Optimization of organotypic hippocampal slice cultures for adult neurogenesis research: exploring pharmacological interactions in newborn dentate granule cells

by

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Abstract

The organotypic hippocampal slice culture technique is an *in vitro* system for explanting the hippocampus and incubating tissue for up to four weeks. This method is useful because the tissue is maintained in a state closely resembling what is found *in vivo*. Previous studies have used organotypic cultures to study hippocampal physiology, but attempts to use the model specifically for adult neurogenesis research have not convincingly shown that newborn neurons can be efficiently studied and quantified.

In this thesis, we optimized organotypic slice cultures for neurogenesis research. We used the model to study extrasynaptic GABAergic signaling in newborn dentate granule neurons. Following treatment with a selective extrasynaptic $GABA_AR$ agonist, neuronal differentiation and maturation was promoted in slice cultures, but this effect was abolished following treatment with a selective NMDAR antagonist. Our results highlight the benefit of organotypic cultures for studying adult neurogenesis and describe an efficient technique for future neurogenesis studies.

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List of Abbreviations

ACSF- Artificial cerebrospinal fluid

AN- Adult neurogenesis

APV- (2R)-amino-5-phosphonovaleric acid

BrdU- 5'-Bromo-2-deoxyuridine

CA1- Cornu Ammonis 1

CA3- Cornu Ammonis 3

CaBP- Calbindin protein

CldU- 5-Chloro-2′-deoxyuridine

CNS- Central nervous system

DAPI- 4’,6’-diamidino-2-phenylindole

DCX- Doublecortin

DG- Dentate gyrus

DGC- dentate granule cell

DIV- Days in vitro

GABA- Ɣ-aminobutyric acid

$GABA_AR$- GABA Receptor, Type A

δ-$GABA_AR$- δ-subunit containing $GABA_AR$

GCL- Granule Cell Layer

GFAP- Glial Fibrillary Acidic Protein

HBSS- Hank's Balanced Salt Solution

h- hour

LTP- Long-term potentiation

MEM- Minimum Essential Medium

NeuN- Neuronal nuclei protein

PFA- Paraformaldehyde

PBS- Phosphate buffered saline

SGZ- sub granular zone

THIP- 4,5,6,7-tetrahydroisoxazolo-[5,4-c] pyridin-3-ol

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1 Introduction

1.1 History of Adult Neurogenesis in the 20th Century

The discovery of adult neurogenesis in the mammalian brain challenged long held notions about the structure and function of the nervous system, and introduced a series of questions that have garnered significant attention from the neuroscience community over the past four decades. In order to understand the scientific impact of research on adult neurogenesis, it is important to be familiar with the historical background of this field. By covering a historical perspective, we will be able to situate our discussion of the novel findings of this thesis in the relevant scientific literature.

In the late 19th century, the great Spanish neuroscientist, Santiago Ramón y Cajal, undertook pioneering work which led to the establishment of the neuron doctrine: a fundamental insight that the nervous system is composed of discrete, individual computational units, neurons, that constitute the functional building blocks of the nervous system (Ramón Y Cajal, 1995). In addition, Cajal described the concept of functional synapses as the junction point for one neuron to communicate with another. Cajal was interested in the regenerative capacity of the central nervous system (CNS) (Ramón y Cajal, 1928). However, his work also established the principle that neurons are exclusively generated during embryonic development.

Clinical observations of patients with neurological diseases can easily lead to the conclusion that no new neurons are born in the adult brain. Unlike trauma or insults to other organ systems, the nervous system appears highly susceptible to permanent and irreversible loss of function, and has limited capacity for effective regeneration. Building

from this observation led Cajal to the disappointing conclusion that “everything may die, nothing may be regenerated” (Ramón y Cajal, 1928). Additionally, the experimental assays of cell proliferation and neuronal turnover used in modern neurogenesis studies were not available to Ramon y Cajal. Despite Cajal’s seminal contributions to inaugurating neuroscience, his work also gave rise to the dogma that there was no adult neurogenesis.

New techniques for investigating cell proliferation would allow the first major breakthroughs in neurogenesis research. In 1960, Schultze and Oehlert published their findings on the utility of tritiated hydrogen-containing thymidine for the autoradiographic detection of DNA synthesis. Thymidine is a DNA nuceloside that becomes incorporated in newly synthesized DNA. Tritiated thymidine is created by pairing a radioactive hydrogen atom (^{3}H) to the thymidine nuceloside, which can be used to assay cell proliferation during the time between administration and sacrifice of the animal (Schultze and Oehlert, 1960). Cells labeled with ^{3}H-thymidine and can be subsequently visualized using autoradiography.

Beginning in 1962, Joseph Altman from the Massachusetts Institute of Technology began using ^{3}H-thymidine autoradiography to detect new cells in the adult mammalian brain (Altman, 1962). Altman’s earliest studies provided evidence for the proliferation of predominantly non-neuronal cells types, such as glia and vascular cells, but also gently suggested that neurons containing the ^{3}H-thymidine label were derived from undifferentiated cells, which underwent mitosis after the administration of the label. In 1965, Altman and Das published evidence that mitotically active neuroblasts in the rat brain incorporated tritiated thymidine in two defined regions, the olfactory bulb and the

hippocampal dentate gyrus (Altman and Das, 1965a). Further, they noted that neuroblast precursors gave rise to cells that closely resembled granule cell neurons of the dentate gyrus (Altman and Das, 1965b). However, they were cautious not to definitively proclaim the labeled cells as neurons, which was likely due to a limitation of tritiated thymidine autoradiography: it is not amenable to co-labeling with other cell-type specific markers (Gould, 2007a).

The observations reported by Altman and colleagues challenged long held beliefs regarding post-natal neurogenesis. Altman also made a prescient conjecture in the 1965 paper, especially interesting considering that the notion of neural plasticity was yet to be fully articulated. He posited that the “macroneurons” with long axons represented the fixed, stable components of the nervous system while smaller, short axon “microneurons,” such as dentate granule cells, could be “modulatory and plastic elements” that allow animals to adapt to their environment. Five decades of subsequent studies have confirmed the notion that the nervous system has potential for structural plasticity; adult neurogenesis represents a robust example of how the brain changes over the lifespan of an animal.

Following Altman’s studies, scientists continued to question the validity of claims regarding adult neurogenesis. The sociological dimensions of how this story unfolds are difficult to capture by referring exclusively to the published scientific literature. A valuable historical account of this story and the evolution of the field was provided by Altman in a 2008 memoir entitled, “The Discovery of Adult Mammalian Neurogenesis” (Altman, 2008). Eventual acceptance of adult neurogenesis by the broader scientific community took nearly 30 years. One possible explanation for the delay is that the

available experimental techniques for studying neurogenesis, for example ^{3}H-thymidine autoradiography, created an entry barrier for new investigators. Altman postulates that the difficult nature of the liquid emulsion technique required for successful tritiated thymidine autoradiography prevented interested researchers from entering the field in the 1970's and 1980's (Altman, 2008). Novel techniques for visualizing and studying adult-born neurons allowed more investigators to begin studying neurogenesis.

First, 5'-Bromo-2-deoxyuridine (BrdU) was introduced as a replacement for ^{3}H-thymidine (Gratzner, 1982). BrdU labeling is a rapid technique for labeling proliferating neurons. Compared to tritiated thymidine, use of BrdU does not require significant expertise. An important use of BrdU was for diagnostic labeling of proliferating tumour cells in cancer patients. Another major advance in the neurogenesis field was the increasing availability of cell-type specific immunohistochemical markers. For example, proteins found in granule cell neurons could be stained for using antibodies and readily available immunohistochemical protocols. Newborn neurons can be phenotypically characterized when BrdU^{+} cells co-label with other neuronal markers (Wojtowicz and Kee, 2006).

In the early 1990's, newborn neurons were identified in several studies investigating the effects of hormones on the central nervous system (Gould et al., 1992, Gould and McEwen, 1993, Cameron and Gould, 1994). These results continued to dislodge the "no neurogenesis" dogma from the prevailing scientific opinion. Additionally, evidence that psychosocial stress modulated hippocampal neurogenesis in the tree shrew resonated with Altman's early suggestion that neurogenesis confers structural plasticity and adaptability in dynamic environments (Gould et al., 1997). The following year, evidence

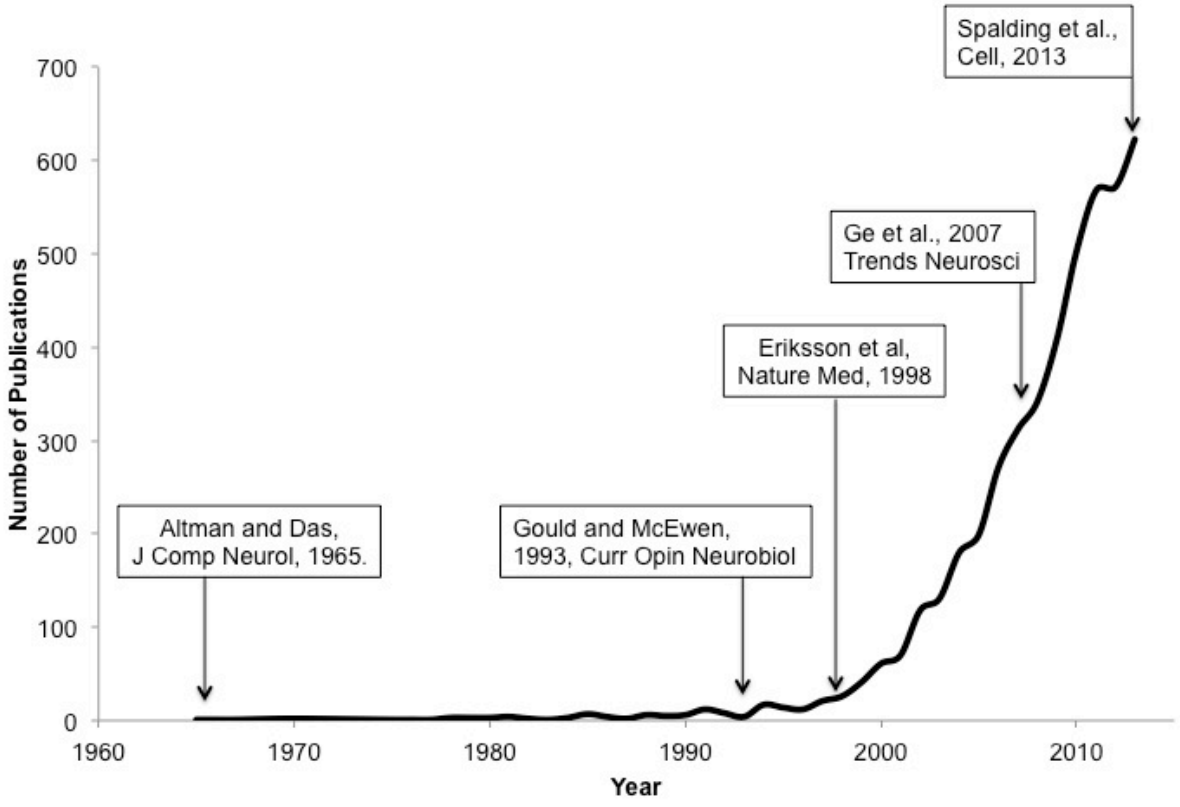
emerged that stressful experiences also negatively regulate neurogenesis in adult marmoset monkeys (Gould et al., 1998). The first report that neurogenesis occurred in the adult human hippocampus followed shortly thereafter (Eriksson et al., 1998). Eriksson and colleagues used post-mortem tissue samples from patients injected with BrdU for diagnostic purposes during cancer treatment. BrdU assays in conjunction with neuron specific immunohistochemical markers were used to provide compelling evidence that neurogenesis occurred throughout the adulthood, even in patients up to seventy years old.

Focusing on the hippocampus, researchers have now characterized adult neurogenesis in many mammalian species, including mice, rats, cats, rabbits, macaques, and humans (Altman and Das, 1965a, Kaplan and Hinds, 1977, Gould et al., 1997, Gould et al., 1998, Gould et al., 1999, Kornack and Rakic, 1999). In the 21st century, scientists are investigating questions ranging from the evolutionary basis of adult neurogenesis to the role of adult-born neurons in neural function in health and disease. As a point of comparison, the PubMed database indicates two papers were published containing the keyword “hippocampal OR hippocampus AND neurogenesis” in the year 1970, sixty-one in 2000, and six hundred new publications in 2013 alone (Figure 1).

Fundamental assumptions regarding brain plasticity have to be reconciled with emerging scientific evidence. The history of adult neurogenesis reveals an exciting account that is reminiscent of the three phases of a paradigm shift described by (Kuhn, 1962). The process of adult neurogenesis is now widely accepted by the neuroscience community and has entered the popular scientific literature (Yong, 2011, Hughes,

2013). However, the paradigm shift that occurred in the adult neurogenesis field involved a tumultuous transition to household knowledge.

Figure 1.1: Visual representation of publications in adult neurogenesis field. Some important publications in the field are highlighted, beginning with first publication by Altman and Das in 1965 for reference. Source: National Library of Medicine, PubMed data on publications containing keywords "hippocampal OR hippocampus AND neurogenesis" from 1965 to 2013.



1.2 Characteristics of Adult Neurogenesis in Mammals

1.2.1 Defining the “neurogenic zone”

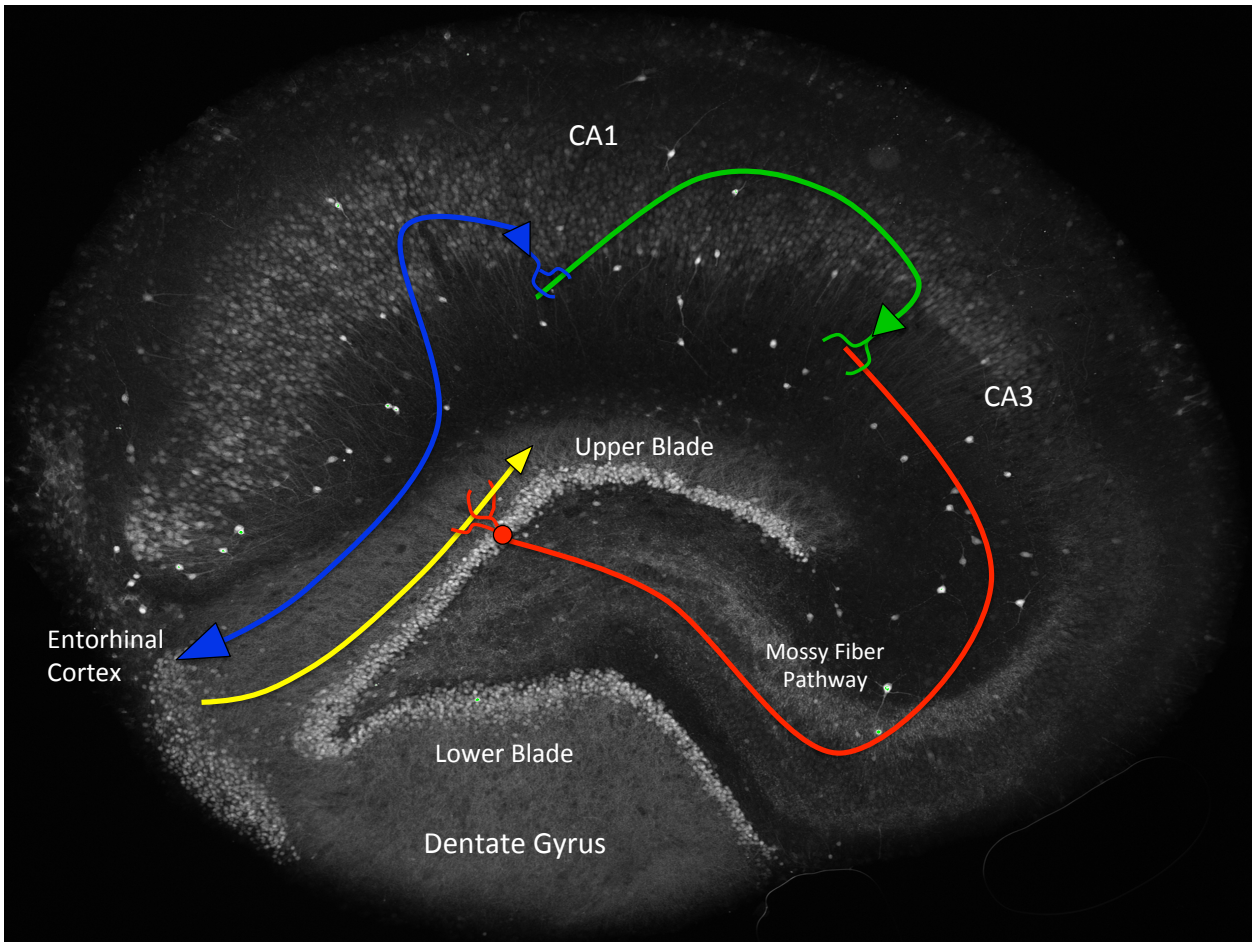
Mammalian adult neurogenesis in physiological conditions is spatially restricted to two distinct environments that are often referred to as “neurogenic” zones. These include the sub-ventricular zone of the lateral ventricles, which give rise to olfactory bulb interneurons and the sub-granular zone (SGZ) of the hippocampal dentate gyrus (DG) (Jordan et al., 2007). The focus of this thesis will be on hippocampal neurogenesis. While experimental evidence for the functional role of hippocampal neurogenesis in humans is still lacking, extrapolation from animal studies suggests that this process is important for neural function in health and disease (Lazarov and Marr, 2013).

The hippocampus is located in the medial temporal lobe of the cortex and plays a critical role in various forms of learning and memory (Nadel and MacDonald, 1980, Squire, 2004). Some of the proposed functions of the hippocampus include pattern separation (Aimone et al., 2009, Sahay et al., 2011), spatial memory (Snyder et al., 2005, Luo et al., 2007) contextual learning (Wojtowicz et al., 2008) and cognitive adaptation to novel experiences (Kempermann et al., 2008). The dentate gyrus has been characterized as a major site of input to the hippocampal formation and assists in pattern separation of incoming information from the overlying cortex.

The hippocampus has distinct anatomical regions. The dentate gyrus and cornus ammonis (CA) are the two most important regions for the present study. The dentate gyrus (DG) contains three distinct zones: upper blade, lower blade, and hilus region. The CA region is subdivided into CA3, CA2 and CA1 (Andersen, 2007). The various

anatomical regions of the hippocampus create the topographical organization that provides a structural foundation for the hippocampal “tri-synaptic circuit” (see Figure 1.2 for a depiction of the anatomy and circuit organization of the hippocampus.

Figure 1.2: Diagrammatic representation of the hippocampal tri-synaptic circuit highlights the important anatomical structures for hippocampal function: CA1, CA3 and the neurogenic zone, the Dentate Gyrus (upper and lower blade). Fluorescence micrograph shows representative transverse coronal section of hippocampus from Sprague Dawley rat brain grown in organotypic culture for 12 days in vitro. Structures are visualized with antibody for Calbindin protein (20x magnification).



1.2.2 Comparing human and rodent neurogenesis

Many animal species including birds, rodents and humans exhibit adult neurogenesis in the dentate gyrus (Lazarov and Marr, 2013). However, given the current lack of reliable studies on adult neurogenesis in humans, it is necessary to utilize experimental models. While neurogenesis has been investigated in a variety of animal species (Grandel and Brand, 2013), rodent studies have proven very effective in determining the molecular, cellular, and electrophysiological properties of adult-born neurons.

One important question is how closely the experimental laboratory conditions mirror real-life conditions. The relatively simple housing environment of experimental animals does not resemble the complex environments of rodents in the wild. However, despite these environmental differences, a study that investigated the relative rates of neurogenesis in age-matched laboratory and wild rats found essentially the same level of hippocampal neurogenesis (Epp et al., 2009, Wojtowicz, 2011).

Experimental evidence in rodents has pointed to unique contributions of adult-born DGCs to hippocampal function. These include processes such as pattern separation (Clelland et al., 2009, Sahay et al., 2011) and cognitive flexibility in spatial learning tasks (Kempermann et al., 1997). An important consideration in comparing adult neurogenesis in different species is the relative number of new neurons compared to mature dentate granule cells. The ratio of immature and mature cells, and the rate of neuronal turnover, likely have important consequences for the functional impact of adult neurogenesis (Kempermann, 2012).

Another prevalent concern in the field is whether adult hippocampal neurogenesis in rodents aligns closely enough with human neurogenesis to validate extrapolation of experimental findings to people. The dynamics of human adult neurogenesis are elusive because experimental techniques employed in animals are difficult to translate to human studies. Recently, researchers devised a creative strategy for retrospectively birth dating DGCs through analysis of their uptake of the Carbon-14 (^{14}C) radioisotope, which was released in high quantities during Cold War nuclear bomb testing and eventually spread over the entire globe (Spalding et al., 2013). Radioactive ^{14}C was incorporated into plants as CO_2 during photosynthesis, where it entered the food chain and eventually became ingested by humans. Bioavailable carbon is incorporated as one of the building blocks of newly synthesized DNA, and therefore ^{14}C from nuclear bomb testing became a constitutive element of adult-born neurons.

By comparing ^{14}C levels in post-mortem brain tissue with known atmospheric levels during the 20^{th} century, the authors were able to generate a model of cell turnover dynamics in the adult hippocampus. They reported that DGCs are generated at a rate of approximately 1400 cells per day in adult humans, and that aging results in a modest reduction in neurogenesis over the life span of the individual. Understanding the differences and similarities between mammalian species in the levels of neurogenesis is important for translational research. Spalding and colleagues suggest that previous studies over-estimated the decline of neurogenesis in aging humans (Kuhn et al., 1996, McDonald and Wojtowicz, 2005). By characterizing human cell turnover dynamics the authors provide evidence that many of the physiological and cognitive benefits attributed to adult-born DGCs have a reasonable basis for interpretation in humans.

Lastly, as evidence that there remain significant questions to address in the field, the same group of researchers used the ^{14}C-dating method to show, for the first time, that human adult neurogenesis also occurs in the striatum and leads to an annual turnover rate of 2.7% in the renewing fractions in adults (Ernst et al., 2014).

1.2.3 Proliferation, differentiation, and survival of dentate granule cells

The incorporation of adult-born neurons into the hippocampal circuitry is not a single event but a complex progression from neural stem cell (NSC) to mature neuron (Abrous et al., 2005, Dieni et al., 2013). This process requires a fine balance between providing beneficial structural and functional plasticity with potentially harmful destabilization of the hippocampal network. Below, the important characteristics of DGC proliferation, differentiation and survival are reviewed.

1.2.3.1 Proliferation

NSCs are characterized by the general properties of being capable of producing neural tissue, retain a capacity for self-renewal, and giving rise to other cells via the process of asymmetric cell division (Gage, 2000). The proliferating NSCs are self-renewing multipotential stem cells and can give rise to neuronal, glial, and endothelial cell types (Palmer et al., 2000) Studies utilizing BrdU and ^{3}H-thymidine have identified the sub-granular zone, a region of the dentate gyrus between the granule cell layer (GCL) and hilus, as the major proliferative zone for NSCs (Kuhn et al., 1996, Filippov et al., 2003), and approximately 9000 new cells are generated in the dentate gyrus each day (Cameron and McKay, 2001). Spalding and coworkers recently analyzed post-mortem human tissue for the incorporation of nuclear-bombed test derived ^{14}C isotope. By

comparing known atmospheric levels of ^{14}C with levels found in the hippocampus, the authors suggested that approximately 1400 new DGCs are added per day in humans (Spalding et al., 2013).

1.2.3.2 Differentiation

NSCs (Type I Cells) give rise to neural progenitor cells (Type II Cells), which have a more restricted cell fate and limited potential for self-renewal (Gage, 2000). Type II cells progress along a pathway of differentiation to become post-mitotic granule cell neurons. After differentiating into neurons, these cells become functionally integrated into the dentate gyrus granule cell layer. If the DGCs do not undergo programmed cell death within the first four weeks after birth, they are more likely to become functionally integrated into circuits and survive long term (Dayer et al., 2003).

1.2.3.3 Survival

The process of neuronal turnover in the dentate gyrus is a putative mechanism for establishing a renewable pool of young neurons in the hippocampus. The death of granule cells that is observed in the adult hippocampus is offset by the production of newborn DGCs along the life course. As dentate granule cells mature, they will receive input and form synapses with neighboring DGCs, hippocampal interneurons, and perforant pathway fibers arriving from the entorhinal cortex. Thus, there is a degree of activity dependent integration and survival is partially mediated by successfully forming these connections (Tozuka et al., 2005). Despite the production of new neurons in adulthood, there is a parallel process of apoptosis that removes a substantial number

Type II progenitors, as well as newborn neurons. (Gould et al., 2001, Dayer et al., 2003, Sierra et al., 2010).

1.3 Functional characteristics of hippocampal neurogenesis

1.3.1 Learning, memory, and enhanced synaptic plasticity

The functional contribution of adult-born DGCs is an area of active investigation. Some insight has been gained into their role in learning and memory. The rate of new DGC production appears to be related to learning and memory; there is a reciprocal relationship between learning and neurogenesis (Abrous, 2008).

Experimental protocols that enhance neurogenesis have been shown to improve learning ability. In the late 1990's, a series of studies showed that exposure to an "enriched" environment simultaneously increased neurogenesis and performance in the Morris water maze, a test for spatial learning ability (Kempermann et al., 1997, van Praag et al., 1999). Additionally, the neurogenesis and memory promoting effect was conserved throughout the life course, with both young (Koo et al., 2003) and adult rats (Nilsson et al., 1999) positively responding to enriched environment.

Another hypothesized role of adult neurogenesis is the encoding of temporal information for new memories (Aimone et al., 2006). Young adult-born DGCs possess unique physiological properties like enhanced membrane excitability and synaptic plasticity (Snyder et al., 2001, Ge et al., 2007). These qualities introduce the possibility that during a transient period between initial recruitment in the tri-synaptic circuit and the

stage of full maturation, DGCs may allow for the encoding of episodic memories that occur in close temporal proximity.

For example, given two unrelated events that occur during the same week, such as attending a championship-sporting event and buying a car, the same subset of newborn DGCs would be activated during the encoding of each event. Many years later, watching a highlight reel of the game may lead to the spontaneous recollection of purchasing the new car. Such temporal associations between unrelated memories are a frequent occurrence in everyday life, but understanding how the brain allows us to remember when things happened remains elusive. According to this theory, pertinent time-related information becomes preferentially encoded by young DGCs. Since DGCs are continuously added to the hippocampus, this model posits that the intrinsic rate of neurogenesis and turnover of DGCs is a necessary element that allows mammals to encode information about time. Future testing of this hypothesis can help clarify the extent to which adult neurogenesis is involved in providing a “time-signature” to episodic memories.

While enriched environments can promote neurogenesis and learning, various other factors, such as normal aging and stress, negatively influence these two processes (Gould, 2007b, Knoth et al., 2010). In laboratory settings, various experimental approaches for reducing or ablating neurogenesis have been used to study the functional significance of adult-born neurons. Using techniques such as irradiation, pharmacological ablation, or transgenic animals, investigators have shown that impairing neurogenesis leads to memory deficits (Shors et al., 2001, Winocur et al., 2006, Imayoshi et al., 2008). Interestingly, blocking neurogenesis impacts specific types

of hippocampus dependent memory functions while sparing other cognitive tasks (Lazarov and Marr, 2013). Future studies are required to more carefully assess the functional role of adult neurogenesis in hippocampus-dependent memory tasks.

1.3.2 Modulation of emotional behavior

Adult neurogenesis appears to also play a role in the pathogenesis of cognitive impairments related to anxiety and depression (Sapolsky, 2004). The link between depression and neurogenesis was first suspected following an observation that the selective serotonin reuptake inhibitor, fluoxetine (Prozac), which is commonly prescribed for major depression, led to a significant increase in hippocampal adult neurogenesis (Jacobs et al., 2000). Interestingly, the authors suggested that anti-depressant medications that enhance serotonergic neurotransmission might act by enhancing endogenous hippocampal neurogenesis. Subsequent studies further elucidated the role of neurogenesis in depression and posited that while reduced neurogenesis may not be sufficient to cause depression, it nevertheless serves a critical role in the therapeutic effect of anti-depressant medication (Drew and Hen, 2007).

Systemic glucocorticoids released in response to stressful experiences are also involved in the neurobiology of depression and the functional regulation of hippocampal neurogenesis (Cameron and Gould, 1994, Wong and Licinio, 2001) (McEwen, 2007). Recent studies have shown that hippocampal neurogenesis is directly involved in modulating depressive illness and that neurogenesis-deficient mice fail to exhibit the normal expression of the endocrine and behavioral aspects of the stress response (Snyder et al., 2011). Interestingly, the clinical benefit of fluoxetine in depression

treatment involves a 3-4 week delay (Wong and Licinio, 2001), which coincides with the developmental timeline of peak spine growth and functional maturation in dentate granule cells (Zhao et al., 2006)

1.3.3 Enriched environment, exercise, and the neurogenic reserve

Adult neurogenesis is a dynamic phenomenon that is highly responsive to environmental factors. Plasma levels of the chemokine, CCL11, are elevated in healthy aging humans and negatively regulate adult neurogenesis (Villeda et al., 2011). Villeda and colleagues highlighted an attractive area of future investigation in the neurogenesis field: How do age-related molecular changes in systemic circulation influence and accompany age-related cognitive decline? Their findings illustrate an interaction between the systemic “milieu” and the nervous system; neurogenesis may represent a sensitive gauge of an organism’s health and vitality.

Age-related cognitive decline has been linked to various early-life parameters. For example, in the 1990’s, the Nun Study of Aging and Alzheimer’s disease provided new information on the relationship between early life educational attainment and the risk of developing Alzheimer’s or other cognitive disability in old age (Snowdon et al., 1996, Snowdon, 1997). The study involved 678 American Roman Catholic sisters from the School of Sisters of Notre Dame. Snowdon and colleagues relied on the precise record keeping of the School of Sisters for their research study; they reviewed autobiographical essays that had been written by the nuns upon joining the Sisterhood many years prior. At the time of writing, the average age of the nuns was twenty-two years old. Careful analysis of the essays showed that lower linguistic ability (measured by complexity,

vivacity, fluency etc.) and low educational attainment early in life (determined from biographical records) was a strong predictor of age-related cognitive decline and the appearance of Alzheimer's disease later in life. Snowdon and co-workers found that among nuns whose writing contained complex language, only 10% later developed the disease, but among the nuns lacking linguistic density in their essays, roughly 80% went on to develop age-related cognitive decline and Alzheimer's.

Following the nun study, several other investigators have shown that educational attainment as well as physical exercise throughout life contributes to a neurocognitive reserve. This concept posits that traits in early, mid, and late life can either positively or negatively influence the cognitive performance in old age (Andel et al., 2008, Aberg et al., 2009, Puccioni and Vallesi, 2012)

Combining these findings with the notion that hippocampal neurogenesis is important for certain types of learning and memory led Kempermann (2008) to question the evolutionary advantage of experientially regulated neuroplasticity in the form of adult neurogenesis. This led to the 'neurogenic reserve' hypothesis: life-long cognitive engagement and physical activity, especially early in life, provides individuals with a retained capacity for cognitive flexibility when faced with novel information and challenges –a desirable feature of healthy mental aging.

In experimental settings, an enriched environment has also been shown to positively affect neurogenesis. Adult mice exposed to environments with numerous littermates, toys, and mazes had a threefold increase in progenitor cell proliferation after four months (Nilsson et al., 1999) and one year of enrichment compared to control animals

(Kempermann et al., 1998). In summary, adult neurogenesis is a phenomenon that recapitulates a genetic neurodevelopmental program de novo while simultaneously responding to and reflecting activity and experience along the life course.

1.4 Factors influencing neuronal maturation and survival

1.4.1 Functional convergence of embryonic- and adult-born DGCs

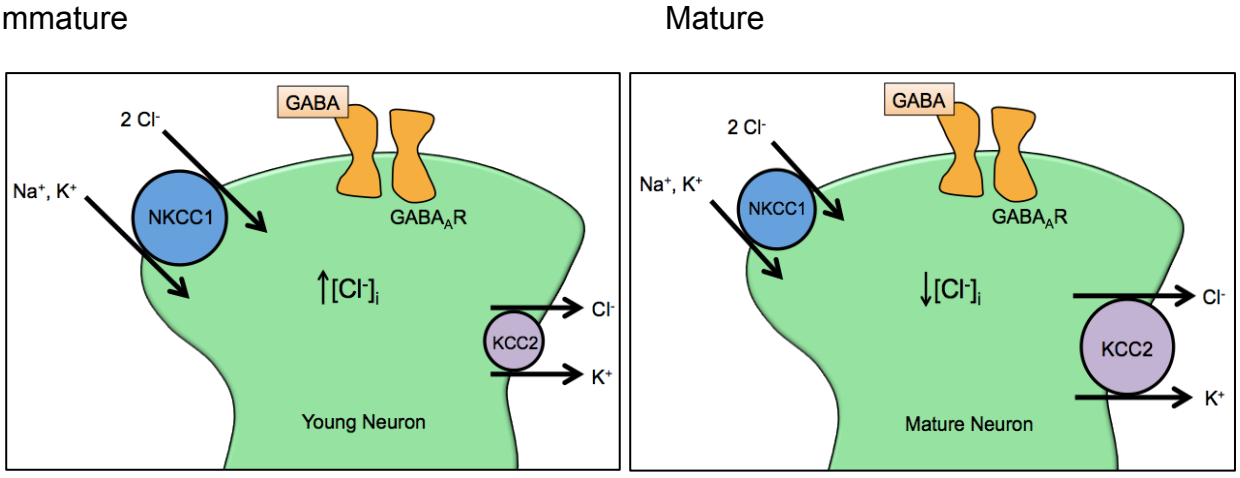
The process of hippocampal adult neurogenesis recapitulates many of the same steps that cells proceed through in the process of embryonic CNS development (Esposito et al., 2005). Importantly, once integrated into the dentate gyrus, adult-born neurons become morphologically indistinguishable from their more mature neighbors (Zhao et al., 2006). However, determining if adult-born cells are functionally equivalent has been less clear. Several studies have provided evidence that adult-born DGCs mature along a similar time course and share electrophysiological characteristics, such as firing behavior and response to excitatory neurotransmission, with embryonically generated neurons (Laplagne et al., 2006, Stone et al., 2011). These features of adult-born DGCs are referred to as ‘functional convergence’ and suggest that while new neurons may be continuously added to the hippocampus, mature DGCs in this structure are functionally homogenous. While the endpoint of becoming functionally integrated into the hippocampus may be conserved, compared to mature neurons, young newborn DGCs have unique characteristics with important implications for their function.

1.4.2 Dynamic chloride gradients in developing neurons

The concentration of intracellular chloride ($[Cl^-]i$) in immature neurons is determined by the sodium-potassium-chloride co-transporter (NKCC1), which increases the intracellular $[Cl^-]$ and creates a concentration gradient (Yamada et al., 2004). The expression of NKCC1 in immature neurons is eventually compensated by increased expression of a chloride and potassium co-transporter, KCC2, which reduces $[Cl^-]i$ (Figure 1.2).

One important quality of immature neurons is the polarity of cellular responses to the neurotransmitter Y-aminobutyric acid (GABA). The accumulation $[Cl^-]i$ in newborn DGCs leads to GABA-mediated depolarization because the ionic reversal potential for chloride is significantly more positive than the resting membrane potential of the neuron (Ben-Ari et al., 2007, Dzhala et al., 2012). The lower $[Cl^-]i$ of mature neurons leads to cellular hyperpolarization in response to GABA (Rivera et al., 1999). The developmental switch that occurs in newborn cells determines whether GABA provides depolarizing trophic support or hyperpolarizing synchronous network activity, which is critical for the timing of cellular events in the hippocampus (Raimondo et al., 2012). The timeline of transition between these two modes of response are conserved in newborn neurons and depolarizing GABA response transitions to a mature phenotype before approximately the 4^{th} week after birth (Dieni et al., 2013).

Figure 1.3: Intracellular chloride concentrations in dentate granule cells are dictated by the competing actions of NKCC1 and KCC2. In immature cells, KCC2 expression is low and NKCC1 predominates, leading to greater net Cl^- influx, which raises $[Cl^-]i$. Under these conditions, the reversal potential for chloride is more depolarized than resting membrane potential and when GABA binds there is a net Cl^- efflux, which leads to depolarization.



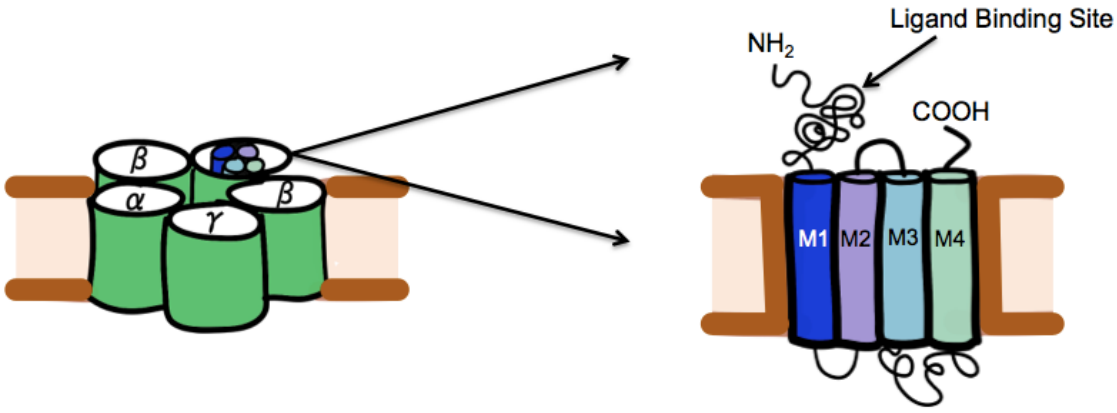
1.4.3 GABAergic signaling and $GABA_A$Rs

Gamma-Aminobutyric acid (GABA), one of the principal neurotransmitters in the nervous system, acts on either ionotropic or metabotropic receptors. The metabotropic receptors comprise the $GABA_B$ receptors ($GABA_BR$), which are G-protein coupled receptors that mediate the action of potassium channels (Turgeon and Albin, 1993). The ionotropic $GABA_A$ receptors ($GABA_AR$) mediate Cl^- movement across the plasma membrane in response to GABA binding in both synaptic and extrasynaptic locations.

$GABA_A$Rs are ionotropic (ion-conducting) ligand-gated ion channels composed of up to 19 different subunits: 6α, 3β, 3γ, δ, ε, π, θ, and 3ρ (Sieghart, 1995, Olsen and Sieghart, 2008, 2009). They are pentameric receptors meaning they have 5 subunits that are organized around a central Cl^- permeable pore and are predominantly composed of 2α, 2β and one γ or δ subunit (Figure 1.4). Each subunit consists of four trans-membrane domains and the GABA binding site is located on the extracellular surface at the interface between α and β subunits (Johnston, 1996). In mature neurons, the established ion gradient for Cl^- dictates that the anion will enter a cell, along its concentration gradient, and lead to a cellular hyperpolarization, which makes it more difficult for the cell to fire and action potential. Conversely, immature neurons have Cl^- gradient that favors anion efflux, leading to cellular depolarization (Payne et al., 2003). Neurons generated during embryonic development also pass through this switch in GABA response, but GABA generally inhibits mature neurons. One important feature of newborn DGCs in the adult hippocampus is that they recapitulate the stages of GABA response during their maturation.

The role of GABA-mediated depolarization in young DGCs is an area of significant interest for scientists seeking to understand the cellular and molecular signals that promote neuronal maturation during adult neurogenesis. One important insight into the role of GABA in DGC maturation involves the activity of GABA-releasing hippocampal interneurons, in modulating the growth and maturation of DGCs (Ben-Ari et al., 2007, Ge et al., 2007).

Figure 1.4: Schematic representation of the $GABA_A$ ionotropic ligand gated ion-channel. The 5 subunits (2α, 2β, 1γ) of the $GABA_AR$ are arranged around a central ion-permeable pore (left). Each subunit is composed of four trans membrane domains (M1, M2, M3, M4) and one α-subunit is shown to highlight the ligand binding site located on the n-terminus of domain M1 (right).



2 Hypotheses and Rationale

2.1 Hypothesis 1: Organotypic hippocampal slice cultures can be optimized to generate an *in vitro* model for studying adult mammalian neurogenesis.

2.1.1 Rationale for Hypothesis 1

The organotypic hippocampal slice culture method is an *in vitro* system for explanting the hippocampus and incubating slices of tissue on a sterile membrane to study various physiological properties. The primary goal of organ cultures is to maintain a tissue explant in a state that closely resembles what is found *in vivo.* Using this technique it is possible to preserve some features of the spatial, structural, and/or synaptic organization of the original tissue. This method provides an efficient model for studying hippocampal neurogenesis for three main reasons: 1) the characteristic topographical organization of the hippocampus is retained for up to 3 weeks (Stoppini et al., 1991); 2) slice cultures allow for pharmacological studies that exclude complex variables such as the deep anatomic location of the hippocampus and blood brain barrier physiology (Buchs et al., 1993); and 3) the protocol is relatively straightforward and, with the exception of culture membranes and a sterile dissection chamber, most materials and products are readily available.

Previous studies have used the slice culture model to study synaptic development and physiology (Muller et al., 1993), gliogenesis (del Rio et al., 1991, Subramanian et al., 2011, Ziemka-Nalecz et al., 2013), ischemic brain damage (Sadgrove et al., 2005, Strassburger et al., 2008), neuroprotection and neurorepair (Noraberg et al., 2005, Cho et al., 2007, Wise-Faberowski et al., 2009) as well as epilepsy (Staley et al., 2011, Koyama et al., 2012, Berdichevsky et al., 2013).

Various investigators have used organotypic cultures to study adult hippocampal neurogenesis (Kamada et al., 2004, Raineteau et al., 2004, Raineteau et al., 2006, Namba et al., 2007, Lee et al., 2012). Despite the utility of slice cultures, one limitation of this method for studying adult neurogenesis is the well-characterized degeneration that occurs in explanted adult tissue after 14 days *in vitro* (DIV) (Legradi et al., 2011, Kim et al., 2013). For this reason, slice cultures are typically prepared from early post-natal (P5-P10) mice or rat pups. This capitalizes on the improved viability of early postnatal brain tissue for culturing (Sadgrove et al., 2006). There are well-characterized differences between the early postnatal and adult brain with regards to synaptic physiology, and the expression of specific neuronal subtypes and neurotransmitter receptors (Fabian-Fine et al., 2000, Mielke et al., 2005). While physiological differences exist on the level of the entire hippocampus, there is still remarkable conservation of the developmental program that newborn DGCs undergo. This is the case during early post-natal or adult ages when the electrophysiological properties of newborn DGCs in cultures are similar to those of immature DGCs in the acute slice preparation (Laplagne et al., 2006) (Lee et al., 2012).

Raineteau and colleagues devised a method for administering BrdU (0.5 μM for 3 days) to slice cultures after ~14 DIV to label dividing cells in culture using a roller tube culture technique. The roller tube technique continuously rotates the explanted tissue, alternately bathing the tissue in nutrient medium and exposing it to the gaseous content of the tube. This rotational motion caused layers of cells to slough off, resulting in thin explant slices between 50-100 μm in thickness (Raineteau et al., 2004). Some of the metrics used for quantifying neurogenesis in this report do not conform to the current

standards in the neurogenesis field i.e. stereological quantification (Fritsch, 1981). For example, when reporting the co-expression of BrdU and NeuN positive cells, they indicate a total number of cells “per culture” instead of providing comparatively useful information regarding tissue area or volume.

Bunk et al. (2010) improved on the hippocampal organotypic culture technique and were able to section the cultured slices down to individual 10 μm sections. This improved visualization and immunohistochemistry (IHC) protocols by allowing antibodies to more readily permeate the tissue slice. They reported double labeling with BrdU (10 μM for 3 days) as the number of co-labeled cells per 10 μm section, but again did not provide important information about the comparative area or specific hippocampal region studied. Moreover, the confocal and fluorescence microscopy images did not convincingly demonstrate whether hippocampal morphology was successfully maintained.

Both of these previous studies used a long BrdU exposure period of 3 days. This exposure has associated drawbacks. BrdU toxicity has been well-characterized. Its use has been shown to cause morphological and behavioral abnormalities (Morris, 1991, Kolb et al., 1999) and negative effects on cell cycle, differentiation, migration and survival of neural stem cells (Bannigan and Langman, 1979, Breunig et al., 2007, Duque and Rakic, 2011). Thus, prolonged administration of BrdU in the previous studies may have introduced confounding variables that altered hippocampal physiology. While some side effects are unavoidable following BrdU administration, we chose to expose the tissue to thymidine analogues for a defined 2-hour window in order to limit some of these complications. We chose to use 5-Chloro-2′-deoxyuridine (CldU) instead of BrdU,

because it exhibited better aqueous solubility than BrdU. CldU exhibited a similar toxicity profile to BrdU and the two-hour CldU incubation was chosen to limit toxicity.

Namba et al (2007) provided valuable data on labeling methods in organotypic slice cultures by comparing the level of neuronal production following two different methods of BrdU application. They compared intraperitoneal (I.P.) injection of BrdU (50mg per kg body weight) in P5 rats with *in vitro* cultures that received culture medium containing 1μM BrdU for 30 minutes immediately following explantation of tissue. They report no statistically significant difference between *in vivo* and early *in vitro* BrdU injection in cultured tissue. The authors did not present clear images outlining the hippocampal structure but they reported BrdU immunoreactive cell numbers as percentages of total cells in the granule cell layer. Providing a more direct measure by area would have been helpful, but they do employ stereological counting techniques.

During our preliminary review of the literature on organotypic cultures, the choice of culture insert membrane used to support the explant appeared as a potential area of improvement. A commonly used insert was the Millpore Millicell polycarbonate insert. Stoppini and colleagues used this insert and cited the low-protein binding characteristic as favourable for their studies. The first culture experiments we performed also used the Millipore inserts. Following the culturing period, we attempted to use a vibratome to section the cultured tissue into 30 μm sections for IHC. The low-protein binding that was advantageous for Stoppini and colleagues was actually a limitation for our studies because cultured hippocampal slices did not adhere to the insert membrane. Therefore, tissue slices needed to be directly affixed to the vibratome cutting stage using an adhesive. The drawback of using an adhesive to affix the slice was that any tissue

exposed to the adhesive would be subsequently unusable for IHC i.e. only sections near the top of the slice could be used because the bottom of the slice would be damaged from the adhesive.

Thermo Scientific Nunc culture inserts coated with Nunclon delta surface were our next choice for culture membrane inserts. The Nunclon delta treated membrane exhibited better tissue adhesion and allowed us to affix the membrane and cultured tissue directly to the vibratome stage as a single unit. This improved adhesion of the slice allowed us to collect more useable sections for IHC because fewer sections were damaged from the adhesive. Another advantage was that the organotypic structure of the hippocampal slices was better preserved with the Nunc-treated inserts.

Previous studies have introduced organotypic cultures as a desirable *in vitro* system for studying neurogenesis, but the protocol can be optimized specifically for the requirements of neurogenesis research (e.g. to improve post-culture tissue processing). To determine if organotypic slices are suitable for adult neurogenesis research we attempted to satisfy the following three criteria: 1) generate cultures that maintain the characteristic features of hippocampal morphology after 10-21 DIV, 2) standardize a technique to quantify newborn DGCs using standard immunohistochemical markers, and 3) use field and intracellular recordings to test if cultures maintain electrophysiological properties at 10-21 DIV. Our approach for optimizing the slice culture technique for neurogenesis studies is outlined in the Methods and Discussion sections.

2.2 Hypothesis 2: The δ-subunit containing $GABA_AR$ preferring agonist, THIP, enhances neurogenesis by promoting extrasynaptic depolarizing GABA responses during a critical window of DGC maturation.

2.2.1 Rationale for Hypothesis 2

Previous studies have found that GABA-mediated depolarization affects neuronal differentiation, regulation of proliferation, neuronal development, plasticity of developing synapses, and DGC survival (LoTurco et al., 1995, Tozuka et al., 2005, Ge et al., 2007). GABA signaling through extrasynaptic $GABA_AR$ generates tonic depolarizing current in immature neurons, which is critical for the progression of NPCs into integrated, electrophysiologically viable, adult DGCs. Tonic currents are mediated by extrasynaptic $GABA_AR$'s (Nusser et al., 1998, Koyama et al., 2012) and in the mammalian dentate gyrus, extrasynaptic δ- subunit containing $GABA_ARs$ ($\delta GABA_AR$) generate the majority of tonic GABAergic current (Farrant and Nusser, 2005, Belelli et al., 2009) The δ-subunit containing $GABA_ARs$ have high affinity for GABA and are weakly desensitizing (Saxena and Macdonald, 1994), though the latter characterization has been questioned (Bright et al., 2011). While tonic current provides an important source of early electrochemical activity in adult-born DGCs, it is unknown whether GABA agonists that preferentially activate extrasynaptic $GABA_ARs$ enhance hippocampal neurogenesis *in vitro*.

In this study, 4,5,6,7-tetrahydroisoxazolo [5,4-c] pyridin-3-ol (THIP), which is a $\delta GABA_AR$-preferring agonist, was used to study the role of $\delta GABA_ARs$ in newborn DGC maturation and survival. THIP was developed as an experimental sleep-aid drug and produces analgesia and hypnosis in humans and rodents (Krogsgaard-Larsen et al., 2004). THIP is a remarkably potent agonist of $\alpha_4\beta_3\delta$, and $\alpha_6\beta_3\delta$ -containing receptors

(Storustovu and Ebert, 2006) and has been shown to elicit GABA currents at nanomolar concentrations (Meera et al., 2011). The putative binding site for THIP is the GABA binding site (Bergmann et al., 2013) and it undergoes competition for this site, which is supported by evidence that GABA attenuates THIP-mediated enhancement of tonic inhibition (Houston et al., 2012).

Based on previous studies in our laboratory, THIP was shown to promote neurogenesis and hippocampus dependent learning *in vivo* (Whissell et al., 2013). The present studies used a THIP concentration of 5 μM, which was previously shown to act on δGABA$_A$R without inducing behavioral deficits (Herd et al., 2009, Vinkers et al., 2009, Mortensen et al., 2010) and is below the ~10 μM threshold concentration for activating synaptic $\alpha_4\beta\gamma2$ GABA$_A$Rs (Brown et al., 2002). However, while low doses of THIP appear to have selective effects on extrasynaptic receptors, there is evidence that THIP also has effects at extrasynaptic non-δ subunit containing GABA$_A$R's. For example, low concentrations of THIP (~5 μM) elicited a tonic current and produced analgesic effects in δ subunit knockout mice (Bonin et al., 2011), suggesting nonselective actions of this drug. Nevertheless, GABA$_A$R's containing the δ-subunit exhibit a significant increase in THIP sensitivity (Meera et al., 2011). The activation of extrasynaptic GABA$_A$R's is hypothesized to promote DGC maturation and survival in hippocampal slice cultures by directly activating immature newborn neurons.

2.3 Hypothesis 3: Blocking glutamate transmission mediated by NMDA receptors will prevent THIP-mediated promotion of neuronal differentiation stage of neurogenesis.

2.3.1 Rationale for Hypothesis 3

Glutamate is the most abundant excitatory neurotransmitter in the CNS (Meldrum, 2000) and plays an important role in the development of newborn granule cells. The N-methyl-D-aspartate (NMDA) ionotropic glutamate receptor (NMDAR) facilitates cation (i.e. Ca^{2+}) permeability and serves as an important regulator of glutamatergic signaling in immature granule cells. Glutamatergic signaling mediated by NMDARs helps regulate adult neurogenesis (Cameron et al., 1995, Gould et al., 1997). While NMDARs are ligand-gated ion channels, they also possess a voltage-dependent Mg^{2+} pore blocker that prevents other permeant cations from entering the cell at resting membrane potentials (Mayer et al., 1984, Nowak et al., 1984). The cation pore in the center of the NMDAR will only allow ions to flow when the following two conditions are satisfied simultaneously: the post-synaptic cell membrane is depolarized, and glutamate is bound to the receptor. This quality of NMDARs is termed “coincidence detection because it allows glutamate binding and cell depolarization on the same target cell to be temporally and spatially associated with each other (Seeburg et al., 1995). One important characteristic of NMDARs for the present study is that the voltage dependent Mg^{2+} blockade can be relieved by depolarizing GABAergic currents in immature neurons with elevated $[Cl^-]_i$ (Leinekugel et al., 1997, Wang and Kriegstein, 2008).

Komuro and Rakic (1993) provided evidence that in newborn neurons, NMDARs play an important role in modulating calcium-dependent cell migration, influencing where neurons move and how they establish synaptic partners. Blocking NMDAR on newborn

neurons with specific antagonists led to impeded cellular migration, whereas activating NMDARs led to increased migration. Fluctuations in $[Ca^{2+}]_i$ drive cytoskeletal changes that are precursors for motility and migration of neurons.

GABA mediated cellular depolarization in immature DGCs has been shown to act "synergistically" with NMDARs containing predominantly the NR2B subunit (Paoletti and Neyton, 2007).This form of signaling plays a critical role in activity dependent survival of DGCs and blocking GABAergic depolarization (Jagasia et al., 2009) or NMDAR inactivation through genetic silencing (Tashiro et al., 2006) can lead to granule cell apoptosis. During a critical survival window between 1-3 weeks after cell birth, DGCs require depolarizing GABA currents to drive NMDAR activation, which serves as a precursor to synapse unsilencing and eventual functional integration into the dentate gyrus (Ge et al., 2006).

The relationship between extrasynaptic GABAergic signaling in immature granule cells and NMDARs is less well characterized. In order to address the issue of functional synergy between $GABA_A$Rs and NMDARs, we co-applied THIP with a competitive antagonist of NMDARs, (2R)-amino-5-phosphonovaleric acid (APV, 10μM), in order to determine if NMDARs were necessary for GABA-mediated promotion of adult neurogenesis.

3 Materials and Methods

Ethics Disclosure: All animal procedures were approved by the Animal Care Committee and conformed to the animal health and welfare guidelines of the Department of Comparative Medicine at the University of Toronto.

3.1 Subjects:

Thirty-eight early post-natal (P7) Sprague Dawley rat pups from Charles River were used for the experiments described in this thesis. Organotypic hippocampal slice cultures were generated from these animals from November 2012 to January 2014 in a series of 6 culture 'batches.' Reported "n" values in the results section correspond to number of animals i.e. "n=12" signifies 12 animals were used. Each animal brain generated between 15-20 hippocampal slices for culturing.

3.2 *In vitro* organotypic hippocampal slice cultures

The following protocol for organotypic slice cultures was modified from (Stoppini et al., 1991) for neurogenesis research and provides step-by-step instructions for using this method:

Table 1: Materials

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
5-chloro-2'-deoxyuridine (CldU)	MP Biomedicals	105478	Hazardous, Carcinogenic
Cell culture inserts, 30mm diameter, 0.4µm pore size	Thermo scientific	140660	Nuclon delta coating on these inserts provides better tissue adhesion and improves slice quality.
Conical Centrifuge tubes (sterile)	Fisher Scientific	14-432-22	
Dissector scissors (angled to side)	Fine Science Tools	14082-09	
Minimum essential medium (MEM)	Gibco	11095; liquid	Store at 4°C
Eclipse Ni-U fluorescent microscope	Nikon		
Glue for tissue	Krazy Glue	KG585	Use minimum amount of glue to achieve adhesion as any tissue exposed to glue will be unusable for IHC.
Hank's Balanced Salt Solution (HBSS) (500 mL)	Gibco	14025-092	Store at 4°C
Horse Serum Heat Inactivated (500 mL)	Gibco	16050-122	Make 50 mL aliquots and store at -20°C
Kimwipes	Kimberly-Clarke	TW 31KYPBX	
Modified glass pipettes (bottom of Pasteur pipette removed and edge smoothed with Bunsen flame)			
Petri Dish (100mm x 15mm) and (60mm x 15mm)	Fisher Brand	FB0875712 and FB0875713A	
Scalpel blades #11	Fine Science Tools	10011-00	
Scalpel handle #3	Fine Science Tools	10003-12	
Serological Pipettes	Sorfa Medical Plastic Co.	P8050	

Standard Pattern forceps	Fine Science Tools	11000-12	
Sterile vacuum filter	Thermo-Scientific	565-0020	
Surgical Scissors	Fine Science Tools	14054-13	
Syringe driven filter unit	Millipore-Millex	SLGP033RS	
Tissue chopper with moveable stage	Stoelting	51425	
Fine tip paintbrush			

Table 2: Solutions and medium for organotypic cultures

Solution	Ingredients and Instructions
1) Dissection solution:	a) 500 mL of Hank's Balanced Salt Solution (HBSS) (Gibco-14025-092). b) Add 2.2 g D-glucose. c) Add 0.5 g Sucrose. d) Add 1.787 g HEPES. e) Mix for 30 minutes with magnetic stir plate. f) Use pH meter to ensure solution has a final pH= 7.4. g) Use osmometer to ensure final osmolality= 320-330 mOsm. h) Sterilize solution in sterile laminar flow hood using vacuum filtration through 0.2 µm filter.
2) Serum-containing culture medium: 100 mL Minimum Essential Medium (MEM) (Gibco 11095), 50 mL Horse serum (Gibco 16050-122), 50 mL HBSS.	a) Add the following to 50 mL HBSS in beaker and dissolve in 37°C water bath. Mix with magnetic stir plate. b) 1.3 g D-glucose. c) 36 mg $MgSO_4$. d) 17.6 mg Ascorbic acid. e) 5 µL of 2M $CaCl_2$ stock solution. f) Add 50 µL Antibiotic-Antimycotic (100x stock, sterile; Gibco 15140-062). g) 1 µg/mL Insulin. h) Sterilize above solution by filtration through a 0.2 µm filter. i) Mix filtered solution with 100 mL MEM and 50 mL Horse serum in laminar flow hood. Make 50 mL aliquots in sterile conical centrifuge tubes (Fisher Scientific-14-432-22) and store at 4°C.
3) 4% Paraformaldehyde fixative solution.	a) Prepare phosphate buffered saline (PBS) by adding the following to 300 mL of distilled H_2O and mixing on magnetic stir plate. b) Add 2.7 g sodium phosphate monobasic (NaH_2PO_4). c) Add 11.5 g sodium phosphate dibasic ($NaHPO_4$). d) Add 9.0 g sodium chloride (NaCl). e) Heat 700 mL of distilled H_2O to 55 °C and turn off heat. f) Add 40 g paraformaldehyde (PFA) and stir into 700 mL of water using magnetic stir plate.
4) 0.1% Sodium Azide Solution	a) Add 1g of powdered sodium azide (NaN_3) to 1 L of PBS solution. b) Mix using magnetic stir plate and store at 4°C.

1. Preparation of Hippocampal Slices

1.1. Sterilize the following instruments using the dry autoclave at 125°C. When sterile, put the instruments into a sterile container and keep covered until use. Immediately before dissection, immerse instruments in 70% ethanol: H_2O solution.

Scalpel handle (#3) (2).

Standard pattern forceps, large (1).

Small dissector scissor (angled to side) (1).

Micro spoon (spoon and flat spatula ends) (1).

Micro-spatulas (rounded and rounded tapered ends) (2).

Fine paintbrush (1).

Fire polished Pasteur pipette (2).

Gauze squares, 2 x 2 in. (5).

1.2. Prepare a 6-well culture plate before beginning dissection procedure by adding 1 mL of culture medium/well and storing plate in the incubator at 35 °C and 5% CO_2

2. Arrange dissection tools in sterile laminar flow hood

2.1. Spray laminar flow hood with 70% ethanol: H_2O and remove sterilized dissection instruments from alcohol. Allow instruments to dry while resting on sterile Petri dish to avoid contact between alcohol and dissected brain tissue.

2.2. Deposit 5-7 mL of sterilized ice-cold dissecting solution in 2 large sterile Petri dishes. One dish will chill and clean the head (dirty), the other one for

cooling and rinsing the scooped out brain (clean). Place a sterilized filter paper in one of the Petri dish lids for dissecting out the brain.

2.3. Deposit 3-5 mL of sterilized ice-cold dissecting solution in 2 small, sterile Petri dishes. One dish will hold the scooped out hippocampus, one will hold sections during separation of hippocampal sections under dissection microscope. Place a small, sterilized filter paper in one of the small Petri dish lid for dissecting out the hippocampus.

2.4. Prepare Stoelting tissue chopper by taping a piece of sterilized filter paper to the cutting stage and mounting sterile razor blade. Wet the filter paper with sterilized dissecting solution.

2.5. Spray a clean bio-bag with alcohol and place in laminar flow clean bench.

2.6. Refer to Figure 3.2 for sample set-up of dissection instruments.

3. Hippocampal Dissection

3.1. Spray the P7 Sprague Dawley rat pup with 70% ethanol: H2O outside of the laminar flow clean bench and quickly decapitate the animal using large sterile surgical scissors inside the laminar flow bench. Let the head drop into ice-cold dissecting solution in one of the Petri-dishes.

3.2. In the Petri dish, rinse off the blood and quickly transfer the head to sterilized filter paper, ventral side down.

3.3. Using the scalpel, cut along the dorsal surface in the sagittal plane to expose the underlying skull. Cut through the skin, but not the underlying bone, which is soft and easily penetrable in rats of this age. Set aside this “dirty” scalpel and do not use on brain tissue.

3.4. Using the small dissector scissors (angled to side) and forceps, cut open the

skull along sagittal suture of the skull to bregma, the anatomical point on the skull where the coronal suture is intersected perpendicularly by the sagittal suture. Use forceps to pull skull flaps up and away from the midline of the skull.

3.5. Place the micro spoon on the underside of the brain, beneath the brain stem, to gently lift the brain out of the skull. Lifting the brain will expose the optic nerves and olfactory bulb on basal surface of brain. These structures must be cut with small scissors to fully detach the brain from skull. Remove and transfer the intact brain to the other large Petri dish containing ice-cold dissecting solution.

3.6. Using the micro spoon, transfer the brain to a small Petri-dish lid containing sterile filter paper. With a sterile Pasteur pipette, rinse the brain with a few drops of dissecting solution to keep tissue moist.

3.7. Using a “clean” scalpel blade, cut the two hemispheres apart. Transfer the left hemisphere back to large Petri dish with micro spoon and place hemisphere pia side down in ice-cold dissecting solution for subsequent use.

3.8. View the medial face of the right hemisphere and identify the edge fimbria, a prominent band of white matter along the medial edge of the hippocampus. Using a sterile scalpel, make a sagittal cut through the fimbria, but take care because only 0.5 cm of the scalpel tip will be sufficient to cut the fimbria.

3.9. Using 2 micro-spatulas, remove the first hippocampus from right hemisphere by placing the right-hand-spatula on the brain stem and lifting the overlying cortex with the left-hand spatula. Gently lift the cortex to reveal

the lateral ventricle and medial surface of the hippocampus. A white curved line, the fimbria, should now be visible.

3.10. Align curvature of spatula with curvature of the fimbria and gently press spatula under the fimbria. Slide spatula left and ride along rostral-caudal axis and then lift spatula in dorsal direction to remove hippocampus.

3.11. Transfer the hippocampus to a 2nd small Petri dish with ice-cold dissecting solution. Repeat the same procedure on the left hemisphere to remove left hippocampus.

3.12. Using a micro spatula, carefully transfer the hippocampi to the Stoelting tissue chopper stage. Arrange them adjacent and parallel to each other and perpendicular to the axis of the chopper blade. Use a paintbrush to position the tissue and add a few drops of the dissecting solution on top of the hippocampi.

3.13. Cut the tissue in 400 µm slices without pausing to remove individual slices (usually they will not adhere to the blade). After the whole hippocampus has been cut, use the paintbrush to gently transfer the sections to 2nd small Petri dish with dissection solution.

3.14. Under a dissecting microscope, carefully separate the floating slices using a micro-spatula and paintbrush.

3.15. Remove the pre-prepared culture plate from the incubator and place in laminar flow hood.

3.16. Using a fire-polished Pasteur pipette, draw 4-5 slices into the pipette and transfer slices to the apical surface of culture insert membrane. Next, adjust positioning with a paintbrush and leave space between individual sections

and the border of the culture insert.

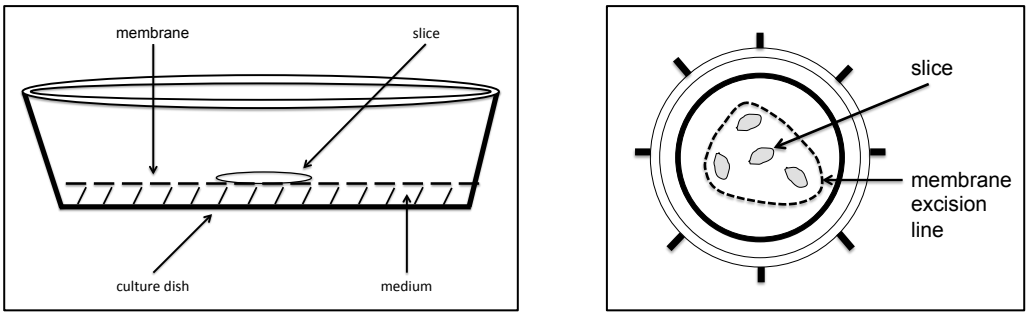
3.17. Using a sterile Pasteur pipette, remove the excess dissecting solution from apical surface of membrane.

Caution: While removing solution avoid drawing tissue sections into pipette. Alternatively, use regular pipette (P200 or P1000) with sterilized pipette tips to slowly remove solution.

3.18. Place the culture plate with serum-containing culture medium and the hippocampal slices back in the incubator at 35 °C and 5% CO_2.

Figure 3.1: A. Diagrammatic representation of the slice culture insert from side (left) and top-view (right). **B.** Illustration of sequential steps for hippocampal dissection in laminar flow clean bench. Dissection steps proceed from left to right (1-8): decapitation of animal (1), removal of brain (2), transfer of brain to ice-cold dissection solution (3), dissection hippocampus from left and right hemispheres (4), storage of dissected hippocampi in ice-cold dissection solution (5), transfer of both hippocampi to Stoelting tissue chopper and sectioning at 400 µm (6), separation of individual slices under dissecting microscope (7), and plating tissue on cell culture inserts (8).

A.



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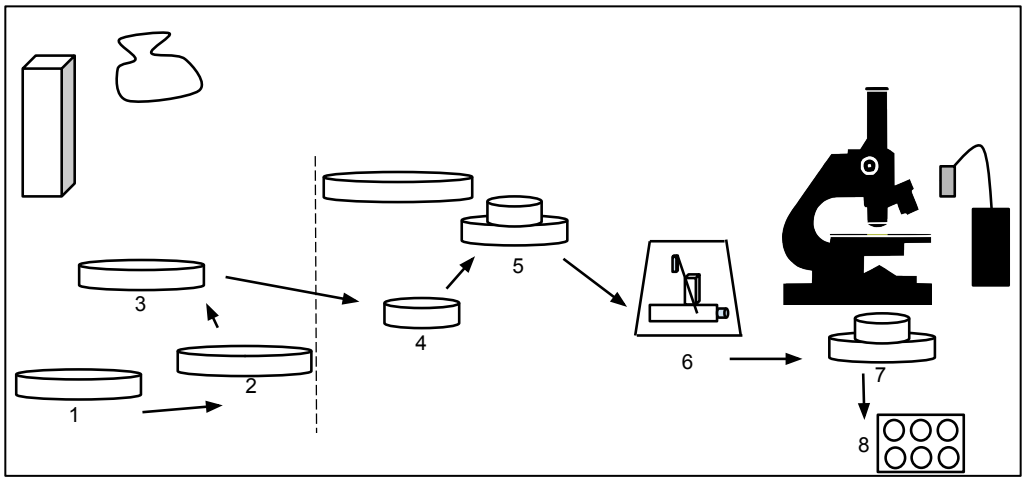
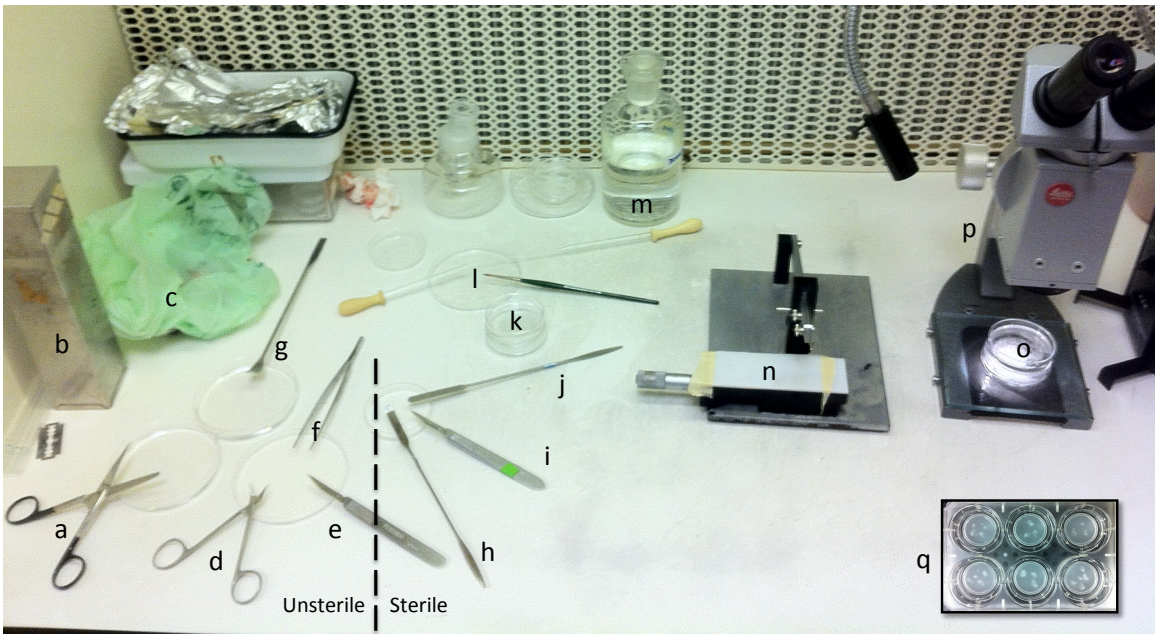


Figure 3.2: Photograph of instrument set-up for dissection. Arrangement of instruments helps avoid contamination of tissue. Letters identify instruments and objects needed for dissection (a-q): Surgical scissors (a), sterile container for glass Pasteur pipettes (b), bio-bag for discarding rat cadavers (c), dissector scissors (d), 'dirty' scalpel for skull (e), standard pattern forceps (f), Fisher Scientific micro-spoon for removing brain (g), Fisher Scientific micro-spatula for lifting cortex (h) and removing hippocampus (j), 'clean' scalpel for cutting fimbria (i), small Petri dish containing ice-cold dissecting solution to hold removed hippocampi (k), lid of large Petri dish for resting Pasteur pipettes and autoclaved paintbrush (l), ice-cold dissecting solution (m), Stoelting tissue chopper (n), small Petri-dish containing ice-cold dissecting solution for separating hippocampal slices under microscope (o), dissecting microscope (p), culture 6-well plate for storing hippocampal slices (q).



4. Feeding and Maintaining Organotypic Slices

4.1. Feed cultures in sterile laminar flow clean bench.

4.2. The first feeding of the cultured sections should be done 2 days post-dissection. Aspirate old culture medium using sterilized glass pipette.

4.3. Use sterile 5 mL serological pipette to add 1 mL of fresh, sterile, serum-containing medium to the wells.

4.4. Gently replace the culture insert and take care to remove any air bubbles that may have formed underneath membrane surface.

4.5. After first feeding, change medium every other day.

5. Incubating tissue slices with thymidine analogues to label newborn neurons

5.1. In order to study the maturation and integration of dentate granule cells in the hippocampus, organotypic slices can be incubated with a thymidine analogue, such as Bromodeoxyuridine (BrdU) or Chlorodeoxyuridine (CldU). In the present experiments, CldU was used because of its higher solubility in the culture medium.

5.2. After 3 DIV, add 10 µg/mL of BrdU for every 1 mL of culture medium. Add medium to culture wells and incubate tissue with CldU-containing medium for exactly 2 h.

5.3. Following 2 h incubation, remove CldU-containing medium and replace with regular feeding medium to resume normal feeding schedule (outlined above).

6. Tissue Fixation and storage

6.1. Establish timeline for applying treatments and fixing tissue samples before starting culture experiments. **(Figure 3.2)**.

6.2. At a predetermined day post-dissection, prepare the following in a laboratory fume hood: 1) 10-50 mL beaker containing 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS); 2) empty beaker for discarded culture medium; 3) small forceps; and 4) 1000 mL pipette with disposable tips.

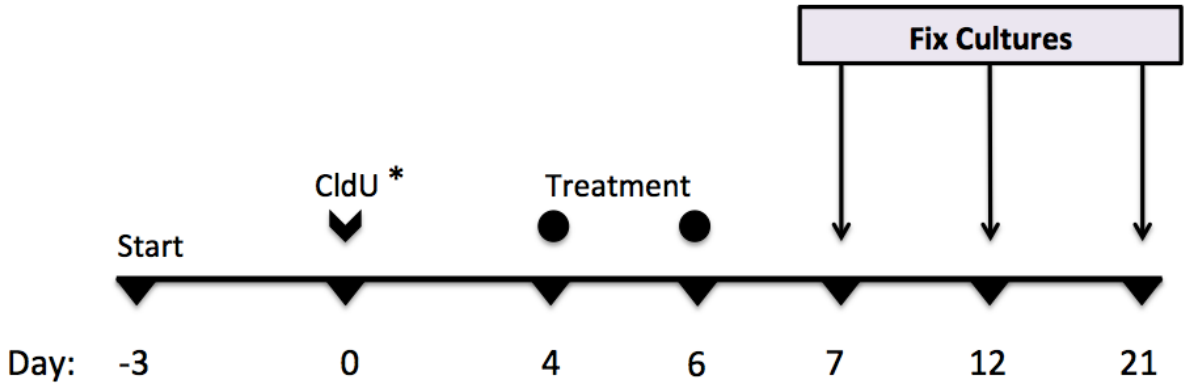
6.3. Remove culture plate(s) from incubation chamber and transfer to fume hood. Lift individual well plate inserts with forceps. Next, use pipette to remove culture medium and transfer to disposal beaker. Tilting the culture plate on an angle can help ensure that all culture medium is removed.

6.4. Finish removing medium for one culture plate at a time. When medium has been removed, add 1 mL of 4% PFA to each culture well and seal well plate with parafilm. Repeat for as many culture plates as necessary and transfer plates to refrigerator at 4°C for 24 hours (h).

6.5. After 24 h, prepare the following in a fume hood: 1) 10-50 mL beaker containing 0.1% Sodium Azide in PBS; 2) an empty beaker for discarded PFA (follow safety precautions when discarding toxic substances); 3) small forceps; and 4) 1000 mL pipette with disposable tips.

6.6. Follow procedure outlined in 6.4 for adding PFA, but add 1 mL of sodium azide in PBS instead. Once completely transferred, seal well plates for future use by wrapping edges in parafilm and storing in refrigerator at 4°C.

Figure 3.3: **Experimental timeline employed for slice culture experiments.** Asterisks indicate the administration of CldU (10 µg/ mL) for a 2 h incubation period. Treatment groups within the cultures were as follows: 1) Control vs. THIP (5 µM), 2) THIP vs. THIP + APV (10 µM), 3) Control vs. THIP vs. APV. **Note**: culture medium was changed every other day following the second DIV.



7. Sectioning tissue for immunohistochemistry

7.1. Tissue sectioning is performed using a Leica VT 1000S vibratome. The following series of steps help maximize the yield of usable tissue sections from organotypic cultures.

7.1.1. Note: Immediately following hippocampal dissection and plating of slices, the tissue has a thickness of approximately 400 µm. However, after 2-3 weeks in the incubation chamber, tissue slices will begin to flatten, resulting in a final section thickness of 150-300 µm.

7.2. Prepare the following items to section cultured tissue: 1) #11 scalpel blade and handle, 2) glass Petri dish containing ice cold PBS, 3) micro-dissection forceps, 4) clean vibratome cutting stage to mount tissue.

7.3. Tissue will adhere to the membrane of Nunclon-treated Thermo Scientific cell culture insert (30 mm diameter) insert after 7 DIV, which allows the membrane to be directly attached to the cutting stage of vibratome using glue. Use scalpel to carefully cut along the perimeter of the circular insert membrane so that it can be detached from the plastic insert housing. Take care to leave ample space between the cultured slice and the scalpel.

7.4. Transfer detached insert membrane to Petri dish containing PBS. After rinsing in PBS, use forceps to transfer the membrane to the vibratome mounting stage.

7.5. Next, use the scalpel to eliminate excess membrane surrounding cultured slices and cut away excess material to create clean edges. This step will ensure the membrane is flat and can easily adhere to the cutting surface.

7.6. Place 1-2 drops of Krazy Glue (KG585) on the vibratome cutting stage and

spread in even layer using a 22-gauge needle. Spread Krazy Glue into rectangle shape with long edge parallel to the cutting blade of the vibratome. This step must be performed quickly.

7.7. Use forceps to transfer trimmed membrane containing hippocampal slices to the cutting stage and gently position membrane on glue and ensure that there are no air bubbles.

7.8. As the superglue dries, transfer vibratome stage with glued membrane back to the PBS containing Petri dish. Prepare the vibratome blade and a 48 well plate containing sodium azide to store tissue sections.

7.9. Use vibratome to generate 30 μm sections of the organotypic slice tissue and transfer to a 48 well plate containing sodium azide for storage and subsequent immunohistochemical staining.

8. Optimizing the slice culture protocol for adult neurogenesis studies

Cultures were incubated with CldU for exactly 2 h at 3 DIV to label dividing neural stem cells. The narrow time window was chosen to improve the likelihood that labeled neurons constituted a homogeneous population of cells at approximately the same maturational stage. Following CldU administration, the timeline of application of pharmacological agents was chosen to target newborn DGCs during particular developmental windows. The GABA agonist, THIP, was applied at 4 and 6 days post-CldU injection because $CldU^+$ cells of that age exhibit high $[Cl^-]i$ and would therefore be depolarized by GABA (Dieni et al, 2013).

The protocol presented above for sectioning cultured slices into 30 µm sections for IHC has not been described previously. This optimization was thought to improve penetration of antibodies for fluorescence microscopy and thus improve visualization of the hippocampal slices to permit analysis of cellular and morphological features of organotypic slice cultures.

Organotypic cultures provide an opportunity to test the effect of various pharmacological agents as hippocampal DGCs pass through distinct developmental stages during maturation. To test the effect of various drugs on neuronal proliferation, migration, and growth, compounds can be applied directly to the hippocampal slice cultures. The present experiments aimed to characterize the effect of extrasynaptic GABAergic signaling on DGC proliferation and survival during a critical window of depolarizing GABA responses from 4-7 days after birth. We also wanted to determine if NMDARs were necessary for mediating the effect of GABA signaling. For this reason, THIP (5 µM) and APV (10 µM), an NMDAR blocker, were dissolved directly into the culture

medium and incubated with the culture tissue for 4 days. This modification of the organotypic culture protocol can be used in future experiments to test other pharmacological agents and timelines.

Another important modification is that we removed culture inserts from the incubation chamber and fixed the hippocampal tissue at different time points after CldU application (Figure 3.3). This strategy allowed us to determine the impact of THIP, APV, or a combination of treatments on the differentiation and survival of DGCs at three different times (7, 12, and 21 days post CldU application).

3.3 Field and intracellular recordings in slice cultures

A short series of experiments sought to characterize the electrophysiological properties of hippocampal slice cultures and determine if they retained some of the network characteristics of acute hippocampal slices. Briefly, between 6 days and 2 weeks *in vitro*, culture inserts were removed from the incubation chamber and individual hippocampal slices were carefully excised from the surrounding culture membrane using a scalpel. Slices were bathed in ~30°C artificial cerebrospinal fluid (ACSF) containing the following ingredients (in mM): 125 NaCl, 2.5 KCl, 1.25 Na2PO4, 2 CaCl2, 1 MgCl2, 25 NaHCO3, and 25 D-glucose perfused with a 95%O_2 /5% CO_2 carbogen mixture at a rate of 2 mL/min. Before experiments started, individual slices were transferred to the electrophysiological recording chamber.

3.3.1 Field recordings

Field recordings measure electrophysiological characteristics from afferent synapses and provide information about populations of granule cells. The recording areas for

these experiments are summarized in Figure 3.4. Bipolar electrodes were inserted into the medial perforant pathway of hippocampal slices to deliver a predefined current that was recorded by glass micropipette recording electrodes. The recording electrodes were prepared using an electrode puller (Sutter Instruments, Model P-87) and consistently had a tip diameter between 5-10 μm, which conferred electrode resistance of ~1 MΩ. Recording the initial slope (0-0.4 ms) after stimulation provides a measurement of the response magnitude for field excitatory postsynaptic potentials (fEPSP), which is a common measure of synaptic physiology employed in acute slice experiments.

Acute hippocampal slices have a well-characterized capacity for long-term potentiation (LTP) that involves a short-term protein synthesis independent (Ko et al., 1997) and long-term synthesis dependent phase (Nguyen and Kandel, 1996)). After allowing the slice to acclimatize to the recording chamber for 15 minutes, we sought to establish a stable baseline recording. The slice was stimulated with the following baseline parameters: train interval of 20,000 ms, train duration of 50 ms, and pulse interval of 40 ms. This protocol produced twin pulse stimulation every 20 seconds. Following approximately 20 minutes of stable recordings, the following LTP induction parameters were used: train interval of 20,000 ms, train duration of 500 ms, and pulse interval of 10ms. This protocol produced brief, high frequency (100 Hz) trains delivered every 20 seconds. Four such trains were used to induce LTP.

3.3.2 Intracellular recordings

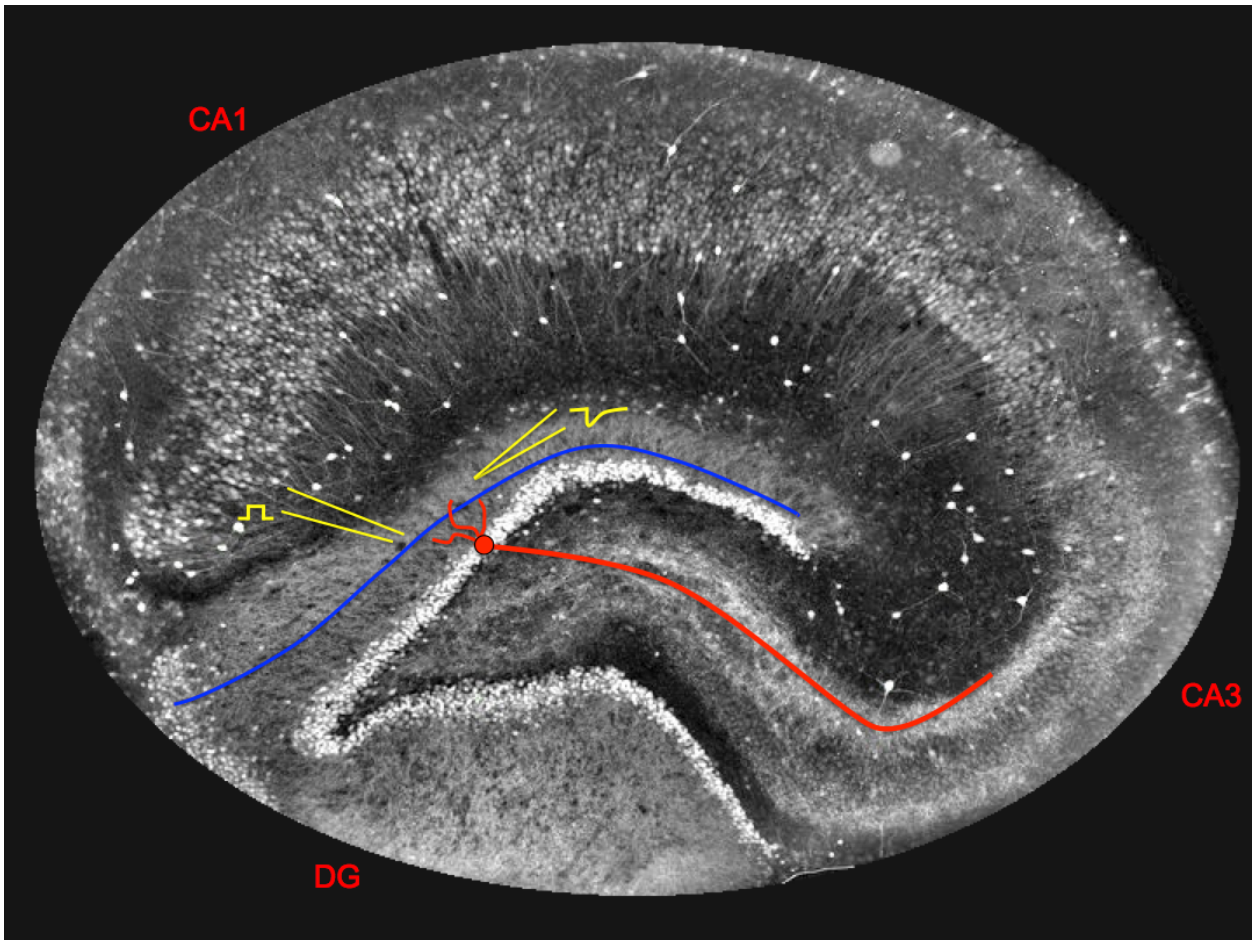
Intracellular recordings were performed in the same conditions as field recordings, except the recording microelectrode diameter was between 0.5 – 1.0 µm, which yielded an electrode resistance of ~10 MΩ. In this case, we filled pipettes were with intracellular recording solution with the following ingredients (in mM): 130 K gluconate, 23 NaCl, 15 $KMeSO_4$, 10 HEPES, 0.1 EDTA, 2 Mg-ATP, 0.2 GTP. The pH was adjusted with to 7.3 with NaOH and the osmolality was adjusted to 300-310 mOsm.

Additionally, we report a pharmacological experiment using the selective $GABA_AR$ agonist, THIP. THIP (5 µM) was dissolved into the ACSF and bubbled with 95%O_2 /5% CO_2 before being added to the perfusing solution after approximately 20 minutes of stable baseline recording. Following 20 minutes of exposure, THIP was washed out with fresh ACSF and recording continued for approximately 1 hour.

3.3.3 Data analysis for electrophysiology experiments

The fEPSP were reported as a percentage relative to the stable baseline responses attained ~5-10 minutes before LTP induction.

Figure 3.4: Schematic representation of the hippocampal formation and approximate positions of electrophysiological recordings in slice cultures. Pictured is a CaBP immunostained section of slice tissue with overlaid anatomical identifiers. The principal cell layers, CA3 and CA1 and the dentate gyrus (DG) can be clearly identified.



3.4 Immunohistochemistry

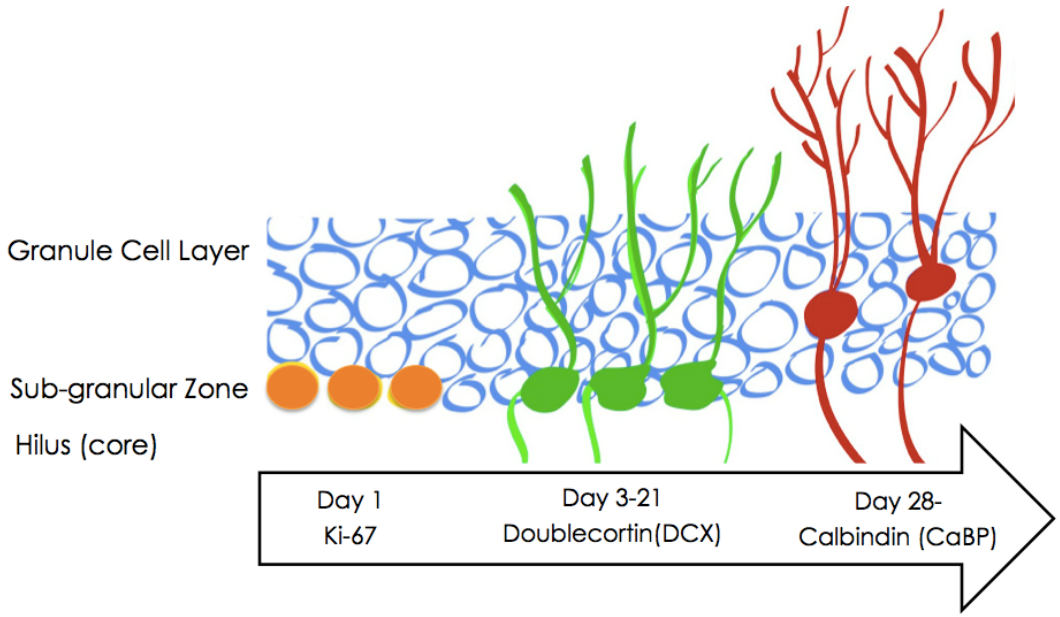
3.4.1 Rationale for using immunohistochemistry

As previously mentioned, early studies in the field suffered from the lack of specific endogenous cellular markers of adult neurogenesis. The rationale behind using IHC in neurogenesis studies is that it provides a means for examining the morphological and biochemical properties of adult generated DGCs. A diagrammatic representation of the time window that DGCs express specific markers is provided in Figure 3.4.

Doublecortin (DCX) is a microtubule-associated protein (Gleeson et al., 1999) that is expressed by transiently amplifying neural progenitor cells as well as immature neurons from approximately 3 to 14 days old (Brown et al., 2003). While the specific function of DCX has not been fully determined, evidence suggests that it plays an important role in the migration of neurons as well as promoting dendritic growth in newborn cells (Koizumi et al., 2006, Cohen et al., 2008).

Calbindin D28K (CaBP) is an endogenous cytoplasmic protein that can be used to quantify neuronal maturation. CaBP is a calcium binding protein that is fully expressed in neurons 28 days post-mitosis (Celio, 1990). In neurogenesis studies, CaBP can also help identify the maturational stage of newborn DGCs and is particularly useful in combination with a thymidine analogue such as BrdU.

Figure 3.5: Dentate granule cells express characteristic endogenous markers at various maturational stages. Ki-67 is a marker of neuronal proliferation and can be detected in cells undergoing mitosis but is absent from resting cells. Doublecortin (DCX) is predominantly expressed by post-mitotic cells and can be visualized in immature neurons 3-21 days old. The mature neuronal marker, Calbindin (CaBP), after the fourth week post-mitosis and is present in mature DGCs.



3.4.2 Immunohistochemistry antibodies and staining protocols

Sections generated from cultures or from whole-brain sections were used for IHC. Free-floating 30µm sections were washed in PBS 3 times for 5 minutes on a well-plate shaker. Antibodies (AB) were diluted to final concentration in PBS containing 0.3% Triton X-100. Secondary antibodies were diluted to [1:200] and then incubated with tissue samples at room temperature for 2-4 h. **Table 3** contains the relevant information for all immunohistochemical experiments performed in this study.

Table 3: Antibodies used for immunohistochemistry

Antibody	**Company**	**Catalog Number**	**[Concentration]/Incubation Time/Secondary Antibody**
Doublecortin (DCX): Primary goat anti-DCX	Santa Cruz Biotechnology	sc-8067	[1:200]/ 24 h at 4°C/ Alexa Fluor 488 Donkey Anti-goat (A-11055).
Glial Fibrillary Acidic Protein (GFAP): Primary mouse anti-GFAP	Millipore	AB5804	[1:2000]/ 24 h at 4°C/ Alexa Fluor 594 donkey anti-rat (A-21209) or Alex 488 goat anti-rat (A-11006)..
Calbindin (CaBP): Primary rabbit anti-CaBP **pre-incubate sections with blocking solution: PBS + 0.3% Triton X-100 +10% normal donkey serum (NDS) for 2 h**	Abcam	ab11426	[1:200]/ 72 h at 4°C / Alexa Fluor 488 goat anti-rabbit (A-11034) or Alexa Fluor 594 goat anti-rabbit (A31632)
Neuronal nuclei (NeuN): Primary mouse anti-NeuN	Abcam	ab138452	[1:200]/ 24 h at 4°C / Alexa fluor 488 goat anti-mouse (A-11001)
BrdU/CldU (same antibody): Primary rat anti-Brdu **pre-incubate sections with 1N HCL for 30 minutes at 45°C to denature DNA**	AbD Serotech	OBT0030	[1:500]/ 24 h at 4°C/ Alexa Fluor 594 donkey anti-rat or Alexa Fluor 488 goat anti-rat

Note: In double-labeled sections, BrdU staining preceded the staining protocol for the second marker of interest. Secondary antibody choice depends on double labeling protocol.

3.5 Quantifying neurogenesis in slice cultures: fluorescence and confocal microscopy

3.5.1 Fluorescence microscopy

Tissue samples were illuminated with X-cite series 120 Lumen Dynamics lamp and visualized using a Nikon Eclipse N-i fluorescence microscope with Nikon DS QiMc camera. Images were subsequently processed using NIS elements software in order to obtain area measurements of the dentate gyrus, as well as to optimize brightness and contract. In order to avoid double-counting cells adjacent sections were not counted.

3.5.2 Confocal microscopy

Samples were analyzed using a Leica TCS-SL confocal microscope with a 40x Oil immersion objective. The light source used to excite the conjugated secondary antibodies for this microscope was an Argon 488 nm and Helium-Neon 568 nm laser. Confocal microscopy allowed for quantification of cells that co-labeled for CldU and other endogenous neuronal markers. Assessment of double-labeling was achieved by examining suspected cells through the z-axis and generating “stack” images that could confirm if $CldU^+$ nuclei co-labeled with endogenous markers. Cell counts for all markers were performed in the upper blade of the dentate gyrus and the area of the upper blade was recorded for all examined tissue.

3.5.3 Data analysis and statistics

Following tissue analysis and microscopy, data was first reviewed using Microsoft Excel for preliminary analysis followed by more detailed statistical analysis on Sigma Plot. The

statistical differences in cell counts were assessed using t-tests with a confidence interval set at 95% and ANOVA with Holm-Sidak *post-hoc* comparisons. Statistical significance was reported for $p < 0.05$.

4 Results

4.1 Testing Hypothesis 1: Organotypic hippocampal slice cultures can be optimized to generate an *in vitro* model for studying adult mammalian neurogenesis.

4.1.1 Optimized culture technique yields slices that maintain the characteristic morphology and topographical organization of acute hippocampal slices after 10 to 21 DIV.

Microscopic analysis of cultured tissue sectioned at 30 µm reveals that the anatomic subregions of the hippocampal tri-synaptic are clearly identifiable. Comparison with hippocampal tissue from age matched *in vivo* controls shows that the dentate gyrus, CA3, and CA1 regions can be easily located. Four commonly used immunohistochemical markers demonstrate the maintenance of granule cell layer and pyramidal cell layer; NeuN and CaBP (Figure 4.1), DCX (Figure 4.2), and GFAP (Figure 4.3).

Sample images (Figure 4.1.B and C) highlight the difference in cultured tissue processed by different techniques. In Figure 4.1.B, the hippocampal slice was removed from the culture membrane and glued directly to the vibratome surface before sectioning at 30 µm. In Figure 4.1.C, the entire membrane with attached culture slice was excised from the surrounding insert and the membrane was carefully glued directly to the vibratome surface.

4.1.2 Newborn granule cells can be “birth dated” in a quantitative manner using widely-accepted *in vivo* techniques for studying neurogenesis

In addition to immunohistochemical staining for the thymidine analogues BrdU and CldU, various endogenous markers of neurogenesis can be visualized in hippocampal slice cultures. The presence of DCX^+ neurons in the granule cell layer after 14 DIV

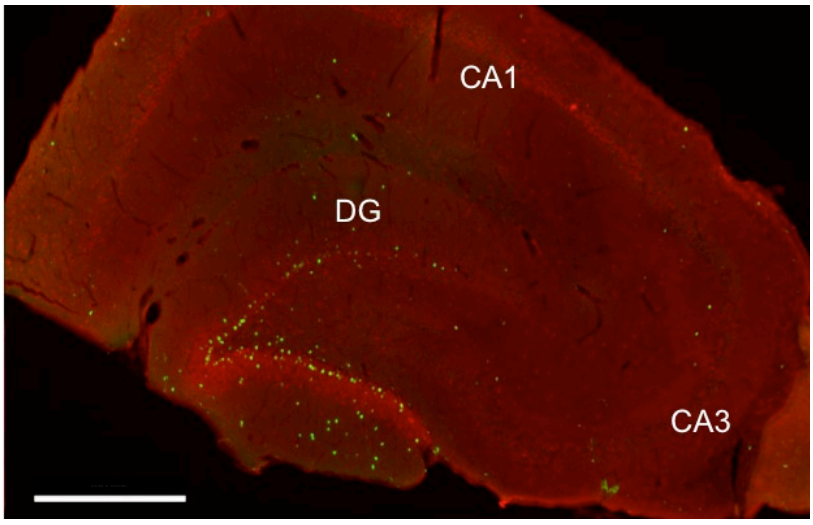
indicates presence of immature neurons. Co-expression of CldU and DCX indicates cells were born after the dissection protocol and administration of CldU (Figure 4.2.A). The mature neuronal marker, CaBP, can be used to measure the number of mature “newborn” neurons per area (Figure 4.2.B). Refer to Figure 3.2 for experimental timeline.

4.1.3 Field and intracellular recordings indicate that cultures maintain electrophysiological properties of acute slices at 10-21 DIV

Field and intracellular recording experiments on hippocampal slice cultures reveals that the electrophysiological properties of acute slices can be observed after 10-21 days post-dissection. A tetanizing stimulus can produce long-term potentiation in the slice cultures, a hallmark feature of hippocampal physiology (Figure 4.3.A). This effect is observed in the absence of any specific GABA antagonists e.g. bicuculline. Application of THIP (5 μM) attenuated post-synaptic potentials in intracellular recording, indicating sensitivity to THIP (Figure 4.3.B). We attempted electrophysiological recordings on 41 organotypic slices as a ‘proof of principle’ for future studies. 76% of the experiments yielded responses for more than 5 minutes and the average length of recordings was 22 minutes. Out of the 41 experiments, the longest recording period was for 108 minutes cultured tissue at 12 DIV.

Figure 4.1: A. Transverse section from an *in vivo* brain from P21 rat pup. Immunostained for CaBP (red) and CldU (green). Note hippocampal morphology of section as point of comparison with culture tissue in b and c. DG = dentate gyrus, CA1,3 = Cornu Ammonis 1, 3. **B.** Section from culture grown for 21 days on Millipore Millicell insert. Stained for NeuN (green) and CldU (red) (opposite colour scheme of Figure 4.1.A). Representative sample of tissue prepared by initial post-culture processing method. *In vivo* morphology is maintained but note widespread CldU labeling over the whole surface of section. **C.** Culture tissue after 12 DIV. Immunostained for CaBP (red) and CldU (green). Grown on Thermo Scientific Nunclon treated culture insert. Representative sample of tissue prepared by second post-culture processing method. Note well-defined borders and $CldU^{+}$ nuclei predominantly restricted to dentate gyrus. Scale bar = 500 μm.

A.



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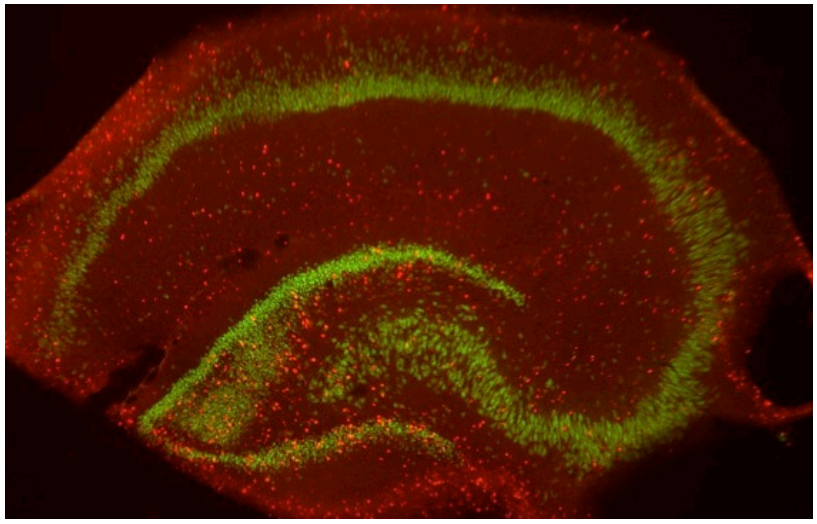

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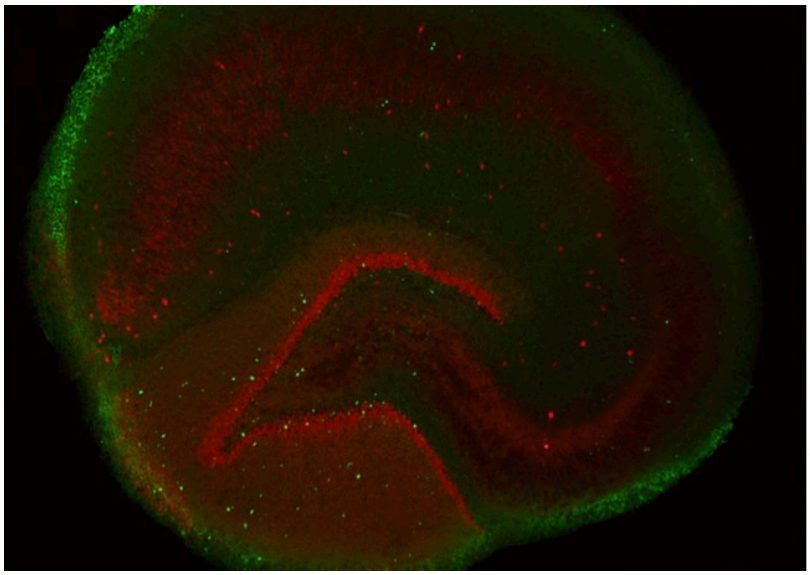
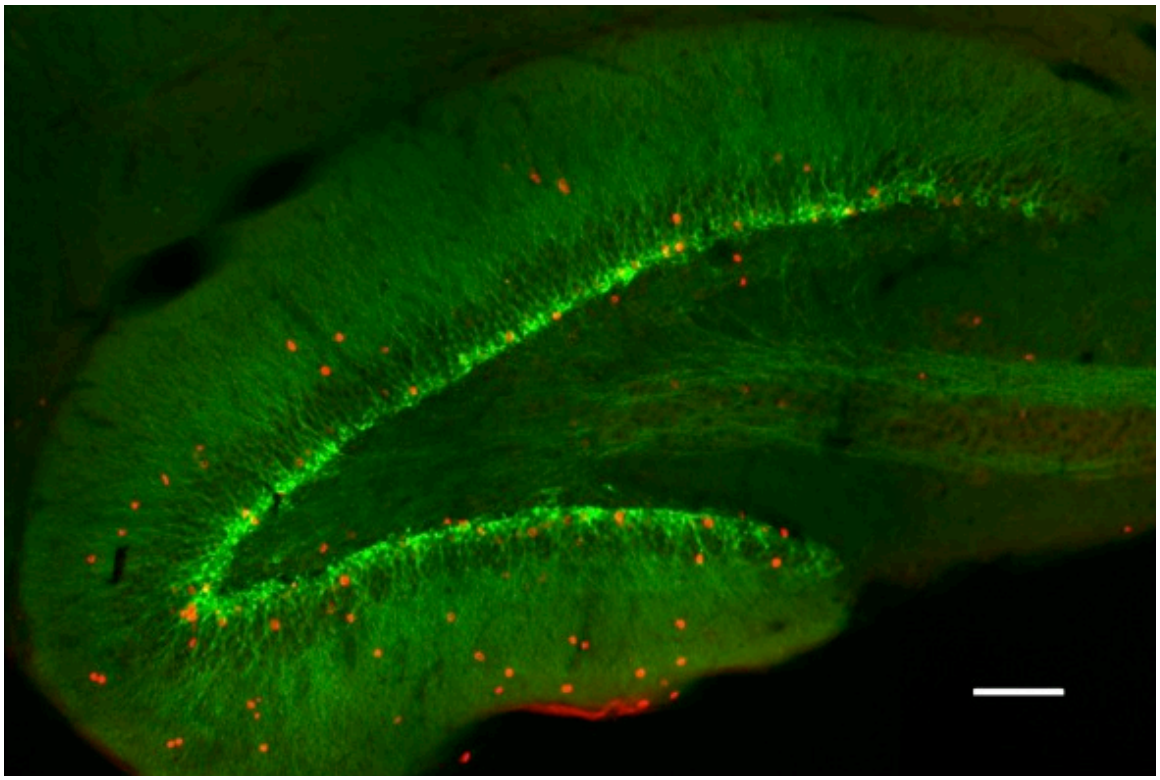


Figure 4.2: A. Transverse section (30 µm) from an *in vivo* brain from P21 rat pup. Immunostained for CldU (red) and DCX (green). Note hippocampal morphology as point of comparison with culture tissue in Figure 4.1.B and C. **B.** Transverse section (30 µm) from culture tissue after 14 DIV immunostained for CldU (red) and DCX (green), 20x air fluorescence micrograph composite image. Scale bar = 100 µm.

A.



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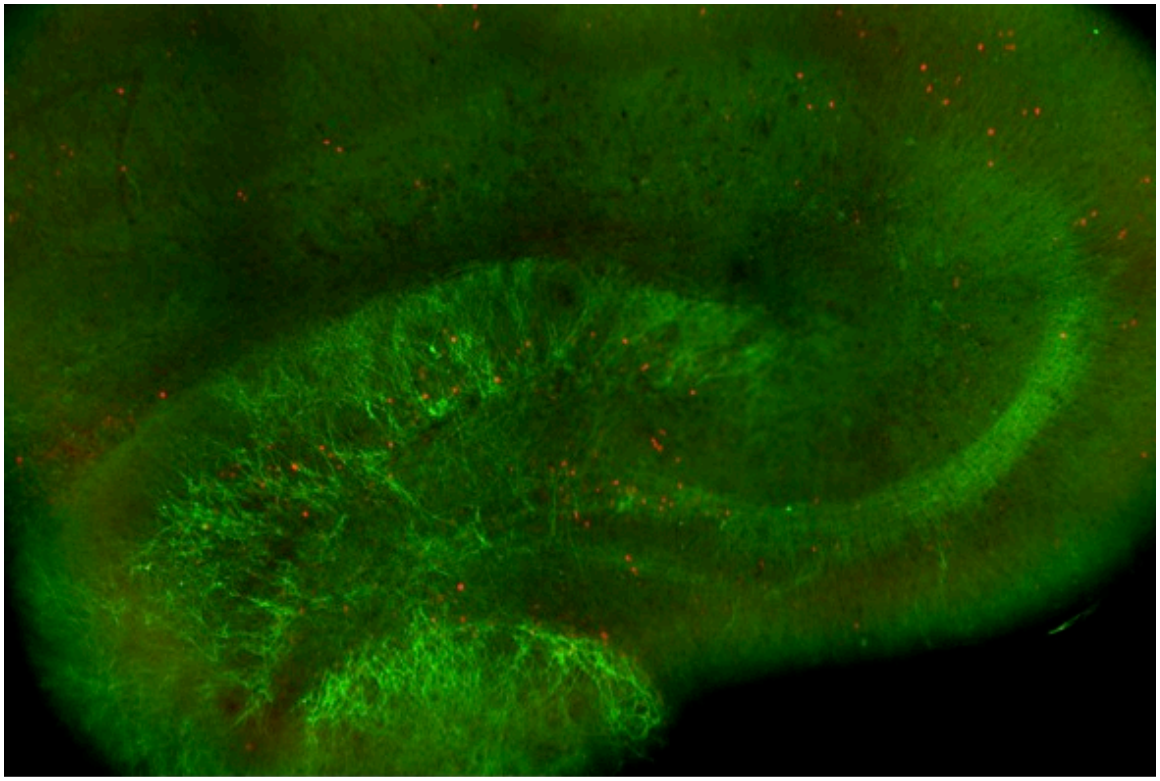
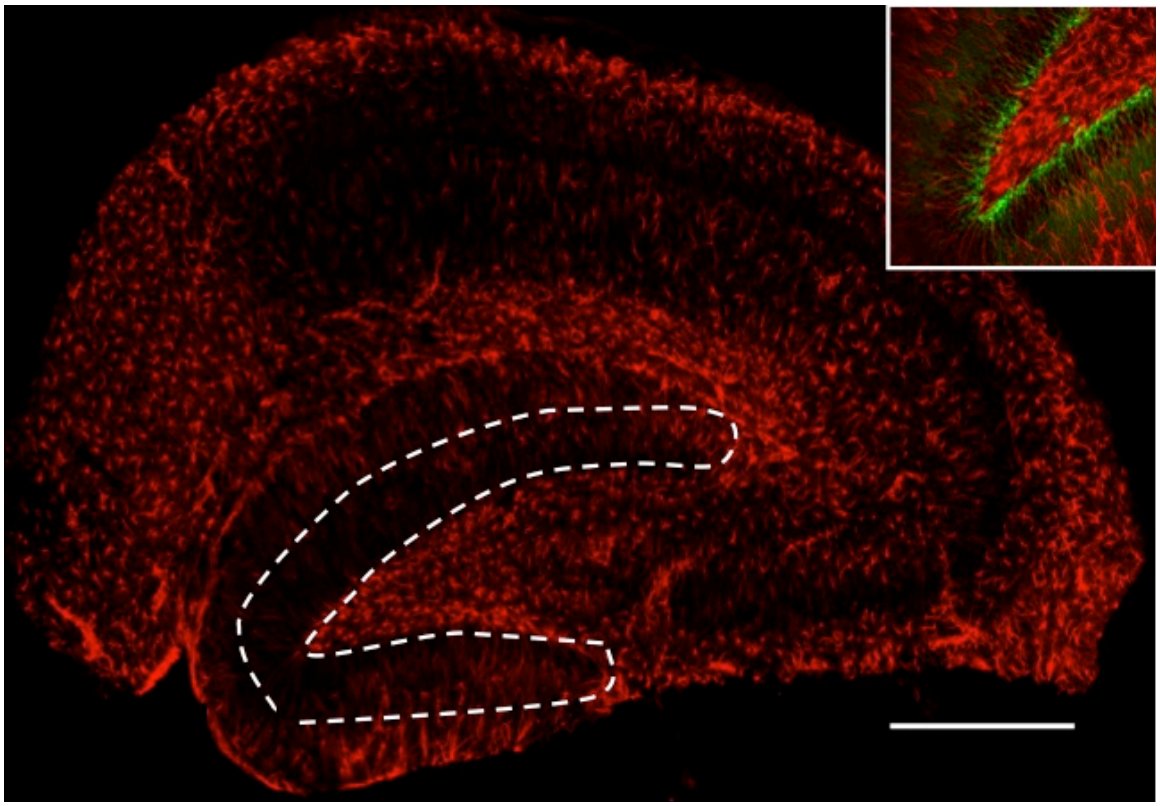


Figure 4.3: **A.** *In vivo* tissue from P21 rat pup immunostained for the astrocytic marker, Glial Fibrillary Acidic Protein (GFAP). Note low GFAP immunoreactivity in GCL (white-dotted line) where DGCs are born (Inset, DCX^+ DGCs in GCL). **B.** Culture tissue from 14 DIV immunostained for GFAP (10x air, fluorescence micrograph composite image). Note similar pattern of GFAP expression compared to *in vivo* but with higher expression in the GCL. Scale= 500 μm.

A.



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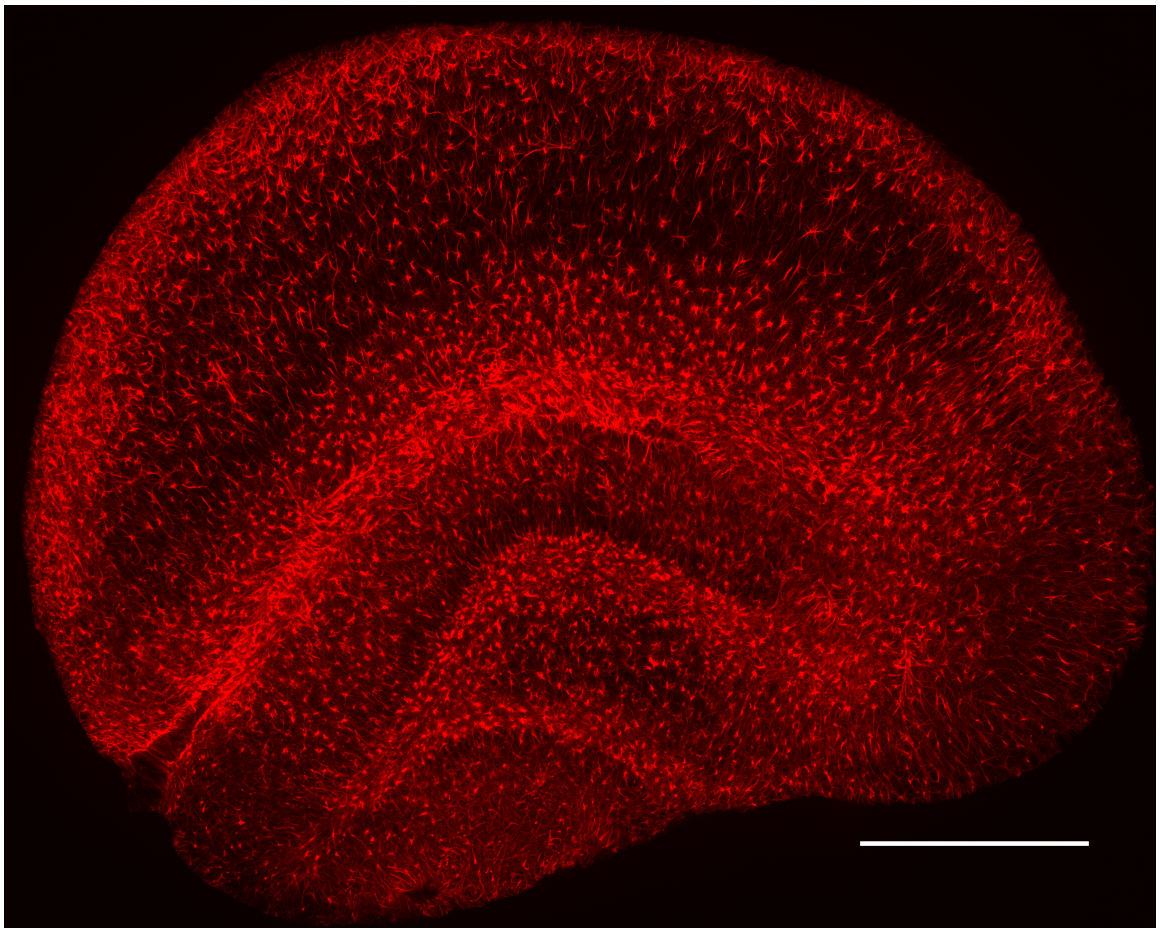
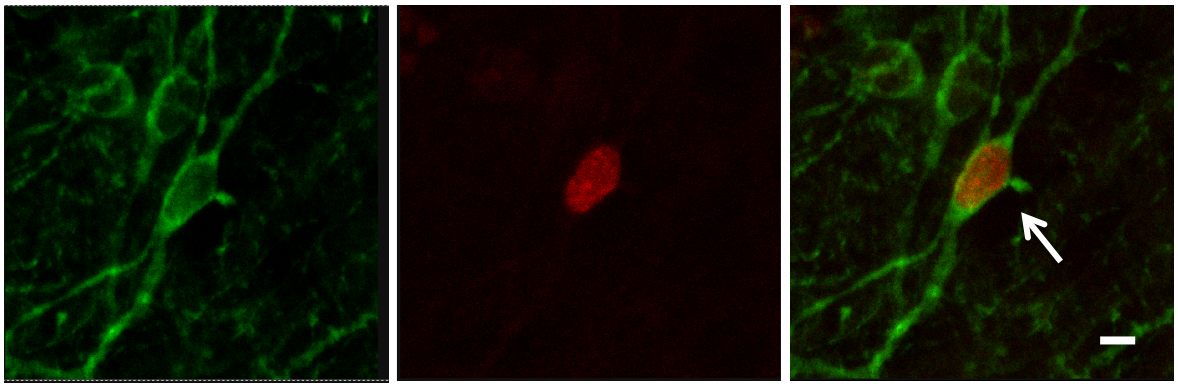


Figure 4.4: Newborn DGCs in organotypic cultures can be identified and their numbers quantified by confocal microscopy and stereological counting. **A**. DGCs co-expressing DCX (green) and CldU (red) are counted as newborn neurons 3-14 days old. Arrow indicates a double-labeled cell. (40x oil immersion confocal micrograph). **B.** The numbers of mature "newborn" DGCs are quantified by counting neurons co-expressing Calbindin (red) and CldU (green). Comparison of relative numbers of $DCX^+/CldU^+$ and $CaBP^+/CldU^+$ gives a measure of cell maturation (40x fluorescence micrograph). Scale bar= 10 μm.

A.



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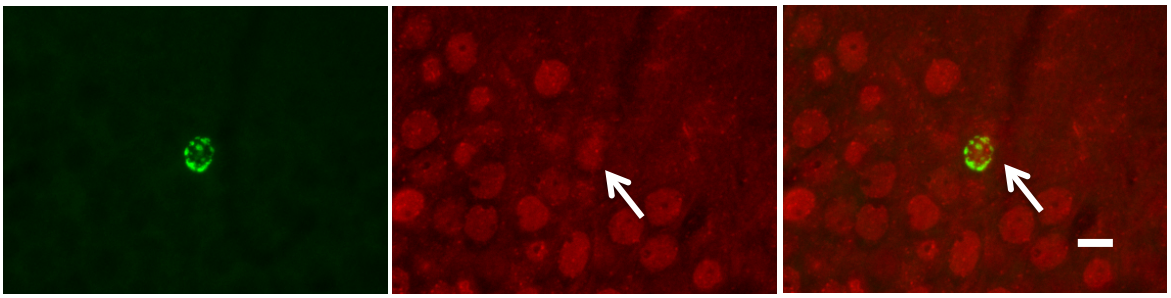
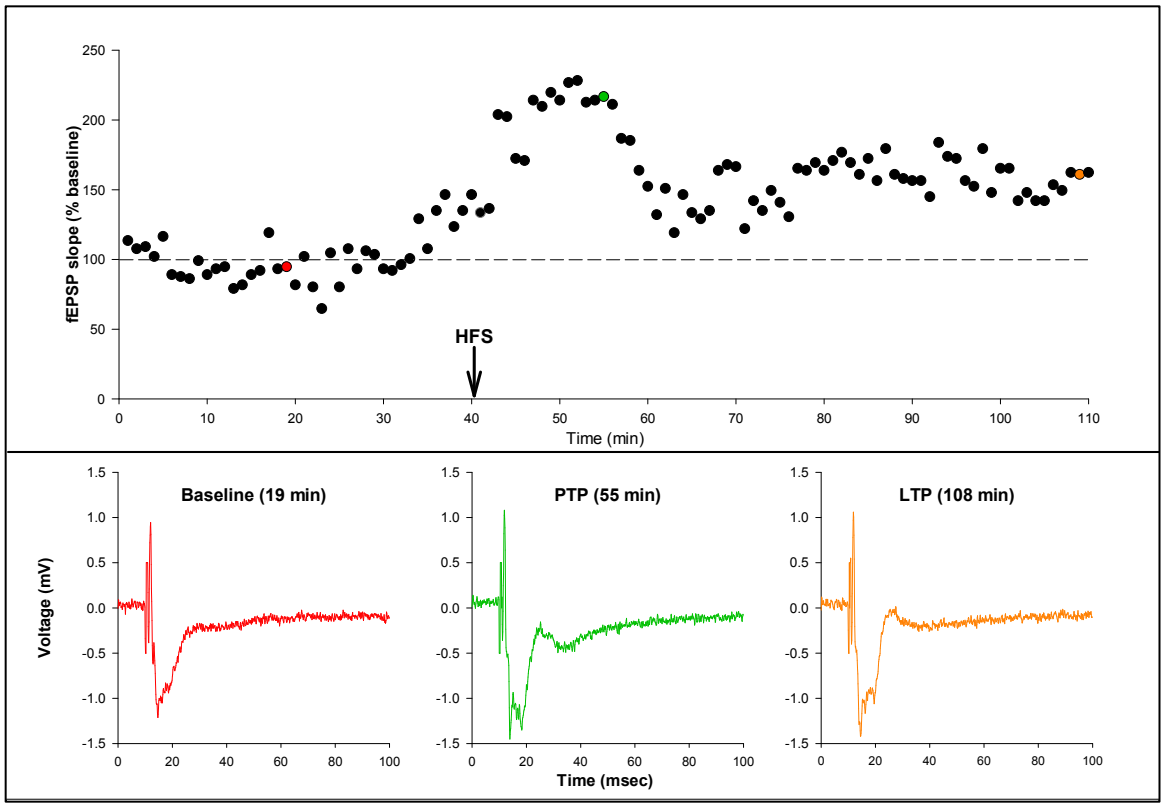
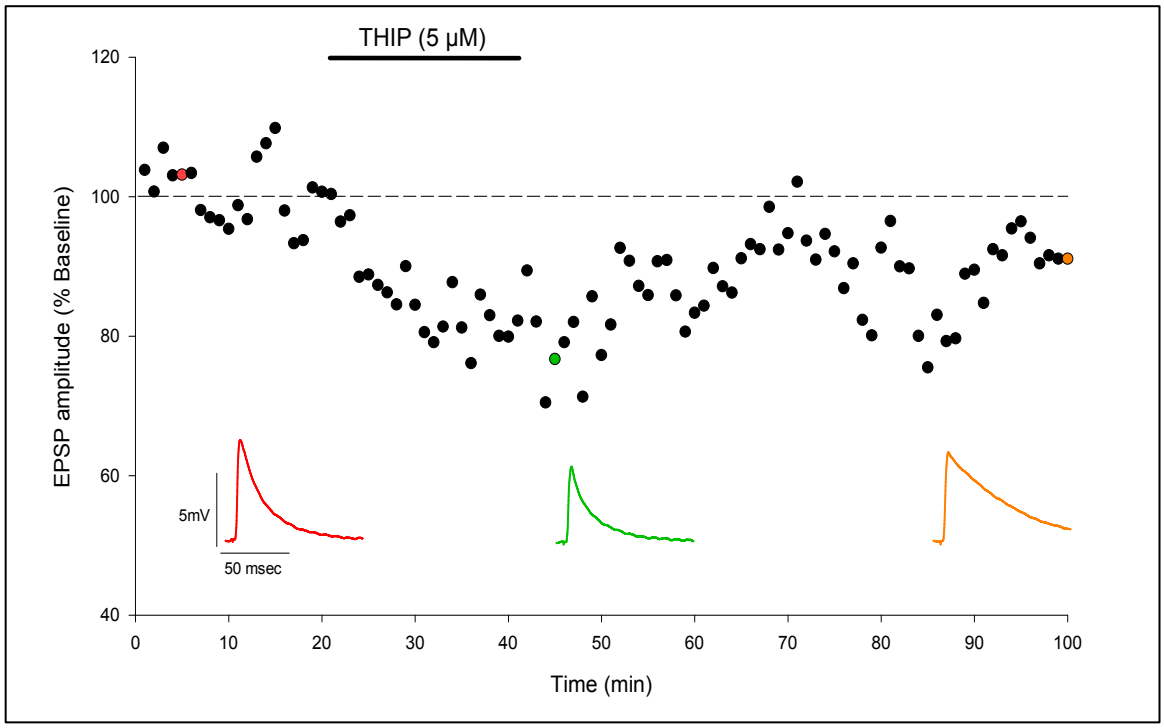


Figure 4.5: Field and intracellular recordings reveal cultures maintain electrophysiological properties at 10-21 DIV. **A**. LTP demonstrated with field recording in culture after 12 DIV. Colored dots on top tracing highlight specific trials at 19 min (red), 55 min (green) and 108 min (orange). HFS= high frequency stimulation. **B.** Effect of bath application of THIP demonstrated with intracellular recordings 10-21 DIV. Colored dots on top tracing highlight specific trials at 15 min (red), 39 min (green) and 61 min (orange).

A.


fEPSP slope (% baseline)
HFS
Time (min)
Baseline (19 min)
PTP (55 min)
LTP (108 min)
Voltage (mV)
Time (msec)

B.


THIP (5 µM)
EPSP amplitude (% Baseline)
5mV
50 msec
Time (min)

4.2 Testing Hypothesis 2: The δ-$GABA_AR$ preferring agonist, THIP, enhances neurogenesis during a critical window of DGC maturation.

4.2.1 THIP promotes differentiation of neural progenitors into DCX^+ young neurons but has no effect on survival of $CldU^+$ cells.

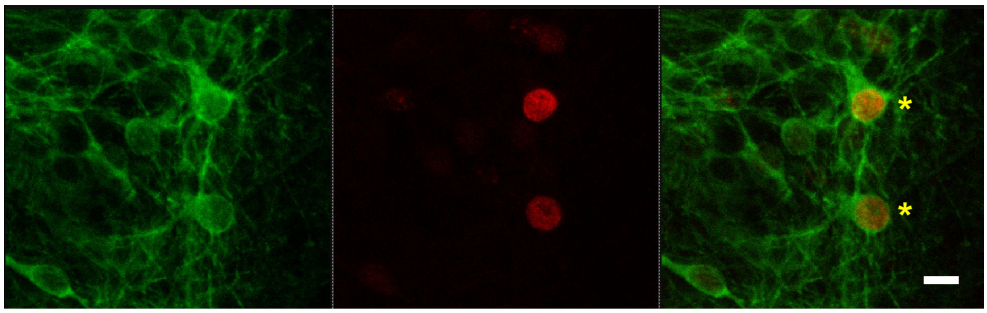
We measured the numbers of $CldU^+/DCX^+$ co-labeled cells at 3 survival times following the CldU application (7, 12, and 21 days). Figure 4.6 presents confocal micrographs of DGCs in Control and THIP conditions. Note the similarities in overall appearance of the DGCs but comparatively higher number of co-labeled cells in THIP-treated cultures. Figure 4.7.A presents data showing an increase in co-labeled cells at 12 days in the THIP-treated cultures. This result indicates an enhancement of neuronal differentiation as measured by DCX expression. Next, we assessed the survival of $CldU^+$ cells by measuring the number of $CldU^+$ nuclei at 7, 12, and 21 days and found no significant differences between the Control and THIP-treated cultures (Figure 4.7.B) ($p=0.002$, Two-way ANOVA, Control n=12, THIP n=12).

4.2.2 Newborn DGCs in culture mature between 7 to 21 DIV and THIP promotes DGC maturation compared to controls.

There is a statistically significant difference in the proportion of $CldU^+$ cells co-expressing CaBP between 7- 21 DIV after treatment with THIP, but no significant difference exists between these time points in control cultures, $*p<0.05$. Comparing THIP and control groups at 21 days revealed a trend towards increased maturation in presence of THIP, but this effect did not achieve significance ($p=0.18$).

Figure 4.6: THIP promotes differentiation of neural progenitors into DCX^+ young neurons and has no effect on survival of $CldU^+$ cells. Sample confocal microscope photograph showing tissue from Control (**A**) and THIP-treated (5 μM) **(B**) cultures with $DCX^+/CldU^+$ cells are labeled (yellow asterisks). Scale bar= 10 μm.

A.



B.

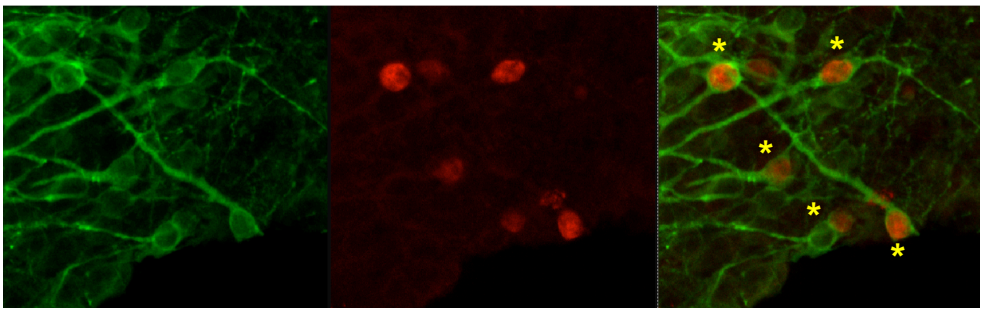
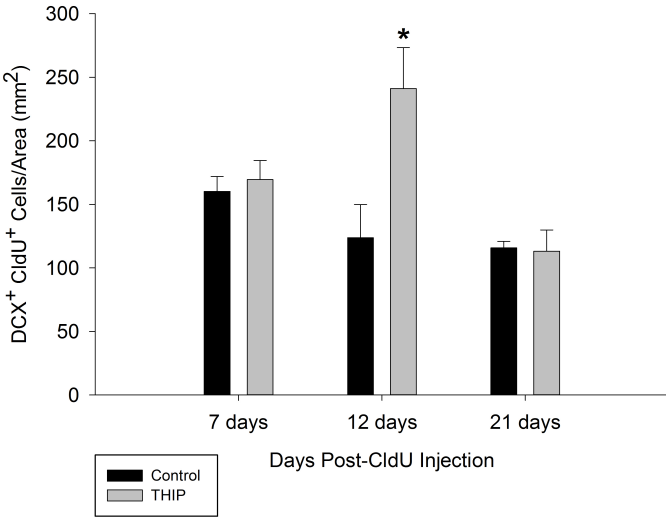
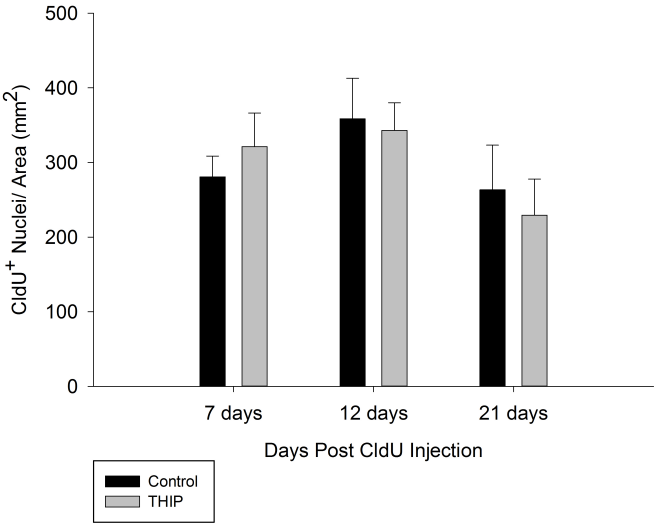


Figure 4.7: A. THIP administration led to a significant increase in the number of $CldU^+$ cells that co-expressed DCX at 12 days post-CldU injection in the dentate gyrus upper blade ($p<0.05$) **B.** Comparison of the total number of $CldU^+$ cells in both treatment conditions revealed no significant differences between groups ($p>0.05$). **C.** The proportion of $CldU^+$ cells that expressed DCX was significantly higher following treatment with THIP in culture tissue analyzed at 12 days post CldU-application (Control n=12, THIP n=12 for all figures).

A.


300
250
200
150
100
50
0
DCX+ CldU+ Cells/Area (mm2)
*
7 days
12 days
21 days
Days Post-CldU Injection
Control
THIP

B.


500
400
300
200
100
0
CldU+ Nuclei/ Area (mm2)
7 days
12 days
21 days
Days Post CldU Injection
Control
THIP

C.

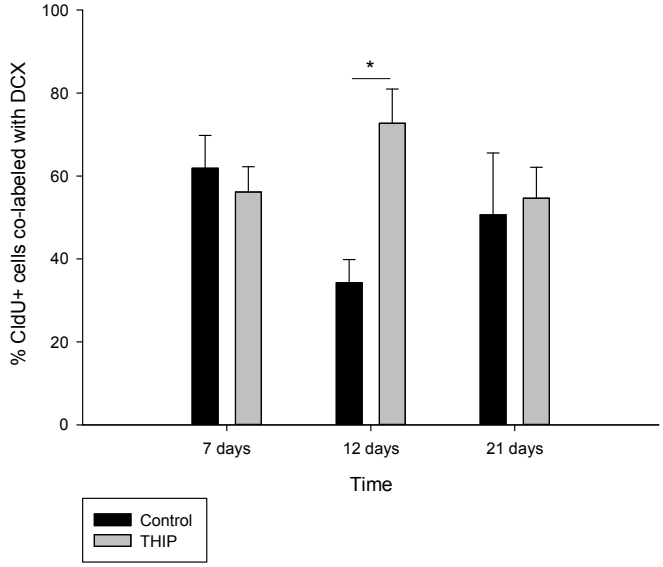
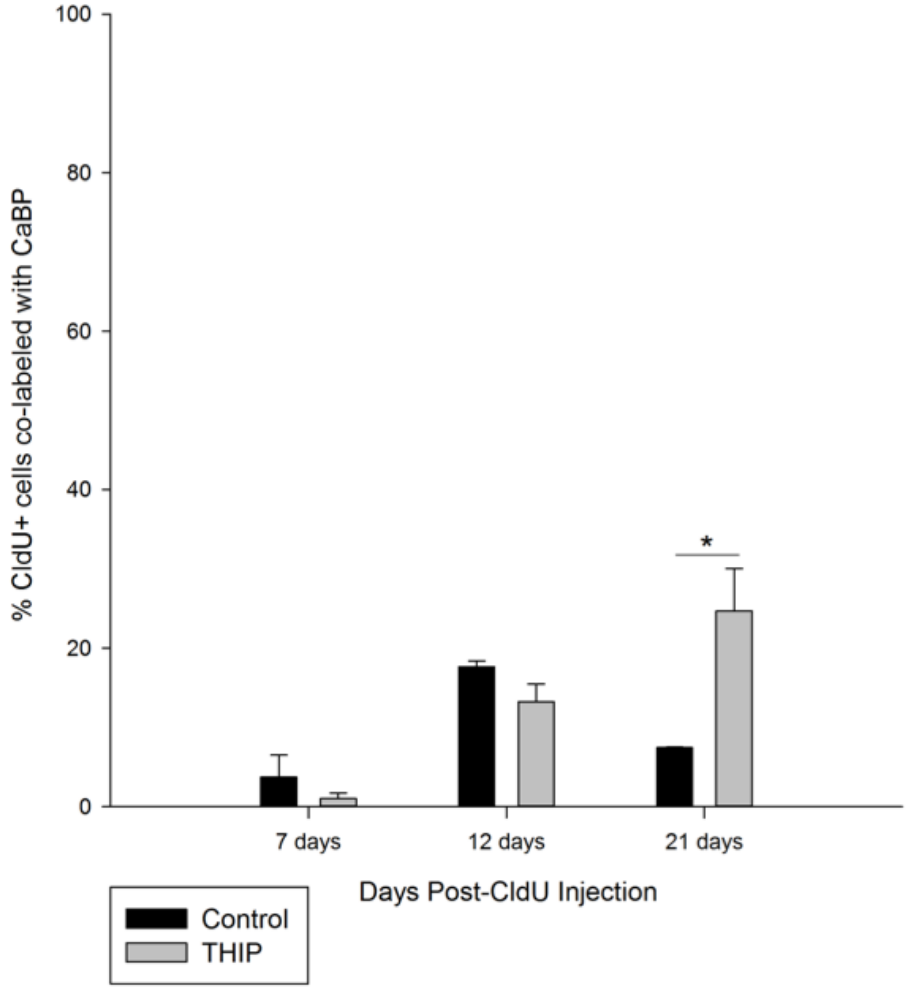

100
80
60
40
20
0
% CldU+ cells co-labeled with DCX
*
7 days
12 days
21 days
Time
Control
THIP

Figure 4.8 THIP increases the percentage of $CldU^+$ cells that co-express the mature neuronal marker, CaBP, between 7 to 21 DIV. Statistically significant differences exist between THIP-groups at 7 and 21 days but not in control cultures during same time window ($p<0.05$, Two-way ANOVA, Control n= 3, THIP n= 3).

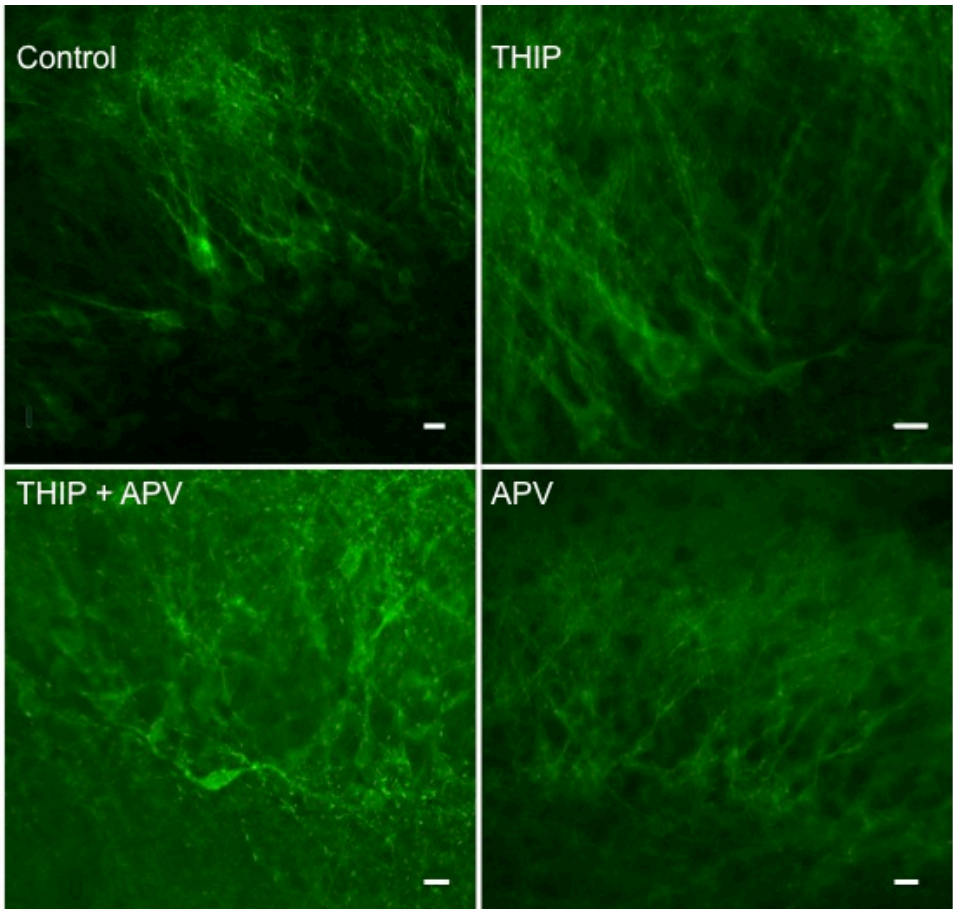

100
80
60
40
20
0
% CldU+ cells co-labeled with CaBP
*
7 days
12 days
21 days
Days Post-CldU Injection
Control
THIP

4.3 Testing Hypothesis 3: Blocking glutamate transmission mediated by NMDA receptors will prevent THIP-mediated promotion of neuronal differentiation stage of neurogenesis.

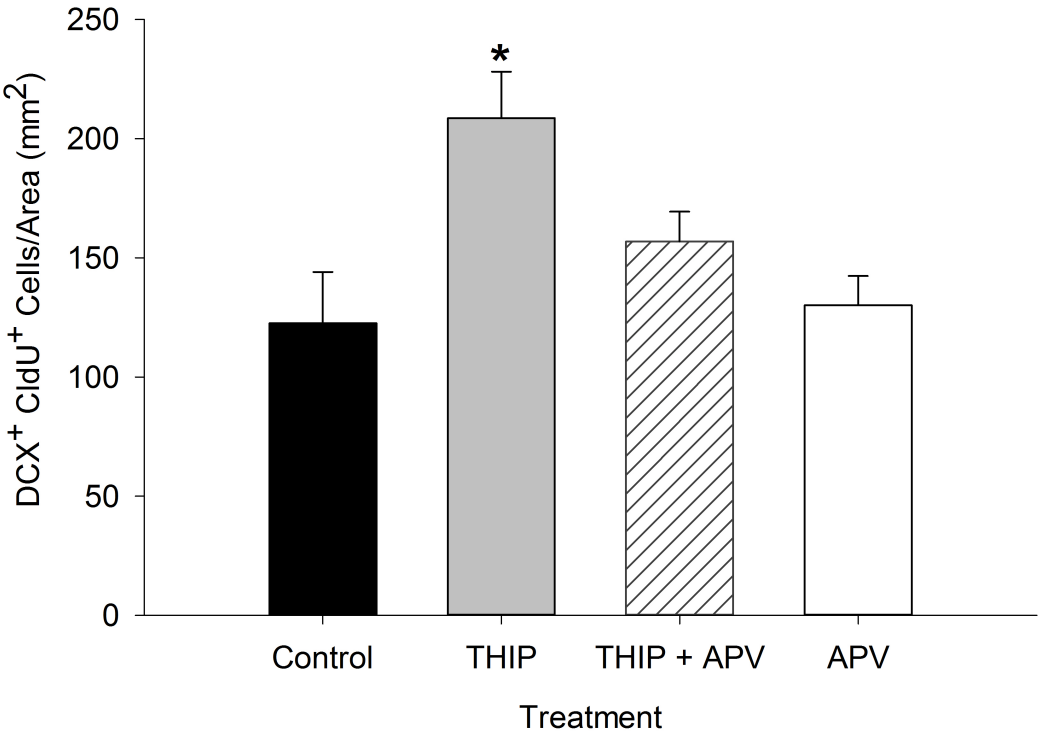
Results from the experiment described in Section 4.2 (Figure 4.7.A), revealed that THIP-mediated promotion of differentiation was confined to cultures fixed at 12 days post-CldU injection. Thus, this time point was used to study the influence of APV. In the presence of APV, the effect of THIP on increasing DCX-expressing $CldU^+$ cell numbers was not observed (Figure 4.9.B). In addition, APV applied individually did not cause a significant change in the number of $DCX^+/CldU^+$ cells per area (Two-way ANOVA, $p=0.02$, Control n=12, THIP n=12, THIP+APV n=6 and APV n=3).

Figure 4.9: A. Fluorescence microscopy photograph showing that application of APV resulted in no gross effect on general appearance of DGCs in any treatment condition i.e. there was no toxic effect of APV that could have prevented the effect of THIP. Cells are located in upper blade of the hippocampal dentate gyrus. Scale bar = 10 μm. **B.** Application of APV prevents THIP-mediated promotion of neuronal differentiation at 12 days post-CldU injection. Results reveal a significant increase in the number of cells co-expressing DCX and CldU after application of THIP ($p= 0.02$), but co-application of APV or APV alone abolished this effect. Measurements of co-labeled cells were done on dentate gyrus upper blade (Control n=12, THIP n=12, THIP+APV n=6 and APV n=3).

A.


Control
THIP
THIP + APV
APV

B.


250
200
150
100
50
0
*
DCX+ CldU+ Cells/Area (mm2)
Control
THIP
THIP + APV
APV
Treatment

5 Discussion

5.1 Optimizing slice cultures for neurogenesis research

The results presented in section 4.1 highlight the utility of organotypic hippocampal slice cultures and outline our attempts to optimize an *in vitro* technique for studying adult neurogenesis. Determining if organotypic cultures would be suitable for our studies required that they satisfy three main criteria: 1) that slices maintain characteristic features of hippocampal slices after 10-21 DIV, 2) that newborn DGCs can be quantified using standard immunohistochemical markers, and 3) that cultures maintain electrophysiological characteristics of acute hippocampal slice preparation.

Regarding the first criterion, the distinctive morphology of the hippocampus and dentate gyrus must be sufficiently maintained to recapitulate the cellular environment of newborn granule cells. Each step of the dissection and culture protocol plays an important role in ensuring the proper maintenance of the tissue. This includes choosing the right products for the experiment. Initial experiments were performed using the Millipore Millicell polycarbonate inserts. This product provided insufficient membrane-tissue adhesion for sectioning prior to immunohistochemical staining. In order to prepare tissue from these inserts, the cultured slice was removed using a paintbrush and directly affixed to the vibratome cutting surface with an adhesive. This technique resulted in significant loss of tissue as any tissue exposed to the glue was subsequently unusable. Also, transferring slices to the cutting surface was very time intensive so producing enough sections for IHC staining was challenging. The process of gluing the tissue to the vibratome cutting surface also damaged tissue borders. To address this problem, we experimented with Thermo Scientific Nunc culture inserts coated with

Nunclon delta surface. The Nunclon delta treated membrane resulted in better tissue adhesion, which significantly improved post-culture tissue processing time and raised the yield of usable tissue for staining. Establishing a method for generating tissue sections of consistent size and border integrity was a requirement for these experiments. Furthermore, qualitative analysis of CldU immunoreactivity between both types of culture plates revealed that CldU staining was localized to the dentate gyrus in Nunc inserts, but in Millicell inserts there were $CldU^+$ cells dispersed through most of the tissue. This observation is important considering that *in vivo* samples show localization patterns consistent with the Nunc inserts and the presence of CldU nuclei in non-neurogenic zones, such as CA3 and CA1, may be indicative of non-neuronal, degenerative cellular proliferation of astrocytes or microglia.

During one experiment, tissue contamination led to the development of bacterial colonies inside of the culture inserts (data not shown). The contamination was confined to a single insert on the six-well culture plate, but microscopic analysis of this tissue revealed significant degeneration of the slice periphery. Ensuring that proper sterile technique was observed helped maintain the slice cultures for up to 21 DIV. If no contamination was observed, the culture tissue was incubated until the end of the experiment (7, 12, or 21 days post-CldU).

Comparison of culture slices with tissue freshly obtained from age-matched *in vivo* controls revealed that the immunohistochemical staining patterns were very similar. These qualitative comparisons provide a foundation upon which to base quantitative measurements of adult neurogenesis in a manner analogous to *in vivo* studies.

Regarding the second criterion, one test for determining that slice cultures provide a good model for neurogenesis research is the capacity to stain and quantify newborn neurons in the hippocampus. The two main findings that support the use of slice cultures for post-natal neurogenesis research were that microscopic analysis revealed immunohistochemical reactivity for CldU as well as endogenous protein markers of neurogenesis, such as DCX and CaBP.

While thymidine analogues are commonly employed in neurogenesis studies, adapting this technique for organotypic cultures required us to determine conditions sufficient to label new neurons without inducing cytotoxic damage to the tissue. Previous studies using slice cultures for neurogenesis research exposed the cultures to BrdU for 3 days at a concentration of 0.5 and 10 μM (Raineteau et al., 2004, Bunk et al., 2010), respectively). One critical feature of neurogenesis for hippocampal function is that at any given time there is a heterogeneous population of dentate granule cells at various maturational stages (Wang et al., 2000, Stone et al., 2011). Using a 2 h pulse exposure, we managed to label a discrete, relatively homogenous population of DGCs. Section 4.1 provides definitive evidence that immunohistochemical protocols commonly used for *in vivo* tissue analysis can be adapted for slice cultures. The phenotype of $CldU^+$ cells was determined using the endogenous markers DCX and CaBP with stereological counting on fluorescence and confocal microscopes.

Regarding the third criterion, we found that organotypic cultures retain electrophysiological characteristics. Using field population and intracellular recordings from cultures between 10-21 DIV, we obtained preliminary evidence that cultures exhibit LTP following brief tetanic stimulation. We also showed that this phenomenon occurs

without application of GABA antagonists such as bicuculline, as is required to elicit LTP in acute slices from the adult dentate gyrus (Wang et al., 2000). The presence of LTP in cultured slices suggests that the majority of cells in the dentate gyrus were immature and disinhibited, which is reasonable given the age of the animal at the time of dissection. Bath application of THIP (5 μM) to the ACSF solution during intracellular recording attenuated post-synaptic potentials in the dentate gyrus and indicated that cultured slices were responsive to THIP between 10 to 21 DIV.

Developing new techniques for studying the brain is an area of active interest. By satisfying the three criteria outlined above, we have shown that organotypic cultures can be utilized for researching adult neurogenesis and testing new pharmacological agents for treating neurological disorders that involve hippocampal neurogenesis.

5.2 Promoting extrasynaptic GABAergic signaling mediated by the δ-$GABA_AR$ enhances neurogenesis during a critical window of granule cell maturation.

We investigated the action of extrasynaptic GABAergic signaling in post-natal neurogenesis for two reasons: 1) while the importance of GABAergic depolarization has been well characterized, the role of specific $GABA_AR$ subunits, such as δ, in this early stage is less well known and 2) these characteristics have not been characterized in organotypic slice cultures.

An important component of development is the phase of GABAergic depolarization mediated by elevated $[Cl^-]_i$. The data presented highlights the effect of THIP during a period in development frequently referred to as the “critical window” (Anderson et al., 2011). By applying THIP 3 days after injection of CldU, we attempted to influence the

development of neurons that were approximately 3-5 days old. The three time points for fixation at 7, 12, and 21 days post-CldU were intended to reveal the short, medium, and longer-term consequences of THIP application. Our results indicate that a significant effect is only observed in tissue from the 12-day cohort. If the maturational timeline of DGCs in slice culture is consistent with previously established timelines *in vivo*, then cells co-expressing DCX and CldU at this time point would be in the middle of the critical period (Dieni et al., 2013). Our results are consistent with previously published findings showing that extrasynaptic GABAergic signaling can determine the rate of morphological maturation in DGCs (Duveau et al., 2011). As previously mentioned, the chloride ion transporter NKCC1 is crucial for the depolarizing GABA response observed in immature neurons. Ge et al. showed that knocking down NKCC1 in newborn neurons resulted in cells with less developed dendritic tress and delayed onset of glutamatergic synaptogenesis (Ge et al., 2006). Tozuka et al. provided evidence that GABAergic excitation promotes neural progenitor cells to leave the cell cycle and commit to neuronal cell fate (Tozuka et al., 2005). Consistent with our findings, if THIP had a similar effect in organotypic cultures then this would be detected as increased expression of the immature marker, DCX.

We did not observe an increase in $DCX^+/CldU^+$ cells in THIP-treated fixed at 21 days post-CldU application. One potential explanation for this observation could be that cells initially quantified, as $DCX^+/CldU^+$ did not survive to 21 days and underwent apoptosis. Incorporation of CldU in the cell nuclei could lead to detrimental effects on cell survival.

Using neurosphere cultures, Lehner et al. (2011) found that 1 μM BrdU increased expression of the apoptotic marker lactate dehydrogenase and significantly increased

cell death. However, the investigators used a BrdU pulse duration of 48h, which is significantly longer than what was used in the present study. In our study, we observed a decline in $DCX^+/CldU^+$ co-expression, but no difference in the total number of $CldU^+$ cells at any of the time points studied. This suggests that cell death is not primarily responsible for the observed decline. Subsequent immunohistochemical studies employing markers of apoptosis could help to more definitively resolve this issue.

A more plausible explanation for "loss" of $DCX^+/CldU^+$ cells could be that extrasynaptic GABA signaling promoted by THIP enhanced neuronal maturation and drove newborn DGCs to become $CaBP^+$ faster than would occur in control conditions. In support of this hypothesis, several investigators have suggested that GABA-mediated depolarization is necessary for promoting the maturational "switch" from a depolarizing to hyperpolarizing GABA response, and that this effect relies on elevating $[Ca^{2+}]_i$ in newborn neurons (Ganguly et al., 2001, Fiumelli et al., 2005). Ganguly and colleagues provided early evidence that GABA-mediated depolarization is involved in regulating the expression of the Cl^- transporter, KCC2, that is elevated in mature neurons and is prerequisite for establishing the reduced $[Cl^-]_i$ of mature neurons. The data presented in Figure 4.5 support the hypothesis that THIP promotes maturation of DGC because drug application markedly increases the percentage of $CldU^+$ cells expressing CaBP between days 7 and 21, whereas this was not observed in control cultures.

GABA is involved in DGC physiology from the period when newborn neurons are produced to the stage of functional integration and engagement in hippocampal processing (Dieni et al., 2013). However, the heterogeneity of $GABA_AR$ subtypes makes it challenging to identify which specific subunits might be responsible for the various

actions of GABA on DGCs. From review of the existing literature, and to the best of our knowledge, this is the first study to address the role of tonic depolarizing currents through extrasynaptic $GABA_A$Rs, and suggest a role for δ-subunit containing $GABA_A$Rs for promoting neuronal proliferation and maturation in organotypic slice cultures.

5.3 Blocking NMDARs with APV prevents the neurogenesis-promoting effect of GABA-mediated depolarization in immature DGCs

The results presented in section 4.3 highlight the complex nature of GABAergic signaling in the development of newborn DGCs. We provide evidence for the existence of a relationship between extrasynaptic GABA signaling, possibly mediated through δ-subunit containing $GABA_A$Rs, and NMDARs. The neurogenesis promoting effect of THIP was abolished in the presence of the selective NMDAR antagonist, APV. This suggests a possibility that GABA depolarization results in NMDAR mediated Ca^{2+} influx that in turn drives growth and branching of dendrites (Konur and Ghosh, 2005) and cytoskeletal rearrangements characteristic of granule cell maturation (Komuro and Rakic, 1993). In order for NMDARs to be involved in GABAergic signaling in cultures there would need to be ambient glutamate during THIP application. The observation that slice cultures exhibit spontaneous miniature excitatory post-synaptic potentials after 14 DIV provides evidence that there is likely ambient or synaptically released glutamate present during THIP application (Dyhrfjeld-Johnsen et al., 2010).

While NMDARs allow Ca^{2+} influx from the extracellular space, there are other important sources of Ca^{2+} for DGC development. Recently, Lee and colleagues (2012) used organotypic slice cultures to study calcium transients in newborn DGCs. They found that L-type voltage dependent calcium channels are essential components of GABA-

mediated calcium signaling. GABA-induced Ca^{2+} influx contributed to axon outgrowth tha they observed in newborn granule cells. The recorded calcium transients were most strongly inhibited by L-type channel blockers. But even in the presence of these agents, there was still Ca^{2+} influx due to GABA-mediated depolarization. One potential explanation for the residual calcium transients reported by Lee et al. could be NMDARs, which would be consistent with previous findings (Paoletti and Neyton, 2007).

To more thoroughly characterize the observed effect of APV on THIP-mediated promotion of neurogenesis, subsequent culture studies should utilize specific channel blockers, such as nifedipine, which blocks L-type Ca^{2+} channels (Lee et al., 2012) and is independent of NMDARs. Applying the calcium chelator, BAPTA, would also help determine if intracellular calcium stores are necessary or sufficient for driving THIP-mediated promotion of neurogenesis.

Future studies are required to characterize the mechanisms whereby APV blocks the differentiation of neural progenitors that was observed with THIP alone. The effect of APV was not examined at the 21-day time point using CaBP IHC. But as APV blocked differentiation at the early time points, it is reasonable to conclude that there would be fewer $CldU^{+}/CaBP^{+}$ following administration of APV. The data we have obtained are consistent with experiments previously performed in the laboratory with *in vivo* adult rats. These similarly showed that THIP did not promote proliferation or survival when co-applied with APV (Rosenzweig, 2012).

6 Conclusions

6.1 Findings and Results

The organotypic slice culture technique represents a valuable tool for studying early postnatal neurogenesis. The well-characterized similarities between early postnatal and adult neurogenesis lend support to the notion that slice cultures from young animals represent a valid model of hippocampal neurogenesis, especially with regard to the differentiation, maturation, and functional integration of newborn granule cells. The present study provides evidence that the organotypic culture method can be used to study neurogenesis and generate important information regarding the effect of various pharmacological manipulations on dentate granule cell development.

One limitation of the present model is that we are not able to track the developmental progress of individual neurons. As the immunohistochemistry protocols require the tissue to be fixed prior to staining, any time point comparison must be done on different slices of tissue from the same culture plate. For example, if the culture well plate holds 6 inserts, and we wanted to study the effect of “compound X” at three time points, we removed two inserts from each 6-well plate on three different days. By fixing the tissue immediately with 4%-PFA followed by long-term storage in sodium azide solution, we aimed to capture a snapshot of the slice at these different times. A single well plate held hippocampal slices from one animal, and we relied on the assumption that there was not significant variability between hippocampal slices on each insert. An alternative strategy for tracking the development of newborn DGCs in a single slice of hippocampal tissue would be to briefly apply a viral vector for Green Fluorescent Protein that would incorporate the protein into a small cohort of newborns DGCs. Using time-lapse

fluorescence microscopy in a sterile environment, one could conceivably track the development of granule cells both in control and experimental conditions. The benefit of this technique over the method presently employed would be that neurons could be followed from the stage of neuronal precursor to synaptically integrated mature DGC.

Another limitation of the current method is the restriction to an early post-natal dissection period. If cultured hippocampal slices could be generated from the adult rodent brain then a more direct comparison between adult neurogenesis *in vivo* could be made. Recently, Kim and coworkers (2013) provided evidence that adult mice can be successfully used to generate organotypic cultures. The authors performed time point comparisons of neuronal viability in different culture conditions. By assessing tissue expression of 4', 6'-diamidino-2-phenylindole (DAPI), a marker of neuronal nuclei, they showed that cultures grown in serum free medium experienced less decline in neuronal numbers after 30 DIV compared to cultures with serum and those transferred from serum to serum-free at 4 DIV. Building from this experimental evidence that serum-free medium preserved neuronal viability, the authors examined whether adult organotypic cultures displayed evidence of neurogenesis. Further analysis showed that despite the presence of NeuN positive cells, there was minimal DCX immuno-reactivity in the dentate gyrus. This led the authors to conclude that the hippocampal neurons in culture consist of mature 'original' cells from before dissection instead of newly generated DGCs. Kim and colleagues cite the highly resilient characteristics of the neonatal brain as a contributing factor that might prevent active neurogenesis in adult slice cultures. Perhaps incorporating trophic factors or other neurogenesis promoting compounds in the experimental protocol would allow for preservation of the *in vivo*

characteristics that allow for hippocampal neurogenesis. However, our experiments used rats because, in comparison to mice, previous studies have indicated that newborn neurons mature faster, are less likely to undergo programmed cell death, and are ten times more likely to be recruited into learning circuits (Snyder et al., 2009).

Preliminary experiments in our laboratory attempted to use 3-month-old adult rats for hippocampal cultures. This proved to be difficult because of the strong and well-developed cranium that delayed timely removal of the brain and transfer to ice cold dissecting solution. Efficiently removing the brain and transferring to ice-cold solution is important for maintaining the health of culture slices following dissection. Anatomical differences between mice and rats, specifically with regards to skull thickness, may have allowed Kim and colleagues to efficiently remove the brain and perform culture experiments for up to 30 DIV. Future experiments are needed to definitively determine if organotypic cultures can be successfully prepared from the adult rat hippocampus.

6.2 Future Directions

The importance of these studies and the contribution they make to the field is that it helps build evidence for the utility of the slice culture protocol for studying neurogenesis and for future pharmacological studies. Optimizing slice cultures to study adult neurogenesis will allow investigators to readily test the effect of drugs suspected to promote or inhibit various stages of neurogenesis. The experimental data presented on the role of THIP and APV in promoting neuronal differentiation provides an example of how to measure and interpret results from pharmacology experiments in hippocampal slice cultures. Future studies combining immunohistochemistry with electrophysiological

studies can be used to assess both the morphological and functional characteristics of newborn DGCs in culture.

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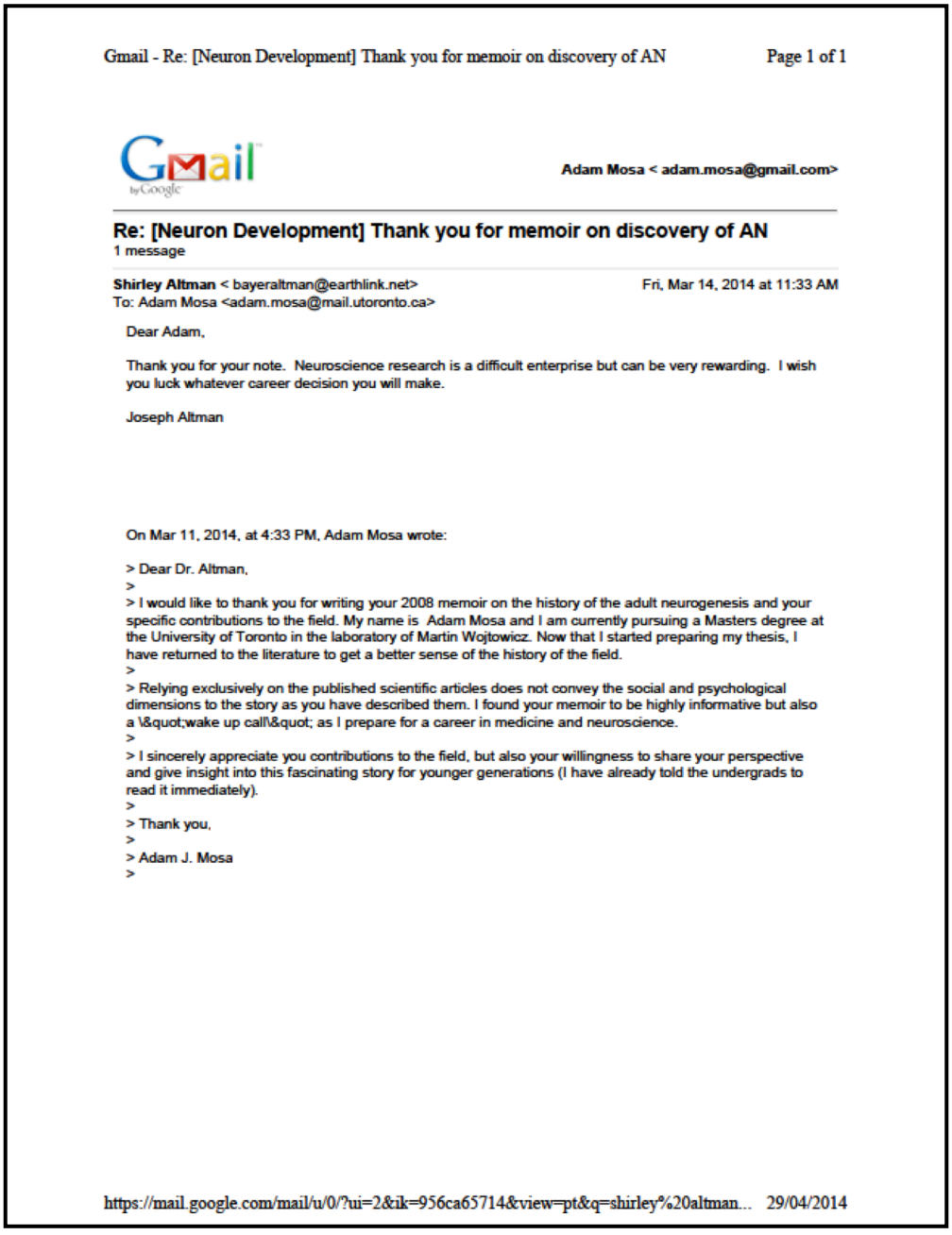
Appendix

1. Email correspondence from Professor Joseph Altman, discoverer of adult neurogenesis.



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Gmail by Google

Adam Mosa < adam.mosa@gmail.com>

Re: [Neuron Development] Thank you for memoir on discovery of AN
1 message

Shirley Altman < bayeraltman@earthlink.net> Fri, Mar 14, 2014 at 11:33 AM
To: Adam Mosa <adam.mosa@mail.utoronto.ca>

Dear Adam,

Thank you for your note. Neuroscience research is a difficult enterprise but can be very rewarding. I wish you luck whatever career decision you will make.

Joseph Altman

On Mar 11, 2014, at 4:33 PM, Adam Mosa wrote:

> Dear Dr. Altman,
>
> I would like to thank you for writing your 2008 memoir on the history of the adult neurogenesis and your specific contributions to the field. My name is Adam Mosa and I am currently pursuing a Masters degree at the University of Toronto in the laboratory of Martin Wojtowicz. Now that I started preparing my thesis, I have returned to the literature to get a better sense of the history of the field.
>
> Relying exclusively on the published scientific articles does not convey the social and psychological dimensions to the story as you have described them. I found your memoir to be highly informative but also a \"wake up call\" as I prepare for a career in medicine and neuroscience.
>
> I sincerely appreciate you contributions to the field, but also your willingness to share your perspective and give insight into this fascinating story for younger generations (I have already told the undergrads to read it immediately).
>
> Thank you,
>
> Adam J. Mosa
>

https://mail.google.com/mail/u/0/?ui=2&ik=956ca65714&view=pt&q=shirley%20altman... 29/04/2014

2. Limmerick on the history of adult neurogenesis. Written and performed for the “60 second challenge” for Frontiers in Physiology 2014, Department of Physiology, University of Toronto.

Santiago Ramon y Cajal
Is the father whose work starts it all
Our system of nerves
Is made up, he observes
Of neuronal cells, discrete and small

Once born, would neurons be able
To renew, or do things stay quite stable?
Dr. Cajal did observe
A life-long loss of verve:
Neurogenesis, then, seemed a fable.

Decades later there was quite a breakthrough
Altman found new adult brain cells that grew!
But his ideas were rejected
And he sulked, quite dejected,
And then ran off in tears to Perdue.

But Eriksson’s work closed the rift
Showing Altman had gotten short shrift
The handy hippocampus
Can clearly revamp us
Thus completing the paradigm shift.