AMERICAN UNIVERSITY OF BEIRUT

ELECTRICAL AND CHEMICAL MODULATION OF ADULT HIPPOCAMPAL NEUROGENESIS

by
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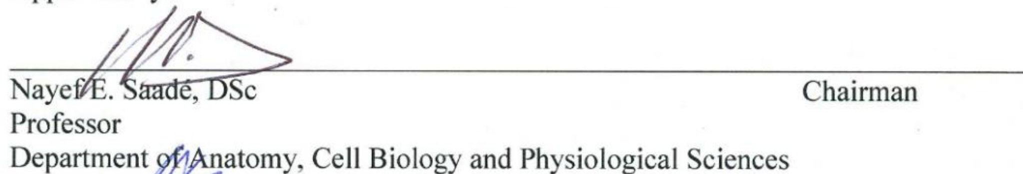

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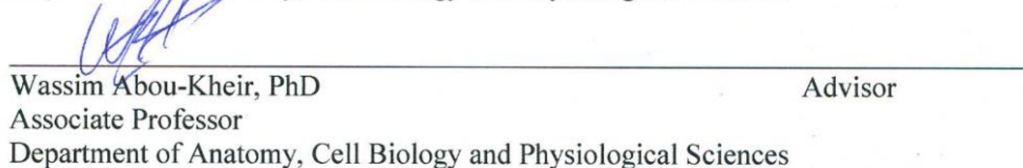

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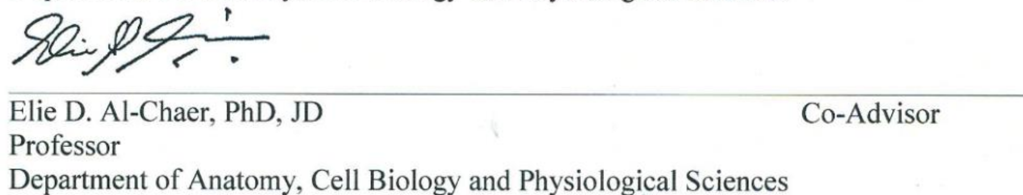

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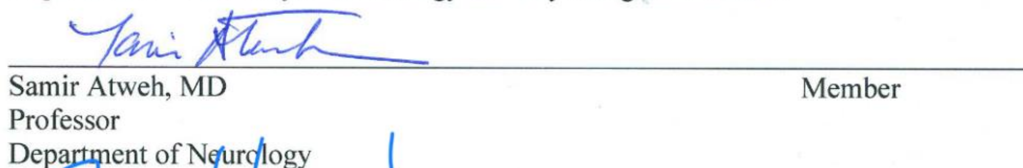

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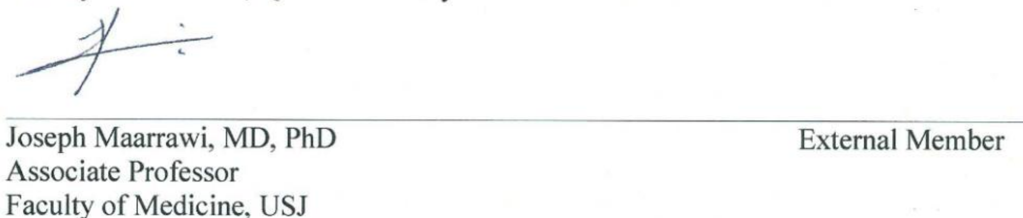

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AN ABSTRACT OF THE THESIS OF

Farah Othman Chamaa for PhD in Biomedical Sciences
Discipline: Neuroscience

Title: Electrical and Chemical Modulation of Adult Hippocampal Neurogenesis

Background: Despite the advances in technology that has steered clinical trials to new approaches and surgical modalities, the basic scientific knowledge of how these technologies work is still controversial. Deep brain stimulation (DBS), for example, has developed during the past 20 years, providing substantial clinical benefit for a variety of movement disorders such as Parkinson's disease, essential tremor and dystonia. It is, however, still unknown how DBS alters neural activity and the neuronal electrophysiology to induce beneficial outcomes. One area of interest is to investigate a possible role for modulation of adult hippocampal neurogenesis in mediating DBS effects. The hippocampus is a structural and functional component of the limbic system and is known to be a neurogenic niche containing neural stem/progenitor cells in the subgranular zone (SGZ) of the dentate gyrus (DG). It possesses extensive interconnections with the anteromedial thalamic nucleus (AMN) proposing that electrical stimulation of the AMN conveys physiological fluctuations to the hippocampus and possibly elicits neurogenesis. The specificity of the electrical signal is dubious, however, and it results in inadvertent stimulation to nearby regions and to the passing fibers. Therefore, a more specific targeting of the AMN cell bodies might be done through chemical stimulation. This is accomplished by using low doses of Kainic acid (KA), a direct agonist of the Glutamic Kainate receptors, to evoke sustained neuronal activation without causing seizures.

Objective: The aim of this study is to examine the effect of AMN stimulation in modulating adult hippocampal neurogenesis at early and later stages to follow the fate of stem/progenitor cells proliferation. This study includes single as well as multiple sessions of electrical stimulation of the AMN using two types of electrodes (cupper or platinum) and chemical stimulation or continuous KA micro-perfusion of that nucleus by an implanted mini-osmotic pump.

Methods: Adult Sprague-Dawley male rats were divided into five groups: the first group (n=6) received unilateral electrical stimulation in the right AMN, the second group is the sham group (n=5) that underwent surgery of electrode implantation with no current delivery. The third group received electrical stimulation in the ventral posterolateral thalamic nucleus (VPL nucleus) that has no known direct connections to the hippocampus. The fourth and fifth groups (n=5 each) received mini-osmotic pump implants in the right AMN that released KA (500pM) or saline, respectively, at a rate of 1µl /hr for 7 days. The groups of electrical stimulation were duplicated to use copper and platinum electrodes. All

groups received 3 injections (66mg/Kg/injection) of 5'-bromo-2'-deoxyuridine (BrdU) 4 days post-surgery and were euthanized on two time points. Early stages of neurogenesis were examined at 5 days post-surgery and the late stages after 4 weeks. The fractionator method was used together with confocal microscopy analysis to count BrdU positive cells in the dentate gyrus (DG) of the hippocampus at 5 days. Double labeling of BrdU with NeuN was examined at the later stages. The Novel Arm Exploration test was performed using a Y-maze to detect for enriched hippocampal skills following stimulation.
Results: Focal neurogenesis was induced in the ipsilateral DG 7 days after a single session of AMN electrical stimulation using copper electrode and it translated into a 76% increase in proliferation of neural stem/progenitor cells. A more prominent increase in proliferation was detected with the use of platinum electrodes which showed a 1.9-fold increase from the sham group. The increase was sustained at 4 weeks post-stimulation, where BrdU-positive mature neurons, co-labeled with NeuN, were 2.5 folds higher than the sham. The number of BrdU-positive mature neurons further increased to 3 folds after multiple sessions of stimulation. The VPL nucleus stimulated group showed no significant differences. Continuous micro-perfusion of KA increased the number of proliferating BrdU-positive cells to 1.6 folds more than the vehicle group at 1 week, and the mature neurons at 4 weeks were 4-folds higher than the vehicle. The Y-maze test showed that both electrical as well as chemical stimulation to the AMN enhanced Novel Arm Exploration at 4 weeks after stimulation.

Conclusions: The current study presents hippocampal neurogenic responses to electrical as well as chemical stimulations. It also reveals a translational behavioral enhancement of hippocampal-related skills following stimulation. This study highlights the importance of glutamic kainate receptor activation in the AMN nucleus in modulating hippocampal neurogenesis and suggests a possible role for kainate receptors activation in mediating DBS effects.

Keywords: Deep Brain Stimulation, Neurogenesis, stem/progenitor cells, Hippocampus, Chemical Stimulation, Kainate Receptors.

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LIST OF ABBREVIATIONS

AMN: Anteromedial Thalamic Nucleus

ANP: Amplifying Neural Progenitors

BDNF: Brain-Derived Neurotrophic Factor

BrdU: 5'-Bromo-2'-Deoxyuridine

CA: Cornu Ammonis

CNTF: Ciliary Neurotrophic Factor

DBS: Deep Brain Stimulation

DG: Dentate Gyrus

DHEA: Dehydroepiandrosterone

ES: Electrical Stimulation

FGF: basic Fibroblast Growth Factor

GC: Granule Cells

GCL: Granular Cell Layer

GCN: Granular Cell Neuron

GFAP: Glial Fibrillary Acidic Protein

HICAP: Hilar Commissural-Associational Pathway-related cells

HIPP: Hilar Perforant Path-associated cells

i.p.: Intraperitoneal

IPC: Intermediate Progenitor Cells

KA: Kainic Acid

LTP: Long-Term Potentiation

ML: Molecular Layer

NGF: Nerve Growth Factor

NGS: Normal Goat Serum

NMDA: N-Methyl-D-Aspartate

NSPCs: Neural Stem/Progenitor Cells

NT: Neurotrophins

OB: Olfactory Bulb

PBS: Phosphate Buffered Saline

QNP: Quiescent Neural Progenitors

RGL: Radial Glia-Like cells

SGZ: Sub Granular Zone

SOX-2: Sex Determining Region Y-box 2

SVZ: Sub Ventricular Zone

TAPs: Transit Amplifying Cells

tDCS: transcranial Direct Current Stimulation

TMS: Transcranial Magnetic Stimulation

VEGF: Vascular Endothelial Growth Factor

VPL Nucleus: Ventral Posterolateral Thalamic Nucleus

INTRODUCTION

A. Adult Neurogenesis

1. Overview

The mammalian brain was well acknowledged throughout most of the twentieth century as structurally stable and static. It was conventional that the mammalian brain acquires its full neuronal counterpart mostly before birth, and that each population of neurons has its specific developmental time window during that period. Adult structural remodeling and plasticity of neural circuits and connections was even argued against (Cajal and May, 1928). It was described by the outstanding histologist Cajal as: "Once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centers, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated." (Cajal and May, 1928). This notion was upheld due to the absence of compelling evidence and the lack of technical advances to be contradicted. The pioneer report by Altman and his colleagues provided the earliest anatomical proof of adult neurogenesis in the postnatal rat hippocampus (Altman and Das, 1965). Thereafter, the first evidence of recruitment of newborn neurons into functional circuits was shown in songbirds (Paton and Nottebohm, 1984). The nucleotide analog bromo-deoxyuridine (BrdU) was then introduced to trace cell lineage and this set off the field of adult neurogenesis in animals (Gratzner, 1982; Kuhn et al., 1996). Adult neurogenesis in humans, however, still remains a controversial issue. In 1998, a pioneer study by Gage provided evidences for the occurrence of adult neurogenesis in human DG

(Eriksson et al., 1998). It was followed by supporting studies that showed birth-dating of the new cells (Spalding et al., 2005) and reported an extensive rate of neurogenesis throughout adulthood (Spalding et al., 2013). On the other hand, opposing studies claim that not "everything that glitters is gold" and that there still remains methodological issues in the field leading to loopholes in the conclusions (Breunig et al., 2007; Gould, 2007). Moreover, a recent study remarkably showed a drop of neurogenesis in humans to undetectable levels in adults (Sorrells et al., 2018). All in all, adult neurogenesis may be a flexible process susceptible to modulation by several external influences in addition to internal challenges. The role of adult hippocampal neurogenesis is still challenging to be properly defined, but experimental outcomes have shown that interfering with neurogenesis may lead to behavioral and genetic changes, the value of which is not yet recognized.

2. History of Neurogenesis Detection

The acceptance of the concept of adult neurogenesis required almost a century of research and it is still debatable till now. The progress was extremely slow due to the limited technical resources at that time. The techniques were almost restricted to histological stains such as Nissl and silver impregnation that can demonstrate the dendritic arborizations of neurons. Detection of underdeveloped arborizations by these stains was considered either to be a histological artifact or a process of cellular differentiation due to plastic changes (Gil-Perotin et al., 2009). There were some hypotheses speculating that these are potentially newly formed neurons but this lacked enough scientific support and were, thereby, overlooked by the scientific community. However, in the late 1950s, tritiated thymidine (3HT) was discovered as a specific marker for cell proliferation (Sidman et al.,

1959). It gets incorporated into nucleic acid during DNA synthesis at the S-phase of the cell cycle and is detected by autoradiography. Adult neurogenesis was incidentally discovered while trying to perfect the 3HT autoradiographic technique (Altman, 1962). The technical advancement in the electron microscope led to the differentiation between neurons and glial cells labeled with 3HT and ultimately to the confirmation of the existence of neurogenesis (Kaplan, 1981; Kaplan et al., 1985). Finally, bromodeoxyuridine (BrdU) was discovered as a synthetic thymidine analog that is non-radioactive (Gratzner, 1982). It can be administered orally or intraperitoneally where it gets incorporated into newly synthesized DNA instead of thymine DNA base during cellular division. The dividing cells can then be detected by a specific antibody that binds to the incorporated BrdU but not to thymidine and it can be used in conjugation with other cellular markers to confirm neuronal identity of proliferating cells. The use of BrdU first started in cancer research to estimate the growth of tumors (Waldman et al., 1988) and after that, professor Gage foresaw its incorporation into new brain cells. He finally succeeded in identifying adult neurogenesis in post mortem brain samples of cancer patients that took in BrdU, and this was a revolutionary finding in the research field of neurogenesis (Eriksson et al., 1998). In 2005, a study used radio-carbon dating techniques to determine the age of cells where they tested ^{14}C levels in genomic DNA (Spalding et al., 2005) and the same team later showed steady dynamics of neurogenesis during adulthood (Spalding et al., 2013).

3. Neurogenic Niche

Initially, persistent adult neurogenesis was observed in the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus (Altman and Das, 1965; Kaplan and

Hinds, 1977), and also in the subventricular zone (SVZ) lining the lateral ventricles (Altman, 1969; Doetsch et al., 1997; Kornack and Rakic, 2001a, b). These two areas house adult neural stem/progenitor cells (NSPCs) and provide the desirable micro-environment for generating new functional neurons. NSPCs are multipotent self-renewing cells (Bonaguidi et al., 2011; Doetsch et al., 1999a) that undergo proliferation regulated by niche-derived and/or intrinsic signals (Zhao et al., 2008). Besides the presence of precursor cells, the niche microenvironment usually contains their progeny of transit amplifying cells and neuroblasts, in addition to glia, endothelial cells and extracellular matrix. The niche is bound by a basal membrane (Mercier et al., 2002) and it comprises essential vasculature that plays a chief role in regulating the proliferation of adult neural precursors (Palmer et al., 2000). Astrocytes send their end-feet closely to surround the vasculature by forming gap junctions. This is not only important for blood-brain-barrier formation and maintenance, but also creates a kind of a modulating interface where astrocytes secrete and express a number of membrane-bound factors that are essential for regulating proliferation and differentiation of neural precursors in addition to maturation, neuronal migration and synapse formation (Barkho et al., 2006). Interestingly, this is specific for astrocytes of the neurogenic hippocampus and SVZ but not for the non-neurogenic other central nervous system regions such as the spinal cord (Lim and Alvarez-Buylla, 1999).

Although the neurogenic niches in both the hippocampus and the olfactory bulb are relatively similar, there remains significant differences between the two. Hippocampal neurogenesis is confined to the dentate gyrus, whereas olfactory neurogenesis includes two areas the SVZ and the olfactory bulb that communicate through a process of migration. They both are enriched with a dense network of neurons but each has its own dynamic

circuitry regulation. Future studies of cellular and molecular mechanisms are needed to segregate and identify the role of individual niche components in controlling developmental stages.

While neurogenesis is restricted to these two zones under normal or healthy conditions, recent evidence suggests that insults to the brain set off the proliferation of neural stem/progenitors that reside in these areas as well as in other unpredicted regions such as the spinal cord (Johansson et al., 1999; Kehl et al., 1997; Weiss et al., 1996) and in other brain regions including the cornu ammonis (CA) (Nakatomi et al., 2002), striatum (Kobayashi et al., 2006), cortex (Gould et al., 1999b), amygdala (Bernier et al., 2002) and substantia nigra (Zhao et al., 2003). This is still controversial and needs further investigation for more conclusive evidence (Bhardwaj et al., 2006; Frielingsdorf et al., 2004; Gould, 2007; Kornack and Rakic, 2001a). However, the discovery that "non-neurogenic" regions also contain NSPCs with neurogenic abilities may serve as a basis for potential endogenous regenerative therapy in cases of CNS damage.

B. Olfactory Bulb

1. Olfactory Bulb Neurogenesis

The olfactory system in most organisms is important for sensing and analyzing odor cues in the environment, needed for determining essential behaviors. The odorant receptors of the olfactory sensory neurons transfer odor information to the olfactory bulb (OB), the primary processing center. Complex synaptic connections occur in the OB whereby local inhibitory interneurons play a major role in determining the output projection. The distinguished regeneration of these interneurons by the process of adult

neurogenesis plays an important role in experience-induced plasticity, and therefore helps organisms in adapting to contemporary cues. This neurogenic process may be a mandatory mechanism for fine tuning OB network in order to improve flexibility of olfactory functions.

Olfactory bulb neurogenesis is marked by the migration of neural progenitor cells from the SVZ towards the OB (Doetsch and Alvarez-Buylla, 1996). Although the route to reach the final destination is long, it is properly delineated by the Rostral Migratory Stream (Lois and Alvarez-Buylla, 1994). Cells then mature in the OB, differentiate and get incorporated in the olfactory circuitry for odor processing (Breton-Provencher et al., 2009). In brief, this process begins by the slow proliferation of radial astrocytic type B cells residing in the SVZ. They express the glial fibrillary acidic protein (GFAP) as well as neural stem cell markers, namely sex determining region Y-box 2 (Sox2), Nestin, Musashi, and LeX/ssea-1 (Brazel et al., 2005; Doetsch et al., 1997; Ellis et al., 2004; Kaneko et al., 2000; Nam and Benezra, 2009). Upon proliferation, they produce transit-amplifying progenitors (Type C) (Doetsch et al., 1999b) that transiently divide, rapidly generate up to 30,000 type A neuroblasts on a daily basis and densely assemble as chain-like aggregates (Alvarez-Buylla et al., 2001; Doetsch and Alvarez-Buylla, 1996; Doetsch et al., 1999a; Kornack and Rakic, 2001b). Type A neuroblasts migrate as packed chains along the Rostral Migratory Stream where they are bounded by an astrocytic scaffold. The role of these glial tubes is to physically restrict migration and to provide directional cues to ultimately ensure the entry to the OB (Ghashghaei et al., 2007; Lepousez et al., 2013; Lois et al., 1996). There, they dissociate and become fully mature granule cells, in addition to few periglomerular interneurons (Lledo et al., 2006; Lois and Alvarez-Buylla, 1994; Lois et al.,

1996). Both are inhibitory GABAergic interneurons that participate in olfactory learning and discrimination (Breton-Provencher et al., 2009; Gheusi et al., 2000; Sakamoto et al., 2011). Adult neurogenesis in the olfactory bulb is considered a form of neural plasticity for refinement of odor in an environment that constantly varies (Gheusi et al., 2000; Moreno et al., 2009). More studies are being conducted to define the role and function of neurogenesis per se in the olfactory bulb.

2. *Functional Role of OB Neurogenesis*

It is well known that neurogenesis in the OB is a very dynamic proliferative process and roughly half of those newly formed neurons become integrated in the pre-existing circuitry, while the rest undergo apoptosis during the maturation process (Lledo et al., 2006). Interestingly, the olfactory experience by itself plays a chief role in determining the apoptotic fate of the new cells. This was approached by two interrelated studies, the first showed that sensory stimuli deprivation decreased the survival of new neurons, while the second showed that olfactory enrichment increased the survival of these neurons (Petreanu and Alvarez-Buylla, 2002; Rochefort et al., 2002). The time window of 14 to 28 days is the interval for new neurons to form synapses and integrate into the circuitry (Kelsch et al., 2008; Yokoyama et al., 2011), this overlaps with the critical period of sensory stimulation that is needed for promoting the survival of the newly formed neurons (Yamaguchi and Mori, 2005). The importance of these new cells is to provide long-term synaptic plasticity for the OB, since the former or "older" neurons tend to lose plasticity as they mature (Nissant et al., 2009). Neurogenesis in the OB therefore plays an important role in olfaction plasticity.

C. Dentate Gyrus

1. Structure of the Dentate Gyrus

The dentate gyrus lies in the curved *Cornu Ammonis* (CA) that is subdivided into three hippocampal regions CA1, CA2 and CA3 (Fig. 1). It is formed of three layers; the granular cell layer (GCL), the molecular layer and the hilus. The GCL or the middle stratum contains the principal neuronal cell bodies of the granule cells (GCs) packed together in 6 to 8 layers. It is V-shaped in structure where the tip or the apex interconnects supra- and infra-pyramidal arms located atop and beneath the CA3 region, respectively (Amaral et al., 2007). The granular cells possess spiny apical dendrites that project into the superficial molecular layer or stratum moleculare of the DG. These dendrites form synapses with axons there that originate from the entorhinal cortex, and are considered as part of the perforant pathway. Additionally, the molecular layer contains GABAergic interneurons and afferent inputs from extrinsic regions. The hilus or the polymorphic layer is the innermost layer containing axons of the granular cells, in addition to mossy cells which are interneurons providing GABAergic or glutamatergic inputs (Chancey et al., 2014; Scharfman, 2016). The SGZ region of adult neurogenesis is located at the border between the GCL and the hilus and it contains, in addition to neural stem/progenitor cells, GABAergic basket cells that form a basket-like plexus around the GC cell bodies.

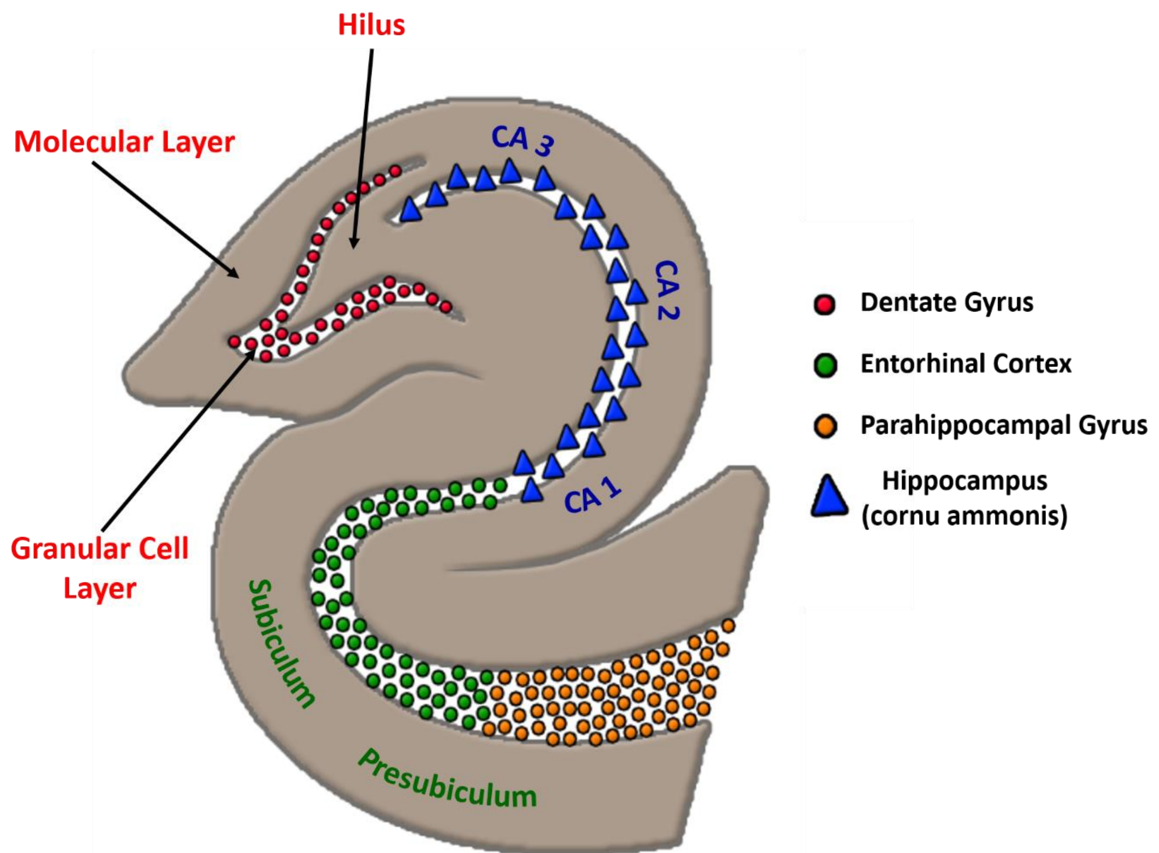

Figure 1: Representation of the structure of the dentate gyrus in the hippocampus. The drawing shows the three layers of the DG (molecular, granular and hilus) in addition to its location in the curved CA of the hippocampus. The parahippocampal gyrus and entorhinal cortex project into the CA3.

2. Circuitry of the Dentate Gyrus

a. Output Pathways

Distinctive axons of the granule cells project along the mossy fibers to conduct all output pathways from the DG. These projections pass through the hilus before entering the stratum lucidum of the CA3 region. Mossy fibers are characterized by irregularly shaped and large terminals that form sparse yet very effective synapses to activate pyramidal cell targets in the CA3 region (Amaral et al., 1990; Henze et al., 2002). They also possess 6-7

thin-terminal collaterals per fiber that form synapses with mossy cells in the hilus and GABAergic interneurons (Acsady et al., 1998). This disynaptic circuit (mossy fiber–GABA interneuron–CA3) is important for a potent feedforward inhibition regulating the excitability of the CA3 (Torborg et al., 2010).

b. Input Pathways (Trisynaptic Circuit)

The dentate gyrus is a main landmark in the trisynaptic hippocampal circuitry. This network is mostly unidirectional and is made up of three main projections (Fig. 2). The first is known as the perforant path and conveys sensory glutamatergic input from layer 2 axons of the entorhinal cortex to the DG. Then, mossy cell fibers from the DG synapse on pyramidal cells in the CA3 region, which carries the connections namely through Schaffer collaterals to CA1 pyramidal cells and finally back into the entorhinal cortex and subiculum. This connection is impinged, as the perforant pathway also possess direct connections from the entorhinal cortex to the CA3 region, thereby bypassing the DG. The sensory information is employed for the ultimate production of episodic memory. Connections from the entorhinal cortex to the dentate gyrus are structurally branched out in an organized way where certain areas of the cortex innervate distinct portions of the GCs. The medial (layer II in addition to a portion of layer III, IV and V) and the lateral entorhinal cortex respectively supply the middle and the outer thirds of the dendritic tree of GCs in the molecular layer (van Strien et al., 2009). The inner third of the GC dendrites is mostly connected with associational and commissural fibers arising from glutamatergic mossy cells that originate in the ipsilateral and contralateral hilus (Buckmaster et al., 1992; Frotscher et al., 1991). In addition to the glutamatergic inputs, GCs also receive

GABAergic innervation from different interneurons including basket cells of the SGZ. These mostly make direct contacts with the granule cell bodies or with proximal segments of both, the axons in the hilus and the dendrites in the molecular layer (Amaral, 1978; Freund and Buzsaki, 1996; Han et al., 1993; Hosp et al., 2014; Houser, 2007). The other two types of GABAergic interneurons are the hilar perforant path-associated (HIPP) cells and the hilar commissural-associational pathway-related (HICAP) cells. The former projects to the outer two-thirds and the latter to the inner third of the molecular layer.

In addition to the trisynaptic circuit inputs, the DG receives extrinsic innervation from regions beyond the hippocampus. It receives glutamatergic inputs from the presubiculum, parasubiculum and from the supramammillary area. It also receives GABAergic and cholinergic input from the septal nuclei. Other types of projections mainly supply the hilus and emanate from the locus coeruleus (noradrenergic input), ventral tegmental area (dopaminergic input) and raphe nucleus (serotonergic input).

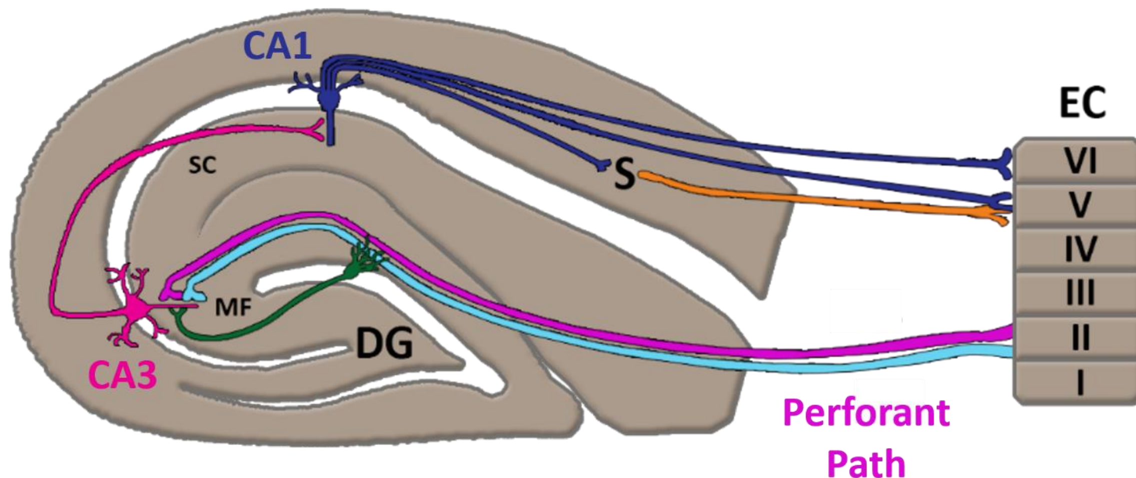

Figure 2: Trisynaptic Circuit Connections. The Perforant Path conveys main input from axons of layer II in the Entorhinal Cortex (EC) to the granular cells of the Dentate Gyrus (DG). The Granular cells project to CA3 pyramidal neurons via mossy fibers (MF). CA3 pyramidal neurons project to CA1 via schaffer collaterals (SC). Finally, CA1 projects back to subiculum (S) and EC**.** Adapted and modified from (Patten et al., 2015).

c. Papez Circuit and the Trisynaptic Pathway

As discussed in the previous section, the entorhinal cortex ensues major input into the hippocampal formation. What is interesting is that it is considered as part of both connections; the trisynaptic pathway and Papez circuitry, and it connects both circuitries together (Fig. 3). The circuit of Papez connects various brain structures and it starts and terminates in the hippocampus. It was first described in 1937 by James Papez (Papez, 1995) as the circuit for emotional processing, connecting the hypothalamus to the limbic lobe, but the functions were revised to include a special role in learning and memory (Vertes et al., 2001). The connections were later anatomically refined through fiber dissection techniques to delineate the anatomical regions involved in Papez circuit and to show the limbic system as a whole in three-dimensions (Shah et al., 2012). The main subtleties of Papez are the

mammillary bodies, the anteromedial thalamic nucleus and the cingulate gyrus as well as the hippocampal formation. It mainly traces the following neural pathway: hippocampal formation (subiculum) → fornix → mammillary bodies → mammillothalamic tract → anteromedial thalamic nucleus → cingulum → parahippocampal gyrus (entorhinal cortex) → hippocampal formation.

The hippocampal formation is buried in the medial temporal lobe and is known to be a structural and functional component of the limbic system. Its involvement in memory is undeniable as it has lots of connections and they mostly serve for communicating mnemonic signals. One other role for the hippocampal formation that is complementary to memory is spatial navigation (Buzsaki and Moser, 2013). Both circuitries; Papez and the trisynaptic must be intact for efficient spatial processing, and damages to either one results in disruption of spatial performance (Jarrard, 1983; Olton et al., 1978). Spatial navigation is mainly processed through a circuitry of connections that link the medial diencephalon with the hippocampus (Clark and Taube, 2012). Within the medial diencephalon is the anteromedial thalamic nucleus (AMN) which forms an integral component of Papez circuit (Fig. 3). Projections from the hippocampal formation to the AMN can be both, directly via the fornix and indirectly through the mammillary bodies via the mammillothalamic tract (Swanson and Cowan, 1977). Similarly, the connections from the AMN back to the hippocampus can be direct through the cingulum bundle (Shibata, 1993) and indirect through the retrosplenial cortex and mammillary bodies (Aggleton and Brown, 1999; van Groen et al., 2002). Such direct and indirect connections are important for the regulation of neural functions in a bidirectional manner. The anterior thalamus contains a large number of "head direction cells" compelling for a role in spatial navigation. These special cells emit

discharges when the animal steers its head for a certain direction (Taube, 1995) and they maintain the firing-tendencies in novel environments (Taube and Burton, 1995). Lesions of the AMN were associated with spatial processing impairment and deterioration of navigation skills (Warburton and Aggleton, 1999) in addition to losses in episodic memory (Aggleton et al., 2010; Clarke et al., 1994; Dusoir et al., 1990).

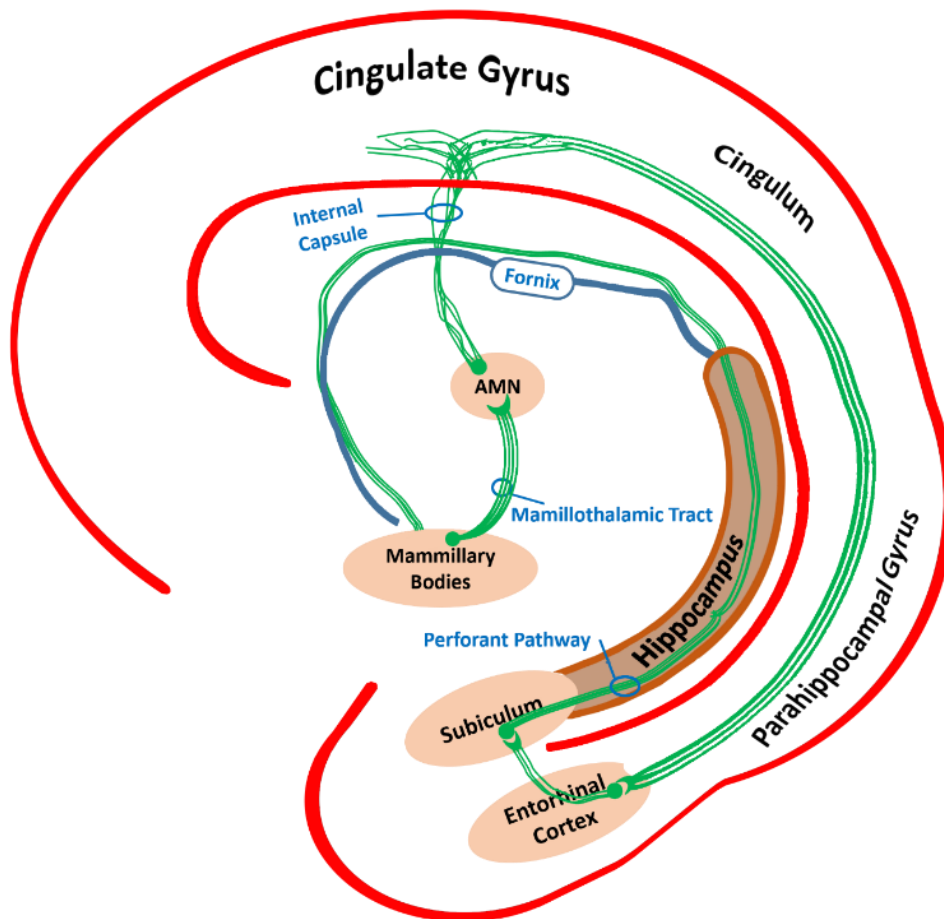

Figure 3: Papez Circuit. Mammillary Bodies send connections to the AMN and then to the cingulate gyrus. Output from cingulate cortex projects to the entorhinal cortex and subiculum through the trisynaptic pathway into the hippocampus and the latter conveys information back to the hypothalamus.

3. Neurogenesis in the Dentate Gyrus

Adult neurogenesis in the hippocampal DG emerges in the SGZ, principally considered the germinal layer with a stem-cell-containing niche (Altman and Das, 1965; Eriksson et al., 1998; Gould et al., 1998). The NSPCs reside in this thin layer that provides a unique milieu permissive for the proliferation and differentiation of radial glia-like type-1 stem cells. The stem cells go through different developmental stages to ultimately generate newly integrated and functional granule cells (Kempermann et al., 2004). This excitatory granule cell is the one principally engendered in the DG and evidence for the generation of other cell types are still uncertain (Liu et al., 2003; Rietze et al., 2000). In brief, the process of neurogenesis in the SGZ starts by the activation of type 1 cells. They then produce non-radial proliferating transit amplifying cells (TAPs) which tend to amplify the neurogenic pool of type-2 cells. In turn, these cells differentiate into immature neurons that migrate short distances into the granule cell layer (Fig. 4). The maturation process needs around four to five weeks for cells to become functional dentate granule cell neurons (Lucassen et al., 2010; Zhao et al., 2008). Migration is accompanied by the branching out of processes that will eventually develop into large dendritic arbors from one end and into axons from the other end. Dendrites receive their input mainly from the entorhinal cortex through the perforant path while axons send excitatory projections along the mossy fiber tract to the pyramidal cells in the CA3 region (Toni et al., 2008; Zhao et al., 2006). They have sparse firing activity that is substantially modulated by interneurons in the DG and in the hilus area. These projections are needed for the full integration of newly generated granule cells into pre-existing hippocampal circuitry. Such a process is highly regulated and is systematically affected by each and every step starting from cellular activity and ending in

the connectivity with pre-existing networks (Deng et al., 2010; Kelsch et al., 2010; Toni et al., 2008; Toni and Sultan, 2011; Zhao et al., 2006).

a. Maturation of Neural Stem Cells in the DG

As the adult born GCs mature, they tend to express different markers which aid in separating the process of division and maturation into classified steps (Kronenberg et al., 2003). The first phase includes type I neural stem cells which send apical processes through the GCL and branch out into the molecular layer. This gives them the radial morphology appearance and usually they express certain markers such as Nestin, Sox2 and GFAP. These stem cells do not undergo frequent divisions, but whenever they do, their division is asymmetrical and produce two types of cells; a self-renewed neural stem cell and an intermediate progenitor cell (IPC) (Bonaguidi et al., 2012). IPCs are the type 2 cells that rapidly proliferate and, in contrast to neural stem cells, exhibit short processes. They are divided into type 2a and type 2b cells, the first also expresses Nestin and Sox2 while the second expresses TBR2. As these proliferate, they generate type 3 cells that lose TBR2 expression and gain the immature neuronal markers, namely DCX and Prox1. Therefore, they become committed to the neuronal lineage as they start developing a short vertical process, and ultimately differentiate into mature GCs. The neuronal markers NeuN and Calbindin are expressed at maturation and the mature cells extend their axons to the CA3 region and branch their dendrites into the molecular layer (Fig. 4).

Two weeks following division, the differentiated GCs form their first output synapses, marking the active integration process and its functional consequences. Synaptic inputs, on the other hand, develop prematurely as early as in the radial glia-like (RGL) cells

time point, where those express receptors for GABA and glutamate (Tozuka et al., 2005; Wang et al., 2005). GABAergic synaptic input on RGL cells ensures their quiescence, similar inputs on IPCs on the other hand promote cell survival, maturation and differentiation. All in all, synaptic input plays a chief role in regulating stem cell proliferation. (Song et al., 2012).

The maturation process includes, in addition to proliferation of progenitor cells, an intricate route of neuronal commitment and functional integration, which is mostly characterized by synaptic connections to pre-existing circuitry (Encinas and Sierra, 2012; Sierra et al., 2010). Throughout this process, 50% or even more of the cells do not get through to the end of maturation (Biebl et al., 2000; Cameron et al., 1993; Hattiangady and Shetty, 2008; Kempermann et al., 2003). They undergo active apoptosis and are selectively eliminated by the first few weeks (Sierra et al., 2010; Sun et al., 2004). The rest of the cells develop synapses and form interconnections which increase their chances of survival (Gould et al., 1999a; Kempermann et al., 1997). Therefore, this process creates a kind of balance between apoptosis and survival and it is properly controlled by the milieu of neuronal activity and by cognitive experiences (Dupret et al., 2007).

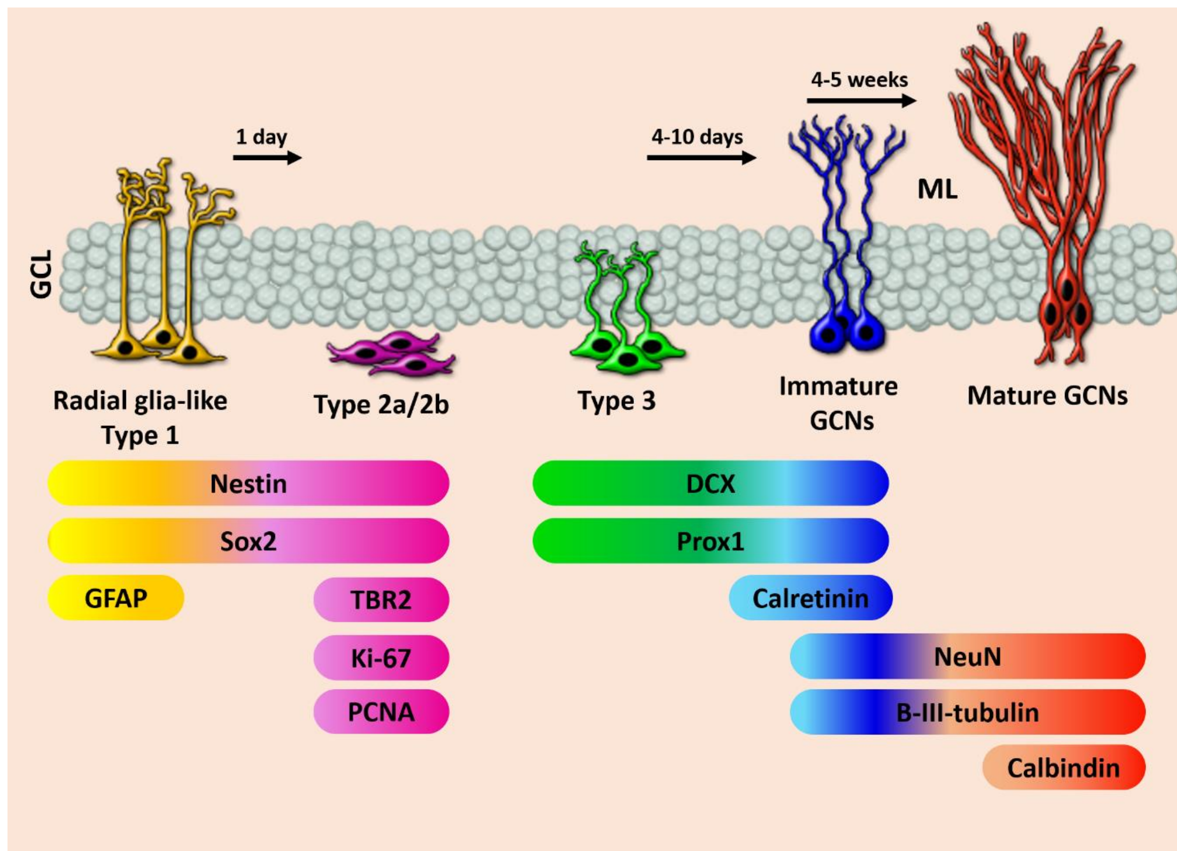

Figure 4: Maturation of Neural Stem Cells in the DG. Radial glia-like type 1 cells express Nestin, Sox-2 and GFAP. They produce type-2 cells which loose GFAP and gain the expression of TBR2, Ki-67 and PCNA. These cells differentiate into type 3 and immature neurons that migrate short distances into the granule cell layer (GCL) and start expressing DCX and Prox1. Immature granule cell neurons (GCNs) express calretinin. Around four to five weeks of maturation is needed for cells to become functional dentate granule cell neurons that express NeuN, B-III-tubulin and Calbindin. The migration process is accompanied by the branching out of processes into the molecular layer (ML). Adopted and modified from (Knoth et al., 2010).

b. Functional Role/Significance of DG Neurogenesis

The contribution of adult neurogenesis to learning and memory is better understood with the knowledge of the DG function as a whole. New DG neurons can influence behavior after forming synapses and integrating into the DG network. This is their process of maturity where they tend to share similar properties with the old DG cells, although there is no consensus on a unified function between the "new" and the "old" cells

of the DG (Toni and Schinder, 2015). Electrophysiological characteristics differ between mature and adult-generated neurons. Newborn neurons are characterized by their increased excitability, decreased LTP-induction (long-term potentiation) threshold and they can even be depolarized by GABA (Ge et al., 2006; Karten et al., 2006; Schmidt-Hieber et al., 2004; Snyder et al., 2001; Wang et al., 2000). During their immature status, the newborn cells' excitability and plasticity are mostly impactful between 4 and 6 weeks after division (Hastings and Gould, 1999; Toni et al., 2007; Zhao et al., 2006). This is the critical period that might be influenced by environmental inputs and modified by cognitive demands. The connectivity and the integration of new neurons into hippocampal networks can be molded by the experiences achieved during this period of early maturation (Bergami et al., 2015; Goncalves et al., 2016; Piatti et al., 2011; Zhao et al., 2006). It is therefore more likely that distinct demands result in specific connections that would lead to particular functions of the new neurons. The exact mechanism of how newborn cells enhance DG function is not yet identified. Recently, evidence of connections between newborn neurons and inhibitory circuits in CA3 was revealed (Restivo et al., 2015), suggesting the complex and dynamic inhibitory networks of the newborn neurons. This ultimately suggests a role for neurogenesis in eliminating the more distant memories by either increasing the inhibition on mature DG cells or possibly due to the new rewiring of DG outputs (Akers et al., 2014).

As it is still elusive, massive experimental approaches are continuously attempting to pinpoint the functional relevance of adult hippocampal neurogenesis. One possible hypothesis considered a role for new neurons in replacing the older dying cells of the hippocampus. It appeared later that the adult-generated populations themselves are the ones that mostly die and not the "embryonic/mature" ones, therefore, it is unlikely for the adult

neurons to perform such a function (Dayer et al., 2003). It is more plausible that neurogenesis helps in the process of modulation, refinement and plasticity of existing DG circuitry, collectively serving as a support system for hippocampal functions that cannot be solely accomplished by the existing neurons (Imayoshi et al., 2008). One way to study hippocampal neurogenesis is by altering the process or the rate of neurogenesis, and this has resulted in some cognitive deficits. Reduction in the number of newborn cells affected spatial memory in general and long-term memory in the Morris water maze test in particular (Ben Abdallah et al., 2013; Manns et al., 2010). Yet another possible function for neurogenesis is involved in decreasing memory interferences of distinct memories formed at different time intervals (Rangel et al., 2014). It also features cognitive flexibility that helps in resolving new challenges faced in previously learned tasks, such as submerging the earlier identified platform in Morris water maze tests (Rangel et al., 2014). This can be also attributed to improved spatial memory and contextualization.

c. Experimental Approaches

One common approach followed to investigate the function of hippocampal neurogenesis is through the ablation of adult-born neurons and subsequently examination of the altered cognitive performances in animals. This may be done through the use of chemical ablation (Garthe et al., 2009; Shors et al., 2001), focal X-ray irradiation to spare SVZ neurogenesis as much as possible (Clelland et al., 2009; Santarelli et al., 2003) or by genetic ablation where SVZ neurogenesis is not spared (Garcia et al., 2004; Saxe et al., 2006). These procedures collectively help in highlighting hippocampal related functions of neurogenesis, yet certainly result in unsolicited side effects. Other more targeted protocols

have used virus-based strategies (Clelland et al., 2009; Jessberger et al., 2009) to minimize side effects and seek out specific populations of adult-generated neurons at specific times during their maturation (Imayoshi et al., 2008; Revest et al., 2009; Zhang et al., 2008).

Further support for the functional implications of hippocampal neurogenesis in spatial learning and memory comes from performing related-behavioral tasks and checking thereafter the effect on the increase in hippocampal neurogenesis. Studies have reported that training in several mazes tend to increase hippocampal neurogenesis. The most commonly used mazes are the Morris Water Maze (Epp et al., 2007; Gould et al., 1999a) and the Barnes Maze (Paul et al., 2009).

i. Spatial Learning

It is of common knowledge to link hippocampal functions to spatial learning, yet categorizing its involvement in specific learning tasks remains complicated. What makes this even more ambiguous is the assorted functional distribution of the hippocampus along its sub-regions (Rolls and Kesner, 2006) and its medio-lateral or rostro-caudal axis (Bannerman et al., 2004; Moser and Moser, 1998) as well as the inevitable plasticity and neurogenesis (Lamprecht and LeDoux, 2004; Nottebohm, 2002). Moreover, the newly formed neurons represent a small portion of the hippocampus as a whole, making the delineation of the functional significance of neurogenesis very critical to be obtained experimentally.

In spite of these difficulties in delineating the specific hippocampal neurogenesis-related functions, studies have tried to surpass them by manipulating around the time of spatial learning as well as the proficiency of tasks. It is interesting to note that spatial

learning undertakings help in increasing the survival of only the 1-week old new neurons, and did not affect the survival of both the younger and the older neurons (Ambrogini et al., 2000; Dupret et al., 2007; Epp et al., 2007; Gould et al., 2000). Moreover, hippocampal neurogenesis is up regulated following high proficiency learning, without being affected by low proficiency learning (Sisti et al., 2007). Age-dependent reduction in hippocampal neurogenesis, on the other hand, is associated with hindrances in learning the Morris water maze task (Drapeau et al., 2003; Driscoll et al., 2006). Moreover, irreversible abolishment of hippocampal neurogenesis by means of low dose irradiation results in long-term retention deficits in the Morris water maze but did not affect acquisition (Snyder et al., 2005; Wojtowicz et al., 2008). In brief, manipulation of hippocampal neurogenesis at both edges (upregulation or downregulation) correspondingly leads to enhancement or impairment, providing evidence for the involvement of hippocampal neurogenesis in spatial learning.

ii. Spatial Recognition Memory

The hippocampus is quite known for having an important contribution to spatial memory and mapping. Expectedly, neurogenesis-deficits of the hippocampus have been correlated with underperformances in several spatial tasks. One of the highly used models for spatial memory assessment is the Morris water maze. Consistent data show that there are no motor differences in the performance of animals, with and without neurogenic deficits, in the training period and the probe trials of the test. However, when given more complicated tasks, such as long delays or tortuous patterns to relocate a previously established spatial site, behavioral disparities would be detected between the different

groups (Ben Abdallah et al., 2013; Garthe and Kempermann, 2013; Snyder et al., 2005). Another model that is analogous to the Morris water maze minus the water is the Barnes maze. Similar to the observation in the water maze, neurogenesis deficient animals show slower spatial strategical skills than normal ones (Raber et al., 2004). Yet another test that is considered as powerful in the assessment of spatial recognition memory is the Y-maze test (Dellu et al., 1992). The importance of it lies in the natural tendency for rats to investigate and explore their surrounding environment, and it was reported that spatial memory performances in the Y maze were impaired in rats exposed to hippocampal lesions. They showed lower exploration drives in the novel arm of the Y maze (Conrad, 2010; Conrad et al., 1996).

iii. Pattern Separation

One new area of focus that is being extensively studied is the involvement of newly developed neurons in the process of pattern separation (Agis-Balboa and Fischer, 2014; Aimone et al., 2010; Aimone and Gage, 2011; Deng et al., 2010; Groves et al., 2013; Sahay et al., 2011b). This is a highly intricate process needed for separation of events and for reducing interferences between similar inputs or memories acquired at different spatial and temporal proceedings. It is also needed for the categorization of subsequent outputs. The observations seen in many studies of contextual discrimination make the definition of pattern separation quite simpler. Commonly, the freezing reaction time needed by mice when introduced to a new object is evaluated as contextual discrimination behavior. Impairing neurogenesis in mice and putting them in similar contexts in order to study contextual discrimination, showed that decreased neurogenesis was associated with slower

differential freezing reaction to analogous frameworks. However, this was ascertained to be highly dependent on the resemblance of the contexts because as they differed, the differential freezing became normal (Kheirbek et al., 2012; Nakashiba et al., 2012). In other experiments, mice with increased neurogenesis were correlated with a quicker discrimination between analogous contexts (Sahay et al., 2011a). Similar observations were seen in the cued water maze, where irradiated rats had more errors in performing a high-interference task that contained similar cues, but their performances were comparable to normal animals when given an uncued low-interference task (Winocur et al., 2012). Such an interference-impairment in cases of similar stimulants was also detected in animals lacking neurogenesis, when subjected to non-spatial tasks. Rats of this group had no trouble learning a list of odor pairs but errors started amassing when given a second list that contained some repeats of the same odors (Luu et al., 2012). Taken together, these studies try to classify the function of new neurons in high cognitive functions involving discrimination between similar inputs and separation between similar memories.

4. Regulatory Mechanisms of Adult Neurogenesis

Adult neurogenesis is a well regulated process that is dynamically controlled by physiological and pathological factors. These factors set a limit to the extensive progenitor cell division and control the number of new neurons in the DG. Some of the questions that rise here are: how is adult neurogenesis regulated, how is it affected by the environment and what are the influencing factors? To answer those questions, we need to distinguish between two major determining factors that are important in controlling the number of new granule cells. The first one is the proliferation of progenitor cells and the second is the

survival of those newly formed cells. It is quite hard to separate between the two processes and the fact that regulators often affect both make it even more complicated. But, there is a sort of a more biased regulation (having a higher effect) on either the former or the latter edge. Herein is a list of regulators that affect the rise or drop of hippocampal neurogenesis (summarized in *Table 1*), but other regulators are also involved and the list is still expanding (Ming and Song, 2005).

a. Genetic Variation and Environmental Enrichment

It is highly expected to have a genetic impact on the variations in hippocampal neurogenesis. Basal levels of neurogenesis can vary between inbred animals at the level of both proliferation and survival (Kempermann et al., 2006). Moreover, there also exists a sex-dependent variation between male and female rats at the level of proliferation (Chamaa et al., 2016). In addition to the influential role of genetics on hippocampal neurogenesis, enriched environment also plays a crucial role in increasing it. Adult mice housed together for several weeks in enriched environments containing toys, showed improved learning skills attributed to the increased number of new neurons in the DG (Kempermann et al., 1997). This was mostly due to the increased survival of newborn neurons as they were being constantly stimulated and thereby underwent lower apoptotic rates. It is important to note that the period of 2 to 3-weeks after division is the most susceptible timeframe for new neurons to be affected by the enriched environment and they usually respond by showing higher survival rates (Tashiro et al., 2007). While environmental enrichment mainly promotes the survival, physical activity has higher effects on progenitor cell division. The

increase in the number of newborn neurons was coupled with bettered performance in learning tasks (van Praag et al., 1999).

b. Aging

Adult neurogenesis is highly affected by aging, where the survival of newborn neurons drops drastically in comparison to youngsters. This is justified by the decrease in the proliferation rate as well as in neuronal differentiation (Van Praag et al., 2005). Corticosteroids exhibit an important role in this decline (Cameron and McKay, 1999), but luckily, physical activity as well as environmental enrichment still help in rescuing such a decline (Kempermann et al., 2002; Van Praag et al., 2005).

c. Neurotransmitters

Whether it is direct or due to secondary changes in the surrounding milieu, the effect of most neurotransmitters on adult neurogenesis is evident. The effect mostly targets the number of new neurons formed. However, studying the effect of neurotransmitters such as GABA and glutamate on neurogenesis is still puzzling. For example, the EC mostly sends glutamatergic input into the DG and interrupting the perforant pathway results in an increase in neurogenesis. Moreover, systemic blocking of the N-methyl-D-aspartate (NMDA) receptors showed comparable results (Nacher and McEwen, 2006). Preliminary work from our lab also showed that nitrous oxide, an NMDA receptor antagonist increases neurogenesis (Chamaa et al., 2018b). This mainly implies a suppressive neurogenic role for glutamate. On the other hand, cell proliferation is increased following the activation of kainic acid glutamate receptors (Gray and Sundstrom, 1998). Besides that, depolarization

of progenitor cells increases neuronal differentiation (Tozuka et al., 2005). Altogether, this explains that the interconnections in the DG are complex and we cannot directly assume that glutamate or excitation inhibits neurogenesis. It is rather an intricate circuitry that involves other connections including GABAergic interneurons whose activation by glutamate can add up to the interplay. This suggests that levels of neurogenesis may be regulated through the proper balance between excitation and inhibition (Deisseroth et al., 2004) especially that GABA is highly important in proliferation and differentiation (Ge et al., 2006; Tozuka et al., 2005).

The DG receives additional input from various brain regions other than the EC and conveys many neurotransmitters that also modulate neurogenesis levels. Specific cholinergic lesions in the forebrain leads to a decrease in neurogenesis (Cooper-Kuhn et al., 2004). Increasing serotonin release from the raphe nucleus (Gould, 1999) or by certain antidepressant drugs (Santarelli et al., 2003; Warner-Schmidt and Duman, 2006) also enhances neurogenesis. Other neurotransmitters such as dopamine and norepinephrine from the ventral tegmental area and locus coereleus, respectively, also have a role in altering neurogenesis (Abrous et al., 2005).

d. Neurotrophic Factors

Collectively, neurotrophic factors are proteins which bind to transmembrane receptors and exert various cellular effects depending on the cell type they bind to. They ultimately aid in proliferation, maturation and trophic support. Brain-derived neurotrophic factor (BDNF) acts on its TrkB receptor for promoting survival of new neuroblasts (Bath et al., 2008). Nerve growth factor (NGF) increases the cholinergic milieu and promotes the

survival of new hippocampal neurons (Frielingsdorf et al., 2007). Hippocampal neurogenesis is endorsed by neurotrophin NT-3 which also helps in improving long-term potentiation (LTP) and spatial memory (Shimazu et al., 2006). Basic fibroblast growth factor FGF-2 functions as a positive modulator of the mitotic effects in neurogenesis and its receptor is essential for LTP and memory consolidation (Zhao et al., 2007). It is important to note the essential role played by the surrounding vasculature in adult neurogenesis and that vascular endothelial growth factor (VEGF) promotes the survival of newborn cells (Sun et al., 2003). Knocking out ciliary neurotrophic factor (CNTF) in mice results in reduction of neurogenesis (Yang et al., 2008). All in all, the involvement of each of these neurotrophic factors is essential for the different stages of neurogenesis and further identification of their roles is needed to better understand the underlying mechanisms of neurogenesis.

e. Hormones, Dietary Restriction and Sleep Deprivation

Another regulator of adult neurogenesis is the hormonal change in the body. Dehydroepiandrosterone (DHEA), the most abundant neuroactive steroid in the CNS, can increase neurogenesis while corticosterone inhibits it (Abrous et al., 2005; Wada et al., 2014). Hormonal changes are also affected by the dietary intake, and dietary restriction increases proliferation in the DG (Lee et al., 2002; Prolla and Mattson, 2001). An additional regulator is sleep deprivation; an overnight increases neurogenesis while chronic sleep deprivation results in its drastic suppression (Grassi Zucconi et al., 2006; Guzman-Marin et al., 2005).

5. Pathological Alteration of Adult Neurogenesis

a. Stress and Depression

Stress is an extensive negative modulator of progenitor cell proliferation and it appeared that psychosocial stress suppresses neurogenesis (Gould et al., 1997). This suppression is mainly mediated by stress-induced increase in corticosteroids from the adrenal gland, and prevention of this increase by adrenalectomy eliminates the stress-induced decline in neurogenesis (Tanapat et al., 2001). Due to the extensive correlation between dampened neurogenesis and stress, it is now assumed that the decline in hippocampal neurogenesis may be vitally involved in the progression of the depressive disorder. It was even noticed that the size of hippocampus in patients with major depression was extremely reduced (Sapolsky, 2000). Moreover, to further verify the association, it was proven that the decline in neurogenesis was counteracted with antidepressant treatments (Warner-Schmidt and Duman, 2006). Interestingly, the time interval taken for the efficiency of the treatments was in line with the time taken for the new dividing cells to integrate. Moreover, the behavioral effects of the antidepressant fluoxetine were abolished when hippocampal neurogenesis was inhibited, showing the importance of neurogenesis for the efficacy of the drug (Santarelli et al., 2003).

b. Seizures and Ischemia

Epileptic discharges have opposing effects to stress and depression. They massively increase hippocampal neurogenesis as well as integration of new neurons into the circuitry (Parent, 2002). Although the number of new neurons increase directly after the seizure, it was seen that these numbers drop dramatically to become even lower than the

control levels several days after the initial insult (Hattiangady et al., 2004). This is not well understood but it can be explained by either the "exhaustion" of progenitor cells or to changes in the neurogenic niche. It was also observed that the new neurons, generated after the seizure onset, develop abnormal basal dendrites (Shapiro and Ribak, 2006) that form immature synapses. The functional impact of those and their overall stability is not well known. It is even argued that these new neurons may be involved in exacerbating the epileptogenic disease process (Parent, 2002).

Ischemic insults result in increased neurogenesis in both the DG and the SVZ (Zhang et al., 2005). New neurons of the SVZ migrate into the injured area for a possible repair mechanism, but the rationale behind the increased hippocampal neurogenesis is not well understood (Lu et al., 2017).

c. Irradiation and Inflammation

Tumors of the central nervous system are often controlled by irradiation. This treatment has detrimental effects on hippocampal neurogenesis and the results are unfortunately long-lasting (Monje et al., 2002). Irradiation is always coupled with inflammatory responses that aggravate the effects on neurogenesis, and treatments with anti-inflammatory drugs rescue neurogenesis to a certain limit (Monje et al., 2003). Recent work from our group also confirmed that endotoxin-induced neuroinflammation reduces hippocampal neurogenesis and the effect is reversed by pretreatment with anti-inflammatory agent (Chamaa et al., 2018a).

d. Neurodegenerative diseases and drugs

As the concept of neurogenesis emerged, it's alteration have been checked on a number of neurodegenerative diseases. Indeed, neurogenesis has been reported to be disturbed in a number of neurodegenerative diseases and these observations were detected in animal experimental models. It was shown to be reduced in Alzheimer's disease (Jin et al., 2004), in Huntington's and in Parkinson's disease, as well as many others (Abrous et al., 2005). It is still unclear, however, what is causing these alterations that are subsequently leading to deterioration in cognitive behavior and dementia.

Neurogenesis can also been affected by the abuse of many drugs including ethanol, opioids and cannabinoids, barbiturates and benzodiazepines, as well as nicotine (Abrous et al., 2005). These addictive drugs mostly affect the proliferation of new DG cells and show varied effects on neuronal survival, differentiation and death (Chambers, 2013).

Table 1:

Enhancing Neurogenesis	**Reducing Neurogenesis**
Environmental Enrichment	Aging
Physical Exercise	Corticosteroids
Anti-depressant Drugs	Depression
Serotonin	Stress
Neurotrophic Factors	Sleep Deprivation
Dehydroepiandrosterone (DHEA)	Seizures
Ischemia	Irradiation
Anti-inflammatory Drug	Inflammation
	Drug Abuse
	Neurodegenerative Diseases

D. Electrical Stimulation

Electrical stimulation (ES) is a modern methodology used for the treatment of a number of neurological, and lately psychiatric diseases, where the list is still expanding. There are several modes of ES such as electroconvulsive therapy (Sackeim et al., 2000), transcranial magnetic stimulation (TMS), vagal nerve stimulation (Smith et al., 2005), epidural stimulation (Jahanshahi et al., 2013), transcranial direct current stimulation (tDCS) and deep-brain stimulation (DBS). Regardless of the ambiguity in the mechanism of action, ES practice persists for its impact in functional recovery, which has been clinically achieved in many disorders. This improvement can be explained by a combination of factors including changes in cortical excitability (Ludemann-Podubecka et al., 2014), permeability of the blood-brain barrier (Levi et al., 2012), neuronal apoptosis (Borsody et al., 2014; Wang et al., 2014) and neuronal plasticity (Boggio et al., 2011) that includes new synapses as well as neurogenesis (Lendvai et al., 2000; Nithianantharajah and Hannan, 2006).

1. Deep Brain Stimulation

Deep brain stimulation (DBS) is an invasive surgical technique that involves electrode implantation into specific brain regions. High frequency stimulation ranging from 130Hz - 200Hz is commonly used for different brain targets (Breit et al., 2004). DBS was first practiced for the treatment of Parkinson’s disease where the electrode was implanted in the thalamic nucleus ventralis intermedius (Benabid et al., 1991). It then became more frequently used in Parkinson’s disease and in other movement disorders such as essential tremor and primary dystonia (Koller et al., 1997; Vidailhet et al., 2005). Its clinical success

in calming the tremors of such disorders overextended DBS usage to other neurological psychiatric disorders such as obsessive compulsive disorder and treatment of resistant depression (Bewernick et al., 2010; Chang, 2004). Studies using DBS have reported significant behavioral improvements in rodents experimental models of several disorders such as stroke dementia and depression. DBS in those studies were applied to a range of brain areas such as the medial septum (Jeong et al., 2014), ventromedial prefrontal cortex (Liu et al., 2015), entorhinal cortex (Stone et al., 2011) and nucleus accumbens (Schmuckermair et al., 2013). All these studies collectively proposed that neurogenesis has an important role in mediating the behavioral amelioration detected following DBS (Jeong et al., 2014; Liu et al., 2015; Morimoto et al., 2011; Schmuckermair et al., 2013; Stone et al., 2011). Similar to ES, the underlying mechanism of DBS is still unraveled. The incidental reporting that DBS possess similar therapeutic effects to lesions of the same area makes scientist propose that they both exhibit similar mechanisms of action (Aziz et al., 1991; Koller et al., 1997). It is proposed that both modalities tend to inhibit the abnormal activity of the targeted area and therefore decrease the output from it, but DBS is superior to lesions in its reversibility and adjustability. Such an inhibitory effect may be induced by the stimulation of inhibitory afferents synapsing with the targeted area (Dostrovsky and Lozano, 2002) or by deactivating voltage-dependent channels thereby blocking depolarization capacities (Beurrier et al., 2001), or even by reducing neurotransmitter bioavailability at the synaptic level (Kringelbach et al., 2007).

The effects of DBS on functions in memory and cognition have disclosed new targets envisaged by the Papez memory circuitry. These main brain targets include the anterior nucleus of the thalamus (Hamani et al., 2011), the entorhinal cortex (Stone et al.,

2011; Toda et al., 2008) and the fornix (Laxton et al., 2010). Although these regions are considered main parts of the circuitry and are all connected, it is still unclear if stimulating each area would result in the same or in unique behavioral effects. AMN is an integral part of Papez circuitry and is mostly targeted for stimulation in studies of epilepsy treatment (Fisher et al., 2010).

2. *DBS and Neurogenesis*

Neurogenesis may be a possible mediator for the beneficial outcomes of DBS (Lozano and Lipsman, 2013; Stone et al., 2011); although not the sole mediator, especially that other mechanisms including electrophysiological manipulations, neurotransmitter alterations and synaptic plasticity are indispensable. Research done in our laboratory has shown that DBS to the AMN increases hippocampal stem cell proliferation (Chamaa et al., 2016). This is in line with previous studies that have provided evidence linking DBS pro-cognitive enhancements with increased hippocampal stem/progenitor proliferation and functional integration into pre-existing circuitry (Encinas et al., 2011; Stone et al., 2011). Moreover, hippocampal neurogenesis which was identified as secondary to entorhinal cortex stimulation was associated with improved spatial memory formation. Coupled with these experiments, was the blocking of neurogenesis by means of the DNA-alkylating agent Temozolomide at the time of stimulation, leading to abolishment of the improved spatial memory performances (Stone et al., 2011). However, the underlying mechanism still remains elusive and further investigation is undoubtedly needed to delineate the exact spectrum for the therapeutic effects of DBS.

E. Chemical Stimulation

Kainic acid (KA) is a potent neuro-excitatory amino acid that acts as a direct agonist to glutamic kainate receptors; a class of glutamate receptors where glutamate is the principle excitatory neurotransmitter in the CNS. At larger doses, KA is considered a neurotoxin that induces immediate neuronal death mainly by overstimulating the neurons and leading to exitotoxicity. However, diluted small doses of kainic acid is known to chemically stimulate neurons (McGeer et al., 1978). It is used to study the neurons in a specific area as KA excites cell bodies without activating axons of passage (Guidotti and Fidia Research Foundation., 1990).

The use of chemical or electrical stimulation to specifically identify brain function mechanisms is undoubtedly a non-physiological manipulation. This is mostly negated by the fact that the brain is a complicated system that works as a whole, where integration of connections and synapses in the related regions are all involved in the studied phenomenon. In other words, no region is completely isolated from the other. Therefore, the use of local stimulation can be very critical especially when trying to come up with the final interpretation. However, one cannot deny the importance of these techniques in uncovering many functional neuronal systems of the brain or the central nervous system. These techniques allow scientists to accurately stimulate targeted brain areas. While electrical stimulation is easier to apply and modify, chemical stimulation is more exigent in terms of finding the right solution to solubilize, optimizing the proper concentration and of course retaining the chemical stability. As discussed before, electrical stimulation effects are mostly widespread and the underlying mechanism of action is yet unidentified. The specificity of the electrical signal is dubious and it results in inadvertent stimulation to

nearby regions and to the passing fibers (Ranck, 1975). This makes interpretation of ES effects at the cellular level quite hard. Alternatively, chemical stimulation specifically targets the region of interest within the brain and makes a clearer interpretation at the cellular level (McGeer et al., 1978). Several modes can be used for chemical stimulation, such as cannulas, microinjections and syringes.

In our study we are using continuous micro-perfusion of low doses of KA, using osmotic mini-pumps to evoke sustained neuronal activation in the AMN without causing seizures and without affecting the fibers of passage. This will give us insight into the mechanism of chemical stimulation and that of electrical stimulation by comparing the effects seen by both neuromodulation procedures to the same area.

F. Aim of The Study

We hypothesize that stimulation of the AMN will facilitate adult hippocampal neurogenesis by inducing proliferation of stem/progenitor cells followed by their differentiation into neurons and integration into the dentate gyrus.

- **Specific Aim 1:** To investigate the differences in stem/progenitor cell proliferation between males and females, and study the effect of AMN electrical stimulation on induced-proliferation in a sex-dependent manner.
- **Specific Aim 2:** To examine the effect of electrical stimulation in the AMN using two types of electrodes, copper or platinum, on proliferation (day 5) and differentiation (week 4) of stem/progenitor cells in the DG.
  - *Sub-aim 1:* To study the effect of a single session of electrical stimulation on proliferation and differentiation.
  - *Sub-aim 2:* To study the effect of multiple sessions of electrical stimulation on proliferation and differentiation.
  - *Sub-aim 3:* To investigate the functional implications of the increase in hippocampal neurogenesis through studying spatial exploration in the Y-maze and show the behavioral correlation between stimulation and neurogenesis.
- **Specific Aim 3:** To test for the specificity of AMN targeting in inducing stem/progenitor cell proliferation by stimulating another thalamic nucleus, VPL nucleus, that do not possess direct connections to the hippocampus.

- **Specific Aim 4:** To examine the effect of a single injection or continuous micro-perfusion of kainic acid on proliferation (day 8) and differentiation (week 4) of stem/progenitor cells in the DG.
  - *Sub-aim 1:* To study the effect of a single injection of kainic acid on proliferation and differentiation.
  - *Sub-aim 2:* To study the effect of continuous micro-perfusion of kainic acid for 7 days on proliferation and differentiation.
  - *Sub-aim 3:* To investigate the functional implications of the increase in hippocampal neurogenesis through studying spatial exploration in the Y-maze and show the behavioral correlation between chemical stimulation and neurogenesis.

MATERIALS AND METHODS

A. Experimental Design

In all groups, rats underwent stereotaxic surgeries for multiple experimental conditions including: implantation of copper or platinum electrodes, insertion of Hamilton syringe for the sake of one injection of Kainic Acid (KA) or insertion of brain infusion cannula attached to mini-osmotic pump for continuous micro-perfusion with KA. All implants were done unilaterally in the right anteromedial thalamic nucleus (AMN), and rats with implanted electrodes were given a 3-day of rest period post-surgery. In one group, copper electrodes were inserted in the ventral posterolateral (VPL) nucleus of the thalamus and served as a control for the target-specific effects of AMN stimulation. Electrical stimulation was done 3 days after surgery while chemical stimulation started directly at the end of the implantation procedure. Four days after stimulation, the proliferation marker 5-Bromo-2'-deoxyuridine (BrdU) was given as 3 intraperitoneal (ip) injections spaced by 2-h intervals to maximize the availability and incorporation. The injections were performed 4 days after stimulation or sham procedures based on a previous report showing that the highest proliferation rate is attained 3 to 5 days post stimulation (Toda et al., 2008). Behavioral assessments of spatial navigation using the Y-maze were done at both the early and the later stages (4 days and 4 weeks). This behavioral test was chosen to study hippocampus-dependent spatial exploration and the possible role of the newly proliferated and integrated cells in enhancing performance. We have used the novel arm exploration task as it highly depends on the innate preference of rats to explore novel environments

over familiar ones (Dellu et al., 1992; Vuillermot et al., 2011; Wright and Conrad, 2005). To detect the effects on proliferation, rats were euthanized 24 h after the last BrdU injection (5 days after surgery), as this is the duration needed to achieve a complete cell cycle (Cameron and McKay, 2001). To check for the survival of stem/progenitor cells, rats were euthanized 4 weeks after BrdU injection (Fig. 5). At both the early and the late time points, brain tissue was sampled and processed, as described in section B below, to check for proliferation (5 days) and survival (4 weeks) of BrdU-incorporated cells. Experiments were carried out by individuals blinded to the identity of the groups (sham versus experimental).

B. Experimental Groups

To investigate the stimulation-induced effects on the proliferation and survival stages of adult neurogenesis, rats were divided into different groups as follows and each set of groups served to attain the corresponding specific aims (also see *Table 2*).

Specific Aim 1: To investigate the differences in stem/progenitor cell proliferation between males and females, and study the effect of AMN electrical stimulation on induced-proliferation in a sex-dependent manner.

- ***Group 1*:** control group that served to assess and compare the basal levels of stem/progenitor cell proliferation in the DG between male (n=4) and female (n=4) rats. This group only received 3 injections of BrdU (ip) without any surgical procedure and rats were perfused 24h later.

- ***Group 2*****:** sham group that was designed to assess if electrode insertion by itself in the AMN causes any effect on proliferation of stem/progenitor cells. Male (n=4) and female rats (n=3) underwent surgery with copper electrode insertion in the right AMN and were given 3 BrdU injections after 3 days from electrode implantation but were not subjected to any current or stimulation. Rats were perfused 24 h after the last BrdU injection.
- ***Group 3*****:** designed for the evaluation of stem/progenitor cell proliferation following AMN stimulation with copper electrodes. The induced effects were compared between male (n=6) and female (n=6) rats to check for sex-dependent differences in the stimulation-induced effects. Rats had surgery with copper electrode insertion which was followed in 3 days by 1h single stimulation. 3 BrdU injections were given 4 days after stimulation. Rats were perfused 24 h after the last BrdU injection (day 5) (Fig. 5A).

Specific Aim 2: To examine the effect of electrical stimulation in the AMN using two types of electrodes, copper or platinum, on proliferation (day 5) and differentiation (week 4) of stem/progenitor cells in the DG.

- *Sub-aim 1:* To study the effect of a single session of electrical stimulation
- *Sub-aim 2:* To study the effect of multiple sessions of electrical stimulation
- *Sub-aim 3:* To investigate the functional implications of the increase in hippocampal neurogenesis through studying spatial exploration in the Y-

maze and show the behavioral correlation between stimulation and neurogenesis.

- ***Groups 4 and 5*****:** designed for the evaluation of stem/progenitor cell proliferation in the DG following AMN stimulation with platinum electrodes. Male rats (n=5) underwent surgery with platinum electrode insertion and was followed in 3 days by 1h stimulation session. 3 BrdU injections were given 4 days after stimulation. Rats were also tested in the Y-maze at day 4 after stimulation to investigate the functional implication of proliferation and perfused the day after (5 days). Group 5 is the sham group (n=4) that was subjected to the same procedures including wires connecting to the electrodes except for electrical current delivery (Fig. 5A).
- ***Groups 6 and 7:*** designed to track the fate of the stem/progenitor cells that showed proliferation at day 5 following AMN stimulation with copper electrodes. Therefore, the survival of these cells were monitored at 4 weeks after stimulation to check for their integration in the DG. Male rats (n=5) underwent surgery with copper electrode insertion and was followed in 3 days by 1h single stimulation. 3 BrdU injections were given 4 days after stimulation. Rats were tested in the Y-maze twice, at day 4 and at 4 weeks after stimulation to investigate the functional implication of neurogenesis. They were perfused at 4 weeks. Group 7 is the sham group (n=4) that was subjected to the same procedures including connecting wires to the electrodes except for electrical current delivery (Fig. 5A).

- ***Groups 8 and 9:*** designed to track the fate of the stem/progenitor cells that showed proliferation at day 5 following AMN stimulation with platinum electrodes. Therefore, the survival of these cells were monitored at 4 weeks after stimulation to check for their integration in the DG. Male rats (n=5) underwent surgery with platinum electrode insertion and was followed in 3 days by 1h single stimulation. 3 BrdU injections were given 4 days after stimulation. Rats were tested in the Y-maze twice, at day 4 and at 4 weeks after stimulation to investigate the functional implication of neurogenesis. They were perfused at 4 weeks. Group 9 is the sham group (n=3) that was subjected to the same procedures including connecting wires to the electrodes except for electrical current delivery (Fig. 5A).
- ***Groups 10 and 11:*** To simulate clinical practice of DBS, we opted to induce multiple stimulation in the right AMN using platinum electrodes. Three days after surgery and platinum electrode insertion, male rats (n=5) were stimulated once per day (1h each session) for 6 consecutive days. BrdU was given daily along the time points for stimulation (6 times, 50mg/kg/ injection) and the animals were tested in the Y-maze at day 8 and week 4 to investigate the functional implication of neurogenesis. The animals were perfused at 4 weeks. Group 11 is the sham group (n=3) that was subjected to the same procedures including connecting wires to the electrodes except for electrical current delivery (Fig. 5B).

Specific Aim 3: To test for the specificity of AMN targeting in inducing stem/progenitor cell proliferation by stimulating another thalamic nucleus, VPL nucleus, that do not possess direct connections to the hippocampus.

- ***Group 12*:** served as the negative control to check for the selectivity of site stimulation in causing stem/progenitor cell proliferation in the DG. Adult male rats (n=4) underwent copper electrode implantation and stimulation (3 days after) in the right VPL nucleus. Rats were given BrdU injections 4 days after stimulation and perfused 1 day later (day 5).

Specific Aim 4: To examine the effect of a single injection or continuous micro-perfusion of kainic acid on proliferation (day 8) and differentiation (week 4) of stem/progenitor cells in the DG.

- *Sub-aim 1:* To study the effect of a single injection of kainic acid
- *Sub-aim 2:* To study the effect of continuous micro-perfusion of kainic acid for 7 days
- *Sub-aim 3:* To investigate the functional implications of the increase in hippocampal neurogenesis through studying spatial exploration in the Y-maze and show the behavioral correlation between chemical stimulation and neurogenesis.

- ***Group 13*:** designed to compare the effects of chemical stimulation on the proliferative activity in the SGZ. Chemical stimulation was induced through a single microinjection of 1 μL Kainic acid (KA) of molarity 500 pM into the

right AMN of female rats (n=3). 3 BrdU injections were given 4 days after injection and rats were perfused 24 h later (day 5).

- ***Groups 14 and 15:*** for continuous chemical stimulation of AMN neurons, mini-osmotic pumps containing low doses of KA were used (500pM, 1µl per hour injection for 7 days). 3 BrdU injections were given to male rats (n=6) after 8 days of the surgery. The animals were tested in the Y-maze at day 8 to investigate the functional implication of proliferation. The animals were perfused at day 9 to check for proliferation of stem/progenitor cells. Group 14 is the sham group (n=4) that was subjected to similar procedures with mini-osmotic pumps containing saline (Fig. 5C).
- ***Groups 16 and 17:*** for continuous chemical stimulation of AMN neurons, mini-osmotic pumps containing low doses of KA were used (500pM, 1µl per hour injection for 7 days). 3 BrdU injections were given to male rats (n=6) after 8 days of the surgery. The animals were tested in the Y-maze at day 8 and week 4 to investigate the functional implication of neurogenesis. The animals were perfused at week 4 to check for the survival of the stem/progenitor cells that previously proliferated. Group 16 is the vehicle group (n=3) that was subjected to similar procedures with mini-osmotic pumps containing saline (Fig. 5C).

A.

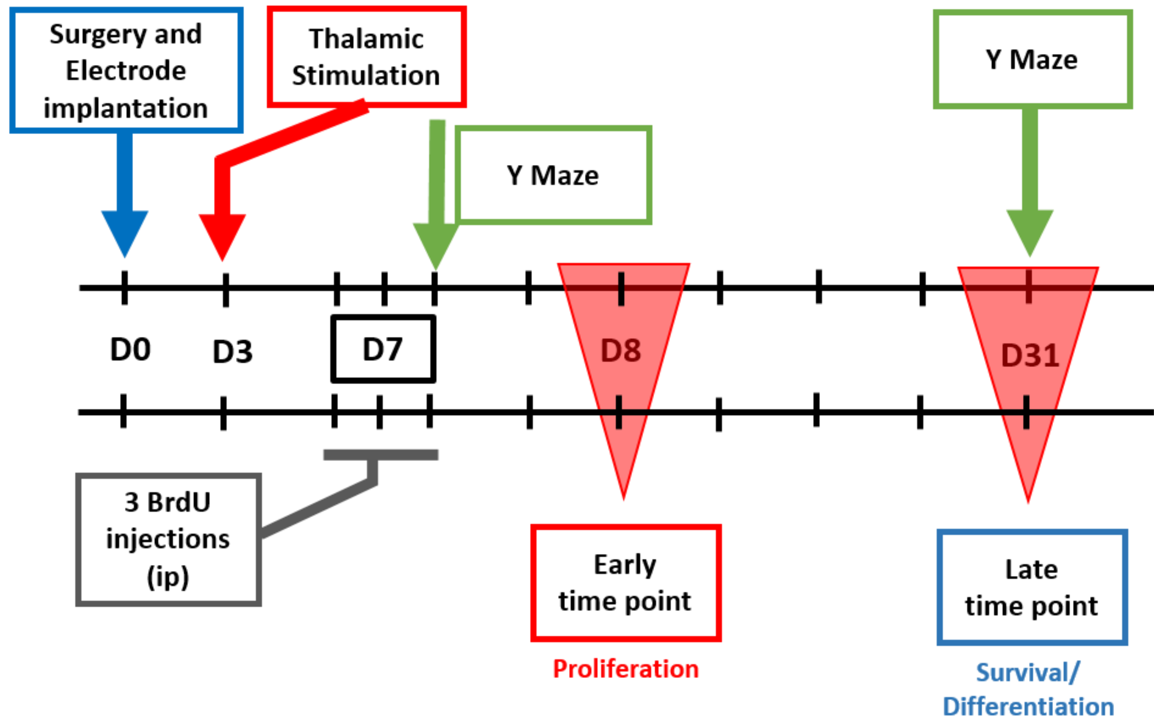

Figure 5A: Timeline followed in the protocols for a single session of electrical stimulation using copper or platinum electrodes.

B.

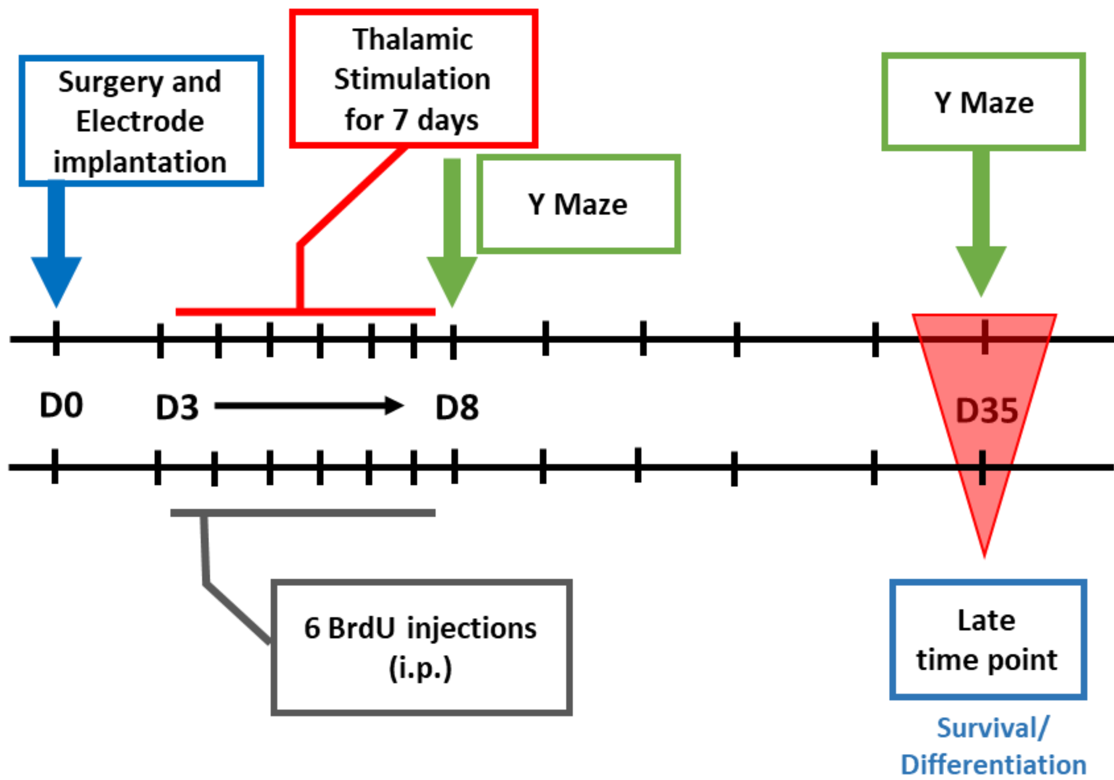

Figure 5B: Timeline followed in the protocols for multiple sessions of electrical stimulation using platinum electrodes.

C.

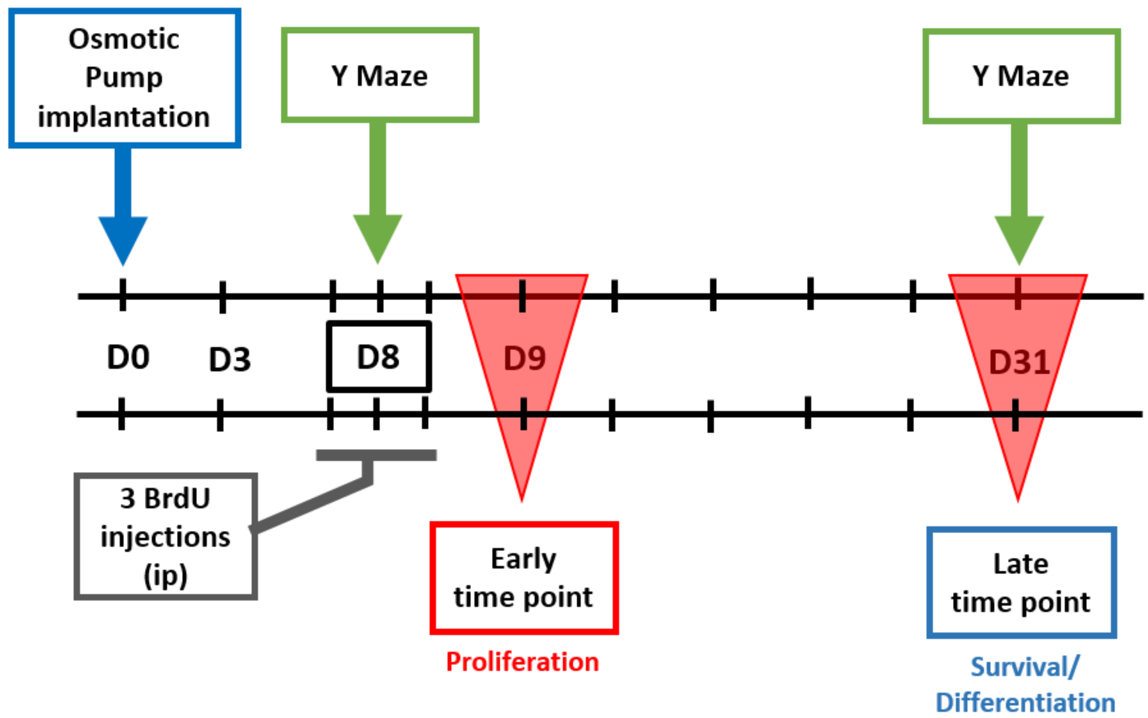

Figure 5C: Timeline followed in the protocols for continuous micro-perfusion of kainic acid using osmotic pumps.

Table 2:

Groups	Description	Rats	Treatment	BrdU Injection	Perfusion
Group 1	Basal neurogenesis levels in males and females	n = 4 males n = 4 females	No Implantation / No Stimulation	3 BrdU injections (66mg/kg/injection/ 3h; *ip*)	24 h after last BrdU injection
Group 2	Electrode implantation effects on proliferation of stem/progenitor cells in males and females (Sham)	n = 4 males n = 3 females	Copper electrode implantation in AMN / No stimulation	3 BrdU injections (66mg/kg/injection/ 3h; *ip*) given 3 days after implantation	24 h after last BrdU injection
Group 3	AMN electrical stimulation effects on proliferation of stem/progenitor cells in DG	n = 6 males n = 6 females	Copper electrode implantation in AMN / Single session of stimulation	3 BrdU injections (66mg/kg/injection/ 3h; *ip*) given 4 days after stimulation	24 h after last BrdU injection
Groups 4&5	AMN electrical stimulation effects on proliferation of stem/progenitor cells in DG	n = 5 males n = 4 sham	Platinum electrode implantation in AMN / Single session of stimulation	3 BrdU injections (66mg/kg/injection/ 3h; *ip*) given 4 days after stimulation	24 h after last BrdU injection
Groups 6&7	AMN electrical stimulation effects on survival of the proliferated cells in DG	n = 5 males n = 4 sham	Copper electrode implantation in AMN / Single session of stimulation	3 BrdU injections (66mg/kg/injection/ 3h; *ip*) given 4 days after stimulation	4 weeks after last BrdU injection

Groups 8&9	AMN electrical stimulation effects on survival of the proliferated cells in DG	n = 5 males n = 3 sham	Platinum electrode implantation in AMN / Single session of stimulation	3 BrdU injections (66mg/kg/injection/ 3h; *ip*) given 4 days after stimulation	4 weeks after last BrdU injection
Groups 10&11	AMN electrical stimulation effects on survival of the proliferated cells in DG	n = 5 males n = 3 sham	Platinum electrode implantation in AMN / Multiple sessions of stimulation	6 BrdU injections (50mg/kg/injection/ day; *ip*) given at each day of stimulation	4 weeks after last BrdU injection
Group 12	VPL nucleus electrical stimulation effects on proliferation of stem/progenitor cells in DG (for target specificity)	n = 4 males	Copper electrode implantation in VPL nucleus / Single session of stimulation	3 BrdU injections (66mg/kg/injection/ 3h; *ip*) given 4 days after stimulation	24 h after last BrdU injection
Group 13	AMN chemical stimulation effects on proliferation of stem/progenitor cells in DG	n = 3 females	Kainic Acid injection in AMN / Single injection	3 BrdU injections (66mg/kg/injection/ 3h; *ip*) given 4 days after injection	24 h after last BrdU injection
Groups 14&15	AMN chemical stimulation effects on proliferation of stem/progenitor cells in DG	n = 6 males n = 4 vehicle group	Kainic Acid continuous injections in AMN / (1µl/h/7days)	3 BrdU injections (66mg/kg/injection/ 3h; *ip*) given 8 days after surgery	24 h after last BrdU injection

Groups 16&17	AMN chemical stimulation effects on survival of the proliferated cells in DG	n = 6 males n = 3 vehicle group	Kainic Acid continuous injection in AMN / (1µl/h/7days)	3 BrdU injections (66mg/kg/injection/ 3h; *ip*) given 8 days after surgery	4 weeks after last BrdU injection

C. Experimental Methods

1. Animals Used

All the experimental procedures and surgeries were pre-approved by the Institutional Animal Care and Use Committee at the American University of Beirut. The animals used in the experiments were adult male and female Sprague Dawley rats weighing 250-300 g. All animals were housed in a controlled environment with a 12 h light/dark cycle, at a constant temperature of 23 ± 2 °C, along with standard rodent chow and water provided ad libitum. The rats were placed individually in deep cages to prevent them from twitching each other's electrodes and stitches, and to further avoid electrode entanglement with the cage grating.

2. Anesthesia

Surgical procedures were performed under deep anesthesia, whereby rats were given intraperitoneal injections (400µl/ip injection) containing a mixture of xyla (Xylazine®; 12 mg/Kg) and ketamine (Ketalar®; 50 mg/kg). Deep anesthesia was attained when no reflexes or pain was detected after pinching of tails.

3. Stereotactic Surgery

Under deep anesthesia and aseptic conditions, the head of the rat was firmly fixed on a stereotaxic frame (DKI, USA), the skin of the scalp was shaved and an incision was made to expose the skull. A hole was drilled into the skull above the target area and implantation with the modality of interest (copper or platinum electrodes / brain infusions / Hamilton syringe) was made stereotaxically into the right AMN, using micromanipulators for accurate placement of the tip of electrodes or microinjection devices. The following stereotaxic coordinates were used: -1.4 mm caudal to bregma, +0.8 mm lateral, with reference to midline, and 6 mm vertical from the surface of the brain (Paxinos and Watson, 1998). In the VPL nucleus stimulated group, copper electrodes were implanted at the following coordinates: -2.7 mm from bregma, -3 mm lateral and 6 mm vertical from the surface of the brain (Paxinos and Watson, 1998). The electrodes / brain infusions were fixed to the skull with acrylic glue and the skin was sutured with silk thread. A topical antibiotic was administered (Baneocin, Sandoz), and the rats were returned to their home cages. Rats were given 3 days of post-surgical recovery before starting electrical stimulation. Chemical stimulation, however, started directly at the end of the implantation procedure as discussed later. Postoperative surveillance for the behavior and body weight were monitored during experiments.

4. Electrical Stimulation: DBS

For the delivery of AMN stimulation, our laboratory has designed a stimulator capable of delivering balanced biphasic charge with constant current at 100 μA (Chamaa et al., 2016). High-frequency electrical stimulation was delivered using the stimulator at the

following parameters (100 μA, 125 μs, 130 Hz), with 2.5V amplitude which approximates the settings used in clinical practice (Stone et al., 2011; Toda et al., 2008). The output cables of the stimulator were connected to the implanted electrodes. Rats were awake and allowed to roam freely in their cage during the period of stimulation. The behavior of rats was observed during and after stimulation. Sham rats were subjected to all procedures, including connecting wires to chronic electrodes, except for delivering electrical current. At the end of the session, rats went back to their standardized conditions and were daily observed.

a. Copper Electrodes

For the copper electrodes, we used concentric bipolar copper electrodes (200 μm diameter with 0.3 mm of exposed tip) inserted into a stainless steel guide cannula. The stimulation session was 1h long; each rat was subjected to one stimulation session, except for the sham group. The reason why we did not use these electrodes for multiple stimulation is because copper electrodes have minimal resistance to corrosion or oxidation and they were reported to elicit possible inflammatory responses in tissue when implanted for a long time period (Geddes and Roeder, 2003).

b. Platinum/Iridium Electrodes

Concentric bipolar platinum/iridium electrodes (60 μm diameter with 0.3 mm of exposed tip, Platinum 10% Iridium, Material #: 100167, California Fine Wire Company) were used and inserted into a stainless steel guide cannula. The stimulation session was 1h long, but there were two regimens for stimulation in the platinum electrode groups. The

first group received a one-hour stimulation session. The second group was subjected to 6 one-hour stimulation sessions, at the rate of one session per day for a period of six days, to mimic multiple stimulation procedures used clinically. Platinum electrodes were used for multiple stimulations primarily because they are coated with iridium, which enhances the charge transfer capabilities of the electrodes. Moreover, both platinum and iridium have highly stable atomic configurations and are known to be corrosion-resistant (Geddes and Roeder, 2003).

5. Chemical Stimulation: Kainic Acid Administration

Chemical stimulation of the AMN was done using either a single microinjection of a low dose of KA (1µl injection, 500 pM) or continuous micro-perfusion of the same concentration using mini-osmotic pumps (1µl / h injection) for 7 days. KA solution was prepared freshly on the same day of surgery. KA powder (Tocris Bioscience) was weighed and diluted in sterile saline at an initial concentration of 1mg/ml, and was further diluted to reach a final concentration of 500 pM in each 1µl injection. The vehicle group of rats were given saline instead of KA either as one injection or as continuous micro-perfusion with mini-osmotic pumps.

a. Single Injection (Hamilton Syringe)

A 5µL Hamilton syringe (Hamilton Company, Nevada, USA), filled with a solution of KA (500pM in sterile saline), was lowered stereotaxically into the AMN and left for 2 min. A volume of 1µl of KA solution was injected into the AMN at a very slow rate over a period of 2-3 min. The tip of the needle was maintained in place for another 2-3

min following the injection. The syringe was removed, the scalp was sutured and the rat was allowed to wake up and recover before being returned to the animal cage. The selection of the dose of KA was based on data from the literature showing that the injected amount can evoke sustained neuronal activation without leading to seizures (Montgomery et al., 1999).

b. Continuos Micro-Perfusion with Mini-Osmotic Pumps

The stereotactic surgery to fulfill this procedure is similar to the ones described above, except that brain infusion cannulas (Brain Infusion Kit 1, Alzet, Durect Corporation, Cupertino) were implanted in the AMN (Fig. 6). The main purpose for this procedure is to keep sustained stimulation of the cells in the AMN for a period of one week. First, the solutions of KA or sterile saline (vehicle) were prepared to fill in the mini-osmotic pump (Mini-Osmotic Pump, 1μl per hour for 7 days, Model 2001, Alzet, Cupertino, USA) as well as the catheter following the procedure prescribed by the manufacturer. Second, the cannula was stereotaxically implanted in the brain and fixed on the skull following the same procedures described in the previous sections. Finally, it was connected to the flow moderator and the pump was implanted under the skin of the abdomen. At the end of the implantation procedures the opened skin of the skull and the abdomen were sutured and covered by ointment containing antibiotics (Baneocin, Sandoz).

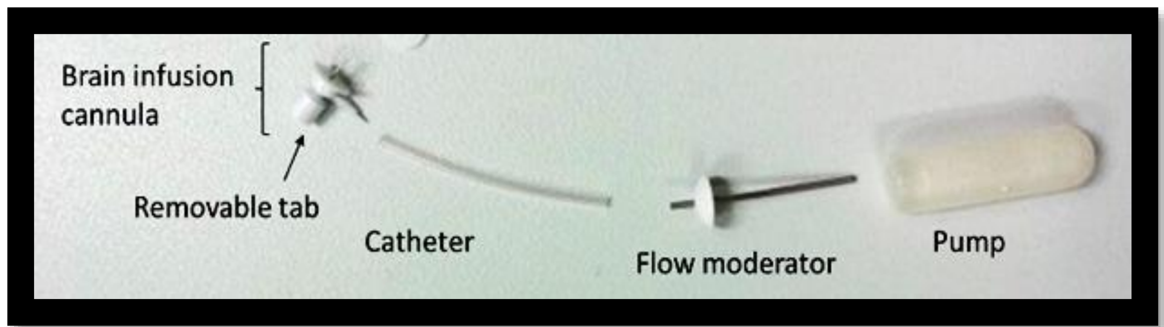

Figure 6. Components of the mini-osmotic pump.
The brain infusion kit contains the cannula, removable tab and the catheter. The mini-osmotic pump kit contains the flow moderator and the pump.

6. *BrdU Administration*

BrdU powder (Sigma-Aldrich, B5002) was weighed and dissolved in 0.9% warm sterile saline at a final concentration of (200 mg/kg in 900µl injection for each rat). All rats received 3 BrdU injections (66mg/Kg/ 300µl injection, ip).

7. *Novel Arm Exploration*

The Y-maze apparatus was used and it consists of three identical arms (10 cm wide and 40 cm long) that are equally spaced (120° apart). No intra-maze cues were added, but different objects were placed at a range of distances outside the maze that were visible to the rats and served as extra-maze cues. The test mainly consisted of two trials that were 1 h apart (Fig. 7). In the first training or acquisition trial, one of the arms was blocked and rats were placed in the "Start" arm. A period of 10 min was timed for the rats to explore and familiarize themselves to both the "start" (S) and the "familiar" arm (F). In the second trial or the test trial (retention trial), the closed/novel arm (N) was opened and rats were also placed in the "S" arm. The rats were allowed to roam and explore the three arms for 5

min whereby the time spent in each arm was recorded by two observers blind to the treatment conditions. The floor and walls of the maze were wiped with 70% alcohol at the end of trial with each rat to avoid odor cues.

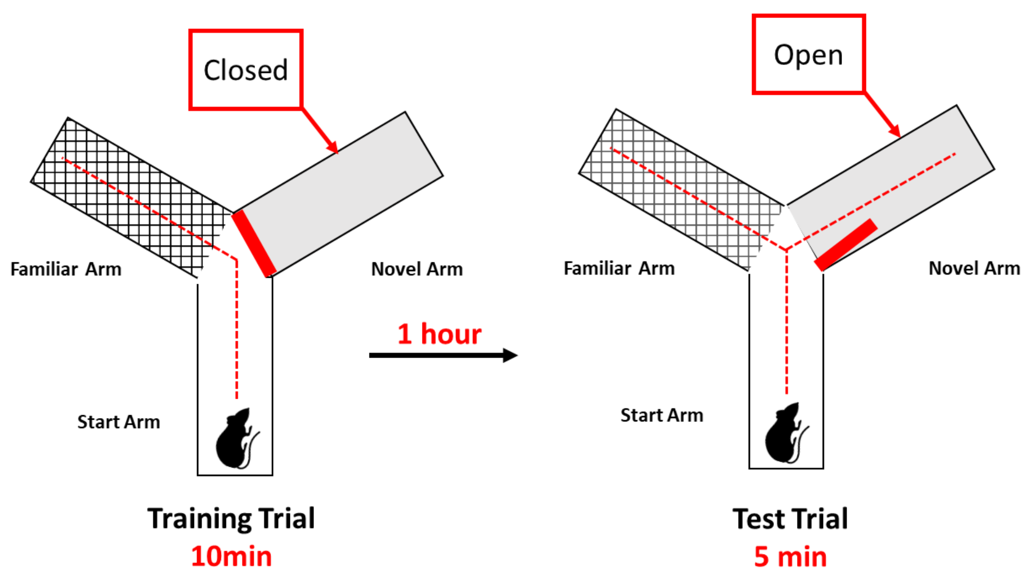

Figure 7: Diagram showing the description of the Novel Arm Exploration test using the Y maze. The first diagram shows the training trial where the Novel arm is closed and the second diagram shows the test trial where the Novel arm is open.

8. Tissue Sampling and Processing

a. Perfusion and Brain Removal

At the end of the observation period scheduled for each group, rats were deeply anesthetized and perfused transcardially with 200 ml of 0.9% saline followed by the same volume of 4% formalin. The chest cavity was opened to expose the heart and a perfusion

cannula was introduced in the left ventricle. The clearing of the red color of the liver is usually an indication that the amount of blood has decreased from the body and that the perfusion is going well. Once cleared, formaldehyde (4%) was allowed to flow in. The skull was exposed through a midline incision and opened with a bone cutter. The brain was carefully removed and placed in a 50ml conical containing 4% paraformaldehyde.

b. Tissue Preparation for Stereology

Brains were fixed overnight in 4% paraformaldehyde and then transferred to 30% sucrose solution in 0.1M PBS and stored at 4 °C until full impregnation (typically 3 days). For unbiased cell stereology, systemic-random sampling of brain sections were completed following the Fractionator principle (Gundersen et al., 1999) (Fig. 8). In brief, 40 μm coronal sections were cut serially using a freezing microtome, from the rostral to the caudal extent of the DG at the following rostro-caudal coordinates covering the whole hippocampal formation (-2.12 to -6.3 mm relative to bregma). To highlight the topographic correspondence of BrdU distribution, the DG region was divided into three areas as follows: rostral ranging from -2.12 to -3.7 mm relative to bregma, intermediate ranging from -3.7 to -4.9 and caudal ranging from -4.9 to -6.3 (Paxinos and Watson, 1998). Sections were serially collected in 24 well plates as 6 sets containing 7 rostral, 5 intermediate and 6 caudal sections per set, where each section is 240 μm apart from the next and each set represents the whole region of interest (Fig. 8). Within the cutting procedure, a needle was inserted every now and then to pierce the right side of the brain. This piercing helps in identifying the right and left sides of the brain sections. All sections were collected and stored in 0.1M PBS solution containing sodium azide (15mM).

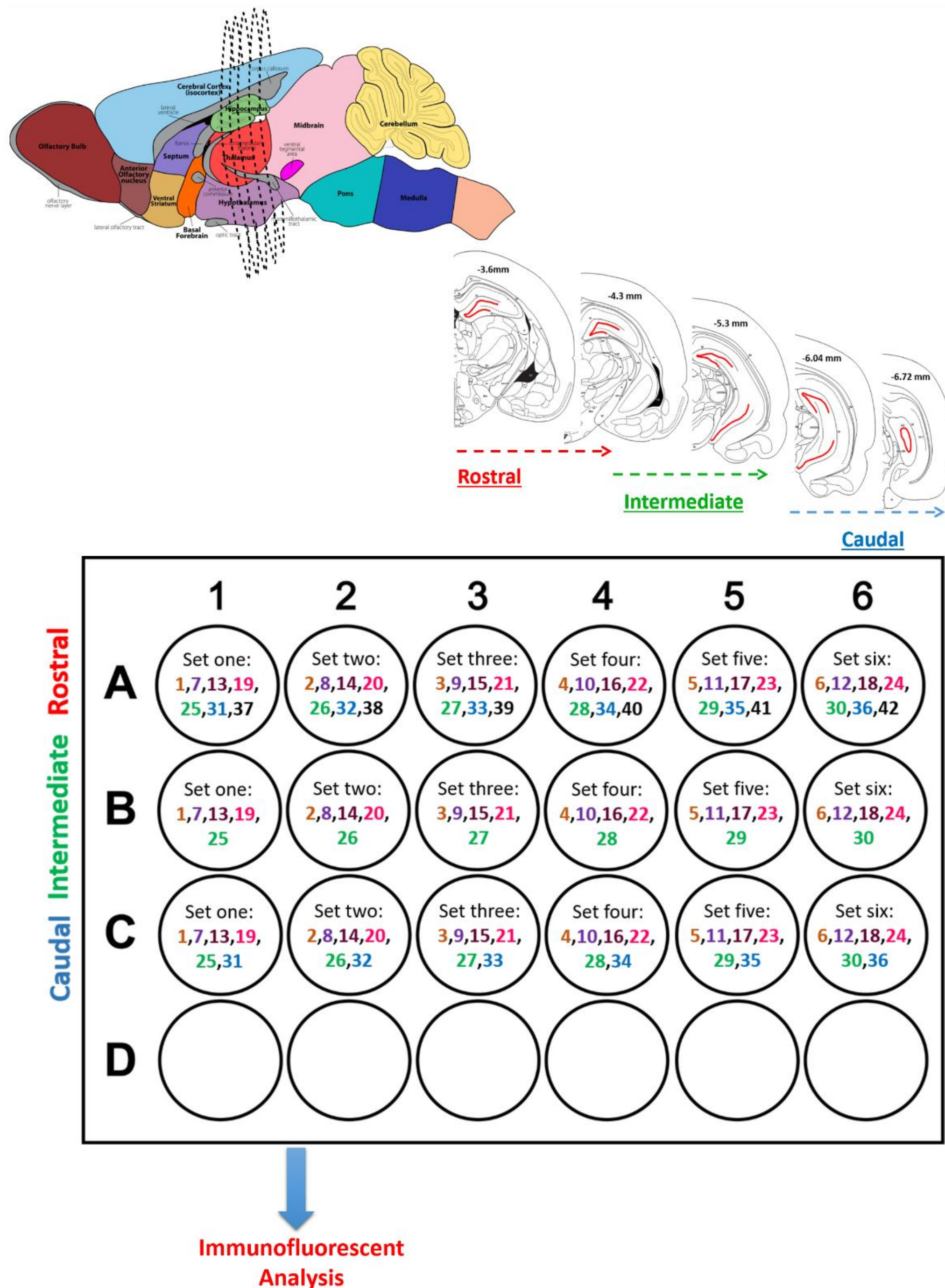

Figure 8. Schematic diagram of the Fractionator Method. Free floating coronal sections are distributed in a 24 well plate. The first section will be placed in the first well, and the following sections will follow in the adjacent wells reaching the 6th slice in well number 6. The 7th slice will be placed back with the first section in well number 1 and this procedure continues all through until the whole hippocampus is covered. This will provide a representative collection of sections encompassing the whole hippocampus whereby each section is 240µm apart from the next one in the same well/set.

c. Cresyl Violet Staining

To verify the correct location of the tip of electrode / brain infusion cannula / needle placement in the AMN or VPL nucleus, cresyl violet staining was performed on selected 40 µm free floating sections that were in the area of insertion. In brief, the free floating sections were washed first with 0.1M PBS then were mounted on Starfrost slides (Starfrost, Knittel Glass, Germany) and dried at 50 °C for 30 m. The slides were then stained with 0.2% cresyl violet solution for 2 m, then rinsed with distilled water 2 times, 5 s each. Dehydration followed using increased ethanol concentration (2 times in 95%, 30 s each and 2 times in 100%, 1 m each) and finally sections were dipped in xylene for 3 m and then slides were mounted with coverslip. Images were captured using a bright field microscope (Fig.9). Rats with incorrect electrode implantation were not included in the study.

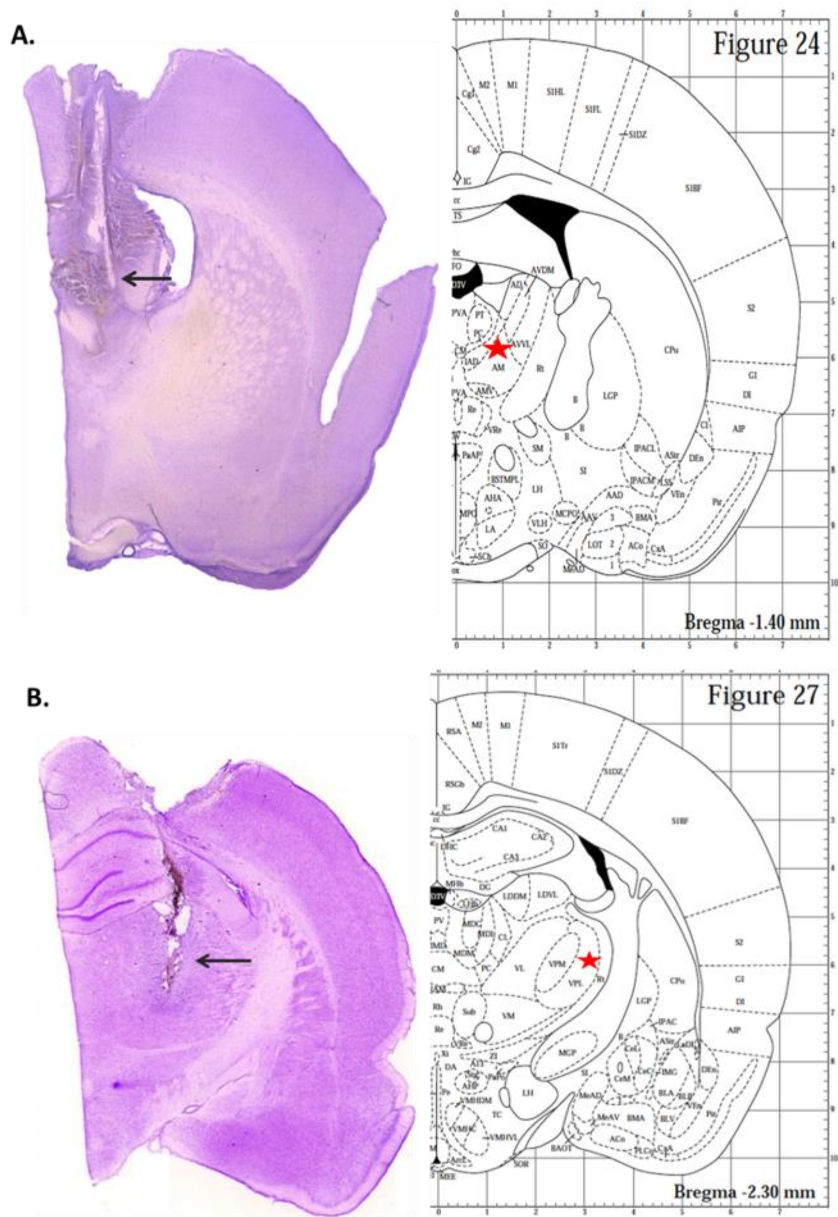

Figure 9. Cresyl Violet staining for verification of electrode implantation.
The location of the electrode in the AMN and VPL groups shown by the headed arrow (A) and (B), respectively. Images were taken using Light microscopy. Pictures on the right are adopted and modified from (Paxinos & Watson, 1998).

d. Immunofluorescence and Confocal Microscopy

One set from each brain region (rostral, intermediate and caudal) of each rat was chosen randomly from the 6 sets. Free-floating sections were washed 3 times (5 min each) with 0.1M PBS in 24-well plate. For BrdU detection, DNA was denatured by incubating the sections in 2N HCl for 30 min at 37°C. Sections were then rinsed with 0.1M PBS for 5 min and washed with 0.1 M Sodium Borate (pH 8.5) for 10min at RT. Tissues were washed 2 X with 0.1M PBS and transferred to the blocking and permeabilization solution (10% NGS, 10% BSA, 0.1% Triton-X diluted in PBS) for 1h at 4°C. In order to minimize non-specific cross labeling between different primary antibodies, we opted to sequentially stain the sections. Therefore, sections were incubated overnight at 4°C with primary antibody rat monoclonal anti-BrdU (1:100; BioRad) diluted in PBS with 3% NGS, 3% BSA, 0.1% Triton-X. The next day, sections were washed 3 times with 0.1M PBS and incubated in the dark with fluorochrome-conjugated secondary antibody Alexa Fluor-568 anti-rat (1:200; Molecular Probes, Invitrogen) diluted in PBS with 3% NGS, 3% BSA and 0.1% Triton-X for 2 h at room temperature on a shaker. Sections were then washed 3 times (5min each) with 0.1M PBS and incubated with mouse monoclonal anti-NeuN (1:500; Millipore) at 4°C overnight diluted in the same solute. The next day the sections were washed 3 times and incubated in the dark, for 2h at RT on a shaker, with the fluorochrome-conjugated secondary antibody Alexa Fluor-488 anti-mouse (1:250; Molecular Probes, Invitrogen) diluted also in the same solution as before. Hoechst stain (1:10,000 in PBS; Molecular Probes, Invitrogen) was added to the sections during the last 10min of the final incubation. After that, the sections were washed with PBS and mounted onto slides with Fluoro-Gel without DAPI (Electron Microscopy Sciences, USA) and covered with a thin glass

coverslip. Microscopic analysis was performed using Zeiss LSM 710 confocal microscope. Cell counting and images were acquired and analyzed using the Zeiss ZEN 2009 image-analysis software.

Double immunofluorescence staining was performed for the evaluation of co-localization of BrdU with another cellular marker for proliferation, namely Ki-67. For this purpose, random selection was made to stain for BrdU and Ki-67 (1:500, Abcam) in several sets of tissues taken from the early time points (day 5) of each group. Moreover, the glial fibrillary acidic protein (GFAP, 1:500, Abcam) was also used as a marker for radial type I neural stem cells. The staining for those antibodies was similar to the ones discussed before. Finally, Z-stack images for BrdU cells were obtained and maximal intensity projection was done to detect co-localization with Ki-67. On the other hand, co-localization of BrdU with NeuN (the mature neuronal marker) was evaluated at the level of survival or late stages (week 4).

e. Cell Stereology

For the analysis of cell proliferation and for survival in the DG, one set out of the six was randomly selected for each region and BrdU immunofluorescence analysis was performed, as described above. Cell stereology was confined to the GCL and SGZ of the DG. BrdU+ cells were counted in every set using 40X-oil objective. The total number of positive cells from the sections of one set was multiplied by 6 (the number of sets per rat), to denote the overall number of BrdU+ cells in each region (rostral, intermediate and caudal) of the DG for each rat (refer to formulas below)

- *Formula-1:* Total number of BrdU+ in rostral DG = number of BrdU+ cells counted in one set (7 sections) x 6 (number of sets).
- *Formula-2:* Total number of BrdU+ in intermediate DG = number of BrdU+ cells counted in one set (5 sections) x 6 (number of sets).
- *Formula-3:* Total number of BrdU+ in caudal DG = number of BrdU+ cells counted in one set (6 sections) x 6 (number of sets).
- To calculate the final number of BrdU+ cells in the whole DG of each rat:

  Total number of BrdU+ in DG = *Formula-1* + *Formula-2* + *Formula-3*

f. Microscopic Analyses

Microscopic analysis was performed using Zeiss LSM 710 confocal microscope and images were acquired using the 40-X oil objective as Z-stack to show the BrdU+ cells distributed within the 40µm section and as tile scan to show the dentate gyrus of each region. The images were analyzed using the Zeiss ZEN 2009 image-analysis software and they were processed with maximal intensity projection. For the purpose of consistency, BrdU+ cells were counted by a single-blinded researcher and images were acquired under the same laser and microscopic parameters.

g. Statistical Analyses

Cell count data were presented as mean ± SEM. The determination of the significance of differences was done using two-tailed t-test or ANOVA, when appropriate. ANOVA was followed by Bonferroni multiple comparisons test. The following variables

were taken into consideration: sex (female / male groups), stimulation (stimulated /sham) side of stimulation (contralateral / ipsilateral to stimulation), and region of DG (rostral / intermediate / caudal). Correlation studies were done using Pearson Correlation test when appropriate. The P value of < 0.05 was considered as the limit of significance of differences. Statistical analysis and plotting of figures were made using Prism 6 GraphPad package (GraphPad software, Inc., CA, USA).

RESULTS

The results show two time points in our study based on the times of proliferation and differentiation of newly dividing stem/progenitor cells. BrdU is known to intercalate into dividing cells during the S phase of the mitotic cycle, making it a good proliferation marker that can be used also to trace the fate and survival of labeled cells during their maturation process. The first time point is the short one of 5 or 8 days where we showed the results of stem/progenitor cells that proliferated at 1-day post BrdU injection. The second is the longer time point of 4 weeks where we showed the differentiation and maturation of stem cells into NeuN-positive neurons.

Basal Levels of Stem/Progenitor Cell Proliferation

Our study revealed differential expression in basal levels of stem/progenitor cell proliferation in the DG of female and male rats, thereby showing sex-dependent differences. Experiments done on naïve female and male rats showed significant variations in the basal levels of stem/progenitor cell proliferation between the different sex groups (Fig. 10A). Remarkably, the average number of BrdU-positive cells in the DG of the male group was 948.75 ± 10 significantly higher than the average number counted in the female group 724.5 ± 14; $P < 0.001$ (Fig. 10B).

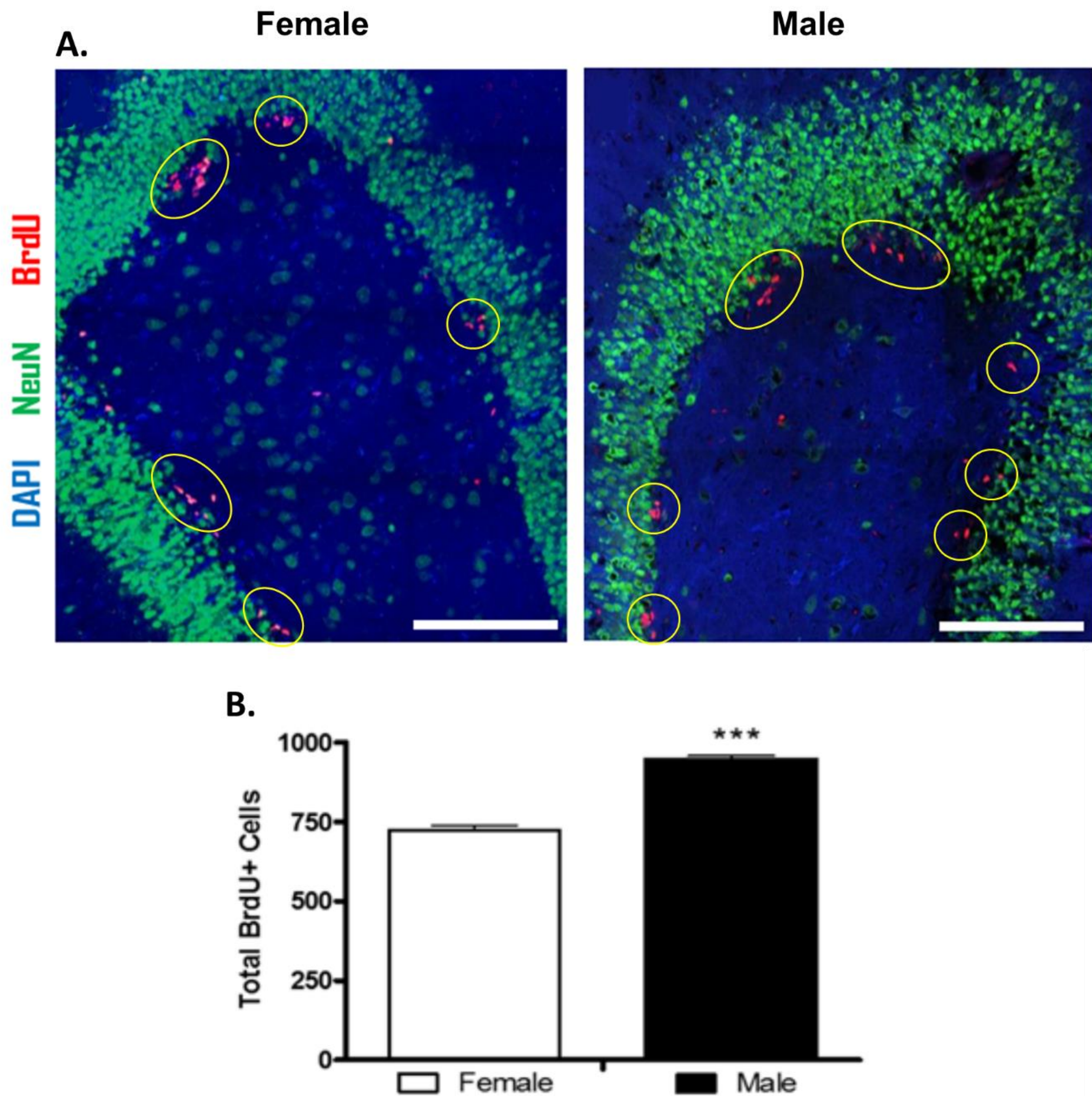

Figure 10. Basal levels of stem/progenitor cell proliferation are higher in male than in female rats. (A) Photomicrographs of the DG neurons labeled with NeuN (*green*) in naive groups sacrificed one day after BrdU injection to detect basal levels of stem/progenitor cell proliferation. These images show BrdU-positive cells (*red*) in females (n=4) and males (n=4). Scale bar = 200µm. Values in the graph (B) represent the mean number of BrdU-positive cells ± SEM showing more BrdU-positive cells in males than in females (***P < 0.001).

A. Short and Long Term Effects of One Session of Electrical Stimulation

1. Stimulation with Copper Electrode

a. Effect at 5 Days Post Stimulation

i. Increased Cellular Proliferation in the Dentate Gyrus

A single session of one-hour unilateral high frequency stimulation by copper electrode placed in the right AMN, resulted in an increase in the number of BrdU-labeled cells in the DG of male and female rats. The number of BrdU-positive cells in the ipsilateral DG, measured at 5 days after stimulation, increased from 952.5 ± 70.59 in the male sham group to 1663 ± 59.22 in the stimulated group ($P < 0.01$) and from 756 ± 24 in the female sham group to 1353 ± 209 in the stimulated group ($P < 0.01$, Fig. 11 and 12). The observed increase in the contralateral DG was significantly less than the ipsilateral DG in both groups. There were BrdU-positive cells around the DG, specifically in the hilar region, but only the ones that were at the SGZ were included in the count as they represent the proliferation of stem/progenitor cells of the DG (Fig. 12).

ii. Stimulation-Induced Increase is Sex-Independent

Although there were significant variations in the basal levels of stem/progenitor cells proliferation between male and female rats, the stimulation-induced proliferation in both sex groups increased equivalently. Despite the final numbers of BrdU-positive cells counted in both sex groups, the rate of increase from the corresponding sham of each group was the same, reaching 78.9% in females and 74.6% in males (Fig.11A and B).

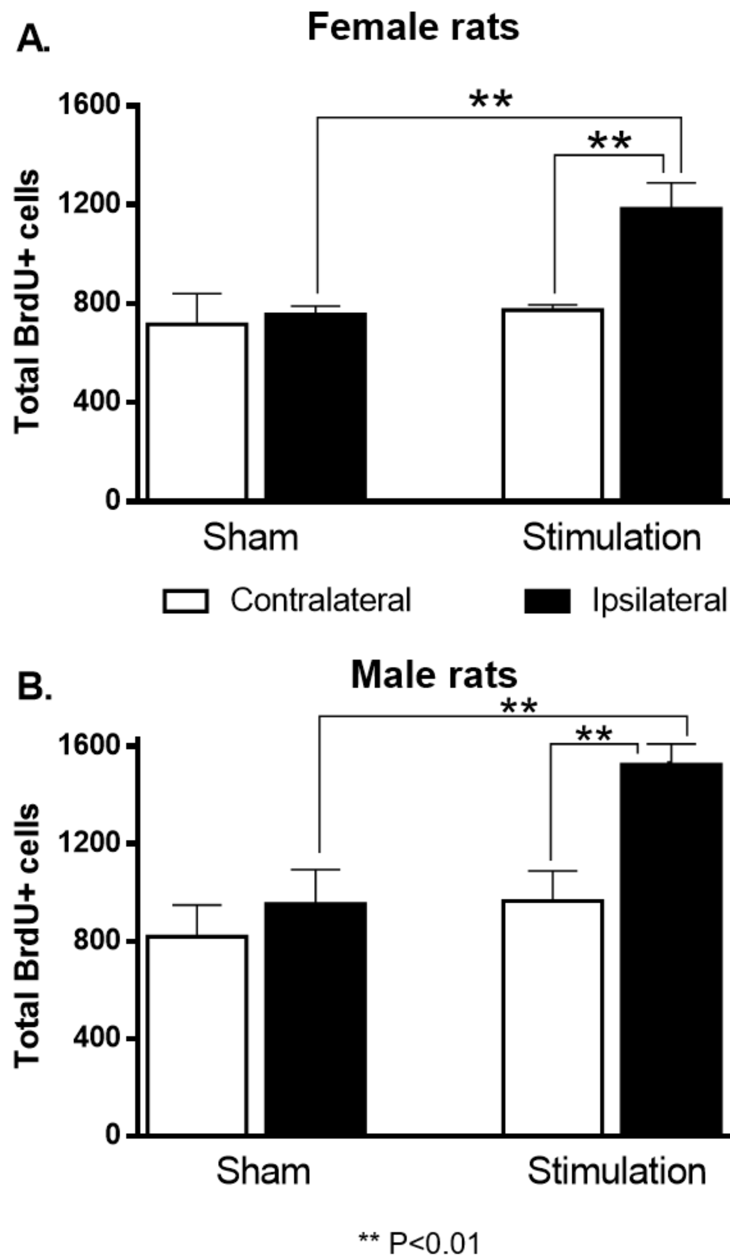

Figure 11. Stimulation placed in the AMN induced comparable proliferation of stem/progenitor cells in the dentate gyrus of male and female rats. Stereological quantification of BrdU-labeled cells in the DG of stimulated (n=6) and sham (n=4) groups of females (A) and males (B). Each bar represents the average ± SEM of BrdU quantification. The red bar represents the similarities of DBS-induced neurogenesis in males and females. The determination of significance of each value was made with reference to the contralateral side of stimulation and sham by ANOVA followed by Bonferroni post hoc test (**$P < 0.01$).

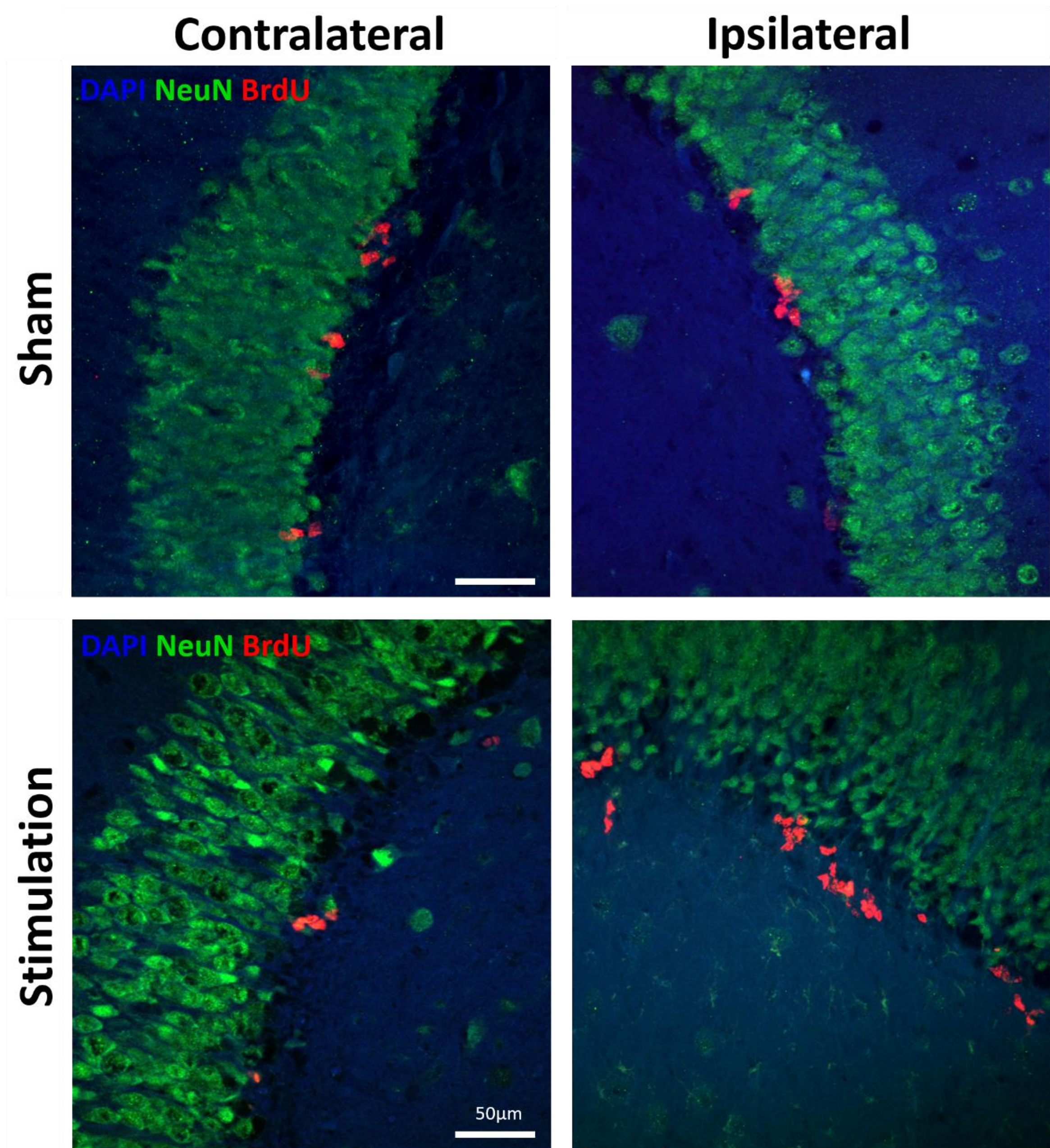

Figure 12. Stimulation of the AMN, using copper electrodes, induced an increase in stem/progenitor cell proliferation in the ipsilateral dentate gyrus. The photomicrographs show immunofluoresence labeling of granular neurons by NeuN (green) and of proliferating cells by BrdU (red) in the contralateral and ipsilateral DG of sham and stimulated groups at 1 week following stimulation. Scale bar = 50µm.

iii. Spatial Distribution of the Increased Proliferation

Our results showed a differential distribution of BrdU-positive cells along the three rostro-caudal regions of the DG attaining a spatial pattern. The rostral DG had the lowest numbers of BrdU-positive cells, the intermediate DG had slightly higher numbers and the highest population was detected in the caudal DG (Fig. 13 and 14). While this representation was seen in the sham group (216 ±19.13 in rostral, 343.5 ± 25.14 in intermediate and 500 ± 52.1 cells in caudal), the same pattern of distribution was also conserved in the stimulated group (219 ± 6.34 in rostral, 498 ± 26.25 in intermediate and 807 ± 46.55 cells in caudal). The vast increase in proliferation following stimulation was confined to the caudal and intermediate regions of the DG, whereby the increase was significant when compared to the respective regions of the sham group ($P < 0.01$, Fig.13).

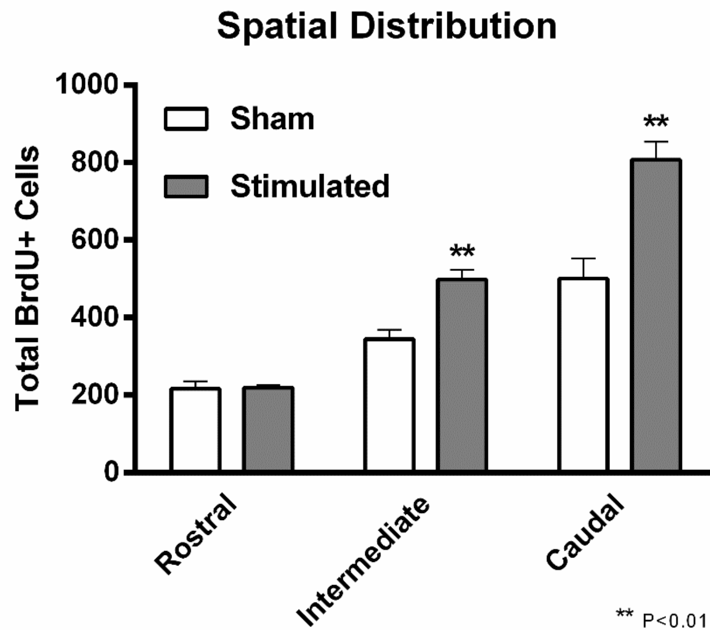

Figure 13. Spatial distribution of increased stem/progenitor cell proliferation in the ipsilateral dentate gyrus following AMN stimulation with copper electrodes. Graph illustrates spatial distribution of the total number of BrdU-labeled cells in rostral, intermediate, and caudal segments of the DG in stimulated (n=6) and sham (n=4) groups of male rats at 5 days. Each bar represents the average ± SEM of BrdU quantification and the determination of significance was made using two tailed t-test where the sham values were compared to the copper stimulated groups for each DG region (**$P < 0.01$).

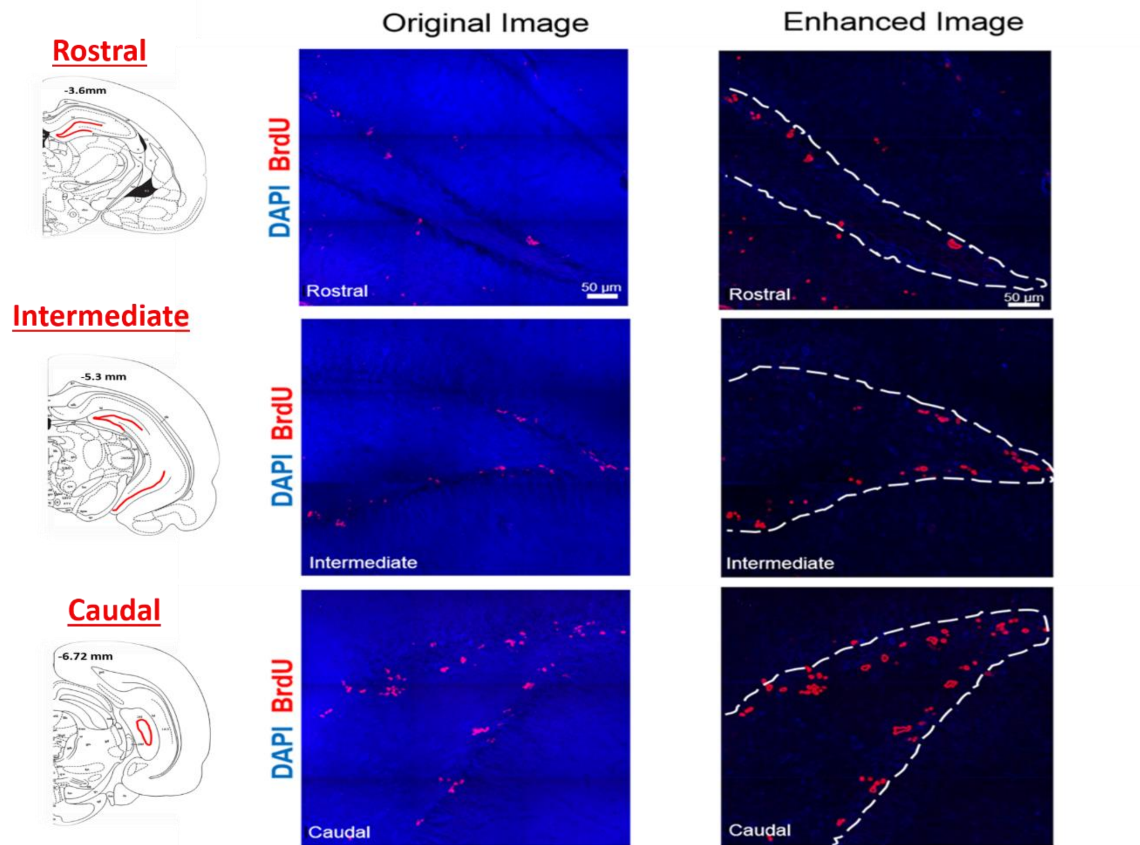

Figure 14. Spatial distribution of stem/progenitor cell proliferation along the rostro-caudal regions of the hippocampus. The drawings on the left side are atlas reconstruction of representative transverse sections at the rostro-caudal levels indicated in each panel. Hippocampus contours on each drawing are shown in red lines. The photomicrographs show immunofluorescence labeling of rostral, intermediate and caudal DG by DAPI and BrdU, where BrdU-positive cells are more prominent in the caudal segment versus the intermediate and rostral segments.

b. Effect on the Development of Labeled Cells at 4 Weeks Post Stimulation

i. Increased Neurogenesis in the Dentate Gyrus

One hour of unilateral stimulation with copper electrodes showed an increased number of BrdU-positive cells in the whole DG at 4 weeks. The number of labeled cells in the sham group (1600 ± 137.58 in the DG ipsilateral to site of electrode insertion and 1794 ± 138.95 in the contralateral DG; n=4) became 3-4 folds higher in the stimulated group (6883.5 ± 521.86 in the ipsilateral DG; P < 0.001 and 5316 ± 308.44 in the contralateral DG; P < 0.001; n=5). It is interesting to note that although the count in both sides showed an increase in BrdU-positive cells at 4 weeks, the numbers in the ipsilateral region were significantly higher than the contralateral ones (P < 0.05) (Fig. 15 and 16).

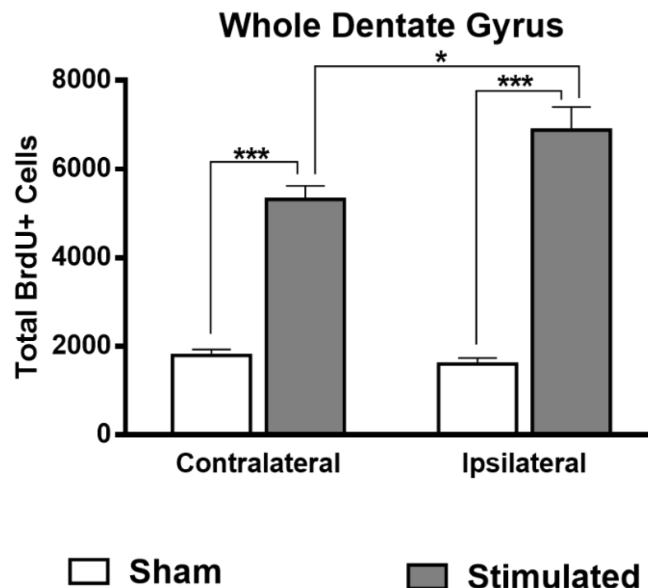

Figure 15. Sustained bilateral increase of neurogenesis induced by AMN stimulation with copper electrodes. Each bar represents the average ± SEM of BrdU quantification, at 4 weeks, in the DG contralateral and ipsilateral to stimulation. Two-tailed t-test was used to determine the significance between stimulated (n=5) and sham (n=4) groups in each hemisphere (***P < 0.001) and between ipsilateral and contralateral sides (*P < 0.05).

ii. Fate of Stimulation-Induced Stem/Progenitor Cells

Tracing the fate of BrdU- positive cells until 4 weeks after stimulation showed that these cells were now co-labeled with the neuronal marker NeuN and that they migrated deeper into the granular zone. This indicated the survival of those cells, their differentiation and their integration into the granular zone (Fig. 16).

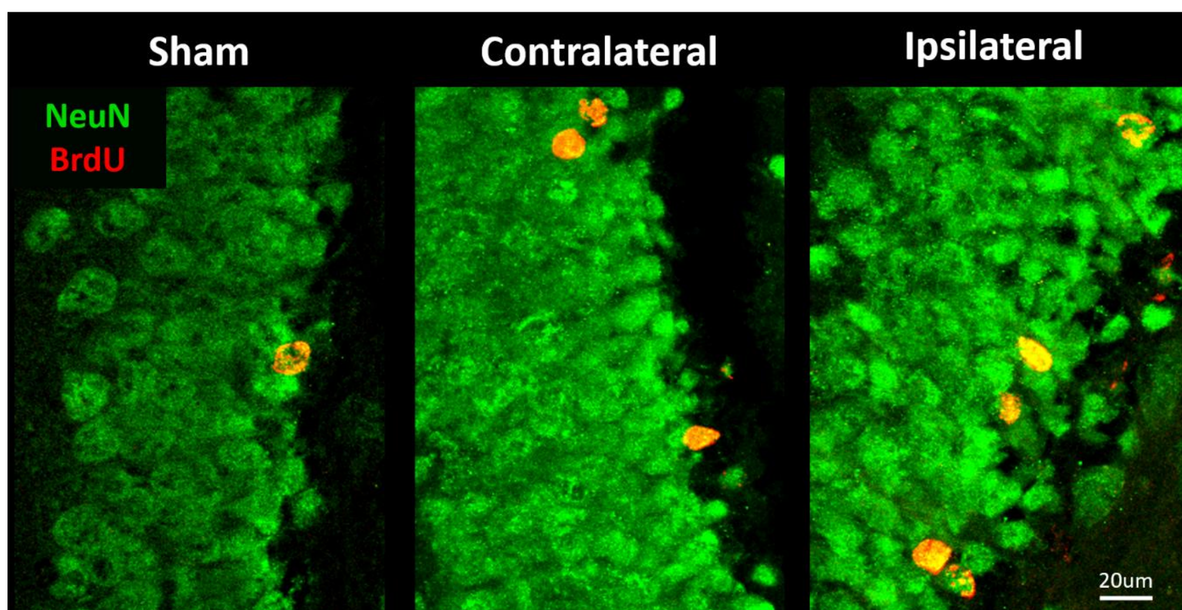

Figure 16. Sustained bilateral increase of neurogenesis induced by AMN stimulation with copper electrodes. Representative confocal images showing immunofluorescence labeling of NeuN and BrdU in the DG of stimulated (n=5) and sham (n=4) groups at 4 weeks. BrdU-positive cells are integrated into the granular zone and co-labeled with NeuN. They are more prominent in the stimulated groups ipsilateral and contralateral to site of stimulation. Images were taken as Z stacks using 40X-oil objective.

iii. Spatial Distribution of Stimulation-Induced Cells

The distribution of labeled cells in different regions of the DG after 4 weeks of stimulation significantly increased at all levels of the DG. The ipsilateral rostral hippocampus showed a significant increase (1562.4 ± 313.87) as compared to the sham

group (518 ± 92.07; $P < 0.05$). The intermediate region also significantly increased (1824 ± 148.21) in comparison to the sham (416 ± 73.51; $P < 0.001$). However, the most significant increase was still preserved in the caudal region of the DG (3634 ± 320.48 in stimulated versus 666 ± 103.46 in sham; $P < 0.001$, Fig. 17A and 18). The effects were not only restricted to the ipsilateral side of stimulation, but also included the contralateral side. Comparisons of the contralateral side of electrode insertion between sham and stimulated groups showed significant increases in the numbers of BrdU-positive cells in the rostral (704 ± 38 versus 1662 ± 246.16; $P < 0.05$), intermediate (350 ± 89.02 versus 1383 ± 113.22; $P < 0.001$) and caudal DG (740 ± 90.42 versus 2107.5 ± 147.11; $P < 0.001$, Fig. 17B and 18).

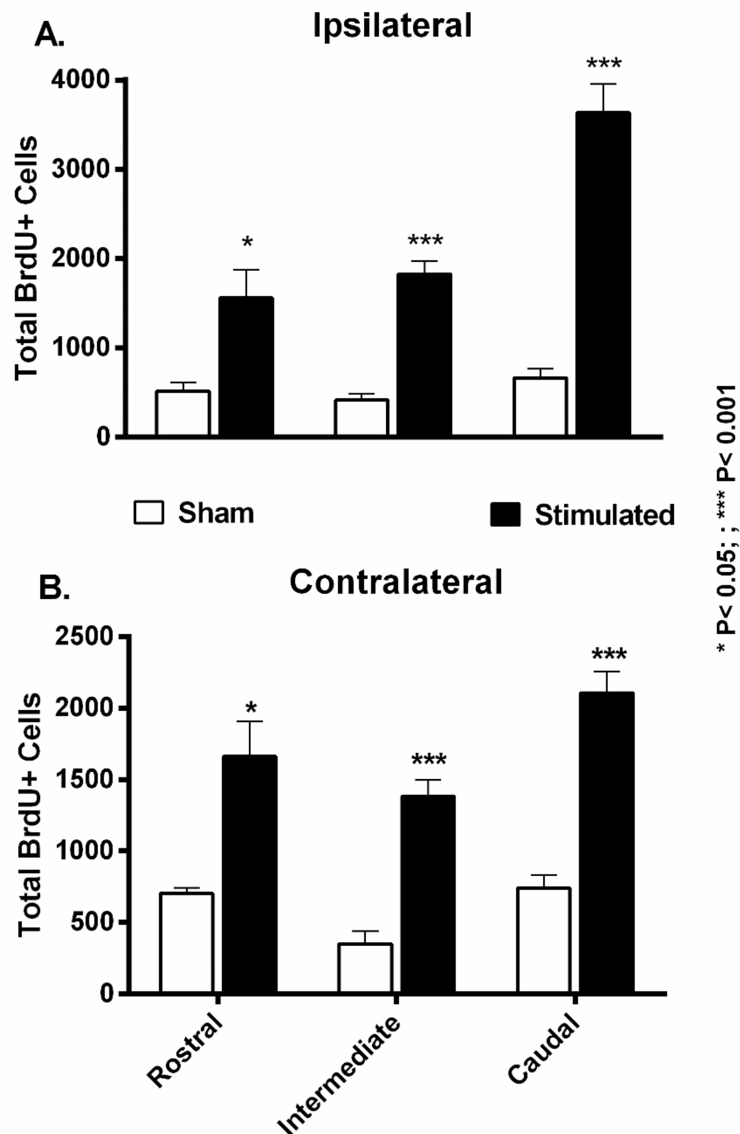

Figure 17. Sustained bilateral increase of neurogenesis induced in the dentate gyrus by AMN stimulation with copper electrodes. The graphs illustrate the spatial distribution of the total number of BrdU-labeled cells in rostral, intermediate, and caudal segments of the ipsilateral (A) and contralateral (B) DG in stimulated (n=5) and sham (n=4) rats at 4 weeks. Each bar represents the average ± SEM of BrdU quantification and the determination of significance was made using two-tailed t-test where the values of sham and stimulated groups were compared between each DG region.

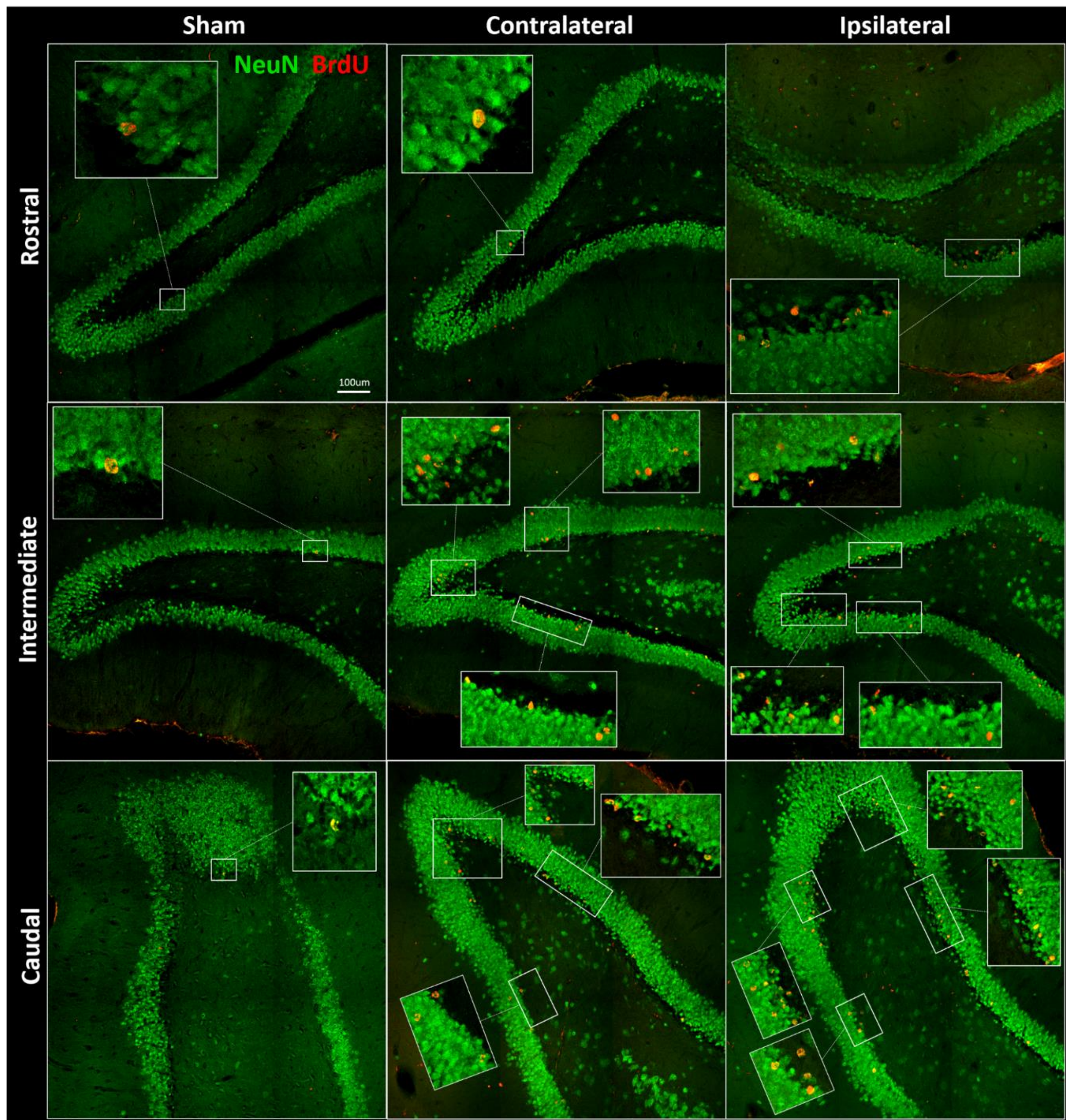

Figure 18. Sustained bilateral increase of neurogenesis in the rostral, intermediate and caudal dentate gyrus by AMN stimulation with copper electrodes. The figure shows confocal images illustrating spatial distribution of BrdU-labeled cells the DG of stimulated (ipsilateral and contralateral side) and sham rats at 4 weeks. Scale bar is 100µm.

c. Preference of the Novel Arm at Week 4 Post Stimulation

In the Y-maze test, there was no difference in the total time spent exploring the novel arm between sham and stimulated groups at week 1 (57.67 ± 3.3 s and 52.4 ± 12.05 s, respectively). However, 4 weeks after stimulation, stimulated rats significantly spent more time exploring the novel arm (187.4 ± 21.61 s) versus the familiar arm (62 ± 6.16 s; $P < 0.001$) and versus the total time spent by sham animals in exploring the novel arm (99.11 ± 9.44 s; $P < 0.01$, Fig. 19).

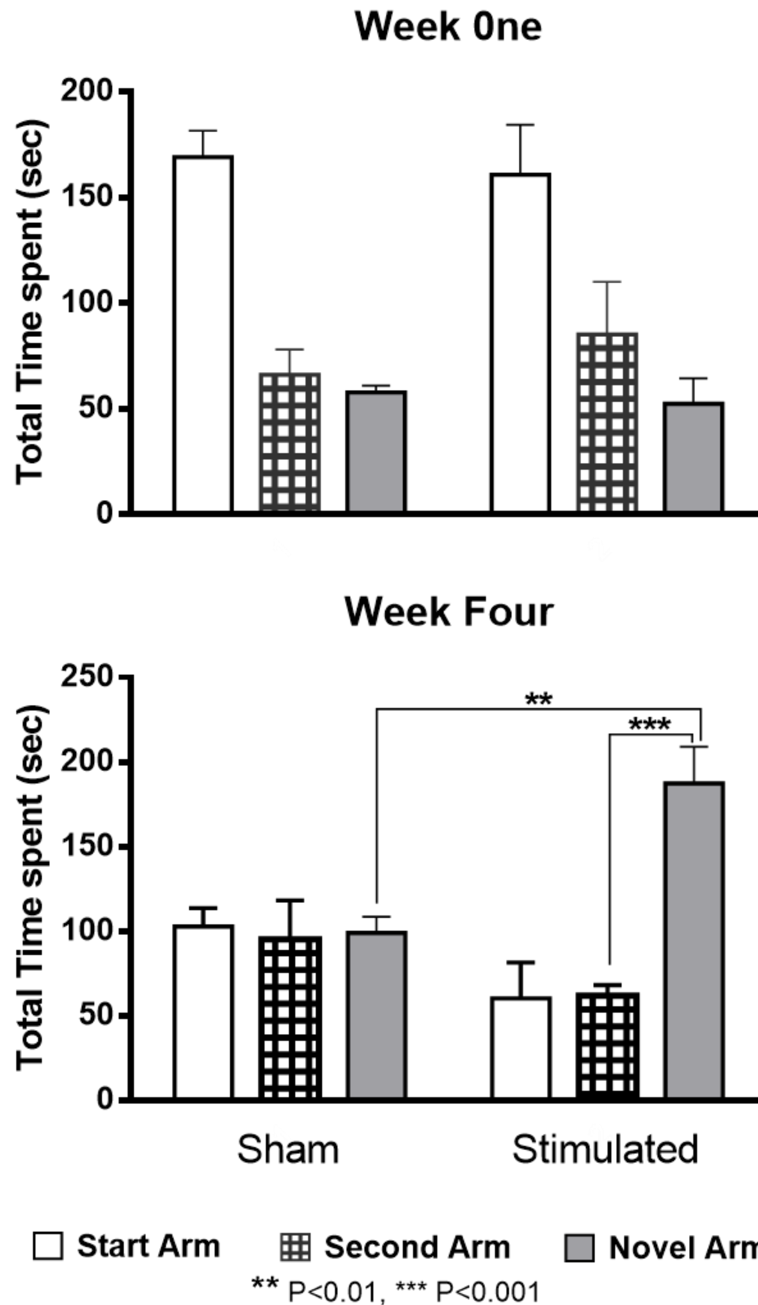

Figure 19. Enhanced exploratory behavior at 4 weeks post stimulation of AMN using copper electrodes. Graph representing the mean ± SEM of the total time spent in the novel, familiar (second) and start arms. The test was done at 1 week and 4 weeks following sham procedure or electrical stimulation. In every time point, two-tailed t-test was performed to compare the differences between total time spent in novel arm measured in sham (n=4) and stimulated (n=5) group and between familiar and novel arm exploration within the same group.

d. VPL nucleus Stimulation

Stimulation using the same parameters and the same type of copper electrodes was applied to the thalamic ventral posterolateral nucleus, instead of the AMN. VPL nucleus stimulation did not alter the baseline of stem/progenitor cell proliferation and thereby demonstrated specificity of the stimulation-induced effects to the AMN and served as a control group to site specificity. The number of BrdU-labeled cells remained equivalent to that observed in sham groups (Fig. 20). In addition, both sides, ipsilateral and contralateral to the site of stimulation exhibited comparable number of BrdU-labeled cells.

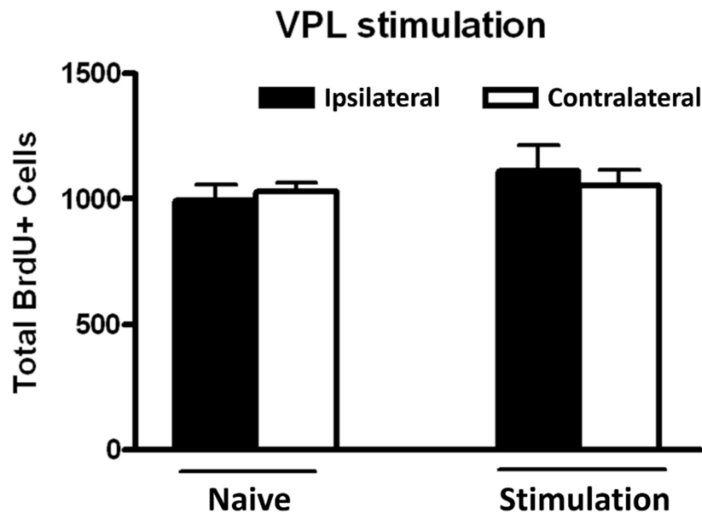

Figure 20. Electrical stimulation, placed in the ventral posterior lateral (VPL) thalamic nucleus, did not induce significant alteration in the proliferation of progenitor cells in the dentate gyrus. Stereological quantification of BrdU-labeled cells in the DG of stimulated (n=4) and naïve (n=4) groups of male rats. Each bar represents the average ± SEM of BrdU quantification in both regions ipsilateral and contralateral to the side of stimulation.

2. Stimulation with Platinum Electrode

a. Effect at 5 Days Post Stimulation

i. Increased Cellular Proliferation in the Dentate Gyrus

The number of BrdU-positive cells in the SGZ of the DG highly increased when rats were subjected to a single session (1h) of electrical stimulation using unilateral platinum electrodes placed in the right AMN. BrdU-positive cells in the ipsilateral DG counted at 5 days after stimulation increased from (1783.75 ± 196.65; n=4) in the sham group to (3391.5 ± 348.53; n=5) in the stimulated group ($P < 0.01$). The contralateral DG also exhibited an increase in the levels of BrdU-positive cells compared to the sham group (2168 ± 132.59 versus 1730 ± 51.05 in sham), but this increase did not reach significant levels (Fig. 21 and 22). There were BrdU-positive cells around the DG, specifically in the hilar region, but only the ones that were at the SGZ were included in the count as they represent the proliferation of stem/progenitor cells of the DG.

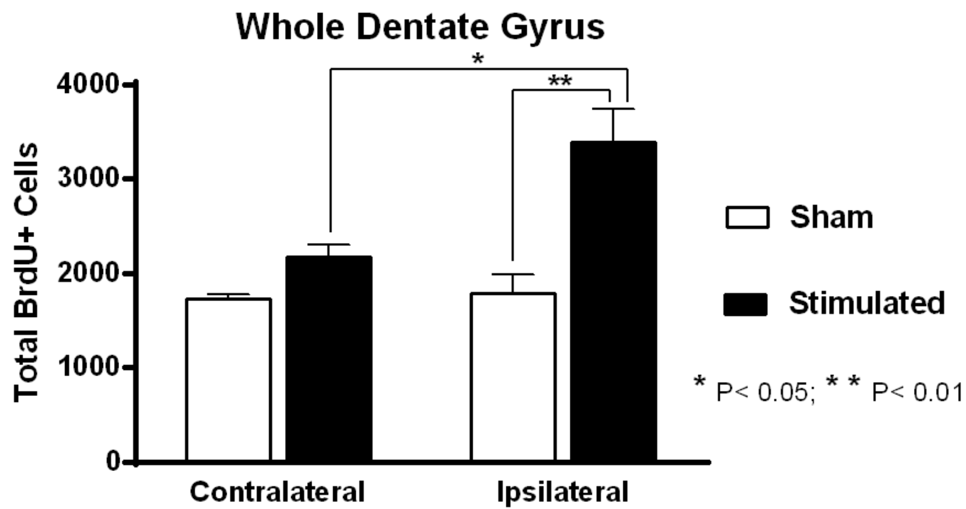

Figure 21. Alteration of the proliferation of stem/progenitor cells in the dentate gyrus at 1 week following unilateral stimulation of the AMN by platinum electrodes. Stereological quantification of BrdU-labeled cells in the DG of stimulated and sham rats at 1 week. Each bar represents the average ± SEM of BrdU quantification. Two-tailed t-test was used to determine the significance between stimulated (n=5) and sham (n=4) groups in each hemisphere.

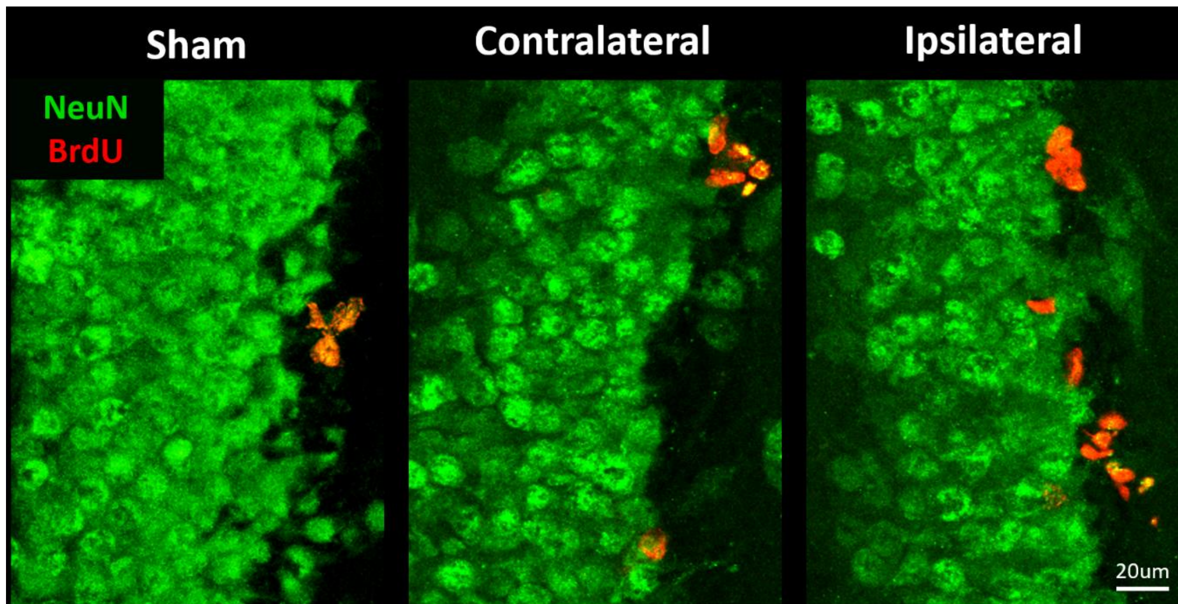

Figure 22. Increased proliferation of stem/progenitor cells in the ipsilateral dentate gyrus at 1 week following stimulation of the AMN by platinum electrodes. Representative confocal images showing immunofluorescence labeling of NeuN and BrdU in the DG of stimulated and sham groups at 1 week. BrdU-positive cells are more prominent in the stimulated group ipsilateral to site of stimulation. Images were taken as Z stacks using 40X-oil objective.

ii. Spatial Distribution of the Increased Proliferation

Spatial distribution of BrdU-positive cells in the hippocampus ipsilateral to electrode insertion showed higher population of cells in the caudal DG of sham animals (1023 ± 158.5) while the numbers were comparable in the rostral and intermediate regions (468 ± 76.17 and 402 ± 72.7, respectively). Stimulation-induced increase in BrdU-labeled cells was mostly confined to the caudal (2107.5 ± 160.37; $P < 0.01$) and the intermediate region of the DG (898.8 ± 100.42; $P < 0.01$; Fig. 23A and 24). There were no significant changes detected in the contralateral region (Fig. 23B and 24).

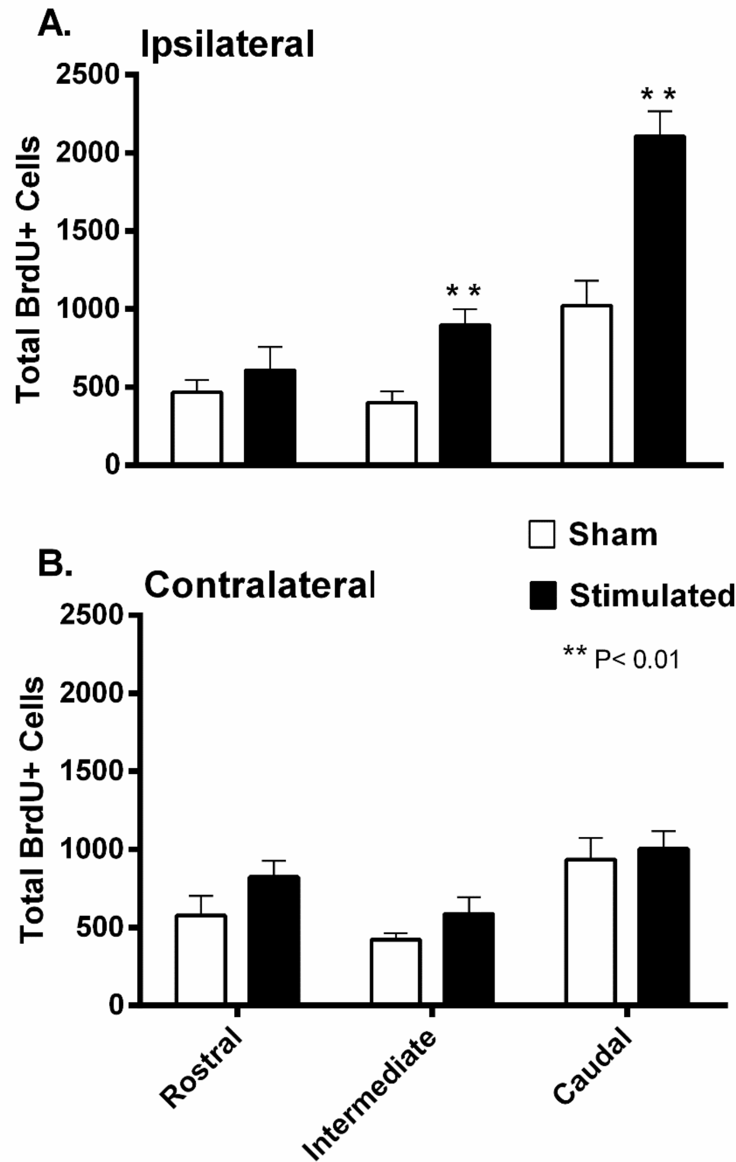

Figure 23. Spatial distribution of increased neurogenesis in the dentate gyrus following AMN stimulation with platinum electrodes. The graphs illustrate spatial distribution of the total number of BrdU-labeled cells in rostral, intermediate and caudal segments of the ipsilateral (A) and contralateral (B) DG in stimulated (n=5) and sham (n=4) groups at 1 week. Each bar represents the average ± SEM of BrdU quantification and the determination of significance was made using two-tailed t-test where the sham values were compared to the platinum stimulated groups for each DG region.

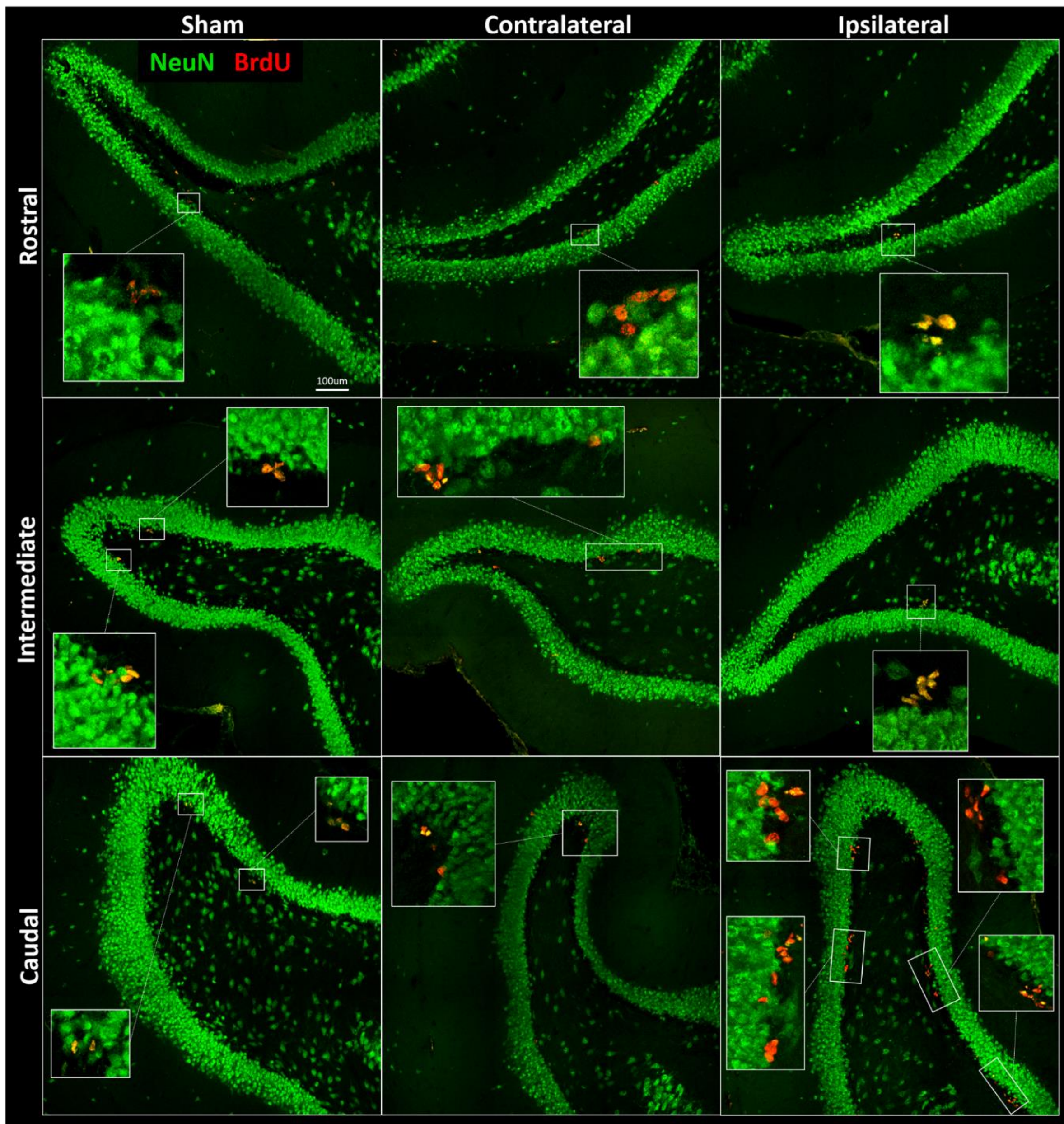

Figure 24. Spatial distribution of increased neurogenesis in the dentate gyrus following AMN stimulation with platinum electrodes. The figure shows confocal images illustrating spatial distribution of BrdU-labeled cells in rostral, intermediate and caudal segments of the DG in stimulated (ipsilateral and contralateral side) and sham rats at 1 week. Scale bar is 100μm.

b. Effect on the Development of Labeled Cells at 4 Weeks Post Stimulation

i. Increased Neurogenesis in the Dentate Gyrus

One hour of unilateral stimulation with platinum electrodes showed an increased number of BrdU-positive cells in the whole DG at 4 weeks. The number of BrdU-labeled cells was 439.33 ± 49.78 in the DG ipsilateral to the site of electrode insertion in the sham group (n=3) and became 1.8 folds higher ($P < 0.01$, Fig. 25 and 26) in the stimulated (n=5) group (804.24 ± 49.54). A significant difference in the increase was detected between the ipsilateral and the contralateral DG of the stimulated group (552.48 ± 50 in the contralateral DG; $P < 0.01$). There were no significant differences in the number of BrdU-positive cells between the contralateral DG of sham and stimulated group (544 ± 66.3 in sham versus 552.48 ± 50 in stimulated group) (Fig. 25 and 26).

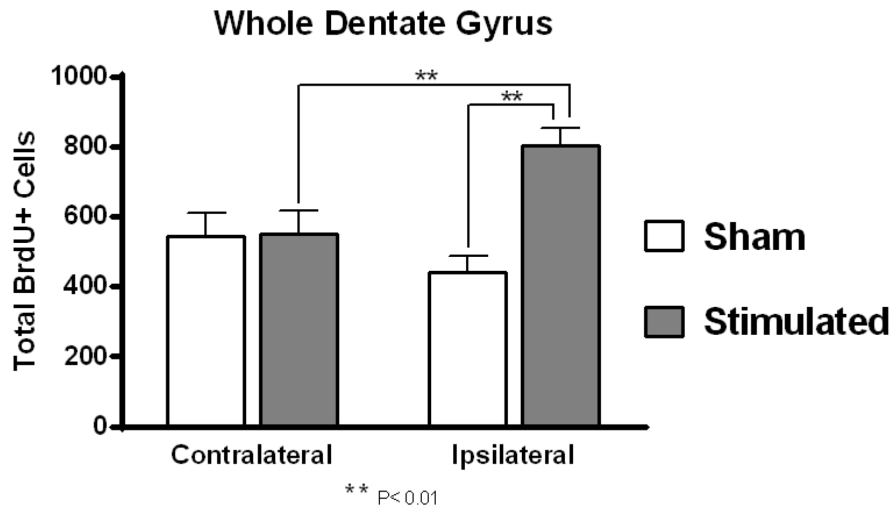

Figure 25. Sustained increase of adult neurogenesis in the dentate gyrus ipsilateral to AMN stimulation with platinum electrodes. Stereological quantification of BrdU-labeled cells in the DG of stimulated (n=5) and sham (n=3) groups at 4 weeks. Each bar represents the average ± SEM of BrdU quantification in the DG contralateral and ipsilateral to stimulation. Two-tailed t-test was used to determine the significance between stimulated and sham groups as well as ipsilateral and contralateral DG.

ii. Fate of Stimulation-Induced Stem/Progenitor Cells

Tracing the fate of BrdU- positive cells until 4 weeks after stimulation showed that these cells were now co-labeled with the neuronal marker NeuN and that they migrated deeper into the granular zone. This indicated the survival of those cells, their differentiation and their integration into the granular zone (Fig. 26).

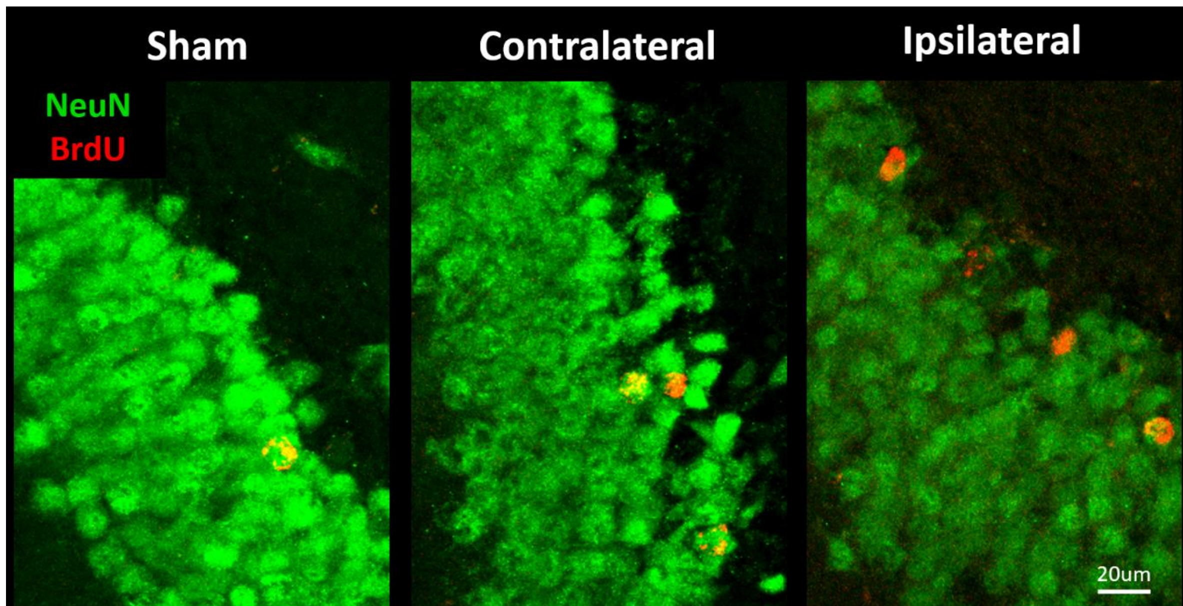

Figure 26. Sustained increase of neurogenesis in the dentate gyrus ipsilateral to AMN stimulation with platinum electrodes. Representative confocal images showing immunofluorescence labeling of NeuN and BrdU in the DG of stimulated and sham groups at 4 weeks. BrdU-positive cells are more prominent in the stimulated groups ipsilateral to site of stimulation. Images were taken as Z stacks using 40X-oil objective.

iii. Spatial Distribution of Stimulation-Induced Cells

The distribution of labeled cells in different regions of the DG after 4 weeks of stimulation showed that the number of BrdU-positive cells increased along the whole ipsilateral DG. The ipsilateral rostral hippocampus shows a significant increase (225.12 ± 20.74) as compared to the sham group (102 ± 24.2; $P < 0.05$). The intermediate region also significantly increased (172.56 ± 17.8) in comparison to the sham (81.33 ± 11.1; $P < 0.01$). Moreover, there was a significant increase in the caudal region of the DG (508.8 ± 28.52 in stimulated versus 255 ± 25 in sham; $P < 0.001$, Fig. 27A and 28). There were no significant changes detected in the contralateral side (Fig. 27B and 28).

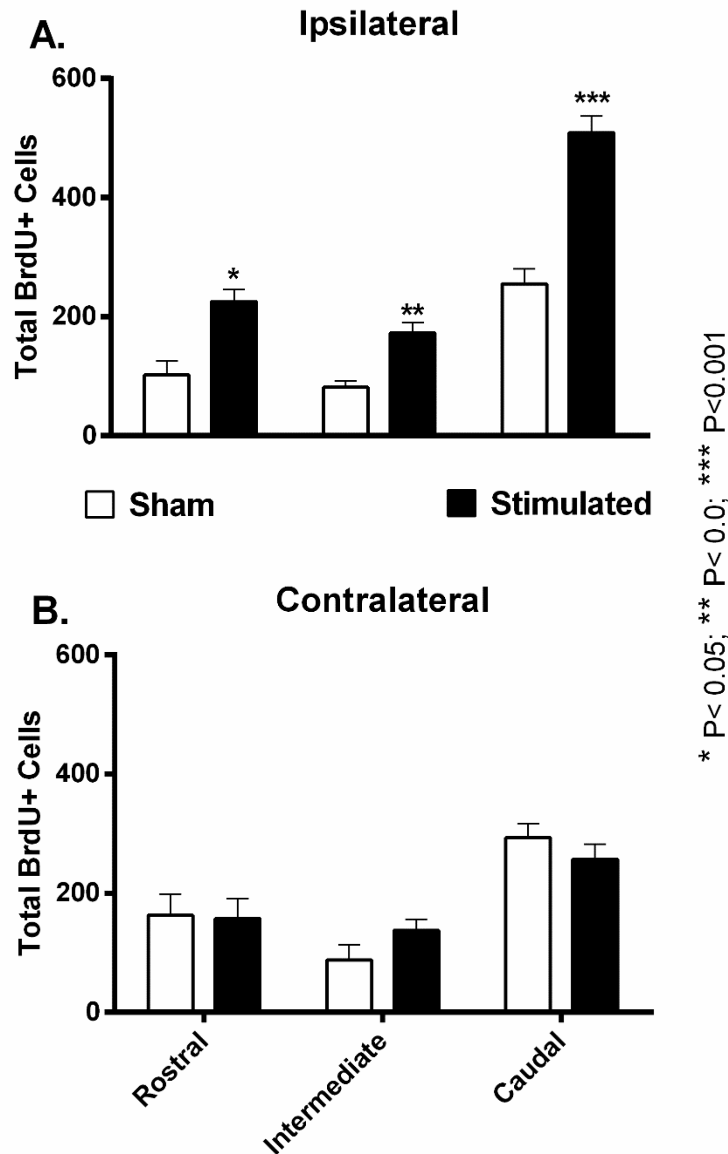

Figure 27. Sustained increase of neurogenesis in the rostral, intermediate and caudal dentate gyrus ipsilateral to AMN stimulation with platinum electrodes. The graphs show the total number of BrdU-labeled cells in rostral, intermediate and caudal segments of the ipsilateral (A) and contralateral (B) DG in stimulated (n=5) and sham (n=3) rats at 4 weeks. Each bar represents the average ± SEM of BrdU quantification and the determination of significance was made using two-tailed t-test where the values of sham and stimulated groups were compared for each DG region.

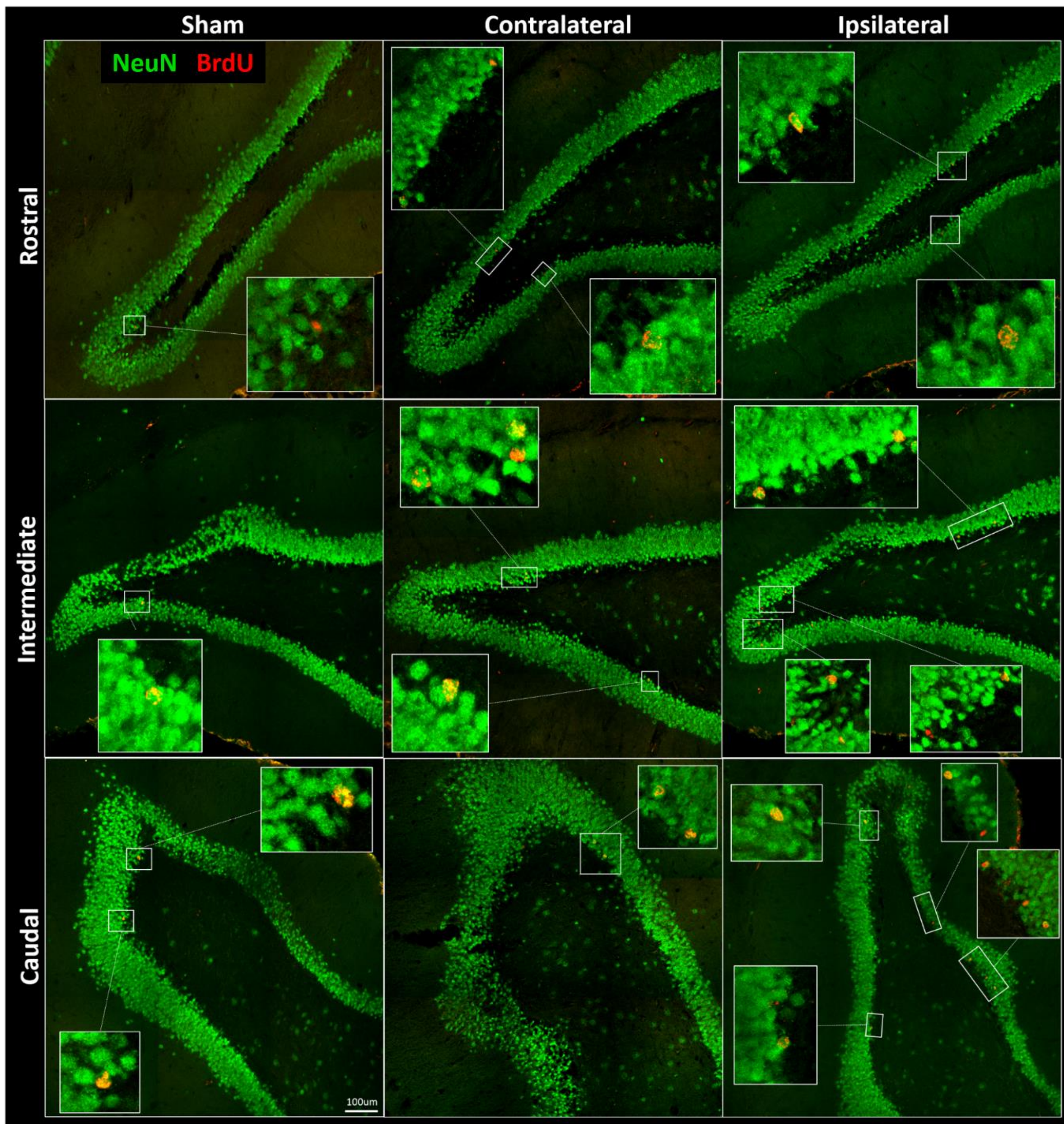

Figure 28. Sustained increase of neurogenesis in the rostral, intermediate and caudal dentate gyrus ipsilateral to AMN stimulation with platinum electrodes. The figure shows confocal images illustrating spatial distribution of BrdU-labeled cells in different segments of DG in stimulated (ipsilateral and contralateral side) and sham rats at 4 weeks. Scale bar is 100µm.

c. Preference of the Novel Arm at Week 4 Post Stimulation

In the Y-maze test, there was no difference in the total time spent exploring the novel arm between sham and stimulated groups at week 1 (111.31 ± 10.85 s and 106.71 ± 10.54 s, respectively). However, exploration of the novel arm was significantly different between the two groups at 4 weeks after stimulation. The stimulated rats significantly spent more time in exploring the novel arm (191.67 ± 20.09 s) versus the familiar arm (27 ± 8.22 s; $P < 0.001$) and versus the total time spent by sham animals in the novel arm (119±5.2; $P < 0.05$, Fig. 29).

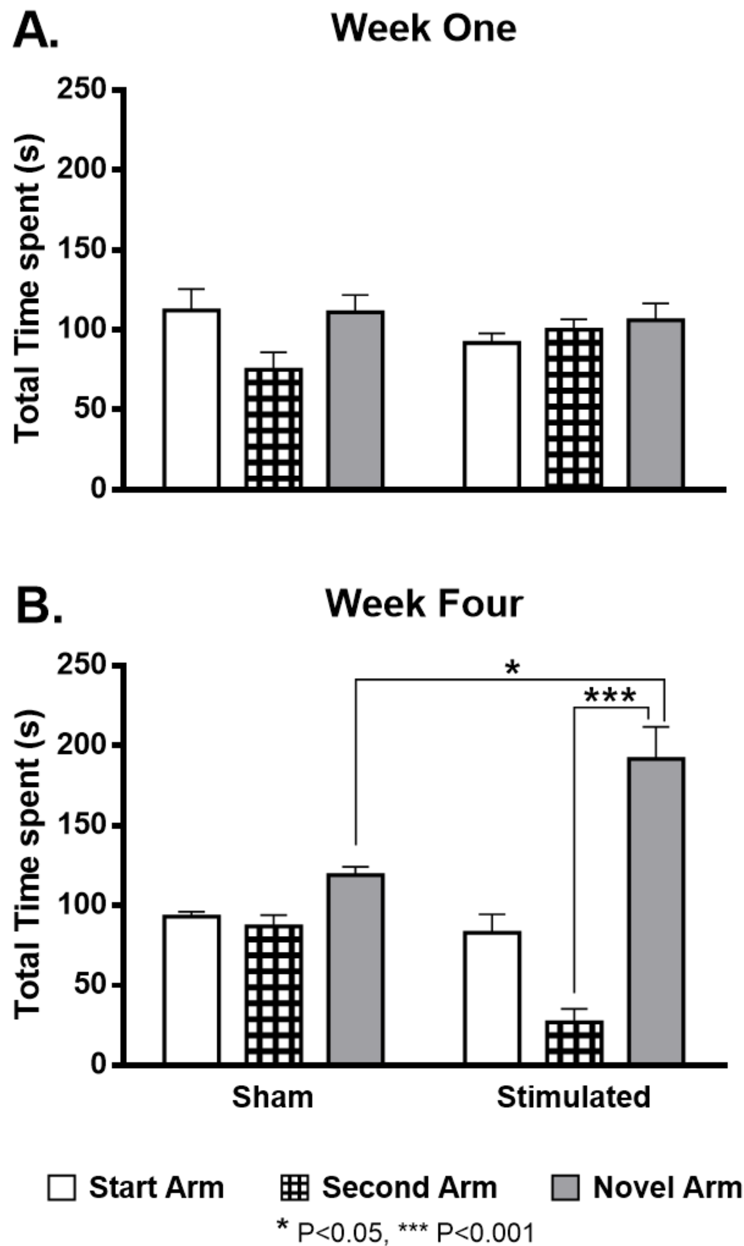

Figure 29. Enhanced exploratory behavior at 4 weeks post stimulation of AMN using platinum electrodes. Graph representing the mean ± SEM of the total time spent in the novel, familiar (second) and start arms. The test was done at 1 week and 4 weeks following sham procedure or electrical stimulation. In every time point, two-tailed t-test was performed to compare the differences between total time spent in novel arm measured in sham (n=3) and stimulated (n=5) group and between familiar and novel arm exploration within the same group.

B. Long Term Effects of Multiple Sessions of Electrical Stimulation to the AMN by Platinum Electrodes

1. Effect on the Development of Labeled Cells at 4 Weeks Post Multiple Sessions of Stimulation

a. Increased Neurogenesis in the Dentate Gyrus

The number of integrated BrdU-labeled cells in the sham group (n=3) reached 544 ± 66.3 in the DG contralateral to electrode insertion and 439.33 ± 49.8 in the ipsilateral DG at 4 weeks. Following multiple sessions of unilateral stimulation with platinum electrode (n=5), the number of BrdU-positive cells significantly increased by 2-3 folds to reach 1230.3 ± 120.4 in the contralateral DG ($P < 0.01$) and 1338.24 ± 150.53 in the DG ipsilateral to stimulating electrodes ($P < 0.01$, Fig. 30 and 31).

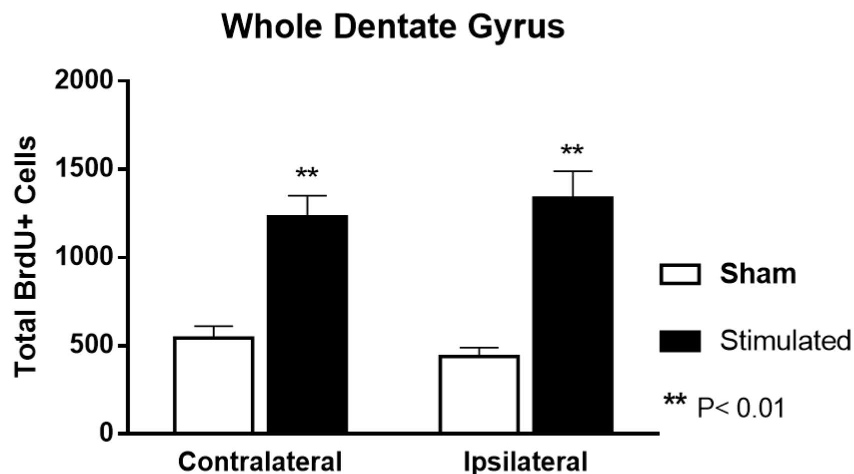

Figure 30. Sustained increase of neurogenesis in the ipsilateral dentate gyrus following multiple stimulation of the AMN by platinum electrodes. Stereological quantification of BrdU-labeled cells in the DG of stimulated (n=5) and sham (n=3) groups at 4 weeks. Each bar represents the average ± SEM of BrdU quantification. Two-tailed t-test was used to determine the significance between stimulated and sham groups.

b. Fate of Stimulation-Induced Stem/Progenitor Cells

Multiple sessions of high frequency stimulation to the AMN using platinum electrodes showed BrdU-positive cells co-labeled with the neuronal marker NeuN at 4 weeks post stimulation. Confocal images clearly show that the BrdU-labeled cells deeply migrated into the granular zone of the DG indicating the survival of those cells until day 40. This indicated cellular differentiation into neurons and their integration into the granular zone (Fig. 31).

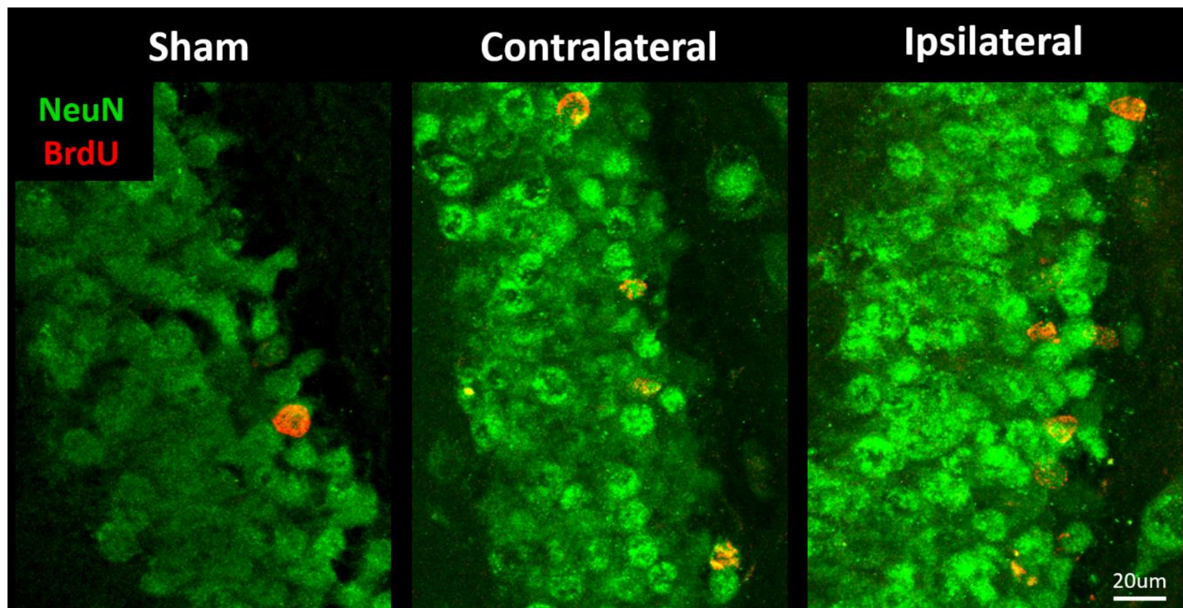

Figure 31. Sustained increase of neurogenesis in the ipsilateral dentate gyrus following multiple stimulation of the AMN by platinum electrodes. Representative confocal images showing immunofluorescence labeling of NeuN and BrdU in the DG of stimulated and sham groups at 4 weeks. BrdU-positive cells are more prominent in the stimulated groups ipsilateral to site of stimulation. Images were taken as Z stacks using 40X-oil objective.

c. Spatial Distribution of Stimulation-Induced Cells

The distribution of labeled cells in different regions of the DG, after 4 weeks of stimulation, showed that the increase in the number of positive cells was distributed along the whole DG. The ipsilateral rostral hippocampus showed a significant increase (265.2 ± 19.29 as compared to sham 102 ± 24.2; $P < 0.01$). The intermediate region also showed significant increased (254.16 ± 30.73 as compared to sham 81.33 ± 11.1; $P < 0.01$). A more significant increase was observed in the caudal region of the DG (944.4 ± 75.6 in stimulated versus 229.3 ± 39.06 in sham; $P < 0.001$, Fig. 32A and 33). In the contralateral side, the increase was also significant in the intermediate region (173.52 ± 20.58 versus 88 ± 25.53 in sham; $P < 0.05$) and mostly significant in the caudal part (703.44 ± 25.32 versus 293.33 ± 23.33 in caudal sham; $P < 0.001$, Fig. 32B and 33).

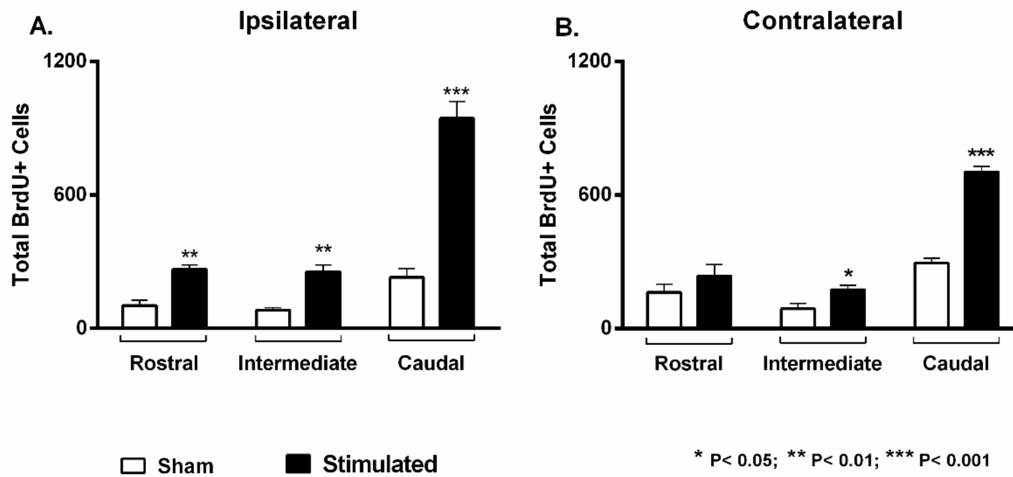

Figure 32. Sustained increase of neurogenesis in the ipsilateral and contralateral dentate gyrus following multiple sessions of stimulation of the AMN by platinum electrodes. The graph illustrates the total number of BrdU-labeled cells in rostral, intermediate and caudal segments of the ipsilateral (A) and contralateral (B) DG in stimulated (n=5) and sham (n=3) rats at 4 weeks. Each bar represents the average ± SEM of BrdU quantification and the determination of significance was made using two-tailed t-test where the values of sham and stimulated groups were compared for each DG region.

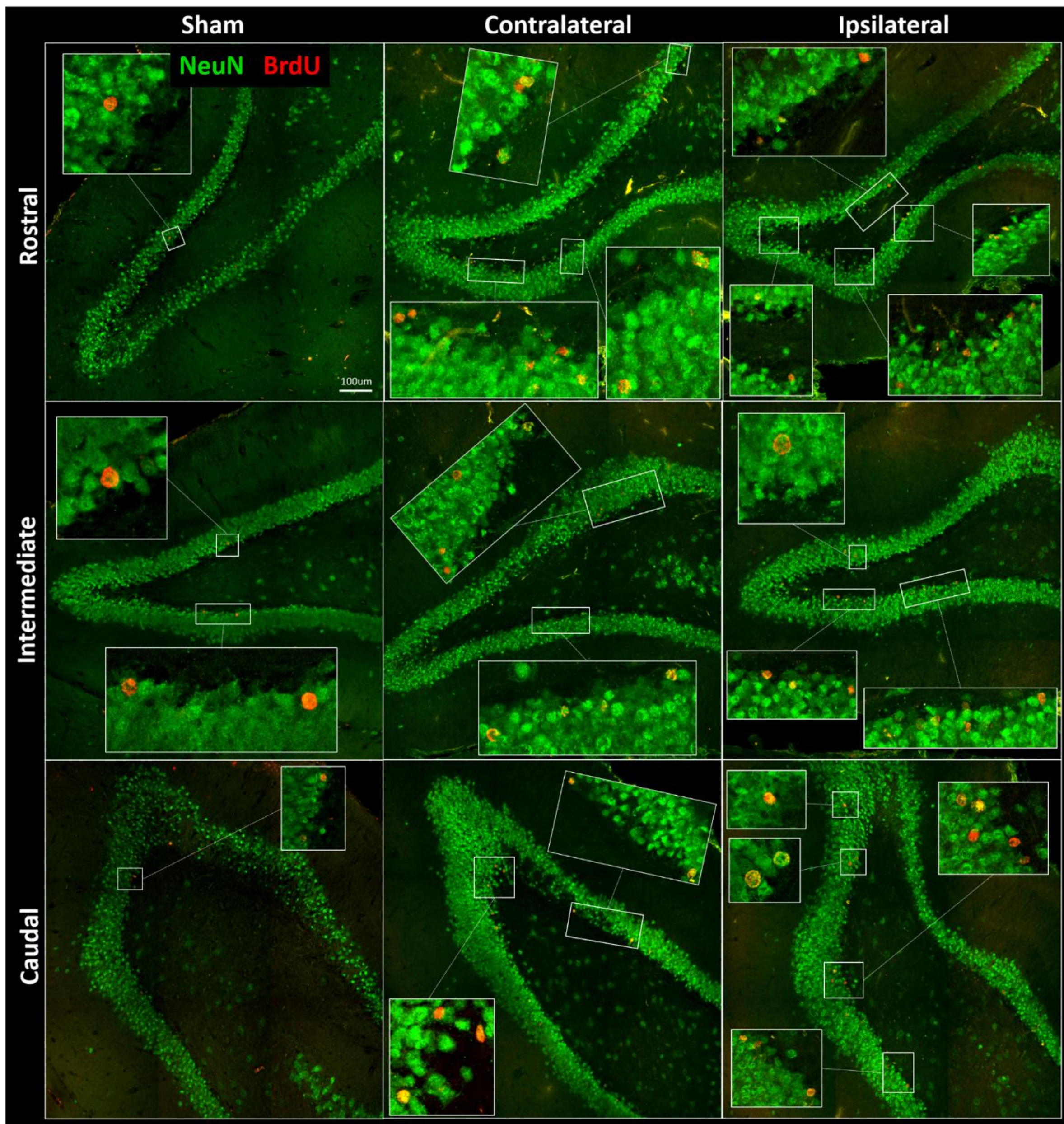

Figure 33. Sustained increase of neurogenesis in the dentate gyrus following multiple sessions of stimulation of the AMN by platinum electrodes. The figure shows confocal images illustrating spatial distribution of BrdU-labeled cells in rostral, intermediate, and caudal segments of the DG in stimulated (ipsilateral and contralateral side) and sham rats at 4 weeks. Scale bar is 100μm.

d. Preference of the Novel Arm at Week 4 Post Stimulation

In the Y-maze test, multiple stimulation induced an early difference in the total time spent exploring the novel arm between animals of the sham (n=3) and stimulated (n=5) groups at week 1 (108.29 ± 10.77 s in sham to 168.4 ± 19.03 s in stimulated animals; P < 0.05). Moreover, the stimulated animals spent significantly more time exploring the novel arm (168.4 ± 19.03 s) versus the familiar arm (44.4 ± 5.05 s; P < 0.001). At 4 weeks after stimulation, the exploration of the novel arm stayed significantly higher. The stimulated rats significantly spent more time in exploring the novel arm (156.2 ± 9.5 s) versus the familiar arm (53 ± 9.86 s; P < 0.001) and versus the time spent by sham animals in the novel arm (113.33 ± 2.73; P < 0.05, Fig. 34).

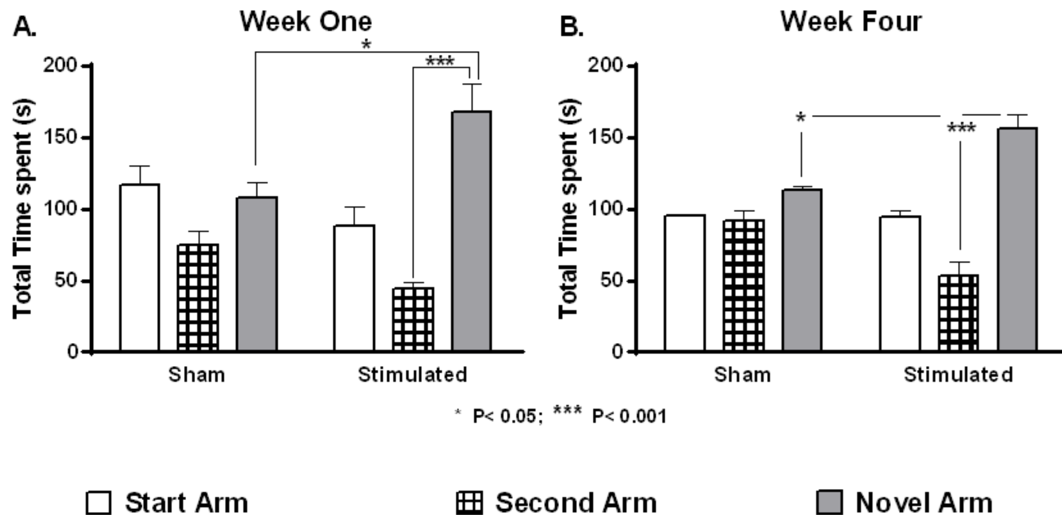

Figure 34. Enhanced exploratory behavior at 1 and 4 weeks post multiple sessions of stimulation of AMN using platinum electrodes. Graph representing the mean ± SEM of the total time spent in the novel, familiar (second arm) and start arms. The test was done at 1 week and after 4 weeks following sham procedure or electrical stimulation. In every time point, two-tailed t-test was performed to compare the difference between total time spent in novel arm in sham (n=3) and stimulated (n=5) group and between familiar and novel arm exploration within the same group.

e. Positive Correlation between Neurogenesis and Novel Arm Exploration at Week 4 Post Multiple Sessions of Stimulation

The relationship between the number of BrdU-positive cells or neurogenesis and the exploratory behavior in the novel arm showed a significantly positive correlation at 4 weeks after multiple sessions of stimulation (Pearson r = 0.92; P < 0.01, Fig. 35).

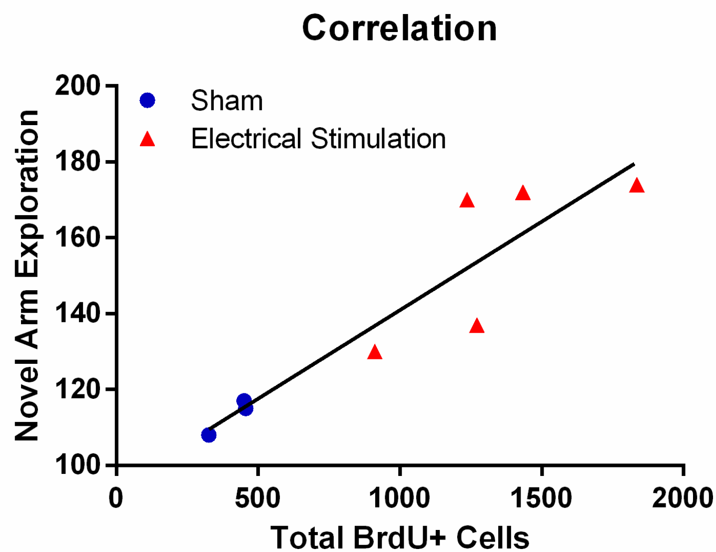

Figure 35. Positive correlation between neurogenesis and the exploratory behavior at 4 weeks post multiple sessions of stimulation in AMN using platinum electrodes. Graph plotting the number of BrdU-positive cells versus the time spent in novel arm for each rat in the sham (blue dots) and stimulated (red triangles) groups. The line represents linear regression of data (y = 93.97x + 8.95; r^2= 0.85).

C. Short Term Effects of a Single Injection of Kainic Acid

One microinjection of 1µl of KA (500pM) stereotaxically placed in the AMN did not reveal any prominent increase in BrdU levels in the DG of injected rats. No significant differences were found between the ipsilateral and contralateral side as well.

D. Short and Long Term Effects of Continuous Micro-Perfusion of Kainic Acid

1. *Effect at 8 Days Post KA*

a. <u>Increased Cellular Proliferation in the Dentate Gyrus</u>

The number of BrdU-positive cells in the SGZ of the DG highly increased when rats were subjected to continuous micro-perfusion (1µl per h for 7 days) of low doses of KA (500pM) in the right AMN. The number of BrdU-positive cells in the ipsilateral DG counted at 8 days after micro-perfusion increased from (1449.00 ± 93.48) in the right DG of the vehicle (n=4) group to (2133.5 ± 110.91) in the KA-perfused (n=6) group ($P < 0.01$, Fig. 36 and 37). The contralateral DG also exhibited a significant increase in the levels of BrdU-positive cells, when compared to the vehicle group (1410.00 ± 84.04 in the contralateral DG of the vehicle-injected versus 2081.5 ± 197.97 in perfused group; $P < 0.05$). There were BrdU-positive cells around the DG, specifically in the hilar region, but only the ones that were at the SGZ were included in the count as they represent the proliferation of stem/progenitor cells of the DG.

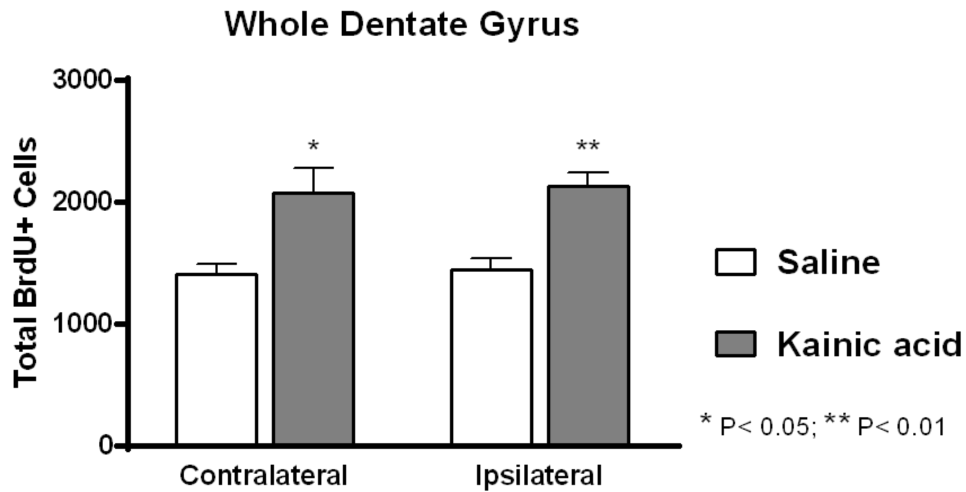

Figure 36. Increased proliferation of stem/progenitor cells in the ipsilateral dentate gyrus at 1 week following continuous micro-perfusion of kainic acid in the AMN. Stereological quantification of BrdU-labeled cells in the DG of KA (n=6) and saline (n=4) injected groups at the end of perfusion. Each bar represents the average ± SEM of BrdU quantification. Two-tailed t-test was used to determine the significance between KA and saline injected groups in each hemisphere.

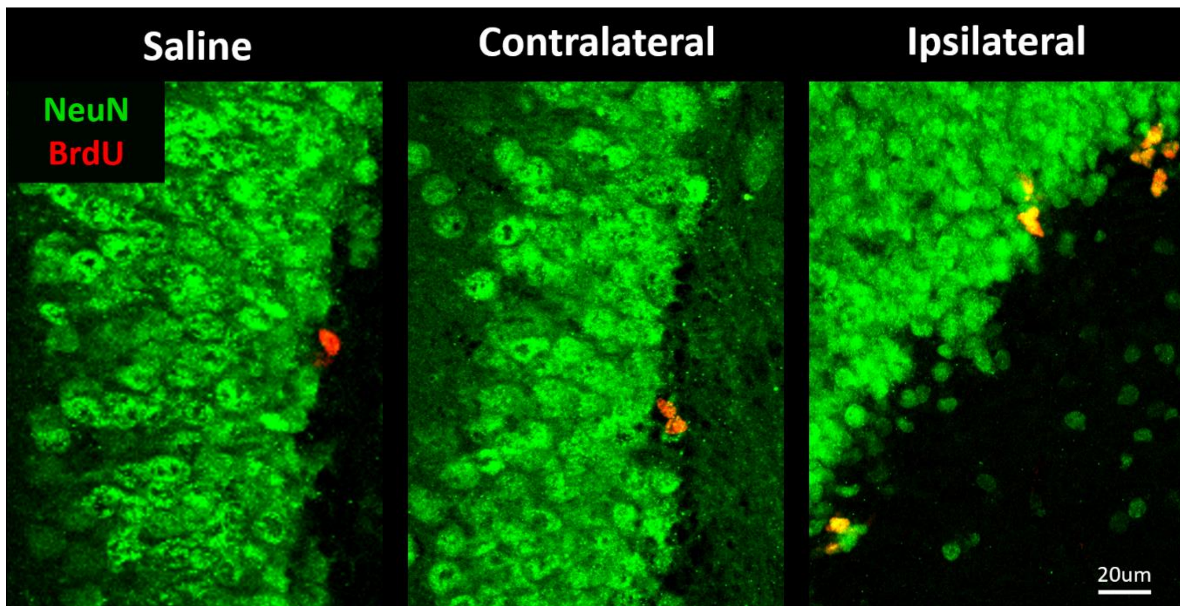

Figure 37. Increased proliferation of stem/progenitor cells in the ipsilateral dentate gyrus at 1 week following continuous micro-perfusion of kainic acid in the AMN. Representative confocal images showing immunofluorescence labeling of NeuN and BrdU in the DG of KA and saline injected groups at the end of perfusion. BrdU-positive cells are more prominent in the KA group ipsilateral to site of injection. Images were taken as Z stacks using 40X-oil objective.

b. Spatial Distribution of the Increased Proliferation

The spatial pattern of BrdU-positive cells in the hippocampus ipsilateral to osmotic pump insertion showed higher population of cells in the caudal DG of saline-injected (n=4) animals (712.5 ± 77.67) while the numbers were comparable in the rostral and intermediate regions (351 ± 24.06 and 385.5 ± 29.84, respectively). KA micro-perfusion (n=6) most significantly increased the number of BrdU-positive cells in the caudal DG (1481.83 ± 150 versus 712.5 ± 77.67 in saline-perfused area; $P < 0.01$, Fig. 38A and 39). The same pattern was detected in the contralateral DG, where KA significantly increased the number of BrdU-positive cells in the caudal segment (1202.67 ± 149.89 versus 672 ± 56.76 in the corresponding vehicle group; $P < 0.05$, Fig. 38B and 39).

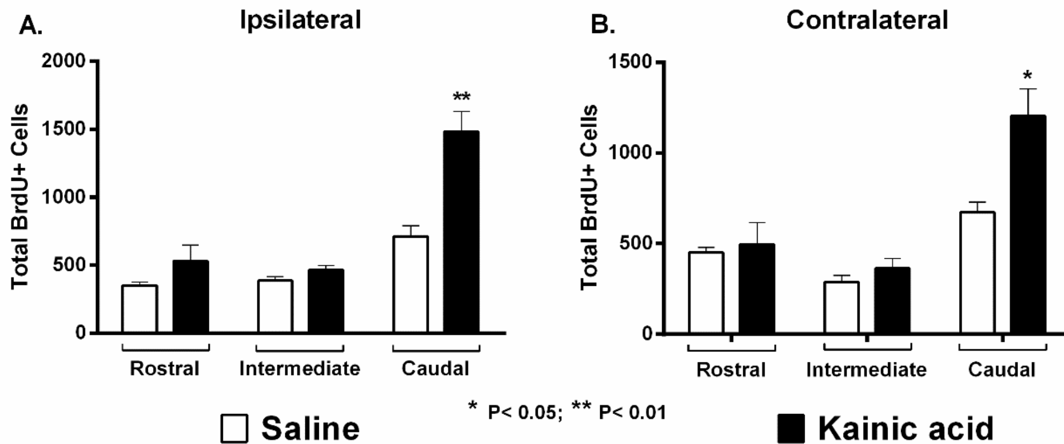

Figure 38. Spatial distribution of increased neurogenesis in the dentate gyrus following continuous micro-perfusion of kainic acid in the AMN. The graphs illustrate the distribution of the total number of BrdU-labeled cells in segments of the ipsilateral (A) and contralateral (B) DG of saline (n=4) and KA-injected (n=6) rats. Each bar represents the average ± SEM of BrdU quantification and the determination of significance was made using two-tailed t-test where the values of the saline and KA groups were compared for each DG region.

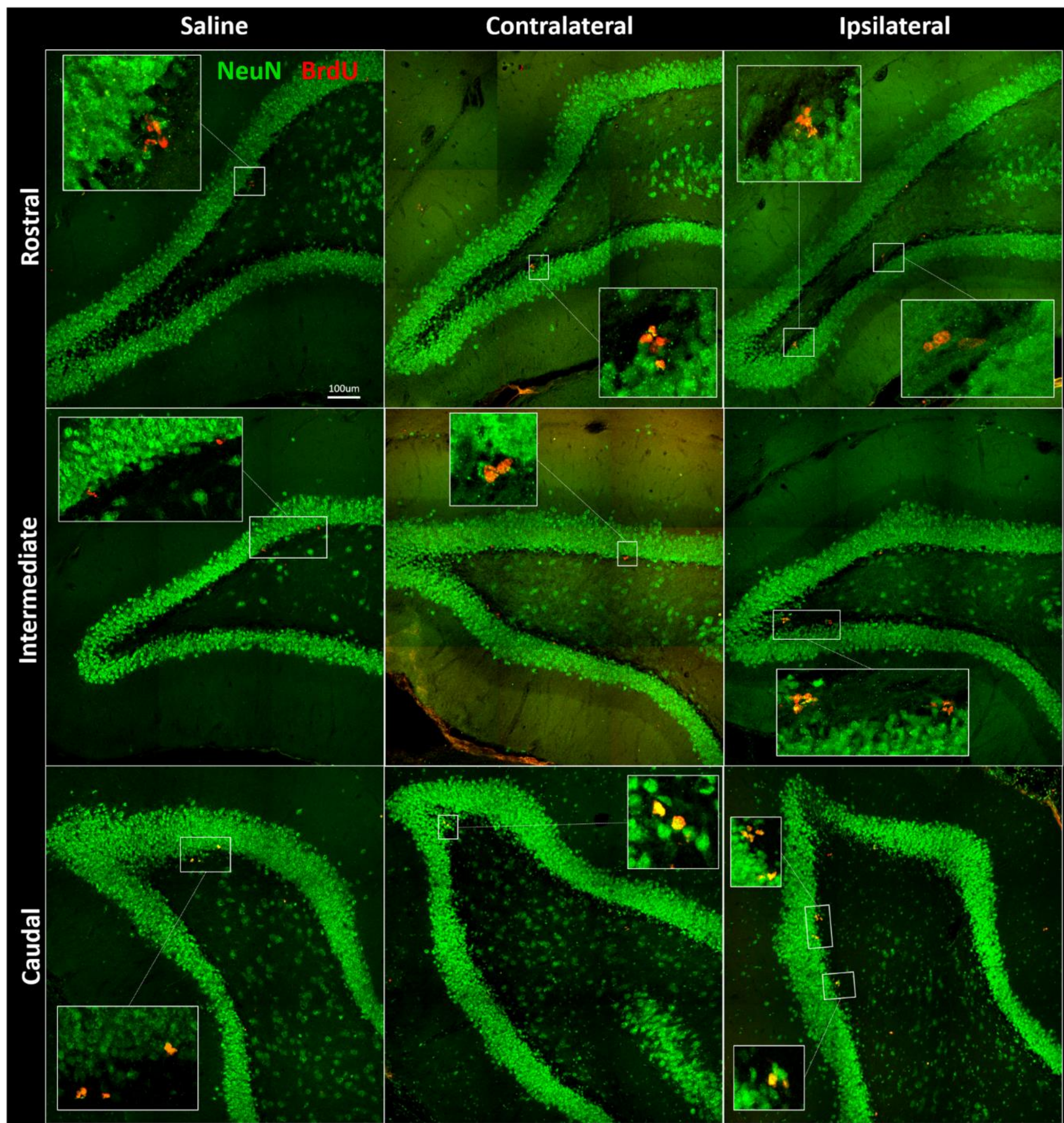

Figure 39. Spatial distribution of the increased stem/progenitor cell proliferation in the ipsilateral dentate gyrus following continuous micro-perfusion with kainic acid. The figures show confocal images illustrating BrdU-labeled cells in rostral, intermediate, and caudal segments of the DG in saline and KA-injected rats (ipsilateral and contralateral sides). Scale bar is 100µm.

2. *Effect on the Development of Labeled Cells at 4 Weeks Post Continuous Micro-Perfusion*

a. Increased Neurogenesis in the Dentate Gyrus

The number of BrdU-labeled cells that got integrated into the DG granular zone counted at 4 weeks after KA-perfusion was higher than the number counted in the saline-injected group. The number of integrated BrdU-labeled cells in the saline-injected group reached 482.67 ± 52.53 in the contralateral DG and 581.33 ± 32.09 in the ipsilateral DG. Following unilateral KA injections, the number of BrdU-positive cells significantly increased by 3 folds to reach 1730.17 ± 214.06 in the contralateral region ($P < 0.01$) and 1868.5 ± 184.3 in the ipsilateral region ($P < 0.001$, Fig. 40 and 41).

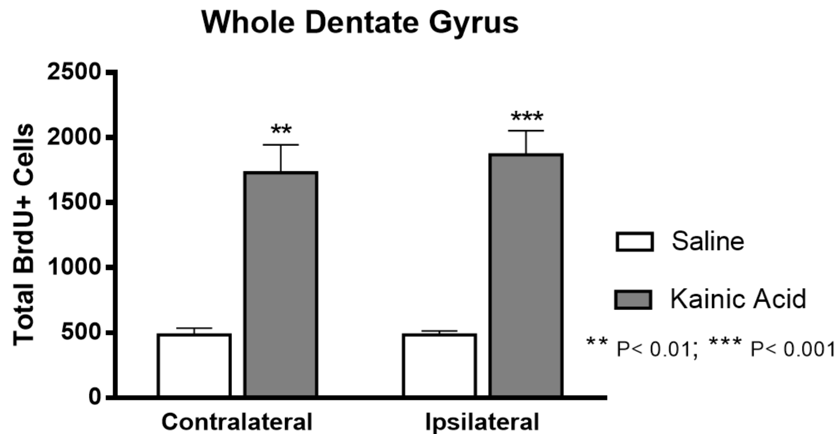

Figure 40. Sustained increase of neurogenesis in the ipsilateral and contralateral dentate gyrus following continuous micro-perfusion with kainic acid in the AMN. Stereological quantification of BrdU-labeled cells in the DG of KA (n=6) and saline (n=3) injected groups at 4 weeks. Each bar represents the average ± SEM of BrdU quantification. Two-tailed t-test was used to determine the significance of difference between KA and saline injected groups in each hemisphere.

b. Fate of KA-Induced Stem/Progenitor Cells

Continuous micro-perfusion of the AMN with KA for a period of one week showed an increased number of BrdU-positive cells co-labeled with the neuronal marker NeuN at 4 weeks. The confocal images show that the BrdU-labeled cells deeply migrated into the granular zone of DG indicating the differentiation and survival of these cells until 4 weeks (Fig. 41).

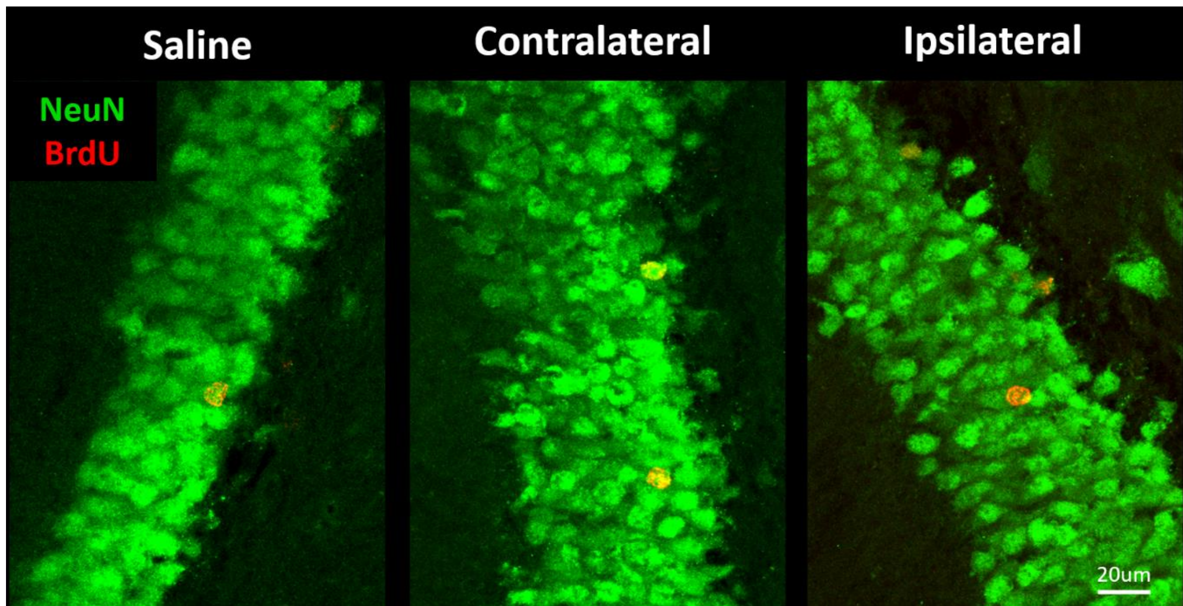

Figure 41. Sustained increase of neurogenesis in the ipsilateral and contralateral dentate gyrus following continuous micro-perfusion with kainic acid in the AMN. Representative confocal images showing immunofluorescence labeling of NeuN and BrdU in the DG of KA and saline injected groups at 4 weeks. BrdU-positive cells are more prominent in the KA group ipsilateral and contralateral to site of injection. Images were taken as Z stacks using 40X-oil objective.

c. Spatial Distribution of KA-Induced Cells

Looking into the three rostro-caudal regions of the DG in the KA-injected group 4 weeks after micro-perfusion showed that the highest increase, in both the ipsilateral and contralateral side of perfusion, was confined to the intermediate and caudal regions of the DG. The number of BrdU-positive cells in the intermediate region significantly increased from 122 ± 22.27 in ipsilateral saline group to 279.5 ± 40.16 after KA perfusion ($P < 0.05$, Fig. 42A and 43) and from 132 ± 22.72 in contralateral saline group to 270 ± 23.74 after KA perfusion ($P < 0.05$, Fig. 42B and 43). The number of BrdU-positive cells in the caudal region significantly increased from 218.67 ± 35.26 in ipsilateral saline group to 1246.17 ± 150 cells after KA perfusion ($P < 0.001$, Fig. 42A and 43) and from 185.33 ± 26.84 in contralateral saline group to 1027 ± 120 after KA stimulation ($P < 0.001$, Fig. 42B and 43).

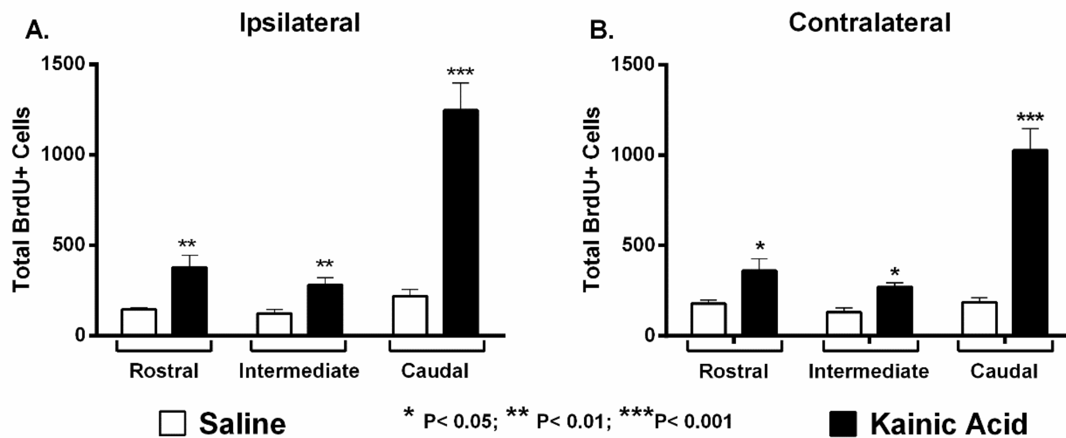

Figure 42. Spatial distribution of neurogenesis showing sustained increase in the ipsilateral and contralateral dentate gyrus following continuous micro-perfusion of kainic acid in the AMN. The graph illustrates the total number of BrdU-labeled cells in rostral, intermediate and caudal segments of the ipsilateral (A) and contralateral (B) DG in saline (n=3) and KA-injected (n=6) rats at 4 weeks. Each bar represents the average ± SEM of BrdU quantification and the determination of significance of differences was made using two-tailed t-test where the values of the saline and KA groups were compared for each DG region.

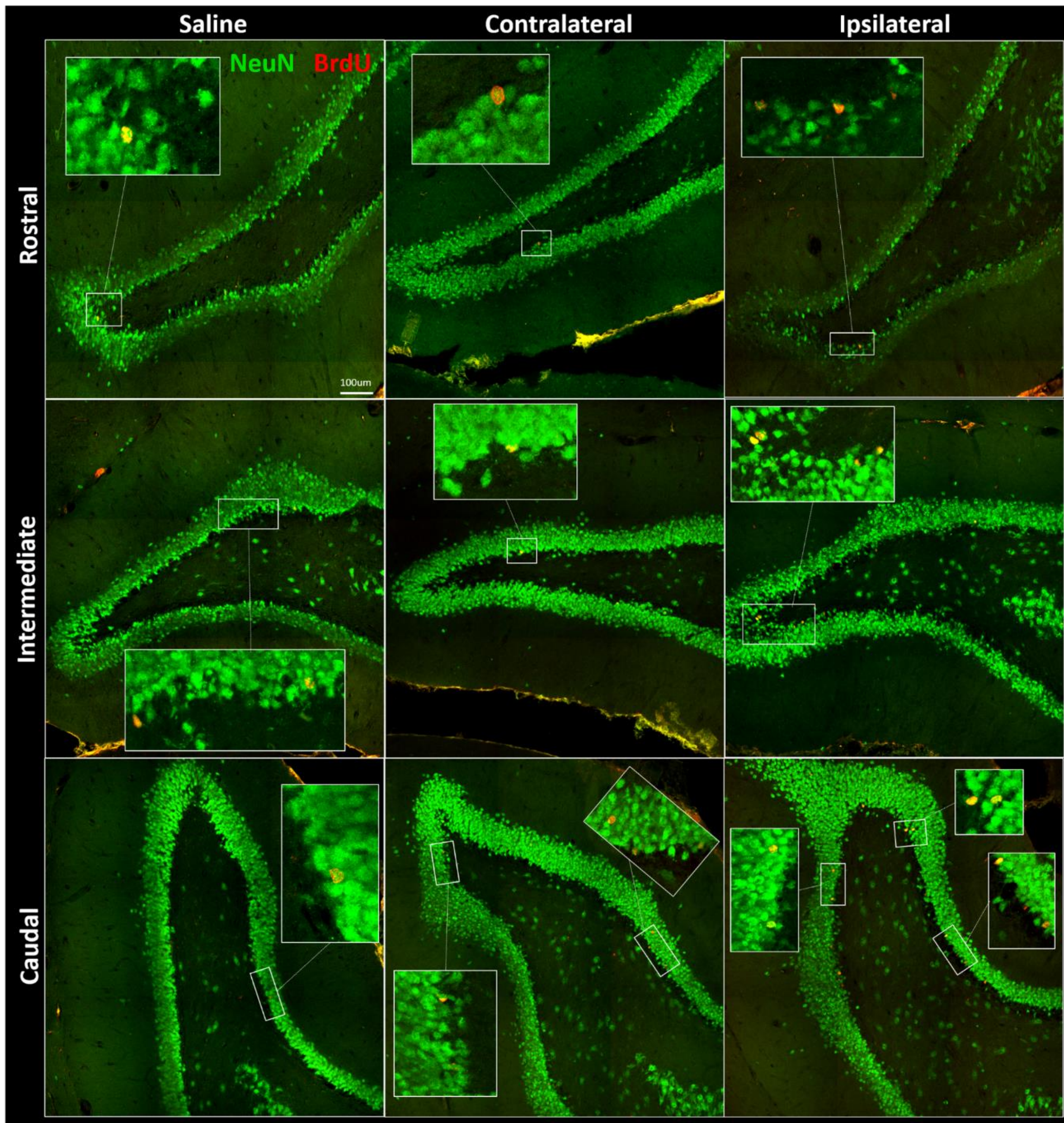

Figure 43. Sustained increase of neurogenesis in the dentate gyrus following continuous micro-perfusion of kainic acid in the AMN. The figure shows confocal images illustrating distribution of BrdU-labeled cells in different segments of the DG in KA- (ipsilateral and contralateral sides) and saline-injected rats at 4 weeks. Scale bar is 100µm.

d. Preference of the Novel Arm at Week 4 Post KA

In the Y-maze test, continuous micro-perfusion of kainic acid induced an early difference in the total time spent exploring the novel arm between the saline-injected animals and the KA-injected group at week 1 (93.94 ± 7.76 s in saline to 146.81 ± 13.71 s in KA-injected rats; $P < 0.01$). Moreover, KA-injected rats spent significantly more time exploring the novel arm (146.81 ± 13.71 s) versus the familiar arm (80.13 ± 8.93; $P < 0.001$). At 4 weeks after KA, the exploration of the novel arm stayed significantly higher. The KA-injected rats significantly spent more time in exploring the novel arm (144.58 ± 11.32 s) versus the familiar arm (88.44 ± 10.22 s; $P < 0.001$) and versus the total time spent by vehicle-injected animals in the novel arm (64.38 ± 5.81; $P < 0.001$, Fig. 44).

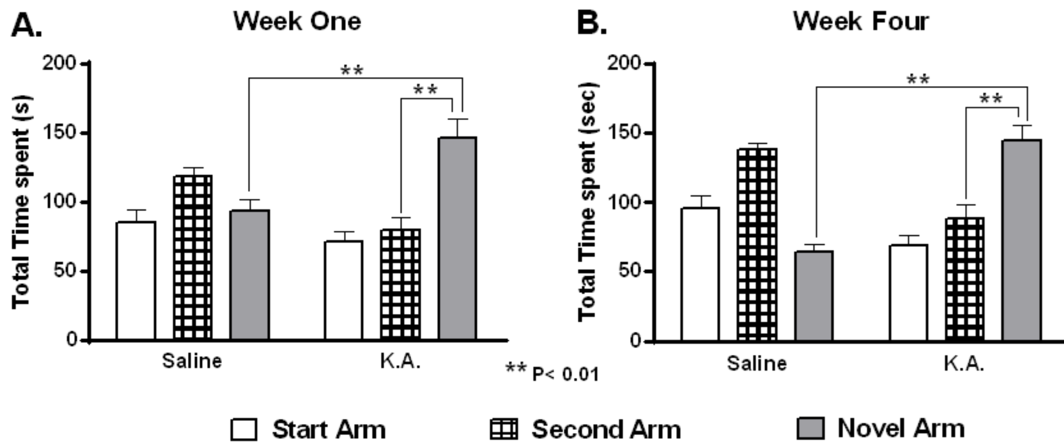

Figure 44. Enhanced exploratory behavior at 1 and 4 weeks following continuous micro-perfusiom of Kainic Acid in the AMN. Graph representing the mean ± SEM of the total time spent in the novel, familiar (second arm) and start arms. The test was done at 1 week and after 4 weeks following saline or KA micro-perfusion. In every time point, two-tailed t-test was performed to compare the differences between the total time spent in novel arm in vehicle (n=3) and KA-perfused (n=6) group and between familiar and novel arm exploration within the same group.

e. Positive Correlation between Neurogenesis and Novel Arm Exploration at Week 4 Post KA

The relationship between the number of BrdU-positive cells or neurogenesis and the exploratory behavior in the novel arm showed a significantly positive correlation at 4 weeks after continuous micro-perfusion with KA (Pearson r = 0.97; P < 0.001, Fig. 45).

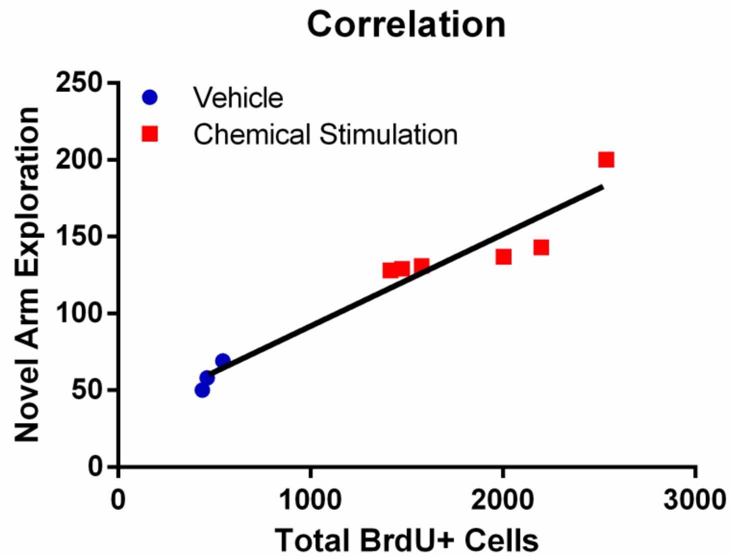

Figure 45. Positive correlation between neurogenesis and the exploratory behavior at 4 weeks post continuous micro-perfusion of kainic acid in the AMN. Graph plotting the number of BrdU-positive cells versus the time spent in novel arm for each rat in the vehicle-injected (blue dots) and KA-injected (red squares) groups. The line represents linear regression of data ($y = 31.92x + 9.49$; $r^2 = 0.93$).

DISCUSSION

The main aim of the present study was to investigate the short and long term effects of activation of the anterior medial nucleus (AMN) of the thalamus on adult hippocampal neurogenesis. For this purpose, we used, in addition to the traditional electrical stimulation, the method of micro-perfusion of excitatory substances, mainly kainic acid. Both methods elicited significant increase in hippocampal progenitor cells that showed important variations depending on the duration of activation and on their rostro-caudal location in the hippocampus. Long term observation of the effects, moreover, allowed to trace the ultimate differentiation of stem cells into neurons integrating hippocampal neuronal circuits. It is worth noting also the specificity of AMN stimulation, when compared to the effects of stimulation of another thalamic nucleus VPL and the possible correlation of the increased neurogenesis and the improved performance of rats in a behavioral test of spatial exploration.

A. Neurogenesis Basal Levels in Male/Female Rats and Sex-Independent Effects of Electrical Stimulation.

Sex is a biological variable in experiments done on animals (Clayton, 2018). Therefore, we looked at basal levels of stem/progenitor cell proliferation in the DG of both female and male rats. BrdU labels mitotic cells during the S phase of the mitotic cycle and its bioavailability after each injection is around 2 hours (Packard et al., 1973). Three injections of BrdU, spaced by 2-h intervals, were administered to each rat to maximize the detection of proliferating BrdU-labeled cells. The proliferation levels were examined one

day after the injection and we have shown significant differences in proliferation of stem/progenitor cells between male and female rats. Our data are in line with a previous report which showed an increased number of proliferating stem/progenitor cells in male more than in females rats (Perfilieva et al., 2001). Another study, however, has shown that estrogen induces an increase in levels of proliferation in adult female rats, precisely during pro-estrus phase of the cycle where estrogen is highest (Tanapat et al., 1999). In fact, gonadal hormones can directly affect hippocampal neurogenesis in females (Galea, 2008). Moreover, and to our interest, the increased cell proliferation rate in males is correlated with increased spatial learning abilities (Beatty, 1984; Williams and Meck, 1991) where they outperform female rats in maze tests (Roof and Havens, 1992; Williams and Meck, 1991). Therefore, male rats were used to avoid possible interference of estrous cycle with the observed changes in neurogenesis.

Despite basal proliferation differences between female and male groups, one-hour session of electrical stimulation in the AMN induced comparable increases in the number of proliferating stem/progenitor cells in both sex groups. To the best of our knowledge, this is the first study to demonstrate that electrical stimulation in the AMN elicits a sex-independent increase in proliferation of stem/progenitor cells (Chamaa et al., 2016).

B. Short Term Effects of Electrical Stimulation

To test for the effect of electrical stimulation on the proliferation of stem/progenitor cells, we administered BrdU at 4 days after stimulation and the rats were killed one day after BrdU injections. This total time of 5 days until perfusion was not chosen randomly; it was previously reported that the maximum number of labeled cells was

observed between 3 and 5 days after stimulation where the frequency of 130 Hz was optimal to induce cellular proliferation with no detected apoptosis (Toda et al., 2008). We used similar stimulation parameters but added some refinements to the method. For instance, we applied unilateral instead of bilateral AMN stimulation and this allowed us to check for laterality differences. Moreover, it helped us in using each rat as its own control for basal proliferation levels compared to naïve and sham rats (Chamaa et al., 2016). More importantly, the stimulation was conducted on unrestrained rats, meaning that they were awake and free of anesthesia. This was done to minimize undesirable factors, such as stress and anesthesia, which might interfere with neurogenesis (Danzer, 2012; Stratmann et al., 2010). Our data showed that unilateral electrical stimulation in the AMN induced a significant increase in proliferation of stem/progenitor cells that was restricted to the ipsilateral dentate gyrus (DG) when using either our lab-designed copper electrodes (Chamaa et al., 2016) or the platinum electrodes.

Screening of the DG as three rostro-intermediate-caudal regions showed a spatial distribution of proliferation mostly dispersed as follows: the rostral region contained the fewest number of proliferating stem/progenitor cells, the numbers were slightly elevated in the intermediate region and were highest in the caudal part (Chamaa et al., 2016). Electrical stimulation using either type of electrodes did not change the spatial distribution pattern, but significantly increased the number of proliferating cells in the intermediate and the caudal areas. While similar observations were reported by another study based on the stimulation of the entorhinal cortex (Stone et al., 2011), none however, have revealed a spatial configuration of neurogenesis induced by AMN stimulation. The most common terms for hippocampal divisions in the literature are the dorsal and ventral hippocampus,

whereby the ventral comprises the intermediate and caudal areas. A suggested role for the ventral hippocampus in spatial memory may explain this anatomical-functional segregation (Loureiro et al., 2012). This patterned increase in proliferation may be explained by a preferential connection of the AMN with the intermediate and caudal DG and might possibly reflect their functional interaction involved in the processing of spatial orientation (Mendez-Couz et al., 2015). Further investigation of this phenomenon might extend our knowledge on the functional implication of the anatomical preferences of stimulation-induced effects.

Moreover, at 1 week, the rats were tested in the Y maze to compare behavioral exploration differences between sham and stimulated groups. It was observed that both groups spent comparable time in exploring the novel arm. Although there was an increase in the number of proliferating stem/progenitor cells, a single session of electrical stimulation was not enough to instigate changes in exploration of novel arm at one week.

C. Long Term Effects of Electrical Stimulation

The long term time point was chosen to follow the fate of progenitor cells that previously took-in the BrdU when dividing at the first week following stimulation. The maturation process needs around 4 weeks for the progenitor cells to commit to the neuronal lineage, differentiate and functionally integrate into the DG circuits (Encinas and Sierra, 2012; Sierra et al., 2010). The cells that survive and mature will be expressing the mature neuronal marker NeuN in addition to BrdU. Our data showed that, after stimulation by either type of electrodes, BrdU positive cells were co-labeled with NeuN at 4 weeks and they were integrated into the granular zone. Moreover, stimulation induced an increase in

the number of integrated neurons when compared to the sham. These data suggest that a large portion of the newly born cells has developed into mature neurons and the total number of new neurons has increased after stimulation. This is consistent with previous reports that showed a stimulation-induced increase in neurogenesis (Encinas et al., 2011; Toda et al., 2008). Platinum electrodes induced an ipsilateral increase in neurogenesis. The use of copper electrodes for stimulation, however, has induced an increase in the survival or integration of neurons at both, the ipsilateral and the contralateral side of stimulation. Moreover, further comparisons between the sham groups showed higher numbers of newly generated BrdU-labeled neurons when copper electrodes were used versus platinum electrodes. Copper electrodes may undergo corrosion and oxidation when left for a long time in the implanted tissue (Geddes and Roeder, 2003) and this might account for the differences seen with the use of copper electrodes. Other studies have reported a regulatory function for copper in neurogenesis (Fu et al., 2015) and this is an interesting finding that should be more investigated where copper deposition from the electrodes should be tested.

The spatial distribution of newly formed neurons at 4 weeks was different from the previously observed spatial distribution of proliferating stem/progenitor cells. The increased levels following stimulation were dispersed along the three regions of the DG without any detected preference to a specific region.

The behavioral exploration measurements were different from the ones previously reported in the first week following stimulation. The Y maze test was repeated at 4 weeks and the results have shown that a single stimulation to the AMN increased the total time spent by the rats to explore the novel arm versus the familiar arm. Moreover, there were significant differences in exploration of novel arm between the sham and stimulated rats.

Remarkably, the increase in exploration time was detected at 4 weeks after stimulation which is the time needed for newborn neurons to get integrated into the granular zone layer and for synapses to start forming. This is in concordance with previous reports that did hippocampal lesion or induced stress on rats and accordingly showed lower exploration drives in the novel arm (Conrad, 2010; Conrad et al., 1996). It is important to note that further behavioral experiments that involve other mazes are of increased value to our reported results. For example, the Morris Water maze can be utilized to further assess spatial learning and memory which will give additional strength to the behavioral translation of electrical stimulation and the subsequent hippocampal neurogenesis.

Our study went further to mimic current clinical use of electrical stimulation where we opted to check for the effects of multiple sessions of electrical stimulation using platinum electrodes. The results of platinum and copper electrodes were comparable in the short time point, but not after 4 weeks. This might be due to the instability of copper electrodes at the longer time points, especially that they tend to induce inflammation and necrosis in implanted tissue (Geddes and Roeder, 2003). Our data have shown an increase in the number of new neurons in both sham and electrical stimulation groups when copper electrode was inserted for the longer time point. Although the stimulated group had significantly higher rates of neurogenesis, there is still doubt in the validity of using copper electrode for longer time points especially that the sham group itself has high numbers. Therefore, we used the platinum electrodes for multiple sessions of stimulation. They are coated with iridium which enhances the charge transfer capabilities and both metals have high stable atomic configurations and are corrosion-resistant (Geddes and Roeder, 2003). Following multiple sessions of stimulation, the number of differentiated newborn cells

were 1.6 folds higher than one session of stimulation. It is important to draw attention to that difference because clinical studies of stimulation undergo multiple or chronic stimulations (Hodaie et al., 2002) and our results show that the increase in the number of stimulation sessions induce even more increase in hippocampal neurogenesis. Moreover, the increase was not only restricted to the ipsilateral side, but also affected the contralateral side and it was dispersed throughout the hippocampus. The increase in neurogenesis was positively correlated with progress in exploratory behavior where multiple sessions of stimulation induced an increase in exploration time of the novel arm that began during the first week and continued to week 4 after stimulation. All in all, we have succeeded in applying multiple sessions of stimulation parameters comparable to ones used in clinical settings. This will help in taking the stimulation setup a step forward for future studies on rat models of stress, neurodegenerative diseases or depression; all reporting decreased levels of hippocampal neurogenesis.

D. Short and Long Term Effects of KA Stimulation

Despite the extensive use of electrical stimulation and reports on the beneficial effects in many disorders, little attention was paid to what specifically is being stimulated. Thereby, the exact mechanism of action of electrical stimulation is still unclear. Previous reports have described lowest thresholds in myelinated axons and higher thresholds in unmyelinated axons, dendrites and cell bodies (Ranck, 1975). After that, it was shown that electrical stimulation sparsely activates cells contained within 4 mm of the surrounding region of the electrode, and the number of stimulated cells in this sphere increase as the current amplitude increases (Histed et al., 2009). What is even more complicated is

delineating whether electrical stimulation acts in an inhibitory or in an excitatory mechanism. Some suggest long-term inactivation of neurons adjacent to the stimulating electrode (Boon et al., 2009; Skarpaas and Morrell, 2009), possibly due to the comparable clinical outcomes of ablation and electrical stimulation (Benabid, 2007). However, other reports have demonstrated activation of the surrounding neurons (Histed et al., 2009; Miocinovic et al., 2006). Altogether, this suggests a complex mixture of activation and inhibition responses that can be produced as a consequence of electrical stimulation. Therefore, our study tried to demarcate one of the possible mechanisms of electrical stimulation by specifically activating the cell bodies in the same compass of the electrode region. The dose selection of KA was based on data from the literature which showed sustained neuronal activation without leading to seizures (Montgomery et al., 1999). We have seen that one injection of KA was not sufficient to induce any changes on proliferation of stem/progenitor cells. Continuous micro-perfusion of low doses of kainic acid, however, provided continuous activation of the Glutamic Kainate receptors in the AMN cell bodies for 7 days and remarkably, induced significant changes in proliferation and neurogenesis that were comparable to electrical stimulation. The increase in neurogenesis after chemical stimulation was correlated with the increase in neurogenesis seen in multiple sessions of electrical stimulation. Just like electrical stimulation, continuous micro-perfusion of KA induced an ipsilateral increase in proliferation mostly at the caudal region of the hippocampus at 1 week. The effects at 4 weeks were comparable to multiple sessions of electrical stimulation, where there was a bilateral increase in hippocampal neurogenesis along the whole DG areas and a subsequent progress in the exploratory behavior that also started at the first week and continued till 4 weeks. The

increase in neurogenesis was positively correlated with the increased exploratory behavior of rats at week 4. These observations have served to highlight that activating cell bodies in the AMN can be one possible mechanism underlying the action of electrical stimulation. While there is much work to be done and multiple other molecules of excitation or inhibition to be examined, this study unravels one important line to follow in defining the mechanisms of electrical stimulation. It can be further explored for its possible use in clinical practice as it better simulates the physiological environment, provided that the side effects of such a paradigm are extensively studied.

E. Specificity of AMN Selection

General targets of DBS depend on therapeutic application where research continues to identify the mechanism of action of DBS and extends to characterize the specific function for each stimulated area. Most stimulation done in areas related to Papez circuitry improve spatial learning and memory-related functions. For example, stimulation to entorhinal cortex facilitates spatial memory (Stone et al., 2011; Suthana et al., 2012), stimulation in fornix results in memory enhancement (Hamani et al., 2008) and AMN stimulation enhances spatial learning and memory in addition to cognitive improvements (Ferreira et al., 2018; Hamani et al., 2011; Oh et al., 2012). Stimulation to these key nodes in Papez circuit, not only influenced neural activity in memory related functions, but is also associated with generating new neurons in the hippocampus. The anatomical location of the hippocampus in the limbic circuitry makes its neurogenic niche a particularly relevant field for the exploration of DBS effects. Therefore, giving potential hope to neuro-regeneration induced by stimulation. For example, DBS in the AMN has succeeded in ameliorating

epilepsy in patients (Andrade et al., 2006; Kerrigan et al., 2004) where increased hippocampal neurogenesis is highly suggested to be involved in contributing to the therapeutic effects.

Given the widespread effects of DBS, even when targeting a specific area, led us to examine stimulation of another thalamic nucleus that has no known direct connections to Papez circuit or the hippocampus specifically. While other studies have stimulated sites distant from AMN such as frontal association area of the cortex (Encinas et al., 2011) and cerebellar lobules (Stone et al., 2011) as a control for target specificity, we opted to stimulate the VPL nucleus since it is anatomically adjacent to the AMN. We detected no significant enhancement in DG proliferation following VPL nucleus stimulation, thereby demonstrating the specificity of connections in DBS-induced neurogenesis and the importance of stimulating centers of Papez circuit in inducing hippocampal neurogenesis. Notably, although the trajectory of the electrode passes through the hippocampus, there was no significant increase in baseline hippocampal neurogenesis.

F. Y maze Selection for Spatial Exploration

The electrode insertion or the KA micro-perfusion was in the AMN which has large numbers of “head direction cells” that compel for a role in spatial navigation and these specialized cells emit discharges when the animal steers its head in a certain direction (Taube, 1995) and maintain firing-tendencies in novel environments (Taube and Burton, 1995). The AMN is a key relay in Papez circuitry that connects extensively with the hippocampal formation (Papez, 1995) and the latter has a role in memory and spatial navigation (Buzsaki and Moser, 2013). Papez and the trisynaptic circuitries are important

for spatial processing and damages to either one results in disruption of spatial performance (Jarrard, 1983; Olton et al., 1978). Spatial navigation is mainly processed through a circuitry of connections that link the medial diencephalon containing the AMN with the hippocampus (Clark and Taube, 2012). In view of this, we decided to do the novel arm exploration test using the Y maze, which exploits the natural tendency for rats to investigate their surrounding environment. And indeed, we succeeded in detecting differences in the exploratory behavior between sham and experimental groups, whether it was a single session of stimulation, multiple sessions of stimulation or continuous micro-perfusion of KA.

G. Conclusions

In summary, one session of unilateral electrical stimulation to the AMN induces hippocampal stem/progenitor cell proliferation that is later manifested as an increase in hippocampal neurogenesis. Multiple sessions of unilateral electrical stimulation, simulating clinical setting, further increased the number of proliferating cells and the subsequent number of newly differentiated neurons. Moreover, activating the cell bodies of the AMN by continuous micro-perfusion of kainic acid induced similar outcomes, suggesting a role for kainite receptor activation in AMN neurons in contributing to neurogenesis. The increase in neurogenesis was correlated with an increase in rats' exploratory behavior in the Y maze. Altogether, this study showed that electrical stimulation can induce cumulative effect on neurogenesis that increased with the increase in the number of stimulation sessions. This is one of the first studies to use chemical stimulation as a model to understand the mechanism underlying electrical stimulation. The

importance of chemical stimulation is that it is target-specific and the molecules used better simulates the physiology of the brain. Indeed, testing other molecules of activation or inhibition is needed to delineate the mechanism underlying electrical stimulation or even test the possible clinical use of chemical stimulation. All combined, this leads us to land proof the hypothesis that stimulation to the AMN facilitates adult hippocampal neurogenesis by inducing proliferation of stem/progenitor cells followed by their differentiation into neurons and integration into the dentate gyrus. Moreover, the induced neurogenesis is correlated with behavioral outcomes in the Y-maze. Future studies are needed to complement these findings with more extensive behavioral examinations and the inclusion of neurogenesis-reduced animal models that involve cognitive impairments.

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