AMERICAN UNIVERSITY OF BEIRUT

EFFECT OF INTRANIGRAL INJECTION OF ENDOTOXIN ON NEURONAL HOMEOSTASIS IN THE HIPPOCAMPUS

by
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ABSTRACT OF THE THESIS

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Title: Effect of intranigral injection of endotoxin (ET) on neuronal homeostasis in the hippocampus

Background

Neurogenesis in the hippocampus has been shown to be prone to alterations in response to a variety of physiological and pathological factors, one of which is neural inflammation. Inflammation has been implicated in changes observed in neurodegenerative diseases such as Parkinson's and Alzheimer's. Previous studies are in favor of the hypothesis that overt or discrete brain inflammation can result in reduced neurogenesis and/or neuronal death associated with cognitive decline and other functional disorders. We aim to show that mild inflammation induced by unilateral Endotoxin (ET) injection in the substantia nigra (SN) and ventral tegmental area (VTA) can alter neurogenesis in the Dentate Gyrus (DG) of the hippocampus. For this purpose, 2ug/2ul of Endotoxin were stereotaxically injected into the right SN of adult rats. The effect of ET administration on lateralized motor function was assessed using the Rota Rod and the Cylinder test. The effect on neurogenesis in the hippocampus was assessed by quantifying the number of proliferating cells in the Dentate Gyrus (DG).

Methods

Adult Sprague Dawley female rats (250-300g) received unilateral injection of Endotoxin (2ug/2ul saline) or sterile saline (2ul) in the right SN. Rats then received 3 consecutive intraperitoneal injections of 5'-Bromo-2'-deoxyuridine (BrdU) (66mg/kg/injection) separated by 2 hours intervals. The rats were perfused on day 3, 6, or 9 post injection. Behavioral observations included cylinder test and Rotarod tests. The fractionator method was used together with confocal immunofluorescent analysis to probe for BrdU and NeuN positive cells in the left and right DG. Moreover, Tyrosine hydroxylase (TH) and Ox-42 immunostaining were performed to check for the effect on dopaminergic neurons and microglial activation, respectively. The number of TH cells was quantified and the intensity of Ox-42 was recorded in the right and left SN.

Results

The cylinder test showed significant ipsilateral bias (with reference to the side of injection) in the rats that received ET injection compared to the sham and Naïve rats at days 2, 3, 6, and 8 with the most prominent effect at day 3 ($p<0.0049$). The Rotarod test showed significant decrease in the latency to fall for ET injected rats at day 2 ($p<0.0059$), which was partially reversed on day 3 and back to normal by day 6. ET

injection in the SN led to a significant bilateral decrease of BrdU positive cells ($p<0.05$) in the right and left DG at day 3 compared to sham and there was no significant difference between sham and naïve rats. This decrease in neurogenesis persisted at days 6 and 9 post injection. Moreover, there was an ipsilateral 24% decrease in TH in the right SN at day 3 post injection which was accompanied by a significant activation of microglia. These changes were not observed at days 6 and 9 following the injection.

Conclusion

This study demonstrates the detrimental effects of neuroinflammation and provide an animal model for the study of the development of neurodegenerative diseases.

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ABBREVIATIONS

BBB: Blood brain barrier

BMP: Bone morphogenic protein

BrdU: 5-Bromo-2$^{'}$-deoxyuridine

DA: Dopamine

DG: Dentate Gyrus

EC: Entorhinal cortex

ET: Endotoxin

GCL: Granule cell layer

i.c.v: intra-cerebro ventricular

IPC: intermediate progenitor cells

i.p: Intraperitoneal

LPS: Lipopolysaccharide

MG: Microglia

NSCs: Neural Stem Cells

NPCs: Neural Progenitor cells

6-OHDA: 6-hydroxydopamine

PD: Parkinson's disease

SGZ: Sub granular zone

SNc: Substantia nigra compacta

SVZ: Subventricular Zone

TH: Tyrosine Hydroxylase

VTA: Ventral tegmental area

CHAPTER 1

INTRODUCTION

A. Neurogenesis:

1. Overview

In the 1960s, the first evidence was provided about the ability of the mature adult brain to continuously generate and provide new neurons throughout its life (Altman & Das, 1965). Accumulating evidence over the years completely challenged and changed the previously held dogma that the mammalian brain is refractory and incapable of generating new neurons or has 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 et al., 1996 ; Kempermann et al., 2003 ; Kuhn et al., 1996 ; Rietze & Reynolds, 2006; Spalding et al., 2013; Zhao et al., 2006).

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 to 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 to the existing circuitry (Vivar et al., 2012; Vivar & van Praag, 2013; C. Zhao et al., 2008). 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.

The hippocampus is part of the limbic system that is involved in cognition. It plays a crucial role in memory, learning and spatial coding. These functions are probably owed to its continuous neuronal turnover within the SGZ niche. As for the SVZ niche, it contains a much larger germinal layer harboring NSCs than the SGZ (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 and it is worth noting that in humans this migratory stream is much more complex and controversial. In fact, humans are unique among mammals in that they do not seem to have a neurogenic niche in the OB

(Bergmann et al., 2012; Sanai et al., 2011). Therefore, the fate of the newly generated neuroblasts in the SVZ have not yet been well characterized in humans. So far, the real functional significance of adult neurogenesis is still not fully clear. 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 DG and control the behavior in contextual and spatial memory (Itaru 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 facilitates synaptic plasticity and the induction of long term potentiation (LTP) (Schmidt 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. In other words, the newly formed cells represent a very malleable source to a variety of inputs, stimuli or factors offering a chance for additional functions and roles in the brain.

Adult 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 prompted by learning and learning new things by itself promotes neurogenesis (Gould et al., 1999). During the first postnatal life of humans, there is a dramatic decrease in neuroblast formation in the SVZ and hippocampus which is later followed by sustained neuronal generation that decreases with aging. The rate of neurogenesis witnesses an

age-dependent drop which is more pronounced in rodents than humans (Spalding et al., 2013). This decrease is exponentially expressed with aging (Kuhn et al., 1996) but with a net additive neurogenesis that leads to an increase in DG cells of rodents. In humans, on the other hand, there is a net loss of DG cells in adult life.

The origin of NSCs is still not completely understood. According to the prevailing hypothesis, these NSCs originate from the neuro-epithelium of the DG. In this epithelium, the embryonic granule cells are generated. However, an opposing hypothesis exists and suggests that the NSCs pool originate during late gestation in the ventral hippocampus and later their descendants relocate to the dorsal hippocampus to finally settle in the SGZ of the DG (Li et al., 2013). There are many questions left unanswered about the origin of NSCs. It is still not known whether the adult neural cells originate from the neural precursors responsible for embryonic neurogenesis or, derived from a quiescent pool of cells reserved aside since development and programmed to have a delayed significant function in adult years.

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 NeuN (neuronal nuclear protein), for glial cells such as GFAP (glial fibrillary acidic protein) 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 (Jules R. Selden, 1993). Furthermore, an important advancement to the field is the use of carbon 14 dating, paving the way for better insight on the dynamics of adult neurogenesis (Ernst & Frisén, 2015). This technique allows accurate measurement of the radioactive carbon 14 isotope in genomic DNA.

2. The Dentate Gyrus: Properties and Circuitry

The dentate gyrus (DG) is part of the hippocampus. It 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. 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 (Fig. 2). 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. This region receives a dense vascular network (O'Reilly & McClelland, 1994). BrdU labeling proved that progenitor cells divide at the borders between the hilus and the granular cell layer. These cells will migrate into the GCL and mature into NeuN calbindin-D28 expressing neurons (Kuhn et al., 1996).

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 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 **(EC → DG → CA3 → CA1)**. The newly formed Granule cell (GC) 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, from cholinergic neurons in the septum, 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.

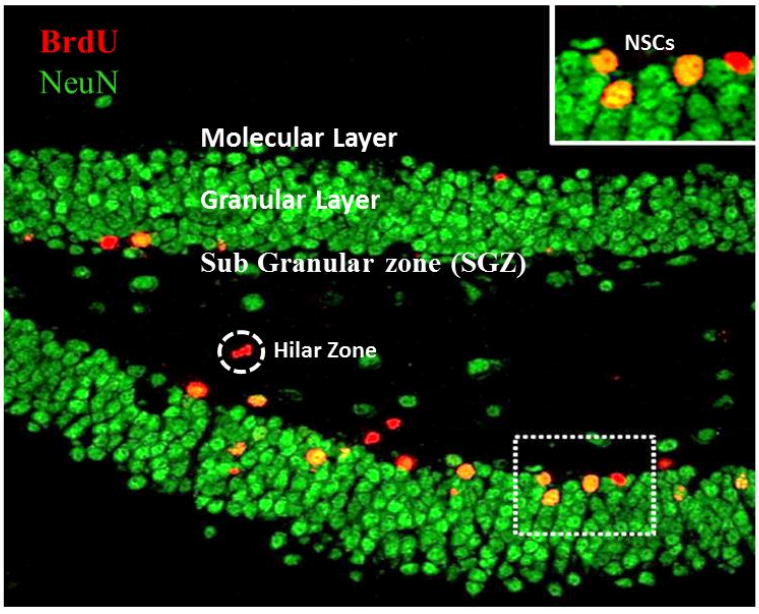


Figure 1: The dentate gyrus. Immunofluorescence image showing the layers of the DG and the position of NSCs. Adopted and modified from reference(Huang et al., 2012).

3. The Neurogenic Niche

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. In adult humans, nearly all DGC turn over (Spalding et al., 2013). However, it takes time for a newly born cell to be fully integrated into the circuitry of the DG and this largely depends on a critical time window (1-3 weeks) when added neurons are still immature while displaying high plasticity and unique electrophysiology. Generally, it takes 4-6 weeks for proliferating progenitor cells to become GCs and takes even more time to reach full maturation and incorporation into the functional network (C. Zhao et al., 2008). The SGZ resident NSCs are known as type I radial glia-like (RGL) cells. They are known as such because they extend radial processes to the Molecular layer and retain stem/pluripotency markers such as nestin, 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 GCs (Lugert S et al., 2010). These cells differ from each other physiologically, morphologically and by several molecular characteristics (Fig. 2). 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 GC layer. During the second week, these cells start extending their dendrites into the ML and the hilar interneurons start providing synaptic input to the newly born neurons. The glutamatergic input into the GCs from the EC, that is critical for their survival and integration, develops around a period of one month. Reaching the third week, the neurons start to resemble mature GCs and spontaneous

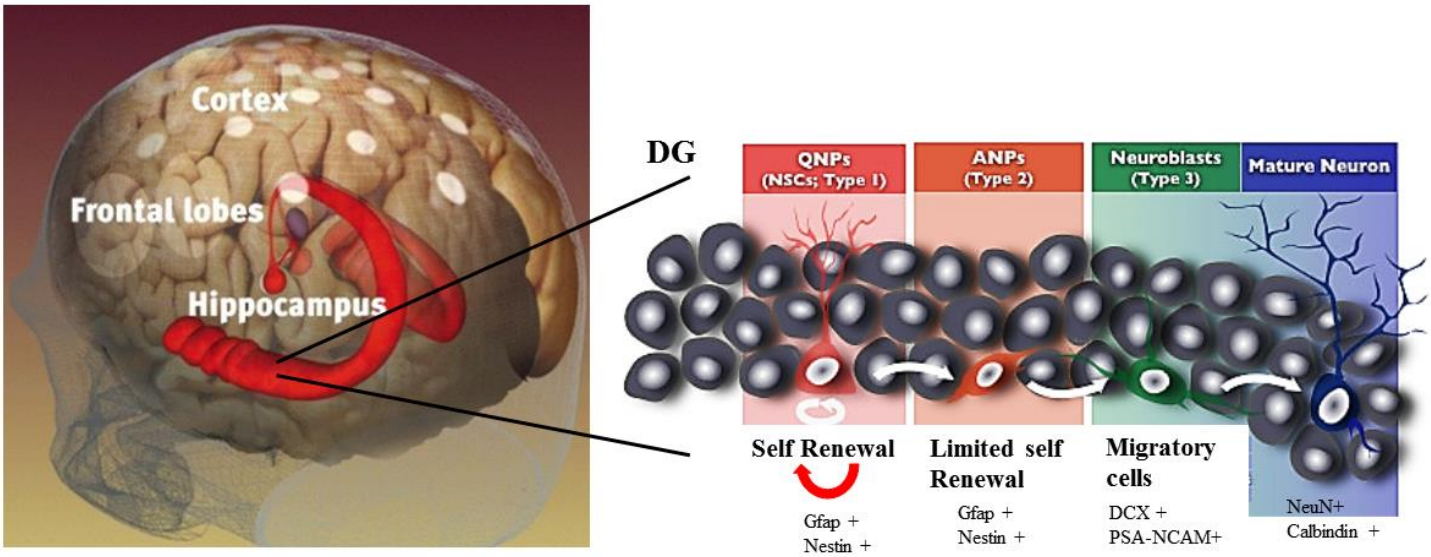


Figure 2: Schematic representation of neurogenesis in the DG. Proliferation, migration and integration of the mature neurons from a population of quiescent neuronal precursors (QNPs). Adopted and modified from reference (Pinheiro et al., 2011;"The Hippocampus," 2017).

synaptic activity is detected.

4. Regulation of Neurogenesis: Intrinsic Factors

Adult NSCs go through different stages before reaching their terminal fate or destination and at every stage there are specific extrinsic and 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 extrinsic factors and conditions. Niche factors such as Notch, bone morphogenic protein (BMP) (Lugert et al., 2010;

Mira et al., 2010) and the neurotransmitter GABA are considered mediators for their 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. On the contrary, Notch can have the opposite effects depending on the developmental context and stage (I. Imayoshi & Kageyama, 2011). Sonic hedgehog signaling 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). 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 (Esther Maier 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). Moreover, it has been recently reported that there is an age-dependent increase in BMP levels which may be correlated to the decreased levels of neurogenesis (Yousef et al., 2015). 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 (CX3CL1) which is highly expressed in neurons can suppress microglia (MG) simply through binding to its receptors thus keeping these cells in a ramified state. The 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). None the less, 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). A remarkable feature of neurogenesis is the temporal relation and the differential recruitment of these various pathways and their interaction reaching the final purpose of initiating and regulating the process of neurogenesis in the adult brain.

It should be noted that around 50% of adult newly born GCs are lost 2 weeks into their maturation (Dayer et al., 2003 ; kempermann et al., 2003). Moreover, more than 95% of the cell population in the DG do not fire under any environment. Therefore, most of the DG cells are non-functional while only a small portion is functional. However, those cells that do live to mature are very stable and very likely to be used and become integrated in the circuitry and even replace the old mature GCs (Dayer et al., 2003). A contributor to this phenomena is the fact that the disparity in the proportions of cells such as the DG contains much more principle cells than the regions of its input and output (O'Reilly & McClelland, 1994).

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 are enhancing for the maturation and integration of these new neurons (Ser et al., 2001 ; Song et al., 2002). 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).

5. Modulation of Neurogenesis: Extrinsic Factors

One of the most intriguing characteristics of neurogenesis is that it can be manipulated. Neurogenesis is prone to modulation in response to a variety of factors: genetic, epigenetic, transcriptional factors, environmental factors, acute and chronic illnesses, inflammation, aging, and psychiatric disorders including depression. A boost in neurogenesis, induced by environmental factors such as exercise and diet, has been associated with improved memory and cognitive thinking (Creer, Romberg, Saksida, Praag, & Bussey, 2010; Stangl & Thuret, 2009). On the contrary, a decrease in neurogenesis as a consequence of events such as γ- or X- radiation (Clelland et al., 2009; Snyder, Hong, McDonald, & Wojtowicz, 2005), aging (Spalding et al., 2013), genetic factors or pharmacological interventions (opiates, adrenal hormones,..) (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 for 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). There is evidence to support that NSCs in the neurogenic niches respond to injury. This is mostly evident in seizures and brain ischemia where cell proliferation and migration promptly increases (Jin K, 2001). However, the point in

question is whether to consider such an increase as a beneficial compensatory mechanism or a brain-self repair process or perhaps a trigger for further pathology.

6. Neurogenesis and neurodegenerative diseases

One of the aspects 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 and in aging (Carlesimo et al., 2012; Lazarov & Marr, 2010; Marxreiter et al., 2013; Simpson et al., 2011; Small et al., 2011). This indicates that in addition to the adult brain loosing certain existing neuronal cells in diseases, its endogenous capacity for self-renewal and gaining functional advantage from adding new neurons is either compromised or somehow lost. Neurogenesis in humans can only be assessed in postmortem tissues but neuroimaging techniques have been used to provide some insight of the brain structure and function and its correlation with the cognitive function and memory of patients.

There has been increasing knowledge and updates in the field of neurogenesis as it holds promise for understanding diseases and possibly treating neurodegenerative diseases and certain complications of aging. The fact that there is neurogenesis in the adult brain holds the promise that such an endogenous source for new healthy cells can be used for treating neurodegenerative diseases especially those that target specific groups of cells (Lie et al., 2004).

B. Inflammation and Neurogenesis:

Inflammation is not a brain disorder by itself but rather an important process associated with many brain disorders. Indeed, inflammation is almost an ultimate implication in aging and neurodegenerative diseases (Liu et al., 2003; Nelson et al., 2002) and is thought to contribute to the propagation of neurodegenerative processes. Even small inflammatory processes can eventually cause changes in brain function. Adult neurogenesis is one process that gets strongly inhibited by inflammation (Bastos et al., 2008; Ekdhal et al., 2003) and this has been shown to be reverted by anti-inflammatory drugs (Monje et al., 2003;Ekdahl et al., 2003). The disruption in neurogenesis following inflammation might explain the memory deterioration, deficits in learning and cognition seen in many of these inflammation-associated diseases. This disruption in neurogenesis could be mediated through microglial (MG) release of inflammatory cytokines and factors. One cannot mention brain inflammation without bringing up the role and involvement of MG. The ability of adult NSCs to divide and differentiate depends largely on the composition of the neurogenic niche in which they are immersed in and microglial cells contribute to the molecular and cellular composition of that niche. In a healthy brain, microglial cells are usually involved in constant surveillance functions and defense mechanisms against infectious agents. These cells are abundant in large numbers in the brain and are not uniformly distributed across the different brain regions (Lawson et al., 1990). Under normal conditions, MG would be in a resting ramified state (Nimmerjahm et al., 2005). Resting microglia are dynamic cells characterized by their high motility which enables them to effectively survey and control the brain microenvironment. They have motile protrusions that are supposed to serve for continuous sampling of the surrounding microenvironment. In the

DG, the surveilling microglia have high phagocytic potential for apoptotic newly born cells.

However, in the case of an insult, microglial cells shift their morphology from such resting state to an amoeboid activated shape (Kettenmann, Hanisch, Noda, & Verkhratsky, 2011; Streit, Walter, & Pennell, 1999; Thored et al., 2009) characterized by increased expression of histocompatibility complex molecules (MHC class II), type 3 complement receptor (CR3 or CD11b/ CD18) (Stence et al., 2001;Kreutzberg, 1996), CD40, and ICAM-1. The amoeboid structure is due to swelling of the cell body. This dynamic pronounced change is unique to MG among other cells in the CNS such as astrocytes. Microglial activation is an innate immune response that is fast and non-specific. Unlike a brain insult, there is no increase in neurogenesis following LPS-induced inflammation. In fact, if a brain insult is associated with microglial activation, any increase in neurogenesis gets attenuated (Tang et al., 2016;Ekdahl et al., 2003; Monje et al., 2003). The effect on neurogenesis not only involves proliferation and survival of cells, but also the integration of the newly born cells into the neuronal circuitry.

Though there are other variety of cell types that could regulate the inflammatory process, MG cells are the primary immune cells to carry out this burden. Microglial response to inflammation involves increase in their proliferation, retraction of their processes, and change in their morphology to an amoeboid form and of course the production of inflammatory mediators such as cytokines, chemokines, ROS and NO (Kettenmann et al., 2011; Ransohoff & Perry, 2009; Stence et al., 2001). Beyond activation, there is a re-gain of motility after a new set of protrusions become expressed which are highly dynamic and undergo rapid extension (protruding) and retraction (not

protruding). This is called the locomotor phase as MG begin to translocate within the tissue (Stence et al., 2001). An important feature of activated MG is that its morphology depends on the type of the inflicted pathology. However, little is known about the heterogeneity of microglial responses to the different pathological events (Hanisch & Kettenmann, 2007). Most reports following bacterial endotoxin (ET) injection, explicitly show and describe an amoeboid activated structure following the induced inflammation (Castano et al., 2002; Herrera et al., 2000).

In response to ET exposure in the brain, microglial activation can start as early as 12 hours (Reinert et al., 2014) and cytotoxic factors can be released in as early as 3 hours post exposure in as low as 1ug/5ul concentration. Even in low tolerable doses, LPS has a toxic effect. LPS, a potent inflammogen, activates MG through binding to toll-like receptor-4 (TLR4) molecules (Palsson-McDermott & O'Neill, 2004). The LPS receptor (TLR4) expressed on microglial cells is thought to transduce the microglial activation process. TLR4 is a recognition receptor that binds to pathogen-associated molecular patterns in LPS and initiates a signaling cascade through nuclear factor *κB* (NF-*κ*B)-dependent and NF-*κ*B-independent pathways (Chen et al., 2012; Palsson-McDermott & O'Neill, 2004). LPS and MG activation ultimately induces the release of several pro-inflammatory cytokines including interleukin-1β (IL-1β) (Green et al., 2012), tumor necrosis factor-α (TNF-α) (Seguin et al., 2009), and interleukin-6 (IL-6) among other inflammatory molecules (Saade et al., 2003). Evidence suggests that these classic pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α contribute to the inflammation-related decrease in neurogenesis (Tang et al., 2016). For example, overexpressing IL-6 in a mouse transgenic model causes a decrease in proliferation levels of NSCs in the DG as shown by BrdU tracking one day after injection (Vallieres

et al., 2002). Activated microglia following LPS injection impairs hippocampal neurogenesis and administering MG inhibitor, minocycline, restores the survival of newly born cells (Monje et al., 2003) (Ekdahl et al., 2003). The LPS affects the proliferation (Fujioka & Akema, 2010) the survival and the integration (Jakubs et al., 2008) of newly born cells. Furthermore, the suppression of neurogenesis by neuro-inflammation occurs in an LPS-dose dependent manner (Bastos et al., 2008). Induced chronic inflammation diminishes the role of new neurons in the processing of contextual memory which is yet another feature of the effect of inflammation on neurogenesis and its related functions (Belarbi et al., 2012).

Activated MG are not a definite inhibitor of neurogenesis. The role of activated MG in response to injury and insults can be either toxic to the tissue or could exert a neuroprotective role depending on several factors at play (Nimmerjahn et al., 2005). Under certain conditions they can have a neuroprotective role (Ransohoff & Perry, 2009) and might act as promotors of neurogenesis depending on the secretion of anti-inflammatory molecules within the niche (Vukovic, J. et al., 2012). Therefore, the effect induced by the MG depends largely on the balance between the pro- and anti-inflammatory secreted molecules (Battista, D. et al. 2006) and whether the inflammation is acute or chronic. For example, TGF-β is one anti-inflammatory molecule that supports neurogenesis (Battista, D. et al. 2006). Thus, microglial activation though plays an important factor in the inflammation induced neurogenesis suppression, this does not exclude the fact that there are other factors involved. For example, there are reports supporting the notion that inflammation increases the inhibitory signaling to the new cells thereby having a suppressive control over neurogenesis.

C. The substantia Nigra:

1. Anatomy & Features of the SN

The substantia nigra (SN) is a darkly pigmented midbrain structure that is rich in melanin and dopaminergic neurons and constitutes a part of the basal ganglia. The SN region is divided into two main parts; the pars compacta which is a long thin dense sheet of dopaminergic neurons and the pars reticulata composed mainly of GABAergic neurons. Dopaminergic neurons in the brain are mainly housed in the SN and the adjacent ventral tegmental area (VTA). The loss of dopaminergic neurons in the SN pars compacta is a major hallmark of Parkinson's disease (PD) and its' observed symptoms. In advanced PD stages, the SN becomes indistinguishable from its surroundings as it loses its pigmentation. Dopamine is an important neurotransmitter that is released in the SN as the starting point for the nigrostriatal pathway which is one of the four major dopamine releasing pathways in the brain (Bjorklund & Dunnett, 2007b). It is the cells of the compacta that contain the dark pigment; melanin and synthesize dopamine. These cells are known as the A9 dopaminergic cell groups and they mainly send terminal projections to the neostriatum; the caudate and putamen (Bjorklund & Dunnett, 2007a). The dopamine released by these DA neurons serves to reduce the influence of the indirect pathway while boosting that of the direct pathway in the Globus pallidus of the basal ganglia. This control and fine tuning is very important for balancing motor function of the body since even a small reduction in dopamine levels can impair one's ability to execute smooth controlled movements and can result in parkinsonian-like behavior. Dopamine even plays a role in the developing brain as it controls the activity of the dividing NSCs (Cameron et al., 1998). It is thought that the effect and regulation done by dopamine during development might be persisting to adult

life. In addition to controlling movement, dopaminergic neurons in the SN have important roles in motivation and controlling the reward pathway. In addition to that, the SN acts as a separate memory system independent of that of the hippocampus (Da Cunha et al., 2003).

The hippocampus and the olfactory bulb are two main regions that get affected during the course of PD (Braak et al., 2003; Carlesimo et al., 2012; Weintraub et al., 2011). Clinical reports state that PD patients in the early phase of the disease present with impaired learning of habit tasks while having preserved episodic memories. Large scale epidemiological studies report that 20% of PD patients at the onset of the disease have cognitive impairment (Aarsaland et al., 2009) where as 80% of patients at advanced stages suffer from dementia (Hely et al., 2008). Altered neurogenesis might be one mechanism driving the decline in cognitive function.

2. The interconnection between the SN and the hippocampus

There are dopaminergic fibers extending all the way to the dentate gyrus in a topographically organized way (Gasbarri et al., 1997). The importance of such dopaminergic input to the hippocampus comes from the role that dopamine plays in learning and synaptic plasticity. It is thought that these DA projections direct the choice of memory encoding in the hippocampus (Du et al., 2016). Dopaminergic input has been shown to promote hippocampal reactivation and spatial memory persistence, through optogenetic stimulation (McNamara CG, 2014). Moreover, the activation of dopamine D1/D5 receptors facilitate the induction of presynaptic LTP at hippocampal synapses (Roggenhofer et al., 2010).

The subiculum and the CA1 mainly receive afferents from the medial part of the substantia compacta (Gasbarri et al., 1994). A combined retrograde tracing and TH staining reveal a major mesolimbic projection towards the hippocampal formation with 15-18% of these projections being of dopaminergic nature. Interestingly, the ventral tegmental area, a region rich in dopaminergic cells adjacent to the SN, projects 10 % of the dopaminergic neurons to the contralateral hippocampal formation. Such contralateral projection is noticeably not evident in the SN (Gasbarri et al., 1994). In addition, endogenous dopamine was detected in isolated hippocampal nerve terminals of rats and guinea pig. The dopamine was found to be released in a gradual way and is thought to be released from large, dense- cored vesicles (Verhage et al., 1992). There has been evidence on the presence of D1 and D2- expressing neurons in the hippocampus and the D1 neurons being GABAergic interneurons scattered in the CA3 and CA1 regions (Gangarossa et al., 2012). Moreover, D1-, D2 and even D-4 like receptors were found on the intrinsic post synaptic neurons whereas none were detected on the presynaptic (Tarazi et al., 1998). All this implies that an insult applied in the SN might be capable of affecting homeostasis in the hippocampus as these two regions seem to have important connections. However, the mechanisms mediating the interconnections requires further study.

3. SN and neurogenesis

Double labeling with BrdU and tyrosine hydroxylase for dopaminergic cells shows a topographic organization of how the dopaminergic SN cells reach out to the to the proliferating cells in the SVZ (Freundlieb et al., 2006). The dopaminergic fibers in the sub-ependymal zone are in close contact with 80% of BrdU positive cells there and the

disruption of such connections drastically affects the proliferation rate. This suggests that an intact nigro-SVZ path is necessary for sustained normal neurogenesis (Freundlieb et al., 2006).

The SNpc sends its axonal projections to the striatum, which is located in the forebrain. The NSCs residing in the SVZ are not far from the striatum that is rich in dopaminergic afferents from the SNpc and VTA. In 2014, Ernst et al. used carbon 14 dating and showed that in adult humans, new neurons get integrated into the striatum from the nearby neurogenic niche of the SVZ (Ernst et al., 2014). This suggests that the newly generated SVZ neurons are destined to give rise to new interneurons in the striatum. In addition to that, the striatum could have its own neurogenic homeostatic niche (Ernst et al., 2014).

Decreased neurogenesis and impaired state and survival of the newly generated neurons are observed in several animal models of PD (Marxreiter et al., 2013). Overexpression of the alpha-synuclein gene that gets accumulated in PD leads to increased neurogenesis paralleled by increased cell death (Winner et al., 2004). It is even suggested that this effect on neurogenesis was directed by the protein itself as this protein not only accumulates in the SN and striatum but also in the hippocampus (Winner et al., 2012). Moreover, structurally different species of alpha-synuclein have different effects on the proliferation, maturation and integration of dentate gyrus neurons (Winner et al., 2011).

Following a 6-OHDA lesion, there would be loss of dopaminergic neurons and an increase in the NPCs of the SVZ. The NSCs of the SVZ can migrate and differentiate into dopaminergic neurons in the SN following lesion. However, this is specific mostly to the cells of the SVZ and not the SGZ of the hippocampus (Xie et al., 2017). In a

unilateral 6-OHDA model of PD, the nigral dopaminergic neurons in the SNpc were destroyed and there was a subsequent depletion of dopamine in the striatum. Interestingly, the injection had an effect on the SVZ and SGZ neurogenic capacity as there was seen a decreased proliferation in the progenitor cells in these regions. The effect was noted in the ipsilateral side to the injection in both the SVZ and the SGZ (Suzuki et al., 2010). This is very important as it indicates a possible involvement for dopaminergic pathways in neurogenesis. It should be noted that there are no direct anatomic connections from the SNc to the hippocampus but it was recently proven that the Dopaminergic neurons from the VTA and SNpc innervate the lower and upper blade of the DG (Hoglinger et al., 2014) which means that the newly born DG cells receive dopaminergic input. In this context, it has been shown that chronic stimulation of DA neurons using the D_2/D_3 dopamine agonist, Pramipexole (PPX) significantly enhances the proliferation in the DG by 42% (Salvi et al., 2016). Therefore, stimulating dopaminergic neurotransmission has the potential to enhance adult neurogenesis in the SVZ and the DG most likely via D_2/D_3 dopamine receptors. This further supports the relation between dopaminergic neurotransmission and neurogenesis in the adult brain which could explain certain cognitive aspects or changes that occur when SN dopaminergic neurons are lost. In other settings, dopaminergic lesioning was shown to impair hippocampal neurogenesis by distinct modification of the alpha-synuclein strain (C. Kim et al., 2016; Schlachetzki et al., 2016). Moreover, an ET injection in the SN leads to oxidative stress in the hippocampus and has a negative effect on behavioral performance in Y-maze and the radial arm maze (Hritcu & Ciobica, 2013).

There has been growing evidence that that dopaminergic enhancing compounds such as levodopa or DA agonists have the potential to boost neurogenesis in the SVZ

and the hippocampus. Experimental depletion of dopamine in rodents decreases the proliferation of precursor cells in both the subependymal zone of the SVZ and the SGZ of the DG. Interestingly, the proliferation of cells there gets restored back to normal after administration of selective D2-like receptor agonists which also directly increases proliferation in neurosphere culture (Hoglinger et al., 2004).

4. Inflammation in the SN

Mechanisms of neuro-inflammation have been reported very often in the pathogenesis of PD and has been established as an essential process involved in either the onset or progression of the disease. Inflammation in the SN and inflammatory related mechanisms are indicated as some of the idiopathic causes of PD (De Virgilio, 2016). Postmortem and experimental studies of PD have found evidence of neuro-inflammation induced by activated MG and/ or infiltrated peripheral immune cells along with their neurotoxic products (nitric oxide, reactive oxygen species, and proinflammatory cytokines). Activated MG were found in the striatum and SN of PD patients and pro-inflammatory cytokines such as TNF, IL-1β, IL-6 and iNOS were found to be increased in the cerebrospinal fluid (CSF) of patients with PD (Walker et al., 2016).

The SN is one of the brain regions that are highly packed with microglia making it the most sensitive to inflammatory insults than other regions such as the striatum for example. A hallmark of inflammation in the CNS is microglial activation. Previous data from *invivo* and *invitro* studies have shown that LPS injected whether in the hippocampus, cortex, or SN of adult rats, will give rise to neurodegeneration only in

the SN. Such region-specific susceptibility to LPS seems to be driven and determined by the abundance of microglia within that specific region (Kim et al., 2000).

D. The LPS/ET Model: Different Versions and Characteristics

1. About the LPS model

Inflammation is an important defense mechanism that is involved in many diseases. It is important to understand the role and effect of inflammation in the CNS and from there comes the need for inflammatory based animal models (Walker et al., 2016). A well-established inflammatory model is the LPS model which was developed for gaining a broader knowledge on the immune-mediated responses and events that occur in degenerative processes. The LPS model is a purely bacterial-based experimental design for recapitulating a possibly similar milieu of inflammation. Such a model represents an important tool needed to study the involvement of inflammation in the progression of diseases and uncover the precise mechanisms of neuro-inflammation mediated cell death or dopaminergic neuronal loss (Herrera et al., 2000). LPS is a potent inflammogen that has been well characterized in inducing nigrostriatal dopaminergic degeneration (Hunter et al., 2009; Qin et al., 2007). This agent stimulates the resident astrocytes and microglia in the CNS to secrete cytokines such as TNF-α, IL-6, and IFN-γ. It can bind to the plasma membrane of microglia resulting in LPS-loaded vesicles and accumulation in the Golgi apparatus (Pei et al., 2007). TLR4 is a major player in the response to LPS and it is mainly expressed in MG. An important feature of LPS is that it does not have any direct effect on neurons probably because they lack the expression of TLR4, therefore making LPS an excellent approach for studying the

inflammation-mediated DA neurodegeneration (Lehnardt et al., 2003). In previous studies, the effect of ET was shown to be specific to dopaminergic neurons, leaving the GABAergic and serotonergic neurons intact (Castano et al., 2002; Herrera et al., 2000). In cases of sepsis, the BBB becomes leaky and might allow the passage of LPS to the cerebrospinal fluid (CSF). In addition, a leaky BBB in PD patients, allowing the entry of ET form the gastrointestinal to the brain, has been proposed as an idiopathic cause of PD (Lange et al., 2003). Due to its specificity for dopaminergic neurons and its ability to cross the BBB, ET has been considered as a risk factor for PD or PD-like disorders. Some cases of PD present after a previous episode of septicemia and some are associated with head trauma and encephalitis further supporting the notion of an inflammatory component involved in PD and the importance of such a model (Fang et al., 2012).

There are differences in the effects produced by LPS in studies even though similar doses have been used. Differences in strain, sex, route of administration or even the coordinates of injection in the SN could alter the response to LPS. Using different doses of LPS yields different results. A pilot study applied different doses of LPS as intra-nigral administration to check at which dosage damage to the SN starts. A dose of 3μg elicited nigral microgliosis without affecting motor behavior nor the TH optical density in the injected and un-injected side. Using an 8μg LPS dose adds a significant difference in the stepping test (contralateral steps) to the observed microgliosis. Going up to 10μg LPS causes changes in TH optical density in the right versus left SNc and in the whisker test for contralateral placings in addition to the previously observed nigral microgliosis and contralateral stepping at lower dose. There is no effect seen in the corridor or rotation test (Hoban et al., 2013).

Injections of LPS and their effect in the SN have been investigated in systemic and central LPS injection models.

2. Systemic LPS administration

Systemic intraperitoneal (*i.p.*) injections of LPS were previously attempted to discern the mechanism of inflammatory transfer from the periphery to the brain (Qin et al., 2007). Intraperitoneal single injection of LPS of 1mg/kg shows long term effect on spatial memory in Morris Water maze and a sustained decrease in neurogenesis (Valero et al., 2014). A dose of 5 mg/kg LPS results in increased expression of brain pro-inflammatory factors (i.e., TNFa, MCP-1, IL-1b, and NF-κB) (Qin et al., 2007). Although the LPS would not be directly administered in the SN, it can still cause gradual decrease in dopaminergic neurons and activation of MG in the SN. In the short term, a high dose of 5mg/kg LPS (*i.p*), causes gradual decrease in TH immunoreactivity starting 3 hours post injection and reaching a maximum decrease after 12 hours accompanied by reactive MG appearance (Reinert et al., 2014). Furthermore, another approach to systemic administration is through intravenous (*i.v)* injection which ultimately results in morphological activation of microglia, neutrophil infiltration, and increased mRNA/protein expression of inflammatory mediators in the SN. Such effect can be seen shortly within 4-8 h post injection, and subsides within 1-3 days. Interestingly, TH loss is not detectable until 8 days after the injection (Jeong et al., 2010). Therefore, the microglial activation and induction of inflammatory mediators can precede dopaminergic neuron loss shedding light on the temporal relation between induction of inflammation and the loss of these cells. Moreover, the systemic LPS administration even though it causes brain inflammation, it does not necessarily drive

dopaminergic cell death in the SN (Jeong et al., 2010). In other words, the acute induction of systemic inflammation does indeed trigger inflammation in the SN, but this may or may not be sufficiently toxic to induce neuronal injury in that region.

3. Intra-nigral or intra-striatal LPS

Direct injection of LPS in the SN leads to progressive loss of SN dopaminergic neurons. A single injection of LPS can lead to up to 50 % loss of DA neurons. The effect and damage in the SN is both time and concentration dependent. Usually, LPS is injected as a single low dose into the nigrostriatal pathway or the striatum or the SN regions to assess the role of inflammation in PD. The effect on the SN neurons can be induced by an LPS injection of as low as 0.5µg/2µl which results in a 20% loss of TH-positive neurons. Higher concentrations of 2.5µg/2µl and 5µg/2µl result in higher and more permanent damage in the SN presented as a 45% and 95% loss of TH positive neurons respectively (Bin Liu, 2000).

The LPS causes a prominent activation of MG in the SN which is far more sensitive to the inflammatory stimulus than the striatum. Performing the LPS injection in the striatum creates a less intense activation of MG compared to the SN with no loss of TH immunoreactivity at any time point. The early hours post-injection are associated with significant increase in mRNA levels of TNFα, iNOS, IL-10, and GAD65 with differences in the latency and percentage of increase between the SN and striatum, further proving that the SN is more susceptible to inflammatory changes than the striatum (Pintado et al., 2011). Unilateral intra-nigral injection of LPS in higher dosages (10µg/2µl) leads to localized microgliosis and neurodegeneration of the nigrostriatal pathway in addition to spontaneous stable motor deficits (Hoban et al., 2013). On the

contrary, applying this injection in the striatum instead of the SN, does not lead to nigrostriatal degeneration but results in localized microgliosis and only transient motor dysfunction. Interestingly, intranigral administration of LPS does not induce any microgliosis in the striatum despite the nigrostriatal lesion extending to that site. As for LPS (2µg/2µl) applied in the medial forebrain bundle, it was shown to have no effect on TH immunoreactivity in both the SN and the striatum with no associated glial activation (Herrera et al., 2000).

Neuroinflammation-induced mitochondrial malfunction is one suggested pathway involved in the progressive degeneration of nigral dopamine neurons (Choi et al., 2009). Interestingly, upon introducing inflammatory pathogens, only the dopaminergic neurons in the SN gets affected while the GABAergic and serotonergic neurons remain intact. In addition to *invivo* models, LPS's effect on dopaminergic and MG cultures have been studied extensively to determine dosage dependent effects and specific mediators for the occurring damage.

E. Aim of the Study

Neurogenesis in the dentate gyrus (DG) of the hippocampus is susceptible to changes. The present study was undertaken to examine whether an inflammation - induced unilateral lesion in the SNc, could affect the number of proliferating cells in the SGZ of the dentate gyrus in the adult rat brain. For this purpose, we studied the neuropathological and behavioral impact of unilateral intra-nigral administration of low dose of ET (2ug/2ul of sterile saline) by assessing its effect on certain motor behavior, on the dopaminergic and microglial cells in the SNpc and on neurogenesis in the DG.

The SN and DG are both highly sensitive to inflammatory stimuli where the SN has a substantial amount of microglia and the hippocampus expresses inflammatory mediator receptors such as IL-1β in high density (Green & Nolan, 2014). Previous reports state that intranigral, or systemic injections of ET have an effect on dopaminergic neurons and microglia in the SN in a dose dependent manner. However, their effect on neurogenesis in the hippocampus has not been investigated in those studies. Therefore, we hereby present an evidence for effect of acute inflammation in the SN on neurogenesis in the hippocampus.

CHAPTER II

MATERIALS & METHODS

Adult female Sprague-Dawley rats (250-300g) were used in the experiments and all experimental procedures were approved by the Institutional Animal Care and Use Committee at the American University of Beirut and followed the ethical guidelines for experimental pain on conscious animals. 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. Intranigral injection of endotoxin (ET)

The head of the anesthetized rat was firmly fixed on a stereotaxic frame. The skin of the scalp was shaved and a small skin incision was made to expose the skull bone to allow needle penetration at the stereotaxic coordinates. Using a stereotaxic frame, a hole was drilled into the skull (-5.5mm caudal to bregma and 1.5 mm lateral to the midline) and a 10ul Hamilton Syringe (Hamilton, Town state country), was used to inject 2µl of ET (Lipopolysaccharide from Salmonella typhosa; Sigma, St. Louis, MO, USA) or 2µl sterile saline in the right substantia nigra. ET was dissolved at a concentration of 2µg/2µl saline and 2ul were injected at a rate of 1µl/min. The needle

was left in place for 3 min after the injection before slow retraction. Sham rats received the equivalent volume of saline into the right SN. The injection was done at a position: caudal to bregma 5.5mm, lateral 1.5mm and vertical 8 mm, according to Paxinos and Watson's atlas (Watson, 2007) (Fig. 3 and 4).

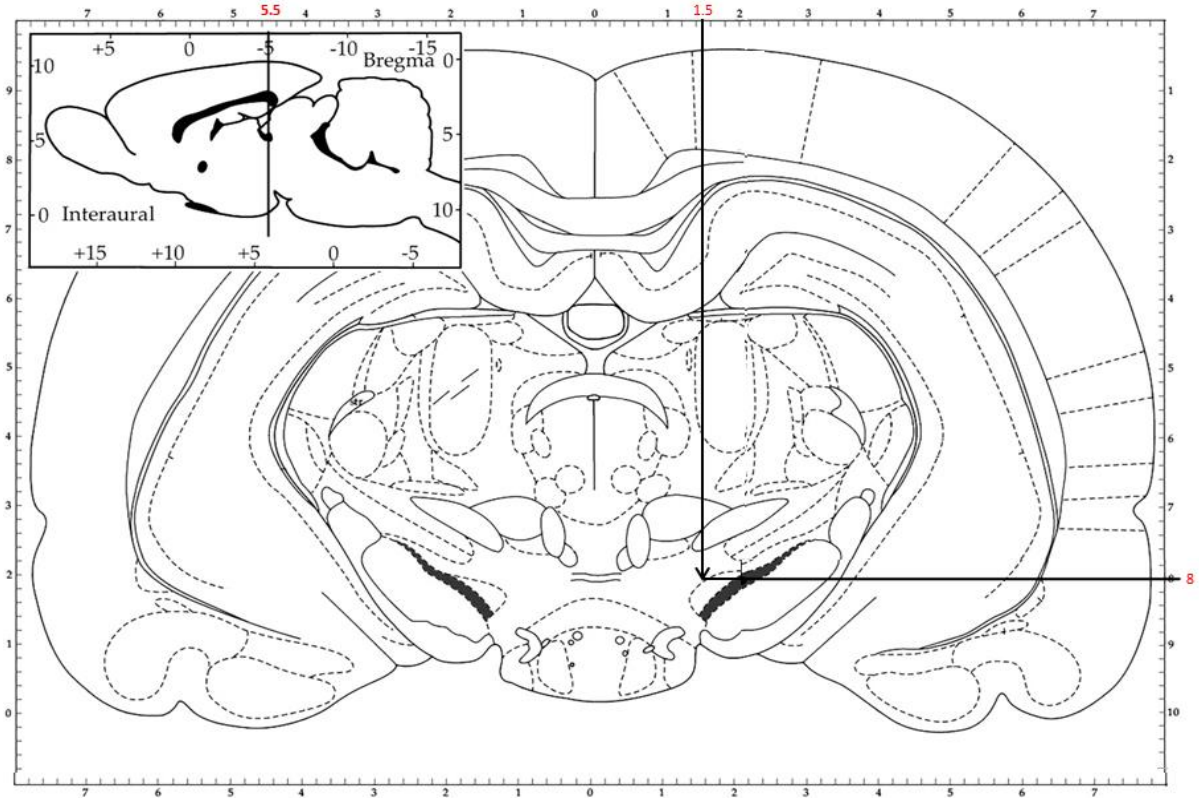


Figure 3: Stereotaxic coordinates of intranigral ET injection in the rat brain. Coordinates are: 5.5mm bregma, 1.5mm lateral, and 8mm vertical. Adopted and modified from (Watson, 2007).

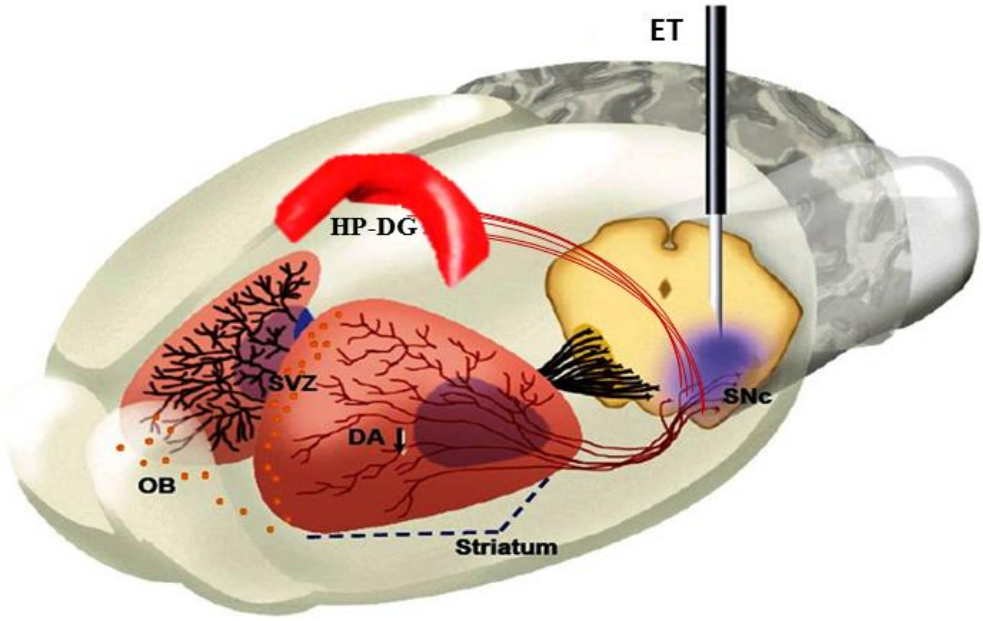


Figure 4: Needle insertion Position. A schematic diagram showing the insertion of the syringe injecting the Endotoxin (ET) into the substantia nigra (SN) of a rat brain and the connections of the SN that might be later involved in the effect. OB; olfactory bulb, DA; dopaminergic afferents, SVZ; subventricular zone, SNc; Substantia Nigra compacta, HP-DG; hippocampus-Dentate Gyrus, ET; Endotoxin. Adopted and modified from (Arias-Carrion, 2008).

B. Experimental Design

1. Experimental Groups

Rats were divided into three experimental sets according to the time of sacrifice following the intra-nigral injection. The first set was sacrificed at day 3, the second at day 6 and the third at day 9 post injection. Each experimental set had three groups distributed as ET, shams and Naïve rats with a total of n=5 in each group (Fig. 5). The ET rats received an ET injection, the sham received a saline injection instead while the naïve remained intact.

2. BrdU Administration

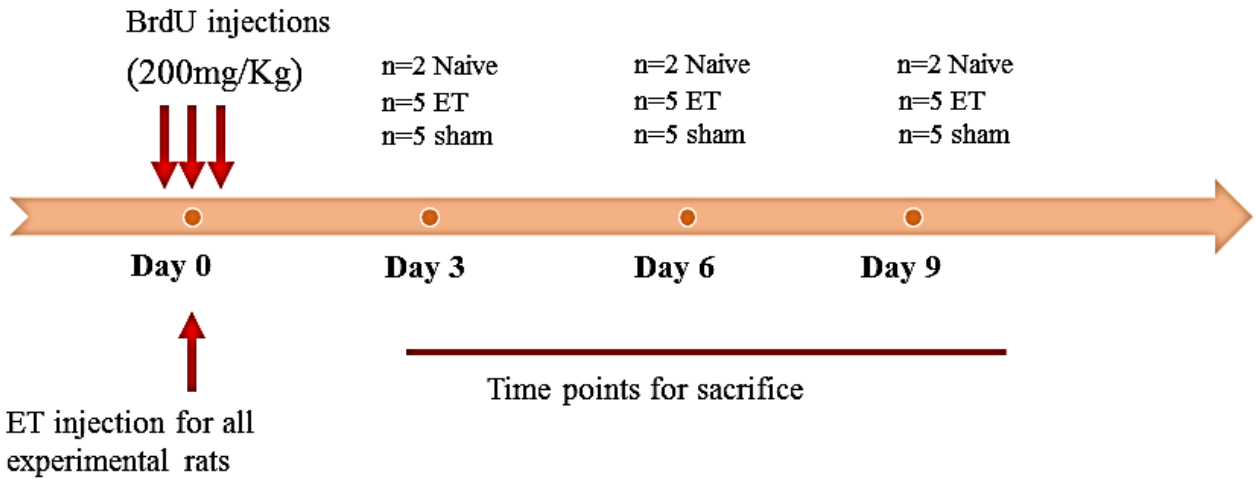


Figure 5: Experimental Design. The time points of injection (day 0) and sacrifice (days 3, 6 or 9).

Bromodeoxyuridine (BrdU) is a synthetic thymidine analog that gets incorporated into the DNA during the S-phase cycle of mitosis. BrdU signal 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. BrdU injections were administered on the same day of the surgical procedure. After intra-nigral administration of either saline or ET, all rats were injected with BrdU three times at a 2 h interval (66mg/kg/ 300µL/ injection, *ip*) to insure maximal availability.

C. Behavioral Testing

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 be familiarized with the testing environment. Two independent observers, of whom only

one was informed about the experimental protocol and the animal treatment, recorded the scores of the tests. For the behavioral tests, the rats of the different groups were combined and used on the respective days of the tests. The last time point for the tests had the least number of rats as most of them were sacrificed at their respective time points.

1. Rotarod

Motor Coordination and balance were measured starting 2 days after the surgical procedure, using a commercially available rat rotarod apparatus. All rats were pretrained for 1 day in order to reach a stable performance. Each rat was given three independent trials on the rod daily, each lasting for 120 seconds and separated by at least a 10 min inter-trial period. Rats were placed on the rod and the apparatus was turned on to a fixed speed of 5 rpm. The latency to fall off the rotating rod was recorded. The results were expressed as the retention time over the three test trials and the average of the 2nd and 3rd trials was *taken. The test was performed on days 2, 3 and 6 post injection.*

2. Cylinder test

Forelimb use during explorative activity was analyzed at 2 days post-surgery. Rats were placed individually in a glass cylinder (20 cm diameter and 20 cm height). Only weight bearing wall-contacts made by each forelimb were recorded. Cylinder wall exploration was expressed in terms of the percentage of unimpaired ipsilateral forelimb (left) wall contacts relative to the total of contacts made by either forelimb (Right +

Left). The following formula was used to calculate the laterality preference, with reference to the side of the lesion, manifested by each animal:

% of ipsi-laterality= (Right wall contacts/ (right + left wall contacts))*100

(Schaar, Brenneman, & Savitz, 2010). This test was performed on days 2, 3, 6 and 8 post injection.

D. Animal Sacrifice

At 3, 6 or 9 days after the nigral injection, rats were deeply anesthetized and perfused trans-cranially with 200ml of 0.9% saline followed by the same volume of 4% formalin. Brains were removed and post-fixed in 4% paraformaldehyde (PFA) overnight and later switched to a 30% sucrose solution in PBS for cryo-protection and stored at 4°C until impregnation which requires about 3 days.

E. Tissue Processing

1. Collection of the hippocampus

Perfused brains were sectioned coronally at 40μm intervals using a freezing microtome. The right side of the brain, site of injection, was marked by a small pierce for comparison with the intact left side. Sections were sliced serially from the rostral to the caudal extent of the DG of the hippocampus following the rostro- caudal coordinates of -2.12 to -6.6 mm relative to bregma. The hippocampus was topographically divided into 3 sub regions; rostral, intermediate and caudal following the coordinates of -2.12 to -3.7 rostral, -3.7 to -4.9 intermediate and -4.9 to -6.3 caudal to bregma (Chamaa et al.,

2016). Systemic sampling of the brain sections was achieved following the fractionator method for unbiased stereology (Hof, 2007; Schmitz et al., 2014) (Fig. 6). Following this method, sections were collected in six wells, and each well contained a specific number of slices designated to the region (rostral, intermediate and caudal). The 1st section was placed in the first well, the 2nd section in the second well and the 6th in the sixth well and this was repeated so that the 7th section gets placed in the first well and the difference between the 1st and 7th section would be 300µm. Consequently, each well represents a random depiction of each topographic area of the hippocampus (Chamaa et al., 2016). Therefore, a random well was picked from each region for immunofluorescent staining. 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. All sections were collected in well plates containing 15mM sodium azide dissolved in PBS.

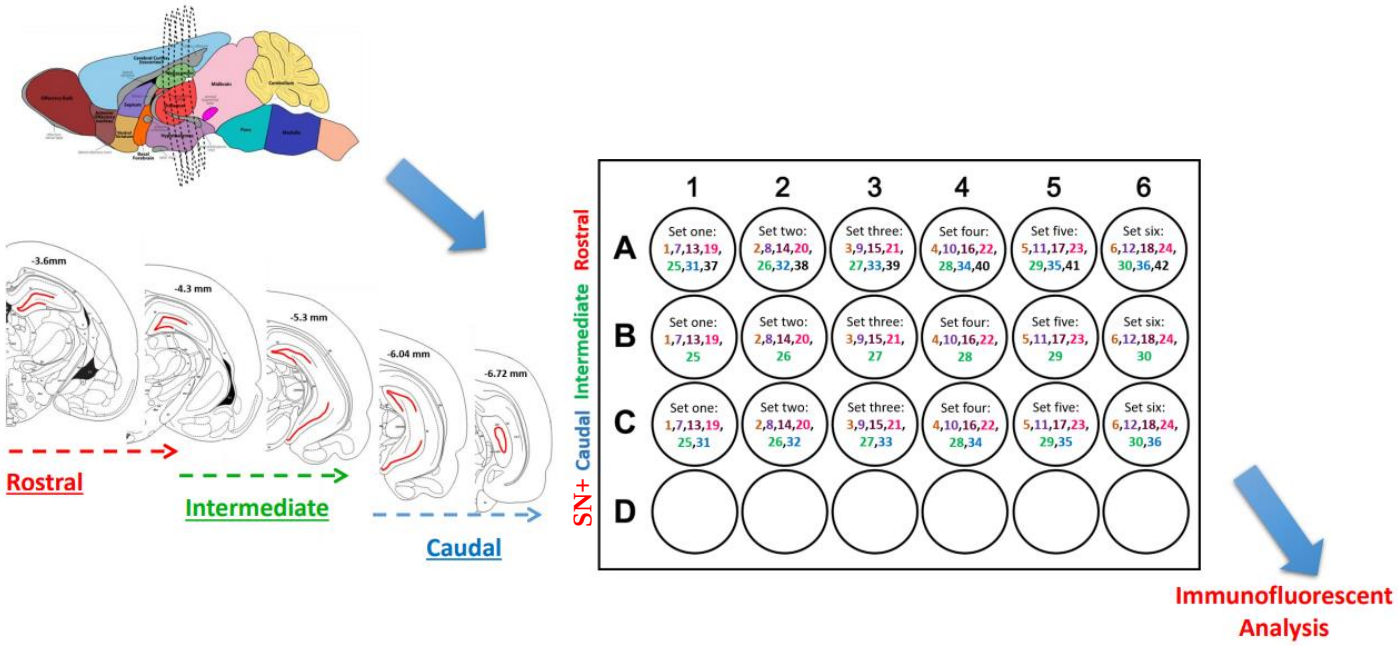


Figure 6: The fractionator Method: free floating 40µm coronal sections were distributed in a 24-well plate based on the topographical region (rostral, intermediate, caudal). The SVZ region was taken as well for future studies and examination. The Caudal region of the HP in this way contains the SN region. The numbers in the wells designate the number of the section that is put in the well while cutting a brain.

2. Collection of the substantia nigra

The substantia nigra coordinates of -4.52 to -6.30 mm relative to bregma were included within the intermediate and caudal coordinates of the hippocampus. Thus, the entire region of the SN was taken in the wells of the intermediate and caudal areas with the same principle of the fractionator method and 40µm sections applied. Representative random wells were chosen from each region for immunofluorescent staining of the SN with tyrosine hydroxylase.

F. Verification of Needle Insertion Position

A 40µm free floating section was selected from the site of needle injection from each ET and sham rats. Cresyl Violet staining was performed on the sections and these were later observed on a light microscope (Fig. 7).

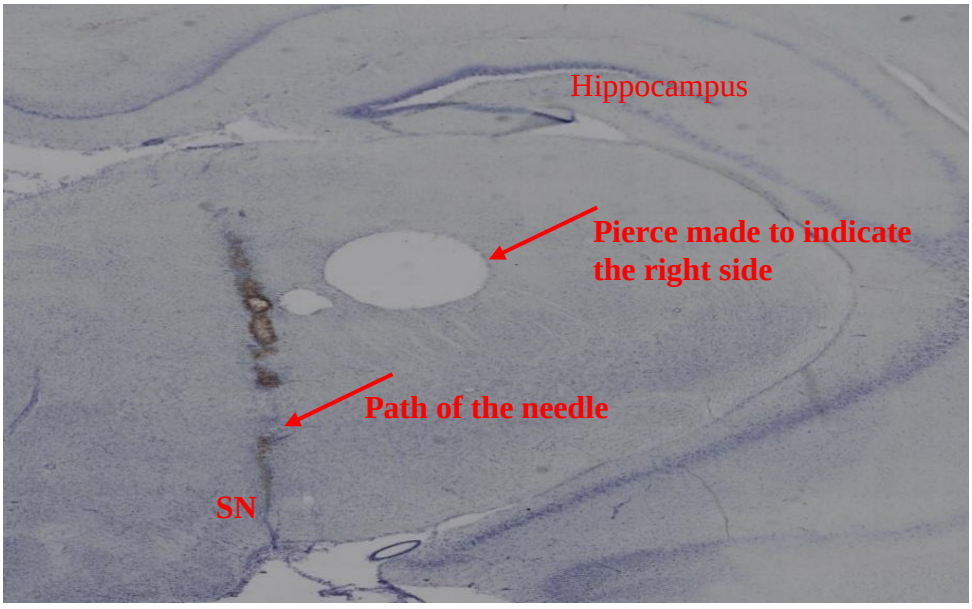


Figure 7: Cresyl Violet stain for needle insertion verification. An image showing the path of the needle in a section collected from an ET treated rat at day3 post-surgery

G. Immunofluorescence

1. Staining for BrdU positive cells

One representative well of each topographic region was chosen randomly. The free-floating sections were washed three times with 0.1 phosphate buffer saline (PBS) for 5 minutes each. Tissues were then incubated at 37°C with 2N HCL for 30 minutes to denature DNA so that the primary anti-BrdU antibody can have access to the incorporated BrdU from the previously administered animal injections. Following denaturation, a washing step with PBS was followed by sodium borate administration (0.1 M, 8.5 PH) for 10 min at room temperature to neutralize the previously added acidity. Tissues were again washed in PBS 3 times for 5 minutes each and later transferred to a 10 % blocking solution (10% NGS, 10% BSA and 0.1% Triton X diluted in PBS) for 1 hour at 4°C to minimize non-specific bindings. The samples were then directly incubated with primary rat BrdU antibody (1:100; Bio-Rad) and kept overnight at 4°C. On the next day, tissues were washed three times with 0.1 M PBS for 5 minutes and were later incubated in the dark with the secondary goat anti-rat 568 (Alexa Fluor 1:200, Invitrogen). The tissues were kept in secondary antibody on a shaker for 2 hours and later were washed three times with 0.1M PBS before being transferred to the second primary antibodies; mouse NeuN (1:400, Millipore) and kept overnight for the same procedure as followed for the BrdU. The secondary antibodies used were goat anti-mouse (Alexa Fluor 1:250, Invitrogen) and goat anti-rabbit (Alexa Fluor 1:250, Invitrogen). All primary and secondary antibodies were diluted in 3% block (3% NGS, 3% BSA and 0.1% Triton X). Hoechst stain (1:10000, Invitrogen) was added for 10 min followed by three 5 min washes. Finally, sections were mounted on

slides with anti-fade mounting media without DAPI and covered with thin glass coverslips.

2. Staining for dopaminergic neurons and microglia

Random wells were chosen from the intermediate and caudal designated wells containing the desired SN region. These sections were stained for TH and Ox-42; a dopaminergic neuronal marker and a microglial marker, respectively. No HCL pre-treatment was required and the tissues were directly washed, blocked and transferred overnight to the primary antibodies; chicken TH (1:500, Abcam) and mouse Ox-42 (1:50, BD Pharmingen). Secondary antibodies used for these were: goat anti-chicken (Alexa Fluor 1:250) and goat anti-mouse (Alexa Fluor 1:250).

H. Cell Stereology and Confocal Microscopy

1. BrdU Quantification

To quantify the number of stem/progenitor cells in the SGZ, BrdU positive cells where counted using confocal microscopy on the 40X-oil objective. The counting was done on sections from the chosen representative well, therefore, the final number of BrdU positive cells was multiplied by 6 (the number of representative wells) to estimate the full count in the whole DG of the hippocampus. 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. The right versus left BrdU counts were also summed and compared to each other. Data were represented as the % of the BrdU positive cells in the experimental ET rats from the shams of the same group. Moreover, % of the laterality of the effect, as in the % of BrdU in the right hippocampus versus the left, was

calculated as such: (the BrdU count in the right/BrdU count in the Right +Left) *100 and same for the left side and these were compared.

Confocal microscopy (Zeiss LSM 710) was used for acquiring images and data quantification. BrdU counts in the DG as well as image capturing was done using the Zeiss ZEN 2009 image-analysis software. Tile scan and Serial z-stacks for BrdU with maximal projection intensity were taken at 40X oil objective.

2. TH and Ox-42 Quantification

Confocal microscopy was also used for acquiring tile scans of the SN region from 4 or more representative sections of the region for each rat. Images were taken for TH, DAPI and Ox-42 under the same laser and microscopic parameters for consistency. Quantification of the TH cells was done using Image J software. TH was represented as the % of the right SN TH counts over the left (% Laterality of TH= number of TH positive cells in the right SN/number of TH cells in the left*100). As for Ox-42, the signal intensity for Ox-42 was calculated in the region of the SN and the area measured was the same for all SN sections.

I. Statistical Analysis

The behavioral results and labelled cell counts (BrdU, TH and Ox-42) were analyzed at every time point and expressed as the mean (X) ± the standard error of the mean (SEM). Determination of the statistical significance was made using student t-test for comparison between the sham and experimental groups. Repetitive measure ANOVA was used to compare the performance of the ET injected rats in the behavioral tests within the tested time points. A P value of <0.05 was considered significant

difference. Plotting of figures were performed using Prism 5 GraphPad package (GraphPad Software, Inc., CA, USA). All statistical analysis were performed using the SPSS v. 20 statistics software.

CHAPTER III

RESULTS

A. Behavioral observations

1. The effect of Intranigral ET injection on motor coordination

To study motor performance and coordination, the Rota Rod test was used to monitor the latency of falling off the rod. Day 0 represents the basal level of performance of the rats which was tested before the injection. A prominent effect on performance was noticed at day 2; ET-injected rats showed a significant lower latency to fall (41.41 ±7.31 sec; $P<0.0001$) compared to the sham group (101.30 ± 4.21 sec). The same rats had an improved but still poor performance at day 3 (60.61±14.76 sec; $P=0.002$) compared to the sham. By day 6, ET-injected rats reached a stable performance (107.11±4.74 sec; $P=0.416$) similar to that of the sham (Fig. 8). The number of rats within the ET groups are variable because some rats were sacrificed at their respective time points. Repeated measure ANOVA with sphericity assumed determined that the mean ET latency differed significantly between the time points ($F(2, 4)=7.144$), $P=0.048$).

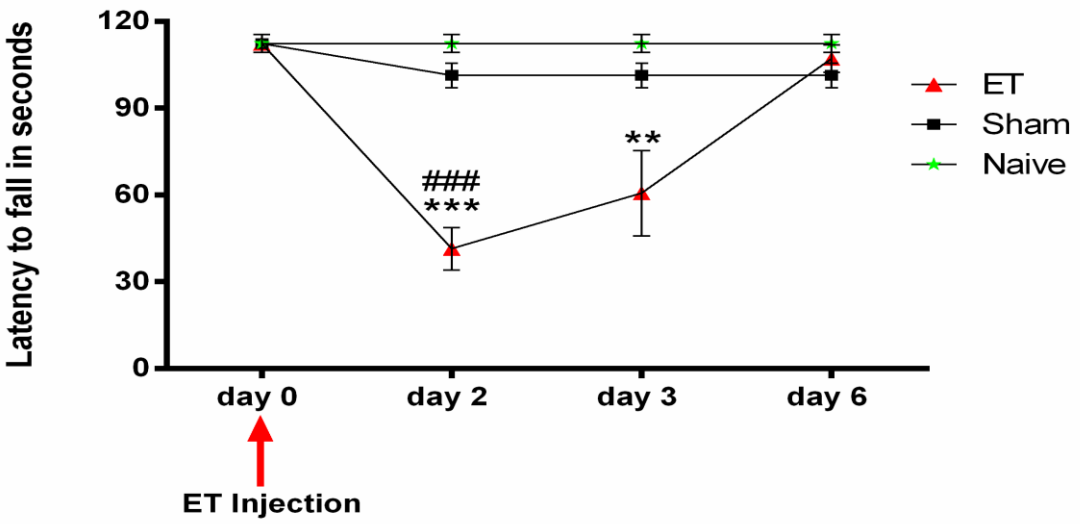


Figure 8: Motor Coordination task performance. Two days post-surgery, ET injected rats (n=12) had a significant much lower latency to fall compared to shams (n=15) and Naives (n=13). There was a slight improvement in performance witnessed at day 3 (n=3). At day 6, the ET rats (n=7) showed a rebound in performance back to the basal levels seen in the shams and naïve. Significance with reference to sham is noted by * and with reference to naïve as #.

2. The effect of Intranigral ET injection on forearm laterality

The cylinder test was performed to check for any laterality effect or bias towards using one forearm over the other; the ipsilateral forearm (right) or the one contralateral (left) to the site of injection. Naïve rats had a stable performance recoding a 52.29% ± 3.33 laterality at all time points and this percentage was detected in all animals prior to surgery as well. After surgery, the shams had a similar performance to Naïve (59.36% ± 2.96). ET injected rats had a constant significant bias for using the ipsilateral right forearm at all days of recording; days 2 (81.36% ±4.67; $P<0.0001$), 3 (84.62% 4.36; $P<0.0001$), 6 (82.93% ±4.76; $P<0.0001$) and 8 (97.56% ±1.38; $p<0.0001$) (Fig. 9). Repeated measure ANOVA with Greenhouse-Geisser correction determined that the

mean ET ipsilaterality did not differ significantly between the time points (F (1.149,

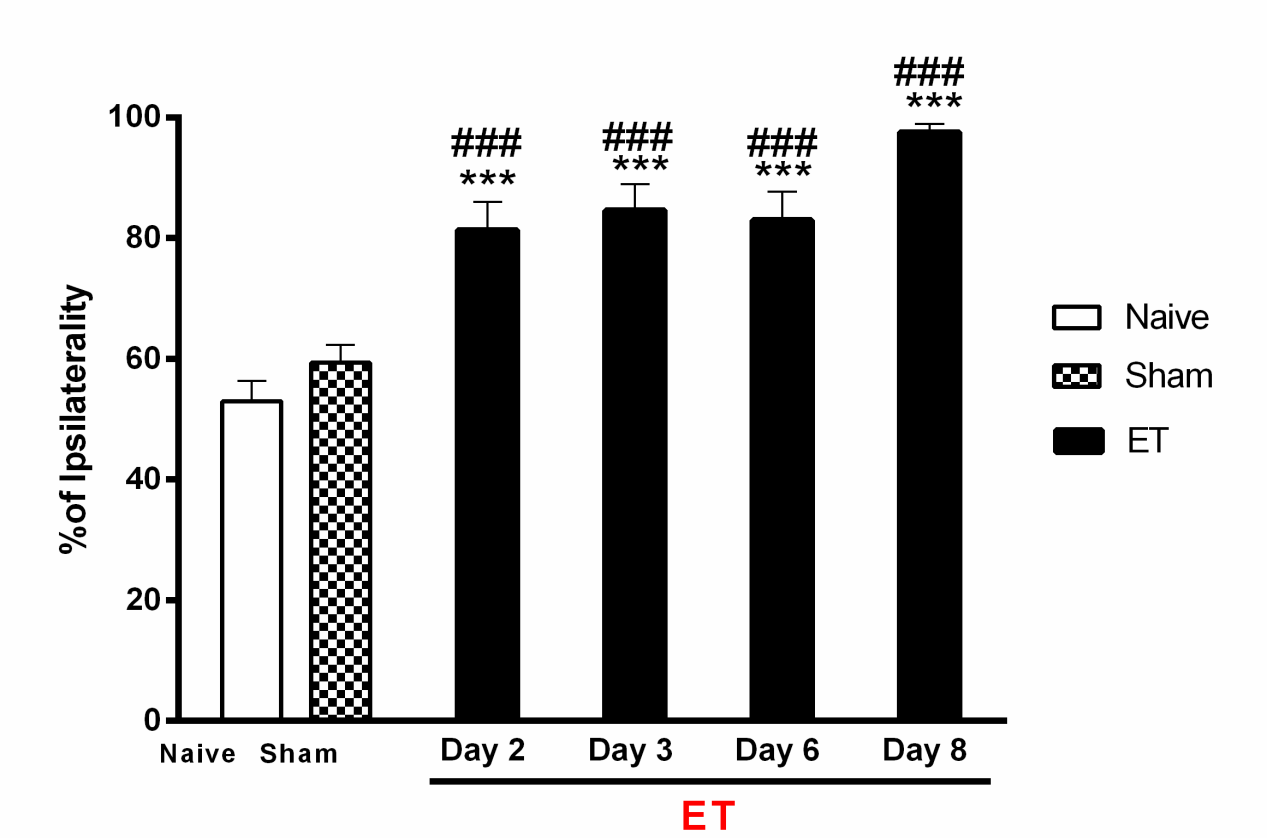


Figure 9: Percentage of ipsilateral forelimb use. Rats injected with ET recorded more than 50% laterality for right forearm use at all experimental days, day2 (n=14), day 3 (n=12), day 6 (n=8), and day 8 (n=3). There is no significance between the sham (n=22) and naïve (n=25). Significance with reference to sham is noted by * and with reference to naïve as #

8.041) =0.035), P=0.885).

B. Effect of intranigral ET-injection on the SN

1. A Decrease in the number dopaminergic neurons at day 3 post injection

In the vehicle injected animals, tyrosine hydroxylase (TH) staining in the SN was the same in the ipsilateral and contralateral sides of the injection. However, at day 3, the ET injected rats recorded a 25.54 ± 1.13 % decrease in TH cells in the right SN compared to sham right SN and a 22.56 ± 1.13 % decrease compared to the left SN of

the ET. This decrease in TH was restored almost back to normal (sham values) at day 6 and day 9 (figure 10 and 11).

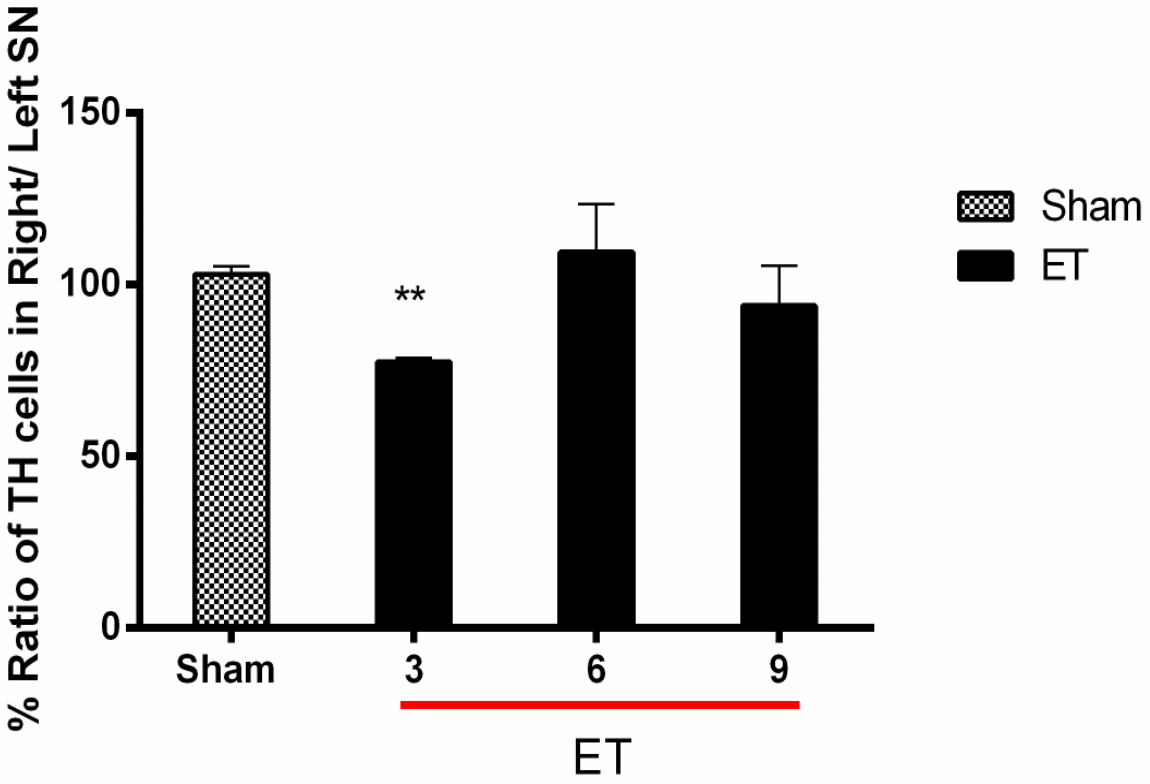
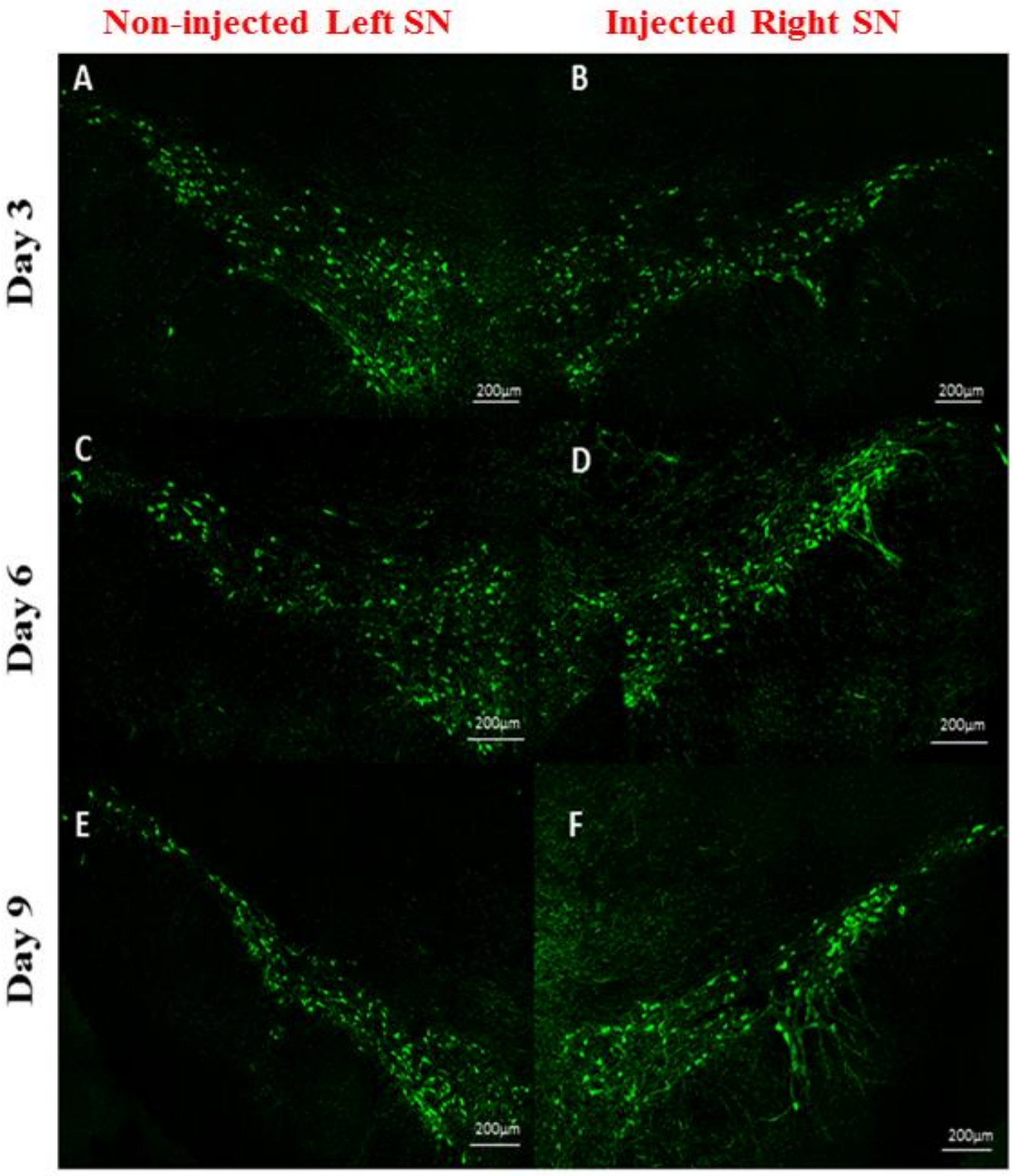


Figure 10: Effect on TH in the ipsilateral side at day 3. A marked decrease in % ratio of TH in the right injected SN seen only at day 3 compared to sham ($P<0.01$, n=4 ET rats). TH % ratio in the injected SN is restored at days 6 and 9 to a similar ratio to that of sham.


Non-injected Left SN
Injected Right SN
Day 3
Day 6
Day 9
A
B
C
D
E
F
200μm
TH

2. Microglial Activation

Two procedures were followed to assess MG activation; this consisted of observing the morphology of the Ox-42-positive cells and the measurement of the signal intensity in the right versus left SN. MG activation was evident at day 3 post injection by a striking overall shift from a ramified MG structure characterized by several extensions to a structure that is circular-like and described in the literature as amoeboid (Fig. 12A & B).

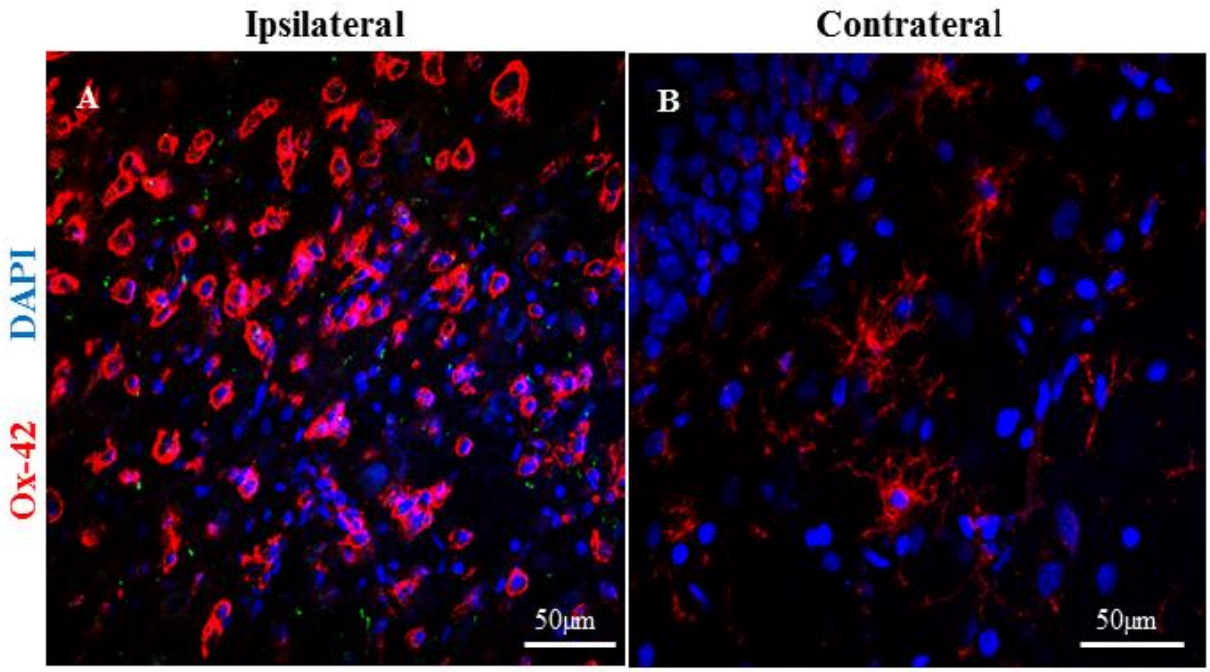


Figure 12: Activated and non-activated microglia at day 3. Immunofluorescent showing double labeling of the microglial marker (Ox-42) and the neuronal marker (DAPI) in the injected SN (A) and non-injected side (B).

The amoeboid form of MG appeared only in the right SN ipsilateral to the injection and almost all MG cells had this form with only a few ramified cells being present at the site if any at all (Fig. 13). Meanwhile, the left SN had no change in the structure of MG in all the sections that were stained for all the rats in that group (Fig. 14). Furthermore, the density and number of MG cells appeared higher in the injected SN than the non-injected (left). Therefore, to add further validation of increased MG activation in the injected SN, in addition to the observed morphological difference, we compared the signal optical intensity of Ox-42 MG marker at day 3 for both the injected and non-injected SN. The comparison showed a striking increase in the intensity of Ox-42 in the injected SN (52.34% ± 11.17) in a constant area measured for all SN sections (area=495,541.5± 108.16). Moreover, activated MG were also present along the needle tract at all the time points studied. By day 6 and 9, MG retains back its ramifications with only a few traces of the amoeboid MG near the SN (Fig. 15 and 16).

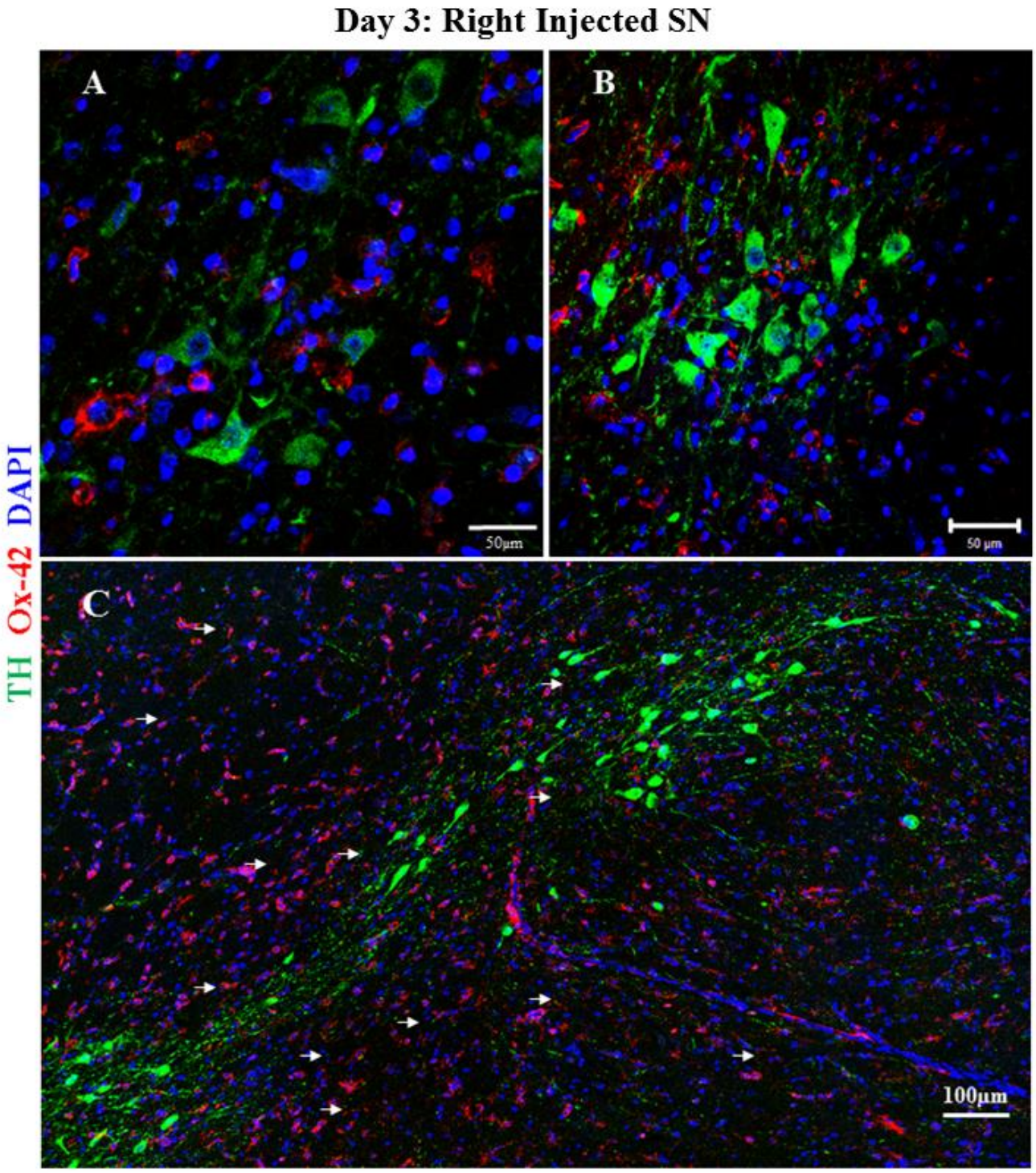


Figure 13: Activated MG in the injected SN at day 3. (A) and (B) are snap images in the right SN region showing activated MG with amoeboid structure and its co-localization with DAPI (blue) and TH for DA cells (green). (C) A tile scan representing an overview of the right SN housing the activated MG. White arrows indicate the position of some activated MG.

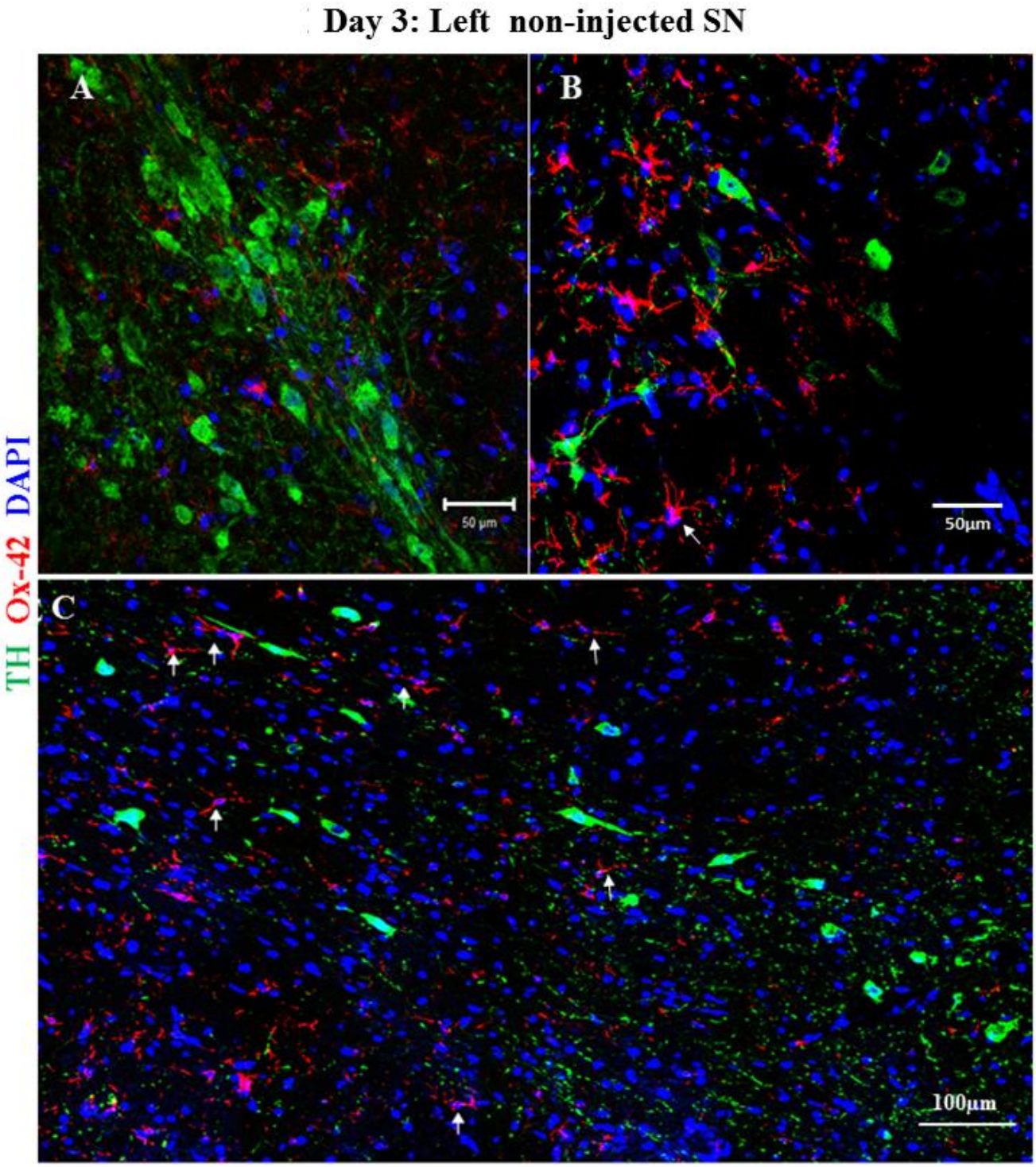


Figure 14: Absence of microglial activation in the left non-injected SN at day 3. (A) and (B) are snap images in the non-injected SN showing the ramified structure of MG. (C) A tile scan representing an overview of the MG cells in the non-injected SN. White arrows indicate position of some non-activated MG.

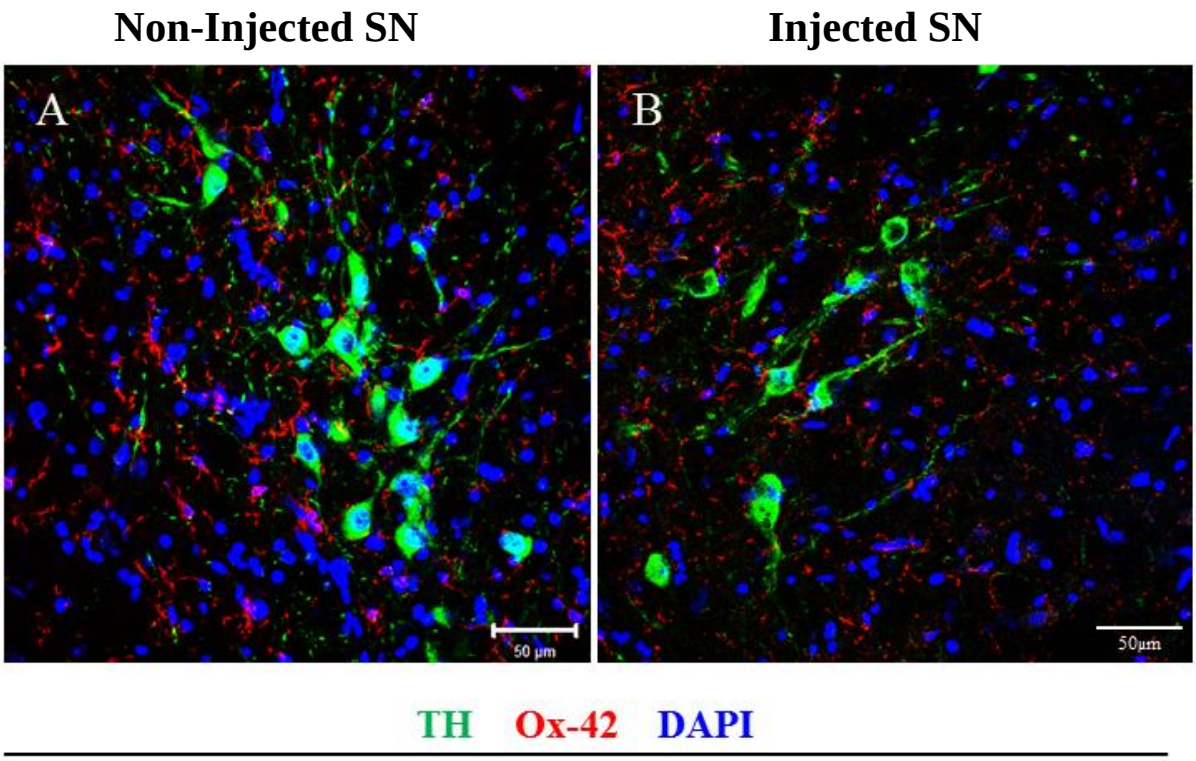


Figure 15: Microglia at day 6. (A) A snap image in the SN region showing a ramified structure of MG (red) and its co-localization with DAPI (blue) and TH (green). (B) A snap image in the right injected SN showing non-activated MG.

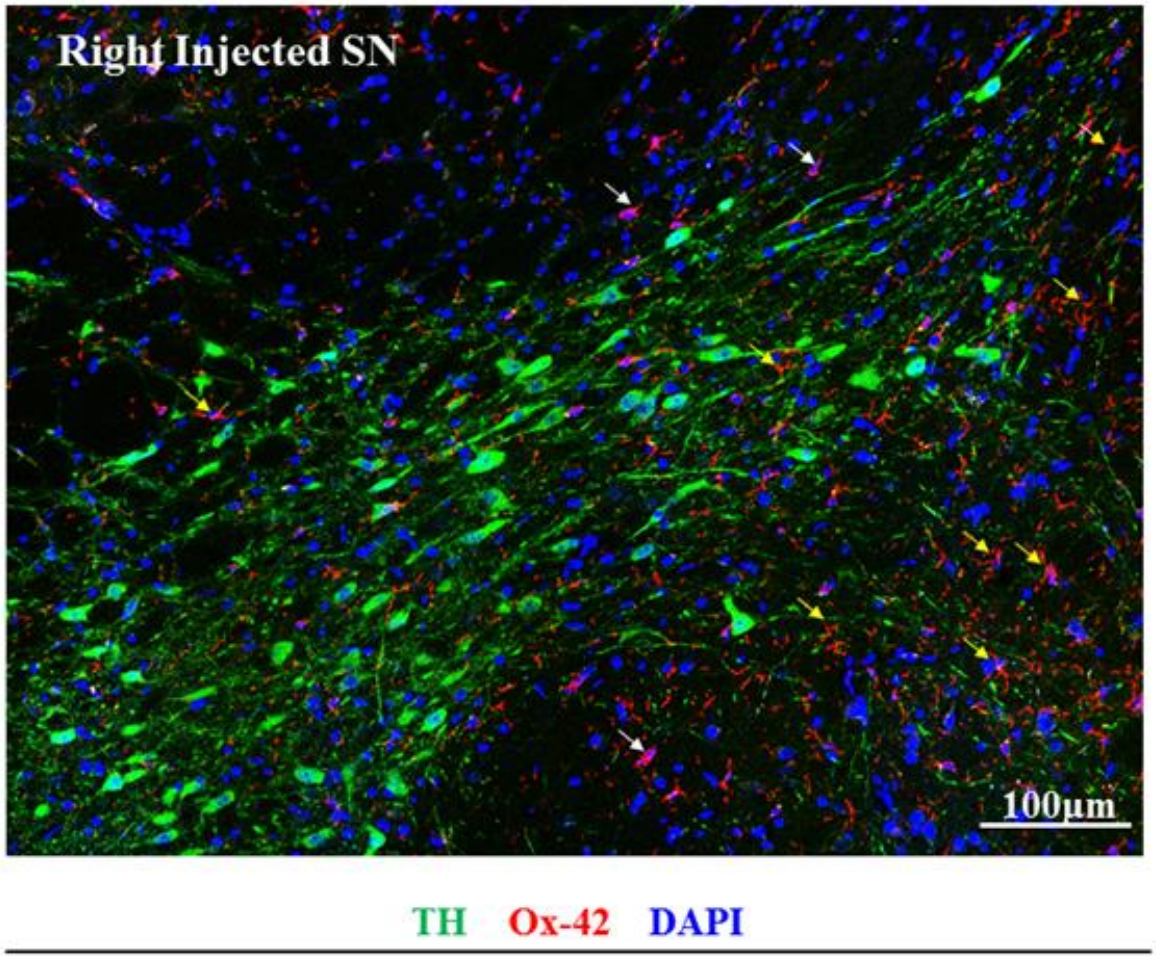


Figure 16: Microglia at day 9. Tile scan of right SN showing microglial ramified structure (red) at day 9. White arrows indicate traces of amoeboid MG and white arrows indicate ramified MG.

C. Effect of ET-intranigral injection on Hippocampal Neurogenesis

1. Global effect on hippocampal Neurogenesis

A single ET intranigral injection of 2µg/2µl leads to a prominent decrease in adult hippocampal neurogenesis at day 3, 6 and 9 post injection (Fig. 17). The most prominent effect was seen at day 3 (62.80± 5.78%) compared to the sham (115.27± 7.38%, P<0.0001)) and naïve (100± 7%, P<0.001). At day 6, the % of total BrdU positive cells of ET rats (70.88± 4.66%) was also significantly lower compared to the shams of that day (113.88± 10.29%) and to naïve (100± 7%, P<0.01). This effect persisted till day 9 and the number of BrdU positive cells in the ET group (78.83± 3.16%) was still significantly lower than the shams (108.84± 10.11%, P<0.01) and the naive (100± 7%, P<0.01). There was no significant difference between the shams of the different days when compared to the naïve (Fig.18 A and B). All in all, there was a total decrease in neurogenesis of 52.47% (Fig.19A) at day 34.3% at day 6 (Fig. 19B) and 30.01 % at day 9 (Fig. 20) compared to the shams of these days. Compared to the naïve, the decrease in neurogenesis is 37.2 %, 29.12 %, and 21.17% at days 3, 6 and 9 respectively. In either way, the pattern of decrease from day 3 till day 9 is relatively maintained.

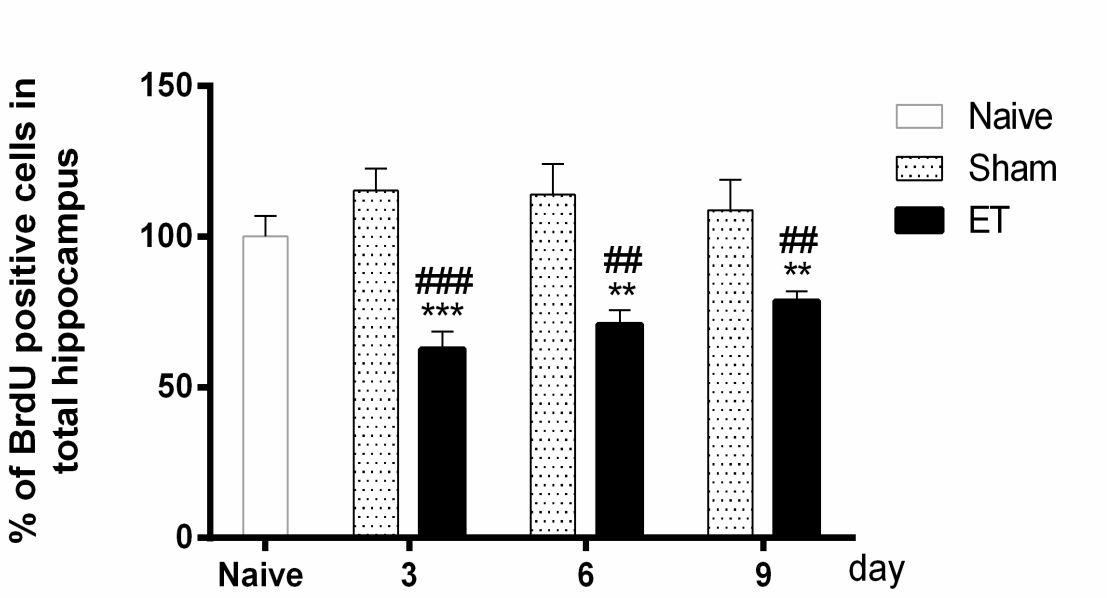


Figure 17: Time course of BrdU alteration in the DG of the ET injected rats. Neurogenesis is significantly reduced at days 3 (n=5), 6 (n=6) and 9 (n=5) following the ET intranigral injection. Each bar represents the average ± SEM of BrdU % in the different groups at the indicated time. The noted significance was made with reference to the shams (*) and the naives (#). There was no statistical significance between the shams (n=5 per group) of the different days and the naives (n=7).

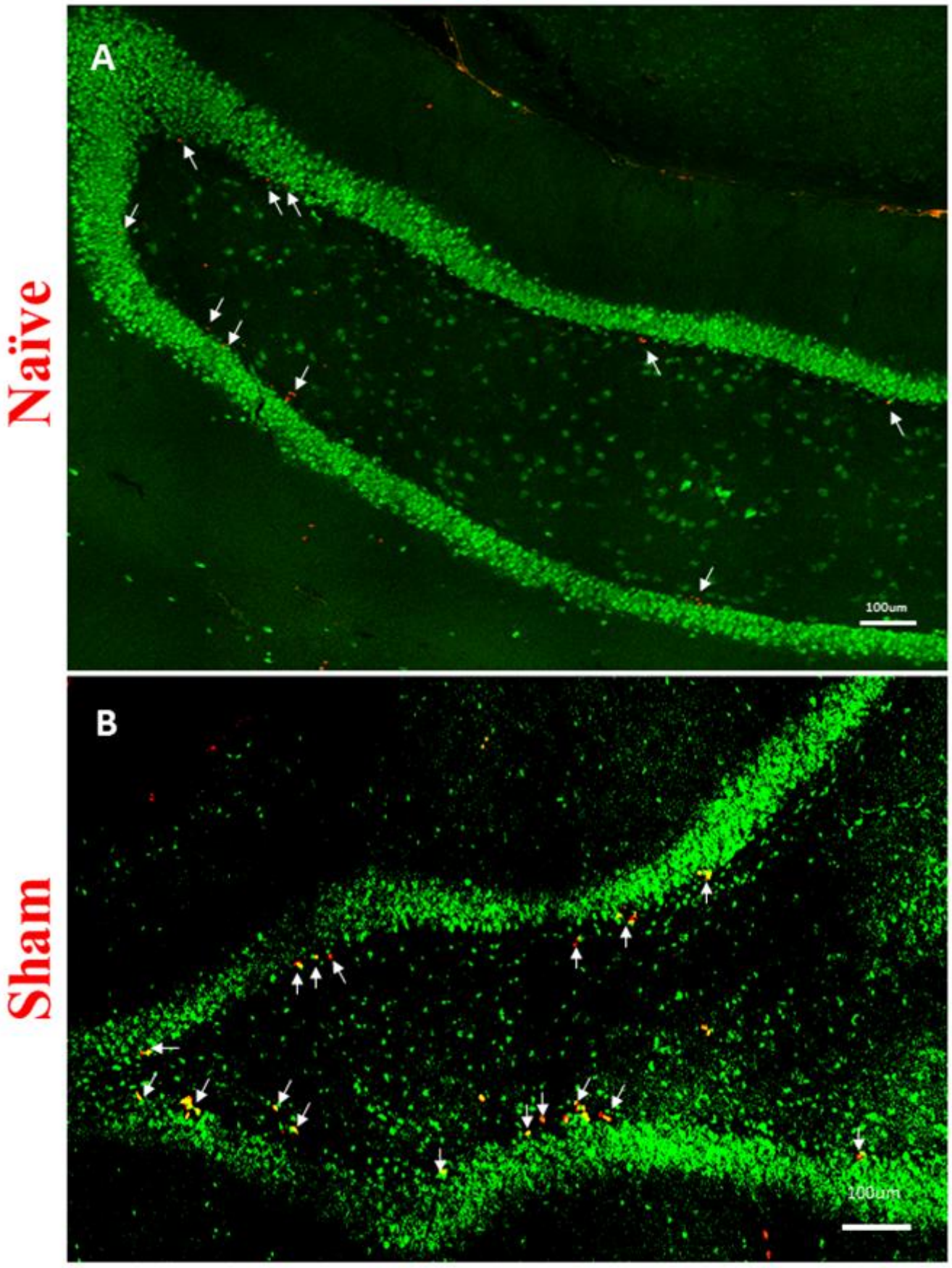


Figure 18: Basal Level of NPC proliferation in the DG of the adult rat hippocampus. Immunofluorescence labeling of the DG by the Neuronal marker NeuN (Green) and BrdU (red) for proliferating cells showing the basal level of proliferation in naïve (A) and sham (B) groups. White arrows indicate the location of BrdU positive cells. Tile scan and z-stacks taken at 40X oil objective.

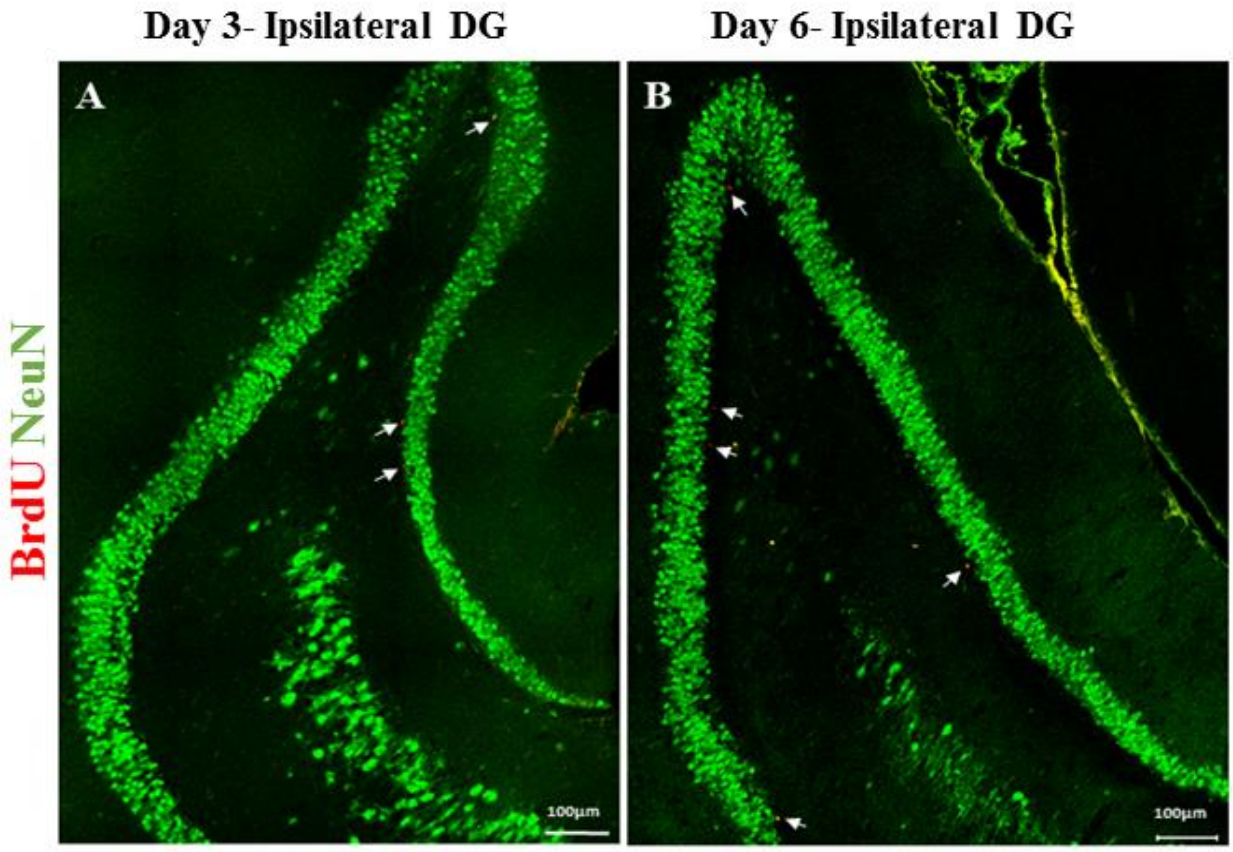


Figure 19: Decrease in Neurogenesis at day 3 and 6 post injection. Tile scan-Z-stack for the DG of the Caudal hippocampi of ET injected rats of day 3 (A) and day 6 (B). White arrows indicate the position of BrdU positive cells (red) on the SGZ stained with NeuN

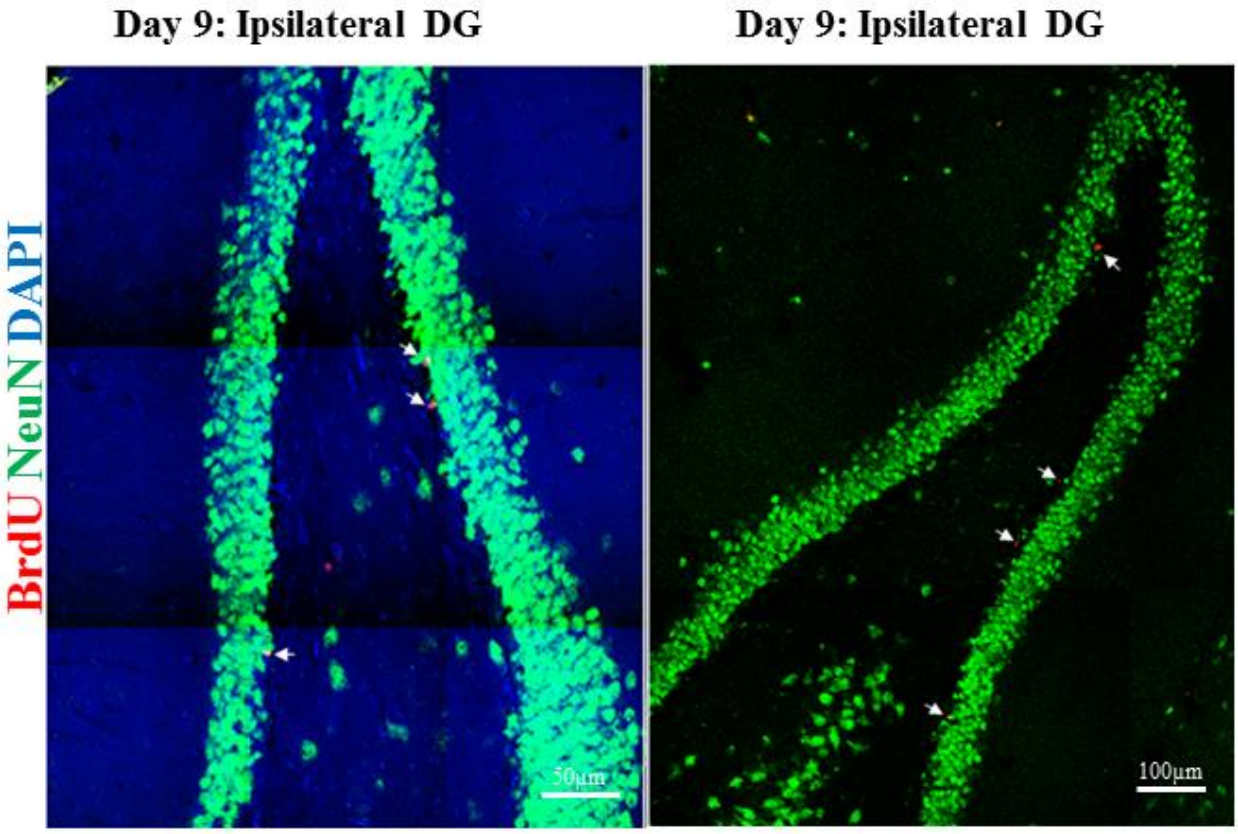


Figure 20: Decrease in Neurogenesis at day 9 post injection. A tile scan-z-stack view on a Caudal (A) and rostral (B) DG at day 9. BrdU (red), NeuN (green) and DAPI nuclear specific stain (blue). The image with Dapi (A) is an example of confirming BrdU identity with Dapi double labeling.

2. Laterality of the ET effect on neurogenesis

Graphs of figure 21 show the % distribution of BrdU positive cells in the right ipsilateral and left contralateral hippocampi in each group. The comparison is made with reference to the shams of the same group and the naïve rats. The right DG of ET-injected rats is compared to the right sham and right naïve. The left DG of ET-injected rats is compared to the left sham and left naïve. Overall, there was a significant decrease between the right ET DG and the right naïve at all 3-time points and the same for the left DG.

Furthermore, to investigate whether there is a preference for decrease in the ipsilateral DG, the two DG sides of the same ET group were compared to one another. The overall effect on neurogenesis affected both the right and the left sides of the hippocampus and therefore showed no laterality. At day 3 (Fig. 21A), the percentage of BrdU positive cells was similar in the right (58.78 ± 8.59 %) and left (62.18 ± 8.18 %) hippocampi and showed no significant differences between the two sides. When compared to the sham and naïve, these numbers were lower by approximately 40 %. As for day 6 (Fig. 21B), the % of BrdU positive cells in the right (71.08 ±6.08 %) and left (79.68 ± 6.51%) DG were also similar with no significant difference between the two sides. However, at day 9 (Fig. 21C), there was a significant difference between the % of BrdU positive cells in the right (73.05± 3.48%) and left DG (85.24± 4.78 %) reflecting a preference for decrease in the ipsilateral side.

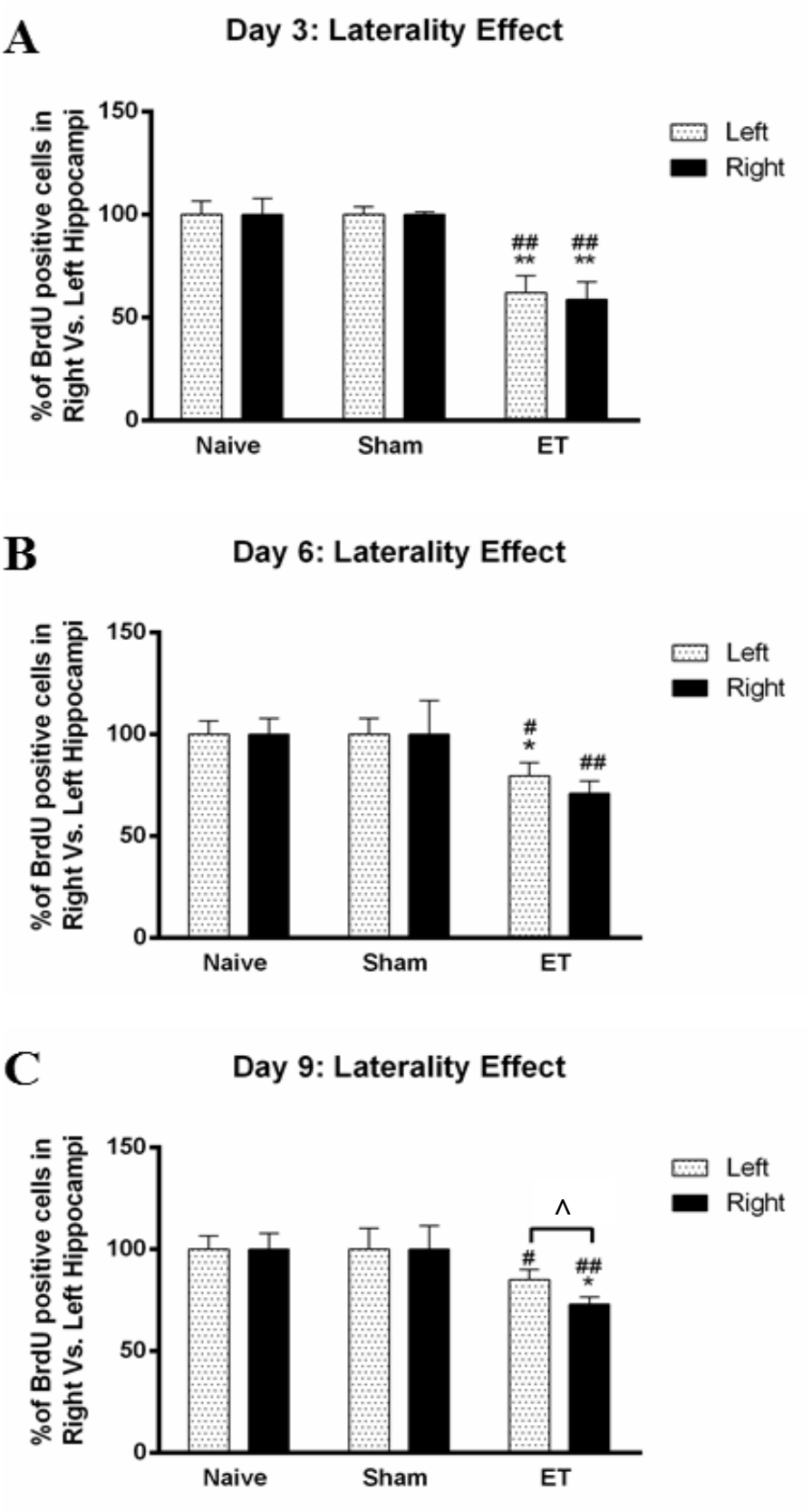


Figure 21: BrdU % distribution in the right (ipsilateral) and left (contralateral) hippocampi at days 3 (A), 6 (B) and 9 (C). # and * marks the significance of the same side with reference to the naïve and sham respectively. The significance between right and left of ET are marked with ^ above a line. Results are expressed as % ± SEM

D. Effect of ET intranigral injection on hippocampal microglia

Since we witnessed a significant effect on hippocampal neurogenesis following the ET inflammation, we suspected whether microglial cells could have mediated this remote response. Therefore, we observed the morphology and signal intensity of Ox-42 positive cells in the ET-injected rats in both dentate gyri and compared these to the sham sections. All microglia were ramified in structure. There was absolutely no presence for any amoeboid structured microglia. There was a notable increase in the number of microglia in the right and left hippocampus compared to the sham sections (Fig. 22). Furthermore, the intensity of Ox-42 was compared at day 3 for the right and left dentate gyrus. The Ox-42 signal intensity in the right dentate gyrus at day 3 (28.87±3.18) was much more significant and intense than that in the left dentate gyrus (18.38± 1.74; P=0.007) (Fig. 23).

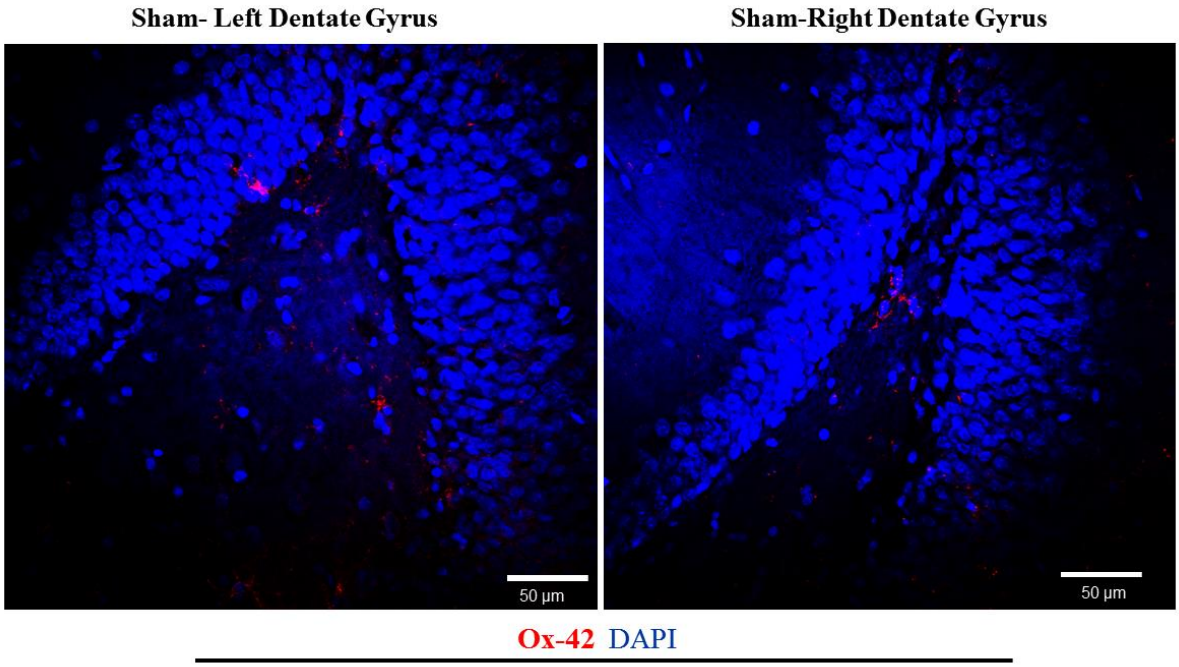


Figure 22: Microglia in the sham dentate gyrus. Sections were stained with Ox-42 and with DAPI. Snap images were taken using a confocal microscope.

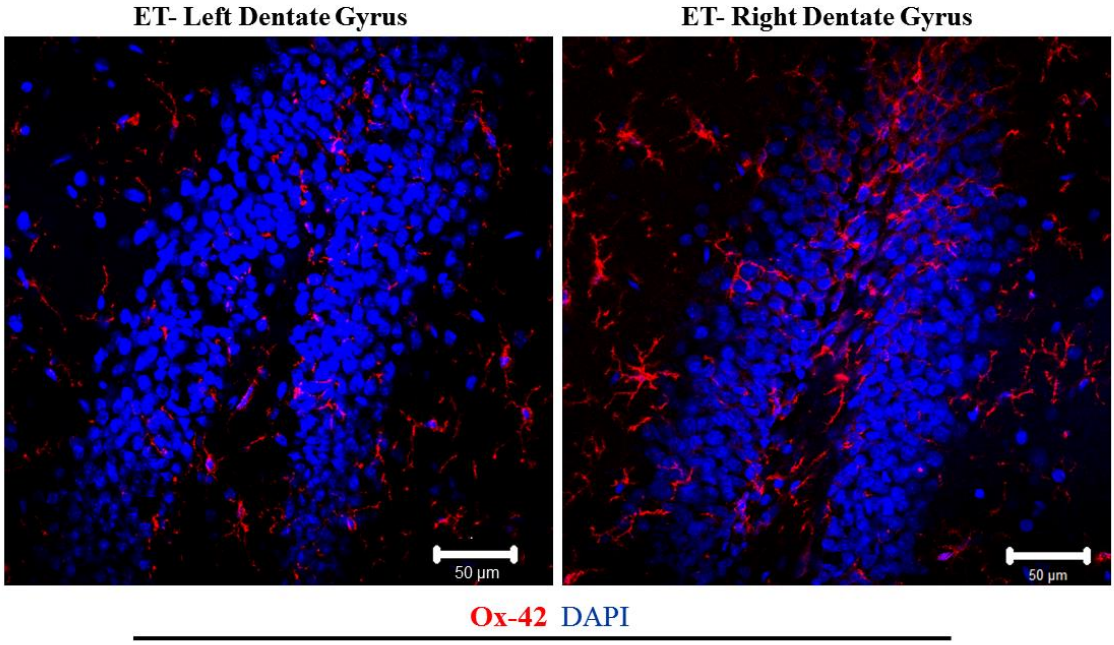


Figure 23: Microglia in the dentate gyrus of ET injected rats. Sections from day 3 group were stained with Ox-42 and with DAPI. Snap images were taken using a confocal microscope.

CHAPTER IV

DISUSSION

The present study was undertaken to examine whether an inflammation-induced unilateral lesion in the SNc, could affect the number of proliferating cells in the SGZ of the dentate gyrus in the adult rat brain. Here, we demonstrate that endotoxin (ET) - induced inflammation in the right substantia nigra (SN) causes an ipsilateral partial loss of dopaminergic neurons, activates microglia in the site of injection, and impairs neurogenesis on both sides of the dentate gyrus of the hippocampus.

Following the injection, we needed to establish the efficacy of the model to affect the SN and therefore motor behavior. Motor performance and motor laterality were evaluated by conducting Rota Rod and Cylinder test, respectively, on the rats. The effect seen on the Rotarod did not persist and the impairment was only partial. This is probably due to the low LPS concentration that was used (2ug/2ul) but may also be due to learning and training that leads to improved performance at later time points. As for the laterality effect tested in the cylinder test, there was a significant bias and dependence towards using the ipsilateral forearm and this effect lasted till day 8 post-lesion. The observed ipsi-laterality indicates a possible weakness in the left contralateral forearm. This makes sense as the contralateral side of the body would be affected following a central lesion to the right side. The laterality effect seen in the cylinder test continues even beyond the restoration of the tyrosine hydroxylase (TH) positive cells seen at days 6 and 9. However, behavioral observations may not always directly reflect observations at the cellular level. Although we are targeting the SN by this model, this doesn't make it a PD model and the behavioral symptoms that were observed in either

the Rota rod or the cylinder test were rather parkinsonian-like symptoms and are not of any replicative feature of PD.

TH stain was used to detect dopaminergic neurons in the SN. TH is the rate limiting enzyme that converts tyrosine to L-DOPA (L-3, 4-dihydroxyphenylalanine) and is considered as a standard for detecting dopaminergic neurons. Analysis of the TH-positive neuron count in the SN shows that at day 3 there was a significant decrease in the number of TH positive cells in the SNc ipsilateral to the lesion. This effect was not seen on the contralateral side and the observed decrease was abolished by day 6 and 9. Therefore, the ET had a mild effect on the dopaminergic cells of the SNc that soon recovered rapidly 6 days after the injection. This could indicate that the cells that were lost at day 3 did not necessarily undergo cell death but rather were dysfunction or might have exhibited a downregulation in TH expression that may have been induced by changes in the milieu of the SN imposed by the ET injection. Therefore, the loss of dopaminergic cells was not progressive possibly owing to the low dosage of ET that was administered. The loss of TH cells and their subsequent restoration is similar to what is present in the literature when using similar concentrations of ET injection (Bin Liu, 2000; Castano et al., 1998). Another thing to suspect regarding the restored TH signal is a process of new neuron generation in the SN. However, the concept of new neuron generation in the adult SN is still a matter of controversy (Frielingsdorf et al., 2004; M. Zhao et al., 2003). Zhao *et al.* suggested that newly born cells in the SVZ would migrate along the ventral midline to the injured SN, where they will differentiate into dopaminergic cells. However, this migration-differentiation process of the newly born cells to TH expressing cells would take at least 3 weeks. Therefore, the restoration

of TH expression by days 6 and 9, was probably independent of any neurogenic restorative process.

A hallmark of neuroinflammation is microglial activation. Microglia are the resident immune cells of the CNS and accordingly they have surveillance and phagocytic properties. A striking feature of MG is their extreme plasticity and fast activation in a very short time following an insult or injury. We applied Ox-42 staining to check for inflammatory microglial reaction taking place in the SN post injection. The Ox-42 antibody recognizes the CR3 receptor (CD11B/ CD18) that is expressed by microglia in rodents. The microglia exhibited a ramified appearance with extensions and processes in both the right and left SN of the sham rats at all time points that were studied. This morphology was maintained in the left contralateral SN at day 3 post-injection. However, in the right SN, we observed that Ox-42 positive microglia adopt an amoeboid structure which is typically indicative of their activation as there was almost a complete absence of ramified microglia in the right SN, thereby; implying a possible full activation of these cells and preparation for engulfing activity. It is important to note that it is possible that some of these cells might be infiltrated macrophages as these cells are also stained by Ox-42, but these would not replace the microglia. If the microglia weren't activated we would expect to see a mixed population of ramified microglia and circular macrophages, but this was not the case. Interestingly, the microglial shift in structure and activation in the right SN at day 3 was accompanied by an ipsilateral decrease in TH positive cells on that same day. This suggests that microglial phagocytic activity and/or inflammatory mediator secretions might be involved in the marked cell or signal loss. Furthermore, the activated microglia (amoeboid) were compactly surrounding the lesion reaching to the SN and were even

spread to nearby regions but not the hippocampus. At days 6 and 9 post injection, the dominant morphology of microglia in the right SN was ramified with only a few cells that were still amoeboid in structure. The return of microglia to their ramified state was associated with a recovery from dopaminergic cell loss. All that might imply that preventing microglial activation might be an intervening therapeutic target in neurodegenerative diseases such as PD. The transformation of microglia to an amoeboid state comes in agreement with previous reports on microglial activation followed by a particular time point of recovery that depends on the concentration of ET used (Gao HM, 2002).

In the literature, applying unilateral injections of ET seem to be the cause of progressive dopaminergic neuronal loss in the SN due to chronic inflammation as well as sustained microglial activation (Reinert et al., 2014). Microglial activation is a process involved in inflammation-driven neurodegeneration starting from an early stage. The microglial activation could be explained as an inevitable event due to the high density of microglia in the SN that makes it more susceptible to ET inflammation (Kim et al., 2000). In previous studies, microglial activation was reported to precede the neuronal degeneration but does not always necessarily mean that the dopaminergic cells will undergo cell death. In this model, it could be that the microglia were activated in the hours after the surgery or even at day 1 or day 2, preceding dopaminergic cell loss at day 3. In the 6-OHDA injection model in the SN, which directly induces apoptosis of the dopaminergic neurons, analysis of microglia and dopaminergic cell death time points show that microglia get activated for phagocytic functions before the induction of dopaminergic cell death and these activated microglia apparently attach to the morphologically normal TH-expressing cells (Marinova-Mutafchieva et al., 2009).

The remote effects of this transient and localized nigral inflammation were translated by the decrease in neurogenesis that seemed to be recovering with time. Therefore, our data showed that inflammation in the substantia nigra has an inhibitory effect on basal levels of neural stem cell proliferation in the DG of the hippocampus. This might be due to the high expression of inflammatory receptors in the hippocampus (Green & Nolan, 2014). In addition, there is a significant number of MG residing there that might have favored and mediated the suppression in neurogenesis. The observed decrease in neurogenesis can be correlated with the important direct and indirect dopaminergic supply and effects on the hippocampus. One way of describing the events is that the inflammation in the SNpc affected the afferents projecting from the right injected SN to the ipsilateral hippocampus. The functions of these afferent connections to the hippocampus, which include but are not limited to synaptic endings from dopaminergic neurons, could have been modified. The inflammation could have disrupted the communication between the cells in the SN and subsequently changed the firing pattern of the afferents to the hippocampus. For the dopaminergic afferent connections in particular, the insult could have affected their level of dopamine secretion. Any possible changes occurring in the DG of the hippocampus are sensed by the microglial cells residing there. Microglia are important for nurturing neural stem cells and mediating the communication between them. We noted an increased number of microglial cells in the right ipsilateral hippocampus. This indicates either an increased level of proliferation and/or microglial cell migration from nearby locations to the hippocampus. Microglial proliferation is one way through which these cells can react to the changes in the DG milieu. The increase in microglia was noted on both sides

which supports the bilateral effect seen. These microglia are not amoeboid in both hippocampi probably because the ET is not present there, so the microglia will not be phagocytosing. The noted decrease in neurogenesis can be due to a disruption in the communication between the cells and the release of pro-inflammatory factors such as IL-1, IFN-γ and TNF-α or anti-inflammatory factors such as IL-10 and TGF-β. For example, an increased release of TNF-α could lead to ceramide-dependent NF-κβ-mediated apoptosis (Duty & Jenner, 2011).

Moreover, since the injection was applied unilaterally targeting only the right SN, we were interested in checking whether the observed effect would be ipsilateral to the injected side. Therefore, we compared the number of BrdU positive cells counted in the right and left DG of each rat and checked whether there was bias towards one side over the other. Although, motor and cellular manifestations of the nigral inflammation were unilateral, the hippocampal effects were bilateral with slightly delayed predominant unilateral effect. We found that the effect was almost equally and homogenously distributed in both the right and left DG except on day 9, when there was a significant bias towards a decrease in the right DG over the left. This bias was there from the start but was not statistically significant. Perhaps increasing the number of rats might show this glimpse of laterality at earlier time points and with statistical significance. The laterality, however, does not mean that the entire decrease would be on one side, rather that it would be distributed in both DG but with a tendency to decrease to a larger extent on one side over the other. However, with the 6-OHDA, the effect on neurogenesis appears to be specific to the ipsilateral SGZ and/or the SVZ (Suzuki et al., 2010). The bilaterality of the effect could be because the hippocampus is part of the limbic system where the concept of lateralization no longer stands. Laterality

in the limbic system becomes more diffused. This is not like motor laterality in the case of SN lesioning. In motor laterality, the motor pathways are well known and are connected in such a way that there is a motor decussation at the level of the medulla and crossing over of the pathways to the contralateral side. In addition to that, in the contralateral hippocampus, there was also a notable increase in microglial cells. This contralateral effect could have been mediated by the connections that the right hippocampus has with the left hippocampus. Thus, we can say that the bilaterality of the effect could have been mediated through the inter-hippocampal connections and the connections that the VTA (ventral tegmental area) of the injected side has with the contralateral hippocampus. Such connections might have played a role in translating the effect to the contralateral hippocampus. The connections of the VTA are an important factor especially that the injection was applied near the VTA region and we saw microglial activation in the right VTA, as noted by the increased intensity of Ox-42 and the amoeboid structure of the cells. In conclusion, the observed bilaterality of the effect can reflect the complexity and redundant circuitry connections of the limbic system.

To our knowledge, this is the first attempt to check for the effect of unilateral intranigral LPS injection on neurogenesis. Previous studies have shown that MPTP (Schlachetzki et al., 2016) or 6-OHDA (Suzuki et al., 2010) injections can lead to degeneration of dopaminergic neurons and impairment of neurogenesis in the hippocampus. In comparison with 6-OHDA injections, the latter causes faster and much intense microglial response and TH loss. A single unilateral injection of 6-OHDA into the rat SNc causes almost complete loss of TH immunoreactivity throughout the striatum and the SNc, as well as reductions in the number of TH-positive cells and fibers in the VTA. One would expect to observe an obvious widespread effect following

the administration of specific toxins that damage the dopaminergic supply to the brain. Our LPS/ET model, however, induced minimal effects on the SN unlike the drastic effects seen with the use of the 6-OHDA or MPTP models. This strategy presents the advantage of investigating the remote effects of acute, moderate and transient inflammation on hippocampal plasticity/neurogenesis. The LPS/ET model is considered to be a glial activation model because a key hallmark of PD is reactive microgliosis in the SN that accompanies the loss of dopaminergic cells there (McGeer et al., 1988; McGeer et al., 2008). Despite the limitations of ET model in how it can mimic PD, it is still a good strategy to study inflammation change in PD and how limiting the inflammation might limit the progression of the disease.

Epidemiological and post-mortem studies on PD brains have provided increasing evidence about the involvement of inflammation in the pathogenesis of the disease (Liu et al., 2006 ; McGeer et al., 1988). Sustained microglial activation and the accumulation of pro-inflammatory and neurotoxic factors are thought to be mediating the progression of the disease. The concept behind using ET to model certain aspects of PD is its specific targeted activation of microglia. The ET is a lipopolysaccharide that binds to CD14 located on the plasma membrane of microglia. Signal transduction occurs when the activation of the transmembrane toll-like receptor 4 (TLR-4) and the extracellular accessory protein MD-2. The activation of TLR-4 leads to activation of various intracellular pathways that eventually leads to kinases activation, upregulation of a variety of proinflammatory transcription factors and generation of free radicals. Since this process occurs in microglia and not neurons, this allows the ET model to represent and delineate the interaction between microglial activation and dopaminergic cell degeneration. This becomes of clinical significance as some brief exposure to

inflammatory incidents can predispose or directly drive dopaminergic cell degeneration. Studies show that people with episodes of septicemia have been shown to be more prone to develop PD than others (Fang et al., 2012). Furthermore, those who have encephalitis lethargica in early life, eventually develop parkinsonism later in their life (Vilensky et al., 2007). Inflammatory insults can also occur after traumatic brain injuries or even after stressful events and all these can be contributors to be risk factors of parkinsonism (Cruz-Haces et al., 2017). It has been shown that exposure to the bacterial ET generates a distinct strain of alpha-synuclein fibril, the protein that gets accumulated in brains of PD patients. The generated fibrils are self-renewable that consistently induces specific patterns of synucleinopathies in mice (Ren WQ 2016).

All in all, the recorded effects all support the validity of this model for the study of neuroinflammation on brain plasticity and functions. Our main concern wasn't to further characterize LPS injection as a model of PD, but rather to focus on the implications of inflammation by itself on dopaminergic neurons and on neurogenesis in the DG of the adult rat brain hippocampus. Therefore, what can be concluded is that silent and moderate neuroinflammation can have remote effects and impair brain function. The findings of this study help define the sensitive relation between a region that is critical for motor functions and another region that is critical for cognitive functions and memory. This could in part explain the memory deterioration symptoms that are present in some PD patients and in patients with lewy body pathology spreading in the SN.

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