β-FUNALTREXAMINE EFFECTS ON LIPOPOLYSACCHARIDE-INDUCED BEHAVIOR DEFICITS AND INFLAMMATION IN MICE

By

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DEFICITS AND INFLAMMATION IN MICE

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Abstract:

Inflammation plays a pivotal role in neurological and peripheral disorders. Specifically, inflammation is one of the common factors in diseases such as anxiety, depression, Alzheimer's disease (AD), Parkinson's disease (PD), inflammatory bowel disease (IBD), and many others that have all been linked to inflammatory changes in our central or peripheral systems. Thus, exploring potential treatments geared toward the abatement of inflammation is crucial to the continuation of treatment development. One pharmacological agent researched for its anti-inflammatory effects is β-funaltrexamine (β-FNA), a selective *mu*-opioid receptor (MOR) antagonist. Preclinical studies using *in vitro* human astroglial cells showed that β-FNA inhibited inflammatory signaling, NF-κB signaling, and chemokine expression in a mechanism unrelated to MOR. Also, the neuroprotective effects of β-FNA were discovered in a preclinical model of lipopolysaccharide (LPS)-induced neuroinflammation and sickness-like behavior when administered before LPS. This study determines the effects of β-FNA (50 mg/kg, i.p.) on LPS-induced (0.83 mg/kg i.p.) sickness-like behavior using a 10 min open field test and anxiety-like behavior using a 5 min elevated plus maze in male and female C57BL/6J mice. Depending on the study, it also assesses the effects on LPS-induced neuro and peripheral inflammation when β-FNA is administered immediately, 4 h or 10 h post-LPS. Tissue collected included the whole brain, hippocampus, prefrontal cortex, cerebellum/brain stem, spleen, liver, small intestine, large intestine, and plasma. Levels of inflammatory cytokines/chemokines (TNF-α, IL-6, IL-1β, CXCL10, CCL2) were measured using an enzyme-linked immunosorbent assay (ELISA), and inflammatory factors (NF-κB-p65, TAK1, p38 MAPK, GFAP) were measured using a western blot analysis. Also, to our knowledge, this is the first time the effects of β-FNA on female mice has been assessed. Differential effects of β-FNA were found between the whole brain vs. brain regions, central vs. peripheral nervous system, inflammatory factors, sexes, and temporal differences. Overall, this study will provide insight into the protection offered by β-FNA in both the central and peripheral systems and provides further exploration into additional therapeutic options for neurological disorders.

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CHAPTER I

INTRODUCTION

Inflammation is present in a multitude of neurological disorders [1, 2]. Anxiety and mood disorders are diagnosed in over 40 million people; this is roughly 18 % of the adult population yearly in the United States alone. Inflammation, however, is not only centralized in the brain but can also occur in the periphery, such as in the spleen, liver, and intestines during certain infections, cirrhosis, inflammatory bowel disease (IBD), Crohn's disease (CD), and ulcerative colitis (UC) [3-6]. For this reason, targeting inflammation has emerged as a viable option for the potential treatment of such disorders. One potential agent shown to have beneficial effects is β-funaltrexamine (β-FNA), a selective *mu*-opioid receptor (MOR) antagonist. Previous data indicated that a single, i.p. dose of β-FNA prior to LPS administration inhibits LPS-induced anxiety-like behavior in adult male C57BL/6J mice [2]. In addition, previous studies have seen the alteration of cellular events due to LPS which led to neuroinflammation (and behavioral deficits) [2, 7, 8]. This is why we set out to establish the extent to which β-FNA is protective when treatment occurs hours after LPS administration and determine whether the effects are sex-specific, as well as identify the extent to which β-FNA administration is protective in whole brain regions (hippocampus, prefrontal cortex, cerebellum/brain stem), plasma, and peripheral tissue (spleen, liver, proximal small intestine, distal small intestine, large intestine). We hypothesized that β-FNA affects LPS-induced behavioral deficits as well as tissues (whole brain, brain regions, spleen, liver, and intestines).

To test our hypotheses, we conducted behavioral testing, which included an open field test (OFT) (to quantify sickness-like behavior) and an elevated plus maze (EPM) (to quantify anxiety-like behavior). C57BL/6J mice were assessed at the end of their treatment. Tissue was collected and homogenized to assess for inflammatory factors using an enzyme-linked immunosorbent assay (ELISA) and immunoblotting assay (western blot) in both central and peripheral tissue. Overall, this study aims to provide further exploration of the therapeutic effects of β-FNA on neurological and peripheral inflammation.

CHAPTER II

REVIEW OF LITERATURE

This work was previously published in Journal of Inflammation with the following citation:

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Neuroinflammation in the central nervous system

One of the similarities present in a multitude of neurological disorders is inflammation [1, 2, 9-12]. Inflammation in the central nervous system has been associated with an increase in inflammatory molecules such as cytokines and chemokines, can develop during infection or trauma, and may occur during neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS) [13-15]. Furthermore, inflammation can be categorized as an innate immune response meaning it is the first line of defense and a local response of a tissue caused by an infection or injury that may result in swelling or pain [16]. The frontliner of the innate immune system are glial cells called microglia cells, which are macrophages that reside in the brain and spinal cord and makeup 10-20% of all glial cells in the CNS [17]. Microglia defend against foreign pathogens, eliminate debris, and when activated, can produce pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1β, (IL-1β), interleukin-6 (IL-6), and p38 mitogen-activated protein kinase (p38 MAPK) [13, 18, 19]. In addition, astrocytes are the most abundant glial cells in the central nervous system.

They play a pivotal role in the conservation of neuronal functions, help maintain brain homeostasis, and provide metabolites and growth factors to neurons. Astrocytes are critical regulators in innate and adaptive immune responses and can produce various cytokines and chemokines such as interferon γ-induced-protein (IP-10, also known as CXCL10); monocyte-chemotactic-protein 1 (MCP-1, also known as CCL2); interleukin-6, (IL-6); interleukin-1β, (IL-1β); and tumor necrosis factor-alpha, (TNF-α) [20].

Although neuroinflammation plays a role in neurological disorders, there are still benefits of neuroinflammation that arise such, as neurogenesis, axonal regeneration, remyelination, and neuroprotection [21]. Overall, a balanced system is required for all cells to function adequately, and it is when diseases formulate that imbalances arise in the system.

Inflammation in the peripheral system

Peripheral inflammation involves activating the innate or adaptive immune system that will then aid in releasing pro-inflammatory cytokines against various hazards. Inflammation is present in several peripheral conditions, such as hepatic and spleen inflammation and inflammatory bowel disease (IBD), Crohn's disease (CD), and ulcerative colitis (UC) [3-6]. Also, peripheral inflammation associated with infection has been linked to being a factor in the development of deterioration of CNS diseases such as AD, MS, PD, and stroke in both preclinical and clinical studies, and this could be a connection between the increased susceptibility of the blood-brain barrier (BBB) in these diseases [22]. There are also reports connecting brain disorders and peripheral diseases with their role in IBD and anxiety/depression [23-25]. Overall, there is a need to further understand the role of inflammation in the peripheral system and its connection to the CNS.

Inflammatory Factors

Cytokines

Cytokines are small proteins released by cells to perform a specific effect and interaction to aid in the communication between cells [15]. Cytokines are also classified as a lymphokine when they are made by lymphocytes; chemokines, which are cytokines that have chemotactic activities; another type of cytokines are interleukins, which are produced by leukocytes, in addition can also act on other leukocytes and cell types.

Cytokines can initiate a cascade of reactions; one cytokine stimulates its target cells, additional cytokines can be produced, and they can be pro-inflammatory or anti-inflammatory [26]. Cytokines are produced by macrophages and helper T cells, and an effect can occur when there is a binding to the receptor of a target cell leading to the signaling cascade that can alter the gene expression of the target cell [27]. Glial cells such as microglial and astrocytes produce cytokines in the CNS. In contrast, in the periphery, the activation of macrophages, endothelial cells, Schwann cells, and mast cells can lead to the production of pro-inflammatory cytokines [28, 29].

Chemokines

Specific cytokines that induce chemotaxis are identified as chemokines. These chemotactic cytokines are small proteins secreted by cells that influence the immune system [30]. Chemotaxis is the response that allows for the movement of cells in response to a chemical (chemokine) gradient, which would enable direct cell migration, adhesion, and activation. Cells respond to chemokines due to their receptors which are part of the G-protein coupled receptors (GPCRs). GPCRs are seven transmembrane receptors that bind to extracellular ligands and form intracellular signaling. This is followed by the binding to the receptor, allowing for further activation and chemokines to play a pivotal role in the immune system development and inflammatory responses

[30]. Chemokines are named by their amino acid composition and cysteine (C) residues within the protein allowing for subfamilies: CC, CXC, CX3C, and C [15].

Tumor necrosis factor-α (TNF-α)

Tumor necrosis factor-α plays a pivotal role in inflammatory responses and homeostatic processes; it can be found in glia and neurons and is secreted by macrophages [31-33]. The two primary TNF-α cell surface receptors in signaling pathways are TNFR1 and TNFR2, which can regulate NF-κB activation of inflammation, activate stress-activated protein kinases (SAPKs), and apoptotic signaling pathways [26]. Specifically, TNFR1 is activated when TNF-α binds to it; then, the activation of caspases will activate cell survival pathways such as the NF-κB pathway and apoptotic signaling pathways. In contrast, TNFR2 signaling only activates NF-κB [13]. Studies have shown that an increased level of TNF-α can be found in Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), and mood disorders such as anxiety [13, 34]. TNF-α is a pro-inflammatory cytokine, and its ability to increase the permeability of the BBB makes it a key player in neuroinflammation [34]. TNF-α also has been shown to be a factor in intestinal inflammation and has been linked with diseases such as ulcerative colitis (UC), Crohn's disease (CD), and inflammatory bowel disease (IBD) [35].

Interleukin-1β (IL-1β)

IL-1β is a pro-inflammatory cytokine that has been associated with an increase in inflammation, pain, and autoimmune conditions [36]. Elevated levels of IL-1β in the hippocampus and prefrontal cortex during stress can contribute to learning impairments produced by stress [37]. In addition, the expression of IL-1B is associated with diseases such as mood disorders, anxiety AD, PD, ALS, MS, and inflammatory bowel disease [13, 35, 38].

Interleukin-6 (IL-6)

IL-6 is a pro-inflammatory cytokine important for host defense that can stimulate immune responses. Levels of circulating IL-6 have been previously linked to stress responses, mood disorders, depression, and increased with psychosocial stress [39-41]. It also has been found to play a role in patients with IBD, CD, AD, and MS [13, 35]. Studies have also linked the blockade of IL-6 signaling as a potential treatment in models of inflammatory diseases where IL-6 is elevated [42].

Interferon-γ-inducible protein 10 (IP-10/CXCL10)

Interferon-γ-inducible protein 10 is a chemokine of the C-X-C family. CXCL10 binds to the CXCR3 receptor, a seven transmembrane-spanning GPCR in a paracrine (cell acts on nearby cell) or autocrine (cell acts on the same cell) action [43]. CXCL10 is known for being a chemoattractant that can activate and recruit T cells to sites of inflammation [44]. CXCL10 is expressed by glia and neurons and has been linked to several neurological and peripheral diseases such as anxiety, AD, MS, and peripheral inflammation [43, 45]. More recently, increased concentrations of CXCL10 have been associated with inflammatory diseases and may be involved in the disease progression of SARS-CoV-2 infection (COVID-19) through the activation of the CXCR3 receptor, which is expressed on macrophages, T lymphocytes (T cells), B cells, dendritic cells (DCs), and natural killer (NK) cells [46].

Monocyte chemoattractant protein (MCP-1/CCL2)

Monocyte chemoattractant protein MCP-1, also known as CCL2, is a chemokine belonging to the C-C family. Previous studies have linked the increase of CCL2 expression in combination with inflammatory cells in the brain [47]. This would imply that glial cells, such as microglia and astrocytes, are a source of CCL2. Elevated levels of CCL2 have been connected with mood disorders and are thought to be related to neuroendocrine activity [48, 49]. Given that CCL2 is a

chemokine, it is also chemoattractant and can initiate the recruitment of immune cells such as T lymphocytes, natural killer cells, and dendritic cells [50, 51].

Nuclear factor kappa B p65

Nuclear factor kappa B p65 (NF-κB-p65) is one of the five components that form the NF-κB/RE1 family: (NF-κB1 p50, NF-κB2 p52, p65 (Rel-A), c-Rel, and Rel-B proteins) [52]. Homodimers or heterodimers will be formed and remain inactive complexes with IκB inhibitory proteins in resting cells. Once IκB proteins are phosphorylated by IKK, the release of the NF-κB subunits (p65 and p50) is initiated for translocation into the nucleus, where they bind to promoters and induce the expression of specific pro-inflammatory protein genes [39]. The elevation of p65 in the cell has been linked to neurodegenerative diseases, rheumatoid arthritis, and inflammatory bowel disease [52, 53].

Transforming growth factor-beta-activated kinase 1 (TAK1)

Transforming growth factor-beta-activated kinase 1 (TAK1) is a critical kinase in pro-inflammatory cytokine signaling pathways such as the NF-κB signaling pathway. TAK1 is activated by tumor necrosis factor receptor-associated factor 6 (TRAF6). TRAF6 is an adaptor protein that is a RING domain ubiquitin ligase and it's synthesis is aided by lysine 63-linked polyubiquitin chains [54]. TAK1 has regulatory subunits, TAB1, TAB2, and TAB3, of which TAB2 and TAB3 bind to polyubiquitin chains, activating TAK1. Once activated, TAK1 activates the downstream kinase IKK, causing the phosphorylation of IκBα and initiating NF-κB activation [39]. TAK1 also functions as a protein in signaling TLRs and various cytokines, such as TNF-α and IL-1β. It has been linked to pro-inflammatory signaling in hepatocytes in the liver [55-57].

p38 Mitogen-activated protein (MAP) kinase (p38 MAPK)

The protein kinase, p38 Mitogen-activated protein (MAP) kinase (p38 MAPK), is reactive to stress stimuli such as inflammatory cytokines and can be activated by LPS [58]. This kinase is part of the p38 MAPK signaling pathway, which aids cells in recognizing external signals, provides communication between cells, and is involved in inflammation, cell growth/death, and cell differentiation [59, 60].

Glial fibrillary acidic protein (GFAP)

Intermediate filaments (IFs) are components of the nuclear and cytoplasmic cytoskeleton. Glial fibrillary acidic proteins (GFAP) are intermediate filament (IFs) proteins of astrocytes, which are the most abundant glial cells in the central nervous system that maintains homeostasis, regulate flood flow, and provide the building blocks for neurotransmitters [61]. In addition, an increase in the expression of GFAP is linked to increased inflammation, stress, oxidation, immune activation, and many other changes that can negatively impact the brain [62].

Lipopolysaccharide (LPS)

LPS is a significant component of the gram-negative bacterial cell wall and is widely used to systematically stimulate the immune system and cause physiological/behavioral changes – such as inflammation. The structure of, LPS is composed of an inner and an outer portion of core oligosaccharides, an O-antigen portion, and a phosphoglycolipid Lipid A component. The Lipid A component is the endotoxic portion of LPS and is one of the locations where modifications to LPS can be made. Bacterial LPS has been typically used to study inflammation due to its physiological changes and effects at the toll-like receptor 4 (TLR4), which induces a cascade of events that leads

to cytokine release [63]. Additional studies have also provided insight into LPS-induced production of cytokines such as TNF-α, IL-6, and IL-1β, as well as the expression of CCL2 and p38 MAPK [2, 7, 8, 63-65].

Toll-like Receptors (TLR)

In the case of toll-like receptors (TLRs) they are described as pattern recognition receptors (PRRs) that can detect microbes, including bacteria, viruses, and fungi. TLRs are part of the innate immune system in which there is an immediate response to a pathogen; however, the response is less specific and can cause damage to tissue if prolonged [66]. TLRs' interaction with ligands induces an immune response by activating downstream signaling pathways and producing inflammatory mediators, inflammatory cytokines, and type I interferons (IFN) [67]. TLRs can also detect endogenous molecules released during tissue injury/death (damage-associated molecular patterns, DAMPs); this is followed by the activation of the innate immune system, and an inflammatory response [67]. In addition, host-derived DAMPs such as heat shock proteins (HSPs), plasma membrane constituents, and deoxyribonucleic acid (DNA) can also be detected by TLRs [67, 68].

TLRs are type I transmembrane glycoproteins composed of an a single transmembrane helix, a C-terminal cytoplasmic signaling domain, and N-terminal ligand recognition domain; they can be found in the plasma membrane and endosomes [69]. There are different types of TLRs; for example, surface TLRs include 1, 2, 4, 5, 6, and 10; they can bind molecules created by extracellular bacteria, whereas intracellular bacterial/viral proteins are detectable by TLRs 3, 7, 8, and 9 [70]. Interestingly the most recognizable TLR2 and TLR4 are the most efficient in recognizing a wide range of ligands, particularly TLR4 for gram-negative derived LPS [68].

Nuclear Factor Kappa B Signaling Pathway (NF-κB)

NF-κB transcription factors regulate a multitude of genes associated with inflammatory and immune responses and are composed of five members (NF-κB1 p50, NF-κB2 p52, p65 (Rel-A), c-Rel, and Rel-B proteins) [52, 71]. NF-κB proteins are located in the cytoplasm and are bound to inhibitory proteins such as those in the nuclear factor of kappa light polypeptide gene enhancer in the B-cells inhibitor alpha (IκBα) family [52].

There are two activation pathways for NF-κB the canonical (classic) pathway and the non-canonical (alternative) pathway. The canonical pathway reacts to cytokine receptors, TNF receptor (TNFR) superfamily members, immune receptors (T-cell and B-cell), and pattern-recognition receptors (PRRs), and the stimulation of this pathway cascades the phosphorylation of the IκB kinase (IKK) complex which consist of two subunits (IKKα and IKKβ) [39, 71].

The phosphorylation of IκB through the kinase IKK releases NF-κB allowing the active NF-κB transcription factor subunits (p65, p50) to translocate into the nucleus, bind to specific gene promoters, and cause target gene expression, which is likely the expression of pro- and anti-inflammatory proteins [39]. In comparison, the non-canonical pathway will react to ligands of the TNFR superfamily members, such as the CD40 ligand. In addition to regulating inflammatory responses, NF-κB has also been linked to playing a role in regulating inflammasome activation.

LPS/TLR4/NF-κB Signaling Pathway

TLRs have also been connected to the activation of the NF-κB pathway, specifically TLR4 for gram-negative bacteria-derived lipopolysaccharides (Fig. 1) [68]. LPS binding protein (LBP), lymphocyte antigen 96 (MD2) cluster of differentiation 14 (CD14), all aid in the binding as well as

the activation of LPS to TLR4 [72]. After LPS binds to TLR4, the conformational change allows the Toll/interleukin-1 receptor/resistance protein (TIR) domain to activate, permitting signaling to begin [73].

The activation of the MyD88-dependent signaling allows for molecules such as MyD88 adaptor-like (MAL) (TLR2/TLR4 signaling only) and myeloid differentiation primary response gene 88 (MyD88) to engage [74]. After MyD88 molecules are recruited, they initiate the activation of interleukin-1R-associated kinase-1 (IRAK1) and interleukin-1R-associated kinase-4 (IRAK4). IRAK1/IRAK4 phosphorylates TNF-receptor-associated receptor 6 (TRAF6), will then activate the transforming growth factor β-associated kinase 1 (TAK1) [74]. This activation of TAK1 is followed by the downstream kinase IKK, which phosphorylates IκBα initiating the NF-κB activation where translocation of subunits p50 and p65 into the nucleus occurs, and then then bind to specific gene promoters, causing a target gene expression releasing pro-inflammatory proteins [39].

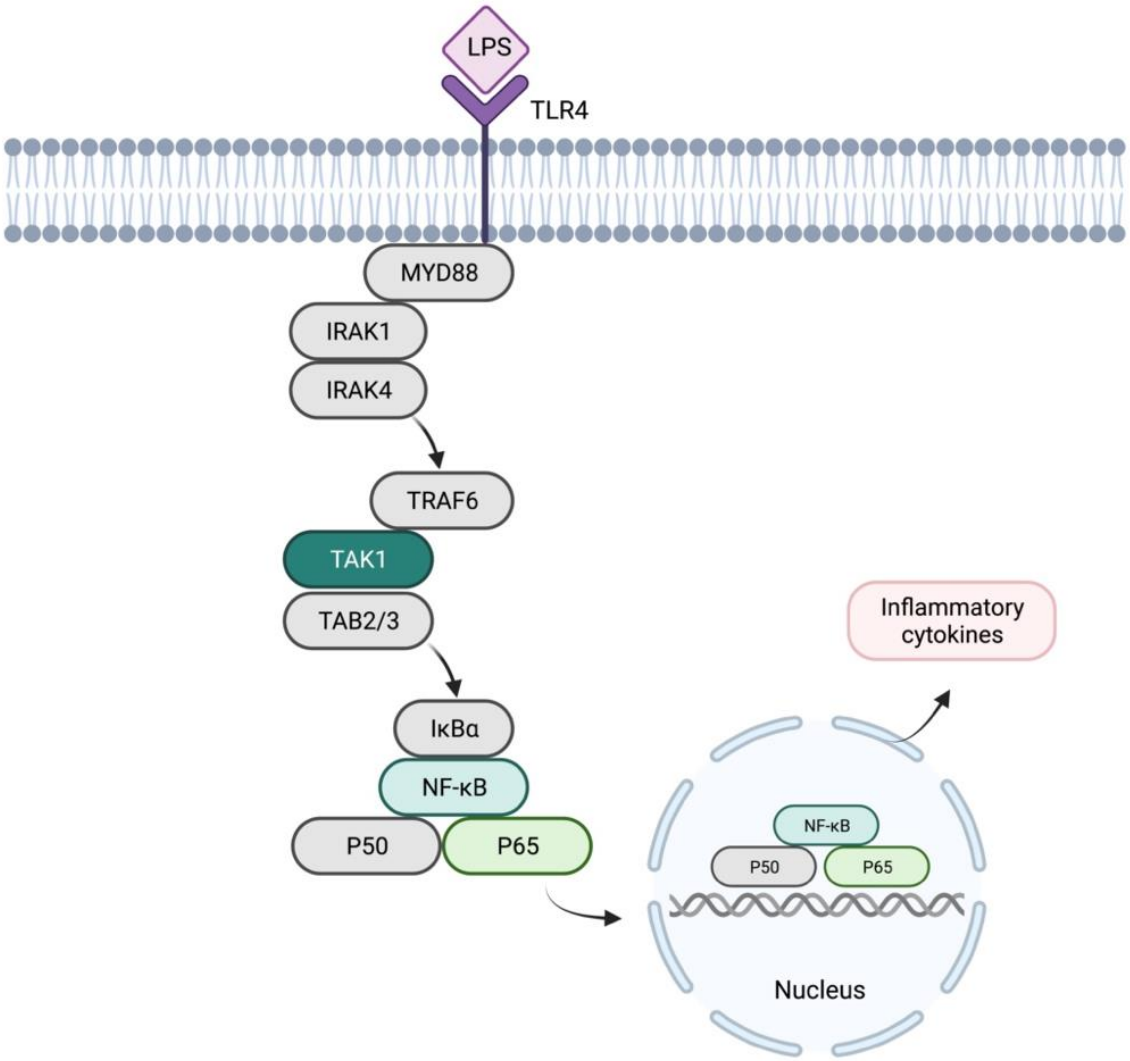


Fig 1: NF-κB Signaling Pathway. Created with BioRender.com

Opioid Receptors

Opioid receptors are GPCRs that are involved in the response of most hormones, drugs, and neurotransmitters and are also involved in sensory perception of taste, vision, and olfaction [75]. GPCRs have three subunits: alpha, beta, and gamma, and they can be differentiated by the type of signaling pathway they activate [75]. Gs protein-coupled receptors are stimulatory receptors and stimulate adenylate cyclase, causing the increase of cyclic adenosine monophosphate (cAMP) production, thus allowing the activation of Protein Kinase A (PKA) [76]. PKA can phosphorylate proteins, enzymes, or ion channels. Gi protein-coupled receptors are inhibitory and can stop cAMP activation [76]. Activation occurs when the G-alpha subunit binds to guanosine triphosphate (GTP), and at resting state G-alpha subunit binds to guanosine diphosphate (GDP); no activation will take place. G-alpha binding to GTP allows the G protein to stay active, and the G-beta-gamma subunit dissociates. Once GTP has been hydrolyzed back to GDP, the subunits form the inactive heterotrimer [77]. Endogenous opioids are natural ligands of opioid receptors that can assist in neurotransmission and pain. Interestingly, during stress and exercise, beta-endorphin, an agonist for *mu*-opioid receptors (MORs), is secreted by the hypothalamus and can induce euphoria, inhibit muscular fatigue, and after-workout pain [78]. Whereas exogenous ligands include drugs that have pharmacological benefits, as well as, drugs that can have adverse side effects [78].

There are at least three specific opioid receptors: mu, kappa, and delta.

<u>Mu (μ) Opioid Receptor (MOR)</u>

In the central nervous system, MORs can be found in the hypothalamus, cerebral cortex, thalamus, and interpeduncular nucleus. In contrast, in the periphery, MORs can be located in the gastrointestinal tract, liver, and certain smooth muscles [79-81]. MORs are also involved in analgesia, hypothermia, constipation, sedation, respiratory depression, cognitive dysfunction,

nausea, vomiting, euphoria, and physical dependence [80]. The MOR also plays a vital role in decreasing stress response by inhibiting the release of norepinephrine (NE) from locus coeruleus (LC), and there is also a connection between high levels of MOR in the limbic system, which is responsible for mood and emotions [76].

Kappa (κ) Opioid Receptor (KOR)

In the central nervous system, the kappa opioid receptors are found in the hippocampus, thalamus, cerebral cortex, and substantia nigra [81]. Studies have found KOR in the gastrointestinal tract, spleen, and liver of guinea pigs [82]. KORs also are vital in modulating serotonin (5-HT), dopamine (DA), and glutamate release in the CNS and can mediate analgesia [83, 84].

Delta (δ) Opioid Receptor (DOR)

In the central nervous system, the nucleus accumbens olfactory bulb, amygdala, and cerebral cortex are all locations where delta opioid receptors can be found [81]. In the periphery, DORs are also found in the gastrointestinal tract, liver, and spleen [85, 86]. Delta receptors mediate analgesia and can also play a role in mood disorders such as anxiety and depression [87].

Opioids

There are several groups of opioids: endogenous opioid peptides (occur naturally, ex. β-endorphins or enkephalins), opium alkaloids (natural plant alkaloids, ex. opium from the opium poppy (morphine, codeine), semi-synthetic opioids (meaning they are made in a lab from natural opiates ex. oxycodone, heroin, hydrocodone, and hydromorphone), and synthetic derivatives (meaning man-made ex. fentanyl, and methadone) [75]. Opioids are drugs that originate from the opium of a poppy plant; they have different effects on the brain but are mostly known for their ability to

mediate analgesia. Some of the common side effects that arise when using opioids include constipation, drowsiness, confusion, nausea, vomiting, respiratory depression, sedation, and euphoria. At a more critical stage, there is also the possibility of developing a tolerance to the medication, which could lead to physical dependence and, in extreme cases, addiction [88].

Opioid Agonists

Opioid agonists at the MOR are ligands that activate opioid receptors in the central and peripheral nervous systems. They are generally classified as analgesic medications that block pain sensation and bind predominantly to the *mu* receptor. An example of an agonist is morphine, a natural plant alkaloid known to have analgesic effects. Studies have previously shown that morphine can generate a response of analgesia, constipation, and respiratory depression; however, when using knock-out mice lacking the *mu* receptor, these effects were not seen with the administration of morphine [89].

Opioid Partial Agonists

Opioid partial agonists will bind to an opioid receptor and will not produce the full agonist response. An example of this is buprenorphine, a semi-synthetic opioid analgesic with a partial response at the *mu* receptor [90]. The benefit of using a partial agonist is that it can offer analgesic effects without the ceiling effect, where the drug's effect on the body has plateaued.

Opioid Antagonists

Opioid antagonists will block an opioid agonist effect by binding to the opioid receptor without activating the receptor. An example is naltrexone, a synthetic opioid antagonist used to reverse opioid-induced respiratory depression and sedation and treat opiate and alcohol addiction [91].

Another example is β-FNA, an irreversible selective mu-opioid receptor antagonist that cannot be competed off and will bind permanently to the receptor.

β-FNA and naltrexone are antagonists; however, naltrexone is a non-selective "reversible" opioid receptor antagonist, whereas β-FNA is characterized as a selective opioid receptor antagonist [92, 93]. β-FNA is also known to readily cross the BBB and is well-tolerated [94-97]. Previous preclinical work has further explored the therapeutic effects of β-FNA and found that it inhibited inflammatory signaling *in vitro* in human astroglial cells, which are known to play a role in CNS homeostasis [7]. Further work on the effects of β-FNA on human astrocytes found that it inhibits NF-κB signaling and chemokine expression in a mechanism unrelated to MOR [8]. The neuroprotective effects of β-FNA have also been examined in a mouse model of LPS-induced neuroinflammation and sickness-like behavior, where it was found to be protective [2]. Given β-FNA characteristics, it became of interest as an agent of anti-inflammatory properties.

CHAPTER III

METHODOLOGY

Ethics Statement

The Oklahoma State University Center for Health Sciences Institutional Animal Care and Use Committee approved the protocol (#2020-1236) for all experimental processes and animal manipulations.

Animals

Oklahoma State University-Center for Health Sciences (OSU-CHS), USDA-approved facilities, housed seven-week-old male and female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME). There was an assignment of three mice per plastic cage with the dimensions of (10 cm × 17 cm × 28 cm). Within the cage *ad libitum* food and water was provided, pine chip bedding, and cardboard tubes for environmental stimulus. An ambient room temperature of 21°C was maintained, and a steady 12:12 light: dark cycle was placed for the animals. A total of 144 animals were approved, and the mice were provided with a 7-day acclimation period before the initiation of experiments and monitored daily.

Experimental protocol

Mice (n = 5-8 per group or n=11-12 depending on the experiment) were injected with an intraperitoneal (i.p.) dose of LPS (0.83 mg/kg dissolved in saline; *Escherichia coli* O55:B5; Sigma). As previously documented, this LPS dose was shown to effectively induce behavioral deficits, such as sickness-like behaviors, anxiety-like behavior, and neuroinflammation in mice [1, 98-101]. Treatment with β-FNA (50 mg/kg, dissolved in saline, i.p.; National Institute on Drug Abuse reagent supply program) was administered immediately, 4 h post-LPS, or 10 h post-LPS depending on the study, and control mice were administered vehicle (200 µl saline). The dose used for β-FNA treatment was established due to the effectiveness of the dose and drug in our previous work and previous literature [6, 8, 98, 102, 103].

Behavioral measures

Two behavioral assays were conducted to assess the impact of LPS and β-FNA on sickness-like and anxiety-like behavior in male and female C57BL/6J mice: the Open Field Test and the Elevated Plus Maze.

Open Field Test

An open-field test (OFT) was used to assess exploratory locomotor activity, a behavioral marker of acute sickness-like behavior (Fig.2-3) [1, 98-101, 104]. Ethovision Software was used to monitor and record for 10 min as mice were placed in the center of the OFT arena (40 cm × 40 cm). Depending on the study distance moved (cm) or the duration (sec.) in the center 35 cm × 35

cm region of the arena was used as a dependent measure; in addition, reduced distance moved (compared to controls) was suggestive of sickness-like behavior [1, 99-101, 104].

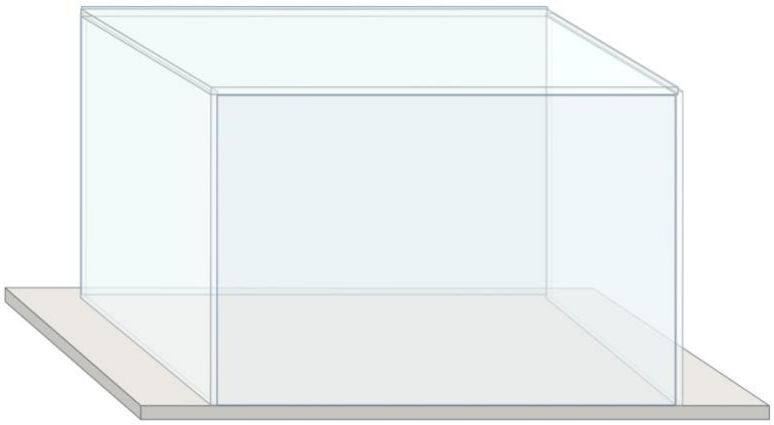


Fig 2: Open Field Test used to assess for assessment of sickness-like behavior. Created with BioRender.com.

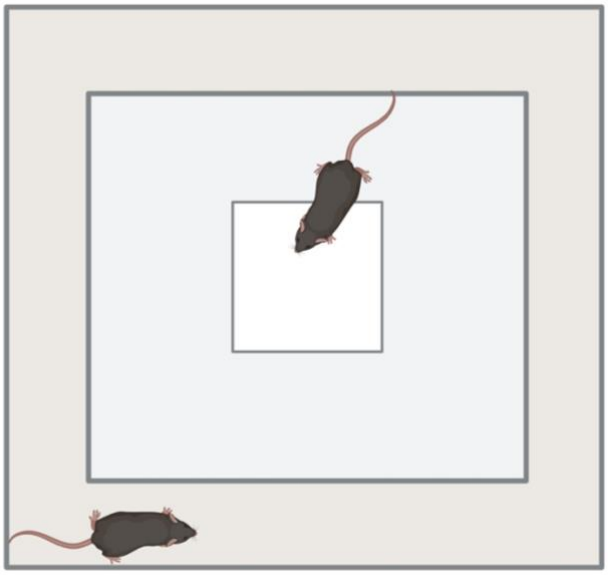


Fig 3: Top view of open Field Test arena used to assess for assessment of sickness-like behavior. Created with BioRender.com.

Elevated Plus Maze

To assess anxiety-like behavior, an elevated plus maze (EPM) was composed where there were two open arms (25 cm × 5 cm × 0.5 cm), two enclosed arms (25 cm × 5 cm × 16 cm), and a center area (5 cm × 5 cm × 0.5 cm) (Fig.4) [105]. Ethovision Software was used to monitor (and record) for 5 minutes as mice were placed in the center of the maze. Anxiety-like behavior was deduced as reduced time (sec.) spent in the open arms (compared to control mice) [105].

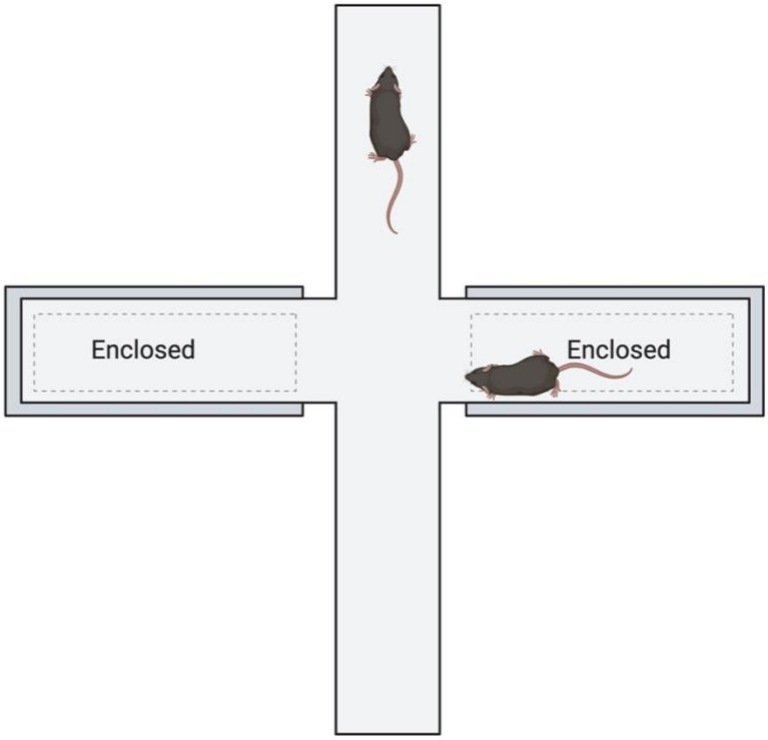


Fig 4: Elevated plus maze used to assess for assessment of anxiety-like behavior**.** Created with BioRender.com.

Tissue collection

At the completion of the behavioral analyses, the animals were immediately euthanized by CO_2 inhalation and subsequent decapitation. Depending on the experiment, the following tissues were collected: trunk blood, brain, spleen, liver, small intestines, and large intestine. All tissue samples were collected into ice-cold tubes and were instantly placed on ice. Centrifugation of (17,000 × g, 15 min., 4°C) allowed for plasma collection. In select experiments, the hippocampus, prefrontal cortex, and cerebellum/brain stem were dissected from the whole brain. All samples were stored in a −80° C freezer until assays were performed. As previously described, the bicinchoninic acid (BCA) protein assays were used to determine total protein [106]. In summary, a Sonic Dismembrator Model 100 (Fisher Scientific) was used to homogenize the tissue in ice-cold triple detergent lysis buffer containing ice-cold HALT Protease/Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) [7]. Centrifugation for samples occurred at (20,000 × g, 20 min, 4 °C) whereafter the aqueous phase was collected and stored.

Enzyme-linked immunosorbent assay (ELISA)

In select experiments, through the use of enzyme-linked immunosorbent assay (ELISA Development Kit, Peprotech), inflammatory chemokines/cytokines (CXCL10; CCL2; IL-6; IL-1β; and TNF-α) in plasma and tissue homogenates were quantified according to the manufacturer's instructions and previously described [107]. Absorbance was read at 450 nm using a BioTek Synergy 2 Multimode microplate reader, a Gen5 microplate reader, and imager software was also used (Agilent, Santa Clara, CA).

Immunoblotting assay (Western Blot)

Western blot analysis was used to measure the expression of NF-κB-p65, p38 MAPK, and GFAP in tissue homogenates depending on the experiment. Total protein (100 µg) was separated by 7.5% SDS polyacrylamide gel electrophoresis and transferred to a PVDF membrane as previously described [5, 108]. The membrane was incubated overnight at 4°C in target-specific 1° antibody [p65 (1:1000), Cat# 4764S; p38 (1:1000), Cat# 9212S; GFAP (1:1000), Cat#3670S; β-tubulin (1:1000), Cat# 2146S; Cell Signaling Technology]. Subsequently, the membrane was washed six times in Tris-buffered saline using 0.1% Tween (TBST), followed by incubation in 2° antibody [Goat-anti-Rabbit IgG (1:10,000) IRDye-680, Cat# 925-32211; Donkey-anti-Mouse IgG (1:10,000) IRDye-800, CAT# 926-32212; Li-Cor) for 2h at room temperature, followed by a final wash. Licor-CLX Odyssey was used to image western blots where direct detection was performed using secondary antibodies labeled with near-infrared fluorescent dyes. Stripping buffer (Cat# 21059; Thermo Fisher Scientific) was used for normalization of membranes, then re-probed with β-tubulin (1:1000, Cat# 2146S; Cell Signaling Technology), labeled with 2° antibody. For the relative quantification of protein signals, NIH Image J was used.

Statistical analysis

Data were analyzed by two-way analysis of variance (ANOVA) (treatment × sex), one-way ANOVA, linear regression, or multiple linear regression depending on the experiment, and Fisher's LSD was used for pairwise comparisons. Data are presented as mean ± SEM, and p-values < 0.05 are considered statistically significant, and data presentation normalized to control value. For data analysis and figure preparation Prism™ Version 9 software (GraphPad Inc, San Diego, CA) was used.

Overall experimental design

To assess the impact of LPS and β-FNA on sickness-like and anxiety-levels behavior in male and female C57BL/6J mice, two experimental processes were conducted:

Experiment 1 focused on the temporal importance of β-FNA treatment in a preclinical model of LPS-induced neuroinflammation (Fig.5). Male C57BL/6J mice were randomly assigned to one of four treatment groups (n=5-8). Male mice were administered LPS (i.p.) followed by treatment with β-FNA (i.p.) immediately or 4 h post-LPS. At 8 or 24 h post-LPS, sickness behavior was assessed using a 10-min open-field test, followed by termination and collection of brain, spleen, and plasma. Levels of inflammatory chemokines (interferon γ-induced protein, CXCL10; monocyte chemotactic protein 1, CCL2; and interleukin-6, IL-6) in tissues were measured using an enzyme-linked immunosorbent assay (ELISA). The expression of NFκB-p65, p38 MAPK, and glial fibrillary acidic protein (GFAP) levels in tissue homogenates were assessed using western blot analysis.

Table 1.
Experimental Design for β-FNA Effects at 8 & 24 hours

Group	Treatment
1	Vehicle + Vehicle
2	LPS + Vehicle
3	LPS + β-FNA Immediately post-LPS
4	LPS + β-FNA at 4-hour post-LPS

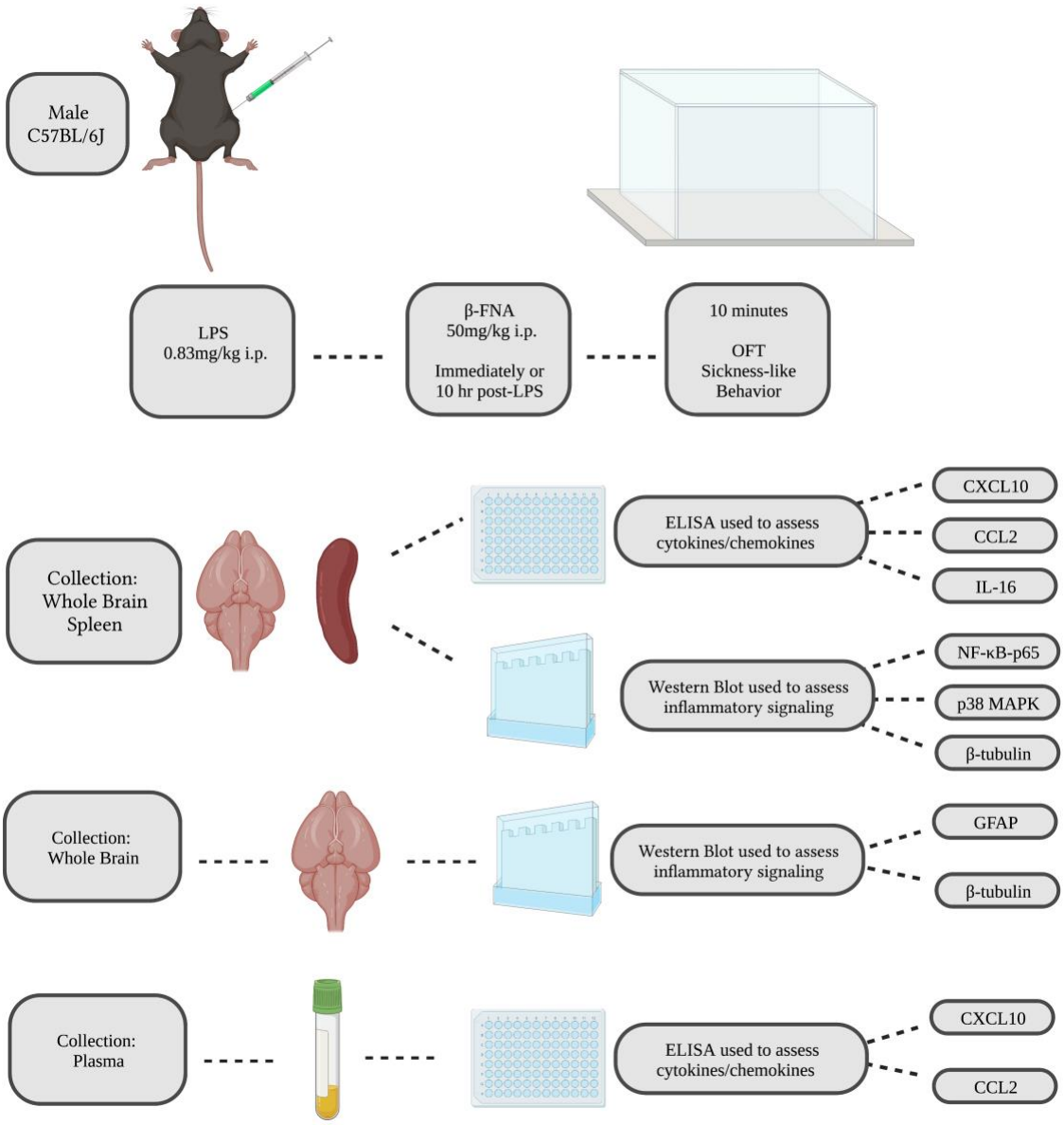


Fig 5: Experimental Design for β-FNA Effects at 8 & 24 hours. Created with BioRender.com.

Experiment 2 focused on the protective effects of β-FNA when treatment occurs 10 h after LPS administration and is the first-ever investigation of the sex-dependent effects of β-FNA on LPS-induced inflammation and behavioral deficits (Fig.6). Male and female C57BL/6J mice were administered LPS (i.p.) followed by treatment with β-FNA (i.p.) immediately or 10 h post-LPS (n=5-6 or n=11-12). Sickness-like behavior was assessed using a 10-min open-field test, and anxiety-like behavior was assessed by a 5-min elevated plus maze test. This was followed by the collection of the whole brain, hippocampus, prefrontal cortex, cerebellum/brain stem, spleen, liver, proximal small intestine, distal small intestine, large intestine, and plasma. Levels of inflammatory chemokines/cytokines (interferon γ-induced protein, CXCL10; monocyte chemotactic protein 1, CCL2; interleukin-6, IL-6; interleukin-1β, IL-1β, and Tumor Necrosis Factor Alpha, TNF-α) in tissues were measured using an enzyme-linked immunosorbent assay. The expression of NF-κB-p65 in tissue homogenates was determined by western blot analysis.

Table 2.
Experimental Design for β-FNA Effects at 24 hours

Group	Treatment
1	Vehicle + Vehicle
2	LPS + Vehicle
3	LPS + β-FNA Immediately post-LPS
4	LPS + β-FNA at 10-hour post-LPS

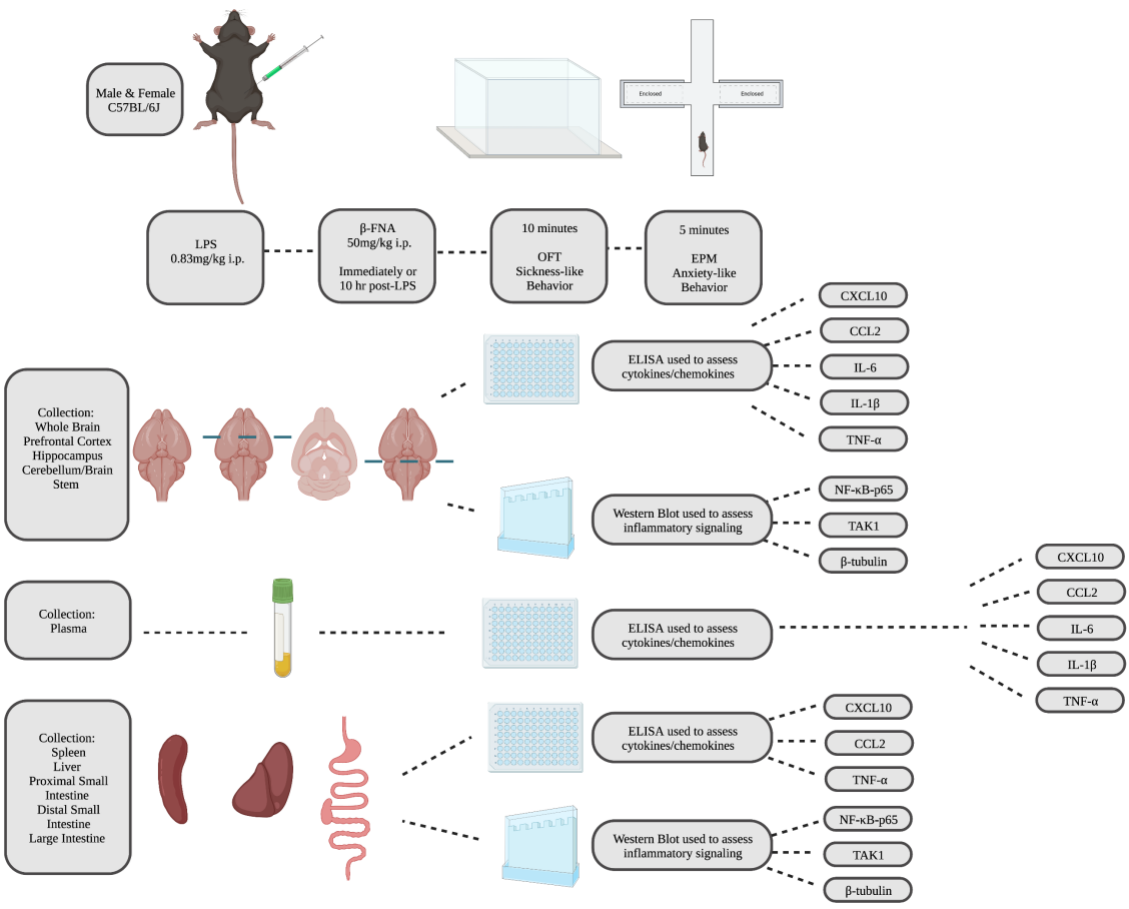


Fig 6: Experimental Design for β-FNA Effects at 24 hours. Created with BioRender.com.

CHAPTER IV

ANTI-INFLAMMATORY ACTIONS OF β-FUNALTREXAMINE IN A MODEL OF LIPOPLYSACCHARIDE-INDUCED INFLAMMATION

This work was previously published in Inflammopharmacology with the following citation:

Myers, S., McCracken, K., Buck, D. J., Curtis, J. T., & Davis, R. L. (2022). Anti-inflammatory actions of β-funaltrexamine in a mouse model of lipopolysaccharide-induced inflammation. *Inflammopharmacology*, 1-10.

Abstract

Neuroinflammation is involved in a wide range of brain disorders, thus, there is great interest in identifying novel anti-inflammatory agents to include in therapeutic strategies. We previously determined that lipopolysaccharide (LPS)-induced sickness behavior and neuroinflammation in mice are prevented by pretreatment with beta-funaltrexamine (β-FNA), a selective mu-opioid receptor (MOR) antagonist. Herein we investigated the temporal importance of β-FNA treatment in this preclinical model of LPS-induced neuroinflammation. Adult male C57BL/6J mice were administered an i.p. injection of LPS followed by treatment (an i.p. injection) with β-FNA immediately or 4 h post-LPS. Sickness behavior was assessed using an open-field test, followed by assessment of inflammatory signaling in the brain, spleen, and plasma. Levels of inflammatory chemokines/cytokines (interferon γ-induced protein, CXCL10; monocyte chemotactic protein 1, CCL2; and interleukin-6, IL-6) in tissues were measured using an enzyme-linked immunosorbent

assay and nuclear factor-kappa B (NF-κB), p38 mitogen-activated kinase (p38 MAPK), and glial fibrillary acidic protein (GFAP) expressions were measured by western blot. LPS-induced sickness behavior and chemokine expression were inhibited more effectively when β-FNA treatment occurred immediately after LPS administration, as opposed to 4 h post-LPS, and β-FNA-mediated effects were time-dependent as evidenced by inhibition at 24 h, but not at 8 h. The inhibitory effects of β-FNA on chemokine expression were more evident in the brain versus the spleen or plasma. LPS-induced NF-κB-p65 and p38 MAPK expression in the brain and spleen were inhibited at 8 and 24 h post-LPS. These findings extend our understanding of the anti-inflammatory effects of β-FNA and warrant further investigation into its therapeutic potential.

Introduction

Anxiolytic, anti-depressant, and anti-psychotic medications generally target neurotransmitter signaling, yet often possess limited anti-inflammatory properties [2, 101, 109, 110]. Neurological conditions, including mood disorders, often present with neuroinflammation, thereby elevating interest in the therapeutic potential of anti-inflammatory agents [1-5]. We previously discovered that β-funaltrexamine (β-FNA), a selective *mu*-opioid receptor (MOR) antagonist, inhibits cytokine-induced expression of cytokines/chemokines *in vitro* in human astroglial cells [7, 106, 107]. Further investigation revealed that the anti-inflammatory actions were not dependent upon actions at the MOR [106]. While the exact mechanism by which β-FNA inhibits inflammatory signaling has yet to be fully elucidated, *in vitro* studies in astroglial cells suggest that disruption of key inflammatory signaling pathways are involved [7, 8, 106, 107, 111]. More specifically, we demonstrated that β-FNA inhibited activation of both p38 mitogen-activated kinase (p38 MAPK) and nuclear factor-kappa B (NF-κB) in human astroglial cells [7, 8, 107]. Investigations are ongoing to further define the anti-inflammatory mechanism of action for β-FNA. Importantly, we have also

determined that β-FNA inhibits bacterial lipopolysaccharide (LPS)-induced neuroinflammation in adult male C57BL/6J mice [2]. In this previous study, β-FNA was administered i.p. immediately prior to the i.p. injection of LPS, and we limited our assessment of inflammatory signals to cytokines/chemokines in the brain and plasma at 24 h post-LPS [2]. In the present study, we extend and expand this line of investigation to assess the benefit of delayed β-FNA treatment, the temporal effects of treatment, and additional inflammatory signaling molecules in both the brain and spleen. Furthermore, we determined the effects of β-FNA on sickness behaviors. Together, these findings will advance our understanding of the neuroprotective actions and potential therapeutic benefits of β-FNA in treating neuroinflammation-associated conditions, including mood disorders.

Methodology

Animal

Seven-week-old male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained in USDA-approved facilities at Oklahoma State University-Center for Health Sciences (OSU-CHS). Mice were assigned three per plastic cage (dimensions 10 cm × 17 cm × 28 cm). Each cage contained pine chip bedding, environmental stimulus was provided by cardboard tubes, and *ad libitum* access to food and water was provided. Room temperature was maintained at 21° C with a 12:12 light:dark cycle. A total of 48 animals were approved for this study and were acclimated to housing conditions for 7 days prior to initiation of experiments, and they were monitored daily. Animal manipulations and handling processes for all experiments were approved by the OSU-CHS Institutional Animal Care and Use Committee.

Experimental protocol

Mice (n = 5-8 per group) were administered LPS (*Escherichia coli* O55:B5; Sigma) i.p. at a dose of 0.83 mg/kg dissolved in saline [2]. This LPS dose is routinely used in mice to induce neuroinflammation, and behavioral deficits, including anxiety-like and sickness behaviors [101, 109, 110]. After the LPS injection, mice were administered β-FNA (National Institute on Drug Abuse reagent supply program) at a dose of 50 mg/kg, i.p dissolved in saline or saline vehicle (200 µl) immediately or 4 h after LPS. The β-FNA dose was established based on the effectiveness observed in our previous work [2, 103].

Behavioral measures

Behavioral analysis was done using an open-field test (OFT) where locomotor activity was used as an index of acute sickness behavior [101, 109, 110]. For this OFT, each mouse was individually placed in the open-field arena, measuring 40 cm × 40 cm, then monitored and recorded with Ethovision Software for 10 minutes. The dependent measures included distance moved (cm) and duration (sec.) in the center 35 cm × 35 cm region of the arena. Sickness behavior included decreased distance moved and decreased time spent in the center region (away from the walls) [101, 109, 110].

Tissue collection

Immediately after behavioral testing, mice were euthanized by CO_2 inhalation and subsequent decapitation. Trunk blood, brain, and spleen were collected into ice-cold tubes and immediately placed on ice. Plasma was collected after centrifugation (17,000 × g, 15 min., 4 °C). All samples were then stored at −80° C until assays were performed. Tissue was homogenized in ice-cold triple detergent lysis buffer containing HALT Protease/Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) using a Sonic Dismembrator Model 100 (Fisher Scientific) [112]. The sample was then centrifuged (20,000 × g, 20 min, 4° C), followed by the collection of the aqueous phase. Homogenized samples were used for the quantification of inflammatory factors. Total protein

levels were measured using a bicinchoninic acid (BCA) protein assay as previously described for subsequent determination of sample loading volume in western blot analyses and normalization of protein quantification data [113].

Measurement of inflammatory mediators

Standard dual-antibody solid-phase immunoassays were performed according to the manufacturer's instructions (ELISA Development Kit, Peprotech) to quantify chemokine/cytokine [interferon gamma-induced protein 10, IP-10/CXCL10; monocyte chemoattractant protein-1, MCP-1/CCL2; interleukin-6, IL-6] levels in plasma and tissues. Absorbance of the final product was read on a BioTeck Synergy 2 Multimode plate reader at 450 nm using a Gen5 microplate reader and imager software (Agilent, Santa Clara, CA).

Western blot analysis was used to measure NF-κB-p65, p38 MAPK, and glial fibrillary acidic protein (GFAP) levels in tissue homogenates. Beta-tubulin was also measured for normalization. The total protein (100 µg) was analyzed by SDS polyacrylamide gel electrophoresis (PAGE) and transferred to the PVDF membrane. The membrane was incubated in target-specific 1° antibody [p65 (1:1000), Cat# 4764S; p38 (1:1000), Cat# 9212S; GFAP (1:1000), Cat#3670S; β-tubulin (1:1000), Cat# 2146S; Cell Signaling Technology], washed 6 times in Tris-buffered saline with 0.1% Tween (TBST), then incubated in 2° antibody [Goat-anti-Rabbit IgG (1:10,000) IRDye-680, Cat# 925-32211; Donkey-anti-Mouse IgG (1:10,000) IRDye-800, CAT# 926-32212; Li-Cor). To image western blots, Licor-CLX Odyssey was used where direct detection was performed using secondary antibodies labeled with near-infrared fluorescent dyes, and protein signals were analyzed using NIH Image J for relative quantification.

Statistical analysis

Data were analyzed by a two-way ANOVA (treatment × time); select pairwise comparisons were further evaluated using Fisher's LSD. Data are presented as mean ± SEM, with p-values < 0.05

considered statistically significant, and data presentation normalized to control value. Prism™ Version 9 software (GraphPad Inc, San Diego, CA) was used to analyze data and prepare figures.

Results

Effects of β-FNA on LPS-induced sickness behavior

Two-way ANOVA for distance moved (Fig. 7A) indicated significant main effects of treatment ($F_{3, 40} = 22.87$, $p < 0.0001$) and time ($F_{1, 40} = 39.61$, $p < 0.0001$), as well as a significant interaction ($F_{3, 40} = 6.273$, $p < 0.002$) between main effects. Pairwise analyses using Fisher's LSD revealed sickness behavior at the 8 and 24 h time points in the LPS groups as indicated by significantly reduced distance moved compared to the respective control group for each time point. At 8 h, the distance moved for the LPS + β-FNA and LPS + β-FNA 4 h groups were also significantly reduced relative to the control group. At the 24 h time point, sickness behavior was observed in the LPS and LPS + β-FNA 4 h groups as indicated by significantly reduced distance moved compared to the control group. Whereas protection was observed in the LPS + β-FNA group as indicated by the increased distance moved at 24 h.

Fig. 7

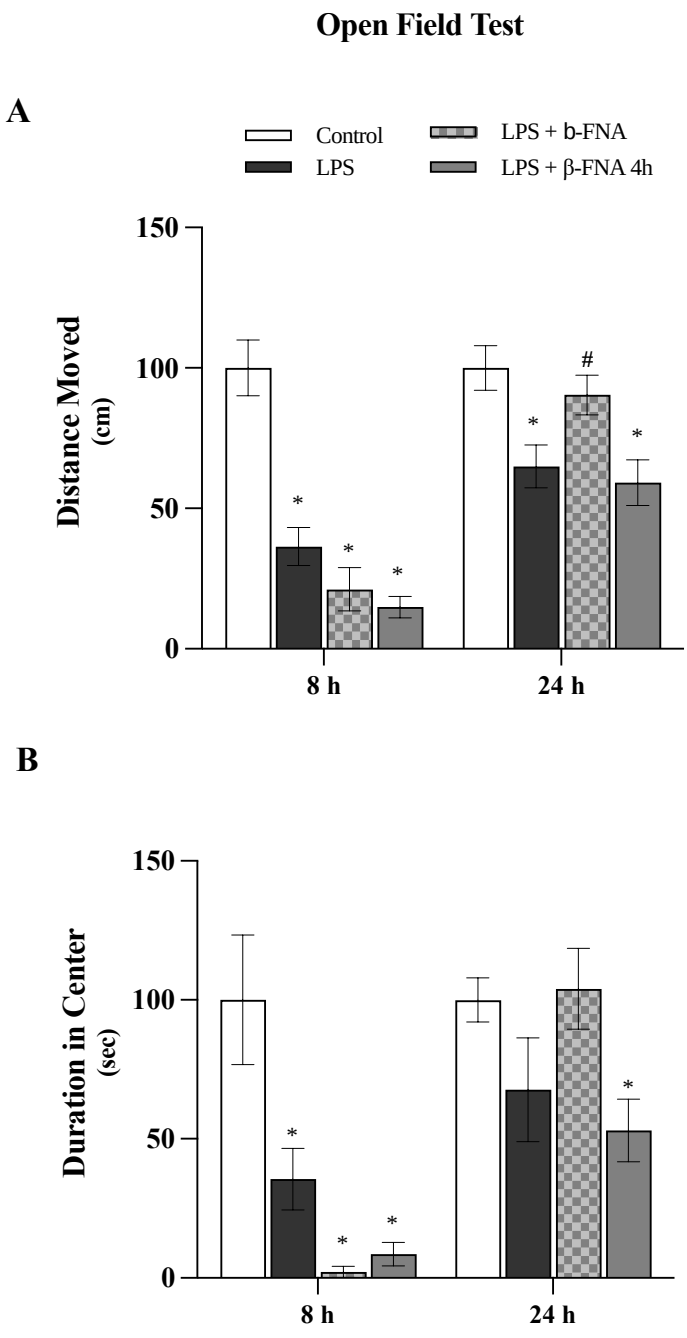

Open Field Test
A
Control
LPS
LPS + b-FNA
LPS + β-FNA 4h
Distance Moved
(cm)
150
100
50
0
8 h
24 h
B
Duration in Center
(sec)
150
100
50
0
8 h
24 h

Fig. 7. Effects of β-FNA on LPS-induced sickness behavior in male C57BL/6J mice. Mice (n = 5-8/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 4 h post-LPS (LPS + β-FNA 4 h). At 8 or 24 h post-LPS, mice were assessed in a 10 minute open-field test (OFT). Endpoints measured include distance moved (A) and duration in the center area of the chamber (B). Data are reported as mean ± SEM. Two-way ANOVA (treatment × time) for distance moved revealed significant effects of both treatment ($p < 0.0001$) and time ($p < 0.0001$); and a significant interaction of treatment and time ($p < 0.002$). Two-way ANOVA for duration in the center area indicated significant effects of both treatment ($p < 0.001$) and time ($p < 0.0001$); and a significant interaction of treatment and time ($p < 0.02$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates significantly different ($p < 0.05$) from the control group at the same time point; # indicates $p < 0.05$ vs. LPS group at the same time point.

Further analysis of OFT data by two-way ANOVA of duration in the center region (Fig. 7B) revealed significant main effects of treatment ($F_{3, 38} = 7.754$, $p < 0.001$) and time ($F_{1, 38} = 17.84$, $p < 0.0001$), as well as a significant interaction ($F_{3, 38} = 3.716$, $p < 0.02$) between main effects. Pairwise comparisons suggested sickness-like behavior 8 h after LPS administration, as indicated by the reduced time spent in the center for the LPS group compared to the control group. Similarly, both β-FNA treatment groups (LPS + β-FNA and LPS + β-FNA 4 h) spent significantly less time in the center region at 8 h post-LPS compared to the control group. At 24 h, the LPS group seemed to spend less time in the center region compared to the control group, yet the difference was not significant ($p = 0.09$). The LPS + β-FNA 4 h group spent significantly less time in the center region compared to the control group.

Effects of β-FNA on LPS-induced cytokine and chemokine expression in brain, spleen, and plasma.

In the brain, CXCL10 and CCL2 expression were significantly affected by treatment ($F_{3, 40} = 50.43$, $p < 0.001$ and $F_{3, 40} = 17.31$, $p < 0.0001$), whereas levels of CXCL10, CCL2, and IL-6 were significantly affected by time ($F_{1, 40} = 126.9$, $p < 0.001$; $F_{1, 40} = 59.03$, $p < 0.0001$; and $F_{1, 40} = 59.28$, $p<0.0001$) (Fig. 8). Furthermore, there were significant interactions between treatment and time for CXCL10, CCL2 and IL-6 in the brain ($F_{3, 40} =14.44$, $p < 0.001$; $F_{3, 40} = 11.74$, $p < 0.0001$; and $F_{3, 40} = 6.392$, $p < 0.002$). Pairwise analyses revealed CXCL10, CCL2, and IL-6 were increased at 8 h in the LPS group compared to the control group. At 8 h, levels of CXCL10, CCL2, and IL-6 in the brain of both the LPS + β-FNA and LPS + β-FNA 4 h groups were significantly higher than in the brain of the control groups. At 24 h, CXCL10 levels in the LPS and LPS + β-FNA 4 h groups were significantly higher than in the control group. The CXCL10 level in the of LPS + β-FNA group was significantly lower compared to the LPS group and approached the level in the control group. CCL2 levels were significantly higher in the LPS group at 24 h compared to controls. β-FNA was protective against CCL2 at 24 h in both the LPS + β-FNA and LPS + β-FNA 4 h group. The levels of IL-6 in the brain were similar among all groups at 24 h.

Fig. 8

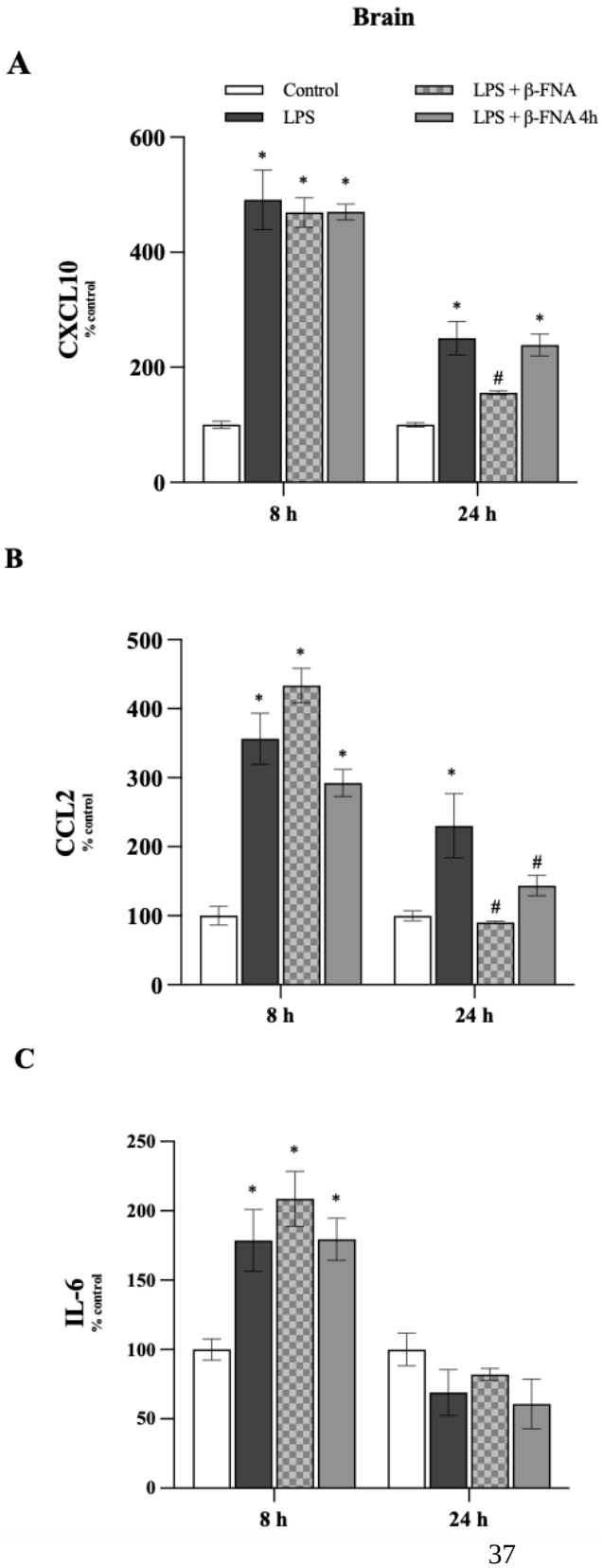

Brain
A
Control
LPS
LPS + β-FNA
LPS + β-FNA 4h
CXCL10
% control
600
400
200
0
8 h
24 h
B
CCL2
% control
500
400
300
200
100
0
8 h
24 h
C
IL-6
% control
250
200
150
100
50
0
8 h
24 h

Fig. 8. Effects of β-FNA on LPS-induced cytokine/chemokine expression in the brain of male C57BL/6J mice. Mice (n = 5-8/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 4 h post-LPS (LPS + β-FNA 4 h). At 24 h post-LPS, mice were terminated followed by collection of brain. Levels of CXCL10 (**A**), CCL2 (**B**), and IL-6 (**C**) in whole brain homogenates were measured by ELISA. Data are reported as mean ± SEM. Two-way ANOVA (treatment × time) revealed a significant effect of treatment on CXCL10 ($p < 0.001$) and CCL2 ($p < 0.0001$); and time significantly affected CXCL10 ($p < 0.001$), CCL2 ($p < 0.0001$), and IL-6 ($p < 0.0001$). There was a significant interaction between treatment and time for all three inflammatory factors ($p < 0.002$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates significantly different ($p < 0.05$) from the control group at the same time point; # indicates $p < 0.05$ vs. LPS group at the same time point.

In the spleen, CXCL10, CCL2, and IL-6 expression were significantly affected by treatment ($F_{3, 40} = 43.20$, $p < 0.0001$; $F_{3, 39} = 32.68$, $p < 0.0001$; $F_{3, 40} = 31.56$, $p < 0.0001$) and time ($F_{1, 40} = 335.2$, $p < 0.0001$; $F_{1, 39} = 132.4$, $p < 0.0001$; $F_{1, 40} = 301.4$, $p < 0.0001$) and there was a significant interaction ($F_{3, 40} = 36.36$, $p < 0.0001$; $F_{3, 39} = 17$, $p < 0.0001$; $F_{3, 40} = 33.11$, $p < 0.0001$) between these main effects (Fig. 9). Pairwise analyses indicated increased CXCL10, CCL2 and IL-6 at 8 h in the LPS group compared to controls. Similarly, CXCL10, CCL2, and IL6 levels in the spleen were significantly elevated in the spleen of both the LPS + β-FNA and LPS + β-FNA 4 h groups compared to controls. At the 24 h time point, only levels of CCL2 were significantly increased in the LPS group compared to the control group. Spleen CCL2 levels in the LPS + β-FNA and LPS + β-FNA 4 h treated mice were significantly decreased compared to the LPS group approached the level and were similar to levels in the control group.

Fig. 9

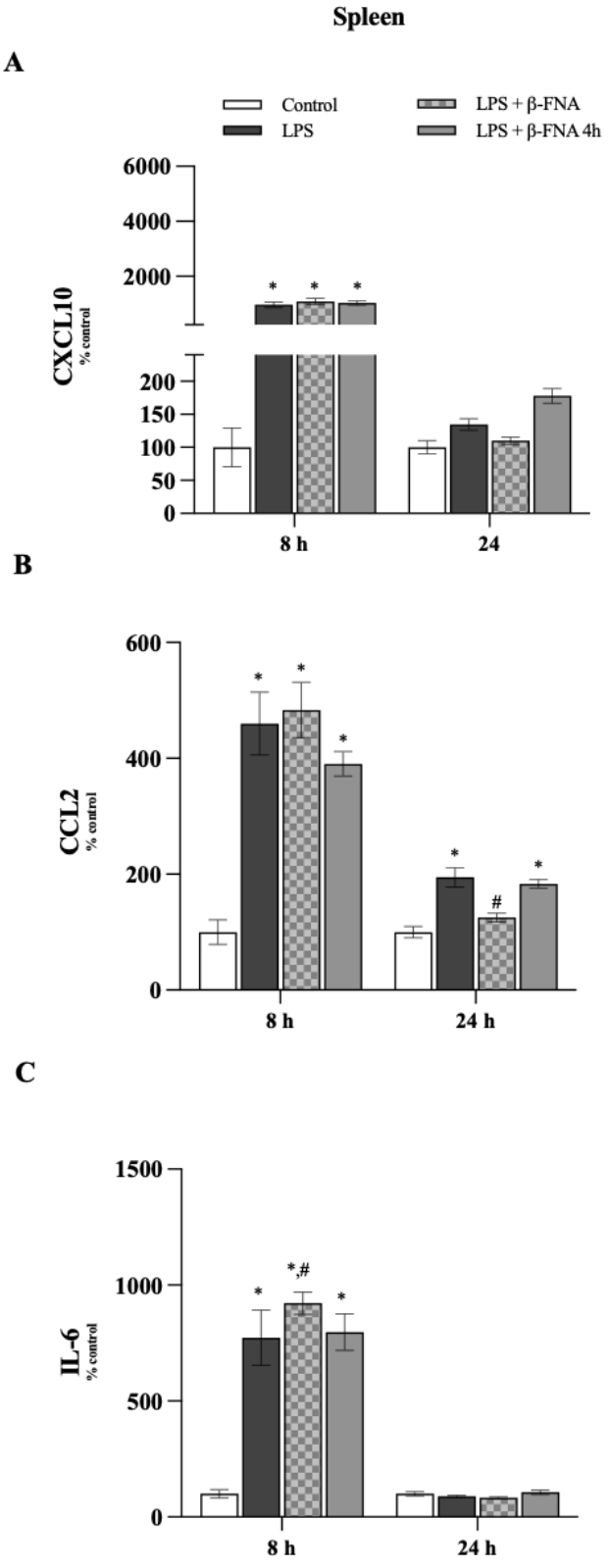

Spleen
A
Control
LPS
LPS + β-FNA
LPS + β-FNA 4h
CXCL10
% control
6000
4000
2000
200
150
100
50
0
8 h
24
B
CCL2
% control
600
400
200
0
8 h
24 h
C
IL-6
% control
1500
1000
500
0
8 h
24 h

Fig. 9. Effects of β-FNA on LPS-induced cytokine/chemokine expression in the spleen of male C57BL/6J mice. Mice (n = 5-8/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 4 h post-LPS (LPS + β-FNA 4 h). At 24 h post-LPS, mice were terminated followed by collection of spleen. Levels of CXCL10 (**A**), CCL2 (**B**), and IL-6 (**C**) in whole spleen homogenates were measured by ELISA. Data are reported as mean ± SEM. Two-way ANOVA (treatment × time) revealed a significant effect of both treatment and time on CXCL10, CCL2, and IL-6 ($p < 0.0001$ in all instances). There was a significant interaction between treatment and time for all three inflammatory factors ($p < 0.0001$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates significantly different ($p < 0.05$) from the control group at the same time point; # indicates $p < 0.05$ vs. LPS group at the same time point.

The levels of CXCL10 and CCL2 in the plasma were significantly affected by both treatment ($F_{3, 40} = 13.91$, $p < 0.0001$; $F_{3, 38} = 4.964$, $p < 0.01$) and time ($F_{1, 40} = 10.27$, $p < 0.003$; $F_{1, 38} = 25.85$ $p < 0.001$) and there were significant interactions ($F_{3, 40} = 7.020$, $p < 0.001$; $F_{3, 38} = 4.985$, $p < 0.001$) between these main effects (Fig. 10). Pairwise analyses of 8 h data revealed that compared to controls, CXCL10 levels in the plasma were only significantly elevated in the LPS + β-FNA group. Plasma CCL2 levels at 8 h were significantly increased in the LPS and LPS + β-FNA groups compared to the control group; while seemingly increased, CCL2 levels in the LPS + β-FNA 4 h mice were not statistically different from control levels ($p = 0.0519$). Plasma CXCL10 levels were significantly increased at 24 h in both the LPS and LPS + β-FNA 4 h groups compared to controls, whereas CXCL10 levels in the LPS + β-FNA group were statistically different from the LPS group. Plasma CCL2 levels at 24 h were not significantly different among the treatment groups. Due to limited sample volume, IL-6 levels were not assessed in the plasma.

Fig. 10

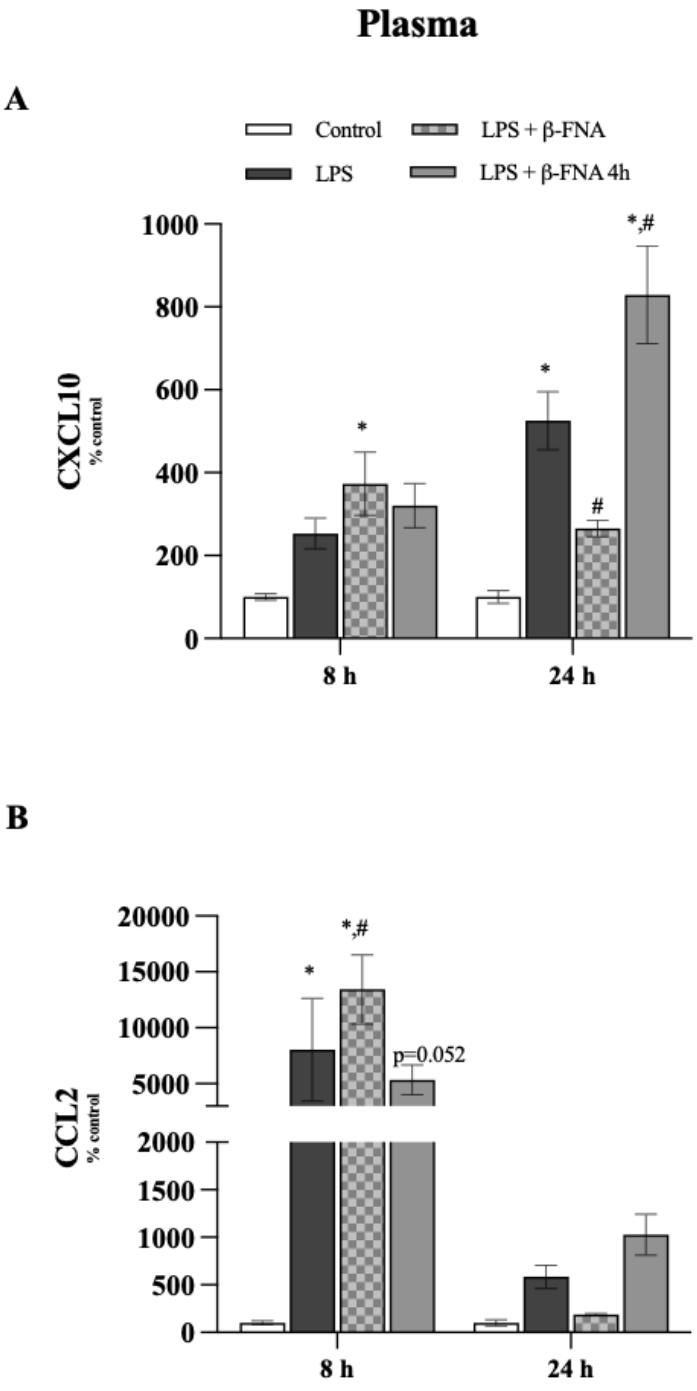

Plasma
A
Control
LPS
LPS + β-FNA
LPS + β-FNA 4h
CXCL10
% control
1000
800
600
400
200
0
*
*
#
*,#
8 h
24 h
B
CCL2
% control
20000
15000
10000
5000
2000
1500
1000
500
0
*
*,#
p=0.052
8 h
24 h

Fig. 10. Effects of β-FNA on LPS-induced chemokine expression in the plasma of male C57BL/6J mice. Mice (n = 5-8/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 4 h post-LPS (LPS + β-FNA 4 h). At 24 h post-LPS, mice were terminated followed by collection of plasma. Levels of CXCL10 (**A**), and CCL2 (**B**) in the plasma were measured by ELISA. Data are reported as mean ± SEM. Two-way ANOVA (treatment × time) revealed a significant effect of treatment on CXCL10 ($p < 0.0001$) and CCL2 ($p < 0.01$) as well as a significant effect of time on these factors ($p < 0.003$). There was a significant interaction between treatment and time for both CXCL10 and CCL2 ($p < 0.001$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates significantly different ($p < 0.05$) from the control group at the same time point; # indicates $p < 0.05$ vs. LPS group at the same time point.

Effects of β-FNA on LPS-induced NF-κB-p65, p38 MAPK, and GFAP expression in brain and spleen.

The levels of NF-κB-p65 and p38 MAPK in the brain were significantly affected by treatment ($F_{3, 40} = 4.970$, $p < 0.01$) ($F_{3, 40} = 3.115$, $p < 0.05$) (Fig. 11). The expression of p38 MAPK in the brain was also significantly impacted by time ($F_{1, 40} = 4.124$, $p < 0.05$). There were no significant interactions between treatment and time on expression of these signaling factors in the brain. Pairwise analyses determined that NF-κB-p65 levels in the brain were significantly increased in the LPS group at 8 and 24 h relative to controls. At both time points, brain NF-κB-p65 levels in the LPS + β-FNA and LPS + β-FNA 4 h groups were similar to control with LPS + β-FNA 4 h being significant different from the LPS group. The levels of p38 MAPK in the brain were increased significantly at 8 h in the LPS group compared to controls, whereas expression in the LPS + β-FNA 4 h group was not significantly different from the control group. At 24 h, p38 MAPK expression in the brain was similar among all four groups. The expression of GFAP in the brain was not

significantly affected by either treatment ($F_{3, 40}$ = 1.177, p = 0.3305) or time ($F_{1, 40}$ = 1.941, p = 0.1713) (Fig. 12).

Fig. 11

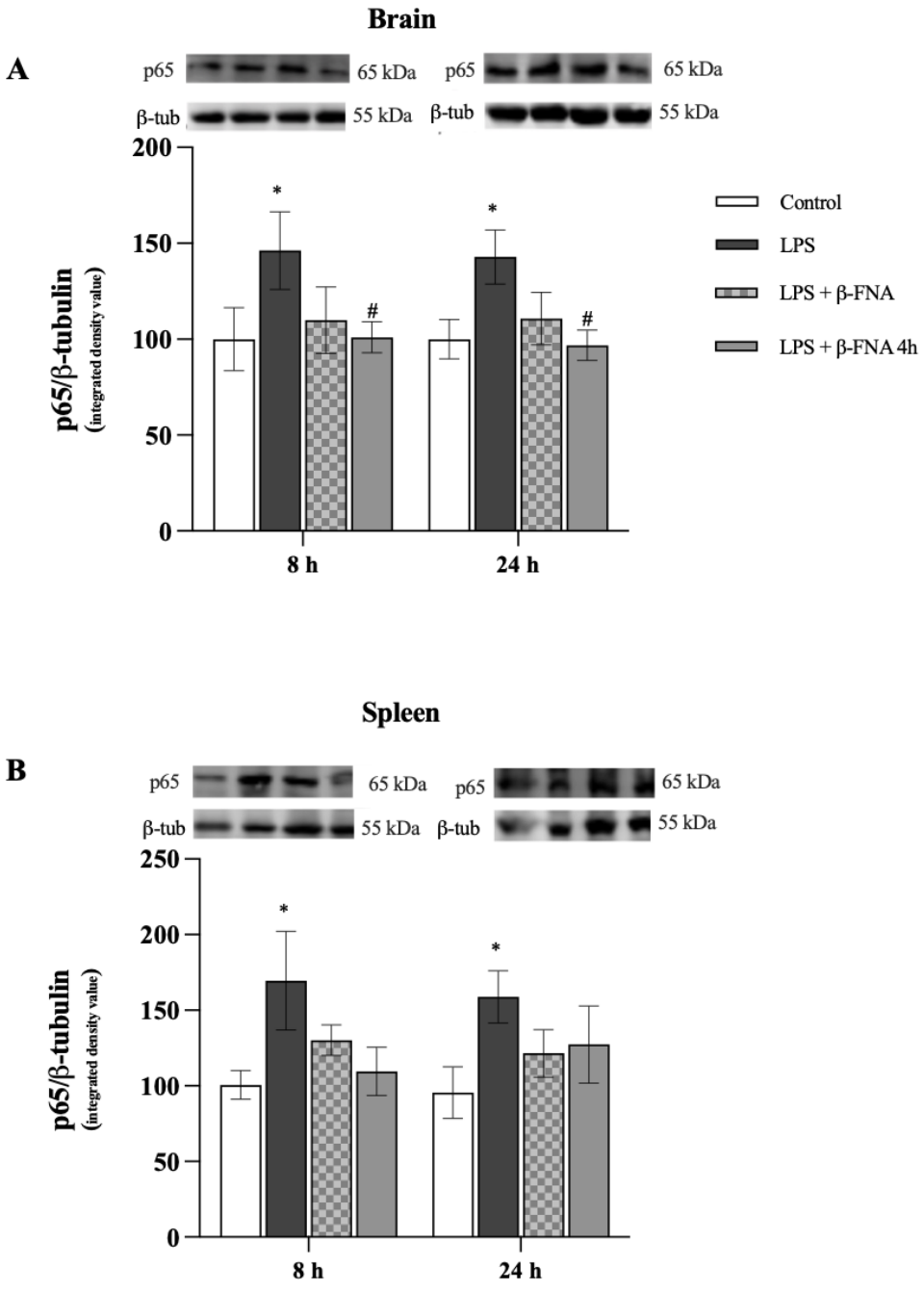


Fig. 11. Effects of β-FNA on LPS-induced NF-κB-p65 expression in the brain and spleen of male C57BL/6J mice. Mice (n = 5-8/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 4 h post-LPS (LPS + β-FNA 4 h). At 24 h post-LPS, mice were terminated followed by collection of brain and spleen. Levels of NF-κB-p65 and beta-tubulin in the brain (**A**) and spleen (**B**) were determined by western blot analysis. NF-κB-p65 levels are expressed relative to beta-tubulin levels and are reported as mean ± SEM. Two-way ANOVA (treatment × time) revealed a significant ($p < 0.01$) effect of treatment on NF-κB-p65 in the brain; but no effect of time; and no significant interaction of treatment and time. Pairwise comparisons were assessed using a Fisher's LSD test; * indicates significantly different ($p < 0.05$) from the control group at the same time point; # indicates $p < 0.05$ vs. LPS group at the same time point.

Fig. 12

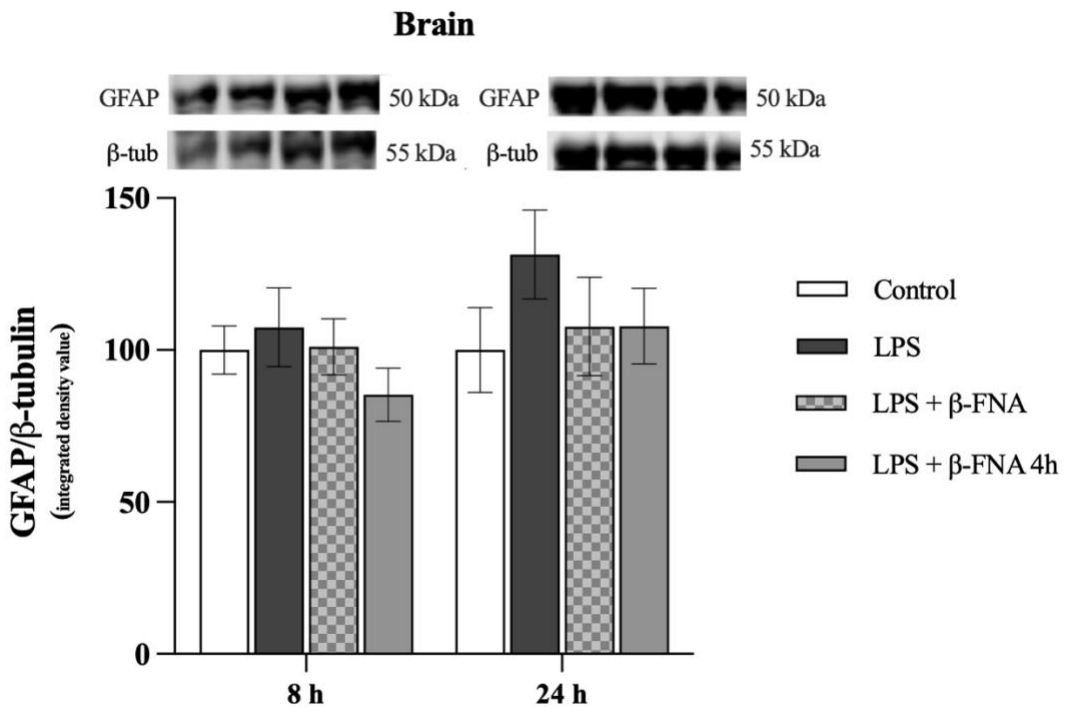


Fig. 12. Effects of β-FNA on LPS-induced GFAP expression in the brain of male C57BL/6J mice. Mice (n = 5-8/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 4 h post-LPS (LPS + β-FNA 4 h). At 24 h post-LPS, mice were terminated followed by collection of brain. Levels of GFAP and beta-tubulin in the brain were determined by western blot analysis. Levels of GFAP are expressed relative to beta-tubulin levels and are reported as mean ± SEM. Two-way ANOVA (treatment × time) did not reveal any significant main effects or interactions.

The levels of NF-κB-p65 and p38 MAPK in the spleen were significantly affected by treatment ($F_{3, 40} = 3.626$, $p < 0.05$; $F_{3, 40} = 6.525$, $p < 0.01$), but not by time ($F_{1, 40} = 0.01294$, $p = 0.91$; $F_{1, 40} = 0.5655$, $p = 0.4565$) (Fig. 13). Mice administered LPS had significantly greater levels of NF-κB-p65 in the spleen at 8 and 24 h compared to control mice. The levels of NF-κB-p65 in both the LPS + β-FNA and LPS + β-FNA 4 h groups were similar to controls at 8 and 24 h post-LPS. At 8 h, p38 MAPK levels in the LPS group were significantly elevated compared to the control mice, whereas the levels of p38 MAPK in the LPS + β-FNA and LPS + β-FNA 4 h groups were significantly different from levels in the LPS group. At 24 h, p38 MAPK expression in the spleen was similar among all four groups.

Fig. 13

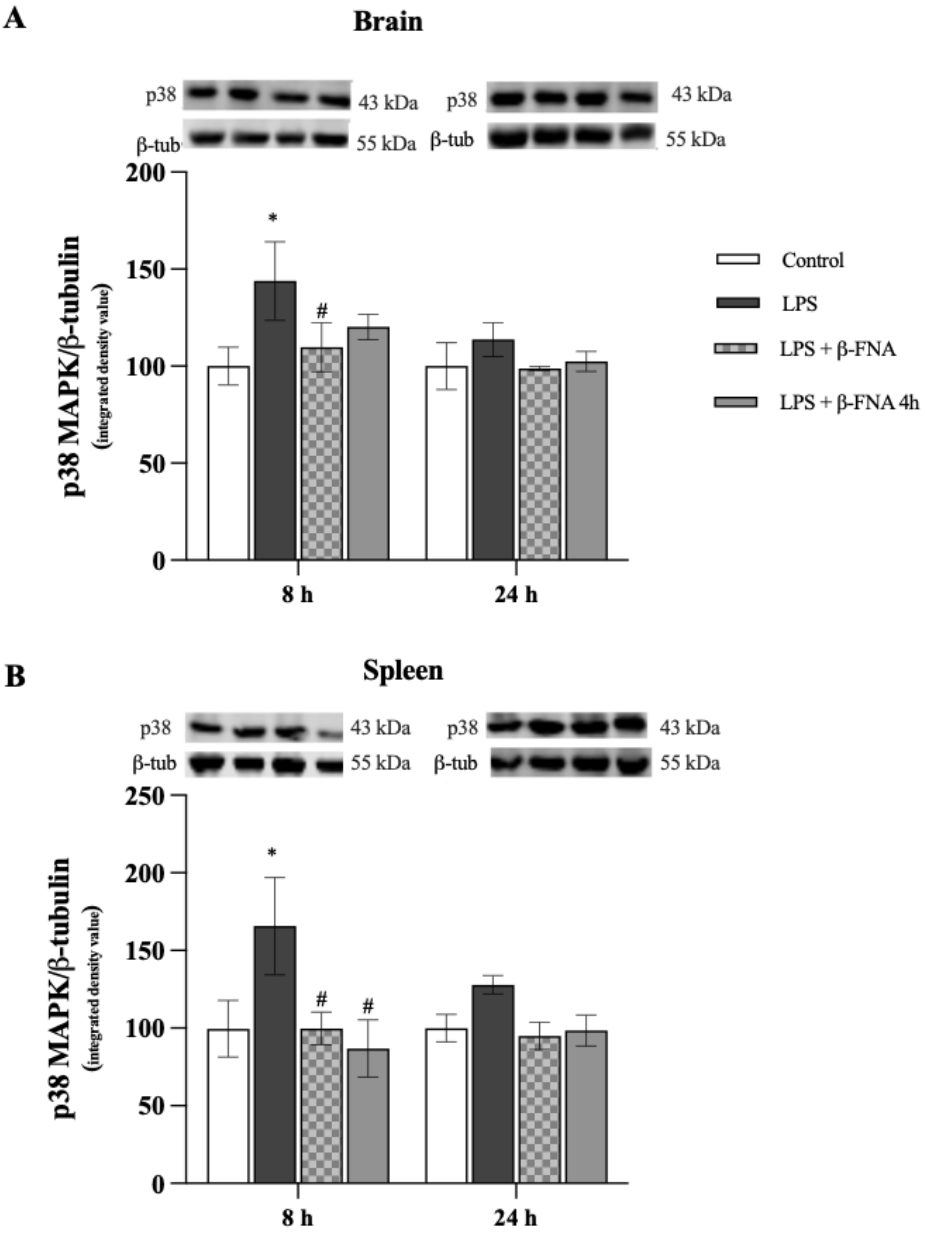

A
Brain
p38
43 kDa
β-tub
55 kDa
p38 MAPK/β-tubulin
(integrated density value)
200
150
100
50
0
*
#
8 h
24 h
Control
LPS
LPS + β-FNA
LPS + β-FNA 4h
B
Spleen
250

Fig. 13. Effects of β-FNA on LPS-induced p38 MAPK expression in the brain and spleen of male C57BL/6J mice. Mice (n = 5-8/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 4 h post-LPS (LPS + β-FNA 4 h). At 24 h post-LPS, mice were terminated followed by collection of brain and spleen. Levels of p38 MAPK and beta-tubulin in the brain (**A**) and spleen (**B**) were determined by western blot analysis. Levels of p38 MAPK are expressed relative to beta-tubulin levels and are reported as mean ± SEM. Two-way ANOVA (treatment × time) revealed a significant effect of both treatment ($p < 0.05$) and time ($p < 0.05$) on p38 MAPK in the brain; but no significant interaction of these main effects. Pairwise comparisons were assessed using a Fisher's LSD test; * indicates significantly different ($p < 0.05$) from the control group at the same time point; # indicates $p < 0.05$ vs. LPS group at the same time point.

Discussion

Neurological conditions, including central nervous system infections, neurodegenerative diseases, and psychiatric disorders involve neuroinflammation [13-15, 100, 114-117]. Evidence of neuroinflammation in these conditions includes elevated expression of pro-inflammatory cytokines and chemokines [1, 15, 98]. Peripheral (i.p.) LPS administration in mice is an established preclinical model of neuroinflammation in which pro-inflammatory cytokines/chemokines are elevated in the brain. For instance, we demonstrated that CXCL10 and CCL2 levels in the brain correlated with LPS-induced behavioral deficits [2]. We and others have used this model to investigate the therapeutic potential of pharmacologic agents [2, 8, 98, 99, 118]. Indeed, we previously determined that LPS-induced CXCL10 and CCL2 expression in the brain of C57BL/6J mice was prevented by pretreatment with β-FNA [2]. We now expand on these findings and show that β-FNA administration is also protective when administered after LPS injection.

Reduced locomotor activity and wall-hugging in the OFT are both reliable indicators of sickness-like behavior, and as expected, LPS-induced sickness-like behavior in both measures was more

pronounced at 8 h than at 24 h post-LPS. Sickness behavior following a single LPS injection (i.p.) at 0.83 mg/kg often resolves by 24 h, however, in this study, decreased locomotor activity indicated that some level of sickness-like behavior remained at 24 h. Treatment with β-FNA immediately after the LPS injection prevented the sickness- behavior at 24 h, whereas a 4 h delay in β-FNA treatment was not protective. The sickness behavior observed at 8 h was not prevented by β-FNA treatment regardless of the timing of administration, suggesting this dose of β-FNA is insufficient to prevent this more severe LPS-induced sickness. It will be important in future studies to assess other behavior deficits to more fully appreciate the protective effects of β-FNA.

IL-6, CXCL10, and CCL2 are among the pro-inflammatory cytokines/chemokines that are increased in the CNS following brain injury, infection and in mood/behavior disorders and are integral to neuropathogenesis [15, 99, 119-122]. Therefore, we are particularly interested in the effects of β-FNA on the expression of these cytokines/chemokines in the brain in this preclinical model of LPS-induced neuroinflammation. LPS-induced expression of IL-6, CXCL10, and CCL2 in the brain was more pronounced at 8 h compared to 24 h post-injection, which is consistent with the relative severity of sickness behavior observed at these two time points. β-FNA failed to inhibit LPS-induced cytokine/chemokine expression in the brain at this early time point, which is consistent with the lack of an effect on sickness behavior at 8 h. The anti-inflammatory effects of β-FNA were most pronounced when administered immediately following the LPS injection, as opposed to delaying treatment by 4 h. Although, β-FNA treatment 4 h post-LPS was also sufficient to inhibit LPS-induced CCL2 expression in the brain. It remains unclear why CCL2, but not CXCL10, is sensitive to delayed β-FNA, but it may be related to differences in transcription/translational control of these two chemokines, yet further investigation is needed. It is also interesting to note that IL-6 is not elevated at 24 h and likely plays a minimal role in the sickness-like behavior.

Thus far, these findings suggest that β-FNA is likely inhibiting a relatively early event in the LPS-induced inflammatory pathway. Indeed, β-FNA inhibited LPS-induced NF-κB-p65 expression in the brain and spleen early on at 8 h and at 24 h post-LPS. Inhibition of p38 MAPK expression in both the brain and spleen by β-FNA at 8 h, but not 24 h post-LPS, provides additional evidence that inflammatory signaling is being disrupted.

The differential effects of β-FNA among tissues and dosing regimen are interesting. As in our previous report, β-FNA effects on LPS-induced chemokine expression are generally more pronounced in the brain than in the periphery. However, in our previous study, peripheral tissue was limited to plasma, and in the current study, we also assessed levels in the spleen [2]. In the spleen, only LPS-induced CCL2 expression (not CXCL10 or IL-6) was sensitive to the inhibitory effects of β-FNA and only when treatment occurred immediately after LPS administration. Also, in the present study, LPS-induced plasma CXCL10 levels, but not CCL2 levels, were decreased by immediate β-FNA (and not by β-FNA at 4 h post LPS). Altogether, it appears that treatment with β-FNA immediately after LPS administration is more effective than treatment prior to LPS or delayed several hours. However, one limitation to this conclusion is the fact that we did not specifically test β-FNA pre-treatment versus post-LPS treatment in the same study. It will be interesting in future studies to investigate additional β-FNA doses and treatment regimens. It will also be critical to determine the effects of β-FNA on neuroimmune signaling in specific brain regions (i.e., hippocampus and prefrontal cortex).

We expected that LPS would activate astroglia, as indicated by increased GFAP expression [123-125]. However, while there was an apparent increase in GFAP expression 24 h following LPS administration (but not in β-FNA-treated mice), the increase did not reach the level of statistical significance ($p = 0.08$). One explanation for the modest LPS-induced increase in GFAP expression is that whole brain tissue was assessed rather than the hippocampus or cortex, as reported by others

[124, 125]. It will be crucial in future studies to determine the extent to which β-FNA differentially affects LPS-induced astrocyte activation across select brain regions.

This study was not designed to specifically assess the mechanism of action for β-FNA-mediated inhibition of inflammatory signaling. However, we have substantial information from *in vitro* studies in human astrocytes that suggest β-FNA inhibits NF-κB-p65 and p38 MAPK signaling pathways resulting in decreased chemokine expression [7, 8, 107]. β-FNA seems to be impacting the early signaling events at 8 h, whereas the effects on secreted factors (i.e., cytokines/chemokines) were observed at a later time point when expression typically peaks. Importantly, investigations are ongoing to fully elucidate the molecular mechanism of action for the anti-inflammatory effects.

Conclusion

In summary, we have advanced this line of investigation by further demonstrating the anti-inflammatory and neuroprotective effects of β-FNA in a preclinical model of LPS-induced neuroinflammation. β-FNA treatment reduces LPS-induced sickness behavior and inflammatory signaling. The beneficial effects of β-FNA are greatest when treatment occurs immediately after LPS administration compared to treatment 4 h post-LPS, and the effects seem to be more pronounced in the brain compared to the spleen and plasma. These findings are expected to advance therapeutic strategies for brain disorders involving neuroinflammation.

CHAPTER V

ANTI-INFLAMMATORY EFFECTS OF β-FUNALTREXAMINE ARE SEX-DEPENDENT IN A PRECLINICAL MODEL OF LPS-INDUCED INFLAMMATION

This work was previously published in Journal of Inflammation with the following citation:

Myers, S., McCracken, K., Buck, D. J., Curtis, J. T., & Davis, R. L. (2023). Anti-inflammatory effects of β-FNA are sex-dependent in a pre-clinical model of LPS-induced inflammation. *Journal of Inflammation, 20*(1), 1-25.

Abstract

Inflammation is present in neurological and peripheral disorders. Thus, targeting inflammation has emerged as a viable option for treating these disorders. Previous work indicated pretreatment with beta-funaltrexamine (β-FNA), a selective *mu*-opioid receptor (MOR) antagonist, inhibited inflammatory signaling *in vitro* in human astroglial cells, as well as lipopolysaccharide (LPS)-induced neuroinflammation and sickness-like behavior in mice. This study explores the protective effects of β-FNA when treatment occurs 10 h after LPS administration and is the first-ever investigation of the sex-dependent effects of β-FNA on LPS-induced inflammation and behavioral deficits. Male and female C57BL/6J mice were administered LPS i.p., followed by treatment with β-FNA i.p. immediately or 10 h post-LPS. Sickness- and anxiety-like behavior were assessed using an open-field test and an elevated-plus maze test, followed by the collection of whole brain, hippocampus, prefrontal cortex, cerebellum/brain stem, plasma, spleen, liver, large intestine (colon), proximal small intestine, and distal small intestine. Levels of inflammatory

chemokines/cytokines (interferon γ-induced-protein, IP-10 (CXCL10); monocyte-chemotactic-protein 1, MCP-1 (CCL2); interleukin-6, IL-6; interleukin-1β, IL-1β; and tumor necrosis factor-alpha, TNF-α) in tissues were measured using an enzyme-linked immunosorbent assay. Western blot analysis was used to assess nuclear factor-kappa B (NF-κB) and phosphorylated/total transforming growth factor beta-activated kinase 1 (TAK1) expression. β-FNA inhibited LPS-induced inflammation in a sex-dependent manner, and there were differential effects across brain regions, peripheral tissues, and dose timing. Overall, CXCL10, CCL2, TNF-α, and NF-κB were most effectively downregulated by β-FNA. β-FNA reduced LPS-induced anxiety-like behavior most effectively in female mice. These findings provide novel insights into the sex-dependent anti-inflammatory effects of β-FNA and advance this agent as a potential therapeutic option for reducing neuroinflammation as well as intestinal inflammation.

Introduction

Neurological disorders, such as anxiety, stress, and other mood disorders, are often in combination with some level of inflammation [1, 98-100, 109, 126]. Inflammation also occurs in the spleen, liver, and/or intestines during certain infections, cirrhosis, inflammatory bowel diseases (IBD) such as Crohn's disease (CD), and ulcerative colitis (UC). [3-6]. Therefore, there is strong interest in identifying anti-inflammatory agents as therapeutic options for such disorders. We are interested in the potential benefits of β-funaltrexamine (β-FNA), a selective *mu*-opioid receptor (MOR) antagonist. Interestingly, we previously discovered that β-FNA inhibits inflammatory signaling *in vitro* in human astroglial cells [2, 7, 106]. More specifically, β-FNA inhibited cytokine-induced expression of cytokines/chemokines and activation of both nuclear factor-kappa B (NF-κB) and p38 mitogen-activated kinase (p38 MAPK) in human astroglial cells [7, 8, 106, 107, 111]. Importantly, these anti-inflammatory actions were not dependent on the actions at the MOR [106].

An *in vivo* approach with adult male C57BL/6J mice was used to determine β-FNA's ability to inhibit bacterial lipopolysaccharide (LPS)-induced neuroinflammation [2]. We determined that β-FNA administered (i.p.) immediately before LPS injection resulted in reduced neuroinflammation as indicated by suppressed cytokine/chemokine expression in the brain 24 h after treatment [2]. The present study advances this line of investigation by exploring the impact of delayed β-FNA treatment (10 h post LPS injection) on neuroinflammation and sickness and anxiety-like behavior. We also assessed brain region-specific anti-inflammatory effects of β-FNA and the impact on inflammation in peripheral tissues. Also, for the first time, we investigated the extent to which the anti-inflammatory effects of β-FNA are sex-dependent; this is particularly important as the prevalence of anxiety, and depression is higher in females than in males [127-129]. In combination, the findings from this study provide novel insights into the anti-inflammatory actions of β-FNA and foster potential therapeutic options for treating not only neurological disorders but also other disorders involving inflammation.

Methodology

Animals

USDA-approved facilities at Oklahoma State University-Center for Health Sciences (OSU-CHS) were used to house ninety-six seven-week-old male (n = 48) and female (n = 48) C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME). Each cage (10 cm × 17 cm × 28 cm) housed three mice and was equipped with pine chip bedding, *ad libitum* access to food and water, and cardboard tubes for environmental stimulus. The room was placed on a 12:12 light: dark cycle with a steady ambient temperature of 21°C. Before the initiation of experiments, the mice were afforded a 7-day acclimation period. OSU-CHS Institutional Animal Care and Use Committee approved all experimental processes and animal manipulations, and all mice were monitored daily.

Experimental protocol

Mice (n = 11-12 per group) were injected with an intraperitoneal (i.p.) dose of LPS (0.83 mg/kg dissolved in saline; *Escherichia coli* O55:B5; Sigma) as previously described (Davis et al., 2017). This dose of LPS was previously documented to effectively induce behavioral deficits, such as sickness behaviors and anxiety-like behavior, and neuroinflammation in mice [2, 101, 109, 110]. β-FNA (50 mg/kg, dissolved in saline, i.p.; National Institute on Drug Abuse reagent supply program) was administered immediately or 10 h post-LPS, and control mice received vehicle (200 µl saline). The β-FNA dose used in this study was derived from our previous work [2, 103]

Behavioral measures

Sickness and anxiety-like behavior were assessed 24 h post-LPS administration using the open-field test (OFT) and elevated plus maze (EPM), respectively, as previously described [2, 101, 109, 110]. For the OFT, the mouse was placed in the center of the arena (40 cm × 40 cm) and then monitored (and recorded) for 10 min using Ethovision Software. Distance moved (cm) was used as a dependent measure, where reduced distance moved (compared to controls) was indicative of sickness behavior [101, 109, 110]. The EPM was composed of two closed arms (25 cm × 5 cm × 16 cm) and two open arms (25 cm × 5 cm × 0.5 cm), and a center area (5 cm × 5 cm × 0.5 cm) [130]. Each mouse was placed in the center of the maze, then monitored and recorded for 5 min using Ethovision Software. Reduced time (sec.) spent in the open arms (compared to control mice) was interpreted as anxiety-like behavior [130].

Tissue and total protein levels collection

Immediately following behavioral analyses, mice were euthanized by CO_2 inhalation and subsequent decapitation. The following tissues were collected: trunk blood, brain, spleen, liver,

colon, and small intestines. All tissue samples were collected into ice-cold tubes and maintained on ice. Plasma was collected immediately by centrifugation (17,000 × g, 15 min., 4 °C). Plasma and tissues were then stored at −80 °C. In select experiments, the hippocampus, prefrontal cortex, and cerebellum/brain stem were dissected from the whole brain and stored at −80 °C. Tissue preparation for biochemical assays was done as previously described [113]. Briefly, tissues were homogenized with a Sonic Dismembrator Model 100 (Fisher Scientific) in ice-cold triple detergent lysis buffer containing HALT Protease/Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) [112]. The aqueous phase was collected after centrifugation (20,000 × g, 20 min, 4 °C). Total protein levels were determined using the bicinchoninic acid (BCA) protein assays as previously described [113].

Cytokine/chemokine levels

IL-1β, IL-6, TNF-α, CXCL10, and CCL2 in plasma and tissue homogenates were quantified using dual-antibody solid-phase immunoassays (ELISA Development Kit, Peprotech) according to the manufacturer's instructions. A BioTek Synergy 2 Multimode plate reader was used to read the absorbance at 450 nm in conjunction with a Gen5 microplate reader and imager software for data analysis.

NF-κB-p65 and TAK-1 levels

The expression of NF-κB-p65, p-TAK-1, and TAK-1 in tissue homogenates was determined by western blot analysis. Protein (100 µg) was separated by 7.5% SDS polyacrylamide gel electrophoresis and transferred to a PVDF membrane as previously described [8, 131]. The membrane was incubated overnight at 4°C in an NF-κB-p65 1° antibody (1:1000; Cat# 4764S, Cell Signaling Technology), p-TAK-1 1° antibody (1:500; Cat# 9339, Cell Signaling Technology), or TAK-1 1° antibody (1:1000; Cat# 4505, Cell Signaling Technology). The membrane was then washed six times in Tris-buffered saline with 0.1% Tween (TBST); then

incubated in 2° antibody [Goat-anti-Rabbit IgG (1:10,000) IRDye-680, Cat# 926-32212; Li-Cor) for 2 h at room temperature. Membranes were washed, then images were obtained using a Licor-CLX Odyssey, where direct detection was performed using secondary antibodies labeled with near-infrared fluorescent dyes. For normalization, membranes were stripped of antibody with stripping buffer (Cat# 21059; Thermo Fisher Scientific), then re-probed with β-tubulin (1:1000, Cat# 2146S; Cell Signaling Technology), labeled with 2° antibody, and imaged as above. NIH Image J was used for the relative quantification of protein signals.

Statistical analysis

A two-way ANOVA (treatment × sex) and a one-way ANOVA were used to analyze data, and Fisher's LSD was used for pairwise comparisons. Linear region analysis and a multiple linear regression was used to determine correlations between tissues and behavioral deficits. Data are presented as mean ± SEM, and p-values < 0.05 are considered statistically significant, and data presentation normalized to control value. Prism™ Version 9 software (GraphPad Inc, San Diego, CA) was used for data analysis and figure preparation.

Results

β-FNA effects on LPS-induced sickness-like behavior

Two-way ANOVA of OFT data indicated a significant main effect of treatment ($F_{3, 84} = 8.64$, $p < 0.0001$) but no main effect of sex ($F_{1, 84} = 0.968$, $p = 0.33$) or interaction ($F_{3, 84} = 2.49$, $p = 0.07$) (Fig. 14A). Pairwise comparisons revealed a significant reduction in distance moved by LPS-treated female mice compared to saline-treated female mice ($p < 0.002$). The distance moved by female LPS + β-FNA and LPS + β-FNA 10 h mice was less than that by saline female mice ($p < 0.0001$), but similar to female LPS mice ($p > 0.05$). For male mice, the distance moved by LPS

mice was seemingly lower than that of saline male mice, but not to the level of significance ($p = 0.078$). Regardless of the timing, β-FNA treatment did not significantly affect the distance moved by LPS-treated male mice ($p > 0.05$). Our conclusion from this experiment was that LPS-induced sickness-like behavior was more pronounced in females than in males, and β-FNA did not significantly impact LPS-induced sickness-like behavior.

Fig. 14

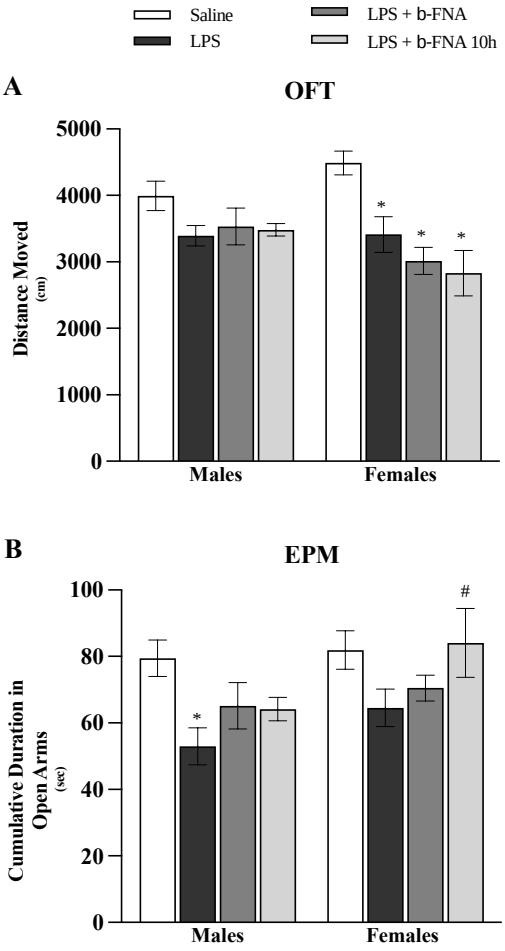


Fig. 14 β-FNAs effects on LPS-induced behavioral effects in male and female C57BL/6J mice. Mice (n = 5-6/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), and LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted via (**A**) 10 minute open-field test (OFT) and (**B**) 5

minute elevated plus maze (EPM). Data are reported as mean ± SEM. Two-way ANOVA indicated a significant main effect of treatment ($p < 0.0001$) on distance moved in the OFT, but no significant effect of sex ($p = 0.33$) and no significant interaction of treatment and sex ($p = 0.07$). Two-way ANOVA revealed significant main effects of sex ($p < 0.03$) and treatment ($p < 0.01$) on time in the open arms in the EPM, but no significant interaction ($p = 0.52$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group.

β-FNA effects on LPS-induced anxiety-like behavior

Two-way ANOVA of EPM data indicated significant main effects of both treatment ($F_{3, 83} = 4.53$, $p < 0.01$) and sex ($F_{1, 83} = 4.99$, $p < 0.03$), but no significant interaction ($F_{3, 83} = 0.761$, $p = 0.52$) (Fig. 14B). Pairwise comparisons revealed that male LPS mice spent less time in the open arms than did the male saline mice ($p < 0.004$). Whereas male LPS + β-FNA and LPS + β-FNA 10 h mice spent a similar duration in the open arms as did male saline mice ($p \geq 0.09$). For females, LPS mice spent less time in the open arms compared to saline females; however, not to the level of significance ($p = 0.051$). The LPS + β-FNA female mice spent similar time in the open arms compared to LPS females, whereas the LPS + β-FNA 10 h females spent more time in the open arms than the LPS females ($p < 0.03$). This experiment suggests that LPS induces anxiety-like behavior in male and female mice, and β-FNA is protective, particularly in LPS + β-FNA 10 h females.

Effects of β-FNA on LPS-induced cytokine and chemokine expression in the brain

As determined by two-way ANOVA, there was a significant main effect of treatment on CXCL10 levels in the whole brain ($F_{3, 39} = 17.51$, $p < 0.0001$), but no significant effect of sex ($F_{1, 39} = 1.25$, $p = 0.27$) and no interaction ($F_{3, 39} = 0.227$, $p = 0.88$) (Fig. 15A). Pairwise comparisons showed a significant increase in CXCL10 levels in male and female LPS mice compared to same-sex saline mice ($p < 0.0001$ and $p < 0.001$, respectively). For both males and females, CXCL10 levels in LPS

+ β-FNA and LPS + β-FNA 10 h mice were significantly higher compared to same-sex saline mice ($p < 0.01$ in all instances).

Fig. 15

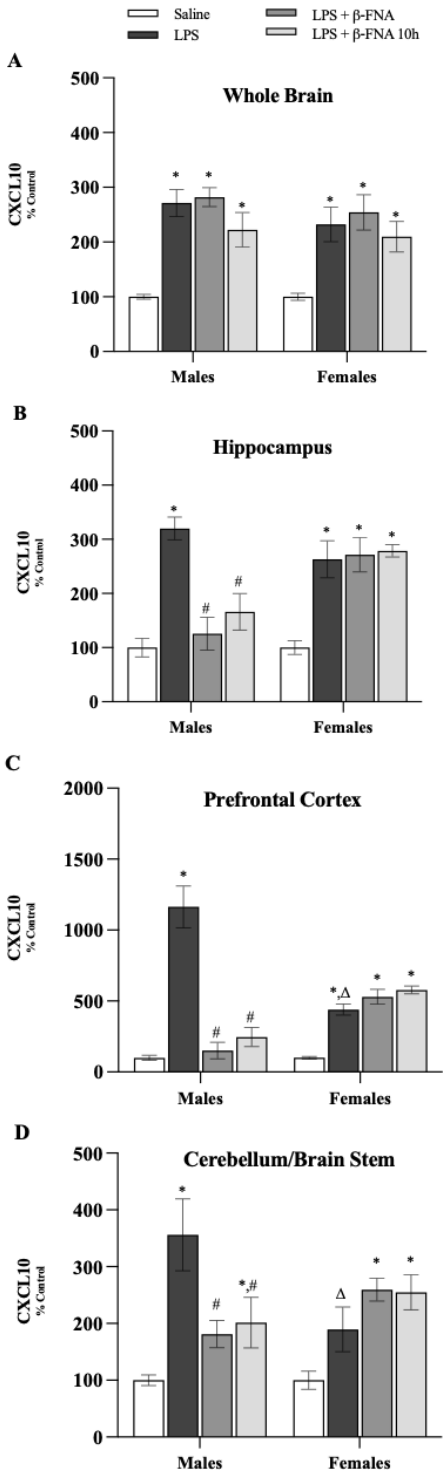


Fig. 15 β-FNAs effect on LPS-induced CXCL10 expression in the whole brain and brain regions (hippocampus, prefrontal cortex, and cerebellum/brain stem) of male and female C57BL/6J mice. Mice (n = 5-6/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, mice were terminated followed by tissue collection. Levels of CXCL10 in whole brain (**A**), hippocampus (**B**), prefrontal cortex (**C**), and cerebellum/brain stem (**D**) were measured by ELISA. Data are reported as mean ± SEM. Two-way ANOVA indicated a significant main effect of treatment ($p < 0.0001$) on CXCL10 levels in the whole brain; but no significant effect of sex ($p = 0.27$), nor a significant interaction ($p = 0.88$). Two-way ANOVA determined CXCL10 in the hippocampus had a significant main effect of sex ($p < 0.01$), treatment ($p < 0.001$), and interactions ($p < 0.001$). In the prefrontal cortex two-way ANOVA determined CXCL10 had a significant main effect of treatment ($p < 0.0001$) and interactions ($p < 0.0001$), but not sex ($p = 0.94$). Two-way ANOVA determined in the cerebellum/brain stem that CXCL10 had a significant main effect of treatment ($p < 0.0001$), and interactions ($p < 0.01$), but not sex ($p = 0.73$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group; Δ indicates $p < 0.05$ vs. males LPS.

There were significant main effects of treatment ($F_{3, 40} = 18.92$, $p < 0.001$) and sex ($F_{1, 40} = 7.68$, $p < 0.01$) on CXCL10 levels in the hippocampus, and a significant interaction of treatment and sex ($F_{3, 40} = 6.80$, $p < 0.001$) (Fig. 15B). Pairwise comparisons indicated that CXCL10 levels in the hippocampus of male LPS mice were significantly increased relative to male saline mice ($p < 0.0001$), whereas CXCL10 levels in male LPS + β-FNA and LPS + β-FNA 10 h mice were similar to levels in male saline mice ($p > 0.05$). In the hippocampus of female mice, CXCL10 levels were increased in LPS mice relative to saline mice ($p < 0.0001$); and levels in LPS + β-FNA and LPS + β-FNA 10 h mice were similar to the levels in LPS mice ($p > 0.05$).

Two-way ANOVA revealed a significant main effect of treatment ($F_{3, 38} = 45.67$, $p < 0.0001$) on CXCL10 levels in the prefrontal cortex, no main effect of sex ($F_{1, 38} = 0.005885$, $p = 0.94$), and a

significant interaction of treatment and sex ($F_{3, 38}$ =34.12, $p < 0.0001$) (Fig. 15C). Pairwise comparisons indicated that in male mice, CXCL10 levels in the prefrontal cortex of male LPS mice were significantly higher than in saline mice ($p < 0.0001$), whereas CXCL10 levels in male LPS + β-FNA and LPS + β-FNA 10 h mice were similar to levels in male saline mice ($p > 0.05$). LPS-induced CXCL10 expression was significantly less in female mice's prefrontal cortex than in males ($p < 0.05$). In the prefrontal cortex of female LPS mice, CXCL10 levels were significantly higher than in saline mice ($p < 0.001$). CXCL10 levels in the prefrontal cortex of LPS + β-FNA and LPS + β-FNA 10 h mice were similar to the levels in LPS mice ($p > 0.05$).

There was a significant main effect of treatment on CXCL10 expression in the cerebellum/brain stem ($F_{3, 40} = 8.88$, $p < 0.0001$), but no main effect of sex ($F_{1, 40}$ =0.123, $p = 0.73$); and there was a significant interaction between main effects ($F_{3, 40}$ =4.93, $p < 0.01$) (Fig. 15D). CXCL10 levels in the cerebellum/brain stem of male LPS mice were increased compared to male saline mice ($p < 0.0001$) as well as male LPS + β-FNA ($p < 0.002$) and LPS + β-FNA 10 h mice ($p < 0.0001$); levels in LPS + β-FNA were not significantly different from the levels in saline mice ($p > 0.05$). In female mice, CXCL10 levels in the cerebellum/brain stem tended to increase in LPS mice relative to saline mice, but not to the level of significance ($p = 0.079$); and compared to male LPS mice, CXCL10 levels were significantly less in female LPS mice ($p < 0.002$). The levels of CXCL10 in the cerebellum/brain stem of female LPS + β-FNA and LPS + β-FNA 10 h mice were similar to the levels in female LPS mice ($p > 0.05$). This experiment revealed that LPS upregulated CXCL10 in the brain of male and female mice, and the increase in the prefrontal cortex and cerebellum/brain stem was more pronounced in males compared to females. Also, the treatment with β-FNA downregulated LPS-induced CXCL10 expression in the hippocampus, prefrontal cortex, and cerebellum/brain stem of male mice, but not in those of female mice.

As determined by two-way ANOVA, there was a significant main effect of treatment on CCL2 expression in the whole brain ($F_{3, 39} = 12.69$, $p < 0.001$), but no significant effect of sex ($F_{1, 39} = 0.545$, $p = 0.47$) or an interaction of treatment and sex ($F_{3, 39} = 0.316$, $p = 0.81$) (Fig. 16A). Pairwise comparisons indicated that CCL2 levels in the whole brain of male and female LPS mice were significantly increased compared to same-sex saline mice ($p < 0.005$ and $p < 0.002$, respectively). In male mice, LPS-induced CCL2 expression in the whole brain was not affected by β-FNA treatment ($p > 0.05$). In female mice, LPS-induced CCL2 expression in the whole brain of LPS + β-FNA mice was similar to the levels in LPS mice ($p > 0.05$), whereas CCL2 levels in LPS + β-FNA 10 h mice were similar to the levels in saline mice ($p > 0.05$) (Fig. 16A).

Fig. 16

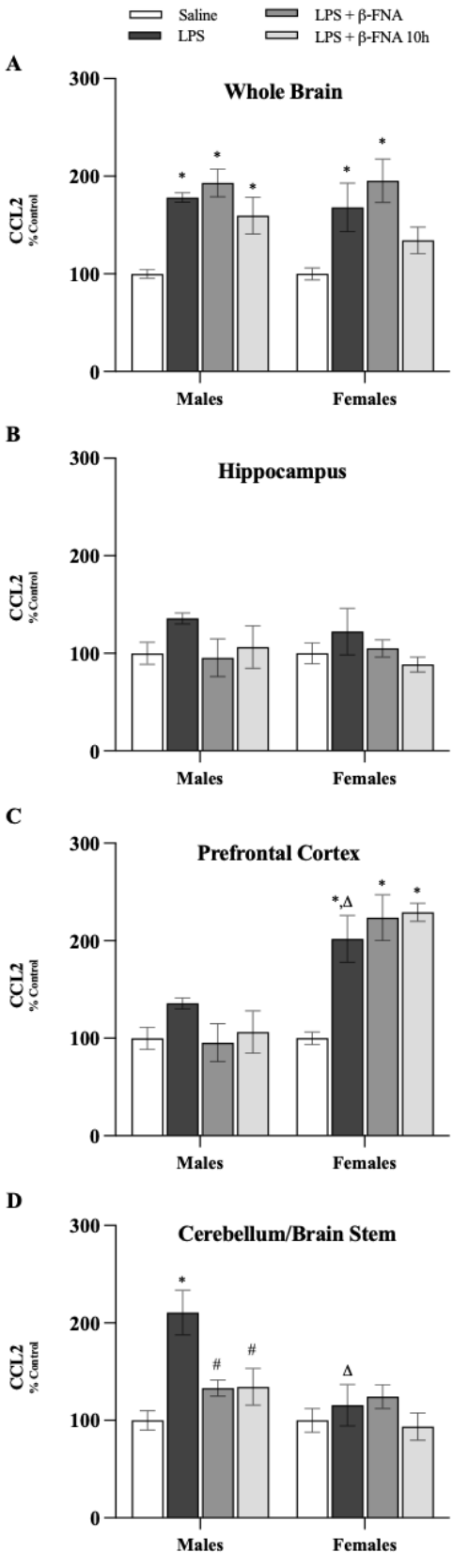

Saline
LPS
LPS + β-FNA
LPS + β-FNA 10h
A
Whole Brain
CCL2 % Control
300
200
100
0
Males
Females
B
Hippocampus
C
Prefrontal Cortex
*,Δ
D
Cerebellum/Brain Stem
#
Δ

Fig. 16 β-FNAs effect on LPS-induced CCL2 expression in the whole brain and brain regions (hippocampus, prefrontal cortex, and cerebellum/brain stem) of male and female C57BL/6J mice. Mice (n = 5-6/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, mice were terminated followed by tissue collection. Levels of CCL2 of whole brain (**A**), hippocampus (**B**), prefrontal cortex (**C**), and cerebellum/brain stem (**D**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Two-way ANOVA indicated a significant main effect of treatment ($p < 0.001$) on CCL2 levels in the whole brain; but no significant effect of sex ($p = 0.47$), nor a significant interaction ($p = 0.81$). Two-way ANOVA determined CCL2 in the hippocampus had no significant main effect of sex ($p = 0.61$), treatment ($p = 0.14$), or interaction ($p = 0.79$). In the prefrontal cortex two-way ANOVA determined CCL2 had a significant main effect of sex ($p < 0.0001$), treatment ($p < 0.001$), and an interaction ($p < 0.005$). Two-way ANOVA determined in the cerebellum/brain stem that CCL2 had a significant main effect of sex ($p < 0.005$), treatment ($p < 0.005$), and an interaction ($p < 0.02$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group; Δ indicates $p < 0.05$ vs. males LPS.

The levels of CCL2 in the hippocampus were not significantly affected by treatment ($F_{3, 40} = 1.95$, $p = 0.14$) or sex ($F_{1, 40} = 0.259$, $p = 0.61$), nor was there an interaction of treatment and sex ($F_{3, 40} = 0.344$, $p = 0.79$) (Fig. 16B).

In the prefrontal cortex, levels of CCL2 were significantly affected by treatment ($F_{3, 40} = 7.69$, $p < 0.001$) and sex ($F_{1, 40} = 44.48$, $p < 0.0001$), with a significant interaction of these main effects ($F_{3, 40} = 6.34$, $p < 0.005$) (Fig. 16C). There was no significant difference between any of the male treatment groups. However, CCL2 levels in the prefrontal cortex were increased in female LPS mice compared to either female saline mice ($p = 0.0001$) or male LPS mice ($p < 0.01$). The levels of CCL2 in the prefrontal cortex of female LPS + β-FNA and LPS + β-FNA 10 h mice were similar to the levels in female LPS mice ($p > 0.05$).

There were significant main effects of treatment ($F_{3, 40}$ =5.88, $p < 0.005$) and sex ($F_{1, 40}$ = 10.50, $p < 0.005$) on CCL2 expression in the cerebellum/brain stem, as well as an interaction between treatment and sex ($F_{3, 40}$ =3.70, $p < 0.02$) (Fig. 16D). Pairwise comparisons indicated a significant increase in CCL2 expression in male LPS mice compared to saline males ($p < 0.0001$) or LPS + β-FNA ($p < 0.002$) and LPS + β-FNA 10 h mice ($p < 0.002$), which were similar to the levels in saline mice. The levels of CCL2 in the cerebellum/brain stem of female mice were not significantly affected by any of the treatments ($p \geq 0.28$), and the levels in female LPS mice were significantly lower than the levels in male LPS mice ($p < 0.0001$). This experiment indicated that LPS-induced CCL2 expression occurs in the whole brain of male and female mice, and the magnitude of induction differs among brain regions in a sex-dependent manner (females higher in the prefrontal cortex; and males higher in cerebellum/brain stem). Also, the effects of β-FNA differed across brain regions, with inhibition only occurring in the whole brain of females treated with β-FNA at 10 h post LPS and in the cerebellum/brain stem of males (regardless of the timing of β-FNA administration).

The levels of IL-6 in the whole brain were not significantly affected by treatment ($F_{3, 39}$ = 0.484, $p = 0.70$) or sex ($F_{1, 39}$ = 0.043, $p = 0.84$), nor was there an interaction of treatment and sex ($F_{3, 39}$ = 0.208, $p = 0.89$) (Fig. 17A). In the hippocampus the levels of IL-6 were significantly affected by treatment ($F_{3, 40}$ = 9.42, $p < 0.0001$) but not by sex ($F_{1, 40}$ = 1.39, $p = 0.25$); and there was no interaction ($F_{3, 40}$ = 0.208, $p = 0.89$) (Fig. 17B). The levels of IL-6 in the hippocampus of male LPS mice were significantly lower than in male saline mice ($p < 0.05$). Male LPS + β-FNA and LPS + β-FNA 10 h mice also had decreased IL-6 levels in the hippocampus compared to male saline mice ($p < 0.005$), yet the IL-6 levels were similar to those in LPS mice ($p > 0.05$). In the hippocampus of female mice, IL-6 levels in LPS-treated mice were similar to the levels in saline mice ($p = 0.15$), whereas the levels of IL-6 in LPS + β-FNA and LPS + β-FNA 10 h mice were significantly lower than the levels in saline mice ($p < 0.01$).

Fig. 17

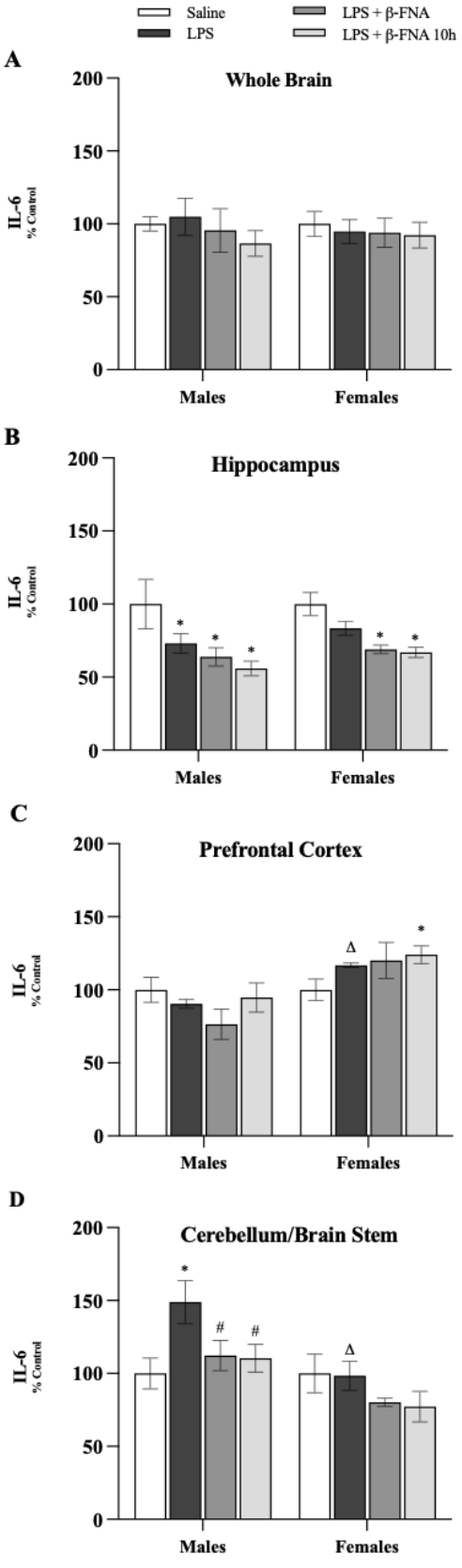

Saline
LPS
LPS + β-FNA
LPS + β-FNA 10h
A
Whole Brain
B
Hippocampus
C
Prefrontal Cortex
D
Cerebellum/Brain Stem
IL-6
% Control
200
150
100
50
0
Males
Females
*
#
Δ

Fig. 17 β-FNAs effect on LPS-induced IL-6 expression in the whole brain and brain regions (hippocampus, prefrontal cortex, and cerebellum/brain stem) of male and female C57BL/6J mice. Mice (n = 5-6/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, mice were terminated followed by tissue collection. Levels of IL-6 in whole brain (**A**), hippocampus (**B**), prefrontal cortex (**C**), and cerebellum/brain stem (**D**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Two-way ANOVA indicated no significant main effect of treatment ($p = 0.70$), sex ($p = 0.84$), or interaction ($p = 0.89$) on IL-6 levels in the whole brain. Two-way ANOVA determined IL-6 in the hippocampus showed significant main effects of treatment ($p < 0.0001$), but not sex ($p = 0.25$), or interaction ($p = 0.89$). In the prefrontal cortex two-way ANOVA determined IL-6 showed significant main effects of sex ($p < 0.001$), but not treatment ($p = 0.56$), or interaction ($p = 0.083$). Two-way ANOVA determined in the cerebellum/brain stem that IL-6 showed significant main effects of sex ($p < 0.001$) and treatment ($p < p < 0.04$) but not interaction ($p = 0.14$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group; Δ indicates $p < 0.05$ vs. males LPS.

There was a significant main effect of sex ($F_{1, 39} = 17.41$, $p < 0.001$) on IL-6 levels in the prefrontal cortex, but no effect of treatment ($F_{3, 39} = 0.701$, $p = 0.56$) and no significant interaction ($F_{3, 39} = 2.39$, $p = 0.083$) (Fig. 17C). The levels of IL-6 in the prefrontal cortex of male mice were not significantly affected by any of the treatments ($p > 0.05$). In females, the levels of IL-6 in the prefrontal cortex of LPS mice were significantly greater than the levels in male LPS mice ($p < 0.04$). The levels of IL-6 in the prefrontal cortex of female saline mice were similar to the levels in both LPS ($p = 0.18$) and LPS + β-FNA ($p = 0.10$) mice, yet less than the levels in LPS + β-FNA 10 h mice ($p < 0.05$).

There were significant main effects of treatment ($F_{3, 40}$ =3.25, $p < 0.04$) and sex ($F_{1, 40}$ =14.47, $p < 0.001$) on IL-6 expression in the cerebellum/brain stem, but no significant interaction of these effects ($F_{3, 40}$ = 1.918, $p = 0.14$) (Fig. 17D). Pairwise comparisons revealed that IL-6 levels in the cerebellum/brain stem of male LPS mice were higher than the levels in male saline mice ($p < 0.01$). The levels of IL-6 in LPS + β-FNA and LPS + β-FNA 10 h mice were significantly less than in LPS mice ($p < 0.03$ and $p < 0.02$, respectively) and similar to the levels in male saline mice ($p > 0.05$). In females, there were no significant differences between any treatment groups in terms of IL-6 expression in the cerebellum/brain stem; however, the levels of IL-6 in female LPS mice were significantly lower compared to the levels in male LPS mice ($p < 0.002$).

Two-way ANOVA indicated that IL-1β levels in the whole brain were significantly affected by treatment ($F_{3, 39}$ = 12.25, $p < 0.001$) and sex ($F_{1, 39}$ = 6.23, $p < 0.02$), but there was not a significant interaction ($F_{3, 39}$ = 1.04, $p = 0.39$) (Fig. 18A). Pairwise comparisons determined that in both male and female mice, IL-1β levels in the whole brain were similar between LPS and saline mice of the same-sex ($p = 0.93$ and $p = 0.25$ for males and females, respectively). Likewise, male and female LPS + β-FNA mice had similar IL-1β levels in the whole brain compared to saline counterparts ($p = 0.11$ and $p = 0.41$, respectively). However, in both males and females, LPS + β-FNA 10 h mice expressed significantly less IL-1β in the whole brain compared to either saline ($p < 0.001$ and $p < 0.02$, respectively) or LPS (both $p < 0.001$) mice.

Fig. 18

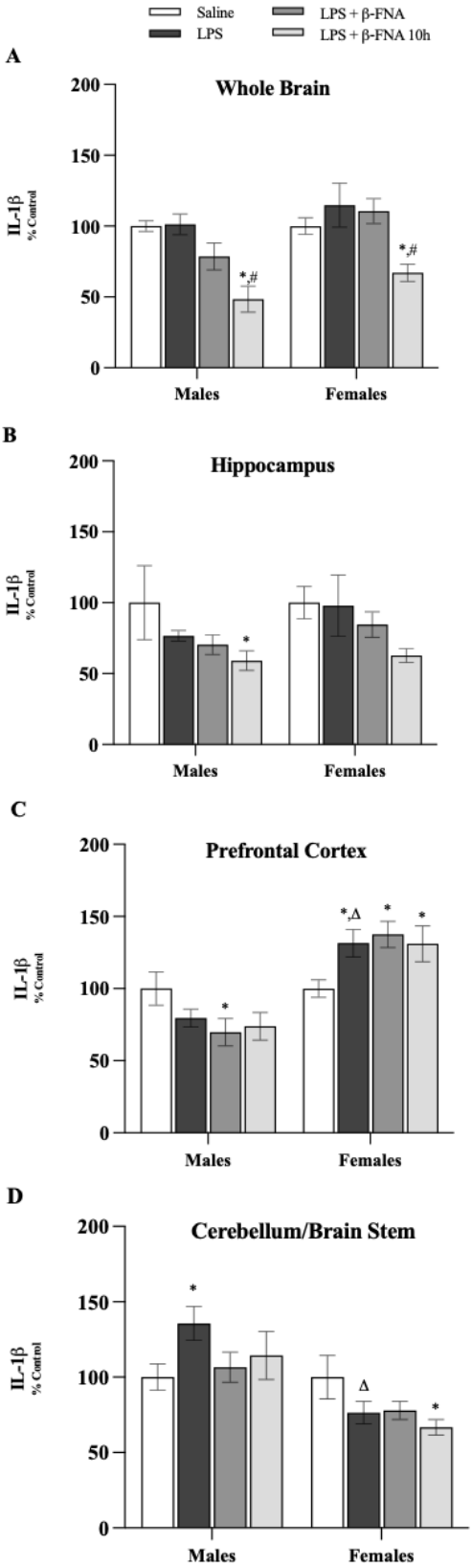

Saline
LPS
LPS + β-FNA
LPS + β-FNA 10h
A
Whole Brain
IL-1β % Control
200
150
100
50
0
*,#
*,#
Males
Females
B
Hippocampus
IL-1β % Control
200
150
100
50
0
*
Males
Females
C
Prefrontal Cortex
IL-1β % Control
200
150
100
50
0
*
*,Δ
*
*
Males
Females
D
Cerebellum/Brain Stem
IL-1β % Control
200
150
100
50
0
*
Δ
*
Males
Females

Fig. 18 β-FNAs effect on LPS-induced IL-1β expression in the whole brain and brain regions (hippocampus, prefrontal cortex, and cerebellum/brain stem) of male and female C57BL/6J mice. Mice (n = 5-6/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, mice were terminated followed by tissue collection. Levels of IL-1β of whole brain (**A**), hippocampus (**B**), prefrontal cortex (**C**), and cerebellum/brain stem (**D**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Two-way ANOVA indicated significant main effects of treatment ($p < 0.001$) and sex ($p < 0.02$) on IL-1β levels in the whole brain, but no significant effect of interaction ($p = 0.39$). Two-way ANOVA determined IL-1β in the hippocampus had a significant main effect of treatment ($p < 0.05$), but not sex ($p = 0.32$) or interaction ($p = 0.86$). In the prefrontal cortex two-way ANOVA determined IL-1β had a significant main effect of sex ($p < 0.0001$) and interaction ($p < 0.01$) but not treatment ($p = 0.95$). Two-way ANOVA determined in the cerebellum/brain stem that IL-1β had a significant main effect of sex ($p < 0.001$) and interaction ($p = 0.04$), but not treatment ($p = 0.43$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group; Δ indicates $p < 0.05$ vs. males LPS.

The IL-1β levels in the hippocampus were significantly affected by treatment ($F_{3, 40} = 2.90$, $p < 0.05$) but not by sex ($F_{1, 40} = 1.02$, $p = 0.32$), and there was no significant interaction of treatment and sex ($F_{3, 40} = 0.252$, $p = 0.86$) (Fig. 18B). The only significant difference detected by pairwise comparisons was IL-1β expression in the hippocampus of male LPS + β-FNA 10 h mice was lower than in male saline mice ($p < 0.05$).

In the prefrontal cortex, IL-1β levels were significantly affected by sex ($F_{1, 40} = 43.42$, $p < 0.0001$), but not by treatment ($F_{3, 40} = 0.121$, $p = 0.95$) (Fig. 18C); and there was a significant interaction of treatment and sex ($F_{3, 40} = 5.07$, $p < 0.01$). In males, IL-1β expression in the prefrontal cortex was significantly lower in LPS + β-FNA mice compared to saline mice ($p < 0.03$), whereas LPS and LPS + β-FNA 10 h mice had IL-1β levels in the prefrontal cortex similar to those in saline mice (p

= 0.14 and p = 0.06, respectively). In females, prefrontal cortex IL-1β expression was increased in LPS mice compared to female saline mice ($p < 0.03$) as well as male LPS mice ($p < 0.001$). Levels of IL-1β in the prefrontal cortex of female LPS + β-FNA and LPS + β-FNA 10 h mice were similar to LPS mice ($p > 0.05$) and greater than saline mice ($p < 0.01$ and $p < 0.05$, respectively).

In the cerebellum/brain stem, IL-1β levels were significantly affected by sex ($F_{1, 40} = 20.75$, $p < 0.001$) and a significant sex x treatment interaction ($F_{3, 40} = 3.02$, $p = 0.04$), but no main effect of treatment ($F_{3, 40} = 0.933$, $p = 0.43$) (Fig. 18D). In males, the levels of IL-1β in the cerebellum/brain stem were increased in LPS mice compared to saline mice ($p < 0.03$), whereas the levels in LPS + β-FNA and LPS + β-FNA 10 h mice were similar to the levels in saline mice ($p = 0.66$ and $p = 0.34$, respectively). In females, IL-1β levels in the cerebellum/brain stem were only significantly affected in LPS + β-FNA 10 h mice, as indicated by reduced expression compared to saline mice ($p < 0.05$). Also, LPS-induced IL-1β expression in the cerebellum/brain stem was less in females than in males ($p < 0.05$).

We conclude from this experiment that LPS-induced IL-1β expression was limited in the brain with upregulation in the prefrontal cortex of females and in the cerebellum/brain stem of males.

Neither treatment nor sex significantly affected TNF-α levels in the whole brain ($F_{3, 39} = 1.28$, $p = 0.29$; $F_{1, 39} = 1.76$, $p = 0.19$, respectively), yet there was a significant interaction ($F_{3, 39} = 3.58$, $p < 0.03$) (Fig. 19A). In males, TNF-α levels in the whole brain were similar among all treatment groups ($p > 0.05$). In females, pairwise comparisons revealed that LPS + β-FNA 10 h mice expressed less TNF-α than did either saline ($p < 0.04$) or LPS ($p < 0.01$) mice. LPS-induced TNF-α expression in the whole brain was significantly greater in females than in males ($p < 0.02$).

Fig. 19

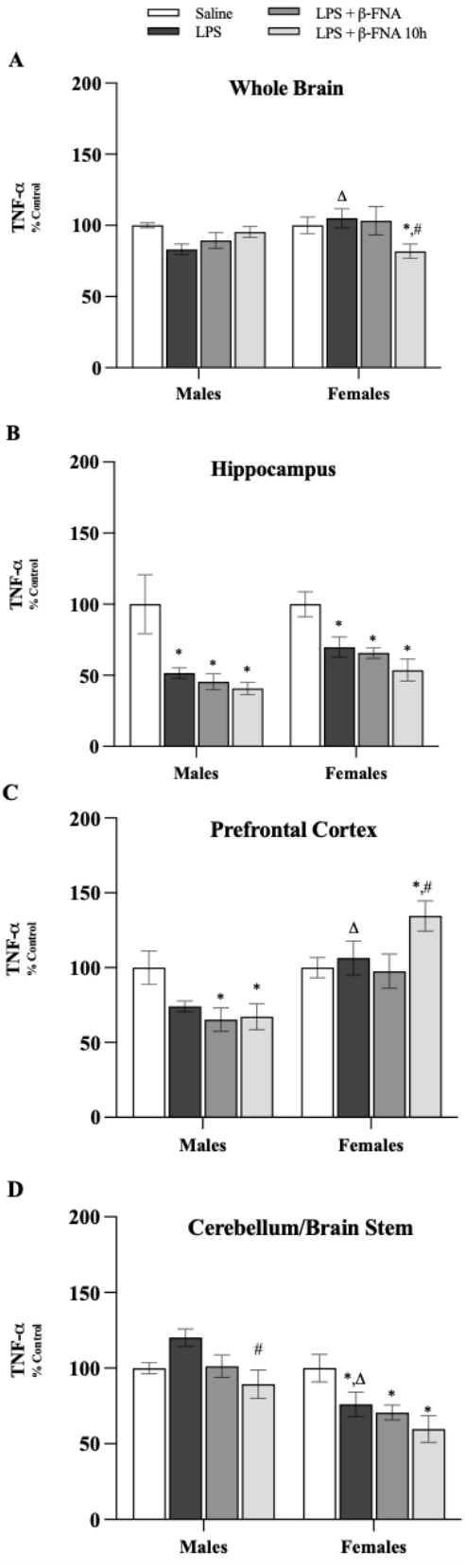

Saline
LPS
LPS + β-FNA
LPS + β-FNA 10h
A
Whole Brain
TNF-α
% Control
200
150
100
50
0
Δ
*,#
Males
Females
B
Hippocampus
*
C
Prefrontal Cortex
D
Cerebellum/Brain Stem
#
*,Δ

Fig. 19 β-FNAs effect on LPS-induced TNF-α expression in the whole brain and brain regions (hippocampus, prefrontal cortex, and cerebellum/brain stem) of male and female C57BL/6J mice. Mice (n = 5-6/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, mice were terminated followed by tissue collection. Levels of TNF-α of whole brain (**A**), hippocampus (**B**), prefrontal cortex (**C**), and cerebellum/brain stem (**D**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Two-way ANOVA indicated no significant main effect of treatment ($p = 0.29$) or sex ($p = 0.19$), but there was a significant interaction ($p < 0.03$) on TNF-α levels in the whole brain. Two-way ANOVA determined TNF-α in the hippocampus had a significant main effect of treatment ($p < 0.001$) but not sex ($p = 0.06$) and interaction ($p = 0.70$). In the prefrontal cortex two-way ANOVA determined TNF-α had a significant main effect of sex ($p < 0.0001$) and treatment ($p < 0.01$) but not interaction ($p = 0.13$). Two-way ANOVA determined in the cerebellum/brain stem that TNF-α had a significant main effect of sex ($p < 0.0001$), treatment ($p < 0.01$), and interaction ($p < 0.04$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group; Δ indicates $p < 0.05$ vs. males LPS.

The expression of TNF-α in the hippocampus was significantly affected by treatment ($F_{3, 40} = 12.52$, $p < 0.001$), but there was no main effect of sex ($F_{1, 40} = 3.75$, $p = 0.06$), and no significant interaction of the main effects ($F_{3, 40} = 0.472$, $p = 0.70$) (Fig. 19B). Levels of TNF-α in the hippocampus of male mice were similar among LPS, LPS + β-FNA, and LPS + β-FNA 10 h mice ($p > 0.05$) and TNF-α levels in all three groups were significantly lower than in saline mice ($p < 0.0007$). Similarly, in females, TNF-α expression in the hippocampus did not differ significantly among LPS, LPS + β-FNA, and LPS + β-FNA 10 h mice ($p > 0.05$), and TNF-α levels in these three groups were significantly lower than in female saline ($p < 0.03$).

In the prefrontal cortex, TNF-α levels were significantly affected by treatment ($F_{3, 40} = 4.45$, $p < 0.01$) and sex ($F_{1, 40} = 25.7$, $p < 0.0001$), but there was not a significant interaction ($F_{3, 40} = 1.99$, $p = 0.13$) (Fig. 19C). Male LPS mice tended to express less TNF-α in the prefrontal cortex than did male saline mice, but not to the level of significance ($p = 0.054$). Male LPS + β-FNA and LPS + β-FNA 10 h mice had significantly lower levels of TNF-α in the prefrontal cortex compared to male saline mice (both $p < 0.02$). Levels of TNF-α in the prefrontal cortex of female LPS mice were greater than the levels in male LPS mice ($p < 0.02$), yet they were similar to the levels in female saline mice ($p = 0.62$) and female LPS + β-FNA mice ($p = 0.86$). However, female LPS + β-FNA 10 h mice expressed significantly more TNF-α in the prefrontal cortex than did either female saline mice ($p < 0.02$) or female LPS mice ($p < 0.04$).

Two-way ANOVA revealed that there were significant main effects of treatment ($F_{3, 40} = 5.11$, $p < 0.01$) and sex ($F_{1, 40} = 24.77$, $p < 0.0001$), as well as a significant interaction ($F_{3, 40} = 3.16$, $p < 0.04$) on TNF-α levels in the cerebellum/brain stem (Fig. 19D). In males, LPS mice appeared to have higher levels of TNF-α in the cerebellum/brain stem than did saline mice, but it was not a significant increase ($p = 0.06$). The only significant difference in cerebellum/brain stem TNF-α expression among males was a decrease in LPS + β-FNA 10 h mice compared to LPS mice ($p < 0.01$). In females, TNF-α expression in the cerebellum/brain stem of LPS, LPS + β-FNA, and LPS + β-FNA 10 h mice were significantly lower than the levels in saline mice ($p < 0.03$, $p < 0.01$, and $p < 0.001$, respectively). Also, LPS-induced TNF-α expression in the cerebellum/brain stem was less in females than in males ($p < 0.0002$).

From this experiment, we concluded that LPS did not upregulate TNF-α expression in the brain. With the exception of the prefrontal cortex, LPS in combination with β-FNA resulted in a downregulation of TNF-α expression, particularly in LPS + β-FNA 10 h mice.

Effects of β-FNA on LPS-induced NF-κB-p65 expression in brain

Two-way ANOVA indicated significant main effects of treatment ($F_{3, 39} = 4.21$, $p < 0.02$) and sex ($F_{1, 40} = 24.77$, $p < 0.0001$) on the levels of NF-κB-p65 in the whole brain, but there was not a significant interaction of these main effects ($F_{3, 39} = 0.423$, $p = 0.74$) (Fig. 20A). Pairwise comparisons revealed that NF-κB-p65 levels were significantly higher in the brain of male LPS mice compared to male saline mice ($p < 0.02$). NF-κB-p65 levels in the brain of male LPS + β-FNA mice were similar to the levels in male LPS mice ($p > 0.05$), yet the levels in male LPS + β-FNA 10 h mice were similar to the levels in male saline mice ($p = 0.45$). In females, NF-κB-p65 expression in the brain was significantly increased in LPS mice compared to saline mice ($p < 0.05$), whereas the expression in LPS + β-FNA and LPS + β-FNA 10 h mice was similar to that in saline mice ($p = 0.50$ and $p = 0.54$, respectively). LPS-induced NF-κB-p65 expression was significantly lower in females compared to males ($p < 0.05$).

Fig. 20

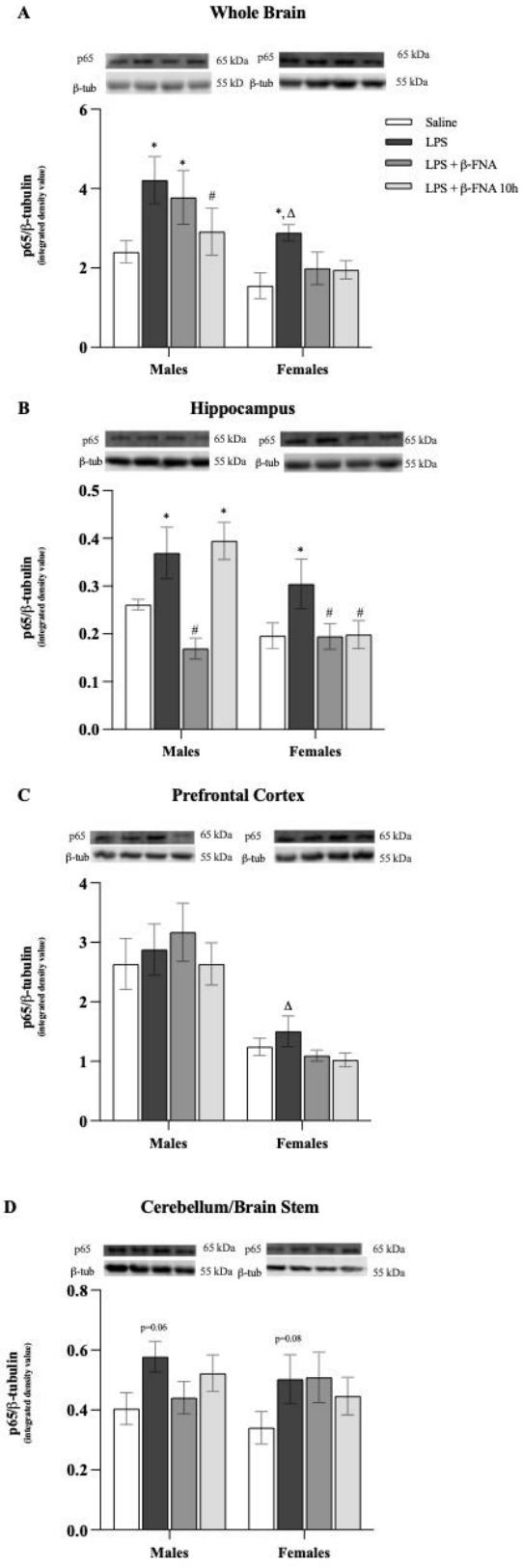

A
Whole Brain
p65
65 kDa
β-tub
55 kD
Saline
LPS
LPS + β-FNA
LPS + β-FNA 10h
p65/β-tubulin (integrated density value)
Males
Females
B
Hippocampus
55 kDa
C
Prefrontal Cortex
D
Cerebellum/Brain Stem
p=0.06
p=0.08

Fig. 20 β-FNAs effect on LPS-induced NF-κB-p65 expression in the whole brain and brain regions (hippocampus, prefrontal cortex, and cerebellum/brain stem) of male and female C57BL/6J mice. Mice (n = 5-6/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, mice were terminated followed by tissue collection. Levels of NF-κB-p65 of whole brain (**A**), hippocampus (**B**), prefrontal cortex (**C**), and cerebellum/brain stem (**D**) of tissue homogenates were measured by western blot analysis (representative western blots are shown above each area analyzed). Data are reported as mean ± SEM. Two-way ANOVA indicated a significant main effect of treatment ($p < 0.02$) and sex ($p < 0.0001$) on levels of NF-κB-p65 in the whole brain; but no significant effect of interaction ($p = 0.74$). Two-way ANOVA determined NF-κB-p65 in the hippocampus had a significant main effect of sex ($p < 0.01$), treatment ($p < 0.001$), and interaction ($p < 0.03$). In the prefrontal cortex two-way ANOVA determined levels of NF-κB-p65 had a significant main effect of sex ($p < 0.0001$), but not treatment ($p = 0.66$) or interaction ($p = 0.68$). Two-way ANOVA determined in the cerebellum/brain stem that NF-κB-p65 had a no significant main effect of sex ($p = 0.42$), treatment ($p = 0.08$), or interaction ($p = 0.62$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group; Δ indicates $p < 0.05$ vs. males LPS.

There were significant main effects of treatment ($F_{3, 40} = 7.585$, $p < 0.001$) and sex ($F_{1, 40} = 9.02$, $p < 0.01$) on NF-κB-p65 levels in the hippocampus, and a significant interaction ($F_{3, 40} = 3.304$, $p < 0.03$) (Fig. 20B). Pairwise comparisons indicated that NF-κB-p65 levels in the hippocampus of males were greater in LPS and LPS + β-FNA 10 h mice compared to saline mice ($p < 0.04$ and $p < 0.02$, respectively); while the levels in LPS + β-FNA mice were significantly less than the levels in LPS mice ($p < 0.001$). In females, NF-κB-p65 expression in the hippocampus was significantly upregulated in LPS mice relative to saline mice ($p < 0.04$), whereas levels in LPS + β-FNA and LPS + β-FNA 10 h mice were downregulated ($p < 0.05$) relative to those observed in saline mice.

A main effect of sex impacted the levels of NF-κB-p65 in the prefrontal cortex ($F_{1, 40} = 49.55$, $p < 0.0001$) but not treatment ($F_{3, 40} = 0.538$, $p = 0.66$), nor was there a significant interaction ($F_{3, 40} = 0.508$, $p = 0.68$) (Fig. 20C). Pairwise comparisons did not reveal any significant differences in NF-κB-p65 levels in the prefrontal cortex of male mice ($p \geq 0.25$). Likewise, in female mice, no significant differences were detected in prefrontal cortex NF-κB-p65 levels among treatment groups ($p \geq 0.57$). However, LPS-induced NF-κB-p65 expression was significantly less in the prefrontal cortex of female mice compared to that in male mice ($p < 0.01$).

NF-κB-p65 levels in the cerebellum/brain stem were not significantly affected by treatment ($F_{3, 40} = 2.38$, $p = 0.08$), sex ($F_{1, 40} = 0.655$, $p = 0.42$), nor was there a significant interaction ($F_{3, 40} = 0.60$, $p = 0.62$) (Fig. 20D). Pairwise comparisons revealed no significant differences; however, both male and female LPS mice tended to have increased NF-κB-p65 levels in the cerebellum/brain stem compared to respective saline mice ($p = 0.06$ and $p = 0.08$, respectively).

Based on these findings, we conclude that the inhibitory effect of β-FNA on LPS-induced NF-κB-p65 expression in the brain is most pronounced in the hippocampus and differs in a sex-dependent manner. Morse specifically, β-FNA treatment immediately post-LPS is most effective in males, whereas, in females, either immediate or delayed (10 h) β-FNA treatment effectively reduces hippocampal NF-κB-p65 expression.

Effects of β-FNA on LPS-induced p-TAK1 expression in brain

Two-way ANOVA indicated a significant main effect of sex ($F_{1, 39} = 4.306$, $p < 0.05$) but not treatment ($F_{3, 39} = 0.2826$, $p = 0.84$) or interaction of these main effects ($F_{3, 39} = 0.01933$, $p = 0.99$) on the levels of p-TAK1 in the whole brain (Fig. 21A). Pairwise comparisons did not reveal any significant differences in p-TAK1 levels in the whole brain of male mice ($p \geq 0.7$). Likewise, in the whole brain of female mice, no significant differences were detected of p-TAK1 levels among treatment groups ($p \geq 0.7$) (Fig. 21A).

Fig. 21

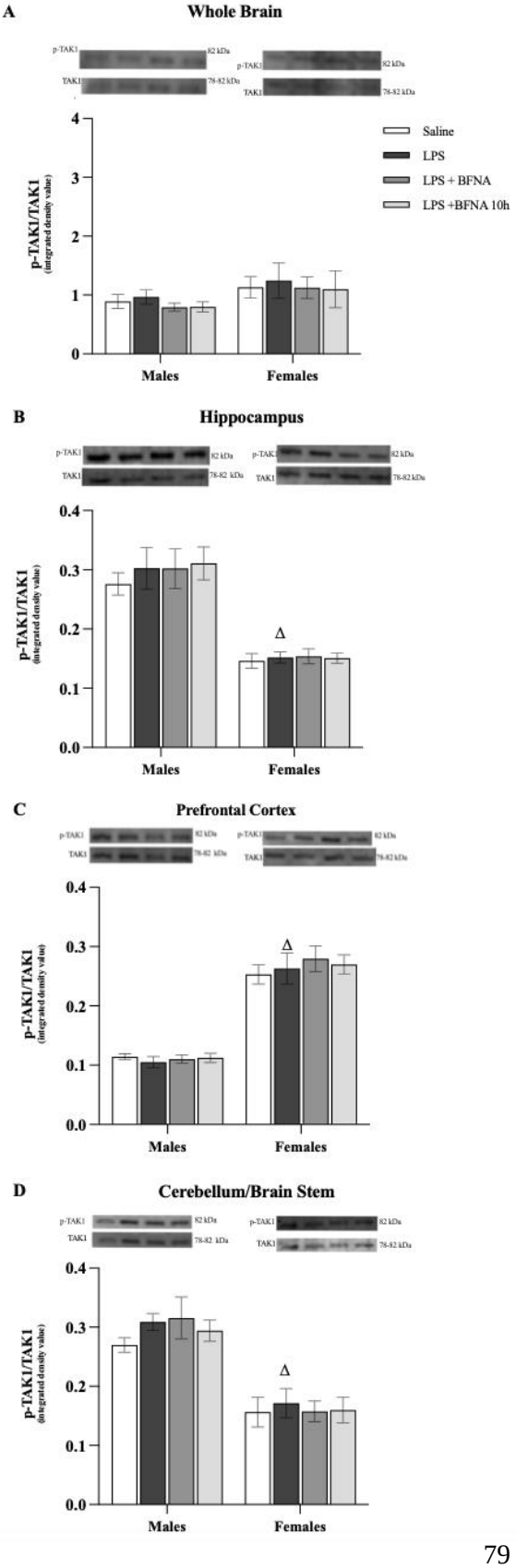

A
Whole Brain
p-TAK1
TAK1
82 kDa
78-82 kDa
Saline
LPS
LPS + BFNA
LPS +BFNA 10h
p-TAK1/TAK1
(integrated density value)
Males
Females
B
Hippocampus
C
Prefrontal Cortex
D
Cerebellum/Brain Stem
Δ

Fig. 21 β-FNAs effect on LPS-induced p-TAK1 expression in the whole brain and brain regions (hippocampus, prefrontal cortex, and cerebellum/brain stem) of male and female C57BL/6J mice. Mice (n = 5-6/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, mice were terminated followed by tissue collection. Levels of p-TAK1 of whole brain (**A**), hippocampus (**B**), prefrontal cortex (**C**), and cerebellum/brain stem (**D**) of tissue homogenates were measured by western blot analysis (representative western blots are shown above each area analyzed). Data are reported as mean ± SEM. Two-way ANOVA indicated significant main effect of sex ($p < 0.05$) but not treatment ($p = 0.84$) or interaction of these main effects ($p = 0.99$) on the levels of p-TAK1 in the whole brain. Two-way ANOVA determined p-TAK1 in the hippocampus had a significant main effect of sex ($p < 0.0001$), but not treatment ($p = 0.81$), and interaction ($p = 0.92$). In the prefrontal cortex two-way ANOVA determined levels of p-TAK1 had a significant main effect of sex ($p < 0.0001$), but not treatment ($p = 0.86$) or interaction ($p = 0.81$). Two-way ANOVA determined in the cerebellum/brain stem that p-TAK1 had a significant main effect of sex ($p < 0.0001$), but not treatment ($p = 0.63$), or interaction ($p = 0.80$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group; Δ indicates $p < 0.05$ vs. males LPS.

There was a significant main effect of sex ($F_{1, 40} = 86.14$, $p < 0.0001$) but not treatment ($F_{3, 40} = 0.3188$, $p = 0.81$) or interaction of these main effects ($F_{3, 40} = 0.1568$, $p = 0.92$) on the levels of p-TAK1 in the hippocampus (Fig. 21B). Pairwise comparisons no significant difference in the levels of p-TAK1 in the hippocampus of male mice ($p > 0.05$). Similarly, females showed no differential effect between groups ($p > 0.05$) (Fig. 21B). LPS-induced p-TAK1 expression was significantly lower in females compared to males ($p < 0.0001$).

A main effect of sex impacted the levels of p-TAK1 in the prefrontal cortex ($F_{1, 40} = 202.0$, $p < 0.0001$) but not treatment ($F_{3, 40} = 0.2430$, $p = 0.86$), nor was there a significant interaction ($F_{3, 40} = 0.3260$, $p = 0.81$) (Fig. 21C). Pairwise comparisons did not reveal any significant differences in p-

TAK1 levels in the prefrontal cortex of male mice ($p \geq 0.7$). Likewise, in female mice, no significant differences were detected in prefrontal cortex p-TAK1 levels among treatment groups ($p \geq 0.2$). However, LPS-induced p-TAK1 expression was significantly less in the prefrontal cortex of male mice compared to that in female mice ($p < 0.0001$).

p-TAK1 levels in the cerebellum/brain stem were significantly affected by sex ($F_{1, 40} = 74.08$, $p < 0.0001$) and were not significantly affected by treatment ($F_{3, 40} = 0.5830$, $p = 0.63$), nor was there a significant interaction ($F_{3, 40} = 0.3339$, $p = 0.80$) (Fig. 21D). Pairwise comparisons no significant difference in the levels of p-TAK1 in the cerebellum/brain stem of male mice ($p > 0.10$). Similarly, females showed no differential effect between groups ($p > 0.60$) (Fig. 21D). LPS-induced p-TAK1 expression was significantly lower in females compared to males ($p < 0.0001$).

We conclude from this experiment that LPS-induced p-TAK1 expression was limited in the brain with upregulation in the hippocampus and cerebellum/brain stem LPS groups of males compared to females.

Effects of β-FNA on LPS-induced TAK1 expression in brain

Two-way ANOVA indicated a significant main effect of sex ($F_{1, 39} = 80.93$, $p < 0.0001$) but not treatment ($F_{3, 39} = 0.1239$, $p = 0.95$) or interaction of these main effects ($F_{3, 39} = 0.1221$, $p = 0.95$) on the levels of TAK1 in the whole brain (Fig. 22A). Pairwise comparisons did not reveal any significant differences in TAK1 levels in the whole brain of male mice ($p \geq 0.4$). Likewise, in the whole brain of female mice, no significant differences were detected of TAK1 levels among treatment groups ($p \geq 0.7$) (Fig. 22A). LPS-induced TAK1 expression was significantly less in the whole brain of female mice compared to that in male mice ($p < 0.0001$).

Fig. 22

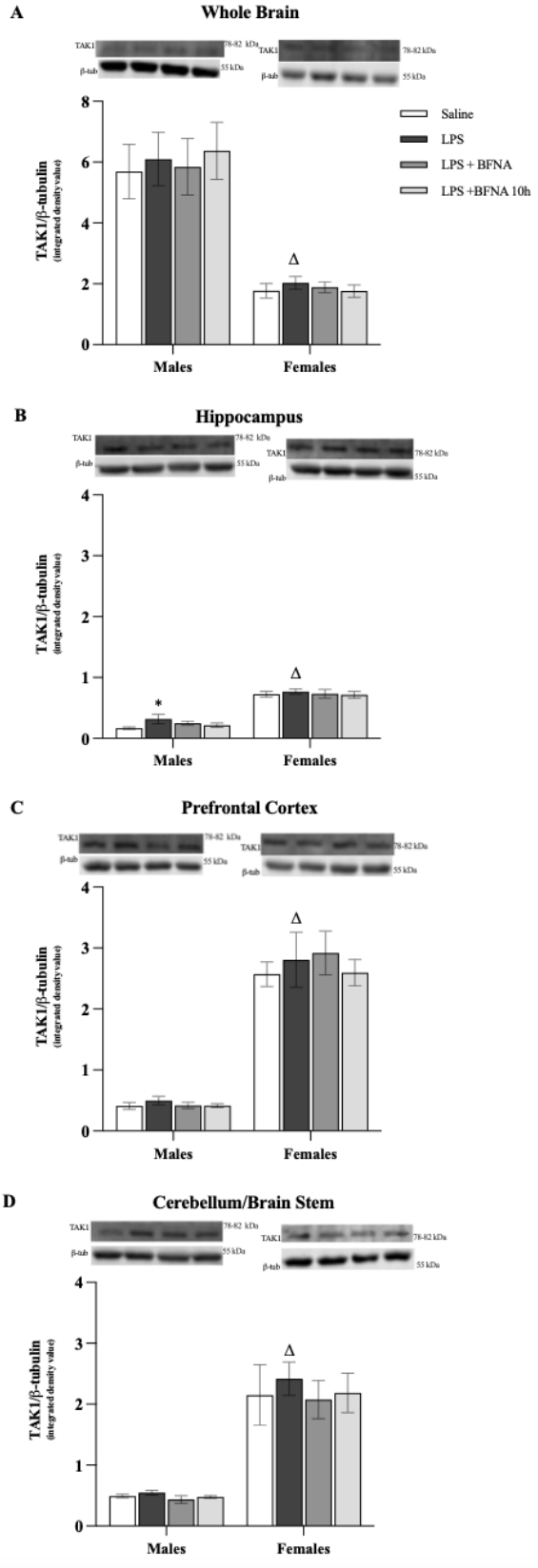

A
Whole Brain
TAK1
78-82 kDa
β-tub
55 kDa
Saline
LPS
LPS + BFNA
LPS +BFNA 10h
TAK1/β-tubulin
(integrated density value)
Δ
Males
Females
B
Hippocampus
*
C
Prefrontal Cortex
D
Cerebellum/Brain Stem

Fig. 22 β-FNAs effect on LPS-induced TAK1 expression in the whole brain and brain regions (hippocampus, prefrontal cortex, and cerebellum/brain stem) of male and female C57BL/6J mice. Mice (n = 5-6/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, mice were terminated followed by tissue collection. Levels of TAK1 of whole brain (**A**), hippocampus (**B**), prefrontal cortex (**C**), and cerebellum/brain stem (**D**) of tissue homogenates were measured by western blot analysis (representative western blots are shown above each area analyzed). Data are reported as mean ± SEM. Two-way ANOVA indicated significant main effect of sex ($p < 0.0001$) but not treatment ($p = 0.95$) or interaction of these main effects ($p = 0.95$) on the levels of TAK1 in the whole brain. Two-way ANOVA determined TAK1 in the hippocampus had a significant main effect of sex ($p < 0.0001$), but not treatment ($p = 0.29$), and interaction ($p = 0.77$). In the prefrontal cortex two-way ANOVA determined levels of TAK1 had a significant main effect of sex ($p < 0.0001$), but not treatment ($p = 0.80$) or interaction ($p = 0.88$). Two-way ANOVA determined in the cerebellum/brain stem that TAK1 had a significant main effect of sex ($p < 0.0001$), but not treatment ($p = 0.84$), or interaction ($p = 0.97$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group; Δ indicates $p < 0.05$ vs. males LPS.

There were significant main effects of sex ($F_{1, 40} = 189.1$, $p < 0.0001$) but not treatment ($F_{3, 40} = 1.303$, $p = 0.29$) or interaction of these main effects ($F_{3, 40} = 0.3703$, $p = 0.77$) on the levels of TAK1 in the hippocampus (Fig. 22B). Pairwise comparisons revealed that levels of TAK1 in the hippocampus of male mice were similar among LPS, LPS + β-FNA, and LPS + β-FNA 10 h mice ($p > 0.05$) and TAK1 levels in the LPS group were significantly different than in saline mice ($p < 0.05$). Females showed no differential effect between groups ($p > 0.05$) (Fig. 22B). LPS-induced TAK1 expression was significantly lower in males compared to females ($p < 0.0001$).

A main effect of sex impacted the levels of TAK1 in the prefrontal cortex ($F_{1, 40} = 193.5$, $p < 0.0001$) but not treatment ($F_{3, 40} = 0.3283$, $p = 0.80$), nor was there a significant interaction ($F_{3, 40} = 0.2261$,

p = 0.88) (Fig. 22C). Pairwise comparisons did not reveal any significant differences in TAK1 levels in the prefrontal cortex of male mice ($p \geq 0.7$). Likewise, in female mice, no significant differences were detected in prefrontal cortex TAK1 levels among treatment groups ($p \geq 0.5$). However, LPS-induced TAK1 expression was significantly less in the prefrontal cortex of male mice compared to that in female mice ($p < 0.0001$).

TAK1 levels in the cerebellum/brain stem were significantly affected by sex ($F_{1, 40} = 88.85$, $p < 0.0001$) and were not significantly affected by treatment ($F_{3, 40} = 0.2795$, $p = 0.84$), nor was there a significant interaction ($F_{3, 40} = 0.084$, $p = 0.97$) (Fig. 22D). Pairwise comparisons no significant difference in the levels of TAK1 in the cerebellum/brain stem of male mice ($p > 0.8$). Similarly, females showed no differential effect between groups ($p > 0.4$) (Fig. 22D). LPS-induced TAK1 expression was significantly lower in males compared to females ($p < 0.0001$).

Based on these findings, we conclude that the inhibitory effect of β-FNA on LPS-induced TAK1 expression in the brain is most pronounced in the hippocampus and differs in a sex-dependent manner.

Effects of β-FNA on LPS-induced cytokine and chemokine levels in plasma

Two-way ANOVA indicated that there were significant main effects of treatment ($F_{3, 81} = 13.60$, $p < 0.0001$), sex ($F_{1, 81} = 20.94$, $p < 0.0001$), and a significant interaction ($F_{3, 81} = 3.07$, $p < 0.04$) of treatment and sex on CXCL10 levels in the plasma (Fig. 23A). Pairwise comparisons revealed a significant increase in CXCL10 in the plasma of male LPS mice compared to male saline mice ($p < 0.002$), whereas the levels in LPS + β-FNA and LPS + β-FNA 10 h mice were not significantly different from those of saline males ($p > 0.05$). In females, the levels of CXCL10 in the plasma were significantly increased in LPS, LPS + β-FNA, and LPS + β-FNA 10 h mice versus saline mice (all $p < 0.0001$) with no significant differences among these three treatment groups.

Fig. 23

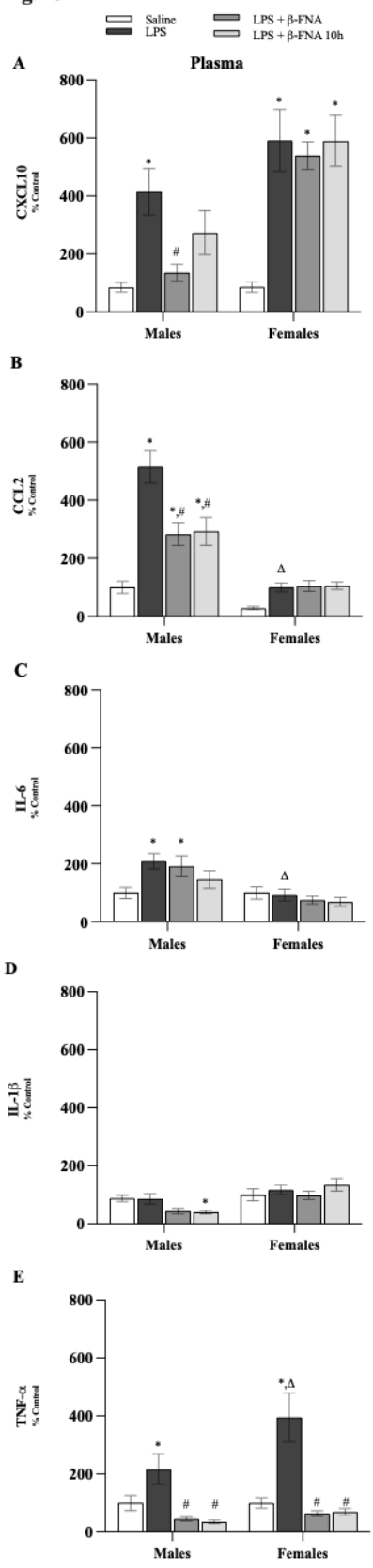

Saline
LPS
LPS + β-FNA
LPS + β-FNA 10h
A
Plasma
CXCL10
% Control
800
600
400
200
0
Males
Females
B
CCL2
% Control
C
IL-6
% Control
D
IL-1β
% Control
E
TNF-α
% Control

Fig. 23 β-FNAs effect on LPS-induced CXCL10, CCL2, IL-6, IL-1β, and TNF-α expression in the plasma of male and female C57BL/6J mice. Mice (n = 11-12/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, mice were terminated followed by plasma collection. Levels of CXCL10 (**A**), CCL2 (**B**), IL-6 (**C**), IL-1β (**D**), and TNF-α (**E**) of plasma were measured by ELISA. Data are reported as mean ± SEM. Two-way ANOVA indicated a significant main effect of treatment ($p < 0.0001$), sex ($p < 0.0001$), and interaction ($p < 0.04$) on CXCL10 levels in the plasma. Two-way ANOVA determined CCL2 had a significant main effect of sex ($p < 0.0001$), treatment ($p < 0.0001$), and interaction ($p < 0.0001$). Two-way ANOVA determined IL-6 had a significant main effect of sex ($p < 0.0001$) but not treatment ($p = 0.14$) and interaction ($p = 0.06$). Two-way ANOVA determined IL-1β had a significant main effect of sex ($p < 0.0001$) but not treatment ($p = 0.28$) and interaction ($p = 0.07$). Two-way ANOVA determined TNF-α had a significant main effect of sex ($p < 0.04$) and treatment ($p < 0.0001$) but not interaction ($p = 0.08$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group; Δ indicates $p < 0.05$ vs. males LPS.

Plasma CCL2 levels were significantly affected by treatment ($F_{3, 86} = 19.28$, $p < 0.0001$) and sex ($F_{1, 86} = 88.56$, $p < 0.0001$), with a significant interaction ($F_{3, 86} = 10.19$, $p < 0.0001$) (Fig. 23B). Male LPS mice had significantly higher plasma CCL2 levels than did male saline mice ($p < 0.0001$). The levels of plasma CCL2 were significantly lower in male LPS + β-FNA and LPS + β-FNA 10 h mice compared to male LPS mice (both $p < 0.0001$). There were no significant differences in plasma CCL2 levels among the groups of female mice ($p \geq 0.09$). However, LPS upregulation of plasma CCL2 was significantly lower in female mice compared to male mice ($p < 0.0001$).

IL-6 levels in the plasma were significantly impacted by sex ($F_{1, 85} = 20.31$, $p < 0.0001$) but not by treatment ($F_{3, 85} = 1.85$, $p = 0.14$); nor was there an interaction of treatment and sex ($F_{3, 35} = 2.55$, $p = 0.06$) (Fig. 23C). Pairwise comparisons indicated that plasma IL-6 levels were significantly increased in male LPS and LPS + β-FNA mice relative to male saline males ($p < 0.01$), whereas the levels in LPS + β-FNA 10 h mice were not significantly greater than in saline mice ($p = 0.19$). There were no significant differences in plasma IL-6 levels among the groups of female mice ($p \geq 0.40$), yet LPS upregulation of plasma IL-6 was significantly lower in female mice than in male mice ($p < 0.001$).

There was a significant main effect of sex ($F_{1, 82} = 17.63$, $p < 0.0001$) on IL-1β levels in the plasma, but not a significant effect of treatment ($F_{3, 82} = 1.30$, $p = 0.28$), and there was no interaction ($F_{3, 82} = 2.4$, $p = 0.07$) (Fig. 23D). The only significant difference among the male groups was a lower level of plasma IL-1β in LPS + β-FNA 10 h mice compared to saline mice ($p < 0.05$); and there were no significant differences among the female groups.

Plasma TNF-α levels were significantly affected by treatment ($F_{3, 86} = 20.30$, $p < 0.0001$) and sex ($F_{1, 86} = 4.58$, $p < 0.04$), but there was not a significant interaction ($F_{3, 86} = 2.31$, $p = 0.08$) (Fig. 23E). Male LPS mice had significantly higher levels of plasma TNF-α than did saline males ($p < 0.04$), LPS + β-FNA ($p < 0.002$) or LPS + β-FNA 10 h ($p < 0.002$). Likewise, in female mice, plasma TNF-α levels were significantly upregulated in LPS mice compared to saline mice and the LPS + β-FNA or LPS + β-FNA 10 h mice (all $p < 0.05$, respectively). Also, LPS-induced elevation in plasma TNF-α level was significantly more pronounced in females than in males ($p < 0.002$).

These findings indicate that LPS-mediated increases in plasma CXCL10, CCL2, and TNF-α levels are most responsive to the inhibitory effects of β-FNA. Also, the inhibitory actions of β-FNA are more apparent in males, with the exception of TNF-α, which is inhibited in both sexes.

Effects of β-FNA on LPS-induced cytokine and chemokine levels in the spleen

The levels of CXCL10 in the spleen were significantly affected by treatment ($F_{3, 86} = 12.71$, $p < 0.001$) and sex ($F_{1, 86} = 799.0$, $p < 0.0001$); and there was a significant interaction ($F_{3, 86} = 7.52$, $p < 0.001$) (Fig. 24A). CXCL10 levels in the spleen were significantly greater in male LPS, LPS + β-FNA and LPS + β-FNA 10 h mice compared to male saline mice (all $p < 0.0001$), and there were no significant differences among the LPS-treated mice ($p \geq 0.1$). There were no significant differences in spleen CXCL10 levels among the female groups ($p \geq 0.30$), and LPS-induced upregulation of CXCL10 in the spleen was significantly less in female mice than in male mice ($p < 0.0001$).

Fig 24

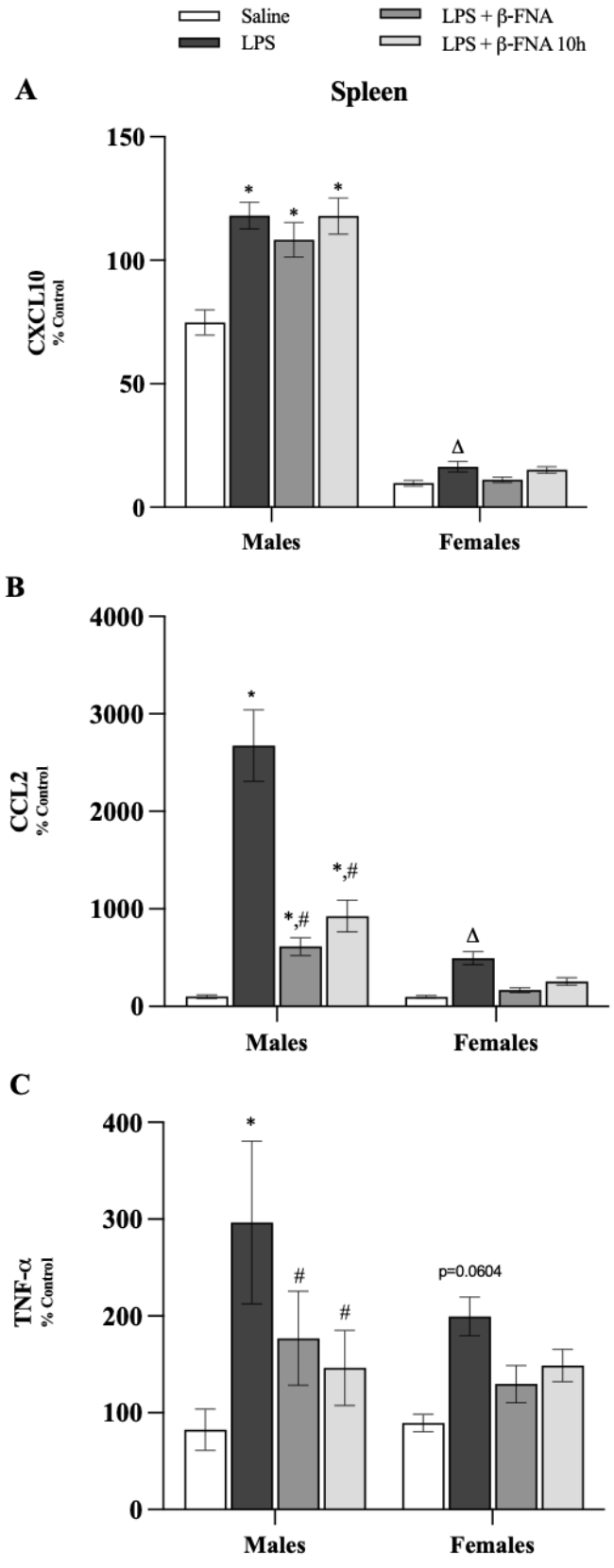

Saline
LPS
LPS + β-FNA
LPS + β-FNA 10h
A
Spleen
CXCL10
% Control
150
100
50
0
Males
Females
B
CCL2
% Control
4000
3000
2000
1000
0
C
TNF-α
% Control
400
300
200
100
0
p=0.0604

Fig. 24 β-FNAs effect on LPS-induced CXCL10, CCL2, and TNF-α expression in the spleen of male and female C57BL/6J mice. Mice (n = 11-12/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, mice were terminated followed by tissue collection. Levels of CXCL10 (**A**), CCL2 (**B**), and TNF-α (**C**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Two-way ANOVA indicated a significant main effect of treatment ($p < 0.001$), sex ($p < 0.0001$), and interaction ($p < 0.001$) on CXCL10 levels in the spleen. Two-way ANOVA determined CCL2 had a significant main effect of sex ($p < 0.0001$), treatment ($p < 0.0001$), and interaction ($p < 0.0001$). Two-way ANOVA determined TNF-α had a significant main effect of treatment ($p < 0.003$), but not sex ($p = 0.25$) or interaction ($p = 0.54$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group; Δ indicates $p < 0.05$ vs. males LPS.

There were significant main effects of treatment ($F_{3, 85} = 37.05$, $p < 0.0001$) and sex ($F_{1, 85} = 59.16$, $p < 0.0001$) on CCL2 expression in the spleen, and a significant interaction ($F_{3, 85} = 19.93$, $p < 0.0001$) (Fig. 24B). The levels of CCL2 were significantly increased in male LPS mice compared to male saline mice ($p < 0.0001$) and CCL2 expression was significantly decreased in male LPS + β-FNA and LPS + β-FNA 10 h mice compared to male LPS mice ($p < 0.02$ and $p < 0.001$, respectively). In females, there were no significant differences in spleen CCL2 levels of saline mice and any other group ($p \geq 0.06$), and LPS-mediated upregulation of CCL2 in the spleen was significantly less in female mice than in male mice ($p < 0.0001$).

TNF-α levels in the spleen were significantly affected by treatment ($F_{3, 85} = 5.30$, $p < 0.003$), but no significant main effect of sex ($F_{1, 85} = 1.37$, $p = 0.25$) and no significant interaction ($F_{3, 85} = 0.722$, $p = 0.54$) (Fig. 24C). In males, LPS mice had higher levels of TNF-α levels in the spleen than did saline, LPS + β-FNA or LPS + β-FNA 10 h mice ($p < 0.001$, $p < 0.04$, and $p < 0.01$, respectively).

TNF-α levels in the spleen of female mice were not significantly different among treatment groups ($p \geq 0.06$).

We concluded from these findings that LPS-induced cytokine/chemokine expression in the spleen was most pronounced in male mice, and only CCL2 and TNF-α levels were inhibited by β-FNA.

Effects of β-FNA on LPS-induced cytokine and chemokine levels in the liver

There were significant main effects of treatment ($F_{3, 85} = 7.01$, $p < 0.001$) and sex ($F_{1, 85} = 127.3$, $p < 0.0001$) on CXCL10 expression in the liver, but no significant interaction ($F_{3, 85} = 0.617$, $p = 0.61$) (Fig. 25A). In males, both LPS and LPS + β-FNA 10 h treated mice had significantly greater levels of CXCL10 in the liver than did male saline mice ($p < 0.001$ and $p < 0.04$, respectively), and the levels in LPS + β-FNA mice were significantly less than the levels in LPS mice ($p < 0.03$). CXCL10 levels in female LPS mice were significantly elevated compared to female saline mice ($p < 0.02$), yet similar to the levels in female LPS + β-FNA and LPS + β-FNA 10 h mice ($p < 0.05$). Also, LPS-induced liver CXCL10 expression was significantly lower in female mice than in male mice ($p < 0.0001$).

Fig. 25

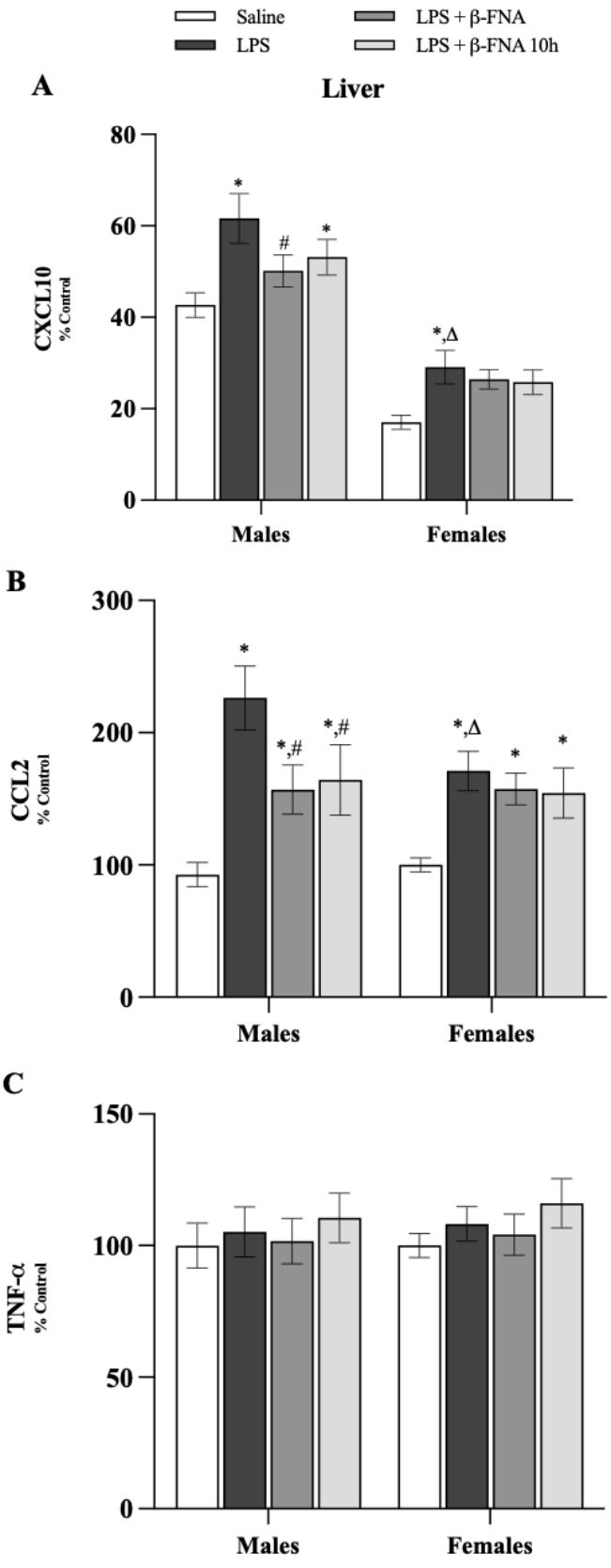

Saline
LPS
LPS + β-FNA
LPS + β-FNA 10h
A
Liver
CXCL10
% Control
80
60
40
20
0
Males
Females
B
CCL2
% Control
300
200
100
0
Males
Females
C
TNF-α
% Control
150
100
50
0
Males
Females

Fig. 25 β-FNAs effect on LPS-induced CXCL10, CCL2, and TNF-α expression in the liver of male and female C57BL/6J mice. Mice (n = 11-12/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, mice were terminated followed by tissue collection. Levels of CXCL10 (**A**), CCL2 (**B**), and TNF-α (**C**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Two-way ANOVA indicated a significant main effect of treatment ($p < 0.001$) and sex ($p < 0.0001$) on CXCL10 levels in the liver; but no significant effect on interaction ($p = 0.61$). Two-way ANOVA determined CCL2 had a significant main effect of treatment ($p < 0.0001$), but not of sex ($p = 0.25$) or interaction ($p = 0.28$). Two-way ANOVA determined TNF-α showed no main effects of sex ($p = 0.63$), treatment ($p = 0.41$), or interaction ($p = 0.99$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group; Δ indicates $p < 0.05$ vs. males LPS.

CCL2 levels in the liver were significantly affected by treatment ($F_{3, 84} = 11.33$, $p < 0.0001$) but not by sex ($F_{1, 84} = 1.32$, $p = 0.25$), nor was there a significant interaction of treatment and sex ($F_{3, 84} = 1.31$, $p = 0.28$) (Fig. 25B). Liver CCL2 levels were significantly increased in male LPS mice compared to male saline ($p < 0.0001$), LPS + β-FNA ($p < 0.01$), and LPS + β-FNA 10 h ($p < 0.02$) mice. In females, LPS ($p < 0.01$), LPS + β-FNA ($p < 0.03$), and LPS + β-FNA 10 h ($P < 0.03$) were all increased relative to saline mice. LPS-induced CCL2 levels were significantly lower in the liver of female mice compared to male mice ($p < 0.03$).

The levels of TNF-α in the liver were not significantly affected by treatment ($F_{3, 86} = 0.982$, $p = 0.41$) or sex ($F_{1, 86} = 0.228$, $p = 0.63$), nor was there a significant interaction ($F_{3, 86} = 0.039$, $p = 0.99$) (Fig. 25C).

This experiment revealed that LPS-induced CXCL10 and CCL2 expression in the liver is inhibited by β-FNA (either immediate or 10 h post-LPS administration), but only in male mice.

Effects of β-FNA on LPS-induced cytokine and chemokine levels in the intestines

Two-way ANOVA indicated significant main effects of treatment ($F_{3, 80} = 5.31$, $p < 0.01$) and sex ($F_{1, 80} = 31.73$, $p < 0.0001$) on CXCL10 levels in the colon, as well as a significant interaction ($F_{3, 80} = 4.27$, $p < 0.01$) (Fig. 26A). Pairwise comparisons showed a significant increase in CXCL10 levels in the colon of male LPS mice compared to male saline mice ($p < 0.0001$). Colon CXCL10 levels were significantly lower in male LPS + β-FNA ($p < 0.03$), and LPS + β-FNA 10 h ($p < 0.001$) mice compared to male LPS mice; with levels in LPS + β-FNA 10 h mice similar to the levels in saline mice ($p = 0.20$). In female mice, there were no significant differences among the treatment groups ($p \geq 0.79$), and LPS-induced CXCL10 expression in the colon of female mice was significantly lower than in male mice ($p < 0.0001$).

Fig. 26

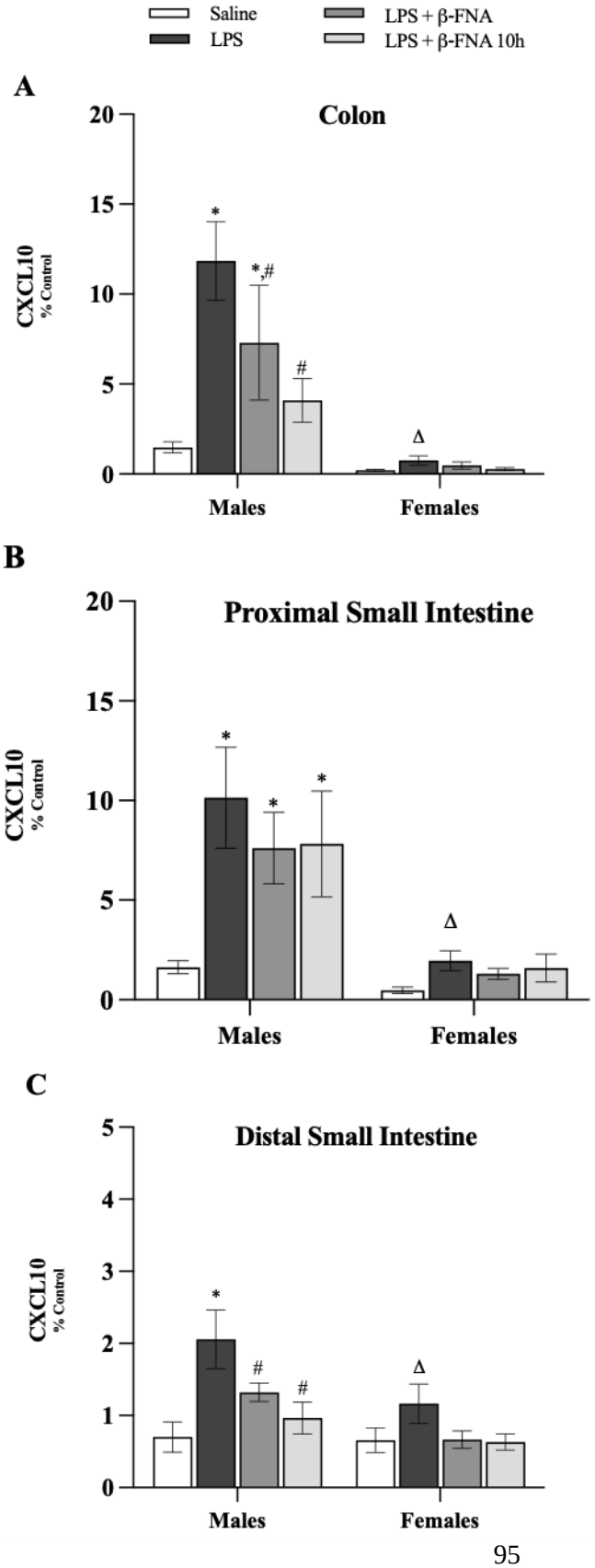

Saline
LPS
LPS + β-FNA
LPS + β-FNA 10h
A
Colon
CXCL10
% Control
20
15
10
5
0
*
*,#
#
Δ
Males
Females
B
Proximal Small Intestine
CXCL10
% Control
20
15
10
5
0
*
*
*
Δ
Males
Females
C
Distal Small Intestine
CXCL10
% Control
5
4
3
2
1
0
*
#
#
Δ
Males
Females

Fig. 26 β-FNAs effect on LPS-induced CXCL10 expression in the colon, proximal small intestine (PSI), and distal small intestine (DSI) of male and female C57BL/6J mice. Mice (n = 11-12/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, mice were terminated followed by tissue collection. Levels of CXCL10 of the colon (**A**), PSI (**B**), and DSI (**C**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Two-way ANOVA indicated a significant main effect of treatment ($p < 0.01$), sex ($p < 0.0001$), and interaction ($p < 0.01$) on CXCL10 levels in the colon. In the proximal small intestine two-way ANOVA determined CXCL10 showed main effects of sex ($p < 0.0001$) and treatment ($p < 0.02$), but not interaction ($p = 0.14$). In the distal small intestine two-way ANOVA determined CXCL10 showed main effects of sex ($p < 0.005$) and treatment ($p < 0.001$) but not interaction ($p = 0.28$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group; Δ indicates $p < 0.05$ vs. males LPS.

In the proximal small intestine, CXCL10 levels were significantly affected by treatment ($F_{3, 77} = 3.77$, $p < 0.02$) and sex ($F_{1, 77} = 26.85$, $p < 0.0001$), but there was not a significant interaction ($F_{3, 77} = 1.89$, $p = 0.14$) (Fig. 26B). In male mice CXCL10 levels in the proximal small intestine were significantly greater in LPS, LPS + β-FNA, and LPS + β-FNA 10 h compared to saline mice ($p < 0.001$, $p < 0.01$, and $p < 0.01$, respectively). There were no significant differences among the female groups ($p \geq 0.49$), and LPS-induced CXCL10 expression in the proximal small intestine of female mice was significantly lower than in male mice ($p < 0.0003$).

Main effect of treatment significantly impacted CXCL10 levels in the distal small intestine ($F_{3, 76} = 6.67$, $p < 0.001$) and sex ($F_{1, 76} = 8.78$, $p < 0.005$), but there was no significant interaction ($F_{3, 76} = 1.30$, $p = 0.28$) (Fig. 26C). Levels of CXCL10 in the distal small intestine were significantly higher in LPS male mice compared to male saline mice ($p < 0.0001$) and relative to male LPS + β-FNA ($p < 0.03$) and LPS + β-FNA 10 h ($p < 0.002$), both of which had levels similar to saline mice

($p > 0.05$). There were no significant differences in distal small intestine CXCL10 levels among the female groups ($p \geq 0.11$), and LPS-induced CXCL10 expression in the distal small intestine of female mice was significantly lower than in male mice ($p < 0.01$).

In the colon, CCL2 levels were significantly affected by treatment ($F_{3, 80} = 9.40$, $p < 0.0001$) and sex ($F_{1, 80} = 13.19$, $p < 0.001$), and there was a significant interaction ($F_{3, 80} = 5.66$, $p < 0.002$) (Fig. 27A). Pairwise comparisons yielded a significant difference in CCL2 levels in the colon male LPS mice compared to saline, LPS + β-FNA, and LPS + β-FNA 10 h mice (all $p < 0.0001$), and levels in both β-FNA-treated groups were similar to saline males ($p > 0.05$). CCL2 levels in the colon of female mice were statistically similar among treatment groups ($p \geq 0.47$), and the levels in LPS mice were lower than in male LPS mice ($p < 0.0001$).

Fig. 27

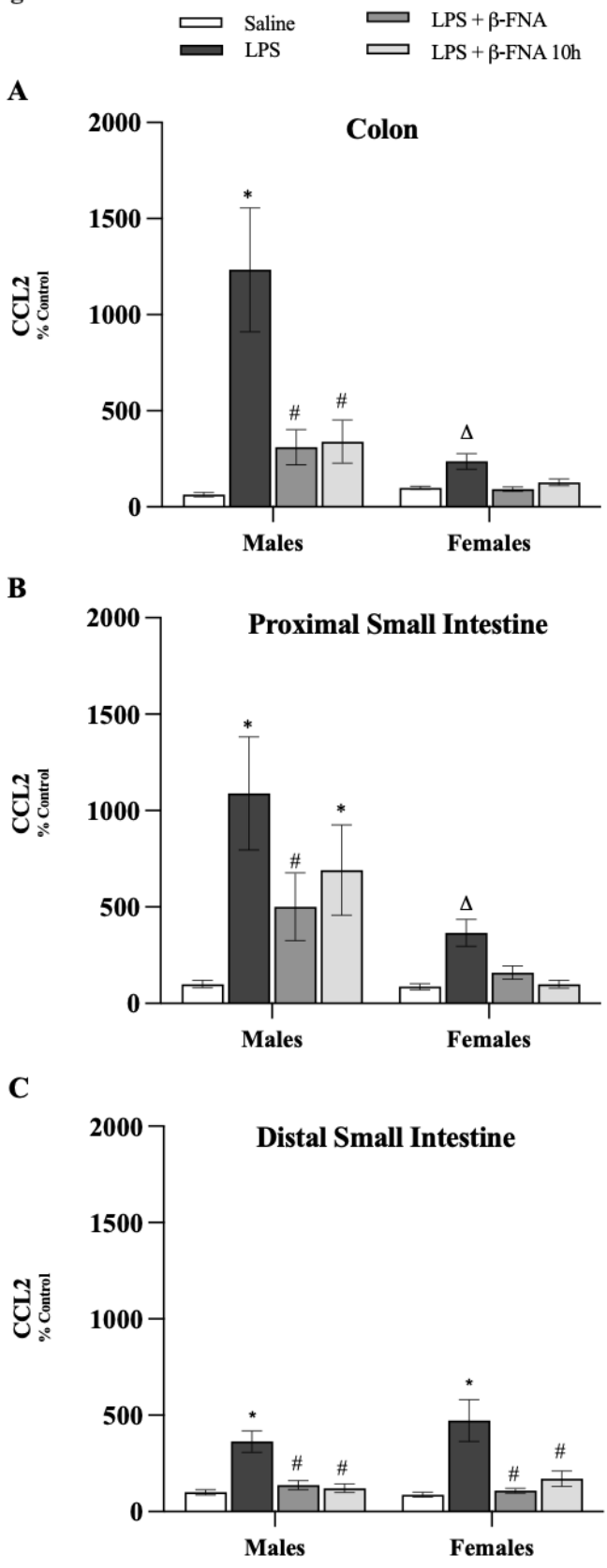

Saline
LPS
LPS + β-FNA
LPS + β-FNA 10h
A
Colon
CCL2
% Control
2000
1500
1000
500
0
Males
Females
B
Proximal Small Intestine
C
Distal Small Intestine

Fig. 27 β-FNAs effect on LPS-induced CCL2 expression in the colon, proximal small intestine (PSI), and distal small intestine (DSI) of male and female C57BL/6J mice. Mice (n = 11-12/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, mice were terminated followed by tissue collection. Levels of CCL2 of the colon (**A**), PSI (**B**), and DSI (**C**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Two-way ANOVA indicated a significant main effect of treatment ($p < 0.0001$), sex ($p < 0.001$), and interaction ($p < 0.002$) on CCL2 levels in the colon. In the proximal small intestine two-way ANOVA determined CCL2 had a significant main effect of sex ($p < 0.001$) and treatment ($p < 0.002$) but not interaction ($p = 0.13$). In the distal small intestine two-way ANOVA determined CCL2 had a significant main effect of treatment ($p < 0.0001$), but not sex ($p = 0.37$) or interaction ($p = 0.42$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group; Δ indicates $p < 0.05$ vs. males LPS.

The levels of CCL2 in the proximal small intestine were significantly affected by treatment ($F_{3, 82} = 5.54$, $p < 0.002$) and sex ($F_{1, 82} = 14.07$, $p < 0.001$), however, there was not a significant interaction ($F_{3, 82} = 1.97$, $p = 0.13$) (Fig. 27B). CCL2 levels in male LPS mice were significantly greater than the levels in male saline ($p < 0.0001$) or LPS + β-FNA mice ($p < 0.01$); and the levels in LPS + β-FNA mice were not significantly different from the levels in saline mice ($p = 0.08$). CCL2 levels in the proximal small intestine of female mice were statistically similar among treatment groups ($p \geq 0.22$), and the levels in LPS mice were lower than in male LPS mice ($p < 0.002$).

In the distal small intestine, CCL2 levels were significantly impacted by treatment ($F_{3, 80} = 21.67$, $p < 0.0001$), but not by sex ($F_{1, 80} = 0.825$, $p = 0.37$), nor was there a significant interaction ($F_{3, 80} = 0.945$, $p = 0.42$) (Fig. 27C). In male mice, distal small intestine CCL2 levels were significantly higher in LPS mice compared to saline ($p < 0.0001$), LPS + β-FNA ($p < 0.001$), and LPS + β-FNA 10 h mice ($p < 0.001$); and both β-FNA-treated groups were similar to saline mice ($p \geq 0.5$). The pattern was similar in female mice as described for males. In females, CCL2 levels in the distal

small intestine were significantly higher in LPS mice compared to saline, LPS + β-FNA, and LPS + β-FNA 10 h female mice (all $p < 0.0001$); and both β-FNA-treated groups were similar to saline mice ($p \geq 0.2$).

After repeated attempts, it was determined that TNF-α levels in the intestines of female mice were below the level of detection; therefore, only data for males are reported. One-way ANOVA indicated a significant main effect of treatment ($F_{3, 39} = 16.57$, $p < 0.0001$) on colon TNF-α levels in male mice (Fig. 28A). Pairwise comparisons revealed that the levels of TNF-α in the colon were significantly greater in LPS mice compared to saline, LPS + β-FNA, and LPS + β-FNA 10 h males (all $p < 0.0001$); and both β-FNA-treated groups were similar to saline males ($p \geq 0.06$).

Fig. 28

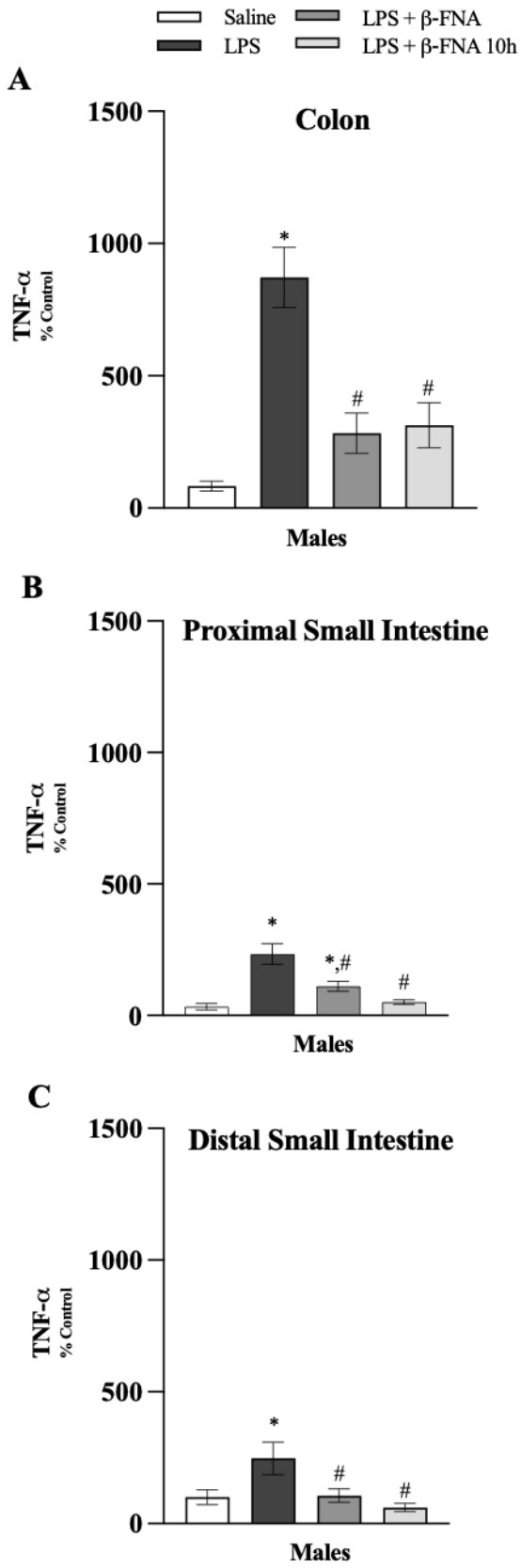

Saline
LPS
LPS + β-FNA
LPS + β-FNA 10h
A
Colon
TNF-α
% Control
1500
1000
500
0
*
#
#
Males
B
Proximal Small Intestine
TNF-α
% Control
1500
1000
500
0
*
*,#
#
Males
C
Distal Small Intestine
TNF-α
% Control
1500
1000
500
0
*
#
#
Males

Fig. 28 β-FNAs effect on LPS-induced TNF-α expression in the colon, proximal small intestine (PSI), and distal small intestine (DSI) of male C57BL/6J mice. Mice (n = 11-12/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, mice were terminated followed by tissue collection. Levels of TNF-α of the colon (**A**), PSI (**B**), and DSI (**C**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. One-way ANOVA indicated a significant main effect of treatment ($p < 0.0001$) on TNF-α levels in the colon, proximal small intestine ($p < 0.0001$), and distal small intestine ($p < 0.01$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group; Δ indicates $p<0.05$ vs. males LPS.

The level of TNF-α in the proximal small intestine was significantly affected by treatment ($F_{3, 40} = 13.73$, $p < 0.0001$) (Fig. 28B). TNF-α levels in the proximal small intestine were significantly increased in LPS males compared to saline ($p < 0.0001$), LPS + β-FNA ($p < 0.001$), and LPS + β-FNA 10 h males ($p < 0.0001$); and the levels of TNF-α in LPS + β-FNA 10 h males were similar to the levels in saline males ($p = 0.64$).

One-way ANOVA indicated a significant effect of treatment ($F_{3, 41} = 4.711$, $p < 0.01$) on distal small intestine TNF-α levels in male mice (Fig. 28C). Pairwise comparisons revealed that the levels of TNF-α in the distal small intestine were significantly greater in LPS mice compared to saline ($p < 0.01$), LPS + β-FNA ($p < 0.02$), and LPS + β-FNA 10 h males ($p < 0.002$); and both β-FNA-treated groups were similar to saline males ($p \geq 0.4$).

We concluded from these experiments that LPS-induced expression of CXCL10, CCL2, and TNF-α in the intestines was lower in females than in males, and treatment with β-FNA effectively inhibits the induction of these cytokine/chemokines in males. Inhibition of CXCL10 and TNF-α expression is more pronounced in mice treated with β-FNA 10 h-post LPS.

β-FNA effects on LPS-induced NF-κB-p65 expression in the spleen and liver

Two-way ANOVA indicated that there was a significant main effect of sex ($F_{1, 39}$ = 51.36, $p < 0.0001$) on NF-κB-p65 levels in the spleen, yet there was no significant main effect of treatment ($F_{3, 39}$ = 1.79, p = 0.16) nor was there a significant interaction ($F_{3, 39}$ =0.596, p = 0.62) (Fig. 29A). Pairwise comparisons revealed that male LPS mice expressed more NF-κB-p65 in the spleen than did male saline mice ($p < 0.05$). β-FNA treated mice immediately or at 10 h were not significantly different from controls ($p \geq 0.10$). In females, NF-κB-p65 levels in the spleen were not significantly different among treatment groups; however, the levels in female LPS mice were significantly greater than in male LPS mice ($p < 0.01$).

Fig. 29

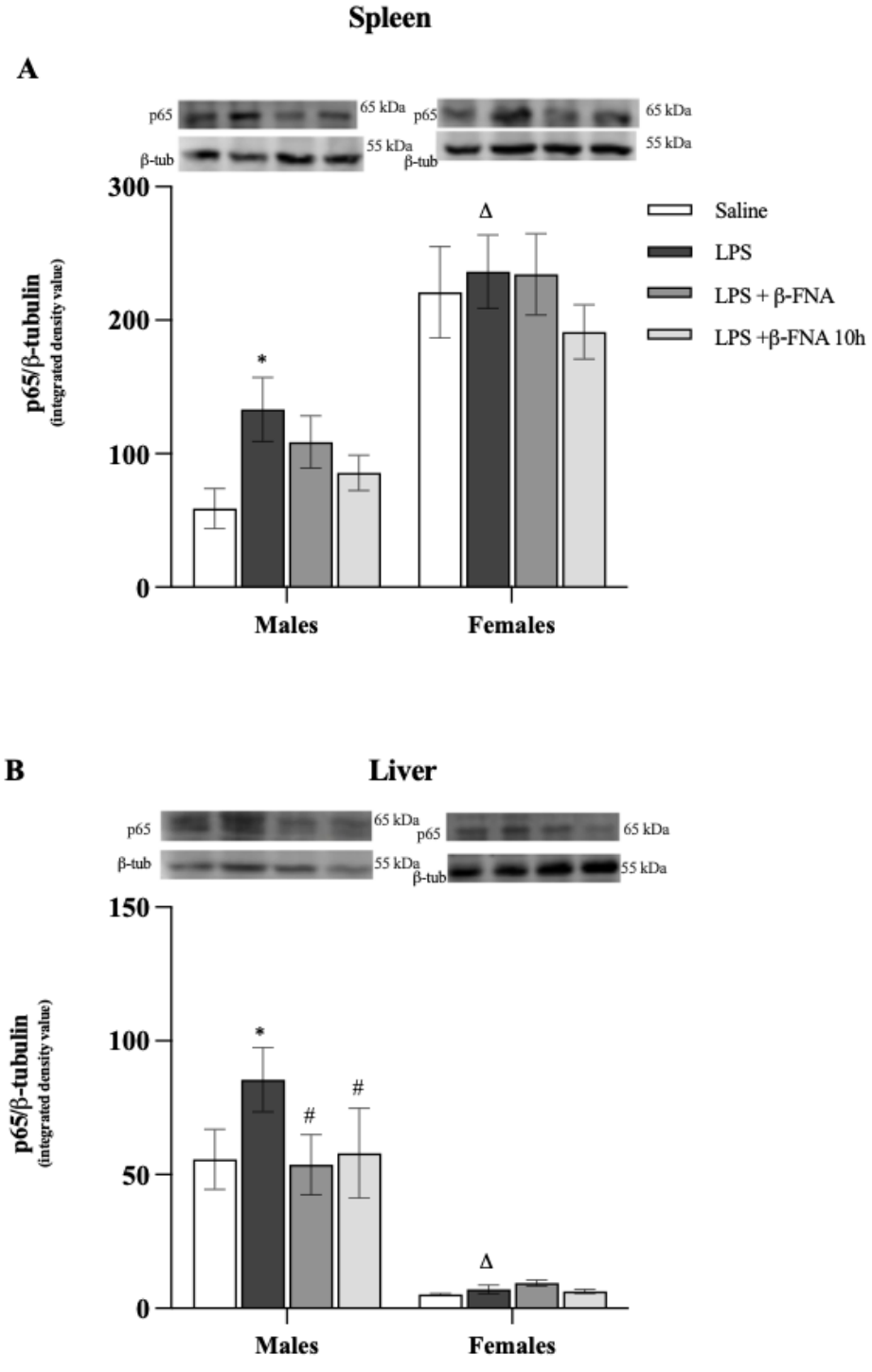

Spleen
A
p65
65 kDa
β-tub
55 kDa
p65
65 kDa
β-tub
55 kDa
p65/β-tubulin
(integrated density value)
300
200
100
0
*
Δ
Saline
LPS
LPS + β-FNA
LPS +β-FNA 10h
Males
Females
B
Liver
p65
65 kDa
β-tub
55 kDa
p65
65 kDa
β-tub
55 kDa
p65/β-tubulin
(integrated density value)
150
100
50
0
*
#
#
Δ
Males
Females

Fig. 29 β-FNAs effect on LPS-induced NF-κB-p65 expression in the spleen and liver of male and female C57BL/6J mice. Mice (n = 5-6/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, mice were terminated followed by tissue collection. Levels of NF-κB-p65 of spleen (**A**) and liver (**B**) of tissue homogenates were measured by western blot analysis (representative western blots are shown above each area analyzed). Data are reported as mean ± SEM. Two-way ANOVA indicated a significant main effect of sex ($p < 0.0001$) on levels of NF-κB-p65 in the spleen; but no significant effect of treatment ($p = 0.16$), nor a significant interaction ($p = 0.62$). In the liver two-way ANOVA determined levels of NF-κB-p65 had a significant main effect of sex ($p < 0.0001$) but no significant effect of treatment ($p = 0.28$), nor a significant interaction ($p = 0.26$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group; Δ indicates $p<0.05$ vs. males LPS.

In the liver, NF-κB-p65 expression was significantly affected by sex ($F_{1, 39} = 74.60$, $p < 0.0001$), but there was no significant main effect of treatment ($F_{3, 39} = 1.34$, $p = 0.28$), nor was there a significant interaction ($F_{3, 39} = 1.39$, $p = 0.26$) (Fig. 29B). NF-κB-p65 expression in the liver was significantly increased in male LPS mice relative to saline ($p < 0.05$), LPS + β-FNA ($p < 0.02$), and LPS + β-FNA 10 h mice ($p < 0.05$); and both β-FNA-treated groups were similar to saline mice ($p \geq 0.80$). NF-κB-p65 levels in the spleen of females were not significantly different among treatment groups ($p \geq 0.74$); however, the levels in female LPS mice were significantly lower than in male LPS mice ($p < 0.0001$). Based on these findings, we concluded that NF-κB-p65 expression is both sex-dependent and tissue specific. Also, β-FNA-mediated inhibition of NF-κB-p65 expression in the spleen and liver is limited to male mice.

β-FNA effects on LPS-induced p-TAK1 and TAK1 expression in the spleen

Two-way ANOVA indicated significant main effect of sex ($F_{1, 38}$ =58.07, $p < 0.0001$), but not treatment ($F_{3, 38}$ = 2.051, $p = 0.12$) or interaction of these main effects ($F_{3, 38}$ =2.033, $p = 0.13$) on the levels of p-TAK1 in the spleen (Fig. 30A). Pairwise comparisons reveal significant differences in p-TAK1 levels in the spleen of male mice LPS group ($p < 0.05$) and LPS + β-FNA ($p < 0.01$) compared to the saline group. There was also a difference between male LPS + β-FNA 10 h group compared to saline; however, this did not reach a level of significance ($p = 0.07$). In the spleen of female mice, no significant differences were detected of p-TAK1 levels among treatment groups ($p \geq 0.9$) (Fig. 30A). LPS-induced p-TAK1 expression was significantly less in the spleen of female mice compared to that in male mice ($p < 0.0001$).

Fig. 30

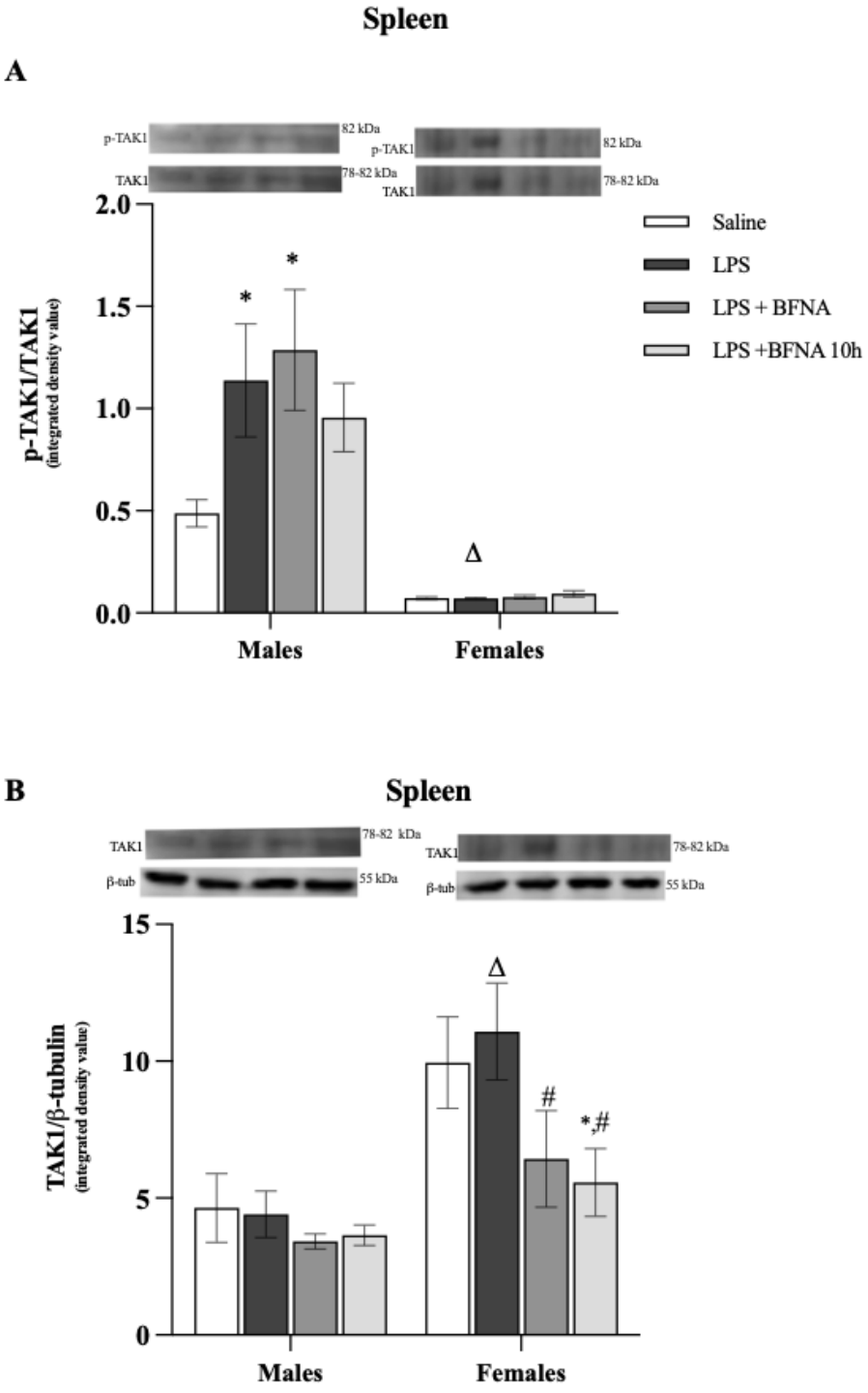

Spleen
A
p-TAK1
82 kDa
TAK1
78-82 kDa
Saline
LPS
LPS + BFNA
LPS +BFNA 10h
p-TAK1/TAK1
(integrated density value)
2.0
1.5
1.0
0.5
0.0
*
*
Δ
Males
Females
B
Spleen
TAK1
78-82 kDa
β-tub
55 kDa
TAK1/β-tubulin
(integrated density value)
15
10
5
0
Δ
#
*,#
Males
Females

Fig. 30 β-FNAs effect on LPS-induced p-TAK1 and TAK1 expression in the spleen of male and female C57BL/6J mice. Mice (n = 5-6/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, mice were terminated followed by tissue collection. Levels of p-TAK1 of spleen (**A**) and levels of TAK1 of spleen (**B**) of tissue homogenates were measured by western blot analysis (representative western blots are shown above each area analyzed). Data are reported as mean ± SEM. Two-way ANOVA indicated significant main effect of sex ($p < 0.0001$) but not treatment ($p = 0.12$) or interaction of these main effects ($p = 0.13$) on the levels of p-TAK1 in the spleen. Two-way ANOVA determined TAK1 in the spleen had a significant main effect of sex ($p < 0.0001$) and treatment ($p < 0.05$), but not interaction ($p = 0.25$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group; Δ indicates $p < 0.05$ vs. males LPS.

There were significant main effects of sex ($F_{1, 39} = 21.72$, $p < 0.0001$) and treatment ($F_{3, 39} = 3.159$, $p < 0.05$), but not interaction of these main effects ($F_{3, 39} = 1.434$, $p = 0.23$) on the levels of TAK1 in the spleen (Fig. 30B). Pairwise comparisons revealed that levels of TAK1 in the spleen of male mice were similar among saline, LPS, LPS + β-FNA, and LPS + β-FNA 10 h mice ($p > 0.05$). Females showed significant differential effects between spleen saline and the LPS + β-FNA 10 h ($p < 0.05$). Also, female LPS + β-FNA ($p < 0.05$) and LPS + β-FNA 10 h ($p < 0.001$) were significantly different from the LPS group (Fig. 30B). LPS-induced TAK1 expression was significantly lower in males compared to females ($p < 0.005$).

Based on these findings, p-TAK1 expression is more pronounced in males than in females with, minimal protection from β-FNA in the LPS + β-FNA 10 h group in males. We can also conclude that the inhibitory effect of β-FNA on LPS-induced TAK1 expression in the spleen is more pronounced in females, and there are differences between sexes.

β-FNA effects on LPS-induced p-TAK1 and TAK1 expression in the liver

Two-way ANOVA indicated a significant main effect of sex ($F_{1, 39}$ =31.99, $p < 0.0001$) but not treatment ($F_{3, 39}$ = 0.3231, $p = 0.81$) or interaction of these main effects ($F_{3, 39}$ =0.1014, $p = 0.96$) on the levels of p-TAK1 in the liver (Fig. 31A). Pairwise comparisons reveal no significant differences in p-TAK1 levels between groups in the liver of male mice ($p > 0.5$). Similarly, there were no differences between groups in female livers ($p > 0.7$). LPS-induced p-TAK1 expression was significantly less in the liver of female mice compared to that in male mice ($p < 0.005$).

Fig. 31

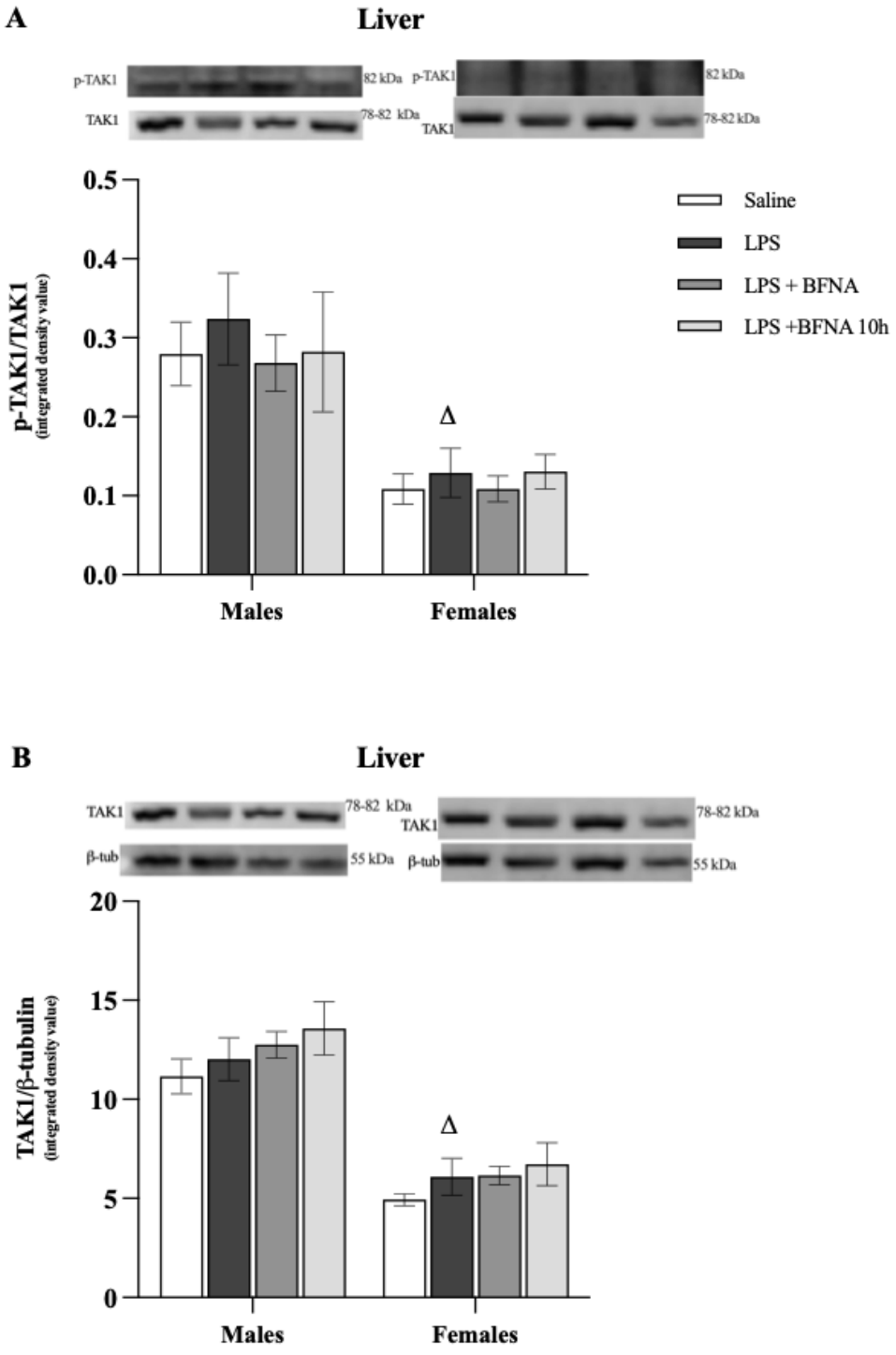

A
Liver
p-TAK1
82 kDa
TAK1
78-82 kDa
p-TAK1/TAK1
(integrated density value)
0.5
0.4
0.3
0.2
0.1
0.0
Δ
Saline
LPS
LPS + BFNA
LPS +BFNA 10h
Males
Females
B
Liver
TAK1
78-82 kDa
β-tub
55 kDa
TAK1/β-tubulin
(integrated density value)
20
15
10
5
0
Δ
Males
Females

Fig. 31 β-FNAs effect on LPS-induced p-TAK1 and TAK1 expression in the liver of male and female C57BL/6J mice. Mice (n = 5-6/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, mice were terminated followed by tissue collection. Levels of p-TAK1 of liver (**A**) and levels of TAK1 of liver (**B**) of tissue homogenates were measured by western blot analysis (representative western blots are shown above each area analyzed). Data are reported as mean ± SEM. Two-way ANOVA indicated significant main effect of sex ($p < 0.0001$) but not treatment ($p = 0.81$) or interaction of these main effects ($p = 0.96$) on the levels of p-TAK1 in the liver. Two-way ANOVA determined TAK1 in the liver had a significant main effect of sex ($p < 0.0001$), but not treatment ($p = 16$), but not interaction ($p = 0.96$). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group; Δ indicates $p < 0.05$ vs. males LPS.

There were significant main effects of sex ($F_{1, 39} = 98.16$, $p < 0.0001$), but not treatment ($F_{3, 39} = 1.794$, $p = 0.16$), and interaction of these main effects ($F_{3, 39} = 0.1007$, $p = 0.96$) on the levels of TAK1 in the liver (Fig. 31B). Pairwise comparisons revealed that levels of TAK1 in the liver of male mice were similar among saline, LPS, LPS + β-FNA, and LPS + β-FNA 10 h mice ($p > 0.05$). Females did not show a differential effect between groups in the liver ($p > 0.1$) (Fig. 31B). LPS-induced TAK1 expression was significantly lower in females compared to males ($p < 0.005$).

Based on these findings, p-TAK1 expression is more pronounced in males than in females. A similar trend can be seen in TAK1 levels of the liver, with males having a more pronounced level of LPS compared to females.

Correlation between LPS-induced CXCL10 levels in whole brain, plasma, spleen, and liver with measures of OFT-distance moved in C57BL/6J male and female mice

Linear regression analysis revealed that in males, CXCL10 levels in the whole brain were significantly correlated with distance moved in the OFT ($r^2 = 0.19$, $F = 4.81$, $p < 0.05$) (Fig. 32A). Similarly, in females, CXCL10 levels in the whole brain were negatively correlated with distance moved ($r^2 = 0.20$, $F = 5.19$, $p < 0.05$) (Fig. 32A). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (distance moved).

Fig. 32

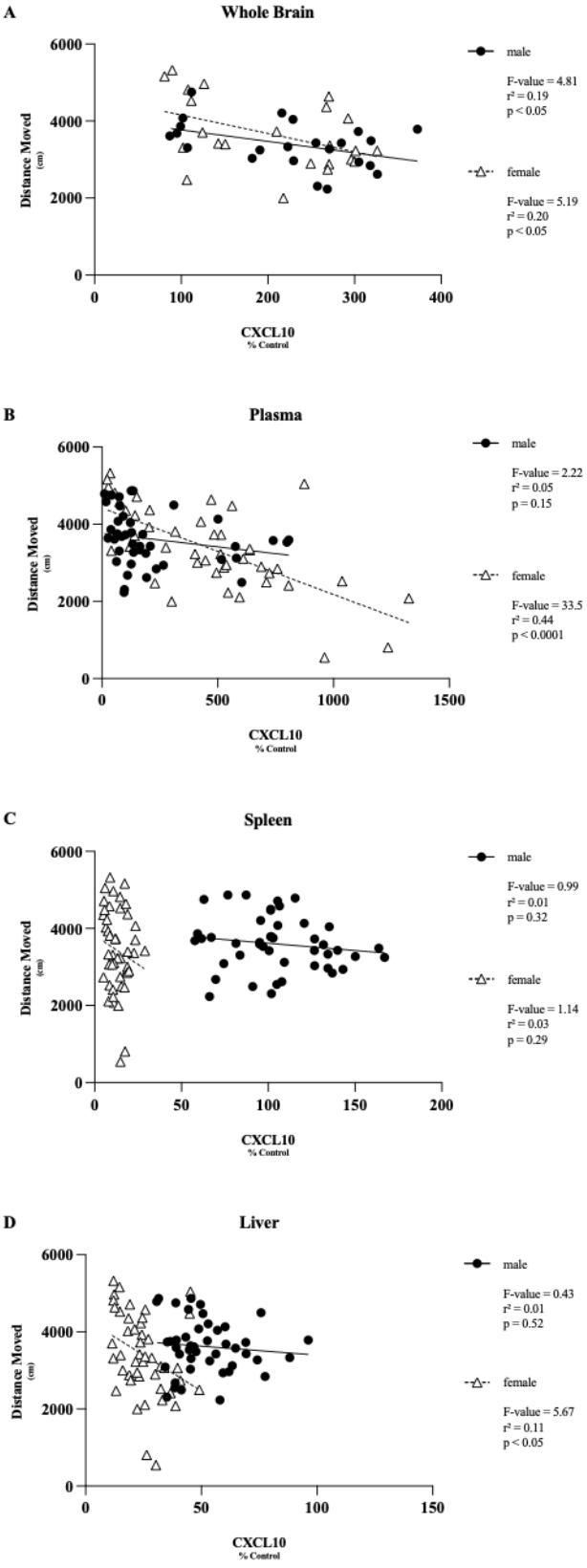

A
Whole Brain
Distance Moved (cm)
CXCL10 % Control
male
F-value = 4.81
r² = 0.19
p < 0.05
female
F-value = 5.19
r² = 0.20
p < 0.05
B
Plasma
Distance Moved (cm)
CXCL10 % Control
male
F-value = 2.22
r² = 0.05
p = 0.15
female
F-value = 33.5
r² = 0.44
p < 0.0001
C
Spleen
Distance Moved (cm)
CXCL10 % Control
male
F-value = 0.99
r² = 0.01
p = 0.32
female
F-value = 1.14
r² = 0.03
p = 0.29
D
Liver
Distance Moved (cm)
CXCL10 % Control
male
F-value = 0.43
r² = 0.01
p = 0.52
female
F-value = 5.67
r² = 0.11
p < 0.05

Fig. 32 LPS-induced CXCL10 levels in whole brain, plasma, spleen, and liver are differentially correlated with measures of OFT-distance moved in C57BL/6J mice. Mice (n = 23-24/group or n = 47-48/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted via 10 minute open-field test (OFT), mice were then terminated followed by tissue collection. Levels of CXCL10 of whole brain (**A**), plasma (**B**), spleen (**C**), and liver (**D**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Linear regression analysis was used to assess the correlation of whole brain (**A**), plasma (**B**), spleen (**C**), and liver (**D**) CXCL10 levels with OFT-distance moved. Linear regression statistics and symbols are provided in figure.

Linear regression analysis revealed that in males, CXCL10 levels in the plasma were not significantly correlated with distance moved in the OFT ($r^2 = 0.05$, $F = 2.22$, $p = 0.15$) (Fig. 32B). However, in females, CXCL10 levels in the plasma were negatively correlated with distance moved ($r^2 = 0.44$, $F = 33.5$, $p < 0.0001$) (Fig. 32B). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (distance moved).

Linear regression analysis was used to assess whether male CXCL10 levels in the spleen were correlated with distance moved. In both males and females, CXCL10 levels in the spleen were not correlated with distance moved ($r^2 = 0.01$, $F = 0.99$, $p = 0.32$; $r^2 = 0.03$, $F = 1.14$, $p = 0.29$) (Fig. 32C). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (distance moved).

In males, the overall regression was not statistically significant between CXCL10 levels in the liver and distance moved ($r^2 = 0.01$, $F = 0.43$, $p = 0.52$) (Fig. 32D). In females, the overall regression was statistically significant between CXCL10 levels in the liver and distance moved ($r^2 = 0.11$, $F = 5.67$, $p < 0.05$) (Fig. 32D). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (distance moved).

These results indicate that regarding CXCL10 levels in the plasma. spleen, and liver, the only significant correlation with distance traveled was with plasma and liver levels and only in females, whereas in the whole brain correlations were present for both males and females.

Correlation between LPS-induced CXCL10 levels in colon, proximal small intestine, and distal small intestine with measures of OFT-distance moved in C57BL/6J male and female mice

Linear regression analysis was used to assess whether male CXCL10 levels in the colon were correlated with distance moved. In both males and females, CXCL10 levels in the colon were not correlated with distance moved ($r^2 = 0.01$, $F = 0.58$, $p = 0.55$; $r^2 = 0.05$, $F = 2.16$, $p = 0.15$) (Fig. 33A). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (distance moved).

Fig. 33

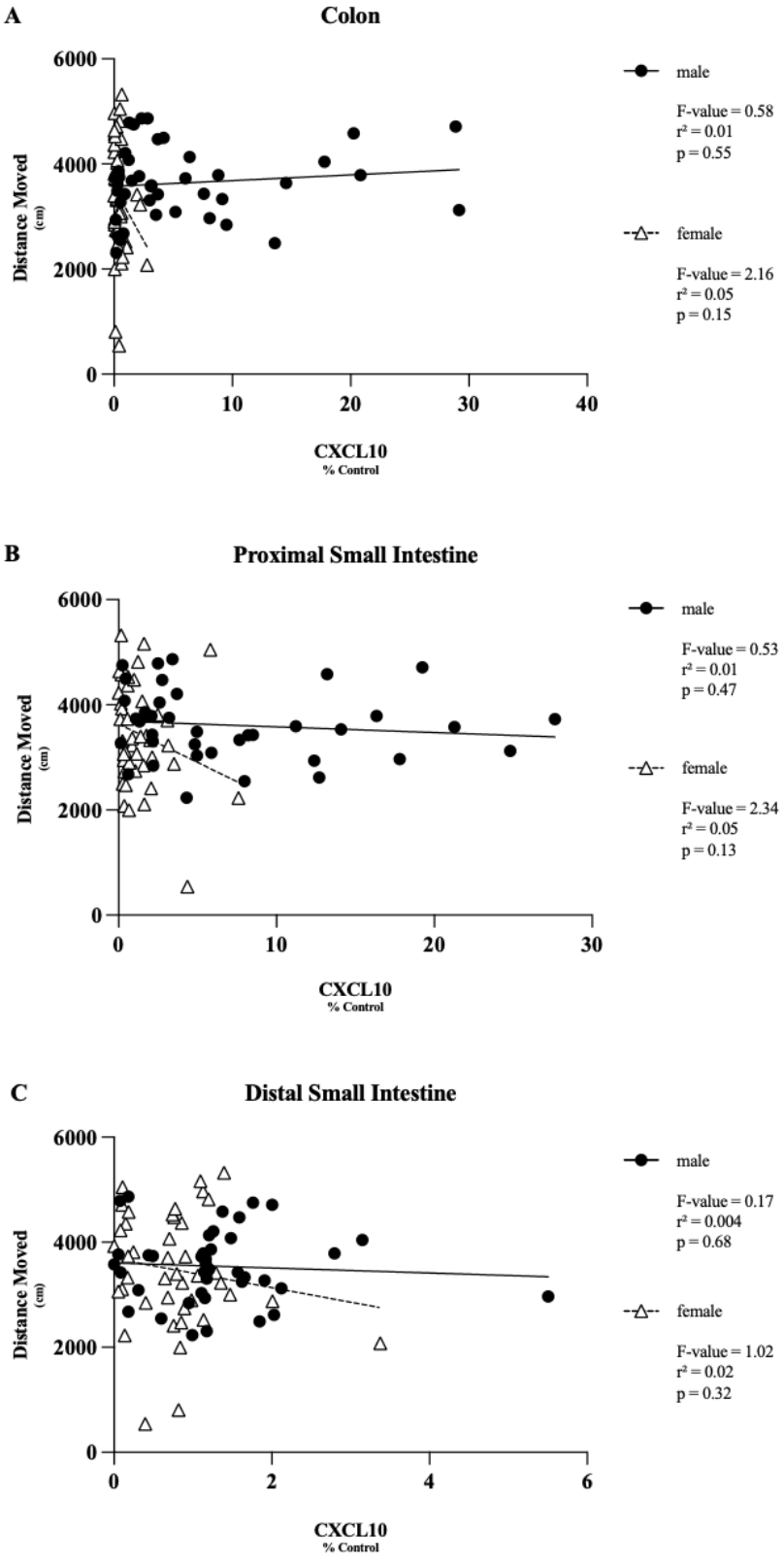

A
Colon
Distance Moved (cm)
6000
4000
2000
0
0
10
20
30
40
CXCL10
% Control
male
F-value = 0.58
r² = 0.01
p = 0.55
female
F-value = 2.16
r² = 0.05
p = 0.15
B
Proximal Small Intestine
Distance Moved (cm)
6000
4000
2000
0
0
10
20
30
CXCL10
% Control
male
F-value = 0.53
r² = 0.01
p = 0.47
female
F-value = 2.34
r² = 0.05
p = 0.13
C
Distal Small Intestine
Distance Moved (cm)
6000
4000
2000
0
0
2
4
6
CXCL10
% Control
male
F-value = 0.17
r² = 0.004
p = 0.68
female
F-value = 1.02
r² = 0.02
p = 0.32

Fig. 33 LPS-induced CXCL10 levels in colon, proximal small intestine, and distal small intestine are differentially correlated with measures of OFT-distance moved in C57BL/6J mice. Mice (n = 47-48/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted via 10 minute open-field test (OFT), mice were then terminated followed by tissue collection. Levels of CXCL10 of colon (**A**), proximal small intestine (**B**), and distal small intestine (**C**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Linear regression analysis was used to assess the correlation of colon (**A**), proximal small intestine (**B**), and distal small intestine (**C**) CXCL10 levels with OFT-distance moved. Linear regression statistics and symbols are provided in figure.

Linear regression analysis was used to assess whether male CXCL10 levels in the proximal small intestine were correlated with distance moved. In both males and females, CXCL10 levels in the proximal small intestine were not correlated with distance moved ($r^2 = 0.01$, $F = 0.53$, $p = 0.47$; $r^2 = 0.05$, $F = 2.34$, $p = 0.13$) (Fig. 33B). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (distance moved).

Linear regression analysis was used to assess whether male CXCL10 levels in the distal small intestine were correlated with distance moved. In both males and females, CXCL10 levels in the distal small intestine were not correlated with distance moved ($r^2 = 0.004$, $F = 0.17$, $p = 0.68$; $r^2 = 0.02$, $F = 1.02$, $p = 0.32$) (Fig. 33C). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (distance moved).

Based on these findings, there was no significant correlation between the CXCL10 levels in the intestine tissues and the distanced moved regardless of sex.

Correlation between LPS-induced CXCL10 levels in whole brain, plasma, spleen, and liver with measures of EPM-cumulative duration in open arms in C57BL/6J male and female mice

Linear regression analysis revealed that in males, CXCL10 levels in the whole brain were not significantly correlated with cumulative duration in open arms in the EPM ($r^2 = 0.13$, $F = 3.08$, $p = 0.11$) (Fig. 34A). However, in females, CXCL10 levels in the whole brain were negatively correlated with cumulative duration in open arms ($r^2 = 0.20$, $F = 5.02$, $p < 0.05$) (Fig. 34A). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

Fig. 34

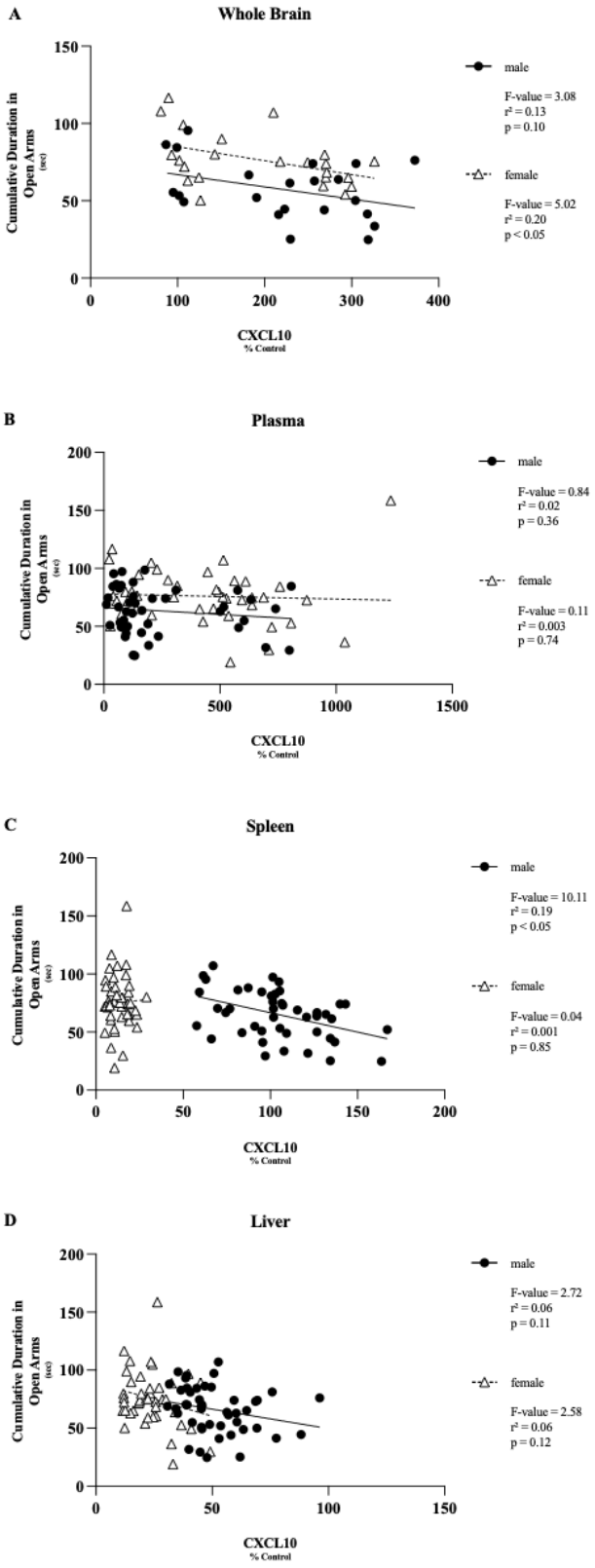

A
Whole Brain
Cumulative Duration in Open Arms (sec)
CXCL10 % Control
male
F-value = 3.08
r² = 0.13
p = 0.10
female
F-value = 5.02
r² = 0.20
p < 0.05
B
Plasma
Cumulative Duration in Open Arms (sec)
CXCL10 % Control
male
F-value = 0.84
r² = 0.02
p = 0.36
female
F-value = 0.11
r² = 0.003
p = 0.74
C
Spleen
Cumulative Duration in Open Arms (sec)
CXCL10 % Control
male
F-value = 10.11
r² = 0.19
p < 0.05
female
F-value = 0.04
r² = 0.001
p = 0.85
D
Liver
Cumulative Duration in Open Arms (sec)
CXCL10 % Control
male
F-value = 2.72
r² = 0.06
p = 0.11
female
F-value = 2.58
r² = 0.06
p = 0.12

Fig. 34 LPS-induced CXCL10 levels in whole brain, plasma, spleen, and liver are differentially correlated with measures of EPM-cumulative duration in open arms in C57BL/6J mice. Mice (n = 23-24/group or n = 47-48/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted via 5 minute elevated plus maze (EPM), mice were then terminated followed by tissue collection. Levels of CXCL10 of whole brain (**A**), plasma (**B**), spleen (**C**), and liver (**D**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Linear regression analysis was used to assess the correlation of whole brain (**A**), plasma (**B**), spleen (**C**), and liver (**D**) CXCL10 levels with EPM-cumulative duration in open arms. Linear regression statistics and symbols are provided in figure.

Linear regression analysis was used to assess whether male CXCL10 levels in the plasma were correlated with cumulative duration in open arms. In both males and females, CXCL10 levels in the plasma were not correlated with cumulative duration in open arms ($r^2 = 0.01$, $F = 0.84$, $p = 0.36$; $r^2 = 0.003$, $F = 0.11$, $p = 0.74$) (Fig. 34B). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

Linear regression analysis revealed that in males, CXCL10 levels in the spleen were significantly correlated with cumulative duration in open arms in the EPM ($r^2 = 0.19$, $F = 10.11$, $p < 0.05$) (Fig. 34C). However, in females, CXCL10 levels in the spleen were not correlated with cumulative duration in open arms ($r^2 = 0.001$, $F = 0.04$, $p = 0.85$) (Fig. 34C). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

Linear regression analysis revealed that in males, CXCL10 levels in the liver were not significantly correlated with cumulative duration in open arms in the EPM ($r^2 = 0.06$, $F = 2.72$, $p = 0.11$) (Fig.

34D). In females, CXCL10 levels in the liver were not correlated with cumulative duration in open arms ($r^2 = 0.06$, $F = 2.58$, $p = 0.12$) (Fig. 34D). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

Together these findings indicated that in females, CXCL10 levels in whole brain are negatively correlated with duration in the open arms of the EPM, whereas in males a negative correlation between CXCL10 levels in spleen and duration in the open arms is present.

Correlation between LPS-induced CXCL10 levels in colon, proximal small intestine, and distal small intestine with measures of EPM-cumulative duration in open arms in C57BL/6J male and female mice

Linear regression analysis was used to assess whether male CXCL10 levels in the colon were correlated with cumulative duration in open arms in the EPM. In both males and females, CXCL10 levels in the colon were not correlated with cumulative duration in open arms ($r^2 = 0.03$, $F = 1.44$, $p = 0.24$; $r^2 = 0.01$, $F = 0.58$, $p = 0.45$) (Fig. 35A). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

Fig. 35

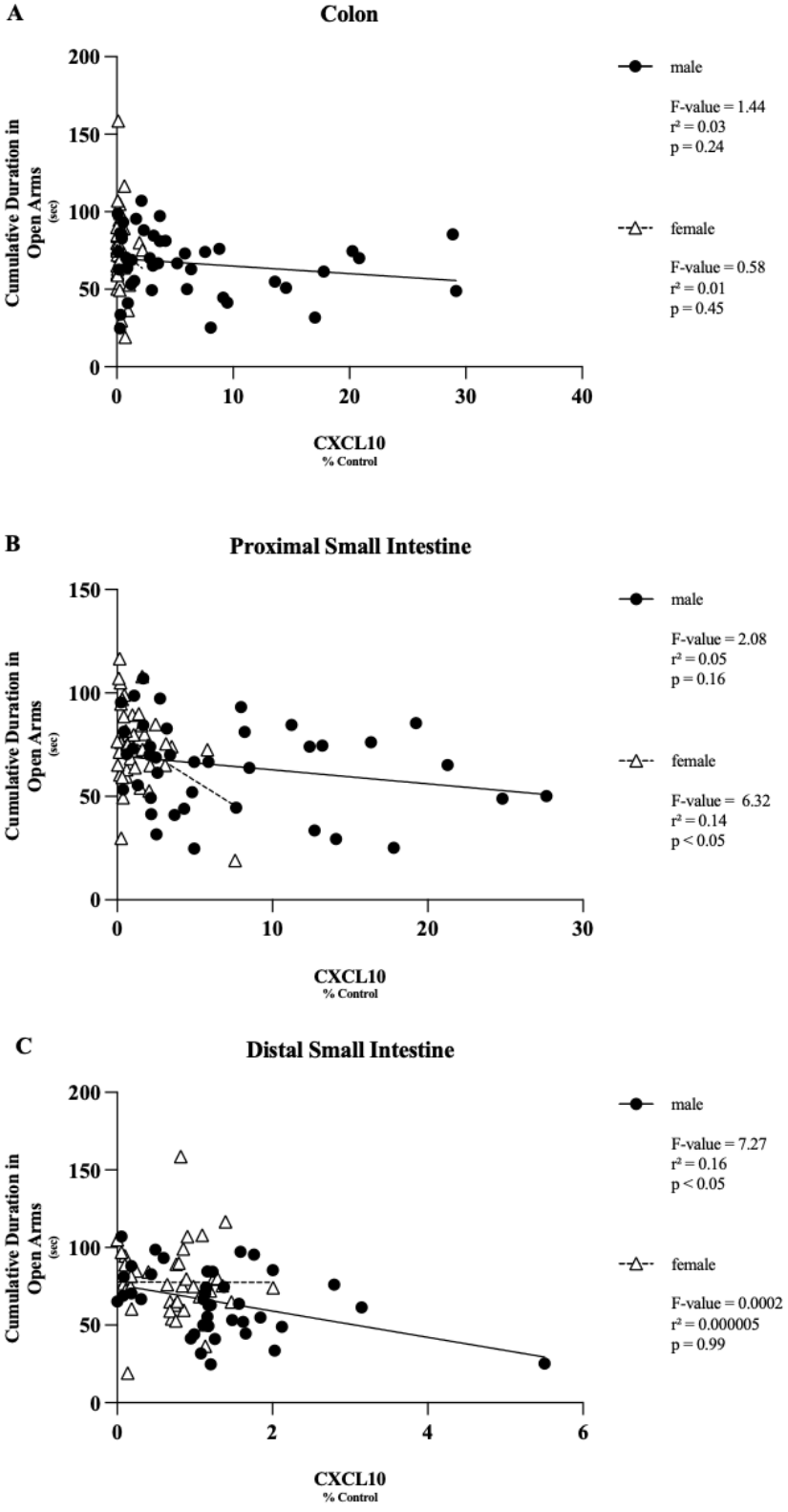

A
Colon
Cumulative Duration in Open Arms (sec)
CXCL10 % Control
male
F-value = 1.44
r² = 0.03
p = 0.24
female
F-value = 0.58
r² = 0.01
p = 0.45
B
Proximal Small Intestine
Cumulative Duration in Open Arms (sec)
CXCL10 % Control
male
F-value = 2.08
r² = 0.05
p = 0.16
female
F-value = 6.32
r² = 0.14
p < 0.05
C
Distal Small Intestine
Cumulative Duration in Open Arms (sec)
CXCL10 % Control
male
F-value = 7.27
r² = 0.16
p < 0.05
female
F-value = 0.0002
r² = 0.000005
p = 0.99

Fig. 35 LPS-induced CXCL10 levels in colon, proximal small intestine, and distal small intestine are differentially correlated with measures of EPM-cumulative duration in open arms in C57BL/6J mice. Mice (n = 47-48/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted via 5 minute elevated plus maze (EPM), mice were then terminated followed by tissue collection. Levels of CXCL10 of colon (**A**), proximal small intestine (**B**), and distal small intestine (**C**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Linear regression analysis was used to assess the correlation of colon (**A**), proximal small intestine (**B**), and distal small intestine (**C**) CXCL10 levels with EPM-cumulative duration in open arms. Linear regression statistics and symbols are provided in figure.

Linear regression analysis revealed that in males, CXCL10 levels in the proximal small intestine were not significantly correlated with cumulative duration in open arms in the EPM ($r^2 = 0.05$, $F = 2.08$, $p = 0.16$) (Fig. 35B). However, in females, CXCL10 levels in the proximal small intestine were correlated with cumulative duration in open arms ($r^2 = 0.14$, $F = 6.32$, $p < 0.05$) (Fig. 35B). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

Linear regression analysis revealed that in males, CXCL10 levels in the distal small intestine were significantly correlated with cumulative duration in open arms in the EPM ($r^2 = 0.16$, $F = 7.27$, $p < 0.05$) (Fig. 35C). However, in females, CXCL10 levels in the distal small intestine were not correlated with cumulative duration in open arms ($r^2 = 0.000005$, $F = 0.002$, $p = 0.99$) (Fig. 35C). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

Based on these findings, there was a significant correlation between the CXCL10 levels in female's proximal small intestine and the male's distal small intestine with cumulative duration in open arms.

Correlation between LPS-induced CCL2 levels in whole brain, plasma, spleen, and liver with measures of OFT-distance moved in C57BL/6J male and female mice

Linear regression analysis revealed that in males, CCL2 levels in the whole brain were significantly correlated with distance moved in the OFT ($r^2 = 0.19$, $F = 5$, $p < 0.05$) (Fig. 36A). Similarly, in females, CCL2 levels in the whole brain were negatively correlated with distance moved ($r^2 = 0.2$, $F = 5.16$, $p < 0.05$) (Fig. 36A). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (distance moved).

Fig. 36

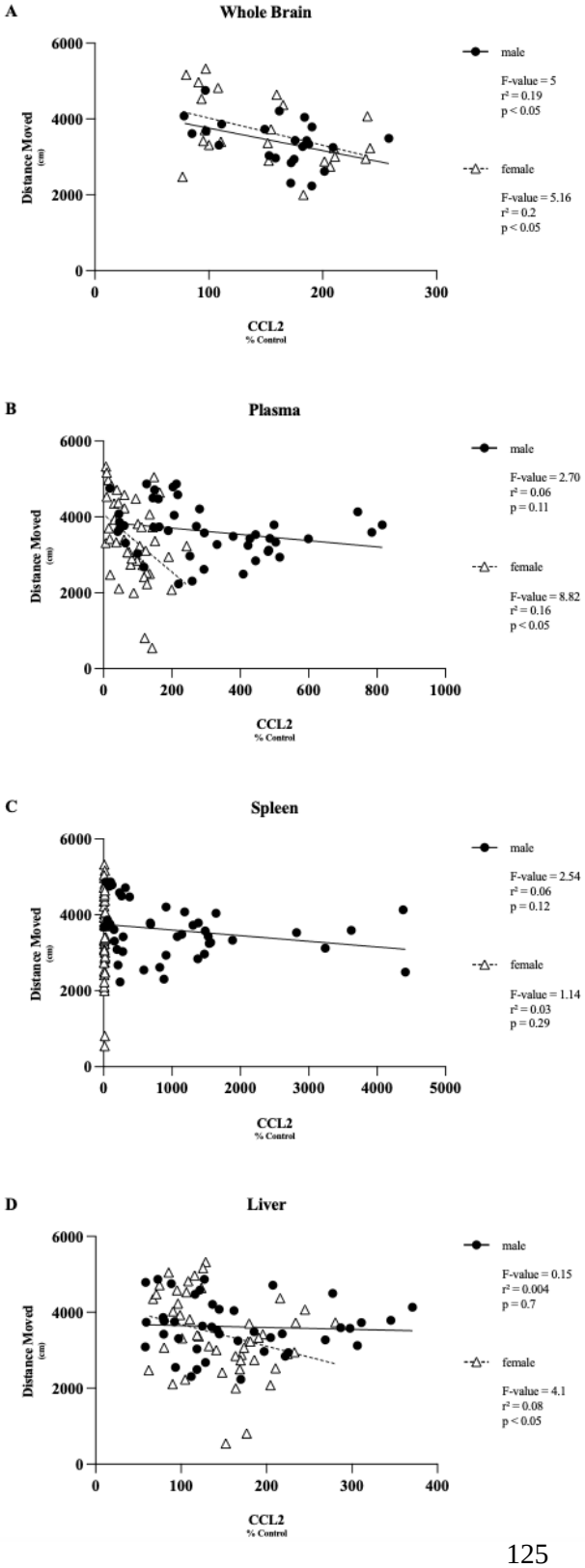

A
Whole Brain
Distance Moved (cm)
CCL2 % Control
male
F-value = 5
r² = 0.19
p < 0.05
female
F-value = 5.16
r² = 0.2
p < 0.05
B
Plasma
male
F-value = 2.70
r² = 0.06
p = 0.11
female
F-value = 8.82
r² = 0.16
p < 0.05
C
Spleen
male
F-value = 2.54
r² = 0.06
p = 0.12
female
F-value = 1.14
r² = 0.03
p = 0.29
D
Liver
male
F-value = 0.15
r² = 0.004
p = 0.7
female
F-value = 4.1
r² = 0.08
p < 0.05

Fig. 36 LPS-induced CCL2 levels in whole brain, plasma, spleen, and liver are differentially correlated with measures of OFT-distance moved in C57BL/6J mice. Mice (n = 23-24/group or n = 47-48/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted via 10 minute open-field test (OFT), mice were then terminated followed by tissue collection. Levels of CCL2 of whole brain (**A**), plasma (**B**), spleen (**C**), and liver (**D**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Linear regression analysis was used to assess the correlation of whole brain (**A**), plasma (**B**), spleen (**C**), and liver (**D**) CCL2 levels with OFT-distance moved. Linear regression statistics and symbols are provided in figure.

Linear regression analysis revealed that in males, CCL2 levels in the plasma were not significantly correlated with distance moved in the OFT ($r^2 = 0.06$, $F = 2.70$, $p = 0.11$) (Fig. 36B). Whereas, in females, CCL2 levels in the plasma were negatively correlated with distance moved ($r^2 = 0.16$, $F = 8.82$, $p < 0.05$) (Fig. 36B). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (distance moved).

Linear regression analysis revealed that in males, CCL2 levels in the spleen were not significantly correlated with distance moved in the OFT ($r^2 = 0.06$, $F = 2.55$, $p = 0.12$) (Fig. 36C). Likewise, in females, CCL2 levels in the spleen were not correlated with distance moved ($r^2 = 0.03$, $F = 1.14$, $p = 0.29$) (Fig. 36C). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (distance moved).

Linear regression analysis revealed that in males, CCL2 levels in the liver were not significantly correlated with distance moved in the OFT ($r^2 = 0.004$, $F = 0.15$, $p = 0.7$) (Fig. 36D). However, in females, CCL2 levels in the liver were not correlated with distance moved ($r^2 = 0.08$, $F = 4.1$, $p < 0.05$) (Fig. 36D). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (distance moved).

Based on these findings, there is a correlation between tissues and the distanced moved, and there are differences based on sex, with males and females showing a correlation between the whole brain and distance moved and females showing a negative correlation in the plasma and liver.

Correlation between LPS-induced CCL2 levels in colon, proximal small intestine, and distal small intestine with measures of OFT-distance moved in C57BL/6J male and female mice

Linear regression analysis was used to assess whether male CCL2 levels in the colon were correlated with distance moved. In both males and females, CCL2 levels in the distal small intestine were not correlated with distance moved ($r^2 = 0.04$, $F = 1.81$, $p = 0.19$; $r^2 = 0.005$, $F = 0.22$, $p = 0.64$) (Fig. 37A). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (distance moved).

Fig. 37

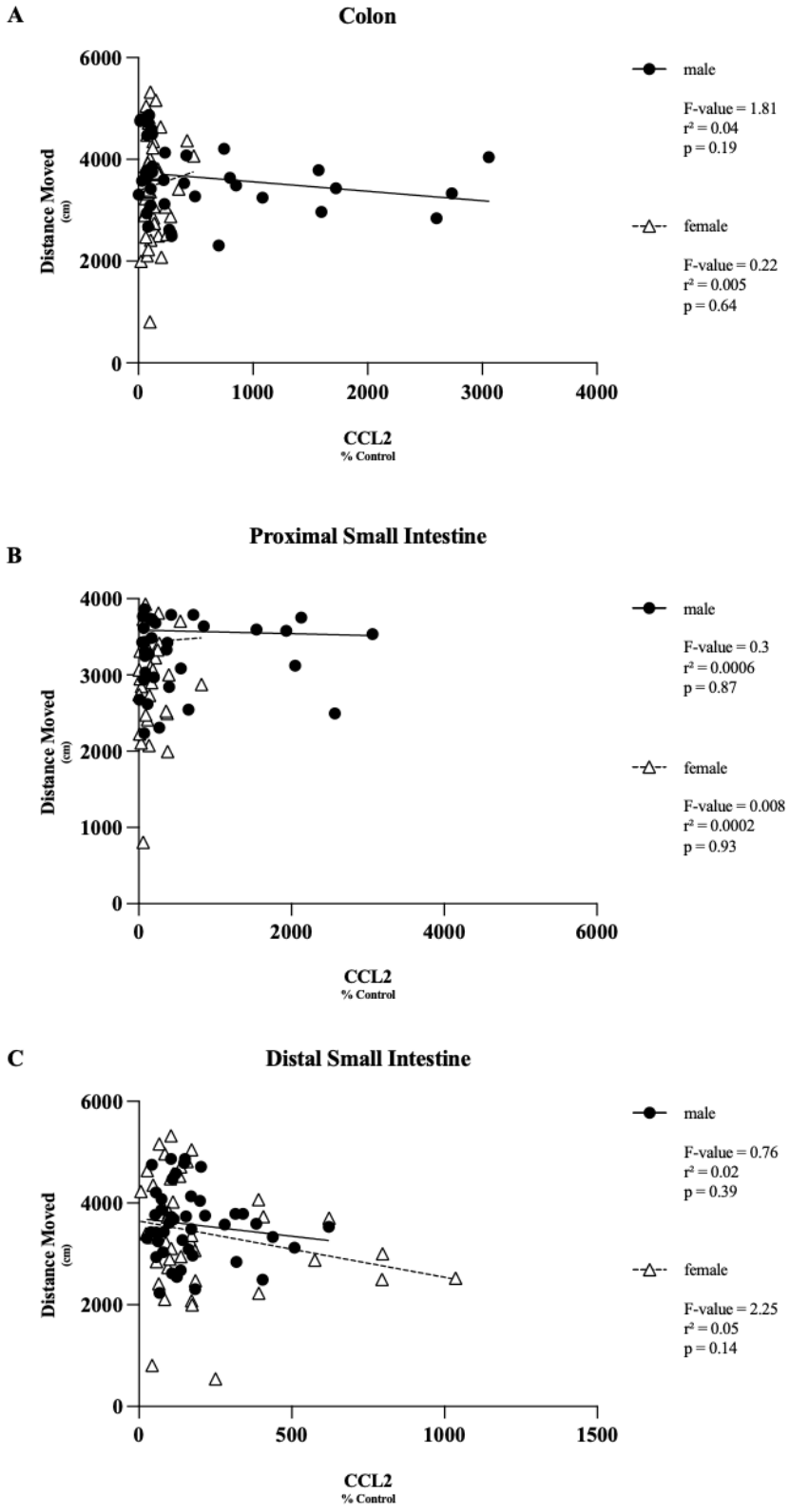

A
Colon
Distance Moved (cm)
6000
4000
2000
0
0
1000
2000
3000
4000
CCL2
% Control
male
F-value = 1.81
r² = 0.04
p = 0.19
female
F-value = 0.22
r² = 0.005
p = 0.64
B
Proximal Small Intestine
Distance Moved (cm)
4000
3000
2000
1000
0
0
2000
4000
6000
CCL2
% Control
male
F-value = 0.3
r² = 0.0006
p = 0.87
female
F-value = 0.008
r² = 0.0002
p = 0.93
C
Distal Small Intestine
Distance Moved (cm)
6000
4000
2000
0
0
500
1000
1500
CCL2
% Control
male
F-value = 0.76
r² = 0.02
p = 0.39
female
F-value = 2.25
r² = 0.05
p = 0.14

Fig. 37 LPS-induced CCL2 levels in colon, proximal small intestine, and distal small intestine are differentially correlated with measures of OFT-distance moved in C57BL/6J mice. Mice (n = 47-48/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted via 10 minute open-field test (OFT), mice were then terminated followed by tissue collection. Levels of CCL2 of colon (**A**), proximal small intestine (**B**), and distal small intestine (**C**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Linear regression analysis was used to assess the correlation of colon (**A**), proximal small intestine (**B**), and distal small intestine (**C**) CCL2 levels with OFT-distance moved. Linear regression statistics and symbols are provided in figure.

Linear regression analysis revealed that in males, CCL2 levels in the proximal small intestine were not significantly correlated with distance moved in the OFT ($r^2 = 0.006$, $F = 0.3$, $p = 0.87$) (Fig. 37B). In females, CCL2 levels in the proximal small intestine were not correlated with distance moved ($r^2 = 0.0002$, $F = 0.008$, $p = 0.93$ (Fig. 37B). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis indicates the dependent value (distance moved).

Linear regression analysis revealed that in males and females, CCL2 levels in the distal small intestine were not significantly correlated with distance moved in the OFT ($r^2 = 0.02$, $F = 0.76$, $p = 0.39$; $r^2 = 0.05$, $F = 2.25$, $p = 0.14$) (Fig. 37C). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (distance moved).

Based on these findings, there were no significant correlations between the CCL2 levels in the intestines of male or female mice with distance moved.

Correlation between LPS-induced CCL2 levels in whole brain, plasma, spleen, and liver with measures of EPM-cumulative duration in open arms in C57BL/6J male and female mice

Linear regression analysis revealed that in males, CCL2 levels in the whole brain were significantly correlated with cumulative duration in open arms in the EPM ($r^2 = 0.24$, $F = 6.19$, $p < 0.05$) (Fig. 38A). However, in females, CCL2 levels in the whole brain were not correlated with cumulative duration in open arms ($r^2 = 0.16$, $F = 4.02$, $p = 0.06$) (Fig. 38A). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

Fig. 38

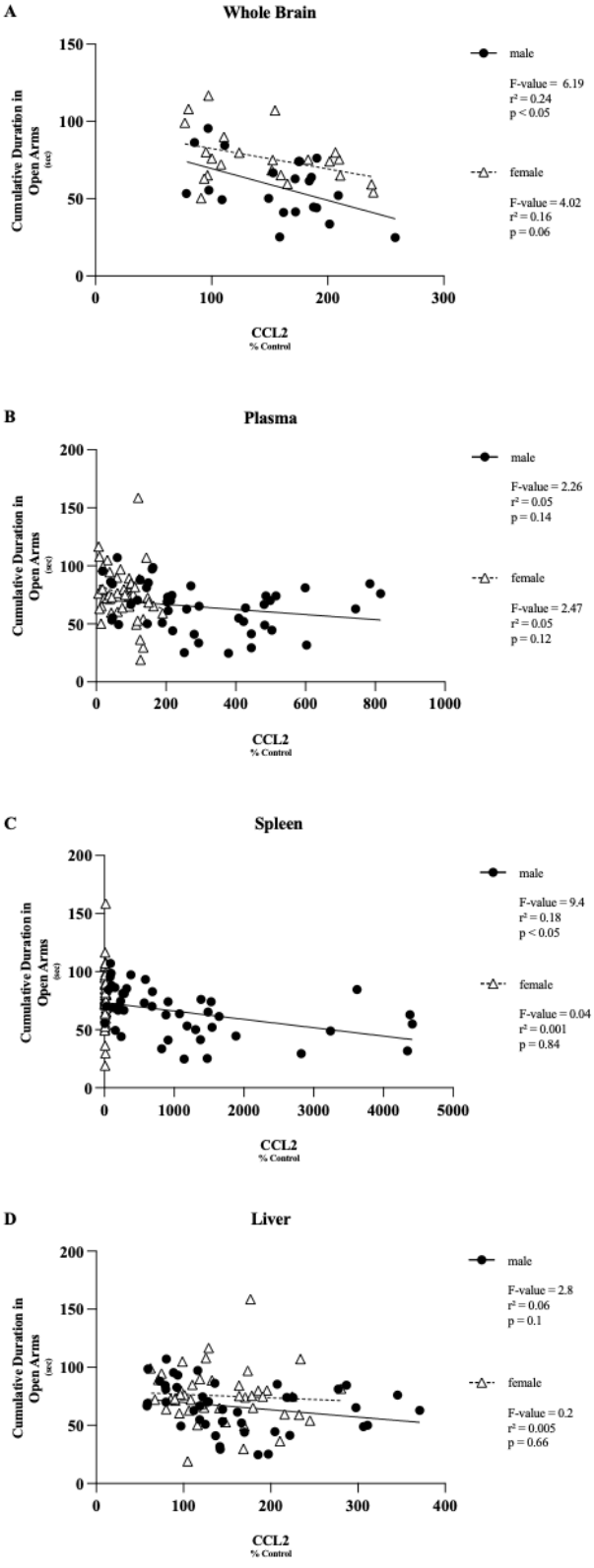

A
Whole Brain
Cumulative Duration in Open Arms (sec)
CCL2 % Control
male
F-value = 6.19
r² = 0.24
p < 0.05
female
F-value = 4.02
r² = 0.16
p = 0.06
B
Plasma
male
F-value = 2.26
r² = 0.05
p = 0.14
female
F-value = 2.47
r² = 0.05
p = 0.12
C
Spleen
male
F-value = 9.4
r² = 0.18
p < 0.05
female
F-value = 0.04
r² = 0.001
p = 0.84
D
Liver
male
F-value = 2.8
r² = 0.06
p = 0.1
female
F-value = 0.2
r² = 0.005
p = 0.66

Fig. 38 LPS-induced CCL2 levels in whole brain, plasma, spleen, and liver are differentially correlated with measures of EPM-cumulative duration in open arms in C57BL/6J mice. Mice (n = 23-24/group or n = 47-48/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted via 5 minute elevated plus maze (EPM), mice were then terminated followed by tissue collection. Levels of CCL2 of whole brain (**A**), plasma (**B**), spleen (**C**), and liver (**D**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Linear regression analysis was used to assess the correlation of whole brain (**A**), plasma (**B**), spleen (**C**), and liver (**D**) CCL2 levels with EPM-cumulative duration in open arms. Linear regression statistics and symbols are provided in figure.

Linear regression analysis was used to assess whether male CCL2 levels in the plasma were correlated with cumulative duration in open arms in the EPM. In both males and females, CCL2 levels in the plasma were not correlated with cumulative duration in open arms ($r^2 = 0.05$, $F = 2.26$, $p = 0.14$; $r^2 = 0.05$, $F = 2.47$, $p = 0.12$) (Fig. 38B). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

Linear regression analysis revealed that in males, CCL2 levels in the spleen were significantly correlated with cumulative duration in open arms in the EPM ($r^2 = 0.18$, $F = 9.4$, $p < 0.05$) (Fig. 38C). However, in females, CCL2 levels in the spleen were not correlated with cumulative duration in open arms ($r^2 = 0.001$, $F = 0.04$, $p = 0.83$) (Fig. 38C). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

Linear regression analysis revealed that in males, CCL2 levels in the liver were not significantly correlated cumulative with duration in open arms in the EPM ($r^2 = 0.06$, $F = 2.8$, $p = 0.1$) (Fig. 38D). Similarly, in females, CCL2 levels in the liver were not correlated with cumulative duration

in open arms ($r^2 = 0.005$, $F = 0.2$, $p = 0.66$) (Fig. 38D). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

These findings show that there are correlations between CCL2 levels in the whole brain and spleen of male mice with cumulative duration in open arms, however, no correlations are found in females.

Correlation between LPS-induced CCL2 levels in colon, proximal small intestine, and distal small intestine with measures of EPM-cumulative duration in open arms in C57BL/6J male and female mice

Linear regression analysis was used to assess whether male CCL2 levels in the colon were correlated with cumulative duration in open arms in the EPM. In males, CCL2 levels in the colon were correlated with cumulative duration in open arms ($r^2 = 0.14$, $F = 6.76$, $p < 0.05$) (Fig. 39A). In females, CCL2 levels in the colon were not correlated with cumulative duration in open arms ($r^2 = 0.02$, $F = 1.01$, $p = 0.32$) (Fig. 39A). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

Fig. 39

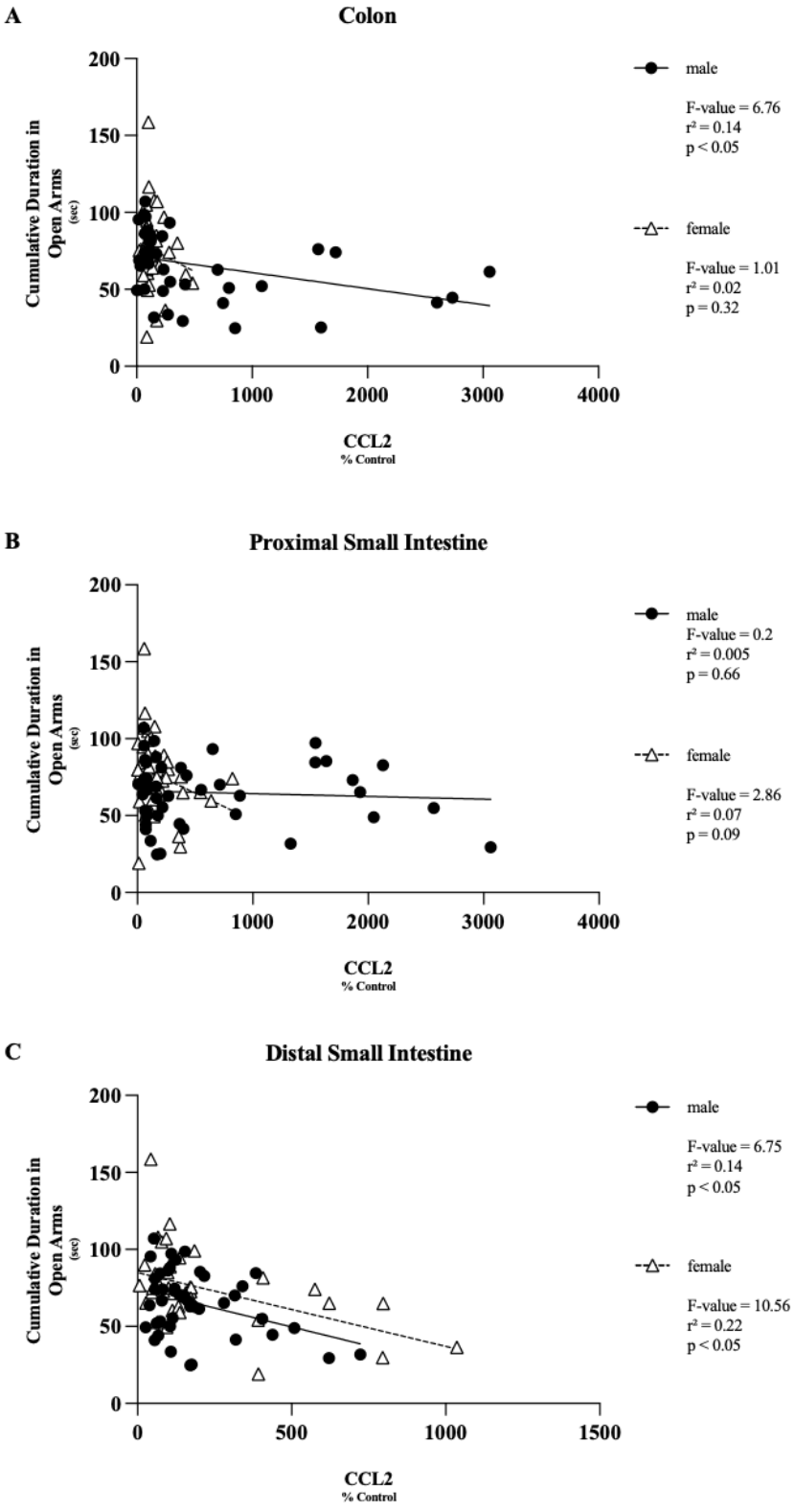

A
Colon
Cumulative Duration in Open Arms (sec)
200
150
100
50
0
0
1000
2000
3000
4000
CCL2
% Control
male
F-value = 6.76
r² = 0.14
p < 0.05
female
F-value = 1.01
r² = 0.02
p = 0.32
B
Proximal Small Intestine
Cumulative Duration in Open Arms (sec)
200
150
100
50
0
0
1000
2000
3000
4000
CCL2
% Control
male
F-value = 0.2
r² = 0.005
p = 0.66
female
F-value = 2.86
r² = 0.07
p = 0.09
C
Distal Small Intestine
Cumulative Duration in Open Arms (sec)
200
150
100
50
0
0
500
1000
1500
CCL2
% Control
male
F-value = 6.75
r² = 0.14
p < 0.05
female
F-value = 10.56
r² = 0.22
p < 0.05

Fig. 39 LPS-induced CCL2 levels in colon, proximal small intestine, and distal small intestine are differentially correlated with measures of EPM-cumulative duration in open arms in C57BL/6J mice. Mice (n = 47-48/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted via 5 minute elevated plus maze (EPM), mice were then terminated followed by tissue collection. Levels of CCL2 of colon (**A**), proximal small intestine (**B**), and distal small intestine (**C**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Linear regression analysis was used to assess the correlation of colon (**A**), proximal small intestine (**B**), and distal small intestine (**C**) CCL2 levels with EPM-cumulative duration in open arms. Linear regression statistics and symbols are provided in figure.

Linear regression analysis was used to assess whether male CCL2 levels in the proximal small intestine were correlated with cumulative duration in open arms in the EPM. In both males and females, CCL2 levels in the proximal small intestine were not correlated with cumulative duration in open arms ($r^2 = 0.005$, $F = 0.2$, $p = 0.66$; $r^2 = 0.07$, $F = 2.86$, $p = 0.09$) (Fig. 39B). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

Linear regression analysis revealed that in males, CCL2 levels in the distal small intestine were significantly correlated with cumulative duration in open arms in the EPM ($r^2 = 0.14$, $F = 6.75$, $p < 0.05$) (Fig. 39C). Similarly, in females, CCL2 levels in the distal small intestine were correlated with cumulative duration in open arms ($r^2 = 0.22$, $F = 10.56$, $p < 0.05$) (Fig. 39C). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

These findings suggest that there is a correlation between CCL2 levels in the male colon and cumulative duration in open arms. Also, in both males and females, there is a correlation between CCL2 levels in the distal small intestine and cumulative duration in open arms.

Correlation between LPS-induced CXCL10 levels in plasma, spleen, and liver with measures of CXCL10 levels in the whole brain in C57BL/6J male and female mice

Linear regression analysis revealed that in males, CXCL10 levels in the plasma were significantly correlated with CXCL10 levels in the whole brain ($r^2 = 0.22$, $F = 16.83$, $p < 0.0005$) (Fig. 40A). Similarly, in females, CXCL10 levels in the plasma were positively correlated with CXCL10 levels in the whole brain ($r^2 = 0.71$, $F = 52.65$, $p < 0.0001$) (Fig. 40A). The x-axis is indicative of the independent value (plasma levels), and the Y-axis is indicating the dependent value (whole brain levels).

Fig. 40

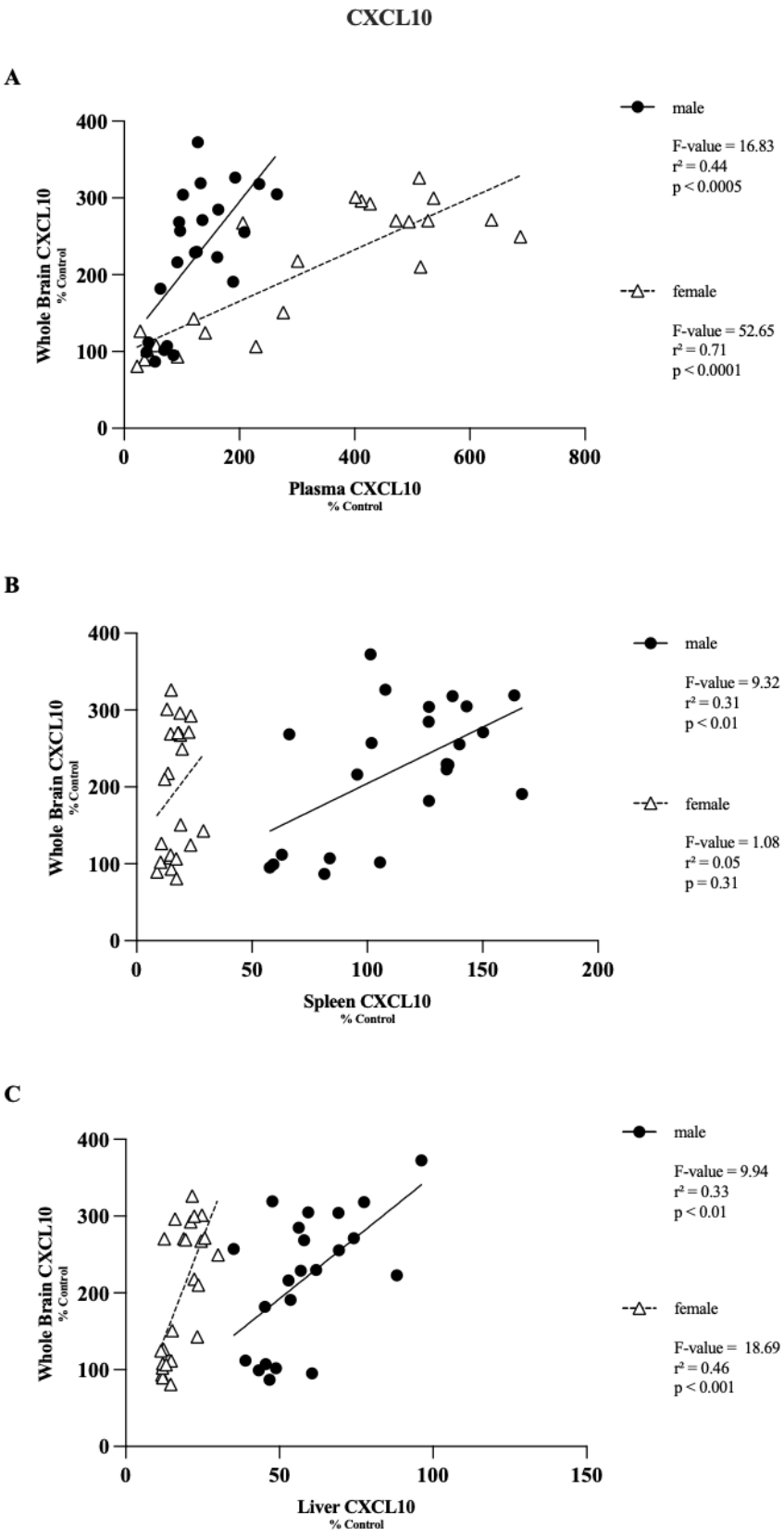

CXCL10
A
Whole Brain CXCL10
% Control
Plasma CXCL10
% Control
male
F-value = 16.83
r² = 0.44
p < 0.0005
female
F-value = 52.65
r² = 0.71
p < 0.0001
B
Whole Brain CXCL10
% Control
Spleen CXCL10
% Control
male
F-value = 9.32
r² = 0.31
p < 0.01
female
F-value = 1.08
r² = 0.05
p = 0.31
C
Whole Brain CXCL10
% Control
Liver CXCL10
% Control
male
F-value = 9.94
r² = 0.33
p < 0.01
female
F-value = 18.69
r² = 0.46
p < 0.001

Fig. 40 LPS-induced CXCL10 levels in plasma, spleen, and liver are differentially correlated with measures of CXCL10 levels in the whole brain in C57BL/6J mice. Mice (n = 23-24/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS mice were then terminated followed by tissue collection. Levels of CXCL10 of plasma (**A**), spleen (**B**), and liver (**C**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Linear regression analysis was used to assess the correlation of plasma (**A**), spleen (**B**), and liver (**C**) CXCL10 levels with CXCL10 levels in the whole brain. Linear regression statistics and symbols are provided in figure.

Linear regression analysis revealed that in males, CXCL10 levels in the spleen were significantly correlated with CXCL10 levels in the whole brain ($r^2 = 0.31$, $F = 9.32$, $p < 0.01$) (Fig. 40B). However, in females, CXCL10 levels in the spleen were not correlated with CXCL10 levels in the whole brain ($r^2 = 0.05$, $F = 1.08$, $p = 0.31$) (Fig. 40B). The x-axis is indicative of the independent value (spleen levels), and the Y-axis is indicating the dependent value (whole brain levels).

Linear regression analysis revealed that in males, CXCL10 levels in the liver were significantly correlated with CXCL10 levels in the whole brain ($r^2 = 0.22$, $F = 9.94$, $p < 0.01$) (Fig. 40C). Similarly, in females, CXCL10 levels in the liver were positively correlated with CXCL10 levels in the whole brain ($r^2 = 0.46$, $F = 18.69$, $p < 0.001$) (Fig. 40C). The x-axis is indicative of the independent value (liver levels), and the Y-axis is indicating the dependent value (whole brain levels).

Based on these findings, there is a correlation between plasma, spleen, and liver and the whole brain CXCL10 expression, except for the female spleen, which showed no correlation between the two tissues.

Correlation between LPS-induced CXCL10 levels in colon, proximal small intestine, and distal small intestine with measures of CXCL10 levels in the whole brain in C57BL/6J male and female mice

Linear regression analysis was used to assess whether male CXCL10 levels in the colon were correlated with CXCL10 levels in the whole brain. In both males and females, CXCL10 levels in the colon were not correlated with CXCL10 levels in the whole brain ($r^2 = 0.06$, $F = 1.26$, $p = 0.28$; $r^2 = 0.02$, $F = 0.34$, $p = 0.57$) (Fig. 41A). The x-axis is indicative of the independent value (colon levels), and the Y-axis is indicating the dependent value (whole brain levels).

Fig. 41

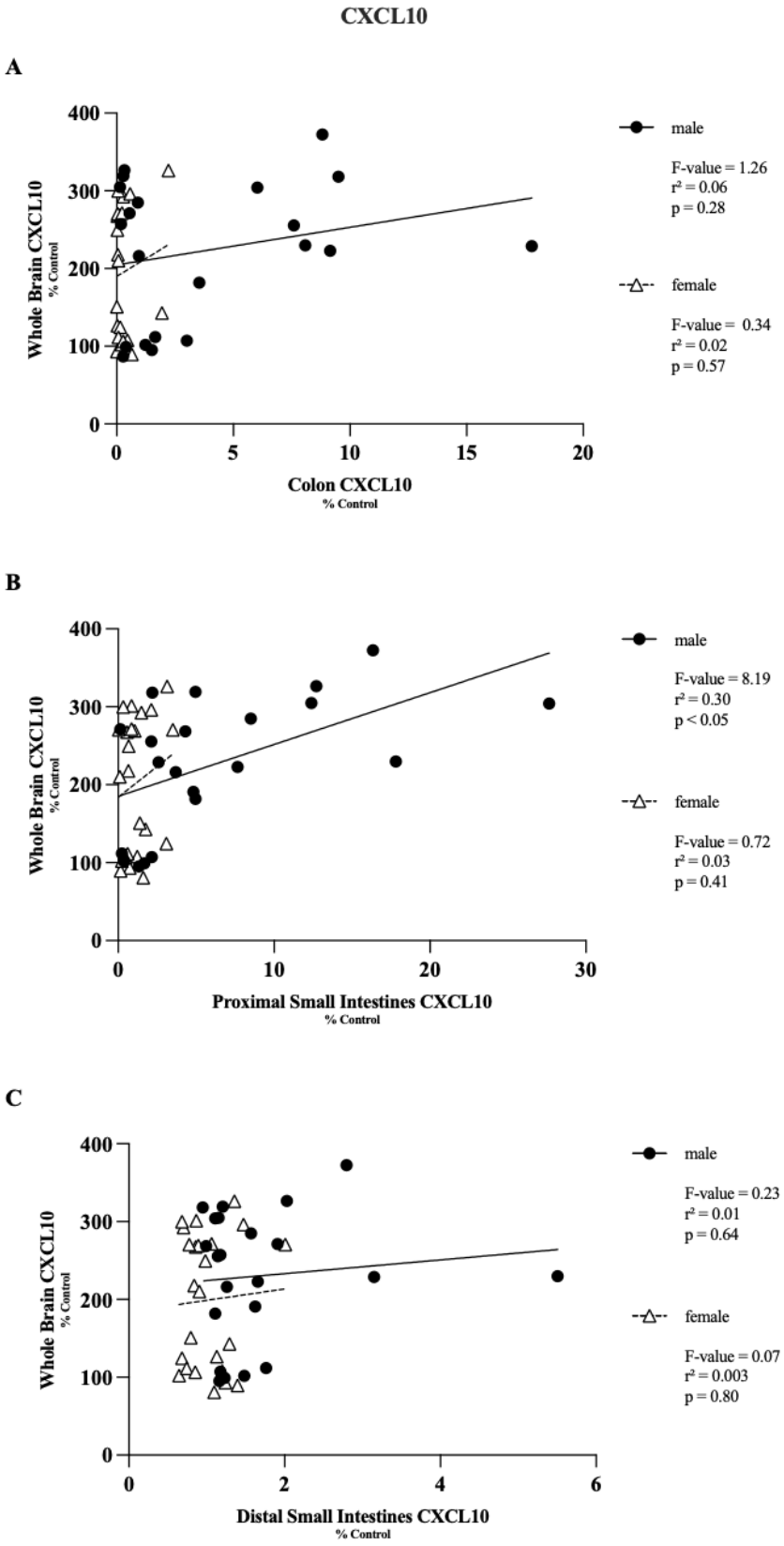

CXCL10
A
Whole Brain CXCL10
% Control
Colon CXCL10
% Control
male
F-value = 1.26
r² = 0.06
p = 0.28
female
F-value = 0.34
r² = 0.02
p = 0.57
B
Whole Brain CXCL10
% Control
Proximal Small Intestines CXCL10
% Control
male
F-value = 8.19
r² = 0.30
p < 0.05
female
F-value = 0.72
r² = 0.03
p = 0.41
C
Whole Brain CXCL10
% Control
Distal Small Intestines CXCL10
% Control
male
F-value = 0.23
r² = 0.01
p = 0.64
female
F-value = 0.07
r² = 0.003
p = 0.80

Fig. 41 LPS-induced CXCL10 levels in colon, proximal small intestine, and distal small intestine are differentially correlated with measures of CXCL10 levels in the whole brain in C57BL/6J mice. Mice (n = 23-24/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS mice were then terminated followed by tissue collection. Levels of CXCL10 of colon (**A**), proximal small intestine (**B**), and distal small intestine (**C**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Linear regression analysis was used to assess the correlation of colon (**A**), proximal small intestine (**B**), and distal small intestine (**C**) CXCL10 levels with CXCL10 levels in the whole brain. Linear regression statistics and symbols are provided in figure.

Linear regression analysis revealed that in males, CXCL10 levels in the proximal small intestine were significantly correlated with CXCL10 levels in the whole brain ($r^2 = 0.30$, $F = 8.19$, $p < 0.05$) (Fig. 41B). However, in females, CXCL10 levels in the proximal small intestine were not correlated with CXCL10 levels in the whole brain ($r^2 = 0.03$, $F = 0.72$, $p = 0.41$) (Fig. 41B). The x-axis is indicative of the independent value (proximal small intestine levels), and the Y-axis is indicating the dependent value (whole brain levels).

Linear regression analysis was used to assess whether male CXCL10 levels in the distal small intestine were correlated with CXCL10 levels in the whole brain. In both males and females, CXCL10 levels in the distal small intestine were not correlated with CXCL10 levels in the whole brain ($r^2 = 0.01$, $F = 0.23$, $p = 0.64$; $r^2 = 0.003$, $F = 0.07$, $p = 0.80$) (Fig. 41C). The x-axis is indicative of the independent value (distal small intestine levels), and the Y-axis is indicating the dependent value (whole brain levels).

Based on these findings, there was a significant correlation between the CXCL10 levels in proximal small intestine of male mice and the whole brain.

Correlation between LPS-induced CCL2 levels in plasma, spleen, and liver with measures of CCL2 levels in the whole brain in C57BL/6J male and female mice

Linear regression analysis revealed that in males, CCL2 levels in the plasma were significantly correlated with CCL2 levels in the whole brain ($r^2 = 0.51$, $F = 21.61$, $p < 0.005$) (Fig. 42A). Similarly, in females, CCL2 levels in the plasma were positively correlated with CCL2 levels in the whole brain ($r^2 = 0.66$, $F = 43.51$, $p < 0.0001$) (Fig. 42A). The x-axis is indicative of the independent value (plasma levels), and the Y-axis is indicating the dependent value (whole brain levels).

Fig. 42

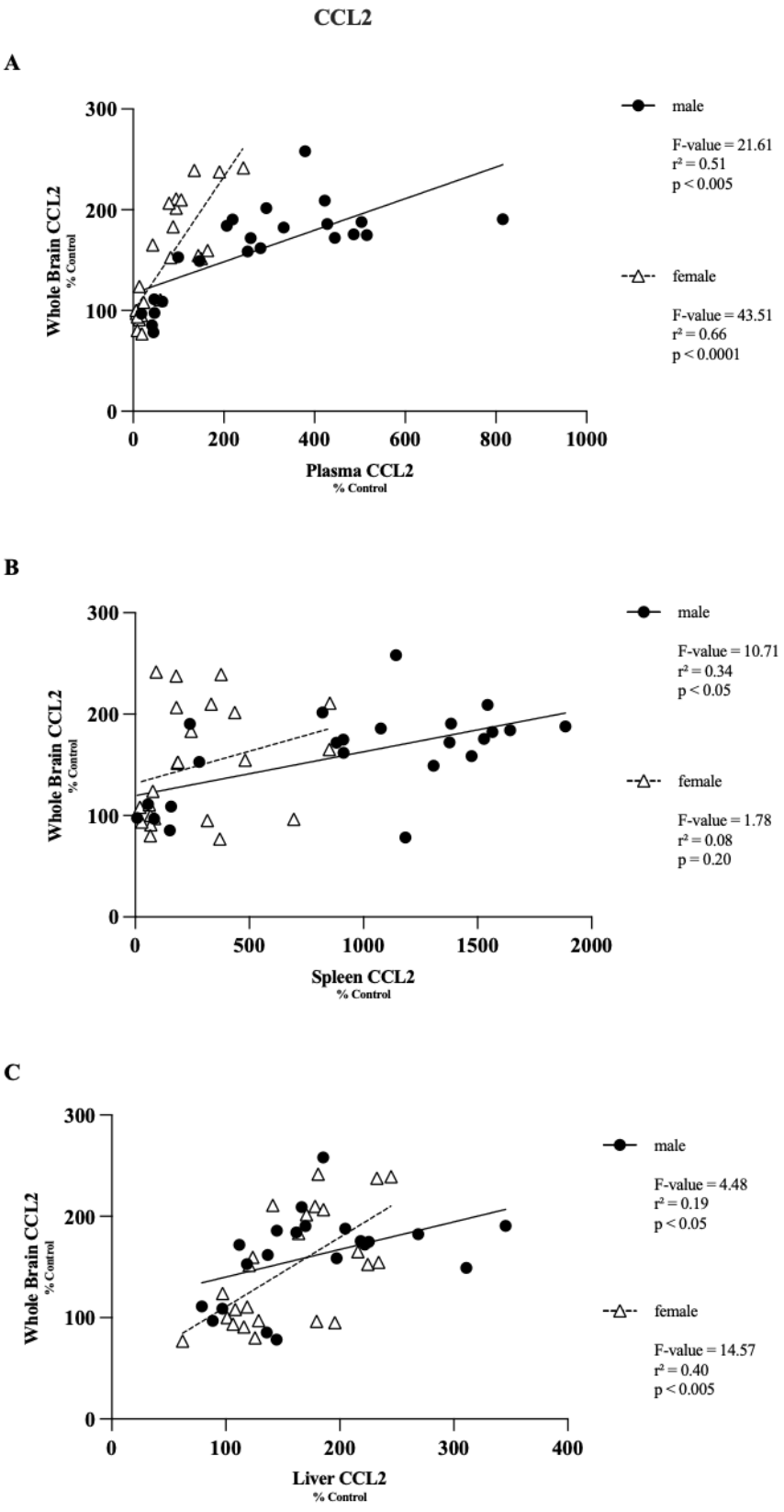

CCL2
A
Whole Brain CCL2
% Control
Plasma CCL2
% Control
male
F-value = 21.61
r² = 0.51
p < 0.005
female
F-value = 43.51
r² = 0.66
p < 0.0001
B
Whole Brain CCL2
% Control
Spleen CCL2
% Control
male
F-value = 10.71
r² = 0.34
p < 0.05
female
F-value = 1.78
r² = 0.08
p = 0.20
C
Whole Brain CCL2
% Control
Liver CCL2
% Control
male
F-value = 4.48
r² = 0.19
p < 0.05
female
F-value = 14.57
r² = 0.40
p < 0.005

Fig. 42 LPS-induced CCL2 levels in plasma, spleen, and liver are differentially correlated with measures of CCL2 levels in the whole brain in C57BL/6J mice. Mice (n = 23-24/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS mice were then terminated followed by tissue collection. Levels of CCL2 of plasma (**A**), spleen (**B**), and liver (**C**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Linear regression analysis was used to assess the correlation of plasma (**A**), spleen (**B**), and liver (**C**) CCL2 levels with CCL2 levels in the whole brain. Linear regression statistics and symbols are provided in figure.

Linear regression analysis revealed that in males, CCL2 levels in the spleen were significantly correlated with CCL2 levels in the whole brain ($r^2 = 0.34$, $F = 10.371$, $p < 0.05$) (Fig. 42B). However, in females, CCL2 levels in the spleen were not correlated with CCL2 levels in the whole brain ($r^2 = 0.08$, $F = 1.78$, $p = 0.20$) (Fig. 42B). The x-axis is indicative of the independent value (spleen levels), and the Y-axis is indicating the dependent value (whole brain levels).

Linear regression analysis revealed that in males, CCL2 levels in the liver were significantly correlated with CCL2 levels in the whole brain ($r^2 = 0.19$, $F = 4.48$, $p < 0.05$) (Fig. 42C). Similarly, in females, CCL2 levels in the liver were positively correlated with CCL2 levels in the whole brain ($r^2 = 0.40$, $F = 14.57$, $p < 0.005$) (Fig. 42C). The x-axis is indicative of the independent value (liver levels), and the Y-axis is indicating the dependent value (whole brain levels).

Based on these findings, there was no correlation between CCL2 levels in the female spleen and the whole brain; however, correlations were present for all other tissue in both males and females.

Correlation between LPS-induced CCL2 levels in colon, proximal small intestine, and distal small intestine with measures of CCL2 levels in the whole brain in C57BL/6J male and female mice

Linear regression analysis was used to assess whether male CCL2 levels in the colon were correlated with CCL2 levels in the whole brain. In both males and females, CCL2 levels in the colon were not correlated with CCL2 levels in the whole brain ($r^2 = 0.19$, $F = 3.99$, $p = 0.06$; $r^2 = 0.06$, $F = 1.27$, $p = 0.27$) (Fig. 43A). The x-axis is indicative of the independent value (colon levels), and the Y-axis is indicating the dependent value (whole brain levels).

Fig. 43

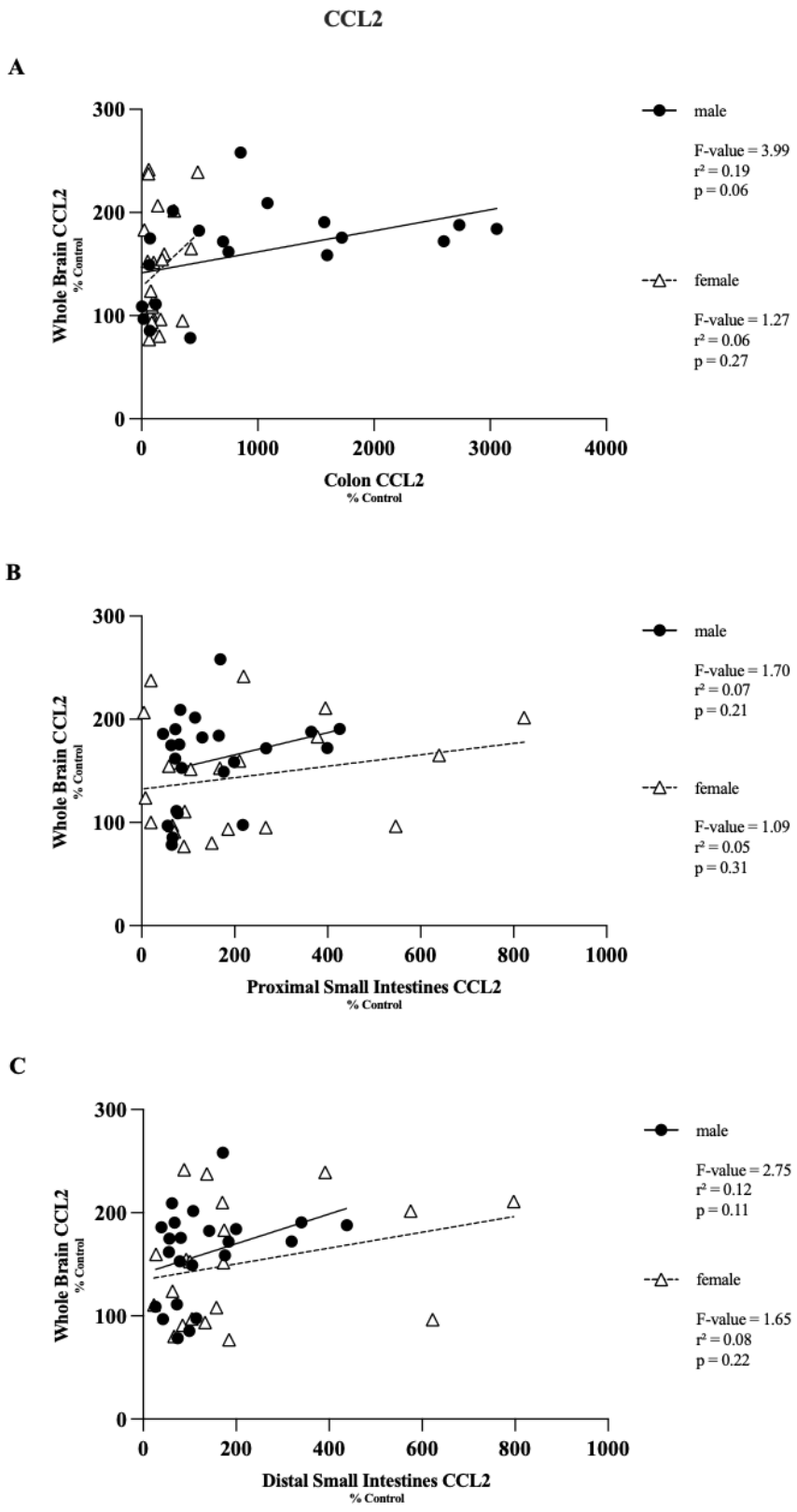

CCL2
A
Whole Brain CCL2
% Control
Colon CCL2
% Control
male
F-value = 3.99
r² = 0.19
p = 0.06
female
F-value = 1.27
r² = 0.06
p = 0.27
B
Whole Brain CCL2
% Control
Proximal Small Intestines CCL2
% Control
male
F-value = 1.70
r² = 0.07
p = 0.21
female
F-value = 1.09
r² = 0.05
p = 0.31
C
Whole Brain CCL2
% Control
Distal Small Intestines CCL2
% Control
male
F-value = 2.75
r² = 0.12
p = 0.11
female
F-value = 1.65
r² = 0.08
p = 0.22

Fig. 43 LPS-induced CCL2 levels in colon, proximal small intestine, and distal small intestine are differentially correlated with measures of CCL2 levels in the whole brain in C57BL/6J mice. Mice (n = 23-24/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS mice were then terminated followed by tissue collection. Levels of CCL2 of colon (**A**), proximal small intestine (**B**), and distal small intestine (**C**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Linear regression analysis was used to assess the correlation of colon (**A**), proximal small intestine (**B**), and distal small intestine (**C**) CCL2 levels with CCL2 levels in the whole brain. Linear regression statistics and symbols are provided in figure.

Linear regression analysis was used to assess whether male CCL2 levels in the proximal small intestine were correlated with CCL2 levels in the whole brain. In both males and females, CCL2 levels in the proximal small intestine were not correlated with CCL2 levels in the whole brain (r^2 = 0.07, F = 1.70, p = 0.21; r^2 = 0.05, F = 1.09, p = 0.31) (Fig. 43B). The x-axis is indicative of the independent value (proximal small intestine levels), and the Y-axis is indicating the dependent value (whole brain levels).

Linear regression analysis was used to assess whether male CCL2 levels in the distal small intestine were correlated with CCL2 levels in the whole brain. In both males and females, CCL2 levels in the distal small intestine were not correlated with CCL2 levels in the whole brain (r^2 = 0.12, F = 2.75, p = 0.11; r^2 = 0.08, F = 1.65, p = 0.22) (Fig. 43C). The x-axis is indicative of the independent value (distal small intestine levels), and the Y-axis is indicating the dependent value (whole brain levels).

Based on these findings, there were no significant correlations between the CXLC10 levels in the colon, proximal small intestine, and distal small intestine and CXCL10 levels in the whole brain.

Multiple linear regression between LPS-induced CXCL10 levels in whole brain, plasma, spleen, and liver with measures of OFT-distance moved in C57BL/6J male and female mice

A multiple linear regression model was conducted to determine if sexes differ in sickness-like behavior response as the concentration of CXCL10 in the brain changes. The results of the multiple linear regression suggest that distance moved was predicted by change in CXCL10 levels in the brain ($F(2, 43) = 5.64$, $p < 0.01$) (Fig. 44A). However, in sexes, their behavioral responses did not differ based on changes in CXCL10 levels in the brain ($F(1, 43) = 0.7460$, $p = 0.39$). The x-axis indicates the independent value (CXCL10 level), and the Y-axis shows the dependent value (distance moved).

Fig. 44

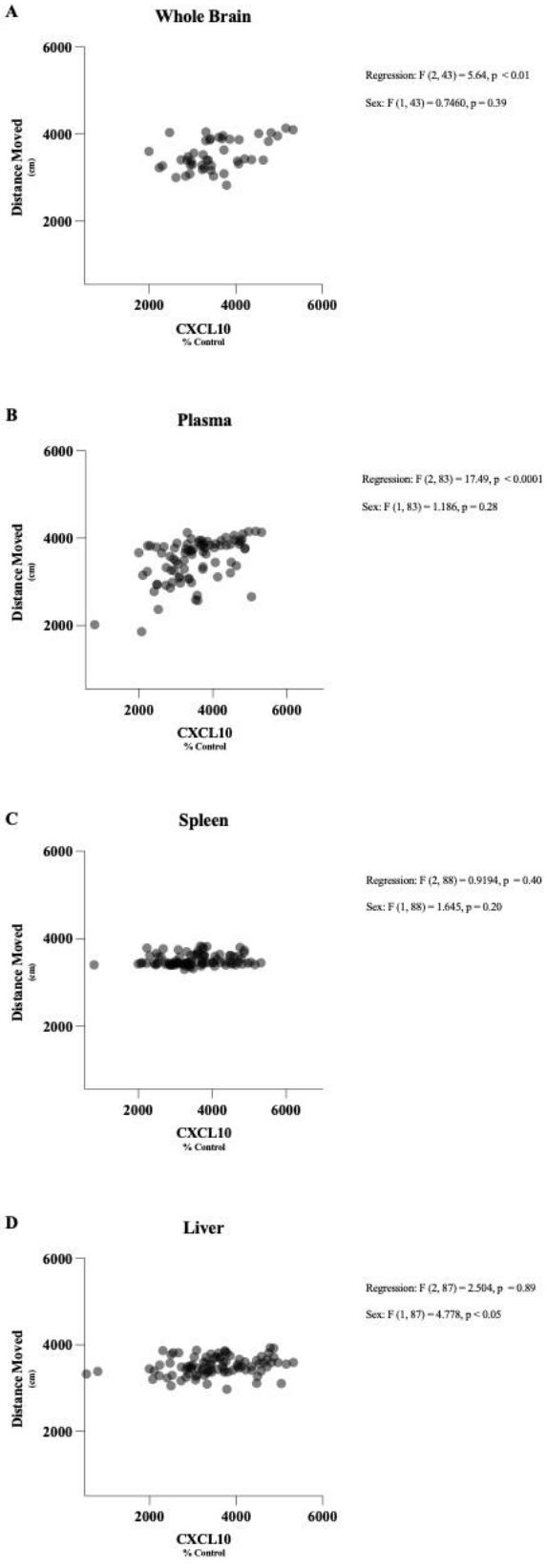

A
Whole Brain
Distance Moved (cm)
6000
4000
2000
2000
4000
6000
CXCL10 % Control
Regression: F (2, 43) = 5.64, p < 0.01
Sex: F (1, 43) = 0.7460, p = 0.39
B
Plasma
Distance Moved (cm)
6000
4000
2000
2000
4000
6000
CXCL10 % Control
Regression: F (2, 83) = 17.49, p < 0.0001
Sex: F (1, 83) = 1.186, p = 0.28
C
Spleen
Distance Moved (cm)
6000
4000
2000
2000
4000
6000
CXCL10 % Control
Regression: F (2, 88) = 0.9194, p = 0.40
Sex: F (1, 88) = 1.645, p = 0.20
D
Liver
Distance Moved (cm)
6000
4000
2000
2000
4000
6000
CXCL10 % Control
Regression: F (2, 87) = 2.504, p = 0.89
Sex: F (1, 87) = 4.778, p < 0.05

Fig. 44 Multiple linear regression between male and female C57BL/6J mice LPS-induced CXCL10 levels in whole brain, plasma, spleen, and liver with measures of OFT-distance moved. Mice (n = 23-24/group or n = 47-48/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted via 10 minute open-field test (OFT), mice were then terminated followed by tissue collection. Levels of CXCL10 of whole brain (**A**), plasma (**B**), spleen (**C**), and liver (**D**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Multiple linear regression analysis was used to assess the correlation of whole brain (**A**), plasma (**B**), spleen (**C**), and liver (**D**) in male and female mice and CXCL10 levels with OFT-distance moved. Multiple linear regression statistics and symbols are provided in figure.

A multiple linear regression model suggests that distance moved was predicted by change in CXCL10 levels in the plasma ($F (2, 83) = 17.49$, $p < 0.0001$), but in sexes, their behavioral responses did not differ based on changes in CXCL10 levels in the plasma ($F (1,83) = 1.186$, $p = 0.28$) (Fig. 44B). The x-axis indicates the independent value (CXCL10 level), and the Y-axis shows the dependent value (distance moved).

In the spleen, a multiple linear regression model did not show changes in CXCL10 levels and distance moved ($F (2, 88) = 0.9194$, $p = 0.40$) (Fig. 44C). When sex was assessed as a factor, no statically significant results were found ($F (1,88) = 1.645$, $p = 0.20$). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis indicates the dependent value (distance moved).

Multiple regression indicates changes in CXCL10 levels in the liver were not correlated with distance moved ($F (2, 87) = 2.504$, $p = 0.89$) (Fig. 44D). However, there was a significant difference between sexes and their behavioral responses based on changes in CXCL10 levels ($F (1,87) =$

4.778, $p < 0.05$) (Fig. 44D). The x-axis indicates the independent value (CXCL10 level), and the Y-axis shows the dependent value (distance moved).

These results indicate that sexes differ in sickness-like behavior response as the concentration of CXCL10 in the liver change.

Multiple linear regression between LPS-induced CXCL10 levels in colon, proximal small intestine, and distal small intestine with measures of OFT-distance moved in C57BL/6J male and female mice

A multiple linear regression model was conducted to determine if sexes differ in sickness-like behavior response as the concentration of CXCL10 in the colon changes. The results of the multiple linear regression suggest that distance moved was not predicted by change in CXCL10 levels in the colon ($F (2, 82) = 0.9421$, $p = 0.39$) (Fig. 45A). Similarly, in sexes, their behavioral responses did not differ based on changes in CXCL10 levels in the colon ($F (1, 43) = 0.7460$, $p = 0.39$). The x-axis indicates the independent value (CXCL10 level), and the Y-axis shows the dependent value (distance moved).

Fig. 45

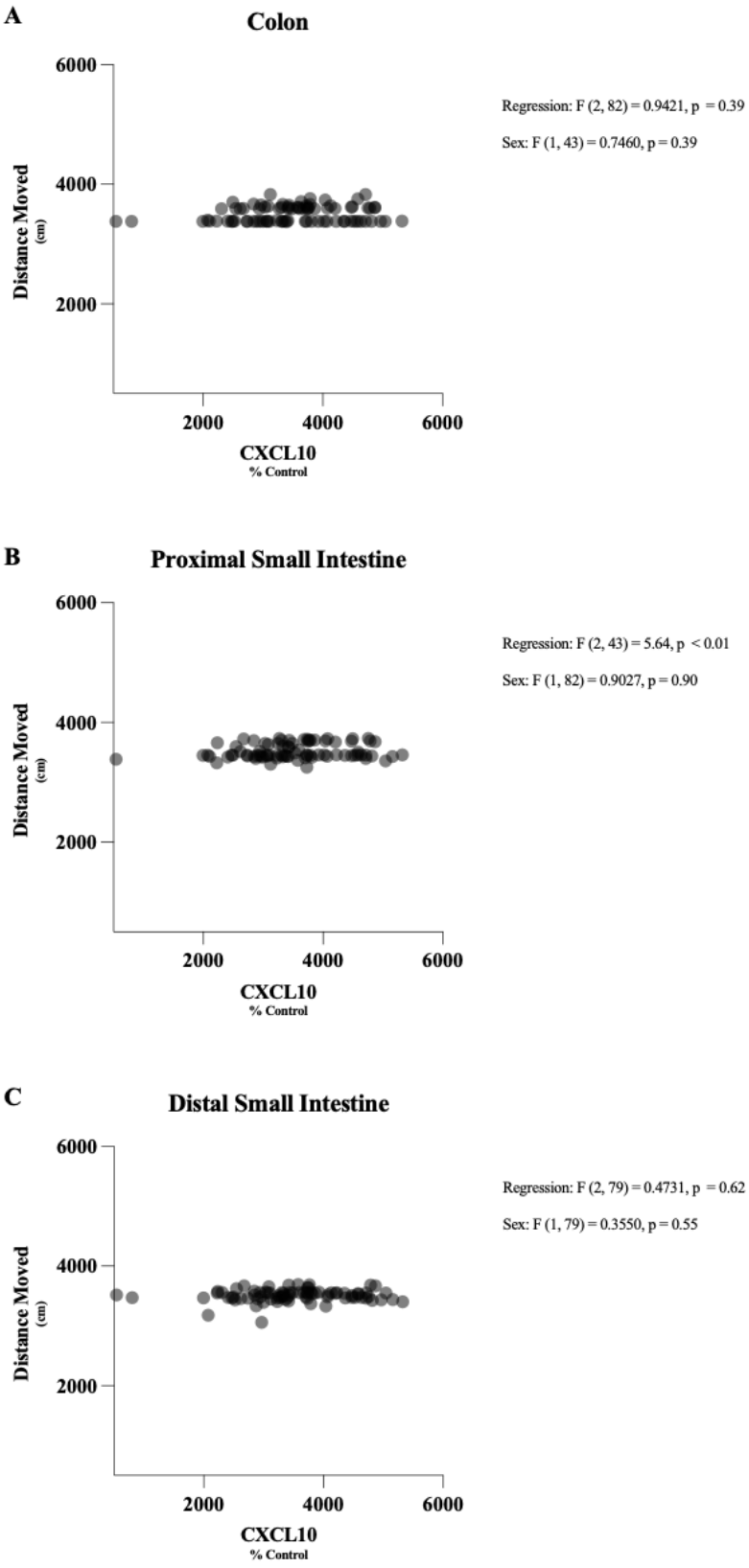

A
Colon
6000
4000
2000
Distance Moved
(cm)
2000
4000
6000
CXCL10
% Control
Regression: F (2, 82) = 0.9421, p = 0.39
Sex: F (1, 43) = 0.7460, p = 0.39
B
Proximal Small Intestine
6000
4000
2000
Distance Moved
(cm)
2000
4000
6000
CXCL10
% Control
Regression: F (2, 43) = 5.64, p < 0.01
Sex: F (1, 82) = 0.9027, p = 0.90
C
Distal Small Intestine
6000
4000
2000
Distance Moved
(cm)
2000
4000
6000
CXCL10
% Control
Regression: F (2, 79) = 0.4731, p = 0.62
Sex: F (1, 79) = 0.3550, p = 0.55

Fig. 45 Multiple linear regression between male and female C57BL/6J mice LPS-induced CXCL10 levels in colon, proximal small intestine, and distal small intestine with measures of OFT-distance moved. Mice (n = 47-48/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted via 10 minute open-field test (OFT), mice were then terminated followed by tissue collection. Levels of CXCL10 of colon (**A**), proximal small intestine (**B**), and distal small intestine (**C**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Multiple linear regression analysis was used to assess the correlation of colon (**A**), proximal small intestine (**B**), and distal small intestine (**C**) in male and female mice and CXCL10 levels with OFT-distance moved. Multiple linear regression statistics and symbols are provided in figure.

A multiple linear regression model suggests that distance moved was predicted by change in CXCL10 levels in the proximal small intestine ($F(2, 43) = 5.64$, $p < 0.01$), but in sexes, their behavioral responses did not differ based on changes in CXCL10 levels in the proximal small intestine ($F(1,82) = 0.9027$, $p = 0.90$) (Fig. 45B). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (distance moved).

In the distal small intestine, a multiple linear regression model did not show changes in CXCL10 levels and distance moved ($F(2, 79) = 0.4731$, $p = 0.62$) (Fig. 45C). When sex was assessed as a factor, no statically significant results were found ($F(1,79) = 0.3550$, $p = 0.55$). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis indicates the dependent value (distance moved).

These results indicate that sexes did not differ in sickness-like behavior response as the concentration of CXCL10 in the colon, proximal small intestine, or distal small intestine changed.

Multiple linear regression between LPS-induced CXCL10 levels in whole brain, plasma, spleen, and liver with measures of EPM-cumulative duration in open arms in C57BL/6J male and female mice

A multiple linear regression model was conducted to determine if sexes differ in anxiety-like behavior response as the concentration of CXCL10 in the brain changes. The results of the multiple linear regression suggest that cumulative duration in open arms was predicted by change in CXCL10 levels in the brain ($F(2, 42) = 11.12$, $p < 0.0001$) (Fig. 46A). Similarly, in sexes, their behavioral responses did differ based on changes in CXCL10 levels in the brain ($F(1, 42) = 10.73$, $p < 0.01$). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

Fig. 46

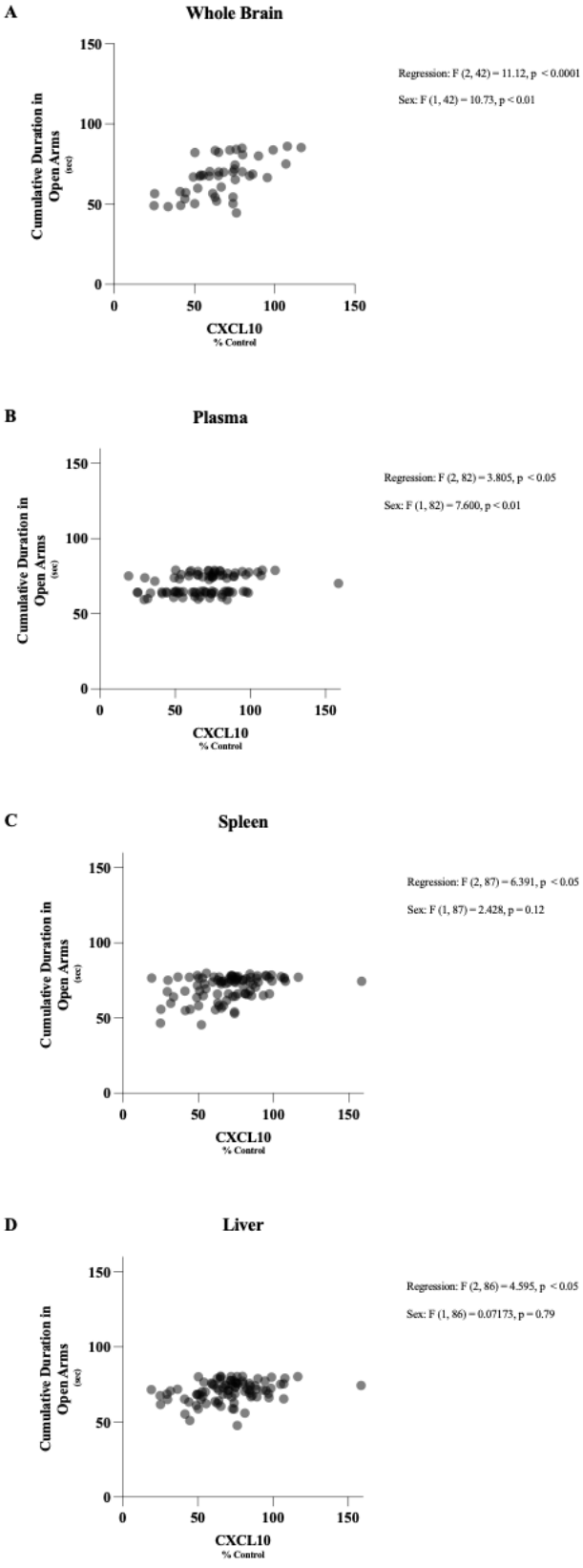

A
Whole Brain
Cumulative Duration in Open Arms (sec)
CXCL10 % Control
Regression: F (2, 42) = 11.12, p < 0.0001
Sex: F (1, 42) = 10.73, p < 0.01
B
Plasma
Cumulative Duration in Open Arms (sec)
CXCL10 % Control
Regression: F (2, 82) = 3.805, p < 0.05
Sex: F (1, 82) = 7.600, p < 0.01
C
Spleen
Cumulative Duration in Open Arms (sec)
CXCL10 % Control
Regression: F (2, 87) = 6.391, p < 0.05
Sex: F (1, 87) = 2.428, p = 0.12
D
Liver
Cumulative Duration in Open Arms (sec)
CXCL10 % Control
Regression: F (2, 86) = 4.595, p < 0.05
Sex: F (1, 86) = 0.07173, p = 0.79

Fig. 46 Multiple linear regression between male and female C57BL/6J mice LPS-induced CXCL10 levels in whole brain, plasma, spleen, and liver with measures of EPM-cumulative duration in open arms. Mice (n = 23-24/group or n = 47-48/group were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted via 5 minute elevated plus maze (EPM), mice were then terminated followed by tissue collection. Levels of CXCL10 of whole brain (**A**), plasma (**B**), spleen (**C**), and liver (**D**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Multiple linear regression analysis was used to assess the correlation of whole brain (**A**), plasma (**B**), spleen (**C**), and liver (**D**) in male and female mice and CXCL10 levels with EPM-cumulative duration in open arms. Multiple linear regression statistics and symbols are provided in figure.

A multiple linear regression model suggests that cumulative duration in open arms was predicted by change in CXCL10 levels in the plasma ($F(2, 82) = 3.805$, $p < 0.05$), and in sexes, their behavioral responses did differ based on changes in CXCL10 levels in the plasma ($F(1,82) = 7.600$, $p < 0.01$) (Fig. 46B). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

In the spleen, a multiple linear regression model did show changes in CXCL10 levels and cumulative duration in open arms ($F(2, 87) = 6.391$, $p < 0.05$) (Fig. 46C). When sex was assessed as a factor, no statically significant results were found ($F(1,87) = 2.428$, $p = 0.12$). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

Multiple regression indicates changes in CXCL10 levels in the liver were correlated with cumulative duration in open arms ($F(2, 86) = 4.595$, $p < 0.05$), however, there was no difference between sexes and their behavioral responses based on changes in CXCL10 levels ($F(1,86) =$

0.07173, p = 0.79) (Fig. 46D). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

These results indicate that the sexes did differ in anxiety-like behavior response as the concentration of CXCL10 in the whole brain and plasma changed.

Multiple linear regression between LPS-induced CXCL10 levels in colon, proximal small intestine, and distal small intestine with measures of EPM- cumulative duration in open arms in C57BL/6J male and female mice

A multiple linear regression model was conducted to determine if sexes differ in anxiety-like behavior response as the concentration of CXCL10 in the colon changes. The results of the multiple linear regression suggest that cumulative duration in open arms was not predicted by change in CXCL10 levels in the colon (F (2, 82) = 1.972, p = 0.15) (Fig. 47A). Similarly, in sexes, their behavioral responses did not differ based on changes in CXCL10 levels in the colon (F (1, 82) = 0.7475, p = 0.39). The x-axis indicates the independent value (CXCL10 level), and the Y-axis shows the dependent value (cumulative duration in open arms).

Fig. 47

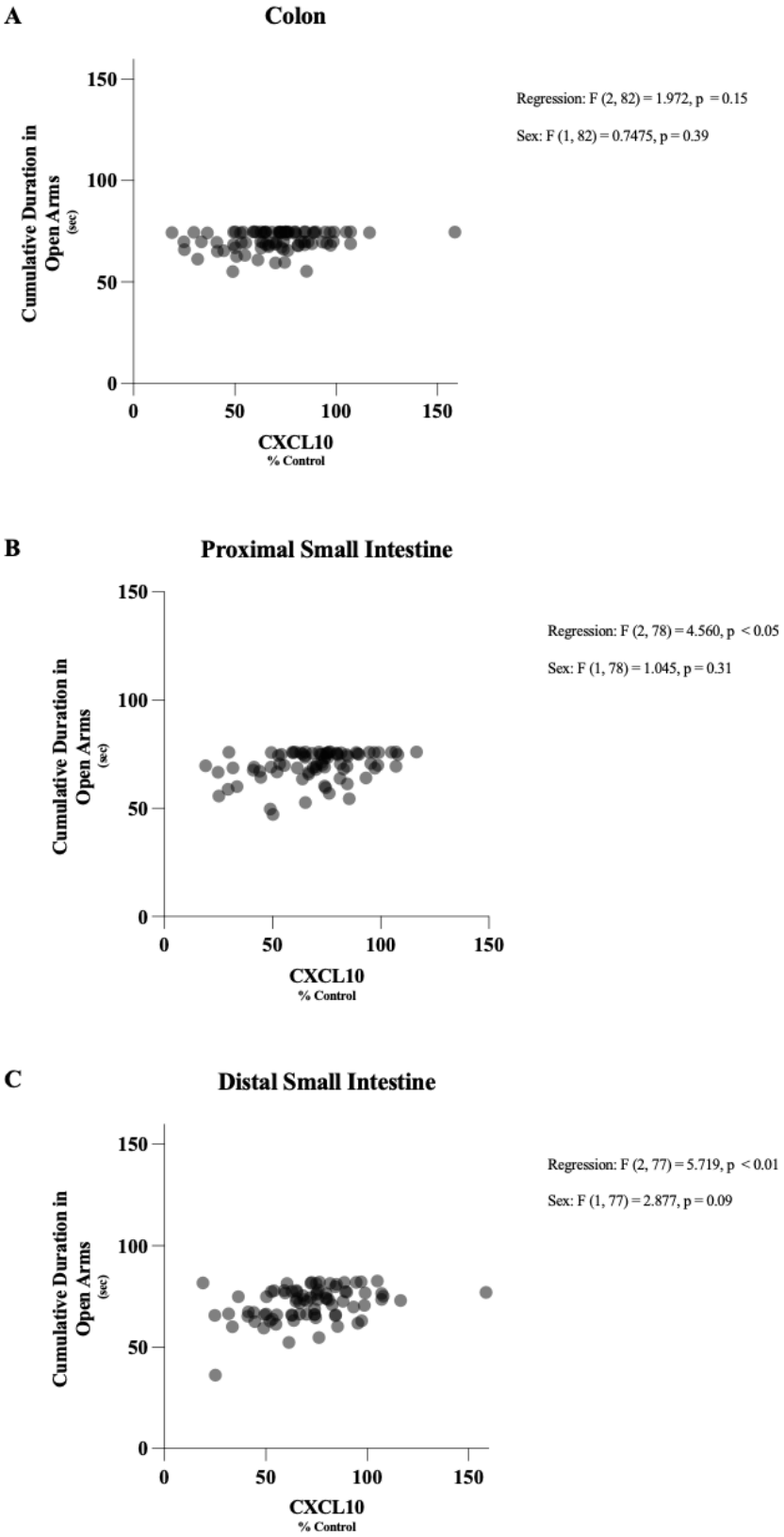

A
Colon
Cumulative Duration in Open Arms (sec)
150
100
50
0
0
50
100
150
CXCL10
% Control
Regression: F (2, 82) = 1.972, p = 0.15
Sex: F (1, 82) = 0.7475, p = 0.39
B
Proximal Small Intestine
Cumulative Duration in Open Arms (sec)
150
100
50
0
0
50
100
150
CXCL10
% Control
Regression: F (2, 78) = 4.560, p < 0.05
Sex: F (1, 78) = 1.045, p = 0.31
C
Distal Small Intestine
Cumulative Duration in Open Arms (sec)
150
100
50
0
0
50
100
150
CXCL10
% Control
Regression: F (2, 77) = 5.719, p < 0.01
Sex: F (1, 77) = 2.877, p = 0.09

Fig. 47 Multiple linear regression between male and female C57BL/6J mice LPS-induced CXCL10 levels in colon, proximal small intestine, and distal small intestine with measures of EPM-cumulative duration in open arms. Mice (n = 47-48/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted via 5 minute elevated plus maze (EPM), mice were then terminated followed by tissue collection. Levels of CXCL10 of colon (**A**), proximal small intestine (**B**), and distal small intestine (**C**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Multiple linear regression analysis was used to assess the correlation of colon (**A**), proximal small intestine (**B**), and distal small intestine (**C**) in male and female mice and CXCL10 levels with EPM-cumulative duration in open arms. Multiple linear regression statistics and symbols are provided in figure.

A multiple linear regression model suggests that cumulative duration in open arms was predicted by change in CXCL10 levels in the proximal small intestine ($F (2, 78) = 4.560$, $p < 0.05$), but in sexes, their behavioral responses did not differ based on changes in CXCL10 levels in the proximal small intestine ($F (1,78) = 1.045$, $p = 0.31$) (Fig. 47B). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

In the distal small intestine, a multiple linear regression model did show changes in CXCL10 levels and cumulative duration in open arms ($F (2, 77) = 5.719$, $p < 0.01$) (Fig. 47C). When sex was assessed as factor no statically significant results were found ($F (1,77) = 2.877$, $p = 0.09$). The x-axis is indicative of the independent value (CXCL10 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

These results indicate that sexes did not differ in anxiety-like behavior response as the concentration of CXCL10 in the colon, proximal small intestine, or distal small intestine changed.

Multiple linear regression between LPS-induced CCL2 levels in whole brain, plasma, spleen, and liver with measures of OFT-distance moved in C57BL/6J male and female mice

A multiple linear regression model was conducted to determine if sexes differ in sickness-like behavior response as the concentration of CCL2 in the brain changes. The results of the multiple linear regression suggest that distance moved was predicted by change in CCL2 levels in the brain ($F(2, 43) = 5.6964$, $p < 0.01$) (Fig. 48A). However, in sexes, their behavioral responses did not differ based on changes in CCL2 levels in the brain ($F(1, 43) = 0.8793$, $p = 0.35$). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (distance moved).

Fig. 48

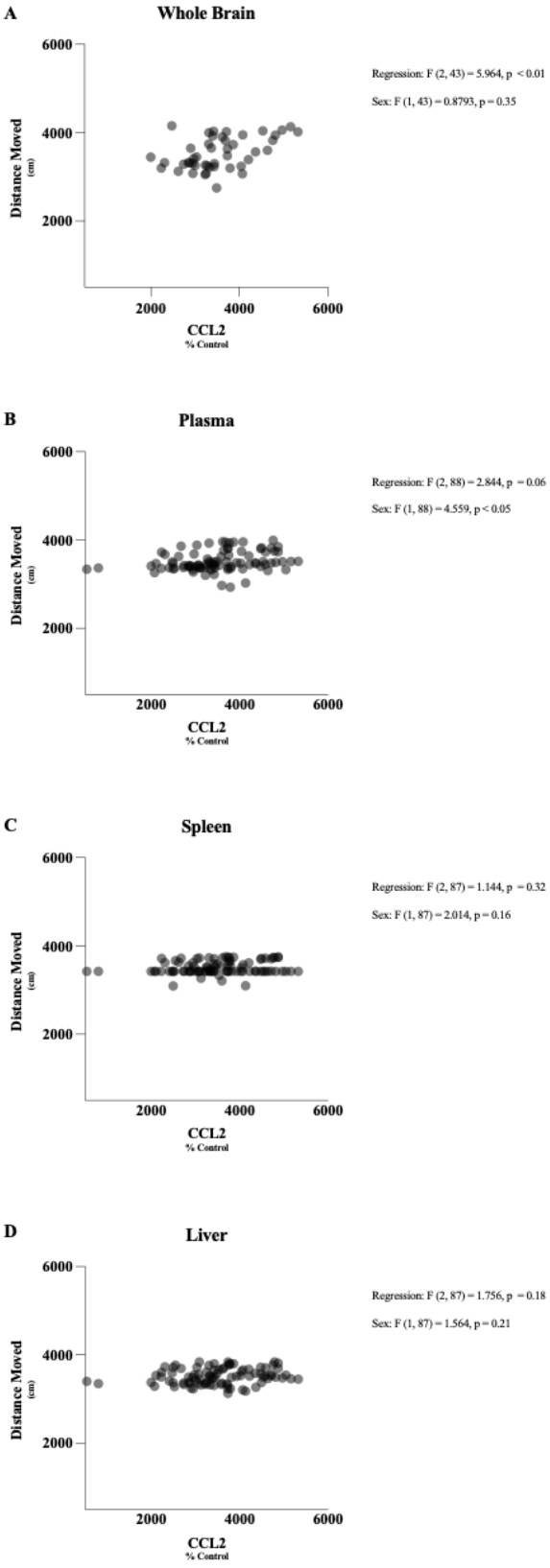

A
Whole Brain
Distance Moved (cm)
6000
4000
2000
2000
4000
6000
CCL2 % Control
Regression: F (2, 43) = 5.964, p < 0.01
Sex: F (1, 43) = 0.8793, p = 0.35
B
Plasma
Distance Moved (cm)
6000
4000
2000
2000
4000
6000
CCL2 % Control
Regression: F (2, 88) = 2.844, p = 0.06
Sex: F (1, 88) = 4.559, p < 0.05
C
Spleen
Distance Moved (cm)
6000
4000
2000
2000
4000
6000
CCL2 % Control
Regression: F (2, 87) = 1.144, p = 0.32
Sex: F (1, 87) = 2.014, p = 0.16
D
Liver
Distance Moved (cm)
6000
4000
2000
2000
4000
6000
CCL2 % Control
Regression: F (2, 87) = 1.756, p = 0.18
Sex: F (1, 87) = 1.564, p = 0.21

Fig. 48 Multiple linear regression between male and female C57BL/6J mice LPS-induced CCL2 levels in whole brain, plasma, spleen, and liver with measures of OFT-distance moved. Mice (n = 23-24/group or n = 47-48/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted via 10 minute open-field test (OFT), mice were then terminated followed by tissue collection. Levels of CCL2 of whole brain (**A**), plasma (**B**), spleen (**C**), and liver (**D**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Multiple linear regression analysis was used to assess the correlation of whole brain (**A**), plasma (**B**), spleen (**C**), and liver (**D**) in male and female mice and CCL2 levels with OFT-distance moved. Multiple linear regression statistics and symbols are provided in figure.

A multiple linear regression model suggests that distance moved was not predicted by change in CCL2 levels in the plasma ($F(2, 88) = 2.844$, $p = 0.06$), but in sexes, their behavioral responses did differ based on changes in CCL2 levels in the plasma ($F(1,88) = 4.559$, $p < 0.05$) (Fig. 48B). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (distance moved).

In the spleen, a multiple linear regression model did not show changes in CCL2 levels and distance moved ($F(2, 87) = 1.144$, $p = 0.32$) (Fig. 48C). When sex was assessed as a factor, no statically significant results were found ($F(1,87) = 2.014$, $p = 0.16$). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (distance moved).

Multiple regression indicates changes in CCL2 levels in the liver were not correlated with distance moved ($F(2, 87) = 1.756$, $p = 0.18$); similarly, there was no difference between sexes and their behavioral responses based on changes in CCL2 levels ($F(1, 87) = 1.564$, $p = 0.21$) (Fig.48D). The

x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (distance moved).

These results indicate that the sexes did differ in sickness-like behavior response as the concentration of CCL2 in the plasma changes.

Multiple linear regression between LPS-induced CCL2 levels in colon, proximal small intestine, and distal small intestine with measures of OFT-distance moved in C57BL/6J male and female mice

A multiple linear regression model was conducted to determine if sexes differ in sickness-like behavior response as the concentration of CCL2 in the colon changes. The results of the multiple linear regression suggest that distance moved was not predicted by change in CCL2 levels in the colon ($F(2, 82) = 0.8122$, $p = 0.45$) (Fig. 49A). Similarly, in sexes, their behavioral responses did not differ based on changes in CCL2 levels in the colon ($F(1, 82) = 1.213$, $p = 0.27$). The x-axis indicates the independent value (CCL2 level), and the Y-axis shows the dependent value (distance moved).

Fig. 49

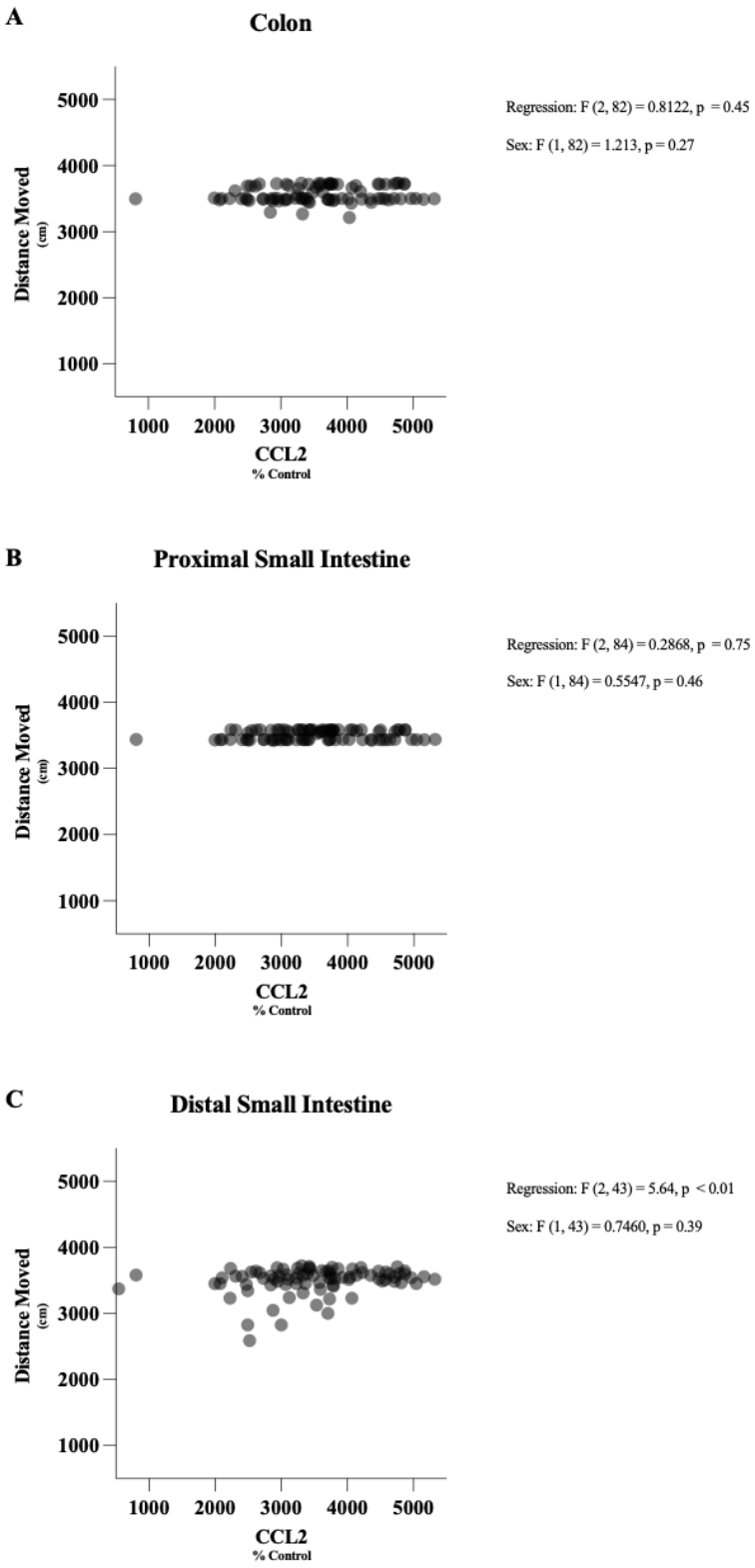

A
Colon
Distance Moved (cm)
5000
4000
3000
2000
1000
1000 2000 3000 4000 5000
CCL2
% Control
Regression: F (2, 82) = 0.8122, p = 0.45
Sex: F (1, 82) = 1.213, p = 0.27
B
Proximal Small Intestine
Distance Moved (cm)
5000
4000
3000
2000
1000
1000 2000 3000 4000 5000
CCL2
% Control
Regression: F (2, 84) = 0.2868, p = 0.75
Sex: F (1, 84) = 0.5547, p = 0.46
C
Distal Small Intestine
Distance Moved (cm)
5000
4000
3000
2000
1000
1000 2000 3000 4000 5000
CCL2
% Control
Regression: F (2, 43) = 5.64, p < 0.01
Sex: F (1, 43) = 0.7460, p = 0.39

Fig. 49 Multiple linear regression between male and female C57BL/6J mice LPS-induced CCL2 levels in colon, proximal small intestine, and distal small intestine with measures of OFT-distance moved. Mice (n = 47-48/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted via 10 minute open-field test (OFT), mice were then terminated followed by tissue collection. Levels of CCL2 of colon (**A**), proximal small intestine (**B**), and distal small intestine (**C**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Multiple linear regression analysis was used to assess the correlation of colon (**A**), proximal small intestine (**B**), and distal small intestine (**C**) in male and female mice and CCL2 levels with OFT-distance moved. Multiple linear regression statistics and symbols are provided in figure.

A multiple linear regression model suggests that distance moved was not predicted by change in CCL2 levels in the proximal small intestine ($F(2, 84) = 0.2868$, $p = 0.75$); similarly, in sexes, their behavioral responses did not differ based on changes in CCL2 levels in the proximal small intestine ($F(1,84) = 0.5547$, $p = 0.46$) (Fig. 49B). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (distance moved).

In the distal small intestine, a multiple linear regression model did show changes in CCL2 levels and distance moved ($F(2, 43) = 0.5.64$, $p < 0.01$) (Fig. 49C). When sex was assessed as a factor, no statically significant results were found ($F(1,43) = 0.7460$, $p = 0.39$). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (distance moved).

These results indicate that sexes did not differ in sickness-like behavior response as the concentration of CCL2 in the colon, proximal small intestine, or distal small intestine changed.

Multiple linear regression between LPS-induced CCL2 levels in whole brain, plasma, spleen, and liver with measures of EPM-cumulative duration in open arms in C57BL/6J male and female mice

A multiple linear regression model was conducted to determine if sexes differ in anxiety-like behavior response as the concentration of CCL2 in the brain changes. The results of the multiple linear regression suggest that cumulative duration in open arms was predicted by change in CCL2 levels in the brain ($F(2, 42) = 12.37$, $p < 0.0001$) (Fig. 50A). Similarly, in sexes, their behavioral responses did differ based on changes in CCL2 levels in the brain ($F(1, 42) = 11.30$, $p < 0.01$). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

Fig. 50

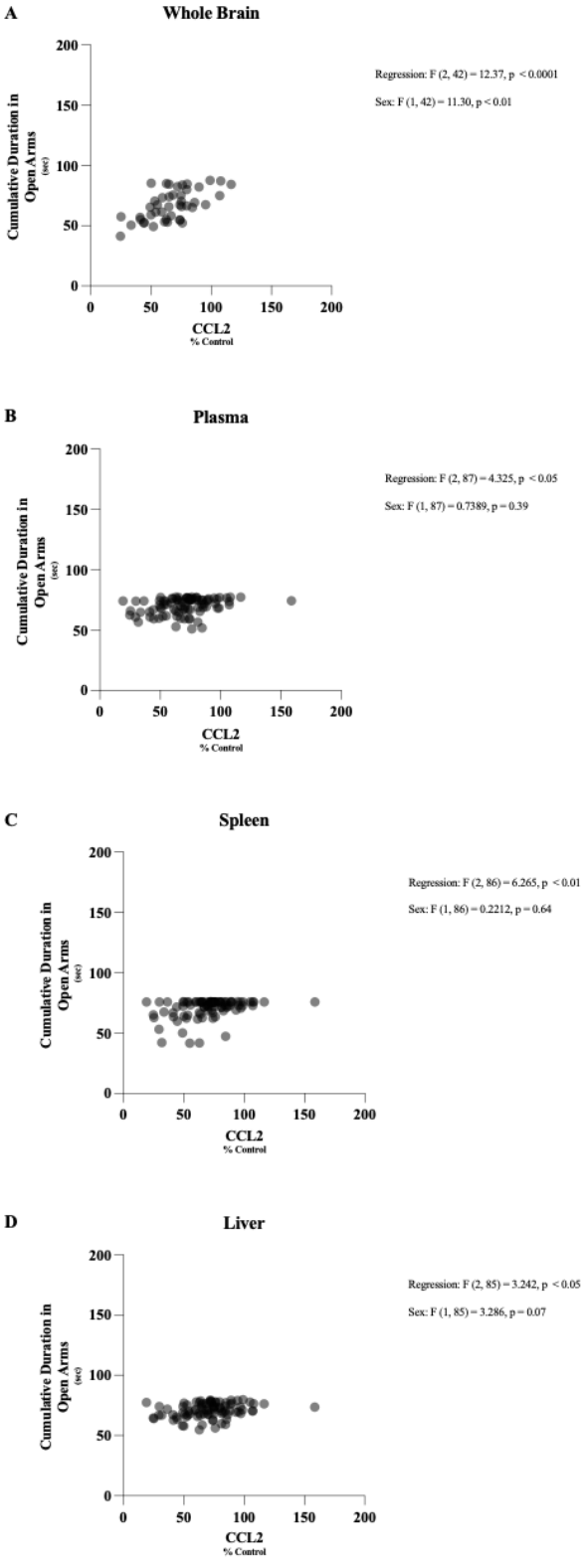

A
Whole Brain
Regression: F (2, 42) = 12.37, p < 0.0001
Sex: F (1, 42) = 11.30, p < 0.01
Cumulative Duration in Open Arms (sec)
CCL2 % Control
B
Plasma
Regression: F (2, 87) = 4.325, p < 0.05
Sex: F (1, 87) = 0.7389, p = 0.39
Cumulative Duration in Open Arms (sec)
CCL2 % Control
C
Spleen
Regression: F (2, 86) = 6.265, p < 0.01
Sex: F (1, 86) = 0.2212, p = 0.64
Cumulative Duration in Open Arms (sec)
CCL2 % Control
D
Liver
Regression: F (2, 85) = 3.242, p < 0.05
Sex: F (1, 85) = 3.286, p = 0.07
Cumulative Duration in Open Arms (sec)
CCL2 % Control

Fig. 50 Multiple linear regression between male and female C57BL/6J mice LPS-induced CCL2 levels in whole brain, plasma, spleen, and liver with measures of EPM-cumulative duration in open arms. Mice (n = 23-24/group or n = 47-48/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted via 5 minute elevated plus maze (EPM), mice were then terminated followed by tissue collection. Levels of CCL2 of whole brain (**A**), plasma (**B**), spleen (**C**), and liver (**D**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Multiple linear regression analysis was used to assess the correlation of whole brain (**A**), plasma (**B**), spleen (**C**), and liver (**D**) in male and female mice and CCL2 levels with EPM-cumulative duration in open arms. Multiple linear regression statistics and symbols are provided in figure.

A multiple linear regression model suggests that cumulative duration in open arms was predicted by change in CCL2 levels in the plasma ($F(2, 87) = 4.325$, $p < 0.05$), and in sexes, their behavioral responses did not differ based on changes in CCL2 levels in the plasma ($F(1,87) = 0.7389$, $p = 0.39$) (Fig. 50B). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

In the spleen, a multiple linear regression model did show changes in CCL2 levels and cumulative duration in open arms ($F(2, 86) = 6.265$, $p < 0.01$) (Fig. 50C). When sex was assessed as a factor, no statically significant results were found ($F(1,86) = 0.2212$, $p = 0.64$). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

Multiple regression indicates changes in CCL2 levels in the liver were correlated with cumulative duration in open arms ($F(2, 85) = 3.242$, $p < 0.05$), however, there was no difference between sexes and their behavioral responses based on changes in CCL2 levels ($F(1,85) = 3.286$, $p = 0.07$) (Fig.

50D). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

These results indicate that the sexes did differ in anxiety-like behavior response as the concentration of CCL2 in the whole brain changed.

Multiple linear regression between LPS-induced CCL2 levels in colon, proximal small intestine, and distal small intestine with measures of EPM- cumulative duration in open arms in C57BL/6J male and female mice

A multiple linear regression model was conducted to determine if sexes differ in anxiety-like behavior response as the concentration of CCL2 in the colon changes. The results of the multiple linear regression suggest that cumulative duration in open arms was predicted by change in CCL2 levels in the colon ($F(2, 82) = 5.143$, $p < 0.01$) (Fig. 51A). However, in sexes, their behavioral responses did not differ based on changes in CCL2 levels in the colon ($F(1, 82) = 1.247$, $p = 0.27$). The x-axis indicates the independent value (CCL2 level), and the Y-axis shows the dependent value (cumulative duration in open arms).

Fig. 51

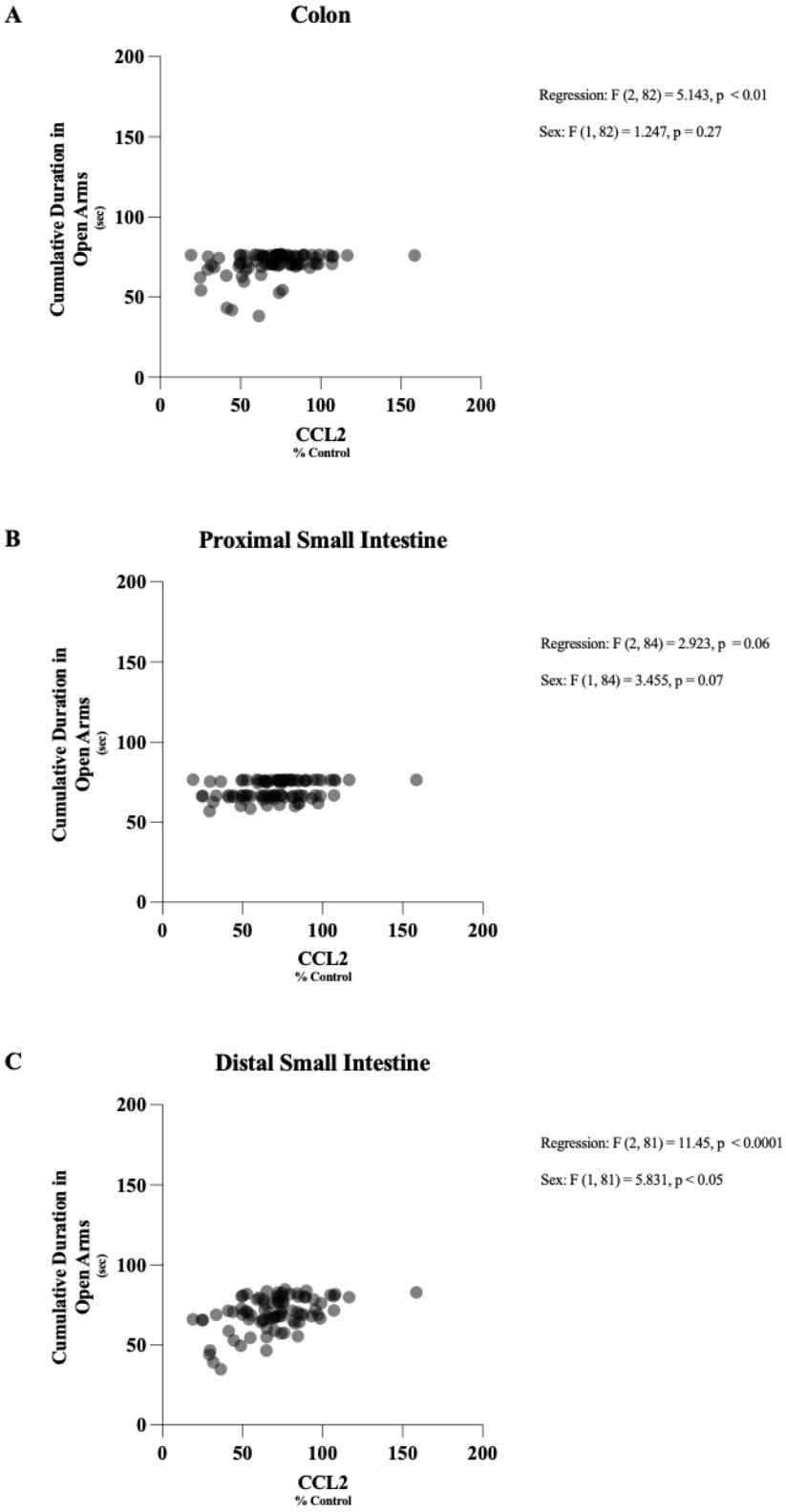

A
Colon
Cumulative Duration in Open Arms (sec)
CCL2 % Control
Regression: F (2, 82) = 5.143, p < 0.01
Sex: F (1, 82) = 1.247, p = 0.27
B
Proximal Small Intestine
Cumulative Duration in Open Arms (sec)
CCL2 % Control
Regression: F (2, 84) = 2.923, p = 0.06
Sex: F (1, 84) = 3.455, p = 0.07
C
Distal Small Intestine
Cumulative Duration in Open Arms (sec)
CCL2 % Control
Regression: F (2, 81) = 11.45, p < 0.0001
Sex: F (1, 81) = 5.831, p < 0.05

Fig. 51 Multiple linear regression between male and female C57BL/6J mice LPS-induced CCL2 levels in colon, proximal small intestine, and distal small intestine with measures of EPM-cumulative duration in open arms. Mice (n = 47-48/group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted via 5 minute elevated plus maze (EPM), mice were then terminated followed by tissue collection. Levels of CCL2 of colon (**A**), proximal small intestine (**B**), and distal small intestine (**C**) of tissue homogenates were measured by ELISA. Data are reported as mean ± SEM. Multiple linear regression analysis was used to assess the correlation of colon (**A**), proximal small intestine (**B**), and distal small intestine (**C**) in male and female mice and CCL2 levels with EPM-cumulative duration in open arms. Multiple linear regression statistics and symbols are provided in figure.

A multiple linear regression model suggests that cumulative duration in open arms was not predicted by change in CCL2 levels in the proximal small intestine ($F(2, 84) = 2.923$, $p = 0.06$), but in sexes, their behavioral responses did not differ based on changes in CCL2 levels in the proximal small intestine ($F(1,84) = 3.455$, $p = 0.07$) (Fig. 51B). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

In the distal small intestine, a multiple linear regression model did show changes CCL2 levels and cumulative duration in open arms ($F(2, 81) = 11.45$, $p < 0.0001$) (Fig. 51C). When sex was assessed as a factor statically significant results were found ($F(1,81) = 5.831$, $p < 0.05$). The x-axis is indicative of the independent value (CCL2 level), and the Y-axis is indicating the dependent value (cumulative duration in open arms).

These results indicate that the sexes did differ in anxiety-like behavior response as the concentration of CCL2 in the distal small intestine changed.

Assessment of estrus cycle and OFT-distance moved in C57BL/6J female mice

One-way ANOVA indicated a significant effect of treatment ($F_{3, 43} = 7.939$, $p < 0.0001$) (Fig. 52A). Pairwise comparisons revealed a significant reduction in distance moved by LPS-treated female mice compared to saline-treated female mice ($p < 0.002$). Similarly, LPS + β-FNA and LPS + β-FNA 10 h female mice showed a reduction in distance moved compared to the saline group ($p < 0.0001$). Females were also separated into subgroups based on specific cycle stage: estrus (Fig. 52B), proestrus (Fig. 52C), and metestrus (Fig. 52D); however, no statistical analyses were performed due to small sample sizes ($n \leq 3$).

Fig. 52

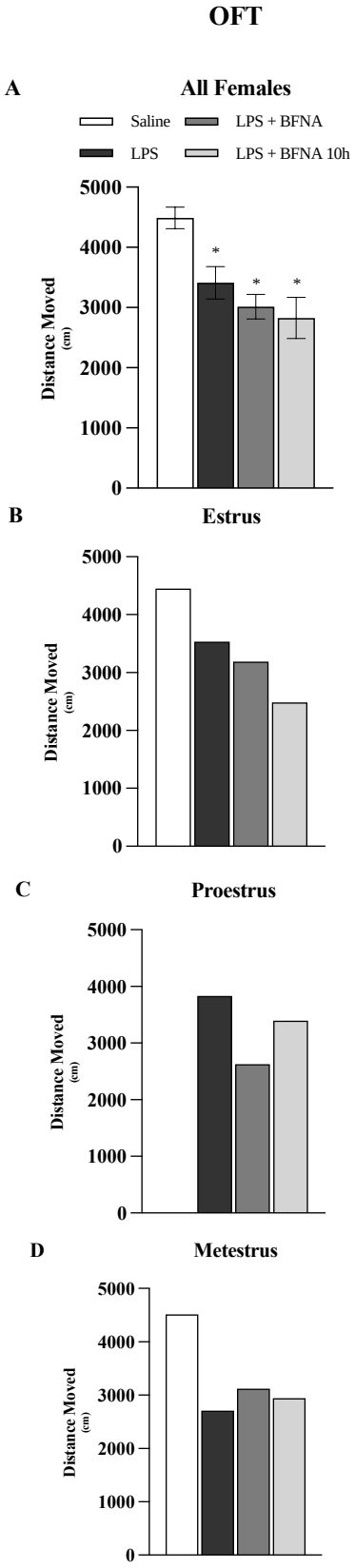

OFT
A
All Females
Saline
LPS + BFNA
LPS
LPS + BFNA 10h
Distance Moved (cm)
5000
4000
3000
2000
1000
0
*
*
*
B
Estrus
C
Proestrus
D
Metestrus

Fig. 52 Assessment of estrus cycle and OFT-distance moved in C57BL/6J female mice. Mice (n = 2-6 group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), and LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted via (**A**) 10 minute open-field test (OFT). Data are reported as mean ± SEM. (**A**) One-way ANOVA indicated a significant main effect of treatment ($p < 0.0001$) on distance moved in the OFT. Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group. Females were separated per cycle estrus (**B**), proestrus (**C**), and metestrus (**D**) no statistical analysis was conducted due to the $n \leq 3$.

These findings suggest that regardless of the estrus cycle stage a similar trend is shown to that of distance moved females' mice in the OFT, with the exception of the proestrus group, which had no control females during that cycle stage.

Assessment of estrus cycle and EPM-cumulative duration in open arms in C57BL/6J female mice

One-way ANOVA of EPM data indicated no significant main effect of treatment ($F_{3, 41} = 1.844$, $p = 0.15$) (Fig. 53A). Pairwise comparisons revealed no significant difference in cumulative duration in open arms by LPS-treated female mice compared to saline-treated female mice ($p > 0.05$). The LPS + β-FNA female mice spent similar time in the open arms compared to LPS females. However, the LPS + β-FNA 10 h females spent more time in the open arms than the LPS females ($p < 0.05$). Females were separated into their specific cycle stage estrus (Fig. 53B), proestrus (Fig. 53C), and metestrus (Fig. 53D); however, no statistical analyses were performed due to small sample sizes ($n \leq 3$).

Fig. 53

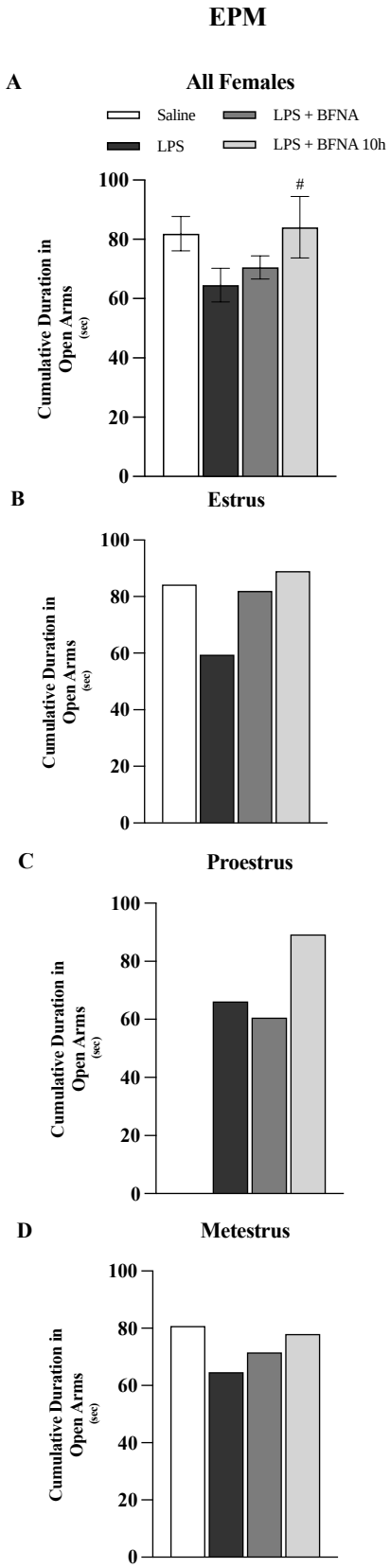

EPM
A
All Females
Saline
LPS
LPS + BFNA
LPS + BFNA 10h
Cumulative Duration in Open Arms (sec)
100
80
60
40
20
0
#
B
Estrus
C
Proestrus
D
Metestrus

Fig. 53 Assessment of estrus cycle and EPM-cumulative duration in open arms in C57BL/6J female mice. Mice (n = 2-6 group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), and LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted via (**A**) 5 minute elevated plus maze (EPM). Data are reported as mean ± SEM. (**A**) One-way ANOVA indicated no significant main effect of treatment ($p > 0.05$) in the cumulative duration in the open arm (EPM). Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group. Females were separated per cycle estrus (**B**), proestrus (**C**), and metestrus (**D**) no statistical analysis was conducted due to the $n \leq 3$.

These findings suggest that regardless of the estrous cycle stage a similar trend is shown to that of cumulative duration in the open arm of female mice in the EPM, except for the proestrus group, which had no females from the saline group during that cycle stage.

Assessment of estrus cycle and CXCL10 level in the hippocampus in C57BL/6J female mice

One-way ANOVA of CXCL10 levels in the hippocampus data indicated a significant main effect of treatment ($F_{3, 20} = 11.80$, $p < 0.001$) (Fig. 54A). Pairwise comparisons indicated that CXCL10 levels in the hippocampus in female mice were increased in LPS mice relative to saline mice ($p < 0.001$); and levels in LPS + β-FNA and LPS + β-FNA 10 h mice were similar to the levels in LPS mice ($p > 0.05$). Females were separated into their specific cycle stage estrus (Fig. 54B), proestrus (Fig. 54C), and metestrus (Fig. 54D); however, no statistical analysis was conducted due to the small n value ($n \leq 3$).

Fig. 54

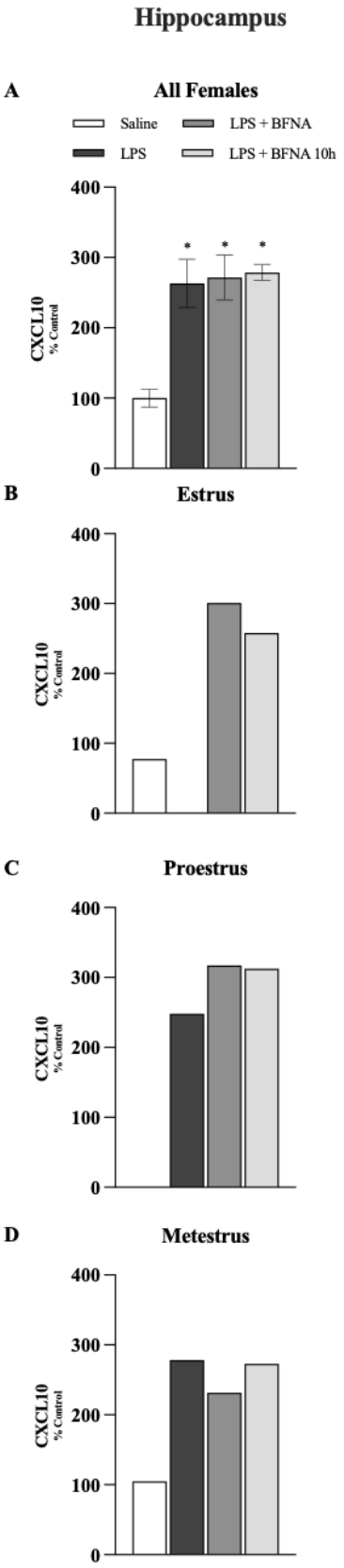

Hippocampus
A
All Females
Saline
LPS + BFNA
LPS
LPS + BFNA 10h
CXCL10
% Control
400
300
200
100
0
*
*
*
B
Estrus
C
Proestrus
D
Metestrus

Fig. 54 Assessment of estrus cycle and CXCL10 levels in the hippocampus in C57BL/6J female mice. Mice (n = 2-6 group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), and LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted followed by tissue collection. Data are reported as mean ± SEM. (**A**) One-way ANOVA indicated significant main effect of treatment ($p > 0.001$) in CXCL10 levels in the hippocampus. Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group. Females were separated per cycle estrus (**B**), proestrus (**C**), and metestrus (**D**) no statistical analysis was conducted due to the $n \leq 3$.

The trend in hippocampal CXCL10 levels is similar across the cycle stage to that observed in the combined group of females. However, no females were present in the LPS group of the estrus cycle, and no females were present in the saline group of the proestrus cycle.

Assessment of estrus cycle and CCL2 level in the hippocampus in C57BL/6J female mice

One-way ANOVA of CCL2 levels in the hippocampus data indicated no significant main effect of treatment ($F_{3, 20} = 0.9515$, $p = 0.43$) (Fig. 55A). Pairwise comparisons indicated that CCL2 levels in the hippocampus in female mice were increased in LPS mice relative to saline mice, however, not to a level of significance ($p > 0.05$). Likewise, levels of LPS + β-FNA and LPS + β-FNA 10 h mice were similar to the levels in saline mice ($p > 0.05$). Females were separated into their specific cycle stage estrus (Fig. 55B), proestrus (Fig. 55C), and metestrus (Fig. 55D); however, no statistical analysis was conducted due to the small n value ($n \leq 3$).

Fig. 55

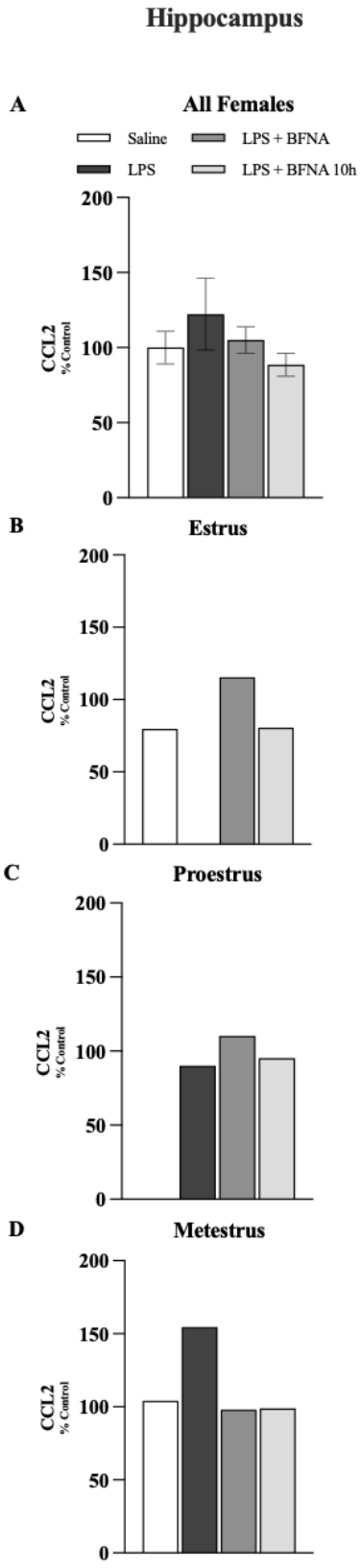

Hippocampus
A
All Females
Saline
LPS + BFNA
LPS
LPS + BFNA 10h
CCL2
% Control
200
150
100
50
0
B
Estrus
C
Proestrus
D
Metestrus

Fig. 55 Assessment of estrus cycle and CCL2 levels in the hippocampus in C57BL/6J female mice. Mice (n = 2-6 group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), and LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted followed by tissue collection. Data are reported as mean ± SEM. (**A**) One-way ANOVA indicated no significant main effect of treatment ($p = 0.43$) in CCL2 levels in the hippocampus. Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group. Females were separated per cycle estrus (**B**), proestrus (**C**), and metestrus (**D**) no statistical analysis was conducted due to the $n \leq 3$.

These findings suggest that regardless of the estrous cycle stage a similar trend is shown to that of CCL2 levels in the hippocampus of female mice. However, no females were present in the LPS group of the estrus cycle, and no females were present in the saline group of the proestrus cycle.

Assessment of estrus cycle and TNF-α level in the hippocampus in C57BL/6J female mice

One-way ANOVA of TNF-α levels in the hippocampus data indicated a significant main effect of treatment ($F_{3, 20} = 7.705$, $p < 0.05$) (Fig. 56A). Pairwise comparisons indicated that in females, TNF-α expression in the hippocampus did not differ significantly among LPS, LPS + β-FNA, and LPS + β-FNA 10 h mice ($p > 0.05$), and TNF-α levels in these three groups were significantly lower than in female saline ($p < 0.03$). Females were separated into their specific cycle stage estrus (Fig. 56B), proestrus (Fig. 56C), and metestrus (Fig. 56D); however, no statistical analysis was conducted due to the small n value ($n \leq 3$).

Fig. 56

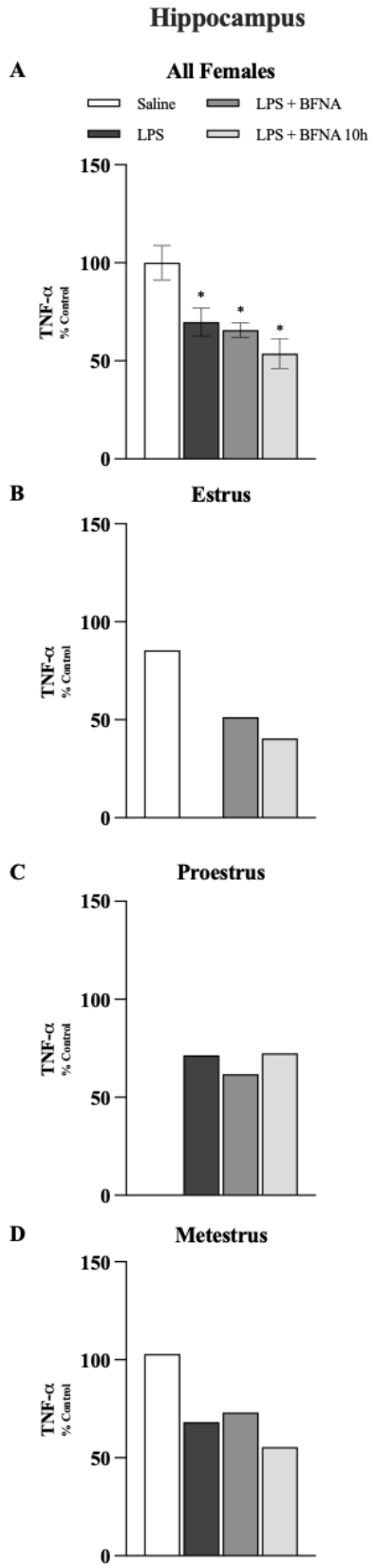

Hippocampus
A
All Females
Saline
LPS
LPS + BFNA
LPS + BFNA 10h
TNF-α
% Control
150
100
50
0
*
*
*
B
Estrus
C
Proestrus
D
Metestrus

Fig. 56 Assessment of estrus cycle and TNF-α levels in the hippocampus in C57BL/6J female mice. Mice (n = 2-6 group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), and LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted followed by tissue collection. Data are reported as mean ± SEM. (**A**) One-way ANOVA indicated significant main effect of treatment ($p < 0.05$) in TNF-α levels in the hippocampus. Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group. Females were separated per cycle estrus (**B**), proestrus (**C**), and metestrus (**D**) no statistical analysis was conducted due to the $n \leq 3$.

Regardless of the estrous cycle stage, a similar trend is shown to that of TNF-α levels in the hippocampus of female mice. With the exception that there were no females present in the LPS group of the estrus cycle, and no females present in the saline group of the proestrus cycle.

Assessment of estrus cycle and NF-κB-p65 level in the hippocampus in C57BL/6J female mice

One-way ANOVA of NF-κB-p65 levels in the hippocampus data indicated no significant main effect of treatment ($F_{3, 20} = 2.317$, $p = 0.11$) (Fig. 57A). Pairwise comparisons indicated that in females, NF-κB-p65 expression in the hippocampus was significantly upregulated in LPS mice relative to saline mice ($p < 0.04$), whereas levels in LPS + β-FNA and LPS + β-FNA 10 h mice were downregulated ($p < 0.05$) relative to those observed in saline mice. Females were separated into their specific cycle stage estrus (Fig. 57B), proestrus (Fig. 57C), and metestrus (Fig. 57D); however, no statistical analysis was conducted due to the small n value ($n \leq 3$).

Fig. 57

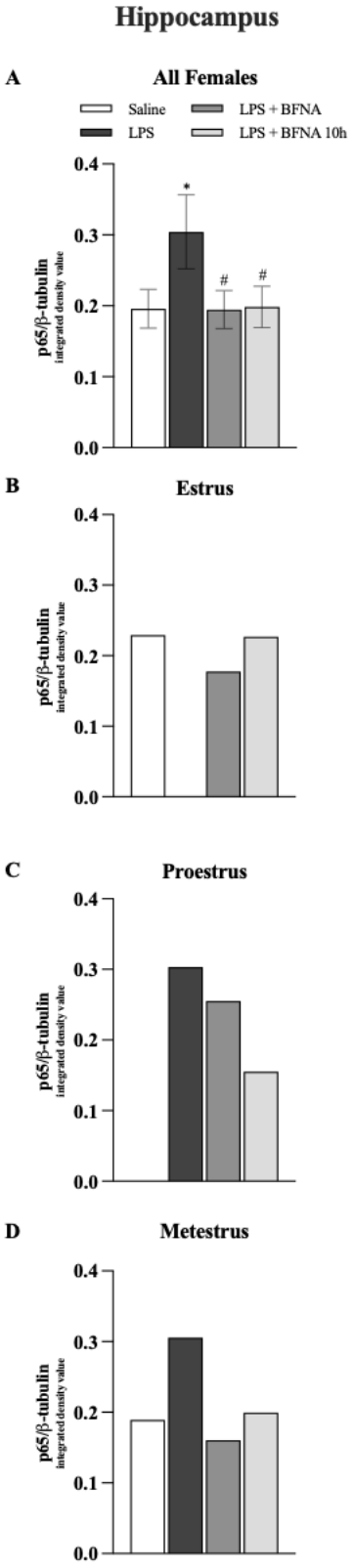

Hippocampus
A
All Females
Saline
LPS
LPS + BFNA
LPS + BFNA 10h
p65/β-tubulin
integrated density value
0.4
0.3
0.2
0.1
0.0
*
#
#
B
Estrus
C
Proestrus
D
Metestrus

Fig. 57 Assessment of estrus cycle and NF-κB-p65 levels in the hippocampus in C57BL/6J female mice. Mice (n = 2-6 group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), and LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted followed by tissue collection. Data are reported as mean ± SEM. (**A**) One-way ANOVA indicated no significant main effect of treatment ($p = 0.11$) NF-κB-p65 levels in the hippocampus. Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group. Females were separated per cycle estrus (**B**), proestrus (**C**), and metestrus (**D**) no statistical analysis was conducted due to the $n \leq 3$.

These findings suggest that regardless of the estrous cycle stage, a similar trend is shown to that of NF-κB-p65 levels in the hippocampus of female mice. However, no females were present in the LPS group of the estrus cycle, and no females were present in the saline group of the proestrus cycle.

Assessment of estrus cycle and CXCL10 level in the liver in C57BL/6J female mice

One-way ANOVA of CXCL10 levels in the liver data indicated a significant main effect of treatment ($F_{3, 43} = 4.034$, $p < 0.05$) (Fig. 58A). Pairwise comparisons indicated that CXCL10 levels in female LPS mice were significantly elevated compared to female saline mice ($p < 0.02$). Females were separated into their specific cycle stage estrus (Fig. 58B), proestrus (Fig. 58C), and metestrus (Fig. 58D); however, no statistical analysis was conducted due to the small n value ($n \leq 3$).

Fig. 58

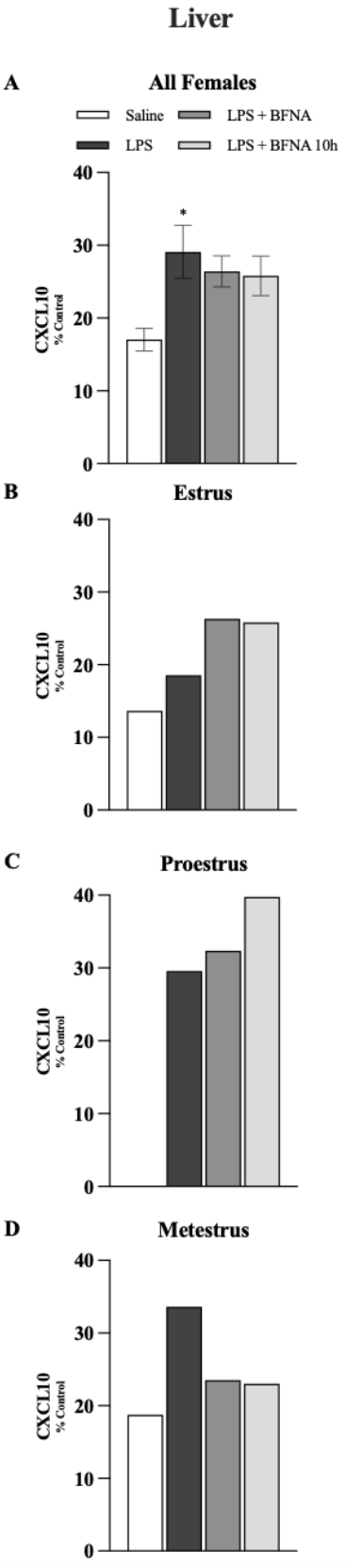

Liver
A
All Females
Saline
LPS + BFNA
LPS
LPS + BFNA 10h
CXCL10
% Control
40
30
20
10
0
*
B
Estrus
C
Proestrus
D
Metestrus

Fig. 58 Assessment of estrus cycle and CXCL10 levels in the liver in C57BL/6J female mice. Mice (n = 2-6 group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), and LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted followed by tissue collection. Data are reported as mean ± SEM. (**A**) One-way ANOVA indicated significant main effect of treatment ($p < 0.05$) CXCL10 levels in the liver. Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group. Females were separated per cycle estrus (**B**), proestrus (**C**), and metestrus (**D**) no statistical analysis was conducted due to the $n \leq 3$.

These findings suggest that regardless of the estrous cycle stage, a similar trend is shown to that of CXCL10 levels in the liver of female mice. However, no females were present in the saline group of the proestrus cycle.

Assessment of estrus cycle and CCL2 level in the liver in C57BL/6J female mice

One-way ANOVA of CCL2 levels in the liver data indicated a significant main effect of treatment ($F_{3, 44} = 5.2$, $p < 0.05$) (Fig. 59A). Pairwise comparisons indicated that in females, LPS ($p < 0.01$), LPS + β-FNA ($p < 0.03$), and LPS + β-FNA 10 h ($p < 0.03$) were all increased relative to saline mice. Females were separated into their specific cycle stage estrus (Fig. 59B), proestrus (Fig. 59C), and metestrus (Fig. 59D); however, no statistical analysis was conducted due to the small n value ($n \leq 3$).

Fig. 59

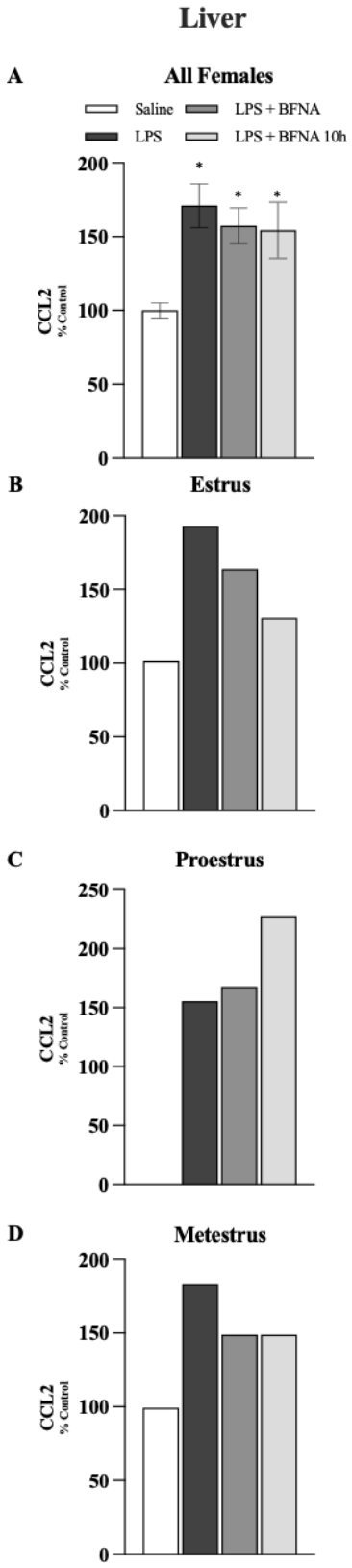

Liver
A
All Females
Saline
LPS
LPS + BFNA
LPS + BFNA 10h
CCL2
% Control
200
150
100
50
0
*
*
*
B
Estrus
C
Proestrus
250
D
Metestrus

Fig. 59 Assessment of estrus cycle and CCL2 levels in the liver in C57BL/6J female mice. Mice (n = 2-6 group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), and LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted followed by tissue collection. Data are reported as mean ± SEM. (**A**) One-way ANOVA indicated significant main effect of treatment ($p < 0.05$) CCL2 levels in the liver. Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group. Females were separated per cycle estrus (**B**), proestrus (**C**), and metestrus (**D**) no statistical analysis was conducted due to the $n \leq 3$.

These findings suggest that regardless of the estrous cycle stage, a similar trend is shown to that of CCL2 levels in the liver of female mice. However, no females were present in the saline group of the proestrus cycle.

Assessment of estrus cycle and CXCL10 level in the distal small intestine in C57BL/6J female mice

One-way ANOVA of CXCL10 levels in the distal small intestine data indicated no significant main effect of treatment ($F_{3, 39} = 2.01$, $p = 0.13$) (Fig. 60A). Pairwise comparisons indicated no differences in distal small intestine CXCL10 levels among the female groups ($p > 0.05$). Females were separated into their specific cycle stage estrus (Fig. 60B), proestrus (Fig. 60C) and metestrus (Fig. 60D), however, no statistical analysis was conducted due to the small n value ($n \leq 3$).

Fig. 60

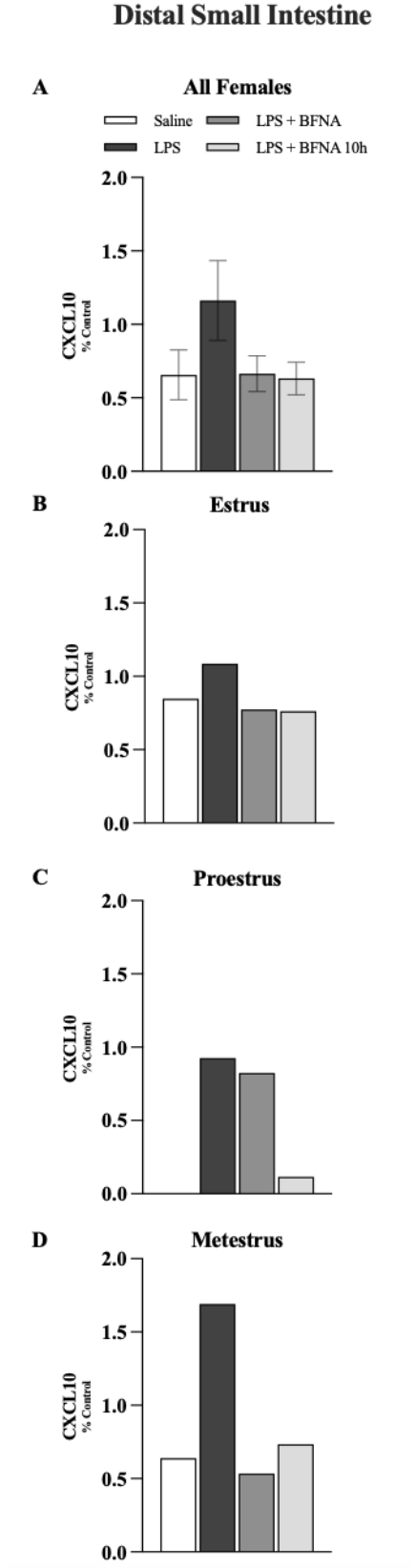

Distal Small Intestine
A
All Females
Saline
LPS + BFNA
LPS
LPS + BFNA 10h
CXCL10
% Control
2.0
1.5
1.0
0.5
0.0
B
Estrus
C
Proestrus
D
Metestrus

Fig. 60 Assessment of estrus cycle and CXCL10 levels in the distal small intestine in C57BL/6J female mice. Mice (n = 2-6 group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), and LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted followed by tissue collection. Data are reported as mean ± SEM. (**A**) One-way ANOVA indicated no significant main effect of treatment ($p = 0.13$) CXCL10 levels in the distal small intestine. Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group. Females were separated per cycle estrus (**B**), proestrus (**C**), and metestrus (**D**) no statistical analysis was conducted due to the $n \leq 3$.

Regardless of the estrous cycle stage, a similar trend is shown to that of CXCL10 levels in the distal small intestine of female mice. However, no females were present in the saline group of the proestrus cycle.

Assessment of estrus cycle and CCL2 level in the distal small intestine in C57BL/6J female mice

One-way ANOVA of CCL2 levels in the distal small intestine data indicated a significant main effect of treatment ($F_{3, 39} = 9.997$, $p < 0.0001$) (Fig. 61A). Pairwise comparisons indicated that in females, CCL2 levels in the distal small intestine were significantly higher in LPS mice compared to saline, LPS + β-FNA, and LPS + β-FNA 10 h female mice (all $p < 0.001$); and both β-FNA-treated groups were similar to saline mice ($p \geq 0.2$). Females were separated into their specific cycle stage estrus (Fig. 61B), proestrus (Fig. 61C), and metestrus (Fig. 61D); however, no statistical analysis was conducted due to the small n value ($n \leq 3$).

Fig. 61

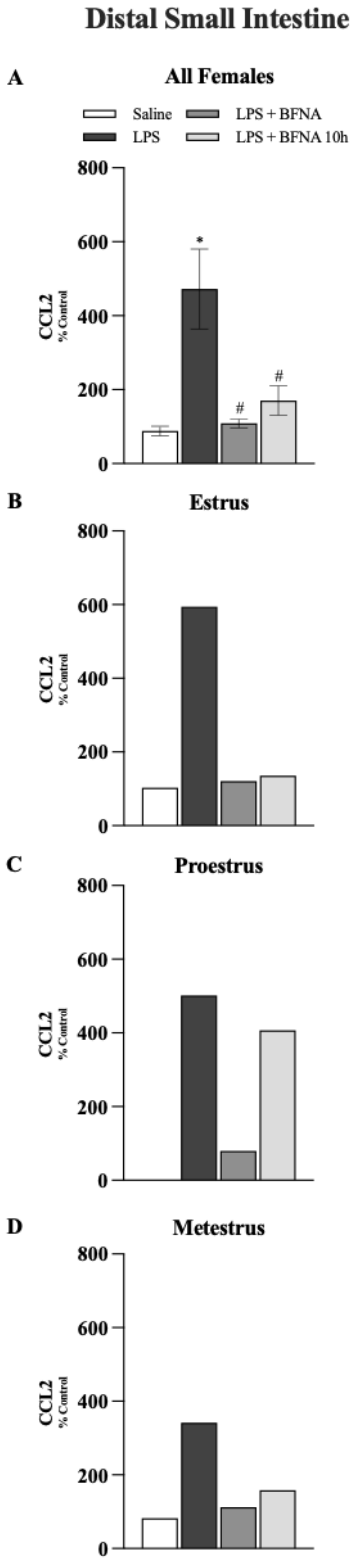

Distal Small Intestine
A
All Females
Saline
LPS
LPS + BFNA
LPS + BFNA 10h
CCL2
% Control
800
600
400
200
0
*
#
#
B
Estrus
C
Proestrus
D
Metestrus

Fig. 61 Assessment of estrus cycle and CCL2 levels in the distal small intestine in C57BL/6J female mice. Mice (n = 2-6 group) were injected (i.p.) with saline (control), LPS (0.83 mg/kg), and LPS followed immediately by β-FNA treatment (50 mg/kg; i.p.; LPS + β-FNA), or LPS followed by β-FNA 10 h post-LPS (LPS + β-FNA 10 h). 24 h post-LPS, assessment of mice was conducted followed by tissue collection. Data are reported as mean ± SEM. (**A**) One-way ANOVA indicated significant main effect of treatment ($p < 0.0001$) CCL2 levels in the distal small intestine. Pairwise comparisons were assessed using a Fisher's LSD test; * indicates $p < 0.05$ vs. saline group; # indicates $p < 0.05$ vs. LPS group. Females were separated per cycle estrus (**B**), proestrus (**C**), and metestrus (**D**) no statistical analysis was conducted due to the $n \leq 3$.

These findings suggest that regardless of the estrous cycle stage, a similar trend is shown to that of CCL2 levels in the distal small intestine of female mice. However, no females were present in the saline group of the proestrus cycle.

Summary of β-FNA effects on LPS-induced behavioral deficits and inflammation in mice

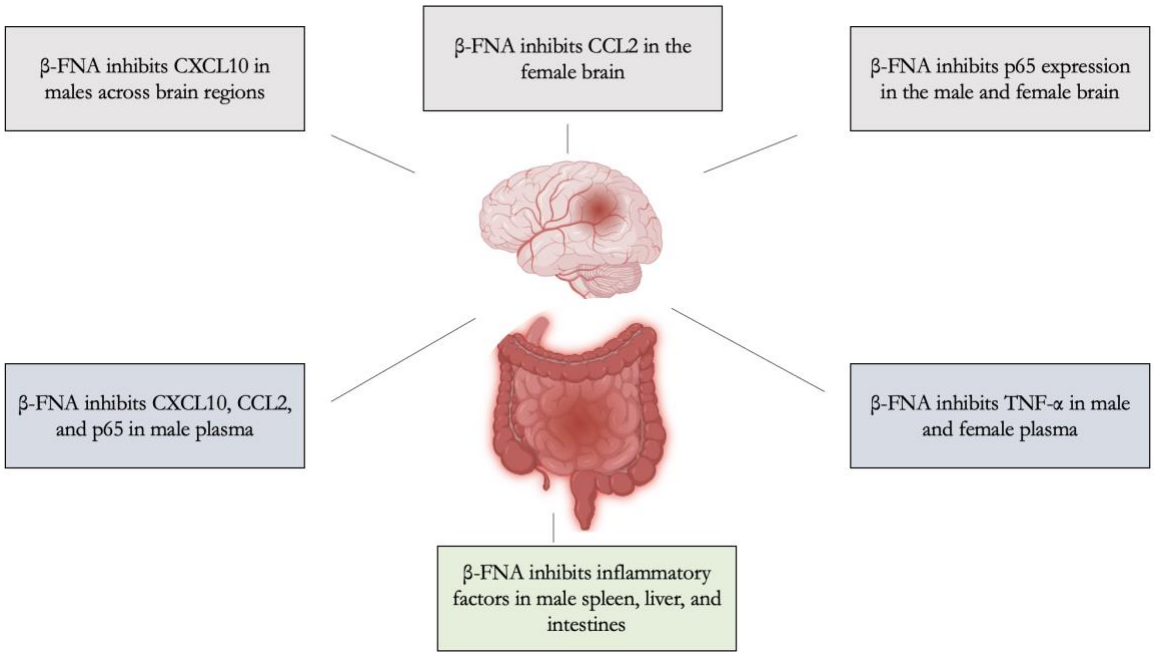


Fig 62: Schematic summary effects of β-FNA on LPS-induced inflammation. Created with BioRender.com

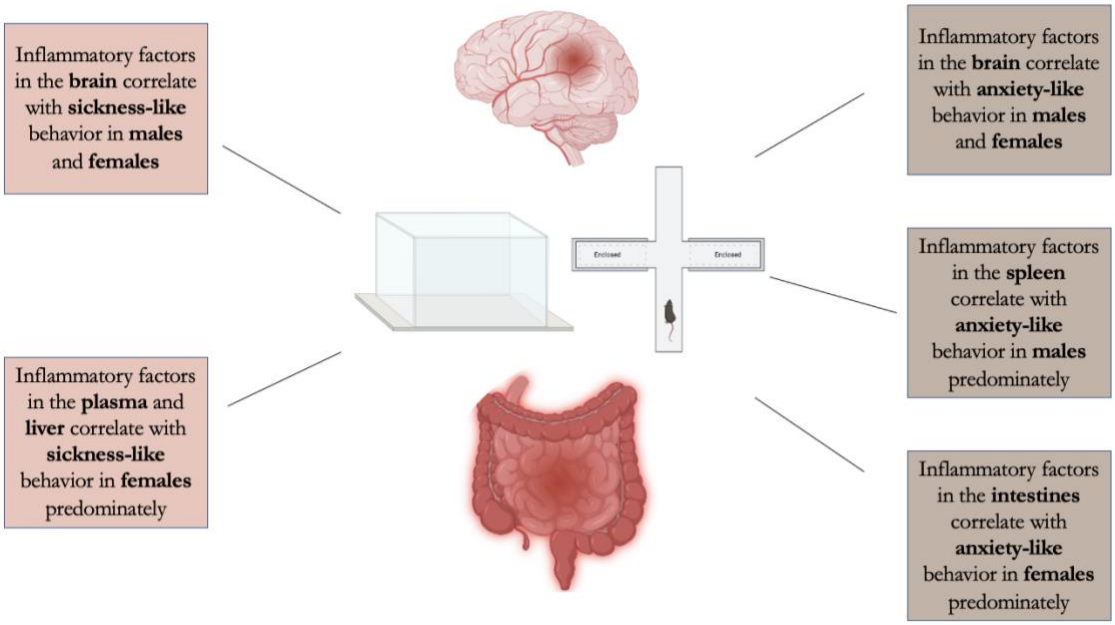


Fig 63: Schematic summary effects of correlations between β-FNA effects on LPS-induced inflammatory factors and behavior. Created with BioRender.com

Table 3: Summary of β-FNA effects on LPS-induced inflammation on signaling factors in the brain

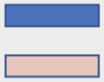
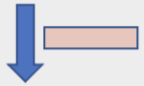
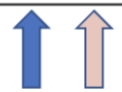
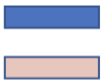
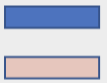
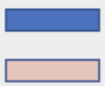
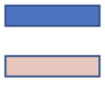
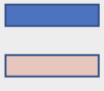
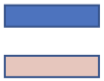
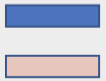
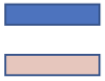
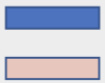
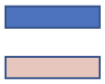
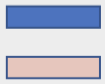
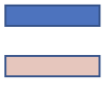
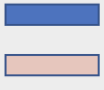
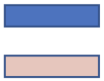
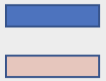
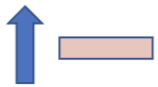
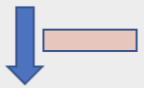
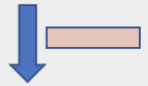
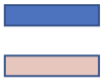
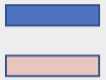
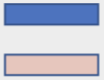
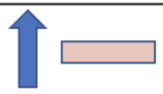
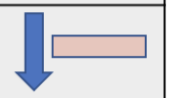
		Whole Brain	B-FNA	Hippocampus	B-FNA	Prefrontal Cortex	B-FNA	Cerebellum/ Brain Stem	B-FNA
Key	CXCL10								
Males	CCL2								
Females	TNF-α								
Increase inflammatory factor ⇧	IL-6								
Decrease inflammatory factor ⇩	IL-1β								
No effect ▭	p65								
Did not run ⊘	p-TAK1								
Below detection level ○	TAK1								

Table 4: Summary of β-FNA effects on LPS-induced inflammation on signaling factors in peripheral tissue

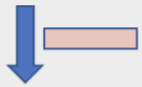
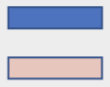
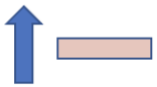
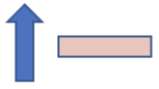
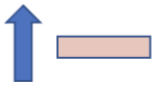
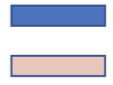
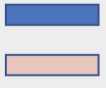
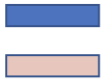
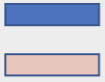
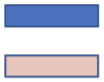
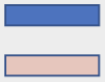
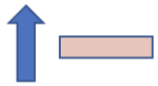
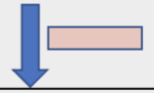
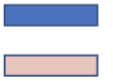
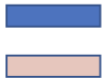
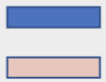
		Plasma	B-FNA	Spleen	B-FNA	Liver	B-FNA
Key	CXCL10						
Males	CCL2						
Females	TNF-α						
Increase inflammatory factor ⇧	IL-6						
Decrease inflammatory factor ⇩	IL-1β						
No effect ▭	p65						
Did not run ⊘	p-TAK1						
Below detection level ○	TAK1						

Table 5: Summary of β-FNA effects on LPS-induced inflammation on signaling factors in the intestines

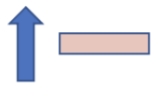
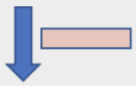
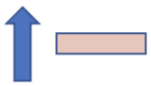
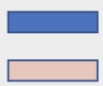
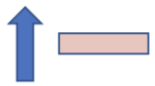
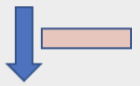
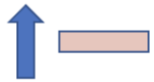
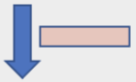
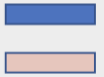
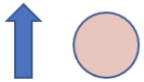
		PSI	B-FNA	DSI	B-FNA	Colon	B-FNA
Key	CXCL10						
Males	CCL2						
Females	TNF-α						
Increase inflammatory factor ⇧	IL-6						
Decrease inflammatory factor ⇩	IL-1β						
No effect ▭	p65						
Did not run ⊘	p-TAK1						
Below detection level ○	TAK1						

Table 6: Summary of correlations between β-FNA effects on LPS-induced inflammatory factors and behavior				
CXCL10 whole brain vs. OFT	**M/F**		CCL2 whole brain vs. OFT	**M/F**
CXCL10 plasma vs. OFT	**F**		CCL2 plasma vs. OFT	**F**
CXCL10 spleen vs. OFT	-		CCL2 spleen vs. OFT	-
CXCL10 liver vs. OFT	**F**		CCL2 liver vs. OFT	**F**
CXCL10 PSI vs. OFT	-		CCL2 PSI vs. OFT	-
CXCL10 DSI vs. OFT	-		CCL2 DSI vs. OFT	-
CXCL10 colon vs. OFT	-		CCL2 colon vs. OFT	-
CXCL10 whole brain vs. EPM	**F**		CCL2 whole brain vs. EPM	**M**
CXCL10 plasma vs. EPM	-		CCL2 plasma vs. EPM	-
CXCL10 spleen vs. EPM	**M**		CCL2 spleen vs. EPM	**M**
CXCL10 liver vs. EPM	-		CCL2 liver vs. EPM	-
CXCL10 PSI vs. EPM	**F**		CCL2 PSI vs. EPM	-
CXCL10 DSI vs. EPM	**M**		CCL2 DSI vs. EPM	**M/F**
CXCL10 colon vs. EPM	-		CCL2 colon vs. EPM	**F**

Discussion

Inflammation is involved in numerous neurological disorders, including anxiety, depression, and other mood disorders. It is also well-established that diseases in peripheral tissues, such as the liver and intestines, involve inflammation. Furthermore, there is emerging evidence of the interplay between brain disorders and peripheral diseases, and inflammatory signaling is a primary link. Indeed, the literature is replete with reports of anxiety/depression exacerbating IBD and vice versa [9, 10, 132]. The literature seems to suggest that the prevalence of these diseases is greater in women than in men, but clearly, both men and women suffer from anxiety/depression and IBD [11, 132]. Studies in preclinical models of anxiety/depression and IBD have revealed differential severity of symptoms, disease progression, and response to treatment among males and females [133-136]. There are also reports of sex-dependent differences in inflammatory signaling [137-139]. Therefore, there remains much to learn about the role of sex in inflammatory diseases and the pharmacologic treatment of these disorders. In fact, it is expected that tailoring therapeutic strategies in a sex-specific manner will be effective for certain diseases, including anxiety, depression, and IBD [12, 139, 140].

In the present study, we assessed male and female mice in a preclinical model of LPS-induced inflammation to advance our understanding of the anti-inflammatory effects of β-FNA. We identified sex-dependent differences in behavioral deficits, inflammatory responses, and protective effects of β-FNA. In this section, we will discuss these key findings.

Consistent with our previous report, a single i.p. LPS injection resulted in anxiety-like behavior at 24 h [2]. Importantly, we observed LPS-induced behavioral deficits in both male and female mice, which is particularly informative given that related studies reported in the literature have predominantly used male mice. However, more recently, there has been an increase in the number of investigations assessing both sexes. For instance, Dockman et al. (2022) found that LPS (0.3 mg/kg, i.p.) resulted in more anxiety-like behaviors in females than in males [133]. Whereas others

administered a similar dose of LPS (0.3 mg/kg) via oral gavage and observed increased anxiety-like behavior in both male and female mice [138]. Interestingly, these researchers suggested that LPS-induced anxiety-like behavior may be regulated in part by sex-specific mechanisms [138]. We now report for the first time that β-FNA effectively reduces LPS-induced anxiety-like behavior. Follow-up studies with a more in-depth assessment of anxiety-like behavior, as well as other behaviors (e.g., depressive-like behavior), are warranted to gain a more robust appreciation of the effects of β-FNA.

Cytokines/chemokines are among the inflammatory mediators thought to contribute to the pathogenesis of anxiety/depression and mood disorders and have emerged as potential biomarkers of mood and anxiety disorders [141-143]. We previously reported that LPS-induced anxiety-like behavior in adult male mice positively correlated with IL-6 and CCL2 levels in the plasma and chemokine (CXCL10 and CCL2) expression in the brain [2]. Our present study supports these earlier findings of LPS-upregulated cytokine/chemokine expression in the brain. Importantly, we have extended our findings to include LPS-induced expression of inflammatory mediators in select brain regions of both male and female mice. We observed sex-dependent differences in cytokine/chemokine expression in the brain. For instance, LPS-induced CXCL10 expression in the prefrontal cortex and cerebellum/brain stem was more pronounced in males than in females. Whereas CCL2 expression was greater in the prefrontal cortex of females compared to males; yet CCL2 levels in the cerebellum/brain stem were lower in females relative to males. IL-6 was only significantly elevated in the cerebellum/brain stem of male mice. Although, IL-6 was not significantly elevated in the whole brain, it was in the plasma of male mice. This is consistent with previous studies that reported IL-6 levels returning to baseline by 24 h in the brain and IL-6 concentrations peaking in the plasma within 6 h and remaining elevated at 24 h in male mice [2, 144]. The sex-dependent differences are very interesting but not unexpected given the considerable

evidence of sexual dimorphism in neuroimmune activation, including differences in microglia activation, cytokine expression, and immune-related receptors [139, 145, 146].

While we know that β-FNA can inhibit LPS-induced CXCL10 and CCL2 expression in the brain, we now show that the inhibition of these two chemokines is sex-dependent and brain region specific. Specifically, β-FNA inhibited CXCL10 across brain regions, but only in male mice, whereas CCL2 was inhibited only by β-FNA in the cerebellum/brain stem and again only in male mice. LPS-induced IL-6 expression was only elevated in the cerebellum/brain stem and only in male mice; and downregulated by β-FNA. Sex-dependent differences in drug effects are not novel *per se*, particularly in terms of anti-depressants and anti-inflammatory agents [136, 147]. For example, there are sex differences in the effectiveness and tolerability of anti-TNF agents [105, 148]. In another example, glucosamine inhibited post-ischemic inflammation more effectively in male mice [108]. However, it is certainly noteworthy that the anti-inflammatory actions of β-FNA are sex-dependent.

The β-FNA-mediated anti-inflammatory effects in the hippocampus and prefrontal cortex are promising in terms of novel therapeutic approaches for anxiety/depression, given the importance of these regions in the neuropathogenesis of anxiety/depression [23-25]. For instance, inhibition of neuroinflammation in the hippocampus and prefrontal cortex was associated with reduced anxiety/depressive-like behavior [149-152]. However, the cerebellum is generally less studied in the context of anxiety/depression, but increasing evidence implicates this brain region in anxiety/depression and is thus a potential therapeutic target [153-155].

It is crucial not only to understand the effects of β-FNA on neuroinflammatory signaling, but also the impact on inflammatory mediators in peripheral tissues. For instance, intestinal inflammation exacerbates anxiety/depression symptoms, which has been widely reported in the context of IBD [9, 10, 132]. Others found that CXCL10 expression is increased in colonic mucosa of patients with

active IBD [156]. Cluny et al. (2022) recently reported that liver and gut inflammation leads to neuroinflammation as indicated by increased TNF, IL-1B, and CCL2 levels in the brain [157]. These findings are consistent with the LPS-induced neuroinflammation coupled with inflammatory cytokine/chemokine expression in the spleen, liver, and intestines. We are particularly intrigued by the pronounced anti-inflammatory effects of β-FNA on LPS-induced CXCL10, CCL2, and TNF-α expression in the intestines and the fact that these inhibitory effects were largely limited to the male mice. These results are promising, considering the reported benefits of anti-inflammatory/immunomodulatory agents in treating IBD and associated anxiety and depression were recently reported by Siebenhuner et al. (2021) [158]. These investigators revealed that anti-TNF therapy combined with immunomodulatory therapy (azathioprine, 6-mercaptopurine, or methotrexate) reduced both disease activity and symptoms of anxiety/depression in patients with IBD. Thus, we are interested in further exploring the anti-inhibitory and protective effects of β-FNA in a preclinical model of IBD. The transcription factor NF-κB is instrumental in the upregulation of inflammatory mediators, including the cytokines/chemokines assessed in this study. Interestingly, Mei et al. (2022) very recently demonstrated in a preclinical experimental model, that the CCL2/NF-κB signaling pathway is implicated in the pathogenesis of colitis and suggested as a therapeutic target [159]. Thus, β-FNA-mediated inhibition of CCL2 and NF-κB expression in both the brain and intestines is quite compelling. We also found that β-FNA inhibited LPS-induced CCL2 and TNF-α expression in the spleen; while in the liver, CXCL10 and CCL2 were inhibited. Furthermore, NF-κB-p65 expression in the spleen and liver was inhibited by β-FNA. Interestingly, β-FNA inhibited these factors in a male-specific manner, which may prove to be useful information in terms of targeting therapeutic strategies in male patients. Whereas levels of p-TAK1 and TAK1 were not altered between groups regardless of sex. However, follow-up studies are required to determine whether this holds true.

In contrast to the spleen and liver, β-FNA downregulated LPS-induced NF-κB-p65 expression in the brain of male and female mice, and the hippocampus seemed to be most responsive. Interestingly, the timing of β-FNA administration influenced the anti-inflammatory effect. For instance, in males, β-FNA was only effective when administered immediately after LPS (and not when delayed by 10 h). Although in females, β-FNA inhibited NF-κB-p65 expression regardless of the dosing regimen. In addition, levels of p-TAK1 and TAK1 were not altered between groups regardless of sex, with the exception of TAK1 in male's hippocampus where there is some protection from β-FNA. While these findings are promising in terms of showing that the NF-κB pathway is impacted by β-FNA, they are limited in part by the fact that we did not measure NF-κB activation *per se*. However, we have determined *in vitro* that β-FNA does in fact inhibit NF-κB activation and subsequent production of chemokines in astrocytes [7, 8, 107].

We were particularly interested in the association between cytokine/chemokine expression in the whole brain, plasma, spleen, liver, colon, proximal small intestine, distal small intestine, and LPS-induced sickness and anxiety-like behavior; therefore, linear regression analyses of LPS-treated mice across β-FNA and saline-alone controls was performed. Levels of CXCL10 in the whole brain were negatively correlated with sickness-like behavior (distance moved) in male and female mice. A similar correlation can be seen in the plasma, where distance moved by female mice decreases as CXCL10 levels increase. This is interesting because it is consistent with previous literature that has also documented that distance moved is not correlated with increased CXCL10 levels in the plasma. [2, 8]. The spleen and liver of male mice were not correlated with CXCL10 levels, and distance moved. In females, however, only the liver showed a correlation between distance moved and not the spleen. No correlation was demonstrated for the colon, proximal small intestine, and distal small intestine for male or female CXCL10 levels and distance moved. The lack of correlation between some of the tissue brings to light the possibility that β-FNAs effects might be tissue-selective.

Levels of CXCL10 in the whole brain were negatively correlated with anxiety-like behavior (cumulative duration in the open arms) in female mice. Although males showed a similar correlation, it did not reach a significance level. As for the spleen of male mice, there was a negative correlation between CXCL10 levels and cumulative duration in the open arms, but not for females. Whereas, for males and females, there was no correlation between CXCL10 inflammation and anxiety-like behavior in the plasma and liver. Similarly, in the colon and proximal small intestine, there is no correlation between male CXCL10 level and cumulative duration in the open arms. However, this is a correlation present in the male distal small intestine. In females, the only correlation was in the proximal small intestine and cumulative duration in the open arms. Overall, there were tissue-specific correlations between males and females and anxiety.

Levels of CCL2 in the whole brain were negatively correlated with sickness-like behavior (distance moved) in male and female mice. This was also the case for plasma levels in female mice, however, not for males. This of which is consistent with previous literature, which has also stated that CCL2 levels in male whole brain and sickness-like behavior are negatively correlated and that there was no significant correlation between CCL2 levels in males' plasma and distance moved [2]. In the spleen, no correlation is established, regardless of sex. In the liver only, females have a negative correlation between CCL2 levels and distance moved. No correlation was presented in the colon, proximal small intestine, or distal small intestine regardless of sex in CCL2 and distance moved.

Levels of CCL2 in the whole brain were negatively correlated with anxiety-like behavior (cumulative duration in the open arms) in male mice and not females. Although females showed a similar correlation, it did not reach a level of significance ($p = 0.06$). In the plasma, no correlation is established, regardless of sex. While males showed a negative correlation in the spleen, females showed no correlation between CCL2 levels and cumulative duration in the open arms. Interestingly, both males and females in the liver showed a negative correlation, although not to a

level of significance. In the intestines, only the colon and distal small intestine in males, and the distal small intestine in females, showed a negative correlation between CCL2 levels and cumulative duration in the open arms.

Levels of CXCL10 in the plasma and liver were positively correlated with CXCL10 in the whole brain in male and female mice. A similar correlation can be seen in the spleen for males, where CXCL10 levels increase as CXCL10 in the whole brain increases. This was the same trend for female spleens, however, there was no correlation. Whereas the males in the proximal small intestine had a positive correlation to CXCL10 levels in the whole brain, and while the colon and distal small intestine showed a similar trend, they did not reach a significance level. No correlation was present for female CXCL10 levels in the colon, proximal small intestine, distal small intestine, and CXCL10 levels in the whole brain.

CCL2 in the plasma and liver were positively correlated with CCL2 in the whole brain in male and female mice, and CCL2 levels in the spleen in males were also positively correlated with the whole brain but not females. Similarly, no significant correlation was present in CCL2 levels in the colon, proximal small intestine, distal small intestine, and whole brain, regardless of sex.

When assessing these correlations further, a multiple regression model was used to determine if sexes differ in sickness-like behavior response as the concentration of CXCL10 and CCL2 increase. This was visible in the liver only for CXCL10 and in the plasma for CCL2, but no other tissue. Additionally, a multiple regression model was also used to assess if sexes differ in sickness and anxiety-like behavior response as the concentration of CXCL10 and CCL2 changes. This was visible in the whole brain and plasma for CXCL10 concentrations and in the whole brain and distal small intestine for CCL2.

To our knowledge, these are the first comparisons of the association between cytokine/chemokine expression in the whole brain, plasma, spleen, liver, colon, proximal small intestine, distal small intestine, and LPS-induced sickness and anxiety-like behavior conducted in females. These are the first reports to examine central and peripheral inflammation related to CXCL10 and CCL2. One of the interesting concepts of this study is the use of female mice. Due to this, it was essential to assess females further and explore the possibility that changes in the estrous cycle might influence some of the correlations we are seeing. Also, the correlations indicated that many of the effects of β-FNA might be tissue-specific and might be having an effect on a specific region not on a global level.

This study was the first to our knowledge to assess the β-FNA effect on LPS-induced inflammation and behavioral deficits in female mice. For this reason, investigating the trends seen in females when separated into their specific cycles can provide some insight into potential differences that will benefit further work. The female estrous cycle is divided into four stages (proestrus, estrus, metestrus, and diestrus). Specifically, the females in our study were only categorized into three stages proestrus, estrus, and metestrus, and we had no females in the diestrus stage of the estrous cycle at the time of evaluation.

The estrogen within each stage functions differently based on the mouse's four-day cycle. The stage in the cycle with the highest estrogen is the proestrus phase, whereas the stages with the lowest estrogen level are the metestrus or diestrus stage [160]. Interestingly, the role of estrogen in inflammation is still exploratory; some studies found that estrogen suppresses lung inflammatory responses in mice and that estrogen caused the decrease of pro-inflammatory mediator expression in IBD [161, 162]. Other studies found that inflammation developed from traumatic brain injuries in mice was not affected by estrogen levels; however, beneficial effects were seen in the spleen [163].

For our study, no statistical analyses were done on the data comparing the different cycle trends in female mice because of the small n. However, as an initial pass, we did find that when females from the OFT-distance moved analysis were separated into their specific cycle stage (proestrus, estrus, or metestrus), there did not seem to be a change in the pattern of response regardless of the stage. Interestingly, the proestrus group had no female control mice in this cycle stage. A similar trend is seen for the EPM-cumulative duration in open arms when females are separated into different cycle groups; all groups follow the same trend except for the proestrus group, which had no control females.

When assessing the female cycles in CXCL10 in the hippocampus, a similar trend is followed by all stages except for the estrus cycle, which had no females in the LPS group, and the proestrus cycle, which had no females in the control group. Similarly, for CCL2 in the hippocampus, all the cycles follow the same trend as seen for CCL2 in the hippocampus for all females; however, no females were in the LPS group for the estrus cycle, and there were no females in the control group in the proestrus cycle stage. In the hippocampus, TNF-α levels in all separate female cycle groups showed the same trend as the group of TNF-α and all females, there are no females present in the LPS group for the estrus cycle, and there are no females in the control group in the proestrus cycle. As for NF-κB in the hippocampus, when females were separated into their specific stages, they all seemed to follow a similar trend; however, there were no females in the LPS group for the estrus cycle or control group in the proestrus cycle.

When assessing the different female cycles compared to all the females in CXCL10 in the liver, regardless of the cycle, all females followed the same trend except for the proestrus group, which had no control females. Similarly, for CCL2 in the liver, all the cycles follow the same trend as seen for CCL2 in the liver for all females, except the proestrus group, which had no control females.

In CXCL10 in the distal small intestine, the same trend can be seen for all cycles compared to all females, and no control females were accounted for in the proestrus group. For CCL2 in the distal small intestine, all the cycles follow the same trend as seen for CCL2 in the distal small intestine, except for the proestrus cycle, which had no females in the control group.

Specifically, CXCL10, CCL2, TNF-α, and NF-κB in the hippocampus, as well as CXCL10 and CCL2 in the liver and distal small intestine, were all chosen to assess female estrous cycle trends because these were inflammatory markers and tissues were β-FNA had an effect on male mice. This would allow for a comparison between cycles differing from the female cycle trend and the trends seen in males. However, in our study, regardless of the female estrous cycle, all trends seemed to follow a similar trend to the female group. Further investigation would be warranted to make further conclusions.

Conclusion

In summary, these studies have advanced our understanding of the anti-inflammatory actions of β-FNA in a preclinical model of LPS-induced inflammation. The inclusion of male and female mice in the study allows for the identification of sex-dependent differences in inflammatory signaling and β-FNA-mediated effects. Inhibition of both neuroinflammation and intestinal inflammation, as well as reduced anxiety-like behavior, by β-FNA is promising in terms of potential therapeutic options for anxiety/depression and/or IBD. The sex-dependent effects of β-FNA require further investigation but may be particularly translational in developing sex-specific treatments. Subsequent investigations to further define inflammatory signaling events that are modulated by β-FNA and obtain additional mechanistic insights will be beneficial. Future studies on repeated β-FNA administration are warranted in this model as well as other models of inflammation (e.g.,

IBD) and in combination with other pharmacologic agents such as secreted serotonin reuptake inhibitors.

CHAPTER VI

CONCLUSION

Inflammation is one of the critical components to further discovering a potential treatment for neurological and peripheral disorders. One of the aims of this dissertation was to determine the effects of β-FNA on LPS-induced sickness and anxiety-like behavior when treatment occurs hours after LPS administration in male and female C57BL/6J mice. A second aim was set to determine β-FNA effects on LPS-induced inflammation. This was conducted by determining the effects of β-FNA on LPS-induced neuroinflammation in the whole brain in male and female C57BL/6J mice, determining the effects of β-FNA on LPS-induced neuroinflammation in select brain regions in male and female C57BL/6J mice, and determining the effects of β-FNA on LPS-induced peripheral inflammation in male and female C57BL/6J mice. This work concludes that there are differential effects in the whole brain vs. brain regions, peripheral vs. central effects, inflammatory factors, and sex effects. All of these are contributions that will allow our field to further explore the beneficial effects of β-FNA and for the continued development of potential treatments for disorders involving inflammation.

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APPENDICES

APPENDICES A: LIST OF ABBREVIATIONS

β-FNA: β-Funaltrexamine
LPS: Lipopolysaccharide
CNS: Central Nervous System
IBD: Inflammatory Bowel Disease
CD: Crohn's Disease
UC: Ulcerative Colitis
GI: Gastrointestinal
OFT: Open Field Test
EPM: Elevated Plus Maze
CXCL10 (IP10): Interferon γ-Induced-Protein
CCL2 (MCP1): Monocyte-Chemotactic-Protein 1
IL-6: Onterleukin-6
IL-1β: Interleukin-1 Beta
TNF-α: Tumor Necrosis Factor Alpha
NF-κB: Nuclear Factor of Kappa Light Chain Enhancer of B Cells
NF-κB-p65: p65 Nuclear Factor of Kappa Light Chain Enhancer of Activated B Cells
NF-κB1 p50: p50 Nuclear Factor of Kappa Light Chain Enhancer of Activated B Cells
NF-κB2 p52: p52 Nuclear Factor of Kappa Light Chain Enhancer of Activated B Cells
p38 MAPK: p38 Mitogen-Activated Protein Kinase
GFAP: Glial Fibrillary Astrocytic Protein
TAK1: Transforming Growth Factor β-Activated Kinase 1
TAB1- TGF-Beta Activated Kinase 1
TAB2 - TGF-Beta Activated Kinase 2
TAB3 - TGF-Beta Activated Kinase 3
TRAF6: Tumor Necrosis Factor Receptor-Associated Factor 6
AD: Alzheimer's Disease
PD: Parkinson's Disease
MS: Multiple Sclerosis
ALS: Amyotrophic Lateral Sclerosis
CSF: Cerebrospinal Fluid
NE: Norepinephrine
LC: Locus Coeruleus

APPENDICES A: LIST OF ABBREVIATIONS

GPCRs: G-Protein Coupled Receptors
ANOVA: Analysis of Variance
ELISA: Enzyme-Linked Immunosorbent Assay
min: Minutes
h: Hours
T Cell: T Lymphocytes Cell
NK cells: Natural Killer Cell
DCs: Dendritic Cell
MOR: Mu Opioid Receptor
TLRs: Toll-Like Receptors
TLR4: Toll-Like Receptor 4
COVID-19: SARS-CoV-2 Infection
(Rel-A): REL-Associated
c-Rel: c-Rel- Associated
Rel-B: RELB Proto-Oncogene, NF-KB Subunit
IκBα: NF of Kappa Light Polypeptide Gene Enhancer in B-cells Inhibitor Alpha
PAMPs: Pathogen-Associated Molecular Patterns
DNA: Deoxyribonucleic Acid
RNA: Ribonucleic Acid
DAMP: Damage-Associated Molecular Patterns
LBP: Lipopolysaccharide-Binding Protein
IFN: Interferon
AC: Adenylate Cyclase
cAMP: Cyclic Adenosine Monophosphate
CD14: Cluster of Differentiation 14
PKA: Protein Kinase A
GTP: Guanosine Triphosphate
GDP: Guanosine Diphosphate
MAL: MyD88 Adapter-Like
IRAK: Interleukin Receptor Associated Kinase
MyD88: Myeloid Differentiation Primary Response Gene 88
IRAK-1: Interleukin Receptor-Associated Kinase-1
IRAK-4: Interleukin Receptor-Associated Kinase-4
IKK: I Kappa B Kinase
5-HT: Serotonin
DOR: *Delta* Opioid Receptor
DA: Dopamine
BBB: Blood-Brain Barrier
BCA: Bicinchoninic Acid
KOR: Kappa Opioid Receptor
TBST: Tris-Buffered Saline-Tween

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