AMERICAN UNIVERSITY OF BEIRUT

STUDYING THE EFFECTS OF PERIPHERAL INFLAMMATION, SPECIFICALLY URINARY TRACT INFECTIONS, ON NEURPLASTICITY

by

RAMI DAOUD ARNAOUT

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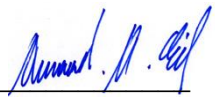

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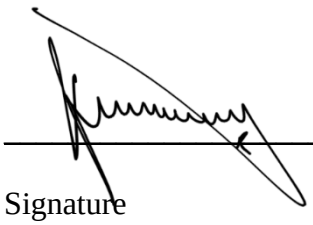

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ABSTRACT

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Title: Understanding the Effects of Peripheral Inflammations, Specifically Urinary Tract Infections, on Neuroplasticity

Several clinical cases have reported that urinary tract infections (UTI) cause cognitive deficits mainly in elderly patients increasing the chances of developing neuropsychiatric disorders. A study has shown that UTIs reduced neurogenesis, caused inflammation to the urinary tract and induced visceral pain. In this project, adult male Sprague-Dawley rats received transurethral instillation of LPS to their bladders to mimic UTI-associated inflammation. Here, we aim to investigate the effect of peripheral inflammation in the urothelium on adult hippocampal neurogenesis. Control groups were instilled with sterile saline. Bromodeoxyuridine (BrdU) analog (200mg/kg, i.p) was injected 24 hours before the euthnasia in order to assess hippocampal neural stem cells (NSCs) proliferation. Rats were euthanized 3 days following LPS instillation. Behavioral tests including, Y-maze, and Novel Object Recognition, were used to assess reference and recognition memory respectively. Thermal and mechanical sensitivity were assessed following the instillation of LPS. RT-qPCR was done on bladder and hippocampal tissue to measure the mRNA transcription level of cytokines and neurotrophins. Brain sections were stained using fluorescent probes to count the number of BrdU-positive cells. Rats that received LPS were more sensitive to pain in both thermal and mechanical test. In addition, the LPS group had a higher level of IL-1β mRNA in the bladder. However, both groups had similar numbers of BrdU-positive cells in the dentate gyrus of the hippocampus and performed similarly in both the Y-maze and Novel Object Recognition test.

TABLE OF CONTENTS

ILLUSTRATIONS

Figure

TABLES

Table

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

IL: Interleukin

IPC: Intermediate Precursor cells

NeuN: Neuron nuclear protein

NGF: Nerve growth factor

NPC: Neural Progenitor Cells

NSCs: Neural Stem Cells

SCI: Spinal cord injury

SGZ: Sub-granular Zone

SVZ: Subventricular zone

UPEC: Uropathogenic E. coli

UTI: Urinary Tract Infection

CHAPTER I

INTRODUCTION

A. Overview on neuroplasticity

The brain is constantly making new connections and new networks between neurons as part of a process referred to as neuroplasticity. This process is crucial for engraving new information and experiences along many other important cognitive functions. It is the mechanism in which the brain goes through functional and structural changes after being exposed to external and internal stimuli. These changes are the result of two processes: neuronal regeneration and collateral sprouting as well as functional reorganization (Mateos-Aparicio & Rodriguez-Moreno, 2019; Puderbaugh & Emmady, 2021). This process happens in most neuronal cells in the brain and it is regulated and overseen by glial cells. Astrocytes uptake and clear neurotransmitters and they also regulate the function of synapses (Bourgognon & Cavanagh, 2020; Halassa & Haydon, 2010; Sattler & Rothstein, 2006; Schousboe & Waagepetersen, 2005). On the other hand, microglia are involved in neuroplasticity by modulating and monitoring neuronal activity and participating in synaptic pruning (Dissing-Olesen et al., 2014; Y. Li, Du, Liu, Wen, & Du, 2012). This process is particularly important in case of brain injury to help in recovery. Aside from synaptic plasticity and neuronal sprouting, neurogenesis is an important contributor to plasticity in the brain.

B. Neurogenesis

1. Defining Neurogenesis

Neurogenesis is the plastic capacity of the brain to continuously generate new neurons throughout adult life (Begega, Alvarez-Suarez, Sampedro-Piquero, & Cuesta,

2017; Kempermann et al., 2010). This process is very active during infancy but decreases with age (Kuhn, Dickinson-Anson, & Gage, 1996). Interestingly, neurons in the brain cannot regenerate after postnatal life but there remain certain brain regions that maintain the capacity to generate and integrate new neurons into their circuitry. These regions are referred to as neurogenic niches and are limited to three main regions in the adult brain. These regions are: the dentate gyrus (DG) of the hippocampus, and the subventricular zone (SVZ) (P. S. Eriksson et al., 1998).

2. Neurogenic niches

A neurogenic niche constitutes the microenvironment that contains all the necessary components that supports the growth of neural stem cells (NSCs) as well as a variety of cells such as immature cells, glial cells, immune cells and an extracellular matrix. Each one of these components play a role in the regulation of the growth of these NSCs and neural progenitor cells (NPCs) (Kempermann, Song, & Gage, 2015; Mercier, Kitasako, & Hatton, 2002; Plumpe et al., 2006; Shapiro, Korn, Shan, & Ribak, 2005). The two neurogenic niches in humans are the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricle. In rodents, the olfactory bulb is another additional neurogenic niche, however, in humans it is still controversial.

a. Dentate gyrus

Most of the research on neurogenesis focuses on the DG of the hippocampus as it houses the neurogenic niche that is responsible for some of the major cognitive functions in the brain such as memory, learning, spatial orientation and emotional behavior (Kempermann et al., 2015). In the dentate gyrus, the precursor cells or neural stem cells are located in a thin layer, between the hilus and the granule cell layer, called

the subgranular zone (SGZ) (Figure 1). The newly generated neurons will migrate from SGZ to the granular layer and later extend their processes to the CA3 region of the hippocampus (Altman & Das, 1965; P. S. Eriksson et al., 1998; Kempermann et al., 2015). Information from the entorhinal cortex is transported through the axonal projections of these granule cells, making the mossy fiber tract, and terminating in the CA3 area of the hippocampus as "boutons" and their firing is regulated by interneurons in the dentate gyrus(Altman & Das, 1965; Kempermann et al., 2015). All this sets a unique circuitry that characterizes the dentate gyrus. The newly formed neurons in the

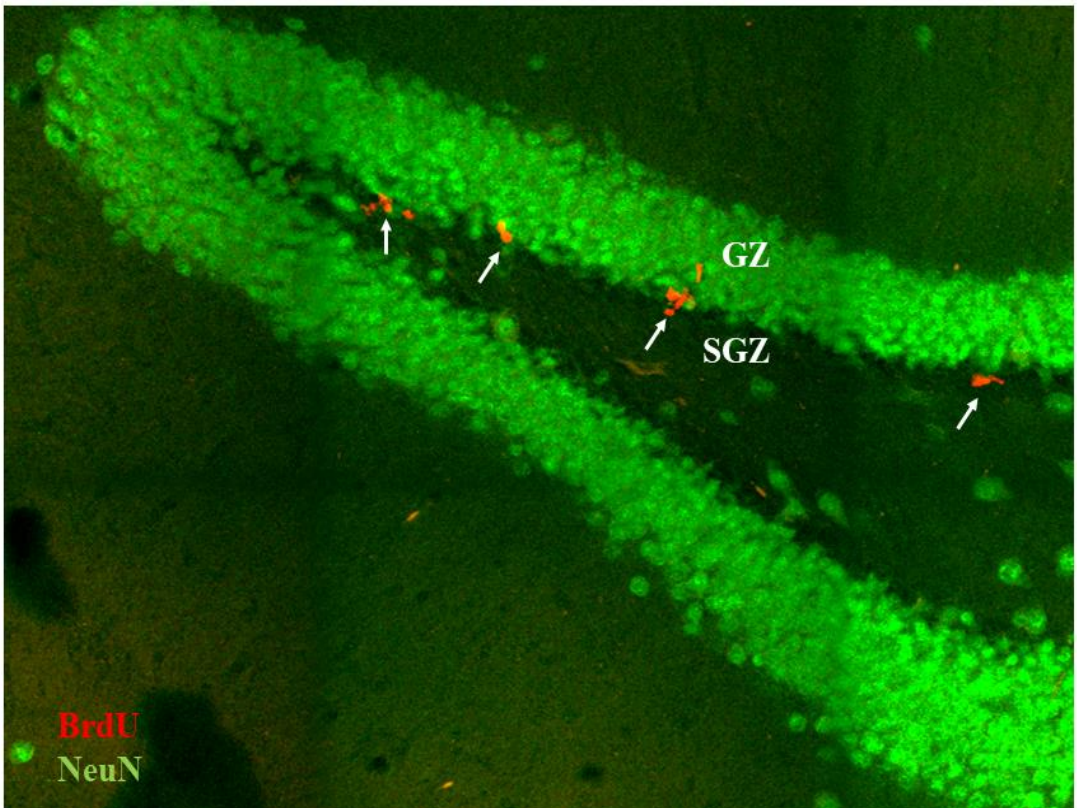

Figure 1. The dentate gyrus. Immunofluorescence image showing the layers of the DG stained with mature neuronal marker; NeuN (green) and the position of NSCs stained with BrdU (red, indicated by white arrows) in the SGZ. GZ: Granular zone; SGZ: Subganular zone.

DG have an increased capacity for synaptic plasticity in the early weeks following their development (Chesnokova, Pechnick, & Wawrowsky, 2016; Ge, Yang, Hsu, Ming, & Song, 2007; Kempermann et al., 2015). Over the course of 4-5 weeks, NSCs will go through several stages of maturation. Radial glia-like type 1 cells get activated and start

dividing to give rise to type 2 cells. They express the markers, Nestin, Sox2 and GFAP. Type 2 cells proliferate rapidly and stop expressing GFAP as they begin to express TBR2 which an essential transcription factor that regulates neurogenesis. Subsequently, Type 3 cells are produced, and they start to express neuronal markers such as doublecortin (DCX) and Prox1 which indicate their commitment to the neuronal lineage. Finally, once fully mature, the cells express the Neuronal nuclear protein (NeuN) and Calbindin (Bonaguidi, Song, Ming, & Song, 2012; Farah Chamaa, Darwish, Saadé, & Abou-Kheir, 2021; Lv et al., 2019).

b. Subventricular zone

The SVZ is part of the lateral wall of the lateral ventricles. The main cells found are neuroblasts, astrocytes and ependymal cells (Garzon-Muvdi & Quinones-Hinojosa). The neurogenic niche in the SVZ has larger germinal layer of NSCs as compared to the sub-granular zone of the dentate gyrus (Doetsch & Alvarez-Buylla, 1996). The rate of neurogenesis, the type of cells produced, and their fate and functions vary among these two niches. In the SVZ, the newly born cells go through a migratory path towards the olfactory bulb (OB) where they mature into interneurons (Ming & Song, 2011). This path is called the rostral migratory stream (RMS) (Bergmann et al., 2012; Sanai et al., 2011).

3. Tracing Neurogenesis

Bromodeoxyuridine (BrdU) is the golden standard used in the literature to study and detect neural stem cells (NSCs) and neural progenitor cells (NPCs). BrdU is a synthetic bromo-thymidine analog that gets incorporated into the DNA of replicating cells and can later be detected by specific immunofluorescent staining using antibodies

directed for BrdU. (P. S. Eriksson et al., 1998). Eriksson et al. studied postmortem brains of humans treated with BrdU, he found that cells in the hippocampus that were labeled for BrdU were also expressing neuronal markers such as NeuN (Apple, Fonseca, & Kokovay, 2017). This provided one of the early pieces of evidence that there are proliferating cells in the hippocampus of adult humans that would eventually differentiate into new neurons (P. S. Eriksson et al., 1998; Kempermann et al., 2015).

4. Significance & Role of Neurogenesis

sNeurogenesis is involved in learning and memory, thus any changes in the formation of new neurons will have an effect on cognitive abilities, leading to cognitive deficits (Costa, Lugert, & Jagasia, 2015) as well as problems with spatial learning and memory retrieval (Chesnokova et al., 2016). Thus, neurogenesis is highly associated with cognition. Cognition represents the ability to understand the world around, knowing ones

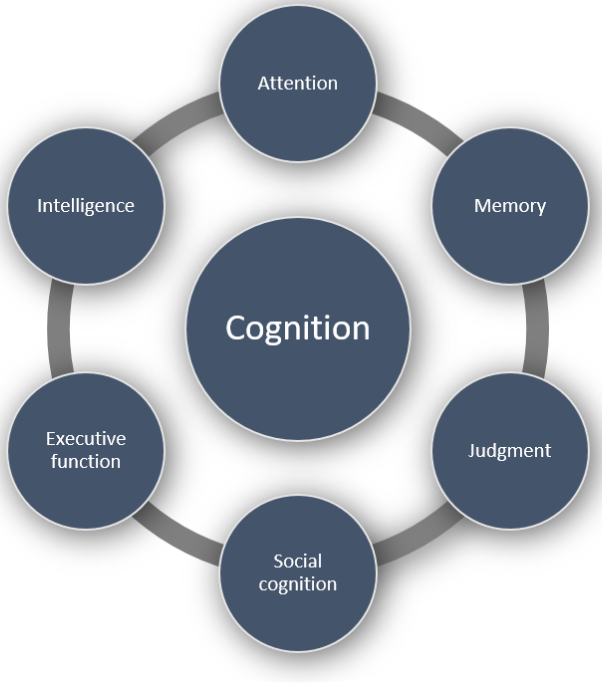

Figure 2. The main components of cognition: Cognition comprises several aspects which include: memory, attention, judgement, intelligence and others

needs, but also gaining knowledge. The main components of cognition are: the formation of new memories (Ge et al., 2007), spatial learning (Deng, Aimone, & Gage, 2010), pattern separation (Sahay et al., 2011), cognitive flexibility, associating new memories with old ones (Jessberger & Gage, 2014; Kohman & Rhodes, 2013), attention, knowledge, decision-making, planning, reasoning, judgment, perception, comprehension, and language (Dhakal & Bobrin, 2021) (Figure 2).

Some of these processes are made possible through neurogenesis. Newly formed granule cells are still easily excitable and easily changed morphologically. One of their primary functions is to influence and facilitate the acquisition and consolidation of new memories and the learning of new knowledge through their integration to the network and the growth of new connections. They also contribute to a better mental wellbeing, playing a role in regulating stress and anxiety, in addition to reducing the incidence of neurodegenerative disorders (Farah Chamaa et al., 2021; Kempermann et al., 2015; von Bohlen Und Halbach, 2007). However, this organized process is delicate and can be disrupted and modulated in different ways in response to different factors.

5. Modulation of neurogenesis

Numerous stimuli affect and control neurogenesis. This process is tightly controlled by the milieu and capacity to achieve a balance between survival and apoptosis (Dupret et al., 2007). During development, 50% of NSC do not mature and they undergo apoptosis during the first 2 weeks (Farah Chamaa et al., 2021; Kempermann, Gast, Kronenberg, Yamaguchi, & Gage, 2003; Sun et al., 2004). Similar to other nervous pathways, neurogenesis in the DG is regulated by neurotransmitters released by other neurons. Interestingly, the SGZ is connected to numerous regions of the brain and

receives several synaptic inputs of different types: dopaminergic from the ventral tegmental area, serotonergic inputs from the raphe nuclei, acetylcholinergic from the septum, and GABAergic from interneurons. Additionally, pharmacological or physical manipulation of these neurotransmitters and inputs, can have an effect on neurogenesis (Bengzon et al., 1997; Cooper-Kuhn, Winkler, & Kuhn, 2004; Dominguez-Escriba et al., 2006; Kempermann et al., 2015). Furthermore, stimulation of the supra-mammillary nucleus (SuM) promotes neurogenesis and the proliferation of NSCs (Y.-D. Li et al., 2022). Deep Brain stimulation of the thalamic nucleus can also boost neurogenesis (F. Chamaa et al., 2021; F. Chamaa, Sweidan, Nahas, Saade, & Abou-Kheir, 2016).

Moreover, several physiological processes control neurogenesis. Insulin-like growth factor-1 (IGF-1) initiates the protection of the brain by promoting neurogenesis after a traumatic brain injury, by affecting NSC proliferation and differentiation as well as maintaining and enhancing dendritic growth (Williams, Carlson, & Saatman, 2022). Cell to cell contact can also play a role in the fate and maturation of cells. Neighboring cells surrounding NSC can interfere in their fate and promote neuronal instead of astrocytic maturation. This also promotes the expression of the Notch pathway which also promotes cell proliferation (McIntyre, Karimzadeh, Riazalhosseini, Khazaei, & Fehlings, 2022). Moreover, certain chemicals and drugs, modulate neurogenesis. The compound cannabidiol (CBD) reduced depressive-like behaviors due to stress, by stopping radial NSC overactivation and their differentiation to astrocytes, thus promoting the neuronal lineage, and increasing neurogenesis (Hou et al., 2022). Interestingly, due to the circadian rhythm, hormonal and chemical variations along the day can also modulate neurogenesis. In addition, there is an established association between neurogenesis and depression as depression has been shown to suppressed neurogenesis (Wang et al., 2015).

Most importantly, it is key to mention that certain genes play a role in the variations in the rate of neurogenesis. DISC1 is a gene whose translocation increases the chances of developing psychiatric disorders, such as schizophrenia. The ablation of this gene was also associated with decreased neurogenesis, changes in the formation and distribution of granule cells in addition to changes in hippocampus-dependent behaviors in rodents (Duan et al., 2007; Gonçalves, Schafer, & Gage, 2016; Kvajo et al., 2008).

Moreover, neurotrophins and cytokines also play major roles in the modulation and regulation of neurogenesis.

a. Neurotrophins

Neurotrophic factors are involved in the regulation of certain processes in the nervous system. In fact, they are implicated in the process of neurogenesis and long term potentiation (LTP) (Kohman & Rhodes, 2013). Important neurotrophins are Brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and insulin-like growth factor (IGF) and Fibroblast growth factor 2 (FGF-2).

In the brain, NSC are regulated by FGF-2 when they start maturing to type II cells (Jin et al., 2003). Similarly, BDNF, is a commonly found neurotrophin in the brain. It plays an important role in the protection and care of neurons as well as in the maturation and differentiation of NSCs and NPCs.

On the other hand, Nerve growth factor (NGF) plays a role in the pain sensation. It increases the sensitivity of neurons, and help in the development of hyperalgesia. This neurotrophin is involoved in the immune response, since it is secreted by immune cells and triggers the release of cytokines and the activation of more mast cells (Duh et al., 2018; Liu et al., 2014).

b. Cytokines

Major modulators of neurogenesis are cytokines. Under normal physiological conditions, cytokines such as IL-1β and IL-6 play important roles in maintaining and regulating plasticity and specifically cognitive functions in the brain (Bourgognon & Cavanagh, 2020) (Table 1).

Table 1. Effects of pro-inflammatory cytokines on plasticity in normal physiological conditions

	Interleukin-1β (IL-1β)	**Interleukin-6 (IL-6)**
Physiological conditions	- Synaptic plasticity (Avital et al., 2003; del Rey, Balschun, Wetzel, Randolf, & Besedovsky, 2013) - Memory retention (Song, Merali, & Anisman, 1999; Zalcman et al., 1994) - Spatial and contextual memory (Depino et al., 2004; Goshen et al., 2007; Labrousse et al., 2009) - Long Term Potentiation maintenance (Avital et al., 2003; del Rey et al., 2013)	- Synaptic plasticity (Balschun et al., 2004; Braida et al., 2004) - Memory maintenance (Balschun et al., 2004; Braida et al., 2004) - Spatial memory (Balschun et al., 2004; Braida et al., 2004)

However, under inflammatory conditions, cognitive capabilities are decreased and neurogenesis is affected. A potential factor for this decrease is the increase in inflammatory cytokines following inflammation (Table 2) (Bourgognon & Cavanagh, 2020).

Table 2. Effects of pro-inflammatory cytokines on plasticity in inflammatory conditions

	IL-1β	**IL-6**
Inflammatory conditions	- Reduced plasticity (Hein et al., 2010; Moore, Wu, Shaftel, Graham, & O'Banion, 2009) - Spatial memory deficit (Taepavarapruk & Song, 2010) - Working memory impairment (Matsumoto, Yamaguchi, Watanabe, & Yamamoto, 2004; Ohno, Yamamoto, & Watanabe, 1992) - Contextual memory (Hein et al., 2010; Moore et al., 2009) - Neural toxicity (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008; Perry, Newman, & Cunningham, 2003)	- Learning and memory impairment (Sparkman et al., 2006) - Depression (Chourbaji et al., 2006; Weaver et al., 2002) - Sickness behavior (Bluthe, Michaud, Poli, & Dantzer, 2000) - Astrocyte and microglia activation (Klein et al., 1997; Penkowa, Molinero, Carrasco, & Hidalgo, 2001)

C. Inflammation and Neurogenesis:

The main response to an injury or an infection is an inflammatory response. It is an adaptive process in which a succession of events happen in a timely manner (Varela, Mogildea, Moreno, & Lopes, 2018). The main five signs of inflammation are: redness, swelling, heat and pain (Varela et al., 2018) and potentially loss of function (Stewart & Beart, 2016).

Inflammations are divided into two types: acute and chronic. Acute inflammations are divided in to two main phases: vascular and immune. The vascular phase is characterized by increased vascular permeability and vasodilation, to increase blood flow to the affected area. Simultaneously immune cells (monocytes, neutrophils, T-cells) are recruited to the site of injury, forming the immune phase. Cytokines, chemokines, lipids, enzymes and several other molecules will work during both phases to resolve the injury. These acute inflammations usually do not leave long lasting negative outcomes (Filipov, 2019; *Fundamentals of Inflammation*, 2010). When inflammations are not resolved quickly and become uncontrolled, they enter the chronic phase. They can be caused by several factors and they originate in several organ systems and present themselves in different ways as such as gastrointestinal disturbances, liver diseases, cardiovascular and respiratory diseases, as well as others (Filipov, 2019; *Fundamentals of Inflammation*, 2010).

Both acute and chronic inflammations can appear in two localizations. Any inflammations that happens outside the central nervous system (CNS) are characterized as peripheral and inflammations of the CNS are characterized as neuroinflammation. In case of peripheral or neuroinflammations microglia, astrocytes are activated and they will produce molecules that attract monocytes from the periphery to infiltrate the brain (Ransohoff, Kivisakk, & Kidd, 2003). They go through activated endothelium that allow the extravasation of neutrophils without erythrocytes escaping the circulation (Medzhitov, 2008; Pober & Sessa, 2007). Neutrophils get activated from contact with pathogens or from activation due to cytokines (Medzhitov, 2008; Nathan, 2006).

In all cases of inflammation, cytokines are produced by microglia, astrocytes, neurons, peripheral inflammatory cell, endothelial cells, pericytes and the choroid plexus.

Receptors to these cytokines are found on neurons, microglia, astrocytes, vascular endothelial and perivascular cells (Kennedy & Silver, 2016; Konsman, Drukarch, & Van Dam, 2007; Verma, Nakaoke, Dohgu, & Banks, 2006). This will activate the later and will also produce cytokines to activate other cells. For example, TNF-α, IL-1β and IL-6 can stimulate each other's production from immune cells and they will work together (Donzis & Tronson, 2014). This affects neuronal response, memory, learning, plasticity (Bourgognon & Cavanagh, 2020).

Inflammation at the periphery affects the CNS and has been shown to interfere with neurogenesis (Ben-Hur et al., 2003; Borsini, Zunszain, Thuret, & Pariante, 2015; Darwish et al., 2022; Monje, Toda, & Palmer, 2003; S. Zonis et al., 2013; Svetlana Zonis et al., 2015) and neuroplasticity. The main consequence is changes in behavioral functions (Wang et al., 2015), and impairment in cognition, learning, and memory (Deng et al., 2010; Kempermann et al., 2018; Saxe et al., 2006; Winocur, Wojtowicz, Sekeres, Snyder, & Wang, 2006). Furthermore, inflammation has been associated with depression and other neurological diseases. Neurodegenerative disorders such as Alzheimer's disease (AD) or Parkinson's disease (PD) are associated with inflammatory processes and are established to affect neurogenesis (Dhakal & Bobrin, 2021). Moreover, brain injuries such as traumatic brain injuries (TBI) are also characterized by inflammation and cognitive deficits. However, the inflammation in the periphery is not the same as neuroinflammation in the CNS. Both involve different mechanisms of immune activation and have different degrees of impact on cognitive functions.

1. Neuroinflammation & Neurogenesis:

Inflammation in CNS is harder to detect compared to peripheral inflammation as the typical signs of inflammation there are difficult to interpret. One of the main signs of

inflammation in the brain are edema or swelling and breakdown of the blood brain barrier, as well as the production of inflammatory mediators and the involvement of microglia and astrocytes (Stewart & Beart, 2016).

It is important to mention that it was thought before that the brain is "immune privileged" and could tolerate the introduction of antigens without eliciting an immune response. It was also thought that it was deprived of NK cells, T- and B-lymphocytes and a lymphatic system (Stewart & Beart, 2016). However, it was found that the nervous system is in constant communication with the immune system and mast cells (Khakh & Sofroniew, 2015; Lau et al., 2012). The CNS is mainly protected by a small array of cells that include microglia, astrocytes and mast cells, that also produce cytokines, chemokines and prostaglandins (53) (Figure 3).

Inflammation of the CNS is an important factor for the suppression of neurogenesis and the decrease in neuronal survival (Bastos, Moriya, Inui, Katura, & Nakahata, 2008; Kohman & Rhodes, 2013). As a matter of fact, spinal cord injuries (SCI), lead to substantial inflammation inside the CNS. This causes the blood brain barrier (BBB) to be more permeable allowing the infiltration of immune cells to the spinal cord and the site of injury. Several studies reported that SCI negatively affect neurogenesis and congitive abilities (Jure, De Nicola, Encinas, & Labombarda, 2022; Jure, Pietranera, De Nicola, & Labombarda, 2017; Wu et al., 2016; Wu et al., 2014).

Two important cytokines involved in neurological disorders are interferons-α and IL-6 (Stewart & Beart, 2016). IL-6 activates a cascade that produces the p21 protein in hippocampal cells. Through the cyclin-dependent kinase inhibitor p21, cell proliferation is arrested and as a result the formation of new neurons (Chesnokova et al., 2016; S. Zonis et al., 2013).

Moreover, a decrease in neurogenesis can be a causative agent for some neurological and psychiatric diseases, such as schizophrenia, and major depression, addiction and anxiety (Gonçalves et al., 2016). Alzheimer's disease, which is associated with neuroinflammation has been associated with reduction in neurogenesis that leads to learning and memory impairments (Hollands et al., 2017). Similarly, schizophrenia, which is also associated with neuroinflammation, is characterized with cognitive deficits that was found to be linked to impairment in neurogenesis (Duan et al., 2007; Gonçalves et al., 2016; Kvajo et al., 2008). Interestingly, a smaller size of the hippocampus has been associated with major depression as well as cognitive defects (Gonçalves et al., 2016; Miller & Hen, 2015).

In cases of neuroinflammation, microglia are the main cells that mediate the neuroinflammatory response (Kohman & Rhodes, 2013; Wolf, Boddeke, & Kettenmann, 2017). They originate from the yolk sac from the myeloid lineage and migrate to the brain during the period of early development, and increase their number through proliferation (Ajami, Bennett, Krieger, Tetzlaff, & Rossi, 2007; Alliot, Godin, & Pessac, 1999; Kohman & Rhodes, 2013). Similar to peripheral macrophages, microglia can have different roles associated with different phenotypes. Their roles include, but are not limited to surveying the environment, removing cellular debris or pathogens and support regenerative processes. Different microglial phenotypes are activated depending on signals from the surrounding. In case of inflammation, the microglia turn on their reactive inflammatory phenotype, or the classically activated phenotype (M1). They start by proliferating, then swelling their cell body and retracting their processes. They will also produce inflammatory molecules, including cytokines, chemokines, reactive oxygen species (Moreno-Jiménez, Terreros-Roncal, Flor-García, Rábano, & Llorens-Martín) and

nitric oxide (NO) (Kettenmann, Hanisch, Noda, & Verkhratsky, 2011; Kohman & Rhodes, 2013). The M1 phenotype produces TNF-α, IL-1β, IL-6, and IL-12 as well as reactive oxygen species (Moreno-Jiménez et al.). These molecules will have harmful effects on the different parts of the CNS. Activation of microglial cells and the resulting secretion of such cytokines are well known to impair hippocampal neurogenesis (Augusto-Oliveira et al., 2019).

When the brain is healthy and uninjured, microglia are in the resting state (M2), the cell body is small with numerous and thin processes. (Kettenmann et al., 2011; Kohman & Rhodes, 2013). They survey the space around them to find any damage or infection in the hippocampus. Moreover, they also play a role in neurogenesis by phagocytosing damaged cells and particles and this supports the growth of new neurons (Kohman & Rhodes, 2013; Sierra et al., 2010). Under normal conditions, microglia have been found to release certain factors to help in the rescue and differentiation of neuronal cells and neuroblasts (Walton et al., 2006). On the contrary to M1, the M2 phenotype will produce IL-4, IL-10 and TGF-β1, which are anti-inflammatory cytokines, reducing the damage on the nervous tissue, and leading regulatory T-cells to reduce the inflammation in the brain (Huang, Hussain, & Chang, 2021). The M2 microglia protect the blood-brain barrier and promote repair of the nervous tissue (Huang et al., 2021). Furthermore, the anti-inflammatory cytokines produced by the M2 phenotype, such as TGF-β, contribute to the maintenance of neurogenesis in the SVZ (Kohman & Rhodes, 2013; Mathieu, Piantanida, & Pitossi, 2010).

It should be noted that as part of its function to combat inflammation, microglia will alternate between two phenotypic states: M1 and M2, to preserve homeostasis (Khakh & Sofroniew, 2015; Lau et al., 2012; Stewart & Beart, 2016).

The distribution of microglia is not the same in all areas of the brain as some regions have denser microglial populations than others. For example, the hippocampus, the striatum, the substantia nigra and the cortex have higher concentrations of microglia compared to other areas of the brain (Filipov, 2019; Lawson, Perry, Dri, & Gordon, 1990). It is important to mention that the areas that contain higher densities of microglia are more sensitive to inflammatory insults and are prone to damage in neurodegenerative diseases (De Lucia et al., 2016; Filipov, 2019; Moehle & West, 2015). The hippocampus, due to its high expression of IL-1 receptor and high microglial density, is one of the highly sensitive regions in the brain to inflammatory insults.

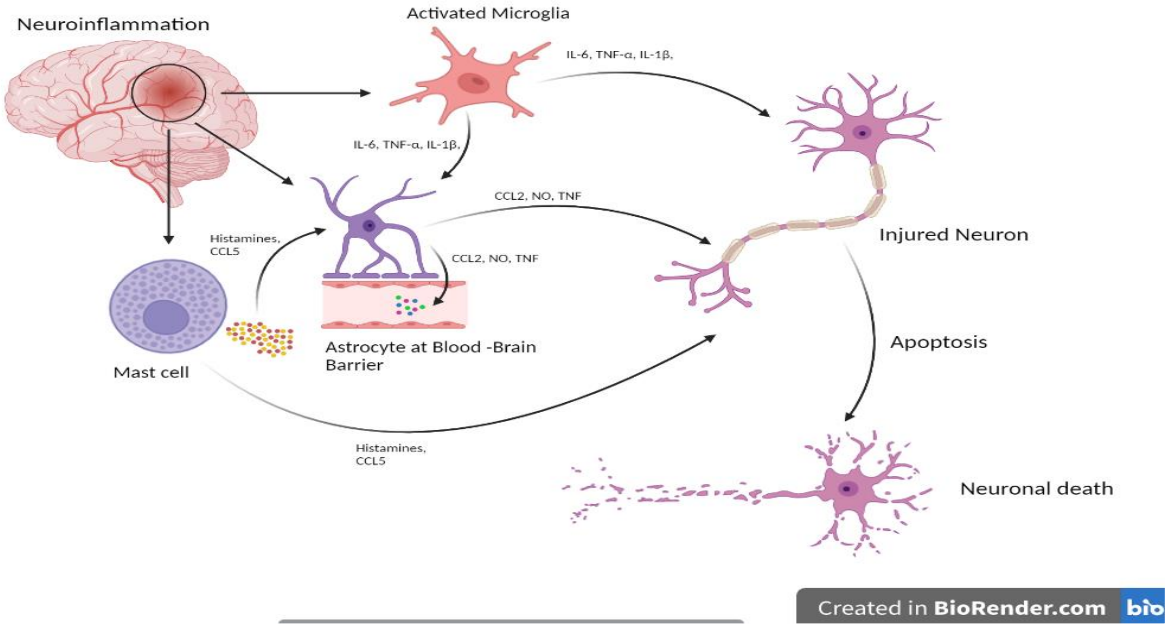

Figure 3. The inflammatory process in the CNS. In cases of neuroinflammation immune cells will get activated and will release cytokines and immunological factors. The latter will interfere on the development of neurons which will eventually cause their death by apoptosis.

2. Peripheral inflammation & Neurogenesis:

Peripheral inflammation involves the activation of the immune system both innate and adaptive to defend the body against pathological stimuli through the release of proinflammatory cytokines outside the CNS (Huang et al., 2021). It has been agreed on

that dilation of blood vessels, itchiness and pain are signs of peripheral inflammation (Stewart & Beart, 2016). Recently, the interaction between the peripheral and central immune system has gained a lot of attention and interest. Peripheral inflammation could lead to the disruption of the BBB thus allowing cells from the periphery and released cytokines to infiltrate the brain. More research is needed to understand the extent of the role of peripheral inflammation on the integrity and the functioning of the BBB and how much they affect CNS (Huang et al., 2021; Troncoso-Escudero, Parra, Nassif, & Vidal, 2018).

Infections that lead to peripheral inflammation are major contributors for the development and the progression of neurodegenerative diseases such as AD, PD, multiple sclerosis (MS), and stroke. (Huang et al., 2021). In AD patients, it has been found that the level of β-amyloid are increased after peripheral inflammation (Huang et al., 2021). Such findings promoted speculations that peripheral inflammation could affect brain homeostasis and possibly lead to damage to the CNS due to various mechanisms that happen as a result of the peripheral inflammations.

There are various mechanisms that could lead to the aggravation of the damage to the CNS post peripheral inflammation. Tight junctions (TJ) are modified and degraded as a result of the action of inflammatory molecules such as cytokines including, IL-6, IL-1β, IL-9, TNF-α, IL-12. These cytokines affect the permeability of the BBB by downregulating main proteins, such as Claudin-5, that hold TJs together and disrupt the high selectivity of the BBB to pathogens and molecules (Huang et al., 2021). Peripheral inflammation will also upregulate the production of adhesive molecules, such as vascular cell adhesion molecule 1 (VCAM-1) or intracellular adhesion molecule (ICAM-1), which increase infiltration of peripheral immune cells leading to more inflammation in nervous

tissue (Huang et al., 2021). IL-1β was found to also disrupt the BBB by affecting cell-cell junctions and cell-matrix adhesion (Huang et al., 2021). Injury to CNS endothelial cells (EC), an important constituent of the BBB, following peripheral inflammation has also been previously documented and shown to lead to further damage. Astrocytes are also activated in cases of peripheral inflammation. Their role depends on several factors including the trigger behind the inflammation. They may produce pro or anti-inflammatory molecules, affecting the BBB in different ways.

Furthermore, peripheral inflammation leads to activation of the hypothalamic–pituitary–adrenal axis (HPA). The overactivation of HPA axis constitutes another pathway through which peripheral inflammation could affect neurogenesis. Cytokines will increase the levels of glucocorticoids in the blood and glucocorticoids themselves are well known strong suppressors of NPC proliferation (Cameron & Glover, 2015; Chesnokova et al., 2016). In fact, patients receiving glucocorticoid treatments have been shown to have memory deficits (Chesnokova et al., 2016; Wolkowitz, Reus, Canick, Levin, & Lupien, 1997).

In this context, one of the most common causes of peripheral inflammation that affect and disrupt cognitive processes are urinary tract infections (UTIs).

a. Urinary Tract Infections & Cognitive Changes

UTIs are relatively common causes for peripheral inflammation. They can be classified into two types: lower urinary tract infections that involve infection of the urethra or bladder (cystitis) or both and upper urinary tract infections that involve the kidneys (pyelonephritis) (Figure 4). Due to the anatomical reasons, the lower urinary tract is more prone to infections. Pathogens are located around the opening of the urethra, and they routinely enter it, but they are cleared at the time of micturition. Sometimes bacteria reach upper levels in the urethra then the bladder and start to grow (Foxman, 2010).

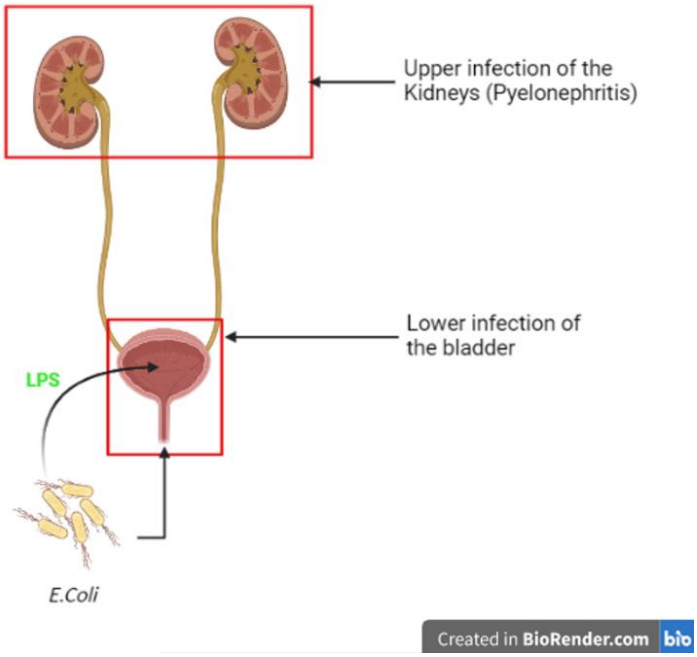

Figure 4. Categories of Urinary Tract Infections. UTIs are separated into two types: upper and lower infections. The lower infection of the bladder is mostly due to *E. coli* bacteria that produce LPS as part of the infection and invasiveness

The prevalence of UTIs is interestingly high in very young individuals, and the risk increases with age. The prevalence is also higher in women compared to men in the age group 60 and above, mainly due to differences in the anatomy of the urogenital organs. The reason for this difference is due to the shorter urethra of the female urinary

tract which allows pathogens to reach the bladder faster and proliferate before getting cleared (Foxman, 2010). In addition to gender, several other factors affect the incidence of UTIs, these include anatomical and functional abnormalities, genetics and behaviors or actions that facilitate the entry of pathogens to the bladder such as sexual intercourse and the strain of the bacteria entering the urinary tract.

The main symptoms of a urinary tract infection, if any, are: dysuria, polyuria, odor and suprapubic pain. In some cases, patients can feel fever and vomiting. If the UTI starts to become severe, new symptoms can appear, such as dizziness, hypotension, and altered mental status, mainly due to the inflammation of the bladder and potentially the kidneys (Dubbs & Sommerkamp, 2019). A large percentage of UTIs are usually self-limited (Foxman, 2010).

The main causative agents are strains of E. coli referred to as uropathogenic *E. coli* (UPEC). These are gram negative facultative anaerobic bacteria and are efficient in forming biofilms to evade the host immune system by using a glycolipid on their surface called Lipopolysaccharide (LPS).

The resulting inflammation from UTIs has been suspected in several clinical studies to be behind some cognitive changes and altered mental status of patients with UTIs (Balogun & Philbrick, 2014; Chiang et al., 2015; I. Eriksson, Gustafson, Fagerström, & Olofsson, 2011; Manepalli, Grossberg, & Mueller, 1990; Mayne, Bowden, Sundvall, & Gunnarsson, 2019). And the main mediator of these inflammations is LPS (Alpizar et al., 2020). The endotoxin is also used by studies as an inflammogen that elicits inflammation.

D. LPS and Neurogenesis

LPS is found on the envelope of Gram-negative bacteria. This glycolipid plays a role in the activation of the immune system; hence it is referred to as an endotoxin, but it also helps in the resistance of Gram-negative bacteria to numerous antibiotics (Bertani & Ruiz, 2018).

1. Injection of LPS in the CNS

Injections of LPS in the CNS are used to mimic cases of neuroinflammation. Injections of LPS to the cortex leads to a decrease of neurogenesis, while cell proliferation is not affected (Ekdahl, Claasen, Bonde, Kokaia, & Lindvall, 2003; Kohman & Rhodes, 2013). Similar results were found in intraperitoneal injections of LPS (Kohman & Rhodes, 2013; Monje et al., 2003). Intracerebroventricular injection of LPS has been found to impair hippocampal NSC proliferation through a possible mechanism mediated by microglial activation (F. Chamaa, Bitar, Darwish, Saade, & Abou-Kheir, 2018). Furthermore, intranigral injection of LPS has also been shown to impair neurogenesis also through a possible association with microglial activation (Darwish, Chamaa, Al-Chaer, Saade, & Abou-Kheir, 2019).

Interestingly, following LPS injection to the brain, neurogenesis rate is dependent on the proximity and level of activation of microglia. High levels of activation reduce neurogenesis, while far and lesser activated microglia affect neurogenesis less. Moreover, it is also possible to find an increase in the birth of new neurons, as a compensatory effect, to replenish lost neurons (Ekdahl et al., 2003).

Injection of LPS directly into the CNS causes sickness behavior as well as depressive-like behaviors (Fu et al., 2010; Tang, Lin, Pan, Guan, & Li, 2016). Cases of

spatial memory disruption, depression-like behavior and anxiety are likely to be caused by the inflammation caused by LPS and its associated suppression of neurogenesis (Chesnokova et al., 2016; Tang et al., 2016).

2. Injection of LPS in the periphery

In case of LPS-induced inflammation in the periphery, astrocytes are activated. One way of doing so is changing their end-feet structure as well as changes in the expression of specific genes leading to a disruption of the BBB (Huang et al., 2021). This leads to organelles damage in endothelial cells, which may eventually lead to apoptosis. When both the BBB and endothelial cells are negatively affected, it becomes easier for harmful molecules to enter the CNS and further cause more disease, and this facilitates the transport of immune cells to the brain. (Huang et al., 2021). For example, it has been shown that amyloid precursor protein transgenic mice injected with LPS to elicit peripheral inflammation, have increased permeability in their BBB to IL-6 and TNF-α which leads to inflammation in the brain and the development of diseases (Apple et al., 2017; Huang et al., 2021).

Increased levels of circulating cytokines will force newly formed neurons to go into apoptosis, (Ben-Hur et al., 2003; Chesnokova et al., 2016; Iosif et al., 2006; Monje et al., 2003) and they will also increase the levels of oxidative stress which will damage neurons in development. Because, in case of inflammation, NPC express receptors for pro-inflammatory cytokines (Chesnokova et al., 2016; Green et al., 2012), administration of LPS to the periphery leads to detrimental effects on the brain and specifically, NPC proliferation (Chesnokova et al., 2016; Kohman & Rhodes, 2013; S. Zonis et al., 2013). All this would subsequently affect hippocampal neurogenesis.

E. AIM

In this study we aim at understanding the effects of UTI-associated inflammation in the bladder on NSCs proliferation. For this, we instilled rats with LPS and analyzed the outcome post instillation. Next we aim at studying the consequence of the instillation on cognition and behavior, thus we exposed the rats to cognitive tests. Moreover, we aimed at inspecting the physiological variations of cytokines and neurotrophins that arise from the LPS instillation and their involvement in the pathways that affect neurogenesis and proliferation.

CHAPTER II

MATERIALS AND METHODS

A. Animals

3 months old Adult male Sprague-Dawley rats (450-500g) were used in the experiments. The rats were kept at a 12h light/dark cycle with water and food *ad libitum* at a temperature of 20-22°C. "All experiments were approved by the institutional Animal Care and Use Committee at the American University of Beirut.". Surgical procedures were performed under general anesthesia using 0.2ml of Xylazine and 0.8ml Ketamine using intraperitoneal (*ip)* injections. Rats were subjected to constant observation postoperatively and their weight were taken during the light part of the cycle.

B. Intra-urethral Instillation of Lipopolysaccharide (LPS)

Rats were positioned on their back exposing their abdomen and thorax while under isoflurane anesthesia. Using two forceps, the penis of the rats was exposed and an intravenous catheter was inserted in order to reach the end of the urethra. Experimental rats were instilled with 0.5ml of 0.5mg/ml of LPS (Lipopolysaccharide L2630 from Escherichia. *Coli*) directly to the urinary bladder. Sham rats were instilled with sterile saline at a volume of 0.5 ml. In order to induce further inflammation and prevent regurgitation of the LPS volume, we followed a successive up and down motion for the needle while performing the instillation. The catheter was slowly retracted at the end to prevent leaking. After the instillation, the abdomen of rats was shaved to expose their skin for the future abdominal sensitivity testing.

C. Experimental Design

The rats were divided into two groups, one experimental, one control, with a total n=6 in each group. LPS starts affecting the organs at least 2 hours after the injection (Meneses et al., 2018), and the day of the instillation was considered day 0 (Figure 5).

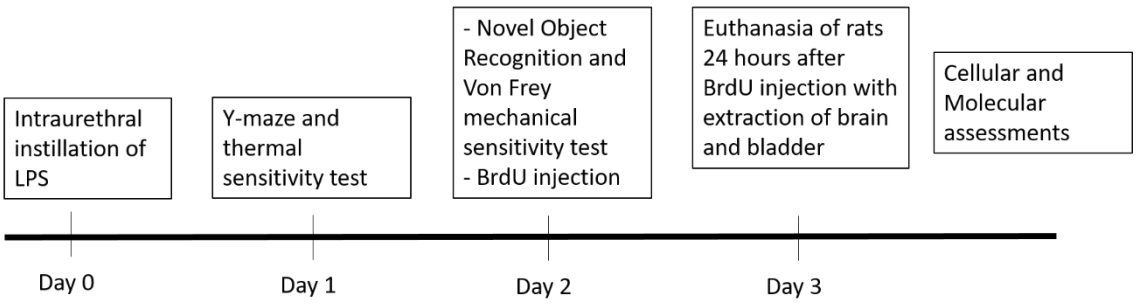

Figure 5. Experimental design. Time points of the LPS instillation, behavioral tests, and euthanasia (Day 3).

D. BrdU injection

Bromodeoxyuridine (BrdU) is a synthetic thymidine analogue used to detect and probe actively proliferating cells to track proliferation and neurogenesis. The BrdU is taken up by the cells and integrated into the DNA during the S-phase of mitosis and is retained in cells while they divide, allowing for the detection of proliferating cells. Rats were weighed and the proper mass of BrdU powder (BOC Sciences, B2706-004257) was diluted in 0.9% saline at a final concentration of 200mg/kg/rat. It was administered intraperitoneally along 3 injections (66mg/kg/300μl/injection) separated by 2 hours on day 3 post-instillation, which was 24 hours before the euthanasia.

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E. Sensitivity Testing

Two sensitivity tests were performed in order to assess the sensitivity of the lower abdomen of rats to heat and mechanical stimuli. It has been shown that Urinary tract

infections and the resulting inflammation from them are associated with visceral abdominal pain (Darwish et al., 2022). Here, we did not do an infection, but instead used an LPS inflammogen to elicit the inflammation similar to UTIs. Both tests were performed during the light phase of the cycle. Rats were placed in the testing area for 30 minutes to allow habituation to the environment and the testing area.

1. Thermal sensitivity testing

Rats are placed on the Ugo Basile thermal aesthesiometer apparatus. When their abdomen came in contact with the glass, thermal heat at the intensity of 36 infrared units was applied (intensity range of 1-99). The latency to make a withdrawal reflex in response to the heat stimulus was recorded. Each measurement was repeated 5 times at an interval of 10 minutes. The results of the 5 trials were averaged and reported as mean ± SEM.

2. Mechanical sensitivity testing (Von Frey)

Rats were placed on the Ugo Basile Von Frey aesthesiometer metal wire mesh platform. Similar to the thermal sensitivity, the stimulus was applied when the abdomen of the rats touched the platform. A series of 5 taps separated by few seconds were applied divided into three trials. Any reflex or the arching of their back was considered as a positive response. The results of the 3 trials were averaged and reported as mean ± SEM.

F. Behavioral Testing

All rats were subjected to a series of behavioral tests to assess several aspects of their behavior. Each test explored a specific characteristic of rat behavior. These tests were performed during the dark phase of the cycle. Rats were already in the room several hours before, making them habituated to the environment.

1. Novel Object Recognition (NOR)

The test is performed in an open field apparatus, where two identical objects are placed in the corners of the apparatus. In the adaptation stage, rats are placed in the apparatus facing the objects and are allowed to roam for 5 minutes for familiarization. Then, rats are returned to their cages, the apparatus and objects were wiped with 70% ethanol. After 5 minutes, in the experimental stage, one object is changed and the rats were placed again in the open field while their performance was being video recorded for a period of 5 minutes. The ANY-maze^{TM} software was used for analysis and several parameters were recorded that include: the time spent in the novel object zone, the time spent in the familiar object zone and the number of entries to each zone.

2. Y-Maze

This maze consists of three identical arms arranged in the shape of a "Y". The test is used to assess the spatial reference memory of rats. The test is divided into two phases: the familiarization phase and the testing phase. During the familiarization phase, rats are allowed to roam freely between the start arm and the familiar arm for 15 minutes. The third arm, also called the novel arm, is closed off preventing the rat from entering it. After the familiarization phase, each rat is allowed to rest for 1 hour, during which the remaining rats are allowed to get familiar with the apparatus. After the 1 hour, rats are placed back in the maze for testing. During this testing phase, the novel arm is opened and rats can enter it and they are video recorded for 5 minutes. The videos are later analyzed using the ANY-maze^{TM} software to determine the number of entries and the time spent in each arm in addition to the latency to entering the novel arm.

G. Euthanasia and tissue extraction

After 24 hours from BrdU administration, rats were euthanized under isoflurane anesthesia. The thorax of the rat is opened and the diaphragm is cut. A longitudinal cut is made to expose the lower abdomen and subsequently the urinary tract. The urethra and bladder were extracted and snap frozen in liquid nitrogen. The prostate was also extracted but it was fixed in paraformaldehyde (PFA). Afterward, the heart is exposed in order to prepare for saline perfusion of the animal. A needle is inserted into the left ventricle of the heart and the right atria is dissected. Saline is circulated through body of the rat entering from the left ventricle allowing to clear the blood from the body. Once the saline runs out clear and all the blood has been completely drained, the brain of rats is removed. Following the extraction, the brains are cut along the longitudinal fissure to separate the right and left lobes. One half is fixed in PFA and the second half is used for the extraction of hippocampus. Each hippocampus is snap frozen in liquid nitrogen to be used for RNA and protein extraction.

H. Brain sectioning and Tissue processing

For cellular analysis, specifically NSC proliferation, rat half brains were processed to prepare them for sectioning. After fixation in PFA, brains were transferred to 30% sucrose for dehydration. They were left for 24 hours until they sink to the bottom of the conical indicating complete dehydration. Brains were prepared for sectioning on the microtome as they were brought to a temperature between -30 and -40°C and positioned on their caudal side and cut at 40 μm thick coronal sections. The hippocampus was divided into three regions: rostral, intermediate, and caudal as follows: 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 (F. Chamaa et al., 2018; Darwish et al., 2022; Paxinos, 2004). All sections were collected and stored in sodium azide solution (15mM in 0.1M PBS).

To properly organize the sections and get the most information from the studied region, we collected the tissue sections using the fractionator method. This method allows us to obtain 6 representative wells for each region, while being able to have proper data from the counting of BrdU positive cells from one representative well per region. Sections are distributed to each well in the fashion represented in figure 6.

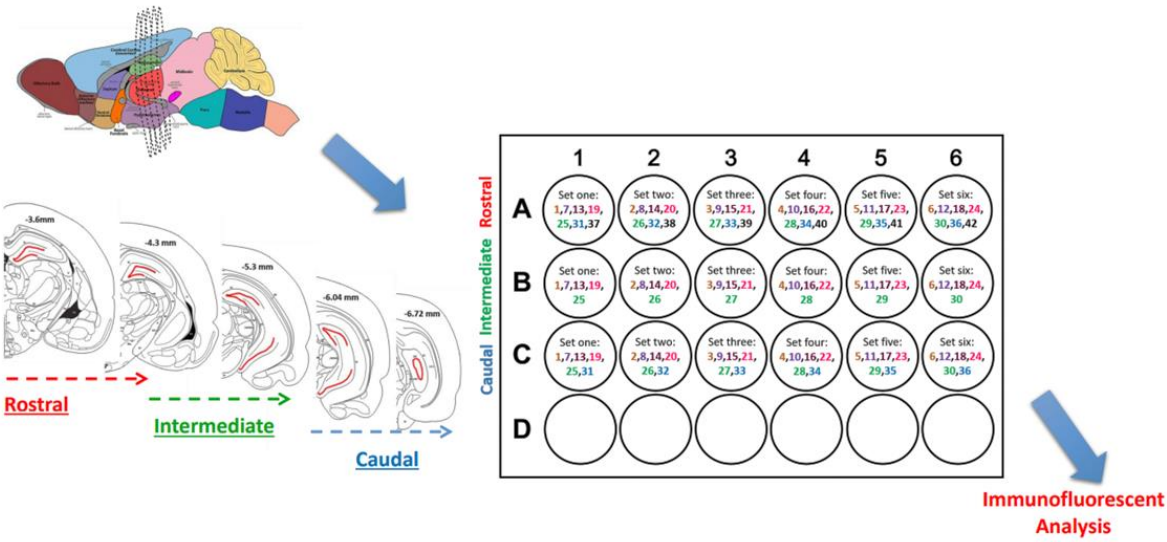

Figure 6. The Fractionator Method. Schematic Diagram showing the distribution of coronal sections of the dentate gyrus (red) in wells following the fractionator method of cutting and counting (Chamaa et al., 2016b).

I. Immunofluorescence:

After sectioning the brain, one random well from each region is chosen randomly for staining. The tissues are placed in a 24 well plate and the sections are washed with 0.1M Phosphate Buffered Saline (PBS) three times for 5 minutes each. Afterwards, sections are incubated for 30 minutes in 2N HCl at 37°C in order to open up the DNA double helix and increase the permeability to the nucleus to allow entry of the staining

antibodies. To neutralize the sections, they are washed once in 0.1M PBS for 5 minutes and incubated for 10 minutes at room temperature in sodium borate (0.1M, 8.5 pH). Tissues are then washed 3 times in PBS for 5 minutes each, and then blocked for 1 hour at 4°C in 10% blocking solution (10% Bovine Serum Albumin (BSA), 10% Normal Goat Serum (NGS), 0.1% Triton X diluted in PBS) in order to reduce non-specific binding. Finally, tissues are incubated overnight with the primary antibodies prepared in 3% block (3% BSA, 3% NGS, 0.1% Triton X diluted in PBS). Two antibodies have been used, a Mouse anti-BrdU primary antibody (1:250, Santa Cruz) and a Rabbit anti-NeuN antibody (1:750, Neuromics). Following overnight incubation, tissues are washed three times in 0.1M PBS for 5 minutes each and secondary antibodies are added and the tissues are incubated for 2 hours at room temperature on shaker. The secondary antibodies used are Goat-anti-mouse 568 (Alexa Fluor 1:250, Invitrogen) and Goat anti-rabbit 488 (Alexa Fluor 1:250, Invitrogen). The sections are then washed three times in 0.1M PBS for 5 minutes and they are mounted on slides. Finally, anti-fade mounting media with DAPI is added and slides are covered with thin glass coverslips.

J. RNA extraction

Once properly stored, hippocampus and bladder tissues are processed for RNA extraction. Tissues are placed in 750 µl of Qiazol (QiaGen) in order to break down the tissues; ceramic and metal beads are also added to the vial to facilitate the breaking of tissues, increasing the efficiency of the extraction with the help of the OMNI Bead Ruptor^{TM} machine. Following the homogenization of the tissues, RNA is separated from other material using 300 µl of chloroform. Vials are vortexed for 1 minute to mix the chloroform and Qiazol and they are left 1 minute at room temperature. They are then

centrifuged for 20 minutes at 4°C and at 15,000g. This will separate the aqueous and organic phases. RNA will be found in the aqueous phase on top and the rest of the materials, such as debris, proteins, and Qiazol will be found in the organic phase. DNA is found between both layers in the form of a white foamy line. The aqueous phase is removed and placed in a new vial for further processing. Isopropanol is added at a ratio of 350 µl of chloroform in order precipitate RNA into a pellet, they are mixed together by gentle pipetting and the vial is centrifuged at 4°C and at 15,000g for 30 minutes. Once the RNA has precipitated, the pellet can be found at the bottom of the tube. The supernatant is removed and discarded and the RNA is washed with 600 µl of 70% ethanol to remove any remaining isopropanol found in the pellet. The content is mixed properly and it is then centrifuged for 20 minutes at 12500g and at 4°C. The ethanol is then removed and the washing step is repeated but the second centrifugation is done at 7500g for 5 minutes at 4°C. The second wash is removed and the pellet is allowed to air dry by keeping the cap open. Once fully dry, the pellet is diluted in RNase/DNase free water and stored at -80°C for long term storage.

K. cDNA synthesis

Once RNA has been extracted, it is converted to complementary DNA (cDNA) for RT-qPCR. The concentration and purity of extracted the RNA is measured using the ThermoScientificTM NanoDrop 2000TM and using this concentration, the proper volume of RNA to use in the synthesis is determined as to obtain and use 2µg of RNA for cDNA synthesis. 2 µl of genomic DNA wipeout buffer is added to the volume of RNA and RNase/DNase free water is added until a total volume of 14 µl is reached and the vials are incubated at 42°C for 2 minutes. Then a master mix containing, reverse

transcriptase (RT), RT buffer and primers (1 µl, 4 µl, 1 µl, respectively) are added and the obtained 20 µl volume is incubated for 2 hours at 42°C. Then the reaction is stopped by incubating for 3 minutes at 95°C. Finally, the obtained 20 µl of cDNA are diluted in a 1:10 ratio in RNase/DNase free water.

L. RNA Extraction and Quantitative Real-time PCR

The levels of mRNA in the extracted tissues of hippocampus and bladder were analyzed using RT-qPCR (Bio-Rad CFXTM Manager Software; cat #1845000). RNA was extracted from hippocampi and bladder tissues using Qiazol (Qiazol®, QiaGen) and following the manufacturer's protocol for RNA extraction from tissues. In brief, 1ml of Qiazol reagent was added to tissues gradually and tissues were homogenized on ice, then 0.2mL chloroform was added followed by centrifugation at 12,000 rpm for 20 min at 4°C. Isolated RNA phase was mixed with 0.35mL isopropanol, incubated for 10 min at room temperature then centrifuged at 15,000 rpm for 30 min at 4°C, then washed twice with 70% ethanol and the pellet was left to air dry before suspending it in RNase-free H2O. The list of primers used, and their sequences are listed in table 3. cDNA synthesis was performed using QIAGEN QuantiTect reverse transcription kit and following the manufacturer's protocol. cDNA was diluted in a 1:10 volume ratio. Concentrations and integrity (RNA integrity number—RIN) of isolated RNA were determined using ThermoScientific™ NanoDrop 2000™ and Agilent BioAnalyzer 2100™, respectively. The mRNA expression of sham and UTI hippocampi samples were analyzed by RT-PCR (Bio-rad CFX™ Manager Software; cat #1845000) using the $\Delta\Delta C_t$ method and the SYBR green system (Applied Biosystems; cat #A46111). The PCR reaction consisted of a DNA denaturation step at 95°C for 5 min, followed by 40 cycles (denaturation at 95°C for 10

s), then annealing at the appropriate temperature of 57°C for each primer for 30 s, and finally an extension step at 72°C for 10 min. For each experiment, reactions were performed in duplicates, and the expression of individual genes was normalized to the housekeeping gene *Gapdh*. Gene expression was calculated through the following equation: $\Delta\Delta C_t = \Delta C_{t\,(target)} - \text{Average}\,[\Delta C_{t\,(Sham)}]$, where $\Delta C_t = C_{t\,(target)} - C_{t\,(GAPDH)}$. The amount of endogenous target gene relative to a calibrator (*GAPDH*) became $2^{-\Delta\,\Delta Ct}$.

Table 3. List of primers used in qRT-PCR in the study. Brain-derived neurotrophic factor: Bdnf; Glyceraldehyde-3-Phosphate Dehydrogenase: Gapdh; Nerve growth factor: Ngf; Fibroblast growth factor 2: Fgf2.

Rattus norvegicus Primers	**Sequence (5'->3')**	**Product Length**
Gapdh	F: TCACCATCTTCCAGGAGCGA R: GGCGGAGATGATGACCCTTT	149
IL-1β	F: AGGCTGACAGACCCCAAAAG R: GGTCGTCATCATCCCACGAG	264
Bdnf	F: CTCCGCCATGCAATTTCCAC R: CAGCCTTCATGCAACCGAAG	279
Ngf	F: CATCGCTCTCCTTCACAGAGTT R: TCTGTGTACGGTTCTGCCTG	222
Fgf2	F: AGGATCCCAAGCGGCTCTAC R: TACCGGTTCGCACACACTC	166

M. Statistical Analysis:

Statistical analysis and plotting of figures were made using Prism 7 GraphPad package (GraphPad software, Inc., CA, USA). Unpaired student t-test was used to assess statistical significance of difference between the two groups; sham and rats injected with LPS. Unpaired student t-test was also used to analyze the following parameters between these two groups: time spent in each arm of the y-maze, latency to enter the novel arm, time spent exploring objects in novel object recognition test, and fold change of mRNA levels of *IL-1β, Bdnf, Ngf* and *Fgf*. All data were averaged per group and presented as

mean ± standard error mean (SEM). The P value of < 0.05 was considered as the limit of significance of differences at 95% confidence interval.

CHAPTER III

RESULTS

A. Abdominal Sensitivity

1. Intra-urethral LPS instillation increases abdominal sensitivity to thermal stimulus

After intra-urethral instillation of LPS, rats were tested for the sensitivity to a thermal stimulus on their abdominal area overlying the bladder. On day 1 post instillation, rats that received the LPS instillation had a significantly lower withdrawal latency (7.75 ± 0.56 sec; p=0.0034) as compared to the sham group (12.77 ± 0.91 sec) who received a saline instillation (Figure 7).

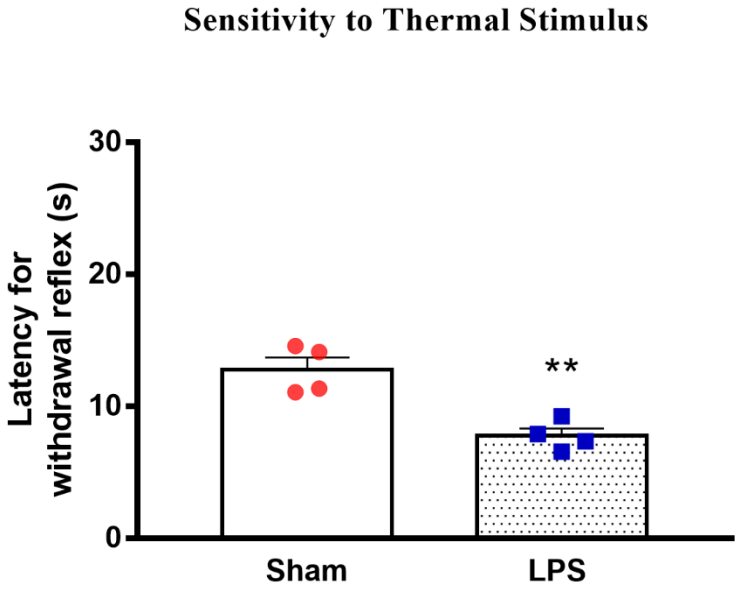

Figure 7. Increased abdominal sensitivity to thermal stimulus following intra-urethral LPS instillation. On Day 1 post-instillation, the rats that received LPS (n=4) were more sensitized when exposed to heat stimulus as compared to sham (n=4) as they displayed withdrawal reflexes that indicate pain. Data is expressed as average ± SEM. (p=0.0034)

2. The effect of LPS instillation on abdominal sensitivity to mechanical stimulus

A similar effect is seen on day 2 afer the instillation, when the Von Frey mechanical sensitivity test was performed. The LPS group had a significantly higher frequency of abdomen withdrawal (0.5175 ± 0.05735 per 5 taps; P=0.0151) compared to the control group (0.318 ± 0.03247) (Figure 8).

B. LPS intra-urethral instillation causes increased expression of IL-1β in bladder

In the group that received LPS intra-urethral injection, we noticed a trend of increase in the expression of IL-1β in the bladder (3.36 ± 1.38 fold change) on day 3 following the instillation of LPS as compared to the levels in the sham group (1.05 ± 0.18 fold change) (Figure 9).

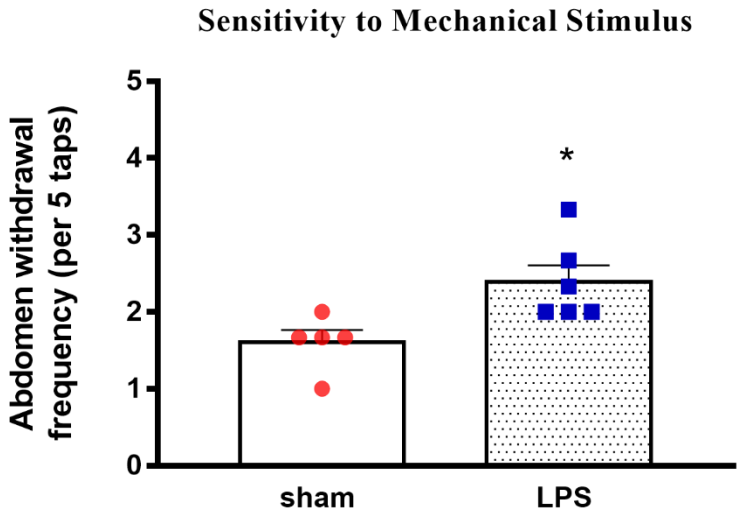

Figure 8. Increase in abdominal sensitivity to mechanical stimulus. On Day 2 post-instillation the rats that received LPS (n=6) were more sensitized when exposed to mechanical stimulus as compared to sham (n=5) and displayed reflexes that indicate pain. Data is expressed as average ± SEM. (p=0.0210)

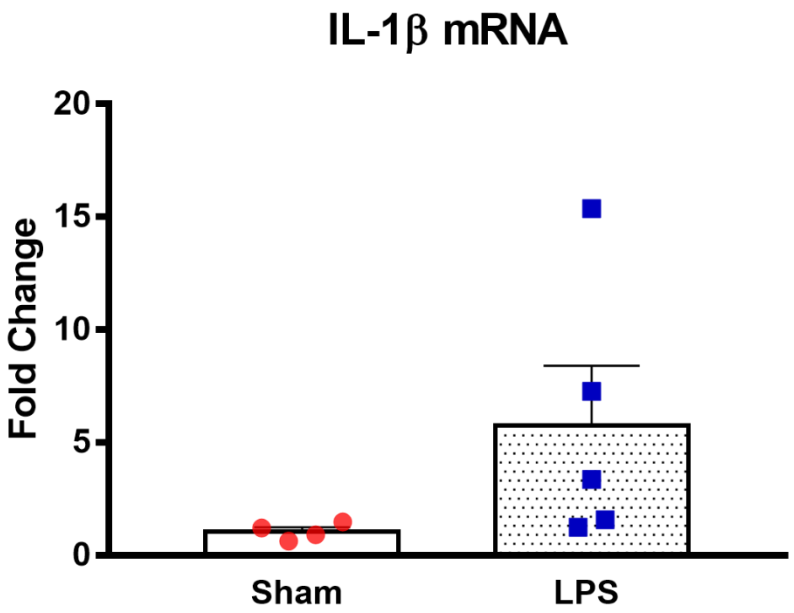

Figure 9. A trend of increase in IL-1β expression in bladder of LPS-instilled rats. Rats that received LPS (n=5) intra-urethral injection had higher level of expression of IL-1β as compared to sham (n=4), though not significant due to high variation in data. Data is expressed as average ± SEM.

C. Effect of intra-urethral instillation of LPS on hippocampal NSCs proliferation and neurotrophins

1. LPS intra-urethral instillation does not affect the basal level of NSCs proliferation in the DG of the hippocampus

BrdU positive cells were counted in hippocampal brain sections to understand the effect of intra-urethral instillation of LPS on proliferation of NSCs in the DG. The rats that received intra-urethral LPS instillation had a comparable number of BrdU positive cells (2545 ± 294 cells) to the sham group that had an average of 2775 ± 194 BrdU positive cells (Figure 10 A & B).

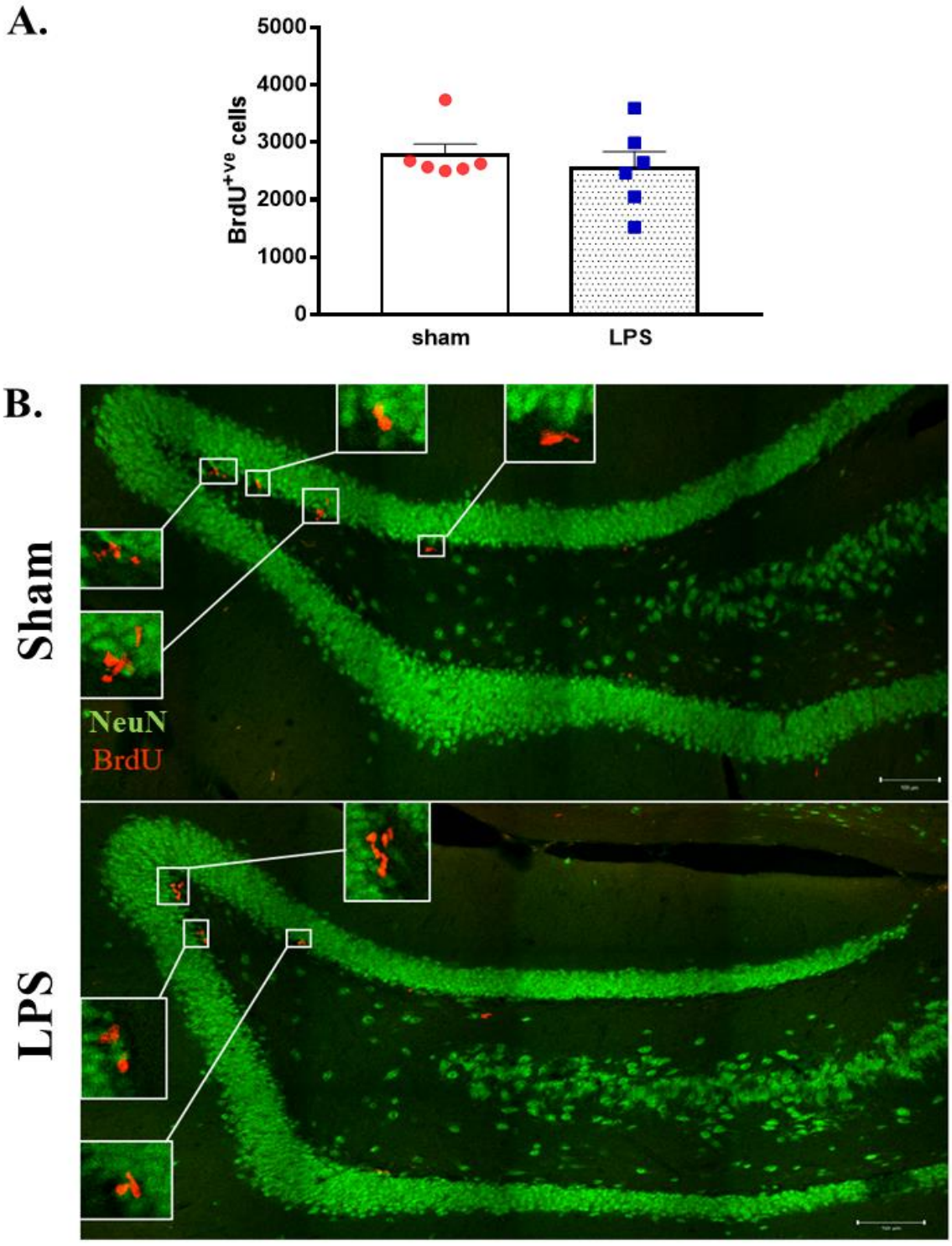

Figure 10. Intra-urethral instillation of LPS did not affect the basal level of proliferation of hippocampal NSCs. (A) The Number of BrdU positive cells in rats that received LPS intra-urethral instillation (n=6) was comparable to sham rats (n=6). Data is expressed as average ± SEM. **(B)** Representative confocal images showing immunofluorescence labeling of NeuN (green) and BrdU (red) in the DG of sham and rats with that received LPS intra-urethral instillation. Images were taken as Z stacks and tile scan using 40X-oil objective.

2. *LPS intra-urethral instillation does not affect the expression of hippocampal neurotrophic factors*

The mRNA levels of *Bdnf*, *Ngf*, and *Fgf* in the hippocampi of rats with LPS instillation (1.24 ± 0.11; 0.85 ± 0.09; 0.98 ± 0.14) were all comparable to levels in sham rats (1.01 ± 0.08, 1.06 ± 0.21, and 1.03 ± 0.13, respectively) (Figure 11 A, B & C).

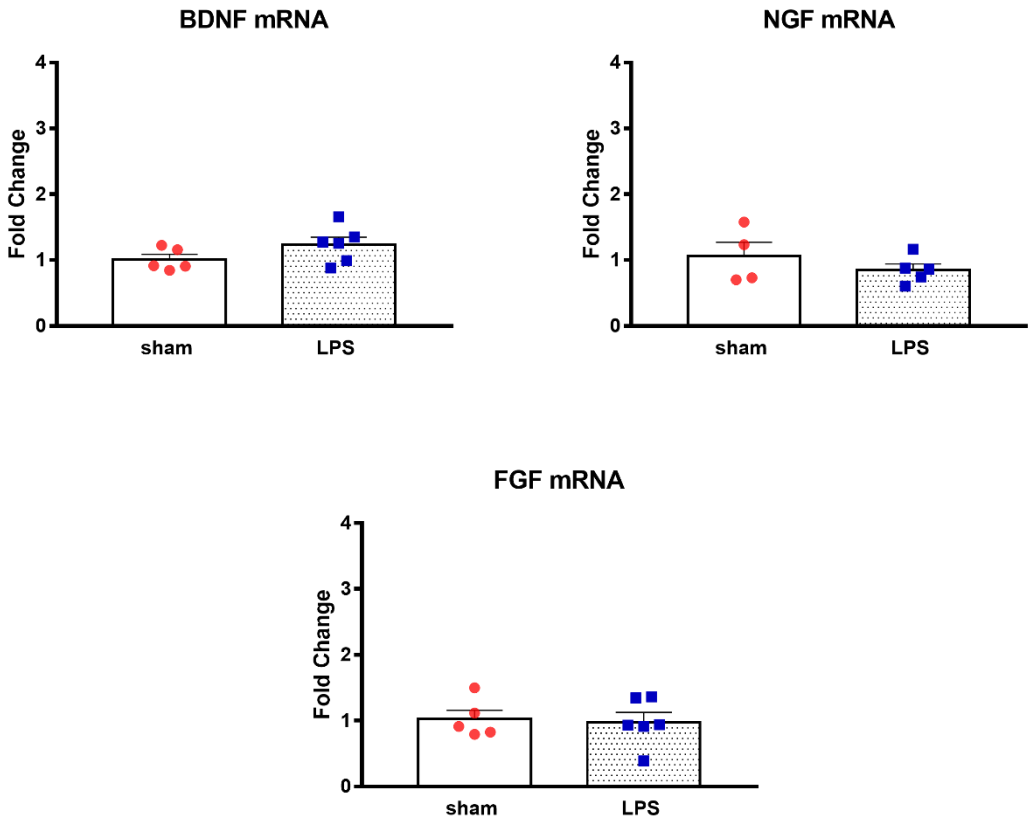

Figure 11. No changes in the expression of hippocampal neurotrophic factors following LPS intra-urethral instillation. The expression of **(A)** *Bdnf*, **(B)** *Ngf*, and **(C)** *Fgf* were all comparable between sham (n=5) and LPS (n=6) rats. Data is expressed as average ± SEM.

D. Effect of intra-urethral instillation of LPS on Rats' Cognitive Behavior

1. LPS intra-urethral instillation does not affect spatial reference memory in Y-Maze

Y-maze test was performed on both sham and rats with LPS injection to investigate the effect of LPS intra-urethral instillation on hippocampal reference memory. The LPS group spent similar time exploring the start arm (156.32s ± 19.83), familiar arm (67.18s ± 17.18), and the novel arm (76.5s ± 10.76) as compared to the sham group (196.5s ± 21.3; 35.56s ± 8.82; and 67.94s ± 13.65, respectively) (Figure 12).

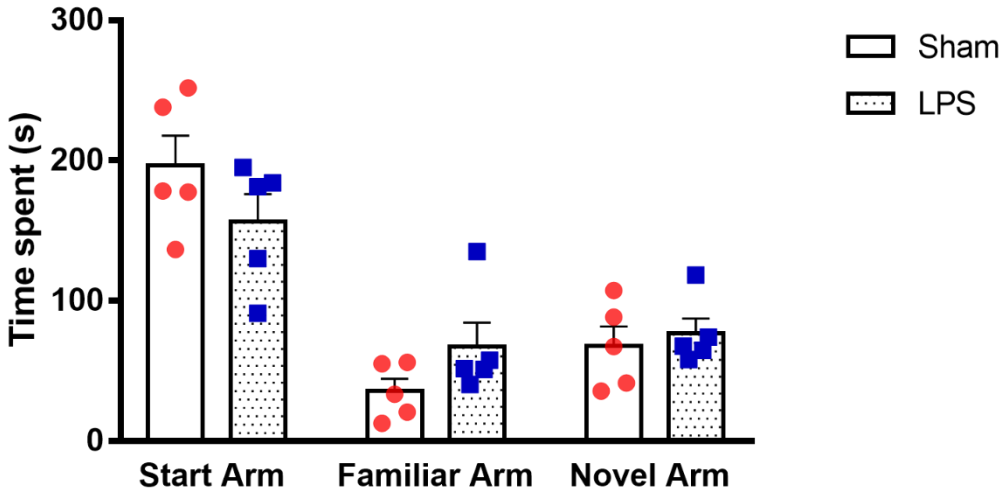

Figure 12. Spatial reference memory is not affected following LPS intra-urethral instillation in the Y-maze test. Similar time was spent exploring the start, familiar and novel arm by both sham rats (n=5) and rats with LPS (n=5) intra-urethral instillation. Unpaired student t-test was used to assess significance between the two groups in each arm individually. Data is expressed as average ± SEM.

2. LPS intra-urethral instillation does not affect recognition memory in NOR

The recognition memory of rats was tested using the Novel Object Recognition test. LPS rats spent similar time exploring the familiar object (56.64s ± 13.02) as compared to the sham group (42.56 ± 8.48 sec). Similarly, both sham (38.74s ± 1.55) and LPS (47.14s ± 10.65) groups spent comparable time exploring the novel object (Figure 13).

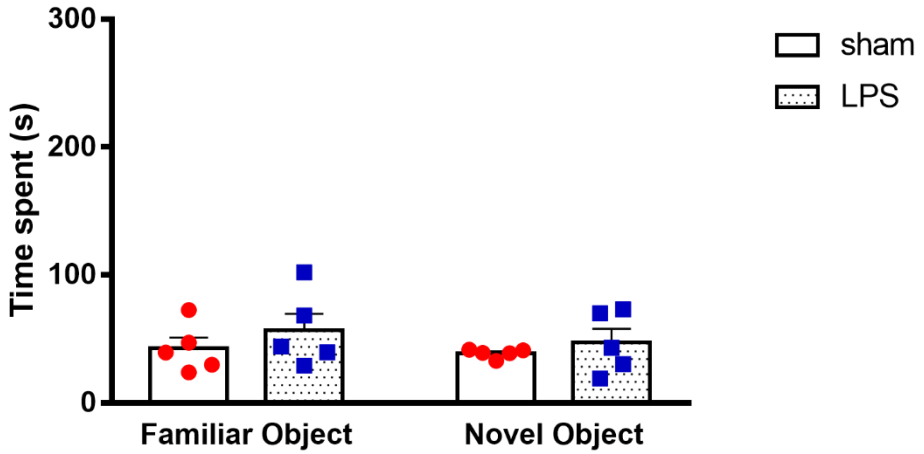

Figure 13. Recognition memory was not affected following LPS intra-urethral instillation in the NOR test. Similar time was spent exploring the familiar and novel objects by both sham rats (n=5) and rats with LPS (n=5) intra-urethral instillation. Unpaired student t-test was used to assess significance between the two groups for each object individually. Data is expressed as average ± SEM.

CHAPTER IV

DISCUSSION

It was hypothesized that intraurethral instillation of LPS to the bladder would have an impact on NSC proliferation. We are trying to understand the impact of inflammation in the urinary tract on NSC proliferation without the presence of bacteria in order to comprehend the mechanism of action that is affecting neurogenesis. This came following a study that showed that bacterial infection of the urinary tract led to an inflammation in the urinary tract, a pain response and a decrease in neurogenesis (Darwish et al., 2022). We optimized an animal model in which we performed an intravesicular instillation of LPS. The data we collected showed that the transcription levels of mRNA for IL-1β in the bladder were increased as well as the sensitivity of LPS rats to thermal and mechanical stimuli. However, the proliferation of NSC was not affected by the intraurethral instillation of LPS. Additionally, reference and recognition memory of rats was also not affected.

The strain of bacteria used in the previous study was the uropathogenic *E. coli* (UPEC). This Gram-negative bacterium has the glycolipid LPS on its surface. The latter is characterized as an endotoxin, which indicates that it elicits an immune response (Bertani & Ruiz, 2018). We used LPS to induce an inflammation of the bladder. The effect of the instillation was monitored by performing sensitivity testing on the abdomen of rats. It was noticed that LPS rats showed referred visceral pain. This reveals that the LPS instillation induces abdominal pain as a common symptom to UTI and this could be a due to an inflammatory response following the LPS instillation. Also, an increase in the transcription of IL-1β in the rats that received LPS instillation indicates that LPS has

caused changes in the expression of inflammatory cytokines. This is consistent with previous studies (Kogan et al., 2018; Yura, Bradley, Ramesh, Reeves, & Bond, 2009; Zhou, Fan, Gu, Yu, & Chai, 2022).

On the other hand, the number of BrdU-positive cells is not affected by the LPS instillation. This is contrasting with the results of previous studies as they have reported that proliferation and neurogenesis are reduced in cases of LPS-induced neuroinflammation (F. Chamaa et al., 2018; Chesnokova et al., 2016; Darwish et al., 2019; Perez-Dominguez, Ávila-Muñoz, Domínguez-Rivas, & Zepeda, 2019; Valero, Mastrella, Neiva, Sanchez, & Malva, 2014). Moreover, systemic and peripheral inflammations induced by intraperitoenal injection of LPS also cause disturbances in proliferation and neurogenesis (Smith, Hagberg, Naylor, & Mallard, 2014). Moreover, LPS-induced systemic inflammations suppress the proliferation of type 2 intermediate precursor cells (IPC) in the hippocampus (Melo-Salas, Pérez-Domínguez, & Zepeda, 2018). This contrast between our results and these studies could be caused by several factors. As a matter of fact, we have instilled LPS in one specific location, the bladder. The localization of the inflammation may impead the systemic spread of the inflammation. Furthermore, we instilled a low dose of LPS and with one single instillation, suggesting that the effect was not substantial enough to have a noticeable effect on proliferation.

However, the mRNA transcription levels of BDNF, NGF and FGF-2 in the hippocampi of rats, is in line with the previously discussed results. Both BDNF and NGF are involved in memory and learning (Eu et al., 2021; Miranda, Morici, Zanoni, & Bekinschtein, 2019). This is consistent with the similarity in the results of the behavioral tests as well as the results of the BrdU-positive cells.

Despite the presence of visceral pain, the brain is not affected by the activity of the endotoxin on the bladder. The results suggest that the neuronal pathway has been activated, through the sensitization of sensory afferent due to pain cause by LPS instillation. The activation of this pathway may not be sufficient in affecting the rates of maturation and proliferation of NSC and NPC (Chiu et al., 2013; Rosen & Klumpp, 2014; Rudick et al., 2010).

Possible reasons for the discrepancy may be due to the difference of mechanisms involved in bacterial infections and LPS-induced inflammation. Both the humoral and the neural pathways play a role in the reduction of neurogenesis when rats were instilled with bacteria. Cytokines, such as IL-1β, and neurotrophic factors, such as BDNF, NGF and FGF-2, were involved in the reduction in the number of BrdU-positive cells. (Darwish et al., 2022).

Interestingly, no effect was seen on neurogenesis, when rats were instilled with LPS, which leads us to think that the ativation of the nociceptive pathway is not sufficient to have a considerable effect on the hippocampus and NSC proliferation. However, an ELISA analysis of protein extracts from the bladder might give us a better picture, mainly through the quatification of cytokines which might help suggest other reasons for these results.

In the case of bacterial infection, the production and the spread of LPS was constant; as a matter of fact, bacteria are continuously proliferating in the bladder. This uninterrupted stimulation of the tissue will presumably cause a tougher immune response as well as a more pronounced nociceptive response, hence the more substantial effect on proliferation. This leads to suggest that the additive effect of both pathways leads to a stronger influence on NSC proliferation and maturation.

Further work is needed to fully understand the impact of UTI-associated inflammations. Increasing the dose of LPS administered could reveal a more significant impact on proliferation, cognitive abilities of rats and neurotrophins. Likewise, increasing the frequency of instillations can be a closer model to the UTI model used in the previous study (Darwish et al., 2022). Moreover, further analysis the tissues might give us a clearer picture of the pathways involved and how both systems communicate in case of inflammation. Finally, increasing the sample size will reduce the variation in the groups thus potentially giving more significant results.

In conclusion, this study showed that a single dose of LPS, causes an increase in the transcription of IL-1β in the bladder but did not have a significant effect on NSC proliferation, neither did it have an effect on the memory of rats. Only the nociceptive pathway was activated but its influence was not sufficient to induce consequential changes. Further investigations are needed to further understand the mechanisms involved.

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