Dissertation

Effects of immune modulation on brain function and behaviour in the context of the microbiota–gut–brain axis

submitted by

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Declaration

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

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Abbreviations and Definitions

1MT	1-methyl-D,L- tryptophan
3HK	3-hydroxykynurenine
ACTH	adrenocorticotropic hormone
AD	antibody diluent
ANOVA	analysis of variance
BBB	blood brain barrier
BCG	Bacille Calmette-Guérin
BNSTd/v	bed nucleus of the stria terminalis dorsal/ventral
CeA	central amygdala
CNS	central nervous system
CORT	corticosterone
CRF	corticotropin-releasing factor
CVO	circumventricular organ
DAB	diaminobenzidine
DG	dentate gyrus
DVC	dorsal vagal complex
EIA	enzyme immunoassay
EPM	elevated plus maze
FCA	Freund's complete adjuvant
FST	forced swim test
h	hour
HPA	hypothalamic-pituitary-adrenal
HPLC	high performance liquid chromatography
IDO	indoleamine 2,3 dioxygenase
iE-DAP	γ-D-glutamyl-meso-diaminopimelic acid
IFN	interferon
IKK	inhibitor of nuclear factor-κB-kinase
IL	interleukin
i.p.	intraperitoneal
KO	knockout
KYN	kynurenine
KynA	kynurenic acid
LPS	lipopolysaccharide

LRR	leucine-rich-repeat
MAMP	microbial-associated molecular pattern
MAPK	mitogen-activated protein kinase
MDP	muramyl dipeptide
MI	memory index
min	minute
MPO	myeloperoxidase
MyD88	myeloid differentiation primary-response protein 88
NALP	NACHT, LRR and PYD domains-containing protein
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NLR	nucleotide-binding domain and leucine rich repeat containing receptors / Nod-like receptor
NMDA	N-methyl-D-aspartate
NOD	nucleotide-binding and oligomerization domain
NORT	novel object recognition test
NPY	neuropeptide Y
NTS	nucleus tractus solitarii
OFT	open field test
OTU	operational taxonomic unit
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PFA	paraformaldehyde
PMSF	phenylmethylsulfonyl fluoride
PP	pancreatic polypeptide
PRR	pattern recognition receptor
PVN	paraventricular nucleus of the hypothalamus
PYY	peptide YY
ROI	regions of interest
rpm	rounds per minute
RT-PCR	reverse transcription polymerase chain reaction
QA	quinolinic acid
s	second
SFO	subfornical organ
SO	supraoptic nucleus

SP	sucrose preference
TAK1	transforming growth factor-β- activated kinase
TLR	Toll-like receptor
TNF-α	tumour necrosis factor alpha
TRP	tryptophan
TST	tail suspension test
VEH	vehicle
WB	wash buffer
WT	wild type
YLDs	years lived with disability

Abstract in German

Entzündungsfördernde Zytokine stehen in einem ursächlichen Zusammenhang mit psychiatrischen Erkrankungen wie Depressionen oder Angststörungen. Während verschiedene Mechanismen für die erhöhte Prävalenz von Immunmarkern in psychiatrischen PatientInnen verantwortlich sein könnten, ist die Gesamtheit der Mikroorganismen, die den Darm bewohnen – das intestinale Mikrobiom – eine potentielle Quelle immunaktivierender Substanzen, die unser systemisches Immunsystem beeinflussen. Bakterielle Zellwandbestandteile aktivieren das Immunsystem, indem sie Rezeptoren wie die „Toll-Like Receptors“ (TLRs) oder „Nuclear-Binding Domain (NOD)-Like Receptors“ (NLRs) aktivieren. Lipopolysaccharide (LPS) sind Bestandteile der äußeren Membran gram-negativer Bakterien und induzieren durch Aktivierung des TLR4 eine starke Immunantwort, die mit Verhaltensveränderungen wie Krankheitsverhalten, Ängstlichkeit und depressionsartigem Verhalten einhergehen. Im Gegensatz dazu lösen NOD-Agonisten eine schwächere Immunantwort aus, sind aber in der Lage, die durch LPS ausgelöste Zytokinausschüttung der Immunzellen zu verstärken.

Da Verhaltensänderungen durch eine Koaktivierung von NOD und TLR4 bisher nicht im Detail untersucht wurden, hatte diese Dissertation zum Ziel, die immunologischen und zentralen Auswirkungen des NOD1-Agonisten FK565 und des NOD2-Agonisten Muramyl-Dipeptid (MDP) allein und in Kombination mit LPS an C57BL/6N Mäusen zu untersuchen. Während die alleinige Verabreichung der ausgewählten NOD-Agonisten nur geringfügige Auswirkungen auf das Verhalten hatte, löste eine intraperitoneale Injektion von FK565 (0,001 oder 0,003 mg/kg) oder MDP (1 oder 3 mg/kg) 4 Stunden vor jener von LPS (0,1 oder 0,83 mg/kg) ein - im Vergleich zu LPS allein - deutlich gesteigertes und verlängertes Krankheitsverhalten aus, das sich durch eine Einschränkung der Lokomotion, Exploration, Nahrungsaufnahme und Körpertemperatur ausdrückte. Dieses verstärkte Krankheitsverhalten war mit erhöhten Werten entzündungsfördernder Zytokine (IFN-γ, IL-1β, IL-6, TNF-α) im Gehirn und Blut, sowie erhöhter zirkulierender Kynurenin-Werte verbunden. Der immunhistochemische Nachweis von c-Fos, einem Marker neuronaler Aktivitätsveränderung, deutete darauf hin, dass ein Synergismus zwischen NOD2 und TLR4 die Aktivität jener Hirnregionen beeinflusste, die eine Rolle in der Reaktion des Körpers auf Immunaktivierung spielen.

Die hauptsächlichste Quelle von TLR- und NOD-Agonisten im Körper stellt das intestinale Mikrobiom dar. Deshalb interessierte, welche verhaltensmäßigen Effekte eine durch Antibiotikagabe ausgelöste Dsybiose des Darmmikrobioms hat. Nach Testung

verschiedener Antibiotika stellte sich heraus, dass die Verabreichung einer Kombination aus Ampicillin (2 mg/ml), Bacitracin (5 mg/ml), Meropenem (1 mg/ml), Neomycin (5 mg/ml) und Vancomycin (0.3 mg/ml) mit dem Trinkwasser das Mikrobiom des Kolon nahezu vollständig auslöschte. Gleichzeitig wurden anxiolytische und depressionsartige Verhaltenseffekte beobachtet und die Kognition beeinträchtigt. Außerdem führte die Zugabe der genannten Antibiotika in das Trinkwasser zu einer Einschränkung der Flüssigkeitsaufnahme und zu Gewichtsverlust.

Zusammenfassend konnte gezeigt werden, dass NOD1 und NOD2 synergistisch mit dem TLR4 zu einer Verstärkung der immunologischen und verhaltensmäßigen Effekte einer peripheren Immunstimulierung führen. Weiters wurden durch die Beseitigung des intestinalen Mikrobioms Verhaltensänderungen erzielt, die dem Verhalten keimfreier Mäuse ähneln. Ob die Veränderungen des Mikrobioms ursächlich an den beobachteten Verhaltensänderungen beteiligt sind, kann zum jetzigen Zeitpunkt nicht eindeutig beantwortet werden, da die potentiellen Nebenwirkungen der Antibiotikabehandlung noch genauer untersucht werden müssen.

Abstract in English

Pro-inflammatory cytokines have been demonstrated to be causally involved in the pathogenesis of psychiatric disorders including major depression and anxiety. While various mechanisms may underlie the increased prevalence of heightened immune markers in psychiatric patients, the microbial community of the intestinal tract, the intestinal microbiota, has emerged as a potential source of immune activating agents being capable of affecting systemic immunity. Bacterial cell wall components activate the immune system by binding to specialized receptors including Toll-like receptors (TLRs) and nuclear-binding domain (NOD)-like receptors (NLRs). The outer membrane component of Gram-negative bacteria, lipopolysaccharide (LPS), is commonly known as a strong immune activator, activating TLR4 and leading to behavioural signs of sickness, anxiety and depression. While the immune activating potency of NOD agonists is less marked, they are able to prime immune cells augmenting LPS-induced cytokine production.

As the behavioural sequelae of NOD and TLR4 co-activation have been little studied, the effects of the NOD1 agonist FK565 and the NOD2 agonist muramyl dipeptide (MDP), alone and in combination with LPS, were investigated in C57BL/6N mice with regard to immune and brain activity markers. In addition, the effects of a disturbance of the intestinal microbiota by oral antibiotics targeting Gram-positive, Gram-negative and/or anaerobic microorganisms on anxiety and depression-like behaviour as well as learning and memory were investigated.

While the NOD agonists under study given alone had only minor behavioural effects, intraperitoneal injection of FK565 (0.001 or 0.003 mg/kg) or MDP (1 or 3 mg/kg) 4 h before LPS (0.1 or 0.83 mg/kg) significantly aggravated and prolonged the LPS-evoked sickness behaviour as revealed by a decrease in locomotion, exploration, food intake and body temperature. The exacerbated behavioural response was accompanied by elevated plasma and cerebral levels of pro-inflammatory cytokines (IFN-γ, IL-1β, IL-6, TNF-α) as well as raised plasma levels of kynurenine. Immunohistochemical labelling of c-Fos, a marker indicating changes in activity within neurons, indicated that NOD2 synergism with TLR4 modulated activation of cerebral nuclei relevant to the sickness response.

The main source of TLR and NOD agonists in the body is the intestinal microbiota. For this reason I was interested in examining the behavioural effects of an antibiotic-induced dysbiosis of the gut microbiota. Administration of various antibiotics revealed that a combination of ampicillin (2 mg/ml), bacitracin (5 mg/ml), meropenem (1 mg/ml), neomycin (5 mg/ml) and vancomycin (0.3 mg/ml) administered via the drinking water

depleted the colonic microbiota and exerted anxiolytic and depressogenic effects, while impairing learning and memory. At the same time, however, addition of the antibiotics to the drinking water resulted in a decrease in fluid intake and body weight.

In summary, these results demonstrate that NOD1 or NOD2 synergizes with TLR4 in aggravating the immune-related and behavioural responses to peripheral immune activation. Furthermore, depletion of the intestinal microbiota succeeded in inducing behavioural changes comparable to those seen in germ-free mice. However, a causal involvement of the microbiota on the behavioural alterations cannot be warranted from the current results, due to potential side effects of the antibiotic treatment.

1 Introduction

1.1 Activation of the immune system and its influence on mood and behaviour

Both humans and animals respond to an infection with symptoms of sickness, which comprise anorexia, fatigue, anhedonia and fever (Hart 1988, Dantzer et al. 2008, McCusker, Kelley 2013). Benjamin L. Hart was the pioneering scientist coining the concept that sickness behaviour is not just an uncomfortable side effect of infection, but an adaptive motivational state, enabling recovery from infection (Hart 1988).
However, while in the acute context of infection the activation of the immune system is useful and beneficial, immune activation has also been demonstrated to be causally involved in the development of major depression and other psychiatric disorders. In 1991 R.S. Smith first proposed "the macrophage theory of depression", reviewing the evidence that cytokines produced by macrophages such as interleukin-1 (IL-1) could play a substantial role in the development of major depression (Smith 1991). This theory was confirmed by Michael Maes, who demonstrated increased levels of circulating cytokines in depressed patients (Maes et al. 1995). In addition, also medically ill patients with disorders that comprise the activation of the immune system show an increased prevalence of clinical depression (Evans et al. 2005). Moreover, the tumour necrosis factors alpha (TNF-α) antagonist etanercept has been found to improve the symptoms of depression in patients with the immune-mediated skin disorder psoriasis (Tyring et al. 2006). In a more recent study performed in depressed but otherwise medically healthy patients the effects of the TNF-α blocker infliximab on mood were tested. Intriguingly, infliximab improved symptoms only in those patients who presented with higher inflammatory markers before treatment, while in those patients who had low levels of peripheral inflammation at baseline infliximab had rather negative effects when compared to the placebo-treated group (Raison, Miller 2013). Thus, there seems to be a U shaped relationship between inflammation and depression, where both very low and high levels of inflammation potentially induce depressive symptoms.
A causal relationship between immune activation and depression was also provided by the use of pro-inflammatory cytokines which have antiviral and antineoplastic properties and therefore are used to treat patients suffering from cancer or hepatitis C infection. Clinical studies revealed that most of the cytokine-treated patients show signs of sickness,

including fatigue, pain, and decreased appetite, starting shortly after the start of the therapy. In contrast, only a subset of these patients (30 – 50 %) develops signs of major depression such as feelings of worthlessness, guilt and also suicidal thoughts at later stages (Capuron et al. 2002a, Dantzer et al. 2011).

This scenario can also be modelled in rodents by the administration of immune activating agents. In these models it has been demonstrated that immune activation can induce sickness behaviour, which can be assessed by a decrease of locomotor activity or social interaction (Frenois et al. 2007). As seen in the clinical setting, also in rodents the acute sickness response can evolve into depression-like behaviour which manifests itself in an increased duration of immobility in the tail suspension test (TST) or forced swim test (FST), which is assumed to reflect behavioural despair (Frenois et al. 2007). In addition, decreased sucrose consumption in the sucrose preference (SP) test is believed to reflect anhedonia, another aspect of depression-like behaviour (Frenois et al. 2007).

1.1.1 Cytokines affecting behaviour – Evidence from rodent models

Animal studies revealed that pro-inflammatory cytokines are the predominant mediators of sickness behaviour and among those IL-1β and TNF-α represent the most important cytokines responsible for the induction of sickness (Dantzer et al. 2008). Likewise, peripheral injection of the bacterial component lipopolysaccharide (LPS) dose dependently leads to central expression of several cytokines including IL-1β and TNF-α (Quan et al. 1999). Studies applying a TNF-α antibody in IL-1 receptor type I (IL-1R1) knockout (KO) mice further demonstrated that sickness behaviour can be induced by either IL-1β or TNF-α, but when both IL-1β and TNF-α are blocked, LPS-induced sickness is likewise inhibited (Bluthe et al. 2000a, McCusker, Kelley 2013). Finally, various routes of administration of IL-1β or TNF-α dose-dependently increase cytokine expression within the brain and induce the full spectrum of sickness behaviour (Bluthe, Dantzer & Kelley 1991, Churchill et al. 2006, Anisman, Gibb & Hayley 2008). While IL-1β has been demonstrated to exert behavioural effects via activation of the IL-1R1 (Bluthe et al. 2000a), TNF-α acts via the TNF-R1 (Palin et al. 2009a). Both the IL-1R1 and the TNF-R1 are expressed on neurons within the central nervous system (CNS) and are likely to modify behaviour by directly changing neuronal activity (McCusker, Kelley 2013).

Interestingly, while there is no direct evidence for IL-1β in mediating depressive-like behaviour, TNF-α has been shown to be involved in depressive-like behaviour (Simen et al. 2006, Kaster et al. 2012).

In contrast to IL-1β and TNF-α, IL-6 induces sickness related responses such as fever, but does not induce behavioural changes characteristic for sickness (Lenczowski et al. 1999). However, despite the lack of a sickness response to IL-6 administration, IL-6 is necessary for a full sickness response induced by LPS or IL-1β and is a prerequisite for amplifying cytokine levels within the brain (Bluthe et al. 2000b, McCusker, Kelley 2013). Furthermore, IL-6 KO mice show lower levels of depression-like behaviour (Chourbaji et al. 2006). Interestingly, a recent study demonstrated that the expression of the serotonin transporter was reduced in the hippocampus after central administration of IL-6, while IL-6 KO mice present with increased serotonin transporter expression (Kong et al. 2015). Similar to IL-6, while there is no evidence for a direct role of IFN-ɣ in inducing sickness behaviours, IFN-ɣ has been shown to be implicated in depressive-like behaviours following immune activation (O'Connor et al. 2009a).

1.1.2 Propagation of peripheral immune activation to the brain

In order to induce behavioural changes, peripheral immune activation has been proposed to induce a "mirrored immune response" within the brain (McCusker, Kelley 2013). The propagation of peripheral immune activation to the brain involves humoral and neuronal communication routes. Activation of the neuronal pathway takes place via microbial components [so called microbial-associated molecular patterns (MAMPs)] or cytokines, which are able to activate primary afferent neurons. This pathway is able to induce a rapid propagation of the peripheral signal to the brain (Dantzer et al. 2008). As part of the humoral pathway, cytokines and MAMPs enter the circulation and act at the blood-brain interface (McCusker, Kelley 2013). The circumventricular organs (CVOs), such as the area postrema and the subfornical organ (SFO) represent an important blood-brain interface, since they lie outside the blood brain barrier (BBB) and neurons and immune cells are located in close proximity within these structures (Goehler, Erisir & Gaykema 2006). The BBB hinders free leakage of most soluble substances from the blood into the brain (Banks, Erickson 2010), yet circulating MAMPs are able to induce the production of cytokines at the BBB and thus affect brain function (Verma et al. 2006). Since cytokines are rather large proteins, they also do not easily penetrate the BBB (Haroon, Raison & Miller 2012). However, they are able to impact the brain via several pathways:

1. saturable cytokine transporters at the BBB transport cytokines into the brain (Banks, Kastin & Durham 1989)

2. activation of cytokine receptors, which are located on cerebral endothelial cells and perivascular macrophages, induce the release of cytokines into the brain (Konsman et al. 2004)
3. recruitment of peripherally activated immune cells into the brain induces the production of cytokines by these cells inside the brain (D'Mello, Le & Swain 2009)

1.1.3 Mechanisms leading to cytokine-induced behavioural changes

Cytokines are able to induce a plethora of neurochemical changes in the brain that comprise altered function of monoamine, glutamate, and neuropeptide systems (Felger, Lotrich 2013). While deficits of monoaminergic neurotransmission such as serotonergic neurotransmission have been implicated in the pathogenesis of depression, activation of indoleamine 2,3 dioxygenase (IDO), an enzyme that converts tryptophan to kynurenine, has emerged as a crucial mediator of the depressogenic effects of cytokines (Dantzer et al. 2011). Thus, immune activation decreases circulating tryptophan levels while increasing those of kynurenine. IDO is expressed in several cell types such as macrophages, dendritic cells, microglia, astrocytes, and neurons (Guillemin et al. 2005, Haroon, Raison & Miller 2012). Accordingly, LPS has been demonstrated to activate IDO both peripherally and centrally concomitant with the occurrence of depression-like behaviour (Lestage et al. 2002). A causal role of IDO activation in the depressogenic effects of immune challenge was also demonstrated by the finding that 1-methyl-D,L- tryptophan (1MT), a IDO antagonist, was able to block LPS-induced depression-like behaviour, while the levels of pro-inflammatory cytokines and sickness behaviour were not affected (O'Connor et al. 2009b).

Since tryptophan is the rate limiting factor for serotonin synthesis, studies suggested that the decreased tryptophan concentrations would lead to a decreased tryptophan bioavailability and hereby limit serotonin production (Capuron et al. 2002b). Intriguingly, while tryptophan levels in rodents decreased in the periphery in response to LPS, in the brain even an increase of tryptophan was observed (O'Connor et al. 2009b). Furthermore, while 1MT, in addition to attenuating depression-like behaviour, also decreased kynurenine levels in response to LPS, it did not affect the increased tryptophan levels in the brain (O'Connor et al. 2009b). While kynurenine is readily transported across the BBB, tryptophan has to compete with other neutral amino acids to cross the BBB (Fukui et al. 1991, van Donkelaar et al. 2011). In addition, about 90 % of the circulating tryptophan is bound to albumin, which hinders its transport across the BBB (van Donkelaar

et al. 2011). The mechanism that leads to the increase in central tryptophan levels in response to immune activation and its consequences are still unknown, but compensation of the decrease in circulating tryptophan by the brain has been proposed (Dantzer et al. 2011). In addition, the authors observed an increase in brain serotonin turnover by LPS, which was also not affected by 1MT (O'Connor et al. 2009b). In summary, these results lead the authors to conclude that inflammation-induced decreases of tryptophan leading to decreased serotoninergic neurotransmission are not responsible for depression-like behaviour in response to immune activation. In contrast, their results indicated that the increased levels of kynurenine account for the depressogenic effects of immune activation. While kynurenine itself is inactive, its metabolites, 3-hydroxy kynurenine (3HK) and quinolinic acid (QA) have been shown to be neurotoxic. They exert their harmful effects by acting as N-methyl-D-aspartate (NMDA) receptor agonists and by generating oxidative radicals. In contrast, another metabolite called kynurenic acid (KynA) is considered to be neuroprotective and acts as a NMDA and alpha-7 nicotinic acetylcholine receptor antagonist (Dantzer et al. 2011). It has further been proposed that imbalances in the degradation of kynurenine to its different metabolites may account for its depressogenic effects during inflammation (Muller, Schwarz 2008). Indeed, depressed patients show a reduction in KynA/3HK and/or KynA/QA ratios (Savitz et al. 2015).
In fact, the NMDA receptor antagonist, ketamine has been demonstrated to abrogate LPS-induced depression-like behaviour, without affecting the sickness response in mice (Walker et al. 2013). Intriguingly, ketamine is able to induce rapid antidepressant effects in patients with treatment refractory depression, highlighting the potential of treating mood disorders by targeting glutamatergic neurotransmission (Salvadore, Singh 2013).

1.1.3.1 The NPY family as protectors against behavioural disturbances in response to immune activation

The neuropeptide Y (NPY) family consists of NPY, peptide YY (PYY) and pancreatic polypeptide (PP), exerting their actions via Y receptors (in particular Y1, Y2, Y4 and Y5), which belong to the group of G-protein-coupled receptors. While NPY is primarily found in the central and peripheral nervous system, PYY and PP are expressed in the digestive system (Holzer, Reichmann & Farzi 2012). Apart from the difference in their expression, the affinities for the Y receptor types differ between the members of the NPY family. Thus, full length NPY and PYY are agonists of the Y1, Y2 and Y5 receptor subtypes, while PP is mainly activating the Y4 receptor. In addition, NPY and PYY can be cleaved

by dipeptidyl peptidase 4 yielding truncated NPY_{3-36} and PYY_{3-36}, respectively, which thereby lose their affinity to the Y1 receptor subtype (Mentlein et al. 1993). NPY is renowned for its potent orexigenic and energy storing effects (Zhang, Bijker & Herzog 2011), while PYY and PP rather reduce food intake (McGowan, Bloom 2004, Field, Chaudhri & Bloom 2010). Apart from NPY's effects on food intake and metabolism, the NPY family also affects other functions of the nervous system and also exerts homeostatic effects on the immune system (Farzi, Reichmann & Holzer 2015). The immunological effects of the NPY family are complex and can be both, pro- or anti-inflammatory in a context-dependent manner. In the brain, however, NPY exerts consistent anti-inflammatory actions, while in the intestine NPY can be considered as pro-inflammatory (Farzi, Reichmann & Holzer 2015). Additionally, the general effects of NPY on brain function and behaviour can be summarized as beneficial, as NPY exerts neuroprotective, antiepileptic, anxiolytic, and stress-buffering effects (Heilig 2004, Malva et al. 2012, Farzi, Reichmann & Holzer 2015).

Importantly, NPY is a vasoconstrictor and is also able to attenuate LPS-induced hypotension and to increase the survival from LPS-induced septic shock (Hauser et al. 1993) (Figure 1). Furthermore, NPY has been demonstrated to stabilize body temperature in response to LPS (Felies et al. 2004). NPY also stabilizes LPS-induced disturbances of granulocyte and T-lymphocyte numbers and attenuates leukopenia in response to LPS (Nave et al. 2004). Finally, NPY is able to blunt LPS-induced increase of TNF-α (Stadler et al. 2011).

Work from my lab has further demonstrated that deletion of NPY, PYY, or both NPY and PYY in mice aggravates and prolongs weight loss evoked by immune challenge with attenuated mycobacterium Bacille Calmette–Guérin (BCG) (Painsipp et al. 2013). As the weight loss of the KO mice outlasted the decrease in food intake, anorexia does not seem to be the only reason underlying the aggravated weight loss in the KO mice. Thus, both NPY and PYY are likely to play a physiological role in maintaining energy homeostasis in response to immune activation (Painsipp et al. 2013).

The NPY family is also able to protect against behavioural disturbances in response to immune challenge (Farzi, Reichmann & Holzer 2015). In more detail, Y2 and Y4 receptor KO mice are more susceptible to the acute and long term actions of LPS, leading to the conclusion that activation of the Y2 and Y4 receptor attenuates inflammation-induced sickness and depression-like behaviour (Painsipp, Herzog & Holzer 2008, Painsipp, Herzog & Holzer 2010).

With regard to the microbiota-gut-brain-axis, intake of specific prebiotic fibres, which are mainly fermented by the intestinal microbiota to products including short-chain fatty acids, have been demonstrated to increase satiety and the release of PYY (Cani et al. 2009, Delzenne et al. 2011). Furthermore, PYY producing enteroendocrine cells in the gut epithelium express the G-protein-coupled receptor, Gpr41, a receptor recognizing short-chain fatty acids. Interestingly, it has been demonstrated that colonization of mice with a fermentative microbial community increases circulating levels of PYY, which is not observed in Gpr41 KO mice. Gpr41 KO mice further were leaner than their wild type (WT) littermates and showed a reduced capacity to harvest energy from the diet (Samuel et al. 2008). These results therefore demonstrate that there may be an important crosstalk between the microbiota and PYY-expressing cells along the microbiota-gut-brain axis (Holzer, Farzi 2014).

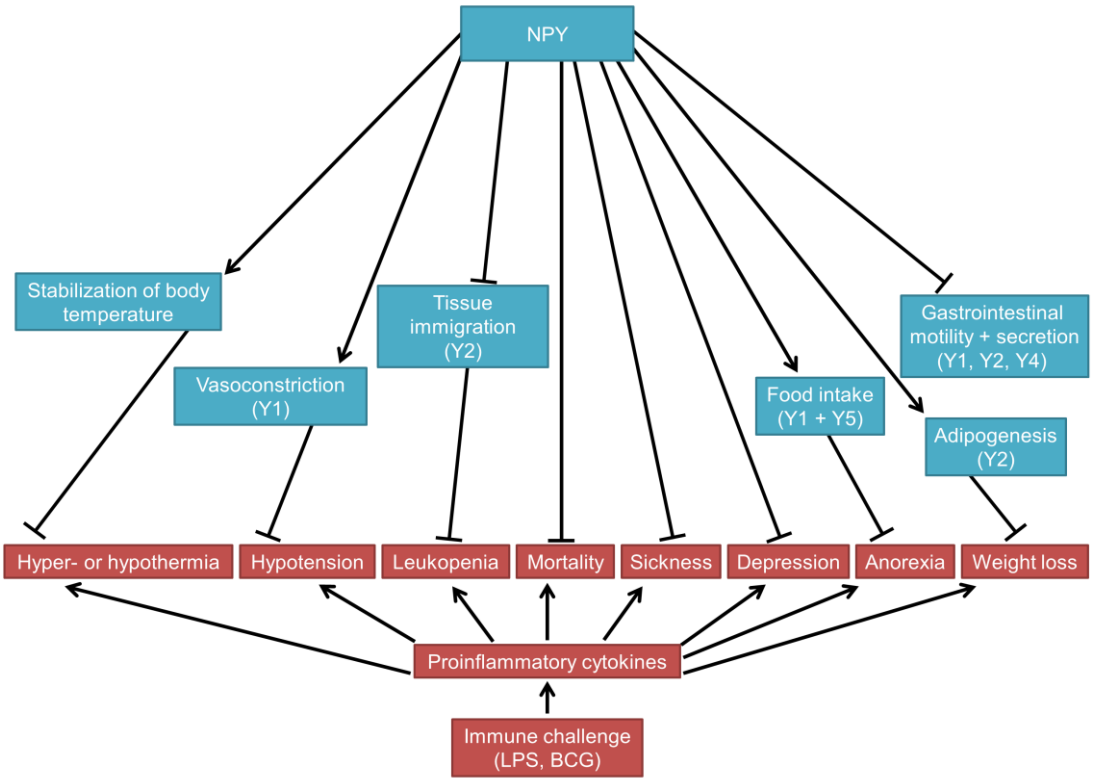

Figure 1 Homeostatic roles of NPY in disturbances of physiological systems caused by peripheral immune challenge. Major receptors involved are bracketed. The arrow symbols denote stimulation; the tack symbols denote inhibition. BCG, Bacille Calmette-Guérin; LPS, lipopolysaccharide (Taken from Farzi, Reichmann & Holzer 2015).

1.1.4 Neuroanatomy of mood-related signalling and c-Fos as a marker for neuronal activation

In the late 1980s it was discovered that stimulated neurons transiently express the immediate early gene *c-fos* (Sagar, Sharp & Curran 1988). Activation of the *c-fos* gene results in the expression of the c-Fos protein, which is located in the cell nucleus. c-Fos expression in neurons occurs in response to various stimuli such as neurotransmitters, growth factors and various stressors and can therefore be used to assess changes in activity within neurons (Hoffman, Lyo 2002). Characteristically c-Fos expression after stimulation is transient and does not persist in response to chronic stimuli. In addition, in some brain areas neuronal activation can occur without c-Fos expression, while other neurons show constitutive c-Fos expression (Hoffman, Lyo 2002).

LPS challenge also induces c-Fos expression in viscerosensory and autonomic network nuclei of the brain with a peak occurring 3 h after injection (Wan et al. 1993, Rivest, Laflamme 1995, Sagar et al. 1995). The nucleus tractus solitarii (NTS) is an important viscerosensory nucleus, which receives information from visceral structures such as the gut via the vagus nerve. In addition it is part of the dorsal vagal complex (DVC), which also includes the area postrema and the dorsal motor nucleus of the vagus, containing motor neurons, which receive input from the NTS and innervate visceral structures such as the gut wall (Marvel et al. 2004). Following intraperitoneal (i.p.) injection of LPS, the vagal nerve is activated and signals to the NTS, which is located in the lower brain stem (McCusker, Kelley 2013). Injection of LPS causes a rapid increase in c-Fos immunoreactivity within the NTS and its projection areas, such as the hypothalamus and the limbic system (Wan et al. 1993). Thus, the NTS has been described as a "relay structure" between the viscera and the brain (Konsman et al. 2008). The importance of the viscerosensory nuclei with regard to sickness behaviour has been demonstrated by the findings that inactivation of the DVC can prevent signs of sickness behaviour (Gaykema et al. 2008, Gaykema, Goehler 2011).

LPS typically leads to increased c-Fos expression in brain regions that are associated with stress and autonomic adjustments such as the paraventricular nucleus of the hypothalamus (PVN), bed nucleus of the stria terminalis (BNST), and central amygdala (CeA) (Gaykema, Goehler 2011). In contrast, LPS has been demonstrated to reduce c-Fos expression in regions involved in exploratory behaviour such as the hippocampus (Gaykema, Goehler 2011). The precise neurocircuits that underlie the distinct dimensions

of sickness behaviour remain elusive, however work mainly coming from Ronald P.A. Gaykema and Lisa E. Goehler's laboratory have shed light on some pathways that might be involved in behavioural changes induced by immune activation. Thus, their work pointed to the possible involvement of changes in neuronal activity in the hypothalamus and hippocampus in the locomotor effects of LPS (Gaykema, Goehler 2011). In contrast, they demonstrated that the activation of the CeA and BNST is rather not involved in the effects of LPS on locomotion (Gaykema, Goehler 2011).

1.1.5 Reciprocal relationship of immune activation and the hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis is a neuroendocrine system characterized by the secretion of corticotropin-releasing factor (CRF) and vasopressin from the PVN, which induce the secretion of adrenocorticotropic hormone (ACTH) from the pituitary, which finally triggers the secretion of glucocorticoids (corticosterone in rodents) from the adrenal cortex (Pariante, Lightman 2008). Glucocorticoids classically induce a negative feedback response to suppress CRH and ACTH production and thereby control HPA axis activity.

H. Besedovsky first described activation of the HPA axis by i.p. injection of IL-1β to rodents (Besedovsky et al. 1986). Subsequently, also other pro-inflammatory cytokines, including IL-6 and TNF-α, were demonstrated to trigger HPA axis activation (Dunn 2000). Likewise, injection of LPS induces activation of the HPA axis and leads to elevated blood levels of ACTH and corticosterone (CORT) (Beishuizen, Thijs 2003). As LPS is not able to induce CRF secretion from hypothalamic explants, again cytokines such as IL-1, IL-6 and TNF-α are proposed to stimulate the HPA axis at different levels in response to LPS (Turnbull, Rivier 1999). However, controversies exist regarding the exact sites of LPS-induced stimulation of the HPA axis (Beishuizen, Thijs 2003). Thus, some studies reported direct stimulatory effects of LPS on adrenocortical cells and cells of the pituitary, while others did not observe any effects of LPS at these levels (Beishuizen, Thijs 2003).

In addition to cytokines, also other immune-related mediators such as prostaglandins and nitric oxide (NO) are able to modulate the HPA axis (Beishuizen, Thijs 2003, Zimomra et al. 2011).

On the one hand, the release of corticosteroids in the context of immune activation provides negative feedback on the immune system and thereby prevents hyperactivity of the immune system (Dunn 2000). On the other hand, the HPA axis plays a role with regard

to mood disorders as depressed patients consistently present with HPA axis hyperactivity (Pariante, Lightman 2008). A deficiency in feedback inhibition by glucocorticoids (glucocorticoid resistance) is thought to underlie the increase in HPA axis activity as the synthetic glucocorticoid dexamethasone is not able to suppress the HPA axis in depressed patients (Pariante, Lightman 2008). Thus inflammatory cytokines could be involved in the development of glucocorticoid resistance by disrupting glucocorticoid receptor signalling (Wang, Wu & Miller 2004).

1.2 PRRs, PAMPs, and the production of cytokines

Cells of the innate immune system are armed with pattern recognition receptors (PRRs), which are responsible for recognizing non-self molecular motifs. These motifs are conserved within classes of microbes and are referred to as MAMPs, or pathogen-associated molecular patterns (PAMPs) (Moreira, Zamboni 2012). After recognition of a PAMP, PPRs lead to the activation of the innate immune system, which initially leads to the production of pro-inflammatory cytokines (Akira, Uematsu & Takeuchi 2006). Besides, the innate immune system can be activated in the absence of a pathogen. In this case, damage-associated molecular pattern molecules (DAMPs), which are cellular proteins, getting released in response to injury and "danger", are also able to activate certain PRRs and induce an inflammatory response (Seong, Matzinger 2004).
Toll-like receptors (TLRs) and Nod-like receptors (NLRs) are among the most important PRRs and are distinguished from each other by their location within the cell, by the PAMPs they recognize and by the signalling pathways they activate (Akira, Uematsu & Takeuchi 2006).

1.2.1 LPS, the prototypical PAMP activating TLR4

LPS is an outer membrane component of Gram-negative bacteria and counts among the best studied immunostimulants (Lu, Yeh & Ohashi 2008). LPS consists of three components, the O side chain (1), a core oligosaccharide (2), and the main immune activating component lipid A (3) (Lu, Yeh & Ohashi 2008). In order to activate TLR4, LPS needs to interact with 3 proteins. Once in the bloodstream, LPS binds to an acute phase protein named LPS binding protein. Subsequently this complex associates with the cluster of differentiation 14, which is expressed on phagocytes. Finally, LPS is transferred to myeloid differentiation factor 2, a molecule that is associated with TLR4 and a prerequisite for TLR4 activation (Shimazu et al. 1999). Because of its ability to induce a strong

cytokine release, LPS has emerged as one of the most popular PAMPs to induce behavioural changes of sickness and depression-like behaviour (Bluthe et al. 2000a, Lestage et al. 2002, O'Connor et al. 2009b).

1.2.2 The TLR family with emphasis on TLR4

The TLR family consists of 12 known members that recognize various PAMPs and play important roles in the activation of the innate immune system. They are membrane glycoproteins, composed of leucine-rich-repeats (LRR) at the extracellular domain and a cytoplasmic signalling domain termed the Toll/IL-1R homology domain (Akira, Uematsu & Takeuchi 2006). Jules A. Hoffmann and Bruce A. Beutler were awarded the Nobel Prize in Physiology or Medicine in 2011 for their discoveries concerning the involvement of the Toll gene in fighting infections (Lemaitre et al. 1996) and for identifying TLR4 as the receptor recognizing LPS (Poltorak et al. 1998). In addition, a TLR4-independent sensing of LPS has been reported recently (Kayagaki et al. 2013).

Among the different TLRs, TLR4 is the only member which activates two distinct signalling pathways: the myeloid differentiation primary-response protein 88 (MyD88)-dependent and -independent pathway (Akira, Takeda 2004). Induction of the adaptor protein MyD88, among others, leads to the activation of transforming growth factor-β-activated kinase (TAK1). TAK1 then phosphorylates both mitogen-activated protein kinases (MAPK) and the inhibitor of nuclear factor-κB kinase (IKK) complex. The phosphorylation of the IKK complex in turn enables translocation of the transcription factor nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) to the nucleus. NF-κB plays a central role in cytokine production by controlling the transcription of genes encoding inflammatory cytokines (Vallabhapurapu, Karin 2009). In contrast, the MyD88-independent pathway activates the IFN-regulatory factor 3 and leads to a late phase NF-κB activation. The MyD88-independent pathway thereby leads to the production of IFN-β and the expression of IFN-inducible genes (Akira, Takeda 2004).

Apart from LPS, TLR4 recognizes several other ligands, such as viral components and DAMPs (e.g. heat shock proteins) (Akira, Uematsu & Takeuchi 2006). Interestingly, opioids such as morphine have also been demonstrated to interact with TLR4 signalling both in the nervous system and intestinal tract (Hutchinson et al. 2010, Meng et al. 2013, Farzi et al. 2015b).

TLR4 is expressed on the cell surface of various immune cells such as macrophages, neutrophils (Prince et al. 2011) and B-cells (Gerondakis, Grumont & Banerjee 2007). Nonimmune cells including endothelial (Raschi et al. 2014) and epithelial cells (Michelsen, Arditi 2007) express TLR4 as well. In addition, TLR4 has been shown to be expressed in cells of the nervous system, including microglia and neuronal cells (Tang et al. 2007, Okun, Griffioen & Mattson 2011). Importantly, TLR4 is also expressed in brain CVOs, structures surrounding the brain ventricles and lacking a functional BBB (Chakravarty, Herkenham 2005). In the intestinal tract, TLR4 is expressed on neurons of the myenteric and submucosal plexus (Rumio et al. 2006, Barajon et al. 2009) and to a small extent on the basolateral surface of intestinal epithelial cells (Kubinak, Round 2012). Interestingly, recognition of commensal microbes by TLRs within the intestine does not always result in inflammation and among others depends on which site of the cell TLR activation takes place (Kubinak, Round 2012). Thus, under physiological conditions, sensing of commensal bacteria by TLRs is important for maintaining colonic homeostasis. Furthermore, TLR4 signalling modulates intestinal motility and sublethal concentrations of LPS are frequently used to model sepsis-induced ileus (De Winter, De Man 2010).

1.3 NLRs - The nucleotide-binding domain and leucine-rich repeat containing receptor gene family

The NLR family is another group of receptors which play important roles in innate immunity. The members of the NLR family are characterized by the presence of a nucleotide-binding and oligomerization domain (NOD) and LRRs (Ting et al. 2008). In mice the NLR family includes 30 members, which are distinguishable by their N-terminal effector domains (Ting et al. 2008). These N-terminal domains have been used to divide the NLR gene family into five subfamilies:

1. NLRA, containing an acidic transactivation domain
2. NLRB, containing baculoviral inhibitory repeat-like domains
3. NLRC, containing a caspase recruitment domain
4. NLRP, containing a pyrin domain
5. NLRX, members with no strong homology to the N-terminal domain of any other NLR subfamily member (Ting et al. 2008).

1.3.1 NOD1 and NOD2

NOD1 (designation for human) / Nod1 (designation for mouse) and NOD2 / Nod2 belong to the NLRC subfamily (Ting et al. 2008). They represent the first members of the NLR family and were identified in the laboratory of Gabriel Nuñez about 15 years ago (Inohara et al. 1999, Ogura et al. 2001b). They represent one of the most studied NLRs and play important roles in pathogen recognition and the activation of the immune system (Moreira, Zamboni 2012). NOD1 and NOD2 function as intracellular receptors for bacterial peptidoglycan fragments, with NOD1 recognizing the dipeptide, γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) (Chamaillard et al. 2003, Girardin et al. 2003a) and NOD2 detecting muramyl dipeptide (MDP) (Girardin et al. 2003b, Grimes et al. 2012). Furthermore, NOD1 mainly senses products from Gram-negative bacteria, since most Gram-positive bacteria do not contain a peptidoglycan incorporating iE-DAP (Strober et al. 2006). However, specific Gram-positive bacteria such as *Listeria* and *Bacillus spp.* do contain iE-DAP and thus can be sensed by NOD1 (Strober et al. 2006). In contrast, MDP is present in all bacterial peptidoglycans, and thus NOD2 can be considered as a general sensor of both Gram-positive and Gram-negative bacteria (Strober et al. 2006). Interestingly, NOD2 is also involved in antiviral responses and has been demonstrated to bind virus-derived single-stranded RNA (Sabbah et al. 2009). Furthermore, MDP is involved in the activation of other NLRs, such as NACHT, LRR and PYD domains-containing protein 3 (NALP3) (Martinon et al. 2004) and NALP1 (Hsu et al. 2008).
The localization of NOD1 and NOD2 expression also varies significantly. Thus, NOD1 is more widely expressed than NOD2 and can be found in a wide variety of haematopoietic and non-haematopoietic cell types (Inohara et al. 1999). In contrast, NOD2 expression is rather restricted to haematopoietic cells (Ogura et al. 2001b). In addition, NOD2 expression can be found within cells of the gastrointestinal tract (Ogura et al. 2003).
NOD activation typically occurs in the course of an infection with an intracellular pathogen (Girardin et al. 2001). However, NOD activation can also take place without a cellular infection by a bacterial pathogen (Philpott et al. 2014).
The mechanisms underlying the internalization of iE-DAP or MDP and NOD activation when injected in the absence of a bacterial infection are not completely understood. In the human epithelial cell line HEK293T, however, it has been demonstrated that the NOD agonists enter cells through endocytosis in a clathrin-dependent manner (Lee et al. 2009). In addition, the plasma membrane transporter, hPepT1, has been demonstrated to

translocate MDP into colonic epithelial cells and oocytes from *Xenopus laevis* (Vavricka et al. 2004, Ismair et al. 2006). Whether similar mechanisms take place in innate immune cells has not been reported so far (Moreira, Zamboni 2012). Phagocytosis would be a likely process leading to internalization of NOD ligands in immune cells. However, a study has demonstrated that polymeric peptidoglycans are more effective in activating the NOD receptors than peptidoglycan monomers such as MDP (Iyer, Coggeshall 2011). These findings led the authors to conclude that innate immune cells are more efficient in recognizing peptidoglycan in its polymeric form. However, potential contaminations of the polymeric peptidoglycan with other MAMPs have been considered to have contributed to the stronger immunologic response (Philpott et al. 2014).

The activation of the signalling pathways downstream of NOD1 and NOD2 requires the adaptor protein receptor-interacting serine/threonine-protein kinase 2 (Park et al. 2007). This is followed by activation of the TAK1 complex leading to the translocation of NF-κB to the nucleus (Hasegawa et al. 2008) and initiation of MAPK-dependent gene transcription, which induce the expression of pro-inflammatory factors (Philpott et al. 2014).

NOD2 has especially drawn the interest of gastroenterologists since 2001 as it was the first gene associated with Crohn's disease (Hugot et al. 2001, Ogura et al. 2001a). Crohn's disease-associated mutations in NOD2 are loss-of-function mutations (Inohara et al. 2003) and NOD2 has been demonstrated to play a protective role in the development of intestinal inflammation (Philpott et al. 2014). Conversely, data mainly coming from animal models suggest that NOD1 or NOD2 activation may play detrimental roles in several other inflammatory diseases such as asthma, arthritis, insulin resistance and experimental autoimmune encephalitis, an animal model of multiple sclerosis (Philpott et al. 2014).

1.3.2 Crosstalk between NOD and TLR signalling

MDP is a synthetic peptide which came into focus when identified as an active component in Freund's complete adjuvant (FCA) (Ogawa, Liu & Kobayashi 2011). FCA comprises heat-killed mycobacterial components and is one of the strongest immunological adjuvants being able to boost the immune response. However, due to strong toxicity its use is rather limited to animal models. Therefore, MDP and its derivatives are regarded as promising adjuvants, which boost the immune response of the recipients (Ogawa, Liu & Kobayashi 2011).

Interestingly, monomeric muramyl peptide attracted the attention of sleep researchers, as they were isolated from rabbit brain and human urine and identified as a sleep regulatory substance called “Factor S” (Pappenheimer et al. 1975, Krueger, Bacsik & Garcia-Arraras 1980, Zielinski, Krueger 2011). As seen for the other behavioural effects, pro-inflammatory cytokines turned out to mediate the alteration of sleep by bacterial pathogens (Zielinski, Krueger 2011). In addition, there are some reports that MDP induces sleep and anorexia (Johannsen et al. 1990, Fosset et al. 2003, von Meyenburg et al. 2004).
However, while the immunostimulatory potency of MDP is rather weak, pre-treatment with MDP has been demonstrated to trigger a potentiated cytokine production in response to LPS (Parant et al. 1990, Le Contel et al. 1993, Parant et al. 1995, Wolfert et al. 2002, Netea et al. 2005). In fact, both NOD1 and NOD2 agonists are able to prime the LPS response of monocytic cells and dendritic cells *in vitro* (Chamaillard et al. 2003, Fritz et al. 2005, Uehara et al. 2005, Park et al. 2007). Similarly, priming with MDP enhances LPS-induced cytokine production as well as anaphylactoid reactions and lethality (Takada, Galanos 1987, Takada et al. 1990, Parant et al. 1995). Intriguingly, while the synthetic NOD1 agonist FK565 induces lower levels of pro-inflammatory cytokines when compared to LPS, it leads to NO synthase-II activity, vascular hypotension and septic shock *in vivo* (Cartwright et al. 2007).
With regard to behavioural alterations, some reports demonstrate a slight MDP-induced attenuation of food intake and locomotion in rats, and aggravation of the LPS-induced decrease of locomotion and food intake by pre-treatment with MDP (Langhans et al. 1990, Engeland, Kavaliers & Ossenkopp 2003). In contrast, the effects of NOD1 activation with or without TLR4 activation on mood and behaviour have remained unexplored.
The mechanism of the enhancing effect of NLRs on TLRs is not fully understood. However, changes in the expression of key components in the respective signal-transduction pathways are likely to contribute to the synergistic effects (Strober et al. 2006). Thus, LPS upregulates the expression of NOD1 and NOD2 (Gutierrez et al. 2002, Takahashi et al. 2006) and, in turn, MDP is able to upregulate the expression of MyD88 (Yang et al. 2001). In addition, MDP was demonstrated to stimulate the production of pro-IL-1β (but not mature IL-1β) in macrophages and downregulate the expression of a negative-feedback regulator of TLR4 signalling, termed suppressor of cytokine signalling 1 (Shikama et al. 2011).
In contrast, the interaction of NOD2 and TLR4 in the colon is of a different nature. Here NOD2 stimulation reduces TLR-dependent cytokine production and attenuates

experimental colitis (Watanabe et al. 2008). Downregulation of the responses to TLR activation by MDP in the colon have further been demonstrated to be due to activation of IFN-regulatory factor 4, an inhibitor of TLR signalling (Watanabe et al. 2008).

1.4 Potential sources of inflammation in the absence of infection – stress and microbiota-gut-brain axis

Stress is one of the major risk factors contributing to major depression (Felger, Lotrich 2013). Intriguingly, both physical and psychological stressors are able to induce the release of pro-inflammatory cytokines and thereby induce behavioural alterations (Koo, Duman 2008). Interestingly, stress-induced inflammatory responses of depressed patients have been demonstrated to be exaggerated, especially in those who experienced early life stress (Pace et al. 2006). One mechanism whereby stress might translate into immune activation could be ‘‘sterile inflammation’’. Thus, DAMPs such as heat shock protein-72, uric acid, and ATP are thought to be released during stress and to activate various PRRs including TLRs and NLRs entailing cytokine production (Fleshner 2013).

Another potential mechanism might be stress-induced disruption of intestinal tight junctions leading to increased translocation of the gut microbiota or bacterial components, and thereby inducing an immune response. Indeed, a probiotic treatment has been reported to prevent stress-induced increases of intestinal permeability and circulating LPS and CORT levels (Ait-Belgnaoui et al. 2012).

In fact, the intestinal microbiota is increasingly attracting the interest of neurobiologists, neurogastroenterologists as well as psychiatrists (Cryan, Dinan 2012, Mayer et al. 2014). The potential influence of the microbiota on the human organism is already highlighted by its vast amount. Thus, the number of the microorganisms inhabiting the human gastrointestinal tract is estimated to be 10 times that of the number of human cells in our bodies and the gut microbiota consists of more than 1000 species (Qin et al. 2010). Increasing evidence demonstrates that the intestinal microbiota is relevant to immune function, digestion, metabolism and even brain function and behaviour(Lee, Mazmanian 2010, Delzenne et al. 2011, Cryan, Dinan 2012). Thus, the gut microbiota is required for a proper development of the immune system and “educates” both the mucosal and the systemic immune system (Shanahan 2013).

Importantly, a study by Clarke et al. (Clarke et al. 2010) demonstrated that peptidoglycan translocates from the gut microbiota to the systemic circulation and bone marrow. Furthermore gut derived peptidoglycan systemically primed the innate immune system in a

NOD1 (but not NOD2 or TLR4) dependent way and was required for proper neutrophil function (Clarke et al. 2010).

Studies examining the behavioural phenotype of mice reared in a germ-free environment provided evidence for the impact of the intestinal microbiota on brain function and behaviour. Thus, while gastrointestinal infection with a pathogen induces anxiety (Goehler et al. 2008), germ-free mice are less anxious and show higher locomotion than mice who harbour intestinal microbiota (Diaz Heijtz et al. 2011). In addition, germ-free mice showed a higher turnover of neurotransmitters including serotonin. Another study reported defective memory and learning in germ-free mice together with decreased expression of brain-derived neurotrophic factor (BDNF) in the hippocampus (Gareau et al. 2011). Furthermore, manipulation of the gut microbiota of mice by oral antibiotics decreased anxiety-like behaviour and modulated central BDNF expression independent of changes in circulating inflammatory cytokines, gastrointestinal neurotransmitters or integrity of the vagus nerve (Bercik et al. 2011a). Finally, probiotics have been demonstrated to exert beneficial effects on brain function and behaviour which are proposed to be mediated by the vagus nerve (Bercik et al. 2011b, Bravo et al. 2011).

1.5 Aims of the project

Both depression and anxiety disorders are often associated with increased biomarkers of inflammation, especially pro-inflammatory cytokines (Capuron, Miller 2011). These cytokines can be released in the context of infection and medical conditions which are associated with inflammation, but also tissue injury and stress. Furthermore, the colonization of our intestinal tract by 10^{13}–10^{14} commensal microbes, referred to as the intestinal microbiota, is gaining importance as an important regulator of innate and adaptive immunity within and outside the intestine. As an example, lack of microbial homeostasis has been proposed to induce changes in gastrointestinal barrier function, which leads to the translocation of bacterial cell wall components into the bloodstream causing systemic immune activation (Maranduba et al. 2015). Thus a high fat Western-style diet has been demonstrated to induce an increase in plasma levels of the TLR4 agonist LPS, also termed endotoxemia (Pendyala, Walker & Holt 2012). In addition, changes in the composition of the gut microbiota, gut barrier dysfunction and endotoxemia are increasingly recognized to contribute to the metabolic syndrome (e.g., obesity and type 2 diabetes), a disorder also associated with inflammation (Everard, Cani 2013). Interestingly, not only LPS but also peptidoglycan has been demonstrated to be able to

cross the intestinal barrier and activate neutrophils via NOD1 activation (Clarke et al. 2010).

While peripheral administration of LPS is frequently used to induce behavioural changes in animal models (Dantzer et al. 2008), the consequences of NOD activation on brain function and behaviour have been little studied.

Likewise, while combined NOD and TLR4 activation induces a potentiated immune response, it is largely unknown whether this interaction at the immune level has a bearing on behaviour and related parameters. Since NLRs and TLRs may be activated in parallel in several contexts, it was the primary aim of this work to examine the effects of NOD1 and NOD2 activation, alone and in combination with the TLR4 agonist LPS, on sickness, anxiety- and depression-like behaviour. Furthermore, cerebral c-Fos expression was assessed in order to visualize some of the brain nuclei relevant to the observed effects.

In order to analyse potential mechanisms behind any synergistic effects of NOD and TLR4 activation on mood and behaviour, inflammatory indices such as peripheral and central cytokine production, circulating CORT and the plasma kynurenine/tryptophan ratio were assessed. Furthermore colonic MPO levels were measured as an inflammatory marker and the number of boli expelled were regarded as a measure of intestinal motility.

Secondly, as the NPY family has been demonstrated to attenuate the (behavioural) effects of immune challenge, a further aim of this work was to assess the effects of NOD2 and TLR4 activation in $NPY^{-/-}$;$PYY^{-/-}$ double KO mice.

Finally, as the gut microbiota might be a potential source of various MAMPs, in an inverted approach, the third aim of this work was to assess the behavioural effects of modulation of the intestinal microbiota by non-absorbable oral antibiotics. To address this issue, the effects of antibiotics with target specificity (e.g. disrupting Gram-negative or Gram-positive bacteria), broad spectrum antibiotics and different antibiotic combinations were studied. Additional gut-related parameters (colonic microbiota, MPO, PYY) were examined for the antibiotic combination showing the most pronounced behavioural effects.

2 Materials and Methods

The description of Materials and Methods is partly reused from Farzi et al. (2015a) with modifications.

2.1 Experimental animals

Animal experiments were performed with adult C57BL/6N mice obtained from Charles River Laboratories at the age of 8 weeks (Sulzfeld, Germany). $NPY^{-/-}$;$PYY^{-/-}$ double KO mice on a mixed C57BL/6:129/SvJ (1:1) and their respective controls were generated at the Neurobiology Research Program of the Garvan Institute of Medical Research (Sydney, Australia) (Zhang et al. 2012) and bred at the institutional animal facility. Temperature (set point 21^{o}C) and relative air humidity (set point 50 %) were controlled tightly with a 12 h light/dark cycle (lights on at 6:00 h, lights off at 18:00 h). Standard laboratory chow and tap water were provided ad libitum throughout the study.

2.2 Ethics statement

The experiments were approved by an ethical committee at the Federal Ministry of Science and Research of the Republic of Austria (BMWF-66.010/ 0119-II/3b/2011 and BMWF-66.010/0026-II/3b/2014) and conducted according to the Directive of the European Communities Council of 24 November 1986 (86/609/EEC) and 22 September 2010 (2010/63/EU). The experiments were designed in such a way that the number of animals used and their suffering was minimized.

2.3 Reagents

M-TriDAP (MurNAc-L-Ala-gamma-D-Glu-mDAP, catalogue number tlrl-mtd, InvivoGen/Eubio, Vienna, Austria) was used as a synthetic NOD1/NOD2 agonist.
iE-DAP (γ-D-Glu-mDAP, catalogue number tlrl-dap, InvivoGen/Eubio) was used as a synthetic NOD1 agonist.
The chemically synthesized NOD1 agonist **FK565** was provided by Astellas Pharma Inc. (Ibaraki, Japan) (Watanabe et al. 1985).
MDP (N-acetylmuramyl-L-alanyl-D-isoglutamine hydrate, catalogue number A9519, Sigma–Aldrich, Vienna, Austria) was used as synthetic NOD2 agonist.
LPS extracted from Escherichia coli 0127:B8 (purified by gel-filtration chromatography, catalogue number L3137, Sigma–Aldrich) was used as a TLR4 agonist.

Ampicillin sodium salt (catalogue number A9518-5G, Sigma–Aldrich) was used as a ß-lactam antibiotic against both Gram-positive and Gram-negative bacteria.
Cefoperazone (Cefobid, Pfizer Pharmaceuticals New York, NY, USA) was used as a broad spectrum cephalosporin antibiotic.
Bacitracin (catalogue number 11702-5G, Sigma–Aldrich) was used to disrupt Gram-positive bacterial cell wall synthesis at the level of peptidoglycan.
Meropenem (Optinem, AstraZeneca, Vienna, Austria) is an ultra-broad-spectrum antibiotic belonging to the subgroup of carbapenems.
Metronidazole (catalogue number M1547-5G, Sigma–Aldrich) is a nitroimidazole antibiotic disrupting anaerobic bacteria.
Neomycin trisulfate salt hydrate (catalogue number N5285-25G, Sigma–Aldrich) is an aminoglycoside antibiotic with activity against Gram-negative bacteria, and to a lesser extent against Gram-positive bacteria.
Vancomycin hydrochloride from Streptomyces orientalis (catalogue number 94747-250MG, Sigma–Aldrich) was used as a glycopeptide antibiotic mostly effective against Gram-positive bacteria.

2.4 Behavioural tests

For all behavioural experiments mice were allowed to habituate to the animal facility for at least 2 weeks before starting the experiments. Prior to the behavioural tests, the mice were allowed to adapt to the test room for at least one day. Except for the experiments performed with the LabMaster system, the movements of the mice during the test period were tracked by a video camera and recorded and analysed with the VideoMot2 software (TSE Systems, Bad Homburg, Germany).

2.4.1 LabMaster and sucrose preference test

The pattern of locomotion, exploration, feeding as well as SP were assessed with the LabMaster system (TSE Systems, Bad Homburg, Germany), allowing continuous recording of the animals without intervention by any investigator (Painsipp et al. 2013). The LabMaster system consists of six recording units, each unit comprising a test cage (type III, 42.0 × 26.5 × 15.0 cm, length × width × height), two external infrared frames and a cage lid fitted with three weight transducers (Figure 2). These devices are connected to a personal computer in order to collect and analyse the data with the LabMaster software.

For recording locomotion and exploration, the two external infrared frames were positioned in a horizontal manner above one another at a distance of 4.3 cm, with the lower frame being fixed 2.0 cm above the bedding floor. The bottom frame was used to record horizontal locomotion of the mice, whereas the top frame served to record vertical movements (rearing, exploration). The measures of activity (locomotion, exploration) were derived from the light beam interruptions (counts) of the corresponding infrared frames. The three weight transducers were employed to quantify ingestive behaviour. To this end, a feeding bin was filled with standard rodent chow (Altromin 1324 FORTI, Altromin, Lage, Germany). The system was configured in such a way that 1440 values of each test parameter were collected over a 24 h interval. In order to assess SP, one drinking bottle was filled with tap water and one with a 1 % sucrose solution and the bottles were each attached to a transducer on the cage lid for the total duration of the experiment. SP was calculated using the formula: sucrose intake / (sucrose intake + water intake). In a few cases in which the fluid bottles got obstructed, the data were excluded from analysis. Activity scores and food intake recorded during the day before injection were set as 100 %, and the daily scores measured post-injection were expressed as a percentage of the pre-injection score. This methodological description was also published in a similar fashion in an original article (Farzi et al. 2015a).

Figure 2 Setup of the LabMaster system

2.4.2 Open field test

The open field (OF) consisted of a box (50 × 50 × 30 cm) made of opaque grey plastic and was illuminated by 35 lux at floor level (Painsipp et al. 2013). The ground area of the box was divided into a 36 × 36 cm central area and the surrounding border zone. Mice were

individually placed in the centre of the OF, and their behaviour during a 5-min test period was tracked and analysed (Figure 3). This methodological description was also published in an original article (Farzi et al. 2015a).

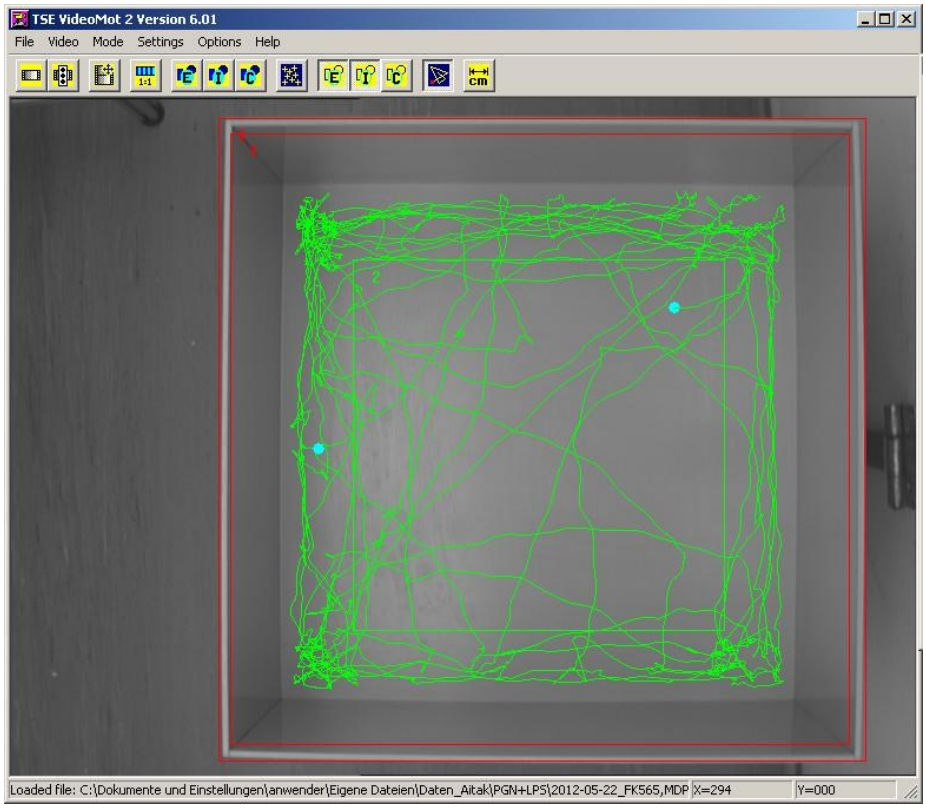

Figure 3 VideoMot software with representative tracks of movement patterns in the OF

2.4.3 Elevated plus-maze test

The animals were placed in the centre of a maze with four arms arranged in the shape of a plus. The maze consisted of a central quadrangle (5 × 5 cm), two opposing open arms (30 cm long and 5 cm wide) and two opposing closed arms of the same size but equipped with 15-cm high walls at their sides and the far end (Figure 4) (Painsipp et al. 2010). The device was made of opaque grey plastic and elevated to 55 cm above the floor. The light intensity was 20, 30, and 5 Lux at the central quadrangle, on the open arms, and in the closed arms, respectively. At the beginning of each trial, the animals were placed on the central quadrangle facing an open arm. The movements of the animals during a 5-min test period were tracked and analysed. The number of entries into the open and closed arms, the time spent on the open and closed arms, and the total distance travelled in the open and closed arms during the test session were determined. Entry into an arm was defined as the instance when the centre of the body of the mouse crossed the border to the arm. Locomotion was quantified by measuring the total distance travelled in the open and

closed arms and the total number of entries into any arm during the 5-min test session. Anxiety-related behaviour was deduced from the time spent on the open arms and the number of entries into the open arms. This methodological description was also published in an original article (Brunner et al. 2014).

Figure 4 Top down view on the EPM with mouse exploring the open arm

2.4.4 Forced swim test

Mice were individually placed in glass beakers (inner diameter 18 cm, height 27 cm, capacity 5 l) containing tap water at 25 ºC (Painsipp et al. 2011). The water depth was 20 cm, which prevented the mice from touching the bottom of the beaker with their paws or the tail. Mice were tested for 6 min and the time of immobility, swimming and climbing was scored by a trained observer blind to the treatment. Mice were considered immobile, when floating passively in the water performing only those movements required to keep their heads above the water level (Cryan, Markou & Lucki 2002). This methodological description was also published in an original article (Farzi et al. 2015a).

2.4.5 Tail suspension test

Mice were suspended by their tail with a 1.9 cm wide strapping tape (Leukotape classic; BSN Medical S.A.S., Le Mans, France) to a lever for 6 min, and their behaviour was recorded by a video camera (Hassan et al. 2014). A trained blinded observer analysed the video recordings with the VideoMot2 software (TSE Systems) event monitoring module for 3 types of behaviour: swinging, curling and immobility (Berrocoso et al. 2013). The mouse was considered swinging when it continuously moved its paws while keeping the body straight and/or moving the body from side to side. The mouse was considered curling when the mouse twisted its trunk. The time spent swinging, curling and being immobile

was calculated. Mice which climbed over their tails were excluded as they had learnt that escape is possible (Cryan, Mombereau & Vassout 2005). This methodological description was also published in an original article (Farzi et al. 2015a).

2.4.6 Novel object recognition test

The novel object recognition test (NORT) was performed in the OF box as described for the open field test (OFT).

In **experiment 6.3**, mice were habituated to the OF for 5 min on the day before the NORT. On the test day, two identical objects were placed on the centreline of the OF, 9 cm from each box end (Redrobe et al. 2004, Painsipp et al. 2008). The animals were allowed to explore the two objects for 5 min during which the exploratory activity directed at each object was tracked and recorded. After a delay of 15 min, the animals were re-exposed to one familiar object together with a novel object not used in the acquisition phase, and the exploratory behaviour directed at each object recorded during another 5-min test period.

In **experiment 6.4**, mice were habituated to the OF each day during 3 consecutive days. On the fourth day mice were allowed to explore two identical objects for 5 min and after a delay of 70 min the animals were re-exposed to one familiar object together with a novel object.

The objects to be discriminated were a rough metal tube (outer diameter 3.1 cm, inner diameter 1.9 cm, length 3.8 cm) and a rough Teflon column (3.2 × 2.9 × 5.8 cm, length × width × height) with a hole (diameter 1.3 cm) at the upper half. The position of each object was alternated between the trials, and the object chosen to be familiar and novel was changed from mouse to mouse. Exploration of the objects was tracked by a blinded examiner. The performance of each mouse was expressed by the memory index (MI) which was calculated according to the formula $MI = (t_{new} - t_{old}) / (t_{new} + t_{old})$, where t_{old} represents the time exploring the familiar object and t_{new} represents the time exploring the novel object (Redrobe et al. 2004, Painsipp et al. 2008).

2.5 Experimental protocols

Six different protocols were used to address the questions under study. Protocol 1 – 4 aimed at examining the effects of NOD and TLR4 agonists on behaviour (Figure 5). Protocol 5 investigated the effects of NOD2 and TLR4 activation on behaviour of $NPY^{-/-}$;$PYY^{-/-}$ double KO mice. Protocol 6 set out to analyse the effects of dysbiosis of the intestinal microbiota on rodent behaviour.

2.5.1 Protocol 1 - Short and long term effects of NOD agonists

The aim of protocol 1 was to evaluate short and long term effects of the NOD1/NOD2 agonist M-TriDAP and the NOD2 agonist MDP on body temperature, body weight, locomotion, anxiety-like behaviour, as well as depression like behaviour. Three separate experiments with different cohorts of group-housed female mice were carried out (Figure 5). Female mice were used in this set of experiments as affective disorders are more prevalent in women than in men (Gorman 2006).

The first experiment (experiment 1.1) analysed the effects of 5 mg/kg of M-TriDAP administered i.p. (starting at 9:00 am) on body temperature (5 h post-treatment), body weight (1 and 7 days after treatment), behaviour in the OFT (starting at 8:00 am) and FST (starting at 10:30 am) 1 day after treatment and elevated plus-maze test (EPM) (starting at 9:00 am) 7 days after treatment (n = 7).

In the 2nd experiment (experiment 1.2) 10 mg/kg of MDP was administered i.p. (starting at 10:30 am) and body temperature was assessed 5 h and 2 days after treatment. Body weight was monitored before the injection, as well as 1 day and 7 days after injection. One day after treatment locomotion was assessed with the OFT (starting at 8:00 am) and depression-related (stress coping) behaviour with the FST (starting at 10:30 am), while exploratory and anxiety-like behaviour was evaluated with the EPM (starting at 9:00 am) 7 days after the treatment (n = 8).

In the 3rd experiment (experiment 1.3) mice were injected with a higher dose of 30 mg/kg of MDP (starting at 9:30 am). Body temperature was assessed after 5 h and body weight was measured before as well as 1 and 7 days after the treatment. The OFT was conducted 1 day after injection (starting at 9:00 am), while the EPM (starting at 8:30 am) and FST (starting at 11:00 am) were performed 7 days after treatment (n = 6).

2.5.2 Protocol 2 - Effects of NOD agonists and LPS on circadian behaviour

Protocol 2 (**experiment 2.1**) was used to compare the effects of NOD1 and NOD2 activation alone with the combined activation of NOD1 or NOD2 and TLR4. The LabMaster system (TSE Systems) was employed to analyse the effects of MDP (1 mg/kg), FK565 (0.001 mg/kg), LPS (0.1 mg/kg), MDP + LPS and FK565 + LPS on the daily pattern of locomotion, exploration, feeding and SP in singly-housed male mice (Painsipp et al. 2013). Male mice were used in this and the following protocols, as female rodents are sensitive to social isolation (Martin, Brown 2010).

The animals were habituated to the drinking bottles used in the LabMaster system and to single housing for 7 days before placing them in the cages of the LabMaster system (Figure 5). Another 3 days of habituation were provided in the test cages of the LabMaster system before injection of PRR agonists (n = 8). For details on the choice of dosing and timing of injections see Sections 2.6 “Dosing” and 2.7 “Timing of injections”.

2.5.3 Protocol 3 - Effects of NOD agonists and LPS (0.83 mg/kg) on sickness and c-Fos expression

Protocol 3 was used to carry out 2 separate experiments (Figure 5).

Experiment 3.1 (experiment 1 of protocol 3) was designed to investigate the effects of MDP (3 mg/kg), FK565 (0.003 mg/kg), and the frequently used dose of LPS (0.83 mg/kg), as well as of MDP + LPS and FK565 + LPS on the sickness response. For this purpose body temperature and weight were measured immediately before treatment and body temperature was measured again 4 h post-injection. An additional measurement of body weight was taken 21 h post-injection after the animals had been subjected to the OFT (n = 8).

After euthanasia on day 3 post-treatment, full-thickness pieces of the distal colon were excised, shock-frozen in liquid nitrogen and stored at -70°C until the measurement of myeloperoxidase (MPO) content.

In experiment 3.2 mice were euthanized 3 h after injection of PRR agonists (Figure 5) and the brains were collected for immunohistochemical visualization of c-Fos expression in select brain regions (n = 3–5). Following euthanasia the brains were removed, put on dry ice and stored at -70°C until use.

2.5.4 Protocol 4 - Effects of NOD agonists and LPS (0.1 mg/kg) on mood and mood related parameters

Protocol 4 was used in 3 separate experiments (Figure 5) in which the effects of MDP and FK565 in combination with the lower dose of LPS (0.1 mg/kg) were investigated.

In experiment 4.1 body temperature and weight were measured before treatment and the body temperature was measured again 4 h post-injection. The OFT was conducted 21 h after the treatment and body weight was measured after the OFT. Subsequently the animals were subjected to the TST for 6 min (25.5 h post-injection) and euthanized 30 min after start of the TST. Blood was sampled to measure the plasma levels of cytokines, CORT, kynurenine and tryptophan (Figure 5). In addition, the brains were collected, frozen in -

70°C cold 2-methyl butane (Fisher Scientific, Leicestershire, UK) and stored at this temperature until measurement of cytokines (n = 7–8).

Furthermore full-thickness pieces of the distal colon were excised, shock-frozen in liquid nitrogen and stored at -70°C until the measurement of MPO content.

In experiment 4.2 singly-housed mice were subjected to the FST 21 h post-injection, since depression-like behaviour has been shown to be modified by different housing conditions (Painsipp et al. 2011) (n = 7–8).

In a further experiment **(experiment 4.3)** group-housed mice were euthanized 3 h after injection of PRR agonists to record the levels of circulating and brain cytokines and circulating CORT without interference by any behavioural test (n = 7–8). This methodological description was also published in an original article (Farzi et al. 2015a).

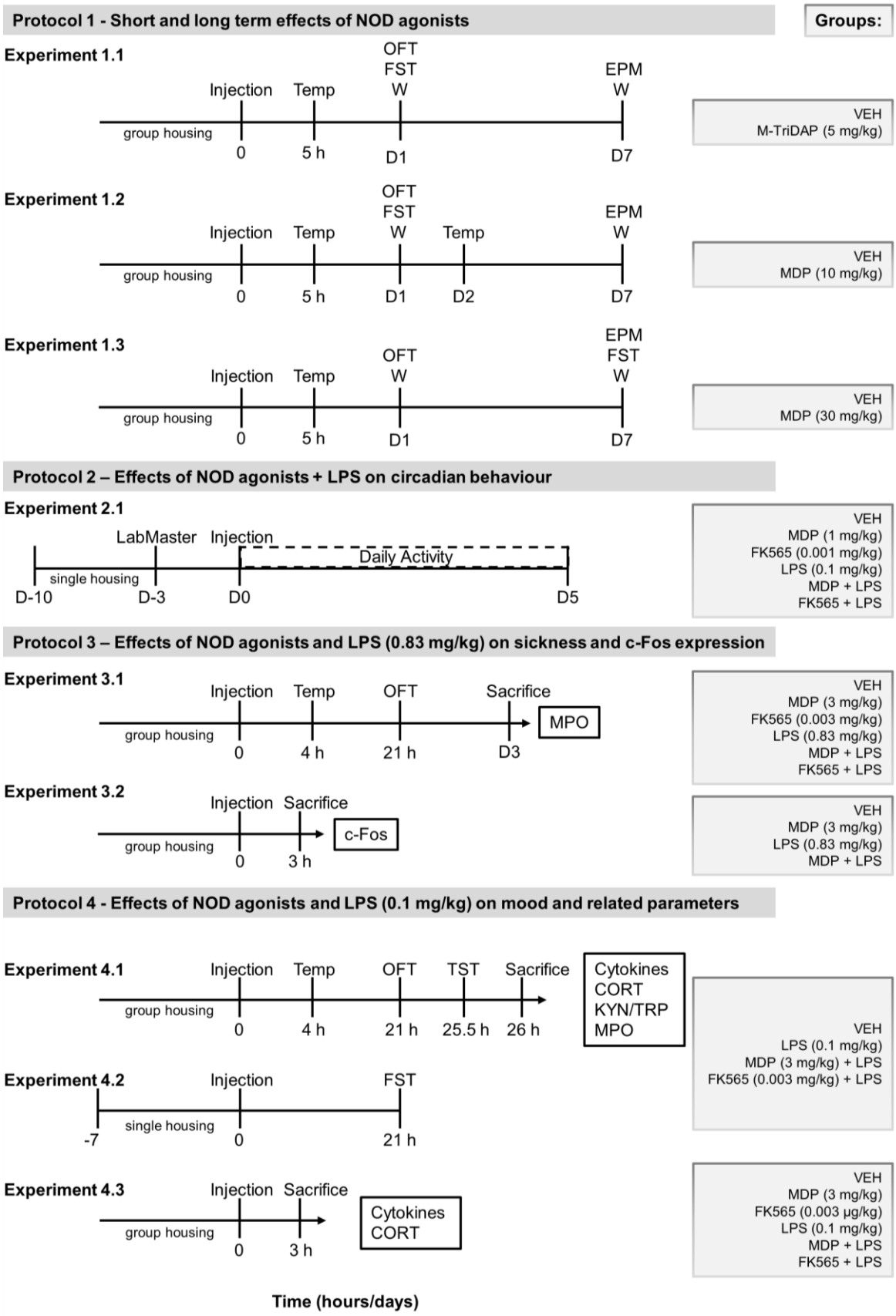

Figure 5 Experimental groups and time lines of the protocols examining the effects of NOD and TLR4 activation in C57BL/6N mice. In protocol 1, short- and long-term effects of NOD1 and/or NOD2 activation was assessed with regard to sickness and affective behaviour. In protocol 2, the effects of MDP and FK565

with or without LPS on daily activity were assessed in the LabMaster system. In protocol 3, the effects of MDP and FK565 alone and the combination of MDP or FK565 with the higher dose of LPS (0.83 mg/kg) on sickness and central c-Fos expression were evaluated. In protocol 4, the effects of MDP and FK565 alone and the combination of MDP or FK565 with the lower dose of LPS (0.1 mg/kg) on sickness, mood and inflammation-related parameters were analysed. Time zero represents the time of injection. Abbreviations: CORT = corticosterone, D = day, EPM = elevated plus maze, FST = forced swim test, h = hour, KYN/TRP = kynurenine/tryptophan ratio, MPO = myeloperoxidase, OFT = open field test, Temp = temperature, TST = tail suspension test. A similar figure has also been published in an original article (Farzi et al. 2015a).

2.5.5 Protocol 5 – Involvement of NPY and PYY

Protocol 5 was used to study the impact of KO of the neuroendocrine peptide NPY and the gut hormone PYY on the effects of immune challenge by MDP and LPS on the sickness response and behaviour. For this purpose adult male WT and $NPY^{-/-}$;$PYY^{-/-}$ double KO mice on a mixed C57BL/6:129/SvJ (1:1) background received i.p. injections of MDP (3 mg/kg) (starting at 8:00 am) or sterile saline and additional injections of LPS (0.83 mg/kg) 4 h after the first injection.

Body temperature was assessed 7 h as well as 1 day (starting at 4:45 pm) after treatment. Body weight was measured 1 and 2 days after treatment. Locomotor and anxiety-related behaviour was evaluated with the OFT 3 h after treatment, while depression-like behaviour was evaluated with the FST 1 day after treatment (starting at 10:00 am) (n = 8).

2.5.6 Protocol 6 - Effects of intestinal microbiota modification with antibiotics on murine behaviour

Protocol 6 was used to study the effects of intestinal microbiota modification on murine behaviour. For this purpose 4 different experiments were performed with different cohorts of mice. Male C57BL/6N mice were treated with the antibiotics administered via the drinking water (Figure 6). Control mice received drinking water without antibiotics. The water bottles were changed every 2 – 3 days, their initial weight and their weight upon change being determined in order to evaluate fluid intake. Fluid intake is expressed as a percentage of the initial bottle weight, divided by the number of mice per cage.

2.5.6.1 Treatment groups of Protocol 6

Experiment 6.1:

- Group 1: Neomycin (5 mg/ml) (to disrupt Gram-negative microbiota)
- Group 2: Vancomycin (1 mg/ml) (to disrupt Gram-positive microbiota)

E**xperiment 6.2**:

- Group 1: Ampicillin (2 mg/ml) (to disrupt Gram-positive microbiota)
- Group 2: Metronidazole (2 mg/ml) (to disrupt anaerobic microbiota)
- Group3: Ampicillin (1 mg/ml), metronidazole (1 mg/ml), neomycin (1 mg/ml), and vancomycin (0.5 mg/ml)

Experiment 6.3:

- Group 1: Cefoperazone (1 mg/ml) (broad spectrum)
- Group 2: Neomycin (10 mg/ml)
- Group 3: Ampicillin (2 mg/ml), cefoperazone (0.5 mg/ml), neomycin (5 mg/ml)

Experiment 6.4: Ampicillin (2 mg/ml), bacitracin (5 mg/ml), meropenem (1 mg/ml), neomycin (5 mg/ml), vancomycin (0.3 mg/ml)

2.5.6.2 Readouts of Protocol 6

The time points and behavioural tests applied are given in Figure 6. In general, anxiety-related behaviour was assessed by the OFT and/or EPM test. Depression-like behaviour was assessed with the FST or TST. Learning and memory were assessed by the NORT. The weight of the animals was assessed before and after antibiotic treatment. Additionally, spleen and caecum weight were determined after sacrifice. In experiment 6.4., blood was sampled to measure the plasma levels of PYY. Additionally full-thickness pieces of the distal colon were excised, shock-frozen in liquid nitrogen and stored at -70°C until the measurement of MPO content. Furthermore a piece of the colon including luminal content was stored at -70°C until analysis of the colonic microbiota.

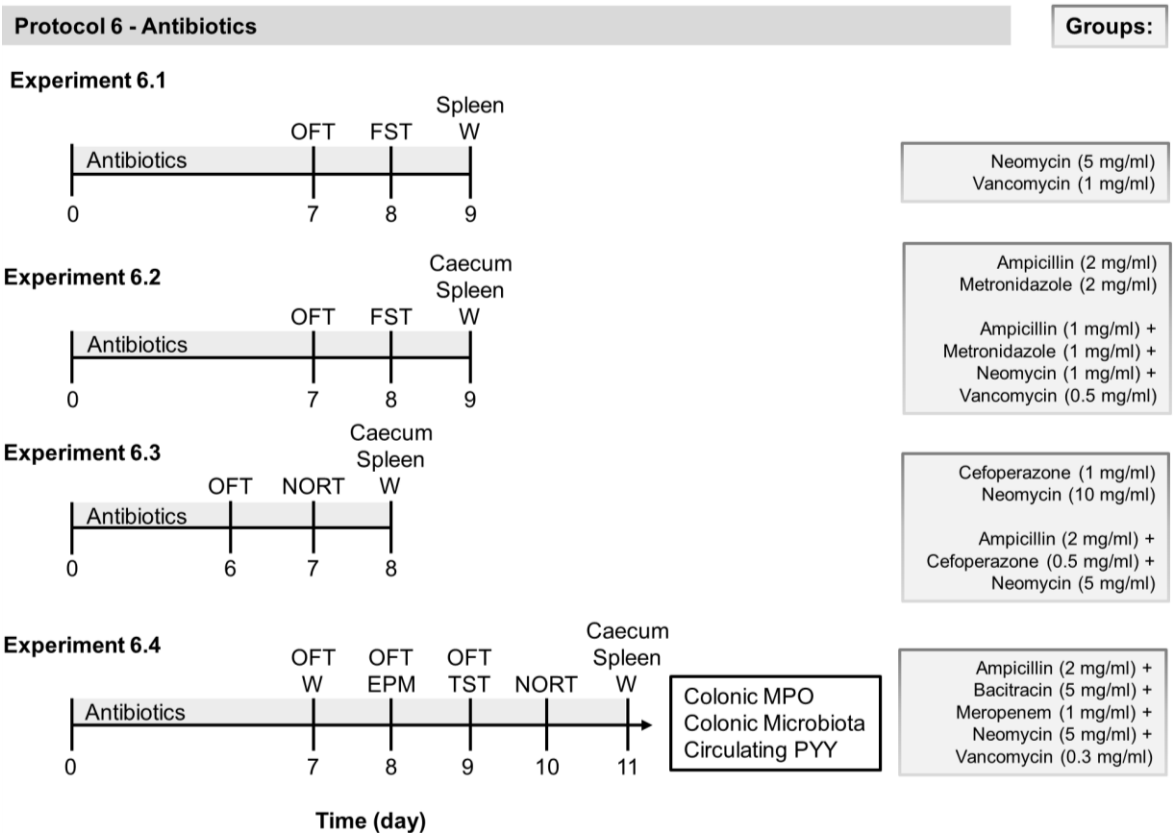

Figure 6 Treatment groups and time lines of the experiments examining the effects of dysbiosis of the intestinal microbiota on behaviour of male C57BL/6N mice.

The time points of the different readouts are outlined in the left scheme. The treatment groups of the different experiments are given in the boxes on the right side. Abbreviations: EPM = elevated plus maze, FST = forced swim test, MPO = myeloperoxidase, NORT = novel object recognition test, OFT = open field test, TST = tail suspension test, W = weight.

2.6 Dosing

All compounds injected i.p. were dissolved in pyrogen-free sterile saline (0.9 % NaCl) and pyrogen-free sterile saline at the same volume (50 µl/10 g body weight) was used as vehicle (VEH) control.

To the best of my knowledge, M-Tri DAP (5 mg/kg) has not been used in experiments *in vivo,* however a related NOD1 agonist (iE-DAP) has been used in comparable doses (Tukhvatulin et al. 2011).

For the analysis of the interaction between NOD agonists and LPS, two doses of LPS were examined. First, the widely used dose of 0.83 mg/kg LPS inducing the full spectrum of sickness (Frenois et al. 2007, Painsipp et al. 2011) was used. Since, in combination with the NOD agonists, this dose of LPS led to a marked decrease in body temperature and locomotion, while a ceiling effect was observed with other parameters, a lower dose of 0.1

mg/kg LPS was also tested. The doses of the NOD agonists were chosen on the basis of their immunological effects *in vivo* (Parant et al. 1995, Shikama et al. 2011) and the results of pilot experiments. Thus, doses of 1 mg/kg (LabMaster studies) and 3 mg/kg (ex LabMaster studies) of MDP and 0.001 mg/kg (LabMaster studies) and 0.003 mg/kg (ex LabMaster studies) of FK565 were used in order to minimize adverse event rates when combined with the 0.83 mg/kg dose of LPS. Lower doses of the PRR agonists were administered in the LabMaster system because pilot experiments had shown that mice kept singly in the LabMaster system were more sensitive to the treatments than group-housed animals. This methodological description was also published in an original article (Farzi et al. 2015a).

The doses of neomycin (1-10 mg/ml) and bacitracin (5 mg/ml) were based on the study of Bercik et al. (Bercik et al. 2011a). The doses of ampicillin (1-2 mg/ml), vancomycin (0.3-1 mg/ml) and metronidazole (1-2 mg/ml) were based on the study of Bereswill et al. (Bereswill et al. 2011). The doses of cefoperazone (0.5-1 mg/ml) were based on the study of Reeves et al. (Reeves et al. 2011), while the chosen dose of meropenem (1 mg/ml) was higher than the doses used in other studies (Moller et al. 2005, Gadjeva et al. 2010).

2.7 Timing of injections

Mice receiving just one of the compounds (MDP, FK565 or LPS) were first injected with saline followed by the respective compound 4 h later. The first injection was given 3 h after start of the light phase. In experiments involving combination treatments, MDP + LPS and FK565 + LPS were given with a time lag of 4 h between injection of the NOD and TLR agonist, since this timing has been shown to have the strongest priming effect on the immune system (Takada, Galanos 1987, Takada et al. 1990). Sickness responses were examined 3–4 h after injection and depression-like behaviour 21–26 h post-treatment. The time points for the recording of the sickness responses were chosen according to the known time course of the sickness response to MDP or LPS (Engeland, Kavaliers & Ossenkopp 2003, Frenois et al. 2007). Sickness behaviour has been shown to pass into depression-like behaviour 1 day after injection of LPS (0.83 mg/kg), which was the reason for choosing the second time point (Frenois et al. 2007). Since MDP or FK565 alone did not induce behavioural changes outside the LabMaster and only induced a modest cytokine response 3 h post-treatment, the single treatment with MDP or KF565 was not investigated in experiments 4.1 and 4.2. This methodological description was also published in an original article (Farzi et al. 2015a).

2.8 Body temperature

The temperature of the mice was measured with a digital thermometer (BAT-12, Physitemp Instruments, Clifton, New Jersey, USA) equipped with a rectal probe for mice. This methodological description was also published in an original article (Farzi et al. 2015a).

2.9 Blood sampling

Mice were deeply anaesthetized with pentobarbital (150 mg/kg i.p.). Blood was sampled by cardiac puncture using citrate (3.8 %) as an anticoagulant. Following centrifugation for 10 min at 4 ºC and 1000×g, blood plasma was collected and stored at -70 ºC until assay. This methodological description was also published in an original article (Farzi et al. 2015a).

2.10 Circulating corticosterone

The plasma levels of CORT were determined with an enzyme immunoassay (EIA) kit (Assay Designs, Ann Arbor, Michigan, USA) (see 6.1 Corticosterone - EIA). According to the manufacturer's specifications, the sensitivity of the assay is 27 pg/ml, and the intra- and inter-assay coefficient of variation amounts to 7.7 % and 9.7 %, respectively. This methodological description was also published in an original article (Farzi et al. 2015a).

2.11 Circulating PYY levels

The plasma levels of PYY were determined with an EIA kit (Catalogue number EK-059-03, Phoenix Pharmaceuticals, Inc, Karlsruhe, Germany) (see 6.2 PYY - EIA). According to the manufacturer's specifications, the sensitivity of the assay is 0.07 ng/ml with a linear range between 0.07 - 0.64 ng/ml.

2.12 Circulating kynurenine and tryptophan

Kynurenine and tryptophan were measured in plasma samples by high-performance liquid chromatography (HPLC) with ultraviolet detection (Herve et al. 1996). 100 µl plasma samples were deproteinized by adding of 100 µl of 5 % (v/v) perchloric acid. After vortexing and 5 min centrifugation at 11000×g, 20 µl of the clear supernatant was injected in the chromatographic system. Separations were achieved on a Chromolith RP18e column (100 × 4.6 mm, 5 µm, Merck Darmstadt, Germany) at 30 ºC by isocratic elution with a mobile phase consisting of 50 mmol/l ammonium acetate, 250 mol/l zinc acetate and 3 %

(v/v) acetonitrile (pH 4.9) at a flow rate of 0.8 ml/min. Kynurenine and tryptophan were detected on a LaChrom UV-Detector Merck HITACHI L-7400 at 235 nm. Acquisition and processing of the chromatograms were performed using Merck Hitachi LaChrom®-D-7000 HPLC-System Manager software (VWR International GmbH/Scientific Instruments, Darmstadt, Germany). The concentrations were determined by peak-height measurement against external standards. This method has been validated according to international guidelines (Center for Veterinary Medicine 2001). All reagents were of p.A. grade (Merck). The within-day coefficient of variation (CV) at different concentrations ranged from 1.7 % to 4.3 %, for kynurenine and 0.7 % to 2.9 % for tryptophan. The between day CVs were 2.0–5.4 % and 6.3–9.3 %, respectively. This measurement was performed by a collaborator (Andreas Meinitzer and team, Clinical Institute of Medical and Chemical Laboratory Diagnostics) and the methodological description was also published in an original article (Farzi et al. 2015a).

2.13 Cytokine protein levels in blood and brain

Concentrations of IFN-ɣ, IL-1β, IL-6 and TNF-α were simultaneously quantified in plasma and brain using the ProcartaPlex™ immunoassay (eBioscience, San Diego, CA, USA) (see 6.4 Procarta Multiplex Immunoassay – Protocol).

Circulating cytokine concentrations were determined using analyte-specific capture beads coated with target-specific capture antibodies according to the manufacturer's specifications. The analytes were detected by biotinylated analyte-specific antibodies. Following binding of the fluorescent detection label (SA-PE), the reporter fluorescent signal was measured with the Bio-Plex 200 multiplex suspension array system employing Luminex xMAP technology in combination with the Bio-Plex 5.0 Software (Bio-Rad, Hercules, CA). Standard curves for each analyte were generated by using the reference analyte concentration supplied and concentrations were calculated using a five-parameter logistic curve-fitting method. Cytokines that were not detected were assigned a value of zero. The sensitivity for the respective cytokines was: IFN-ɣ: 0.09 pg/ml, IL-1β: 0.14 pg/ml, IL-6: 0.21 pg/ml, TNF-α: 0.39 pg/ml. Plasma samples of experiment 4.3 were run in duplicate. Since the coefficient of variance for the duplicate samples was small, single samples were run subsequently. This methodological description was also published in an original article (Farzi et al. 2015a).

The cerebral levels of cytokines were measured in the frontal cortex, which was microdissected on a cold plate (Weinkauf Medizintechnik, Forchheim, Germany) set at

-20°C. The isolated frontal cortex was homogenized in RIPA buffer containing 50 mmol/l Tris-HCl (pH 8), 150 mmol NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate, and 10 % protease inhibitor phenylmethylsulfonyl fluoride (PMSF). The protein concentration in each sample was measured with the Pierce BCA Protein Assay Kit (Thermo Scientific) (see 6.3 Protein extraction). Brain samples were diluted with sample dilution buffer provided by the kit to yield a concentration of 10 mg protein/ml.

2.14 RNA extraction and reverse transcription

LPS has been reported to induce a ubiquitous upregulation of cytokine mRNA expression in discrete brain regions (O'Connor et al. 2009b). Therefore, one part of a hemibrain (Bregma +0.50 to -2.70) weighing 50–60 mg was dissected on a cold plate and homogenized in MagNA Lyser bead tubes (Catalogue number 03358 941 001, Roche Diagnostics, Rotkreuz, CH) using the MagNA Lyser centrifuge (Roche Diagnostics). Total RNA was extracted in TRIzol reagent (Catalogue number 15596018, Life Technologies, Carlsbad, CA) (see 6.5 TRIzol extraction) and randomly tested for quality on the BioAnalyzer BA2100 (Agilent; Foster City, CA) with the RNA 6000 Nano LabChip Kit (Catalogue number 5067-1511, Agilent, Foster City, CA). The RIN (RNA Integrity Number) of all tested samples ranged between 7.9 and 8.7. The total RNA was also randomly tested on the Qiaxpert System (Qiagen, Hilden, Germany). Minor impurities were visible. Four samples were chosen as controls without reverse transcription (RT) based on the results of the Qiaxpert System.
RNA (2 µg per sample) was reverse transcribed simultaneously in the Thermocycler 'MyCycler' (Bio-Rad Laboratories, Hercules, CA), using the High Capacity cDNA Reverse Transcription Kit (Catalogue number 4368813, Life Technologies) according to the manufacturer's instructions (see 6.6 cDNA synthesis). This methodological description was also published in an original article (Farzi et al. 2015a).

2.15 Real-time RT-PCR

Real-time RT-polymerase chain reaction (PCR) was performed on an LightCycler®480 System using Taqman®gene expression assays for TNF-α (Mm00443258_m1), IL-1β (Mm00434228_m1), IFN-ɣ (Mm01168134_m1), IL-6 (Mm00446190_m1), Gapdh (Mm999999_g1) and Actb (Mm00607939_s1) purchased from Life Technologies (Catalogue number 4331182). For the real-time RT-PCR setup the TaqMan Gene Expression Master Mix (Catalogue number 4369016, Life Technologies) was used (see 6.7

PCR). Reactions were carried out in triplicates according to the manufacturer's instructions using a 20 ng cDNA template for each reaction.

Table 1 PCR cycling conditions

Step	Temperature (°C)	Duration (min)	Cycles
Uracil-DNA-Glykosilase Incubation	50	02:00	HOLD
AmpliTaq Gold®, UP Enzyme Activation	95	10:00	HOLD
Denature	95	00:15	40 Cycles
Anneal/Extend	60	01:00	

As negative controls, amplifications without reverse transcription or template were included. All negative controls remained negative. Quantitative measurement of target gene levels relative to controls was performed with the $2^{-\Delta\Delta Ct}$ method (Schmittgen, Livak 2008). Gapdh and Actb were used as endogenous housekeeping genes. This methodological description was also published in an original article (Farzi et al. 2015a).

2.16 Immunohistochemistry

The activation of neurons in select nuclei and cortical areas of the brain was visualized by c-Fos immunohistochemistry 3 h after injection of PRR agonists as c-Fos expression is maximal at this timepoint after LPS injection (Rivest, Laflamme 1995).
Immunohistochemistry was performed according to a slightly modified version of the protocol provided by Sundquist and Nisenbaum (Sundquist, Nisenbaum 2005) and described by Reichmann et al. (Reichmann, Painsipp & Holzer 2013).
Coronal sections (20 µm) were cut from the mouse forebrain with the Microm HM 560 cryostat (Microm, Walldorf, Germany) and mounted on Superfrost Plus slides (Menzel, Braunschweig, Germany). Every sixth section was used for immunohistochemistry. The sections were surrounded with a hydrophobic barrier pen (ImmEdge Pen, Vector Laboratories) and incubated in 4 % paraformaldehyde (PFA) (Sigma-Aldrich) in 1X phosphate buffered saline (PBS) for 10 min (see 6.8 Reagents for immunohistochemistry). Afterwards, slides were washed three times for 5 min in washing buffer (WB; 0.05 % Tween 20 (Roth) in 1X PBS) and incubated in 0.3 % hydrogen peroxide (H_2O_2) in methanol for 15 min. After three further washes in WB, the tissues were incubated with 10 % normal goat serum in antibody diluent (AD; 1X PBS containing 0.05 % Tween 20 and 1

% bovine serum albumin) for 5 min and then with the primary antibody in AD (rabbit polyclonal anti-c-Fos SC-52, 1:2000, Santa Cruz Biotech, Santa Cruz, USA) overnight at 4°C. On the next day the sections were washed three times in WB and incubated for 30 min in AD containing the biotinylated secondary antibody (goat anti-rabbit IgG 1:200, Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, USA) at room temperature. After three further washes in WB they were incubated for 30 min in avidin-biotin complex. Subsequently the tissues were rinsed three times in WB and developed with 3,3-diaminobenzidine (DAB) substrate (DAB substrate kit, Vector Laboratories). Finally the sections were washed three times for 5 min in distilled water, air-dried overnight, cleared in xylene (Merck-Millipore) and coverslipped with entellan (Merck-Millipore).

2.17 Cell counting and quantification of c-Fos expressing cells

The c-Fos labelled brain sections were examined with a light microscope (Axiophot, Zeiss, Oberkochen, Germany) coupled to a computerized image analysis system (MCID Basic, version 7.0, Imaging Research Inc., Brock University, St. Catharines, Ontario, Canada) (Reichmann, Painsipp & Holzer 2013). The investigator was blind to the treatment groups under investigation and brain regions of interest (ROIs) were identified with the help of the mouse brain stereotaxic atlas of Paxinos and Franklin (Paxinos, Franklin 2001).
MCID Basic was used to measure mean intensity values of three background measurements per region. Subsequently, the background threshold was defined such that the maximum number of c-Fos labelled cells was counted without inclusion of any background staining
While in the PVN and the granular cell layer of the dentate gyrus (DG) all c-Fos positive cells were counted, the number of c-Fos labelled cells in the other ROIs was quantitated within a square of 200 × 200 µm, and the c-Fos labelled cells of the SFO were quantitated within a square of 400 × 400 µm. One section was counted bilaterally to quantitate the number of c-Fos positive cells in the dorsal part of the BNST (BNSTd) (Bregma +0.38 to +0.14), while two consecutive sections were counted bilaterally to quantitate the number of c-Fos positive cells in the ventral part of the BNST (BNSTv) (Bregma +0.50 to +0.14), the PVN (Bregma -0.58 to -0.94), the insula (Bregma +0.38 to +0.14), and the SFO (Bregma -0.58 to -0.70). Three consecutive sections were counted bilaterally to quantitate the number of c-Fos positive cells in the CeA (Bregma -1.34 to -1.70), the supraoptic nucleus (SO) (Bregma -0.70 to -1.06), and the DG (Bregma -1.34 to -1.94). The cell counts obtained for each ROI in the different sections of each animal were averaged to calculate

the mean number of c-Fos-positive cells within a particular brain region of that animal. These average values/brain region of each animal were used for statistical analysis.

2.18 Myeloperoxidase levels in the colon

Tissue levels of MPO were quantified in order to assess inflammation-associated infiltration of predominantly neutrophils into the tissue (Krawisz, Sharon & Stenson 1984, Reichmann, Painsipp & Holzer 2013).

The frozen colon tissues were weighed and placed, at a ratio of 1 mg per 20 µl, in MPO lysis buffer. The samples were homogenized on ice with an Ultraturrax (IKA, Staufen, Germany) and subsequently subjected to two centrifugation steps at 6000×g and 4°C for 15 min. The MPO content of the supernatant was measured with an enzyme-linked immunosorbent assay (ELISA) kit specific for the rat and mouse protein (Hycult Biotechnology, Uden, The Netherlands) (see 6.9 MPO - ELISA). The sensitivity of this assay is 1 ng/ml at an intra- and inter-assay variation of around 10 % (Reichmann, Painsipp & Holzer 2013).

2.19 Microbiome analysis

2.19.1 DNA isolation and PCR amplification

Frozen colon tissues including luminal content and weighing 70 mg on average were homogenized on a MagNA Lyser Instrument using MagNA Lyser Green Beads (Roche Diagnostics GmbH, Mannheim, Germany), and bacterial DNA was extracted with the Power Lyzer® Power Soil® DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA concentration was first determined with a Nanodrop ND 1000 spectrophotometer (Peqlab Biotechnology, Erlangen, Germany). Subsequently the DNA concentration was determined using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies) according to the manufacturer's instructions and measured with a fluorometer (Synergy4, BioTek Instruments, Winooski, VT, USA). Bacterial 16S rRNA was amplified with the Rotor-Gene SYBR Green PCR Kit (Qiagen, Hilden, Germany) using fusion primers with the template-specific sequence F27—AGAGTTTGATCCTGGCTCAG and R357—CTGCTGCCTYCCGTA. A sample specific nucleotide barcode sequence was included on primer F27 to allow for a simultaneous analysis of multiple samples during the sequencing run. In addition to the tissue samples a sample devoid of any tissue (blank) was also

included in the PCR run in order to assess contaminations of the reagents used and imported during the workflow.
PCR conditions were as follows: For each sample, a PCR mix of 25 µl was prepared containing 20 ng DNA from each sample, 12.5 µl of 2X Rotor-Gene SYBR Mastermix (Qiagen) and 1 µl of the primer mix (25 µM of forward and reverse primer). Thermal cycling conditions were:

- Initial denaturation at 95°C for 5 min
- 1 cycle at 95°C for 5 s
- 50 cycles touchdown PCR from 65°C to 55°C with a decrease of 1°C every second cycle
- Melting curve from 55°C to 95°C with a 1°C increment every 5 s
- Final step of 25°C for 5 min

PCR products were separated on a 2 % SeaKem ME agarose gel (Cambrex, Rockland, ME, USA) and specific bands (~ 450 bp) were excised and gel extracted using the Qiagen gel extraction kit (Qiagen) and eluted in 30 µl of distilled water.
Amplicon DNA concentrations were determined using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies) according to the manufacturer's instructions.
Sequencing was performed by using Ion PGM Sequencer (Life Technologies) with an Ion Sequencing 400 Kit (Life Technologies). Sequencing was performed by Andrea Thüringer at the Institute of Pathology of the Medical University of Graz.

2.19.2 Sequence analysis

Contaminating human sequences were removed by aligning all sequences to the human reference genome and removing all sequences that map to the human genome.
Acacia error correction was applied on all reads using standard parameters (Bragg et al. 2012). Briefly, Acacia collapses all homopolymers in all reads and determines homopolymer differences in similar collapsed sequences. If sequences are only different in homopolymer length these are assumed to be sequencing errors and are corrected to give equal homopolymer length.
Usearch algorithm was then applied to detect chimeras in a reference based and a de-novo approach. Detected chimeras were removed from the sequence file.

The resulting bam file was introduced into the QIIME (v1.8.0) 16S workflow (www.qiime.org). Briefly, sequences were grouped by similarity using a 97 % identity as threshold which roughly corresponds to species level (operational taxonomic unit, OTU picking script). For each cluster of sequences (OTU) the representative sequence was determined and mapped to a database of known sequences using the Ribosomal Database Project classifier (http://rdp.cme.msu.edu/) (assign taxonomy script). OTU tables were constructed using the make_otu_table script. Furthermore, the alpha diversity (i.e. number of OTUs) and beta diversity (i.e. interindividual variability) were calculated and visualized with the core diversity script. Group differences were assessed with the ADONIS test, while the differences of OTU frequencies were assessed with the Kruskal–Wallis test. Sequence analysis was performed by Karl Kashofer at the Institute of Pathology of the Medical University of Graz.

Table 2 Applied scripts within the QIIME (v1.8.0) 16S workflow

Task	Script
OTU picking	parallel_pick_otus_uclust_ref.py -i output/seqs.fna -O 4 -s 0.97 -o otus/uclust_picked_otus -r /home/worker/software/qiime-1.8/gg_otus-13_8-release/rep_set/97_otus.fasta
Assign taxonomy	parallel_assign_taxonomy_uclust.py -i otus/rep_set/seqs_rep_set.fasta -o otus/uclust_assigned_taxonomy -T --jobs_to_start 4
OTU tables	make_otu_table.py -i otus/uclust_picked_otus/seqs_otus.txt -t otus/uclust_assigned_taxonomy/seqs_rep_set_tax_assignments.txt -o otus/otu_table.biom
Core diversity	core_diversity_analyses.py -a -O 4 -i otus/otu_table.biom -o core_diversity -m ../mapping.txt -e 2000 -t otus/rep_set.tre

2.20 Statistics

Statistical evaluation of the results was made with SPSS 20 (SPSS Inc., Chicago, Illinois, USA).

Differences between two independent groups were analysed with the two sample t-test. If more than two groups were compared, the data were analysed by one-way or two-way analysis of variance (ANOVA), as appropriate, and in some cases for repeated measurements. Two-way ANOVA was performed with the NOD agonists (VEH, MDP, FK565) and LPS (VEH, LPS) as the between subject variables in order to reveal significant main factor effects or interactions denoted as NOD × LPS interactions. The homogeneity of variances was assessed with the Levene test. In case of sphericity violations the Greenhouse–Geisser correction was applied. Post-ANOVA analysis of group differences was performed with the Tukey HSD (honestly significant difference) test, when the variances were homogeneous, and with the Games–Howell test, when the variances were unequal. In case of a non-parametric distribution of the parameters, statistical differences among groups were determined with the Kruskal–Wallis test and post-hoc analysis of group differences was performed with the Mann–Whitney test. p-Values were adjusted for multiple comparisons with the Bonferroni correction. Probability values of $p < 0.05$ were regarded as statistically significant and of $p < 0.1$ were regarded as a trend. Data are presented as means ± SEM, n referring to the number of mice in each group.

3 Results - Findings

3.1 Neither MDP (10 mg/kg or 30 mg/kg) nor M-TriDAP (5 mg/kg) exert short or long term effects on sickness-related parameters, mood and behaviour (Protocol 1)

3.1.1 NOD2 agonism with MDP induces an increase in body weight of female mice

I.p. injection of the NOD1/NOD2 agonist M-TriDAP (5 mg/kg) did not induce any changes in body temperature or weight in adult female C57BL/6N mice. Thus, body weight was not affected 1 day ($t_{(12)} = 0.125$, $p = 0.903$) or 7 days ($t_{(12)} = 0.180$, $p = 0.860$) post-treatment (n = 7). Likewise body temperature was comparable between the control and treatment group 5 h post-treatment ($t_{(12)} = -0.831$, $p = 0.422$) (Table 3, Experiment 1.1).

Similarly, the NOD2 agonist, MDP (10 mg/kg) did not induce any significant changes of body temperature as measured 5 h post-treatment ($t_{(14)} = -1.084$, $p = 0.297$) (n = 8). Interestingly, however, MDP induced an increase of body weight measured 1 day after treatment ($t_{(14)} = -2.226$, $p = 0.043$). Seven days post-treatment there was no difference in body weight between the VEH and MDP-treated group ($t_{(14)} = -1.167$, $p = 0.263$). (Table 3, Experiment 1.2).

The higher dose of MDP (30 mg/kg) did not exert any significant effects on body weight of the animals, neither 1 day ($t_{(10)} = -1.111$, $p = 0.293$) nor 7 days ($t_{(10)} = -1.005$, $p = 0.339$) post-treatment (n = 6). However, body weight in the MDP-treated group was nominally higher than in the controls. As seen with the lower dose of MDP, body temperature was not influenced by MDP (30 mg/kg) 5 h post-treatment ($t_{(10)} = 0.745$, $p = 0.474$) (Table 3, Experiment 1.3).

Table 3 Effects of the NOD2 agonist MDP and the NOD1/NOD2 agonist M-TriDAP on body temperature and weight in female mice

E	Readout + time	VEH	MDP	M-TriDAP
1.1	Temp 5h	36.63 ± 0.25	----	36.89 ± 0.18
	Weight D1	0.047 ± 0.40	----	- 0.051 ± 0.67
	Weight D7	- 0.662 ± 0.98	----	- 0.909 ± 0.96
1.2	Temp 5h	36.35 ± 0.27	36.69 ± 0.15	----
	Temp D2	36.63 ± 0.15	36.94 ± 0.24	----
	Weight D1	- 0.005 ± 0.81	**+ 2.150 ± 0.53**	----
	Weight D7	- 1.606 ± 0.86	- 0.087 ± 0.97	----
1.3	Temp 5h	36.67 ± 0.22	36.37 ± 0.34	----
	Weight D1	1.068 ± 0.48	2.709 ± 1.40	----
	Weight D7	1.572 ± 0.71	3.195 ± 1.45	----

Mice were injected i.p. with saline (VEH), M-TriDAP (E 1.1: 5 mg/kg) or MDP (E 1.2: 10 mg/kg, E 1.3: 30 mg/kg) in 3 separate experiments. The table shows the body in temperature 5 h post-treatment and the change of body weight 1 and 7 days post-treatment. The weight change induced by the treatment is expressed as a percentage of the body weight measured pre-treatment. The values are means ± SEM, n = 6–8. Significant results ($p < 0.05$ versus VEH) are bolded. Abbreviations: D = day, E = experiment, h = hour, Temp = temperature.

3.1.2 NOD1 and/orNOD2 agonism does not affect locomotion or anxiety-like behaviour one day post-treatment in the OFT

Neither the NOD1/NOD2 agonist M-TriDAP (5 mg/kg) nor the NOD2 agonist MDP (10 – 30 mg/kg) induced any changes in locomotion or anxiety-like behaviour as measured in the OFT 1 day post-treatment in female C57BL/6N mice.

In more detail, M-TriDAP (5 mg/kg) had no effect on the time spent in the central area ($t_{(11)} = -0.209$, $p = 0.838$), the number of entries into the central area ($t_{(11)} = -0.075$, $p = 0.942$), the total travelling distance ($t_{(11)} = -0.274$, $p = 0.789$) and the number of boli expelled during the test ($t_{(11)} = -0.487$, $p = 0.636$) (n = 6-7) (Table 4, Experiment 1.1).

Likewise, MDP (10 mg/kg) had no effect on behaviour of the mice in the OFT (time spent in the central area: $t_{(14)} = -0.683$, $p = 0.506$; number of entries into the central area: $t_{(14)} = -1.566$, $p = 0.140$; total travelling distance: $t_{(14)} = -1.283$, $p = 0.220$; number of boli expelled: $t_{(14)} = 0.202$, $p = 0.843$) (n = 8) (Table 4, Experiment 1.2).

Also the higher dose of MDP (30 mg/kg) failed to affect the parameters assessed during the OFT (time spent in the central area: $t_{(9)} = -0.230$, $p = 0.823$; number of entries into the

central area: $t_{(9)} = -0.642$, $p = 0.537$; total travelling distance: $t_{(9)} = -0.336$, $p = 0.744$; number of boli expelled: $t_{(9)} = -1.199$, $p = 0.261$) (n = 5-6) (Table 4, Experiment 1.3).

Table 4 Effects of the NOD2 agonist MDP and the NOD1/NOD2 agonist M-TriDAP on locomotion and anxiety-like behaviour of female mice in the OFT

E	Parameter	VEH	MDP	M-TriDAP
1.1	Time in centre (s)	90.00 ± 12.56	----	94.65 ± 17.44
	Central entries	41.00 ± 4.31	----	42.00 ± 11.77
	Total distance (m)	23.37 ± 2.26	----	25.07 ± 5.36
	Boli	1.50 ± 0.34	----	2.00 ± 0.90
1.2	Time in centre (s)	69.87 ± 6.66	77.20 ± 8.42	----
	Central entries	30.50 ± 2.24	35.75 ± 2.49	----
	Total distance (m)	26.70 ± 1.23	29.05 ± 1.35	----
	Boli	1.63 ± 0.80	1.38 ± 0.94	----
1.3	Time in centre (s)	92.92 ± 18.28	99.42 ± 20.73	----
	Central entries	30.40 ± 2.44	34.00 ± 4.66	----
	Total distance (m)	26.37 ± 2.77	27.73 ± 2.87	----
	Boli	0.80 ± 0.58	2.33 ± 1.05	----

Mice were injected i.p. with saline (VEH), M-TriDAP (E 1.1: 5 mg/kg) or MDP (E 1.2: 10 mg/kg, E 1.3: 30 mg/kg) in three separate experiments. The OFT was conducted 1 day after treatment. The table shows the time spent in the central area, the number of entries into the central area, the total distance travelled, and number of boli expelled during the 5-min test session. The values are means ± SEM, n = 5–8.
Abbreviations: E = experiment, m = metre, s = seconds

3.1.3 NOD1 and/orNOD2 agonism does not affect short- or long-term depression-like behaviour in the FST

The FST was employed to assess changes in depression-like behaviour, where an increase in the time spent immobile is proposed to reflect a depressive phenotype of rodents. While the FST was conducted 1 day after treatment with M-TriDAP (5 mg/kg) and the lower dose of MDP (10 mg/kg), the test was carried out 7 days after injection of the higher dose of MDP (30 mg/kg) in order to assess potential long-term changes in depression-like-behaviour. M-TriDAP (5 mg/kg) had no effect on behaviour of the female mice in the FST (time spent immobile: $t_{(11)} = -1.596$, $p = 0.139$; time spent swimming: $t_{(11)} = 0.744$, $p = 0.473$; time spent climbing: $t_{(11)} = 1.543$, $p = 0.151$) (n = 6-7) (Table 5, Experiment 1.1).

Likewise, MDP (10 mg/kg) had no effect on time spent immobile ($t_{(12)}$ = -1.366, p = 0.197), the time spent swimming ($t_{(12)}$ = -0.642, p = 0.533) and the time spent climbing ($t_{(12)}$ = 1.377, p = 0.194) (n = 7) (Table 5, Experiment 1.2).
Also the higher dose of MDP (30 mg/kg) failed to affect the behaviour of the mice in the FST when measured 7 days post-treatment (time spent immobile: $t_{(10)}$ = 0.271, p = 0.792; time spent swimming: $t_{(10)}$ = -0.174, p = 0.866; time spent climbing: $t_{(10)}$ = -0.171, p = 0.867) (n = 6) (Table 5, Experiment 1.3).

Table 5 Effects of the NOD2 agonist MDP and the NOD1/NOD2 agonist M-TriDAP on depression-like behaviour of female mice in the FST

E	Parameter	VEH	MDP	M-TriDAP
1.1	Immobility	267.92 ± 20.83	----	305.14 ± 6.06
	Swimming	29.11 ± 7.80	----	25.79 ± 8.28
	Climbing	62.98 ± 19.59	----	29.08 ± 5.84
1.2	Immobility	286.94 ± 5.37	287.86 ± 6.02	----
	Swimming	39.06 ± 3.29	36.46 ± 3.98	----
	Climbing	31.42 ± 4.89	31.57 ± 8.47	----
1.3	Immobility	258.26 ± 25.70	249.99 ± 16.46	----
	Swimming	34.50 ± 6.47	37.14 ± 13.76	----
	Climbing	67.26 ± 27.37	72.88 ± 18.07	----

Mice were injected i.p. with saline (VEH), M-TriDAP (E 1.1: 5 mg/kg) or MDP (E 1.2: 10 mg/kg, E 1.3: 30 mg/kg) in three separate experiments. The FST was conducted 1 day (E 1.1. and 1.2) or 7 days (E 1.3) after treatment. The duration of immobility, swimming and climbing during the 6-min test session is expressed in seconds. The values are means ± SEM, n = 6–8. Abbreviations: E = experiment.

3.1.4 NOD1 and/orNOD2 agonism does not affect long-term anxiety-like behaviour on the EPM

The EPM test was performed to assess anxiety-related behaviour, where a decrease of the time spent on the open arms and the number of entries into the open arms reflect an anxious phenotype. None of the treatments induced changes in anxiety-related behaviour as assessed 7 days post-injection in female mice (Table 6).
Thus, M-TriDAP (5 mg/kg) had no effect on the time spent on the open arms ($t_{(12)}$ = 0.932, p = 0.370), the number of entries into the open arms ($t_{(12)}$ = -1.003, p = 0.335), the total

number of entries into any arm ($t_{(12)}$ = -0.735, p = 0.476) and the total travelling distance ($t_{(12)}$ = -0.387, p = 0.705) (n = 7) (Table 6, Experiment 1.1).
Likewise, MDP (10 mg/kg) failed to affect the behaviour of the mice on the EPM (time spent on the open arms: $t_{(14)}$ = -0.103, p = 0.919; number of entries into the open arms: $t_{(14)}$ = -1.131, p = 0.277; total number of entries into any arm: $t_{(14)}$ = -0.875, p = 0.396; total travelling distance: $t_{(14)}$ = -1.083, p = 0.297) (n = 8) (Table 6, Experiment 1.2).
Also the higher dose of MDP (30 mg/kg) had no effect on anxiety-like behaviour of the mice on the EPM 7 days post-treatment (time spent on the open arms: $t_{(10)}$ = 1.623, p = 0.136; number of entries into the open arms: $t_{(10)}$ = -0.102, p = 0.921; total number of entries into any arm: $t_{(10)}$ = -0.456, p = 0.658; total travelling distance: $t_{(10)}$ = -0.577, p = 0.577) (n = 6) (Table 6, Experiment 1.3).

Table 6 Effects of the NOD2 agonist MDP and the NOD1/NOD2 agonist M-TriDAP on anxiety-like behaviour and locomotion of female mice on the EPM

E	Parameter	VEH	MDP	M-TriDAP
1.1	Time on open arms (%)	18.74 ± 5.46	----	12.60 ± 3.70
	Open arm entries	11.57 ± 1.91	----	15.57 ± 3.50
	Total entries	25.43 ± 2.57	----	29.57 ± 5.01
	Total distance (m)	8.94 ± 0.59	----	9.37 ± 0.92
1.2	Time on open arms (%)	8.72 ± 2.78	9.08 ± 2.14	----
	Open arm entries	7.63 ± 1.31	9.50 ± 1.02	----
	Total entries	25.75 ± 2.32	28.25 ± 1.67	----
	Total distance (m)	13.25 ± 1.13	14.75 ± 0.80	----
1.3	Time on open arms (%)	11.36 ± 3.34	5.50 ± 1.38	----
	Open arm entries	10.17 ± 2.12	10.50 ± 2.49	----
	Total entries	27.33 ± 4.67	30.67 ± 5.63	----
	Total distance (m)	5.13 ± 0.32	5.58 ± 0.72	----

Mice were injected i.p. with saline (VEH), M-TriDAP (E 1.1: 5 mg/kg) or MDP (E 1.2: 10 mg/kg, E 1.3: 30 mg/kg) in three separate experiments. The EPM test was conducted 7 days after treatment. The table shows the time spent on the open arms, number of entries into the open arms, total number of entries into open and closed arms and the total distance travelled. Time spent on the open arms is expressed as a percentage of the 5-min test duration. The values are means ± SEM, n = 6–8. Abbreviations: E = experiment, m = metre.

3.2 NOD1 or NOD2 activation amplifies the behavioural effects of TLR4 activation (Protocol 2 – 4)

3.2.1 Effects of MDP, FK565 and LPS on daily levels of locomotion, exploration, food intake and SP (Protocol 2)

MDP, FK565 and LPS altered locomotion, exploration, food intake and SP in a compound-, combination- and time-dependent manner (Figure 7). Repeated measures ANOVA revealed a significant interaction of NOD (VEH, MDP, FK565) × LPS (VEH, LPS) × time (days post-treatment) for the variation in locomotion ($F_{(5.661,116.05)} = 2.457$, $p < 0.05$). The same was true for exploratory behaviour ($F_{(5.250,110.25)} = 2.470$, $p < 0.05$). Likewise, there was a significant NOD × LPS × time interaction for the differences in food intake ($F_{(5.025,105.52)} = 5.244$, $p < 0.001$). SP depended on time ($F_{(1.130,39.55)} = 27.838$, $p < 0.001$), with a significant interaction with LPS ($F_{(1.130,39.55)} = 18.397$, $p < 0.001$) and an interaction with the NOD agonists by trend ($F_{(2.260,39.55)} = 2.339$, $p = 0.10$). Post-hoc analysis revealed significant NOD × LPS interactions on day 1 and 2 post-treatment. While MDP (1 mg/kg) and FK565 (0.001 mg/kg) alone did not induce any significant changes in locomotion, LPS (0.1 mg/kg) led to a decrease of locomotion for 2 days after injection when compared with the VEH-treated group. Combination of MDP + LPS attenuated locomotion compared to treatment with MDP or LPS alone during day 1 and 2 post-treatment (Figure 7A). Likewise, the combination of FK565 + LPS significantly decreased locomotion when compared with FK565 or LPS alone. Post-hoc analysis of the changes in exploration disclosed a significant NOD × LPS interaction on day 1 and 3 post-treatment, while there was only a trend for interaction on day 2 ($p = 0.10$). Specifically, MDP + LPS and FK565 + LPS decreased exploration when compared with LPS or MDP and FK565, respectively (Figure 7B). A significant NOD × LPS interaction was evident for food intake on day 1 and 2 post-treatment (Figure 7C). While the effect of FK565 did not reach statistical significance after correcting for multiple testing, LPS diminished food intake 1 day after treatment when compared to VEH. Again, MDP + LPS and FK565 + LPS further attenuated food intake 1 day post-treatment compared to MDP and FK565, respectively. Both combinations also led to a decrease of food intake when compared with LPS (Figure 7C). On day 2 post-treatment food intake was still decreased in the FK565 + LPS group compared to the FK565 or LPS groups, while the effect of MDP + LPS did not reach

significance after correcting for multiple testing. Unlike LPS, MDP + LPS or FK565 + LPS led to a nominal decline of SP on day 1 post-treatment, but the interaction of LPS with the NOD agonists did not reach statistical significance (Figure 7D). These findings have been published in an original article (Farzi et al. 2015a).

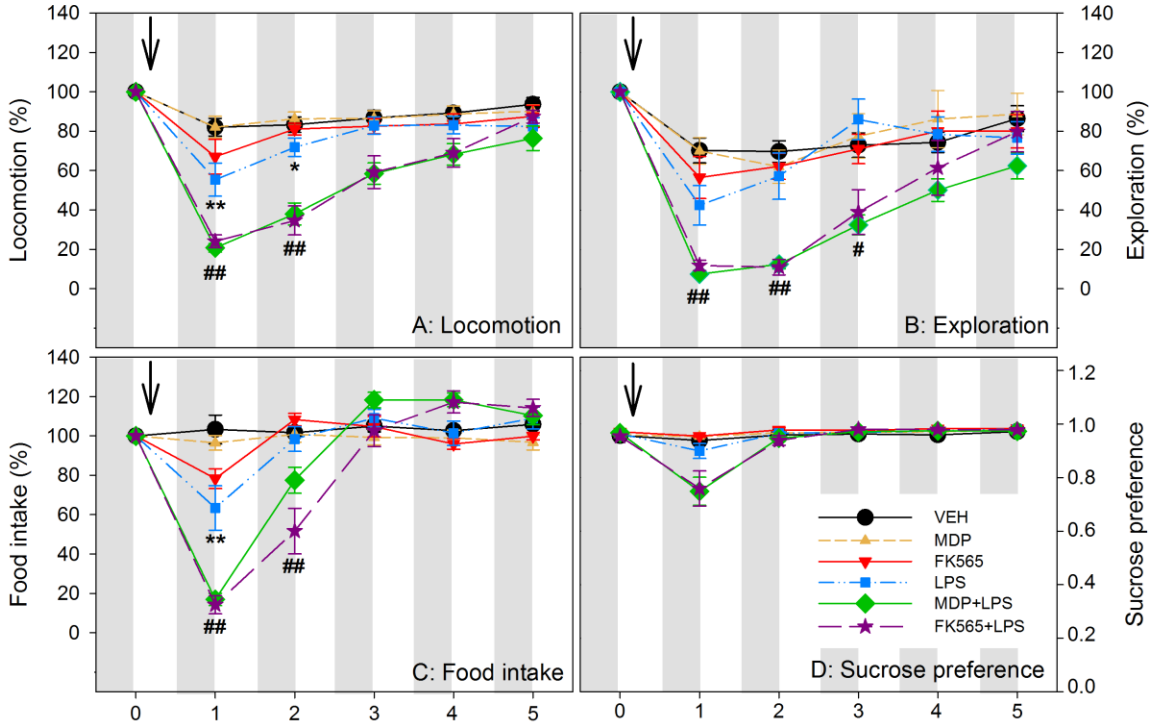

Figure 7 Effects of MDP, FK565 and LPS on daily levels of locomotion (A), exploration (B), food intake (C) and sucrose preference (D) in male mice.

Saline (VEH), MDP (1 mg/kg), FK565 (0.001 mg/kg), LPS (0.1 mg/kg), MDP + LPS or FK565 + LPS were injected i.p. as indicated. The graphs show the daily levels of activity for 1 day before (set as 100 % in A, B and C) and 5 days after injection. The values are means ± SEM, n = 8. Post-hoc analysis of significant NOD × LPS interactions in 2-way ANOVA: *$p < 0.05$, **$p < 0.01$, versus VEH. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, MDP + LPS or FK565 + LPS versus MDP, FK565 or LPS. These findings have been published in an original article (Farzi et al. 2015a).

3.2.2 Effects of MDP and FK565 alone and in combination with LPS on body temperature and weight (Protocol 3 and 4)

MDP, FK565 and LPS interacted with each other in modifying body temperature but not body weight (Figure 8). Two-way ANOVA revealed a significant NOD × LPS interaction for the changes in body temperature ($F_{(4,65)} = 20.413$, $p < 0.001$) (Figure 8A). Post-hoc analysis showed that neither MDP (3 mg/kg), FK565 (0.003 mg/kg) nor the two doses of LPS induced changes of body temperature 4 h post-treatment. In contrast, combined treatment with MDP + LPS (0.83 mg/kg) and FK565 + LPS (0.83 mg/kg) evoked a strong hypothermic response compared to single treatment with the NOD agonists or LPS (Figure 8A). Also the combination of MDP or FK565 with the lower dose of LPS (0.1 mg/kg) slightly decreased body temperature, the effect of MDP + LPS (0.1 mg/kg) reaching statistical significance when compared to MDP alone (Figure 8A). The effects on body weight differed from those on body temperature. Thus, a NOD × LPS interaction was not evident for the differences in weight (Figure 8B). Two-way ANOVA showed that weight loss depended solely on LPS ($F_{(2,67)} = 166.200$, $p < 0.001$) (Figure 8B). These findings have been published in an original article (Farzi et al. 2015a).

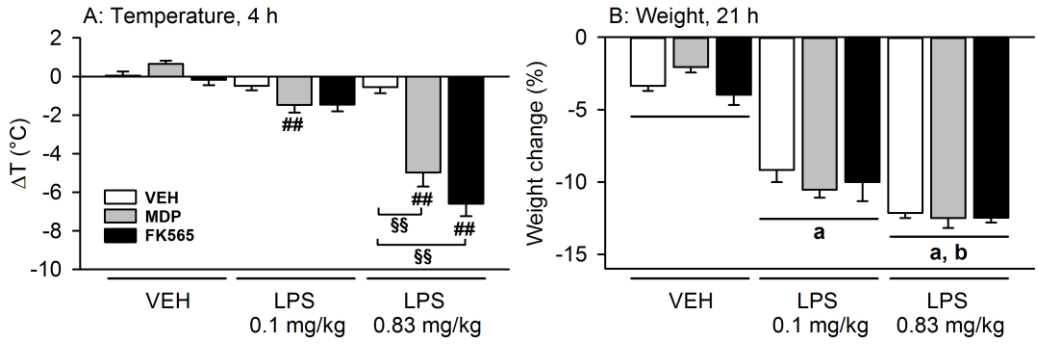

Figure 8 Effects of MDP (3 mg/kg), FK565 (0.003 mg/kg) and LPS (doses as indicated) to reduce body temperature (A) and weight (B) in male mice.

The graphs show the change in temperature 4 h post-treatment and body weight 21 h post-treatment. Body temperature and weight were measured before treatment and 4 and 26 h post-injection, respectively. The weight loss induced by the treatment is expressed as a percentage of the body weight measured pre-treatment. The values are means + SEM, n = 15 for VEH (merged from 2 separate experiments), n = 7–8 for other groups. Post-hoc analysis of significant NOD × LPS interactions in 2-way ANOVA: $^{\#\#}p < 0.01$, MDP + LPS versus MDP or FK565 + LPS versus FK565. $^{\S\S}p < 0.01$, MDP + LPS and FK565 + LPS versus LPS (0.83 mg/kg). Main factor effects without NOD × LPS interactions: $^{a}p < 0.01$, LPS versus VEH. $^{b}p < 0.01$, LPS 0.83 mg/kg versus LPS 0.1 mg/kg. These findings have been published in an original article (Farzi et al. 2015a).

3.2.3 Effects of MDP and FK565 alone and in combination with LPS on behaviour in the OF (Protocol 3 and 4)

The behaviour in the OF was modified by MDP, FK565 and LPS in a compound-, combination- and time-dependent manner (Figure 9). The OFT was used to assess anxiety-like behaviour as deduced from the time spent in the central area and the entries made to the central area of the OF and locomotion as deduced from the travelling distance (Figure 9). In experiments with the higher dose of LPS (0.83 mg/kg), two-way ANOVA revealed a significant NOD × LPS interaction for the changes in locomotion ($F_{(2,42)} = 3.168$, $p \leq 0.05$). Post-hoc analysis showed that while the NOD agonists did not impact on locomotion, treatment with LPS (0.83 mg/kg) slightly decreased the travelling distance in the OF (Figure 9C). Moreover, combined treatment with MDP (3 mg/kg) + LPS (0.83 mg/kg) or FK565 (0.003 mg/kg) + LPS (0.83 mg/kg) further diminished the distance travelled when compared with LPS, MDP and FK565 alone, respectively (Figure 9C). The entries made into the centre of the field depended on LPS ($F_{(1,42)} = 31.001$, $p < 0.001$), while the effect of the NOD agonists and their interaction with LPS did not reach significance (Figure 9B). The time spent in the central area of the OF was not significantly affected by any of the compounds (Figure 9A). In experiments with the lower dose of LPS (0.1 mg/kg), LPS alone, MDP + LPS (0.1 mg/kg) as well as FK565 + LPS (0.1 mg/kg) reduced the time spent in the central area of the field (Figure 9D) and the entries made to the central area (Figure 9E) without affecting the total distance travelled (Figure 9F). The combination of FK565 + LPS had the most pronounced effects. While the time in the central area was reduced in all groups ($F_{(3,25)} = 7.176$, $p = 0.001$) (Figure 9D), the entries made to the central area of the field were solely reduced by FK565 + LPS ($F_{(3,25)} = 6.256$, $p < 0.01$) (Figure 9E). These findings have been published in an original article (Farzi et al. 2015a).

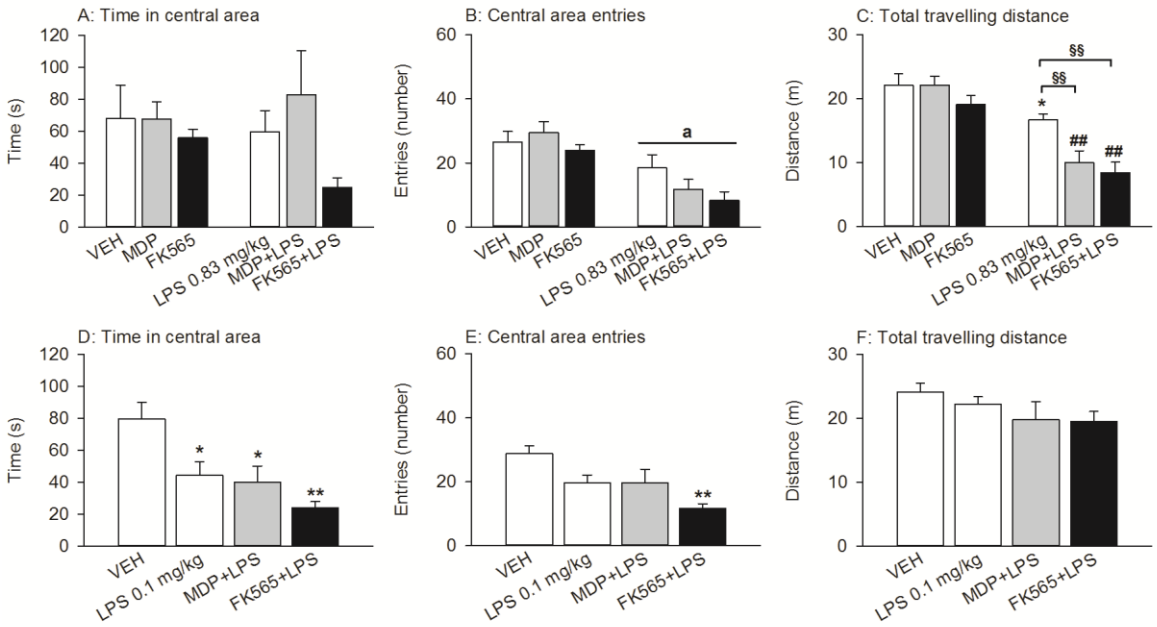

Figure 9 Effects of MDP (3 mg/kg), FK565 (0.003 mg/kg) and LPS (doses as indicated) on behaviour in the OF 21 h post-treatment in male mice.

The graphs show the time spent in the central area (A + D), the number of entries into the central area (B + E) and the total distance travelled (C + F) during the 5-min test session. The values are means + SEM, n = 7–8. A–C: Post-hoc analysis of significant NOD × LPS interactions in 2-way ANOVA: *p < 0.05, versus VEH. $^{\#\#}$p < 0.01, MDP + LPS versus MDP or FK565 + LPS versus FK565. §§p < 0.01, MDP + LPS or FK565 + LPS versus LPS. Main factor effects without NOD × LPS interactions: ^{a}p < 0.01, LPS versus VEH. D–F: One-way ANOVA: *p < 0.05, **p < 0.01, versus VEH. These findings have been published in an original article (Farzi et al. 2015a).

3.2.4 Effects of MDP, FK565 and LPS alone and in combination on depression-like behaviour in the FST and TST (Protocol 4)

LPS (0.1 mg/kg) did not change any behavioural parameter in the FST. In contrast, combined treatment with MDP + LPS and FK565 + LPS slightly increased immobility and decreased the duration of the time spent swimming, but these changes did not reach statistical significance (Table 7). Likewise, in the TST there were no significant changes in the duration of immobility, swinging or curling by any of the treatments (Table 7). These findings have been published in an original article (Farzi et al. 2015a).

Table 7 Effects of LPS (0.1 mg/kg), MDP (3 mg/kg) + LPS and FK565 (0.003 mg/kg) + LPS on depression-like behaviour in the FST and TST 1 day post-treatment in male mice

	Parameter	VEH	LPS	MDP + LPS	FK565 + LPS
FST	Immobility	237.70 ± 30.34	242.14 ± 22.55	285.91 ± 13.11	292.27 ± 9.16
	Swimming	108.25 ± 28.95	102.15 ± 21.77	65.71 ± 13.06	53.95 ± 9.57
	Climbing	14.07 ± 2.04	15.75 ± 4.38	8.40 ± 2.15	13.81 ± 4.86
TST	Immobility	261.25 ± 11.71	241.93 ± 7.33	252.43 ± 12.72	232.01 ± 10.19
	Swinging	40.58 ± 6.89	55.13 ± 7.48	60.19 ± 15.74	56.94 ± 6.41
	Curling	58.19 ± 5.49	62.96 ± 5.79	47.40 ± 6.55	71.07 ± 8.36

Mice were injected i.p. with saline (VEH), LPS, MDP + LPS or FK565 + LPS in two separate experiments. The mice subjected to the FST (21 h post-treatment) were singly-housed, while the mice exposed to the TST (25.5 h post-treatment) were kept in groups of 2. The duration of immobility, swimming, climbing, swinging and curling during the 6-min test session is expressed in seconds. The values are means ± SEM, n = 7–8. These findings have been published in an original article (Farzi et al. 2015a).

3.2.5 Effects of MDP, FK565 and LPS alone and in combination on circulating cytokines (Protocol 4)

MDP, FK565 and LPS, alone and in combination, had distinct effects to enhance the circulating levels of pro-inflammatory cytokines (Figure 10). Three h after injection, there was a significant NOD × LPS interaction with regard to the circulating levels of IFN-ɣ ($F_{(2,39)} = 6.004$, $p < 0.01$), IL-1β ($F_{(2,40)} = 6.274$, $p < 0.01$), IL-6 ($F_{(2,40)} = 7.092$, $p < 0.01$) and TNF-α ($F_{(2,40)} = 7.665$, $p < 0.01$) (Figure 10A–D). Post-hoc analysis revealed that treatment with MDP (3 mg/kg) or FK565 (0.003 mg/kg) alone did not induce significant increases in the plasma levels of the cytokines measured (Figure 10). LPS (0.1 mg/kg) alone increased circulating IL-1β and IL-6 levels compared to VEH (Figure 10B and C). In contrast, treatment with MDP or FK565 + LPS increased the levels of all circulating cytokines under study relative to MDP and FK565, respectively (Figure 10A–D). In addition, the cytokine levels in the MDP + LPS group were significantly higher than in the LPS group and with regard to IL-6 and TNF-α were even larger than in the FK565 + LPS group (Figure 10C and D). The cytokine levels in the FK565 + LPS group were increased compared to LPS for all measured cytokines except TNF-α. Twenty-six h after treatment, the circulating levels of IFN-ɣ, IL-1β, IL-6 and TNF-α had largely decreased in all groups studied and were below the detection limit in many samples (Figure 10E–H). As seen 3 h post-treatment, the highest levels of IFN-ɣ, IL-1β and TNF-α were recorded in the MDP + LPS treatment group, with a trend being evident for TNF-α ($U = 7.000$, $p = 0.054$, Bonferroni correction). IL-6 remained significantly increased in all treatment groups (LPS: $U = 3.000$, $p = 0.018$, Bonferroni correction; MDP + LPS: $U = 2.000$, $p = 0.018$, Bonferroni correction; FK565 + LPS: $U = 2.000$, $p = 0.012$, Bonferroni correction), comparable levels being seen in the MDP + LPS and FK565 + LPS treatment groups (Figure 10G). These findings have been published in an original article (Farzi et al. 2015a).

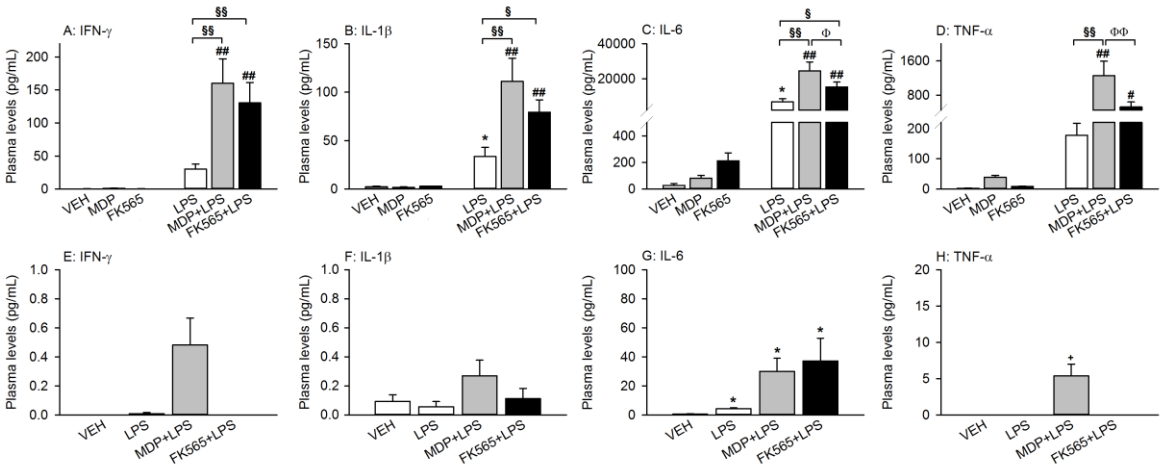

Figure 10 Effects of MDP (3 mg/kg), FK565 (0.003 mg/kg) and LPS (0.1 mg/kg) on circulating cytokine levels 3 h (A–D) and 26 h (E–H) after treatment of male mice.

Mice were injected i.p. with saline (VEH), MDP, FK565, LPS, MDP + LPS or FK565 + LPS. Plasma was collected 3 h (A–D) or 26 h (E–H) later. Circulating levels of IFN-ɣ (A + E), IL-1β (B + F), IL-6 (C + G), and TNF-α (D + H) were measured. Note that the scale of the ordinate is different between the 2 time points. The values are means + SEM, n = 7–8. A–D: Post-hoc analysis of significant NOD × LPS interactions in 2-way ANOVA: *$p < 0.05$, versus VEH. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, MDP + LPS versus MDP or FK565 + LPS versus FK565. $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, MDP + LPS or FK565 + LPS versus LPS. $^{\Phi}p < 0.05$, $^{\Phi\Phi}p < 0.01$, MDP + LPS versus FK565 + LPS. E–H: One-way ANOVA: *$p < 0.05$, **$p < 0.01$, versus VEH. These findings have been published in an original article (Farzi et al. 2015a).

3.2.6 Effects of MDP, FK565 and LPS alone and in combination on cytokine mRNA expression in the brain (Protocol 4)

The expression of cytokine mRNAs in the brain was measured 3 and 26 h after injection of the PRR agonists in order to analyse cytokine expression at the time of predominant sickness and depression-like behaviour, respectively (Figure 11). When cytokine mRNA was assessed 3 h post-treatment, two-way ANOVA revealed a NOD × LPS interaction for the expression of IFN-ɣ mRNA ($F_{(2,42)} = 5.911$, $p < 0.01$) and a trend for IL-6 mRNA expression ($F_{(2,42)} = 2.774$, $p = 0.07$). Post-hoc analysis disclosed that while neither MDP (3 mg/kg), FK565 (0.003 mg/kg) nor LPS (0.1 mg/kg) alone increased mRNA expression of IFN-ɣ or IL-6, combined treatment with MDP + LPS or FK565 + LPS increased IFN-ɣ and IL-6 mRNA expression compared to LPS, MDP and FK565, respectively (Figure 11A and C). In contrast, expression of IL-1β mRNA depended on LPS ($F_{(1,42)} = 24.984$, $p < 0.001$) and the NOD agonists ($F_{(2,42)} = 3.174$, $p \leq 0.05$) without a significant interaction (Figure 11B). Likewise, TNF-α mRNA expression depended on LPS ($F_{(1,42)} = 25.735$, $p <$

0.001) and the NOD agonists ($F_{(2,42)} = 8.535$, $p < 0.001$) without a significant interaction (Figure 11D).

Twenty-six h after treatment, cerebral IFN-ɣ mRNA expression had returned to basal levels in all treatment groups (Figure 11E). Conversely, the expression of IL-1β mRNA remained significantly increased in response to MDP + LPS and FK565 + LPS ($F_{(3,26)} = 11.341$, $p < 0.001$) and enhanced by trend in the LPS group ($p = 0.085$). In addition, IL-1β mRNA expression was significantly higher in the MDP + LPS group compared to the LPS group (Figure 11F). Likewise, TNF-α mRNA expression was increased in every treatment group ($F_{(3,26)} = 9.588$, $p < 0.001$), with the highest expression seen in the MDP + LPS group (Figure 11H). In contrast, IL-6 mRNA expression was decreased in all treatment groups ($F_{(3,26)} = 13.621$, $p < 0.001$) (Figure 11G). These findings have been published in an original article (Farzi et al. 2015a).

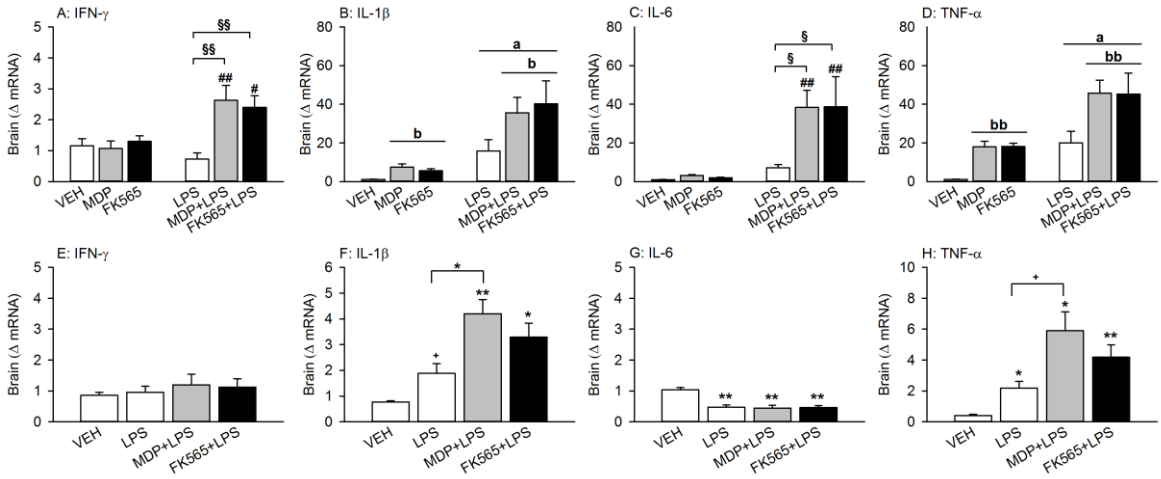

Figure 11 Effects of MDP (3 mg/kg), FK565 (0.003 mg/kg) and LPS (0.1 mg/kg) on cytokine mRNA expression in the brain 3 h (A–D) and 26 h (E–H) after injection in male mice.

Mice were injected i.p. with saline (VEH), MDP, FK565, LPS, MDP + LPS or FK565 + LPS. Expression of IFN-ɣ (A + E), IL-1β (B + F), IL-6 (C + G), and TNF-α (D + H) was measured 3 h (A–D) or 26 h (E–H) after injection. Note that the scale of the ordinate is different between the 2 time points. The values are means + SEM, n = 7–8. A–D: Post-hoc analysis of significant NOD × LPS interactions in 2-way ANOVA: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, MDP + LPS versus MDP or FK565 + LPS versus FK565. $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, MDP + LPS and FK565 + LPS versus LPS. Main factor effects without NOD × LPS interactions: $^{a}p < 0.01$, LPS versus VEH. $^{b}p < 0.05$, $^{bb}p < 0.01$, NOD agonists versus VEH. E–H: One-way ANOVA: $^{+}p < 0.1$, $*p < 0.05$, $**p < 0.01$, versus VEH or as indicated by the brackets. These findings have been published in an original article (Farzi et al. 2015a).

3.2.7 Effects of MDP, FK565 and LPS alone and in combination on cytokine protein expression in the brain (Protocol 4)

Protein levels of cytokines in the frontal cortex were measured 3 and 26 h after injection of the PRR agonists in order to analyse cytokine levels at the time of predominant sickness and depression-like behaviour, respectively (Figure 12). While protein levels of IFN-γ were not detectable in any group, MDP or FK565 alone did not increase the brain levels of IL-1β, IL-6 or TNF- α 3 h after injection (Figure 12A-C).

Two-way ANOVA revealed that cerebral IL-1β protein levels depended on LPS ($F_{(1,42)}$ = 7.202, p = 0.01) without a significant NOD × LPS interaction (Figure 12A). In contrast, the cerebral protein levels of IL-6 and TNF- α were significantly increased by the combination of MDP + LPS and FK565 + LPS (Figure 12B,C). Thus, there was a significant NOD × LPS interaction for the expression of IL-6 ($F_{(2,42)}$ = 5.122, p = 0.01) and TNF- α ($F_{(2,42)}$ = 5.384, p = 0.008).

Twenty-six h after treatment, the brain levels of IL-1β and IL-6 had returned to basal values and in every treatment group were indistinguishable from those measured in the VEH-treated group (Figure 12D,E). The cerebral TNF-α levels had decreased below the detection limit at this time point.

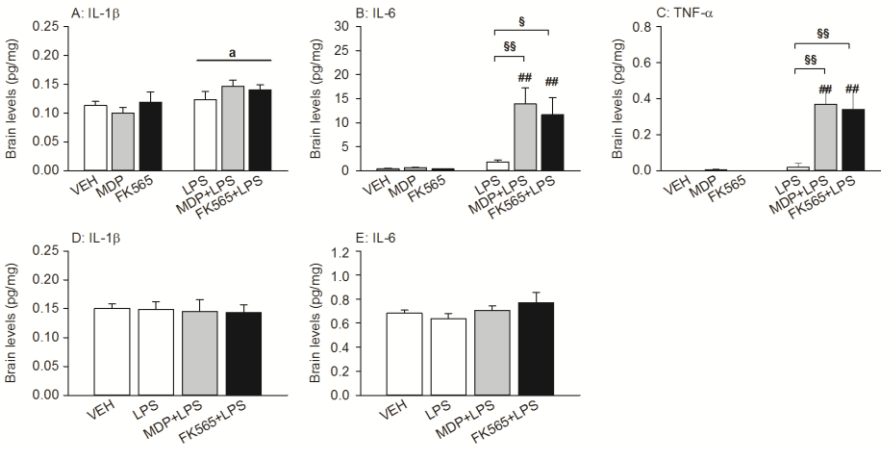

Figure 12 Effects of MDP (3 mg/kg), FK565 (0.003 mg/kg) and LPS (0.1 mg/kg) on cytokine protein levels in the frontal cortex 3 h (A-C) and 26 h (D+E) after injection in male mice.

Mice were injected i.p. with saline (VEH), MDP (3 mg/kg), FK565 (0.003 mg/kg), LPS (0.1 mg/kg), MDP + LPS or FK565 + LPS. Brains were collected 3 h (A–C) or 26 h (D + E) later. The frontal cortex was dissected and protein levels of IL-1β (A + D), IL-6 (B + E) and TNF-α (C) were measured. Note that the scale of the ordinate is different between the 2 time points. The values are means + SEM, n = 7–8. A–C: Post-hoc analysis of significant NOD × LPS interactions in 2-way ANOVA: $^{\#\#}p < 0.001$, MDP + LPS versus MDP or FK565 + LPS versus FK565. $^{\S}p < 0.01$, $^{\S\S}p < 0.001$, MDP + LPS and FK565 + LPS versus LPS. Main factor effects without NOD × LPS interactions: $^{a}p \leq 0.01$, LPS versus VEH.

3.2.8 Effects of MDP, FK565 and LPS alone or in combination on circulating levels of corticosterone and the kynurenine/tryptophan ratio (Protocol 4)

The PRR agonists under study had a distinct effect to enhance the plasma levels of CORT as measured 3 h after injection. Two-way ANOVA revealed a significant main factor effect for LPS ($F_{(1,40)} = 76.581$, $p < 0.001$) and the NOD agonists ($F_{(2,40)} = 16.608$, $p < 0.001$) without a significant interaction. Post-hoc analysis of the main factor effects disclosed that FK565 increased circulating CORT compared to VEH and MDP (Figure 13A). One day after treatment, the plasma levels of CORT were examined 30 min after exposure to the TST. Under these conditions, the circulating levels of CORT were enhanced in the MDP + LPS treatment group relative to the vehicle (but not LPS) treatment group and remained unchanged in the LPS and FK565 + LPS treatment groups ($F_{(3,26)} = 4.282$, $p < 0.05$) (Figure 13B).

The plasma kynurenine/tryptophan ratio, measured 26 h after treatment, was significantly increased following injection of LPS, MDP + LPS and FK565 + LPS ($F_{(3,26)} = 10.160$, $p < 0.001$), this increase being more pronounced after treatment with MDP + LPS. Particularly, the plasma kynurenine/tryptophan ratio in the MDP + LPS treatment group was significantly larger than in the LPS-treated group (Figure 13C).

A similar picture emerged for the circulating levels of kynurenine (Figure 13D). Kynurenine levels were increased by LPS, MDP + LPS and FK565 + LPS ($F_{(3,26)} = 12.098$, $p < 0.001$). As for the kynurenine/tryptophan ratio, the kynurenine levels in the MDP + LPS group were significantly higher than in the LPS group, while the levels in the FK565 + LPS group were increased by trend only compared to LPS alone ($p = 0.077$). The levels of tryptophan were increased by MDP + LPS and FK565 + LPS, while LPS alone did not change the plasma tryptophan levels ($F_{(3,26)} = 11.207$, $p < 0.001$) (Figure 13E). Furthermore, the tryptophan levels in the FK565 + LPS group were significantly higher than in the LPS group. These findings have been published in an original article (Farzi et al. 2015a).

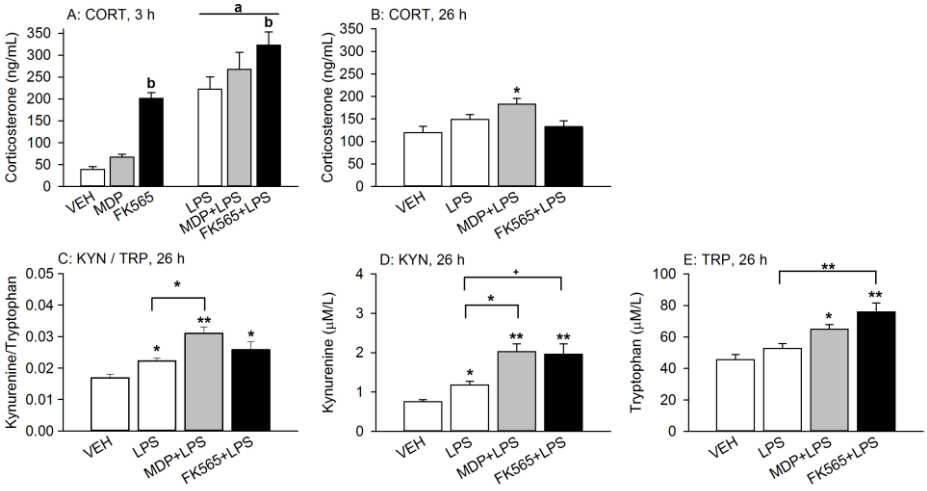

Figure 13 Effects of MDP (3 mg/kg), FK565 (0.003 mg/kg) and LPS (0.1 mg/kg) on circulating corticosterone (CORT) levels 3 h (A) and 26 h (B) after treatment, as well as on the kynurenine/tryptophan (KYN/TRP) ratio (C) and circulating levels of kynurenine (D) and tryptophan (E) measured 26 h after treatment of male mice.

Plasma CORT was measured 3 h after treatment (A) and 26 h after treatment, 30 min following exposure to tail suspension stress (B). Likewise, plasma kynurenine and tryptophan (C–E) were determined 26 h after treatment, 30 min following exposure to tail suspension stress. The values are means + SEM, n = 7–8. A: Main factor effects without NOD × LPS interactions: $^{a}p < 0.001$, LPS versus VEH. $^{b}p < 0.001$, FK565 versus VEH or MDP. B–E: One-way ANOVA: $^{+}p < 0.1$, $*p < 0.05$, $**p < 0.01$, versus VEH or as indicated by the brackets. These findings have been published in an original article (Farzi et al. 2015a).

3.2.9 Effects of MDP and LPS (0.83 mg/kg) alone and in combination on c-Fos expression in the brain (Protocol 3)

In order to analyse brain circuits that are associated with the observed effects of MDP (3 mg/kg) and LPS (0.83 mg/kg), the expression of c-Fos was studied by immunohistochemistry in select brain areas involved in sickness. Two-way ANOVA revealed a significant NOD × LPS interaction in the PVN ($F_{(1,11)} = 18.810$, $p < 0.001$), insula ($F_{(1,13)} = 6.940$, $p < 0.05$) and SO ($F_{(1,13)} = 17.496$, $p \leq 0.001$) and an interaction approaching significance in the BNSTv ($F_{(1,15)} = 4.257$, $p = 0.057$). Post-hoc analysis disclosed that MDP alone did not change c-Fos expression in these areas, while LPS alone increased c-Fos expression in the BNSTv and PVN compared to VEH (Figure 14A and C). In contrast, MDP + LPS increased c-Fos expression in all 4 areas relative to MDP or LPS (Figure 14A,C,E and F). While LPS had a significant main factor effect in all other areas under study (BNSTd: $F_{(1,13)} = 16.883$, $p < 0.001$; CeA: $F_{(1,15)} = 80.556$, $p < 0.001$; SFO: $F_{(1,14)} = 11.334$, $p < 0.01$; DG: $F_{(1,15)} = 39.727$, $p < 0.001$), a significant main factor effect

of the NOD agonist MDP was evident in the CeA ($F_{(1,15)} = 14.296$, $p < 0.01$) and by trend in the BNSTd ($F_{(1,13)} = 3.237$, $p < 0.1$) (Figure 14B,D,G and H). The effect of MDP + LPS to increase the number of c-Fos positive cells in the SFO, relative to LPS, was statistically not significant (Figure 14G). Representative micrographs showing the effects of MDP, LPS and MDP + LPS on the expression of c-Fos in the cerebral areas under study are shown in Figure 15. These findings have been published in an original article (Farzi et al. 2015a).

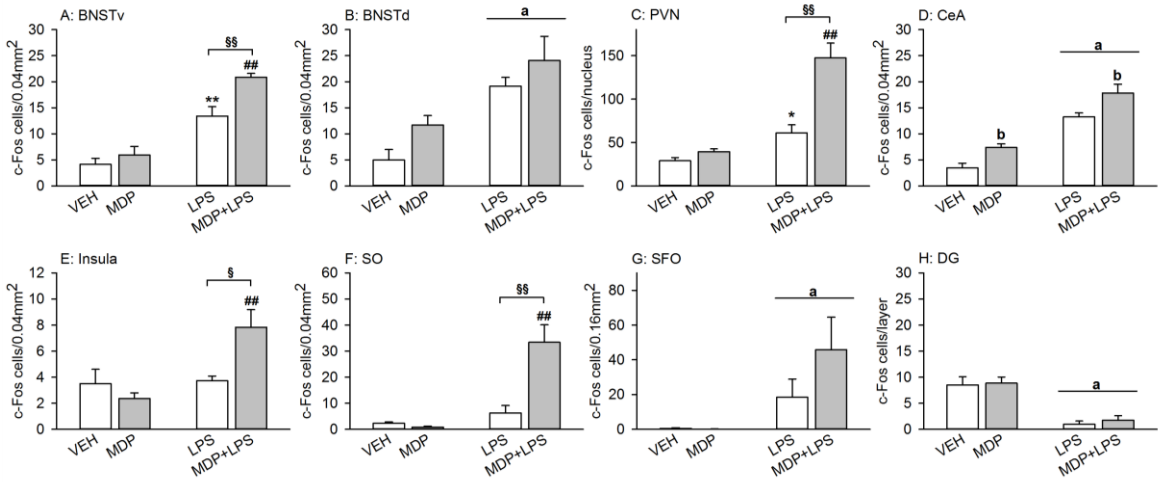

Figure 14 Effects of MDP (3 mg/kg), LPS (0.83 mg/kg) and MDP + LPS on c-Fos expression 3 h after treatment of male mice. Mice were injected i.p. with saline (VEH), MDP, LPS or MDP + LPS.
Brains were collected 3 h post-treatment and the expression of c-Fos, a marker of neuronal activation, was measured by immunohistochemistry. The values are means + SEM, n = 3–5. Post-hoc analysis of significant NOD × LPS interactions in 2-way ANOVA: *$p < 0.05$, **$p < 0.01$, versus VEH. $^{\#\#}p < 0.01$, MDP + LPS versus MDP. $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, MDP + LPS versus LPS. Main factor effects without NOD × LPS interactions: $^{a}p < 0.01$, LPS versus VEH. $^{b}p < 0.01$, NOD agonist versus VEH. Abbreviations: BNSTd/v = bed nucleus of the stria terminalis dorsal/ventral, CeA = central amygdala, DG = dentate gyrus, PVN = paraventricular nucleus of the hypothalamus, SFO = subfornical organ, SO = supraoptic nucleus. These findings have been published in an original article (Farzi et al. 2015a).

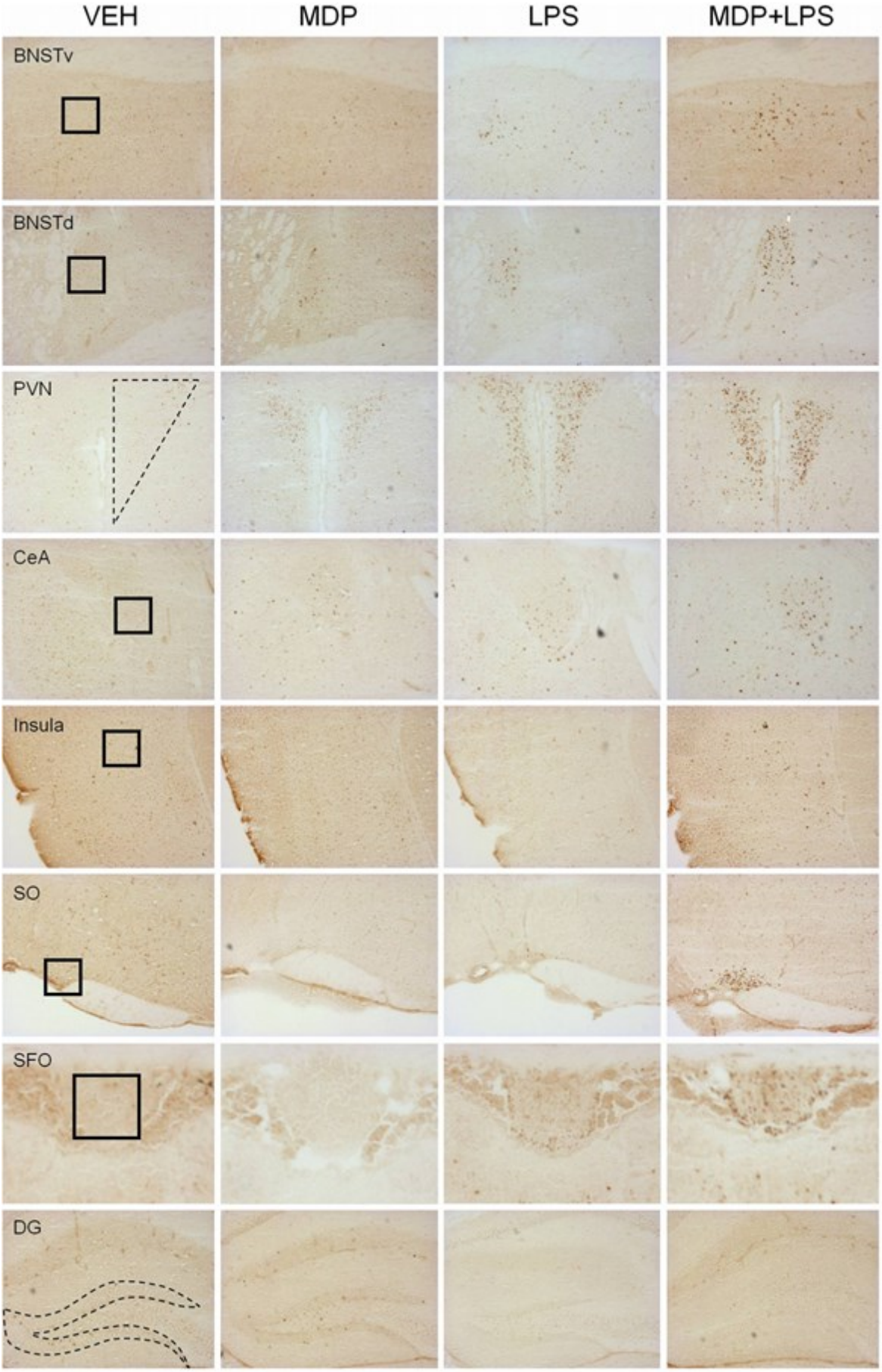
VEH
MDP
LPS
MDP+LPS
BNSTv
BNSTd
PVN
CeA
Insula
SO
SFO
DG

Figure 15 Representative micrographs of forebrain regions illustrating c-Fos immunoreactivity induced by MDP (3 mg/kg), LPS (0.83 mg/kg) and MDP + LPS in male mice.

The left column panels show micrographs of forebrain regions taken from saline (VEH)-treated mice euthanized 3 h after injection. The second column panels depict micrographs of the same brain regions taken from MDP-treated mice, while the third column panels show micrographs of LPS-treated mice. The right column panels represent c-Fos immunolabeling induced by MDP + LPS 3 h after treatment. The squares in the left column represent the position and size of the ROIs. Abbreviations: BNSTd/v = bed nucleus of the stria terminalis dorsal/ventral, CeA = central amygdala, DG = dentate gyrus, PVN = paraventricular nucleus of the hypothalamus, SFO = subfornical organ, SO = supraoptic nucleus. These findings have been published in an original article (Farzi et al. 2015a).

3.2.10 Effects of MDP, FK565 and LPS alone and in combination on colitis (Protocol 3 and 4)

In order to assess colonic inflammation, tissue levels of MPO, an enzyme mainly found in neutrophils (Krawisz, Sharon & Stenson 1984), were quantified.

Two-way ANOVA of the results with the higher dose of LPS (0.83 mg/kg) demonstrated that there was a significant NOD × LPS interaction for the colonic MPO levels measured 3 days post-treatment ($F_{(2,40)} = 3.839$, $p = 0.030$) (Figure 16A). Post-hoc analysis of the main factor effects disclosed that FK565 + LPS (0.83 mg/kg) induced an increase of colonic MPO compared to FK565, LPS (0.83 mg/kg) and MDP + LPS (Figure 16A).

Likewise, the result of the experiment with the lower dose of LPS (0.1 mg/kg) disclosed that treatment with FK565 + LPS (0.1 mg/kg) resulted in an elevation of colonic MPO content compared to LPS (0.1 mg/kg) alone when assessed 1 day after treatment ($F_{(3,26)} = 4.158$, $p = 0.016$) (Figure 16B).

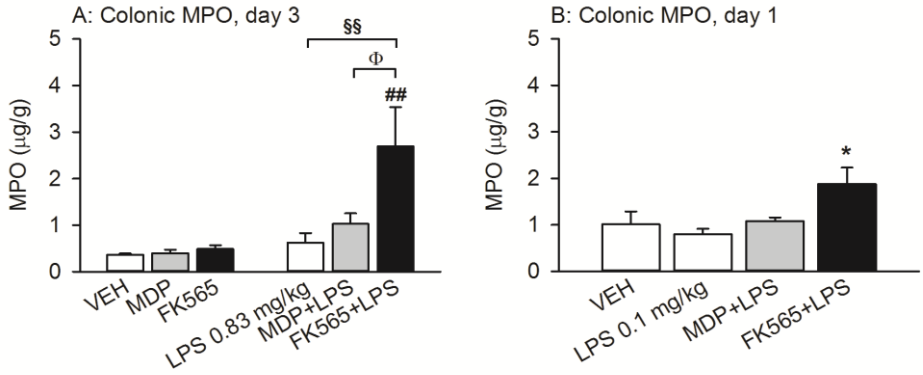

Figure 16 Effects of MDP (3 mg/kg), FK565 (0.003 mg/kg) and LPS (doses as indicated) on colonic myeloperoxidase (MPO) content of male mice.

Mice were injected i.p. with saline (VEH), MDP, FK565, LPS, MDP + LPS or FK565 + LPS. Colonic MPO content was quantified 3 days (A) or 1 day (B) after injection. The values are means + SEM, n = 6–8. A: Post-hoc analysis of significant NOD × LPS interactions in 2-way ANOVA: $^{\#\#}p < 0.001$, FK565 + LPS versus FK565. $^{\S\S}p \leq 0.001$, FK565 + LPS versus LPS. $^{\Phi}p < 0.05$, FK565 + LPS versus MDP + LPS. B: One-way ANOVA: $^{*}p < 0.05$ versus LPS.

3.2.11 Effects of MDP, FK565 and LPS alone and in combination on faecal boli expelled during the OFT (Protocol 3 and 4)

While the number of faecal boli expelled during the OFT is generally used as a further evidence of anxiety, it is also likely to reflect motor function of the intestinal tract. The NOD × LPS interaction with respect to the number of boli expelled during the OFT did not reach significance in the experiments with the higher dose of LPS ($F_{(2,40)} = 1.900$, $p = 0.163$) (n = 7–8) (Figure 17A). However, one-way ANOVA of the experiments with the lower dose of LPS demonstrated that the MDP + LPS-treated mice expelled less boli during the OFT than LPS-treated mice ($F_{(3,24)} = 4.487$, $p = 0.012$) (n = 6–7) (Figure 17B).

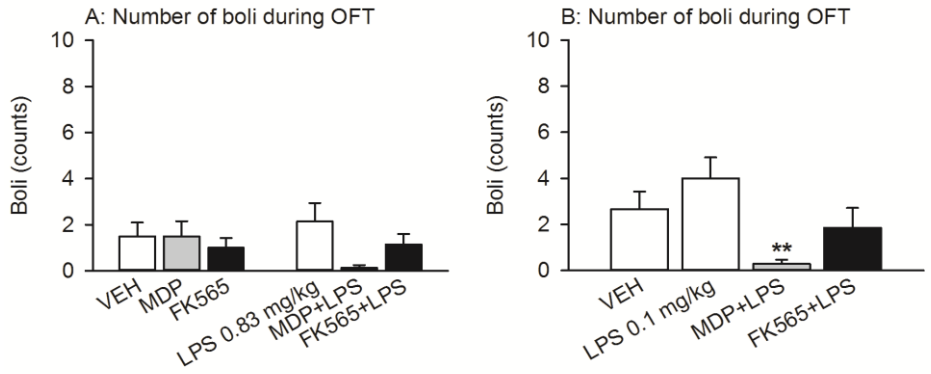

Figure 17 Effects of MDP (3 mg/kg), FK565 (0.003 mg/kg) and LPS (doses as indicated) on the number of boli expelled during the OF 21 h post-treatment in male mice.

The graphs show the number of boli expelled during the 5-min test session of the experiments applying the higher dose of LPS (0.83 mg/kg) (A) and the lower dose of LPS (0.1 mg/kg) (B). The values are means + SEM, n = 6–8. **p < 0.01, versus LPS (0.1 mg/kg).

3.3 The sickness response of NPY$^{-/-}$;PYY$^{-/-}$ double KO mice to NOD2 and TLR4 activation (Protocol 5)

3.3.1 Effects of LPS with or without MDP on body temperature and weight in male NPY$^{-/-}$;PYY$^{-/-}$ double KO mice

Studies administrating NPY or knocking out NPY, PYY or their respective receptors have demonstrated that the NPY family is able to attenuate the sickness response to immune challenge (Farzi, Reichmann & Holzer 2015). Therefore the response of NPY$^{-/-}$;PYY$^{-/-}$ double KO mice to LPS, on the one hand, and MDP + LPS, on the other hand were investigated.

Two-way ANOVA demonstrated that the body temperature measured both 7 and 33 h post-treatment differed solely with treatment (7 h: $F_{(2,41)} = 26.272$, $p < 0.001$, 33 h: $F_{(2,42)} = 4.856$, $p = 0.013$), without a significant interaction between genotype and treatment (Figure 18A,B).

The weight change of the animals 1 day after treatment depended on their genotype ($F_{(1,42)} = 18.180$, $p < 0.001$) and on the treatment ($F_{(2,42)} = 3.934$, $p = 0.027$), without a significant interaction between these factors (Figure 18C). The change in body weight on the second day after treatment depended on the treatment solely ($F_{(2,42)} = 6.271$, $p = 0.004$), without a significant interaction between genotype and treatment (Figure 18D).

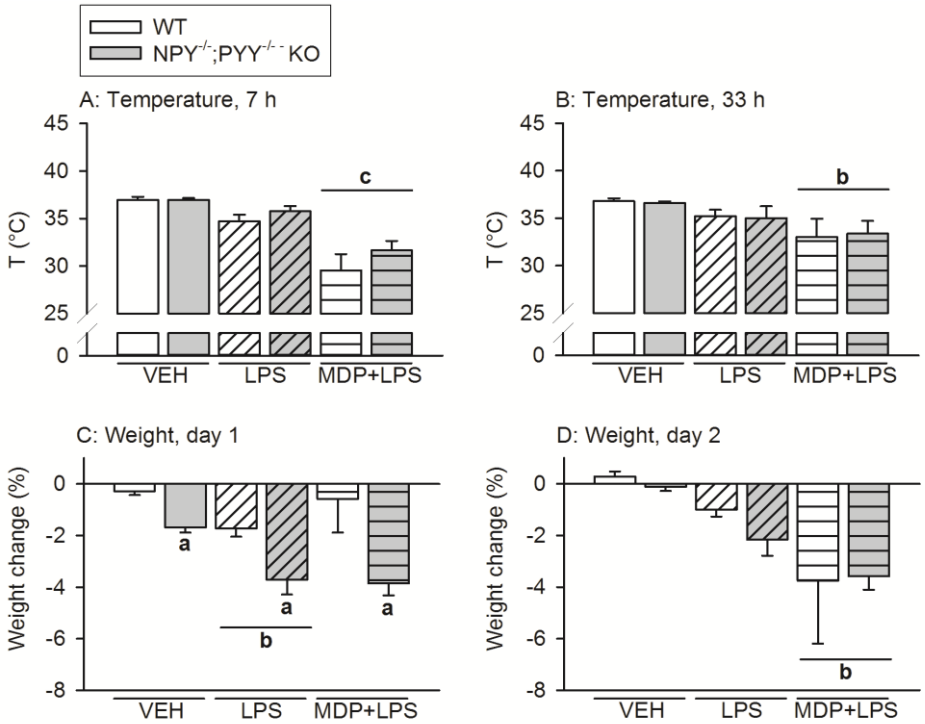

Figure 18 Effects of MDP (3 mg/kg) and LPS (0.83 mg/kg) to reduce body temperature (A + B) and weight (C + D) in male mice.

The graphs show the change in temperature 7 h (A) and 33 h (B) post-treatment and body weight 1 day (C) and 2 days (D) post-treatment. Body weight was measured before treatment and the weight loss induced by the treatment is expressed as a percentage of the body weight measured pre-treatment. The values are means + SEM, n = 8. Main factor effects without genotype × treatment interactions: $^{a}p < 0.01$, KO versus WT. $^{b}p < 0.05$, indicated treatment versus VEH. $^{c}p < 0.001$, indicated treatment versus VEH and LPS.

3.3.2 Effects of LPS with or without MDP on anxiety and locomotion of male NPY$^{-/-}$;PYY$^{-/-}$ double KO mice

The behaviour of the mice in the OFT as assessed 3 h post-treatment depended on their genotype and treatment with respect to the total travelling distance (genotype: $F_{(1,38)} = 4.263$, $p = 0.046$, treatment: $F_{(2,38)} = 20.181$, $p < 0.001$) and central area entries (genotype: $F_{(1,38)} = 5.218$, $p = 0.028$, treatment: $F_{(2,38)} = 16.540$, $p < 0.001$) without an interaction between genotype and treatment (Figure 19B,C). Analysis of the time spent in the central area of the OF revealed a significant interaction between genotype and treatment ($F_{(2,38)} = 3.918$, $p = 0.028$). Specifically, KO mice treated with MDP + LPS spent significantly less time in the central area of the OF than MDP + LPS-treated WT mice (Figure 19A). The number of boli expelled during the OFT depended on the treatment ($F_{(2,37)} = 13.283$, $p < 0.001$) without an interaction with the genotype of the mice (Figure 19D).

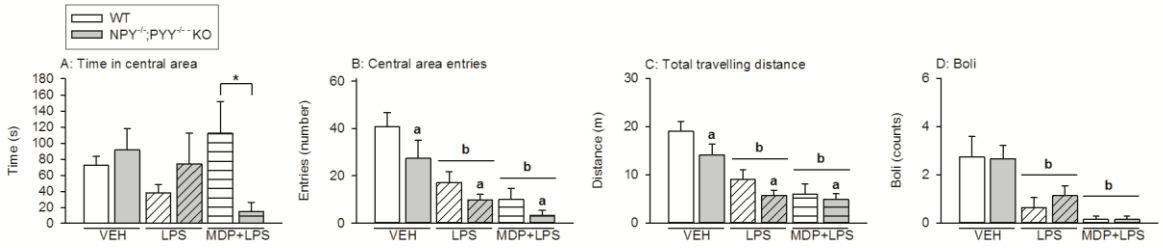

Figure 19 Effects of MDP (3 mg/kg) and LPS (0.83 mg/kg) on behaviour in the OF 3 h post-treatment in male mice.

The graphs show the time spent in the central area (A), the number of entries into the central area (B), the total distance travelled (C) and the number of boli expelled (D) during the 5-min test session. The values are means + SEM, n = 6–8. (A): Post-hoc analysis of significant genotype × treatment interactions in 2-way ANOVA: *$p < 0.05$ as indicated by the bracket. Main factor effects without genotype × treatment interactions: [a]$p < 0.05$, KO versus WT. [b]$p < 0.01$, indicated treatment versus VEH.

3.3.3 Effects of LPS with or without MDP on depression-like behaviour of male NPY$^{-/-}$;PYY$^{-/-}$ double KO mice

The behaviour of WT and NPY$^{-/-}$;PYY$^{-/-}$ mice in the FST 1 day post-treatment differed with genotype but not treatment with regard to the time spent immobile ($F_{(1,40)} = 50.756$, $p < 0.001$) and time spent swimming ($F_{(1,40)} = 21.789$, $p < 0.001$). The time spent climbing was affected by the genotype ($F_{(1,40)} = 32.562$, $p < 0.001$) and by the treatment ($F_{(2,40)} = 4.127$, $p = 0.023$) (Figure 20). There was no interaction between genotype and treatment for any factor.

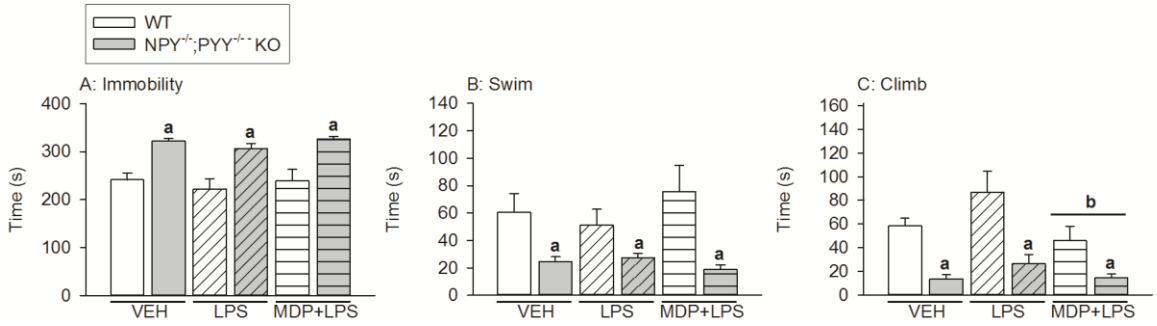

Figure 20 Effects of MDP (3 mg/kg) and LPS (0.83 mg/kg) on depression-like behaviour in the FST 1 day post-treatment in male mice.

The graphs show the time spent immobile (A), swimming (B), and climbing (C) during the 6-min test session. The values are means + SEM, n = 6–8. Main factor effects without genotype × treatment interactions: [a]$p < 0.001$, KO versus WT. [b]$p < 0.05$, indicated treatment versus LPS.

3.4 Mood disturbances by alterations of the intestinal microbiota (Protocol 6)

3.4.1 Oral treatment with neomycin or vancomycin does not affect behaviour in male mice

A one-week treatment with neomycin (5 mg/ml) or vancomycin (1 mg/ml) via the drinking water did not modulate behaviour of adult male mice in the OFT (time spent in the central area: $F_{(2,27)} = 0.953$, $p = 0.398$; number of entries into the central area: $F_{(2,27)} = 2.391$, $p = 0.111$; total travelling distance: $F_{(2,27)} = 1.643$, $p = 0.212$; number of boli expelled: $F_{(2,27)} = 1.932$, $p = 0.164$) ($n = 10$) (Table 8).

Likewise, depression-like behaviour as assessed with the FST 8 days after start of the treatment was not changed by neomycin or vancomycin (time spent immobile: $F_{(2,27)} = 0.984$, $p = 0.387$; time spent swimming: $F_{(2,27)} = 0.059$, $p = 0.943$; time spent climbing: $F_{(2,27)} = 0.950$, $p = 0.399$) ($n = 10$) (Table 8).

Similarly, neither body weight ($F_{(2,27)} = 0.268$, $p = 0.767$) nor spleen weight ($F_{(2,27)} = 2.331$, $p = 0.116$) were affected by treatment with neomycin or vancomycin 9 days after start of the treatment ($n = 10$) (Table 8).

Table 8 Treatment with neomycin or vancomycin does not affect locomotion, mood or body weight

Test	Readout + time	NT	Neomycin	Vancomycin
OFT	Time in centre (s)	83.10 ± 10.33	65.85 ± 9.48	77.76 ± 7.00
	Central entries	34.70 ± 2.55	27.00 ± 2.31	33.80 ± 3.23
	Total distance (m)	28.38 ± 1.31	24.36 ± 1.47	26.19 ± 1.88
	Boli	6.60 ± 0.81	6.00 ± 1.23	4.20 ± 0.51
FST	Immobility (s)	332.22 ± 3.84	325.28 ± 3.75	329.88 ± 3.03
	Swimming (s)	16.87 ± 1.87	16.87 ± 2.18	16.02 ± 1.99
	Climbing (s)	10.93 ± 4.08	17.87 ± 3.77	14.13 ± 2.69
Weight	Body D9	0.041 ± 0.02	0.031 ± 0.01	0.043 ± 0.01
	Spleen D9	0.264 ± 0.01	0.244 ± 0.01	0.232 ± 0.01

Mice were subjected to neomycin (5 mg/ml) or vancomycin (1 mg/ml) administered via the drinking water for 9 days. OFT was performed 7 days after start of the antibiotic treatment. FST was conducted 8 days after the start of treatment, while body and spleen weight were assessed 9 days after start of the treatment. The table shows the parameters measured during the 5-min test session of the OFT, the 6-min test session of the FST, the change of body weight and spleen weight. The weight change is expressed as a percentage of the body weight measured pre-treatment and spleen weight as a percentage of the body weight. The values are means ± SEM, n = 10. Abbreviations: D = day, m = metre, NT = no treatment, s = seconds.

3.4.2 Behavioural effects of ampicillin, metronidazole and the combination of ampicillin, metronidazole, neomycin and vancomycin

While the antibiotic solution containing ampicillin (2 mg/ml) was tolerated well, the metronidazole (2 mg/ml) solution and the quadruple treatment containing ampicillin (1 mg/ml), metronidazole (1 mg/ml), neomycin (1 mg/ml), and vancomycin (0.5 mg/ml) led to a decrease of water consumption by more than 50 % during the first 3 days of treatment ($F_{(3,4)} = 25.165$, $p = 0.005$) (Table 9). In accordance with this observation a significant weight loss of the animals of these two treatment groups was observed on day 9 of the treatment ($F_{(3,29)} = 26.447$, $p < 0.001$) (n = 8-9) (Table 9). Antibiotic treatment led to a decrease of spleen weight in all treatment groups ($F_{(3,29)} = 24.589$, $p < 0.001$), while the caecum weight was increased in all treatment groups ($F_{(3,29)} = 23.272$, $p < 0.001$) (n = 8-9) (Table 9).

One-way ANOVA of the OFT performed on day 7 of the treatment did not demonstrate any significant effects with regard to the time spent in the central area ($F_{(3,29)} = 1.494$, $p = 0.237$), the total travelling distance ($F_{(3,29)} = 1.536$, $p = 0.226$), or the number of boli

expelled ($F_{(3,28)}$ = 0.495, p = 0.689) (n = 8-9) (Table 9). However, the number of entries into the central area approached significance ($F_{(3,29)}$ = 2.512, p = 0.078) and the quadruple treatment lead to a decrease of the central area entries by trend (p = 0.082) (n = 8-9) (Table 9).

None of the treatment regimens had an effect on behaviour of the mice in the FST (time spent immobile: $F_{(3,27)}$ = 2.662, p = 0.068; time spent swimming: $F_{(3,27)}$ = 0.243, p = 0.865; time spent climbing: $F_{(3,27)}$ = 2.396, p = 0.090) (n = 6-9) (Table 9). The nominal decrease of the time spent floating and the nominal increase of the time spent climbing in the metronidazole and the quadruple treatment group was likely due to difficulties in floating caused by the ruffled fur as observed in these treatment groups.

Table 9 Effects of ampicillin, metronidazole and the combination of ampicillin, metronidazole, neomycin and vancomycin

Test	Readout + time	NT	Ampicillin	Metronidazole	AMNV
OFT	Time in centre (s)	54.88 ± 9.92	46.38 ± 11.55	50.88 ± 8.29	29.01 ± 6.59
	Central entries	26.13 ± 2.78	23.00 ± 4.40	24.44 ± 3.75	**13.63 ± 2.65^{+}**
	Total distance (m)	18.64 ± 1.27	15.94 ± 1.92	16.69 ± 1.04	14.65 ± 0.91
	Boli	0.63 ± 0.38	1.63 ± 0.93	1.00 ± 0.38	1.00 ± 0.50
FST	Immobility (s)	274.65 ± 6.68	261.81 ± 15.95	225.71 ± 16.94	225.33 ± 20.41
	Swimming (s)	21.05 ± 2.91	20.80 ± 4.23	25.01 ± 7.14	26.19 ± 5.73
	Climbing (s)	64.31 ± 8.59	77.41 ± 12.94	109.31 ± 13.90	108.50 ± 24.12
Weight	Fluid intake D1-3	12.465 ± 0.639	11.415 ± 1.775	**4.540 ± 0.366***	**2.156 ± 0.630***
	Body D9	1.719 ± 0.24	1.420 ± 0.45	**-2.721 ± 0.60****	**-3.121 ± 0.60****
	Spleen D9	0.323 ± 0.02	**0.259 ± 0.01***	**0.192 ± 0.02****	**0.145 ± 0.02****
	Caecum D9	0.794 ± 0.06	**1.735 ± 0.09****	**1.522 ± 0.13****	**1.735 ± 0.06****

Mice were subjected to ampicillin (2 mg/ml), metronidazole (2 mg/ml) or a combination of ampicillin (1 mg/ml), metronidazole (1 mg/ml), neomycin (1 mg/ml), and vancomycin (0.5 mg/ml) administered via the drinking water for 9 days. The OFT was performed 7 days after start of the antibiotic treatment while the FST was conducted 8 days after the start of treatment. Body, spleen and caecum weight were assessed 9 days after start of the treatment. The table shows the parameters measured during the 5-min test session of the OFT, the 6-min test session of the FST, as well as fluid intake during the first 3 days of treatment and the change of body, spleen, and caecum weight. The body weight change is expressed as a percentage of the weight measured pre-treatment. Spleen- and caecum weight are expressed as a percentage of the body weight. The values are means ± SEM, n = 6-9. Significant results ($^{+}p < 0.1$, $*p < 0.05$, $**p < 0.001$, versus VEH) are bolded. Abbreviations: AMNV = ampicillin + metronidazole + neomycin + vancomycin, m = metre, D = day, NT = no treatment, s = seconds.

3.4.3 Behavioural effects of cefoperazone, neomycin and the combination of ampicillin, cefoperazone and neomycin

One-way ANOVA of the OFT performed on day 6 of the treatment did not demonstrate any significant effects of cefoperazone (1 mg/ml), neomycin (10 mg/ml) or the triple combination of ampicillin (2 mg/ml), cefoperazone (0.5 mg/ml) and neomycin (5 mg/ml) with regard to the time spent in the central area ($F_{(3,24)} = 1.159$, $p = 0.346$), the number of entries made into the central area ($F_{(3,24)} = 1.242$, $p = 0.317$) and the number of boli expelled ($F_{(3,24)} = 1.213$, $p = 0.326$) (n = 6-8). However, a nominal increase of the time spent in the central area was evident in the treatment group receiving the triple antibiotic treatment (Table 10). Moreover, the total travelling distance was decreased in the neomycin-treated group ($F_{(3,24)} = 3.690$, $p = 0.026$).

Learning and memory as assessed by the MI in the NORT was not significantly different between the treatment groups ($F_{(3,21)} = 2.153$, $p = 0.124$) (n = 6-7). However, in the triple antibiotic treatment group, the MI was nominally lower compared to the other groups ($p = 0.153$) (Table 10).

Triple treatment with ampicillin, cefoperazone and neomycin also led to a decrease of spleen weight, while no change was induced by the single antibiotic treatments ($F_{(3,24)} = 4.485$, $p = 0.012$) (n = 6-8). In contrast, all treatment groups showed an increase of caecum weight ($F_{(3,24)} = 23.906$, $p < 0.01$) (n = 6-8) (Table 10).

Table 10 Effects of cefoperazone and the combination of ampicillin, cefoperazone and neomycin

Test	Readout + time	NT	Cefoperazone	Neomycin	ACN
OFT	Time in centre (s)	65.28. ± 5.76	68.23 ± 7.61	68.58 ± 14.89	92.62 ± 13.18
	Central entries	27.83 ± 3.70	26.14 ± 3.45	22.75 ± 3.27	31.00 ± 2.14
	Total distance (m)	22.14 ± 1.61	20.64 ± 1.21	**16.59 ± 1.48***	21.30 ± 0.86
	Boli	0.17 ± 0.17	0.71 ± 0.36.	0.38 ± 0.26	0.86 ± 0.26
NORT	Memory index	0.39 ± 0.20	0.16 ± 0.17	0.37 ± 0.19	-0.18 ± 0.14
Weight	Body D8	1.563 ± 0.21	2.031 ± 0.65	0.393 ± 0.24	0.584 ± 0.27
	Spleen D8	0.274 ± 0.01	0.248 ± 0.01	0.245 ± 0.01	**0.226 ± 0.01***
	Caecum D8	0.780 ± 0.07	**2.130 ± 0.12****	**1.960 ± 0.11****	**1.844 ± 0.15****

Mice were subjected to cefoperazone (1 mg/ml), neomycin (10 mg/ml) or a combination of ampicillin (2 mg/ml), cefoperazone (0.5 mg/ml) and neomycin (5 mg/ml) administered via the drinking water for 8 days. The OFT was performed 6 days after start of the antibiotic treatment while the NORT was conducted 7 days after the start of treatment. Body, spleen and caecum weight were assessed 8 days after start of the treatment. The table shows the parameters measured during the 5-min test session of the OFT, the 5-min test session of the NORT, as well as the change of body, spleen, and caecum weight. The body weight change is expressed as a percentage of the weight measured pre-treatment. Spleen and caecum weight are expressed as a percentage of the body weight. The values are means ± SEM, n = 6-8. Significant results (*$p < 0.05$, **$p < 0.001$, versus VEH) are bolded. Abbreviations: ACN = ampicillin + cefoperazone + neomycin, D = day, m = metre, NT = no treatment, s = seconds.

3.4.4 Quintuple antibiotic treatment induces behavioural changes reminiscent of germ-free mice

3.4.4.1 Administration of a quintuple antibiotic combination induces an anxiolytic phenotype on the EPM

In order to amplify the effects seen with the triple antibiotic treatment, bacitracin and meropenem were added to the combination treatment and cefoperazone was replaced by vancomycin. Thus the quintuple antibiotic treatment consisted of ampicillin (2 mg/ml), bacitracin (5 mg/ml), meropenem (1 mg/ml), neomycin (5 mg/ml) and vancomycin (0.3 mg/ml) and was applied via the drinking water for 11 days.

Following this treatment, mice displayed an anxiolytic phenotype on the EPM, 8 days after start of the treatment (Figure 21). In detail, the number of entries made into the open arms

of the maze was significantly higher in the antibiotic-treated group compared to the control group ($t_{(16)}$ = -2.162, p = 0.046) (n = 8–10) (Figure 21B). Similarly, the time spent on the open arms was higher by trend in the antibiotic-treated group ($t_{(16)}$ = -1.859, p = 0.082) (n = 8–10) (Figure 21A). While the total entries made into any arm were not different between the groups ($t_{(16)}$ = 0.023, p = 0.982) (Figure 21C), the total travelling distance was decreased by trend in the antibiotic-treated group ($t_{(16)}$ = 1.853, p = 0.082) (Figure 21D).

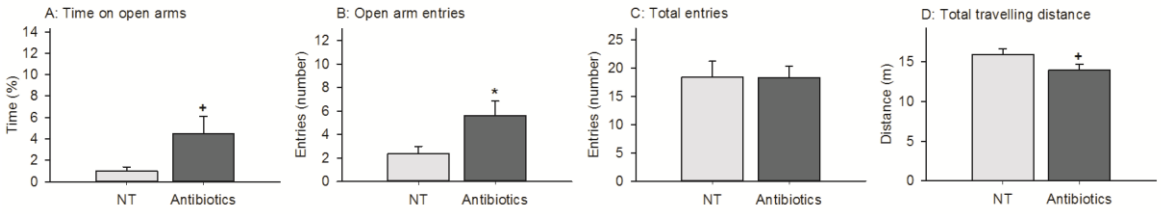

Figure 21 Effects of the combination of ampicillin (2 mg/ml), bacitracin (5 mg/ml), meropenem (1 mg/ml), neomycin (5 mg/ml) and vancomycin (0.3 mg/ml) on behaviour on the EPM 8 days after initiation of antibiotic treatment in male mice.

The graphs show the time spent on the open arms (A), the number of entries into the open arms (B), the total entries made into any arm (C) and the total distance travelled (D) during the 5-min test session. Time spent on the open arms is expressed as a percentage of the 5-min test duration. The values are means + SEM, n = 8–10. $^{+}p < 0.1$, $*p < 0.05$, versus NT (no treatment).

3.4.4.2 Quintuple antibiotic treatment leads to deficits in learning and memory in the NORT

The NORT carried out on day 10 of the antibiotic treatment revealed a decreased MI in the antibiotic-treated mice, pointing to deficits in learning and memory ($t_{(10)}$ = 2.343, p = 0.041) (Figure 22) (n = 5-7). Mice who explored the objects less than 1 s were excluded from the analysis. The OFT performed on 3 days before the NORT in order to habituate the mice to the OF revealed antibiotic-induced changes in locomotion and number of boli expelled during the test in the antibiotic-treated group (Table 11). Thus, antibiotic-treated mice tended to increased locomotion. Furthermore, the number of boli expelled during the 2nd and 3rd OF session was decreased in the antibiotic-treated group (n = 8–10) (Table 11).

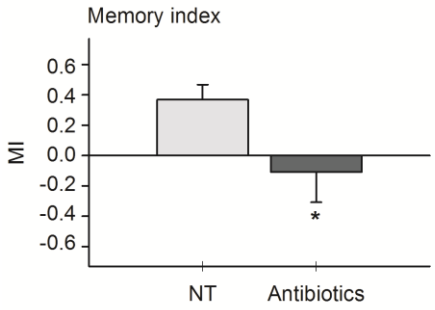

Figure 22 Effects of the combination of ampicillin (2 mg/ml), bacitracin (5 mg/ml), meropenem (1 mg/ml), neomycin (5 mg/ml) and vancomycin (0.3 mg/ml) on learning and memory in the NORT 10 days after initiation of antibiotic treatment in male mice.

The graph shows the MI that was calculated according to the formula MI = $(t_{new} - t_{old})/(t_{nne} + t_{old})$, where t_{old} represents the time exploring the familiar object and t_{new} represents the time exploring the novel object. The values are means ± SEM, n = 5–7. *p < 0.05, versus NT (no treatment).

Table 11 Effects of quintuple antibiotic combination on behaviour in the OFT

Test	Parameter	NT	Antibiotics	Statistics
OFT 1	Time in centre	70.52 ± 13.21	78.87 ± 7.87	($t_{(16)}$ = -0.568, p = 0.578)
	Central entries	28.00 ± 2.90	29.60 ± 2.84	($t_{(16)}$ = -0.390, p = 0.702)
	Total distance	22.52 ± 0.92	24.96 ± 1.51	($t_{(16)}$ = -1.297, p = 0.213)
	Boli	1.25 ± 0.65	0.40 ± 0.22	($t_{(16)}$ = 1.357, p = 0.194)
OFT 2	Time in centre	42.42 ± 15.18	44.29 ± 9.48	($t_{(16)}$ = -0.109, p = 0.915)
	Central entries	15.75 ± 3.14	18.10 ± 3.04	($t_{(16)}$ = -0.533, p = 0.601)
	Total distance	16.96 ± 1.35	**20.36 ± 1.32**$^{+}$	($t_{(16)}$ = -1.783, p = 0.094)
	Boli	2.75 ± 0.73	**0.33 ± 0.24****	($t_{(16)}$ = 3.328, p = 0.005)
OFT 3	Time in centre	26.95 ± 8.69	25.26 ± 3.71	($t_{(14)}$ = 0,179, p = 0.860)
	Central entries	9.75 ± 3.10	7.75 ± 0.92	($t_{(14)}$ = 0,618, p = 0.547)
	Total distance	11.01 ± 01.39	13.14 ± 0.80	($t_{(14)}$ = -1,330, p = 0.205)
	Boli	3.000 ± 1.05	**0.38 ± 0.18***	($t_{(13)}$ = 2,643, p = 0.020)

Mice were subjected to a combination of ampicillin (2 mg/ml), bacitracin (5 mg/ml), meropenem (1 mg/ml), neomycin (5 mg/ml) and vancomycin (0.3 mg/ml) administered via the drinking water for 11 days. Three sessions of the OFT were performed on days 7 - 9 after start of the antibiotic treatment. The table shows the parameters measured during the 5-min test session of the OFT. The values are means ± SEM, n = 8-10. Significant results ($^{+}$p < 0.1, *p < 0.05, **p < 0.001, versus NT) are bolded. Abbreviations: NT = no treatment.

3.4.4.3 Quintuple antibiotic treatment provokes a depression-like phenotype

Immobility in the TST on day 9 after treatment initiation was increased by trend in the antibiotic-treated group ($t_{(14)}$ = -1.904, p = 0.078) (n = 8) (Figure 23A). Swinging ($t_{(14)}$ = 1.445, p = 0.170) and curling ($t_{(14)}$ = 0.516, p = 0.614) were not significantly affected by the antibiotic treatment (Figure 23B,C).

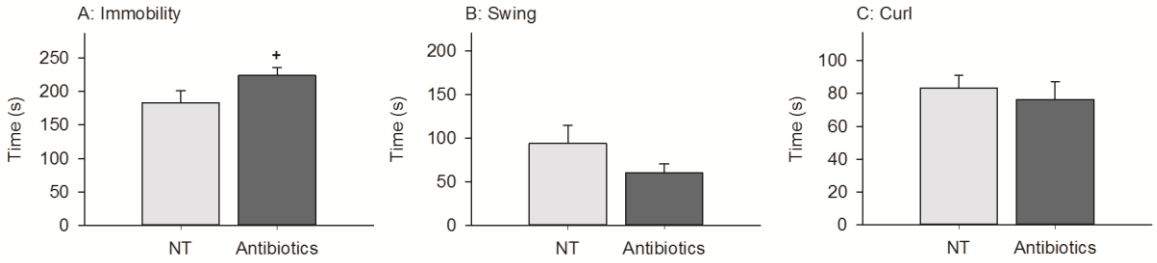

Figure 23 Effects of the combination of ampicillin (2 mg/ml), bacitracin (5 mg/ml), meropenem (1 mg/ml), neomycin (5 mg/ml) and vancomycin (0.3 mg/ml) on depression-like behaviour in the TST 9 days after initiation of antibiotic treatment in male mice.

The graphs show the duration of immobility (A), swinging (B) and curling (C) during the 6-min test session, expressed in seconds. The values are means + SEM, n = 8. $^{+}p < 0.1$, versus NT (no treatment).

3.4.4.4 Quintuple antibiotic treatment induces changes in body, spleen, and caecum weight

As a side effect, mice lost weight by an average of 5 % of their body weight, based on a restriction of intake of the antibiotic solution, especially at the beginning of the treatment (Figure 24A).

Repeated measures ANOVA revealed a significant interaction between the treatment and time for the variation in fluid intake ($F_{(4,16)}$ = 6.944, p = 0.002). Post-hoc analysis showed that during the first 2 days of treatment fluid intake was diminished in the antibiotic-treated group, while it started to increase during the course of the treatment and was higher than in the control group at the end of the treatment (Figure 24A).

Body weight of the antibiotic-treated mice was markedly decreased on day 7 ($t_{(16)}$ = 6.902, p < 0.001) and had partly recovered on day 11 of the treatment ($t_{(14)}$ = 4.122, p = 0.001) (n = 8-10) (Figure 24B,C). Relative spleen weight of the antibiotic-treated mice was likewise decreased ($t_{(14)}$ = 5.734, p < 0.001), while the caecum weight was significantly increased ($t_{(13)}$ = -8.128, p < 0.001) (n = 7-8) (Figure 24C,D).

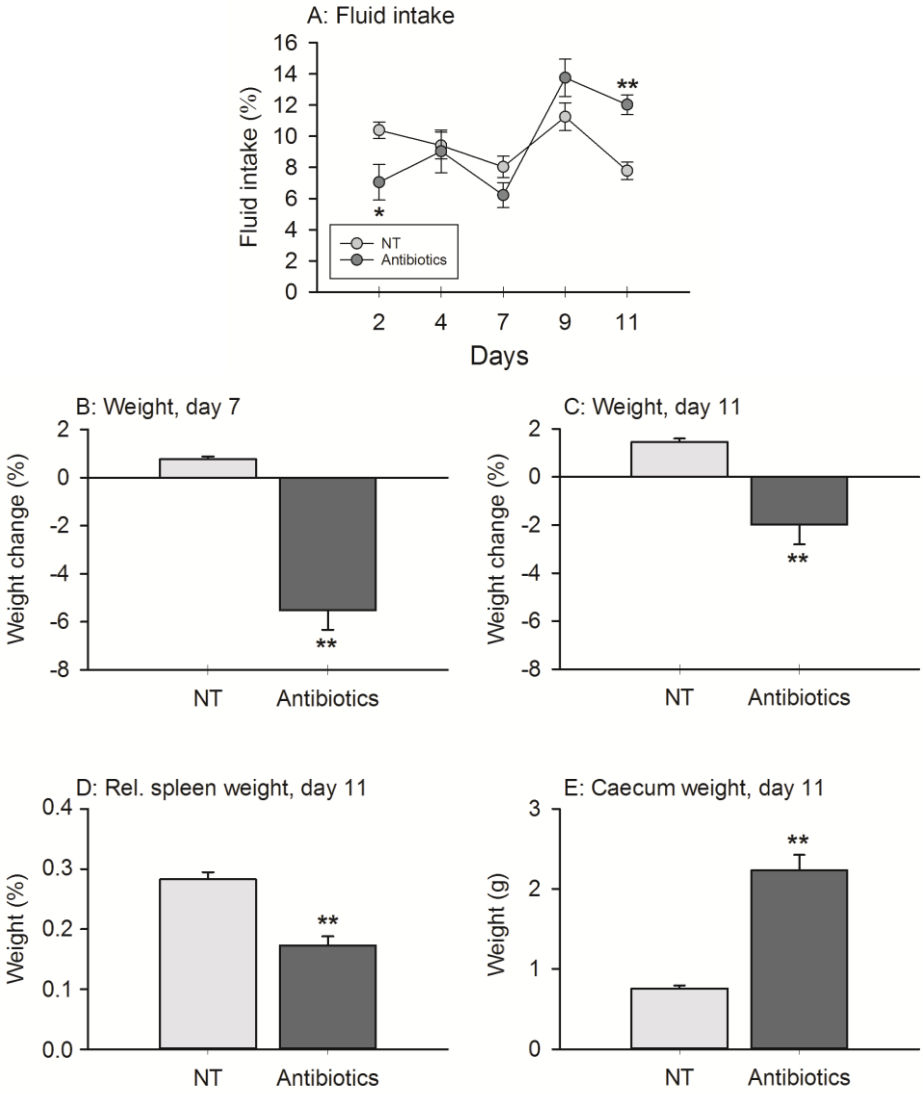

Figure 24 Effects of the combination of ampicillin (2 mg/ml), bacitracin (5 mg/ml), meropenem (1 mg/ml), neomycin (5 mg/ml) and vancomycin (0.3 mg/ml) on fluid intake and body, spleen, and caecum weight 11 days after initiation of antibiotic treatment in male mice.

The graphs show the fluid intake during the whole treatment period(A), the change in body weight on day 7 (B) and 11 (C) of antibiotic treatment, as well as spleen (D) and caecum weight (E). The weight loss induced by the treatment is expressed as a percentage of the body weight measured pre-treatment. Spleen and caecum weight are expressed as a percentage of the body weight. The values are means + SEM, n = 7–10. *$p < 0.05$, **$p \leq 0.001$, versus NT (no treatment) (A: no Bonferroni correction).

3.4.4.5 Colonic MPO and circulating PYY levels are not significantly affected by quintuple antibiotic treatment

In order to assess whether the antibiotics induced colitis, colonic MPO levels were measured. Levels of the inflammatory marker MPO in the colon were not significantly increased in the antibiotic-treated group ($t_{(14)} = -1.008$, $p = 0.330$) ($n = 8$) (Figure 25A). As there is some evidence for microbiota-induced changes of circulating PYY in the context of the microbiota-gut-brain axis, plasma levels of PYY were measured. Similarly, circulating levels of PYY were not significantly changed by the antibiotics ($t_{(10)} = -1.634$, $p = 0.133$) ($n = 6$) (Figure 25B).

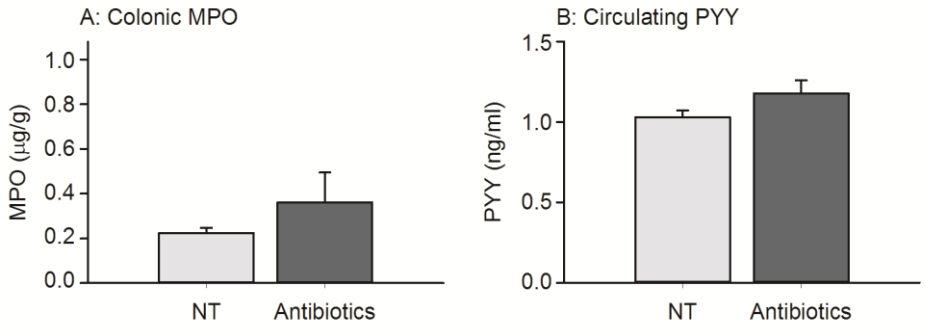

Figure 25 Effects of the combination of ampicillin (2 mg/ml), bacitracin (5 mg/ml), meropenem (1 mg/ml), neomycin (5 mg/ml) and vancomycin (0.3 mg/ml) on colonic MPO content (A) and circulating PYY levels (B) 11 days after initiation of antibiotic treatment in male mice.

Mice were administered a combination of 5 different antibiotics via the drinking water over a period of 11 days. Subsequently colonic MPO content (A) and circulating PYY levels (B) were quantified. The values are means + SEM, n = 6–8. Abbreviation: NT = no treatment.

3.4.4.6 Quintuple antibiotic combination depletes the colonic microbiota

The specific band of the 16S rRNA PCR product of approximately 450 bp already revealed that the colonic microbiota of the antibiotic-treated mice are strongly reduced compared to untreated controls (Figure 26A). This picture was confirmed by the rarefaction analysis, which demonstrated a decreased microbial richness of the antibiotic-treated group, which was comparable to that of the blank sample, which was devoid of any colonic tissue (Figure 26B). The bacterial strains detected in the blank can be explained by contaminations of the PCR reagents, which are produced in bacterial systems. Principle component analysis, which was based on a weighted UniFrac, accounting for the relative abundance of each of the taxa, revealed that the colonic microbiota of untreated and antibiotic-treated mice were significantly different ($p < 0.001$, ADONIS test) and that the microbiota of the antibiotic-treated mice was comparable with the blank sample (Figure 26C).

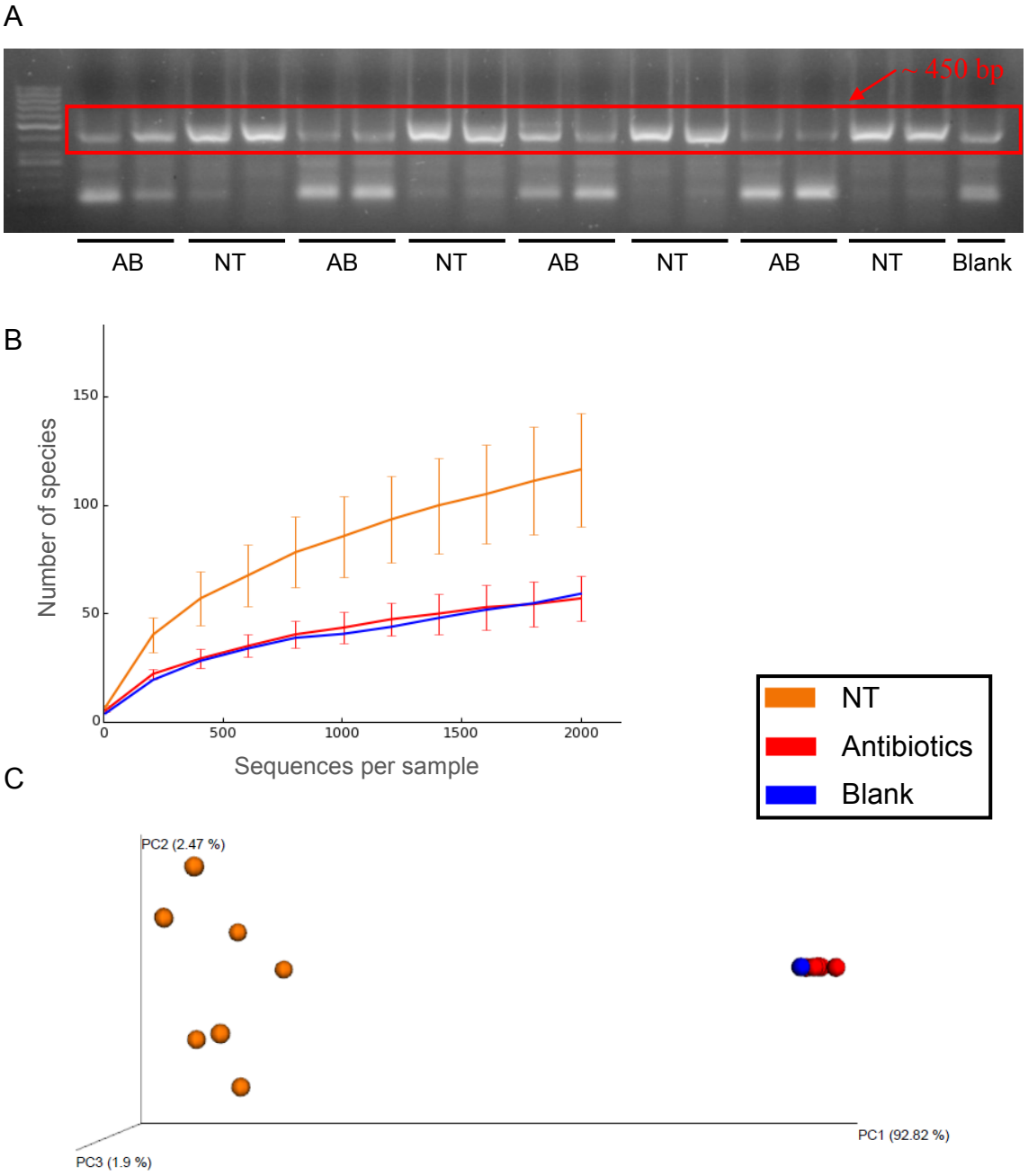

Figure 26 Quintuple antibiotic combination leads to depletion of colonic microbiota of male mice. The graphs show the picture of the agarose gel electrophoresis with the specific band of the 16S rRNA PCR product framed in red (A) and the rarefaction analysis of colon samples of untreated (NT) and antibiotic (AB)-treated mice, as well as one sample lacking colonic tissue (Blank) showing the number of species in relation to the number of sequences (B). Principal component (PC) analysis of colon samples shows the clustering of untreated and antibiotic-treated mice. Each dot represents the microbial community of 1 sample. PC1 explains 92.82 % of the difference of control and antibiotic-treated microbiota (C).

The colonic microbiota of the untreated mice consisted mainly of the phyla *Bacteroidetes* (60.2 %) and *Firmicutes* (39.4 %), which were represented by Bacteroidales (60.2 %), Lactobacillales (25.0 %) and Clostridiales (14.3 %) at the order level. The microbial distribution of antibiotic-treated mice was strikingly different and consisted mainly of the order Burkholderiales (96.6 %), which belong to the phyla *Proteobacteria,* which made up 99.5 % of the colonic phyla in the antibiotic-treated mice. The colonic microbiota of the antibiotic-treated mice was comparable to that found in the blank control, where 99.8 % of the phyla consisted of *Proteobacteria* (Figure 27). Kruskal-Wallis test showed that there was a statistically significant difference between the colonic microbial community of untreated and antibiotic-treated mice at phylum and order level ($p < 0.05$ after Bonferroni correction).

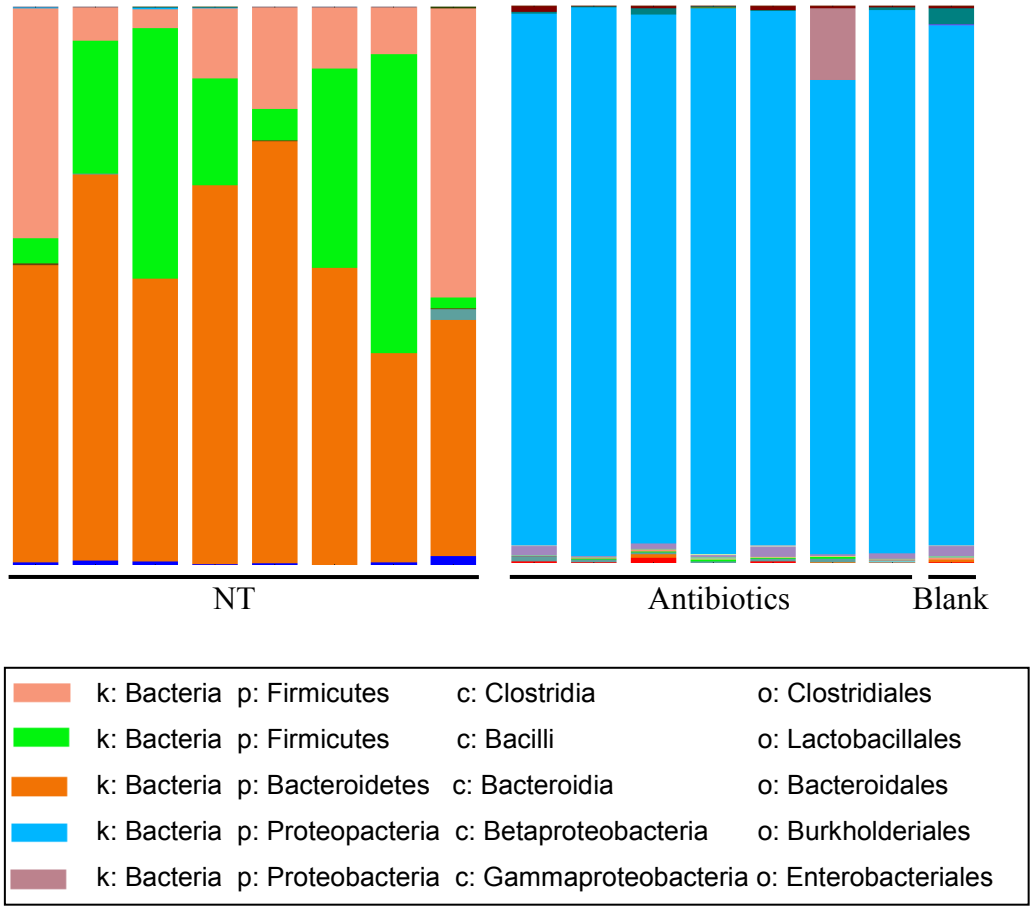

Figure 27 Quintuple antibiotic combination disrupts the community structure of colonic microbiota.
The graphs show the distribution of the colonic microbiota at order level of untreated (NT) and antibiotic-treated mice as well as one sample lacking colonic tissue (Blank).
Abbreviations: k = kingdom, p = phylum, c = class, o = order.

4 Discussion

4.1 Rationale of the project

According to a systematic analysis for the Global Burden of Disease Study 2010, which aims to quantify the burden of non-fatal health outcomes, mental and behavioural disorders are the main contributors to the global years lived with disability (YLDs) (Vos et al. 2012). Furthermore, the largest categories within mental and behavioural disorders are depressive disorders, while anxiety disorders are another major cause. Interestingly, mental and behavioural disorders show a comparable prevalence across different regions. Importantly, the YLDs have increased for mental and behavioural disorders by 37 % from 1990 to 2010 highlighting the need for a better understanding and more effective treatment strategies for this complex global health problem (Vos et al. 2012). Apart from other factors that may increase the likelihood of developing major depression, there is increasing evidence that inflammation is involved in the pathophysiology of neuropsychiatric diseases in a subset of patients (Capuron, Miller 2011, Raison, Miller 2013).

In this context, the effects of the bacterial cell wall component and TLR4 agonist, LPS, on mood and behaviour and its potential translocation across the intestinal barrier have been frequently studied (Cani, Delzenne 2011, Haroon, Raison & Miller 2012). In contrast, the behavioural effects of peptidoglycan constituents, another prominent component of the bacterial cell wall, have not been studied in great depth. Therefore, this work aimed at examining the effects of i.p. injection of the peptidoglycan constituent and NOD1 agonist FK565 as well as the NOD2 agonist MDP, in combination with or without LPS, on mood and behaviour. Furthermore, as the intestinal microbiota is the biggest bacterial source and both peptidoglycan and LPS have been demonstrated to be able to cross the intestinal barrier and affect systemic immunity (Clarke et al. 2010), the second part of this work assessed whether modulation or depletion of the intestinal microbiota by non-absorbable oral antibiotics are able to affect mood and behaviour.

4.2 Effects of NOD1 and NOD2 agonism

Administration of M-TriDAP (5 mg/kg; NOD1/NOD2 agonist), FK565 (0.001–0.003 mg/kg; NOD1 agonist) or MDP (1–30 mg/kg; NOD2 agonist) alone indicated that activation of NOD1 and/or NOD2 does not induce a strong sickness response in mice. In more detail, M-TriDAP, FK565 or MDP did not affect body temperature as measured 4–5

h after treatment. Likewise M-TriDAP, FK565 or MDP did not decrease body weight, when assessed 1 day post-treatment. Similarly, NOD1 or NOD2 activation did not impact on anxiety- or depression-like behaviour at any time point. However, while locomotion measured in the OFT was likewise not affected by any of the NOD agonists 1 day post-treatment, continuous assessment of the effects of the NOD1 agonist FK565 and the NOD2 agonist MDP revealed that treatment with FK565 induced a nominal decrease of locomotion when assessed in the LabMaster system. To the best of my knowledge, this is the first report that NOD1 activation has some effects on home cage activity in male mice. In contrast, MDP (1 mg/kg) did not lead to a reduction of locomotion in the LabMaster system. These findings are in contrast to other studies, which have demonstrated that NOD2 activation is able to induce slight reductions of locomotion in male rats (Engeland, Kavaliers & Ossenkopp 2003, Fosset et al. 2003). In more detail, i.p. injection of 1.5 mg/kg MDP to male rats increased resting time during the third h after treatment, while activity was decreased nominally (Fosset et al. 2003). Another study demonstrated that a dose of 1.6 mg/kg MDP decreases locomotion 2 h after treatment in both male and female rats, as measured in the OF for 30 min (Engeland, Kavaliers & Ossenkopp 2003). Several factors might underlie these diverging results. First, in the present study, locomotion was assessed over a period of 24 h, while the reported decreases of locomotion were assessed within hours after MDP administration. Second, the dose applied in this study (1 mg/kg) is somewhat lower than the one reported to induce changes in locomotion. Third, species differences in the sensitivity to MDP might be another possible explanation for the observed difference. Thus, murine macrophages have been demonstrated to be less susceptible to MDP compared to rat macrophages (Nagao et al. 1990). In line with this notion, while MDP induces fever in rats and other species (Kamerman, Mitchell & Laburn 2002), to the best of my knowledge there is no report of MDP-induced fever induction in mice.

Similar to the lack of effect of MDP on locomotion in the LabMaster system, food intake remained likewise unaltered by MDP. This finding is in keeping with studies in which MDP failed to reduce body weight in rats (Engeland, Kavaliers & Ossenkopp 2003, Cloutier et al. 2012). Thus, in one study, injection of 1.6 mg/kg of MDP to male Long–Evans rats did not induce significant weight loss 24 h post-treatment compared to VEH-treated mice (Cloutier et al. 2012). However, body weight in the MDP-treated group was nominally lower than in the VEH-treated group (Cloutier et al. 2012). Also Engeland et al. did not observe any weight loss in MDP (1.6 mg/kg)-treated male or female rats, 24 h post-

treatment (Engeland, Kavaliers & Ossenkopp 2003). Other studies, however, demonstrated that 1.6 mg/kg of MDP reduced food intake and weight gain in rats 24 h after injection, while a dose of 0.6 mg/kg did not affect ingestion (Langhans et al. 1990, Biberstine, Rosenthal 1994, Fosset et al. 2003).

Thus, while one could speculate that the dose of 1 mg/kg MDP administered in the LabMaster experiments might have been too low to modulate food intake, mice treated with higher doses of MDP (3-30 mg/kg) outside the LabMaster system did not lose more weight than VEH-treated controls within 1 day after treatment either. Surprisingly, female mice treated with 10 mg/kg MDP even presented with more weight than their respective controls 1 day-post treatment (Experiment 1.2). While the mechanism of MDP-induced weight gain in female mice was not further investigated in this study, low grade inflammation has been implicated in the pathogenesis of obesity (Cullberg et al. 2014). Interestingly, high fat diet-induced obesity has been demonstrated to lead to increased NOD2 expression (Kim, Choi & Han 2011). Therefore it is tempting to speculate that certain doses of MDP might induce low-grade inflammation and play a role in the development of obesity. However, as we did not investigate the mechanism of MDP-induced weight gain, this interpretation should be dealt with caution. In addition, the question arises whether there is any sex difference in the effects of low-grade inflammation on body weight as reported in humans (Thorand et al. 2006), since body weight gain was solely observed in female mice. However, as the same dose was not tested in male mice, this question cannot be answered from the current results. In summary, in contrast to the studies performed in rats it can be concluded that doses of up to 30 mg/kg of MDP did not induce any weight loss in mice in the current work.

In contrast to MDP, FK565 (0.001 mg/kg) reduced food intake by trend as observed in the LabMaster system. In contrast, no appreciable weight loss was evident 21 h after injection of a higher dose of FK565 (0.003 mg/kg) in double-housed mice outside the LabMaster. While reports regarding the effects of NOD1 activation on food intake and body weight are scarce, it has previously been reported that an injection of 6 mg/kg FK565 decreases body weight in mice (Izumi et al. 1983).

In line with the notion that pro-inflammatory cytokines mediate sickness responses following the injection of PAMPs, MDP or FK565 failed to significantly augment circulating cytokine levels when measured 3 h after injection. This finding complies with other *in vitro* and *in vivo* studies, which also demonstrated that NOD1 or NOD2 activation does not induce a strong cytokine release (Wolfert et al. 2002, Fritz et al. 2005, Uehara et

al. 2005). Against this background, it was all the more surprising that FK565 significantly increased circulating CORT levels, while MDP was devoid of such an effect. Since cytokine levels were generally low in the FK565-treated group and comparable between FK565 and MDP, the FK565-evoked rise of plasma CORT is unlikely due to pro-inflammatory cytokine signalling. The mediator of the FK565-induced rise of CORT was not further investigated in this work. However, apart from cytokines, prostaglandins and NO and other mediators are also involved in the LPS-induced rise of CORT (Gadek-Michalska, Bugajski 2004, Gadek-Michalska, Spyrka & Bugajski 2005). Interestingly, NOD1 activation has been demonstrated to induce the expression of IL6 and the subsequent production of prostaglandin in subcutaneous adipose tissue (Lappas 2014). In addition, FK565 is more potent than MDP in inducing NO, the second putative mediator of HPA axis activation (Cartwright et al. 2007). Therefore, prostaglandins and/or NO might indeed be involved in FK565-induced CORT release. However, it awaits further studies to identify the mechanism behind the FK565-induced HPA axis activation.

In summary, it can be concluded that stimulation of NOD1 and NOD2 alone with the doses of the agonists applied in the current study is insufficient to evoke an overt sickness response. However, among the NOD agonists tested, FK565 induced slight signs of sickness, when continuously assessed inside the LabMaster system.

4.3 Effects of combined NOD1 or NOD2 plus TLR4 agonism in causing sickness

NOD agonists have been demonstrated to boost the LPS-induced production of various cytokines both *in vitro* (Chamaillard et al. 2003, Fritz et al. 2005, Uehara et al. 2005, Park et al. 2007) and *in vivo* (Parant et al. 1995, Shikama et al. 2011). In addition, LPS-induced reduction of ingestion and locomotion in rats has been reported to be aggravated, when rats were pre-treated with MDP (Langhans et al. 1990, Engeland, Kavaliers & Ossenkopp 2003). In contrast, to the best of my knowledge, the behavioural effects of combined NOD1 and TLR4 activation remained as yet unexplored.

The ability of NOD1 and NOD2 agonism to aggravate and prolong the sickness response to LPS was highlighted in this work by the LabMaster data, murine behaviour in the OFT, as well as the induction of hypothermia by MDP + LPS and FK565 + LPS.

Specifically, in the LabMaster LPS (0.1 mg/kg) alone was only able to decrease locomotion and food intake, whereas the combination of FK565 + LPS and MDP + LPS aggravated the effects of LPS on all parameters tested (locomotion, exploration, ingestion,

SP). While SP was decreased for a short period of time, FK565 + LPS and MDP + LPS significantly decreased locomotion, exploration (rearing) and food intake of the mice for 2–3 days. Interestingly, differences were observed inside and outside the LabMaster system with regard to the duration of hypo-locomotion. Thus, in contrast to the LabMaster data, novelty-induced locomotion in the OF was not altered 1 day after treatment with 0.1 mg/kg LPS, as reported by others (Frenois et al. 2007). Several factors might underlie the prolonged hypo-locomotion in response to the PRR agonist inside the LabMaster system. First, single housing has been reported to modulate the effects of immune challenge on mood and behaviour and thus might have made the animals more vulnerable by the PRR agonists (Painsipp et al. 2011). Second, while the LabMaster monitors home cage activity, the OFT assesses novelty-induced locomotion, which might mask general fatigue and lethargy. A further explanation might be the timing of the experiments with regard to the light–dark cycle. Thus the behavioural tests outside the LabMaster were performed during the light phase when activity levels are generally lower. In contrast, home cage behaviour in the LabMaster system was recorded during the whole light–dark cycle.

Despite the lack of changes in locomotion by the lower dose of LPS (0.1 mg/kg), an anxiogenic effect of LPS was uncovered. In line with the LabMaster data, the increase in anxiety-like behaviour was most pronounced after treatment with FK565 + LPS. In contrast, the combination of FK565 or MDP plus 0.83 mg/kg LPS decreased novelty-related locomotion in the OF 21 h post-treatment compared to LPS.

As changes of locomotion can influence and mask indices of anxiety no conclusion can be drawn as regards the effects of FK565 or MDP plus 0.83 mg/kg LPS on anxiety-like behaviour.

In general, the SP test assesses anhedonia, which is a typical aspect of depression-like behaviour (Frenois et al. 2007). However, as a prerequisite food intake should not be altered, as a general decrease of calorie intake would also affect the intake of sucrose. The LabMaster data of my work indicate that the anorexic effect of LPS outlasts its anhedonic effect. While some studies were able to demonstrate a decrease in SP following LPS injection at a time point when food intake had already normalized (Frenois et al. 2007), others also found that the duration of LPS-induced sickness overlaps with that of depression-related behaviour (Biesmans et al. 2013). In summary, my results are rather in line with those reported by Biesmans et al., as it proved difficult to clearly distinguish sickness behaviour from “pure” depression-like behaviour.

As decreased locomotion and exploration can reflect visceral pain (Schwartz et al. 2013) and LPS can induce hyperalgesia (Karshikoff et al. 2014), the question arises whether the hypo-locomotion observed in the LabMaster system was due to inflammatory visceral pain. This question cannot be answered unequivocally from the results obtained so far, as specific pain-related behaviours or molecular markers of pain were not further investigated. Surprisingly, however, the only study that investigated the effects of MDP and FK565 on nociception, reported an analgesic effect of these compounds, when assessing the number of writhing movements after acetic acid injection (Sato et al. 2010). Moreover, it was proposed that opioid receptors are involved in NOD-induced analgesia, as naloxone, a non-selective opioid receptor antagonist, blunted the analgesic effects (Sato et al. 2010). Interestingly, also LPS has been demonstrated to lead to analgesia in a time-dependent manner via activation of opioid receptors (Yirmiya et al. 1994). Thus, LPS injection enhanced pain sensitivity in the hot-plate test 30 min after administration, while 2-30 h after drug administration an analgesic response was evident in the hot-plate and tail-flick test (Yirmiya et al. 1994). Similar to the effects of the NOD agonists, naltrexone, another opioid antagonist, blocked the analgesic effects of LPS (Yirmiya et al. 1994). *In vitro* studies further demonstrated that LPS, acting via TLR4, are able to induce the secretion of opioid peptides from leukocytes and thereby lead to antinociception (Sauer et al. 2014). In summary, LPS can lead to both hyper- and hypo-analgesia in a context- and time-dependent manner. While further work is needed to investigate the potential involvement of pain in the decreased locomotion seen in the LabMaster, a state of chronic visceral pain in response to one injection of NOD and TLR4 agonists seems rather unlikely.

Twenty-four h after injection of LPS (0.83 mg/kg) enhanced depression-like behaviour has been observed in mice, while sickness behaviour had largely vanished at this time point (Frenois et al. 2007). The dose of 0.1 mg/kg LPS, which was used in my study, was too low to induce depression-like behaviour in the FST, which is in line with other studies (Deak et al. 2005). In contrast, the combination of the NOD agonists with LPS (0.1 mg/kg) nominally prolonged the time spent immobile in the FST, which suggests that NOD agonists might facilitate the development of depression when combined with low doses of LPS. The pronounced immobility as seen in the control group might impede a significant increase of the time spent immobile in the treatment groups. This effect might in part be attributed to the C57BL/6N mouse strain, which displays a lower activity and increased anxiety-like behaviour compared to the C57BL/6J mouse strain (Simon et al. 2013).

Interestingly, also group or single housing affects the time spent immobile in the FST in response to immune challenge. Thus, contrary to the typical increase of immobility mostly seen in singly-housed mice, group housing has also been demonstrated to modulate depression-like behaviour in the FST in response to immune challenge (Painsipp et al. 2011).

Single and combined administration of NOD and TLR agonists exerted strikingly different effects on body temperature. Especially the combination of the NOD agonists with 0.83 mg/kg LPS induced overt hypothermia, while LPS alone and the combination with 0.1 mg/kg LPS lead only to a slight decrease of body temperature.

The change in body temperature in response to LPS depends on the route of administration, the dose of LPS applied and the ambient temperature (Rudaya et al. 2005). Ambient temperatures of 31°C to 33°C have been demonstrated to be thermoneutral for mice (Rudaya et al. 2005). Thus, the ambient temperature of our mouse facility is clearly below thermoneutrality for mice, which has been demonstrated to facilitate hypothermia in response to LPS (Rudaya et al. 2005). Interestingly, while hypothermia increases mortality rates in human septic patients (Peres Bota et al. 2004), it increases survival in septic rodents (Leon et al. 2015). In contrast to the pronounced effects of the NOD agonists with 0.83 mg/kg LPS on body temperature, body weight was mainly affected by LPS and was not modulated by addition of a NOD agonist. This result is rather surprising, as the combination of MDP or FK565 with LPS decreased food intake compared to treatment with LPS alone. As an explanation, the concurrent decrease in locomotion might have decreased energy expenditure and thereby prevented body weight loss.

4.4 Potential mechanisms of the synergistic effects of NLR and TLR agonists on sickness

In order to analyse potential mechanisms whereby FK565 and MDP aggravate LPS-induced sickness, several peripheral and cerebral factors were assessed.

c-Fos is a product of an immediate early gene, which can be transiently expressed following neuronal activation, and thus its expression is also induced in certain brain regions in response to LPS (Rivest, Laflamme 1995, Frenois et al. 2007). Therefore the numbers of c-Fos positive cells were assessed in order to evaluate whether the exaggerated sickness response following treatment with MDP + LPS (0.83 mg/kg) is reflected by respective changes of neuronal activity in certain brain areas. c-Fos immunohistochemistry demonstrated that priming with MDP enhanced the number of c-Fos positive neurons in

response to LPS in an additive or synergistic manner depending on the brain area evaluated. Thus c-Fos expression was increased in the BNSTd, CeA and SFO in a rather additive manner. In the BNSTv, PVN, insula and SO synergistic increases of c-Fos expression were observed.

The CeA, BNST, and the PVN have been proposed to integrate internal challenges (e.g. immune challenge) with autonomic, neuroendocrine, and behavioural responses (Sawchenko, Li & Ericsson 2000, Goehler et al. 2008). With regard to behaviour, these three nuclei have also been implicated in the mediation of fear and anxiety (Walker, Toufexis & Davis 2003). Indeed, in the context of gastrointestinal infection there is evidence that the CeA, PVN and BNST serve as interfaces between gastrointestinal inflammation and behavioural responses to stress (Goehler et al. 2008). Similarly, the insula is another cortical brain area that is proposed to integrate internal sensory information and emotion (Goehler, Lyte & Gaykema 2007). The SO is best known for producing vasopressin, a peptide hormone inducing water retention, which is released in response to decreased blood volume or blood pressure. Indeed, LPS has been demonstrated to induce vasopressin release and antidiuresis (Palin et al. 2009b). Interestingly, however, there is also some evidence that vasopressin is involved in anxiety- and depression-like behaviours (Neumann, Landgraf 2012).

The c-Fos expression seen within the SFO, a circumventricular organ lacking a BBB, is likely indicating that the inflammatory signals induced by LPS and MDP + LPS are also affecting the brain via the humoral pathway.

Finally, the observation that LPS, with or without MDP, decreased the number of c-Fos positive cells in the dentate gyrus of the hippocampus was of particular interest. Thus, decreased c-Fos counts in this area have already been associated with a decrease of exploratory behaviour following LPS administration (Gaykema, Goehler 2011). Moreover the involvement of the hippocampus in the pathophysiology of depression has gained attention over the last years. Thus, depressed patients present with decreases in hippocampal volume, which is proposed to occur due to loss of existing neurons or reduced hippocampal neurogenesis (Haase, Brown 2015). In this context, LPS and other inflammatory agents have been demonstrated to inhibit central levels of neurotrophins such as BDNF, as well as neurogenesis, pointing to an important role of immune activation in affecting neuroplasticity (Lapchak, Araujo & Hefti 1993, Wu et al. 2013, Khan et al. 2014).

In summary, these observations indicate that the synergistic effect of NOD2 and TLR4 stimulation on the sickness response is related to altered neuronal activation in relevant brain nuclei.

While increased CORT levels are frequently observed in depressed patients and are proposed to be involved in the pathophysiology of depression (Pariante, Lightman 2008), in the acute setting of immune activation they exert rather beneficial effects by attenuating and thereby preventing an exaggerated immune response (Dunn 2000). This notion is in line with the current assessment, where the combination of FK565 or MDP with LPS did not increase the rise of circulating CORT when compared with LPS treatment alone. Therefore, it can be concluded that activation of the HPA axis did not contribute to the aggravation of sickness by combined NOD + TLR agonism.

In contrast, the pro-inflammatory cytokines measured in the plasma and brain are likely mediators of the exacerbation of sickness due to NOD plus TLR activation. In the current study, the LPS-induced increase of plasma cytokines 3 h post-treatment was synergistically augmented by pre-treatment with FK565 or MDP. This finding is consistent with the synergism of FK565 and MDP with LPS in innate immune cells (Le Contel et al. 1993, Wang et al. 2001, Wolfert et al. 2002, Netea et al. 2005). Similarly, the cerebral mRNA expression of IFN-γ and IL-6 was synergistically enhanced by FK565 + LPS and MDP + LPS, whereas the increase of cerebral IL-1β and TNF-α mRNA expression occurred in an additive manner. The protein levels of the cytokines in the brain coincided with those assessed at the mRNA level 3 h post-treatment. Interestingly, while MDP or FK565 did not induce any notable increase of the circulating cytokines, their central expression was somewhat comparable to that induced by LPS.

One day post-treatment the evoked increase in plasma cytokines had largely waned, whereas the cerebral mRNA expression of IL-1β and TNF-α mRNA was still elevated, although to a lesser degree than 3 h post-treatment. In contrast, no increase in the central protein levels could be detected at this time point. Surprisingly, while the plasma levels of IL-6 remained elevated 1 day after treatment with LPS or the NOD agonists + LPS, the cerebral expression of IL-6 mRNA was reduced 26 h post- treatment. Decreases of central IL-6 mRNA in response to LPS have also been reported by others (Andre et al. 2008, Bay-Richter et al. 2011). In addition, although the majority of clinical trials demonstrate increased levels of IL-6, also decreased IL-6 levels in the cerebrospinal fluid of patients with acute depression have been observed (Levine et al. 1999). Further work is required to

answer, whether the reduced IL-6 mRNA expression in the brain is of relevance for the behavioural effects of immune challenge.

It can be suggested from the current findings that the sickness response to combined NOD and TLR activation is triggered by immune stimulation which in turn activates secondary mechanisms that perpetuate the malady. Kynurenine is a likely mediator, as its plasma level was elevated 26 h post-treatment and was significantly higher in mice treated with the NOD agonists + LPS than those receiving solely LPS. In fact, a pivotal role for kynurenine in inducing depression-like behaviour has been demonstrated by the finding that blockade of the enzyme IDO, which converts tryptophan into kynurenine, blocks LPS-induced depression-like behaviour without affecting brain cytokine expression (O'Connor et al. 2009b). Surprisingly, while one would anticipate decreased levels of tryptophan in response to cytokine-induced IDO activation, an increase in plasma tryptophan was observed. While the mechanism of this tryptophan increase cannot be identified from the results generated, the TST employed 30 min before blood sampling might play a role in this regard. Thus, stress has been reported to increase circulating tryptophan levels, while the underlying mechanisms are not understood (Dunn 1988, Malyszko et al. 1995). As kynurenine levels were increased in the treatment groups, a blockade of IDO is unlikely to be responsible for the observed increase of tryptophan. It thus appears as tryptophan derives from an unidentified source. Interestingly, it has been proposed that in cases of proteolysis, amino acids including tryptophan may be released from the tissue (Moffett, Namboodiri 2003). Indeed, there is evidence for psychosocial stress-induced proteolysis (Moussa et al. 2013), offering an explanation for stress-induced increases of tryptophan. This novel hypothesis, however, requires further studies.

4.5 Differences and similarities of NOD1 and NOD2 activation

In general, both NOD1 and NOD2 agonists potentiated the sickness response to LPS in a grossly similar manner, although slight differences in distinct aspects of the sickness response could be observed.

Thus, cytokine levels were generally higher in the MDP + LPS group compared to the FK565 + LPS group, which may be related to the preferential expression of NOD2 in monocytes (Ogura et al. 2001b). Consistent with the cytokine levels of the MDP + LPS group, the CORT levels and the kynurenine/tryptophan ratio were likewise highest in this group 1 day post-treatment. Thus there seems to be a relationship between cytokine expression, CORT release and kynurenine formation.

In contrast, in the FK565 + LPS-treated group, the behavioural effects were tendentially more pronounced than those in the MDP + LPS-treated group. These disparities might be explained by the broader expression of NOD1, which can be detected in peripheral and cerebral tissues (Inohara et al. 1999). Importantly, NOD1 is expressed in the choroid plexus and other circumventricular organs, while NOD2 is not expressed at these sites (Inohara et al. 1999). Furthermore, in contrast to LPS, MDP failed at inducing a rise of intracellular calcium in microglial cells of the area postrema, which again demonstrates a lack of NOD2 expression at this circumventricular organ (Wuchert et al. 2008). Therefore, the interaction of NOD1 with TLR4 at the blood–brain interface might account for the somewhat more pronounced effects of FK565 in the brain.

In line with this contention, there is some evidence that also the cerebral effects of peripherally injected LPS depends rather on TLR4 expression by CNS resident cells than on the effects of systemic cytokines (Chakravarty, Herkenham 2005, Murray, Skelly & Cunningham 2011).

A striking difference could be observed with regard to the effects of the NOD agonists on colitis as assessed by colonic MPO levels and intestinal motility as discerned by the number of faecal boli expelled during the OFT. Thus, while NOD1 + TLR4 activation induced a rise in MPO, NOD2 + TLR4 activation decreased the number of faecal boli expelled during the OFT. These results point to distinct effects of NOD1 and NOD2 activation on colonic immune activation and motility. Interestingly, a study demonstrated that especially intestinal epithelial cell lines are highly responsive to NOD1 activation and that NOD1 agonists are particularly triggering the recruitment of neutrophils (Masumoto et al. 2006). These results are in good agreement with the results of this study, demonstrating a special involvement of NOD1 activation in the rise of colonic MPO, which is mainly synthesized in neutrophils. Furthermore, to the best of my knowledge, this is the first report describing a distinct effect of NOD2 + TLR4 stimulation on gut motility. While several cell types and mediators are involved in inflammation-induced ileus, residential macrophages and cytokines might be likely candidates responsible for the observed effects of NOD2 + TLR4 activation on intestinal motility (Gutierrez et al. 2002, De Winter, De Man 2010). The exact mechanisms however require further investigations.

4.6 Involvement of NPY and PYY

As the NPY family has been demonstrated to attenuate several sequelae of immune challenge (Painsipp et al. 2013, Farzi, Reichmann & Holzer 2015), the consequences of a KO of both NPY and PYY in the context of NOD2 and TLR4 agonism were assessed. While changes of body temperature were not affected by the KO, $NPY^{-/-}$;$PYY^{-/-}$ KO mice lost more weight than WT controls 1 day after injection irrespective of the treatment. This result is in accordance with a previous report of aggravated BCG-induced weight loss in $NPY^{-/-}$;$PYY^{-/-}$ KO mice (Painsipp et al. 2013). Interestingly, however, also the VEH-treated KO mice lost more weight than VEH-treated WT mice in the current work. Thus $NPY^{-/-}$;$PYY^{-/-}$ KO seem to be less resilient to the stress exposure in the context of an i.p. injection.

The OFT revealed that, on the one hand, KO mice show a decrease of locomotion independent of the treatment, while LPS or MDP + LPS decrease locomotion independent of the genotype as deduced from the number of central area entries and the total travelling distance. This finding is in accordance with other studies, reporting decreased locomotion of $NPY^{-/-}$;$PYY^{-/-}$ KO mice (Edelsbrunner, Herzog & Holzer 2009). Interestingly, a significant interaction between genotype and treatment was evident for the time spent in the central area of the OF. Thus, the MDP + LPS treatment reduced the time spent in the centre in KO mice only, indicative of increased anxiety in the KO mice in response to MDP + LPS. However, the decrease in locomotor activity might bias the time spent in the centre of the OF.

As reported previously (Painsipp et al. 2011), $NPY^{-/-}$;$PYY^{-/-}$ KO mice displayed increased depression-like behaviour, as deduced by an increase in immobility and a decrease in swimming and climbing in the FST. While neither LPS nor MDP + LPS induced any changes in the time spent immobile, which represents the main marker of depression-like behaviour, the time spent climbing was decreased by MDP + LPS independent of the genotype, suggesting that MDP + LPS led to some depressive-like behaviour.

In summary, although the NPY system has been repeatedly demonstrated to blunt behavioural effects of immune challenge, KO of both NPY and PYY did not reveal a major change in the responsivity to immune challenge. As $NPY^{-/-}$;$PYY^{-/-}$ KO present with a marked phenotype at baseline, it is likely that a ceiling effect is reached with regard to the behavioural phenotype, masking further effects of immune activation on behaviour. Furthermore, compensatory changes are likely to occur due to germline KO of both NPY

and PYY, which again might influence the responsivity of these mice to immune challenge. However, $NPY^{-/-}$;$PYY^{-/-}$ KO mice reacted with stronger weight loss and pronounced anxiety-like behaviour in response to MDP + LPS, which is in agreement with the buffering effects of the NPY family in response to immune challenge and stress (Farzi, Reichmann & Holzer 2015).

4.7 Effects of antibiotic-evoked dysbiosis on behaviour

As LPS and NOD1 agonists are components of preferentially Gram-negative bacteria, while NOD2 senses both Gram-negative and Gram-positive bacteria, as a first approach the behavioural effects of oral treatment with antibiotics disrupting either Gram-negative (neomycin) or Gram-positive (vancomycin, ampicillin) bacteria of the intestinal tract were tested, but did not induce any behavioural alterations. Likewise, treatment with cefoperazone, a broad spectrum antibiotic, did not affect murine behaviour. Care was taken to choose antibiotics with low oral bioavailability, warranting that behavioural alterations are due to modulation of the intestinal microbiota and not induced by systemic side effects of the antibiotics. However, in order to disrupt anaerobic bacteria, metronidazole was applied orally. In contrast to the other antibiotics under study, metronidazole has a high oral bioavailability and is absorbed systemically to a great extent (Jensen, Gugler 1983). Accordingly, visual inspection revealed that metronidazole treatment led to a ruffled fur of the mice, indicating that the treatment was not tolerated well. While the mechanism behind this undesirable effect was not further investigated, systemic effects of metronidazole, on the one hand, and the decreased fluid intake and concomitant weight loss in the metronidazole-treated group, on the other hand, could be involved. Therefore it can be concluded that metronidazole is not a well-qualified antibiotic to assess the effects of microbial dysbiosis on brain function and behaviour due to undesirable side effects. This conclusion is affirmed by neurotoxic effects of metronidazole observed in humans (Sarna, Furtado & Brownell 2013).

Among the antibiotics tested, the quintuple combination consisting of ampicillin (2 mg/mL), bacitracin (5 mg/mL), meropenem (1 mg/mL), neomycin (5 mg/mL) and vancomycin (0.3 mg/mL) induced the most pronounced behavioural changes. Interestingly, the antibiotic-induced phenotype was similar to that seen in germ-free mice, which also display an anxiolytic phenotype and deficits in learning and memory, while to the best of my knowledge, changes in depression-like behaviour have not been assessed in germ-free mice (Diaz Heijtz et al. 2011, Gareau et al. 2011). Interestingly, while the first germ-free

mice were raised at the beginning of the 20th century, it took another century until the interest in their behavioural phenotype aroused (Smith, McCoy & Macpherson 2007). In contrast, early studies focussed on vitamin deficiencies in germ-free animals (Gustafsson 1959), along with other abnormalities such as underdevelopment of the immune system, and changes in intestinal morphology and function (Smith, McCoy & Macpherson 2007). As an example, increases of the caecum and decreases of the spleen weight are characteristic of germ-free mice (Wostmann, Bruckner-Kardoss 1959, Moghadamrad et al. 2015), and were induced by several antibiotics in the current study. These changes have also been reported to evolve in response to oral antibiotic administration and are suggested to occur due to under-stimulation of the host immune system by commensal microbes (Reikvam et al. 2011). Furthermore, as it has been observed that oral antibiotics increase the accumulation of water in the intestinal lumen, it is proposed that the increase in caecum weight and volume in antibiotic-treated mice is due to a lack of certain microbiota involved in promoting water transport across the intestinal epithelium (Savage, Dubos 1968).
Not only did the behavioural and morphological features of the quintuple antibiotic-treated mice resemble that of the germ-free state, also microbial community analysis revealed that the quintuple treatment practically depleted the colonic microbiota. While the levels of colonic MPO did not indicate an inflammatory process, other indices of intestinal inflammation were not assessed and cannot be excluded. As a side effect, however, the antibiotic-treated group refused to drink the antibiotic solution at the beginning of the treatment and subsequently displayed profound weight loss. Importantly, both decreased fluid and food intake are able to affect behaviour. Thus, while reports regarding the effects of water restriction on affective behaviour are rare, 7 days of food restriction increased the entries made and the time spent on the open arms of the EPM and increased locomotor activity of male rats (Genn et al. 2003). Similarly, food restriction in C57BL/6 mice reduced anxiety- and depressive-like behaviours as assessed on day 8 of food restriction with the EPM and FST, respectively (Yamamoto et al. 2009). In addition, 28 days of food restriction impaired memory of mice assessed in the NORT (Carlini et al. 2008). In contrast, however, 2 weeks of water restriction rather promoted explorative and cognitive performance of mice (Tucci, Hardy & Nolan 2006). As it cannot be excluded from the current results that the observed decrease in fluid intake and concomitant weight loss contributes to the behavioural changes induced by the antibiotic treatment, an alternative route of administrating the antibiotics may be a more promising strategy aiming at avoiding changes in water intake.

A study by Bercik et al. also assessed the behavioural effects of a combination of neomycin, bacitracin, and the fungicide pimaricin (Bercik et al. 2011a). As seen in the current work, antibiotics induced an anxiolytic phenotype, while the amount of fluid intake and the weight of the mice are not mentioned (Bercik et al. 2011a). Interestingly, the same study reported that transfer of the intestinal microbiota from a non-anxious mouse strain to an anxious mouse strain is able to alter the behavioural phenotype of the recipient mouse towards less anxious behaviour, providing further evidence that the intestinal microbiota is able to modulate behaviour in adult mice (Bercik et al. 2011a).

The potential systemic effects of the antibiotics under study are another aspect that needs further attention. While the oral bioavailability of all but one antibiotic of this quintuple combination is less than 5% (Armstrong, Wilson 1995, Craig 1997, Weinstein et al. 1999, Craig, Stitzel 2004), ampicillin has an oral bioavailability of 30-40% (Lafforgue et al. 2008). Therefore, further analysis is warranted to examine the systemic and central levels of ampicillin. This aspect might be all the more important as a recent study demonstrated that lack of gut microbiota goes along with an increased BBB permeability and impairment of tight junction proteins (Braniste et al. 2014).

As, apart from immune signalling, communication along the microbiota-gut-brain-axis can occur via neural, endocrine, and humoral pathways, the circulating levels of the gut hormone PYY were assessed as a potential mediator of microbiota-brain signalling. While levels of PYY were not changed in the current model, other gut hormones or bacterial metabolites might participate in the behavioural effects of microbiota depletion. Thus, blood metabolites of germ-free mice differ greatly from those of conventional mice (Wikoff et al. 2009) and a recent clinical study reported correlations between depression and the levels of the volatile fatty acid isovaleric acid in stool samples (Szczesniak et al. 2015).

In summary, while treatment with single antibiotics did not trigger any behavioural changes, combined treatment with 5 different antibiotics induced a behavioural phenotype resembling several aspects of germ-free mice. However, further research is required to reveal whether the observed behavioural changes are caused by the disruption of the intestinal microbiota or other side effects of the treatment. Furthermore, the mechanisms and pathways of communication underlying the microbiota – mood axis need to be clarified.

4.8 Conclusions

In conclusion, this study provides a multivariate assessment of the effects of NOD1 and NOD2, alone and in combination with the TLR4 agonist LPS, on immune, cerebral, neuroendocrine and behavioural parameters of sickness and mood in C57BL/6N mice. The results revealed that NOD1 and NOD2 activation alone has only minor effects on cytokine production and sickness behaviour but potently synergizes with TLR4 stimulation in aggravating and prolonging illness. As aggravation of sickness was associated with enhanced production of pro-inflammatory cytokines in the periphery and brain, increased kynurenine formation and activation of immune responsive brain nuclei, these parameters are likely the mediators of the behavioural consequences of immune challenge.

As under conditions of a dysfunctional microbiota-host interaction, NLRs and TLRs are likely to be targeted in parallel by multiple PRR agonists, we assessed the effects of a disturbance of the intestinal microbiota, which is the major source of PRR, on mood and behaviour. While depletion of the intestinal microbiota succeeded in inducing behavioural changes comparable to those seen in germ-free mice, a causal involvement of the microbiota cannot be warranted, due to side effects of the antibiotic treatment on body weight.

Further studies are required to analyse the exact sites of interaction between NOD1, NOD2 and TLR4. Moreover, it remains to be investigated whether the concentrations of PRR agonists occurring in different settings give rise to a similar synergism of NLRs and TLRs as seen in the current work. Further work is warranted to explore to which extent the intestinal microbiota is able to affect systemic immunity, metabolism and behaviour. This work proposes that the interaction of NLRs and TLRs in triggering a sickness response reflects an important immunological and neurobiological mechanism of protection from microbial invasion.

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6 Appendix

6.1 Corticosterone - EIA

Preparations

- Dilute assay buffer: 10 ml assay buffer concentrate / 100 ml H_2O
- Dilute wash buffer: 7.5 ml wash buffer concentrate / 150 ml H_2O
- Dilute serum 1:200 with assay buffer

Standard Preparation

- Diluted standards should be used within 60 min of preparation
- Label 5 tubes # 1 through # 5
- Pipet 900 µl of assay buffer into tube # 1
- Pipet 800 µl of assay buffer Into tubes # 2 through # 5
- Add 100 µl of the 200 000 pg/ml standard to tube # 1
- Vortex thoroughly
- Add 200 µl of tube # 1 to tube # 2 and vortex
- Add 200 µl of tube # 2 to tube # 3 and vortex
- Continue for tube # 4 and # 5

Assay Procedure

Bring all reagents to room temperature for at least 30 min prior to opening the kit

All standards and samples should be run in duplicate

1. Pipet 100 µl of assay buffer into appropriate wells according to the assay layout sheet
2. Pipet 100 µl of standards # 1 through # 5 into the appropriate wells
3. Pipet 100 µl of the samples into the appropriate wells
4. Pipet 50 µl of blue conjugate into appropriate wells
5. Pipet 50 µl of yellow antibody into appropriate wells
6. Incubate the plate at room temperature on a plate shaker for 2 h at 500 rounds per minute (rpm)
7. Empty the contents of the wells
8. Wash 3 x by adding 400 µl of wash solution to every well
9. Add 5 µl of the blue conjugate to appropriate wells

10. Add 200 µl of the pNpp substrate to the wells and incubate 1 h at room temperature without shaking
10. Add 50 µl of stop solution to every well
11. Read immediately on microplate reader (BIO-TEK) at 405 nm

6.2 PYY - EIA

Preparations

- Bring the kit and samples to room temperature
- Dilute the 20X assay buffer concentrate with distilled water
- Centrifuge and dilute the peptide standard with 1 ml of assay buffer and vortex
- Add 5 ml of assay buffer to primary antibody
- Add 5 ml of assay buffer to biotinylated peptide
- Rehydrate positive control with 200 µl of assay buffer
- Dilute samples (serum) 1:2 with assay buffer

Standard Preparation

- Use the standard vial as tube # 1
- Prepare 5 tubes with 900 µl assay buffer
- Make a serial dilution by pipetting 100 µl from tube # 1 to tube # 2, mix well
- Pipette 100 µl from tube # 2 to tube # 3
- Continue until tube # 6

Assay Procedure

1. Leave blank well empty
2. Pipette 50 µl of assay buffer into appropriate wells according to the assay layout sheet
3. Add 50 µl of standards, positive control and prepared samples into appropriate wells in duplicate
4. Add 25 µl of primary antibody into each well except the blank
5. Add 25 µl of biotinylated peptide into each well except the blank
6. Seal the plate and incubate for 2 h at room temperature on an orbital shaker at 300-400 rpm
7. Dilute SA-HRP 1:1000 with assay buffer
8. Wash the plate 4 x with 350 µl assay buffer
9. Add 10 µl of SA-HRP solution into each well

10. Seal the plate and incubate for 1 h at room temperature on an orbital shaker at 300-400 rpm
11. Wash the plate 4 x with 350 µl assay buffer
12. Add 100 µl of TMB solution – protect from light
13. Incubate on an orbital shaker for 1 h at room temperature on an orbital shaker at 300-400 rpm
13. Add 100 µl 2 N HCl into each well to stop the reaction
14. Measure the plate within 20 min with microplate reader (BIO-TEK) at 450 nm

6.3 Protein extraction

Preparations

- Fill tubes with beads, label (+weigh)
- Put samples into tube (+ weigh)
- Label additional tubes (2 per sample)
- Defrost standards (A-I) (+ Procarta cell lysis buffer)
- Cool down centrifuge to 4°C
- Dilute reagent A+B of the Pierce BCA protein assay kit 50:1 and calculate needed amount (200 µl per sample)

Procedure

- Add 150 µl RIPA buffer
- Homogenize twice (programme 2) (put samples on ice between the 2 runs)
- Centrifuge samples: 4°C, 13 000 rpm, 10 min
- Transfer supernatant into new tube
- Add 54 µl of NaCl (0.9 %) into another new tube and add 6 µl of supernatant (1:10 dilution) on ice
- Store rest of supernatant in -70°C
- 25 µl of standard and sample into appropriate well
- 200 µl of reagent A+B into well
- Shake for 2 min
- Cover and incubate for 30 min at 37°C
- Read the plate with microplate reader at 562 nm

6.4 Procarta Multiplex Immunoassay – Protocol

Preparations - Day 1:

- Prepare plate (mark standards, samples) (magnetic plate)
- Thaw samples (plasma samples are used undiluted)

Wash Buffer:

- Dilute 1:10 (20 ml buffer 10X + 180 ml distilled water)
- Store at 4°C (stable for 6 months)

Standard Preparation

- Spin down vials in a microcentrifuge before opening: 2000×g, 10 s
- Add 250 µl of universal assay buffer
- Vortex gently (30 s)
- Incubate on ice (5-10 min)
- Take provided 8-tube strip
- 200 µl of antigen standard into tube # 1
- 150 µl of universal assay buffer into tubes# 2-7
- Transfer 50 µl from tube # 1 into tube # 2
- Mix by pipetting 10 times, change tip
- Transfer 50 µl from tube # 2 into tube# 3 and continue until tube # 7

Procedure

1. Vortex magnetic beads (IL-1β, IL-6, IL-10, TNF-α, IFN-γ) for 30 s
2. Add 50 µl to each well (multi) (for each cytokine)
3. Insert into plate washer – wait 2 min
4. Remove liquid and blot
5. Repeat for all cytokines
6. Wash: 150 µl of 1X wash buffer into each well – wait 30 s - remove liquid and blot
7. Add standards and samples
    - 25 µl of universal assay buffer into each well
    - 25 µl standards or sample into appropriate well
    - 25 µl of universal assay buffer to blank
8. Seal with plate seal
9. Remove from plate washer
10. Cover with black lid
11. Shake at 500 rpm for 30 min at room temperature

12. Transfer to 4°C – incubate over night

Day 2:

1. Shake plate for 30 min at room temperature
2. Prepare detection antibody for 96 tests:
    - Label a vial with “Detection antibody mixture”
    - Pipette 60 µl of each detection antibody concentrate into vial
    - Fill up to 3000 µl with detection antibody diluent
3. Wash:
    - Insert plate into plate washer – wait 2 min
    - Remove plate seal
    - Remove solution – do not blot
    - 150 µl of 1X wash buffer into each well – wait 30 s
    - Remove liquid
    - Repeat 3 x – blot just last time
4. Add 25 µl detection antibody into each well
5. Seal with plate seal
6. Remove from plate washer
7. Cover with black lid
8. Shake at 500 rpm for 30 min at room temperature
9. Use the 30 min break to turn on and calibrate the Bioplex (see descriptions below)
10. Wash 3 x
11. Add 50 µl SA-PE into each well
12. Seal with plate seal
13. Remove from plate washer
14. Cover with black lid
15. Shake at 500 rpm for 30 min at room temperature
16. Wash 2 x

Analysis:

1. 120 µl of reading buffer into each well
2. Seal with plate seal
3. Remove from plate washer
4. Cover with black lid
5. Shake at 500 rpm for 5 min at room temperature

6. Remove plate seal prior to reading

Bioplex

- Turn on Bioplex (2 switches on back, right) + PC
- Make sure that sheath fluid bottle is full and the waste bottle is empty
- Open BioPlex Manager 6.1
- View → Quick Guide → Startup and calibrate → follow the instructions on the screen

Calibration:

- Use Bio-Plex MCV Plate IV
- Compare control numbers, DD target, C1 target, CL2 target and RP1 target with values on bottles of calibration beads CAL1 and CAL2
- Add 70 % isopropanol and dH_2O into respective wells
- Add CAL1 and CAL2 in respective wells (3-5 drops each)
- Vortex calibration bead bottles before use
- Start calibration

To run measurement:

- New → Select analytes → add panel (name→add
- Type in cytokines and respective regions (region = bead number on certificate of analysis) → OK
- Add all (transfer to the right window)
- Format plate → Mark wells with standards, blank and samples
- Standard info → Add concentration S1 (e.g. IL-10:10000; TNF-α:15000; etc…)
- Enter dilution factor (e.g. 4)
- Enter unit of measurement (e.g. pg/ml)
- Sample Info → name samples (e.g. VEH; LPS;…)
- Run protocol
- Start → reader opens → remove calibration plate → place sample plate in reader

6.5 TRIzol extraction

Preparation:

- Label MagNA Lyser tubes (Roche)
- Label 1.5 ml low binding Eppendorf tubes and add 500 µl isopropyl alcohol (Merck)

- Cool TRIzol
- Cool down centrifuge (4°C)
- Prepare 80 % EtOH (store at -20°C) (Ethanole absolut, Merck, diluted with distilled water)

Procedure:

1. Transfer brain tissue into MagNA Lyser tubes and place the tubes into liquid nitrogen
2. Add 700 µl of cooled TRIzol
3. Homogenize samples 3 times with the MagNA Lyser centrifuge (6500 rpm, 20 s)

a. Place the tubes on ice for 1 min between the 3 runs

4. Centrifuge for 5 s at 600 rpm
5. Place the tubes on ice
6. Add 300 µl TRIzol
7. Shake vigorously and incubate for 5 min at room temperature on the rotation wheel
8. Add 200 µl chlorophorm (Merck)
9. Shake by hand for 15 s
10. Incubate for 3 min at room temperature on the rotation wheel
11. Centrifuge for 15 min at 12200 rpm and 4°C
12. Transfer upper phase to Eppendorf tubes containing 500 µl isopropyl alcohol
13. Shake vigorously for 15 s
14. Incubate for 10 min at room temperature on the rotation wheel
15. Centrifuge for 10 min at 13500 rpm and 4°C
16. Remove supernatant
17. Wash pellet with 1 ml of 80 % EtOH
18. Centrifuge for 5 min at 13500 rpm and 4°C
19. Repeat step 16 and 17
20. Remove supernatant and dry RNA pellet
21. Dissolve RNA pellet in 30µl RNase-free water
22. Store at 4°C overnight
23. Measure RNA concentration with Eppendorf Bio-Photometer

6.6 cDNA synthesis

1. Dilute RNA 1:10
2. Insert 2 µg RNA in 10 µl RNase free water (in 96 well plates)

3. Add 10 µl of Mastermix to each sample

Mastermix components	**Volume per sample / reaction**
10X RT buffer	2 µl
25X dNTPs	0.8 µl
10X random hex primers	2 µl
MultiScribe RT (50 U/µl)	1 µl
H_2O	4.2 µl
Final volume	**10 µl**

4. Spin down plate
5. Load into thermal cycler (Biorad, MyCycler)

Thermal cycler conditions:					
		Step1	Step2	Step3	Step4
Temperature		25°C	37°C	85°C	4°C
Time		10 min	120 min	5 s	∞

6. Set reaction volume to 20 µl
7. START the run

6.7 PCR

1. Dilute cDNA 1:20 = 20 ng/4 µl (inserted for one reaction)
2. Add 6 µl of Master Mix per sample
   - Master Mix per sample:
     - TaqMan Gene Expression Master Mix 5 µl
     - Taqman®gene expression assays 0.5 µl
     - RNase free water 0.5 µl
3. Load into thermal cycler (LightCycler®480)

6.8 Reagents for immunohistochemistry

1X PBS

- 7.59 g NaCl
- 1.246 g $Na_2HPO_4 \cdot 2H_2O$
- 0.14 g $NaH_2PO_4 \cdot 1H_2O$
- ad 1 L distilled water,

- Adjust pH to 7.4 with NaOH

4 % Paraformaldehyde in 1X PBS

- 40 g PFA
- 800 ml distilled water
- Stir and heat to 55°C
- Add 100 µl of NaOH in a cumulative manner until solution gets clear
- 100 ml 10X PBS
- ad 1 l distilled water
- Cool down to room temperature
- Adjust pH to 7

6.9 MPO - ELISA

Lysis buffer

- 200 mM NaCl (5.84g)
- 5 mM EDTA (0.93g)
- 10 mM Tris (0.605g)
- 10 % glycerol (50 ml)
- 0.1 mM PMSF (2.17 ml [10 mg PMSF/2.5 ml isopropyl alcohol])
- 1 µg/ml leupeptin (0.5 mg)
- 28 µg/ml aprotinin (14 mg)
- ad 500 ml H_2O; pH 7.4

Homogenization

- Add 200 µl lysis buffer to 10 mg tissue before homogenization
- Homogenize with the Ultraturrax (2 x 1 min)
- Centrifuge samples twice: 7000 rpm, 15 min, 4°C.
- Store supernatant at -70°C

Reagent preparation

Wash buffer: 20 ml concentrated wash buffer + 380 ml H_2O

Dilution buffer:

- Mix 5 ml of the 10X dilution buffer A with 20 ml H_2O
- Mix 5 ml of the 10X dilution buffer B with 20 ml H_2O
- Combine both solutions equally and mix well

Standard solution:

The standard is reconstituted by pipetting the amount of dilution buffer mentioned on the certificate of analysis in the standard vial

- Use the standard vial as Tube # 1
- Prepare 7 tubes with 225 µl dilution buffer
- Make a serial dilution by pipetting 225 µl from tube # 1 to tube # 2, mix well
- Pipette 225 µl from tube # 2 to tube # 3
- Continue until tube # 7
- Tube # 8 is only filled with dilution buffer

Tracer solution:

- The tracer is reconstituted by pipetting 1 ml H_2O
- Dilute the reconstituted 1 ml tracer with 11 ml dilution buffer

Streptavidin-peroxidase solution:

Mix 125 µl streptavidin-peroxidase conjugate with 12.375 ml dilution buffer

Sample dilution:

- DSS-treated samples 1:20 or 1:50
- Control samples 1:2

Procedure

1. Bring all reagents to room temperature
2. Centrifuge samples at 7000 rpm for 15 min
3. Transfer 100 µl of blank, standard and samples into appropriate wells in duplicate.
4. Cover the plate and incubate for 1 h at room temperature
5. Wash the plate 4 x with 200 µl wash buffer
6. Add 100 µl of diluted tracer to each well
7. Cover the tray and incubate for 1 h at room temperature
8. Wash the plate 4 x with 200 µl wash buffer
9. Add 100 µl of diluted streptavidin-peroxidase to each well
10. Cover the plate and incubate for 1 h at room temperature
11. Wash the plate 4 x with 200 µl wash buffer
12. Add 100 µl of TMB substrate to each well
13. Cover the tray with aluminium foil and incubate the tray for 20-30 min
14. Stop the reaction by adding 100 µl of stop solution to each well
15. Read the plate within 30 min with microplate reader (BIO-TEK) at 450 nm