Dissertation

Diverse action of bacterial metabolites on immune activation, brain, and behavior

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 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".

Disclosure

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Abbreviations and definitions

ACTB	beta actin
ACTH	adrenocorticotropic hormone
BBB	blood-brain barrier
CA	central area
CARD	caspase recruitment domain-containing protein
CCL2	chemokine (C-C motif) ligand 2
CD	cluster of differentiation
CLDN5	claudin 5
CNS	central nervous system
CRH	corticotropin releasing hormone
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
EU	endotoxin unit
FCA	Freund’s complete adjuvant
GAPDH	glycerinaldehyde-3-phosphate-Dehydrogenase
HEK	human embryonic kidney
HPA	hypothalamic-pituitary-adrenal
hTLR	human Toll-like receptor
IBD	inflammatory bowel disease protein
IDO	indoleamine 2,3 dioxygenase
IFN	interferon
IL	interleukin

i.p.	intraperitoneal
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAP	mitogen activated protein
MDP	muramyl dipeptide
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	nucleotide-binding domain, leucine rich repeat containing receptor
NOD	nucleotide-binding oligomerization domain
NR3C1	nuclear receptor subfamily 3 group C member 1
OCLN	occluding
OF	open field
PAMP	pathogen associated molecular patterns
PFCT	prefrontal cortex
PPIL3	peptidyl-prolyl cis-trans isomerase-like 3
PRR	pattern recognition receptor
TJP1	tight junction protein 1
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
SEM	standard error of the mean
SLC6A4	solute carrier family 6 (neurotransmitter transporter)

Zusammenfassung

Pattern Recognition Receptors (zu Deutsch in etwa Mustererkennungsrezeptoren), wie Toll-like Rezeptor 4 (TLR4) erkennen mikrobielle Metaboliten, die Effekte auf das Immunsystem, das Gehirn und Verhalten haben können. Während die Wirkung des TLR4 Agonisten Lipopolysaccharid (LPS) auf Immunsystem und Gehirn gut erforscht ist, gebührte anderen Toll-like Rezeptoren und ihren Agonisten weniger Aufmerksamkeit. In der vorliegenden Dissertation wurde daher folgende Hypothese aufgestellt: der TLR2 Agonist Lipoteichonsäure (LTA) aktiviert das Immunsystem peripher sowie zentral, stimuliert die Hypothalamic-Pituitary-Adrenal (HPA)-Achse und hat nachteilige Effekte auf Blut-Hirn-Schranke und emotionales Verhalten.

Da kommerzielle LTA Produkte häufig mit LPS kontaminiert sind, wurden für diese Studie ein Extrakt der LTA ($LTA_{extract}$), eine gereinigte Form der LTA (LTA_{pure}), und eine gereinigte Form des LPS ($LPS_{ultrapure}$) verwendet und miteinander verglichen. Alle drei Substanzen wurden auf ihre Effekte auf molekularer Ebene und Verhaltensparameter untersucht, 3 Stunden nach intraperitonealer Injektion bei männlichen C57BL/6N Mäusen. Um Reinheit und Spezifität der Substanzen zu untersuchen, wurde der HEK-Blue® Reporter Zell Test sowie der TLR4 Antagonist TAK-242 verwendet.

Im Open Field Test zeigte $LTA_{extract}$ (20 mg/kg) eine anxiogene Wirkung, erhöhte die Zytokinspiegel im Blut und Gehirn und verringerte die Expression der Tight Junction Proteine im Gehirn. Die Menge an LPS Kontamination in $LTA_{extract}$ wurde ermittelt, und eine entsprechende Dosis $LPS_{ultrapure}$ zeigte eine Wirkung korrelierend mit der von $LTA_{extract}$ auf molekulare Marker und Verhalten. LTA_{pure} (20 mg/kg) erhöhte die Menge an Tumor Necrosis Factor-α (TNF-α), Interleukin-6 und Interferon-γ im Plasma, sowie die Expression von TNF-α und Interleukin-1β mRNA und weiterer Zytokine in Amygdala und Präfrontalkortex. Die Expression der Tight Junction Proteine Claudin 5 und Occludin im Gehirn war in Folge der Behandlung mit LTA_{pure} verringert. Des Weiteren erhöhte LTA_{pure} zirkulierendes Kortikosteron, ohne dabei einen Effekt auf das Verhalten auszuüben.

Die vorliegende Arbeit zeigt, dass Aktivierung von TLR2 durch LTA Effekte auf das Immunsystem hat und neuroinflammatorische Prozesse im Gehirn initiiert. Die neuroinflammatorischen Prozesse gehen einher mit einer Schwächung der Blut-

Hirn-Schranke und Aktivierung der HPA-Achse, ohne jedoch Einfluss auf das emotionale Verhalten zu haben. Außerdem weisen die Ergebnisse auf eine gegenseitig verstärkende Wirkung von TLR2 und TLR4 hin. Mögliche Auswirkungen auf eine langfristige Neuroinflammation, Beeinträchtigung der Blut-Hirn-Schranke und daraus resultierende negative Effekte auf die psychische Gesundheit machen diese Erkenntnisse relevant für weitere Studien.

Abstract in English

Pattern recognition receptors (PRRs) are a crucial factor of the first-boarder defense against invasion by microbes. One broadly studied member of the PRRs is the lipopolysaccharide (LPS)-recognizing Toll-like receptor 4 (TLR4) which stimulates not only the peripheral immune system when activated, but affects brain and behavior. While the effects of LPS on the communication of the immune system and the brain are well understood, effects of other TLR agonists are insufficiently studied. Therefore, the current study aimed to elucidate the wide-ranging effects of the TLR2 stimulating bacterial cell-wall compound lipoteichoic acid (LTA) and hypothesized that LTA leads to immune activation in periphery and brain, activates the hypothalamic-pituitary-adrenal (HPA) axis and affects behavior as well as the integrity of the blood-brain barrier (BBB). Since commercially available LTA is often found to be contaminated with LPS that might be the true cause of LTA effects studied thus far, an extract of LTA ($LTA_{extract}$), purified LTA (LTA_{pure}), and pure LPS ($LPS_{ultrapure}$) were studied with emphasis on their effects 3 h following intraperitoneal (i.p.) injection to male C57BL/6N mice. To assess the purity and specificity of the TLR2 and TLR4 stimulating compounds under study, HEK-Blue® reporter cell assay as well as TAK-242, a TLR4 antagonist, were used.

$LTA_{extract}$ (20 mg/kg) showed adverse effects on behavior in the open field and affected molecular parameters, as it induced anxiety-like behavior and raised cytokine levels in the peripheral circulation and cytokine mRNA expression in the brain. Tight junction-associated proteins were down-regulated in their expression on mRNA level. By measuring endotoxin/LPS contamination of $LTA_{extract}$ the corresponding $LPS_{ultrapure}$ dose was determined and used to reproduce numerous effects of $LTA_{extract.}$ In contrast, although effective in elevating corticosterone levels within the periphery, LTA_{pure} (20 mg/kg) failed to induce anxiety-like behavior and change locomotor activity in the open field. Furthermore, LTA_{pure} affected transcription of tight junction-associated proteins, namely claudin 5 and occludin in the brain while upregulating transcription of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1 β), among other cytokines, in the prefrontal cortex and amygdala. Concerning circulating cytokines, LTA_{pure} elevated interleukin-6 (IL-6), TNF-α, and interferon-γ (IFN- γ).

Taken together, this study provides a broader insight into the effects of LTA acting on TLR2. These effects include stimulation of the peripheral immune system and induction of neuroinflammatory processes within the brain that go along with reduced mRNA expression of BBB tight-junction constituents as well as the activation of the HPA axis. As $LTA_{extract}$ showed more adverse effects when compared to LTA_{pure}, including the induction of anxiety-like behavior among others, a facilitatory interaction of LTR2 and TLR4 activation by endotoxin contamination of $LTA_{extract}$ is assumed. These data inform further studies to elucidate possible long-term inflammatory effects in the brain, disturbance of the integrity of the BBB as well as effects on mental health.

Introduction

The immune-brain axis: interplay between the immune system, brain, and behavior

The observation that mammals respond to infection with sickness behavior including fatigue, anhedonia, and depression has been stated in medical literature since the 19th century (Bested et al., 2013). While the activation of the immune system and the subsequent sickness behavior serve the positive purpose of enabling recovery from disease, the immune system might also be directly responsible for depressive symptoms and those depressive symptoms are furthermore a predictor for heart disease and other chronic illnesses or an indicator for worse outcome of such chronic diseases (Evans et al., 2005). Studies showing effectiveness in the treatment of depression with the tumor necrosis factor α blocker/antibody etanercept/infliximab further strengthen this hypothesis, even more so considering that infliximab was only effective in depressed patients with high inflammatory markers before treatment onset (Raison and Miller, 2013; Schmidt et al., 2014). Vice versa, treatment modalities involving the cytokine interferon α, as used for certain cancers, have also been observed to induce depression in patients (Capuron, 2002; Dantzer et al., 2011). Taking into account the constant influx and exchange of immune stimuli from the microbiota, it is not surprising the hypothesis of the microbial-immune-brain axis gained traction over the last three decades, along with advances in microbiome research techniques (Qin et al., 2010). While the debate over the ratio of bacterial versus mammalian cells in the human body has not been settled since 1972 (Luckey, 1972), the current estimate is 1:1 (Sender et al., 2016). Furthermore, the human microbiome is estimated to contain more than 10 million genes (Li et al., 2014). Considering there are 10^{13}-10^{14} bacterial as well as mammalian cells in the human body, it is hypothesized that a vast signal transfer is taking place between the microbial population and the immune system (Schroeder and Bäckhed, 2016).

The crosstalk between the gut and the central nervous system (CNS) takes place via various signaling systems (De Palma et al., 2014) which can be grouped in five major pathways (Figure 1). (1) Microbial metabolites, secreted by the microbiota display pathogen associated molecular patterns (PAMPs). PAMPs get recognized by pattern recognition receptors (PRRs) such as Toll-like receptors (TLR) on

macrophages and dendritic cells and upregulate genes responsible for the expression of cytokines (Takeuchi and Akira, 2010). Microbial metabolites also exert a number of other effects. Butyric acid is an agonist at G-protein coupled receptors 41 and 43, produced by the microbiota via fermentation of dietary fiber. Butyric acid was shown to reduce symptoms of depression and Huntington's disease (Ferrante et al., 2003; Schroeder et al., 2007). The inflammatory and cytokine inducing effects of the TLR5 agonist flagellin are widely used in vaccine development (Cuadros et al., 2004; Honko et al., 2006; Newton et al., 1991; Turley et al., 2011). (2) Cytokines are an important means of communication between the immune system and the brain following microbial insult (Bluthé et al., 1994; Dantzer, 2004; Dantzer et al., 2008; Holzer and Farzi, 2014). In addition to being an effect of microbial insult, they can also have a positive impact on neurogenesis and synaptic remodeling (Yirmiya and Goshen, 2011). In the brain, inflammatory effects of cytokines mainly affect the monoamine, glutamate, and neuropeptide systems (Felger and Lotrich, 2013). Furthermore, the enzyme converting tryptophan to kynurenine, indoleamine 2,3 dioxygenase (IDO), was shown to be a major contributor to the depression inducing effects of cytokines (Dantzer et al., 2011) The vagus nerve is another important means for relaying cytokine induced signaling to the brain (Schweighöfer et al., 2016). Zielinski et al. (2013) showed that the effect of peripheral TNF-α to induce TNF-α and IL-1β mRNA expression in the brain was attenuated by vagotomy.

(3) The secretion and recognition of gut hormones is heavily influenced by the gut microbiota. Enteroendocrine cells are constantly scanning the milieu of the gut, sensing metabolites secreted or produced by the microbiota, such as short-chain fatty acids, to respond to the host with an array of gut hormones (Clarke et al., 2014; Greiner and Bäckhed, 2016). Gut hormones such as peptide YY, pancreatic polypeptide, glucagon-like peptide 1, glucagon, and cholecystokinin regulate satiety, blood pressure, insulin secretion, and behavior (Afsar et al., 2016; Field et al., 2010; Greiner and Bäckhed, 2016; Holzer et al., 2012; Skibicka and Dickson, 2013). Gut hormones can act either on vagal afferent neurons, close to the enteroendocrine cells they originate from, or get relayed to the brain via the circulation (Dockray, 2013). (4) Vagal as well as spinal afferent neurons represent a “fast signaling“ route between the gastrointestinal tract and the CNS. Intrinsic (enteric nervous system) and extrinsic sympathetic, parasympathetic (pelvic nerves

and vagus nerves), and sensory (spinal and vagal pathways) neurons are distributed throughout the gastrointestinal tract (Uesaka et al., 2016). The vagus nerve has been shown to be an important route for the interaction between the gut microbiome and the brain (Bluthé et al., 1994; Bravo et al., 2011; Forsythe et al., 2014). Bercik et al. (2011) showed that Bifidobacterium longum NCC3001 exerts anxiolytic activities by decreasing excitability of enteric neurons, and this effect is relayed to the brain via vagal pathways. Vagal afferent neurons furthermore sense gut hormones such as cholecystokinin and glucagon-like peptide 1 secreted in response to nutrient intake and mechano-distension (de Lartigue and Diepenbroek, 2016; Webster and Beyak, 2013). (5) The hypothalamic-pituitary-adrenal (HPA) axis represents a truly bi-directional information system between the brain and gut (Figure 1) because, on the one hand, information from the brain is transmitted via cortisol (corticosterone in rodents) to the periphery and, on the other hand, cortisol and corticotropin-releasing hormone (CRH)-related peptides, formed in the gut, are feeding back information to the brain. CRH, together with peptides of the urocortin family, are responsible for mounting an appropriate coping response to stress (Ourailidou and Grammatopoulos, 2017). CRH as well as urocortin have been shown to induce stress-like stimulatory actions on colonic propulsive motor activity via activation of myenteric cholinergic neurons expressing CRH_1 receptors (Taché et al., 2005).

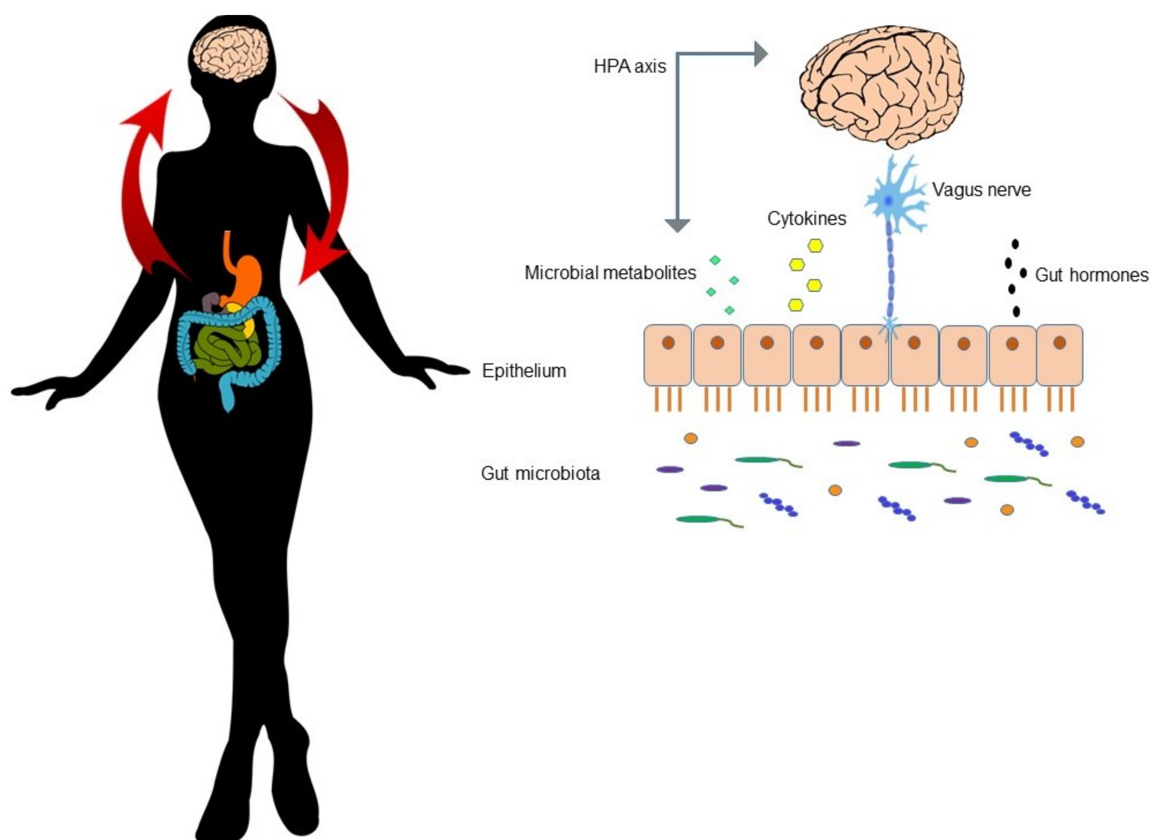


Figure 1 Factors influencing the microbiota-gut-brain pathways.

Pro-inflammatory cytokines and their effects on behavior

Bacterial infection of peripheral tissues has been shown to cause innate immune cells to produce pro-inflammatory cytokines which act on the brain to cause sickness as well as molecular and behavioral perturbations (Dantzer, 2006; Dantzer et al., 2008). This sickness behavior, such as anhedonia, anorexia, and fatigue are a means of fighting an infection by reserving all resources for coping with invading pathogens via fever and mounting an appropriate immune response, as well as isolating the infected animal from others to halt the spread of the pathogen (Hart, 1988). Upon activation of the PRRs by invading pathogens, an inflammatory response is induced by the host immune system (Takeuchi and Akira, 2010). The major players in this innate immune response are cytokines. The cytokines best characterized regarding their sickness inducing effects are IL-1β, IL-6, TNF-α, and interferons (Dantzer, 2006). Peripheral administration of lipopolysaccharide (LPS) has been shown to induce interleukin-1β (IL-1β) and TNF-α expression (Quan et al., 1999). The behavioral effects of LPS are also well demonstrated (Bluthé et al., 2000;

Farzi et al., 2015b; Frenois et al., 2007; McCusker and Kelley, 2013). Depressive-like behavior in mice following LPS administration was displayed in the tail suspension test, forced swim test, sucrose preference test, as well as motor activity. Furthermore, LPS-induced cytokine secretion has been shown to act on the HPA axis via stimulation of adrenocorticotropic hormone (ACTH) secretion (Rivier, 1993).

Neuroinflammatory processes in the brain

The effects of peripheral immune activation to alter brain function and behavior are thought to be mediated by neuroinflammatory processes in the brain in which activation of microglial cells, generation of an array of pro- and anti-inflammatory cytokines, and the recruitment of peripheral leukocytes are involved, these processes ultimately leading to a change in neuronal function and even neuronal loss in distinct brain circuits. Importantly, neuroinflammation appears to be a pathogenetic factor relevant to many neuropsychiatric as well as neurodegenerative diseases (Becher et al., 2016; Skaper, 2007). Neuroinflammatory processes involve the brain's own resident immune system, in which glial cells (microglia, astrocytes, and oligodendrocytes) engage in "inflammatory" processes that defend the brain from pathogens and help it to recover from stress and injury (Skaper, 2007). If these glial functions result in a more severe and chronic neuroinflammatory cycle, neurodegenerative disease may ensue (Skaper et al., 2014). More detailed analysis has revealed that different types of neuroinflammatory reactions do exist, depending on which triggers are involved and whether tissue invasion and destruction by blood-borne leukocytes takes place (Becher et al., 2016). Alterations in tissue homeostasis, trauma or ischemic damage initiate glial cell responses within the brain, which include the production of an array of cytokines. In contrast, in acute infectious conditions or chronic inflammatory diseases, blood-borne leukocytes invade the CNS and also represent a major source of inflammatory mediators including cytokines (Becher et al., 2016). Consequently, the outcome and severity of neuroinflammation depends on whether cytokines produced predominantly by brain-resident cells or CNS-invading cells are involved (Becher et al., 2016).

Coping with stress: the hypothalamic-pituitary-adrenal axis

The HPA axis is a major pathway in processing stress stimuli. Stress, in general, can be defined as a disruption of homeostasis. In a pathologic context, psychological as well as molecular perturbations are of relevance (Ulrich-Lai and Herman, 2009). The general loop, shown in Figure 2, entails secretion of corticotropin-releasing hormone (CRH) and vasopressin from the paraventricular nucleus (PVN), following secretion of ACTH from the anterior pituitary, inducing the secretion of glucocorticoids (corticosterone in rodents, cortisol in humans) from the adrenal cortex. The negative feedback loop is mediated by glucocorticoids to suppress CRH and ACTH production and thereby control HPA axis activity (Smith and Vale, 2006). Immune stimulation is considered to be a type of internal stress that leads to activation of the HPA axis and, most likely, the glucocorticoid feedback loop. It has previously been reported that activation of murine TLR4 by LPS enhances the circulating levels of corticosterone (Farzi et al., 2015b; Lehmann et al., 2013), an effect that indirectly contributes to the behavioral alterations associated with peripheral immune stimulation. Furthermore, corticotropin-releasing hormone secreted from the paraventricular nucleus of the hypothalamus activating CRH receptor 1 has been shown to induce anxiety (McCall et al., 2015; Zhang et al., 2016).

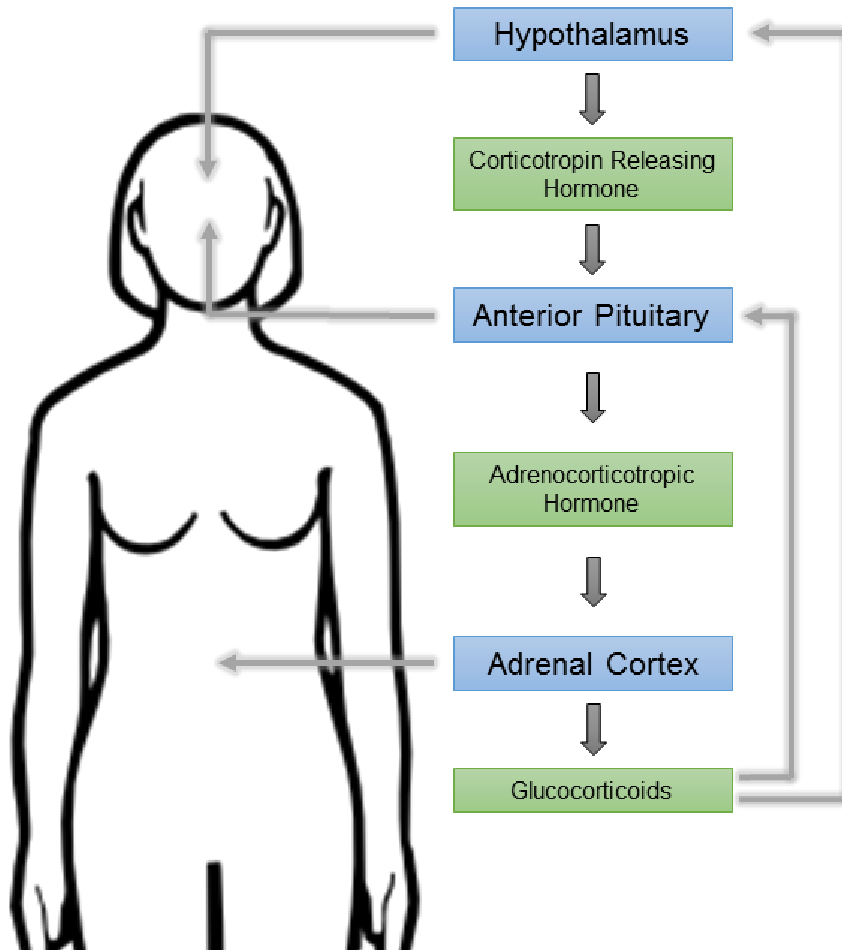


Figure 2 A schematic drawing of the feedback loop of the HPA axis.

Lipopolysaccharide and its action on Toll-like receptor 4

The immune system senses bacterial intrusion via PRRs which recognize PAMPs, evolutionarily highly conserved structures on pathogens. TLRs represent the best characterized family of PRRs which are expressed on the cell surface and thus can initiate a first-line immune response (Figure 3) against invading pathogens (Medzhitov et al., 1997).

TLR4, expressed on the plasma membrane of macrophages and dendritic cells, for instance, is responsible for the recognition of lipopolysaccharide (LPS) on the cell wall of gram-negative bacteria (Poltorak, 1998; Takeuchi and Akira, 2010). Upon binding of LPS to TLR4, which requires the presence of myeloid differentiation 2, the signaling cascade targets myeloid differentiation primary response protein 88 and results in the release of pro-inflammatory cytokines (Kawai et al., 1999). If LPS

is endocytosed, the TRIF-related adaptor molecule/TIR-domain-containing adapter-inducing interferon-β pathway is activated and causes release of type 1 interferons (Kawai and Akira, 2010) (Figure 3). Through these immune mediators, LPS is known to cause sickness and evoke signs of anxiety- and depression-like behavior in rodents (Bluthé et al., 1994; O'Connor et al., 2009; Painsipp et al., 2010; Sulakhiya et al., 2016). In humans, symptoms of depression and anxiety are correlated with LPS exposure and subsequent cytokine release (Vogelzangs et al., 2016), and increased IL-6 and INF-α levels correlate with severity of depression and anxiety (Capuron et al., 2009; Raison et al., 2006).

The family of TLRs comprises 12 members (10 in humans) (Pandey et al., 2015) which are targeted by different PAMPs (Kawai and Akira, 2010). Under conditions of bacterial invasion, it is likely that different PRRs are activated in parallel and that the ensuing immune and brain responses are the result of the positive and/or negative interactions between the PRR-mediated reactions. For instance, the sickness response to LPS is enhanced by synergism between TLR4 and the nuclear-binding domain (NOD)-like receptors NOD1 and NOD2, which recognize peptidoglycan elements (Farzi et al., 2015b).

Lipoteichoic acid and its action on Toll-like receptor 2

In contrast to LPS, lipoteichoic acid (LTA) is a major cell wall component of gram-positive bacteria and a PAMP that is primarily recognized by Toll-like receptor 2 (TLR2) (Hermann et al., 2002) (Figure 3). LTA is a surface-associated adhesion amphiphile composed of a soluble polymer, consisting of polyhydroxy alkane units, such as ribitol and glycerol, attached to the cell membrane with a diacylglycerol. The sequence of glycerol and ribitol repeat units varies between species (Schneewind and Missiakas, 2014). Bacteriolysis leads to the release of LTA into the bloodstream, which occurs in response to β-lactam antibiotic treatment (van Langevelde et al., 1998). TLR2 is expressed on the cell surface of monocytes, macrophages, dendritic cells, and mast cells and with the help of the class B scavenger protein CD36 specifically recognizes LTA (Abe et al., 2010; Hennessy et al., 2010). LTA induces the secretion of cytokines such as IL-1β and TNF-α, which can contribute to the disruption of the blood-brain barrier (BBB) (Boveri et al., 2006). In addition, LTA is required for anchoring microorganisms to brain microvascular endothelial cells that disrupt the BBB (Sheen et al., 2010).

While there are several important barriers in the CNS (De Bock et al., 2014), and a TLR mediated impact of bacterial metabolites on the choroid plexus and the brain-cerebrospinal fluid barrier has been observed (Stridh et al., 2013), the work at hand is based on the studies of Braniste et al. (2014) and Wittmann et al. (2015), who showed that LPS and other bacterial metabolites stemming from the microbiota negatively impact the BBB.

Despite the deleterious impact mediated by LTA on BBB function, the effects of this PAMP on molecular changes in the immune-brain axis and on behavior have not yet been explored.

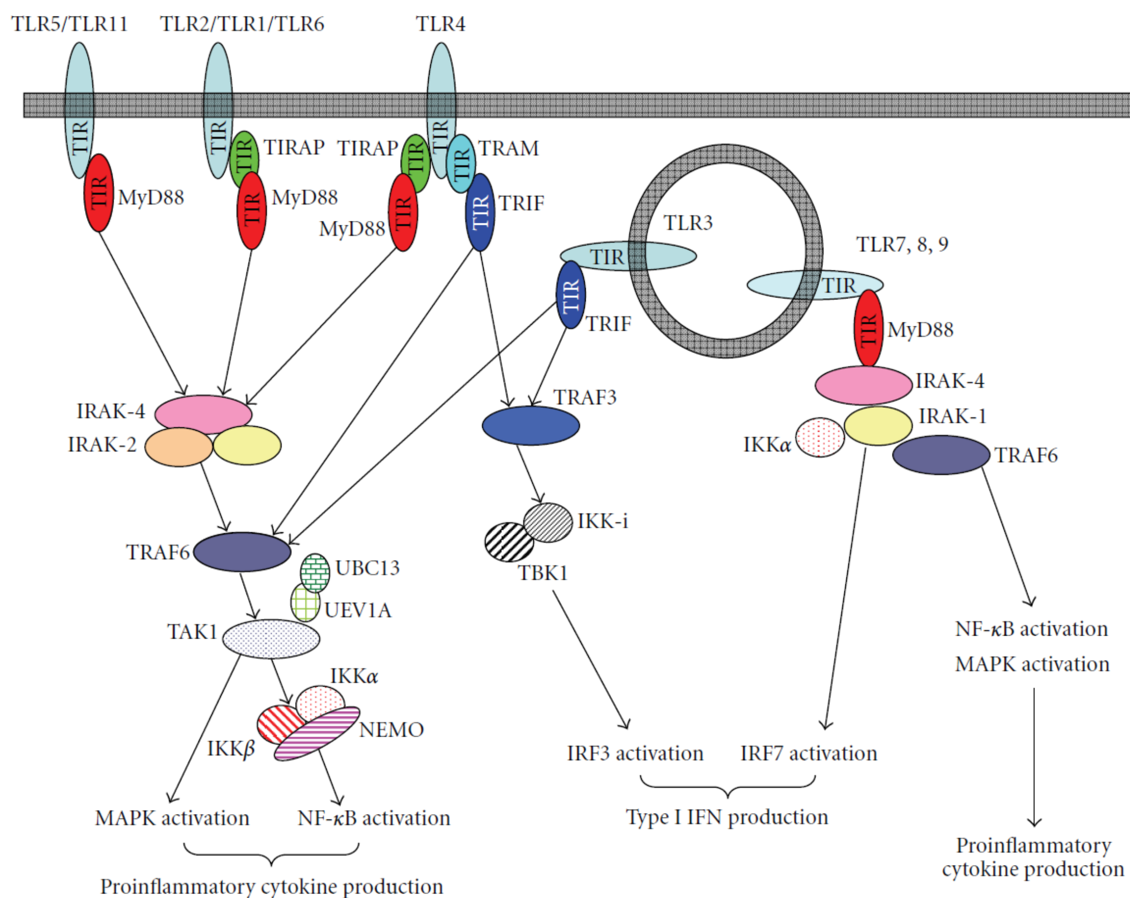


Figure 3 TLR-mediated signaling pathways. TIRAP and MyD88-dependent pathways result mainly in the production of proinflammatory cytokines; TRIF and TRAM-mediated signaling. Reprinted with permission from Hindawi Publishing Corporation [Gastroenterology Research and Practice] (Yamamoto and Takeda, 2010), copyright 2010. Legend adapted from (Yamamoto and Takeda, 2010).

Nucleotide-binding domain and leucine-rich repeat containing gene family – NLRs

NLRs are pattern recognition receptors sensing microbial compounds intracellularly either following phagocytosis or as result of cell stress in the form of damage-associated molecular patterns. Activation of the NLRs is a crucial part of the innate immune response. Depending on the specific type of NLR and the respective microbial compound involved, the activation is either caspase-1 or NF-κB- and MAP-kinase mediated. There are over 34 NLR genes in mice (23 in humans) (Franchi et al., 2009). According to their N-terminal domain, NLRs are divided into 4 subfamilies:

- NLRA (A for acidic transactivation domain)
- NLRB (B for baculoviral inhibitory repeat-like domains)
- NLRC (C for caspase recruitment domain)
- NLRP (C for pyrin domain)
- NLRX (X for no significant homology to any N-terminal domain) (Ting et al., 2008)

Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) belongs to the NLRC subfamily and is responsible for the recognition of muramyl dipeptide (MDP) (Girardin, 2003).

NOD2 and the recognition of muramyl dipeptide

NOD2 is also known as caspase recruitment domain-containing protein 15 (CARD15) or inflammatory bowel disease protein 1 (IBD1) and is connected to a predisposition to Crohn's disease and Blau syndrome (Girardin et al., 2003). Upon stimulation of NOD2, NF-κB and mitogen-activated protein kinases (MAPKs) are activated by MDP. MDP is part of peptidoglycan, a polymer on the outer layer of the membrane of gram-positive and gram negative bacteria (Strober et al., 2006). Peptidoglycan consists of glycan chains of alternating N-acylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) units cross-linked by peptides. NOD2 is furthermore involved in the antiviral immune response by binding to virus-derived single-stranded RNA (Sabbah et al., 2009). The expression of NOD2 is limited to monocytes and macrophages and intestinal epithelial cells (Oh et al., 2005).

Reciprocity between NOD and TLR

The immunostimulatory potential of MDP was discovered in the course of experiments involving Freund's complete adjuvant (FCA). FCA is made of heat-killed *Bacille Calmette Guerin* and MDP is an immune stimulating agent in this preparation. As FCA has strong toxicity, MDP was regarded a promising alternative (Ogawa et al., 2011). MDP alone has weak immune boosting activities, however, if used as a priming agent in combination with other PAMPs, MDP is capable of potentiating the immunostimulatory action of the PAMP in question (Takada et al., 2002; Wolfert et al., 2002; Yang et al., 2001). Parant et al., (1990) were the first to show an increased secretion of TNF following MDP and LPS treatment in mice. The combination of MDP and LPS was shown to increase cytokine and corticosterone secretion, as well as the kynurenine/tryptophan ratio; MDP in combination with LPS was furthermore shown to increase immobility in the forced swim test, an indicator for depression in mice (Farzi et al., 2015b). In cultured human dendritic cells, MDP in combination with lipid A was shown to significantly increase IL-12 and INF-γ production (Tada et al., 2005).

Aims of the thesis

Bacillus subtilis is an omnipresent microbe, found as a commensal in soil (Han et al., 2014; van Dijl and Hecker, 2013) as well as the human intestinal tract (Fakhry et al., 2008; Hong et al., 2009; Tam et al., 2006), is used in probiotics (Hanifi et al., 2015; Lefevre et al., 2017; Oggioni et al., 1998) on the one hand and has been found as the cause of nosocomial infections and sepsis on the other hand (La Jeon et al., 2012; Matsumoto et al., 2000; Oggioni et al., 1998). Gram-positive related nosocomial infections and sepsis have increased since the 1980s and gram-positive, particularly methicillin-resistant *Staphylococcus aureus* infections now make up a majority of sepsis cases (Jiang et al., 2017; Martin, 2012; Martin et al., 2003; Qin et al., 2017). Cognitive impairment, depression, and anxiety are widespread consequences of sepsis (Jaenichen et al., 2012; Jones and Griffiths, 2013; Kapfhammer, 2016). While the effects of endotoxin from gram-negative bacteria on mood, behavior, and the BBB have been widely studied, the impact of LTA on behavior and the brain is largely unknown (Bluthé et al., 2000; Farzi et al., 2015b; Layé et al., 1994; Lestage et al., 2002; O'Connor et al., 2009). Furthermore, the colonization of the human body with 10^{13}-10^{14} microbial cells, the microbiome,

presents a constant influx of microbial metabolites into the system, influencing host immune system and behavior (Qin et al., 2010; Schroeder and Bäckhed, 2016). Increased biomarkers of inflammation, such as proinflammatory cytokines, are considered major players in inducing depression and anxiety following microbial insult (Capuron and Miller, 2011). LTA has been shown to induce the release of proinflammatory cytokines such as TNF-α and IL-1β as well as to be involved in the breakdown of the BBB, though possible behavioral effects of these events have not been investigated (Boveri et al., 2006; Sheen et al., 2010). TLR2 is the main receptor responsible for LTA detection and plays a crucial role in immune system activation following infection with gram-positive bacteria (Hermann et al., 2002). More importantly, TLR2 activation is crucial for the initiation of an appropriate immune response after CNS infection of bacteria crossing the BBB (Böhland et al., 2016; Echchannaoui et al., 2002).

As issues regarding the purity and activity of commercial LTA preparations have been raised (Gao et al., 2001; Morath et al., 2001), the study at hand was performed with three different immune-stimulatory agents. A crude extract of LTA from *Bacillus subtilis* ($LTA_{extract}$), a purified preparation of LTA from *Bacillus subtilis* (LTA_{pure}), and an ultrapure preparation of LPS from *E.coli* ($LPS_{ultrapure}$), as LPS is a common contaminant of LTA products (Gao et al., 2001). Effects of $LTA_{extract}$ and LTA_{pure} on the immune-brain axis were compared with the effects of $LPS_{ultrapure}$, in order to establish a robust protocol to assess true LTA activity and rule out LPS confounding actions.

Against this background, this thesis set out to pursue 8 specific aims related to the effect of LTA on the interaction between peripheral immune system, on the one hand, and brain function and behavior, on the other hand.

The first aim was to examine, in a comparative manner, the effects of $LTA_{extract}$, LTA_{pure}, and $LPS_{ultrapure}$ on behavior in the open field, a common paradigm to assess anxiety and sickness. $LTA_{extract}$, LTA_{pure}, and $LPS_{ultrapure}$ were injected i.p. prior to exposure to the open field.

As these experiments revealed both differential and overlapping changes in behavior, the second aim was to analyze the specificity and purity of $LTA_{extract}$, LTA_{pure}, and $LPS_{ultrapure}$ as TLR2 and TLR4 agonists, respectively. This goal was

addressed with the HEK-Blue® reporter cell assay in vitro and with the TLR4 inhibitor TAK-242 in vivo.

The third aim was to investigate immune system activation in the periphery and brain by determining the levels of cytokines in the plasma as well as their expression in the amygdala and prefrontal cortex in response to i.p. administration of $LTA_{extract}$, LTA_{pure}, and $LPS_{ultrapure}$.

Cytokine induction caused by immune stimulation may in turn trigger secondary mechanisms that contribute to alterations in brain function. For instance, formation of the tryptophan catabolite kynurenine by IDO is enhanced by cytokines, kynurenine being considered to contribute to the anhedonic and anxiogenic effects of immune stimulation (Haroon et al., 2012; O'Connor et al., 2009; Salazar et al., 2012). This contention is deduced from the ability of IDO blockade to attenuate LPS-induced depression-like behavior without altering the expression of cytokines in the brain (O'Connor et al., 2009). For this reason, the fourth aim of the thesis project was to measure the circulating tryptophan and kynurenine levels following $LTA_{extract}$ exposure.

Tryptophan is the principal substrate for the synthesis of the neurotransmitter serotonin (5-hydroxytryptamine). As serotonin-mediated neurotransmission is regulated by reuptake of this indoleamine into the presynaptic nerve terminals via the serotonin transporter encoded by the gene SLC6A4 (solute carrier family 6 (neurotransmitter transporter), the fifth aim of my thesis was to explore the effect of LTA_{pure}, $LTA_{extract}$, and $LPS_{ultrapure}$ on SLC6A4 mRNA expression in the amygdala and prefrontal cortex. In addition, the expression of the glucocorticoid receptor encoded by the gene NR3C1 (nuclear receptor subfamily 3 group C member 1) in these brain regions was also assessed in order to assess any glucocorticoid feedback on the brain (Howell and Muglia, 2006).

Extrinsic and intrinsic stressors are known to affect the HPA axis, immune activation being considered to impose endogenous stress as deduced from the ability of LPS to stimulate the HPA axis (Borrow et al., 2016; Farzi et al., 2015b; Lehmann et al., 2013). The sixth aim was therefore to compare the effects of $LTA_{extract}$, LTA_{pure}, and $LPS_{ultrapure}$ on the circulating corticosterone levels.

Given that both LPS and LTA have been found to affect the BBB (Boveri et al., 2006; Sheen et al., 2010), the seventh aim of the dissertation was to address the effects of $LTA_{extract}$, LTA_{pure}, and $LPS_{ultrapure}$ on BBB composition. BBB integrity was assessed in relation to the transcriptional regulation of the tight junction-associated proteins claudin 5 (CLDN5), occludin (OCLN) and tight junction protein 1 (TJP1) in the amygdala and prefrontal cortex. Since the expression of some of these proteins was altered, I also evaluated BBB functionality by measuring Evans Blue extravasation.

The eighth aim of this thesis addressed the possible interactions of several PAMPs, given that the combination of MDP and LPS has been found to synergize in immune stimulation and in the activation of the immune-brain axis (Farzi et al., 2015b). To this end, the combination of $LTA_{extract}$, which was found to include both LTA and LPS, and MDP was investigated with respect to cytokine expression in the periphery and brain as well as behavior.

Methods and materials

The description of Materials and Methods is partly reused from Mayerhofer et al. (2017).

Experimental animals

The experiments were performed with 10-week-old male C57BL/6N mice (n=204; 22-27 g body weight) obtained from Charles River (Sulzfeld, Germany). The animals were housed in pairs in a vivarium under controlled conditions: temperature set point at 22 °C, air humidity set point at 50 % and a 12 h light/dark cycle. Tap water and standard laboratory chow consisting of 19.2 % protein, 4.1 % fat, 6.1 % fiber, 6.9% ash (Altromin 1324 forti, autoclavable maintenance diet for rats and mice; Altromin, Germany) were provided *ad libitum* throughout the experiment. Parts of this methodological description was also published in an original article (Mayerhofer et al., 2017).

Ethics statement

The experimental procedure and number of animals used were approved by the ethical committee at the Federal Ministry of Science, Research, and Economy of the Republic of Austria (BMWF-66.010/0026-WF/II/3b/2014) and conducted according to the Directive of the European Parliament and of the Council of September 22, 2010 (2010/63/EU). The experiments were designed in such a way that both the number of animals used and their suffering was minimized. This methodological description was also published in an original article (Mayerhofer et al., 2017).

Reagents

- **LTA from *Bacillus subtilis*** in the form of a crude cell wall extract was obtained from Sigma-Aldrich (Vienna, Austria; catalog number L3265), from here on referred to as $LTA_{extract}$.
- **LTA from *Bacillus subtilis*** in a purified formulation obtained from Invivogen (Toulouse, France; catalog number tlrl-lta), from here on referred to as LTA_{pure}.
- **LTA from *Staphylococcus aureus*** of unspecified purity was obtained from Sigma-Aldrich (catalog number L2515), from here on referred to $LTA_{extract}$ S.aureus.

- **LPS from *Escherichia coli*** O111:B4 extracted by successive enzymatic hydrolysis steps and purified by the phenol-TEA-DOC extraction protocol was obtained from Invivogen (catalog number tlrl-3pelps), from here on referred to as $LPS_{ultrapure}$.
- **TAK-242**, a TLR4 antagonist for TLR4 antagonism experiments, was obtained from Calbiochem/Merck Millipore (Darmstadt, Germany; catalog number US1614316, resatorvid, ethyl (6R)-6-[N-(2-chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate) (Ii, 2006; Kawamoto et al., 2008).
- **MDP** (N-acetylmuramyl-L-alanyl-D-isoglutamine hydrate), an activator of NOD2, was purchased from Sigma–Aldrich (catalogue number A9519).

Quantitation of endotoxin in $LTA_{extract}$ with the EndoLISA® endotoxin detection assay

To determine the amount of endotoxin present in $LTA_{extract}$, the EndoLISA® (Hyglos, Bernried am Starnberger See, Germany) endotoxin detection assay with a measurement range of 0.05 - 500 EU/ml was used. The assay was performed according to the manufacturer's instructions. This methodological description was also published in an original article (Mayerhofer et al., 2017).

Experimental groups and timelines of the *in vivo* experiments

The animals were given two weeks to get accustomed to the vivarium at the institute before any experiments were performed. To reduce extrinsic stressors affecting the experiments, they were transferred to the behavioral test room at least 16 h (overnight) before behavioral testing or euthanization.

Preliminary experiments involving 7 groups (n=6-8 per group) of mice were conducted to study the effect of $LTA_{extract}$ *B. subtilis* in the open field test (Figure 4) with regard to dosage and timeline. Initially, $LTA_{extract}$ (0.15 mg and 0.5 mg per mouse) or its vehicle (pyrogen-free sterile saline, 8 ml/kg) was administered i.p. 3 h before the open field test was performed. Next, 0.5 mg $LTA_{extract}$ (corresponding to 20 mg/kg in a 25 g mouse) or its vehicle was administered i.p. to test for any behavioral effect in the open field test 6 h and 27 h post-injection. All further experiments were conducted with the 20 mg/kg LTA dose injected i.p. 3 h prior to behavioral testing or organ collection.

Furthermore, a pilot experiment to assess the anxiogenic potential of LTA of *S.aureus* was performed. Mice (n=8 per group) were administered either 0.5 mg of $LTA_{extract}$ *S.aureus* or its vehicle (pyrogen-free sterile saline, 8 ml/kg) i.p. 3 h before the open field test was performed. As $LTA_{extract}$ *S.aureus* did not elicit any anxiogenic effect, further experiments were performed with LTA from *B.subtilis*.

To compare the effects of LTA_{pure}, $LTA_{extract}$, and $LPS_{ultrapure}$, relative to their vehicle on behavior in the open field, six groups (n=6-8 per group) of mice were employed.

For the collection of blood and brains, six additional groups (n=5-9 per group) treated with LTA_{pure}, $LTA_{extract}$, $LPS_{ultrapure}$ or their vehicle were used to exclude any potential confounding effects of behavioral tests on molecular marker expression. All three TLR agonists were dissolved in pyrogen-free sterile saline right before administration and injected i.p. LTA_{pure} and $LTA_{extract}$ were administered at a dose of 20 mg/kg and a volume of 5 ml/kg, while $LPS_{ultrapure}$ was given at a dose of 1938 EU/kg and a volume of 7.75 ml/kg, corresponding to the amount of 97 EU/mg endotoxin detected in the 20 mg/kg $LTA_{extract}$ dose by means of the EndoLISA® endotoxin quantification assay. The vendor of $LPS_{ultrapure}$ does not provide any information on the EU/weight ratio of this preparation. According to a formula given by the vendor of the EndoLISA® assay (1 EU ≈ 100 pg *E. coli* LPS; www.hyglos.de) the dose of 1938 EU/kg $LPS_{ultrapure}$ would roughly correspond to 0.19 mg/kg. This quantity appears plausible, as a dose of 0.1 mg/kg LPS has previously been found to affect behavioral parameters in the LabMaster system (Farzi et al., 2015b). As control treatment, pyrogen-free sterile saline was used at a volume of 5 ml/kg. Due to the experimental schedule, a maximum of 16 animals could be used per day. Therefore, the experiments were performed in a consecutive manner, each treatment group (LTA_{pure}, $LTA_{extract}$, $LPS_{ultrapure}$) being compared with a separate vehicle group, which is also reflected in the statistical analysis (independent samples *t* test). Injections were made between 9:00 am and 12:00 pm, and the open field test and the plasma/organ collections were performed 3 h after the injection of the respective TLR agonist or its vehicle.

For the *in vivo* assessment of the effects of TAK-242 on corticosterone levels, six additional groups (n=7-8 per group) of animals were used. The animals received 4 mg/kg of TAK-242 dissolved in 11% DMSO i.p. at a volume of 10 ml/kg, and 30 min

later the animals received either 20 mg/kg LTA_{pure} or $LTA_{extract}$ as well via i.p. injection. As control treatments, either 11% DMSO (vehicle for TAK-242) or pyrogen-free sterile saline (vehicle for LTA_{pure} or $LTA_{extract}$) were used, respectively (Farzi et al., 2015a). Blood was collected 3 h after the injection of LTA_{pure} or $LTA_{extract}$. Parts of this methodological description were also published in an original article (Mayerhofer et al., 2017).

To study the effects of 20 mg/kg $LTA_{extract}$, 3 mg/kg MDP or a combination of both (all injected i.p.), relative to vehicle on open field behavior (experiment 1), circulating corticosterone, cytokines in plasma, as well as on cytokine expression in the brain (experiment 2), and tryptophan and kynurenine levels (experiment 3) twelve groups (n=5-9 per group) of mice were used. The assessments were performed 3 h after i.p. treatment.

To gauge crude effects on BBB disruption by $LTA_{extract}$, a perfusion experiment including Evans blue was performed (Jangula and Murphy, 2013; Üllen et al., 2013). Animals received either 20 mg/kg $LTA_{extract}$, 3 mg/kg MDP or a combination of both via i.p. injection. Sterile saline was used as control. Each of the four experimental groups included 6-9 mice. Simultaneously they received 120 µl/30 g Evans blue solution (3% in sterile saline) i.p. Three hours after the injections, the animals were transferred to the perfusion laboratory and anesthetized with 200 µl of pentobarbital. After cessation of reflexes, the thorax was cut open and a hole was cut into the left and right ventricle of the heart. A needle connected to a perfusion pump was inserted into the left ventricle and secured with a clamp. Subsequently, 25 ml of ice cold 0.1 M phosphate buffered saline (PBS; pH 7.4) was used to perfuse the mouse in an approximate time frame of 6 minutes. Afterwards, the head was removed immediately and the brain was collected and placed in 5 ml dimethylformamide (DMF) to extract Evans Blue from the tissue.

Activation of TLR2 and TLR4 in the HEK-Blue® reporter cell assay

HEK-Blue® (Invivogen, Toulouse, France) hTLR2 and hTLR4 cells were grown in Dulbecco's Modified Eagle Medium (Gibco, ThermoFisher, Waltham, MA, USA) containing 4.5 g/l glucose, 2 nM L-glutamine, 10% fetal bovine serum, 1% penicillin-streptomycin and 1 x HEK-Blue® Selection at 37 °C and 5% CO_2. After confluency was reached, cells were seeded into 24-well plates, 2.5 x 10^5 cells/well. Cells were

then treated with 10^2 pg/ml, 10^4 pg/ml, or 10^6 pg/ml of LTA_{pure}, $LTA_{extract}$, or $LPS_{ultrapure}$ and incubated for 24 h. Sterile, distilled H_2O was used as control.

To assess the TLR4 specificity of the agonists, cells were incubated overnight (12 h) with 3 µM TAK-242 dissolved in DMSO. DMSO was used as control at a concentration that did not exceed 0.2%. Following the overnight treatment with TAK-242 or DMSO, $LTA_{extract}$ (10^6 pg/ml) or $LPS_{ultrapure}$ (10^4 pg/ml, or 10^6 pg/ml) was added, after which the cells were incubated for 24 h.

For quantitation of TLR2 and TLR4 activation, 180 µl of HEK-Blue® Detection medium was added to a 96-well plate, and 20 µl of supernatant from the treated cells was added. Alkaline phosphatase activity was subsequently measured with a Victor plate reader (PerkinElmer, Rodgau, Germany) at 655 nm. This methodological description was also published in an original article (Mayerhofer et al., 2017).

Open field test

The open field consisted of an opaque grey box (50 x 50 x 30 cm, B x W x H), illuminated by 35 lx at floor level (Figure 4). The central area (CA) was defined as 36 x 36 cm square in the middle, leaving a 7 cm boarder zone on each side. The test was performed individually with each mouse. The animals were placed in the center of the box, and their behavior (time in CA, CA visits, and total traveling distance) was tracked for 5 min by a video camera mounted above the open field and recorded with the VideoMot2 (TSE Systems, Bad Homburg, Germany) software (Figure 4). This methodological description was also published in an original article (Mayerhofer et al., 2017).

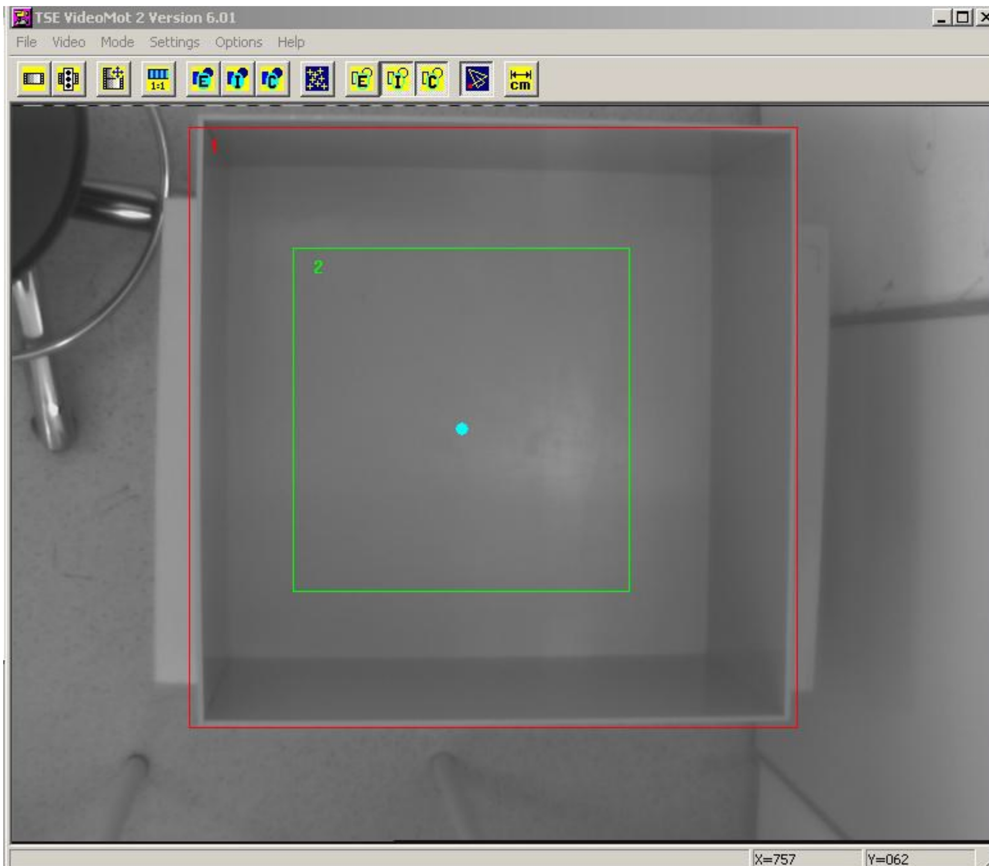


Figure 4 Screenshot of the VideoMot software including the open field box.

Blood sampling and collection of brains

Animals were deeply anesthetized with pentobarbital (150 mg/kg i.p.), and blood was collected via cardiac puncture. One hundred µl of 3.8% sodium citrate was used as anticoagulant in each single-use syringe. The samples were centrifuged at 7000 rpm and 4 °C for 15 min, then plasma was collected and stored at -70 °C until further processing. After blood collection, brains were collected and immediately frozen for 5 s in 2-methylbutane on dry ice. Brains were wrapped in aluminum foil and stored at -70 °C. This methodological description was also published in an original article (Mayerhofer et al., 2017).

Circulating corticosterone

Corticosterone levels in plasma were determined via an enzyme-linked immunosorbent assay (Assay Designs, Ann Arbor, Michigan, USA). In the current experiments, 5 µl of plasma was added to 995 µl assay buffer and processed according to the manufacturer’s instructions. The optical density was measured in a

plate reader at 405 nm. All samples were measured in duplicates. The manufacturer's specifications state a sensitivity of 27.0 pg/ml and intra- and inter-assay coefficients of variation of 7.7% and 9.7%, respectively. This methodological description was also published in an original article (Mayerhofer et al., 2017).

Circulating cytokines in plasma

To determine plasma levels of IL-1β (catalog number EPX01A-26002), IL-6 (EPX01A-20603), IL-10 (EPX01A-20614), INF-γ (EPX01A-20606), and TNF-α (EPX01A-20607), the magnetic bead-based ProcartaPlex™ immunoassay (catalog number EPX010-20440-901, eBioscience, San Diego, CA, USA) was used. The assay is based on the principle of analyte-specific magnetic beads coated with target-specific antibodies exhibiting a fluorescent signal upon excitation. The fluorescent signal was quantified with the Bio-Plex 200 multiplex suspension array system equipped with Luminex® xMAP® technology and the Bio-Plex 5.0 software (BioRad, Hercules, CA, USA). A reference analyte concentration was supplied by the manufacturer for the generation of a standard curve, and concentrations were determined via a five-parameter logistic curve-fitting method (Reichmann et al., 2015). The manufacturer states a sensitivity for each cytokine as follows: IL-1β: 0.14 pg/ml, IL-6: 0.21 pg/ml, IL-10: 0.69 pg/ml, TNF-α: 0.39 pg/ml, IFN-γ: 0.09 pg/ml. In the current experiments, 25 µl of undiluted plasma with sodium citrate as anticoagulant was used. The assay was performed according the manufacturer's instructions. This methodological description was also published in an original article (Mayerhofer et al., 2017).

Circulating tryptophan and kynurenine

This measurement was performed by Andreas Meinitzer and team (Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz), and the description of the method was published in Farzi et al. (2015b). Kynurenine and tryptophan levels were determined in plasma samples by high-performance liquid chromatography (HPLC) via ultraviolet detection (Hervé et al., 1996). In the current experiments, 100 µl of 5 % (v/v) perchloric acid was added to 100 µl plasma to deproteinize the sample, followed by vortexing and 5 min centrifugation at 11000×g. A volume of 20 µl of clear supernatant was injected into the chromatographic system. Chromolith RP18e columns (100 × 4.6 mm, 5 µm, Merck Darmstadt, Germany) at 30 ºC were used for separations 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. A LaChrom UV-Detector Merck HITACHI L-7400 at 235 nm was used for detection of kynurenine and tryptophan. Acquisition and processing of the chromatograms were executed using the Merck Hitachi LaChrom®-D-7000 HPLC-System Manager software (VWR International GmbH/Scientific Instruments, Darmstadt, Germany). Peak-height measurements against external standards were performed to determine concentrations. The method used has been validated according to international guidelines (Center for Veterinary Medicine 2001) and the reagents used were of p.A. grade (Merck).

Extraction of Evans Blue from brain tissue

The brains collected in 5 ml DMF were homogenized using a stick homogenizer. The homogenized brains in DMF were then placed on a tilting plate and kept in constant motion for one hour to allow extraction of Evans Blue from the tissue. The amount of Evans Blue extravasation was determined spectrophotometrically at 620 nm (Jangula and Murphy, 2013; Üllen et al., 2013).

Microdissection of amygdala and prefrontal cortex

The brains were microdissected by a trained researcher on a cold plate (Weinkauf Medizintechnik, Forchheim, Germany) at -20 °C (Brunner et al., 2014). The instruments were cleaned with RNase AWAY (Carl Roth, Karlsruhe, Germany) before and in between uses. The prefrontal cortex (Bregma +3.20 to -0.22) and amygdala (Bregma -0.58 to -2.54) were microdissected under a stereomicroscope. The dissected brain samples were transferred to micro packaging tubes with Precellys beads (Peqlab, Erlangen, Germany) and stored at -70 °C until further processing. This methodological description was also published in an original article (Mayerhofer et al., 2017).

Reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qPCR) of amygdala and prefrontal cortex

Amygdala and prefrontal cortex sections were homogenized with the Precellys 24 homogenizer (Peqlab, Erlangen, Germany). Subsequently, RNA was extracted according to the manufacturer’s instructions using the RNeasy lipid tissue mini kit (Qiagen, Hilden, Germany). The RNA concentration in each sample was determined via NanoDrop (Thermo Scientific, DE, USA). Afterwards, 2 µg of RNA were reverse-

transcribed with the high capacity cDNA reverse transcription kit (Fisher Scientific, Vienna, Austria) according to the manufacturer's instructions, using the Mastercycler Gradient (Eppendorf, Hamburg, Germany). Relative quantitation of mRNA levels was performed via qPCR using a LightCycler 480® system with TaqMan gene expression assays for CLDN5 (catalog number Mm00727012_s1), OCLN (Mm00500912_m1), TJP1 (Mm00493699_m1), CCL2 (Mm00441242_m1), IL-1β (Mm00434228_m1), IL-6 (Mm00446190_m1), IL-10 (Mm01288386_m1), INF-γ (Mm01168134_m1), TNF-α (Mm00443258_m1), SLC6A4 (Mm00439391_m1), and NR3C1 (Mm00439391_m1), and a master mix (catalog number 4369510, Fisher Scientific, Vienna, Austria). Controls without reverse transcriptase were included for each brain area and treatment group. ACTB (Mm00607939_s1), GAPDH (Mm99999915_g1), and PPIL3 (Mm00510343_m1) were used as endogenous reference genes. The $2^{-\Delta\Delta Ct}$ method was used to quantitate target gene levels relative to controls. Differences in treatment groups were expressed as fold changes. This methodological description was also published in an original article (Mayerhofer et al., 2017).

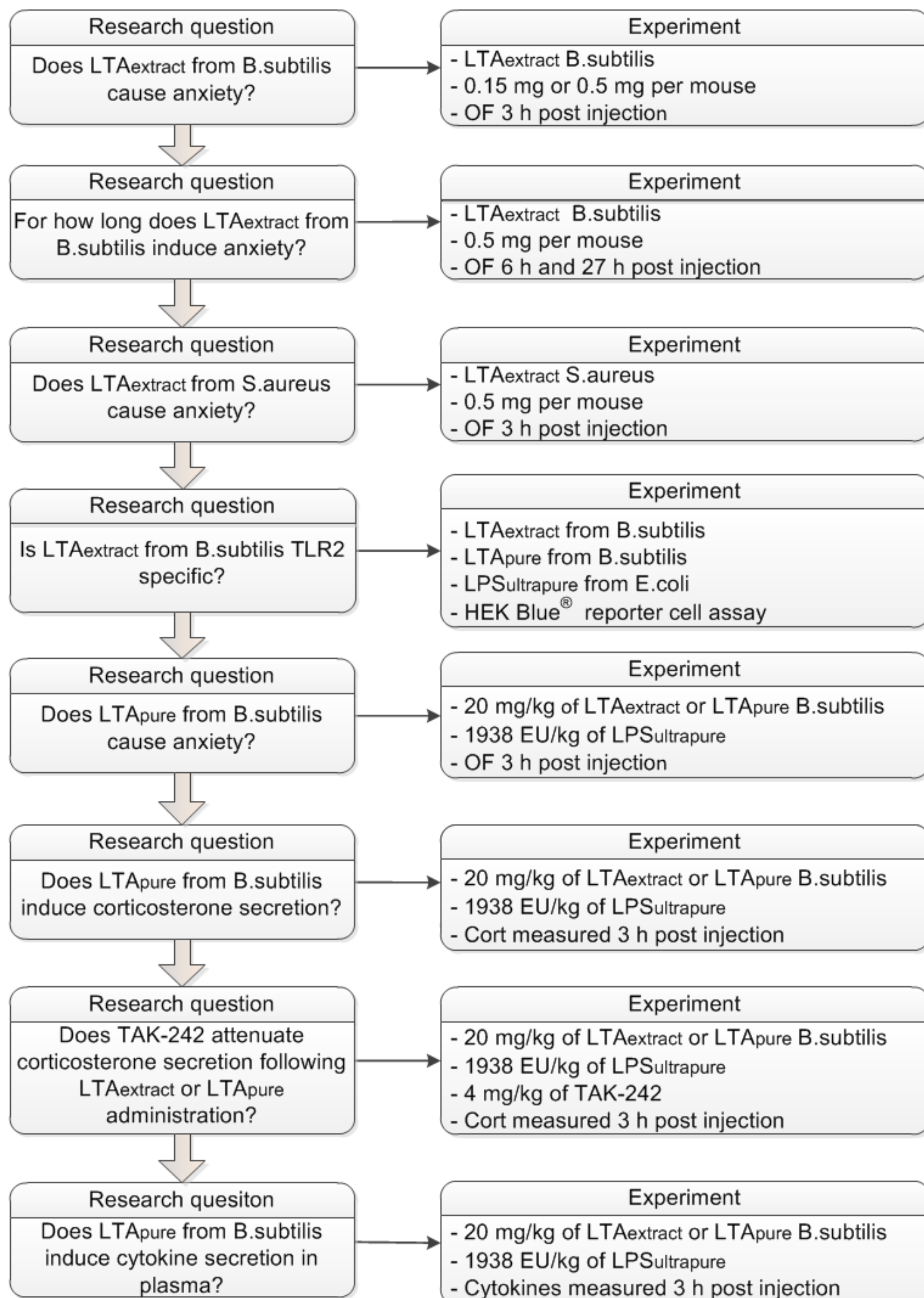
Statistics

Results were statistically analyzed using GraphPad® Prism5 (GraphPad Software Inc., La Jolla, CA, USA) or SPSS 22 (SPSS Inc., Chicago, IL, USA). Homogeneity of variances was assessed with the Kolmogorov-Smirnov test. Differences between groups were analyzed with one-way ANOVA and the unpaired samples *t* test was used where ANOVA was not permitted due to the consecutive setup of the experiments. In case a non-parametric test was required, the Mann–Whitney *U* test or Kruskal Wallis test was used. Probability values of $p \leqslant 0.05$ were regarded as statistically significant. Data are presented as means, SEM or SD being given as indicated, n referring to the number of mice in each group. This methodological description was also published in an original article (Mayerhofer et al., 2017)

Results

Research questions

Figure 5 lists 16 research questions according to the aims I addressed in my thesis project and gives information on the experimental approach to answer the questions.



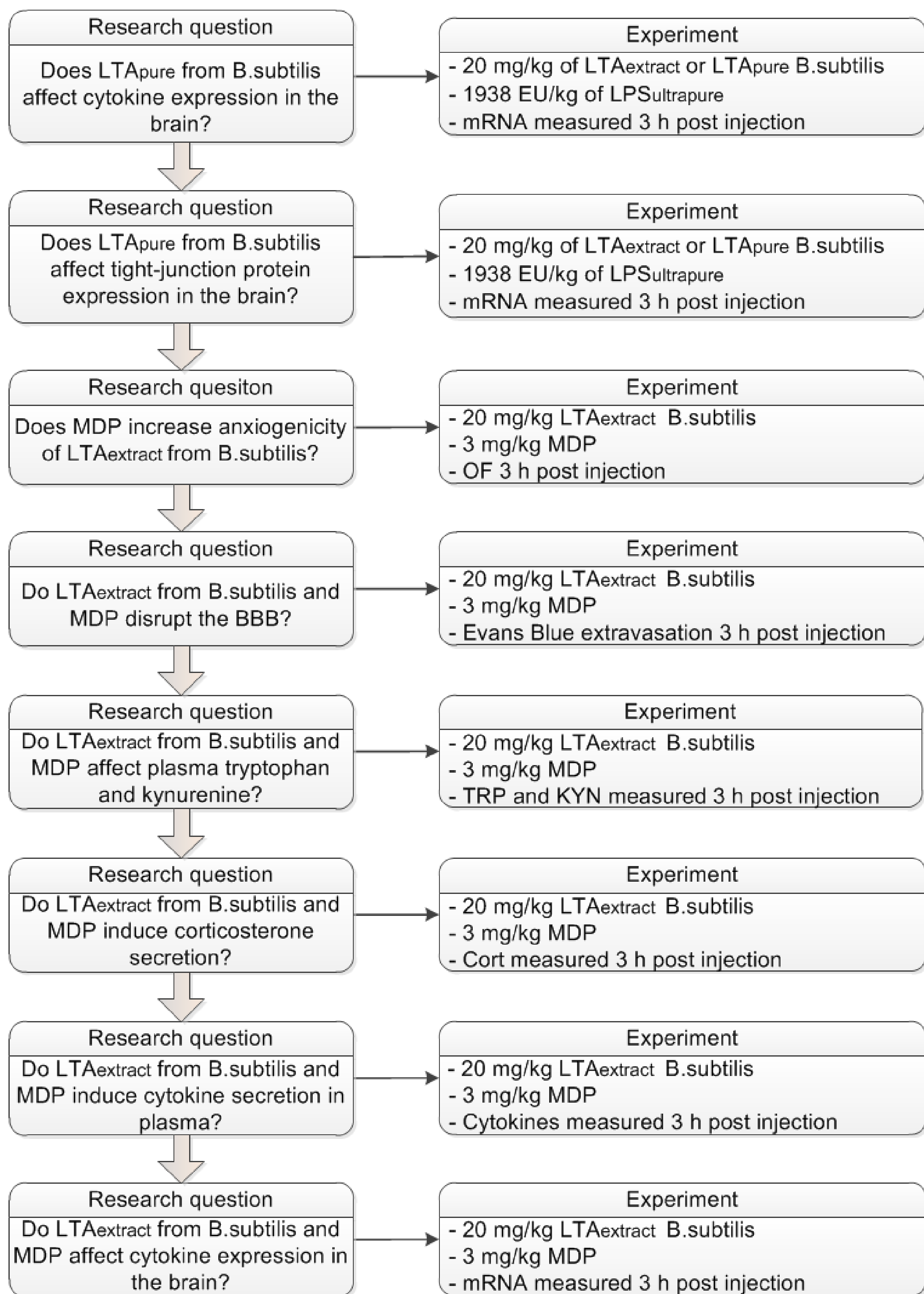


Figure 5 Flowchart of the experiments performed. The chart highlights research questions leading to the experiments performed to answer the questions and serves to guide the reader through the Results section.

$LTA_{extract}$ induces anxiety-like behavior in the open field test

To determine anxiety-like behavior following injection of the TLR agonists under study, the open field test was performed and time spent within the CA, as well as the frequency of CA visits were employed to measure anxiety-related behavior. In this respect, a prolonged time spent in the CA and an enhanced number of visits to the CA were valued as indicators for diminished anxiety, whereas locomotor activity determined by the total traveling distance was used to score sickness behavior. $LTA_{extract}$ enhanced anxiety-like behavior when administered at a dose of 0.5 mg/mouse (20 mg/kg in a 25 g mouse) 3 h (Figure 6), but not at 6 h or 27 h post injection (Table 1), nor when administered at the lower dose of 0.15 mg/mouse at any time point of behavioral testing (Figure 6).

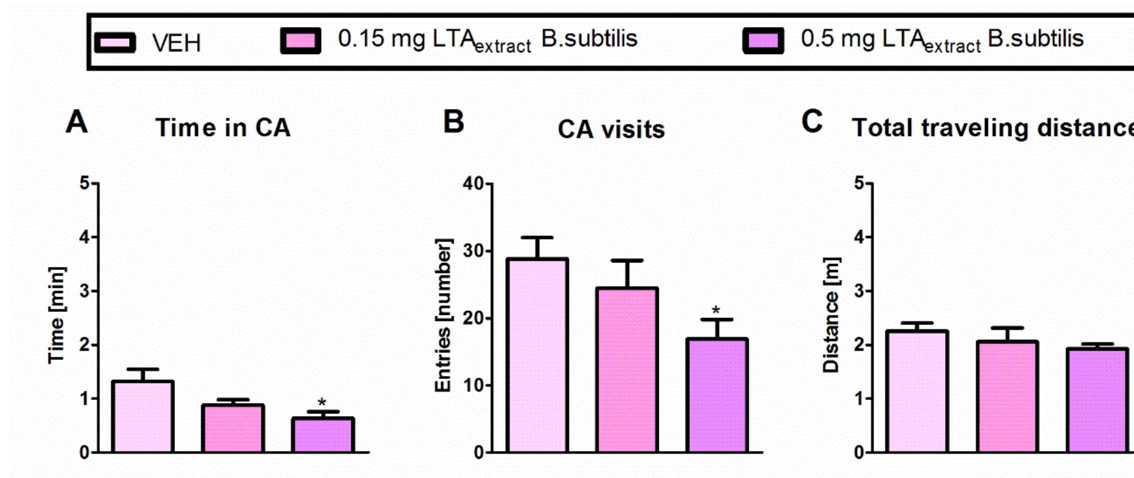


Figure 6 Effect of 0.15 mg and 0.5 mg $LTA_{extract}$ from *B. subtilis* on anxiety-like behavior in the open field test. The graphs depict the time spent in the central area (CA), the number of CA visits, and the total traveling distance during a 5 min test period. The open field test was performed 3 h after i.p. treatment with 0.15 mg or 0.5 mg $LTA_{extract}$ or the respective vehicle (VEH). The bars represent means + SEM, n = 6-8; $^*p \leq 0.05$ compared to VEH treated mice, one-way ANOVA with Tukey post-hoc test.

Treatment	VEH	$LTA_{extract}$	VEH	$LTA_{extract}$
Dose injected [mg]		0.5		0.5
Time of open field test post injection [h]	6		27	
Time in central area [min]	0.98	1.08	0.85	1.06
Central area visits	17.57	20.71	20.17	22.43
Total traveling distance [m]	2.08	2.03	2.29	2.04

Table 1 Lack of dose- and time-dependent effect of $LTA_{extract}$ from *B. subtilis* on anxiety-like behavior in the open field test as evaluated 6 and 27 h after i.p. treatment of mice with $LTA_{extract}$ at the dose/mouse indicated or its vehicle (VEH). The figures represent means, n = 6-8; independent samples *t* test. These findings have been published in an original article (Mayerhofer et al., 2017).

$LTA_{extract}$ from *S.aureus* has no effect on anxiety-like behavior

To assess the anxiogenic potential of $LTA_{extract}$ from *S.aureus*, 0.5 mg of $LTA_{extract}$ from *S.aureus* was administered 3 h prior an open field test. $LTA_{extract}$ from *S.aureus* had no effect on time spent in the CA, CA visits, and total traveling distance (Figure 7).

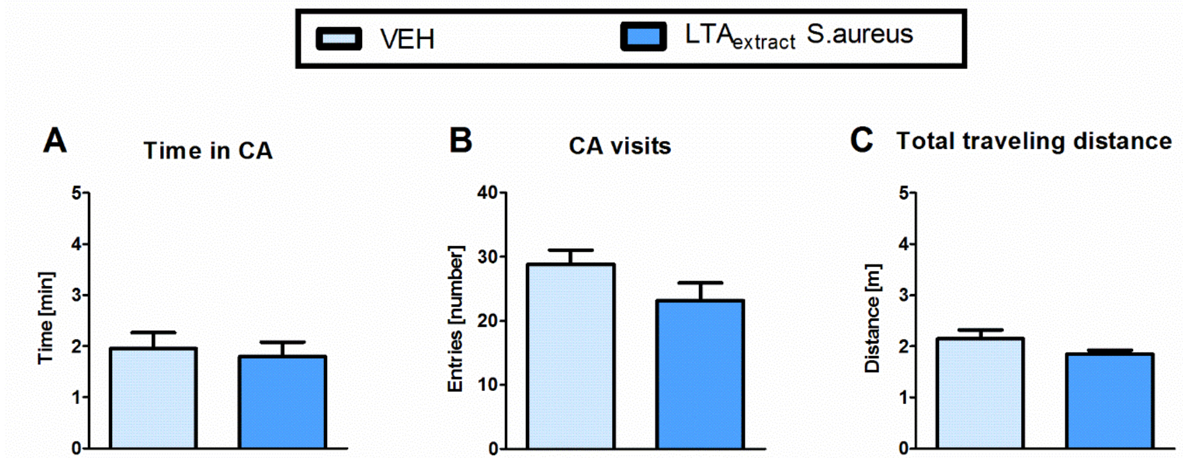


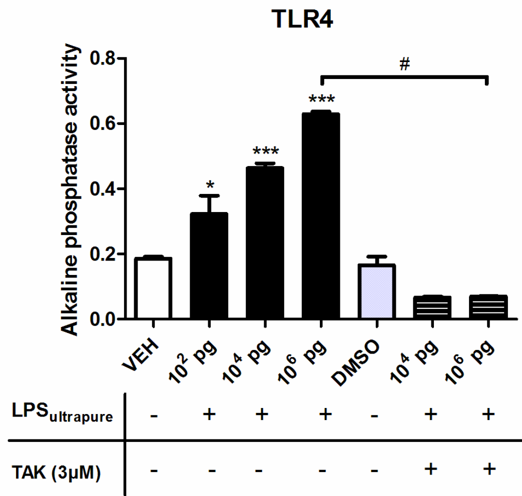
Figure 7 Effect of 0.5 mg $LTA_{extract}$ from *S. aureus* on anxiety-like behavior in the open field test. The graphs depict the time spent in the central area (CA), the number of CA visits, and the total traveling distance during a 5 min test period. The open field test was performed 3 h after i.p. treatment with 0.5 mg $LTA_{extract}$ or the vehicle (VEH). The bars represent means + SEM, n = 6; independent samples t test.

LTA_{pure} activates TLR2 but not TLR4 in the HEK-Blue® reporter cell assay, while $LTA_{extract}$ activates TLR4, but fails to activate TLR2

Since the purity of commercially available LTA was considered a subject of concern in publications of other groups in the past (Gao et al., 2001; Morath et al., 2001), the TLR selectivity of the TLR agonists $LTA_{extract}$, LTA_{pure}, and $LPS_{ultrapure}$ was examined by using HEK-Blue® reporter cell assay to clarify how much a contamination with LPS might contribute to the effects of the two LTA preparations.

First, $LPS_{ultrapure}$ was used to confirm the validity of the TLR4 system and the compound was shown to increase the secretion of alkaline phosphatase in a dose-dependent manner, as expected. This effect could be prevented by the TLR4 inhibitor TAK-242 (Figure 8A). TLR2 was dose-dependently activated to secrete alkaline phosphatase by the corresponding LTA_{pure}, while $LPS_{ultrapure}$ had no effect (Figure 8B). HEK-Blue® cells expressing the TLR4 receptor did not show alkaline phosphatase expression following LTA_{pure} treatment (Figure 8C). Furthermore, $LTA_{extract}$ proved to be ineffective in inducing TLR2 signaling (Figure 8D), but did activate TLR4 signaling in a concentration-dependent manner. This effect could be

diminished to baseline level when TAK-242 was present (Figure 8E). These findings have been published in an original article (Mayerhofer et al., 2017).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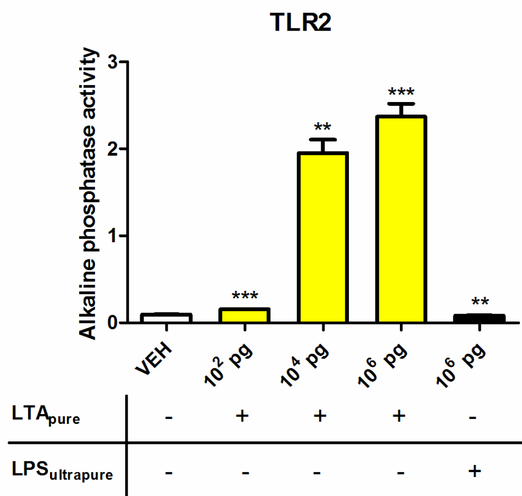
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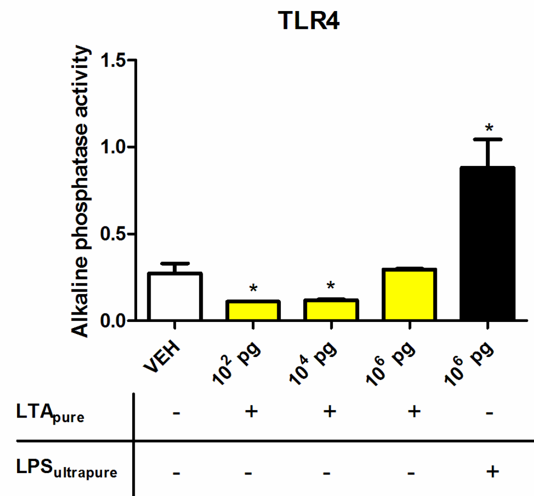
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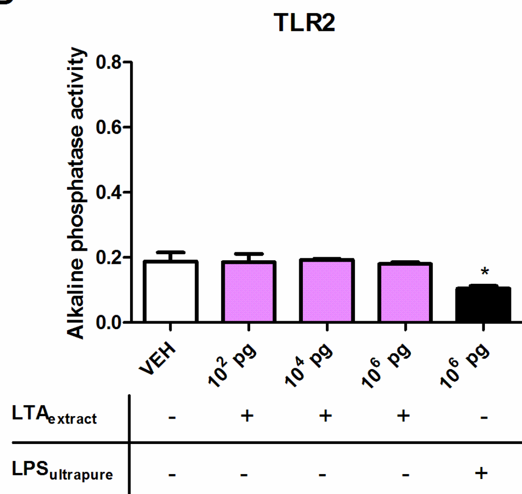
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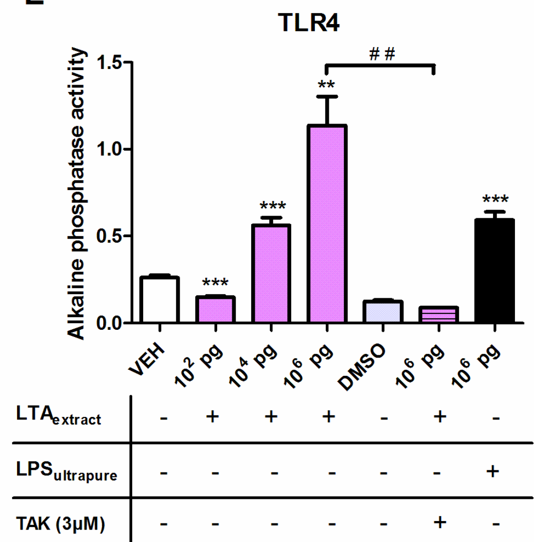
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TAK (3µM) - - - - - + -

Figure 8 Effect of LTA_{pure}, $LTA_{extract}$, and $LPS_{ultrapure}$ on alkaline phosphatase release in HEK-Blue® TLR2 and TLR4 reporter cells. HEK-Blue® TLR4 (A,C,E) and TLR2 (B,D) cells were incubated with $LPS_{ultrapure}$ (10^2 pg/ml, 10^4 pg/ml, 10^6 pg/ml; A), LTA_{pure} (10^2 pg/ml, 10^4 pg/ml, 10^6 pg/ml; B,C), or $LTA_{extract}$ (10^2 pg/ml, 10^4 pg/ml, 10^6 pg/ml; D,E). After a 24 h incubation, alkaline phosphatase activity was determined at 655 nm on a plate reader. TAK-242 (TAK; 3 µM) was added to the cell suspensions 12 h prior incubation with $LTA_{extract}$ or $LPS_{ultrapure}$. The bars represent means + SD from one representative experiment performed in triplicates; $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$ compared to vehicle (VEH) treated cells; $^{\#}p \le 0.05$ compared to 10^6 pg/ml $LPS_{ultrapure}$; $^{\#\#}p \le 0.01$ compared to 10^6 pg/ml $LTA_{extract}$. The data in panels A,B,E were analyzed with the independent samples *t* test, those in panels C,D with the non-parametric Kruskal-Wallis test. These findings have been published in an original article and are reused here with permission (Mayerhofer et al., 2017).

Lipopolysaccharide, but not pure lipoteichoic acid, induces anxiety-like behavior in the open field test

While comparing the effects of all three TLR agonists in a further set of open field tests, I found that LTA_{pure} did not influence behavior of the animals in a significant manner (Figure 9A). Regarding the time spent in the CA, systemic treatment with $LTA_{extract}$ clearly tended to induce anxiety ($p=0.0941$), while $LPS_{ultrapure}$ significantly triggered anxiety-like behavior (Figure 9B,C). The number of CA visits as well as total traveling distance were unaffected by $LTA_{extract}$ and $LPS_{ultrapure}$ (Figure 9B,C). Taken together, $LTA_{extract}$ administration leads to behavioral changes similar to $LPS_{ultrapure}$ which acts as an anxiogenic stimulus, while LTA_{pure} shows no such effect. These findings have been published in an original article (Mayerhofer et al., 2017).

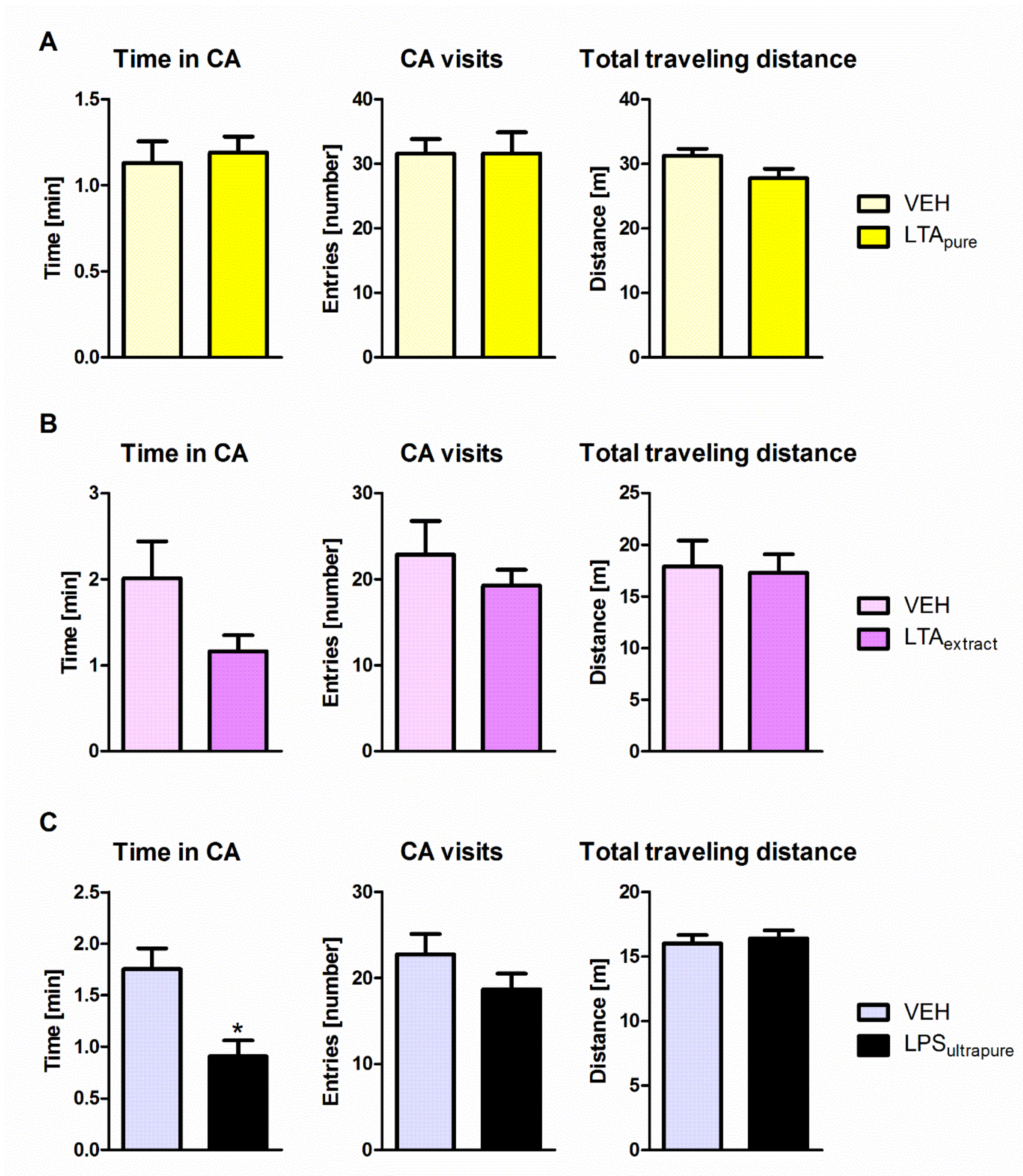


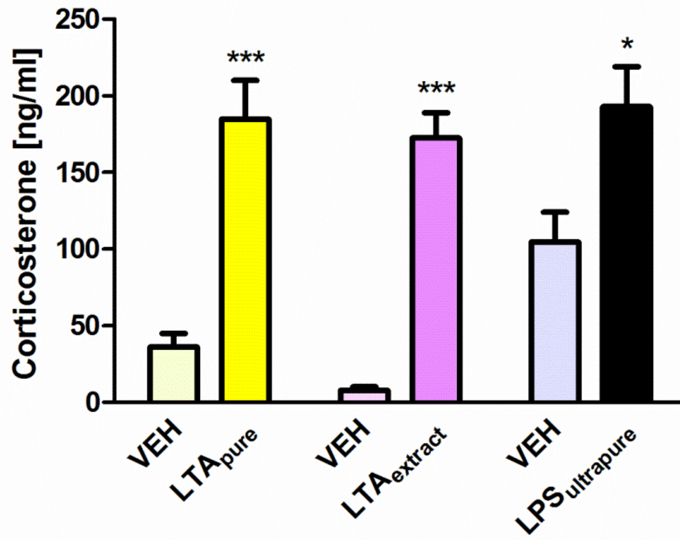
Figure 9 Effect of LTA_{pure}, $LTA_{extract}$, and $LPS_{ultrapure}$ on anxiety-like behavior in the open field test. The graphs depict the time spent in the central area (CA), the number of CA visits, and the total traveling distance during a 5 min test period. The open field test was performed 3 h after i.p. treatment with LTA_{pure} (20 mg/kg; A), $LTA_{extract}$ (20 mg/kg; B), $LPS_{ultrapure}$ (1938 EU/kg ≈ 0.19 mg/kg; C) or the respective vehicle (VEH). The bars represent means + SEM, n = 8-10; *$p \leq 0.05$ compared to VEH treated mice; independent samples *t* test. These findings have been published in an original article and are reused here with permission (Mayerhofer et al., 2017).

LTA_{pure}, $LTA_{extract}$, and $LPS_{ultrapure}$ increase circulating corticosterone

LPS is known to trigger corticosterone release into the circulation (Farzi et al., 2015b). To assess whether the three TLR agonists of interest in this study show effects on HPA axis activity, plasma concentrations of corticosterone were measured following LTA_{pure}, $LTA_{extract}$ and $LPS_{ultrapure}$ injection. Compared to samples drawn from mice treated with vehicle, all three agonists led to a significant increase of plasma corticosterone (Figure 10A). These findings have been published in an original article (Mayerhofer et al., 2017).

The TLR4 antagonist TAK-242 attenuates the increase in plasma corticosterone levels induced by $LTA_{extract}$ but not LTA_{pure}

To assess any in vivo effects of the TLR2 agonists LTA_{pure} and $LTA_{extract}$ on TLR4 signaling, the antagonist TAK-242 (4 mg/kg) was injected i.p. 30 minutes before administration of LTA_{pure} or $LTA_{extract}$, after which circulating corticosterone levels were measured. Compared to the vehicle control, corticosterone levels were slightly, but significantly increased by TAK-242 (Figure 10B). While LTA_{pure} was not inhibited in its ability to elevate plasma corticosterone when injected posterior to TAK-242 administration, application of this antagonist significantly reduced the capacity of $LTA_{extract}$ to induce HPA axis activity (Figure 10B). These findings have been published in an original article (Mayerhofer et al., 2017).



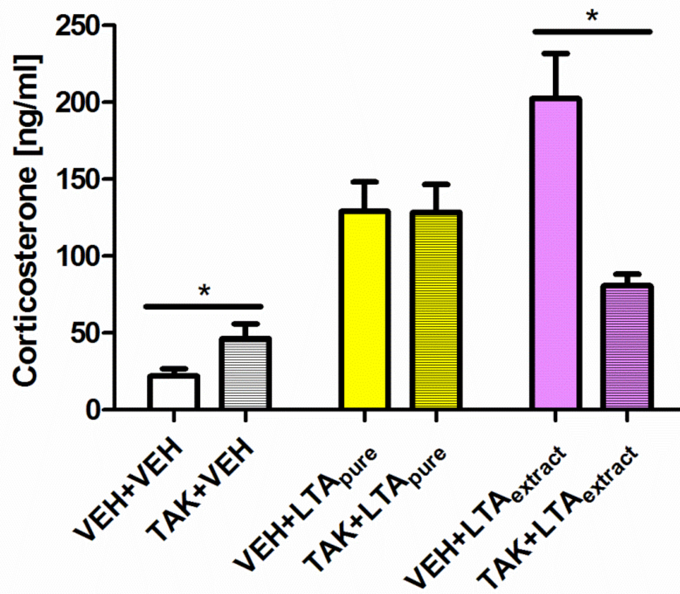


Figure 10 Effect of LTA_{pure}, $LTA_{extract}$, and $LPS_{ultrapure}$ on plasma corticosterone levels. Panel A depicts circulating corticosterone levels measured 3 h after i.p. treatment with LTA_{pure} (20 mg/kg), $LTA_{extract}$ (20 mg/kg), $LPS_{ultrapure}$ (1938 EU/kg ≈ 0.19 mg/kg) or the respective vehicle (VEH). Panel B depicts circulating corticosterone levels measured 3 h after i.p. treatment with LTA_{pure} (20 mg/kg) or

$LTA_{extract}$ (20 mg/kg), TAK-242 (TAK; 4 mg/kg) or its VEH being administered 30 min before LTA_{pure} or $LTA_{extract}$ was given. The bars represent means + SEM, n = 6-8; *$p \leq 0.05$, ***$p \leq 0.001$ compared to VEH treated mice; independent samples *t* test. These findings have been published in an original article and are reused here with permission (Mayerhofer et al., 2017).

LTA_{pure}, $LTA_{extract}$, and $LPS_{ultrapure}$ increase circulating cytokines

To estimate the ability of LTA_{pure}, $LTA_{extract}$ as well as $LPS_{ultrapure}$ to stimulate the peripheral immune system, plasma levels of cytokines were measured. Treatment with all three TLR agonists lead to significantly elevated concentrations of IL-6, TNF-α, and INF-γ 3h post injection (Figure 11B,D,E). Plasma samples of animals treated with LTA_{pure} and $LPS_{ultrapure}$ or the corresponding vehicles did not show detectable IL-1β and IL-10 concentrations (Figure 11A,C). $LTA_{extract}$, on the other hand, increased both IL-1β and IL-10 levels significantly relative to vehicle (Figure 11A,C).

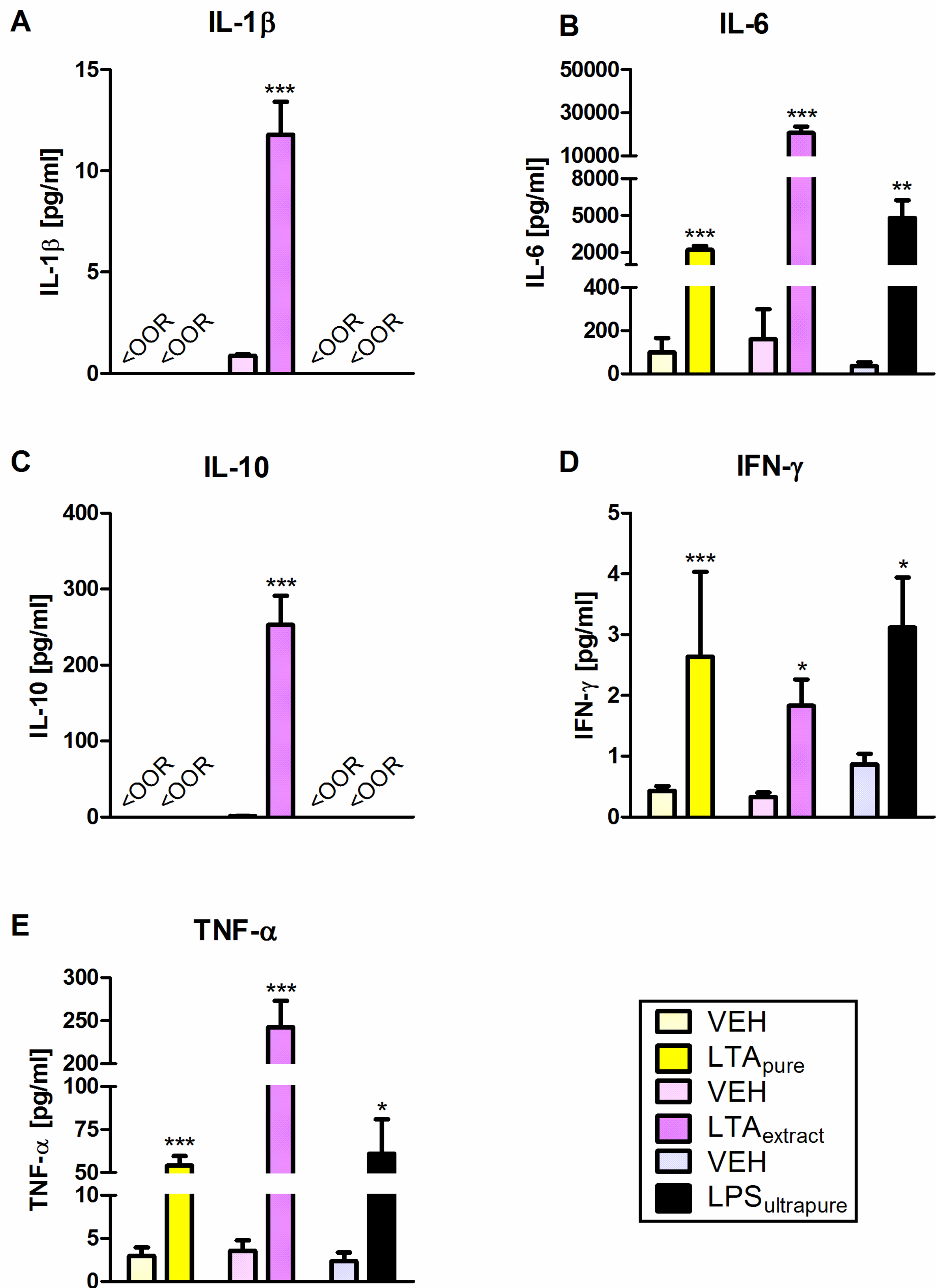


Figure 11 Effect of LTA_{pure}, $LTA_{extract}$, and $LPS_{ultrapure}$ on plasma cytokine levels. The graphs depict circulating levels of interleukin-1β (IL-1β; A), interleukin-6 (IL-6; B), interleukin-10 (IL-10; C), interferon-γ (INF-γ; D), and tumor necrosis factor-α (TNF-

α; E). LTA_{pure} (20 mg/kg), $LTA_{extract}$ (20 mg/kg), $LPS_{ultrapure}$ (1938 EU/kg ≈ 0.19 mg/kg) or the respective vehicle (VEH) was administered 3 h before the cytokines were assayed. The symbol <OOR refers to values below the detection limit. The bars represent means + SEM, n = 6-9; $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ compared to VEH treated mice; independent samples *t* test. These findings have been published in an original article and are reused here with permission (Mayerhofer et al., 2017).

LTA_{pure}, $LTA_{extract}$, and $LPS_{ultrapure}$ differentially affect transcription of cytokines and tight junction-associated proteins in the amygdala and prefrontal cortex

Given their involvement in anxiety-related brain circuits and their role in processing fearful stimuli, the amygdala and prefrontal cortex were chosen as the two brain regions of interest in this study (Maroun, 2013; Robinson et al., 2016). Cytokines expressed in the brain represent not only indices of neuroinflammatory processes but are also known to influence mood and anxiety (Vogelzangs et al., 2016). Therefore, expression patterns of cytokines in the amygdala and prefrontal cortex were evaluated following TLR agonist injection. LTA_{pure} treatment significantly elevated the expression of IL-1β, TNFα, and CCL2 mRNA in both brain regions studied (Figures 12A,E,F and 12A,E,F), while the IL-6 mRNA level was found to be enhanced in the prefrontal cortex only (Figure 13B). $LTA_{extract}$ also increased cytokine mRNA expression, however, to a larger extent regarding certain cytokines and/or in additional brain areas (Figures 12 and 13). IL-1β, IL-6, TNFα, and CCL2 mRNA expression was significantly elevated in the amygdala as well as in the prefrontal cortex following $LTA_{extract}$ treatment (Figures 12A,B,E,F and 13A,B,E,F). IL-10 mRNA expression was in addition found to be enhanced in the prefrontal cortex (Figures 13B). By and large, the $LTA_{extract}$ appeared to be nominally more effective in elevating cytokine mRNA expression than LTA_{pure}, although this differential activity did not prove statistically significant. Specifically, IL-6 and CCL2 mRNA expression levels in the amygdala were raised to a higher extent in $LTA_{extract}$ treated than in LTA_{pure} treated mice (Figure 12B,F). Likewise, IL-6, IL-10, TNFα and CCL2 mRNA expression in the prefrontal cortex attained appreciably higher levels following $LTA_{extract}$ injection than after LTA_{pure} injection (Figure 13B,C,E,F). As

shown in Figures 12A,B,E,F and 13A,E,F, $LPS_{ultrapure}$ raised IL-1β, IL-6, TNFα and CCL2 mRNA expression in the amygdala, as well as IL-1β, TNFα and CCL2 mRNA expression in the prefrontal cortex.

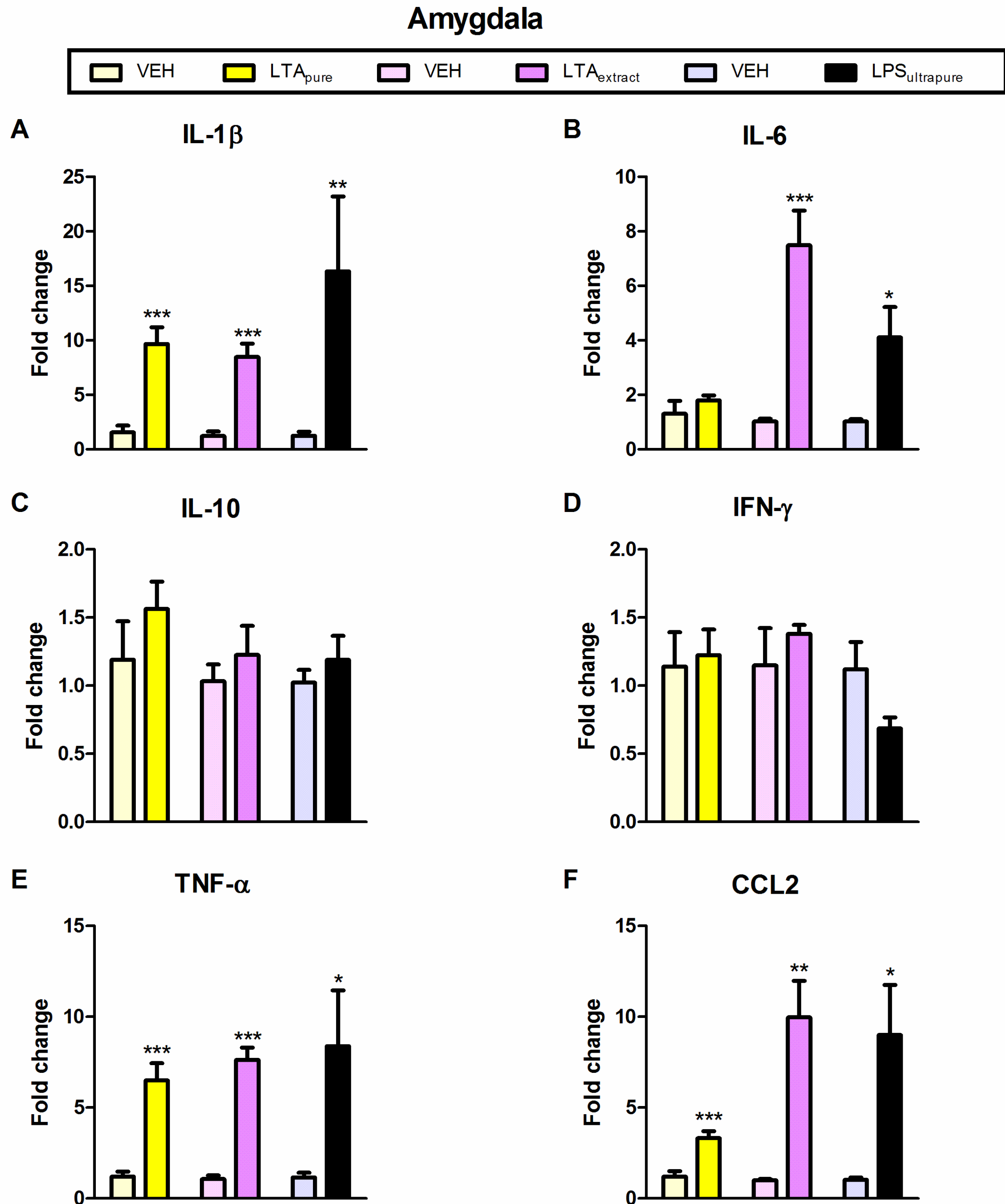


Figure 12 Effect of LTA_{pure}, $LTA_{extract}$, and $LPS_{ultrapure}$ on cytokine mRNA expression in the amygdala. The graphs depict the expression of interleukin-1β (IL-1β; A), interleukin-6 (IL-6; B), interleukin-10 (IL-10; C), interferon-γ (INF-γ; D), tumor

necrosis factor-α (TNF-α; E), and chemokine (C-C motif) ligand 2 (CCL2; F) mRNA. LTA_{pure} (20 mg/kg), $LTA_{extract}$ (20 mg/kg), $LPS_{ultrapure}$ (1938 EU/kg ≈ 0.19 mg/kg) or the respective vehicle (VEH) was administered 3 h before the cytokines were assayed. mRNA transcription is expressed as fold change relative to VEH treated mice. The bars represent means + SEM, n = 5-7; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ compared to VEH treated mice; independent samples *t* test. These findings have been published in an original article and are reused here with permission (Mayerhofer et al., 2017).

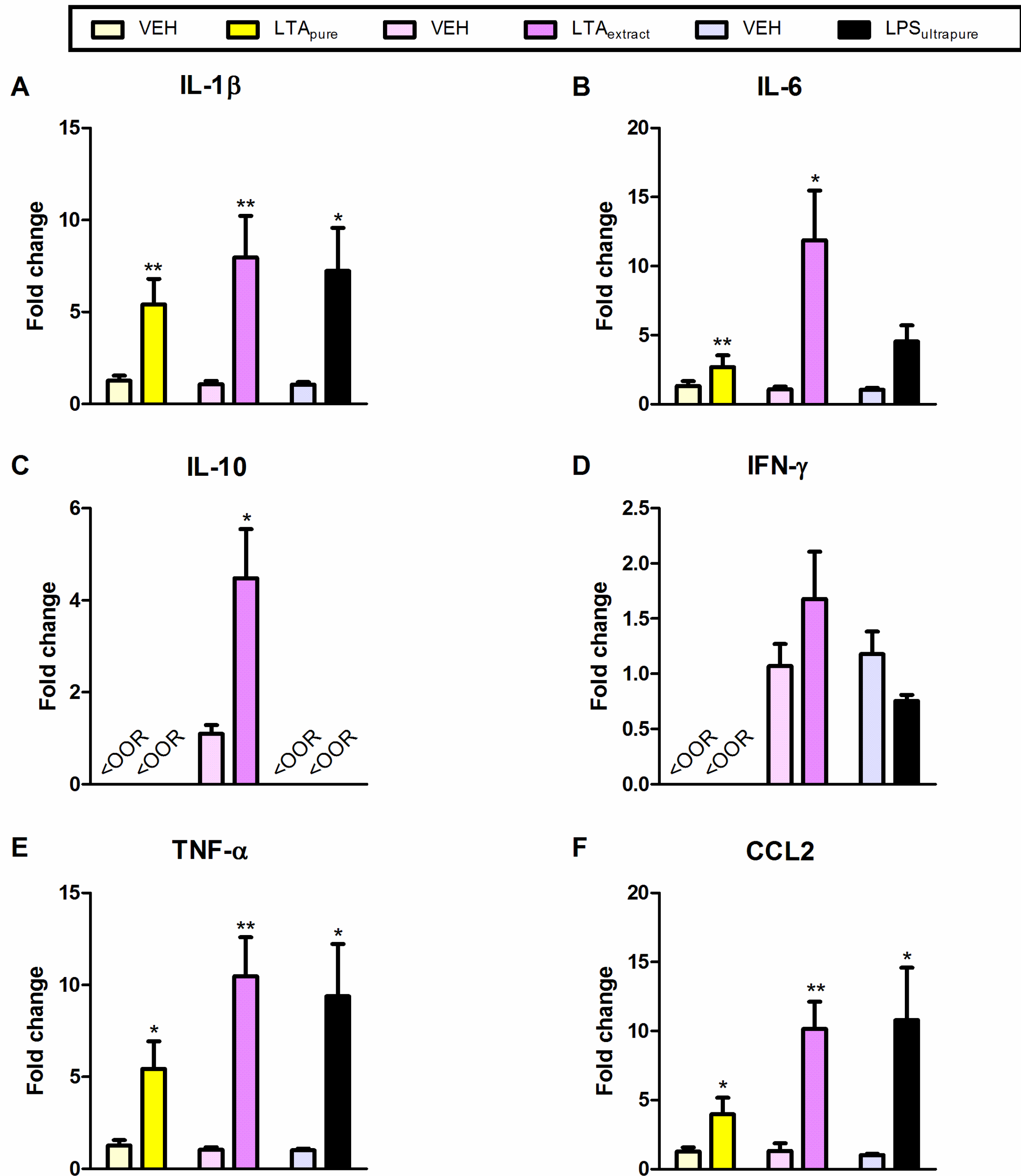


Figure 13 Effect of LTA_{pure}, $LTA_{extract}$, and $LPS_{ultrapure}$ on cytokine mRNA expression in the prefrontal cortex. The graphs depict the expression of interleukin-1β (IL-1β; A), interleukin-6 (IL-6; B), interleukin-10 (IL-10; C), interferon-γ (INF-γ; D), tumor necrosis factor-α (TNF-α; E), and chemokine (C-C motif) ligand 2 (CCL2; F) mRNA. LTA_{pure} (20 mg/kg), $LTA_{extract}$ (20 mg/kg), $LPS_{ultrapure}$ (1938 EU/kg ≈ 0.19 mg/kg) or the respective vehicle (VEH) was administered 3 h before the cytokines were assayed. The symbol <OOR refers to values below the detection limit. mRNA

transcription is expressed as fold change relative to VEH treated mice. The bars represent means + SEM, n = 5-7; *$p \leq 0.05$, **$p \leq 0.01$ compared to VEH treated mice; independent samples *t* test. These findings have been published in an original article and are reused here with permission (Mayerhofer et al., 2017).

As immune signaling has an impact on BBB integrity, the tight junction-associated components of the BBB CLDN5, OCLN, and TJP1 were assessed regarding their mRNA expression pattern following TLR agonist treatment. Quantification by qPCR revealed a significant decrease of CLDN5 mRNA in the amygdala and prefrontal cortex post LTA_{pure} and $LTA_{extract}$ injection. $LPS_{ultrapure}$, on the other hand, significantly elevated CLDN5 mRNA expression in both brain areas (Figure 14A,B). While all three TLR agonists decreased OCLN expression at the mRNA level in the amygdala (Figure 14C), only $LTA_{extract}$ and $LPS_{ultrapure}$ decreased mRNA levels of OCLN within the prefrontal cortex (Figure 14D). TJP1 mRNA expression in the prefrontal cortex remained unaffected by treatment with all three TLR agonists under study, whereas LTA_{pure} and $LTA_{extract}$ both decreased TJP1 mRNA levels in the amygdala (Figure 14E,F).

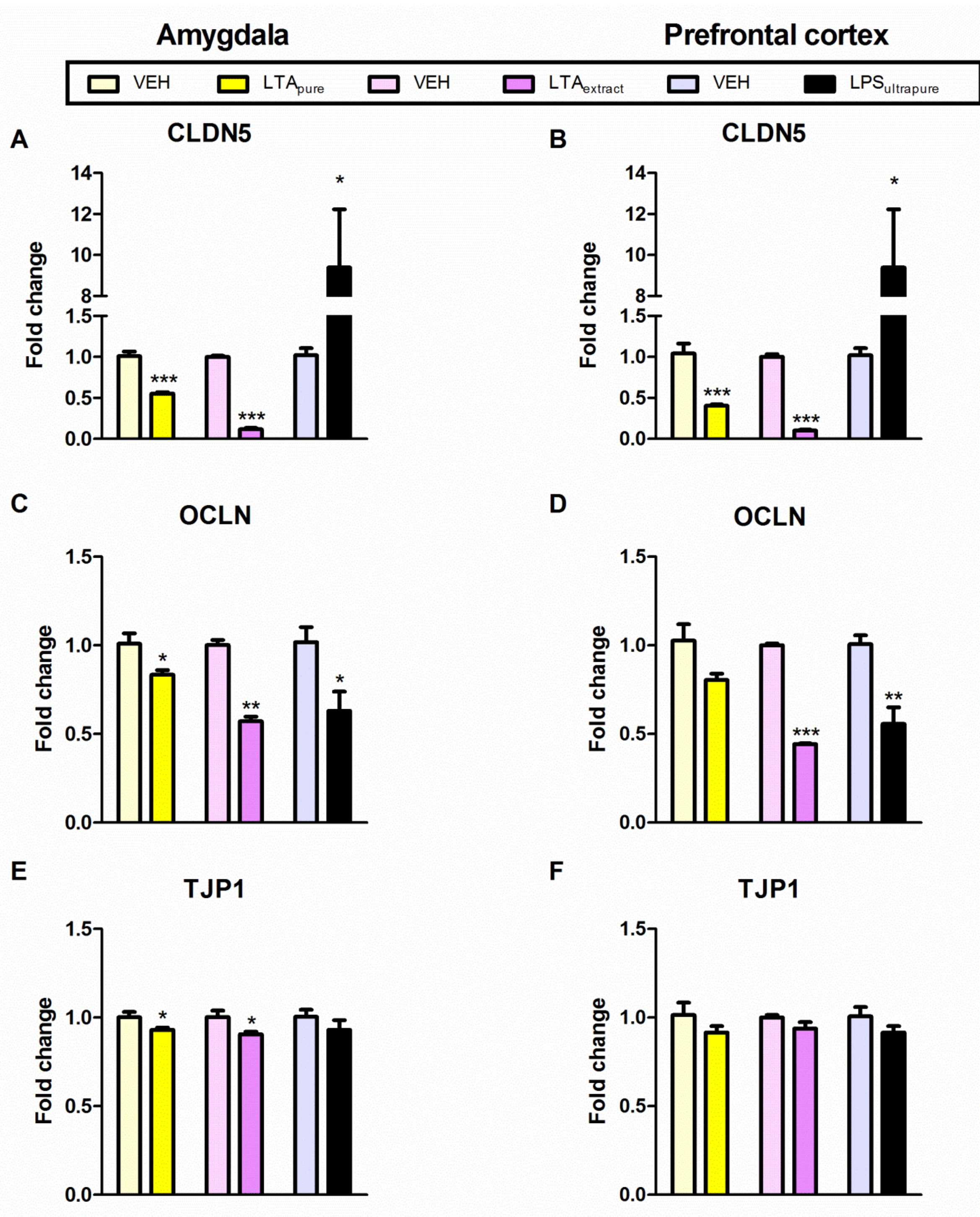


Figure 14 Effect of LTA_{pure}, $LTA_{extract}$, and $LPS_{ultrapure}$ on tight junction-associated protein mRNA expression in the amygdala (A,C,E) and prefrontal cortex (B,D,F). The graphs depict the expression of claudin 5 (CLDN5; A,B), occludin (OCLN; C,D), and tight junction protein 1 (TJP1; E,F) mRNA. LTA_{pure} (20 mg/kg), $LTA_{extract}$ (20 mg/kg), $LPS_{ultrapure}$ (1938 EU/kg ≈ 0.19 mg/kg) or the respective vehicle (VEH) was administered 3 h before the tight junction-associated proteins were assayed. mRNA

transcription is expressed as fold change relative to VEH treated mice. The bars represent means + SEM, n = 5-7; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 compared to VEH treated mice; independent samples *t* test. These findings have been published in an original article and are reused here with permission (Mayerhofer et al., 2017).

LTA_{pure}, $LTA_{extract}$, and $LPS_{ultrapure}$ do not affect the expression of the glucocorticoid receptor NR3C1 and the serotonin transporter SLC6A4 in the amygdala and prefrontal cortex

The mRNA expression patterns of the glucocorticoid receptor NR3C1 and the serotonin transporter SLC6A4 following administration of TLR agonists was assessed. Quantification by qPCR showed that administration of TLR agonists had no gross effect on the expression patterns of NR3C1 and SLC6A4 mRNA in amygdala (Figure 15A,C) and prefrontal cortex (Figure 15B,C). The only significant change that was observed concerned a diminution of NR3C1 mRNA expression in the prefrontal cortex evoked by $LTA_{pure.}$

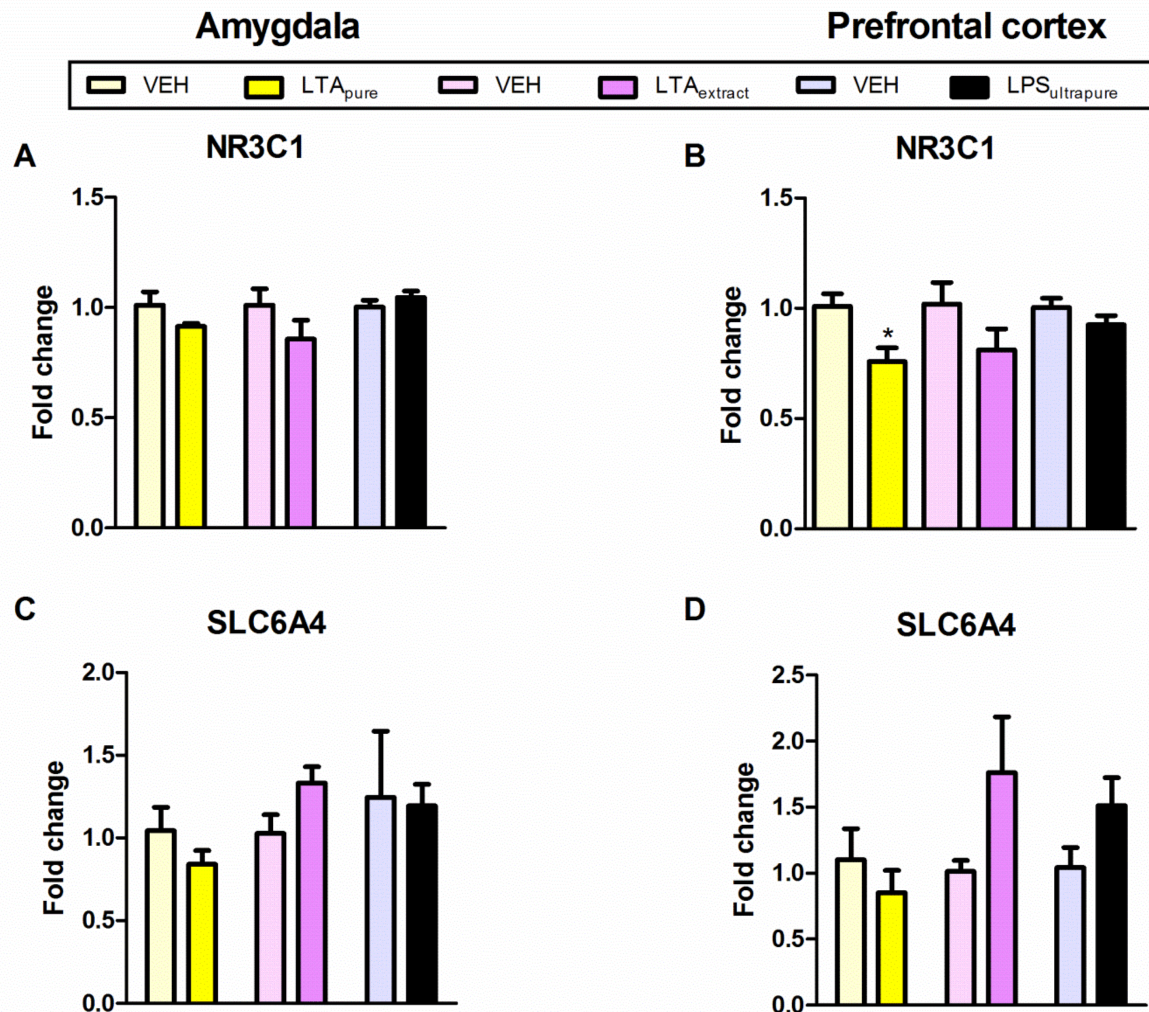


Figure 15 Effect of LTA_{pure}, $LTA_{extract}$, and $LPS_{ultrapure}$ on NR3C1 and SLC6A4 mRNA expression in the amygdala (A,C) and prefrontal cortex (B,D). LTA_{pure} (20 mg/kg), $LTA_{extract}$ (20 mg/kg), $LPS_{ultrapure}$ (1938 EU/kg ≈ 0.19 mg/kg) or the respective vehicle (VEH) was administered 3 h before NR3C1 and SLC6A4 were assayed. mRNA transcription is expressed as fold change relative to VEH treated mice. The bars represent means + SEM, n = 5-7; $*p \leq 0.05$ compared to VEH treated mice, independent samples t test. These findings have been published in an original article and are reused here with permission (Mayerhofer et al., 2017).

$LTA_{extract}$, MDP as well as $LTA_{extract}$ + MDP affect behavior in the open field, but have no effect on BBB integrity assessed via Evans Blue

The open field test was used to assess the effects of 20 mg/kg $LTA_{extract}$ and 3 mg/kg MDP or a combination of both on behavior in the open field test (Figure 16). Animals

of all three treatment groups developed anxiety-like behavior to a certain extent, as mice injected with either of the three treatments spent significantly less time within the CA when compared with vehicle treated animals (Figure 16A). While the number of CA visits was significantly decreased by $LTA_{extract}$ and the combined treatment, MDP injection had no effect on this parameter (Figure 16B). Importantly, only a combination of $LTA_{extract}$ and MDP treatment significantly decreased the total distance traveled within the open field when compared to vehicle or MDP treated mice (Figure 16C).

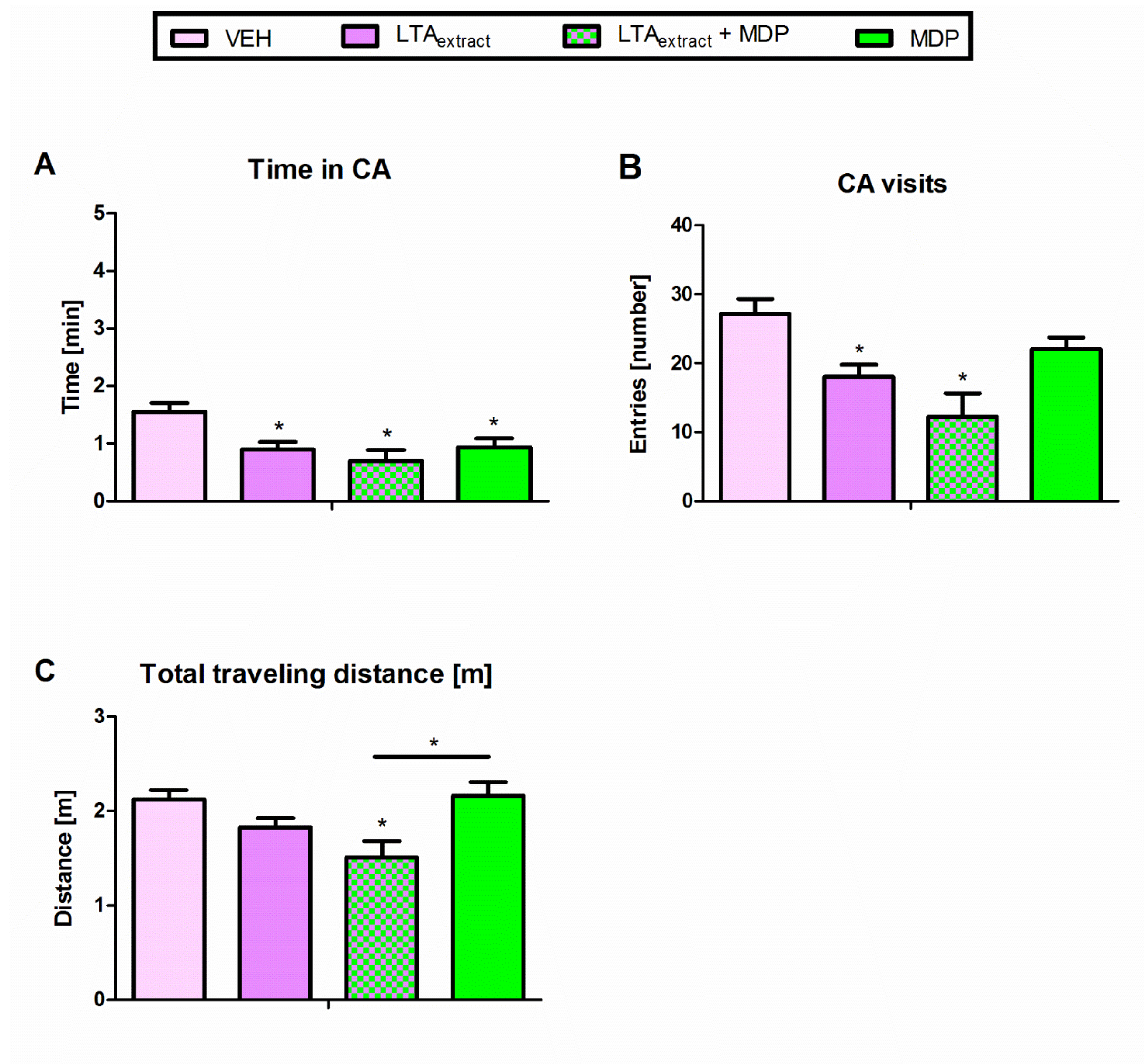


Figure 16 Effects of 20 mg/kg $LTA_{extract}$ from *B. subtilis,* 3 mg/kg MDP and a combination of both on anxiety-like behavior in the open field test. The graphs depict the time spent in the central area (CA), the number of CA visits, and the total traveling distance during a 5 min test period. The open field test was performed 3 h

after i.p. treatment with 20 mg/kg $LTA_{extract}$, 3 mg/kg MDP, a combination of both, or the vehicle (VEH). The bars represent means + SEM, n = 8-16; *$p \leq 0.05$ compared to VEH treated mice; statistically significant differences between groups as determined by one-way ANOVA; Tukey and Dunnett-T post-hoc test.

Evans Blue accumulation in brain tissue was used to gauge disruption of the BBB 3 h following i.p. treatment with 20 mg/kg $LTA_{extract}$, 3 mg/kg MDP or a combination of both. Extravasation of Evans Blue was determined spectrophotometrically. Neither injection of $LTA_{extract}$, $LTA_{extract}$ + MDP, nor MDP led to a higher extravasation rate when compared to vehicle treated animals. Therefore, no disruption of the BBB could be detected following any of the treatments (Table 2).

	Blank	LTA	LTA + MDP	MDP
Evans Blue [µg/ml]	0.017	0.018	0.019	0.023
SEM	0.0	0.0007	0.0024	0.0047

Table 2 Effects of 20 mg/kg $LTA_{extract}$ from *B. subtilis*, 3 mg/kg MDP and a combination of both on BBB integrity measured by Evans Blue extravasation. The figures represent means + SEM, n = 6-9; independent samples t test.

Circulating kynurenine and tryptophan remain unaltered by PRR agonist treatment

Treatment with $LTA_{extract}$, $LTA_{extract}$ + MDP, and MDP was ineffective in altering kynurenine and tryptophan levels in the plasma 3 h post injection (Figure 17A,B) and did not affect the kynurenine/tryptophan ratio (Figure 17C).

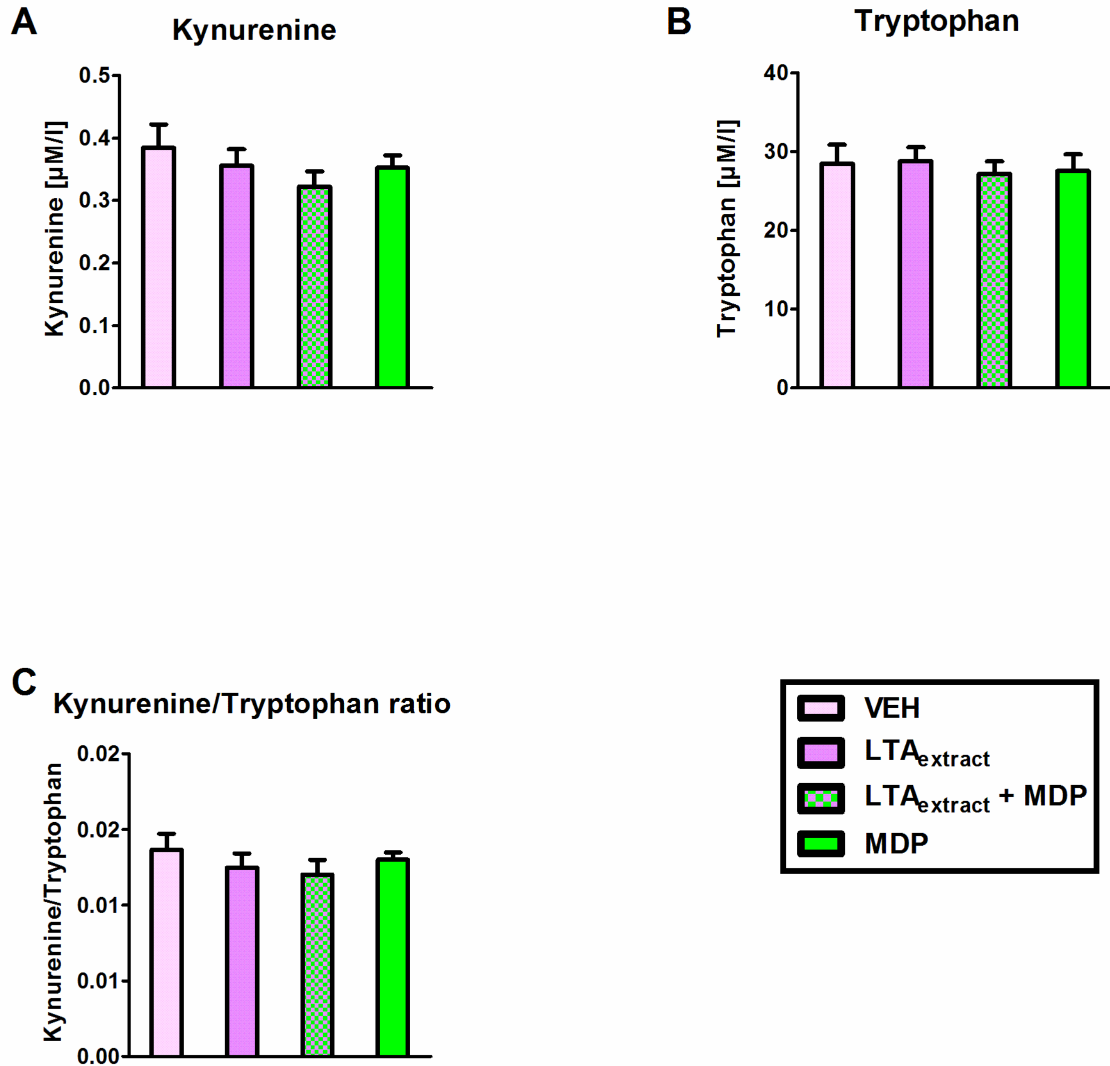


Figure 17 Effects of $LTA_{extract}$ from *B.subtilis,* MDP and a combination of both on circulating levels of kynurenine (A), tryptophan (B), and kynurenine/tryptophan ratio (C). $LTA_{extract}$ (20 mg/kg), MDP (3 mg/kg) or the respective vehicle (VEH) was administered 3 h before the plasma was assayed. The bars represent means + SEM, n=8; one-way ANOVA.

$LTA_{extract}$ and $LTA_{extract}$ + MDP affect circulating corticosterone and cytokine levels

To assess the effect of PRR agonists under study on circulating corticosterone and cytokine concentrations, plasma samples were taken 3 h post i.p. injection. Treatment with $LTA_{extract}$ elevated corticosterone levels to a significant degree when

compared to vehicle treated control mice, $LTA_{extract}$ + MDP injected, and MDP treated animals (Figure 18). $LTA_{extract}$ + MDP administration also significantly increased corticosterone levels in the plasma, when compared both to vehicle as well as MDP treated animals, but was significantly less effective than $LTA_{extract}$ administration alone (Figure 18). MDP injection alone failed to raise circulating corticosterone levels (Figure 18).

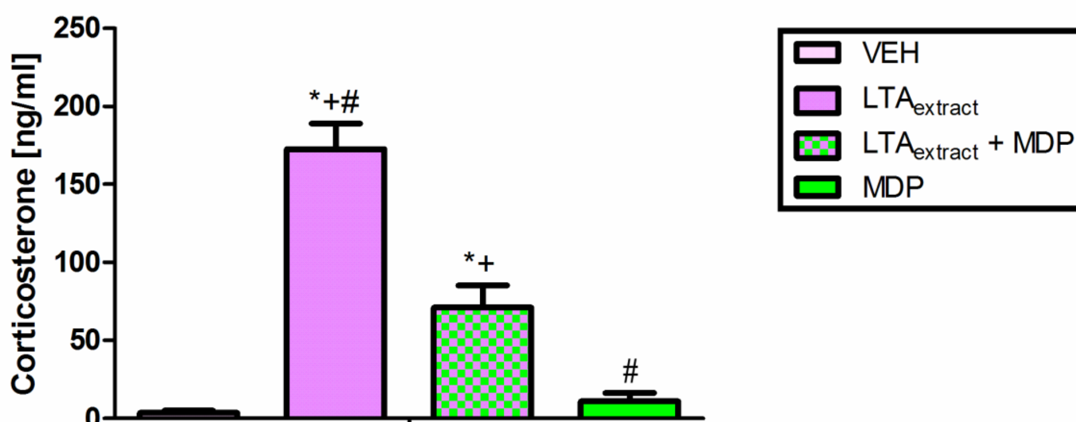
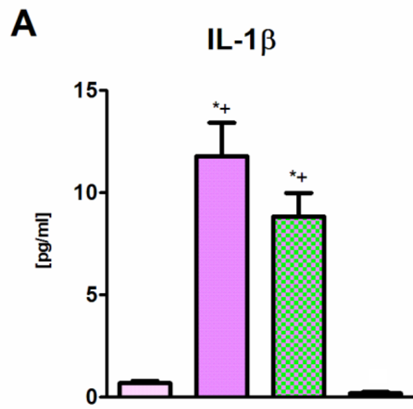


Figure 18 Effects of 20 mg/kg $LTA_{extract}$ from *B. subtilis*, 3 mg/kg MDP and a combination of both on plasma corticosterone levels. The graph depicts circulating corticosterone levels measured 3 h after i.p. treatment with 20 mg/kg $LTA_{extract}$, 3 mg/kg MDP, a combination of the two, or the vehicle (VEH). The bars represent means + SEM, n = 9-13; *$p \leq 0.05$ compared to VEH treated mice, +$p \leq 0.05$ compared to MDP treated mice, #$p \leq 0.05$ compared to $LTA_{extract}$ + MDP treated mice; one-way ANOVA with Tukey and Dunnett-T post-hoc test.

Cytokine levels were determined in the plasma 3 h after injection of vehicle or PRR agonists (Figure 19A-E). Both treatment with $LTA_{extract}$ alone and co-treatment with $LTA_{extract}$ + MDP elevated the plasma concentrations of all cytokines measured (IL-1β, IL-6, IL-10, IFN-γ and TNF-α) when compared to treatment with vehicle and treatment with MDP alone (Figure 19A-E). There was a general trend that injection of $LTA_{extract}$ alone was more effective than combined injection of $LTA_{extract}$ + MDP to increase the plasma levels of IL-1β, IL-10, IFN-γ and TNF-α, but the activity of MDP

to reduce the efficacy of $LTA_{extract}$ in enhancing the circulating cytokine levels reached statistical significance only in the case of TNF-α (Figure 19E).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IL-1β
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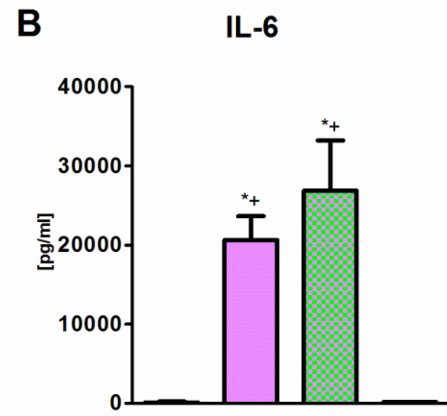
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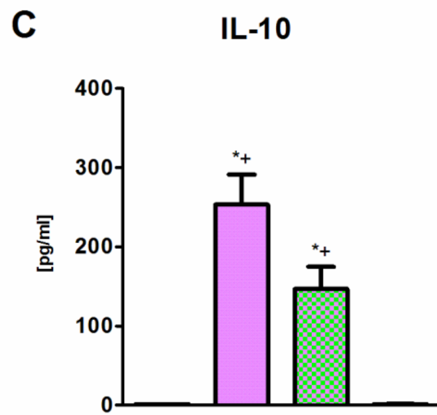
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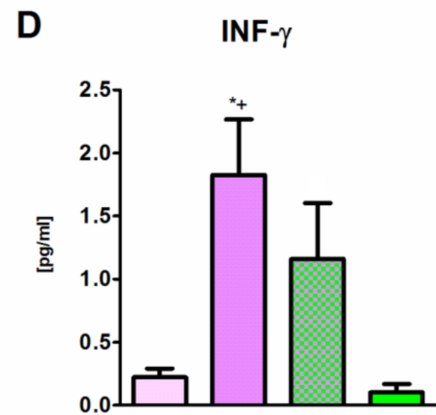
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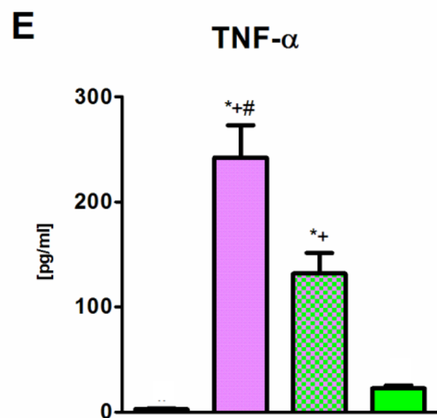
E
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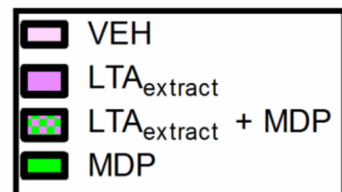

VEH
LTAextract
LTAextract + MDP
MDP

Figure 19 Effect of $LTA_{extract}$ from *B. subtilis,* MDP and a combination of both on plasma cytokine levels. The graphs depict circulating levels of interleukin-1β (IL-1β; A), interleukin-6 (IL-6; B), interleukin-10 (IL-10; C), interferon-γ (INF-γ; D), and tumor necrosis factor-α (TNF-α; E). $LTA_{extract}$ (20 mg/kg), MDP (3 mg/kg), the combination of both or the respective vehicle (VEH) was administered 3 h before the cytokines were assayed. The bars represent means + SEM, n = 8-9; *p ≤ 0.05 compared to VEH treated mice, +p ≤ 0.05 compared to MDP treated mice, #p ≤ 0.05 compared to $LTA_{extract}$ + MDP treated mice; one-way ANOVA with Tukey and Dunnett-T post-hoc test.

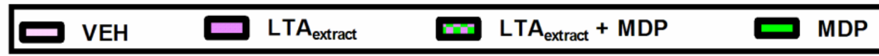
$LTA_{extract}$, $LTA_{extract}$ + MDP, and MDP treatment change mRNA cytokine expression in the amygdala and prefrontal cortex, but fail to alter NR3C1 mRNA levels in these brain areas

Cytokine mRNA expression analysis in the amygdala 3 h post treatment with $LTA_{extract}$, MDP or $LTA_{extract}$ + MDP showed a significantly upregulated IL-1β, TNF-α and CCL2 mRNA expression following all three treatments compared to vehicle treatment (Figure 20A,B,E,F). In contrast, only co-treatment with $LTA_{extract}$ + MDP caused a significant increase of the anti-inflammatory cytokine IL-10 mRNA expression in the amygdala when compared with vehicle treatment (Figure 20C), whereas none of the treatments altered the INF-γ mRNA expression pattern in the amygdala to any significant extent (Figure 20D). The IL-1β and IL-10 mRNA upregulation in the amygdala of animals co-treated with $LTA_{extract}$ + MDP was particularly worth noting, as the expression was significantly increased relative to animals treated either with $LTA_{extract}$ or MDP alone (Figure 20A,C). A similar picture emerged when analysing TNF-α mRNA expression, as in mice treated with $LTA_{extract}$ + MDP it was significantly higher than in $LTA_{extract}$ treated animals (Figure 20E).

Within the prefrontal cortex, only $LTA_{extract}$ + MDP induced IL-1β, IL-6, and IL-10 mRNA expression in a significant manner compared to vehicle (Figure 21A,B,C). In contrast, TNF-α mRNA was significantly increased by $LTA_{extract}$ + MDP and MDP alone, relative to vehicle, 3 h after injection, the effect of $LTA_{extract}$ + MDP being significantly larger than that of $LTA_{extract}$ alone (Figure 21E). CCL2 mRNA expression

was elevated in LTAextract + MDP and MDP treatment groups compared to vehicle treated mice (Figure 21F).

Amygdala



A IL-1β

Fold change

B IL-6

Fold change

C IL-10

Fold change

D INF-γ

Fold change

E TNF-α

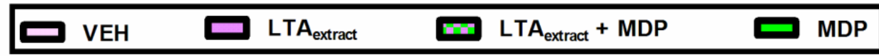
Fold change

F CCL2

Fold change

Figure 20 Effect of $LTA_{extract}$ from *B. subtilis,* MDP and a combination of both on cytokine mRNA expression in the amygdala. The graphs depict the expression of interleukin-1β (IL-1β; A), interleukin-6 (IL-6; B), interleukin-10 (IL-10; C), interferon-γ (INF-γ; D), tumor necrosis factor-α (TNF-α; E), and chemokine (C-C motif) ligand 2 (CCL2; F) mRNA. $LTA_{extract}$ (20 mg/kg), MDP (3 mg/kg) or the respective vehicle (VEH) was administered 3 h before the cytokines were assayed. mRNA transcription is expressed as fold change relative to VEH treated mice. The bars represent means + SEM, n = 5; *$p \leq 0.05$ compared to VEH treated mice, #$p \leq 0.05$ compared to $LTA_{extract}$ + MDP treated mice; one-way ANOVA with Tukey and Dunnett-T post-hoc test.

Prefrontal cortex



A IL-1β

Fold change

30 20 10 0

*

B IL-6

Fold change

150 100 50 0

*

C IL-10

Fold change

20 15 10 5 0

*

D INF-γ

Fold change

3 2 1 0

E TNF-α

Fold change

40 30 20 10 0

* #*

F CCL2

Fold change

40 30 20 10 0

* *

Figure 21 Effect of $LTA_{extract}$ from *B.subtilis,* MDP and a combination of both on cytokine mRNA expression in the prefrontal cortex. The graphs depict the expression of interleukin-1β (IL-1β; A), interleukin-6 (IL-6; B), interleukin-10 (IL-10; C), interferon-γ (INF-γ; D), tumor necrosis factor-α (TNF-α; E), and chemokine (C-C motif) ligand 2 (CCL2; F) mRNA. $LTA_{extract}$ (20 mg/kg), MDP (3 mg/kg) or the respective vehicle (VEH) was administered 3 h before the cytokines were assayed. mRNA transcription is expressed as fold change relative to VEH treated mice. The bars represent means + SEM, n = 5; *$p \leq 0.05$ compared to VEH treated mice, #$p \leq 0.05$ compared to $LTA_{extract}$ + MDP treated mice; one-way ANOVA with Tukey and Dunnett-T post-hoc test.

Furthermore, expression of the glucocorticoid receptor encoded by the gene NR3C1 was assessed in the amygdala and prefrontal cortex 3 h after injection of vehicle, $LTA_{extract}$, MDP or $LTA_{extract}$ + MDP. As is shown in Figure 22A,B, there was no change in NR3C1 mRNA expression following any of the PRR agonist treatments.

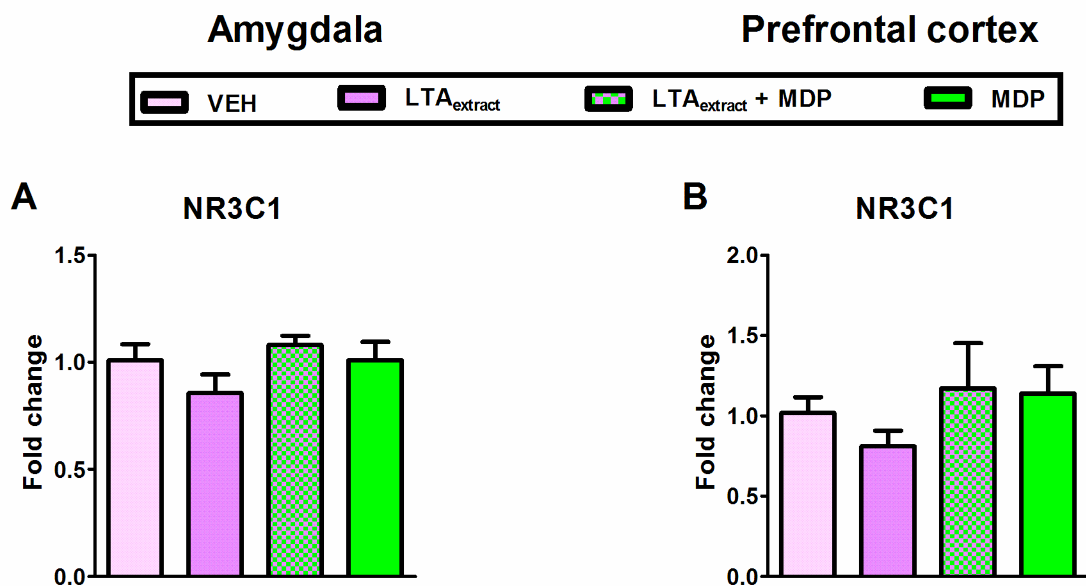


Figure 22 Effect of $LTA_{extract}$ from *B.subtilis,* MDP and a combination of both on NR3C1 mRNA expression in the amygdala and prefrontal cortex. $LTA_{extract}$ (20 mg/kg), MDP (3 mg/kg) or the respective vehicle (VEH) was administered 3 h before

NR3C1 mRNA levels were assayed. mRNA transcription is expressed as fold change relative to VEH-treated mice. The bars represent means + SEM, n = 5; one-way ANOVA.

Discussion

Rationale of the thesis

As considered in detail in the Introduction, immune activation and inflammatory processes both in the periphery and brain are considered to be relevant to the development of several mental disorders including anxiety disorders and major depressive disorder (Capuron and Miller, 2011). The recognition of the gut microbiota as a rich source of immunologically active molecules, which may leak into the circulation, has spurred research on the relationship between immune activation and the pathophysiology of neuropsychiatric disorders (Julio-Pieper and Bravo, 2016; Kelly et al., 2015). Bacteria shed several cell wall components that act as microbe-associated molecular patterns (MAMPs) or PAMPs to activate PRRs of the innate immune system. While in this context the effect of the TLR4 agonist LPS on brain function, mood and behaviour has extensively been studied, the study of many other MAMPs and PAMPs in their impact on the brain lags behind. There is good reason to assume that under pathophysiological conditions several PAMPs interact with each other in their effect on the immune system and CNS. This has been exemplified by the synergistic action of peptidoglycan-evoked NOD1 and NOD2 stimulation with LPS-evoked TLR4 stimulation in causing peripheral immune stimulation, neuroinflammation and sickness behavior (Farzi et al., 2015b).

Against this background, my thesis project focused on LTA, another MAMP that has been shown to stimulate the immune system preferentially via activation of TLR2. Although there is some information that LTA affects the BBB and may have effects on the brain, its impact on molecular and behavioral aspects of brain function has not yet been systematically addressed. The present data reveal new insights into this relationship and show that the activation of peripheral TLR2 by purified LTA obtained from *Bacillus subtilis* (LTA_{pure}) leads to an upregulated expression of cytokines in plasma as well as in the prefrontal cortex and amygdala. Furthermore, the immune response triggered by LTA-induced TLR2 activation involves stimulation of the HPA axis, as reflected by increased corticosterone release, as well as changes in the expression pattern of BBB associated proteins in the brain. However, in contrast to agonism at other PRRs like TLR4, which causes sickness and anxiety-like behavior, TLR2 stimulation does not lead to behavioral alterations in the doses under study. By comparing effects of a crude LTA extract with those of

LTA_{pure} and LPS on the immune system and brain I was able to unveil a facilitatory interaction of the two involved PRRs, namely TLR2 and TLR4. Furthermore, my data demonstrate that contaminations with LPS/endotoxin found in insufficiently purified LTA preparations make a distinct contribution to the biologic effects of the TLR2 agonist.

Dosage and purity of LTA

Earlier studies have shown that LTA of undeclared purity stimulates peripheral and cerebral (microglial) immune cells (Huang et al., 2013; Lim et al., 2013; Neher and Brown, 2007; Oberg et al., 2011). In line with that, the TLR2 agonist causes the formation of a pro-inflammatory environment by an upregulated release of cytokines (Medzhitov and Janeway, 1998). My *in vivo* mouse study supports these data, as i.p. injection of LTA_{pure} and $LTA_{extract}$ (20 mg/kg) significantly elevated plasma levels of IL-6, INF-γ, and TNF-α 3 h following administration. Preliminary data, presented in Table 1, were used to choose time points of measurement and dosage. $LTA_{extract}$ affected behavior in the open field test 3 h after treatment as it reduced the time spent in the CA, but had no effect 6 or 27 h post treatment. Previous studies have shown that cytokine release and sickness behavior peak 1-6 h after administration of the TLR4 agonist LPS (Dantzer et al., 2008; Layé et al., 1994). A minimum of 3 h between injection and analysis was considered necessary to prevent any confounding effect of the handling and injection procedure on the behavioral outcome (Mayerhofer et al., 2017).

Alike the stimulation of other PRRs such as TLR4 (Farzi et al., 2015b; Lehmann et al., 2013), LTA activates the HPA axis and thereby elevates circulating corticosterone levels, which can be observed following both $LTA_{extract}$ and LTA_{pure} treatment. To clarify whether endotoxin contaminations of commercially available LTA could be involved in the effects on HPA axis activity seen in LTA treated mice (Gao et al., 2001; Morath et al., 2001), the animals were pretreated with the TLR4 antagonist TAK-242 (Ii, 2006; Kawamoto et al., 2008) at a dose of 4 mg/kg (Farzi et al., 2015a; Wang et al., 2013). The antagonist prevented the $LTA_{extract}$ induced rise of circulating corticosterone, but failed to blunt the activation of the HPA axis by LTA_{pure}. Based on the selectivity of TAK-242 for TLR4 I concluded that $LTA_{extract}$ broadly acts via TLR4 stimulation due to contaminations by the respective agonist LPS. The results of the TLR2/TLR4 HEK-Blue® reporter assays supported my

suspicion when I saw that the $LTA_{extract}$ stimulated TLR4 expressing cells in a similar manner as $LPS_{ultrapure}$. LTA_{pure}, on the other hand, did not activate TLR4, but stimulated TLR2, an effect which was not blunted by the selective TLR4 antagonist TAK-242. The antagonist did, however, prevent the activation of TLR4 by $LTA_{extract}$ and $LPS_{ultrapure}$. To assess the immunologic, cerebral and behavioral effects of LTA_{pure} and $LTA_{extract}$, which displayed such diverse receptor affinities for TLR2 and TLR4, I conducted a series of experiments in which the two LTA preparations were tested simultaneously and their effects were compared with each other and $LPS_{ultrapure}$.

It remains to be elucidated why $LTA_{extract}$ did not stimulate TLR2 in the HEK-Blue® reporter assay (Figure 8D), while activating solely TLR4 (Figure 8E). This unexplained observation could be due to the presence of unidentified components in the $LTA_{extract}$ preparation that negatively influence the interaction of LTA with TLR2. To stimulate TLR2, an active form of LTA must be present (Lebeer et al., 2010), and stimulation of immune cells via TLR2 is amplified by CD-14 and LPS-binding protein but independent of TLR4 and the co-receptor MD-2 (Schröder et al., 2003). Furthermore, TLR2 blocking antibodies have been used to show that LTA does not need to act via TLR2 in order to stimulate IL-8 secretion (Hattar et al., 2006). Therefore, three explanations why $LTA_{extract}$ failed to stimulate TLR2 in the reporter assay can be taken into account: (1) Unknown components are present in the preparations of $LTA_{extract}$ that prevent TLR2 activation and signal transduction. (2) The conformation of LTA is incorrect and, for this reason, LTA fails to interact with TLR2. (3) TLR2 signaling is negatively regulated by excess LPS in the reporter system (Mayerhofer et al., 2017). The HEK-Blue® reporter cell assay revealed the presence of relevant amounts of LPS in the $LTA_{extract}$ preparation, which was confirmed by the ability of the TLR4 inhibitor TAK-242 to blunt the effect of $LTA_{extract}$ in TLR4 expressing cells (Figure 8E).

The contamination by LPS/endotoxin is a confounding factor in the analysis of the in vivo effects of LTA preparations. As my experiments showed, however, it is possible to tackle this problem by a careful analysis of the TLR selectivity of the components present in impure LTA preparations. Using the EndoLISA® endotoxin quantification assay I was even able to quantitatively estimate the LPS/endotoxin content of impure LTA preparations and design experiments in which the

contribution of the LPS/endotoxin contamination to the overall in vivo effects of the impure LTA preparation could be quantitated. In addition, a comparative analysis of the in vivo effects of $LTA_{extract}$, LTA_{pure} and $LPS_{ultrapure}$ enabled me to disclose potential (synergistic or antagonistic) interactions between TLR2 and TLR4 stimulation in their impact on immune system, brain and behavior.

Effect of LTA on circulating and cerebral cytokine induction

After sorting out the important issue of the TLR2/TLR4 selectivity of commercial LTA preparations it was an important aim to gain insight into the immunologic reactions evoked by TLR2/TLR4 agonism in the periphery and brain. This goal was addressed by determining the levels of cytokines in the plasma as well as their expression in the amygdala and prefrontal cortex in response to i.p. administration of $LTA_{extract}$, LTA_{pure}, and $LPS_{ultrapure}$. In line with a preceding study in which a low dose of LPS (0.1 mg/kg) was reported to elevate circulating cytokine levels (Farzi et al., 2015b), I found that a slightly higher dose of LPS (0.83 mg/kg) was distinctly able to enhance the plasma levels of IL-6, INF-γ and TNF-α. The spectrum of plasma cytokines induced by LPS was very similar to that generated by LTA_{pure} and $LTA_{extract}$, which likewise increased the circulating levels of IL-6, INF-γ and TNF-α. My findings are in gross agreement with previous work by Kang and colleagues who found that pure LTA from *S. aureus* elevates plasma levels of pro-inflammatory cytokines such as CCL2, TNF-α, INF-γ (Kang et al., 2012) as well as IL-1, IL-5, IL-6 and IL-8 (Ginsburg, 2002). Particularly worth noting in the current study was that the production of IL-1β and the anti-inflammatory cytokine IL-10 was stimulated by $LTA_{extract}$, but not by LTA_{pure} and $LPS_{ultrapure}$. Furthermore, $LTA_{extract}$ elevated the circulating IL-6 and TNF-α levels to a higher extent than LTA_{pure} or $LPS_{ultrapure}$. Given that $LTA_{extract}$ contains both LTA and LPS, this comparative analysis of the circulating cytokine spectrum strongly hints at a facilitatory interaction between LTA and LPS, i.e. TLR2 and TLR4 activation, as well as other PAMPs that might be present in $LTA_{extract}$ preparations. In cellular systems, other groups have previously observed such facilitatory interactions between LPS-induced activation of TLR4 and stimulation of TLR2 (Beutler et al., 2001; Xu et al., 2007).

In a further experiment, I was able to show that the LTA-induced immune stimulation not only affected the peripheral immune system but also extended to the brain. This conclusion is based on my findings that in the CNS the expression of cytokines as

well as of tight junction-associated proteins at the mRNA level was changed significantly. In detail, the expression of IL-1β, TNFα and CCL2 mRNA was found increased by LTA_{pure} both in the prefrontal cortex and amygdala, while the IL-6 transcript was upregulated in the prefrontal cortex only. These observations indicate that LTA_{pure} is capable of inducing neuroinflammatory processes in those two brain areas which were selected for analysis because both the amygdala and prefrontal cortex are involved in emotional-affective behavior such as anxiety (Maroun, 2013; Robinson et al., 2016). A comparison of the effect of LTA_{pure} to raise circulating cytokine levels with that to stimulate cytokine mRNA expression in the brain reveals distinct differences in the cytokine spectrum affected. While in the periphery IL-6, INF-γ and TNF-α were preferentially induced by LTA_{pure}, it was predominantly IL-1β, TNFα and CCL2 that were expressed in the brain. This differential effect of the TLR2 agonist on the peripheral and cerebral cytokine spectrum indicates that the peripheral and cerebral cytokine induction caused by LTA_{pure} originated from different cell populations.

It is worth emphasizing that a comparison of the effects of LTA_{pure} and $LPS_{ultrapure}$ on cytokine expression in the amygdala and prefrontal cortex also discloses distinct commonalities. This is particularly true for TNF-α, the expression of which was increased by all TLR agonists under study, LTA_{pure}, $LTA_{extract}$, and $LPS_{ultrapure}$. This discovery is consistent with the contention that TNF-α is one of the most potent cytokines capable of transferring immune signaling from the periphery to the CNS. This hypothesis is supported by previous work showing that, in TNF receptor-knockout mice, cytokine mRNA levels were not altered in the brain following peripheral administration of LPS or TNF-α (Qin et al., 2017). Since the TLR agonists under study also stimulated the expression of IL-1β in both amygdala and prefrontal cortex, and the expression of IL-6 in most cases, these cytokines might likewise play master roles in the transfer of peripheral immune activation to neuroinflammatory processes in the brain. This conclusion is in line with previous work showing that the IL-1 receptor is of prime importance to the cytokine signaling cascade that results in the activation of microglia and vascular endothelial cells to recruit peripheral leukocytes (Basu et al., 2002). IL-6 is likewise an important player in pro-inflammatory processes within the brain and in the mental disturbances that

accompany neuroinflammation (Burton et al., 2013; Qian et al., 2014; Spooren et al., 2011).

A comparison of the effects of $LTA_{extract}$ with that of LTA_{pure} and $LPS_{ultrapure}$ on cerebral cytokine expression hints at particular interactions between TLR2 and TLR4 signaling in the brain. Such an interaction is deduced from the ability of $LTA_{extract}$ to enhance the induction of IL-6 and CCL-2 in the amygdala and that of IL-6, TNF-α and CCL2 in the prefrontal cortex to a larger degree than LTA_{pure} and, in some instances, $LPS_{ultrapure}$. This observation hints at a synergism between TLR2 and TLR4 signaling in the brain, an interaction that also applies to the TLR2- and TLR4-mediated activation of the peripheral immune system. Particularly worth noting is the capacity of $LTA_{extract}$ to stimulate the expression of the anti-inflammatory cytokine IL-10 in the prefrontal cortex, an activity that was not seen with LTA_{pure} and $LPS_{ultrapure}$. It may be speculated that the induction of pro-inflammatory cytokines in the brain in response to $LTA_{extract}$ is so strong that it triggers a counter-regulatory reaction.

Effect of LTA on tight junction-associated proteins of the BBB

Since both LPS and LTA have been found to affect the BBB (Boveri et al., 2006; Sheen et al., 2010), I conducted a comparative analysis of the effects of LTA_{pure}, $LTA_{extract}$ and $LPS_{ultrapure}$ on the transcription of the tight junction-associated proteins CLDN5, OCLN and TJP1 in the amygdala and prefrontal cortex. Immune activation by $LTA_{extract}$ and, to a lesser extent, by $LTA_{pure,}$ led to a decrease of the mRNA levels of CLDN5 and OCLN in both amygdala and prefrontal cortex, while TJP1 transcription was only marginally diminished. $LPS_{ultrapure}$ also reduced OCLN mRNA expression in both brain regions but elevated the CLDN5 mRNA levels, an effect that remains unexplained in the present context. In an attempt to evaluate whether these transcriptional alterations have a bearing on BBB functionality, I measured Evans Blue extravasation in brain tissue as an index of BBB disruption. However, the results of this experiment did not provide any evidence for a disturbance of the BBB after treatment with $LTA_{extract}$.

While the current study does not provide any indication that the transcriptional changes of CLDN5 and OCLN are associated with alterations in BBB function and integrity, other studies indicate that stimulation of TLR2 and TLR4 and the resulting

shift in tight junction-associated protein mRNA expression does affect BBB integrity and is related to neuroinflammation (Boveri et al., 2006). In line with this contention, it has been shown *in vitro* that endothelial cell monolayers treated with TNF-α, IL-1β, and IL-6 are increased in their permeability (de Vries et al., 1996), which suggests that cytokines can alter BBB function. As these cytokines were also induced by the TLR agonists studied here, I speculate that they may have contributed to the changes in CLDN5 and OCLN mRNA expression in the brain. It needs to be added that CCL2, another cytokine found elevated in the present study, is not only critical for inflammatory processes within the periphery and brain (Bennett et al., 2003) but has also a deleterious impact on the BBB via activation of C-C chemokine receptor type 2 which leads to myosin light chain hyperphosphorylation (Yao and Tsirka, 2014).

Another study using an in vitro model with bovine brain capillary endothelial cells co-cultured with rat primary glial cells has shown that LTA from *S. aureus* purified to a high degree impacts on barrier integrity in a time- and concentration-dependent manner by activating glia cells (Boveri et al., 2006). Furthermore, activated glial cells have been observed to affect the BBB by TNF-α, IL-1β, and nitric oxide release (Boveri et al., 2006), although the same study failed to show significantly altered CLDN and OCLN expression following treatment with highly purified LTA. However, other work hints at TNF-α, IL-1β and IL-6 as major mediators of disrupted BBB integrity (de Vries et al., 1996), and Singh et al. (2007) could observe reduced OCLN transcription in endothelial cells following treatment with LTA (purity not specified). In addition, LTA has been found to be a crucial factor for *S. aureus* to adhere to and infect brain endothelial cells (Sheen et al., 2010), which is also likely to have a negative influence on BBB functionality. Despite the inconsistent reports regarding the effect of LTA on BBB constituents, the findings of my thesis project suggest that neuroinflammation triggered by TLR2 stimulation is related to molecular changes in BBB composition. This inference is based on the significant reduction of CLDN5 and OCLN transcription observed in the prefrontal cortex and amygdala and on the pronounced elevation of pro-inflammatory cytokine expression in these brain areas. As LTA obtained from *E. hirae* ameliorates impairment of intestinal tight junctions (Miyauchi et al., 2008), it needs to be considered that the effect of this TLR2 agonist

on paracellular permeability depends on the origin and structure of LTA as well as on the type of barrier under study.

Effect of LTA on sickness and anxiety-like behavior

As discussed before, neuroinflammatory processes in the brain impact on neuronal function and behavior, and one of the central aims of my thesis project was to examine, in a comparative manner, whether $LTA_{extract}$, LTA_{pure}, and $LPS_{ultrapure}$ affect behavior in the open field, a common paradigm to assess anxiety and sickness. Anxiety is deduced from a reduction of the time spent in the CA of the open field and/or the number of entries into the CA, while a reduction in locomotion (total distance traveled during the test session) is considered to reflect sickness. The "sickness response" (sometimes also called "illness response") refers a characteristic behavioral reaction to TLR stimulation by PRR agonists such as LPS and comprises fever, anorexia, somnolence, decreased locomotion, blunted exploration, and suppressed social interaction, enhanced anxiety and hyperalgesia (Capuron and Miller, 2011; Dantzer et al., 2008; Watkins et al., 1994; Wieczorek et al., 2005). These behavioral effects are mediated by multiple signaling mechanisms which include activation of vagal afferent neurons and the induction of neuroinflammatory processes in the brain (Bluthé et al., 1994; Dantzer, 2004; Goehler et al., 2000; Watkins et al., 1994; Wieczorek et al., 2005). Depending on the dose of LPS administered, the sickness response sets in quickly, reaches a peak within a few hours and subsequently resides within one day post treatment. The transient sickness response may be followed by a more prolonged disturbance of emotional-affective behavior such as the induction of depression-like behavior (Dantzer et al., 2011, 2008; O'Connor et al., 2009; Painsipp et al., 2010)

In the present study, I confirmed that a low dose of $LPS_{ultrapure}$ (1938 EU/kg ≈ 0.19 mg/kg) changed behavior in the open field in a manner that is indicative of increased anxiety, whereas locomotion was not significantly altered, which indicates that this aspect of sickness behavior was not affected. It has previously been shown that the behavioral response to low doses of LPS depends to a good deal on the experimental conditions (Farzi et al., 2015b). The behavioral response to $LPS_{ultrapure}$ in the open field was reproduced by $LTA_{extract}$, but not by LTA_{pure}. In view of the other findings related to the comparative analysis of the effects of LTA_{pure}, $LTA_{extract}$ and $LPS_{ultrapure}$ I conclude that TLR2 signaling evoked by LTA_{pure} does not result in

behavioral changes indicative of anxiety or sickness, whereas the anxiogenic effect of $LTA_{extract}$ is most likely due to the contamination of this preparation with LPS. Furthermore, the data support the contention that LTA_{pure}, $LTA_{extract}$ and $LPS_{ultrapure}$, at the doses studied here, were too low to evoke a degree of sickness that would manifest itself in a shortening of the total traveling distance in the open field (Painsipp et al., 2010). The inability of LTA_{pure} to evoke a behavioral response is unexpected inasmuch as pure LTA was able to induce immunologic reactions both in the periphery and brain and to alter the expression of tight junction-associated proteins at the BBB. It remains unclear whether the neuroinflammatory reactions to LTA_{pure} were too weak to extend to behavioral alterations or whether counter-regulatory mechanisms prevented the manifestation of behavioral disturbances. There is scarce information in the literature as to whether treatment with LTA has an effect on particular dimensions of emotional-affective behavior. Some evidence obtained in a murine schizophrenia model hints at a role of TLR2 agonism in anxiety-like behavior, as genetic deletion of TLR2 is associated with blunted anxiety-like behavior in the open field and elevated plus maze tests (Park et al., 2015).

The comparative analysis of the effects of LTA_{pure}, $LTA_{extract}$, and $LPS_{ultrapure}$ was conducted with LTA preparations obtained from *B.subtilis*, after preliminary experiments had shown that $LTA_{extract}$ from *B.subtilis*, but not $LTA_{extract}$ from *S.aureus* (purity undefined), induced anxiety-like behavior in the open field test. In addition, it was found that the change of behavior manifested itself 3 h, but not 6 or 27 h post injection. Thus, the behavioral effect of LTA preparations depended on the bacterial source of LTA, the dose of LTA, the time of assessment and, as this study also established, on the degree of contamination with LPS/endotoxin. That the bacterial source of LTA is related to its immunologic activity has been observed in different bacterial species (Lebeer et al., 2010). It is nevertheless an important conclusion that I would like to draw from the current results, namely, that bacterial source, structure and purity of LTA need to be considered as important determinants of its biologic activity.

Effect of LTA on HPA axis activity

It has been observed some time ago that immunologic stimuli can trigger a stress response as shown by a rise of HPA axis activity, resulting in increased blood levels of ACTH and corticosterone (Dunn, 1993; Dunn et al., 1999). This is consistent with

the concept that not only extrinsic stressors but also intrinsic stressors such as immune activation or inflammation are able to stimulate the HPA axis (Borrow et al., 2016; Farzi et al., 2015b; Lehmann et al., 2013; Painsipp et al., 2008). As I found LTA to evoke immunologic reactions in the periphery and brain, I wondered whether peripheral immune stimulation by LTA would also raise the circulating concentrations of corticosterone. The pertinent experiments revealed that treatment with LTA_{pure}, $LTA_{extract}$ and $LPS_{ultrapure}$ elevated the plasma levels of corticosterone to a similar extent as assessed 3 h following injection. These observations demonstrate that both TLR2 and TLR4 activation reflects "immune stress" that leads to activation of the HPA axis (Dunn, 1993; Dunn et al., 1999; Farzi et al., 2015b; Lehmann et al., 2013; Painsipp et al., 2008). Furthermore, my findings are in keeping with a number of rodent studies in which stimulation of TLR4 by LPS has been reported to enhance the activity of the HPA axis. In contrast, there is only one study in the literature to show that LTA (1 mg/kg; purity not specified) is also able to induce HPA axis activity (Bergt et al., 2013).

As increased activity in the HPA axis is known to have an impact on anxiety and other dimensions of emotional-affective behavior (Borrow et al., 2016; Jacobson, 2014), it is of note that LTA_{pure} did not alter anxiety-like behavior, although it enhanced circulating corticosterone levels. Peripheral immune stimulation has been reported to affect many neurotransmitters in the brain, notably the monoaminergic systems (Dunn et al., 1999; Felger and Lotrich, 2013). Neurotransmission through the monoamine serotonin (5-hydroxytryptamine) is controlled by reuptake of the indoleamine into the presynaptic nerve terminals via the serotonin transporter encoded by the gene SLC6A4. However, this aspect of serotonin-mediated neurotransmission remained unchanged by TLR2 and TLR4 signaling, as treatment with LTA_{pure}, $LTA_{extract}$, and $LPS_{ultrapure}$ had no effect on SLC6A4 mRNA expression in the amygdala and prefrontal cortex.

Another potential link between HPA axis activity, corticosterone and stress-induced behavior involves the glucocorticoid feedback to the brain, given that glucocorticoid receptor stimulation in the brain is known to influence emotional-affective behavior (Howell and Muglia, 2006; McEwen, 2007). In view of this relationship I hypothesized that enhanced plasma concentrations of corticosterone due to immune stress could alter the expression of the glucocorticoid receptor encoded by

the gene NR3C1 in the amygdala and prefrontal cortex. The results showed, however, that LTA_{pure}, $LTA_{extract}$, and $LPS_{ultrapure}$ failed to grossly affect the expression of NR3C1 in the amygdala, whereas in the prefrontal cortex LTA_{pure} (but not $LTA_{extract}$, and $LPS_{ultrapure}$) diminished NR3C1 expression to a significant extent. This observation warrants further study involving prolonged treatment with TLR2 and TLR4 agonists to analyze the impact of chronic immune stress on transmission and signaling pathways in an extended range of brain circuits.

Immune-brain communication evoked by simultaneous TLR2, TLR4 and NOD2 agonism

The analysis of the immunologic and behavioral effects of $LTA_{extract}$ (containing LTA contaminated with LPS/endotoxin), relative to those of LTA_{pure} and $LPS_{ultrapure}$, disclosed a potential synergistic interaction between TLR2 and TLR4 in their impact on immune system and CNS. Since under pathophysiological conditions several PRR agonists shed from commensal and/or pathogenic bacteria will be simultaneously present and simultaneously stimulate various PRRs, I considered it important to explore such interactions in their influence on the immune system and brain. It has previously been demonstrated that peptidoglycan components stimulating NOD1 and NOD2 synergize with LPS stimulating TLR4 in causing peripheral immune activation, neuroinflammation and sickness behavior (Farzi et al. 2015). For this reason, a systematic analysis of the effects of $LTA_{extract}$, being both a TLR2 and a TLR4 stimulant due to the LPS contamination, combined with the NOD2 agonist MDP was conducted, the effects of the combination being compared with those of vehicle, $LTA_{extract}$ alone and MDP alone, respectively.

While treatment with $LTA_{extract}$ alone, MDP alone and $LTA_{extract}$ + MDP induced anxiety-like behavior to a certain degree, only the combination of $LTA_{extract}$ + MDP was able to reduce locomotion, a sign of sickness behavior. These findings indicate that TLR2, TLR4 and NOD2 signaling can synergize with each other in causing behavioral disturbances. In an attempt to explain some mechanisms underlying this behavioral outcome I analyzed a series of molecular and immunologic markers in the periphery and brain. Unlike in the behavioral study, MDP exerted an antagonistic effect on the ability of $LTA_{extract}$ to elevate circulating cytokine and glucocorticoid levels, because the rise of plasma IL-1β, IL-10, IFN-γ, TNF-α and corticosterone was higher in response to $LTA_{extract}$ alone than in response to co-treatment with

$LTA_{extract}$ + MDP, while MDP alone had virtually no effect on circulating cytokine and corticosterone concentrations. The action of MDP to blunt the rise of plasma TNF-α and corticosterone evoked by $LTA_{extract}$ reached statistical significance. In the brain, however, the type of interaction between MDP and $LTA_{extract}$ was completely different as the two PRR agonists synergized in enhancing the expression of IL-1β, IL-6, IL-10 and TNF-α mRNA in the amygdala as well as of IL-1β and TNF-α mRNA in the prefrontal cortex. These findings not only provide an explanation for the ability of $LTA_{extract}$ + MDP to cause sickness behavior but also indicate once more that the mechanisms whereby PRR agonism causes induction of cytokines in the plasma and cytokine expression in the brain are distinct from each other.

The observations regarding plasma cytokine levels, cytokine expression in the brain, activation of the HPA axis and behavioral disturbances point to a dual synergistic/antagonistic interaction between TLR2 and TLR4, on the one hand, and NOD2, on the other hand. Further molecular analyses did not contribute to the elucidation of the underlying mechanisms. Specifically, no interaction between $LTA_{extract}$ and MDP was noted in altering the circulating levels of tryptophan and kynurenine, which were analyzed because the formation of the tryptophan catabolite kynurenine by IDO can be enhanced by cytokines, and kynurenine is thought to contribute to the behavioral effects of immune stimulation (Haroon et al., 2012; O'Connor et al., 2009; Salazar et al., 2012). Likewise, $LTA_{extract}$ and MDP failed to affect BBB integrity as assessed by Evans Blue extravasation and did not alter the expression of the glucocorticoid receptor encoded by the gene NR3C1 in the amygdala and prefrontal cortex. Although a further in-depth investigation was beyond the scope of this work, the current findings open up a new window for exploration into how different classes of PRR agonists synergize and/or antagonize in their impact on immune-brain cross-talk.

Conclusions

My work has demonstrated that the effects of the TLR2 agonist LTA on immune-brain communication depend to a significant extent on the microbial source and analytic purity of the LTA preparation under study. Importantly, I was able to overcome this limitation by the use of a highly purified LTA preparation, biologic validation of its selectivity as a TLR2 agonist and careful analysis of the effects that

potential LPS/endotoxin contaminations may contribute (Mayerhofer et al., 2017). This approach enabled me to unveil that selective stimulation of TLR2 by purified LTA not only causes peripheral immune activation but also initiates neuroinflammatory processes in the brain as mirrored by transcriptional up-regulation of pro-inflammatory cytokines. These neuroinflammatory processes take place in parallel with a transcriptional down-regulation of tight junction-associated proteins, which points to a disruption of the molecular composition of the BBB (Mayerhofer et al., 2017). In the framework of related studies, I conclude that TNF-α, IL-1β and IL-6 may be major regulators of the transition from peripheral immune stimulation to neuroinflammation and molecular disruption of the BBB. The molecular changes evoked by TLR2 activation extend to activation of the HPA axis but have little impact on emotional behavior, unless TLR2 is concomitantly activated with TLR4, which appears to boost peripheral and cerebral immune activation and to enhance anxiety (Mayerhofer et al., 2017).

In a translational perspective, the mutual interactions between various PRRs are important to consider in order to understand the multiple immune-brain signaling pathways that operate simultaneously under pathophysiological circumstances such as bacterial invasion or translocation of microbial factors across biologic barriers. In this context, I was able to reveal novel synergistic and antagonistic interactions between TLR2 and TLR4, on the one hand, and NOD2, on the other hand, in immune-brain communication. Constituents of the vast intestinal microbiota will also cause immune activation if the intestinal mucosal barrier is disrupted and allows microbe-derived molecules to pass into the lamina propria to reach the mucosal immune system (Garrett et al., 2010; Inman et al., 2012; Kelly et al., 2015; Maslanik et al., 2012). There is increasing evidence that a disturbed interaction between the gut microbiota and the intestinal immune system has an impact on mental health (Kelly et al., 2015; Sampson and Mazmanian, 2015).

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