Dissertation

Protective potential of neuropeptide Y to maintain brain function and behavior disturbed by viral or bacterial immune stimulation in the periphery

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz".

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Disclosures

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Abbreviations

Actb	actin beta
ACTH	adrenocorticotropic hormone
AL	ad libitum
ANOVA	analysis of variance
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
bp	base pairs
CCL2	chemokine (C-C motif) ligand 2 (MCP-1)
CD	cluster of differentiation
CLDN	claudin
CNS	central nervous system
CORT	corticosterone
CRH	corticotropin-releasing hormone
CSF	cerebrospinal fluid
CVO	circumventricular organ
CXCL 1	C-X-C motif ligand 1
DAMP	damage-associated molecular patterns
DC	dendritic cells
DPP	dipeptidyl peptidase
dsRNA	double-stranded RNA
GR	glucocorticoid receptor

GRIN2B	glutamate [NMDA] receptor subunit epsilon-2
h	hour
HK	housekeeping gene
HPA	hypothalamic-pituitary-adrenal
i.c.v	intracerebroventricular
IF	intermittent fasting
IFN	interferon
IKK	inhibitor of nuclear factor-κB kinase
IL	interleukin
i.p.	intraperitoneal
IRAK	IL-1R-associated kinase
IRF	interferon regulatory factor
KO	knockout
LBP	LPS-binding protein
LPS	lipopolysaccharide
LRR	leucine-rich-repeat
MCP-1	monocyte chemotactic protein 1 (CCL2)
MD	myeloid differentiation factor
MDA	melanoma differentiation-associated gene
min	minutes
MyD	myeloid differentiation primary-response protein
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NMDA	N-methyl-D-aspartate
NPY	neuropeptide Y

NR3C1	nuclear receptor subfamily 3 group C member 1
OCLN	occludin
OF	open field
PAMP	pathogen-associated molecular pattern
Poly(I:C)	polyinosinic-polycytidylic acid
Ppil	peptidylprolyl isomerase
PRR	pattern recognition receptor
PTSD	post-traumatic stress disorder
qPCR	quantitative real-time PCR
RIG	retinoic acid inducible gene
RM	repeated-measures
RIP	receptor-interacting protein
rpm	revolutions per minute
s	second
SEM	standard error of mean
TIR	Toll/IL-1R homology
TJP	tight-junction protein
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAF	TNF receptor-associated factor
Tubb	tubulin beta class I
Ube2d2	ubiquitin conjugating enzyme E2 D2
VEH	vehicle (NaCl)
Y	NPY receptor

Ywhaz	14-3-3 protein zeta/delta

Zusammenfassung

Über mehrere Signalwege interagieren das periphere Immunsystem und das Gehirn kontinuierlich miteinander. So induzieren viszerale Entzündungen nicht nur eine Immunantwort in der Peripherie, sondern auch die Freisetzung von pro-inflammatorischen Zytokinen und anderen Modulatoren im Gehirn. Diese Mechanismen gehen mit Verhaltensänderungen wie beispielsweise reduziertes exploratorisches Verhalten in neuer Umgebungen einher. Obwohl dieses krankheitsbezogene Verhalten während einer akuten Infektion als adaptive Reaktion die Eliminierung des Pathogens unterstützt, kann die übermäßige oder chronische Stimulation der Immun-Hirn-Achse neuropsychiatrische Folgen haben. So gibt es eine Gruppe an Patient*innen mit neuropsychiatrischen Erkrankungen wie Depressionen, die erhöhte Entzündungswerte sowie eine aktivierte Hypothalamus-Hypophysen-Nebennierenrinden (HHN)-Achse aufweisen und schlechter auf herkömmliche Behandlungen reagieren. Deshalb wird zurzeit intensiv nach Strategien gesucht, um die Übertragung pro-inflammatorischer Signale von der Peripherie ins Gehirn einzuschränken.

Die meisten Forschungsarbeiten zum Thema Immun-Hirn-Achse haben sich bisher mit den Auswirkungen peripher applizierter bakterieller Faktoren auseinandergesetzt. Dazu wird Versuchstieren ein Bestandteil der äußeren Membran gramnegativer Bakterien injiziert, sogenannte Lipopolysaccharide (LPS), die den „Toll-like receptor 4" (TLR4) aktivieren. Über andere mikrobielle Faktoren, beispielsweise viraler Herkunft, ist in diesem Zusammenhang noch vergleichsweise wenig bekannt. Deshalb war das erste Ziel dieser Dissertation, die akuten (neuro-) immunologischen und verhaltensverändernden Effekte eines peripher in Mäuse injizierten viralen Faktors zu untersuchen. Dazu wurde das dsRNA Molekül „polyinosinic:polycytidylic acid" (Poly(I:C)), das TLR3 aktiviert, in einer Dosierung von 12 mg/kg eingesetzt. Zusätzlich wurde analysiert, ob Poly(I:C) das Verhalten der Mäuse langzeitig verändert und etwa Ängstlichkeit oder depressionsähnliche Zustände verursacht, wie es bei LPS Behandlung beobachtet wurde. In weiteren Versuchen wurde elaboriert ob intermittierendes Fasten die Effekte von Poly(I:C) einschränken könnte, da diese Intervention in anderen Entzündungs-Modellen vorteilhafte Auswirkungen aufweist. Zusätzlich wurde analysiert, ob die intranasale Gabe des anti-inflammatorisch wirkenden Neuropeptid Y (NPY) in einer Dosierung von 100 µg die Aktivierung der Immun-Hirn-

Achse durch periphere Applikation von LPS nach 3 und 21 Stunden positiv beeinflussen kann.

Die Versuche zeigen, dass Poly(I:C) klare, krankheitsbezogene Verhaltensänderungen induziert, die nach etwa 4 Stunden ihren Höhepunkt erreichen und ohne beobachtbare Langzeiteffekte abklingen. In den Experimenten zum Wirkungsausmaß von peripher injiziertem Poly(I:C) und LPS wurde erhoben, dass eine vergleichsweise hohe Dosis des viralen Faktors notwendig ist, um annähernd gleich starke Verhaltenseffekte wie mit LPS zu erzielen.

Überraschenderweise verstärkte vorausgehendes Fasten die akuten Auswirkungen von Poly(I:C) auf Verhaltensebene und es wurden erhöhte Werte zirkulierender Zytokine (IL-6, IFN-γ, TNF-α, IFN-α, MCP-1, IL-10) im Blut gemessen. Im Gegenzug schwächte eine intranasale Vorbehandlung der Mäuse mit NPY die akuten Verhaltensänderungen nach LPS-Injektion ab und verringerte den LPS-induzierten Gewichtsverlust. Zusätzlich wurde durch die Gabe des Neuropeptids einer übermäßigen Aktivierung der HHN-Achse entgegengewirkt, gemessen an den geringeren Kortikosteron-Werten im Blut nach 21 Stunden.

Zusammengefasst zeigt diese Studie, dass intermittierendes Fasten das durch TLR3-Stimulation hervorgerufene Krankheitsverhalten verstärkt und dieser Effekt wahrscheinlich durch die erhöhten Zytokinwerte im Blut verursacht wird. Zudem konnte gezeigt werden, dass intranasal appliziertes NPY in der Lage ist verhaltensbezogene und die HHN-Achse betreffende Auswirkungen von LPS abzuschwächen.

Abstract

The peripheral immune system and the brain continuously interact with each other via several routes. Thus, visceral inflammation evokes an immune reaction within the periphery which also induces the expression of pro-inflammatory cytokines and other modulators within cerebral tissue. These effects are associated with a behavioral sickness response, reflected by reduced motivation to explore new environments and to interact with other individuals, as well as decreased ingestive behavior. While these sickness-evoked molecular and behavioral alterations are physiologic responses to an acute infection, under certain circumstances an overshooting or chronic stimulation of the immune-brain axis has detrimental effects. Thus, a subgroup of patients suffering from neuropsychiatric disorders, like major depressive disorder, present with elevated circulating inflammatory markers as well as an activated hypothalamus-pituitary-adrenal (HPA) axis and are often treatment-resistant. Therefore, strategies to interfere in the transmission of certain pro-inflammatory processes from the periphery to the brain are urgently needed.

Many studies in this field thus far used peripheral toll-like receptor 4 (TLR4) activation by bacterial factors like lipopolysaccharide (LPS) to stimulate the immune-brain axis, however, little is known about the effects of virus-associated molecular patterns. The first aim of this study was therefore to systematically analyze the acute (neuro-) immune and behavioral effects of intraperitoneally applied polyinosinic:polycytidylic acid (Poly(I:C)) (12 mg/kg), a viral dsRNA mimic and TLR3 agonist, in mice. Additionally, the behavioral effects of the TLR3 agonist were compared to the effects evoked by a comparably lower dose of LPS (0.03 mg/kg). Furthermore, it was investigated whether Poly(I:C) might induce anxiety- and depression-related behavioral changes when the acute sickness response had already abated, effects that were previously reported in studies involving LPS. Another set of experiments aimed to elucidate whether a time-restricted feeding regimen, which is thought to beneficially influence some inflammation-related disorders, might impact the detrimental effects induced by Poly(I:C). Furthermore, it was analyzed whether intranasal pretreatment with the anti-inflammatory neuropeptide Y (NPY) (100 µg), a peptide with anti-inflammatory effects in the brain, could interfere with the behavioral and immunological effects in response to LPS at 3 h and 21 h post-treatment.

This report shows that Poly(I:C) evokes a clear acute behavioral sickness response, which peaks around 4 h after application in a homecage-like environment and is not followed by

anxiety- or depression-related behavior. Furthermore, a comparably high dose of Poly(I:C) elicits a relative mild behavioral sickness response when compared to LPS as assessed within the LabMaster system. Surprisingly, preceding fasting aggravated Poly(I:C)-induced increase in circulating cytokine levels (IL-6, IFN-γ, TNF-α, IFN-α, MCP-1, IL-10) as well as the behavioral sickness response observed at 4 h following immune stimulation during the open field (OF) test.

Intranasal pretreatment with NPY, on the other hand, potently abated the acute behavioral sickness response 3 h following LPS treatment, attenuated LPS-induced weight loss and reduced the HPA axis activation as reflected by reduced circulating corticosterone (CORT) levels at the later time point under study.

In summary, the findings of this study highlight an adverse influence of intermittent fasting on TLR3-mediated immune stimulation and sickness behavior associated with a boost in peripheral cytokine levels, and a favorable impact of NPY, infused to the nasal cavity, on the behavioral and HPA axis effects of peripheral LPS challenge.

1. Introduction

1.1 The immune-brain axis

Along the immune-brain axis, information is exchanged from the peripheral immune system to the brain in a bi-directional manner. Thus, while on the one hand persisting psychological stress can contribute to a battery of pro-inflammatory effects in the peripheral immune system, vice versa, peripheral inflammation stresses the brain (Holzer et al., 2017). Peripheral inflammation is evoked, for example, by invading pathogens that present pathogen-associated molecular patterns (PAMPs) which activate the innate immune system's pattern recognition receptors (PRRs) (Kawai and Akira, 2009, Holzer et al., 2017). One of the most frequently used PAMP to study the immune-brain-axis is lipopolysaccharide (LPS), an outer cell-wall component of gram-negative bacteria, which triggers an immune response via the activation of Toll-like receptor (TLR) 4 (Poltorak et al., 1998, Farzi et al., 2015b). TLRs are part of the PRR-family and comprise 12 receptors in mammals, which recognize specific microbe-derived PAMPs. In addition, some of the TLRs are activated by damage-associated molecular patterns (DAMPs), molecules that are released from the organism itself during high stress (Akira et al., 2006). The stimulation of an organism's innate immune system is associated with an elevated secretion of pro-inflammatory cytokines and increased circulating corticosterone (CORT) levels during the acute phase of the immune reaction and to a lesser extent at later time points (Farzi et al., 2015b, Mayerhofer et al., 2017). Importantly, the inflammatory response evoked by PAMPs within the periphery is not locally restricted, given that the central nervous system (CNS) can be affected in its function resulting in behavioral changes (Capuron and Miller, 2011). Consequently, the molecular and behavioral effects of inflammation are summarized as the general term "sickness response". This response can be detected in all mammals, including rodents, and is associated with reduced locomotor activity and ingestion, sleep impairment, decreased motivation to interact with other individuals, anxiety, fatigue and cognitive dysfunction (Wieczorek et al., 2005, Frenois et al., 2007, Dantzer et al., 2008, Capuron and Miller, 2011). Interestingly, many of these symptoms overlap with characteristics of neuropsychiatric disorders, such as major depression, while alterations in immune function have been observed in a subset of psychiatric patients (Capuron and Miller, 2011). Additionally, while the dampened motivation for physical activity as observed in individuals suffering from acute infections is mostly adaptive and helps to promote recovery, chronic

inflammation is associated with somatic as well as psychiatric disorders being responsible for an impaired quality of life (Kelley et al., 2003). Importantly, while it is well known that the immune system and the brain are tightly interconnected, the crucial mechanisms by which inflammation might facilitate neuropsychiatric disorders, or vice versa, remain widely elusive.

1.1.1 Inflammation in neuropsychiatric disorders

Studies have shown that infections or sterile inflammation that activate the immune system via the release of PAMPs or DAMPs can influence the expression patterns of neurotransmitter systems, which could in turn harm neuropsychological balance (Sergeyev et al., 2001, Felger and Lotrich, 2013, Haroon et al., 2018). For example, cytokines and other inflammatory mediators are thought to enhance the release of glutamate and additionally reduce its reuptake from the synaptic cleft or extrasynaptic compartments by the downregulation of glutamate transporters on glial cells (Pitt et al., 2003, Ida et al., 2008). More specifically, *in vitro* human oligodendrocytes treated with tumor necrosis factor (TNF)-α reduced their expression of the glutamate transporter, excitatory amino acid transporter-1, which inhibited glutamate uptake by over 75% (Pitt et al., 2003). In turn, extrasynaptic NMDA receptors are stimulated by the excess glutamate which was found to reduce the production of brain derived neurotrophic factor (BDNF) and mediate excitotoxic effects by the amino acid (Hardingham et al., 2002, Capuron and Miller, 2011). Interestingly, the downregulation of BDNF was specifically observed by activation of extrasynaptic N-methyl-D-aspartate (NMDA) receptors, while synaptic NMDA receptors on the other hand induced BDNF gene expression (Hardingham et al., 2002). Although the pathogenic mechanisms are not defined yet, the inhibition of BDNF is thought to not only affect neuroplasticity, but might also be involved in the development or exacerbation of psychiatric disorders, including depression (Pittenger and Duman, 2008).

Further evidence for the thorough interaction of the stimulated peripheral immune system with the brain and thereby induced alterations in mood in humans was provided by the cytokine treatment strategy that was applied for hepatitis C patients until 2014 and involved interferon (IFN) injections (Mettke et al., 2018). IFN treatment of these patients was found to bring along an elevated risk to develop major depressive disorder (MDD) and was associated with increased adrenocorticotropic hormone (ACTH) and cortisol levels in circulation (Capuron et al., 2003, Raison et al., 2005, Raison et al., 2006). Interestingly,

symptoms for depressive-like disorders were ameliorated by treatment with a serotonin reuptake inhibitor that is used in depressed patients, which suggests an interaction between the neurotransmitter system and cytokines (Musselman et al., 2001, Capuron and Miller, 2011).

Thus far, basic research in the field focused mostly on how bacterial PAMPs might shape the communication between the peripheral immune system and the brain, and therefore, LPS was frequently applied in animal models (Poltorak et al., 1998, Kawai and Akira, 2011). Thereby, one of the crucial mechanisms implicated in the immune-brain axis was found to be the transmission of inflammatory signals from the periphery to the brain, where cytokine expression is found enhanced a few hours (h) post peripheral LPS treatment (Poltorak et al., 1998, Dantzer et al., 2000, Kawai and Akira, 2011, Farzi et al., 2015b).

1.1.2 Cytokines and stimulated immune cells act as direct messengers along the immune-brain axis

One mechanism by which inflammation is transferred from the periphery to the CNS involves blood-borne cytokines that act directly on the brain via three main routes (Fig. 1). First, cytokines that travel to the brain via the circulation can act on the blood-brain barrier (BBB) by stimulating astrocytes and endothelial cells from the neurovascular unit, which in turn release secondary messengers. Second, cytokines can enter the brain via circumventricular organs (CVOs) that present with a more fenestrated BBB, while third, some cytokines are actively transferred to the CNS by specific transporters (Banks and Erickson, 2010, Capuron and Miller, 2011, Burfeind et al., 2016). Under physiological conditions, the BBB prevents the passage of molecules from peripheral circulation to the brain. This property of the BBB was first described by Ehrlich and colleagues in the early 20th century, who found that peripherally applied dyes are unable to stain the CNS (Banks, 2015). Later that century, the structure of the BBB was revealed and tight junctions were found to provide a barrier function, preventing uncontrolled leakage into and from the brain (Banks and Erickson, 2010). However, high levels of systemic pro-inflammatory cytokines or PAMPs can damage the BBB and lead to its disruption by the down-regulation of tight-junction protein expression (Mayerhofer et al., 2017). Furthermore, endothelial cells of the BBB express TLR2, 3, 4, and 6, as well as receptors that are stimulated by cytokines such as TNF-α and interleukin (IL)-1, and the activation of those receptor pathways are thought to be involved in the propagation of inflammatory states from the periphery to the brain

(Cunningham and De Souza, 1993, Hartz et al., 2006, Nagyoszi et al., 2010, Banks, 2015). Additionally, human cerebral endothelial cells stimulated by IL-1β or TNF-α were found to increase their expression levels of TLRs 2 and 3. Activation of TLR2 in turn was found to reduce the expression of the tight-junction proteins occludin (OCLN) and claudin (CLDN)-5 (Nagyoszi et al., 2010), which might further promote cerebral pro-inflammatory processes by leakage of inflammatory factors from the periphery to the brain. In response to those immune stimulating signals, microglial cells, neurons and astrocytes within the brain release a similar pattern of cytokines as observed in the periphery, which amplifies central inflammation (Capuron and Miller, 2011).

Furthermore, peripheral immune cells, such as monocytes, that are stimulated by strong inflammation can traffic to the brain (Fig. 1). For this route of immune-to-brain communication, microglial activation and an elevated cerebral expression of monocyte chemotactic protein 1 (MCP-1, or chemokine (C-C motif) ligand 2; CCL2) in response to the peripheral stimulus is crucial (D'Mello et al., 2009). Interestingly, peripheral TNF-α release is also necessary for this recruitment of monocytes by activated microglial cells (D'Mello et al., 2009). Moreover, repeated high social stress was found to induce anxiety-related behavior in rodents which was associated with trafficking of peripheral monocytes to the brain via perivascular space, effects that were observed to depend on intact CCL2 signaling (Wohleb et al., 2013).

An important brain area for orchestrating the cerebral response to peripheral immune stimulation is the hypothalamus, as it controls changes in body temperature as well as ingestive behavior and is crucially involved in the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Burfeind et al., 2016). Especially TNF-α and IL-1β are thought to play a central role in the cytokine-induced sickness response coordinated by the hypothalamus. For example, systemic immune challenge was repeatedly shown to elevate IL-1β expression in several brain areas including the hypothalamus, an effect accompanied by reduced food intake, which can be blunted by central applications of IL-1 receptor antagonists (Gabellec et al., 1995, Layé et al., 2000, Farzi et al., 2015b, Mayerhofer et al., 2017). Furthermore, the development of fever is induced by the IL-1β dependent activation of cyclooxygenase-2 and the subsequent release of pyrogenic prostaglandin E2 (Gourine et al., 1998, Cartmell et al., 1999, Burfeind et al., 2016). While peripheral TNF-α plays an important role in enhancing the release of other circulating cytokines like IL-1 and IL-6 during infections,

intracerebroventricular (i.c.v) application of TNF-α alone was found to also reduce food intake and increase body temperature (Fong et al., 1989, Layé et al., 2000, Romanatto et al., 2007). Although TNF-α and IL-1β seem to be inevitable factors to induce sickness in response to immune challenge, IL-6 is also necessary in order to evoke inflammation-related behavioral changes. Thus, inhibition of central IL-6 signaling was found to support recovery from sickness and rodents lacking IL-6 presented with a decreased LPS-induced sickness response (Bluthé et al., 2000b, Burton et al., 2011, McCusker and Kelley, 2013).

In addition to their ability to circulate to the brain and directly act on the CNS, blood-borne cytokines can influence cerebral functions by stimulating afferent fibers, including those of the vagus nerve (Fig. 1).

1.1.3 The vagus nerve acts as a translator from the periphery to the brain

Numerous reports, dissecting the roles of the vagus nerve in the transmission of pro-inflammatory signals from the periphery to the brain, found that the intact vagus nerve is necessary for a full behavioral sickness response, but not for the development of fever following peripheral IL-1β or LPS injections in rats (Bluthe et al., 1996, Hansen and Krueger, 1997, Konsman et al., 2000, Luheshi et al., 2000). Furthermore, the interruption of signaling along the vagus nerve by vagotomy was also found to impact the HPA axis response to peripheral LPS treatment, as reflected by lower circulating adrenocorticotropic-hormone (ACTH) levels (Dantzer et al., 2000). However, vagotomy does not seem to influence HPA axis activation at all levels to the same extent, as circulating CORT levels were not persistently affected, which might be due to immune-activating factors that directly stimulate the adrenal cortex within the periphery (Dantzer et al., 2000, Hansen et al., 2000).

Signaling from the periphery to the brain via the vagus nerve is induced by the stimulation of PRRs, like TLR4, and cytokine receptors that are expressed on the subdiaphragmatic vagus nerve. Subsequently, afferent fibers transfer the stimulus to activate cerebral catecholaminergic neurons within the nucleus tractus solitarii (NTS) and their projections transmit the signal from there to other brain areas whose activity is changed (Goehler et al., 2000, Raison et al., 2006, Barajon et al., 2009). Importantly, some of these projecting neurons transfer the stimulus from the NTS to the hypothalamus, which is thought to be critically involved in the subsequent activation of the HPA axis (Goehler et al., 2000).

1.1.4 The hypothalamic-pituitary-adrenal (HPA) axis provides a link between inflammation, brain, behavior and mood

An important link between neuropsychiatric disorders, like MDD, and chronic inflammation is that in both disorders an activation of the HPA axis can be observed (Fig. 1). This is reflected by increased secretion of corticotropin-releasing hormone (CRH), elevated circulating levels of ACTH as well as cortisol (in humans) (Haroon et al., 2012). Thus, a study evaluating melanoma patients who received IFN-α therapy found elevated ACTH and cortisol levels in response to initial IFN treatment, which was accompanied by symptoms of major depression (Capuron et al., 2003). Similarly, application of cytokines or PAMPs is known to induce HPA axis activation in animal models (Berkenbosch et al., 1987, Farzi et al., 2015b, Matsuwaki et al., 2017).

Following the first report of IL-1β triggering the activation of the HPA axis in 1986, numerous studies were conducted to provide evidence for other cytokines, including TNF-α, to induce HPA axis activation (Besedovsky et al., 1986, Dunn, 1992, Ando and Dunn, 1999). Interestingly, IL-1β was found to most potently enhance the release of ACTH and CORT, while TNF-α seems to prolong the activation of the HPA axis (Dunn, 2000). Furthermore, inflammation, especially when occurring over a longer period, is thought to enhance and stabilize the activation of the HPA axis, which in turn amplifies the stress-response and probably symptoms of depression (Dantzer et al., 2008). This might be due to a cytokine-induced resistance and decreased responsiveness of glucocorticoid receptors to its agonist and thereby the feedback loop. These effects are partly mediated by enhanced relative expression of the inactive β glucocorticoid receptor (GR) isoform, which was found to attenuate the activation of glucocorticoid-sensitive genes *in vitro* (Webster et al., 2001, Pace et al., 2007). As a consequence to this resistance, anti-inflammatory effects of glucocorticoids are dampened and furthermore, the negative feedback loop by corticosteroids that suppress the central release of CRH, might be abrogated, reflected by increased circulating cortisol concentrations (Holsboer, 2000, Webster et al., 2001, Pace et al., 2007, Dantzer et al., 2008, Capuron and Miller, 2011). However, it should be noted at this point that the exact impact of isotype GR expression on the development or maintenance of MDD remains elusive. Nevertheless, there are studies that indicate a correlation between decreased GR-dependent transcription activity and enhanced nuclear factor 'kappa-light-

chain-enhancer' of activated B-cells (NF-κB) -mediated pro-inflammatory gene activation and mood disorders (Bekhbat et al., 2017).

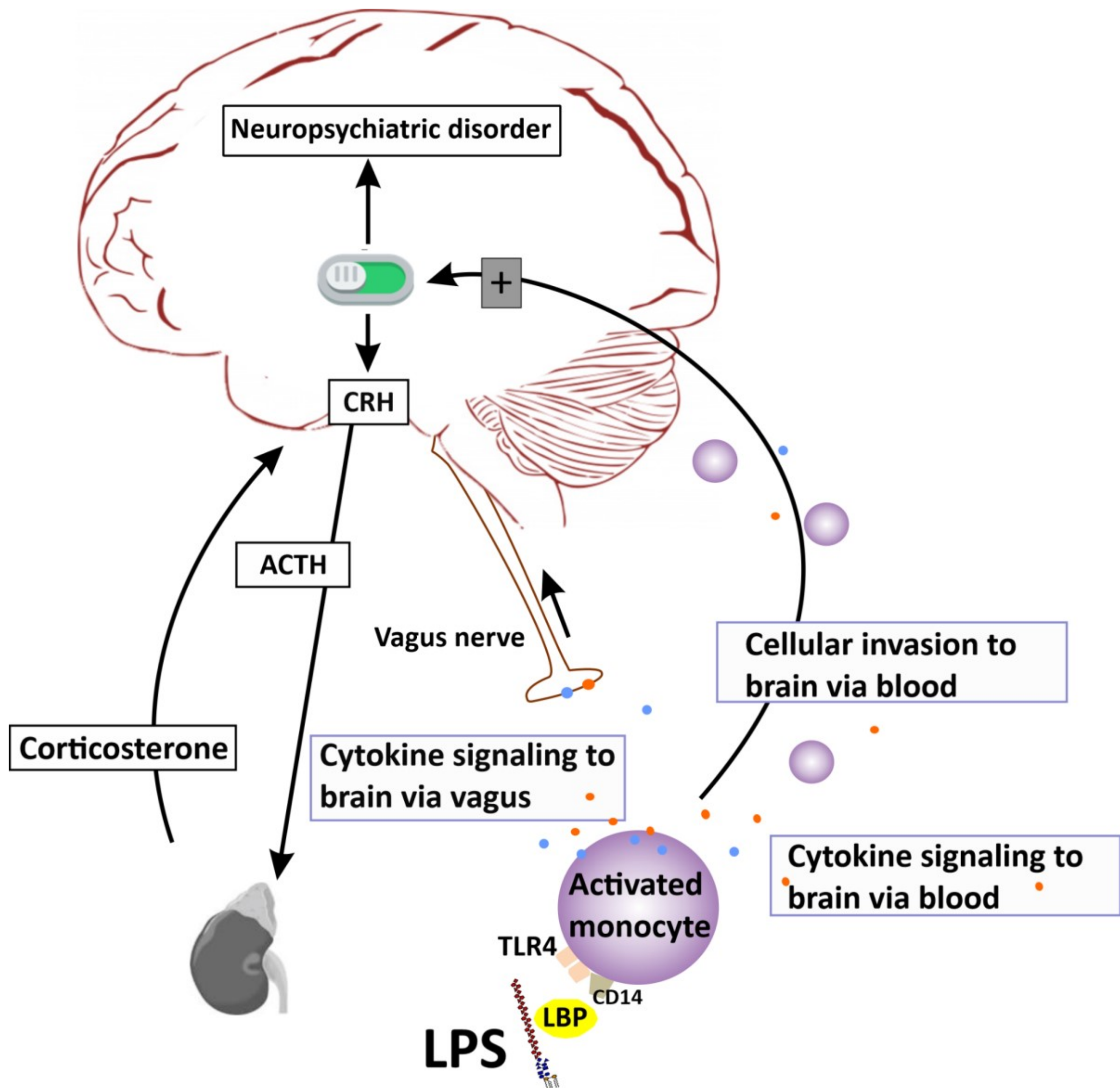

Figure 1 Scheme of immune-to-brain signaling by pro-inflammatory stimuli. Cytokines released by stimulated peripheral immune cells travel by circulation to the central nervous system and enter the brain, or they activate receptors present at fibers of the vagus nerve. Stimulated immune cells can also travel to the brain and induce a central immune reaction. In turn, the hypothalamus-pituitary-adrenal axis is activated. Abbreviations: ACTH=adrenocorticotropic hormone, CD=cluster of differentiation, CRH=corticotropin-releasing hormone, LBP=LPS-binding protein, LPS=lipopolysaccharide, TLR=toll-like receptor.

1.2 TLR 3 and 4 and their signaling pathways

Invading pathogens, or more specifically their characteristic PAMPS, stimulate PRRs on cells of the innate immune system which in turn leads to the release of pro-inflammatory cytokines. TLRs are a subgroup of PRRs that are expressed on various immune cells as well

as epithelial cells and fibroblasts, however, their expression is not static but shifts with the appearance of pathogens and the occurrence of specific cytokines (Akira et al., 2006).

Structurally, TLRs contain extracellular leucine-rich-repeats, while their cytoplasmic domain consists of the Toll/IL-1R homology (TIR) domain, homologous to the interleukin 1 receptor (Akira et al., 2006). Their stimulation initiates signaling cascades that eventually lead to the release of cytokines and other immune modulators. The first step in this cascade involves the recruitment of specific adaptor proteins to the TIR domain of the receptor, like myeloid differentiation factor 88 (MyD88) in the case of TLR4 or TIR-domain-containing adaptor protein inducing interferon-β (TRIF) in the case of TLR3 and TLR4 (Yamamoto et al., 2003). MyD88 then interacts with IL-1R-associated kinase (IRAK) 1 and 4 as well as TNFR-associated factor (TRAF) 6, which activates the inhibitor of NF-κB kinase (IKK) complex and allows the release of NF-κB from its inhibitor. In turn, NF-κB is translocated to the nucleus and stimulates the transcription of pro-inflammatory cytokines (Fig. 2) (Akira and Takeda, 2004, Akira et al., 2006).

TLR3 activation within the endosome of cells belonging to the innate immune system induces a MyD88-independent pathway. First, TRIF is recruited that interacts with receptor-interacting protein 1 (RIP1) and activates TRAF6 and TRAF3 as well as IKK-*I,* which phosphorylates interferon regulatory factor (IRF)-3 and IRF-7 (Yamamoto et al., 2003, Fitzgerald et al., 2003). The homodimers formed by phosphorylated IRF-3 and IRF-7 enter the nucleus to initiate type I IFN gene expression (Fig. 2) (Honda et al., 2005, Akira et al., 2006).

While functional innate immunity is vital for accurate protection against invading pathogens, overshooting TLR responses were found to contribute to detrimental health conditions that are associated with chronic inflammation.

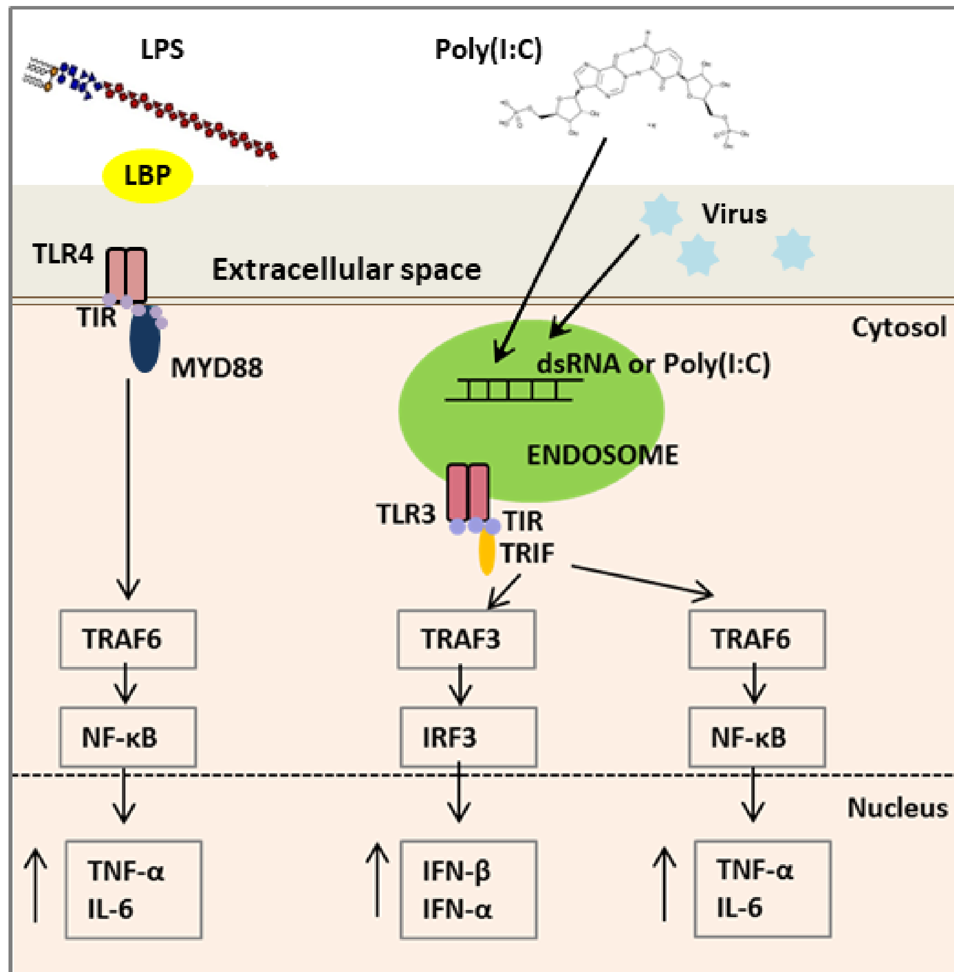

Figure 2 Signaling pathways of Toll-like (TLR) receptor 3 and TLR 4 activation. The bacterial factor LPS and the viral mimic Poly(I:C), or virus, stimulate distinct signaling pathways by activating TLR4 and TLR3, respectively. Abbreviations: IFN=Interferon, IL=Interleukin, IRF=Interferon regulatory factor, LBP=LPS-binding protein, LPS=Lipopolysaccharide, MyD=myeloid differentiation factor, NF-κB=Nuclear factor-κB, Poly(I:C) =Polyinosinic:polycytidylic acid, TIR=Toll/IL-1R homology domain, TLR=Toll-like receptor, TNF=Tumor necrosis factor, TRAF=TNFR-associated factor, TRIF=TIR-domain-containing adaptor protein inducing interferon-β.

1.2.1 TLR3 and its agonist polyinosinic:polycytidylic acid (Poly I:C)

In 2001, Alexopoulou and colleagues first discovered TLR3 as the responsive receptor for non-self double stranded RNA (dsRNA) molecules that occur in most viruses during the replication cycle (Alexopoulou et al., 2001, Perales-Linares and Navas-Martin, 2013). TLR3 is expressed in endosomal compartments of macrophages and myeloid dendritic cells (DCs) as well as both intracellularly and on the cell surface of epithelial cells and fibroblasts (Koyama et al., 2008, Watanabe et al., 2011). The delivery to the endosome is thought to be mediated by cluster of differentiation (CD) 14 and clathrin (Watanabe et al., 2011, Jensen

and Thomsen, 2012). In 2005, the crystal structure of the ectodomain of human TLR3 was published and revealed a horseshoe-like shape (Choe et al., 2005). Choe and colleagues already conceded that dsRNA molecules might be recognized by TLR3 in conjunction with another protein, which indeed was identified in 2016. Mex-3 RNA Binding Family Member B, a co-receptor associated to TLR3 is thought to be involved in the process of binding dsRNA molecules to the receptor (Zhu et al., 2016).

The most widely studied TLR3 agonist is certainly polyinosinic:polycytidylic acid, in short Poly(I:C), which is a synthetic mimic of dsRNA molecules. TLR3 does not bind certain sequences within dsRNA ligands and in the last two decades it was more and more recognized that the length and quality of the synthetic dsRNA mimic is crucial for its specificity in binding to TLR3. Accordingly, the double-stranded oligonucleotide only acts on TLR3 with a minimum length of 40-45 base-pairs (bp) (Botos et al., 2009). Besides binding to TLR3, dsRNA also acts via retinoic acid-inducible gene I (RIG I) and melanoma differentiation-associated gene 5 (MDA5), cytosolic viral RNA sensors in DCs and macrophages (Koyama et al., 2008). However, while RIG-I binds short and preferably 5' triphosphated dsRNA (and ssRNA) molecules and MDA5 recognizes dsRNA strands that contain more than 2000 bp, specific binding to TLR3 occurs by dsRNA molecules between 200-1000 bp length. Furthermore, dsRNA molecules bind TLR3 in acidic environments, alike that found within the endosomal compartment and more specifically, optimal binding occurs at a pH below 6.5 (Kato et al., 2008, Botos et al., 2009). Upon stimulation, TLR3 signaling classically enhances Type I IFN secretion, meaning IFN-α and IFN-β (Kato et al., 2008).

Behavioral and immune effects of TLR3 stimulation by Poly(I:C) in rodents

Both rats and mice are frequently used to study the effects of TLR3 stimulation *in vivo*. Several groups observed reduced locomotor and exploratory activity in mice 3, 4, 6, 8 and 12 h following intraperitoneal (i.p.) injections of the viral mimic Poly(I:C) (Cunningham et al., 2007, Konat et al., 2009, Michalovicz and Konat, 2014, Murray et al., 2015). This change in behavior was accompanied by increased circulating IFN-β, IFN-α, TNF-α, IL-6 and MCP-1 levels as observed in mice treated with doses between 10-12 mg/kg (Cunningham et al., 2007, Weintraub et al., 2014, Kong et al., 2015). Furthermore, i.p. injections of Poly(I:C) activate the HPA axis, reflected by higher CORT plasma levels 3 h and 6 h post-treatment

(Kong et al., 2015) and induce the expression of cytokines at mRNA level in the brain, including TNF-α, IL-6, IFN-β, MCP-1 and IL-1β (McLinden et al., 2012, Michalovicz and Konat, 2014).

Apart from the acute effects of Poly(I:C) on cytokine expression and behavior, there is evidence from rat models that the viral mimic could trigger depression and anxiety-related behavior. Anhedonia, a symptom associated with depression, reflected by reduced sucrose-intake was observed in rats 24 h following i.p. Poly(I:C) (6 mg/kg) injections but had returned to normal after 72 h (Gibney et al., 2013). Another long-term effect found in mice treated consecutively with 7 injections of the viral mimic is the accumulation of amyloid-beta in the hippocampus and impaired contextual fear memory (Weintraub et al., 2014). The same study also reported that in contrast to LPS, repeated treatment with Poly(I:C) does not lead to any habituation effect influencing the acute sickness response (Randow et al., 1995).

1.2.2 TLR4 and lipopolysaccharide (LPS)

Two years after the Toll gene was discovered to be involved in the immune response to invading pathogens, TLR4 was the first receptor to be identified as a specific target of the bacterial PAMP, LPS (Lemaitre et al., 1996, Poltorak et al., 1998). LPS is composed of three parts; the O side chain, the core oligosaccharide and lipid A, which is thought to be the main part interacting with the immune system (Raetz and Whitfield, 2002, Miller et al., 2005). LPS classically stimulates TLR4 expressing mononuclear phagocytes that in turn produce and release pro-inflammatory cytokines to the circulation, inducing several changes in brain function and behavior (as described below) (Kelley et al., 2003). Furthermore, the LPS receptor is also expressed by other cells including neutrophils, B-cells, cells of the epithelium as well as neurons and microglial cells within the CNS (Gerondakis et al., 2007, Michelsen and Arditi, 2007, Okun et al., 2011, Prince et al., 2011). TLR4 is presented at the cell surface in a complex with myeloid differentiation factor 2 (MD2), and binds circulating LPS associated to LPS-binding protein (LBP). Furthermore, CD14 is involved in delivering the LPS-LBP complex to TLR4-MD2 (Kawai and Akira, 2010) (Fig. 2).

Behavioral and immune effects of TLR4 stimulation by LPS in rodents

Generally, LPS induces the expression and release of several cytokines, including IL-6, IFN-γ and TNF-α which is thought to be one of the mechanisms by which it mediates the sickness response (Dantzer et al., 2008, Farzi et al., 2015b, Mayerhofer et al., 2017). While some

effects of i.p. injected LPS on behavior were found to be dampened by preceding application with an IL-1 receptor antagonist (Kelley et al., 2003), blocking both IL-1β and TNF-α signaling pathways inhibits LPS-induced sickness (Bluthé et al., 2000a, McCusker and Kelley, 2013).

LPS has emerged to be the PAMP of choice to potently induce inflammation in the periphery and brain leading to behavioral changes observed in various protocols (Dantzer, 2001, Lestage et al., 2002, Dantzer et al., 2008, Farzi et al., 2015b, Moraes et al., 2017). For instance, sickness and anhedonic behavior were found to be induced time-and dose dependently as measured in the OF test and the sucrose preference test. These behavioral alterations were accompanied by increased secretion of cytokines, chemokines and CORT (Biesmans et al., 2013, Martin et al., 2013, Biesmans et al., 2016). In its effectiveness to induce a sickness response, LPS is more potent when compared to viral factors such as Poly(I:C), as circulating levels of pro-inflammatory cytokines, including TNF-α and IL-6, induced by LPS immune stimulation are up to 30-fold higher, although the dosage used for the bacterial factor is usually lower (Kong et al., 2015). Furthermore, behavioral effects like decreased ingestion and wheel running as well as locomotor activity in the homecage are more pronounced in rodents treated peripherally with LPS when compared to Poly(I:C) (Hopwood et al., 2009). Interestingly, the HPA axis seems to be stimulated similarly by LPS and Poly(I:C) in rodents, as circulating CORT levels induced by the bacterial factor were found to match those levels observed in animals treated with the viral factor (Kong et al., 2015).

1.3 Interventions targeting immune-brain axis stimulation

Given the role of immune challenge in the development and maintenance of neuropsychiatric disease, increasing interest is brought towards finding a way to interfere in the detrimental behavioral and immunological effects of peripheral inflammation.

1.3.1 Fasting

Several fasting approaches have been studied in their ability to beneficially impact health issues including diabetes, obesity and autoimmune disorders (Farzi et al., 2018). These feeding regimens include food intake-related calorie restriction down to 70% of the normal rate, alternate-day fasting (referred to as intermittent fasting here; IF), or periodic fasting, meaning fasting for 1-2 days a week (Anton et al., 2018, Farzi et al., 2018). Interestingly, IF

was shown to similarly benefit health when compared to continuous calorie restriction, while requiring less commitment to count calories during the feeding period (Anton et al., 2018, Farzi et al., 2018). Some of the beneficial effects of fasting also affect the brain. For example, within the CNS, the fasting-induced periodic shift to ketone utilization in aging mice was found to preserve white matter integrity as well as long-term memory (Guo et al., 2015). Furthermore, BDNF expression was shown to be upregulated by IF, which in turn boosts synaptic plasticity and thereby supports learning processes and memory formation (Anton et al., 2018). Interestingly, neuroprotective effects of IF that are observed in young rodents were found to be impaired by increasing age (Arumugam et al., 2010), which is important to consider when interpreting fasting-related data, since most studies used young animals. The above-mentioned study investigated the effects of an IF regimen for 4-5 months prior to an experimentally induced stroke, which did not reduce infarct volume in old mice (16 months). Furthermore, the improved IF-induced stress response observed in young mice, reflected by an increased level of protein chaperones and antioxidant enzymes, was abolished in old mice. Additionally, enhanced insulin sensitivity by IF was found to be ablated in older mice when compared to younger mice (Arumugam et al., 2010).

Fasting induced attenuation of inflammatory processes are also increasingly put into focus. For example, in the context of a high-fat diet feeding regimen similar to a western-style diet, mice that were timely restricted in their access to food showed reduced mRNA expression levels of pro-inflammatory cytokines when compared to ad libitum (AL) fed controls (Hatori et al., 2012, Chaix et al., 2014, Farzi et al., 2018). Furthermore, restricted feeding regimens were studied in their ability to reduce sickness in response to bacterial infection or LPS treatment (Matsuzaki et al., 2001, Godinez-Victoria et al., 2014, Radler et al., 2014, Zenz et al., 2019). For example, mice restricted to 50% of their regular calories showed blunted microglial activation as well as suppressed sickness behavior after LPS treatment, which was associated with altered neurotransmitter expression patterns within the hypothalamus. Interestingly, raised levels of the orexigenic and centrally anti-inflammatory acting neuropeptide Y (NPY) were observed (MacDonald et al., 2011, Radler et al., 2015, Zenz et al., 2019).

1.3.2 Neuropeptide (Y)

For a very long period of time it was believed that the transmission of information from one neuron to another was conducted entirely through electrical impulses. It was only during the

first quarter of the 20th century when Otto Loewi, Chair of the Institute of Pharmacology at the University of Graz at that time, conducted his probably most famous experiment: He discovered that neurons transduce signals not only electrically, but also communicate by the release of chemicals, namely neurotransmitters. Besides monoamines and amino acids, also peptide transmitters – neuropeptides - were found to be involved in neuronal signaling. Neuropeptides are the only neurotransmitters that are not taken back into the neurons once released, but are degraded by specific enzymes named peptidases and are therefore inactivated or shifted in their affinity to bind to specific receptors. Furthermore, neuropeptides are packed in large dense-core vesicles within the neuron and can diffuse to distant brain areas by volume transmission upon secretion (van den Pol, 2012). One of the most abundant neuropeptides in the CNS is NPY, which affects metabolism, ingestion, mood, stress coping as well as immune processes, mostly via 4 G-protein coupled receptors Y1, Y2, Y4 and Y5 (Reichmann and Holzer, 2016). NPY consists of 36 amino acids and is cleaved at the N-terminus in the periphery by aminopeptidase P to its $NPY_{2\text{-}36}$ fragment, while dipeptidyl peptidase (DP) 4 (also CD26) cleaves NPY to its $NPY_{3\text{-}36}$ fragment. Within the CNS mostly DP 8/9 truncates NPY, however, with lower substrate turnover compared to the peripherally occurring DP4 (Bjelke et al., 2006, Dimitrijevic et al., 2008, Wagner et al., 2015). Especially the $NPY_{3\text{-}36}$ fragment is thought to selectively stimulate the Y2 receptor, whereas the intact NPY molecule activates preferentially Y1 (Reichmann and Holzer, 2016). This shift in receptor affinity is important because stimulation of these two receptor subtypes is thought to, at least partly, have opposing effects. For example, while Y2 receptor stimulation within the brain might rather induce anxiety-related behavior, Y1 activation is thought to hold anxiolytic effects (Heilig, 2004, Wagner et al., 2015, Reichmann and Holzer, 2016). One explanation is the differential location at cerebral synapses; the Y2 receptor is expressed pre-synaptically and therefore inhibits the secretion of NPY and co-transmitters, whereas Y1 decreases neuronal activity postsynaptically (Holzer et al., 2012). However, the opposing effects of Y1 and Y2 activation might be at least partly brain-region dependent, as microinjections of NPY to the locus coeruleus were effective to reduce anxiety-related behavior, whereas a non-peptide small molecule Y1 antagonist was not very effective to inhibit this effect, pointing at a role of Y2 receptors in this region to mediate anxiolytic effects (Kask et al., 2000, Heilig, 2004).

NPY in (immune-) stress

NPY is thought to hold stress-buffering effects, promote resilience and beneficially influence behavioral disturbances evoked by immune stimulation (Ferreira et al., 2012, Radler et al., 2014, Farzi et al., 2015a). Evidence for this hypothesis was generated by the use of transgenic rodents, lacking NPY, that present with an increased anxious phenotype (Heilig, 2004). Also in humans, NPY seems to play an important role in stress resilience. For example, veterans exposed to combat who developed post-traumatic stress disorder (PTSD) were found to have lower levels of NPY in their cerebrospinal fluid (CSF) compared to healthy individuals and furthermore, a follow-up study revealed that veterans without PTSD presented with higher CSF levels of NPY compared to veterans who developed the mental illness (Sah et al., 2009, Sah et al., 2014, Reichmann and Holzer, 2016). Another study reported that a high-stress military training increased NPY levels in plasma in healthy male soldiers, which was correlated with an enhanced ability to adapt to stressful situations (Morgan et al., 2000). Furthermore, lower expression of functional NPY in individuals carrying a single nucleotide polymorphism was correlated to higher activation of the amygdala when they were exposed to threat-related facial expressions (Zhou et al., 2008).

In rodent models, NPY was additionally studied in its function to promote resilience to the detrimental effects of immune challenge. For example, *in vitro* studies showed that IL-1β-induced microglial motility and LPS-induced microglial IL-1β and TNF-α release as well as phagocytosis are suppressed by NPY (Ferreira et al., 2011, Ferreira et al., 2012, Li et al., 2014). Furthermore, NPY may convey neuroprotective effects by suppressing NMDA receptor expression and reducing NMDA receptor activity via its ability to diminish the release of IL-1β and TNF-α (Li et al., 2014). While the anti-inflammatory effects of NPY on microglial cells are thought to be conducted via Y1 receptor activation, other receptors stimulated by NPY might also be involved in the buffering of immune stress. Thus, *in vivo* studies analyzed knockout (KO) mice lacking Y2 receptors and found aggravated short- as well as long-term behavioral effects induced by peripherally injected LPS (Painsipp et al., 2008, Painsipp et al., 2010). In line with these results, NPY was found to protect dopaminergic cells in an animal model of Parkinson's disease, an effect that was diminished in mice lacking the Y2 receptor or in animals that were treated with an Y2 antagonist (Decressac et al., 2012). Stress buffering effects of NPY were furthermore associated with decreased stimulation of the HPA axis, which plays a crucial role in immune stress as well

(Antonijevic et al., 2000, Tasan et al., 2016). Furthermore, pretreatment with NPY protected rats from hypotension and stabilized body temperature following peripheral immune stimulation by LPS (Felies et al., 2004).

However, not only in rodent immune stress models, but also in patients suffering from psychiatric disorders, an accumulating body of evidence hints at the regulatory potential of NPY (Yehuda et al., 2006, Zhou et al., 2008). For example, intravenous injections of NPY were found to improve sleeping quality in both healthy volunteers and depressed patients and to reduce plasma ACTH and cortisol release in healthy volunteers (Antonijevic et al., 2000, Held et al., 2006).

Intranasal application of NPY

In order to facilitate NPY delivery to the brain and apply the neuropeptide in a non-invasive manner, NPY can be infused intranasally (IN) which has been studied both in rodents as well as humans (Lochhead and Thorne, 2012, Serova et al., 2013, Laukova et al., 2014, Sayed et al., 2018). Interestingly, IN infusion of NPY in a rodent model of PTSD potently reduced traits of anxiety and depressive-like behavior, which was accompanied by a suppressed release of ACTH and CORT to the circulation (Serova et al., 2013). Among the benefits of applying compounds IN are the rapid delivery to the brain, the circumvention of the BBB as well as the bypass of liver metabolism and the prevention of degradation of the peptide within the gastrointestinal tract (Molero et al., 2018, Sayed et al., 2018). Recently, a small dose-range study analyzed the safety and efficacy of IN applied NPY in twenty-six PTSD patients. Anxiety symptoms were reduced by NPY treatment in a dose-dependent manner with the most anxiolytic effect in the group receiving the highest dose of 9.6 mg with no obvious detrimental side effects (Sayed et al., 2018). In rodent models, IN applied peptides like NPY were shown to reach most brain areas, including the hypothalamus, within 30 min (Lochhead and Thorne, 2012, Laukova et al., 2014, Fatoba et al., 2018). Interestingly, in a rodent model of PTSD, the infusion of NPY was found to dampen the HPA axis activation and to suppress behavioral alterations (Serova et al., 2013, Laukova et al., 2014).

Recently, another compound that is applied IN was approved by the FDA and is found effective in treatment-resistant MDD, namely *S*-ketamine (esketamine) (Canuso et al., 2018, Daly et al., 2018, FDA, 2019). Interestingly, previous studies had found a higher

bioavailability of IN applied ketamine compared to orally applied ketamine (45% and 20%, respectively) (Yanagihara et al., 2003, Molero et al., 2018).

1.4 Aims of this dissertation

With this study, composed of two projects, I (I) evaluated the acute and long-term behavioral alterations induced by peripheral immune challenge with the viral mimic Poly(I:C), (II) analyzed the effects of an every-other-day fasting regimen on acute Poly(I:C)-evoked inflammatory processes, (III) compared viral and bacterial compounds in their ability to induce sickness behavior and (IV) elucidated the impact of IN infused NPY on the short-term and long-term effects of LPS-induced stimulation of the immune-brain axis.

1.4.1 Project 1: Analysis of Poly(I:C)-induced sickness response and the effects of preceding intermittent fasting

Dietary restrictions are increasingly investigated in their ability to mitigate bacterial infection-induced inflammation (Matsuzaki et al., 2001, Godinez-Victoria et al., 2014, Radler et al., 2014). However, little is still known about how fasting could alter the effects of PAMPs that stimulate TLRs other than TLR4. Therefore, it was assessed how IF for 9 days impacts the sickness response to the dsRNA mimic Poly(I:C) at a dose of 12 mg/kg at the immune and behavioral level (Zenz et al., 2019). The alternate-day fasting regimen was chosen because it is a rather mild approach, given that calorie restricted rodents are usually provided once daily with a certain amount of calories and will thereby run without food for the rest of the day after finishing their meal, which might enhance the fasting effect. Furthermore, intermittent calorie intake for one day on every other day was shown to similarly benefit several health issues when compared to continuous calorie restriction (Gotthardt et al., 2016). The first aim of this project was to analyze the time course of the behavioral sickness response induced by intraperitoneal (i.p.) injections of the viral mimic Poly(I:C) in a homecage-like environment. Furthermore, it was assessed whether peripheral injections of the viral mimic Poly(I:C) might induce depressive-like or anxiety-related behaviors after the acute phase of sickness has abated. The third aim and main focus of this project was to evaluate the effects of IF on TLR3 activation-induced alterations of exploratory behavior in a new environment, circulating cytokines and hypothalamic cytokine expression at the time point when the response to Poly(I:C) peaks (as evaluated by the first two sets of experiments) (Zenz et al., 2019).

1.4.2 Project 2: Effects of intranasal NPY pretreatment on LPS-evoked immune and sickness response

Common anti-depressants, such as serotonin reuptake inhibitors, fail to effectively treat PTSD or MDD in up to 50% of cases. NPY could be a promising candidate especially to overcome treatment-resistance in patients suffering from mental illness combined with elevated inflammatory markers (Reichmann and Holzer, 2016, Sabban and Serova, 2018, Sayed et al., 2018). This inflammatory state is reflected by increased levels of cytokines as well as an activated HPA axis and can be induced by infections but also stress and tissue injury (Capuron and Miller, 2011). In order to interfere with the inflammation-induced behavioral alterations and neuroimmune processes, I chose to use the anti-inflammatory and anxiolytic neurotransmitter NPY (Farzi et al., 2015a). Given the rapid transport of substances from the nostril cavity to the brain and presumably quick therapeutic effect of IN applied compounds (Sayed et al., 2018), the effects of IN applied NPY on immune-stimulation by LPS were assessed. LPS was used in this set of experiments because it was found to have stronger sickness-evoking effects when compared to Poly(I:C) in the LabMaster system (Results section 3.1.8). IN infused NPY has been previously reported to reach several brain areas, including the hypothalamus, within 30 min following its application (Lochhead and Thorne, 2012, Laukova et al., 2014, Fatoba et al., 2018), and therefore the neuropeptide was administered at a frequently used dose of 100 µg 30 min before the i.p. injection of LPS (Serova et al., 2013, Laukova et al., 2014).

First, I assessed whether ingestive behavior as well as basic locomotion and exploration within the homecage-like environment of the LabMaster were disturbed by IN NPY applications. Secondly, I examined the effectiveness of NPY pretreatment to interfere with the activation of the immune-brain axis at the behavioral and immune level during the acute phase of the immune response (3 h post-injections) as well as at a later time point when the acute phase had abated (21 h). Therefore, peripheral and central cytokine expression patterns were assessed, HPA axis activation was analyzed by circulating CORT levels and the central expression of CRH. Furthermore, the expression levels of NPY and its receptors Y1, Y2 and Y5 were evaluated at both time points under study. In order to assess exploratory behavior within a new environment, which is usually reduced in animals exposed to immune-stress, mice were subjected to the OF test at 3 h and at 21 h post-injections.

2 Materials and Methods

The Materials and Methods section is in part identical to the information that was published in Zenz et al (2019) and to the Materials and Methods chapter of another study submitted for publication.

2.1 Experimental animals

An ethics committee at the Federal Ministry of Science, Research and Economy of the Republic of Austria approved all experiments as well as the number of animals used (BMWFW-66.010/0122-WF/V/3b/2017 and BMBWF-66.010/0185-V/3b/2018). All experiments were designed to reduce the total number of animals used and special care was taken to keep suffering to an absolute minimum. In line with that, all experiments were performed following the Directive of the European Parliament and of the Council of 22 September 2010 (2010/63/EU). Male C57BL/6N mice were purchased from Charles River Laboratories (Sulzfeld, Germany) at the age of 8 weeks. For at least one week after arrival, animals were habituated to the new environment before any procedure was undertaken. Unless mentioned otherwise, free access to both standard laboratory chow and tap water was provided and mice were housed in pairs under a strict 12 h light/dark cycle (lights on 6:00 am, lights off 6:00 pm). The relative air humidity as well as room temperature were tightly controlled, like all other housing conditions, and set to 50% and 22°C, respectively. Mice were habituated to the test environment for at least 24 h previous to testing, and temperature, light/dark cycle as well as relative air humidity were identical to those in the housing facility as mentioned above.

2.2 Reagents and dosing

Poly(I:C), the synthetic dsRNA analog, and the bacterial factor LPS were obtained from Invivogen (Toulouse, France). Poly(I:C): catalog number tlrl-picw; free from microbial contaminants; low molecular weight: 0.2 kb to 1 kb, LPS: catalogue number tlrl-3pelps, ultrapure, E. coli 0111:B4) (Zenz et al., 2019). Both reagents were dissolved in sterile pyrogen-free saline, provided by Invivogen, as recommended by the manufacturer. Poly(I:C) was applied at a dose of 12 mg/kg which by others was reported to induce sickness-like symptoms in rodents (Cunningham et al., 2007, Michalovicz and Konat, 2014). LPS was applied at a subseptic dose of 0.03 mg/kg in order to evoke a mild sickness response

comparable to the effects observed in Poly(I:C)-treated animals. Control groups were applied with pyrogen-free sterile saline (0.9% NaCl). All animals of all treatment groups were injected with the same volume of 10 µl/g body weight.

Lyophilised NPY, purchased from Phoenix Pharmaceuticals (Karlsruhe, Germany, catalogue number 049-03), was dissolved in sterile distilled water (10 µg/µl) just before the IN infusions of 100 µg per mouse (Serova et al., 2013).

2.3 Protocols

Some of the protocols used here were published by Zenz et al. (2019). In addition, another manuscript has been submitted for publication including parts of the protocols described below.

2.3.1 Project 1: Analysis of Poly(I:C)-induced sickness response and the effects of preceding intermittent fasting

Experimental setup

In order to analyze when the sickness behavior in response to i.p. applied Poly(I:C) peaks, mice were submitted to the LabMaster system (TSE Systems, Bad Homburg, Germany) where locomotion, exploration and ingestive behavior were recorded (Fig. 3 a). Furthermore, anhedonic and anxiety-like behaviors were assessed at a time point when the acute sickness response has already abated to analyze long-term effects of the viral mimic Poly(I:C) (Fig. 3 b, c).

In a second set of experiments, the behavioral and immune response to peripherally applied Poly(I:C) was evaluated following an intermittent fasting (IF) regimen (Fig. 3 d). In more detail, mice were fed every other day for 9 consecutive days and were injected with the viral mimic Poly(I:C) on day 10. In order to reinforce the effect of fasting, food was withdrawn at 6:00 am on that day, which is in line with a schedule used by others to test the effects of fasting on the response to LPS (MacDonald et al., 2011). Sickness behavior was assessed 4 h after i.p. Poly(I:C) injections (11:00 am) within the OF (3:00 pm), after which mice were sacrificed and plasma and brains were collected for molecular analysis.

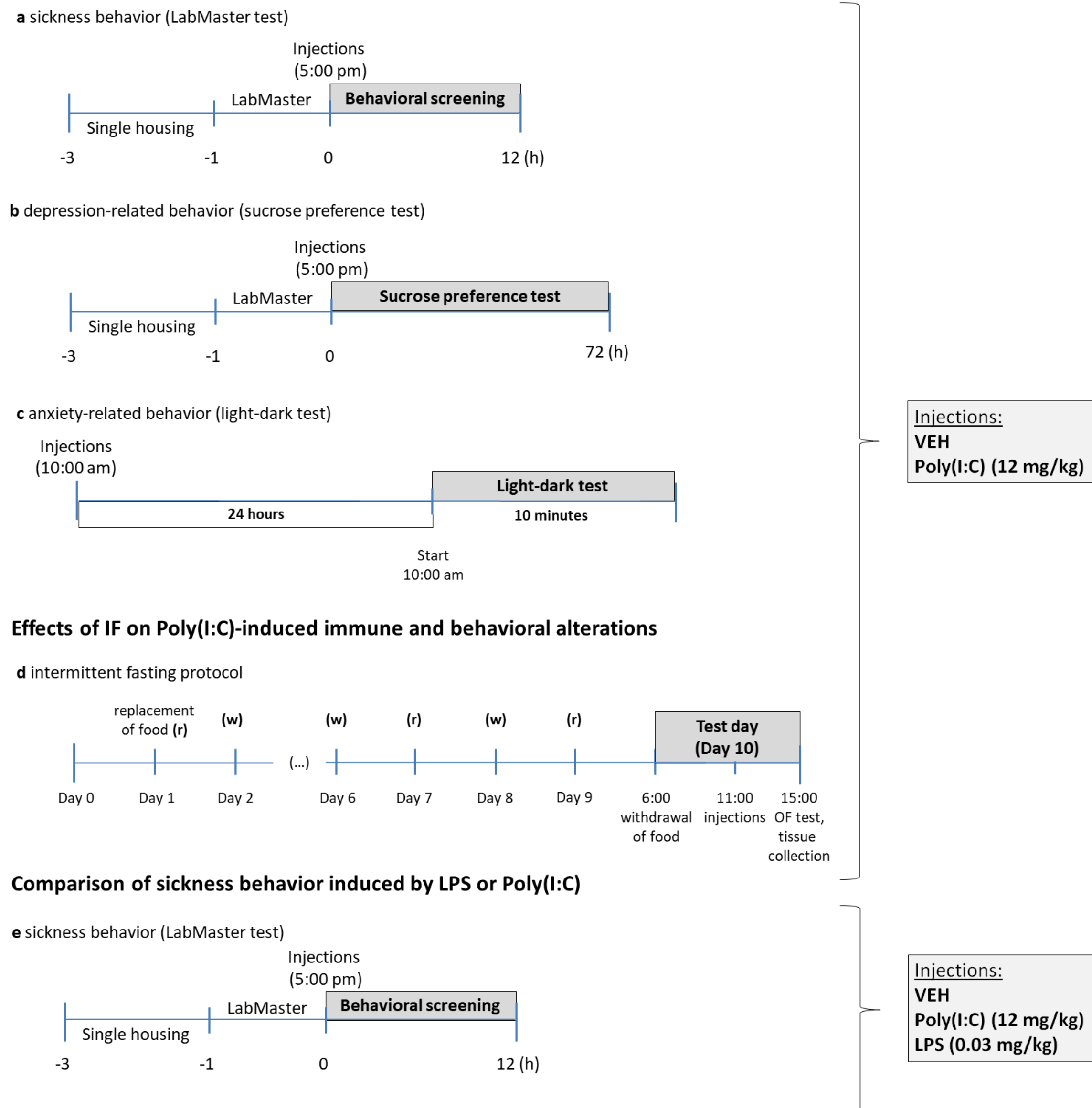

Figure 3 Protocol schemes for Project 1. Treatment groups are shown on the right side of the scheme, the time lines to the experiments on the left. (a) The time course of sickness response to i.p. injections of Poly(I:C) (12 mg/kg) was evaluated compared to VEH treated mice in the LabMaster system. (b) Assessment of depression-related behavior in the sucrose preference test and (c) assessment of anxiety-related behavior in the light-dark box test. (d) Effects of intermittent fasting (IF) for 9 days on immune and behavioral response to Poly(I:C) was evaluated on day 10. (e) Comparison of LPS- versus Poly(I:C)-induced sickness behavior. Abbreviations: w=withdrawal of food, r=replacement of food, LPS=lipopolysaccharide, OF=open field, Poly(I:C)= polyinosinic:polycytidylic acid, VEH=vehicle. Panels a-d were reproduced from Zenz et al. (2019).

LabMaster test

Before placing the 9-10 week-old mice into the LabMaster cages, the animals were habituated for at least 72 h to the drinking bottles used in the homecage-like LabMaster system (TSE Systems, Bad Homburg, Germany). Furthermore, mice were placed one by one in the LabMaster cages 24 h before the experiment, to habituate the animals to single housing as well. Single housing was necessary in order to ensure accurate measurements of activity. Animals were randomly assigned to one treatment group and were i.p. injected with either saline (VEH) or Poly(I:C) at 5:00 pm, one h before the start of the dark cycle during which mice are typically more active (Fig. 3 a, e). Ingestive behavior (water and food intake) as well as locomotion and exploratory rearing were recorded in the LabMaster for 12 h, starting from one h before to 11 h after the injection of either VEH, Poly(I:C) or LPS.

Horizontal locomotion and vertical rearing were sensed by the interruption of laser beams that were sent out by two infrared frames surrounding the transparent LabMaster cages (type III, length 42.0 x width 26.5 x height 15.0 cm). The upper infrared frame to recognize rearing was positioned 6.3 cm above the cage floor, while the lower frame was positioned 2.0 cm above the cage floor to recognize locomotion. In order to collect data about the ingestive behavior of the rodents, drinking bottles and feeding bin filled with standard rodent chow were equipped with weight transducers. The recording devices were connected to a personal computer equipped with the LabMaster software.

Sucrose preference test

The sucrose preference test was performed in the homecage-like environment of the LabMaster (described above) during 72 h following Poly(I:C) treatment, whereas a second drinking bottle filled with 1% sucrose solution in tap water was installed per cage (Figure 3 b) (Farzi et al., 2015b). The preference for water or sucrose solution was calculated as follows: sucrose intake / (sucrose intake + water intake).

Light-dark box test

In order to evaluate anxiety-related behavior evoked by injections with the viral mimic Poly(I:C), mice were subjected to the light-dark box test 24 h after Poly(I:C) treatment, when the behavioral sickness response had already abated (Figure 3 c). During the light-dark box test, animals were able to freely explore a box (37 x 21 x 20.5 cm) consisting of one dark

compartment (with black acrylic walls) and one light compartment. The light compartment was illuminated by light set to 300-400 lux (Brunner et al., 2014) and each mouse was placed individually into the light compartment at the beginning of the test. Mice could commute from one compartment to the other through a 4.5 x 6 cm door and explore the light-dark box for 10 min. The ActiMot2 laser-beam frame recording system of TSE Systems was applied to record the animals' behavior and the time spent within the light or dark compartment as well as the number of transitions between the dark and light compartment were assessed. Furthermore, the total distance moved during the exploration time was evaluated. Although mice favor to avoid light places, they tend to explore novel environments, thus only very anxious animals would stay in the dark compartment of the light-dark box throughout the whole experiment.

OF test

The OF test was conducted to analyze locomotor, exploratory and anxiety-like behavior in a new environment (Figure 3 d). Mice at the age of 11 weeks were individually placed to one corner of the opaque gray plastic OF box (length 50 x width 50 x height 30 cm), illuminated by 35 lux at floor level (Painsipp et al., 2013). Centrally above the OF, a video camera was attached to record the exploratory behavior of mice during the 5 min spent in the new environment. Collected data were analyzed with EthoVision XT (Noldus, Wageningen, The Netherlands). Following each trial, the OF box was cleaned with ethanol (70%) and with water afterwards.

2.3.2 Project 2: Effects of intranasal NPY pretreatment on LPS-induced immune and sickness response

Since LPS seemed to influence the behavior of animals for a longer period in the LabMaster test when compared to Poly(I:C), I decided to focus on LPS in the second project.

Experimental setup

In the first set of experiments, mice were screened for behavioral changes within the OF test (as described in 2.3.1) following IN infusion of NPY and i.p. LPS injections. More specifically, mice assigned randomly to a treatment group were anesthetized lightly with isofluorane and infused IN with water (10 µl) or 100 µg NPY dissolved in 10 µl water as described by others (Serova et al., 2013). To slowly infuse the fluids to the nostrils, mice

were held in a tilted backward position. By using a pipette the fluids were applied by approximately 5 droplets, while avoiding contact with the mucosa. To avoid discharge and promote NPY uptake from the nasal cavity into the brain, mice were kept in that position for another 20 seconds (s). After the IN application, mice were closely monitored and placed into their cages only when they had fully recovered from anesthesia, approximately within 5 min.

Mice received an injection of VEH or LPS into their peritoneal cavity 30 min following IN infusions with water or NPY (Fig. 4 a, b, c).

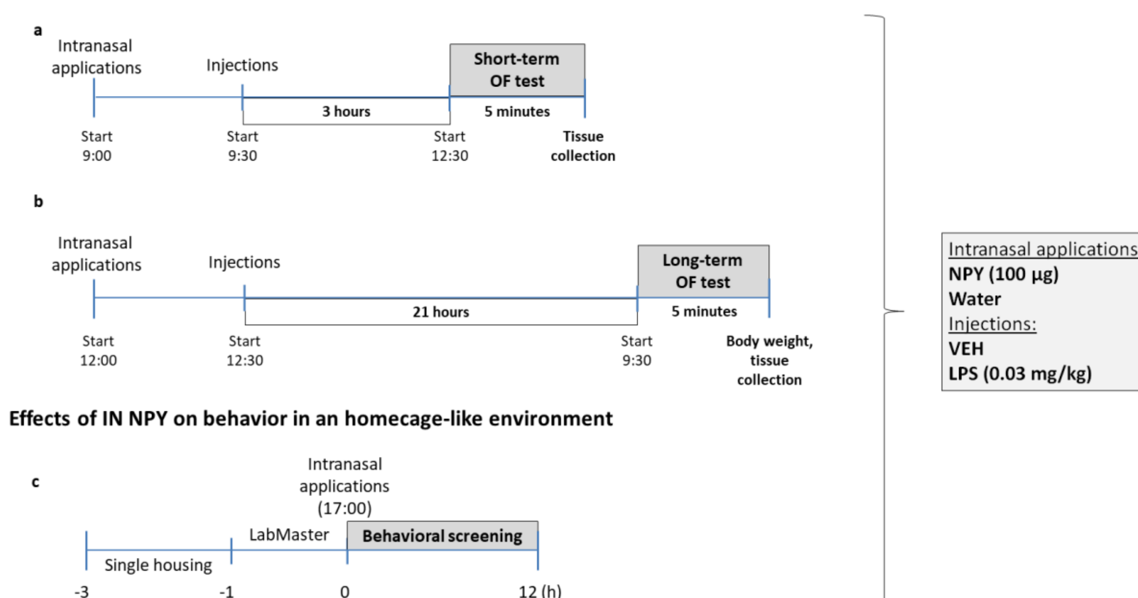

Figure 4 Protocol schemes for Project 2. Treatment groups are shown on the right side of the scheme, the time lines of the experiments on the left. (a) Experiment to assess short-term effects of IN NPY in combination with LPS treatment, (b) experiment to assess long-term effects of IN NPY in combination with LPS treatment, (c) assessment of behavioral changes due to IN NPY infusion in the homecage-like LabMaster system. Abbreviations: IN=intranasal, LPS=lipopolysaccharide, OF=open field, Poly(I:C)=polyinosinic:polycytidylic acid, VEH=vehicle.

Short Term and Long Term OF test

In order to analyze exploration, locomotion as well as anxiety-like behavior in an unfamiliar environment, mice were subjected to the OF test (Fig. 4 a, b and as described in section 2.3.1). In this set of experiments, locomotion, rearing and anxiety-like behavior was assessed at two time points post i.p. injections with VEH or LPS. The acute sickness response,

referred to as the *short term* effects, to LPS was evaluated 3 h after injection, while *long term* effects were evaluated 21 h post-treatment. EthoVision XT (Noldus, Wageningen, The Netherlands) was used in this set of experiments to produce heatmaps, which depict the tracks of exploration and visualize the duration that mice spent in the different compartments of the OF box. A representative heatmap per treatment group is shown in the Results part (Fig.15 e).

Evaluation of ingestive behavior following NPY infusion

To analyze altered food and water intake of mice following IN application of NPY, ingestive behavior was screened within the LabMaster system (Figure 4 c, as described in section 2.3.1). Mice were infused IN with NPY at approximately 5:00 pm and subsequently placed into the LabMaster cages, where their behavior was recorded for 12 h.

2.3.3 Molecular readouts

Blood and brain tissue harvesting

Mice were anesthetized deeply directly after the OF tests with 150 mg/kg pentobarbital i.p. Using a syringe filled with 100 µl of the anticoagulant sodium citrate (3.8%), cardiac puncture was performed to draw blood. In order to gain plasma for the analysis of circulating cytokines and other inflammatory markers, blood was centrifuged at 1000 x g for 15 min at 4°C. Plasma was stored at -70°C until further use. Subsequently after blood was drawn, brains were collected and immediately transferred to 2-methylbutane (Sigma-Aldrich, Vienna, Austria) on dry ice for 10 s. Brains were stored at -70°C until microdissection.

Circulating cytokines and CORT

Concentrations of cytokines in the plasma samples were evaluated by the use of ProcartaPlex™ immunoassays (eBioscience, San Diego, CA, USA) according to the manufacturer's specifications and as described previously (Farzi et al., 2015b). The following cytokines were measured in duplicates: IFN-α, IFN-β (catalogue number EPX020-22187-901), IL-1β, TNF-α, IL-6, IFN-γ, MCP-1, IL-10 (custom panel). Quantification of fluorescent signals was performed by using the Bio-Plex 200 multiplex suspension array system and Luminex® xMAP® technology and the Bio-Plex 5.0 software (BioRad, Hercules, CA, USA) was used to analyze the data. Whenever cytokine levels were below detection level, they were assigned a value of zero. Mice of all groups of the IF protocol

presented with IL-1β levels that were too low to be detected and therefore, IL-1β will not be displayed in the results of that project. Circulating levels of CORT were analyzed with an enzyme immunoassay kit with a sensitivity of 0.027 ng/ml (Assay Designs, Ann Arbor, Michigan, USA) according to the manufacturer's specifications and as previously described (Farzi et al., 2015b).

Microdissection of brain tissue

In order to analyze expression patterns of cytokines and other molecular entities, brains were microdissected for hypothalamic regions (Bregma +0.38 to -2.92, Fig. 5). Dissection was performed under a stereomicroscope on a cold plate with the temperature set to -20°C (Weinkauf Medizintechnik, Forchheim, Germany) as previously described (Farzi et al., 2015b). Before the dissection of each brain, dissection instruments and working area were cleaned with RNase AWAY (Carl Roth, Karlsruhe, Germany). MagnaLyser bead tubes purchased from Roche Diagnostics (catalogue number: 03358 941 001, Rotkreuz, Switzerland) were filled with Precellys beads (Peqlab, Erlangen, Germany) and used to collect hypothalamic brain areas. The tissue was stored at -70°C until RNA extraction.

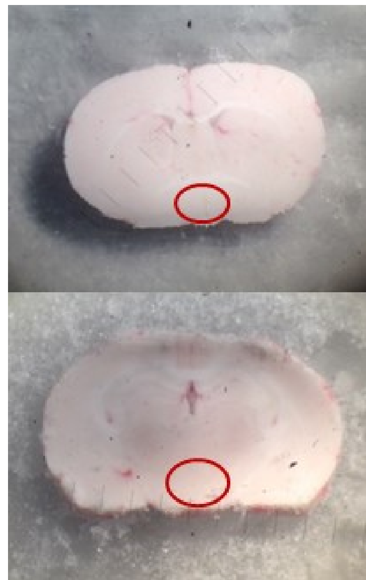

Figure 5 Photographs of two representative consecutive murine brain sections that illustrate the microdissection procedure. Red cycles illustrate the hypothalamic brain areas that were harvested and analyzed for mRNA expression. Note that usually more than 2 sections contained hypothalamic areas and were used for analysis.

RNA extractions

RNA was extracted from brains of all protocols and treatment groups by the use of RNeasy lipid tissue mini kit (Qiagen, Hilden, Germany) and RNA concentrations were determined with NanoDrop (Thermo Fisher Scientific, Vienna, Austria).

Reverse transcription and quantitive real-time PCR (qPCR) of hypothalamic brain tissue

Brains harvested from mice assigned to the IF protocol, were analyzed for expression patterns of cytokines by qPCR. First, 2 µg of RNA were reverse-transcribed by the use of the high capacity cDNA reverse transcription kit purchased from Thermo Fisher Scientific (Vienna, Austria) and the Mastercycler Gradient (Eppendorf, Hamburg, Germany) (Appendix 6.1). In order to quantify mRNA levels, each sample was analyzed in triplicates via qPCR using TaqMan gene expression assays and the LightCycler 480® system (Appendix 6.2). For qPCR, the TaqMan gene expression master mix (Thermo Fisher Scientific) as well as the following assays were used: IFN-γ (Mm01168134_m1), IL-1β (Mm00434228_m1), IL-6 (Mm00446190_m1), NPY (Mm03048253_m1) and TNF-α (Mm00443258_m1). For each group, negative controls from the reversed transcription without the enzyme added were included. The following endogenous housekeeping genes (HK; reference genes) were used: Ppil3 (Mm00510343_m1) and Actb (Mm00607939_s1). Mean values of the vehicle treated group were used as a calibrator and quantification of target gene levels relative to controls were performed by the use of the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Differences between treatment groups were expressed as fold changes.

Nanostring analysis

Nanostring analysis was used to assess mRNA expression patterns in the hypothalamus of mice assigned to the IN NPY protocol as suggested by the supplier's instructions and as reported by others (Ayers et al., 2017). For this method, total mRNA nCounter PlexSet probe and oligonucleotide design for 24 genes was obtained from NanoString Technologies (NanoString Technologies, Seattle, WA, USA). The HK genes included Ppil3, Ywhaz, Tubb5 and Ube2d2 (Appendix Table 3), and oligonucleotides were synthesized at Integrated DNA Technologies (Leuven, Belgium). In order to determine the required RNA amount for

hybridization, titration was performed with 20 ng, 100 ng and 200 ng according to the supplier's instructions. For the 20 h PlexSet hybridization reaction, 1190 ng RNA per sample was used, following the manufacturer's guidelines. An nCounter MAX prep station (NanoString Technologies, Seattle, WA, USA) at the Core Facility Molecular Biology of the Center for Medical Research (Medical University of Graz) was used to process samples. A generation II nCounter Digital Analyzer (NanoString Technologies, Seattle, WA, USA) was used to scan cartridges. nCounter data files (RCC files) were used for data analysis and were imported into the NanoStrings nSolver 4.0 analysis software. Pre-processing and normalization of raw data was performed for standard procedures as suggested by the manufacturer (positive normalization to geomean of top3 positive controls, codeset content normalization using HK Ppil3, Ywhaz, Tubb5, Ube2d2 and codeset calibration with the reference sample).

To calculate fold-change differences in the gene expression between the treatment groups, means of mRNA values of the control group (water/VEH) were used.

2.3.4 Statistics

Statistical analysis was performed by the use of GraphPad® Prism6 (Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com) and SPSS 20 (SPSS Inc., Chicago, Illinois, USA).

Project 1: Analysis of Poly(I:C)-induced sickness response and the effects of preceding intermittent fasting

LabMaster experiments to evaluate behavioral effects of Poly(I:C)

LabMaster data, including the sucrose preference test, were analyzed by repeated measures (RM) two-way analysis of variance (ANOVA). Post-hoc Bonferroni's multiple comparison test was used to compare values for each time point under study.

Light-dark box test

RM two-way ANOVA with Bonferroni's multiple comparison test was performed to evaluate the time mice spent within the dark versus the light compartment of the box. Furthermore, unpaired t-tests were performed to evaluate whether animals injected with VEH or Poly(I:C) differed in the numbers of transitions and the total distance traveled.

Intermittent fasting experiments

Data obtained from the IF experiment were analyzed with two-way ANOVA. For planned comparison, Sidak's multiple comparison test was performed to compare between the treatment groups and the AL/VEH control group. Extreme outliers were excluded, meaning values that lay more than three times the interquartile range off a quartile. This was applied in the analysis of four circulating cytokines and CORT: IL-6 (one extreme outlier, in both vehicle treated groups), IFN-β (one extreme outlier, in the IF/Poly(I:C) group), IL-10 (one extreme outlier, in the AL/VEH group), IFN-γ (one extreme outlier, in the AL/VEH group), CORT (one extreme outlier, in the IF/Poly(I:C) group).

LabMaster experiments to compare effects of Poly(I:C) and LPS

RM two-way ANOVA followed by Bonferroni's post-hoc test was performed to analyze the data of all LabMaster experiments, values of p-≤ 0.05 were regarded significant.

Project 2: Effects of intranasal NPY pretreatment on LPS-induced immune and sickness response

Intranasal NPY Experiments

Two-way ANOVA followed by Tukey's multiple comparison post-hoc test in cases of significant interactions ($p \leq 0.05$) between IN x i.p. treatment was performed to analyze the data of all experiments.

3 Results

3.1 Project 1: Analysis of Poly(I:C)-induced sickness response and the effects of preceding intermittent fasting

The results described in this part of the dissertation were published in Zenz et al. (2019).

3.1.1 Behavioral sickness peaks around 4 h following i.p. Poly(I:C) injections

Behavioral sickness, as assessed by reduced exploratory activity in the homecage-like environment of the LabMaster system, was found to peak between 4-6 h after i.p. Poly(I:C) injections. This specific time course was observed in all parameters under study. Thus, RM two-way ANOVA and Bonferroni's multiple comparison post-hoc test revealed that horizontal locomotion, vertical exploratory behavior (rearing) as well as food and water intake were altered by Poly(I:C) treatment (Fig. 6). During the second interval (4-6 h post-injection), Poly(I:C)-injected animals differed the most significantly in their behavior from the vehicle-treated group. Furthermore, while food intake was already significantly reduced within the first interval (Fig. 6 d), both water intake as well as rearing behavior were reduced until 7-9 h following i.p. injections with Poly(I:C) when compared to the control group (Fig. 6 b, c). These results were recently published in (Zenz et al., 2019).

Taking into account both the previous work by others (Cunningham et al., 2007, Michalovicz and Konat, 2014, Murray et al., 2015) and the clear sickness response 4 h post-treatment found in this study, further experiments to study Poly(I:C)-evoked sickness were conducted at that time point (Zenz et al., 2019).

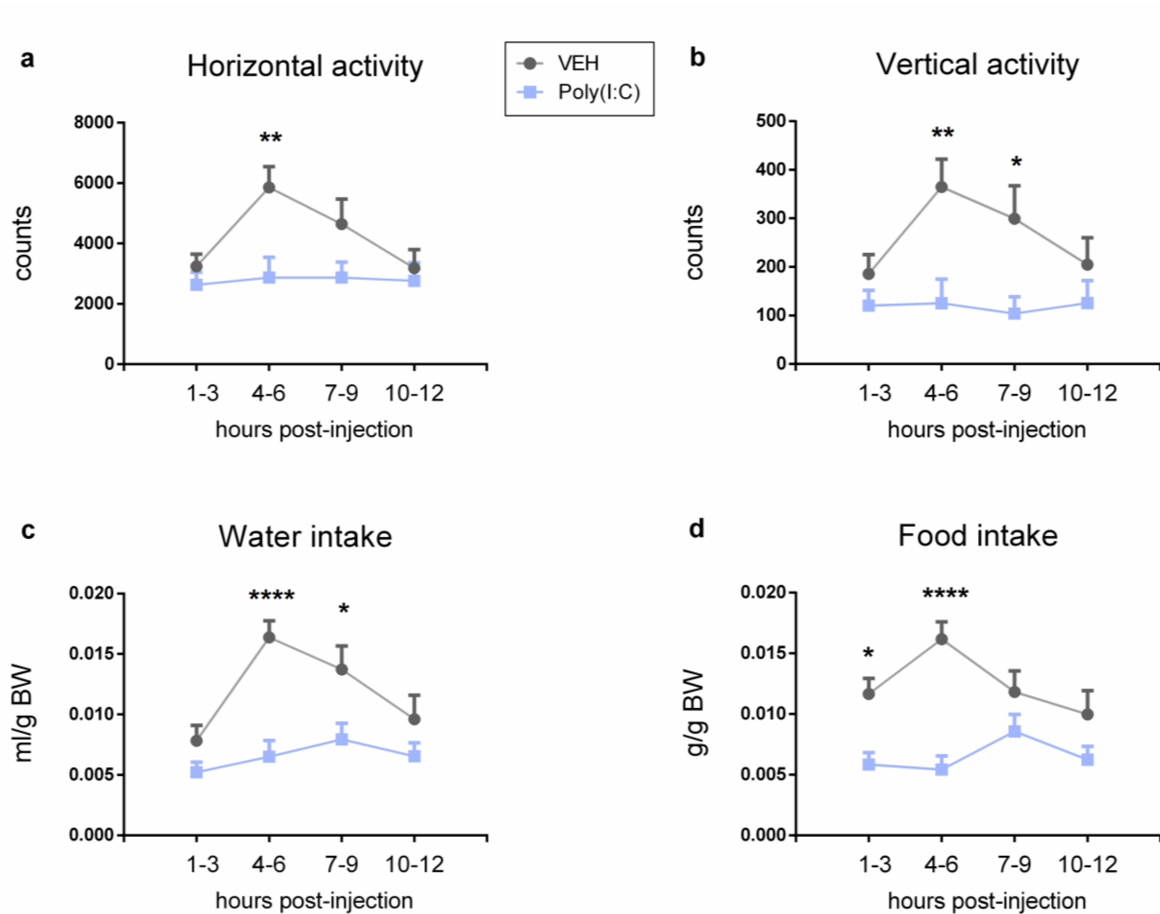

Figure 6 Effects of Poly(I:C) injected i.p. on recorded beam breaks by horizontal locomotor behavior (a), beam breaks by vertical exploratory behavior (rearing) (b), water intake (c) and food intake (d). At 0 h, Poly(I:C) (12 mg/kg) or vehicle (VEH) was applied and all parameters under study were recorded during the subsequent 12 h. Values represent means + SEM, n=6; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ compared to VEH at the time point indicated (Bonferroni's multiple comparison test). RM two-way ANOVA results: interaction of time and treatment in horizontal locomotion; $F(3,102)=2.559$, $p=0.0592$, vertical behavior (rearing); $F(3,102)=1.992$, $p=0.1199$, water intake; $F(3,102)=2.661$, $p=0.0521$, food intake; $F(3,102)=3.256$, $p \leq 0.05$. Abbreviations: BW, body weight. These findings were published in an original article (Zenz et al., 2019).

3.1.2 Poly(I:C) treatment does not induce anhedonic or anxiety-related behavior

Depression-like behavior was assessed by the sucrose preference test, which aims to evaluate long-term anhedonic behavior after the acute phase has already abated (Farzi et al., 2015b), as non-depressed mice usually favor the sweet taste of the sucrose solution over normal water. There was no significant difference between the two treatment groups in their sucrose

intake and thus, Poly(I:C)-injected animals were not found to develop depression-related behavior (Fig.7).

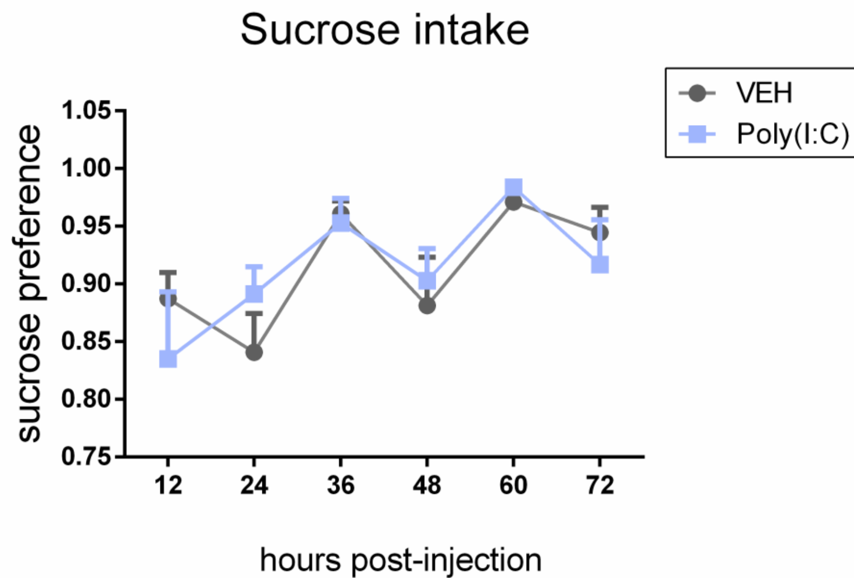

Figure 7 Sucrose preference during 72 h following i.p. treatment with Poly(I:C). Mice were injected with Poly(I:C) (12 mg/kg) or VEH at time point 0 and their intake of tap water and 1% sucrose solution was recorded for 72 h. Intervals represent intake of water or sucrose solution during periods of 12 h each following i.p. treatment. Sucrose preference was calculated with the following formula: sucrose intake / (sucrose intake + water intake). Values represent means + SEM, n=7-9; RM two-way ANOVA results: interaction of time and treatment; $F(5,70)=0.8302$, $p=0.5325$, there were no significant differences between treatment groups as analyzed by Bonferroni's post-hoc test.

At 24 h following i.p. injections with Poly(I:C), mice were subjected to the light-dark box test to evaluate their anxiety-related behavior at a time point when the acute phase of sickness had already abated (see Fig. 6). Mice did not show any anxiety-related behavior due to treatment with the viral factor, as Poly(I:C) injected animals spent a similar amount of time exploring the two compartments as their VEH-treated controls (Fig. 8 b). Furthermore, the transitions between the dark and the light compartment of the light-dark box as well as the distance traveled throughout the experiment were unaltered by Poly(I:C) injections at that time point (Fig. 8 a, c).

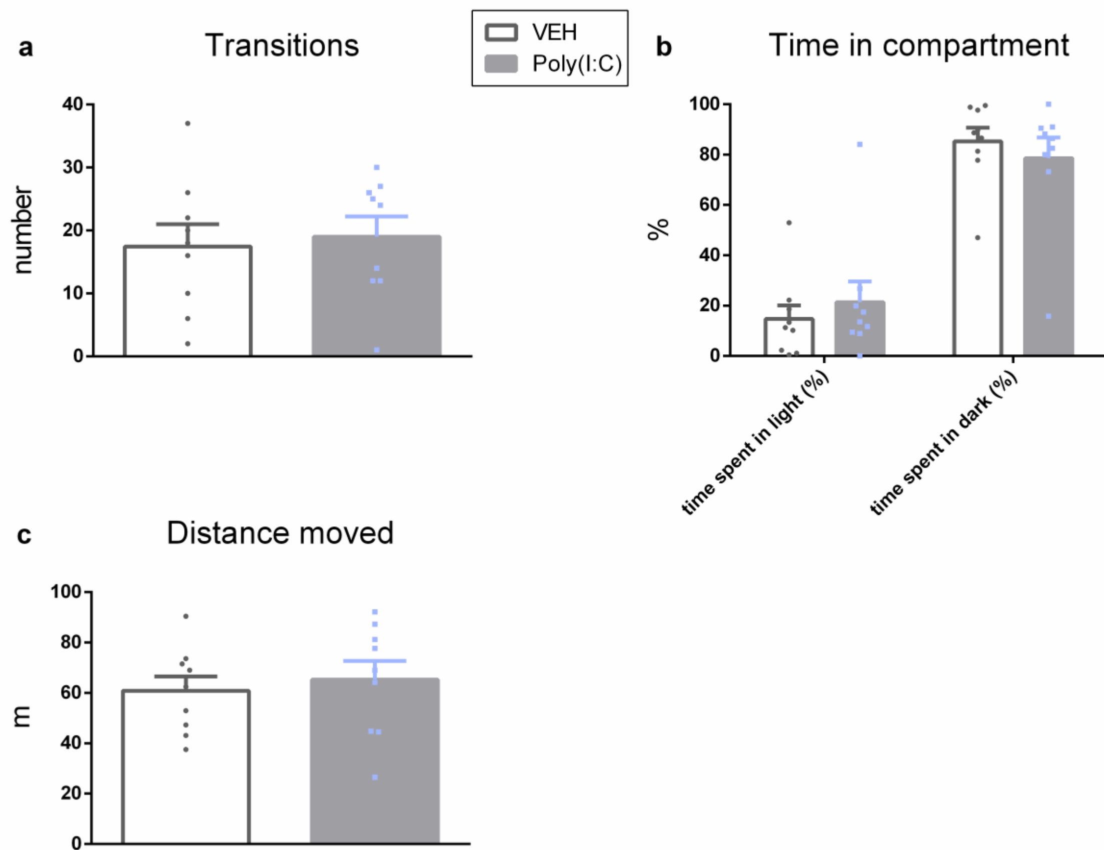

Figure 8 Light-dark box test 24 h following i.p. Poly(I:C) treatment. Mice were injected with Poly(I:C) (12 mg/kg) or VEH and their behavior within the light-dark box was recorded 24 h thereafter. Values represent means + SEM, n=9; (a) t-test analysis revealed that there was no difference between the treatment groups in the numbers of transitions: p=0.7496, (b) two-way ANOVA results: there was no interaction of time spent within the compartments x treatment; F(1,32)=0.9014, p=0.3495, (c) t-test results for the distance moved: p=0.6450.

3.1.3 The overall body weight is stable in intermittently fasted mice

Mice were weighed before the beginning of the fasting intervention and at the day of behavioral experiments (Day 10) at 6:00 am, when mice from the IF-group were deprived of food. The body weight of mice was not changed by the IF protocol when compared to the AL feeding regimen as analyzed by RM two-way ANOVA and Bonferroni's multiple comparison test **(**Fig. 9**)**, which is in line with observations by others (Gotthardt et al., 2016).

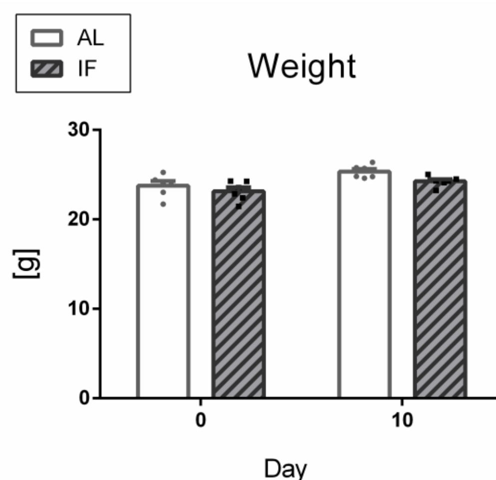

Figure 9 Body weight of animals under ad libitum (AL) feeding or intermittent fasting (IF) regimen. Mice were fed AL or kept on an alternate-day fasting regimen (IF) for 9 days. Weight of the mice was recorded at 6:00 am (Day 0 and Day 10), values represent means + SEM, n=7-8; there was no significant difference between AL and IF animals (Bonferroni's multiple comparisons test). RM two-way ANOVA: interaction of time x feeding on body weight; $F(1,10)=0.7946$, $p=0.3937$. These findings were recently published in an original article (Zenz et al., 2019).

3.1.4 Poly(I:C)-induced sickness behavior in the OF test is apparent only in fasted mice

The OF test was applied to evaluate exploratory behavior, which is known to be motivated by presentation of a novel environment. As depicted in Fig. 10, only mice subjected to IF and treated with the viral mimic Poly(I:C) showed inhibition of exploratory behavior in the OF. In more detail, all four parameters, distance moved, the time spent immobile, the frequency of central area visits and the speed of movements were changed by both IF and Poly(I:C) treatment (Fig. 10 a-d). Planned comparison furthermore showed that AL fed animals injected with Poly(I:C) did not present significant signs of sickness behavior when compared to saline-injected mice under the same feeding regimen (Fig. 10 a-d). However, the administration of Poly(I:C) in combination to preceding IF significantly reduced the traveled distance (Fig. 10 a), extended the time spent immobile (Fig. 10 b), decreased the interest to visit the central area (Fig. 10 c) and reduced the speed of movements, when compared to mice injected with saline and fed AL (Fig. 10 d).

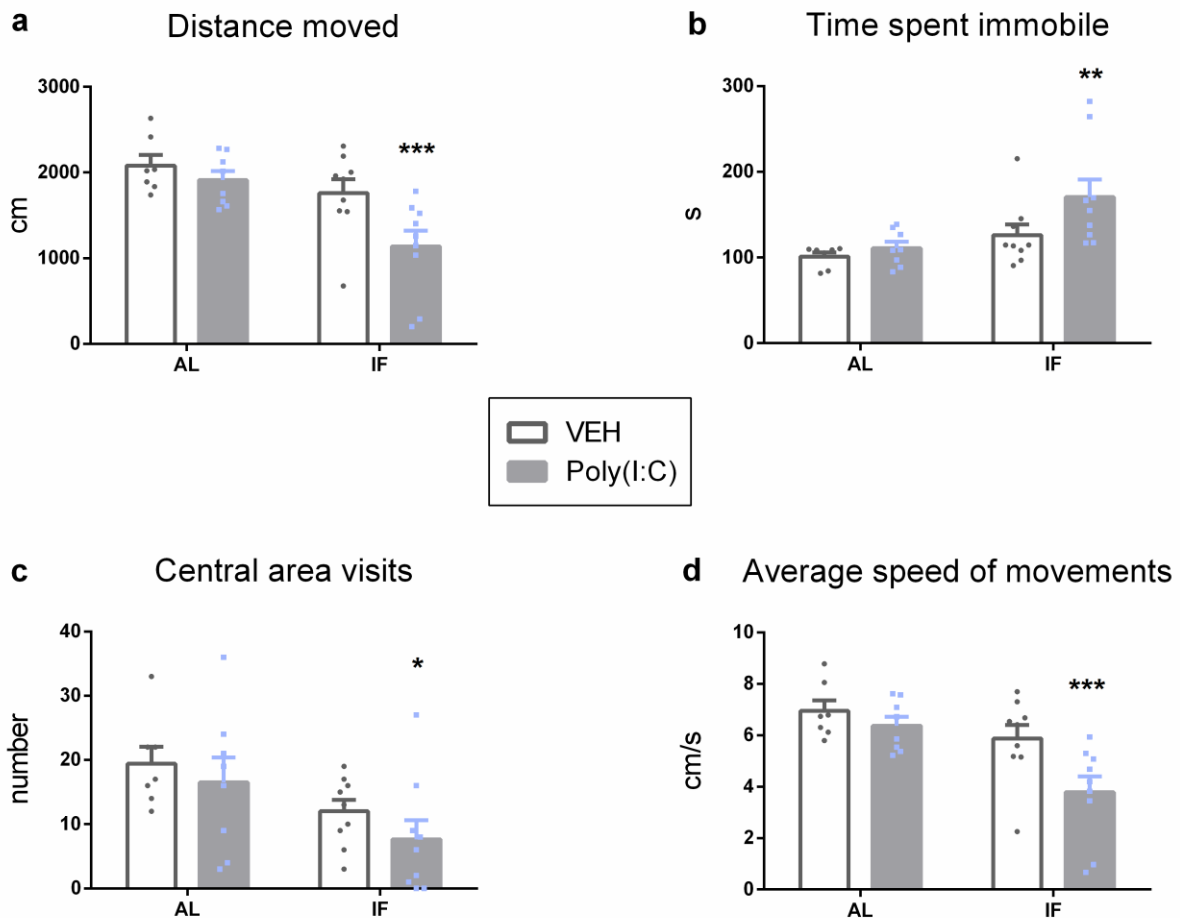

Figure 10 Exploratory behavior following intermittent fasting (IF) and Poly(I:C) treatment. Mice were fed ad libitum (AL) or kept on an alternate-day fasting regimen (IF) for 9 days. Animals were treated i.p. with saline (VEH) or Poly(I:C) (12 mg/kg) on day 10 and subjected to the new environment of the open field (OF) to evaluate sickness-related behavior 4 h following injections: (a) distance traveled, (b) time spent immobile, (c) visits to the central area of the OF, (d) average velocity of movements. Values represent means + SEM, n=7-9; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ compared to AL/VEH (Sidak's multiple comparison test). Two-way ANOVA: interaction of feeding x i.p. treatment on distance traveled; $F(1,29)=2.164$, $p=0.1521$, on time spent immobile; $F(1,29)=1.575$, $p=0.219$, on the number of central area visits; $F(1,29)=0.05753$, $p=0.8121$, the speed of movements; $F(1,29)=2.163$, $p=0.1521$. These findings were published in an original article (Zenz et al., 2019).

3.1.5 Intermittent fasting amplifies plasma cytokine levels that are elevated in response to Poly(I:C) treatment and increases circulating CORT levels

Plasma levels of cytokines were found to be elevated by Poly(I:C) treatment at 4 h after injection, an effect further enhanced by preceding fasting, relative to vehicle-injected

animals fed AL (Fig. 11). Significant differences between the groups, as evaluated by planned comparison, were found in the IF/Poly(I:C) group compared to AL/VEH treated animals for plasma levels of TNF-α, IL-6, IL-10, IFN-α and IFN-γ (Fig. 11 a, c, d, e, g). It is important to note that these cytokines were not significantly increased by Poly(I:C) treatment with preceding AL access to food.

Circulating levels of MCP-1 and IFN-β, however, were found increased in Poly(I:C)-treated animals of both feeding regimens relative to the AL/VEH group (Fig 11 b, f).

Two-way ANOVA followed by Sidak's multiple comparison test revealed a significant difference of circulating CORT levels between treatment groups (Fig. 11 h) and IF was found to enhance the release of CORT into the plasma. Thus, CORT levels were significantly elevated in both IF groups when compared to the AL/VEH controls, while Poly(I:C) treatment did not influence CORT levels to a significant extent (Fig. 11 h).

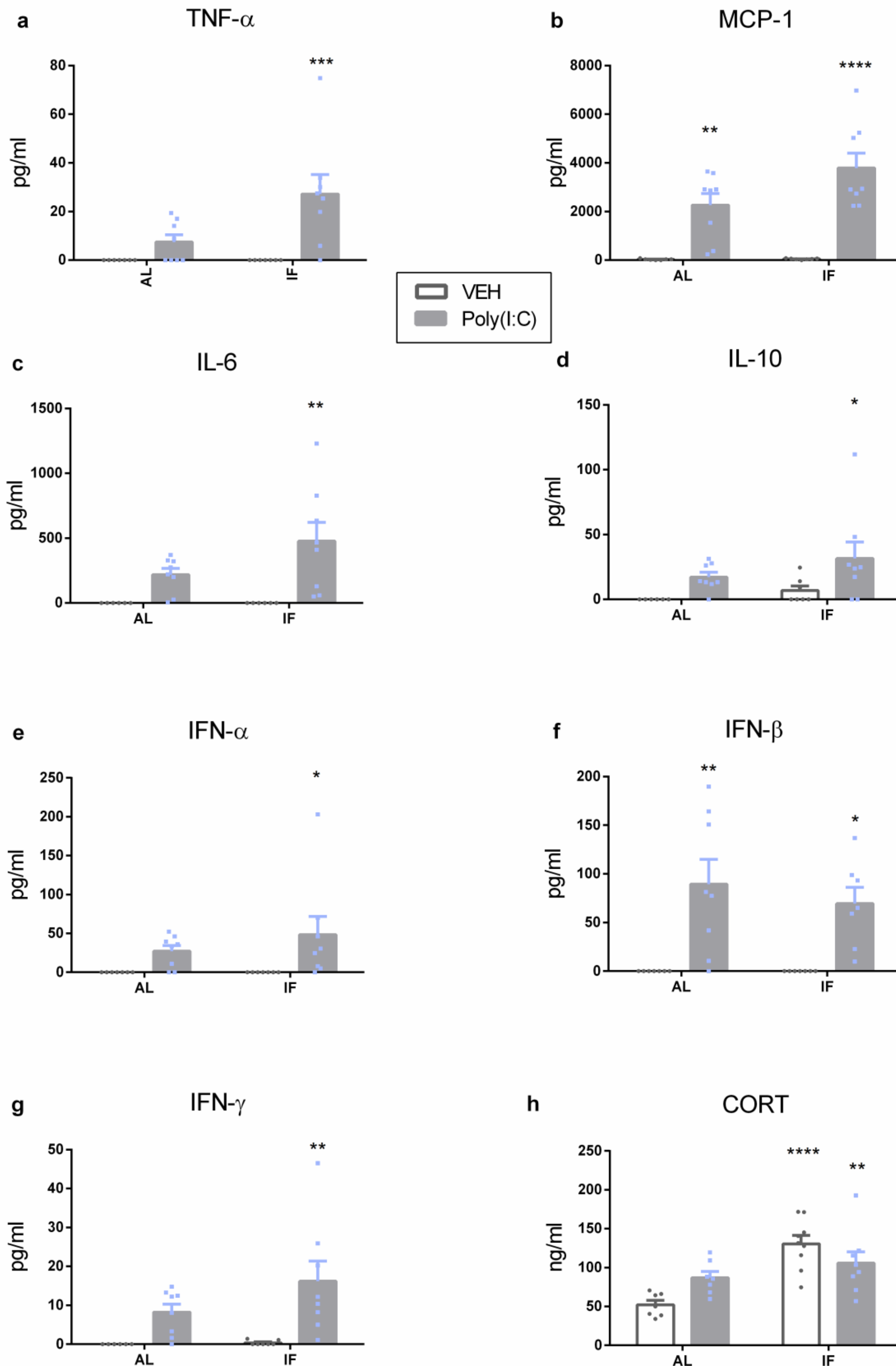

Figure 11 Plasma cytokine and corticosterone (CORT) levels following intermittent fasting (IF) and Poly(I:C) treatment. Mice were fed ad libitum (AL) or kept on an alternate-day fasting regimen (IF) for 9 days. Animals were treated i.p. with saline (VEH) or Poly(I:C) (12 mg/kg) on day 10. Blood was drawn 4 h

following injections to evaluate plasma cytokine and CORT levels. Values represent means + SEM, (a,e,f) n=6-8, (b,c,d,g) n=7-8, (h) n=7-9. a-h: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$ compared to AL/VEH (Sidak's multiple comparison test). Two-way ANOVA: interaction of feeding x i.p. treatment on plasma TNF-α; $F(1,26)=4.636$, $p \leq 0.05$, on plasma MCP-1; $F(1,26)=3.222$, $p=0.0843$, on IL-6; $F(1,24)=2.062$, on IL-10; $F(1,25)=0.2464$, $p=0.6240$, on IFN-α; $F(1,26)=0.6399$, $p=0.4310$, on IFN-β; $F(1,25)=0.3809$, $p=0.5427$, on IFN-γ; $F(1,25)=1.492$, $p=0.2334$, on CORT; $F(1,27)=7.638$, $p \leq 0.05$. These findings were published in an original article (Zenz et al., 2019).

3.1.6 Peripheral Poly(I:C) injections induce cytokine mRNA expression in the hypothalamus

Pro-inflammatory cytokine mRNA expression levels were found altered by peripherally injected Poly(I:C) in both AL and IF animals (Fig. 12). In more detail, planned comparison revealed that mRNA levels of IFN-γ, TNF-α, IL-6 and IL-1β were significantly elevated in the hypothalamus by peripheral Poly(I:C)-treatment, independently of feeding regimen (Fig. 12 a-d).

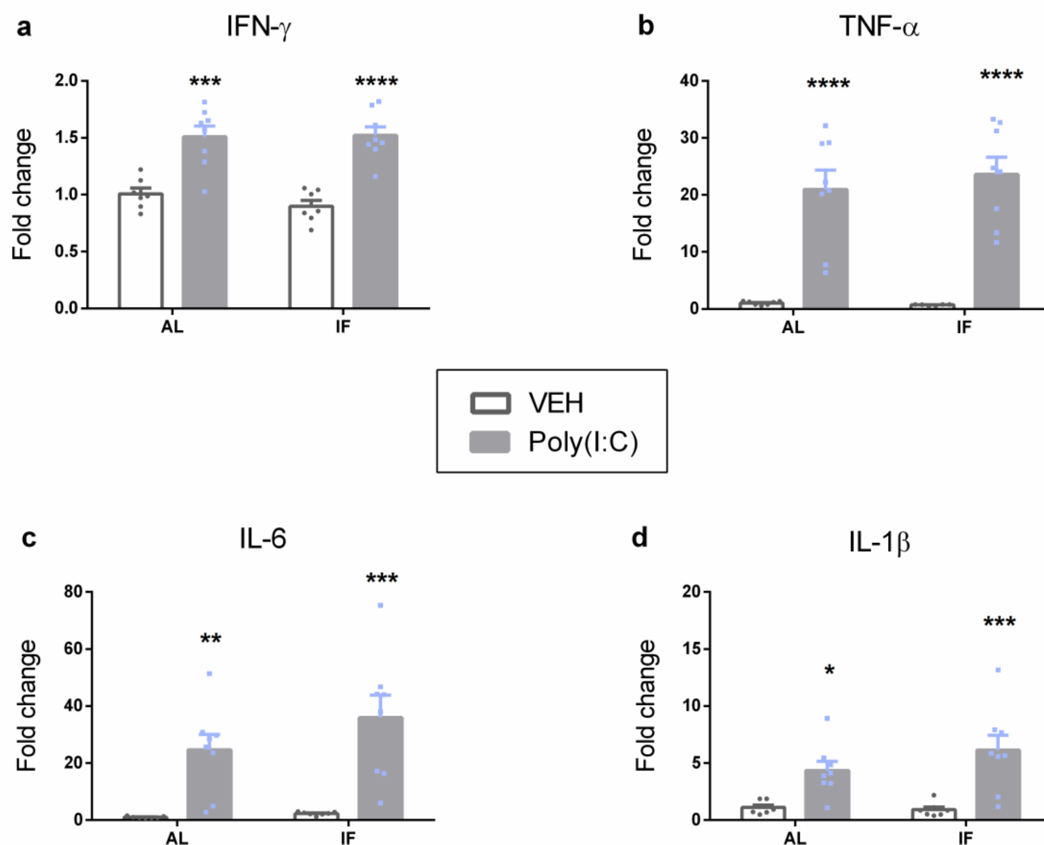

Figure 12 Hypothalamic mRNA expression patterns following intermittent fasting (IF) and peripheral Poly(I:C) treatment. Mice were fed ad libitum (AL) or kept on an alternate-day fasting regimen (IF) for 9

days. Animals were treated i.p. with saline (VEH) or Poly(I:C) (12 mg/kg) on day 10 and brains were extracted 4 h later to analyze hypothalamic mRNA expression of cytokines. Values represent means + SEM, n=7-8; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p≤0.0001 compared to AL/VEH (Sidak's multiple comparison test). Two-way ANOVA: interaction of feeding x i.p. treatment on hypothalamic IFN-γ; $F(1,26)=0.6824$, $p=0.4163$, on TNF-α; $F(1,25)=0.3376$, $p=0.5664$, on IL-6 $F(1,26)=0.9556$, $p=0.3373$, on IL-1β; $F(1,26)=1.401$, $p=0.2473$. These findings were published in an original article (Zenz et al., 2019).

3.1.7 NPY mRNA expression is enhanced by intermittent fasting in the hypothalamus

NPY mRNA expression levels in the hypothalamus were found to differ between the treatment groups and it was shown that fasting affected hypothalamic NPY levels, as both IF/VEH and IF/Poly(I:C) treated animals presented with significantly higher hypothalamic NPY mRNA levels when compared to AL/VEH controls (Fig. 13).

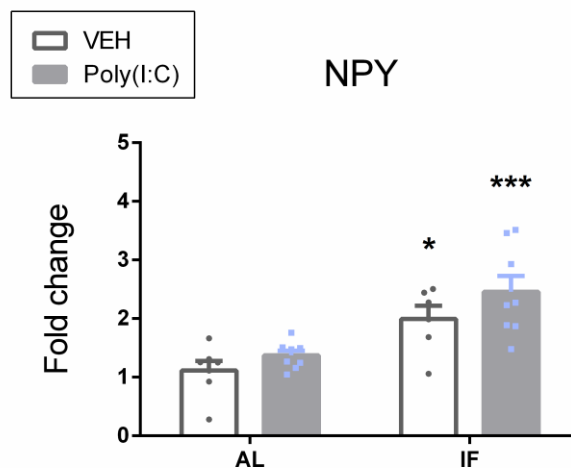

Figure 13 Hypothalamic neuropeptide Y (NPY) expression following intermittent fasting (IF) and Poly(I:C) treatment. Mice were fed ad libitum (AL) or kept on an alternate-day fasting regimen (IF) for 9 days. Animals were treated i.p. with saline (VEH) or Poly(I:C) (12 mg/kg) on day 10 and brains were extracted 4 h later to analyze hypothalamic mRNA expression of NPY. Values represent means + SEM, n=6-8; *p ≤ 0.05, ***p ≤ 0.001 compared to AL/VEH (Sidak's multiple comparison test). Two-way ANOVA: interaction of feeding x i.p. treatment on NPY mRNA expression; $F(1,25)=0.2708$, $p=0.6074$. These findings were published in an original article (Zenz et al., 2019).

3.1.8 LPS is more potent in inducing behavioral sickness compared to Poly(I:C)

Given the relatively mild effects of Poly(I:C) treatment without preceding fasting on behavior in the OF test as described above (in 3.1.4), I wanted to know whether the sickness-inducing effects of the viral mimic are comparable to those observed in rodents treated with LPS. Therefore, mice were injected with either Poly(I:C) at the same dose used in the IF experiments or with LPS at a subseptic dose (0.03 mg/kg) and subsequently subjected to the LabMaster system to screen the animals' behavior during 12 h following treatment (Fig. 14). RM two-way ANOVA revealed an interaction between time x i.p. treatment on horizontal locomotor behavior and a trend for an interaction between time x i.p. treatment with LPS on vertical rearing behavior. Post-hoc Bonferroni's multiple comparison test revealed that LPS at that comparably low dose was more potent than Poly(I:C) to depress exploratory behavior, as reduced locomotion and rearing could be observed for a longer period in mice injected with LPS (Fig. 14 a, b). Therefore, I decided to conduct the experiments in the second part of this thesis with LPS.

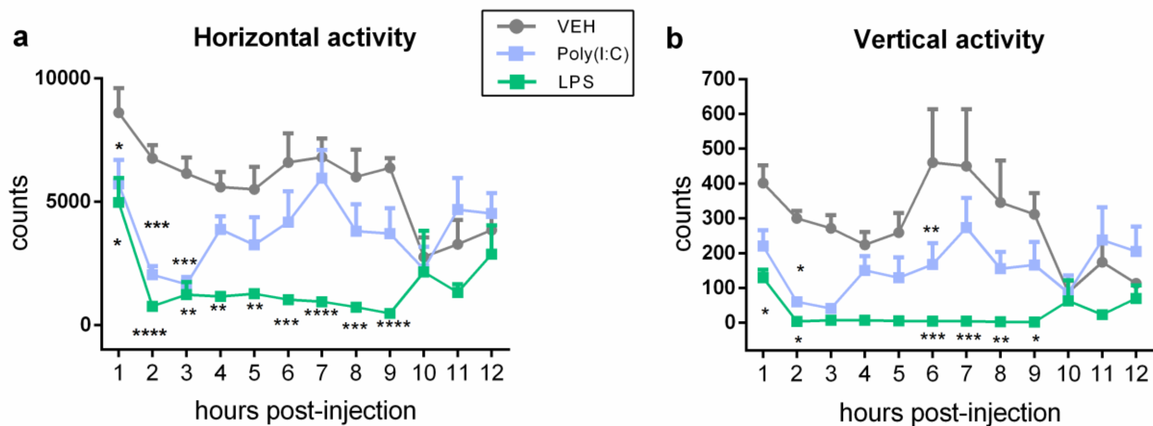

Figure 14 Effects of Poly(I:C) and LPS injected i.p. on beam breaks by horizontal locomotor activity (a) and by vertical exploratory behavior (rearing) (b). At time point 0, Poly(I:C) (12 mg/kg), LPS (0.03 mg/kg), or vehicle (VEH) was injected i.p. to mice and parameters under study were recorded during the subsequent 12 h. Values represent means + SEM, n=4-8; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$ compared to VEH at the time point indicated (Bonferroni's multiple comparison test). Statistically significant differences between Poly(I:C) and VEH are depicted above the blue curve, significant differences between LPS and VEH are depicted below the green curve. RM two-way ANOVA: interaction between time x i.p. treatment on horizontal activity; $F(22,165)=2.230$, $p \leq 0.01$, on vertical activity; $F(22,165)=1.538$, $p=0.0678$.

3.2 Project 2: Effects of intranasal NPY pretreatment on LPS-induced immune and sickness response

3.2.1 Intranasal pretreatment with NPY abates behavioral sickness response to LPS 3 h following treatment

Exploratory behavior within the new environment of the OF was significantly different between the treatment groups under study 3 h following peripheral injection of VEH or LPS (Fig. 15). A significant interaction was revealed by two-way ANOVA between IN NPY pretreatment and i.p. LPS injections in all parameters under study, namely the distance traveled, the time spent within the center zone of the OF, the time spent immobile, as well as the number of visits to the central area of the OF (Fig. 15 a-d). Post-hoc Tukey's multiple comparison test furthermore revealed a significant difference in the behavioral parameters under study only in water/LPS treated mice when compared to water/VEH controls (Fig. 15 a-d). As opposed to this, the NPY/LPS group did not present with significant behavioral alterations in comparison to the water/VEH control group. Thus, IN pretreatment with NPY prevented LPS-induced behavioral sickness response 3 h post-injections, as depicted in Figure 15.

By 21 h following i.p. LPS injections, the acute signs of sickness had abated, when no statistically significant difference between the treatment groups could be observed (Fig. 16).

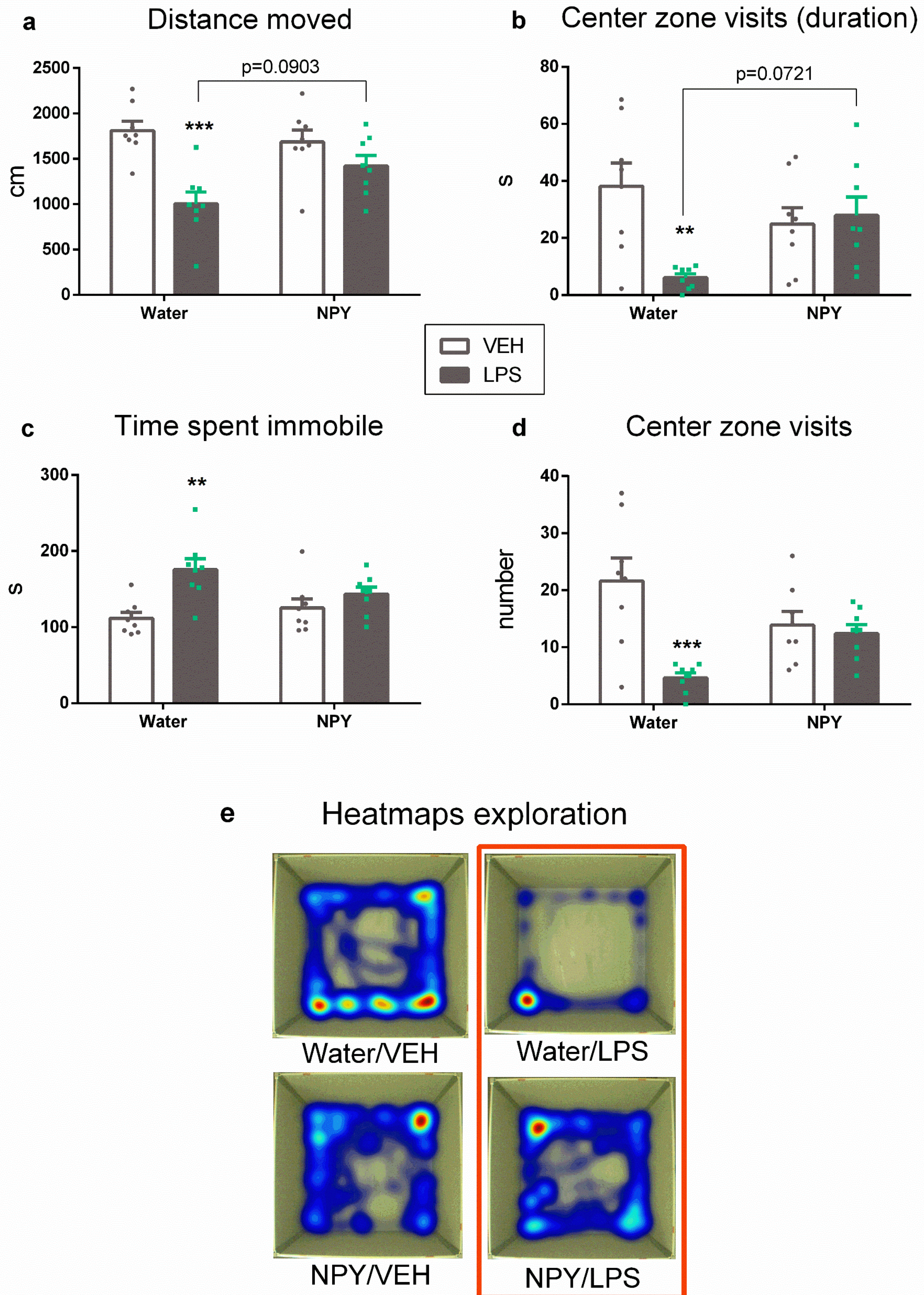

Figure 15 Effects of pretreatment with intranasal (IN) NPY and i.p. injections of LPS on behavior in the open field (OF) test 3 h after treatment. Mice were IN infused with either water or NPY (100 µg), and 30 min later injected i.p. with saline (VEH) or LPS (0.03 mg/kg). The OF test was performed 3 h later in order to analyze sickness-related behavior: (a) distance traveled, (b) time remained within the center zone, (c) time remained immobile, (d) number of visits to the central area, (e) representative heatmaps of exploratory behavior as recorded 3 h after treatment. Values represent means + SEM, n=8; $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ vs. water/VEH group (Tukey's post-hoc test). Two-way ANOVA: interaction of IN x i.p. treatment on distance traveled;

F(1,28)=5.079, p≤0.05, on the time spent within the center zone; F(1,28)=8.476, p≤0.01, on time spent immobile; F(1,28)=4.185, p≤0.05, on the number of central area visits F(1, 28)=9.552, p≤0.05. These findings have been submitted for publication.

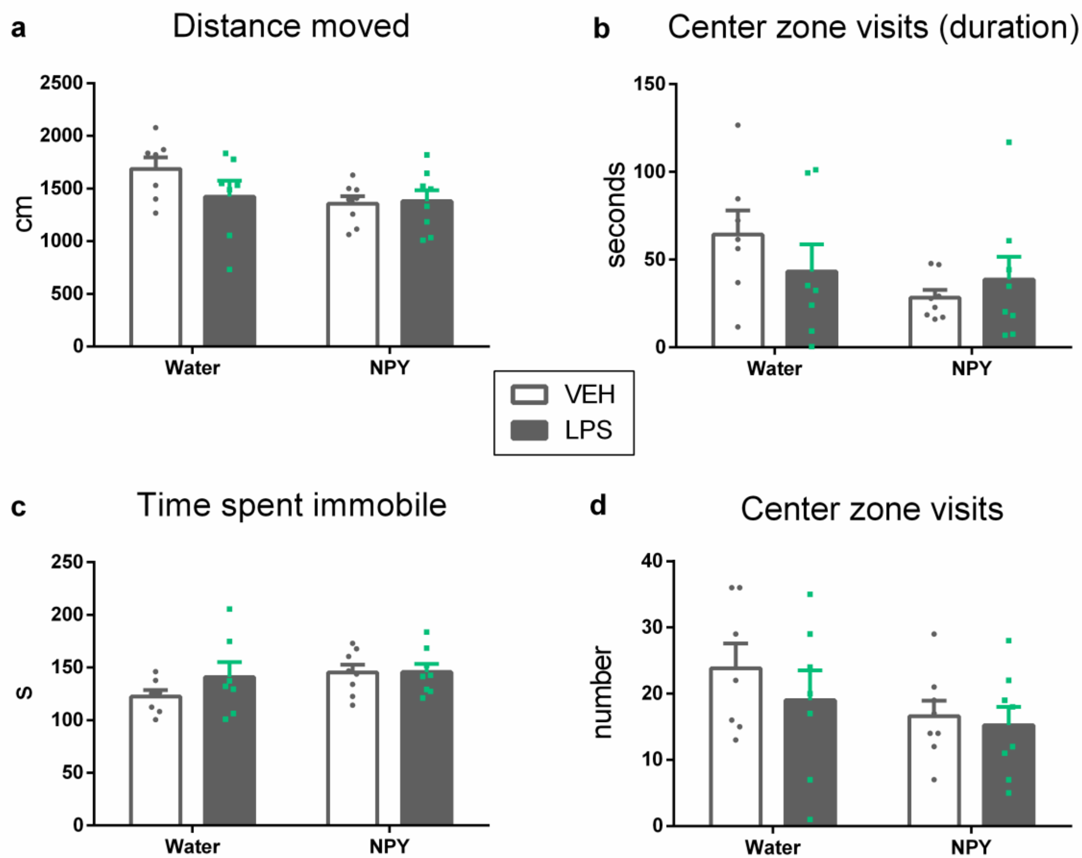

Figure 16 Effects of pretreatment with intranasal (IN) NPY and i.p. injections of LPS on behavior in the open field (OF) test 21 h after treatment. Mice were IN infused with either water or NPY (100 µg), and 30 min later injected i.p. with saline (VEH) or LPS (0.03 mg/kg). The OF test was performed 21 h later in order to analyze exploratory behavior: (a) distance traveled, (b) time remained within the center zone, (c) time remained immobile, (d) number of visits to the central area. Values represent means + SEM, n=8; there were no interactions of IN x i.p. treatment on parameters under study (Two-way ANOVA): interaction of IN x i.p. treatment on distance traveled; F(1,26)=1.707, p=0.2028, on the time spent within the center zone; F(1,26)=1.692, p=0.2047, on time spent immobile; F(1,26)=0.9930, p=0.3282, on the number of central area visits F(1,26)=0.2707, p=0.6073) . These findings have been submitted for publication.

3.2.2 Circulating cytokine levels are elevated by LPS treatment, but left undisturbed by intranasal NPY pretreatment

Peripheral LPS injection elevated plasma cytokine levels 3 h post-treatment (Fig. 17). Two-way ANOVA revealed a main effect of LPS treatment, which strongly induced the release

of MCP-1, IFN-γ, IL-6, TNF-α, IFN-β and IL-10, but no interaction between IN pretreatment with NPY and i.p. LPS injections was found. This demonstrates that the effect of LPS to increase circulating levels of the above mentioned cytokines is independent of previous IN NPY applications. The only cytokine that was shown to be affected by both i.p. LPS treatment as well as IN NPY infusion was IL-1β, as there was a significant interaction found between IN NPY and i.p. LPS. As depicted in Figure 17 d, post-hoc analysis revealed higher cytokine levels in the NPY/LPS group compared to the groups pretreated with water and injected with VEH or LPS. However, it is important to note that all measured values for IL-1β were below 3 pg/ml, and therefore comparably low.

Until 21 h following treatment, only plasma MCP-1 levels remained significantly elevated in both groups of LPS-injected mice (Figure 18). All other cytokines under study were not detectable at this time point and had returned to baseline level (data not shown).

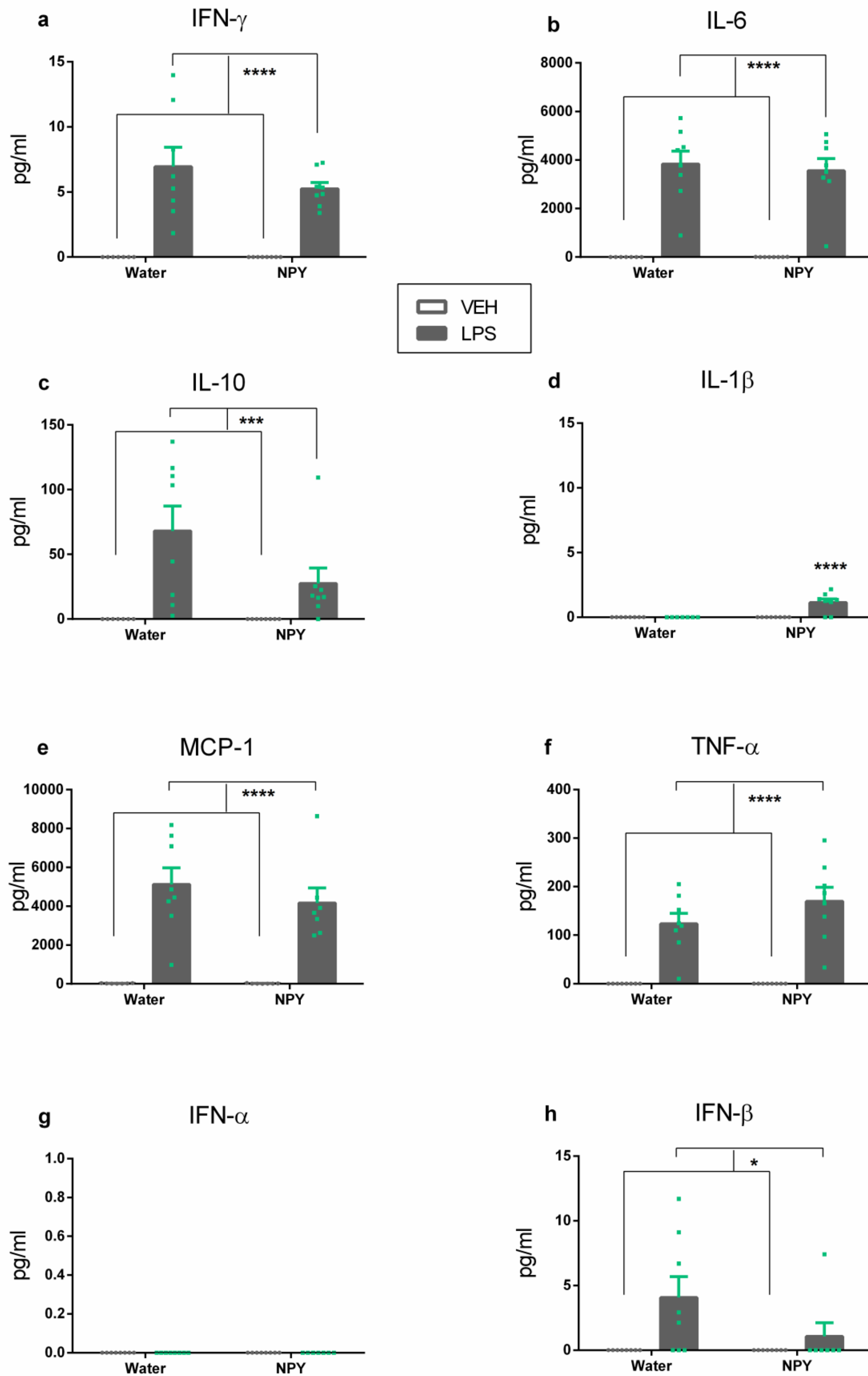

Figure 17 Effects of pretreatment with intranasal (IN) NPY and i.p. injections of LPS on release of circulating cytokines into the plasma 3 h after immune challenge. Mice were IN infused with either water

or NPY (100 µg), and 30 min later injected i.p. with saline (VEH) or LPS (0.03 mg/kg). Animals were sacrificed 3 h later and plasma samples were analyzed for cytokine levels. Values represent means + SEM, n=7-8; *p≤0.05, ***p≤0.001, ****p≤0.0001 vs. water/VEH group (two-way ANOVA main i.p. effects) or as indicated (Tukey's post hoc test). Two-way ANOVA: interaction of IN x i.p. treatment on circulating levels of IFN-γ; $F(1,27)=1.080$, $p=0.3080$, IL-6; $F(1,27)=0.1271$, $p=0.7242$, IL-10; $F(1,27)=2.989$, $p=0.0952$, IL-1β; $F(1,27)=16.20$, $p≤0.001$, MCP-1; $F(1,25)=0.6165$, $p=0,4397$, TNF-α; $F(1,28)=1.659$, $p=0.2083$, IFN-β; $F(1,27)=2.462$, $p=0.1283$. MCP=monocyte chemoattractant protein, IL=interleukin, IFN=interferon, TNF=tumor necrosis factor. These findings have been submitted for publication.

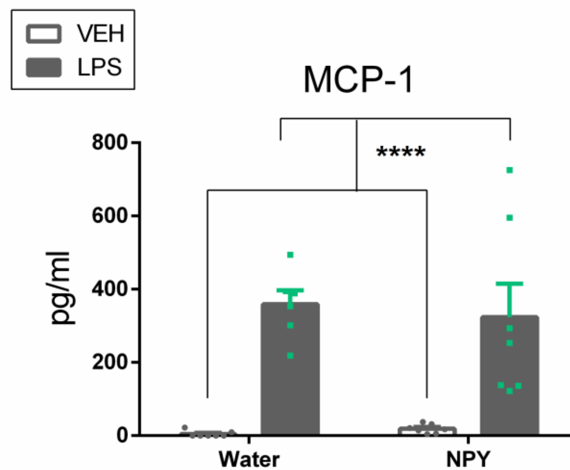

Figure 18 Effects of pretreatment with intranasal (IN) NPY and i.p. injections with LPS on release of circulating MCP-1 into the plasma 21 h after immune challenge. Mice were IN infused with either water or NPY (100 µg), and 30 min later injected i.p. with saline (VEH) or LPS (0.03 mg/kg). Animals were sacrificed 21 h later and plasma samples were analyzed for MCP-1 levels. Values represent means + SEM, n=7; ****p≤0.0001 (Two-way ANOVA main i.p. effect) as indicated. Two-way ANOVA: interaction of IN x i.p. treatment on circulating MCP-1; $F(1,23)=0.2394$, $p=0.6292$. MCP=monocyte chemoattractant protein. These findings have been submitted for publication.

3.2.3 Intranasal NPY pretreatment blunts the LPS-induced release of circulating CORT 21 h following treatment

Plasma CORT levels were found significantly higher during the acute phase of behavioral sickness response in both LPS-injected groups, regardless of IN infusion of water or NPY (Fig. 19 a). Therefore, pretreatment with IN NPY did not seem to affect LPS-induced rise of circulating CORT found 3 h after treatment. However, a significant interaction was found between IN NPY infusion and i.p. LPS application 21 h after the treatment. Post-hoc analysis revealed that only LPS-injected mice that were pretreated with water still presented with significantly elevated circulating CORT levels at that later time point when compared to the

water/VEH control group (Fig. 19 b). In contrast, LPS-treated animals that were IN infused with NPY prior to i.p. LPS injection did not show elevated plasma CORT levels 21 h post-injection (Fig. 19 b).

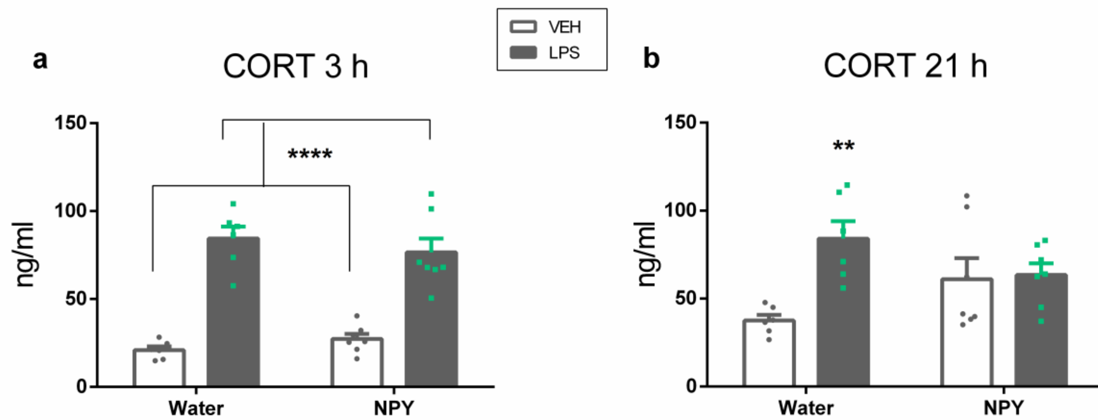

Figure 19 Circulating corticosterone (CORT) levels at 3 h and 21 h following i.p. treatment with LPS. Mice were IN infused with either water or NPY (100 µg), and 30 min later injected i.p. with saline (VEH) or LPS (0.03 mg/kg). Animals were sacrificed 3 h or 21 h later and plasma samples were analyzed for CORT levels. Values represent means + SEM, n=6-7; ** $p \leq 0.01$ vs. water/VEH group (Tukey's multiple comparisons test), ****$p \leq 0.0001$ (Two-way ANOVA main i.p. effect) as indicated. Two-way ANOVA: interaction of IN x i.p. treatment on circulating CORT levels at 3 h; $F(1,22)=1.590$, $p= 0.2205$, on circulating CORT levels at 21 h; $F(1,22) = 6.244$, $p \leq 0.05$. These findings have been submitted for publication.

3.2.4 Intranasal NPY pretreatment abates LPS-induced body weight loss

Twenty-one h following i.p. LPS injections, mice of both groups treated with LPS presented with significantly less body weight when compared to the water/VEH group (Fig. 20). However, two-way ANOVA followed by Tukey's post-hoc test disclosed that pretreatment with IN NPY protected the animals from excessive weight-loss, as water/LPS treated mice lost significantly more weight compared to NPY/LPS treated animals (Fig. 20).

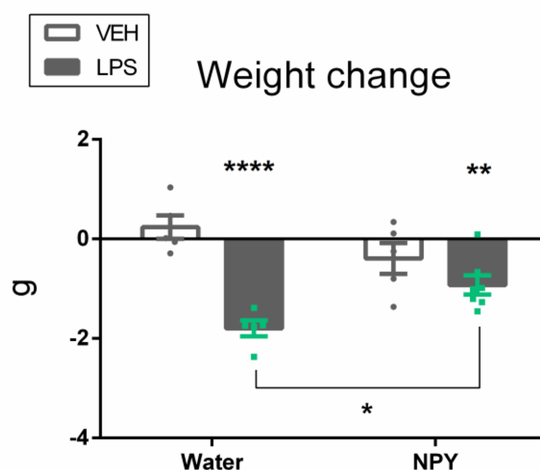

Figure 20 Weight change as measured 21 h post i.p. LPS injections. Mice were IN infused with either water or NPY (100 µg), and 30 min later injected i.p. with saline (VEH) or LPS (0.03 mg/kg). Animals were weighed 21 h after i.p. LPS injections. Values represent means +/- SEM, n=5-7; * $p \leq 0.05$, ** $p \leq 0.01$, ****$p \leq 0.0001$ vs. water/VEH group or as indicated (two-way ANOVA + Tukey's multiple comparisons test). Two-way ANOVA: interaction of IN x i.p. treatment on body weight; $F(1,18)=10.66$, $p \leq 0.01$. These findings have been submitted for publication.

3.2.5 Hypothalamic mRNA expression patterns of cytokine and tight-junction proteins are differentially modified by intranasal infused NPY and i.p. LPS treatment 3 h post-injections

Peripheral treatment with LPS significantly influenced expression patterns of cytokine and tight-junction protein mRNA in the hypothalamus 3 h post-injection. However, most of the gene-expression was not affected by IN NPY pretreatment (Fig. 21, 22).

A main effect of LPS treatment was found by two-way ANOVA for mRNA expression of the following proteins: CCL2 (which was referred to as MCP-1 in the description of circulating cytokine levels), IL-6, IL-1β, CLDN-5, TJP1, OCLN and NRC31. In more detail, mRNA expression of the cytokines CCL2, IL-1β and IL-6 was upregulated by i.p. LPS (Fig. 21 a, c, d), while other cytokines under study (IL-10, IFN-γ, TNF-α, IFN-α, IFN-β; Fig. 21 b, e, f, g, h) were left undisturbed. In contrast, the tight-junction proteins CLDN-5, TJP1 and OCLN as well as GR NR3C1 were downregulated in their expression levels by peripheral treatment with LPS (Fig. 22 a, c, d, e). It should be noted at this point that there was a trend towards an interaction of IN NPY infusion and i.p. LPS injection for the hypothalamic expression of CRH (Fig. 22 h).

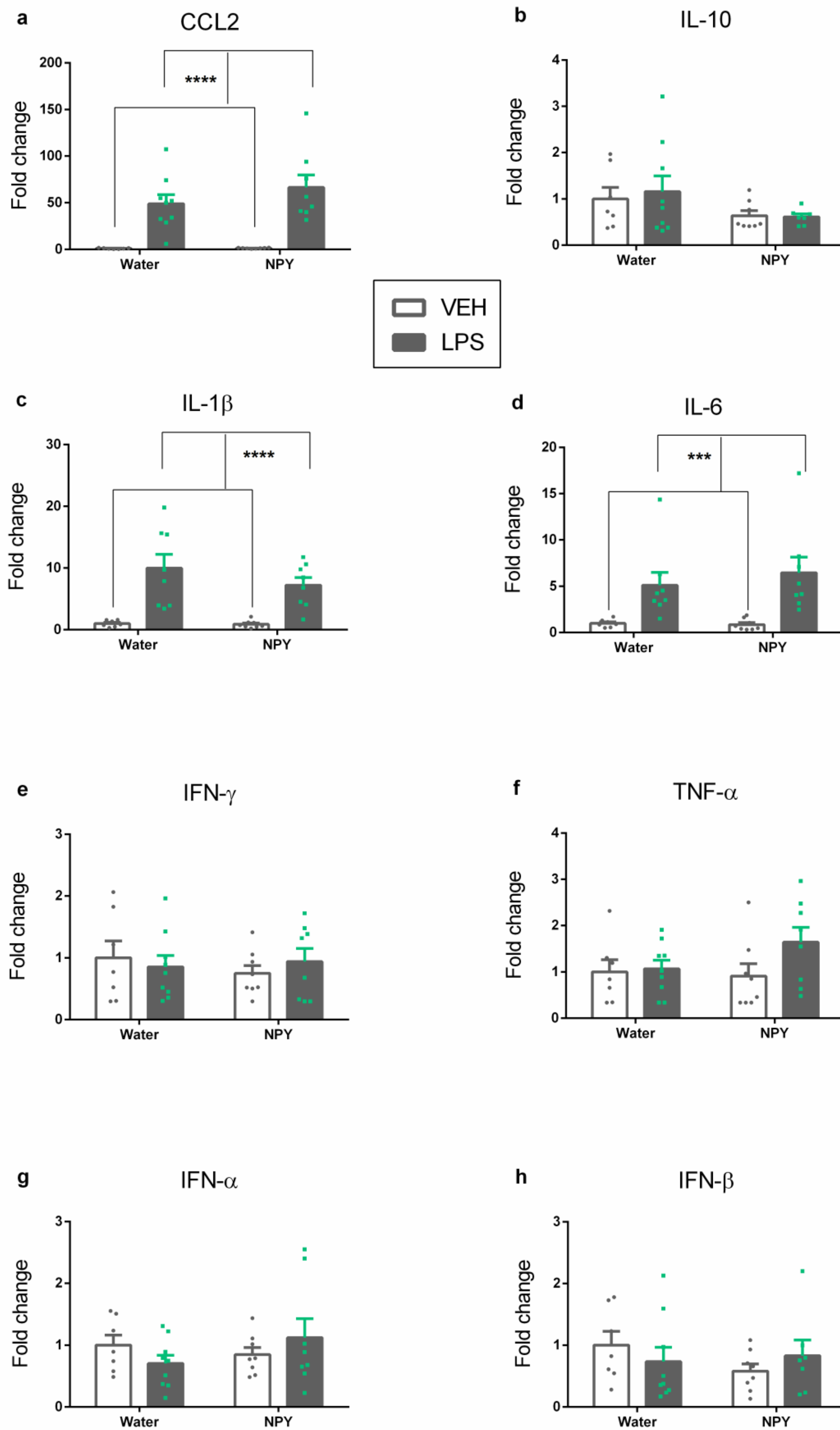

Figure 21 Hypothalamic cytokine mRNA expression following LPS injection 3 h post-treatment. Mice were IN infused with either water or NPY (100 µg), and 30 min later injected i.p. with saline (VEH) or LPS

(0.03 mg/kg). Animals were sacrificed 3 h later and extracted brains were microdissected. Hypothalamic samples were analyzed for mRNA cytokine expression levels. Values represent means + SEM, n=7-9; ***$p \leq 0.001$, ****$p \leq 0.0001$ as indicated (two-way ANOVA main i.p. effects). Two-way ANOVA: interaction of IN x i.p. treatment on mRNA expression levels of CCL2; $F(1,28)=0.9551$, $p=0.3368$, IL-10; $F(1,27)=0.1496$, IL-1β; $F(1,27)=0.9762$, $p=0.3319$, IL-6; $F(1,27)=0.4267$, $p=0.5191$, IFN-y; $F(1,28)=0.7053$, $p=0.4081$, TNF-α; $F(1,28)=1.599$, $p=0.2165$, IFN-α; $F(1,28)=2.154$, $p=0.1534$, IFN-β; $F(1,27)=1.462$, $p=0.2372$. CCL2=CC-chemokine ligand 2, IL=interleukin, IFN=interferon, TNF=tumor necrosis factor. These findings have been submitted for publication.

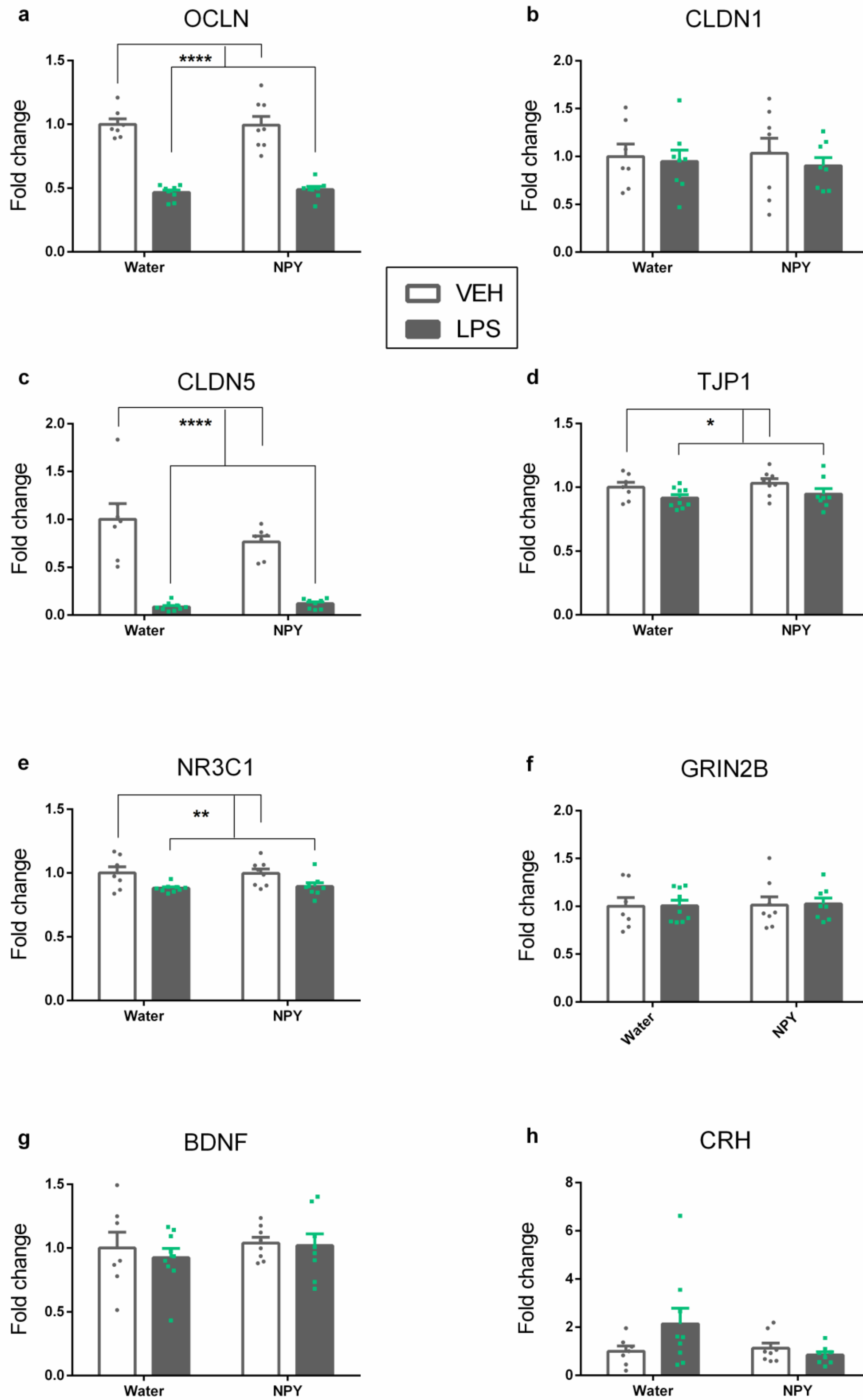

Figure 22 Hypothalamic tight junction proteins and NR3C1 mRNA expression levels following IN NPY infusion and LPS injection 3 h post-treatment. Mice were IN infused with either water or NPY (100 µg),

and 30 min later injected i.p. with saline (VEH) or LPS (0.03 mg/kg). Animals were sacrificed 3 h later and extracted brains were microdissected. Hypothalamic samples were analyzed for mRNA expression levels. Values represent means + SEM, n=7-9; *$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.0001$ as indicated (two-way ANOVA main i.p. effects). Two-way ANOVA: interaction of IN x i.p. treatment on mRNA expression levels of OCLN; $F(1,27)=0.1071$, $p=0.7460$, CLDN1; $F(1,27)=0.09742$, $p=0.7573$, CLDN5; $F(1,27)=2.750$, $p=0.1088$, TJP1; $F(1,28)=0.0001402$, $p=0.9906$, NR3C1; $F(1,28)=0.06777$, $p=0.7965$, GRIN2B; $F(1,28)=0.002555$, $p=0.9600$, BDNF; $F(1,28)=0.1065$, $p=0.7466$, CRH; $F(1,28)=3.078$, $p=0.0903$. OCLN=occludin, CLDN=claudin, TJP=tight junction protein, NR3C1= nuclear receptor subfamily 3 group C member 1, GRIN2B= glutamate [NMDA] receptor subunit epsilon-2, BDNF=brain-derived neurotrophic factor, CRH=corticotropin-releasing hormone. These findings have been submitted for publication.

3.2.6 Hypothalamic mRNA expression patterns of cytokine and tight-junction proteins are differentially modified by intranasal infused NPY and i.p. LPS treatment 21 h post-injections

The mRNA expression patterns of some cytokines and tight-junction proteins were long-lastingly affected by LPS until 21 h post-treatment with LPS (Fig. 23, 24). Both CCL2 and IL-1β mRNA levels were still significantly higher in the LPS-treated animals than in VEH-injected mice, and additionally, hypothalamic mRNA expression of TNF-α was found upregulated by i.p. LPS at the later time point under study (Fig. 23 c, f). For the expression of IL-10 and IFN-β, two-way ANOVA revealed a significant interaction between IN NPY and i.p. LPS treatments 21 h post-injections (Fig. 23). Post-hoc Tukey's test showed that IL-10 was upregulated in the NPY/LPS group when compared to the VEH/LPS group (Fig. 23 b). Furthermore, IFN-β was downregulated by LPS treatment within the water-pretreated groups and IN NPY significantly decreased IFN-β mRNA levels within the VEH-injected groups (Fig. 23 f). In addition, while CLDN-5 mRNA levels still were elevated by i.p. LPS 21 h following treatment, all other tight-junction proteins under study as well as NR3C1 were expressed at basal levels at this time point (Fig. 24 c).

It is worth noting that some genes were unaffected in their mRNA expression by i.p. LPS application and IN NPY infusion at both time points under study. This includes IFN- α, IFN-y (Fig. 21and 23 e, g) and tight-junction protein CLDN1, glutamate receptor GRIN2B, BDNF, as well as CRH (Fig. 23 and 24 b, f, g, h).

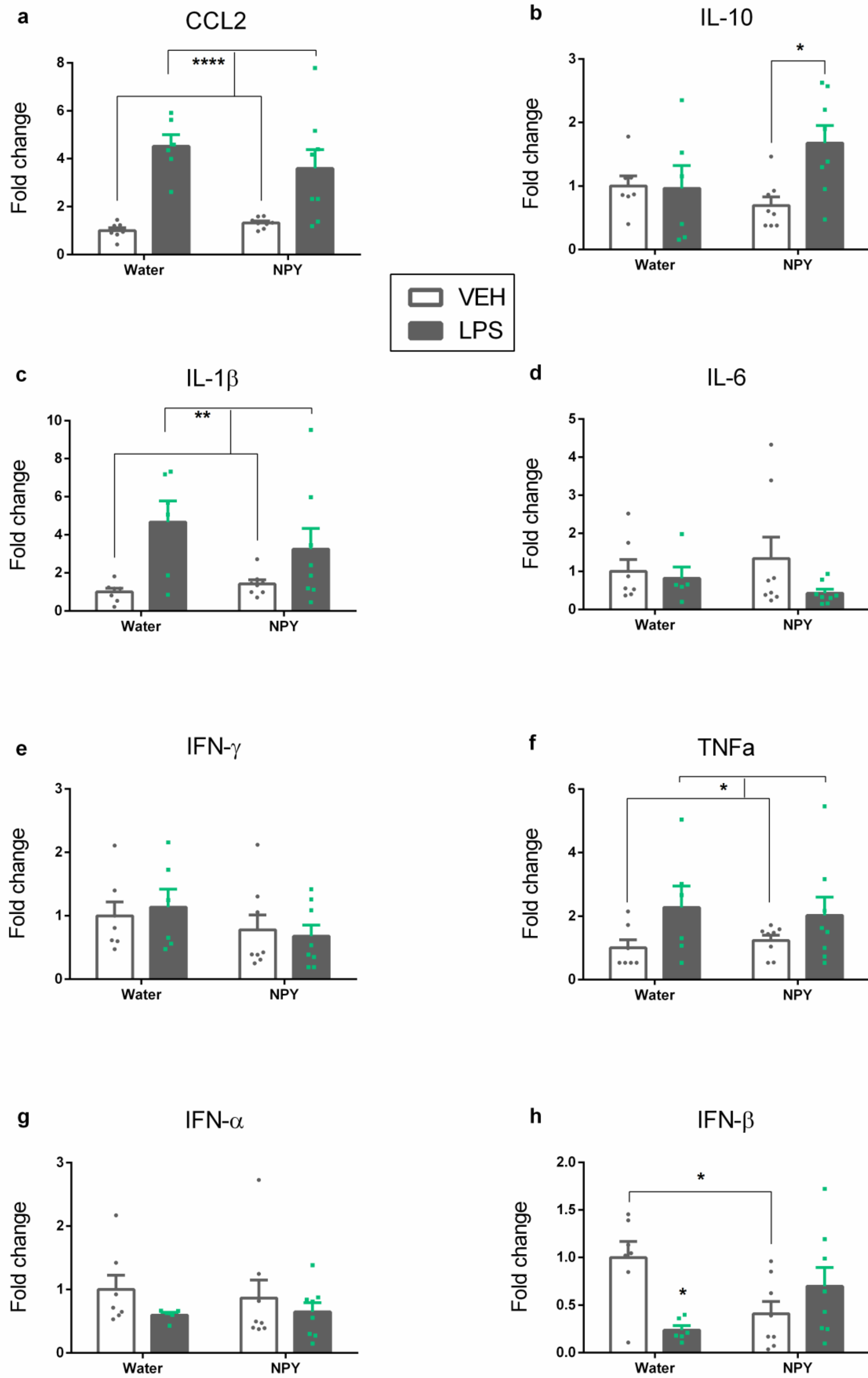

Figure 23 Hypothalamic cytokine mRNA expression 21 h following IN NPY infusion and LPS injection. Mice were IN infused with either water or NPY (100 μg), and 30 min later injected i.p. with saline (VEH) or

LPS (0.03 mg/kg). Animals were sacrificed 21 h later and extracted brains were microdissected. Hypothalamic samples were analyzed for cytokine mRNA expression levels. Values represent means + SEM, n=6-8; *$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.0001$ as indicated (two-way ANOVA main i.p. effects or Tukey's multiple comparison test). Two-way ANOVA: interactions of IN x i.p. treatment on mRNA expression levels of CCL2; $F(1,25)=1.614$, $p=0.2156$, IL-10; $F(1,25)=4.569$, $p \leq 0.05$, IL-1β; $F(1,25)=1,415$, $p=0.2455$, IL-6; $F(1,24)=0.8861$, $p=0.3559$, IFN-y; $F(1,25)=0.2772$, $p=0.6032$, TNF-α; $F(1,25)=0.2851$, $p= 0.5981$, IFN-α; $F(1,24)=0.1745$, $p=0.6799$, IFN-β; $F(1,25)=11.22$, $p \leq 0.01$. CCL2=CC-chemokine ligand 2, IL=interleukin, IFN=interferon, TNF=tumor necrosis factor. These findings have been submitted for publication.

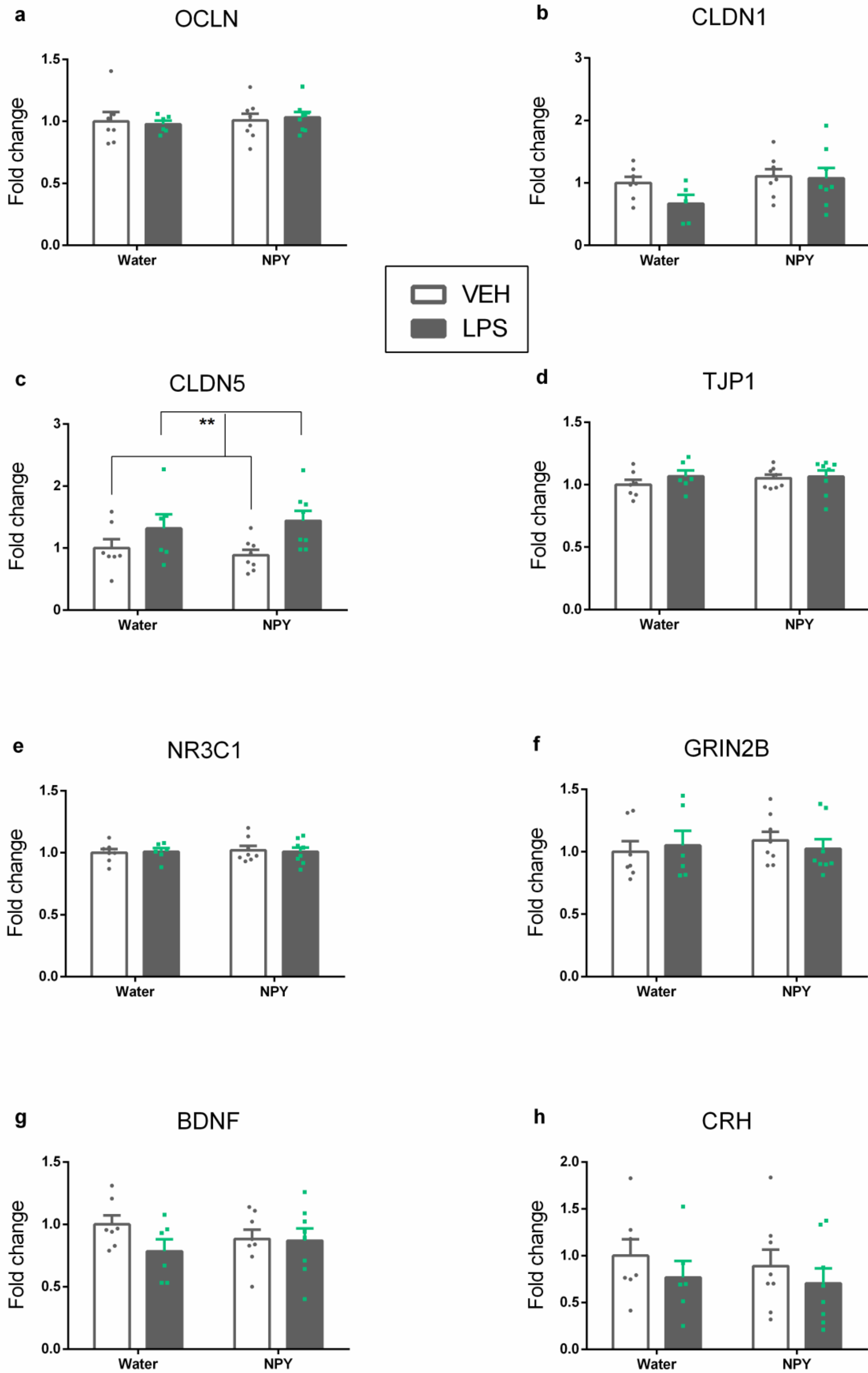

Figure 24 Hypothalamic tight junction protein mRNA expression 21 h after IN pretreatment with NPY and i.p. LPS injections. Mice were IN infused with either water or NPY (100 µg), and 30 min later injected

i.p. with saline (VEH) or LPS (0.03 mg/kg). Animals were sacrificed 21 h later and extracted brains were microdissected. Hypothalamic samples were analyzed for mRNA expression levels. Values represent means + SEM, n=7-9; **$p \leq 0.01$ as indicated (Two-way ANOVA main i.p. effects). Two-way ANOVA: interaction of IN x i.p. treatment on mRNA expression levels of OCLN; $F(1,25)=0.1847$, $p=0.6710$, CLDN1; $F(1,24)=1.194$, $p=0.2853$, CLDN5; $F(1,25)=0.5765$, $p=0.4548$, TJP1; $F(1,25)=0.4137$, $p=0.5260$, NR3C1; $F(1,25)=0.1081$, $p=0.7451$, GRIN2B; $F(1,25)=0.4763$, $p=0.4964$, BDNF; $F(1,25)=1.373$, $p=0.2523$, CRH; $F(1,25)=0.01946$, $p=0.8902$. OCLN=occludin, CLDN=claudin, TJP=tight junction protein, NR3C1=nuclear receptor subfamily 3 group C member 1, GRIN2B=**g**lutamate [NMDA] receptor subunit epsilon-2, BDNF=brain-derived neurotrophic factor, CRH=corticotropin-releasing hormone. These findings have been submitted for publication.

3.2.7 Hypothalamic NPY mRNA expression is left undisturbed by intranasal NPY and i.p. LPS treatment, while Y5 and Y2 receptor mRNA is altered 3 h and 21 h post-injection

Three h post-injection, two-way ANOVA revealed that Y5 receptor mRNA expression was significantly enhanced by i.p. LPS treatment when compared to VEH-injected mice, an effect that was independent of pretreatment with either water or NPY. At the later time point under study, this effect was no longer detectable and there was no significant difference in the Y5 mRNA expression between the treatment groups (Fig. 25 and 26).

In contrast, Y2 mRNA expression was reduced in LPS-injected mice that had been pretreated with IN NPY 21 h before, as analyzed by two-way ANOVA and Tukey's post-hoc test (Fig. 26 c). At the earlier time point under study, however, Y2 receptor mRNA expression was left undisturbed by the preceding treatment (Fig. 25 c).

Both NPY and Y1 mRNA expression remained unaltered by either i.p. LPS injection or IN NPY infusion at either time point under study (Fig. 25 and 26 a, b).

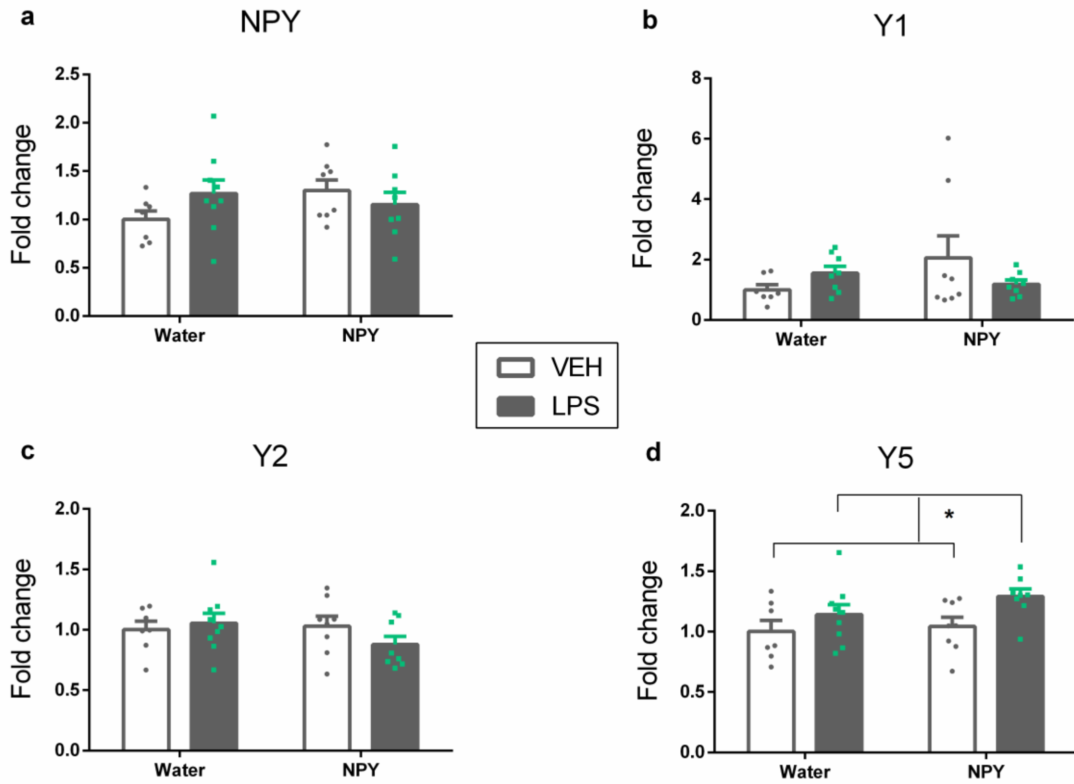

Figure 25 Hypothalamic neuropeptide Y (NPY) and Y receptor mRNA expression 3 h after IN NPY infusion and i.p. LPS treatment. Mice were IN infused with either water or NPY (100 µg), and 30 min later injected i.p. with saline (VEH) or LPS (0.03 mg/kg). Animals were sacrificed 3 h later and brains were microdissected. Hypothalamic samples were analyzed for mRNA expression levels of NPY and its receptors. Values represent means + SEM, n=7-9; *$p \leq 0.05$ as indicated (two-way ANOVA main i.p. effect). Two-way ANOVA interaction between IN x i.p. treatment on expression levels of NPY; $F(1,28)=2.855$, $p=0.1022$, Y1; $F(1,27)=3.017$, $p=0.0938$, Y2; $F(1,28)=1.747$, p=0,1970, Y5; $F(1,28)= 0.4612$, $p=0.5026$. NPY=neuropeptide Y, Y= NPY receptor. These findings have been submitted for publication.

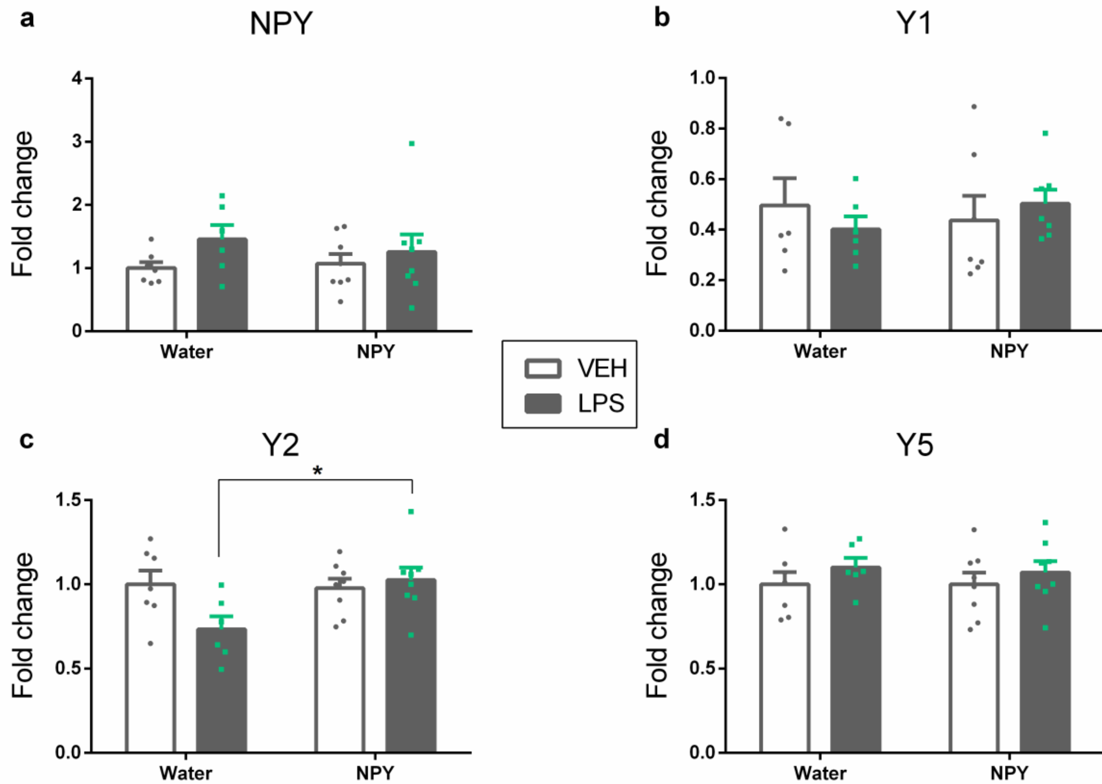

Figure 26 Hypothalamic neuropeptide Y (NPY) and Y receptor mRNA expression 21 h after IN NPY infusion and i.p. LPS treatment. Mice were IN infused with either water or NPY (100 µg), and 30 min later injected i.p. with saline (VEH) or LPS (0.03 mg/kg). Animals were sacrificed 21 h later and extracted brains were microdissected. Hypothalamic samples were analyzed for mRNA expression levels of NPY and its receptors. Values represent means + SEM, n=7-9; *$p \leq 0.05$ as indicated (Tukey's multiple comparison test). Two-way ANOVA: interactions of IN x i.p. treatment on mRNA expression of NPY; $F(1,25)=0.4273$, $p=0.5193$, Y1; $F(1,22)=0.9603$, $p=0.3378$, Y2; $F(1,25)=4.781$, $p \leq 0.05$, Y5; $F(1,25)=0.04914$, $p=0.8264$. NPY=neuropeptide Y, Y= NPY receptor. These findings have been submitted for publication.

3.2.8 Ingestive and exploratory behavior in a homecage-like environment was not altered by intranasal NPY infusion

Mice were treated IN with NPY and subsequently placed into the LabMaster cages for 12 h, in order to record whether infusion of NPY alters exploratory or ingestive behavior during that time period.

Both horizontal locomotion as well as vertical exploratory behavior (rearing) were not affected by IN infusion of NPY (Fig. 27 a, b). Also water and food intake were left undisturbed by IN infusion with NPY (Fig 27 c, d), although during the 9th h after IN

infusion, NPY treated mice ingested less chow when compared to the water infused control animals (Fig. 27 c).

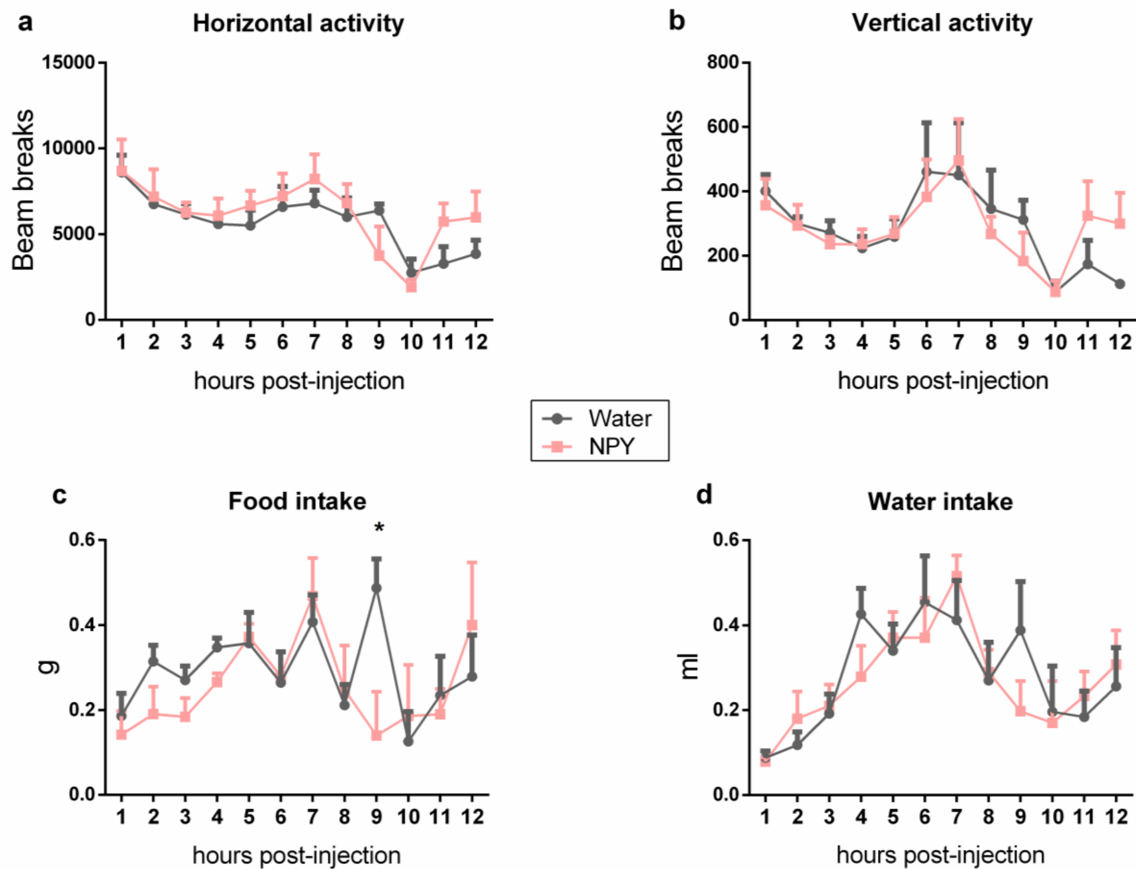

Figure 27 Exploratory and ingestive behavior in the homecage-like environment of the LabMaster system after IN NPY treatment. Horizontal (a) and vertical (b) exploration, as well as water intake (d) were left undisturbed by IN NPY infusion. Food intake was only changed by IN NPY infusion 9 h following treatment (n=5-7). Values represent means + SEM, $*p \leq 0.05$, compared to water treated mice at the time point indicated (Bonferroni's multiple comparison test). RM two-way ANOVA: interaction between time x i.p. treatment on horizontal activity; $F(11,121)=1.190$, $p=0.3009$, on vertical activity; $F(11,121)=0.7949$, $p=0.6448$, on food intake; $F(11,110)=1.313$, $p=0.2267$, on water intake; $F(11,110)=0.6972$, $p=0.7388$. These findings have been submitted for publication.

4 Discussion

Chronic stimulation of the peripheral immune system, for example by invading pathogens such as viruses or bacteria, is associated with an elevated expression of pro-inflammatory cytokines within the brain as well as alterations in brain function and behavior (Holzer et al., 2017). This includes changes in expression patterns of neurotransmitter systems, reduced motivation to explore the environment and engage in physical activity, decreased appetite and a dampened interest to interact with other individuals, symptoms that overlap with those observed in depressed or anxious patients (Capuron and Miller, 2011). While this adaptation is beneficial in terms of recovery from acute infections, the life quality of chronically ill people, for example, can be dramatically reduced (Kelley et al., 2003). Thus, an increasing number of studies aims to elaborate the underlying mechanisms behind the communication between the peripheral immune system and the brain. Furthermore, recent research in this area has started to focus more and more on how to interfere in the transmission processes of pro-inflammatory signaling from the periphery to the brain. With this current study, I evaluated two potential interventions in their capability to abrogate the detrimental effects of peripheral immune stimulation by viral or bacterial factors on brain and behavior.

4.1 Project 1: Analysis of Poly(I:C)-induced sickness response and the effects of preceding intermittent fasting

An accumulating body of evidence suggests that restricted feeding might benefit various health issues, for example autoimmune disease or some subtypes of cancer by stimulating repair mechanisms in response to the metabolic intervention (Lee et al., 2012, Lv et al., 2014, Choi et al., 2016, Choi et al., 2017). Additionally, some studies have proposed that a restriction of caloric intake to a certain daily amount could alleviate the LPS-induced sickness response (Inoue and Luheshi, 2010, MacDonald et al., 2011, Zenz et al., 2019). Thus, while the effects of fasting on bacterial immune-stimulation have already been elaborated to some extent, this is the first study to interrogate how restricted feeding might interfere with inflammatory processes evoked by a viral mimic. Therefore, the influence of an IF regimen on the expression patterns of cytokines within the periphery and brain as well as the behavioral manifestations of Poly(I:C)-induced immune stimulation were evaluated.

First, I investigated the time point at which the viral mimic evokes apparent behavioral alterations. Therefore, four behavioral parameters (locomotion, exploration, food and water

intake) were recorded in the homecage-like LabMaster system during 12 h following Poly(I:C) treatment and depression- as well as anxiety-related behavioral changes were assessed at a time point when acute sickness had already abated. I was interested in these long-term effects by TLR3 stimulation, because peripheral activation of other TLRs, such as bacterial factors, were found to induce behavioral alterations after the acute sickness response had abated (Raison et al., 2006, Dantzer et al., 2008).

While Poly(I:C) injection caused an alteration of all parameters studied in the LabMaster 4 h following treatment, which is in line with other reports (Cunningham et al., 2007, Michalovicz and Konat, 2014, Weintraub et al., 2014), no depression- or anxiety-like behavior was induced by the viral mimic. One explanation for the absence of these long-term effects by stimulation of TLR3 signaling could be that the dsRNA mimic is degraded quickly by ubiquitous RNases following peripheral application (Krasowska-Zoladek et al., 2007, Fil et al., 2011, Konat, 2016, Zenz et al., 2019). Since LPS, on the other hand, is thought to enter circulation once applied peripherally, and was detected in the serum of i.p. LPS-injected rats hours after treatment (Hansen et al., 2000), the bacterial factor could act on various sites additionally to the site of injection, inducing a stronger immune response with longer lasting effects that also differentially impact brain and behavior (Lenczowski et al., 1997, Romanovsky et al., 2000). This could also explain the weaker overall sickness behavior induced by the viral mimic (12 mg/kg) when compared to LPS (0.03 mg/kg) in the LabMaster test as observed in this current study (section 3.1.8). Interestingly, some studies did report Poly(I:C)-evoked depressive- and anxiety-like behavior in rats that were i.p. injected with a dose of 6 mg/kg (evaluated by sucrose preference and the OF test) (Gibney et al., 2013). One reason for why this finding could not be reproduced might be that in the current study, endotoxin-free Poly(I:C) was applied and even very small amounts of endotoxin contamination can induce comparably strong behavioral effects (Mayerhofer et al., 2017). Additionally, other factors such as a distinct length of the dsRNA mimic and differences in experimental animals or housing conditions can affect the outcome of immune stimulation (Kato et al., 2008, Pasquarelli et al., 2017).

In order to analyze whether IF could interfere with the effects of peripheral immune stimulation by Poly(I:C), further experiments were conducted at the time point when mice showed a pronounced sickness response to Poly(I:C) in the LabMaster system. Therefore,

the impact of the 9-day IF regimen on the immune and behavioral sickness response to the dsRNA mimic Poly(I:C) was evaluated 4 h post-injection on day 10 (Zenz et al., 2019).

4.1.1 Poly(I:C)-induced sickness response is enhanced by intermittent fasting

Unlike observed in the LabMaster test, within the novel environment of the OF, peripheral Poly(I:C) injections alone failed to alter exploratory behavior. Similar observations were already made in previous studies which showed a stronger behavioral sickness response to peripheral immune challenge in the homecage-like LabMaster compared to the OF test (Farzi et al., 2015b). This might be due to the intrinsic motivation of rodents to explore novel environments which could have overruled the moderate sickness behavior observed within the LabMaster cages to which the animals were allowed to habituate prior to the test (Zenz et al., 2019).

Nevertheless, Poly(I:C)-injected animals that were held under a strict IF regimen prior to treatment, showed particular signs of behavioral sickness during the OF test. It should be noted at this point that the fasting regimen itself did not influence the studied parameters and the animals maintained their body weight over time which was already reported by others (Gotthardt et al., 2016).

Thus, in contrast to beneficial effects of caloric restriction on the LPS-induced sickness and immune response, this report shows that behavioral and molecular effects of Poly(I:C) dependent TLR3 stimulation were exaggerated by IF (Zenz et al., 2019). It is therefore proposed that metabolic changes induced by IF could amplify the behavioral effects observed in the OF test by boosting the immune reaction to peripheral Poly(I:C). Interestingly, others have shown that LPS treated rats are more prone to develop anorexia when compared to Poly(I:C) treated animals in the acute phase of sickness, which could point at a differentially regulated metabolism during bacterial versus viral insults and the need for caloric intake during viral infection (Hopwood et al., 2009). Accordingly, Wang and colleagues have shown that a high dose of Poly(I:C) (30 mg/kg) injected retro-orbitally to mice was lethal only in animals that were co-treated i.p. with 2-deoxy-D-glucose, which is an inhibitor of glycolysis, whereas all animals of the control cohort injected with vehicle survived Poly(I:C)-treatment (Wang et al., 2016). Interestingly, this exacerbating effect of 2-deoxy-D-glucose was not found to parallel an increased inflammatory response, therefore,

this is the first report to show that IF enhanced the immune response to the dsRNA mimic, which is reflected by the increased levels of circulating cytokines and could explain the exaggerated sickness response (Zenz et al., 2019).

The exaggerated behavioral sickness response to Poly(I:C) observed in intermittently fasted mice could be triggered by the elevated levels of circulating cytokines

Cytokine expression patterns were evaluated both within the periphery and the hypothalamus. This brain area was chosen for the analysis of inflammatory marker expression as it has implications in the central response to peripheral immune stimulation as well as energy balance (Guijarro et al., 2006, Zenz et al., 2019). For instance, several studies have shown that i.p. administration of LPS enhances the expression of c-Fos in the hypothalamus and other brain areas, which indicates that neuronal activity in this cerebral regions is enhanced by peripheral immune stimulation (Wan et al., 1993, Castanon et al., 2003, Farzi, 2015). Furthermore, peripherally applied LPS was shown to influence neuropeptide expression in the hypothalamus, and the expression of anorexigenic peptides, including Proopiomelanocortin and Cocaine-and amphetamine-regulated transcript, is increased. These processes are involved in the development of anorexia as observed in immune challenged animals (Sergeyev et al., 2001). Additionally, altered neuronal activity in the hypothalamus and hippocampus is associated with reduced locomotor activity caused by peripherally applied LPS (Gaykema and Goehler, 2011). Another link between peripheral immune stimulation and the hypothalamus is that peripheral injections of LPS were found to increase c-Fos immunoreactivity within the Nucleus tractus solitarii as well as its projection areas, including the hypothalamus (Wan et al., 1993)

Interestingly, cytokine levels in the plasma of Poly(I:C) treated mice with AL access to food were lower than reported in other studies applying the same dose of the viral mimic (Cunningham et al., 2007, Weintraub et al., 2014, Murray et al., 2015), which might be due to the quality and length of the dsRNA molecule applied (as described above in 4.1).

IF exaggerated the cytokine release within the periphery following TLR3 stimulation and only mice that were previously fasted presented with significantly higher levels of circulating TNF-α, IL-6 and IFN-α compared to the control group. TNF-α is one crucial mediator to transfer peripheral pro-inflammatory signals to the brain and, therefore, the

exacerbated release of this cytokine by IF/Poly(I:C) could be a key mechanism (Bluthé et al., 2000a, D'Mello et al., 2009, Zenz et al., 2019). This argument is supported by the finding that plasma TNF-α was not elevated to a significant extent by Poly(I:C) treatment alone, which could explain the absence of a clear behavioral sickness response in the AL/Poly(I:C) group during the OF test. In contrast, under conditions of fasting, both IL-6 and IFN-α were increased by the dsRNA mimic, which might further add to the sickness-enhancing effects of TNF-α. This surmise is based on the observation that hypoactivity is thought to be induced by additive effects of circulating IL-6 and IFN-α and IL-6 to enhance sickness behavior induced by systemic inflammation (Bluthé et al., 2000b, Murray et al., 2015). Furthermore, only fasted mice presented with significantly increased circulating IFN-γ levels, which might enhance the expression and release of further cytokines, as was described in experimental models of chronic stress by others (Litteljohn et al., 2010, McCusker and Kelley, 2013). The simultaneous increase in circulating IL-10 is in accordance with other reports in which LPS was applied to calorie-restricted rats and enhanced levels of this anti-inflammatory cytokine were observed (MacDonald et al., 2014), which could reflect an attempt to limit the inflammatory process.

Although others proposed a central role for IL-1 in the development of sickness behavior following peripheral immune stimulation by bacterial and viral factors (Kelley et al., 2003, Fortier et al., 2004), neither Poly(I:C) (Project 1) nor LPS (Project 2) treated mice presented with elevated circulating IL-1 levels. Thus, some of the effects by Poly(I:C) seen in other studies might be due to contaminations of Poly(I:C) and LPS with other factors that additionally stimulate the immune system (Mayerhofer et al., 2017).

Within the hypothalamus, cytokine mRNA expression patterns were unaltered by fasting, while Poly(I:C) treatment significantly enhanced all cytokines under study. These findings suggest that circulating cytokines, which were enhanced in the IF/Poly(I:C) group, influenced the behavioral response to Poly(I:C) treatment to a larger extent than central cytokine expression. Interestingly, others have reported that preceding IF for 30 days blunts hippocampal cytokine release induced by intravenous LPS (1 mg/kg) injections in rats. This effect included IL-1β and TNF-α and was measured 2 h post immune challenge (Vasconcelos et al., 2014). Furthermore, IF in the above-mentioned study did not only blunt the LPS-induced increase of cerebral cytokines, but also reduced serum levels of IL-1β following immune challenge (Vasconcelos et al., 2014). This is contrary to what was

observed in the current study, as IF prior to Poly(I:C) treatment further increased circulating cytokine levels and fasting failed to blunt hypothalamic cytokine mRNA expression. Therefore, the effects of peripheral immune stimulation seem to be influenced differently at the peripheral and cerebral level by preceding fasting, depending on whether it was induced by LPS or Poly(I:C).

4.1.2 Circulating CORT is elevated by intermittent fasting

In line with other studies that reported a rise in circulating CORT levels following short or long periods of restricted calorie intake (Wan et al., 2003, Lowette et al., 2014, MacDonald et al., 2014), in the present set of experiments the fasting regimen significantly enhanced CORT plasma levels in vehicle-treated mice. Furthermore, also the IF/Poly(I:C) group presented with markedly increased circulating CORT levels, however to a lesser extent than the IF/VEH group. These observations suggest that immune stress triggered by Poly(I:C) somehow dampened the fasting-induced activation of the HPA axis, which is in line with another report that observed lower CORT levels in IF mice following bacterial infection when compared to non-infected animals (Godinez-Victoria et al., 2014, Zenz et al., 2019). Furthermore, the current data show that, although glucocorticoids are known to have anti-inflammatory properties, the rise of circulating CORT levels was not sufficient to dampen the overall immune-response in Poly(I:C)-injected mice. Given that the HPA axis was stimulated by IF prior to the immune-challenge, these results are in line with other reports showing that administration of glucocorticoids prior to LPS treatment could exacerbate the immune response, which suggests that stimulation of the HPA axis might time-dependently augment inflammation (Frank et al., 2010, Coutinho and Chapman, 2011). For example, one study showed that subcutaneous CORT administration both 2 and 24 h prior to peripheral LPS challenge exacerbated pro-inflammatory cytokine expression 4 h following immune stimulation within the liver and the hippocampus in rats (Frank et al., 2010). In line with this, others have proposed that stress could have a priming effect if occurring prior to immune challenge and might thereby exacerbate the response (Sorrells et al., 2009). Thus, immune cell mobility is promoted by the stress-induced high levels of circulating CORT (Sorrells et al., 2009). Interestingly, some studies show that glucocorticoids affect the cerebral inflammatory response to immune challenge in a brain region-dependent manner. Thus, peripheral LPS injection following chronic stress was reported to induce NF-κB activation and cytokine expression within the frontal cortex and the hippocampus, while the

opposite effect was observed in the hypothalamus (Munhoz et al., 2006, Sorrells et al., 2009). The stress-induced increase of neuroinflammation was abrogated by pretreatment with an antagonist for glucocorticoid receptors and, therefore, stimulation of glucocorticoid receptor pathways is thought to be required in order to exacerbate inflammatory signaling (Munhoz et al., 2006). The underlying mechanisms leading to opposing responses in different brain regions remain to be elucidated (Sorrells et al., 2009).

4.1.3 Intermittent fasting elevates NPY mRNA expression within the hypothalamus

In this current study, IF increased hypothalamic expression of the orexigenic neurotransmitter NPY, which is in accordance with a vast majority of studies investigating the effects of fasting on central NPY expression (White and Kershaw, 1990, Chua et al., 1991, Marks et al., 1992, Vogelzangs et al., 2016). However, while others have shown that inflammation triggered by bacterial factors like LPS or Freund's adjuvant also have an impact on central NPY expression (Ji et al., 1994, Kim et al., 2007), immune-stimulation by the viral dsRNA mimic alone did not significantly alter NPY expression (Zenz et al., 2019). However, the impact of peripheral immune stimulation on central NPY expression might be time-dependent as, for example, one study reported a decreased NPY expression 2 h following peripheral LPS application (Kim et al., 2007).

Furthermore, although others have suggested that some of the beneficial effects of fasting on inflammation, namely the dampened LPS-induced microglial activation, could indirectly be mediated by an increased central NPY release (Radler et al., 2015), the elevated expression of NPY found in this study did not beneficially impact the sickness response to the viral mimic. Given that a metabolic change influences the organism at several levels, it would not be feasible to draw any conclusions about the ability of NPY to attenuate Poly(I:C)-evoked sickness at this point, and it would be worthwhile to further explore this question. Nevertheless, glucose deprivation was previously demonstrated by others to differentially impact LPS- and Poly(I:C)-induced immune activation, as it promotes survival during septic bacterial inflammation, but has deleterious effects during septic viral immune-stimulation (Wang et al., 2016). Thus, the upregulated endogenous expression of NPY observed in this study might not be sufficient to overrule those deleterious metabolic effects evoked by fasting.

Additionally, given that LPS and Poly(I:C) induce different routes to signal immune challenge from the periphery to the brain and that there are differences in the mechanisms by which the two factors evoke behavioral changes and immune activation (Hopwood et al., 2009, Kong et al., 2015, Wang et al., 2016), NPY could affect these disturbances in a distinct manner. For example, while peripheral LPS is thought to enter the blood stream and thereby can access several areas of the organism, including the brain, Poly(I:C) is degraded quickly by RNases and only acts on TLR3 that are present at the site of injection (Lenczowski et al., 1997, Romanovsky et al., 2000, Konat, 2016).

Furthermore, intermittent fasting might shift the circadian clock to some extent, which in turn could also impact the immune response to infection, as the magnitude of LPS-evoked inflammation was found to depend on the time point of feeding during the day (Cissé et al., 2018).

4.2 Project 2: Effects of intranasal NPY pretreatment on LPS-induced immune and sickness response

LPS, an outer cell-wall component occurring in gram-negative bacteria and a TLR4 agonist, is frequently used to study mechanisms by which the peripheral immune system and the brain communicate with each other. The activated innate immune system is well known to evoke a battery of behavioral alterations, referred to as sickness response, which can be associated with the development of depression- and anxiety-related behavioral changes when sickness has abated (Dantzer et al., 2008, Capuron and Miller, 2011). For project 2 of this current study, the murine immune system was chosen to be stimulated by LPS instead of Poly(I:C), because the bacterial factor was found to induce behavioral alterations of larger magnitude and for a longer period of time when compared to immune challenge with the dsRNA mimic (see section 3.1.8). This is in line with the observation by others that cytokine levels are upregulated to a larger extent in LPS-treated mice than in Poly(I:C) treated mice (Kong et al., 2015).

By triggering the TLR4 signaling cascade, LPS evokes a strong sickness response, considering that comparably low doses like 0.2 mg/kg (Mayerhofer et al., 2017), 0.1 mg/kg (Farzi et al., 2015b), and in this study even 0.03 mg/kg, readily decrease exploratory behavior, locomotion and ingestion in rodents. This reaction is associated with upregulated levels of pro-inflammatory cytokines within circulation and the brain, the stimulation of the

HPA axis and altered brain function reflected by a change in behavior as described above. In humans, this is of relevance because a reduced barrier function of the intestinal wall, which can be found in some individuals exposed to chronic stress, may promote the translocation of LPS or other microbial factors derived from the intestinal microbiota (Dantzer et al., 2008, Kell and Pretorius, 2015, Mayerhofer et al., 2017). Stimulation of the innate immune system by excess LPS could in turn play a role in the development of MDD and other neuropsychiatric disorders that are associated with neuroinflammatory processes (Capuron and Miller, 2011). NPY, which is a resilience-promoting neurotransmitter, is thought to at least partly ameliorate these stress responses induced by visceral inflammation, which entails the dampened activation of the HPA axis by the neuropeptide (Antonijevic et al., 2000, Mayerhofer et al., 2017). Additionally, previous work by my group and others have shown that adverse behavioral effects evoked by peripheral LPS application are enhanced in murine KO models lacking the NPY receptors Y2 or Y4, which is in line with the notion that NPY promotes resilience to detrimental manifestations of immune challenge and neuroinflammation (Painsipp et al., 2008, Painsipp et al., 2010, Farzi et al., 2015a). Furthermore, microglial IL-1β release and IL-1β-induced phagocytosis as well as motility of cultured microglial cells were found to be inhibited *in vitro* by NPY (Ferreira et al., 2010, Ferreira et al., 2011, Ferreira et al., 2012). However, protective effects against LPS-induced sickness by NPY have not yet been demonstrated and, therefore, this study aimed to explore the possible effect of NPY in immune-brain signaling evoked by peripheral LPS injections. The neuropeptide was applied via the non-invasive IN route, because thereby compounds reach the CNS quickly and do not have to penetrate the BBB. Interestingly, the IN application of esketamine was recently approved in the US for treating patients suffering from treatment-resistant MDD. Nevertheless, given the potential for side-effects, including its addictive properties, dissociative symptoms, increased blood pressure and dizziness (Garay et al., 2017, Daly et al., 2018, Molero et al., 2018), NPY might in future be an alternative, especially for those patients presenting with inflammatory markers. Furthermore, patients suffering from PTSD were excluded from some studies where esketamine was applied IN (Daly et al., 2018) and also for this subgroup of patients, NPY could be of interest in future.

4.2.1 Intranasal NPY dampens the sickness response to peripheral LPS

To my knowledge, this is the first study to show that IN pretreatment with the exogenous neurotransmitter NPY can interfere with the development of sickness symptoms evoked by peripheral LPS. Thus, NPY prevented the depression of exploratory behavior during the acute phase of the sickness response, promoted the regression of circulating cortisol levels, and mitigated LPS-evoked weight loss. I chose to apply NPY IN because drugs administered by this route are rapidly delivered to the brain in a non-invasive manner that bypasses the BBB as even larger molecules than NPY (4.27 kDa), like insulin-like growth factor-I (7.65 kDa) or horseradish peroxidase (40 kDa), were found to reach the CNS (Thorne et al., 2004, Hanson and Frey, 2008, Lochhead and Thorne, 2012, Khan et al., 2017).

This set of data suggests that NPY dampens HPA axis activity in response to stress, which is in line with work from others reporting an inhibitory effect of NPY on CRH neurons within hypothalamic areas as well as decreased circulating levels of both ACTH and glucocorticoids in humans (Antonijevic et al., 2000) and rodents (Krysiak et al., 1999, Serova et al., 2013, Baldock et al., 2014). Accordingly, individuals with low stress resilience were found to present with reduced circulating NPY levels as well as impaired HPA axis function, both of which play a role in stress recovery (Tasan et al., 2016). Furthermore, decreased CSF and plasma levels of the neuropeptide were found in patients that suffer from PTSD when compared to healthy controls (Yehuda et al., 2006, Sah and Geracioti, 2013).

Interestingly, it was previously demonstrated by others that IN treatment with NPY could dampen the behavioral and molecular response to a rodent stress protocol which induces PTSD-like symptoms (Serova et al., 2013, Laukova et al., 2014). Additionally, in a mouse model for Huntington's disease, IN application of a truncated version of NPY targeting the Y2 receptor was found to ameliorate microglial activation as well as TNF-α expression (Fatoba et al., 2018).

In the current study, LPS at a comparably low dose of 0.03 mg/kg potently evoked a sickness response reflected by reduced locomotor and exploratory behavior 3 h after peripheral immune stimulation. Interestingly, this effect of the TLR4 agonist was only observed in mice pretreated with water, but not in NPY infused animals that did not show any signs of behavioral sickness response. This demonstrates that IN infused NPY protected animals from LPS-induced behavioral alterations.

In order to address the question about where IN applied NPY might interfere with the immune-brain axis stimulated by peripheral LPS, the impact of NPY and LPS treatment on the BBB, the expression of pro-inflammatory cytokines within the CNS and the periphery, as well as HPA axis activation was analyzed.

4.2.2 Circulating cytokine levels are enhanced by LPS treatment

Immune stimulation by LPS induced cytokine levels in the plasma at 3 h following treatment in a similar pattern as previously reported by my lab and others (Dantzer et al., 2008, Farzi et al., 2015b, Mayerhofer et al., 2017), however by 21 h post-injection all cytokines were back to a normal level, except for MCP-1. This rise in circulating cytokines during the acute phase of sickness response was not abated by pretreatment with NPY, which implies that the IN infused neuropeptide did not affect the TLR4-mediated peripheral immune stimulation. One explanation for this might be that NPY does not reach the periphery to a sufficient amount when applied IN, which is in line with the work by others who reported that plasma concentrations of NPY remained unaltered in rodents 30 min following IN NPY application, whereas within the CSF NPY concentrations were increased (Serova et al., 2014). Surprisingly, the current results show an increase of circulating IL-1β levels in animals that were treated with both NPY and LPS, however, the measured concentrations in this group still were comparably low (below 2.2 pg/ml). At this point it should be mentioned that NPY was reported by others to induce the release of IL-1β by peripheral macrophages and human whole blood cells (Hernanz et al., 1996, De la Fuente et al., 2001) and future studies will clarify whether and to what extent IN applied NPY could affect the peripheral immune system after all.

4.2.3 Central cytokine expression is increased by peripheral immune stimulation with no effect of pretreatment with intranasal NPY

This current study shows that central pro-inflammatory cytokine mRNA expression was elevated by peripheral TLR4 stimulation, which is in line with previous work by my lab and others who observed an activation of cerebral microglial cells and an altered cytokine expression pattern within the CNS (Quan et al., 1999, Dantzer et al., 2008, Sandiego et al., 2015, D'Mello and Swain, 2017, Holzer et al., 2017). Gene expression was analyzed within the hypothalamus because this brain region is involved in the response to peripheral immune challenge and other conditions, including chronic stress, which play a role in mental health

(Beishuizen and Thijs, 2003, Guijarro et al., 2006). The overall rise of central pro-inflammatory cytokine expression remained unaltered by pretreatment with NPY and was observed until 21 h after immune stimulation. This prolonged effect of LPS on cerebral mRNA cytokine levels is in accordance with a previous report that observed pro-inflammatory markers at mRNA level 26 h after i.p. LPS injection (Farzi et al., 2015b).

Nevertheless, pretreatment with NPY did increase the mRNA expression of the anti-inflammatory cytokine IL-10 in LPS-treated mice 21 h following injection, which is in accordance with the anti-inflammatory profile of NPY within the brain (Sah and Geracioti, 2013, Farzi et al., 2015a). Furthermore, in absence of peripheral LPS treatment, IFN-β expression was found to be decreased by NPY at the later time point under study, which might be one of the factors by which the neuropeptide conveys its neuroprotective effects.

4.2.4 Tight junction protein expression is impaired by LPS, while neuron-relevant genes are left undisturbed by LPS treatment

The integrity of the BBB is an important factor to control pro-inflammatory signaling from the periphery to the brain. Furthermore, neuroinflammation that brings along an elevated expression pattern of cytokines has been shown to interrupt the BBB (de Vries et al., 1996, Rivest et al., 2000, Bennett et al., 2003, Yao and Tsirka, 2014, Banks, 2015, Holzer et al., 2017). Therefore, one aim of this study was to analyze the expression pattern of CLDN5, TJP1 and OCLN, which are tight-junction proteins involved in the paracellular permeability of the BBB (Tietz and Engelhardt, 2015). While LPS treatment induced a downregulated expression of all tight-junction proteins under study 3 h following LPS injection, IN infusion with NPY did not alter tight-junction protein expression at mRNA level. This indicates that the neuropeptide failed to interfere with the deleterious effects of peripheral LPS challenge on the integrity of the BBB. Interestingly, while CLDN5 expression was downregulated 3 h following immune stimulation, this tight-junction protein was upregulated in its expression 21 h post-injection, which could be a compensatory effect induced to restore the full integrity of the BBB after the acute immune response has abated. By knocking out the gene for CLDN5 others have previously shown that mice lacking this tight-junction protein have a higher permeability for molecules smaller than 800 Da, which suggests an important role for CLDN5 and probably the whole claudin family, to establish an intact BBB and restricting its permeability (Nitta et al., 2003, Dyrna et al., 2013).

No change in the expression of hypothalamic BDNF induced by IN NPY (or i.p. LPS) was observed which is in line with other studies that did not find a correlation of the neuroprotective effects by NPY and an altered BDNF expression (Decressac et al., 2012). Furthermore, the glutamate receptor subunit GRIN2B was not affected in its expression by either peripheral immune stimulation, nor by IN NPY infusion at both time points under study, which indicates a minor role for the excitatory neurotransmitter in this set of experiments.

While the expression of NPY and its receptor Y1 was left undisturbed by i.p. LPS, Y5 receptor mRNA was upregulated 3 h after i.p. LPS treatment and Y2 receptor mRNA was downregulated in water/LPS mice 21 h post-injections when compared to NPY/LPS treated animals. This is reminiscent of another study, in which a similar shift in expression was found by cytokine treatment of human umbilical vein endothelial cells *in vitro* (Silva et al., 2003).

4.2.5 Dampening of HPA axis activation by LPS might be one of the mechanisms involved in sickness mitigation by intranasal NPY

Peripheral immune stimulation and inflammation induced by TLR signaling pathways are linked to an activation of the HPA axis, which is reflected at all three levels of the axis. Thus, within the hypothalamus, an increased expression of CRH can be observed shortly after immune stimulation, subsequently the pituitary gland releases ACTH into the blood stream and, in turn, the adrenal cortex activated by ACTH secretes CORT into the circulation (Lehmann et al., 2013). Activation of the HPA axis is thought to be mediated mostly by cytokines such as TNF-α, IL-1β and IL-6 that are secreted from LPS-stimulated macrophages (Takemura et al., 1997, Beishuizen and Thijs, 2003). Interestingly, while some studies report that a full-blown HPA axis stimulation by peripheral LPS challenge depends on an intact hypothalamus and its ability to release CRH, others have also observed a hypothalamus-independent response and direct stimulation of the adrenal cortex as well as the pituitary gland induced by treatment with the bacterial factor (Takemura et al., 1997, Beishuizen and Thijs, 2003). Increased HPA axis activity and especially elevated circulating cortisol levels are observed in several stress-related disorders, including PTSD and MDD, and in a mouse-model of ACTH-induced and treatment-resistant depression, the cerebral application of NPY reduced depressive-related symptoms (Britton et al., 2000, Loh et al., 2015). Furthermore, others have studied the interaction between NPY and CRH in emotional

responsiveness to stress and found that i.c.v injections of NPY antagonized the anxiogenic effects of CRH in rats, pointing at opposing effects of these two neurotransmitters in stress-induced behavioral alterations (Britton et al., 2000). Thus, HPA axis activation state was analyzed at several levels at both time points of the current study.

This report shows that pretreatment with NPY protected mice from a prolonged HPA axis stimulation observed 21 h post immune challenge, which is reflected by the blunted release of CORT into circulation at that time point. Interestingly, at the earlier time point under study (3 h post LPS), NPY failed to blunt the elevated secretion of CORT induced by peripheral LPS injection. It follows that HPA axis marker levels are affected by IN NPY pretreatment at a time point between 3 h to 21 h following TLR4 activation, or even thereafter, and future studies will elaborate the exact time-course of how IN NPY impacts the HPA axis. Nevertheless, the dampened CORT response at the later time point was accompanied by an alleviated body weight loss, which may be attributed to an enhanced resilience to immune stress evoked by NPY. Likely this effect of NPY is mediated by the energy conserving ability of the neuropeptide (Loh et al., 2015) rather than enhanced food intake which was found to be unaltered by IN NPY in this current study in the LabMaster test (see section 3.2.8). The protection from weight-loss observed in NPY pretreated TLR4 stimulated animals reported here is in line with a previous study in which Bacille Calmette–Guérin infected mice lacking NPY were more prone to lose weight when compared to wild type controls (Painsipp et al., 2013).

Peripheral immune challenge during the acute phase of sickness decreases hypothalamic expression of NR3C1, which is a characteristic of the negative feedback loop mediated by CORT (McEwen, 2007). Surprisingly, LPS used at a dose of 0.03 mg/kg did not significantly upregulate CRH mRNA levels within the hypothalamus, although there was a trend towards an interaction between IN NPY and i.p. LPS treatment on CRH expression levels. Thus, it will be worthwhile to conduct further studies to elucidate the exact point of interaction between the NPY system and the HPA axis and to analyze ACTH expression and release following immune stimulation in combination with NPY treatment. Fig. 28 provides a graphical summary of the effects induced by IN NPY to blunt the acute sickness response evoked by LPS and to interfere with HPA axis activation.

Taken together, the interference of NPY with LPS-evoked HPA axis stimulation might contribute to the dampened sickness response, given that others have shown that antagonism

of glucocorticoid receptors inhibits the stress-induced exacerbation of inflammation by peripheral immune challenge (Munhoz et al., 2006, Sorrells et al., 2009). The ability of NPY to interfere with HPA axis activation might be mediated via activation of inhibitory Y1 receptors expressed by hypothalamic melanocortin neurons which represses the release of pro-opiomelanocortin, the precursor protein of ACTH (Beishuizen and Thijs, 2003, Hokfelt et al., 2003). In turn, the release of CORT from peripheral adrenal glands would be reduced.

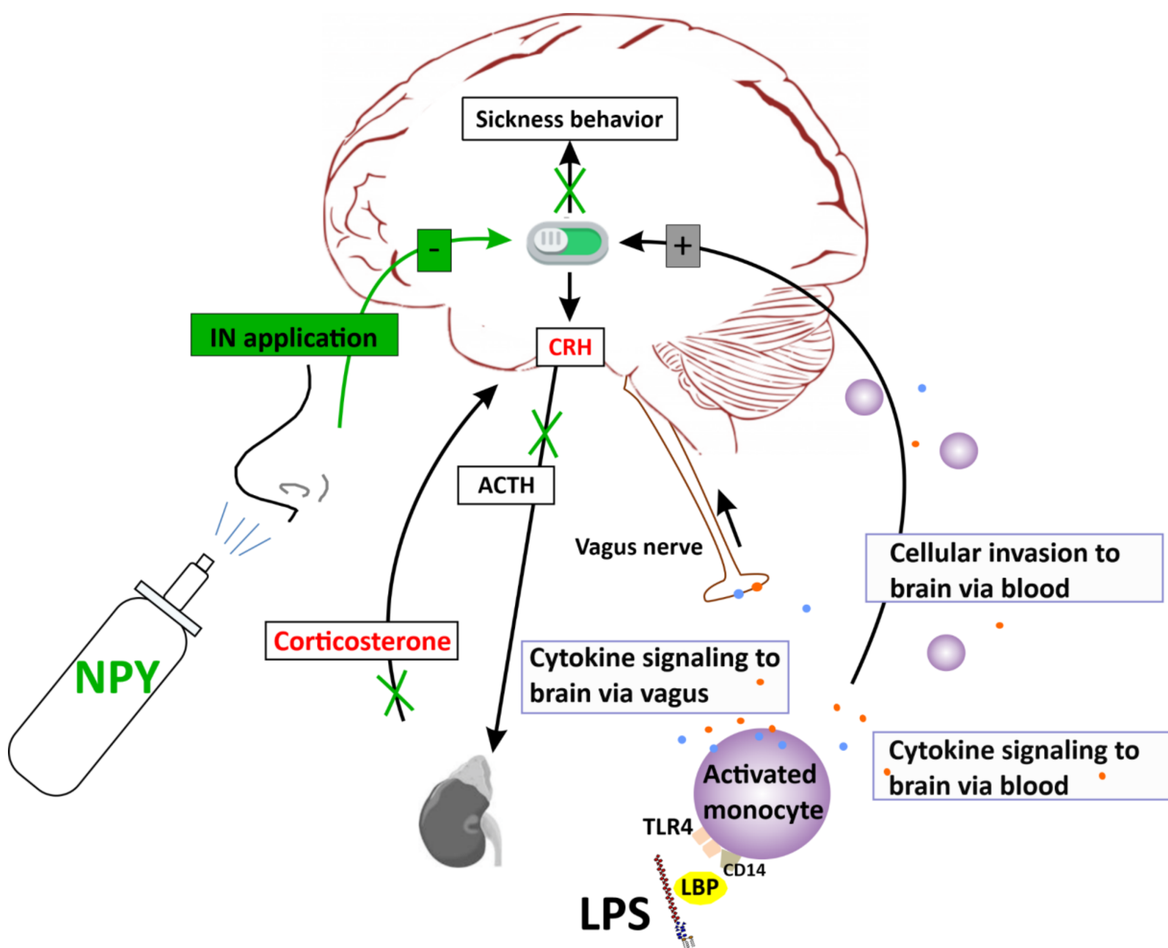

Figure 28 Proposed impact of intranasal (IN) neuropeptide Y (NPY) on hypothalamus-pituitary-adrenal (HPA) axis activation and feedback as well as sickness in response to peripheral lipopolysaccharide (LPS) challenge. Cytokines released by stimulated peripheral immune cells travel by circulation to the central nervous system and enter the brain, or they activate receptors present at fibers of the vagus nerve. Stimulated immune cells can also travel to the brain and induce a central immune reaction. Following IN application, NPY acts to blunt HPA axis activation and to mitigate sickness behavior. Abbreviations: ACTH=adrenocorticotropic hormone, CD=cluster of differentiation, CRH=corticotropin-releasing hormone, IN=intranasal, LBP=LPS-binding protein, LPS=lipopolysaccharide, NPY=neuropeptide Y, TLR=toll-like receptor.

4.3 Limitations and open questions

My thesis has provided a number of novel insights into how the effects of peripheral immune stimulation on brain function and behavior are modified by dietary and pharmacologic interventions. At the same time, these advances raise several new questions and need to undergo critical consideration with regard to limitations in their interpretation. For instance, it is difficult to compare the activity of Poly(I:C) and LPS in stimulating the innate immune system, given that their molecular size is ill-defined and the PRRs activated by them differ in type, cellular expression and extra-/intracellular location. As a consequence, different pathways of immune-brain interaction are likely to operate. Peripheral immune stimulation is known to give rise to a neuroinflammatory response in the brain, consisting in a disruption of the BBB, influx of leukocytes and intracerebral expression of cytokines by activated microglia. One limitation of this study is that the specific role of microglia cells in the cerebral reaction to peripheral immune stimulation was not analyzed and future studies based on this thesis need to focus on this issue. Furthermore, it will be interesting to analyze the impact of IN applied NPY on LPS-induced behavioral alterations other than those assessed in the OF test. For example, it would be worthwhile to evaluate whether depressive-like behavior (evoked by peripheral LPS treatment at a higher dose than was used in this thesis) might be prevented by preceding NPY infusion. Additionally, while cerebral gene expression was assessed in the hypothalamus, this thesis did not evaluate alterations in other brain areas that might be differently affected by the treatments applied in this study. Common to most studies using animal models, this study included one single mouse strain and only male mice in one facility. Therefore, environmental effects as well as effects of genetic background and gender differences on the interventions studied here remain unknown.

4.4 Conclusions

This current study shows that the TLR3-induced increase of peripheral cytokine release as well as the acute sickness response to Poly(I:C) is exacerbated by preceding fasting. Although hypothalamic mRNA expression of the anti-inflammatory NPY and circulating CORT levels were elevated by IF, those factors did apparently not influence the detrimental impact of IF on viral immune challenge. Thus, fasting aversively affects the outcome of sickness induced by Poly(I:C), which is in contrast to the effects observed in calorie restricted rodents subjected to bacterial immune stimulation. Therefore, it is likely that the

neuronal and neuroendocrine mechanisms controlling the response to viral or bacterial insults are differently affected by feeding restriction.

In the second part of my dissertation I show that IN pretreatment with NPY protects mice from the development of a behavioral sickness response and excessive HPA axis stimulation in response to TLR4 activation, which indicates an important role for the neuropeptide in resilience to immune challenge-evoked stress. These results could also have implications for patients presenting with chronically upregulated inflammatory markers and neuropsychiatric disorders.

Taken together, this study shows that (I) fasting does not ameliorate, but exacerbates the viral mimic Poly(I:C)-induced behavioral sickness response in the OF test, and (II) this is accompanied by an exacerbated peripheral secretion of pro-inflammatory cytokines. Furthermore, I report that (III) the bacterial factor LPS is able to induce a stronger and longer-lasting behavioral sickness response than Poly(I:C) in a homecage-like environment and (IV) IN pretreatment with NPY blunts the acute behavioral sickness response, (V) which is associated with the ability of the neuropeptide to interrupt a full blown HPA axis activation due to peripheral immune stimulation with LPS.

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6 Appendix

6.1 cDNA synthesis PCR program

RNA from hypothalamic brain samples were reverse transcribed according to the manufacturer's suggestions and the following PCR program. PCR was performed in a thermal cycler (Biorad, MyCycler).

Table 1 Thermal cycler conditions for reverse transcription.

Step	1	2	3	4
Temperature	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 s	∞
Cycles	1	1	1	

6.2 RT qPCR program

RT qPCR was performed as suggested by the manufacturer and cDNA was inserted at a dilution of 1:10, a thermo cycler (LightCycler®480) was used for the reaction.

Table 2 Thermal cycler conditions for qPCR

Step	1	2	3	4
Temperature	50°C	95°C	95°C	60°C
Time	2 min	10 min	15 s	60 min
	1	1	40	

6.3 nCounter PlexSet Details

Table 3 nCounter PlexSet Design Genes and Target Sequences. IFN=interferon, IL=interleukin, TNF=tumor necrosis factor, NPY=neuropeptide Y, CRH=corticotropin releasing hormone, BDNF=brain-derived neurotrophic factor, TJP1=tight-junction protein1, OCLN=occludin, CLDN=claudin, Ppil3=peptidyl-prolyl cis-trans isomerase-like 3 Reporter gene, Ywhaz=tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide, Tubb5=tubulin beta 5 class, UBE2D2=ubiquitin-conjugating enzyme E2 D2 reporter gene, NR3C1=glucocorticoid receptor, GRIN2B=glutamate receptor NMDA2B, CCL2=CC-chemokine ligand 2, HK=housekeeper.

nCounter PlexSet Details (Mus musculus)										
Report Date: 17-Apr-18										
Name	**Accession**	**Position**	**Target Sequence**	**A Tm**	**B Tm**	**Flags**	**HUGO Gene**	**NSID**	**Tag**	**Design Remarks**
IFN-γ	NM_008337.1	96-195	CTAGCTCTGAGACAATGAACGCTACACACTGCATCTTGGCTTTGCAGCTCTTCCTCATGGCTGTTTCTGGCTGTTACTGCCACGGCACAGTCATTGAAAG	82	79		Ifng	NM_008337.1:95	T001	
IFN-α	NM_010502.2	355-454	CTGCAAGGCTGTCTGATGCAGCAGGTGGGGGTGCAGGAATTT	88	87	X	Ifna1	NM_010502.2:354	T002	also targets several other interferon alpha

			CCCCTGACCCAGGAAGATGCCCTGCTGGCTGTGAGGAAATACTTCCACAGGATCACTG							genes @ >90%
IFN-β	NM_010510.1	336-435	GATGAACTCCACCAGCAGACAGTGTTTCTGAAGACAGTACTAGAGGAAAAGCAAGAGGAAAGATTGACGTGGGAGATGTCCTCAACTGCTCTCCACTTGA	79	82		Ifnb1	NM_010510.1:335	T003	
IL-6	NM_031168.1	41-140	CTCTCTGCAAGAGACTTCCATCCAGTTGCCTTCTTGGGACTGATGCTGGTGACAACCAC	82	82		Il6	NM_031168.1:40	T004	

			GGCCTTCC CTACTTCA CAAGTCC GGAGAGG AGACTTCA CAG							
IL-1β	NM_0083 61.3	109-208	TTGACAG TGATGAG AATGACCT GTTCTTTG AAGTTGA CGGACCC CAAAAGA TGAAGGG CTGCTTCC AAACCTTT GACCTGG GCTGTCCT GATGAGA GCAT	81	85		Il1b	NM_ 0083 61.3: 108	T005	
TNF-α	NM_0136 93.2	515-614	TGGATCTC AAAGACA ACCAACTA GTGGTGC CAGCCGA TGGGTTG TACCTTGT CTACTCCC AGGTTCTC TTCAAGG	85	85		Tnf	NM_ 0136 93.2: 514	T006	

			GACAAGG CTGCCCC GACTACG TGCT							
IL-10	NM_0105 48.2	251-350	GACAACA TACTGCTA ACCGACTC CTTAATGC AGGACTT TAAGGGT TACTTGG GTTGCCA AGCCTTAT CGGAAAT GATCCAG TTTTACCT GGTAGAA GTGA	81	80		Il10	NM_ 0105 48.2: 250	T007	
NPY	NM_0234 56.2	231-330	GACACTA CATCAATC TCATCACC AGACAGA GATATGG CAAGAGA TCCAGCCC TGAGACA CTGATTTC AGACCTCT TAATGAA GGAAAGC	85	85		Npy	NM_ 0234 56.2: 230	T008	

			ACAGAAAACGC							
NPY receptor Y1	NM_010934.4	311-410	TTCTCCACTTCTGGCTTTTGAAAATGATGACTGCCACCTGCCCTTGGCTGTGATATTCACCTTGGCTCTCGCTTATGGGGCGGTGATTATTCTTGGCGTC	83	79		Npy1r	NM_010934.4:310	T009	
NPY receptor Y2	NM_001205099.1	591-690	TCTACAGCCTTTCCACCCTGCTCATCCTGTACGTTTTGCCTCTGGGCATCATATCTTTCTCCTACACCCGTATCTGGAGTAAGCTGAGGAACCACGTCAG	82	83		Npy2r	NM_001205099.1:590	T010	

NPY receptor Y5	NM_016708.3	725-824	CCAGTGTTTCACAGCCTTGTGGAACTTAAGGAAACCTTTGGCTCAGCATTGCTAAGCAGCAAGTATTTGTGTGTTGAGTCATGGCCCTCTGATTCATACA	80	79		Npy5r	NM_016708.3:724	T011	
CRH	NM_205769.3	657-756	CGCTGGAGAGGGAGAGGCGGTCGGAGGAGCCGCCCATCTCTCTGGATCTCACCTTCCACCTTCTGCGGGAAGTCTTGGAAATGGCCCGGGCAGAGCAGTT	92	89		Crh	NM_205769.3:656	T012	
BDNF	NM_007540.4	640-739	GTTCCACCAGGTGAG	78	81		Bdnf	NM_0075	T013	

			AAGAGTGATGACCATCCTTTTCCTTACTATGGTTATTTCATACTTCGGTTGCATGAAGGCGGCGCCCATGAAAGAAGTAAACGTC					40.4:639		
TJP1	NM_009386.1	3566-3665	GAGCAGCCGTCATACAGGTATGAGGTCTCAAGCTACACAGACCAGTTTTCTCGGAACTATGACCATCGCCTACGGTTTGAAGATCGAATCCCTACCTATG	81	82		Tjp1	NM_009386.1:3565	T014	
OCLN	NM_008756.2	1421-1520	CTGGGTCAGGGAATATCCACCTATCACTTC	84	83		Ocln	NM_008756.2:1420	T015	

			AGATCAACAAAGACAACTCTACAAGAGAAATTTTGATGCAGGTCTGCAGGAGTATAAGAGCTTACAGGCA							
CLDN1	NM_016674.4	411-510	CTTCGACTCCTTGCTGAATCTGAACAGTACTTTGCAGGCAACCCGAGCCTTGATGGTAATTGGCATCCTGCTGGGGCTGATCGCAATCTTTGTGTCCACC	82	82		Cldn1	NM_016674.4:410	T016	
CLDN5	NM_013805.4	555-654	CTCTGCTGGTTCGCCAACATCGTTGTCCGCGAGTTCTATGATCC	80	92		Cldn5	NM_013805.4:554	T017	

			GACGGTG CCGGTGT CACAGAA GTACGAG CTGGGCG CGGCGCT GTACATC GGCTGGG							
Ppil3	NM_0273 74.3	571-670	GAGGAGG TAGCAGC ATCTGGG CCAAAAA GTTTGAG GATGAAT ACAGTGA ATATCTGA AGCACAA TGTTCGA GGTGTTG TATCTATG GCTAATA ATGGCCC	83	80	HK	Ppil3	NM_ 0273 74.3: 570	T018	
Ywhaz	NM_0117 40.2	456-555	AACGTTG TAGGAGC CCGTAGG TCATCGTG GAGGGTC GTCTCAA GTATTGA GCAGAAG	80	82	HK	Ywhaz	NM_ 0117 40.2: 455	T019	

			ACGGAAGGTGCTGAGAAAAAGCAGCAGATGGCTCGAGAATACA							
Tubb5	NM_011655.5	407-506	AGCCACAGGTGGCAAGTATGTCCCTCGAGCTATCTTGGTGGATCTAGAACCTGGGACTATGGACTCCGTTCGCTCAGGTCCTTTTGGCCAGATCTTCAGA	85	83	HK	Tubb5	NM_011655.5:406	T020	
UBE2D2	NM_019912.1	2245-2344	TTCATGTGAACTAGGCTAGTTACCTCCCCTCGTCCCCTTTCCTAACCTAAATGTAAGCCAGGCCAGCTT	80	83	HK	Ube2d2a	NM_019912.1:2244	T021	

			GAAGGCC AATGCTCT TTAGTCAC CAAGATCT							
NR3C 1	NM_0081 73.3	1801- 1900	ACCAGGA TTCAGAA ACTTACAC CTGGATG ACCAAAT GACCCTTC TACAGTAC TCATGGA TGTTTCTC ATGGCAT TTGCCCTG GGTTGGA GATCATAC AGA	81	81		Nr3c1	NM_ 0081 73.3: 1800	T022	
GRIN2 B	NM_0081 71.3	1741- 1840	GAAGAGG ATCTACCA GTCTAACA TGCTGAA TAGGTAT CTGATCAA CGTCACTT TTGAAGG GAGAAAC CTGTCCTT CAGTGAA GATGGCT	82	82		Grin2 b	NM_ 0081 71.3: 1740	T023	

			ACCAGATGCAT							
CCL2	NM_011333.3	416-515	TCTTCAGCACCTTTGAATGTGAAGTTGACCCGTAAATCTGAAGCTAATGCATCCACTACCTTTTCCACAACCACCTCAAGCACTTCTGTAGGAGTGACCA	79	82		Ccl2	NM_011333.3:415	T024	