UNIVERSITY OF CALIFORNIA, SAN DIEGO

The role of experience dependent newborn neurons in the adult dentate gyrus of the mammalian hippocampus

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

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The Dissertation of Gregory Dane Clemenson Jr is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

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2012

DEDICATION

This thesis work is dedicated to my Mom, Dad, Sister and Brother for their steadfast support and love.

And to my good friend, Louis Nguyen.

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LIST OF ABBREVIATIONS

BDNF: Brain-derived neurotrophic factor

BrdU: 5-bromo-2-deoxyuridine, a thymidine analog used to visualize dividing cells

CA3: Cornu Ammonis 3 subregion of the hippocampus

CFC: Contextual fear conditioning

cFos: a protein encoded for by the immediate early gene FOS (FBJ murine osteosarcoma viral oncogene)

CldU: 5-chloro-2-deoxyuridine, a thymidine analog used to visualize dividing cells

CON: Control

CR: Conditioned response

CS: Conditioned stimulus

DCX: Double cortin, a protein expressed in immature neurons

DG: Dentate gyrus, subregion of the hippocampus

dnWnt: Dominant negative Wnt, a dominant negative protein used to inhibit Wnt signaling

EDNN: Experience dependent newborn neurons, new neurons whose survival is dependent on an enriched environment exposure

EE: Enriched environment

GABA: γ-Aminobutyric acid, a neurotransmitter

GC: Granule cell

GCL: Granule cell layer

GFP: Green fluorescent protein, often used to label proteins or cells

HCL: Hydrochloric acid

IdU: 5-iodo deoxyuridine, a thymidine analog used to visualize dividing cells

IEG: Immediate early gene, genes transiently expressed in response to a variety of stimuli, often used as a histological marker of neuronal activation

IR: Irradiation, a treatment involving exposure to X-rays that is capable of knocking out neurogenesis

LTP: Long-term potentiation

LV: Lentivirus, used to express a gene or protein of choice into all local cell types

mCFC: Modified contextual fear conditioning paradigm

MWM: Morris water maze, a hippocampus dependent behavioral task

NeuN: Neuronal nuclei, a neuron-specific nuclear antigen used to label mature neurons

NGF: Nerve growth factor

NMDA: N-methyl-D-aspartic acid, an amino acid derivative agonist of NMDA receptors

NPC: Neural precursor cell

NSC: Neural stem cell

peCFC: Pre-exposure contextual fear conditioning

PFA: Paraformaldehyde

SGZ: Sub granular zone

SVZ: Sub ventricular zone

US: Unconditioned stimulus

VEGF: Vascular endothelial growth factor, subfamily of platelet-derived growth factor family and signaling protein involved in angiogenesis

Wnt: A family of signaling proteins involved in embryonic and adult neurogenesis

XdU: A combination of both IdU and CldU

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ABSTRACT OF THE DISSERTATION

The role of experience dependent newborn neurons in the adult dentate gyrus of the mammalian hippocampus

by

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Doctor of Philosophy in Biology

Professor Fred Gage, Chair

This work explores the functional role of experience dependent newborn neurons (EDNN) in the dentate gyrus of the adult mammalian hippocampus. Adult hippocampl neurogenesis is the generation of newborn neurons that occurs throughout life in mammals. EDNNs are a specific population of newborn neurons whose survival is dependent on an exposure to an enriched environment, suggesting an intimate connection between these EDNNs and the enriched environment. To probe the functional role of these EDNNs, I have employed a combination of immunohistochemistry, lentivirus mediated-knockdown, multiple enriched environments, and two commonly used hippocampus dependent behavioral tasks; the Morris watermaze and contextual fear conditioning. With the use of these methods, the work presented in this thesis provides evidence for 3 distinct functional role of EDNNs. First, EDNNs appear to play a role in the long-term memory retention of the Morris watermaze. Using a lentivirus mediated knockdown of adult neurogenesis,

animals devoid of EDNNs did not exhibit a preference for the target quadrant at 1 week after training, suggesting a deficit in long-term memory. Second, EDNNs encode information specifically about previously experienced environments such that a re-exposure of the environment elicits a response by the EDNNs. By labeling two populations of EDNNs and exposing animals to two distinct enriched environments, we found that EDNNs encode information about the environment they experienced during an early critical period. Third, EDNNs are potentially involved in the ability of animals to decode their surrounding environment. When subjected to a more difficult, contextual fear conditioning paradigm, where animals are given extremely small amounts of time to explore the context, enriched animals are able to discriminate between two different contexts better than controls. This phenotype suggests that enriched animals are better at deciphering their surroundings than control animal, indicating a possible relationship between EDNNs and the ability of animals to decode their environment. The work presented in this thesis provide evidence for a functional role of EDNNs in how they benefit the animal through its ability to remember old experiences and encode new and novel ones.

CHAPTER 1: Introduction

1.1 Introduction to neurogenesis

Neural stem cells (NSCs) proliferate and differentiate into all cell types of the nervous system and are unique in their unlimited self-renewal and multi-lineage potential. NSCs generate neural progenitor cells (NPCs), which are limited in proliferation and differentiate into neurons or glia [1]. Newborn neurons migrate to their target location, extend their dendrites and axons, and integrate into the local circuit as neurons communicate with one another via synapses to function efficiently and effectively. From the development of the nervous system and for the entire lifespan of the individual, neurons are continuously undergoing synaptic plasticity and remodeling as they extend and withdraw their processes, forming new and severing old targets.

Neurogenesis, the generation of new neurons, is a key contributor to synaptic plasticity in the adult brain. While NPCs exist in many regions throughout the adult brain [2], they produce new neurons in only two specific regions of the adult brain under physiological conditions: the subventricular zone (SVZ) lining the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus. NPCs in the SVZ supply new neurons to the olfactory bulb, while

neurons born in the SGZ integrate into the existing hippocampal circuit. The extent to which newborn neurons contribute to the functions of the olfactory bulb and hippocampus remain controversial [3, 4], but newborn neurons confer synaptic plasticity to two regions involved in learning and memory.

1.2 Integration and maturation of newborn neurons

Newborn neurons in the adult DG undergo a maturation process similar to those generated during development and eventually integrate into the existing circuit of the hippocampus [5-9]. This process begins with the initial proliferation of NPCs in the SGZ, which are found clustered among blood vessels, indicative of an intimate relationship between the vasculature and neurogenesis [10]. In the first 2 weeks, new cells migrate from the SGZ and into the granule cell layer where they begin to extend their axons towards the CA3 pyramidal cells of the hippocampus [8, 11, 12]. At 2-4 weeks of age, the morphological appearance of these immature neurons begin to resemble that of their neighboring mature neurons as they now display elaborate dendritic arborizations and have developed dendritic spines [8]. A striking difference between the newly differentiated and mature neurons remains in their physiological properties, specifically in connection with long-term potentiation (LTP) and GABAergic inputs. LTP refers to the enhanced postsynaptic potential following a brief pulse of synaptic activation and is thought to be an integral component of learning and memory. Newborn immature neurons exhibit a lower threshold for LTP,

which may be important for both integration into the local network and contributing to hippocampal functions such as learning and memory [13, 14]. GABA is typically known as an inhibitory neurotransmitter for neurons; however, immature neurons initially receive excitatory GABAergic synaptic inputs from local interneurons, which eventually become inhibitory synaptic inputs as the neurons mature. During this time, the neurons begin to receive excitatory glutamatergic inputs from the entorhinal cortex [5, 6]. At 4-6 weeks of age, the newborn neurons are characterized by a critical period of synaptic plasticity, similar to that found during development, with enhanced excitability, larger LTP amplitudes, and reduced induction thresholds [13]. Eventually, the new neurons adopt the same morphological and physiological characteristics to that of the pre-existing neurons and contribute to hippocampal functions.

1.3 Function of the hippocampus and more specifically, the DG

It is generally accepted that the hippocampus plays a crucial role in spatial reference learning and memory. With the use of hippocampal lesions and complete removal of the hippocampus, hippocampus-dependent behavioral tasks have been designed to test this function [15, 16]. The Morris Water Maze (MWM) is the most widely used and is considered a standard for hippocampus-dependent behavior. In this spatial navigation task, the animal is placed in a circular pool and trained to swim to a hidden platform just below the surface of the water using spatial cues placed around

the room. Latency to reach the platform is used as a measure of spatial learning. In general, once the animal is successfully trained to find the hidden platform, the platform is removed from the pool and probe trials are used to assess the animal's ability to remember the spatial location of the platform. During the probe trial, the animal is allowed to swim freely for a set period of time. If the animal has learned the task using a spatial strategy (relying on the hippocampus), the animal spends the majority of its time swimming in the quadrant, which contained the previously hidden platform. Time spent in the target quadrant during the probe trial is indicative of spatial memory. Animals without an intact hippocampus are unable to learn the location of the platform [15]. If the hippocampus is inactivated after training, the animals exhibit impaired spatial memory.

In addition to MWM, contextual fear conditioning (CFC) is commonly used as a secondary complement to MWM. While both tasks are considered hippocampus-dependent, CFC uses different motor and sensory components and allows for another method to independently test hippocampal function. The CFC test is based on Pavlovian conditioning, where a conditioned stimulus (CS) becomes associated with an unconditioned stimulus (US). The level of association learning can be measured by the conditioned response (CR) in response to presentation of the CS only. In the CFC task, animals must make an association between an aversive experience (foot shock - US) and a spatial context (CS). Briefly, the animal is placed in a box (context 1) and administered a foot shock. Following the experience, the animal is either placed again in context 1 or into a new context 2 to determine whether the animal makes an

association between the foot shock and the specific spatial context. Memory, in this case, is measured by the animal's natural response of freezing (CR), which only occurs in context 1 if the animal has learned the task appropriately. Probe trials can be administered to test for retention of the contextual memory.

Animals may vary quite a bit in their response and ability to discriminate in contextual fear conditioning, many features can be manipulated in the CFC paradigm, including the presentation of tones, number of trials, and timing of retention trials, to influence behavioral performance.

Based on the hippocampus-dependent nature of both the spatial learning and memory aspects of the MWM and CFC, studies investigating the functional role of hippocampal neurogenesis have utilized these behavioral paradigms to determine if the absence of neurogenesis negatively impacts spatial learning and memory [22, 34, 37]. To test this, these adult born neurons need to be specifically targeted and inhibited. Many approaches have been used to specifically ablate adult neurogenesis, however, they all vary in regards to their effectiveness and selectivity [41, 51]. One common and widely used technique is focal X-irradiation, involving several low-dose treatments spread throughout multiple days [51]. Although the effectiveness of irradiation in blocking adult neurogenesis is high, results have been mixed. Some studies show deficits in spatial memory and CFC whereas others have not. This variability that is observed may be attributed to the specific paradigms used in each behavioral task, but also to possible off target effects of irradiation. In order to be sure that adult born neurons are specifically targeted without affecting the integrity of the

surrounding cells, we have used a lentivirus expressing the dominant negative form of the WNT protein. WNT has been shown to be critical for neuronal proliferation and maturation, and by locally injecting this lentivirus into the DG, dividing neural progenitor cells can be specifically targeted and inhibited without affecting the local granule cell populations [41, 48]. Using this method to knockdown neurogenesis in rats, it was shown that these rats have deficits in spatial memory of MWM, correlating with viral coverage in the DG [41].

Although CFC and MWM are standard hippocampus-dependent behavioral tasks and have proven useful in many ways, there is still a need for more sensitive tasks. While it is clear that neurogenesis persists in the hippocampus and these newborn neurons functionally integrate themselves into the existing hippocampal circuitry, the contribution that these newborn neurons make to the overall function of the hippocampus is still unclear. Although a few studies did not find a correlative relationship between neurogenesis levels and hippocampal dependent behavior, the majority of studies have reported a positive correlation [17]. Many of the discrepancies seen with neurogenesis levels and hippocampus-dependent behavior could be attributed to slight variations in each of the studies (strain, species, side-effects of manipulations, age, etc…) but perhaps we simply need more sensitive tasks. Neurogenesis is restricted to the DG and therefore it is highly likely that the contribution of newborn neurons is more crucial to DG function specifically. Aside from spatial learning and memory, the hippocampus, and primarily the DG, has also been implicated in spatial pattern separation [18, 19]. Spatial pattern separation refers

to the ability to discriminate between two closely related but distinct pieces of spatial information. Recently, studies have suggested that as two pieces of spatial information become more similar, neurogenesis becomes necessary to distinguish between the two. If the two pieces of spatial information are relatively different, even animals without neurogenesis can distinguish between the two [20, 21]. To test spatial pattern separation, a recent study has developed a novel touch screen task that appears to be very sensitive to changes in neurogenesis [20].

One of the main sources of variability in behavioral tests is the investigator who handles the animals and administers the tests. For example, in the MWM, investigators differ in the methods of introducing the mouse into the water, how they guide the mouse to the platform during training, and how the mice are handled after each trial. These tiny manipulations have a tremendous impact on the behavioral performance of the animals. One of the benefits to using mouse touchscreen operant chambers is that each individual trial is run by the mouse and therefore eliminates any variability introduced by the investigator. Each mouse touchscreen chamber is outfitted with an infrared touchscreen, a reward dispenser (either pellet or liquid) with light illumination to let the animal know a reward has been given, and head entry detectors so the animals can activate the next trial.

A spatial pattern separation task using mouse touch screens has been shown to be especially sensitive to changes in neurogenesis levels [20]. The two-choice spatial discrimination task is designed to test the spatial discrimination ability of the mouse. Briefly, mice are presented with 2 illluminated boxes during each trial. Mice are

trained to nose-poke one of the boxes, which is fixed in its spatial location until a certain criteria was met. After criterion is met, reversal trials are performed, and mice are now rewarded only when choosing the other box. By manipulating the degree of separation between the two boxes, this task becomes sensitive to neurogenesis levels. If the boxes are spaced farther apart (high separation), mice with or without neurogenesis perform equally well on this task. However, at low degrees of separation, where the boxes are spaced in close proximity to each other, mice without neurogenesis are severely impaired in discriminating between the two spatial locations. This study not only highlights the advantages to using the touchscreens, but also provides evidence for a task that is highly sensitive to neurogenesis levels.

1.4 Effects of running and enrichment on neurogenesis

Neurogenesis is a dynamic process and is regulated by many factors. Manipulations of the environment, such as enrichment and exercise, have been shown repeatedly to increase neurogenesis [22, 23]. Environmental enrichment can be defined as an environment that enhances cognitive, sensory, social, and motor stimulation. In many cases, this includes an enlarged housing cage consisting of toys, tunnels, larger social groups (allowing for more social interaction), and, in some cases, running wheels. Previously, most enriched environments included running wheels for motor stimulation; however, it was not until one study identified voluntary exercise as one of the most salient components of environmental enrichment that the effects of

each were investigated separately [24]. Both voluntary exercise and environmental enrichment enhance adult hippocampal neurogenesis albeit through different mechanisms. Voluntary exercise increases cell proliferation in the DG, leading to an overall total increase in the number of newborn neurons, whereas environmental enrichment promotes the survival of newborn neurons. Both environmental manipulations are consistent with increases in trophic factors, such as BDNF and nerve growth factor (NGF), as well as increases in spine density (see [25] for review), which could partially explain the observed increase in neurogenesis and enhanced learning and memory. The reasons for why each manipulation acts differently to increase neurogenesis is still unclear, but one clear difference between voluntary exercise and environmental enrichment is the rigorous physical activity animals undertake when allowed access to a running wheel [26]. This increase in motor activity is associated with changes in the vasculature of not only the brain but also the periphery, resulting in increased blood flow and BBB permeability. This change in both brain and peripheral vasculature could help increase the circulation of pro-neurogenic factors such as VEGF to other areas of the brain [27].

The effects of voluntary exercise are well established and well documented, however, there is still debate over enrichment and its affect on adult neurogenesis and hippocampal function. Studies have shown a positive effect of enrichment on learning and memory of spatial or object dependent information, and may even prevent cognitive declines associated with aging [22, 34, 37]. Although this enhancement in spatial hippocampal dependent tasks is associated with an increase in neurogenesis,

one study separating enrichment and running claims that the increase of neurogenesis and the neurotropic factor BDNF can be attributed mainly to running and not to enrichment [46]. Animals that received running alone or enrichment in conjunction with running resulted in significant increases in both cell proliferation and cell survival, however, no significant effects of enrichment were observed. Using irradiation as a method to almost completely knockout adult hippocampal neurgenesis, one study reports that the beneficial effects of enrichment on spatial learning and memory is independent of neurogenesis [51]. Enriched irradiated mice performed just as well as control enriched sham mice in MWM. Both irradiated and sham treated mice that received enrichment performed better than irradiated and sham treated controls. In this particular study, however, the MWM paradigm used does not appear to be sensitive to neurogenesis due to the fact that irradiated control animals do not exhibit deficits in the task when compared to sham controls.

Although multiple studies report conflicting results on the effects of enriched environment, it is possible that these inconsistencies are due to the way the enriched environment is setup. These environments vary in many ways including size, types and number of toys and running wheels, however, it is unclear which features of the environment or most salient to the mice.

1.5 Activity dependent survival of newborn neurons

Survival of newborn neurons is promoted by experiences such as environmental enrichment, suggesting that their survival may be regulated in an experience-dependent manner [55, 28]. As theses newborn neurons mature, they experience a critical period of their survival that is dependent on NMDA activation and is susceptible to the effects of environmental enrichment [28, 57]. The selective experience-dependent survival of these newborn neuron suggest that they may play a direct role in learning and memory processes. To determine if specific neural networks are involved in particular learning and memory processes or unique experiences the animals have, immediate early genes have been used in conjunction with behavior or enrichment, as a marker of neuronal activation [43, 57]. Immediate early genes are genes that are transiently activated in response to a variety of stimuli and are commonly used as a histological marker of neuronal activation. By exposing animals to a short experience, for example a probe trial of a watermaze or a short exposure to an enriched environment, and then sacrificing the animal immediately, you can essentially "catch" neurons that are presumably responding to the last experience they had. This is a transient effect, however, and will only last for a couple hours.

Newborn cells were labeled with BrdU and different groups were trained on the MWM at different stages during the maturation of these BrdU cells in order to determine what the age of neurons that are involved in MWM learning [43]. Recruitment was determined by co-localization of BrdU with the immediate early gene marker, cFos. Neurons that were 4-6 weeks old during MWM training showed

the greatest amount of activated neurons (BrdU+/cFos+) in response to a probe trial, suggesting that new neurons 4-6 weeks of age are preferentially incorporated and involved in MWM learning.

1.6 Experience dependent newborn neurons

Newborn neurons go through an extended maturation process, however there is evidence to suggest that these newborn neurons also experience a critical period during their development where they are most susceptible to experiences such as enrichment [57]. In this study, 4 groups of animals all received BrdU injections to label a newborn cell population. Each group was then exposed to an enriched environment in staggered 1 week periods, in order to test the effects of enriched environment when it is presented during different 1 week stages of their maturation. Newborn neurons that experience an enriched environment 1-2 weeks after birth, correlating with the hyper excitability of immature neurons, showed the greatest number of surviving BrdU when compared to the group that received enrichment immediately after birth (0-1 weeks) and at later stages of maturation (2-3 week and 3-4 weeks). At the end of the experiment, each group received a re-exposure to the enriched environment and immediate early genes (genes that are transiently expressed in response to a variety of stimuli) were used to identify neurons that responded to the re-exposure of the previously experienced enriched environment. Newborn neurons that experienced the enriched environment during the 1-2 week "critical period"

responded more to a re-exposure of the same environment than when the enrichment was presented at other times during maturation. These newborn neurons that respond to the previously experience environment and whose survival is dependent upon the enriched environment experience are what I will refer to as experience dependent newborn neurons (EDNNs).

1.7 A computational model of the function of adult neurogenesis

Supporting the results of the previously described experiment, Aimone et al took a computational approach to modeling the function of adult neurogenesis [29]. Using a bottom up approach and detailed characteristics about neurogenesis and the DG from the literature, they created a DG network with multiple layers (populations of cells) including a neurogenic granule cell layer. After "growing" this virtual DG to full size (mimicking the development of the GC), the DG was exposed to 5 different virtual environments for 40 days each in order to simulate what an animal's DG may experience if it were to live in 5 different enriched environments. At the end of the experiment the DG was re-exposed to each of the 5 environments. Interestingly, distinct populations of neurons responded preferentially to each re-exposure of past environments and the neurons that responded to the re-exposure, experienced that environment during their early maturation. Because of the change in excitability of the immature neurons and their preferential response to experiences occurring during the stages of hyper excitability, this allows the DG to encode experiences in a time-

dependent manner as well as respond to familiar experiences differently than novel ones.

My PhD work has focused on EDNNs and the possible roles they play in relation to hippocampal function. EDNNs are dependent on the experience they receive from the enriched environment and the fact that their survival is actively being promoted, suggests that their involvement is crucial to the animal, either immediately or in the future. Although it is unclear exactly what functional role these EDNNs are performing, it is evident that they have a unique connection to environmental experiences, perhaps either in the spatial aspects of living in a much more enriched space or specific features unique to each environment the animal experiences. Each of my 3 aims explores a unique function these EDNNs may participate in: Aim 1 explores the participation of these EDNNs in a hippocampus dependent learning/memory task, the MWM, aim 2 explores the ability of these EDNNs to encode information about previously experienced enriched environments, aim 3 explores a more unique role of these EDNNs in the deciphering of their surrounding environment.

CHAPTER 2: EDNNs role in hippocampal dependent learning and memory

2.1 Introduction

Although it is generally accepted that the hippocampus is involved in the processing of spatial information, it remains debatable how neurogenesis plays a role. Numerous methods for knocking down neurogenesis and increasing neurogenesis have been used to determine the specific role it plays in hippocampus dependent tasks, however, the results have been somewhat inconclusive. Environmental enrichment has been shown to promote the survival of newborn neurons, presumably because these newborn neurons are helpful to the animal in the spatial context of the environment. We hypothesized that promoting the survival of these newborn neurons, which would play a more general role in the overall function of the hippocampus, would enhance their ability to perform in hippocampus dependent tasks. In this experiment, a lentivirus expressing dominant negative WNT, which has previously been shown to effectively knockdown neurogenesis [42, 49], was utilized in conjunction with enrichment to specifically knockdown the EDNN population and determine their contribution to hippocampal function.

2.2 Materials and Methods

Animals and lentivirus injection

All animal procedures were performed in accordance with animal guidelines at The Salk Institute for Biological Studies. A total of 40 female C57/Bl6 mice (Harlan, San Diego) 7-8 weeks old were used. Animals were separated into 4 experimental groups: 1 control groups (n=10) injected with the control lentivirus GFP (GFP), 1 enrichment control group (n=10) injected with the control lentivirus GFP and enrichment (GFPEE), 1 experimental group (n=10) injected with the dominant negative Wnt lentivirus (dnWnt), and 1 experimental enrichment group (n=10) injected with the dominant negative Wnt lentivirus (dnWnt EE). Briefly, animals were anesthesized deeply with a ketamine/xylazine cocktail and placed into a stereotaxic apparatus. The dentate gyrus of the hippocampus was targeted using the coordinates (from bregma): -2 (a/p), ± 1.5 (m/l), -2.3 (d/v). A total of 1.5ul of virus was injected into each hemisphere. 3 weeks after lentivirus injections, animals were injected interperitoneally with 4 injections (over 2 days) of 50mg/kg of bromodeoxyuridine (BrdU).

Lentivirus vectors and virus preparation

We used lentiviral vectors previously described (Lie et al. 2005) that are CMV-driven dnWnt followed by an internal ribosomal entry site (ires) GFP. The control vector CSC.cPPT.hCMV.eGFP.Wpre

Immunohistochemistry

Mice were anesthetized with a ketamine/xylazine mixture and transcardially perfused with 0.9% NaCl solution followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. Brains were postfixed overnight in 4% PFA at 4^0C and then transferred into 30% sucrose solution. 40um coronal sections were cut on a sliding microtome and all immunohistochemistry was performed on free-floating sections. Tissue from animals was first incubated with antibodies against neuronal nuclei (NeuN) for 48 h at 4^0C followed by incubation with secondary antibodies, post-fixed (4 % PFA, 20 min at room temperature), and then treated with 1M HCl for BrdU detection followed by incubation with an antibody against BrdU. Primary antibodies used were rat anti-BrdU (1:500; Accurate), mouse anti-NeuN (1:10, Bobbi's Clone A60), and rabbit anti-GFP (1:250; Invitrogen, Carlsbad, CA). The total number of BrdU-positive cells was quantified using every 6th section (240 μm apart for young animals). BrdU-labeled cells were counted throughout the rostrocaudal extent of the granule cell layer (GCL) using a 20 x objective and an upright epifluorescence microscope (E800; Nikon, Tokyo, Japan). The co-labeling of BrdU-positive cells with NeuN was analyzed using a confocal microscope (Radiance 2100; Bio-Rad, Hercules, CA). All analyses were performed in sequential scanning mode, and double labeling was confirmed by 3-dimensional reconstructions of z-series. Per experimental group, 50 BrdU-positive cells were randomly picked throughout the GCL and analyzed for NeuN co-labeling.

Environmental enrichment

1 week after animals had been injected with BrdU, two groups (CON EE and dnWnt EE) were placed into 2 separate enriched environments. Enrichment cages were custom made with plexi-glass, 36in x 36in x 12in, and contained food, water, transparent plastic tunnels and igloos. Enriched animals lived in the enriched environments during the enrichment period. After 2 weeks of enrichment, enriched animals were placed back into standard mouse housing.

Behavioral testing

1 week after enrichment (or standard housing for controls), animals were tested in the Morris Water Maze. Briefly, animals were trained to find a hidden underwater platform in a pool of water for 10 days, 2 trials/day. At 24 hours and 1 week after the last trial, the platform was removed from the pool and mice were allowed to swim in the pool for 60 seconds. Time spent in each of the 4 quadrants was recorded.

2.3 Results

Exposure to an enriched environment increases neurogenesis and injection with a dnWnt lentivirus leads to a reduction in neurogenesis

In total, 4 groups were used in this experiment. Two control groups were injected with a lentivirus GFP, one received enrichment and the other without

enrichment (GFPEE and GFP), and two experimental groups injected with a lentivirus dnWnt, one received enrichment and the other without (dnWntEE and dnWnt). 3 weeks after receiving the GFP or dnWnt virus, animals were injected with BrdU to label a newborn population of cells. After enrichment, animals were placed back into standard housing and subjected to Morris Water Maze testing (fig. 2.1). Animals that received the LV-dnWnt injections showed fewer DCX (an immature neuronal marker) cells than their LV-GFP injected counterpart controls (fig. 2.2a, 2.2b). Exposure to an enrichment environment partially restored the loss in DCX (dnWnt EE) numbers (~%80 of controls). As expected, exposure to an enriched environment promoted the survival of newborn cells as demonstrated by BrdU numbers (fig. 2.2c). The enrichment control group (GFP EE) had significantly more BrdU surviving cells than all other groups. Although enrichment partially enhanced the number of BrdU cells in the knockdown group (dnWnt EE), it was not to the same extent as the control enriched group (GFP EE).

EDNNs enhance learning ability in the Morris Water Maze

Although all groups demonstrated the capacity to learn the location of the hidden underwater platform in the MWM, the control enrichment group (GFP EE) showed an enhancement in learning (fig. 2.3a). The control enrichment group took significantly less time to reach the hidden platform over the 10 days of training than the remaining 3 groups. As another measure of learning ability, the control

enrichment group exhibited a shorter path length to get to the platform, indicating that they used a more direct route to the platform than the other 3 groups (fig. 2.3b). Although enrichment of the knockdown group (dnWntEE) exhibited a trend towards slight improvement in the speed at which the dnWnt group was able to learn the task, it was not significantly different.

EDNNs are required for long-term memory retention

Both 24 hours and 1 week after the last training trial, animals were subjected to probe trials to measure their short and long-term memory retention for the hidden platform. 24 hours after the last trial, all groups with the exception of the knockdown group (dnWnt) showed a preference for the target quadrant (fig. 2.4a). In other words, these 3 groups spent significantly more time exploring the quadrant that contained the hidden platform than the other 3 quadrants. The knockdown group (dnWnt) was the only group that did not show a preference for any of the 4 quadrants consistent with the idea that neurogenesis is necessary for memory retention of the MWM. 1 week after the last trial, only the control enrichment group (GFP EE) showed a preference for the target quadrant suggesting that enrichment and EDNNs are involved in the long-term memory retention of the MWM (fig. 2.4b).

2.4 Discussion

In this study, we were determined to elucidate the functional role of experience dependent newborn neurons in hippocampus dependent behavior. Experience dependent newborn neurons are a population of newborn neurons whose survival is dependent on the enrichment experience and it is likely that they play a key role in the function of the hippocampus because their survival is actively being promoted by the enrichment experience. To test the role of this specific population of EDNNs, we used a LV-dnWnt, which efficiently knocked down neurogenesis. Animals that received a combination of lentivirus and housing conditions were tested in the MWM, in which animals were trained to locate a submerged platform in a water maze using external-maze cues. Only the control enriched group showed enhancement during the training/learning phase of the MWM. When measuring latency to platform as well as distance traveled during trials, enriched control groups (GFPEE) were significantly faster than all other groups, suggesting that EDNNs are involved in enhanced learning of MWM. Memory was tested using probe trials where the underwater platform was removed and mice were allowed to swim in the pool for a set amount of time. Performance in memory retention was assessed using time spent in the target quadrant (quadrant that contained the platform during training). These GFPEE animals were the only group able to successfully show a preference for the target quadrant 1 week after the last training trial, suggesting that EDNNs contribute to long-term memory retention.

One interesting result from this experiment is when we enriched the knockdown group (dnWntEE). The dnWnt and dnWntEE group showed no differences in the acquisition of the MWM task, however, enrichment seemed to rescue the short-term memory deficit observed 24 hours after the last training trial. This may suggest the possibility of non-neurogenesis dependent effect of enrichment. Interestingly, this phenomenon has previously been observed by [51]. In this study, neurogenesis in mice was blocked using x-focal irradiation, which has been shown previously to almost completely knockout neurogenesis, placed in an enriched environment and subsequently subjected the animals to MWM. Although the experimental paradigm is different from the one we performed in this current study, they also found neurogenesis independent effects of environmental enrichment, consistent with the results of our study. 24 hours after the last trial of MWM, irradiated enriched animals showed a preference for the target quadrant same as the sham treated control animals, indicating that neurogenesis is not required for the short-term retention of the MWM task.

Although the irradiation study only performed a short-term measure of memory retention, it appears that EDNNs may play a more distinctive role in long-term memory. Exposure to the enriched environment promotes the survival of newborn neurons and these newborn neurons participate in the memory retention of the MWM.

Chapter 2, is currently being prepared for submission for publication. Clemenson GD, Gage FH. The dissertation author was the primary investigator and author of this material.

2.5 Figures

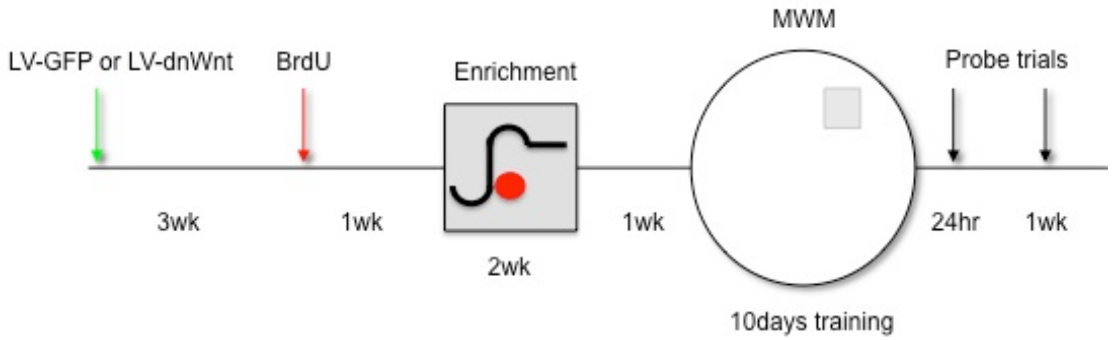

Figure 2.1
Paradigm of experiment

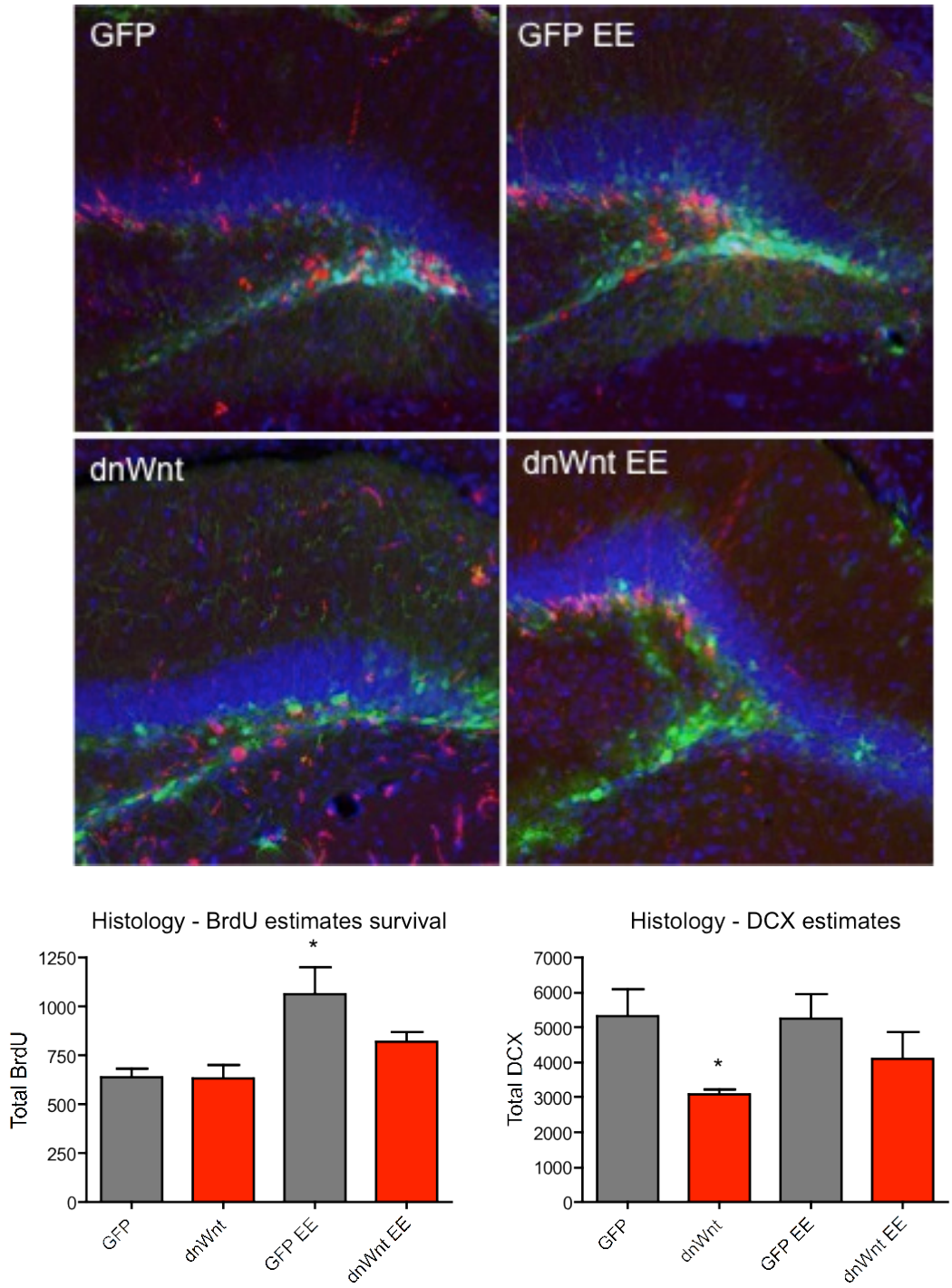

Figure 2.2
a) Example of histology from each of the 4 groups, stained with GFP, DCX, and NeuN
b) Total estimated DCX quantifications from all 4 groups
c) Total estimated surviving BrdU cells from all 4 groups
*$p < 0.05$

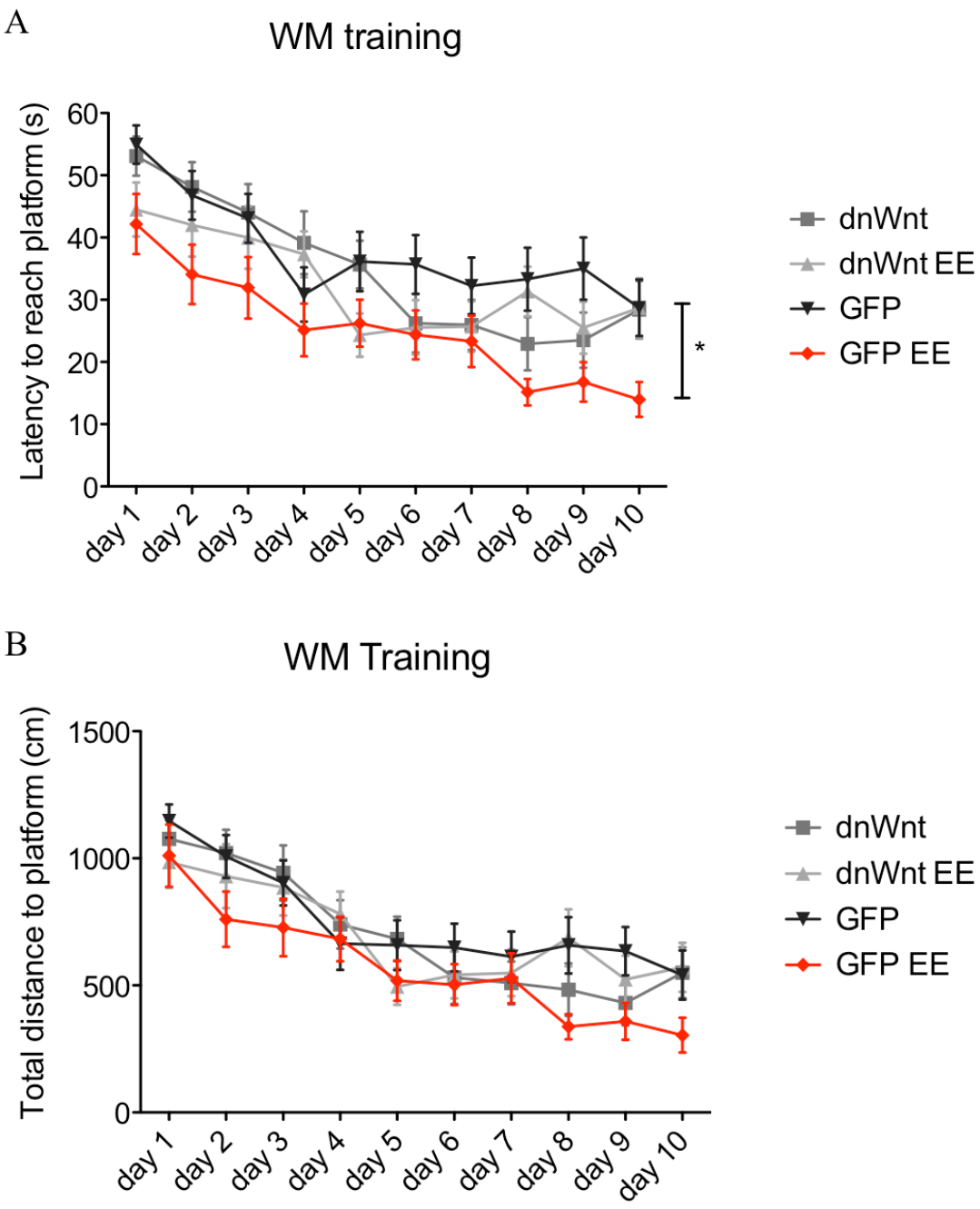

Figure 2.3
a) Latency to platform training trials of MWM by all 4 groups
b) Distance to platform training trials of MWM by all 4 groups
*p <0.05

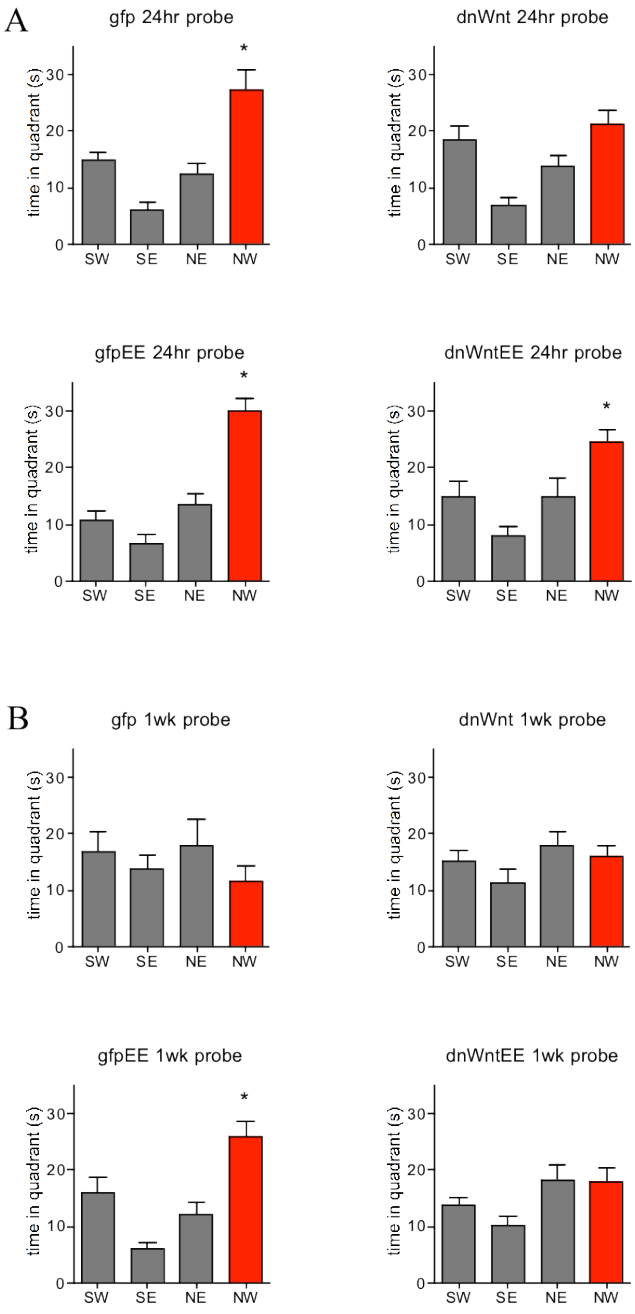

Figure 2.4
a) 24 hour probe trial of 4 groups. Highlighted bar is the amount of time spent in the target quadrant.
b) 1 week probe trial of 4 groups. Highlighted bar is the amount of time spent in the target quadrant.
*$p < 0.05$

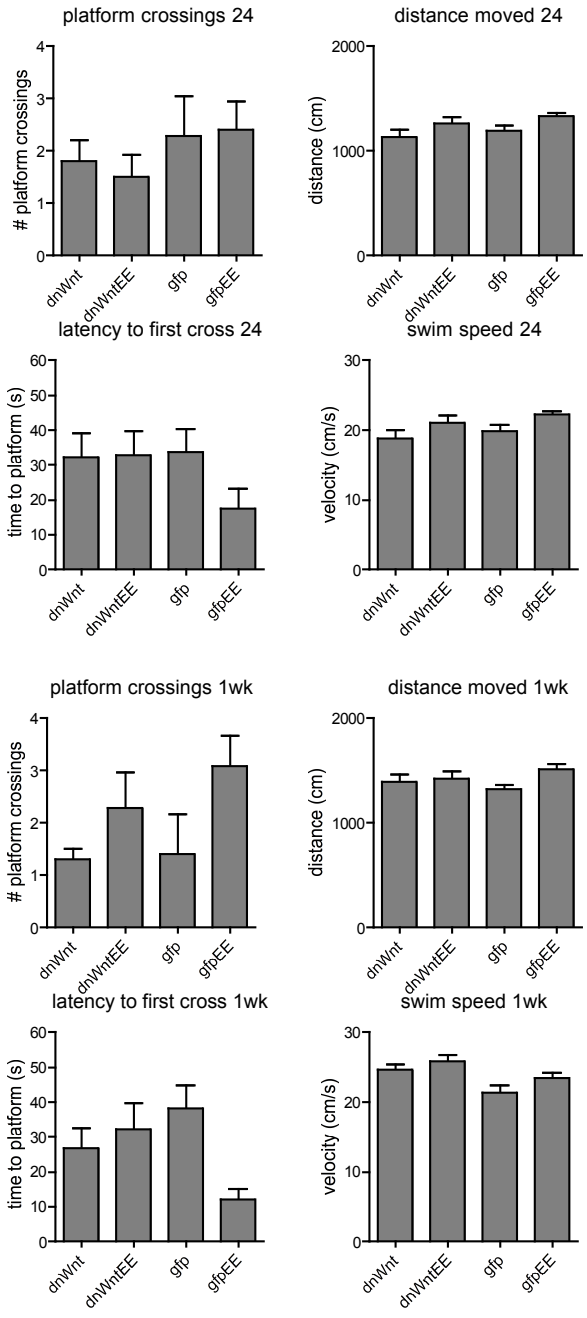

Figure 2.5
a) 24 hour probe trial data, platform crossings, distance moved, latency to first cross, and swim speed.
b) 1 week probe trial data, platform crossings, distance moved, latency to first cross, and swim speed.

CHAPTER 3: EDNNs role in the encoding of previously experienced environments

3.1 Introduction

The study by Tashiro et al [57] brought forth two interesting points about newborn neurons and environmental enrichment; 1) Newborn neurons experience a "critical period" during their maturation (when the newborn neurons are 1 week old) such that they are most susceptible to the survival effects of enriched environment, 2) newborn neurons that experience the enriched environment during the "critical period" respond specifically to a re-exposure of the same, previously experienced enriched environment. These two points hint at an interesting role newborn neurons may play in relation to past experiences the animal has. Aimone et al [29] suggests that because of the hyper excitability that these newborn neurons experience (correlating with the "critical period") during their maturation, these newborn neurons learn something about the experience creating a temporal scale of past events and experiences that can be recalled at a later point in time. In other words, the continuous process of neurogenesis and creation of new populations of neurons, encode current events and experiences at a particular time point correlating with the "critical period" of these newborn neurons. To test the hypothesis that newborn neurons encode information about past environments and experiences animals have, we designed an experiment that would label two distinct cell populations within the same animal and give this

animal two distinct enriched environment exposures, each exposure timed with the "critical period" of either labeled population of neurons. At the end of the experiment, we will re-expose each animal to either of the two environments and use immediate early genes as a method for determining which cell population responds to a re-exposure of which environment.

3.2 Materials and Methods

Animals

All animal procedures were performed in accordance with animal guidelines at The Salk Institute for Biological Studies. A total of 110 female C57/Bl6 mice (Harlan, San Diego) 7-8 weeks old were used for 4 separate experiments. All animals received a total of 8 interperitoneal injections of both iodo-deoxyuridine (IdU) or chlorodeoxyuridine (CldU) equivalent to a concentration of 50mg/kg of BrdU

Immunohistochemistry

Mice were anesthetized with a ketamine/xylazine mixture and transcardially perfused with 0.9% NaCl solution followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. Brains were postfixed overnight in 4% PFA at 4^0C and then transferred into 30% sucrose solution. 40um coronal sections were cut on a sliding

microtome and all immunohistochemistry was performed on free-floating sections. Tissue from animals was first incubated with antibodies against neuronal nuclei (NeuN) or cFos for 48 hours at 4^0C followed by incubation with secondary antibodies, post-fixed (4 % PFA, 20 min at room temperature), and then treated with 1M HCl, for both IdU and CldU detection, followed by incubation with an antibody against IdU and CldU. Because of the strong reactivity of the antibody used against CldU, all tissue was first stained for IdU followed by a postfix of 4% PFA prior to CldU staining. Primary antibodies used were rat anti-CldU (1:500; Accurate), mouse anti-IdU (1:100, BD), mouse anti-NeuN (1:10; Bobbi's Clone A60), and rabbit anti-cFos (1:1000, Calbiochem). The total number of IdU/CldU-positive cells was counted using every 6th section (240 μm apart for young animals). IdU/CldU-labeled cells were counted throughout the rostrocaudal extent of the granule cell layer (GCL) using a 20 x objective and an upright epifluorescence microscope (E800; Nikon, Tokyo, Japan). The co-labeling of IdU/CldU-positive cells with either NeuN or cFos was analyzed using a confocal microscope (Radiance 2100; Bio-Rad, Hercules, CA) and a 40x oil objective. All analyses were performed in sequential scanning mode, and double labeling was confirmed by 3-dimensional reconstructions of z-series.

Environmental enrichment

1 week after animals had been injected with either IdU or CldU, animals were placed into 1 of 2 different enriched environments (EE1 and EE2) where they lived for

a period of 2 weeks. Enrichment cages were custom made with plexi-glass, 36in x 36in x 12in, and contained food, water, plastic tunnels and igloos. Enrichment animals were housed continuously in the enriched environments for 2 weeks while the counter-part controls were housed in standard mouse housing cages. Immediately prior to sacrifice, animals were given a short 2 hour exposure to either EE1 or EE2 (to induce cFos gene expression).

Experiment 1: Encoding of EE1 and EE2

Two distinct populations of newborn cells were labeled within each animal, using CldU and IdU (two thymidine analogs), and each animal received two unique environmental enrichment experiences (E1 and E2) (fig. 3.2), coinciding with the "critical period" of each labeled population of newborn neurons (fig. 3.1). The experiment was counter balanced (fig. 3.3) to ensure that there was no bias of either presentation of the environments or age of each labeled population of newborn neurons. Briefly, animals received the first injection (CldU/IdU) and 1 week later placed into the first enriched environment (EE1/EE2) where they lived for 2 weeks time. Animals received the second injection (IdU/CldU) 1 week after the end of the first enrichment experience, and 1 week after the second injection, animals received the second enrichment experience (EE2/EE1). In both combinations of injections and EE exposure, newborn neurons are 1-3 weeks old while the animals are housed in the

EE. After the second enrichment experience, we waited 5 weeks before re-exposing animals to either EE1 or EE2 for 2 hours and sacrificed immediately after.

Experiment 2: encoding by young neurons versus older neurons

To compare the encoding by 1-3 week (young) old neurons versus 5-7 (older) week old neurons, we used a modified experimental paradigm of experiment 1 (fig. 3.9a). Briefly, animals received the first injection (CldU/IdU) to label the older 5-7 week population and 4 weeks later, received the second injection to label the younger 1-3 week population. 1 week after the second injection, animals were placed in EE1 for 2 weeks. 5 weeks after the EE1 experience, animals were re-exposed for 2 hours to either EE1 or EE2 and sacrificed immediately afterwards.

Experiment 3: Encoding by mature neurons

To determine whether fully mature neurons are capable of encoding information about previously experienced environments, we used a modified experimental paradigm of experiment 1 (fig. 3.9b). Briefly, animals received an injection of CldU to label a population of newborn neurons. We waited 10 weeks to allow the population to fully mature before exposing them to EE1 as their environmental experience. 5 weeks later, animals were re-exposed for 2 hours to either EE1 or EE2 and sacrificed immediately afterwards.

Experiment 4: Maximum encoding

To determine if there is a maximum level of encoding by EDNNs, we used a modified experimental paradigm of experiment 1 (fig. 3.9c). Briefly, animals received the first injection (CldU/IdU) and 1 week later placed into EE1 where they lived for 2 weeks time. Animals received the second injection (IdU/CldU) 1 week after the first exposure to EE1 and 1 week after the second injection, animals were given a second 2 week exposure to EE1. 5 weeks later, animals were re-exposed for 2 hours to either EE1 or EE2 and sacrificed immediately afterwards.

3.3 Results

Encoding of either EE1 or EE2 by 2 distinct populations of EDNNs within the same animal

To determine whether EDNNs encode information about previously experienced environments, 2 distinct populations of EDNNs were labeled in the same animal and all animals were give 2 unique environmental enrichment experiences 1-2 weeks after the last CldU/IdU injection, correlating with the "critical period" of each labeled EDNN population (fig. 3.1). Control groups were used to show that the re-exposure was necessary to induce cFos expression and to also show that EDNNs are necessary to respond to the re-exposure.

Exposure to either EE1 or EE2 effectively promoted the survival of both populations of EDNNs as shown by total IdU and CldU numbers (fig. 3.5). Animals that did not receive the initial exposures to EE1 or EE2 had significantly less surviving IdU and CldU populations. Exposure to either EE1 or EE2 also enhanced the amount of neuronal differentiation (fig. 3.6) compared to the control groups that did not receive the initial exposures to EE1 or EE2.

Animals that were re-exposed to either EE1 or EE2 had significantly more cFos positive cells than animals that did not receive the re-exposure (fig. 3.7), consistent with findings that cFos activity is transiently increased upon immediate exposure to external stimuli. Interestingly, animals that received the initial EE exposures and the re-exposure had more overall cFos positive cells than animals that did not receive the initial EE exposures but did receive the re-exposure.

In this experiment we defined 3 separate groups of EDNNs. These 3 groups are:1-3 week encoders, 5-7 week encoders, and unseen encoders. The 1-3 week encoders are the group that experienced the enriched environment when the labeled neurons were 1-3 weeks of age. The 5-7 week encoders are the group that experienced the enriched environment when the labeled neurons were 5-7 weeks of age. The unseen group represents the EDNN population that was exposed to the opposite enriched environment that was re-exposed. Fig. 3.8a illustrates the amount of double labeled XdU and cFos positive cells, which is a measure of how many cells, of each population of EDNNs, is responding to the re-exposed environment. 1-3 week encoders responded more to a re-exposure of the previously seen enriched

environment when compared to both 5-7 week encoders and unseen encoders. In other words, we found roughly twice as many labeled EDNNs that co-expressed cFos than in either 5-7 week encoders or unseen encoders. Interestingly, both 5-7 week encoders and unseen encoders had significantly more co-labeled XdU/cFos cells than control animals that never received the initial exposures, suggesting that encoding of either of the environments may be occurring at both timepoints but perhaps a larger population encodes at the earlier timepoint. As another way of quantifying the data, we looked at the entire population of activated cells (total cFos) within every animal and determined what percentage of the entire activated cell population is from the 1-3 week encoders, 5-7 week encoders, and unseen encoders (fig. 3.8b). Confirming our previous findings, we discovered that of the 3 defined encoding groups, the 1-3 week encoders made up the majority of the active population when compared to the other 2 groups. In all instances, 1-3 week encoders responded significantly more to a re-exposure of the previously seen environment that all other groups. In control animals that never received the initial exposures to the enriched environment, we did not find a single cell co-labeled XdU/cFos positive cell, suggesting that the initial exposure to the enriched environment is necessary to encode something about the environment.

To be sure that the effects we see are due to a preferential response by the EDNNs, we performed some simple calculations to determine the probability of finding a co-labeled XdU/cFos positive cell by chance. If we assume that an enriched adult mouse brain contains roughly 310,000 neurons [22], based on our histology we can estimate that there are roughly 1500 activated cells upon re-exposure and there are

roughly 2000 XdU cells that survive after an enrichment period. With this data, we can estimate the probability of finding a neuron that is activated upon re-exposure (1500/310,000) and the probability of finding a neuron that is labeled with XdU (2000/310,000). If we multiply these two values together, we can obtain an estimate for the probability of finding a neuron that is labeled with XdU and is active upon re-exposure of an experience, by chance. According to this math, the chance of finding one of these neurons by chance is %0.0028 or roughly 9 cells in the entire dentate gyrus. This number may demonstrate the reason why we do not see any cells in the control animals that did not receive the initial enrichment exposures. Perhaps if we counted more neurons, we might eventually be able to find more of these 9 cells. This does suggest, however, that the EDNNs respond to a re-exposure of a previously seen environment at both 1-3 weeks and 5-7 weeks.

Encoding by 1-3 week old neurons versus encoding by 5-7 week old neurons

During the analysis of the previous experiment, we discovered that although it is clear that 1-3 week old neurons responded more to a re-exposure of the previously experienced environments than 5-7 week old neurons, 5-7 week old neurons are no different in their response to either enriched environment than the unseen responders. Unseen responders are the labeled population of cells that were exposed to one enriched environment and then re-exposed to the other unseen enriched environment. Because of this unexpected result, we designed a new paradigm to distinctly separate

encoding by 1-3 week old neurons and encoding by 5-7 week old neurons (fig. 3.9a). Exposure to an enriched environment during the 1-3 week critical period promotes the survival of newborn neurons when compared to an exposure during 5-7 weeks of age (fig. 3.10a). Preliminary results suggest that neurons that experienced the enriched environment at 1-3 weeks of age, responded greater to a re-exposure of the previously seen environment, than neurons that experienced the enriched environment at 5-7 weeks of age (fig. 3.10b). These results trend in the same direction as the previous experiment, however the results are preliminary and more quantifications well be necessary to determine if the trend is real. We also re-exposed groups to an environment that was not previously seen, however it did not appear that either population of neurons responded preferentially to this re-exposure (fig. 3.10c).

Encoding by 10 week old mature neurons

Although it is clear that newborn neurons encode information at 1-3 weeks of age as well as 5-7 weeks of age, it remains to be determined if fully mature neurons are capable of encoding. To explore this idea, we labeled a newborn population of cells with CldU and allowed them to mature for 10 weeks before exposing them to an enriched environment. After a 2 week exposure to an enriched environment, animals were re-exposed to either the same previously seen enriched environment or a novel enriched environment (fig 3.9b). Preliminary results of this experiment, as indicated by total surviving CldU cells, show that exposure to an enriched environment when

cells are fully mature (10 weeks old) does not promote their survival to the same extent as if immature neurons are exposed to an enriched environment (fig. 3.11a). We were also not able to find any co-localized cFos/CldU cells in any of the animals, however, we found very few CldU cells to begin with (fig. 3.11b). More cell counts will need to be achieved before we can make an accurate conclusion about the results of this experiment.

A maximum level of encoding

To determine a maximum level of encoding by EDNNs we gave animals multiple exposure to the same environment before giving them a re-exposure at the end of the experiment. Results of this experiment are preliminary, however, based on the number of surviving XdU+ cells (fig. 3.9c), two exposures to the same enriched environment does not enhance the survival of newborn neurons above one exposure to an enriched environment (fig. 3.5, fig. 3.12a). It is unclear whether EDNNs show an enhanced response to a re-exposure of the previous environment that was experienced at two separate events (fig. 3.12b). More quantifications are necessary to make an accurate conclusion.

3.4 Discussion

In this study we were determined to understand the role of EDNNs in the encoding of previously experienced environments. By labeling two distinct cell populations within all animals and pairing these populations with enriched environments, presented during the "critical period" of newborn maturation, we were able to show that EDNNs do in fact encode some information about their environment such that a re-exposure of the same environment elicits a response, specifically from the EDNN population. By comparing different time points when the labeled populations were exposed to an enriched environment, we were able to determine that EDNNs respond to the environment that was experienced during their critical period. This suggests that not only is enrichment/experience during the "critical period" necessary for survival, but these EDNNs perhaps encode necessary information about the specific environment/experience that may be used at a later time point. Interestingly, when compared to control animals that never received the initial environmental enrichment experience, EDNNs still respond to a re-exposure of the previously seen environment even when presented with initial exposure to the enriched environment at a later period of maturation (5 weeks after birth). There appears to be a cut off, however, to the level of maturation at which EDNNs can still learn something about the environment and a re-exposure elicits a preferential response. As shown by the mature encoding experiment, newborn neurons that experience an enriched environment 10 weeks after birth, when they are considered

fully mature, do not respond preferentially to the same environment. These studies show that EDNNs, exposed to an enriched environment during their critical period, encode information specifically about that environment. These EDNNs are still plastic even at later stages of maturation (5 weeks) and will still respond to previously seen environment, although not to the same extent as the "critical period" exposure. This plasticity of the newborn neurons to learn about the environment is eventually lost once the cells mature to a certain stage.

Chapter 3, is currently being prepared for submission for publication. Clemenson GD, Gage FH. The dissertation author was the primary investigator and author of this material.

3.5 Figures

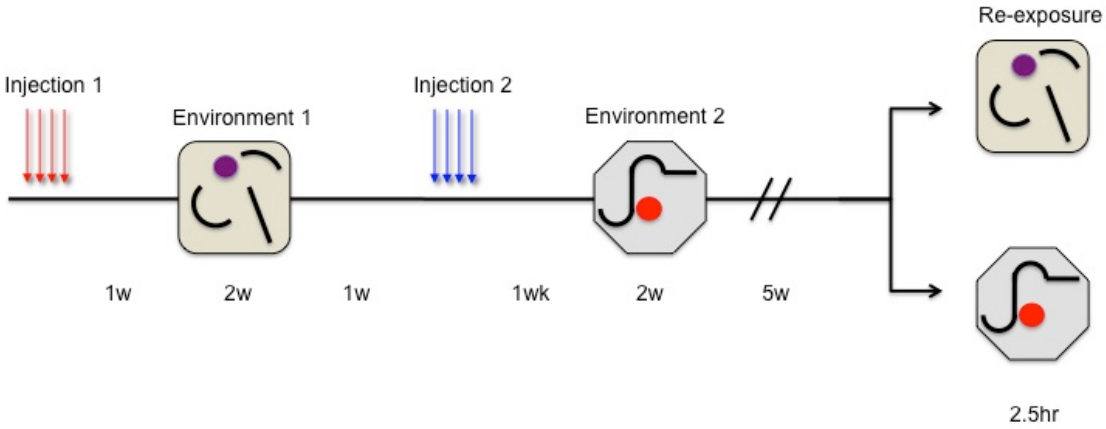

Figure 3.1
Experimental paradigm

Figure 3.2
Two enriched environments used for the duration of the experiment

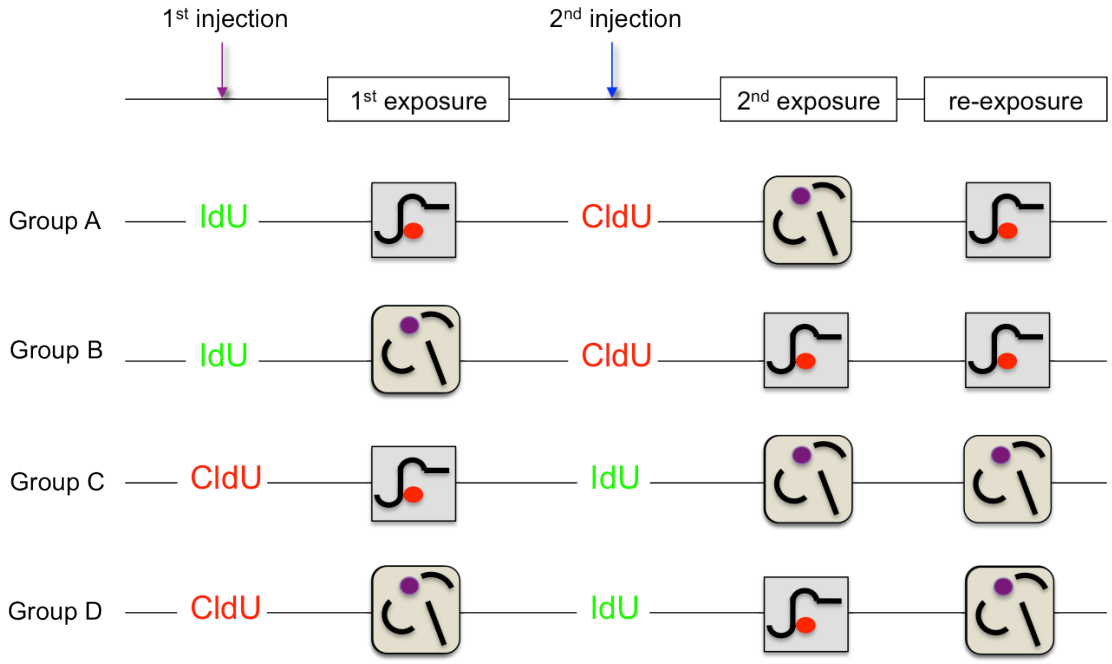

Figure 3.3
Counterbalancing of experiment to control for injection of CldU and IdU, and for presentation of environment 1 or environment 2.

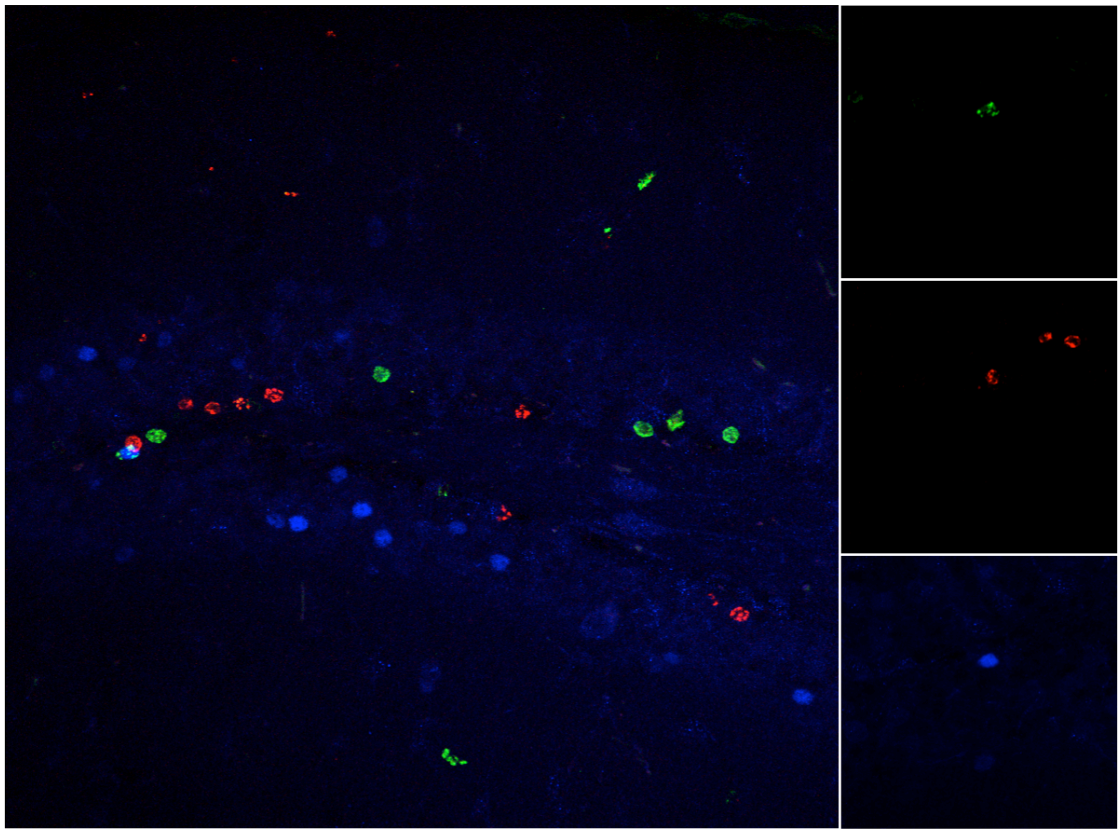

Figure 3.4
Example of immunohistochemistry demonstrating specificity of CldU and IdU antibodies

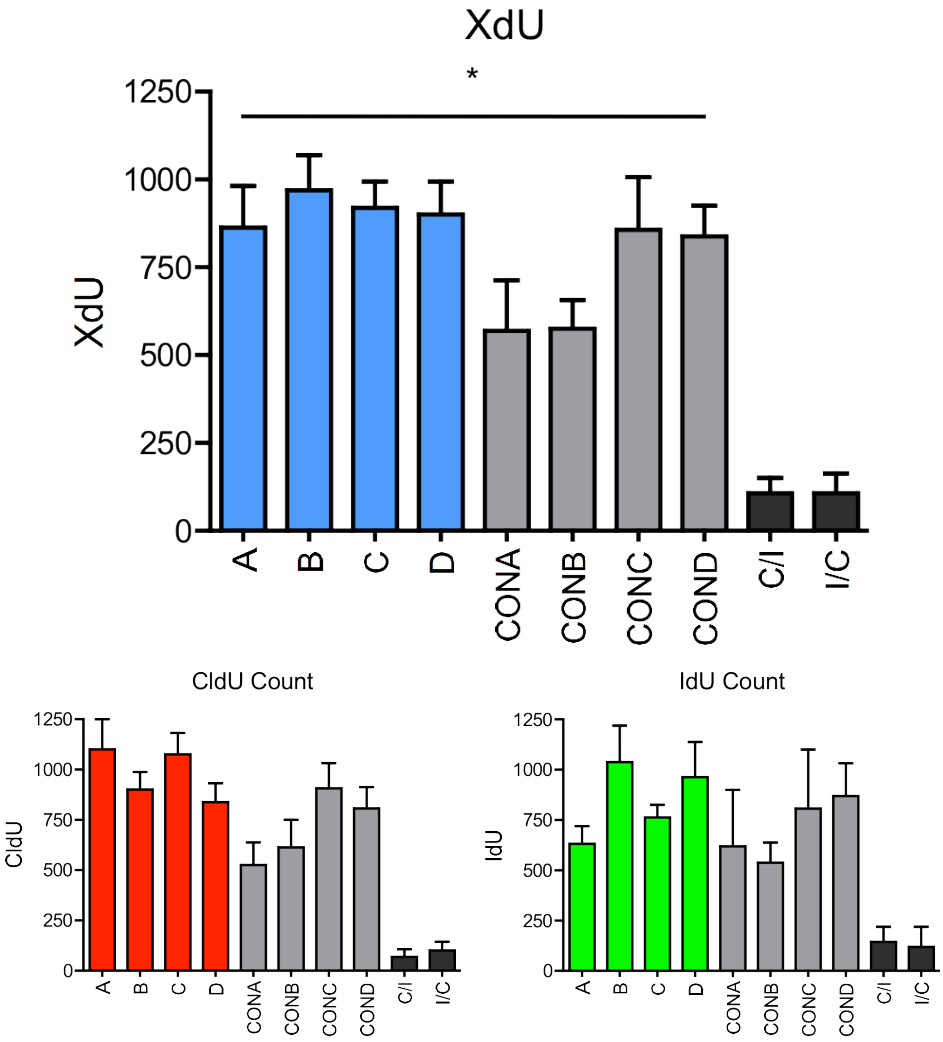

Figure 3.5
a) Estimated total number of XdU cells in the DG of experimental (A-D), control of re-exposure (CONA-COND), and control of initial exposure (C/I and I/C).
b) Estimated total number of CldU cells in the DG of experimental (A-D), control of re-exposure (CONA-COND), and control of initial exposure (C/I and I/C).
c) Estimated total number of IdU cells in the DG of experimental (A-D), control of re-exposure (CONA-COND), and control of initial exposure (C/I and I/C).
*$p < 0.05$

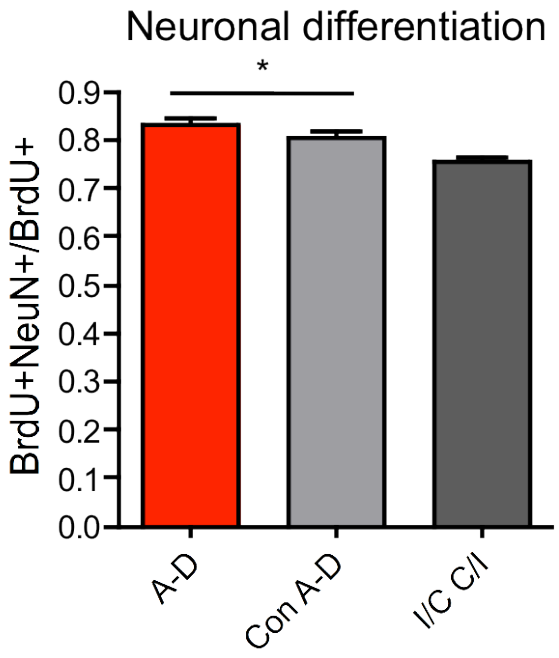

Figure 3.6
Neuronal differentiation of experimental (A-D), control of re-exposure (CONA-COND), and control of initial exposure (C/I and I/C).
*$p < 0.05$

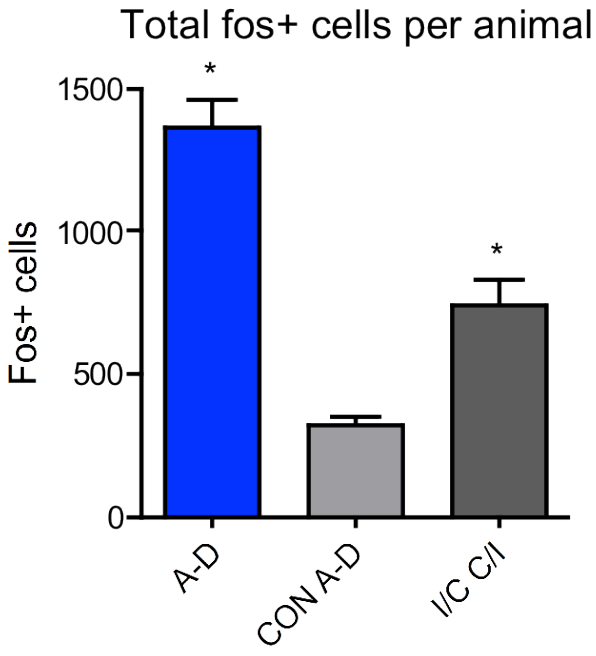

Figure 3.7
Estimated total number of XdU cells in the DG of experimental (A-D), control of re-exposure (CONA-COND), and control of initial exposure (C/I and I/C).
*$p < 0.05$

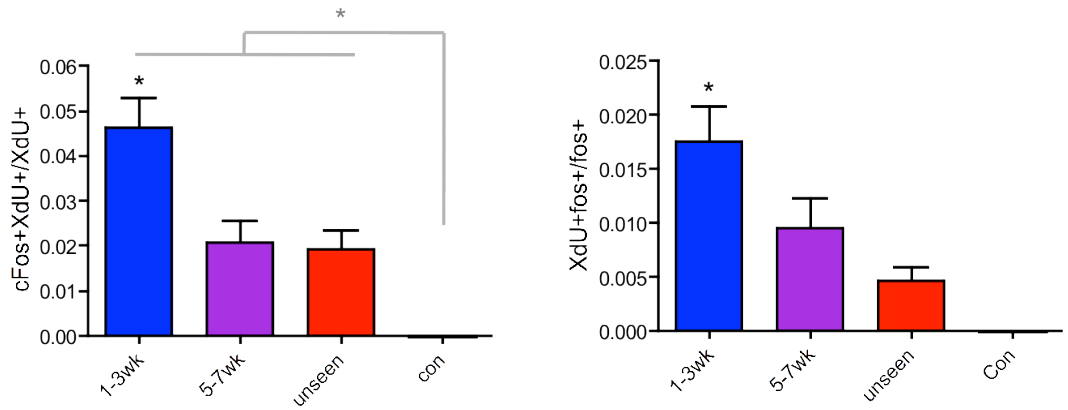

Figure 3.8
a) Percentage of colocalized cFos and XdU within total XdU population of 1-3 week encoding group, 5-7 week encoding group, unseen encoding group, and control of initial exposure group.
b) Percentage of colocalized cFos and XdU within total cFos population of 1-3 week encoding group, 5-7 week encoding group, unseen encoding group, and control of initial exposure group.
*$p < 0.05$

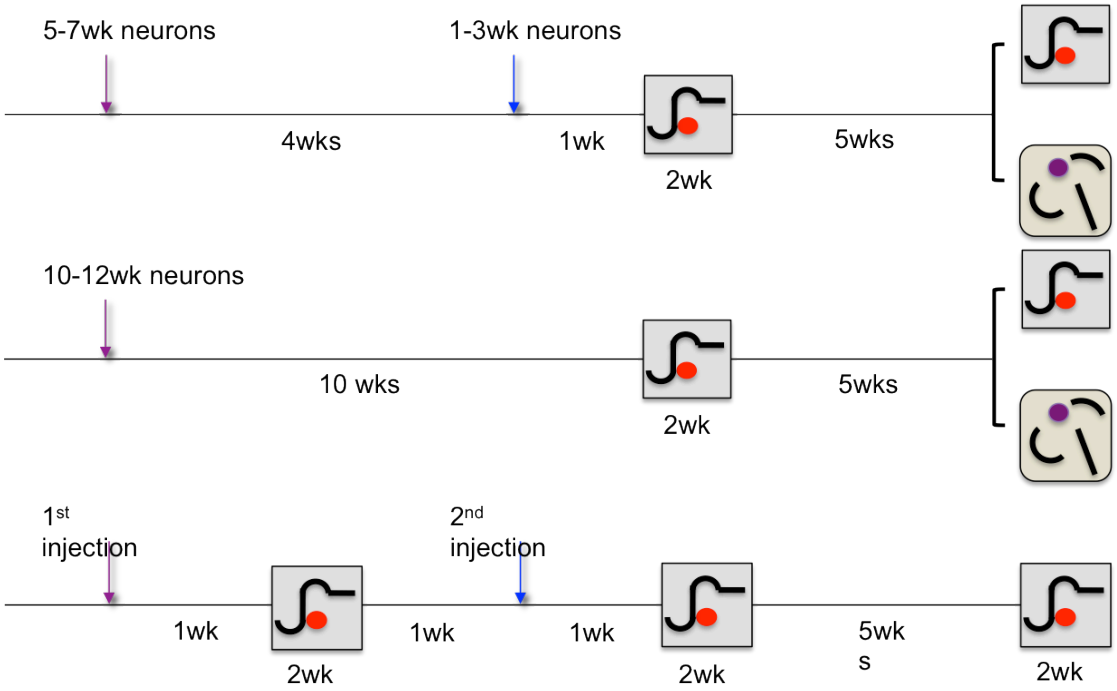

Figure 3.9
a) Paradigm early encoders versus late encoders to similar and different environments
b) Paradigm mature encoders to similar and different environments
c) Paradigm maximum encoding by early encoders

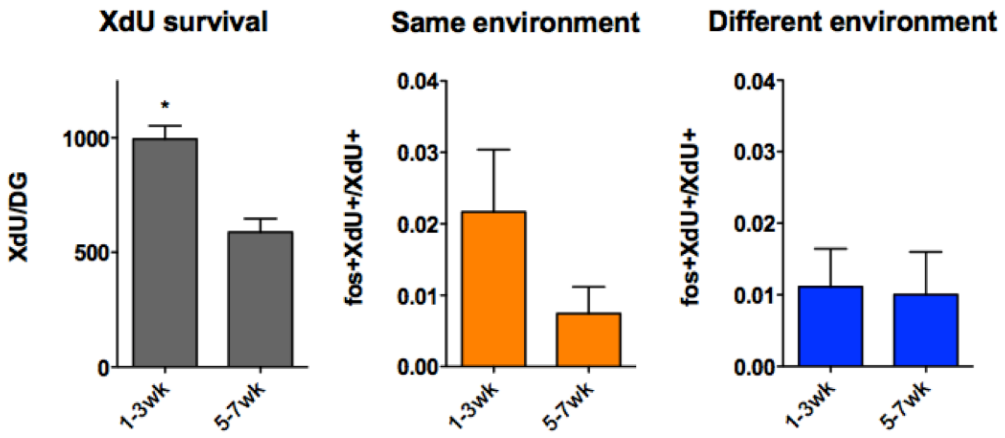

Figure 3.10
a) Total XdU survival
b) % co-localized fos/XdU cells of the total XdU population, same environment
c) % co-localized fos/XdU cells of the total XdU population, different environment
*$p < 0.05$

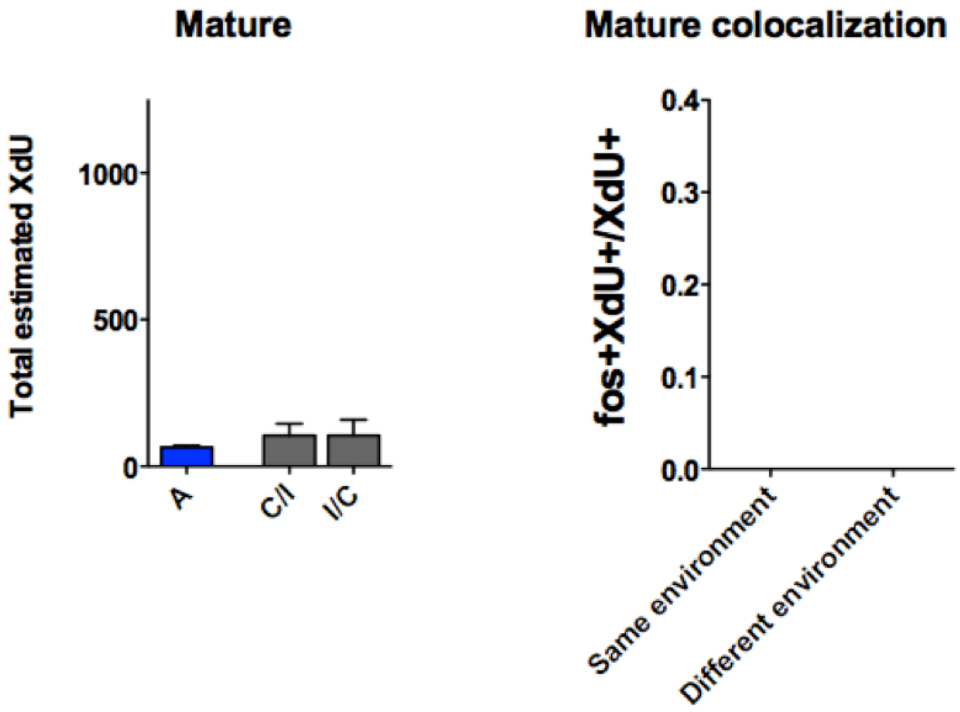

Figure 3.11
a) Total estimated number of XdU cells compared to controls
b) % co-localized fos/XdU cells in same environment and different environment conditions

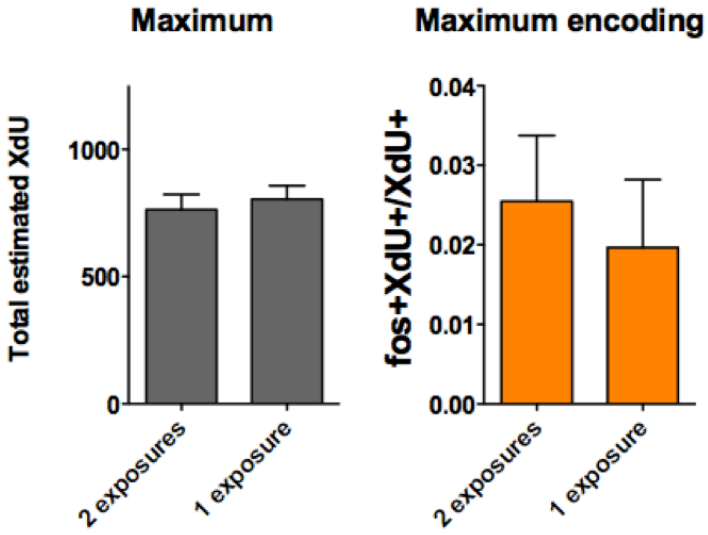

Figure 3.12
a) Total estimated number of XdU cells
b) % co-localized fos/XdU cells in 2 environmental exposures and 1 environmental exposure

CHAPTER 4: EDNNs role in decoding new and novel environments/contexts

4.1 Introduction

Both environmental enrichment and exercise both promote neurogenesis in the hippocampus of mice. This enhancement in levels of neurogenesis has been correlated with an improvement in spatial tasks such as the MWM and contextual fear conditioning. Interestingly, however, environmental enrichment, which increases neurogenesis levels in mice, has not been correlated with contextual fear conditioning. Surprisingly, when animals that received enrichment were subject to a simple paradigm of contextual fear conditioning, control animals appeared to perform the task better than enriched animals. Control animals demonstrated a clear discrimination phenotype between the shock context and the novel context whereas enriched animals did not. In fact, when freezing was measured in both contexts, enriched animals appeared to freeze to both the shocked context and the novel context. This simple behavior may be interpreted in two ways. One, the enriched animals cannot discriminate between two different contexts, or two, enriched animals are generalizing the fear response from the shocked context to the novel context. Generalization, very simply, can be defined as when two non-identical stimuli elicit the same response. In this case, it is possible that the mice perceive the novel context the same as the shocked context and therefore freeze to a presentation of either context. We hypothesized that because enriched animals received a prior exposure to a spatially

complex environment, they are infact quicker at deciphering their surrounding environment. To determine which of these two interpretations is correct, we designed a novel, modified contextual fear conditioning (mCFC) that would test animal's ability to understand their environment within a limited time window.

4.2 Materials and Methods

Animals

All animal procedures were performed in accordance with animal guidelines at The Salk Institute for Biological Studies. A total of 110 female C57/Bl6 mice (Harlan, San Diego) 7-8 weeks old were used for 4 separate experiments. All animals received a total of 8 interperitoneal injections of iodo-deoxyuridine (IdU) or chlorodeoxyuridine (CldU) equivalent to a concentration of 50mg/kg of BrdU

Immunohistochemistry

Mice were anesthetized with a ketamine/xylazine mixture and transcardially perfused with 0.9% NaCl solution followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. Brains were postfixed overnight in 4% PFA at 4^0C and then transferred into 30% sucrose solution. 40um coronal sections were cut on a sliding microtome and all immunohistochemistry was performed on free-floating sections.

Tissue from animals was first incubated with antibodies against neuronal nuclei (NeuN) or the immediate early gene cFos for 48 hours at 4^0C followed by incubation with secondary antibodies, post-fixed (4 % PFA, 20 min at room temperature), and then treated with 1M HCl, for both IdU and CldU detection, followed by incubation with an antibody against IdU and CldU. Because of the strong reactivity of the antibody used against CldU, all tissue was first stained for IdU followed by a postfix of 4% PFA prior to CldU staining. Primary antibodies used were rat anti-CldU (1:500; Accurate), mouse anti-IdU (1:100, BD), mouse anti-NeuN (1:10; Bobbi's Clone A60), and rabbit anti-cFos (1:1000, Calbiochem). The total number of IdU/CldU-positive cells was counted using every 6th section (240 μm apart for young animals). IdU/CldU-labeled cells were counted throughout the rostrocaudal extent of the granule cell layer (GCL) using a 20 x objective and an upright epifluorescence microscope (E800; Nikon, Tokyo, Japan). The co-labeling of IdU/CldU-positive cells with either NeuN or cFos was analyzed using a confocal microscope (Radiance 2100; Bio-Rad, Hercules, CA) and a 40x oil objective. All analyses were performed in sequential scanning mode, and double labeling was confirmed by 3-dimensional reconstructions of z-series.

Environmental enrichment

1 week after animals had been injected with either IdU or CldU, animals were placed into 1 of 2 different enriched environments (EE1 and EE2) where they lived for

a period of 2 weeks. Enrichment cages were custom made with plexi-glass, 36in x 36in x 12in, and contained food, water, plastic tunnels and igloos. Enrichment animals were housed continuously in the enriched environments for 2 weeks while the counter-part controls were housed in standard mouse housing cages.

Running cages

Animals were housed in a rat cage where where they had free access to 2 running wheels. 3 mice were housed in each running cage.

Contextual fear conditioning paradigm – pre-exposure (peCFC)

3 different contexts are used in this FC paradigm, A', A, and B. Context A' and context A differ only in the fact that A has a square piece of plastic covering the metal bars where the animals receive the foot shock. A' is only used during the shock trial, otherwise animals always receive context A as the "shocked context". On the first day, animals are given a pre-exposure to the "shocked context" where they are placed in context A for 10 minutes. On the second day, animals are given a 30 second shock trial where the animals receive a small 2 second foot shock, 5 seconds into the shock trial. 30 minutes later animals are given a discrimination trial where animals are given 3 minutes in context A and 3 minutes in context B in a counterbalanced manner

(fig. 4.1a). Time spent freezing in both context A and context B is used to determine if they successfully discriminated between the two contexts.

Modified contextual fear conditioning (mCFC) paradigm – no pre-exposure

A total of 4 different contexts were used for the mCFC paradigm. Context A is defined as the standard fear conditioning operant chamber. Context A' and context A differ only the fact that A has a square piece of plastic covering the metal bars where the animals receive the foot shock. A' is only used during the shock trial, otherwise animals always receive context A as the "shocked context". Context B is also set within the fear conditioning operant chambers with the addition of a curved piece of plastic placed at the back of the chamber to create a different shape than Context A. Context B also contains 5 posters placed within the operant chamber to vary the visual stimuli. Context C is defined as activity monitor chambers that are completely different than the fear conditioning operant chambers. The activity monitoring chambers, context C, are also located in an entirely different room than the fear conditioning operant chambers. On the first day, animals receive a shock trial lasting 30 seconds where animals are placed into the shock context A', receive a short 2 second foot shock (7mA) 15 seconds into the trial and remain in context A' for the remainder of the 30 seconds. 30 minutes after the shock trial, a post-exposure trial was performed where animals were placed into context A for 3 minutes. On the second day of the mCFC paradigm, animals were given a discrimination trial where

they were placed either in context A for 3 minutes or context B for 3 minutes. To counterbalance presentation of either context, half of the animals received context A first and the other half, context B (fig. 4.2b). Time spent freezing was recorded and used to determine whether animals were able to associate the shock with the context. If animals correctly associated context A with the shock, during the discrimination trial we expected that animals would freeze more in an exposure to context A than an exposure to context B.

In order to determine the characteristics of the mCFC, following experiments were designed to systematically test the effects of the 30 minute post-exposure. Animals received one of the following: post-exposure to A (fig. 4.4a), post-exposure to B (fig. 4.4b), post-exposure to C (fig. 4.4c), post-exposure to both A and B (both A then B or B then A) (fig 4.4d), or no post-exposure (fig. 4.3). One group of animals received a discrimination task on the second day between A and C (fig. 4.6a).

4.3 Results

Enriched animals do not discriminate in the peCFC paradigm

When enriched animals were subjected to the peCFC paradigm (fig. 4.1a), although control animals were able to discriminate between context A and context B, enriched animals showed similar levels of freezing when placed in context A and context B (fig. 4.1b). Surprisingly, although enriched animals showed enhanced levels

of neurogenesis, which has been correlated with an improvement in discrimination (exercise), in the peCFC paradigm we did not observe any discrimination. We hypothesized that perhaps this particular fear conditioning paradigm was not difficult enough for these enriched animals. In other words, because of the previous environmental exposure these animals received they are better at interpreting their surroundings and they were able to tell that context A and context B were actually the same box. We decided to design a more difficult modified fear conditioning paradigm by utilizing the immediate shock deficit phenomenon. Simply, the immediate shock deficit states that if animals are given a small foot shock (US) immediately upon exposure to the context (CS), they have not had enough time to sufficiently explore the context and therefore do not make an association between the two. With this in mind, we designed a modified fear conditioning paradigm where the pre-exposure was removed and less time was given to form the association between the context and the shock. If enriched animals are better at deciphering their surrounding, we hypothesize that they will require less time in the context, prior to the shock than control animals, to make the association.

Enriched animals discriminate in a no pre-exposure mCFC paradigm

We decided to test enriched animals in the mCFC paradigm (fig. 4.2b). With just 15 seconds to explore the shocked context before the shock, control animals showed no difference in freezing to context A versus freezing to context B indicating

that control animals did not discriminate in this particular task. Surprisingly, enriched animals froze significantly more in context A than in context B, suggesting that they were able to associate the shock with context A while receiving only a 15 second exposure to the shocked context (fig. 4.2c). Within 15 seconds of exploring the context, enriched animals appeared to have learned sufficient information about the context such that they were able to recognize it at a later time point and realize that they were previously shocked in it. This discrimination phenotype of enriched animals was also observed at both 5 seconds and 25 seconds of exploration before the shock.

Post-exposure to the shocked context is required for enriched animals to make a discrimination in mCFC paradigm

We were surprised by the results of the enriched animals in the previous experiment. When given less time to explore the shock context before the shock, enriched animals were still capable of discriminating between context A and context B, indicating that they are somehow "better" at gathering information about their surroundings. Next, we decided to determine how crucial the 30 minute post exposure of the mCFC paradigm is for the enriched animals to make the discrimination 24 hours later. We have already determined that by giving animals a post-exposure to the shocked context (A), enriched animals were able to discriminate in this task whereas control animals were not. When we changed the context of the post-exposure to

context B, control animals were still not able to discriminate and interestingly, enriched animals had lost their ability to discriminate between A and B (fig. 4.4b). Enriched animals showed equal amounts of freezing to both context A and B, similar to controls. When we changed the context of the post-exposure to a completely different context, context C, both control animals and enriched animals were not able to discriminate between context A and context B during the discrimination trial (fig. 4.4c). When we exposed animals sequentially to both context A and B during the post-exposure, control animals did not discriminate, however once again, the enriched animals demonstrated discrimination (fig. 4.4d). These data suggests that a 30 minute post-exposure to the shocked context (A) is necessary for the enriched animals to make a discrimination 24 hours later, between context A and B. In any case, control animals were never able to discriminate between context A and B suggesting that this mCFC paradigm yields an enrichment specific phenotype. It still remains unclear whether this phenotype is attributed to enrichment-induced neurogenesis or if it is merely an effect of environmental enrichment, independent of neurogenesis.

Discrimination in the no pre-exposure, mCFC paradigm is specific to enrichment

The ability to discriminate between two contexts in the mCFC paradigm appears to be specific to enrichment, however, it is not clear if the phenotype we observe is dependent on neurogenesis. Although enrichment has been known to enhance levels of neurogenesis, it has been suggested that many of the beneficial

effects of enrichment are independent of neurogenesis [51]. To elucidate this question, we decided to test aged animals and exercised animals. Aged animals show a dramatic reduction in the levels of neurogenesis and exercise has been shown to enhance neurogenesis. Both young (3 months) and aged (14 months) animals were placed into 3 groups; young/aged controls, young/aged enriched, and young/aged runners (fig. 4.5a, 4.5b). In young animals, confirming previous results, control animals did not show any discrimination whereas enriched animals did. Interestingly, exercised animals were no different than controls in this particular task, exhibiting no discrimination. Unfortunately in the aged animals, all 3 groups (control, enriched, runner) froze less than 10% in both context A and context B, indicating that perhaps the task was too hard regardless of whether they received enrichment or not. It has also been shown that aged animals have deficits in sensory ability suggesting that they do not perform well in fear conditioning tasks because they have trouble perceiving the shock itself.

24 hours of enrichment is not enough time to demonstrate discrimination in the mCFC paradigm

Although the data to this point may suggest a phenotype that is specific to enrichment, it remains unclear if it is dependent on enrichment-induced neurogenesis. To determine whether the phenotype we observe is due to neurogenesis-independent effects of enrichment, we decided to give animals a much shorter exposure to an

enriched environment so that they would get the enrichment exposure but presumably not have any enrichment-induced neurogenesis (fig. 4.6b). Instead of receiving 2 weeks of enrichment, animals were placed in the enriched environment for 24 hours. When these animals were given the mCFC paradigm, enriched animals were not significantly different than control animals, indicating that 24 hours of enrichment is not enough time to demonstrate the discrimination phenotype.

4.4 Discussion

Through this series of experiments, we have discovered, for the first time, an enrichment specific behavioral phenotype. It has been well established, in our lab as well as others, that exposure to an enriched environment promotes the survival of newborn neurons. As demonstrated in Tashiro et al [57], newborn neurons are most susceptible to the survival effects of enrichment when they receive the enrichment experience during week 1-2 of maturation, indicating a possible "critical period" of newborn neuronal survival. As shown in Aim 2, one possible functional role of these EDNNs is to encode information specifically about previously experienced environments. Although this represents only a fraction of the population of EDNNs (~%5), we hypothesize that there is another subset of the EDNN population that are devoted to helping the animal decode and decipher new and novel environments the animal encounters. We have often used the peCFC and consistently find that animals are able to discriminate between the context so we were surprised to discover that

enriched animals did not discriminate between the two contexts. In fact, the enriched animals appeared to freeze equal amounts in context A versus B. We hypothesized that perhaps the task was too easy for the group of enriched animals. By having the prior environmental enrichment experience, they are "trained to explore" new and novel environments so they can easily tell that context A and B are not that different. With this hypothesis in mind, we wanted to determine a way to test that enriched animals are better at deciphering their surroundings. We decided to utilize the immediate shock deficit theory that states if an animal does not have enough time to explore the context before a shock, it will not be able to form an association of the shock with the context [39]. We hypothesized that enriched animals would require less time to explore the context than control animals and associate the context with a shock. Without giving animals a pre-exposure to the shocked context, we decided to vary the amount of time prior to receiving the shock, during the shock trial. What we discovered is that when giving limited amounts of time prior to the shock, control animals were never able to discriminate between the shocked context and another separate context.

When freezing time was measured, control animals did not freeze more in shocked context A than in a different context B. Animals that experienced an enriched environment, however, were able to discriminate between the 2 contexts with as little as 5 seconds prior to receiving the shock. This indicates that enriched animals do indeed decipher their surroundings much faster than control animals. It remains to be determined whether this enrichment specific behavioral phenotype is dependent on

neurogenesis. Thus far, the data is inconclusive. Currently an experiment is in progress to knockout neurogenesis using irradiation to determine, the involvement of neurogenesis (fig. 4.7).

Chapter 4, is currently being prepared for submission for publication. Clemenson GD, Lee SW, Gage FH. This dissertation author was the primary investigator and author of this material.

4.5 Figures

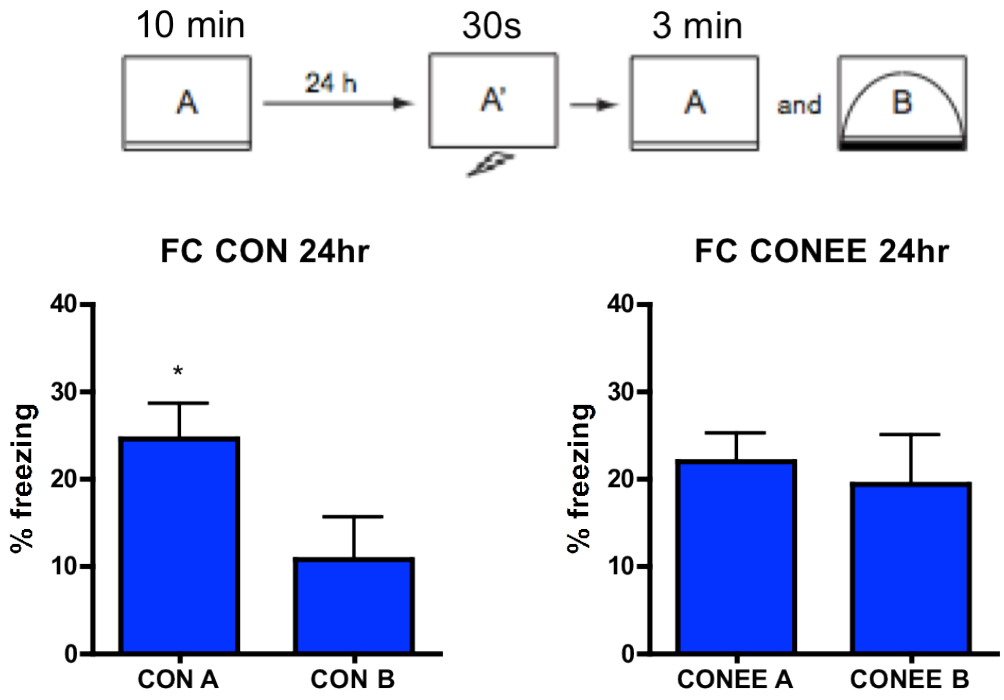

Figure 4.1
a) paradigm of fear conditioning paradigm with 10 minute pre-exposure
b) amount of freezing in context A and context B in control and enriched animals
*$p < 0.05$

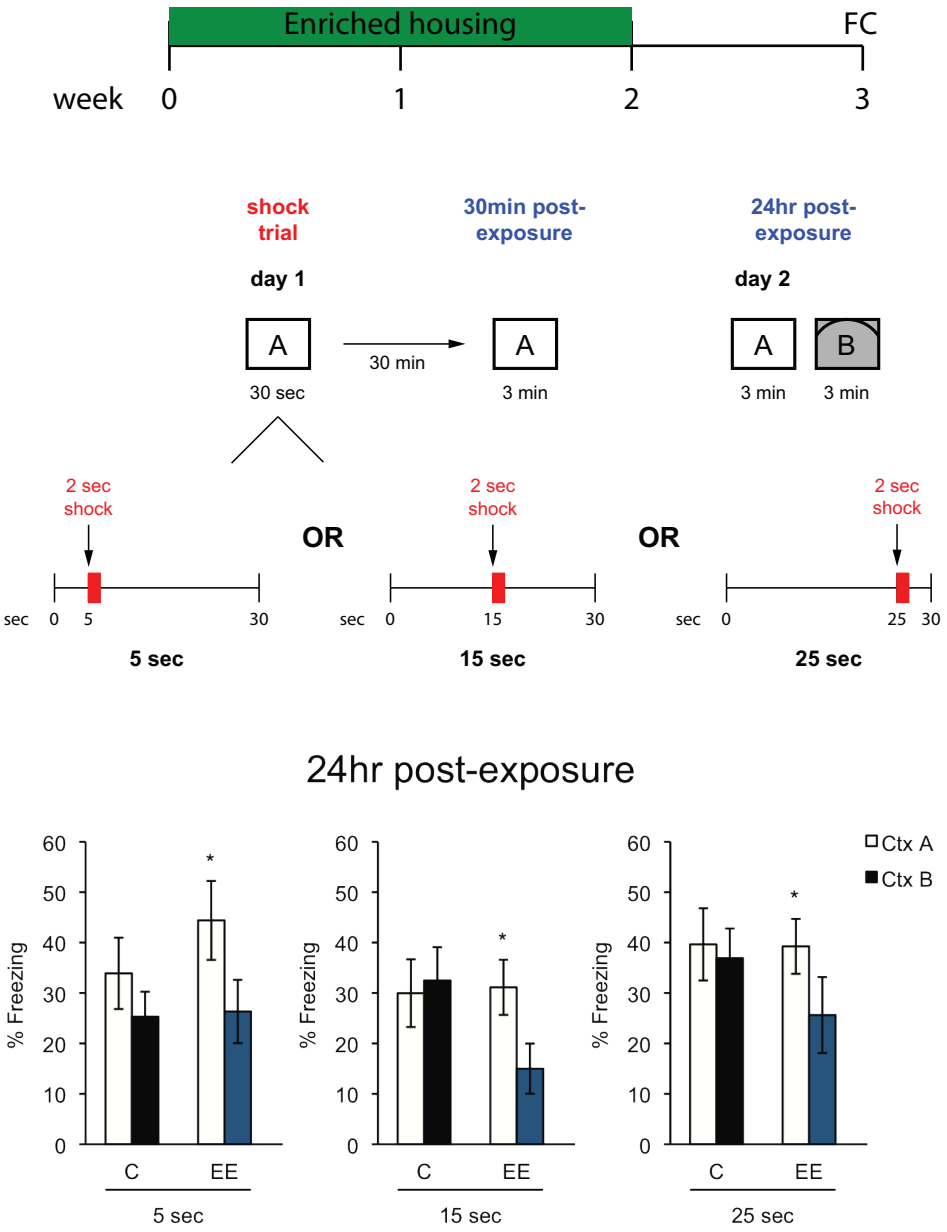

Figure 4.2
a) Enrichment paradigm. Animals live in the enriched environment for 2 weeks and 1 week later, subjected to fear conditioning.
b) Modified fear conditioning paradigm without pre-exposure. Animals were given shock 5 seconds, 15 seconds, or 25 seconds after 5 seconds of being transferred into the context.
c) Percent freezing of control and enriched animals during the 24 hour context discrimination task for 5 second, 15 second, or 25 second groups.
*$p < 0.05$

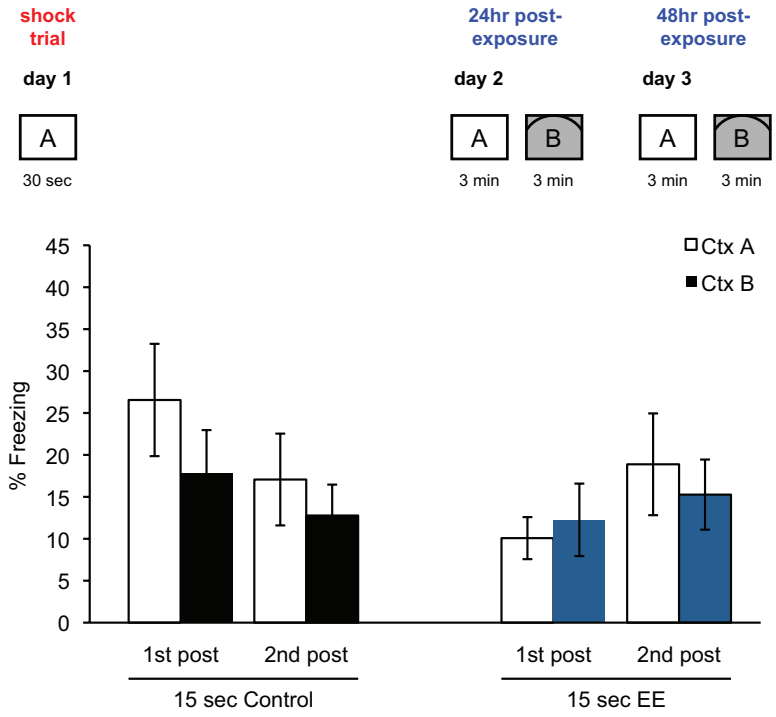

Figure 4.3
a) no pre-exposure fear conditioning without a postexposure
b) % freezing of control and enriched animals 24 and 48 hours post shock trial

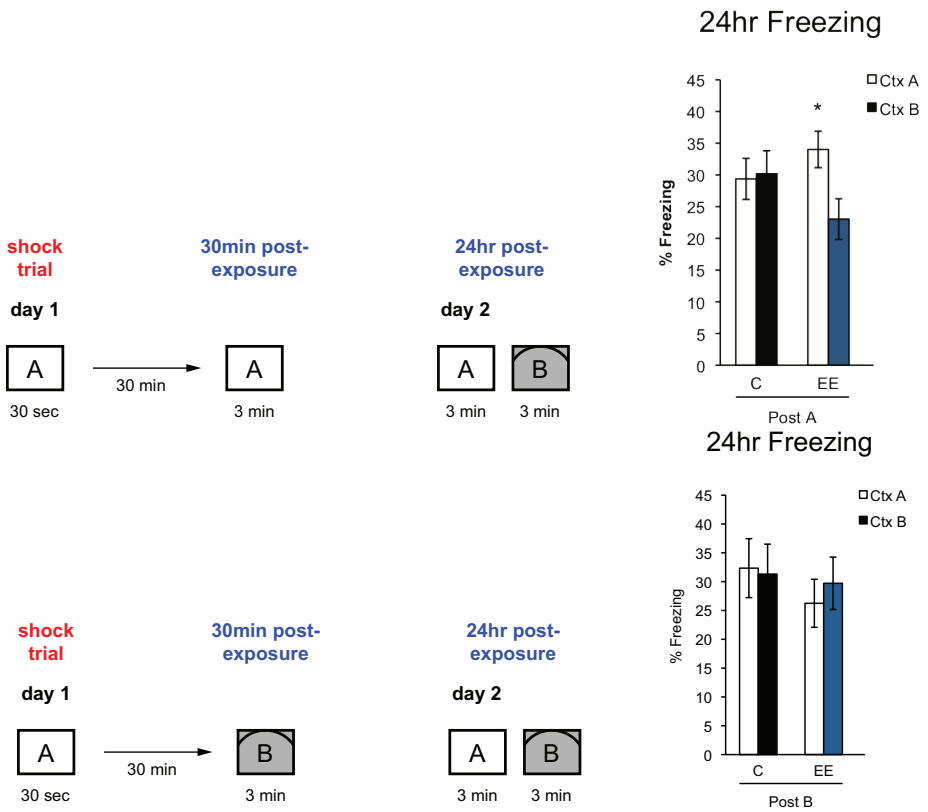

Figure 4.4
a) no pre-exposure paradigm, post exposure to A
b) no pre-exposure paradigm, post exposure to B
*$p < 0.05$

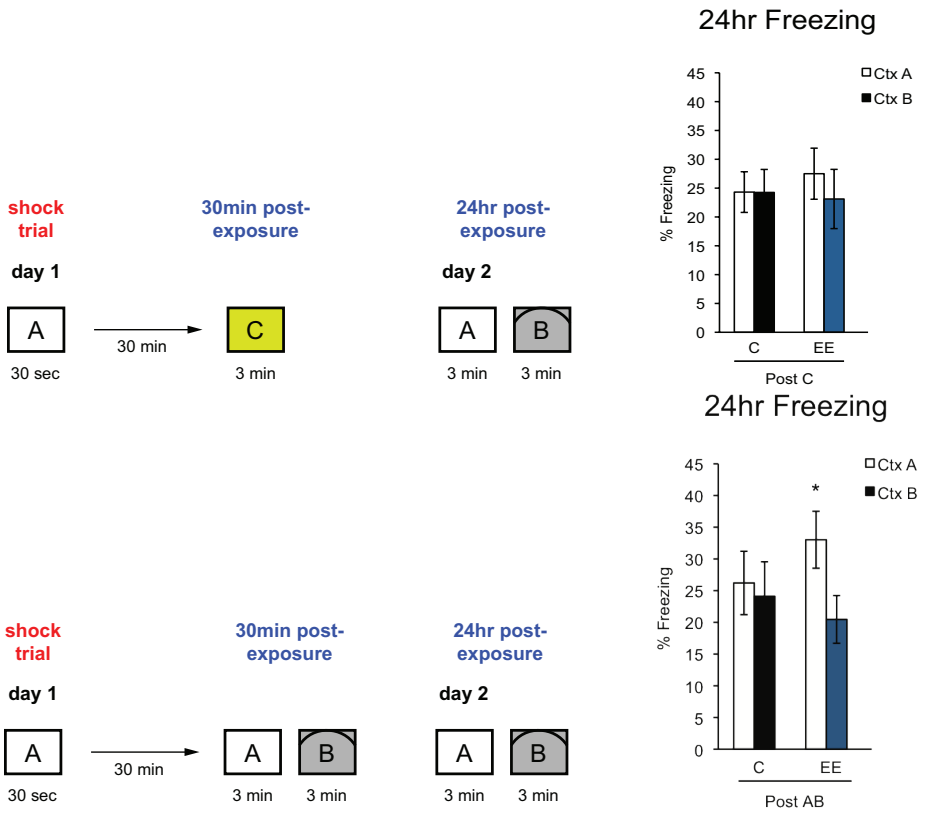

Figure 4.4 continued.
c) no pre-exposure paradigm, post exposure to C
d) no pre-exposure paradigm, post exposure to A and B
*$p < 0.05$

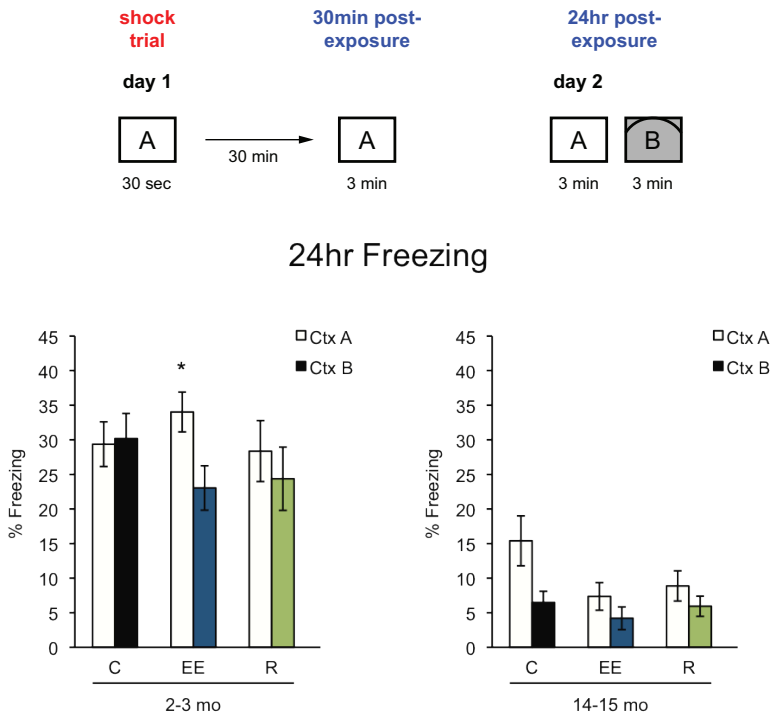

Figure 4.5
a) Young controls, enriched and runners tested in the no pre-exposure fear conditioning paradigm
b) Aged controls, enriched and runners tested in the no pre-exposure fear conditioning paradigm
*$p < 0.05$

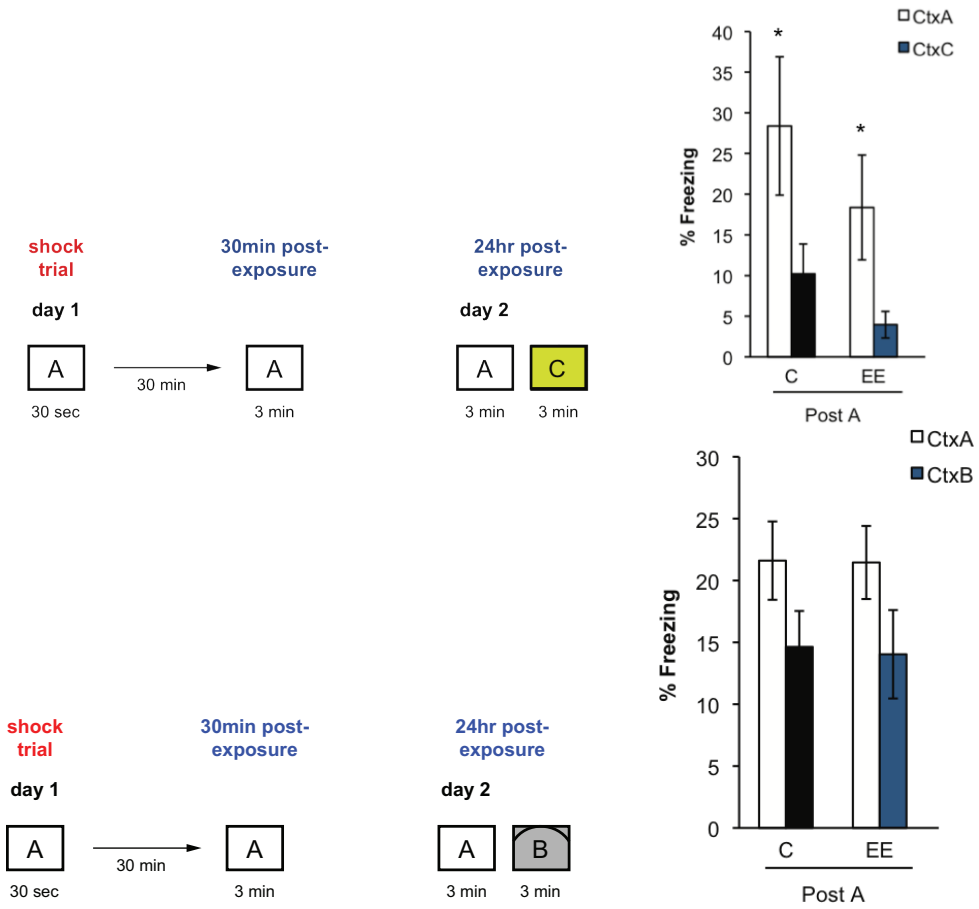

Figure 4.6
a) Animals are tested using a discrimination task of 2 very different contexts
b) Animals that received 24 hours of enrichment are tested in the no pre-exposure paradigm
*$p < 0.05$

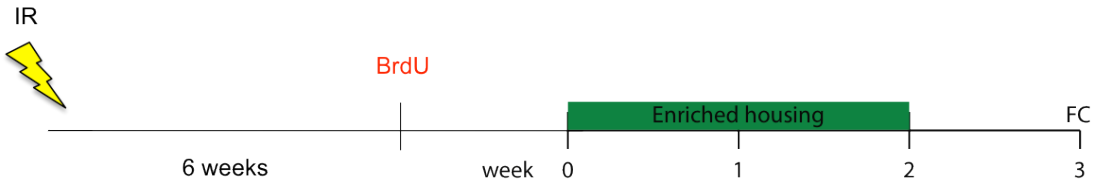

Figure 4.7
Irradiation paradigm

CHAPTER 5: Conclusion

My work has focused on determining the functional role experience dependent newborn neurons play in the adult mouse. The survival of this specific population of newborn neuron is dependent on the enrichment experience they receive during the early stages of their maturation, signifying the potential importance of their unknown function. It is clear, however, that this newborn population has a unique bond with the way in which the animal interacts with their environment and surrounding. This may be through the spatial aspects of the enriched environment, particular features unique to each environment the animal experiences, and/or similarities and differences between multiple environments the animal experiences. I have explored 3 possible roles for this population of newborn neurons in the way that adult mice interact with their surrounding environment and overall hippocampus dependent memory tasks.

The first aim suggests a more general role of these EDNNs in the participation of long-term memory of a hippocampus dependent task, the MWM. Exposure to an enriched environment promotes the survival of EDNNs ultimately leading to an enhancement of the MWM task, in both the acquisition and long-term memory retention. When EDNNs were knocked down through lentiviral delivery, animals lost their ability to remember the target quadrant. Perhaps because of the previous exposure to an enriched environment, these EDNNs are specialized in their ability to gain and retain spatial information. These environments are much larger and spatially enriching, requiring the animal to retain more information as the environment is

explored. In the same manner, these EDNNs enhance the animals ability to learn and remember the spatial cues and location of the hidden platform. Without this population of EDNNs, the animals no longer have the advantage of specialized, spatial neurons and perform no better than animals that never received the enriching experience.

The second aim suggests that EDNNs participate in the encoding of previously experienced enriched environments during the early stages of their maturation. EDNNs that experience an enriched environment during the critical period of their maturation, respond preferentially to a re-exposure of the same environment. During this period of hyper excitability, these EDNNs are responsive to all the sensory inputs they receive as the animal explores the enriched environment. As these EDNNs mature and because their survival is dependent on their activity, their response tightens and hone's into specificities about the environment they experienced. Due to the continuous process of neurogenesis and because of the temporal separation and extended maturation of these newborn neurons, distinct populations of EDNNs arise to encode multiple and temporally distinct environmental exposures the animal experiences.

The third aim suggests a novel role for these EDNNs in the decoding of new and novel environments an animal encounters. In a difficult, modified version of contextual fear conditioning, animals that experienced an enriched environment were faster at learning a context and associating it with a shock, than animals that never received an enrichment experience. Environmental enrichment promotes the survival

of EDNNs and a portion of these EDNNs are tuned to respond to previously experienced environments (as demonstrated in aim 2), however, another subset of these EDNNs are primed to decipher the surroundings of new environments that animal experiences. As we saw previously, only a fraction (~%5) of EDNNs are preferentially responding to a re-exposure of a previously seen environment, however, it would be advantageous to the animal to support the survival of other EDNNs to account for new novel experiences the animal may encounter. By keeping a portion of these EDNNs in a "primed" state, they can be quickly accessed and used to encode important, and possibly brief, events or experiences that may be beneficial to the animal at a later time.

Adult hippocampal neurogenesis is a highly regulated and dynamic process. Interestingly, simple external manipulations appear to have a tremendous impact on the process of adult neurogenesis. The beneficial effects of exercise have been well studied both dependent and independent of adult neurogenesis, from mice all the way to humans. Less is known about the effects of enrichment partially because it is not clear what "enrichment" really means. In mice, because of the lifestyle they lead in the laboratory setting, it is understandable that any other experience they have in their routine and mundane lives, is enriching. An exposure to an enriched environment where they are exposed to toys, space, smells, and other cage mates may be more indicative of what wild mice experience. Interestingly, a handful of studies have looked at rodents caught from the wild [31-33, 41, 46]. As expected, because of the lifestyles of many different species of rodents in nature, the rates of neurogenesis vary

significantly from laboratory mice whose lifestyle is much more controlled. For example, the size of territories that different species of rodents patrolled correlated with the amount of basal cell proliferation in the hippocampus [32, 33]. Interestingly, the robust effect of voluntary exercise in laboratory mice on cell proliferation does not translate to rodents caught in the wild [41]. This, however, would be expected because of the limited space laboratory mice are given whereas rodents in the wild have endless space to roam. These studies highlight the important fact that although the laboratory setting, because of the controlled environment, is a great tool to test general functions and behaviors, we must be careful in our interpretations of the results and how they translate to real-world experiences.

It is clear that this enrichment promotes the survival of a population of newborn neurons indicating that this population is somehow intimately connected with the experience they received. From my research I have explored the involvement of these newborn neurons and how they connect the mouse with its surrounding environment, however, it remains unclear how this applies to humans. In humans, we are constantly being enriched purely by the fact that the world we live in and experience on a daily basis from birth, changes constantly. New places we explore, new people or faces that we surround ourselves with, or even new skills that we acquire will always retain a certain value to us as individuals and we will need a constant supply of new neurons to add these to our experience library. This supply of new neurons and their integration into our existing circuitry allows us to make new

connections between the past experiences and new and novel experiences, just incase we come across them again.

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