AMERICAN UNIVERSITY OF BEIRUT

URINARY TRACT INFECTIONS AND HIPPOCAMPAL NEUROGENESIS: IS THERE A LINK?

by
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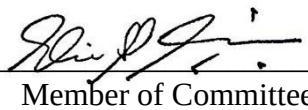

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ABSTRACT
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Previous studies have suggested a link between urinary tract infections (UTIs) and cognitive impairment in elderly patients. These patients have a higher risk for dementia and/or neuropsychiatric disorders. One possible contributing factor for UTI-induced cognitive changes, that has not yet been investigated, is a potential alteration in hippocampal neurogenesis. In this study, we aim to investigate the effect of UTI on hippocampal neurogenesis in a rat model of UTI. Adult male Sprague rats received intra-urethral injection of an *Escherichia coli* (*E. coli)* clinical isolate (10^8 CFU/mL) to induce UTI. Behavioral tests including the open field, Y-maze, T-maze, and novel object recognition were performed to assess anxiety-like behavior, exploration, spatial reference memory, cognitive ability and working memory, respectively. Rats were later injected with Bromodeoxyuridine analog (200mg/kg, i.p) and sacrificed at different timepoints. Levels of pro-inflammatory cytokines in bladder and urethra were measured by ELISA. Neurotrophins and proinflammatory cytokines mRNA expression in the hippocampus were determined by RT-PCR. Rats had confirmed infection as seen with CFU/mL $\geq 10^5$ and had elevated concentrations of pro-inflammatory cytokines (IL-8 and IL-1β). Furthermore, rats with confirmed UTI exhibited higher thermal sensitivity on the abdominal skin overlying their urinary tract area. We found that UTI reduces proliferation of neural stem cells (NSCs) at an early time point post-infection (p.i.) (day 4) and neurogenesis at a later time point (day 34). This was associated with decreased expression in mRNA of BDNF, NGF, and FGF2 and elevated expression of IL-1β in the hippocampus at 6 hours post infection, but with no changes in optical intensity of microglia and astrocytes. In addition, infected rats spent less time exploring a novel arm in the Y-maze test on day 2 p.i. Treatment with anti-inflammatory drug did not revert the effect on NSCs, while treatment with antibiotic further decreased basal level of their proliferation. This study presents novel findings on the impact of urinary tract infections on hippocampal neurogenesis that could be correlated with cognitive impairment.

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ABBREVIATIONS

BBB: Blood Brain Barrier
BDNF: Brain derived neurotrophic factor
BMP: Bone morphogenic protein
BrdU: Bromo-deoxy Uridine
CNS: Central Nervous System
DG: Dentate Gyrus
FGF: Fibroblast growth factor
GFAP: Glial fibrillary acidic protein
Hes1: Hairy-enhancer-of-split 1
IF: immunofluorescence
IHC: Immunohistochemistry
IL: Interleukin
IPC: Intermediate progenitor cells
LTP: Long term potentiation
LUT: Lower Urinary Tract
NeuN: Neuron nuclear protein
NGF: Nerve growth factor
NPC: Neural Progenitor Cells
NSCs: Neural Stem Cells
OB: Olfactory bulb
PAMPs: Pathogen-associated molecular pattern
PMC: Pontine micturition center
QNP: Quiescent neuronal precursors
PARs: protease activated receptors
PGE_2: Prostaglandin E_2
RMS: Radial migratory stream
SCI: Spinal cord injury
SGZ: Sub-granular Zone
Shh: Sonic hedgehog
SVZ: Subventricular zone
UPEC: Uropathogenic E. coli
UT: Urinary Tract
UTI: Urinary Tract Infection
vEGF: Vascular endothelial growth factor

CHAPTER I

INTRODUCTION

A. Neurogenesis

1. Overview

It was previously thought that the adult brain has no capacity for regeneration and that it was rather fixed and immutable. Ramon Cajal, known as the father of neuroscience, has previously stated this once famous dogma: “Once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centers, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated.” (Cajal & May, 1928). However, this was based on histological staining alone and was inferred at a time when advanced technological tools were absent. Such tools were later developed and have served to negate this dogma and reshape the field of neuroscience and brain research. In the 1960s, the first evidence was provided about the ability of the mature rat adult brain to continuously generate and provide new neurons throughout its life (Altman & Das, 1965). Accumulating evidence over the years completely challenged and changed this previously held dogma that the mammalian brain is refractory and incapable of generating new neurons or has a limited ability to do so (Eriksson et al., 1998). This ability to continuously generate new neurons is known as adult neurogenesis. Although it took several years to confirm its presence, today, it is widely established that adult neurogenesis occurs in humans and most mammals, and some vertebrates as well (Eriksson et al., 1998; Gage, 2000; Kempermann et al., 2003; Kuhn et al., 1996; Rietze & Reynolds, 2006; Spalding et al., 2013). Lately,

there has been debate over its existence in humans but the evidence for its presence, even in the elderly, cannot be negated (Boldrini et al., 2018; Kempermann et al., 2018; Tobin et al., 2019). Most importantly, the role of neurogenesis in humans has been the central focus of scientists for years. Ongoing research aims to understand the functional significance of the newly added neurons, how they can be manipulated and how they contribute to specific or overall brain activities. The presence of neurogenesis in the adult brain holds great promise for curing brain diseases and supports the hope of stem cell based-therapeutic approaches (Collin et al., 2005). A key factor for taking advantage of neurogenesis lies in understanding what factors regulate the process and how it gets affected in case of diseases or pathology.

2. Defining Neurogenesis

Neurogenesis is the plastic capacity of the brain to continuously generate new neurons and integrate them into its own pre-existing circuitry (Ide et al., 2008). It is the sequence of events that leads to the formation of new neurons from stem cells or progenitor cells. The defining characteristics of neural stem cells (NSCs) are their infinite capacity for self-renewal through cell cycle division and their ability to differentiate into a variety of specialized cell types known as a multi-potent capacity (Gage, 2000). NSCs can give rise to the different cell lineages, neurons, astrocytes, and oligodendrocytes. In the process, NSCs divide asymmetrically giving rise to one daughter progenitor cell and one stem cell having the same properties. The progenitor cells can, later on, divide asymmetrically producing daughter cells that either differentiate into astrocytic or neuronal lineage and one progenitor cell that retains the capacity to divide multiple times (Morrison & Kimble, 2006). The process of neurogenesis can be summarized in four main events: proliferation,

differentiation, maturation, and integration in the existing circuitry (Vivar et al., 2012; Vivar & van Praag, 2013). During development and in postnatal life, neurogenesis is very crucial as it gives rise to all components of the central nervous system (CNS). In the adult life, however, this capacity becomes limited to only two brain regions: the sub-ventricular zone (SVZ) of the lateral ventricles and the sub-granular zone (SGZ) of the DG of the hippocampus. These two main neurogenic niches continuously generate new neurons from a built-in reservoir of neural stem cells that is housed within.

3. Neurogenic Niches

A neurogenic niche is the dynamic specialized microenvironment that houses, nourishes, and supports the NSCs, or progenitor cells. The niche can either stay dormant or keep on dividing.

a. Subventricular zone

The neurogenic niche in the SVZ contains a larger germinal layer harboring NSCs than the sub-granular zone of the dentate gyrus (Doetsch & Alvarez-Buylla, 1996). The rate of neurogenesis, the type of cells produced, and their fate varies among these two niches. In the SVZ, the number of new cells produced is higher and the cells produced there are destined for a migratory path towards the olfactory bulb (OB) where they mature into interneurons (Ming & Song, 2011). This is referred to as the rostral migratory stream (RMS) and it is worth noting that in humans this migratory stream is much more complex and controversial (Bergmann et al., 2012; Sanai et al., 2011). Cells generated in the SVZ are classified into three different types of lineages: type B, C, and A cells. Type B cells express the astrocytic marker GFAP (Gage, 2000). These slowly dividing cells produce the transit-amplifying type C cells. The rapidly dividing type C cells eventually give rise

to type A cells, which in turn are doublecortin positive neuroblasts that migrate in chains through the RMS (Ming & Song, 2011; Zhu et al., 2018). In the OB, these cells differentiate into different subtypes of interneurons such as GABAergic granule neurons and tyrosine hydroxylase positive dopaminergic periglomerular neurons which all eventually integrate into the OB circuitry (Zhu et al., 2018). The neural stem cells in the SVZ are organized in a complex 3D structure that looks like a pinwheel structure in the center of the SVZ. The NSCs in the center are surrounded directly by ciliated ependymal cells that serve to support and maintain the NSCs by producing several factors such as Noggin (Lim et al., 2000). On the apical side, the NSCs are in direct contact with the CSF where they can sense environmental cues in the CSF. Furthermore, SVZ-NSCs cells send processes that are in direct contact with blood vessels which enables these cells to respond to factors in the circulation (Zhu et al., 2018). Therefore, the NSCs of the SVZ are controlled by many dynamic factors.

There is evidence to support that NSCs in the neurogenic niches respond to injury. This is most evident in seizures and brain ischemia where cell proliferation and migration promptly increase (Jin K, 2001). An interesting characteristic of neuroblasts born in the SVZ is their strong migratory capacity. SVZ-neuroblasts usually migrate through a fixed path through the RMS to the OB. However, there is notable evidence that suggests that these cells could change their path and migrate towards a position of a lesion or cellular degeneration and differentiate into functional neurons in order to repopulate the injured tissue or maintain a function. Such migratory capacity of SVZ neuroblasts has proven to be essential for regenerating and replacing neurons in remote areas of the brain (Kaneko et al., 2017). However, these neuroblasts can help regenerate only to a certain extent and are incapable of replacing the number of cells lost in cases of injury or degenerative

diseases. It is important to note that the direction and paths of neuroblasts migration vary between rodents and humans as they are more complex in primates. In humans, the number of migrating neuroblasts seems to be restricted, and much is still unknown about them (Kaneko et al., 2017).

b. Dentate Gyrus of the Hippocampus

The hippocampus is part of the limbic system that is involved in cognition. It plays a crucial role in memory, learning, and spatial coding. Adult hippocampal neurogenesis is the process of formation and integration of new neurons from the NSCs housed within the neurogenic niche of the dentate gyrus of the hippocampus (Kuhn et al., 2018). The dentate gyrus houses the neurogenic niche of the hippocampus and is thus characterized by a special network of connections. It is molded into three main layers: the molecular outer layer, the granule cell layer, and the sub-granular inner deepest layer (Figure 1). The molecular layer contains the dendrites of the granular layer and the axons that originate from several other sources. The granule cell layer (GCL) is highly compacted with mature granular NeuN-expressing neurons. As for the sub-granular zone, it is a unique thin layer between the GCL and the hilar zone that provides a permissive milieu and a unique microenvironment for the nourishment and maintenance of the adult neural stem cell (aNSC) population.

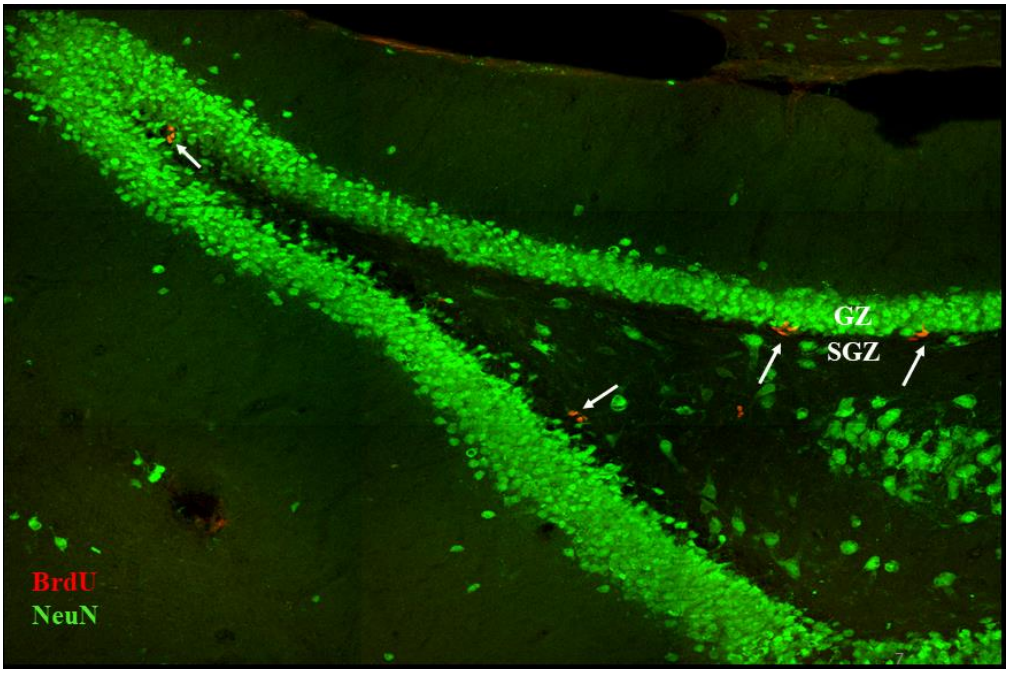

Figure 1. The dentate gyrus. Immunofluorescence image showing the layers of the DG stained with mature neuronal marker; NeuN (green) and the position of NSCs stained with BrdU (red) in the SGZ. GZ: Granular zone; SGZ: Subganular zone.

The various connections of the DG reflect the various roles that it plays. The granule cells in the DG receive their main direct input from the perforant fibers in layer II of the medial entorhinal cortex and the lateral entorhinal cortex (EC) while forming themselves a loop of unidirectional mossy fibers extending to the pyramidal cells of the nearby CA3 region of the hippocampus. The lateral entorhinal cortex provides input about external cues while the medial entorhinal cortex gets information on spatial cues. Moreover, there are projections from the entorhinal cortex that form synapses not only on the granule cells but also on the CA3 cells, creating the complex tri-synaptic hippocampal circuit. Therefore, the information seems to be passed on from the EC to the DG then from the DG to CA3, and from CA3 to CA1 to be ultimately stored in the cortex. The newly formed granule cell neurons have their own unique afferents (Treves et al., 2008). They receive direct feedback input from the CA3 region, transiently from the mature GCs, and

dominantly from the Lateral entorhinal cortex (Vivar et al., 2012). The newly born cells also receive input from the contralateral hippocampus, cholinergic neurons in the septum, and dopaminergic neurons in the midbrain (Schlachetzki et al., 2016), glutamatergic mossy cells, inhibitory interneurons of the hilus as well as the granular layer and molecular Layer. Moreover, a variety of modulatory inputs innervate the DG. It receives intensive glutamatergic innervations coming from the entorhinal cortex and some from the supra-mammillary area. In addition, the DG also receives modulatory cholinergic and GABAergic inputs from septal nuclei, serotonergic inputs from the raphe nuclei, noradrenergic inputs from the nucleus locus coeruleus, and dopaminergic input from the ventral tegmental area (Toda & Gage, 2018).

4. Hippocampal Neurogenesis

The integration of newly born neurons into the pre-existing circuitry of the dentate gyrus provides functional and structural plasticity in the hippocampal circuitry (Toda et al., 2018). However, it takes time for a newly born cell to be fully integrated into the circuitry of the dentate gyrus and this largely depends on a critical time window of 1 to 3 weeks, when added neurons would still be immature while displaying high plasticity and unique electrophysiology. Generally, it takes 4-6 weeks for proliferating progenitor cells to become granule cells and takes even more time to reach full maturation and incorporation into the functional network (Zhao et al., 2008). The sub-granular zone resident NSCs are known as type I radial glia-like cells. They are known as such because they extend radial processes to the Molecular layer and retain stem/pluripotency markers such as Nestin, glial fibrillary acidic protein (GFAP), and Sox2. These type I cells are activated under certain conditions and once activated they will give rise to the progeny of

proliferating intermediate progenitor cells (IPC, type II) under the effect of local niche factors such as FGF-2 (fibroblast growth factor 2) (Jin et al., 2003), sonic hedgehog (Shh) (Lai et al., 2003), vascular endothelial growth factor (vEGF) (Lai et al., 2003), and Wnt7a (Qu et al., 2010). These type II IPCs differentiate by asymmetric division in the neuronal lineage expressing NeuroD and Prox1 and eventually giving rise to the migratory neuroblasts (type III cells) that express PSA-NCAM, calretinin, and doublecortin before subsequently reaching their final maturation stage into granule cells (Lugert et al., 2010). It is worth noting that not all cells exit quiescence as some will retain their stem cell markers. In the first week of maturation, the neural lineage-committed cells start their migration into the inner granule cell layer. During the second week, these cells start extending their dendrites into the molecular layer and the hilar interneurons start providing synaptic input to the newly born neurons. Reaching the third week, the neurons start to resemble mature granule cells and spontaneous synaptic activity is detected. The glutamatergic input into the granule cells from the Entorhinal cortex, which is critical for their survival and integration, develops around a period of one month.

5. Tracing Neurogenesis

The golden standard for tracking NSCs is the use of the synthetic analog of thymidine; 5-bromo-2$^{'}$-deoxyuridine (BrdU) (Eriksson et al., 1998). This molecule gets incorporated into replicating DNA and can thus be tracked by the use of BrdU targeted antibodies (Kuhn et al., 1996). BrdU can be detected by immunohistochemistry (IHC) or immunofluorescence (IF) allowing stereological quantification of newly born cells with clear visualization of their morphology. BrdU is commonly used with other specific markers for mature neurons such as neuronal nuclear protein (NeuN), for glial cells such as GFAP, or for proliferating cells such as Ki67 to confirm the cells' identity. This

technique though widely used, has its own limitations as it requires DNA denaturation and tissue fixation and cannot be done in live cells. Moreover, the added nucleotide analog can be incorporated not just into proliferating cells but also into the damaged nicked DNA of cells undergoing repair but on a much smaller scale (Selden et al., 1993). While BrdU has been the golden standard for detecting and studying neurogenesis in rodents, there are more advanced techniques that have been utilized recently. An important advancement in the field is the use of carbon 14 dating, paving the way for better insight into the dynamics of adult neurogenesis (Ernst & Frisén, 2015). This technique allows accurate measurement of the radioactive carbon 14 isotopes in genomic DNA.

New technological advances in the field include the use of transgenic models. Transgenic approaches have been among the most recent powerful tools for visualizing the steps of neurogenesis. In addition, such tools allowed understanding the impact of genetic and environmental factors on adult hippocampal neurogenesis. Transgenic mouse models use fluorescent proteins or Cre recombinase enzymes under the control of cell-type-specific promoters such as Nestin, Sox2, Hes5, and GFAP that selectively label neural stem progenitor cells and their progeny. Other transgenic mice models specifically delete target genes in these cellular populations (Kuhn et al., 2018). Moreover, viral tools such as retroviral and lentiviral tools have been used to trace adult newly born cells in their population. These viral tools could also express optogenetic and chemo-genetic proteins that could selectively activate neuronal populations.

However, all the above techniques would still constrain the knowledge on NSCs to what can be seen in a fixed tissue taken at a single time frame. Therefore, a new technological tool was employed to address this challenge and that was to image the DG

in awake mice using multiphoton microscopy (Kuhn et al., 2018). Such live imaging and documentation of the dynamics of adult neurogenesis and neuronal maturation uncovered new information on survival rate and fate commitment which were difficult to estimate from fixed tissues and staining. In parallel with such progress in techniques comes the single-cell RNA sequencing with optimized next-generation sequencing which offers an exclusive look into the cellular heterogeneity and function of individual cells in the neurogenic niche.

It should be noted that in humans, the study of neurogenesis is more complicated and challenging; since it is mainly restricted to post-partum analysis and histological examinations of fixed tissues which have several limitations such as the delay time for fixation and its duration. More recently, new technologies have been introduced that rely on magnetic resonance imaging that uses the PET tracer: 3′-deoxy-3′-[^{18}F] fluoro-L-thymidine ([^{18}F] FLT) (Sierra et al., 2011; Tamura & Kataoka, 2017).

Regardless of all the emerging advanced techniques, the use of BrdU remains the gold standard for studying neurogenesis in rodents as it offers the benefit of affordability, feasibility, and ease of use.

6. Role and Significance of Adult Hippocampal Neurogenesis

So far, the complete functional significance of adult hippocampal neurogenesis is still not fully uncovered. It is firmly established that this process is instrumental for learning, memory encoding, and cognitive function (Toda et al., 2018). As far as we know, NSCs are there to contribute to memory formation and some cognitive functions and they could have a possible role in repair mechanisms (Lie et al., 2004). It is suggested that these newly born neurons contribute to fine pattern separation, which is the ability to

discriminate between similar inputs such as memories, events, or experiences, into different representations (Treves & Rolls, 1994). Moreover, they allow fine-tuning of the neural circuitry in the dentate gyrus and control the behavior in contextual and spatial memory (Imayoshi et al., 2008). During maturation, the new neurons exhibit enhanced properties of plasticity and excitability which is suggested to be very useful in the formation of new memories (Treves et al., 2008). The young granule cells are characterized by isolated calcium spikes and sodium fast boosts of action potential that facilitate synaptic plasticity and the induction of long-term potentiation (LTP) (Schmidt-Hieber et al., 2004). This is further supported by the notion that these newly born cells receive innervations from regions that have important memory-related functions. An important aspect of neurogenesis is that its alteration can be linked to cognitive changes associated with several brain pathologies. Indeed, impaired neurogenesis seems to be a common hallmark in several neurodegenerative diseases such as Parkinson's, Alzheimer's, and Huntington's in addition to aging (Carlesimo et al., 2012; Lazarov & Marr, 2010; Marxreiter et al., 2013; Simpson et al., 2011; Small et al., 2011).

In comparison to the total number of DG neurons, adult-born neurons represent only a minor population out of the total number. However, the numbers here do not entail significance. This is shown as the continuous addition of these newly born neurons (approximately 700 cells/day in humans) has been proven to add substantial structural and functional plasticity to the tri-synaptic circuit of the hippocampus (Spalding et al., 2013; Toda & Gage, 2018). Newly added cells represent new encoding units capable of effectively attracting new inputs to new cells and in a way contributing to separating contexts in different hippocampal cellular representations (Baptista & Andrade, 2018). Immature neurons in the DG have been shown to have an important function in forming

temporal associations for events occurring closer in time (Morris et al., 2013). It is theorized that neurogenesis can also help and yield in the clearance of old associations as it leads to better integration of new information while disintegrating some of these old associations (Frankland et al., 2013).

In rodents, neurogenesis mainly contributes to memory encoding, contextual learning, spatial navigation, mood regulation, and recognition memory among others (Kozareva et al., 2019).

7. Modulation of Neurogenesis

Around 50% of adult newly born GCs are lost within 2 weeks of their maturation (Dayer et al., 2003; kempermann et al., 2003). It should be noted that these newly born cells are more likely to be lost than the cells born during development (Spalding et al., 2013). The survival of these newly born neurons is strongly prompted by learning and learning new things by itself promotes neurogenesis (Gould et al., 1999). There are many other factors that affect and regulate the process of neurogenesis. Hence, along the process of neurogenesis, there is a significant opportunity for modulation. Adult NSCs go through different stages before reaching their terminal fate or destination and at every stage, there are specific extrinsic and/or intrinsic factors involved in regulating neurogenesis (Goncalves et al., 2016). These NSCs are quiescent stem cells as in they enter their cell cycle only under specific factors and conditions. In fact, one of the most intriguing characteristics of hippocampal neurogenesis is that it is highly prone to changes in response to a variety of intrinsic and extrinsic factors (Toda & Gage, 2018). A variety of factors could drive such modulation including molecular, genetic, epigenetic,

transcriptional factors, environmental factors, acute and chronic illnesses, inflammation, aging, and psychiatric disorders including depression.

a. Intrinsic Factors

Intrinsic factors such as Notch, bone morphogenic protein (BMP) (Lugert et al., 2010; Mira et al., 2010), and the neurotransmitter GABA are considered mediators for NSCs activation (Song et al., 2012). Notch signaling has different important roles in regulating cell fate by promoting astrocyte differentiation, inhibiting oligodendrocyte maturation, and regulating neuroblast migration and neurite morphology. Notch 1 is required for maintaining the proliferating properties and Nestin expression of adult NSCs in the hippocampus (Imayoshi & Kageyama, 2011). Shh is another major player characterizing the adult niches in the brain. It is hypothesized that NSCs arise from an initial population of Shh-responsive cells in the ventral hippocampus (Li et al., 2013). Furthermore, in the adult neurogenic niche, BMP is secreted by NSCs, mature granular cells, and some other cells in the niche and acts on these cells differently depending on certain receptor expression. The major role of BMP is to create an equilibrium between quiescence and proliferation (Maier et al., 2011). Chordin, Noggin, and Neuro-genesin -1 are inhibitors of BMP and such inhibition is what regulates its dual function in the niche (Lim et al., 2000). In addition, Wnt signaling also has a dual role in the niche as it promotes proliferative capacity in the early stages of neurogenesis versus promoting differentiation of intermediate progenitor cells in mid and late stages of neurogenesis (Varela-Nallar & Inestrosa, 2013). Furthermore, Fractalkine signaling has been proposed to have a role in adult neurogenesis through mediating neuron-microglia crosstalk in the neurogenic niche (Bachstetter et al., 2011). Nonetheless, several neurotrophic factors, neurotransmitters

and growth factors have been reported in several scenarios as being part of the regulatory control over neurogenesis (Goncalves et al., 2016). Brain-derived neurotrophic factor (BDNF) is an extensively studied neurotrophic factor that is abundantly expressed in the brain. BDNF has a pivotal role in preserving and maintaining neurons in the CNS, in addition to important roles in the differentiation/maturation of NSCs/NPCs.

The role of microglial cells in regulating adult neurogenesis has recently emerged along with their many other functions. Microglia contributes to homeostatic maintenance of the brain and influences cognitive processes and memory. A critical role for microglia in hippocampal neurogenesis involves synaptic pruning of newborn cells during the first sensitive period for survival (Gemma & Bachstetter, 2013). Ramified microglia have the crucial function of phagocytosing apoptotic newborn cells. On the other hand, activated microglia have been associated with the impairment of adult neurogenesis (Augusto-Oliveira et al., 2019). Thus, microglial cells maintain the homeostatic baseline of the neurogenic cascade (Sierra et al., 2010).

Astrocytes are another type of cells that are present in the niche and are important for supporting the differentiation of progenitor cells. The synapse-like connections that the astrocytes make with the dendrites and spines of new GCs enhance the maturation and integration of these new neurons (Song et al., 2002; Seri et al., 2001). Moreover, it is thought that the formation of dendritic spines starting at day 16 might be under the control of local astrocytes (Zhao et al., 2006). Furthermore, the unique neural circuits innervating the DG allow several brain regions to be involved in the process of regulating neurogenesis upon the activation of these circuits following certain cues such as an emotional experience or state.

Thus, an overall remarkable feature of neurogenesis is the temporal relation between all the processes that regulate it. There are various pathways involved and the differential recruitment of these pathways and their interaction results in initiating and regulating the process of neurogenesis in the adult brain.

b. Extrinsic Factors

Extrinsic factors that are extensively studied include environmental factors, stress, exercise, drugs, traumatic brain injury, diet, and nutritional factors, such as omega 3, among many others. A boost in neurogenesis, induced by environmental factors such as exercise and diet, has been associated with improved memory and cognitive thinking (Creer et al., 2010; Stangl & Thuret, 2009). The same effect has been documented following electrical stimulation in several different brain regions (Wu et al., 2020; Chamaa et al., 2021). On the contrary, a decrease in neurogenesis, as a consequence of events such as γ- or X- radiation (Clelland et al., 2009; Snyder et al., 2005), aging (Spalding et al., 2013), genetic factors or pharmacological interventions (Eisch et al., 2000; Gould et al., 1992), is associated with deficient memory function. Regulation can occur at any level throughout the neurogenesis process, from proliferation or differentiation to even the survival and fate of the newly generated neurons. However, it is still unknown whether distinct NSC populations respond differently to the same stimuli and if they share common molecular mechanisms for their activation and differentiation. Defects in neurogenesis have been previously associated with many psychiatric and neurological illnesses. Seizures for example accelerate the integration of new GCs and increase adult neurogenesis (Parent et al., 1997). Moreover, there have been reports of increased neurogenesis following brain transient ischemia (Liu et al., 1998), single and

intermittent limbic seizures (Bengzon et al., 1997), and stroke (Arvidsson et al., 2001). However, the point in question is whether to consider such an increase as a beneficial compensatory mechanism, a brain-self repair process, or perhaps a trigger for further pathology.

Among the many factors modulating neurogenesis, inflammation is a well-known strong suppressor of adult hippocampal neurogenesis (Chamaa et al., 2018; Darwish et al., 2019). Inflammation, whether general or localized in the brain, can have detrimental effects on neurogenesis.

All in all, newly formed cells in the adult brain represent a very malleable source to a variety of inputs, stimuli, or factors offering a chance for additional functions and roles in what used to be thought a fixed immutable brain.

B. Inflammation, Neurogenesis, and Cognition

Inflammation is an important process implicated in many brain disorders and diseases, including traumatic brain injury and neurodegenerative diseases. Indeed, inflammation is almost an inevitable implication in aging and is thought to contribute to the propagation of neurodegenerative diseases (Liu & Hong, 2003; Nelson et al., 2002). Even minor inflammatory processes can eventually cause changes in brain function. Adult neurogenesis is one process that gets strongly inhibited by inflammation (Bastos et al., 2008) and this has been shown to be reverted by anti-inflammatory drugs (Ekdahl et al., 2003; Monje et al., 2003). The disruption in neurogenesis following inflammation might explain the memory deterioration, and deficits in learning and cognition seen in many of these inflammation-associated diseases. This disruption in neurogenesis could be mediated through the microglial release of inflammatory cytokines.

Inflammation, whether located in the periphery or the central nervous system has a detrimental effect on neurogenesis and cognition. Neuroinflammation is defined as the inflammatory response within the central nervous system, the brain, and the spinal cord (DiSabato et al., 2016). On the other hand, inflammation in the periphery is referred to as peripheral inflammation.

1. Neuroinflammation and Neurogenesis

A hallmark of neuroinflammation is microglial recruitment and activation. Microglia are the resident immune cells of the CNS and accordingly, they have surveillance and phagocytic properties. A striking feature of microglial cells is their extreme plasticity and fast activation in a very short time following an insult or injury. The microglial response includes increased proliferation, morphological changes, and the release of several cytokines, chemokines, reactive oxygen species, and nitric oxide (Kettenmann et al., 2011). The microglial response can have both protective and harmful effects depending on the type of insult and the microglial phenotypic state of activation. In a healthy uninjured brain, microglia are found in a resting ramified morphology state. In the DG, these resting surveilling microglia have high phagocytic potential for apoptotic newly born cells. Furthermore, microglial cells release factors that rescue neuroblasts and instruct differentiation (Kohman & Rhodes, 2013). However, in the case of inflammation, the classical phenotype activation of microglial cells has been documented to significantly impair hippocampal neurogenesis (Bastos et al., 2008; Monje et al., 2003).

Activated microglia in the brain release inflammatory cytokines, mainly TNFα and IL-1β with each having their specific consequent anti-neurogenic effects. NSCs exposed to TNFα have been shown to have increased expression of the anti-neurogenic gene hairy-

enhancer-of-split 1 (Hes1) that decreases neuronal differentiation by suppressing the expression of pro-neural genes (Keohane et al., 2010). Moreover, TNFα seems to drive the differentiation of NSCs towards having an astrocytic fate thereby affecting the differentiation process of new neurons (Johansson et al., 2008). In addition, elevated levels of IL-1β also affect the proliferation and differentiation of NSCs as it also increases the probability of astrocytic fate differentiation (Green et al., 2012; Wu et al., 2012;). It should be noted that NPCs express type 1 IL-1 receptor which allows IL-1β to act directly on these cells and directly elicit its effects (Green et al., 2012). Moreover, IL-1β can suppress progenitor cell proliferation by slowing the cell cycle through reducing cyclin D1, a protein involved in regulating mitotic events (Kohman & Rhodes, 2013). Similarly, IL-6 is another proinflammatory cytokine involved in reducing NSCs proliferation, survival, and differentiation (Vallières et al., 2002). It should also be noted that astrocytes are also involved in the immune response in response to inflammatory insults as they secrete pro- and anti-inflammatory cytokines, and chemokines.

An example of neuroinflammation is spinal cord injury (SCI) which is associated with a strong inflammatory response and results in increased immediate permeability of the blood-spinal cord barrier. The injury leads to the recruitment of macrophages, microglia, and $CD45^+$ leukocytes to the site of injury. It is well noted that chronic SCI is associated with severely reduced neurogenesis and impaired cognition (Jure et al., 2022; Jure et al., 2017; Wu et al., 2016; Wu et al., 2014). SCI is also associated with impaired immunity and increased susceptibility to infections. Interestingly, urinary tract infections are potential strong contributors to the impaired hippocampal neurogenesis associated with SCI (Sun et al., 2010). Thus, while an inflammatory response is essential for defense, it

has detrimental effects on neurogenesis especially if the inflammation is prolonged or chronic.

2. Effect of Peripheral Inflammation on Brain and Cognition

The nervous system and the immune system communicate in a bidirectional manner. Now that it has been established that the brain is not an "immune-privileged" organ as previously assumed, studies have been directed to focus on the interplay between the peripheral and central immune responses. Peripheral inflammation has been associated with neurodegenerative and neuropsychiatric diseases (Litteljohn & Hayley, 2012). In addition, peripheral inflammation is responsible for causing remote global gene expression changes at the level of the brain (Chamaa et al., 2016a; Thomson et al., 2014).

In recent years, it is becoming more and more evident that the immune response plays a critical role in modulating behavior. Recent studies revealed that certain molecules or cellular components of the immune system are involved in neurological functions such as learning, memory, anxiety, and social behavior (Avital et al., 2003; Derecki et al., 2010; Filiano et al., 2016). Mast cells, for example, are abundant in the meninges and the brain and are crucial for neurogenesis. It has been previously reported that a deficiency in mast cells is associated with anxiety and deficits in learning and memory (Nautiyal et al., 2008). Behavioral disorders such as autism, schizophrenia, and depression have all been associated with dysfunction in the immune system, in particular, altered levels of cytokines (Kirsten et al., 2018). It should be noted that the exact mechanisms that modulate such correlation between peripheral inflammation and brain function remain unclear. However, there have been some theories and evidence that suggest different mechanisms for crosstalk.

One proposed mechanism is that innate immune response could affect glutamate release and signaling, which in turn may result in synaptic and circuit dysfunction that is relevant to behavioral pathology (Miller et al., 2017). Moreover, peripheral inflammatory stimuli can affect basal ganglia circuits thereby contributing to motivational and motor deficits (Miller et al., 2017). In addition, peripheral inflammation induces weakening in the blood-brain barrier and increases its permeability, and all together leads to microglial activation. Cytokines cannot readily cross the BBB, however, there are leaky regions in the barrier where cytokines can cross. However, certain inflammatory cytokines secreted in the periphery such as IL-6 and TNF-α are capable of readily crossing the blood-brain barrier and gaining access to the brain. Otherwise, these cytokines and others can interact with receptors expressed by cerebral endothelial cells and induce the production of prostaglandin E2 into the brain parenchyma (Cerejeira et al., 2014; Matsumura & Kobayashi, 2004). Furthermore, as peripheral inflammation elicits the activation of microglial cells, this in turn could further recruit circulating monocytes from the periphery (D'Mello et al., 2009). A notable infiltration of macrophages and myeloid cells of peripheral origin into the hippocampus is detected following peripheral inflammation (Gampierakis et al., 2021; Schain & Kreisl, 2017).

Central, local or systemic administration of the bacterial endotoxin lipopolysaccharide (LPS) has been the most extensively used model to elicit inflammation and microglial response. Interestingly, systemic intraperitoneal LPS administration has been shown to have different vast effects at the level of the brain. Early studies have shown that LPS given intraperitoneally causes a rapid increase in *c-fos* in the nucleus tractus solitaries, brain stem nuclei, and the hypothalamus (Wan et al., 1993). More recently, it was further shown to cause a disruption in the BBB, oxidative stress, microglial activation, neuronal

loss, and white matter damage in different brain regions (Pires et al., 2020). Most importantly, LPS administered intraperitoneally has been strongly established to cause a decrease in NSCs proliferation, new cell survival, differentiation, and the overall neurogenesis process (Ekdahl et al., 2003; Fujioka & Akema, 2010; Saraiva et al., 2019). Moreover, it has been shown that neurons that mature in an inflammatory environment, resulting from such intraperitoneal LPS injections, exhibit increased inhibitory input from synapses that may potentially alter the function of these newly added neurons (Jakubs et al., 2008; Kohman & Rhodes, 2013). Therefore, collective data suggest that microglial activation and the resulting secretion of cytokines such as IL-6 and IL-1β create an environment that does not favor neurogenesis and the survival of the newly born neurons (Kohman & Rhodes, 2013). However, the exact mechanisms that lead to microglial inflammation in the brain post peripheral LPS challenge are still not completely understood and known.

In addition to LPS models, there are several diseases and disorders that are associated with peripheral inflammation and have been shown to affect the brain and cognition. An example of peripheral inflammation that alters neurogenesis is intestinal inflammation such as inflammatory bowel disease or chronic colitis (Zonis et al., 2015). Chronic intestinal inflammation is clinically associated with cognitive dysfunction, depression, and increased risk for psychiatric disorders. During the acute phase of intestinal inflammation, there are elevated levels of IL-1β and IL-6 in the hippocampus along with increased expression of the microglial marker IBA-1. In addition to inflammatory cytokines, there is a decreased expression of the stem/progenitor markers such as ki67 and Nestin and the neuronal marker doublecortin (Zonis et al., 2015).

More recently, there has been focus on the peripheral inflammation induced by COVID-19 and the behavioral cognitive changes that were reportedly associated with the viral infection. A state of dementia-like cognitive impairment has been increasingly reported as a complication of COVID-19 infection. Some common neurologic symptoms/ or complications that were reported in patients with COVID-19 include loss of taste or smell, delirium, stroke, and in some rare cases seizures (Zhou et al., 2021). Cognitive impairment can be detected in COVID-19 patients as shown by poor preliminary neuropsychological assessments and/or changed behaviors such as agitation, confusion, inattention, and disorientation (Rogers et al., 2020). Even in the previous outbreak of the severe acute respiratory syndrome (SARS-COV-1), it was documented that 20% of recovered patients had ongoing memory impairment (Rogers et al., 2020). A significant association has been reported between COVID-19 and neuroinflammation (Zhou et al., 2021). However, the exact mechanisms underlying such changes in cognition and behavior in COVID-19 patients remain unclear and need further exploration. Regardless, the resulting neuroinflammation and rise in cytokine levels are strongly suspected as mediators of such changes (Alnefeesi et al., 2021).

In addition, peripheral inflammation of the sciatic nerve leads to an increase in the levels of inflammatory cytokines such as IL-1β, IL-8, and IL-6 remotely in the dorsal root ganglion of the spinal cord (Saab et al., 2009). Furthermore, injury to the sciatic nerve induces a bilateral increase in TNFα and IL-1β in the central nervous system in the spinal cord (DeLeo et al., 1997) and in various brain regions (Al-Amin et al., 2011; Chamaa et al., 2016a; Covey et al., 2002; Sarkis et al., 2011). An increase in TNFα is reported in the hippocampus of rats with sciatic nerve injury and is thought to involve α_2-adrenergic receptor and the locus coeruleus (Covey et al., 2000).

Thus, taken altogether, peripheral inflammation seems to be causing significant effects and changes in central immune response and therefore causing changes at the level of the brain and consequently effects on cognitive functions and behavior.

3. Mechanisms Behind Inflammation-Induced Cognitive Deficits

Activation of the immune system leads to well-documented behavioral changes. "Sickness behavior" is the known term used to describe some of these adaptive behavioral changes that are associated with inflammation and that could facilitate recovery. Elevated levels of TNFα in the CNS, post-acute systemic inflammation, are responsible for the transient change in behavior that is referred to as sickness behavior. Sickness behavior experienced during a viral or bacterial infection is characterized by constellations of symptoms that include decreased motricity, reclusion, anorexia, hypotension, somnolence, hyperalgesia, and a fever (Dantzer, 2004; Kohman & Rhodes, 2013; McCusker & Kelley, 2013). Such behavioral changes are thought to contribute to energy conservation aiming to expedite recovery. However, not all of the observed behavioral alterations are considered adaptive responses. In addition to sickness behavior, immune activation has been strongly linked to impairment in certain aspects of cognitive function. Such changes in cognitive performance are thought to be a consequence of some remnant effects of the excessive immune activation that persist beyond the sickness behavior (Kohman et al., 2007). Notably, the cognitive deficits induced by inflammation are related to hippocampal-dependent tasks and functions such as contextual fear conditioning and memory consolidation (Kranjac et al., 2012). Intraperitoneal administration of a relatively high dose of LPS was shown to be correlated with depressed-like and anxiogenic-like behavior that was distinct from sickness behavior

(McCusker & Kelley, 2013). The exact reasons and mechanisms through which inflammation impairs cognitive functions remain unknown. However, there are several processes that play important roles in learning and memory that have been shown to be impaired following inflammation.

Behavioral changes post-peripheral inflammation are thought to be particularly attributed to increased levels of IL-1β and TNFα (Thomson et al., 2014). It has been documented that, patients with depression or neuropsychiatric disorders have higher serum levels of proinflammatory cytokines such as IL-1β and TNFα which further emphasized the role of these cytokines on cognition and behavior (Ng et al., 2018; Raison et al., 2006). There has been evidence suggesting that inflammation could be impairing cognition functions by disrupting the process of long term potentiation (LTP) which is crucial for learning and memory (Cunningham et al., 1996; Murray & Lynch, 1998). Elevated levels of IL-1β and TNFα are thought to inhibit LTP in the DG and thus suggesting a modulatory role for these cytokines in the hippocampus (Cunningham et al., 1996). They do so through the mitogen-activated protein kinase (MAPKs), p38, and JNK pathways via inhibition of calcium channels (McCusker & Kelley, 2013).

Moreover, another important mechanism through which inflammation could disrupt cognitive functions is through altering levels of neurotrophic factors that are essential for neurogenesis and LTP such as BDNF, nerve growth factor (NGF), and insulin-like growth factor (IGF) (Kohman & Rhodes, 2013). In addition, neuroinflammation could interfere with the process of learning by reducing event-induced neuronal activation (Hein et al., 2010).

All the aforementioned mechanisms are shared reasons for causing the decrease in hippocampal neurogenesis which is translated to cognitive and learning deficits.

C. Urinary Tract Infections & Cognitive Behavior

Recently, there has been more focus on the interplay between the urinary tract and the brain (Tahamtan et al., 2021), and the effects of UTIs on cognition are increasingly becoming evident (Björkelund et al., 2006; Mayne et al., 2019). Previous studies have shown that UTIs are among the most commonly occurring bacterial infections that are correlated with cognitive impairment (Björkelund et al., 2006; Mayne et al., 2019). The state of distressing behavior associated with UTI is referred to as delirium, which can develop in as little as one to two days and can range from agitation and restlessness to hallucinations and delusions (Manepalli et al., 1990). Among elderly patients, delirium is considered a non-specific symptom of UTI and is the most common reason for suspecting UTIs for their age (Balogun & Philbrick, 2014; Juthani-Mehta et al., 2009). So far, it is well known that elderly patients with UTIs experience delirium, might develop dementia, or even aggravate pre-existing dementia (Cerejeira et al., 2014). However, such knowledge has been based on clinical observation without understanding the physiological or molecular mechanisms leading to such changes.

1. UTIs: Epidemiology and Increasing Threat

A urinary tract infection is an infection that can occur in any part of the urinary tract which includes the kidneys, ureters, bladder, and urethra. UTIs could be caused by gram-negative or gram-positive bacteria or even certain fungi. UTIs are one of the most common infectious diseases occurring worldwide, where an estimated 150 million cases are yearly reported (Flores-Mireles et al., 2015; Foxman, 2003). This is associated with a significant economic and social burden, especially since the recurrence rate of UTIs is continuously increasing along with antimicrobial resistance. Serious sequelae of UTIs

include frequent recurrences of the infection and complications resulting from frequent antibiotic use. The frequent use of antibiotics is becoming a huge threat to the health care system and an increasing cause of mortality. Antibiotics can cause long-term alteration in the urothelium, the normal microbiota of the vagina, and the gastrointestinal tract (Kostakioti et al., 2012; Ramirez et al., 2020; Zhang & Chen, 2019). The most notable serious sequalae of administering antibiotics is manifested in the alarming increase in the number of multidrug-resistant organisms.

The prevalence of UTIs increases with age and is more common in females than males due to anatomical differences (Medina & Castillo-Pino, 2019). However, while women experience UTIs more frequently than men, men develop more severe symptoms and chronic infections. One in every three women develops UTI before the age of 24 and more than half of all women will experience at least one UTI episode in their lifetime (Hayes & Abraham, 2016).

2. Anatomy of the Lower Urinary Tract

The urinary tract consists of upper and lower tracts. The lower urinary tract is where most infections occur and constitute the urethra and the bladder. As for the upper urinary tract, it includes the kidneys and ureters leading to them. Due to its proximity to the rectum, the lower urethra is the most constantly exposed to gut-resident uropathogens. On the other hand, the bladder is considered to be the most sterile part of the urinary tract.

The urinary bladder wall is made up of three layers: the tunica mucosa, tunica muscularis propria, and tunica adventitia. The tunica mucosa consists of transitional epithelium and a lamina propria (Gonzalez et al., 2014). This mucosal lining of the bladder wall facing the lumen is made of epithelial layers connected by strong tight

junctions referred to as the urothelium which makes the bladder the most impenetrable tissue in the body (Hickling et al., 2015; Lacerda Mariano & Ingersoll, 2020). The transitional epithelial cells in the urinary bladder making the urothelium are arranged in basal, intermediate, and apical cell layers (Gonzalez et al., 2014). The urothelium consists of the following cellular layers in order from the innermost closest to the lumen to the outermost: umbrella cells or facet cells, intermediate cells, and basal cells. The umbrella cells are covered by proteins called uroplakins which have important physical barrier properties against toxins and pathogens. However, bacterial pili type 1 or fimbriae specifically bind to these uroplakins to gain access and colonize the urothelium. Thus, an important mucus layer situated above the umbrella cells provides a physical barrier and prevents the penetration of bacteria. This layer situates above these cells and is the surface layer in direct contact with the bladder lumen. The mucosal layer is composed of proteoglycans and glycosaminoglycans that further contribute to bladder wall impermeability and acts as an anti-adherence factor. Most importantly, this layer is a defense mechanism and helps protect the urothelium from accumulated toxins in urine. The extracellular matrix of the lamina propria contains diverse constituents such as interstitial cells, nerve terminals, and vasculature (Gonzalez et al., 2014). Due to its components and innervations, this layer serves to integrate epithelial and smooth muscle function.

The urethra lies directly beneath the urinary bladder and similarly to the bladder it has three layers: the tunica mucosa, tunica muscularis propria, and the tunica adventitia (Gonzalez et al., 2014). In the urethra, the tunica mucosa consists of transitional epithelium proximal to the urinary bladder followed by nonkeratinized, stratified squamous epithelium (Gonzalez et al., 2014).

3. Neural Control of the Lower Urinary Tract

The lower urinary tract (LUT) receives supraspinal, spinal, and peripheral nervous system control (PNS) input to control and maintain switch-like patterns of storage and elimination (de Groat et al., 2015). It receives a bilateral efferent innervation from the thoracic and lumbosacral segments of the spinal cord. The sympathetic control of the bladder involves urine storage and filling while the parasympathetic involves contraction and voiding to release urine. Efferent axons are carried in three sets of peripheral nerves: sacral parasympathetic (pelvic nerves), thoracolumbar sympathetic (hypogastric nerves and sympathetic chain), and sacral somatic nerves (the pudendal nerves) (de Groat et al., 2015). Sympathetic postganglionic neurons release noradrenaline, which activates β3 adrenergic receptors to relax bladder smooth muscle and activates α_1 adrenergic receptors to contract urethral smooth muscle which allows urine filling. Parasympathetic postganglionic axons in the pelvic nerve release acetylcholine which produces a bladder contraction by stimulating M3 muscarinic receptors in the bladder smooth muscle which allows urine voiding.

The bladder wall is lined with mechanoreceptors that synapse on spinal interneurons. These receptors serve to activate the visceral afferent (Aδ) fibers' activity during urine storage. The spinal interneuron pathways then activate preganglionic sympathetic fibers from the lower thoracic (T10) through the upper lumbar (L2) spinal cord (Gonzalez et al., 2014). The preganglionic fibers then synapse on the prevertebral inferior mesenteric ganglia or paravertebral ganglia and travel along the hypogastric and pelvic nerves, respectively (Gonzalez et al., 2014). These continue through the hypogastric nerves to synapse on α-adrenergic receptors in the bladder neck (internal sphincter) and proximal urethra, and β-adrenergic receptors in the bladder fundus. The

smooth muscle β-adrenergic receptors promote bladder wall relaxation and urine filling which is further facilitated by the activation of the α-adrenergic receptors on the internal urethral sphincter resulting in its contraction.

For the parasympathetic, these arise from the detrusor nucleus at the sacral level of the spinal cord (S2-S4) and are carried by the pelvic nerves to cholinergic parasympathetic neurons in the ganglia in the detrusor. The activation of parasympathetic neurons and the release of acetylcholine causes the activation of M2 and M3 muscarinic receptors and the subsequent contraction of the detrusor muscle. The parasympathetic also innervates the proximal urethra and upon activation, nitric oxide is released and causes urethral smooth muscle relaxation (Dorsher & McIntosh, 2012).

In addition, voluntary control is involved in the micturition reflex and that requires the integration of autonomic and somatic afferent pathways to coordinate the bladder activity. The striated muscle of the external urethral sphincter is directly innervated by axons reaching from motoneurons in the spinal cord (de Groat et al., 2015). The somatic innervation passes from the pudendal (Onuf's nucleus) through the pudendal nerves reaching the striated external urethral sphincter. This somatic system is under voluntary control from higher supra-spinal centers. These higher centers produce an excitatory influence on the pedundal nucleus during filling and an inhibitory descending influence during voiding of the bladder (Dorsher & McIntosh, 2012).

The brain adds voluntary control over this spinal reflex via ascending and descending pathways. The ascending pathways carry input and information from the stretch receptors regarding the degree of fullness of the bladder. A certain degree and amount of urine in the bladder create the urge to urinate. As for descending pathways from the brain, they give the ability to initiate or prevent micturition. The micturition

center is located at the level of the pons and is known as Barrington's nucleus or M-region. The pontine micturition center (PMC) receives parasympathetic and sympathetic input and modulates their opposing effects to send the appropriate response to the lower urinary tract. Another major center related to micturition is the Periaqueductal gray (PAG) which receives sensory information from ascending pathways regarding the bladder state. In other words, the two major CNS centers of micturition are the PAG and the PMC. The PAG plays the role of the receiver (input) from ascending sensory pathways and the PMC plays the role of the output sending descending pathways to the bladder detrusor and sphincters. Moreover, other CNS centers involved in micturition are the prefrontal cortex, anterior cingulate gyrus, and the insula (Dorsher & McIntosh, 2012).

Nonetheless, while the urothelium mainly acts as a barrier, it also has been recently viewed as having neuron-like properties through its interaction with afferent nerves. Urothelial cells display properties similar to sensory neurons (nociceptors/mechanoreceptors). A number of receptors and ion channels are associated with neurons in the urothelium. Examples of these include receptors for bradykinin, neurotrophins, purines, norepinephrine (α and β), acetylcholine (muscarinic and nicotinic), protease-activated receptors (PARs), amiloride/mechanosensitive Na^+ channels, prostaglandin E_2 (PGE_2) receptors, and TRP channels (TRPV1, TRPV2, TRPV4, TRPM8, and TRPA1) (de Groat et al., 2015). Upon activation of these receptors/ ion channels in response to mechanical or chemical stimulation, urothelial cells release chemical mediators such as nitrous oxide (NO), ATP, acetylcholine, prostaglandins, and substance P. Thus, the urothelium in this way can have the ability to respond to local chemical stimuli and send signals to bladder afferent nerves which convey the relayed

information to the CNS (de Groat et al., 2015). In the context of inflammation, the urothelium releases NO which is thought to have a role in modulating inflammatory and nociceptive pathways (de Groat et al., 2015).

Recent evidence from functional imaging studies such as fMRIs has added more knowledge on the patterns of neural activation that happen in response to bladder-related tasks such as urinary voiding or filling, and even on bladder-related sensations. Some of the brain regions that have demonstrated activation in response to bladder filling are the frontal cortex, anterior cingulate cortex, insula, parahippocampal gyrus, cerebellum, thalamus, and brainstem (Roy & Green, 2019). As for bladder filling, some of the regions reported to show activation include: the cingulate cortex, medial frontal cortex, occipito-parietal regions, insula, parahippocampal gyrus, and pons (Roy & Green, 2019).

4. UTI Pathogenesis and Uropathogenic Strains

UTIs can be categorized based on the organ infected as either a lower UTI (cystitis) or an upper UTI (pyelonephritis). Moreover, UTIs are clinically classified based on their severity as complicated or uncomplicated UTIs. Uncomplicated UTIs are infections that occur in healthy individuals that have anatomically normal urinary tracts. Complicated UTIs are infections that occur in individuals with compromised urinary tracts or host defense due to factors that increase susceptibility to colonialization such as urinary obstruction, renal failure, pregnancy, immunosuppression, and others (Flores-Mireles et al., 2015; Lichtenberger & Hooton, 2008).

The majority of UTI cases are caused by a group of *Escherichia coli* (*E. coli*) strains referred to as the uropathogenic *E. coli* (UPEC) (70-80% of cases). This strain is part of the larger group of extra-intestinal pathogenic *E. coli* (Flores-Mireles et al., 2015). UPEC

strains lead in being the most prevalent causative agents in both complicated and uncomplicated UTIs (Flores-Mireles et al., 2015). Moreover, UPEC are believed to originate from the intestines where these bacteria are normal components of the gastrointestinal microbiome (Hayes & Abraham, 2016).

The anatomical design of the bladder, along with its anti-microbial secretions, allows it to be resistant to active bacterial colonization. Despite such natural defenses, pathogens such as UPEC, are still capable of invading and colonizing the urinary tract (Hayes & Abraham, 2016). UPEC strains are more likely to survive in the vicinity of the uroepithelium as they produce toxins and proteases that further promote their invasion. Different strains of UPEC can significantly vary in their ability to colonize the urinary tract and cause symptoms or disease. This is associated with their expression of a broad spectrum of virulence factors, including adhesive molecules, capsular antigens, pili, fimbriae, and flagella that contribute to their capacity to cause disease (Terlizzi et al., 2017). Flagella and pili are the required appendages for pathogen migration towards the bladder, an event that follows the colonization of the urethra by that pathogen. Adhesins are considered one of the most important determinants for pathogenicity, as they promote bacterial invasion first by enabling the binding of UPEC to host cells within the urinary tract and second by preventing their clearance through the rapid urine flow. Moreover, adhesins trigger host and bacterial cell pathways that contribute to virulence and even facilitate the delivery of bacterial products or toxins to host cells. Bacterial adhesins bind to receptors on the bladder epithelium and promote bacterial colonization of the bladder. The subsequent host-pathogen interactions determine whether the pathogens would be able to survive and be successful in colonizing the urothelium or will be eliminated by the host defenses. If left untreated, a pathogen would continue to multiply and invade

until eventually it ascends to the kidneys and consequently would be able to cross the tubular epithelium and gain access to the blood stream initiating bacteremia (Flores-Mireles et al., 2015).

5. Natural defenses and the immune response to infection

A rapid and vigorous immune response is essential for guarding against bacterial pathogens especially UPEC, which tend to bypass the natural defenses of the UT. The innate immune response defends the uroepithelium from bacterial invasion through an expulsion mechanism that mainly depends on the Toll-like receptor 4 (TLR-4) which is extensively expressed on uroepithelial cells. TLR-4 is one of the pattern-recognition receptors (PRR) that recognize pathogen-associated molecular patterns (PAMPs). These receptors are activated by pathogens like UPEC within minutes and induce an inflammatory response and cytokine release. The receptors are highly expressed on the surface of superficial epithelial cells and can immediately recognize bacterial pathogens. In addition to immune cells releasing cytokines as part of the innate immune response, epithelial cells of the bladder and urethra have characteristic secretions of IL-1β, IL-8, and IL-6 mainly. These interleukins are essential for recruiting neutrophils and clearing bacterial infections (Engelsöy et al., 2019). Several of these interleukins are released via the canonical NF-κB pathway mediated by the adaptor myeloid differentiation primary response gene 88 (MyD88).

IL-1β has been shown to play a crucial role in UPEC clearance, while IL-8 is essential for recruiting neutrophils during a UTI (Engelsöy et al., 2019). The rapid surge in IL-1β and IL-6 post-infection results in a vigorous influx of immune cells to the urothelium to combat bacterial infections. Thus, inflammatory cells migrate from the circulation to the

urothelium to further amplify the inflammatory response and eliminate invading bacteria. Neutrophils are without a doubt the first immune cells to be recruited to the site of infection. Recruited neutrophils exit the blood vessels and migrate through the lamina propria to penetrate the basement membrane (Hayes & Abraham, 2016). After that, neutrophils penetrate through the epithelial layers before reaching the inner lumen. It should be noted that neutrophil recruitment is often vigorous and proportional to the bacterial load. Neutrophil counts start to peak at 6 hours post-infection and tend to decrease once the bacterial clearance begins (Haraoka et al., 1999; Hayes & Abraham, 2016). The way neutrophils clear bacteria are through phagocytosis as well as by releasing reactive oxygen species which are highly toxic to bacteria (Wu et al., 2017). Nonetheless, the immune response is a multicomponent defense program involving other cells such as macrophages and mast cells.

During UPEC infection, superficial urothelial cells exfoliate, helping to eliminate intracellular bacteria but also giving bacteria access to deeper layers of the urothelium. An evidence for such exfoliation is sometimes seen in the presence of bacterial epithelial cells being shed and flushed out in the urine (Hayes & Abraham, 2016). Moreover, the urinary tract constitutively secretes antimicrobial proteins that help prevent infection. Antimicrobial peptides are produced in addition to cytokines by the urothelium during UTI, which can directly kill bacteria and/or recruit inflammatory cells. An example of such proteins is the uromodulin, also called the Tamm-Horsfall protein, which is produced in the kidneys. This protein works by competing with type 1 pili for uroplakins binding, thus preventing bacterial adhesion to the host urothelium (Pak et al., 2001). Another antimicrobial peptide is beta-defensin which is produced by epithelial cells of the kidneys rather than the bladder (Hayes & Abraham, 2016). In addition, the urothelium

produces secretory immunoglobulin A which promotes pathogen clearance through a process of pathogen agglutination called immune exclusion. This process is mediated through antibody cross-linking of antigens and entrapment of pathogens in the mucus (Mantis et al., 2011).

Nonetheless, regardless of the immune defense mechanisms, UPEC strains harbor the characteristic to behave as an opportunistic intracellular pathogen, which enables them to invade, hide and eventually replicate inside superficial bladder epithelial cells. To evade expulsion from bladder epithelial cells via TLR-4-dependent mechanism, UPEC forms aggregates of cytosolic clusters of bacteria termed intracellular bacterial communities (IBC) (Klein & Hultgren, 2020). Thus, UPEC can survive in the urinary tract within a protected niche unbothered by the host defenses and even protected from antibiotic treatment. These UPEC protected reservoirs contribute to recurrent infections and serious complications (Flores-Mireles et al., 2015; García Méndez et al., 2016).

6. UTIs and Pain

Pelvic pain is a serious component of the morbidity associated with UTIs. Pain and hyperalgesia are defining characteristic features of interstitial cystitis/ bladder pain syndrome. However, it should be noted that the exact mechanisms behind UTI-associated pain are poorly understood. The pain associated with UTI is transient and usually the resolution of the infection leads to the resolution of pain. C-fiber afferents are the prime sensory neurons contributing to hyperalgesia (Yoshimura et al., 2014). The stimulation of bladder sensory afferents causes the sensation of pain and this might correlate with bacteria-induced inflammation and with released LPS and bacterial toxins. The bacterial product, LPS, released during the course of a bacterial infection, can by itself influence

visceral sensitivity (Watkins et al., 1994; Yirmiya et al., 1994). Mast cells were reported to be an important contributing factor to mediating bladder-induced pelvic pain. Mast cells release pro-inflammatory cytokines that act on afferent neurons in a positive feedback loop resulting in the release of neuropeptides which further induce mast cells degranulation and inflammatory response. This is referred to as the nerve-mast cell interaction axis. A persistent stimulation of afferent nerves could lead to altered neural plasticity and central nerve sensitization in the dorsal root ganglia and upper spinal cord (Akiyama et al., 2020). Nerve growth factor (NGF) may contribute to hyperalgesia by increasing neuronal hypersensitivity. NGF is produced by inflammatory cells, mainly macrophages, and mast cells, and NGF in turn stimulates the release of cytokines and further activation of mast cells. NGF increases the trafficking of transient receptor potential vanilloid 1 (TRPV1) receptor to the cell membrane surface in bladder urothelium in cystitis model (Duh et al., 2018; Liu et al., 2014). The TRPV1 receptor is a temperature-sensitive nociceptive receptor on C fibers which gets upregulated in bladder walls of cystitis patients. Thus, NGF has a substantial role in pain associated with cystitis or UTIs.

Inflammation has a suggested role in partaking in part of the resulting pain post-UTI. Inflammation leads to the release of downstream sensitizing agents and C-fiber hyper-excitability (Yoshimura et al., 2014). However, inflammation alone is not sufficient to induce the resulting pain. The pain response is also interestingly independent of the level of bladder colonization or the presence of bacterial pili. Thus, whether a pain response exists or not post UTI is not correlated with urinary tract and bladder colonization, rather it could be intrinsic to LPS of *E. coli* and dependent upon the LPS receptor TLR4 (Rosen & Klumpp, 2014). It has been shown that LPS can initiate inflammation and hyperalgesia

through a mechanism involving IL-1β, independently of peripheral sympathetic nervous system involvement (Safieh-Garabedian et al., 2002). Nonetheless, it should be noted that the pain response post-infection seems to be more specifically associated with UPEC strains than others (Rudick et al., 2010).

7. UTIs and the Brain: Effect on Cognition and Behavior

UTIs are among the most common bacterial infections that have been associated with acute psychosis (Mostafa, 2014). The activation of the immune system post-infection leads to neuroendocrine and behavioral changes (Yirmiya et al., 1994). Once an infection has occurred in the periphery, both cytokines and secreted bacterial toxins deliver this information to the brain using both humoral and neuronal routes of communication (McCusker & Kelley, 2013). Cytokines synthesized in the periphery, released in response to infections, initiate a response from endothelial cells of the BBB or directly reach the brain by crossing the leaking BBB through circumventricular organs. This initiates pro-inflammatory cytokines release in the CNS and leads to a set of behavioral changes.

There have been several meta-analyses and systematic reviews documenting clinical reports of cognitive changes, in particular delirium, associated with episodes of UTI (Balogun & Philbrick, 2014; Chiang et al., 2015; Eriksson et al., 2011; Gual et al., 2018; Manepalli et al., 1990; Mayne et al., 2019). A recent meta-analysis confirmed that such association between delirium and UTIs in older adults is significant regardless of confounding factors (Krinitski et al., 2021). Delirium is extremely prevalent in elderly patients with UTI that it has become one of the leading reasons to suspect UTI in elderly people presenting to the clinic with symptoms of confusion. Moreover, symptoms of delirium go beyond the elderly population and affect both males and females (Chae &

Miller, 2015). Studies have found a positive correlation between UTIs and neuropsychiatric disorders, mood disorders, and dementia. There are several theories to explain this. One theory stipulates possible physiological changes in the urothelium in people with neuropsychiatric disorders. A second theory attributes it to the possible lack of personal hygiene in people with mental disorders and in the elderly. Nonetheless, a third theory supports the notion of inflammation-induced cognitive changes which are dependent on the immune system activation and cytokine release in the CNS. Interestingly, in some cases, the UTI precedes dementia or psychiatric disorders which increases the possibility that UTI is a precipitating factor for such cognitive changes. A recent study investigated differences in gene expression in patients with delirium who have UTIs and those with delirium from unknown etiologies. Patients with delirium who also have UTI had significant activation of interferon signaling, upstream cytokines, and transcription regulators, as well as significant inhibition of actin cytoskeleton, integrin, paxillin, glioma invasiveness signaling, and upstream growth factors as compared to those without UTI (Kalantar et al., 2018). The findings of the study are interesting as they could be suggestive of several mechanisms and pathways that might mediate delirium-associated-UTIs. One of those factors could be inflammation and the release of cytokines. Interestingly, it has been reported that patients with interstitial cystitis or painful bladder syndrome show significant cognitive changes (Nickel et al., 2010). Moreover, there has been evidence linking neurotrophins such as BDNF to lower urinary tract function, particularly in regard to bladder pain syndrome/ interstitial cystitis and the stress associated with urinary incontinence (Song et al., 2014).

It should be noted that most of the mentioned studies are from clinical reports. Based on these clinical reports, it seems the association between UTI and delirium is becoming

more significantly evident over the years. Thus, the interest in using animal models of UTI has increased since these models allow to further investigate the exact molecular mechanisms and pathways linking UTIs to delirium. Recently, IL-6 has been reported as a possible mediator of delirium-like phenotypes in a murine model of UTI (Rashid et al., 2021). The cause behind the reported delirium involved cytokine-mediated neuronal dysfunction in both the frontal cortex and the hippocampus (Rashid et al., 2021).

Thus far, there are studies that investigate the effect of urinary tract infection on the brain. All in all, inflammation is a suspected cause of the effects of UTIs on the brain. As previously discussed, inflammation is a well-established strong inhibitor of adult hippocampal neurogenesis (Bastos et al., 2008; Chamaa et al., 2018; Darwish et al., 2019). Thus, it would be interesting to investigate whether inflammation is a possible mediator of the discrete crosstalk between the infected UT and the brain, and in particular on adult hippocampal neurogenesis.

D. Aim of the Study

The main aim of this study is to investigate the effect of UTI on the proliferation of neural stem cells and adult hippocampal neurogenesis. To achieve this, we established a reproducible model for urinary tract infections in rodents and characterized the resulting inflammatory response post-infection. Our next aim was to investigate the functional implications of the decrease in hippocampal neurogenesis on cognition and memory following UTI. For this purpose, we performed several cognitive-behavioral tests to assess different aspects of memory and exploration. Furthermore, aside from investigating the effect of UTI on the brain and cognition, another aim was to investigate possible factors that could mediate the effect on NSCs post urinary tract infections.

Inflammation could be a possible mediator between peripheral infection and hippocampal neurogenesis. Thus, we further investigated the inflammatory response post-infection in the urothelium. Finally, we attempted to investigate the effect of two treatment regimens (antibiotic or anti-inflammatory drugs) on the proliferation of neural stem cells to get more insight on the mechanism linking the bacterial infection to the alteration in proliferation of hippocampal NSCs.

CHAPTER II

METHODS

A. Characterization of clinical isolate E. coli 1176

1. Isolate Collection

The American University of Beirut Medical Center (AUBMC) provided the clinical bacterial isolate included in this study.

2. Antibiotic Resistance Profile for E. coli Clinical Isolate 1176

The clinical isolate used in this study was tested for antibiotic resistance. E. coli 1176 was shown to be sensitive to Colistin, Tigecycline, and Fosfomycin, while being resistant to all other antibiotics (Table 1).

a. Broth microdilution for susceptibility testing to antibiotics

Broth microdilution is done against 14 different antibiotics from different families. Serial dilution took place between columns 1 and 11 to have a concentration ranging from 2048 μg/mL to 2 μg/mL. Half of the wells in column 12 are used as a positive control and the other half as a negative control. For each isolate, a bacterial suspension of 0.5 MacFarland is prepared, followed by dilution to reach a concentration of 5 x 10^6 CFU/ml. This is followed by adding 10 μL of the latter into all the wells between columns 1-11, and in the positive control designated wells in column 12, ending with a final volume of 100 μL in all the wells. The plate is then placed in the incubator at 37 °C for 18 hours after which the negative control is checked to ensure the absence of contamination. The positive control is checked to ensure that the bacterial suspension is properly prepared, and growth took place. Wells 1-11 are checked for bacterial growth,

the well preceding the first well with bacterial growth, is referred to as the well containing the minimal inhibitory concentration (MIC). Experiments are run in duplicates for each bacterial isolate. The results are interpreted according to the CLSI M100 guidelines. The antibiotics are meropenem (AstraZeneca UK limited, Cheshire, United Kingdom); imipenem (Merck Sharp & Dohme B.V., Haarlem, Netherlands); ceftazidime (SIGMA-ALDRICH, St. Louis, USA); cefepime (Gulf Pharmaceutical Industries, Ras Al Khaimah, U.A.E); gentamicin (SIGMA-ALDRICH, St. Louis, USA); ciprofloxacin (HIKMA Pharmaceuticals, Amman – Jordan); levofloxacin (Sanofi-Aventis Deutschland GmbH, Frankfurt, Germany); colistin (SIGMA-ALDRICH, St. Louis, USA); amikacin (ANFARM HELLAS S.A., Kifissia, Greece); piperacillin/tazobactam (Wyeth Lederle S.P.A, Catania, Italy); tetracycline (Wyeth Lederle S.P.A., Catania, Italy); trimethoprim/sulfamethoxazole (SIGMA-ALDRICH, St. Louis, USA); Imipenem (as Imipenem/Cilastatin, Tienam®, Merck & Co., Inc., Whitehouse Station, NJ, United States); Ertapenem (Invanz®, Merck & Co., Inc., Whitehouse Station, NJ, United States), and Meropenem (Meronem®, AstraZeneca, Wilmington, DE, United States).

b. Determination of minimal Inhibitory Concentrations

The minimal inhibitory concentrations are determined via antimicrobial broth microdilution in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2012). Interpretations are made according to the CLSI M100-ED30:2020 Performance Standards for Antimicrobial Susceptibility Testing Guidelines.

Table 1. Antibiotic Resistance profile of E. coli clinical isolate 1176
Abbreviations: **R**, Resistant; **S**, Sensitive

Antibiotics	**MIC (µg/ml)**	**Interpretation**
Meropenem	8	R
Imipenem	8	R
Ertapenem	64	R
Gentamicin	>1024	R
Amikacin	>1024	R
Colistin	1	S
Cefepime	128	R
Ceftazidime	>1024	R
Tigecycline	1	S
Tazocin	512	R
Zerbaxa	1024	R
Fosfomycin	16	S
Ciprofloxacin	128	R
Levofloxacin	32	R

B. *In vitro*: Testing the Effect of E. coli Bacterial Extracts on Normal Human Bladder Epithelial Cells

1. Bacterial culture and secondary metabolite extraction

Bacterial secondary metabolites are directly collected as supernatants from overnight liquid cultures of both *E. coli* 1176 and *E. coli* ATCC 25922. E. coli ATCC 25922 is a reference strain for antibiotic testing mainly. A full loop of bacterial colonies from an overnight grown culture plate is inoculated in LB broth and incubated overnight on a shaker incubator at 37º C at 160 rpm. The broths are centrifuged at 5000 rpm for 5 min, the supernatants collected, and the pellets discarded. Protease/phosphatase inhibitor cocktail was added to the supernatant bacterial extracts before they were filtered twice, first with a 0.5µm filter and then a 0.2 µm filter. The extracts are stored at -20℃ until they were used as treatment for the cells.

2. Cell Culture Assays:

a. SV-HUC epithelial bladder cell line

The SV-HUC normal human bladder epithelial cells are cultured and maintained in Dulbecco's Modified Eagle Media (DMEM) Ham's F-12 (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich) and 1% Penicillin/Streptomycin (Sigma-Aldrich). Cells are incubated at 37° C in a humidified incubator containing 5% CO_2.

b. MTT assay

MTT ([3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]) assay is performed for assessing the anti-proliferative and cytotoxic effects of the bacterial extracts on SV-HUC bladder epithelial cells. Briefly, cells are seeded (2000 cells/well) in 100 µl complete medium in three different 96-well plates - one plate per time point (24 hr, 48 hr, 72 hr) - and incubated overnight at 37° C, 5% CO_2 before being exposed to the different bacterial extracts. Treatment is replenished at 24 and 48 hours. At each time point, media is removed and replaced by fresh media along with 10 µl/well of the MTT yellow dye (Bahmad et al., 2020). Six control wells are included at each time point. Vehicles are included as triplicates; 4µl of Broth is added to vehicle media. The cells are incubated for 3 hours, after which 100 µl of solubilizing agent isopropanol is added to each well for one hour. Absorbance intensity is measured by the microplate ELISA reader (Multiscan EX) at 595 nm. The percentage of cell viability is presented as an optical density (OD) ratio of the treated to the untreated control cells. The data are derived from the mean of duplicate wells of three independent experiments.

C. Experimental Design

1. Animals

Adult male Sprague Dawley rats, 3 months of age and weighing 450-550g were employed. The rats were housed under standard colony conditions in a room maintained at a constant temperature (20-22°C) on a 12 h light/dark cycle with standard rodent chow and water provided ad libitum. Animals were habituated to housing conditions a week prior to the experiment. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the American University of Beirut.

2. Experimental Groups

Figure 2 provides a summary of the different experimental groups and their time of treatment and sacrifice. Rats were sacrificed at 6 and 24 hours (h) post-infection to investigate early inflammatory response while others were euthanized on day 4 or day 34 post-infection to investigate the proliferation of DG neural stem cells and neurogenesis, respectively (Figure 2).

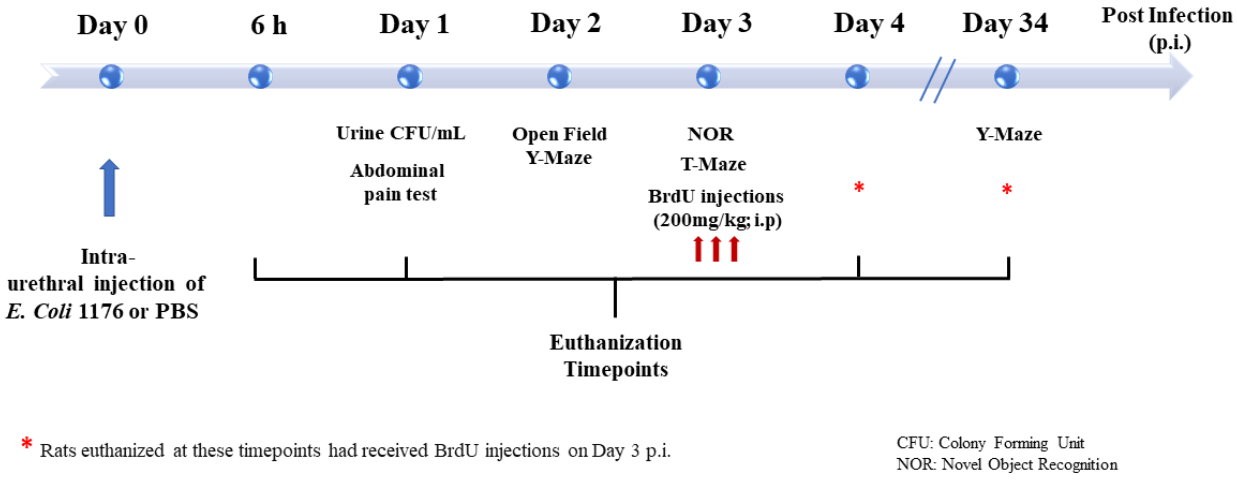

Figure 2. Experimental Design and settings. Schematic Diagram showing the experimental timeline followed for behavioral tests, BrdU injections and sacrifice.

Moreover, rats with UTI and sham received treatment with either the non-steroidal anti-inflammatory drug (NSAID) Piroxicam or the antibiotic drug Fosfomycin and were euthanized on day 4 post-infection. All groups and euthanization time points are listed in Table 2.

Table 2. Experimental Groups as per treatments and sacrifice time points.

Time points of euthanization	Groups / Treatments
6 and 24 hs post-infection	• UTI and sham (n=5 each)
Day 4 post-infection	• UTI and sham (n=9 each) • Treatment with Piroxicam; UTI and sham (n=5 each) • Treatment with Fosfomycin; UTI and sham (n=5 each)
Day 34 post-infection	• UTI (n=6) and sham (n=5)

D. Urinary Tract Infection Model

An *Escherichia coli* (*E. coli*) clinical isolate 1176 obtained from the urine of a patient at the American University of Beirut Medical Center (AUBMC) was used in this study. The experimental procedure for inflicting urinary tract infection was performed as described previously by Zychlinsky Scharff et al., 2017, with the single adjustment of the injection volume from 50 µL in mice to 500 µL in rats. Briefly, a single colony of an overnight culture of *E. coli* 1176 on MacConkey agar was used to inoculate 10 mL of Luria Bertani (LB) broth. The resulting culture was incubated statically overnight at 37 ºC. The liquid culture was centrifuged at 5000 g for 5 min and the bacterial pellet was resuspended in sterile Phosphate buffered saline (PBS) in order to obtain a bacterial suspension with an optical density (OD_{600}) of 0.5 McFarland which is equivalent to 10^8 colony forming units (CFU) per mL. Intraurethral injections were performed under isoflurane anesthesia and a compression is applied on the abdomen overlying the bladder to allow any present urine to be released. Catheters used are the BD Instyle Autoguard shielded IV catheters (24 G, 0.7 mm external diameter, 14 mM long) (Figure 3A). The

catheter should slide smoothly into the urethra, indicating correct placement which was tested on trial rats of the same age by injecting trypan blue (Figure 3B). Once the catheter is completely inside, the injection volume is slowly dispensed while the penis is maintained in a protruded perpendicular position to the animal's body using forceps. Each rat received an intra-urethral injection of 10^8 CFU/mL of the bacteria in 500 µL sterile PBS, while sham rats received an intra-urethral injection of 500 µL of the PBS vehicle. The catheter is slowly retracted to prevent leakage of the inoculum. Rats were deprived of drinking water for a minimum of 4 hours before the injections and another 2 hours post-injection. Urine was collected after 24 hours post bacterial infection in sterile 50ml conical held at a close distance to the rat but not in touch with its body or fur to avoid contamination. Serial dilutions in LB broth were immediately performed to establish CFU counts from urine. Serial dilutions and CFU count were performed under aseptic conditions in a biosafety level 2 cabinet. CFU/mL assay was performed on MacConkey agars through the full plate streaking of different dilutions and plates were kept overnight in an incubator at 37 ℃. Colonies were counted the next day from dilutions that had a number of colonies between 5 and 150 and CFU/mL was calculated as follows: $\text{CFU/mL}_{\text{Urine}} = \frac{\text{N x Dilution Factor}}{\text{Plated volume (ml)}}$, where N is the number of counted bacterial colonies.

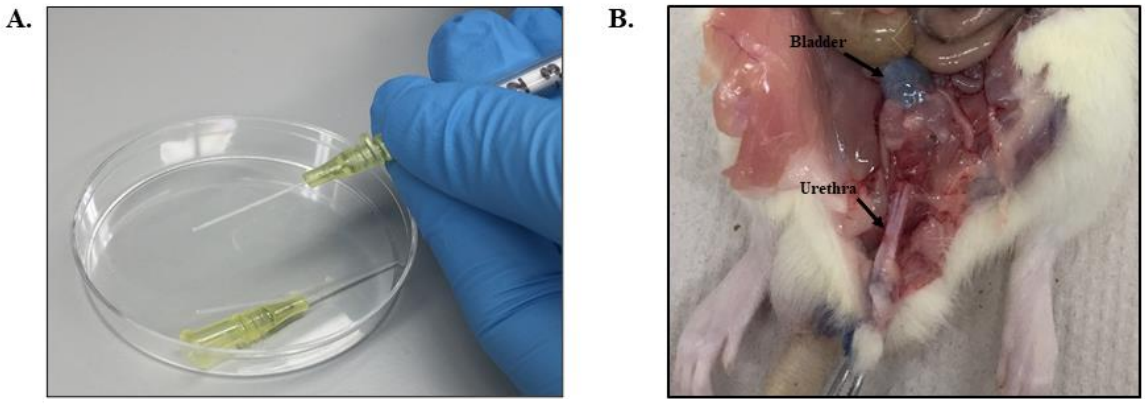

Figure 3. Injection site and catheters used for injection. (A) Catheters used for intraurethral injections. **(B)** Anatomical location showing placement of trypan blue intraurethral instillation in the bladder of a rat used for trial.

E. Treatment Regimens for UTI

Two treatment regimens were adopted; treatment with the antibiotic drug Fosfomycin (selected based on the resistance of *E. coli 1176*) or treatment with the anti-inflammatory drug Piroxicam.

Rats were treated with Fosfomycin (200mg/kg, i.p.) twice daily over 4 days starting from the time of intraurethral bacterial injection. The injections were separated by 8 hours with the first given one-hour post-infection. This treatment regimen is similar to clinical settings and represents adequate dosage exposure for Sprague Dawley rats (Poeppl et al., 2014). Fosfomycin is a broad-spectrum antibiotic used mainly for the treatment of uncomplicated lower urinary tract infections and is effective against multi-drug resistant (MDR) bacterial strains. We selected Fosfomycin since the clinical isolate *E. coli* 1176 used in this study was resistant to all antibiotics except Fosfomycin and Colistin. Colistin was not chosen due to its suspected nephrotoxicity and neurotoxicity (Ordooei Javan et al., 2015; Pogue et al., 2011).

Rats were treated with Piroxicam (10mg/kg, i.p, Feldene©) over 4 days. On the day of infection, the 1st dose of Piroxicam was given 30 minutes before intra-urethral injection of *E. coli* and the second dose was given 5 hours post-infection; this was followed by only one injection over the next 3 days. Piroxicam dosage was based on previous evidence from our group showing that daily injections of 12mg/kg, i.p. do not affect basal levels of neurogenesis (Chamaa et al., 2018a). Moreover, Piroxicam is a nonsteroidal anti-inflammatory (NSAID) drug and a member of the oxicam group. It has a long plasma half-life of approximately 2 days which allows once-a-day dosing (Richardson et al., 1985).

F. Behavioral Tests

Behavioral tests including the thermal sensitivity, open field, Y-maze, T-maze, and novel object recognition were performed to assess response to pain, anxiety-like behavior, exploration, spatial reference memory, cognitive ability, and working memory, respectively.

1. Thermal Sensitivity Test

Rats were placed in transparent plexiglass boxes and were allowed a minimum of 30 minutes before starting each session for familiarization with the environment. A nociceptive radiant heat spot (50 ℃) was projected from a 160-watt light bulb to the shaved lower abdominal surface of the rat and was only applied when the rats were stationary. The rats' abdomens were shaved a day before the test. Each rat was tested twice per session separated by a minimum interval of 5 minutes. The measurements made on all rats in an experimental group were averaged for the session and expressed as mean ± SEM.

2. Open Field

The open-field test was used to assess spontaneous locomotor activity and detect potential signs of anxiety-like behavior induced by UTI (Seibenhener & Wooten, 2015). The experiment was performed in dim light settings to boost further exploration. The rats were allowed 20 to 30 minutes to habituate to the experimental room and then were successively placed in the open field apparatus (no prior exposure to the apparatus) for a 5 min testing that was recorded by a camera. The videos were analyzed using Any Maze™ software and variables such as the number of entries to central zone, total time spent in

the central zone of open field, total distance traveled, average speed, and total time spent immobile were recorded. The central zone is designated as 25% of the area of the open field apparatus (Seibenhener & Wooten, 2015; Sestakova et al., 2013).

3. Novel-Object-Recognition (NOR)

The novel object recognition test was performed in the Open Field apparatus and on the second day after the rats have performed the open field test and became familiar with the apparatus. The test was performed on day 3 post-infection and consisted of 2 phases: a familiarization phase and a testing phase. In the familiarization phase, two identical objects were placed in 2 adjacent corners of the open field then rats were placed in the open field facing the opposite side to the objects and allowed to explore for 5 minutes. In the testing phase, one of the objects is replaced by a different novel object (location is kept the same), and animals were allowed to explore for 5 min in the presence of the novel object. The inter-phase separation time is 5 min when the animal is briefly returned to the cage and the apparatus is wiped with 70% ethanol. The number of times the rat visits the area of the novel object and the familiar object, and the total time spent there are recorded and analyzed on AnyMaze™ software.

4. Y-Maze test

This test was used to assess novel arm exploration in the different groups. The apparatus consists of three identical arms (10 cm wide and 40 cm long) that are equally spaced (120° apart) (Figure 4). No intra-maze cues were added, but different objects are placed at a range of distances outside the maze that would be visible to the rats and serve as extra-maze cues. Training and testing were performed as previously described

(Chamaa et al., 2021). Briefly, the test mainly consists of two phases that are 1 hour apart. In the first training or acquisition phase, one of the arms denoted as the novel arm is closed (shown as red block in Figure 4) and rats are placed in the "Start" arm. A period of 15 min is timed for the rats to explore and familiarize themselves with both the "start" (S) and the "familiar" arm (F). In the second phase or the test phase (retention trial), the closed/novel arm (N) is opened, and rats are also placed in the "S" arm. The rats were allowed to roam and explore the three arms for 5 min and videos were recorded on camera and analyzed using Any Maze™ software whereby the number of entries and the total time spent in each arm were recorded. The floor and walls of the maze were wiped with 70% alcohol at the end of the trial with each rat to avoid odor cues.

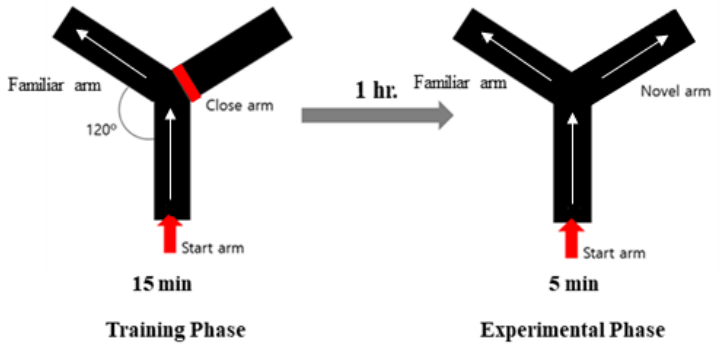

Figure 4. Schematic Diagram showing the Y-maze setup and protocol.
Arrows represent the directions the animal is allowed to explore.

5. T-Maze test

The T-shaped maze has three arms (90° apart); one is denoted as a start arm and the two side arms are denoted as the "goal arms". This test for spontaneous alteration is carried out as previously described by Deacon and Rawlins (Deacon & Rawlins, 2006). Animals are first put in the maze in the start arm facing the maze wall opposite the goal arms. Once the rat chooses and enters a goal arm, it is locked in that arm for 30 seconds.

After the 30 seconds, the lock is lifted, and animals are carried gently and placed in the start arm again facing the wall and then the rats' goal arm of choice is recorded again. An entry in an arm is denoted as a full entry of the animal along with its tail. The rats' natural tendency in a T-maze is to alternate their choice of goal arm. There is a central partition between the goal arms to placed produce more reliability in alteration rates. If the rat chooses the goal arm that it was locked in, then it failed to spontaneously alternate and if it chooses the opposite arm where it wasn't locked then it is recorded as spontaneous alteration. The response on each trial varies according to what the rats have previously chosen. This procedure is repeated four times per rat and the percentage of spontaneous alteration per rat in total is calculated and the average is calculated per group.

G. BrdU Injections

BrdU powder (5'-bromo-2-deoxyuridine, Sigma-Aldrich) was dissolved in 0.9% warm sterile saline and was given over 3 injections (66mg/Kg/ 300 μL injection, i.p.) on day 3 post-infection. The dosage, volume, and time of injection were based on a protocol followed in previous work by our group (Chamaa et al., 2016b).

H. Sacrifice and Tissue Collection

Rats were deeply anesthetized by i.p. injection of ketamine (Ketalar®; 50 mg/kg) and xyla (Xylazine®; 12 mg/Kg). Bladder and urethra were collected in cryovials under sterile conditions, snap-freezed in liquid nitrogen, then transferred to -80 ℃ for later processing and protein extraction. After that, rats were perfused transcardially with a solution of 0.9 % saline followed by 4 % formalin. The brains were carefully removed and fixed in 4 % paraformaldehyde for 24 hours before being transferred to 30 % sucrose

solution in 0.1M PBS to be stored at 4 °C until full impregnation. Brain sections were cut using a sliding microtome and sampled in a systemic manner as 6 sets using the fractionator method as previously described (Figure 5) (Chamaa et al., 2021; Gundersen et al., 1999). In brief, 40 µm coronal sections were cut serially, from the rostral to the caudal extent of the DG at the following rostrocaudal coordinates covering the whole hippocampal formation (-2.12 to -6.3 mm relative to bregma). To highlight the topographic distribution of BrdU positive cells, the DG region was divided into three areas 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 (Chamaa et al., 2018a; Paxinos & Watson, 1998). All sections were collected and stored in sodium azide solution (15mM in 0.1M PBS).

For groups sacrificed at 6 and 24 hours post-infection, aorta excision was performed under anesthesia with no perfusion-fixation. Fresh bladder and urethra tissues were collected on ice as with previous groups. In addition, the brain was extracted, and the hippocampi were cut on ice, snap-freezed with liquid nitrogen, and stored at -80°C for later RNA extraction.

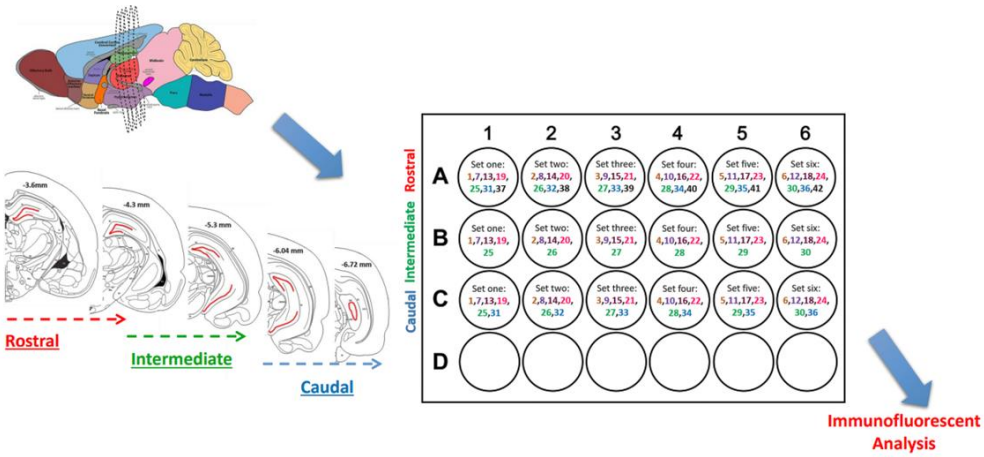

Figure 5. The Fractionator Method. Schematic Diagram showing the distribution of coronal sections of the dentate gyrus (red) in wells following the fractionator method of cutting and counting (Chamaa et al., 2016b).

I. Molecular Analysis

1. Immunofluorescence Assay

The tissues were stained for NSC marker and mature neuronal marker; BrdU and NeuN, respectively. The purpose of this double staining is to allow localization and counting of the newly born BrdU positive cells only in the sub-granular zone at the early time point of proliferation and in the granular zone at the later timepoint for neurogenesis. Wells containing rostral, intermediate, and caudal dentate gyrus regions for each rat were chosen randomly and immunofluorescence was performed as previously described by Chamaa et al. (2016b). Briefly, sections were washed 3 times with PBS and incubated at 37 °C with 2N HCL to allow denaturing of DNA, then 0.1M sodium borate (pH 8.5) was added for 10 min followed by another 3 washes and 1-hour incubation with 10% blocking solution. Tissues were incubated overnight with monoclonal primary antibodies: mouse anti-BrdU (1:500; Santa Cruz) and rabbit anti- NeuN (1:500; Millipore) diluted in PBS with 3% NGS, 3% BSA, 0.1% Triton-X. Incubation with secondary antibodies would follow on the second day, for 2 hours on a shaker at room temperature, using Alexa Fluor-568 goat anti-mouse (1:250; Molecular Probes, Invitrogen) and Alexa Fluor-488 goat anti-rabbit (1:250; Molecular Probes, Invitrogen). Finally, sections were washed and mounted onto slides with Fluoro-Gel with DAPI (Electron Microscopy Sciences, USA). For IBA-1 and GFAP staining, staining was performed as described above except sections were directly washed and placed in 10% blocking solution. For IBA-1, rabbit anti-IBA-1 primary antibody (1:1000; WAKO) and Alexa Fluor-568 goat anti-rabbit secondary antibody were used (1:250; Molecular Probes, Invitrogen). For GFAP, rabbit anti-GFAP primary antibody (1:1000; Abcam) and Alexa Fluor-568 goat anti-rabbit secondary antibody were used (1:250; Molecular Probes, Invitrogen).

2. Cell Counting and Confocal Microscopy

Counting of $BrdU^{+ve}$ cells is strictly confined to the subgranular zone (SGZ) of the DG on day 1 post BrdU injection (proliferation) and the granular cell layer (GCL) on day 32 post BrdU injection (Neurogenesis). $BrdU^{+ve}$ cells were counted manually using a 40X-oil objective. The total number of positive cells counted per rat was multiplied by 6 (the number of sets per rat), to denote the overall number of $BrdU^{+ve}$ cells in each region (rostral, intermediate, and caudal) of the DG per rat. For consistency, $BrdU^{+ve}$ cells were counted by the same researcher, and images were acquired under the same laser and microscopic parameters.

Z-stack and tile scan Images were taken using Zeiss LSM 710 confocal microscope at the 40-X oil objective. Z-stacks were used to show all $BrdU^{+ve}$ cells distributed within the 40µm section in the whole dentate gyrus of each region. The images were analyzed using the Zeiss ZEN 2009 image-analysis software and were processed with maximal intensity projection.

Representative immunofluorescent images for IBA-1 and GFAP were captured using laser screening on the confocal microscope. Per brain section, 3 snapshots covering and spanning the DG region were quantified and averaged for signal intensity per section. A representative example of one DG is shown in figure 6. Per rat, signal intensity was quantified in 5 sections taken from the same region for consistency (intermediate DG) and their average was taken to represent signal intensity for each rat. Signal intensity was represented as the average of values from 9 rats per group.

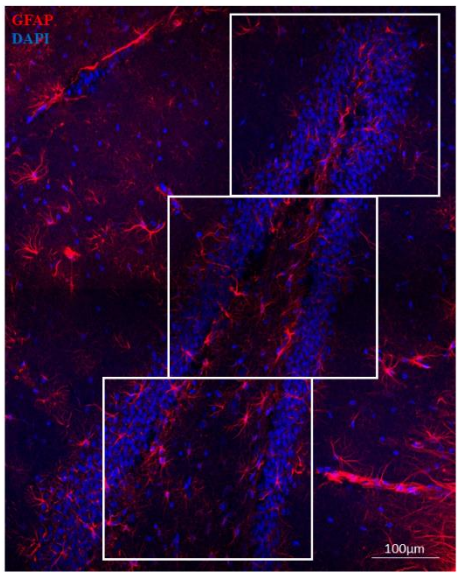

Figure 6. Signal intensity quantification Method. Representative tile scan image of astrocytic cells (red/GFAP) in the DG (blue/DAPI) of the hippocampus. The scan shows schematic snap shots that were similarly used to quantify optical intensity in the different sections.

3. Enzyme-linked immunosorbent assay (ELISA)

Supernatants of the homogenized tissues are used for the detection of IL-1β, and IL-8. The protein concentration of each sample is first determined using the Lowrey protein assay according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Pro-inflammatory cytokine release in response to infection was measured using a two-site sandwich ELISA assay. Pro-inflammatory cytokines: interleukin 8 (IL-8) and interleukin 1beta (IL-1β) were screened in protein extracts of urethra and bladder tissues. ELISA plates were prepared according to the manufacturer in a four-day protocol as described previously (Chamaa et al., 2016a; Saadé et al., 2002). Nunc 96-well Immuno plates were coated with immunoaffinity-purified polyclonal sheep anti-rat IL-1β or IL-8 coating antibodies (100 µl/well; NISBC, England) diluted in coating buffer and kept overnight at 4℃. The next day, three washes were performed followed by one-hour incubation with blocking solution (3% BSA, 0.1% Tween20 in PBS) at 37℃. After three washes, samples and standards were added in duplicates to respective wells to be incubated overnight at 4℃. On day 3, respective biotin-conjugated immunoaffinity-

purified polyclonal antibodies (1:4000; 100 µl/well; NISBC, England) diluted in wash buffer containing 1% Normal Sheep Serum (NSS; Abcam, Cambridge, UK) were added after three washes and incubated at 4 °C overnight. On day 4 of the protocol, three washes were performed then the streptavidin horseradish peroxidase enzyme (Amersham; diluted 1:8000) diluted in wash buffer with 1% BSA was added for 30 min on a shaker. Finally, the samples were incubated with the substrate: tetramethylbenzidine (TMB) along with H_2O_2 for 15 min before the reaction is stopped with the sulfuric acid solution (1 M H2SO4; 100 µl/well). Absorbance intensity was measured using the microplate ELISA reader (Multiscan EX) at 450 nm. Four parameter logistic curve-fit on Prism 7 GraphPad package (GraphPad Software, Inc., CA, USA) was used for obtaining a standard curve to interpolate samples' concentrations. Cytokine levels are expressed as picograms per milligram protein.

4. Conventional PCR

The *E. coli* 1176 clinical isolate is positive for New Delhi Metallo-β-lactamase variant 5 (*NDM-5*) (supplementary Figure S1). Conventional PCR was performed to confirm and monitor the presence of the *E. coli* 1176 bacterial isolate in urine samples over time through the detection of NDM-5 presence. The positive control used in experiments is the extracted DNA of clinical isolate 1176. The primer sequence is in Table 3.

Table 3. PCR primer for NDM-5 used for detecting presence of clinical isolate 1176 in urine samples in conventional PCR. **F:** Forward and **R:** Reverse.

Target Gene	Conventional PCR Primer Sequence (5'→3')
bla$_{NDM-5}$	F: 5′-GGCCAGCAAATGGAAACTGG-3' R: 5′-CAAACCGTTGGAAGCGACTG-3'

5. RNA Extraction and Quantitative Real-time PCR

RNA was extracted from hippocampi tissues using TriZol (TRI reagent®, Sigma) and following the manufacturer's protocol for RNA extraction from tissues. In brief, 1ml of TriZol 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 4. 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 4. 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
IL-6	F: ACAAGTCCGGAGAGGAGACT R: ACAGTGCATCATCGCTGTTC	167
Bdnf	F: CTCCGCCATGCAATTTCCAC R: CAGCCTTCATGCAACCGAAG	279
Ngf	F: CATCGCTCTCCTTCACAGAGTT R: TCTGTGTACGGTTCTGCCTG	222
Fgf2	F: AGGATCCCAAGCGGCTCTAC R: TACCGGTTCGCACACACTC	166

J. 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 with UTI across the following parameters: number of BrdU positive cells, number of BrdU/NeuN double positive cells, parameters in the open field, concentration of cytokines on day 4 post infection, time spent in each arm of the y-maze, latency to enter the novel arm, time spent exploring object in novel object recognition test, optical intensity of IBA-1 and GFAP. One way ANOVA followed by Tukey's multiple comparison was used to test statistical significance whenever comparing a variable across three groups in the study. The measure of statistical significance for IL-1β and IL-8 concentrations in sham and rats with UTI sacrificed at 6 or at 24 hours was analyzed by one-Way ANOVA followed by Tukey's multiple comparison test. Moreover, one way ANOVA followed by Tukey's multiple comparison test was also used to assess statistical significance for the number of

BrdU positive cells between sham, rats with UTI, and rats with UTI treated with Fosfomycin or Piroxicam. 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. Urinary Tract Infection in Rodents: Infection and Inflammation

1. Confirming Infection in male rats subjected to E. coli injections

Rats were subjected to intra-urethral injection of either E. coli 1176 clinical isolate or vehicle (PBS). The infection was monitored through urine sampling at different time points post-infection and CFU/mL assay was performed on day 1 post-infection. Urine samples collected were further tested for expression of NDM-5 (conventional PCR) that is specifically expressed in clinical isolate E. coli 1176. Urethra and bladder tissues were collected upon sacrifice for CFU/mL assay and for checking for NDM-5 expression through conventional PCR. Rats included in the study were confirmed to have UTI as the CFU counts in their urine exceeded 10^5 CFU/mL on day 1 post-infection (Figure 7 A&B) and by detection of bla_{NDM-5} in their urine culture (Figure 7 C&D). Moreover, we detected the presence of bla_{NDM-5} in the urethra tissue homogenate taken from these rats at time of sacrifice (Figure 8 A & B).

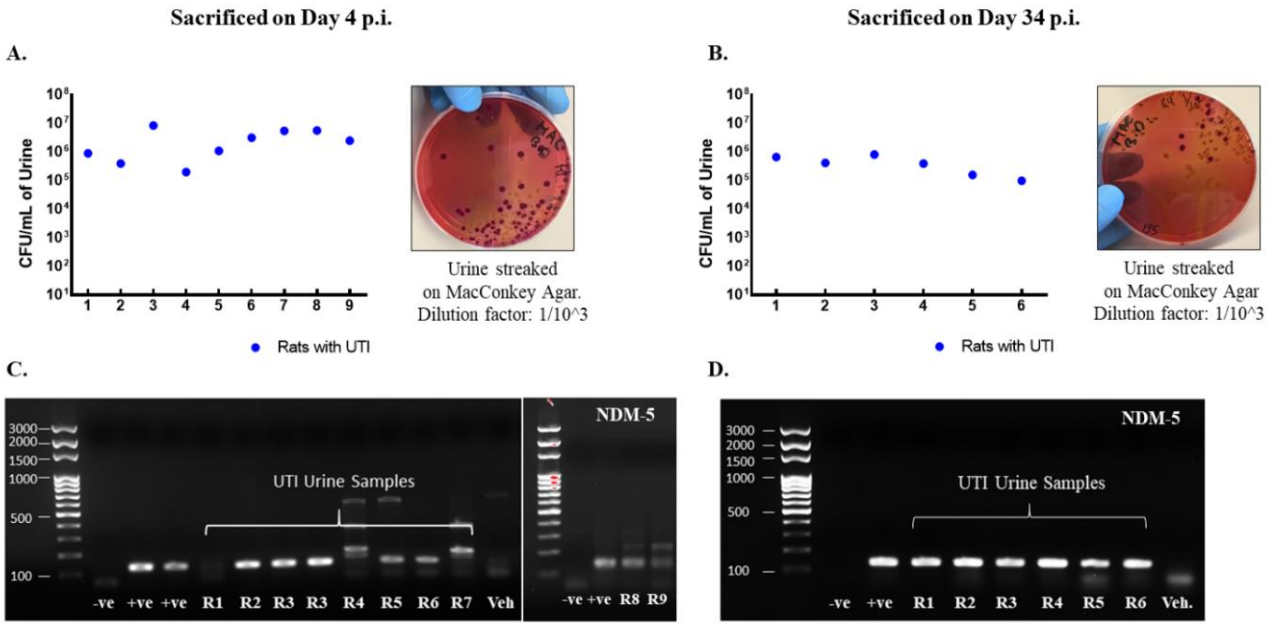

Figure 8. Two methods of confirmation of UTI in rats. Rats with UTI were confirmed for infection by presence of NDM-5 bacterial-specific gene in urine and CFU/mL>105. CFU/ml of urine samples collected on day 1 post infection from rats with UTI that were either sacrificed on **(A)** day 4 or **(B)** day 34 post infection along with representative images for the streaked MacConkey agar plates. Each dot represents the CFU/ml value for one rat. All rats had CFU/ml value above 10^5 confirming infections. Presence of *Ndm-5* gene in urine samples collected on day 1 post infection as confirmed by conventional PCR and shown by gel electrophoresis for rats with UTI sacrificed on **(C)** day 4 post infection and **(D)** day 34 post infection.

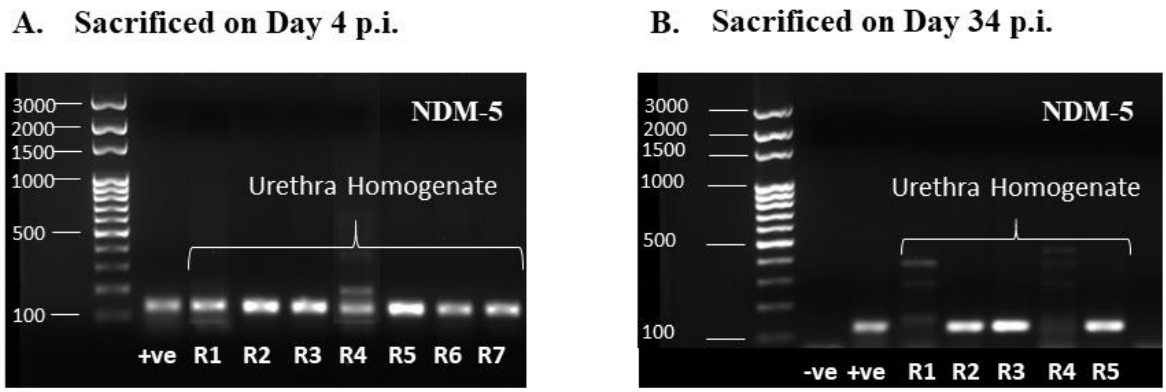

Figure 7. NDM-5 is expressed in urethra tissue homogenates from rats with UTI. Most urethra samples collected at time of sacrifice on day 4 **(A)** and day 34 **(B)** post infection express NDM-5.

2. Increased Concentrations of Cytokines Post Urinary Tract Infection

In addition to CFU and NDM-5 expression, infected rats had increased levels of pro-inflammatory cytokines production in their bladder and urethra as compared to vehicle-treated sham rats (Figure 9).

Levels of pro-inflammatory cytokines were tested at several time points post-infection in urethra and bladder tissues, in addition to plasma (6 hrs., 24 hrs., day 4 p.i. and day 34 p.i) for comparison between rats with UTI and vehicle. Inflammatory cytokines were quantified by ELISA assay for interleukins IL-1β and IL-8.

A tendency for increase in the production of IL-1βwas detected 6 hours post infection (52.29 pg/mg ± 6.05), which continued to reach a significant peak at 24 hours post infection (81.06 pg/mg ± 19.43, p=0.0315) as compared to non-infected sham rats (32.03 pg/mg ± 2.54) ($F(2,12)=4.332$, $P=0.0384$). Similarly, a trend of increase in the production of IL-8 was detected at 6 hours post infection (86.31pg/mg ± 15.93) which later significantly peaked at 24 hours post infection (202.21 pg/mg ± 57.96, p=0.0003) as compared to sham non-infected rats (26.81pg/mg ± 2.38) ($F(2,12)=17.05$, $P=0.0003$). At day 4 post infection, the increase in the production of IL-1β in urethra was maintained (42.76 pg/mg ± 5.9, p= 0.036, unpaired student t-test) and was significantly higher than that of sham non-infected rats (26 pg/mg ± 3.93). However, the levels of IL-8 in urethra at day 4 post infection (23.7 pg/mg ± 5.4) subsided to sham levels (10.02 pg/mg ± 2.98) (Figure 9 A&B).

In bladder tissues, we detected a significant peak in the production of IL-1β only on day 4 post infection (108.38pg/mg ± 7.84, $p<0.0001$) as compared to sham (36.16pg/mg±6.35). There were no significant alterations detected at 6 (49.08pg/mg ± 5.89) and 24 hours (37.8pg/mg± 4.38) as compared to sham (48.55pg/mg ± 6.95)

(F(2,10)=1.431, P=0.2841) (Figure 10 A). A similar trend was seen with IL-8 production in the bladder where a significant increase in IL-8 production was only detected on day 4 post infection (80.92pg/mg ± 4.98, p<0.0001) as compared to sham rats (11.07pg/mg±1.22). However, there was a slight non-significant increase in IL-8 at 24 hours post infection (17.95pg/mg ± 8.2), as compared to IL-8 concentrations at 6 hours (5.45pg/mg ± 0.69) and sham (5.57pg/mg ± 0.9) (Figure 10 B).

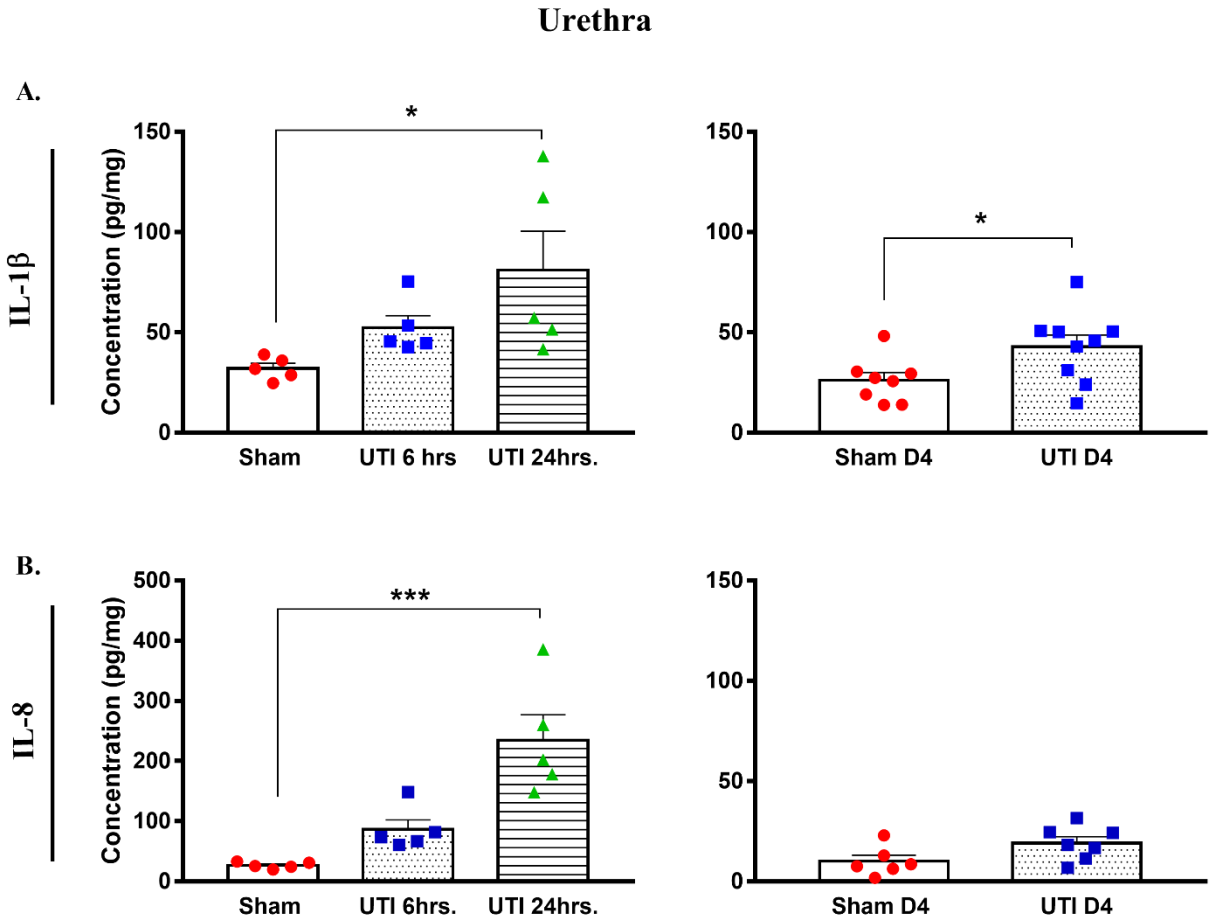

Figure 9. Increased protein concentrations of IL-1β and IL-8 in urethra tissues. Concentration of IL-1β **(A)** and IL-8 **(B)** in urethra tissues of rats with UTI at 6, 24-hours and day 4 post infection, as compared to sham injected with saline. One-way Anova followed by Tukey's multiple comparisons tests was used to determine statistical significance between sham, and rats with UTI at 6 and 24 hours. Student t-test was used to assess significance on day 4 (D4).

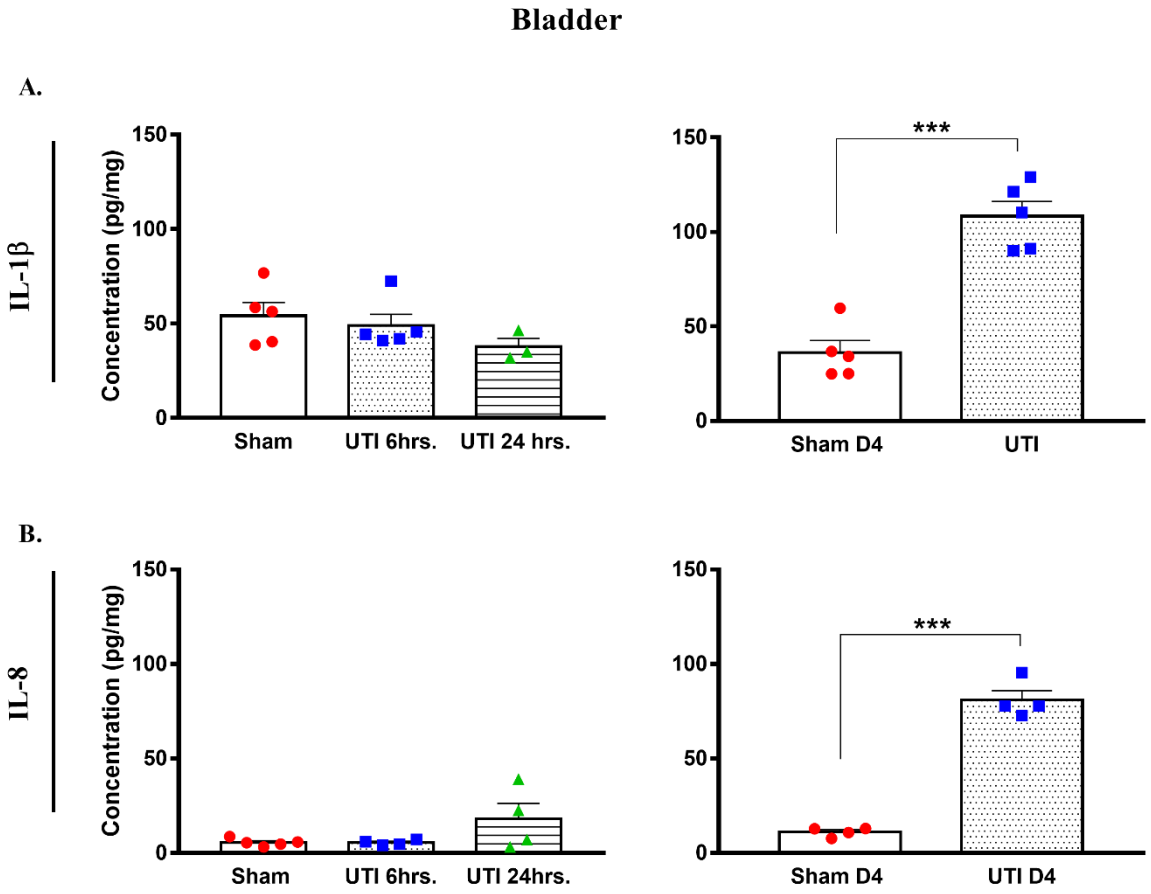

Figure 11. Increased protein concentrations of IL-1β and IL-8 in bladder tissues. Concentration of IL-1β **(A)** and IL-8 **(B)** in bladder tissues of rats with UTI at 6, 24-hours and day 4 post infection, as compared to sham injected with saline. One-way Anova followed by Tukey's multiple comparisons tests was used to determine statistical significance between sham, and rats with UTI at 6 and 24 hours. Student t-test was used to assess significance on day 4 (D4).

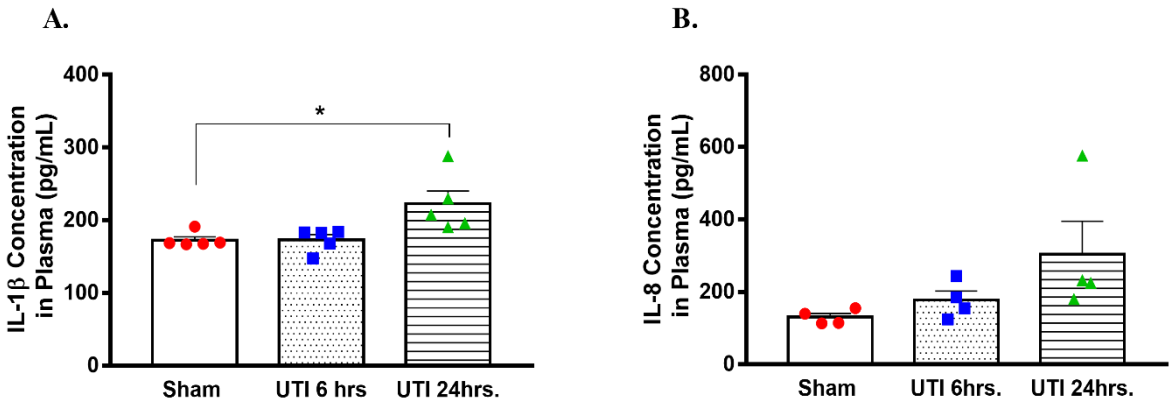

Figure 10. Plasma Concentrations of Interleukins at 6 and 24 hours. Concentration of IL1β **(A)** and IL-8 **(B)** in sham versus rats with UTI sacrificed at 6 or 24 hours post infection. One-way Anova followed by Tukey's multiple comparisons tests was used to determine statistical significance between sham, and rats with UTI at 6 and 24 hours.

Furthermore, we measured the concentration of IL1β and IL-8 in the plasma of sham and rats with UTI at 6 and 24 hours. We detected an increase in IL-1β levels in plasma of rats with UTI at 24 hours (222.4 pg/mg± 17.77, p=0.023), but not at 6 hours (173 pg/mg ± 15.72), as compared to sham (172.6 pg/mg ± 4.63) (F (2,12)= 6.362, P=0.0131) (Figure 11 A). However, there was no significant increase in concentrations of IL-8 in plasma of rats with UTI at 6 (176.95 pg/ml ± 29.5) and 24 (303.53 pg/ml ± 91.64) hours as compared to sham (130.32pg/ml ± 10.15) (Figure 11 B).

The concentration of TNFα in urethra of rats with UTI at 6 (23.36 pg/mg ± 4.11) and 24 (26.31 pg/mg ± 1.64) hours post infection were comparable to sham concentrations (18.58 pg/mg ± 2.41) (Figure 12 A). Furthermore, we did not detect an increase in TNFα at 6 (2.18 pg/mg ± 0.32) and 24 (1.25 pg/mg ± 0.32) hours post infection in bladder tissues compared to sham (1.74 pg/mg ± 0.25) (Figure 12 B).

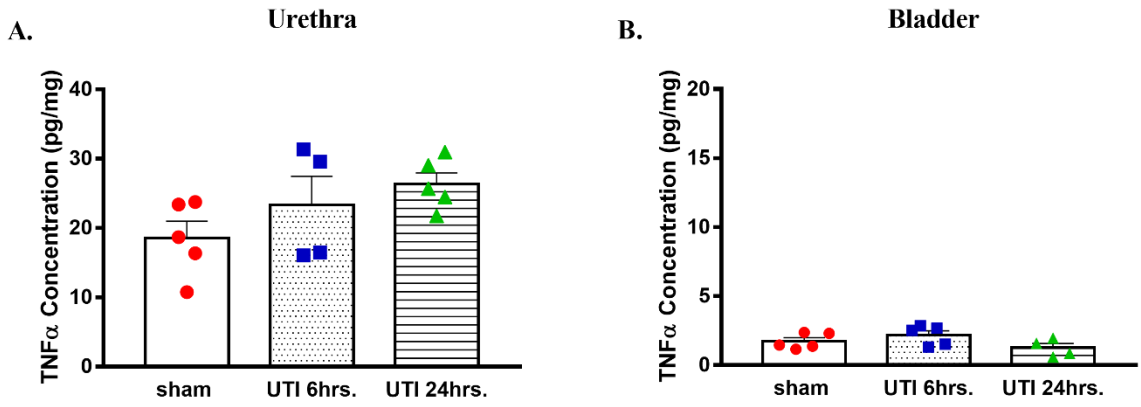

Figure 12. Expression of TNFα in bladder and urethra. There was no significant alteration in concentration by ELISA of TNFα in urethra **(A)** and bladder **(B)** of sham and rats with UTI at 6 and 24 hours post infection.

3. Urinary Tract Infection in Female Rodents

Given the fact that UTIs are more prevalent in females more than males, we wanted to establish the same UTI model in female Sprague Dawley rats in an attempt to assess and compare differences in response to infection between males and females. It is known that while females experience UTIs more frequently than males, males develop more severe symptoms and chronic infections. Interestingly, we found that female rats were more resistant to infections than male rats as shown by the fast clearance of bacteria from urine.

a. <u>NDM-1 expression in urine and urethra samples collected on day 7 post infection</u>

Only one out of five female rats had positive expression of NDM-1 in both urine and urethra post infection with E. coli 1176 clinical isolate (Figure 13).

Figure 13. NDM-1 expression in urine and urethra samples collected on day 7 post infection. One out of 5 female rats expressed NDM-1 in both its urine and urethra samples. -ve: negative control; +ve: positive control; Veh: vehicle; p.i.: post infection.

b. Variable ranges in CFU/mg in the urethra of female infected rats

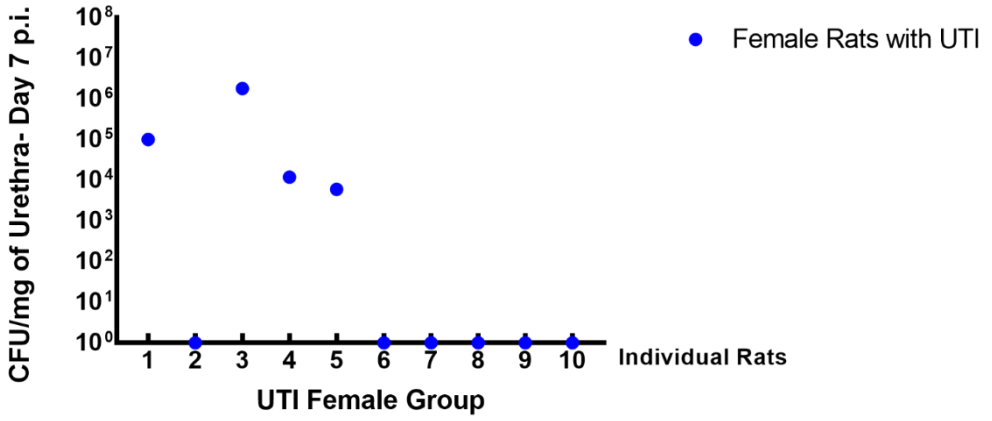

Figure 14. Most female rats have cleared the infection after a week. CFU/mg of urethra homogenate samples from female adult rats collected on day 7 post infection with E. coli 1176 clinical isolate. Rats R2 and R6-R10 had zero CFU/mg. each dot represents value for one rat.

The CFU/mg of urethra from female infected rats show variable range of colonies ranging from zero to 10^3 and 10^6 colonies counts and 6 of the infected rats had already cleared infection from urethra. No bacterial growth was observed when urethra homogenate from rats R2, R6-R10 was streaked on MacConkey agars (Figure 14).

4. Toxicity of E. coli 1176 bacterial extract on normal human bladder cell line SVHUC

While we used in this study *E. coli* 1176 strain, we were interested in comparing whether there might be differences between bacterial strains in regard to their toxicity and effects on normal bladder epithelial cells. The supernatants would supposedly contain bacterial metabolites, toxins and LPS. Thus, we proceeded invitro to investigate whether the bacterial supernatants of E. coli 1176 and E. coli ATCC 25922 would have differences in their toxic effects on bladder epithelial cells' proliferation.

Different increasing concentrations of the bacterial extract supernatant of *E. coli* 1176 (25, 50, 100, and 200 µg) were screened for their toxicity effect on normal bladder epithelial cell line SV-HUC at two different time points after treatment: 48 and 72 hours. The bacterial extract of *E. coli* 1176 significantly decreased cell proliferation of SV-HUC at 200µg concentration of the supernatant at 48 hours (45.95 ± 7.1 % of control; p=0.0034) and at 100µg (63.02 ± 4.14 % of control; p=0.045) and 200µg (30.24 ± 7.59 % of control; p=0.0004) concentrations at 72 hours after treatment (Figures 15, 16 and 17).

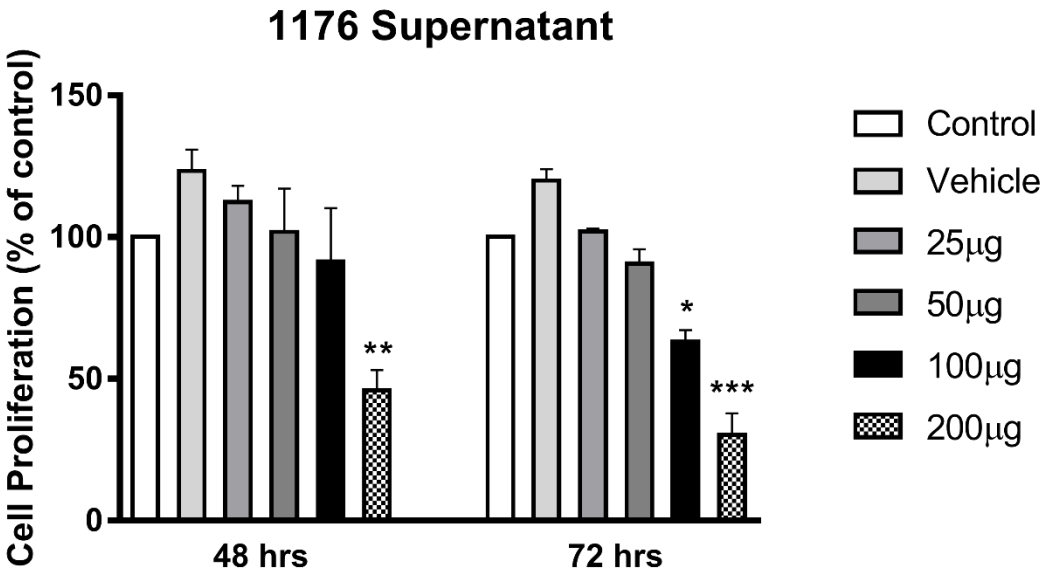

Figure 15. The effect of increasing concentrations of bacterial extract *E. coli* 1176 on SV-HUC cell proliferation. After incubation of the cell line (SV-HUC) for 48 and 72 hours with or without bacterial extracts of increasing concentrations, cell proliferation was determined using MTT assay. *E. coli* 1176 supernatant significantly decreases SV-HUC cell proliferation in a time-dependent manner. Results are expressed as a percentage of the treated group compared to its control. Data represented is an average of two independent experiments. The data are reported as mean ± SEM (Two-way ANOVA; *p<0.05, **p<0.05, *** p<0.001; different treatment concentrations compared to control, Bonferroni's post hoc multiple comparison test).

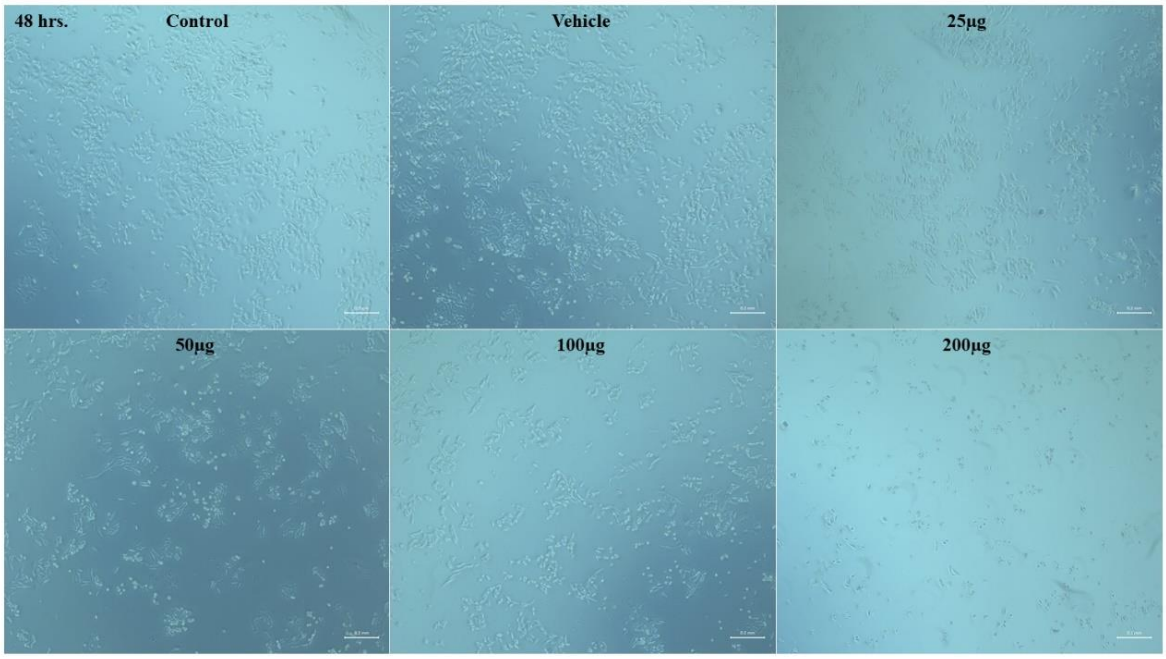

Figure 16. The effect of increasing concentrations of bacterial extract *E. coli* 1176 on SV-HUC cell proliferation at 48 hours. Representative bright-field images of non-treated control SV-HUC cells and that of cells treated with 25 µg, 50 µg, 100µg and 200µg of *E. coli* 1176 bacterial extract at 48 hours post treatment. Images taken at 5x objective, scale bar: 200µm.

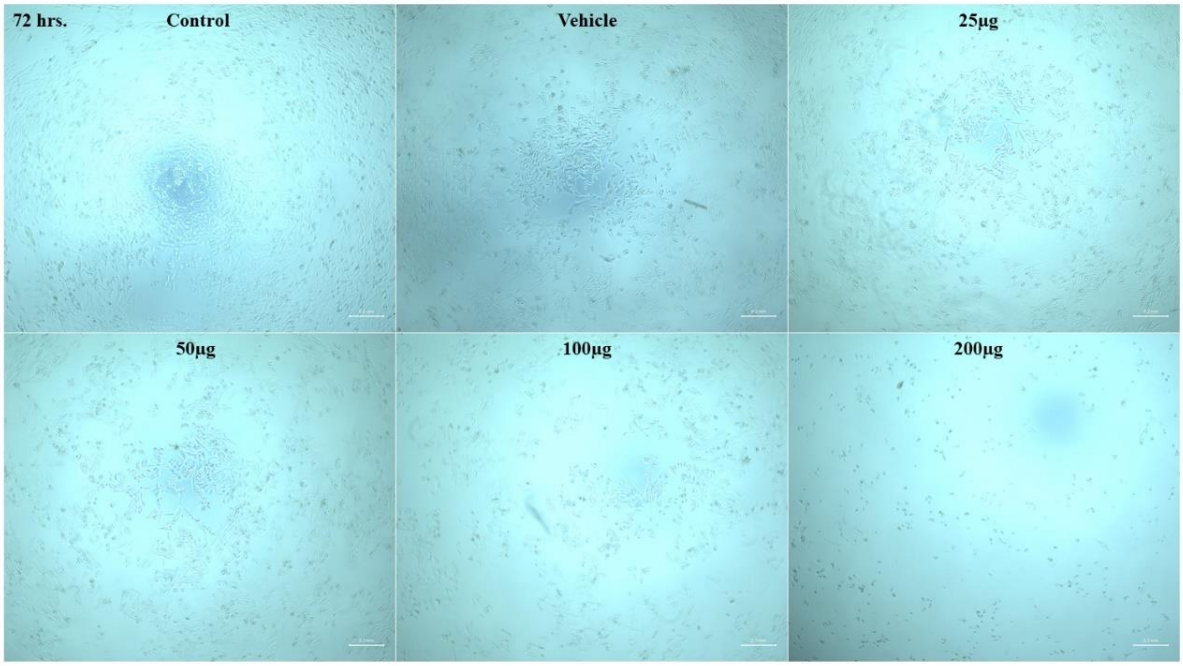

Figure 17. The effect of increasing concentrations of bacterial extract *E. coli* 1176 on SV-HUC cell proliferation at 72 hours. Representative bright-field images of non-treated control SV-HUC cells and that of cells treated with 25 µg, 50 µg, 100µg and 200µg of *E. coli* 1176 bacterial extract at 72 hours post treatment. Images taken at 5x objective, scale bar: 200µm.

B. The Effect of Urinary Tract Infection on Hippocampal Neurogenesis

1. UTI decreased proliferation of Neural Stem Cells in the DG at 4 days post infection

Since rats were sacrificed 24 hours post BrdU injection, as expected the BrdU positive cells were not co-stained with the mature neuronal marker; NeuN. Rats with confirmed UTI had a significantly decreased number of BrdU positive cells (2452 ± 243; $p<0.001$, unpaired student t-test) in their DG as compared to sham rats (4544 ± 303) at day 4 post infection (Figure 18 A & B).

Topographically, the decrease in BrdU positive cells was significantly noted in all regions of the DG of rats with UTI; rostral DG (343±93 versus 845±189; $p=0.03$), intermediate DG (413±73 versus 757±68; $p=0.0033$), and caudal DG (1684±187 versus 2943±298; $p=0.0025$), as compared to sham rats, respectively (Figure 19 A&B).

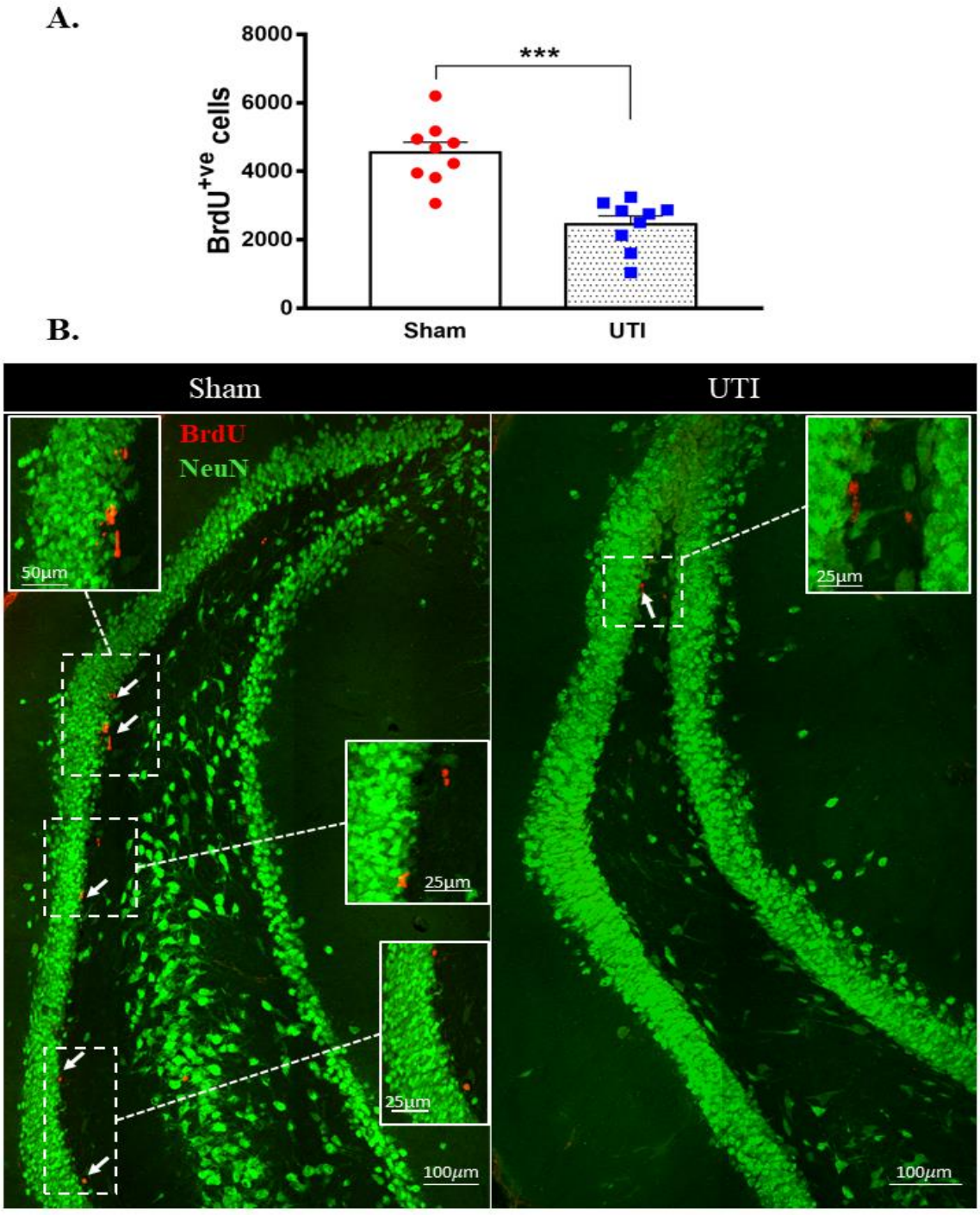

Figure 18. Decrease in the proliferation of DG NSCs on day 4 post infection.
(A) Stereological quantification of BrdU-labeled cells in the DG of sham (n=9) and rats with UTI (n=9) sacrificed on day 4 post infection. Unpaired student t-test was used to determine the statistical significance between sham and UTI groups, ***p<0.001. Each bar represents the average ± SEM of BrdU positive cells per group and each dot represents measured number in each rat. **(B)** Representative confocal images showing immunofluorescence labeling of NeuN and BrdU (arrows) in the DG of sham and rats with UTI. Images were taken as Z stacks and tile scan using 40X-oil objective.

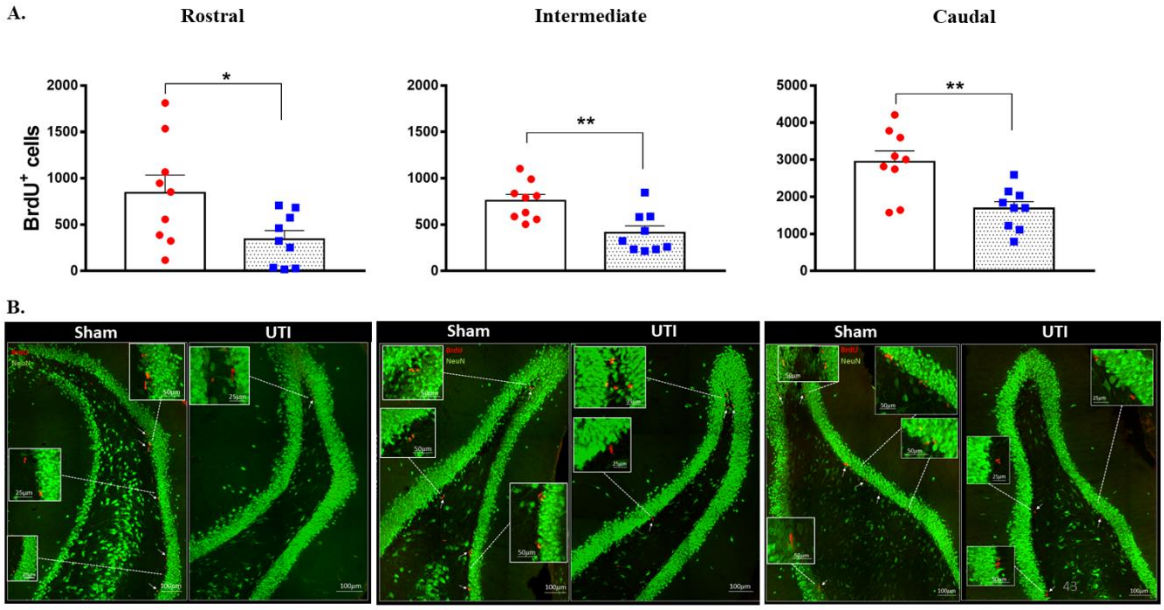

Figure 19. Topographical Distribution of BrdU positive cells in the DG.
(A) Numerical topographical distribution of BrdU positive cells in the rostral, intermediate and caudal regions of the DG of rats sacrificed on day 4 post infection. **(B)** Representative confocal images showing immunofluorescence labeling of NeuN and BrdU (arrows) in the rostral, intermediate and caudal regions of the DG of sham rats and rats with UTI. Determination of significance of differences was generated using two-tailed unpaired student t-test.

2. UTI decreased neurogenesis in the DG at 34 days post infection

As neurogenesis takes around 30 to 40 days, some of the BrdU positive NSCs that were born at time of BrdU injection have become mature NeuN positive neurons. Total BrdU positive cells were counted to know total count of BrdU positive cells present at time of sacrifice. Rats with confirmed UTI had a decreased number of BrdU positive cells (829 ± 101 cells; p=0.04) as compared to that in DG of sham rats (1477 ± 280 cells). It is expected that a number of the newly born cells at time of BrdU injection would die as normal part of neuronal turnover and a number would differentiate into either astrocytes or oligodendrocytes. Thus, the number of BrdU/NeuN double positive cells were counted to assess the number of BrdU positive cells that have specifically differentiated into

neurons. The number of BrdU/NeuN double positive cells in the DG rats with UTI (764 ± 90 cells; p=0.03) was significantly lower than that of sham rats ((1374 ± 248 cells) (Figure 20A, B &D). Both sham (93 ± 1%) and UTI rats (93 ± 2%) had comparable percentage of BrdU/NeuN double positive cells out of total BrdU-positive labelled cells (Figure 20C). While the total number of BrdU positive cells was reduced, the ratio of committed cells to neuronal fate was no affected.

Topographically in the DG, the decrease in BrdU positive cells was significantly notable in the intermediate (152 ± 23; p=0.04) and caudal DG (434 ± 50; p=0.02) of rats with UTI as compared to sham rats (292 ± 59 and 821 ± 139, respectively). As for the rostral DG, the total number of BrdU positive cells was comparable between sham (365 ± 89) and UTI rats (233 ± 41) (Figure 21 A& B).

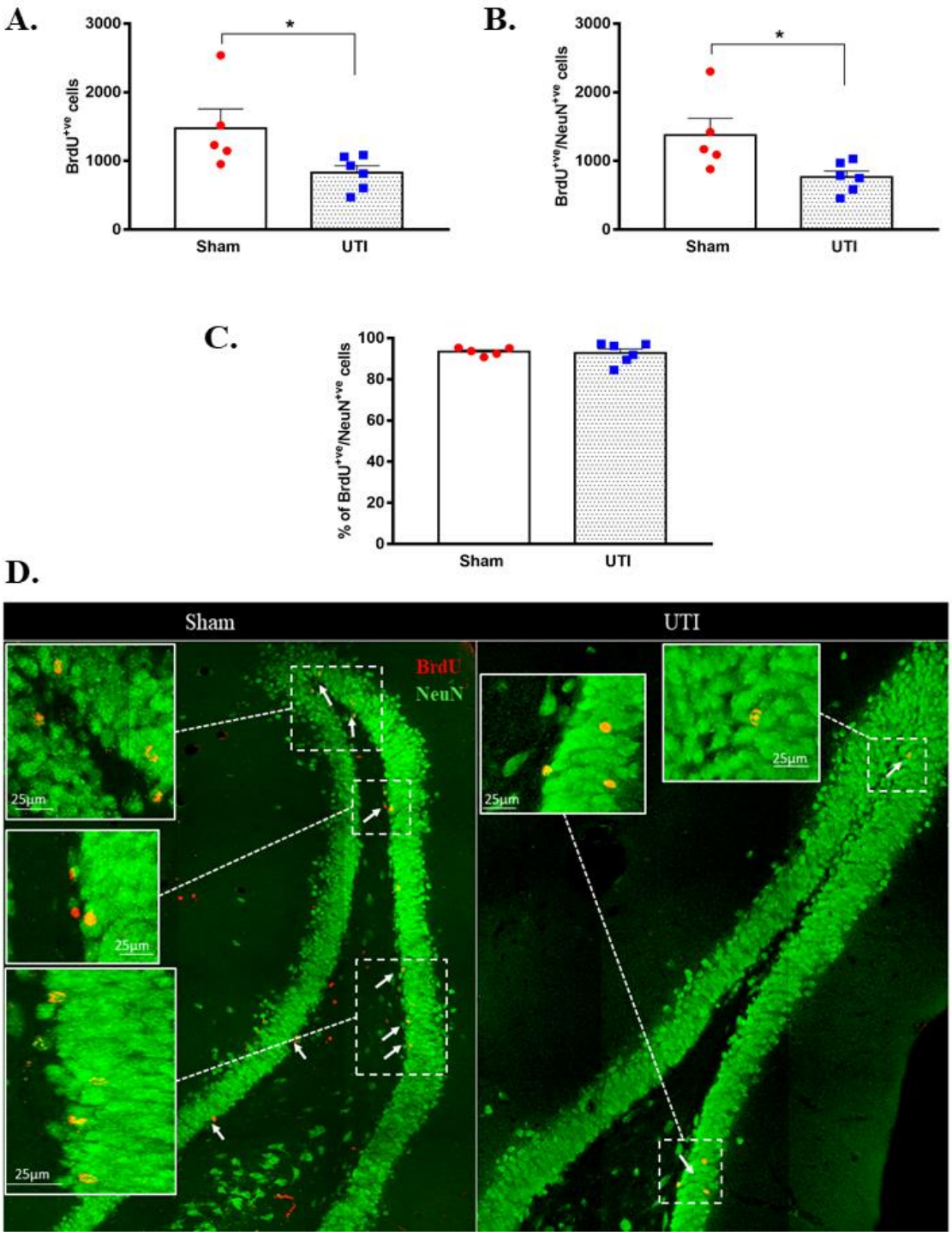

Figure 20. Decrease in neurogenesis persists on day 34 post infection.
(A) Stereological quantification of BrdU-labeled cells in the DG of sham rats (n=5) and rats with UTI (n=6) sacrificed on day 34 post infection. **(B)** Stereological quantification of BrdU/NeuN double labeled cells in the DG of sham and rats with UTI. **(C)** Percentage of BrdU/NeuN double labeled cells out of total BrdU positive cells in the DG of sham and rats with UTI. Unpaired student t-test was used to determine the significance between sham and UTI groups. **(D)** Representative confocal images showing immunofluorescence labeling of NeuN and BrdU (arrows) in the DG of sham and rats with UTI. Images were taken as Z stacks and tile scan using 40X-oil objective.

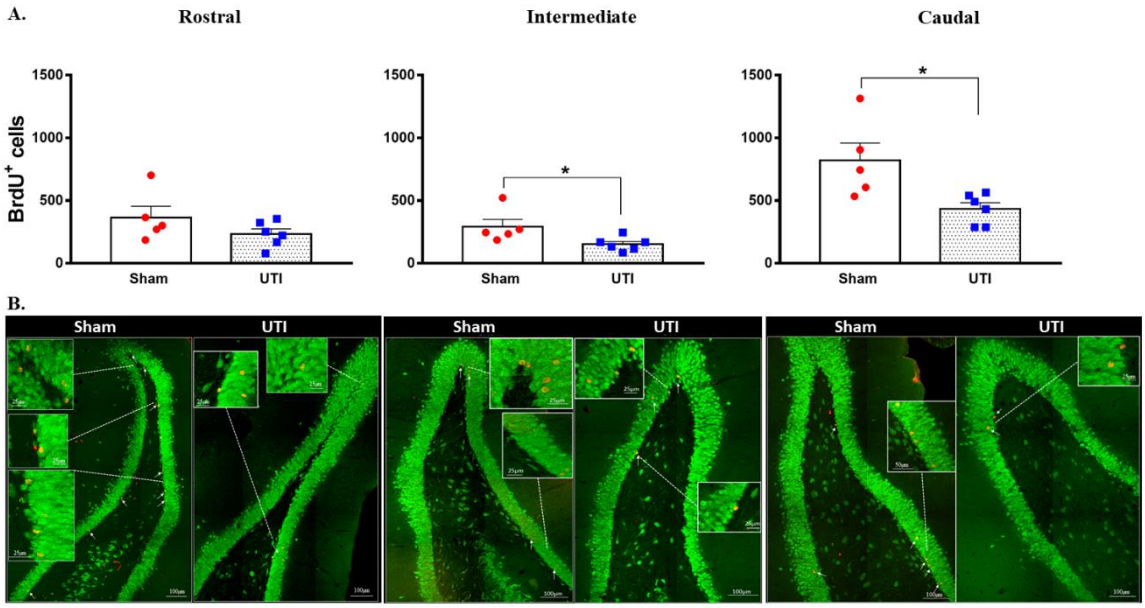

Figure 21. Topographical Distribution of BrdU positive cells in the DG.
(A) Numerical topographical distribution of BrdU positive cells in the rostral, intermediate and caudal regions of the DG of rats sacrificed on day 34 post infection. **(B)** Representative confocal images showing immunofluorescence labeling of NeuN and BrdU (arrows) in the rostral, intermediate and caudal regions of the DG of sham rats and rats with UTI. Determination of significance of differences was generated using two-tailed unpaired student t-test.

3. Treatment with the antibiotic drug Fosfomycin decreased basal levels of proliferation of NSCs

Both sham (1556 ± 120; p<0.0001) and rats with UTI (1092 ± 204; p<0.0001) treated with Fosfomycin (200mg/kg, i.p.) had significantly decreased numbers of BrdU positive cells as compared to untreated sham rats (4544 ± 303) (F (3,24) =33.88; P<0.0001) (Figure 22 A & B). Fosfomycin injection cleared the infection after one day of treatment as seen with CFU assay (Figure 23). No colonies were detected on MacConkey agars that were streaked with urine from all the infected rats treated with Fosfomycin.

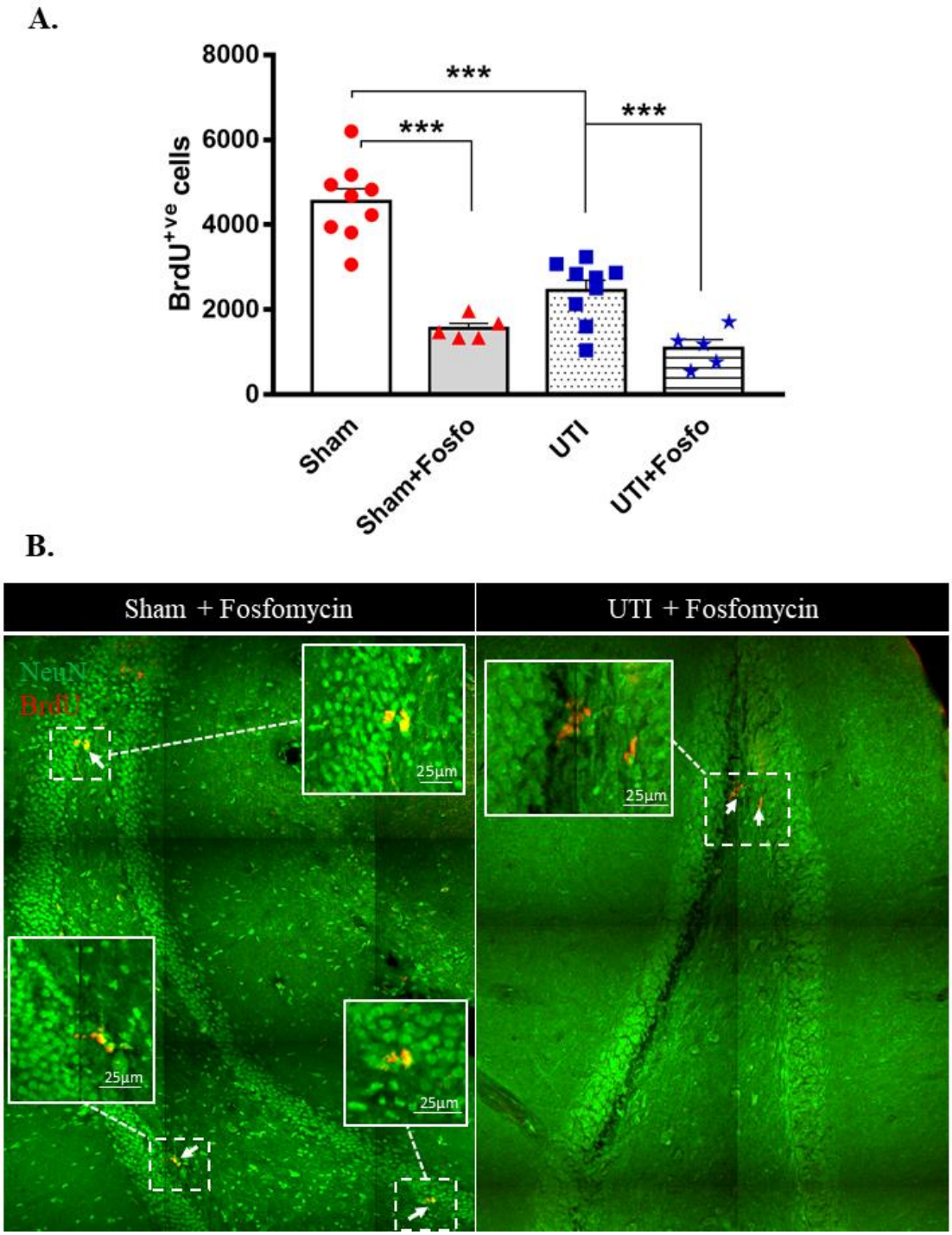

Figure 22. Treatment with Fosfomycin (fosfo) decreases the basal level of BrdU-positive cells and does not revert the decrease in NSCs on day 4 post infection. **(A)** Stereological quantification of BrdU-labeled cells in the DG of Fosfomycin-treated sham and UTI rats sacrificed on day 4 post infection. Determination of statistical significance of differences was generated using one-way Anova followed by Tukey's multiple comparison test. **(B)** Representative confocal images of the DG of Fosfomycin-treated sham (n=5) and UTI rats (n=5) showing BrdU positive cells (arrows). Images were taken as Z stacks and tile scan using 40X-oil objective.

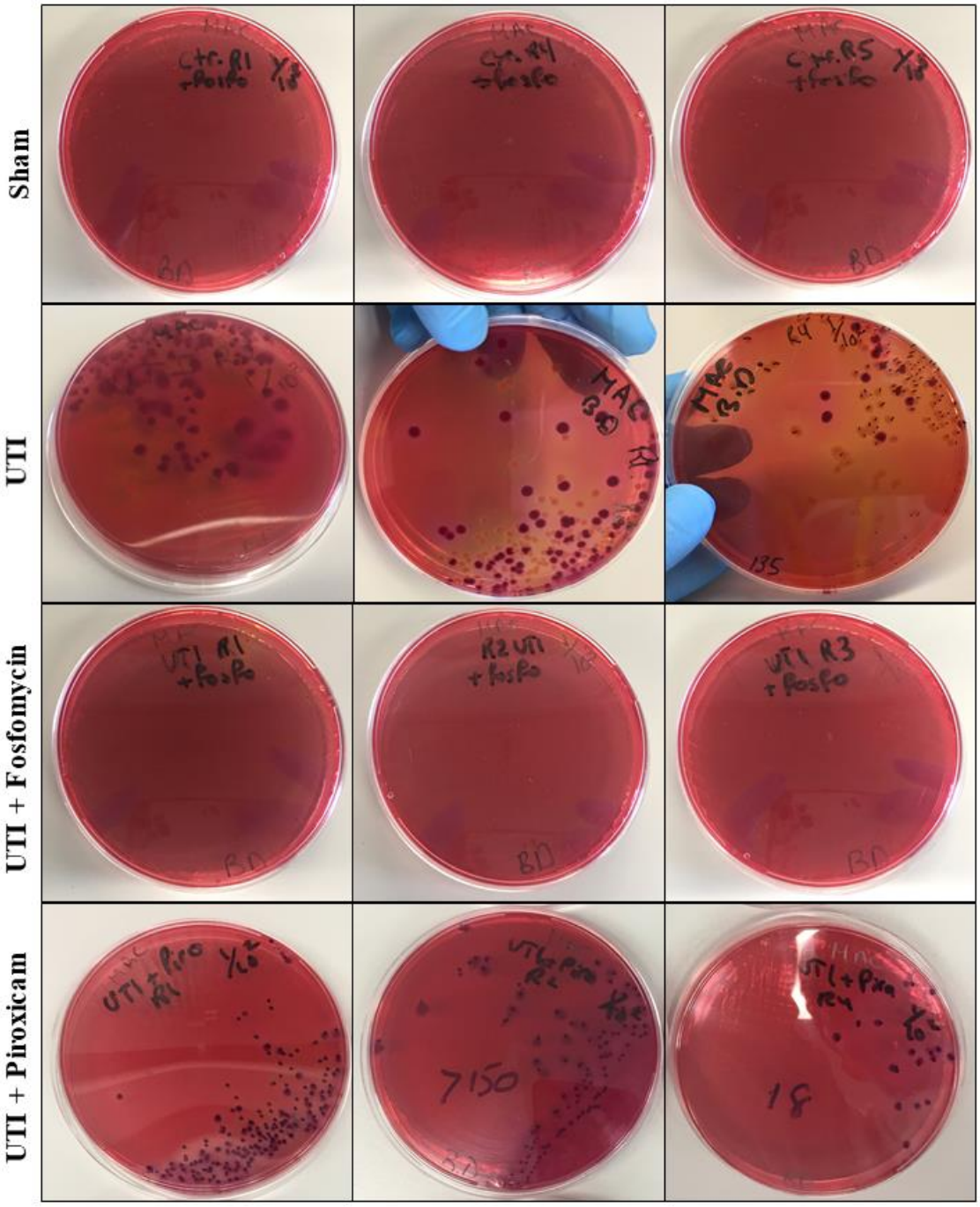

Figure 23. Infection in rats with UTI was cleared infection by Fosfomycin treatment. MacConkey agars showing CFU/mL from urine samples of sham rats, rats with UTI, rats with UTI treated with Fosfomycin and rats with UTI treated with Piroxicam.

4. Treatment with the anti-inflammatory drug Piroxicam did not alter the number of NSCs in sham and rats with UTI

Treatment with the NSAID Piroxicam did not induce significant alteration in the number of BrdU-positive cells in sham and rats with UTI (Figure 24A). Sham rats treated with Piroxicam (3790± 198) still had significantly higher number of BrdU positive cells as compared to untreated rats with UTI (2452 ± 243; p= 0.0154). Rats with UTI that were treated with Piroxicam had comparable number of BrdU positive cells to untreated rats with UTI (2140 ± 243 versus 2452 ± 243, respectively). Moreover, number of BrdU positive cells in rats with UTI treated with Piroxicam (2140 ± 243) was significantly lower than the number of BrdU positive cells in sham treated with Piroxicam (3790± 198; p=0.0078) and in untreated sham rats (4544±303; p<0.0001) ($F(3,24)=17.7$; $P<0.0001$) (Figure 24A and B). Treatment with Piroxicam did not affect the CFU assay from urine indicating the infection persisted (Figure 23).

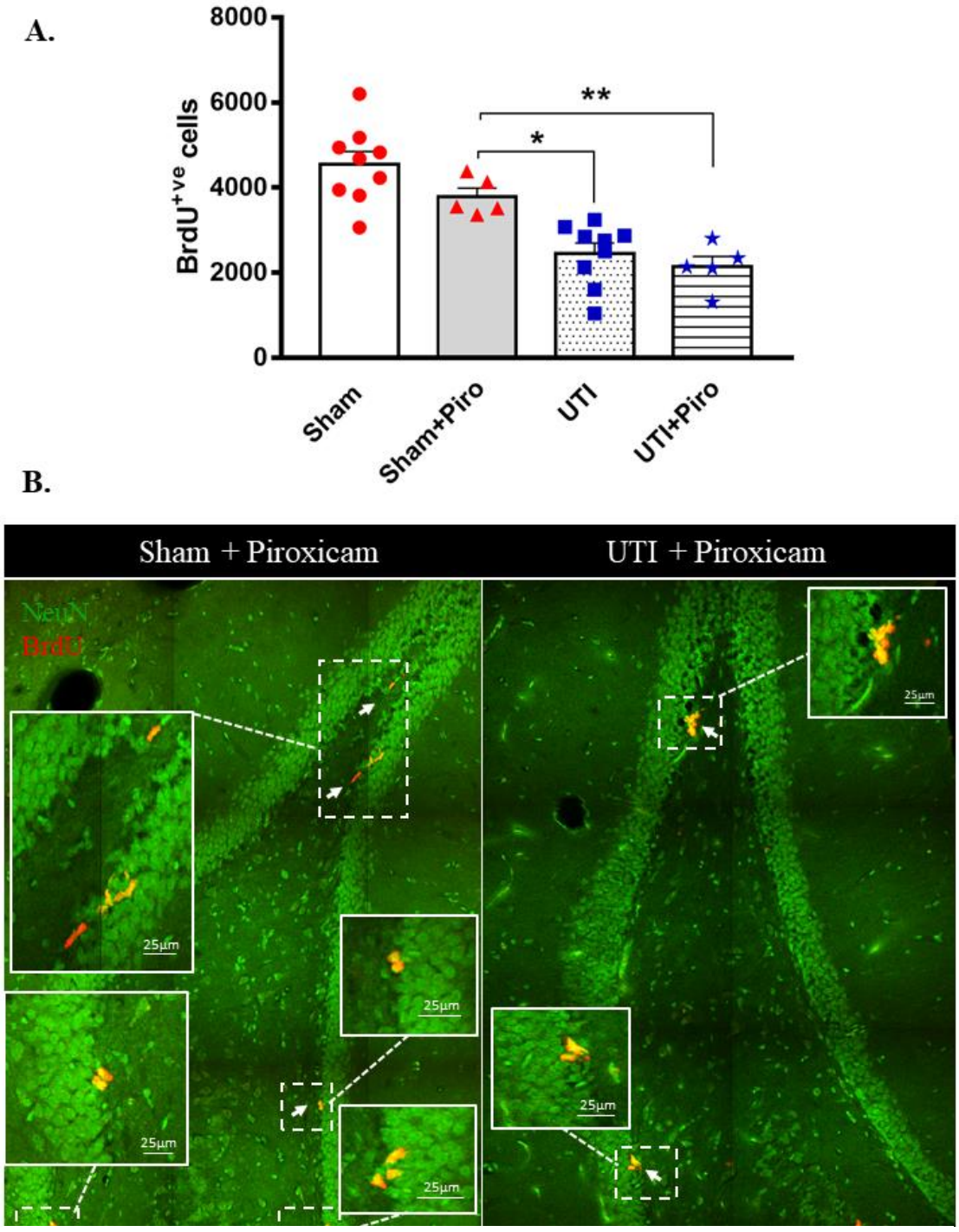

Figure 24. Treatment with Piroxicam (Piro) does not revert the decrease in NSCs on day 4 post infection.

(A) Stereological quantification of BrdU-labeled cells in the DG of Piroxicam-treated sham and UTI rats sacrificed on day 4 post infection. Each bar represents the average ± SEM of BrdU positive cells per group and each dot represents measured number for each rat. Determination of statistical significance was generated using one-way Anova followed by Tukey's multiple comparison test. **(B)** Representative confocal images of the DG of Piroxicam-treated-vehicle and rats with UTI showing BrdU positive cells (arrows). Images were taken as Z stacks and tile scan at 40X-oil objective.

5. UTI elevated the mRNA expression of Il-1β and decreased that of Bdnf, Ngf and Fgf2

There was a significant increase in the levels of *Il-1β* mRNA detected at 6 h post infection in the hippocampi of rats with UTI (1.97±0.28, p=0.025) as compared to mRNA levels in the hippocampi of sham rats (1.02±0.12). This increase was no longer sustained at 24 h post infection (1.36±0.44) (Figure 25 A). There were no significant changes in the mRNA levels of *Il-6* in hippocampi of rats with UTI at 6h (1.13±0.2) and 24 h (1.67±0.55) as compared to sham (1.05±0.18) (Figure 25 B).

On the other hand, mRNA levels of *Bdnf, Ngf,* and *Fgf* were significantly lower in the hippocampi of rats with UTI at 6 h post infection (0.36± 0.17, p=0.034; 0.45±0.17, p=0.049; and 0.6± 0.07, p=0.007, respectively) as compared to expression level in sham hippocampi (1.03±0.13, 1.04±0.15, and 1.22±0.11, respectively). Basal levels were recovered at 24 h post infection (0.78±0.15, 1.02±0.13, and 0.95±0.12, respectively) ($F(2,11)=4.469$, $P=0.038$ for *Bdnf*; $F(2,10)=5.066$, $P=0.0302$ for *Ngf*; $F(2,10)=8.253$, $P=0.0076$ for *Fgf2*) (Figure 25 C, D &E).

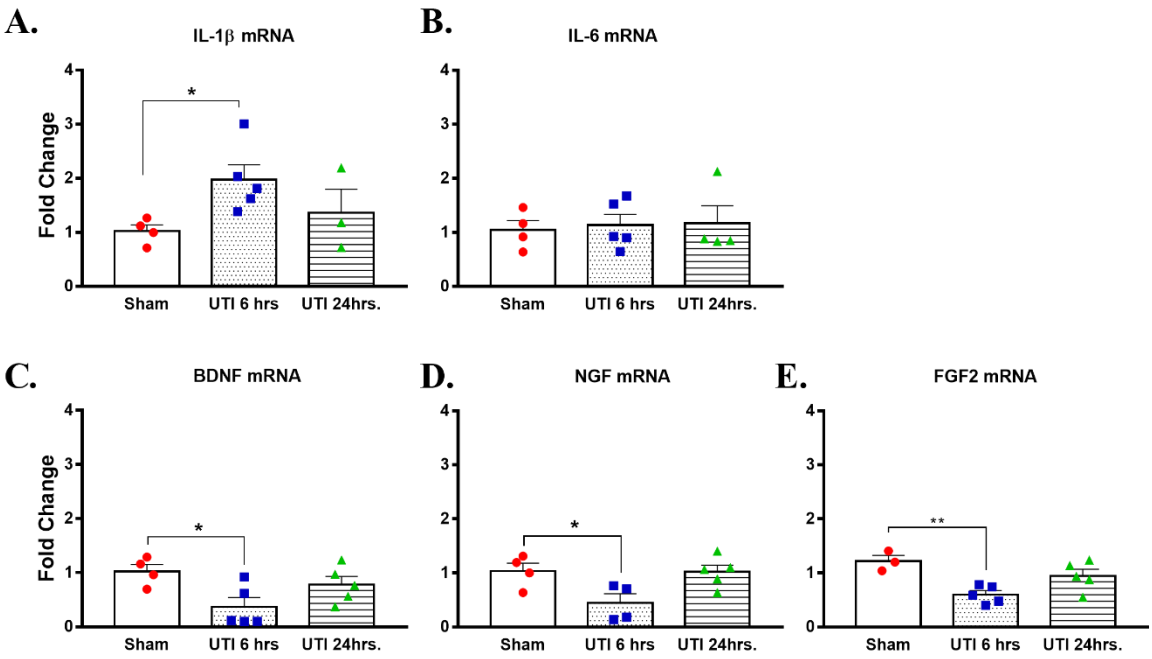

Figure 25. UTI elevated mRNA expression of Il-1β and decreased that of Bdnf, Ngf and Fgf2. (A&B) Increased expression of *Il-1β* mRNA with no changes in *Il-6* in hippocampi of rats with UTI versus sham. **(C, D, &E)** Decreased expression of *Bdnf*, *Ngf* and *Fgf2* mRNA in hippocampi of rats with UTI versus sham hippocampi detected at 6 hours post infection. One-way Anova followed by Tukey's multiple comparison test, p=0.034, p=0.049 and p=0.007, respectively.

6. UTI did not induce significant changes in microglial and astrocytic cells

We quantified the signal intensity of IBA-1 staining for microglial cells and GFAP staining for astrocytic cells in order to assess whether there are differences in the density of these cells in the DG of sham versus that of rats with UTI on day 4 post infection. An increase in signal intensity would imply an increase in proliferation of these cells which would refer to recruitment or activation. The signal intensity of microglial cells stained with IBA-1 in the DG was comparable between rats with UTI (23.78 A.U. ± 1.36) and sham rats (23.6 A.U. ± 1.33) (Figure 26 A&B). Similarly, the signal intensity for GFAP staining astrocytic cells in the DG was also comparable between rats with UTI (23.32 A.U. ± 1.08) and sham rats (24.7 A.U. ± 1.45) (Figure 27 A&B).

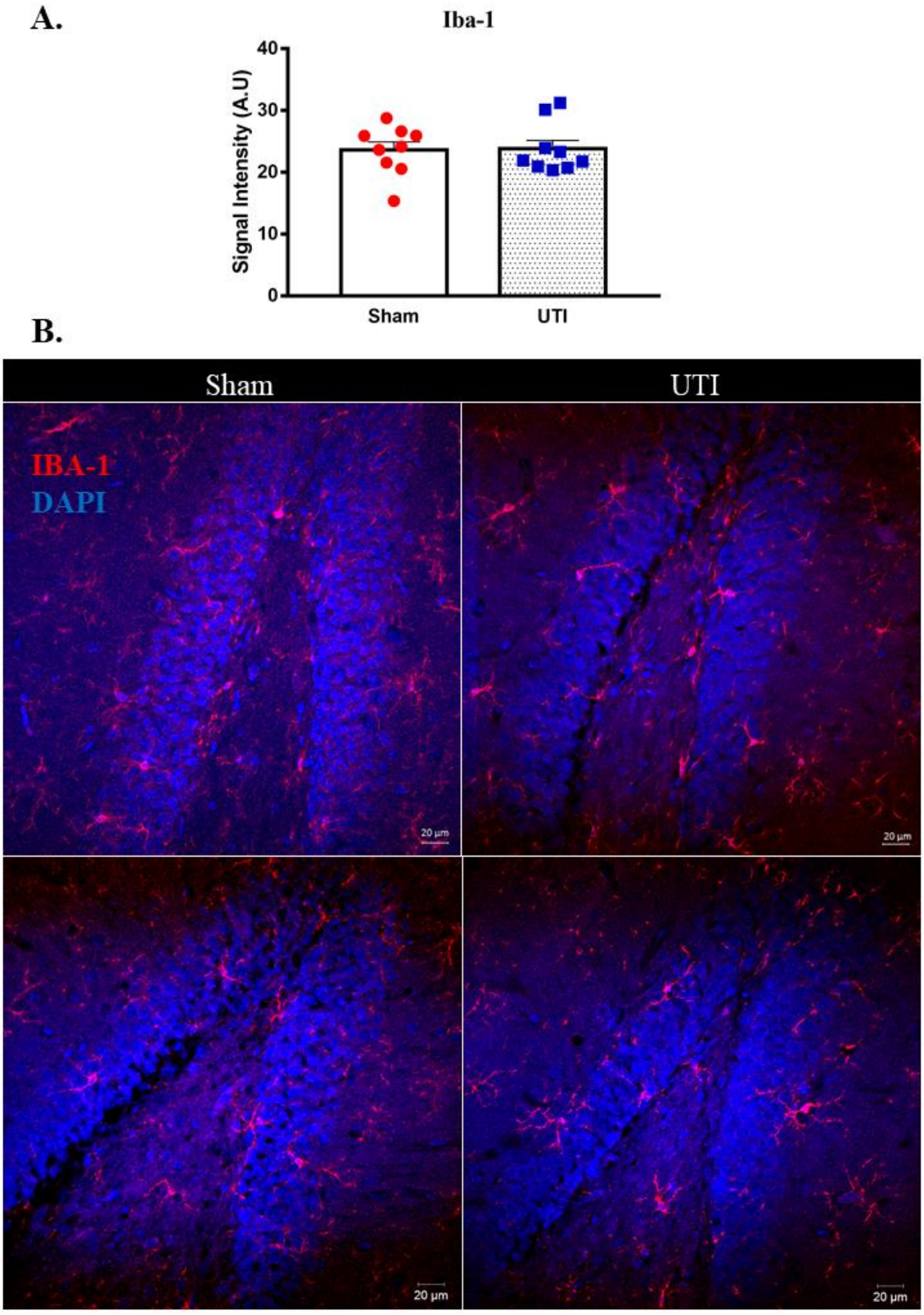

Figure 26. **UTI does not induce significant alteration in microglial cells. (A)** signal intensity quantification for IBA-1 positive cells in the DG of sham and rats with UTI. Signal intensity was quantified using Zeiss LSM 710 laser scanning confocal microscope. A.U., Arbitrary unit. **(B)** Representative confocal images taken at 40X oil.

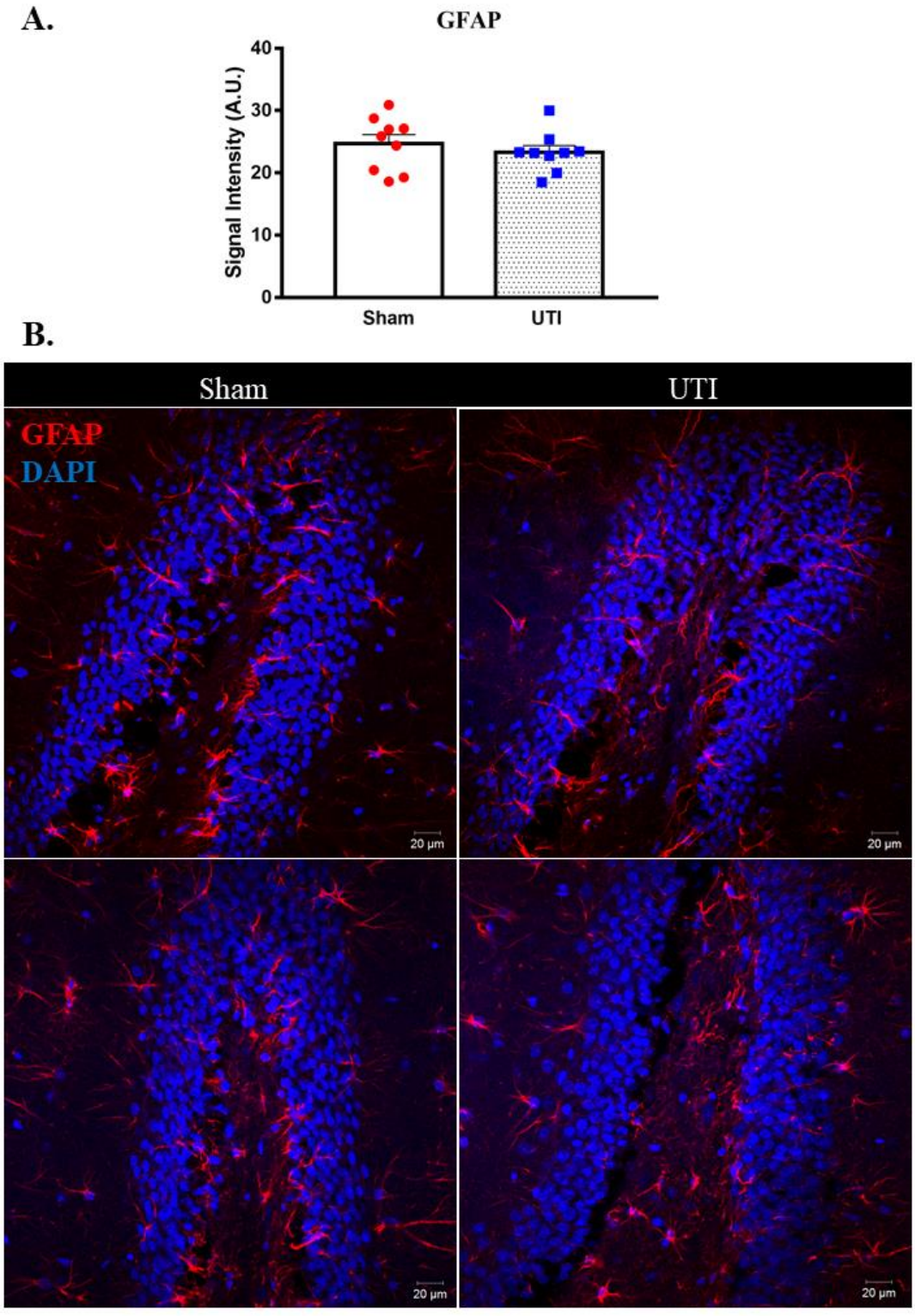

Figure 27. UTI does not induce significant alteration in astrocytic cells.
(A) Signal intensity quantification for GFAP positive cells in the DG of sham and rats with UTI. Signal intensity was quantified using Zeiss LSM 710. A.U., Arbitrary unit. **(B)** Representative confocal images for GFAP positive cells in the DG of sham and rats with UTI taken at 40X oil.

C. Increased Heat Sensitivity in Urinary Tract Infected Rats

Rats with UTI displayed a shorter latency of withdrawal reflex in reaction to nociceptive heat (8.02 ± 0.39) as compared to vehicle-treated sham rats (22.6s ± 1.3; $p<0.001$). This decrease in latency denotes a heat hyperalgesia induced at the level of the abdominal skin of rats with UTI (Figure 28).

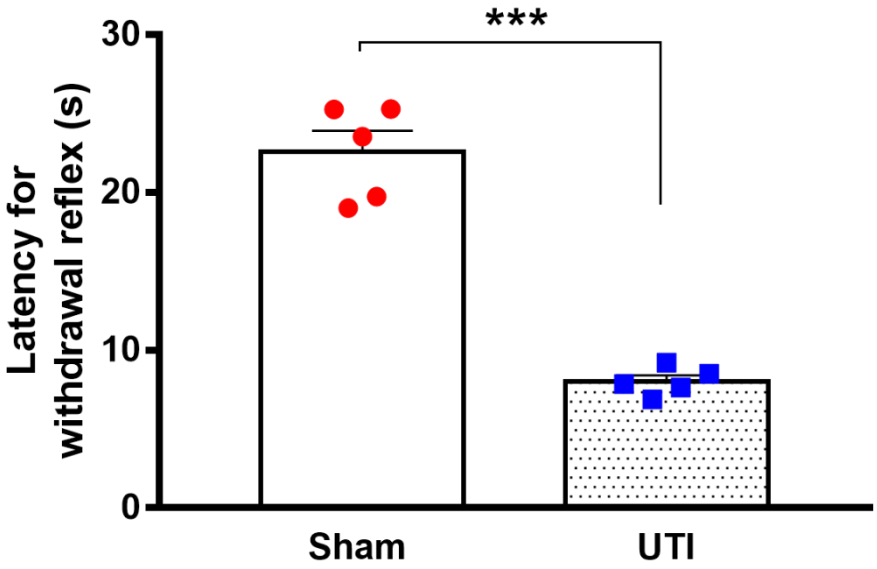

Figure 28. Rats with UTI displayed abdominal thermal hyperalgesia. The latency for abdominal wall contraction was lower in rats with UTI (n=5) versus sham rats (n=5). Determination of significance of differences was generated using student t-test.

D. Effect of Urinary Tract Infection on Rats' Behavior

To assess the impact of UTI on exploratory and motor behavior, several motor and memory-related behavioral tests were performed. Exploration and anxiety were assessed through the Open Field test and Elevated Plus Maze respectively. Working and reference recognition memory were assessed in the Y-maze and novel object recognition tests.

1. Rats with UTI displayed normal spontaneous locomotor activity and exploratory behavior

Rats with UTI scored comparable measurements for the following indicators as compared to sham rats, respectively: total time spent in the central zone (27.63s ± 4.66 in UTI versus 23.72s± 5.14 in sham), latency to enter central zone (18.8s ± 4.36 versus 15.22s ±4.56), average speed (0.036m/s± 0.003 versus 0.038m/s± 0.0034), total distance traveled (10.67m ±1 versus 11.59m ± 1.01), total mobility time (150.39s± 15.36 versus 184.07s± 12.98) and total immobility time (149.69s±15.36 versus 115.93±12.98s) (Figure 29).

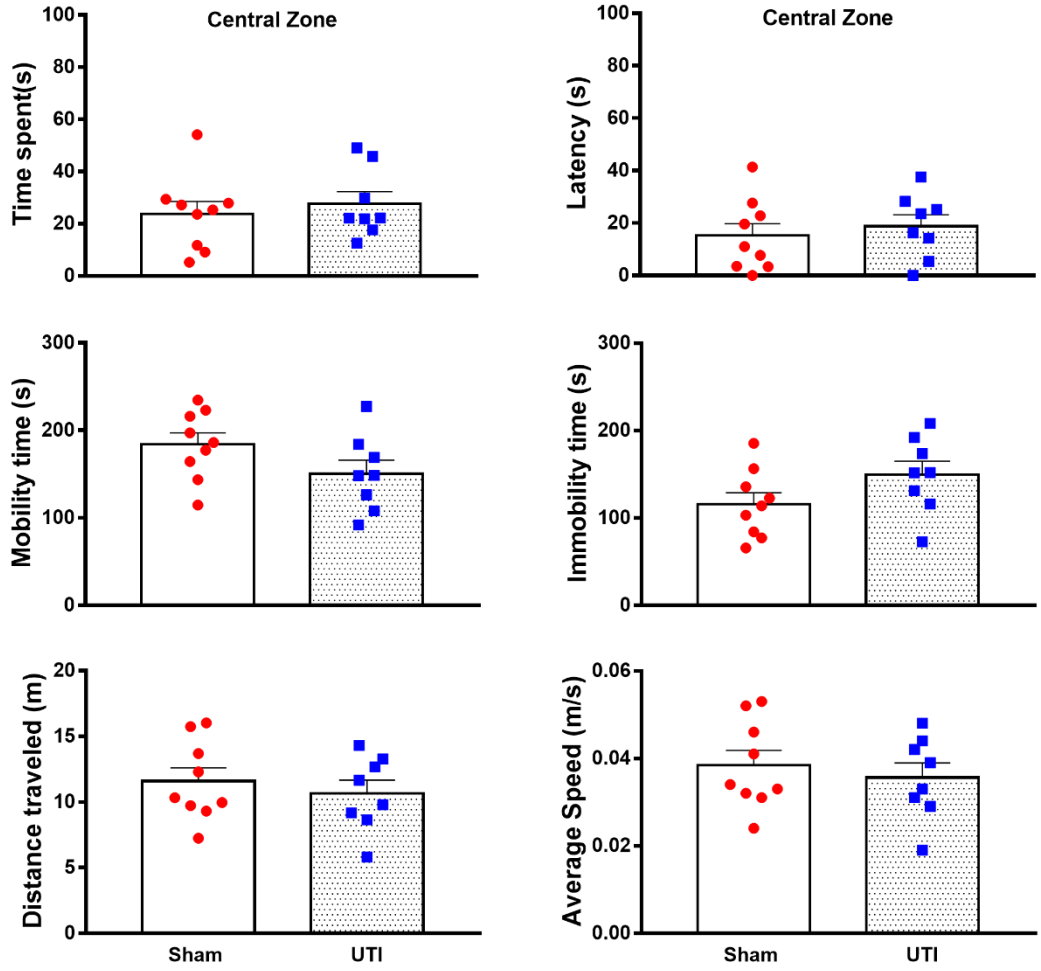

Figure 29. Rats with UTI display normal exploration and locomotor activity in open field test. Parameters tested in open field test: Central zone exploration, mobility, immobility, total distance traveled, average speed and latency to enter central zone were all comparable between rats with UTI and sham rats.

2. *Rats with UTI had similar tendency to explore a novel object as sham rats*

Rats with UTI spent comparable time exploring the novel object (59.44s ± 6.58) as the sham rats (75.73s ± 10.46) in the novel object recognition test. Similarly, the total time spent exploring the familiar object by rats with UTI (63.81s ± 7.81) was comparable to sham rats (54.63s ± 10.12) (Figure 30).

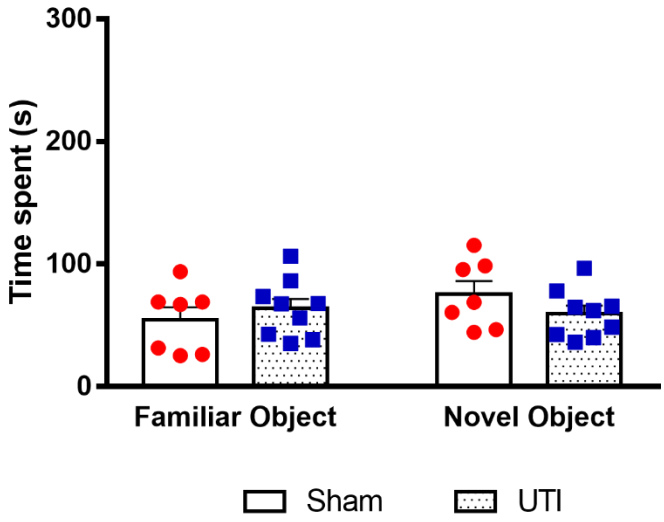

Figure 30. Rats with UTI spent comparable time to sham rats in exploring a novel object in the novel object recognition test. Total time spent by sham and rats with UTI in zones of Familiar and Novel objects in the Novel object recognition task.

3. *Rats with UTI spent less time exploring the novel arm in the Y-maze test*

On day 2 post infection, the total time spent by rats with UTI in the novel arm (51s ± 5, p=0.0245) was significantly less than that scored by sham rats (84s ± 11.65). On the other hand, the total time spent by rats with UTI in the start arm (139s ± 20) and familiar arm (97 ± 23) was comparable to that spent by sham rats (116s ±18 and 86s ± 11, respectively) (Figure 31A). Moreover, rats with UTI spent significant longer time to first enter the novel arm (42s ± 10, p=0.0358) as compared to sham rats (13s ± 6) (Figure 31B).

On day 33 post infection, the total time spent by rats with UTI in the start arm (134s ± 31), familiar arm (87s ± 21) and novel arm (68s ± 19) were all comparable to that spent by sham rats (114s ± 17, 82s ± 11, and 85s ± 16, respectively) (Figure 32).

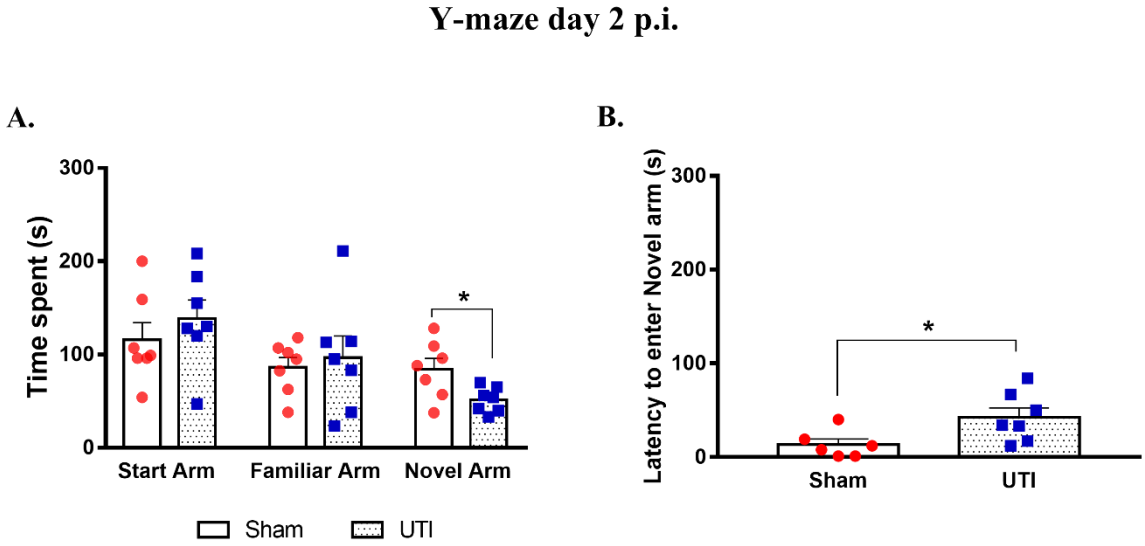

Figure 31. Rats with UTI spend less time exploring the novel arm in the Y-maze on day 2 post infection. (A) The total time spent in the novel arm by rats with UTI is significantly lower than that spent by sham rats in Y-maze on day 2 post infection (p.i.). **(B)** Rats with UTI had longer latency to enter the novel arm as compared to sham. Statistical significance for latency and time spent in novel arm between sham and rats with UTI was assessed using unpaired student t-test; * $p<0.05$.

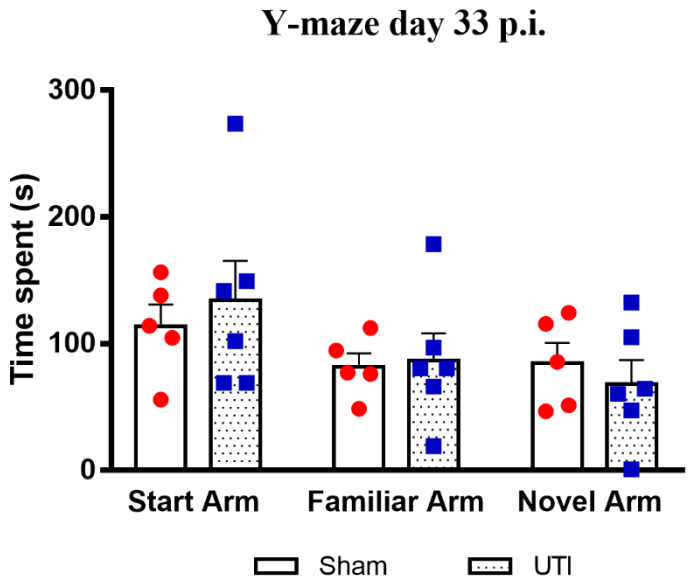

Figure 32. Rats with UTI had improved performance in the Y-maze test after 33 days from infection. Rats with UTI spent comparable time in all arms of the Y-maze as compared to sham on day 33 post infection.

4. Rats with UTI had less tendency to spontaneously alternate in the T-maze test

Rats with UTI recorded balanced alternation rate of 47% ± 10 (p=0.034) which is significantly lower than that recorded by sham rats 86% ± 5 (Figure 33).

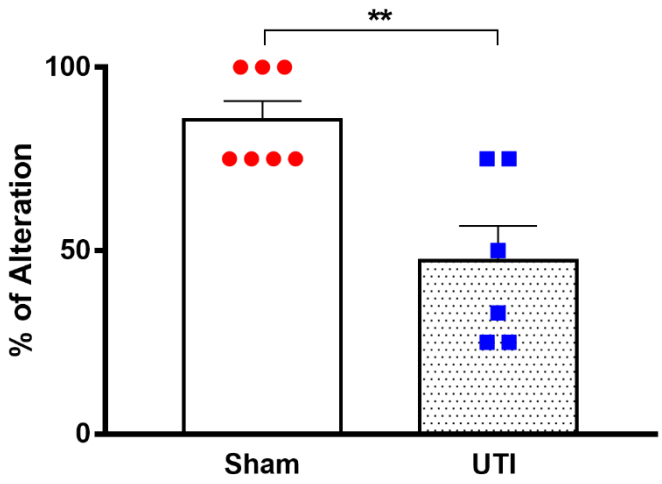

Figure 33. Rats with UTI scored a significantly lower percentage of correct alteration in the T-maze test as compared to sham rats. Percentage of alteration in the T-maze test in sham versus rats with UTI. Unpaired student t-test, *p<0.05.

5. Rats with UTI treated with Fosfomycin had decreased locomotor activity

Rats with UTI (9.47±1.04) traveled a similar distance to sham rats (10.2 m±0.95). However, rats with UTI that were treated with Fosfomycin (3.703 m± 0.87; p=0.0034) had significantly lower distance traveled as compared to sham rats (10.2 m±0.95). As for rats with UTI that were treated with Piroxicam, these recorded a lower (7.24 m± 0.48), though not significant, distance traveled as compared to sham rats (10.2 m±0.95) (Figure 34).

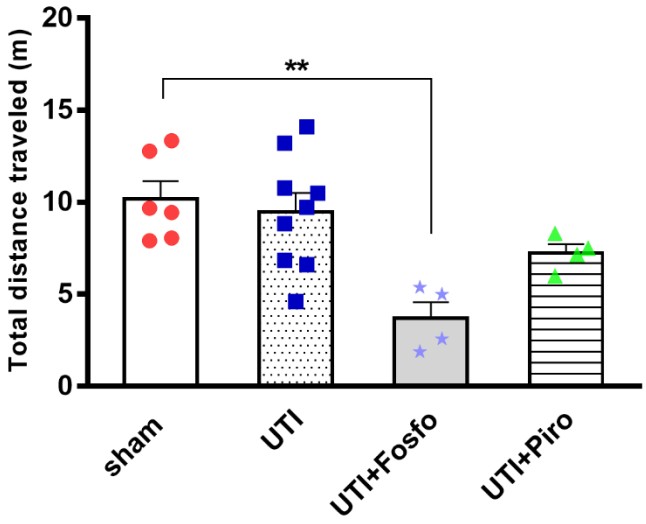

Figure 34. Locomotor activity was affected in rats treated with Fosfomycin but not in rats with UTI. Statistical significance was determined using One-way ANOVA test (P=0.0029), followed by Tukey's multiple comparisons test.

6. Treatment with Piroxicam did not improve exploratory behavior in the Y-maze test

On day 2 post infection, the total time spent by rats with UTI in the novel arm treated with Piroxicam (44.66± 11.33) was less than that scored by sham rats (84s ± 11.65), though not statistically significant (Figure 35).

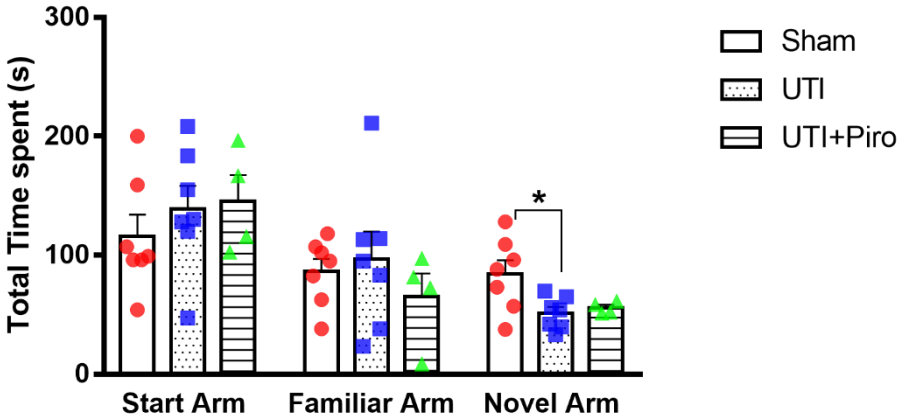

Figure 35. Treatment with Piroxicam did not restore the exploratory behavior of rats with UTI. Rats with UTI treated with Piroxicam spent similar time in the novel arm as the untreated rats with UTI.

CHAPTER IV

DISCUSSION

In this study, we hypothesized that urinary tract infections could influence hippocampal neurogenesis based on clinical observations of the non-conventional symptoms of UTIs in elderly patients such as confusion and delirium. Our aim was to investigate whether UTIs influence hippocampal neurogenesis, as the latter has critical roles in memory encoding and cognition. For this purpose, we designed and characterized a new model of UTI in male rats, by intravesical infiltration of a resistant strain of E coli bacteria. Here, we present evidence that UTIs cause a decrease in the proliferation of neural stem cells in the dentate gyrus of the hippocampus at an early time point post-infection and a decrease in neurogenesis a month post-infection. This was accompanied by decreased expression of neurotrophic factors in the hippocampi of infected rats and decreased exploration of the novel arm in the Y-maze test. Neither treatment with antibiotics nor NSAIDs was able to restore the basal levels of NSCs proliferation in rats with UTI.

Urinary tract infections are one of the most common infectious diseases occurring worldwide, where an estimated 150 million cases are yearly reported (Flores-Mireles et al., 2015; Foxman, 2003). The majority of UTI cases are caused by a group of *E. coli* strains referred to as the uropathogenic *E. coli* (UPEC). In this study, we used a UPEC multi-drug resistant (MDR) clinical isolate from a patient with UTI in order to mimic clinical settings where there is an alarming increased occurrence of MDR pathogens (World Health Organization, 2021).

As mentioned in the Methods section, our model was based on a mouse UTI model described previously with modifications related to the pathogen used and its dosage for rats (Scharff et al., 2017). The validity of this slightly modified model was confirmed by the following findings: CFU from urine ($>10^5$), PCR screening for the presence of NDM gene variant bla_{NDM-5}, and increased levels of proinflammatory cytokines in the urethra and urinary bladder. Moreover, rats with UTI showed persistent signs of infection and inflammation for a long period of time, and they exhibited referred visceral inflammatory pain depicted by increased heat sensitivity of the skin of the lower abdomen covering the urinary tract. In the present study, we opted to establish UTI in male rodents as we have shown that female rats have less prominent infections compared to males. This is consistent with similar findings in the literature where infections in female mice were cleared faster than in males which were attributed to higher Interleukin 17 production in females (Zychlinsky Scharff et al., 2019).

Our results show that UTIs cause a decrease in the proliferation of BrdU-positive neural stem cells in the dentate gyrus of the hippocampus. This effect is further manifested a month post-infection as a decrease in the number of BrdU/NeuN double-positive cells and thus decreased adult hippocampal neurogenesis. This finding is in line with accumulated evidence showing a causal link between decreased neurogenesis and neuroinflammation (Chamaa et al., 2018; Chesnokova et al., 2016; Darwish et al., 2019; Valero et al., 2014) or peripheral inflammation in general (Melo-Salas et al., 2018; Zonis et al., 2015). In addition to decreased proliferation of NSCs, we also observed a decrease in the mRNA expression of *Bdnf*, *Ngf*, and *Fgf2* in the hippocampus of rats with UTI. BDNF and NGF are both neurotrophic factors that are well characterized for their critical role in learning and memory (Eu et al., 2021; Miranda et al., 2019). FGF2 is a potent

multi-functional growth factor implicated in the regulation of neural stem/progenitor cells in the neurogenic niche of the dentate gyrus (Woodbury & Ikezu, 2014). In addition, increased FGF2 levels are also associated with spatial memory learning (Stevens et al., 2012). On the other hand, an increase in the mRNA expression of *Il-1β*, but not *Il-6*, was detected in hippocampi of rats with UTI. Such robust increase in IL-1β mRNA transcription has been similarly documented before, in the hypothalamus, as early as 2 hours after intraperitoneal LPS challenge (Skelly et al., 2013). IL-1β is synthesized and released by both neurons and glial cells of the brain. Traditionally considered pro-inflammatory, this cytokine is not only involved in inflammatory pathways but also has a well-established role in the brain as a neuromodulator (Hewett et al., 2012; Park et al., 2018; Schneider et al., 1998). IL-1β is required for neuronal differentiation and normal regulation of hippocampal plasticity and memory (Labrousse et al., 2009; Takemiya et al., 2017). However, excessive production of IL-1β in the brain, especially the hippocampus, has been previously reported to impair hippocampal-dependent learning and synaptic plasticity (Gibertini et al., 1995; Hein et al., 2010; Moore et al., 2009). It is even reported that increased IL-1β induces a cognitive decline in Alzheimer's Disease patients (Holmes et al., 2003). In conclusion, both the decreased expression of *Bdnf*, *Ngf*, and *Fgf* and the increased expression of *Il-1β* can be considered key players in the observed reduction of neurogenesis.

Traditionally, glial cells are considered as a first potential contributor to the observed alteration in the expression of neurotrophic factors and cytokines. Looking into the signal intensity of microglia and astrocytes, we did not detect significant changes between rats with UTI and sham rats. We also did not notice overt morphological changes in these cells between sham and rats with UTI. However, this does not completely reflect

the activity of these cells. There could be changes at the level of transcriptional profile that would not be detected by signal intensity.

A rapid and vigorous immune response is essential for guarding against bacterial pathogens such as UPEC, which sometimes tend to bypass the natural defenses of the urinary tract. Epithelial cells of the bladder and urethra have characteristic secretions of IL-1β, IL-8, and IL-6 mainly. These interleukins are essential for recruiting neutrophils and clearing bacterial infections (Engelsöy et al., 2019). We detected peaks in the production of the pro-inflammatory cytokines IL-1β and IL-8 at 6 and 24 hours and even day 4 post-infection in the urethra and bladder tissues. Such findings are consistent with the literature and are expected after an infection. Moreover, we detected a peak in IL-1β production in plasma 24 hours post-infection. As for TNFα, we were not able to detect the peak of TNFα production at the earliest time point that we chose which was 6 hours. However, TNFα is characterized by an early robust and short-term lasting peak post infections. Thus, it is possible that TNFα might have peaked at earlier time points post-infection and was restored to sham levels by 6 hours.

All in all, the above-mentioned findings leave an open question on the mediator between UTIs and decreased neurogenesis. Once an infection is initiated in the periphery, both the released cytokines and LPS, convey this information to the brain using both humoral and neuronal routes of communication (McCusker & Kelley, 2013). It might be speculated, also, that this link could be due to neural mechanisms related to the activation of the hypothalamic-pituitary-axis (HPA) due to inflammation and nociception (Haddad et al., 2002). The release of pro-inflammatory cytokines in the periphery activates the HPA and results in elevated levels of glucocorticoids which are well-known established suppressors of hippocampal neurogenesis (Cameron & Glover, 2015; Kohman & Rhodes,

2013). Prostaglandins can also mediate some of the inflammation-induced effects on hippocampal neurogenesis (Monje et al., 2003).

Nonetheless, we found that infected rats had higher sensitivity to thermal pain in their abdominal area overlying the urinary tract. This is in line with previous reports on UTIs and pain response (Rosen & Klumpp, 2014; Rudick et al., 2010). The bacterial pathogens, in particular the LPS on their surface, can produce pain by directly activating sensory neurons that modulate inflammation (Chiu et al., 2013). On the other hand, the hippocampus is emerging as a key brain region involved in the processing of pain as multiple evidence shows altered hippocampal plasticity and cytokine expression in animal models of chronic pain (Apkarian et al., 2016; Sarkis et al., 2011). Interestingly, research has shown that pain is associated with suppressed neurogenesis and altered short-term synaptic plasticity in a way similar to stress does (Grilli, 2017). Thus, it is possible that both the activation of the HPA with its consequences and the activation of pain pathways could have mediated the effect seen at the level of NSCs proliferation.

It is already established that the central and peripheral nervous systems interact with immune mediators (Waldburger & Firestein, 2010). A large body of evidence has shown how the brain and the immune system are intertwined and could affect each other in a bidirectional manner (Haddad et al., 2002; Kwon, 2022; Salvador et al., 2021). The HPA axis, as previously mentioned, is one major player in such interaction as it provides essential feedback on inflammation. The sensory and autonomic peripheral nervous systems are also involved in this communication. The notion of peripheral inflammation affecting inflammatory mediators in the central nervous system has been previously reported (Bay-Richter et al., 2011; Chamaa et al., 2016a; Quan et al., 1999; Riazi et al., 2008; Turrin et al., 2001). Recently there has been emerging emphasis on the role of

cytokines in influencing host behavior even in the absence of infection (Salvador et al., 2021; Willis et al., 2022). Moreover, cytokine receptors have been shown to be expressed on sensory neurons. Cytokines, such as IL-1β and TNFα, can activate dorsal root ganglia and lead to generating action potentials. Thus, this reflects one way of how changes in the peripheral cytokine milieu can be sensed by the peripheral nervous system, which further relays information to the brain (Salvador et al., 2021). Furthermore, IL-8 is a chemoattractant cytokine that activates the sympathetic nervous system and has the potential to form a bidirectional communication between the nervous system and the immune system (Cunha et al., 1991; Gonzalez et al., 2014; Watkins & Maier, 1999). Thus, IL-8 could be considered as another important mediator between peripheral inflammation and the brain (Sullivan et al., 2020). The interplay between the brain and the peripheral immune system has been the focus of recent studies including one that shows that the brain's insular cortex stores immune-related information and carries neuronal representations of inflammatory information (Koren et al., 2021). Interestingly, the activation of neurons in the insular cortex was able to start an intestinal immune response during inflammation of the colon (Koren et al., 2021). Moreover, activation of neurons in the ventral tegmental area was shown to boost the immune response and slow lung tumor growth (Ben-Shaanan et al., 2018). Based on the findings of this study, we suggest that such interaction between peripheral inflammation and the nervous system could be mediating the effect on neural stem cells in the hippocampus and consequently leading to cognitive changes.

It is worth noting that the infection did not alter the rats' normal spontaneous locomotor activity and exploration in an open field test. These findings are not in favor of the hypothesis attributing the impaired performance of rats in tests for exploration and

memory due to sickness behavior as a result of the infection. Rats with UTI seem to have impaired spatial reference memory as shown by their performance in the Y-maze test. We have previously reported similar findings relating levels of NSCs proliferation and neurogenesis to performance in a Y-maze test (Chamaa et al., 2021). By contrast, performance in the novel object preference/recognition test was comparable between rats with UTI and sham suggesting there is no impairment in the rats' recognition memory. This could be expected as the novel object recognition task evaluates the rats' non-spatial learning of an object's identity, which also involves several brain regions (Denninger et al., 2018). In addition, rats with UTI had a considerably lower percentage of spontaneous alteration in the T-maze test which evaluates the short-term working memory. This is in line with previous reports showing altered performance in the T-maze following peripheral inflammation (Cho et al., 2018). Thus, the cellular and molecular changes in the brain were paralleled by changes in cognitive behavior in some memory-performance tasks.

To investigate the possible factors playing a role in the interplay between urinary tract infections and the brain, we opted for two different kinds of treatments: treatment with NSAIDs and treatment with antibiotics. Treating the rats with Fosfomycin was able to clear the bacterial infection, on one hand, however, it reduced basal levels of hippocampal NSCs proliferation. It has been previously shown that Fosfomycin penetrates the cerebrospinal fluid (CSF) even in presence of an intact BBB and is even suggested for the treatment of CNS infections (Pfeifer et al., 1985; Tsegka et al., 2020). This could explain why the use of this drug had an effect on hippocampal NSCs proliferation. Nonetheless, it should be noted that such effects of Fosfomycin at the level of the brain and neurogenesis, in particular, have not been previously reported or

investigated in the literature. Thus, in this case, it is difficult to draw a conclusion on whether treatment with antibiotics could have recovered NSCs proliferation since Fosfomycin by itself further decreased their proliferation. Moreover, cognitive-behavioral assessment of the effect of Fosfomycin would have confounding factors since rats with UTI treated with Fosfomycin traveled less distance as compared to sham rats and untreated rats with UTI. Due to this decreased mobility during testing, we cannot assess cognitive exploratory behavior as it would be impossible to assess whether rats might be exploring less due to sickness behavior or the effect on hippocampal NSCs. Thus, it would make it possible that rats would explore less in cognitive-behavioral tests due to side effects of the drug that affect mobility, not just cognitive abilities.

The effect of NSAIDs has been recently investigated in clinical trials for the treatment of UTIs and has yielded variable results from recovery to worsening of symptoms (Carey et al., 2020; Yu Zhang et al., 2020). We used the NSAID, Piroxicam, as we have previously established that it doesn't affect the basal levels of hippocampal neurogenesis (Chamaa et al., 2018). Piroxicam's mechanism of action involves inhibition of cyclooxygenases (COX1 and COX2). This inhibition causes the peripheral downregulation of prostaglandin synthesis, which usually works to promote inflammation, pain, and fever. Piroxicam was given as prophylactic treatment before injecting the bacteria in an attempt to dampen the immune response after infection. Since the immune response and cytokine release are suspected mediators for the effect at the level of the brain and neurogenesis, we opted to dampen the immune response by giving Piroxicam as a prophylactic treatment before bacterial injection. Treatment with Piroxicam did not reverse the reduction in proliferation of NSCs in rats with UTI nor did it affect the basal levels of proliferation in sham rats. This was also reflected in

exploratory behavior as well. Treatment with Piroxicam did not recover, though not significantly, the exploratory behavior of rats with UTI as seen in the Y-maze test. It is possible that the prophylactic administration of anti-inflammatory drugs might exacerbate the infection with time. This hypothesis receives support from the effect of NSAIDs on the infection. It should be noted that giving Piroxicam as a prophylactic treatment worsened the infection as seen by CFU and NDM-5 expression from urine samples and urinary incontinence. Thus, in the absence of a strong immune response to infection, the bacteria might have thrived. Further experiments that use LPS intra-urethral injection would be beneficial in addressing whether inflammation in the urinary tract, minus bacterial infection, would have the same effect on the proliferation of NSCs.

Interestingly, we found that the strain we used, *E. coli* 1176, affected the proliferation of epithelial bladder cells in vitro while the other virulent reference strain used did not have any effect. Thus, there may be underlying causes of why one bacterial strain may be more toxic to the epithelium than the other. Thus, this might lead to a discrepancy in their effects on the urinary tract and in their ability to elicit the same immune response. In future studies, it would be interesting to compare whether uropathogenic strains that are not resistant to antibiotics would elicit the same effect on the proliferation of NSCs in *invivo* settings. It would also be interesting to investigate this with non-uropathogenic strains that are common among UTIs.

This study sheds light on the effects of peripheral inflammation on brain and cognition, in particular on hippocampal neurogenesis. All in all, the mediator between urinary tract infection and the brain remains ambiguous. There could be two routes of communication between the urinary tract and the brain that could have led to the results of this study: Humoral and Neural. The humoral route consists of the released cytokines

and depends on their ability to cross the BBB. The neuronal route of communication comprises the sensory afferents innervating the bladder and projecting to different levels of the CNS on one hand and the autonomic efferents on the other hand. The released bacterial LPS and/ or bacterial toxins lead to activation of the sensory afferents and could lead to central sensitization. Thus, it could probably be a combination of the activation of both of these routes of communication that lead to neurotrophic factors and neurogenesis being affected at the level of the brain, in addition to cognitive changes. Here, we show that neurotrophic factors in the hippocampus could be possible mediators of the effect on NSCs, however, what leads to the decrease in their expression remains ambiguous. As for microglia and astrocytes, we cannot overrule their involvement as a mediator based only on their morphology and optical intensity. Thus, their role and participation remain questionable. A summary of all changes and possible mediators between the urinary tract and the hippocampus is mentioned in Figure 36.

In conclusion, we present novel findings on the effect of urinary tract infections on hippocampal neurogenesis and cognitive behavior. This could, at least, partly explain symptoms of confusion and delirium seen during episodes of UTI, especially in elderly patients. Peripheral inflammation is a suspected factor for changes seen at the level of the brain. It has been previously reported that peripheral inflammation, in particular Toll-like receptor-induced inflammation, induces remote global gene expression changes in the brain (Thomson et al., 2014). However, the exact mechanism for the effect of peripheral inflammation on the brain requires further investigation. The findings of this study might lead to considering neurogenesis as a potential target in the dynamic crosstalk between the infected urinary tract and the brain.

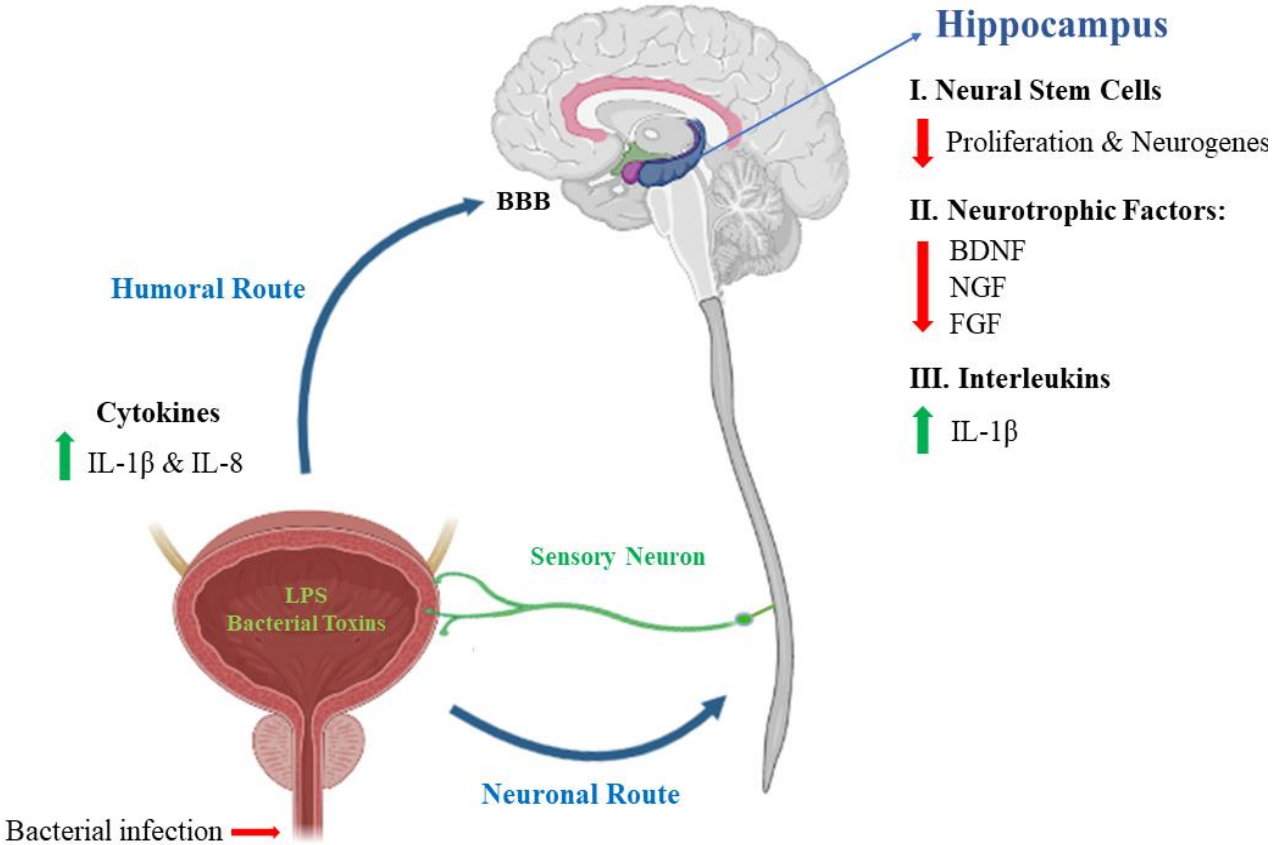

Figure 36. Schematic representation of possible mediators between the urinary tract infection and hippocampal neurogenesis. Created on BioRender. BBB: Blood Brain Barrier; BDNF: Brain derived neurotrophic factor; FGF: Fibroblast growth factor; IL: Interleukin; LPS: Lipopolysacharide; NGF: Nerve growth factor.

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