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THALAMIC STIMULATION IN AWAKE RATS INDUCES NEUROGENESIS IN THE HIPPOCAMPAL FORMATION

by
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ABSTRACT OF THE THESIS

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Title: Thalamic stimulation in awake rats induces neurogenesis in the hippocampal formation

Background and Aims: Deep brain stimulation has the potential to provide substantial clinical benefits for a variety of movement disorders, and is currently under investigation for the treatment of cognitive disorders. However, the molecular mechanisms mediating these effects remain elusive. One possible underlying mechanism might be the regulation of adult hippocampal neurogenesis. The present study investigates the effects of unilateral anteromedial thalamic nucleus stimulation on adult hippocampal neurogenesis in awake and unrestrained rats.

Methods: Our study was based on the following 5 experimental groups. *Group 1:* adult female rats (n=4) were subjected to unilateral stimulation in the right anteromedial thalamic nucleus **(AMN).** *Group 2:* adult male rats (n=6) received unilateral stimulation in the right AMN. *Group 3*: adult female (n=2) and male (n=4) sham rats were subjected to electrode implantation only without current delivery. *Group 4:* adult male rats (n=2) received unilateral stimulation in the right ventral posterolateral thalamic nucleus. *Group 5:* adult female rats (n=3) received a single injection of KA (500pM) in the right AMN. All rats, except those in group 5, received 4 injections (50mg/Kg/injection) of 5'-bromo-2'-deoxyuridine **(BrdU)** 3 days after surgery and euthanized 24 h later. Rats in group 5 received single injection of BrdU (50mg/kg) for 5 days and were euthanized 3 h after the last injection. Confocal immunofluorescent analysis of BrdU, GFAP and NeuN was performed. Stereological counting of positive cells was done in the dentate gyrus and hilar zone of the hippocampal formation, using the fractionator method.

Results: Stimulation of the right AMN induced focal neurogenesis in the ipsilateral (right) dentate gyrus. Stimulation-induced effects were gender-independent and were translated by an increase in prolifereation rate at the level of amplifying neural progenitors. Importantly, increased-neurogenesis rate was limited to the caudal region of the dentate gyrus. Furthermore, this increase was specific for the AMN stimulation and not any other thalamic nucleus. Neurogenesis as the level of the hilar and the olfactory zones was not affected by anteromedial thalamic nucleus stimulation.

Conclusion: Our results are in favor of a role for hippocampal neurogenesis in the mechanisms underlying the effects of deep brain stimulation. The exclusivity of these effects to AMN stimulation suggests a key role of the components of the Papez circuit in the treatment of cognitive and behavioral disorders. The behavioral implications of short and long term deep brain stimulation can constitute a subject for further investigation in awake and unrestrained animals.

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ABBREVIATIONS

ANP: Amplifying Neural Progenitors

AMN: Anteromedial Thalamic Nucleus

BrdU: 5'-Bromo-2'-Deoxyuridine

CA: Cornu Ammonis

DG: Dentate Gyrus

DGC: Dentate Granule Cells

DGL: Dentate Granule Layer

DBS: Deep Brain Stimulation

EC: Entorhinal Cortex

GFAP: Glial Fibrillary Acidic Protein

i.p.: Intraperitoneal

KA: Kainic Acid

ML: Molecular Layer

NGS: Normal Goat Serum

PBS: Phosphate Buffered Saline

QNP: Quiescent Neural Progenitors

VPL: Ventral Posterolateral Thalamic Nucleus

SGZ: Sub Granular Zone

SVZ: Sub Ventricular Zone

CHAPTER I

INTRODUCTION

A. Adult Neurogenesis

Neurogenesis, introduced as the division, differentiation and functional integration of newborn neurons from precursor cells, was traditionally restricted to prenatal and perinatal stages (Ming & Song, 2005). Adult neurogenesis, continued to be an unorthodox theory, until 1965 where it was recognized in rodents (Altman & Das, 1965) and successively proven in other mammals including monkeys (Gould et al., 1999) and humans (Eriksson et al., 1998). Fifty years since the discovery, persistent neurogenesis is now fully established as a lifetime process that arises from neural precursors residing within two permissive neurogenic areas, the subventricular zone (SVZ) of the lateral ventricles (Altman, 1969; Doetsch, Garcia-Verdugo, & Alvarez-Buylla, 1997; Kornack & Rakic, 2001a; Kornack & Rakic, 2001b; Pencea, Bingaman, Freedman, & Luskin, 2001) and the subgranular zone (SGZ) of the dentate gyrus (DG), a sub region of the hippocampal formation (Altman & Das, 1965; Kaplan & Hinds, 1977). The traditional doctrine of immutability in the central nervous system that was firmly established by Santiago Ramón y Cajal in 1913 (Cajal, 1913), was challenged through the advent of innovative techniques capable of tracing and visualizing dividing neural progenitors. One of the essential techniques arose with the discovery of triated thymidine ([H3] –thymidine) by Sidman and colleagues in the late 1950s (Sidman, Miale, & Feder, 1959) as a marker

for dividing cells. In 1965, Jennifer Altman used this discovery and, in combination with autoradiography, successfully presented solid evidence picturing neurogenesis in the hippocampus of juvenile rats (Altman & Das, 1965). Bromodeoxyuridine (BrdU) was finally discovered as a synthetic thymine analog (Gratzner, 1982) and was used in conjugation with other cellular markers to confirm neuronal identity of proliferating cells. The aforementioned techniques, however, are capable of detecting cellular division with limited functional relevance. Emerging, yet limited, electrophysiological studies are being utilized to examine the physiological aspects and connectivity profiles of the newly formed DG cells and their functional relevance among existing circuits (Belluzzi, Benedusi, Ackman, & LoTurco, 2003; van Praag et al., 2002).

While the lifetime persistence of neurogenesis is well accepted, consensus lacks on the functional relevance of adult-born cells. Growing evidence suggests that persistent neurogenesis during adulthood in the SGZ and SVZ is an activity-dependent process that contributes to the functional plasticity required for learning and adaptation (Becker & Wojtowicz, 2007; Kempermann, 2008; Ma, Ming, Gage, & Song, 2008). This evolutionary preserved process can be enhanced by learning, exercise or exposure to novel environment (Fabel & Kempermann, 2008; van Praag, Kempermann, & Gage, 2000).

B. Adult Neurogenic Proliferative Zones

1. Olfactory system

The major neurogenic niche of the adult brain is the SVZ (Doetsch & Alvarez-Buylla, 1996). Newly formed neurons in the SVZ migrate in chain-like aggregates through the

rostral migratory system to the olfactory bulb where they mature into granule and periglomerular interneurons (Lois & Alvarez-Buylla, 1994; Lois, Garcia-Verdugo, & Alvarez-Buylla, 1996). Once sufficiently mature, newborn cells are thought to be incorporated into existing olfactory circuitry and implicated in odor processing (Breton-Provencher, Lemasson, Peralta, & Saghatelyan, 2009). The constant cell renewal in the olfactory bulb has been linked to neural plasticity needed for the discrimination and adjustment of odors in a continuously changing environment (Gheusi et al., 2000; Moreno et al., 2009). However, ongoing investigations are being conducted to gain more insight into this conspicuous process for a better understanding of its origin, regulation and functional contribution.

The subependymal region of the SVZ contains four distinct cell types: ependymal cells (type E cells), astrocytes (type B), transit-amplifying cells (type C), and migrating neuroblasts (type A) (Doetsch et al., 1997; Ihrie & Alvarez-Buylla, 2011). Olfactory neurogenesis starts with radial astrocytic type B cells, that stain positive for 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 & Benezra, 2009). Following a slow proliferation rate, type B cells produce transit-amplifying progenitors (type C) (Doetsch, Garcia-Verdugo, & Alvarez-Buylla, 1999). As the name suggests, these proliferative precursors rapidly divide into type A neuroblasts and converge into chain-like aggregates (Doetsch & Alvarez-Buylla, 1996; Kornack & Rakic, 2001b). Neuroblasts migrate few millimeters along the rostral migratory system, a narrow path delineated by a sheath of

astrocytic processes (Lois et al., 1996). Remarkably, this migration occurs in rodents and primates excluding humans (Sanai et al., 2004). As they reach the olfactory bulb, chain-forming neuroblasts dissociate and undergo full maturation into granule cells and few periglomerular cells (Lledo, Alonso, & Grubb, 2006). These two types of cells are inhibitory GABAergic interneurons and are reported to participate in olfactory learning and discrimination (Breton-Provencher et al., 2009; Gheusi et al., 2000; Sakamoto et al., 2011).

2. Hippocampal Formation

The Hippocampus is a structural and functional component of the limbic system, found in the medial temporal lobe. Propelled by advanced methodologies, scientists assigned vital roles for the hippocampus in the formation and association of episodic (Vargha-Khadem et al., 1997) and declarative (Squire, 1992) memories, contextual associational memory (Rudy & Sutherland, 1995) and spatial orientation (O'Keefe & Dostrovsky, 1971). The hippocampus continues to generate new neurons throughout lifetime. The dentate gyrus, a sub region of the hippocampus holds the privilege of this distinguishing feature (Altman & Das, 1965).

Neurogenesis persists during adulthood from neural stem cells residing in the germinal layer of the DG, the SGZ (Altman & Das, 1965; Eriksson et al., 1998; Gould, Tanapat, McEwen, Flugge, & Fuchs, 1998). This process of morphogenesis produces a "stratified granular cell layer", composed of adult-born dentate granule cells (DGCs) at the inner leaflet and perinatal cells at the outermost one (Zhao, Deng, & Gage, 2008). Adult-

generated DGCs resemble DGCs formed during developmental stages in the aspect of functional incorporation into pre-existing hippocampal circuitry (Kee, Teixeira, Wang, & Frankland, 2007; Schmidt-Hieber, Jonas, & Bischofberger, 2004; Toni et al., 2007; Toni et al., 2008). Being the major supply of afferent input into the hippocampus, mounting evidence supports the function of adult-born DGC in hippocampal mnemonic tasks (Kempermann, 2002).

C. Features of Adult Hippocampal Neurogenesis

1. Connectivity patterns of adult rodent hippocampal formation

a. Trisynaptic circuit

The Hippocampal formation is divided into four cytoarchitectural regions: DG, cornu ammonis (CA) (split into the subareas CA1, CA2 and CA3), the subicular complex (subiculum, presubiculum and parasubiculum) and the entorhinal cortex (EC). The major conduit for transmission of polymodal sensory information from neocortical regions to the hippocampus is the EC. The hippocampal components communicate through unidirectional projections forming a trisynaptic circuit for processing information (Andersen, Bliss, Lomo, Olsen, & Skrede, 1969) (Fig. 1). Axons of layer 2 of the EC form the perforant pathway and projects predominantly to the DG. The dendrites of DGCs make excitatory synaptic contact with axons of the perforant path. Axons of the DGCs called mossy fibers, project to CA3 pyramidal cells (Amaral & Lavenex, 2007; Ribak, Seress, & Amaral, 1985) which then projects back to CA1 via Schaeffer collaterals (axons of CA3). Axons of CA1 project back to EC and subiculum. Other subcortical structures reciprocate

synapses with the hippocampus; the medial thalamus connects with the hippocampal formation extensively via hippocampal –thalamic circuit or Papez's circuit (Ribak et al., 1985). Harm to either the medial temporal lobe or the medial thalamus has been associated with anterograde amnesia (inability to retain recent information in long-term memory (Aggleton & Brown, 1999). This study will be exploiting, in adult rats, the strong correlation between the hippocampal formation anteromedial thalamic nucleus (AMN) to get more insights into adult neurogenesis.

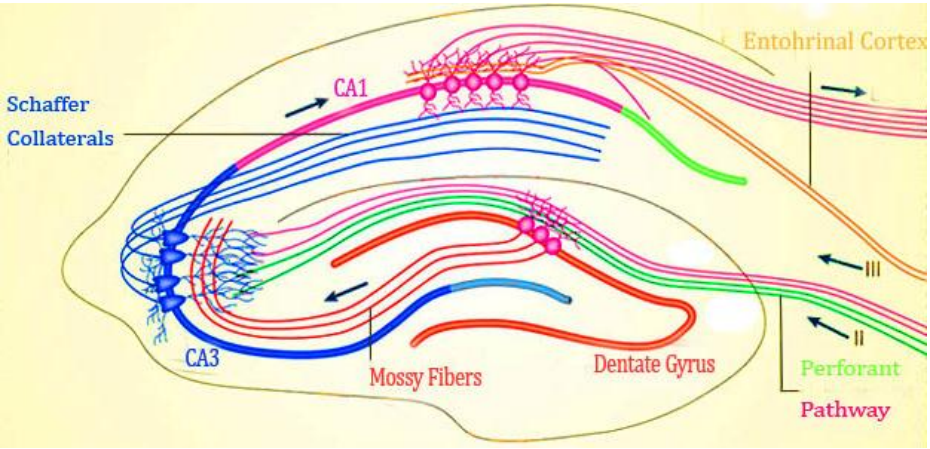

Figure 1. Trisynaptic Circuit.
EC projects to the GCL of the DG mainly through the perforant pathway formed of layer II and III efferents**.** DGCs project to CA3 pyramidal neurons via mossy fibers (Red). CA3 pyramidal neurons project to CA1 via schaffer collaterals (blue). Finally, CA1 (pink) projects back to EC and subiculum**.** Adopted and modified from (Deng, Aimone, & Gage, 2010).

b. Papez circuit

While it is deemed as the visceral brain for emotional processing (Papez, 1995), current electrophysiological studies suggest that Papez circuitry (Fig. 2) supports the processing of episodic memory (Deng et al., 2010) and spread of seizures. The circuitry "permits information to be set in its spatial and temporal context" (Aggleton & Brown, 1999). Evidence for this theory stems from the extensive direct and indirect connectivity between the hippocampus and the AMN.

The hippocampus projects to the AMN both directly through the fornix and indirectly through the mammillary bodies and mammillothalamic tract (Swanson & Cowan, 1977). In a similar fashion, the AMN project directly to the hippocampus through the cingulum bundle (Shibata, 1993)and indirectly via the retrosplenial cortex and mammillary bodies (Aggleton & Brown, 1999; van Groen, Kadish, & Wyss, 2002). Thus, these structures display the capacity to regulate the neural functions of each other in bidirectional manner (Fig.2).

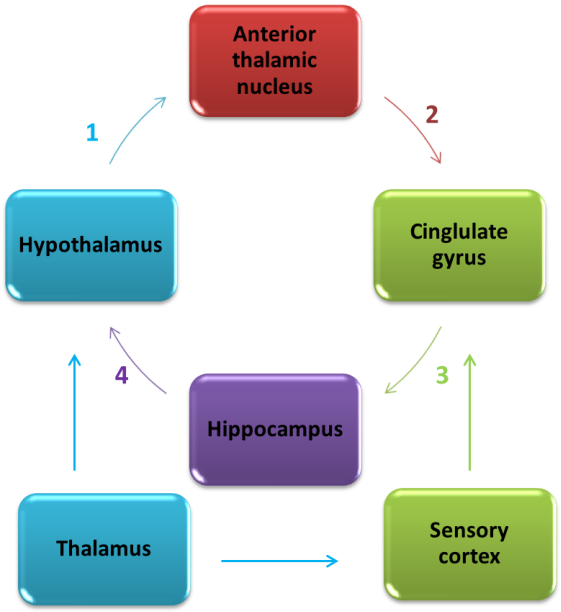

Figure 2. Papez Circuit.
Hypothalamus send connections to the AMN and then to the cingulate gyrus. Output from cingulate cortex projects to the hippocampus and the latter conveys information back to the hypothalamus.

The presence of "head direction cells" in the anterior dorsal nucleus (Taube, 1995) of the AMN and synchronized theta-modulated firing cells in the anterior ventral nucleus of the AMN (Vertes, Albo, & Viana Di Prisco, 2001) with theta rhythm of the hippocampal formation provides solid evidence of an "extended hippocampal-diencephalic" system for the processing of information, with a pivotal role for AMN (Aggleton & Sahgal, 1993). Lesions or pathologies of the AMN have been associated with remarkable loss of episodic memory, the cardinal symptom of diencephalic amnesia (Aggleton et al., 2010; Clarke et al., 1994; Dusoir, Kapur, Byrnes, McKinstry, & Hoare,

1990) and impairment of spatial processing and navigation skills(Warburton & Aggleton, 1999). Based on data from electrophysiological and anatomical studies, circuit tracing and targeted lesioning, it is postulated that AMN is crucial for spatial learning and processing.

2. Neurogenesis in the DG

a. Anatomy of the DG

As it is considered a critical component of hippocampal circuitry, the DG contains a substantial pool of granule neurons, reaching one million. The DG is formed of three individual layers: the polymorphic cell layer (hilus), which is the innermost layer and contains excitatory mossy cells, inhibitory interneurons and DGC axons; the second layer is the intermediary located granule cell layer (GCL) and the third layer is an outer-positioned acellular molecular layer (ML) which is located above the GCL. The principal layer is the GCL, populated densely with DGC. DGCs are bipolar neurons with axons extending to hilar region and dendrites spreading apically to the ML. As previously mentioned, perforant path synapses with the dendrites of granule cells, located in the ML. The area of interface between the hilar region and the GCL is termed the SGZ where populations of precursor cells reside (Fig.3). Neurogenesis in adult rodent brains is possible in the SGZ, generating less than1% of the total granule neurons reserve per day (McDonald & Wojtowicz, 2005).

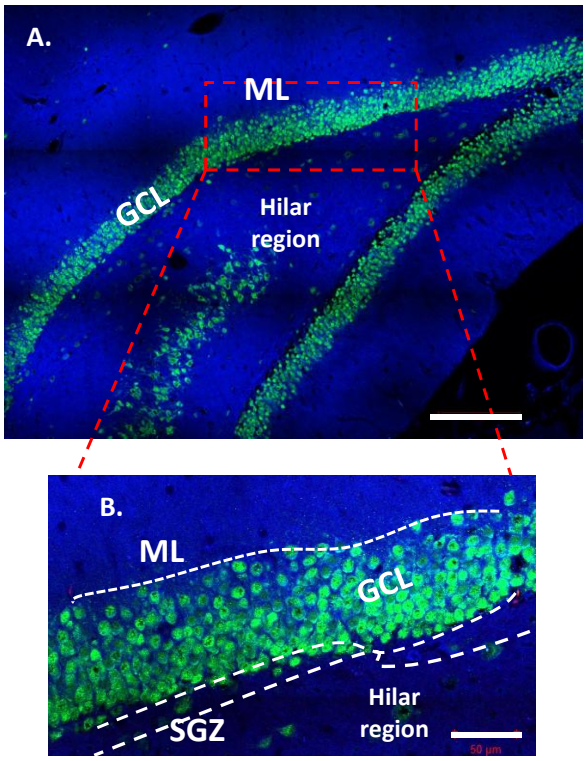

Figure 3. Anatomy of the DG.
Representative confocal images of the DG stained by NeuN (A). Tile scan covering the area was taken at 40X oil objective. (B) is an enlargement of (A) showing the 3 layers of the DG: the innermost is the Hilar region, the middle is the GCL and ML is the outermost. Scale bar = 200μm for A and 50 μm for (B).

a. Neurogenic niche

The neurogenic niche of an adult brain includes endothelial cells, astrocytes, microglias, mature neurons and adult neural precursors (Gotz & Barde, 2005; Zhao et al., 2008)Neural progenitors in the SGZ termed quiescent neural progenitors (QNP) or type 1 cells are radial-glial like cells exhibiting astroglial features with proliferative capacity

(Gotz & Barde, 2005; Kempermann, Jessberger, Steiner, & Kronenberg, 2004) (Fig. 4). Their radial processes have been observed under electron microscope, extending to the top of the subgranular layer (SGL) and the basal ML. Despite lack of consensus on conclusive cell markers for neural stem cells, QNPs uniquely express a panel of markers including GFAP, Nestin, brain lipid- binding protein and SOX2 (Fukuda et al., 2003).

Type 1 cells are believed to undergo asymmetric cell division, giving birth to self-renewing progenitors, usually defined as a type-2 cell and reverting back to the quiescent stage. The recurrence of quiescent state is a conservative process to maintain the neurogenic pool and minimize probabilities of mutations upon mitosis (Garcia, Doan, Imura, Bush, & Sofroniew, 2004). Type-2 cells are highly proliferative and are termed transit amplifying neural progenitor cells (ANP) (Fukuda et al., 2003). ANPs denote the shift from astrocytic like cells (type-2a nestin and BLBP positive) to cells with neuronal destination (type-2b) co-expressing nestin and doublecortin (DCX) (Fukuda et al., 2003). Once destined to neuronal fate, type-2b cells cease to express nestin and migrate short distances to the GCL, giving rise to type 3 cells or neuroblasts (Fig. 4). At this stage, DCX is down-regulated and mature neuronal markers such as NeuN and Calbindin begin to appear and to be up-regulated. Gradually, adult-generated DGCs achieve mature granule cell morphology, marked by elongation of dendrites and axons, and electrophysiological properties analogous to postnatal-created DGCs, implying functional capacity for integration(Esposito et al., 2005; Song et al., 2005). Identifying markers specific for each developmental stage does not only contribute to our knowledge of neurogenesis, but will also prove helpful when developing therapeutic interventions (Fig. 4).

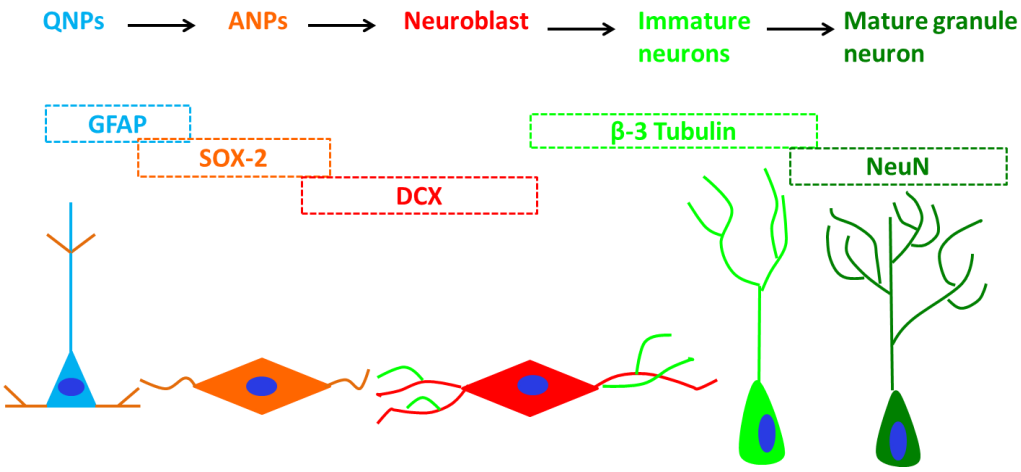

Figure 4. Hippocampal Neurogenesis.
QNPs identified by GFAP divide asymmetrically to produce ANPs lacking GFAP but positive for SOX-2 and Nestin. The highly proliferating ANPs start expressing DCX toward the end of the cell cycle. Neuroblasts, the progeny of ANPs continue the cell cycle and are positive for DCX. Neuroblasts give rise to post-mitotic immature neurons that start to express NeuN and cease the expression of DCX. Finally, NeuN is abundantly expressed to signal the mature neuronal fate.

D. Development, regulation and function of adult-generated granule cells

1. Structural development

Within the first week of maturation, some neuroblasts migrate short distances toward GCL, and the majority remain close to the hilar zone (Fukuda et al., 2003; Garcia et al., 2004; Gotz & Barde, 2005). Recapitulating an immature neuron during embryonic development, these newborns express GABAergic and glutamergic receptors with no synaptic connections at this point (Fukuda et al., 2003; Garcia et al., 2004; Gotz & Barde, 2005; Kempermann et al., 2004). By the second week, synaptogenesis is facilitated by the formation of dendritic spines and axons (Zhao, Teng, Summers, Ming, & Gage, 2006).

Early synaptic input is exclusively GABergic and through the third week, glutamergic axons of the perforant pathway innervate the adult-generated DGCs (Ge et al., 2006). As of the fourth week, apical dendrites of new DGCs arborize into the ML and axonal terminals project to interneurons of the hilar zone and dendritic spines of CA3 region (Zhao et al., 2006). Six- to eight weeks after the initiation of neurogenesis cascade, adult-generated neurons approach the electrophysiological properties of existing mature DGCs and integrate in local functional circuits (Ge, Yang, Hsu, Ming, & Song, 2007).

2. Development of the electrical behavior

Compared to perinatal granule neurons, newborn DGCs, ranging in age from two to six weeks, exhibit distinctive electrical membrane properties, typical to those observed in developing immature neurons (Wang, Scott, & Wojtowicz, 2000). Young DGCs exhibit elevated input resistance, reduced induction thresholds and greater tendency for long-term potentiation, enabling them to fire action potentials in response to few excitatory currents (Wang et al., 2000). The hyper-excitability and enhanced long-term potentiation are the hallmarks of a "critical period "during which new neurons are characterized by enhanced synaptic plasticity (Ge et al., 2007). At the end of the maturation phase, the electrophysiological discrepancies among the adult-born DGCs and existing mature neurons are reduced until the basic membrane properties such as passive membrane features and kinetics of input are similar in both (van Praag et al., 2002). Therefore, by continuously generating cohorts of new neurons, the adult hippocampus is able to retain an

enhanced form of plasticity in a population of DGCs throughout adulthood (Kempermann, 2008).

3. Regulation of adult neurogenesis in the hippocampus

Active neurogenesis in the adult hippocampus is a finely-adjusted dynamic process and regulated by intrinsic and extrinsic factors at different stages. The genetic background of the animal constitutes the intrinsic factor affecting levels of basal neurogenesis and hippocampal dependent cognitive function (Kempermann & Gage, 2002). Extrinsic factors as well exhibit a wide array of influence on the proliferation of neural precursors, via signals from the neurogenic niche of the SGZ, including mature neurons, astrocytes, microglia and endothelial cells of blood vessels (Ma et al., 2008). An extensive network of capillaries lies in close proximity with dividing precursors in the SGZ (Palmer, Willhoite, & Gage, 2000). This prominent vasculature facilitates the delivery of neurotransmitters, growth factors, cytokines, hormones and drugs to the microenvironment of the SGZ (Encinas, Hamani, Lozano, & Enikolopov, 2011a). Notably, microglia are thought to be key players in orchestrating adult hippocampal neurogenesis through apoptosis-coupled phagocytosis of new neurons to maintain homeostatic levels of the neurogenic cascade (Sierra et al., 2010). A small subset of newly formed cells survives and matures into DGCs predominantly.

Moreover, adult neurogenesis is influenced by physiological and pathological stimuli. Enriched environment (Kempermann, Kuhn, & Gage, 1997) promotes survival of new neurons and the expansion of cell proliferation is further enhanced by physical exercises (van Praag, Kempermann, & Gage, 1999). Above all, seizures remain the most known pro-neurogenic factor that increases neurogenesis in the DG, as well as other non-neurogenic regions, in an ectopic, aberrant and non-beneficial manner (Parent et al., 1997; Parent, Elliott, Pleasure, Barbaro, & Lowenstein, 2006). Interestingly, rodent brains exposed to doses of radiation are associated with suppressed hippocampal neurogenesis (Monje, Mizumatsu, Fike, & Palmer, 2002). Together, hippocampal neurogenesis is a dynamic process that responds to local network activity cues arising from internal and external stimuli.

4. Functional relevance of adult generated granule cells

Despite the intensive analyses at the electrophysiological, circuitry and behavioral levels, scientists could not assign a widely accepted role of adult hippocampal neurogenesis. Characteristics of long lasting survival and stable synaptic integration of newly generated DGCs into local circuitries, prompted scientists to investigate a role for adult-generated neurons in hippocampal dependent learning and memory activities.

Data from spatial navigation, water maze training and recalling tasks revealed an up regulation in immediate early genes rate, co-expressed with BrdU marker. This is consistent with the functional recruitment and involvement of newly formed DGCs into hippocampal circuitries concerned with spatial functions(Alme et al., 2010; Jessberger &

Kempermann, 2003). Findings provided substantial evidence of the incorporation of adult-born DGCs in hippocampal circuits concerned with novelty recognition, contextual fear conditioning, and spatial orientation and memory formation (Kee et al., 2007). A hippocampal network model system derived from computational modeling demonstrated a constant neuronal rejuvenation to help create separate memory cues for extremely comparable patterns (Becker, 2005). The persistent addition of new neurons empowers the hippocampus to adapt better with innovation and to fine-tune synaptic involvement of the DG for the processing of novel and complex conditions (Kempermann, 2002; Snyder, Kee, & Wojtowicz, 2001).

E. Deep brain stimulation

1. Overview

Deep brain stimulation (DBS) is a surgical therapeutic modality, whereby high frequency stimulation, ranging from 130Hz - 200Hz, is passed from a subcutaneously implanted stimulator to specific brain targets through unipolar or bipolar electrodes (Breit, Schulz, & Benabid, 2004). Accurate positioning of electrodes in the brain is achieved with the guidance of noninvasive neuroimaging techniques such as MRI, electroencephalography and recordings of neural activity by external electrodes. The choice of DBS is adopted in concert with a number of factors involving the physiological characteristics of brain, geometric conformation of the electrode and surrounding region, and parameters of stimulation. The first electrode implantation and chronic stimulation was pioneered by Benabid et. al in 1991 for treatment of Parkinson's disease where the

thalamic nucleus ventralis intermedius was stimulated (Benabid et al., 1991). Since then, different sites for stimulation were implicated and new targets emerged for treating a wide array of movement disorders such as essential tremor**,** Parkinson's disease**,** and primary dystonia (Koller et al., 1997; Vidailhet et al., 2005). In motor disorders it is well established that by addressing the neurophysiological activity of its targets, DBS restores, to a certain extent, a coordinated neural activity approximating that of a normal brain. The clinical success achieved prompted scientists to extend the margin of DBS application to psychiatric and neurologic disorders including depression and obsessive compulsive disorder (Chang, 2004). To date, clinical trials proved the efficacy of DBS in conferring an antidepressant activity in patients with treatment-resistant depression (Bewernick et al., 2010). Limited yet mounting evidence suggests that DBS might exert pro-cognitive improvements through increased adult hippocampal neurogenesis and functional lifelong integration of newly generated neurons into hippocampal networks (Encinas, Hamani, Lozano, & Enikolopov, 2011b; Stone et al., 2011). However, the exact mechanism underlying the full spectrum of therapeutic effects in motor and behavioral disorders remains elusive and under extensive investigation.

2. Mechanism of action

The most targeted structures for DBS in the treatment of movement disorders are the subthalamic nucleus, globus pallidus internus, and thalamus. Due to the similarity in therapeutic effects obtained after lesioning or stimulating these targets (Aziz, Peggs, Sambrook, & Crossman, 1991; Koller et al., 1997), it was postulated that DBS inhibits the abnormal activity of the target and decreases its output, in a reversible and controllable

manner. The lesioning effects approximated that of the DBS with the additional benefits of adjustability and reversibility. Several mechanisms were proposed to explain the inhibitory effect and they include: stimulation of inhibitory afferents to the target and thus inducing synaptic inhibition (Dostrovsky & Lozano, 2002), blockage of depolarization essential for neuronal firing after deactivation of voltage-dependent channels (Beurrier, Bioulac, Audin, & Hammond, 2001), or reducing the bioavailability of neurotransmitters at the synaptic level (Kringelbach, Jenkinson, Owen, & Aziz, 2007). The spread of the stimulation effects from the site of stimulation to distal regions is complex and conflicting; however it is usually separate from local tissue effects.

In an attempt to extend the therapeutic window of this minimally invasive procedure, researchers are exploring its potential application in psychiatric disorders including depression (Mayberg et al., 2005) and obsessive compulsive disorder (Abelson et al., 2005). Remarkably, DBS in patients with refractory depression exhibited antidepressant-like improvements, approximating that of the pharmacotherapy. Moreover, in a promising clinical trial on patients with Alzheimer disease, DBS to limbic targets was accompanied by an amelioration of dementia-associated cognitive decline (Laxton et al., 2010). Importantly, an increase in the neuronal activity of hippocampal region and entorhinal cortex was recorded in patients (Laxton et al., 2010). While the underlying mechanisms mediating the therapeutic effects of DBS remain elusive, enhanced hippocampal neurogenesis might account for the improved cognitive function of DBS. The newly formed neurons are thought to be functional and readily integrative as discussed above.

F. Neurogenesis: a therapeutic mechanism of deep brain stimulation

DBS of the AMN succeeded in decreasing the propensity of partial seizures and refractory epilepsy (Fisher et al., 2010). The subgenual cingulate cortex, the anterior limb of the internal capsule, and the nucleus accumbens are candidates under investigation for treating depression as well. The role of the hippocampus in the limbic circuitry and its functional connectivity with limbic structures propelled the theme of hippocampal neurogenesis as a mechanism of DBS effects.

Within this context, a compelling question is whether the intimate connections between the hippocampus and the AMN will affect the neurogenesis levels in the hippocampal formation upon stimulating the AMN.

G. Aim of Study

The objective of this study is to determine the effect of DBS of the AMN on hippocampal neurogenesis in awake male and female rats. To address this issue, we examined the stimulation-induced effects on the proliferative activity of the DG using a combination of experiments. In the first set of in vivo experiments we aimed at measuring the levels of neurogenesis in the DG of stimulated rats using BrdU incorporation. To prove target specificity in inducing neurogenesis, we stimulated the VPL of the thalamus using the same parameters. Finally, we compared the effects of electrical stimulation to that of chemical stimulation by Kainic acid as an attempt to restrict the electrophysiological effects to the cell body or axons. This is a novel approach toward gaining insights into the molecular mechanisms mediating the neurogenesis effects of DBS. The findings in this study build upon our current understanding of the mechanisms facilitating stimulation-induced effects on neurogenesis and the molecular mechanism(s) of this process.

CHAPTER II

MATERIALS AND METHODS

Experiments were performed on adult male and female Sprague–Dawley rats (250-300 g) and were approved by the Institutional Animal Care and Use Committee at the American University of Beirut and followed ethical guidelines (Zimmermann, 1983). Animals were housed under standard colony conditions in a room maintained at a constant temperature (20–22°C) on a 12 h light/dark cycle with standard rodent chow and water provided ad libitum. Surgical procedures were performed under deep anesthesia in two steps: injection of the pre-anesthetics, atropine (atropine sulfate, Laboratoire Aguettant, dilution 1:10 in saline 0.05 mg/kg, i.p.) and chlorpromazine (Largactil®, 8 mg/kg, i.p.), followed, ten minutes later, by the anesthetic ketamine (Ketalar®, 50mg/kg, i.p.) to achieve deep anesthesia of the rats. Postoperative behavioral and body weight monitoring was conducted during the light phase of the cycle.

A. Surgical Procedure and Stimulation

1. Stereotactic surgery

The head of the anesthestized rat was rigidly fixed on a sterotaxic frame (DKI), the skin of the scalp was shaved and an incision was made to expose the skull bone. A hole was drilled into the skull andconcentric bipolar electrodes were implanted unilaterally into the right anteromedial thalamic nucleus according to the following stereotaxic coordinates:

-1.4 mm from lambda, +0.8 mm lateral and 6 mm vertical from the surface of the brain (Paxinos & Watson, 1998). In the negative control group, electrodes were implanted in the ventral posterolateral thalamic nucleus at the coordinates: -2.7 mm from lambda, -3 mm lateral and 6 mm vertical from the surface of the brain (Paxinos & Watson, 1997). The electrodes were fixed to the skull with acrylic glue and the skin was sutured with silk thread. A topical antibiotic was administered, and the rats were returned to their home cages. Rats were given 3 to 5 days of post-surgical recovery before the stimulation or sham procedure, as described below.

2. Electrical stimulation: DBS

High-frequency electrical stimulation was delivered using a stimulator (ANS,model 3510, Plano, TX) for 1 h. The output cables of the stimulator were connected to the implanted electrodes and rats were allowed to roam freely in their cages during stimulation. The current intensity and frequency of stimulation were set at 100±10μA and 130 Hz, respectively, with 120 μsec pulse width and 2.5V amplitude which approximates the settings used in clinical practice. The behavior of rats was observed throughout the stimulation period and no abnormal behavioral or motor signs were recorded. Sham rats underwent the same stereotaxic surgery and wires were connected to implanted electrodes without current delivery. At the end of the session, rats were transported back to their standard colony condition and daily observed for a period of 3 days.

3. *Chemical stimulation: Kainic acid administration*

In an attempt to demonstrate that the activation of the anterior medial thalamic nucleus involves mainly the stimulation of neuronal cell bodies a group of rats (n=3) received microinjections of a solution of kainic acid (KA). Two month old Sprague-Dawley female rats were deeply anesthetized for craniotomy. KA (500 pM) was prepared freshly on the same day of surgery. Using the same surgical procedures and stereotaxic coordinates, a 5µL Hamilton syringe filled with 1 µL KA was lowered slowly into the AMN and left for 2 min. A volume of 1µl of KA solution was injected into the AMN at very slow rate over a period of 2-3 min. The tip of the needle was maintained in place for another 2-3 min before removing the syringe and suturing the skin of the skull. 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, Bardgett, Lall, Csernansky, & Csernansky, 1999).

B. BrdU Administration

To explore the basal and stimulation-induced effects on the levels of neurogenesis, rats of all groups received a total of 4 BrdU (Sigma-Aldrich B5002-1G) (50mg/Kg/injection,i.p.), spaced 3 h apart for maximal availability. BrdU powder was weighed and dissolved in 0.9% warm saline. The solution was properly dissolved and filtered using a 0.2µm filter unit. The injections were performed 3days after stimulation; based on the report by Lozano, et.al (Toda, Hamani, Fawcett, Hutchison, & Lozano, 2008) showing that the highest proliferation rate is attained 3 to 5 days post stimulation. Rats

were euthanized 24 h after the last BrdU injection, which is the duration needed to achieve a complete cell cycle (Cameron & McKay, 2001).

C. Verification of Electrode Placement

50 μm free floating sections were selected from the area of electrode insertion and needle injection to assess the location of electrode placement. In brief, sections were washed first with 0.1M PBS then rinsed with distilled 2 times, 5 sec each. Sections were then stained with 0.2% cresyl vilolet solution for 2 min. Dehydration followed using increased ethanol concentration (2 times in 95%, 30 sec each and 2 times in 100%, 1 min each) and finally sections were dipped in xylene for 3 min and then slides were mounted. Images from electrical stimulation of the AMN and VPL are shown in Fig.5 and images from chemical stimulation are shown in Fig.6. Images were captured using a bright field microscope. Rats with incorrect electrode implantation were not included in the study.

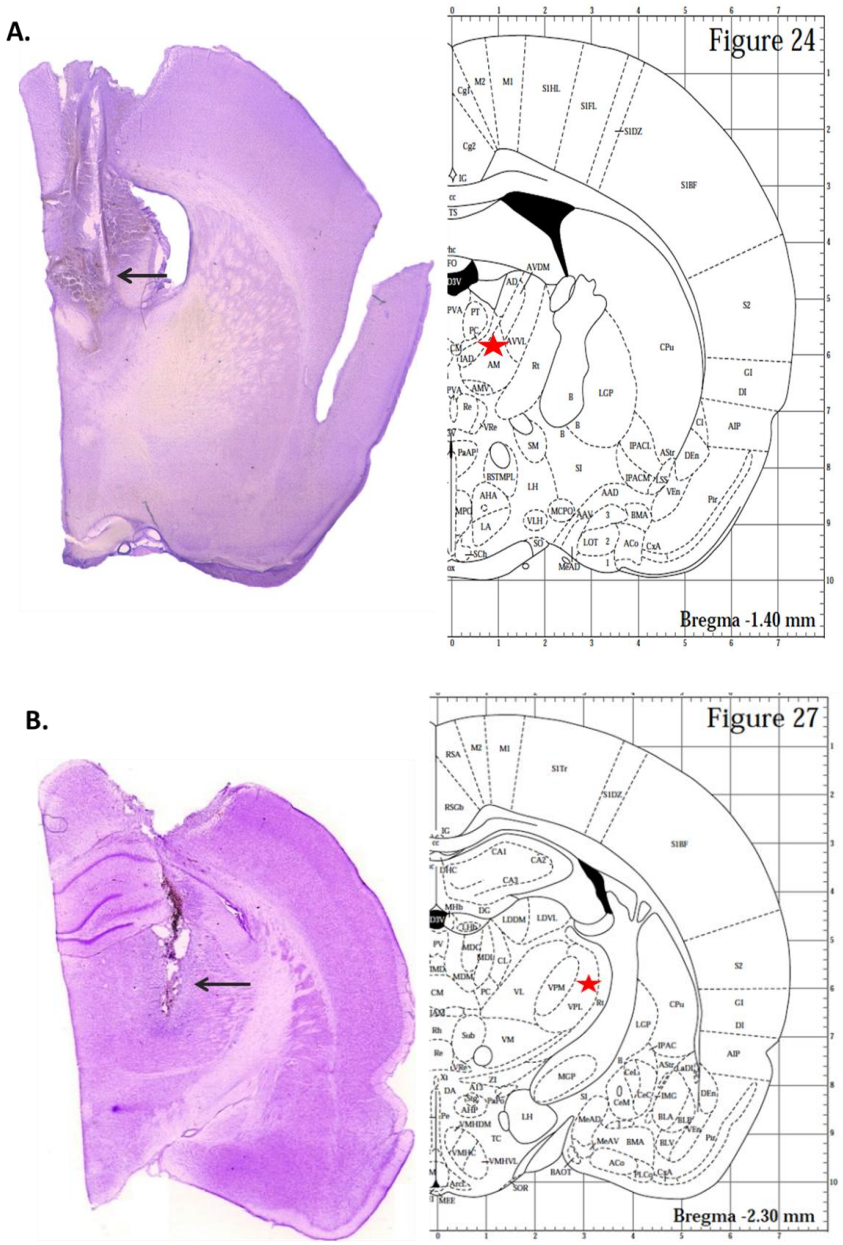

Figure 5. Cresyl Violet staining for verification of electrode implantation.
The location of electrode from electrical stimulation of the AMN and VPL group is shown by the headed arrow (A) and (B), respectively. Images were taken using Light microscopy. Pictures on the right are adopted and modified from (Paxinos & Watson, 1998).

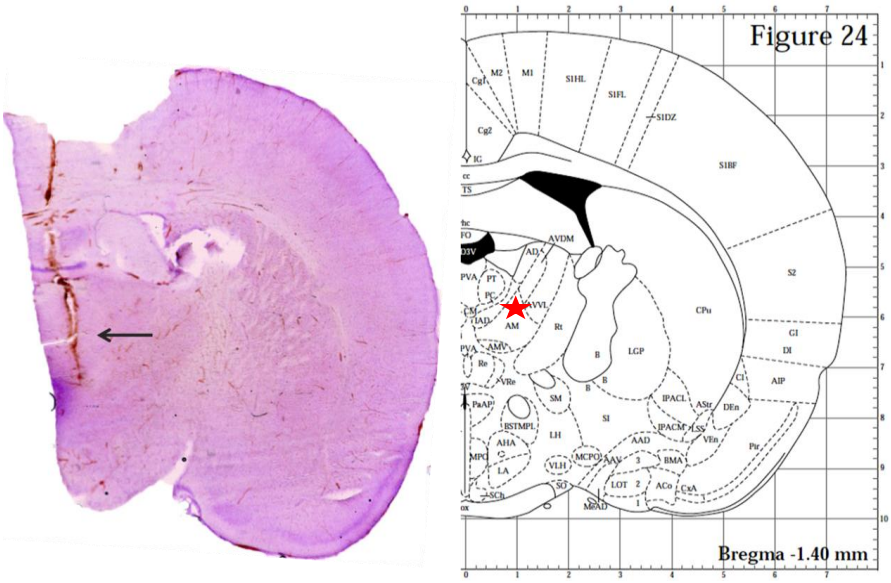

Figure 6. Cresyl Violet staining for verification of needle insertion.
The location of the syringe insertion in the AMN is shown by the headed arrow. Images were taken using Light microscopy. Pictures on the right are adopted and modified from (Paxinos & Watson, 1998).

D. Experimental Design

To investigate the stimulation-induced effects on adult neurogenesis in rodents, female and male rats were divided into different groups as follow (also see *Table 1*).

Group 1: this group was designed for the evaluation of the stimulation-induced effects on hippocampal neurogenesis following unilateral DBS. For this purpose, rats (males n=6 and females n=6) were stimulated unilaterally for 1h.

Group 2: this group was designed to serve as the sham. For this purpose, Rats (males n=4 and females n=3) underwent electrode insertion in the right AMN and subjected to all procedures except for current delivery.

Group 3: this group was designed to assess the basal level of neurogenesis. Adult rats (males n=4 and females n=4) received 4 injections of BrdU (i.p.) without any surgical procedure and were perfused 24h later.

Group 4: this group was designed to serve as the negative control in order to assess the off targeting in causing neurogenesis in the DG. Adult male rats (n=4) underwent electrode implantation and stimulation in the right VPL nucleus.

Group 5: this group was designed to compare the effects of chemical stimulation versus electrical stimulation on the proliferative activity in the SGZ. Chemical stimulation was induced through microinjection of 1 μL Kianic acid (KA) of molarity 500pM into the right AMN of female rats (n=3).

Table 1. *Summary of the procedures performed on the different experimental groups.*

Experimental group	Treatment	BrdU injection	Perfusion
Baseline neurogenesis	No Implantation/No stimulation	A total of 4 BrdU injections (50mg/Kg/injection; i.p), every 3 h	24 h after last BrdU injection
AMN-S induced effects on DG neurogenesis	Unilateral AMN-stimulation (Right hemisphere)	3d after AMN-S: A total of 4 BrdU injections (50mg/Kg/injection; i.p), every 3 h	24 h after last BrdU injection
Sham	Unilateral electrode implantation in AMN (Right Hemisphere) /No stimulation	3d after implantation: A total of 4 BrdU injections (50mg/Kg/injection; i.p), every 3 h	24 h after last BrdU injection
VPL-S for target specificity	Unilateral VPL-stimulation (Right hemisphere)	3d after VPL-S: A total of 4 BrdU injections (50mg/Kg/injection; i.p), every 3 h	24 h after last BrdU injection
KA-induced effects on DG proliferation	Unilateral KA injection into right AMN	Daily: A total of 5 BrdU injections (50mg/Kg/injection; i.p)	3 h after last BrdU injection

E. Experimental Procedures

1. Sacrifice and tissue preparation for stereology

24 h after the last BrdU injection rats were deeply anesthetized and perfused transcardially with 200 ml of 0.9% saline followed by the same volume of 4% formalin. Brains were removed, 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). The brains were then cut sagittally into 2 halves (stimulated and non-stimulated

hemisphere) and were then sliced separately. Using a freezing microtome, 50 μm coronal freezing sections were cut serially from the caudal to the rostral extent of the DG at the following caudo-rostral coordinates -6.3 to -3.3 mm relative to bregma for use in immunostaining. The free floating sections from each hemisphere were distributed in 6 well plates using the fractionator method (Gundersen, Jensen, Kieu, & Nielsen, 1999) for unbiased cell stereology (Fig. 7). Basically, the 1st section is placed in the first well, the 2nd in the second well and the 6th in the sixth well. As for the 7th section it was placed in the first well, such that the difference between the 1st and 7th section is 300 μm. Six parallel sets including 12 sections each from each hemisphere were collected for every rat. Accordingly, each set comprised a systematic random sample representative of the entire DG for use in quantification analysis. All Sections were collected in 0.1 M PBS and additional sets were stored in a PBS solution containing 15 mM Sodium Azide for future processing.

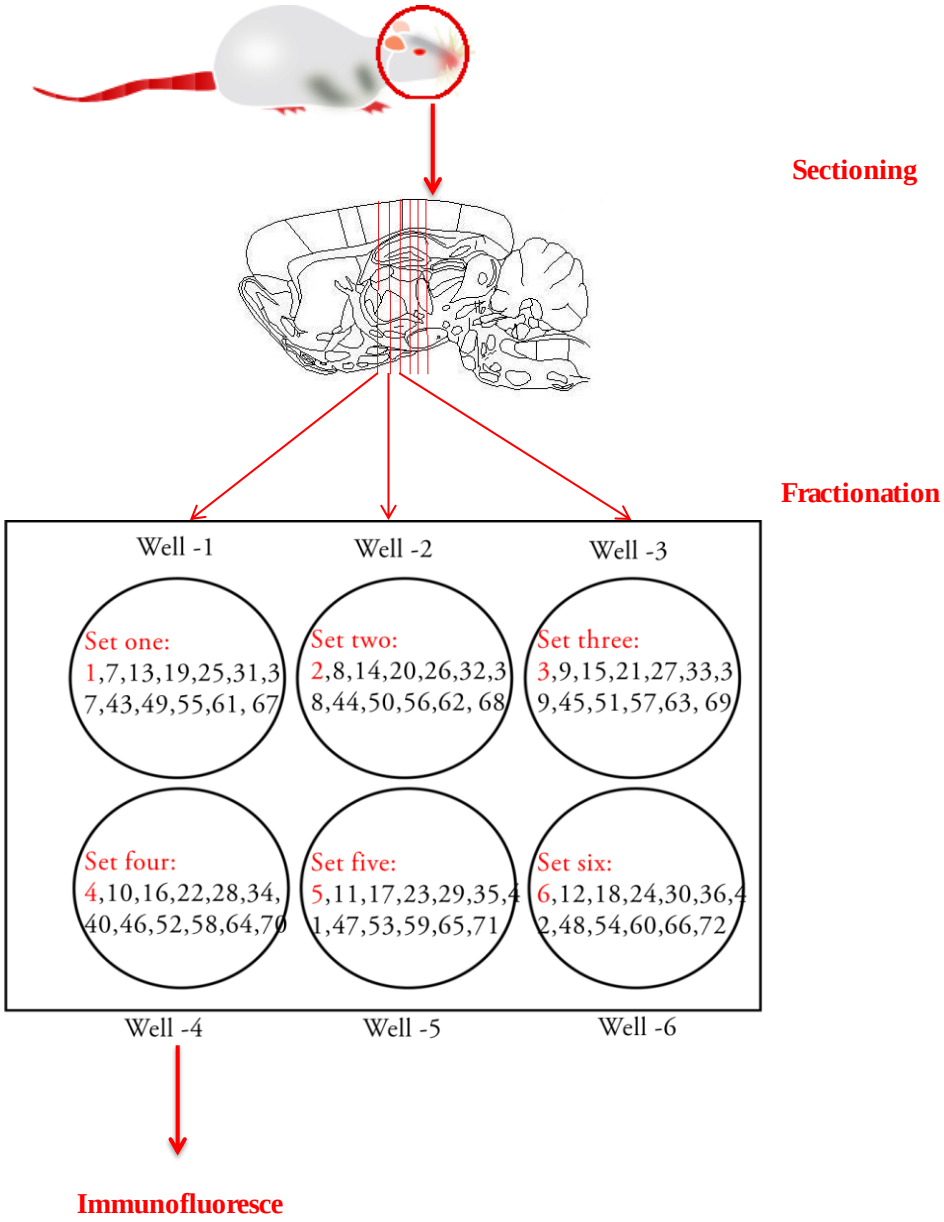

Figure 7. Fractionator Method.
Free floating coronal sections are distributed in a 6 well plate. The first section will be placed in the first well, and the following sections will be placed in the adjacent wells reaching the 6th slice in the 6th well. The 7th section will be placed in the first well so that each slice is 300µm apart from the next slice in the same well.

2. Immunofluorescence and confocal microscopy

One set comprising 12 sections was chosen randomly from the 6 sets, representing the whole DG area in each rat. Free-floating sections were washed 3 times (5 min each) with 0.1M PBS in 12-well plate. For the purpose of BrdU detection, DNA was denatured by incubating the sections in 2N HCl for 1.5 h 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, 3% 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 NeuN and/or GFAP diluted in PBS with 10% NGS, 0.1% Triton-X (see *Table 2*). The following day sections were washed 3 times with 0.1M PBS and incubated in the dark with fluorochrome-conjugated secondary antibodies diluted in PBS with 10% NGS and 0.1% Triton-X for 2 h at room temperature on a rotator. Sections were then washed with 0.1M PBS and incubated with primary BrdU antibody at 4°C overnight and the next day the secondary antibody was applied as before. After the last wash with PBS, sections were mounted onto slides with Fluoro-Gel containing 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.

Table 2. *Summary of the antibodies applied in immunofluorescence and their corresponding dilutions.*

Primary antibody	Source	Concentration	Secondary antibody	Source	Concentration
NeuN	MILLIPORE	1:1000	Alexa Fluor® 488 Goat Anti-Mouse	Invitrogen	1:250
BrdU	SEROTEC	1:100	Alexa Fluor® 568 Goat Anti-Rat	Invitrogen	1:100
GFAP	SIGMA-ALDRICH	1:500	Alexa Fluor® 660 Goat Anti-Rabbit	Invitrogen	1:250

3. Cell stereology

For the analysis of cell proliferation in the DG, one set out of the six was randomly selected 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 6^{th} section (300µm apart) using 60X-oil objective. The total number of positive cells from the 12 sections of one set was multiplied by 6 (the number of sets per rat), to denote the overall number of BrdU+ cells in the entire DG of each rat (refer to formula -1)

Formula-1: Total number of BrdU+ in DG = average of BrdU+ in one set (12 sections) x 6 (number of sets per hemisphere).

Double immunofluorescence staining was performed for the evaluation of co localization of BrdU with other cell markers. For this purpose, 2 sets of tissues were randomly chosen from stimulated (n=4) and sham rats (n=2) of the female group; one set was stained for BrdU and NeuN and the other for BrdU and GFAP. Z-stack images for BrdU cells were obtained and maximal intensity projection was done to detect co-localization with NeuN or GFAP. The total number of BrdU+/GFAP+, BrdU+/NeuN+ and BrdU+/GFAP- cells was calculated according to the following formula-2 where X symbolizes the other markets:

Formula-2: Total number of BrdU+/X in DG = total number of BrdU+/X in one set (12 sections) x 6 (number of sets per hemisphere).

For the purpose of highlighting the topographic organization of BrdU distribution, the DG region was divided into three thirds, the caudal begins from -6.30 to -5.10, intermediate from -5.10 to -3.90 and rostral from -3.90 to -2.70 according to bregma. Thus in a well of 12 tissues, every 4 sections represent a region of the DG. The total BrdU cells in each region were calculated using formula-3 where Y symbolizes one region of the DG:

Formula-3: Total # of BrdU in region Y of DG: Average BrdU cells in 4 sections x 24 (total sections per region)

For the purpose of consistency, images of BrdU+ cells were acquired under the same laser and microscopic parameters. BrdU quantification was done by two observers. First, a dependent observer exposed to all experimental conditions performed quantification of BrdU+ cells. Then, another independent observer, blinded to

experimental conditions performed quantification for the same subjects. In this study, we report the mean values of the two observations.

F. Statistical Analyses

Cell count data are presented as mean ± SD as calculated from different experiments.The determination of the significance of differences was done using t-test or ANOVA, when appropriate. ANOVA was followed by Bonferroni multiple comparisons test. The following variables were taken into consideration gender (female / male groups), stimulation (stimulated /sham) side of stimulation (contralateral / ipsilateral to stimulation), and region of DG (rostral / intermediate / caudal). The P value of <0.05 was considered as the limit of significance of differences. Statistical analysis and plotting of figures were made using Prism 4-5 GraphPad package (GraphPad software, Inc., CA, USA).

CHAPTER III

RESULTS

A. Adult Neurogenesis in the Hippocampus

Adult neurogenesis has been reported in several studies and shown to vary depending on the brain areas. Working on the hippocampal formation our study elicited changes in the level of neurogenesis between male and female rats. Our first aim, therefore, was to document further this variation and to investigate whether it might show changes following electrical stimulation.

1. Gender-dependent variations in adult hippocampal neurogenesis

Intraperitoneal injections of BrdU (200mg/kg) in control (naïve) male (n=4) and female (n=4) rats, one day prior to their sacrifice, revealed gender variations in the basal levels of hippocampal neurogenesis. The average number of BrdU positive cells in the DG of females was 724.5 ± 14, compared to 948.75 ±10 cells in their male counterparts ($p<0.01$). However, no significant gender differences were observed in the BrdU levels of the hilar region. Fig. 8 shows clusters of BrdU cells present in the DG of females (A) and males (B).

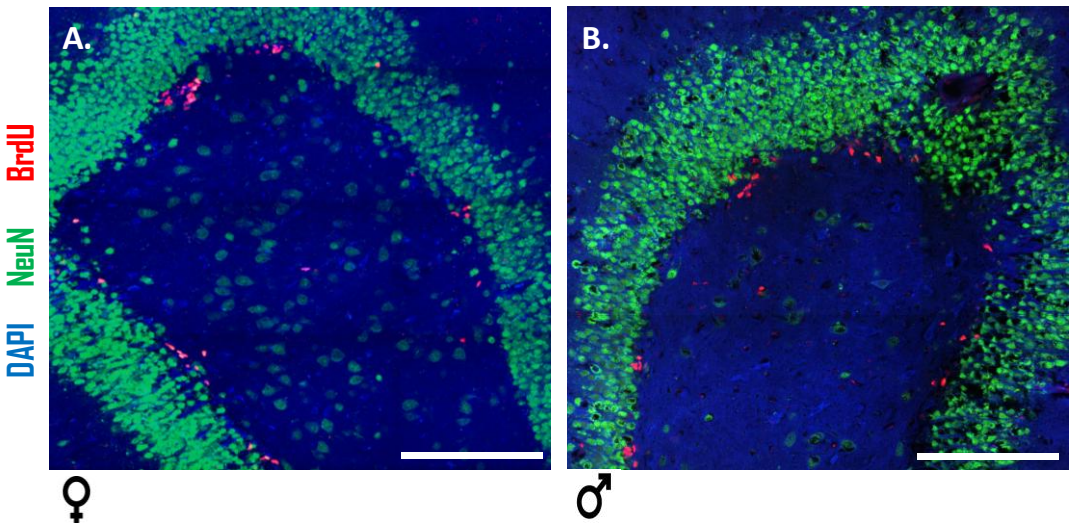

Figure 8. Representative images of baseline adult hippocampal neurogenesis. Immunofluorescence labeling of the DG by NeuN (green) and proliferating cells by BrdU (red) was performed on indicated groups. Tile scan covering the area was taken at 40X oil objective. Scale bar = 200µm.

B. Electrical stimulation of the AMN increases adult hippocampal neurogenesis

1. Increased cell proliferation in the DG

To evaluate stimulation-induced changes on the levels of neurogenesis, 6 female and 6 male rats were subjected for 1h to high frequency stimulation of their right thalamic AMN. One way ANOVA test revealed a substantial increase in BrdU stained cells evoked by stimulation. Number of BrdU positive cells was enhanced in females reaching 1183±209 ($P< 0.01$) and in males reaching 1524 ±85 ($P< 0.001$) (Fig. 9), when compared to their respective sham groups (756±33.94 and 952.5±141.17) . Analysis within each subject revealed that the DG contralateral to stimulation had a mild increase in BrdU levels that are notably lower than that observed in the ipsilateral DG, and slightly higher than the

levels in sham groups. Immunofluorescent analysis reveals the difference in neurogenesis levels between sham (Fig.10-A) and stimulated (Fig.10-B) groups.

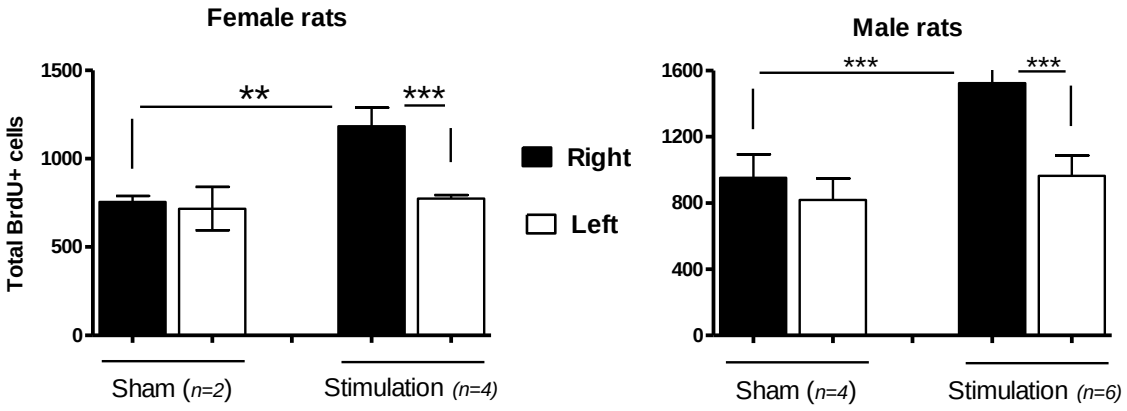

Figure 9. Quantification of stimulation-induced effects on neurogenesis in adult DG. Each bar in each panel represents the average ± SD of BrdU quantification made on the indicated groups. The determination of significance of each value was made with reference to contralateral side of stimulation (Left) and sham group by ANOVA followed by Bonferroni multiple comparisons test. (**, P<0.01; ***, P<0.001).

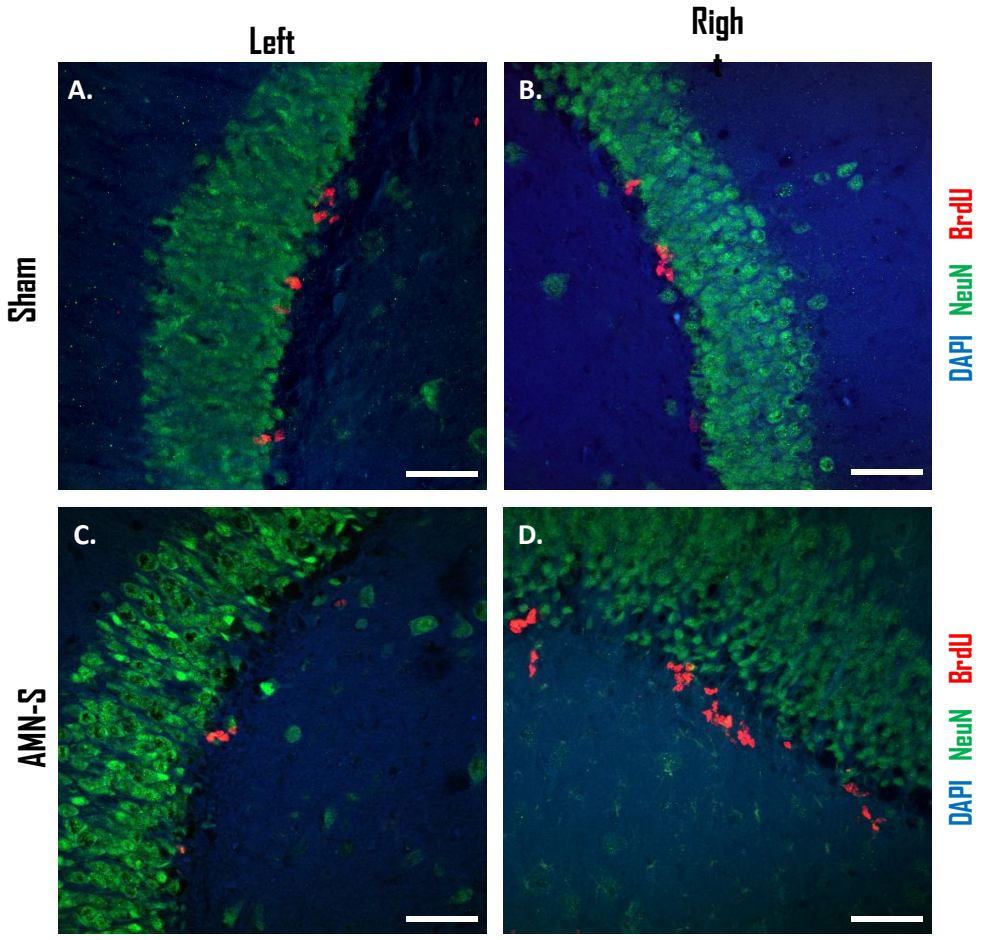

Figure 10. Representative images of stimulation-induced neurogenesis in the SGZ of stimulated rats.

Immunofluoresence labeling of GCL by NeuN (green) and proliferating cells by BrdU (red) was performed and representative images for the indicated groups are shown. (A) and (B) are confocal microscopic images of the left and right side (side of electrode implantation) of the DG of a sham rat, respectively. (C) and (D) are confocal microscopic images of the left and right side (side of stimulation) of the DG of a stimulated rat. Scale bar = 50μm.

2. Stimulation-induced increase is gender-independent

Knowing that gender-dependent variations exist for baseline neurogenesis in adult rodent hippocampus and that it is influenced by neural activity, we asked whether stimulation-induced proliferation will be subject to similar gender-dependent variations. The total number of BrdU positive cells was higher in the DG of stimulated male rats, compared to stimulated females of the same age and strain (males=1524 ±85; Females= 1183±209, $P< 0.01$). Despite this difference, the rate of DBS-induced neurogenesis was the same, reaching 56.4% in females and 60% in males.

3. Spatial changes in stimulation-induced neurogenesis in the DG

In order to explore the spatial distribution of DBS-induced neurogenesis, we divided the DG into equal thirds from the rostral to the caudal poles. The number of BrdU positive cells in sham rats elicited a progressive increase from the rostral to the most caudal segment of the DG. This pattern was conserved following DBS and became more evident in both female and male rats (Fig. 11). While the most rostral DG had the lowest BrdU expression and was not significantly altered by stimulation, the intermediate DG exhibited a moderate increase in BrdU population. Notably, the highest increase in BrdU population was confined to the caudal DG (Fig. 11 and Fig. 12).

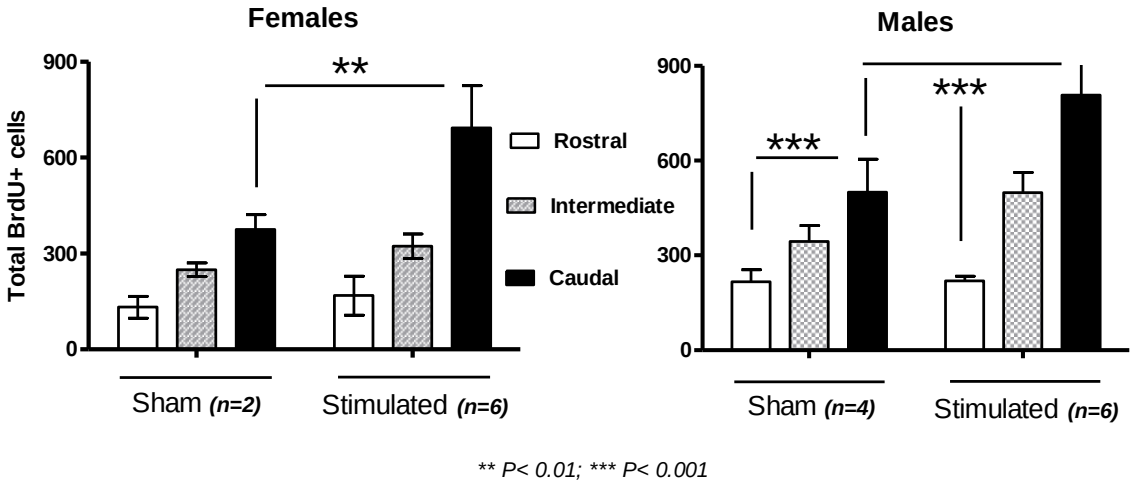

Figure 11. Topographical quantification of BrdU.
Each bar in each panel represents the average ± SD of BrdU quantification made on indicated groups. The determination of significance of each value was made either within subject with reference to DG region or between groups with reference to sham rats (**p< 0.01, ***p< 0.001).

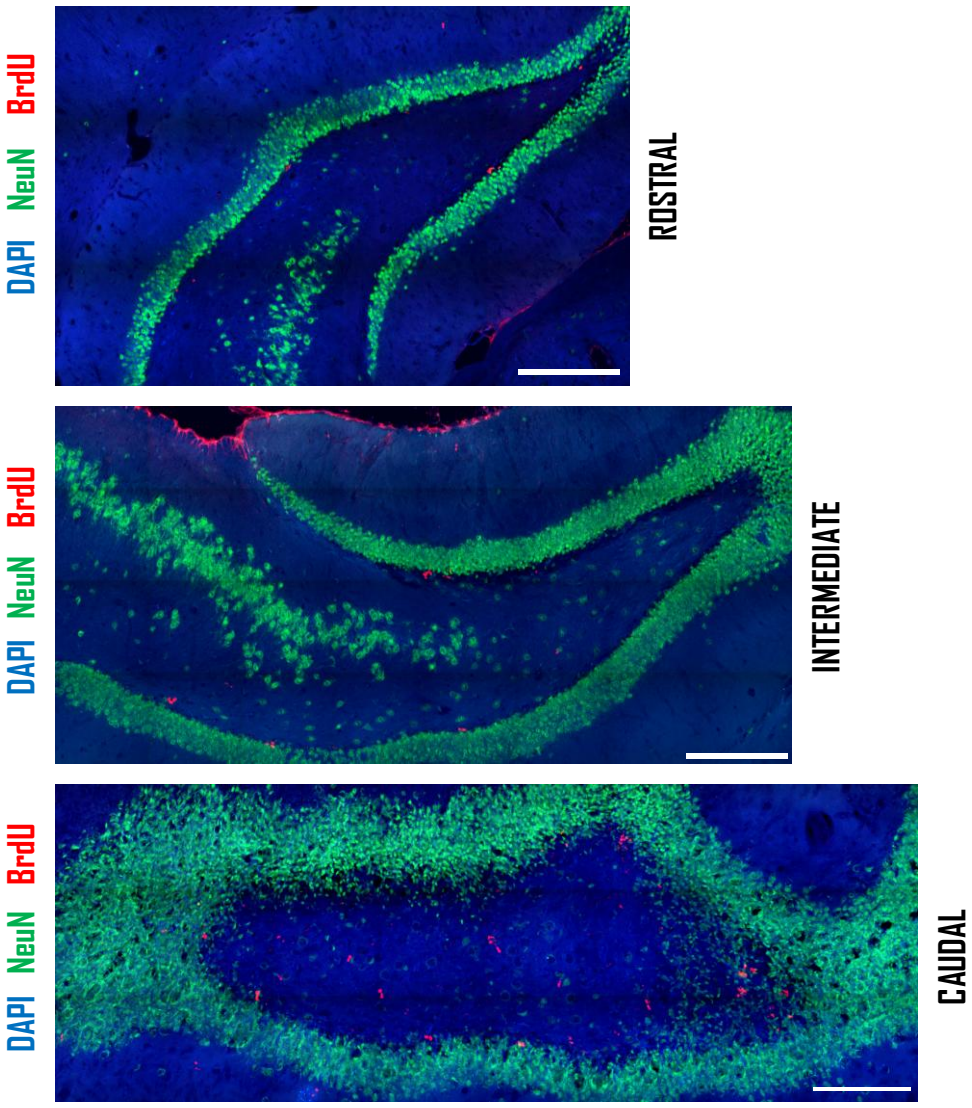

Figure 12. Representation of the topographical BrdU distribution in the DG. Immunofluoresence labeling of GCL by NeuN (green) and proliferating cells by BrdU (red) was performed and representative images of stimulated male rat is shown. In order to cover the different DG areas and to minimize loss of information due to the thickness of each section (50 μm), a tile scan image for each region was acquired in the XYZ projection and was later subjected to maximal intensity projection and presented above. Images are shown from the rostral to the caudal extent of the DG. Scale bar = 200μm.

4. Stimulation increased the proliferation of neural progenitors

Using a model of endogenously and exogenously expressed cellular markers, we were able to determine the identity of proliferating cells. We made use of BrdU in combination with GFAP to stain proliferating quiescent neural progenitors, BrdU alone to label amplifying neural progenitors, DCX in combination with Ki-67 to stain neuroblasts and BrdU with NeuN to mark neuronal fate. The number of BrdU+/GFAP+ cells was not altered in the stimulated female group compared with the sham. However, the number of amplifying neural progenitors stained only with BrdU was significantly higher than sham group (Fig. 13).

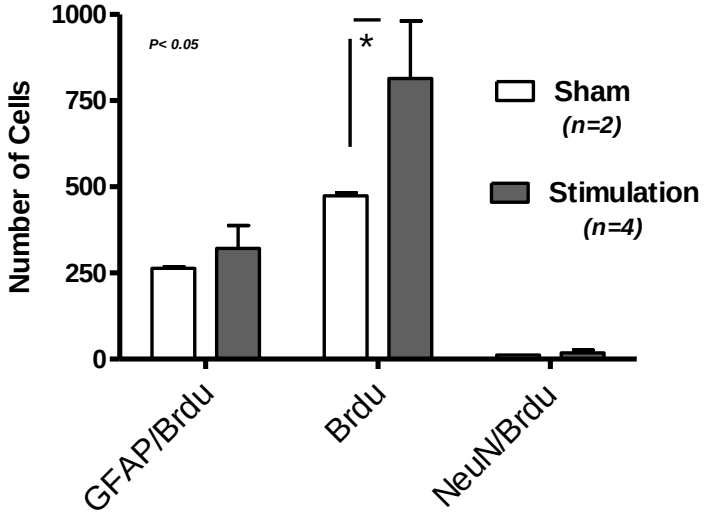

Figure 13. Quantification of stimulation-induced effects on neural progenitors. Each bar in each panel represents the average ± SD of BrdU quantification made on the indicated groups. The determination of significance of each value was made with reference to sham group.

BrdU positive cells were seen either in chains extending parallel to the SGZ (Fig. 14-A) or as clusters in the SGZ (Fig. 14-B).

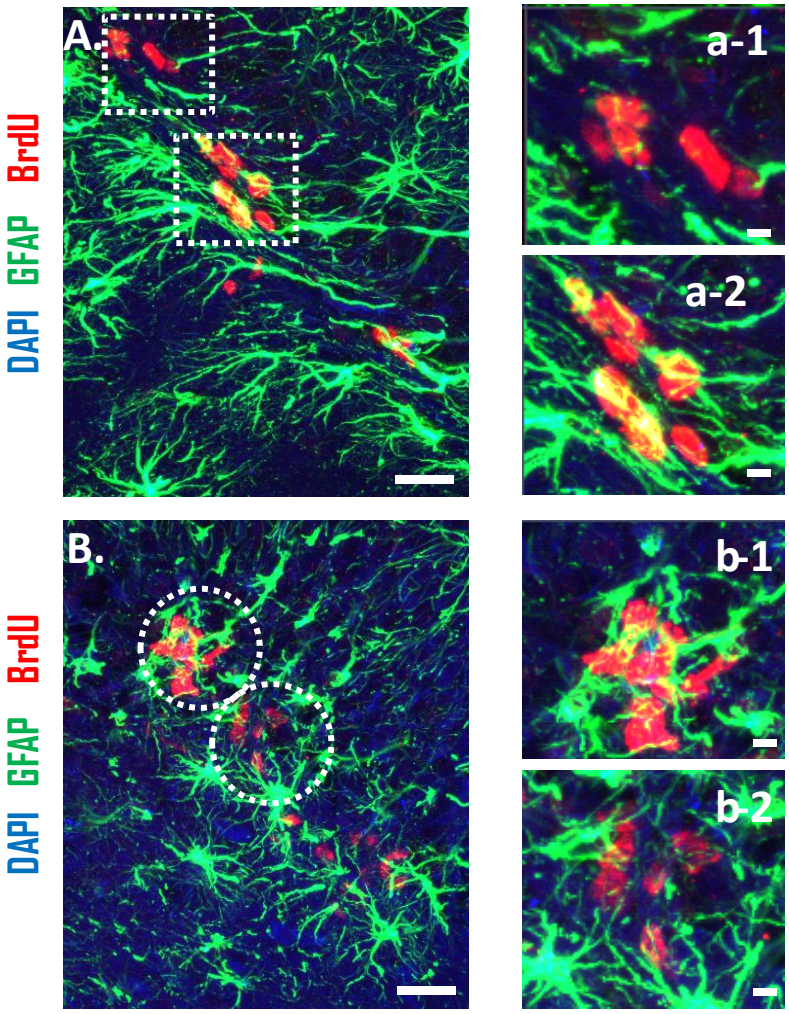

Figure 14. Representation of the Stimulation-induced effects on neural progenitors. Immunofluorescence labeling of chains (A, dotted square) or clusters (B, dotted circle) of proliferating cells by BrdU (red) and GFAP (Green). a-1 and a-2 are magnified view of image A. b-1 and b-2 are magnified view of image B. Serial optical sections along the Z-axis were acquired and were later subjected to maximal intensity projection and presented above. Scale bar, A-B = 20μm and a-b = 5μm.

The morphology of proliferating cells was recorded. Quiescent neural progenitors co-expressed BrdU and GFAP and they were small and elongated (Fig. 15-A). However amplifying neural progenitors were larger, rounded and more numerous (Fig. 15-B).

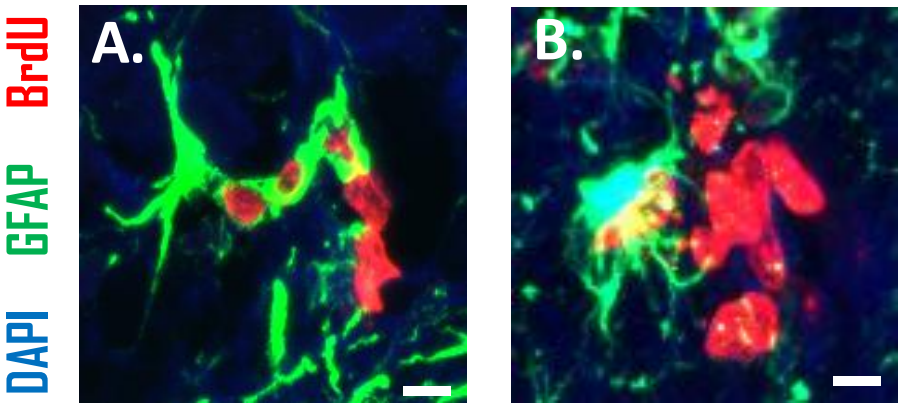

Figure 15. Representation of quiescent and amplifying neural progenitors during proliferation.
Immunofluorescence labeling of proliferating cells by BrdU (red) and GFAP (Green). Quiescent neural progenitors (A) were labeled by co-expression of BrdU (red) and GFAP (green) and amplifying neural progenitors (B) were labeled only with BrdU. Serial optical sections along the Z-axis were acquired and were later subjected to maximal intensity projection and presented above. Scale bar = 5μm.

The 3 days duration between stimulation and last BrdU injection is too short to observe any stimulation-induced changes at the level of Neuroblasts or NeuN committed cells. At the time of sacrifice, the number of BrdU+ NeuN+ cells was approximately the same among stimulated and non-stimulated groups. Confocal images were obtained for

BrdU+ NeuN+ cells (Fig.16). It is noteworthy to mention that NeuN appeared in some BrdU+ cells at a weak signal.

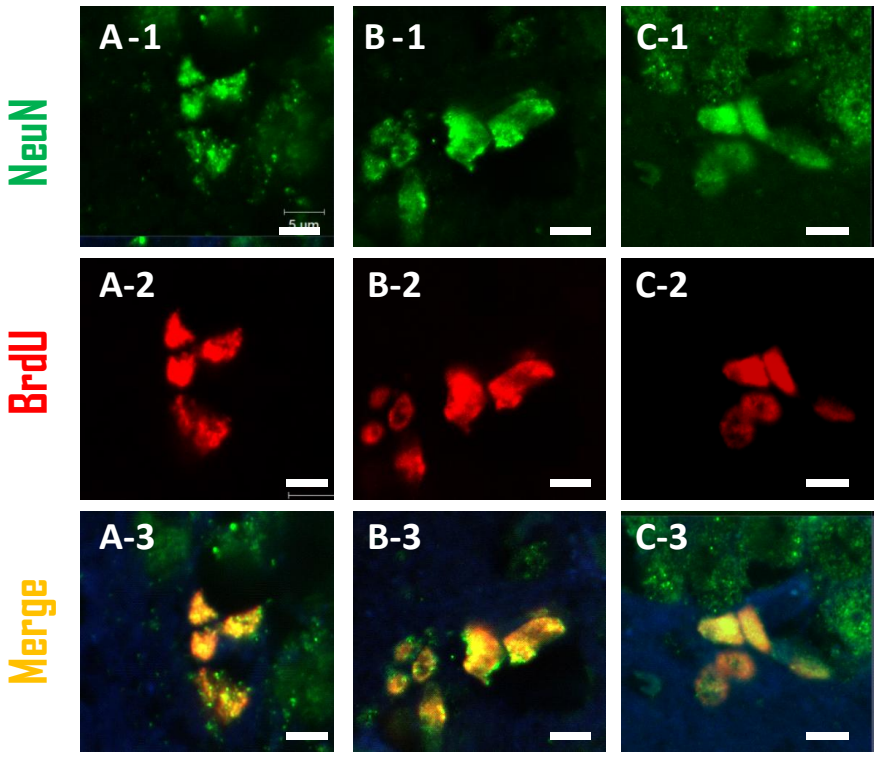

Figure 16. Representation of the neuronal fate of proliferating cells.
Immunofluorescence labeling of proliferating cells by NeuN (green) and BrdU (red) are shown in A1-C3. Serial optical sections along the Z-axis were acquired and were later subjected to maximal intensity projection and presented above. Scale bar = 5µm.

C. Basal Levels of Proliferation in hilar zone and SVZ are not Altered by AMN Stimulation

1. Neurogenesis in the lateral wall of the ventricles

The level of neurogenesis was not altered in the subventricular zone after stimulation. The overall density of BrdU positive cells was similar in the sham (Fig.17-A) and stimulated rats (Fig.17-B). Moreover, no apparent difference in olfactory neurogenesis was noticed between the ipsilateral and contralateral side of stimulation.

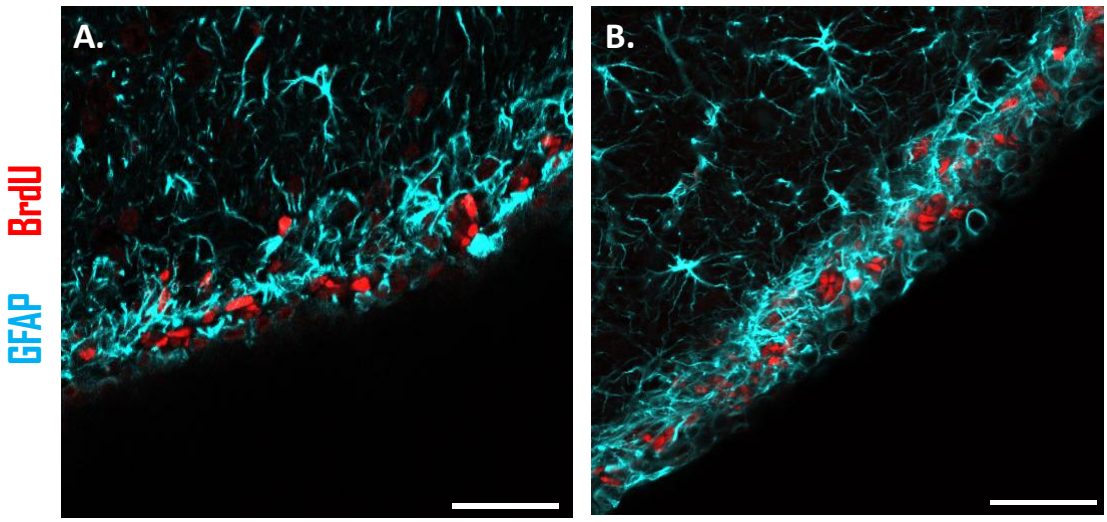

Figure 17. Representation of neurogenesis in SVZ after stimulation.
Immunofluorescence labeling of SVZ by GFAP (Blue) and BrdU (red) was performed on coronal sections from sham (A) and stimulated (B) groups. Images were taken using 40X oil objective. Scale bar = 50 µm.

2. *Neurogenesis in hilar zone*

A population of BrdU+ cells was found repeatedly in the hilar zone as doublets of round cells in both hemispheres (Fig. 18-A). Moreover, BrdU+ cells did not express GFAP (Fig. 18-B) or NeuN (Fig. 18-C). Their morphology and pattern was distinct from that of the SGZ. The doublets were dispersed in the hilar region, had a round shape and were few in numbers.

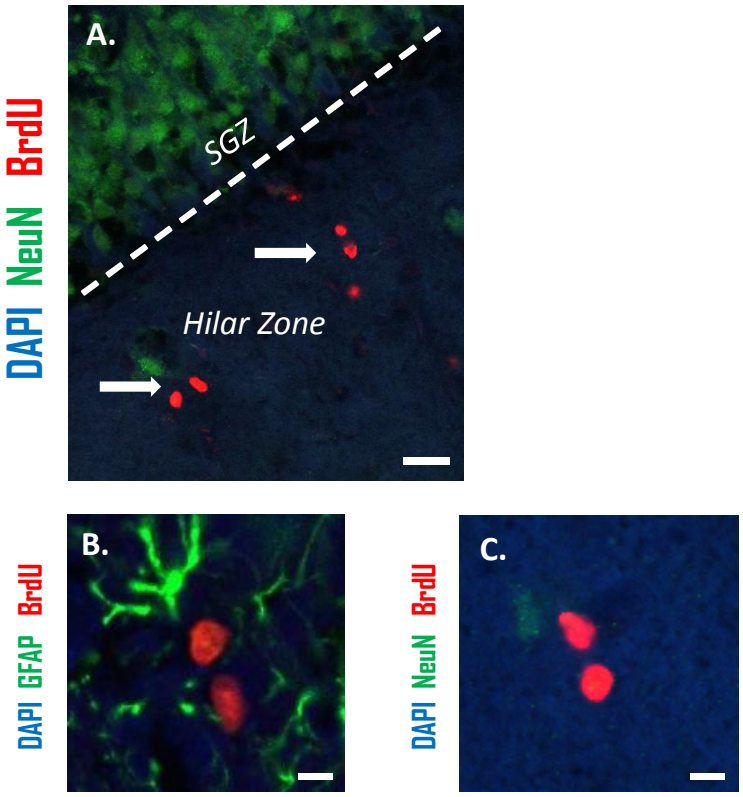

Figure 18. Representation of stimulation-induced effects on neurogenesis in the hilar zone. Immunofluoresence labeling of proliferating cells in hilar zone was done by BrdU (red), shown in the dotted circles (A) as doublets. Proliferating cells were GFAP negative (B) and NeuN negative (C). Images were taken using 40X oil objective. Scale bar = 20µm.

Quantification of BrdU revealed no changes at the level of proliferation rate in the stimulated males and females, in comparison with the sham (Fig.19). Strikingly, the female and male groups had similar mitotic activity in the hilar region, as opposed to the SGZ.

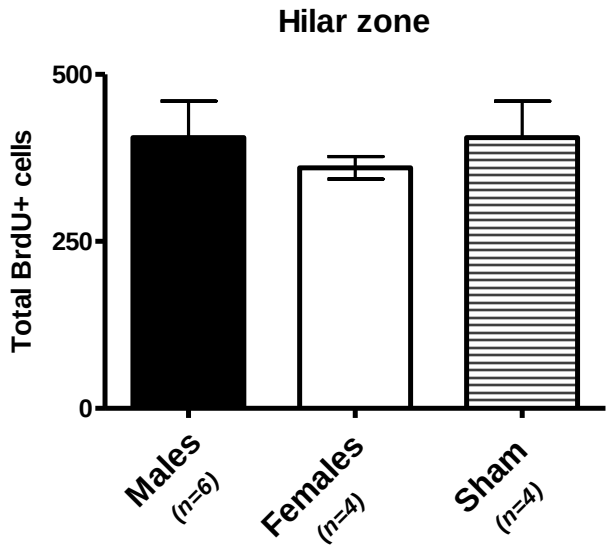

Figure 19. **Quantification of BrdU positive cells in hilar region.**
Each bar in each panel represents the average ± SD of BrdU quantification made on the indicated groups.

D. Effect of the Injection of Low Doses of Kainic Acid in the AMN

A group of female rats (n=3) was subjected to microinjection of 1μl of KA (500pM) stereotaxically placed in the AMN. Stereological counting did not reveal any prominent increases in BrdU levels in the DG of injected rats. No significant differences were found between the ipsilateral and contralateral side as well.

E. Stimulation of VPL Nucleus Does not Alter Baseline DG Neurogenesis

To demonstrate the specificity of the effects of AMN stimulation, a group of male rats (n=2) was subjected to stimulation, using the same parameters and the same type of electrodes, placed in the thalamic ventral posterolateral nucleus. VPL stimulation did not alter the baseline neurogenesis levels, which 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.

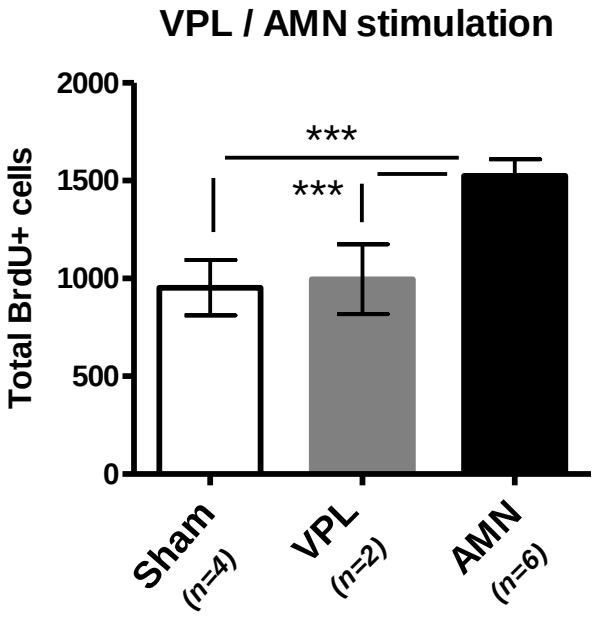

Figure 20. Comparative quantification for the specificity of AMN targeting.
Each bar in each panel represents the average ± SD of BrdU quantification made on the indicated groups. The determination of significance of each value was made between subjects with reference to sham or VPL-stimulated rats. (***p< 0.001).

CHAPTER IV

DISCUSSION

The primary aim of this study was to examine the influence of unilateral thalamic anteromedial nucleus (AMN) stimulation on adult hippocampal neurogenesis in awake and unrestrained rats. The results of the present study can be summarized as follows. First, a basal level of continuous neurogenesis exists in the dentate gyrus (DG) of the hippocampus and a further comparison between genders reports higher levels in males than females. Second, the targeted stimulation of the AMN induced focal neurogenesis in the DG ipsilateral to electrode side that is gender- independent and confined to the caudal extent of the DG. Third, the stimulation-induced effects were translated as an augmented proliferation rate of amplifying neural progenitors (ANP). Fourth, stimulation did not alter baseline neurogenesis in the subventricular zone (SVZ) and hilar regions. Fifth, stimulation of ventral poster-lateral thalamic nucleus (VPL) did not induce significant alteration of basal level neurogenesis. Together, these results suggest that the therapeutic effects of DBS might be attributed, at least partly, to the enhanced neurogenesis in the hippocampus.

Our results on basal neurogenesis levels are in line with previous findings documenting the existence of adult neurogenesis in two neurogenic zones, the SVZ and the DG (Zhoa, 2008). We also found a continuous mitotic activity in the hilar region which is in accordance with early reports describing a process of "Cell genesis" in the hilar zone (Kuhn et al., 1996; McEwen 1996). The superior male basal neurogenesis levels are

supported by early studies on hippocampal formation of rats reporting a gender-dependent variations in the size (Diamond, 1987), neurogenesis rate (Beatty, 1984; Perfilieva, Risedal, Nyberg, Johansson, & Eriksson, 2001) and function of the hippocampus (Beatty, 1984). However, our work shows that this gender-dependent difference is restricted to the DG and does not involve the mitotic activity in the hilar zone.

DBS has been shown to exert pro-neurogenic effects in adult hippocampus after bilateral stimulation of the AMN (Toda et al., 2008). One advantage of our study was using unilateral stimulation, which made the contralateral DG as a within-animal control of baseline neurogenesis. Increased-neurogenesis was restricted to the DG ipsilateral to stimulation. Laterality in DBS-induced effects provided clear cut proof on the effectiveness of DBS in eliciting distinctive and organized response, specific for the side of stimulation. More importantly, our demonstration of enhanced neurogenesis is based on the application of electrical stimulation on unrestrained, awake animals and free of anesthesia. This was done to mimic clinical trials on one hand and to screen for any abnormal reactions during stimulation on the other hand.

While many studies have not sought to compare stimulation-induced neurogenesis between genders, to the best of our knowledge, this is the first study to demonstrate that DBS elicits gender-independent neurogenesis effects post stimulation. Additionally, our results on the increased proliferation rate at the level of ANPs come in parallel with other reports supporting the hypothesis that stimulation-induced neurogenesis is mediated by the augmented symmetric division of ANPs and not the division of QNPs (Encinas et al., 2011b).

The preferential effect of stimulation-induced neurogenesis on the caudal DG area was reported by Stone et. al., (2011) following entohrinal cortex stimulation and not AMN. The heterogeneous proliferation spatial pattern might be related to extensive connections occurring mostly between the caudal DG and the AMN. Further investigation on this phenomenon might extend our knowledge on the functional implications of the anatomical preferences of induced neurogenesis.

Our results on the constant levels of mitotic activity in the SVZ and hilar zone are in line with previous reports that examined the stimulation-induced effects in the SVZ (Encinas et al., 2011b; Stone et al., 2011). In the present work, the area of research was extended to include the hilar zone as well. There was no indication of an elevated ectopic hilar neurogenesis as observed during generalized seizures. These results demonstrate the specificity of DBS in inducing neurogenesis effects in the DG and not hilar zone.

Induced-neurogenesis effects were specific for AMN stimulation and not stimulation of any other thalamic nucleus. Previous studies employed distant sites from the AMN as a control for target specificity, such as frontal association area of the cortex (Encinas et al., 2011b) and cerebellar lobules (Stone et al., 2011). However in the present study, the selection of another thalamic nucleus, VPL, as a negative control provides additional advantage. The VPL thalamic nucleus is spatially far from the AMN, which precludes the hypothesis of current spread after stimulation. The absence of enhanced neurogenesis following VPL stimulation indicates that hippocampal neurogenesis is induced mainly by the stimulation of afferents projecting to the DG.

Adult neurogenesis is a morphogenic, strategic and dynamic process designed to

sustain cellular plasticity for the optimal function of DG with age development. One of the major catalysts fueling our focus on adult hippocampal neurogenesis stems from the indispensible need for restoring, or at least ameliorating the cognitive deleterious implications associated with neurodegenerative diseases. Coupled with this unmet challenge, is our efforts to investigate the role of adult neurogenesis as a cell replacement therapy post injuries or degenerative neurological disorders. Should the technical limitation of investigating neurogenesis in vivo be overcome, much of the human studies will be facilitated and we will gain insight to its robust roles and regenerative potentials.

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