AMERICAN UNIVERSITY OF BEIRUT

MODULATION OF HIPPOCAMPAL NEUROGENESIS BY INFLAMMATORYAND ANTI-INFLAMMATORY AGENTS IN ADULT RATS

by
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ABSTRACT OF THE THESIS

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Title: Modulation of hippocampal neurogenesis by inflammatory and anti-inflammatory agents in adult rats

Background: Constant formation of functional neurons from neural stem and progenitor cells in postnatal stages has been observed in two main neurogenic brain regions: the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) in the lateral ventricles. Adult neurogenesis, however, is prone to alterations by different physiological, pathological and pharmacological stimuli. One flaunting factor is neural inflammation which has been implicated in neurodegenerative disorders. Our aim is to demonstrate that inflammation, induced by intracerebroventricular (*icv*) injection of Endotoxin (ET) altersthe neurogenic niche of adult rats by decreasing neurogenesis.

Methods: Adult Sprague-Dawley male rats (250-300g) received stereotaxic *icv* injections of ET (6 µg in 1.5 µl) or sterile saline as described previously (Safieh-Garabedian et al., Neuropharmacology, 2011,60:496-504). Rats then received 3 injections (66mg/Kg/injection; *ip*) of 5'-bromo-2'-deoxyuridine (BrdU) and were perfused at different time intervals (Days 1, 2, 3, 6, and 9). The non-steroidal anti-inflammatory drug (NSAID) Piroxicam was given as daily injections to rats perfused at day 3. Behavioral pain tests were performed and BrdU positive cells were counted in the DG of the hippocampus.

Results: ET injection resulted in a significant decrease ($p < 0.0002$) of adult neurogenesis in rats at day 2 (439.75±81.16) and day 3 (479.87±94.69) when compared to sham (966.16±49.60). This was followed by a rebound at day 6 (1124.80±161.18) then recovered the basal levels at day 9 (997±87.23). These alterations were accompanied by thermal hyperalgesia that peaked at day 3. Daily treatment with Piroxicam (12.5 mg/kg; *ip*) was able to alleviate the ET effects on neurogenesis and reduce hyperalgesia.

Conclusion: The current study sheds light on the negative impact of discrete neuro-inflammation on neurogenesis in the hippocampal formation. Thus, understanding the adverse effects of silent chronic neuro-inflammation on neurogenesis opens a new window for the treatment and management of disorders having inflammation as its hallmark.

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ABBREVIATIONS

SGZ: SubGranular Zone

DG: Dentate Gyrus

SVZ: SubVentricular Zone

ET: Endotoxin

icv: Intracerebroventricular

ip: Intraperitoneally

GCL: Granule Cell Layer

NPC: Neural Progenitor Cells

TAP: Transit Amplifying Progenitor

Shh: Sonic Hedghog

PSC: Progenitor Stem Cells

CHAPTER I

INTRODUCTION

A. Neurogenesis

Neurogenesis is the process of generation of functional neurons from neural stem cells and progenitor cells(Ming and Song, 2011). It has a discrete time window in most of the brain regions during prenatal life yet it resumes in postnatal stages and is spatially restricted, under regular conditions, to two main neurogenic brain regions (Christie and Cameron, 2006, Ming and Song, 2011).These include the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus, where new dentate granule cells are generated, and the subventricular zone (SVZ) of the lateral ventricles where new neurons are generated and then migrate through the rostral migratory stream to the olfactory bulb to become interneurons (McDonald and Wojtowicz, 2005, Ming and Song, 2011).

1. History of Neurogenesis

The first published evidence of adult neurogenesis in rodents was reported by Altman and Das (Altman and Das, 1965). It was not until a few decades later that a paradigm shift was finally attained since neurons were surmised to be refractory to replication(Lois and Kelsch, 2014) owing to their complexity, with their highly branched dendrites and polysynaptic axonal combinations, and to the paradoxical concept of functional integration into the pre-existing circuit(Gage, 2002).

The lack of tentative phenotypic markers of neurons that could be detected with auto-radiographic birth dating and the undermined notion of adult stem cells in the

brain hindered the acceptance of the concept of neurogenesis (Riddle and Lichtenwalner, 2007). Interest in neurogenesis increased in particular during the late 1980s when Nottebohm studied the song system in birds (Nottebohm, 1989). Subsequently, studies to validate neurogenesis in adults spread essentially during the 1990s (Gould et al., 1997, Eriksson et al., 1998, Gould et al., 1999, Kempermann and Gage, 1999, Kornack and Rakic, 1999).

Thus, to study neurogenesis, new methodologies of detection were implemented (Sierra et al., 2011). Primarily, the use ofthymidine-H^3, a radioactive nucleotide that incorporates into the cells during the S phase of the cell cycle, allows the detection of the amount of proliferation (Messier and Leblond, 1960). This was then substituted by its analog, bromodeoxyuridine (BrdU); a halogenated analog of thymidine, which could be detected by a specific antibody to label new-born cells (Miller and Nowakowski, 1988).

Postnatal neurogenesis persists for a limited period, within the white matter tracts and external granular layer of the cerebellum (Zhang and Goldman, 1996).Cells with properties similar to the progenitor cells have also been identified in the striatum (Palmer et al., 1995), cortex (Palmer et al., 1999), spinal cord (Weiss et al., 1996, Shihabuddin et al., 1997), optic nerve (Tropepe et al., 2000) and the substantia nigra (Lie et al., 2002).These cells have been shown to exhibit at least limited self-renewal and produce differentiated cells of the three neural lineages: astroglia, oligodendroglia, and neurons *in vitro* (Emsley et al., 2005).The detection of neural stem cells in the adult brain that gave rise to new neurons, astrocytes, and oligodendrocytes, just as in the postnatal brain, was the essence of the idea of neurogenesis in the adult brain (Gage, 2002).

Neurogenesis may be induced in non-neurogenic regions of the adult brain in response to injury and neuronal death such as that seen in cerebral strokes whereby neurons are generated in the neocortex of rats (Yang et al., 2007). Compelling evidence has shown that the neurogenic capability of some regions in the brain is directed by local environmental cues or by growth-stimulating signals such as the Sonic hedgehog signaling protein (Shh) that is produced by local astrocytes and act directly on progenitor cells (Jiao and Chen, 2008).

2. Role of Neurogenesis

It is estimated that around 700 new neurons are added in each hippocampus of an adult human per day (Spalding et al., 2013, Borsini et al., 2015). This suggests a functional role of hippocampal neurogenesis in adults (Ryan and Nolan, 2015). The role of neurogenesis in the adult hippocampal formation is not yet fully established, however it is postulated that it is involved in memory formation (episodic and spatial memory), learning (Deng et al., 2010) and mood regulation (Ekdahl et al., 2003). It is well known that neurogenesis in rodents is involved in olfaction, learning and memory (Sierra et al., 2011).It is argued that neurogenesis in the adult hippocampus permits a higher capacity for neural plasticity which ultimately promotes the encoding and storage processes including both current and future learning experience (Snyder et al., 2001, Taupin, 2006). The 'learning and memory' theory is in part based on the fact that newly formed neurons, which are not yet fully integrated into the circuitry, have the potential to mold their connections to experiences more adequately as compared to older neurons that were already incorporated into the circuitry (Kohman and Rhodes, 2013). Noticeably, old neurons are able to amend their synapses in response to experiences, however younger neurons may exhibit a higher degree of excitability (Ge et al., 2007) and

plasticity (Kohman and Rhodes, 2013). Electrophysiological evidence is accordant with the idea that newly formed neurons are characterized by elevated plasticity and excitability (Kohman and Rhodes, 2013).

The process of neurogenesis in the adult dentate gyrus of the hippocampus can be divided into three discrete phases whereby newborn cells are subject to multiple regulatory factors influencing the proliferation, maturation, fate and survival of the cells (Kuhn et al., 1996, Bruel-Jungerman et al., 2007). First, neural precursor cells that are found at the border between the hilus and the granule cell layer (GCL) undergo cell division. BrdU or H^3 thymidine are used as markers to detect this proliferation (Sidman et al., 1959, Gratzner, 1982).Second, these precursor cells initiate their migration into the GCL and extend neuronal processes (Kuhn et al., 1996). Third, these cells integrate into the GCL and start expressing neuronal markers (Esposito et al., 2005). It is worth mentioning that neurogenesis in the adult hippocampus varies widely across species in the rate of proliferation, survival, and neuronal maturation (Drew et al., 2013).

3. Topographical Distribution Of Adult Neurogenesis

a. Hippocampal Formation

The hippocampus is a region of the brain that constitutes part of the limbic system located in the brain's medial temporal lobe. It is primarily associated with spatial navigation and episodic (long-term memory) memory (Burgess et al., 2002) and learning (Jarrard, 1993). The SGZ in the DG of the hippocampus constitutes a crucial neurogenic niche (Piatti et al., 2013). Neural progenitor cells(NPCs) in the SGZ are located at the border between the GCL and the hilus of the DG (Riddle and Lichtenwalner, 2007). Not all NPCs survive and reach differentiation (Chesnokova et

al., 2016). Neurons that do persist migrate a short distance into the GCL of the DG and incorporates into the existing neuronal circuitry eventually receiving input from the entorhinal cortex (Chesnokova et al., 2016). Cell bodies, on the other hand, stay at the GCL, dendrites ramify through the molecular cell layer and axons project to the hilus and CA3 regions, as early as 4 to 10 days after their final mitosis (Riddle and Lichtenwalner, 2007).The GCL can change in volume in a range of 5-20% due to changes in the rate of neurogenesis dictated by environmental factors (Kohman and Rhodes, 2013).

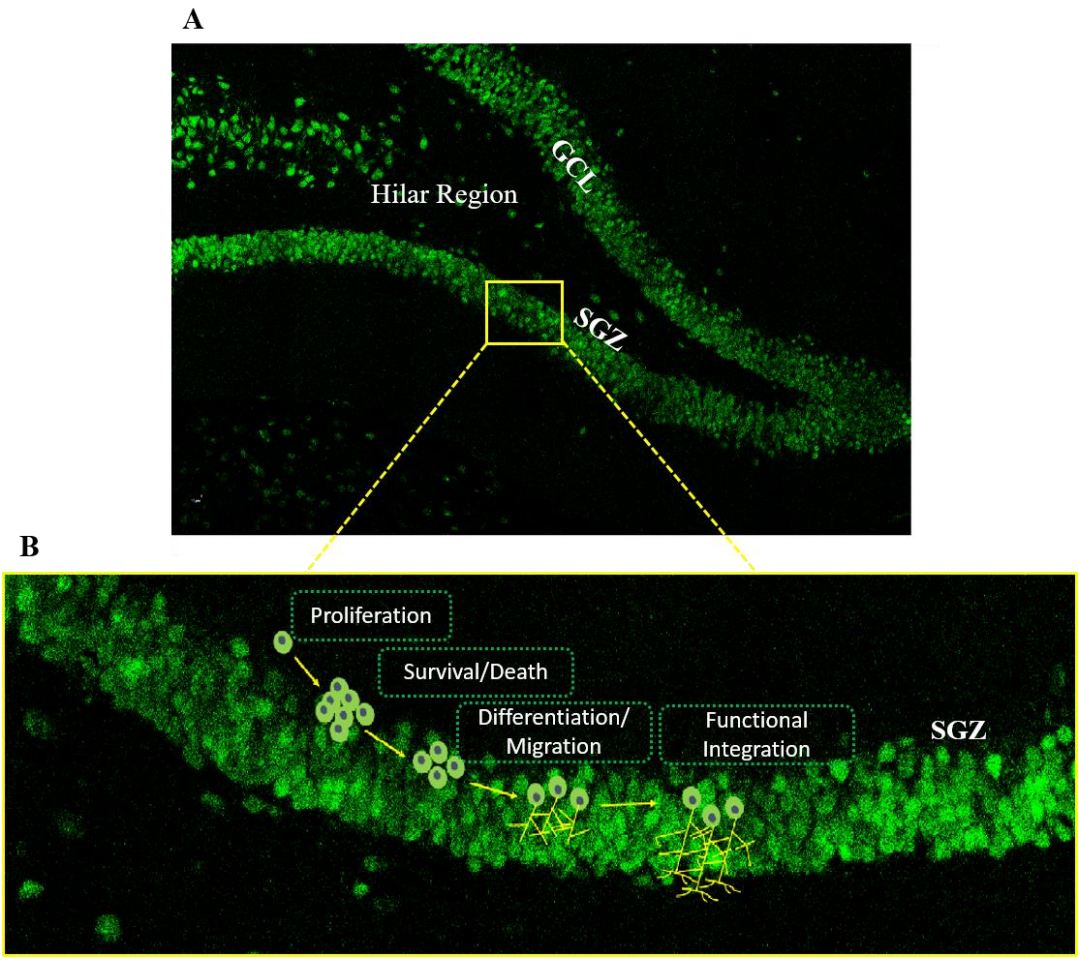

Figure 1: Photomicrograph representation of adult neurogenesis in the dentate gyrus. Stem and precursor cells located in the SGZ (A) give rise to new neurons that integrate into the granule cell layer (GCL) of the DG. They proliferate, differentiate and then integrate into the granule cell layer (GCL) of the DG. They proliferate, differentiate and then integrate into the circuitry to become functional mature neurons (B).

b. Subventricular Zone and Rostral Migratory Stream

Situated throughout the lateral walls of the lateral ventricles, the SVZ represents a significant and chief reservoir of progenitor cells in the adult brain (Alvarez-Buylla and Garcia-Verdugo, 2002). Extensive investigation of this germinal region revealed that it encompasses several cell types including the slowly dividing stem cells, a more rapidly dividing population of transit amplifying progenitor (TAP) cells, neuroblasts, glial cells and a monolayer of ependymal cells that separates it from the ventricle(Riddle and Lichtenwalner, 2007). Note that the stem/progenitor cell populations are not the same as those of the SGZ which lacks true stem cells, containing only more restricted progenitor cells (Riddle and Lichtenwalner, 2007).

c. Olfactory Bulb

New neurons born in the SVZ of adult mammals migrate anteriorly into the olfactory bulb (OB), where they mature into local interneurons. These cells migrate as elongated aggregates of cells called chains without the aid of radial glia or axonal guides (Lois et al., 1996, Doetsch et al., 1997). They migrate in a well-defined pathway composed of neuroblasts ensheathed by slowly proliferating cells expressing Glial Fibrillary Acidic Protein (GFAP) i.e. of astrocytic origin to insure an appropriate microenvironment for migration and cell division (Lois et al., 1996, Riddle and Lichtenwalner, 2007). Several studies reported the birth of new olfactory receptor neurons within the adult olfactory epithelium (Calof et al., 1996, Schwob, 2002).These neurons migrate superficially as they develop their characteristic apical dendrite and project an axon to the glomerular layer of the OB (Riddle and Lichtenwalner, 2007). They may survive a few weeks or months due to the continued damage of the exposed

olfactory mucosa. Hence, neurogenesis in the adult olfactory epithelium is a continuous turnover process as compared to the more selective replacement of new neurons within the granule cell and interneuron populations of the DG and OB respectively (Riddle and Lichtenwalner, 2007).

4. Potential Modulators

Adult neurogenesis is prone to alterations by different physiological, pathological and pharmacological factors (Ma et al., 2009, Walton, 2012) that can profoundly regulate neurogenesis and influence the learning and memory processes organized by the hippocampus (Bruel-Jungerman et al., 2007). These factors can perturb the standard niche in the brain leading to a cascade of molecular and cellular events that interfere with the release of various inflammatory mediators, namely cytokines (Borsini et al., 2015). Ample data support the notion that neurogenesis is essentially modulated by inflammatory cytokines that play a pivotal role in the central nervous system: on one hand, they can provide immune protection that aids the system in getting rid of dead and impaired neurons and, on the other hand, they may act as pro-inflammatory agents (Chesnokova et al., 2016) and have adverse effects on the NSCs (Borsini et al., 2015).Cognitive impairments and mood disorders such as anxiety or depression are attributed to disrupted neurogenesis (Chesnokova et al., 2016).

Extracellular modulators such as Notch and Shh regulate activation and destiny of adult neural precursors (Ming and Song, 2011). Glutamate (Glu), the predominant excitatory neurotransmitter in mature neurons, can also act as an extracellular modulator of neurogenesis (Schlett, 2006).

Intracellular cell cycle regulators, transcription factors and epigenetic regulators are also major directors of adult neurogenesis (Zhao et al., 2008). Stress and the steroid hormone corticosterone are known to impede neuronal proliferation at pre and post-natal stages (Gould et al., 1997, Tanapat et al., 1998).

B. Neuroinflammation and Neurogenesis

Neuroinflammation, an immune response that takes place in the central nervous system (Wohleb and Godbout, 2013), has been associated with several neurodegenerative diseases (Fuster-Matanzo et al., 2013). Furthermore, neuroinflammation has been shown to directly affect adult neurogenesis (Fuster-Matanzo et al., 2013).

Neuroinflammatory responses comprise beneficial outcomes for the CNS by imparting neuroprotection, the preservation of neurogenesis as a mechanism of brain repair, the recruitment of neural precursors for repair, remyelination, and axonal regeneration (Shaftel et al., 2008, Wee Yong, 2010). On the other hand, neuroinflammation can be detrimental for the CNS resulting in neuronal damage (Lee et al., 2008). Benefits and detriments balance rely chiefly on the extent of the immune response (Fuster-Matanzo et al., 2013). Accordingly, we discern between two types of responses in which inflammation has traditionally been categorized these are acute and chronic inflammation.

Acute inflammation encompasses the prompt response to an adverse agent and is essentially a defensive response that facilitates repair of the damaged site. It is usually short-lived and unlikely to be detrimental to long-term neuronal survival (Streit et al., 2004a, Fuster-Matanzo et al., 2013). On the other hand, chronic neural inflammation is

caused by exceptional stimuli designated by the persistence of the inflammation over a longer period (Streit et al., 2004b, Rao et al., 2012).

Both acute and chronic neural inflammation have been associated with neurodegenerative disorders. Stroke and injury often result from acute neural inflammation (Toth et al., 2016)whereas diseases such as multiple sclerosis or Alzheimer disease are associated with the chronic form of inflammation (Wyss-Coray and Mucke, 2002, Cappellano et al., 2013).

Some long-term peripheral illnesses, metabolic disorders and normal aging ultimately cause a state of chronic peripheral inflammation (Donath and Shoelson, 2011, Lampa et al., 2012, Elahy et al., 2015).Such conditions, concomitant with behavioral disturbances including deficits in memory and learning, cognitive decline and psychological deterioration, are induced by disrupted adult hippocampal neurogenesis (Aimone et al., 2009, Jessberger et al., 2009). These diseases engender chronic inflammation either directly by producing inflammation or by eliciting pathological metabolic states, which in turn contribute to inflammatory processes (Chesnokova et al., 2016). Systemic inflammation affects the central nervous system(Lucas et al., 2006).

C. Aim of the study

The hippocampus is highly prone to inflammatory insults, a fact that can be attributed to the presence of a high density of receptors for inflammatory mediators (Green and Nolan, 2014). Interleukin-1β, a key mediator of inflammation and stress in the CNS, had its receptors expressed in pyramidal neurons of a normal and unlesioned hippocampus (Friedman, 2001).

Lipopolysaccharide (LPS) is one agent that has been reported to substantially reduce the regenerative capacity in adult brain and diminish the amount of immature neurons in the hippocampus, when injected intraperitoneally(Valero et al., 2014). It results in acute neural inflammation characterized by microglial activation and up-regulation of the pro-inflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor–α (TNF-α) primarily in the dentate gyrus (Monje et al., 2003b) by binding to the CD14/TLR4/MD2 receptor complex(Chow et al., 1999, Cohen, 2002).

Attempts to reverse inflammation may be a potential approach to induce neurogenesis. Albeit several studies have focused on both pro-and anti-inflammatory cytokines regarding their effects on neurogenesis, there is still a need for further investigating their contribution in attuning cell differentiation and survival (Das and Basu, 2008).Depending on the type of insult that hits the CNS, neurogenesis is either potentiated or hindered (Das and Basu, 2008).

The objective of this study is to investigate the effect of acute localized intracerebral inflammation on the proliferation of progenitor cells in the hippocampal formation. For this purpose, we studied the effects of intracerebroventricular injection of small doses of lipopolysaccharide (1.5 µL per injection of 4 µg ET dissolved in 1 µL of sterile saline) on neurogenesis in the SGZ of the hippocampus of adult rats. As previously mentioned, acute or chronic neural inflammation has been implicated in neurodegenerative disorders. Subsequently, attempts to reverse the effects of the induced inflammation were made by treating with an anti-inflammatory drug.

Part of the results of this study will be reported as an abstract (poster form) in the Annual meeting of Society for Neuroscience (SfN) to be held in San diego, USA, November, 2016 (Bitar et al., 2016).

CHAPTER II

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 250-300g were used in the experiments. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the American University of Beirut and followed ethical guidelines for experimental pain on conscious animals (Zimmermann, 1983). Animals 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. Surgical procedures were conducted under deep anesthesia by intraperitoneal (*ip*) injection of ketamine (Ketalar®; 50 mg/kg) and xyla (Xylazine®; 12 mg/Kg). Postoperative surveillance for the behavior and body weight of the rats was performed during the light phase of the cycle.

A. Stereotactic Surgery

The head of the anesthetized rat was rigidly fixed on a stereotaxic frame (DKI). The skin of the scalp was shaved and a small skin incision was warily made to expose the skull bone to allow needle penetration at the targeted stereotaxic coordinates (fig.2). A hole was drilled into the skull and a Hamilton Syringe (Hamilton, Town state country), was used to inject either 1.5 µL of0.9% sterile saline or Endotoxin (ET) [Lipopolysaccharide (LPS), Sigma, from Salmonella typhosa]in the lateral ventricle of the right cerebral hemisphere (1.5 µL per injectionof 4 µg ET dissolved in 1 µL of sterile saline). The following stereotaxic coordinates were used: lateral, 1.4mm; vertical,

3.3mm, and anterior-posterior, -0.9mmwith reference to bregma (Paxinos and Watson atlas, 1998) (Fig.2).

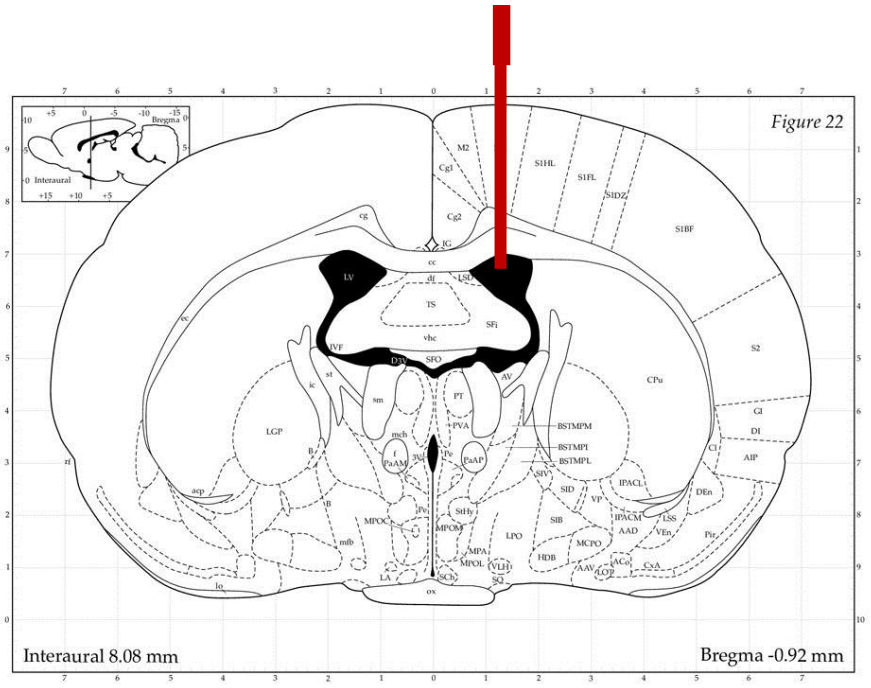

Figure 2: Stereotaxic coordinates of icv injection site in the rat brain. Site of insertion of the Hamilton syringe into the lateral ventricle to deliver the ET/saline. Image adopted and modified from Paxinos and Watson, 1998

B. BrdU Administration

Bromodeoxyuridine (BrdU) a synthetic thymidine analog that gets incorporated into the DNA during the S-phase cycle of mitosis, was used to assess for proliferation/neurogenesis. BrdU powder (Sigma-Aldrich B5002-1G) was weighed and dissolved in 0.9% warm saline. The solution was properly dissolved and filtered using a 0.2µm filter unit. After *icv* administration of either saline or ET, all rats were injected with BrdU three times at a 3 h interval (66mg/kg/ 500 µL/ injection, *ip*) to insure maximal availability.

C. Behavioral Studies

Behavioral tests were carried out during the light phase of the cycle. Animals were transferred to the experimental room at least 1 hour before the test in order to familiarize them with the test environment. Two independent observers of whom only one was informed about the experimental protocol and the animal treatment recorded all scores.

1. Pain Test

Testing of heat reactivity was performed in order to assess pain sensitivity in the rats following surgery and *icv* injection. Rats were placed in transparent plexiglass boxes (16 × 16 × 16 cm) with a floor made of metallic grid (2 × 2 mm). The box was situated on an elevated wire mesh platform to allow vivid observation of the rats and easy access to their paws. A minimum time of 30 minutes was allowed, for familiarization with the environment, before starting each test.

The paw withdrawal latency (PWL) method described by Hargreaves was used (Hargreaves et al., 1988). A radiant heat spot was projected to the plantar surface of the hindpaw from a 160-watt light bulb. PWL was recorded from the instant the beam hit the hindpaw until the animal withdrew its paw. The paw withdrawal duration (PWD) was also measured and assigned an arbitrary value of 0.5 seconds for the brisk reactions seen in normal rats and a maximum of 10 seconds for sustained withdrawal of the paw (Safieh-Garabedian et al., 2003). The decrease of the latency of paw withdrawal or the increase in the duration of this reaction is considered an indication of hyperalgesia. Each rat was subjected to two PW tests per session separated by a minimum interval of 5 minutes.

2. Cylinder Test

Forelimb use during explorative activity was analyzed using the cylinder test. The test was performed at every time point post-surgery during the onset of the light cycle, when rats are considered to be most active. Rats were placed individually in a glass cylinder (10 cm diameter, 20 cm height) in dim light conditions. An investigator who was blinded to the group and to the expected results analyzed all explorative behavior. Only weight-bearing wall contacts made by each forelimb on the cylinder wall were scored. Wall exploration was expressed in terms of the percentage of the total number of times the rat touched the wall with the left or right forelimbs combined (Schaar et al., 2010).

D. Experimental Design

Three different sets of experiments were performed each having its own conditions and groups:

1. Experiment 1: ET icv Injection

This experimental set was designed to evaluate the time course of the effect of ET injection in the lateral ventricles on the proliferation of Progenitor Stem Cells (PSCs) in the SGZ of adult male rats. In this experiment, male rats were given *icv* injections of either the endotoxin or saline. The surgery was followed by BrdU injections on the same day as previously described. Different time points were evaluated; day 1 (n=5), day 2 (n=4, each), day 3 (n=6) and days 6 and 9 (n=4).

2. Experiment 2: ET icv Injection With Piroxicam Treatment

In order to test the ability of Piroxicam to reverse the effect of ET, male rats were injected with ET *icv* similar to the groups in experiment 1. At the same time, these rats received pre-surgical treatment and daily *ip* injections of either Piroxicam or saline. The surgery was also followed by four BrdU injections on the same day.

3. Experiment 3:Sham and control groups

This experimental set is made of 4 control groups aiming to determine the basal level of hippocampal neurogenesis, either in naïve rats (n=4) or in rats receiving sterile saline injections (*icv*, n=3) or (*ip*, n=6) or Piroxicam injection (*ip*, n=5). Rats in the different groups were sacrificed 3 days after BrdU injections (Table 1).

Table 1 : Summary of the experiments performed on the different groups.

Experiment.	**Groups**	**Time (days)**	**Treatment ***
ET *icv*	1 (n=5)	1	
	2 (n=4)	2	
	3 (n=6)	3	
	4 (n=4)	6	
	5 (n=4)	9	
ET *icv* + Piroxicam *ip*	1 (n=4)	3	Daily Piroxicam
Controls	Naïve 1 (5)	3	None
	Sham 1 (n=3)	3	Saline *icv*
	Sham 2 (n=5)	3	Saline *ip*
	Piroxicam *ip* (n=5)	3	Piroxicam
	Saline *icv* (n=5)	3	Piroxicam *ip*

**Note that all the groups received BrdU injections at the time of the treatment*

E. Experimental Procedures

1. Euthanasia And Tissue Preparation For Stereology

Rats were deeply anesthetized and perfused transcardially with 200ml of 0.9% saline until blood was completely drained. Next, 200ml of 4% formalin was perfused. Brains were extracted, fixed in 4% paraformaldehyde for 24hrs at room temperature and then placed in 30% sucrose solution in 0.1M PBS and stored at 4℃ until full impregnation (2-3 days).

Brains were then cut sagittally into left and right hemispheres. The right hemisphere was cut transversally, using a freezing microtome 40 µm thickness of section, from its rostral tip through the brain stem. The SVZ is taken followed by the whole hippocampus with its 3 topographical areas (rostral, intermediate and caudal). The jejunum part of the small intestine of every rat was also taken and cut at the same thickness as a positive control for BrdU.

The fractionator method is a design-based stereological tool used to minimize biased estimates and counting (Schmitz and Hof, 2007). All sections of the SVZ and SGZ were collected in well plates containing sodium azide in PBS (15mM). The hippocampal formation sections were divided according to their topographical distribution in a sense that the rostral, intermediate and caudal was each divided onto 6 well plates. Basically, the 1^{st} section was placed in the first well, the 2^{nd} section in the second well and the 6^{th} in the sixth well. As for the 7^{th} section it was placed in the first well, such that the difference between the 1^{st} and 7^{th} section is 300µm (fig. 3). Six parallel sets from one hemisphere was collected for every rat. Consequently, each well

represents a systematic random depiction of each topographical area of the hippocampus.

Using the method described in the Atlas of the Rat Brain (Gamble, 1980), hippocampal subdivisions were based on the following coordinates: rostral from -2.12 to -3.70, intermediate from -3.70 to -4.90 and caudal from -4.90 to -6.30 in reference to bregma.

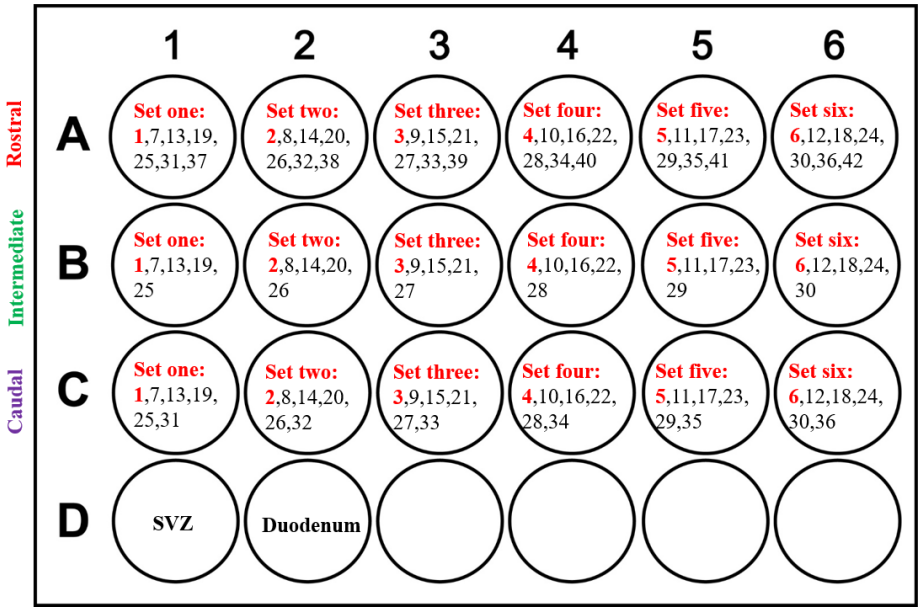

Figure 3 : Fractionator Method. Free floating coronal sections were distributed in a 24-well plate based on the topographical region. The first section was placed in the first well and the following sections in the adjacent wells until the 6th slice is in the 6th well and the cycle repeats. The total number of sections per well were: 7 sections for the rostral region, 5 sections for the intermediate and 6 sections for the caudal.

2. Immunofluorescence And Confocal Microscopy

One well encompassing a whole topographical area was randomly chosen. The free-floating sections were washed 3 times with 0.1 M PBS for 5 minutes each. Next, the tissues were placed in 2N HCl acid for 30 minutes at 37^{o}c in order to denature the

DNA and allow the anti-BrdU to bind to the previously incorporated BrdU and ultimately be detected. Sections were then rinsed once with PBS as a washing step followed by a wash with Sodium Borate (0.1 M) of pH 8.5 for 10 minutes at room temperature in order to neutralize the previously added HCl. The tissues were again washed for 3 times with PBS and transferred to a freshly prepared block that comprises 10% NGS, 10% BSA and 0.1% Triton-X all diluted in PBS for an hour at 4^{o}c to minimize non-specific bindings. The samples then were incubated overnight at 4^{o}c with the primary antibody anti-BrdU (1/100; Bio-Rad) diluted in 10% NGS, 3% BSA, 0.1% Tx and PBS. The next day, the tissues were washed 3 times with 0.1 M PBS (5 min each) and then incubated in the dark with secondary antibody goat anti-rat 568 (1/100; Invitrogen) diluted in 10% NGS, 10% BSA, 0.1% Tx and PBS for 2hrs at RT on a shaker. They were then washed and moved to be incubated with the second primary antibody NeuN (1/500; Millipore) diluted again in 10% NGS, 10% BSA, 0.1% Tx and PBS overnight. Note that the staining procedure was performed sequentially rather than simultaneously since the two antigens are present in the same cellular compartment. On the following day, 3 washes with PBS were performed and then the samples were placed in the secondary antibody Alexa-Fluor 488 goat anti-mouse (1/250; Invitrogen) as previously mentioned. After 2 hours, the tissues were washed and Hoechst stain (1/10000; Invitrogen) was added for 10min. The sections were then mounted on labeled slides with a mounting media and covered with a thin glass coverslip.

Confocal microscopy (Zeiss LSM 710) was used to analyze the obtained results. BrdU counts in the dentate gyrus as well as image capturing was done using the Zeiss ZEN 2009 image-analysis software. Tile scan and serial Z-stack images for BrdU

with maximal projection intensity where taken. BrdU-positive cells appeared either as small foci that tightly grouped in clusters or as chains extending parallel to the SGZ.

3. Cell Stereology

To quantify the amount of stem/progenitor cells in the SGZ, BrdU positive cells where counted under the confocal microscopy using both the 40X-oil and the 63X-oil objective. Since the counting was solely done in 1 representative well, the final number of BrdU positive cells was multiplied by 6 to estimate the full count in the whole hippocampus. Z-stack and tile scan images were taken with maximal intensity projection to represent the BrdU cells. The sum of the number of BrdU +ve cells in the rostral, intermediate and caudal was added to obtain the total number in the hippocampus i.e. in one hemisphere.

Formula: Total number of BrdU +ve cells in the right SGZ =
sum of BrdU +ve cells in 1 well x 6 (wells)

4. Statistical Analysis

The behavioral parameters and BrdU counts were analyzed at every time point and expressed as the mean (X) ± the standard error of the mean (SEM). The following variables were taken into consideration: type of treatment and treated versus non-treated. Determination of the statistical significance was made using the student t-test by comparing values obtained from sham and experimental groups. The data was also statistically evaluated using one-way Analysis of Variance (ANOVA), which reveals the differences among group means. The P value of <0.05 was considered as the limit of

significance of differences. Statistical analysis and plotting of figures were made using Prism 4-5 GraphPad package (GraphPad Software, Inc., CA, USA).

CHAPTER III

RESULTS

A. Behavioral Observations

After one-day recovery from surgery, individual rats in all groups (naive, sham and ET) did not elicit evident signs of abnormal motor behavior and showed normal weight gain throughout the observation period.

The nociceptive heat reactivity was tested in rats of all groups. The test did not elicit significant alteration in saline *icv* injected rats as compared to naïve; however, in *icv* ET-injected groups significant changes in nociceptive reaction were observed whereby heat hyperalgesia was evident during the first week following the injection (Fig.4). Treatment with Piroxicam resulted in a significant attenuation of the ET-induced heat hyperalgesia (Fig.5).

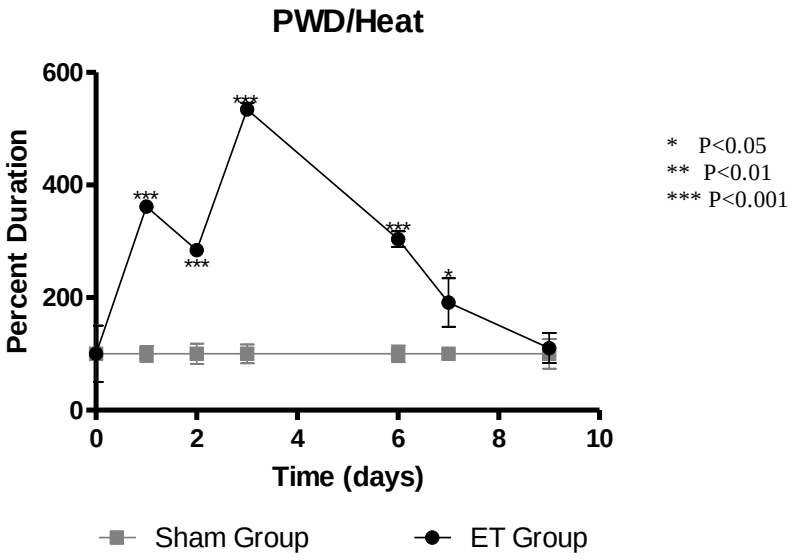

Figure 4: Time course of the heat hyperalgesia induced by icv injection of ET, as compared to saline. Paw Withdrawal Duration (PWD) was measured in several groups of rats (n=4 to 6) and averaged for each time point. The final measurements were plotted as percent variation of heat sensitivity with reference to measurements made on sham group with *icv* injection of saline that did not elicit significant alteration of heat nociceptive thresholds. The determination of significance of each value was made with reference to the sham group.

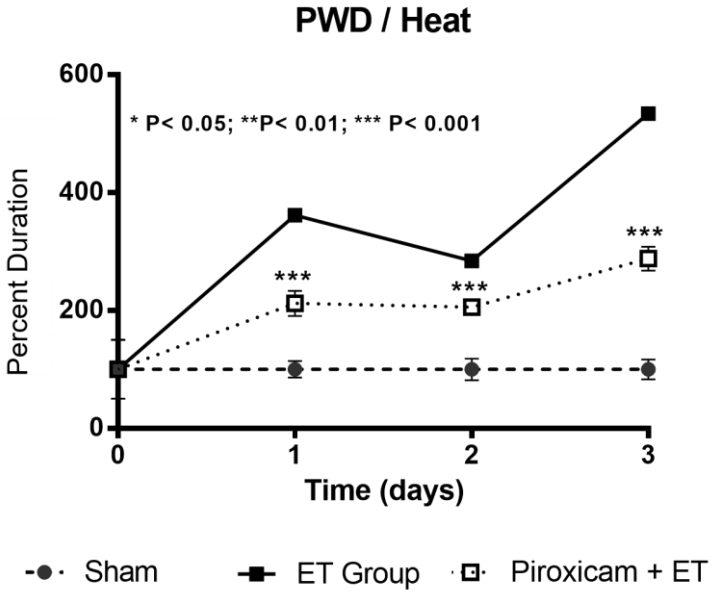

Figure 5 : Daily treatment with Piroxicam (12 mg/kg, ip)resulted in a significant attenuation of the ET-induced hyperalgesia. Piroxicam treatment was made in a group of 5 rats and the measured values of PWD were compared to those observed in ET and saline injections reported in Fig.3.The determination of significance of the values in each time interval was made with reference to the corresponding ET group using the unpaired t test with Welch correction.

The cylinder test showed no significant locomotor asymmetry between the two sides (left and right) and the rats used both forelimbs equally for support during the exploratory behavior (Fig. 6).

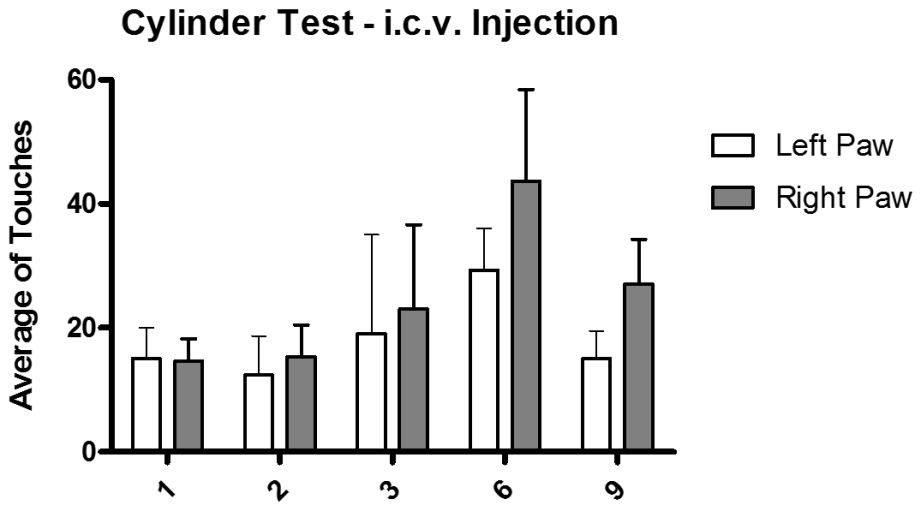

Figure 6: Time course of the cylinder test performed on ET groups. Cylinder test done on ET groups at the time points showed no laterality when comparing the number of paw touches on the walls of the cylinder. The exploratory behavior of the rats was symmetrical between the left and right paws as seen.

B. Hippocampal Neurogenesis In Control Groups

The basal level of generation of hippocampal new progenitor cells was 991.86±55.68, as determined in a group of 5 control/naïve rats (Fig. 7A). Comparable values (941.51±80.31) were observed in sham rats (n=5) receiving an *icv* injection of sterile saline (Fig. 7B).

Since values in both groups did not show significant changes over the days, the reported values in the following paragraph will be the average ± SEM of all measurements made in each group.

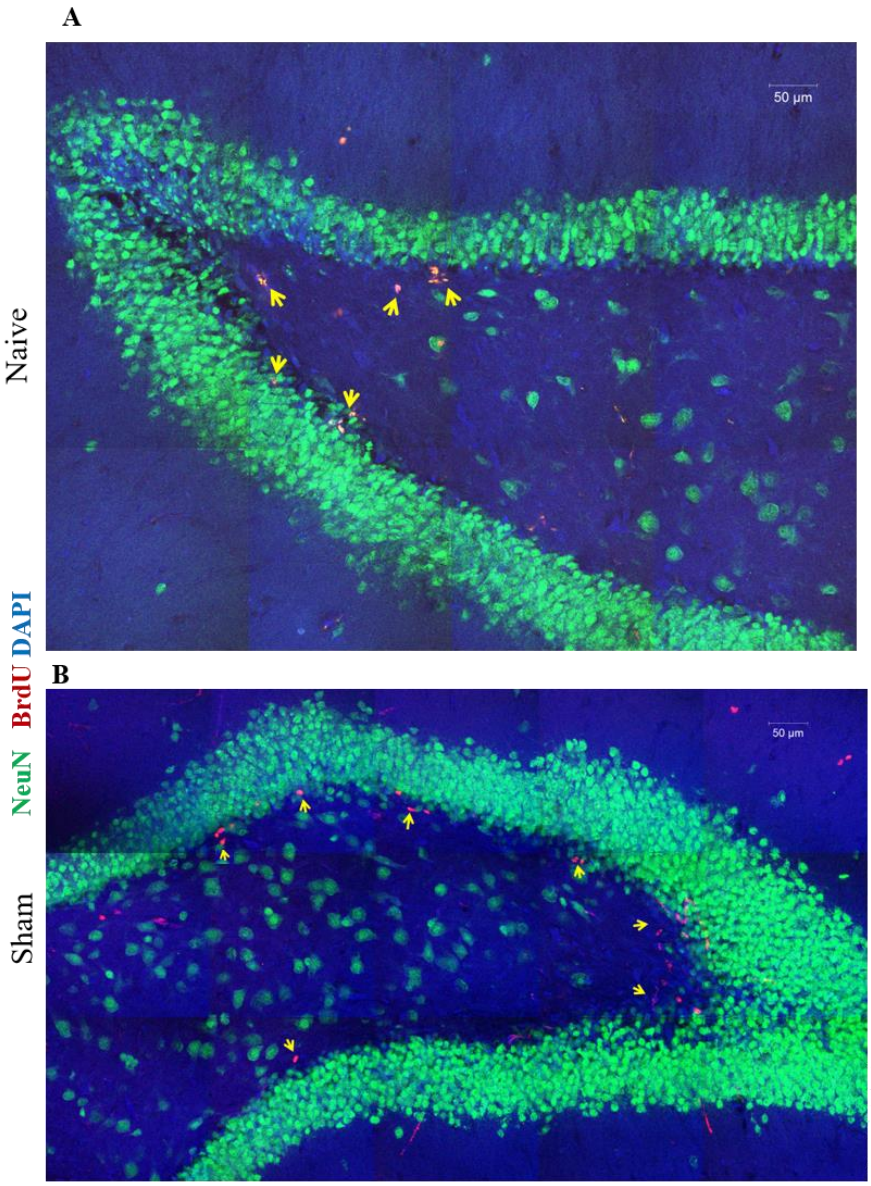

Figure 7: Basal level of generation of new progenitor cells in adult rat hippocampus. Immunofluorescence labeling of the DG by the neuronal marker NeuN (green) and proliferating cells by BrdU (red) showing the baseline level of neurogenesis in naïve (A) and sham (B) groups. The arrows indicate the location of BrdU positive cells. Tile scan and Z-stack were taken at 40X oil objective. Scale bar = 50µm

C. Hippocampal Neurogenesis in ET-*icv* Injected Groups

1. Global Alteration Of Hippocampal Neurogenesis

Following *icv* injection of ET in rat brains (n=5 for day 1; n=4 for days 2, 6 and 9; n=6 for day 3), the amount of BrdU positive cells in the SGZ initially decreased at 24 hours (717.3±143.5) post-surgery (Fig. 8). A notable decrease followed at days 2

(439.8±81.16; P<0.01) and 3 (451.1±77.3; P<0.01). The BrdU counts on day 6, however, is likely a rebound of neurogenesis (1124.8 ±161.2) when compared to naïve and sham groups. A return to basal level (826.5±181.3) was observed at day 9 post injection.

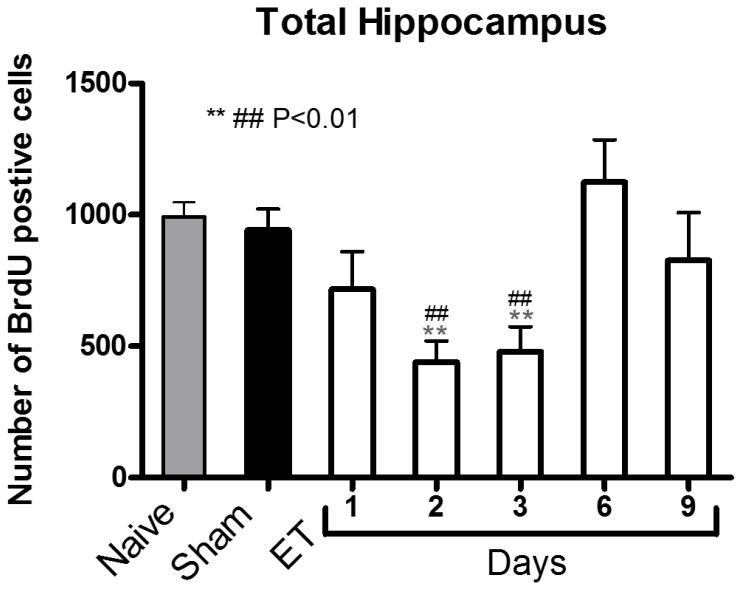

Figure 8: Time course of the alteration of BrdU expression in the DG of ET-injected rats. Neurogenesis is significantly reduced at days 2 and 3 following ET icv injections. Progenitor cell levels surpass the basal levels at day 6 and is restored to normal levels at day 9. Each bar represents the average ± SEM of BrdU count in a different group of rats at the indicated time interval. The determination of significance of each value was made with reference to naïve (*) and sham (#) groups by ANOVA followed by Bonferroni post hoc test.

2. Topographic Distribution of ET-induced Alteration

To investigate the spatial distribution of ET-induced alteration of neurogenesis, we divided the DG into 3 topographical sections ranging from the rostral to the intermediate reaching the caudal area. Generally, the pattern of neurogenesis is least in the rostral and most in the caudal part. Consequently, we would expect the intermediate and caudal regions to be mostly affected. The ET groups were compared to rostral

(168.2±38.1), intermediate (222.4±20.4) and caudal (453.4±25.3) regions of the sham groups.

a. A Decrease In Neurogenesis At Day 1 Post Surgery With A Maximal Effect Seen At Days 2 and 3 post-Injection.

A slight, but significant, decrease in the level of stem/progenitor cells was observed at day 1 in the intermediate (106.7±36.8; $P<0.05$) region of the hippocampus (Fig. 9A) when compared to sham level (222.4±20.4). A more pronounced decrease in cell proliferation was observed at day 2 (Fig. 9B) involving the intermediate (116.25±39.27, $P< 0.05$) and the caudal (294.75±31.43, $P< 0.01$) regions. This decrease continued to day 3 post-injection (intermediate, 145.00±25.00; caudal, 243.36 ±52.96), as illustrated in Fig. 9C.

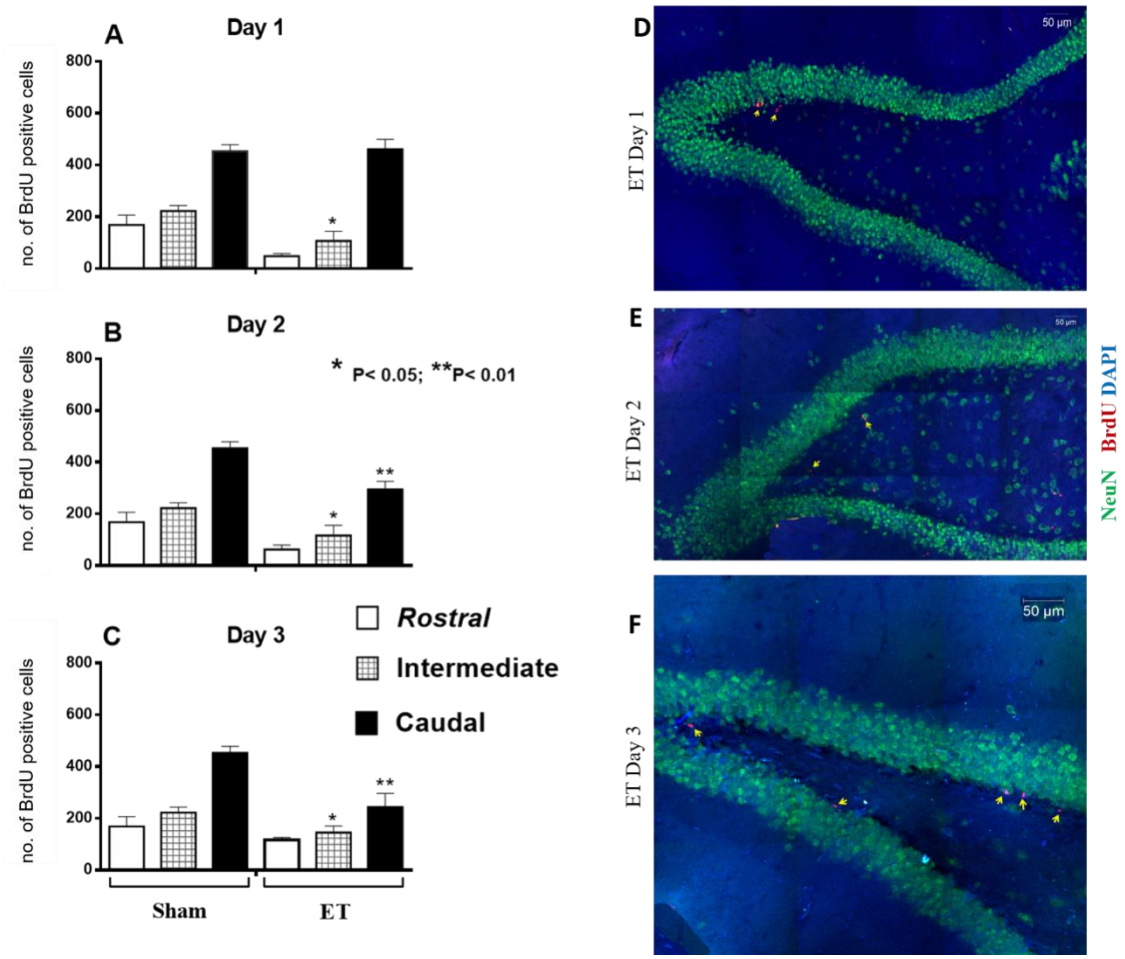

Figure 9: Topographic distribution of the effects of ET injections on cell proliferation in the hippocampal regions. BrdU-labeled cells showed a significant ET-induced decrease at days 1 (A), 2 (B) and 3 (C) mainly at the intermediate and caudal regions of the DG. Each bar represents the average ± SEM of BrdU quantification. The determination of significance of each value was made with reference to a sham group. Immunofluorescence labeling of the DG shows representative confocal images of the ET groups at days 1 (D), 2 (E) and 3 (F) with BrdU positive cells (red) as indicators of proliferating cells and NeuN (green) a marker of mature neurons. Tile scan and Z-stack were taken at 40X oil objective. Scale bar = 50µm

b. Cell Proliferation At Days 6 And 9

As previously mentioned in the total BrdU count in the hippocampus, we observed are bound of PSCs level at day 6 post-*icv* injection of ET. This was mostly restricted to the caudal region (1124.8±161.2) as compared to sham (453.4±25.3, P<

0.01) of the hippocampus (Fig. 10A). A restoration of basal PSCs level was observed at day 9 with values comparable to those of the sham and naïve groups (Fig. 10B).

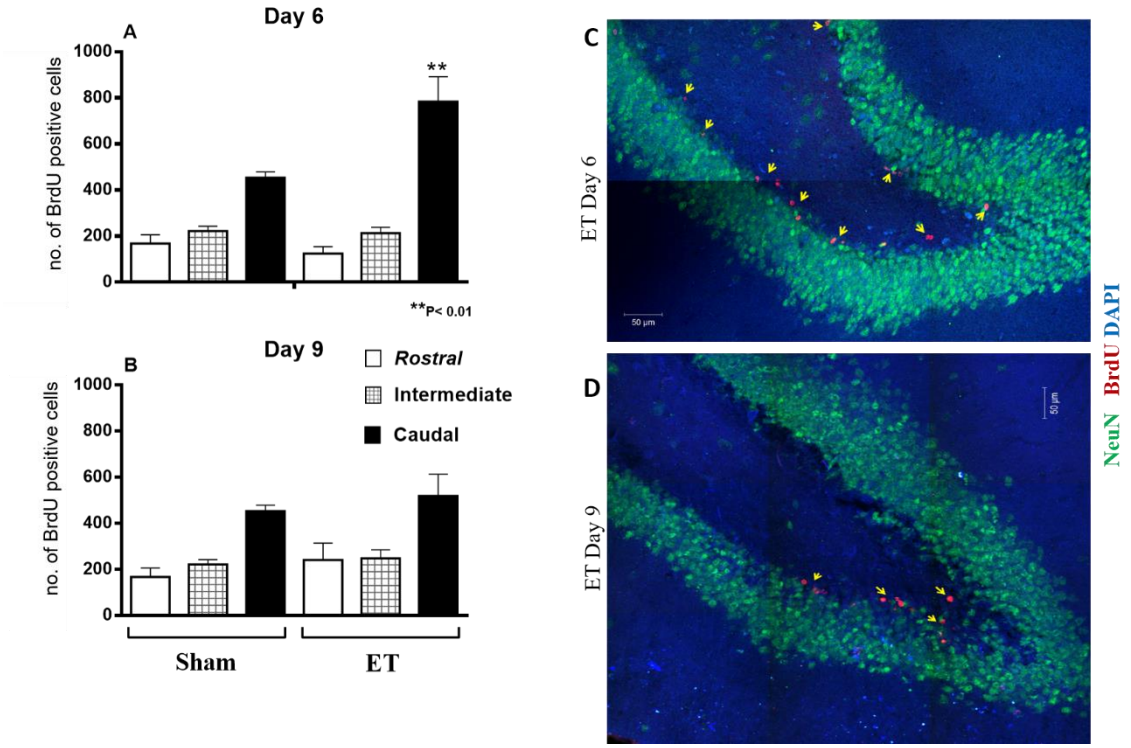

Figure 10: Topographic distribution of the effects of ET injections on cellular proliferation in the hippocampal regions. A rebound of neurogenesis at day 6 in the caudal region and the restoration to basal levels at day 9 following ET injection. Each bar represents the average ± SEM of BrdU quantification. The determination of significance of each value was made with reference to a sham group. Immunofluorescence labeling of the DG in the ET group by the neuronal marker NeuN (green) and proliferating cells by BrdU (red) showing restored neurogenesis levels in ET groups at days 6 (C) and 9 (D) post-surgery. Tile scan and Z-stack were taken at 40X oil objective. Scale bar = 50µm.

D. Treatment with Piroxicam

Piroxicam did not elicit significant alteration of basal BrdU expression, when injected alone (Fig.11). The BrdU counts in both Piroxicam (1184.954±101.5929) and saline (1424.06± 95.14) groups were roughly similar (Fig. 11).

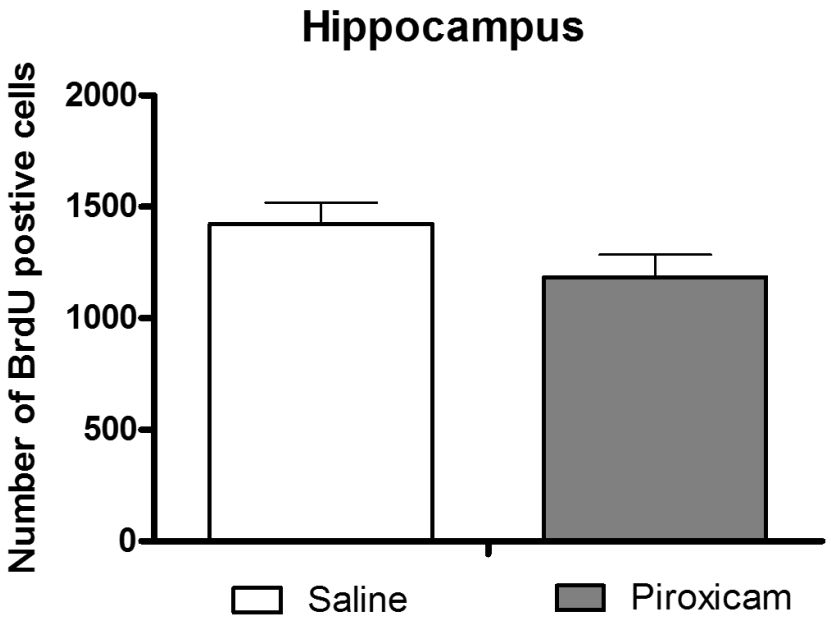

Figure 11: Piroxicam injectiondid not affect basal proliferation of progenitor cells. The BrdU count following ip injections of Piroxicam (n=5) to naïve rats was not affected. Each bar represents the average ± SEM of BrdU positive cells. The determination of significance of each value was made with reference to a sham group given an *ip* injection of saline (n=5).

Daily treatment with Piroxicam of ET-injected rats restored baseline neurogenesis (1192.80±144.85, total count) as shown in fig. 12A. Detailed topographic examination of Piroxicam effects revealed that this treatment cancelled the ET-inducing decrease in the intermediate zone and promoted progenitor cell proliferation in the caudal zone of the hippocampus (Fig.12B).The representative confocal images of the effect of Piroxicam is shown in Figure 13.

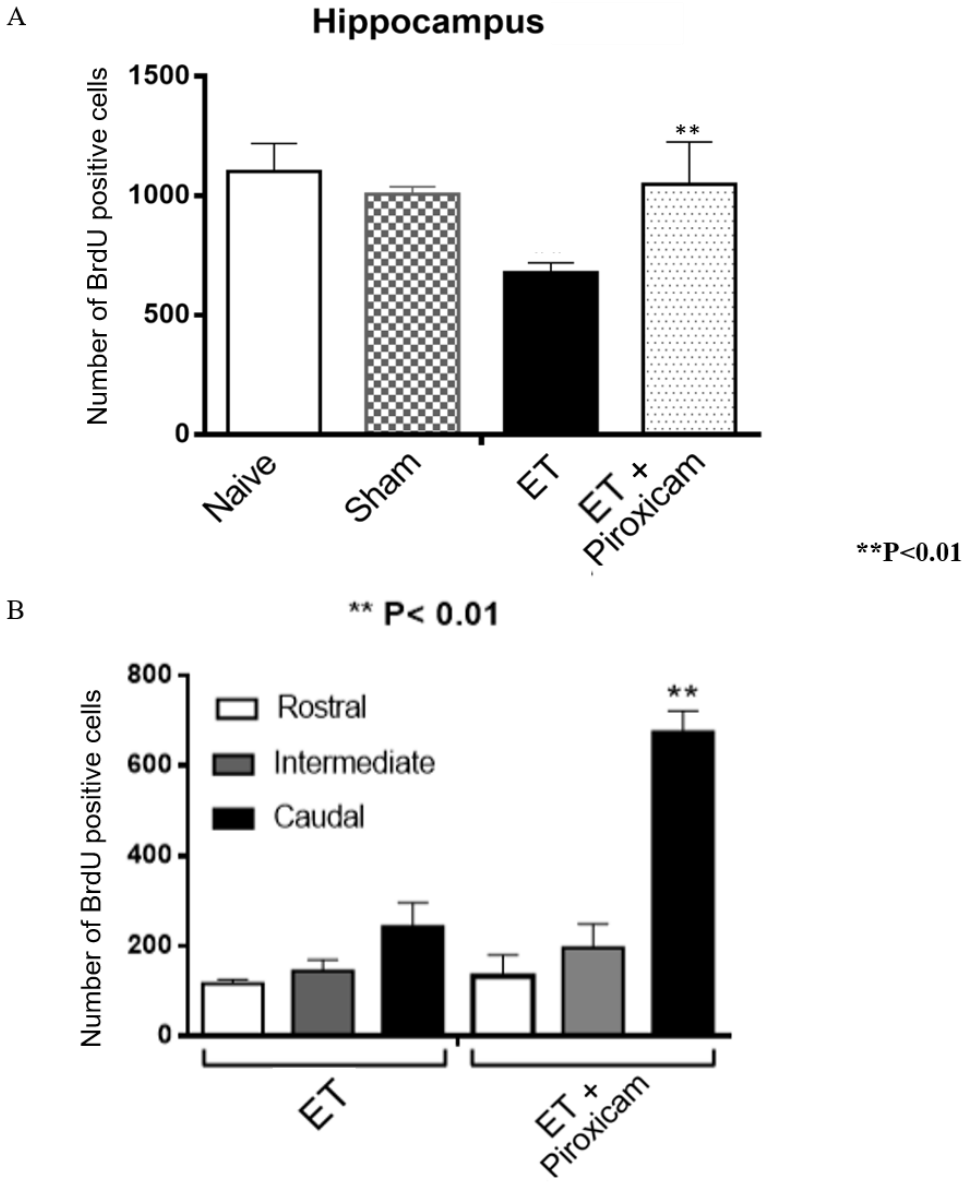

Figure 12: Treatment with Piroxicam reversed the decreasing effects of ET injection on hippocampal progenitor cells. Total BrdU count following ip injections of Piroxicam in ET icv-injected rats is shown in panel A. Topographical quantification shows a significant increase in neurogenesis in the caudal region of the Piroxicam treated ET-group as compared the ET group (B). Each bar represents the average ± SEM of BrdU count in each group

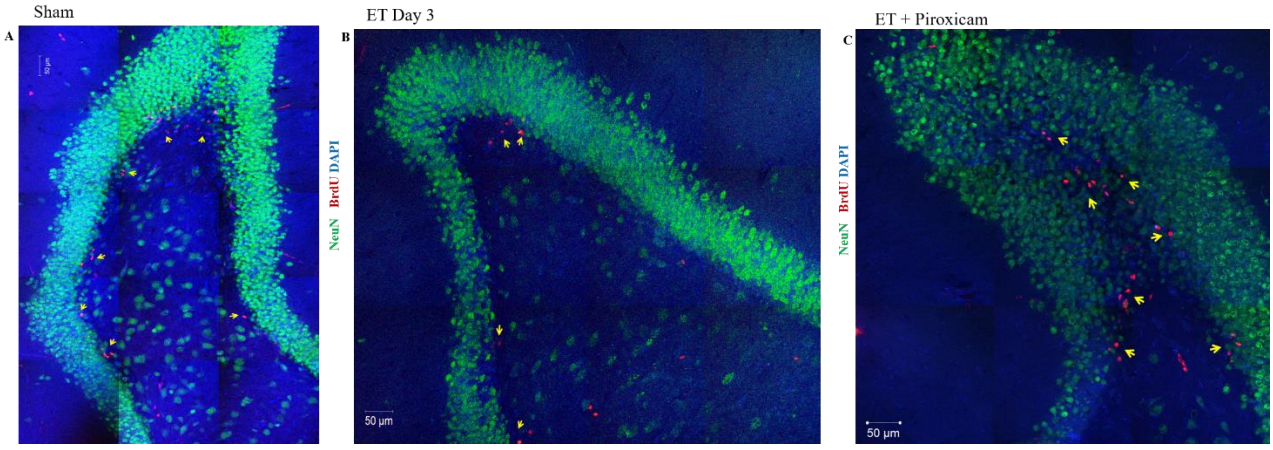

Figure 13: Effect of Piroxicamip injection on neurogenesis following ET-induced neural inflammation. Immunofluorescence labeling of the caudal region of the DG by the neuronal marker NeuN (green) and proliferating cells by BrdU (red) showing decreased BrdU positive cells in the ET group (B) and its restoration in ET groups receiving *ip* injections of Piroxicam at day 3 (C) as compared to the basal level of neurogenesis in sham group (A). Tile scan and Z-stack were taken at 40X oil objective. Scale bar = 50µm

CHAPTER IV

DISCUSSION

The main objective of this study is to examine the influence of neuroinflammation on adult neurogenesis in the dentate gyrus of the hippocampus. In brief, the results obtained showed a marked decrease in the proliferation of progenitor stem cells (PSC) at days 1, 2 and 3 following *icv* injection of endotoxin. Behavioral studies elicited significant heat hyperalgesia during the first 3 days following the injection. Changes in PSC proliferation and heat nociceptive threshold recovered their basal control levels at 9 days post *icv* injection of endotoxin. Pretreatment with Piroxicam (NSAID) prevented the ET-induced decrease in PSC proliferation and heat hyperalgesia. The absence of laterality in the cylinder test is clearly a consequence of the dilution of the endotoxin in the CSF of the ventricle and its distribution to all brain regions.

It is now well established that neuroinflammation is implicated in several neuropathological syndromes ranging from sickness behavior to cognitive decline, depression and neurodegenerative disorders (Akiyama et al., 2000, Hirsch et al., 2012). The expanding attentiveness on the involvement of inflammation in neurodegenerative disorders lead to the rerouting toward approaches that target the immune system (Amor et al., 2014) albeit the causal link between inflammation and these disorders remains to be elucidated. Different studies have attempted to provide cellular and molecular substrates of the association between neuroinflammation and the reported neuronal and behavioral disorders; one study has shown that elevated TNF-α increases the levels of

neuronal receptors for glutamate (Ye et al., 2013) which can lead to neuronal death by excitotoxicity(Leonoudakis et al., 2004). It has been shown also that TNF-α-mediated inflammation can alter the normal function of astrocytes, thus indirectly impairing the regulation of glutamate(Coulter and Eid, 2012) leading to its accumulation and the over-sensitization of neurons (Belarbi et al., 2012).

Another study highlights the reduction in microglial cells' normal ability to help repair and maintain neuronal connections (Harry and Kraft, 2008, Belarbi and Rosi, 2013).

Finally, behavioral disorders (such as depression) induced by inflammation has been associated, in several recent reports, with the impairement of hippocampal neurogenesis (Snyder et al., 2011, Akers et al., 2014).

Previous work on ET *icv* injected rats showed thermal hyperalgesia accompanied by increased expression of pro-inflammatory cytokines such as TNF-α, IL1-β and IL-6 in various brain areas (Safieh-Garabedian et al., 2011). These effects were reversed by treatment with a thymic hormone, thymulin or by a synthetic analogue (PAT), which has been shown to exert an anti-inflammatory action by decreasing the levels of pro-inflammatory cytokines and increasing the expression of anti-inflammatory cytokines (Safieh-Garabedian et al., 2003).

Increased expression of cytokines in the brain has been associated with several neurodegenerative diseases (Holmes et al., 2003, Dik et al., 2005) and seems to affect neural plasticity and normal behavior (Vitkovic et al., 2000, Reichenberg et al., 2001, Felger and Lotrich, 2013, Lewitus et al., 2014). The function of these mediators might not be limited to the observed nociceptive sensations, but probably underlies more

intricate and discrete homeostatic and plastic changes (Chamaa et al., 2016). Moreover, clinical attempts to maintain the expression of pro-inflammatory mediators within normal ranges might open new venues for the treatment and/or prevention of neurodegenerative pathologies (Safieh-Garabedian et al., 2012).

Data from previous studies by our group has shown that *icv* injection of small doses of endotoxin can produce a low level of inflammation in the brain that persists over a period of 2-3 days (Safieh-Garabedian et al., 2003; 2011). This procedure provides a good model for the exploration of the effects of this localized inflammation on repair mechanisms in the brain.

A notable decrease in neurogenesis was detected during the first few days following *icv* injection of endotoxin. This may be considered as a main consequence of the resulting moderate inflammation. Moreover, the observed rebound of proliferation of PSCs at day 6 post injection might reflect an overshoot recovery of the neural repair mechanisms in response to the detrimental effects of neuroinflammation. During the inflammatory process, microglia (the resident macrophage cells of the brain parenchyma) acquire a reactive inflammatory phenotype characterized by its increased proliferation, morphological modifications and release of numerous inflammatory molecules comprising cytokines, chemokines, reactive oxygen species, and nitric oxide (Kettenmann et al., 2011). When combined, these affect the neurogenic niche making them detrimental to neurogenesis (Huang et al., 2012). The injected LPS is known to activate microglia by binding to the toll-like receptor-4 (TLR4) molecules and promoting the release of several pro-inflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) among other inflammatory molecules (Kohman and Rhodes, 2013). Activated microglia play a

dynamic and dual role in brain homeostasis(Ajmone-Cat et al., 2010) and can be considered as major contributors to neurodegeneration, under pathological conditions (Pisanu et al., 2014).

In addition, the obtained results might be explained by the fact that the hippocampus has a high density of receptors for inflammatory mediators making it highly prone to inflammatory insults (Green and Nolan, 2014). Moreover, a subfamily of receptor protein tyrosine kinases, termed 'TAM', expressed in the CNS are involved in different cellular functions, including regulation of immune responses and act as important homeostatic regulators that dictate microglial response to infection or tissue damage (Ji et al., 2015). Lack of these receptors resulted in an increased level of pro-inflammatory cytokines, particularly IL-6, which inhibits progenitor stem cell proliferation and differentiation thus compromising neurogenesis (Ji et al., 2013). TAM receptors play pivotal roles in adult hippocampal neurogenesis by supporting PSC survival, proliferation, and differentiation into immature neurons (Ji et al., 2013).

In adult hippocampal neurogenesis, the fate of new neurons is determined as early as within the first few hours or days following mitosis; although not all of the cells that do express early neuronal markers become mature neurons (Kempermann, Gast et al. 2003). Previous research by Kempermann et al. 2003 has demonstrated that exposure to 'challenging' stimuli in the environment (such as inflammation) increases the number of new neurons by means of a survival-promoting effect. This might explain the rebound of PSC proliferation at Day 6 prior to the restoration of control levels seen at day9 post injection. The fate of these newly formed neurons is partially confirmed by the detection of several $BrdU^{+}/NeuN^{+}$ cells which probably represent newly formed immature neurons that may or may not become mature.

Several neurodegenerative disorders occur without being induced by an injury (Larson et al., 2014). Thus, it is critical to find out how neurogenesis is affected in such cases (Larson et al., 2014). Homeostasis in the adult brain can be the end-result of an equilibrium between neurogenesis and neurodegeneration, which are tightly coordinated to establish and maintain properly functioning neural circuits(Larson et al., 2014). As previously mentioned, several factors interfere with this equilibrium, that range from brain insults to pharmacological manipulations (Cho and Kim, 2010).

The CNS has been regarded as an immune-privileged system however it is now clear that there is a constant and dynamic bi-directional communication with the immune system across the blood–brain barrier and that the healthy adult CNS contains a population of brain macrophages; microglia (Abdipranoto et al., 2008, Huber et al., 2014, Banks, 2015, Louveau et al., 2015). A marked decrease in neurogenesis and increased microglial activation were observed in a status epilepticus model (Ekdahl et al., 2003). Similar results were also seen with the systemic (Monje et al., 2003a) or intra-cortical (Ekdahl et al., 2003) administration of LPS and were reversed by treatment with NSAID or antibiotic (Ekdahl et al., 2003, Monje et al., 2003a).

Inflammation plays an important role in the pathogenesis of ischemic brain injuries (Jin et al., 2010), which results in neuronal cell death and alter the normal pattern of adult neurogenesis (Lipton, 1999). It has been shown that ischemia stimulates cell proliferation within the SGZ by increased birth of dentate progenitor cells (Liu et al., 1998). This enhanced neurogenesis following an ischemic-induced neuronal loss may underlie the partial recovery that occurs after the ischemic episode, although it is presumably not upregulated in an ample manner to cause full recovery(Abdipranoto et al., 2008). Thus the importance of understanding the mechanism by which inflammation

affects neurogenesis is crucial to discern whether impaired neurogenesis contributes to the progression of chronic neurodegenerative disorders. This enables us to exploit the possibility of targeting the compromised neurogenesis as a therapeutic approach.

The Non-Steroidal Anti-Inflammatory Drug Piroxicam (Feldene ©) is a strong non-selective inhibitor of cyclooxygenase, preferentially COX-1, that has an analgesic and an antipyretic effect (Sigurdardottir et al., 2008; Beyer et al., 2012). The efficacy of Piroxicam as an anti-inflammatory agent is mostly attributable to the inhibition of prostaglandins synthesis (Sigurdardottir et al., 2008).Since microglia are important sources of prostaglandins and majorly contribute to decreased neurogenesis, they constitute a likely target for NSAID's in the brain (Minghetti and Levi, 1998). Thus, it seems that the repair mechanism observed following treatment with Piroxicam is most directly linked to microglial activation.

Briefly, a silent and mild neuroinflammation caused by injection of endotoxin into the cavity of the lateral ventricles leads to a decrease in PSCs levels. These effects are reversed when inflammation resolves. Referring to compiled evidence about the role of neurogenesis in the hippocampus and its secondary effect when suppressed, we may consider it as an indicator of brain health. Therefore, further studies to understand the mechanism by which acute or chronic inflammation affect neurogenesis is essential. Additionally, investigating the pattern of how endotoxin affects neurogenesis differently at the topographical levels of the hippocampus is key to understand the differences in connections at these levels.

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