Diploma thesis

Microglial activation in mouse brain following peripheral immune stimulation

written by

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Preamble

“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”

— Marie Curie

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Table of content

Table index

Index of figures

Abbreviations

Actb	actin beta
ANOVA	analysis of variance
BBB	blood-brain barrier
CD	cluster of differentiation
cDNA	complimentary desoxyribunocleic acid
CNS	central nervous system
CRH	corticotropin-releasing hormone
CSF	cerebrospinal fluid
Gapdh	glycerinaldehyd-3-phosphat-Dehydrogenase
HPA	hypothalamic-pituitary-adrenal
IBA	ionized calcium-binding adapter molecule
IDO	Indolamin-2,3-Dioxygenase
IFN	interferon
IL	interleukin
IN	intranasal
IP	intraperitoneal
IRAK	interleukin-1 receptor (IL-1R) associated kinase
IRF	interferon regulator factor
LBP	LPS-binding protein
LPS	lipopolysaccharide
MAPKs	mitogen-activated protein kinases
MyD	myeloid differentiation factor
NF-κB	nuclearfactor-κB
NMDA	N-Methyl-D-Aspartat
NPY	neuropeptide Y

PAMPs	pathogen associated molecular patterns
PCR	polymerase chain reaction
Poly(I:C)	polyinosinic:polycytidylic acid
Ppil3	peptidyl-prolyl cis-trans isomerase-like 3
RNA	ribonucleic acid
RT	reverse transcriptase
TIR	toll/IL-1R homology domain
TIRAP	TIR adaptor protein
TLR	toll-like receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRAF	TNFR-associated factor
TRIF	TIR-domain-containing adaptor protein inducing interferon-β
VEH	vehicle

Zusammenfassung

Es gibt zunehmend Hinweise darauf, dass entzündliche Prozesse an der Entstehung neuropsychiatrischer Störungen beteiligt sein könnten. Obwohl die zugrundeliegenden Mechanismen noch nicht vollständig verstanden sind, haben frühere Arbeiten gezeigt, dass eine periphere Immunstimulation sowohl durch virale als auch bakterielle Faktoren neuroinflammatorische Prozesse auslöst. Dieser Effekt ist nicht nur gekennzeichnet durch eine erhöhte Expression von Zytokinen im Gehirn der Maus und Verhaltenszeichen von Krankheit, sondern auch depressionsartiges Verhalten zu einem Zeitpunkt, an dem die Akutsymptomatik schon abgeklungen ist. In diesem Zusammenhang wird den Mikrogliazellen eine entscheidende Rolle zugeschrieben. Sie sind eine spezialisierte Population von Makrophagen, die unter anderem geschädigte Zellfragmente phagozytieren, um die Homöostase des zentralen Nervensystems aufrechtzuerhalten. Darüber hinaus wird angenommen, dass das Neuropeptid Y (NPY), ein in hohen Konzentrationen vorkommendes Neuropeptid im ZNS, dem negativen Einfluss der Immunstimulation auf Stimmung und Stressresistenz entgegenwirkt. Diese homöostatische Rolle des NPY könnte sich aus seiner Fähigkeit ergeben, die Aktivierung von Mikrogliazellen zu beeinflussen, die durch die Immunaktivierung induziert wird.

Ziel dieser Diplomarbeit war es, die durch bakterielle und virale periphere Immunstimulation hervorgerufene Aktivierung hypothalamischer Mikrogliazellen und die mögliche Wirkung von intranasal appliziertem NPY zu zwei Zeitpunkten zu charakterisieren. Mäuse wurden intranasal mit Wasser oder NPY infundiert und 30 Minuten danach intraperitoneal entweder mit der bakterielle Zellwandkomponente Lipopolysaccharid (LPS) oder dem viralen Agonisten Poly(I:C) injiziert. Daraufhin wurden nach 3h und 21h Gehirne entnommen, um die Mikrogliaaktivierung zu analysieren. Dazu wurden die hypothalamischen Bereiche des Gehirns mikrodissektiert und die RNA extrahiert. Mittels qPCR wurde die Genexpression der mikroglialen Marker Iba1 und der Polarisationsmarker CD86 (M1) und CD206 (M2) ausgewertet.

Interessanterweise zeigte sich in den statistischen Analysen 3h nach kombinierter Injektion mit NPY + Poly(I:C) die stärkste Hochregulation aller drei untersuchten Marker Iba1 ($p=0.0056$), CD206 ($p=0.0033$) und CD86 ($p=0.0019$). Poly(I:C) und LPS führten ebenfalls zu einer Hochregulation sowohl des eher pro-

inflammatorischen Markers CD86 (p=0.0504 bzw. p= 0.0104) und des eher anti-inflammatorischen CD206 (p=0.455 bzw. p=0.0282). Zum Zeitpunkt 21h nach Applikation zeigte sich einzig CD206 in den Behandlungsgruppen Wasser + Poly(I:C) und NPY + Poly(I:C) noch hochreguliert.

Diese Ergebnisse unterstreichen frühere Befunde, die eine starke Stimulation der Mikrogliazellen nach einer peripheren Immunstimulation zeigen. Zusammenfassend und entgegen unserer Arbeitshypothese kann die intranasale Vorbehandlung mit NPY die hypothalamische Expression der mikroglialen Marker Iba1, CD206 und CD86, nach peripherer Gabe von LPS oder Poly(I:C) nicht reduzieren.

Abstract

Emerging evidence indicates that inflammatory processes may be involved in the development of neuropsychiatric disorders. However, while underlying mechanisms are not yet fully understood, previous work in rodents has shown that peripheral immune stimulation by both viral and bacterial factors evokes neuroinflammatory processes. This “mirroring” effect is characterized by an enhanced expression of cytokines in the murine brain, behavioral signs of sickness and depressive-like behavior at a time point when acute sickness has abated. In this context, microglial cells are thought to play a crucial role. They are resident macrophages and constant monitors of brain tissue playing a key role in conserving CNS integrity. Furthermore, Neuropeptide Y (NPY), which is one of the most prevalent peptides in the central nervous system, is thought to counteract such a negative impact of immune stimulation on mood and stress resilience. This homeostatic role of NPY might arise from its capability to influence the activation of microglial cells, which is induced by immune activation.

The aims of this diploma thesis were to characterize hypothalamic microglial cell activation evoked by bacterial and viral peripheral immune stimulation and the possible impact of intranasally (IN) applied NPY at two time points: 3h post peripheral immune challenge (short term) and 21h post peripheral immune challenge (long term). Therefore, mice were infused IN with water or NPY and 30 minutes thereafter, intraperitoneally (IP) injected with either the bacterial cell-wall component lipopolysaccharide (LPS) or the viral mimic Poly(I:C).

Brains were microdissected and RNA was extracted from hypothalamic areas. Gene expression of microglial markers Iba1 and polarization markers CD86 (M1) and CD206 (M2) were evaluated via qPCR. Two-way analysis of variance (ANOVA) was performed for statistical analysis, followed by post-hoc planned comparison with Fisher’s Least Significant Difference (LSD) test.

Interestingly, in the short term paradigm 3h after application, the strongest upregulation of all three markers Iba1 ($p=0.0056$), CD206 ($p=0.0033$) and CD86 ($p=0.0019$) was found after the combined injection of NPY and Poly(I:C).

Poly(I:C) and LPS also led to an upregulation of both the more pro-inflammatory marker CD86 ($p=0.0504$ and $p=0.0104$) and the more anti-inflammatory marker CD206 ($p=0.455$ and $p=0.0282$). In the groups 21h after application only CD206 in the water + Poly(I:C) and NPY + Poly(I:C) combination was still upregulated.

These results underline previous findings of strong stimulation of microglial cells following peripheral immune stimulation. In conclusion and contrary to the hypothesis under study, IN NPY application failed to mitigate microglial marker expression in the hypothalamus following peripheral immune stimulation by LPS or Poly(I:C).

1. Introduction

The exploration of inflammatory mechanisms dates back to over 2000 years, when the cardinal symptoms of inflammation were described by Aulus Cornelius Celsus. Historically, Robert Koch and Louis Pasteur set another milestone in this development towards the end of the 19th century: The germ theory of disease, which states that microbial substances are the main cause of acute inflammatory reactions. Today we know that a reduction to acute conditions would be an insufficient simplification of all the forms and modalities in which inflammatory reactions can appear, although this often serves as a starting point of a long-followed process. Increasing attention has been paid to the broad spectrum of chronic inflammatory conditions to unknown triggers that involve various organ systems and major diseases of the 21st century in industrialized countries, including diabetes and obesity, atherosclerosis, and neurodegenerative diseases to name a few (2).

Over the past decade, evidence indicating that inflammatory processes are involved in the development of neuropsychiatric disorders has risen. In fact, peripheral inflammation has been linked to widely known psychiatric diseases such as major depression (3). In this context, microglial cells, the resident innate immune cells in the brain, including the hypothalamus, the primary regulatory organ of the autonomic and endocrine system, have been given a major role (4).

While the answer to the question why we react and defend ourselves from external agents may make common sense in the first place, namely to eradicate pathogens and ensure homeostasis and subsequently health, the way how and why certain sub-events in this process happen and to which long term consequences they lead are current topics of research since increasing knowledge also gave rise to more, fundamental physiological questions across many disciplines (5).

In the following, a general overview on immune-brain dialogs with special focus on the hypothalamus will be given, starting with an explanation of peripheral immune reaction in the body, followed by its potential progression to the brain and cerebral consequences these processes could imply.

1.1 Unwanted intruders: Peripheral immune stimulation

As a living being, mammals are in constant material exchange with the environment, containing a wide variety of infective pathogens. Therefore, there is always the danger of pathogenic germs entering the organism. The occurrence of such an event would in any case influence homeostasis, which describes a concept of inner physiologic stability that has been developing since the 19th century (6). The mammal's immune system is a complex and multilevel defense system, generally comprising an innate, the first line response which is present from birth and immediately active after pathogen contact, and an acquired immune response.

To recognize a broad spectrum of invading molecular structures unique to microbial metabolism and regardless of their life-cycle stage, the so-called pathogen-associated molecular patterns (PAMPs) have been known for 20 years to be at the start of the first line defense and to induce a downstream signal cascade (7).

The two most common immune stimulants are bacterial and viral entities. To mimic immune stimulation under controlled circumstances lipopolysaccharide (LPS), a component of the outer wall of gram-negative bacteria, as well as the viral mimic polyinosinic:polycytidylic acid (Poly(I:C)) are widely used (8, 9).

1.1.1 Lipopolysaccharide binds to Toll like Receptor 4

LPS, consisting of lipid A as the most immunologic active component and a polysaccharide chain, has long been known for its role as a potent immunostimulant (10). Once administered, LPS has been shown to quickly attach to the acutely induced plasma lipopolysaccharide binding protein (LBP) which is then responsible for their delivery to cluster of differentiation 14 (CD14), a mobile or cell surface glycosylphosphatidylinositol anchor (11, 12). The cellular first aid, the innate immune system, mainly macrophages and dendritic cells, now hold several germline-coded pattern recognition receptors, PRRs, identification scanners of PAMPs, mentioned above. Within these receptors, the family of 10 Toll-like receptors (TLR) in humans were shown to play a vital role (13). Originally discovered in *Drosophilia* their important role in vertebrates, which was first shown in 1997, is becoming increasingly understood (14).

TLRs are type I transmembrane proteins specialized in recognizing different microbial ligands and therefore also vary in cellular localization for accessibility. Expression takes either place on the cell surface, as for TLR4 which is the best characterized LPS binding receptor, or on intracellular vesicles, as for TLR3 recognizing double-stranded RNA. Furthermore, TLR4 is not exclusively expressed by cell types of the myeloid lineage but on some non-immune cells like fibroblasts, intestinal epithelial and endothelial cells (15).

The extracellular domain contains leucine-rich repeats, while the cytosolic domain Toll/IL-1R (TIR) is structurally equivalent to the cytosolic domain of the interleukin 1 (IL-1) receptor. Thus, the same proteins activated during TLR4 signaling, also participate in transduction induced by IL-1, a group of important pro-inflammatory cytokines primarily associated with acute and chronic inflammation (16).

Upon transfer of LBP-LPS to CD14, LPS is parted into monomeric molecules and recognized by TLR4 on the cell membrane as can be seen in Figure 1 (12, 17). The extracellular stimulation complex induces a recruitment and binding of intracellular adaptor proteins containing a TIR domain (TIRAP). From there, TLR signaling is roughly divided into two different reaction chains depending on the involvement of the adaptor molecules, either myeloid differentiation factor 88 (MyD88) or the TIR-domain-containing adaptor protein inducing interferon-β (TRIF). This being said, TLR4 is the only receptor that can activate both ways (18). The TIRAP-MyD88 complex leads to phosphorylation and thus activation of intracellular kinases like for example interleukin-1 receptor (IL-1R) associated kinase (IRAK). Via further steps involving tumor necrosis factor receptor associated factor (TRAF) (19), Nuclear factor-κB (NF-κB) formation and mitogen-activated protein kinases (MAPKs) are initiated. NF-κB then translocates into the cell nucleus where it upregulates the expression of inflammatory cytokines like Interleukin 6 (IL-6), tumor necrosis factor α (TNF-α) and Type I Interferons such as Interferon-α (IFN-α) und IFN-β. Temporally, it has been observed that the MyD88-dependent signaling pathway results in an early phase transcription compared to TRIF-dependent pathways (18, 20).

For the latter, TLR4 undergoes endocytosis upon stimulation and is intracellularly transported with TRIF-related adaptor molecule (TRAM), a small TIR domain-containing protein, to the endosome, where it builds a complex with TRIF (21). TRAF3 then leads to interferon-regulation factors (IRF) translocating to the

nucleus inducing type 1 Interferon formation. Furthermore, the activation of NF-κB and MAPK is also part of the TRIF-dependent pathway in the late phase (18).

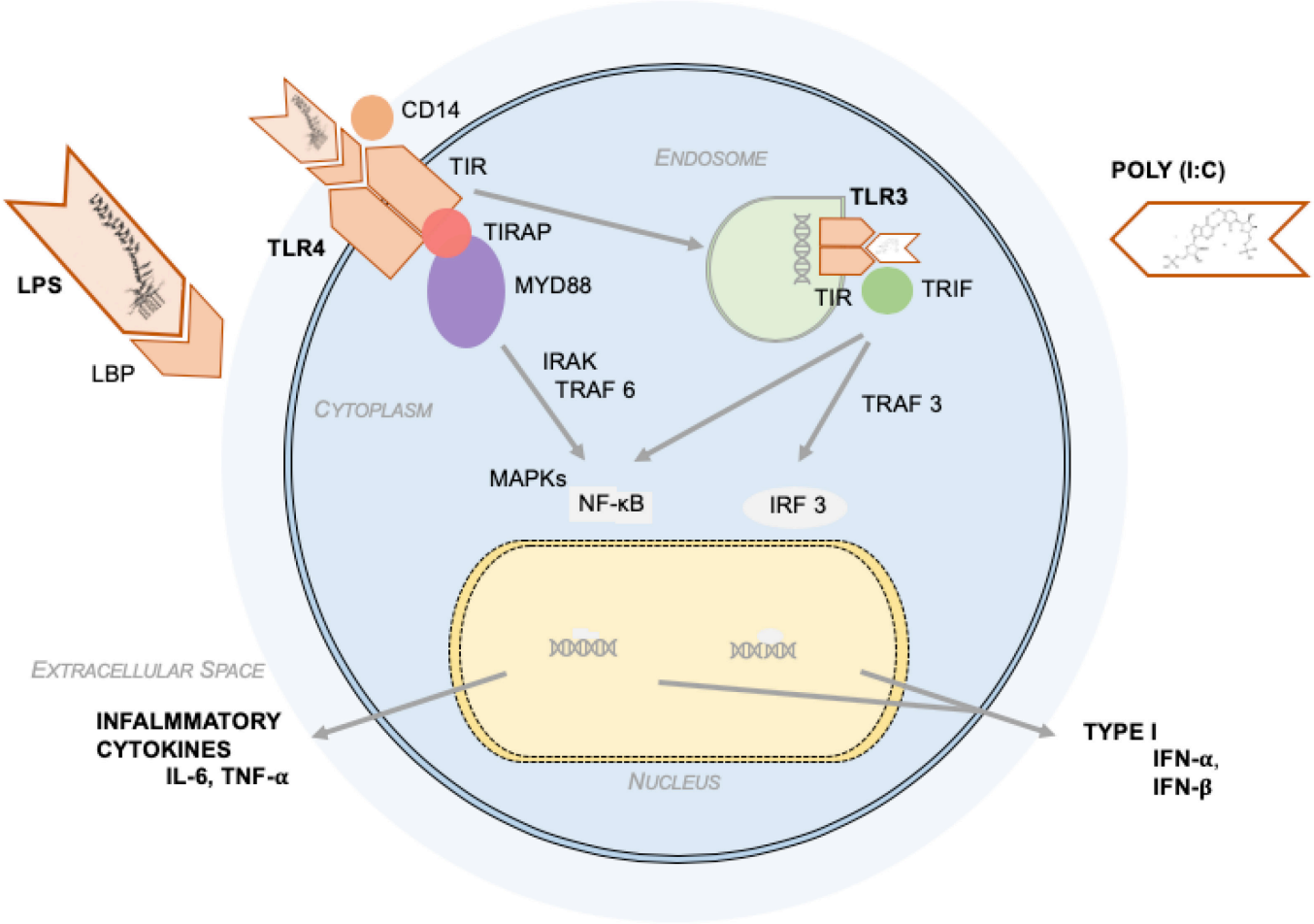

Figure 1: Toll-like (TLR) receptor 3 and 4 signaling pathways. The bacterial factor LPS and the viral mimic Poly(I:C) stimulate TLR4 and TLR3, respectively, and thereby induce intracellular signaling cascades, which possibly end in the release of pro-inflammatory signals. TLR=Toll-like receptor, LPS=Lipopolysaccharide, LBP=LPS- binding protein, MyD=myeloid differentiation factor, MAPKs=mitogen-activated protein kinases, IRF=Interferon regulator factor, IRAK=interleukin-1 receptor(IL-1R)associated kinase, NF-κB=Nuclear factor-κB, TIR=Toll/IL-1R homology domain, Poly(I:C) =Polyinosinic:polycytidylic acid, TNF=Tumor necrosis factor, TNFR= Tumor necrosis factor receptor, TRAF=TNFR-associated factor, TRIF=TIR-domain-containing adaptor protein inducing interferon-β, TIRAP=TIR adaptor protein, IFN=Interferon, IL=Interleukin

Alterations were also observed in epithelial cells that are permanently exposed to microorganisms and appear to suppress TLR activation response to commensal microbiota. In these cells the ability of changing receptor localization and quantity has been observed (22). TLR expression is by no means static, but is rapidly adapted in response to pathogens, a variety of cytokines and environmental stressors (20). Due to its potential lethal role in septic shock, immune responses to LPS have been topic of research for a long time. Thereby a so called endotoxicity tolerance or LPS desensitization phenomenon has been observed in survivors. By exposing macrophages to a sublethal dose of LPS the cells become refractory for a following exposure to a high dose of LPS (23). Although TLR4 is known to bind to LPS, it is noteworthy that other ligands such as paclitaxel, proteins of Respiratory Syncytial Virus (RSV), fibronectin, heat shock proteins and also endogenous molecules can act as TLR4 agonists (18).

1.1.2 Polyinosinic:polycytidylic acid binds to Toll like receptor 3

While LPS is a stable amphipathic molecule that is quickly transported from the peritoneum to the bloodstream (24) and stimulates the innate immune cells at more distant sites including the blood brain barrier (BBB), and the circumventricular organs (CVO).

Fairly different behaves Poly(I:C): Also stimulating the innate immune system it is a large, charged molecule, that is easily degraded by ubiquitous RNAses throughout the body (25). Therefore, Poly(I:C) leads to elevated blood-borne inflammatory mediators at the site of entry (9, 26).

Of all nucleic-acid sensitive TLRs, TLR3 has most often been identified for its ability to recognize double stranded ribonucleic acid (dsRNA), derived from viruses, virus-infected cells or the viral mimic Poly(I:C) (18). As a viral receptor, TLR3 detects molecular replication patterns and has a unique intracellular localization (27). Like TLR4, it is expressed not only in immune cells, where it is localized in the endosome, but also in fibroblasts, epithelial cells and neurons, where TLR3 could additionally be found on the cell surface. TLR3-mediated signaling is initiated, nevertheless, intracellularly by the endosomal compartments in both cell types (28). Receptor formation takes place in the endoplasmic reticulum, from where it is taken up into the endosomes by transmembrane proteins (29). After a distinct molecular analysis in 2005, TLR3 is structurally

known for its large size and surface area to facilitate binding of the N-terminal and C-terminal region of dsRNA (30). Hereby, sufficient stability to form a homodimer across the C-terminal TIR is provided upon ligand connection on the ectodomain. TLR3, as TLR4, initiates immune responses directly by activation of the TRIF-dependent signaling pathway and via NF-κB and IRF3 through a consecutive production of type I interferon and inflammatory cytokines (31). This result is the common characteristic that TLR3 and TLR4 share. However, TLR3 is the only one that does not use the MYD88 as an adaptor protein, but exclusively TRIF (18). Upon Poly(I:C) stimulation, histological analysis demonstrated CNS responses via elevated IL-1β expression by microglia as well as IL-1β-induced fever induction (9).

1.1.3 Communication packets: Cytokines

Derived from the latin inter = "between" and greek leukos = "white" the family of interleukins are communication signals between white blood cells. Currently, more than 15 different subtypes are known. It is a very heterogeneous group of mediators, which all have different functions throughout the organism and are secreted by different cells. Many cytokines interact with each other or regulate the synthesis and release of other cytokines (32). IL-1, IL-6 and TNF-α can be summarized as the most important pro-inflammatory cytokines, leading to induction of fever via the hypothalamus and the synthesis of acute-phase proteins in the liver (32).

IL-1, TNF-α, IL-6 and IL-12 are also known for their role in chronic inflammation and the influence on various metabolic functions (33).

Interleukins were numbered according to the date of their discovery, which explains why IL-1 is the first described interleukin. IL-1 has two active forms, encoded on different genes, binding to the same receptor (34). As mentioned above, the cytosolic domain of the IL-1 receptor is the TIR domain equal to TLRs. After cloning the murine IL-1 receptor (35), it could be repeatedly proven that the TIR domain is essential for signal transduction in IL-1 production in 1992 (36) (37). Although relevance and connection to the TIR domain is known, cellular production and secretion mechanisms of IL-1β are not yet entirely understood. The endoplasmic reticulum and the Golgi do not seem to be involved in its protein completion, as is classically the case, but rather requires precursor protein

modification through the inflammasome complex, detection protein packs, and membrane permeabilization (18, 38-40). In short, numerous studies could show a significant increase of IL-1β levels after immune stimulation (18, 41, 42).
In general, cytokines are expected to induce adequate immune responses as well as behavioral changes to promote recovery and at the same time stimulate anti-inflammatory processes that attenuate the inflammatory response once the thread is under control or has been eliminated (8). It is important to note that anti-inflammatory does not mean that the inflammatory environment is immediately dissolved. Responsible mediators, such as glucocorticoids or IL-10, cause a 10-15% repression of those genes that induce cytokines (2, 5).

So far, various functions of cellular components of our innate immune system, which include macrophages, granulocytes, dendritic cells, monocytes, mast cells and natural killer cells, have been investigated. As part of the immune response, monocytes are recruited into the tissue to differentiate into macrophages. However, there are different macrophages resident in all organs such as Langerhans cells in the skin, Kupffer cells in the liver and microglia in the brain, which will be the topic of interest in the following chapters (43).

1.2 Highways to the brain: From the periphery to the CNS

How the brain and the periphery exchange immune-related information has not yet been fully understood up to this date, although some important mechanisms have already been elucidated. Afferent neural and humoral pathways enable a constant exchange of peripheral immune signals with the CNS (Figure 2) (44).

1.2.1 The vagus nerve as the central neural pathway

The autonomic nervous system is characterized by fast and subconscious transmission of stimuli. In 2002 the term "inflammatory reflex" was introduced, which describes a neural response to acute inflammation that activate central control circuits. Both afferent and predominantly efferent transmission processes with anti-inflammatory properties have been described. In contrast to all other cranial nerves, its afferent and efferent fibers are not restricted to the head and neck area but extend into the abdominal cavity. General and specific viscerosensitive fibers end in the first CNS synapse in the medulla oblongata, or

more precisely in dorsal vagal complex, including the nucleus tractus solitarii (NTS). NTS, in turn, is a group of sensory nuclei exhibiting a unique diversity of neurotransmitters and their receptors that differentiate inputs and project, among others, onto the limbic system, thalamus and hypothalamus, and form circuits that contribute to autonomous regulation (45). In recent years, this 10th cranial nerve has shown increasing involvement in the bidirectional gut-brain-axis and also in the hypothalamic-pituitary-adrenal (HPA) axis, which results in a connection of emotional and cognitive centers of the brain with peripheral intestinal functions (46). In terms of neurally mediated effects of inflammation an activation of primary afferent nerves by PAMPs and locally produced cytokines has been observed (44). Central and peripheral administration of LPS was shown to not only elevate corticosterone levels but to significantly increase c-Fos mRNA expression, a transcription factor indicating recent neural activity, in the hypothalamus as well as other brain nuclei (47). In response to intravenous application of IL-1ß, activation of vagal afferents projecting to the NTS to activate neuronal circuitries could be monitored. Within the course of its nerval structures phagocytes were found (48). In the periphery, more precisely in the liver and its macrophages, the Kupffer cells, LPS administration likewise produces a release of IL-1ß via the mechanism described above. This is particularly interesting because these cells show a special propinquity to the afferent fibers of the vagus nerve, which can be affected by the produced cytokines (49). Remarkably, vagal afferents detect even low levels of endotoxin or IL-1, which are not transported to the CNS via the blood stream (50). Moreover, it could be shown that vagotomy stops the behavioral effects of IL-1, when injected intraperitonially (51).

1.2.2 Humoral Pathway: CEC and CVO and cytokines

A highly selective semipermeable BBB envelops the brain parenchyma in a unique immunological environment to separately protect it from peripheral threats. Its structure is composed of the capillary endothelium, held together by tight junctions with space for the diffusion barrier between them, basement membrane, pericytes and astrocytes end-feet (52). For a long time, the brain would be seen in this privileged position, although the BBB is far from unconditionally tight and its integrity fluctuates depending on age, brain area and health status (44). A special feature of the BBB are the so-called CVOs, whose structure is characterized by a

fenestrated endothelium and thus allows the transfer of molecular substances from the blood into the brain. Vitkovic et al 2000 showed a propagation of cytokines to the brain via volume diffusion through the BBB (53). Accordingly, increased c-Fos mRNA expression could be detected in CVO areas after systemic TNF-α application (44).

Immune cells in the brain, macrophages and dendritic cells, resident in the choroid plexus and meninges, equally express TLRs and likewise react to circulating PAMPs and TNF-α with de novo expression of inflammatory cytokines (54, 55). While IL-6 showed indirect effects, TNF-α and IL-1β receptors could be localized on the surface of cerebral endothelial cells (CEC) in venules, but also on neurons and neuroglia all over the brain, and found to induce the release of prostaglandins and nitric oxide via NF-kB (3, 5, 44). Fever is a well-known symptom and yet another example of how an inflammatory reaction somewhere in the body, mainly via IL-1ß, can cause an increase in body temperature via the hypothalamus (56).

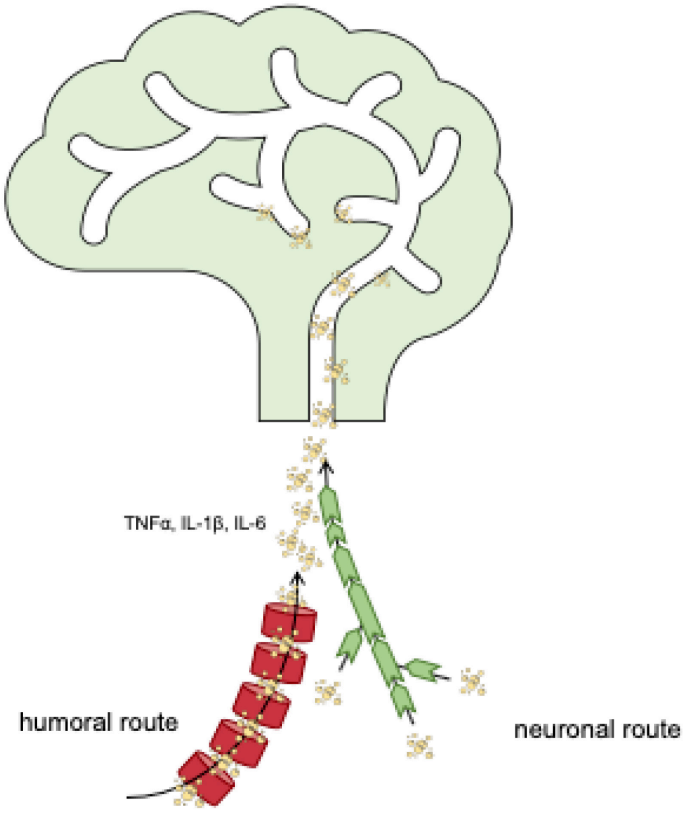

Figure 2: Peripheral immune signaling to the brain. Humoral route IL-1, IL-6 and TNF-α enter the brain via the bloodstream, cerebral endothelial cells (CECs), circumventricular organs (CVOs) and the choroid plexus. The neuronal inflammatory route acts via afferent viscerosensitive fibers of the vagus nerve which end in the nucleus tractus solitarii (NTS) in the medulla oblongata. From there signals are transmitted to numerous areas of the brain like the limbic system, the diencephalon with the thalamus and the hypothalamus and also affect the autonomous regulation. (44, 45)

1.3 Resident macrophages within brain: Microglia cells

Not only through the above-mentioned pathways but under some pathological conditions, parts of the BBB may lose their protective function leading to increased passage of peripheral mediators, including TNF-α, and IL-1ß (52). Microglia cells exist as resident macrophages and are constant monitors of brain tissue playing a key role in conserving CNS integrity and contributing their share to neurogenesis. Although experiments suggest that development out of erythromyeloid progenitors starts very early, they are classified as part of the innate immune system (57). Accordingly, it has been repeatedly shown that cerebral TLR4 binding in microglia cells boosts their central production of pro-inflammatory cytokines (3, 44, 58). While TNF-α activation of microglia is viewed as a paracrine-mediated process, endotoxemia has also been shown to induce CD14 expression in microglia in numerous areas of the brain (59).

In cell lines, endotoxins have repeatedly been shown to increase cell microglial motility, enabling them to react to pathogens and move towards the site of action. In this process, IL-1ß also plays a key role (60, 61). Over the past century there has been a dramatic increase in research on microglial involvement in systemic inflammation and their influence on neurodegenerative diseases (62).

1.3.1 Classification of microglia plasticity, resting or active, has changed in recent years

While under healthy conditions, microglia retain a branched structure, they change it under threatening circumstances by resembling that of an amoeba, which can move through extensions (59). This plasticity of microglia led to the identification of functional polarization states (classically activated M1 type and alternatively activated M2 type) which are dependent on the extracellular environment. M1 and M2 polarization states of macrophages play an important role in controlling the balance between pro-inflammatory, mainly M1, and anti-inflammatory, mainly M2, conditions (63) In recent years, a dynamic continuum of overlaps and exceptions of this traditional classification has been established. Nevertheless, there is a clear tendency that LPS stimulation induces the formerly M1, pro-inflammatory, phenotype and promotes TNF-α and IL1-beta production. (64) Although the data for Poly(I:C) do not appear too impressive, Poly(I:C) doses do cause central IL-1ß producing effects and are able to exaggerate neurodegenerative diseases (62).

1.3.2 CD86, CD206 and Iba1

Different immune cell types have their own characteristic surface protein profile. In order to characterize microglial cells, numerous markers have proven to be helpful. On the basis of surface properties, cells can be immunophenotypically and according to their activation state classified into the so-called clusters of differentiation (CD). Among them are CD86 and CD206. *In vivo* investigations of morphological changes demonstrated significantly higher expression of CD86 in microglial cells compared to astrocytes in an inflammatory environment, while other CD molecules were low or absent. Interestingly, expression levels of CD86 and clinical conditions corresponded in the autoimmune encephalomyelitis model. Upon activation, CD86 contributes to the initiation of the adaptive immune response, mainly by T-cells (64, 65).

CD206 acts as a pattern recognition receptor on the cell surface of both the adaptive and the innate immune response (66). The transmembrane glycoprotein is primarily expressed by microglial cells, astrocytes but also endothelial cells and appears to be selective for microbial substances mediating the first recognition and their endocytosis (67, 68). Both *in vivo* and *in vitro* experiments have shown that microglia have strong capacities for phagocytosis and higher expression of CD206 in the brain (68). Cytokine stimulation of murine microglial cells showed expression of CD206 and preferred tendencies towards an anti-inflammatory milieu, associated with the previously described M2 state of microglia (66). In further studies, CD206 was linked to cerebral cell adhesion of not only macrophages but region-dependently also of neurons, synaptogenesis and neurite outgrowth due to homologies in the involved ligands like acetylcholinesterase and sugars of the surface of microorganisms (68). Interestingly, CD206 was observed in samples from depressed patients more strongly than in manic states (67).

Ionized calcium-binding adapter molecule 1 (Iba1) or allograft inflammatory factor 1 (AIF1) was first isolated in 1996. As a segment of the major histocompatibility complex region class III on the cell surface, Iba1 is seen to transmit calcium signals. Its specificity for microglia cells in the brain could already be seen at the time of its discovery (69). Today, Iba1 is one of the most frequent utilized microglial activity markers (55). It highlights branches and cell shape characteristics and has been able to detect IL-1β associated morphological alterations to active states of microglial cells in the hypothalamus (64).

1.4 Neuropsychiatric disorders and inflammation

Neuropsychiatry studies cognitive and mental disorders and forms a bridge between neurology and psychiatry. The characteristic of the discipline is to investigate pathophysiological and molecular backgrounds of behavioral changes.
When people feel ill, a specific pattern of behavior develops: They withdraw, have loss of appetite, lack drive, feel weak and their cognitive abilities are also impaired. This pattern of "sickness behavior" shows clear parallels to depressive symptoms. Even before the analysis of neurobiological mechanisms, pro-inflammatory cytokines were shown to have the ability to induce acute sickness behavior and potentially depression (3). At the end of the 20th century the "macrophage theory of depression" was formulated, which was based on findings of elevated blood levels of inflammatory markers in severely depressed patients. Thus, the hypothesis was put forward that depression is related to an acute-phase inflammatory reaction (3). Under the same assumption, pro-inflammatory cytokines were clinically not only linked to depression, but also a hyperactive HPA axis, serotonin disbalance and neurovegetative manifestations (3). Strikingly, in severe depression shifts in immunological activity have been reported for some time, whereas current trends suggest that the underlying behavioral changes may be induced by elevated pro-inflammatory cytokines, namely IL-1α and IL-1β, IL-6 and TNF-α (3, 8). In line with the behavioral changes, LPS-induce immune activation leads to increased c-Fos immunoreactivity of cells in the amygdala, hippocampus and hypothalamus (paraventricular nucleus (PVN) and the arcuate nucleus (ARC) of rodents. Interestingly, the most activated structure was the NTS (70). While the involvement of the amygdala in neuropsychiatric diseases is commonly argued on their characteristics of cognitive, emotional and motivational alterations (71), hypothalamic involvement usually refers to disorders of the HPA axis (5).
Another interesting mechanism that activated microglia cells induce by pro-inflammatory cytokines is the stimulation of indoleamine 2, 3-dioxygenase (IDO) and a consequent lack of tryptophan, which leads to lower serotonin levels as well as glutamate and dopamine turbulences. Since current clinical therapies mainly treat this cause of depression, this fact links to the theory of neuroinflammation (72, 73). Investigations of the cerebrospinal fluid revealed elevated concentrations of kynurenine. That is of relevance because neurotoxic, metabolic products of

kynurenine act as agonists at the N-methyl-D-aspartate (NMDA) receptor. This glutamate receptor is also sensitive to glutamate that is released by activated microglia cells. This is interesting because it has been shown that ketamine, in its function as NMDA antagonist, could block LPS-stimulated depression-like behavior (73).

1.5 Centralized control: Hypothalamus

In 1936, Hans Selye, first published on the concept of stress, or "general adaptation syndrome", as he named it (74), while observing a “common sign” in all "sick" patients being lethargic and depressed in one way or another. He insisted on the fact that such stressors can be numerous and nonspecific and put forward a concept as to how the body reacted to these stressors. At this time the first molecular mediators of the stress reaction were identified, namely glucocorticoids (75). The mechanism we know of today is their production via the HPA axis, centrally starting from the hypothalamus, where its corticotropin-releasing hormone (CRH) affects the production of adrenocorticotropic hormone (ACTH) in the adenohypophysis which further stimulates the adrenal cortex with the production of glucocorticoids (1). Connecting the HPA axis to the immune system, the administration of Piromen, another bacterial polysaccharide of gram negative bacteria, showed an increase in ACTH and an induction of adrenal activity in murine experiments already in 1958. It was further demonstrated that this effect did not occur if a pituitary glandectomy had been performed previously (76). In 1986 Besedovsky et al. were able to clone IL-1β and to first show, among others, thar cytokines have the ability to activate the HPA axis in a mouse model (5, 77). To date, it has been repeatedly demonstrated that after acute immune stimulation, activation of the HPA axis, measured by elevated circulating glucocorticoid levels, is part of an elementary inflammatory response. (3, 78-80) In turn, glucocorticoids are known for their anti-inflammatory effect, which can often be observed in clinical routine as immunosuppressants. They do this by inhibiting the majority of cytokines in their production. Temporally, this effect is not seen as immediate but as a delayed immune reaction and the more prominent the immune reaction the bigger is the anti-inflammatory effect of glucocorticoids. (5)

Increasing evidence shows a connection between hyperactivity of the HPA axis and, as a consequence, glucocorticoids and depression. In depressive states a

high secretion of ACTH and glucocorticoids was found, where pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α have been shown to be involved in its induction (5, 46, 81).

Anatomically, the hypothalamus can roughly be divided into 3 parts (see Figure 3): Anterior core group (nucleus supraopticus, paraventricularis, suprachiasmaticus and nuclei preoptici), middle core group (nucleus, arcuatus and nuclei tuberales) and posterior core group (nuclei corporis mammillaris). The hypothalamus controls other endocrine glands by hormone production as well as various neuropeptides and dopamine, and thereby regulates autonomic functions of the body to a large extent (1). This basal part of the diencephalon is essential for the maintenance of homeostasis, including temperature (*nuclei praeoptici)*, regulation of food intake (*nucleus arcuatus* and *nucleus paraventricularis*) and water balance (*nucleus paraventricularis* and *nucleus supraopticus)*, immune regulation, circadian rhythm (*nucleus suprachiasmaticus)*, sleep as well as the control of sexual and reproductive behavior (*nucleus praeopticus, nuclei corpora mamillaria* and *nucleus paraventricularis)* (1). Nuclei of the hypothalamus have intensive projections to the limbic system, which influences emotions and acts as the center for memory formation and again regulates autonomic functions (82). Furthermore, the hypothalamus receives numerous projections from sensory centers of the CNS and projects to multiple, especially visceromotor core areas (83).

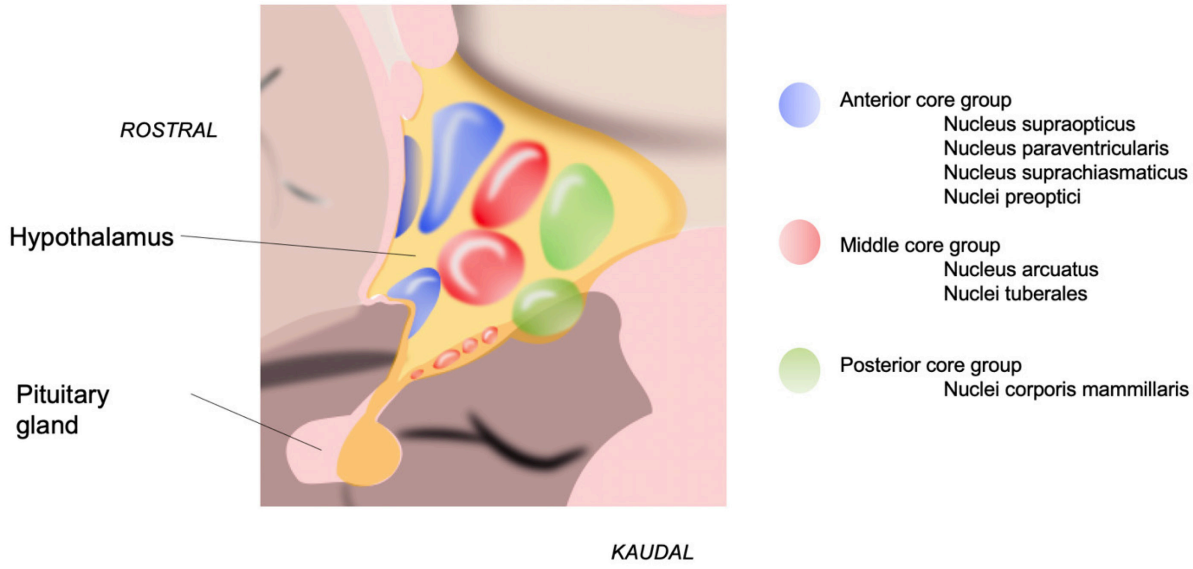

Figure 3: Hypothalmus as part of the dienencephalon controlling various homeostatic functions: Temperature (Nuclei praeoptici), regulation of food (Nucleus arcuatus and Nucleus paraventricularis) intake and water (Nucleus paraventricularis and Nucleus supraopticus) balance, immune regulation, circadian rhythm (Nucleus suprachiasmaticus), sleep as well as the control of sexual and reproductive behavior (Nucleus praeopticus, Nuclei corpora mamillaria and Nucleus paraventricularis).(1)

This can merely be an outline of the elementary role that the hypothalamus plays in the organism. Typically, actions of the endocrine system are strictly regulated to ensure that the body can respond promptly to stressful events and return to homeostasis, just as quickly.

1.6 Neuropeptide Y

Neuropeptide Y (NPY) is one of the most prevalent neurotransmitters of the CNS, whose history dates back to 1982, when Tatemoto et al. initially isolated a new peptide from the porcine brain (84). This group also worked on analytical methodologies which was important for the characterization of NPY's family members pancreatic peptide (PP) and peptide YY (PYY) (84, 85). Structurally comparably short, NPY consists of 36 amino acids in its biologically active form and is seen to form a three-dimensional structure according to computer modeling studies (84, 86). Compared to neurotransmitters, neuropeptides are larger in size and have sustained bioactive effects on peptidergic synapses (87). Best known for its influence on eating behavior, NPY has a broad spectrum of versatile functions at the interface of bodily systems, including its roles in anxiolysis, stress resilience and neuroprotection, which are currently subjects of research (87).

Its G-protein coupled receptor family in mammals includes Y1, Y2, Y4, and Y5, which operate very heterogeneously, while y6 receptor is non-functional (88). In 1999, a study analyzing receptor distribution showed a broad occurrence of Y1,2,4,5 receptors in the rat brain. Specifically, in the hypothalamus, a dominance of Y2 (especially in the area of the nucleus supraopticus and the nucleus arcuatus), followed by Y1 was observed. Y4 and Y5 were found to a moderate extent, with Y4 being especially expressed in the dorsal vagal complex and Y5 showing a tendency to exhibit an area-restricted pattern in the rat brain (89). Postmortem analysis and further studies of the human brain likewise revealed a strong presence of Y2 and Y1 in the hypothalamus (90, 91). In summary, the hypothalamus is a place of high NPY activity.

Work from recent years has attributed increasing roles to NPY in the immune-brain-axis (87). In previous experiments, mice with Y2 receptor depletion were found to be especially prone to short-term LPS-induced decline in exploration in the commonly used open field test and changes in social interaction. At the same time, a significant increase in corticosterone concentration in peripheral blood was

found in these experiments after LPS application in the absence of Y4, but not in the absence of the Y2 receptor (92). Conversely, long-term behavioral changes, as well as anxiety and depression-like traits in mouse models could previously be shown to be impeded by NPY administration. These mechanisms are likewise primarily believed to be mediated via Y2 und Y4 (93).
Concerning the effects of NPY on microglial cell lines, Y1 receptor has been involved in a reduction of LPS-induced motility in cortical areas and phagocytosis, while Y2 und Y5 did not show these effects on microglia (60, 61, 94).

1.7 Aims of this project

Against this background layed out briefly it is interesting to ask whether NPY has antagonistic effects on immune mediated hypothalamic microglial activity *in vivo*. To investigate this question, in combination with peripheral immune stimulation we decided to apply NPY intranasally (IN). IN administration of peptides and pharmaceuticals offers the possibility of bypassing the blood-brain barrier, which usually hinders drug transfer to the brain, and provides direct and rapid delivery of substances to the central nervous system (58, 95). For example, nasally administered oxytocin, also a peptide hormone and neuropeptide which is physiologically produced in the hypothalamus, reliably stimulates uterus contractions. Intracerebral concentrations of oxytocin in experimental animal studies are – following IN administration - up to 13 times higher than after intraarterial administration, which is possibly due to the absence of molecular degradation (96). However, IN applied NPY has equally been successful to show buffering effects on psychological discomfort in the past (95).
It has previously been shown in this laboratory that IN administration of NPY can blunt the sickness behavior induced by IP LPS injection but fails to suppress the expression of pro-inflammatory cytokines in the murine hypothalamus (58). The aim of this diploma thesis was to characterize the role of NPY on microglia in the hypothalamus of the mouse brain in response to both viral and bacterial immune stimulation by Poly(I:C) and LPS, respectively. Using molecular biological techniques, the activation of the microglia and its M1/M2 polarization were analyzed at two time-points, namely 3h and 21h following peripheral immune stimulation.

2 Materials and Methods

The ethical committee at the Federal Ministry of Science, Research and Economy of the Republic of Austria (BMWFW-66.010/0122-WF/V/3b/2017) duly approved all of the following animal care and use protocols, including quantity and housing conditions. All experiments were carried out with great effort to minimize animal discomfort. The animals, male C57BL/6N 8-week-old mice, obtained from Charles River Laboratories (Sulzfeld, Germany) got accustomed to the new habitat for another week before any interventions were undertaken. Mice were kept two by two in a vivarium with standard laboratory chow and tap water access ad libitum. The conditions of 22°C room temperature, air humidity of 50% and a 12-hour light/dark cycle were regularly reassured and tightly controlled.

8-week old C67BL76N mice were randomly divided into 2 observation groups: short term (ST, 3h) and long term (LT, 21h) observation after peripheral immune stimulation. Each observation group was then divided into 3 intervention groups receiving either intraperitoneal saline, LPS or Poly(I:C), which were again divided into 2 groups receiving either water or NPY IN (Figure 5). The vehicle-water group will be referred to as the control group.

2.1 Reagents and dosing

LPS (catalogue number tlrl-3pelps, ultrapure, E.coli 0111:B4), extracted from gram negative E. coli bacterial wall, and Poly (I:C) (catalogue number tlrl-picw, free from microbial contaminants), the synthetic double-strained RNA (dsRNA) analog representative for viral stimulation, were both acquired from Invivogen (Toulouse, France) and dissolved in pyrogen-free sterile saline (0.9% NaCl) (58).

In previous experiments, various doses of LPS were tested and it was shown that the bacterial compound readily induces inflammatory responses in murine brain at rather low amounts. Therefore, we chose a dose of 0.03 mg/kg LPS to make effects comparable to Poly(I:C), which has comparably weaker effects (97, 98) Thus, Poly(I:C) was applied at a dose of 20 mg/kg, as this dose has been previously shown to evoke inflammatory responses in the periphery as well as in the central nervous system (9). Both LPS and Poly(I:C) were injected in a volume of 10 µl/g body weight. The vehicle (VEH, saline) control group was treated with pyrogen-free sterile saline solution (0.9%) administered at the same volume (10 µl/g body weight) as LPS and Poly(I:C).

Prior to IN application, the lyophilized NPY (Phoenix Pharmaceuticals, Karlsruhe, Germany, catalogue number 049-03, stored at 4°C) was dissolved in distilled sterile saline solution (10 µg/µl). The selected IN dose was 100µg per mouse based on previous results by others of a successful NPY delivery to the brain (99).

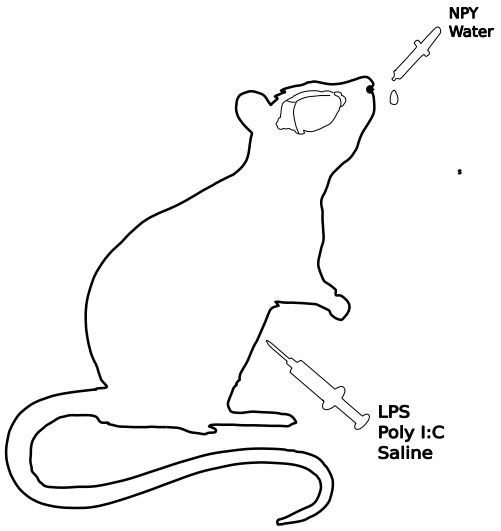

Figure 4: Application IN of 100µg NPY or water and 30 minutes afterwards IP injection either of 0.03 mg/kg LPS, 20 mg/kg Poly(I:C) or vehicle.

2.2 Injections and timing

One at a time, rodents were carefully removed from their cage and put under light isoflurane anesthesia. For slow IN application of fluid, the prepared NPY solution was administered in approximately 5 droplets to the nostrils by using a pipette without touching the mucosa. It is helpful to take advantage of gravity and try to place it high up in the nasal cavity to the olfactory epithelium to enhance NPY access from the nasal cavity to the brain and prevent loss of solution from the nares. Mice were therefore kept in a tilted backward position for about 20 seconds. The control group was infused IN in the same manner and quantity with water. Only when the animals were fully awake again, which mostly took approximately 5 minutes, they were returned to the cage.

Intraperitoneal injections of either VEH, LPS or Poly(I:C) was done 30 minutes after nasal administration of NPY or VEH.

This diploma thesis was carried out under guidance and in connection with a PhD project (98), which was published by Zenz et al. (58). Therefore, the animals included in this diploma thesis were also used for other tests including behavioral

assessments, such as the open field test. Directly after the open field test, mice were sacrificed, and brain tissue was collected for further analysis including the expression of microglial markers which were studied only in the current work.

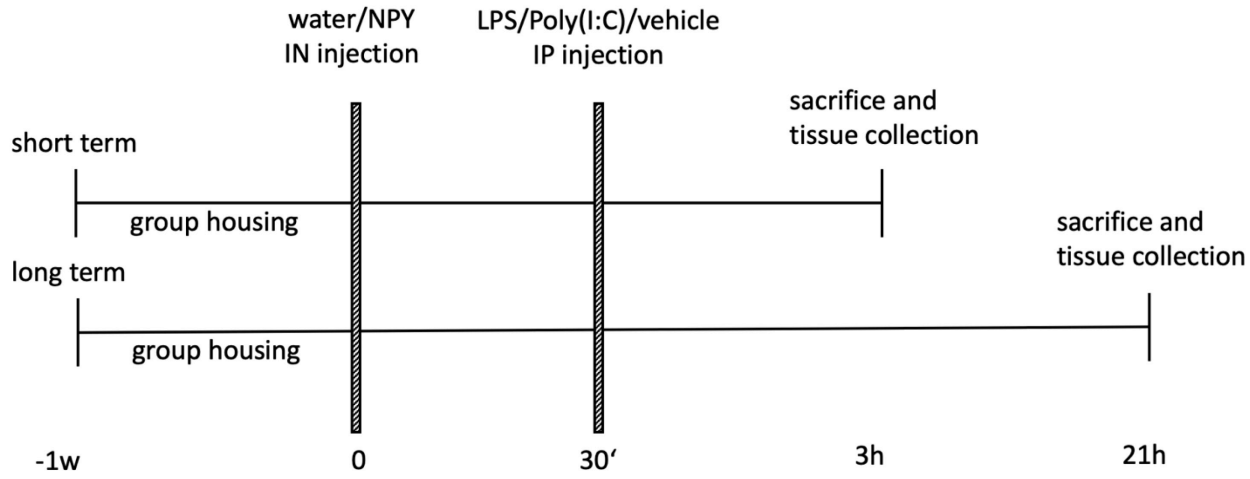

Figure 5: Timeline: After one week of group housing injections were performed. Groups were analyzed for hypothalamic mRNA expression either 3h (short term) or 21h (long term) post injection.

2.3 Brain microdissection

After mice were anesthetized with IP pentobarbital in a dose of 150mg/kg the brain was collected from a posterior cervical approach and immediately frozen in 2-methylbutane (Sigma-Aldrich, Austria) on dry ice for 10 seconds. Finally, the brain tissue was wrapped in aluminum foil and stored at -70°C until the microdissection procedure.

The IP applied LPS has been shown to significantly induce upregulation of cytokine expression in certain brain areas, including the region of interest of this project, the hypothalamus (100). For investigating mainly hypothalamic microglial activation it is first important to isolate this region.

Prior to microdissection, the working area as well as the preparation instruments were wiped with RNase AWAY (Carl Roth, Karlsruhe, Germany) in order to prevent digestion of RNA by ubiquitous RNases. On a cold plate (Weinkauf Medizintechnik, Forchheim, Germany) set at -20°C the dissection (Bregma +0.38 to -2.92) was conducted by a trained researcher under the help of a stereomicroscope. The tissue was homogenized in MagnaLyser bead tubes

(Catalogue number 03358 941 001, Roche Diagnostics, Rotkreuz, CH) using the MagnaLyser centrifuge (Roche Diagnostics).

2.4 RNA purification, RT and qPCR

Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) is a highly sensitive method for quantitative analysis of gene expression as it allows the detection of low abundance RNAs in samples through amplification of specific segments.

First, sample RNA is transcribed into cDNA through reverse transcriptase (RT) enzymes. In the second step, these cDNA templates are amplified exponentially by the polymerase chain reaction (PCR). PCR itself dates back to the early 1980ies and consists of denaturation, primer annealing and elongation.

For this process, RNA was first isolated from all homogenized tissue samples using RNeasy lipid tissue mini kit (Qiagen, Hilden, Germany). RNA purity and concentration were measured with a NanoDrop spectrophotometer from Thermo Fisher Scientific (Vienna, Austria) to continue working with comparable quantities.

For the synthesis of single-stranded cDNA from total RNA we first diluted RNA samples 1:10 and added 2µg RNA to 10µl diszilled water in 96 well plates. The uniform quantity of 2µg of each RNA probe was then reverse transcribed according to the manufacturer's instruction using a high capacity cDNA reverse transcription kit (Thermo Fisher Scientific Vienna, Austria). Mastermix components included a reverse transcription buffer, dNTP mix containing sodium salts of dATP, dCTP, dGTP and dTTP, specific primers as well as reverse transcriptase and sterile H_2O. Taking into account the instability of RNA and potential interferences, pipetting was performed with special care in adequate surroundings (101).

PCR itself was performed in a thermal cycler (Biorad, MyCycler) set to a reaction volume of 20µl according to the following protocol.

Table 1: qPCR Protocol

Step	1	2	3	4
Temperature	50°C	95°C	95°C	60°C
Time	2'	10'	15s	1'
			39x	

All samples were measured in triplicate. CT value was measured, which describes the first time point when the fluorescence signal is statistically significantly higher than the background signal (102). To quantify gene expression, we used the standard curve method. The PCR signal of the target gene is expressed relative to another sample, the control group (103). Control values, amplifications without reverse transcriptase, were included in each group and produced the expected blank result. Gapdh, Actb and Ppil3 were used as reference or so-called housekeeping genes (cell-type independent genes that maintain basic cellular function like glycolysis, cell integrity or protein folding) (104).

As presented in the Introduction, the focus of interest was on the levels of expression of CD206 (Table 2), more likely to be assigned to the formal M1 polarization state, CD86 (Table 2), more likely to be M2, and Iba1 (Mm00479862_g1, Thermo Fischer Scientific, Waltham, MA, USA) as activity marker.

Table 2: qPCR Primers used for CD206, CD86

Gene	Forward Primer	Reverse Primer
CD206	TCTTTGCCTTTCCCAGTCTCC	TGACACCCAGCGGAATTTC
CD86	TTGTGTGTGTTCTGGAAACGGAG	AACTTAGAGGCTGTGTTGCTGGG

2.5 Statistical analysis

For statistical evaluation as well as figure creation GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) was used. Data (altered gene expression) represent fold changes of the target gene relative to the mean of the vehicle (VEH) control group using the comparative CT method, also referred to as the $2^{-\Delta\Delta Ct}$ method (103).

First, extreme outliers found in 8 mice, with a total number of 19 out of 258 values, were excluded (extreme outliers defined as > 3*IQR).

Data were then log10 transformed, and each value was multiplied by a factor of 20 to reach normal distribution. To assess main effects a two-way analysis of variance (ANOVA) was the method of choice. Dependent variables were microglia specific markers CD206, CD86 and Iba1 with the factors LPS, Poly(I:C) or VEH IP and NPY or water IN. To identify the differences between control versus treatment

groups a planned comparison post-hoc Fisher's LSD test was performed. Probability values of ≤0.05 were regarded as statistically significant. N refers to the number of mice in each group.

3 Results

3.1 NPY in combination with Poly(I:C), but not LPS, increases hypothalamic Iba1 expression 3h post injection

As can be seen in Table 3, there is a highly significant difference in Iba1 mRNA expression between the control group, which received water + VEH, and NPY + Poly(I:C), with p=0.0056 and a higher mean value for NPY + Poly(I:C) (M=1.495) than for the control group (M=0.7938) with a mean difference of 0.7011. This significant difference is also illustrated in Figure 6. There is no significant difference between any of the other conditions. The mean difference between the control group and water + LPS (0.4633; p=0.067), which can be discerned as a trend, is larger than between the control group and NPY + LPS (0.3456; p=0.1678). The hypothesis that there is a difference between the conditions under study can therefore only be assumed with regard to the control group and NPY + Poly(I:C).

Taken together, it can be said that neither Poly(I:C) nor LPS alone led to a significant increase in hypothalamic Iba1 expression. Surprisingly, however, pretreatment with IN NPY induced a slight but significant increase of Iba1 expression in response to Poly(I:C).

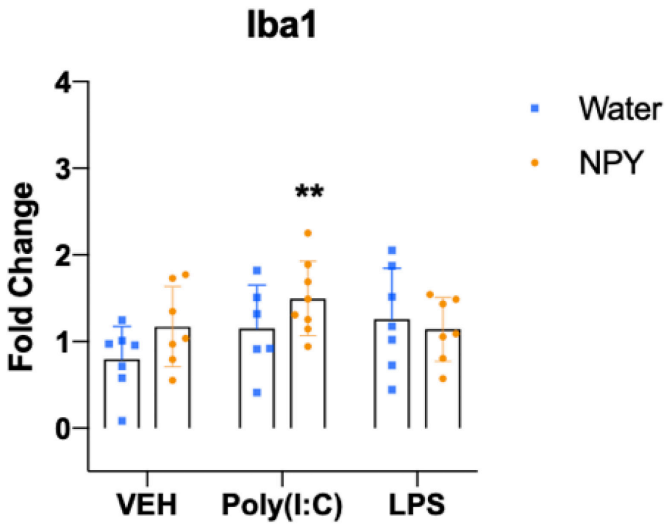

Figure 6: Hypothalamic Iba1 mRNA expression 3h (short term) after IN injection of either NPY or water and IP injection of VEH, LPS or Poly(I:C). Mice were pretreated IN either with water or NPY (100µg) and 30 minutes thereafter injected IP with VEH, LPS or Poly(I:C). 3h afterwards brains were harvested, and hypothalamic tissue was analyzed for microglial mRNA expression levels of Iba1. Poly(I:C) and NPY injection significantly increased Iba1 expression (p=0.0056) compared to the control group. Values represent means ± SEM, n = 6-8 *p ≤ 0.05, **p ≤ 0.01 vs. control (VEH + water) groups (main effect, two-way ANOVA and planned comparison post-hoc Fisher's LSD).

Table 3: Fold change data for Iba1 mRNA expression 3h post injection

Injection	n	M	Mean diff vs. control group	P-value vs. control group	Significance vs. control group
Control group	7	0.7938			
NPY	7	1.171	0.3769	0.1335	ns
Poly (I:C)	6	1.148	0.3543	0.1741	ns
NPY + Poly (I:C)	8	1.495	0.7011	0.0056	**
LPS	7	1.257	0.4633	0.0672	ns
NPY + LPS	7	1.139	0.3456	0.1678	ns

ns, not significant

3.2 *Iba1 expression levels reveal no statistically significant differences 21h post injection compared to the control group*

Looking at the long term results (21h post injection) of Iba1 mRNA expression, there are no statistical differences or trends within the groups compared to the control group detectable (Table 4, Figure 7).

In summary, neither the peripheral immune stimulants LPS and Poly(I:C), nor NPY influenced hypothalamic Iba1 expression 21h after application.

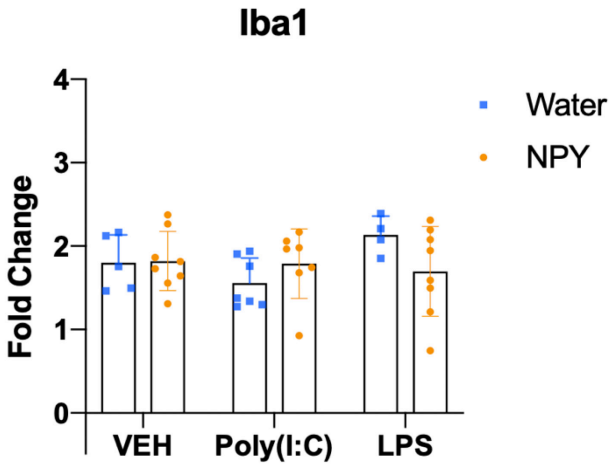

Figure 7: Hypothalamic Iba1 mRNA expression 21h (long term) after IN injection of either NPY or water and IP injection of VEH, LPS or Poly(I:C). Mice were pretreated IN either with water or NPY (100µg) and 30 minutes thereafter injected IP with VEH, LPS or Poly(I:C). 21h afterwards brains were harvested, and hypothalamic tissue was analyzed for microglial mRNA expression levels of Iba1. No statistically significant differences to the control ,group was found. Values represent means ± SEM, n = 4-8 *p ≤ 0.05, **p ≤ 0.01 vs. control (VEH + water) groups (main effect, two-way ANOVA and planned comparison post-hoc Fisher's LSD).

Table 4: Fold change data for Iba1 mRNA expression 21h post injection

Injection	n	M	Mean diff vs. control group	P-value vs. control group	Significance vs. control group
Control group	5	1.800			
NPY	8	1.820	0.01961	0.9306	ns
Poly (I:C)	7	1.557	0.2437	0.2961	ns
NPY + Poly (I:C)	7	1.789	0.01139	0.9607	ns
LPS	4	2.133	0.3323	0.2152	ns
NPY + LPS	8	1.697	0.1030	0.6479	ns

ns, not significant

3.3 *Poly(I:C) + NPY as well as singular LPS or Poly(I:C) increase hypothalamic CD206 expression 3h post injection*

As shown in Table 5 and Figure 8, a highly significant difference in CD206 mRNA expression between the control group and the NPY + Poly(I:C) group (p=0.003) and a higher mean value for NPY + Poly(I:C) (M=1.49) than for the control group (M=0.75), with a mean difference of 0.7527 is found. Furthermore, there is a significant difference between the control group and water + Poly(I:C) (p=0.046) and between the control group and the water + LPS group (p=0.0282). In the group receiving NPY + LPS, a trend vs. the control group is discernible at p=0.0687.

Overall, while Poly(I:C) and LPS individually upregulated hypothalamic CD206, NPY + Poly(I:C) in combination were able to induce the strongest upregulation of CD206.

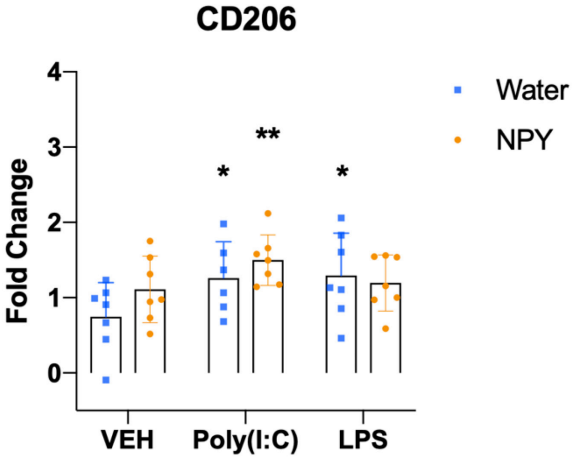

Figure 8: Hypothalamic CD206 mRNA expression 3h (short term) after IN injection of either NPY or water and IP injection of VEH, LPS or Poly(I:C). Mice were pretreated IN either with water or NPY (100µg) and 30 minutes thereafter injected IP with VEH, LPS or Poly(I:C). 3h afterwards brains were harvested, and hypothalamic tissue was analyzed for microglial mRNA expression levels of CD206. Poly(I:C) + NPY (p=0.0033), Poly (I:C) (p=0.0455) and LPS (0.0282) injections significantly increased CD206 expression compared to the control group. Values represent means ± SEM, n = 6-7 *p ≤ 0.05, **p ≤ 0.01 vs. control (VEH + water) groups (main effect, two-way ANOVA and planned comparison post-hoc Fisher's LSD).

Table 5: Fold change data for CD206 mRNA expression i3h post injection

Injection	n	M	Mean diff vs. control group	P-value vs. control group	Significance vs. control group
Control group	7	0.7465			
NPY	7	1.110	0.3639	0.1365	ns
Poly (I:C)	6	1.262	0.5154	0.0455	*
NPY + Poly (I:C)	7	1.499	0.7527	0.0033	**
LPS	7	1.293	0.5468	0.0282	*
NPY + LPS	7	1.195	0.4485	0.0687	ns

ns, not significant

3.4 CD206 expression levels remain elevated only in the Poly (I:C) + water and Poly(I:C) + NPY groups 21h post injection

In the long term paradigm (21h post injection), there is a highly significant difference in CD206 mRNA expression between the control group and water + Poly(I:C) group (p=0.004) and a higher mean value for the control group (M=1.09) than for water + Poly(I:C) (M=1.091) group, with a mean difference of 0.70. A significant difference is also found by comparison of the control group and NPY + Poly (I:C) group (p=0.0277). These observations are detailed in Table 6 and Figure 9.

To summarize the results 21h after injections, CD206 is the only hypothalamic marker that is downregulated in response to Poly(I:C) and NPY + Poly(I:C).

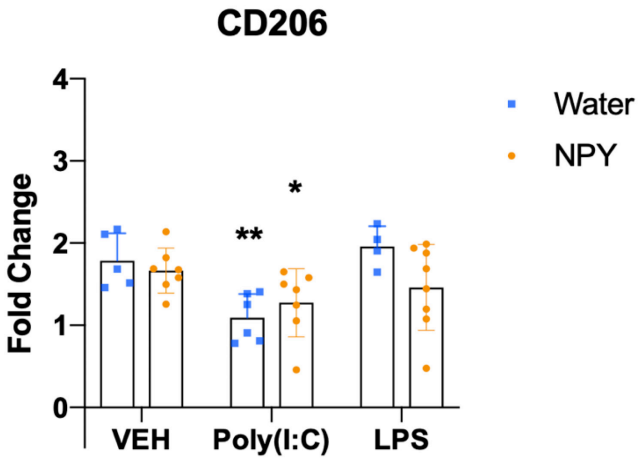

Figure 9: Hypothalamic CD206 mRNA expression 21h (long term) after IN injection of either NPY or water and IP injection of VEH, LPS or Poly(I:C). Mice were pretreated IN either with water or NPY (100µg) and 30 minutes thereafter injected IP with VEH, LPS or Poly(I:C). 21h afterwards brains were harvested, and hypothalamic tissue was analyzed for microglial mRNA expression levels of CD206. Poly(I:C) (p=0.0048) and NPY + Poly (I:C) (p=0.0277) injections significantly increased CD206 expression compared to the control group. Values represent means ± SEM, n = 4-8 *p ≤ 0.05, **p ≤ 0.01 vs. control (VEH + water) groups (main effect, two-way ANOVA and planned comparison post-hoc Fisher's LSD).

Table 6: Fold change data for CD206 mRNA expression 21h post injection

Injection	n	M	Mean diff vs. control group	P-value vs. control group	Significance vs. control group
Control group	5	1.787			
NPY	7	1.666	0.1211	0.5887	ns
Poly (I:C)	6	1.091	0.6960	0.0048	**
NPY + Poly (I:C)	7	1.275	0.5119	0.0277	*
LPS	4	2.958	0.1714	0.5048	ns
NPY + LPS	8	1.461	0.3261	0.1409	ns

ns, not significant,

3.5 CD86 expression levels are upregulated 3h after peripheral administration of LPS or Poly(I:C) and more so when NPY is added

In the short term paradigm (3h post injection), CD86 mRNA expression levels differ to a highly significant degree (p=0.001) between the control group and the Poly(I:C) + NPY group, with a higher mean value for Poly(I:C) + NPY (M=1.60)

than for the control group (M=0.79) and a mean difference of 0.80 (Table 7, Figure 10). A significant difference is also found by comparison of the control group with the LPS + water group (p=0.0104) and between the control group and the NPY + LPS group (p=0.0234). The groups treated with NPY + VEH or water also show trends towards up-regulation of CD86 mRNA, with p-values for NPY (p=0.0862) and Poly(I:C) (p=0.0504) 3h post injection (Table 7).

In conclusion, while LPS and NPY + LPS upregulated hypothalamic CD86, astonishingly NPY + Poly(I:C) together result in the most pronounced elevation of CD86.

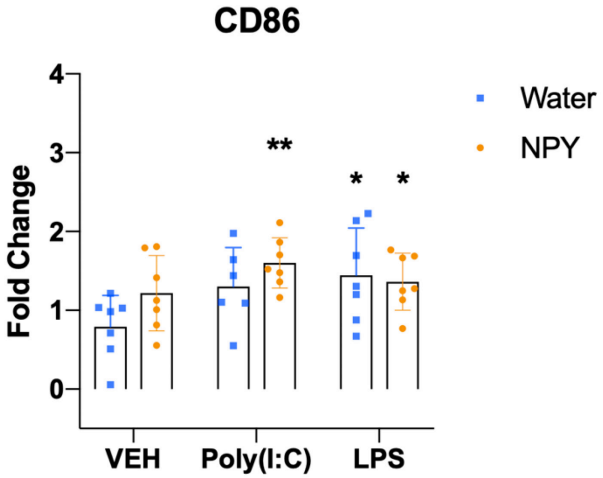

Figure 10: Hypothalamic CD86 mRNA expression 3h (short term) after IN injection of either NPY or water and IP injection of VEH, LPS or Poly(I:C). Mice were pretreated IN either with water or NPY (100µg) and 30 minutes thereafter injected IP with VEH, LPS or Poly(I:C). 21h afterwards brains were harvested, and hypothalamic tissue was analyzed for microglial mRNA expression levels of CD86. NPY + Poly (I:C) (p=0.0019), LPS (p=0.0104) and NPY + LPS (p=0.0234) injections significantly increased CD86 expression compared to the control group. Values represent means ± SEM, n = 6-7 *p ≤ 0.05, **p ≤ 0.01 vs. control (water + VEH groups (main effect, two-way ANOVA and planned comparison post-hoc Fisher's LSD).

Table 7: Fold change data for CD86 mRNA expression 3h post injection

Injection	n	M	Mean diff vs. control group	P-value vs. control group	Significance vs. control group
Control group	7	0.7919			
NPY	7	1.218	0.4257	0.0862	ns
Poly (I:C)	6	1.300	0.5086	0.0504	ns
NPY + Poly (I:C)	7	1.601	0.8092	0.0019	**
LPS	7	1.445	0.6531	0.0104	*
NPY + LPS	7	1.363	0.5716	0.0234	*

ns, not significant

3.6 CD86 expression levels are no longer statistically significantly different 21h post injection

In the long-term paradigm (21h post injection), there is no statistical differences in CD86 mRNA expression between the different treatment groups detectable (Table 8, Figure 11).

Just as Iba1, none of the administrations elevate CD86 expression levels in neither of the long term groups.

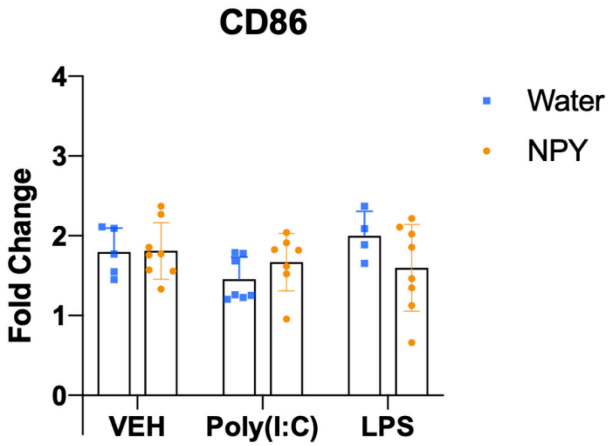

Figure 11: Hypothalamic CD86 mRNA expression 21h (long term) after IN injection of either NPY or water and IP injection of VEH, LPS or Poly(I:C). Mice were pretreated IN either with water or NPY (100µg) and 30 minutes thereafter injected IP with VEH, LPS or Poly(I:C). 21h afterwards brains were harvested, and hypothalamic tissue was analyzed for microglial mRNA expression levels of CD86. No statistically significant difference to the control group was found. Values represent means ± SEM, n = 4-8 *p ≤ 0.05, **p ≤ 0.01 vs. control (VEH + water) groups (main effect, two-way ANOVA and planned comparison post-hoc Fisher's LSD).

Table 8: Fold change data for CD86 mRNA expression 21h post injection

Injection	n	M	Mean diff vs. control group	P-value vs. control group	Significance vs. control group
Control group	5	1.795			
NPY	8	1.811	0.01613	0.9415	ns
Poly (I:C)	7	1.457	0.3384	0.1406	ns
NPY + Poly (I:C)	7	1.670	0.1247	0.5816	ns
LPS	4	2.000	0.2054	0.4295	ns
NPY + LPS	8	1.600	0.1953	0.3774	ns

ns, not significant

4 Discussion

The aim of this study was to investigate whether changes of CD206, CD86 and Iba1 mRNA expression in hypothalamic microglial cells in response to peripheral IP immune stimulation with bacterial LPS or the viral mimic Poly(I:C) are under the influence of IN pretreatment with NPY.

The upregulation of both the more pro-inflammatory marker CD86 ($p=0.0504$ and $p=0.0104$) and the more anti-inflammatory CD206 ($p=0.0455$ and $p=0.0282$) support previous findings of peripheral immune stimulation with Poly(I:C) or LPS leading to central microglial effects (9, 55, 73, 105, 106)

Interestingly, the most dominant upregulation of expression of all markers investigated, Iba1 ($p=0.0056$), CD206 ($p=0.0033$) and CD86 ($p=0.0019$), was observed 3h after treatment with NPY in combination with Poly(I:C). Long term investigations revealed that only CD206 in the Poly(I:C) + water and the Poly(I:C) + NPY groups remained upregulated 21h after injection. These findings indicate that IN pretreatment with NPY enforces the hypothalamic microglial response to peripheral Poly(I:C) rather than that to LPS and that this effect of NPY dwindles with time. The results also show that, contrary to the effect of IN NPY to reduce the sickness response to LPS, IN NPY does not mitigate the effects of IP LPS and Poly(I:C) to enhance microglial marker expression (58). This result is, however, in line with the previous investigation that IN NPY is not able to blunt the rise of intracerebral cytokine expression evoked by IP administration of LPS (58).

The short term (3h post injection) upregulation of microglial markers by peripheral Poly(I:C), in our case whether with or without NPY, are in line with results from others that the neuroinflammatory response to this viral mimic is at its peak 3h after injection and cytokines are rapidly degraded thereafter (9, 107).

Due to the higher stability of LPS compared to Poly(I:C), which is believed to be rapidly degraded, one would expect that this bacterial immune stimulant has a longer-lasting effect on hypothalamic microglia than Poly(I:C). LPS has been shown to have central effects in comparably low doses, which our dose of 0.03 mg/kg can be considered as, while Poly(I:C) requires higher doses to elicit central effects, most probably because its half-life once systemically injected is rather short (9, 47, 106, 108).

It needs to be added that it is difficult to compare the potencies of LPS and Poly(I:C) in their immune-stimulating activities because of their different and complex macromolecular structures and the different molecular targets (PRRs) they act on. Nevertheless, TNF-α and complement factors have previously been detectable more than 21h after application of lower doses of the viral mimic than the one used in this project (9).

Regarding the characteristic changes of microglial cells, the upregulation of Iba1 by NPY + Poly(I:C) ($p=0.0056$) indicates changes in their cell shape towards a more branched structure, whereas a simultaneous, distinct upregulation of CD86 in the same group ($p=0.0019$) is consistent and indicates morphological changes and a change to polarization type M1. Interestingly, CD206 is also elevated in the same group, which acts as PPR, detects phagocytosis and is typical for the polarization type M2. Indications for a M1 polarization state appear likewise in the groups treated with water + LPS ($p=0.0104$) but also with NPY + LPS ($p=0.0234$), which suggests that NPY is not able to block LPS-induced transformations to pro-inflammatory M1 states. Anti-inflammatory M2 microglial polarization expressed through a CD206 upregulation on the other hand is found with both immunostimulants, water + Poly(I:C) ($p=0.0455$) and water + LPS ($p=0.0282$). 21h after application solely water + Poly(I:C) ($p=0.0048$) and again NPY + Poly(I:C) ($p=0.0227$) still show increased CD206 expression indicating anti-inflammatory M2 counter regulation (64, 65, 68).

4.1 Neuropsychiatric influences: It is all in your head, is it literally?

A relationship between peripheral immune activation and neuropsychiatric diseases has been inferred mostly from the results of rodent experiments. However, a study from 2013 could show for the first time that treatment resistant depressive patients with high baseline inflammatory biomarkers improved after treatment with the TNF-α blocker Infliximab (109). In 1996, LPS had already been found to trigger acute sickness behavior and deferred depression-like behavior in rats (110), but also Poly(I:C) has shown remarkable, but less potent, effects in the upregulation of, among others, IL-1β, and in the development of fever in the hypothalamus and lethargy (9). Timewise it is interesting that after LPS stimulation, a cytokine peak as well as a sickness response are established after 3

hours, and until 48h afterwards central gene changes for neurotransmission are present (58). Interestingly, those brain areas responsible for cytokine-induced behavior in sickness and depressive states differ not only in function but also in location. (70) A study of humans who received LPS in a low dose revealed not only increased plasma levels of TNF-α and IL-6 but also central behavioral effects. The level of cytokines correlated positively with anxiety and depressed mood. Cytokine elevation, both centrally and peripherally, is the subject of numerous diseases and often accompanied by depressed and anxious mood patterns. Therefore, cytokines could causally be linked to the pathophysiological mechanisms of disease-related behavioral pathology (111).

Viruses are the second common pathogens which play a major role not only at a very young age, but also at a more advanced age, which makes it worthwhile to take a close look. Interestingly, Poly(I:C) appears to stimulate solely the innate immune system. Concerning its influences on neuropsychiatric conditions, Poly(I:C) is suspected of not only aggravating social disruption and fatigue but also neuropathological events in Alzheimer's disease, seizures or prion disease and impairing cognition (9, 106, 112). On a molecular level, psychosocial stressors combined with the viral immune stimulation potentially exert synergistic effects on circulating cytokines and the HPA axis (112).

In summary, it could be concluded that although mechanisms of neuropsychiatric diseases operate in the brain, they are not independent from stressors and signals from the periphery.

4.2 Microglia involved in many mechanisms

4.2.1 Microglia in time

Many neuropsychiatric diseases typically do not manifest until (later) adulthood and characteristics of ageing include elevated inflammatory states (8). This fact, together with the observation that young "naive" microglia cells behave differently than "primed" microglia, with a prolonged response and a longer process until homeostasis is restored, is worth investigating. Characteristic responses of primed microglia include stronger expression of TNF-α, IL-1β and IL-6, but also IL-10 and TGF-β, which are part of the anti-inflammatory counterregulation and are slowed down in the aged brain after LPS stimulation (72). Increased neuronal damage

was also observed in aged mice (62). It is interesting to note that our 8-week-old mice are in young adult life and therefore results are most likely to be transferable to this age (113).

4.2.2 Microglia in health and disease

Previously, it has been described that inflammatory response of microglia impairs neuronal circuitries. Recent evidence suggests that interleukin levels (including IL-6), microglial function and homeostatic functions in the hypothalamus significantly correlate and this might not be the only influence of microglia on neuronal functions (4). Not only in murine models but also in clinical studies the activity of microglia cells increased due to the presence of TNF-α, but also showed repeated influence on neuronal functions and sickness behavior (8). Conversely, peripheral inflammation coupled with antibiotic therapy prevented microglia activation and altered the cytokine-related behavioral and motivational responses (114).

Increasing evidence further support the idea that HPA axis hormones directly affect microglia. Previously, they have been identified as important mediators in the body's energy balance by influencing neuronal functions in the hypothalamus (115). Under chronic stress, microglia cells express IL-1ß, which in turn triggers the HPA axis and glucocorticoid release. This seems to lower motivation and reward. Also, early life stress, a known risk factor for the development of psychiatric disorders, can alter microglial operation. Furthermore, other environmental factors such as nutrition and alcohol consumption influence microglial functions (72).

In postmortem investigations of both unipolar and manic depression, microglial reactions as well as elevated concentrations of IL-1ß and NF-κB were found (72). An *in vivo* human study suggested that M1 microglia appear dominant in the manic state, while M2-indicating markers such as CD206 are upregulated in the depressive state. However, this study points to the big limitation that the participating patients were treated with medication during this time due to their bipolar disorder (67).

One might conclude the activation of microglia has a purely negative connotation. However, the associated neurotoxicity is countered by the fact that microglia cells can also remove dysfunctional synaptic input, displace synapses and stimulate NMDA receptors and thus can also have a neuroprotective effect (72).

4.2.3 Microglia in inflammatory states

Interestingly, it has been shown that prolonged peripheral inflammation, such as in the liver, for 10 days lead to stronger fatigue behavior and additionally increased microglia activity compared to day 5 (44, 116).

Colitis represents an inflammatory disorder of a peripheral organ, where patients often experience anxious and depressive mood (117). Rodent colitis models show that Iba1 was downregulated in the paraventricular nucleus and in the dentate gyrus, while in other hypothalamic sites Iba1 expression levels were independent of colitis (117). Other parts of the brain, such as the amygdala, showed the expected microglial activation. No general M1 and CD86 or M2 and CD206 allocation could be made, which increases the suspicion that different subgroups of microglia are activated simultaneously. A decrease of Iba1 has also been monitored in response to LPS administration, while cytokine levels were upregulated at the same time (117). Decreased Iba1 expression could represent an anti-inflammatory counterregulation, since pro-inflammatory defense reactions are just as necessary as anti-inflammatory counterregulation to regain homeostasis and prevent major neuronal damage. Interestingly, however, while the quantity of microglia cells did not change, a significant increase of macrophages was found in the brain of the colitis group, which makes them interesting cell population to closely investigate in future projects (117).

In the course of another experiment, in contrast to many studies before, Radler et al. 2015 (105) observed that 50 µg/kg LPS alone did not significantly activate the morphology of microglia in the hypothalamic arcuate nucleus 6h after injections, but microglia tended to be primed, which describes a phenotype which is able to produce exaggerated IL-1ß responses to subsequent immune stimuli. Furthermore, they found a higher density of immune-labeled Iba1, which we observed as a trend towards an Iba1 increase by LPS in our study (p=0.0.02) (105). It cannot be left unsaid here that the Iba1 marker is not able to distinguish microglial cells from recruited macrophages (116). Other studies found the adaptor molecule Iba1 still being expressed over a longer period of time in various brain regions after LPS-stimulation, so this could possibly be an interesting marker in the future. In these studies, arthritis induced by complete Freund's adjuvant containing heat-killed *Mycobacterium butyricum* was applied. The bacterium is known for its intracellular location and was shown to still activate microglial cells

two to four weeks after application. (55). However, reviews also note the fact that immune stimulation with LPS is significantly stronger compared to stimulation with bacteria as a whole and therefore further away from clinical reality (55, 62).

Microbes from the host's intestine as well as their products also have a clear influence on microglia maintenance with a variety-rich microbiome being beneficial. M1 phenotype associated CD86 was shown to be less reactive to immune challenge by LPS in germ-free mice compared to healthy mice, indicating impaired microglial immune response in microbiome deprived mice (57). Pro-inflammatory CD86 in our experiment with healthy mice, however, was significantly upregulated by LPS ($p=0.0104$).

A very interesting remark with regard to chronic inflammation is that over time, in the presence of pathogenic ligands, such as *Candida albicans*, CD206 is being downregulated by them and also the exposure to cytokines, such as IFN-γ, can have an influence on the regulation of the expression of the mannose receptor (68). In any case, the breadth of different markers paired with the heterogeneous states of microglial cells requires further investigations to make clear statements on pathomechanisms along the immune-brain axis (55).

4.3 Chronic inflammation

Chronic activity of the immune system can result from permanent contact with pathogens. The question of where a potential repetitive stimulus for microglia cells can come from, led many research groups to investigating chronic inflammation. In addition to PAMPs, damage-associated molecular patterns (DAMPs) also exist, which are activated when surrounding tissue sends out corresponding signals, for instance under highly stressful conditions (20). Potential sources of microbial agents in various formulations are permanent guests in our organism, such as the microbiome of the gut or on the skin. In response to the emerging question where LPS could enter the bloodstream, multiple influences on intestinal epithelial permeability are being studied (88). It has been shown that bacterial cell wall components can induce epithelial damage and loss of tight junctions to cross this physical barrier and enter the bloodstream (118). Elevated LPS blood levels were found in obese patients and nonalcoholic fatty liver diseases (119). In vivo, this would not be a singular stimulation by one microbial metabolite but would potentially include factors derived from all microbes present in the host microbiome, including lipoteichoic acid (LTA) of gram-positive bacteria acting as

an agonist on TLR2, which is involved in immune-brain stimulation (78). TLR2 is also known for its recognition of *Candida albicans,* a common member of the human intestinal flora, and *Cryptococcus neoformans,* in immunosuppressive states such as high glucocorticosteroid levels (20). In my eyes, it could be worth investigating them as immune stimulants to the circuit of neuroinflammation, since these yeasts are widespread and with high glucocorticoid levels immunosuppression is closely tied to fungal infections (120).

It should not be disregarded that in certain states there is no permanent immune stimulus (any more), such as from the intestine, but that a large number of endogenous metabolic molecules "auto-inflammatorily" induce a conversion of IL-1β into its active form and thus result in elevated blood concentrations (33). An important finding, also with regard to therapeutic and preventive approaches, is that chronically increased inflammatory activity and a concomitant increased susceptibility to the development of neuropsychiatric diseases has also been found recurrently in obese patients (8).

4.4 Hypothalamic roles

In this project, we saw that hypothalamic microglial activity was primarily enhanced in those groups that received the viral mimic Poly(I:C) along with NPY administration. Electrophysiologic studies by others have shown that TLR4 activation of microglia in the hypothalamus by bath-applied LPS *in vitro* influences neuronal activity in the nucleus arcuatus, in an excitatory manner on some neurons, but in an inhibitory fashion on NPY neurons (121). It is therefore worth considering that NPY exerts its effects not primarily on the shape and markers of activated microglial cells, as we did not see buffering effects of NPY upon immune stimulation with LPS and Poly(I:C), but rather on microglial metabolites or their targets. What definitely has been seen in the past is that not only NPY upregulation, but also microglial phagocytotic activity is highly dependent on energy homeostasis and nutritional depletion resulting in such induction (122, 123).

As already mentioned, increasing evidence support the idea that HPA axis hormones affect microglial cells (72). The regulation of stress by the HPA axis, food intake and the site of action of NPY intersect in the hypothalamus. High NPY activity, which can be induced by various stressors, meets in the nucleus arcuatus

and can be communicated through a strong NPY-mediated junction to neurons in the paraventricular nucleus, a major hub of the HPA axis where CRH is synthesized (88). NPY and LPS in combination have been shown to tend to increase CRH, but not glucocorticoid levels (58).

In the hypothalamus, IL-1β induced responses of the HPA axis are impeded if neurons of the paraventricular nucleus have been the subject of preceding iatrogenic interferences, either surgically or with noradrenaline antagonists (5). It is perhaps less well known that one of the glucocorticoid functions is the co-regulation of neuronal survival, formation of neuronal cells and their noticeable effect on memory formation, emotions, attention and vigilance (46, 124). Both preclinical and clinical studies showed the value of adequate HPA reactivity, as mental illness can be linked to both excessive or decreased secretion of glucocorticoids (125). In permanently elevated states, glucocorticoid resistance can develop, and it is not only the usual anti-inflammatory counterpart that is reduced but also the negative signals for inhibition of CRH production in the hypothalamus, which in turn can continue this cycle (3, 8).

4.5 NPY as potential therapeutic target

On immune cells, such as dendritic cells, NPY Y1 receptor activation appears to elicit a pro-inflammatory response initiating migration, adhesion as well as recruiting to the site of inflammation. T cells, on the other hand, receive NPY-mediated anti-inflammatory signals. Y1 but also Y4 and Y5 receptors are involved in NPY's anti-inflammatory signaling (87). In the past, effects of NPY on murine microglia cells have been demonstrated as Y1, Y2 and Y5 receptors are found in the cell membrane of these cells and are upregulated after LPS administration. On the one hand, NPY has been reported to inhibit the release of IL-1β via Y1 receptor while, on the other hand, it has surprisingly also been shown to increase IL-1β in the circulation when stimulated with LPS (58, 87). Results from previous LPS stimulation studies suggested that NPY could indirectly mitigate activation of microglia through its role in temperature regulation (87), which might also be the reason for the absence of a distinct impact on the investigated microglial markers in this project. Owing to the elevation of IL-1β the substantial impact of LPS on fever development in the hypothalamus should not be forgotten and may be co-responsible for NPY's impact on hypothalamic microglial function (9). In their experiments, Radler et al. (2015) found that NPY blocks fever induced by LPS and

saw further that the activation of microglia in the hypothalamic arcuate nucleus was in part depending on local temperature. Mild hypothermia is therefore believed not only to mitigate pro-inflammatory cytokines but also to lower the quantity of activated microglia after LPS injection in their *in vivo* models (105). Previous animal experiments further revealed systemic Poly(I:C) application to induce hyperthermia, activate the HPA axis in rabbits and reduce mouse body weight (9). NPY on the other hand, in a dose of 160 pmol/kg, has in the past been able to exert temperature stabilizing effects for more than 8h upon 0.1 mg/kg intravenous LPS administration, which in comparison to our dose of 0.03mg/kg administered via the IP route is very high (126). In another *in vivo* experimental setting, chicken were put under heat-stress evoking an increase in body temperature, where their food intake decreased while hypothalamic NPY rose (127).

As supported by previous results from this laboratory, it is worth considering that NPY *in vivo* does not (only) interact with the production of cytokines in microglia, indirectly measured by Iba1, CD206, CD86 (58). The neuropeptide may modify microglia or could cross the pathways of cytokines and sickness induced behavioral alterations in other ways. Our results obtained with Poly(I:C) in combination with NPY resulting in the strongest upregulation of microglial Iba1, CD206 and CD86 in the hypothalamus reminds of NPY's pro-inflammatory effects in the gut (87).

Post-traumatic stress disorder is a reactive disorder to prolonged exposure to any kind of stress with symptoms such as anxiety, avoidance behavior, depression or autonomic hyper-responsiveness. It is one of the neuropsychiatric diseases for which there are few drug-based treatment approaches so far available. Experimentally mimicking this disease in mice, IN NPY has successfully proven its resilience-promoting and stress-buffering effects, including reduced HPA axis activity (88, 95). On the other hand, NPY function appears to show different results depending on the target cell type, applied quantity and duration of the effect (61). *In vitro* experiments by de la Fuente et. al. (1993) could show that the influence of NPY on phagocytic processes varies with the microbial stimulant used, the neuropeptide increasing phagocytic activity of *Candica albicans* (128). Meanwhile, effects of NPY in reducing phagocytosis of *E. coli*, whose LPS we used in this experiment, and *Staphylococcus aureus*, a widespread gram-positive bacterium,

could be observed in macrophage cell experiments (94). For the first time, work from this laboratory was able to show that LPS-induced reactions, measured by standard behavioral tests, but neither circulating nor hypothalamic cytokine concentrations could be attenuated by IN applied NPY. Additionally, an accompanying reduction of HPA axis activity could be measured (58). Interestingly, electron microscopic analysis demonstrated interactions between ACTH and NPY neurons with hypothalamic parvocellular neurons expressing CRH (91, 129).

Therefore, despite the lack of straightforward effects of NPY in hypothalamic microglial inhibition in the current mouse model, it cannot be ruled out that NPY generally has no therapeutic effects on immune mediated changes in hypothalamic functions in humans. Various murine studies by others (58, 87, 95) confirmed its potentially beneficial role in neuropsychiatric diseases and make it worthwhile to further investigate and differentiate its effects in both rodents and humans. Thus, on the one hand we see IL-1β associated positive effects of NPY in buffering *in vivo* microglial cell motility and phagocytosis via the Y1 receptor and, on the other hand, we see positive effects of NPY on sickness induced behavior, while the intervening pathophysiological mechanisms remain hidden until now (58, 60, 61) The hypothalamic microglial markers Iba1, CD206 and CD86 investigated in this project do not seem to map these NPY interventions.

4.6 Limitations of this project

As in any experiment, there is no guarantee it is immune to mistakes regarding performance, to being blind to relevant influencing factors or detection biases. A weak point can also be the exclusive examination of male mice and the lack of females. A further limitation can be the restricted investigation to only three microglial markers. Regarding a potential false positive result (104), it is of course always useful to critically reevaluate the selection of housekeeping genes, even though they are well established. Another potential problem could be that the scope of all hypothalamic functions may be too broad. To overcome this limitation, it might be interesting to study the hypothalamic functions divided according to the respective nuclei. One additional limitation within the brain is certainly that only the hypothalamus was examined. Other areas, such as the nuclei of the limbic system, the prefrontal cortex would certainly also be very important to dissect, if

only because of the effects of NPY and its contribution to shaping behavior (8, 88). Furthermore, in this project we restrictively analyzed the transcription, but not the translation of the investigated markers. Since we can only draw conclusions from the investigated markers and have not morphologically examined the microglia themselves, it is not clear whether their shape and function have changed at all.

For more than 2 centuries mice have been used as a simple, inexpensive model in scientific experiments. With their similar genetic profile to humans, numerous elementary insights have been gained. With regard to age, parallels can be drawn in this project between the 8-week-old animals as young adults (113). Needless to say, experiments with older mice would also be very interesting.

With regard to the transferability of the data from the mouse model to humans, species-specific aspects of brain and neurotransmission in quality and quantity must of course be considered. However, a review by Hökfelt et al. (2013) reported that there is good comparability and many neuropeptides have a similar distribution across human and rodent hypothalami. Differences seem to occur rather in the transmission through nitrogen monoxide and glutamate (130).

4.7 Future directions

There are numerous parameters that influence immune responses in humans and mice. Some mechanisms of positive and negative regulation of macrophage functions, for instance, have been uncovered, but some are still unknown. For example, Villa et al. (2018) observed murine microglial cell differences based on gender-specific expression of a significant number of genes (131). After intraperitoneal injection of a mild dosage of 0.63 mg/kg LPS to mice, brains of older females showed the strongest pro-neuroinflammatory response when compared to younger females and older males (132). This is in line with the understanding that females develop enhanced immune responses and are less likely to develop immune tolerances (5). As this project only included males, it would be interesting to ask whether there were different results in female mice.

Although the hypothalamus is a major site of action, it should not be forgotten that IL-1β also modifies ACTH production taking place in the pituitary gland. It would therefore be interesting to study microglial cells at other locations along the HPA axis: Basal, bioactive IL-6 also originates to a large extent from the pituitary gland, which is also affected by LPS, but also other typical pro-inflammatory cytokines

are produced at this site (5). Viral influences play a major role not only at a very young age, but also at a more advanced age. In rodents that received more frequent viral stimulation, 7 per day, no reduction of the inflammatory response was observed. Interestingly, Poly(I:C) appears to stimulate solely the innate immune system (9). Chronic stimulation purely with LPS *in vitro* showed a tolerance development of macrophages, which was reflected in a reduced TNF-α production and could be interrupted and thus reactivated by the administration of a different, in this case gram positive, stimulant. (133).

Neuropsychiatric disorders are often of a chronic nature and as this was only an in vitro experiment, in order to understand the underlying mechanisms better, I would propose to create such chronic, mild immunostimulatory conditions in future *in vivo* mouse models in order to test any effects of NPY. Since our two different microbial stimulants (LPS and Poly(I:C)) yielded different effects on microglial marker expression in the hypothalamus, I would be interested to investigate, for example, stimulants of gram-positive bacteria. Furthermore, as we did not measure thermic conditions or cytokine parameters in our experiment, it would be worthwhile to take thermic influences on hypothalamic NPY activity into account.

5 Conclusion

The immune-brain crosstalk explains that pathogens do not necessarily have to be neuroinvasive, in order to have influences on central neural structures and its functions. Even though IN pretreatment with NPY does not mitigate the effects of viral and bacterial immune stimulants to enhance microglial marker expression in the hypothalamus, both the therapeutic value of NPY and the activity of microglia cells in experiments by others indicate their involvement in neuropsychiatric processes and require further differentiated investigations.

Immunomodulated diseases are in the rise, which is why I conclude it is highly relevant to further investigate ongoing mechanisms. I further propose the idea to not only isolate distinct hypothalamic nuclei but also other areas of the brain in this context and to use different doses and ligands for peripheral immune stimulation. Performing behavioral studies in addition to the same experimental protocol may advance the understanding of the clinical impact. Furthermore, I think even though it is not the simplest way to think, that breaking disciplinary boundaries will be crucial in understanding underlying mechanisms. The thought of what more we will know on this topic in 20 years' time fills me with excitement.

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