assessed as an indicator for the effects of chronic stress on the stress response, since chronic stress was previously shown to cause adrenal hypertrophy following separation from a partner (Bosch et al., 2009). Separation from a partner did not cause an adrenal hypertrophy in separated male and female voles (**Figure 19 C,D**). Plasma corticosterone levels were assessed by an ELISA in male and female voles, as plasma CORT levels were reported to being upregulated following separation from a partner (Bosch et al., 2009) (**Figure 19 E,F**). Separation from a partner and acute stress did not alter basal CORT levels in both male and female prairie voles.

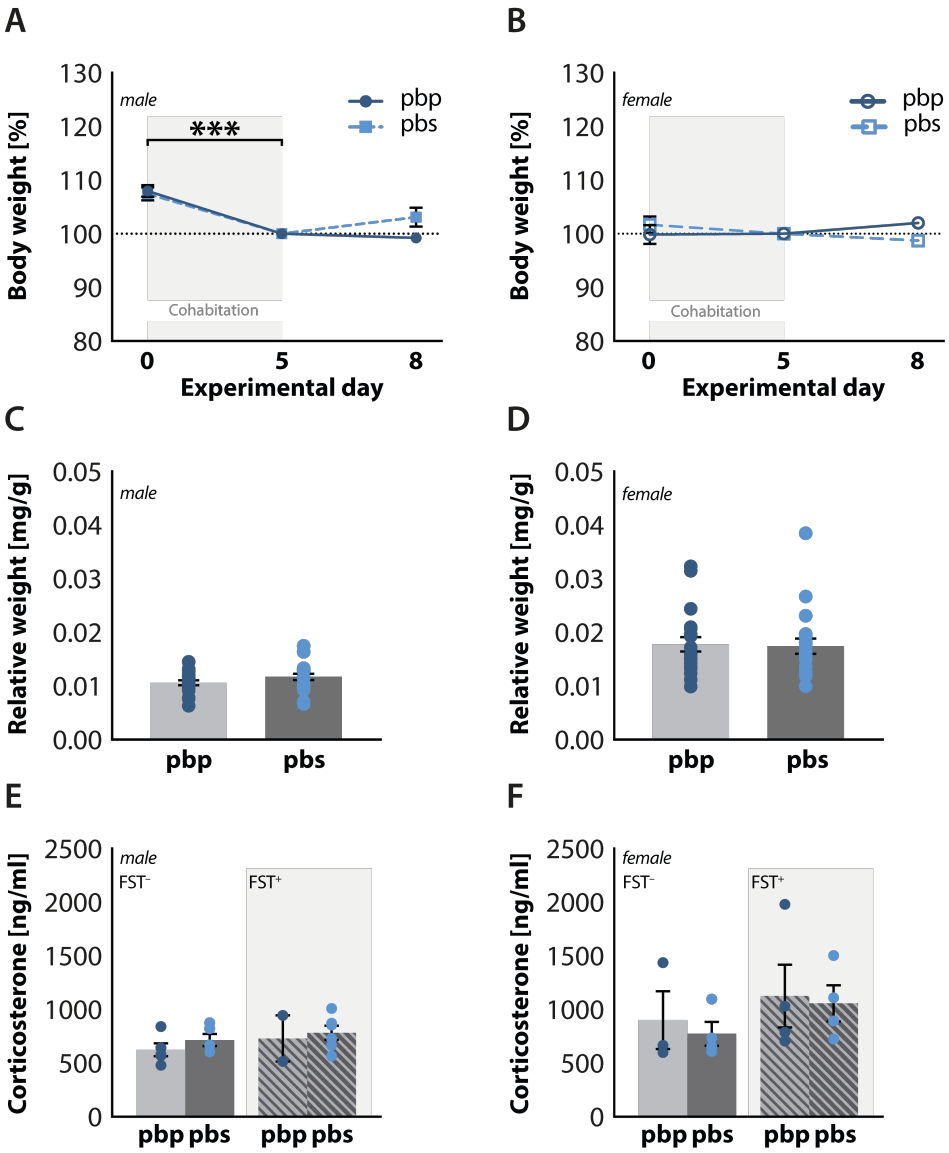

Figure 19 | Physiological parameters following the loss of a partner.
Body weight was assessed on experimental day 0, 5, and 8 in **(A)** male and **(B)** female prairie voles. **(A)** Male body weight differed significantly pairing on experimental day 0 and experimental day 5. Neither separation on experimental day 5, nor continuous pair housing caused a change of body weight in male voles. **(B)** Separation from a partner did not change female body weight between experimental day 5 and 8. **(C)** No difference in the relative adrenal gland weight was observed between *pbp* and *pbs* males and **(D)** females. **(E)** In male prairie voles, neither separation from a partner nor acute stress affected CORT levels. **(F)** No effect of separation and acute stress was observed in female CORT levels. The data is presented in line plots or bar charts with aligned dot plots with mean ± SEM. All groups n=2-20. The main effect of housing condition is indicated above the horizontal line with the respective *post hoc* statistics indicated underneath the horizontal line as ≈ $p \geq 0.7$, (*) $p<0.07$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$). Statistical significance for the main effect of acute stress and *post hoc* statistics for the interaction are indicated above the respective bars with (*) $p<0.07$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Statistics for planned comparisons are indicated above the respective bars with (#) $p<0.07$, # $p<0.05$, ## $p<0.01$, ### $p<0.001$.

Table 18 | Repeated measures ANOVA of body weight development following partner loss.

Sex	*N*	Main effect: Experimental day	*Post hoc* tests		Between-subjects: Housing	Interaction
M	36	$F(1.606,54.607)=32.728$, $p<0.001$, $\eta^2=0.490$	ED1 vs. ED5 ED1 vs. ED8 ED5 vs. ED8	$p<0.001$ $p<0.001$ $p=0.681$	$F(1,34)=1.667$, $p=0.205$, $\eta^2=0.047$	$F(1.606,54.607)=2.785$, $p=0.082$, $\eta^2=0.076$
F	37	$F(1.353,47.344)=0.246$, $p=0.694$, $\eta^2=0.007$			$F(1,35)=0.300$, $p=0.587$, $\eta^2=0.008$	$F(1.353)=2.862$, $p=0.086$, $\eta^2=0.076$

Table 19 | Pairwise comparisons for relative adrenal gland weight following partner loss.

Sex	Treatment	*n*	*M*	*SD*	*df*	*t*	*p*	Effect Size
M	pbp	19	0.011	0.002	37	1.474	0.325	0.473[b]
	pbs	20	0.012	0.003				
F	pbp	20	0.018	0.006	38	0.173	0.864	0.053[a]
	pbs	20	0.017	0.006				

[a] = Cohen's *d*, [b] = Hedge's *g*

Table 20 | ANOVA for basal CORT levels following the loss of a partner.

Sex	*N*	Main effect: Housing	Main effect: FST	Interaction
M	18	$F(3,14)=0.783$, $p=0.391$, $\eta^2=0.053$	$F(3,14)=1.118$, $p=0.308$, $\eta^2=0.074$	$F(3,14)=0.057$, $p=0.815$, $\eta^2=0.004$
F	15	$F(3,11)=0.200$, $p=0.664$, $\eta^2=0.018$	$F(3,11)=1.352$, $p=0.270$, $\eta^2=0.109$	$F(3,11)=0.018$, $p=0.896$, $\eta^2=0.002$

3.2.2 Microglia alterations following partner loss

3.2.2.1 Microglia numbers in specific brain regions

Social and nonsocial stressors were shown to modulate proliferation and activation of microglia in a region-specific manner (Kreisel et al., 2014; Wohleb et al., 2012). Since behavioral differences were observed in the FST following separation from a partner between male and female prairie voles, the question arose whether this could be due to a difference in the proliferation and abundance of microglia cells. Following labelling of microglia cells with the microglial marker IBA1, microglia were counted in the regions of the PrL, the NAcc shell, and the PVN. The percentage was calculated based on the number of microglial cells in relation to the total cell count within each respective region. All statistics including the results of performed two-way ANOVAs, *post hoc* analyses and planned comparisons can be found in **Table 21** or in appendix **Table 35**. Within the PrL of male voles, neither separation nor acute stress affected the percentage of microglia cells (**Figure 20 A**). A two-way ANOVA revealed no main effect of housing condition, acute stress, and a trend towards an interaction between housing condition and acute stress ($F(1,20)=3.963$, $p=0.060$, $\eta^2=0.165$). Bonferroni-corrected *post hoc* tests revealed no further significances for the interaction between housing condition and acute stress. Neither separation nor acute stress impacted the number of microglial cells within the NAcc shell of male voles (**Figure 20 B**). Similarly, no effect of separation

and acute stress was observed within the PVN of male prairie voles (**Figure 20 C**). Partner loss and acute stress did not influence microglia percentages within the PrL of female prairie voles **(Figure 21 A**). Within the NAcc shell of female prairie voles, separation from a partner and acute stress did not impact microglia percentages (**Figure 21 B**). Within the PVN, however, partner loss affected the number of microglia significantly (**Figure 21 C**). A two-way ANOVA revealed a main effect of housing condition ($F(1,20)=4.836$, $p=0.040$, $\eta^2=0.195$), a trend towards a main effect of acute stress ($F(1,20)=3.882$, $p=0.063$, $\eta^2=0.163$), and no interaction between housing condition and acute stress. Bonferroni-corrected *post hoc* tests did not show significant differences between *pbp* or *pbs* voles or FST^- or FST^+.

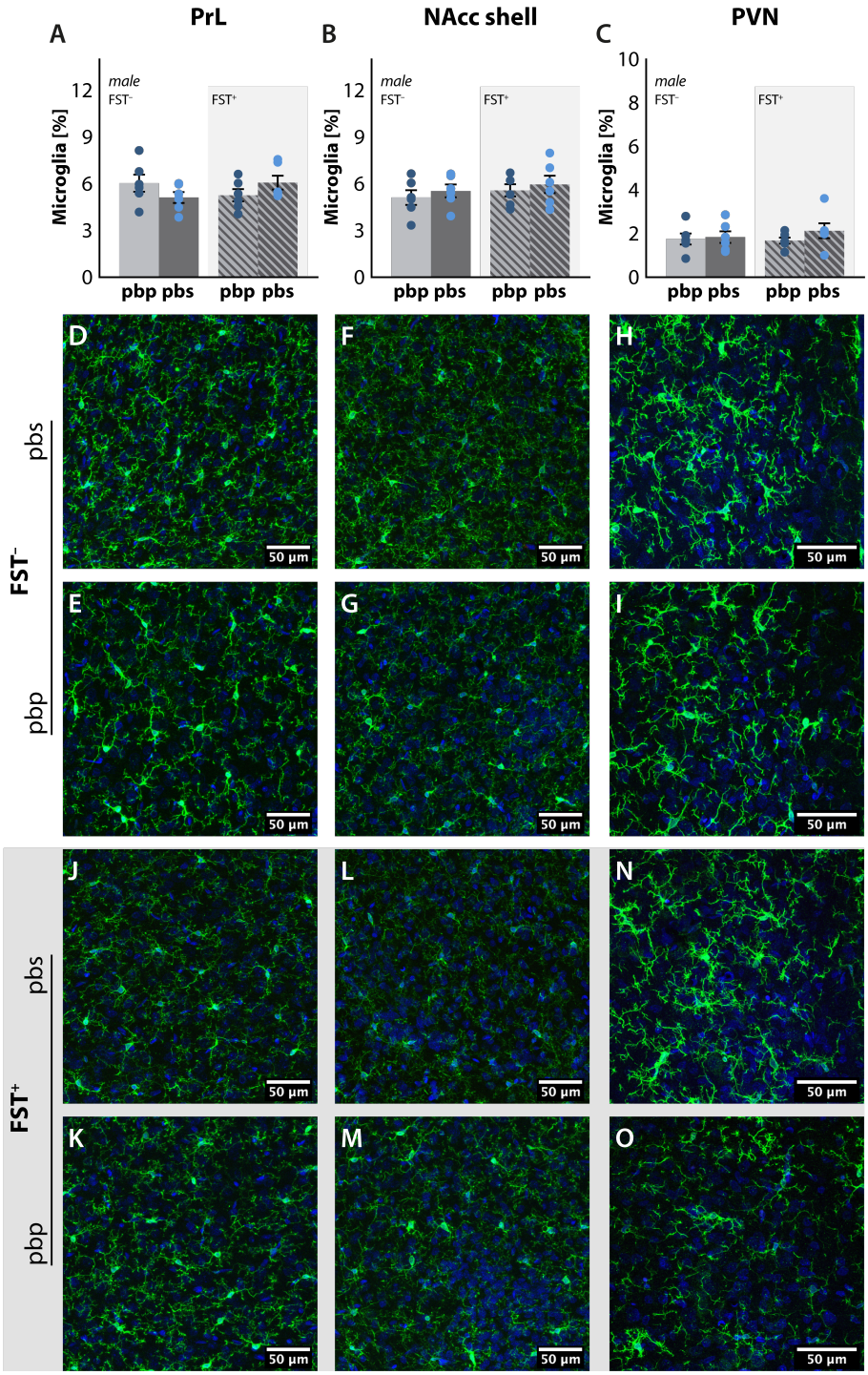

Figure 20 | Microglia percentages following partner loss and/or acute stress in males. Microglia percentage of total cells was assessed in **(A,D,E,J,K)** the PrL, **(B,F,G,L,M)** the NAcc shell, and **(C,H,I,N,O)** the PVN. **(A)** In the PrL of male prairie voles, partner loss and/or acute stress did not affect the percentage of microglia. **(B)** Partner loss and/or acute stress did not affect microglia percentages in the NAcc shell of male voles. **(C)** Partner loss and/or acute stress did not affect microglia percentages in the PVN of male voles. The data is presented as bar charts with aligned dot plots with mean ± SEM. All groups n=6. The main effect of housing condition is indicated above the horizontal line with the respective *post hoc* statistics indicated underneath the horizontal line as ≈ p≥0.7, (*) p<0.07, * p<0.05, ** p<0.01, *** p<0.001). Statistical significance for the main effect of acute stress and *post hoc* statistics for the interaction are indicated above the respective bars with (*) p<0.07, * p<0.05, ** p<0.01, *** p<0.001. Statistics for planned comparisons are indicated above the respective bars with (#) p<0.07, # p<0.05, ## p<0.01, ### p<0.001.

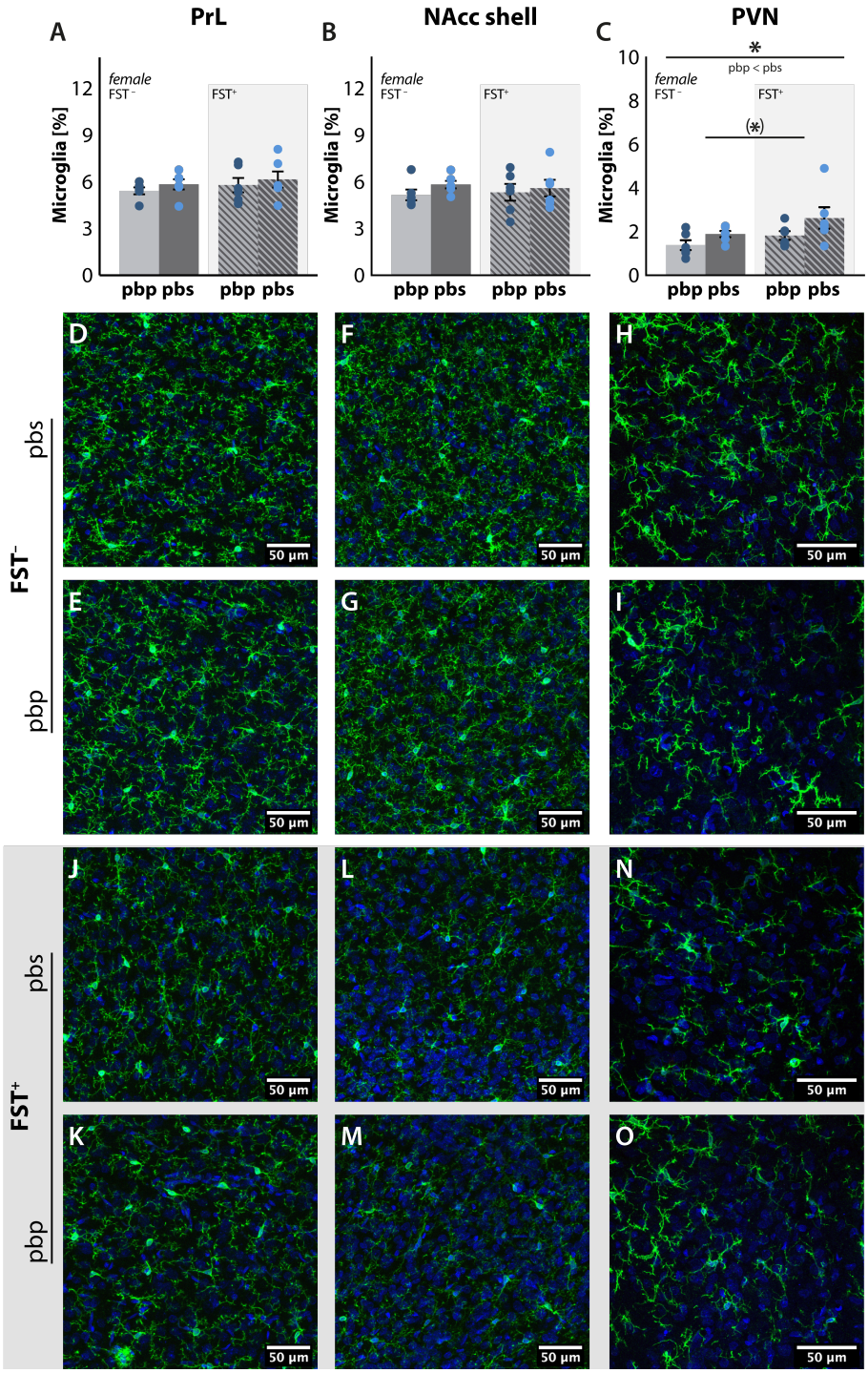

Figure 21 | Microglia percentage following partner loss and/or acute stress in females. Microglia percentage of total cells was assessed in **(A,D,E,J,K)** the PrL, **(B,F,G,L,M)** the NAcc shell, and **(C,H,I,N,O)** the PVN. **(A)** Partner loss and/or acute stress did not affect microglia percentages in the PrL of female voles. **(B)** Microglia percentages in the NAcc shell of female voles were not affected by partner loss and/or acute stress. **(C)** Only partner loss increased microglia percentages in the PVN of female voles. The data is presented as bar charts with aligned dot plots with mean ± SEM. All groups n=6. The main effect of housing condition is indicated above the horizontal line with the respective *post hoc* statistics indicated underneath the horizontal line as ≈ p≥0.7, (*) p<0.07, * p<0.05, ** p<0.01, *** p<0.001). Statistical significance for the main effect of acute stress and *post hoc* statistics for the interaction are indicated above the respective bars with (*) p<0.07, * p<0.05, ** p<0.01, *** p<0.001. Statistics for planned comparisons are indicated above the respective bars with (#) p<0.07, # p<0.05, ## p<0.01, ### p<0.001.

Table 21 | Two-way ANOVA for microglia percentages in specific brain regions.

Region	Sex	*N*	Main effect: Housing	Main effect: Stress	Interaction	*Post hoc* tests	
PrL	M	24	$F(1,20)=0.014$, $p=0.907$, $\eta^2=0.001$	$F(1,20)=0.043$, $p=0.838$, $\eta^2=0.002$	$F(1,20)=3.963$, $p=0.060$, $\eta^2=0.165$	FST^- FST^+ pbp pbs	$p=0.152$ $p=0.200$ $p=0.222$ $p=0.136$
	F	24	$F(1,20)=0.0.955$, $p=0.340$, $\eta^2=0.046$	$F(1,20)=0.721$, $p=0.406$, $\eta^2=0.035$	$F(1,20)=0.006$, $p=0.939$, $\eta^2=0.000$		
NAcc shell	M	24	$F(1,20)=0.779$, $p=0.388$, $\eta^2=0.037$	$F(1,20)=0.917$, $p=0.350$, $\eta^2=0.044$	$F(1,20)=0.002$, $p=0.962$, $\eta^2=0.000$		
	F	24	$F(1,20)=1.175$, $p=0.291$, $\eta^2=0.056$	$F(1,20)=0.005$, $p=0.946$, $\eta^2=0.000$	$F(1,20)=0.216$, $p=0.647$, $\eta^2=0.011$		
PVN	M	24	$F(1,20)=1.091$, $p=0.309$, $\eta^2=0.052$	$F(1,20)=0.179$, $p=0.676$, $\eta^2=0.009$	$F(1,20)=0.496$, $p=0.489$, $\eta^2=0.024$		
	F	24	$F(1,20)=4.836$, $p=0.040$, $\eta^2=0.195$	$F(1,20)=3.882$, $p=0.063$, $\eta^2=0.163$	$F(1,20)=0.250$, $p=0.622$, $\eta^2=0.012$		

3.2.2.2 Classification of microglia morphology following partner loss

Changes in behavior have been linked to changes in microglia morphology and function in previous studies (Kettenmann et al., 2011). Also, exposure to either acute or chronic stress have been described as being potent modulators of microglia activation, and they are also linked to an increase in passive stress-coping behaviors (Stein, Vasconcelos, Albrechet-Souza, Cereser, & de Almeida, 2017). Therefore, since separation from a partner is regarded as a social stressor (Bosch et al., 2016), and changes in passive stress-coping following separation were observed in this study, microglia morphology was assessed following the flow chart as described in 3.2.7.1 The respective ANOVAs and planned comparisons can be found in **Table 22** and **Table 23** for male prairie voles and **Table 24** and **Table 25** for females. Planned comparisons are presented in appendix **Table 36** (PrL), **Table 37** (NAcc shell), **Table 38** (PVN), and **Table 39** (subregions of the PVN).

Males

Neither separation from a partner nor acute stress affected the morphological activation of microglia in the PrL of male voles. Analyzing housing condition and stress as independent between subject factors using a mixed models two-way ANOVA revealed no main effect or interaction between the factors. When analyzing the effect of separation in each stress condition separately using a mixed models one-way ANOVA, no effect of separation from a partner on microglia morphology in the PrL of male voles (**Figure 22 A**) was revealed. Separation from a partner paired with acute stress similarly did not alter microglia morphology (**Figure 22 B**).

In the NAcc shell, an interaction between housing condition and acute stress was observed, resulting in a lower percentage of ramified microglia in paired males following the FST, as revealed

by a mixed models two-way ANOVA (F(1.084,21.689)=4.346, p=0.046, η^2=0.179). Analyzing microglia profiles for each stress condition separately, no effect of separation from a partner on microglia morphology was observed in the NAcc shell of separated males (**Figure 22 C**). This was also apparent in acutely stressed male prairie vole (**Figure 22 D**).

Separation from a partner did not affect morphological microglia activation in the PVN of male prairie voles. Acute stress caused a reduction of reactive microglia within the PVN of male as revealed by a mixed models two-way ANOVA (F(1.439,28.783)=7.374, p=0.006, η^2=0.269). Investigating the impact of separation from a partner for each stress condition separately, no effect was observed in the PVN of male prairie voles (**Figure 22 E**). Focusing on the effects of partner loss within the subregions of the PVN separately, an effect of separation on microglia morphology in the parvo- (PVN_{parvo}) but not in the magnocellular region (PVN_{magno}) was revealed. Analyzing the interaction between housing condition and acute stress in the PVN_{parvo}, a mixed models two-way ANOVA with Greenhouse-Geisser correction revealed that microglia within the PVN_{parvo} showed a trend towards a difference between microglia states following separation (F(1.9737,36.795)=2.932, p=0.067, η^2=0.134) and a reduction of reactive cells following acute stress (F(1.937,36.795)=8.840, p=0.001, η^2=0.502). Separation led to an increase in the percentage of primed microglia (p=0.041). Acute stress caused an increase in primed (p=0.002) and a decrease in reactive microglia (p=0.006) in *pbp* male voles. When analyzing the microglia activation states separately for each basal and acute stress condition (**Figure 23**), separation from a partner induced morphological changes in the PVN_{parvo} of male voles under basal conditions. A mixed models one-way ANOVA revealed an interaction between housing condition and microglia state in the PVN_{parvo} (F(3,30)=2.154, p=0.018, η^2=0.308). Planned comparisons revealed a significant difference in the PVN_{parvo} between *pbp* and *pbs* voles with a significant increase in primed microglia following separation (t(9)=2.872, p=0.018, g=1.739, **Figure 23 C**). In acutely stressed animals, no effect of separation on microglia was observed (**Figure 24 B,D**).

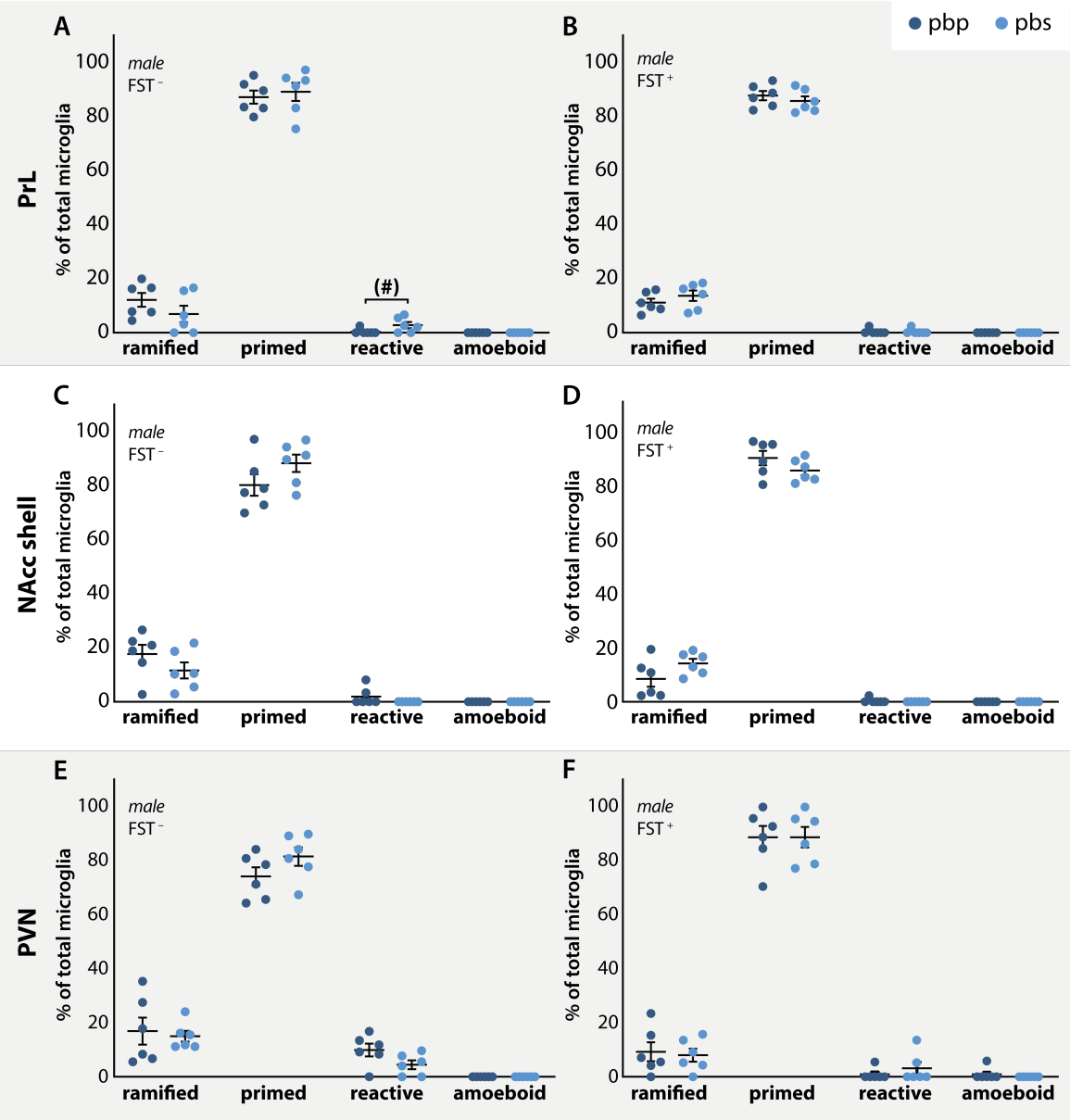

Figure 22 | Microglia activation profiles following separation from a partner under basal conditions and following acute stress in male prairie voles.
The microglia activation states were assessed in the **(A,B)** the PrL, **(C,D)** the NAcc shell, and **(E,F)** the PVN of male prairie voles both under **(A,C,E)** basal conditions and **(B,D,F)** following acute stress in the form of the FST. **(A)** Separation did not affect microglia activation states in the PrL under basal conditions. **(B)** Following acute stress, separation had no effect on the composition of microglia states in the PrL. **(C)** In the NAcc shell of male voles, separation had no effect on microglia composition under basal conditions. **(D)** Microglia activation was not influenced by separation in the NAcc shell following acute stress. **(E)** In the PVN, no effect of separation on microglia activation was observed under basal conditions. **(F)** No effect of separation was observed in the PVN following acute stressor exposure. The data is presented as scatter dot plots ± SEM. All groups n=6. Interaction and between-subjects effects are indicated above the horizontal line with the respective *post hoc* statistics indicated underneath the horizontal line as ≈ $p \geq 0.7$, (*) $p<0.07$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$). Planned comparisons are indicated above the respective bars with (#) $p<0.07$, # $p<0.05$, ## $p<0.01$, ### $p<0.001$.

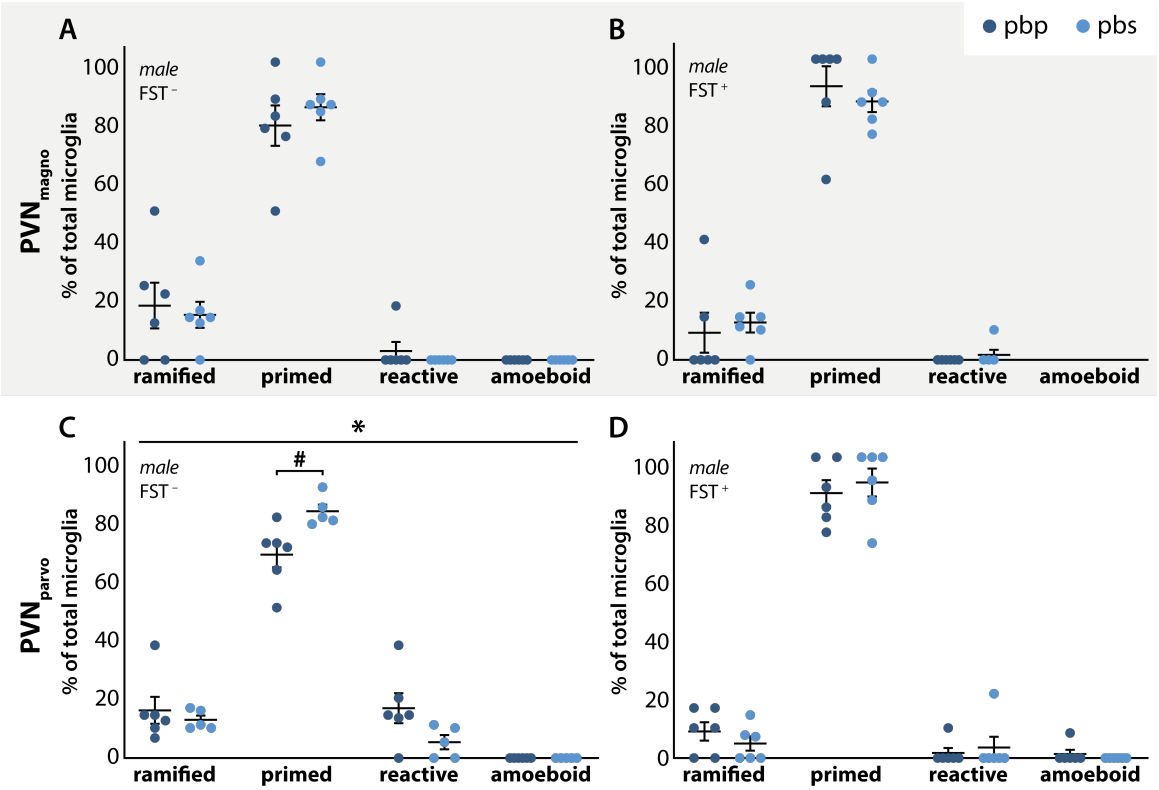

Figure 23 | Microglia activation profiles in subregions of the PVN following separation from a partner under basal conditions and following acute stress in male prairie voles.
Microglia morphology was assessed within **(A,B)** the PVN_{magno} and **(C,D)** the PVN_{parvo} of paired and separated male prairie voles under **(A,C)** basal conditions and **(B,D)** following acute stress. **(A)** In the PVN_{magno}, separation had no effect on the percentage of microglia states under basal conditions. **(B)** Acute stress did not affect microglia activation states following separation in the PVN_{magno}. **(C)** Separation from a partner resulted in significant differences of microglia morphology in the PVN_{parvo} under basal conditions. **(D)** Separation from a partner followed by acute stress did not affect microglia morphology in the PVN_{parvo}. The data is presented as scatter dot plots ± SEM. All groups n=5-6. Interaction and between-subjects effects are indicated above the horizontal line with the respective *post hoc* statistics indicated underneath the horizontal line as ≈ $p \geq 0.7$, (*) $p<0.07$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$). Planned comparisons are indicated above the respective bars with (#) $p<0.07$, # $p<0.05$, ## $p<0.01$, ### $p<0.001$.

Table 22 | Mixed models ANOVA of microglial profiles of male prairie voles.

Region	FST	*N*	Main effect: State	Between-subjects: Housing	Interaction
PrL	FST^-	12	$F(1.140,11.397)=629.770$, $p<0.001$, $\eta^2=0.984$	$F(1,10)=2.365$, $p=0.155$, $\eta^2=0.191$	$F(1.140,11.397)=1.057$, $p=0.336$, $\eta^2=0.096$
	FST^+	12	$F(1.092,10.920)=1715.614$, $p<0.001$, $\eta^2=0.994$	$F(1,10)=1.000$, $p=0.341$, $\eta^2=0.091$	$F(1.092,10.920)=0.859$, $p=0.384$, $\eta^2=0.079$
NAcc shell	FST^-	12	$F(1.102,11.022)=395.614$, $p<0.001$, $\eta^2=0.975$	$F(1,10)=0.011$, $p=0.917$, $\eta^2=0.001$	$F(1.102,11.022)=2.154$, $p=0.170$, $\eta^2=0.177$
	FST^+	12	$F(1.018,10.177)=1036.477$, $p<0.001$, $\eta^2=0.990$	$F(1,10)=2.500$, $p=0.145$, $\eta^2=0.200$	$F(1.018,10.177)=2.620$, $p=0.136$, $\eta^2=0.208$
PVN	FST^-	12	$F(3,30)=251.958$, $p<0.001$, $\eta^2=0.962$	$F(1,10)=0.060$, $p=0.812$, $\eta^2=0.006$	$F(3,30)=1.447$, $p=0.249$, $\eta^2=0.126$
	FST^+	12	$F(1.307,13.069)=376.561$, $p<0.001$, $\eta^2=0.974$	$F(1,10)=1.253$, $p=0.289$, $\eta^2=0.111$	$F(1.307,13.069)=0.130$, $p=0.790$, $\eta^2=0.013$

Table 23 | Mixed models ANOVA of microglial profiles in subregions of the PVN of male prairie voles.

Region	FST	*N*	Main effect: State	Between-subjects: Housing	Interaction	*Post hoc* tests	
PVN_{magno}	FST^-	12	$F(3,30)=117.387$, $p<0.001$, $\eta^2=0.921$	$F(1,10)=0.000$, $p=1.000$, $\eta^2=1.000$	$F(3,30)=0.371$, $p=0.774$, $\eta^2=0.036$		
	FST^+	12	$F(3,30)=191.927$, $p<0.001$, $\eta^2=0.950$	$F(1,10)=0.000$, $p=1.000$, $\eta^2=1.000$	$F(3,30)=0.353$, $p=0.787$, $\eta^2=0.034$		
PVN_{parvo}	FST^-	12	$F(3,30)=157.878$, $p<0.001$, $\eta^2=0.946$	$F(1,10)=2.332$, $p=0.161$, $\eta^2=0.206$	$F(3,30)=2.154$, $p=0.018$, $\eta^2=0.308$	Ramified Primed Reactive Amoeboid	$p=0.553$ $p=0.018$ $p=0.088$ $p=1.000$
	FST^+	12	$F(1.529,15.293)=309.611$, $p<0.001$, $\eta^2=0.969$	$F(1,10)=3.357$, $p=0.097$, $\eta^2=0.251$	$F(1.529,15.293)=0.471$, $p=0.583$, $\eta^2=0.045$		

Females

No statistically significant effect was observed when analyzing the interaction between separation from a partner and acute stress in the PrL of female prairie voles. A Greenhouse-Geisser-corrected two-way mixed models ANOVA revealed no effect of both factors and their interaction. However, when analyzing both stress conditions separately, separation from a partner resulted in a lower microglia activation profile in the PrL of separated female voles that did not undergo acute stress in the FST (**Figure 22 A)**. Mixed models one-way ANOVA revealed a significant interaction between microglia state and housing condition in the PrL of female voles ($F(1.406,14.063)=8.147$, $p=0.008$, $\eta^2=0.449$). Planned comparisons within microglial states revealed significant differences in ramified and primed microglia between paired and separated female voles. Separation caused an increase of ramified cells ($t(6.143)=3.791$, $p=0.009$, $g=2.114$) while a decrease of primed cells ($t(10)=2.750$, $p=0.021$, $d=1.588$) was observed. However, when the animals underwent acute stress in the FST, the previously described effect of separation on microglia morphology was not observed (**Figure 24 B**).

No interaction was observed between separation from a partner and acute stress within the NAcc shell of female prairie voles. Furthermore, analyzing microglia profiles for each stress conditions separately, no difference in morphological activation was observed between paired and separated female voles within the NAcc shell (**Figure 22 C)**. Similarly, acute stress in the FST did also not impact microglia morphology following separation from a partner (**Figure 24 D**).

Within the PVN of female prairie voles, the analysis of the interaction between housing condition and acute stress revealed a trend towards an interaction between microglia activation state, housing condition, and acute stress. A mixed models two-way ANOVA revealed a trend towards an interaction between all three factors ($F(3,57)=2.141$, $p=0.063$, $\eta^2=0.119$). Acute stress led to an increase of ramified microglia and a decrease of primed microglia in the PVN of paired females ($p=0.033$, and $p=0.037$) Comparing microglia percentages within each stress condition independently, separation caused a significant change of microglia morphology in the PVN of female voles that were not subjected to the FST (**Figure 22 E)**. Mixed models one-way ANOVA revealed a significant interaction between microglial states and housing condition ($F(3,30)=3.884$, $p=0.020$, $\eta^2=0.301$). Planned comparisons within microglia states revealed a significant difference between the percentage of ramified microglia of paired and separated females ($U=4$, $p=0.0498$, $r=0.475$). When analyzing microglia morphology following acute stress and separation from a partner, the previously described effect of separation was not observed (**Figure 24 F**). Analyzing the interaction between both separation from a partner and acute stress, no significant differences between groups in the PVN_{magno} or the PVN_{parvo} were observed (**Figure 25**).

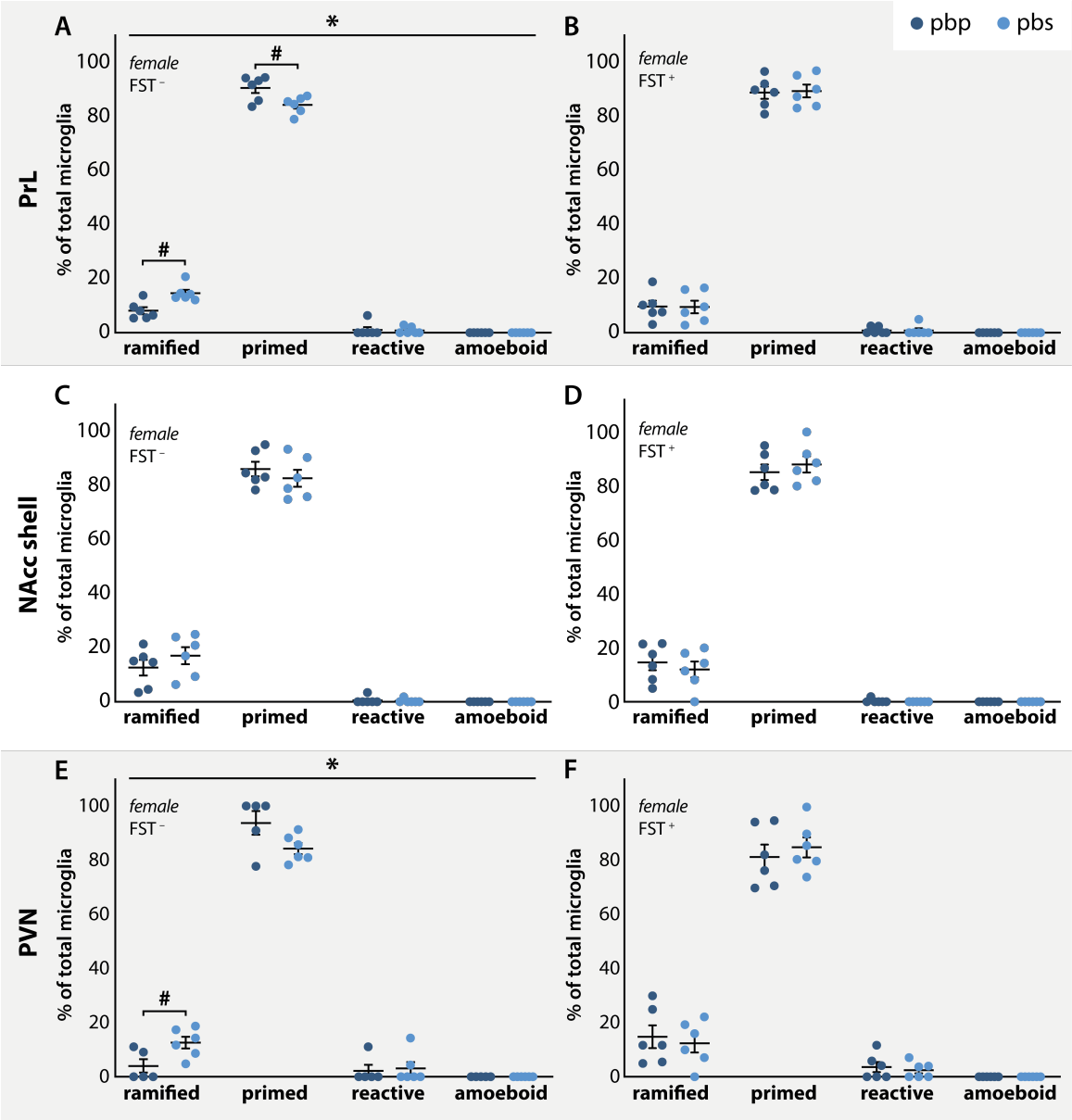

Figure 24 | Microglia activation profiles following separation from a partner under basal conditions and following acute stress in female prairie voles.
The microglia activation states were assessed in the **(A,B)** the PrL, **(C,D)** the NAcc shell, and **(E,F)** the PVN of female prairie voles both under **(A,C,E)** basal conditions and following **(B,D,F)** acute stress in the form of the FST. **(A)** Separation from a partner had a significant effect on microglia activation in the PrL under basal conditions. **(B)** Following acute stress, no effect of separation on microglia activation was observed in the PrL. **(C)** Separation from a partner did not affect microglia activation within the NAcc shell under basal conditions. **(D)** Similarly, no effect of separation was observed following acute stress within the NAcc shell. **(E)** Separation from a partner affected microglia activation in the PVN under basal conditions. **(F)** Following acute stress, no effect of separation was observed in the PVN. The data is presented as scatter dot plots ± SEM. All groups n=5-6. Interaction and between-subjects effects are indicated above the horizontal line with the respective *post hoc* statistics indicated underneath the horizontal line as ≈ $p \geq 0.7$, (*) $p<0.07$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$). Planned comparisons are indicated above the respective bars with (#) $p<0.07$, # $p<0.05$, ## $p<0.01$, ### $p<0.001$.

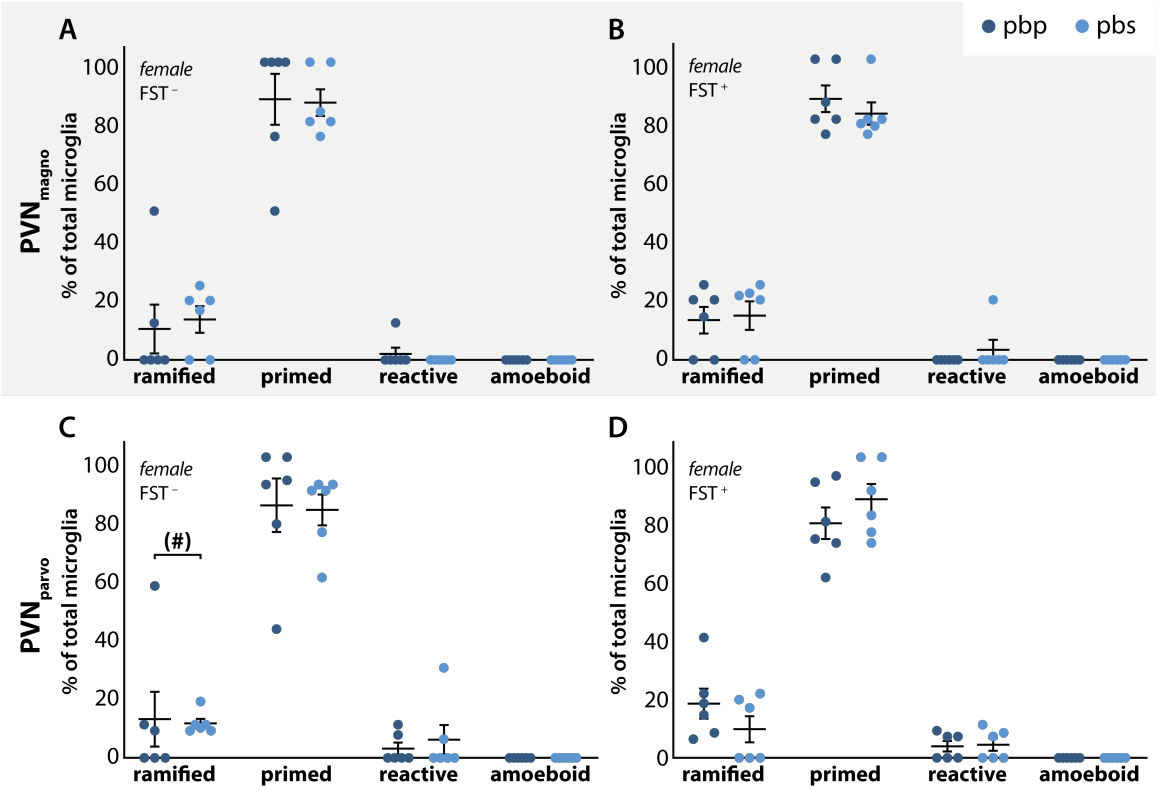

Figure 25 | Microglia activation profiles in subregions of the PVN following separation from a partner and acute stress.

Microglia morphology was assessed within **(A,B)** the PVN_{magno} and **(C,D)** the PVN_{parvo} of paired and separated male prairie voles under **(A,C)** basal conditions and **(B,D)** following acute stress. **(A)** No effect of separation was observed in the PVN_{magno} of female voles. **(B)** Separation in combination with acute stress did not affect microglia morphology in the PVN_{magno}. **(C)** In the PVN_{parvo}, no effect of separation on microglia activation was observed. **(D)** In the PVN_{parvo}, no effect of separation coupled with acute stress was observed. The data is presented as scatter dot plots ± SEM. All groups n=6. Interaction and between-subjects effects are indicated above the horizontal line with the respective *post hoc* statistics indicated underneath the horizontal line as ≈ $p \geq 0.7$, (*) $p<0.07$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$). Planned comparisons are indicated above the respective bars with (#) $p<0.07$, # $p<0.05$, ## $p<0.01$, ### $p<0.001$.

Table 24 | Mixed models ANOVA of microglial profiles of female prairie voles.

Region	FST	*N*	Main effect: State	Between-subjects: Housing	Interaction	*Post hoc* tests	
PrL	FST $^-$	12	$F(1.406,14.063)=2108.431$, $p<0.001$, $\eta^2=0.995$	$F(1,10)=1.000$, $p=0.341$, $\eta^2=0.091$	$F(1.406,14.063)=8.147$, $p=0.008$, $\eta^2=0.449$	Ramified Primed Reactive Amoeboid	$p=0.005$ $p=0.020$ $p=0.857$ $p=1.000$
	FST $^+$	12	$F(1.163,11.631)=1021.784$, $p<0.001$, $\eta^2=0.990$	$F(1,10)=0.991$, $p=0.343$, $\eta^2=0.090$	$F(1.163,11.631)=0.016$, $p=0.927$, $\eta^2=0.002$		
NAcc shell	FST $^-$	12	$F(1.029,10.290)=545.129$, $p<0.001$, $\eta^2=0.982$	$F(1,10)=1.902$, $p=0.198$, $\eta^2=0.160$	$F(1.029,10.290)=0.868$, $p=0.376$, $\eta^2=0.080$		
	FST $^+$	12	$F(3,30)=605.993$, $p<0.001$, $\eta^2=0.984$	$F(1,10)=0.000$, $p=1.000$, $\eta^2=0.000$	$F(3,30)=0.446$, $p=0.722$, $\eta^2=0.043$		
PVN	FST $^-$	11	$F(3,30)=522.573$, $p<0.001$, $\eta^2=0.983$	$F(1,10)=0.868$, $p=0.376$, $\eta^2=0.088$	$F(3,30)=3.884$, $p=0.020$, $\eta^2=0.301$	Ramified Primed Reactive Amoeboid	$p=0.028$ $p=0.067$ $p=0.794$ $p=1.000$
	FST $^+$	12	$F(3,30)=268.529$, $p<0.001$, $\eta^2=0.964$	$F(1,10)=0.297$, $p=0.598$, $\eta^2=0.029$	$F(3,30)=0.286$, $p=0.651$, $\eta^2=0.028$		

Table 25 | Mixed models ANOVA of microglial profiles in subregions of the PVN of female prairie voles.

Region	FST	*N*	Main effect: State	Between-subjects: Housing	Interaction
PVN_{magno}	FST $^-$	12	$F(3,30)=113.448$, $p<0.001$, $\eta^2=0.919$	$F(1,10)=0.000$, $p=1.000$, $\eta^2=1.000$	$F(3,30)=0.086$, $p=0.967$, $\eta^2=0.009$
	FST $^+$	12	$F(3,30)=224.250$, $p<0.001$, $\eta^2=0.957$	$F(1,10)=0.000$, $p=1.000$, $\eta^2=1.000$	$F(3,30)=0.0437$, $p=0.728$, $\eta^2=0.042$
PVN_{parvo}	FST $^-$	12	$F(3,30)=84.397$, $p<0.001$, $\eta^2=0.894$	$F(1,10)=0.188$, $p=0.674$, $\eta^2=0.018$	$F(3,30)=0.058$, $p=0.981$, $\eta^2=0.006$
	FST $^+$	12	$F(3,30)=170.826$, $p<0.001$, $\eta^2=0.945$	$F(1,10)=0.326$, $p=0.581$, $\eta^2=0.032$	$F(3,30)=1.311$, $p=0.289$, $\eta^2=0.116$

3.2.3 Alternative morphological characterization of microglia

3.2.3.1 Sholl analysis

With a change in the morphology of microglia, changes of microglial complexity can be observed (Kettenmann et al., 2011). Previous studies investigating microglial changes following e.g. stress exposure are using sholl analyses to quantify the change of complexity based on intersections between microglia branches and radial circles surrounding each cell. To compare results obtained from the classification as described in **section 2.9.4.2**, sholl analyses were performed for the PrL and the NAcc shell **(Figure 26 A)** of both male and female prairie voles under basal conditions or after acute stress in form of the FST. Starting at a radius of 10 µm, concentric circles with an inter-circle distance of 4 µm were outlined encircling the center of a microglial cell and intersections with each circle were counted in an area covering 42 µm from the center of each cell **(Figure 26 B)**. Detailed statistical descriptions can be found in **Table 26** and **Table 27**.

Separation from a partner did not affect the number of intersections and therefore the complexity of microglial cells in the PrL of male prairie voles (**Figure 26 C**). However, acute stress significantly affected the complexity independent of separation. A mixed models two-way ANOVA revealed no interaction between circles and housing condition, an interaction between circles and acute stress ($F(3.043,60.866)=2.957$, $p=0.039$, $\eta^2=0.129$), and no interaction between circles, housing condition and acute stress. Bonferroni-corrected *post hoc* tests revealed that acute stress tended to increase microglia complexity 30 from the center ($p=0.057$) and significantly increased complexity 34 µm from the center of the microglia cells ($p=0.034$). Within the NAcc of male prairie voles, no effect on cell complexity of either separation or acute stress was observed (**Figure 26 D**). Separation from a partner and acute did not affect microglia the number of intersections within the PrL and the NAcc shell of female prairie voles (**Figure 26 E,F).**

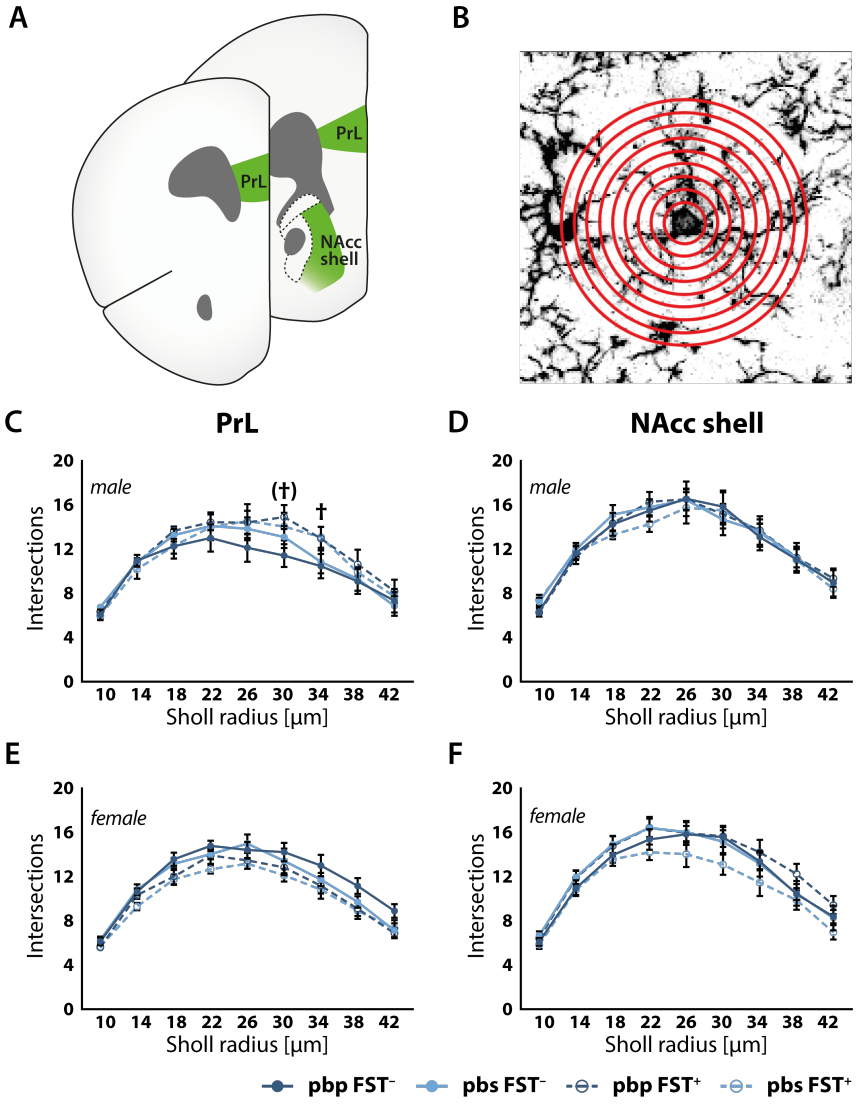

Figure 26 | Microglia complexity following separation from a partner and acute stress.
(A) Assessed regions were the PrL and the NAcc shell as highlighted in green. **(B)** Microglia with sholl analysis circles. The inner circle has a diameter of 10 µm, the distance between circles was set to 4 µm. Intersections between microglia processes and circles were analyzed. **(C)** Acute stress led

Figure 26 | Continued.
to an increase of microglia complexity while separation from a partner had no effect. **(D)** In the NAcc of male prairie voles, neither separation nor acute stress affected microglia complexity. **(E)** Separation from a partner and acute stress did not affect microglia complexity within the PrL of female prairie voles. **(F)** No effect of separation and acute stress on microglia complexity was observed in the NAcc shell of female prairie voles. The data is presented as line plot with mean ± SEM. All groups n=6. Statistical significance for the *post hoc* tests of the interaction between circle intersections and housing condition is indicated above the respective dot plots with (*) p<0.07, * p<0.05, ** p<0.01, *** p<0.001. Statistical significance for the *post hoc* tests of the interaction between circle intersections and acute stress is indicated above the respective dot plots with (†) p<0.07, † p<0.05, † † p<0.01, † † † p<0.001.

Table 26 | Mixed models ANOVA of microglia Sholl analysis of the PrL and NAcc shell of male and female prairie voles.

Region	Sex	*N*	Main effect: Circle*Separation	Main effect: Circle*Stress	Interaction
PrL	M	24	F(3.043,60.866)=0.611, p=0.613, η^2=0.030	F(3.043,60.866)=2.975, p=0.039, η^2=0.129	F(3.043,60.866)=0.608, p=0.615, η^2=0.029
	F	24	F(3.707,74.150)=0.690, p=0.591, η^2=0.033	F(3.707,74.150)=0.395, p=0.797, η^2=0.019	F(3.707,74.150)=1.199, p=0.318, η^2=0.057
NAcc shell	M	24	F(3.194,63.880)=0.856, p=0.320, η^2=0.041	F(3.194,63.880)=0.320, p=0.823, η^2=0.016	F(3.194,63.880)=0.929, p=0.437, η^2=0.044
	F	24	F(2.925,58.492)=1.102, p=0.355, η^2=0.052	F(2.925,58.492)=0.688, p=0.559, η^2=0.033	F(2.925,58.492)=0.229, p=0.871, η^2=0.011

Table 27 | Bonferroni-corrected *post hoc* tests of the interaction between Circle*Stress in the PrL of male prairie voles.

Post hoc	Circle	*p*
FST $^-$ vs. FST $^+$	1	0.450
	2	0.485
	3	0.783
	4	0.566
	5	0.247
	6	0.057
	7	0.034
	8	0.310
	9	0.349

3.2.3.2 Skeleton analysis

Cell complexity of microglia is closely linked o the ramification of their branches and branch number (Kettenmann et al., 2011). During the activation of microglia cells, cell complexity is decreasing. Therefore, analyzing complexity of microglial cells can give insight into changes of microglial activation. To compare the results obtained with previously described techniques, a skeleton analysis was performed which is quantifying the number of endpoints and the branch length. Separation from a partner and acute stress affected the number of endpoints in a sex- and

region-specific manner. Detailed statistical analyses can be found in **Table 28** and **Table 29** and in the appendix **Table 40** (endpoints) and **Table 41** (branch length).

In the PrL of male voles, separation and acute stress did not affect the number of endpoints (**Figure 27 C**). Similarly, neither separation nor acute stress induced changes to the number of endpoints in the NAcc shell of male prairie voles (**Figure 27 D**). When quantifying the number of endpoints of microglia cells in the PrL of female prairie voles, separation did significantly reduce the number of endpoints. Additionally, acute stress tended to also decrease the number of endpoints. Two-way ANOVA revealed a main effect of housing condition ($F(3,20)=5.746$, $p=0.026$, $\eta^2=0.223$), a trend towards a main effect of acute stress ($F(3,20)=3.795$, $p=0.066$, $\eta^2=0.159$), and no interaction between housing condition and acute stress (**Figure 27 E**). In the NAcc shell of female prairie voles, separation affected the number of endpoints. The number of endpoints was significantly reduced following separation from a partner as revealed by a two-way ANOVA with a main effect of housing condition ($F(3,20)=6.298$, $p=0.021$, $\eta^2=0.239$, Bonferroni-corrected *post hoc* tests: FST^- $p=0.021$), no main effect of acute stress, and no interaction between housing condition and acute stress (**Figure 27 F**). Furthermore, the branch length per microglial cell was quantified. Here, separation and acute stress did also affect microglia branch length in a sex- and region-specific manner. In the PrL of male prairie voles, neither separation nor acute stress significantly affected the observed length of microglia branches (**Figure 28 A**). This was also not apparent within the NAcc shell of male voles (**Figure 28 B**). Neither separation nor acute stress affected microglia branch length in the PrL of female prairie voles. A two-way ANOVA revealed no main effect of housing condition, no main effect of acute stress, and no interaction between housing condition and acute stress (**Figure 28 C**). However, planned comparisons showed a trend towards a decrease of branch length following separation ($t(10)=-2.097$, $p=0.062$, $d=1.21$) under basal conditions and a decrease of branch length following acute stress in *pbp* voles ($t(10)=4.916$, $p<0.001$, $d=0.260$). Within the NAcc shell, however, separation from a partner caused differences in the length of microglial branches in female prairie voles (**Figure 28 D**). A two-way ANOVA revealed a main effect of housing condition ($F(3,20)=4.627$, $p=0.044$, $\eta^2=0.188$), no main effect of acute stress, and a trend towards an interaction between housing condition and acute stress ($F(3,20)=3.726$, $p=0.068$, $\eta^2=0.157$). Bonferroni-corrected *post hoc* tests revealed that separation from a partner significantly reduced microglia branch length under basal conditions ($p=0.009$) and acute stress reduced microglia branch length in the NAcc shell of *pbp* females ($p=0.039$).

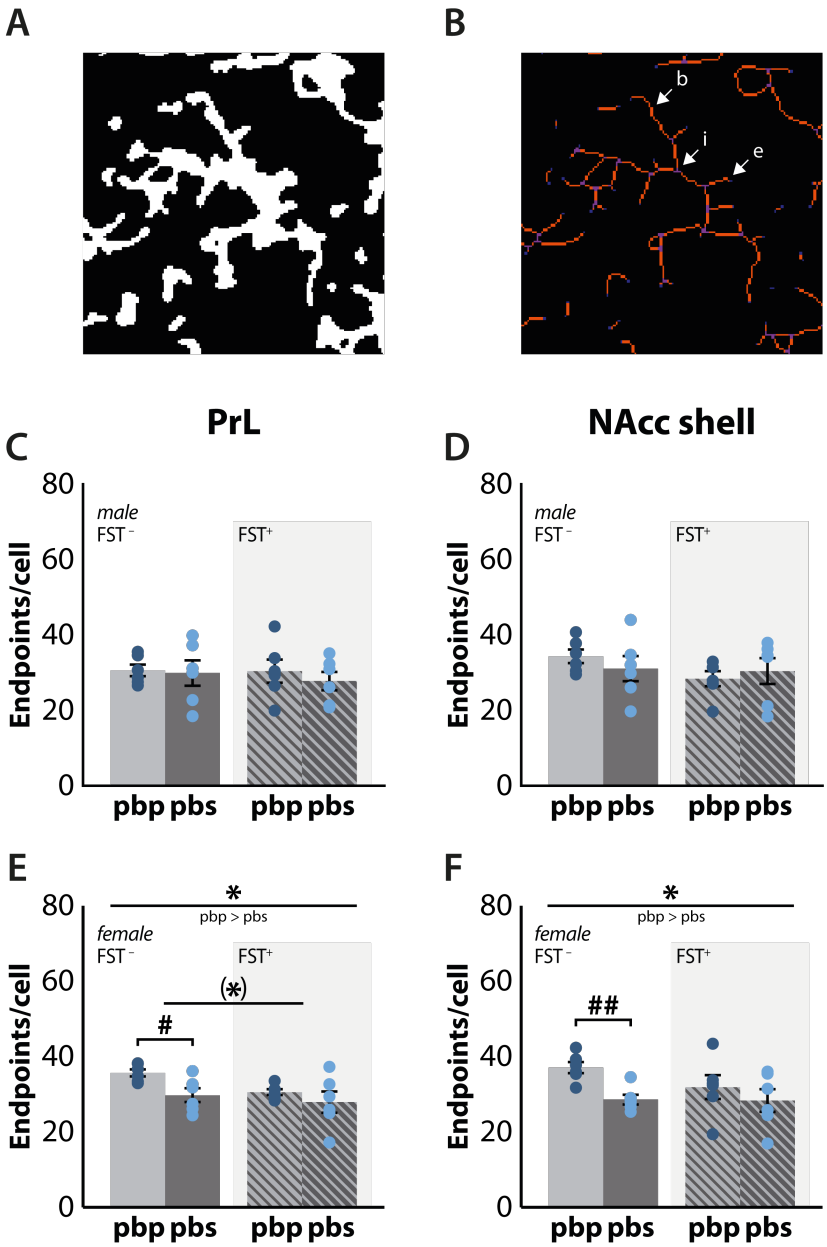

Figure 27 | Endpoints, skeleton analysis of microglia in male and female prairie voles.
(A) Microglia cell following thresholding. **(B)** Skeletonized microglial cell. Branches (b) are marked in red; intersections (i) are marked in blue; endpoints (e) are marked in purple. **(C)** Neither separation from a partner nor acute stress influenced the number of endpoints in the PrL of male voles. **(E)** Separation from partner caused differences in the number of endpoints in the PrL of female prairie voles. **(F)** Separation from a partner caused a decrease of endpoints per microglia cell in the NAcc shell of female voles. The data is presented as bar charts with aligned dot plots with mean ± SEM. All groups n=6. The main effect of housing condition is indicated above the horizontal line with the respective *post hoc* statistics indicated underneath the horizontal line as ≈ $p \geq 0.7$, (*) $p<0.07$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$). Statistical significance for the main effect of acute stress and *post hoc* statistics for the interaction are indicated above the respective bars with (*) $p<0.07$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Statistics for planned comparisons are indicated above the respective bars with (#) $p<0.07$, # $p<0.05$, ## $p<0.01$, ### $p<0.001$.

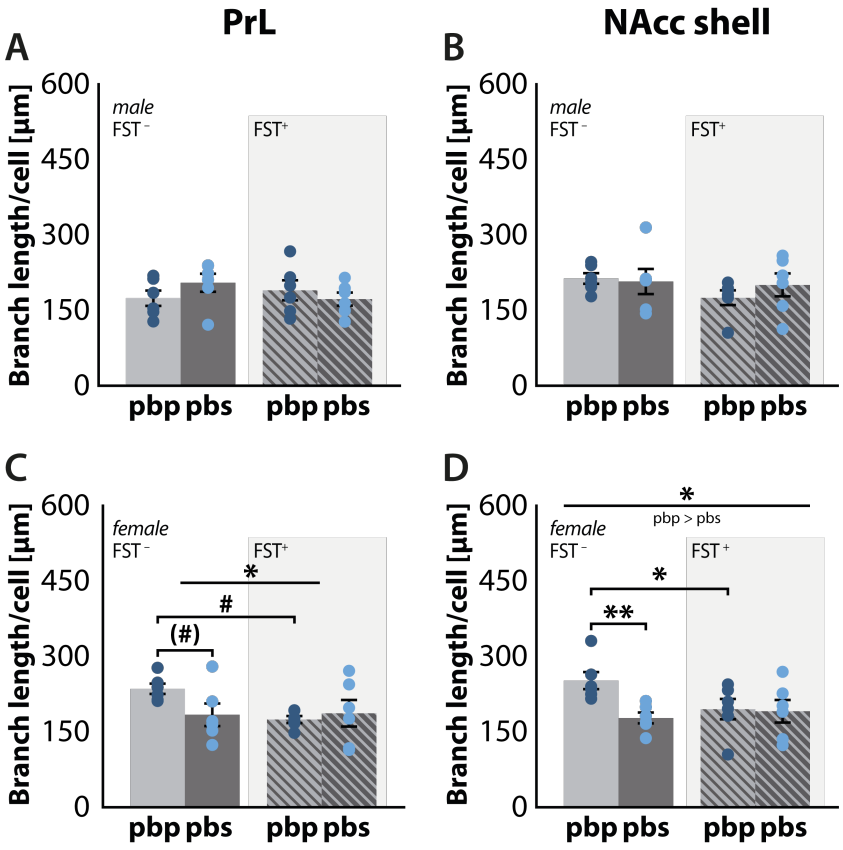

Figure 28 | Branch length per microglial cell in the PrL and NAcc shell.
Microglia branch length following separation from a partner and acute stress was assessed in **(A,C)** the PrL and **(B,D)** the NAcc shell of **(A,B)** male and **(C,D)** female prairie voles. **(A)** Separation or acute stress did not affect microglia branch length within the PrL of male prairie voles. **(B)** Likewise, separation from a partner and acute stress did not affect branch length within the NAcc shell of male prairie voles. **(C)** Separation from a partner tended to, and acute stress reduced microglia branch length in the PrL of female voles following separation and acute stress, respectively. **(D)** Separation from a partner caused differences in the length of microglial branches in the NAcc shell of female voles. The data is presented as bar charts with aligned dot plots with mean ± SEM. All groups n=6. The main effect of housing condition is indicated above the horizontal line with the respective *post hoc* statistics indicated underneath the horizontal line as ≈ p≥0.7, (*) p<0.07, * p<0.05, ** p<0.01, *** p<0.001). Statistical significance for the main effect of acute stress and *post hoc* statistics for the interaction are indicated above the respective bars with (*) p<0.07, * p<0.05, ** p<0.01, *** p<0.001. Statistics for planned comparisons are indicated above the respective bars with (#) p<0.07, # p<0.05, ## p<0.01, ### p<0.001.

Table 28 | Two-way ANOVA for microglial endpoints within the PrL and NAcc shell.

Region	Sex	*N*	Main effect: Housing	Main effect: Stress	Interaction
PrL	M	24	$F(3,20)=0.374$, $p=0.548$, $\eta^2=0.18$	$F(3,20)=0.188$, $p=0.670$, $\eta^2=0.009$	$F(3,20)=0.130$, $p=0.722$, $\eta^2=0.006$
	F	24	**$F(3,20)=5.746$, $p=0.026$, $\eta^2=0.223$**	**$F(3,20)=3.795$, $p=0.066$, $\eta^2=0.159$**	$F(3,20)=0.842$, $p=0.370$, $\eta^2=0.040$
NAcc shell	M	24	$F(3,20)=0.050$, $p=0.825$, $\eta^2=0.003$	$F(3,20)=1.430$, $p=0.246$, $\eta^2=0.067$	$F(3,20)=0.934$, $p=0.345$, $\eta^2=0.045$
	F	24	**$F(3,20)=6.298$, $p=0.021$, $\eta^2=0.239$**	$F(3,20)=1.288$, $p=0.270$, $\eta^2=0.060$	$F(3,20)=1.070$, $p=0.313$, $\eta^2=0.051$

Table 29 | Two-way ANOVA of microglial branch length measures from the PrL and NAcc shell.

Region	Sex	*N*	Main effect: Housing	Main effect: Stress	Interaction	*Post hoc* tests	
PrL	M	24	$F(3,20)=0.158$, $p=0.695$, $\eta^2=0.008$	$F(3,20)=0.247$, $p=0.625$, $\eta^2=0.012$	$F(3,20)=2.066$, $p=0.166$, $\eta^2=0.094$		
	F	24	$F(3,20)=1.147$, $p=0.297$, $\eta^2=0.054$	$F(3,20)=2.487$, $p=0.130$, $\eta^2=0.111$	$F(3,20)=3.028$, $p=0.097$, $\eta^2=0.131$		
NAcc shell	M	24	$F(3,20)=0.261$, $p=0.615$, $\eta^2=0.013$	$F(3,20)=1.400$, $p=0.251$, $\eta^2=0.650$	$F(3,20)=0.668$, $p=0.423$, $\eta^2=0.032$		
	F	24	$F(3,20)=4.627$, $p=0.044$, $\eta^2=0.188$	$F(3,20)=1.415$, $p=0.248$, $\eta^2=0.066$	$F(3,20)=3.726$, $p=0.068$, $\eta^2=0.157$	FST $^-$ FST $^+$ pbp pbs	$p=0.009$ $p=0.878$ $p=0.039$ $p=0.606$

3.2.3.3 Expression of microglia- and stress-related genes

qPCR was performed to assess the impact of separation on microglia-related genes that are altered and involved in the morphological and functional changes of microglia following stress in other rodent models. The assessed genes were chosen based on studies in other model organisms that show a change in expression following stress exposure and are involved in the functional role of microglia (Bollinger et al., 2016; Häusl et al., 2019; Holtman et al., 2015; Wohleb et al., 2011). The investigated regions included the PrL, NAcc shell, and PVN. Target genes were both *CX3CL1* and its receptor *CX3CR1*, as well as *LGALS3*, and *FKBP51*. Detailed statistics for the pairwise comparisons of gene expression can be found in the appendix **Table 42**.

In the PrL of male and female voles, no difference in gene expression was observed following separation from a partner (**Figure 29 A,B**). Also, separation from a partner did not alter the expression of all assessed target genes within the NAcc shell of male and female voles (**Figure 30 A,B**). Similarly, separation did not significantly affect the expression of the analyzed target genes in the PVN of male and female voles **(Figure 31 A,B)**.

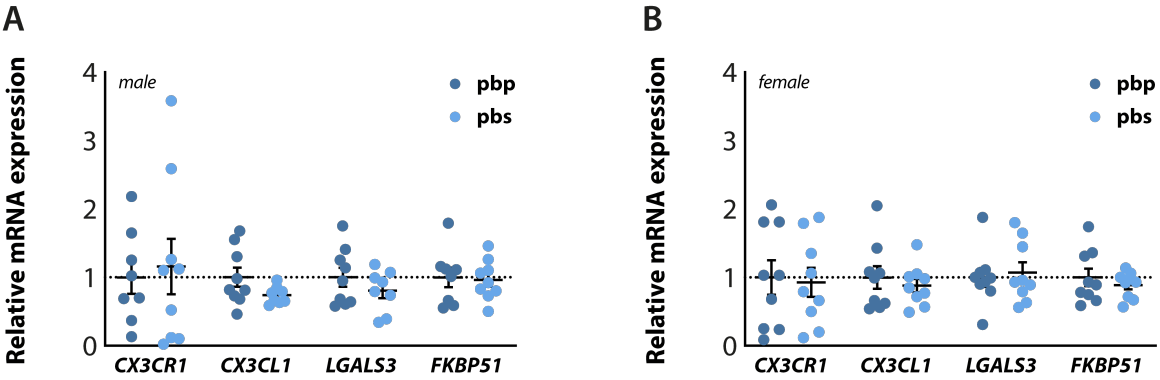

Figure 29 | Relative gene expression in the PrL following the separation from a partner. **(A)** In male prairie voles, partner loss did not affect the expression of *CX3CR1*, *CX3CL1*, *LGALS3*, and *FKBP51*. **(B)** In female prairie voles, separation from a partner did not affect gene expression of *CX3CR1*, *CX3CL1*, *LGALS3*, and *FKBP51*. The data is presented as scatter dot plots with mean ± SEM. All groups n=8-9. Statistical significances for independent two-samples tests are indicated above the respective bars with (#) $p<0.07$, # $p<0.05$, ## $p<0.01$, ### $p<0.001$.

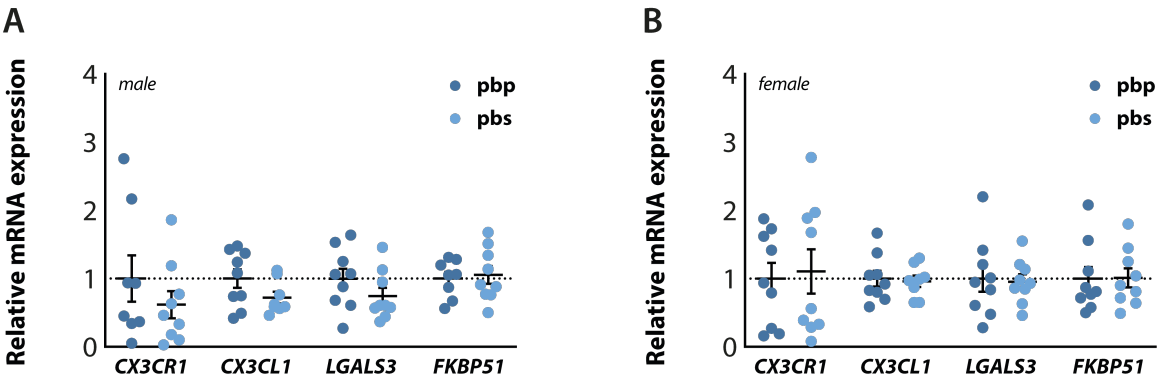

Figure 30 | Relative gene expression in the NAcc shell following separation from a partner. **(A)** Partner loss had no impact on the expression of *CX3CR1*, *CX3CL1*, *LGALS3*, and *FKBP51* of male voles. **(B)** Similarly, in females, separation from a partner did not affect gene expression of *CX3CR1*, *CX3CL1*, *LGALS3*, and *FKBP51*. The data is presented as scatter dot plots with mean ± SEM. All groups n=8-9. Statistical significances for independent two-sample tests are indicated above the respective bars with (#) p<0.07, # p<0.05, ## p<0.01, ### p<0.001.

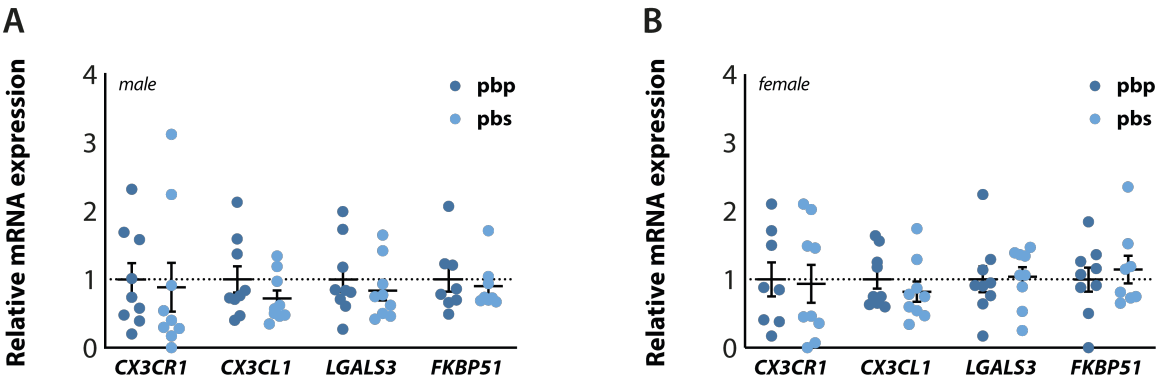

Figure 31 | Relative gene expression in the PVN following separation from a partner. **(A)** Within the PVN of male prairie voles, partner loss did not affect the expression of *CX3CR1*, *CX3CL1*, *LGALS3*, and *FKBP51*. **(B)** In females, separation from a partner did not affect gene expression of *CX3CR1*, *CX3CL1*, *LGALS3*, and *FKBP51*. The data is presented as scatter dot plots with mean ± SEM. All groups n=8-9. Statistical significances for independent two-sample tests are indicated above the respective bars with (#) p<0.07, # p<0.05, ## p<0.01, ### p<0.001.

3.2.4 Measurement of neuronal activation following partner loss

3.2.4.1 Quantification of c-Fos

Microglia are sensitive to neuronal activity and can respond by regulating synaptic activity (York, Bernier, & MacVicar, 2018). Neuronal activation can also be also linked to changes of the release of neurotransmitters and hormones in specific regions. Microglia are sensitive to neurotransmitter and hormone changes and previous studies showed a link between neural activity and microglia morphology and function (Liu et al., 2019). Therefore, it was hypothesized that partner loss might change neuronal activity and that this might be linked to changes of microglia morphology. The PrL, NAcc shell, and PVN were assessed for neuronal activity analyzing the immediate early gene *c-Fos*. Detailed statistical analyses for the ANOVA are presented in **Table 30** and appendix **Table 43** (subregions of the PVN, data not illustrated) and planned comparisons can be found in appendix **Table 44** and **Table 45**.

Separation from a partner caused significant changes in neuronal activity within the PrL of male voles under basal conditions (**Figure 32 A**). Two-way ANOVA revealed no main effect of separation and acute stress, but a trend towards an interaction between housing condition and acute stress ($F(1,18)=3.928$, $p=0.063$, $\eta^2=0.179$). Previously planned comparisons revealed that, under basal conditions, *pbs* males had significantly higher abundance of c-Fos$^+$ cells ($p=0.036$). Separation from a partner, independent of acute stress, did not affect neuronal activation in the NAcc shell of male prairie voles (**Figure 32 B**). In the PVN of male prairie voles, separation had no effect on neuronal activation, however, acute stress increased c-Fos expression (**Figure 32 C**). A two-way ANOVA revealed no main effect of housing condition, a main effect of acute stress ($F(1,20)=10.388$, $p=0.004$, $\eta^2=0.342$), and no interaction between housing condition and acute stress. No effects of separation or acute stress were observed in the PrL of female voles, which was also apparent within the NAcc shell (**Figure 32 D,E**). While separation had no effect on neuronal activation within the PVN of female voles, acute stress caused a significant change in neuronal activity. Two-way ANOVA revealed no main effect of housing condition, a main effect of acute stress ($F(1,20)=62.320$, $p<0.001$, $\eta^2=0.757$), and no interaction between housing condition and acute stress (**Figure 32 F**).

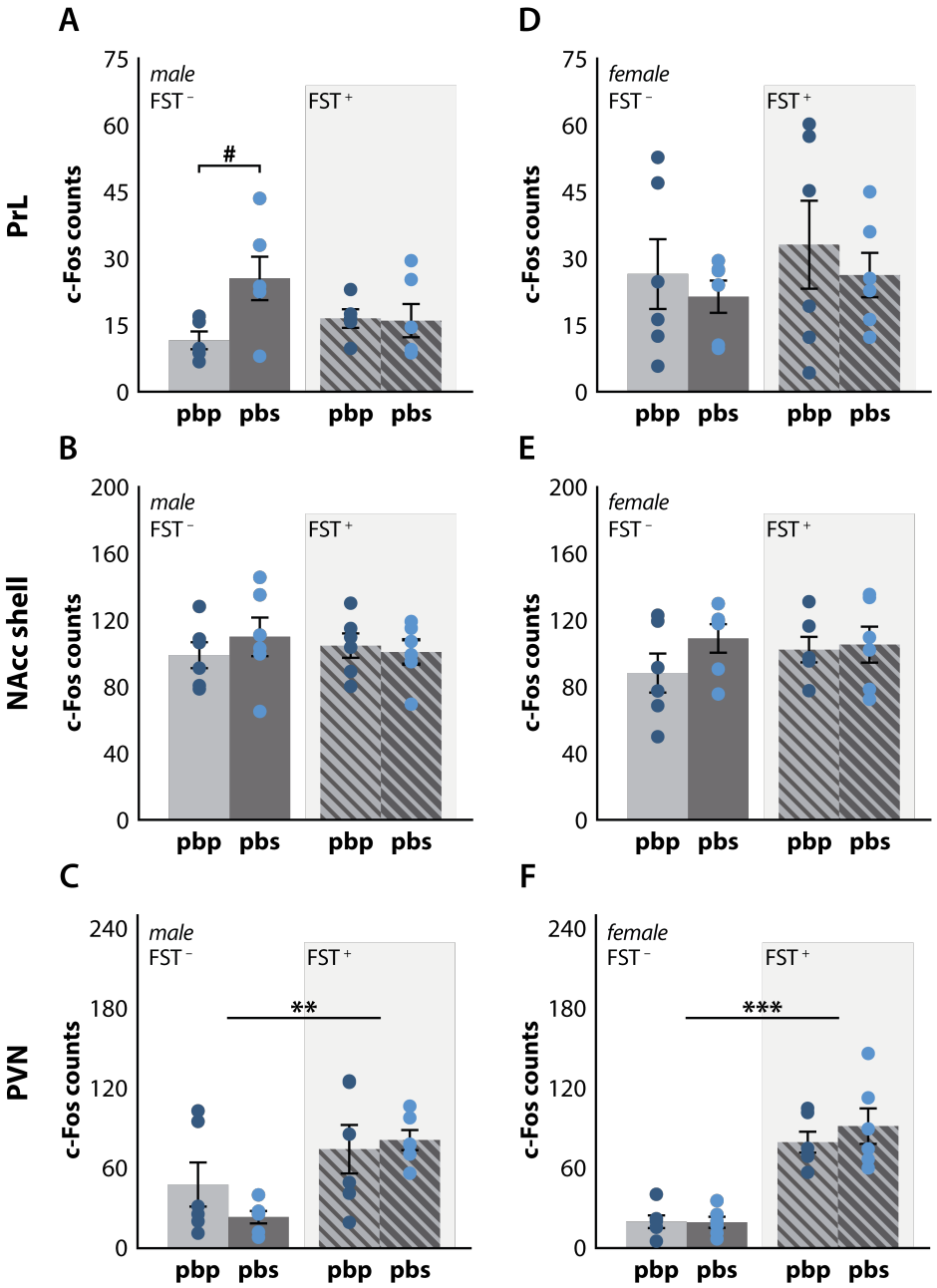

Figure 32 | Quantification of c-FOS⁺ cells.
Neuronal activation within (**A,D**) the PrL, (**B,E**) the NAcc shell, and (**C,F**) the PVN was assessed following separation from a partner in both (**A-C**) male and (**D-F**) female prairie voles. **(A)** In the PrL of male prairie voles, separation from a partner induced higher neural activity under basal conditions. way **(B)** Separation and acute stress did not affect neural activity in the NAcc shell of male voles. **(C)** Acute stress significantly affected the neural activation in the PVN of male voles. **(D)** Separation or acute stress did not affect neuronal activity in the PrL of female voles. **(E)** Neither separation, nor acute stress affected neuronal activation in the NAcc shell of female voles. **(F)** Acute stress but not separation affected neuronal activity in the PVN of female voles. The data is presented as bar charts with aligned dot plots with mean ± SEM. All groups n=5-6. The main effect of housing condition is indicated above the horizontal line with the respective *post hoc* statistics indicated underneath the horizontal line as ≈ $p \geq 0.7$, (*) $p<0.07$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$). Statistical significance for the main effect of acute stress and *post hoc* statistics for the interaction are indicated above the respective bars with (*) $p<0.07$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Statistics for planned comparisons are indicated above the respective bars with (#) $p<0.07$, # $p<0.05$, ## $p<0.01$, ### $p<0.001$.

Table 30 | Two-way ANOVAs for c-Fos counts following separation from a partner and acute stress.

Region	Sex	*N*	Main effect: Separation	Main effect: Stress	Interaction	*Post hoc* tests	
PrL	M	22	$F(1,18)=3.444$, $p=0.080$, $\eta^2=0.161$	$F(1,18)=0.401$, $p=0.535$, $\eta^2=0.022$	$F(1,18)=3.928$, $p=0.063$, $\eta^2=0.179$	FST $^-$ FST $^+$ pbp pbs	$p=0.014$ $p=0.930$ $p=0.373$ $p=0.068$
	F	24	$F(1,20)=0.718$, $p=0.407$, $\eta^2=0.035$	$F(1,20)=0.668$, $p=0.423$, $\eta^2=0.032$	$F(1,20)=0.015$, $p=0.902$, $\eta^2=0.001$		
NAcc shell	M	24	$F(1,20)=0.171$, $p=0.684$, $\eta^2=0.008$	$F(1,20)=0.039$, $p=0.845$, $\eta^2=0.002$	$F(1,20)=0.720$, $p=0.406$, $\eta^2=0.035$		
	F	24	$F(1,20)=1.442$, $p=0.244$, $\eta^2=0.067$	$F(1,20)=0.275$, $p=0.606$, $\eta^2=0.014$	$F(1,20)=0.819$, $p=0.376$, $\eta^2=0.039$		
PVN	M	24	$F(1,20)=0.453$, $p=0.508$, $\eta^2=0.022$	$F(1,20)=10.388$, $p=0.004$, $\eta^2=0.342$	$F(1,20)=1.429$, $p=0.246$, $\eta^2=0.067$		
	F	24	$F(1,20)=0.472$, $p=0.500$, $\eta^2=0.023$	$F(1,20)=62.320$, $p<0.001$, $\eta^2=0.757$	$F(1,20)=0.565$, $p=0.461$, $\eta^2=0.027$		

3.2.5 Measurement of peripheral immune system factors following separation from a partner

3.2.5.1 Plasma cytokine levels

Cytokine levels of pro- and anti-inflammatory cytokines were measured in plasma using Luminex multiplex cytokine detection. In male and female prairie voles, the measured levels of all cytokines (IL-10, IL-12p40, IL-6, IP-10, and TNF-α) were below the limit of detection (data not shown).

3.2.5.2 Bacterial killing assay

Since social stress and social isolation were shown to influence the activity of the peripheral immune system (Scotti et al., 2015; Takahashi, Flanigan, McEwen, & Russo, 2018), it was hypothesized that separation from a partner affects the functioning of the peripheral immune response. To assess the effects of separation and acute stress on the peripheral immune system, the capability of the peripheral immune system to clear the plasma from bacteria was measured using the bacterial killing assay. This assay is relying on the activity of the complement system found within the blood plasma and its capability to reduce the number of CFU. The ANOVA can be found in **Table 31** and the planned comparisons in appendix **Table 46**. In both male voles and female, neither separation nor acute stress affected the peripheral immune system's ability to inhibit *E. coli* growth (**Figure 33 A**).

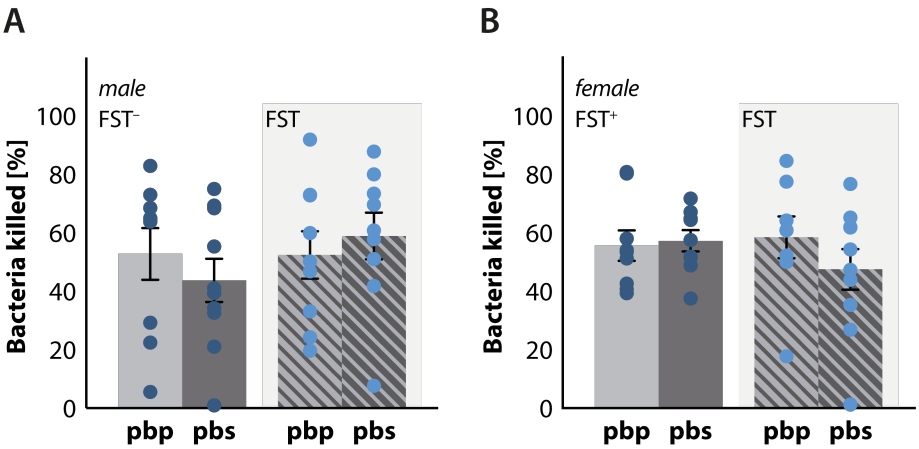

Figure 33 | Results from the bacterial killing test in male and female prairie voles.
Bacteria killing capacity following separation from a partner and acute stress was quantified in both **(A)** male and **(B)** female voles. **(A)** The peripheral immune system of male voles was not affected by separation from a partner and acute stress. **(B)** In female voles, neither separation from a partner nor acute stress had an impact on the peripheral immune response. The data is presented as bar charts with aligned dot plots with mean ± SEM. All groups n=8-10. Main effects of housing condition and acute stress are indicated above the horizontal line with the respective *post hoc* statistics indicated underneath the horizontal line as ≈ p≥0.7, (*) p<0.07, * p<0.05, ** p<0.01, *** p<0.001). Statistical significance for the *post hoc* tests of housing condition and acute stress is indicated above the respective bars with (*) p<0.07, * p<0.05, ** p<0.01, *** p<0.001. Statistical significances for planned comparisons are indicated above the respective bars with (#) p<0.07, # p<0.05, ## p<0.01, ### p<0.001.

Table 31 | Two-way ANOVAs for the bacterial killing test following separation from a partner and acute stress.

Sex	*N*	Main effect: Separation	Main effect: Stress	Interaction
M	37	$F(1,33)=0.025$, $p=0.876$, $\eta^2=0.001$	$F(1,33)=0.882$, $p=0.368$, $\eta^2=0.025$	$F(1,33)=0.921$, $p=0.344$, $\eta^2=0.027$
F	36	$F(1,32)=0.623$, $p=0.436$, $\eta^2=0.190$	$F(1,32)=0.338$, $p=0.565$, $\eta^2=0.100$	$F(1,32)=1.128$, $p=0.294$, $\eta^2=0.034$

3.2.5.3 Splenic immune response

Since the spleen is the largest secondary immune organ (Bronte & Pittet, 2013), and spleen hypertrophy was observed following social stress in mice (Azzinnari et al., 2014), the spleen weight was assessed to measure the potential influence of separation from a partner on the peripheral immune system. In male and female voles, separation from a partner did not affect relative spleen weight on the fourth day following partner loss (**Figure 34 A,B**).

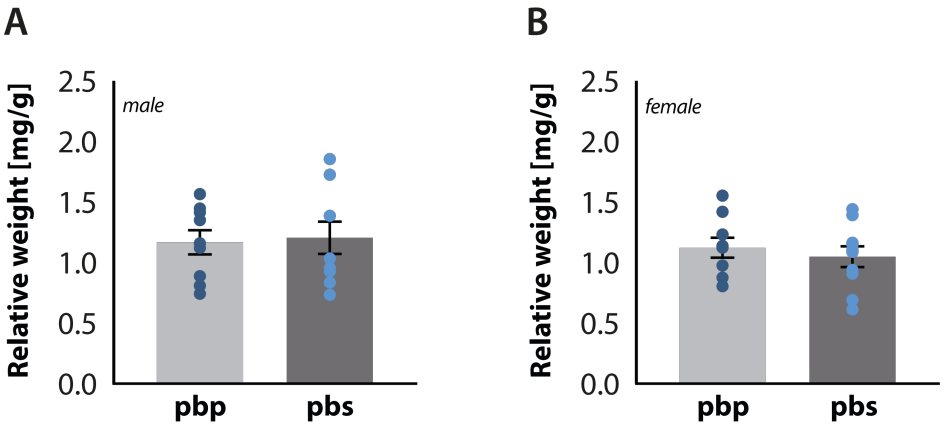

Figure 34 | Relative spleen weight following separation from a partner.
The relative spleen weight was assessed in **(A)** male and **(B)** female prairie voles. **(A)** Separation from a partner did not alter the relative spleen weight in male prairie voles. **(B)** No changes in relative spleen weight were observed in separated female prairie voles. The data is presented as bar charts with aligned dot plots with mean ± SEM. All groups n=9-10. Statistical significances for the independent two-sample t-tests are indicated above the respective bars with (*) p<0.07, * p<0.05, ** p<0.01, *** p<0.001.

Table 32 | Pairwise comparisons of relative spleen weight of male and female prairie voles.

Sex	Treatment	*n*	*M*	*SD*	*df*	*t*	*p*	Effect Size
M	pbp	9	1.167	0.300	16	-0.228	0.822	0.107[a]
	pbs	9	1.205	0.401				
F	pbp	9	1.122	0.248	17	0.617	0.545	0.283[b]
	pbs	10	1.049	0.268				

[a] = Cohen's *d*, [b] = Hedge's *g*

Discussion

4 Discussion

The loss of partner is a far-reaching event that can have detrimental effects on both mental and physical health (Barger, 2013; Cacioppo & Hawkley, 2003; Carey et al., 2014; Gerra et al., 2003; Goforth et al., 2009; Holt-Lunstad & Smith, 2016; Shear & Shair, 2005; Uchino, 2006). As described in the previous sections, the prairie vole has proven useful for elucidating the effects of partner loss on the dysregulations of neuropeptide systems and behavior. Both, the OT and the CRF system are dysregulated following the loss of partner, two systems which are important for the regulation of the stress response and behavior (Bosch et al., 2009; Pohl et al., 2019). Following partner loss, manipulations of the OT system reverse the negative outcomes on stress-coping in prairie voles (Bosch et al., 2016; Bosch et al., 2009). Since the OT release within the NAcc shell is decreased following partner loss, the first part of this study aimed to rescue partner loss-induced stress-coping by reversing the dysregulated release of OT following the separation from a partner.

Moreover, dysregulations of the stress response and neuropeptide systems can impact not only stress-coping behavior, but also the immune response (Slavich & Irwin, 2014; Yirmiya, Rimmerman, & Reshef, 2015). Interestingly, altered immune responses are related to the susceptibility for the development of psychiatric disorders and behavioral changes (Yirmiya et al., 2015). In humans, alterations of the peripheral immune system are present in grieving individuals (Fagundes et al., 2019; Schultze-Florey et al., 2012). However, the impact of the loss of a partner on the neuroinflammatory response has yet to be investigated. Interestingly, complicated grief is heightening the susceptibility for the development of MDD (Sung et al., 2011). Strikingly, MDD is correlated with altered neuro- and peripheral inflammatory responses at basal levels and altered responses towards stressor exposure (Shelton et al., 2011; Torres-Platas, Cruceanu, et al., 2014; Vogelzangs et al., 2012). Therefore, the second study aimed to assess the impact of the dysregulation of neuropeptide systems and stress response on the activation of the neuro- and peripheral immune system and its correlations with altered neuronal activation in brain regions that are important for emotio-behavioral processing.

4.1 The impact of MTII treatment on negative effects of partner loss on the emotio-behavioral response

To rescue the negative effects of the separation from a partner, the first study aimed to elucidate the effects of MTII treatment in conjunction with social interaction on the prairie voles' stress response, emotionality, and social behavior. For the investigation of the stress response, the body weight was tracked throughout the experiment. Furthermore, basal CORT levels and adrenal gland weight were assessed. Because altered emotionality is observed following the loss of a partner (Bosch et al., 2009), passive stress-coping and anxiety-like behavior were assessed in the FST and EPM. Furthermore,

socio-behavioral responses were analyzed on the first day of separation following the onset of the treatment administration and on the last day of treatment to elucidate the acute and chronic effects of MTII treatment on relevant social and non-social behaviors.

4.1.1 MTII treatment with social interaction did not affect physiological and stress-related parameters

Prior research demonstrates that MTII treatment initially has anorexic effects in male rats as it reduces the food intake and increases energy expenditure (van den Heuvel et al., 2015). Similarly, treatment of neonatal male and female prairie voles with daily injections of MTII between postnatal day (PND) 1-7 significantly reduces body weight between PND 2-7 (Barrett et al., 2014). This effect is not observed on PND 21 in the absence of further MTII administration from PND 7 on. Furthermore, the acutely anorexic effect of MTII treatment is attenuated following chronic MTII administration in rats (Raposinho, White, & Aubert, 2003), which is in line with the results in this study following 8 days of MTII treatment in male prairie voles. Here, MTII treatment led to a significant reduction in body weight. In separated male prairie voles, body weight was reduced following 3 days of treatment with MTII compared to VEH-treated pbp and pbs males. Following chronic administration of MTII, no difference in body weight was observed in male prairie voles, which might be due to altered food intake and physiological adaptations.

In female prairie voles, MTII treatment following separation significantly decreased body weight compared to paired VEH-treated females. VEH treatment had no effect on body weight following the separation from a partner. However, the comparisons between separated females receiving either VEH or MTII are mixed. Three days of MTII treatment significantly reduced body weight in separated females with social interaction which is in accordance with the effects observed in males. Treatment with MTII and no social interaction, however, did not significantly affect body weight in comparison to *pbs VEH*-treated females. No effect of social interaction was observed within the MTII treatment groups. It is therefore unclear why MTII treatment without social interaction did not evoke changes in body weight in female prairie voles. On the 8th day of separation, MTII treatment did not affect body weight compared to *pbs VEH*-treated females. This is in line with previously reported effects of chronic MTII treatment on the attenuation of body weight.

4.1.2 MTII treatment with social interaction did not affect endocrine parameters of the stress response

Previous studies report an increase in the relative adrenal gland weight following separation from a partner in male prairie voles (Bosch et al., 2009). MTII treatment and separation from a partner had no effect on relative adrenal gland weight in male and female prairie voles, indicated by the absence of adrenal hypertrophy in both treatment groups. However, an increase in the relative adrenal gland weight was observed in separated males treated with MTII and no social interaction compared to

paired VEH males. This might be due to higher basal activity of the HPA axis in the absence of a social stimulus following MTII treatment. It is moreover possible, that the time point at which the adrenal glands were analyzed might explain the discrepancy between the two studies since the previous study analyzed adrenal gland weight after 5 days of separation from a partner (Bosch et al., 2009). Furthermore, a confounding factor that might have led to differential results in the present study is the route of treatment administration. Daily *i.p.* injections potentially posed as a stressor that affected the adrenal gland weight. The lack of alterations of the adrenal gland weight, therefore, might have been due to a roof effect, since brief restraint and *i.p.* injections can initiate the stress response in mice (Meijer, Spruijt, van Zutphen, & Baumans, 2006). Therefore, a different route of treatment application could prove itself useful to exclude injection-induced stress responses.

4.1.3 Emotionality was not altered by MTII administration with social interaction

Separation from a partner induces an increase in passive stress-coping and anxiety-like behavior in male and female prairie voles (Bosch et al., 2009; Bosch et al., 2018; McNeal et al., 2014; Sun et al., 2014). This is due to an increase of CRFR2 signaling. Agonistic binding of endogenous ligands to CRFR2 located on OT^+ neurons is leading to a reduction of the expression of OT mRNA within the PVN and inhibition of OT release in the NAcc shell. Blocking CRFR2 activation within the NAcc shell by infusion of CRFR2 antagonist increases the release of OT within the NAcc shell which is coupled to a reduction of passive stress-coping in the FST (Bosch et al., 2016). It was therefore hypothesized that an increase of endogenous OT release might counter the negative effects of partner loss.

However, *i.p.* treatment with MTII, which potentiates the release of endogenous OT within the NAcc shell during social interaction, did not affect passive stress-coping of male and female prairie voles. This is most likely due to the absence of increased passive stress-coping following partner loss. However, increased passive stress-coping is reported between 4 days up to 4 weeks following partner loss (Bosch et al., 2016; Bosch et al., 2009; McNeal et al., 2014; Sun et al., 2014).

Furthermore, treatment with MTII and separation from a partner with social interaction did not affect anxiety-like behaviors in the EPM. Moreover, separation from a partner did not increase anxiety-like behavior in VEH-treated male and female prairie voles. In contrast, an increase of anxiety-like behavior following partner loss is reported at different time points in male and female prairie voles (Bosch et al., 2009; Bosch et al., 2018; Sun et al., 2014). Therefore, the presented results are not in agreement with the current literature. This is unlikely due to the time point of the assessment of emotionality in the present study.

It is of importance to mention that both the FST and the EPM are sensitive to prior stressor exposure. As previously stated, the treatment administration in general, independent of either VEH

or MTII, might have posed as a chronic stressor, since *i.p.* injections and acute restraint pose as a stressful experience in mice (Meijer et al., 2006). Chronic stressors differentially affect behavioral parameters in the FST. This is exemplified by chronic restraint and chronic foot shocks evoking depressogenic effects in rats (Consoli, Fedotova, Micale, Sapronov, & Drago, 2005; Suvrathan, Tomar, & Chattarji, 2010) or antidepressant-like effects in mice (Swiergiel, Leskov, & Dunn, 2008; Swiergiel, Zhou, & Dunn, 2007), or no effect (Suvrathan et al., 2010). Similar observations are reported following *i.p.* injections of saline in mice, marked by increased anxiety-like behavior in the EPM (Lapin, 1995). Therefore, daily stress exposure potentially reduced the passive stress-coping behavior, normally observed in prairie voles following the loss of a partner. Accordingly, future studies assessing stress-sensitive parameters should limit the impact of treatment administration on the stress system.

4.1.4 MTII treatment differentially affected social- and non-social behaviors

MTII treatment had no effect on prosocial and affiliative behaviors

MTII is an MC3R and MC4R agonist which enhances the formation of a partner preference in male and female prairie voles (Modi et al., 2015). This effect is modulated by the MTII-induced potentiated release of OT within the NAcc shell during social interactions.

Overall, treatment with MTII did not alter active prosocial behaviors in male and female prairie voles towards an opposite sex stranger in comparison to pair-housed and separated prairie voles receiving VEH treatment. This effect is potentially due to the reduction of prosocial behaviors of pair-housed male and female prairie voles following 8 days of VEH treatment in the present study. On the first day of treatment with either VEH or MTII, separated prairie voles showed less affiliative behaviors compared to non-separated partners, independent of sex. However, no differences between the groups were observed following 8 days of daily injections, regardless of the treatment and sex. This is due to a significant reduction of affiliative behaviors in pair-housed prairie voles. Cohabitating with a partner was shown to be protective against the effect of acute stress exposure (Burkett et al., 2016). Unstressed partners show increased partner-directed prosocial behaviors such as allogrooming towards a stressed partner, indicative of consoling behavior (Burkett et al., 2016). Indeed, affiliative behaviors following acute stress exposure might be a reaction of stress-coping in prairie voles (Blondel & Phelps, 2016) and humans (Taylor, 2006). In the present study, however, both partners were briefly handled and received daily *i.p.* injections for 8 days. It is possible, that repeated exposure towards injection-induced stress of both partners affected the occurrence of affiliative behaviors following the exposure to a stressor. Male and female prairie voles show differential socio-behavioral responses to stress at least in the context of partner preference formation (Curtis & Wang, 2005; DeVries et al., 1995; DeVries et al., 1996). Successively, it remains unclear if the reduction of affiliative behaviors is mutual, or if the reduction stems from the

behavioral response of one partner. All in all, treatment with MTII did not alter prosocial and affiliative behaviors towards an opposite sex stranger following partner loss, and chronic stress might negatively impact social behaviors between two partners or the maintenance of a pair bond.

Aggression was not altered by MTII treatment

Pair bonded prairie voles display selective aggression even towards receptive female strangers (Gobrogge, Liu, Jia, & Wang, 2007). Lower levels of aggression towards a female stranger prairie vole are observed in males after diminishing the bond to their partner following 4 weeks of separation. This behavior is reminiscent of sexually naïve prairie voles prior to pair bond formation (Gobrogge et al., 2007; Gobrogge, Liu, Young, & Wang, 2009; Gobrogge & Wang, 2011; Shapiro & Dewsbury, 1990; Sun et al., 2014).

The levels of displayed aggression were stable throughout the experiment, independent of treatment with either MTII or VEH. Surprisingly, neither VEH-treated nor MTII-treated prairie voles showed aggressive behaviors towards stranger females following separation from a partner, which is standing in contrast to previous reports. However, females that were separated from their partner displayed significantly more aggression towards an opposite sex stranger than towards their mate in the paired group. This is in accordance with previous studies reporting that paired females display increased aggression towards opposite sex strangers (Getz et al., 1981). It is possible, that treatment with either VEH or MTII by *i.p.* injection was conceived as a stressful stimulus and therefore reduced aggression in male voles following separation from a partner, promoting prosocial behaviors. Acute stress was shown to reduce aggressive behaviors in male rats which could potentially explain the lack of aggressive behaviors in male prairie voles towards a female stranger (Yohe, Suzuki, & Lucas, 2012). Male and female prairie voles show differences in the response towards stress; exposure to a stressor or CORT injections were shown to facilitate the formation of new pair bonds in male prairie voles (DeVries et al., 1996) but not female prairie voles (DeVries et al., 1995). It would, therefore, be interesting to speculate that the lack of observed aggression in males towards female strangers stems from differences in the response to stress. Furthermore, in the context of the acute stress-induced aggression, MTII is directly affecting the HPA axis activity as MC4R binding of MTII induced CRF gene transcription within the PVN and was followed by an upregulation of plasma CORT 30 min after administration in rats (Lu, Barsh, Akil, & Watson, 2003). It is therefore likely, that both handling and *i.p.* injections and treatment with MTII might have influenced aggressive behaviors in male, but not female prairie voles.

Explorative behaviors and autogrooming were affected by MTII administration

MTII treatment significantly reduced explorative behaviors in both male and female prairie voles on both days of assessment. Separation from a partner alone did not affect explorative behaviors.

Pair-housed VEH-treated female prairie voles increased exploration in the course of the experiment which can potentially be attributed to the decrease of affiliative and prosocial behaviors. The reduction of explorative behaviors following MTII treatment is most likely due to changes in grooming behaviors that were observed following MTII treatment in both male and female prairie voles. A study that investigated the effects of MTII treatment on exploratory behaviors in male mice reported no alterations of explorative behaviors in the open field test (Minakova et al., 2019). This study assessed explorative behaviors in an open field test (OFT). In the present study, however, behaviors were assessed in the home cage, which potentially altered the exploratory drive in comparison with the previous study, since the OFT is also used for the assessment of anxiety-like behaviors.

Increases of autogrooming, yawning, and stretching are known behavioral effects of melanocortins such as ACTH and α-MSH binding to MC4R within the hypothalamus (Argiolas, Melis, Murgia, & Schioth, 2000; Ferrari, Gessa, & Vargiu, 1963). Moreover, agonistic binding of MC4R within the PVN facilitates the local somatodendritic release of OT (Modi et al., 2015), and OT levels within the CNS positively correlate with autogrooming (Calcagnoli et al., 2013).

MTII treatment increased autogrooming in male and female prairie voles, which is in line with the reported effects of MC4R agonism within the hypothalamus. Therefore, the agonistic binding of MTII to MC4R located on OT^+ neurons is likely. Nevertheless, autogrooming can also be indicative of heightened anxiety in other rodent models (Kalueff et al., 2016). More specific, *i.c.v.* injections of MTII were shown to directly affect the HPA axis activity as MC4R binding of MTII induced CRF gene transcription within the PVN and was followed by an upregulation of plasma CORT 30 min after administration in rats (Lu et al., 2003). While the mechanism of the mediation of those behaviors still needs to be clarified, *i.p.* treatment with MTII likely reached the intended target receptors within the PVN.

4.2 The impact of partner loss on the neuro- and peripheral immune system

In this study, I am describing the impact of separation from a partner and acute stress on the neuro- and peripheral inflammatory response. After the separation from their partner, male and female prairie voles were tested for altered emotionality, physiological, neuroendocrine, and immune parameters of the CNS and peripheral immune system. For the neuroinflammatory part, I developed a novel approach to classify morphological microglia activation states (ramified, primed, reactive, or amoeboid). This was combined with other morphological analyses of microglia, qPCR of microglia- and stress-associated genes (*CX3CR1*, *CX3CL1*, *LGALS3*, and *FKBP51*), and assessment of neuronal activation in the PrL, NAcc shell, and PVN of male and female prairie voles. The peripheral immune system was analyzed for the secretion of cytokines and physiological functioning in the bacterial killing assay in combination with other physiological effects following the loss of a partner.

4.2.1 Partner loss did not alter physiology and the stress response at basal levels

Body weight was not altered following the separation from a partner

To assess the impact of separation from a partner and acute stress, the body weight was measured on the onset of cohabitation, separation, and on the day of euthanasia. Male prairie voles showed a significant decrease in body weight during the cohabitation period, however separation-induced change of body weight between or within groups was not observed. In contrast to those findings, chronic stress has been linked to changes in metabolic functions that may stem from a stress-induced increased feeding behavior, changed caloric intake and body weight, and decreased percentage of body fat (Finger, Dinan, & Cryan, 2011). Sun et al., 2014 reported an increase in body weight reactivity in male prairie voles following 2 and 4 weeks separation from a partner (Sun et al., 2014). It is possible, that this discrepancy stems from the differences in the duration of the partner loss-induced chronic stress. While 4 days of separation from a partner might not be sufficient, longer separation periods might evoke a significant change in body weight.

Basal HPA axis activity was not affected by separation from a partner

The activity of the HPA axis at basal levels was assessed by measuring the relative adrenal gland weight and basal plasma CORT levels. In contrast to previous studies that reported an increase in both parameters (Bosch et al., 2009), no change was observed in the present study. In those studies, partner loss upregulates HPA axis activity at basal levels and following acute stress in male prairie voles, and potentiates HPA axis reactivity in male and female prairie voles (Bosch et al., 2009; McNeal et al., 2014). Interestingly, social isolation of female prairie voles induces depressive-like behaviors, indicated by a decrease of sucrose intake after 4 weeks of isolation, as well as an increase in time spent floating in the FST, which is present up to 6 weeks (Grippo et al., 2007; Grippo, Wu,

Hassan, & Carter, 2008; Lieberwirth, Liu, Jia, & Wang, 2012). In contrast, basal CORT levels do not differ following social isolation of female prairie voles under basal conditions (Scotti et al., 2015). However, when coupled with acute stress, social isolation induces an exaggerated HPA axis reactivity in female prairie voles (Scotti et al., 2015).

Therefore, it is possible that separation from a partner not necessarily increases the basal HPA axis activity but influences the reactivity of the HPA axis to an acute stressor, thereby evoking behavioral and molecular / neuronal alterations when assessing effects of partner loss.

4.2.2 Partner loss led to increased emotionality in male but not female prairie voles

The incapability of an organism to adequately respond to stressors negatively influences psychological and physiological parameters, commonly observed in patients suffering from complicated grief (Everly & Lating, 2019). Following separation from a partner, male but not female prairie voles showed an increase of passive stress-coping compared to the *pbp* group. The impact of partner loss on passive stress-coping in male prairie voles is partially in line with previous studies (Bosch et al., 2009; McNeal et al., 2014), which report an increase in passive stress-coping of separated male and female prairie voles (Bosch et al., 2009; McNeal et al., 2014). However, it is unclear why passive stress-coping remained unaltered in female *pbs* prairie voles, especially since a similar social stressor, social isolation from same-sex individuals, induces increased passive stress-coping in the FST (Grippo et al., 2007; Grippo et al., 2008; Lieberwirth et al., 2012). One explanation could be that brain areas that are controlling stress-coping in the FST were differentially altered between male and female prairie voles following the loss of a partner. Examples for potential areas are the mesocortical and prefrontal cortex which control the mesoaccumbens dopaminergic activity during stress-coping (de Kloet & Molendijk, 2016). Activation of DA neurons within the VTA projecting to the NAcc was shown to reduce passive stress-coping in the FST (Tye et al., 2013). It is therefore interesting to speculate that separation from a partner is altering the connectivity of not only PVN-NAcc shell OT release during acute stress, but also VTA-NAcc DA release or DA receptor distribution within the NAcc shell. Those changes might be due to neuronal remodeling in those respective areas following the separation from a partner, which can be partly facilitated by microglia and potentially dependent on the sex of the individual (Wohleb, Terwilliger, Duman, & Duman, 2018).

4.2.3 The loss of a partner and/or acute stress modulated the neuroinflammatory response in a sex- and region-dependent manner

Chronic and acute stress can manipulate the neuro- and the peripheral immune system (Pocock & Kettenmann, 2007; Slavich & Irwin, 2014; Watters & Pocock, 2014). One mechanism of this manipulation is through the release of GCs following stress exposure from the adrenal glands. GCs

can exert anti- and pro-inflammatory effects on nearly all immune cell types through binding to GR located within them (Cruz-Topete & Cidlowski, 2015). Microglia form the innate immune system of the CNS and are sensitive to a wide range of hormones, neuropeptides, and neurotransmitters, including OT, CRF, and GCs (Kettenmann et al., 2011; Stevens et al., 2003; Yuan et al., 2016). Hence, microglia homeostasis and neuroinflammatory response can be modulated by altered neuropeptide or neurotransmitter release. Changes of the neuroimmune system can consequently lead to behavioral changes in a bi-directional way (Kreisel et al., 2014) and can be assessed by analyzing microglia morphology and gene expression. Importantly, the function of the neuroinflammatory response is closely related to microglia morphology and implied in contributing to the development and outcomes of stress-related psychiatric disorders such as MDD (Torres-Platas, Cruceanu, et al., 2014; Troubat et al., 2020). However, the influence of partner loss on the neuroinflammatory status remains to be observed. Therefore, I analyzed the morphology and gene expression of candidate genes as well as neuronal activation in brain regions which are relevant for emotion regulation and dysregulated following the loss of a partner. Analyzed were the PrL, the NAcc shell, and the PVN of male and female prairie voles. The PrL is a subregion of the mPFC which is involved in decision-making, reward-related behaviors, and the adaptation to chronic stress (McKlveen et al., 2013). Changes of neuronal or microglial homeostasis might be associated with altered stress-coping following the separation from a partner. The NAcc shell is directly connected to the regulation of emotional behavior in the FST and is dysregulated following the loss of a partner (Bosch et al., 2016; Bosch et al., 2009). This also applies to the PVN, which is the center for the initiation of the stress response and shows dysregulations in the synthesis of OT mRNA following the loss of a partner (Bosch et al., 2016).

Separation from a partner increased microglia numbers in the PVN of female prairie voles

Chronic and acute stress can affect microglia numbers and morphology (Sugama et al., 2019). Also, different types of stressors and the stressor duration modulate microglia proliferation in a species- and sex-dependent manner (Kreisel et al., 2014; Walker et al., 2013). In humans, a lower density of glia, which encompasses both astrocytes and microglia, is observed in patients with a history of MDD (Cotter et al., 2002; Smialowska, Szewczyk, Wozniak, Wawrzak-Wlecial, & Domin, 2013). Dynamic microglia alterations are underlying stress-induced depressive-like behavior in male rats (Kreisel et al., 2014). Furthermore, microglia decline following chronic stress is also associated with the development of depressive-like behavior in adolescent male mice (Tong et al., 2017). In the present study, partner loss led to increased microglia percentages only in the PVN of female prairie voles. Furthermore, exposure to an acute stressor did not affect microglia percentages in males and females. The observed effects of partner loss in females but not males are in line with previous research reporting an initial increase of microglia numbers and activation following 2-3 days of

chronic unpredictable stress in rats and mice (Kreisel et al., 2014). However, heightened microglia numbers not necessarily correlate with a shift towards pro-inflammatory responses. It is also feasible that cohabitation with a partner decreased microglia percentages. Hence, separation from a partner led to either no change of microglia numbers or a repopulation in the PVN of female prairie voles. This might imply a resolution of inflammatory processes following partner loss (Rice et al., 2017). For this, however, knowledge about basal microglia percentages before cohabitation with a partner would be helpful for a better understanding of microglia dynamics during partner preference formation and partner loss.

Moreover, the neuroimmune system of prairie voles may respond differently to stressor exposure in comparison to other rodent species. Because prairie voles show a natural GC resistance, previous research suggests differential interactions between the HPA axis and the immune system in prairie voles compared to other animal models (Klein, Taymans, DeVries, & Nelson, 1996). Thereby, prairie voles might serve as a valuable model to study GC resistance in humans and the interactions between GCs and the neuroimmune system. Regarding the influence of the sex of the organism, the present study reports distinctive effects of partner loss in male and female prairie voles on the number of microglia. Although the number of literature addressing the influence of sex on microglia numbers following acute and chronic stress is limited, previous studies did not find differences in microglia numbers in male and female rats (Bollinger et al., 2016).

Furthermore, this is the first study to describe microglia percentages in male and female prairie voles. The microglia percentage was comparatively low (PrL: up to 6.2%, NAcc shell: up to 6.0%, PVN: up to 2.6%). Therefore, the microglia percentages within the PrL and the NAcc shell lie in the range of reports in the literature between 5% within the cortex and corpus callosum and 12% in the substantia nigra of adult female mice (Lawson, Perry, Dri, & Gordon, 1990).

Neuroinflammatory status following separation from a partner and/or acute stress was sex- and brain region-dependent

Morphological alterations of microglia are closely linked to function (Fernandez-Arjona, Grondona, Granados-Duran, Fernandez-Llebrez, & Lopez-Avalos, 2017). Furthermore, stressor exposure and emotional and behavioral dysfunctions are associated with changed neurotransmission, altered regulation of neurotransmitter systems, and microglia functioning (Duman, Sanacora, & Krystal, 2019; Frank et al., 2012; Torres-Platas, Cruceanu, et al., 2014; Yirmiya et al., 2000). Both the OT and CRF system and stress-coping behavior are dysregulated following the loss of a partner (Bosch et al., 2016; Pohl et al., 2019). Hence, I hypothesized a correlation between a dysregulation of those systems and altered microglia function in brain regions which are responsible for the behavioral response to stressors. Changes of microglia morphology were

observed in all of the observed brain regions. This change was dependent on the sex of the individual and the region of the brain. Interestingly, acute stress following the loss of a partner did not significantly affect microglia morphology but reversed the observed alterations induced by partner loss.

In male prairie voles, separation from a partner did not affect microglia activation states within the PrL and the NAcc shell. An increase of primed microglia was only observed in the PVNparvo. None of the investigated brain regions showed changes in the number of intersections in the sholl analysis or the number of endpoints and total branch length. The absence of altered morphological parameters in the PrL and NAcc shell of male prairie voles is further supporting the results from the previous microglia classification.

The findings in the PrL of male prairie voles in the present study are analogous to the absence of microglial activation following chronic stress within the mPFC of male rats (Bollinger et al., 2016). However, the lack of altered morphological microglia activation in the NAcc shell of separated prairie vole males is standing in contrast to the effects of chronic stress in other animal models. Here, chronic stress or chronic variable mild stress increase IBA1 expression and CD11b immunoreactivity, indicative of a pro-inflammatory phenotype, within the NAcc of male rats and mice (Farooq et al., 2012; Schiavone et al., 2009; Tynan et al., 2010). Furthermore, in the context of the sudden disruption of the reward circuitry, withdrawal of nicotine results in a more pro-inflammatory morphology of microglia within the NAcc of male rats (Adeluyi et al., 2019), indicating the involvement of microglia in reward-related alterations. Regarding the PVN, chronic stress through 6 days of repeated social defeat increases the proportional area covered by IBA1 expression in the PVN, indicating a more activated morphology in male mice (Wohleb et al., 2011). The present study is reporting increased primed microglia only within the PVN_{parvo}. Since the other activation states remained unchanged, the directionality of this alteration remains to be elucidated. Nevertheless, a non-significant decrease of reactive microglia is observed within the PVN_{parvo} following partner loss. Together with an increase of primed microglia, this might hint at lower morphological microglia activation following partner loss within this region.

Acute stress had no effect on morphological microglia activation following the separation from a partner but reduced ramified microglia only in the NAcc shell of paired males. While no overall effect of acute stress was observed in the number of intersections of microglia branches within the PrL, they differed 34 μm from the cell center. Although not statistically significant, those results might indicate changes of microglia complexity within the PrL of male prairie voles after the exposure to an acute stressor and might be connected to a more activated (primed) phenotype of microglia.

Accordingly, this is consistent to some extent with studies in male rats describing microglia activation following acute stress as indicated by increased branch intersections and primed morphology in the PFC (Bollinger et al., 2016; Hinwood et al., 2013). While the number of studies investigating the effects of acute stress on PVN microglia activation is limited, the results in the present study are not in support of prior findings. In the hypothalamus of male rats, acute stress increases the immunofluorescence of CD11b, indicating higher microglia activation within one hour following stressor exposure (Sugama, Fujita, Hashimoto, & Conti, 2007).

Separation from a partner reduced morphological microglia activation only within the PrL and the PVN of female prairie voles. This was evidenced by increased ramified and decreased primed microglia percentages. Regarding the alternative morphological descriptions, microglia within the PrL of female prairie voles had significantly fewer endpoints and showed a trend towards reduced total branch length following separation from a partner. Similarly, the number of endpoints and total branch length of microglia were reduced in the NAcc shell.

To some extent, these results are supported by previous literature describing the effects of chronic stressor exposure on morphological changes of microglia (Bollinger et al., 2016; Bollinger et al., 2017). In female rats, the ratio of ramified:primed microglia is shifted towards a more ramified morphology in the mPFC following 10 days of chronic unpredictable mild stress (Bollinger et al., 2016). The reductions of microglia endpoints and total branch length following the separation from a partner might be connected to reduced ramification sprouting and therefore increased ramified microglia (Fumagalli, Perego, Pischiutta, Zanier, & De Simoni, 2015). The results obtained from the alternative morphological analyses are hence supporting the results from the morphological classification of microglia.

In general, acute stress did not affect the percentages of microglia activation states in female prairie voles, and the previously observed effect of separation from a partner within the PrL and PVN was not present following acute stress. However, acute stress reduced the general complexity in the PrL of female prairie voles as indicated by the total branch length. Since the other parameters were not affected, the directionality of this alteration remains elusive. As previously noted, the reduction of total branch length could be due to a shift towards a more ramified phenotype which was not detected using the other assessment methods. It is, therefore, possible, that this finding in the present study is in accordance with previous studies describing a decrease of morphological microglia activation in the mPFC following acute stress in female rats (Bollinger et al., 2016).

Even though previous results obtained from different animal models are in support of the present findings, differential microglia activation might be the result of other assessment methods or differences between the species. Furthermore, the results obtained from male and female prairie

voles are suggesting a distinctive regulation of microglia morphology following the separation from a partner and acute stress. This could be due to differences in the secretion of sex steroids. Estrogen and progesterone have anti-inflammatory and neuroprotective effects by reducing the expression of mediators of the inflammatory response (Sierra et al., 2008). This is directly regulated by microglia as they express receptors for both estrogen and progesterone (Sierra et al., 2008). Therefore, sex-dependent differences in the expression of both steroids during stress might govern anti- or pro-inflammatory effects, indicated by microglia activation.

Besides steroidal mechanisms, other mechanisms could also explain the differences observed in the observed brain regions in the present study. For example, the response of the CRF system is differentially regulated between both sexes, resulting in differences following the exposure to a stressor in rats (Bangasser & Wiersielis, 2018; Iwasaki-Sekino, Mano-Otagiri, Ohata, Yamauchi, & Shibasaki, 2009). Moreover, the mPFC is differentially innervated by DA neurons based on the sex of the individual in rats (Kritzer & Creutz, 2008).

The microglia alterations observed in the PVN_{parvo} of male prairie voles may hint at structural changes and lower activation of CRF^{+} neurons. The morphological shift towards less activated microglia could be explained by the nature of habituating homotypic stressors, which can cause a decrease of CRF mRNA expression within the PVN over time (Babygirija, Bulbul, Yoshimoto, Ludwig, & Takahashi, 2012). Microglia express receptors for both GABA and CRF (Kettenmann et al., 2011). While GABA exerts an anti-inflammatory and neuroprotective effect on microglia, CRF causes microglia activation (Arzt & Holsboer, 2006; Pocock & Kettenmann, 2007). Indeed, locally released CRF during stress is acting on $CRFR1^{+}$ neurons which in turn make inhibitory GABAergic synapses on CRF neurons. This is thereby increasing GABA release and dampening CRF release, attenuating the HPA axis activity (Jiang, Rajamanickam, & Justice, 2019). As a result, an increase of extracellular GABA and a decrease of CRF could be an explanation for the findings in female prairie voles. The effects of acute stress on microglia morphology within the NAcc shell of male prairie voles might be explained by changes of neurotransmission (glutamate/DA) following acute stress, which can lead to microglia activation (Campioni, Xu, & McGehee, 2009; Kettenmann et al., 2011).

Therefore, besides differences in the release of sex steroids, differences in the responses of neurotransmitter systems towards stress exposure might also contribute to the observed differential results in the morphological microglia activation in male and female prairie voles.

Gene expression of microglia- and stress response-related genes was not affected by partner loss

Gene expression of microglia activation- and stress response-related genes was performed for the PrL, the NAcc shell, and the PVN of paired and separated male and female prairie voles. In general,

separation from a partner did not affect the gene expression of the observed genes within each brain region.

The release of pro-inflammatory cytokines such as IL-1β is dependent on CX3CL1–CX3CR1 signaling between neurons and microglia (Rogers et al., 2011). Given the decrease of the inflammatory response of microglia within the PrL and PVN of female prairie voles, a reduction of the interaction between neurons and microglia was expected, resulting in lower levels of *CX3CR1* and *CX3CL1* mRNA expression. However, no changes of *CX3CR1* mRNA expression were detected following separation from a partner in both male and female prairie voles. Nevertheless, the findings in the present study are in support of chronic stress-induced effects in female rats, where acute but not chronic stress leads to a decrease of *CX3CR1* mRNA expression (Bollinger et al., 2016).

As the upregulation of *LGALS3* mRNA expression positively correlates with primed microglia (Holtman et al., 2015), I expected a reduction of *LGALS3* mRNA within the PrL and PVN of female prairie voles following separation from a partner. However, *LGALS3* mRNA expression remained unchanged following separation from a partner, regardless of sex or morphological changes of microglia. It is possible that the magnitude of observed morphological changes was too minor for evoking significant changes in the expression of *LGALS3* or that microglia priming is dependent on the regulation of other genes apart from *LGALS3*.

The protein FKBP51 is fundamentally involved in shaping the stress response by modulating GR sensitivity, directly influencing the release of CRF within the PVN (Häusl et al., 2019; Zannas, Wiechmann, Gassen, & Binder, 2016). Also, by changing the sensitivity of the GR, it can influence the activation of the immune system (Zannas et al., 2016). Furthermore, chronic or acute stress influences the expression of *FKBP51* mRNA in the PVN of mice (Scharf, Liebl, Binder, Schmidt, & Müller, 2011). Therefore, I assessed the change of *FKBP51* mRNA expression following the separation from a partner. The expression of *FKBP51* was not altered following separation from a partner in male and female prairie voles. Therefore, the observed negative behavioral outcomes following separation in male prairie voles might be due to a different mechanism than through FKBP51 signaling. Hence, this finding could point towards differential regulation of the stress response between prairie voles and other rodent species. Since prairie voles have naturally high levels of GCs under basal conditions, other mechanisms may be involved in maintaining GC responsiveness under stressful conditions, rendering the organism capable of responding to stressful stimuli. Moreover, FKBP51 is co-expressed in microglia. A reduction of *FKBP51* mRNA within microglia is associated with more pro-inflammatory microglia profiles following repeated social defeat (Wohleb et al., 2011). Due to the present studies' design, however, it is not possible to differentiate between microglia-expressed FKBP51 or neuron-expressed FKBP51. Future studies

could, therefore, perform cell sorting for microglia followed by cell-type-specific expression analysis of *FKBP51* following the separation from a partner.

Separation from a partner increased neuronal activation within the PrL of male prairie voles

Microglia activation can be linked to a change of neuronal activation and, conversely, microglia can also regulate neuronal activity (Cserép et al., 2020; Szepesi, Manouchehrian, Bachiller, & Deierborg, 2018). Therefore, I hypothesized that the observed morphological changes of microglia following partner loss and/or acute stress correlate with neuronal activation patterns within the PrL, NAcc shell, and the PVN.

A persistent increase of c-Fos expression was observed only in the PrL of male but not female prairie voles following separation from a partner.

The mPFC, which comprises the PrL, is capable of attenuating the stress response (Reser, 2016). It should, therefore, be noted, that only males showed increased passive-stress coping in the FST which might be linked to the basal upregulation of neuronal activation in the mPFC. However, no changes in neuronal activation were observed following acute stress and acute stress coupled with separation from a partner, which is partly in line with previous research. The exposure to a chronic stressor can induce neuronal remodeling within the mPFC, which is potentially linked to changed c-Fos expression (Cook & Wellman, 2004; Radley et al., 2004). c-Fos expression is usually assessed following an acute stressor, hence providing insight into the activation of neuronal circuits that are responsible for the resulting neuroendocrine and behavioral changes (Duman, Adams, & Simen, 2005). However, in the literature, the effects of chronic stress on c-Fos expression are mixed. In male mice, c-Fos expression is upregulated within the mPFC, cingulate cortex, and hypothalamus even 24 h after chronic social defeat stress (Matsuda et al., 1996). Conversely, social isolation decreases *c-Fos* mRNA expression in the PFC of socially isolated male mice (Ieraci, Mallei, & Popoli, 2016). In the present study, acute stress did not alter the expression of c-Fos within the PrL of *pbp* or *pbs* prairie voles. The finding in *pbp* prairie voles is in accordance with another study where neuronal activation does not increase within the PrL of the observer prairie vole following the exposure to a stressor towards the other partner (Burkett et al., 2016). In the context of an acute stressor following chronic pre-stress, pre-exposure to a chronic stressor followed by an acute stressor has attenuating effects on neuronal activation within the mPFC of rats (Moench, Breach, & Wellman, 2019; Ostrander et al., 2009). This was not observed in prairie voles that were subjected to acute stress following separation from a partner in the present study. Interestingly, the attenuation of the stress-evoked neuronal activation within the mPFC is not observed in female rats (Moench et al., 2019), indicating sex-differences in the stress response within the mPFC, which is in line with the results obtained from female prairie voles in this study. Potential explanations for the conflicting results within the literature might be the uniqueness of the loss of a partner as a

chronic stressor, the duration of exposure to a stressor, and also the differences in the stress response between species.

Separation from a partner leads to decreased OT release within the NAcc shell of male prairie voles (Bosch et al., 2016). Therefore, alterations in neuronal activation within the NAcc shell could have been expected following the separation from a partner. However, no changes of neuronal activation were observed in both male and female prairie voles. Therefore, altered behavioral responses are not necessarily associated with neuronal activation within the NAcc shell at basal conditions. These results following the loss of a partner are similar to the effects of chronic social isolation in male rats (Stanisavljevic et al., 2019).

Following acute stress or during consoling behaviors in pair bonded prairie voles, OT is released from the PVN (Smith & Wang, 2014). Furthermore, projections from the PVN innervate the NAcc shell, a region with a high abundance of OTR (Bosch et al., 2016; Young & Wang, 2004). Therefore, altered neuronal activation could have been expected within the NAcc shell after exposure to an acute stressor. Regarding acute stressor exposure, foot shocks can increase the neuronal activation in the NAcc shell of male rats (Funk, Li, & Le, 2006). However, acute stress did not influence neuronal activation within the NAcc shell, and potential consoling behaviors between partners did not alter the expression, in line with previous research in consoling prairie voles (Burkett et al., 2016). This discrepancy could be due to the different nature of the stressor (foot shock vs. forced swimming) or the studied species.

Within the subregions of the PVN, a change of neuronal activation was hypothesized following the separation from a partner, since dysregulations of the OT and CRF systems were previously described following partner loss (Bosch et al., 2016; Bosch et al., 2009). CRF^{+} neurons are predominantly found in the PVN_{parvo} and involved in the initiation of the stress response. No differences were observed in the expression of c-Fos, and therefore neuronal activation, within the PVN and its subregions following separation from a partner in both male and female prairie voles. Differential results were reported about the effect of chronic stress on neuronal activation within the PVN and other brain areas (Matsuda et al., 1996; Ostrander et al., 2009), which might be stressor-dependent. Generally, studies are showing an attenuation of the stress-induced *c-Fos* mRNA expression within the PVN following chronic stress (Ostrander et al., 2009; Shelton et al., 2011). However, chronic stress can also increase c-Fos expression and lead to a sensitization of the stress-induced neuronal activation in the hypothalamus of male mice and the PVN_{parvo} of male rats (Bhatnagar & Dallman, 1998; Matsuda et al., 1996). The reason for the discrepancies within the literature including this study might be associated with the salience or the intensity of either the chronic or the acute stressor. Acutely stressed male and female prairie voles, however, show a robust increase of neuronal activation within the PVN and its subregions, independent of housing

condition. This is in agreement with research in other rodent models which also report an increase of c-Fos expression following an acute stressor (Kovacs, 2008). Prairie voles exert consoling behavior which induces an OT-mediated dampening of the HPA axis activity and therefore might decrease neuronal activation within the PVN (Burkett et al., 2016). One explanation for the absence of partner-induced social buffering effects on neuronal activation within the PVN following acute stress might be that in the present study, both partners were exposed to an acute stressor simultaneously, which potentially interferes with partner-directed consoling behavior. This, however, was not analyzed in the present study and probably needs to be taken into account for future studies.

Therefore, the current findings demonstrate an interesting angle on sex-dependent neuronal activation differences within the PrL in the response to separation from a partner which might be linked to the observed behavioral and neuroinflammatory alterations.

4.2.4 The peripheral immune system was not altered by partner loss and/or acute stress

The peripheral immune system is a complex system that can differentially respond to stressor exposure (Seiler, Fagundes, & Christian, 2020). Plasma cytokine levels were measured using a Luminex multiplex assay. Unfortunately, all of the measured cytokine levels were below the detection limit. However, this absence of detectable cytokine levels might indicate that the activation of the peripheral immune system does not change following the loss of a partner or acute stress. This hypothesis, however, needs to be taken with caution. Concerning human literature, bereavement causes an increase in the pro-inflammatory cytokines IL-1β and soluble tumor necrosis factor receptor type II in the saliva (O'Connor, Irwin, & Wellisch, 2009). In rodent models, the effects of exposure to a stressor on the peripheral immune system vary. On the one hand, circulating pro-inflammatory cytokine levels increase following social or physical stress; Inescapable foot shocks increase plasma cytokine levels of IL-6, TNF-α, IL-10 in male mice, reminiscent of an activated peripheral immune system (Cheng, Jope, & Beurel, 2015). Moreover, chronic stressors, such as chronic social defeat, induce an elevation of plasma IL-6, IL-10, IL-12p40, and TNF-α levels in male mice, further strengthening the link between stress and immune system activation (Razzoli, Carboni, Andreoli, Ballottari, & Arban, 2011). Regarding the present study, increasing the sensitivity of the assay might help detect the desired parameters, hence gaining a better understanding of the peripheral immune system response following separation from a partner and/or acute stress. Furthermore, since other cytokines (e.g. IL-2, IFNγ, IL-4, and IL-5) are also involved in the inflammatory process of the peripheral immune system (Seiler et al., 2020), these might also provide insights into the mechanisms of peripheral inflammation following separation from a partner.

Regarding the functioning of the innate immune system, separation from a partner and/or acute stress did not affect the innate immune system's ability to clear the blood from pathogens as assessed in the bacteria killing assay. This finding is in line with previous research in female, but not male prairie voles which reports suppressed peripheral immune responses following 4 weeks of social isolation in males (Scotti et al., 2015). Also, acute stress following social isolation decreases the bacterial killing ability, indicative of a suppressed innate immune system (Scotti et al., 2015). Although CORT can alter the peripheral immune response, other rodent models show that acute stress does not change immunoglobulin levels in socially isolated male mice and prairie voles, despite elevated plasma CORT levels following the FST (DeVries et al., 1997). Furthermore, although not assessed in the present study, chronic stress can also enhance the response of the acquired immune system while at the same time suppressing the innate immune system in Siberian hamsters (*Phodopus sungorus*) (Chester, Bonu, & Demas, 2010). Therefore, while bacterial killing ability remained unaltered in the present study, this does not exclude the possibility that the peripheral immune system is affected by either acute stress or partner loss at different time points following stressor exposure.

The measurement of the spleen weight is a common methodological approach to evaluate dysregulations of both the innate and acquired peripheral immune system (Calder, 2007). Here, separation from a partner did not influence the relative spleen weight in male and female prairie voles. Those findings are partially in consensus with previous studies, since reported effects of chronic stress on spleen weight and spleen functionality vary, as will be discussed below. On the one hand, splenic weight increases following chronic stress and associated with increased neutrophil production, indicative of an elevated peripheral immune system activation (Wohleb, McKim, Sheridan, & Godbout, 2014). Furthermore, 6 days of repeated social defeat increase spleen weight in male mice, which is coupled to higher blood cell progenitor mobilization into the spleen, leading to elevated innate immune cell maturation (McKim et al., 2018). Moreover, chronic subordinate colony (CSC) housing is accompanied by increased absolute spleen weight after 19 days in male mice, coinciding with decreased splenic GR and increased FKBP51 protein expression, indicating an involvement of the stress response in splenic alterations (Foertsch et al., 2017). Furthermore, increased spleen weight is related to *in vitro* GC resistance of stimulated splenocytes of male and female mice that underwent social disruption (Sheridan, Stark, Avitsur, & Padgett, 2000). On the other hand, studies in rats do not report changes in spleen weight following chronic stress exposure; chronic unpredictable mild stress does not change the spleen weight while altering basal CORT levels after three weeks of stressor exposure (Bielajew, Konkle, & Merali, 2002). Those reports are indicating a potentially temporal-, stress-, and species-specific regulation of the peripheral immune system response towards stress exposure. Although the peripheral immune

system activation correlates positively with grief severity in humans (Fagundes et al., 2019), separation from a partner and acute stress did not affect investigated parameters in the present study. Therefore, further studies might be needed to verify the findings in the present study.

4.3 Limitations

In the first study, social interactions were recorded and analyzed 30 min after the treatment with either VEH or MTII. However, as the *i.p.* injection itself might have posed as an acute stressor, social interactions between partners might have altered the stress response. Therefore, only limited conclusions can be drawn about the influence of *i.p.* injection on prosocial behaviors or the potential occurrence or absence of social buffering behaviors following 8 days of treatment. As previously stated, the route of treatment administration might have posed as an acute stressor. However, this conclusion should be taken cautiously since no handling or non-handling control was included in this experiment. Furthermore, repeated *i.p.* injections might have also increased the activation of the peripheral or neuroimmune system. Therefore, the lack of effects evoked by the separation or treatment with MTII might have been caused by a generally heightened inflammatory response. This is also true for the observed reduction of affiliate behaviors in pair-housed partners.

For the evocation of potentiated OT release following MTII administration, all prairie voles were allowed to socially interact with an opposite-sex stranger. This could potentially be a confounding factor since the behavioral response towards social might have varied between stimulus prairie voles, thereby possibly altering the perceived valence of social encounters.

Furthermore, the effect of MTII treatment on behavioral parameters in paired voles was not assessed in this study, which is limiting the statistical analysis since no definite claim could be made about main effects or interactions. The presented statistical tests are comparing groups of animals in which housing conditions and treatments were combined into one variable.

No partner preference test was conducted following treatment with MTII, which could have been useful in deciphering the effect of the treatment with either MTII or VEH. Thus, it remains unclear if the observed reduction of affiliative behavior between partners is linked to impaired pair bond maintenance or the diminishing of partner preference.

In both studies, male prairie voles were vasectomized. Prior studies show that sterility negatively affects the maintenance of a pair bond two weeks following cohabitation (Curtis, 2010). As partner preferences were not assessed in the present study, the extent to which the reproductive fitness of male prairie voles affected emotional behavior following the loss of a partner remains unclear.

Another potentially limiting factor is the age of the prairie voles used for the analysis of gene expression, which was higher than the age of the experimental prairie voles used for the assessment

of other neuroinflammatory and peripheral immune system factors. Age can influence the morphology and gene expression of microglia cells, resulting in a more pro-inflammatory (primed/reactive) phenotype since aging positively correlates with neuro- and peripheral inflammation (Godbout & Johnson, 2009). Furthermore, aged microglia can show an exaggerated response to immune challenges or stress (Norden & Godbout, 2013). Although it remains to be investigated, the microglia profile and response towards separation from a partner might, therefore, be different in the two parts of this study. Moreover, although there is no study to investigate the link between age and the effect of partner loss, older prairie voles might react differently to the loss of a bonded partner, which might alter the involved physiological and behavioral parameters.

In respect to the morphological classification of microglia into the four activation states, I did not include other classifications which also can be observed following activation of the neuroimmune system. Apart from ramified, primed, reactive, and amoeboid states, other states are also discussed in the literature. Hyper-ramified microglia are likely to fall into the primed category in this analysis. Adding to this, microglia profile parameters were assigned based on studies in mice and humans because of the lack of studies describing microglia morphology in prairie voles (Torres-Platas, Comeau, et al., 2014). This might have impacted the results of the classification of morphological microglia activation states.

4.4 Future directions

To exclude the potentially confounding effect of acute stress evoked by the administration of MTII, future studies should apply a different route of administration. Oral administration of MTII would be a preferred way of treatment since it is mimicking a common route of administration in humans and is relatively stress-free, depending on the method used. For example, the administration of MTII could be achieved through the drinking water, which potentially is the preferred route of administration since it does not require handling of the animals or *via* oral gavage, which is more precise and allows for accurate dosing.

Furthermore, more knowledge about the effects of MTII on other systems apart from the OTergic system and their involvement in the regulation of emotionality and social behavior might be useful for future studies. For example, MC3R and MC4R are located in various brain regions and receptor agonism might influence the functioning of other neuropeptidergic systems. Those systems include the earlier mentioned CRF system, but also the DA system in the VTA or DA receptor 1 expressing medium spiny neurons within the NAcc (Navarro, 2017). Altered activation of those systems could lead to behavioral changes following the administration of MTII. Furthermore, MC4R agonism by α-MSH mediates anti-inflammatory effects through inhibition of NF-κB. This can lead to a reduction of pro-inflammatory cytokines and potential microglia changes within several brain

regions involved in emotional processing. Accordingly, it would be interesting to investigate the effects of MTII treatment on microglial activation and its link to the observed behavioral changes following the separation from a partner.

The findings of the second study remain mostly correlational. Subsequently, future studies should establish a functional link between the pro- or anti-inflammatory microglia activation states and behavioral phenotype. This can be achieved by either manipulation of the activation of microglia locally using either G_q- or G_i-coupled designer receptors exclusively activated by a designer drug, or by systemic manipulation of the inflammatory response by administration of lipopolysaccharide or non-steroidal anti-inflammatory drugs (NSAIDs). This trajectory might give rise to translational implications in humans since NSAIDs are commonly used in human medicine.

Furthermore, OT exerts anti-inflammatory effects on microglia cells *in vitro* and *in vivo* (Yuan et al., 2016). Thereby, OT can locally regulate microglia activation and potentially affect neuronal remodeling in brain areas that are involved in social salience and reward. Therefore, future studies might investigate the link between the OT system, behavior, and neuroinflammation in relevant brain areas such as the PVN, NAcc shell, and the PrL.

Furthermore, chronic stressor exposure dynamically alters microglia activation in rats (Kreisel et al., 2014). It is therefore imaginable that similar processes are also present following an extended period of separation from a partner in prairie voles. Extending the knowledge of the inflammatory response within the CNS and the periphery at different time points will contribute to understanding the acute response and the long-term alterations of those systems which might drive stressor-induced behavioral changes and adaptations.

Furthermore, other cytokines and genes apart from those investigated in this study might be involved in the dysregulation of the CNS and peripheral immune response. Hence, future studies assessing other target cytokines and genes might reveal differences following separation from a partner or acute stress and help to unravel potential mechanisms of activation changes of the CNS and peripheral immune response. This could be useful in the context of detecting a potential biomarker for the pathology of human grief and finding potential treatment to counter the observed inflammatory alterations.

Regarding further investigations of sex differences in the inflammatory response following the loss of a partner, differential effects of acute stress and separation from a partner were observed on behavior and morphological changes. Microglia are known to express receptors for estrogen receptor (ER) alpha, which is involved in the regulation of inflammatory processes. Binding of ER ligands to ERs inhibits nuclear factor-kappa B (NF-κB) transcription, which is involved in the activation of microglia, and inflammatory processes (Frakes et al., 2014; Gottfried-Blackmore, Croft, & Bulloch, 2008). Therefore, future studies should further investigate the interactions

between the effects of the loss of a partner and acute stress with sex steroids to gain a better understanding of the sex-specific responses towards a stressor.

4.5 Summary and conclusions

The first study aimed to investigate the effects of MTII treatment on passive-stress coping, anxiety-like behavior, and social and non-social behaviors following the loss of a partner. MTII treatment did not alter emotionality in the FST and EPM following separation from a partner in male and female prairie voles. This is due to the absence of effects evoked by the separation from a partner in both behavioral tests in VEH-treated voles.

No changes in prosocial behaviors were observed following separation from a partner or treatment with MTII; however, affiliative behaviors were affected by separation but not MTII treatment on the first day of assessment. Daily i.p. injections led to no difference in affiliative behaviors on the last day of treatment. This is due to the general reduction in affiliative behaviors between pair bonded partners. It is possible, that the treatment itself was perceived as a stressor and therefore led to emotional and behavioral alterations. Regarding aggressive behaviors, MTII treatment did not affect male and female prairie voles. Additionally, partner loss led to increased aggression towards an opposite-sex stranger only in female prairie voles. As the daily *i.p.* injections of either VEH or MTII might have been perceived as a stressful experience, the observed differences in behavior of separated male and female prairie voles might have been modulated by differences in response towards an acute or chronic stressor.

Regarding explorative behaviors in the homecage with the presence of an opposite-sex stranger, MTII treatment reduced explorative behaviors, which is most likely due to the observed increase in autogrooming, an effect that can be attributed to MTII treatment.

Taken together, since potentially confounding factors were present and not addressed in this study, protective effects evoked by daily MTII administration and successive increases of endogenous OT release on emotionality remain to be observed.

Furthermore, since the knowledge about the effects of partner loss and its interaction with acute stress on the neuro- and the peripheral immune system is limited, the second study aimed to gain insights into this topic. Behaviorally, separation from a bonded partner had a negatively affected stress-coping only in male prairie voles, indicating that separated partners might adopt sex-dependent coping strategies. On the neuroendocrine basis, separation from a partner did not affect basal plasma CORT levels and adrenal gland weight. However, while the basal HPA axis activity remained unaltered, the HPA axis reactivity following separation from a partner might have been affected in both male and female prairie voles.

This is the first study to describe the effects of separation from a partner in combination with acute stress on the neuroimmune system in prairie voles. In this context, a novel method for the objective evaluation of morphological microglia alterations was developed and used together with other methods to assess morphological microglia activation states and complexity. Partner loss and/or acute stress differentially affect the neuroimmune system within several brain regions. Importantly, those effects are dependent on the nature of the stressor in a sex-specific temporal pattern. Microglia activation was lower within the PrL and PVN of separated female prairie voles, whereas microglia had a more primed phenotype within the PVN_{parvo} of male prairie voles. This is an indicator of an anti-inflammatory effect of separation from a partner. Regarding acute stress, exposure to the FST did not affect microglia morphology in females but reduced the percentage of ramified microglia within the NAcc shell of male prairie voles, indicative for a pro-inflammatory shift of activation, further hinting at sex differences in the response towards acute stress.

Comparing the results from the novel method with those from different approaches for the morphological analysis of microglia activation following both chronic and acute stress resulted in different, yet not necessarily contradicting results. Separation from a partner did not affect microglia branch intersections in the sholl analysis. Acute stress, however, caused an increase of microglia branch intersections in the PrL of male prairie voles, potentially indicating a shift towards a more activated morphological phenotype. An analysis based on the total microglia branch length and endpoints per cell revealed no effect of separation or acute stress in male prairie voles. In female prairie voles, however, a reduction of branch length and branch endpoints was observed in the PrL and NAcc shell. This is hinting at an anti-inflammatory effect of partner loss in connection with the results obtained by the morphological classification of microglia states. **Figure 35** depicts a summary of morphological changes following acute stress or separation from a partner.

In light of those results, it is important to note that observed changes in microglia morphology might be dependent on the method of assessment. The combination of different approaches for the morphological analysis of microglia could potentially aid in gaining a more complete understanding of microglial changes. This, in turn, can help understanding and interpreting the impact of different stressors on neuroinflammatory processes.

Thereby, the present study is adding to the existing literature of neuroinflammation by introducing a novel approach for the classification of microglia activation states and the introduction of the prairie vole using a novel type of social stressor, i.e. partner loss Furthermore, it is also highlighting differences in the neuroinflammatory response between sexes to both a chronic social- as well as an acute physical stressor.

Furthermore, neuronal activation was assessed in the PrL, NAcc shell, and PVN. Separation from a partner evoked a higher neuronal activation within the PrL of male prairie voles, while no other

regions of male and female prairie voles showed changes following separation from a partner. Acute stress caused an increase of neuronal activation within the PVN of separated but not pair housed male prairie voles, while a consistent upregulation was observed within the PVN of female prairie voles. The origin and the influence of the upregulation of neuronal activation within the PrL of male prairie voles on the behavioral and morphological microglia changes are unclear. The morphological microglia activation changes following partner loss observed within the PrL of female prairie voles may influence neuronal activation within the PrL, therefore evoking a protective effect against maladaptive behavioral outcomes. This however remains to be investigated. Regarding the response of the peripheral immune system, no alterations were observed in the assessed parameters following neither separation from a partner, nor acute stressor exposure in male or female prairie voles. As described in the earlier sections, variability in responding to a stressor can stem from the type of stress, e.g. physical (cold stress, immobilization stress, swim stress) or social (e. g. social isolation, social defeat, CSC). Furthermore, differences can arise based on the duration of stress exposure (acute vs. chronic), the model organism, and the sex of the individuals. Therefore, this study adds to the existing body of literature in that it is describing the impact of partner loss on several peripheral immune system-relevant markers. By this, it gives way for drawing further implications on the effects of partner loss and its connections to human grief. Given the higher prevalence of stress-related psychopathologies in women, understanding the interactions between sex, immune factors, and microglia activation can contribute to unravel the sex-dependent effects of stress on the well-being.

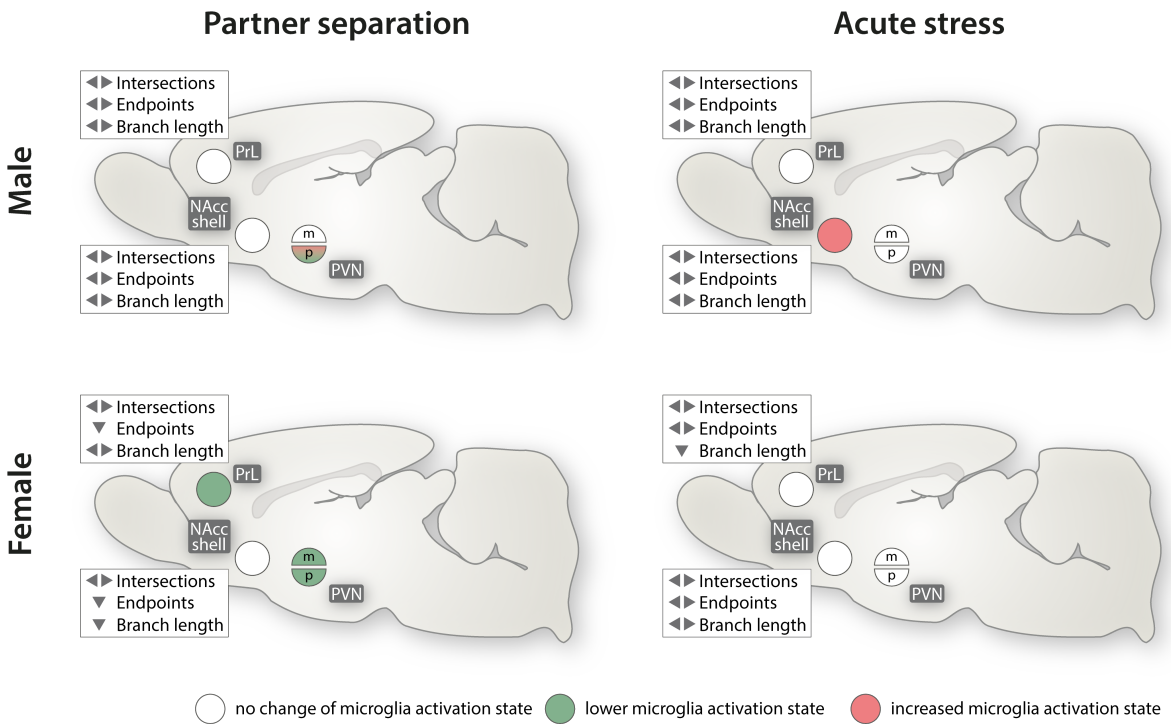

Figure 35 | Morphological microglia alterations following acute stress and separation from a partner in male and female prairie voles.
Complex morphological alterations of microglia were observed following the loss of a partner and acute stress in both male and female prairie voles. Those morphological alterations are dependent on the sex of the individual as well on the type of stressor. In the square boxes, the results of the analysis of microglia complexity of the sholl and skeleton analysis are depicted. The shift of microglia activation is presented in the respective circles; green is representing a lower morphological microglia activation, while red is representing a higher activation. The PVN is split into the magnocellular (m) and parvocellular (p) region.

Appendix

Appendix

Table 33 | *Post hoc* tests for observed experimental group body weight differences on each respective experimental day.

Sex	Exp. Day	Exp. Group	pbp VEH si	pbs VEH ni	pbs VEH si	pbs MTII ni	pbs MTII si
M	0	pbp VEH si	p=1.000	p=1.000	p=1.000	p=1.000	p=1.000
		pbs VEH ni		p=1.000	p=1.000	p=1.000	p=1.000
		pbs VEH si			p=1.000	p=1.000	p=1.000
		pbs MTII ni				p=1.000	p=1.000
		pbs MTII si					p=1.000
	5	pbp VEH si	p=1.000	p=1.000	p=1.000	p=1.000	p=1.000
		pbs VEH ni		p=1.000	p=1.000	p=1.000	p=1.000
		pbs VEH si			p=1.000	p=1.000	p=1.000
		pbs MTII ni				p=1.000	p=1.000
		pbs MTII si					p=1.000
	8	pbp VEH si	p=1.000	p=1.000	p=1.000	p<0.001	p<0.001
		pbs VEH ni		p=1.000	p=1.000	p<0.001	p<0.001
		pbs VEH si			p=1.000	p=0.013	p=0.005
		pbs MTII ni				p=1.000	p=1.000
		pbs MTII si					p=1.000
	12	pbp VEH si	p=1.000	p=1.000	p=1.000	p=1.000	p=1.000
		pbs VEH ni		p=1.000	p=1.000	p=0.512	p=0.830
		pbs VEH si			p=1.000	p=1.000	p=1.000
		pbs MTII ni				p=1.000	p=1.000
		pbs MTII si					p=1.000
F	0	pbp VEH si	p=1.000	p=1.000	p=0.090	p=0.151	p=1.000
		pbs VEH ni		p=1.000	p=1.000	p=1.000	p=1.000
		pbs VEH si			p=1.000	p=1.000	p=1.000
		pbs MTII ni				p=1.000	p=1.000
		pbs MTII si					p=1.000
	5	pbp VEH si	p=1.000	p=1.000	p=1.000	p=1.000	p=1.000
		pbs VEH ni		p=1.000	p=1.000	p=1.000	p=1.000
		pbs VEH si			p=1.000	p=1.000	p=1.000
		pbs MTII ni				p=1.000	p=1.000
		pbs MTII si					p=1.000
	8	pbp VEH si	p=1.000	p=0.803	p=1.000	p=0.002	p=0.001
		pbs VEH ni		p=1.000	p=1.000	p=0.284	p=0.068
		pbs VEH si			p=1.000	p=0.123	p=0.028
		pbs MTII ni				p=1.000	p=1.000
		pbs MTII si					p=1.000
	12	pbp VEH si	p=1.000	p=1.000	p=1.000	p=0.038	p=0.009
		pbs VEH ni		p=1.000	p=1.000	p=1.000	p=0.440
		pbs VEH si			p=1.000	p=0.381	p=0.106
		pbs MTII ni				p=1.000	p=1.000
		pbs MTII si					p=1.000

Table 34 | *Post hoc* tests for observed experimental group body weight differences between experimental days.

Sex	Exp. Group	Exp. Day	0	5	8	12
M	pbp VEH si	0	*p*=1.000	***p*<0.001**	***p*=0.022**	***p*=0.010**
		5		*p*=1.000	*p*=1.000	*p*=1.000
		8			*p*=1.000	*p*=1.000
		12				*p*=1.000
	pbs VEH ni	0	*p*=1.000	***p*<0.001**	*p*=0.118	*p*=0.057
		5		*p*=1.000	*p*=0.669	*p*=1.000
		8			*p*=1.000	*p*=1.000
		12				*p*=1.000
	pbs VEH si	0	*p*=1.000	***p*<0.001**	***p*<0.001**	***p*=0.001**
		5		*p*=1.000	*p*=1.000	*p*=1.000
		8			*p*=1.000	*p*=1.000
		12				*p*=1.000
	pbs MTII ni	0	*p*=1.000	***p*<0.001**	***p*<0.001**	***p*<0.001**
		5		*p*=1.000	***p*<0.001**	***p*=0.040**
		8			*p*=1.000	*p*=1.000
		12				*p*=1.000
	pbs MTII si	0	*p*=1.000	***p*<0.001**	***p*<0.001**	***p*<0.001**
		5		*p*=1.000	***p*<0.001**	*p*=0.112
		8			*p*=1.000	*p*=1.000
		12				*p*=1.000
F	pbp VEH si	0	*p*=1.000	*p*=0.078	*p*=1.000	***p*=0.015**
		5		*p*=1.000	***p*=0.004**	***p*<0.001**
		8			*p*=1.000	*p*=0.110
		12				*p*=1.000
	pbs VEH ni	0	*p*=1.000	*p*=1.000	*p*=1.000	*p*=0.252
		5		*p*=1.000	*p*=1.000	*p*=0.198
		8			*p*=1.000	*p*=0.128
		12				*p*=1.000
	pbs VEH si	0	*p*=1.000	*p*=1.000	***p*=0.033**	***p*<0.001**
		5		*p*=1.000	*p*=0.599	***p*=0.022**
		8			*p*=1.000	***p*=0.011**
		12				*p*=1.000
	pbs MTII ni	0	*p*=1.000	*p*=1.000	*p*=1.000	*p*=1.000
		5		*p*=1.000	*p*=0.290	*p*=1.000
		8			*p*=1.000	*p*=0.157
		12				*p*=1.000
	pbs MTII si	0	*p*=1.000	*p*=1.000	***p*=0.035**	*p*=1.000
		5		*p*=1.000	*p*=0.460	*p*=1.000
		8			*p*=1.000	*p*=0.534
		12				*p*=1.000

Table 35 | Planned comparisons for microglia percentage in specific brain regions of male and female prairie voles.

Parameter	Sex	FST	Treatment	*n*	*M*	*Mdn*	*SD*	*df*	*t*	*U*	*p*	Effect Size
PrL	M	FST⁻	pbp	6	6.05		1.33	10	1.426		0.184	0.827[a]
			pbs	6	5.13		0.85					
		FST⁺	pbp	6		5.15	0.95			9	0.180	0.416[d]
			pbs	6		5.48	1.08					
	F	FST⁻	pbp	6	5.43		0.53	10	1.079		0.306	0.622[a]
			pbs	6	5.85		0.80					
		FST⁺	pbp	6	5.80		1.13	10	0.516		0.617	0.298[a]
			pbs	6	6.16		1.28					
NAcc shell	M	FST⁻	pbp	6	5.12		1.17	10	0.686		0.508	0.396[a]
			pbs	6	5.55		1.00					
		FST⁺	pbp	6	5.59		0.95	10	0.568		0.583	0.327[a]
			pbs	6	5.97		1.37					
	F	FST⁻	pbp	6		4.91	0.85			7	0.093	0.509[d]
			pbs	6		5.73	0.59					
		FST⁺	pbp	6	5.34		1.31	10	0.354		0.731	0.204[a]
			pbs	6	5.61		1.31					
PVN	M	FST⁻	pbp	6	1.76		0.62	10	0.241		0.815	0.140[a]
			pbs	6	1.85		0.65					
		FST⁺	pbp	6	1.69		0.34	10	1.235		0.245	0.712[a]
			pbs	6	2.14		0.84					
	F	FST⁻	pbp	6	1.39		0.55	10	1.913		0.085	1.100[a]
			pbs	6	1.90		0.35					
		FST⁺	pbp	6	1.83		0.49	10	1.506		0.163	0.867[a]
			pbs	6	2.63		1.22					

[a] = Cohen's *d*, [b] = Hedge's *g*, [c] = Glass's *delta*, [d] = Pearson's correlation

Table 36 | Planned comparisons of microglia morphology states in the PrL.

Sex	FST	State	Housing	*n*	*M*	*Mdn*	*SD*	*df*	*t*	*U*	*p*	Effect Size
M	FST⁻	ramified	pbp	6	12.19		6.25	10	1.311		0.219	0.757[a]
			pbs	6	6.97		7.48					
		primed	pbp	6	87.38		5.96	10	0.460		0.656	0.266[a]
			pbs	6	89.29		8.23					
		reactive	pbp	6		0.00	1.05			8	0.106	0.463[d]
			pbs	6		2.38	2.78					
		amoeboid	pbp	6		0.00	0.00			18	>0.999	0.000[d]
			pbs	6		0.00	0.00					
	FST⁺	ramified	pbp	6	11.17		3.65	10	1.038		0.324	0.599[a]
			pbs	6	13.72		4.79					
		primed	pbp	6	87.87		4.18	10	0.828		0.427	0.477[a]
			pbs	6	85.87		4.21					
		reactive	pbp	6		0.00	1.02			17.50	>0.999	0.048[d]
			pbs	6		0.00	1.00					
		amoeboid	pbp	6		0.00	0.00			18	>0.999	0.000[d]
			pbs	6		0.00	0.00					
F	FST⁻	ramified	pbp	6		7.32	3.23			3	0.015	0.694[d]
			pbs	6		13.73	3.13					
		primed	pbp	6	90.73		4.60	10	2.750		0.021	1.589[a]
			pbs	6	84.44		3.19					
		reactive	pbp	6		0.00	2.63			16	>0.999	0.095[d]
			pbs	6		0.00	1.34					
		amoeboid	pbp	6		0.00	0.00			18	>0.999	0.000[d]
			pbs	6		0.00	0.00					
	FST⁺	ramified	pbp	6	9.72		5.27	10	0.055		0.957	0.033[a]
			pbs	6	9.54		5.77					
		primed	pbp	6	89.01		5.59	10	0.186		0.856	0.107[a]
			pbs	6	89.62		5.77					
		reactive	pbp	6		0.00	1.31			16	>0.999	0.095[d]
			pbs	6		0.00	2.04					
		amoeboid	pbp	6		0.00	0.00			18	>0.999	0.000[d]
			pbs	6		0.00	0.00					

[a] = Cohen's *d*, [b] = Hedge's *g*, [c] = Glass's *delta*, [d] = Pearson's correlation

Table 37 | Planned comparisons of microglia morphology states in the NAcc shell.

Sex	FST	State	Housing	*n*	*M*	*Mdn*	*SD*	*df*	*t*	*U*	*p*	Effect Size
M	FST $^-$	ramified	pbp	6	17.72		8.36	10	1.341		0.210	0.775[a]
			pbs	6	11.63		7.33					
		primed	pbp	6	80.38		9.83	10	1.561		0.150	0.901[a]
			pbs	6	88.42		7.91					
		reactive	pbp	6		0.00	3.32			12	0.455	0.276[d]
			pbs	6		0.00	0.00					
		amoeboid	pbp	6		0.00	0.00			18	>0.999	0.000[d]
			pbs	6		0.00	0.00					
	FST $^+$	ramified	pbp	6	8.49		6.96	10	1.741		0.112	1.004[a]
			pbs	6	14.24		4.15					
		primed	pbp	6	90.39		6.47	10	1.478		0.170	0.852[a]
			pbs	6	85.76		4.15					
		reactive	pbp	6		0.00	0.93			15	>0.999	0.138[d]
			pbs	6		0.00	0.00					
		amoeboid	pbp	6		0.00	0.00			18	>0.999	0.000[d]
			pbs	6		0.00	0.00					
F	FST $^-$	ramified	pbp	6	12.68		7.11	10	1.037		0.324	0.598[a]
			pbs	6	17.12		7.73					
		primed	pbp	6	86.26		6.55	10	0.817		0.433	0.472[a]
			pbs	6	82.88		7.73					
		reactive	pbp	6		0.58	1.41			17.50	>0.999	0.032[d]
			pbs	6		0.31	0.76					
		amoeboid	pbp	6		0.00	0.00			18	>0.999	0.000[d]
			pbs	6		0.00	0.00					
	FST $^+$	ramified	pbp	6	14.56		6.90	10	0.630		0.543	0.364[a]
			pbs	6	11.98		7.27					
		primed	pbp	6	85.11		7.12	10	0.700		0.500	0.404[a]
			pbs	6	88.02		7.27					
		reactive	pbp	6		0.00	0.80			15	>0.999	0.138[d]
			pbs	6		0.00	0.00					
		amoeboid	pbp	6		0.00	0.00			18	>0.999	0.000[d]
			pbs	6		0.00	0.00					

[a] = Cohen's *d*, [b] = Hedge's *g*, [c] = Glass's *delta*, [d] = Pearson's correlation

Table 38 | Planned comparisons of microglia morphology states in the PVN.

Sex	FST	State	Housing	*n*	*M*	*Mdn*	*SD*	*df*	*t*	*U*	*p*	Effect Size
M	FST $^-$	ramified	pbp	6	16.75		12.19	10	0.351		0.733	0.204[a]
			pbs	6	14.86		4.89					
		primed	pbp	6	73.39		8.19	10	1.533		0.156	0.884[a]
			pbs	6	80.67		8.28					
		reactive	pbp	6	9.87		5.70	10	1.912		0.085	1.105[a]
			pbs	6	4.46		3.93					
		amoeboid	pbp	6		0.00	0.00			18	>0.999	0.000[d]
			pbs	6		0.00	0.00					
	FST $^+$	ramified	pbp	6	9.30		8.61	10	0.298		0.772	0.173[a]
			pbs	6	8.02		5.97					
		primed	pbp	6	88.80		10.40	10	0.006		0.996	0.003[a]
			pbs	6	88.83		9.42					
		reactive	pbp	6		0.00	2.27			15	0.727	0.138[d]
			pbs	6		0.00	5.55					
		amoeboid	pbp	6		0.00	2.40			15	>0.999	0.138[d]
			pbs	6		0.00	0.00					
F	FST $^-$	ramified	pbp	5		0.00	5.58			4	0.050	0.606[d]
			pbs	6		13.03	5.31					
		primed	pbp	5		100.00	9.75			7	0.173	0.441[d]
			pbs	6		83.48	4.97					
		reactive	pbp	5		0.00	0.00			10	>0.999	0.276[d]
			pbs	6		0.00	1.94					
		amoeboid	pbp	5		0.00	0.00			18	>0.999	0.000[d]
			pbs	6		0.00	0.00					
	FST $^+$	ramified	pbp	6	14.85		10.34	10	0.442		0.668	0.256[a]
			pbs	6	12.45		8.31					
		primed	pbp	6	81.52		11.15	10	0.605		0.559	0.349[a]
			pbs	6	85.07		9.10					
		reactive	pbp	6	3.64		4.71	10	0.511		0.621	0.297[a]
			pbs	6	2.47		2.97					
		amoeboid	pbp	6		0.00	0.00			18	>0.999	0.000[d]
			pbs	6		0.00	0.00					

[a] = Cohen's *d*, [b] = Hedge's *g*, [c] = Glass's *delta*, [d] = Pearson's correlation

Table 39 | Planned comparisons of microglia morphology states in the subregions of the PVN.

Sex	FST	State	Housing	*n*	*M*	*Mdn*	*SD*	*df*	*t*	*U*	*p*	Effect Size
M	FST⁻	ramified	pbp	6	18.29		18.81	10	0.352		0.732	0.203[a]
			pbs	6	15.18		10.69					
		primed	pbp	6	78.68		16.61	10	0.761		0.464	0.440[a]
			pbs	6	84.82		10.69					
		reactive	pbp	6		0.00	7.42			15	>0.999	0.138[d]
			pbs	6		0.00	0.00					
		amoeboid	pbp	6		0.00	0.00			18	>0.999	0.138[d]
			pbs	6		0.00	0.00					
	FST⁺	ramified	pbp	6		0.00	16.20			12	0.383	0.278[d]
			pbs	6		12.70	8.09					
		primed	pbp	6		100.00	16.20			11	0.275	0.324[d]
			pbs	6		85.71	8.50					
		reactive	pbp	6		0.00	0.00			15.00	>0.999	0.138[d]
			pbs	6		0.00	4.08					
		amoeboid	pbp	6		0.00	0.00			18	>0.999	0.000[d]
			pbs	6		0.00	0.00					
F	FST⁻	ramified	pbp	6		0.00	20.03			12	0.372	0.278[d]
			pbs	6		18.33	10.87					
		primed	pbp	6		100	20.92			15.50	0.784	0.114[d]
			pbs	6		81.67	10.87					
		reactive	pbp	6		0.00	5.10			15	>0.999	0.138[d]
			pbs	6		0.00	0.00					
		amoeboid	pbp	6		0.00	0.00			18	>0.999	0.000[d]
			pbs	6		0.00	0.00					
	FST⁺	ramified	pbp	6		17.14	10.78			14.50	0.615	0.161[d]
			pbs	6		20.71	11.56					
		primed	pbp	6		82.86	10.78			11.50	0.349	0.300[d]
			pbs	6		79.29	9.06					
		reactive	pbp	6		0.00	0.00			15	>0.999	0.138[d]
			pbs	6		0.00	8.17					
		amoeboid	pbp	6		0.00	0.00			18	>0.999	0.000[d]
			pbs	6		0.00	0.00					
M	FST⁻	ramified	pbp	6		13.39	10.99			15	>0.999	0.138[d]
			pbs	5		11.11	3.26					
		primed	pbp	6	67.56		10.24	9	2.872		0.018	1.739[b]
			pbs	5	82.01		4.92					
		reactive	pbp	6	16.57		12.21	9	1.911		0.088	1.003[b]
			pbs	5	6.78		5.28					
		amoeboid	pbp	6		0.00	0.00			15	>0.999	0.138[d]
			pbs	5		0.00	0.00					
	FST⁺	ramified	pbp	6	8.89		7.50	10	1.037		0.324	0.600[a]
			pbs	6	4.85		5.88					
		primed	pbp	6	88.06		10.46	10	0.556		0.590	0.321[a]
			pbs	6	91.58		11.43					
		reactive	pbp	6		0.00	0.00			12.50	>0.999	0.932[d]
			pbs	6		0.00	0.00					
		amoeboid	pbp	6		0.00	0.00			15	>0.999	0.138[d]
			pbs	6		0.00	0.00					
F	FST⁻	ramified	pbp	6		4.55	22.25			11	0.314	0.324[d]
			pbs	6		10.56	3.65					
		primed	pbp	6		91.61	21.73			11	0.299	0.324[d]
			pbs	6		88.89	12.53					
		reactive	pbp	6		0.00	4.97			18	>0.999	0.000[d]
			pbs	6		0.00	12.00					
		amoeboid	pbp	6		0.00	0.00			18	>0.999	0.000[d]
			pbs	6		0.00	0.00					
	FST⁺	ramified	pbp	6		16.23	12.17			11.50	0.331	0.300[d]
			pbs	6		8.33	10.60					
		primed	pbp	6	78.02		12.89	10	1.094		0.300	0.631[a]
			pbs	6	85.99		12.35					
		reactive	pbp	6		3.57	3.90			16.50	0.870	0.071[d]
			pbs	6		3.57	5.02					
		amoeboid	pbp	6		0.00	0.00			18	>0.999	0.000[d]
			pbs	6		0.00	0.00					

[a] = Cohen's *d*, [b] = Hedge's *g*, [c] = Glass's *delta*, [d] = Pearson's correlation

Table 40 | Planned comparisons of microglial endpoints within the PrL and NAcc shell.

Region	Sex	FST	Housing	*n*	*M*	*Mdn*	*SD*	*df*	*t*	*U*	*p*	Effect Size
PrL	M	FST $^{-}$	pbp	6	30.62		3.74	10	0.183		0.858	0.106[a]
			pbs	6	29.94		8.23					
		FST $^{+}$	pbp	6	30.42		7.47	10	0.668		0.190	0.386[a]
			pbs	6	27.81		5.97					
	F	FST $^{-}$	pbp	6	35.71		2.33	10	2.877		0.022	1.661[a]
			pbs	6	29.78		4.48					
		FST $^{+}$	pbp	6	30.58		1.90	5.752	0.905		0.402	1.395[c]
			pbs	6	27.93		6.91					
NAcc shell	M	FST $^{-}$	pbp	6	34.36		4.39	10	0.860		0.410	0.495[a]
			pbs	6	31.10		8.20					
		FST $^{+}$	pbp	6	28.43		4.83	10	0.514		0.618	0.296[a]
			pbs	6	30.46		8.40					
	F	FST $^{-}$	pbp	6	37.10		3.62	10	4.299		0.002	2.479[a]
			pbs	6	28.63		3.20					
		FST $^{+}$	pbp	6	31.91		7.73	10	0.809		0.437	0.467[a]
			pbs	6	28.39		7.35					

[a] = Cohen's *d*, [b] = Hedge's *g*, [c] = Glass's *delta*, [d] = Pearson's correlation

Table 41 | Planned comparisons of microglial branch length within the PrL and NAcc shell.

Region	Sex	FST	Housing	*n*	*M*	*Mdn*	*SD*	*df*	*t*	*U*	*p*	Effect Size
PrL	M	FST $^{-}$	pbp	6	174.60		37.13	10	1.303		0.222	0.752[a]
			pbs	6	205.34		44.31					
		FST $^{+}$	pbp	6	190.35		48.76	10	0.732		0.481	0.423[a]
			pbs	6	172.93		31.90					
	F	FST $^{-}$	pbp	6	235.80		24.99	10	2.097		0.062	1.211[a]
			pbs	6	184.20		54.85					
		FST $^{+}$	pbp	6	174.91		17.22	5.709	0.451		0.669	0.713[c]
			pbs	6	187.19		64.49					
NAcc shell	M	FST $^{-}$	pbp	6	213.72		26.00	10	0.216		0.833	0.125[a]
			pbs	6	207.85		61.08					
		FST $^{+}$	pbp	6		185.70	35.68			11	0.310	0.324[d]
			pbs	6		212.10	55.75					
	F	FST $^{-}$	pbp	6	252.28		42.05	10	3.680		0.004	2.124[a]
			pbs	6	177.87		26.18					
		FST $^{+}$	pbp	6	195.41		49.78	10	0.133		0.897	0.077[a]
			pbs	6	191.38		55.18					

[a] = Cohen's *d*, [b] = Hedge's *g*, [c] = Glass's *delta*, [d] = Pearson's correlation

Table 42 | Relative gene expression of target genes in the PrL, NAcc shell, and the PVN following separation from a partner.

Region	Sex	Gene	Housing	*n*	*M*	*Mdn*	*SD*	*df*	*t*	*U*	*p*	Effect Size
PrL	M	*CX3CR1*	pbp	8	0.99		0.68	15	-0.324		0.750	0.160[b]
			pbs	9	1.16		1.22					
		CX3CL1	pbp	9	1.00		0.42	10	1.805		0.102	0.844[a]
			pbs	8	0.74		0.13					
		LGALS3	pbp	9	1.00		0.42	15	1.095		0.291	0.516[b]
			pbs	8	0.81		0.31					
		FKBP51	pbp	8	0.99		0.41	15	0.215		0.833	0.106[b]
			pbs	9	0.96		0.29					
	F	*CX3CR1*	pbp	9	1.00		0.75	16	0.219		0.829	0.103[a]
			pbs	9	0.93		0.64					
		CX3CL1	pbp	9	0.99		0.50	16	0.618		0.545	0.294[a]
			pbs	9	0.88		0.30					
		LGALS3	pbp	9	1.00		0.41	16	-0.363		0.721	0.171[a]
			pbs	9	1.07		0.50					
		FKBP51	pbp	9	1.00		0.39	11.759	0.783		0.449	0.288[c]
			pbs	9	0.89		0.19					
NAcc shell	M	*CX3CR1*	pbp	9	1.00		0.97	15	1.004		0.331	0.471[b]
			pbs	8	0.62		0.59					
		CX3CL1	pbp	9	1.00		0.41	13.284	1.723		0.108	0.680[c]
			pbs	8	0.72		0.25					
		LGALS3	pbp	9	0.99		0.44	16	1.329		0.202	0.628[a]
			pbs	9	0.75		0.36					
		FKBP51	pbp	8	1.00		0.28	15	-0.328		0.748	0.158[b]
			pbs	9	1.05		0.39					
	F	*CX3CR1*	pbp	9	1.00		0.69	16	-0.270		0.790	0.128[a]
			pbs	9	1.11		0.98					
		CX3CL1	pbp	9	1.00		0.34	15	0.268		0.793	0.131[b]
			pbs	8	0.96		0.24					
		LGALS3	pbp	9	1.00		0.57	16	0.198		0.846	0.093[a]
			pbs	9	0.96		0.32					
		FKBP51	pbp	9	1.00		0.51	16	-0.050		0.961	0.023[a]
			pbs	9	1.01		0.42					
PVN	M	*CX3CR1*	pbp	9		0.74	0.71			29	0.340	0.239[d]
			pbs	9		0.40	1.07					
		CX3CL1	pbp	9	1.00		0.58	16	1.243		0.232	0.586[a]
			pbs	9	0.72		0.36					
		LGALS3	pbp	9	0.99		0.55	16	0.702		0.493	0.332[a]
			pbs	9	0.84		0.43					
		FKBP51	pbp	9		0.82	0.50			29.5	0.798	0.228[d]
			pbs	9		0.74	0.35					
	F	*CX3CR1*	pbp	8	1.00		0.70	15	0.175		0.864	0.085[b]
			pbs	9	0.93		0.83					
		CX3CL1	pbp	9	1.00		0.41	16	0.900		0.382	0.421[a]
			pbs	9	0.82		0.44					
		LGALS3	pbp	9	1.00		0.56	16	-0.166		0.870	0.079[a]
			pbs	9	1.04		0.42					
		FKBP51	pbp	9	0.99		0.53	15	-0.549		0.591	0.263[b]
			pbs	8	1.14		0.57					

[a] = Cohen's *d*, [b] = Hedge's *g*, [c] = Glass's *delta*, [d] = Pearson's correlation

Table 43 | Two-way ANOVA for c-FOS counts in the subregions of the PVN.

Region	Sex	*N*	Main effect: Separation	Main effect: Stress	Interaction
PVN_{magno}	M	24	$F(1,20)=0.019$, $p=0.892$, $\eta^2=0.001$	$F(1,20)=12.393$, $p=0.002$, $\eta^2=0.383$	$F(1,20)=2.257$, $p=0.149$, $\eta^2=0.101$
	F	24	$F(1,20)=0.793$, $p=0.384$, $\eta^2=0.038$	$F(1,20)=27.359$, $p<.001$, $\eta^2=0.578$	$F(1,20)=0.674$, $p=0.421$, $\eta^2=0.033$
PVN_{parvo}	M	24	$F(1,20)=1.013$, $p=0.326$, $\eta^2=0.048$	$F(1,20)=7.870$, $p=0.011$, $\eta^2=0.282$	$F(1,20)=0.814$, $p=0.378$, $\eta^2=0.039$
	F	24	$F(1,20)=0.048$, $p=0.829$, $\eta^2=0.002$	$F(1,20)=77.719$, $p<0.001$, $\eta^2=0.795$	$F(1,20)=0.187$, $p=0.670$, $\eta^2=0.009$

Table 44 | Planned comparisons for c-FOS counts in the PrL, NAcc shell, and PVN.

Region	Sex	FST	Treatment	*n*	*M*	*Mdn*	*SD*	*df*	*t*	*U*	*p*	Effect Size
PrL	M	FST^-	pbp	5	11.60		4.51	9	2.459		0.036	1.488[b]
			pbs	6	25.54		11.90					
		FST^+	pbp	5	16.50		4.73	9	0.101		0.922	0.061[b]
			pbs	6	16.04		9.14					
	F	FST^-	pbp	6		20.50				17	0.937	0.462[d]
			pbs	6		25.63						
		FST^+	pbp	6	33.13		24.24	10	0.692		0.552	0.356[a]
			pbs	6	26.29		12.29					
NAcc shell	M	FST^-	pbp	6	99.04		19.03	10	0.785		0.451	0.452[a]
			pbs	6	110.00		28.51					
		FST^+	pbp	6	104.70		18.01	10	0.366		0.722	0.212[a]
			pbs	6	100.90		17.92					
	F	FST^-	pbp	6	88.38		28.82	10	1.425		0.185	0.821[a]
			pbs	6	109.10		21.02					
		FST^+	pbp	6	102.50		18.70	10	0.220		0.831	0.126[a]
			pbs	6	105.40		26.63					
PVN	M	FST^-	pbp	6	47.88		40.43	5.806	1.425		0.206	0.605[c]
			pbs	6	23.42		11.52					
		FST^+	pbp	6	74.42		44.80	10	0.346		0.737	0.199[a]
			pbs	6	81.25		18.38					
	F	FST^-	pbp	6	19.96		11.65	10	0.086		0.934	0.046[a]
			pbs	6	19.42		10.24					
		FST^+	pbp	6	79.75		19.36	10	0.777		0.455	0.448[a]
			pbs	6	91.79		32.66					

[a] = Cohen's *d*, [b] = Hedge's *g*, [c] = Glass's *delta*, [d] = Pearson's correlation

Table 45 | Planned comparisons for c-FOS counts in subregions of the PVN.

Region	Sex	FST	Treatment	*n*	*M*	*Mdn*	*SD*	*df*	*t*	*U*	*p*	Effect Size
PVN_{magno}	M	FST^-	pbp	6	20.08		15.10	10	1.381		0.198	0.797[a]
			pbs	6	10.88		6.23					
		FST^+	pbp	6	31.42		18.63	10	0.848		0.416	0.489[a]
			pbs	6	39.08		11.96					
	F	FST^-	pbp	6	7.25		3.553	10	0.147		0.886	0.085[a]
			pbs	6	7.63		5.159					
		FST^+	pbp	6	31.08		14.03	10	0.881		0.399	0.508[a]
			pbs	6	40.33		21.57					
PVN_{parvo}	M	FST^-	pbp	6	27.79		25.67	5.548	1.417		0.210	0.594[c]
			pbs	6	12.54		6.02					
		FST^+	pbp	6	43.00		27.58	5.999	0.071		0.946	0.030[c]
			pbs	6	42.17		8.76					
	F	FST^-	pbp	6	12.71		8.11	10	0.228		0.824	0.132[a]
			pbs	6	11.79		5.58					
		FST^+	pbp	6	48.67		10.50	10	0.368		0.720	0.213[a]
			pbs	6	51.46		15.31					

[a] = Cohen's *d*, [b] = Hedge's *g*, [c] = Glass's *delta*, [d] = Pearson's correlation

Table 46 | Planned comparisons for the bacterial killing test.

Sex	FST	Treatment	*n*	*M*	*SD*	*df*	*t*	*p*	Effect Size
M	FST $^-$	pbp	9	52.81	26.66	17	0.787	0.442	0.362[b]
		pbs	10	43.76	23.48				
	FST $^+$	pbp	9	52.42	24.34	16	0.576	0.967	0.269[a]
		pbs	9	58.93	23.98				
F	FST $^-$	pbp	9	55.65	15.54	16	0.261	0.798	0.123[a]
		pbs	9	57.30	10.85				
	FST $^+$	pbp	8	58.53	20.24	16	1.094	0.290	0.519[b]
		pbs	10	47.51	22.00				

[a] = Cohen's *d*, [b] = Hedge's *g*

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Curriculum Vitae

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2016 M.Sc. in Biology, **Ludwig-Maximilians-Universität,** Munich, Germany
- Thesis: *Early life stress and stress reactivity as modulators of social behaviour and molecular changes in a mouse model of affective disorders.* Supervised by Prof. Dr. Chadi Touma and PD Dr. Carsten Wotjak (**Max-Planck-Institute of Psychiatry**, Munich, Germany).

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Publications

Pohl, T. T., Young, L. J., & Bosch, O. J. (2019, Feb). Lost connections: Oxytocin and the neural, physiological, and behavioral consequences of disrupted relationships. *Int J Psychophysiol, 136*, 54-63. https://doi.org/10.1016/j.ijpsycho.2017.12.011

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McIlwrick, S., **Pohl, T.**, Chen, A., & Touma, C. (2017). Late-Onset Cognitive Impairments after Early-Life Stress Are Shaped by Inherited Differences in Stress Reactivity. *Front Cell Neurosci, 11*, 9. https://doi.org/10.3389/fncel.2017.00009

Invited presentations

2020 Seminar talk "Mechanisms of Social Behaviour", Cornell University, Ithaca, NY, USA

2020 Data Blitz, Neurobiology of Grief International Network 2020, University of Arizona, Tucson, Arizona, USA

2019 Symposium talk at the 18th National Meeting of the Spanish Society of Neuroscience, Santiago de Compostela, Spai

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