

AMERICAN UNIVERSITY OF BEIRUT

STUDYING THE EFFECT OF CHRONIC PERIPHERAL
INFLAMMATION, SPECIFICALLY URINARY TRACT
INFECTION-LIKE VIA LPS, ON NEUROPLASTICITY

by
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ABSTRACT OF THE THESIS OF

Alaa Khaled Al Mikkawi

for

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Major: Neuroscience

Title: Studying the Effect of Chronic Peripheral Inflammation, Specifically Urinary Tract Infection-like via LPS, on Neuroplasticity

Introduction: Neurogenesis is the process by which new neurons are produced from neural stem cells in the brain. This process might be affected by multiple factors such as neuroinflammation, aging, oxidative stress, and brain injury. Neuroinflammation or systemic inflammation can be potentially caused by several extrinsic or intrinsic factors and are often associated with alterations in neurogenesis. Previous clinical observations report cognitive deficits in patients exhibiting symptoms of urinary tract infections (UTI). In our laboratory, we have shown that E-coli induced UTI caused a significant decrease in neurogenesis and impaired cognitive and memory skills. Consequently, we would expect that lipopolysaccharide (LPS) instillation, an outer membrane component of gram negative bacteria such as E.Coli, into the urinary bladder might also have an adverse impact on neurogenesis. Therefore, more work is needed to explore the effect of chronic inflammation via LPS on neurogenesis and brain plasticity.

Objective: Here, we aim to investigate the effect of LPS-induced urinary bladder inflammation on the general and cognitive-like behavior in rats and its potential correlation with impaired hippocampal neurogenesis.

Methods: Adult male Sprague-Dawley rats received four transurethral instillations of LPS every 24 hours over a period of 4 days, to mimic chronic UTI-associated inflammation. Control/Sham groups were instilled with sterile saline. Bromodeoxyuridine (BrdU) analog was injected 24 hours before euthanizing the rats to assess hippocampal neural stem cells (NSCs) proliferation or 4 weeks before euthanasia to assess hippocampal neurogenesis. In addition, a battery of behavioral tests including heat sensitivity, open field, Y-maze, and Object Recognition (NOR) tests were used to assess thermal pain, spontaneous/exploratory motor behavior, cognitive ability and working memory, respectively.

Results: Chronic LPS-induced inflammation in the urothelium has shown increased thermal sensitivity in the paw and abdomen. Also, LPS injected rats have displayed affected motor and exploratory behavior as well as possible anxiety-like behavior. Additionally, induction of LPS into the urinary bladder has decreased the level of proliferation and neurogenesis of neural stem cells. Moreover, mRNA levels of

cytokines were shown to increase in LPS injected rats in the hippocampus and bladder compared to the sham group.

Conclusion/Future Perspectives: This study demonstrates that chronic inflammation induced by repetitive instillations of LPS in the urinary bladder is associated with decreased hippocampal neurogenesis and impairment of cognitive-like behavior in rats. As such, this will provide experimental evidence about the association of UTI and cognitive disorders reported in clinical observations. This study will open a window for therapeutic approaches based on the link between inflammation of urothelium and the increased levels of inflammatory markers in the brain and in particular, the hippocampal formation.

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ABBREVIATIONS

BBB: blood brain barrier

BDNF: brain derived neurotrophic factor

BrdU: Bromo-deoxy Uridine

CNS: central nervous system

DG: dentate gyrus

FGF: fibroblast growth factor

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GFAP: glial fibrillary acidic protein

IGF: insulin-like growth factor

IL: Interleukin

LPS: lipopolysaccharide

NeuN: neuron nuclear protein

NGF: nerve growth factor

NPC: neural progenitor cells

NSC: neuronal stem cells

PBS: phosphate buffered saline

PNS: peripheral nervous system

RNA: ribonucleic acid

SGZ: sub-granular zone

SVZ: subventricular zone

TNF- α : tumor necrosis factor alpha

UTI: urinary tract infection

CHAPTER 1

INTRODUCTION

1.1. Neuroplasticity and Neurogenesis

1.1.1. Overview on Neuroplasticity

Neuroplasticity is the ability of the nervous system to continuously adapt and be altered in response to changes from the environment over the course of one's lifetime. The central nervous system (CNS) is able to change due to intrinsic or extrinsic stimuli leading to the formation of new neural connections (Mateos-Aparicio & Rodríguez-Moreno, 2019). This could allow the brain to reorganize its connections, thus altering its internal structure and function. Interestingly, research has shown that the brain remains in a dynamic cycle across one's lifespan, contradicting the traditional belief that the brain's structure and function were fixed upon adulthood (Draganski et al., 2004). Moreover, neural plasticity shapes the human behavior, memory, and cognition especially during developmental periods where the brain is sensitive to environmental influences (Kolb & Gibb, 2011).

At the synaptic level, neuroplasticity involves modifications at individual synapses hence altering the strength of their connections. This is an important neurophysiological process as it develops and reorganizes brain networks especially after damage (Stampanoni Bassi et al., 2019). Synaptic plasticity integrates two processes: long-term potentiation (LTP) and long-term depression (LTD). Both LTP and LTD are found to be associated with learning and memory. LTP works to enhance synaptic strength and better its ability to

transmit signals whereas LTD reduces the synapses excitability which weakens the signal transmission efficiency (Escobar & Derrick, 2007). Moreover, LTP and LTD could induce structural changes upon neurons by means of either increasing or decreasing the spine size respectively (Engert & Bonhoeffer, 1999; Matsuzaki et al., 2004; Nägerl et al., 2004; Zhou et al., 2004). Thus, this affects the brain's learning capacity and memory abilities, shaping the brain's overall plasticity and capability to adapt throughout life. A major contributor of neuroplasticity is neurogenesis.

1.1.2. Overview on Neurogenesis

1.1.2.1. Defining Neurogenesis

Neurogenesis is the process of producing new neurons in the central nervous system. This process happens in select regions of the brain: the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus (DG) (Figure 1). Neurogenesis is usually known to occur at higher rates during early rates and declines as we age. Besides humans, it also occurs in various mammalian species (Catlow et al., 2016). Neurogenesis consists of the proliferation of neural stem cells (NSCs) and neural progenitor cells (NPCs) which later differentiate into mature neurons followed by integration into neural circuits. This allows the formation of synaptic connections between hippocampal neurons and fibers to and from the cerebral cortex (Catlow et al., 2016).

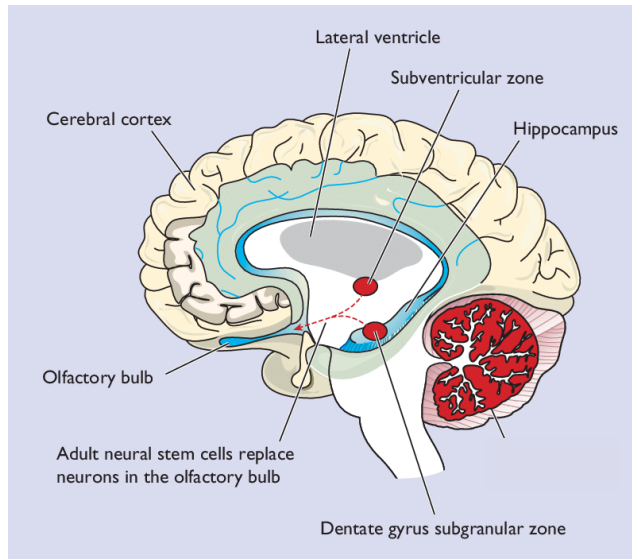


Figure 1. Neurogenic Niches. The two main neurogenic regions in the brain: the subventricular zone (SVZ) and hippocampus (dentate gyrus). Adopted and modified from (Ekonomou et al., 2008).

The topic of neurogenesis is considered to be a controversial topic as some researchers believe that it occurs only in the developmental stages of human life, whereas others consider it to persist in the DG of the hippocampus of old aged human brains (Kumar et al., 2019).

As mentioned before, hippocampal DG neurons are connected to the cerebral cortex which notably allows neurogenesis to play an important role in the aspect of learning, memory, and forgetting (Catlow et al., 2016). This delicate process undergoes multiple stages in which all are necessary for the formation of new neurons (Figure 2).

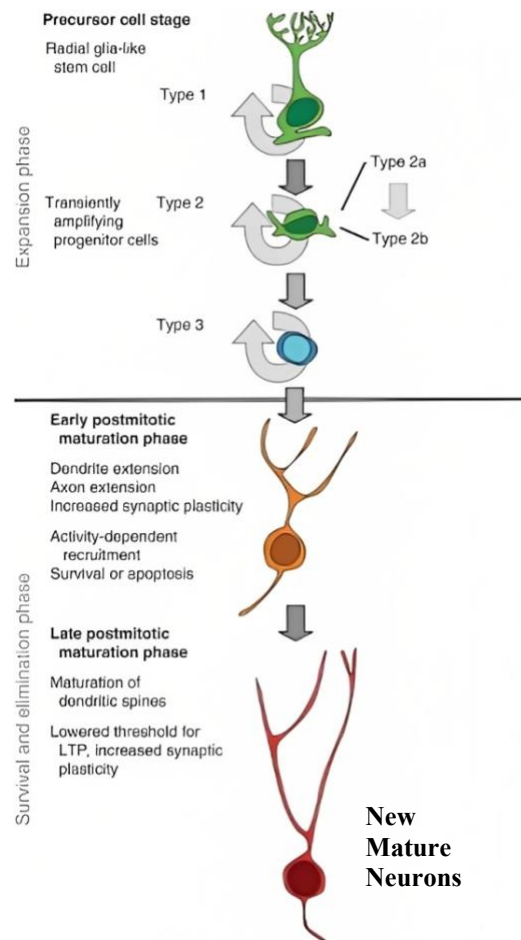


Figure 2. Stages of Adult Hippocampal Neurogenesis. This schematic drawing shows the different stages of neurogenesis. Adopted and modified from (Kempermann et al., 2015).

1.1.2.2. Neurogenic Niches

These regions are the main niches that host neural stem cells (NSCs), neural progenitor cells (NPCs) and their progeny, facilitating their further development into new neurons. The niches are either dormant or active where they function to divide or guide the early stages of differentiation. They are responsible for the generation, growth, and integration of new neurons into existing neural circuits in the brain (Bjornsson et al., 2015; Kumar et al., 2019).

The primary neurogenic niches in the mammalian brain are the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus (DG).

1.1.1.2.1. Subventricular Zone (SVZ)

Neural stem cells found in the SVZ divide to become neuroblasts which then differentiate into neuronal or glial cells that are integrated into existing neural circuits (Bath & Lee, 2010). These neuroblasts tend to replace granule cells found in the DG (Bath & Lee, 2010). Also, cells of the subventricular zone are inclined to divide and then migrate through the rostral migratory stream (RMS) to the olfactory bulb (OB), where the cells differentiate into granular cells and periglomerular cells (Lois & Alvarez-Buylla, 1994; Luskin, 1993).

1.1.1.2.2. Subgranular zone (SGZ) of the Dentate Gyrus (DG)

The subgranular zone of the dentate gyrus is found in the hippocampus, between the hilus and the granular layer of the hippocampus. The SGZ is considered a vital zone of some major cognitive functions in the brain including spatial orientation, emotional behavior, memory, and learning (Kempermann et al., 2015). In this zone, generated neurons differentiate into granule cells where axon projections are formed, extending to a subregion in the hippocampus (Carlén et al., 2002). The granule cells integrate into local circuits at a low rate until adulthood with a lifespan of at least 8 months in rodents and 2 years in humans (Altman & Das, 1965; Dayer et al., 2003; Eriksson et al., 1998).

NSC's in the neurogenic niche go through numerous stages of maturation leading to 3 types of cells (Table 1). Type 1 radial glia-like cells start

proliferating once activated where they express markers such as Nestin, SOX2, and GFAP. This leads to the formation of type 2 cells which divide rapidly replacing the GFAP expression with TRB2 marker, an essential transcription factor for neurogenesis regulation. After that, type 3 cells surface, expressing neuronal markers such as doublecortin (DCX) and Prox1. At last, the cells start to express NeuN (the neuronal nuclear protein) and Calbindin, ensuring their maturation (Bonaguidi et al., 2012; Farah Chamaa et al., 2021; Lv et al., 2019). Monitoring and tracking the development of new neurons and other neural cells is essential to understand certain changes in behavior and cognition.

Table 1. Stages of Neurogenesis. Developmental stages of neurogenesis in adult hippocampus with the expression of their immuno-histological protein markers. Adopted and modified from reference (Kumar et al., 2019)

Stage 1: Proliferation	Glial fibrillary acidic protein (GFAP), Nestin, Pax 6, and SOX2
Stage 2: Differentiation	DCX, PSA-NCAM
Stage 3: Migration	DCX, PSA-NCAM, Tuj-1b, TUC-4, NeuroD
Stage 4: Axonal & Dendritic Targeting	Calretinin, NeuN
Stage 5: Synaptic Integration	Calbindin

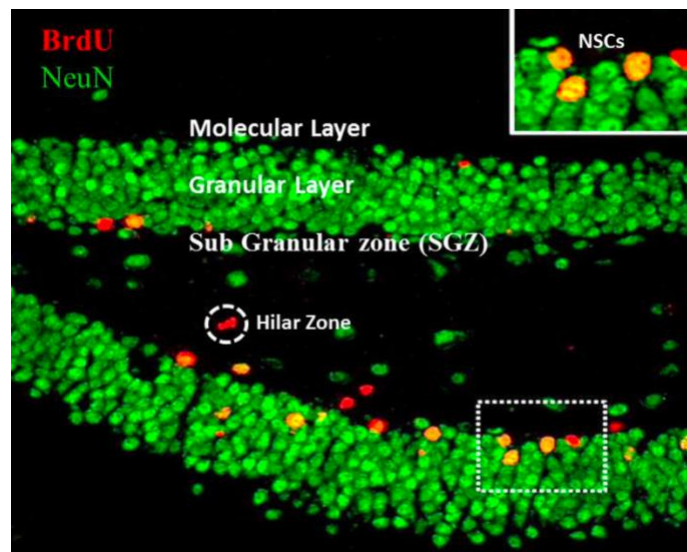


Figure 3. The Dentate Gyrus of the Hippocampus. Immunofluorescence image showing its three layers and the NSCs position. Adapted and modified from reference (Huang et al., 2012)

1.1.2.3. Detecting and Tracking Neurogenesis

Bromodeoxyuridine (BrdU) is a thymidine analog which was developed after the discovery of [3H] thymidine which provided the first autoradiography evidences showing that neurogenesis takes place in specific regions in the brain of rodents: the SVZ, and DG of the hippocampus (Altman, 1969; Altman & Das, 1965). BrdU labels actively proliferating cells in the body and helps in the detection of neural stem cells as well as neural progenitor cells. BrdU is incorporated in the DNA of dividing cells during the S-phase of the cell cycle (Taupin, 2007). Detection of BrdU is done by specific immunofluorescent staining using antibodies that are designed to detect BrdU (Eriksson et al., 1998). BrdU positive cells in the hippocampus were also found to be expressing neuronal markers including NeuN (Eriksson et al., 1998). This shows that proliferating cells are present in the hippocampus of adult humans leading to their differentiation into new neurons (Eriksson et al., 1998; Kempermann et al.,

2015). These newly formed neurons are essential for the proper functioning of the hippocampus.

1.1.2.4. Role of Neurogenesis

Adult hippocampal neurogenesis is known to be heavily involved in learning and memory (Deng et al., 2010; Saxe et al., 2006; Winocur et al., 2006; Zhao et al., 2008). The newly generated granule cells have higher levels of plasticity and excitability. This allows such cells to play an important role in cognitive functions such as producing memories (Farah Chamaa et al., 2021; Ge et al., 2007), pattern separation (Sahay et al., 2011), spatial learning (Deng et al., 2010), cognitive flexibility, and the association between old and new memories (Jessberger & Gage, 2014; Kohman & Rhodes, 2013). In addition to that, granule cells take part in regulating stress and anxiety, which in turn reduces the uprising of neurodegenerative disorders (Farah Chamaa et al., 2021; Kempermann et al., 2015; von Bohlen Und Halbach, 2007). Any disruption in the process of neurogenesis could lead to either cognitive deficits (Costa et al., 2015) such as spatial or contextual memory retrieval (Chesnokova et al., 2016) or CNS pathologies including schizophrenia, mood disorders, autism, and Alzheimer's disease (Costa et al., 2015). Considering the importance of neurogenesis, this process is highly monitored and controlled to ensure a proper functioning of the brain.

1.1.2.5. Factors regulating Neurogenesis

Several factors contribute to the regulation of newly generated neural stem cells or progenitor cells in the brain. This regulation includes the control of neurogenesis as well as its rate and extent of new neuron production. It does so by balancing between the survival and apoptosis of cells due to the milieu and capacity available (Dupret et al., 2007). Within the developing stages of birth, 50% of the NSCs remain premature and undergo apoptosis (Dayer et al., 2003). The remaining cells that reach maturation, differentiate mostly into granule cells with a lifespan of several months in the DG (Kempermann et al., 2003). Such cells receive synaptic inputs with axons extending along the mossy fiber tract that contribute to the maturation of their electrophysiological properties (Hastings & Gould, 1999; Stanfield & Trice, 1988; van Praag et al., 2002; Zhao et al., 2006).

The dentate gyrus's neurogenic niche is mandatory for neuronal development (Kempermann et al., 2015). The latter is controlled and regulated due to the special characteristics of the niche including its extracellular matrix, cell to cell contact, and short and long-range humoral factors (Kempermann et al., 2015). Also, the evolving neurons are in contact with local astrocytes that are important for neuronal development (Plümpe et al., 2006; Shapiro et al., 2005). Astrocytes function to strongly coordinate synapse generation and function, survival of neurons, and axon guidance (Sloan & Barres, 2014). Any defects in the process of forming functional neural circuits would lead to neurodevelopmental disorders (Sloan & Barres, 2014). Moreover, the SGZ is considered unique as it acquires synaptic input from different brain regions such

as the dopaminergic fibers from the ventral tegmental area, serotonergic projections from the raphe nuclei, cholinergic input from septum, γ -aminobutyric acid (GABA)ergic connections from local interneurons (Kempermann et al., 2015). Hence, adult hippocampal neurogenesis is regulated by neurotransmitters. However, any pharmacological or physical manipulations leading to variations in neurotransmitters levels potentially influence neurogenesis (Bengzon et al., 1997; Cooper-Kuhn et al., 2004; Domínguez-Escribà et al., 2006; Kempermann et al., 2015). Furthermore, neurogenesis can be promoted by the stimulation of the supra-mammillary nucleus (SuM) (Li et al., 2022) and deep brain stimulation (DBS) of the thalamic nucleus (F. Chamaa et al., 2021; Chamaa et al., 2016).

After a traumatic brain injury, insulin-like growth factor-1 (IGF-1) is activated and protects the brain by boosting neurogenesis by promoting proliferation and differentiation of NPCs and stimulating dendritic growth (Williams et al., 2022). In addition, specific drugs and chemicals could modulate neurogenesis. Cannabidiol (CBD) compound, for instance, increases neurogenesis by halting radial NSC overactivation and their differentiation to astrocytes which promotes the neuronal lineage (Hou et al., 2022). Also, the circadian rhythm can control behavior, physiology, hormone secretion and brain metabolism which in turn modulates adult hippocampal neurogenesis (Ali & von Gall, 2022).

Additionally, specific genes can influence the rate of neurogenesis. For example, upon knocking out DISC1, which is a gene that increases the risk of psychiatric disorder like schizophrenia, can decrease neurogenesis by changing

the formation and distribution of granule cells as well as the hippocampus-dependent behaviors in rodents (Duan et al., 2007; Gonçalves et al., 2016; Kvajo et al., 2008).

Neutrophils and cytokines also play an important role in the modulation of adult hippocampal neurogenesis.

1.1.2.5.1. Neurotrophins

Neurotrophic factors are a family of biomolecules responsible for the survival of sympathetic and sensory neurons as it regulates the development and function of neurons in both the CNS and PNS (Skaper, 2018). Some vital neurotrophins are the brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), fibroblast growth factor 2 (FGF), and insulin-like growth factor (IGF).

BDNF has an important role in the survival and growth of neurons as it serves as a neurotransmitter modulator and contributes in neuronal plasticity. It is largely expressed in the central nervous system, the gut, and other tissues (Bathina & Das, 2015). BDNF also has a neuroprotective part especially in adverse conditions such as neural toxicity, hypoglycemia, and other (Bathina & Das, 2015). It stimulates NSCs to grow and differentiate into new neurons (neurogenesis). BDNF is highly expressed in specific regions of the brain such as the hippocampus, olfactory bulb, cortex, and other (Bathina & Das, 2015).

FGF plays a role in multiple developmental processes such as brain patterning, branching morphogenesis, and limb development (Beenken &

Mohammadi, 2009). FGF promotes the proliferation of hippocampal NPCs, thus aiding in adult hippocampal neurogenesis (Kang & Hébert, 2015).

Nerve growth factor (NGF) is present in the central nervous system and acts on sympathetic and neural crest derived sensory neurons (Skaper, 2018). It serves to develop and maintain basal forebrain cholinergic neurons (Dreyfus, 1989). In addition to that, NGF contributes to pain sensation in which it increases the sensitivity of neurons and aids in the development of hyperalgesia. NGF plays a part in the immune response where it triggers the release of cytokines and activation of mast cells (Duh et al., 2018; Liu et al., 2014).

1.1.2.5.2. Cytokines

Inflammatory cytokines were found to have a dual role (either positive or negative) on proliferation and neuronal differentiation (Borsini et al., 2015). Cytokines tend to modulate neurogenesis in response to an activated immune response (Borsini et al., 2015). Three types of cytokines are known to be important during an immune response: interleukins such as IL-1 β and IL-6, interferons (IFNs), and tumor necrosis factor (TNFs). Cytokines assist in the protection of the brain by clearing the system from dead and damaged neurons and employing physiological and neuroprotective functions (Pan & Kastin, 2001). There is even evidence showing that during an inflammatory response, cytokines modulate NPC proliferation and thus neurogenesis especially in psychiatric and neurodegenerative disorders (Fuster-Matanzo et al., 2013; Makhija & Karunakaran, 2013). However, cytokines can in parallel affect neurogenesis when activated in the neurogenic niches due to peripheral

inflammation (Dantzer et al., 2008). It can directly alter hippocampal-dependent forms of synaptic plasticity (McAfoose & Baune, 2009) causing cognitive deficits (Wilson et al., 2002).

Once cytokines are released a cascade of events starts systemically, leading to an inflammation.

1.2. Inflammation

Inflammation is the natural response of living organisms to injury, trauma, infection, excessive irritation (neurogenic inflammation), pathological situations (stroke, ischemia, abnormal activation of the immune system). Such inflammation activates the immune system which triggers the secretion of primary inflammatory cells and signaling molecules (Pahwa et al., 2023). Inflammations are of two types: acute or chronic. Acute inflammations are characterized by tissue damage which can be due to noxious compounds, microbial invasions, or others. For a condition to be classified as acute, its symptoms must manifest rapidly and with a severe intensity, within a short period of time. Subacute inflammation is defined as the interphase between acute and chronic inflammation with a span of 2 to 6 weeks (Pahwa et al., 2023). To reach chronicity, the body needs to be exposed to an injury that alters its ability to repair and overcome certain damages caused by the injury. This type of inflammation is slow and long-term, ranging from months to years. Some features of chronic inflammation include vasodilation, capillary permeability, and diapedesis, which is the migration of neutrophils to the infected site through

the capillary wall. As soon as the inflammation worsens, macrophages, lymphocytes, and plasma cells start to replace neutrophils (Pahwa et al., 2023). Once those inflammatory cells are triggered, they begin to secrete cytokines, growth factors, enzymes which aid in fixing the tissue damage and then launch secondary repair such as fibrosis, granuloma formation, and other (Cutolo et al., 2019; Milenkovic et al., 2019; Needham et al., 2019; Yousuf et al., 2019).

Neuroinflammation is a localized inflammation in the central nervous system (CNS) and the peripheral nervous system (PNS).

1.2.1. Central Nervous System (CNS) Inflammation

The CNS was traditionally considered to be ‘immune privileged’ meaning it was neither susceptible to nor contributing to inflammation (Lucas et al., 2006). On the contrary, after significant revision, the CNS was found to exhibit features of inflammation. CNS inflammation could be either symptomatic or asymptomatic which makes detecting it more challenging in comparison to identifying PNS inflammation. CNS inflammation differs from PNS inflammation in ways of initiation and sensitivity to said inflammation (Whitney et al., 2009).

This phenomenon is vital for maintaining the homeostasis of the central nervous system and protect the brain from potential threats (More et al., 2013). For instance, in neurodegenerative diseases, the neuroinflammation cleans out the infection to control the disease’s progression and severity (Kempuraj et al., 2017). However, inflammation in the CNS does have a dual role. From one end,

it can repair injured neurons whereas on the other end it can induce neurodegeneration (Lucas et al., 2006).

Due to its immune privilege, the central nervous system only allows certain molecules such as T cells, macrophages, and dendritic cells to enter and exit under normal conditions (Hickey, 1999). These cells protect the central nervous system (Khakh & Sofroniew, 2015; Lau et al., 2012). Upon damage, the production of inflammatory mediators is activated allowing the involvement of astrocytes and microglia (Figure 3) (Stewart & Beart, 2016) as well as peripheral macrophages and lymphocytes (Whitney et al., 2009). Astrocytes, microglia, macrophages, and lymphocytes activate the secretion of a plethora of anti and pro inflammatory cytokines [i.e tumor necrosis factor alpha (TNF- α), interferon gamma (IFN- γ), interleukin-1 beta (IL-1 β), interleukin-8 (IL-8), interleukin-6 (IL-6), and chemokines], neurotransmitters (i.e. glutamate), and reactive oxygen species (i.e. nitric oxide) (Whitney et al., 2009). Such factors disturb the blood brain barrier and recruit monocytes and lymphocytes to cross through it to reach the site of inflammation (Hickey, 1999; Lossinsky & Shivers, 2004; Taupin, 2008). Additionally, CNS inflammation could vary from acute to chronic inflammation, where the inflammatory factors differ in the two extremes, resulting in the decrease of neurogenesis and neuronal survival (Bastos et al., 2008; Kohman & Rhodes, 2013; Whitney et al., 2009). It has been seen that spinal cord injuries (SCI) leads to inflammation in the central nervous system which causes the BBB to allow immune cells to pass through it towards the site of injury. Studies have shown that SCIs induced neurogenesis reduction,

long term hippocampal neurodegeneration, and cognitive abilities deficits (Jure et al., 2022; Jure et al., 2017; Wu et al., 2016; Wu et al., 2014).

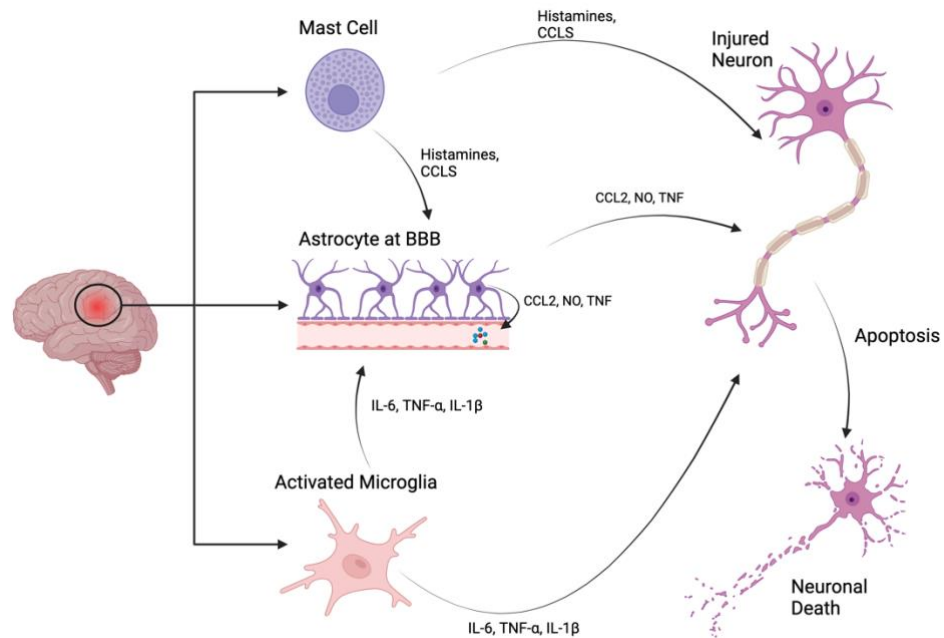


Figure 4. The inflammatory process in the central nervous system. Neuroinflammation leads to the activation of microglia, mast cells, and astrocytes which in turn release a plethora of anti and pro inflammatory markers such as cytokines, reactive oxygen species, and neurotransmitters. Such cells will affect the development of neurons leading to apoptosis and decrease in neurogenesis. This schematic was done using BioRender © 2023.

1.2.2. *Peripheral Nervous System (PNS) Inflammation*

PNS inflammation could be triggered by multiple agents such as injury, infections, autoimmune disorders, and other which could result in neuropathies, pain, sensorimotor disorders. The duality function of such an inflammation is yet to be fully understood as it could easily aid in axon regeneration but also in its degeneration (Mietto et al., 2015). Peripheral inflammation triggers an immune response, leading to the release of pro and anti-inflammatory molecules as well as inflammation-promoting mediators to defend the body against any pathological stimuli (Huang et al., 2021; Mietto et al., 2015). If said inflammation persists, then it

could potentially induce an inflammation in the central nervous system. Peripheral inflammation leads to the disturbance of the blood brain barrier which makes it vulnerable and permeable to peripheral cells and cytokines. Specific cytokines such as IL-6 and TNF- α can cross the BBB reaching the brain. Such cytokines can interact with cerebral endothelial cell receptors triggering the generation of prostaglandin E2 into the brain parenchyma (Cerejeira et al., 2014; Matsumura & Kobayashi, 2004). Peripheral inflammation can induce different tactics that can disrupt the BBB (Figure 5).

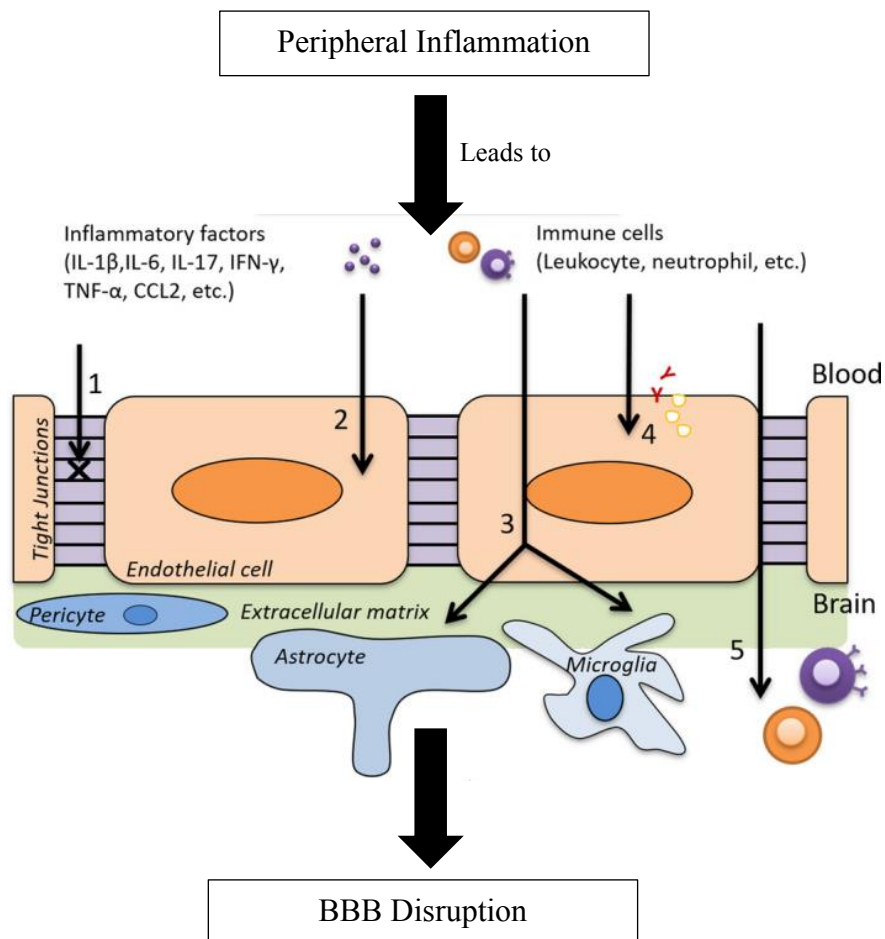


Figure 5. Schematic representation of different mechanisms of peripheral inflammation disrupting the BBB. 1. Tight junction changes; 2. Damage to endothelial cells; 3. Activation of microglia and astrocytes; 4. Modification of various transport pathways and receptors; 5. Penetration of peripheral immune cells. Adopted and modified from (Huang et al., 2021).

Infectious diseases that induce peripheral inflammation could potentially contribute to the rise and progression of neurodegenerative diseases like Alzheimer's disease, Parkinson's, and multiple sclerosis (MS) (Huang et al., 2021). Furthermore, microglial cells are activated which recruit circulating monocytes from the periphery (D'Mello et al., 2009). Also, macrophages and myeloid cells were found to infiltrate the hippocampus following a periphery inflammation which might lead to memory and cognitive deficits (Gampierakis et al., 2021; Schain & Kreisl, 2017).

Urinary tract infection (UTI) is the second most infectious disease in humans and the common cause of peripheral inflammation. UTIs are classified by anatomic site of involvement into two types: lower urinary tract infection and upper urinary tract infections. Lower urinary tract infections constitute an infection in the bladder or urethra or both (cystitis) whereas the upper infection involves the kidneys (pyelonephritis). Inflammation in the lower urinary tract is the most common due to anatomical reasons.

In numerous clinical observations, UTI patients have been showing altered mental statuses and cognitive changes. Among elderly patients, delirium is a non-specific symptom of UTI which could develop in one to two days and can range from agitation and restlessness to hallucinations and delusions (Manepalli et al., 1990). Studies have shown that elderly UTI patients experience delirium and potentially develop dementia or even provoke pre-existing dementia (Cerejeira et al., 2014). Dementia is strongly correlated with UTIs as one third of dementia patients are diagnosed with UTIs (Yourman et al., 2020). In a previous study conducted by our group, urinary tract infection (via E.coli) injected into the urinary

bladder decreased hippocampal neurogenesis which might explain the confusion and delirium seen in elderly UTI patients during UTI episodes (Batoul Darwish et al., 2022).

Uropathogenic *E.coli* (UPEC) strains are one of the main causes of urinary tract infections. Such strains are gram-negative bacteria that exhibit an ability to form biofilms which aids in their evasion of the host immune system. The glycolipid found on the surface of gram-negative bacteria that is responsible for such an evasion is called lipopolysaccharide (LPS).

1.3. Lipopolysaccharide (LPS) Model and Neurogenesis

LPS is known to induce inflammation as it activates the immune response in the host which triggers the release of pro inflammatory cytokines and other inflammatory mediators. LPS-induced inflammation can vary from an acute inflammation to a chronic one (local or systemic) depending on the severity and duration of exposure. Each animal responds to LPS stimuli in various manners depending on age and species (Batista et al., 2019). The outcome also could be influenced depending on the source of the stimulus, its dosage, the route, and the duration of administration (Zakaria et al., 2017). LPS injection can be administered either in the central nervous system or in the periphery.

1.3.1.1. Injection of LPS in the CNS

LPS injections in the central nervous system are meant to simulate cases of neuroinflammation. Different outcomes of such inflammation are seen to be dependent on which region of the CNS is LPS being administered to. Injection of

LPS into the lateral ventricle of the brain is shown to trigger an inflammatory reaction with the involvement of astrocytes and microglia cells in the brain and the release of cytokines (Shigemoto-Mogami et al., 2018).

The increase in the number of microglia and cytokines such as IL-6 and IL-1 β creates an unfavorable milieu which hinders both neurogenesis and the survival of newly generated neurons (Kohman & Rhodes, 2013). This causes neuronal cell loss in multiple brain regions such as the hippocampus, cerebral cortex, substantia nigra, ventral tegmental area, and the thalamus (Fan et al., 2011; Ulmer et al., 2002; Wang et al., 2013; Zhu et al., 2014). In fact, administration of LPS in the cortex has shown to decrease neurogenesis with no effect on cell proliferation (Ekdahl et al., 2003; Kohman & Rhodes, 2013). Furthermore, single intracerebroventricular (i.c.v) injections of LPS heightened IL-1 β levels in all brain regions except the cerebrum as well as induced microglial activation (Batista et al., 2019), thus leading to impairment of neural stem cell proliferation (Chamaa et al., 2018). Also, intranigral injections of LPS demonstrated the potential to decrease neurogenesis possibly due to a link with the microglial activation (Batoul Darwish et al., 2019).

Since LPS administration in the CNS causes neuroinflammation, it has been linked to impaired spatial memory in Sprague-Dawley rats (Haus-Wegrzyniak et al., 1998), depressive-like behaviors (Fu et al., 2010; Tang et al., 2016), and to the development of neurodegenerative diseases such as Alzheimer's disease (Wee Yong, 2010).

1.3.1.2. Injection of LPS in the periphery

LPS-induced inflammation in the periphery has been seen to cause significant effects and alterations in the central nervous system's immune response leading to a disturbance at the level of the brain and thus affecting behavior and cognitive functions. In fact, studies show that intraperitoneal administration of LPS causes a significant reduction in the proliferation of NSCs, the survival of newborn cells, their differentiation, and the overall neurogenesis process (Ekdahl et al., 2003; Fujioka & Akema, 2010; Saraiva et al., 2019). Also, intraplantar injection of LPS in rodents induced a local inflammatory response causing peripheral hyperalgesia and upregulation of proinflammatory cytokines (Kanaan et al., 1996).

Upon the induction of the LPS inflammation model, astrocytes are activated, and a surge of circulating cytokines is present forcing newly born neurons to undergo apoptosis (Ben-Hur et al., 2003; Chesnokova et al., 2016; Iosif et al., 2006; Monje et al., 2003). Additionally, the cytokines will elevate oxidative stress which will result in the impairment of developing neurons. Thus, hippocampal neurogenesis would be affected drastically.

1.4. Research Objective and Aim

Previous work from our group has shown the adverse effect of inducing UTI in the urinary bladder via bacteria (*E.coli*) on neurogenesis. Stemming from these findings, our group continued to induce acute inflammation by injecting LPS (an inflammogen found on *E.coli*) once into the bladder. Subsequently, our study combines the effect of chronic LPS induction into the bladder on the short term (proliferation of NSCs) and long term (neurogenesis of NSCs). We aim to

investigate the inflammation's effect on the proliferation and neurogenesis level of neural stem cells as well as cognitive behavior and nociception. This aids us in assessing the exploratory behavior and memory in rats. Additionally, we inspected the molecular variations resulting from the inflammation on the immune response cells including the expression level of cytokines as well as the neurotrophic factors.

CHAPTER 2

MATERIALS AND METHODS

2.1. Animals

Adult male Sprague-Dawley rats aged 8 weeks and weighing between 450 and 500g were used in this project. The rats were housed in an environment of 12-hour cycle of light and darkness at a temperature of 20-22°C in which water and food *ad libitum* were provided. Rats were allowed over one week to adapt to specific conditions one week prior to the start of experiments. Surgical procedures were conducted using general anesthesia, administered through inhalation of isoflurane. Rats were constantly monitored postoperatively, and their weight was taken during the light part of the cycle. The Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut granted approval to all experiments conducted in this project.

2.2. Intraurethral Instillation of Lipopolysaccharide (LPS) into the Urinary Bladder

Rats were deprived of water for a minimum of 4 hours prior the injections. Intraurethral injections were done under isoflurane anesthesia followed by a compression on the abdomen overlying the bladder to release any urine left. BD Angiocath IV catheters (24 GA, 0.75 IN, 0.7mm external diameter, 19mm long) (Figure 6a) were used. To ensure proper placement, the catheter should slide effortlessly into the urethra with no resistance. This was tested on trial rats of the same age by injection of trypan blue. After the catheter is fully inserted, the injection volume is gradually dispensed while the penis is held in an extended perpendicular position to the animal's body using forceps. The catheter is slowly retracted from the urethra in order to avoid

any leakage. Experimental rats were instilled with 0.5mL of 0.5mg/mL of LPS (Lipopolysaccharide from *Escherichia Coli* 055:B5; InvivoGen), while sham rats were instilled with 0.5mL of sterile saline. To ensure further inflammation, we followed an up and down motion of the catheter. For the induction of chronic inflammation, our model was based on a rat LPS-induced inflammation model previously described with modifications related to the dosage given (Yoshizumi et al., 2021). Moreover, before the start of the experiment, an instillation of trypan blue through the urethra into the urinary bladder was done on a trial rat (Figure 6b).

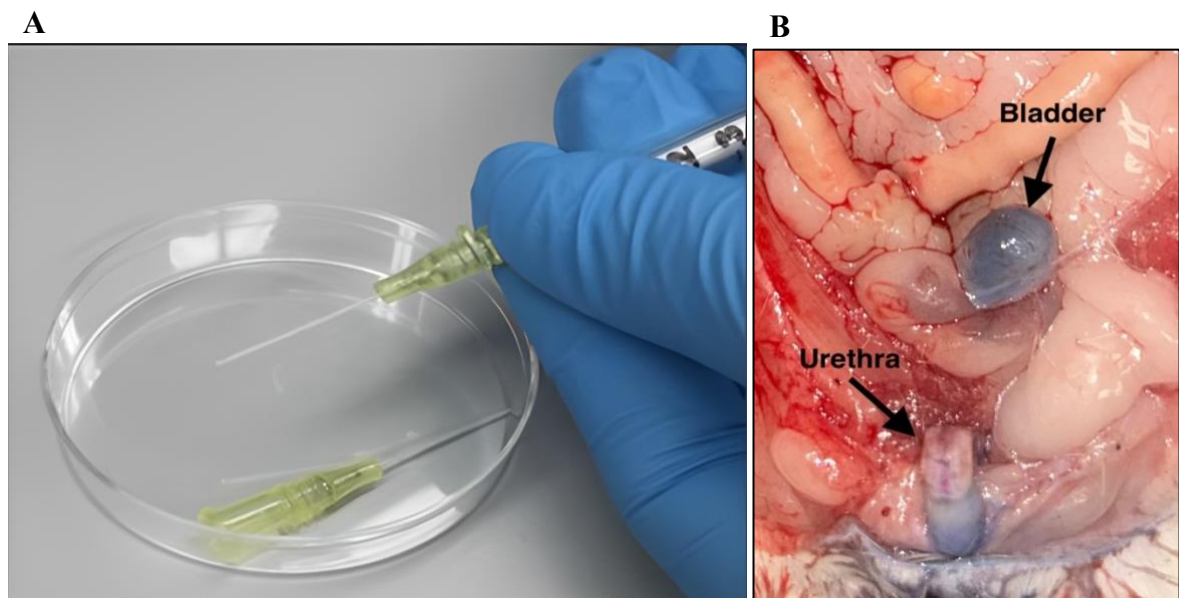


Figure 6. Catheters used and site of injection.

(A) Catheters used for intraurethral injections.

(B) Placement of trypan blue through intraurethral instillation in the bladder of a trial rat to show anatomical location.

2.3. Experimental Design

Rats were divided randomly into three groups: naïve, sham, and experimental (LPS). On day 0, following the pain sensitivity test, the sham group received intraurethral instillation of saline (n=8) into the bladder whereas the experimental group

(n=9) received LPS. This procedure was repeated on days 1, 2, and 3. First batch of rats (n=12) were sacrificed 24hrs after BrdU injection to investigate proliferation of DG neural stem cells. Second batch of rats (n=11) were sacrificed 1 month after BrdU injection to study neurogenesis. Figure 7 presents a summary of the experimental timeline of the proliferation and neurogenesis phase.

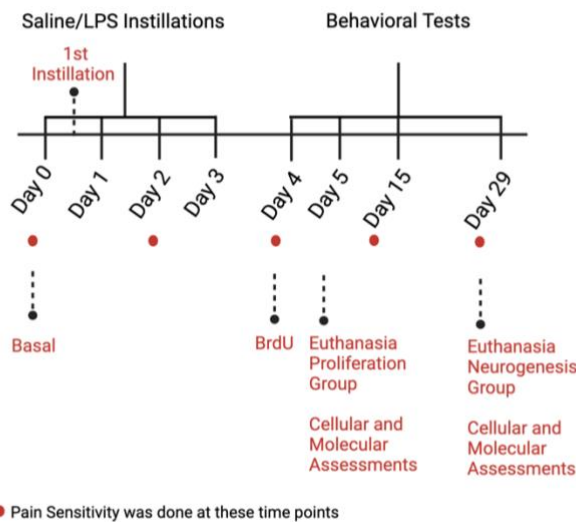


Figure 7. Experimental Timelines. Schematic diagram showing the experimental timeline followed for thermal pain sensitivity testing, behavioral tests, BrdU injections, and euthanasia.

2.4. BrdU Injection

Bromodeoxyuridine (BrdU, 5'-bromo-2-deoxyuridine), a synthetic thymidine analogue, was used to label and probe actively dividing cells to track cell proliferation and neurogenesis. The BrdU powder (BOC Sciences) was dissolved in 0.9% warm sterile saline based on the average weight of rats to get a final concentration of 200mg/kg/rat. It was administered intraperitoneally divided into 3 injections

(66mg/kg/300 μ L/injection) every 2 hours. This protocol was followed according to previous work done by our group (Chamaa et al., 2016).

2.5. Behavioral Tests

Various behavioral tests were performed including the thermal sensitivity (to assess the rat's response to pain), open field (to assess locomotion and exploration), novel object recognition (NOR; to assess recognition and novelty memory), Y-maze (to assess spatial reference and working memory), and elevated plus maze (EPM; to assess anxiety-like behavior). Before the start of the experiment, the rats were settled in the experimental room for 30 minutes for habituation.

2.5.1. Thermal Sensitivity Testing

Rats were placed in their designated transparent plexiglass boxes on the Ugo Basile thermal aesthesiometer apparatus. Before the start of the experiment, they were allowed 30 minutes for familiarization with the environment. A radiant heat was emitted from an infrared light source at the intensity of 36 infrared units (equivalent to approximately 45-50°C) to the shaved lower abdomen surface of the rats. The rats' abdomens were shaved thoroughly one day before for better heat exposure. The latency of withdrawal of the abdomen in response to the radiant stimulus was recorded. The recorded withdrawals of the abdomen should be accompanied by the arching of the back. The trial would be terminated if the animal fails to respond within 20.1s (cut off time). Each measurement was repeated 3 times separated by a time interval of approximately 3 minutes. The results were averaged and reported as mean \pm SEM.

2.5.2. Open Field

The open field apparatus is a square container with a border length of 60cm consisting of four walls that are of sufficient height to prevent the rats from escaping. Individual rats were put in the container facing a designated wall and allowed to roam for a 5-minute testing. The rat's behavior was monitored through a camera. Each video was analyzed using Any MazeTM software and variables including number of entries to central zone, total time spent in the central zone of open field, and total distance traveled were recorded. This experiment is used to assess the rat's spontaneous locomotor activity and anxiety-like behavior (Seibenhener & Wooten, 2015). Also, the novel object recognition (NOR) was done in the open field apparatus, but with the inclusion of two identical objects placed in a corner adjacent to each other. The NOR test is commonly used to investigate the recognition and novelty memory of rats (Chesworth et al., 2018). The rats were given 5 minutes to explore the area for familiarization. Then, rats were returned to their cages for an interval of 5 minutes. One of the identical objects is replaced with a novel object and the apparatus is wiped with 70% alcohol. Rats were then placed for the testing phase for a period of 5 minutes and the results were recorded. Each video was analyzed by the Any MazeTM software and variables such as the number of entries to the familiar and novel object zone as well as the total time spent in each zone were recorded.

2.5.3. Y-Maze

The Y-maze test was utilized to assess the rat's spatial working memory (Prieur & Jadavji, 2019). The maze consists of three identical arms (10 cm wide and 40 cm long) that are equally spaced (120° angle) forming a "Y" shape. During the

familiarization phase, rats were allowed to explore between the start arm and the familiar arm for 10 minutes where the third arm (the novel arm) was closed off. After that, rats were returned to their cages and rested for 1 hour. During the testing phase, the closed/novel arm was opened, and rats were allowed to roam for 5 minutes. The videos recorded of the testing phase were analyzed using the Any Maze™ software in which the number of entries and the total time spent in the novel arm were recorded.

2.5.4. Elevated Plus Maze (EPM)

This maze was used to assess the rat's anxiety-like behavior (Walf & Frye, 2007). The maze consists of 4 identical arms (2 closed arms with high walls and 2 open arms with no walls) that are elevated (height: 100cm). The rat was put at the junction of the 4 arms, facing the open end of the maze, and allowed to roam for 5 minutes. The videos recorded of the testing phase were analyzed using the Any Maze™ software in which the number of entries and the total time spent in each arm were recorded.

2.6. Euthanasia and Tissue Extraction

Rats were deeply anesthetized with isoflurane through inhalation and then euthanized. A group of rats were euthanized using the decapitation method (using a special guillotine) to get fresh tissues for RNA and protein extraction. Fresh bladder and hippocampus tissues were collected in cryovials, under sterile conditions. These vials were snap-frozen in liquid nitrogen and then moved to -80 °C for storage and later processing. Another group of rats were perfused transcardially with a solution of 0.9% saline followed by a solution of 4% formalin. Each rat's brain was extracted carefully and fixed further in 4% paraformaldehyde for 24hrs. The brains were then transferred to

30% sucrose solution in 0.1M PBS to be stored at 4°C for dehydration and then used for sectioning.

2.7. Brain Sectioning and Tissue Processing

The brains were stored in 30% sucrose solution at 4°C until they sink to the bottom of the conical indicating complete dehydration. They were then prepared for sectioning by removing the cerebellum and brain stem and only using the cerebral hemisphere. The area of interest containing the hippocampal formation was put vertically on the stage of the microtome and sliced into sections from rostral to caudal. The sections were sampled in a systemic manner using the fractionator method following a previous protocol used by our group (F. Chamaa et al., 2021) and others (Gundersen et al., 1999). In this way, we obtain 6 representative wells for each region of the hippocampus enabling us to have proper data from counting BrdU positive cells of one representative well per region (Figure 8). Coronal sections (40 µm) were cut serially, from the rostral to the caudal extent of the DG. By following the rostrocaudal coordinates -2.12 to -6.3 mm relative to bregma, this allows us to cover the whole hippocampal formation. The DG region was divided into three areas: rostral ranging from -2.12 to -3.7 mm relative to bregma, intermediate ranging from -3.7 to -4.9, and caudal ranging from -4.9 to -6.3 (Chamaa et al., 2018; Paxinos & Watson, 2006). This allows a better topographic distribution of BrdU positive cells where each well contains representative samples from all hippocampal area. All sections were obtained and stored in sodium azide solution (15mM in 0.1M PBS) at 4°C.

Normal Goat Serum (NGS), 0.1% Triton X diluted in PBS). The latter allows the reduction of non-specific binding. Sections were incubated overnight with the primary antibodies: rabbit anti-NeuN (1:500 Encor, SinoBiological) and mouse anti-BrdU (1:250, Santa Cruz) diluted in 3% blocking solution (3% BSA, 3% NGS, 0.1% Triton X diluted in PBS). On the second day, the brain sections were washed 3 times with 0.1M PBS for 5 minutes and then incubated with the secondary antibodies: goat anti-mouse 568 (1:250, AlexaFluor Invitrogen) and goat anti-rabbit 488 (1:250, AlexaFluor Invitrogen) diluted in 3% blocking solution on the shaker for 2 hours at room temperature. Finally, the sections were washed three times and mounted onto slides with mounting media containing DAPI staining.

2.8.2. Cell Counting and Microscopy

The BrdU positive cells were counted in the subgranular zone (SGZ) of the DG after 24 hours from BrdU injection (proliferation) and in the granular cell layer (GCL) after 4 weeks from the BrdU injection (neurogenesis). The confocal microscope was used to count the BrdU⁺ cells using the 40X-oil objective. The counted total number of positive cells per rat was multiplied by 6 (number of sets per rat). This denotes the overall number of BrdU⁺ cells in each region: rostral, intermediate, and caudal of the DG per rat.

Tile scan and Z-stack images were taken using Zeiss LSM 710 laser scanning confocal microscope at the 40X oil objective. Tile scan was used to acquire a stitched mosaic of the dentate gyrus while the Z-stack was done to display the distribution of all BrdU⁺ cells within the 40 μ m section in the whole DG of each region. The images were analyzed by the Zeiss ZEN 2009 image-analysis software and obtained with

maximal intensity projection. Representative immunofluorescent images for BrdU and NeuN stained slides were captured using laser screening on the confocal microscope of each region.

2.8.3. RNA Extraction

Total RNA was extracted from the hippocampus and bladder tissues using Qiazol (QiaGen). In brief, 750 μ L of Qiazol was added to tissues in vials alongside ceramic and metal beads to break down the tissues. This process was facilitated with the use of the OMNI Bead Ruptor™ machine to increase the efficiency of the extraction. Then, 300 μ L of chloroform was added to the vials followed by centrifugation for 20 minutes at 4°C and at 15,000g. to separate the RNA from the organic phases. The aqueous phase, containing the RNA, was extracted from the vials, and placed in a new vial. Isopropanol was then added to the vials followed by centrifugation for 30 minutes at 4°C and at 15,000g to precipitate the RNA into a pellet. Afterwards, the supernatant was removed and discarded then the vial was washed with 70% ethanol (600 μ L). The vials were centrifuged for 20 minutes at 4°C and at 12,500g and then the washing process was repeated once more. Finally, the supernatant was removed, and the pellet was allowed to air dry. Once fully dry, the pellet was diluted with RNase/DNase free water and then stored at -80°C for long term preservation.

2.8.4. cDNA Synthesis

Upon the extraction of RNA, its concentration and purity were measured by the ThermoScientific™ NanoDrop 2000™. This concentration was then used to calculate the adequate volume of RNA needed to obtain 2 μ g to convert the RNA to

complementary DNA (cDNA). A mixture of 3 μL of genomic DNA wipeout buffer, RNase/DNase free water, and RNA was prepared until a total volume of 15 μL was reached. The vials were incubated for 2 minutes at 42°C followed by the addition of a master mix consisting of reverse transcriptase (RT, 5 μL), RT buffer and primers to reach a final volume of 20 μL . The latter mixture was then incubated for 5 minutes at 25°C, 15 minutes at 55°C, and 2 minutes at 85°C.

2.8.5. Quantitative Real-Time PCR (RT-qPCR)

RT-qPCR (Bio-Rad CFX™ Manager Software; cat #1845000) was performed using the $\Delta\Delta C_t$ method and the SYBR green system (Applied Biosystems; cat #A46111) on the cDNA of the hippocampus and bladder (each in a separate plate) to analyze their mRNA expression. The PCR reactions were performed in duplicates and the expression of individual genes was compared to the standard reference which is the housekeeping gene *GAPDH*. The PCR process consisted multiple steps starting with DNA denaturation at 95°C for 5 minutes followed by 40 cycles (denaturation at 95°C for 10 s). Then, annealing at the specific primer temperature of 57°C for 30 s took place and finally an extension step was done at 72°C for 10 minutes. Gene expression was calculated utilizing the following equation: $\Delta\Delta C_t = \Delta C_{t(\text{target})} - \text{Average} [\Delta C_{t(\text{Sham})}]$, where $\Delta C_t = C_{t(\text{target})} - C_{t(\text{GAPDH})}$. The amount of endogenous target gene relative to a calibrator (*GAPDH*) became $2^{-\Delta\Delta C_t}$.

Table 2. List of primers used in RT-qPCR in the study. Glyceraldehyde-3-Phosphate Dehydrogenase: *Gapdh*; interleukin 1 beta: IL-1 β ; interleukin 6: IL-6; interleukin 8: IL-8; Brain-derived neurotrophic factor: *Bdnf*; necrosis factor: TNF- α ; Prostaglandin-endoperoxide synthase 2: COX-2

Rattus norvegicus Primers	Sequence (5'->3')
<i>Gapdh</i>	F: TCACCATCTTCCAGGAGCGA R: GGCGGAGATGATGACCCTTT
<i>IL-1β</i>	F: AGGCTGACAGACCCCAAAAG R: GGTCGTCATCATCCCACGAG
<i>IL-6</i>	F: ACAAGTCCGGAGAGGAGACT R: ACAGTGCATCATCGCTGTTC
<i>IL-8</i>	F: CCTAGGCATCTTCGTCCGTC R: CAGAAGCTTCATTGCCGGTG
<i>Bdnf</i>	F: CTCCGCCATGCAATTTCCAC R: CAGCCTTCATGCAACCGAAG
<i>TNF-α</i>	F: GGCTTTCGGAACACTCACTGGA R: GGGAACAGTCTGGGAAGCTC
<i>COX-2</i>	F: AAGGCGTTCAACTGAGCTGT R: ACACAGGAATCTTCACAAATGGAAC

2.9. Statistical Analysis

Statistical analysis and plotting of figures were made using Prism 9 GraphPad package (GraphPad software, Inc., CA, USA). One way ANOVA followed by Sidak's multiple comparison was used to test statistical significance when comparing a variable within the same groups. Two-way ANOVA followed by Tukey's multiple comparison was used to test statistical significance when comparing two variables between groups. Unpaired t-test (Welch's Test) was used to test statistical significance when comparing the means of two independent groups. The measure of statistical significance of the number of entries and time spent in the novel arm as well as the number of entries and time spent in the central zone were done by one way ANOVA followed by Sidak's multiple comparison test. Also, one way ANOVA followed by Sidak's multiple comparison test was used to evaluate the statistical significance for the number of entries to the novel object zone as well as the number of BrdU^{+ve} cells between the

naïve, control, and LPS groups. Unpaired t-test (Welch's test) was used to assess statistical significance for *IL-1 β* , *IL-6*, *IL-8*, *COX-2*, *TNF- α* , and *Bdnf* concentrations in the control and LPS groups. Moreover, two-way ANOVA test followed by Tukey's multiple comparison test was used to test the statistical significance for the latency of withdrawal of the thermal sensitivity test. Data were averaged per group and presented as mean \pm standard error mean (SEM). The P value of < 0.05 was considered as the limit of significance of differences at 95% confidence interval.

CHAPTER 3

RESULTS

3.1. General Observations

After four instillations of LPS into the urinary bladder, individual rats in each group (naïve, sham, and experimental) did not show any evident signs of abnormal motor behavior and elicited normal grooming behavior as well as normal weight gain. The rats were subjected to a series of behavioral tests post LPS instillations (1-6 days, short-term period) and 2-4 weeks post LPS instillations (long-term period).

The RT-PCR test was done on the bladder tissues of the sham and LPS groups to determine the mRNA levels of IL-1 β , IL-6, IL-8, and TNF- α . The mRNA levels of IL-6 (Figure 9B) and IL-8 (Figure 9C) in the bladder of the LPS group were significantly higher (4.63 ± 0.009 ; $p=0.015$; 4.93 ± 1.41 ; $p=0.042$, respectively) than that of the sham group (0.91 ± 0.46 ; 0.54 ± 0.29 , respectively).

The mRNA levels of IL-1 β (Figure 9A) and TNF- α (Figure 9D) in the bladder of the LPS group (4.70 ± 3.24 ; 2.45 ± 0.84 , respectively) were higher than that of the sham group (1.11 ± 0.49 ; 1.09 ± 0.27 , respectively). This evidence elicits the presence of inflammation in the bladder of the LPS groups after four instillations of LPS into the urinary bladder.

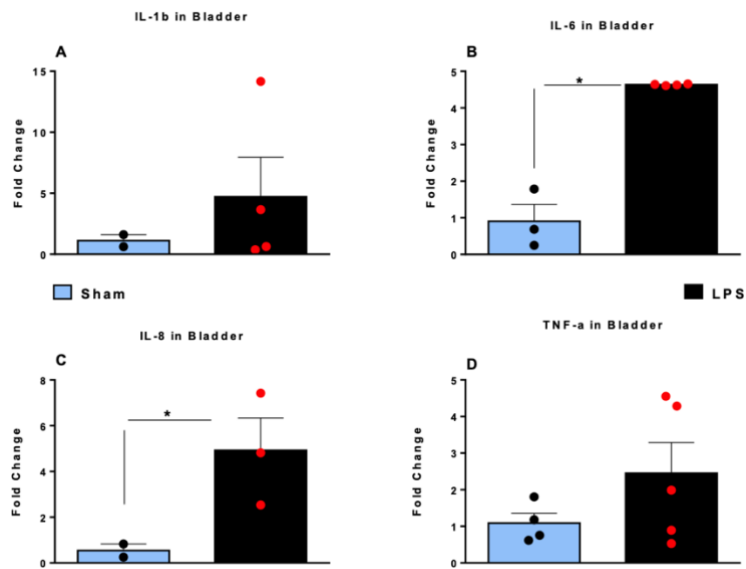


Figure 9. Instillations of LPS increased expression of the mRNA levels of cytokines in the bladder. (A,D) The mRNA levels of IL-1 β and TNF- α in the bladder in the LPS group were higher than that of the sham group. (B,C) The mRNA levels of IL-6 and IL-8 in the bladder of the LPS group were significantly higher when compared to the sham group. Unpaired t-test was used to assess significance between the groups (* $p < 0.05$). Data is expressed as mean \pm SEM

3.2. Behavioral Observations

3.2.1. Thermal Sensitivity

LPS instillations induced immediate and sustained thermal hypersensitivity. On days 1, 3, and 4, the LPS group (n=5) displayed a significant shorter latency withdrawal time (8.78s \pm 0.38; $p=0.0099$, 8.523 \pm 0.41; $p=0.0054$, 6.04s \pm 0.29; $p=0.0224$, respectively) of their abdomen compared to the sham group (n=4) on days 1, 3, and 4 (15.00s \pm 1.95, 12.06s \pm 0.86, 9.79s \pm 0.95, respectively). One month post LPS instillations (day 29), the LPS group (n=4) had a significantly lower latency withdrawal

time (7.47 ± 0.64 ; $p=0.0010$) of their abdomen when compared to the sham group ($n=3$) on day 29 ($13.85s \pm 0.65$) (Figure 10).

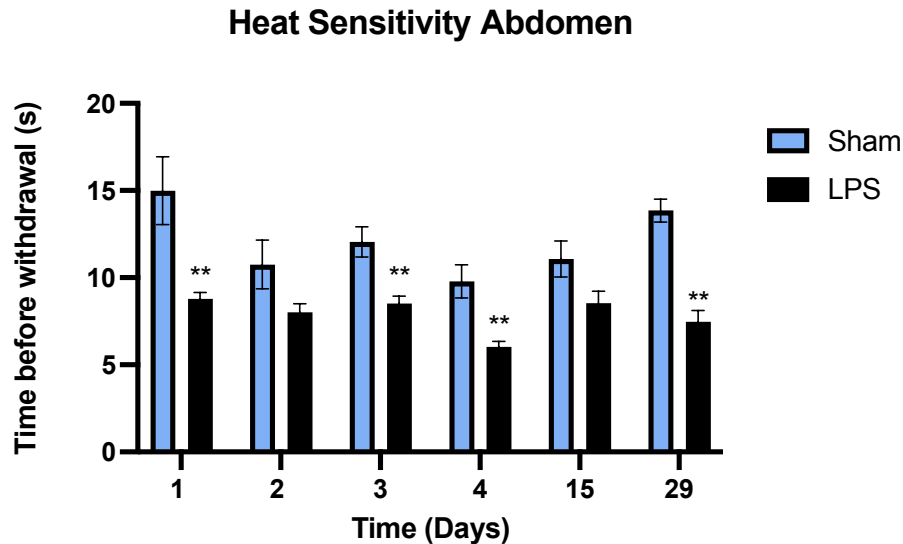


Figure 10. Rats with LPS instillations showed increased thermal sensitivity (hyperalgesia) on the skin of their lower abdomen. On days 1, 3, and 4, the LPS group showed a higher sensitivity to thermal stimuli administered to their abdomen when compared to the sham group on days 1, 3, and 4 respectively. On day 29, the LPS group displayed a lower latency withdrawal time of their abdomen compared to the sham group on day 29. Two-way ANOVA was used to assess significance between the groups (** $p<0.01$.). Data is expressed as mean \pm SEM.

3.3. Alterations in rats' behavior in the field tests induced by instillation of LPS into the urinary bladder

3.3.1. Instillations of LPS moderately affects the spontaneous motor behavior of rats

Two weeks post LPS instillations, LPS injected rats ($n=3$) covered less distance ($6.40m \pm 0.94$; $p=0.048$) than the sham group ($10.72m \pm 1.53$; $n=3$) which indicates that the motricity of the rats was moderately affected (Figure 11A).

The LPS group had a significant lower incidence of entries (4.00 ± 0.58 ; $p=0.031$) to the central zone than the sham group (13.67 ± 2.91) (Figure 11B).

Also, the LPS group has been recorded to spend less time ($5.30s \pm 1.54$; $p=0.023$) in the central zone than the sham group ($16.47s \pm 2.72$) (Figure 11C).

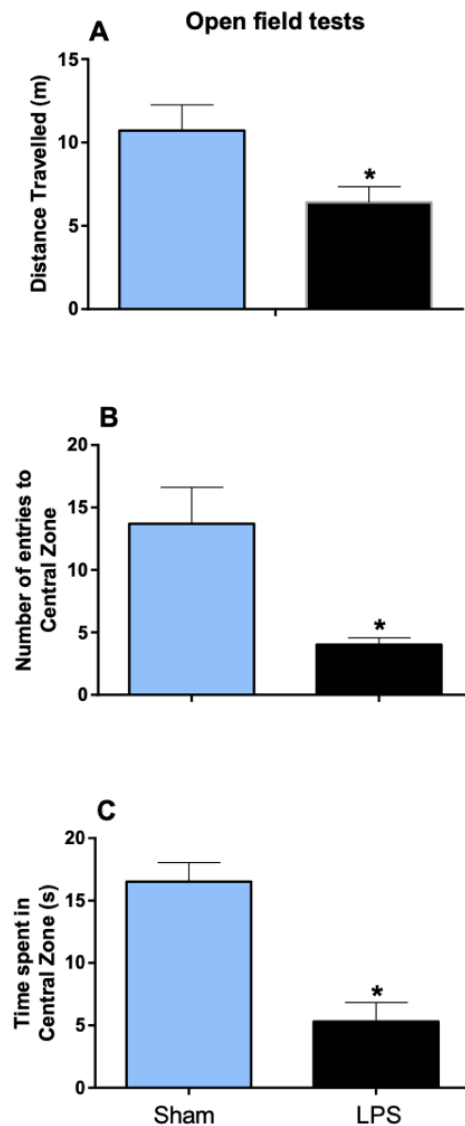


Figure 11. Instillations of LPS moderately affected the rats' motor behavior.

(A) Two weeks post LPS injections, spontaneous motricity was moderately affected in LPS injected rats compared to the sham group.

(B) The LPS group entered the central zone fewer times compared to the sham group.

(C) The LPS group spent less time in the central zone than the sham group.

Unpaired t-test was used to assess significance between the groups (* $p < 0.05$). Data is expressed as mean \pm SEM.

Two weeks post-instillations, the LPS group (3.25 ± 0.25) entered the novel object zone fewer times than the sham group (4.67 ± 1.45), however no significance difference was recorded (Figure 12A).

The LPS group elicited, also, significantly more time ($30.58s \pm 6.97$; $p=0.003$) to enter the novel object zone when compared to the sham group ($3.93s \pm 1.07$) (Figure 12B).

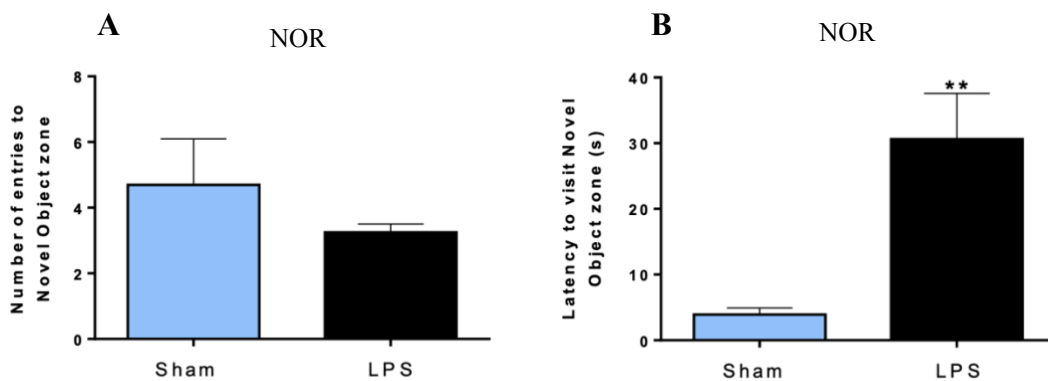


Figure 12. The LPS group had lower incidence of entries to the novel object zone compared to the sham group.

(A) Two weeks post LPS injections, the LPS group entered the novel object zone fewer times than the sham group, however no significance was recorded.

(B) The LPS group significantly took more time to enter the novel object zone when compared to the sham group.

Unpaired t-test was used to assess significance between the groups (** $p<0.01$). Data is expressed as mean \pm SEM.

3.3.2. Instillations of LPS significantly altered the rats' performance in Y-maze test

During the short-term period, the LPS group ($n=4$) significantly spend less time ($33.08s \pm 12.34$; $p<0.01$) in the novel arm compared to the sham group ($79.18s \pm 3.866$; $n=5$) and naïve group ($89.70s \pm 1.646$; $n=3$) (Figure 13A).

The LPS group ($193.1s \pm 57.64$; $p=0.019$) showed to significantly spend more time in the start arm compared to the sham group ($42.62s \pm 5.966$) (Figure 13B).

Also, the LPS group significantly displayed lower incidence of entries (2.75 ± 1.03 ; $p=0.0019$) to the novel arm compared to both the sham group (7.00 ± 0.32) and the naïve group (7.33 ± 0.67) (Figure 13C).

During the long-term period after LPS instillations, no comparable difference is displayed in the time spent in the novel arm between the LPS group ($78.5s \pm 10.65$; $n=3$) and sham group ($91.95s \pm 1.91$; $n=3$) (Figure 13D).

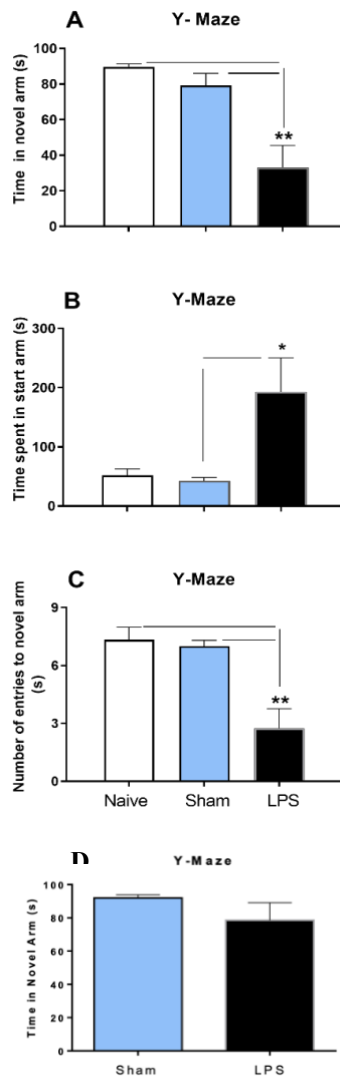


Figure 13. Y-Maze test elicits significant alteration in the behavior of LPS injected rats.

(A) The LPS group spent less time in the novel arm compared to the sham group and the naïve group.

(B) The LPS group spent more time in the start arm compared to the sham group and the naïve group.

(C) The LPS group entered the novel arm less frequently compared to the sham group and the naïve group.

(D) During the long-term period, no significance in the time spent in the novel arm was displayed between the LPS group and sham group.

Ordinary one-way ANOVA followed by Tukey-Kramer's multiple comparison test and unpaired t-test were used to assess significance between the groups (* $p < 0.05$, ** $p < 0.01$). Data is expressed as mean \pm SEM.

3.3.3. Instillations of LPS into the urinary bladder show alterations in the rats' behavior in the EPM test

The EPM test was done between 3-4 weeks post LPS instillations in order to avoid long term adjustment of the rats.

The LPS group (n=3) showed, significantly, fewer entries to the open arms fewer times (3.00 ± 0.57 ; $p=0.05$) than the sham group (6.66 ± 0.33 ; n=3) (Figure 14A).

Also, the LPS group was recorded to spend less time ($43.70s \pm 22.5$) in the open arms than the sham group ($71.5s \pm 9.12$) (Figure 14B).

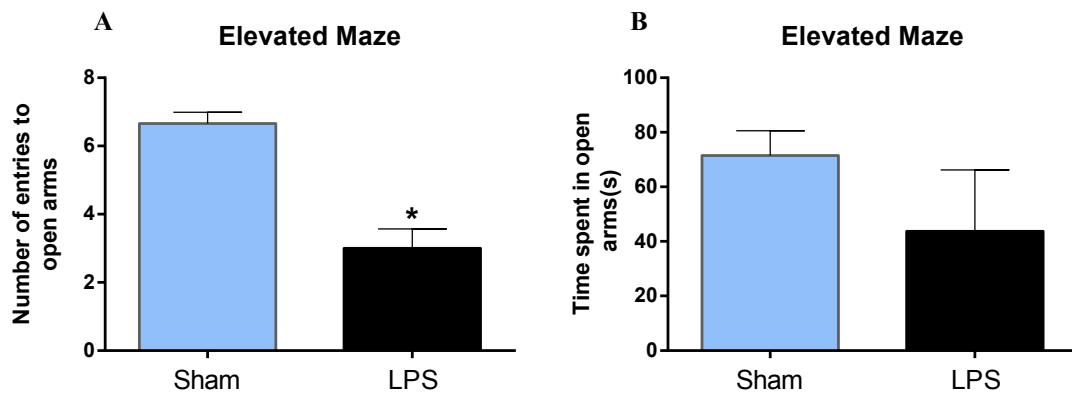


Figure 14. The LPS group displayed lower incidence of entries and less time spent in the open arms compared to the sham group.

(A) The LPS group entered the open arms fewer times compared to the sham group.

(B) The LPS group spent less time in the open arms than the sham group.

Unpaired t-test was used to assess significance between the groups. Data is expressed as mean \pm SEM.

3.4. LPS instillation and Hippocampal neural stem cells proliferation and neurogenesis

3.4.1. LPS instillations in the urinary bladder significantly decreased the proliferation of neural stem cells

BrdU positive cells were counted in hippocampal brain sections to analyze the effect of four instillations of LPS on proliferation of NSCs in the DG. The naïve group and sham group were pooled together due to the limiting number of rats. The LPS-injected group showed a significantly lower number of BrdU positive cells (4761 ± 181.8 cells; $p=0.0048$) compared to that of the naive/sham group (9168 ± 1472 cells) (Figure 15A).

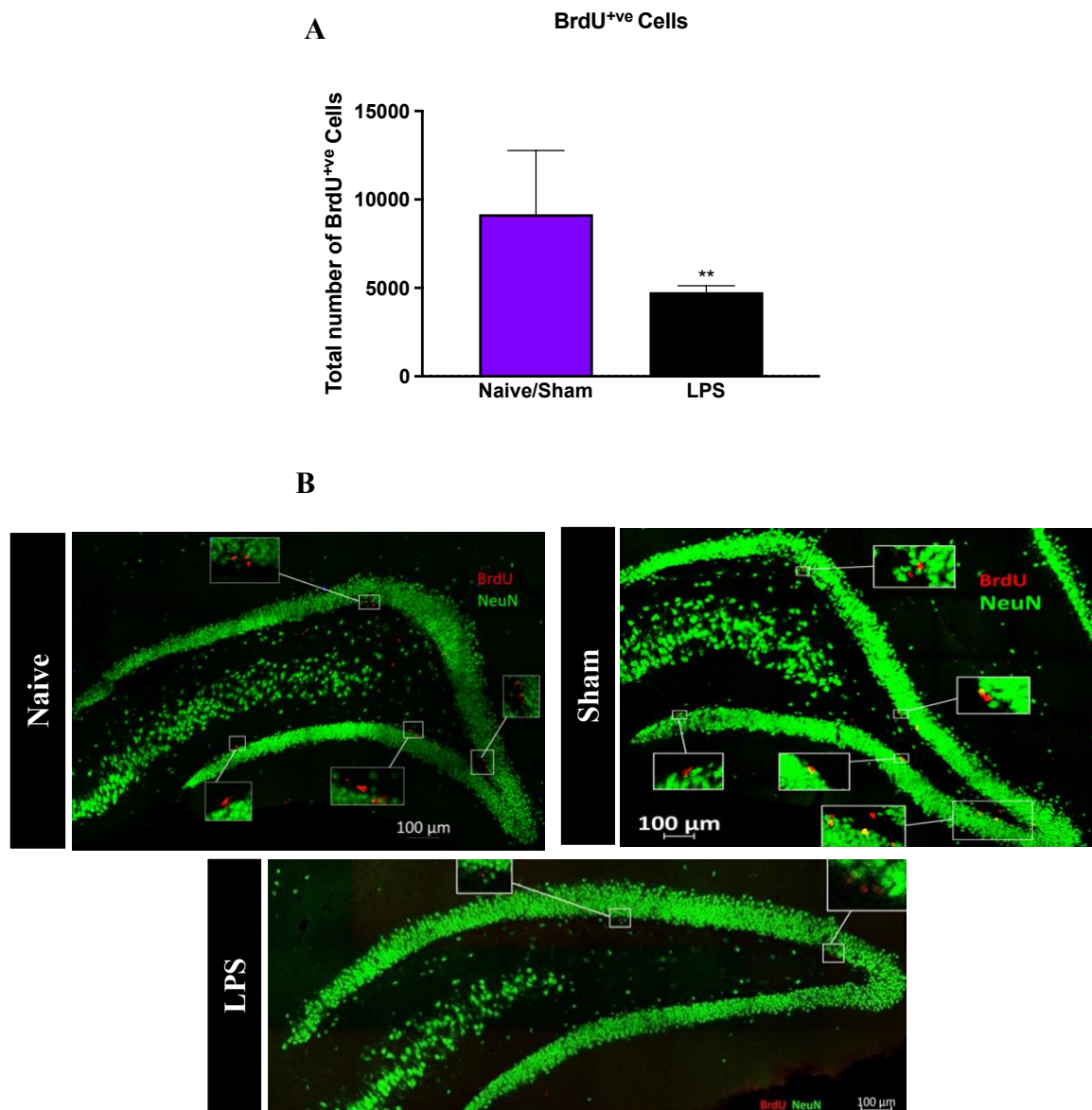


Figure 15. Urinary bladder inflammation induced significant decrease in neuronal stem cells proliferation.

(A) The number of BrdU positive cells in the LPS group (n=5) was significantly less than the naïve/sham group (n=6). Unpaired t-test was used to assess significance between the groups. (**p<0.01). Data are expressed as mean ± SEM

(B) Representative confocal images showing immunofluorescence labeling of NeuN (green) and BrdU (red) in the DG of the naïve, sham, and LPS group. Images were taken using Z stacks and tile scan with 40X-oil objective.

3.4.2. LPS instillations in the urinary bladder significantly decreased the number of neural stem cells

Four weeks post LPS instillations, the number of NeuN-BrdU positive cells in the LPS group (n=5) was significantly lower (3068 ± 280 cells; $p=0.019$) compared to the naive/sham group (4149 ± 347 cells; n=6) (Figure 16A).

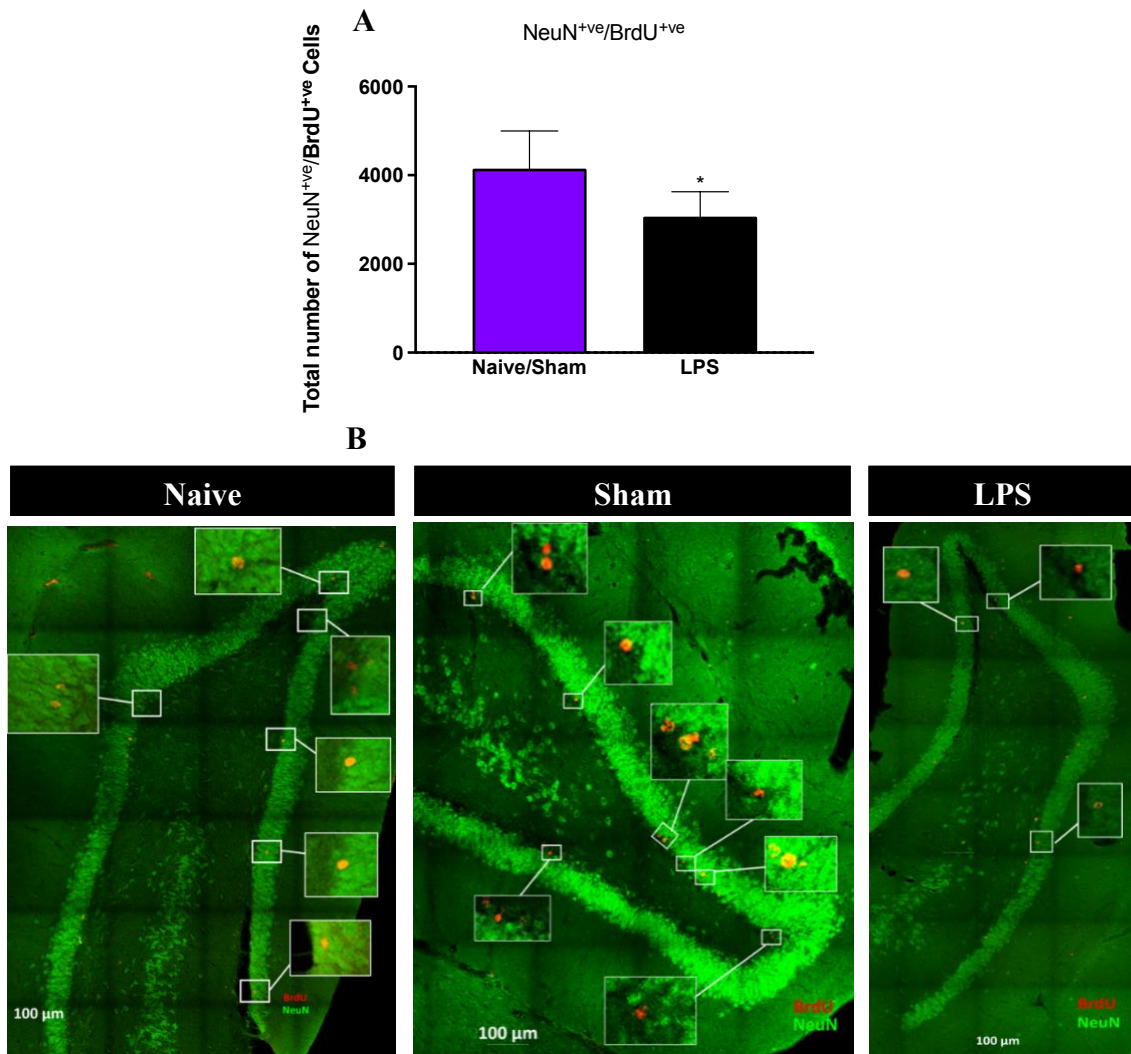


Figure 16. Decrease in the neurogenesis of DG NSCs after LPS instillations.

(A) The number of BrdU positive cells in the LPS group was significantly less than the control/sham group. Unpaired t-test was used to assess significance between the groups. Data are expressed as mean \pm SEM

(B). Representative confocal images showing immunofluorescence labeling of NeuN (green) and BrdU (red) in the DG of the naïve, sham, and LPS groups. Images were taken using Z stacks and tile scan with 40X-oil objective.

3.5. Effect of LPS instillations on cytokines and neurotrophic factors in the hippocampus

The mRNA levels of IL-1 β (1.51 ± 0.19) in the hippocampi of rats with LPS instillation were significantly ($p=0.038$) higher than that observed in the sham group (0.85 ± 0.15) (Figure 17A).

The mRNA levels of IL-6, IL-8, and COX-2, in the hippocampus of the LPS group (1.50 ± 0.35 ; 3.27 ± 1.33 ; 1.49 ± 0.49 ; respectively) were higher than that of the sham group (1.04 ± 0.21 ; 1.54 ± 0.68 ; 1.04 ± 0.17 ; respectively) (Figure 17B, D, E).

No comparable difference was observed in the mRNA levels of TNF- α between the sham group (1.09 ± 0.28) and the LPS group (1.20 ± 0.18) (Figure 17C)

The mRNA levels of BDNF (0.76 ± 0.18) in the hippocampus of the LPS group were comparable to that of the sham group (1.06 ± 0.22) (Figure 17F).

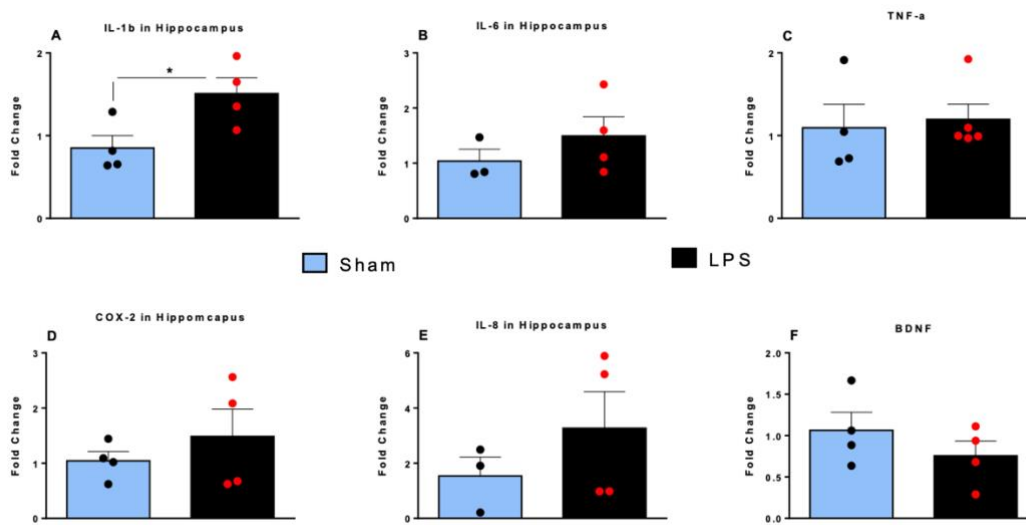


Figure 17. Instillations of LPS increases the mRNA levels of certain cytokines in the hippocampus.

(A) The mRNA levels of IL-1 β in the hippocampus in the LPS group (n=4) is significantly higher than that of the sham group (n=4).

(B, D, E) The mRNA levels of IL-6, COX-2, and IL-8 in the hippocampus in the LPS group were higher than that of the sham group.

(C) No difference is seen in the mRNA level of TNF- α between the sham group and the LPS group.

(F) The mRNA levels of BDNF in the hippocampus in the LPS group is lower than that of the sham group.

Unpaired t-test was used to assess significance between the groups (*p<0.05). Data is expressed as mean \pm SEM.

CHAPTER 4

DISCUSSION

In this study, we hypothesized that chronic intraurethral instillations of LPS into the bladder would influence the proliferation and neurogenesis of neural stem cells. Consequently, we sought to investigate any potential association between these effects and the cognitive abilities of the rats. In fact, the present work was based on data from two previous research studies, recently done by our group. The first one showed that infection induced by instillation of *E. Coli* in the urinary bladder of rats led to an inflammation in the urinary tract and ultimately decreased neurogenesis (Batoul Darwish et al., 2022). The second showed that a single intraurethral instillation of LPS (acute inflammation) could have a potential effect on NSC proliferation (Arnaout, 2022). As mentioned in the Materials and Methods section, we followed an LPS-induced cystitis model (Yoshizumi et al., 2021) on male rats with modifications including the dosage given and repetitive daily injections (over 4 days) aiming to simulate sustained inflammation.

Here, we present evidence that four LPS instillations caused inflammation in the bladder and a significant decrease in the proliferation and neurogenesis of neural stem cells in the DG of the hippocampus. Additionally, an alteration in the expression of trophic and inflammatory markers such as BDNF, IL-8, IL-6, IL-1 β , TNF- α , and COX-2 was observed in different tissues, namely, hippocampus and urinary bladder. These changes were paralleled by an overt disruption in the performance of rats in several behavioral tests.

Two groups of results provide confirmation of urinary bladder inflammation. The first is the thermal hypersensitivity of the skin of the lower abdomen, reflecting hyperalgesia referred to this area of the skin due to inflammation of urothelium, that is a well-documented syndrome known as cystitis (Yoshizumi et al., 2021). The hyperalgesia, maintained throughout the observation period of 30 days, may reflect the persistence of inflammation. The second is the increased level of expression of inflammatory marker observed in the bladder tissues sampled 5-6 days after starting LPS instillation. The fact that increased expression of IL-1 β and TNF- α did not reach significance level can be explained by the early (30-60 minutes) and short-lived (<24 h) increase of both cytokines following local injection of LPS or other irritants described previously by several authors from our group (Saab et al., 2009; Saadé et al., 2002; Safieh-Garabedian et al., 1997). However, the maintained significant increase in the mRNA expression of IL-6 and IL-8, can be considered as enough indication for the maintained urinary bladder inflammation (Skelly et al., 2013).

The data we collected showed that the abdomen of the LPS group has become more sensitized to the heat stimulus compared to the sham group over the acute and long-term periods. Moreover, the mRNA levels of IL-6 and IL-8 in the bladder of the LPS group were significantly higher than that of the sham group. These changes have been observed before (Skelly et al., 2013) indicating that the induction of the chronic inflammation was achieved.

We have also seen that the level of NSCs proliferation and neurogenesis in the dentate gyrus of the hippocampus have been significantly affected by the intravesical injections of LPS. These findings are consistent with previous studies linking reduction in neurogenesis with either neuroinflammation induced by intracerebral LPS injection

(Chamaa et al., 2018; Batoul Darwish et al., 2019) or with peripheral local and systemic inflammation (Chesnokova et al., 2016; B. Darwish et al., 2022; Perez-Dominguez et al., 2019; Valero et al., 2014) .

Our study attempted to correlate between the LPS-induced decrease in neurogenesis and two major observations, based on two sets of results: first, the reported changes in mRNA expression in the hippocampus, and *second*, the alterations of rats' performance in behavioral tests.

First, two out of the cytokines and neurotrophic factors elicited significant differences in their mRNA expression in the hippocampus of LPS-injected rats, IL-1 β and BDNF mRNA expression levels. IL-1 β is vital for normal regulation of hippocampal plasticity and memory as well as neuronal differentiation (Labrousse et al., 2009; Takemiya et al., 2017). On the other hand, increase in the levels of IL-1 β in the brain, namely, hippocampus, has previously been shown to impair hippocampal dependent learning and synaptic plasticity (Gibertini et al., 1995; Hein et al., 2010; Moore et al., 2009). Previous studies have shown significant increase of proinflammatory cytokines in parallel with a significant decrease of trophic factors expression (BDNF and GDNF) and hippocampal neurogenesis following intracerebral injections of LPS (Chamaa et al., 2018; B. Darwish et al., 2019; Safieh-Garabedian et al., 2003). However, apart from a universal consensus about a basal expression of cytokines and trophic factors in the CNS under normal conditions, discrepancies are shown in the intensity (significance) of changes and distribution in the CNS of these markers following peripheral inflammation induced by LPS and other irritants or pathological conditions (Al-Amin et al., 2011; B. Darwish et al., 2022; Safieh-Garabedian et al., 2002)

Second, possible correlation between rats' performance in cognitive behavioral tests with either pain or decreased hippocampal neurogenesis was envisaged in several tests. After four LPS instillations, the LPS group showed that spatial memory was significantly affected in the Y-maze test. This result was previously reported by our group (Farah Chamaa et al., 2021). Moreover, after two weeks of LPS instillations, the rats' novelty memory in the NOR test has been affected as the LPS group took more time to enter the novel object zone when compared to the sham group. These findings supported by the decreased levels of BDNF in the LPS group might explain the results observed in the behavioral test, as BDNF plays a role in learning and memory (Eu et al., 2021; Miranda et al., 2019). Moreover the decreased number of BrdU⁺ cells in the hippocampus have also been associated with decrease performance in behavioral test. (B. Darwish et al., 2022).

The spontaneous motor behavior of the rats in the open field test was affected where the LPS group displayed a significant difference in the number of entries to the central zone and spent less time in it compared to the sham group. Furthermore, upon subjecting individual rats to the EPM test, we saw that the LPS group entered significantly fewer times into the open arms compared to the naïve group suggesting an increase in the rats' anxiety-like behaviors. Pain from inflammation has been associated with increased anxiety-like behaviors (Spinieli et al., 2022) explaining the results of the EPM and Open field test. This study sheds light on the effects of chronic peripheral inflammations on the brain, cognition, behavior, and hippocampal neurogenesis of rats. The hippocampus is shown to be a crucial region of the brain involved in the processing of pain where various evidence shows altered hippocampal plasticity and cytokine expression in chronic pain animal models (Apkarian et al., 2016; Sarkis et al., 2011).

One limitation of the study is the sample size as it should be increased to reduce variability for a better depiction of the results. Further work needs to be done to get a better understanding of the influence of UTI-like inflammations on the brain. For instance, adjusting the LPS dosage administered in the rats might unveil a more significant impact on the rat's cognitive abilities, proliferation, neurogenesis, and their corresponding neurotrophins and inflammatory markers. Moreover, the model used can be divided into female and male groups of rats to study further the effect of peripheral inflammation in both genders, aiming to study the gender dynamics and eliminate any biases that could affect the accuracy of the work. Finally, additional analysis of the tissues could offer a more distinct understanding of the pathways implicated as well as the inflammatory markers at play during instances of inflammation.

In conclusion, this study presented data that repetitive intravesical injections of LPS affected the thermal sensitivity, spatial reference memory, novelty memory, and exploratory behavior of rats. It also showed significant increase in the mRNA levels of IL-1 β in the hippocampus and IL-8 and IL-6 in the bladder. Further investigations need to be done for a better understanding of the influence of UTI-like inflammations on the hippocampal functions and expression of inflammatory mediators.

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