RESPONSE OF NEURAL STEM CELLS TO TRIMETHYLTIN-INDUCED HIPPOCAMPAL INJURY

by

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ABSTRACT OF THE DISSERTATION

Response of neural stem cells to trimethyltin-induced hippocampal injury

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The mammalian brain retains the ability to produce new neurons and glia throughout life. The retention of stem cells in the adult brain is phylogenetically conserved from rodents to humans and probably exists in all mammals. Their functional role and the factors that control their proliferation, migration, and differentiation is currently an intense area of investigation and debate. Although there are broad species-specific differences in brain anatomical and neurochemical structure, two primary neurogenic regions in the adult appear to be consistent: the subventricular zone (SVZ) lining the lateral ventricles which gives rise to new olfactory interneurons, and the subgranular zone (SGZ) of the hippocampus dentate gyrus which gives rise to new granule cell neurons. There is accumulating evidence that SVZ-derived stem cells are capable of responding to environmental cues within the brain, including injury, to influence both their migratory behavior and their lineage fate. Response to injury has been explored in various rodent models, some of which have shown that SVZ-derived cells can proliferate and migrate to the injury site and differentiate into neurons or glia, though they may lack in number and appropriate phenotypic characteristics to qualify as a fully functional repair mechanism. SGZ stem cells are responsive to environmental stimuli involving learning behavior and stress, as well as upregulate proliferation in response to injury. The lineage potential of SGZ neural stem cells is generally limited to producing new granule cell neurons that integrate into the granule cell layer.

Deviation from this pattern can occur under pathological condition such as when granule cell precursors ectopically migrate into the hippocampal molecular layer following chronic epileptic seizures. It is not clearly defined whether SVZ-derived cells in the adult can migrate to the injured hippocampus or contribute to hippocampal repair. We hypothesized that trimethyltin-induced hippocampal injury in mice would induce the response of endogenous subgranular zone stem cells and subventricular zone-derived cells as part of a repair mechanism.

Trimethyltin is a limbic system neurotoxicant that preferentially damages granule cells in the mouse hippocampus dentate gyrus. Stem cell response to TMT injury was examined *in vivo* in C3H mice. Mice were injected intraperitoneally with TMT to determine both dose-response and time course of TMT -induced granule cell injury. Brains were harvested at appropriate timepoints for cryosection and immunostaining. Mice were also injected with bromodeoxyuridine to quantify proliferating cells and follow differentiation of newly born cells. To trace migrating SVZ-derived cells, mice were injected with the fluorescent dye spDiI directly into the lateral ventricle. A steep dose-dependent induction of cell death in the granule cell layer was evident within 48 hours post-TMT injection based on pyknotic nuclei and immunostaining for activated caspase-3, as well as fluorojade C labeling of dying granule cells. The peak period of cell death was at three days post-TMT, with a gradual decline in number of dead or dying cells to near control levels at 8 days post-TMT. No pyknotic or caspase-3^{+} cells were detected at 28 days post-TMT. Glial activation with was prominent and limited to the hippocampus, and coincident with the induction of granule cell death. Immunostaining using markers for microglia (cd11b) and astrocytes (GFAP) indicated microglial activation persisted out to 8 days post-TMT injection before returning to control level, while elevated GFAP expression and astrocyte branching persisted out to 28 days post-TMT. Co-localization of nestin expression was present in some activated astrocytes in TMT-exposed brain, possibly indicative of conversion to a more primitive phenotypic state with

reparative functional roles. TMT injury induced increased Ki-67$^+$ staining of cycling cells in the SGZ in a dose-dependent manner, with a decline above 2.8 mg/kg suggesting possible toxicity to either stem cells or dividing progeny. Quantitative analysis showed that induction of proliferation peaked at 3 to 5 days post-TMT injection and persisted out to eight days post-TMT. Expression of TBR2, a specific marker for neuronal precursors, was significantly increased from 2 to 5 days after TMT injection, indicating induction of a neurogenic response as opposed to gliogenesis. Proliferation of cells in response to TMT injury appeared to be limited to the hippocampus since there was no upregulation in the number of Ki-67$^+$ cells in the subventricular zone of the lateral ventricles. Because neural stem cells occupy a neurovascular niche that may regulate stem cell activity, blood vessel parameters were quantified to determine whether TMT injury induces any overt morphological alterations. In TMT-injured brain there was a prolonged and progressive reduction in blood vessel thickness in the hippocampus, suggesting dysregulation of vessel constriction and dilation that could potentially influence stem cell activity. Icv injection of spDiI exclusively labeled the ependymal/subependymal cells lining the lateral ventricles. 28 days following TMT-induced injury of the hippocampus, spDiI$^+$ cells were detected in the hippocampus dentate gyrus and molecular layer. Very few spDiI$^+$ cells expressed markers of neuronal (NeuN) or glial cells (GFAP, cd11b) and may represent an undifferentiated population of cells. spDiI$^+$ cells were absent in intact uninjured brain, and at the early time point (7d) after TMT injection. To determine whether new cells were critical for recovery from TMT injury, neurogenesis was blocked by dosing mice with 10 Gray gamma radiation prior to TMT exposure. Mice were euthanized 7 days and 1 year after TMT treatment and analyzed for stem cell activity. Time of onset of TMT-induced tremoring activity was unchanged by radiation treatment, but tremors were more severe and prolonged in mice that were irradiated. Hippocampal neurogenesis was reduced by 75% in irradiated mice. Irradiated mice retained the capacity to upregulate

hippocampal neurogenesis following TMT-induced injury but it was significantly reduced compared to unirradiated mice. Only 3% of the newly-born $BrdU^+$ cells in hippocampus generated after TMT injury survived at 1 year post-treatment and co-localized primarily with the neuronal marker NeuN. The number of $Ki\text{-}67^+$ cells in the 1yr hippocampus was only approximately 2% of that measured in 7day post-treatment groups. Overall, the data suggest that stem cells in both subgranular and subventricular zones respond to TMT-induced hippocampal injury and may contribute to behavioral and cellular recovery. The vast majority of newly-born cells generated at the time of injury are not present one year after treatment, though the dentate gyrus in TMT-treated mice are morphologically indistinguishable from that of untreated mice at this time point.

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List of Abbreviations

Abbreviations

AB	Angular bundle
AC	Associational commissural
ADAMs	A disintegrin and metalloproteinase
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
APOER2	Apolipoprotein E receptor-2
ARAC	Cytosine arabinoside
ARC	Activity-regulated cytoskeleton
ATF3	Activating transcription factor-3
ATF4	Activating transcription factor-4
ATP	Adenosine triphosphate
BAPTA-AM	Cell permeant chelator selective for divalent calcium ion
B-cat	Beta-catenin
BDNF	brain derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BLBP	brain lipid-binding protein
BMi-1	B cell-specific Molone murine leukemia virus integration site-1
BMP	bone morphogenetic proteins
BrdU	Bromodeoxyuridine
C3H	C3H/HeJ mouse strain
CA1	Cornus ammonus subregion 1
CA3	Cornus ammonus subregion 3
CatD	Cathepsin D
CBF	Cerebral blood flow
CBV	Cerebral blood volume
CC	Corpus callosum
CD11b	Integrin $alpha_M$
CDK	Cyclin-dependent kinase
CDNF	Ciliary derived neurotrophic factor
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CO2	Carbon dioxide
COX	Cyclooxygenase
Cp	Cortical plate
CREB	Cyclic amp-response element binding protein
CVMS	*caudoventral migratory streams*
CX	Cortex
CXCR4	CXC receptor-4
DAB1	Disabled-1
Dbl1	Disabled-1
DCX	Doublecortin
DG	Dentate gyrus
DNA	Deoxyribonucleic acid

Dsh	Disheveled
EAE	Experimental autoimmune encephalomyelitis
EC	*entorhinal cortex*
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Emx2	Empty spiracles homeobox-2
ErbB4	receptor tyrosine protein kinase-4
EZRIN	Synonym: Cytovillin, villin-2
FGF	Fibroblast growth factor
Fi	Fimbria
FoxG1	Forkhead box protein G1
FOXOs	Forkhead box proteins
GABA	Gamma-aminobutyric acid
GAD	glutamate decarboxylase
Gcl	Granule cell layer
GDNF	Glial-derived neurotrophic factor
GFAP	glial fibrillary acid protein
GLAST	glutamate-aspartate transporter
GLuR	Glutamate receptors
Gp130	Glycoprotein 130
GTPases	Guanine triphosphatase
H2O2	Hydrogen peroxide
Hf	Hippocampal fissure
HICAP	hilar commissural-associational pathway related
HIPP	perforant path-associated cell
ICV	Intracerebroventricular
IGF	Insulin growth factor
IGF-1	insulin-like growth factor-1
IL-1beta	Interleukin-1 beta
IL6	Interleukin-6 beta
IP	Intraperitoneal
IP3	Inositol-3-phosphate
JAK-STAT	Janus kinase/signal transducer and activator of transcription
JNK	c-Jun N-terminal kinase
KA	Kainic acid
Ki67	Antigen Ki67; nuclear protein associated with cellular proliferation
LEF1	Lymphoid enhancer binding factor-1
LIF	leukemia inhibitory factor
LRP6	LDL receptor related protein-6
LTP	Long term potentiation
M2	Macrophage activation alternative (2), anti-inflammatory
MAP	Microtubule associated protein
MASH1	Mammalian archaete scute homolog-1
MF	mossy fibers
MIA	Migration-inducing activity
Ml	Molecular layer

MMP	Matrix metalloproteinase
MOPP	molecular layer perforant path-associated cell
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
N-cad	N-cadherin
NeuN	Neuron specific nuclear protein
NeuroD1	Neurogenic differentiation-1
NFkappaB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NG2	Neural/glial antigen 2
NGF	Nerve growth factor
NGN2	Neurogenin-2
nIPCs	neurogenic intermediate progenitor cells
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NRG1	Neuregulin-1
NSC	Neural stem cells
NT	Neurotrophin
NT3	Neurotrophin-3
OB	Olfactory bulb
OPCs	Oligodendrocyte progenitor cells
P73	Tumor protein 73; family of p53/p63/773 family of transcription factors
PAR-1	Platelet activated receptor-1
Para	*Parasubiculum*
PAX6	Paired box protein 6
PDGF	Platelet-derived growth factor
PEA-15	Phosphoprotein enriched in astrocytes
PirCx	piriform cortex
PKA	Protein kinase-A
PKC	Protein kinase-C
Pl	Polymorphic layer
Pl3	Phosphoinositide triphosphate
Pre	Presubiculum
PSA NCAM	Polysialylated neural cell adhesion molecule
RG	Radial glia
RGC	Radial glial cell
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROBO	Roundabout gene family transmembrane proteins
ROS	Reactive oxygen species
RTK	receptor tyrosine kinase family
SB	Subiculum
SC	Schaffer collateral
SDF1	Stromal cell-derived factor 1
SGZ	Subgranular zone
SH2	Src homology 2
Shh	Sonic hedgehog
SIRT1	Sirtuin-1 (silent mating type information regulation 2 homolog)

Sl	Stratum lucidum
SLIT-1	Slit guidance homolog-1
SLIT-2	Slit guidance homolog-2
Sl-m	*stratum lacunosum-moleculare*
So	Stratum oriens
SOX2	sex determining region
spDiI	(1,1'-Dioctadecyl-6,6'-Di(4-Sulfophenyl)-3,3,3',3'-Tetramethylindocarbocyanine)
Sr	Stratum radiatum
Sub	Subiculum
SVZ	Subventricular zone
T3	Thyroid hormone
TBR1	t-box brain 1
TBT	Tributyltin
TCF/Lef	T-cell factor/lymphoid enhancer factor
TET1	Ten-eleven translocation 1
TGFbeta	Tumor growth factor-beta
TLR2	Toll-like receptor 2
TMT	Trimethyltin
TNF	Tumor necrosis factor
TRK	tropomyosin-related kinase
TRK(A,B,C)	Tropomyosin receptor kinase A, B, or C
TUC4	TOAD/Ulip/CRMP-4
UV	Ultraviolet
VCMS	*ventrocaudal migratory streams*
VEGFR	vascular endothelial growth factor receptor
VEGF	vascular endothelial growth factor A
VEGF-A	vascular endothelial growth factor C
VEGF-C	vascular endothelial growth factor
VLDLR	Very low density lipoprotein receptor
VZ	Ventricular zone

Overview and statement of hypothesis

I. Overview

Many natural and man-made substances are increasingly recognized as having toxicological effects on animal and human populations. Organic and inorganic chemicals released from industrial processes including synthetic compounds (e.g. pesticides, dioxin) and extraction wastes (e.g. mercury, cadmium) present as environmental contaminants pose risks of exposure to the general population. The last quarter century has seen a large number of studies demonstrating the susceptibility of the central nervous system, particularly the brain, to injury from chemical exposures. The neurotoxicological profile of chemicals is influenced by the unique characteristics of the brain, including its highly complex cellular architecture and intercellular communication, high lipid content, blood-brain barrier, high energy requirement/low energy storage, limited immune function, and low rate of cell turnover and repair. The ability of a compound to cross the blood-brain barrier is related to its interaction with transport systems (e.g. lead, manganese, paraquat) and/or lipophilic properties (e.g. methylated organometals) that allows unfacilitated transport across cell membranes. Compounds that act as neurotoxicants disrupt neurological processes including neurotransmission, myelination, energy metabolism, intracellular transport, cell-cell communication, neuroinflammation, cell cycling, and migration. The developing central nervous system is particularly vulnerable to intoxication due to complex arrays of cellular proliferation, migration and differentiation, each modulated by tightly regulated concentration gradients and direct cell-cell signaling. Small perturbations in any of these processes could translate into significant lifetime molecular and structural alterations of the neural network that impact behavior and cognition.

The brain response to injury involves the functional contribution of multiple cell types including neurons, astrocytes, microglia, and cells of the cerebrovascular system such as

endothelial cells and pericytes. As will be discussed, an important reaction to brain injury is the inflammatory response. The extent and type of injury may determine whether a pro- or anti-inflammatory response ensues, and whether it promotes neural repair or exacerbates the injury. The mechanisms and factors that play a role in determining the injury response are not well understood and are currently under intensive research.

Accumulating data indicate that the brain has the potential to invoke a multitude of responses to chemical-induced injury and other insults that result in either containing the extent of the injury, promoting neural survival and repair, inhibiting the repair process (e.g. glial scar formation), or exacerbate the effects of the injury. Additionally, responses may include activation of pathways that protect the brain from potential injury from subsequent insults. For instance, preconditioning mice with a mild ischemic insult can protect against subsequent, more severe insult by upregulating expression of defensive genes (e.g. heat shock proteins), and regulatory pathways such as MAP kinases and ubiquitin-proteasome activity (reviewed by Liu et al., 2009). Other brain responses include glial responses, where reactive astrocytes can perform multiple functions including secretion of trophic factors, removal of excess glutamate or other substances from the extracellular/synaptic environment, and facilitate repair of the blood brain barrier.

Neurons demonstrate remarkable plasticity in the pathological environment that can lead to recovery (e.g. formation of new neurons; synaptic reorganization) or dysfunction (e.g. excessive neurogenesis, mossy fiber sprouting; ectopic migration). Neurogenesis continues into adulthood within discrete neurogenic niches in the subventricular zone and in the hippocampus, and injury can activate stem cells to generate new cells with the potential to migrate into lesion sites as part of a repair response. It was recently shown that the synaptic integration of newly-born hippocampal granule cells is strongly influenced by the characteristics of the pathological environment (Wood et al., 2011). Some neuropathological conditions (e.g. epileptic seizures) can

disrupt the normal migration of granule cell precursors, as well as induce mossy fiber sprouting and abnormal formation of basal dendrites that may deleteriously amplify hyperexcitability. In contrast, new granule cells exhibit reduced excitatory drive and increased inhibitory function which could counteract other plastic changes that increase hyperexcitability in the hippocampus (Jakub et al. 2006). The molecular events mediating this neuronal plasticity and the effects on hippocampal function are poorly understood.

Neurogenesis within the hippocampus and subventricular zone contribute an additional level of plasticity in response to injury. Increased cell cycling activity in the SVZ or SGZ neurogenic niche following injury is emerging as a common theme in many forms of injurious insults to the brain. The role of stem cell activation can include the formation of new neurons to replace dead cells and potentially reduce hyperexcitability through remodeling, or the production of glia that can function to contain the injury and promote healing. It was recently found that stem cells and their undifferentiated descendants may also play non-neurogenic functions by secreting trophic factors into the immediate microenvironment to promote neuronal survival and support differentiation of newly born cells (Butti et al., 2014). Moreover (and surprisingly), neuroblasts have a phagocytic function that is critical for removal of apoptotic neuronal bodies and important for maintaining neurogenesis (Lu et al., 2011). Mosher et al. (2012) showed that neural precursor cells can modulate microglial activation, proliferation, and phagocytosis through the secretion of vascular endothelial growth factor (VEGF), and other non-neurogenic functions of neural stem cells have been described (reviewed in Butti et al, 2014). Stem cell biology, as it relates to brain injury, is a complex and rapidly expanding field of research. Very little is known about how toxicants influence stem cell biology or the factors that determine whether stem cell activation promotes repair and recovery of normal physiological function, or exacerbates the pathology. Understanding how toxicants influence neural stem cells is important to unraveling the

mechanisms underlying neural repair, and represents an important potential contribution of neurotoxicology to the broader neurosciences.

Systematic and detailed studies are required to investigate stem cell functions in neurorepair and must be based on acceptable models that utilize the most contemporary tools, particularly the use of an ever-expanding array of sensitive antibodies.. The vast majority of stem cell studies have been carried out in rodents which are also most commonly used as models for toxicological studies. A large knowledge base has been amassed on stem cell biology in the mouse which makes it a useful tool for investigating neural stem cell response to chemical injury. Many of the observations in mouse models parallel those made in other species including humans. Therefore, enriching our knowledge of stem cell response to chemical injury in mice will have clinical relevance to human medicine.

In this thesis, we examined two neurogenic regions recognized to produce neurons in adulthood (hippocampal subgranular zone and the subventricular zone) and tested the response of these zones to chemically-induced injury by the well-characterized neurotoxicant, trimethyltin. Trimethyltin (TMT) is a potent limbic system neurotoxicant that preferentially induces granule cell death in the murine dentate gyrus. Previous studies (Harry et al., 2004; Ogita et al., 2005) have shown that TMT injury can induce a neurogenic response by endogenous hippocampal stem cells to produce new granule cells. In this thesis, we used the C3H mouse strain for determining the effects of trimethyltin on neural stem cell activity in the hippocampal subgranular zone (SGZ) and the subventricular zone. After initially characterizing the effects of TMT injury in this mouse model, additional studies are described to study the stem cell responses. A variety of techniques were employed including the use of specific markers of proliferation and migration, bromodeoxyuridine and spDiI, respectively; an array of specific antibodies to identify cells; and gamma radiation to inhibit neurogenesis. Little is known about the SVZ stem cell response to

chemical injury to the hippocampus. Increasing our knowledge on how the different neurogenic regions respond, and perhaps coordinate, to promote recovery from hippocampal injury is the overall interest of this project.

II. Statement of hypothesis and specific aims

Hypothesis: **Recovery from the TMT-induced hippocampal injury involves both ventricular and hippocampal stem/progenitor cell migration and proliferation. Further, we hypothesize that brain injury may 'recruit' newly proliferated neural precursor cells to participate in neural recovery.**

To address these hypotheses, the following specific questions were addressed:

I. Do hippocampal stem cells proliferate and differentiate in response to injury?

II. Do cells derived from the ependymal/subventricular zone migrate to the injured hippocampus and contribute to regeneration?

III. What is the fate of new cells that repopulate the hippocampus as part of a repair response?

IV. Does inhibition of neurogenesis effect recovery from trimethyltin-induced injury?

CHAPTER 1. INTRODUCTION

1.1 DENTATE GYRUS: STRUCTURE AND FUNCTION

The dentate gyrus is an integral part of the hippocampal formation of the mammalian brain, where it functions as a major processing center for episodic memory information and temporary storage for short-term memory. Some of the important memory functions associated with the dentate gyrus include pattern separation, the encoding of temporal context to memories such as in trace conditioning tasks that require integration of 'memory events' that occur close in time, and conjunctive encoding that encodes location and time to an event. The anatomical and electrical attributes of the dentate gyrus enable its functional role of filtering cortical sensory input and forming trace memories and pattern separation. For instance, the sparse and selective activation of granule neurons within the densely packed granule cell layer, and the high threshold of developing LTP inherent to the electrical circuitry of the hippocampal granule cells, contribute to the resolution needed to carry out these hippocampal functions (JL Baker, 2003; Deng et al., 2010 The hippocampus also exhibits regional specialization for different functions along the dorso-ventral axis. For instance, the more dorsal aspects play roles in cognition whereas the ventral regions are predominantly involved in emotional function(Bannerman et al., 2002; Fanselow et al., 2010). The hippocampus is one of two regions in the adult brain where neural stem cells actively produce new neurons. The unique properties of new neurons generated in the adult hippocampus may play a vital role in enabling pattern separation and temporal discrimination of new memories (reviewed in Aimone, 2014). Recent studies also implicate the dentate gyrus and neurogenesis in the regulation of emotion and affective behavior, an underlying mechanism for the observed effects of antidepressants. However, the plasticity and

neurogenic activity within the dentate gyrus could impart its susceptibility to disease and injury including excitotoxic, traumatic, neurodegenerative, chemical, seizure activity, or other insults**.**

The focus of this thesis is on neurogenesis and neural migration following TMT-induced hippocampal injury. To understand the consequences of injury and mechanisms of neurorepair, it is appropriate to preface that with an overview of the current concepts of hippocampal structure and function (reviewed by Amaral et al., 2007; Seress Laszlo, 2007; Frotscher et al., Ribak et al., 2007; Li and Pleasure, 2007). Since adult neurogenesis recapitulates many features of neurogenesis and neural migration that take place during mammalian development, a discussion of aspects of neurogenesis and migration during development, early postnatal, and in the adult will be also presented.

The function of the hippocampus in terms of its memory-related roles is preserved from rodents to primates. The mammalian hippocampal formation is one of the oldest (phylogenetically) cortical areas of the brain which can be identified, having an equivalent structure in reptilian species. The hippocampus proper (the pyramidal cell fields CA1 – CA3) is morphologically similar between rodents and nonhuman primates, though there is a general increase in cell number of the pyramidal cell layer to form a thicker multilayered structure in nonhuman primates and humans as compared to mammals lower in the phylogenetic scale. Although, the dentate gyrus shows marked species differences in architecture, cell number, neurochemical characteristics, and afferent/efferent connections. The following features, however, are characteristic of the mammalian dentate gyrus:

i. configuration of the CA3 region of the pyramidal cell layer;

ii. granule cells form a unidirectional pathway to innervate the CA3;

iii. all the cortical afferents which innervate the dentate gyrus also project to the pyramidal cells of the hippocampus CA1 and CA3 areas,

iv. there are far more granule cell neurons than pyramidal cells in the hippocampus;

v. the dentate gyrus “gates” or “tunes” the cortical sensory input from the cortical areas to the hippocampal pyramidal cell layers.

An afferent nerve fiber pathway, the perforant pathway, projects from the entorhinal cortex to the dentate gyrus. This pathway transmits sensory information from the cortex to the dentate gyrus. The absence of counter-synaptic connections projecting from the dentate gyrus back to the entorhinal cortex makes this a unidirectional circuit. In fact, the hippocampal network as a whole is essentially unidirectional (Figure 1.1, 1.2A). The circuitry forms a trisynaptic loop starting with cortical sensory input from the entorhinal cortex layer II to the granule cells of the dentate gyrus. The granule cells then project axons (mossy fibers) that synapse with the CA3 pyramidal cells in the pyramidal cell layer. CA3 Schaffer collaterals (axons originating from CA3 pyramidal cells) send input to the CA1 pyramidal cell layer neurons. The CA1 pyramidal neurons then complete the loop by projecting axons to the subiculum and back to cell layer IV of the entorhinal cortex. The dentate gyrus is a first step for processing of information that leads to the formation of episodic memories. The dentate gyrus ‘processes’ the information from the cortex prior to conveying it to the CA3 subfield of the hippocampus, again, in a unidirectional fashion. As in many structures in the brain, the dentate gyrus is comprised of more than one type of neuron, including the dentate granule cell, the dentate pyramidal basket cell, and the mossy cell, as well as a variety of interneurons. These neurons may be excitatory or inhibitory and carry out specific functions in the

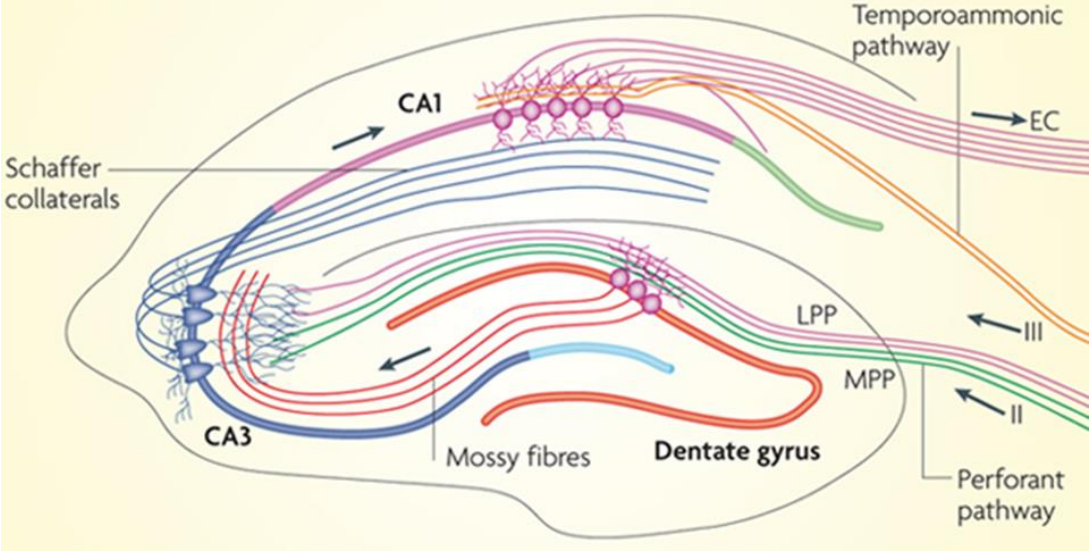


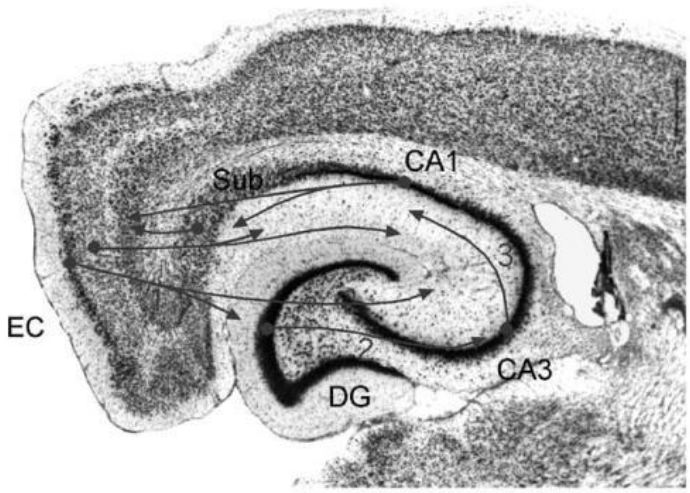
Figure 1.1. The trisynaptic configuration of the hippocampal neural network is largely a glutamate dependent unidirectional flow of information. The different subfields of the hippocampus perform specialized memory functions: the dentate gyrus is important for pattern separation, and the CA3 plays a role in pattern completion. Input originating from neocortical afferents pass information into the entorhinal cortex (EC) and from there projection neurons from cell layers II and III project along the perforant pathway, split into lateral (LPP) and medial MPP) bundles, and synapse into hippocampal subfields: primarily granule cell dendritic processes in the molecular layer of the dentate gyrus, and also form some direct connections with CA3 pyramidal neurons. Mossy fibers from the granule cells project to the CA3 pyramidal cells, which then send axons to pyramidal cells in the CA1 subfield via the Schaffer collateral pathway. The CA1 pyramidal neurons then project onto the subiculum and back to EC cell layer IV. External to the trisynaptic configuration, the EC cell layer III also projects directly to the CA1 subfield and subiculum in the temporoammonic pathway; the functional role of which is unclear. (From Deng et al., 2010)

dentate gyrus including determining number of extent of granule cell activation and LTP formation based on amplitude and frequency of cortical input.

The granule cell layer partially encloses a less cellular region termed the hilus, or polymorphic layer. The most prominent cell occurring within the hilus is the mossy cell. In addition, along the boundary between the hilus and the inner third of the granule cell layer are interneurons such as the pyramidal basket cell.

The hippocampal formation, including the dentate gyrus and other subfields, extends caudally starting from the septal nuclei to the temporal cortex. This axis is frequently termed the septo-temporal axis, and is the longest axis of the hippocampal formation. The 'transverse axis' runs at right angles to the septo-temporal axis. The structure of the dentate gyrus does not have defined subregions along the septo-temporal axis and is a relatively homogeneous structure, though the shape can change slightly to more of a U-shape temporally. The dorsal region of the granule cell layer between the CA3 and CA1 subfields is called the suprapyramidal blade, and the portion opposite to this is referred to the infrapyramidal blade. The point where the super pyramidal and infra pyramidal meet is referred to as the "crest" (Figure 1.2C).

A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3
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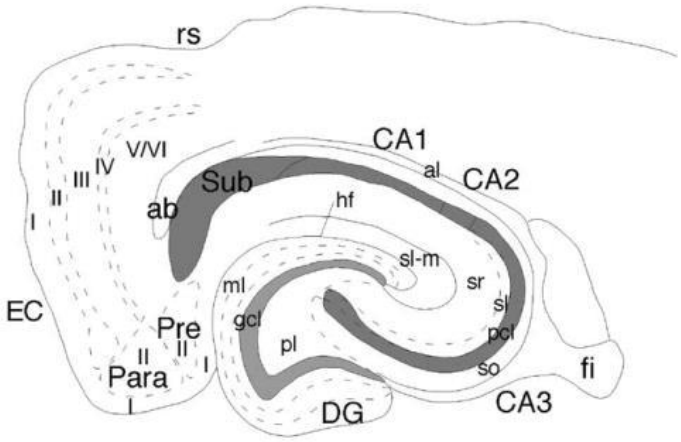
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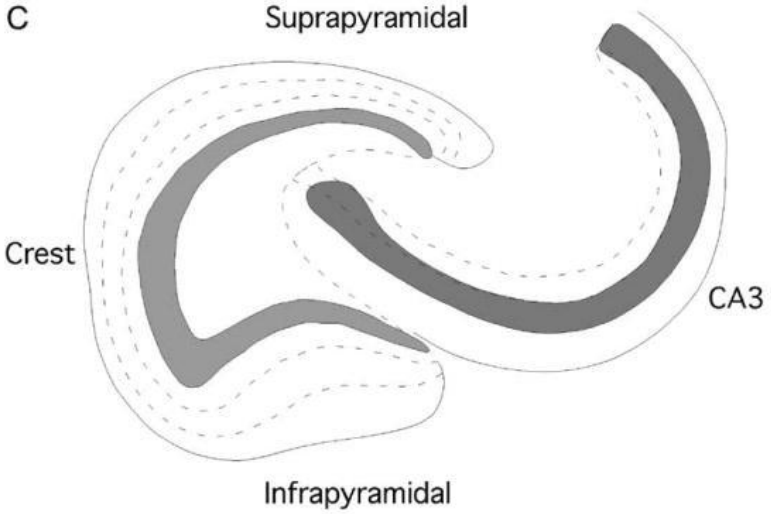

Suprapyramidal
Crest
CA3
Infrapyramidal

Figure 1.2. *The rat hippocampal formation.* ***A****, image of horizontal section of hippocampus that is Nissl stained showing directional flow (arrows) of information in the trisynaptic network. Entorhinal cortex (EC) fibers in the perforant pathway project into dentate gyrus (DG) stratum lacunosum layer to synapse with granule cell dendrites. EC cell layer III also projects into the CA1 subfields and the subiculum. Interneurons in the polymorphic layer and CA3 subfield synapse with mossy fibers projecting from DG granule cells. Pyramidal cell axons in the CA3, i.e. Schaffer collaterals, project to the CA1 subfield.. The CA1 pyramidal cells then provide input to the subiculum and back to the EC cell layer IV.* ***B****, anatomical illustration of the DG displaying its laminar organization comprised of the molecular layer (ml), the granule cell layer (gcl), and the polymorphic layer (pl) or hilus. The molecular layer is further subdivided into three sublayers that differ in input connectivity. The hippocampal pyramidal cell layers are divided into CA3, CA2, and CA1 subfields. EC cell layers are indicated with Roman numerals.* ***C****, illustration of DG and hippocampus showing relative position of suprapyramidal blade, infrapyramidal blade and crest. Abbreviations: ab, angular bundle; a, alveus; fi, fimbria; hf, hippocampal fissure; Para, parasubiculum; Pre, presubiculum; sl-m, stratum lacunosum-moleculare; sl, stratum lucidum; sr, stratum radiatum; Sub, subiculum. (From Amaral et al., 2007)*

Major cell types in the hippocampus dentate gyrus

Perhaps the most distinctive structure within the hippocampus proper is the granule cell layer within the dentate gyrus. Functionally, these cells are critical for forming spatial and discriminatory memories. The granule cell layer is a compact relatively homogeneous cellular structure containing a densely packed organization of excitatory granule cell neurons. In rodents, there are approximately 600,000 to 1 million granule cells within the dentate gyrus; in humans there are 9 to 18 million of these cells, which is roughly proportional to brain weight (reviewed by Seress, 2007). Within the subgranular zone of the granule cell layer is a stem cell niche that functions to generate new granule cell neurons throughout the life of the animal. A steady state in the number of granule cells is maintained despite the continued genesis of mitotic precursor granule cells largely through attrition of non-integrated newly born neurons. Maintenance of the cell population is apparently critical since in certain neurological disease states there is an abnormal expansion in the granule cell population resulting in dysfunction. Since clusters of granule cells contribute to forming episodic memories with unique temporal and spatial

signatures, loss of some granule cells may deteriorate certain aspects of individual memories yet leave essential function or "gate-keeping" intact. However, a large reserve of granule cells exists in the dentate gyrus since there is no pronounced impact on function until there is a >50% loss of cells (Czurko et al., 1997; Czeh et al., 1998, 2001; reviewed by Seress, 2007).

Granule cell soma are only about 15 micrometers in diameter, very compact, and have little cytoplasmic volume (Claiborne et al., 1990), which allows for a high density arrangement within the granule cell layer (Figure 1.3A). However, there appears to be very little synaptic communication between individual cells within the granule cell layer under physiological conditions (Czeh et al., 2005) which prohibits recurrent circuitry among granule cells. Functionally, this protects against hyperexcitability and facilitates unidirectional input from the entorhinal cortex (perforant path) and output via the mossy fiber pathway to the CA3 pyramidal cell layer. Further, the densely packed cellular organization of the granule cell layer and limited local circuitry allows for high resolution memory encoding derived from entorhinal and cortical input. The granule cell excitatory output is modulated by complex interactions with inhibitory and excitatory hilar and molecular layer interneurons.

Granule cells have a single myelinated basal axon, referred to as a mossy fiber, and apical dendrites that project through the granule cell layer to form extensive apical dendritic trees in the molecular layer that arborize with entorhinal cortical fibers (Figure 1.3B). As granule cells differentiate and mature, they transition from being GABAergic to excitatory glutamatergic neurons. While glutamate is the primary transmitter used by granule cells, other neurotransmitters and modulators have been localized in mossy fibers including GABA (likely immature granule cells) and dynorphin (Walker et al., 2002; McGinty et al., 1983;). Some granule cell dendrites have been observed to extend beyond the molecular layer to reach the hippocampal fissure or ventricular surface . Depending on where the cells are localized within the

granule cell layer, either infrapyramidal or suprapyramidal, the number of dendritic spines varies with the density of the perforant path input. The suprapyramidal region tends to have greater entorhinal input and therefore has more extensive dendritic branching and spine density (Desmond and Levy, 1985; Seress, 2007).

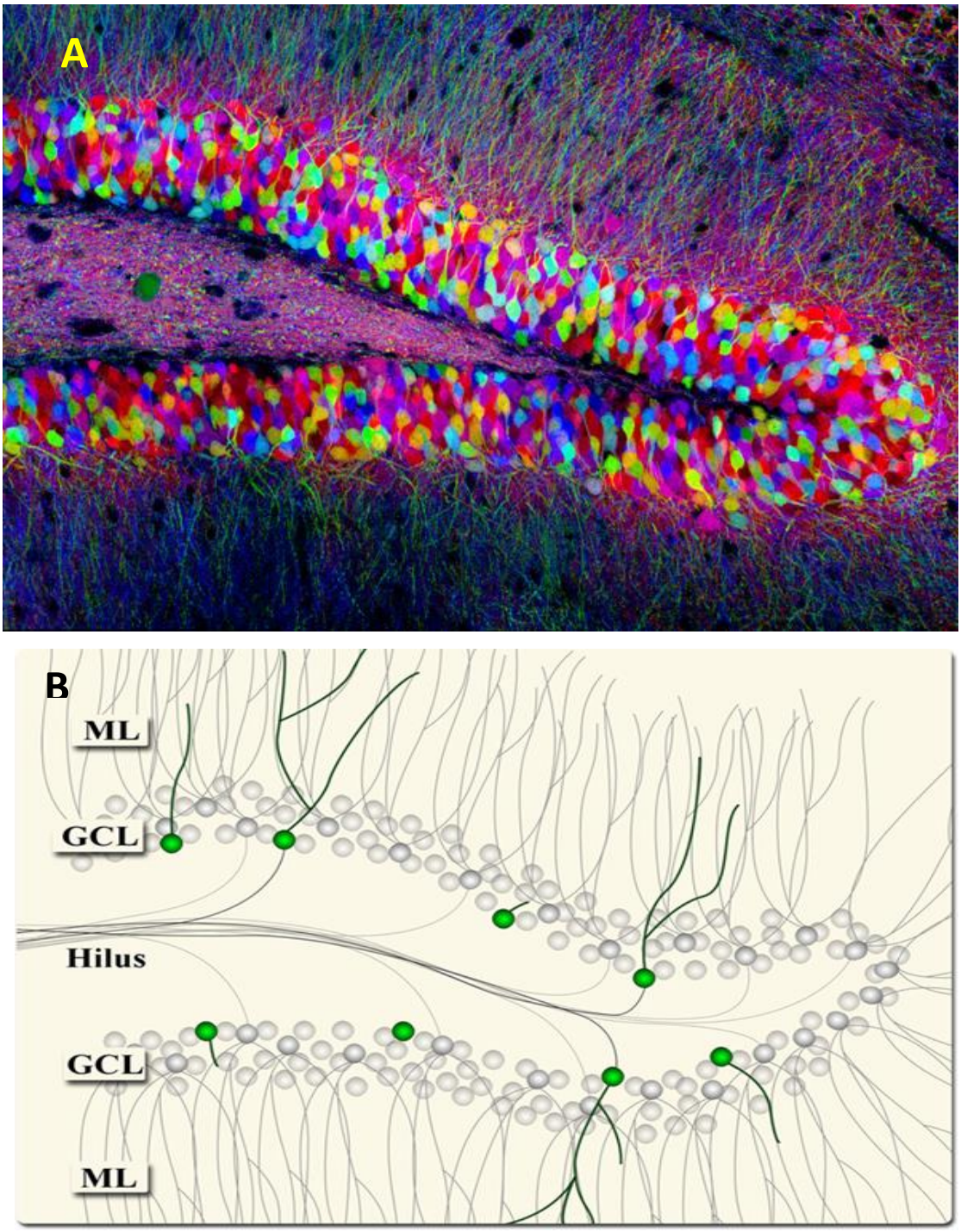


Figure 1.3. A, Individual granule cell neurons of the dentate gyrus rendered fluorescent in "Brainbow" mice showing cellular density and projection of their dendrites through the gcl and into the molecular layer where they receive input from the entorhinal cortex. Neurogenesis occurs along the inside edge of the "V," where neurons are born and then migrate outward toward the dentate gyrus. (From laboratory of Tamily Weissman, Harvard University;

www.weissmanlab.com). B, Illustration showing the organization of granule neurons in the dentate gyrus granule cell layer (GCL) with apical dendrites extending through the GCL into the molecular layer (ML), and basal axons projecting into the hilus. Green cells represent immature, doublecortin-expressing neurons. (From Rosenzweig et al., 2011).

Mossy fibers project into the hilus (i.e. polymorphic layer) where they form synapses with the CA3 pyramidal cell subfield. As a general rule, mossy fibers do not project to the CA2 subfield. Mossy fibers form different myelinated bundles that project to the CA3 pyramidal cell layer depending on whether the granule cell soma is located in the infra- or supra-pyramidal blade of the dentate gyrus. The mossy fiber develops up to seven collaterals as it projects into the hilus to form extensive synapses with various interneurons whose role is to modulate granule cell output to the CA3 (Figure 1.4). Many of the interneurons function as inhibitory GABAergic cells including hilar basket cells and chandelier cells, as well as excitatory hilar mossy cells. Mossy cells play multiple roles in modulating granule cell activity and memory acquisition. Mossy cells synapse with inhibitory interneurons (i.e. basket cells) and activate them to induce inhibition of granule cell activity. However, mossy cells also form recurrent circuitry with distal (contralateral) granule cells to enhance granule cell depolarization. Mossy cells participate in a secondary excitatory circuit involving CA3 pyramidal cell back projections that follow a CA3 pyramidal cell > mossy cell > granule cell path. The sum effects of these apparent conflicting actions on granule cell activity (i.e. both inhibitory and excitatory) are multistep inhibition of proximal and excitation of distal granule cell neurons which is critical in encoding synaptic efficacy and potentiation. Mechanisms have been proposed to describe how these interactions modulate hippocampal memory function including pattern separation and auto/hetero-associative memory (Lisman et al., 2005; Myers and Scharfman, 2011, reviewed by Scharfman and Myers, 2012).

Following seizure activity, mossy fibers can form aberrant collaterals (mossy fiber sprouting) that project to the molecular layer to form a recurrent circuit (Scharfman et al., 2003) and promote granule cell hyperexcitability and potentially propagate chronic seizures. However, a recent study using rapamycin to block mossy fiber sprouting did not prevent seizure frequency, but instead implicated hilar cell loss and ectopic granule cell formation as likely seizure-propagating events (Buckmaster and Lew, 2011).

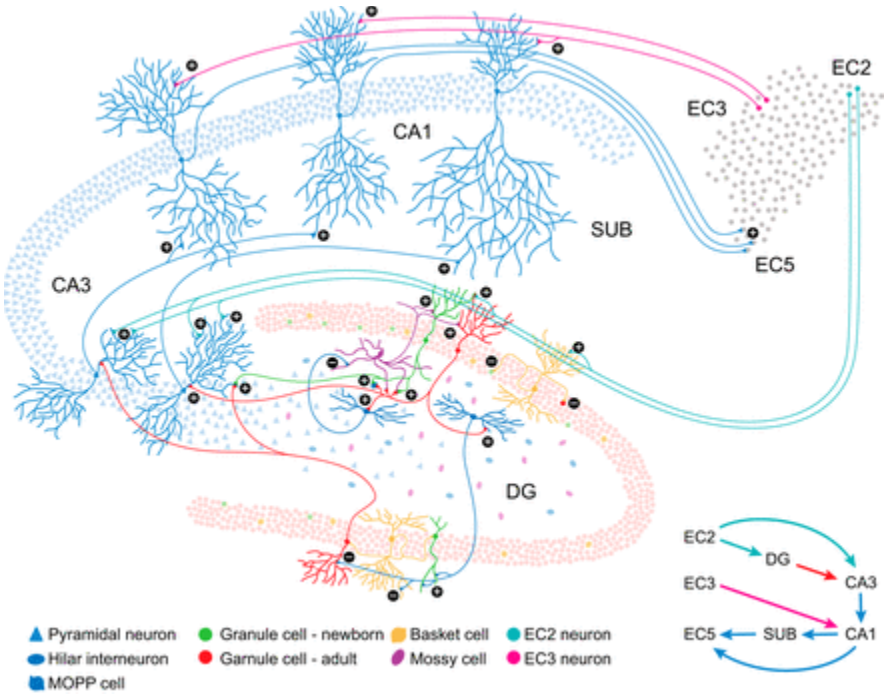


Figure 1.4. *Representation of hippocampal neural network. Granule cell mossy fibers projecting into the hilus and terminate on CA3 pyramidal neurons. Granule cell soma and mossy fibers are innervated by various interneurons that can either inhibit (e.g. basket, MOPP, other hilar cells) or amplify (e.g. mossy cells) the excitatory glutamatergic granule cell function. The mossy fibers transmit the modulated granule cell input to the CA3 region, which is further conditioned via recurrent circuitry. CA3 pyramidal neurons innervate the CA1 pyramidal cells which then project axons back to the specific cell layers in the entorhinal cortex and subiculum, forming the final leg of the trisynaptic loop. (From Aimone et al., 2014)*

Other interneurons in the hilus include the HIPP cell (hilar perforant path-associated cell), other somatostatin-immunoreactive neurons, and multipolar HICAP (hilar commissural-associational pathway related) cells which that are GABAergic and have spiny dendrites that project in the hilus and to the perforant path terminal zone in the molecular layer. Mossy, HIP, and other hilar interneurons are proposed to regulate pattern separation functions in the hippocampus (Myers and Scharfman, 2009).

The molecular layer of the hippocampus is sparsely populated with soma but is enriched with dendrites from granule cells and hilar cells such as pyramidal basket cells. Axons from entorhinal cortical neurons and neurons from other regions (e.g. contralateral mossy cell axons) project into the molecular layer. Cell bodies of at least two neuron types are found in the molecular layer: the MOPP cell (molecular layer perforant path-associated cells) and the axo-axonic cell. Each of these cells project an axon to the granule cell layer where it branches to form terminal synapses with granule cell axons. The axo-axonic cells are responsive to GABA and may exert inhibitory influence on granule cell activity. This inhibitory influence may be significant since a single axo-axonic cell can innervate up to 1000 granule cells (Amaral et al., 2007). MOPP cells also provide inhibitory input to granule cells in the granule cell layer. Recent studies have shown that MOPP cells are activated via perforant path glutamatergic innervation, prior to activation of dentate granule cell neurons, and represent a feedforward inhibition of granule cell activity. It is believed the inhibitory network by both MOPP cells and axo-axonic cells enable the discriminatory function of the hippocampus between similar inputs (Li et al., 2012, Amaral et al., 2007). Interestingly, MOPP cells have been shown to directly innervate newborn granule cell neurons to modulate their activity and contribute to feed forward inhibition in the mouse dentate gyrus (Li, 2013).

The trilaminar structure of the dentate gyrus is conserved across mammalian species. As mentioned previously, variation in the number of dentate granule cells is largely proportional to

brain weight or volume differences between species. Still, some ultrastructural differences are notable. For instance, in rodents, the mature granule cells only project apical dendrites, while the monkey and human also express basal dendrites (Seress, 2007). In rodents, immature granule cells transiently exhibit basal dendrites which are lost as they migrate and fully differentiate. In the non-human primate, the CA3 pyramidal cell field penetrates much more deeply into the polymorphic layer between the blades of the dentate gyrus as compared to the rodent (Seress 2007). Mossy cell morphology also varies between species. In nonhuman primates, a significant volume of mossy cell dendrites project into the hippocampal molecular layer, whereas this rarely occurs in rodents (Seress, 2007). This difference could reflect a significantly greater perforant path input to the mossy cells in monkeys as opposed to rodents.

The functional significance of the differing neuroanatomy of the dentate gyrus between rodents, monkeys and humans is not clearly resolved. Interspecies differences in connectivity pathways may not only affect function, but also response to pathological or toxicological conditions. Hence it must be kept in mind that toxicological insults or injury in rodent models may not directly translate to the same effect in other species.

Functional aspects of the dentate gyrus

During memory formation, the hippocampus encodes a cognitive map of the space which an animal navigates. The encoding of spatial position involves activation of a specific but sparse population of dentate granule cells owing to a combination of granule cell layer density and the inhibitory plexus formed by inhibitory interneurons, as to the hippocampal pyramidal cell network which is neither specific nor sparsely activated. The sparse coding of dentate granule cells is

theorized to be important in information processing and memory formation in the hippocampus in general.

Granule cells of the dentate gyrus exhibit relatively low levels of excitability, (thus differing from other excitatory cells of the hippocampus) due to the extensive interneuronal innervation and recurrent circuitry with the GABAergic plexus. Importantly, granule cells express the inhibitory $GABA_A$ receptor, with a subunit pattern and increased complexity that rarely occurs in other brain regions. Granule cells exhibit both synaptic and extra-synaptic $GABA_A$ receptors with unique properties including a high affinity for GABA that does not undergo desensitization. This lack of desensitization allows for a tonic inhibitory environment or constant responsiveness to physiological levels of GABA in the extracellular space. This generates powerful feedforward and feedback GABAergic inhibition which is characteristic of dentate gyrus circuit function. The resistance of granule cell firing, or the trend to exhibit a reluctance to fire, especially synchronously in network bursts, is due in part to intrinsic factors that (i) promote a hyperpolarized resting membrane potential, (ii) lack of conductance which permit phasic firing or bursting, and (iii) a marked spike frequency adaptation.

The generalized resistance to excitability in the dentate gyrus may serve to filter or gate synchronous excitatory input from the entorhinal cortex and protect it from hyper-activating and damaging susceptible downstream hippocampal structures and triggering seizure activity. As in cognitive mapping within the dentate gyrus, the gating function is also due to powerful feedforward and feedback GABAergic inhibition provided by interneurons (such as MOPP and HICAP cells) as well as the intrinsic properties of granule cells.. In disease states such as epilepsy where there are alterations in $GABA_A$ receptor expression, the gating function of the dentate gyrus is adversely affected. Such alterations could result in loss of inhibitory synaptic receptor function and may disrupt normal operation of the dentate gyrus**.** Indeed, germane to the studies

described in this thesis is the finding that trimethyltin can inhibit $GABA_A$ receptors, and may contribute both to seizure activity and the subsequent effect on cognition following injury (Kruger et al., 2005).

The dentate granule cells express as many as 10 or more $GABA_A$ receptor subunits within a single cell. They express a relatively unique set of $GABA_A$ receptors in which only the thalamus expresses receptors of a similar composition ($alpha_4$-beta-delta). These two brain regions share a functional requirement to fire action potentials in discrete, small populations of neurons, and to resist synchronous firing during information transfer. These requirements are evident as "sparse coding" properties discussed previously for the dentate gyrus where synchronous firing would disrupt or scramble information transfer. This suggests that the $GABA_A$ receptor composition has a critical role in discrete coding of information and prevents synchronous firing. Receptors lacking the $gamma_2$ subunit do not cluster in membrane regions, and they occur at synapses primarily at extra- or peri-synaptic regions. They have high affinity for GABA (nanomolar, compared to the low micromolar range for synaptic receptors), and they exhibit no desensitization. Hence these receptors can respond to ambient or synaptic "spill-over" levels of released GABA and can maintain a tonic current for sustained periods without desensitizing, which defines tonic $GABA_A$ receptors. Tonic and spill-over-mediated $GABA_A$ currents derived from activity of $alpha_4$-beta-delta-comprising receptors are a significant and prevalent feature of dentate granule cell physiological function and are important contributors to the overall function of the dentate gyrus.

Current hypotheses of the dentate gyrus primary function is that it is a vital checkpoint for the regulation of limbic system excitability (Lothman et al., 1992). The dentate gyrus filters and constrains the amplitude duration of afferent inputs as it passes information on to area CA3 of the hippocampus. This gatekeeper function maintains a balance between excitatory and inhibitory actions of glutamate and GABA, respectively. Disruption of this balance can result in

neurons becoming more excitable and prone to the development of seizure activity, as well as compromised memory function. Using tissue slices and voltage sensitive dyes it was shown that the dentate gatekeeper function was compromised when the $GABA_A$ receptors were blocked by an antagonist such as picrotoxin, which blocked both the lower affinity synaptic and higher affinity extra-synaptic $GABA_A$ receptors (Carlson et al. 2002a, Ang et al., 2006). The competitive inhibitor gabazine, which has a more specific inhibitory effect on the lower affinity synaptic $GABA_A$ receptor and not the extra-synaptic $GABA_A$ receptor, did not affect the gatekeeping function of the dentate.

$GABA_A$ receptor expression and function is an important focus in studies of epilepsy. Temporal lobe epilepsy is defined by seizure discharges which activate the temporal lobe, including the hippocampus. It has been shown that there is a doubling in the density of $GABA_A$ receptors in dentate granule cells in both synaptic and whole cell assays, and almost a corresponding doubling in the amplitude of the quantum role in inhibitory synaptic responses. This observation has been shown in multiple models of temporal lobe epilepsy. This is somewhat surprising, since this up-regulation would appear to be contrary to expectations if these receptors are important for gatekeeping, and inconsistent with the hypothesis that seizure activity in epileptic animals is a result of compromised inhibition in the dentate gyrus. However, though there is an increase in the density of $GABA_A$ receptors, there appears to be an alteration in the subunit composition of the receptors based on pharmacological properties and changes in expression patterns of subunits in the granule cells. In the epilepsy models, the synaptic $GABA_A$ receptors become sensitive to zinc blockade as well as a loss of sensitivity to benzodiazepine site agonists. This suggests the alteration in subunit composition of the $GABA_A$ receptors. It's been shown there is a down regulation of expression of $alpha_1$ subunits and an up-regulated expression for $alpha_4$ subunits. This constitutes basically an alpha subunit switch which could explain the enhanced sensitivity to zinc blockade and diminished sensitivity to benzodiazepine site agonists.

The functional significance of the altered zinc sensitivity of $GABA_A$ receptors is an alteration in inhibitory synaptic response.

The dentate gyrus in epileptic animals frequently demonstrates aberrant sprouting of the output axons of granule cells, the mossy fibers. These axons, perhaps in response to loss of targets in the hilus and area CA3, sprout and re-innervate the proximal dendritic tree of other dentate granule cells, creating an abnormal re-entrant excitatory circuit. In addition to releasing glutamate, mossy fibers also co-release large quantities of zinc, which plays a modularly role. Therefore in the epileptic brain, there is a combination of the appearance of an enhanced zinc delivery system from ectopic sprouting of mossy fibers, as well as up-regulated expression of zinc-sensitive synaptic $GABA_A$ receptors. Simultaneous occurrence of these alterations led to the hypothesis that during periods of repetitive afferent activation of the dentate gyrus, which triggers zinc release, there may be a zinc-induced collapse of augmented GABAergic inhibition that would promote seizure initiation (Cohen et al., 2003). In accordance with this hypothesis is the finding that in epileptic animals there is a reduction in tonic $GABA_A$ current in granule cells, which is accompanied by down-regulation of expression of delta subunits. This down regulation could potentially compromise function of the dentate gyrus as a gatekeeper despite that there is a concomitant up-regulation of synaptic $GABA_A$ receptors.

1.2. ADULT NEUROGENESIS IN THE SUBVENTRICULAR AND HIPPOCAMPAL SUBGRANULAR ZONES

Overview of neurogenesis in the brain

Neurogenesis in the adult brain and the integration of the new neurons was first identified in songbirds (Paton and Nottebohm, 1984). It was discovered soon after that stem cells and

neurogenesis occurred in the adult mammalian brain as well (Reynolds and Weiss, 1992). The advent of new technology to identify stem cells and proliferating progenitor cells (e.g. bromodeoxyuridine labeling) and trace their lineage and positioning commitment has enabled a deeper understanding of the biological/functional significance of adult neurogenesis.

Stem cells can be characterized by two primary features: (1) the ability to replicate themselves via symmetrical cell division creating two identical daughter cells; and (2) undergo asymmetrical division in which one of the daughter cells differentiates into a specialized cell, or a progenitor cell with more limited lineage potential to generate specialized cells (Gage et al., 2000).

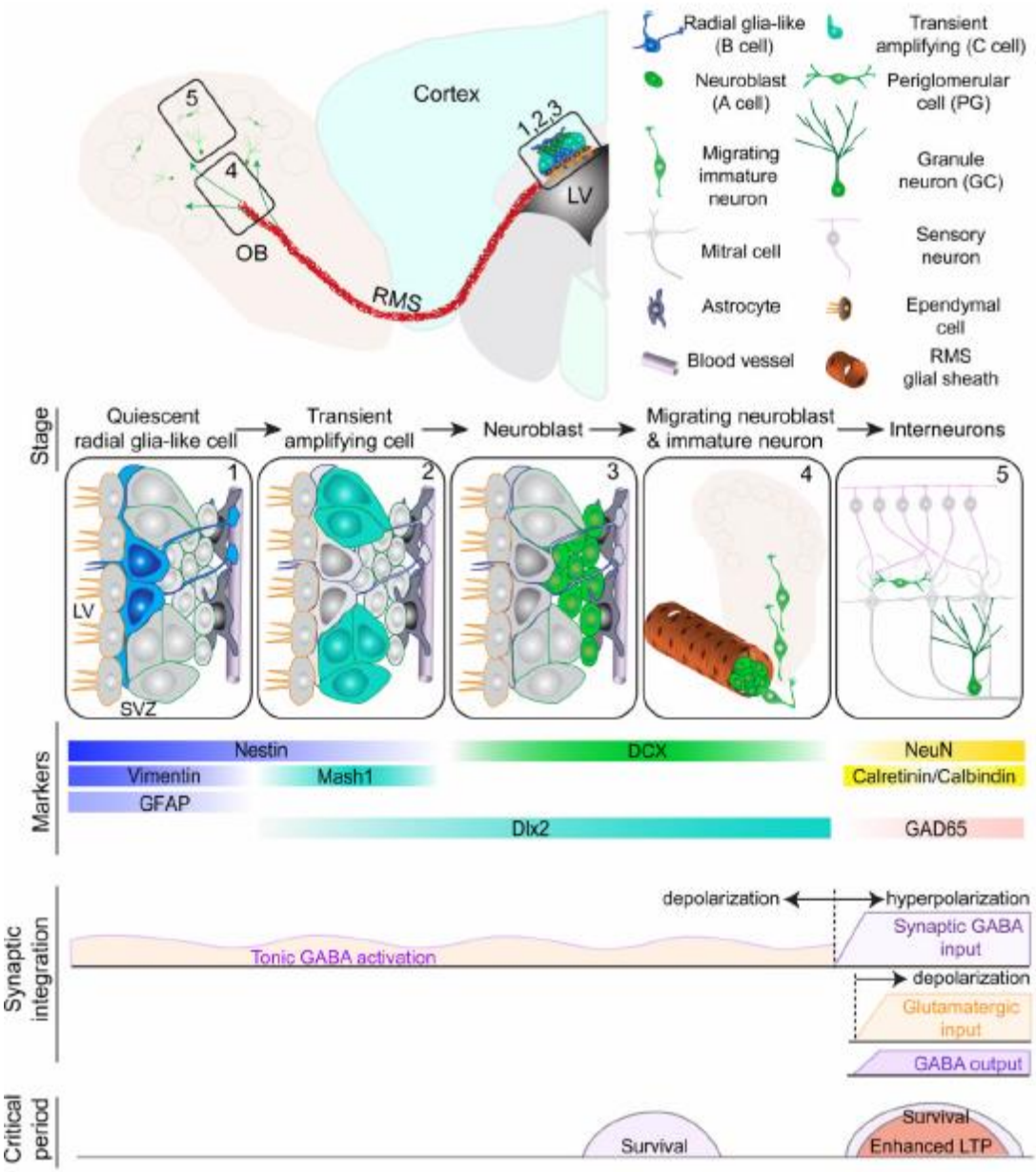


Figure 1.5. *Neurogenesis in the subventricular zone gives rise to precursor cells that migrate along the rostral migratory stream to the olfactory bulb. The SVZ neurogenic niche harbors stem cells and intermediate precursors that communicate with blood vessels, astrocytes, and ependymal cells to regulate the neurogenesis. Specific markers are shown that help identify the various stages of developing neuroblasts and immature neurons in the SVZ. (From Ming and Song, 2011)*

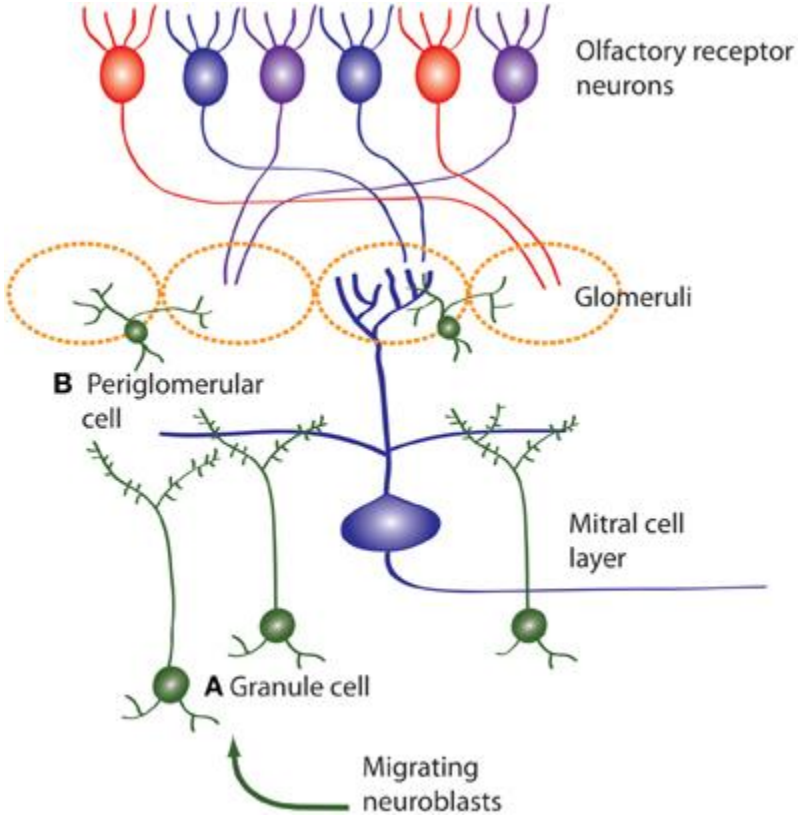


Figure 1.6. *Subventricular zone neuroblasts migrate into the olfactory bulb and differentiate into olfactory granule cells and periglomerular interneurons (A and B). These cells exert inhibitory influence on mitral cell activity that receive input from the excitatory olfactory receptor neurons, each of which expresses a single type of olfactory receptor. Axons derived from olfactory neurons expressing the same olfactory receptor converge within glomeruli and synapse with mitral cells which then project input processes to the olfactory cortex. (From Konefal et al., 2013)*

In the hippocampus, neural precursors born in the SGZ form new granule cells that migrate and integrate into the granule cell layer of the dentate gyrus. *In vivo*, the lineage fate of subgranular zone stem cells is limited to the formation of granule cells. However, *in vitro* manipulation of these cells suggest they have multipotential capability (Figure 1.7). Although *in vitro* studies have been very useful for discovering stem cells in brain and their biology, the *in vitro*

behavior of the cells does not necessarily reflect that occurring in the intact brain. Indeed, addition of certain growth factors to culture conditions can induce cells to undergo differentiation pathways that would not ordinarily occur in vivo (Gabay et al., 2003; Palmer et al., 1999). The new granule cells form axonal and dendritic processes that integrate into the neural circuitry of the hippocampus: branched dendrites project across the granule cell layer into the molecular layer, and axons project into the hilus and synapse with CA3 pyramidal cell neurons (Zhao et al., 2006). Electrophysiological studies have demonstrated that some of these cells integrate with the established hippocampal circuitry. Initially, the newly born granule cells are activated by GABA released by proximal interneurons that have synaptic input to the new cells (Bhattacharyya et al., 2008). Later, the granule cells become responsive to glutamate derived from newly formed glutamatergic synaptic inputs (Ge et al., 2006; Overstreet-Wadiche et al., 2006). The granule cells develop mossy fiber (axonal) processes characteristic of mature granule cells that synapse with hilar interneurons and CA3 pyramidal cell neurons (Faulkner et al., 2008; Toni et al., 2008). Interestingly, recent studies suggest that the new adult-born granule cell neurons have distinct functional properties that differentiate them from older granule cell neurons under physiological conditions (Danielson et al., 2016), and exhibit integration plasticity under pathophysiological conditions that may attenuate hyperexcitability (Wood et al., 2011).

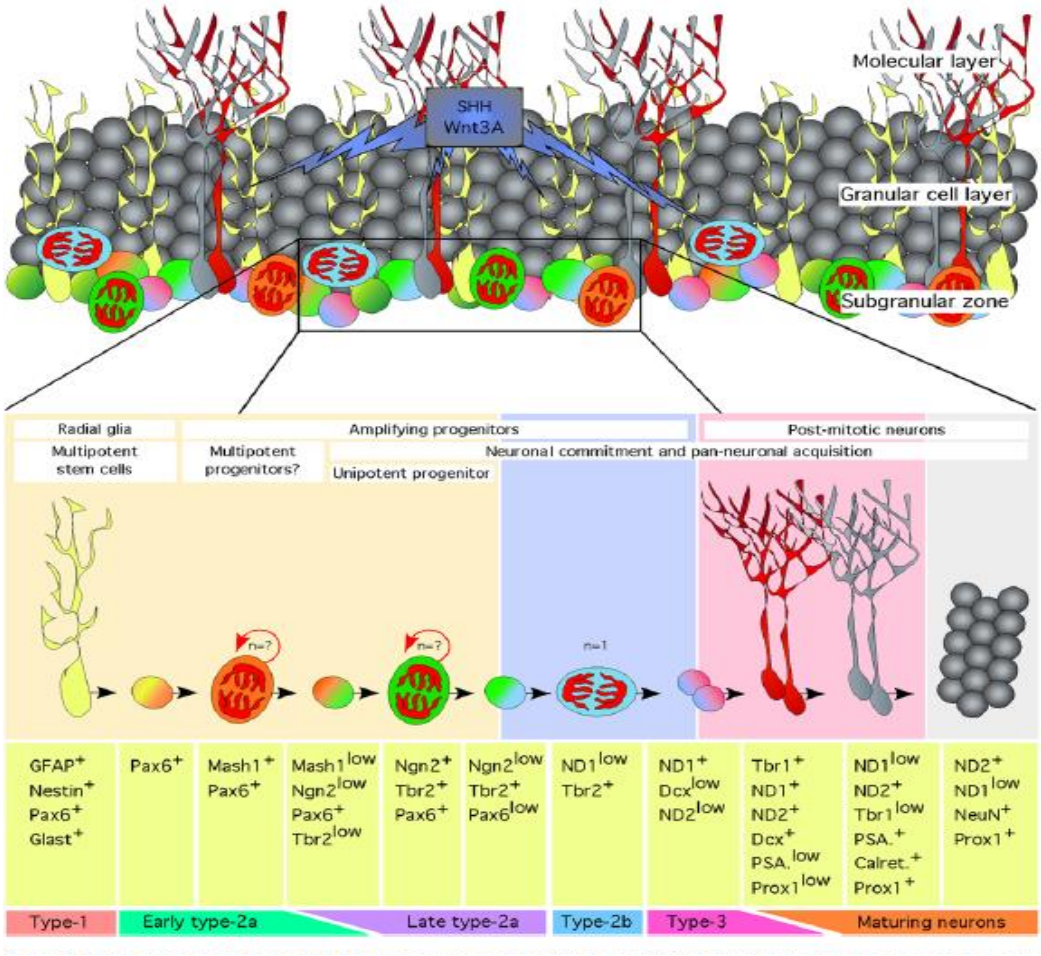


Figure 1.7. *Illustration of the structural organization of the dentate gyrus granule cell layer showing stem cells and precursors cells residing in the subgranular zone. The multipotent stem cells and intermediate precursors express specific antigens, some of which overlap, that can be used to identify the stages of neural development with this neurogenic niche. The earliest stem cell projects apical glial-like processes deep into the granule cell layer which retract as they undergo cell division. Depicted are both asymmetric and symmetric cell division where chromosomal are arranged parallel or perpendicular to the long axis of the granule cell layer during metaphase, respectively. Many of the antigens represent transcription factors including Pax6, Mash1, NeuroD, Tbr1/2, and others. (From Roybon et al., 2009)*

Stem cells in the subventricular zone are radial glia-like cells that express the astrocytic marker GFAP. After undergoing multiple intermediate stages of development, the new cells become neuroblasts that undergo tangential chain migration toward the olfactory bulb, where they disperse radially and differentiate into interneurons. Some of the migrating cells also migrate into

the corpus callosum where they differentiate into oligodendrocytes (reviewed in Alvarez-Buylla and Lim, 2004). Hippocampal SGZ stem cells are also radial glia-like cells that express GFAP, and give rise to intermediate neuronal precursors that migrate into the granule cell layer where they integrate as new granule cells in the dentate gyrus. Depletion of rapidly dividing intermediate precursor cells using chemical or radiation treatment results in the upregulation of stem cell activity to replace the lost cells. Linear tracers and fate-mapping demonstrated that radial glial-like stem cells are the progenitors of the newly formed cells in the adult brain (Guo-li Ming and Hongjun Song, 2012; Doetsch et al., 1999; Seri et al., 2001; Dhaliwal and Lagace, 2011).

It is not clear if multiple subtypes of neural stem cells exist in the SGZ, or that they are a more homogeneous population that gives rise to a heterogeneous population of neural and glial precursors (Lugert et al., 2010). An alternative model for adult hippocampal neurogenesis proposes that a Sox2 expressing stem cell, rather than a radial glial-like cell, gives to neurons and glia (Suh et al., 2007). These cells express the sex determining region Y-box 2 (Sox2), and through asymmetric cell division, give rise to both new Sox2-expressing cells, as well as neuronal and glial precursors. Sox2 is an intracellular signaling molecule that plays an important role in maintaining the precursor cell pool within the subgranular zone. Deletion of Sox2 activity results in the accelerated proliferation and differentiation of stem cells and results in depletion of the stem cell pool. Sox2 mediates this activity through Notch signaling and downstream interaction with sonic hedgehog (Ehm et al., 2010).

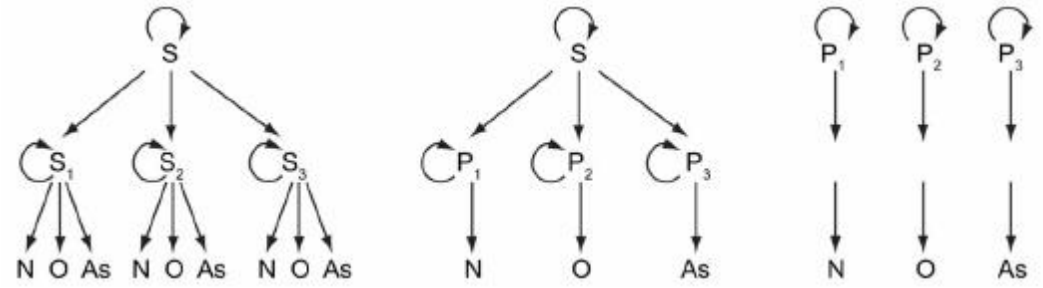


Figure 1.8. *Models of hippocampal neurogenesis depicting three lineage fates of neural stem cells. In the first, the radial glial-like stem cells (S) can produce more stem cells with multipotential to directly generate neurons and glia. In the second model, stem cells generate different populations of progenitor cells with limited potential that give rise to neurons or glia. In the third model, rather than a single multipotential stem cell population, there exists multiple populations of progenitor cells with limited potential to give rise to neurons or glia. As, astrocyte; N, neuron; O, oligodendrocyte; P, progenitor cell; S, stem cell.* (From Ming and Song, 2011)

As suggested in the lineage diagram (Figure 1.8), there are a few different model pathways suggested for the terminal differentiation of neurons, oligodendrocytes and astrocytes (Ming and Song, 2011). However, the most recent studies suggest that there are subpopulations of stem cells in the neurogenic niche that are partially dependent on the spatial positioning of the stem cells within the SGZ and subventricular zone. The phenotypic fate of the new olfactory bulb interneuron precursors that arrive via the rostral migratory stream (rms) is dependent on the rostro-caudal location of the SVZ radial glial-like cells from which the particular cell is derived (Merkle et al., 2007). Furthermore, the relative position of neuroblasts along the rms can also influence the effect of growth factors and other effectors on their migratory destination and differentiation fate.

A difficulty in assessing the identity and other biological characteristics of stem cells is due to the fact that they infrequently undergo cell division or are essentially quiescent, which limits the efficacy of using proliferation markers such as the deoxynucleotide marker bromodeoxyuridine (BrdU) or retroviruses. Long-term exposure to nucleotide analogs to label slowly dividing cells may be necessary, such as by administration through drinking water, may have toxic side effects. (Doetsch et al., 1999; Morshead et al., 1994; Mo Costandi, 2011). It has also been proposed that ependymal cells, which are normally quiescent under physiological conditions, possess stem cell properties and are a source for generating new neuronal cells after injury (Johansson et al., 1999; Carlen et al., 2009; Coskun et al., 2008; Mirzadeh et al., 2008).

Neurogenesis in the subventricular zone

The primary neurogenic zone in the adult brain is in the subventricular zone (sometimes referred to as the subependymal layer), which is a much larger reservoir of proliferative cells compared to the SGZ neurogenic region. The neuronal precursors that are generated in the SVZ also must migrate much longer distances to reach their final destination at the olfactory bulb. The SVZ neurogenic niche consists of both slowly dividing stem cells and rapidly dividing transit amplifying cells that give rise to neuroblasts that migrate as chains of cells to the olfactory bulb. Perinatally, the olfactory bulbs have an open olfactory ventricle which closes as the animal matures and forms the glial rostral migratory stream used by SVZ-derived neuroblasts in the adult. Additionally, radial glial cells that exist developmentally in the subventricular zone, disappear during the early postnatal period and differentiate into astrocytes that take residence in the cortex, white matter, and subventricular zone (Malatesta et al., 2000; Noctor et al., 2001; Tramontin et al., 2003). Some of the radial glial cells also differentiate into olfactory interneurons (Ventura and Goldman, 2007). Within the subventricular zone the astrocytes retain stem cell characteristics, giving rise to neural progenitors throughout the life of the animal (Doetsch et al., 1999; Capela and Temple, 2002; Chiasson et al., 1999; Garcia et al., 2004; Laywell et al., 2000).

There are similarities and differences between the subventricular zone neurogenic niche and the neurogenic niche existing in the dentate gyrus. One of the similarities is the communication between the neural stem cells and blood vessels. The subventricular zone is highly vascularized with blood vessels that run parallel to the direction of the migrating neuroblasts in the rostral migratory stream. In fact all of the cell types that comprise the neurogenic niche have been shown to make contact with blood vessels (Eichmann and Thomas, 2012). Type B astrocytes, the putative

stem cell in the subventricular zone, has a long basal process that makes contact with blood vessels and migrating chains of neuroblasts (Mirzadeh et al., 2008). Recent studies suggest that blood-borne factors and blood vessels play an important role in guidance of neuroblasts to the olfactory bulb (Shen et al., 2008).

The subventricular zone is comprised of multiple cell types, each playing a role in the maintaining the neurogenic niche:

- ciliated ependymal cells (type E cells),
- radial-glial-like astrocytes (type B1 cells) with stem cell features
- transit amplifying cells (type C cells),
- neuronal progenitors (type A cells) that migrate along the rms stream and continue to proliferate during chain migration;
- type B2 cells, the astrocytes that form the rms and form the tubes within which the migrating neuroblasts travel;
- microglia, which also reside in the subventricular zone but only represent 1.5% of the total population in this region. Recent studies suggest microglia play an important role in regulating neurogenesis and that this function is coordinated with T cells (Ziv et al., 2006).

The cells comprising the SVZ neurogenic niche form an organized unit with an integral role in regulating neurogenesis through both structural support and secretion of regulatory molecules (Figure 1.9;Table 1.1). The type B cells have an apical process that contacts the surface of the ventricular lumen, and basal processes that make contact with blood vessels and migrating chains of neuroblasts. These contacts may confer a level of control by the stem cells on the proliferation and differentiation migrating cells. Since the B cells make contact with both the ventricular cerebral spinal fluid and the blood vessels, factors from both of these regions may be integrated to provide a control mechanism on the production and migration of the precursor population.

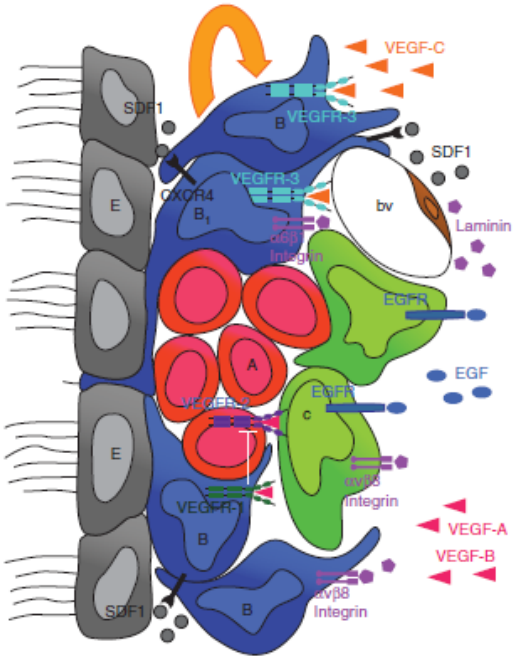


Figure 1.9. *Model showing the intercellular relationships within the SVZ neurogenic/neurovascular niche. Astroglial (B) and astroglial stem cells (B1) at the subependymal cell border communicate with CSF, ependymal cells, and blood vessels. Type C mitotic intermediate precursors preferentially associate with blood vessels, and type A cells (in red) migrate as neuroblasts in contact with astrocytes as they enter the rostral migratory stream. Precursor cells express receptors to angiogenic factors such as VEGF and EGF secreted by astrocytes and endothelial cells to regulate proliferation in the SVZ. Extracellular matrix proteins such as laminin bind integrins (avβ8) expressed by astrocytes to regulate angiogenesis, blood-barrier formation, and precursor cell migration and differentiation. SDF1 expressed by ependymal and blood vessels binds CXCR4 receptor to influence stem cell proliferation, cell adhesion, and precursor cell migration. ; A, neuroblasts; B, astrocyte; B1, stem cell; bv, blood vessel; C, proliferating progenitors; E, ependymal cell; EGF, epidermal growth factor; EGFR, EGF receptor; SDF1, stromal cell derived factor-1; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.* (From Eichmann and Thomas, 2012*)*

Type A cells proliferate and migrate along the rostral migratory stream (Lois et al., 1996). The type A cells are derived from the transit amplifying proliferating type C cells. The type A cells are neuroblasts, but are also referred to as neuronal progenitors. The type A cells are a heterogeneous population of neuroblasts, with some expressing doublecortin and some that do not (Nam et al., 2007). They migrate tangentially within the rostral migratory stream in a migration pattern identified as chain migration or also referred to as homophilic migration (to be

described later). Type A cells migrate in the adult brain within the glial tubes formed by the type B2 astrocytes, however, migration of these cells also has been observed in neonates even though they lack a developed astrocytic ensheathment of the rostral migratory stream. The neuroblasts in neonates migrate as single cells rather than in chains, which may be related to the fact that they do not express the cell surface protein PSA-NCAM until several days after birth. Hence streaming of neuroblasts towards the olfactory bulb is not absolutely dependent on type-B2 cells forming a glial scaffold. Instead, the glial tubes that develop later may serve to separate the migrating neuroblasts from factors present in the less permissive parenchyma in the maturing and adult brain.

The speed with which SVZ precursor (type A) cells can migrate has been measured to be between 20 to 100 microns/hr., but is not a continuous movement (reviewed by Cayre et al., 2009; Bolteus and Bordey, 2004; Davenne et al., 2005; De Marchis et al., 2001; Lois and Alvarez-Buylla, 1994). Migration within the rostral migratory stream is comprised of periods of active migration and periods of relatively little activity punctuated with apparent exploratory behavior (Davenne et al., 2005 Nam et al., 2007) and is probably a result of heterogeneous movement of individual neuroblasts. As Type A cells migrate through the rostral migratory stream, they extend a leading process oriented toward the direction of migration followed by translocation of the cell nucleus and detachment of the trailing celular process. The cell attaches to a new position to re-start the motive cycle.

The migratory speed may be regulated by GABA receptor activation in that the type B2 ensheathing astrocytes express GABA transporters that may control the level of receptor activation in the migrating cells. Astrocytes also express a protein called migration-inducing activity (MIA) that promotes neuronal migration (Mason et al., 2001). Additional control of neurogenesis and migration may involve communication between blood vessels and ependymal

cells. There is an extravascular basal lamina that is associated with the blood vessels that extends to the ependymal cells which could play a role in regulating neurogenesis and cell migration in the adult subventricular zone (Mercier et al., 2002). Once the migrating neuroblasts reach their destination at the olfactory bulb, they detach from the rostral migratory stream and migrate into the granule and periglomular cell layers. When the cells reach their final position they form GABAergic and dopaminergic interneurons (Belluzzi et al., 2003; Carleton et al., 2003; Doetsch and Alvarez-Buylla, 1996).

Other possible sites of secondary neurogenesis in adult mammals have been identified such as in the amygdala (Bernier et al., 2002; Fowler et al., 2003; Park et al., 2006; Keilhoff et al., 2006) and hypothalamus (Huang et al., 1998; Pencea et al., 2001; Kokoeva et al., 2005) . In these regions new cells have been identified and in the case of the piriform cortex one study suggests that the SVZ neuroblasts in adult rodents migrate from the subventricular zone through the subcortical white and gray matter to differentiate within the piriform cortex (Shapiro et al., 2007). However, another study suggested that rather than migrating from the subventricular zone to the piriform cortex, that endogenous precursor cells within this cortex gave rise to the newly born cells (Rivers et al., 2008).

It has been shown that adult humans also maintain sites of neurogenesis in the SVZ and SGZ. Intravenous injection of the proliferative marker bromodeoxyuridine (BrdU) into brain cancer patients labeled cells in the dentate gyrus in addition to some subventricular zone cells. This confirmed that two neurogenic germinative zones exist in the human adult brain. Though the human adult subventricular zone may be a reservoir of neural stem cells, there is controversy regarding the existence of a rostral migratory stream. No clear chain migrating neuroblasts to the olfactory bulb have been identified (Sanai et al., 2004). One study suggested that a portion of the

lateral ventricle extends to the olfactory bulb in the adult human brain, creating a pathway for SVZ neuroblasts to migrate into the olfactory bulb (Curtis et al., 2007).

Neurogenesis in the hippocampal subgranular zone

As described earlier, granule neurons of the dentate gyrus originate from progenitor cells that radially migrate from the neuroepithelium lining the lateral ventricle across the developing hippocampus (Altman and Bayer, 1990; Rickmann et al., 1987). Some of these early progenitor cells then take residence within the early developing dentate gyrus and form a secondary germinative area to fully populate the granule cell layer. Many of these progenitor cells persist within the dentate and are localized in the subgranular zone in the adult where they continue to give rise to new neurons throughout life of the animal. The mitotically active cells in the subgranular zone are comprised of two cell types. Firstly, the stem cells, labeled as "Type-1" cells, have characteristics similar to radial glia. Commonly referred to as radial glia-like astrocytes, these cells can be immunologically identified by a combination of markers including GFAP, Nestin, Pax6, brain lipid-binding protein (BLBP), and glutamate-aspartate transporter (GLAST). The type-1 cells extend long processes that traverse the width of the granule cell layer to the outer edge of the granule cell layer and bordering the molecular layer. Additionally, the cells have tangentially directed processes that line the inner border of the granule layer adjacent to the polymorphic layer. The type-1 cells are also mitotically active at a very low frequency and give rise to mitotic intermediate precursors that occupying the subgranular zone. The type 2 and type 3 intermediate precursors are sometimes collectively referred to as transient progenitors or "D" cells. These intermediates are often further categorized based upon expression levels of specific markers defining "type 2a", type 2b", "type 2a", "type 3" (refer to Fig. 1.7) in order of maturity. These D

cells occupy the subgranular zone and are equivalent to the transit amplifying cells that occur within the subventricular zone. The D cells are early neuronal progenitors that proliferate to give rise to neuronal precursors that are not mitotically active, but which differentiate to express various markers of the immature neuroblasts including doublecortin, Tuc4, and PSA-NCAM on their way to terminally differentiating into mature neurons. The young neurons remain tightly associated with the long B-cell processes as they tranverse to the granule cell layer until they mature into granule cells (Figure 1.10). All three of the cell types, B cells, D cells and neuroblasts comprise clusters of proliferative zones in the subgranular zone from which the neuroblasts migrate short distances into the granule cell layer (Figure 1.11). The cells migrate tangentially within the subgranular zone before turning radially to migrate deeper into the granule cell layer. During this migration, the cells leave a trailing process (basal dendrites) within the proliferative zone of the subgranular zone, and then develop radially-oriented processes toward the granule layer (which later become apical dendrites) as the cell bodies position themselves within the granule cell layer (Seki et al., 2007).

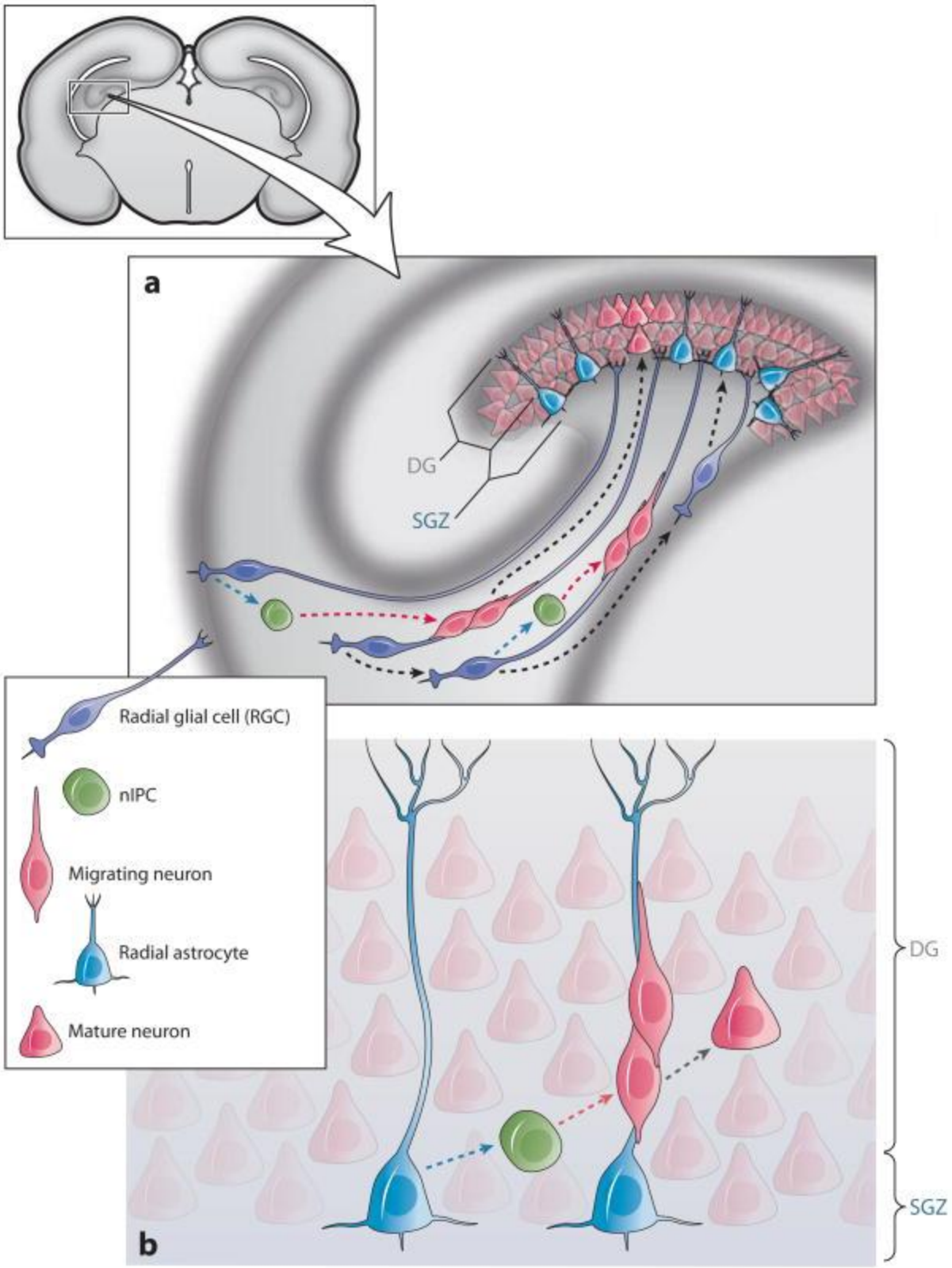


Figure 1.10. Schematic depicting the progenitor cell types in the hippocampus of the developing and adult mammalian brain. a, shows the relationship between radial glial cells (RGC) from the

ventricular zone and the developing hippocampus in which RGC's extend processes and migrate to the SGZ of the hippocampus while giving rise to neural intermediate precursors cells (nIPC) and migrating neurons (i.e. neuroblasts). The RGC's take residence in the SGZ and remain there into adulthood as a pool of radial astrocytes, or type 1 cells, that give rise to nIPC's that migrate and differentiate into the granule cell layer as new granule cells. The long processes of the RGC's and SGZ radial glia serve as a scaffold directing migration of neuroblasts. DG, dentate gyrus; nIPCs, neurogenic intermediate progenitor cells; RG, radial glia; SGZ, subgranular zone; VZ, ventricular zone. (From Kriegstein and Alvarez-Buylla, 2009)

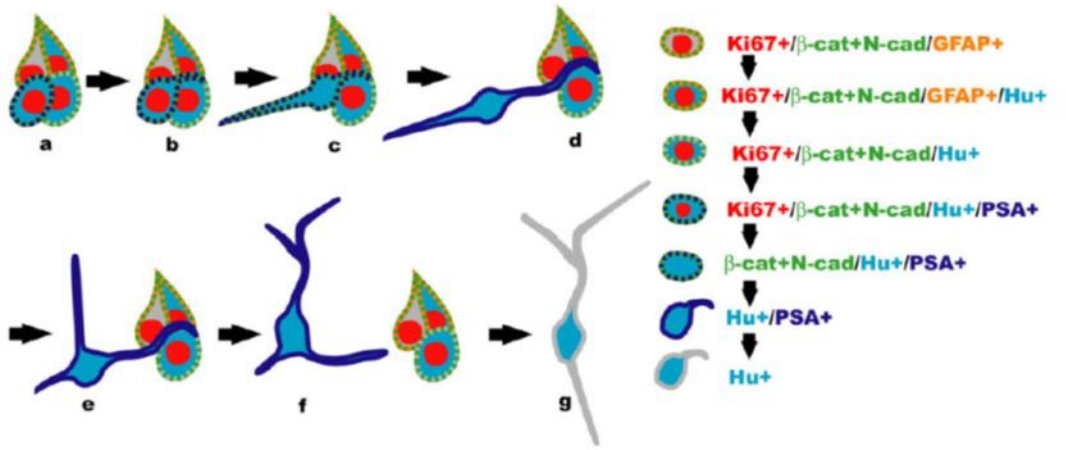


Figure 1.11 A model depicting dividing cells ($Ki67^+$) and formation of new granule cells in the SGZ of adult mouse hippocampus. Prior to terminal differentiation, all the intermediate cells within the cluster express cell adhesion complex beta-catenin/N-cadherin. Hu is a proneural marker expressed in immature and mature neurons. In (a), dividing cells in the SGZ neurogenic niche form clusters comprised of early progenitors ($GFAP^+$ and $GFAP^+/Hu^+$) and neuroblasts (Hu^+ and Hu^+/PSA^+). PSA-NCAM (PSA) expression increases and plays a role promoting cell migration; (b,c) as a new cell matures, tangential processes form and interact with neighboring cells; (d,e,f) as the cell begins to migrate tangentially, they form radial apical processes that eventually develop into dendrites; (g) the mature granule cell with apical dendrites and basal mossy fiber axon. (From Seki et al., 2007)

Mammals, produce new dentate granule cell neurons are generated throughout life, though at a significantly declining rate with aging, including humans and non-human primates (Altman and Das, 1965; Kaplan and Hinds, 1977; Cameron et al., 1993; Kuhn et al., 1996; Amrein and Lipp, 2009; Eriksson et al., 1998; Gould et al., 1998; Kornack and Rakic, 1999; Schlessinger et al., 1975; Kuhn et al, 1996). In the rat, approximately 9000 new granule cells in the dentate are added daily

with 6% of the granule cell layer comprised of newly born neurons (Cameron and McKay, 2001). In both the SVZ and SGZ neurogenic niches, the microvasculature is an integral component with poorly understood roles in modulating neurogensis. Neural precursors within the dentate SGZ are as intimately associated with blood vessels as they are with the radial processes of the B cells in the SVZ. The early precursors and progeny form clusters and cell-cell interactions (Seki, 2002) with the microvasculature to create a microenvironment that promotes the formation of new neurons throughout the life of the animal, and directs not only lineage commitment but also regulates the migration pattern of the newly generated neurons (Palmer, 2000). The cells ultimately dissociate from the glial and vascular scaffolds once they are localized to their final position within the granule cell layer and differentiate into mature granule cells, and if survive, integrate with the established hippocampal network (Bruel-Jungerman et al., 2005; Snyder et al., 2001; van Praag et al, 2002; Wang et al., 2000).

Hippocampal neural stem cells or progenitor cells give rise to transit amplifying precursors to expand the number of neuroblasts that will ultimately form new dentate granule cells. The stem cells reside in the subgranular zone (SGZ) of the dentate gyrus and express glial fibrillary acid protein (GFAP) and nestin (Seri et al., 2001; Filippov et al., 2003). The stem cells have a radial glial-like astrocyte morphology. The stem cells proliferate and give rise to clusters of transit amplifying cells or transit amplifying precursors that further divide to produce neuroblasts. Each stage of the development of the new cells can be characterized through various markers that are expressed at each stage (reviewed in Kempermann et al., 2004). The dividing progenitor cells and early immature neuronal cells express doublecortin (DCX) and polysialylated neural cell adhesion molecule (PSA-NCAM), which continues to be expressed in the early post mitotic neurons. As the post mitotic neurons mature, they begin to express calretinin and Prox1. The fully mature neurons continued to express Prox1 and are also immunoreactive for calbindin and NeuN.

Neural stem cells in the SGZ versus SVZ in the adult brain

There are similarities and differences in the neurogenic niches of the SVZ and the SGZ (Figure 1.12). In recent years there has been progress delineating the sequential steps in neurogenesis from the earliest stem cell to the immature neuron to the adult integrated mature neuron. (Alvarez-Buylla and Lim, 2004; Duan et al., 2008; Lledo et al., 2006).

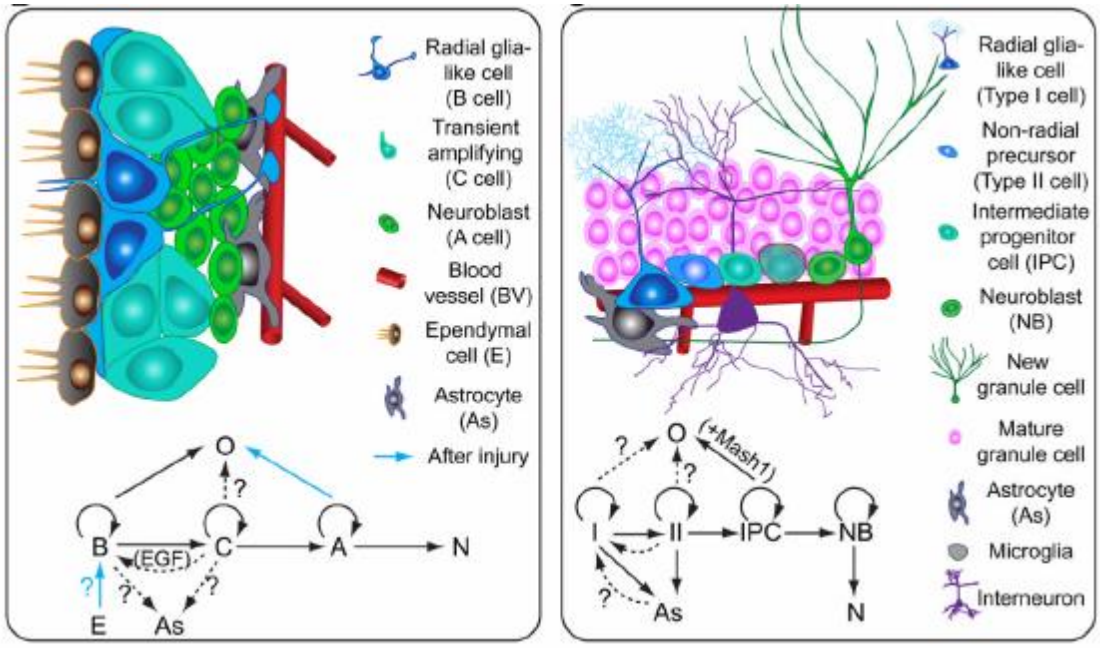


Figure 1.12. *Representation of SVZ and SGZ comparing the neurogenic niches and formation of new neurons in these two regions. In both zones, radial glia produce intermediate stage precursor cells that give rise to migrating neuroblasts. Astrocytes and blood vessels are important supporting elements during neurogenesis by providing regulatory signals and scaffold infrastructure for migrating neuroblasts. Ependymal cells play a unique role in the SVZ by influencing the proliferative status of the adjacently positioned stem cells through the BMP-noggin-notch signaling pathway. Question marks in the fate diagrams indicate unknown potentials including whether ependymal cells can act as stem cells, and exactly how gliogenesis (astrocyte and oligodendrocyte generation) may fit into the scheme.* (From Ming and Song, 2011)

As mentioned earlier, many of the processes involving neurogenesis at the embryonic stage are recapitulated in neurogenesis in the adult brain. The development of new techniques for following lineage development and birth dating, including the use of BrdU to label proliferating cells (Kuhn et al., 1996) and retroviral labeling (van Praag et. al., 2002) has allowed the characterization of the neurogenesis events in the adult and the developing embryo. Retroviral labeling of adult hippocampal progenitor cells to express green fluorescent protein enabled tracing of neuronal progeny *in vivo* for weeks. This allowed investigators to study the morphological and functional development and maturation of these cells (Esposito et al., 2005). The stages of maturation of neural stem cells is similar in both adult and perinatal brain:

1- silent synapse formation;
2- expression of GABA receptors and responsive to GABA input ;-
3- expression of GLU receptors and effectively glutamatergic neural identity;
4- responsive to GABA inhibitory tone.

The process and time course for maturation of the hippocampal granule neuron is a critical period not only for the long-term survival of the neuron, but important for the function of the newborn neurons in memory encoding (Deng, W., 2010; Aimone et al., 2011):

A) 3-day old immature neurons express functional GABA and glutamate receptors but no GABAergic synaptic events were detected;
B) 7-day: GABAergic synaptic events that induce depolarization of the cell rather than hyperpolarization as is characteristic of the mature neuron;
C) 21-day: glutamatergic inputs detectable; few GABAergic inhibitory inputs;
D) over the course of several weeks there is increasing GABAergic inhibitory input to the new neurons due to both increased number and synaptic strength of inhibitory synapses (Li et al., 2012).

As reviewed by Guo-Li Ming (2011), there are a number of general characteristics that can be applied to adult neurogenesis: (1) the stages of neuronal development from the radial glial-like stem cell is highly conserved in the embryonic, early postnatal, and adult animal. Neural precursors pass through a stage in which they respond to GABAergic input before they become

responsive to glutamatergic input. (2) There is a differential rate of maturation of neural precursors in the adult versus the embryo with an increased duration of time it takes to generate the mature neuron in the adult animal (Overstreet-Wadiche et al., 2006; Zhao et al., 2006). It is unclear why there is a delayed maturation compared to the embryo but it has been proposed that in the adult there is a higher density of cellular milieu in the brain parenchyma through which the neurons must migrate in addition to the higher complexity of the established neuronal network in the adult that the new neurons must integrate into. The new neurons also exhibit significant plasticity in the course of forming new glutamatergic inputs (Ge et al., 2007; Nissant et al., 2009; Schmidt-Hieber et al., 2004). This plasticity may confer a selective advantage for the new granule cell neurons over older established granule cells to form stable afferents and efferent synaptic connections (Tashiro et al., 2006 Toni et al.; 2007; Ming and Song, 2011). (3) The doublecortin-expressing neuroblasts in the subventricular zone exhibit plasticity in terms of lineage commitment, especially under pathological or injurious conditions. For instance, they can be induced to undergo differentiation into oligodendrocytes when there is a demyelination injury in the corpus callosum. The neuroblasts within the rostral migratory stream can be diverted to the injury site and differentiate into oligodendrocytes to reform new myelin in an attempt to repair the injury (Jablonska et al., 2010).

Neurogenesis in the subgranular zone and the SVZ share temporally critical periods that are pivotal points for the survival of the new cells. As one would expect, the critical periods through which new cells must transit for long-term survival include the intermediate stage of development of the precursor cells and the late stage integration process after the cell has migrated to its final position either in the olfactory bulb or the dentate granule cell layer (Tashiro et al., 2006; Toni et al., 2007).

There are also similarities and differences between the SGZ and the SVZ as it relates to the neurogenic niche where the stem cells reside. Both the SGZ and the SVZ stem cells communicate with the vascular system and their respective brain regions such that they form clusters around microvascular processes (Palmer et al., 2000), especially at branch points. It is becoming increasingly clear that blood-borne factors and endothelial derived factors strongly influence neurogenesis in the adult brain. *In vitro* studies have shown that endothelial cells, when co-cultured with subventricular zone cells, induce increased neuronal differentiation (Leventhal et al., 1999). Astrocytes form part of the blood-brain barrier by physically interacting with capillaries and may contribute to cell-cell communication with local stem cells and precursor cells that also interact with the same astrocytes. However, adult subventricular zone precursors can also interact with blood vessels in absence of astrocytic intermediaries, suggesting that direct communication with blood-borne factors also significantly influences neurogenesis. It has been shown that BrdU-labeled clusters of newly born neurons in the dentate gyrus subgranular zone are frequently localized in close proximity or in contact with blood vessels. Following radiation injury, dissociation from blood vessels or increasing distances between the stem cells and blood vessels, may result in dysfunctional neurogenesis and preference of new cells toward a glial fate (Fike et al., 2005). In both the subventricular zone and subgranular zone blood vessels also provide a substrate for migration of neuroblasts. In concert with radial glial-like processes that project through the granule cell layer, blood vessels form a parallel arrangement with those processes and together form a scaffold along which immature granule cell neurons can migrate into the granule cell layer. Blood vessels are also an important substrate along which subventricular zone-derived neuronal precursors can migrate when induced to dissociate from normal chain-type homophilic migration following injury. Under these conditions, the diverted

SVZ-derived neuroblasts can leave the rostral migratory stream and track blood vessels to the injury site (Kojima et al., 2010).

Astrocytes also play an important role in maintaining the neural stem cell pool in the adult brain in both the SVZ and the SGZ zone, as well as play a regulatory role in the fate specification of neural stem cells. Astrocytes secrete factors that induce neural precursor proliferation and promote differentiation along a neural lineage fate (Lim and Alvarez-Buylla, 1999; Song et al., 2002). As mentioned earlier, astrocytes are also an important substrate for facilitating migration of neuroblasts to their destination. Astrocytes also play a role in the maturation and synapse formation of the newly born cells (Barkho et al., 2006). Some of the factors astrocytes express include Robo receptors that function to influence migration patterns of neuroblasts in the rostral migratory stream that express Slit1 (Kaneko et al., 2010). Neurotransmitter expression (e.g. glutamate) by astrocytes also plays a role in promoting the survival of migrating SVZ neuroblasts (Platel et al., 2010).

The finding that astrocytes play a regulatory role in the fate specification of neural stem cells was unexpected, since during development neurons are formed before astrocytes are generated. It is postulated that early in development, the forming brain is very permissible for migration, while later as the brain forms, astrocytes function to facilitate signaling and migration of new neurons through a less permissible and increasingly populated cellular matrix. Interestingly, astrocytes from different regions in the CNS have a differential gene expression profile. Astrocytes in neurogenic regions of the brain express neurogenesis-promoting factors; whereas astrocytes localized in areas of the CNS that are not neurogenic, express factors that inhibit neuronal differentiation (Barkho et al., 2006). For example, the extracellular factors noggin and neurogenesin-1, expressed by astrocytes prevent loss of neural precursors through rapid proliferation and differentiation. These factors antagonize the effect of bone morphogenetic

proteins (BMP's) that promote gliogenesis and inhibit neurogenesis. Clearly a balance in expression of factors that either induce or antagonize glio- and neurogenesis in the adult brain are critical in maintaining a balance between the production of new cells while retaining (i.e. avoid depletion of) a functional population of neural stem cells throughout the life of the animal. Other factors that participate in this balancing act include ephrins and Eph receptors, which regulate cell proliferation in the adult subventricular zone (Genander and Frisen, 2010). Sonic hedgehog is an important extracellular mediator that interacts with radial glia-like cells and plays a significant role in maintaining the neural stem cell population in both the SGZ and the SVZ (Ahn and Joyner, 2005; Han et al., 2008). Another astrocyte factor that influences neural stem cell proliferative activity and lineage commitment to neuronal fate is Wnt3 (Lie et al., 2005; Song et al., 2002). In both the subgranular zone and the subventricular zone, Notch activation contributes a primary role in activation of radial glia-like cells to differentiate into intermediate progenitors (Imayoshi et al., 2010; Pierfelice et al., 2011). Activated astrocytes also produce inflammatory cytokines, IL-1beta and IL-6, that influence neurogenesis. While inflammation generally inhibits neurogenesis, it is now known that low concentrations of these cytokines in combination with other factors will promote the differentiation of neural stem cells. Other astrocytic factors can have an inhibitory effect on neuronal differentiation including insulin-like growth factor binding protein-6 and the extracellular proteoglycan, decorin (Barkho et al., 2006), which inhibit IGF (insulin growth factor) and TGFbeta-2.

Hence, in both the subventricular zone and subgranular zone neurogenic niches, there is a complex and exquisitely controlled expression of factors (cytokines, chemokines, growth hormones) that serve to regulate the neuronal stem cell population by modulating symmetric or asymmetric cell division, migration, and fate specification. Astrocytes play a critical role in intact adult brain and after injury, by participating in the production of growth factors and inflammatory

proteins that may regulate the neurogenic niche or neural precursor cells directly to influence neurogenesis and survival of new neurons. Table 1.1 A and B lists some of the factors that are involved with regulating neurogenesis and migration of neuronal stem cells and precursors, but is not an exhaustive list of factors playing a role in neurogenesis.

The neurotrophins are small proteins that consist of non-covalently linked homodimers that bind to tropomyosin-related kinase (TRK) transmembrane receptors, which are members of the receptor tyrosine kinase family (RTKs). Secretion of these factors is activity-dependent, and under physiological and pathological conditions, function to promote neuron differentiation, growth, survival, and integration (Griesbeck et al, 1999; Hartmann et al., 2001; Egan et al., 2003; Goggi et al., 2003; Brigadski et al., 2005). The specificity of response is governed by the expression profile for the receptor and ligand. For instance, post-synaptic cell terminals secrete neurotrophic factors that bind to specific receptors expressed by pre-synaptic cells, inducing neurite growth to form new synapses. Interestingly, the expression of receptors in an environment with insufficient neurotrophin ligand to activate the receptor results in apoptotic cell death (Lesman et al., 2003; Dekkers et al., 2013). Hence, the activity-based secretion of neurotrophic factor prevents a "default" induction of apoptotic cell death through unoccupied neurotrophic receptors. Neurotrophins are broadly expressed in the CNS under various conditions by multiple cell types including neurons, astrocytes, oligodendrocytes and microglia.

Nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) are two highly characterized neurotrophins that occur in the CNS. Neurotrophins are inclusive of several other factors including neurotrophin-3 (NT-3) and neurotrophin-4/5. In general, neurotrophins are synthesized as a prohormone that is converted to the active form following cleavage by convertase enzymes such as furin. Binding of neurotrophins to their specific receptors, TrkA, TrkB, and TrkC, confers the biological activity, though there is significant overlap in receptor binding

between the various neurotrophin. NGF binds with high affinity to TrkA and has low affinity binding to TrkB; BDNF binds TrkB; NT-4/5 binds both TrkA and TrkB; and NT-3 binds TrkB and TrkC. Receptor dimerization induces autophosphorylation to activate recognition sites (i.e. SH2 receptor domains) for the binding of intracellular target proteins including phospholipase C, p85, and Shc. Receptor activation induces signaling cascades involving MAP kinases and phosphorylation of cyclic AMP-response element binding protein (CREB).

TRK receptors are expressed in both a full-length functional form as well as a more truncated form lacking the kinase domain. Although the truncated receptors are missing a functional domain, they still play a role in growth and development. These receptors can act as competitive inhibitors against the full-length TRK receptor under pathological or other conditions, and may play an important role in the cell response to injury and repair mechanisms. . For instance, Frisen et al (1993) showed that astrocytes express truncated TRK receptors following injury. This upregulation of truncated receptors may effectively sequester BDNF (Biffo et al., 1995; Roback et al., 1995; Alderson et al., 2000) and modulate neuronal vulnerability to cell death (Saarelainen et al., 2000). Other studies have shown that truncated TRK receptors play an active role in calcium signaling in glia (Climent et al., 2000; Rose et al., 2003).

NGF is expressed in regions of the brain where cholinergic neurotransmission is prominent and acts to support the survival of cholinergic neurons (sensory and sympathetic). The cortex, striatum, and pyramidal cell layer and dentate gyrus of the hippocampus are regions that can express high levels of NGF depending on status of neuronal activity. NGF expression in the hippocampus dentate gyrus increases dramatically and transiently when status epilepticus is induced in the pilocarpine model; whereas the receptor expression is not affected. ICV injection of NGF antibodies or NGF inhibitory peptides block kindling-induced mossy fiber sprouting (Van der Zee et al., 1995).). In contrast, Adams et al., 1997 showed that ICV injection of NGF facilitates

hippocampal cell death and increases mossy fiber sprouting. Blocking the signaling of TRKA by inhibition of downstream effector Ras inhibits kindling and kindling-associated mossy fiber sprouting (Li et al., 2005).

BDNF is widely distributed throughout the brain, including all primary neurons in the hippocampus (Enfors et al., 1990; Hofer et al., 1990; Wetmore et al., 1990). TrkB is the primary receptor for BDNF and is expressed in both full-length and truncated forms in hippocampus. The truncated form is also expressed on ependymal cells (Yan et al., 1994; Anderson et al., 1995).

BDNF is strongly expressed in dentate granule neuron mossy fibers (Connor et al., 1997) and is distributed by both retrograde and anterograde transport. It plays important roles in neurodevelopment and cell survival (pro-survival) as well as synaptic plasticity. Neuronal activity stimulates secretion of BDNF with a direct pre- to post-synaptic transneuronal transfer. Synaptic remodeling, such as occurs during certain pathological conditions such as epilepsy and chronic pain, is modulated by excessive levels of secreted BDNF. However, the trophic properties of BDNF have been thought to be potentially therapeutic in neurodegenerative and neuropsychiatric disease.

Neurogenesis in adult brain has been shown to be modulated and enhanced by BDNF, which also acts as a pro-neural differentiation factor. BDNF affects neurogenesis by binding to the TRKB receptor and activating MAP kinase and PI3-kinase pathways (Barnebie-Heider and Miller, 2003), and post-translation modification of basic helix-loop-helix transcription factors (Ito et al., 2003). In studies in which BDNF is injected ICV or via adenoviral-induced BDNF activity results in an increased number of neurons in the adult olfactory bulb, septum, and thalamus (Zigova et al., 1998; Benraiss et al., 2001; Pencea et al., 2001). Injection of BDNF into the hippocampus of adult rats increases neurogenesis in the SGZ, but also increases the frequency of ectopic granule cells

(Scharfman et al., 2005). BDNF increases the number of granule cells by increasing the generation of new cells and promoting their survival (Lee et al., 2002).

There have been various reports that reduced levels of BDNF occurs in neurological disease (Murer et al., 2001). BDNF gene transcripts are reduced in the hippocampus of Alzheimer-diseased brain (Phillips et al., 1991; Ferrer et al., 1999), and Huntington's (Zuccato et al., 2001), although there is an up-regulation of BDNF expression in glial cells associated with amyloid plaque in animal models (Burbach et al., 2004). In Parkinson disease, there is a decrease in BDNF protein in the substantia nigra (Howells et al., 2000). In models of Alzheimer and Parkinson disease, increasing BDNF levels promotes neuronal survival.

NT-3 is widely distributed throughout the CNS in the adult brain and can bind to both TrkA and TrkB receptors, though the preferred receptor is TrkC. The functional effects of neurotrophic factor NT3 parallels that with BDNF in many aspects. However, unlike NGF and BDNF, the level of NT-3 expression decreases in dentate gyrus granule neurons after seizures. TrkC expression may go up or down depending on the model used. NT-3 also plays a role in neurogenesis by promoting the survival and differentiation of newly born neuronal precursors (Barnabe-Heider and Miller, 2003).

NT-4/5 neurotrophin is expressed at low levels in brain under basal conditions and is not increased during seizures. The roles of NT-4/5 include enhancing excitatory synapses in hippocampal neurons (Lessmann et al., 1994) and neuroprotection from energy deprivation or corticosteroid depletion via adrenalectomy (Cheng et al., 1994; Qiao et al., 1996).

Although there are many similarities in neurogenic niche functions between the subventricular zone and the hippocampus, there are clear differences. In the case of the hippocampus, the neurogenic niche is positioned adjacent to the terminal position of the newly formed mature neurons in the granule cell layer. Hence the distance that the neuroblasts must

migrate is relatively short from the SGZ to the granule cell layer of the dentate gyrus. Additionally, the SGZ neurogenic niche containing the stem cells and intermediate precursors is embedded in an environment which includes hippocampal circuitry that is enriched with multiple neuronal subtypes, intricate intercellular contacts and synapses, and neurotransmitters. This is not the case for the SVZ neurogenic niche, whereby the source of the new cells in the SVZ niche is distantly located from the final position where the new neurons integrate in the olfactory bulb. Additionally, the neuronal circuitry surrounding the subventricular zone is relatively sparse compared to that of the subgranular zone in the hippocampus. Unlike the SGZ neurogenic niche, the SVZ neuronal precursors are arranged in an organized pinwheel-like architecture throughout the adult subependymal region in the adult (Mirzadeh et al., 2008).

Table 1.1A. *Factors that regulate adult neural stem cells and progenitors in SVZ and SGZ (From Zhao et al., 2008)*

Extracellular morphogens		sonic hedgehog (shh)	
		Notch	
		Wnts	
		BMPs	
		Ephrins	
	Cytokines and chemokines	IL-1 beta	
		IL-6	
		TNF alpha	
	Hormones	Corticosteroids	
		Estrogen	
		Thyroid hormone (T3)	May modulate stem cell neural commitment and migration via regulating expression of SOX2
	Neurotrophins/ Growth factors	NGF	
		BDNF	
		CDNF	
		NT3	
		GDNF	
	Neurotransmitters	Glutamate	Regulates cell survival of SVZ neuroblasts and SGZ immature neurons via NMDAR-dependent mechanisms
		GABA	In SGZ, promotes dendritic growth, synapse formation, and survival through CREB signaling. In SVZ, promotes neuroblast migration and inhibits cell division.
		Acetylcholine	
		Antidepressants (via serotonin and norepinephrine)	
Cell cycle regulators	p16, p21, p5		
Transcription factors	SOX2		Critical for maintaining neural stem cell pool; regulates transcription of pluripotency factors. Sox2-expressing cells can undergo both symmetric and asymmetric proliferation.
	NeuroD		
	Prox1		
	Pax6		
	DLX-2		
	FOXOs	Neuregulins	
	Olig-1	Slits	
	Kruppel-like factor 9		
	Orphan nuclear receptor TLX		
Epigenetic Regulators	DNA methylation	Methyl-CpG biding domain protein	Suppresses expression of FGF-2 and miRNAs; modulates proliferation vs. differentiation

			during adult hippocampal neurogenesis.
	DNA demethylation	Gadd45b, TET1	Promotes BDNF and FGF1 expression. Deletion of Gadd45b reduces dendritic new granule cells and reduces activity-induced proliferation of neural progenitors in the adult hippocampus.
		BMi-1	Part of complex that represses gene expression; critical role maintaining self-renewal capacity of neural stem cells
	Histone modifiers	HDACs	
		SIRT1	Plays a role in embryonic neural stem cell fate determination; limits proliferation of stem cell pool; SIRT1 inactivation increases oligodendrocyte precursor pool.
		Histone acetyltransferases	
	Non-coding RNA's	Micro-RNAs (miR24, 137, 184)	
Adhesion Molecules		Beta1-integrin	
		PSA-NCAM	
		Tenascin-R	
Other	Slit-1		Regulate RMS neuroblast migration via ROBO receptors
	Reelin		Regulates migration of neuroblasts; depletion of Reelin cause ectopic positioning of new granule cells to hilus (polymorphic layer of dentate gyrus)
	Neuregulins	NRG1	

Table 1.1B. *Supplemental data).Extrinsic and intracellular pathways that play a role in regulating neural stem cell proliferation, migration, differentiation in SVZ and SGZ. (From Zhao et al., 2008)*

Factors and pathways	Proliferation	Differentiation	Survival	Neurogenesis	Other functions	References
Extrinsic factors						
Growth factors & neurotrophins						
Amphiregulin	+					Falk and Frisen, 2002
BDNF	+ (SVZ)		+ (SGZ)	+		Henry et al., 2007; Sairanen et al., 2005; Scharfman et al., 2005; Young et al., 2007
EGF	+				maintenance of NSCs	Doetsch et al., 2002; Ghashghaei et al., 2007; Kuhn et al., 1997; Reynolds and Weiss, 1992
FGF2	+				maintenance of NSCs	Jin et al., 2003; Palmer et al., 1995; Zhao et al., 2007
NGF			+	+		Frielingsdorf et al., 2007
IGF-1	+ (aging brain)	+ (oligodendrocyte)				Hsieh et al., 2004a; Lichtenwalner et al., 2001
PDGF	+	+ (astrocyte)				Jackson et al., 2006
PEDF	+					Ramirez-Castillejo et al., 2006
TGFα	+					Cameron et al. 1998
TGFβ				+		Battista et al., 2006
VEGF	+	+ (neuron)		+		Cao et al., 2004; Fabel et al., 2003; Jin et al., 2002
Morphogens						
BMP		+ (astrocyte)				Nakashima et al., 1999; Yanagisawa et al., 2001
Neurogenesin-1		+ (neuron)				Ueki et al., 2003
Noggin		+ (neuron)		+		Lim et al., 2000
Shh	+					Ahn and Joyner 2005; Banerjee et al., 2006; Lai et al., 2003; Machold et al., 2003; Palma et al., 2005
Wnt3	+			+		Lie et al., 2005
Cytokines						
CNTF				+	self-renewal of adult NSCs	Chojnacki et al., 2003; Emsley and Hagg 2003; Shimazaki et al., 2001
G-CSF		+ (neuron)				Schneider et al., 2005
IFNγ				+		Wong et al., 2004
IL-6	-			-		Valleres et al., 2002
LIF		+ (astrocyte)				Nakashima et al., 1999
TNFα					+ (cell death)	Wong et al., 2004
Neurotransmitters						
Acetylcholine			+			Kotani et al., 2006; Hartist et al., 2004; Mechawar et al., 2004; Mohapel et al., 2005
Dopamine	+ or -					Coronas et al., 2004; Hoglinger et al., 2004; Kippin et al., 2005a; van Kampen et al., 2004
GABA					differentiation of type 2 cells in SGZ	Tozuka et al., 2005
Glutamate	+ or -					Cameron et al., 1995; Deisseroth et al., 2004; Gould et al., 1997; Nacher et al., 2001
Nitric oxide	+ or -					Cheng et al., 2003; Moreno-Lopez et al., 2004; Packer et al., 2003; Reif et al., 2004; Zhang et al., 2001; Zhu et al., 2003; etc
Norepinephrine						see table 5
Serotonin						see table 5
Hormones						
Corticosterone	-			-		Cameron et al., 1994
Estrogen				+ or no effect		Lagace et al., 2007; Tanapat et al., 1999
Male pheromone				+		Mak et al., 2007
Nandrolone	-					Brannvall et al., 2005
Drugs						
Nicotine	-			-		Abrous et al., 2002
Opiate	-			-		Eisch et al., 2000; Kahn et al., 2005
Other extrinsic factors						
Ccg	+			+		Taupin et al., 2000
Complement factor C3a				(+)		Rahpeymai et al., 2006
Docosahexaenoic acid (DHA)				+		Kawakita et al., 2006
Galectin	+					Ishibashi et al., 2007
PACAP	+					Mercer et al., 2004
sAPP	+					Caille et al., 2004
Intracellular pathways						
Receptors						
Smoothened	(+)			(+)		Balordi and Fishell, 2007
TNF-R1	(-)			(-)		Iosif et al., 2006
Toll-like receptor 2				(+)		Rolls et al., 2007
Toll-like receptor 4	(-)	(-) (neuron)				Rolls et al., 2007
Transcription factors						
Bmi-1					maintenance of adult NSCs	Molofsky et al., 2005
NeuroD					formation of DG	Liu et al., 2000
Olig2		+ (oligodendrocyte)				Hack et al., 2005
Pax6		+ (neuron)				Hack et al., 2005
TLX					maintenance of adult NSCs	Shi et al., 2004; Sun et al., 2007
Epigenetic regulations						
MBD1		(+) (neuron)			genome stability	Zhao et al., 2003
Histone deacetylase	+ (with TLX)	- (neuron)				Hsieh et al., 2004b; Sun et al., 2007
Cell cycle regulation						
P16INK4a	(-)					Molofsky et al., 2006
p21	(-)					Kippin et al., 2005b
p53	(-)					Gil-Perotin et al., 2006
Other intracellular pathways						
NR2E dsRNA		+ (neuron)				Kuwabara et al., 2004
Retrotransposition						Muotri et al., 2005
Telomere	(+)					Ferron et al., 2004

This table is a partial list of the factors that have been reported to regulate adult NSCs. The precise roles of these factors need to be further investigated. Because of the heterogeneous nature of the current culturing methods to maintain NSCs in vitro and the lack of definitive markers to identify NSCs in vivo, the target cells of these factors are mostly unclear. (-): enhanced by loss of the gene; (+): decreased or inhibited by loss of the gene. See supplementary material for the complete list of references.

Studies using retrograde tracers and mitotic labeling studies using BrdU demonstrated that new granule cell neurons formed in the adult eventually become morphologically indistinguishable from established granule cells, including formation of mossy fibers that terminate in the CA3 area of the hippocampus pyramidal cell layer (Stanfield and Trice, 1988; Hastings and Gould, 1999; Markakis and Gage, 1999). It was also demonstrated that new adult-born granule cells exhibit electrophysiological characteristics of mature dentate granule cells based on recordings taken from hippocampal slices (Song et al., 2002; van Praag et al., 2002; Overstreet-Wadiche et al., 2005; Ge et al., 2006). Similar to what occurs during development, new granule cells in the adult brain receive GABAergic synaptic input one week after formation and are depolarized in response to GABA. Glutamatergic input becomes established within two weeks. Interestingly, new granule cells formed in the adult are much slower to develop (i.e. form mature dendritic arborization patterns) compared to granule cells formed during development, taking up to 3 to 6 months (van Praag et al., 2002). Dentate granule cells born in the adult have higher input resistance but a sub-threshold calcium conductance that may enhance their excitability compared to older granule cell neurons. The new cells have a decreased threshold for LTP or LDP, and a higher amplitude for LTP.

Evidence suggests that the newly born dentate granule cells in the adult support a role in learning and memory functions, but the precise function of the cells is as yet not fully understood (reviewed in Doetsch and Hen, 2005). It was shown that when neurogenesis is stimulated, such as by exercise or environmental enrichment, there is improved performance on some hippocampal learning tasks (Bruel-Jungerman et al., 2005; van Praag et al., 2005; Meshi et al., 2006). Additionally some strains of mice that have a higher basal level of neurogenesis perform better on some learning tasks (Kempermann and Gage, 2002). Conversely, blocking neurogenesis has seen opposing effects on learning and memory (Shors eta al., 2001, 2002; Winocur et al.,

2006). This supports the finding that LTP is more easily developed in the newly born cells, and they also have a higher amplitude for LTP (Wang et al., 2000; Schmidt-Hieber et al., 2004).

Neurogenesis in the adult brain is modulated by many endogenous intracellular and extrinsic factors. Touched on earlier in this thesis was a description of how environmental cues external to the animal can influence neurogenesis such as learning experiences. Certain behaviors can regulate adult neurogenesis including physical exercise, stress, and aging (Figure 1.13). Mice housed in an enriched environment as well as exposure to learning tasks generally increases the survival of new neurons in the hippocampus (Nilsson et al., 1999; Kempermann et al., 1997, 1998, 2002; Drapeau et al., 2007; Mouret et al., 2008). Physical exercise also stimulates neurogenesis but unlike environmental enrichment, the increase in neurogenesis is due to increased proliferation and not survival (van Praag et al., 1999). Physical exercise and activities that are mediated through hippocampal-dependent function, alter neurogenesis primarily in the hippocampal neurogenic niche and not the subventricular zone neurogenic niche.

Experimental or pathological conditions can have profound effects on neurogenesis and are often used to unravel mechanisms regulating stem cell responses or to determine the biological significance of neurogenesis in disease. Models that are used to study neurogenesis and neurorepair include brain trauma, stroke (ischemia), neurodegenerative disease, chemical injury (e.g. trimethyltin), radiation, surgical procedures that affect cerebrovascular or hormonal function (e.g. middle cerebral artery occlusion, adrenalectomy) or removal of sources of migration/differentiation factors such as olfactory bulbectomy, and medicinal agents (e.g. anti-depressants). Stem cell response and its contribution to neural repair is limited and variable. Whether the response has no significant biological effect, restores function, or exacerbates a pathological condition, is dependent on the type of injury, brain region affected, and status of the

animal. Multiple complex steps are interweaved in determining the outcome of a stem cell response which involves not only modulation of cell cycling, but also migration, appropriate differentiation, integration, and ultimately survival of newly born cells. For instance, in models of focal or global ischemia to simulate stroke, there is an induction of neurogenesis in the SVZ, and some of the neuroblasts detach from the rostral migratory stream to migrate to the lesion site (e.g. striatum). They may differentiate into new neurons (reviewed in Lindvall and Kokaia, 2003) at the lesion, however the vast majority of the new neurons do not survive long term (Arvidsson et al., 2002).

The discussion of epilepsy earlier in this thesis becomes relevant here since much of the early studies and abundance of data on stem cell responses to injury is derived from models of epilepsy. The effects of epileptic seizure activity on hippocampal neurogenesis has already been described, but it is worth mentioning again that even brief seizure activity potently stimulates an increase in proliferation. Repeated seizure activity alters normal migration and morphological development of newly born granule cells and may exacerbate the condition and propagate further seizure activity (Kron et al., 2010; Parent et al., 1997). Specifically, ectopic migration of immature granule cells to the hilus and aberrant dendritic growth such as formation of excitatory hilar basal dendrites may promote recurrent excitatory circuitry. Additionally, alterations in the electrophysiological properties (i.e. GABAergic and glutamatergic synaptic inputs) of both newly born (Jakubs et al., 2006) and older established granule cells (e.g. mossy fiber sprouting) occurs with chronic seizures. Blocking neurogenesis via infusion of the antimitotic AraC to reduce the number of ectopic granule cells reduces spontaneous recurrent seizure activity (Jung et al., 2004).

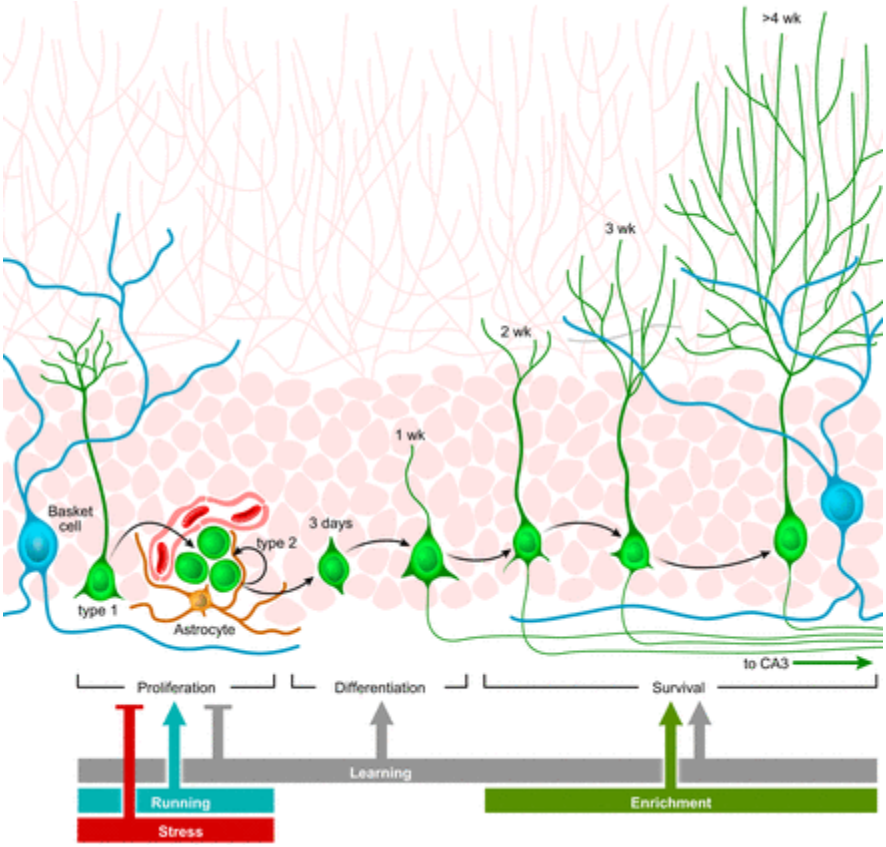


Figure 1.13 Diagrammatic representation of various behavioral effects and timeline on neurogenesis in the hippocampus. Running induces proliferation of hippocampal precursors while enrichment of the environment promote survival of new granule cell precursors. Learning behavior promotes differentiation and survival of the granule cell precursors while possibly inhibiting or activating cell division within the stem cell niche. Stress inhibits proliferation of neural stem cells. (From Aimone et al., 2014)

Another major factor that can have negative as well as positive consequences on neurogenesis is neuroinflammation. Inflammatory reactions result from essentially any insult on the brain whether it be traumatic or chemical-induced injury. Trimethyltin intoxication induces a strong inflammatory reaction that includes activation of microglia and astrocytes and the secretion of various inflammatory factors (Lefebvre d'Hellencourt et al., 2005; Yoneyama et al., 2011) including interferon-gamma, TNF alpha, IL-1beta, IL-6, and nitric oxide (Table 1.2). The pro-inflammatory cytokine TNF alpha has been shown in *in vitro* studies to have a negative influence on the survival and differentiation of hippocampal neural precursor cells (Cacci et al., 2005).

However, recent studies also show that TNF alpha contributes to the directional migration of the hippocampal neural precursors into the granule cell layer (McPherson et al., 2011). TNF alpha exerts its cellular effects in the activation of two different receptors: TNFp55R and TNFp75R. Different cellular responses can result depending on the relative expression levels of each of these two receptors. TNFp75R activation can invoke a trophic response and promote cell survival (Shen et al., 1997; Yang et al., 2002), whereas the TNFp55R receptor subtype can transduce apoptotic cues to initiate cell death (Thorburn, 2004; McPherson et al., 2011). A major source of the inflammatory cytokines in the pathologically inflamed brain are from activated microglia, although astrocytes also present a minor source of some of these factors, including TNF alpha. Microglia are also a source of neurotrophic factors that can promote proliferation of hippocampal neural precursor cells (Morgan et al., 2004). Other factors secreted by microglia including insulin-like growth factor-1 (IGF-1), can increase stem cell proliferation in the subgranular zone (Choi et al., 2008) as well as play a role in stroke-induced neurogenesis in the subventricular zone (Thored et al., 2009). Activated microglia have been shown to have a disruptive effect on neurogenesis in the adult dentate gyrus. Neural inflammation induced by radiation, seizures, or lipopolysaccharide decrease the survival of new granule cells in the dentate gyrus. Inhibition of microglial activity using minocycline or indomethacin can partially reverse this affect (Ekdahl et al., 2003; Monje et al., 2003). The phenotypic status of microglia may therefore govern what factors are predominantly secreted (inflammatory or neurotrophic) and determine their effect on neurogenesis and neural survival.

Hormones also regulate neurogenesis. Stress hormones reduce neurogenesis in the dentate gyrus in adults (reviewed by Mirescu and Gould, 2006). Acute or chronic stress decreases neurogenesis in the dentate gyrus of young adult rodents and primates (Mirescu and Gould, 2006). Stress hormones, particularly corticosteroids, also have a negative influence on

neurogenesis by decreasing the proliferation of neural stem cell precursors. Circulating levels of corticosteroids tend to increase with age and may be tied to significantly reduced neurogenesis which is characteristic of the older adult brain. Procedures that reduce levels of corticosteroid production, such adrenalectomy, have been shown to increase hippocampal neurogenesis (Cameron et al., 1994; Montaron et al., 1999). However, at least one study suggests the increase may be transient and there is a corresponding increase in cell death in the dentate gyrus (Spanswick et al., 2011). Removal of the olfactory bulbs in rats reduces the proliferation of both subventricular zone neural precursors and hippocampal precursors in rats (Keilhoff et al., 2006). There is also an increase in cell death in SVZ-derived precursors following bulbectomy which is not observed in the hippocampus (Keilhoff et al, 2006).

Endogenous factors/enzymes	Proliferation	Differentiation	Migration
IFNγ	↓	↑	↑
TNFα	↑ or ↓	↓ or ↑	↑
IL-1β	↑ or ↓	–	–
IL-6	↓	↓	–
Glutamate	↑ or ↓	↑	–
NOS1 (nNOS)	↓	↑	–
NOS2 (eNOS)	↑	↑	–

↑, enhancement ; ↓, attenuation ; –, no report.

Table 1.2. Activation or down-regulation of proliferation, differentiation, or migration in response to endogenous cytokines. (From Yoneyama et al., 2011)

Many studies have shown that aging negatively affects neurogenesis, resulting in a decrease in subventricular zone and hippocampal neurogenesis (reviewed in Rossi et al., 2008). Evidence has been mounting that the dentate gyrus is particularly vulnerable to aging. Aged animals exhibit a reduction in hippocampal neurogenesis (Seki and Arai, 1993; Kuhn et al., 1996) and formation

of new dentate granule cells (reviewed by Artegiani et al., 2012). Across species, methods using magnetic resonance imaging show that the cerebral blood volume (CBV) directly correlated with regional energy metabolism and memory performance, and is decreased in the dentate gyrus with aging (Small et al. 2004).

Hippocampal neurogenesis declines rapidly with aging, which has been theorized to be due to reduced precursor proliferation and altered differentiation, or delayed maturation of progeny (Kempermann et al., 1998; Hattiangady et al., 2005). There is a marked decline in new neurons and mitotically active progenitor cells within the subgranular layer beginning at middle age (Kuhn et al., 1996; Hattiangady et al., 2005). Though there is a continuous decline in stem cell activity from middle age to senescence, the proportion of new neuroblasts that survive and differentiate into mature granule cells remains stable (Hattiangady, 2005). Learning behavior induces hippocampal neurogenesis in young, as well as the aged but at a reduced level In addition to sheer numbers of newly born cells being reduced with aging, other factors may play a role in the cognitive decline in normal aging including integration of new neurons into existing hippocampal circuitry. Figure 1.14 diagrammatically displays some of the current concepts proposed to explain the age-related decline in hippocampal neurogenesis (Artegiani et al., 2012). Elevation in circulating corticosteroids has been proposed to play a role since adrenalectomy can reverse the age-dependent reduction in neurogenesis (Cameron and McKay, 1999). Infusion of growth factors can also stimulate neurogenesis in aged mice (Jin et al., 2003; Lichtenwalner et al., 2001). Growth factors contribute to the regulation of neurogenesis as demonstrated by blocking uptake of insulin-like growth factor 1 (IGF-1) or vascular endothelial growth factor (VEGF) which prevents stimulation of neurogenesis by exercise or environmental enrichment (Trejo et al., 2001; Fabel et al., 2003; Cao et al., 2004). Administration of these growth factors can increase neurogenesis in

the dentate gyrus but it is unclear whether it is through increased proliferation or survival (Aberg et al., 2000; Jin et al., 2002; Cao et al., 2004; Schanzer et al., 2004).

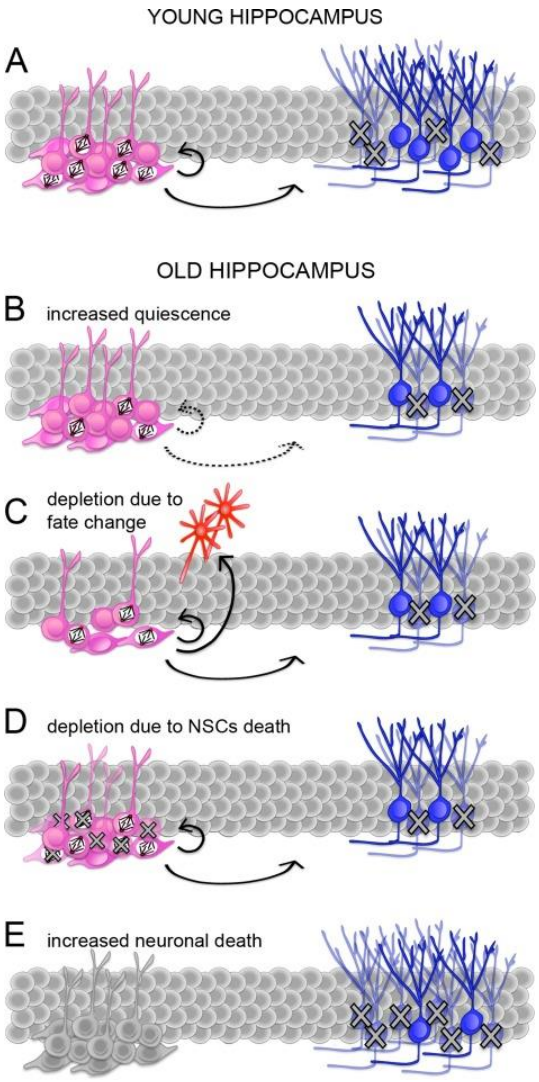


Figure 1.14. Representations of possible mechanisms by which neurogenesis declines in the aging hippocampus. A, and the young hippocampus active stem cell proliferation gives rise to neuroblasts that differentiate into granule cells as well as symmetric proliferation to renew the stem cell pool. B, the aged hippocampus harbors increasing numbers of amitotic stem cells resulting in fewer neuroblasts and reduced symmetric proliferation to renewal the stem cell pool. C, with aging, some of the stem cells are committed to an alternate lineage such as glia

that reduces the number of neuroblasts and potentially reduces the amount of symmetric proliferation. This could be due to alterations in expression of differentiation factors within the older hippocampus. D, the number of stem cells decreases with aging as a result of limited number of potential cell cycles. The stem cells may undergo apoptotic cell death or alternatively terminally differentiate and become amitotic, thus reducing the stem cell pool and the number of newly born granule cells. E, loss of stem cells due to the above mechanisms or other factors, and/or reduced survival of neuroblasts and granule cell precursors due to alterations in growth factors or reduced integration. (From Artegiani et al., 2012)

In normal aging, hippocampal-based memory processes can become vulnerable to disruption. Alterations in structure, function, and gene expression have been observed in aged granule cells that can lead to consequences for the hippocampal function and cognition. Cognitive decline with aging is not necessarily the result of global loss of neurons in rat, monkey, and human (Sterio, 1984; West, 1993, 1994; Morrison and Hof, 1997), but may be the result of alterations in synaptic efficiency. For example., approximately one-third of the medial entorhinal synaptic input to the dentate gyrus is lost with senescence, which is not associated with a loss of cells in the entorhinal cortex, granule cell layer of the dentate gyrus, or CA pyramidal cells (Rapp and Gallagher, 1996; Rasmussen et al., 1996; West, 1993; Peters et al., 1996; Merrill et al, 2000, 2001; Rapp et al., 002). There are reports that there is a reduction in the number of synapses. Using stereological techniques, Geinisman et al. (1992) showed that there is a loss of axospinous synapses in the middle third of the molecular layer of the dentate gyrus in memory-impaired old rats. Smith et al. (2000) demonstrated that there is a loss of synaptophysin in the entorhinal cortical layer II projection which innervates both the molecular layer and the dentate gyrus. There is no evidence of decreased granule cell dendrites with aging. Studies comparing young versus old mice have shown that there is no reduction in spine density in the apical dendrites of the upper blade of the dentate gyrus and area CA1 (von Bohlen and Halbach et al., 2006). However, the mean spine length did show reduction in the dentate gyrus and CA1 in young compared to old mice. It is possible that some regions of the dentate gyrus could undergo reduction of the dendritic tree but

be compensated for in other regions in order to preserve homeostasis of neuronal function (Samsonovich and Ascoli, 2006).

Factors involved in regulation of adult hippocampal neurogenesis

In the adult brain, neurogenesis persists throughout life in the hippocampus to produce new hippocampal granule cells (Altman and Das, 1965; Gould and Cameron, 1996; Eriksson, 2003). The stem cell niche in the dentate gyrus is comprised of neurons and neural progenitors, astrocytes, cerebrovascular elements, and microglia (Wurmser et al., 2004). Each of these components can play a regulatory role in adult neurogenesis and are differentially affected by many physiological, environmental, and pathological factors (Jessburger and Gage, 2008). Aging, stress, exercise, growth factors, neurotransmitters, hormones, and injury and disease all have significant impacts on neurogenesis.

New cells are born in the subgranular layer of the dentate gyrus and migrate into the granule cell layer (Kuhn et al., 1996) where the surviving cells integrate in the hippocampal circuitry and eventually develop similar electrophysiological properties as older established granule cells (van Praag et al., 2002; Ribak et al., 2004; Song et al., 2005). The newly born cells have been shown to functionally integrate in the granule cell layer and form dendritic processes that project to the molecular layer. The new cells express the plasticity-related immediate-early gene *Arc* in response to the animal performing spatial exploration, indicating that the cells are integrated into the hippocampal neuronal network (Ramirez-Amaya et al., 2006). Shors et al. (2001) showed that neurogenesis occurring in the dentate gyrus correlates with effective hippocampal-dependent memory function. Neurogenesis is correlated with preserved spatial memory in older rats (Drapeau et al., 2003). Exercise-induced increases in neurogenesis are also associated with improved Morris water maze performance (van Praag et al., 2005). However some studies failed

to show correlation between neurogenesis and behavior (Bizon and Gallagher, 2003) and there are some studies that suggest a negative correlation between neurogenesis and behavior (Bizon et al., 2004). One hypothesis proposed for the function of new granule cells is that as they develop new synapses, they create a "time tag" that becomes integrated with the formation of new memories (Aimone et al., 2006). Thus the new cells contribute to the mechanism of forming temporal association to new memories.

Neuronal activity and the release of neurotransmitters are known to impact neurogenesis (reviewed in Berg et al., 2013). Glutamatergic input suppresses neurogenesis. NMDA receptor activation reduces neurogenesis, whereas an antagonist to this receptor has the opposite effect and increases granule cell numbers and cellular density of the granule cell layer (reviewed by Berg et al., 2013). Neurogenesis in the dentate gyrus is also increased following lesioning of entorhinal cortex excitatory (glutamatergic) input to the dentate gyrus (Cameron et al., 1995). Serotonin also has effects on neurogenesis: chronic treatment with antidepressant drugs increases proliferation in the dentate gyrus through blockade of serotonin reuptake. (Malberg et al., 2000). Similarly, inhibition of serotonin synthesis decreases proliferation. Indeed, it was shown that dentate granule cell neurogenesis in the adult is necessary for the therapeutic effect of antidepressants (Santarelli et al, 2003). Nitric oxide (NO) is a player in multiple processes in brain and vasculature, acting as both neurotransmitter and regulator of other cell processes, including vasodilation and microglial function. Induction of nitric oxide synthase is generally stimulated by intracellular calcium flux such as during NMDA receptor activation in neurons. Because NO is capable of diffusing freely between and within cells, it can exert influence over relatively large distances from its production site. Neuronal precursors are sensitive to the effects of nitric oxide. Nitric oxide reduces proliferation in the dentate gyrus by reducing proliferation of neural precursors, but promotes neuronal differentiation (Packer et al., 2003).

Astrocytes play a primary role in regulating neurogenesis in the adult, including cell proliferation, migration and differentiation (Lim and Alvarez-Buylla, 1999; Song et al., 2002). Astrocytes produce various factors including growth factors, cytokines, and stress factors that can modulate proliferation rate, migration and differentiation of adult-born neurons.

Various studies have implicated the vascular system as a major part of the stem cell niche owing to the proximity of stem cells residing next to blood vessels in the dentate gyrus in the SVZ and SGZ (Palmer et al., 2000; Shen et al., 2004). In fact, angiogenesis has been closely linked with neurogenesis in the brain. Cranial irradiation can disrupt angiogenesis which is implicated in the suppressive effect of radiation on neurogenesis (Monje et al., 2002). Endothelial cells are particularly sensitive to irradiation and either undergo alteration or cell death following exposure to therapeutic levels of radiation. Infusion of the angiogenesis factor VEGF into brain, which increases endothelial cell proliferation, also increases production of dentate gyrus granule cells (Jin et al., 2002).

1.3 CELL MIGRATION IN THE DEVELOPING AND ADULT BRAIN

During development, brain formation occurs through a number of successive phases: first, proliferation of embryonic neural stem cells in the ventricular neuroepithelium generate the early template of the developing brain. Second, the neurogenic phase produces cortical neurons that migrate in a highly organized pattern in an inside-out migration pattern to form the outer cortical layers. The third phase is the proliferation and differentiation of glia followed by the final phase, which includes the process of myelination, synaptic (Haas and Frotscher, 2010) pruning, synaptic stabilization, and apoptosis to refine the neural network. The rate and extent of developmental neurogenesis is drastically reduced by the time of birth, with the exception of continued proliferation of new cells in the subventricular zone and granule cell proliferation in the

hippocampal dentate gyrus (Cameron et al., 1993; Seki, 2002). Neuronal integration in the hippocampus continues for weeks postnatally.

Neural and glial precursor cells use various physical mechanisms to help navigate through the developing or adult parenchymal matrix in order to reach their final destination (Figure 1.15). This may include using certain structures in the brain that orient in the direction of migration (e.g. blood vessels), or, the collective formation of the neuronal precursors themselves create the migration scaffold (e.g. chain migration). In the developing and adult CNS, there are two general types of migration patterns: radial and tangential migration (Kriegstein and Noctor, 2004; reviewed in Cooper, 2013). Radial migration refers to cells that migrate parallel to the direction of radial glial fibers. Tangential migration occurs generally perpendicular to the ventricular wall and follows a course anti-parallel to radial glial fibers. Tangential migration is the primary directional type of migration taking place during positioning cortical interneurons, as well as migrating neuroblasts within the rostral migratory stream. During development, neural precursors arise from the ventral forebrain and migrate tangentially, or parallel to white matter tracts in the intermediate zone between the subventricular zone and the cortical plate (which ultimately forms the white matter in the adult brain). The migrating interneurons then change direction to migrate radially into the cortex (Marin and Rubenstein, 2003). SVZ-derived interneuron precursors migrating tangentially to the olfactory bulb detach from the chain of migrating cells in the bulb and migrate radially into their final positions.

There are two major modes of neuronal migration: one consists of nuclear translocation, which predominates in early development for positioning short distances; the second mode is the glial-guided (gliophilic) mechanism that allows neurons or neuronal precursors to translocate long distances. During development the dentate gyrus contains a radial-glial scaffold which forms a network of glial matrices upon which neuronal cells can migrate. This radial-glial scaffold persists

postnatally but rapidly disappears early after birth. The cells that comprise this scaffold retract and differentiate into astrocytes in the molecular layer, with some localized in the SGZ and identified to have stem cell properties in the adult animal. Since neurogenesis persists in the adult in the absence of the radial-glial scaffold, the mode of migration differs from that which occurs earlier in development. The importance of the radial-glial scaffold has been demonstrated in some mouse mutants, particularly in reelin mutants, in which radial-glial cells take on aberrant morphology and positioning, which in turn results in altered positioning and disrupted layers of granule cells (Katsuyama and Terashima, 2009) . There is also a significant decrease in neurogenesis of granule cells in dentate gyrus and an increase in gliogenesis. Interestingly, reelin, an extracellular matrix protein, is necessary in the adult brain to maintain regional laminar organization and the compact granule cell structure in the dentate gyrus. Hence it would appear that final positioning of neural cells and structural organization in the adult brain is not necessarily stable or static without active maintenance provided by specific effectors such as reelin.

Gliogenesis occurs primarily outside the areas of the neurogenic niche but represents the largest population of mitotically active cells in the brain. Glial progenitor cells are not known to migrate under normal physiological conditions (Roy et al., 1999; Dawson et al., 2003; Assanah et al., 2006) and glial cells do not migrate in chains as do neuronal cells. Also unlike neurons, glial cells continue to proliferate at a high frequency during migration. There is extensive gliogenesis occurring in the lateral and dorsal regions of the subventricular zone.

During early postnatal development gliogenesis persists in the subventricular zone, giving rise to new astrocytes and oligodendrocytes. These glial progenitors migrate in various pathways depending on their destination, which can include white matter, neocortex, striatum, and hippocampus. For example, progenitors destined to migrate into the dorsal hippocampus migrate radially along glial processes. Hence, radial glial cells function not only as a scaffold for migration

of neural progenitor cells but also for glial progenitor cells. Recent studies have shown that these radial glial cells also maintain the progenitor cells in an undifferentiated, migratory state until they stop and detach at or near their final destination (Goldman et al., 1997). These cells may remain undifferentiated and retained as a pool of progenitors that are potentially reactive to injury and promote repair. Another pathway by which glial cells can migrate, which enables translocating from one hemisphere to the other, is by traveling in parallel to axons along the corpus callosum. In this case, the cells do not utilize radial glial cells as a substrate for migration support (Kakita et al., 2003).

Similar to the neuronal pattern of migration into the developing cortex, glial precursor cells can either continue migrating all the way into the cortical layers or stop within the underlying white matter. However, glial progenitors destined to migrate to more lateral regions of the cortex will tend to migrate tangentially along white matter tracts initially, and then turn to migrate radially into the neocortex as appropriate to their final destinations. Another similarity in migration pattern between neural and glial precursors is both have been demonstrated to migrate along blood vessels which often align themselves in parallel with radial glial processes. Communication between the blood vessels and blood-borne factors may play an important role not only in angiogenesis, but in neurogenesis and migration patterns of neural and glial precursor cells. Little is known about factors which control the migration of astrocyte progenitors or glial cells in general, though this is an area of current research.

An area of intensive research is focused on determining how migrating cells find their target destination in both the developing brain and the adult brain. Molecular guidance factors including chemoattractant, chemorepulsive, detachment, and stop signals all play a role controlling cell migration. In addition, the migrating progenitor cells must express receptors that are sensitized to the appropriate environmental cues and activate appropriate pathways to induce changes in

cellular physiology to promote migration. Models of brain disorders and diseases have been useful tools to examine the mechanisms of cell migration.

In the developing brain, the neural stem cells are identified as radial glial cells which reside in the ventricular zone where they extend long processes from the ventricular wall to the pial surface of the brain. These processes provide a scaffold for the radial migration (termed gliophilic migration) of the first layer of cells to the outermost surface of the brain which consist of Cajal-Retzius cells and cortical pyramidal cells. "Reeler" mice are a model extensively used to study cell migration in the brain. First described in 1951 (Falconer et al., 1951), these mice lack the extracellular matrix protein reelin. Reelin is an important chemotactic factor that controls the radial migration and correct positioning of neuroblasts migrating from the ventricular wall and through the cortical layers of neurons during the inside-out development of the cortex (reviewed by Forster, 2014). Profound effects on migration and brain patterning occur in reeler mice. They exhibit hypoplasia of the cerebellum, disorganization of neurons in the cortical laminar layers, and they lack a compact granule cell layer in the hippocampus. Various factors in addition to reelin play an important role in regulating the radial migration of the cells into the cortical plate during development including bone morphogenetic proteins, sonic hedgehog, and CDK

s including CDK5.

Various other extracellular matrix (ECM) molecules are expressed within the rostral migratory stream that play roles in migration and stabilization including chondroitin sulfate proteoglycans, tenascin-C, and laminin. In general, the adult brain is not permissive for migration of new cells, hence a mechanism must exist to modify the microenvironment to allow migration. The expression of matrix metalloproteinases that cleave extracellular matrix molecules is necessary in order to allow for migration in the adult brain. Matrix metalloproteinases are expressed by cells along the rostral migratory stream and are also expressed by migrating neuroblasts. Matrix

metalloproteinases are necessary for radial migration in which cells migrate individually and not as chain;, however, deletion of matrix metalloproteinases does not inhibit migration of cells undergoing homophilic chain migration (Bovetti et al., 2007).

ECM proteins are increasingly recognized to have significant roles in the formation and maintenance of the neurogenic niche, proliferation and differentiation of neural stem cells, and the migration and survival of neural precursors (reviewed by Gattazzo et al., 2014). Some important ECM proteins that contribute to the regulation of cellular migration in the brain in addition to reelin include ADAMs, integrins, laminin, tenascins, and proteoglycans. ADAMs are transmembrane proteins that possess metalloproteinase activity and participate in breaking down of the extracellular matrix to allow migration of neuroblasts. ADAM21 is expressed by ependymal cells and subventricular zone cells. These proteins also interact with integrins and have a role in neurogenesis and neuroblast migration. Integrins are cell surface proteins that bind to extracellular matrix as well as to receptors on adjacent cells. Beta-1 integrin and laminin, which binds to this integrin, promotes the formation of migrating neuroblast chains in the rostral migratory stream, as well as for maintaining the rostral migratory stream astrocytic structure (Belvindrah et al., 2007).

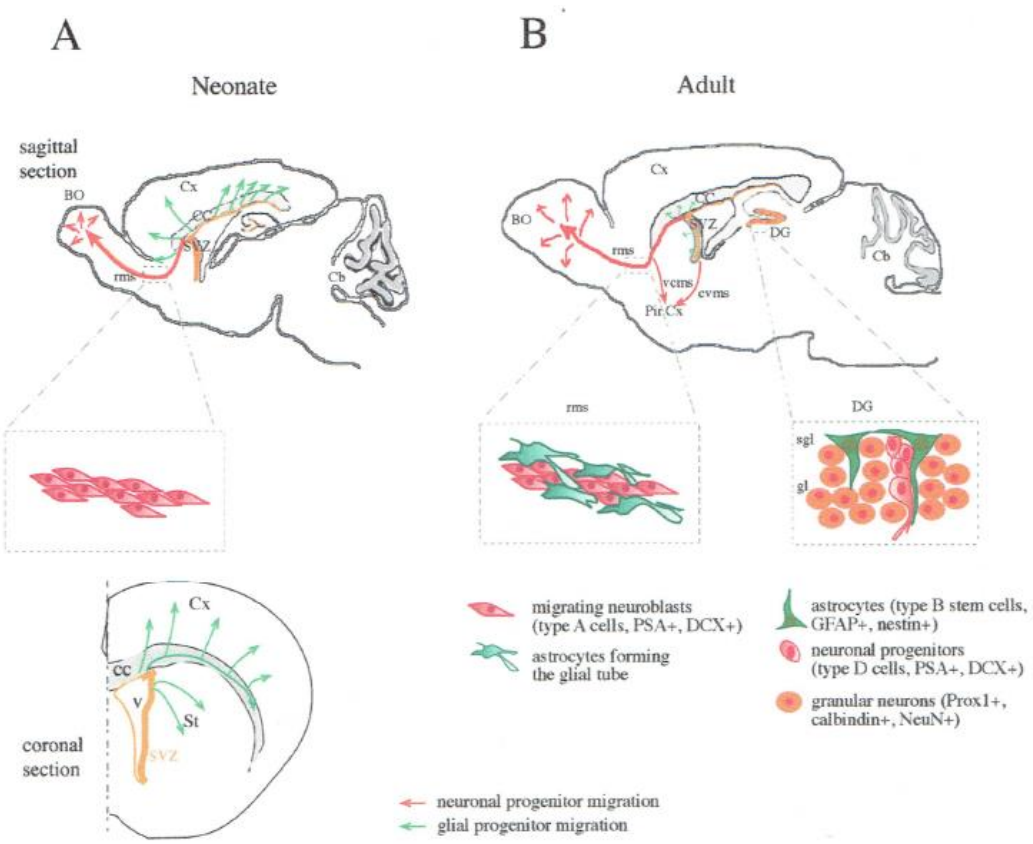


Figure 1.15 In the neonate brain, stem cells along the anterior ventricular subventricular zone gives rise to neuroblasts that migrate as chains (homophilic migration) to the olfactory bulb (OB), while progenitors to glial cells migrate caudally along nerve fibers and perhaps blood vessels toward the corpus callosum (CC) and the cortex (Cx) to differentiate into astrocytes and oligodendrocytes. B, As the neonate develops into an adult the rostral migratory stream (rms) formed by glial cells creates a path for the tangential migration of neuroblasts from the subventricular zone to the olfactory bulb. Some neural precursors also migrate tangentially into the piriform cortex (PirCx) via the caudoventral (cvms) or ventrocaudal migratory stream (vcms). Some cell migration of OPCs continues from the subventricular zone into the corpus callosum and stratum in the adult, particularly in response injury. Migration waves of glial progenitors becomes sparse with more localized clonogenic proliferation predominating in the adult. Neural precursor cells in the adult dentate gyrus (DG) subgranular zone (sgl) express markers of differentiation states as they migrate in the subgranular zone and the short distance into the granule cell layer (gl) where they eventually express mature granule cell markers (Prox1, calbindin, NeuN). (From Cayre et al., 2009)

Hippocampal and cortical neurons are encompassed by a reticular web of ECM molecules including laminins and proteoglycans referred to as a perineural net (reviewed in Giamanco and Matthews, 2012). Studies of the PNN have shown its importance in maintaining cortical and

hippocampal function in memory (Hylin et al., 2013), neuronal survival in disease and after pathological insults (Bonneh-Barkey and Wiley, 2009). Laminin was shown to play an important role in the survival of neurons following excitotoxic insult, and is part of a dynamic extracellular matrix that can be modified under various conditions to either promote cell survival or neurodegeneration (Chen et al., 2003).

The regulation of cellular migration is controlled by multiple factors that are continuing to be discovered. Proteins involved in cell-cell recognition that are bound to the plasma membrane, as well as secreted proteins that act as chemoattractant or chemo repellents, are important factors regulating and modulating migration of neural progenitors. In many instances, factors and mechanisms playing roles in development are recapitulated in the adult brain following injury or in other pathologies that stimulate a stem cell response. In fact, the pattern of neuronal migration during the early postnatal period is very similar to that in the adult, but different mechanisms may modulate migration and neurogenesis in the adult brain. For instance, neuroinflammation in the adult brain has been found to play not only a negative role but also a positive role in terms of recruiting stem/progenitor cells to a site of injury by affecting cell migration patterns. Secreted growth factors and some inflammatory cytokines can act as chemoattractants for neural precursors.

As described above, the SVZ and SGZ give rise to neural progenitors that migrate into specific structures under physiological conditions. There is a distinctive difference between the proliferation rates and the distances the SVZ- and SGZ-derived neuroblasts migrate. For example the subventricular zone is more highly proliferative under physiological conditions, giving rise to 30,000 to 80,000 new cells every day that migrate into the olfactory bulb and represent approximately 1% of the total granule cell population (Kaplan et al., 1985; Peterson, 2002). These new cells migrate long distances, several millimeters, in the rostral migratory stream to reach

their olfactory destination. On the other hand, only 9000 newly born cells are generated daily in the dentate gyrus (Cameron and McKay, 2001), and these cells only migrate short distances along radial glial processes where they are restricted to the dentate granule cell layer. These newly born cells represent only 0.03% of the neuronal cells in the dentate gyrus. While in both cases, cells derived from the subventricular zone and the dentate gyrus subgranular zone differentiate and integrate into the appropriate neural circuitry of the target destination, a full understanding of their functional role is still under intensive investigation (van Praag et al., 2002; Carleton et al. 2003; Mizrahi, 2007). Various studies have demonstrated that neurogenesis in the adult is important for maintaining olfactory bulb interneuron populations, and that new granule cell neurons may play a role in hippocampal dependent memory (Imayoshi et al., 2008).

The neurogenic niche and migration in the dentate gyrus

One of the major distinctions of development of the hippocampus dentate gyrus versus other cortical regions is a population of stem cells (originating from the subventricular stem cell niche) that are translocated to the dentate to initially form the supra-pyramidal blade of the dentate gyrus and are necessary for later expansion of the dentate granule cell layer. During midgestation, precursor cells and immature granule cells migrate along a subpial stream at the boundary between the fimbria and the meninges. This migration is dependent on the radial glial network in the developing hippocampal region and is sometimes referred to as a dentate migratory stream. The chemokine SDF1 is present in this migratory route and directs migration of stem cells through binding and activation of the CXCR4 receptor expressed on dentate precursors. Inhibiting SDF1 signaling as a chemoattractant in the subpial migratory stream results in a diminished population of precursors in the forming dentate gyrus. Deficits or mutations that alter the dentate

migratory stream organization often cause malformations in the infrapyramidal blade. Mutations in integrin signaling can also induce a similar result. After the dentate neuroepithelial structure is specified by various morphogenic factors, the next process is to populate the dentate with stem cells to further develop the dentate gyrus. Migration of precursors from the secondary germinal site in the future dentate hilus generates the neuroblasts that migrate to form the infrapyramidal blade. Any direct disruption of the radial glial network causes alterations in dentate gyrus morphogenesis. For example, a mutation in fibroblast growth factor receptor-1 causes a defect in the radial glial scaffold and results in a diminished precursor or stem cell population. In Frizzled mutants (g-protein coupled receptors that mediate wnt signaling), there is an increase in apoptosis of dentate precursors as they migrate from the neuroepithelium to the dentate, which causes a depletion of adult granule cell numbers (Zhou et al., 2004). The role of Wnt signaling in dentate gyrus morphogenesis will be discussed in the following section. While most of these stem cells are lost postnatally, a small population migrates to the subgranular zone and is retained as a source of new cells in the adult to form new granule cells in both the infra- and supra-pyramidal blades.

Finally, the migrating precursors must undergo appropriate differentiation into mature dentate granule cells. The differentiation process involves interplay between various transcription factors and extracellular signaling from the surrounding cellular milieu. Prox1 is a homeobox gene that is expressed specifically in mature granule cells of the dentate and is often used as a specific marker to identify the cells. Calbindin is another marker used to identify dentate granule cells. NeuroD is a basic helix–loop–helix transcription factor that is important for granule cell differentiation. Mutations in NeuroD have been described (Liu et al., 200) that are specific for causing abnormal granule cell morphology. NeuroD may also play a role in formation of the radial glial scaffold, as mutants in NeuroD typically exhibit a more dispersed granule cell layer.

Appropriate migration of progenitors in the adult brain requires a combination of factors that coordinate proliferation with signals that convey guidance cues to specific target sites. Guidance cues can be both repellent and attractive, and also include attachment and de-attachment signals from relevant scaffolding substrates. It is a complex orchestration of processes that control the movement of cells (Kaverina et al, 2002).. Adhesion and detachment mechanisms regulated by expression patterns of the substrate extracellular matrix, cell receptors, and other binding molecules on the cell surface play critical roles in cell movement. Expression and post-translational modification of intracellular proteins (e.g. microtubule-related factors) in response to extracellular cues initiate the necessary cellular machinery for cell motility. Some of these factors were described earlier and presented in Tables. 1.1A and 1.1B. It was previously mentioned that reelin expression is critical for maintaining the compact granule cell layer in the hippocampus, and disruption of reelin signaling in the adult brain results in dispersion of granule cells (Haas and Frotscher, 2010) Furthermore, it was recently shown that loss of reelin expression by hilar interneurons results in the ectopic migration of differentiated granule cells into the hilus following kainic acid-induced seizures (Orcinha et al., 2016).

Morphogenesis of the Dentate Gyrus

Stem cells from the subventricular zone migrate into what will later be the hilus (termed the tertiary matrix by Altman and Bayer, 1990) and the subgranular zone of the developing dentate gyrus. These stem cells create a 'secondary' germinal center that will then give rise to the infrapyramidal blade of the dentate gyrus. Massive proliferation in the matrix of the hilus further populates the blades of the dentate gyrus in the first week of postnatal life. It is also during this time that subgranular zone radial glial cells orient fibers through the granule cell layer to create a scaffold for migration of the rapidly forming new granule cell neurons. The radial glial cell bodies

are positioned in the subgranular zone while the long glial fibers extend to the pial surface. By the 10th postnatal day the cellular matrix in the hilus has reorganized, with the dividing progenitor cells localized in the subgranular zone and no longer in the hilus. Production of new neurons persists into adulthood, but decreases with age (reviewed by Li and Pleasure, 2007).

Diffusible cues are released from specialized signaling centers that are localized to specific regions of the developing brain and direct migration of the cellular components to form the brain structure (reviewed by Li and Pleasure, 2007). The most caudal-medial signaling center is the cortical hem that occurs within the neural epithelium near where the dentate gyrus will develop (reviewed by Li and Pleasure, 2007). The hem is a longitudinal stripe running rostro-caudally along the medial faces of each telencepahlic hemisphere, or in other words, the most medial-pallial domain of the cortex adjacent to the region forming the neocortex. In the developing brain, the cortical hem initiates the cellular development of the dentate gyrus and hippocampus by directing the migration of precursors from the neuroepithelium. However, the hem is not considered cortical in that it does not contribute to the hippocampus or neocortex. Rather, it releases diffusible factors into the cellular compartment that drives a gradient of activated transcription factors responsible for specifying the development of the hippocampus and other cortical regions. The importance of the cortical hem for development of the dentate gyrus was demonstrated when deletion of the hem ablated the formation of the dentate gyrus as well as reducing cortical volume (reviewed by Li and Pleasure, 2007; Monuki et al., 2001; Yoshida et al., 2006). Factors that are released by the cortical hem include Wnt and BMP factors, in addition to other molecules that provide axon guidance. The cortical hem also gives rise to Cajal-Retzius cells which as mentioned previously, are neurons that are critical for neuronal positioning via the release of the ECM protein and guidance factor reelin. Reelin is vital in the development of the hippocampus, as well as for maintenance of the dentate gyrus granule cell layer compact structure throughout

life as demonstrated by the loss of reelin expression and subsequent granule cell dispersion following kainate-induced seizures (Orcinha et al., 2016).. Cajal-Retzius cells migrate tangentially to cover the superficial cortical layer of the cerebral cortex called the marginal zone. These neural cells are among the earliest to form from the neuroepithelium and are adjacent to the region that will generate the dentate granule neurons. The Cajal-Retzius cells migrate and respond to the chemokine factor SDF1, which is produced by the meninges, and which maintains these cells in their superficial cortical position. SDF1 also controls the migration of cells to the dentate gyrus later in development.

The hem produces factors of the Wnt family that are critical for hippocampal formation (reviewed by Li and Pleasure, 2007). Transcription factors activated by diffusible effectors (e.g. Wnt, BMP, SDF1, LRP6) modulate the migration and differentiation of granule cell precursors. Some of the important diffusible factors including Emx2, Lhx2/5, p73, Foxg1, beta-catenin, Tcf/Lef, and NeuroD. Wnt binding to its G-protein coupled receptor, Frizzled, induces phosphorylation/activation of the intracellular protein, disheveled (Dsh). Dsh relays receptor activation down three known pathways: (1) Dsh inhibits GSK3-mediated phosphorylation of beta-catenin, which protects it from proteasome degradation via ubiquitination. Beta-catenin can then accumulate and enter the nucleus where it interacts with the Tcf/Lef family of transcription factors to alter gene expression (the canonical pathway). (2), the non-canonical planar cell polarity pathway in which Dsh activates the G-protein Rho to regulate cytoskeletal structure including actin polymerization; (3), the non-canonical calcium pathway, in which a trimeric G-protein interfaces with Dsh leading to activation of PLC or a cGMP-specific phosphodiesterase. Activation of PLC can lead to release of calcium from endoplasmic stores, which in turn can activate PKC that can phosphorylate CDC42, an important regulator of ventral patterning during development. Additionally the increased calcium in the cell can activate other calcium-dependent

kinases that can activate transcription factors such as NFAT, which plays a role in cell migration and cell adhesion.

Mutations that specifically impact the canonical pathways (those involving beta-catenin signaling) of this family produce hippocampal deformity. The non-canonical pathways regulate expression of genes that play a role in cytoskeletal and axon guidance control. Mutation in Wnt proteins can severely affect hippocampal morphogenesis largely due to reduction in neural precursors in the medial pallium. Additionally, in adult brain, wnt signaling is important for normal hippocampal-mediated functions in learning and memory (Ortiz-Matamoros et al., 2013). Mutation of the Lef1 transcription factor causes a complete loss of development of granule cell neurons in the hippocampus. Mutation of the LRP6 (a Frizzled co-receptor required for cell signaling) has a similar phenotype as Lef1 mutants where it results in a relatively specific deficiency in the expansion of early dentate precursors that fail to populate the dentate gyrus with stem cells. The mutation also causes a defect in prenatal radial glial scaffolding.

The cortical hem also produces members of the BMP family, but at levels lower than that produced by the choroid plexus. BMPs are important for development of the choroid plexus, as well as regulating Fox-G1 expression. Early in development the BMPs are important for cortical patterning, with ligand mutants exhibiting defects in the development of the medial cortical wall. BMPs also play a critical role in the early development of the hippocampus.

1.4 NEURAL INJURY AND REPAIR

There are a number of factors that can affect both the function of the dentate gyrus and neurogenesis. Some of the important factors include neuroinflammation, steroid stress hormones (corticosteroids), neurotrophic factors, and aging (reviewed by Cayre et al., 2009).

Studies increasingly show many of these factors contribute important roles in normal development as well as in injury and repair processes in the mature animal CNS. Aging also has impacts on function and neurogenesis in the hippocampus.

Neuroinflammation characterizes many acute and chronic neurological conditions (reviewed by Perry et al., 2010). Depending on the nature and duration of the inflammatory response to the pathology, the effects on neurogenesis can either be beneficial or detrimental (Ekdahl et al., 2009; Whitney et al., 2009). Pro-inflammatory cytokines are constitutively expressed in the physiologically normal CNS. In chronic conditions such as Alzheimer's disease, multiple sclerosis, infection, and acute injury such as ischemia and stroke, elevated levels of pro-inflammatory cytokines are expressed within the CNS. Pro-inflammatory cytokine receptors occur throughout the hippocampus, and there is evidence to suggest that the functions of the cytokines are regionally distinct within the hippocampus.

As the primary innate immune cells of the CNS, microglia are a key source of regulators of both pro- and anti-neuroinflammatory cytokines, as well as chemokines and growth factors. Hence, these cells are well positioned when activated under pathological conditions to modulate neurogenesis (Whitney et al., 2009). Pro-inflammatory factors (e.g. TNF alpha) and reactive oxygen species can inhibit neurogenesis and induce neural precursor cell apoptosis (Cacci et al., 2008; Ekdahl et al., 2003; Hoehn et al., 2005; Monje et al., 2003) through a NFkappaB-dependent mechanism (Guadagno et al., 2013). It has been reported that other microglia-derived factors (anti-inflammatory factors, growth factors, chemokines) can promote the proliferation and recruitment of neural progenitor cells to sites of injury (Aarum, 2003; Walton, et al., 2006). Interestingly, in a study using TNFR2 (p75) receptor knock-out mice (McPherson et al., 2011), subgranular zone neural precursor cells failed to migrate into the granule cell layer following trimethyltin-induced lesion of the dentate gyrus. This demonstrates that at least in some forms

of brain injury, pro-inflammatory factors such as TNF alpha may contribute to neurogenesis and neurorepair. TNF alpha is known to play a role in synaptic plasticity in the dentate gyrus in addition to playing a role in apoptosis. Microglia are a major source of TNF alpha in injury conditions and a key mediator in the ischemia pathology (Nimmerjahn et al., 2005; Takahashi et al., 2005), and also act as a neuromodulator in the brain (Beattie et al., 2002; Stellwagen et al., 2006).

Increased levels of TNF alpha have been observed in several pathological states that are associated with learning and memory deficits such as Alzheimer disease. However, the effects of TNF alpha are complex and often contradictory. The effects of TNF alpha are dependent on various parameters including TNF alpha concentration, timing of secretion and duration, TNF alpha receptor expression, and the physiological/cellular context in which the TNF alpha is presented. In addition, specific neuronal populations in the dentate gyrus and CA3 can be selectively activated by TNF alpha, with outer regions of the granule cell layer exhibiting greater NFkappaB expression than the inner one to two layers. This suggests that TNF alpha can specifically affect granule cell neuronal function in a region-dependent fashion.

TNF alpha plays a role regulating development of the hippocampus. Knockout of TNF alpha in mice results in a reduction of apical dendrite arborization at the CA1 and CA3 regions as well as causing accelerated development in the dentate gyrus (Golan, et al., 2004). This may be related to the increased levels of NGF and decreased BDNF levels in the TNF alpha knockout. Some studies suggest that TNF alpha impairs learning and memory processes. Transgenic mice with deletion of TNF alpha perform better in spatial memory and learning in a Morris water maze task compared to wildtype mice (Golan 2004).

Many studies have demonstrated the complex involvement of TNF alpha in neurotoxicity and ischemic injury (Bruce et al., 1996; Gary et al., 1998; Sullivan et al., 1999; Marchetti et al., 2004;

Pradillo et al., 2005). TNFα can have both neurodegenerative and neuroprotective roles during brain injury and inflammation (Sriram and O'Callaghan, 2007). TNF alpha is produced in the brain by astrocytes, microglia, and some neurons. It is rapidly released under various pathological conditions including ischemia and inflammatory disease and as rapidly as one hour after brain injury and before neuronal death. TNF alpha plays an important role in neurotoxicity. Although originally identified as a likely neurodegeneration-inducing factor, it has been also shown to have functions other than inducing or facilitating cell death. For instance, TNF alpha has been shown to activate caspase-3-mediated apoptotic cell death in hippocampal cell cultures, whereas microglial-derived TNF alpha plays a protective role against neuronal cell death in permanent middle cerebral artery occlusion (Lambertsen et al., 2009).

TNF alpha receptors are constitutively expressed by neurons and glia in the CNS. TNF alpha signaling occurs via two receptors: TNFR1 and TNFR2. Receptor binding can activate various signal transduction pathways (reviewed by Pickering et al., 2005). The composition of the coupled downstream effectors, have been suggested to determine whether there is a neurotoxic or neuroprotective effect (Botchkina et al., 1999). Another layer of complexity with the TNF alpha pathway is contributed by the expression of receptor and peptide in neurons versus glia, and the effects of interacting cell populations (Eskes et al. (2003). Trimethyltin (TMT) added to microglial cultures induced TNF alpha secretion, which was attenuated when co-cultured with astrocytes. In both cases, no activation of microglia or astrocytes was morphologically apparent. However, when microglia were co-cultured with neurons, TMT more potently induced TNF alpha secretion and resulted in transformation of microglia into an activated state. TMT-induced neuronal cell death was enhanced from 30% in enriched neuronal cultures to 100% in neuron-microglia co-cultures. The TNF alpha release is dramatically enhanced in the presence of microglia (Bezzi et al., 2001).

In general, neural excitotoxicity is linked to excessive activation of glutamate receptors, especially the N-methyl-D-aspartate (NMDA) receptor. Impaired uptake of excessive glutamate by glial cells is known to be a contributor to cell death via over-activation of glutamate receptors. Evidence supporting this is derived from EAAT2/GLT-1 knockout mice (an excitatory amino-acid transporter). The knockout mice develop epilepsy, and are more susceptible to injury as a result of excessive accumulation of extracellular glutamate (Tanaka, 1997). Interestingly, this transporter is regulated both positively and negatively by NFkappaB where NFkappaB binds to the EAAT2 promoter. Epidermal growth factor increases EAAT2 expression by promoting binding of NFkappaB to the promoter. However TNF alpha can also decrease expression of the transporter through the classical IkappaB degradation pathway to trigger NFkappaB nuclear translocation and DNA binding to repress EAAT2 expression. This increases susceptibility to excitotoxicity through elevated extracellular glutamate accumulation (Zou and Crews, 2005).

IL-1 beta and IL-18 have been implicated in neurodegeneration, decreased learning and memory function, and other brain pathologies involving elevated pro-inflammatory processes. Functionally, IL-1 beta attenuates synaptic plasticity and LTP possibly by blocking glutamate release and loss of arachidonic acid (due to elevated ROS) as a retrograde messenger necessary for LTP formation. IL-1 also inhibits calcium flux and NMDA receptor activation which dampens LTP across various neural network pathways including the dentate gyrus mossy fiber-CA3 pathway, the Schaeffer colateral-CA1, and the MPP-dentate granule cell pathways. The highest levels of IL-1 receptors occur in the molecular and granular layers of the hippocampus. IL-18 is expressed in the hippocampus and other brain regions primarily in astrocytes and microglia. These mediators induce changes in gene expression similar to the activation of TNFR2 receptors including growth factors, adhesion molecules, cytokines, and various kinases (p38 MAP-kinase, jun kinases, p42/44). In contrast to the detrimental effects of IL-1, genetic deletion of IL-1

receptor in mice causes a deficit in some learning paradigms including LTP, suggesting that IL-1 beta has a positive role in learning and memory.

Compared to other brain regions, the dentate gyrus is exquisitely sensitive to concentrations of corticosteroid hormones that go beyond the physiological range. Corticosteroids are critical to maintaining dendritic morphology and synaptic transmission. However, prolonged exposure to excessive levels of corticosterone (such as under stress) compromises the survival of dentate granule cells and makes them susceptible to delayed cell death. Neurogenesis in the dentate gyrus is also suppressed by prolonged corticosteroid exposure inherent with chronic stress.

Dentate granule cells express high levels of receptors for the stress hormone corticosterone which include the high-affinity mineralocorticoid receptor, and the glucocorticoid receptor which is activated after stress. The dentate gyrus is unique in that it requires corticosteroid receptor activation to prevent apoptosis and to preserve neuronal integrity. Dentate granule cells are particularly sensitive to hormone levels, and in the absence of corticosteroids, proliferation and apoptotic cell death are significantly increased. Exogenous administration of low dose of corticosterone at a concentration where only binding occurs to high-affinity mineralocorticoid receptors but not glucocorticoid receptors, can reverse the apoptosis of granule cells. This suggests that mineralocorticoid receptors play the prominent role in dentate granule cell maintenance. Interestingly, not all dentate granule cells are sensitive to the removal of corticosterone, suggesting some subpopulations are more susceptible to the depletion of this hormone. One study suggests that young adult-born granule cells are more resistant to adrenalectomy-induced apoptosis (Cameron and Gould, 1996). Calcium influx is enhanced, resulting in increased vulnerability of older neurons to undergo cell death, whereas relatively young granule cells may be able to tolerate these adverse conditions probably because they have

a smaller calcium influx to start with and are able to increase expression of survival genes such as Bcl-2.

Adrenalectomy promotes neurogenesis in the dentate gyrus, but also increases cell death of mature granule cells (Gould et al., 1991, 1992; Rodriguez et al., 1998; Cameron and Mckay, 1999; Nichols et al., 2001; Sloviter et al., 1989, 1993; Roy et al., 1990; McNeill et al., 1991). The process may involve alterations in entorhinal input and NMDA receptor-dependent transmission (Cameron et al., 1995). The increased neurogenesis following adrenalectomy requires mineralocorticoid activation as well as glucocorticoid activation (Wong and Herbert, 2005). BDNF and beta FGF are both modulated by corticosteroids (Hansson et al., 2000) which may be involved in the effects of corticosteroids on neurogenesis and neuronal survival. There is a 30% loss in excitatory postsynaptic potential in the molecular layer of the dentate gyrus a few days after adrenalectomy (Stienestra et al., 1998). An extensive synaptic reorganization seems to occur which largely alters synaptic properties of the surviving cells. This process of synaptic reorganization begins prior to the initiation of apoptosis.

In summary, physiological levels of corticosteroids are needed to maintain mineralcorticoid receptor activation to prevent apoptosis and the upregulation of neurogenesis. However, repeated exposure to high levels of corticosterone reduces the number of newborn cells in the dentate gyrus, as well as a reduction in survival of newborn granule cells (Wong and Herbert, 2004) This may or may not lead to a large reduction in overall number of cells or volume of the dentate gyrus. Prolonged ongoing stress could substantially change the composition of the dentate gyrus granule cells leading to cognitive disturbances (Pham et al., 2003; Westenbroek et al., 2004; Heine et al., 2004).). Studies have shown that chronic stress is a risk factor for psychiatric diseases including depression. In support of the role that neurogenesis may have in psychiatric disorders such as depression, anti-depressants have been shown to promote

hippocampal neurogenesis in concert with their therapeutic effects in animal models (Santarelli et al., 2003).

1.5. CELL MIGRATION IN THE INJURED OR DISEASED BRAIN

In many pathological conditions of the brain, cell migration and proliferation in the neurogenic regions are often disrupted. It is not clear whether these alterations in neurogenesis and migration play a major role in the development of psychiatric disorders, or are merely secondary effects. Cell migration is observed to be altered in various disease models including schizophrenia, neurodegenerative disease, and brain injury via trauma or chemically-induced (reviewed by Cayre et al., 2009). Dying neurons may directly influence neurogenesis in the adult brain, such as shown for apoptotic cortical neurons which promote the formation of new cortical thalamic or corticospinal projection neurons (Magavi et al., 2000; Chen J., Magavi, S. et al., 2004). *In vitro* studies support the contention that dying neurons release factors that can influence neurogenesis. Agasse et al. (2004) showed that media from cultured adult apoptotic cortex neurons could activate proliferation of subventricular zone-derived neurospheres, whereas conditioned medium from healthy cortex had an inhibitory effect on growth.

Migration requires a multistep process of events that involve the modification of the extracellular matrix and intracellular alterations of the cytoskeleton to promote motility. The mature brain is a tightly packed cellular tissue that leaves little room for migration of cells. The extracellular space in the adult brain are submicrometer, much tighter than the space that exists in the developing brain or other tissues. This spatially constrained physical structure creates a barrier that can impede migrating precursor cells. Part of the strategy to circumvent this barrier to migrating cells is to produce factors that interact with the extracellular matrix including

tenascin, laminin, fibronectin, osteopontin, and the expression of matrix metalloproteinases. Their activity will modify the extracellular matrix and promote interaction with the leading edge of the migrating cells (which express molecules such as integrins that interact with the extracellular matrix components) to facilitate penetration through the parenchymal matrix. Binding of integrins to the cellular matrix facilitates migration through brain tissue. Migration of glial cells is dependent on calcium flux to induce cytoskeletal modification, which is required for cell motility. Inhibition of calcium entry (which is activated via AMPA receptors) or calcium signaling inhibits cell migration. Hence glutamate signaling may play an important role in migration. Another important factor that plays a role in saltatory migration is myosin-II, which facilitates directional movement of both nucleus and the cell body. In animal models of schizophrenia, there is a decrease in hippocampal stem cell proliferation (Liu et al., 2006). Ectopic positioning of granule cells in the dentate gyrus is observed to occur (Duan et al., 2007). The protein disrupted-in-schizophrenia-1 (DISC1) is down-regulated in this disorder. This protein has a nuclear localization sequence but primarily localizes in the mitochondria of cells and is also found in the nucleus, cytoplasm, synapses, and axons. DISC1 has been implicated in playing roles in a multitude of cell functions including migration, proliferation, and differentiation of neural stem cells during development and in the adult brain. Aberrations in DISC1, in particular, a single nucleotide substitution, has been associated with patients with schizophrenia. DISC1 activity is not enzymatic, rather, it interacts with proteins to modulate their activity. It modulates the activity of dynein and influences microtubule polymerization and stabilization, which in turn modulates cell shape and intracellular transport. DISC1 also modulates neurite outgrowth.

In schizophrenia patients, the expression of reelin is decreased, and genetic variation in neuregulin-1 and its receptor (Erb4) are also associated with this disorder (Eastwood and

Harrison, 2006; Fatemi, 2005; Norton et al., 2006). Both reelin and the NRG1 system are important regulators of cell migration in the CNS.

As discussed earlier, epileptic seizure activity induces a profound reorganization or remodeling within the hippocampus including involvement of mossy fibers, granule cell dispersion, and ectopic repositioning of granule cells. Seizures induce increasing proliferation in the dentate as observed in pilocarpine-induced models of epilepsy. However, this model also demonstrated the migration of adult-born neuroblasts to ectopic positions within the hilus (Parent et al., 1997; Scharfman et al, 2000). It suggested that the ectopic granule cells may contribute to seizure generation and propagation since they have been shown to exhibit hyperexcitability (Parent et al., 2007). The ectopic granule cell migration may be the result of loss of reelin-secreting interneurons (Gong et al., 2007). There is also an increase in subventricular zone proliferation in the pilocarpine model. However, these newly formed migrating neuroblasts deviate from the rostral migratory stream and can enter the striatum, corpus callosum, hippocampus, and cortex (Parent and Lowenstein, 2002; Parent et al., 2006). The cells do not give rise to new neurons; rather, they differentiate into oligodendrocytes and astrocytes. Similar observations were observed in epileptic human brain samples with rostral migratory stream-derived neuroblasts migrating into adjacent white matter (Gonzalez-Martinez et al., 2007).

Alterations in neurogenesis in neurodegenerative disease has been reported but are often inconsistent. In human Alzheimer patients there is an increase in hippocampal neurogenesis (Jin et al., 2004). However, in a mouse model for Alzheimer disease there is a decrease in hippocampal neurogenesis. Animal models are not always consistent with what is observed in the human disease, thus making interpretation of the role of neurogenesis in Alzheimer disease difficult (Galvan and Bredesen, 2007). In Huntington disease, the observed effects on neurogenesis are more consistent than that of Alzheimer disease. The subventricular zone layer is expanded due

to increased progenitor cell proliferation, and the severity of the disease is correlated with the degree of increased SVZ proliferation (Curtis et al., 2003). This is observed in both humans and animal models. It is suggested that tangential migration of subventricular zone progenitors in the rostral migratory stream may be disrupted, as it has been observed that some cells "leak" into the adjacent striatum in Huntington disease animal models (Batista et al., 2006). Unlike Huntington disease, there is actually a decrease in SVZ proliferation in Parkinson disease, as well as in the dentate gyrus (Hoglinger et al., 2004). However, as in Huntington disease, there is an observed redirection of migrating subventricular zone neuroblasts into the striatum when injury is induced in rodent models using 6-hydroxydopamine (Baker et al., 2004; Hoglinger et al., 2004; Winner et al., 2008; Cooper and Isacson, 2004; Fallon et al., 2000). Dopamine itself is a positive regulator of adult neurogenesis (Baker et al., 2004; Borta and Hoglinger, 2007; Freundlieb et al., 2006; Liu et al., 2006). The lineage fate of the SVZ-derived progenitors that migrate into the subventricular zone is controversial. Some studies report differentiation of the cells into dopaminergic neurons once positioned into the 6-OHDA-lesioned striatum, whereas other studies have indicated that the subventricular zone-derived cells differentiate into glia and not neurons (Cameron and McKay, 2001; Cooper and Isacson, 2004; Winner et al, 2008). Hence, the role that SVZ-derived migrating progenitor cells play in the repair of the diseased striatum is not clear.

In rodent studies, global ischemia induces the proliferation of neural progenitors both in the dentate gyrus and the subventricular region. These cells migrate toward the damaged CA1 region of the hippocampus (Nakatomi et al., 2002; Liu et al., 1998). However, in focal ischemia, migration of subventricular zone cells is limited to the striatum (Arvidsson et al., 2002; Jin et al., 2003; Zhang et al., 2001). The increased number and proliferation of subventricular zone progenitors is a result of both increased symmetric proliferation of the progenitors as well as the proliferation and transformation of ependymal or subependymal cells into radial glia (Zhang et al., 2004, 2007).

The migration of neuroblasts into the surrounding parenchyma following ischemic injury may be the result of damage to the glial barrier created by the rostral migratory stream tubule structure. Immature migrating cells normally constrained to the stream can venture into the surrounding parenchyma as chains of migrating cells (Zhang et al., 2004), or as individual cells (Winner et al., 2008). When the migrating cells approach the lesion site, they disperse and migrate into the lesion where they differentiate into primarily medium spiny neurons (Arvidsson et al., 2002; Teramoto et al., 2003).

The ectopic migration of neuroblasts into the injury site following ischemia can continue for relatively long periods of time (up to one year after injury), although the increase proliferation is more transient and ends within a few weeks. The functional effect these ectopically positioned new striatal neurons have in the adult brain is unclear. They typically do not survive for long, possibly as a result of the inflammatory state within the lesion. Only 0.2% of the population of dead striatal neurons are replaced by these newly formed neurons (Ardvisson et al., 2002). Ectopic migration of neuroblasts into the striatum is not observed in nonhuman primates (Tonchev et al., 2005) and their normal migratory path to the olfactory bulb is retained. This is in contrast to what is observed in epileptic patients in which there is a redirection of neuroblasts into the striatum.

The increase in proliferation and migration induced by seizures is also observed in aged animals, indicating that the mechanisms responsible for this induction are retained, and also the cells retain the capacity to upregulate neurogenesis (Kuhn et al., 1996; Luo et al., 2006). The size of the lesion is correlated with the degree of activation in recruitment of new striatal neurons and is not disrupted by the age of the animal (Darsalia et al., 2005; Kokaia et al., 2006).

Oligodendrocyte progenitor cells (OPCs) are scattered throughout the adult brain (in both white and grey matter) and under normal physiological conditions the cells do not migrate. OPCs

are believed to be activated to replace oligodendrocytes that are lost to maintain myelination in their immediate vicinity (McTigue and Tripathi, 2008). Oligodendrocytes consist of a population that may be +/- for NG2 expression, and also express PDGFR and Nkx2.2 (Dawson et al., 2003; Reynolds and Hardy, 1997). Cells that are lacking in NG2 expression may be at a more advanced stage of lineage progression. NG2 is a proteoglycan that interacts with integrin and other extracellular matrix molecules, the interaction of which induces a cascade of cell signaling events that alter the cytoskeletal structure to promote motility (Karram et al., 2005). Disease or injury in which there may or may not be involvement of the myelination, can activate the proliferation and migration of the oligodendrocyte precursor cells towards the site of injury. The cells then differentiate into myelin-producing mature oligodendrocytes. In the case of focal ischemia, where oligodendrocytes are lost in the peri-infarct area causing a reduction in myelination, the oligodendrocyte precursors migrate towards the lesion to replace the lost cells and re-myelinate the affected nerves (Tanaka et al., 2003).

Therapeutic strategies that may improve neurological outcome from brain insult and injury include use of neurotrophins to stimulate neurogenesis, induce migration of neuroblasts to the lesion site, and promote differentiation to the appropriate phenotype. The challenge for successful therapy is the need to specifically induce all three of these events. A single, small molecule for therapeutic purposes may be insufficient to affect appropriate migration and differentiation. Additionally, treatments targeting specific neurotrophic receptors may be necessary depending on the type of injury so there is an adequate stem cell induction to create sufficient numbers of new neuronal cells of the desired phenotype.

1.6. ORGANOTINS: TRIMETHYLTIN

The organotin, trimethyltin, is a limbic system neurotoxicant used in the studies described herein to investigate the response of endogenous stem cells to hippocampal injury. Trimethyltin is a validated and reproducible model for studying chemically-induced hippocampal injury. As will be presented in detail in this section, and later sections, trimethyltin potently induces cell death of hippocampal dentate granule cell neurons in mice, and stimulates proliferation of subgranular zone stem cells.

History and applications of organotins

Organotins are a widespread environmental contaminant throughout the United States, Europe and Japan that can accumulate up the ecological food chain in terrestrial and marine environments (Alzieu et al., 1991; Borghi and Porte, 2002; Fent and Hunn, 1991; Holloway et al., 2008; Teuten et al., 2009; Tolosa et al., 1992; Ueo et al., 1999). There has been a global increase in the production of methyltins (Hoch, 2001) with the expected rise in human exposure to these toxicants (Tang et al., 2008, 2010). The organotins are a family of organometallic compounds that consist of a tin (Sn) atom linked to carbon atoms through covalent bonds. Many organometallic compounds have been created since the first discovery of the chemistry in 1830 that generated the metal-carbon bond. (Figure 1.16) The carbon-tin covalent bond is less stable and easier to break compared to carbon-carbon or other carbon covalent bonds in organic structures. However, under ordinary physiological conditions the compound does not degrade as a result of instability of the chemical structure.

The first organotin was described and studied in the 1850s (Lowig, 1852; Frankland, 1853; 1854). The earliest study on the toxicity of alkyltins was published in 1858 (Buckton, 1858). It was not until a hundred years later that systematic studies of organotin toxicity were performed during which the number and kind of organic groups covalently linked to tin were found to correlate with the toxicity of the compound. In particular, fungicidal and bactericidal properties were discovered to be potentially useful for industrial and medicinal applications. Interestingly, the first industrial use of organotin was in 1925 as a mothproofing agent. This was soon followed in 1932 by its use in the energy sector for the manufacture of capacitors and transformers where organotins were used for stabilizing chlorinated benzenes and biphenyls (Champ and Bleil, 1988). By 1936 it was found that dibutyltin could be used in plastics manufacturing and as a PVC stabilizer. In 1954 an attempt was made to utilize the biocidal properties of organotins to medicine. The results were disastrous since the toxicity of the compounds were poorly evaluated. Over one hundred patients with staphylococcal infection died when organotin was administered as part of a treatment plan (Saxena, 1987). Moving away from medicinal applications, the broad biocidal properties of organotins (van der Kerk and Luijten, 1954) were utilized for agricultural and industrial use. The potent fungicidal properties of organotins were exploited in agriculture to control fungus causing the leaf blight on crops such as beets, carrots, onions, rice and sugar. They were also used as fungicides to control infection in cocoa, pecans, peanuts, and coffee. The fungicidal properties of dibutyltins are used in wood preservatives, and in paints used in bathroom fixtures, decks, exterior siding, and fences. It was also used by textile industry for preventing growth of odor-causing fungi in various garments including socks.

In the 1960's the molluscicidal activity of two organotins (tributyltin oxide and tributyltin fluoride) was discovered and soon exploited by the shipping industry. Hull fouling by barnacles and other marine organisms presents a chronic and challenging issue in the military and

commercial shipping industry. Decreased speed and significantly increased fuel consumption spurred the search for compounds that would inhibit growth of barnacles, seaweeds, or tubeworms that coat the ship's hull and contribute to its inefficiency. In 1961, tributyltin was utilized as an additive to boat hull paints to act as an anti-fouling agent. Various types of paints were produced that differed in how the biocides were incorporated into the paint as well as the mechanism by which the paint interacted with the environment. In general there were three types: (1) a free association in which the biocide was mixed with the paint with the biocide leaching from the paint surface in contact with the seawater; (2) another type of paint was referred to as 'ablative', in which the paint shed or peeled away small particles over time from its surface revealing an active layer of biocidal material; and (3) copolymer paints were developed and most commonly used in the 1990s. In the latter case, tributyltin was chemically bonded with the paint polymer which yielded a much lower release rate of the organotin into the aquatic environment hence increasing its longevity to 5 to 7 years. It also relatively confined the biocide to the immediate surface of the paint layer, which gradually degraded to release the active biocidal agent. The use of organotins in antifouling paints was utilized internationally for naval vessels as well as commercial and recreational vessels, yielding significant savings and improved efficiency. (Champ and Seligman, 1996).

However, unintended environmental consequences of the use of organotins became recognized in the marine environment. Tributyltin (TBT) exposure to non-target organisms such as oysters caused growth abnormalities and thickened shells. TBT induced imposex in the common dogwhelk, and other non-target marine mollusks (Mensink et al., 2003; Horiguchi et al., 2006), causing development of male reproductive organs in the female member of the species. The abnormalities in oysters and other mollusks correlated with the number of boats in an area with highest frequency of defects observed along marinas and boat moorings. The toxic effects

were suggested to impact early life stages of mollusk larvae resulting in higher mortality. These ecotoxic effects resulted in the current international ban on the use of organotins in aquatic paints.

The most prevalent application of organotins presently is in their use as stabilizing agents for PVC polymers. Specifically, diorganotin (dimethyltin) compounds are used for this application (Guess and Stetson, 1968). Without a stabilizing agent, PVC tends to lose its optical clarity over time as a result of exposure to heat or ultraviolet light. Using an organotin in the manufacturing process stabilizes the PVC to retain its transparency for long periods. The mechanism for the stabilization is through removal of allylic chloride groups from the polyvinylchloride, and by absorbing and preventing the dissociation of hydrogen chloride. Organotin compounds were included in about 10% of all PVC products produced in the 1970s. When in combined with PVC, the alkyltin compounds are not toxic, although there is increasing concern that methyltins could leach out of these materials and contaminate drinking water and food (Richardson and Edwards, 2009; Hoch, 2001).

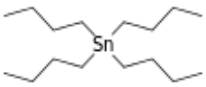
Trimethyltin

Trimethyltin is not utilized commercially for its biocidal activity (although it does possess biocidal properties). Rather, trimethyltin at the industrial level is a secondary or intermediate product (precursor) of the synthesis of dimethyltin. The synthesis of dimethyltin for use in manufacturing of plastics results in the formation of dimethyltin (80%), trimethyltin (8%), and monoethyltin (4%). Trimethyltin is also sometimes used as a substrate for chemical reactions to produce other compounds such as vinyltrimethylstannane, or to form new tin-tin or tin-carbon containing compounds. While production of trimethyltin salts is essentially limited to use for

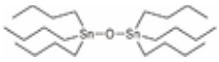
research (such as neurotoxicology and neurodegeneration), there is still potential for occupational exposure since organotins are still being produced as heat stabilizers in polyvinylchloride, as agricultural biocides, and as preservatives for some wood and textile products (Fent, 1996; Ohno et al., 2002; Piver, 1973; Rudel, 2003; Teuten et al., 2009).

Trimethyltin chloride can be synthesized directly by the following chemical reaction: a redistribution reaction (Kocheshkov reaction) of tetramethyltin with tin tetrachloride:

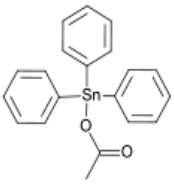
SnCl4 + 3 SnMe4 → 4 Me3SnCl



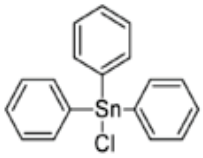
Tetrabutyltin starting material for the



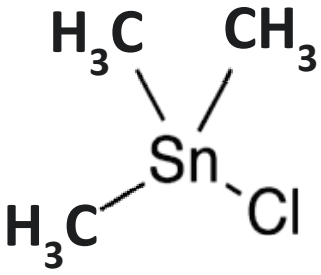
Tributyltin oxide, a colorless to pale yellow liquid used in wood preservation



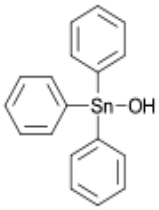
Triphenyltin acetate, an off-white crystalline solid, used as an insecticideand a fungicide



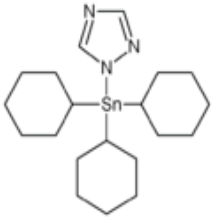
Triphenyltin chloride, a white crystalline solid, used as a biocide and an intermediate in chemical

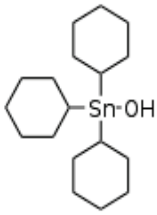


Trimethyltin chloride also a

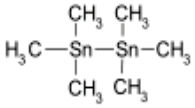


Triphenyltin hydroxide, an off-white powder, used as a fungicide and to sterilize insects

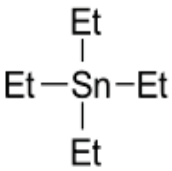




Cyhexatin, a white crystalline solid, used as an acaricide and



Hexamethylditin used as an intermediate in chemical



Tetraethyltin, boiling point 63–65° /12 mm is a catalyst [18]

Figure 1.16. *Common organotin compounds*

Trimethyltin Toxicity

Early studies found that triorganotins were the most biologically active, and that their toxicity correlated with the number of alkyl groups bonded to the tin (Laughlin et al., 1985; Walsh, 1986). The activity also correlated with the increased lipid solubility of alkyltins with alkyl group substitution, enabling greater movement across biological membranes.

TMT can readily cross the blood brain barrier and cell membranes owing to its solubility in both water and lipid. Because of this, and its relatively high volatility, routes of exposure to TMT can be via inhalation, ingestion, or through skin contact. Human exposure to trimethyltin has been associated with occupational activity or ingestion of contaminated food (Kimbrough, 1996; Borghi and Porte, 2002). Indirect exposure to trimethyltin has been proposed to be the result of endogenous methylation following exposure to dimethyltin (Jiang et al., 2000; Yoo et al., 2007; Furuhashi et al., 2008; Tang et al., 2013), though further analytical studies need be performed to confirm *in vivo* methylation. Many of the pathological sequelae found in human cases resemble those in animal models including loss of hippocampal dentate granule cell neurons, as well as neuronal loss in spinal ganglia, hippocampal pyramidal cells, cerebral cortex and Purkinje cell layer of the cerebellum (Besser et al, 1987). Electron microscopy revealed increased frequency of lysosomal dense bodies and dispersion of rough endoplasmic reticulum in neurons (Bouldin et al., 1981; Brown et al., 1979; Nolan et al., 1990; Kreyberg et al., 1992; Reuhl et al., 1985). The long-term prognosis of high exposure based on behavioral sequelae (i.e. hyperkinesia, severe memory deficits and aggressiveness) is poor (Rey et al., 1984). Memory loss by exposed individuals has been observed six months after exposure (Yanofski et al., 1991).

Administration of trimethyltin to experimental animals causes CNS neuronal toxicity primarily in the limbic system (amygdala, entorhinal cortex, hippocampus, olfactory tubercle, and piriform cortex). Various behavioral sequelae develop after exposure is dependent on dose and species. Typically, seizure/tremor activity occurs with loss of hippocampal neurons and long-term cognitive deficits are observed (Table 2.1). The hippocampal formation is particularly susceptible to trimethyltin toxicity resulting in either acute or delayed neurodegeneration depending on the model species (i.e. mouse versus rat, respectively). In mice, the TMT-induced lesion selectively affects the dentate gyrus granule cells which undergo apoptotic-mediated cell death (Fiedorowicz et al, 2001; Morita et al., 2008). Studies have indicated the primary mode of cell death is via apoptosis (as it is in the kainic acid model for neurodegeneration), which distinguishes the trimethyltin model from some other models used to study neurodegeneration such as ischemic or pilocarpine models, in which necrosis is the primary mechanism of cell death. Additionally, both pilocarpine and kainic acid models mediate the toxicity through excitotoxic mechanisms: pilocarpine via muscarinic and NMDA receptor involvement; and ionotropic glutamate receptor activation for kainic acid toxicity (Curia et al., 2008; Zheng et al., 2010). While excitotoxicity from release of endogenous excitotoxins (Sloviter et al., 1986) is a likely component of trimethyltin-induced neuronotoxicity (Koczyk, 1996; Shuto et al., 2009), other cellular mechanisms are involved which have not yet been fully delineated and will be discussed in this chapter.

Human LD50 (estimated) = 3.0 mg/kg (Aldridge et al., 1981)
Rat LD50 (oral) = 12.6 mg/kg (Brown et al., 1979)
Rat LD50 (oral) = 9.2 mg/kg (Reuhl and Cramer, 1984)

Parameter	Phenotypic changes	Period (days) [a]
General behavior	Hyperactivity, convulsion and aggressiveness	1～3
Appetite	Decrease in food and water intakes	～2
Body weight	Decrease (recovered —7 days)	～2
Object memory	Decrease (recovered at 14 days)	4[b]
Histology in the CA1	No significant changes	～30
Histology in the DG	Severe neurodegeneration	～3 (～54)
	Decrease in molecular markers of neurons	～3 (～54)
	Increase in granule cells	4～7
	Increase in genes related to neurogenesis	4～5
Plasma corticosterone	Increase	1～3[b]

a) Period when phenotypic changes were observed. *b*) Time course analysis was not performed.

Table 2.1. Phenotypic changes in mice injected with a high dose of TMT. (From Shintani et al., 2006)

Acute exposure (human)

There is a latent period of 1 to 3 days after exposure, during which symptoms and signs are negligible or absent. With time, adverse effects appear which can include as described by Besser et al., 1987:

- disorientation
- confabulation
- retrograde and anterograde amnesia
- disturbances of sexual behavior, complex partial seizures
- ataxia
- hearing loss
- paresthesis in the legs

Addition effects were described by Kreyberg et al. (1992) including:

- tinnitus or deafness;
- impaired memory
- aggressiveness
- Lightheadedness and episodes of unresponsiveness
- respiratory depression and coma
- mild slowing of sensory nerve conduction without reflex changes or sensory loss

Chronic exposure (human)

Industrial workers involved in organotin production such as in PVC manufacturing industry and chemical production industries are most vulnerable to exposure. Medical histories suggest chronic low-level exposure results in nonspecific symptoms such as headache, insomnia, memory issues, fatigue and avolition. In addition, alternating depressive behavior and aggressive behavior have been observed with a duration of only several hours to days (Ross et al., 1981).

Two human case studies are described below and represent excerpts reproduced directly from IPSCS inchem.org. The first case study was originally described by Feldman et al., 1995; the second case study was originally described by Yanofsky and Nierenberg, 1991:

I. "A 48-year-old woman and her friend drank some wine that was later shown to be contaminated with TMT. Ten minutes after the first mouthfuls they both noticed tinnitus and felt "strange". Her friend only drank one or two mouthfuls but symptoms continued and she was hospitalized because of tinnitus and vertigo a few days later. She suffered from loss of memory for several months but she is now back to work without sequelae. The other subject drank one glass or more and became gradually restless with episodes of unresponsiveness. After three hours she became agitated and started to yell and scream. She also lost control of her bowels. She remained restless and agitated when she was admitted to the hospital the next morning. She presented with hypokalemia and leukocytosis. Her condition deteriorated rapidly with development of metabolic acidosis and liver derangement. She was given sedatives and treated with a respirator until she died five days later."

II. "A 23-year-old male chemistry student presented to the emergency department with burns of the face, hand and chest. He had been working in the laboratory with bis-trimethyl-stannyl acetylene and ether when a flash fire occurred, igniting the substance. He immediately washed under the laboratory shower. The patient did well until 48 hours later when a friend noticed that he was not acting like himself, repeating himself frequently and not able to remember events in the past. Over the course of the next two days he exhibited memory problems and confusion. The patient had a gradual improvement over the next several months."

Mechanisms of TMT cytotoxicity in experimental animals

The neuronotoxicity of trimethyltin primarily affects the limbic system in rodents with hippocampal structures particularly sensitive. TMT intoxication results in the development of seizures, high frequency tremor, behavior alterations including hyperactivity, tail mutilation and aggression, and long-term cognitive defects including memory loss and learning impairment. Interestingly, the sensitivity to trimethyltin intoxication varies with species, strain, age, dose and route of administration. In mice, toxic doses are lower than those of rats with the dentate granule cells being particularly vulnerable, while progressive loss of pyramidal neurons of Ammon's horn subfield CA3 and CA1 occurs in rats. Additionally, rats manifest a delayed and progressive loss of hippocampal pyramidal neurons over the course of several weeks after exposure, as opposed to mice that exhibit rapid cytotoxicity of hippocampal granule neurons (within 24-48 hours) but no effect on the pyramidal cell layer at sublethal concentrations of the toxicant. The rat model for trimethyltin-induced neurodegeneration is seen as a useful model in studies of neurodegenerative diseases including Parkinson and Alzheimer disease where there is progressive loss of neurons over time and long-term neurological/behavioral effects. Trimethyltin has a long half-life in rat tissue and blood where TMT is present in tissues up to 80 days after administration (Tang et al., 2013). Half-life for elimination of TMT in blood was 1.5 days in mice (Ekuta et al., 1998), compared to 12-15 days in rats (Lipscomb, et al., 1989). This difference in effective dose between rats and mice is thought to be associated with the differential affinity that trimethyltin has for hemoglobin. In the case of rats, there is a higher affinity of TMT to hemoglobin compared to mice, which may explain the delayed and prolonged progressive loss of neurons in rats (Hasan, et al., 1984).

The mechanisms underlying the vulnerability of different neuronal subpopulations to trimethyltin between species or within species have not definitively identified. The selectivity of intoxication cannot be ascribed to a preferential distribution *in vivo* to sensitive areas since tin distributes uniformly across brain regions following TMT exposure (Cook et al., 1984). Calretinin and parvalbumin-expressing interneurons are particularly resistant to trimethyltin toxicity (Geloso et al., 1996, 1997, 1998) which may be due to the calcium-buffering activity of these two proteins (intracellular calcium flux and overload is a feature of TMT-induced toxicity are discussed below). Another potential mechanism that may confer selectivity to TMT-induced toxicity has been proposed to involve the protein stannin. Stannin expression, which is localized to membranes of mitochondria and endoplasmic reticulum (Billingsley et al., 2006; Davidson et al., 2004), can bind trimethyltin directly (Buck et al., 2004). Stannin is phylogenetically highly conserved across vertebrates (Dejneka et al., 1997) with unclear function. Inhibition of stannin expression using antisense treatment is protective against trimethyltin-induced cell death (Thompson et al., 1996). One hypothesis is that trimethyltin is demethylated to dimethyltin when binding to stannin, inducing a conformational change that initiates apoptosis, possibly by compromising the mitochondrial membrane function (Figure 1.17). *In vitro* studies have shown that trimethyltin reduces ADP-stimulated mitochondrial oxygen consumption and promotes the release of cytochrome C (Misiti et al., 2008) resulting in activation of apoptosis.

Various pathways have been implicated as causative for the neurotoxicity of TMT, though none are definitively identified as the initiating event for TMT-mediated cell death. Using inhibitors of

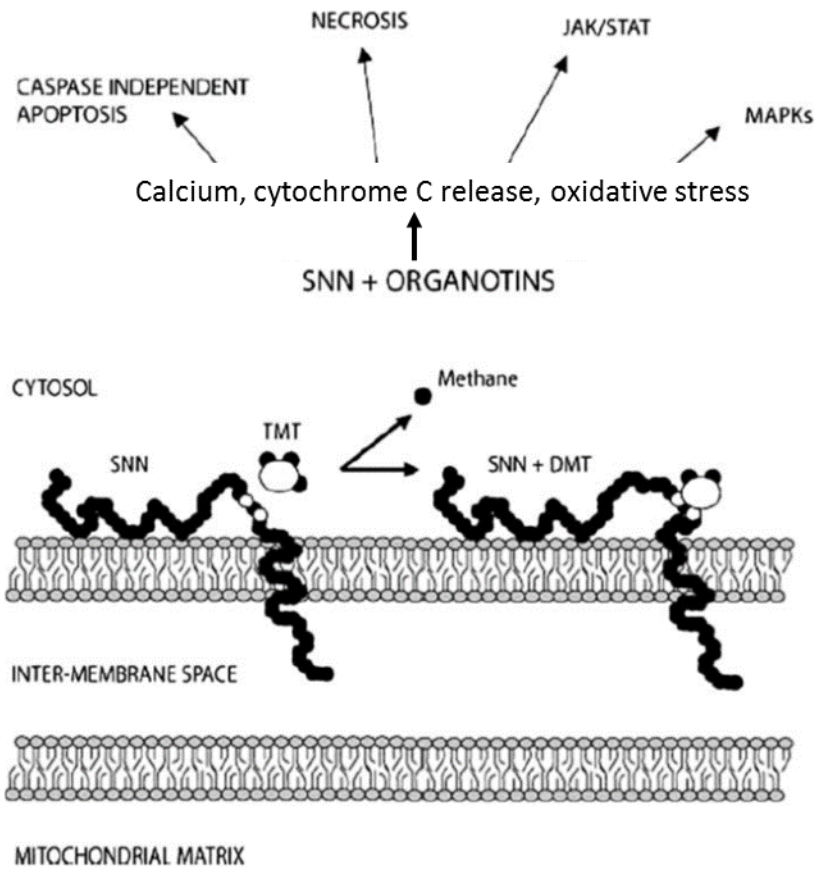


Figure 1.17 A possible role of ogranotin inducing injury by interactivng with mitochondrial membrane is depicted. An organotin (trimethyltin or dimethyltin) associates with stannin (SNN) in the outer mitochondrial membrane and subsequent dealkylation. High affinity binding to stannin may activate pro-apoptotic mechanisms inducing downstream events to mitochondrial damage including release of calcium stores and cytochome C. Increased intracellular calcium can activate proteases such as calpain and cathepsin D that can lead to activation of autophagy or programmed cell death. The production of reactive oxygen species increases oxidative stress and lipid peroxidation that can lead to apoptosis via activation of caspases, release of cytochrome C, and/or direct mitochondrial injury. Release of calcium stores in the endoplasmic reticulum can also occur either through a stannin-mediated mechanism or from release of cytochrome C (Modified from Davidson et al., 2004)

specifically affected pathways independent (e.g. radical scavengers, glutamate receptor antagonists) can independently provide neuroprotection or attenuate cognitive impairment when administered in conjunction with TMT. However, it should be noted that therapeutic strategies that reduce the deleterious cognitive effects of TMT intoxication may not effectively act as

neuroprotective agents, such as the norepinephrine reuptake inhibitor atomoxetine (Tamburella et al., 2012) and the $GABA_A$ activator phenobarbital (Ishikura et al., 2001; Zimmer et al., 1985). This suggests that cognitive dysfunction may be independent of the acute neuronotoxic effect of TMT (Corvino et al., 2013). The progressive 'cognitive-toxicity' subsequent to the neurotoxic effect of TMT (particularly in the rat model) led to the proposed usefulness of this model in neurodegenerative disorders such as Alzheimer's disease (Woodruff and Baisden, 1994).

Calcium overload

Trimethyltin induces dysregulation of calcium homeostasis in cells, which is thought to be an important mechanism in its toxicity (Cristofol et al., 2004; Florea et al., 2005). *In vitro* studies have demonstrated that hippocampal cells exposed to trimethyltin dose-dependently releases calcium from intracellular stores (Piacentini et al., 2008). Additionally, it was shown that in the early phase of trimethyltin intoxication there is an enhanced extracellular calcium influx through L-type calcium channels in endoplasmic reticulum. The induction of apoptotic cell death by TMT-induced calcium overload can be prevented by treating with the calcium chelator BAPTA-AM, indicating that a calcium overload has an important role in cell death following trimethyltin exposure. The mechanisms by which calcium overload initiates apoptosis likely involves activation of calpains and caspases (esp. caspase-12), upregulation of pro-apoptotic genes, and oxidative stress (Chan and Mattson 1999; Arundine and Tymianski 2003; Mattson 2003, 2007; Orrenius et al., 2003; Hernandez-Fonseca and Massieu 2005; Verkhratsky 2005; Chinopoulos and Adam-Vizi 2006; Bano and Nicotera 2007). Additionally, it has been proposed that low concentrations of cytochrome C (released from mitochondria following TMT exposure) can bind to IP3 on the endoplasmic reticulum and promote the release of intracellular calcium stores (Boehning et al., 2003). This

elevated intracellular calcium then triggers massive release of cytochrome C from mitochondria, resulting in the activation of the cell death pathway through caspase and endonuclease activation (Boehning et al., 2003). The selective vulnerability of different neuronal cells within the hippocampus may be attributed to the expression levels of calcium binding proteins such as calretinin, calbindin and parvalbumin that can buffer elevated intracellular calcium levels. As noted earlier, it had been shown that cells expressing parvalbumin and calretinin are resistant to the toxic effects of trimethyltin (Geloso et al., 1996, 1997, 1998).

Gene expression analyses

Gene expression profiling of injured mouse hippocampus following trimethyltin intoxication (reviewed in Lattanzi et al., 2013) shows up-regulation of pathways involved with inflammation, neurodegeneration and neurogenesis, apoptosis, and calcium homeostasis (Figure 1.18). The gene expression profile of hippocampal cells following TMT exposure parallels that of cells undergoing adaptive responses to hypoxic stress, given that many of the genes affected by TMT *in vitro* are target genes for hif-1 (hypoxia-inducible factor-1).

There also appears to be a generalized decrease in transcriptional activity with TMT intoxication, including down-regulation of some genes that are involved with calcium homeostasis such as calbinin-28k, calmodulin and calcineurin. Overall, genetic profiling studies support hypotheses that an alteration in calcium homeostasis, apoptosis, and inflammatory response plays a key role in trimethyltin toxicity in mice. In a genetic study by Lefebvre d'Hellencourt and Harry (2005), TMT-affected hippocampus exhibited a significant increase of gene expression pathways involved with neuronal differentiation and astrocyte activation (NeuroD1 and PEA-15) within six hours after TMT treatment. This effect was limited to dentate granule cell neurons and

not other cells of the hippocampus. The investigators also found a significant increase in the expression these gene categories in the dentate gyrus following TMT exposure:

- inflammatory response (T-cell antigen receptor and CD3 antigen zeta)
- cell adhesion (cadherin 5 and CD14)
- apoptosis (DNA-damage inducible transcript 3, DDIT3)
- neuronal survival (ATF3 and ATF4)
- cell cycle activation (cyclin D2, CDK5, and CDK7)
- migration (MIP1 alpha and beta)
- differentiation (HSP70-5, inhibin beta-A and hairless).

Overall, genetic profiling suggests that there is an early inflammatory phase followed by a stress-induced activation of genes in an attempt to promote survival, activation of cell cycle genes, and pathways involved with neuronal differentiation and glial activation.

In another genetic profiling study of the TMT-injured hippocampus (24hrs post-exposure) there was a significant activation of genes involved in inflammatory and cell death pathways including TNF alpha, NFkappaB, caspase-3/8, and IL-6-related pathways (Funk et al., 2011). Though this study examined the entire brain rather than specifically the hippocampus, they found a significant increase in gene expression associated with cell survival and inhibition of apoptosis (Hras1, Fos, and Stat3). A later TMT study (McPherson et al., 2011) restricted genetic analysis to the hippocampus and compared adult mice to adolescent (postnatal day 21). An age-dependent differential expression pattern occurred in which an upregulation of IL-1-related gene expression occurred 48 hours after trimethyltin exposure in young mice, whereas adult mice exhibited a significant activation of IL-6-mediated pro-inflammatory gene expression. The IL-1 alpha-related signaling in adolescent mice (which involves NFkappaB among other genes) may activate a putative alternative neurogenesis pathway (McPherson et al., 2011; reviewed in Lattanzi et al., 2013).

Genes	TMT (% of control)
TNFα	576 ± 124**
IL-1β	259 ± 84*
IL-6	182 ± 35*
IFNγ	175 ± 61
NOS1 (nNOS)	70 ± 34
NOS2 (iNOS)	221 ± 46*
NOS3 (eNOS)	86 ± 10
STAT3	116 ± 15
FasL	87 ± 25
Pax6	123 ± 36*
COX1	117 ± 29
COX2	94 ± 13

Figure 1.18. Changes in gene expression in the hippocampus at an early step of neurogenesis following granule cell loss induced by TMT treatment. Data (n=4) are semi-quantitative RT-PCR of whole brain from 3 day post-TMT treated versus saline control mice. (From Yoneyama et al., 2011)

In yet another gene expression study comparing trimethyltin-treated wild type mice versus transgenic mice lacking the p50 subunit of NFkappaB (Kassed et al., 2004), the central role of NFkappaB in the genetic response to trimethyltin injury was demonstrated. NFkappaB is one of the most prolific activators of gene expression, playing roles in inflammation cell survival as well as cell death. In the central nervous system, NFkappaB regulates processes involved in plasticity including synaptic activity, learning, memory, survival, and apoptosis (Sarnico et al., 2009; Kaltschmidt et al., 2015). Mice lacking the p50 subunit of NFkappaB exhibited an increase in neurodegeneration after chemical-induced hippocampal injury (Kassed et al., 2002; Yu et al., 1999). In other models of injury, including ischemic and excitotoxic, NFkappaB activity is correlated with increased neuronal death in striatum. Therefore the role of NFkappaB in neurological injuries is dependent on brain regions involved and mechanism of injury. Kassed et al. (2004) induced hippocampal injury using TMT in mice and found differential expression of NFkappaB activated genes associated with neuronal survival, neurite outgrowth, and

synaptogenesis. Expression of NFkappaB appeared to be limited to neurons and was not seen in resting or activated astrocytes before or after injury. Genes playing a role in immune function, as well as neuroprotection and lysosomal enzymes were upregulated with TMT injury, some of the details of which are shown in Figure 1.19 from the study published by Kassed et al. (2004). In transgenic mice where NFkappaB was inactivated, these genes were not upregulated in response to trimethyltin injury. The heat shock proteins (HSP) assist in protein folding and act as chaperones for protein transport; their increased expression is correlated with increased neuronal protection against ischemic assaults and other brain injury models (Song et al., 2001; Truettner et al., 1999, 2002). CD53 increases glutathione levels when expressed by immune cells, hence improving their resistance against oxidative stress-induced apoptosis (Voehringer et al., 2000) and also activates survival pathways (Yunta et al., 2003). Calcium/calmodulin dependent protein kinase II activity induces neurite outgrowth (Caran et al., 2001) and hence may contribute to neurorepair.

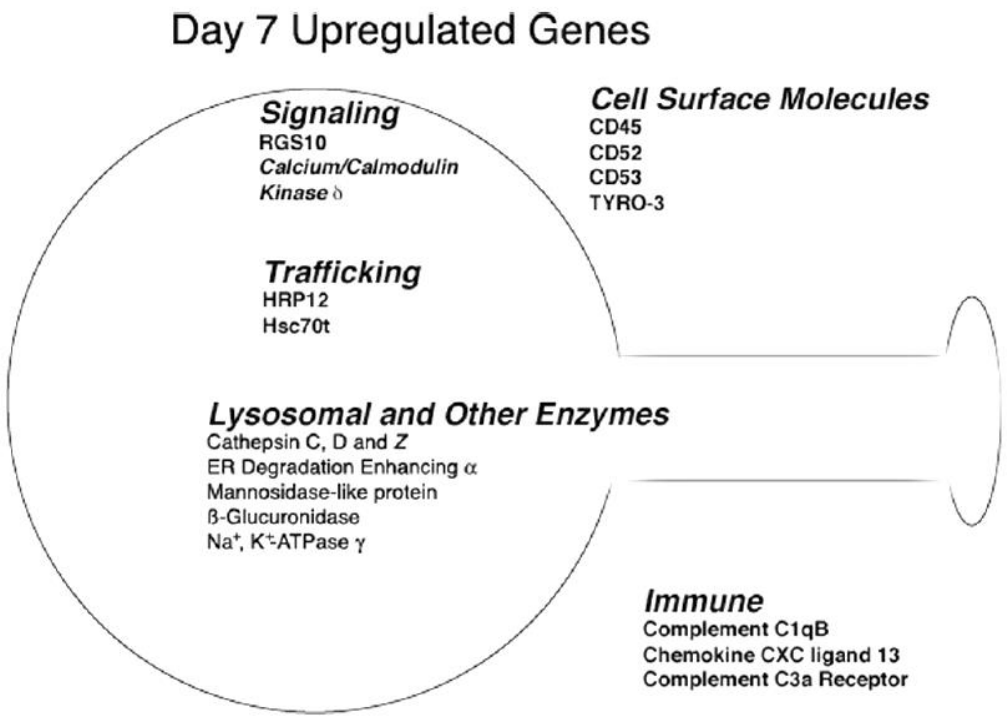


Figure 1.19. *NFkappaB p50-modulated genes upregulated in mice seven days after TMT treatment.* (From Kassed et al., 2004)

The expression of cathepsin D (CatD), a lysosomal aspartyl protease, is upregulated with trimethyltin-induced neurodegeneration (Kassed et al., 2004), yielding elevated protease activity in neuronal and glia in the rat hippocampus where it was determined to play a crucial role in the neurodegeneration process(Ceccariglia et al., 2011). Using the calcium ion chelator BAPTA-AM and inhibitors to CatD and calpain (pepstatin A and calpeptin, respectively), it was shown that TMT-induced neuronal loss was mediated through CatD in a calcium-calpain dependent mechanism (Ceccariglia et al., 2011). Cat D plays a role in a number of metabolic, immune, and cell death pathways. Importantly, it functions to degrade intracellular "waste" products and help prevent deleterious accumulation of excess or nonessential proteins. CatD expression may represent an adaptive response to either cope with intracellular accumulated protein waste products, or, activate programmed cell death through cytochrome c release to eliminate the

dysfunctional cell. Various factors can cause leakage of lysosomal contents including CatD, which can activate apoptosis or apoptosis-like death pathways (Figure 1.20). Rupture of lysosomes and rapid release of CatD also induces rapid necrotic cell death. In an *in vitro* model for Niemann-Pick type C (NPC) disease, (Amritraj et al., 2013) demonstrated that a primary function of upregulated CatD was the initiation neuronal apoptotic death in hippocampal neurons. CatD expression increased in both neurons and microglia. Interestingly, extracellular CatD (released from dying cells or possibly secreted from microglia), was also found to activate cell death in neurons by activating autophagy rather than through a conventional caspase/apoptotic mechanism (Amritraj et al. 2013).

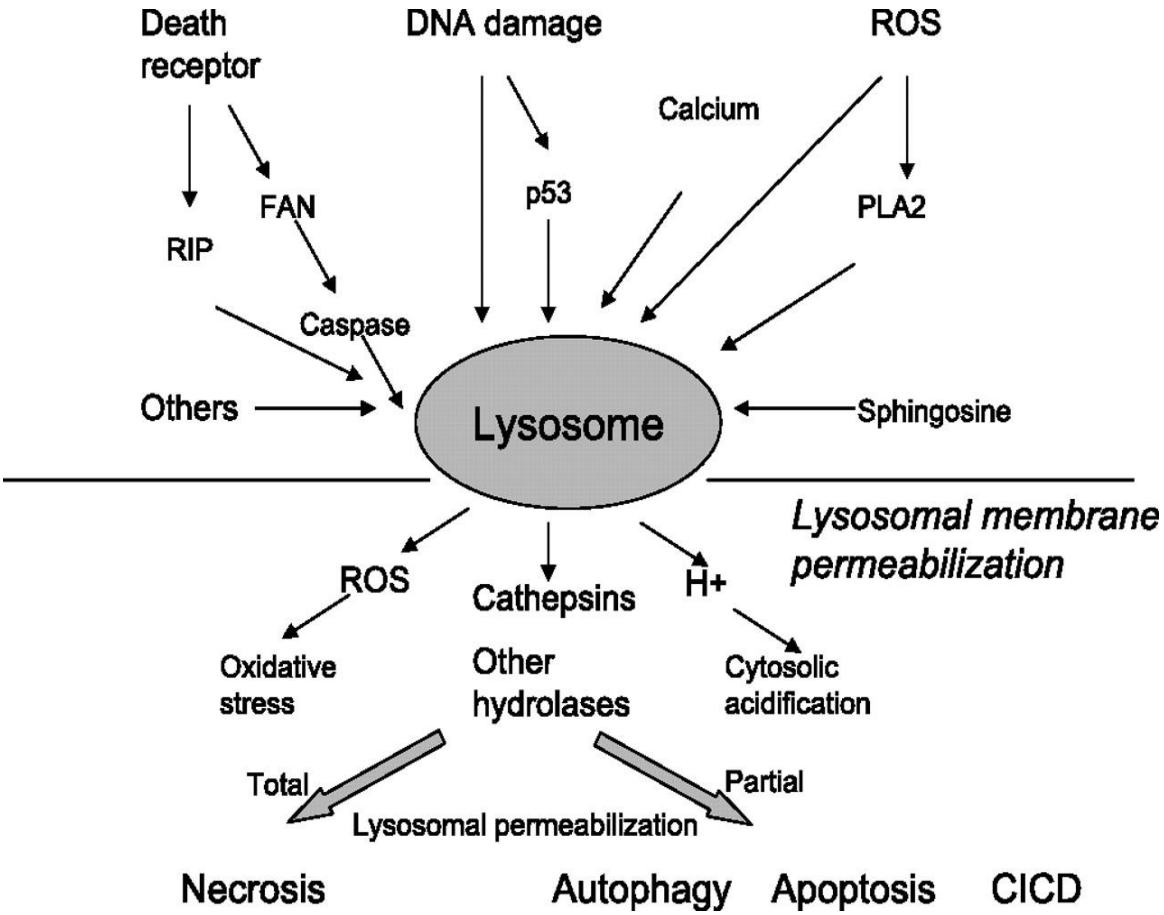


Figure 1.20. *Representative diagram showing factors that can induce lysosomal leakage of components that potentially induce apoptotic mechanisms.* (From Tang et al., 2008)

Inflammatory Mechanisms

Some studies suggest that the neurotoxicity of trimethyltin is primarily mediated through inflammatory processes involving upregulation and release of a number of inflammatory factors. The range of inflammatory processes involved is extensive and involves numerous cytokine mediators, reactive oxygen/nitrogen species, and the involvement of activated microglia and astrocytes at the injury site. Inflammatory responses harbor both positive and negative consequences during the extent of the injury and the recovery. The early phase of the trimethyltin injury induces a strong pro-inflammatory response that serves to remove cellular debris following cell death. Activation of glial cells has positive effects on recovery including secretion of trophic factors, chemokines that may promote neurogenesis and neurite growth, and synaptogenesis during the recovery phase.

Neuroinflammation has been proposed as a major component of trimethyltin-induced neurotoxicity. TMT markedly activates microglia with significant elevation of microglial markers indicative of reactivity (Brabeck et al, 2002; Fiedorowicz et al., 2008 Geloso et al., 2004; Harry et al., 2008; Rohl and Sievers, 2005), and in mice, is localized to site of injury in the hippocampus dentate gyrus. Microglia activated to the M1 pro-inflammatory state produce a number of pro-inflammatory cytokines including IL-1's and IL-6, TNFalpha, as well as nitric oxide and reactive oxygen species (Bruccoleri and Harry, 2000 ; Harry et al, 2008 ; Maier et al., 1997; Ali et al., 1992; Wang et al., 2008; Zhang et al., 2006; Viviani et al., 2001). Morphological and transcriptional changes in microglia indicative of a pro-inflammatory response occur very early after trimethyltin treatment and prior to neuronal damage (d'Hellencourt and Harry, 2005). Hence the early phases

of trimethyltin neurotoxicity could be modulated by the activity these secreted inflammatory factors (Rohl and Sievers, 2005). Co-culturing neurons with microglia potentiates the neurotoxic effect of trimethyltin. Trimethyltin can activate microglia directly (Kim et al., 2015) or indirectly through interaction with astrocytes (Rohl and Sievers, 2005; Rohl et al., 2009) and rapidly stimulate expression of markers indicative of an inflammatory response including CD11b, CD11a, CD11c, CD18 and others. Both *in vitro* and *in vivo* studies have shown that ROS generation dose-dependently accompanies TMT injury, and that antioxidants or upregulation of intracellular glutathione is neuroprotective (Cookson et al., 1998; Shin et al., 2005). Various forms of reactive oxygen and nitrogen species have been detected following TMT treatment which frequently involves activation of nitric oxide synthase (Ali et al., 1992; Wang et al., 2008; Zhang et al., 2006; Viviani et al., 2001). Activation of nitric oxide synthase plays a primary role in TMT neurotoxicity since the NOS inhibitor L-NAME can block the formation of reactive oxygen species and block both necrotic and apoptotic cell death of cerebellar granule neurons (Gunasekar et al., 2001).

It was found that the production of ROS in microglia was dependent on the activity of NADPH oxidase (Kim et al., 2015) (Figure 1.21). Interestingly, inhibition of NADPH oxidase not only prevented ROS production, but also prevented the activation of MAP kinases and NFkappaB, which are upstream effectors that target the production of inflammatory factors in microglia (He et al., 2011; Lull and Block, 2010). Both nitric oxide and TNF alpha production were inhibited when NADPH oxidase was blocked. Furthermore, inhibition of NADPH oxidase reduced the TMT-induced phosphorylation and activation of p38 and JNK which are upstream effectors of NFkappaB and MAPK. Hence, oxidative stress mediated by the activation of NADPH oxidase is an upstream activator of the cascade of kinases and activated transcription factors that induce the production of inflammatory mediators. It has been demonstrated that protein kinase C plays a role in the activation of NADPH oxidase (Raad et al., 2009). Since increased intracellular calcium

is an early indicator of trimethyltin toxicity, the activation of PKC in this environment is likely involved in promoting oxidative stress by phosphorylating NADPH oxidase to an active state, which in turn induces the inflammatory response. It should be noted that NFkappaB is also activated in astrocytes following trimethyltin exposure (Reali et al., 2005).

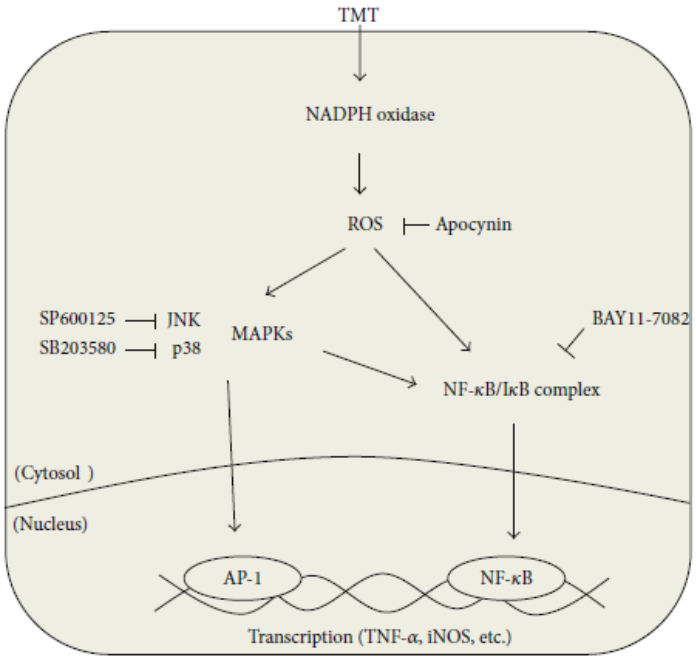


Figure 1.21. Effect of TMT on oxidative stress and cell signaling in the macrophage cell line BV2 cells. Exposure cells to TMT resulted in an increase in reactive oxygen species primarily to the activation of NADPH oxidase as well as secretion of inflammatory factors TNFalpha and iNOS. Activation of p38 and JNK map kinases occurred upstream of activation of NFkappaB and up regulation of NfkappaB-mediated inflammatory genes TNFalpha and iNOS. Inhibition of NADPH oxidase using apocynin blocked all downstream TMT-induced kinase activation and gene transcription. Inhibition of NFkappaB using Bay11-7082 did not prevent map kinase activation with TMT exposure, whereas inhibition of MAPKs prevented NfkappaB activation, indicating a TMT>MAPKs>NfkappaB signaling pathway. (From Kim et al., 2015)

Other studies have suggested that activation of the NMDA glutamate receptors is a primary mechanism behind TMT neurotoxicity and generation of oxidative stress (Koczyk, 1996; Patel et

al., 1990); however, inhibition of this receptor using MK-801 fails to block apoptosis or the formation of oxidative species at lower TMT concentrations (Gunasekar et al., 2001). This indicated that glutamate receptors are likely not involved in trimethyltin-induced apoptosis. Furthermore, a genetic profile study by Harry et al. (2008) showed that genes differentially expressed during hypoxia/ischemia and glutamate activation were not altered in the TMT model of hippocampal damage. In contrast, where higher concentrations of trimethyltin are used, necrotic cell death could be ameliorated by inhibition of both NMDA and non-NMDA glutamate receptors (MK-801 and MCPG, respectively).

As mentioned previously, TMT-induced oxidative stress may be dependent on the activation of protein kinase C (PKC). Blockade of PKC activity using the inhibitor chelerythrine prevents the formation of reactive oxygen species following trimethyltin treatment. PKC positively regulates nitric oxide synthase activity via phosphorylation (Bredt and Snyder, 1992). Hence the oxidative stress generated by trimethyltin is at least in part the result of PKC-mediated activation of NOS in neurons (Gunasekar, 2001). Catalase pretreatment is also neuroprotective for TMT-induced apoptosis, but does not protect against necrosis at higher TMT concentrations. In sum, oxidative stress is present during trimethyltin-induced apoptotic and necrotic neuronal cell death, and involves a PKC-mediated activation of nitric oxide synthase. However, apoptosis at low concentrations of TMT is independent of activation of glutamate receptors, whereas necrosis at higher TMT concentrations is dependent on glutamate receptor activation (both NMDA and metabotropic glutamate receptors). PKC, in addition to phosphorylating and activating nitric oxide synthase, also has a role in a wide range of cellular functions including mitotic activity and migration, secretion, apoptosis, contraction, hypertrophy, and apoptosis (Igumenova, 2015). The role that PKC plays varies depending on the PKC isoform profile in the cell, the cell or tissue type, and the context of the stimulus. The activation of PKC is initiated by the activation of

phospholipase C (which itself is activated by elevated calcium concentrations in the cell) to generate diacylglycerol from catalysis of cell membrane phospholipids. The source for elevated intracellular calcium can be both from intracellular stores (mitochondrial and/or endoplasmic reticulum release) and extracellular (Thore et al., 2005). Phospholipase C-dependent activation of PKC also includes via activation of receptors to growth factors and hormones. Binding of diacylglycerol to PKC significantly increases its affinity for calcium and recruitment to the cell membrane. PKC plays a role in modulating both extrinsic (receptor-mediated) and intrinsic (drugs, chemicals, radiation) pathways of apoptotic cell death and can be either anti- or pro-apoptotic depending on the cell type and the initiating signal. Furthermore, specific responses to stimuli may be mediated by activation (i.e. phosphorylation) of specific PKC isoforms. It is becoming clear that the isoform profile is particularly important in whether PKC activation is pro-apoptotic or pro-survival. As described above in the case of trimethyltin-induced neurotoxicity, PKC activity plays an important role as a pro-apoptotic mediator of cell death.

Role of astrocytes and microglia in Trimethyltin toxicity

Astrocytes and microglial are reactive cells in CNS injury, and their morphological and molecular profiles are hallmarks of diverse forms of neuroinjury. Both cells types express a variety of pro- and anti-inflammatory mediators, as well as chemokines and neurotrophins (Fig. 2.8). The mechanism of trimethyltin intoxication is unclear; partially the result of often contradictory observations between *in vitro* studies and their relevance to *in vivo* effects of TMT. Trimethyltin intoxication elicits a strong astrocyte response *in vivo*, characterized by increased density of astrocytic cells, hypertrophy of astrocytic processes and increased expression of GFAP, transient expression of vimentin and nestin (Andersson et al., 1994; Geloso et al., 2004), secretion of TNF

alpha, expression of PARs (Pompili et al., (2004) (esp. PAR-1), P2X2R purinergic receptor (Latini et al., 2010) as well as trophic genes including NGF and TrkA (Koczyk and Oderfeld-Nowak, 2000). Astrocytes have been implicated in both mediating trimethyltin toxicity as well as preventing trimethyltin-induced neuronal death by modulating the level of oxidative stress in the neuronal population (Gunasekar et al., 2001). The TMT-induced astrocytic reactivity in mice occurs during the cell death of granule cells, and they remain reactive at least 7 to 10 days after TMT-induced hippocampal damage. However, in one vitro study, it was suggested that astrocytes mediate the activation of microglia following TMT exposure (Rohl et al., 2005). Astrocytes in homogeneous cell culture were relatively resistant to TMT toxicity whereas microglial cells were much more sensitive to TMT. TMT was found to induce morphological changes in both microglia and astrocytes in culture, but only when the cells were in co-culture did the two cell types become activated, with increased expression of nitic oxide (Rohl et al., 2005), a marker for microglial activation. It was proposed that astrocytes were responsible for modulating the activity of microglia following TMT exposure. In contrast, another study showed that TMT added to pure microglial cultures induced the production of TNFalpha without changes in morphology (Eskes et al., 2003), while co-culturing with astrocytes resulted in reduced TNFalpha production. Astrocytes attenuated microglial inflammatory response to TMT in this *in vitro* system and hence could act to reduce TMT toxicity. Kim et al. (2014) showed that astrocyte primary cultures treated with TMT had upregulated expression of iNOS, arginase-1, IL-1β, TNF-α, and IL-6. They also found that iNOS, IL-1β, TNF-α, and IL-6 expression was significantly increased in BV2 microglial cultures following TMT treatment. When neuronal cells were co-cultured with microglia, there was an increase in microglial reactivity and TNFalpha production, which was proposed to increase the neuronotoxicity of TMT (Eskes et al., 2003) While it has been proposed that glia mediate the toxic effects of TMT, some studies suggest astrocytes exert a neuroprotective effect by reducing the

oxidative stress induced by TMT (Gunsekar et al., 2001) and reduce secretion of TNFalpha by microglia.

Nestin is an intermediate filament used as a marker for the identification of neural stem cells and stem cells of other tissues. Recent research has shown that brain injury (including ischemic and traumatic) can induce the expression of nestin in as subpopulation of astrocytes (Geloso, et al., 2004). In the adult brain, nestin is only expressed in stem cells localized to the two neurogenic regions: the subventricular zone and the subgranular zone. The expression of nestin does not always correlate with the upregulated expression of GFAP in reactive astrocytes. It has been postulated that astrocytes revert to a more primitive form in response to injury, re-expressing genes that are normally down regulated in the adult, which includes the expression of both nestin and neural stem cell transcription factor Sox-2 (Geloso, et al., 2004). The expression of these genes in astrocytes has been proposed to play a role in the structural remodeling of these cells in response to injury (Cho et al., 2013).

Overall, the role of astrocytes and microglia in TMT toxicity is unclear. Both inflammatory and anti-inflammatory factors are expressed by these cells (Zhang and Zhu, 2011) following TMT exposure and in vitro data is equivocal on the neuroinflammatory and neuroprotective effects. Variability in culture conditions, cell types, concentrations of TMT, and other factor likely contribute to the diversity in results. Further studies and perhaps better in vitro models are needed to evaluate TMT mechanisms of toxicity that are relevant to in vivo effects of trimethyltin intoxication.

Although the mechanism of TMT-induced granule cell death in murine hippocampus is not clearly identified, the TMT model is a validated and reproducible model for studying responses to hippocampal injury. For these reasons, as well as the desirable cytotoxic effects and subsequent

neural repair that occurs in the hippocampus following TMT intoxication, this model was used to test the hypothesis posed in this thesis.

Re-Statement of Hypothesis

The preceding Introduction provided an overview of the structure and function of the hippocampus. Additionally, an overview of neural stem cell biology in the adult mammalian brain in context with physiological and pathological conditions was discussed. This is to provide a better understanding of the hypothesis and the studies described in the following sections of this thesis. The detailed description of the neurotoxicant trimethyltin including historical profile and proposed mechanisms of toxicity should provide the reader with a background understanding of the rationale for using this compound to induce hippocampal injury for studying neural stem cell responses.

We hypothesis that **recovery from the TMT-induced hippocampal injury involves both ventricular and hippocampal stem/progenitor cell migration and proliferation. Further, we hypothesize that brain injury may 'recruit' newly proliferated neural precursor cells to participate in neural recovery.**

To address these hypotheses, the following specific questions were addressed:

I. Do hippocampal stem cells proliferate and differentiate in response to injury?

II. Do cells derived from the ependymal/subventricular zone migrate to the injured hippocampus and contribute to regeneration?

III. What is the fate of new cells that repopulate the hippocampus as part of a repair response?

IV. Does inhibition of neurogenesis effect recovery from trimethyltin-induced injury?

CHAPTER 2: RESPONSES OF STEM CELLS TO TMT INJURY IN C3H MICE

Abstract

The mechanisms regulating adult neurogenesis and the role of new neurons in the adults are currently intense areas of research. One role of neurogenesis in adults may be to compensate for cell loss due to injury or aging. In this study, the response of stem cells and their progeny to injury was studied in two-month old male C3H mice treated with the limbic system neurotoxicant trimethyltin (TMT). TMT exhibits a steep dose-response relationship between minimal behavioral effect (i.e. tremors) and morbidity. Neural stem cell response to injury can vary depending on multiple factors including the toxicant dose-response, mouse strain, or other factors, thus making it necessary to characterize the effect in the TMT model. Pyknotic and caspase-3^+ cells were visible at 2.0 mg/kg TMT and drastically increased at doses above 2.6 mg/kg. A significant inflammatory reaction, including microglial activation and astrocytosis, occurred within 48 hours of exposure. Microglial response detected using the microglial marker cd11b was rapid and peaked at 5 days post-TMT exposure, then declined at 8 days to near normal levels at 28 days. Astrocyte reactivity was induced within 48 hrs of TMT exposure, but remained significantly elevated out to 28 days post-TMT injection. TMT stimulated mitotic activity in the hippocampus dentate gyrus dose-dependently, but there was a reduction in $Ki67^+$ cells at concentrations above 2.6 mg/kg, likely due to toxicity and death of dentate neurons. Activation of neuronal mitosis occurred within 48 hrs post-TMT injection and began to decline at 8 days. The number of $TBR2^+$

neural precursor cells increased at 3-5 days post-TMT, and then returned to control levels after 28 days. Bromodeoxyuridine (BrdU) labeling showed a similar temporal profile as the Ki67 marker, revealing a significant number of BrdU-labeled cells in the dentate gyrus granular and subgranular zones, and in the molecular layer of the hippocampus. $BrdU^+$ cells exhibited NeuN colocalization at 5 days post-TMT indicating differentiation toward a neuronal phenotype. Ki67 labeling in the lateral ventricle subventricular zone (SVZ) was unchanged with TMT dose indicating a lack of dose-response effect on SVZ proliferative activity.

Introduction

Prenatal morphogenesis of the rodent brain involves the production of neuronal and microglial cells arising from two germinal centers: the periventricular zone, and a secondary neurogenic niche in the hippocampal dentate gyrus. Gradients of morphogens, neurotrophins, differential patterns of receptor expression, and mediators of migration orchestrate the fate of new cells arising from these germinal centers to sculpt the developing brain structure organization. Stem cells residing in these germinal centers initially give rise to migrating precursors of neurons and specialized radial glia, followed by astrocytes and oligodendrocytes that function to support the developing neural network (Malatesta et al., 2000; Noctor et al., 2001; Tramontin et al., 2003; Ventura and Goldman, 2007). As prenatal development nears its end, the number and proliferative activity of the stem and progenitor cells diminish. Reduction of the mouse ventricular germinal centers ends at embryonic day 20 with differentiation of some stem cells into post-mitotic epithelial ependymal cells lining the lateral ventricles, and others localized to the subependymal region as subventricular zone (SVZ) astrocytes. In the mouse, cell

proliferation in the hippocampus continues through the first postnatal week, after which developmental neurogenesis is essentially complete.

In the adult mouse, neurogenesis persists in the developmental germinal centers to produce new cells to sustain normal brain function. The SVZ retains a population of ventricular stem cells that continuously generates neuroblasts which migrate anteriorly to the olfactory bulb to replenish glomerular and granule cell layer interneurons. Numerous reports have shown that neurogenesis also occurs in the granule cell layer of the hippocampus dentate gyrus (Altman and Das, 1965; Cameron et al., 1993; Kuhn et al., 1996). Newly generated cells migrate from the subgranular stem cell niche and integrate into the granule cell layer where they exhibit characteristics of mature granule cell neurons (reviewed by Kempermann et al, 2015). The importance of hippocampal neurogenesis in learning and memory is suggested by the severe cognitive deficits associated with progenitor cell dysfunction following cranial X-irradiation (Monje, 2002; Saxe et al., 2006). Other experimental models in which neural stem cell activity in the SGZ of the dentate gyrus is inhibited or enhanced by genetic, hormonal, or chemical manipulation have provided further evidence of the importance of new neuron formation for neurological/cognitive function (Malberg and Duman, 2003; Levone et al., 2015; Tiwari et al., 2015; Hornsby et al., 2016; Trivino-Paredes et al., 2016) . Various factors have been shown to induce neurogenesis in the dentate gyrus, including growth and neurotrophic factors, dietary restriction, a complex learning environment, exercise, epileptic seizures, and injury by mechanical, ischemic, or chemical insult (reviewed by Aimone et al., 2014). Many of these effects are mediated by downstream factors such as stress hormones (glucocorticoids) that can be either detrimental or beneficial to neural stem cell activity (reviewed by Koutmani, and Karalis, 2015). However, the functional significance of new neurons produced in response to injury or pathological conditions is incompletely understood, especially since neurogenic response varies

depending on type of injury and brain region affected. This can further by complicated by other factors including model species, age, gender, and experimental conditions including, genetic or surgical modification and extraneous addition of trophic factors (Grandel and Brand, 2013; Gould, 2007; Couillard-Després, 2013).

Neural stem cells in non-mammalian vertebrates have the capacity to respond to brain injury by repopulating lesioned areas to regain function. This process of neuro-regeneration requires proliferation of precursor cells, migration, and appropriate differentiation at the injury site for integration. In contrast to the established "post-mitotic brain" concept of the past, current research demonstrates that endogenous stem cells in mammals also have some innate capacity to respond to injury. It was demonstrated in rats that SVZ precursor cells are not necessarily committed to rostral migration to the olfactory bulb, but can be redirected by ischemic injury to the striatum and the CA1 pyramidal subfield of the hippocampus (Nakatomi, 2002; Jin, 2003). Chapter 3 of this thesis will describe a study testing the hypothesis that SVZ stem cells can contribute to hippocampal injury repair.

Chemical injury by trimethyltin generally targets limbic structures of the central nervous system, with the most significant effect of dentate granule cell degeneration in the murine hippocampus. As described earlier, the hippocampus dentate gyrus subgranular zone retains the potential to generate new granule cell neurons. Under physiological conditions turnover of granule cells is slow, but can be accelerated under certain conditions including injury. Physiological production of new granule cells is believed to contribute to certain memory functions including spatial memory and contextual discrimination. In the case of contextual discrimination, the function of the hippocampus in pattern separation is facilitated by newly generated granule cell neurons (Aimone et al.,2014). These cells create an inhibitory feedback mechanism that dampens global remapping within the hippocampal network (McAvoy et al.,

2015), and in so doing limits the number of granule cells that respond to a particular episode. This 'sparseness' of neural response is the basis for allowing discrimination between similar events.

The activity of stem cells and progenitor cells within the SGZ appear to respond to a broader range of stimuli than cells residing in the subventricular zone neurogenic region. For instance, hippocampal stem cells are more responsive to glucocorticoids than are subventricular zone stem cells. Certain activities such as exercise, enriched learning environment, and learning/memory tasks can activate neurogenesis in the hippocampus, but have little impact on subventricular zone neurogenesis. Injury can activate neurogenesis in either the subgranular zone or subventricular zone depending on the nature of the insult. It is postulated that neurogenesis is also part of a repair mechanism that functions to replace neurons lost to damage, though the long-term functional significance is unclear. Neurogenesis in the hippocampus is affected by many factors including stress and exercise that activate the secretion of secondary messengers such as hormones or other factors that directly modulate stem cell activity. The precise factors that activate hippocampal neurogenesis in response to injury are not defined. Stimulating factors released from degenerating neurons or secreted from other cells that respond to the injury, such as microglia or astrocytes, may contribute. Additionally, inflammatory factors released from parenchymal cells or blood vessels may contribute to subgranular zone stem cell activity. Interestingly, blood vessels play an important role in the neurogenic niche and are known to be involved in not only acting as a scaffold for neural precursor cell migration, but also produce factors that regulate both stem cell proliferation and precursor cell migration and differentiation.

In this study, the time course and dose-response effects of TMT on neurogenesis in the hippocampus was studied. Labeling of dividing nuclei with BrdU was used to both quantify proliferation and lineage fate of SGZ and SVZ precursors..

Materials and Methods

Animals and TMT exposure

Male C3H mice aged 1 ½-2 months (Taconic, NY) were housed in polycarbonate cages with wood chip bedding and maintained according to AALAC guidelines. Trimethyltin chloride (TMT, Sigma, St. Louis, MO) was dissolved in normal saline at a stock concentration of 2.5 mg/ml. For the time-course study, a single dosage of TMT was administered (2.8 mg/kg; ip), from a working solution of TMT (0.25 mg/mL) prepared in sterile saline which would induce tremor activity within 24-36 hours and recovery within 48-72 hours. For the dose-response study, a series of working solutions were prepared from the same TMT stock solution so that similar volumes were injected across the different dosage groups (1.5-3.2 mg/kg). Treatment groups of 5-8 mice were used for generating saline- and TMT-injected brain samples allowing for treatment mortality and statistical evaluation. The initiation and cessation of tremor was monitored daily for up to 4 days after treatment.

BrdU labeling

Bromodeoxyuridine (Sigma) was prepared from solid crystalline stock by dissolving to a stock concentration of 9 mg/mL in saline. The solution was sterilized through a 0.2 μm syringe filter. Mice were injected intraperitoneally with the bromodeoxyuridine solution on the same day as trimethyltin injection at a concentration of 50mg/mL. Subsequent daily injections of BrdU were performed for three days. The mice were euthanized by CO2 asphyxiation at various time points after trimethyltin injection. Brains were removed and immediately frozen without perfusion and stored at -70 ^{o}C to retain quality for subsequent immunohistological analysis.

Tissue sectioning and immunohistochemistry

Twelve-micrometer cryostat sagittal sections of the left hemisphere of the brain presenting hippocampus (0.6-1.3 mm lateral to bregma) were prepared from frozen brain tissue, mounted on slides and post-fixed by immersion in 4% para-formaldehyde at room temperature for 15 minutes. H & E staining was performed using standard methods. For antibody staining, antigen retrieval was performed by heating slides in 10 mM sodium citrate (pH 5.5) to boiling for 10 minutes, then cooling to room temperature before mounting onto Shandon coverplates (Thermo Scientific, MA). Samples were incubated overnight at 4^{o}C with primary antibodies prepared in blocking buffer containing 3% donkey serum and 0.3% Tween-20. Primary antibodies used were sheep anti-BrdU (5ug/ml; Research Diagnostics, Inc.), chicken anti-GFAP (1:300; Aves, Inc), mouse anti-NeuN (1:400; Millipore), rat anti-CD11b (1:300; BD Pharmingen), rabbit anti-active caspase-3 (1:300; R&D Systems, 4Inc.) and rabbit anti-collagen IV (1:200; Pierce). Primary antibody incubation was carried out by overnight incubation at 4°C. NeuN primary antibody staining was performed overnight at 4^{o}C after the antigen retrieval step used for BrdU staining. For GFAP and BrdU co-staining, slides were microwaved for 15 minutes in 10 mM sodium citrate (pH 5), cooled to room temperature, and then incubated with BrdU and GFAP primary antibody overnight at 4^{o}C. Slides were then incubated for 45 minutes at room temperature with Alexa Fluor-conjugated secondary antibodies (1:300; Invitrogen, CA) prepared in blocking buffer.

Imaging and cell quantification

Tissue sections were visualized by epifluorescence using a Zeiss Imager A.1 microscope equipped with a Zeiss MRc camera and Axiovision image capture software. Sagittal and coronal

sections of matched anatomical levels along the rostro-caudal axis were used for analysis. Quantification was performed on 8-10 sections/animal with an intersection distance of 90 micrometers. Image analysis was performed on HALO software by IndicaLabs. Algorithms for quantification included branch structure (collagen IV for blood vessels and $GFAP^{+}$ astrocytic processes), area quantification (for GFAP, CD11b, nestin staining), and cytonuclear (for Ki67, caspase, NeuN, BrdU staining). Where appropriate, co-localization was determined using these algorithms. Data were analyzed using Graphpad Prism 6 (Graphpad software version 6.05). All data are expressed as standard error of the mean (SEM). Data were considered statistically significant when $P < 0.05$. Data were compared using two-way ANOVA. If a significant difference was detected, post-hoc analyses were performed using Tukey multiple comparison tests or Dunnetts test.

Results

Trimethyltin was administered in a dose-response study in 3 month old mice to assess effects on granule cell neuron morphology in the hippocampus dentate gyrus. TMT induced tremoring activity of mice 36 hours after exposure to concentrations above 2.6 mg/kg. Mice dosed with the highest concentration of trimethyltin exhibited severe tremoring. All mice were euthanized 48 hours after TMT exposure for tissue harvesting. Hematoxylin and eosin staining tissue sections of the hippocampus of mice treated with1, 1.5, 2.0, 2,.3, 2.6, 2.9, and 3.2 mg/kg trimethyltin showed a dose-dependent increase in pyknotic nuclei starting at 2.0 mg/kg (Figure 1). Large numbers of granule cell pyknotic nuclei were visible in the dentate gyrus granule cell layer of mice treated with 2.9 and 3.2 mg/kg. The CA1-CA3 subfields and molecular layer of the hippocampus appeared to be spared of the effect of TMT based on absence of pyknotic nuclei.

To quantitate cell toxicity based on cell death as well as assess the mode of cell death, tissue sections were stained for active caspase-3, an indicator of caspase-3-mediated programmed cell death (Figure 2). Using primary antibody against active caspase-3, no positive cells were detected in hippocampus of control animals or animals treated with up to 1.5 mg/kg TMT. Sparse positive cells were found in 2.0 mg/kg TMT-treated animals. However, there was a marked increase in active caspase-3 staining in mice treated with 2.3 mg/kg TMT, with a clear dose-dependent increase in numbers of positive cells with the higher concentrations of TMT administered. Quantification of active caspase-3 fluorescence using a Cytofluor algorithm (IndicaLabs) (Figure. 3) showed essentially a linear dose-dependent relationship between 2.3-3.2 mg/kg TMT. The results for the H&E staining and expression of active caspase-3 were in agreement in that there was a distinct increase in cell death starting at the 2.3 mg/kg dosage of TMT. Fluorojade C staining (Figure 4) further demonstrated the TMT effect by staining degenerating neurons in the hippocampus dentate gyrus of TMT-treated animals.

GFAP reactivity was significantly increased with TMT-mediated injury at 48 hrs post-dose (Figure 5), indicating a rapid early astrocytic response at a high dose (3.2mg/kg) of TMT. This astrocytic response was limited to the hippocampus and was not expressed globally in other regions of the brain. Quantification of the astrocytic response was carried out by using an image analysis algorithm for area quantification and a branching algorithm for analyzing $GFAP^+$ astrocyte branching processes (Figures 6 and 7). There was a dose-dependent increase in GFAP reactivity beginning at the 2.3 mg/kg dosage. The dose-response for the astrocyte reactivity was generally weak and limited to high concentrations of TMT likely due to the early time point (48 hrs) at which these samples were taken.

There was a qualitative increase in nestin expression correlated with TMT dosage and astrocytic response. Immunofluorescent labeling using anti-GFAP and anti-nestin antibodies

revealed a significant colocalization of these two markers in TMT-treated mice. (Figure 8). Nestin-expressing astrocytes were confined to hippocampal granule cell layer. $GFAP^+$ astrocytes in other regions of the hippocampus yielded no detectable nestin expression. Essentially all $GFAP^+$ astrocytes appeared to co-express nestin in the dentate gyrus of TMT-treated mice. In control mice, only a small proportion of the $GFAP^+$ cells co-labeled with nestin, which were representative of type 1 stem neural stem cells. The upregulation of nestin expression in a broader pool of $GFAP^+$ astrocytic cells confounded the ability to specifically quantify the neural stem cell population since they share expression of these markers.

TMT induced a significant increase in the number proliferating cells expressing the cell cycling marker Ki-67. $Ki67^+$ cells in the hippocampus were generally restricted to the subgranular zone of the dentate gyrus, with some labeling found within the hilus and sporadic labeling within the granule cell layer and molecular layer (Figure 10). In the trimethyltin dose-response (Figure 9), there is a steep elevation in the number of $Ki\text{-}67^+$ cells in the hippocampus dentate gyrus starting at 2.3 mg/kg. The increase peaked at 2.6mg/kg and then began to decline at higher TMT concentrations. There was approximately a five-fold increase in frequency of proliferating cells in the hippocampus between untreated animals and animals treated with 2.6mg/kg TMT.

To determine the time of onset of toxicity of trimethyltin and its effects, a time-course study was performed in which animals were dosed with TMT and BrdU and brains harvested at different time points. Based on the extent of granule cell injury determined in the dose-finding study, a concentration of TMT of 2.8mg/kg was utilized for the time course study. Pyknotic nuclei were clearly identified in H&E stained tissue sections representative of each time point (Figure 11). The earliest time point, 48 hours, clearly exhibited numerous pyknotic nuclei in the granule cell layer of the dentate gyrus. Pyknotic nuclei were distributed throughout the granule cell layer in both the supra– and infrapyramidal blades. The pyramidal cell layer and the molecular layer of the

hippocampus appeared unaffected. The greatest density of pyknotic nuclei was seen at three and five days post-TMT. By eight days, the number of pyknotic/apoptotic neurons was markedly diminished, and none were visible at 28 days post-TMT. Quantification of cells expressing cleaved caspase-3 in the hippocampus showed a clear time-dependent effect of TMT (Figures 12 and 13). While there were virtually no caspase-3 positive cells in control brain at 48 hrs, there was a significant increase of approximately 40 fold in TMT-treated mice. At 72 hours post-TMT treatment, there was nearly a 200-fold increase in the number of caspase-3^+ cells in the hippocampus compared to control. At five days post-TMT and later, there was a decline in the cell death marker. The number of caspase-3^+ cells had returned to control levels at 28 days. When present, caspase-3^+ cells were restricted to the granule cell layer of the dentate gyrus at all time points (Figures 12 and 13).

To determine the time-course for an inflammatory response to trimethyltin injury, samples were stained for microglia (Cd11b) and quantified for reactive astrocytes (GFAP). Consistent with induction of injury, there was a strong microglial response to TMT in a time-dependent fashion (Figures 14 and 15). The microglial response occurs within 24 hours of TMT exposure and was clearly and significantly observed by the 48 hour time point as quantitated by area quantification of CD11b immunoreactivity within the hippocampus region of interest. The microglial response peaked at five days after TMT exposure with the nearly 6-fold increase in CD11b immunoreactivity. CD11b signal declined significantly by eight days and 28 days after TMT injection. There was still a slightly, but not statistically significant, elevation of microglial activity in the 28 day samples. Microglial immunoreactivity was strongest within the granule cell layer of the dentate gyrus, but there was also increased immunoreactivity within the hilus and the molecular layer between the suprapyramidal blade of the dentate gyrus and the overlying CA1-CA 2 subfields of the pyramidal cell layer.

Astrocyte reactivity to TMT injury was assessed by multiple measures of quantification. Because of inaccuracy in localizing and counting individual cells expressing the cytoplasmic marker GFAP, area of the overall GFAP immunoreactivity relative to the area of the region of interest was quantified, and the percentage of GFAP expression normalized for tissue area was determined for each time point. It was clear that there was a significant increase of GFAP expression at 5, 8, and 28 days after trimethyltin injection (Figures 15 and 16). This was unlike the microglial response, which did not persist for a long period time and was not significant at the 28 day post-TMT time point. Since astrocyte activation includes an increase in process branching patterns of these cells, further analysis of the astrocytic response was performed utilizing a branch structure algorithm (IndicaLabs). Four parameters of the branching processes were measured: the number of branch points, total branch area, total branch thickness, and branch length. The number of branch points and total branch area were normalized for the total area measured on the tissue section. As shown in Figure 17A and B, the number of branch points and total branch area of GFAP positive astrocytes has significantly increased from five days to 28 days post-TMT. Total branch area at the three day time point had also reached statistical significance. The thickness of the branches was not significantly different across all samples (Figure 17C), however branch length had significantly increased at the 5 day through 28 day time points compared to control (Figure 17D).

The time course of activation of mitosis in the hippocampus following trimethyltin injury was determined by Ki-67 staining and detection of bromodeoxyuridine incorporation into cycling cells (Figure 18A, B). The time-dependency profile for the number of Ki-67$^+$ and BrdU $^+$ cells present in the hippocampus was similar. For both markers, at 48 hours post-TMT there was a 2.5- to 3-fold increase in Ki-67$^+$ cells in the hippocampus dentate gyrus. The number of Ki-67$^+$ cells peaked at three days post-TMT (6.5-fold) and then declined afterwards, whereas the number of BrdU$^+$ cells peaked at five days (14.6-fold) before declining. Interestingly, the number of BrdU$^+$ cells declined

rapidly indicating a loss of these cells since BrdU is incorporated into the DNA and not down regulated as could be a protein antigen.

There was a rapid onset of accelerated proliferation following TMT injury based on both Ki-67 and BrdU labeling of cells within the hippocampus. To determine lineage characteristics of these proliferating cells, samples were stained for TBR2, a transcription factor that is specific for type 2 amplifying progenitor cells in the hippocampal subgranular zone (Figure 18C). There was a rapid increase in $TBR2^+$ cells in the hippocampus at 48 hours, and by 72 hours post-TMT there was a significant 250-fold increase in $TBR2^+$ cells. After the 72 hour time point the number of $TBR2^+$ cells declined and by eight days had returned to control levels. Since these cells represent precursors to neuronal granule cells in the hippocampus, the mature phenotype marker NeuN was detected in five day post-TMT brain samples using an anti-NeuN antibody (Figure 19). It was found that BrdU-labeled cells in the dentate gyrus colocalized with NeuN, but not with the astrocytic marker or GFAP (Figure 19).

The effect of trimethyltin on proliferation in the lateral ventricle was assessed in the TMT dose-response study. Using Ki-67 as a marker of proliferating cells it was observed that there was no significant change in mitotic activity in the subventricular zone as the result of trimethyltin-induced injury of the hippocampus (Figures. 20-21). Ki-67 positive cells are located throughout the subventricular zone and can be identified sparsely along the ependymal wall of the lateral ventricle (Figure 21).

Because blood vessels comprise an important component of the neurogenic niche and have been found to be important in regulating neurogenesis, blood vessel morphology was assessed in this study (Figures 22 and 23). To determine any vascular changes, several parameters were analyzed including blood vessel density, vessel area, vessel branching, and number of endpoints of vessel processes. Analysis was carried out using a branching algorithm (IndicaLabs) on blood

vessels immunofluorescently stained for collagen IV. A statistically significant change in blood vessel morphology was shown to be time-dependent following TMT injury (Figure 22.) There was a significant decrease in blood vessel area in the hippocampus dentate gyrus shortly after TMT treatment, accompanied by a decrease in vessel thickness (Figure 22A, B). Interestingly, there was a time-dependent increase in vessel branching and number of vessel process endpoints detected. This suggests there may be a rapid constriction of the cerebrocapillary bed in the dentate gyrus following TMT injury. There was also a significant increase in blood vessel branching and endpoints (Figure 22C, D). While it is possible there could be an increased capillary branching at these early time points, it is more likely that the thinner processes are better resolved, resulting in more branches being scored by the branching algorithm.

Discussion

The goal of this study was to characterize some of the cellular responses to TMT injury, and characterize the response of hippocampal stem cells to the injury. TMT is a limbic hippocampal neurotoxicant that exhibits relatively specific excitotoxicity toward dentate granule cells in mice. Intraperitoneal injection of TMT induces excitotoxicity and other effects (described earlier in this thesis) that result in apoptotic and necrotic death of granule cell neurons. Cell death is initiated dose-dependently within 48 hours after TMT injection, resulting in significant cell loss as was shown by H&E staining (Figures. 1 and 11). A month after TMT exposure the morphological appearance of the hippocampus is essentially indistinguishable from that of untreated animals- the lost cells having been replaced with new neuronal cells in the granule cell layer. Quantitation of activated caspase-3 showed that cell death of granule cells in the dentate gyrus occurs within 48 hours after TMT exposure and persisted for about 8 days, peaking by day 3.

The appearance of pyknotic cells correlated with the time-course and dose-dependency of caspase-3 activation. Within 48 hours post-TMT there was significant positive immunoreactivity for active caspase-3 (Figures 13 and 14). H&E identification of pyknotic nuclei is not a specific indicator of apoptotic versus necrotic cell death, therefore the relative amount of cell death attributed to necrosis is not defined in this study. Fluorojade C staining showed focal degeneration of neurons in the hippocampus dentate gyrus (Figure 4), but could be indicative of both necrotic and apoptotic cell death. However, Gunasekar (2001) showed that higher concentrations of TMT causes glutamate-receptor-mediated cell death, whereas low concentrations elicit oxidative and PKC-mediated mechanisms of apoptotic cell death. The behavioral signs of acute TMT toxicity in mice, tremoring, initiates at about 24 hours post-TMT exposure and continues for up to 72 hours post-dose. This behavioral sign correlated with the timing of appearance of necrotic and apoptotic cells in the dentate gyrus. However, the peak of caspase-3 activation and number of pyknotic cells at 5 days post-TMT exposure was evident at a time after tremor had resolved (refer to Chapter 4; figure 1 for tremor profile). Involvement of non-granule cells in induction of seizure activity or compensatory mechanisms that serve to reduce the granule cell excitatory drive in the TMT-affected dentate gyrus may contribute to the cessation of seizure activity with ongoing granule cell death. For example, it has been shown that basket cells, which are involved in recurrent inhibition in the dentate gyrus, undergo degenerative changes soon after TMT exposure (Chang and Dyer, 1985), possibly contributing to hyperexcitability in the dentate gyrus. Hence, the disconnect observed here between cessation of tremors and the peak time for apoptotic/necrotic granule cell death, suggests that granule cell death may not be the sole mechanism responsible for either initiating or promoting on-going TMT-induced tremors.

New neurons born during TMT injury conditions in the dentate gyrus may help to mitigate neuronal hyperexcitability. Newly generated hippocampal neurons exhibit a higher inhibitory drive as a result of increased afferent plasticity (Jakubs et al., 2008, 2011). The birth and integration of new neurons in a pathological environment, such as during induction of status epilepticus or inflammatory conditions induced by lipopolysaccharide or status epilepticus, will have greater resistance to excitatory input (Wood et al., 2011). Remodeling of the dentate gyrus through increased inhibitory synaptic afferents to newly born granule cells and concomitant reduction in their intrinsic excitability is hypothesized to act as a compensatory mechanism to reduce epileptic seizure severity and propagation through dentate hyperexcitability. TMT injury also induces a significant inflammatory response in the hippocampus dentate gyrus. The finding that new neurons born in a pathological environment (i.e. inflammatory conditions or conditions of significant cell death) exhibit increased plasticity in their afferent synapses may be relevant for many brain injury models. New neurons formed after TMT-induced injury could function not only to repopulate the dentate gyrus with new granule cell neurons but, through remodeling and forming an increased inhibitory drive, function to reduce the extent or duration of hippocampal injury. While this study did not analyze electrophysiological properties of the new cells, this would be an area of interest for future study. In a later chapter of this thesis, the role of new neurons in reducing the behavioral effects of TMT (i.e. tremors) is examined by blocking neurogenesis through gamma irradiation.

The involvement of new neurons in remodeling the dentate gyrus under pathological conditions leads to the question of whether neurogenesis would confer resistance to re-injury caused by repetitive exposure to trimethyltin or other limbic system neurotoxicant. Various studies have shown that preconditioning protocols using sub-threshold levels of ischemic insult provide significant resistance against a subsequent, more severe insult yielding a much smaller

lesion or infarct size in the brain compared to a non-preconditioned subject. The mechanisms that confer this resistance include induction of stress factors, NMDA and adenosine receptors, heat shock proteins, inhibitors of apoptosis, activation of ubiquitin and autophagy pathways, or other pathways (reviewed by Liu et al., 2009). Additionally, Toll-like receptors, traditionally viewed as receptors that invoke inflammatory response to bacterial pathogens and act as part of innate host defense, may also be involved in neuroprotection against ischemic brain injury (Gesuete et al., 2014). TLR2 plays a role in neuroprotection against ischemia conferred by preconditioning, and the mechanism of TLR2 neuroprotection involves the AKT/PI 3 kinase pathway (Hua et al., 2008). Studies looking at specific agonists of TLR2 have shown that activation of this receptor alone can provide neural protection when administered prior to a toxic ischemic insult (Lu et al., 2011). In a separate study it was shown that elevated expression of BDNF in neural stem cells not only plays a role in differentiation, but is neuroprotective against TMT-induced cell injury and death, and that the mechanism for this protection is mediated through the AKT/PI3 kinase and MAP kinase pathways (Casalbore et al., 2010). Though the two neurological insults (ischemia and TMT-induced injury) differ in their toxicological mechanisms, the convergence in the neuroprotective pathways that are activated suggest that preconditioning to either ischemia or low dose of trimethyltin could potentially provide resistance to injury from a second exposure.

TMT intoxication induces a rapid inflammatory response in the hippocampus dentate gyrus granule cell layer and surrounding molecular layers. The response involves microglial and astrocyte activation which was evident as increased expression of CD11b and GFAP markers, respectively. It had been observed in earlier experiments that microglial activation occurs very early, within 24 hours after TMT intoxication, and prior to significant astrocytic activation as measured by GFAP expression (Weig, data not shown). In brain injury, astrocytic activation may

be dependent on the prior activation of microglial cells and their secretion of cytokine factors such as IL-1 beta (Liberto et al., 2004). These cytokine-activated astrocytes (isomorphic astrocytes) can function to limit the injury and promote neurorepair, or in the cases of more severe or prolonged injury, take on a phenotype (anisomorphic astrocytes) that may exacerbate injury by adding to the pro-inflammatory milieu and block neurorepair by formation of the glial scar. In this study, astrocyte response to TMT injury was quantitated using parameters that allowed an unbiased assessment of more intricate morphological details including branch area, length and thickness of processes, and number of branching endpoints. These parameters were useful since characterization of individual astrocytes by GFAP/DAPI colocalization is difficult due to the density of $GFAP^{+}$ astrocytic processes throughout the hippocampus. All the parameters were in agreement (except branch thickness, which was unchanged) that the trimethyltin injury induced a significant astroglial response that persisted to the 28 day post-TMT time point. The upregulation of CD11b and GFAP expression in these cell types correlated with the initiation of granule cell death in the dentate gyrus based on caspase-3 and H&E staining. The significant increase in expression of activated caspase-3 by dentate granule cells indicates this is a major cell death pathway induced by TMT intoxication, which is supported by other reports (Gunasekar et al., 2001; Qing et al., 2013) The significant glial activation following TMT injury (based on CD11b and GFAP immunoreactivity) suggests that necrosis may be a large component of the injury since apoptosis ordinarily does not cultivate a profound inflammatory response. Alternatively, direct effects of the toxicant on glial cells may upregulate the inflammatory response. In an *in vitro* neuron-free co-culture model consisting of astrocytes and microglia, it was shown that TMT could activate microglia at subcytotoxic concentrations (Rohl and Sievers, 2005). In this study, microglial activation declined rapidly by 8 days post-TMT injury and was indistinguishable from control brain by 28 days. Whether the microglial response was modulated by astrocytic factors is unclear, but

data suggest otherwise since microglial reactivity dissipated even though astrocytosis persisted out to the 28day post-TMT timepoint. Further research would be necessary to determine the factors regulating microglial responses in the TMT model.

Astrocytes carry out a variety of functions in the injured brain including modulating inflammatory responses by producing cytokine mediators. Astrocyte activation, as with microglial activation, can be differentially characterized based on the expression profile of various factors that may participate in inflammatory versus reparative mechanisms. For instance, microglia can exhibit an M1 versus M2 phenotypic profile, the former having more of an inflammatory role and potentially exacerbating injury, and the latter (M2) phenotype having anti-inflammatory effects and participating in the repair process. Similarly, astrocytes can express both deleterious factors that can exacerbate or prolong the injury process, or act to reduce the injury and promote recovery. Astrocytes participating in reactive astrogliosis, also termed anisomorphic activation, are usually generated during more severe injury and produce cytokines that tend to promote inflammatory responses. Anisomorphic astrocytes also participate in forming the glial scar which has been generally viewed as a barrier for neuroregeneration and axonal regeneration. However, a recent study suggests that the glial scar plays an important role in supporting axon regeneration (Anderson et al., 2016). Milder injury can activate astrocytes to a lesser extent, (isomorphic activation) and in this condition function more favorably towards recovery from the injury. This may include repair and maintenance of the blood-brain barrier, secretion of neurotrophic factors, uptake of excess glutamate to reduce excitotoxicity, neutralization of toxic reactive oxygen metabolites, sequestration of heavy metals (e.g. manganese), and neuronal remodeling (Liberto et al., 2005; Zhang et al., 2010). The protracted activation of astrocytes in this study may be indicative of a long-term function of reactive astrocytes in the neural repair phase and remodeling of the damaged hippocampus. The biological significance of the prolonged astrocyte reactivity

(i.e. 28 days post-TMT), approximately 3 weeks after the cytotoxic effects on granule cells and attenuation of microglial activation, is unclear but could reflect an extended role in the neural repair process. Astrocytes are known to have a critical regulatory role in neurogenesis in intact and injured adult brain. Ephrin-B2 presented by hippocampal astrocytes instructs neuronal differentiation of neural stem cells in the SGZ of the adult hippocampus via signaling through the EphB4 receptor and downstream activation of beta-catenin and the Wnt signaling pathway (Ashton et al., 2012). It has been reported that astrocyte populations derived from neurogenic brain regions secrete factors, including IL-1 and IL-6, that promote differentiation of neural stem cells into the neuronal phenotype (Barkho et al., 2006; Oh et al., 2010). Activation of the IL-6 receptor is reported to regulate levels of neurotrophins which play a role in neuronal protection and survival (Gadient and Otten, 1994; Otten et al., 2000). It should be noted that trimethyltin increases production of IL-6 and IL-1beta in glial cultures (Harry et al., 2002).

In this study, we demonstrated that $GFAP^+$ astrocytes in the dentate gyrus of the TMT-injured brain also co-localized with nestin. The $GFAP^+$/$nestin^+$ cells were largely confined to the dentate gyrus, whereas $GFAP^+$ astrocytes in other regions of the hippocampus were devoid of nestin labeling. Nestin is an embryonic intermediate filament expressed in neural stem cells (and stem cells of other tissues) in early development, but is down-regulated as GFAP expression becomes dominant. Nestin expression declines later in development, persists in neurogenic regions in the adult brain and is useful as a marker for neural stem cells in the SGZ/SVZ. However, nestin expression has been shown to increase with brain injury, including TMT-mediated brain injury (Lin et al., 1995; Geloso et al., 2004; Harry et al, 2004; Moon et al., 2004; Ogita et al., 2005; Cho et al., 2013), and has been found co-localized with activated astrocytes (Lin et al., 1995; Cho et al., 2013). It is hypothesized that expression of nestin in the astrocytes of the injured brain may allow for their structural remodeling in response to injury. Expression of primitive neural filaments in

astrocytes is also proposed to be indicative of active de-differentiation of astrocytes into neural stem cells with the potential to contribute to regeneration of new neural cells at the injury site (Lee et al. 2014).

Using Ki67 and BrdU markers for cell proliferation, we showed a significant increase in proliferation rate in the TMT-injured hippocampus dentate gyrus beginning at 48 hours after TMT administration, which is in agreement with a previous study (Harry et al., 2005). The increase in number of $Ki67^{+}$ cells in the dentate gyrus persisted up to about eight days after injury and returned to control levels by day 28. Further, the number of $BrdU^{+}$ cells increased between three days and five days post-TMT, and then declined by day 8. By 28 days, the number of cells incorporating BrdU was no longer significantly above control. Since bromodeoxyuridine persists in the DNA of living cells, this loss of BrdU signal after eight and 28 days suggests that there was a significant loss in newborn cells after approximately a week from the time proliferation initiated in response to the injury. This is not entirely unexpected, since it is known that many newborn cells in the hippocampus are culled under physiological conditions. Uncontrolled neurogenesis could be deleterious in the normal hippocampus, as it could serve to disrupt the existing neural network. It is possible that the TMT injury and the resulting loss of older granule cell neurons in the dentate gyrus could result in a substantially greater number of surviving newborn neurons, but such analysis was not undertaken in this study. It should be noted that in chapter 3 of this thesis, in which migrating cells were labeled with BrdU and spDiI, it was observed that the number of BrdU labeled neurons that existed prior to TMT injury was lower in the uninjured brain, suggesting that indeed there is greater survival of newborn neurons within the injured brain. The data in the time-course (Figure 18B) serves to show that there is an increase in number of BrdU labeled cells at five days post- injury and that most of these cells are lost by the 28 day time-point. It should be stressed that the new BrdU-labeled neurons in the time course study were born

during the conditions of TMT injury and associated inflammatory reaction, whereas in the later study in this thesis, in which BrdU was used as a tracer, those cells were born under physiological conditions before the inflammatory response induced by TMT injury. It is possible that the conditions in which new neurons are born define not only their survival duration but perhaps their functional role. In the case in which new neurons are born just prior to injury, those cells may serve as long-term replacements of cells lost to the injury. The cells produced during the surge in proliferation during the injury may be less destined for long-term survival and not be a replacement of new neurons, but may serve to help mitigate the effects of the injury in the short-term. It was noted previously that the newly born neurons have a greater inhibitory drive than old neurons, and the new neurons may provide other as yet unknown neuroprotective properties to the established hippocampal cellular network.

TMT-mediated injury of the hippocampus rapidly induces a significant increase in cell cycling in the SGZ of the dentate gyrus. Ki67, a marker detectable in all active phases of the cell cycle, was infrequently detected in control mouse hippocampus. A significantly larger pool of $Ki67^+$ cells was observed in the SVZ of the lateral ventricle. Within 48 hours of TMT injection there was a significant increase in number of $Ki67^+$ cells in the SGZ of the dentate gyrus. The number of $Ki67^+$ cells peaked at 3 days post-TMT at which time there was a ~9-fold increase relative to control. After the 5 day post-TMT time point there was a rapid decline in $Ki67^+$ mitotic cells, even though cell death was still occurring at 5 days post-TMT. This suggests that the proliferation rate is tightly controlled to prevent run-away proliferation and needless production of new cells once the instigating event inducing cell death has ended. Whether the proliferation response to injury is linked to directly to cell death and degeneration, or indirectly through glial cell activation is not clear.

The burst of cell proliferation in the dentate gyrus following TMT injury vastly increased the pool of neural precursors as indicated by the nearly 300-fold increase in cells expressing the early neural marker TBR2. The number of $TBR2^+$ cells rapidly returned to control levels by 8 days post-TMT. By 5 days post-TMT, $BrdU^+$ cells were predominantly expressing the neuronal phenotype marker NeuN. Hence TMT injury upregulated production of amplifying progenitor cells (type 2 cells) in the SGZ, eventually leading to generation of new granule cell neurons.

Ki67 cells were visible in the SVZ as well as very sparsely among ependymal cells lining the lateral ventricles. Unlike the response of hippocampal neural stem cells to TMT injury, there was no significant effect on cell proliferation in the SVZ of the hippocampus in the TMT dose-response study. This suggests that TMT-induced cell injury to the hippocampus did not induce SVZ stem cells in terms of a proliferative response even at high concentrations of TMT. However, the TMT dose-response study was limited to a 48hr post-TMT time point. Later time points may yield different results if there is a delayed response of SVZ stem cells to TMT injury. TMT intoxication induced caspase-3-mediated cell death in the olfactory bulb, which is also part of the limbic system. Prior studies have shown that injury to the olfactory bulb does not induce increased SVZ stem cell proliferation rates (Liu et al., 2011). Rather, injury to the olfactory bulb can disrupt neuroblast migration in the rostral migratory stream, resulting in RMS enlargement and premature differentiation of neuroblasts into NeuN-expressing neural cells. Whether TMT injury to olfactory bulb causes abnormal migration effects on neuroblasts targeted to the olfactory bulb was not investigated here.

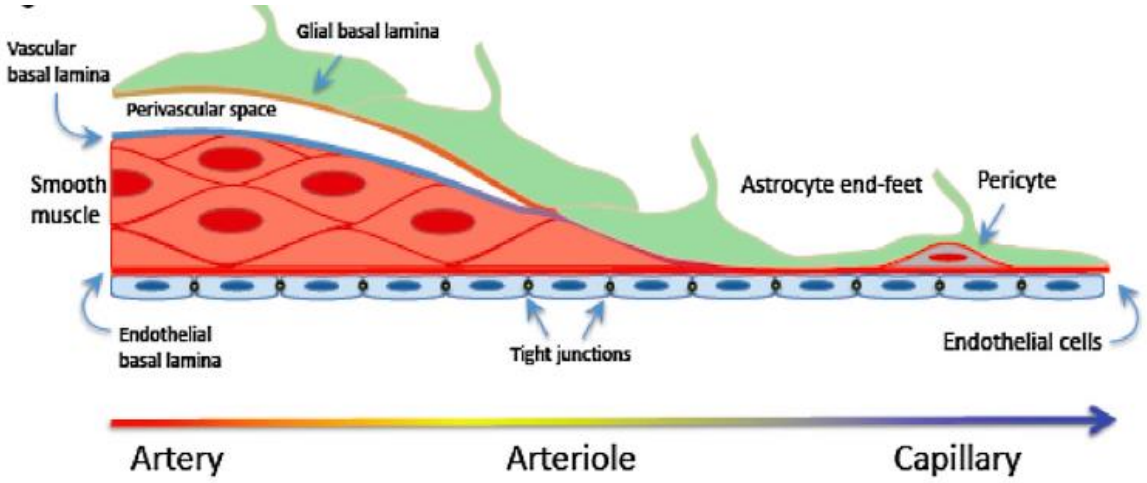
Data in this study showed that TMT caused a rapid and prolonged reduction in blood vessel area and vessel thickness within the hippocampus. Interestingly, there was also a time-dependent increase in blood vessel branch points and blood vessel endpoints. There is limited information on the effects of organotins on the cerebrovascular system. Some early studies of trimethyltin in

mammals indicated cardiovascular toxicity such as disruption of heme metabolism, decreased organ-to-heart mass ratios, and decreased blood pressure as a result of depression of vascular smooth muscle (Nath, 2008). Chen et al. (2012) showed in zebrafish that TMT exposure led to disrupted cardiovascular development and induced altered gene expression including profiles categorized for metabolic and cardiovascular disease. In a rat model, TMT-induced neurodegeneration caused upregulation of aquaporin-4 in astrocytes, which may contribute to alterations and blood-brain barrier permeability. Michetti et al., 2012 described an intense vascular reaction to TMT intoxication which was characterized by hypertrophic vessels with 'abnormal course and dimensions' in the brain (Michetti et al., 2012). Reports of cardiovascular effects of TMT in mice were found; a rodent which may have significant effects from rats due to the different half-life of the toxicant between these rodents, and differing cells targeted for toxicity. Cerebral blood supply is obviously of vital importance for the structural and functional integrity of the brain. As discussed below, the cerebrovasculature also plays important roles in neurogenesis, including maintaining neurogenic niches in the brain, modulating neural stem cell activity and providing scaffolding for migration of new neuroblasts in the adult brain. Any alterations in cerebral blood vessels and blood flow can have a profound impact on neural activity and cognitive function.

Cerebral blood flow (CBF) is physiologically controlled by neural activity, which also consumes most of the brain's energy supply. CBF is increased in localized brain regions exhibiting increased activity in order to meet the oxygen and nutrient demands of activated neurons. This cardiovascular response to neural activity is referred to as neurovascular coupling (Roy and Sherrington, 1890). Neurovascular coupling, which coordinates hemodynamic changes with neural (synaptic) activity, is orchestrated by factors that depend on the concerted responses of neurons and astrocytes and vascular cells. The molecular signals include ions, arachidonic

metabolites, nitric oxide (NO), adenosine, neurotransmitters, and neuropeptides (Drake and Iadecola, 2007). Changes in CBF are mediated by vasoactive molecules that induce vasoconstriction or vasodilation of cerebrovascular processes. Pial arterioles on the brain surface were originally thought to be the key vascular structures for controlling CBF (Iadecola, 2004), however, more recently it has been shown that the pericytes lining capillaries deep within the brain parenchyma are primarily responsible for regulating blood flow within the cerebral hemispheres (Hall et al., 2014). Dysfunctional regulation and diminished blood flow is associated with various neurodegenerative (e.g. Alzheimer disease) and psychiatric disorders ranging from vascular cognitive impairment to dementia (reviewed by Iadecola, 2013).

Pericytes, also called Rouget or mural cells, wrap around blood capillaries and resemble vascular smooth muscle cells because of their contractile fibers. The pericytes can be thought of as replacing the vascular smooth muscle cells present in larger vessels (arteries and arteriole). They are embedded within the basement membrane of the capillaries and are less densely populated along the vascular structure than are smooth muscle cells. They are well positioned to directly communicate with endothelial cells of the capillary as well as glial and neural cells on the parenchymal side of the vessel.



The anatomical arrangement of smooth muscle cells and pericytes in the brain microvasculature. (From Iadecola, 2013).

The role of pericytes in blood vessel formation, maintenance, tumor angiogenesis, control of cerebral blood flow, and role in pathology is becoming increasingly of interest and a focus of research. Similar to vascular smooth muscle cells in arterioles and arteries, pericytes control vasodilation and vasoconstriction of the capillary bed in response to vasoactive molecules. Both astrocytes and neurons contribute to production of vasoactive molecules. Astrocytes generate increases in intracellular calcium in response to neuronal activity (Anderson and Nedergaard, 2003). This increase in calcium stimulates arachidonic acid metabolism and production of vasodilating epoxyeicosatrienoic acids (EETs), vasoconstricting 20-hydroxyeicosatetraenoic acid (20-HETE), and prostaglandins that have can either vasoactive effect (Amruthesh et al., 1993).

Neurotransmitters such as glutamate that are released into extracellular space following synaptic activity, induce vasodilation and increase blood flow. However, the glutamate-mediated vasodilation is dependent on co-existing levels of nitric oxide (Meta et al., 2006). Nitric oxide inhibits synthesis of 20-hydroxyeicosatetraenoic acid (20-HETE) which is a vasoconstricting metabolite of arachidonic metabolism. However, NO also can shift vasoactivity to vasoconstriction by inhibiting CYP epoxygenase that metabolizes arachidonic acid to the

vasodilating epoxyeicosatrienoic acids (EETs). The complexity of the overall effect of the milieu of vasoactive agents released in pathological conditions probably underlies the often contradictive reports of effects of injury on vascular function.

Despite the complexity of the molecular players involved in vascular tone, the role of pericytes has been shown to be of significant importance in CBF, particularly with respect to ischemic conditions such as stroke. Hall et al. (2014) showed that pericytes are vulnerable to ischemic conditions, and upon cell death, indefinitely maintain the involved capillary microvessels in a constricted state that are not responsive to vasodilators. This results in a prolonged and essentially irreversible reduction in CBF which may significantly contribute to the cognitive dysfunction and dementia that develops in up to 50% of relatively young (<50 years) stroke victims (Schaapsmeerders et al., 2013).

The effect of TMT on the blood vessel parameters analyzed in this study could involve not only changes in the production and secretion of vasoactive agents, but could also reflect an alteration in the function of pericytes. While the specific mechanisms of toxicity of TMT in the brain are not well defined, calcium overload and the partial role of NO in oxidative stress, is proposed to be one of the major factors in its toxicity. Hence the increased calcium load in context with NO may alter production of factors to favor vasoconstriction in the mouse model. Further research is needed to determine what specific effects TMT has on production of vasoactive molecules in the mouse brain. Furthermore, it would be of substantial interest to determine whether trimethyltin intoxication has either direct or indirect effects on pericyte functionality.

In addition to the importance of blood vessels in CBF, they also play an important role in neurogenesis in the mouse brain. As discussed earlier, blood vessels form an important component of the neurogenic niche within the brain. Neural stem cells in the subventricular zone

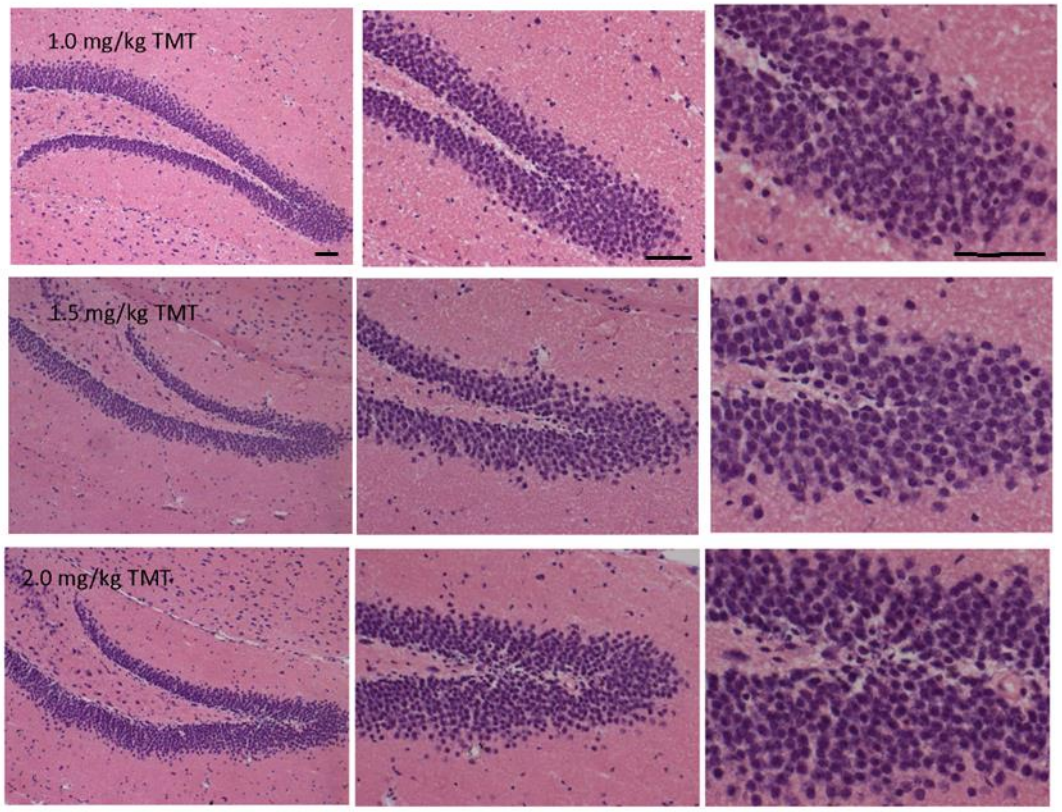
and the subgranular zone of the hippocampus reside in a neurovascular niche. The neurovascular niche of the subventricular zone has been more extensively studied than the hippocampal vascular niche but in both cases the proliferating cells are in close physical proximity to blood vessels (Palmer et al., 2000; Mirzadeh, et al., 2008). Contact between neural stem cells and blood vessels is observed in both neurogenic niches (reviewed in Licht, 2015). The effect of trimethyltin on the cerebrovasculature could have consequences on stem cell activity within the hippocampus. Further research will be required to determine how changes in blood vessel morphology affect the short-term induction of neurogenesis following injury as well as the long-term effects on neurogenic potential.

Trimethyltin had no significant effect on cell proliferation within the subventricular zone at 48 hours post-dose. This suggests that the injury does not induce a mitotic response by stem cells residing along the lateral ventricle. This is not surprising since various studies have shown that injury to the olfactory bulb, the physiological target for migrating neuroblasts originating from the subventricular zone, does not induce significant alteration in stem cell proliferation in the SVZ. A recent study in a rat model showed that a focal lesion can induce subventricular zone proliferation in immature brain but was almost undetectable in adult brain (Goodus et al., 2015). Some earlier studies suggested that ependymal cells in the lateral ventricle respond to injury by proliferation in the production of new neural precursor cells. The proposal that ependymal cells act as stem cells to produce new neurons or glia in response to injury in the adult brain is controversial, and the biological significance of the limited fraction of ependymal cells responding is unclear. In this study, it was observed that some ependymal cells appeared to express Ki-67 following TMT injury. Quantitative analysis of Ki-67 expression among ependymal cells is required. However, given the rarity of Ki-67^{+} ependymal cells, such a quantitative analysis will require a large cohort of animals

to generate informative statistics. In addition, long-term administration of bromodeoxyuridine may be necessary to label the infrequently cycling ependymal cells and to trace their lineage fate.

In sum, TMT-induced injury to the murine hippocampus stimulates both inflammatory and stem cell responses. Stem cell proliferation is limited to SGZ production of new granule cell neurons to replace dead or degenerating granule cells affected by the cytotoxicity of TMT. Morphological recovery of the dentate gyrus granule cell layers appears to have occurred by 28 days post-TMT, though there is continued activation of $GFAP^{+}$ astrocytes above baseline level. Neural repair involves both stem cell and glial cell responses, but specific mediators that regulate the repair process require further study. TMT injury also affect blood vessel morphology, but the biological significance as it relates to injury and repair are not clear and is worthy of more detailed investigation.

Figure 1. H & E staining of hippocampus dentate gyrus from mice treated treated in a Trimethyltin dose-response. Cell death as indicated by increased numbers of pyknotic nuclei are visible starting at 2.3 mg/kg (yellow arrows). Scale bar = 50 micrometers



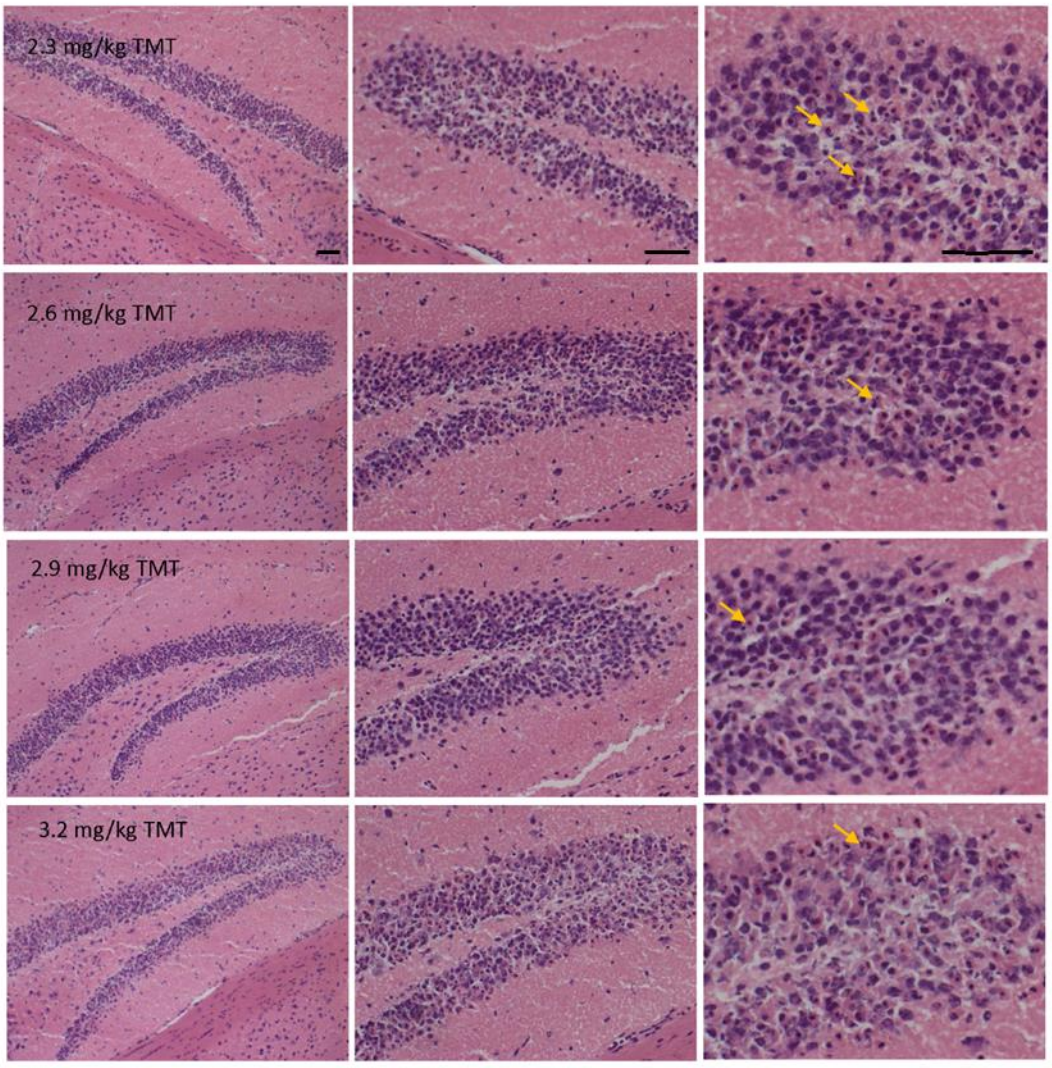
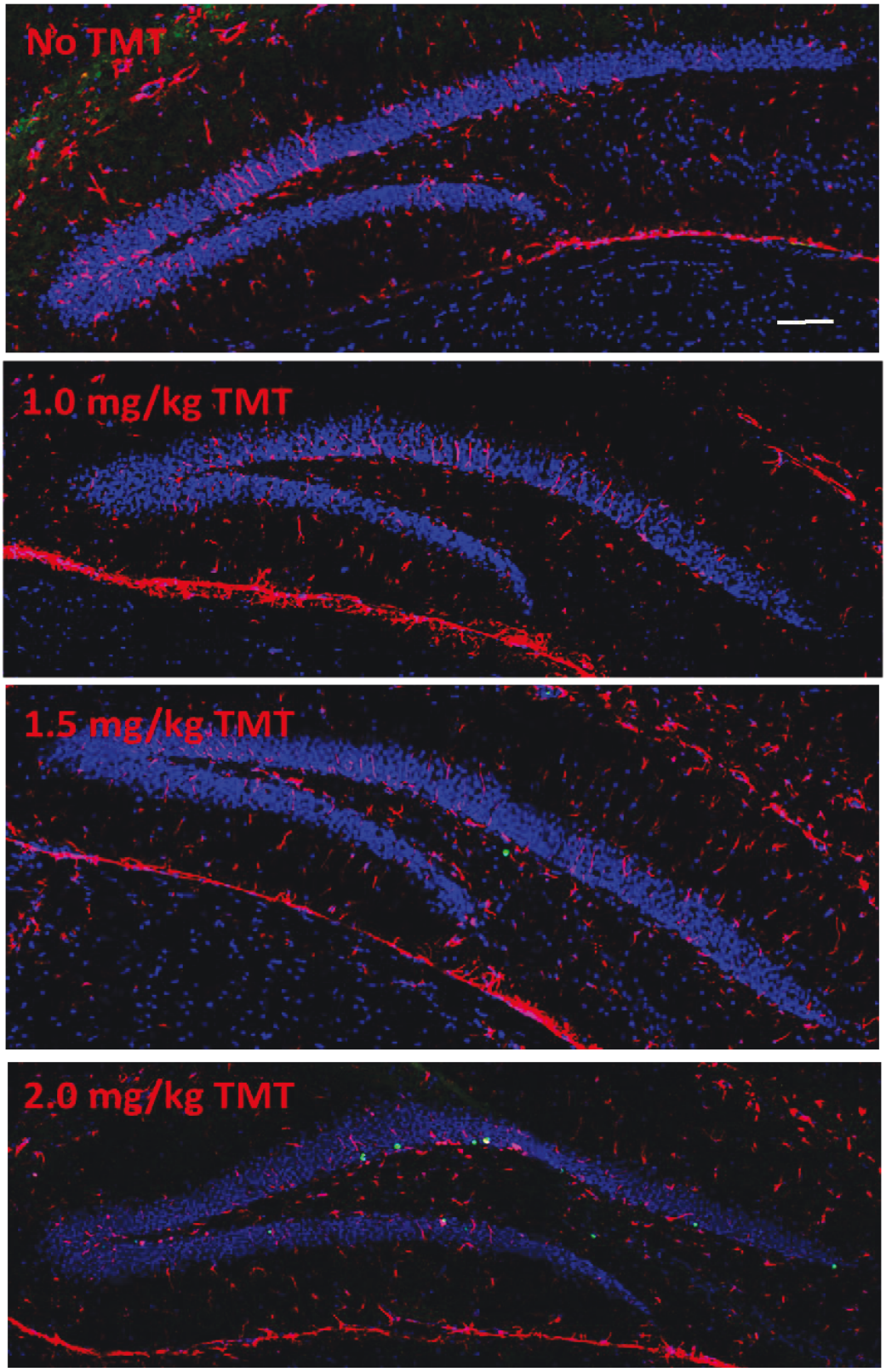

2.3 mg/kg TMT
2.6 mg/kg TMT
2.9 mg/kg TMT
3.2 mg/kg TMT

Figure 2. GFAP and active caspase-3 staining of hippocampus dentate gyrus of mice 48 hours post-TMT treatment. GFAP^{+} astrocytes (red) and active caspase-3 (green) expression upregulated at doses above 2.0 mg/kg TMT. Scale bar = 100 micrometers.



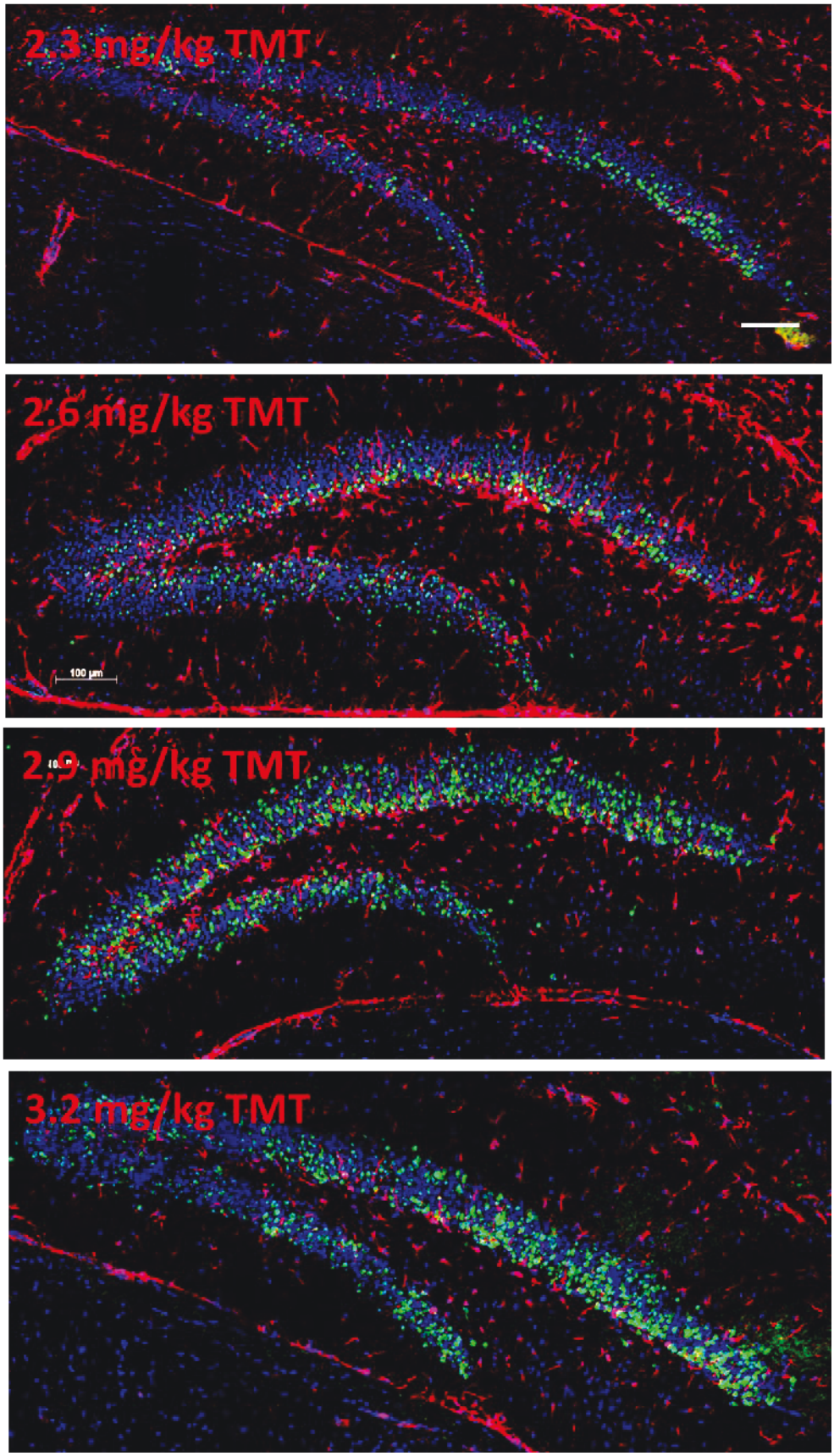
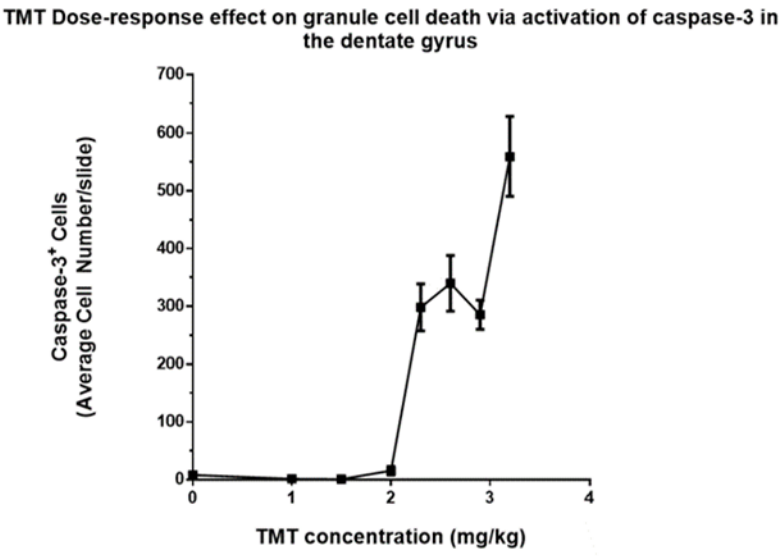
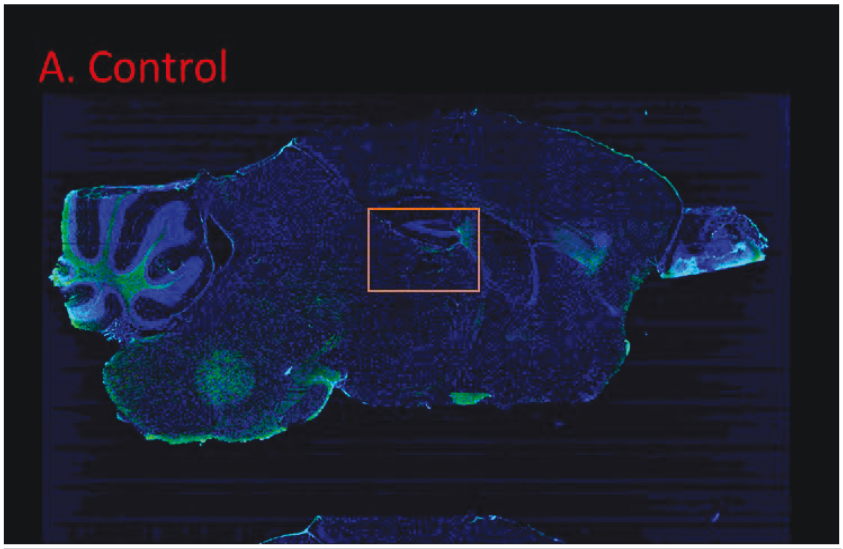

2.3 mg/kg TMT
2.6 mg/kg TMT
100 µm
2.9 mg/kg TMT
3.2 mg/kg TMT

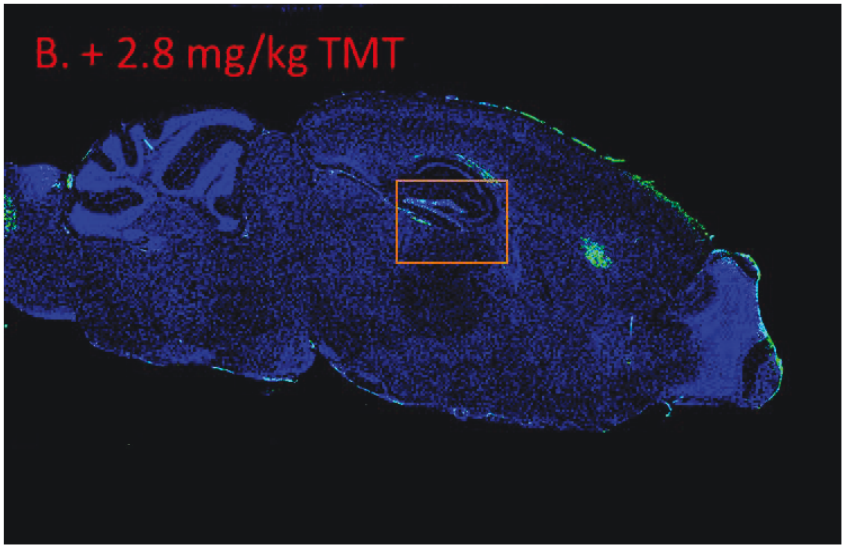
Figure 3. Quantification of active caspase-3 staining in hippocampus dentate gyrus of mice at 48 hours post-TMT treatment. Graph depicts the steep dose-response of of TMT-mediated apoptotic cell death.

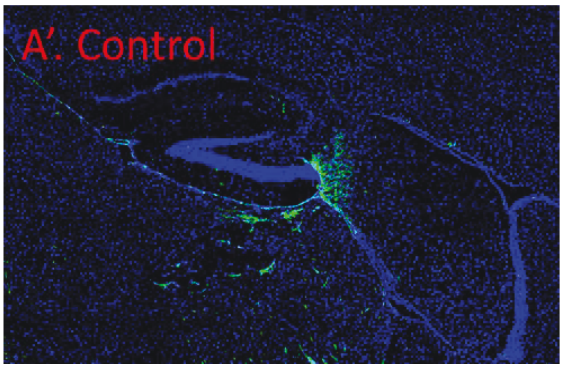


Data points represent average cell counts from 3 animals per time point.

Figure 4. Fluorojade staining of mouse brain treated with 2.8 mg/kg TMT showing degenerating neurons in the hippocampus dentate gyrus. (A and A', Control (whole brain); B and B', 2.8 mg/kg TMT (5x magnification).







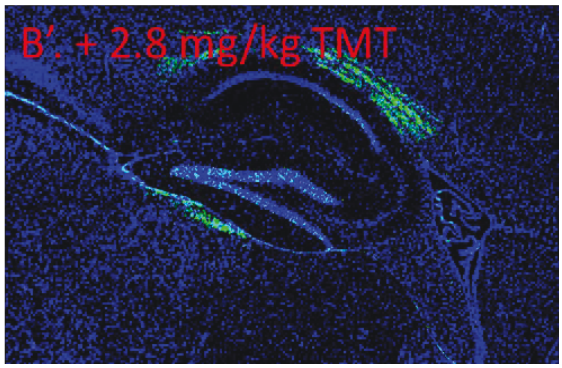
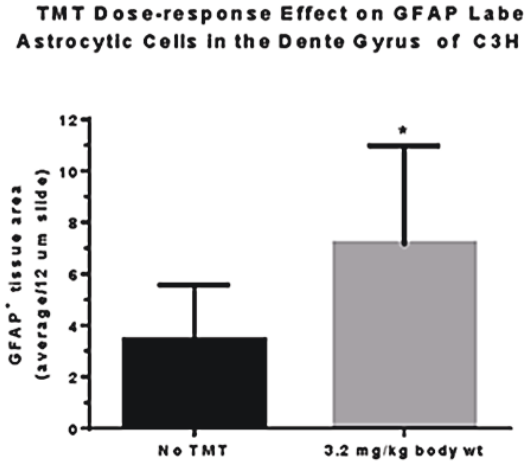
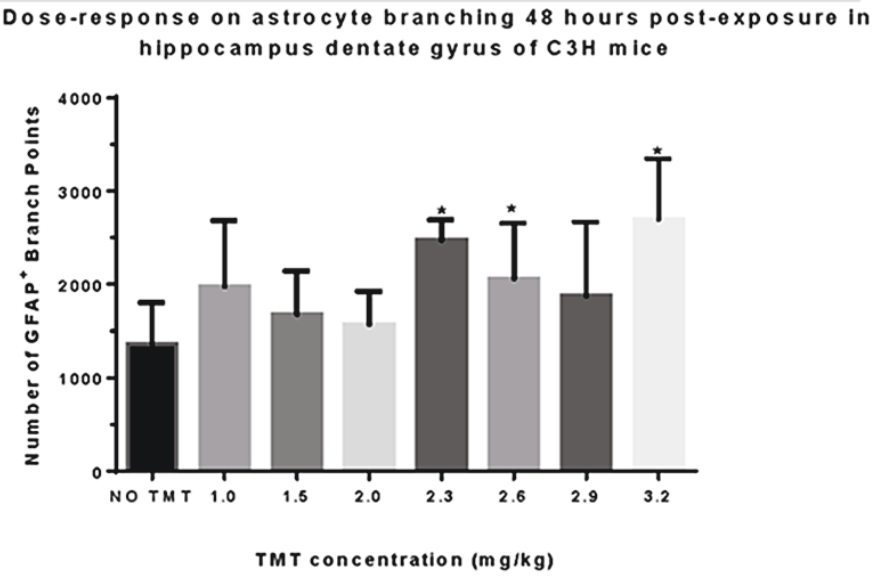


Figure 5. Area quantification of GFAP in hippocampus following 48 hr TMT exposure showing increased reactive astrocytes at 3.2 mg/kg dose.



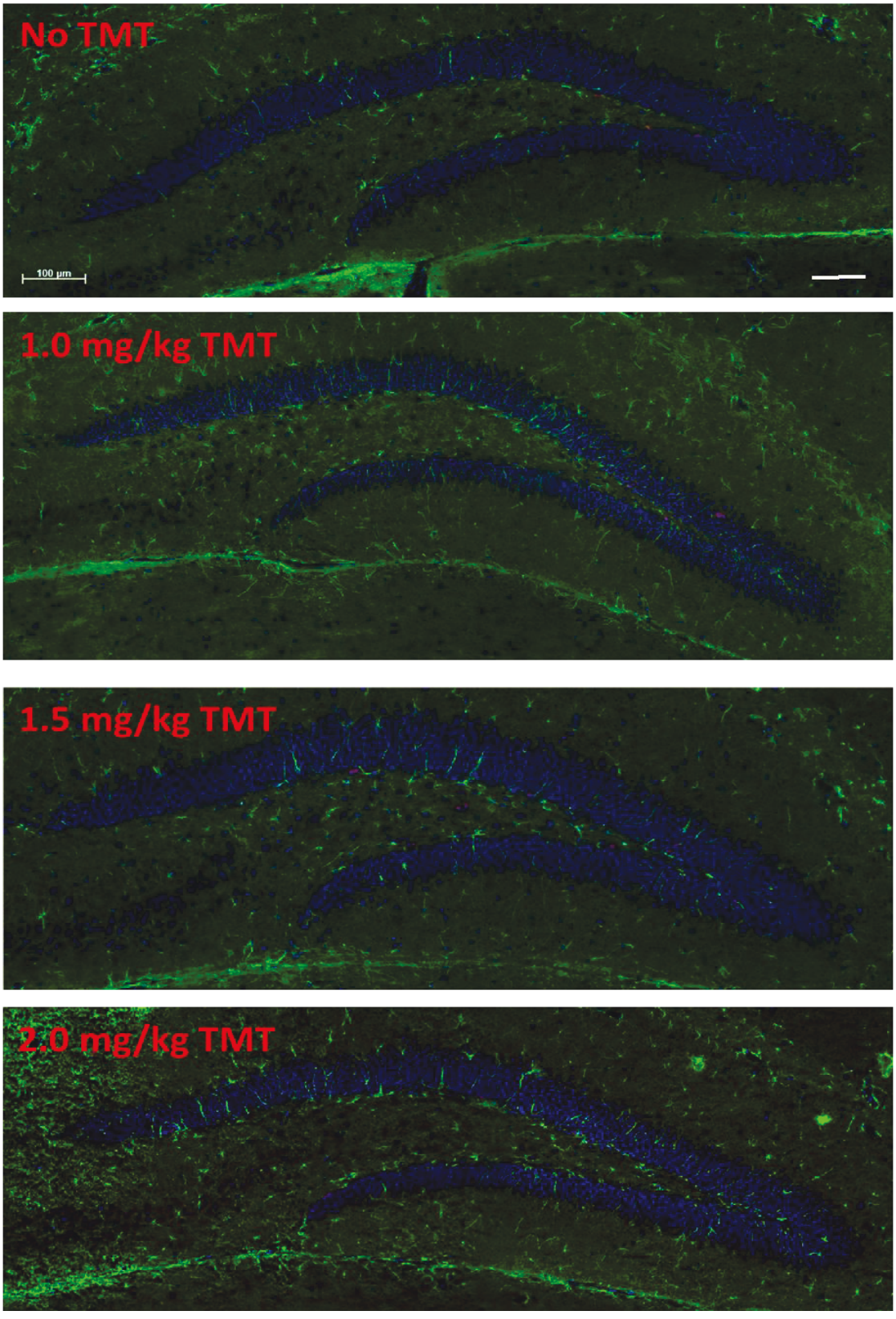
Data are mean of 5 animals per group; unpaired t-test; *P<0.05.

Figure 6. Quantification of GFAP+ astrocyte branching in hippocampus dentate gyrus 48 hours post-TMT dose-response. Increased branching at TMT doses above 2.0 mg/kg at this early time point.



Data are mean of 5 animals per group; one-way ANOVA-Dunnetts test; *P<0.05.

Figure 7. Staining of dentate gyrus for GFAP^{+} astrocytes for image analysis of astrocyte activation 48 hours post-TMT. Scale bars = 100 micrometers.



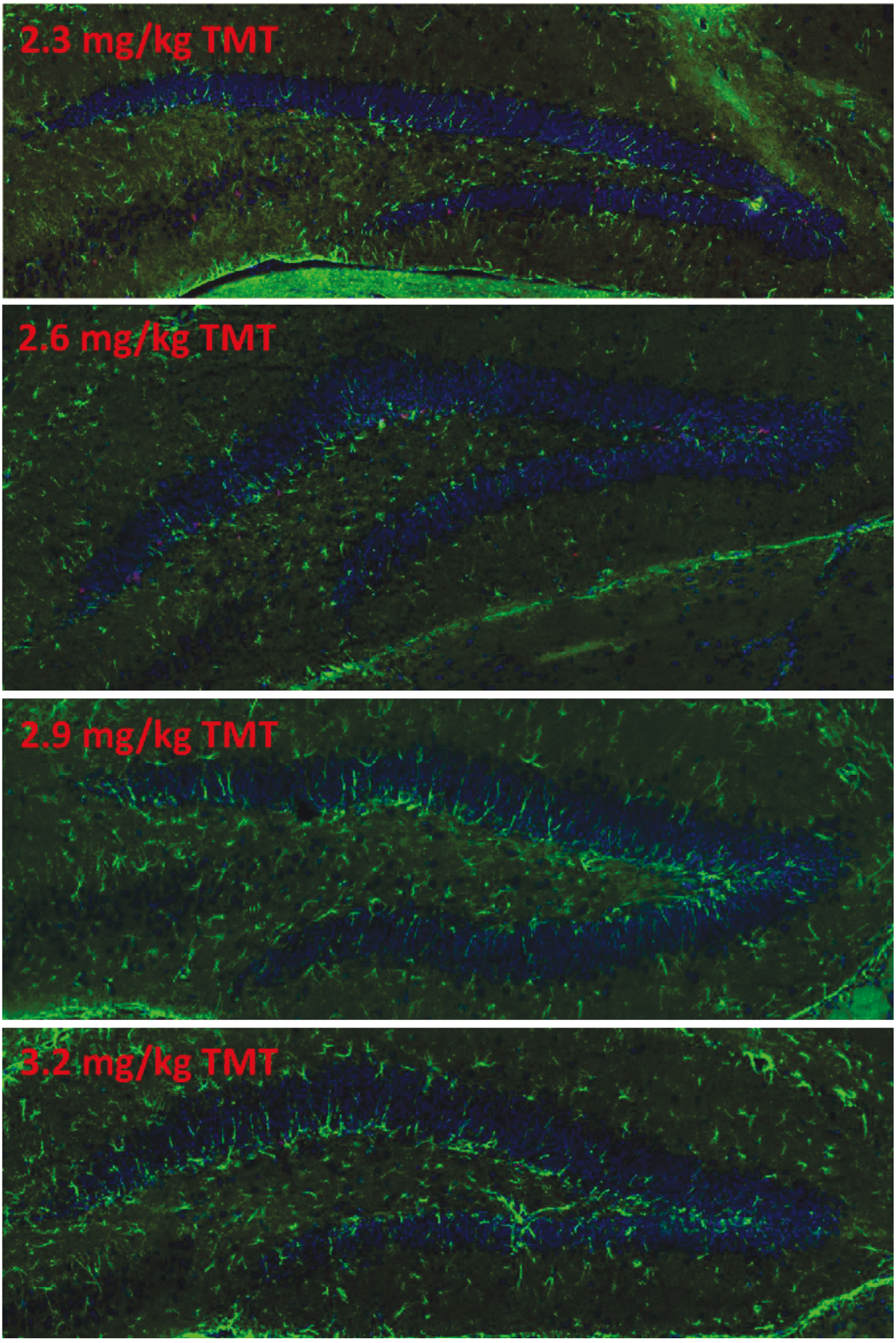
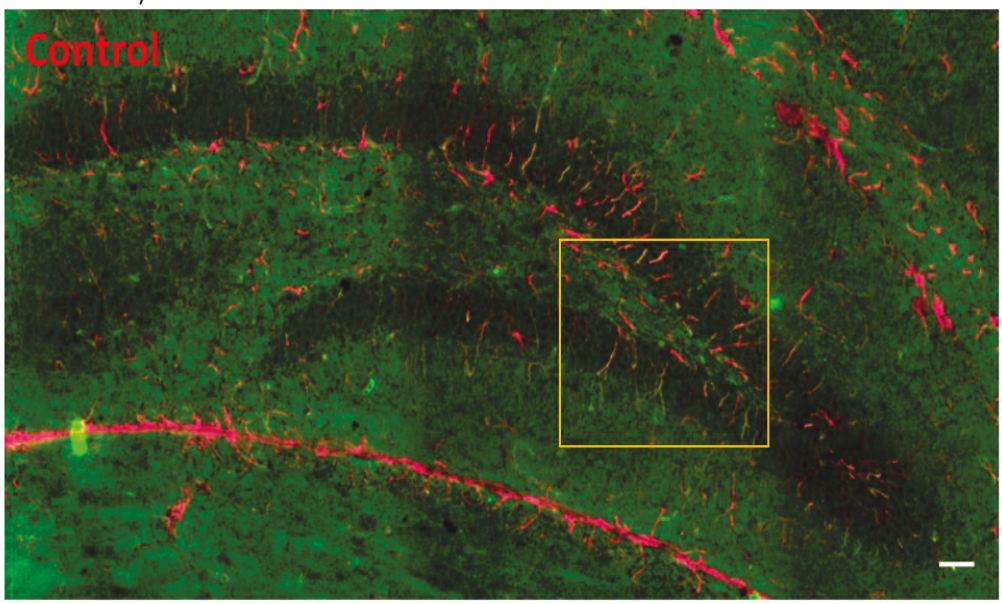
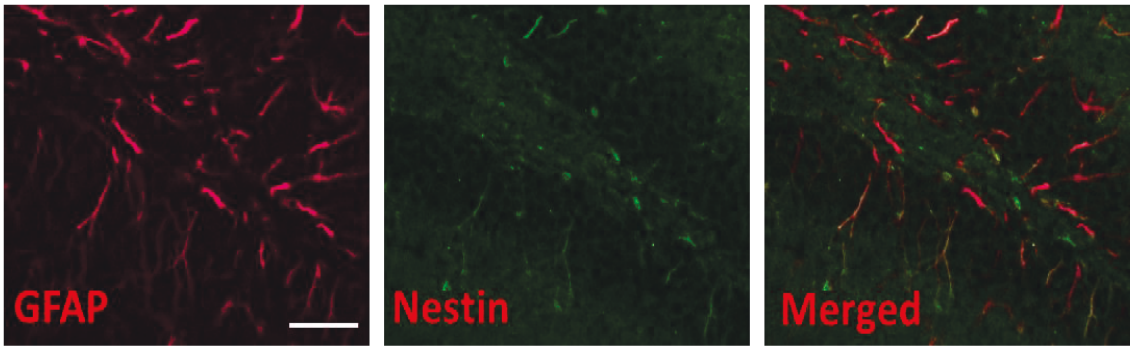

2.3 mg/kg TMT
2.6 mg/kg TMT
2.9 mg/kg TMT
3.2 mg/kg TMT

Figure 8. Effect of TMT on nestin and GFAP expression in the hippocampus dentate gyrus 48 hours post-treatment. Increased expression of nestin in GFAP+ astrocytes localized to hippocampus dentate gyrus following TMT injury.(Nestin, green; GFAP, red; scale bar = 50 micrometers)





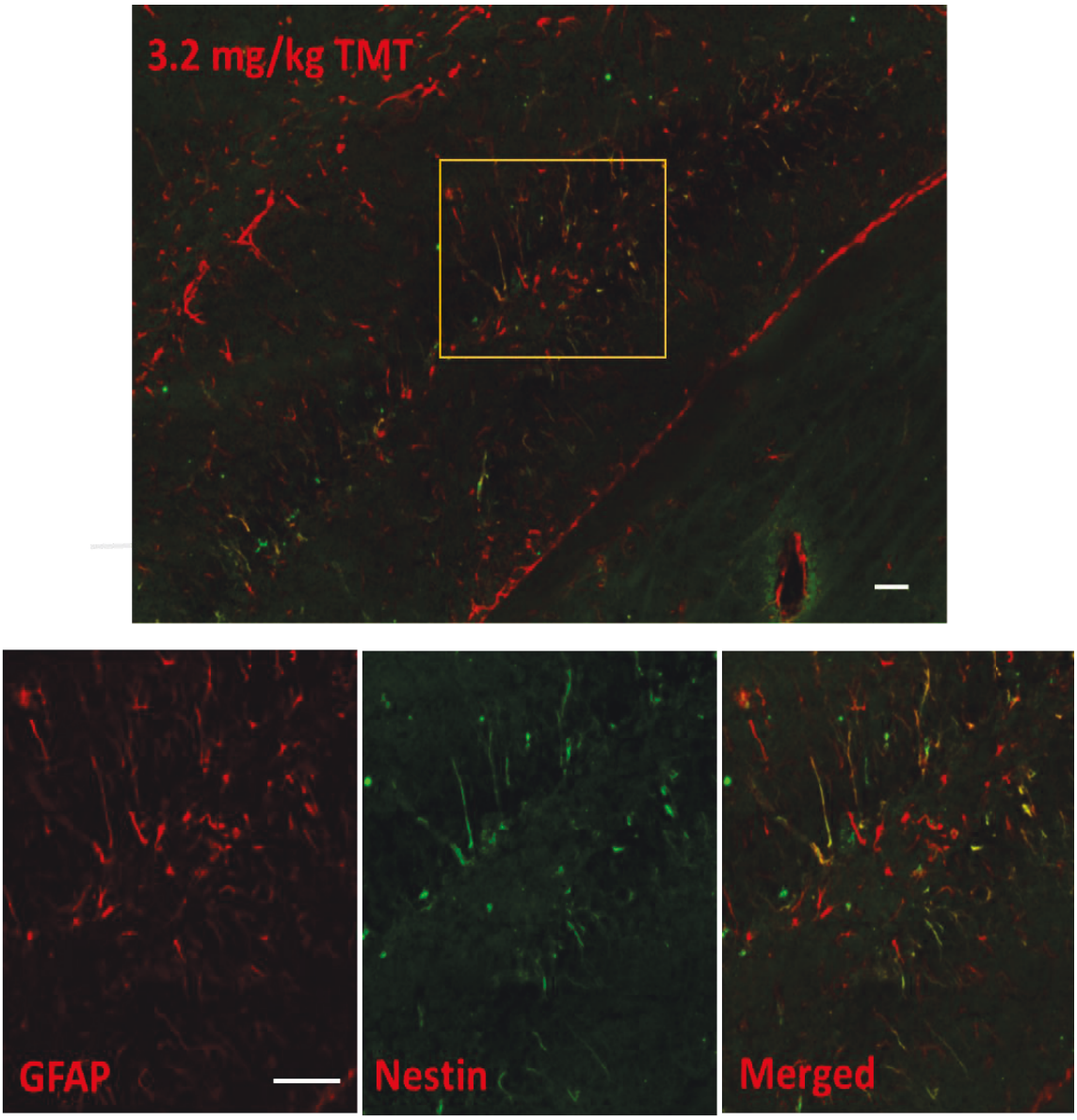
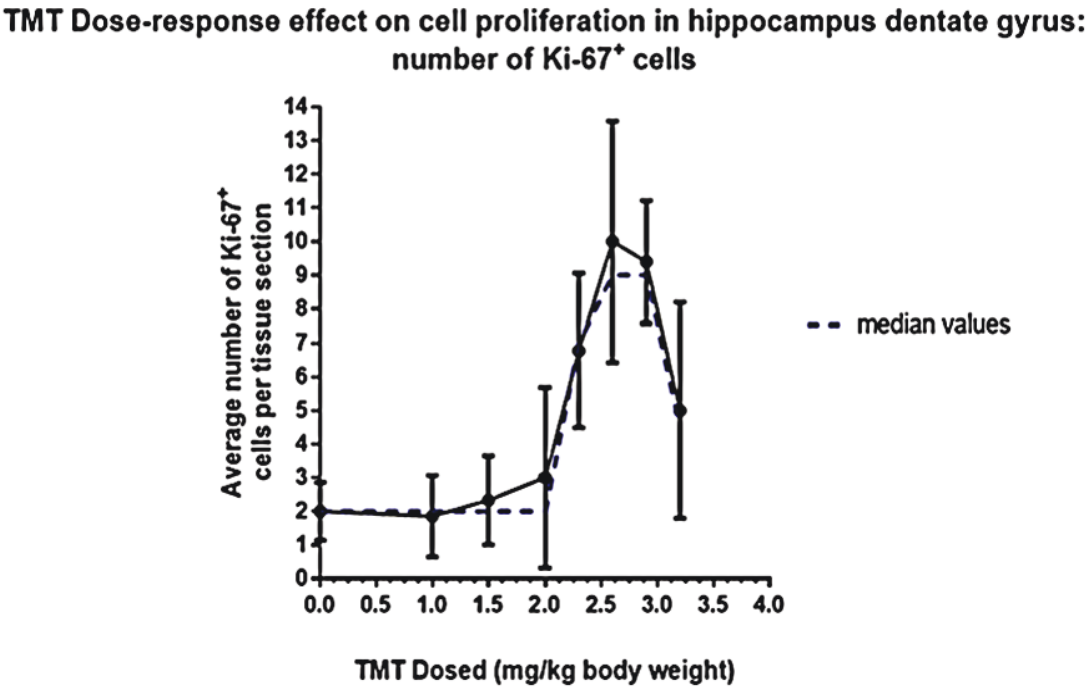
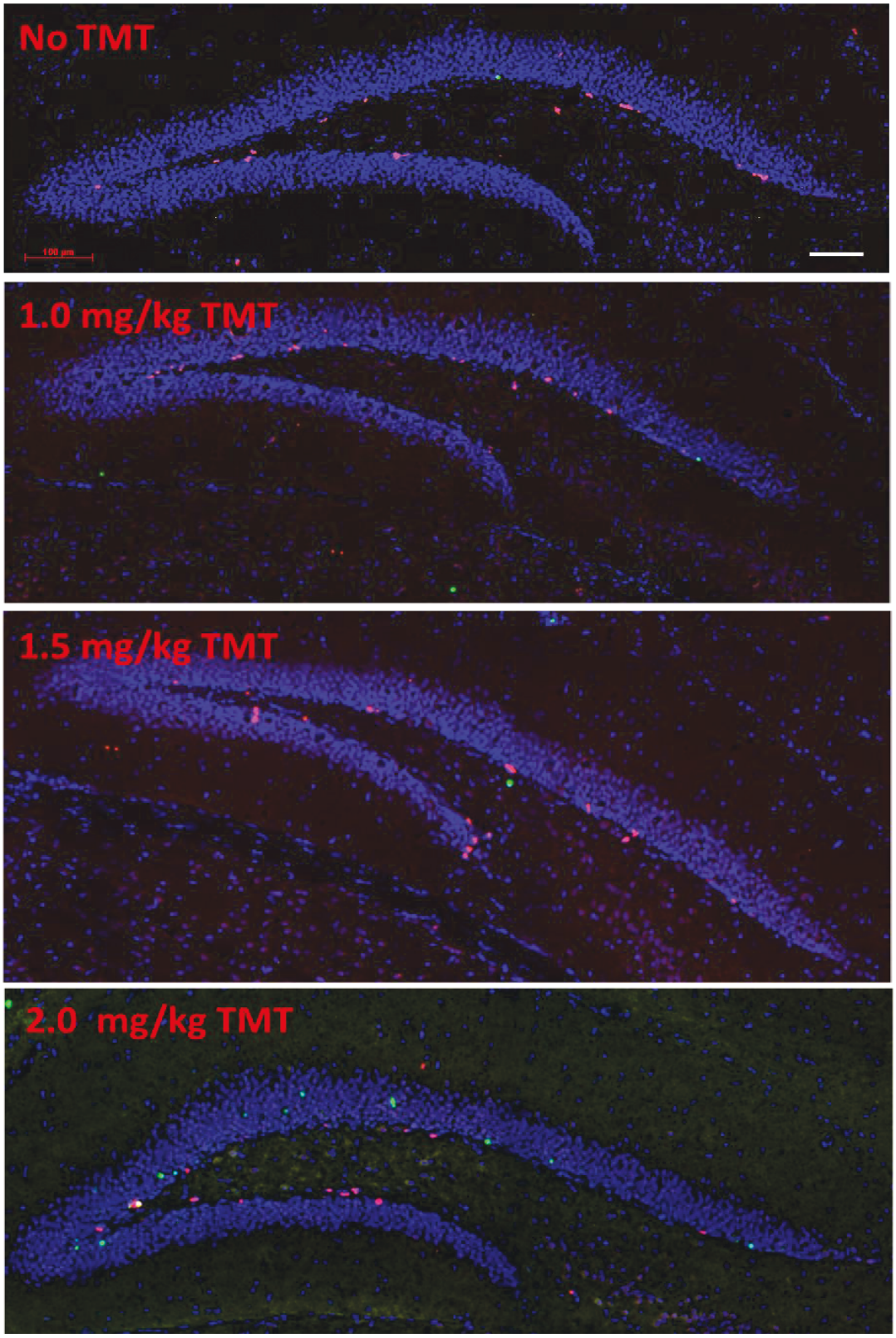

3.2 mg/kg TMT
GFAP
Nestin
Merged

Figure 9. Quantification of Ki67^{+} mitotic cells in the hippocampus dentate gyrus 48 hours post-TMT showing dose-response effects on proliferation.



Data points represent average cell counts from 3 animals per time point.

Figure 10. Ki67+ mitotic cells in hippocampus dentate gyrus 48 hours following TMT exposure and induction of cell death. Ki-67, red; active caspase-3, green; DAPI, blue. Scale bar = 50 micrometers.



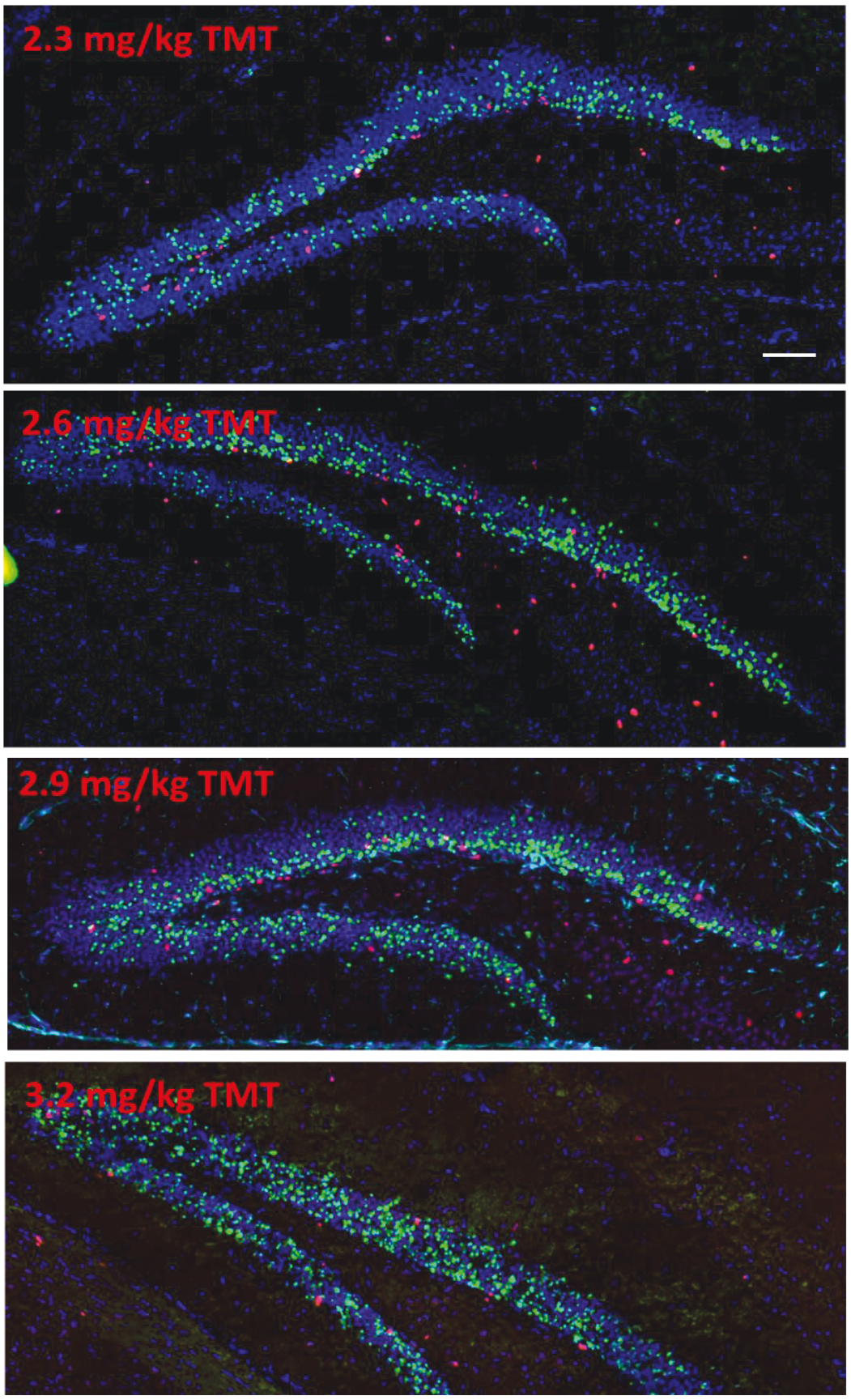

2.3 mg/kg TMT
2.6 mg/kg TMT
2.9 mg/kg TMT
3.2 mg/kg TMT

Figure 11. Hemotoxylin and eosin staining of hippocampus from mice treated with 2.8 mg/kg TMT and euthanized at different time points. Pyknotic nuclei evident in the granule cell layer 2-5 days post-TMT treatment (yellow arrows). Scale bar = 50 micrometer.

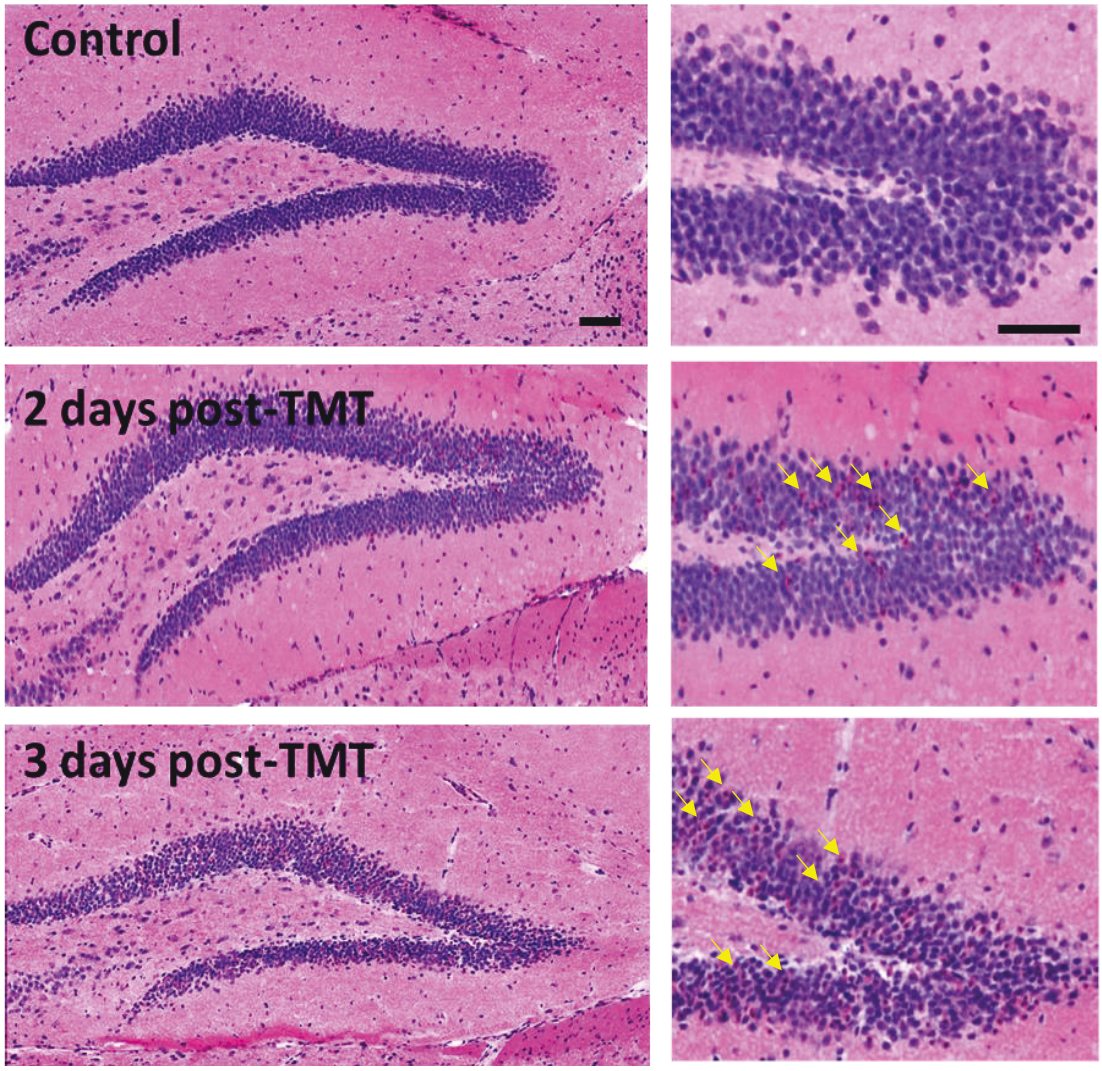


Figure 11 (continued). Hemotoxylin and eosin staining of hippocampus from mice treated with 2.8 mg/kg TMT and euthanized at different time points. Pyknotic nuclei evident in the granule cell layer 2-5 days post-TMT treatment (yellow arrows). Scale bar = 50 micrometer.

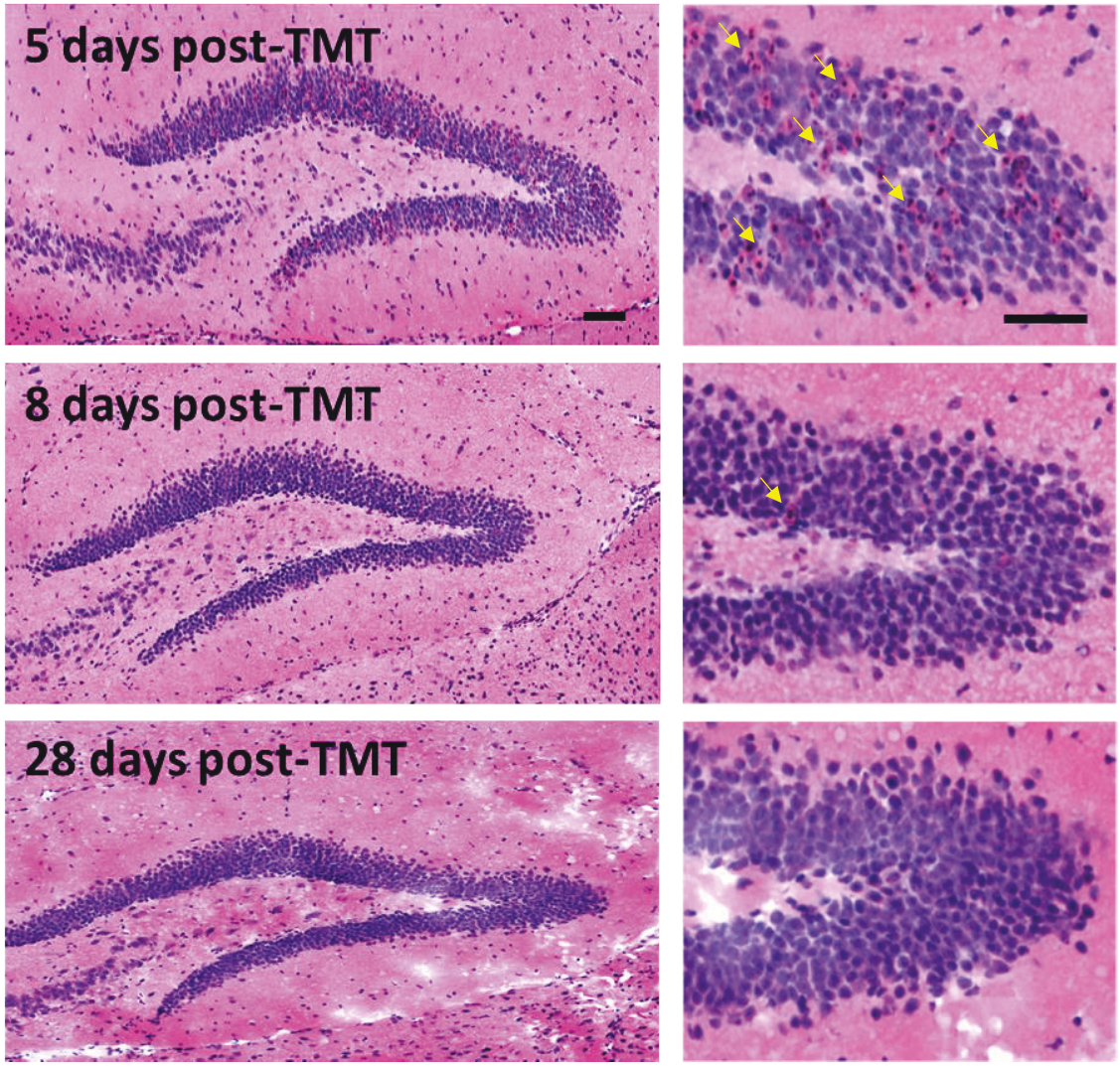
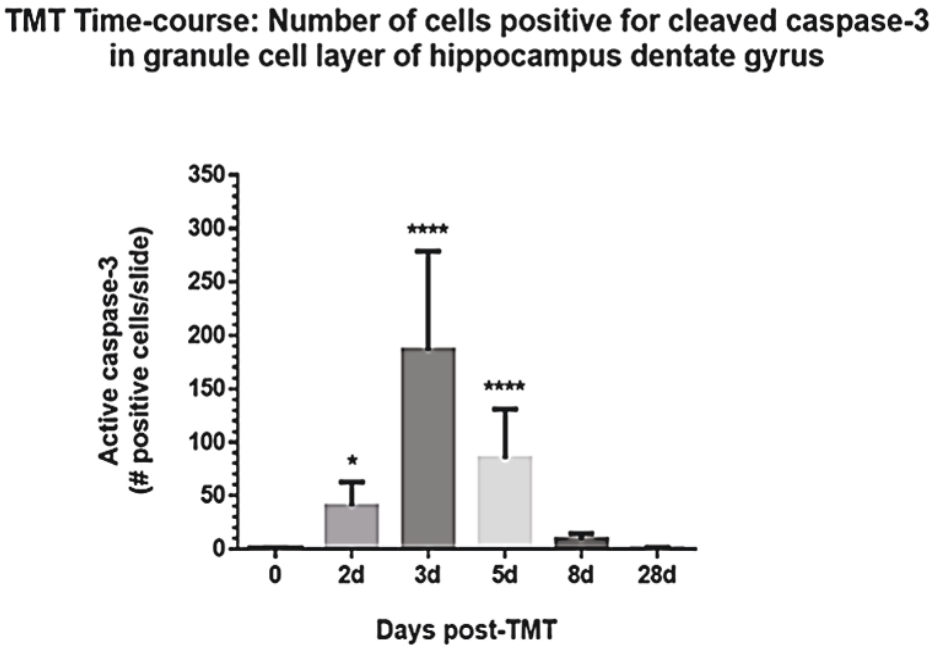
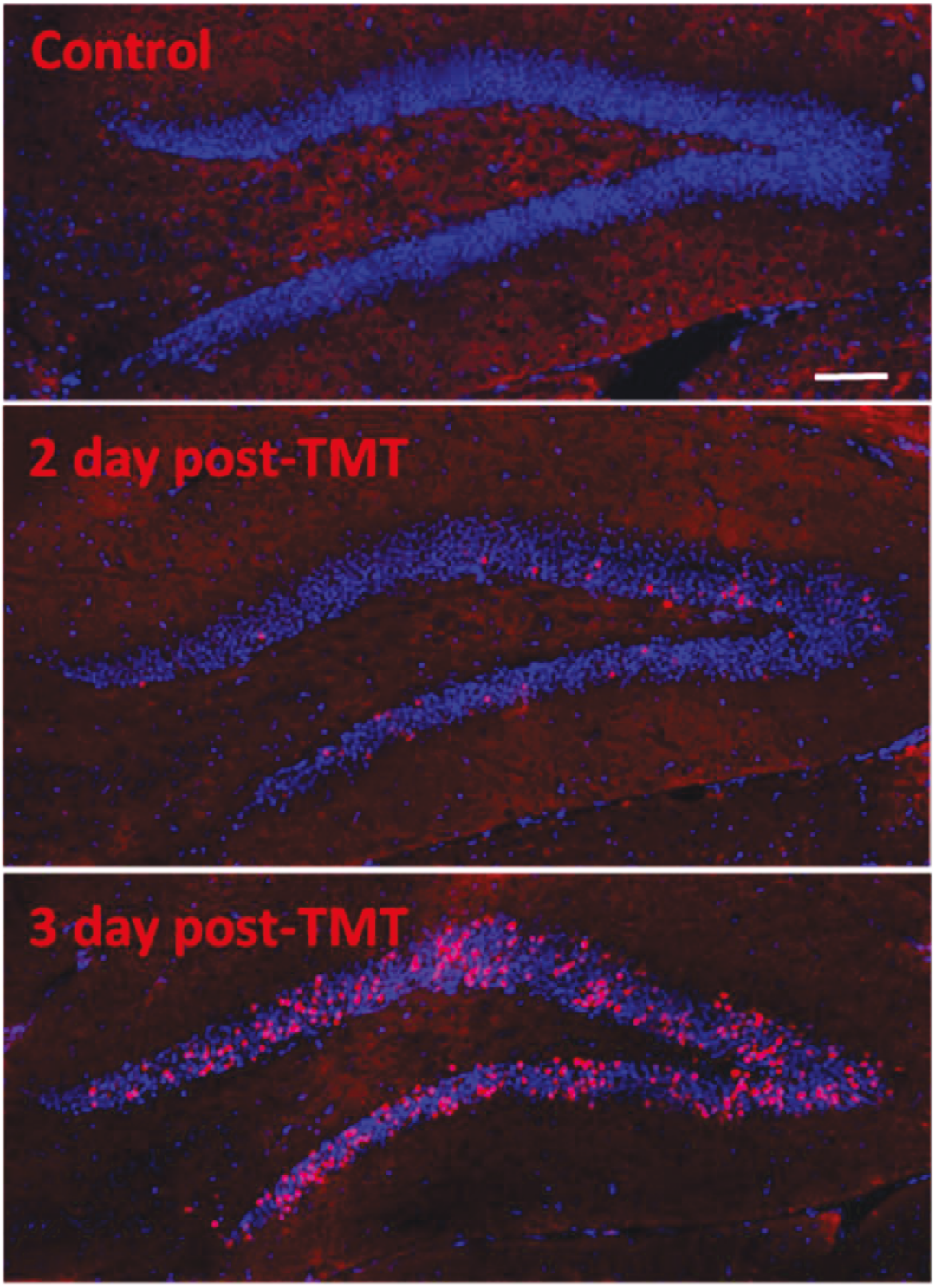


Figure 12. Time-course effect of 2.8 mg/kg TMT on induction of apoptosis in hippocampus dentate gyrus showing peak cell death at 3 days post-TMT exposure.



Data are mean of 5 animals per group; one-way ANOVA-Dunnetts test; *P<0.05.

Figure 13. TMT Time-course effect on apoptotic cell death of granule cell neurons in the hippocampus dentate gyrus. (activated caspase-3, red; DAPI, blue). Scale bare = 100 micrometers



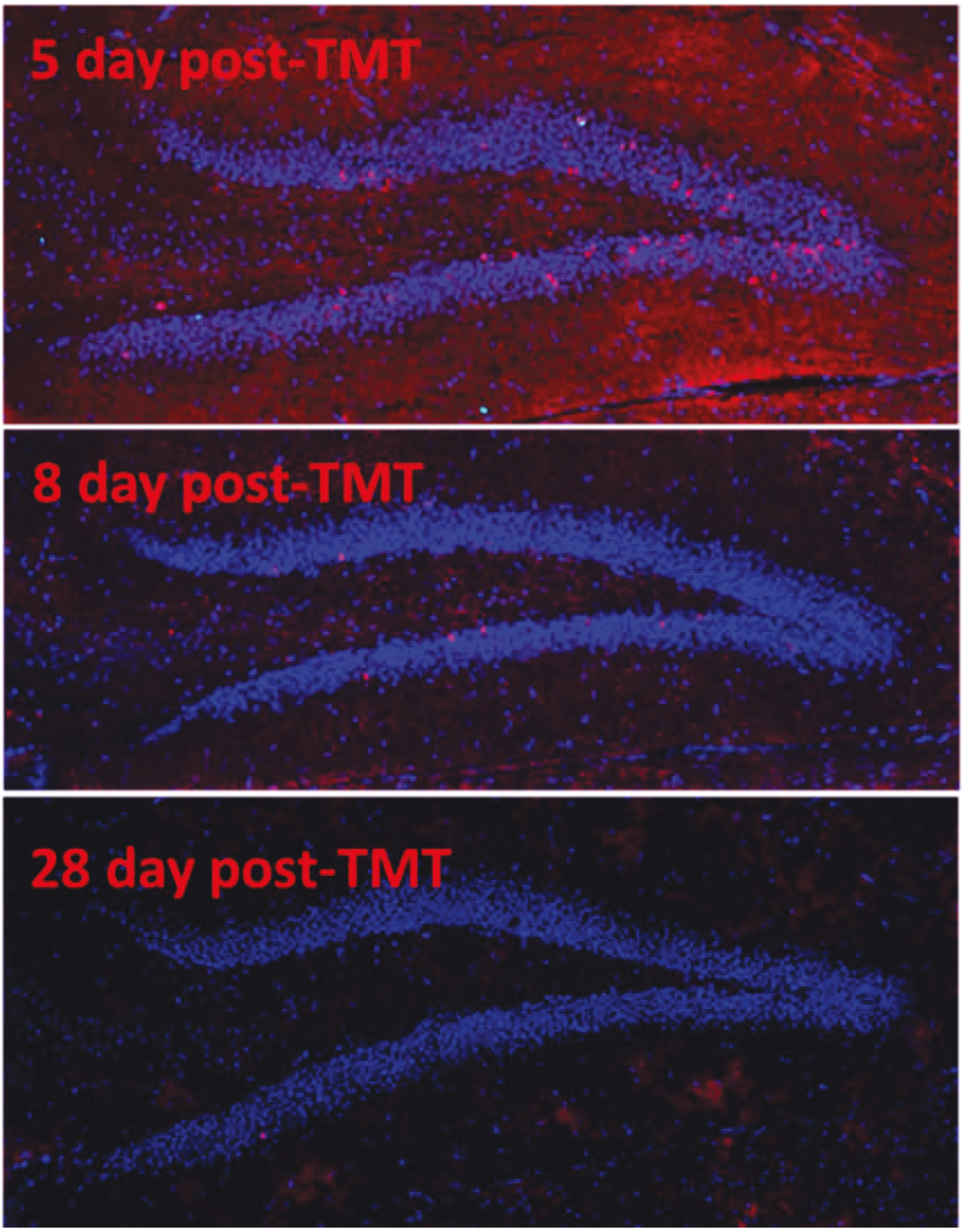
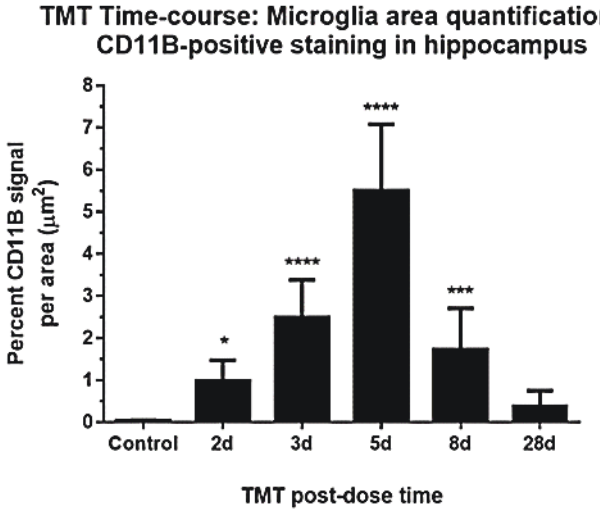

5 day post-TMT
8 day post-TMT
28 day post-TMT

Figure 14.Time-course showing activation of CD11b+ microglia in the hippocampus of mice treated with 2.8 mg/kg TMT.



Data are mean of 5 animals per group; one-way ANOVA-Dunnetts test; *P<0.05.

Figure 15. TMT Time-course effect on microglial and astrocyte activation (CD11b, red; GFAP, green; DAPI, blue)

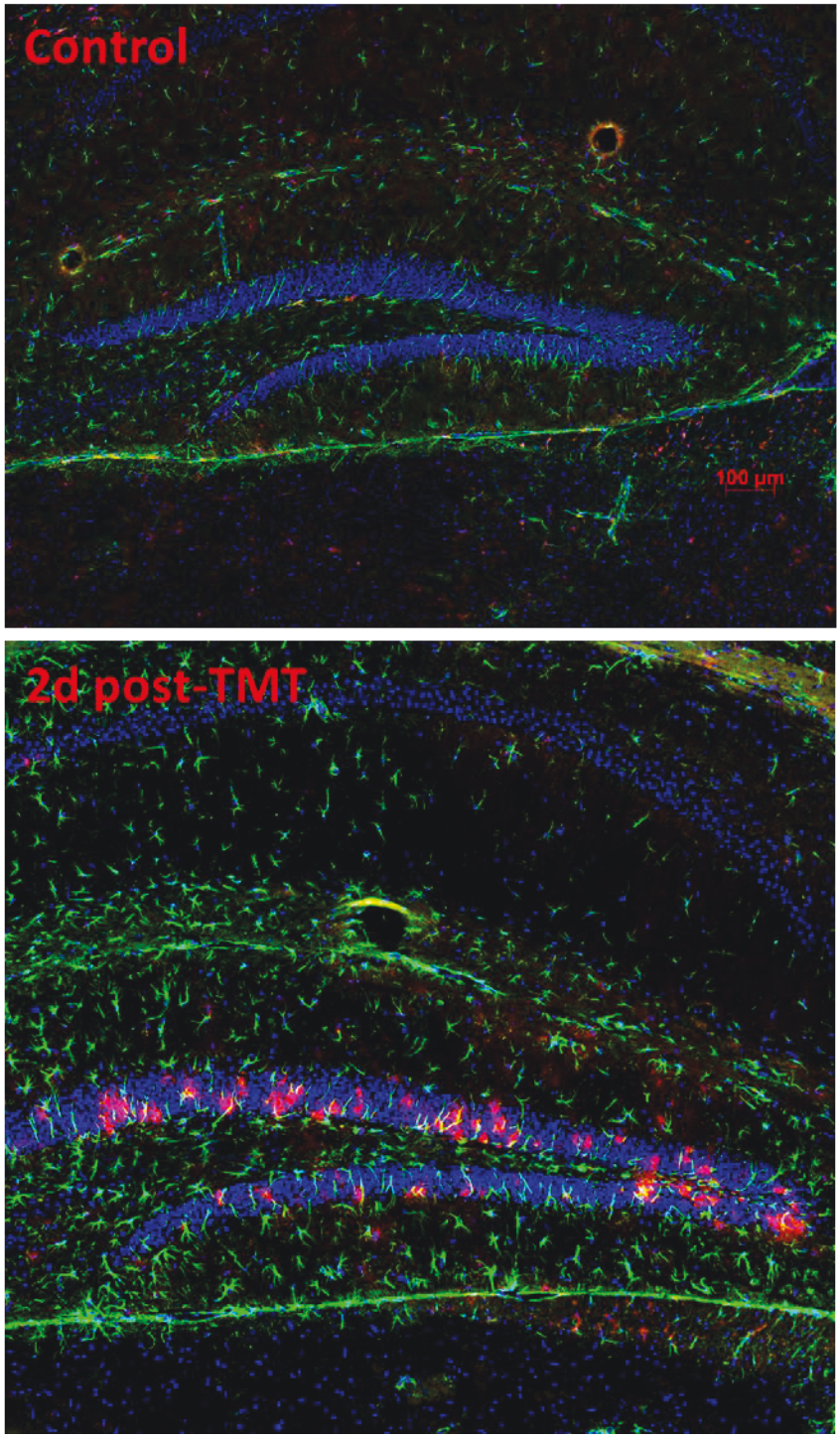


Figure 15. TMT Time-course effect on microglial and astrocyte activation (CD11b, red; GFAP, green; DAPI, blue)

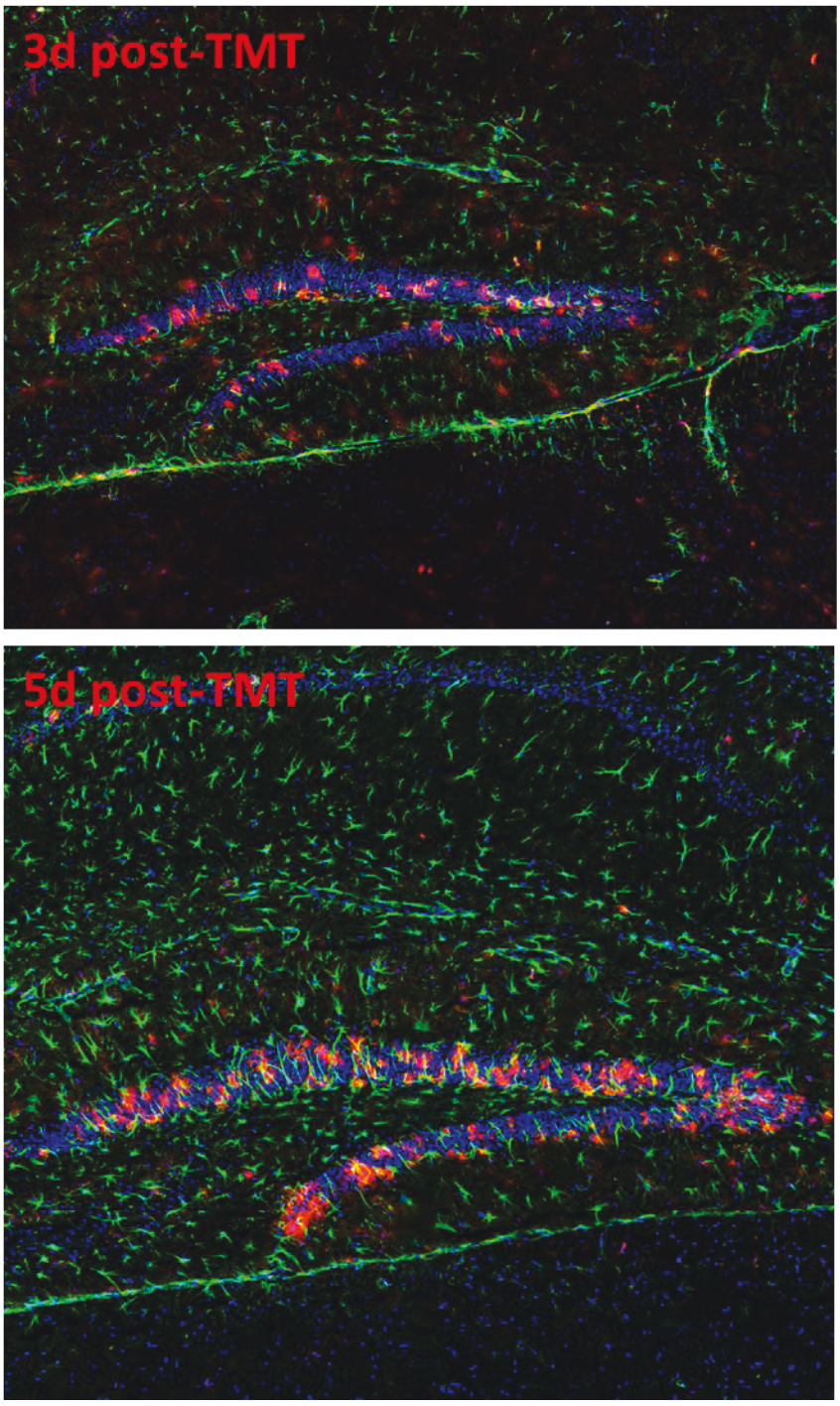


Figure 15. TMT Time-course effect on microglial and astrocyte activation (CD11b, red; GFAP, green; DAPI, blue)

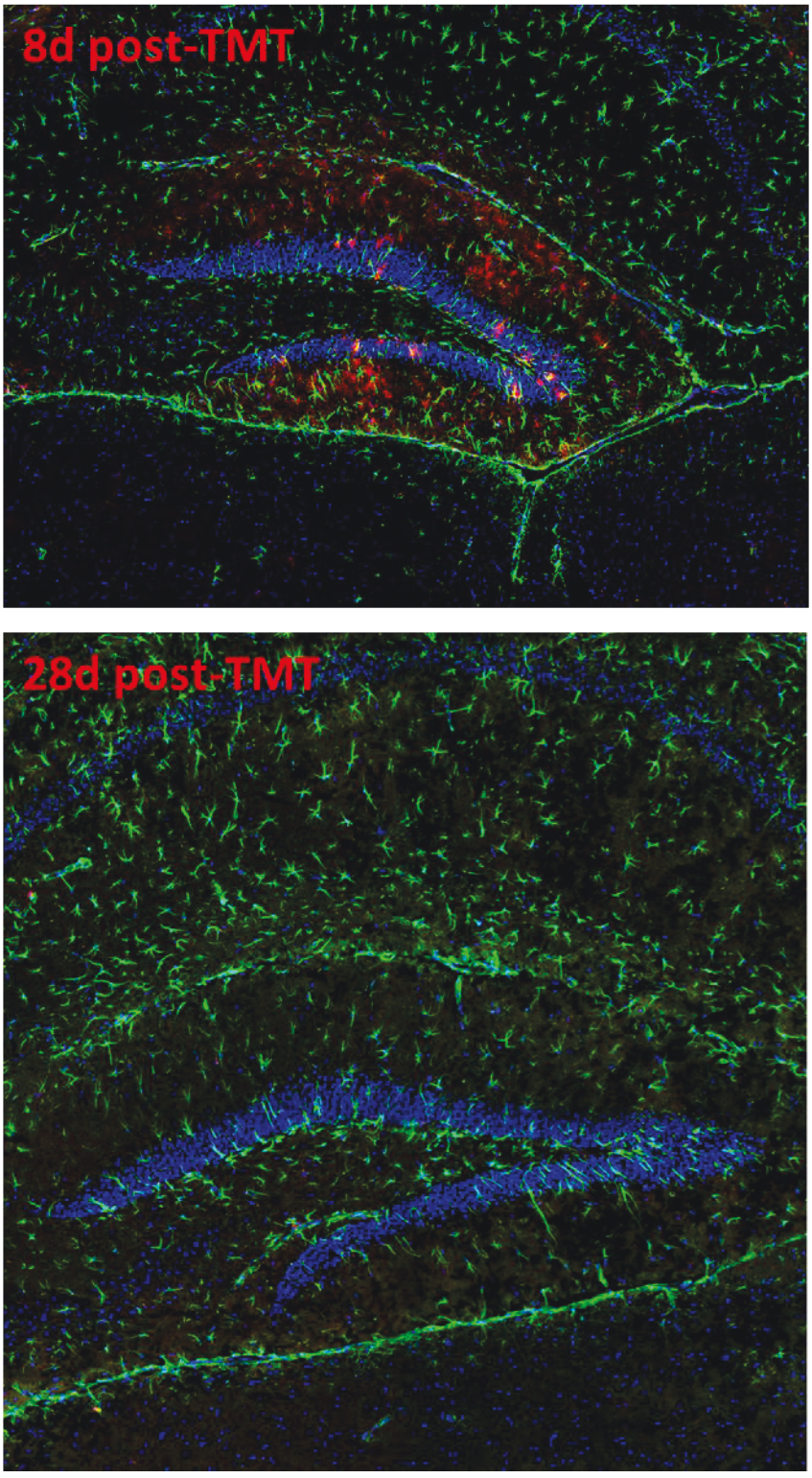
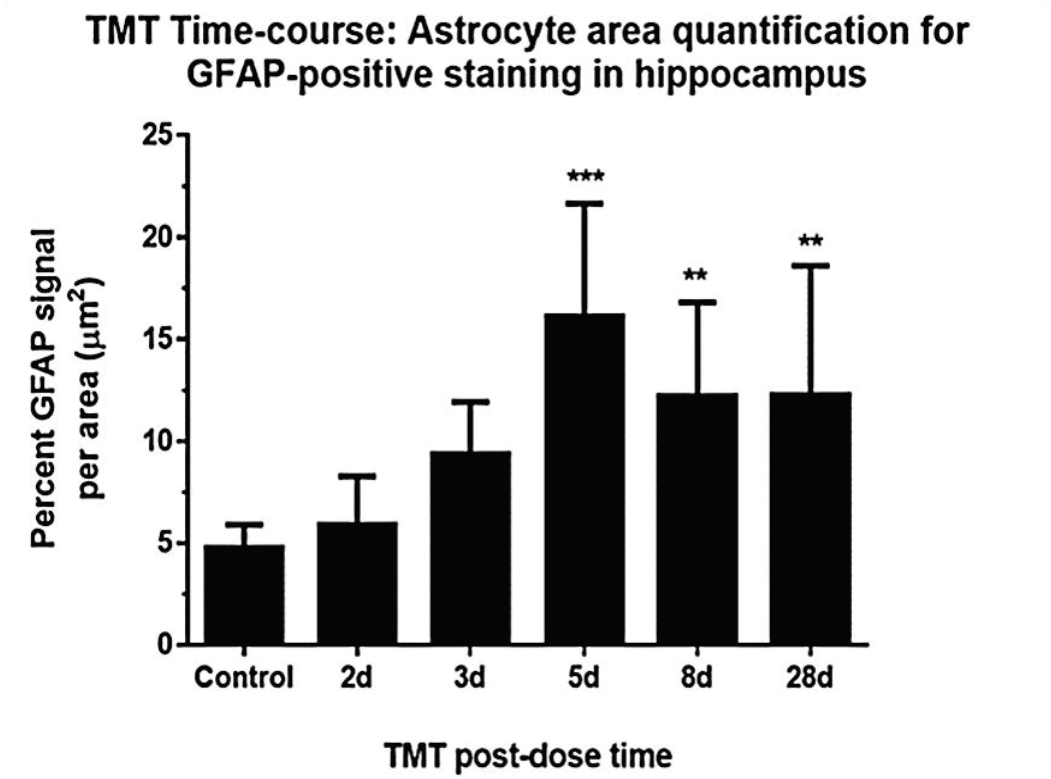


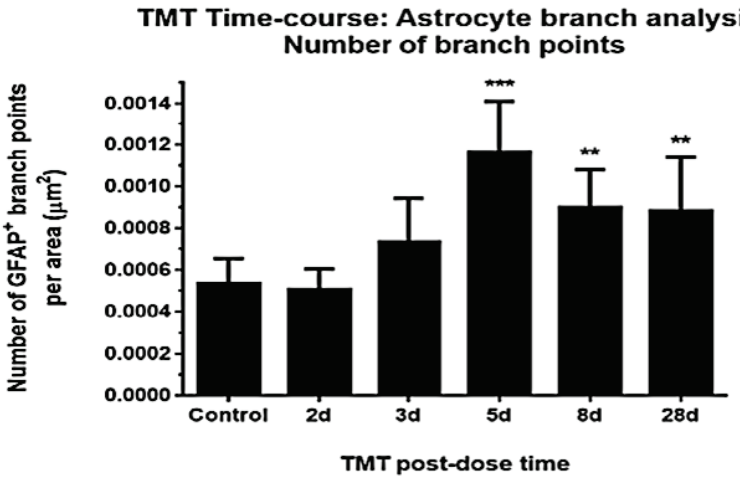
Figure 16. Quantification of GFAP+ immunoreactivity in hippocampus in time-course of TMT-induced injury showing gradual increase and persistence of reactive astrocytes.



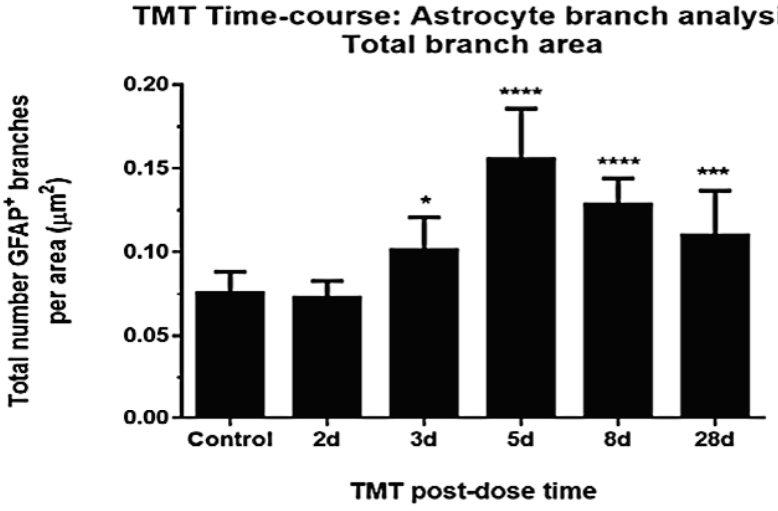
Data are mean of 5 animals per group; one-way ANOVA-Dunnetts test; *P<0.05.

Figure 17. TMT time-course: Branch analysis of GFAP+ astrocyte processes following exposure to 2.8 mg/kg TMT. A, number of branch points; B, total branch area; C, total branch thickness; D, branch length.

A



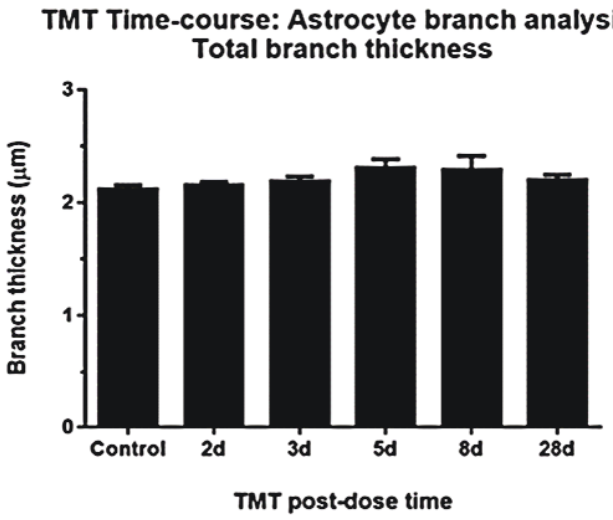
B



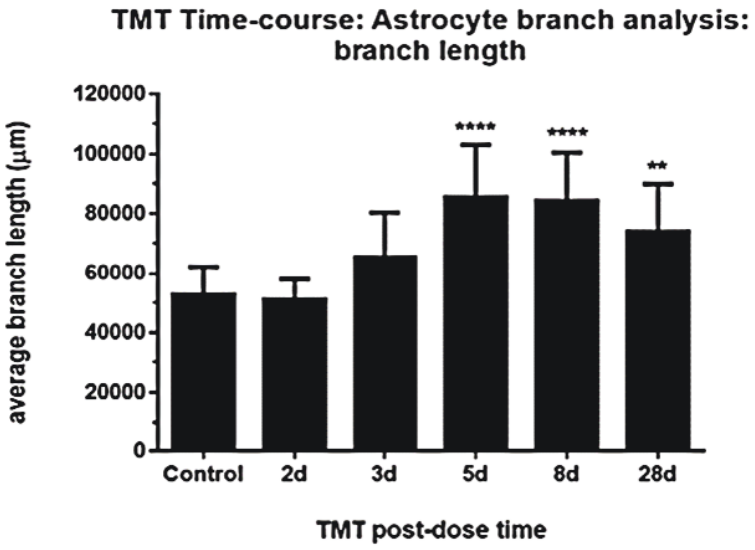
Data are mean of 5 animals per group; one-way ANOVA-Dunnetts test; *P<0.05.

Figure 17 (continued). TMT time-course: Branch analysis of GFAP+ astrocyte processes following exposure to 2.8 mg/kg TMT. A, number of branch points; B, total branch area; C, total branch thickness; D, branch length.

C



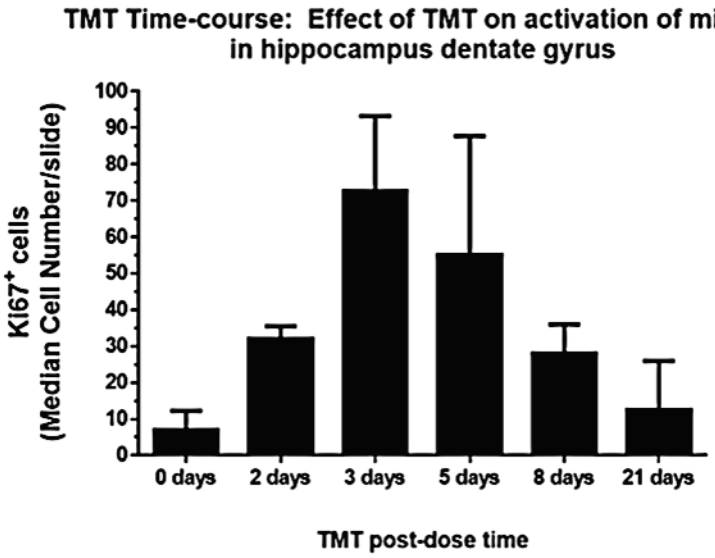
D



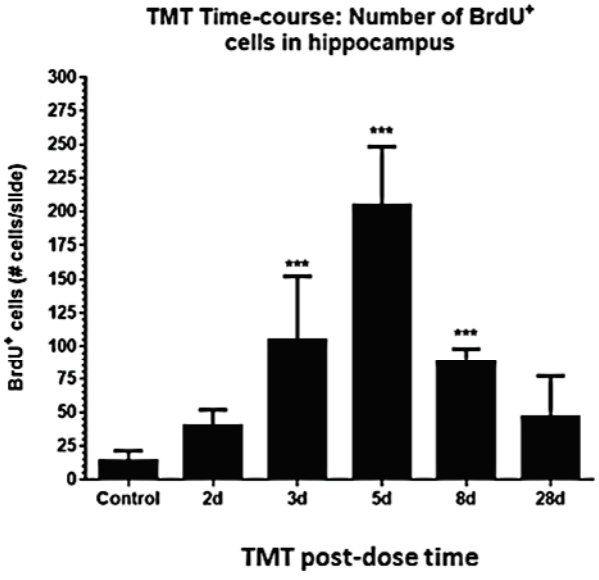
Data are mean of 5 animals per group; one-way ANOVA-Dunnetts test; *P<0.05.

Figure 18. TMT timecourse effect on number of Ki67$^+$ proliferating cells in the hippocampus. *A*, Ki-67$^+$; *B*, BrdU$^+$; and *C*,TBR2$^+$ cells in hippocampus.

A

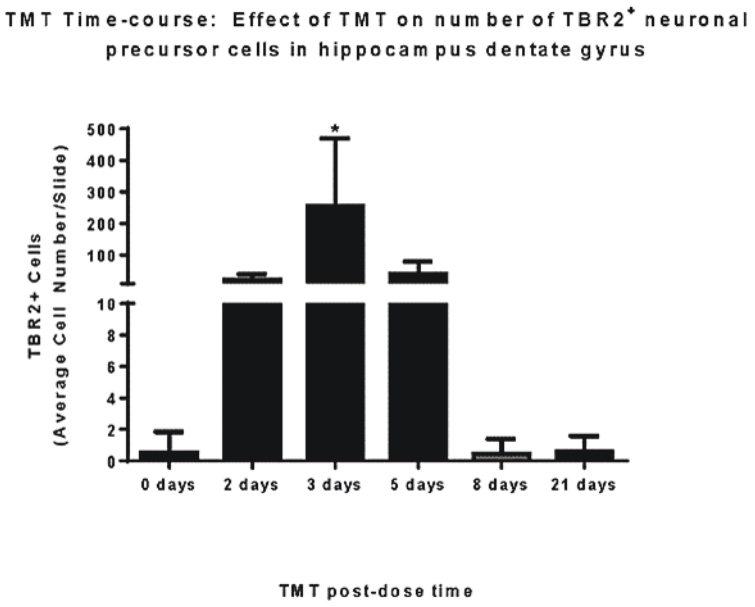


B



Data are mean of 5 animals per group; one-way ANOVA-Dunnetts test; *P<0.05.

C



Data are mean of 5 animals per group; one-way ANOVA-Dunnetts test; *P<0.05.

Figure 19. Colocalization of BrdU^{+} cells with mature phenotype markers for astrocytes (A) and neurons (B) in hippocampus dentate gyrus at 5 days post-TMT. A, BrdU, green; GFAP, red; DAPI, blue. B, BrdU, green; NeuN, red; BrdU+NeuN, yellow; DAPI, blue. Scale bar = 50 micrometers.

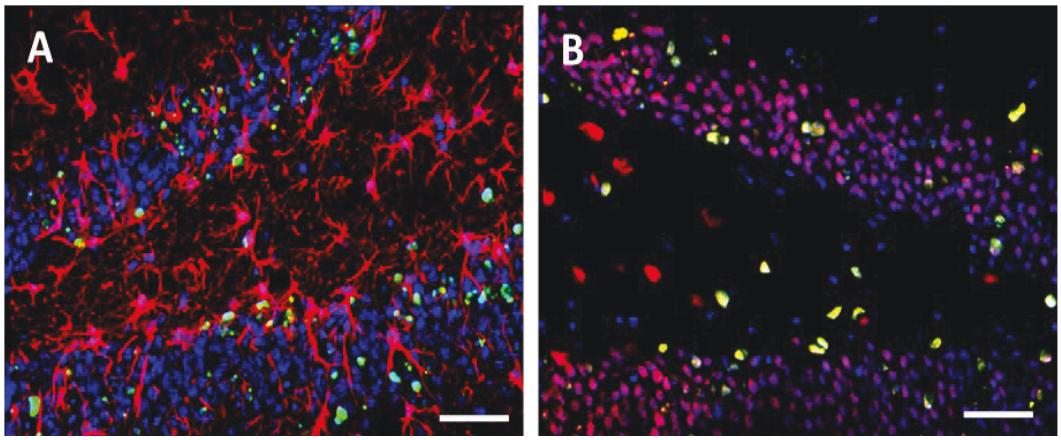
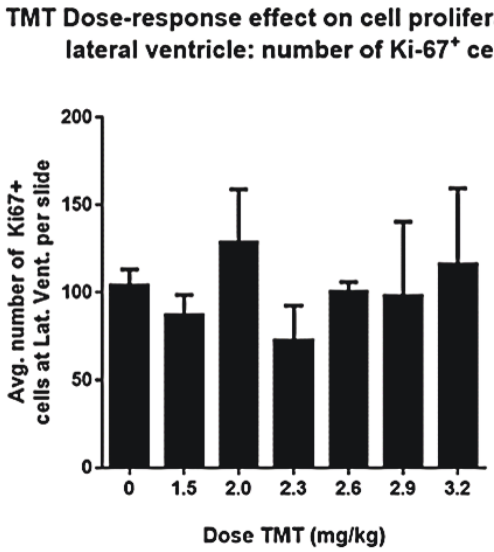


Figure 20. No effect by TMT on cell proliferation at the lateral ventricle subependymal zone at 48 hours post-exposure. The quantification of staining for Ki-67+ cycling cells is graphically represented.



Data are mean of 3 animals per group; one-way ANOVA-Dunnetts test; *P<0.05.

Figure 21. Ki67+ cells along lateral ventricle of mouse brain at 48 hours post-TMT dosing showing Ki67+ cells in SVZ and possibly ependymal cell layer (yellow arrows). Ki67, red; GFAP, green; DAPI, blue. Scale bar = 100 micrometers. LV, lateral ventricle.

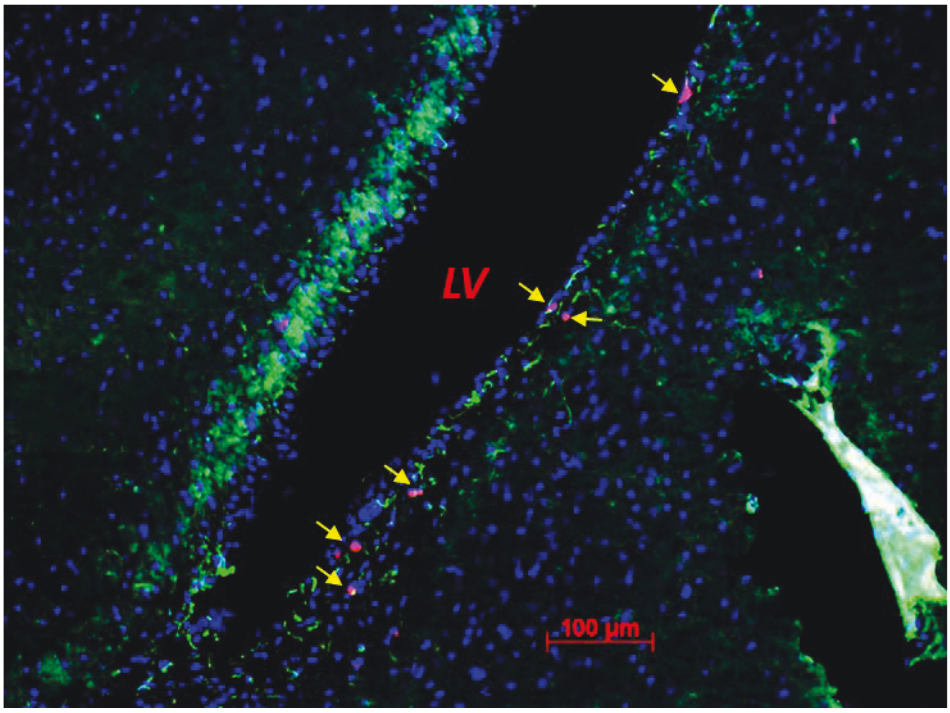


Figure 21 (continued). Ki67^{+} cells along lateral ventricle of mouse brain at 48 hours post-TMT dosing showing Ki67+ cells in SVZ and possibly ependymal cell layer (yellow arrows). Ki67, red; GFAP, green; DAPI, blue. Scale bar = 100 micrometers. LV, lateral venricle.

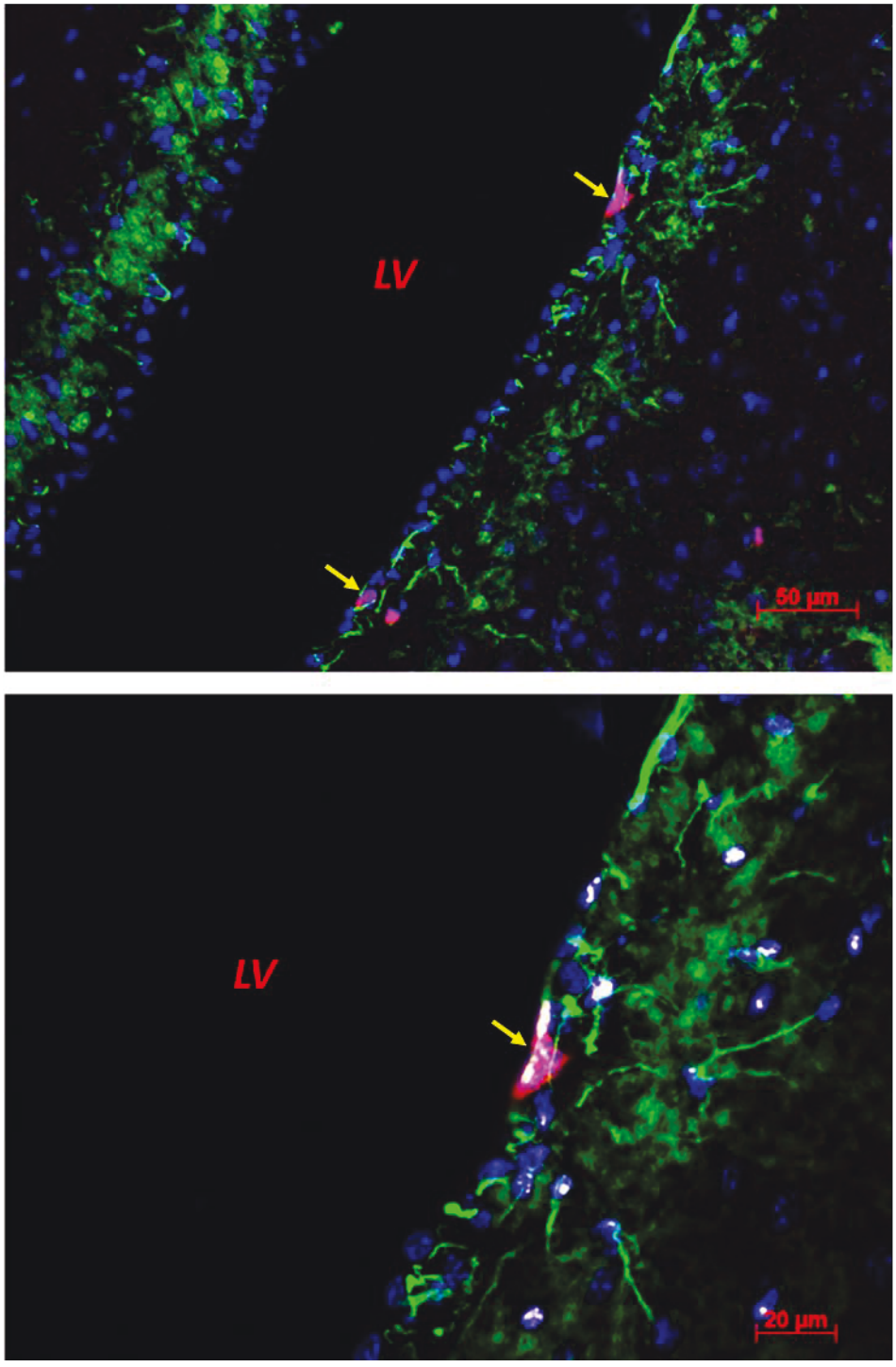


Figure 21 (continued). Ki67^{+} cells along lateral ventricle of mouse brain at 48 hours post-TMT dosing showing Ki67+ cells in SVZ and possibly ependymal cell layer (yellow arrows). Ki67, red; GFAP, green; DAPI, blue. Scale bar = 100 micrometers. LV, lateral venricle; cp, choroid plexus

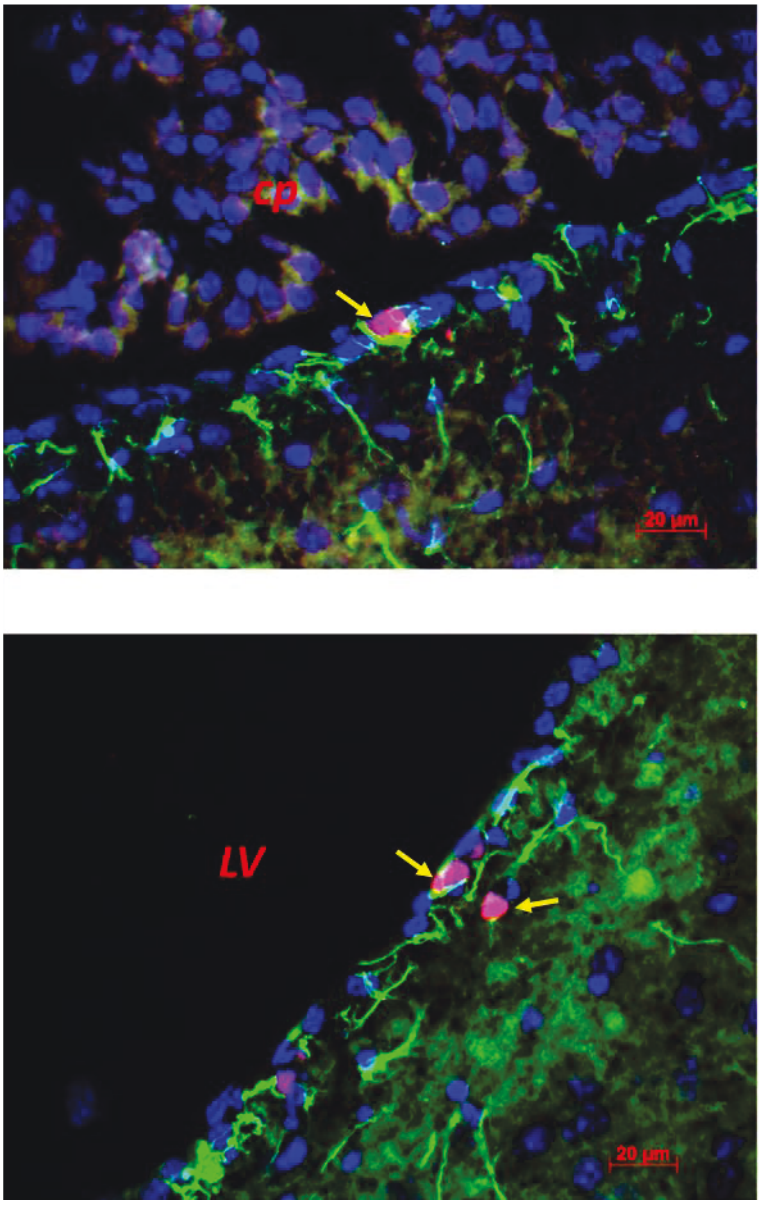
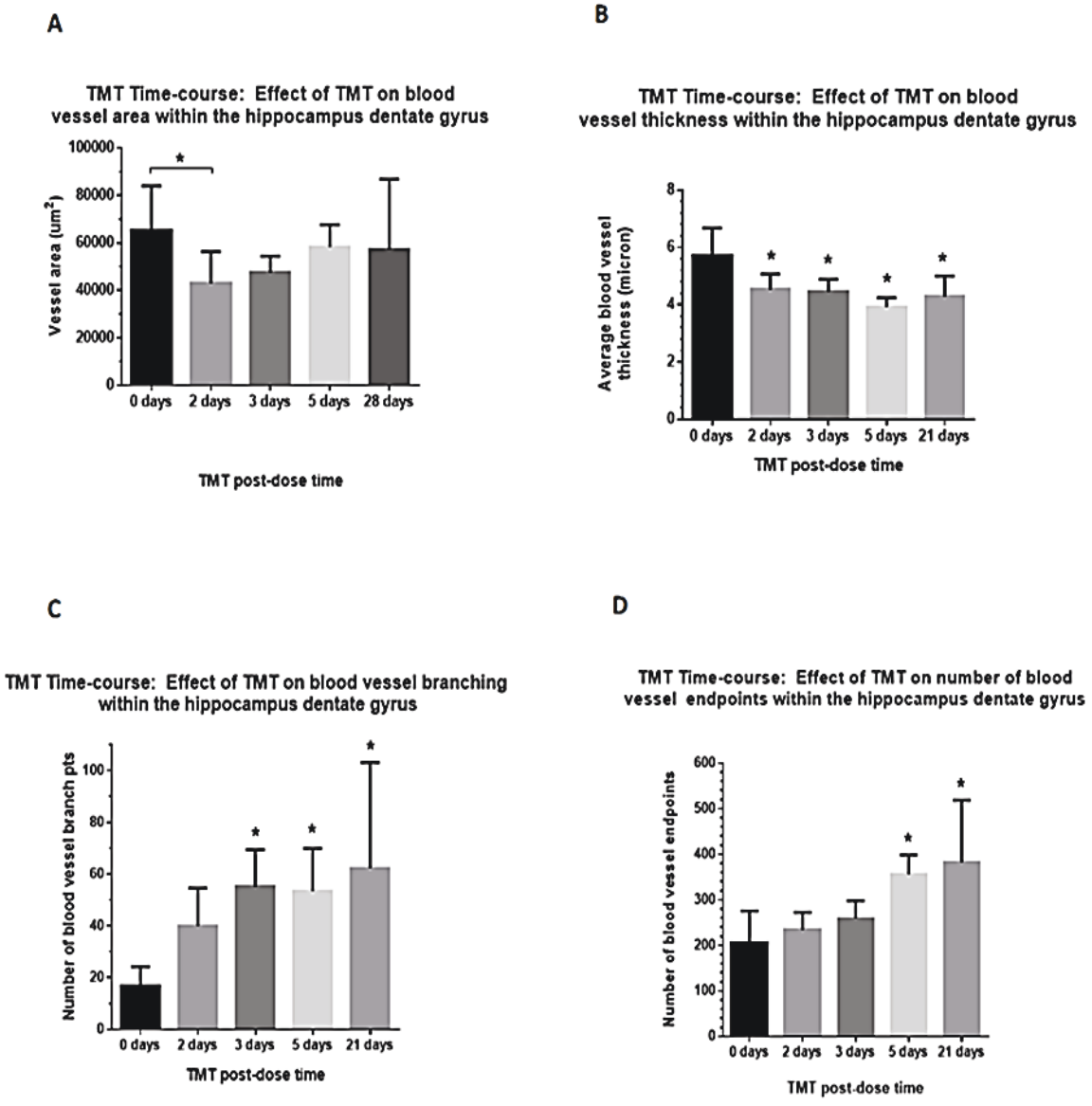
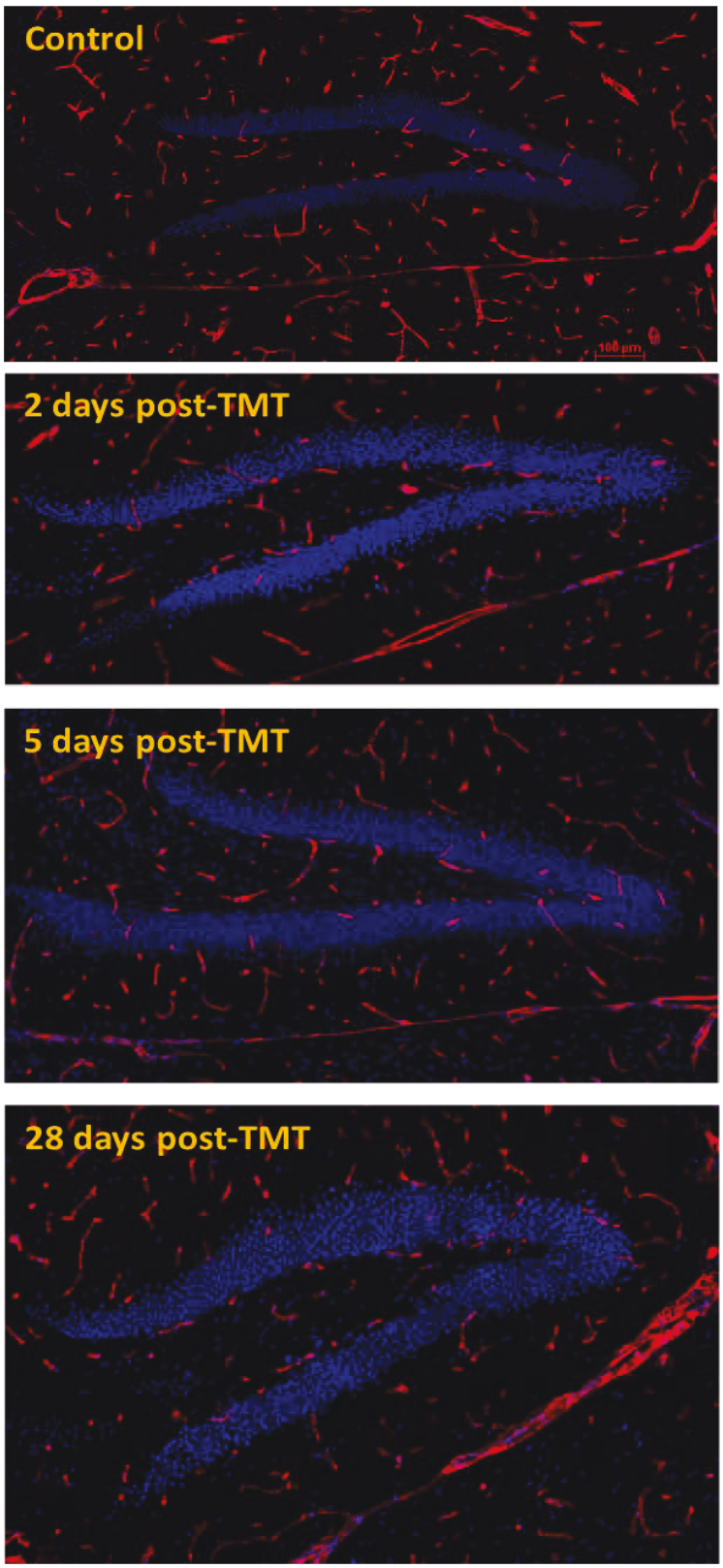


Figure 22. Effect of TMT on blood vessels in the hippocampus dentate gyrus as detected using anti-collagen IV antibody. Various blood vessel morphology parameters were quantified: A, vessel density (area) within the dentate gyrus; B, vessel thickness; C, number of vessel branch points; and D, number of vessel endopints.



Data are mean of 5 animals per group; one-way ANOVA-Dunnetts test; *P<0.05.

Figure 23. Time-course: blood vessel staining in the hippocampus dentate gyrus. Collagen IV, red; DAPI, blue.



*CHAPTER 3. TRIMETHYLTIN INTOXICATION INDUCES THE MIGRATION OF VENTRICULAR/SUBVENTRICULAR ZONE CELLS TO THE INJURED MURINE HIPPOCAMPUS

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Abstract

Following the postnatal decline of cell proliferation in the mammalian central nervous system, the adult brain retains progenitor cells with stem cell-like properties in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampus. Brain injury can stimulate proliferation and redirect the migration pattern of SVZ precursor cells to the injury site. Sublethal exposure to the neurotoxicant trimethyltin (TMT) causes dose-dependent necrosis and apoptosis in the hippocampus dentate gyrus and increases SGZ stem cell proliferation to generate new granule cells. To determine whether SVZ cells also contribute to the repopulation of the TMT-damaged dentate gyrus, 6-8 week old male C3H mice were injected with the carbocyanine dye spDiI and bromodeoxyuridine (80 mg/kg; ip.) to label ventricular cells prior to TMT exposure. The presence of labeled cells in hippocampus was determined 7 and 28 days after TMT exposure. No significant change in the number of $BrdU^+$ and $spDiI^+$ cells was observed in the dentate gyrus 7 days after TMT treatment. However, 28 days after TMT treatment there was a 3-4 fold increase in the number of spDiI-labeled cells in the hippocampal hilus and dentate gyrus. Few $spDiI^+$ cells stained positive for the mature phenotypic markers NeuN or GFAP, suggesting they may represent undifferentiated cells. A small percentage of migrating cells were $BrdU^+/spDiI^+$, indicating some newly produced, SVZ- derived precursors migrated to the hippocampus. Taken together, these

data suggest that TMT-induced injury of the hippocampus can stimulate the migration of ventricular zone-derived cells to injured dentate gyrus.

Keywords

Neurogenesis, trimethyltin, hippocampus, neural stem cells, subventricular stem cells, brain injury

*Chapter 3 reproduced from the manuscript submitted and published (Weig et al., 2016)

Introduction

Cell proliferation in the developing central nervous system of rodents declines rapidly during the perinatal period, with the final stages of hippocampal and cerebellar neurogenesis largely completed by postnatal day 21. However, some neurogenesis persists in the rodent adult brain, primarily through the activity of stem cells localized in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampus (Gage 2000; reviewed by Ming et al., 2011). Slowly dividing radial-glia-derived neural stem cells residing in the subventricular zone of the lateral ventricle give rise to neural precursors that migrate to the olfactory bulbs and once there, disperse radially and differentiate into olfactory interneurons (Merckle et al., 2004). This long distance tangential migration, referred to as the rostral migratory stream, is facilitated by a network of astrocytic tube-like processes that create both an attachment scaffold and molecular guidance cues to migrating neuroblasts (Doetsch et al., 1997; Conover et al., 2000). The migration of SVZ precursors is confined to the rostral migratory stream under physiological conditions, however, injury or neurodegenerative processes can induce progenitor cell proliferation and

migration of SVZ-derived neuroblasts toward affected non-olfactory brain regions (De Marchis et al., 2004; Nakatomi et al., 2002; Arvidsson et al., 2002; Zhang et al., 2004a; Yamashita et al., 2006; Goings et al., 2004).

A second neurogenic environment is located in the subgranular zone (SGZ) of the hippocampal dentate gyrus which generates new cells throughout life (Scharfman 2000). Within the SGZ are quiescent radial glia-like cells (Seri et al., 2001; Filippov et al., 2003), coined type-1 cells, that give rise to mitotically active type 2a multipotent stem cells. These cells produce lineage restricted neural progenitors (type 2b cells) that generate neuroblasts (type 3 cells) destined to become new granule cell neurons. Unlike SVZ-derived neuroblasts, hippocampal neuroblasts only migrate short distances into the granule cell layer (GCL) of the dentate gyrus where they differentiate into granule cells. Progenitor proliferation rate and neurogenesis in the hippocampal SGZ are acutely responsive to injury, neurological and chemical factors including physical activity (van Praag et al., 1999), environmental enrichment (Nilsson et al.,1999), neurotransmitters (Cameron et al., 1995; Brezun and Daszuta 1999; Brazel et al., 2005), gonadal and adrenal hormones (Gould et al., 1992; Cameron and Gould, 1994; Cameron et al., 1998; Galea et al., 2013) and antidepressants (Malberg et al., 2000; Santarelli et al., 2003). The effect of injury on proliferation and neurogenesis in the hippocampus is dependent on the injury model and the extent of the injury. Further, if the injury involves alteration of the neurogenic environment, it can impart abnormal remodeling through ectopic localization of new cells or disproportionate gliogenesis at the expense of neurogenesis (Parent et al. 1998; Ferland et al. 2002; Scott et al., 2000; Monje et al., 2002; Madsen et al., 2003; Scharfman et al., 2000; Gray and Sundstrom, 1998; Rola et al., 2006; Wang et al., 2012).

In vitro studies of isolated hippocampal stem cells reveal multipotency upon exposure to appropriate factors (Palmer et al., 1997), but *in vivo* multipotency has not been clearly demonstrated. Numerous models have been proposed to describe the identity and mechanism of lineage fate progression for neural stem cells (reviewed by Guo-Ming et al., 2011). Both asymmetric and symmetric cell division has been observed; however, it is clear that there is a loss of stem cell numbers with aging. It has been proposed that SGZ stem cells may irreversibly enter a quiescent state, undergo cell death, or leave the stem cell pool after a finite number of cell divisions and subsequently differentiate into a post-mitotic astrocyte (Encinas et al., 2011), resulting in a diminished hippocampal neuronal cell density in the aged rodent. Injury or other extraneous factors may radically accelerate turnover in the stem cell pool, potentially leading to a premature depletion of viable stem cells later in life if no mechanism exists to replenish this pool. Perhaps to conserve this finite stem cell capacity, hippocampal neurogenesis occurs at a much lower frequency in comparison to SVZ neurogenesis, which has been shown to undergo asymmetric division to maintain the stem cell pool (Morshead et al., 1998; Zhang et al., 2004b).

Trimethyltin (TMT) is a potent hippocampal neurotoxicant that preferentially damages regions of the limbic system in rodents (Chang et al., 1983). In adult mice, TMT induces extensive apoptosis and necrosis within the hippocampal dentate gyrus granule cell layer within 24-36 hours after exposure (Chang et al., 1982; Fiedorowicz et al., 2001). Harry et al. (2004) first demonstrated that TMT-induced injury stimulates a strong neurogenic response by endogenous stem cells in the murine hippocampal subgranular zone. Further studies showed the utility of this injury model for studying neurogenesis and repair mechanisms in the hippocampus (Ogita et al., 2005, 2012; Corvino et al., 2005; McPherson et al., 2011; Yoneyama et al., 2014). The current study tests the hypothesis that TMT-induced injury of the mouse dentate gyrus triggers migration of cells from ventricular neurogenic regions to the lesion site as part of a potential repair response.

Materials and Methods

Animals and TMT exposure

Male C3H mice aged 6-8 weeks (Taconic, NY) were housed in polycarbonate cages with wood chip bedding and maintained according to AALAC guidelines. Trimethyltin chloride (TMT, Sigma, St. Louis, MO) was dissolved in normal saline at a concentration of 2.5 mg/ml. A working solution was prepared in saline (0.25 mg/ml) and a single intraperitoneal injection was administered to mice at a dose (2.7 mg/kg) that would induce tremor activity within 24-36 hours and recovery by 72-94 hours post-dose. Groups of 5-8 mice were used for generating saline- and TMT-injected brain samples.

spDiI and BrdU labeling of ependymal/subventricular cells

The method of intraventricular injection of spDiIC18(3) ([1,1-dioctadecyl-6,6-di(4-sulfophenyl)-3,3,3,3-tetramethylindocarbo-cyanine], Life Technologies) was described previously (Johansson et al., 1999). Briefly, mice were sedated by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (12 mg/kg). An incision was made along the midline of the cranial surface and 3ul spDiI (0.2% w/v in DMSO) were injected i.c.v. into one lateral ventricle 2 mm below the dura mater at 0.5 mm posterior and 0.7 mm lateral to bregma (Paxinos and Franklin, 2001). The incision was closed using Vetbond tissue adhesive (3M Animal Care Products) and mice were permitted to recover from surgery for 48 hours prior to TMT injection.

To track the migration of rapidly proliferating cells including mitotically active cells in the SVZ (i.e. type C cells; Doetsch et al., 1997), mice were dosed with bromodeoxyuridine (BrdU; 50 mg/kg) every two hours for twelve hours (ip). Twenty-four hours after the last BrdU dose, the mice were then injected with TMT (2.7 mg/kg; ip) and returned to normal housing. The mice were euthanized by CO_2 asphyxiation 7 days and 28 days later; the brain was removed and flash frozen at -70 ^{o}C for subsequent immunohistological analysis.

Tissue sectioning and immunohistochemistry

Twelve-micrometer cryostat sections were prepared from stereotaxically-defined regions of hippocampus (-1.46 to 2.40 mm relative to bregma), mounted on slides and post-fixed by immersion in 4% para-formaldehyde at room temperature for 15 minutes. Antigen retrieval was performed by heating slides in 10 mM sodium citrate (pH 5.5) to boiling for 10 minutes, then cooling to room temperature before mounting onto Shandon coverplates (Thermo Scientific, MA). Samples were incubated overnight at 4^{o}C with primary antibodies prepared in blocking buffer containing 3% donkey serum and 0.3% Tween-20. Primary antibodies used were sheep anti-BrdU (5ug/ml; Research Diagnostics, Inc.), anti-GFAP (1:100; Sigma) for astrocytes, anti-NeuN (1:100; Chemicon) for neurons, anti-cd11b (1:200; BD Pharmingen) for microglia, and anti-collagen IV (Pierce) for staining blood vessels. For BrdU staining, slides were acid digested in 2.5 M HCl at 37^{o}C for 30 minutes. HCl-digested samples were then neutralized in 10 mM sodium borate solution (pH 8) for 5 min before incubation with sheep anti-BrdU primary antibody in blocking buffer. NeuN primary antibody staining was performed overnight at 4^{o}C after the antigen retrieval step used for BrdU staining. For GFAP and BrdU co-staining, slides were microwaved for 15 minutes in 10 mM sodium citrate (pH 5), cooled to room temperature, and then incubated

with BrdU and GFAP primary antibody overnight at 4^{o}C. Slides were then incubated for 45 minutes at room temperature with Alexa Fluor-conjugated secondary antibodies (1:300; Invitrogen, CA) prepared in blocking buffer.

Imaging and cell quantification

Tissue sections were visualized by epifluorescence using a Zeiss Axiophot microscope equipped with a CoolSnap digital camera and Image-Pro Plus image analysis software (Advanced Imaging Concepts, Princeton, NJ). Coronal sections of matched anatomical levels along the rostro-caudal axis were used for analysis. Quantification was performed only in hemispheres contralateral to spDiI injection (icv) on 8-10 sections/animal with an intersection distance of 90 micrometers. Data were analyzed using Graphpad Prism 6 (Graphpad software version 6.05). All data are expressed as standard error of the mean (SEM) and considered statistically significant when $P < 0.05$. Data compared using two-way ANOVA with two fixed factors: condition (Ctrl vs. TMT-treated) and time (7 day vs. 28 day). Post-hoc analyses were performed using Tukey multiple comparison test with p-values presented in text as multiplicity adjusted p values. The percentage of spDiI^{+} cells that co-localized with BrdU staining was determined by dividing the number of spDiI^{+}/BrdU^{+} cells by the total number of cells positive for spDiI labeling for each of 4 representative slides for each animal in the treatment group, followed by calculation of the mean for the group.

Results

To determine whether periventricular cells (ependymal or subventricular) migrate into the hippocampus in response to TMT injury, the lipophilic carbocyanine dye spDiI was unilaterally injected into the lateral ventricle 48 hrs prior to TMT exposure. In this study, virtually the entire ependymal cell lining of the ventricular system was intensely labeled by spDiI (Fig. 1*A*), with some localized penetration (or transport) of the dye into the underlying 1-2 cell layers of the subependymal region (Fig. 1*C-F*).

Seven days after TMT exposure, labeling in both control and TMT-treated mice was essentially limited to the ventricular system, with rare labeled cells appearing in the rostral migratory stream and olfactory bulb (Fig. 1B). spDiI$^+$ ependymal cells and cells localized outside the ventricular region exhibited a punctate fluorescence pattern, reflecting possible association of the dye with endosomal/lysosomal intracellular compartments as previously reported for other DiI carbocyanine dye analogues (Mukherjee et al., 1999; Zhao et al., 2003). Over time, diffuse membrane labeling of migrated spDiI$^+$ cells diminished but positive cells could be easily discerned by the dense intracellular fluorescent inclusions. In this TMT model, mice showed a time- and treatment-dependent effect on the localization of spDiI$^+$ cells. spDiI$^+$ cells were limited to the ventricular/subventricular zone at the time TMT injection with increasing numbers of cells found in the hippocampal region 28 days following TMT-induced injury. A two-way ANOVA revealed significant main effect of TMT treatment ($F_{1,19} = 9.067$; $P = 0.007$), time ($F_{1,19} = 15.93$; $P = 0.0008$) and interaction between time and treatment ($F_{1,19} = 5.882$; $P = 0.025$). In the 28 day post-injection control group a small number of spDiI$^+$ cells appeared in the hippocampus region, but the 7 day control group ($p = 0.68$). However, brains from mice euthanized 28 days after TMT treatment exhibited a marked and significant increase in number of spDiI$^+$ cells in the hippocampus (Fig. 2F) compared to 28 day untreated mice ($p = 0.003$). Labeled cells were observed in the hippocampal dentate gyrus GCL and SGZ, the polymorphic layer of the hilus, and the molecular layer. spDiI$^+$

cells were often found in the molecular layer between the infra-pyramidal blade of the dentate gyrus granule cell layer and the adjacent ventricular process (Fig. 2*B-D*), suggesting that spDiI$^+$ cells were migrating toward the dentate gyrus from this region. spDiI$^+$ cells were rarely noted in the pyramidal layer (Ammon's horn). Because adult neural stem cells and progenitor cells tend to cluster in neurogenic niches of which the cerebrovasculature is an integral part, we determined if spDiI$^+$ cells in the SGZ were in proximity to blood vessels in the SGZ. Fluorescent staining of blood vessels using anti-collagen IV staining showed that some spDiI$^+$ cells had localized to the cerebrovasculature (Fig. 2E). The number of spDiI$^+$ cells associated with blood vessels was limited and insufficient for statistical analysis.

Counterstaining of spDiI$^+$ cells in the dentate granule layer for the cell-specific markers NeuN and GFAP revealed that some of these cells were putatively neurons or astrocytes, respectively (Fig. 3A,B). Microglial cells, identified by staining with cd11b, represented only a small proportion of the spDiI$^+$ cells in the hippocampus (Fig. 3C). Overall, most spDiI$^+$ cells were negative for these markers, which may indicate they represented a different phenotype or undifferentiated cells.

The duration of bromodeoxyuridine administration (12 hours) yielded labeling of mitotic cells in the SVZ and hippocampus of 7 day control and TMT-treated mice. BrdU$^+$ cells in the hippocampus were present throughout the dentate gyrus, hilus, and molecular layers, but most were localized to the SGZ. Two-way ANOVA showed a significant interaction between treatment and time ($F_{1,18} = 10.68$; $P = 0.0043$), and main effect of treatment ($F_{1,18} = 5.690$; $P = 0.028$), but not a main effect of time ($F_{1,18} = 0.537$; $P = 0.473$). Although there was no significant difference in number BrdU-labeled cells in the hippocampus between the 7 day control, 7 day TMT- and 28 day TMT-treatment groups ($p>0.27$), the number of BrdU$^+$ cells in the hippocampus of the 28-day control mice (Fig. 4D) was significantly lower (75% reduction) compared to the 28 day TMT-treated mice ($p = 0.004$). Furthermore, BrdU$^+$ cells were more frequently observed along the

molecular layer between the infra-pyramidal blade of the dentate gyrus granule cell layer and the meninges in the 28 day TMT treated mice, which paralleled the apparent migration of spDiI$^+$ cells (Fig. 4*A*-*C*). Only a small proportion of the BrdU$^+$ cells in the hippocampus co-labeled with spDiI (10.4%) suggesting either that they were derived from a population separate from origin of the spDiI$^+$ cells, or may represent the same population but were ineffectively labeled with spDiI. Additionally, the BrdU$^+$ cells in the molecular layer did not co-label with mature phenotypic markers for neurons or astrocytes.

Discussion

Studies have shown that TMT injury will induce neurogenesis in the murine hippocampus to repopulate the damaged dentate gyrus (Harry et al., 2004; Ogita et al., 2005, 2012; McPherson et al., 2011). This model is useful for studying activation and proliferation of neural stem cells, as well as factors modulating migration of neuronal precursors (McPherson et al., 2011; Casalbore et al., 2010). While some injury models may irreversibly damage or alter the neurogenic microenvironment resulting in increased gliogenesis at the expense of neurogenesis (Monje et al., 2002; Rola et al., 2006; Ledergerber et al., 2006; Kralic et al., 2005), the TMT-injured hippocampus remains permissive for the formation of new neural cells.

The primary goal of this study was to determine whether endogenous precursor cells derived from the ventricular/subventricular zone (SVZ) migrate into the hippocampus dentate gyrus following trimethyltin-induced injury. Although SVZ-derived precursor cells in the adult CNS are capable of migrating to non-olfactory targets (e.g. striatum, corpus callosum) following injury

(Arvidsson et al., 2002; Menn et al., 2006; Yamashita et al., 2006), it is unclear whether precursors derived from ventricular regions can be directed to the hippocampus dentate gyrus.

Previous studies have shown the utility of carbocyanine dye analogues as *in vivo* tracers of cell migration in CNS injury models. Intracerebroventricular injection of the dye labels ependymal/periventricular cells upon contact, and has been shown in previous studies to enable tracking of DiI$^+$ ependymal or SVZ-derived cells mobilized following spinal, ischemic, EAE, or MPTP-induced insults (Johansson et al., 1999; Picard-Riera et al., 2002; Nakatomi et al., 2002; Zhao et al., 2003). In our studies, we observed stable labeling of periventricular regions with the dye for up to 1 year in control animals (data not shown). Since the hippocampus does not come into direct contact with the dye via icv injection (Picard-Riera et al., 2002), the tracer was essentially confined to the ventricular lining. However, in this study, spDiI$^+$ cells appeared to migrate across the alveus toward the ventral blade of the dentate gyrus at 28 days following TMT injury. This raises the possibility that the TMT injury is recapitulating developmental processes to repopulate the GCL/SGZ with cells derived from the ventricular zone (Li et al., 2009, 2013). Perhaps there exists a mechanism for renewing the population of stem/progenitor cells in the adult hippocampus following acute injury. Whether such a mechanism is activated with injury or under physiologically normal conditions is unknown. Based on recent data, type 1 hippocampal stem cells are proposed to undergo a limited number (3-4) of passages of asymmetric cell division followed by terminal differentiation into astrocytes. This limited duration of 'stemness' is theorized to play a role in the age-related decline in stem cell activity and loss of granule cell layer density (Encinas et al., 2011).

As described above, BrdU was injected into the mice over a 12 hour period prior to the i.c.v. injection of spDiI and TMT exposure. During this short time frame, BrdU is primarily incorporated into the highly mitotically active population of cells including the transit amplifying type "C" cells

in the SVZ and type 2 neural progenitor cells in the hippocampus (Ming et al., 2011). The $BrdU^+$ cells were localized primarily in the SGZ and GCL of the dentate gyrus, as well as the hippocampal molecular layer. TMT had no effect on the number of $BrdU^+$ cells in the hippocampus 7 days after treatment, suggesting that TMT did not induce cell death of newly-born cells. This may be because mature integrated glutamatergic granule cells would potentially be the most vulnerable to excitotoxicity induced by TMT intoxication (He et al., 2013).

In contrast, there was an increased number of $BrdU^+$ cells in the hippocampus in the 28 day post-TMT group compared to the 28 day vehicle control group. This could be explained by (i) cell division of $BrdU^+$ endogenous hippocampal precursors; (ii) migration of $BrdU^+$ SVZ precursors into the hippocampus; or (iii) increased survival of $BrdU^+$ cells in the hippocampus. It is less likely that the number of $BrdU^+$ cells increased as a result of injury-induced mitotic activity since there was no significant difference between the 7 day post TMT and 7 day control groups. The seven-day time point was well past the peak proliferative response to TMT injury based on our observations (data not shown) and others (Ogita et al., 2005). Furthermore, indications of cell cycling of BrdU-labeled cells such as clustering of $BrdU^+$ cells were infrequently observed. The migration of $BrdU^+$ precursors from the subventricular zone into the hippocampus is a possible source of the cells. Indeed, a small proportion of $BrdU^+$ were also $spDiI^+$, indicating that they were exposed to dye in the ventricular region. Since the carbocyanine dye does not necessarily label all SVZ cells, and only those cells undergoing DNA replication at the time of BrdU dosing would incorporate the DNA marker, a low proportion of dual-labeled $spDiI^+/BrdU^+$ would be expected.

With respect to the third possibility, it cannot be discounted that upregulated trophic factors in the damaged hippocampus or other injury-induced modifications of the local microenvironment promoted the survival of new-born cells as part of the neurorepair process (Geloso et al., 2007). Secretion of signaling molecules induced by injury (e.g. inflammatory

mediators, neurotrophic factors) by glial cells (e.g. astrocytes and microglia) has been shown to promote migration and survivability of new-born cells (reviewed by Gemma et al., 2013) that would normally be eliminated. Neuroprotective factors induced by the injury may have contributed to the increased number of $BrdU^+$ cells observed in the hippocampus at the 28d TMT time point. Such a mechanism was proposed in a rat model of TMT injury in which neural stem cells grafted into TMT-injured hippocampus exhibited greater survival versus uninjured brain (Geloso et al., 2007).

Given the relatively short duration of BrdU administration prior to TMT exposure, little labeling of the relatively quiescent radial glial stem cell population in the SVZ would be expected. However, this study does not rule out the possible migration of $spDiI^+$ ependymal cells to the hippocampus following TMT injury. In adult rats, ependymal cells lining the spinal canal proliferate in response to spinal cord injury and differentiate into astrocytes near the lesion site (Mothe et al., 2005). Though the majority of ependymal cells are thought to be post-mitotic and terminally differentiated, some studies suggest that a small subpopulation of ependymal cells in brain may retain stem cell activity (Johansson et al., 1999; Li et al., 2002) or have the potential to proliferate and respond to injury (Chiasson et al., 1999; Belvindrah et al., 2002; Xu et al., 2005; Coskun et al., 2008; Gleason et al., 2008; Carlen et al., 2009).

Specific antibody markers for neurons, astrocytes, and microglia were used to determine the phenotype of the $spDiI^+$ cells. Few $spDiI^+$ cells (but no $BrdU^+$ cells) co-labeled with the cd11b marker for microglia. Uptake of spDiI by microglia may be via direct contact with the dye or following phagocytosis of cells labeled with the dye. $spDiI^+$ cells in the hippocampus rarely expressed markers for mature astrocytes or neurons (GFAP or NeuN, respectively). The lack of expression of these mature phenotype markers 28 days after hippocampal injury suggests that these $spDiI^+$ cells did not mature into the neurons or astrocytes typical of the local cellular

environment but rather retained an undifferentiated state. This is similar to studies in which progenitor cells transplanted or induced to migrate into brain regions where they would not normally be found failed to differentiate appropriately (Cooper et al., 2004). In rats, TMT-induced damage of the CA3/hilus resulted in the migration of $BrdU^+$ cells to the site, but these cells remained in an undifferentiated state, lacking expression of neuronal or astrocytic phenotype markers (Corvino et al., 2005). Similarly, Nakatomi et al. (2002), demonstrated in a rat model of ischemia that DiI- or GFP-labeled ventricular cells migrated into the damaged CA1/CA2 hippocampal subfields 28 days after injury. Importantly, labeled cells were also observed in the dentate gyrus and were postulated to represent undifferentiated cells since they lacked NeuN or GFAP expression. In studies using mouse and rat models of Huntington disease (Kohl et al. 2010; Kandasamy et al., 2015), SVZ-derived neuroblasts were observed to redirect migration away from the olfactory bulb to the degenerating striatum. The cells, however, did not undergo differentiation to express a mature neuronal phenotype marker (NeuN). These observations may complement our observations in the mouse TMT model that ventricular-derived cells may be redirected to migrate to an injured brain region (in this case, the hippocampus), but are unable to differentiate to mature neurons. It is presently unclear why the cells fail to undergo differentiation and integrate into the cellular/synaptic architecture, although this lack of responsiveness may reflect insufficient concentrations of neurotrophic factors in the injured or adult neurogenic microenvironment. Exogenous addition of neurotrophic factors (FGF-2, BDNF) can induce differentiation and integration of SVZ-derived cells migrating into injured striatum, demonstrating that they retain the potential for maturation if appropriate concentrations of signaling molecules are present (Chen et al., 2007).

In the injured brain, stem cells may serve multiple roles in the repair process. It is known that stem cells express neurotrophic factors (Lu et al., 2003), which may promote the survival of the

remaining neurons in the lesion site. Through this activity, stem cells can contribute to repair both through production of new neurons and glia in addition to providing molecular support via secretion of trophic factors (Conti et al., 2006). Whether a similar role may be played by the spDiI^{+} cells that migrate into the TMT-injured hippocampus requires further exploration. Taken in concert, the present study suggests that mechanisms that restore hippocampal function after TMT injury include response of proliferating populations associated with the ventricular region in addition to the previously observed activation of endogenous hippocampal stem cells. Further research is needed to fully characterize the spDiI^{+} cells, the factors that induce their migration, and their ultimate fate once localized to the hippocampus.

Figure 1. Fluorescent labeling of ventricular system 28 days after icv injection with spDil. *A*, spDil labeling of ependymal lining throughout lateral and third ventricles; *B*, spDil^{+} cells in granule cell layer of olfactory bulb (arrowheads); *C, E*, intense spDil labeling contralateral lateral ventricle of ependymal/subependymal region and choroid plexus; *D*, *F*, lateral ventricle stained with toluidine blue corresponding to *C* and *D*, respectively. *cp*, choroid plexus; *ep*, ependymal layer; *lv*, lateral ventricle; *sep*, subependymal layer. Scale bar = 50 micrometers.

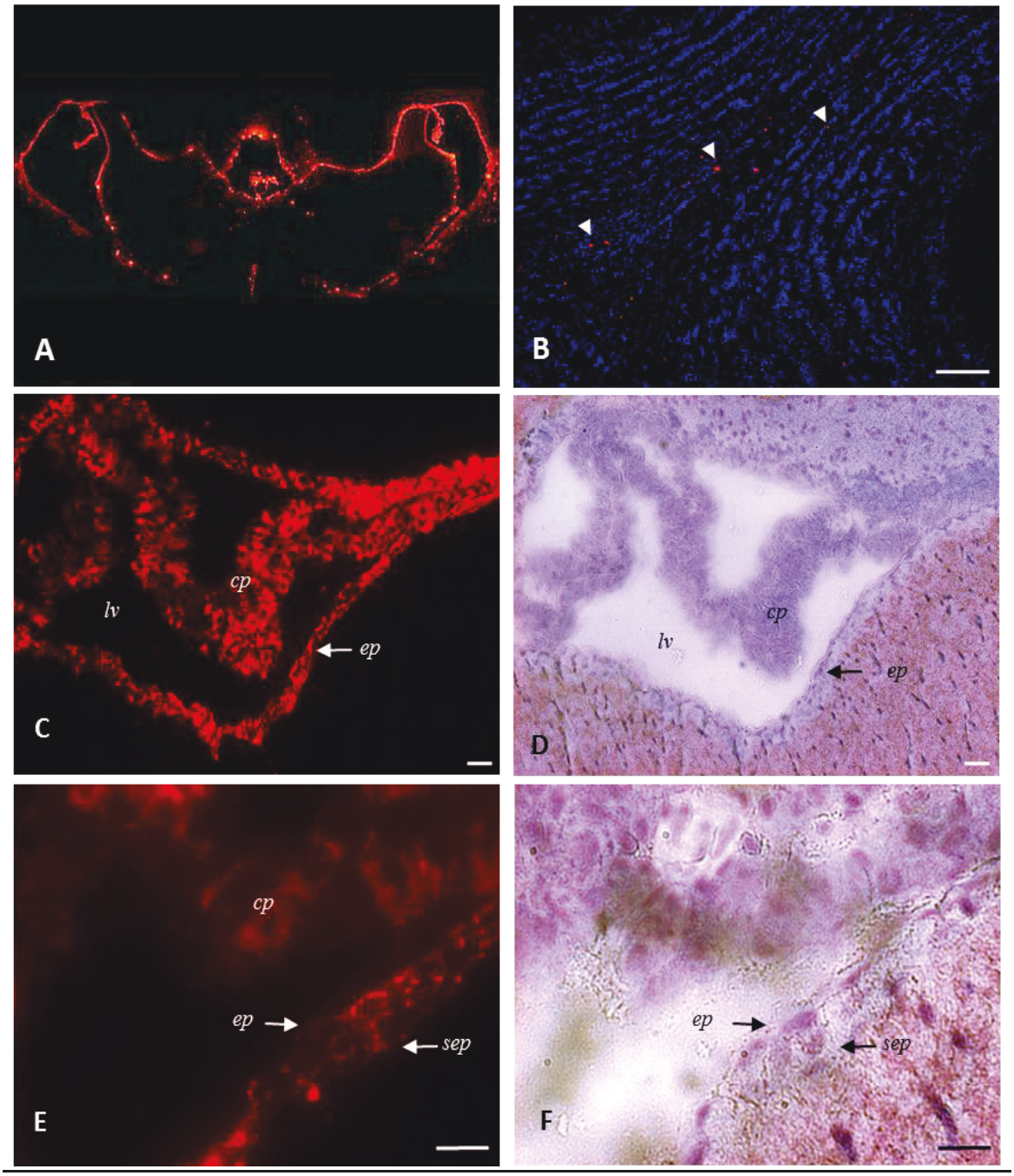

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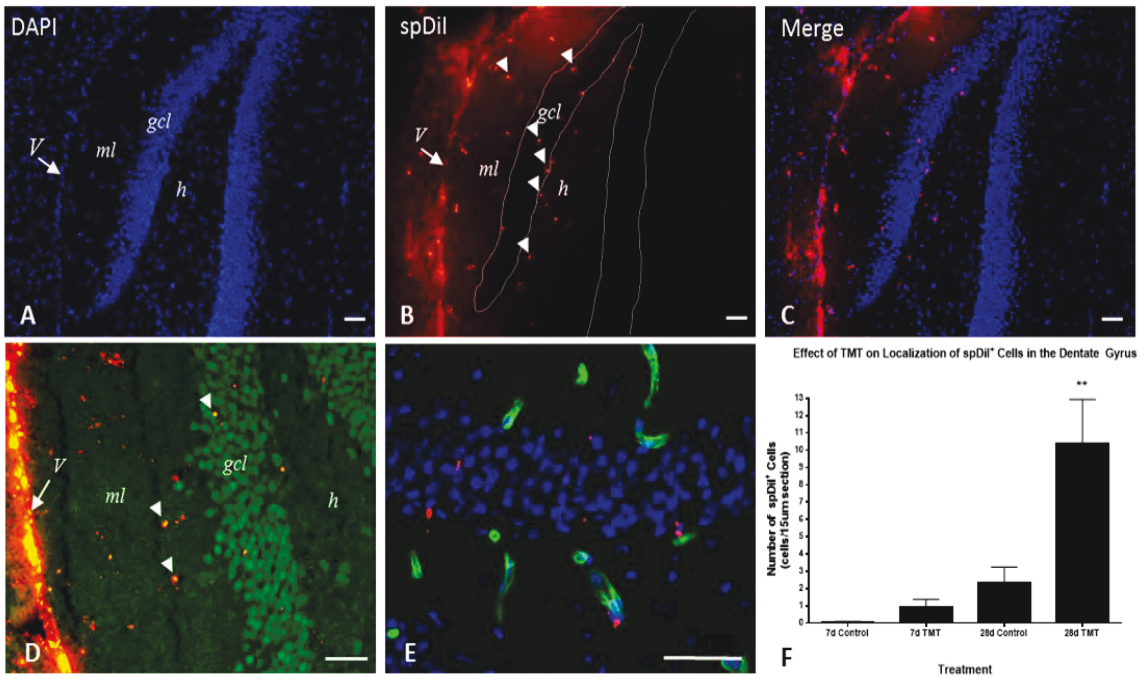
Figure 2. Cells labeled with spDil migrating from the ventricle into the hippocampus dentate gyrus 28 days after TMT injury. *A-C*, spDil positive cells in hippocampus stained with DAPI (blue); *D*, NeuN-labeled neurons (green) in dentate gyus showing DiI^+ cells between ventricle and infrapyramidal blade of dentate gyrus; *E*, $spDiI^+$ cells associated adjacent to lectin-stained blood vessels (green) in hippocampus. F, Quantification of $spDiI^+$ cells in the dentate gyrus. Arrowheads, spDil-labeled cells. *bv*, blood vessel; *gcl*, granule cell layer; *h*, hilus; *ml*, molecular layer; *V*, ventricle. Scale bar = 50 micrometers.



Graph data are means from 7-8 animals per group; one-way ANOVA-Tukey test; **P<0.01.

Figure 3. Migration of spDil^{+} cells into the dentate gyrus granule cell layer co-staining with neuronal (NeuN) or astrocytic (GFAP) phenotype markers. *A*, spDil^{+} cells co-labeled with neuronal cells (NeuN, green) in the granule cell layer. *B*, spDil+ cells not co-localized with astrocyte marker GFAP (green). spDil^{+} cells frequently found in the subgranular zone bordering the hilus of the dentate gyrus and *C*, cd11b+ microglia were infrequently associated with spDil+ cells in the dentate gyrus. *bv*, blood vessel; *gcl*, granule cell layer; *h*, hilus. Scale bar = 50 micrometers.

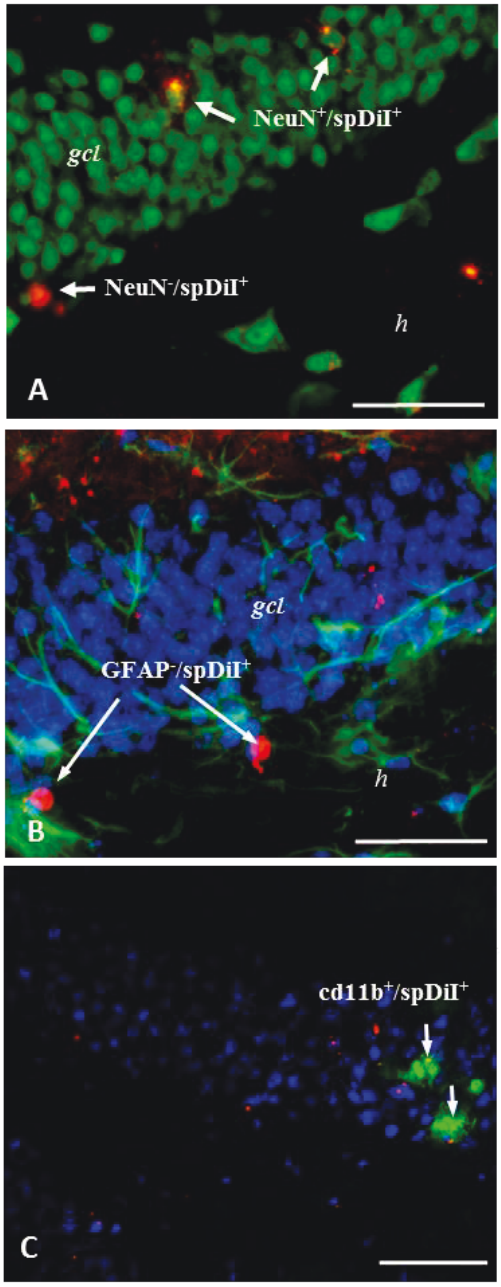
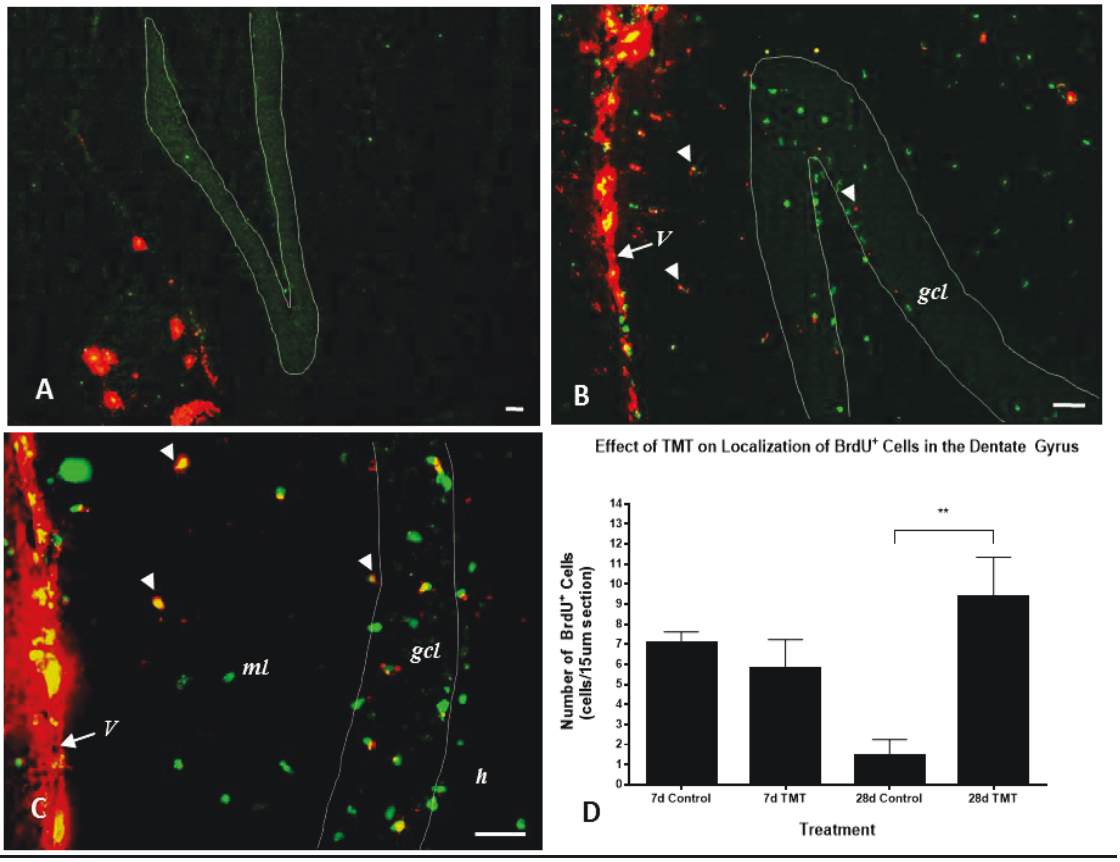


Figure 4. Migration of spDil- and BrdU-labeled ventricular and subependymal cells into the hippocampus 28 days post-TMT exposure. *A*, Control animals showed little BrdU labeling in the hippocampus, and no spDil+ cells were visible in the dentate gyrus. *B*, C at 28d post-TMT treatment increased numbers of spDil^{+} and BrdU^{+} cells found in dentate granule cell layer, hilus and molecular layers. Approximately 10% of spDil labeled cells co-labeled with BrdU. D, Quantification of bromodeoxyuridine (BrdU)-labeled cells (green) in dentate gyrus. *ep*, ependymal layer; *gcl*, granule cell layer; *h* = hilus; *ml*, molecular layer of hippocampus; *V*, ventricle. Scale bar = 50 micrometer.



Graph data are means from 7-8 animals per group; one-way ANOVA-Tukey test; **P<0.01.

CHAPTER 4. TRIMETHYLTIN-INDUCED INJURY IN THE HIPPOCAMPUS IS BLOCKED BY GAMMA-IRRADIATION AND EXACERBATES TREMOR ACTIVITY

Abstract

The hippocampus of rodents retains populations of stem cells that continue to generate new cells throughout the lifetime of the animal. Lying primarily within the subgranular zone of the dentate gyrus, these stem/progenitor cells generate new granule cells that migrate and integrate into the granule cell layer. Neurogenesis can be upregulated via a number of stimuli, and the functional significance may depend on the context of the behavioral parameter being studied. Trimethyltin is a specific limbic system neurotoxicant in rodents that has been used as a model of neural injury and repair. Dose-dependent cell death of granule cells in the hippocampus dentate gyrus occurs during the first 24-48 hours after TMT exposure concomitant with development of whole body tremor. Neurogenesis in the injured hippocampus is markedly increased during this early phase of injury. Tremors resolve within 3-4 days after TMT exposure, and a histologically normal-appearing dentate gyrus is present 30 days after injury. Mice received whole cranial 10G gamma radiation prior to TMT injection to reduce neurogenic activity to test for effects on recovery from TMT injury. Whole brain irradiation ablated TMT-induced upregulation of proliferation in the hippocampus based on bromodeoxyuridine staining, with only background physiological levels of mitotic activity remaining. $Ki67^{+}$ proliferating cells were also reduced at 6 days post-TMT exposure. Tremor activity was both increased in severity and prolonged in duration in irradiated mice. This study was intended to determine whether changes in neurogenesis can be correlated with alteration of the neurogenic/angiogenic environment. This study suggests that neurogenesis plays a role in the recovery of TMT-induced hippocampal injury, and is associated with alteration of the neurogenic environment.

Introduction

Neurogenesis persists in the adult murine brain mainly in the germinal centers of development – the subventricular zone (SVZ) of the lateral ventricles and in the subgranular zone (SGZ) of the hippocampus (Gage, 2000). Neuroblasts are continuously generated in the SVZ and migrate into the olfactory bulb where they differentiate into new glomerular olfactory neurons. Adult hippocampal stem cells localized in the SGZ of the dentate gyrus are putatively identified as radial glial-like cells (Seri et p, 2001; Filippov, et al., 2003) that give rise to new granule cell neurons. Recent studies indicate that hippocampal neurogenesis can be stimulated or inhibited by endogenous and exogenous factors such as adrenal steroids (Gould et al., 1992; Cameron and Gould, 1994), gonadal hormones and analogs (Brannvall et al., 2002;, Galea et al., 2006; Suzuki et al., 2007; Brannvall, 2005), physical activity (Van Praag et al., 1999), an enriched learning environment (Nilsson et al., 1999), neurotransmitters (Cameron et al., 1995; Brezun and Daszuta, 1999; Brazel et al., 2005), antidepressants (Malberg et al., 2000; Santarelli et al., 2003) and ionizing radiation.

Disruption of the neural microenvironment resulting from disease or injury can also influence the rate and paths of differentiation of hippocampal stem cell progeny (Monje et al., 2002; Rola, et al., 2006; Bergsland et al., 2014). Neural and endothelial progenitor cells are sensitive to the effects of ionizing radiation and accumulation of DNA damage that can cause dysfunction and early senescence (Richardson et al., 2009; et al., 2007; Lee et al., 2012), apoptotic cell death or induction of epigenetic changes. X-irradiation induces cellular damage and reversible or irreversible changes of the neurovascular environment that promote proliferation, migration and

differentiation and survival of newly formed neurons in the neurogenic regions of the adult rodent brain (Mizumatsu et al., 2003; Monje et al., 2002; Andres-Mach et al., 2008). Inflammatory response to irradiation may play a role in the delayed secondary effects of radiation on tissue. Studies have shown that thrombomodulin, a regulator of thrombin expressed in endothelial cells, may be an important mediator of the inflammatory and subsequent deleterious effects of radiation (Hauer-Jensen et al., 2004). The loss of thrombomodulin function exacerbates the inflammatory and cellular destruction of gastrointestinal tissue following exposure to high doses of radiation (Hauer-Jensen et al., 2004).

In the brain, x-irradiation of rodents at doses considered equivalent to the graded doses in radiotherapeutic treatment in humans causes marked reduction of cell proliferation in the neurogenic regions. Hippocampal neurogenesis is severely reduced immediately following irradiation and despite cessation of the inflammatory response, long-term effects of radiation on neurogenesis is evident based on continued suppression in proliferation and alteration of terminal lineage commitment (Mizumatsu et al., 2003; Monje et al., 2002). Glial cell proliferation (NG^+) proportionally increases at the expense of the formation of new neurons in the hippocampus (Monje et al., 2002). Inflammatory response, as indicated by expression of activated microglia, astrogliosis and cytokine expression, are increased short-term following gamma irradiation. Data supports that effects of radiation on the cerebrovasculature can induce secondary effects on inflammatory reactions (for example, astrogliosis), which can be attenuated by intravenous administration of anti-TNF alpha antibody (Wilson et al., 2009).

The anti-mitotic effects of cranial irradiation have been used to study the role of neurogenesis in various disease paradigms. For instance, it was shown that the therapeutic effect of antidepressant drugs was lost when neurogenesis was blocked by cranial irradiation (Surget et al., 2008; David et al., 2009; Walker et al., 2015). Conversely, the stimulatory effect of exercise and

environment enrichment on cognitive function tests was found not to be affected by ablation of neurogenesis by irradiation (Meshi et al., 2006). Hence, the role of neurogenesis in function is context-dependent. Another study showed that hippocampal associated memories, such as trace fear memories, are dependent on neurogenesis as shown by the inhibitory effect of cranial irradiation (Achanta et al., 2009).

Trimethyltin (TMT) is a potent hippocampal neurotoxicant that, when injected systemically, will induce neurotoxicity in the rodent limbic system. In adult mice, TMT preferentially damages neurons of the dentate gyrus, where extensive apoptosis and necrosis within the granule cell layer occurs within 24 – 36 hours after exposure (Chang, et al., 1982; Fiedorowicz et al., 2001). Tremor activity in the first 72 hours after exposure to TMT characterizes the immediate early behavioral response to intoxication. TMT injury induces proliferation of precursor cells in the dentate gyrus (Harry et al., 2004; Ogita et al., 2005) which give rise to new granule cell neurons. The new neurons contribute to the recovery of the histological structure of the dentate gyrus; they may contribute to the behavioral recovery as well, which in mice occurs within two weeks after TMT exposure (Ogita et al., 2005). Hence, TMT is a useful tool for studying neurogenesis in neuro repair processes since it reproducibly targets a specific region in the brain and does not disable the neurogenic and microenvironment that promotes new granule cell neuron formation.

The present study was initiated to determine if irradiation affects the neural stem cell response to TMT – induced injury of the mouse dentate gyrus. Neurogenesis in the hippocampus and SVZ, and behavioral response to TMT intoxication were assessed to test for radiation effects. It was found that irradiation significantly reduced the proliferative response to TMT-induced injury and that mice exposed to radiation exhibited a delayed recovery of a behavioral marker of hippocampal injury (i.e. tremor). This suggests that neurogenesis in the hippocampus plays a partial role in the recovery of mice to TMT intoxication.

Materials And Methods

Gamma irradiation of mice

Two month old male C3H mice (Taconic, NY) were housed in polycarbonate cages with wood chip bedding and maintained according to AALAC guidelines. To induce the desired radiation effect to the brain, the mice were treated with whole cranial irradiation at a mean absorbed dose of 10 Gray of ^{137}Cesium using a JL Shepard Mark 1 gamma irradiator. Lead shielding around the mouse body limited radiation exposure to the head region of the mouse (supplemental figure 1). Mice were restrained to limit movement during the irradiation period using a Broome rodent restrainer (Harvard Apparatus, cat. #BS4 52-0460) combined with a rodent restraining bag as an internal liner (Harvard Apparatus, cat.#BS4 72-6409) to reduce odor transfer to the apparatus. The Broome restrainer containing the mouse was inserted into a lead brick specially bored to accommodate the restrainer. The short duration of irradiation and restraint configuration prevented the necessity of anesthetizing the mice which eliminated potential secondary effects on mitotic response to irradiation. Mice were adapted to the restraint device by placing the mice in the device several times prior to the actual experiment. Calculated exposure duration to the radiation source was 12 minutes for a 10 Gray dosage. Immediately after irradiation, the mouse was released from the restrainer and returned to its cage. No pain or discomfort was observed during and after irradiation. 48 hours after irradiation, the mice received a single dose of trimethyltin chloride. The effects of radiation and trimethyltin treatment were determined histologically at 7 day and 1 year time points after TMT treatment.

Trimethyltin and bromodeoxyuridine administration

Trimethyltin chloride (TMT, Sigma, St. Louis, MO) was dissolved in normal saline at a stock concentration of 2.5 mg/ml. Gamma irradiated mice or non-irradiated mice were administered a single dose of TMT (2.8 mg/kg; ip), from a working solution of TMT (0.25 mg/mL) prepared in sterile saline which would induce tremor activity within 24-36 hours and recovery within 48-72 hours. Control mice were administered a comparable volume of saline solution. Treatment groups of 4-5 mice were used for generating saline- and TMT-injected brain samples allowing for treatment mortality and statistical evaluation. The initiation and cessation of tremor was monitored daily for the first week after treatment. Mice tremoring activity was qualitatively assessed based on a five-point scale where "1" = minimally detectable tremoring; "2" = mild tremors when moving; "3" moderate continuous tremors; 4 = strong continuous tremors; and "5" = severe debilitating tremors. In this study mice were dosed with TMT at a concentration that would induce mild to moderate tremoring.

Bromodeoxyuridine (Sigma) was prepared from solid crystalline stock by dissolving to a stock concentration of 9 mg/mL in saline. The solution was sterilized using a 0.2 µm syringe filter. Mice were injected intraperitoneally with the bromodeoxyuridine solution 24 hours after trimethyltin injection at a concentration of 50mg/kg twice daily for 3 days. The mice were euthanized by CO2 asphyxiation at 7 days and 1 year post-trimethyltin injection. Brains were removed and immediately frozen and stored at -70 ^{o}C for subsequent immunohistological analysis.

Tissue sectioning and immunohistochemistry

Twelve-micrometer cryostat sections were prepared from frozen brain tissue, mounted on slides and post-fixed by immersion in 4% paraformaldehyde at room temperature for 15 minutes. H & E staining was performed using standard methods. For antibody staining, antigen retrieval was performed by heating slides in 10 mM sodium citrate (pH 5.5) to boiling for 10 minutes, then

cooling to room temperature before mounting onto Shandon coverplates (Thermo Scientific, MA). Samples were incubated overnight at 4°C with primary antibodies prepared in blocking buffer containing 3% donkey serum and 0.3% Tween-20. Primary antibodies used were sheep anti-BrdU (5ug/ml; Research Diagnostics, Inc.), chicken anti-GFAP (1:300; Aves, Inc), mouse anti-NeuN (1:400; Millipore), rat anti-CD11b (1:300; BD Pharmingen), rabbit anti-active caspase-3 (1:300; R&D Systems, 4Inc.) and rabbit anti-collagen IV (1:200; Pierce). Primary antibody incubation was carried out by overnight incubation at 4°C. NeuN primary antibody staining was performed overnight at 4°C after the antigen retrieval step used for BrdU staining. For GFAP and BrdU co-staining, slides were microwaved for 15 minutes in 10 mM sodium citrate (pH 5), cooled to room temperature, and then incubated with BrdU and GFAP primary antibody overnight at 4°C. Slides were then incubated for 45 minutes at room temperature with Alexa Fluor-conjugated donkey anti-rabbit, -sheep, -chicken- or mouse secondary antibodies (1:300; Invitrogen, CA) prepared in blocking buffer. Double and triple labeling was performed using AlexaFluor secondary antibodies of different spectral profiles including AlexaFluor 488, 594, and 647.

Imaging and cell quantification

Tissue sections were visualized by epifluorescence using a Zeiss Imager A.1 microscope equipped with a Zeiss MRc camera and Axiovision image capture software. Sagittal and coronal sections of matched anatomical levels along the rostro-caudal axis were used for analysis. Quantification was performed on 4-5 sections/animal with an intersection distance of 90 micrometers. Image analysis was performed on HALO software by IndicaLabs. Algorithms for quantification included branch structure (for blood vessels and $GFAP^+$ astrocytic processes), area quantification (for GFAP, CD11b, nestin staining), and cytonuclear (for Ki67, caspase, NeuN, BrdU staining). Where appropriate, co-localization was determined using these algorithms. Data were

analyzed using Graphpad Prism 6 (Graphpad software version 6.05). All data are expressed as standard error of the mean (SEM). Data were considered statistically significant when $P < 0.05$. Data were compared using two-way ANOVA. If a significant difference was detected, post-hoc analyses were performed using Tukey multiple comparison tests or Dunnetts test.

Results

It has been demonstrated in this thesis, and by others (Harry et al., 2005; Ogita et al., 2005) that endogenous hippocampal stem cells are activated following TMT injury which may contribute a functional role of repopulating the hippocampus dentate gyrus with new granule cells. In this study, C3H mice were used to test whether stem cell activation induced after trimethyltin injury is critical for recovery from tremor activity—the behavioral hallmark of trimethyltin-induced hippocampal injury in mice. To block injury-induced upregulation of hippocampal stem cell mitotic activity, mice were irradiated with a single dose of 10G gamma radiation 48 hours prior to TMT injection. Bromodeoxyuridine was administered for a duration of four days, starting the day after trimethyltin injection, to enable tracing of both mitotic activity as well as differentiation and survival of new cells. Two cohorts of mice were used to enable collection of brain samples at two different time points: seven days post-TMT and one year post-TMT.

Whole cranium irradiation was accomplished with a 12 minute exposure within a gamma irradiator. The rest of the mouse body was shielded from radiation via a lead block (see Material supplement Figure 2). After irradiation, the mice expressed normal behavior with no obvious effect on feeding or water intake. However, while the average body weights for the irradiated versus non-irradiated mice were not different at 120 days post-irradiation, the body

weights of the irradiated mice were less variable than the non-irradiated mice. This dispersion in body weights of the non-irradiated mice is attributed to reduced bodyweight of the subset of mice that were dosed with trimethyltin. It is unclear why the irradiated mice did not exhibit a similar distribution in body weight. A repeat study using a larger sample population would be needed to further continue this observation.

One year after irradiation, the irradiated mice had a profound loss of pigment in the cranial hair (at the seven day time point hair coloration was unaffected). The rest of the body, which was protected by lead shielding while in the gamma irradiator, had normal dark brown-pigmented hair, as did the non-irradiated mice. The mice that were irradiated and treated with trimethyltin exhibited some cranial hair graying, but to a much lesser degree than the irradiated mice that did not receive trimethyltin. The darker hair in the trimethyltin treated mice was immediately obvious. Unfortunately the hair coloration was not photographically recorded or quantified, due to restrictions at the study site. Qualitatively, the hair coloration differences were clear to the untrained eye.

Mice dosed with TMT developed tremors within 24 hours of injection. The tremors ranged from mild to moderate in intensity and eventually resolved. There was a distinct difference in timing of onset and recovery from tremor activity depending on whether the mice were subjected to radiation treatment (Figure 1). In non-irradiated mice, the tremor activity initiated at 36 hours after trimethyltin treatment and resolved within 48 hours. Mice that were irradiated exhibited mild to moderate tremors at 24 hours which did not resolve until 72-84 hours after TMT injection. The severity of the tremors tended to be greater in the irradiated versus non-irradiated mice at 24-36 hours time point. All cages contained a small plastic dome to create a shelter and environmental enrichment. Irradiated mice treated with trimethyltin all stayed within the dome shelters when cages were placed on the lab bench, whereas non-irradiated mice exhibited more

exploratory behavior outside of the dome. Irradiated mice were also more excitable when handled compared to non-irradiated mice at 24 to 72 hours post-TMT injection. At the one year time point, mice that had been treated with TMT exhibited greater agitation and nervousness when removed from their cages as opposed to control mice. In contrast to the observation at 2 to 3 days after TMT treatment, at 1 year post-TMT, the trimethyltin-treated, non-irradiated mice were more excitable when handled compared to mice that were irradiated.

As expected, TMT administration induced cell death within the granule cell layer of the hippocampus dentate gyrus (Figure 2). This was evident by pyknotic nuclei dispersed throughout the granule cell layer in the seven day post-TMT mouse groups. Hematoxylin and eosin staining revealed no obvious effect of gamma radiation on the hippocampus or other regions of the brain, nor did radiation appear to impact the extent of injury induced by trimethyltin exposure (Figure 2). Staining for astrocytes using GFAP as a specific phenotype marker indicated that gamma irradiation alone did not result in astrocyte activation (Figure 3).

Quantification of bromodeoxyuridine incorporation into cycling cells in the hippocampus showed that TMT injury induced an approximately 4-fold increase in $BrdU^+$ cells at 7 days post-TMT injection (Figure 4). This is in agreement with characterization data presented in Chapter 2 of this thesis. BrdU labeled cells were primarily localized to the granule cell layer, with some positive staining cells evident in the molecular layers and hilus. A single dose of 10G gamma radiation reduced cell proliferation to levels similar to control uninjured mice (Figure 4B). Quantification of the BrdU-labeled cells was repeated using a combined acid-heat antigen retrieval process followed by quantification using image analysis software to determine $BrdU^+$ cell number (Figures 5A and 6). The addition of this technique was to potentially enhance the detection of bromodeoxyuridine. The modified antigen retrieval step increased the number of $BrdU^+$ cells detected, but the relative increase in number of positive cells due to TMT treatment

was similar to the data collected using the standard method (Figure 4B). Ki-67 staining to detect cycling cells in the hippocampus showed that at the seven day time point, TMT treatment had increased mitotic activity. The number of Ki-67$^+$ cells was reduced by radiation treatment (Figures 5B and 6).

The hippocampus of TMT treated or untreated mice at the one year time point appeared to have normal morphology with no pyknotic nuclei nor other abnormalities (Figure7). The one year post-TMT-treated mice had only approximately 3% of the number of BrdU positive cells in the hippocampus compared to the 7 day post TMT-treated mice (Figure 9). Mice that were treated with TMT harbored more BrdU$^+$ cells in the hippocampus than untreated mice. Furthermore, irradiation resulted in an overall reduction in the number of BrdU$^+$ cells in the hippocampus. Ki-67 staining of proliferating cells showed a similar pattern in which the one year old mice only exhibited 1-2% of the number of Ki-67$^+$ cells compared to the younger cohort at 7 days post-TMT (Figure 13 and 15). Because of the scarcity of the number of Ki67$^+$ cells identified in the 1 year old hippocampus, no statistical determination of differences between groups was possible.

Mouse brains were stained to examine co-localization between BrdU and mature cell markers for neurons and astrocytes (NeuN and GFAP, respectively). In the 7 day post-TMT groups, no mature neuronal markers for neurons or astrocytes (NeuN and GFAP, respectively) were detected that co-localized with bromodeoxyuridine-labeling. The 7-day time point may have been too early to detect expression of NeuN in differentiating cells. However, mice in the one year time point showed co-localization between BrdU and phenotypic markers. BrdU$^+$ cells expressing the neuronal marker NeuN were less frequent in mice that were irradiated (Figure 10). Also, among the mice that were irradiated, there were more BrdU$^+$/NeuN$^+$ cells in the trimethyltin-treated mice compared to the control, but this did not reach statistical significance (Figure 10). There was no difference in number of BrdU$^+$/NeuN$^+$ cells with trimethyltin treatment in the non-irradiated

mice. Interestingly, calculating the ratio of $BrdU^+/NeuN^+$ cells among the total number of $BrdU^+$ cells showed there was a smaller proportion of cells that expressed the mature neuronal marker in the TMT-treated mice (30%) compared to controls (60-65%), regardless of whether the mice were irradiated (Figure 12). Radiation treatment did not appear to alter the lineage fate of the $BrdU^+$ cells. A few $BrdU^+$ cells in hippocampus were found to express the mature astrocytic marker, GFAP. Irradiation resulted in a reduction in the number of these cells, but there was no statistically-significant differential effect of trimethyltin treatment (Figures 8 and 11).

Discussion

This study was designed to test whether gamma irradiation would (i), block TMT-induced upregulation of hippocampal stem cell mitotic activity, and (ii) affect the capacity for cellular and behavioral recovery from the injury. Irradiation has been used as a tool for studying neurogenesis and the biological function of neurogenesis in a variety of paradigms, including effects on learning and memory. For instance, radiation-mediated ablation of stem cell mitotic activity was useful to determine that activation of neurogenesis was important for the activity of anti-depressants (Santarelli et al., 2003), but was less involved in the increased cognition observed when rodents were exposed to enriched environments (Meshi et al., 2006). Few studies have utilized gamma-irradiation for studying the role of neurogenesis in models of chemical injury in the adult brain. Because TMT significantly upregulates neurogenesis of endogenous hippocampal stem cells in the mouse brain, the effects of radiation to deplete neurogenesis should be readily detectable. Additionally, if neurogenesis is an important mechanism for TMT recovery, including the recovery

from tremor activity, irradiation may help to delineate its role in the recovery from TMT intoxication.

TMT induces apoptotic granule cell death in the hippocampus dentate gyrus of mice. Multiple mechanisms have been proposed for mediating the toxicity of TMT including PKC activation, oxygen radical formation, binding to the mitochondrial protein stannin, calcium overload, and excitotoxicity through glutamate receptor activation. Though the toxicological mechanism(s) is unclear, the TMT mouse model is useful for studying neurodegeneration and neurogenesis since the granule cell layer is replenished with new adult-born neurons after the injury. Histologically, the hippocampus exhibits pyknotic nuclei with reactive gliosis in the first week of intoxication, but returns to a normal morphological appearance about a month after exposure (Figure 1). Acute behavioral sequelae develop within 24-36 hours after TMT injection, involving tremor activity of dose-dependent severity. Tremoring generally subsides within 72 hours after TMT injection. The TMT time-course study described in Chapter 2 of this thesis showed that although the tremors begin at about the same time that apoptotic cells appear in the dentate gyrus, cell death continues for several days after the tremors have resolved. Whether neurogenesis in the injured brain is responsible for the cessation of tremors in the context of ongoing cell death is not known, but does not appear likely.

Cranial irradiation was performed in this study to block upregulation of neurogenesis in the TMT injury model to determine if it would have an effect on recovery from tremor activity. Mice were dosed with 10 gray (1000 rad) gamma irradiation 48 hours prior to injection of trimethyltin. The 10 gray dose is approximately equivalent to a clinically relevant, graded radiotherapeutic dose given to humans, and does not induce acute radiation effects on vasculature, myelination, or induction of radionecrosis (Monje et al. 2002). Additionally, mice were exposed to a dosage that should avoid induction of an inflammatory response that would otherwise complicate the study

by causing secondary effects independent of the trimethyltin -induced injury. In this study, no radionecrosis was observed in the 7 day brain sections, and no significant inflammatory response was evident (Figure 3) since there were no changes in astrocyte reactivity (GFAP expression) relative to control non-irradiated mice. 10G gamma irradiation successfully blocked mitotic activity in the hippocampus but did not fully ablate it (Figure 4). Irradiation depressed the number of cells incorporating BrdU in the hippocampus of all mice by 70-75%. Interestingly, TMT-induced a 4-fold increase in mitotic activity (compared to mice not treated with TMT), independent of whether the mice were irradiated. This suggests that the stimulatory effect of trimethyltin-induced injury on proliferation is not fully inhibited by radiation treatment; instead, there may be fewer stem cells capable of responding to whatever pro-mitotic factors were released during the injury.

It was found that cranially irradiated mice tended to have stronger TMT-induced tremors, and the tremors persisted more than 48 hours longer than for non-irradiated mice (Figure 2). Since the irradiation significantly reduced the production of new cells in the hippocampus, it is possible that this contributed to the enhancement of tremoring. As discussed previously in this thesis, it is known that new neurons born in the hippocampus under pathological conditions tend to have an inhibitory effect on excitatory drive. The afferent plasticity of new neurons to form inhibitory synapses as well as their increased threshold for excitatory firing could be a compensatory mechanism to reduce the hyperexcitability caused by excitotoxic injury. However, whether the short time frame of 2 to 3 days after TMT injury is sufficient for synaptic plasticity to have an effect on tremor activity and hyperexcitability in the hippocampus is questionable, particularly if it if it involves newly-born cells. It should be emphasized, however, that the gamma irradiation did not fully inhibit the mitotic activity in the hippocampus. Irradiation blocked the level of TMT-induced stimulation of mitotic activity to near physiological level seen in untreated, non-irradiated mice

(Figure 4B). Regardless of the quantity of $BrdU^+$ new-born cells, it seems unlikely that the short time frame would be sufficient for synaptic plasticity to effectively depress tremor activity. While newly-born cells may begin to express markers of mature neuronal phenotype as early as 4 to 5 days from birth, initial GABAergic synaptogenesis requires at least several days more (reviewed by Ge et al, 2008). Stem cells are known to produce trophic factors, which could pose as another explanation for how they may function to modulate hyperexcitability in the context of hippocampal injury. Newly born cells (or progenitor cells) in the injured hippocampus may provide enhanced trophic support to existing neurons (Butti, et al., 2014). Perhaps a portion of the damaged cells could be 'rescued' by increased levels of neurotrophic factors secreted by new cells. Because the tremor activity eventually subsided in the irradiated mice, albeit at a later time compared to non-irradiated mice, mechanisms independent of stem cell activation could also be involved. For instance, TMT induces the upregulation of microglia and astrocytes in the vicinity of the injury. Though this is most often regarded as an inflammatory response, it could also may play a role in dampening propagation of excessive excitatory transmission. Both microglia and astrocytes produce factors that can promote the survival of neurons under some pathological conditions. While there is ample evidence that pro-inflammatory cytokines such TNF alpha, IL-1 and IL-6 are upregulated following TMT injury, both M1 and M2 phenotypic markers of microglia are upregulated within 24 hours after TMT injection in mice (Kim et al., 2014; McPherson, et al., 2014). This would suggest that there are both pro- and anti-inflammatory responses taking place early in TMT intoxication. Furthermore, activated astrocytes contribute inflammatory cytokines to the TMT-injured hippocampal environment (Kim et al., 2014), but also produces anti-inflammatory factors such as arginase-1 (also an M2 phenotype marker) that can reduce nitric oxide levels (by competing with iNOS for its substrate arginine) and reduce oxidative stress mediated through NO and peroxynitrite formation. In some brain lesions there is reduced

astrocyte-mediated glutamate uptake which can potentially exacerbate excitotoxic injury (Schreiner, et al., 2013). Though it was shown that TMT can inhibit *in vitro* glutamate transport by astrocytes in rat cortical preparations (Karpiak, et al., 2001), it is not known whether glutamate transport is suppressed in murine hippocampal astrocytes or in context with the intact brain. Our understanding of astrocyte biology has increased in recent years with the discovery that astrocytes are heterogeneous, with differential responses to stimuli dependent on brain region or other variables. An example of this was illustrated in Chapter 2 of this thesis where there was an upregulation of nestin expression in a subpopulation of astrocytes in the TMT-injured hippocampus. The vast increase in neural stem cell activity following TMT injury may shift the cellular-molecular balance toward a repair and recovery state. The complexity of the cellular response in the murine hippocampus to TMT injury makes it difficult at this point to determine which factors may be dominant in reducing the tremor activity in the non-irradiated mice, and how this is disrupted in the irradiated mouse brain. Further studies are needed to address these questions.

Neurogenesis in the hippocampus is sensitive to many factors that can change the rate of proliferation by endogenous stem cells including environmental stimuli and injury. For example, Rola et al. (2006) showed that traumatic brain injury reduces neurogenesis in the hippocampus and increased the astrocytic and microglial cell population, suggesting these changes may play a role in cognitive decline. The regulators of neurogenesis have been discussed earlier in Chapter 1 of this thesis and will not be exhaustively discussed here. Among the environmental insults that neural stem cells (and potentially stem cells of all tissues) are particularly sensitive is exposure to ionizing radiation. Relatively low levels of gamma- or x-irradiation exposure, at doses that do not necessarily involve a secondary inflammatory response, will significantly reduce stem cell mitotic activity. In addition, studies have shown that the lineage fate of stem cell progeny born after

irradiation is disrupted, yielding a shift from neurogenesis to gliogenesis (Monje, et al., 2002), and that the niche that fosters the neurogenic microenvironment becomes altered following irradiation (Monje et al., 2002). The proliferation and differentiation of stem cell progeny is severely impacted by radiation. The exact mechanism for this effect is not clear. Since the effects of the radiation linger for months or longer, the mechanisms probably involve more than DNA damage of proliferating cells and induction of programmed cell death. Alterations in cellular architecture may disrupt intercellular communication critical for regulating stem cell activity. Neural stem cells that normally associate in clusters and are closely juxtapositioned along microvascular tissue (i.e. cerebral capillaries) become less intimately connected to capillaries following irradiation (Monje et al. 2002). Since blood-borne and angiogenic factors play crucial roles in modulating stem cell activity, the altered intercellular communication between the microvasculature and stem cells has been proposed to play a role in the deleterious effects of irradiation on neurogenesis. Using grafting and in vitro techniques, Monje (2002) demonstrated that irradiation does not necessarily affect the capability of precursor cells to differentiate into neurons, rather the neurogenic microenvironment in the hippocampus is altered such that it less favorable to neurogenesis. However, a later study has suggested that the neurogenic microenvironment is not altered (Bӧstrom et al., 2013)

In this study, cranial irradiation did not affect the lineage commitment of the newly born cells that were generated during the acute phase of TMT injury. However, it is interesting that at the one year time point, the cells generated in the pathological environment created by TMT intoxication had proportionately lower survival than the cells generated under physiological conditions (Figure 12). This phenomenon was independent of cranial irradiation. Neurons that were generated during the injury may have been produced in excess and did not fully integrate into the neuronal network, ultimately leading to their disappearance. It is also possible that a

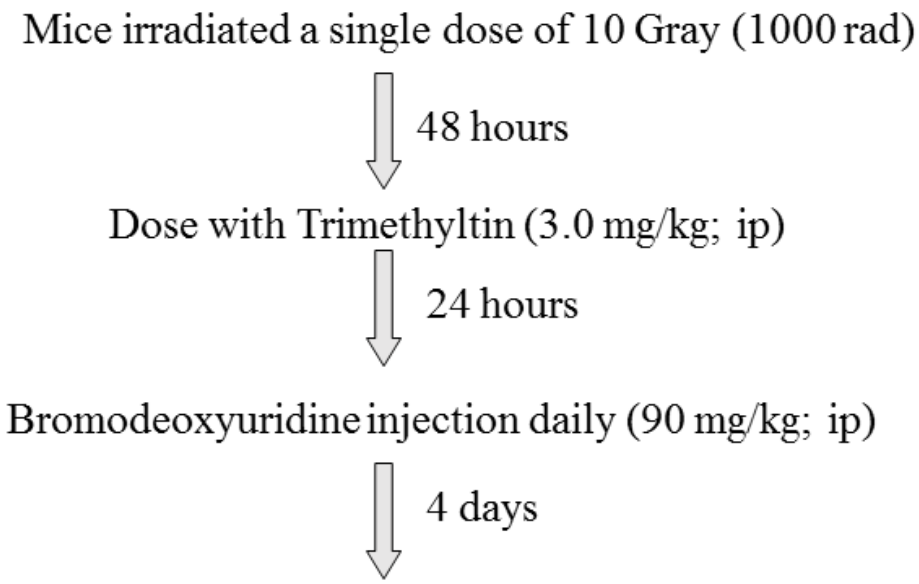
large fraction of the new neurons were physiologically compromised and susceptible to cell death over time, or, TMT injury disrupted the neural microenvironment, making it less favorable for the long-term survival of these neurons.

In addition to the sensitivity of stem cells, endothelial cells of the vasculature are also extremely sensitive to radiation exposure, resulting in cell death or alteration of endothelial cell function, including secretion of inflammatory factors. Inflammation may play a major role in the effect of the secondary effects of radiation injury as well as play a major role in stem cell function. One of the mechanisms found to play a role in the induction of inflammation following radiation exposure involves the protein thrombomodulin. This protein modulates the activity of thrombin, a major inflammatory factor. Radiation injury induces oxidative damage to the primary structure of thrombomodulin and inhibits its anti-inflammatory effect (Glaser et al., 1992; Hauer-Jensen, et al., 2004; Ross et al., 2008). Given that the neurogenic niche incorporates the cerebrovasculature as an important component in modulating stem cell activity, radiation-induced alteration of the normal endothelial cell function could play a role in disrupting neurogenesis.

More research is needed to understand the mechanisms underlying the effects of radiation on stem cell activity. This is particularly relevant to human oncology since progressive cognitive decline frequently occurs in patients having given radiotherapy. It is worth noting that children receiving targeted intracranial radiation to the cerebellum during morphogenesis for medulloblastoma have persistent cerebellar injury and cognitive defects. The decline in neurogenesis in the hippocampus been postulated to play a major role in the effects of radiotherapy on cognition. In this regard, animal models that stimulate neurogenesis such as trimethyltin-induced hippocampal injury, may be very useful for identifying toxicological mechanisms that can be targeted for reducing the secondary effects of radiotherapy.

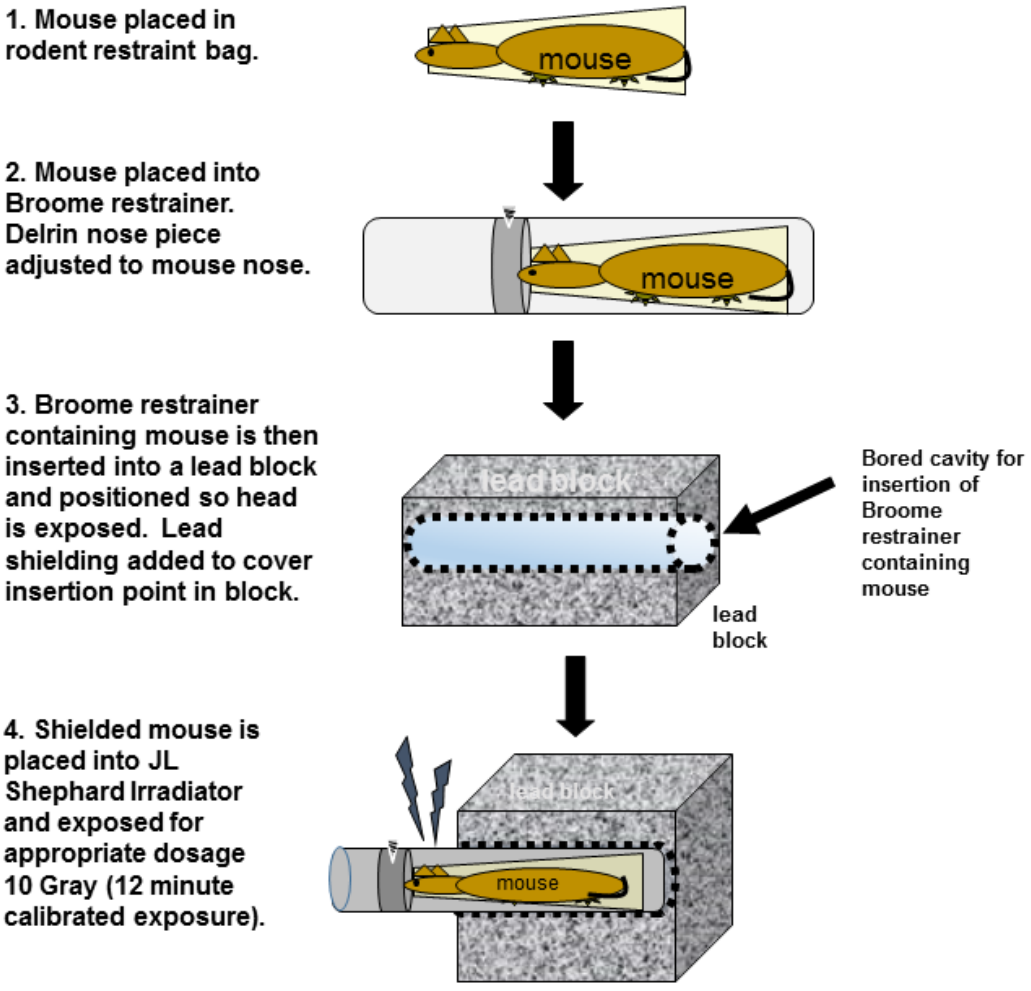
Methods supplement figure 1.

Flow diagram of irradiation and TMT procedure



Methods supplement figure 2.

Schematic diagram of gamma-irradiation procedure



Supplemental data figure 1.

Graph showing body weights of mice at 4 months post-irradiated are less dispersed than body weights of non-irradiated mice

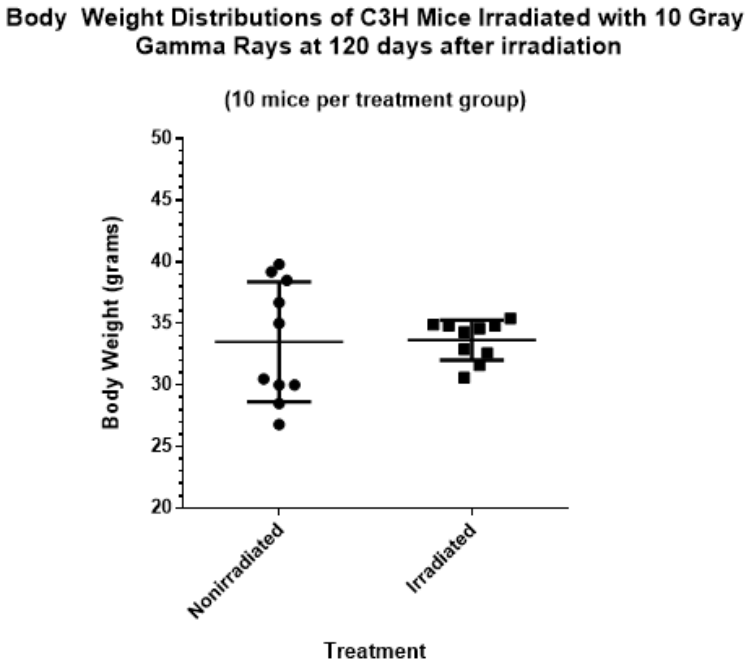


Figure 1. Time-course for TMT-induced tremoring activity in mice with and without gamma irradiation. Mice were assessed daily on severity of tremoring based on a 5pt scale, with 5 being most severe with morbidity (none of the animals responded above level 3). The red horizontal line indicates the group average tremoring response for the indicated day. (NT = not detected)

Mouse Tremor Activity

Days post-TMT injection	Not Irradiated	Irradiated
0	N.T.	N.T.
1	mild	mild
2	N.T.	moderate
3	N.T.	mild
4-7	N.T.	N.T.

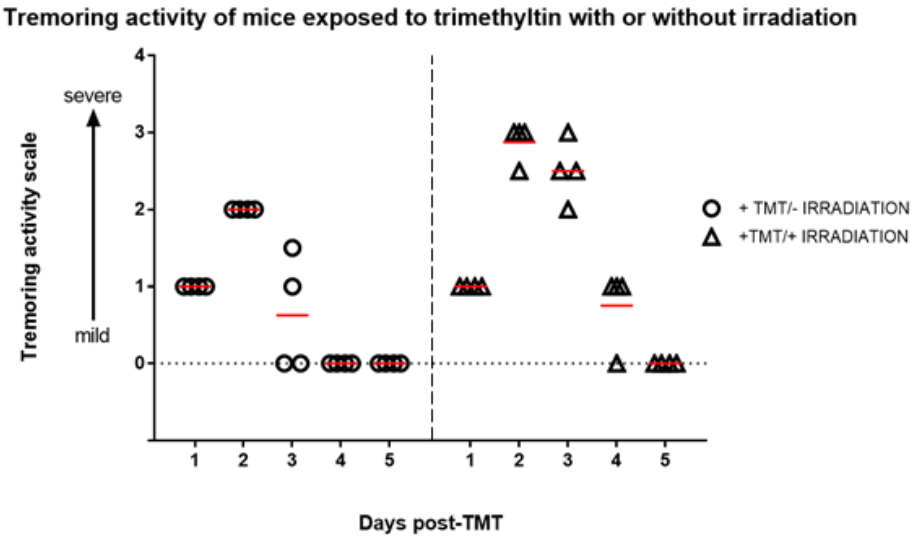


Figure 2. Hematoxylin and eosin staining of hippocampus of mice at 7 days post-TMT +/- exposure to 10gray gamma radiation. Arrows indicate pyknotic nuclei. Scale bar = 50 micrometers.

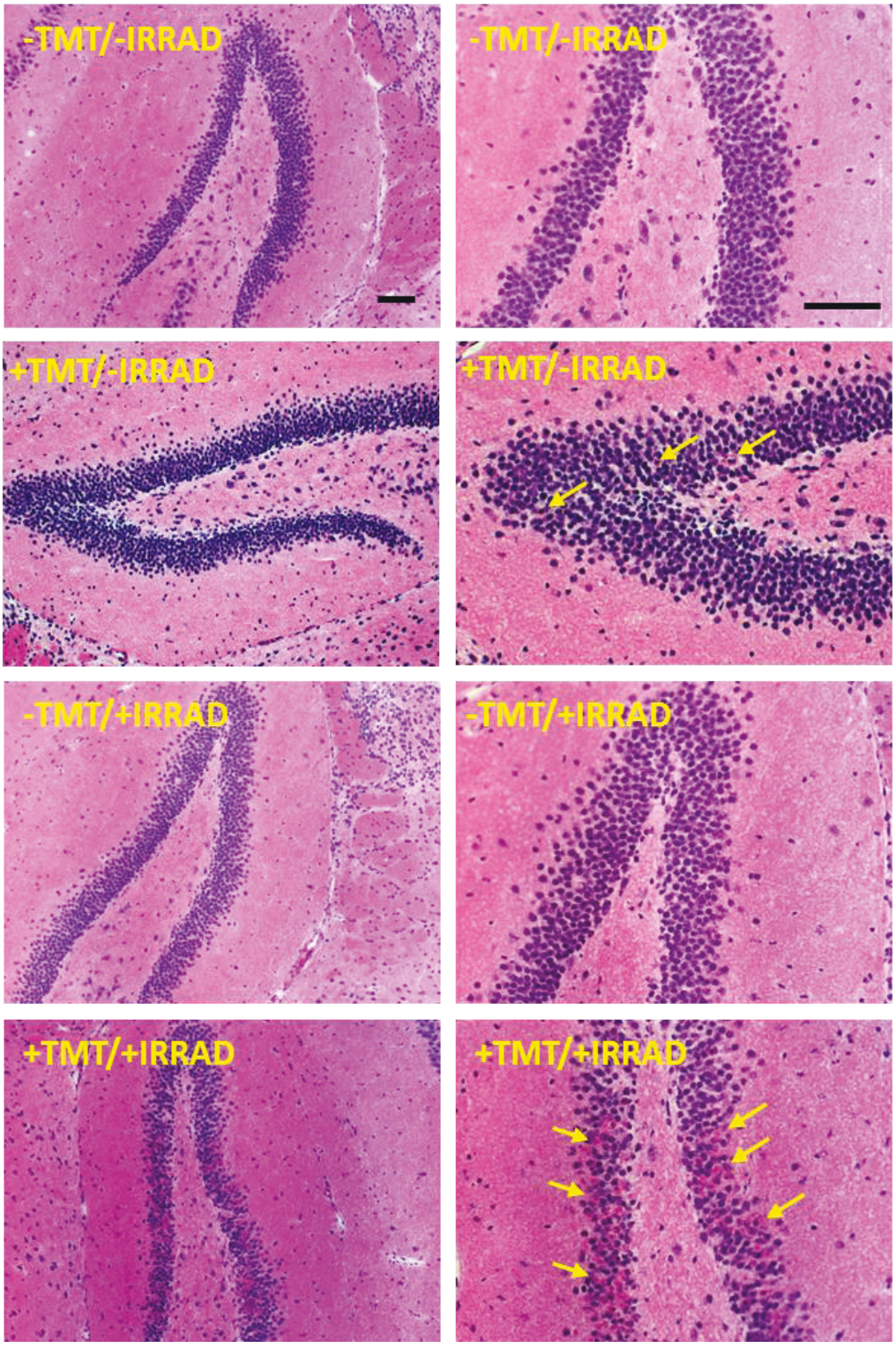


Figure 3. GFAP expression in brain 7 days post-treatment with TMT or gamma irradiation. (GFAP, red; DAPI, blue)

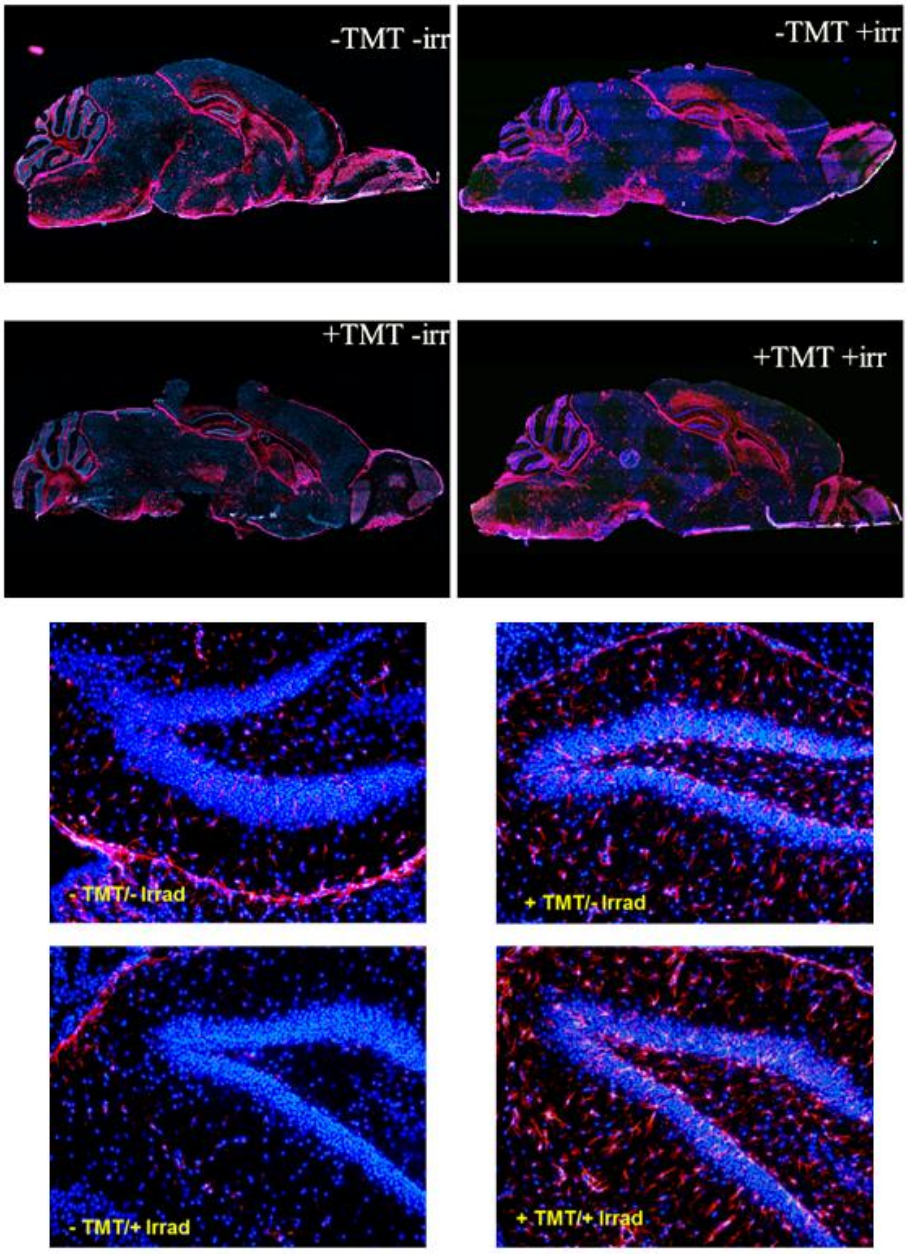
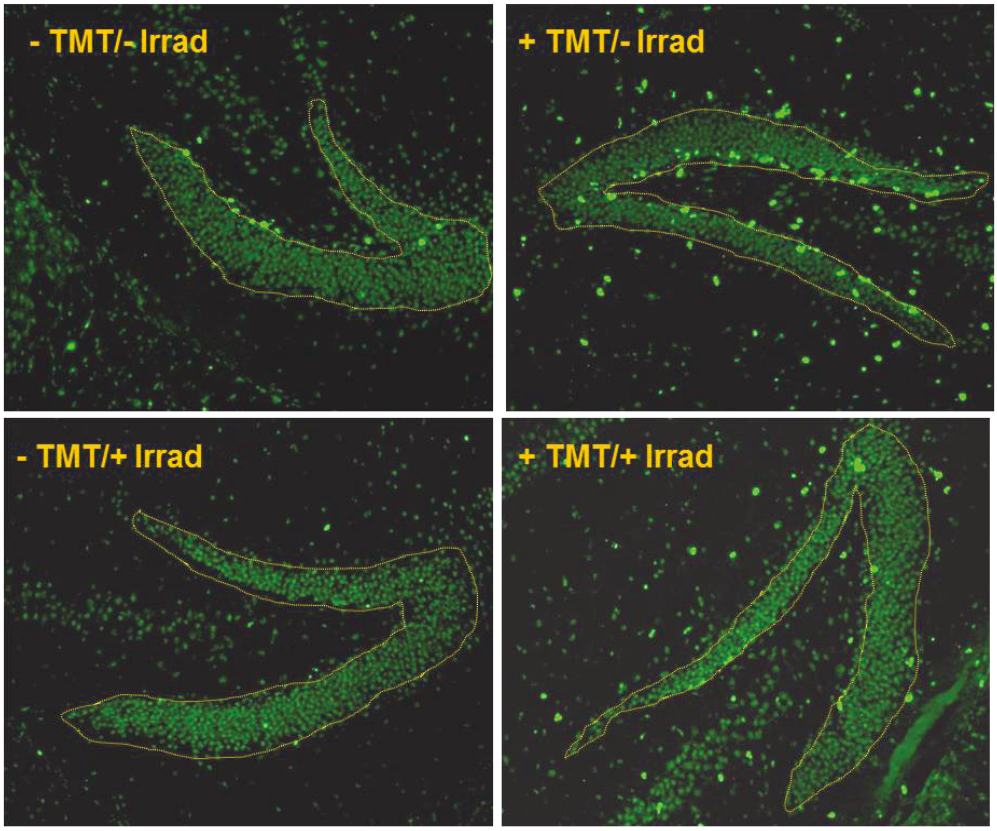


Figure 4. Effect of TMT and irradiation on proliferating cells in the hippocampus dentate gyrus at 7 days post-TMT treatment. *A*, bromodeoxyuridine labeling (green) of cycling cells in mouse brain hippocampus; *B*, quantification of BrdU+ cells in hippocampus. Dentate gyrus granule cell layer outlined for clarity.

A



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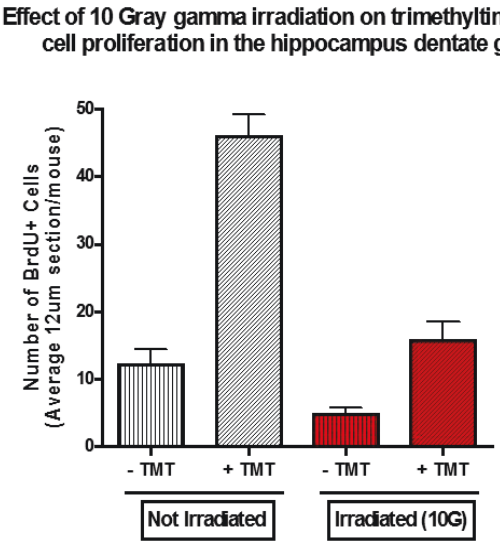
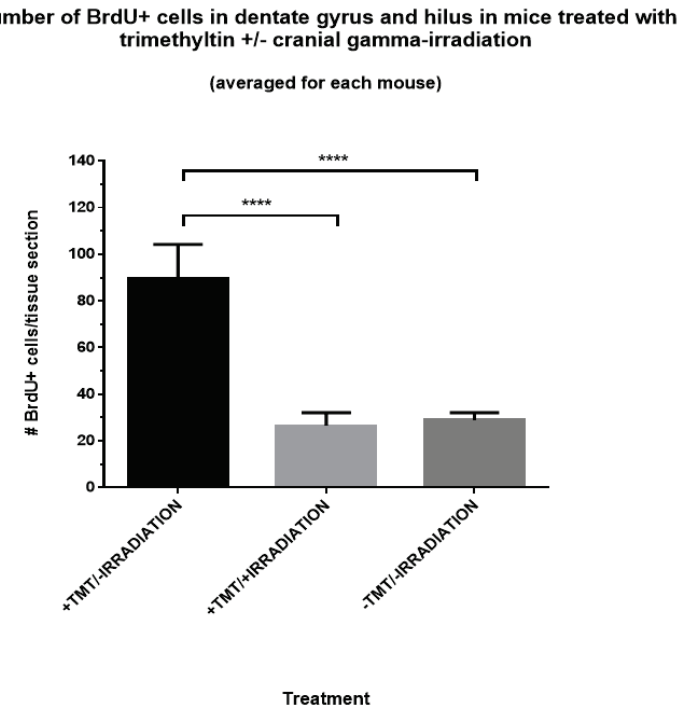


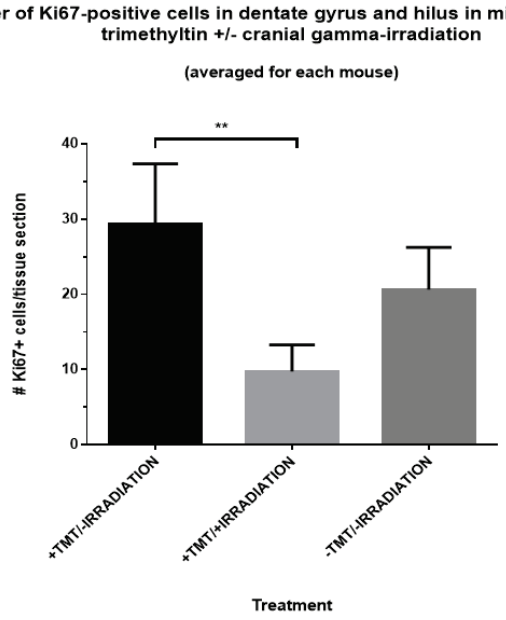
Data are means of 3-4 animals per group; one-way ANOVA-Tukey test

Figure 5. Effect of TMT and irradiation on proliferating cells in the hippocampus dentate gyrus at 7 days post-TMT treatment. *A*, these data represent repeat staining for BrdU for quantification using IndicaLabs image analysis software. BrdU staining quantified total number of mitotic cells occurring during the first 4 days after TMT injection. *B*, Ki-67 staining was performed to quantify proliferating cells at the 7 day post-TMT timepoint.

A



B



Data are means from 3-4 animals per group; one-way ANOVA-Tukey test; **P<0.01.

Figure 6. Ki-67 and BrdU staining of proliferating cells in 7 days post-TMT hippocampus showing TMT-induced increase in cell proliferation and BrdU incorporation with neuron marker co-localization. (Ki-67, red; BrdU, green; NeuN, blue)

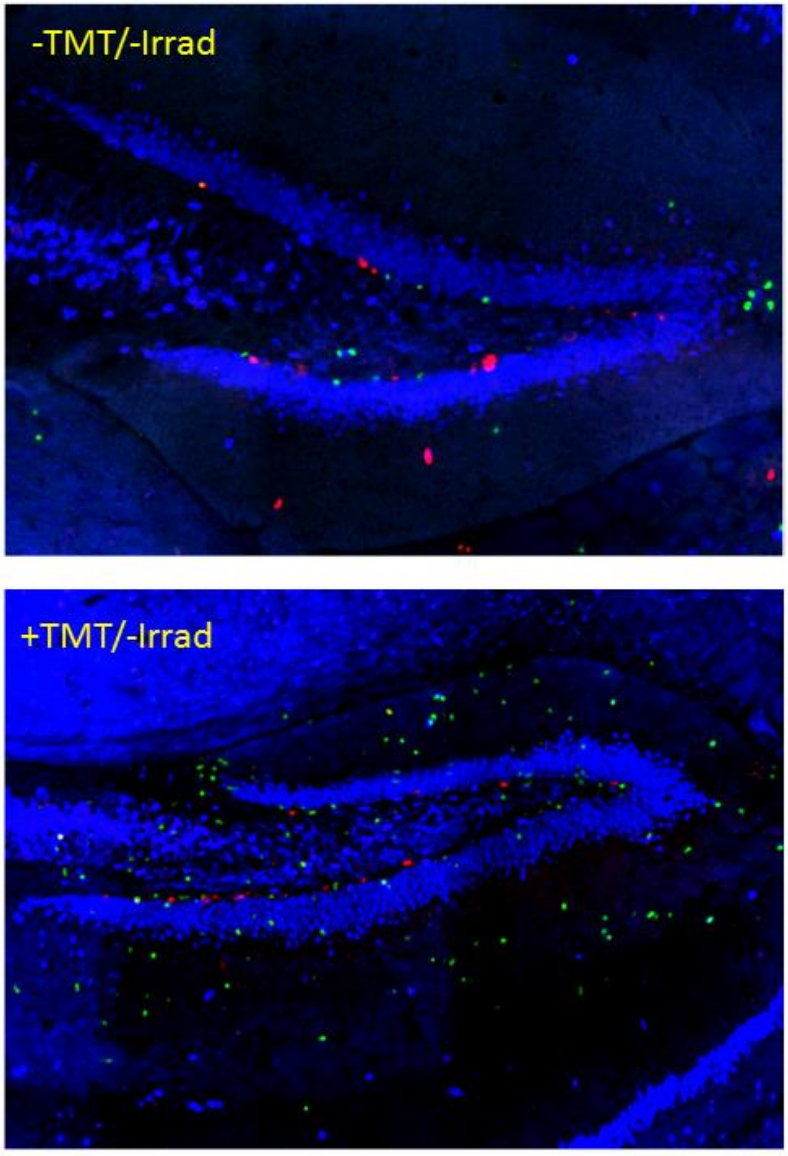


Figure 6. Ki-67 and BrdU staining of proliferating cells in 7 days post-TMT hippocampus. (Ki-67, red; BrdU, green; NeuN, blue)

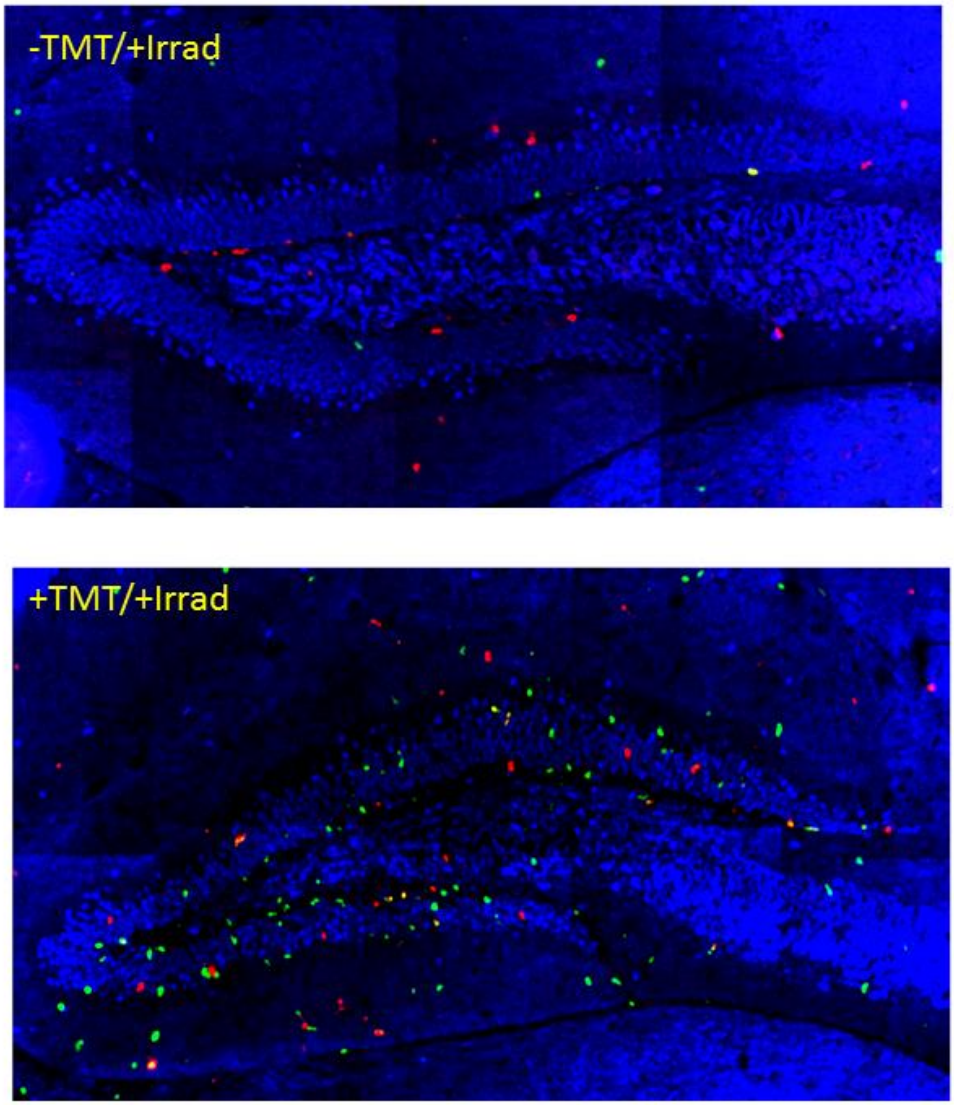


Figure 7. Hemotoxylin and eosin staining of hippocampus dentate gyrus of 1 year post-TMT and +/-exposure to 10G gamma irradiation showing normal morphology and no pyknotic nuclei.

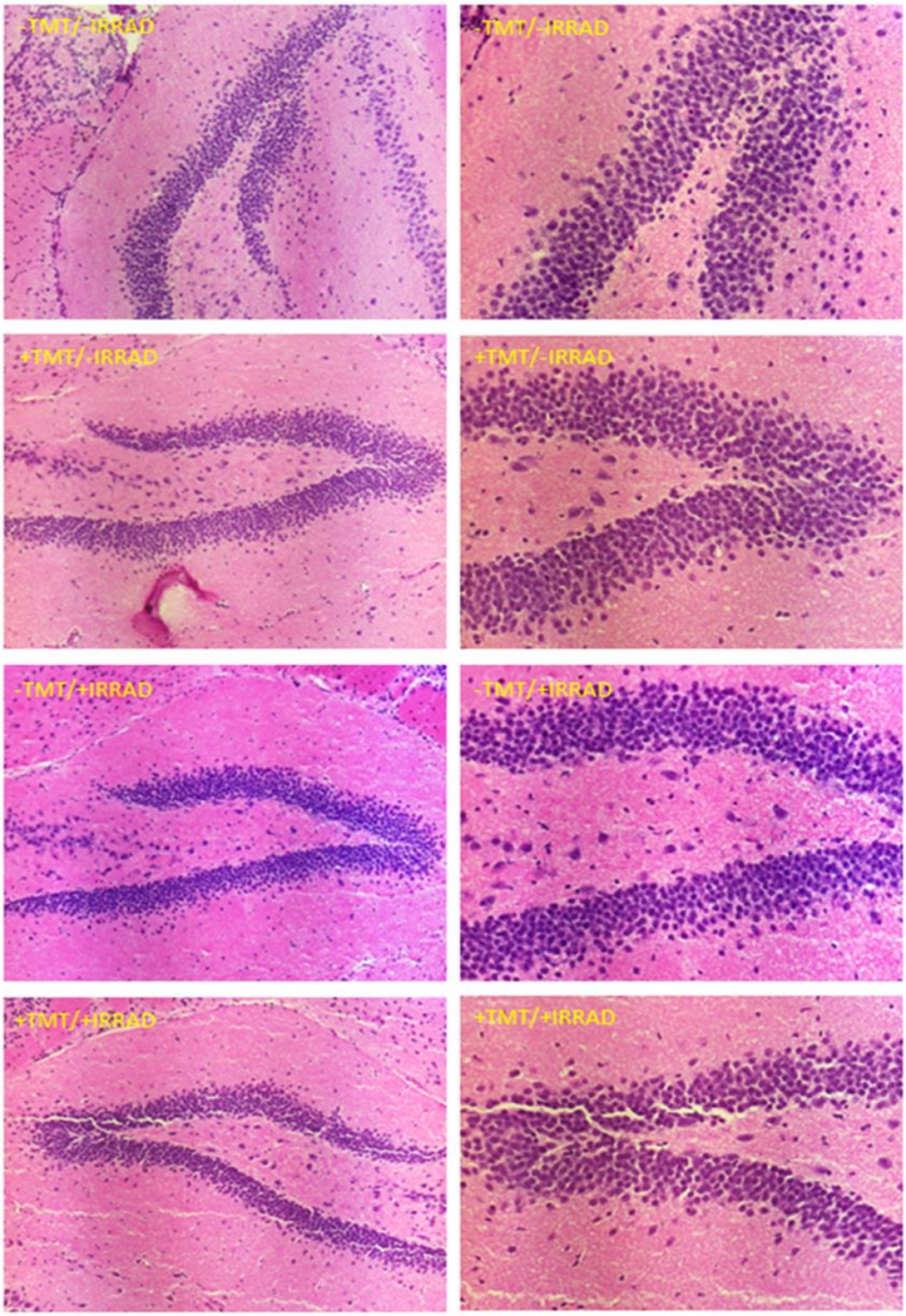


Figure 8. BrdU+, GFAP+, and NeuN+ cells in hippocampus dentate gyrus 1 year following TMT and gamma irradiation treatment showing no difference in GFAP immunoreactivity and few BrdU$^+$ cells remaining in hippocampus. (BrdU, green; NeuN, red; GFAP, magneta)

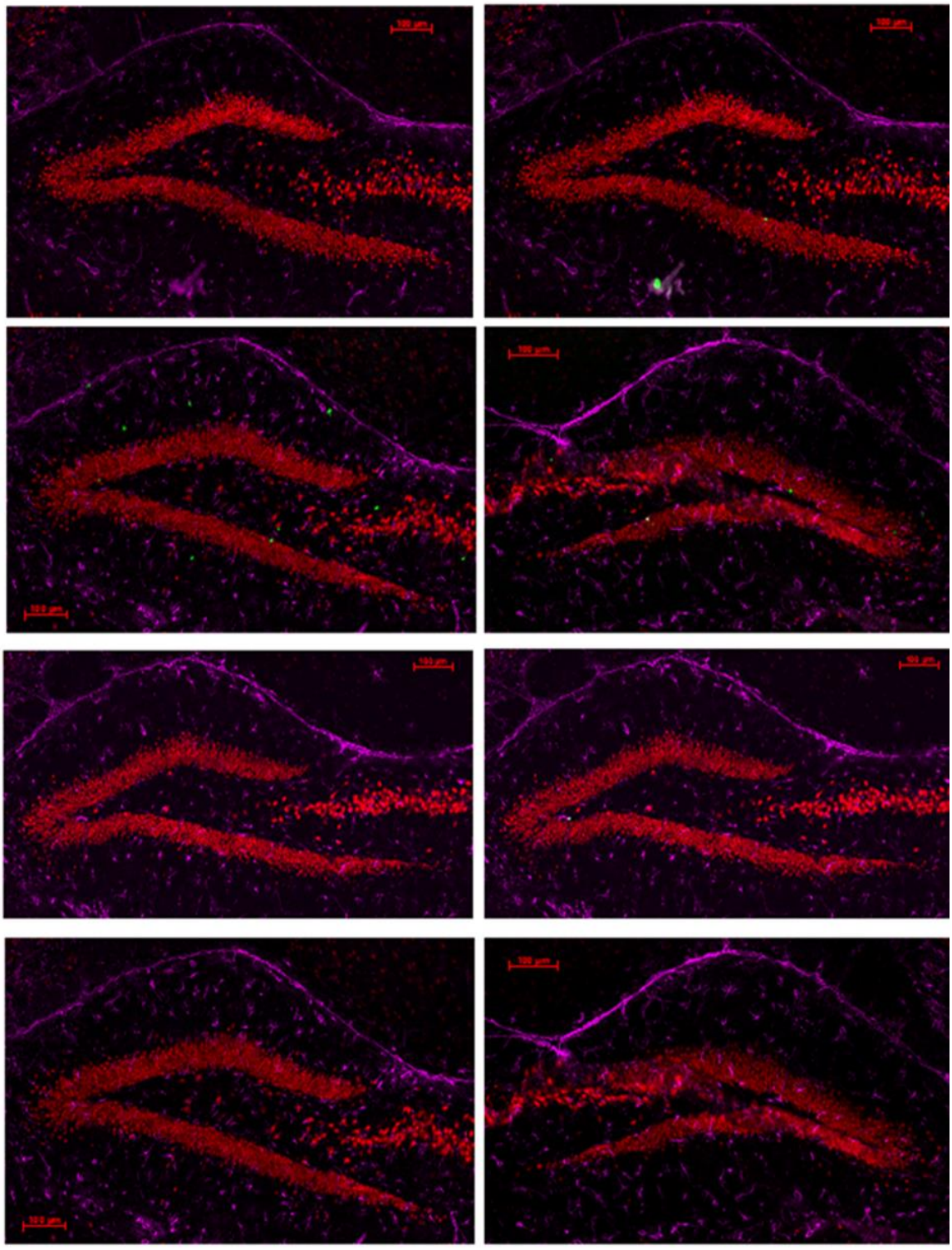


Figure 9. Survival of BRDU$^+$ cells in the hippocampus dentate gyrus one year after TMT and gamma irradiation treatment

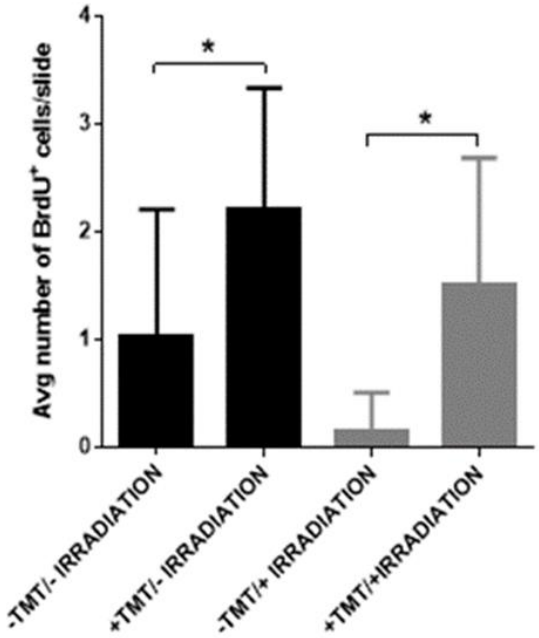
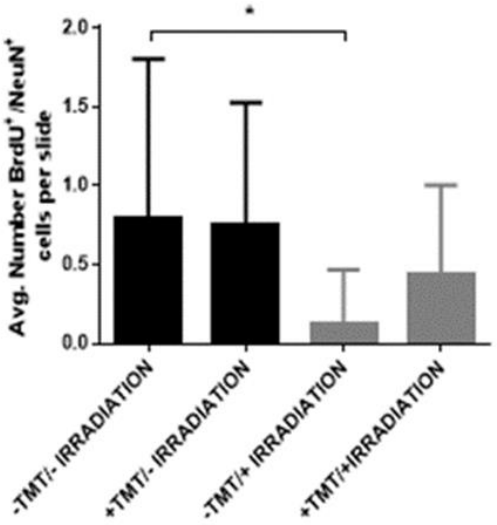


Figure 10. Survival of BRDU$^+$/NeuN$^+$ neuronal cells in hippocampus dentate one year after TMT and gamma irradiation treatment



Data are means from 3-4 animals per group; one-way ANOVA-Tukey test; **P<0.01.

Figure 11. Survival of BRDU$^+$/GFAP$^+$ cells in hippocampus dentate gyrus one year after TMT and gamma irradiation treatment

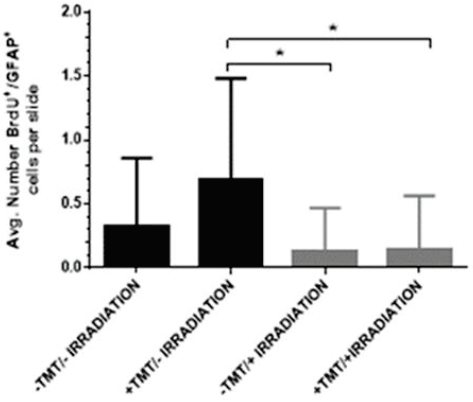


Figure 12. Ratio of NeuN$^+$/BrdU$^+$ to total number of BrdU$^+$ cells in hippocampus dentate gyrus survival one year after TMT and gamma irradiation treatment

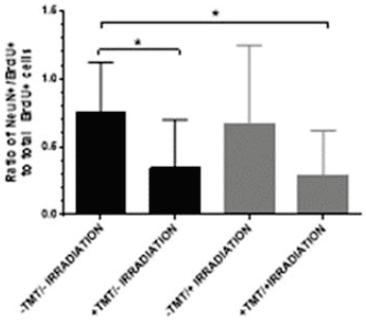
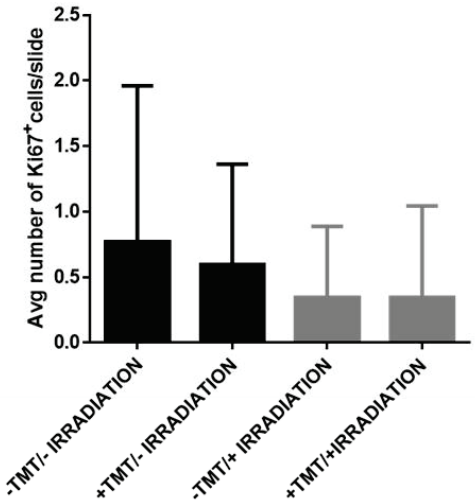
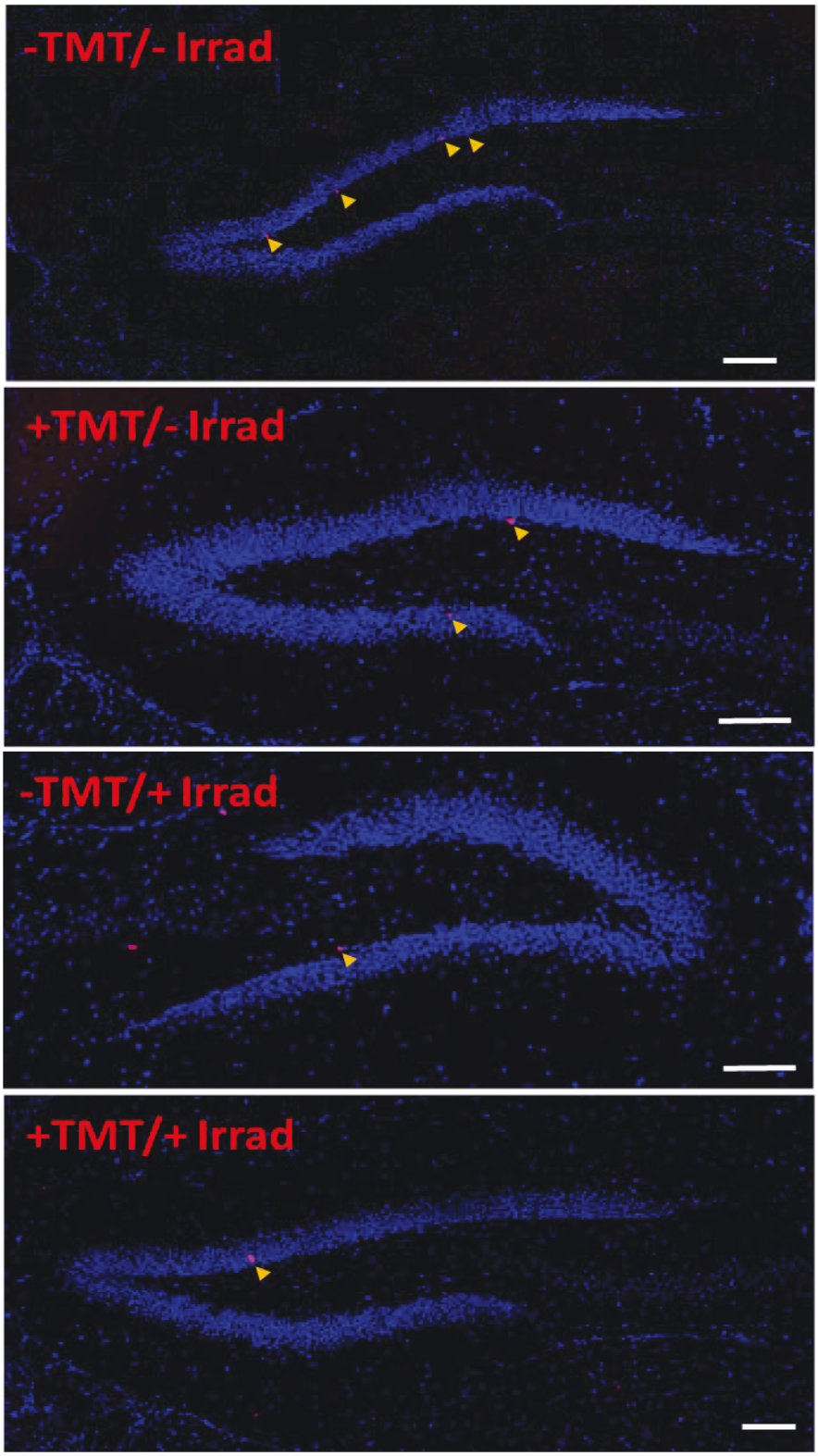


Figure 13. Mitotically active cells in the hippocampus dentate gyrus one year after TMT and gamma irradiation treatment



Data are means from 3-4 animals per group; one-way ANOVA-Tukey test; **P<0.01.

Figure 14. Ki67^{+} cells (arrow heads) in hippocampus dentate gyrus 1year following TMT and gamma irradiation treatment. (Ki67, red; DAPI, blue). Scale bar = 100 micrometers.



CHAPTER 5. SUMMARY AND FUTURE DIRECTIONS

Summary

We analyzed neural stem cell activity in the murine hippocampus following injury by trimethyltin intoxication. The goals were to provide an assessment of trimethyltin-mediated stem cell reactivity in the male C3H mouse model, examine whether neurogenesis plays a role in recovery from trimethyltin injury, and to test the hypothesis that SVZ derived cells are capable of migrating to the injured hippocampus as part of a repair response.

In order to more accurately describe the effects of trimethyltin on stem cell activity in this model, we characterized both the trimethyltin-induced injury effects on the hippocampus, as well as the response by endogenous hippocampal stem cells. Furthermore, we examined whether there was an effect on stem cell proliferation in the subventricular zone following TMT -induced hippocampal injury.

In the C3H mouse model of hippocampal injury, TMT induced a significant neuronotoxicity of granule cells in the hippocampus dentate gyrus. Pyknotic nuclei were clearly visible in H&E stained brain samples and this change was both concentration and time-dependent. Cell death was mediated through an apoptotic mechanism as revealed by immunoreactivity for activated caspase-3 in granule cell neurons. This is in agreement with previous studies indicating that trimethyltin induces apoptotic cell death, in addition to the necrotic cell death occurring at higher TMT doses. An inflammatory response after trimethyltin intoxication involved both astrocytic and microglial activation, and was limited to the site of injury at the hippocampus. Previous studies

have shown that TMT activates an inflammatory response that has both anti-inflammatory and pro-inflammatory components, though it is generally viewed that the inflammatory process is the predominant pathological feature, reflected in elevated levels of TNF alpha and inflammatory cytokines such as IL-1 and IL-6. The role of these inflammatory factors in mediating TMT injury has been demonstrated by blocking inflammatory cytokine activity. At least some of the toxicity of trimethyltin has been attributed to the production of reactive oxygen species. A recent study had shown that NADPH oxidase activation in microglial cells plays a significant role in ROS production and contributes to the downstream effect on cytokine upregulation through the activation of NFkappaB during TMT injury (Kim et al., 2015). Interestingly, some of these inflammatory factors could have dual roles by contributing to the repair process. McPherson et al. (2011) showed in a TMT mouse model that neural precursor cells from the subgranular zone express TNF alpha receptor, and that deletion of the receptor disrupts migration of the cells into the damaged granule cell layer. They suggested the microglial activation could have a supportive role in the repair process. In this study, we showed that GFAP and microglial activation occurs rapidly in the trimethyltin-injured mouse brain. Microglial activation, based on CD11b immunoreactivity, had returned to control levels by eight days after trimethyltin exposure while astrocytic reactivity remained elevated at 28 days post-TMT injury. Astrocytes are known to secrete trophic factors that may help survival of existing neurons as well as promote the differentiation and survival of newly formed neurons. In this study, the prolonged astrocytic reactivity may be an indicator that these cells are actively participating to promote a neural microenvironment more favorable for neuronal survival.

Trimethyltin intoxication in mice causes reversible, high-frequency tremors which are characteristic of the hippocampal injury. The initiation of tremor occurs soon after TMT treatment and temporally corresponds to apoptotic cell death of the hippocampus dentate granule cells.

However, the tremors stop within 72 hours even though apoptotic cell death continues for an additional 48 hours or more. The hippocampal hyperexcitability may be ameliorated via two processes that are activated early during trimethyltin injury: (i) activation of microglia and astrocytes, and (ii) proliferation of SGZ precursors. Microglia and astrocytes may play a role in limiting the extent and severity of the injury (e.g. anti-oxidants, glutamate transport, blood-brain barrier repair) while producing trophic or other factors to promote cell survival.

Trimethyltin injury induces proliferation of new cells in the subgranular zone of the hippocampus. These cells may have both the neurogenic and non-neurogenic functions that provide not only new cells to replace lost neurons, but some may also remain undifferentiated and secrete trophic factors to promote the survival of existing neurons (Butti et al., 2014). Astrocytes, microglia, and the non-neurogenic functions of neural precursor cells, may act in unison to reduce hyperexcitability and promote recovery from the injury.

In this study we showed that gamma irradiation significantly attenuated trimethyltin-induced activation of SGZ stem cell proliferation. Irradiation also resulted in increased severity and duration of tremor activity in the mice. It was beyond the scope of this thesis to determine whether the irradiation also affected astroglial and microglial function beyond the expression of phenotypic markers GFAP and CD11b, respectively. A separate cohort of mice underwent trimethyltin treatment and irradiation, and were maintained for up to one year post-treatment to assess the status of new cells generated at the time of trimethyltin injury, as well as to determine if irradiation affects the overall proliferative activity in the hippocampus dentate gyrus. As expected with aging, there was a reduction in the amount of mitotic activity in the subgranular zone. Interestingly, TMT treatment and irradiation independently reduced the number of Ki-67 positive cells in the hippocampus dentate gyrus. Irradiation can have long-term effects on the hippocampal neurovascular niche or have other effects that result in depleted proliferative

capacity and neurogenic regions (Monje et al., 2002). A recent study by Hellstrom (2009) showed that the subventricular zone and the subgranular zone in rats were initially equally sensitive to 6G irradiation, which resulted in attenuated stem cell proliferation and neurogenesis. However, there was a differential long-term effect of the radiation on stem cell activity in the subventricular zone versus the dentate gyrus. At 9 weeks post-irradiation, the SVZ recovered at least 50% of its proliferative capacity, whereas the dentate gyrus suffered a long-term decrease of proliferation to only 10% of control levels (Hellstrom et al., 2009). There was no persistent inflammatory reaction detected in the hippocampus that could have explained the suppression of proliferation. Importantly, the number of stem cells in the subventricular zone that was $GFAP^{+}/Nestin^{+}$ were unchanged following irradiation, whereas the stem cells in the dentate gyrus had been reduced to 15% of control (Hellstrom et al., 2009). While the mechanism(s) that would mediate this differential effect are unknown, clearly there was a change in the stem cell pool in the dentate gyrus, reflecting vulnerability of this brain region to ionizing radiation. As part of ongoing studies in this thesis, we are interested in quantifying the stem cell pool in both the seven day and one year post-treatment study to determine if these pools are affected by radiation and trimethyltin.

The depletion of proliferative capacity following trimethyltin treatment at one year is interesting. Could the same mechanisms mediating the radiation-induced depletion of stem cell number also be involved in the loss of proliferation in trimethyltin-treated mice? In addition to direct toxicity to stem cells, multiple factors may contribute to the reduced SGZ mitotic activity, including alterations to the neurogenic niche via effects on the cerebrovasculature. For instance, endothelial cells are extremely sensitive to radiation injury, causing altered function that mediates secondary effects of radiotoxicity. In this thesis, preliminary evidence of a quantitative effect of TMT on blood vessel morphology was observed. The biological significance of these morphological changes has yet to be explored, but given the important role that the vasculature

plays as a component of the neurogenic or neurovascular niche, any cardiovascular effect by a toxicological agent could translate to secondary effects on stem cell function. Recent studies indicate the importance of pericytes in modulating blood vessel function including vasoconstriction and vasodilation. In this regard, it was shown in nonhuman primates that grafted neural progenitor cells migrated toward blood vessels and took on the appearance of pericytes (Lundberg, et al., 1996; Lundberg, et al., 1997). Pericytes themselves have been proposed to have neural stem cell activity (Yamashima et al., 2004; Dore-Duffy, 2006). Furthermore, recent research suggests that stem/progenitor cells in the hippocampus cluster around blood vessels and that neurogenesis occurs within an 'angiogenic' microenvironment (Palmer et al., 2000).

In this study, stem cells residing within the subventricular zone appeared not to be activated via proliferation by trimethyltin injury. Various studies have shown that subventricular zone-derived precursor cells are capable of responding to injury by upregulating proliferation and even diverting their migration away from of the normal rostral migratory stream pathway to the olfactory bulb, and instead migrate to the lesion site. Reports describing whether these cells differentiate to a phenotype appropriate to replace those lost to the injury are equivocal, and vary depending on type of injury, age, and whether endogenous or exogenous growth factors are present.

The significantly reduced proliferation in the hippocampus dentate gyrus in animals at one-year post-treatment with TMT could be the result of a depletion of cells with mitotic potential. The differential long-term effect of radiation on SVZ versus SGZ mitotic activity could reflect differences in modes of cell cycling between these two neurogenic regions. Stem cells in the subventricular zone can regenerate themselves through symmetric or asymmetric cell division, thus sustaining the primary stem cell population for future capacity to generate new transit amplifying cells. On the other hand, less is known about the cell division patterns in the

subgranular zone of the hippocampus. In a recent study, it was shown that in early postnatal mice there may exist two populations of amplifying precursor cells, $GFAP^+/Sox2^+$ stem cells in the hippocampus (Figure 1), defined by whether the population co-expresses pro-neural genes including Mash1 and Hu (Namba et al., 2011). The progenitor cells expressing the pro-neural genes are thought of as transit amplifying cells that undergo cell division symmetrically to form neural precursors. The other stem cell population, lacking pro-neural gene expression, are identified as likely primary progenitors or stem cells capable of dividing asymmetrically (producing a progenitor and neural precursor) or self-renew symmetrically. As animals reach adulthood there may be a progressive loss of self-renewal capacity and an increase in the progenitor population expressing GFAP/Sox2 and pro-neural genes (Namba et al., 2011). Hence these cells are predictably more restrictive in their lineage fate (Namba et al., 2011; Seaberg et al., 2002), resulting in eventual depletion of the multipotent stem cell pool. Interestingly, a recent study suggested that there are two populations of transit amplifying cells in the subventricular zone, one of which responds to injury by increasing proliferation (GFAP-/Mash1) and generating neuroblasts, and the other ($GFAP^-/EGFR^+$) capable of de-differentiating to an earlier lineage resulting in a non-proliferative increase in the $GFAP^+$ stem cell population (Thomsen et al., 2014). Hence, the subventricular zone stem cell population may have multiple mechanisms to renew itself. Whether such a process occurs within the hippocampus is unclear, though it is generally accepted that there is a decrease in stem cell activity in the hippocampus with aging, which is consistent with the data in the radiation study presented in this thesis.

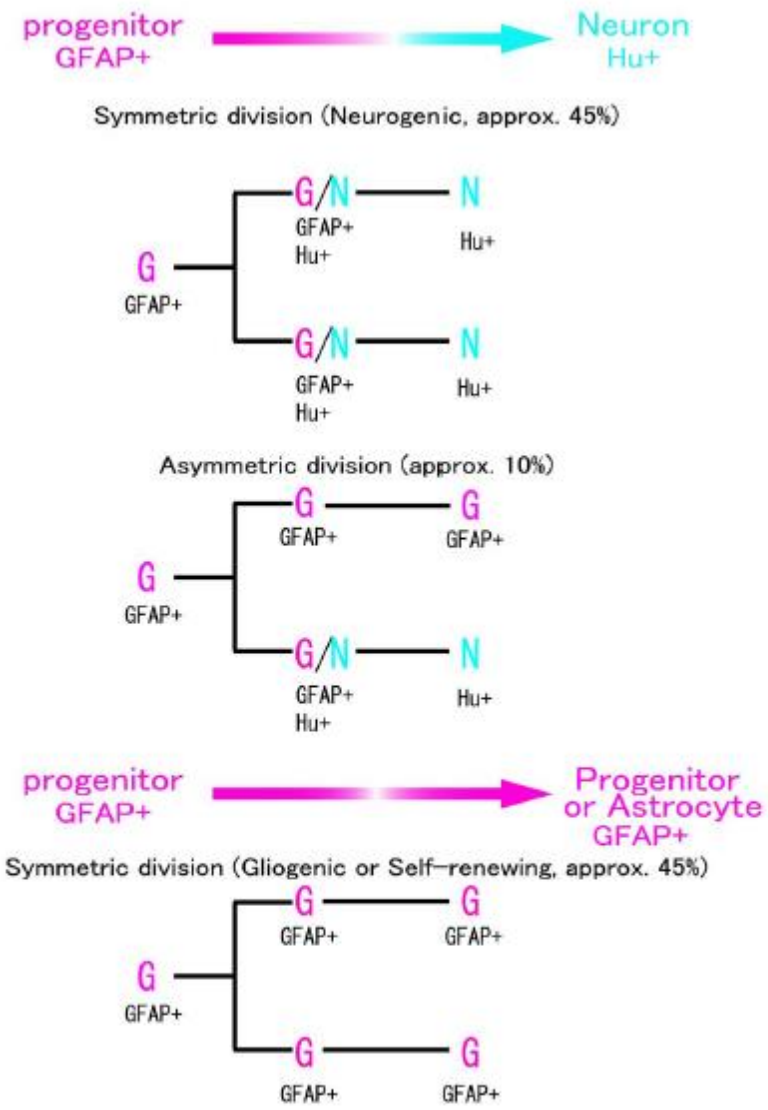


Figure 1. Proposed patterns of cell division by GFAP$^+$ progenitors in the hippocampus dentate gyrus during the early post-natal period. Only a small percentage of GFAP$^+$ progenitors divide asymmetrically to produce new progenitor cells and intermediate neural precursors (GFAP$^+$/Hu$^+$). Most GFAP$^+$ progenitors divide symmetrically to either self-reproduce or give rise directly to intermediate neural precursors. (From Namba et al., 2011)

In addition to the reduced proliferation occurring in the hippocampus at one year following TMT treatment, there was a significant reduction in the percentage of BrdU$^+$ cells that co-labeled with the neuronal marker NeuN (Figure 12; Chapter 4). This effect was independent of whether the animals received radiation. As discussed previously, this could be the result of changes in the

neural microenvironment following TMT injury that not only reduces proliferation in the hippocampus, but also negatively impacts the long-term survival of newly generated cells. The possibility that there is a low-level inflammatory response persisting in mice treated with TMT is part of a plan for future studies. While there is no overt qualitative elevation in astrocyte reactivity based on GFAP detection, it remains to be determined whether other factors derived from astrocytes or microglial are elevated. The finding of reduced long-term survival of neurons may be in contrast with an earlier study. In a rat model of TMT injury, Geloso et al., (2007) demonstrated that neural stem cells grafted into the TMT-injured hippocampus exhibited greater survival versus an uninjured brain. However, this study was done in rats and only went out to 30 days. As mentioned above, astroglial reactivity persisted in this study for at least 28 days post-injury. If the astrocytes are providing support to the new cells it would not be surprising that transplanting precursor cells into this microenvironment would increase the chances of survival up to this time. A time-course extending out to one year post-injury would be informative in resolving the status of new-born and grafted cell survival, as well as define changes that occur in the neurogenic microenvironment.

We demonstrated that there was an induction of migration of spDiI-labeled subventricular cells into the TMT-injured hippocampus dentate gyrus at 28 days. In the 28-day post-TMT group, $spDiI^{+}$ cells migrated across the alveus toward the ventral blade of the dentate gyrus. This area is adjacent to the fimbria and cortical helm region, which during development is the source of migrating neural stem cells (the dentate migratory stream) and morphogenic factors that contribute to generation of the inner layer of the dentate gyrus and the long-lived SGZ stem cells (Li et al., 2009;2013). The localization of some $spDiI^{+}$ cells in the SGZ is intriguing and raises the possibility that the response to TMT injury is recapitulating a developmental process to repopulate the GCL/SGZ with cells derived from the ventricular zone.

The migration of SVZ precursor cells under physiological conditions is confined to the rostral migratory stream as a source of replacement olfactory neurons. However, injury can induce progenitor cell proliferation and migration of SVZ-derived cells toward affected non-olfactory brain regions (De Marchis et al., 2004; Nakatomi et al., 2002; Arvidsson et al., 2002; Zhang et al., 2004a; Yamashita et al., 2006; Goings et al., 2004; Weig et al, 2016). The majority of the studies showed the migration of these cells to extra hippocampal sites such as striatum. Few studies have shown the capacity of subventricular stem cells to migrate to the hippocampus, and of those, the migration is limited to the CA1/CA2 subregions of the pyramidal cell layer (Nakatomi, et al., 2002). However, recently Belmadani et al. (2015) showed that EGFP-CXCR4-labeled ventricular cells migrated into HIV virus-injured hippocampus dentate gyrus in 8 week old mice. The virus induced inflammation and neurodegeneration with the dentate gyrus. Cell migration was traced through the adjacent fimbria as well as ventrally along the meningeal-dentate junction proximal to the infrapyramidal blade of the dentate gyrus. This pattern appears similar to the migration path of spDiI^{+} cells following TMT injury observed in the study described in this thesis, suggesting there may be a common response mechanism for mobilization of ventricular-derived cells to acutely injured hippocampus dentate gyrus.

In conclusion, TMT injury of the murine hippocampus is a useful model for studying cellular events occurring during the acute phase of the injury, and the repair mechanisms that attempt to return the hippocampus to homeostasis. This model allows rapid studies on the mechanism of brain injury and neural repair, and related aspects from stem cell proliferation to new neuron integration and the behavioral consequences. While the early response to TMT intoxication may be primarily geared to limit the extent and severity of the injury and generate new neurons that will integrate into the neural network, there may be long-term consequences that include an altered neurogenic environment and depletion of stem cell function. Trimethyltin may also have

other effects on cerebrovascular and glial function that directly or indirectly affect stem cell activity. In addition, a non-neurogenic function of stem cells may be activated under neuropathological conditions in which new cells remain undifferentiated but secrete neurotrophic mediators that aid in promoting survival of older bystander neurons and newly-born neurons. Further studies are required to delineate the interactions between the various cell types and identify the molecular factors that play key roles in facilitating repair from brain injury. Such knowledge could provide new avenues to study stem cell biology in other tissues and species, as well as elucidating potential clinical applications for stem cell research in other forms of brain injury.

Future Directions

The studies presented in this thesis raise a number of intriguing questions related to the response of neural stem cells to trimethyltin injury. There are many potential avenues for future directions of research, not only directed at the mechanisms modulating the proliferative response of stem cells following injury, but also mechanisms that play a role in inducing and directing the migration of neural precursor cells derived from the hippocampal SGZ and subventricular zone. Clearly, the role played by new cells derived from either of these neurogenic zones needs to be further defined. Do they contribute more than repopulating the lesion site with new replacement cells, or do the precursor cells perform non-neurogenic functions that promote survival of newly born and existing neurons? Do other studies, in addition to the trimethyltin mediated injury presented in this thesis, such as HIV virus-induced injury presented by Belvindrah (2014) or ischemic injury by Nakatomi (2002) suggest that there is a common mechanism in the adult brain

that directs SVZ-derived precursors cells to the injured hippocampus? Do the SVZ-derived cells harbor non-neurogenic activity, and/or neurogenic activity once they are localized to the subgranular zone neurogenic niche? Data in this thesis in addition to prior studies (Belvindrah et al., 2014) may be indicative of a recapitulation of a developmental pattern in the adult in which new cells migrate along a developmental dentate migratory stream to the forming hippocampal dentate gyrus.

The gamma irradiation study presented in this thesis showed that there was a reduction of proliferative capacity by endogenous hippocampal stem cells. Gamma irradiation studies should be further expanded to explore mechanisms by which neurogenesis aids in the recovery from tremor activity and repopulation of the trimethyltin-lesioned dentate granule cell layer.

An intriguing area of research is looking at the long-term effects of trimethyltin and gamma radiation on the dentate gyrus. In this study it was shown that gamma irradiation resulted in the loss of proliferative activity in the dentate gyrus of the one year post-treatment mice. In addition, trimethyltin treatment alone resulted in a similar effect on the loss of proliferative capacity at one year post-treatment. What biological impact did radiation and trimethyltin treatment have that resulted in loss of proliferation at one year after dosing? Was there a loss of stem cells, or, was the neurogenic microenvironment altered in a way that resulted in the loss of the stem cell activity? Did the induction of proliferation by trimethyltin result in the premature depletion of precursor cells that had a limited proliferative capacity during the life of the animal? Radiation and trimethyltin injury had a similar effect on the loss of physiological mitotic activity at one year post-treatment, even though trimethyltin induced a significant upregulation of proliferation at the time of injury, whereas gamma radiation inhibited proliferation. It would be interesting to look at whether the one year post-treatment animals were still capable of undergoing injury-induced upregulation of cell proliferation in the hippocampus following exposure (or re-exposure)

to trimethyltin. Such a study would indicate that stem cells with the capacity to respond to whatever factors activate the proliferative response still exist. A differential effect by trimethyltin injury on inducing proliferation would suggest different mechanisms may be involved in reducing proliferation by the two different types of exposure. Further, *in vitro* analysis of stem cells derived from the hippocampus to study formation of neurospheres and their capacity to produce new neurons would help determine the stem cell status of the two treatments.

Separately from the effect of trimethyltin on neurogenesis and cell proliferation, was the observation that irradiated mice that had been treated with trimethyltin exhibited less alteration in hair pigmentation of the cranium as opposed to animals that were not treated with trimethyltin. This raises questions related to whether pre-conditioning or post-conditioning by a nonlethal dose of trimethyltin could act to protect the animal from subsequent effects of another insult. In other words, did treating irradiated mice with trimethyltin cause physiological changes that protected them from the long-term effects of the radiation exposure? It is well established in rodent models of ischemia/hypoxia that preconditioning with a low level ischemic insult will protect the animal from a primary insult through the upregulated genetic expression of protective factors. Could a similar pre-conditioning effect be happening in the TMT-treated irradiated mice?

There are number of core questions and possible directions of study that would be of interest to pursue, some of which are listed below:

I. What are the factors that induce proliferation of endogenous hippocampal stem cells following trimethyltin injury?

II. Further characterization of early and long-term alterations in the hippocampal neurogenic niche as the result of TMT injury may increase understanding of the mechanisms involved in the neural repair. Is there a reduced number of stem cells as

a result of the 'burst' of proliferation in the SGZ that occurs immediately following injury? As the trimethyltin-treated mice age, do they retain the capacity to have a proliferative burst following injury?

III. Is the toxicity of trimethyltin limited to mature granule cells in the hippocampus dentate gyrus, or do neural precursor populations have a selective sensitivity to the toxicant?

IV. Does trimethyltin-induced injury promote the survival of more newly born cells in the hippocampus, and how does that compare to the long-term survival of cells that that are produced under normal physiological conditions?

V. Additional studies in which proliferation and neurogenesis in the hippocampus is effectively shut down either by more intense radiation or the use of other anti-mitotic agents would help to define the role of new cells in context with trimethyltin-induced injury.

VI. Further study on the effects of trimethyltin on the cerebrovasculature would be very interesting. The preliminary data in this thesis suggests that blood vessels are impacted by trimethyltin intoxication. Since blood vessels play an important role in the neurovascular niche in which neural stem cells reside, trimethyltin-mediated alteration in blood vessel or endothelial cell function could have a secondary effects on neurogenesis in the short- or long-term. Toxicity to pericytes, cells that modulate cerebrovascular function, may be a contributory factor to trimethyltin-induced changes in stem cell function.

VII. Does trimethyltin and/or radiation reduce the volume of granule cell neurons occupying the hippocampus at one year after exposure? No stereological studies of

the murine hippocampus have been performed in mice at extended time windows after trimethyltin exposure (e.g. 6-12 months post-exposure).

VIII. Long-term effects of trimethyltin treatment on behavior, with or without gamma irradiation treatment, would be an important component of further study. It was shown that activation of neurogenesis with chronic lithium treatment in trimethyltin-injured mice protected against cognitive effects from the injury (Yoneyama, 2014). If a similar study were conducted in mice that are irradiated (to block the lithium-induced increase in neurogenesis), would lithium still result in improvement on the trimethyltin-induced effect on cognition?

IX. In this thesis it was shown the trimethyltin injury could induce migration of spDiI-labeled ventricular cells to the hippocampus. What factors play a role in modulating the migration of these cells? Among other factors, Reelin, SDF1, and CXCR4 activity will be of particular interest to investigate to determine if they are upregulated with trimethyltin injury and play a role in directing subventricular zone cells to the injured hippocampus.

X. An alternative to using spDiI for tracing migrating cells may be useful in future research studies. Other fluorescent markers may be brighter and more stable for long-term labeling of migrating cells, such as the use of mCherry (Belvindrah, 2014). Tracers that would allow analysis of cellular architecture, including electron microscopy, might be useful in helping to identify the migrating cells and characterize the surrounding cellular microenvironment.

XI. Further research to optimize the effect of trimethyltin injury on inducing migration of subventricular zone-derived cells would be useful. Parameters that should be

analyzed include age of mice, dose of trimethyltin and extent of injury, and a more extensive time course.

XII. Prior studies have suggested that ependymal cells have the capacity to proliferate in response to injury and have stem cell-like activity (Xu et al., 2005; Coskun et al., 2008). Studies using long-term administration of proliferation markers such as bromodeoxyuridine, may help to reveal whether ependymal cell proliferation occurs in context with trimethyltin injury. Specific labeling of ependymal cells would also be of interest to determine whether they respond and migrate to the hippocampus after trimethyltin injury.

XIII. Stereotaxic transplantation and grafting studies of subventricular zone stem cells into mice treated with trimethyltin may be useful for determining the capacity of these cells to migrate into the injured hippocampus. Differential placement of grafted cells could help elucidate regional dependency on whether the cells are stimulated to migrate in response to any injury-induced secretion of migration factors.

XIV. Organotypic brain slices have proven useful for studying interactions between cells in a semi intact neural environment (reviewed in Humpei, 2015). The technique has proven useful for studies of dopaminergic neurons in the substantia nigra in Parkinson's disease models. The technique may also be useful for looking at trimethyltin effects on induction of stem cell activity, and for following the migration of labeled stem cell precursors into the injury site.

XV. What role do astrocytes play in regulating neurogenesis recovery from trimethyltin injury? A recent study showed that astrocytes mediate neurogenesis through Ephrin-B signaling (Ashton, et a., 2012). Since trimethyltin induces astrogliosis in the murine

hippocampus, these cells are favorably positioned to influence stem cell activity in the injury site.

XVI. Microglial cells are activated following trimethyltin injury. The cells may have both pro-inflammatory and anti-inflammatory functions in the trimethyltin-injured hippocampus. These cells may play a role in both modulating stem cell activity and produce factors that affect the migration of precursor cells. Activated microglia express TNF alpha and other cytokines. In this context, TNF alpha receptors are expressed by SGZ precursor cells, and deletion of this receptor disrupts the migration of the cells into the granule cell layer (McPherson et al., 2011). Other microglia-derived factors may play a role in modulating migration as well as inducing proliferation of stem cells in TMT-injured hippocampus. Does radiation treatment affect the function of microglia and astrocytes, causing them to lose regulatory control of stem cell activity? Hence another direction of future study would be to look at how glial cells modulate neural stem cell activity in a neuropathological environment and whether these cells are susceptible to disruption by radiation and trimethyltin.

XVII. Another potential future study will be to isolate the $spDiI^{+}$ cells that migrate into the TMT injured hippocampus and culture them in vitro. If they are capable of forming neurospheres and responding to growth factors, this would suggest that the cells have stem cell-like activity. If such activity were found, then that may indicate that the biological function of SVZ-derived cells migrating into the hippocampus may be an attempt to repopulate the SGZ stem cell niche. However, the observations in this thesis that trimethyltin results in loss of proliferative capacity one year after

treatment, suggest that those cells are either nonresponsive or fail to survive for the long-term.

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