EXPLORING THE ROLE AND SENSITIVITY
OF THE HIPPOCAMPAL DENTATE GYRUS:
FROM ADDICTION-RELEVANT MEMORIES
TO THE INFLUENCE OF SPACE RADIATION
ON HIPPOCAMPAL NEUROGENESIS

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DEDICATION

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by

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Abstract

The hippocampus and its subregion the dentate gyrus (DG) are involved in learning and memory. Adult hippocampal neurogenesis, which takes place in the DG, is also thought to contribute to learning and memory. Understanding the neural basis of learning and memory could help in a wide range of situations, from helping addicts break the cycle of substance

abuse to ensuring appropriate astronaut action during spaceflight missions. This doctoral dissertation spans this wide range by using animal models relevant to addiction and spaceflight to improve understanding of the DG and adult neurogenesis, and obliquely, of learning and memory. After an introductory chapter, I show morphine-context reward memories are established via drug/context associations (D/CA, Chapter 2), and require adult neurogenesis for extinction of young reward memories (Chapter 3). Using conditioned place preference, a behavioral test classically used to assess drug strength, and the immediate early gene cFos as an indirect marker of neuronal activity, I found that morphine-paired mice sequestered to a morphine-paired context had more DG cFos+ cells than those sequestered to a saline-paired context or other controls. Thus, the retrieval of D/CA memory is accompanied by activation of hippocampal DG neurons. Surprisingly, image-guided cranial irradiation (IG-IR) prevented extinction of young, but not old, morphine D/CA memories without affecting retrieval. These data suggest that deficits in adult neurogenesis may contribute to stronger D/CA reward memory. The second section of my dissertation (Chapter 4) examines the influence of space radiation on adult neurogenesis. I find acute and fractionated space radiation similarly diminish adult neurogenesis, but neither decrease neural stem cell number, the putative source of new neurons. Thus, while

spaceflight mission success may be hampered by space radiation due to diminished neurogenesis, my data raise the possibility that neurogenesis may recover overtime. Taken together, my data show an impaired DG (and perhaps neurogenesis) diminishes extinction of morphine-context reward memories, and that adult neurogenesis is decreased (perhaps reversibly) by space radiation. In my final chapter (Chapter 5), I discuss implications of these data for the fields of learning/memory and neuroscience in general, and suggest future directions that may help addicts recover and allow astronauts to perform optimally during spaceflight missions.

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LIST OF DEFINITIONS

Acquisition/Encoding – A process of learning where percieved sensory information is linked to neuronal plasticity in the brain.

Consolidation– A process of converting short-term to long-term memory.

Retrieval – A process where a previosuly consoldiation memory is conciosuly recalled.

Reconsolidation– A process that occurs after consoldiation, where a memory can become weakend or strengthened based concurrent assocaitions.

Extinction – New learning that requires multiple training days and different contexts/cues.

GCR – Galactic cosmic radiation is a specific type of radiation created by supernovas and massive stars.

HZE particles – High energy, high charged particles that are created with a synchrotorn used to mimick GCR radiation on Earth.

Addiction – A mental illness where neurocircuitry in the brain is altered to favor rewarding experineces.

Conditioned place preference – A behavioral paradigm that is used to examine drug/context associations.

LIST OF ABBREVIATIONS

ABGC, adult-born granule cell

CPP, conditioned place preference

CS, conditioned stimulus

Ctx, cortex

DA, dopamine

DCX, Doublecortin

DG, dentate gyrus

EC, entorhinal cortex

GCL, granule cell layer

GCR, Galactic cosmic radiation

GFAP, glial fibrillary acidic protein

GLAST, Glutamate Aspartate Transporter

HZE, high charge, high energy

LTP, long-term potentiation

LTD, long-term depression

mf, mossy fibers

Mol, molecular layer

MTL, medial temporal lobe

NAc, nucleus accumbens

PFC, prefrontal cortex

pp, perforant path

PTSD, post-traumatic stress disorder

RGC, radial glial cells

sc, Schaffer collaterals

SGZ, subgranular zone

US, unconditioned stimulus

VTA, ventral tegmental area

CHAPTER ONE:

INTRODUCTION

OVERVIEW

The hippocampus and regional neurocircuitry are believed to be involved in the creation and recall of memories. Humans with portions of their medial temporal lobes removed have deficits in the creation and recall of memories. Similarly, animal studies with lesions to the hippocampus also have deficits in learning and memory processes. Within the hippocampus is the unique process of adult neurogenesis, where the birth of new neurons can occur throughout life. Interestingly, adult neurogenesis has been implicated in mediating the learning and memory process by altering hippocampal neurocircuitry. Therefore, understanding the role of adult neurogenesis in learning and memory aspects could help situations that range from developing behavioral based treatments for human addicts to developing appropriate risk assessment for astronauts in deep space. As such, this dissertation aims to examine adult neurogenesis by asking two general questions (which is a general motto of the Eisch lab): 1) what is adult neurogenesis good for, and 2) what is good/bad for adult neurogenesis? Of particular interest to one aspect of my dissertation is the study of episodic memory after “strong” or memorable events caused by reward (Chapter 2) and how cranial irradiation to the hippocampus and

adult neurogenesis alters learning and memory (Chapter 3). The remainder of my dissertation examines the specific conditions of space radiation that can negatively impact adult neurogenesis (Chapter 4). Chapter 5 then discusses how this dissertation has furthered the fields of learning and memory, addiction, and space radiation, and future directions are discussed. Therefore, the introduction provides information on a wide range of topics needed to appreciate the results from the data Chapters (2-4): general hippocampal learning and memory, hippocampal neurocircuitry, adult neurogenesis and its role in learning and memory, the involvement of adult neurogenesis in addiction, and finally the effects of space radiation on adult neurogenesis.

THE HIPPOCAMPUS

Historical Perspective

For many years, philosophers, psychologists, and cognitive neuroscientists have tried to understand how memory formation and retrieval occurs in the brain. For the past 50 years, the field of learning and memory has largely been influenced by one person, Mr. Henry Gustav Molaison (1926-2008, more commonly known as H.M.). H.M. was a patient with debilitating epileptic seizures who underwent a bilateral medial temporal lobectomy in 1953, where over half of the posterior hippocampus was removed as well as the entire amygdala and entorhinal cortex (i.e. all contained

in the medial temporal lobe, MTL) (Scoville and Milner, 1957). While H.M.'s seizures were not as debilitating after surgery, researchers noticed in general that H.M. could no longer create new memories (anterograde amnesia) nor could he remember some events and facts prior to the MTL surgery (retrograde amnesia).

From studying H.M. and others who had similar damage (e.g. R.B. (Zola-Morgan et al., 1986); G.D., L.M., and W.H. (Rempel-Clower et al., 1996)), three basic findings of memory were formed; 1, there are different types of memory; 2, different brain regions are responsible for learning, retrieval, and storage of memories; 3, the hippocampus is largely involved in memory retrieval (reviewed by (Squire and Wixted, 2011)). In regards to point 1, based on H.M.'s MTL surgery, researchers determined that long-term declarative memories (i.e. the conscious recollection of facts and events) prior to H.M.'s surgery were impaired (retrograde amnesia), but not completely lost, while procedural (i.e. nondeclarative) memory (i.e. the unconscious memories which influence perception and personality) remained intact (Cohen and Squire, 1980; Squire and Wixted, 2011). Due to this finding, other subsets of declarative memory types have also been described: semantic (i.e. information factual such as language or world and object knowledge) and episodic (i.e. spatial information that coincides with temporal events) (Squire and Zola, 1998; Squire and Wixted, 2011). In regards to point 2, H.M.'s ability to learn new motor skills, but not create new long-term memories (anterograde amnesia), suggested

that memories are complex and can utilize various brain regions. For example, H.M.'s ability to retrieve some memories prior to surgery could occur, suggests that long-term memory storage and retrieval does not completely rely on the MTL region, and likely involves the neocortex. In regards to point 3, memory impairments were observed (e.g. in R.B., G.D., L.M., and W.H.) with lesions to the hippocampus and it's subregions, such as the CA subfields and the dentate gyrus (DG) (Rempel-Clower et al., 1996; Zola-Morgan et al., 1986). Non-human primates also had impairments in learning and memory after lesions to the hippocampus and hippocampal subregions (Mishkin, 1978; Zolamorgan et al., 1994). Taken together, H.M. truly provided a foundation for future learning and memory experiments to better understand the process and brain regions involved in learning and memory (Squire and Wixted, 2011). Therefore, my dissertation studied episodic memory after "strong" or memorable events caused by reward and how cranial irradiation to the hippocampus altered learning and memory (Chapter 3) (Koob and Volkow, 2010). As I discuss in the conclusion chapter (Chapter 5), the same principle can theoretically be applied to other "strong" events caused by fear (e.g. PTSD).

Hippocampal Neurocircuitry

Briefly, before discussing the neurocircuitry, the structural intricacies of the hippocampus should be clarified since the term "hippocampus" has recently lost its accuracy. Therefore, a brief review of the terminology used in this dissertation is

necessitated. According to “The Hippocampus Book”, the term hippocampal formation contains six structures: the dentate gyrus, hippocampus proper, subiculum, presubiculum, parasubiculum, and the entorhinal cortex (Amaral and Lavenex, 2007). In addition, the hippocampus proper contains the subregions of CA1, CA2, CA3 (i.e. CA, *cornu ammonis*, is latin for Ram’s horn). For clarity, this dissertation uses the term hippocampus to mean hippocampal formation.

Classically, the trisynaptic circuit (i.e. three sequential synapses in the hippocampus via the perforant path [pp], mossy fibers [mf], and Schaffer collaterals [sc]) has been used to define subregion specific functionality in the hippocampus (Becker, 2005). Therefore, to understand the process of learning and memory at the circuit level, a brief description of the hippocampal circuit is provided (**Fig. 1-1**). The major input into the hippocampus is from EC layer II neurons, which send glutamatergic projections (via the pp) to the CA3 subfield as well as the molecular layer (Mol) of the dentate gyrus (DG) (Amaral et al., 2014, McNaughton and Miller, 1984). Granule cell neurons make up a major component of the DG and extend their dendrites into the Mol (i.e. there are also other cell types found in the DG such as glia and interneurons which can also contribute to changes in neurocircuitry). The granule cell layer (GCL) is easily observed since granule cell neurons are densely packed and form a sideways “V” structure (or as you get more posterior, an upside down “V”) when examining coronal sections of the hippocampus (Amaral et al., 2007). The

sideways “V” of the DG GCL can be further divided into an upper and lower blade referred to as the suprapyramidal and infrapyramidal blades found below the hippocampal fissure (Smith, 1902). In between the blades of the GCL “V” is the polymorphic layer, also referred to as the hilar region (hilus). DG GCL neurons from both the supra- and infrapyramidal blades send glutamatergic mf projections to CA3 pyramidal neurons and hilar mossy cells (i.e. another type of glutamatergic neuron found in the DG) (Mori et al., 2007; Scharfman and Myers, 2012). In addition to DG GCL neurons sending mf projections to CA3, there are two other interesting aspects of neurocircuitry at this particular junction (i.e. DG-to-CA3) that require further explanation. First, mossy cells in the hilus send glutamatergic projections to interneurons, which send inhibitory GABAergic projections back onto the DG GCL neurons (Ikrar et al., 2013). Second, CA3 pyramidal cells send excitatory glutamatergic projections back to DG GCL neurons, but it is not clear why (Acsady et al., 1998; Vivar and van Praag, 2013). One theory suggests having back projections to inhibit DG GCL neurons allows activation of a sparse ensemble of DG GCL neurons (Ikrar et al., 2013; Vivar and van Praag, 2013). This DG GCL activation of a sparse ensemble may play a role in distinguishing differences in similar spatial environments (i.e. pattern separation) (Leutgeb et al., 2007; Myers and Scharfman, 2009, 2011; Lee and Kesner, 2004). However, more research is needed to determine the function of back projections in the DG. After the CA3, the CA1 subfield receives the next glutamatergic projections, via the sc from CA3

pyramidal neurons and projections from EC layer III (**Fig. 1-1**) (Chafai et al., 2012; Vago and Kesner, 2008). Finally, the subiculum receives glutamatergic projections from the CA1 (O'mara, 2005) and projects back to the EC layer V. Taken together, the hippocampal trisynaptic circuit generally shows how information is sent through the hippocampus. However, it has become more apparent that the current view of the trisynaptic circuit may be oversimplified and does not take into account local subregion circuitry. Therefore, this dissertation examines the DG and adult neurogenesis, a unique process that can alter the DG neurocircuitry,

ADULT HIPPOCAMPAL NEUROGENESIS

Historical Perspective

The field of adult neurogenesis is relatively new (i.e. 1960's). First discovered in the cerebral cortex (Altman, 1962) and then in the dentate gyrus of young adult rats (Altman, 1963), adult neurogenesis is the process of generating new neurons throughout a mammal's life (e.g. in rodents (Altman, 1963; Altman and Das, 1965), bats (Amrein et al., 2007), birds (Goldman and Nottebohm, 1983), cats (Altman, 1963), non-human primates (Gould et al., 1999), and humans (Eriksson et al., 1998)). Adult neurogenesis was first examined by using a tritiated-nucleoside thymidine (3H-thymidine) in combination with autoradiography, which led to the discovery of two well-studied regions of adult neurogenesis: the subgranular zone

(SGZ) in the dentate gyrus (Altman and Bayer, 1990) and the subventricular zone (SVZ) in the lateral ventricles. Briefly, the cellular machinery during mitosis incorporates 3H-thymidine into the DNA, which can then be visualized with autoradiography film. The advent of nucleoside-analogs (e.g. 5-bromo-2'-deoxyuridine, BrdU and 5-ethynyl-2'-deoxyuridine, EdU) and antibodies specific to cells in the neurogenic regions of the SVZ/SGZ (i.e. using the technique immunohistochemistry, IHC) further helped researchers to visualize the adult born granule cells (ABGCs) and subsequently develop the stages of adult neurogenesis. While the SVZ is not discussed in this dissertation, the stages of adult neurogenesis were first described in the SVZ and later described in the SGZ. The stages of adult neurogenesis, as we currently know them, are discussed below in the "Stages of Neurogenesis (SGZ)" section (**Fig. 1-2**).

During the time of using nucleoside-analogs, a transgenic reporter mouse (Nestin-GFP) was developed by Yamaguchi and colleagues that allowed for a "snap shot" of the early stages of adult neurogenesis (Yamaguchi et al., 2000). Utilizing this mouse and the nucleoside-analog BrdU, Kempermann and colleagues examined the expression of double-labeled BrdU and GFP in the DG and developed the first testable model of the stages of adult hippocampal neurogenesis (Kempermann et al., 2003). With the development of an inducible transgenic reporter mice (Bonaguidi et al., 2011; Dranovsky et al., 2011; Encinas et al., 2011; Imayoshi et al., 2006;

Lagace et al., 2007), the field of adult neurogenesis found a reasonable technique to study the entire process of adult neurogenesis (e.g. nestin-CreER^{T2} x Rosa26R, developed by Dr. Amelia Eisch (Lagace et al., 2007)) rather than examine the process of adult neurogenesis at a specific instant. For example, the inducible Nestin-CreER^{T2} reporter mice are analogous to a movie while transgenic Nestin-GFP reporter mice are analogous to Polaroid pictures. An independent study examining 3 lines of Nestin-Cre transgenic mice found that the K line produced by Lagace and colleagues was the only nestin line with limited expression to adult-born neurons, whereas other lines produced ectopic expression (Dranovsky et al., 2011; Imayoshi et al., 2006; Lagace et al., 2007; Sun et al., 2014). The difference in expression is likely due to the size and sequence elements used in each construct. Sun and colleagues highlight that mouse models of adult neurogenesis can vary between studies and interpretations only in specific mouse models should be considered (Sun et al., 2014).

Stages of Adult Neurogenesis (SGZ)

Radial-glia like cells (RGCs / Type-1 cells)

In the DG, radial-glia like cells (RGCs) are believed to be the origin of adult neurogenesis (i.e. adult neural stem cell) (Kempermann et al., 2004; Ming and Song, 2011). Using the transgenic Nestin-GFP reporter mouse, RGC morphology that consisted of a triangular cell body with a single process that extended through the

granule cell layer (GCL) and terminated with “tufts” in the inner molecular layer (iMol) were termed Type-1 cells (Filippov et al., 2003). In addition to the distinct morphology, nestin-GFP+ Type-1 cells had a similar electrophysiological profile as astrocytes (i.e. a reversal potential close to the K+ equilibrium potential (Filippov et al., 2003)). Interestingly, Type-1 cells were considered distinct from astrocytes since immunoreactivity for a well-known astrocyte marker (S100β) was not found. However, nestin-immunoreactive (+) and glial fibrillary acidic protein (GFAP)+ Type-1 cells were observed (Filippov et al., 2003). Other commonly used markers that typically coincide with nestin, GFAP, and Type-1 morphology include Glutamate Aspartate Transporter (GLAST), nestin, Sox2, and brain lipid binding protein (BLBP) (Vadodaria and Gage, 2014).

Data have shown that the Type-1 adult neural stem cells have the capacity to self-renew and asymmetrically divide to create heterogeneous linages in the SGZ (Bonaguidi et al., 2011; DeCarolis et al., 2013). One recent study has evaluated the known heterogeneous populations of RGCs in the SGZ using Glutamate Aspartate Transporter (GLAST)- and Nestin-Cre transgenic mice to label RGCs and their specific lineages (DeCarolis et al., 2013). Interestingly, after an ablation and enhancement of adult neurogenesis, the GLAST+ RGC lineage was found to contribute to adult neurogenesis while the Nestin+ RGCs lineage did not. The concept of two separate ABGC lineages is further supported by work showing two

distinct populations of ABGCs that can be differentiated based on their input integration profile (i.e. one group is sensitive to narrow input intensity ranges while another group linearly reports input strength) (Brunner et al., 2014). Further work examining these two distinct and overlapping populations of ABGCs may possibly provide a better understanding of the functional role adult neurogenesis (discussed below).

Mitotic progenitors (Type-2/3)

Asymmetric division of a Type-1 cell gives rise to Type-2 cells, or early nonradial transiently amplifying progenitors (TAPs) (Seri et al., 2004). Type-2 early nonradial progenitors have an inverted tear drop cell body, a smooth plasma membrane, and lack any protrusions or projections (Seri et al., 2004). Type-2 cells can be further divided into two groups based on their transcription factor expression of Ascl1 (Type-2a) and Prox1 or NeuroD1 (Type-2b (Lugert et al., 2010)). Fate choice, or the neuronal lineage, is believed to begin in Type-2b cells (Lugert et al., 2010; Roybon et al., 2009). Type-2b cells then divide to generate the late progenitor/neuroblast intermediate cell type (Type-3). Type-3 cells are defined as having an apical dendrite and an axon leading to the CA3 region (Kempermann et al., 2004). Interestingly, 3-14 day-old ABGCs begin to synapse on to CA3 pyramidal neurons (Hastings and Gould, 1999; Vivar and van Praag, 2013; Zhao et al., 2006). Type-3 cells then

develop into immature neurons, which are immunoreactive to Doublecortin (DCX), PSA-NCAM, Tbr2, and Prox1 (Kempermann et al., 2004).

Nearly half of the TAPs that arise during neurogenesis die within 4 weeks of birth (Cameron et al., 1993), likely due to apoptosis or phagocytosis (Dayer et al., 2003; Lu et al., 2011). Studies examining how TAPs are cleared have lead to a debate in the field. One theory states that TAPs make a fate choice, become post-mitotic immature ABGCs (defined in the next section, 66% are post-mitotic by 3 days (Hayes and Nowakowski, 2002)), and then decide whether to live-or-die (i.e. through apoptosis) depending on the incoming signal (i.e. likely through Notch1 and/or NeuroD) to immature neurons (Kempermann et al., 2004; Ma et al., 2009; Ables et al., 2010; Miyata et al., 1999). Another compelling theory states that 1-4 day old ABGCs become phagocytized by surveillant microglia (Sierra et al., 2014; Sierra et al., 2010), but the selectivity of this process is unclear. One more theory states that the DCX+ cells can also phagocytize other DCX+ cells (Lu et al., 2011). Clearly, more studies are warranted to understand how TAPs are cleared, which may provide insight into the selectivity processes of deciding which cells live, die, and integrate into the DG neurocircuitry, a major question in hippocampal research.

Post-mitotic immature and mature granule cell neurons

Immature granule cell neurons are defined as having axons (i.e. mossy fibers) that terminate in the CA3 region and dendrites that reach into the middle Mol (mMoL) (Esposito et al., 2005; Kempermann et al., 2004). There are markers that are thought to exclusively label immature neurons (calretinin, TOAD-64), while other markers label the Type-2/3 and immature neurons (DCX/PSA-NCAM). Over time (~2-3 weeks after calretinin expression) mature ABGCS begin to express calbindin (Kempermann et al., 2004). These maturing (~2-6 week-old) ABGCs are believed to be structurally (Lagace et al., 2007; Toni et al., 2008) and functionally (Kee et al., 2007; Stone et al., 2011; Toni et al., 2008) incorporated into the hippocampal neurocircuitry. In the section below ("The Adult Neurogenic Niche Regulates the Local Circuitry"), the neurocircuitry and cellular activation that dynamically occurs during the process of adult neurogenesis is discussed.

The Adult Neurogenic Niche and the Local Circuit

Classically, the trisynaptic circuit has been used to define subregion specific functionality in the hippocampus (Becker, 2005). However, it has become more apparent that the current view of the trisynaptic circuit may be oversimplified and does not take into account local subregion circuitry or other circuit connections, like EC to CA3 and CA1, which were discovered later. However, using monosynaptic rabies virus-based tracing techniques, the local subregion circuitry was determined

for the process of adult neurogenesis (Deshpande et al., 2013; Vivar et al., 2012; Vivar and van Praag, 2013). Based on these studies, newborn ABGCs were found to be innervated by various cell types in sequential order before the canonical innervation from the entorhinal cortex (EC) occurs (i.e. 3 week old ABGCs, the classical trisynaptic circuit): 1) ~5 day old newborn ABGCs are innervated by local interneurons in the SGZ (possibly parvalbumin-positive basket cells, GABAergic), 2) then hilar interneurons (possibly HIPP cells, GABAergic), 3) and then local mossy cells (glutamatergic) (Deshpande et al., 2013). Surprisingly, in agreement with the innervation of ~5-day-old ABGCs (Deshpande et al., 2013), ABGCs as young as 3-days-old were activated by ambient GABA (Vivar and van Praag, 2013), likely through $GABA_A$ receptors (Ge et al., 2006). It is known that GABA easily excites (depolarizes) and produces LTP in ABGCs (1-14 day old) (Esposito et al., 2005; Ge et al., 2006; Schmidt-Hieber et al., 2004), while more mature granule cell neurons (1-3 weeks old) are excited by glutamate (Schmidt-Hieber et al., 2004). Based on this feature, young ABGCs have been termed “young and excitable” (Doetsch and Hen, 2005). In addition, the immediate early gene, Arc is expressed in 1-7 day-old adult generated cells (Kuipers et al., 2009). I personally speculate that GABA activation of newborn/young ABGCs can induce Arc expression, which allows for fate choice and survival to a mature granule cell neuron. However, this type of experiment has yet to be performed.

As the ABGC develops, it will project an apical dendrite without spines though the GCL (< 10 days old). Eventually three stages occur simultaneously: 1) the dendrite grows to the inner (~10 days old) and middle Mol (~14 days old), 2) the mf axon concurrently synapses on CA3 pyramidal cells (10-14 days old), 3) and the ABGC becomes DCX+ (Esposito et al., 2005; Vivar et al., 2012). At this stage, young (~10-15 days old) ABGCs are innervated by Mol interneurons (possibly MOPP cells, GABA) and receive long-range cholinergic inputs from the medial septum/nucleus of the diagonal band, in addition to all of the aforementioned innervations (i.e. local interneurons and mossy cells) (Deshpande et al., 2013). At ~2 weeks of ABGC development, GABA no longer depolarizes, but instead hyperpolarizes (i.e. via the switch in $GABA_A$-receptor reversal potential and the Na-K-2Cl co-transporter) (Deshpande et al., 2013; Ge et al., 2006).

The stage of ABGC maturation (3-9 weeks old) initially coincides with dendrites reaching the outer Mol (oMol), synapse formation, glutamatergic innervations from the EC and subiculum, and spontaneous postsynaptic activity (an indicator of synaptic input) (Deshpande et al., 2013; Esposito et al., 2005; Vivar et al., 2012). Interestingly, in order for the ABGCs to survive and integrate into the trisynaptic circuit, they must express NMDA receptors (Tashiro et al., 2006) and have primary cilia (Kumamoto et al., 2012). Recently, it has been proposed that that learning must also occur in order for ABGCs to survive (i.e. via egr1/Zif268) (Veyrac et al., 2013).

The dendrites of ~3-week-old ABGCs extend to the oMol, a major region of excitatory input from the lateral EC through the pp. However, the majority of the input to ~3-week-old ABGCs is from hilar mossy cells and back projections from mature granule cell neurons and pyramidal neurons in the DG and CA3, respectively (Myers and Scharfman, 2011; Vivar et al., 2012). It is thought that this overlap of glutamatergic input from the mossy cells, granule cells, and pyramidal cells to maturing ABGCs makes it easier to create LTP than older mature granule cells (Doetsch and Hen, 2005; Vivar et al., 2012). Within a week (~4 weeks old) ABGCs start to lose projections from mature granule cells, but maintain the hilar mossy cell and CA3 projections (Vivar et al., 2012). Interestingly, when EC input (glutamatergic) via the medial pp is stimulated, 28-65 day-old ABGCs observed paired-pulse facilitation (PPF, an indicator of short-term synaptic plasticity), while 90-day-old ABGCs show the characteristic paired-pulse depression (McNaughton, 1980; Vivar et al., 2012). Optogenetic stimulation in the medial EC has also been shown to activate 4-week-old ABGCs (Kumamoto et al., 2012). However, lateral pp stimulation results in PPF in both mature and young ABGCs. Taken together, these data suggest that medial and lateral EC glutamatergic input differentially alters short-term synaptic plasticity during the maturation of ABGCs. Overall, more work is needed examining the adult neurogenic circuit which my aid in understanding learning and memory processes.

Adult Neurogenesis and Learning and Memory

ABGCs have been implicated in a variety of contextual/spatial learning and memory processes (Jessberger et al., 2009), including pattern separation (Nakashiba et al., 2012) and temporal separation (Aimone et al., 2006; Kannangara et al., 2014; Rangel et al., 2014). ABGCs have also been implicated in specific aspects of learning and memory, such as acquisition, retrieval, and extinction. These aspects of learning and memory and their relationship to DG and ABGCs are the focus of Chapters 2 and 3. Therefore, after a brief introduction to the processes of learning and memory, an in-depth literature review on the behavioral changes associated with ABGC reduction/ablation/manipulation is provided below.

Learning and Memory

Learning and memory is proposed to occur in 3 stages: acquisition/encoding, consolidation, and retrieval (Quirk and Mueller, 2008; Buchanan, 2007; Daumas et al., 2005; Winters et al., 2008). Many brain regions are involved during the creation (acquisition/encoding), storage (consolidation), and recall (retrieval) of a memory (Squire and Wixted, 2011; Straube, 2012). However, the hippocampus appears very important, as previously discussed (see “THE HIPPOCAMPUS AND MEMORY, Historical Perspective”). For example, the human MTL, which contains the hippocampus, is largely involved in the creation of new memories (e.g. H.M. had anterograde amnesia) memory recall (e.g. H.M. had retrograde amnesia) (Squire and Wixted, 2011). One way to examine the creation of new memories is to examine

the behavioral process of extinction. Extinction is used to assess the creation of new inhibitory memories over time (i.e. the acquisition and consolidation of a new memory), which is behaviorally observed as a decrease in a specific conditioned response (Radulovic and Tronson, 2010; Suzuki et al., 2004). Extinction itself also contains an acquisition, consolidation, and retrieval phase (Quirk and Mueller, 2008). In addition to the creation of new memories, two ages of memory recall have also been examined: young memories (i.e. testing 1-5 days post-training), and old memories (i.e. testing >7 days post-training) (Frankland and Bontempi, 2005). Behavioral paradigms that use strategies to examine young and old memories are helpful to determine when a memory is hippocampal-dependent and labile or hippocampal-independent and stored/stabilized (i.e. consolidated) (Frankland and Bontempi, 2005; Kitamura et al., 2009). Brain regions such as the cortex are also involved in retrieving a consolidated memory (i.e. theory of systems consolidation) (Frankland and Bontempi, 2005; Kitamura et al., 2009). However, more research is needed to determine the brain regions and subregions involved in the acquisition, retrieval, and extinction of a memory, particularly in regards to the age of a memory during retrieval and extinction.

Hippocampal Neuroplasticity of Memory Formation, Storage, and Retrieval: An Overview

During the acquisition/encoding of a new memory, a sparse yet specific set of neurons is activated by glutamate (i.e. which is necessary for learning and memory) in the DG GCL (Peters and De Vries, 2012). It is believed that this sparse "memory trace" or "engram" of activated neurons is distinct to a unique memory (Denny et al., 2014). For instance, a memory about a recent airplane ride may activate a specific set of neurons in the DG, while a memory of a childhood event will activate another set of neurons. In order for encoding of a memory trace to occur, synaptic plasticity (i.e. long-term potentiation, LTP) must also occur (Denny et al., 2014; Lynch, 2004). LTP can be divided into two phases: an early phase lasting 2-3 hours (i.e. E-LTP, which is responsible for encoding of a memory) and a late phase lasting hours to weeks (i.e. L-LTP, which coincides with memory consolidation) (Kudryashova, 2013; Lynch, 2004). Once a memory is encoded, the memory is believed to remain in the hippocampus and cortex as short-term memory until it becomes stable (Dudai, 2004; Frankland and Bontempi, 2005; Kitamura and Inokuchi, 2014). Interestingly, both L-LTP and the process of consolidation require synthesis of new proteins (Lynch, 2004; Rossato et al., 2007; Young and Nguyen, 2005). The process of consolidation can be further divided into short- and long-term phases, where short-term is believed to occur in the hippocampus through synaptic consolidation (i.e. which is believed to be mediated by L-LTP), and long-term is believed to transfer a memory from the

hippocampus to the cortex for memory storage (i.e. systems consolidation) (Bramham and Messaoudi, 2005; Frankland and Bontempi, 2005; Nadel et al., 2012; Winocur and Moscovitch, 2011). Taken together, the strengthening of a memory is believed to involve the process of creating structural “wiring” or synaptic changes of neurons that, over time, “fire together”.

Once the memory is stored, it can be retrieved through associations and conscious memory recall. During retrieval, memory is believed to undergo a process called reconsolidation where the memory becomes labile and can either be restabilized (reinforced/strengthened) or destabilized (weakened) based on the associations that occur with retrieval (Almeida-Correa and Amaral, 2014). Reconsolidation, by definition, is determined by protein synthesis after retrieval (Lee et al., 2004; Nader, 2003). For example, protein synthesis inhibitors given right after retrieval can produce amnesia for the retrieved memory. Reconsolidation can also be regulated by the immediate early gene Zif268, CREB, and NMDA receptors (Lee et al., 2004; Nader, 2003). The degree of strengthening or weakening of the reconsolidation memory is correlated to how different the context is from the original memory (Almeida-Correa and Amaral, 2014). If the context is slightly different, reconsolidation will occur. If the context is generally different, extinction occurs (discussed below). Taken together, retrieval can undergo a “labilization” process,

which may strengthen/weaken depending on the associations and contexts presented during retrieval (Almeida-Correa and Amaral, 2014).

Retrieval of Young and Old Memories: A Role of ABGCs?

The interval between consolidation and retrieval can provide information regarding the dependency of brain regions on a memory. As mentioned above, one way to examine the hippocampal-dependency of a memory (or memory decay over time) is to use retrieval tasks of young and old memories (Frankland and Bontempi, 2005; Kitamura and Inokuchi, 2014). In fact, ABGCs are believed to mediate the speed at which a hippocampal-dependent memory becomes hippocampal-independent (Kitamura et al., 2009). As also mentioned above, ABGCs are a novel form of hippocampal plasticity that have been implicated in various aspects of learning and memory. Therefore, if the hippocampal-dependency of a memory can be understood, then it may be possible to manipulate memories using adult neurogenesis to treat humans that have PTSD, are depressed, or addicted (Drew and Hen, 2007; Eisch and Petrik, 2012; Snyder et al., 2011). However, animal studies examining the function of ABGCs have found mixed results in regards to retrieval of young and old memories (see Table 1-1). The mixed results may be due to at least four major variables: 1) the degree of ablation in ABGCs, 2) the specificity of ablation (i.e. irradiation or a transgenic mouse), 3) the behavioral tasks being used (Deng et al., 2010), and 4) key intervals, such as the interval between training

and testing, and between ablation and behavior. Clearly, more studies on the role of ABGCs on retrieval of young and old memories are needed, which is the focus of my Chapters 2 and 3.

Despite these mixed results, a role of ABGCs in specific forms of learning and memory has emerged regarding pattern and temporal separation, or the ability to dissociate familiar associations and events in time, respectively (Aimone et al., 2011; Burghardt et al., 2012; Garthe et al., 2009; Nakashiba et al., 2012). In support of this theory, recent work has shown that distinct populations of DG neurons are activated after slightly altering environmental inputs (Deng et al., 2013). In addition, a reduction of ABGCs has been shown to decrease pattern and temporal separation of older memories (Kesner et al., 2014). Taken together, pattern and temporal separation are potential functions of ABGCs, however other potential ABGC functions are currently being explored (e.g. forgetting) (Akers et al., 2014).

Extinction: A Role of ABGCs?

As mentioned above, extinction is the process of new inhibitory learning where a previously learned conditioned response is no longer observed. Based on this diminished conditioned response, it may be possible to manipulate previously formed memories to treat humans that have PTSD or are addicted. However, animal studies examining the function of ABGCs have found mixed results in regards

extinction (see Table 1-1). Interestingly, if extinction studies from Table 1-1 are separated into two groups based on their training-to-testing interval (i.e. young vs old memories), it appears that ABGCs may generally play a role in extinction of young, but not old, memories (Table 1-1). This difference in extinction of young and old memories is likely due to systems consolidation occurring in the older memory (i.e. allowing the memory to become hippocampal-independent), but not in the younger memory (Frankland and Bontempi, 2005; Kitamura and Inokuchi, 2014). However, not all behaviors examined exhibit deficits in extinction of young memories (Table 1-1) (Deng et al., 2009; Ko et al., 2009; Saxe et al., 2006), which may be due to a long interval between ablation/reduction of ABGCs and training (i.e. > 3 weeks). With such a long interval and assuming only a reduction in ABGCs, there is always the potential to recover and restore the impaired behavior (Deng et al., 2009). Taken together, more studies in young versus old memories are needed to determine to what extent ABGCs are involved in the learning and memory aspects of extinction.

ADDICTION

Historical Perspective

The National Institute on Drug Abuse (NIDA) refers to drug addiction as a brain disease where changes to gene expression and neurocircuitry can negatively impact cognition leading to compulsive drug taking, seeking, and use. Seminal studies on

addiction showed that two brain regions were involved in the canonical reward pathway implicated in the process of addiction: the ventral tegmental area (VTA) and the nucleus accumbens (NAc) (Olds, 1954, 1956; Olds and Milner, 1954). One of the first experiments to tease out the canonical reward pathway used an electrode implanted into the NAc of rats which would allow them to self-administer a small electrical stimulus by pressing a lever, thus stimulating neurons in the NAc and providing a pleasurable experience (Olds and Milner, 1954). Since then, studies have shown that the VTA responds to drugs of abuse (e.g. opiates) by triggering dopamine (DA) release into the NAc, which is innervated by neurons from the VTA (Kauer, 2004; Koob and Volkow, 2010). The VTA can also innervate other limbic regions believed to reinforce the addictive behavior by sending dopaminergic efferents to the NAc: prefrontal cortex (PFC), amygdala, and in particular the hippocampus (Fuxe, 1965; Gasbarri et al., 1994; Kauer and Malenka, 2007) (**Fig. 1-3**). Interestingly, DA projections from the substantia nigra and VTA to the SGZ of the dentate gyrus, a region where adult neurogenesis occurs, have also been found (Hoglinger et al., 2004). As mentioned above, glutamate is necessary for most types of learning and memory processes (Peters and De Vries, 2012). This therefore presents a problem for human addicts since enhanced glutamate coincides with DA release and is believed to aid in strengthening an addictive memory leading to the one major problem when treating addiction: preventing relapse (Fuchs et al., 2009; Peters and De Vries, 2012) (**Fig.1-3**). Relapse is partially dependent on the

environment (i.e. contexts and cues) associated with addictive behaviors (Baler and Volkow, 2006; Koob and Volkow, 2010; Taubenfeld et al., 2010). As the hippocampus has a major role in the formation of contextual learning and memory, understanding how learning and memory aspects and hippocampal neuroplasticity are involved during the process addiction is essential to finding better treatments for human addicts. Importantly, this dissertation studies the establishment of a drug/associated context (Chapter 2) and the potential role of adult neurogenesis in the learning and memory mechanisms involved during the process of addiction (Chapter 3).

Neurobiology of Morphine Conditioned Place Preference (CPP)

The Hippocampus and CPP

Having reviewed the basic of aspects of learning and memory above - acquisition, consolidation, and reconsolidation - it is now useful to apply them to reward-based memory and examine the neurocircuitry involved. Many brain regions including the amygdala, hippocampus, hypothalamus, and other various regions of the frontal cortex can innervate the NAc and VTA, and via that innervation can alter the canonical reward pathway neurocircuitry (Koob and Volkow, 2010; Wells et al., 2013) (**Fig. 1-3**). As mentioned above, the hippocampus is important for several learning and memory aspects such as spatial learning (Jessberger et al., 2009) and contextual memory (Hernandez-Rabaza et al., 2008). In regards to reward memory,

the hippocampus has been suggested to play an important role in the acquisition, consolidation, and reconsolidation of hippocampal-dependent addictive behaviors (Han et al., 2010; Lee et al., 2004; Moron et al., 2010). For example, dorsal and complete hippocampal lesions impair conditioned place preference (CPP), a behavioral task used to assess context-dependent reward memory (Bardo et al., 1995; Ferbinteanu and McDonald, 2001; Tzschentke, 2007). Further more, the hippocampal neurocircuitry is required for the consolidation and reconsolidation of a drug-associated context reward memory (Moron et al., 2010). Therefore, the hippocampus and its subregions are well poised to modify the formation and reconsolidation of a drug-associated context reward memory.

As mentioned above, to study reward memory of addictive behaviors, the CPP behavioral paradigm is used since it examines context-dependent learning associations (Bardo and Bevins, 2000; Bardo et al., 1995; Han et al., 2010; Moron et al., 2010; Tzschentke, 2007). Briefly, animals are trained to associate a reward with a context using drugs of abuse (unconditioned stimulus, US) and the drug is continually paired to a specific environment (conditioned stimulus, CS). This pairing strategy allows the animals to associate a CS to a drug-paired environment (CS+) (Tzschentke, 2007). After training, preference for the drug-associated context is determined by measuring the time an animal stays in the CS+ environment minus the time in the environment without drug pairing (CS-). If the animal spends more or

less time in the previous reward context, conditioned preference or aversion was established, respectively.

There are additional factors that should be considered to appropriately interpret CPP behavior. For example, CPP can be performed using a biased or unbiased apparatus as well as a counterbalances or uncounterblanced CPP paradigm (Cunningham et al., 2006). If an apparatus bias is used (i.e. the subject spends significantly more time in one context versus another before pairing), it becomes difficult to interpret the data since “floor”/“ceiling” effects and actual conditioned preference/aversion cannot be dissociated. In addition, if an uncounterbalanced strategy is used (i.e. the subject population was not equally distributed between contexts during pairing), it becomes difficult to determine whether the CS or the rewarding/aversive effects of US contributed to conditioned preference/aversion (Cunningham et al., 2006). Therefore, studies performing CPP should determine whether these variables would affect their overall interpretation before performing the CPP paradigm.

Intracellular Signaling of Morphine CPP

Intracellular mechanisms can provide important information regarding the inception of addiction. Interestingly, the pharmacological effects of morphine alone on the intracellular signaling differ if a rodent is given and single or multiple doses without

an associated context. For example, a single dose of morphine (10 mg/kg, s.c.) administered to a rodent without an associated context leads to significant decreases in pCREB levels in the NAc, VTA, striatum (Str), PFC, hippocampus, and cortex (Ctx). While multiple (5 mg/kg, s.c., 5 days) injections of morphine lead to significant decreases in pCREB levels in the hippocampus and Ctx. In addition, GluA1 levels remained unchanged for single and multiple injections of morphine in both the Ctx and hippocampus. Other drugs of abuse such as delta9-THC have also shown a decrease in the pCREB level in rodents that received repeated exposures, leading to deficits in LTP synaptic plasticity (Fan et al., 2010). Taken together, a context is needed to associate drug use with since drugs of abuse alone do not create the right conditions (i.e. decreased synaptic plasticity) for learning to occur in the hippocampus.

Other studies have suggested that the hippocampus plays a role in mediating morphine CPP (Corrigall and Linseman, 1988; Ferbinteanu and McDonald, 2001). As mentioned above, dorsal and complete hippocampal lesions impair conditioned place preference (CPP) (Bardo et al., 1995; Ferbinteanu and McDonald, 2001; Tzschentke, 2007). In addition, rodents trained with morphine CPP have increased levels in pCREB and pGluA1 in the hippocampus and Ctx, opposite of what was observed in experiments without CPP (Moron et al., 2010). An increase in pCREB in the VTA was also observed after morphine CPP. Further more, an infusion of

CAMKII inhibitor KN-62 to the hippocampus prevented acquisition and retrieval of morphine CPP (Lu et al., 2000). From these results one could hypothesize that learning a drug-context association requires the hippocampus and the canonical reward pathway.

Interestingly, other major intracellular components (e.g. immediate early genes [IEGs]) can mediate synaptic plasticity in the DG (Fig. 1-2). Studies examining amphetamine CPP activate the IEG cFos in the DG (Rademacher et al., 2006). CFos is an IEG that is used as an indirect marker of neuronal activity since cFos is often expressed when neurons fire action potentials, establish LTP, and is associated with intracellular signaling necessary for neural plasticity (Miyamoto, 2006; VanElzakker et al., 2008). In addition, cFos provides a temporal specificity of neuronal activity by forming an AP1 complex with Jun transcription factors, which inhibits subsequent cFos transcription (Reddy and Mossman 2002). In fear memory studies, activation of a specific engram of cFos in the DG is sufficient to recall of a fearful memory (Liu et al., 2012; Liu et al., 2014). In regards to reward memory, one study has also found that cFos in the DG and NAc positively correlate with the number of lever presses a rodent will take for drug (Recinto et al., 2012). Taking intracellular and IEG data together, one could hypothesize that the canonical reward pathway is activating DG neurocircuitry allowing for the formation and/or maintenance of a drug-associated context reward memory.

The Role of Adult Neurogenesis in Reward Memory

To assess the role of ABGCs in reward memory, studies have either increased or decreased neurogenesis and examined the reward behavior. For example, increasing adult neurogenesis via environmental enrichment prevents retrieval of cocaine place preference in mice (Solinas et al., 2008). In addition, the timing of when adult neurogenesis is increased also affects the extinction of a young and old cocaine-context reward memory. If neurogenesis is increased prior to or after training, it takes rodents more or less time to extinguish cocaine place preference, respectively (Mustroph et al., 2011). Paradoxically, an increase in DCX cells, by increasing NeuroD activity, diminished extinction of a morphine reward memory (Zheng et al., 2013). Further confounding the role of adult neurogenesis in reward memory, studies that have ablated/reduced neurogenesis have show mixed results in regards to retrieval and extinction of young and old reward memories (Table 1-1) (Brown et al., 2010; Deschaux et al., 2014; Noonan et al., 2010; Zheng et al., 2013). For example, a reduction of adult neurogenesis by cranial irradiation does not prevent acquisition of cocaine CPP (Brown et al., 2010) or alter retrieval or extinction of old reward memories in rats that previously self-administered cocaine (i.e. an operant behavioral task used to assess drug taking/seeking) (Noonan et al., 2010). However in the same study, cranial irradiation after - but not prior to - cocaine SA had impaired extinction of old reward memories (Noonan et al., 2010). In addition, reduced adult neurogenesis by an inducible knock-down of NeuroD and irradiation

improves and has no effect on extinction of young reward memory using fentanyl CPP and cocaine self-administration (SA), respectively (Deschaux et al., 2014; Zheng et al., 2013). Taken together, it is unclear what the role of adult neurogenesis plays in learning and memory mechanisms of reward memory. Therefore this dissertation aims to clarify at least one role of adult neurogenesis in reward memory by examining aspects of retrieval and extinction in young and old memories with prior cranial irradiation. In summary, adult neurogenesis is located in an ideal region (i.e. the dentate gyrus) and has the potential to influence reward memory during reconsolidation, which may potentially alter rewarding memories to help treat human addicts. Therefore, one aspect of this dissertation aims to determine the role of adult neurogenesis in the learning and memory mechanisms involved in drug-associated context reward memory (Chapter 3).

GALACTIC COSMIC RADIATION

Overview

As humans explore outer, cis-lunar, interplanetary space, and interstellar space they will receive a chronic low dose of galactic cosmic radiation (GCR) that cannot be shielded by any feasible means to date. This poses a potential risk for astronauts during spaceflight missions to the Moon, Mars, and beyond since we do know if long-term GCR can hinder mission success (i.e. the primary objective for

astronauts). Estimating the amount of risk astronauts can have is difficult since there is no human data to estimate risk from GCR (Cucinotta et al., 2012). Therefore, animal models have been used to determine risk by mimicking GCR on Earth using particle accelerators (Nelson, 2003). Of the health risks to astronauts (i.e. reduced gravity, isolation/confinement, closed environment, distance from earth, and increased radiation), the most detrimental appears to be from increased radiation experienced during spaceflight mission. In particular, the CNS, carcinogenesis, cardiovascular disease, and acute radiation syndrome are all important risks that are currently being assessed by NASA (Cucinotta, 2014; Cucinotta et al., 2012). While each risk is important to assess, we examined how space radiation affects the CNS. Therefore this dissertation aims to determine if simulated space radiation effectively ablates adult neurogenesis, which could impact cognition and therefore mission success. In order to proceed, we must first understand what GCR is, how it is formed on Earth, and examine the behavioral effects of this unique radiation.

Simulated "Space Radiation" on Earth

Characteristics of HZE Particles

Based on detectors sent into space, it is known that GCR is composed of various nuclei ranging from protons [^{1}H] to Francium [^{87}Fr] (NASA). The majority of GCR particles are protons (Hydrogen, 90%), alpha particles (Helium, 9%), and the remaining 1% is the heavier nuclei (NASA). Although the 1% percent has a low

abundance, they have a high ionizing energy (contain a charge greater than 2+), meaning they cause much damage by depositing energy into a material (i.e. the absorbed dose). High energy GCR is believed to have originated and been accelerated from the nuclear fusion and explosion of dead stars (i.e. supernovas, > 100 GeV/n), while the source of low energy GCR is thought to be from highly evolved massive stars (< 100 GeV/n) (Meyer et al., 1997; Meyer et al., 1974). While these nuclei can cause damage in living organisms in outer space and beyond, the Earth is generally protected from GCR by the magnetosphere (Melkonian and Bourrieau, 1994). Therefore, the ability to study GCR can only truly occur in outer space.

Luckily, GCR can be simulated on earth by using a particle accelerator which strips electrons from atoms, thereby creating a high-energy (E), high-charge (Z) nuclei/particle (HZE) capable of high-linear energy transfer (LET), or the amount energy a HZE particle deposits into a material per unit distance (International Commission on Radiation and Measurements, 2011; Schimmerling, 2003). The high-LET HZE particle radiation is observed in a non-uniform pattern along a single-track structure or “microlesion” (Nelson, 2003; Todd, 1992). This is fundamentally different than low-LET radiation like X-ray, where the radiation is diffuse and uniform. The average high-LET HZE particle energy is estimated to be 1500 MeV/n, while protons and alpha particles have an average energy in the hundreds range of MeV

(Kim et al., 2009). As expected, the higher the energy of the HZE particle, the faster the velocity a particle will have (Nelson, 2003). The LET along the “microlesion” can be determined by dividing the particles charge squared (z^2) and velocity squared (v^2) (z^2/v^2) (Nelson, 2003; Todd, 1992). As the particle travels through a material it will decrease in velocity. As velocity reaches zero, the LET exponentially increases in the irradiated material. This hallmark of depth versus dose received is referred to as a “Bragg peak” (Nelson, 2003). The depth of material that is irradiated by HZE particles will determine the dose received. Interestingly, when a HZE particle transverses a material, it creates a secondary penumbra of damage created by nuclear reactions (i.e. HZE particle interacting with another molecule/atom to produce protons, alpha particles, and neutrons) and delta rays (i.e. energetic electrons). The damage caused by this secondary penumbra varies with energy, nuclei, and material that are transversed (Cucinotta, 2014; Cucinotta et al., 2012). In general, the damage caused by HZE particle radiation is believed to remodel the extracellular matrix, induce repair machinery for double- and single-stranded breaks in the DNA, and induce cell death (Asaithamby and Chen, 2011; Ehrhart et al., 1996; Nelson, 2003). In addition, HZE particle radiation produces similar parameters (i.e. neuronal signaling and behavior), as aged animals, suggesting that HZE radiation is a model of aging (Casadesus et al., 2005; Joseph et al., 1992; Joseph et al., 1993; Joseph et al., 2000; Shukitt-Hale et al., 2007). Taken together, the characteristics of

HZE particles are complex; making it difficult to mechanistically determine the risk astronauts will have in outer space and beyond.

Space Radiation Risk to the CNS

While there are many risks that astronauts can experience, one that they cannot control is the dose, energy, and nuclei of GCR they will receive during a spaceflight mission. Given the inherent variability of GCR, many ground-based studies also vary in the dose, energy, and nuclei used in their experiments. However, most studies have used the ^{56}Fe HZE particle due to its relatively high abundance in GCR and high ionizing power. Of particular interest to NASA is the risk to the CNS and the subsequent cognition. Several studies examining behavior as an output for cognitive risk have acutely exposed rodents to the HZE particle ^{56}Fe with doses ranging from 10 – 50 cGy (a dose range believed to be acquired by astronauts during a long-term space flight mission, ~18 cGy/year (Cucinotta, 2014)) and found deficits in CNS related behaviors such as novel object recognition, barnes maze, and attentional set-shifting paradigm (Britten et al., 2012; Cherry et al., 2012; Haley et al., 2013; Lonart et al., 2012). However, in the same behavioral studies no change was also found in CNS related behaviors such as morris water maze and contextual fear conditioning (Cherry et al., 2012; Haley et al., 2013; Rosi et al., 2012). Many other behavioral studies using a dose rate of 100 cGy or higher also find deficits and no changes in CNS related behaviors after HZE particle radiation (Manda et al., 2008a,

b; Raber et al., 2011; Shukitt-Hale et al., 2000; Villasana et al., 2010; Villasana et al., 2013). Taken together, it is unclear if low doses of HZE particle radiation are detrimental to the CNS behaviors. It is also possible that another factor, other than dose, may be responsible for the variability in the CNS related behaviors.

Interestingly, many of the studies performed have only examined an acute effect of exposure to HZE particle radiation, which is different than the chronic low-dose of HZE particles astronauts will receive in outer space. In addition, multiple HZE nuclei will irradiate astronauts making it difficult to interpret the behavioral data to model risk (Cucinotta, 2014). Therefore, my dissertation aimed to determine if a difference in acute versus fractionated exposure could be observed in the CNS (Chapter 4). A major future direction for the field of space radiation should utilize both a fractionated and mixed-field HZE particle radiation paradigm to mimic GCR as best as possible on Earth.

QUESTIONS ASKED IN THIS DISSERTATION

Chapter 2

1. Does morphine CPP lead to DG activation?
2. Does the retrieval of the drug context lead to DG activation?

Chapter 3

3. Does adult neurogenesis have a functional role in the retrieval of young/old drug-associated context reward memories?
4. Does adult neurogenesis have a functional role in the extinction of young/old drug-associated context reward memories?

Chapter 4

5. Does an acute or fractionated exposure of HZE particles alter adult neurogenesis?

Figures

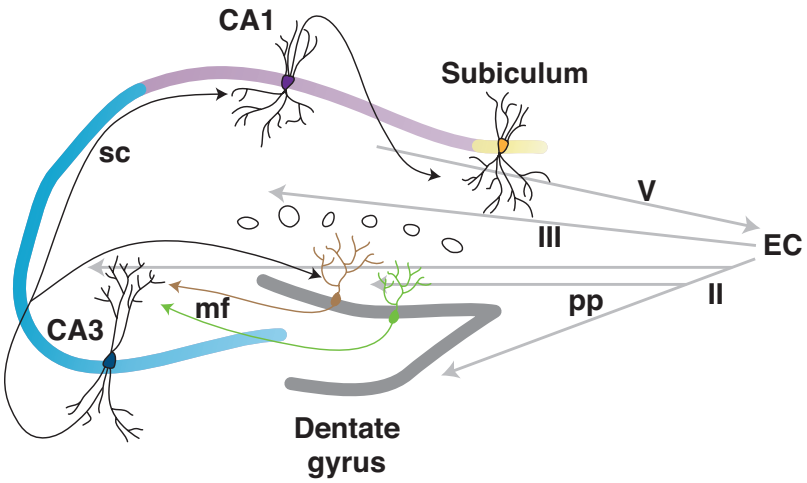

FIGURE 1-1. Hippocampal neurocircuitry.

A simplified diagram of hippocampal neurocircuitry begins with projections from layer II of the entorhinal cortex (EC) to the dentate gyrus (DG, grey region) via the perforant path (pp). Both embryonic- (brown) and adult-generated (green) DG granule cell neurons are innervated by EC projections in the molecular layer. Granule cell neurons next send mossy fibers (mf) to the CA3 subfield (blue region) of the hippocampus. CA3 pyramidal neurons next send Schaffer collateral (sc) projections to the CA1 (purple region). CA1 pyramidal neurons subsequently send projections to the subiculum (yellow region), which sends projections to layer V of the EC. The EC can also send projections from layer III to the CA1 region.

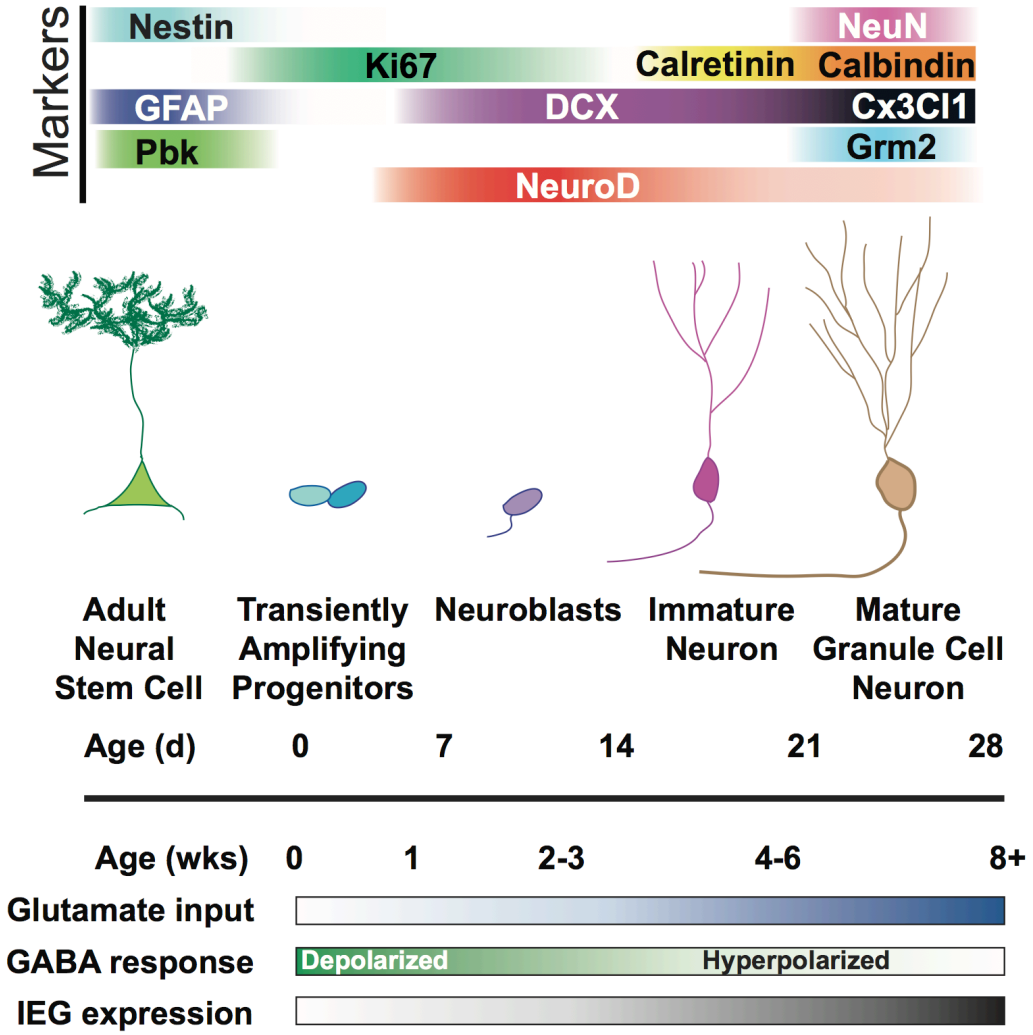

FIGURE 1-2. The process of DG adult neurogenesis.

The process of adult hippocampal neurogenesis takes place in dentate gyrus (DG) and can be visualized using markers to label ABGCs. The putative adult neural stem cell (Type-1 cell) asymmetrically gives rise to transiently amplifying progenitors (TAPs), which give rise to neuroblasts that typically have an observable axon protruding towards the CA3 region. Once the mitotic cells finish dividing, they then

mature into immature neurons and eventually mature granule cell neurons. As ABGCs develop, they differentially receive glutamatergic and GABAergic inputs. ABGCs also respond to these inputs differently as they mature (e.g. young ABGCs are depolarized by GABA, while older ABGCs are hyperpolarized by GABA). These immature and mature adult-generated granule cell neurons are considered functional and structurally integrated into the DG circuitry around 4-6 weeks of age, indicated by the expression of immediate early genes (IEGs) (Kee et al., 2007; Stone et al., 2007).

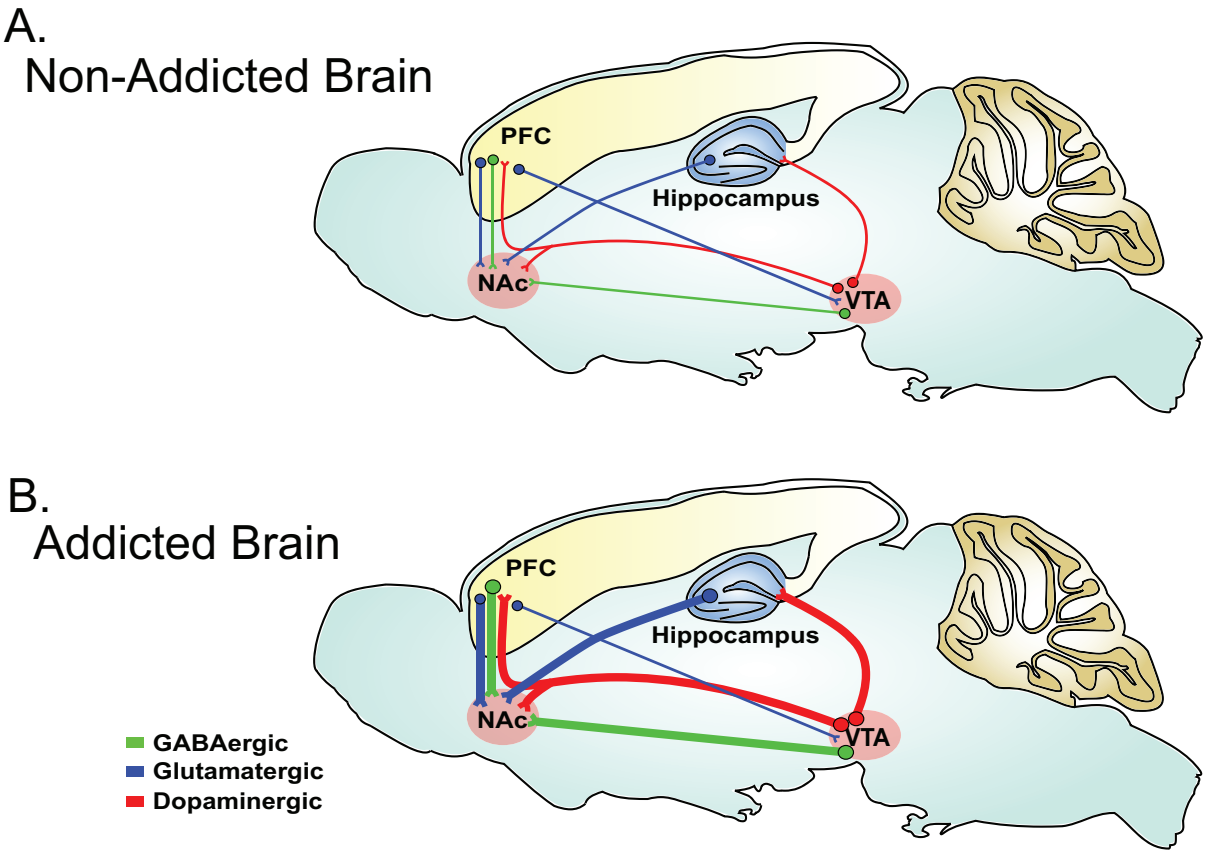

FIGURE 1-3. Hypothetical neurocircuit plasticity in the addicted and non-addicted brain.

Revised mesolimbic dopamine circuitry incorporating the hippocampus (Gill and Grace, 2011). A) Simplified schematic of a normal reward pathway circuitry highlighting major inputs to the nucleus accumbens (NAc) and the ventral tegmental area (VTA) (Dopaminergic projections, red; glutamatergic projections (excitatory), blue; GABAergic projections (inhibitory), green) (Fadda et al., 2003; Lobo and Nestler, 2011). B) Hypothetical schematic of neurocircuitry plasticity in the addicted brain. In general, increased dopamine in the striatum (NAc) is associated with

reward and addiction. The circuitry involved in mediating dopamine release is mainly attributed to the disinhibition of GABAergic neurons in the VTA, which allows for increased dopamine release to the PFC, Hipp, and NAc (Moron et al., 2010). In addition, GABAergic projections from the VTA to local interneurons in the NAc have also been found (Brown et al., 2012). Emphasized in this schematic is the recent hypothesis for how the hippocampus may contribute to addiction-like behaviors (via enhanced hippocampal glutamatergic input to the NAc) (Peters and De Vries, 2012). Recently, the co-release from dopamine and glutamate from dopaminergic neurons have also found (Fortin et al., 2012) which may potentially reinforce addictive-like behaviors (Britt et al., 2012). Omitted from this diagram is the direct/indirect pathway (i.e. the striatonigral and striatopallidal pathways, respectively (Hikida et al., 2010)), amygdalar projections to the hippocampus and NAc (Belujon and Grace, 2011), and the serotoninergic and cholinergic projections (Lobo and Nestler, 2011) believed to be important in the addicted brain. PFC, prefrontal cortex.

Table1. Cited literature involving reduction/ablation of ABGCs and its effects on contextual learning and memory behaviors							
Research field	**Behavioral task**	**How is learning/memory behavior altered by disrupted neurogenesis?**	**Species**	**Approach to disrupt neurogenesis**	**Disrupted neurogenesis and training interval**	**Reference**	**Figure in reference**
Retreival of Young Memory (test occurs 1-5 days post-training)							
L & M	BM	NC	mouse	Genetic ablation	> 1 mon	Imayoshi et al., 2008	Fig. 8c
L & M	BM	NC	mouse	Irradiation	3 mon	Rola et al., 2004	Fig. 5
L & M	BM	NC	mouse	Genetic ablation	KO	Urbach et al., 2013	Fig. 5
L & M	CTA	Deficit	mouse	Genetic ablation	7 wks	Arruda-Carvalho et. al., 2011	Fig. 7h
L & M	DNMP	NC	rat	Genetic ablation	Continuous	Groves et al., 2013	Fig. 6d
L & M	DNMP	Deficit	mouse	Genetic ablation	1 mon	Ko et al., 2009	Fig. 4f
L & M	DNMP	Deficit in difficult task	mouse	Genetic ablation	4 mon	Pan et al., 2013	Fig. 6
L & M	DNMP	NC	mouse	Irradiation	3 mo	Saxe et al., 2006	SI Fig. 5f
L & M	DNMTS	Deficit in long, but not short intervals	rat	Irradiation	4 wks	Winocur et al., 2006	Fig. 3a-d
L & M	MWM	Deficit	mouse	Genetic ablation	7 wks	Arruda-Carvalho et. al., 2011	Fig. 9c
L & M	MWM	NC	mouse	Genetic ablation	4-6 wks	Bergami et al., 2008	SI Fig. 6
L & M	MWM	NC (sham vs. IRR, sedentary)	mouse	Irradiation	~15-17 wks	Clark et al., 2008	Fig. 4b
L & M	MWM	NC	mouse	Genetic ablation	1 wk	Deng et al., 2009	Fig. 5c
L & M	MWM	Deficit	mouse	Genetic ablation	6 wks	Dupret et al., 2008	Fig. 6a-b
L & M	MWM	NC	mouse	Chemotherapy drug	4 wks	Garthe et al., 2009	Fig. 4e
L & M	MWM	Deficit	mouse	Antimitotic toxin	1 d	Goodman et al., 2010	Fig. 2b
L & M	MWM	NC	rat	Genetic ablation	Continuous	Groves et al., 2013	Fig. 5b
L & M	MWM	NC	mouse	Genetic ablation	1 d	Jaholkowski et al., 2009	Fig. 7b
L & M	MWM	Deficit @ >2wks	rat	LV-mediated KD	8-9 wks	Jessberger et al., 2009	Fig. 3
L & M	MWM	NC	rat	Irradiation	~2 wks	Madsen et al., 2003	Fig. 6

L & M	MWM	NC (sham vs. IRR, standard)	mouse	Irradiation	~17 wks	Meshi et al., 2006	Fig. 3c
L & M	MWM	Deficit in reversal probe test, but not probe test	mouse	Genetic ablation	4 mon	Pan et al., 2013	Fig. 3
L & M	MWM	Deficit	mouse	Irradiation	~6 wks	Raber et al., 2004	Fig. 3b
L & M	MWM	Deficit	mouse	Irradiation	3 mon	Rola et al., 2004	Fig. 4b
L & M	MWM	NC	mouse	Irradiation	3 mo	Saxe et al., 2006	SI Fig. 5d
L & M	MWM	Deficit	mouse	Genetic ablation	Continuous	Shimazu et al., 2006	Fig. 7b
L & M	MWM	NC	rat	Irradiation	6 wks	Wojtowicz et al., 2008	Fig. 4b
L & M	MWM	Deficit	mouse	Genetic ablation	4 wks	Zhang et al., 2008	Fig. 7h
L & M	MWM	Deficit	mouse	Genetic ablation	KO	Zhao et al., 2003	Fig. 5b
L & M	MWM	Deficit	mouse	Genetic ablation	4 wks	Zhao et al., 2008	Fig. 5b
L & M	NMTS	NC	rat	Irradiation	4 wks	Winocur et al., 2006	Fig. 2
L & M	PR	Deficits @ 1/3 wks; NC @ 7wks	rat	Irradiation	~2 wks	Madsen et al., 2003	Fig. 5a
L & M	RAM (HML/NI)	NC	mouse	Irradiation	3 mo	Saxe et al., 2007	Fig. 2a
L & M	RAM (LML/HI)	Improved @ 30" and 50" delay	mouse	Irradiation	3 mo	Saxe et al., 2007	Fig. 2b
L & M	RAM (LML/HI)	Improved @ 35" and 55"-135" delay	mouse	Irradiation & genetic ablation	3 mo and continous	Saxe et al., 2007	Fig. 3b & 4b
L & M	SLR	Deficits in "similar", but not "dissimilar" tasks	rat	LV-mediated KD	~4 wks	Bekinschtein et al., 2014	Fig. 1d
L & M	WM-VD	Deficit	mouse	Genetic ablation	7 wks	Arruda-Carvalho et. al., 2011	Fig. 10g
Fear	CD	Deficits in "similar", but not "different" contexts	mouse	Genetic ablation	6 wks	Kheirbek et al., 2012	Fig. 4h-g
Fear	CD	Deficit	mouse	Genetic ablation	Continuous	Scobie et al., 2009	Fig. 10f
Fear	CD	Deficit	mouse	Genetic ablation	Continuous	Tronel et al., 2012	Fig. 2d-e
Fear	CFC	NC	mouse	Genetic ablation	Continuous	Akers et al., 2014	Fig. 4d
Fear	CFC	Deficit	mouse	Genetic ablation	7 wks	Arruda-Carvalho et. al., 2011	Fig. 7d
Fear	CFC	NC	mouse	Irradiation	~15-17 wks	Clark et al., 2008	Fig. 5b
Fear	CFC	Deficit in 180" PSI w/ 180" delay, but not 360" PSI	mouse	Genetic ablation	2 wks	Denny et al., 2012	Fig. 10
Fear	CFC	Deficit in 6 wk w/ 180" delay, but not 2 & 4 wk interval	mouse	Irradiation	2, 4, & 6 wks	Denny et al., 2012	Fig. 8b
Fear	CFC	NC	mouse	Irradiation	2 wks	Denny et al., 2012	Fig. 9

Fear	CFC	NC	mouse	Irradiation	6 - 12 wks	Drew et al., 2010	Fig. 2c
Fear	CFC	Deficit in delayed shock, but not immed. shock	mouse	Irradiation	6 - 12 wks	Drew et al., 2010	Fig. 3b
Fear	CFC	NC in 0.7, 0.4, & 0.3 mA	mouse	Irradiation	6 - 12 wks	Drew et al., 2010	Fig. 4
Fear	CFC	Deficit (no pre-exposure) in 1, but not 2 shocks	mouse	Irradiation	6 - 12 wks	Drew et al., 2010	Fig. 5b
Fear	CFC	NC (pre-exposure) w/ 1 or 2 shocks	mouse	Irradiation	6 - 12 wks	Drew et al., 2010	Fig. 5b
Fear	CFC	NC	mouse	Genetic ablation	6 wks	Dupret et al., 2008	Fig. 5c
Fear	CFC	NC	rat	Genetic ablation	Continuous	Groves et al., 2013	Fig. 4b
Fear	CFC	NC	mouse	Irradiation & genetic ablation	5 wks	Kitamura et al., 2009	Fig. 2m & 4d
Fear	CFC	Deficit	mouse	Genetic ablation	1 mon	Ko et al., 2009	Fig. 4c
Fear	CFC	NC	mouse	Genetic ablation	4 mon	Pan et al., 2013	Fig. 4a
Fear	CFC	NC	mouse	Genetic ablation	1 d	Jaholkowski et al., 2009	Fig. 6a
Fear	CFC	Deficit	mouse	Irradiation & genetic ablation	3 mon	Saxe et al., 2006	Fig. 1e & 2b
Fear	CFC	NC	rat	MAM	n/a	Shors et al., 2002	Fig. 4a
Fear	CFC	Deficit	rat	Irradiation	4 & 8 wks	Snyder et al., 2009	Fig. 4c-d
Fear	CFC	NC	mouse	Irradiation	4 & 8 wks	Snyder et al., 2009	Fig. 4g-h
Fear	CFC	Deficit	rat	Irradiation	6 wks	Warner-Schmidt et al., 2008	Fig. 5a
Fear	CFC	Deficit	rat	Irradiation	4 wks	Winocur et al., 2006	Fig. 4a
Fear	CFC	N/A	rat	Irradiation	5 wks	Wojtowicz et al., 2008	Fig. 3a
Fear	CFC	NC	mouse	Genetic ablation	4 wks	Zhang et al., 2008	Fig. 4b
Fear	PA	NC	mouse	Genetic ablation	4 mon	Pan et al., 2013	Fig. 7
Fear	TC	Improved @ 30" delay	mouse	Genetic ablation	1 d	Jaholkowski et al., 2009	Fig. 6a
Fear	TC	Improved @ 30" delay	rat	MAM	n/a	Shors et al., 2002	Fig. 3a
Reward	CPP, cocaine	NC	rat	Irradiation	10 d or 60 d	Brown et al., 2010	Fig. 3
Retreival of Old Memory (test occurs > 7 days post-training)							
L & M	BM	Deficit	mouse	Genetic ablation	> 1 mon	Imayoshi et al., 2008	Fig. 8d
L & M	MWM	Deficit in posttraining, but not pretraining ablation	mouse	Genetic ablation	~1 wk prior to training, and 1 mon posttraining	Arruda-Carvalho et. al., 2011	Fig. 9i & 9l
L & M	MWM	Deficit	mouse	Genetic ablation	1 wk	Deng et al., 2009	Fig. 5f

L & M	MWM	NC	mouse	Genetic ablation	3.5 or 9 wks	Deng et al., 2009	Fig. 4e & 4g
L & M	MWM	Deficit	mouse	Genetic ablation	1 wk	Deng et al., 2009	Fig. 5f
L & M	MWM	Deficit	mouse	Antimitotic toxin	1 d	Goodman et al., 2010	Fig. 2c
L & M	MWM	Deficits 2-8 wks	rat	LV-mediated KD	8-9 days	Jessberger et al., 2009	Fig. 3b
L & M	MWM	NC @ 1 wk, Deficits @ 2 & 4 wks	rat	Irradiation	4 wks	Snyder et al., 2005	Fig. 3b
L & M	MTP	NC	rat	Irradiation	8 wks	Hernandez-Rabaza et al., 2009	Fig. 2a
L & M	NMTP	NC	rat	Irradiation	2 wks	Hernandez-Rabaza et al., 2009	Fig. 2b
Fear	CFC	NC	mouse	Irradiation & genetic ablation	11 d & 5 wks	Kitamura et al., 2009	Fig. 6c & 2n
Fear	CFC	Deficit	rat	Irradiation	4 wks	Winocur et al., 2006	Fig. 4a
Fear	CFC	Deficit	mouse	Genetic ablation	Continuous	Akers et al., 2014	Fig. 4e & 4j
Fear	CFC	NC	mouse	Genetic ablation	1 d	Jaholkowski et al., 2009	Fig. 5b
Fear	PA	Deficit	mouse	Genetic ablation	4 mon	Pan et al., 2013	Fig. 7
Reward	SA, cocaine	NC	rat	Irradiation	5 wks prior to SA	Noonan et al., 2010	Fig. 7a
Extinction of Young Memory (extinction begins 1-5 days post-training)							
L & M	MWM	NC	mouse	Genetic ablation	1 wk	Deng et al., 2009	Fig. 6d
L & M	MWM	Improved	mouse	LV-mediated KD	1 wk	Zheng et al., 2013	Fig. 5
L & M	TC	Deficit w/ 14 d, but not 6 d of MAM	rat	Antimitotic toxin	2 d	Shors et al., 2001	Fig. 4b & d
Fear	CFC	Deficit @ 1 wk interval, but NC @ 5 wk interval	mouse	Genetic ablation	1 & 5 wks	Deng et al., 2009	Fig. 7c & 7k
Fear	CFC	Deficit	rat	Irradiation	9 wks	Hernandez-Rabaza et al., 2009	Fig. 5b
Fear	CFC	NC	mouse	Irradiation	3 mon	Ko et al., 2009	Fig. 4d
Fear	CFC	NC	mouse	Antimitotic toxin	3 d	Ko et al., 2009	Fig. 2c
Fear	CFC	Deficits	mouse	Genetic ablation	n/a	Kondo et al., 2014	Fig. 2a
Fear	CFC	Deficit	mouse	Genetic ablation	4 mon	Pan et al., 2013	Fig. 4d-e
Reward	CPP, fentanyl	Improve	mouse	LV-mediated KD	1 wk	Zheng et al., 2013	Fig. 4b
Reward	SA, cocaine	NC	rat	LFS	During extinction	Deschaux et al., 2013	Fig. 2b
Extinction of Old Memory (extinction begins > 7 days post-training)							

Fear	CFC	NC	mouse	Irradiation	3 mon	Ko et al., 2009	Fig. 5c
Fear	CFC	NC	mouse	Genetic ablation	1 wk	Deng et al., 2009	Fig. 7g
Reward	SA, cocaine	Deficit in posttraining, but not pretraining ablation	rat	Irradiation	4 wks prior to training, ~1 day post-training	Noonan et al., 2010	Fig. 6b-c
Reward	SA, sucrose	NC	rat	Irradiation	5 wks prior to SA	Noonan et al., 2010	Fig. 5f or 7a

Key
L & M, learning and memory
Fear
Reward
BM, Barnes maze
CD, contextual discrimination
CFC, contextual fear conditioning
CPP, conditioned place preference
CTA, conditioned taste aversion
DNMP, delayed non-matching to position
DNMTS, Delayed Nonmatching-To-Sample Task
HML, high-memory load
LFS, low-frequency stimulation
LI, limited intertrial interference
NI, no intertrial interference
NMTS, Nonmatching-To-Sample Task
PA, passive avoidance task
PAL, paired associates learning (PAL) object- in-place
PR, place-recognition test
SA, self-administration
SLR, spontaneous location recognition
TC, trace eye blink conditioning task
WM-VD, Water maze visual discriminiation
MTP, match-to-place
PSI, placement to shock

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CHAPTER TWO: RETRIEVAL OF MORPHINE-ASSOCIATED CONTEXT INDUCES CFOS IN DENTATE GYRUS NEURONS

Adapted from: **Rivera PD**, Raghavan RK, Yun S, Latchney SE, McGovern M-K, García EF, Birnbaum SG, Eisch AJ. Retrieval of morphine-associated context induces cFos in dentate gyrus neurons. *Accepted to Hippocampus.*

Abstract

Addiction has been proposed to emerge from associations between the drug and the reward-associated contexts. This associative learning has a cellular correlate, as there are more cFos+ neurons in the hippocampal dentate gyrus (DG) after psychostimulant conditioned place preference (CPP) vs. saline controls. However, it is unknown whether morphine CPP leads to a similar DG activation, or whether DG activation is due to locomotion, handling, pharmacological effects, or – as data from contextual fear learning suggests – exposure to the drug-associated context. To explore this, we employed an unbiased, counterbalanced, and shortened CPP design that led to place preference and more DG cFos+ cells. Next, mice underwent morphine CPP but were then sequestered into the morphine-paired (conditioned stimulus+ [CS+]) or saline-paired (CS-) context on test day. Morphine-paired mice sequestered to CS+ had ~30% more DG cFos+ cells than saline-paired mice. Furthermore, Bregma analysis revealed morphine-paired mice had more cFos+ cells

in CS+ compared to CS- controls. Notably, there was no significant difference in DG cFos+ cell number after handling alone or after receiving morphine in home cage. Thus, retrieval of morphine-associated context is accompanied by activation of hippocampal DG granule cell neurons.

Introduction

Drug addiction is a brain disease of compulsive drug taking, seeking, and use with many negative health and societal consequences. The focus of most addiction research is the canonical reward pathway, which includes the nucleus accumbens, ventral tegmental area, and prefrontal cortex (Koob and Volkow, 2010; Nestler, 2005). However, the hippocampus and its dentate gyrus (DG) subregion have also been implicated in addiction (Noonan et al., 2010). For instance, cocaine-dependent humans show cue-induced hippocampal dopamine release (Fotros et al., 2013). Furthermore, conditioned place preference (CPP) in rodents, which involves context-dependent learning associations with drugs (Bardo et al., 1995; Tzschentke, 2007), relies on an intact DG (Hernandez-Rabaza et al., 2008). CPP has also been used to assess hippocampal neuroadaptations that correlate and contribute to drug-reward memories (Hernandez-Rabaza et al., 2008; Koob and Volkow, 2010; Meyers et al., 2006), and are thought to negatively impact cognition and mood (NIDA; Russo and Nestler, 2013). Thus, understanding how the formation of drug-reward memories

alters the neurobiology of the hippocampal DG may shed light on the later and more persistent aspects of addiction.

In learning and memory research, DG expression of the immediate early gene (IEG) cFos, correlates with learning and memory retrieval (Kubik et al., 2007; Lopez et al., 2012), and reactivation of DG cells expressing cFos during an initial fear conditioning experience is sufficient for retrieval of contextual fear (Liu et al., 2012; Liu et al., 2014). Correlative studies in addiction research also show increased DG expression of cFos after retrieval of psychostimulant CPP (Chauvet et al., 2011; Rademacher et al., 2006), suggesting increased DG activity is involved in context-reward memory. In order to determine whether DG cells play a causative role in addiction related behaviors, more fundamental information is needed about the extent to which populations of DG cells are active during context-reward memories. For example, the hippocampus and DG have been proposed to play a role in determining the salience, or importance, of contexts (Penner and Mizumori, 2012), which influences choice behavior for a rewarding context (Kennedy and Shapiro, 2009). However, whether DG cells are involved in defining the salience of a context has yet to be determined. As such, CPP is well poised to examine DG activity, after choice behavior (i.e. free access to CPP chambers on test day) (Tzschentke, 2007) and elimination of choice by sequestration (i.e. confinement to the previously-paired saline or morphine context on test day). Therefore, the involvement of DG activity,

indicated by cFos expression, was examined in mice tested in both choice behavior and sequestration CPP paradigms. The correlative data presented below support the role of DG activity in reward and memory, and strengthen the rationale for future examination of the DG activity contribution to retrieval of reward memory, which can ultimately lead to better treatments for addicted humans.

Methods/Results

Animals

All animal procedures were in accordance with the National Institutes of Health, Guide for the Care and Use of Laboratory Animals, and performed in IACUC-approved facilities at UT Southwestern Medical Center (UTSW). Experiments were designed to minimize animal number, pain and suffering. 6-8 week old male C57BL/6 mice were ordered from Jackson (#000664) and housed 3-4/cage (12-h light/dark cycle) with ad libitum access to food and water.

Conditioned Place Preference (CPP)

Mice were trained in a 3-compartment apparatus (Olson et al., 2005; Taniguchi et al., 2012). The CPP apparatus consisted of 2 large pairing and one smaller middle compartment, each with three distinct environments (Grey compartment [G], grey walls with course mesh flooring and dim lights; Middle compartment [M], white walls with bar flooring and brighter lights; Striped compartment [S], black and white striped

walls with fine mesh flooring and dim lights). No differences in time in middle compartment were observed for all groups tested (data not shown). An unbiased conditioning apparatus and counterbalanced compartment assignment CPP design was used (Tzschentke, 2007). Mice were trained on CPP with a 5-day paradigm inspired by previous studies with rat psychostimulant CPP (Smith and Aston-Jones, 2014; Zarrindast et al., 2002).

Briefly, on day 1 (pretest) mice were placed in the middle compartment (~11-1 P.M.) and had full access to the CPP box for 30 min. Time spent in each compartment was recorded by 8 photo beams per large compartment (i.e. the larger grey and striped compartments; Med Associates Inc.). For each mouse the time in the grey compartment was subtracted from the time in the striped compartment (i.e. difference score). The difference score was then used to assign a mouse to either a grey or striped CS+ compartment, respectively, such that the final pretest CPP score (i.e. difference score) within a cage was close to zero [modified from (Russo et al., 2007)]. All mice within a cage received either saline/saline (Sal/Sal) or saline/morphine (Sal/Mor), which is why we balanced preferences within each cage. An equal number of mice were conditioned in the grey and striped compartments. Mice that spent more than 20% (>6 min) of their CPP score in any compartment were excluded from future analysis to ensure subjects did not have a bias in the

conditioning apparatus (Fig.1, Sal/Sal, n=3; Sal/Mor, n=5; Fig. 3, Sal/Sal, n=3; Sal/Mor, n=8).

During pairing days (D 2-4), the CPP box was divided into the 3 compartments (G, M, S). All mice received saline (subcutaneous administration [s.c.], sterile bacteriostatic 0.9% saline [Hospira]) in the morning (~9-11 A.M.) and were paired to a context (conditioned stimulus, CS-) for 20 min and the number of photo beam breaks was recorded (Bardo et al., 1995). This was repeated in the alternate context (CS+) following saline or morphine (s.c., 15 mg/kg morphine in 0.9% saline; morphine sulfate powder, NIDA) in the afternoon (2-4 P.M.). On day 5 (test day), mice were again allowed access to all 3 compartments of the CPP box for 30 min and time spent in each compartment was recorded. In order to examine the rewarding effects of morphine, any Sal/Mor mouse that had a negative CPP test score was excluded from future analysis (Fig. 1, n=5).

Tissue Collection and IHC

Ninety min after CPP test, mice were killed and perfused (Ables et al., 2010; Lagace et al., 2007). Brains were sectioned coronally on a freezing microtime (Microm) in a 1:9 series at a 30 µm section thickness and were stored in 1X PBS 0.01% sodium azide solution (Lagace et al., 2007).

Free floating IHC was performed as previously described (Ables et al., 2010), with the following differences. Briefly, sections were placed in 1X PBS for 5 min, to help remove any residual sodium azide, incubated in 3.3% H2O2 (30% w/w) in 1X PBS for 30 min, placed into blocking solution (3% normal goat serum with 0.3% TritonX100 in 1X PBS) for 30 min, and incubated in the cFos primary antibody solution (3% normal goat serum with 0.3% Tween20 in 1X PBS with rabbit polyclonal anti-cFos (cFos; 1:25,000; Santa Cruz Biotechnology) overnight at RT for a minimum of 16 hrs. Sections were incubated in biotinylated secondary antibody solution (1.5% normal goat serum in 1X PBS with biotinylated goat anti-rabbit; 1:200; Vector Laboratories) for 2 hrs, VECTASTAIN® Elite® ABC solution (Vector Laboratories) for 1 hr and visualized with the chromophore 3,3′-Diaminobenzidine (Thermo Scientific Pierce). Sections were counterstained with Nuclear Fast Red (Vector Laboratories), washed, mounted in order of Bregma (-0.82 to -4.72 mm) onto charged slides (Fisher) and coverlipped with DPX mountant (VWR).

Cell Quantification and Volume Determination

Regional analysis of cFos+ cells in the DG GCL was determined for the supra and infrapyramidal blade as well as the inner and outer GCL throughout the rostral-caudal extent of the hippocampus (SI Fig. 2). For supra and infrapyramidal blade quantification of cFos+ cells, line made by the blind observer from the apex to apex of the hilus and GCL, respectively, for both the dorsal and ventral GCL of the

hippocampus (modified, (Snyder et al., 2012; Snyder et al., 2009). Cells observed on the line were categorized as being located in the suprapyramidal blade. For inner and outer GCL quantification of cFos+ cells, a line made by the blind observer divided the GCL in half along both the supra and infrapyramidal blades (modified, (Kempermann et al., 2003). Cells observed on the line were categorized as being located in the outer GCL.

Using an Olympus BX-51 microscope (Tokyo, Japan), cFos-immunoreactive (cFos+) cells were quantified using stereological principles (MBF Bioscience). The rare population of cFos+ cells in the dentate gyrus necessitated the use of exhaustive counting of cells in the granule cell layer (GCL) of the dentate gyrus (DG) at 400X magnification (NA 1⁄4 0.90) as previously described (Rivera et al., 2013). Briefly, stereological parameters of cFos+ cells used a height and area sampling fraction set to 1 to account for rare populations of cells within the DG GCL (Dayer et al., 2003; DeCarolis et al., 2013; Jayatissa et al., 2009; Lagace et al., 2010; Lister et al., 2005; Rivera et al., 2013). A section sampling fraction of 1/9 was used and raw counts were multiplied by 9 to estimate the total number of cFos+ cells within the entire DG GCL per section.

The total volume of the DG GCL was calculated using Cavalieri's estimation principles within hippocampal Bregma -0.82 to -4.24 (section evaluation interval = 9,

frame area = 60 × 60 μm) (MBF Bioscience) (Rivera et al., 2013). The coefficient of error (Gundersen, [m=0]) ranged from 3.7% to 7.4%, with an average of 5% and 5.025% for Sal/Sal (n=7) and Sal/Mor (n=4) groups, respectively (Fig. 2).

Statistical Analyses

Data are reported as mean ± SEM for all groups. Statistical analyses were performed using one- and two-way analysis of variance (ANOVA) followed by posthoc analysis using Sidak's multiple comparisons test (denoted within graphs using *). General comparisons between two variables used an unpaired Student t-test (denoted within graphs using #). Statistical significance was defined as $P < 0.05$. All statistical analyses were performed using Prism GraphPad (version 6.0c) software.

Based on the role of DG activity in contextual fear learning (Liu et al., 2014), we hypothesized that an increase in DG activity (i.e. cFos expression) after CPP was due to retrieval of the drug-associated context reward memory. To examine the retrieval of a drug-associated context reward memory, male C57BL/6J mice underwent morphine CPP (5 days, n=6-13, Fig. 1A, see Detailed Methods). Mice paired with morphine (Sal/Mor, days 2-4, s.c.,15 mg/kg, n=6; Fig. 1A) and tested on day 5 had a positive CPP score compared to saline controls (Sal/Sal) on test day (n=13, one-way ANOVA, ***P<0.001, Fig. 1B), therefore validating the use of this

shorter morphine CPP paradigm (Ribeiro Do Couto et al., 2003; Smith and Aston-Jones, 2014; Zarrindast et al., 2002). Mice were then killed 90 min post-test, a time point at which spatial/retrieval stimulus-induced cFos protein levels are high in many brain areas (Clark et al., 2010; Kee et al., 2007).

Due to functional and structural heterogeneity of the DG (Snyder et al., 2009), cFos+ nuclei (Fig. 1C inset) were quantified in DG granule cell layer (GCL) and GCL subregions. In agreement with previous reports with psychostimulants (Chauvet et al., 2011; Rademacher et al., 2006), mice (Sal/Mor) previously paired with saline in one context (CS-) and morphine in another context (CS+) had ~30% more cFos+ cells in the DG GCL than mice that received saline in both contexts (n=5/group, Student's t-test, #P<0.05, Fig. 1D). Analysis between Sal/Sal and Sal/Mor groups for DG GCL cFos+ cells across the coronal septotemporal axis of the hippocampus revealed a main effect treatment ($F(1,154)=25.87$, $P<0.001$) and distance ($F(13,154)=28.28$, $P<0.001$), but had no significant interaction (data not shown). It is unlikely that increased locomotion contributed to the increased DG GCL cFos+ cells during test day, as no difference in total beam breaks during test day was observed between Sal/Mor and Sal/Sal groups (Fig 1E).

Subregional GCL analysis revealed Sal/Mor mice had more cFos+ cells in the suprapyramidal (supra) blade as well as the inner (iGCL) and outer (oGCL)

compared to Sal/Sal controls (n=5/group/region, Student's t-test, supra, #P<0.05; iGCL, #P<0.01; oGCL, #P<0.05; Fig. 3B-D). No significant difference in infrapyramidal (infra) blade cFos+ cell number was found in Sal/Mor compared to Sal/Sal (Fig. 3E). Taken together, these data show that re-exposure to the CPP box after morphine pairing can generally activate DG GCL neurons relative to saline-paired controls, suggesting that drug experience and the context (CS+) can activate the DG.

Previous studies have shown that drug-induced locomotion (Zhang et al., 2006), restraint (Hoffman et al., 2013), and spatial contexts (Lopez et al., 2012) can increase the number of DG cFos-expressing cells. Therefore, we also explored whether the increase in cFos+ cells in the DG GCL (Fig. 1D) was due to these alternative explanations, or even to prior morphine exposure. In regards to locomotion, the total number of beam breaks between Sal/Sal and Sal/Mor mice on pretest and test day was not significantly different, suggesting that locomotor activity did not contribute to the increase in DG GCL cFos+ cells in Sal/Mor mice during test day (Fig. 1E). In addition, no difference in DG GCL volume was observed between Sal/Sal and Sal/Mor after morphine CPP (Fig. 2). In regards to prior morphine exposure, naïve vs. handled (n=9-12, Fig. 4A) and homecage Sal/Sal vs. Sal/Mor groups (n=5-6, Fig. 4C) showed no significant difference in DG GCL cFos+ cells (Fig. 4B, Naïve vs. Handled, Student's t-test, P>0.05; Fig. 4D, Sal/Sal vs. Sal/Mor,

Student's t-test, P>0.05). Taken together, these data show the increase of DG GCL cFos+ cells initially observed after choice behavior (Fig. 1D) was not due to locomotion or other variables that coincided with performing the CPP paradigm, such as handling or effects of prior morphine exposure.

One question that remained was whether the increase in DG GCL cFos+ cells (Fig. 1D) was due to choice behavior and/or retrieval of drug-associated context reward memory. Optogentically-induced cFos activation in the DG GCL is sufficient to recall fear memory (Liu et al., 2012). However, in regards to CPP, it is unclear if the retrieval of a rewarding memory vs. the morphine CPP paradigm itself led to more DG GCL cFos+ cells (Laakso et al., 2002). We considered choice behavior as a possible variable leading to more DG GCL cFos+ cells, and developed a paradigm to sequester mice into their saline-paired (CS-) or morphine-paired (CS+) contexts. Surprisingly, Sal/Sal and Sal/Mor mice sequestered on test day to CS+ or CS- (Fig. 3A) showed a main effect of treatment (n=7-9/group/treatment, Fig. 5B, $F(3,28)=3.311$, $P<0.05$). Posthoc analysis revealed Sal/Mor CS+ mice had more DG GCL cFos+ cells than Sal/Sal CS+ controls [Fig. 5B, M=-1749, 95% CI(-3417,-79.90), *P<0.05]. Analysis between Sal/Sal and Sal/Mor CS- groups for DG GCL cFos+ cells across the coronal septotemporal axis of the hippocampus revealed no significant difference (Fig. 5C). However, analysis between Sal/Sal and Sal/Mor CS+ groups for DG GCL cFos+ cells across the coronal septotemporal axis of the

hippocampus showed a significant main effect of treatment ($F(1,156)=22.92$, ***$P<0.001$) and distance ($F(12,156)=37.03$, $P<0.001$), but no significant interaction ($F(12,156)=1.658$, $P>0.05$). Posthoc analysis of the CS+ group revealed significantly more DG GCL cFos+ cells in Sal/Mor at Bregma points -2.32 [M=-33.57, 95% CI(-66.51,4.084), *$P<0.05$] and -2.92mm [M=-35.57, 95% CI(-68.51,-2.631), *$P<0.05$], compared to Sal/Sal controls (Fig. 5D). To determine if the context was important in establishing reward-associated context memory, analysis between CS- and CS+ in both Sal/Sal (Fig. 5E) and Sal/Mor (Fig. 5F) groups was performed. While both Sal/Sal and Sal/Mor groups showed a main effect of distance or interaction across the coronal septotemporal axis of the hippocampus (Sal/Sal, $F(12,182)=43.09$, $P<0.001$; Sal/Mor, $F(13,196)=60.58$, $P<0.001$), only the Sal/Mor had a main effect of context (Fig. 5F; $F(1,196)=7.45$, **$P<0.01$).

Subregional analysis by one-way ANOVA showed a significant interaction for the infra ($F(3,28)=3.852$, $P>0.05$) and oGCL ($F(3,28)=3.464$, $P>0.05$) regions (Fig. 6B,D). Posthoc analysis revealed Sal/Mor CS+ mice had more cFos+ cells than Sal/Sal CS+ for both infra [M=-690.4, 95% CI(-1285,-95.55), *$P<0.05$, Fig. 6B] and oGCL regions [M=-1237, 95% CI(-2425,-48.74), *$P<0.05$, Fig. 6D]. Notably, on test day there was no difference in total number of beam breaks between all groups (data not shown). Subregional analysis also found significant differences in Sal/Mor CS+ versus Sal/Sal CS+ for supra (Student's t-test, #$P<0.05$, Fig. 6A) and iGCL

(Student's t-test, #P<0.05, Fig. 6C) regions. A significant difference in Sal/Mor CS+ versus Sal/Mor CS- was also observed (Student's t-test, #P<0.05) in the oGCL region. Therefore, our data suggest that after sequestration to a previously-learned drug context, more DG GCL cFos+ cells are observed after retrieval of a drug-associated context reward memory.

Discussion

In sum, using a brief morphine CPP paradigm (3 pairing days, 15 mg/kg morphine) and free access to the CPP chambers on test day, we found a correlation between morphine-paired mice in the morphine-paired context (CS+) and more DG GCL cFos+ cells compared to saline-paired mice. Control experiments showed the increase in DG GCL cFos+ cells was not due to handling or pharmacological effects of morphine. However, additional morphine CPP experiments revealed that, as in the choice behavior experiment, morphine-paired mice sequestered to CS+ on test day had more DG GCL cFos+ cells compared to saline-paired mice. Taken together, these data correlatively suggest that DG cellular activation after morphine CPP appears to be dependent on the drug-associated context. For example, on test day when choice for a salient context was removed and only the drug-associated context was presented, a significant difference in DG GCL cFos+ cells in Sal/Sal and Sal/Mor mice was observed in the CS+ group, but not the CS- group (Fig. 5).

However, we cannot rule out that changes in the contextual environment on test day contribute to DG cFos activation.

It is useful to consider the present results in the context of the literature relevant to context dependent-reward and -fear learning and memory. Thus far, it was presumed that CPP led to rodents making an association between a drug and a context (Bardo and Bevins, 2000). The closest experiment to our shortened sequestration morphine CPP paradigm examined retrieval of drug/texture association using cocaine CPP (Johnson et al., 2010; Zombeck et al., 2008). In both of these studies, mice were paired to different textures (CS+ cocaine-paired texture; CS- saline-paired texture) with black walls throughout, and not given a choice between textures on test day. In conflict with our results where more DG cFos+ cells were observed after retrieval of a drug-associated context (sequestration to CS+), these prior experiments found either fewer DG cFos+ cells in CS+ (Johnson et al., 2010) or no significant difference (Zombeck et al., 2008) compared to CS- controls. The discrepancy with our results may be due to differences in spatial vs. textural contexts (Lopez et al., 2012), number of pairing days (Guzowski et al., 2001; Tzschentke, 2007), and drug type. Fear (contextual fear conditioning) and reward (psychostimulant CPP) learning and memory experiments have also shown that retrieval is sufficient to increase cFos in the DG (Beck and Fibiger, 1995; Rademacher et al., 2006) and septal DG (Chauvet et al., 2011). In addition, region

specific activity in the hippocampus is necessary for the acquisition and expression of cocaine CPP (Meyers et al., 2006). Our data agree with the concept of region specific activity within the hippocampus after retrieval since we observed an increase in cFos in the intermediate region of the DG (Fig. 5D) after retrieval of a morphine-associated context. However, more work is necessary to determine the function(s) of each region and how they may interact with one another during retrieval of a drug-reward memory. Taken together, our data provide an interesting parallel between the formation of reward vs. fear memory, which may be useful in developing treatments for addicted humans similar to those treatment that have been developed for the extinction of fear memory.

Our work lays the foundation for future studies that can test the hypothesis that DG cellular activation is necessary and sufficient to establish drug-associated context. While data are not available on DG activation during retrieval of morphine-paired CPP, recently published electrophysiology data from CA1 show retrieval of morphine-paired CPP correlates with increased basal synaptic transmission, impaired hippocampal LTP, and increased synaptic expression of the NR1 NMDAR subunit in the CA1 region (Portugal et al., 2014). It remains to be tested whether these changes in CA1 are causative, whether they are due to re-exposure-induced activation or modulation of the DG, and to what extent other inputs to the hippocampus or networks are involved in these changes (Otis et al., 2013; Tang and

Dani, 2009). In addicted humans, drug-associated contextual memories are strong and long lasting, making it difficult not to relapse when exposed to familiar associations (Koob and Volkow, 2010). Given the cognitive flexibility and enhanced contextual discrimination afforded by DG neurogenesis (Burghardt et al., 2012; Garthe et al., 2009; Kheirbek et al., 2012), one possible treatment avenue for addiction consists of increasing new DG neuron number or survival in the DG (Clelland et al., 2009). This would be predicted to allow better sparse encoding in the DG (Deng et al., 2010; Leutgeb et al., 2007), which may enhance extinction, or learning that a previous drug-associated context is no longer rewarding. However, it is also possible that aside from DG neurogenesis, DG interneurons (Ikrar et al., 2013) and DG granule-like cells (Williams et al., 2007) may play a role in modulating DG output during retrieval of a drug/associated context reward memory. All of these studies would be made more feasible by a better understanding of the neuroplastic changes specific to the hippocampal DG during CPP retrieval.

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Figures

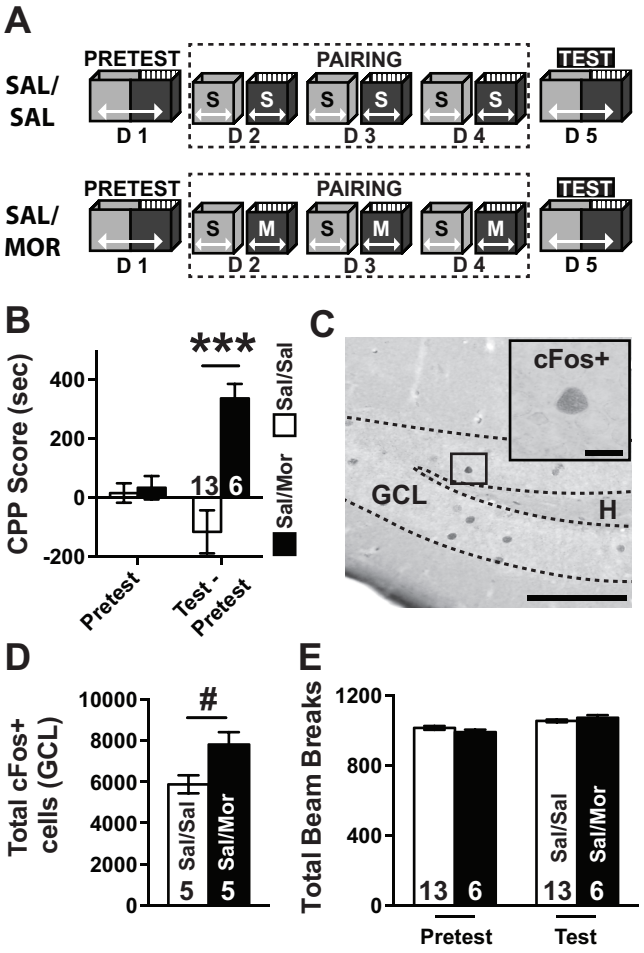

FIGURE 2-1. Morphine CPP produces place preference and results in more DG GCL cFos+ cells.

(A) Time line of morphine CPP experiment and schematic of experimental groups. Using a condensed 5-day morphine CPP paradigm Sal/Sal and Sal/Mor mice are placed into the CPP chamber on day 1 (pretest) and allowed to freely move throughout the CPP chamber. On days 2-4, Sal/Sal and Sal/Mor groups are

administered saline (S) in the morning and saline or morphine (M) in the afternoon, respectively. On the fifth day, mice are tested and allowed to freely move throughout the CPP chamber. (B) CPP score of Sal/Mor compared to Sal/Sal. One-way ANOVA followed by Sidak post hoc comparisons, ***P<0.001; n=6-13/group. (C) Photomicrograph of cFos+ cells in the DG GCL. Inset: cFos+ nuclei. Scale bar: (C) 100 um; (inset) 10 um. (D) Stereological quantification of cFos+ cells in the DG GCL. Student's t-test, #P<0.05; n=5/group. (E) Analysis of total beam breaks on pretest and test day. n=6-13 (mean ± SEM). S=saline, M=morphine, GCL=granule cell layer, H=hilus.

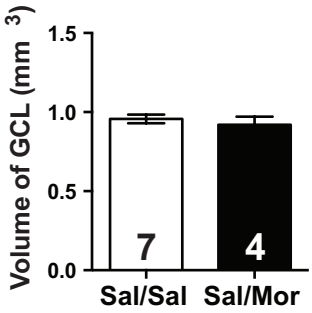

FIGURE 2-2. Morphine CPP does not alter the DG GCL volume.

Cavalieri estimation of DG GCL volume in Sal/Sal and Sal/Mor mice after morphine CPP (n=4-7/group). (mean ± SEM).

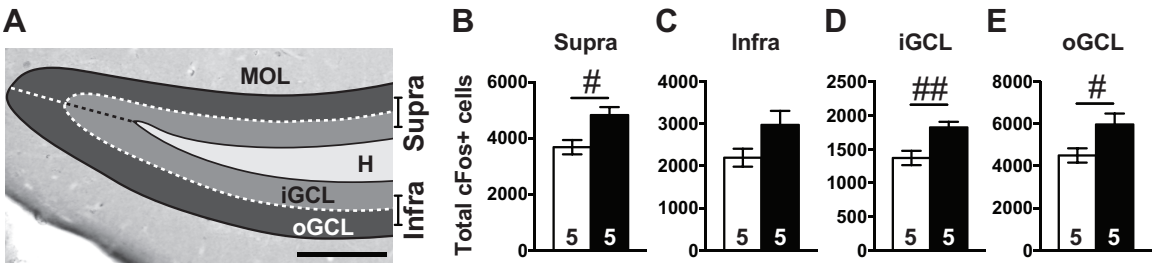

FIGURE 2-3. Morphine CPP results in more cFos+ cells in dentate gyrus subregions.

(A) Diagram representing subregional criteria for supra- infrapyramidal blades and inner- outerGCL. A line from the apex of the hilus to the apex of the GCL divided the supra- and infrapyramidal blade. The GCL region more dorsal to the line was classified as supra, while the region more ventral was classified as infra. A line dividing the GCL in half with the MOL and H providing the upper and lower boundaries of the GCL determined inner (light grey) and outer (dark grey) GCL regions. (B-E) Stereological quantification of cFos+ cells in the supra (B), infra (C), iGCL (D), and oGCL (E) subregions. Student's t-test, #P=0.05, ##P=0.01; n=5/group (mean ± SEM). MOL = molecular layer, H = hilus, iGCL = inner granule cell layer, oGCL = outer granule cell layer.

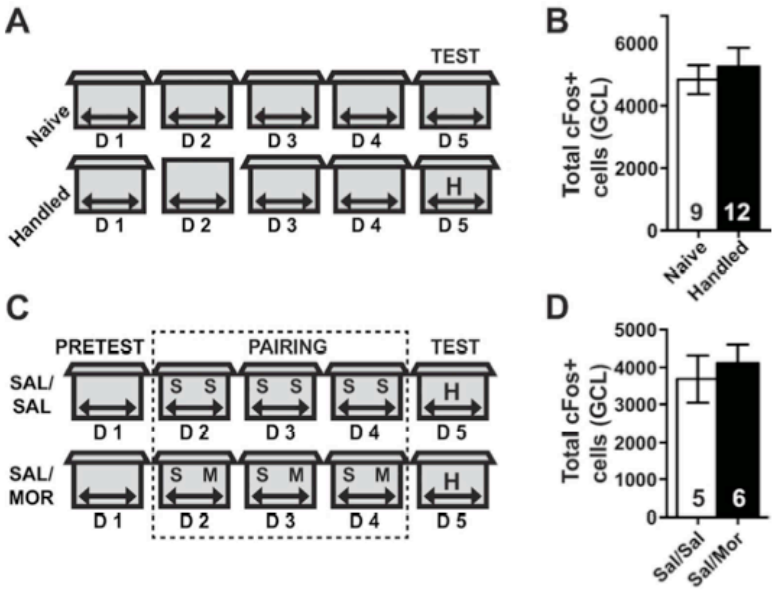

FIGURE 2-4. Home cage control experiments result in no change in DG GCL cFos+ cells.

(A) Time line of naïve vs. handled home cage experiment. Briefly, all mice were kept in home cage for 5 days. On the fifth day, mice were either handled or remained in home cage. (B) Stereological quantification of cFos+ cells in the DG GCL. n=9-12. (C) Time line of Sal/Sal vs. Sal/Mor home cage experiment. Briefly, all mice followed a similar paradigm as in Figure 1A, but in home cage. (D) Stereological quantification of cFos+ cells in the DG GCL. n=5-6 (mean ± SEM).

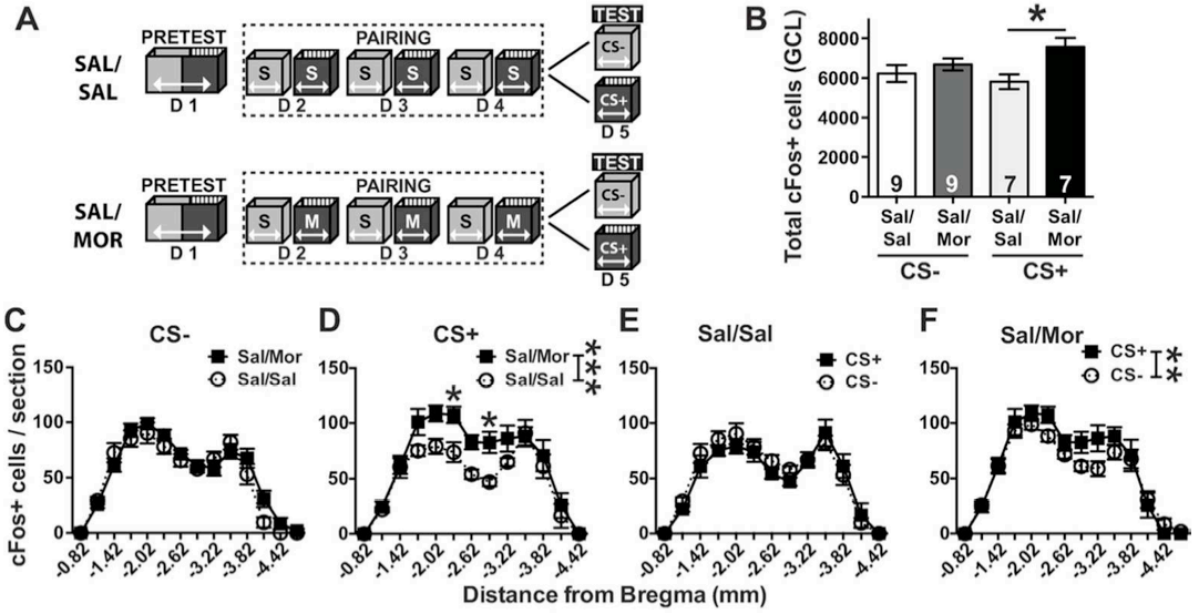

FIGURE 2-5. Sequestration to a previously-paired morphine context (CS+) results in more DG GCL cFos+ cells.

(A) Time line of morphine CPP sequestration experiment. Briefly, a same paradigm in Figure 1A was used; however on the fifth day mice were sequestered into their previously saline- (CS-) or morphine-paired (CS+) context. (B) Stereological quantification of cFos+ cells in the DG GCL comparing Sal/Mor and Sal/Sal in CS- and CS+ groups, respectively. (C-F) Bregma analysis comparing cFos+ cells per section in CS- (C), CS+ (D), Sal/Sal (E), and Sal/Mor (F) groups. One- and Two-way ANOVA followed by Sidak post hoc comparisons, *P<0.05; n=7-9/group (mean ± SEM). FIGURE 2-6. Sequestration to a previously paired morphine context results in more cFos+ cells in dentate gyrus subregions compared to saline controls.

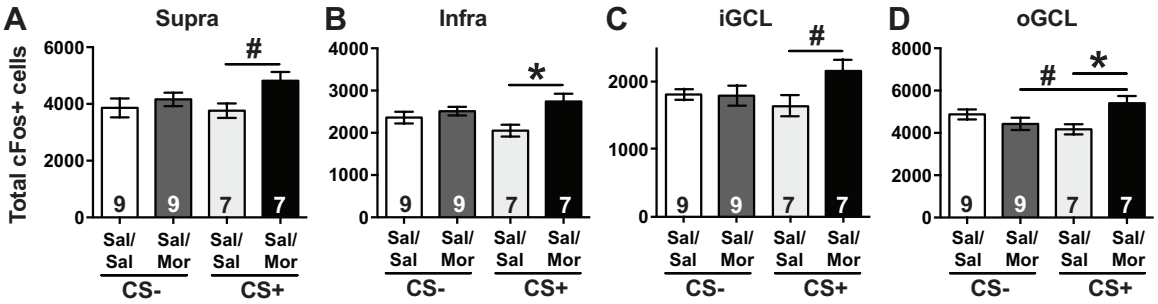

FIGURE 2-6. Sequestration to a previously paired morphine context (CS+) results in more cFos+ cells in dentate gyrus subregions compared to saline controls.

(A-D) Stereological quantification of cFos+ cells in the supra (A), infra (B), iGCL (C), and oGCL (D) subregions. Student's t-test, #P=0.05; One-way ANOVA followed by Sidak post hoc comparisons, *P=0.05; n=7-9 (mean ± SEM).

CHAPTER THREE:

IMAGE-GUIDED CRANIAL IRRADIATION-INDUCED ABLATION OF DENTATE GYRUS NEUROGENESIS DIMINISHES EXTINCTION OF YOUNG, BUT NOT OLD, MORPHINE REWARD MEMORIES

Adapted from: **Rivera PD**, Reynolds RP, Just AL, Birnbaum SG, Eisch AJ. Image-Guided Cranial Irradiation-Induced Ablation of Dentate Gyrus Neurogenesis Diminishes Extinction of Young, But Not Old, Morphine Reward Memories. *In submitted to Hippocampus.*

Abstract

Dentate gyrus adult neurogenesis is implicated in many hippocampal-dependent functions. However, the role of adult neurogenesis in context-dependent memory is unclear, as conflicting data exist on the role for them in retrieval and extinction of fear-associated memories. In addition, the role of adult neurogenesis in performance on reward-based context-dependent paradigms, like conditioned place preference (CPP), is particularly understudied. Here we used image-guided, hippocampal-targeted X-ray irradiation (IG-IR, 15 Gy) and morphine CPP to explore whether the dentate gyrus and in particular adult neurogenesis play a role in the retrieval or extinction of young and older reward memories in adult C57BL/6J male mice. Six weeks post-irradiation or Sham treatment, mice underwent morphine CPP (Pretest,

Day 1; Pairing, Days 2-4 [saline AM, morphine PM, 7 or 15 mg/kg s.c.]). In keeping with prior work in cocaine CPP in rat, retrieval of a young memory of the morphine-paired chamber (Test, Day 5, 24h post-CPP) was similar in IG-IR and Sham mice. In addition, retrieval of an older memory (Test, Day 21, 3 weeks post-CPP) was also similar in IG-IR and Sham mice. However, extinction of a young memory of the morphine-paired chamber (Test, Days 5-24) was significantly and strikingly diminished in IG-IR mice compared to Sham mice. For example, while Sham mice extinguished a young drug-associated context reward memory in 6 days, IG-IR mice took more than twice as long (14 days). In contrast, extinction of an older memory (Test, Days 25-44) was roughly similar in IG-IR and Sham mice. Taken together, these data show that hippocampal-directed irradiation and the associated decrease in dentate gyrus adult neurogenesis interfere with extinction of young context-dependent reward memories. This work suggests that a reduction or loss of adult DG neurogenesis in humans may trigger or perpetuate the cycle of addiction.

Introduction

Increasing evidence suggests that the hippocampus and the dentate gyrus (DG) are important in reward-associated behavior. On a correlative level, hippocampal plasticity is modified by conditioned place preference (CPP) with morphine (Portugal et al., 2014), and DG neurons are stimulated by exposure to a previously-paired drug context after psychostimulant or opiate CPP (Barr and Unterwald, 2015;

Hernandez-Rabaza et al., 2008; Rivera et al., 2014). On a causative level, both the hippocampus and DG are specifically implicated in the acquisition and retrieval of CPP drug/context associations (Hernandez-Rabaza et al., 2008; Meyers et al., 2006; Milekic et al., 2006; Rezayof et al., 2006; Zarrindast et al., 2007). Finally, human and rodent studies show that retrieval of a previously-learned hippocampal-dependent reward context is a key factor in mediating reward behavior (Fuchs et al., 2005; Takano et al., 2010; Wittmann et al., 2005; Wolosin et al., 2012). Such studies support that the hippocampus and DG play a role in learning and memory mechanisms underlying context-dependent reward memory (Ferbinteanu and McDonald, 2001; Milekic et al., 2006; Smith and Bulkin, 2014; Taubenfeld et al., 2010). Given that drug/context association triggers of human addicts that can drive cravings, continued drug use, and/or relapse (Bardo and Bevins, 2000; Franklin et al., 2007; Gould, 2010; Hyman, 2005), clarification how these limbic structures influence reward-based behaviors – particularly in the context of CPP – will significantly benefit the field of addiction research.

One novel aspect of the DG is the process of adult neurogenesis, where adult-born granule cell neurons (ABGCs) are generated throughout life. ABGCs have been implicated in many aspects of hippocampal-dependent function, including learning and retention in the contextual fear conditioning paradigm (CFC) (Akers et al., 2014; Warner-Schmidt et al., 2008; Winocur et al., 2006). ABGCs have begun to be

examined for their potential role in reward-associated learning and memory as well (Canales, 2007; Eisch et al., 2008; Mandyam and Koob, 2012). For example, ABGC deletion via hippocampal-directed irradiation or inducible transgenic mice results in increased self-administration of cocaine and decreased sucrose preference, but does not change operant learning for sucrose (Noonan et al., 2010; Snyder et al., 2011). Given the utility of the CPP paradigm to gain insight into the learning and memory components that contribute to the formation of drug/context associations, and given that manipulations of the DG that modulate neurogenesis also modulate CPP to psychostimulants and opiates (Noonan et al., 2010; Snyder et al., 2011), it is surprising that few studies have examined the role of ABGCs in CPP (Brown et al., 2010; Mustroph et al., 2015; Zheng et al., 2013). While psychostimulants and opiates have distinct mechanisms of action, it is also surprising that no studies have examined the role of ABGCs in morphine CPP.

Given the dearth of CPP studies, it is useful to assess commonalities and knowledge gaps between what is known about the role of ABGCs in context-dependent learning and memory in fear-based tasks (CFC) vs. reward-based tasks (CPP). For example, similar to CFC work (Dupret et al., 2008; Groves et al., 2013; Kitamura et al., 2009), CPP research shows that ablation of ABGCs does not change the retrieval of a cocaine/context association learned 24h earlier (Brown et al., 2010), underscoring that ABGCs are not involved in retrieval of recent or “young” memories. In contrast,

there are conflicting data about whether ablation of ABGCs impairs the extinction of young fear memories in CFC (Deng et al., 2009; Hernandez-Rabaza et al., 2008; Ko et al., 2009; Pan et al., 2013) the role of ABGCs in the extinction of drug/context associations in CPP has not been examined. In fact, given the conflicting data on whether ablation of new neurons influences extinction of reward memories in a self-administration paradigm (Deschaux et al., 2014; Noonan et al., 2010), examination of the role of ABGCs in the extinction of drug/context associations in a CPP paradigm would be helpful in expanding our understanding of new neurons in reward-based learning. A specific aspect of CFC research that has proven profitable in dissecting the role of ABGCs in fear learning is the examination of remote or "older" and young memories as a mechanism to determine when a memory is hippocampal-dependent and labile, or hippocampal-independent and stored for later retrieval in other brain regions such as the cortex (Frankland and Bontempi, 2005; Kitamura et al., 2009; Tayler et al., 2013). From this perspective, it is notable that ablation/reduction of ABGCs prior to retrieval of an older memory generally causes deficits in learning and memory (Arruda-Carvalho et al., 2011; Deng et al., 2009; Imayoshi et al., 2008; Snyder et al., 2005) and CFC (Akers et al., 2014; Pan et al., 2013; Winocur et al., 2006). This suggests a possible role of ABGCs in modulating older hippocampal-dependent memories. However, in regards to CPP, no study has assessed whether ABGCs ablation influences retrieval of older morphine CPP

reward memory. Therefore, a better understanding of ABGC's role in drug-associated context reward memory is needed.

Here we used image-guided cranial irradiation to ablate ABGCs and address the unanswered questions of whether ABGCs play a role in supporting retrieval and extinction of young versus old memories a morphine-associated context reward memory. Based on past research, we hypothesized that ablation of ABGCs would decrease retrieval and extinction of an older drug-associated context reward memory, but not influence retrieval and extinction of a younger drug-associated context memory.

Methods

Animals

Male C57BL/6J mice (6-8 week old) were ordered from Jackson Labs (#000664) and allowed to habituate for 1 week before conditioned place preference (CPP) or image-guided irradiation (IG-IR). Mice were housed at UTSW (4/cage, 12h light/dark cycle) with *ad libitum* access to food and water. All animal procedures and husbandry were in accordance with the National Institutes of Health, Guide for the Care and Use of Laboratory Animals, and performed in IACUC-approved facilities at UT Southwestern Medical Center (UTSW). Experiments were designed to minimize animal number, pain, and suffering.

Image-Guided Cranial Irradiation

Image-guided cranial irradiation (IG-IR, n=50) was delivered via the X-RAD 225Cx self-contained irradiation system (Precision X-Ray)(Clarkson et al., 2011) as described previously (Walker et al., 2014). Briefly, in all experiments, except **Fig. 2**, the hippocampi of anesthetized (1.5-2.5% isoflurane) 7-9 week old mice received diagnostic X-Rays to confirm head alignment and collimator placement (6 x 14 mm rectangular collimator) and were then irradiated (15 Gy, (Moravan et al., 2011; Olschowka et al., 1997)). Sham control mice (Sham, n=47) were exposed to isoflurane for a similar amount of time as IG-IR mice, but were never placed in the irradiator. Six weeks after IG-IR (a time point when ABGCs are significantly decreased (Walker et al., 2014)) or Sham exposure, CPP training was performed.

Drug Treatment

Morphine sulfate powder was provided by the National Institute on Drug Abuse (NIDA, Baltimore, MD). Morphine sulfate powder was dissolved in sterile bacteriostatic 0.9% saline (Hospira) and administered at 7 or 15 mg/kg (s.c.). Saline controls were given sterile bacteriostatic 0.9% saline (s.c., 10 mL/kg).

Conditioned Place Preference

Conditioned Place Preference (CPP) chambers were composed of 3 compartments: a grey compartment, middle, and striped compartment, as previously described (Rivera et al., 2014). Grey and striped compartments were the same size with dimensions 24.5 x 15 x 33 cm. The grey compartment had grey walls and large-grid wire flooring, while the striped compartment had black and white vertical striped walls with small-grid wire flooring. The middle compartment was located between the grey and striped compartments and was twice as small in length (12 x 15 x 33 cm) with white walls and parallel bar flooring. Opaque dividers on each side of the middle compartment were lowered to either confine mice to one compartment (grey or striped) on pairing days, or lifted to allow free access to all compartments during pretest and test/extinction days. Time spent in each compartment was measured by photo beam breaks, which was collected by Med Associates, Inc. software.

During Pretest (D1), mice were placed into the middle compartment and given free access to all 3 compartments for 20 min. Time spent in each compartment was recorded and used to determine CPP score for each mouse (see Data Analysis). After 20 min, mice were returned to their home cage (Rivera et al., 2014).

Based on CPP scores determined from Pretest, unbiased pairing of mice to a drug context (DC) and non-drug context (NDC) was performed for Pairing days (D2-4). Pairing consisted of a morning saline (AM) and afternoon morphine (PM)

conditioning session. During the AM session, each mouse received subcutaneous (s.c.) administration of 0.9% saline and was immediately placed into the NDC for 30 min. In the PM session, each mouse received s.c. morphine and was immediately placed into the DC for 30 min. Following the AM and PM sessions, mice were returned to their home cages (Rivera et al., 2014). Injections were only given during Pairing; the retrieval and extinction sessions described below were performed with no injections (i.e. drug-free).

For retrieval of young reward memories, which are sometimes referred to as recent retrieval (Frankland and Bontempi, 2005; Tayler et al., 2013), mice were placed into the middle compartment ~24h post-Pairing with free access to all 3 compartments for 20 min (Test, D5; **Fig. 1A**). In a separate group of animals, retrieval of older reward memories was tested using the same procedure but performed instead ~21d post-Pairing (Test, D21; **Fig. 1D**). For extinction of young and old reward memories, mice were placed daily into the middle compartment and given 20min with free access to all 3 compartments. Extinction testing of young memories began ~24h post-Pairing and was repeated every day for 7d (Extinct, D5 to D11, **Fig. 2A**) or 20d (Extinct, D5-24, **Fig. 3A**). Extinction testing of old memories began 3 weeks post-Pairing and was repeated every day for 20d (Extinct, D25-44, **Fig. 3D**). For retrieval and extinction sessions, time spent in each compartment was recorded.

Pretest CPP scores were calculated from the difference in the time spent between the DC minus the NDC compartment. For each Sham, IG-IR, or dose treatment group, the difference scores were unbiasedly chosen from individual mice to bring the group sum as close to zero as possible. Any mouse with a CPP score ≥ ± 240 sec was automatically paired to receive drug in the non-preferred context, received Pairing along with the other mice, but their data were not included in data analysis.

Statistical Analyses

Data are reported as mean ± SEM for all groups. All statistical analyses used repeated measures two-way analysis of variance (ANOVA) and Sidak's multiple comparisons posthoc analysis test. Statistical significance was defined as $P<0.05$. All statistical analyses were performed using Prism GraphPad (version 6.0c) software.

Results

Image-guided cranial irradiation does not effect retrieval of young and old morphine-context reward memories.

To examine the influence of hippocampal IG-IR on retrieval of young and old drug/context association reward memories, we first validated that ABGCs (DCX+ cells) were evident throughout the septal and temporal DG of Sham mice, but ablated throughout the DG of IG-IR mice (data not shown). Six weeks post-IG-IR or

Sham treatment, mice were trained on morphine CPP. Young reward memory was examined ~24h post-Pairing (**Fig. 1A**). When the time spent in each context was examined within treatment groups over time, both Sham and IG-IR groups showed significant interactions (Sham, $F_{1,20}$=43.82, *P*<0.001; IG-IR, $F_{1,30}$=132.6, *P*<0.001) and a main effect of context (Sham, $F_{1,20}$=11.78, *P*<0.01; IG-IR, $F_{1,30}$=48.52, *P*<0.001). However, Sham showed no main effect of time ($F_{1,20}$=1.427, *P*=0.25) while IG-IR did ($F_{1,30}$=5.86, *P*<0.05). Sidak's posthoc analysis comparing DC and NDC showed the time spent in the DC was significantly higher on the D5 Test in both Sham (M=327.5, 95% CI [204.9,450.1], *P*<0.001, **Fig. 1B**) and IG-IR (M=422.5, 95% CI [341.0,504.0], *P*<0.001, **Fig. 1C**) mice. These data show that retrieval of a young morphine-context reward memory was intact 6 weeks post-IG irradiation.

In a separate cohort of mice, retrieval of an older reward memory was examined 3 weeks post-CPP training (**Fig. 1D**). When time spent in each context was examined within treatment groups over time, both Sham and IG-IR groups showed significant interactions (Sham, $F_{1,16}$=37.26, *P*<0.001; IG-IR, $F_{1,12}$=55.12, *P*<0.001) and a main effect of context (Sham, $F_{1,16}$=14.72, *P*<0.01; IG-IR, $F_{1,12}$=23.44, *P*<0.001), but neither treatment group had a main effect of time (Sham, $F_{1,16}$=0.9, *P*=0.36; IG-IR, $F_{1,12}$=1.324, *P*=0.27). Sidak's posthoc analysis comparing DC and NDC showed the time spent in the DC was significantly higher on D21 Test in both Sham (M=337.6, 95% CI [217.0,458.3], *P*<0.001, **Fig. 1E**) and IG-IR (M=384.7, 95% CI [271.3,498.0],

P<0.001, **Fig. 1F**) mice. These data show that, similar to young memories, retrieval of an older morphine-context reward memory was intact 6 weeks post-IG irradiation.

Differential extinction of young reward memory after CPP training with 0, 7, or 15 mg/kg.

Prior to performing extinction testing with IG-IR and Sham mice, we wanted to assess a dose-response of extinction learning in naïve mice. Therefore, naïve (non-irradiated) mice were CPP paired to 0, 7, and 15 mg/kg of morphine and the extinction of young reward memory was examined (**Fig. 2**). Differences in time spent in DC and NDC within each treatment dose were examined across extinction trials (over 7 days; **Fig. 2A**). In the 0 mg/kg group, two-way ANOVA did not reveal any interaction or main effects of context and time (data not shown). The 7 mg/kg group revealed a significant interaction ($F_{7,140}$=3.987, *P*<0.001) and main effect of context ($F_{1,20}$=9.25, *P*<0.01), but no main effect of time ($F_{7,140}$=0.76, *P*=0.62). Sidak's posthoc analysis showed that mice spent significantly more time in DC compared to NDC on the first day of extinction, D5 (M=204.5, 95% CI [32.83,376.1], *P*<0.01), and also on D6 (M=229, 95% CI [57.35,400.7], *P*<0.01) and D9 (M=203.8, 95% CI [32.19,375.5], *P*<0.05, **Fig. 2B**). The 15 mg/kg group revealed a significant interaction ($F_{7,126}$=6.217, *P*<0.001), but no main effect of time ($F_{7,126}$=0.28, *P*=0.96) or context ($F_{1,18}$=1.66, *P*=0.21). Sidak's posthoc analysis showed that mice spent significantly more time in DC compared to NDC, only on the first day of extinction,

D5 (M=318, 95% CI [28.54,607.5], P<0.05, **Fig. 2C**). Taken together, these data show pairing with 7 and 15 mg/kg of morphine lead to place preference, but that 7 mg/kg results in a longer extinction of young reward memory compared to 15 mg/kg. Due to the possibility of improved extinction (Zheng et al., 2013), the dose that provided longer extinction (7 mg/kg) was used for the IG-IR extinction experiments.

Image-guided cranial irradiation diminishes extinction of young – but not old – morphine-context reward memories.

Previous contextual fear conditioning research has shown ablation of DG ABGCs leads to impaired extinction of young fear memories (Deng et al., 2009). Therefore, we examined if IG-IR similarly impaired extinction of young and old reward memories after morphine CPP. Mice received IG-IR or Sham treatment 6 weeks prior, were CPP trained with 7 mg/kg of morphine, and extinction of the young and old drug-associated context reward memory was examined (**Fig. 3)**. In the extinction of young reward memory experiment (**Fig. 3A)**, analysis of the data from Sham mice revealed a significant interaction ($F_{10,160}$=19.89, P<0.001) and main effect of context ($F_{1,16}$=5.288, P<0.05), but no main effect of time ($F_{10,160}$=0.23, P=0.99). Sidak's posthoc analysis showed Sham mice spent significantly more time in DC, compared to NDC, on early in extinction: D5-6 (M=385.5, 95% CI[264.8,506.2], P<0.001), D7-8 (M=385.5, 95% CI[105.2,346.6], P<0.001), and D9-10 (M=385.5, 95% CI[20.81,262.2], P<0.05, **Fig. 3B**). Analysis of the data from IG-IR mice also

revealed a significant interaction ($F_{10,200}$=17.72, *P*<0.001) and a main effect of context ($F_{1,20}$=26.08, *P*<0.001), but no main effect of time ($F_{10,200}$=0.61, *P*=0.81). However, Sidak's posthoc analysis showed that IG-IR mice spent significantly more time in DC, compared to NDC, over the first 14 days of extinction testing (D5-6, [M=404.5, 95% CI{294.8,514.2}, *P*<0.001]; 7-8, [M=233.4, 95% CI{123.7,343.1}, *P*<0.001]; 9-10, [M=146.2, 95% CI{36.53,255.9}, *P*<0.01]; 11-12, [M=161.1, 95% CI{51.40,270.7}, *P*<0.001]; 13-14, [M=177.4, 95% CI{67.73,287.1}, *P*<0.001]; 15-16, [M=176.5, 95% CI{66.82,286.1}, *P*<0.001]; 17-18, [M=130.6, 95% CI{20.9,240.2}, *P*<0.01], **Fig. 3C**). Taken together, these data show IG-IR mice take more than twice as long to extinguish a young morphine-context reward memory compared to Sham mice.

In the extinction of older reward memories experiment (**Fig. 3D**), analysis of the data from Sham mice revealed a significant interaction ($F_{10,260}$=8.47, *P*<0.001) and main effect of context ($F_{1,26}$=5.208, *P*<0.05), but no main effect of time ($F_{10,260}$=0.5311, *P*=0.86). Sidak's posthoc analysis showed Sham mice spent significantly more time in DC, compared to NDC, early in extinction testing on D25-26 (M=424, 95% CI[205.4,642.6], *P*<0.001) and D27-28 (M=385.5, 95% CI[98.87,536.0], *P*<0.001, **Fig. 3E**). Analysis of the data from IG-IR mice also revealed a significant interaction ($F_{10,280}$=5.735, *P*<0.001) and a main effect of context ($F_{1,28}$=12.27, *P*<0.01), but no main effect of time ($F_{10,280}$=12.27, *P*=0.82). Sidak's posthoc analysis showed that

mice spent significantly more time in DC, compared to NDC, on D25-26, (M=337.1, 95% CI[187.4,486.9], *P*<0.001), D27-28, (M=215.1, 95% CI[65.35,364.9], *P*<0.001), and D35-36 (M=191.9, 95% CI[42.14,341.7], *P*<0.01, **Fig. 3F**). While the IG-IR mice take 2 more days to extinguish relative to Sham mice, these data show that extinction of older morphine-context reward memories is roughly similar in both Sham and IG-IR mice.

Discussion

Prior to the present work, it was unknown if DG ABGCs could mediate the memory of previously formed reward-associated contexts during retrieval and extinction. As ABGCs may have a role temporal separation of events (Aimone et al., 2006; Deng et al., 2009; Kesner, 2007; Rangel et al., 2014), discrimination of similar contexts (Guo et al., 2011; Tronel et al., 2012), and memory consolidation (Kitamura and Inokuchi, 2014; Zhao et al., 2007), the current study was designed to examine if ablation of ABGCs leads to temporal deficits in learning and memory mechanisms necessary for discriminating drug/context associations. Using mice previously irradiated and trained on morphine CPP (15 mg/kg), we demonstrated that retrieval of young and old reward memories was preserved in both IG-IR and Sham groups (**Fig. 1**). Mice that received either dose of morphine expressed CPP, yet mice that received 7 mg/kg showed significantly longer extinction learning than mice that received 15 mg/kg (**Fig. 2**). Surprisingly, IG-IR mice showed deficits in the extinction of young –

but not old – reward memories (7 mg/kg) compared to Sham mice (**Fig. 3**). As discussed below, the lack of effect of ablated ABGC on morphine CPP retrieval of young or old reward memories are generally consistent with prior work with CPP and even with CFC. However, the ability of image guided cranial irradiation to interfere with young – but not old – reward memories is a highly novel finding, and contrary to some reports with non-reward learning tasks and CFC. Taken together, these data suggest that IG-IR ablation of ABGCs may have a functional role in the extinction of recently formed reward-associated contexts.

Diminished Extinction of Young, but not Old, Reward Memories after IG-IR

Our most striking finding is that IG-IR mice have impaired extinction of young reward memories. This is the first study on IG-IR and morphine CPP, and the first on IG-IR and CPP extinction. Thus, it is are challenging to compare to existing publications that relate to young reward extinction and ABGCs (Deschaux et al., 2014; Noonan et al., 2010; Portugal et al., 2014; Zheng et al., 2013), since only one uses an ablation strategy (Noonan et al., 2010). For example, here we used IG-IR to ablate ABGCs, while another work blunted ABGC number by modulating expression of a neuronal transcription factor in the entire DG (Zheng et al., 2013) or stimulation of a downstream hippocampal region (Deschaux et al., 2014). In addition, the morphine CPP paradigm has also been shown to decrease DG doublecortin (DCX, i.e. a marker of immature neurons) (Zheng et al., 2013). Interestingly, non-reward studies

that reduce ABGCs prior to learning and memory and fear behaviors show deficits in extinction of young memories (Deng et al., 2009; Hernandez-Rabaza et al., 2008; Pan et al., 2013; Shors et al., 2001), similar to our findings. Several non-reward studies have also shown time-dependent deficits in learning and memory and fear behaviors from longer duration of an antimitotic toxin (Shors et al., 2001) to a shorter time interval from genetic ablation to behavioral testing (Deng et al., 2009). Given the time-dependence of extinction learning, along with reduced ABGC numbers, it is possible that ABGCs from other works could recover (Ko et al., 2009). Given the effective ablation of ABGCs in the present study, we suggest that the observed behavioral deficits in the extinction of young reward memories are due to the loss of hippocampal ABGCs.

In contrast to our finding that IG-IR interferes with extinction of young reward memories, we find no obvious change in the extinction of older reward memories. Although extinction of older memories with a prior reduction/ablation of ABGCs has not been thoroughly examined, the existing literature generally supports that new neurons do not play a role in extinction of old memories (Deng et al., 2010; Ko et al., 2009; Noonan et al., 2010). These data suggest that older memories become hippocampal-independent and are stored for later retrieval in other brain regions such as the cortex (Frankland and Bontempi, 2005; Kitamura et al., 2009). Interestingly, deficits in extinction of older reward memories have been seen when

ABGCs were ablated between training and testing, and not prior to training (Noonan et al., 2010). This is similar to our data presented here, where no difference in extinction of older reward memories was observed when ABGCs were ablated prior to morphine CPP training. Given the prominent role of the prefrontal cortex in extinction learning for fear and addiction (Peters et al., 2009), and the strong influence the hippocampus has on the prefrontal cortex (Sotres-Bayon et al., 2012), future work could probe whether manipulation of other brain regions might disrupt the extinction of older reward memories. In agreement with this statement, silenced ventromedial prefrontal cortex pyramidal neurons prevented extinction learning of an old (~3 weeks) cocaine CPP reward memory (Van den Oever et al., 2013). Taken together, we speculate that once reward memories become hippocampal-independent, targeting other brain regions such as the prefrontal cortex or amygdala will have a greater impact in developing extinction based treatments for old reward memories.

Retrieval of Young and Old Drug-Associated Contexts are Unchanged After IG-IR

The lack of effect of ablated ABGCs on retrieval of young and old memories we show here is similar to previously published reward data. For example, cranial irradiation-induced reduction of ABGCs leads to no change in retrieval of young cocaine-associated memories in the rat (Brown et al., 2010) or to retrieval of young (1d) and old (28d) fear memory (Kitamura et al., 2009). Our data are also similar to

retrieval of an old reward memory after cocaine SA (Noonan et al., 2010). Taken together, our data add to the previous literature showing that retrieval of young and old memories may not require the hippocampus (Cowansage et al., 2014; Squire and Wixted, 2011). Instead, successful retrieval of young memories may only require activation of cortical ensembles to retrieve a fear or reward memory (Cowansage et al., 2014). In agreement with other brain region involvement in retrieval, activation of an ensemble of amygdalar neurons may modulate cocaine memory consolidation (Hsiang et al., 2014). However, the contribution of both cortical and amygdalar ensembles in the retrieval of older reward memories has yet to be examined.

Conclusion

This work implicates a temporal role of DG adult neurogenesis in the retrieval of memories necessary for discriminating drug/context associations, which is observed as diminished extinction learning of a young reward memory. As adult neurogenesis is believed to be involved in temporal separation of events (Aimone et al., 2006; Deng et al., 2009; Kesner, 2007; Rangel et al., 2014) and extinction learning is temporally distinct from other forms of learning and memory (Suzuki et al., 2004), future studies should examine if ABGCs alter hippocampal-cortical and hippocampal-amygdalar engrams in learning and memory mechanisms (Cowansage et al., 2014; Hsiang et al., 2014), such as extinction. Therefore, experiments examining the learning and memory mechanisms of addiction after a

reduction/enhancement of ABGCs may provide beneficial information to develop better treatments for addicted humans.

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Figures

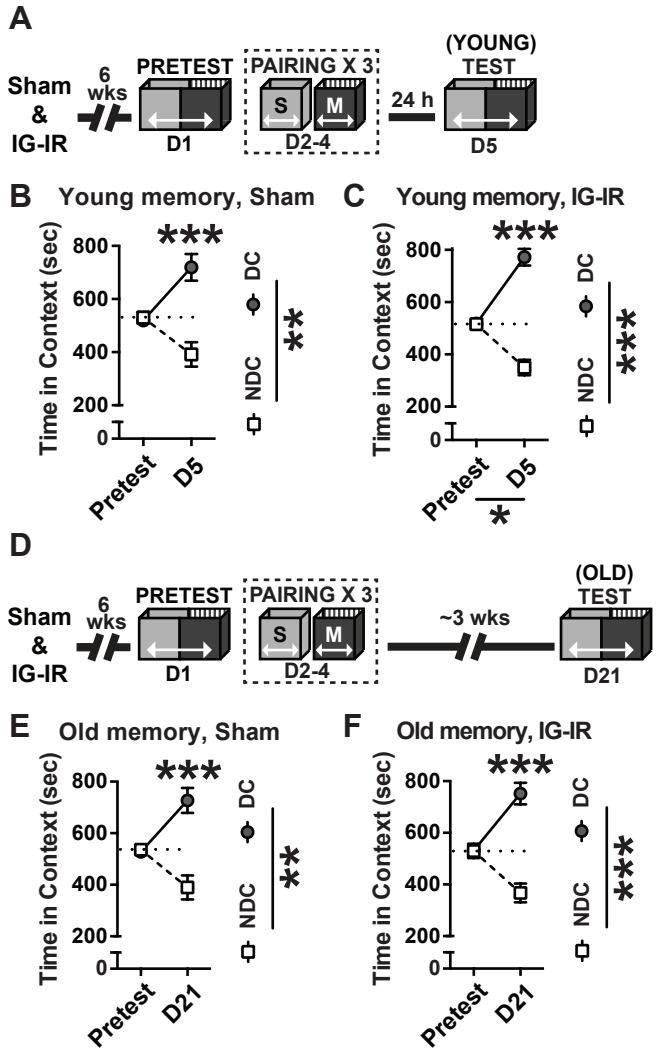

FIGURE 3-1. Image-guided cranial irradiation does not effect retrieval of young or older morphine-context reward memories.

(A-B) Timeline of morphine CPP experiment and schematic of treatment groups. Six weeks post-irradiation, Sham and IG-IR mice were trained on morphine CPP (D1-4) and tested (A) 24h or (D) 3 weeks later to examine young and older reward memories, respectively. (C-F) The total time spent in the DC or NDC was examined

during retrieval of (B-C) young (n=11-16/group) and (E-F) old (n=8-13/group) performance in (B, E) Sham and (C, F) IG-IR mice. Two-way ANOVA, Sidak's post hoc analysis, ***P=0.001, Mean±SEM. DC=drug context, M=morphine, NDC=non-drug context, S=saline.

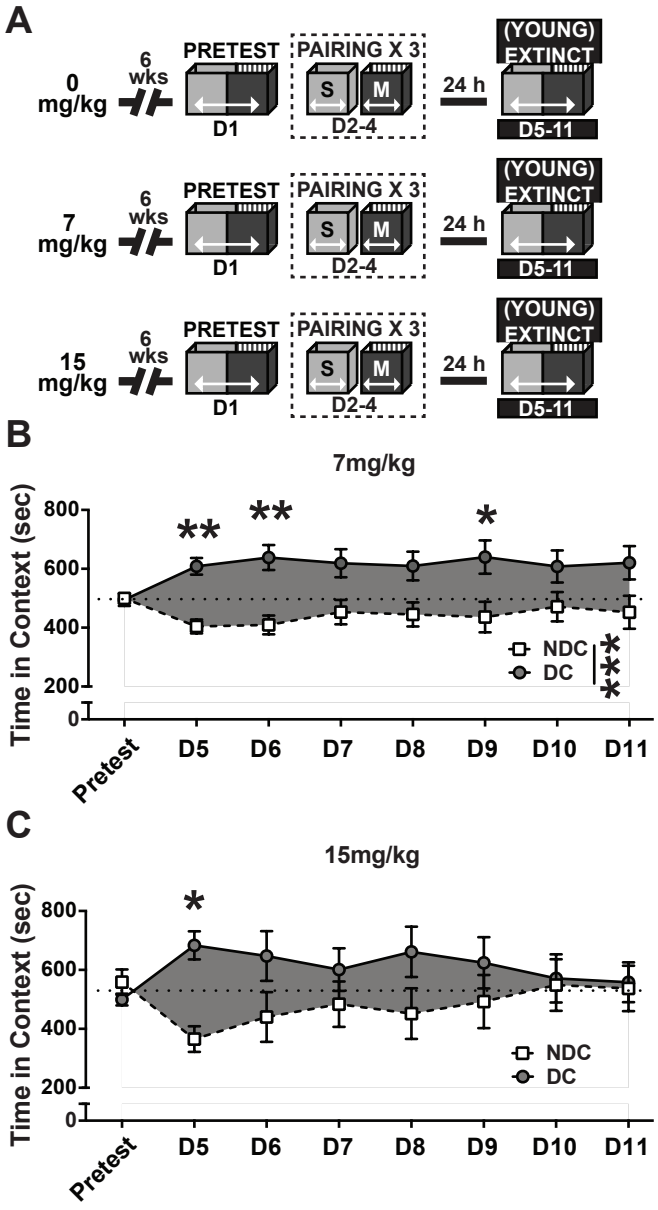

FIGURE 3-2. Dose-response in non-irradiated, morphine CPP paired mice reveals that pairing with 7 mg/kg results in longer extinction than with 0 or 15 mg/kg.

(A) Timeline of morphine CPP extinction experiment and schematic of treatment groups (0, 7, 15 mg/kg morphine). Mice were trained on morphine CPP (D1-4) and 24 h later extinction of young reward memory began. (B-C) Daily extinction of the young reward memory over 7d (D5-11). (B) Mice that received pairing with 7 mg/kg took at least 5d to extinguish the reward memory. (C) Mice that received pairing with the 15 mg/kg treatment group took one day to extinguish the reward memory. Two-way ANOVA, Sidak's post hoc analysis, **P=0.01, *P=0.05; n=8-10/group. Mean±SEM. DC=drug context, M=morphine, NDC=non-drug context, S=saline.

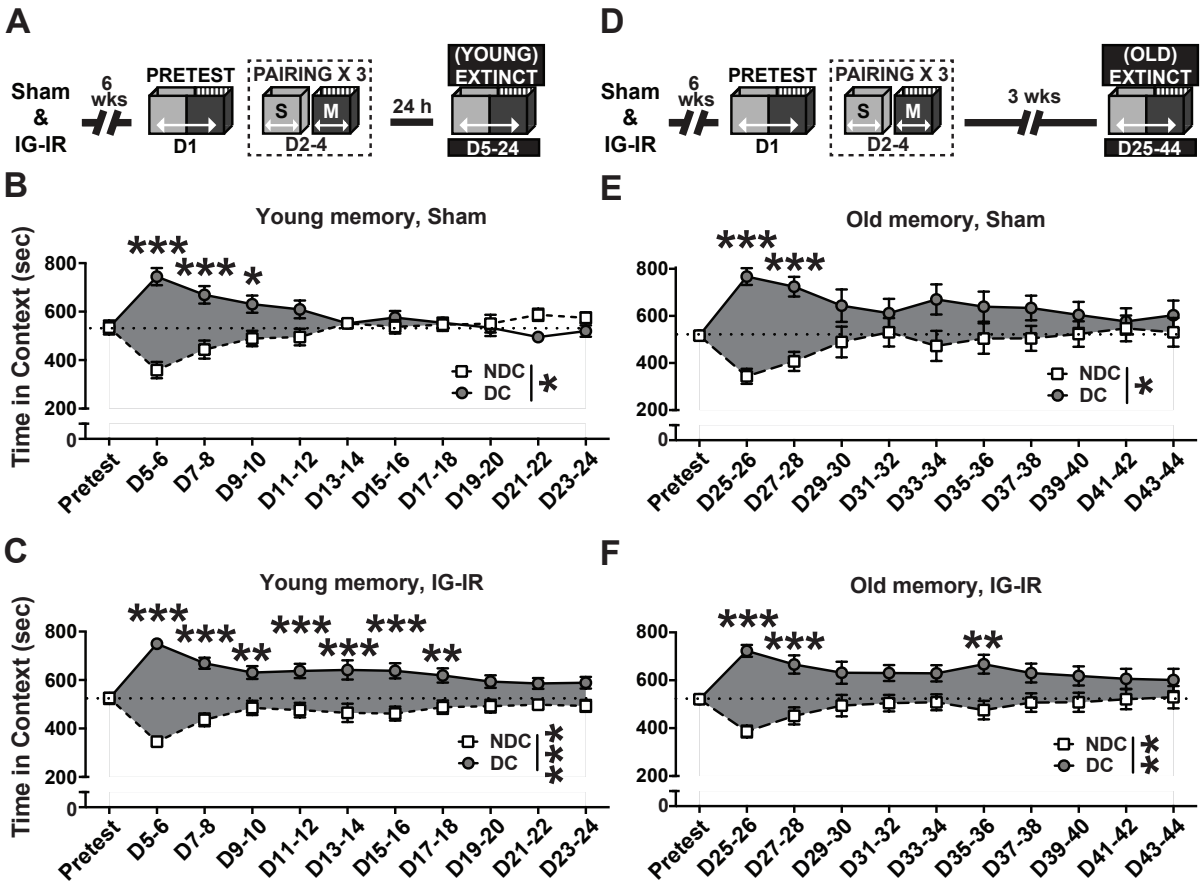

FIGURE 3-3. Image-guided cranial irradiation diminishes extinction of young – but not older – morphine-context reward memories.

(A, D) Extinction timelines of young and old morphine-context reward memories and schematic of Sham and IG-IR treatment groups. Six weeks post-irradiation, mice were trained on morphine CPP (D1-4, 7 mg/kg) and (A) 24h or (D) 3 weeks later extinction was performed for 20d. (B-F) The total time spent in the DC or NDC over 20d for extinction of (B, C) young and (E, F) old reward memories in (B, E) Sham

and (C, F) IG-IR treatment groups. Two-way ANOVA, Sidak's post hoc analysis, ***P=0.001, **P=0.01, *P=0.05. Young memory extinction, n=9-11/group. Old memory extinction, n=14-15/group. Mean±SEM. DC=drug context, M=morphine, NDC=non-drug context, S=saline.

CHAPTER FOUR:

ACUTE AND FRACTIONATED EXPOSURE TO HIGH-LET ^{56}FE HZE-PARTICLE RADIATION BOTH RESULT IN SIMILAR LONG-TERM DEFICITS IN ADULT HIPPOCAMPAL NEUROGENESIS

Adapted from: ***Rivera PD**, *Shih H, LeBlanc JA, Cole MG, Amaral WZ, Ahn F, DeCarolis NA, Chen BPC, and Eisch AJ. Acute and Fractionated Exposure to High-LET 56Fe HZE Particle Radiation Both Result in Similar Long-Term Deficits in Adult Hippocampal Neurogenesis. Radiation Research. 2013, 6(180): p. 658-667.

Abstract

Astronauts on multi-year interplanetary missions will be exposed to a low, chronic dose of high-energy, high-charge particles. Studies in rodents show acute, non-fractionated exposure to these particles causes brain changes – like fewer adult-generated hippocampal neurons and stem cells – that may be detrimental to cognition and mood regulation, and thus compromise mission success. However, the influence of a low, chronic dose of these particles on neurogenesis and stem cells is unknown. To examine the influence of galactic cosmic radiation on neurogenesis, adult-generated stem and progenitor cells in Nestin-CreERT2/R26R-YFP transgenic mice were inducibly labeled to allow fate tracking. Mice were subsequently exposed Sham conditions, acute ^{56}Fe particle exposure of 100 cGyX1, or fractionated ^{56}Fe particle exposure of 20 cGyX5. Adult-generated hippocampal

neurons and stem cells were quantified 24 hours or 3 months later. Both Acute and Fractionated exposure decreased measures of proliferating cells and immature neurons relative to Sham. Unexpectedly, neither Acute nor Fractionated exposure decreased the number of adult neural stem cells relative to Sham. Our findings show that both a single exposure as well as fractionated exposure of particle radiation are similarly detrimental to adult-generated neurons. Implications for future missions and ground-based studies in space radiation are discussed.

Introduction

Astronauts travelling through interplanetary space will be exposed to a chronic low dose (~60 cGy per year) of galactic cosmic radiation (GCR) (Cucinotta and Wilson, 1994; Cucinotta et al., 2003; Vazquez, 1998; Zeitlin et al., 2013). GCR contains penetrating high-energy, high-charge nuclei (HZE) that are damaging to cells and tissues due to the inherent high-linear energy transfer (LET) properties these particles possess (Asaithamby and Chen, 2011; Zeitlin et al., 2013). For example, small mammals sent into space subsequently show deficits in their immune, hematopoietic, reproductive, and central nervous systems (CNS) (Gridley et al., 2003; Haymaker et al., 1975; Masini et al., 2012; Pecaut et al., 2003; Shvets and Portugalov, 1976). Experiments simulating GCR on Earth strongly suggest HZE particles themselves are damaging to these systems (Datta et al., 2012; Sapp et al., 1992; Vlkolinsky et al., 2007), particularly the CNS (Machida et al., 2010). However,

it is unclear whether the results from ground-based experiments (which are primarily done with a single acute exposure) reflect the chronic or “fractionated” exposure that would be experienced in space (Chang et al., 2007; Goldstein et al., 1981). Therefore, an important challenge for the field is to directly compare acute vs. fractionated whole-body exposure to high-LET particles. This would enable optimal translation of findings from ground-based HZE particle experiments into relevant risk assessment for spaceflight.

In regards to the CNS, evidence suggests high-LET particles encountered during spaceflight may damage brain regions critical for mission success. For example, ground-based HZE particle exposure (≥100 cGy) leads to short- and long-term deficits in hippocampal-dependent spatial learning (Manda et al., 2008; Shukitt-Hale et al., 2003; Shukitt-Hale, 2000). Even lower doses of HZE particles (10-100 cGy) lead to persistent deficits in hippocampal-dependent spatial learning (Britten et al., 2012) and the premature appearance of neurodegeneration in a mouse model of Alzheimer’s disease (Cherry et al., 2012). One cellular basis for these acute HZE particle-induced functional deficits is a reduction in adult hippocampal neurogenesis (Encinas et al., 2008; Manda et al., 2008; Rola et al., 2008; Rola et al., 2004; Rola et al., 2005; Rosi et al., 2012; Shukitt-Hale, 2000), a dynamic process where stem cells and neuroblasts/immature neurons give rise to fully mature neurons (Kempermann, 2003; Kempermann et al., 2004a). While the negative effects of acute HZE particle

exposure on neurogenesis are clear, it is unknown if and how adult neurogenesis is influenced by a fractionated exposure. Such information would be important as it would either mitigate or exacerbate the CNS risk assessment for extended missions in space.

The aim of this study was to determine how acute versus fractionated HZE particle exposure differentially influence adult hippocampal neurogenesis. Using transgenic mice in which the dynamic process of adult neurogenesis can be inducibly labeled and tracked (Lagace et al., 2007), hippocampal stem cells and adult-generated neurons were quantified at 24 h and 3 mo after an acute or fractionated whole-body exposure of 100 cGy of 56Fe. Based on prior studies comparing the effect of whole-body acute vs. fractionated exposure on other CNS and non-CNS measures (Chang et al., 2007; Goldstein et al., 1981), we hypothesized that fractionated exposure would be less damaging to adult hippocampal neurogenesis when compared to acute. However, here we report that both acute and fractionated exposure cause long-term decreases in the number of immature and mature adult-generated neurons, while neither change the number of putative hippocampal neural stem cells. These data highlight striking similarities between acute and fractionated HZE particle exposure and their influence on adult hippocampal neurogenesis, and therefore are important to consider for risk assessment for spaceflight on extended missions.

Methods

Animals

All animal procedures and husbandry were in accordance with the National Institutes of Health, Guide for the Care and Use of Laboratory Animals, and performed in IACUC-approved facilities at UT Southwestern Medical Center (UTSW) and Brookhaven National Laboratories' animal facility. Experiments were designed to minimize animal number, pain and suffering. Male and female Nestin-CreERT2/R26R-YFP mice were used (n=68; 34 male, 34 female) (Lagace et al., 2007). Mice were housed at UTSW (2-4/cage, 12-hour[h] light/dark cycle) with ad libitum access to food and water and moderate housing enrichment (Mouse Igloo®, Bio-Serv). To generate the mice for these studies, homozygous Nestin-CreERT2 transgenic mice on a C57BL/6 background ((Lagace et al., 2007); generated by the Eisch Laboratory and bred at UTSW, but also available via Jackson Laboratories, Stock #016261) were crossed with homozygous R26R-YFP knock-in mice (Jackson Laboratories, Stock #006148) to produce the heterozygous mice (Nestin-CreERT2 hemizygous, R26R-YFP heterozygous, designated Nestin-CreERT2/R26R-YFP) that were genotyped as previously described (Lagace et al., 2007).

Drug Treatment

To induce recombination and YFP expression in nestin-expressing stem cells and their progeny (Lagace et al., 2007), Nestin-CreERT2/R26R-YFP mice were given

tamoxifen (TAM, 150-180 mg/kg, i.p., 30mg/mL in 10% EtOH/sunflower oil, all from Sigma Aldrich) during young adulthood (5-6 weeks of age) for 5 days (d)(Lagace et al., 2007)(Fig. 1). To label proliferating cells in the S-phase of the cell cycle (Mandyam et al., 2007), all mice received the thymidine analog bromodeoxyuridine (BrdU, 150 mg/kg, i.p., 10 mg/mL in 0.9% saline, Boehringer Mannheim) 2 h prior to kill (Fig. 1)(Mandyam et al., 2007).

Particle Irradiation

Adult Nestin-CreERT2/R26R-YFP mice (8.6-11.7 weeks of age, 28-35 d post-TAM) were shipped from UTSW to Brookhaven National Laboratory (BNL; Upton, NY) and allowed to acclimate 4 d prior to irradiation (IR, Fig. 1). The reference number for this NASA-sponsored experimental campaign was NSRL-09C. The 5 d experiment began on November 2, 2009 by bringing mice from Brookhaven Laboratory Animal Facility (BLAF) to the NASA Space Radiation Laboratory (NSRL). For each of the 5 d of the experiment, mice in all groups were placed into IR “hotels” (polypropylene, 50 mL Conical Centrifuge Tubes, 30x115mm, Fisher Scientific, ~6-8 5mm air holes drilled throughout length of the tube) for ~20 min (Fig. 1, grey arrowheads) between 9-11 A.M. Mice were split across 3 groups (balanced by weight, birthdate, and sex) to receive different IR treatments (Fig. 1): “Sham” mice (n=21; 9 male, 12 female) received 5 exposures of 0 cGy; “Acute” mice (n=25; 11 male, 14 female) received 4 exposures of 0 cGy and 1 exposure of 100 cGy (5th day, Fig. 1, single black arrow);

“Fractionated” mice (n=22; 14 male, 8 female) received 5 exposures of 20 cGy (Fig. 1, 5 black arrows). The 56Fe26+ ion beam was produced by the AGS Booster Accelerator at BNL and transferred to the experimental beam line in the NSRL. IR at NSRL consisted of whole-body exposure to 56Fe26+ ions at 300 MeV/nucleon (56Fe, LET, 238.8 KeV/micron). A 56Fe beam was delivered at an average dose rate of 20 cGy/min for the Fractionated group and 100 cGy/min for the Acute group. Delivered doses were ±0.5% of the requested value.

Body Weight Measures and Tissue Collection

Weights were recorded for mice 1 month (mo) prior to IR, 24 h post-IR, and 3 mo post-IR. Per UTSW and BNL IACUC-approved policies, mice were euthanized by live decapitation 24 h or 3 mo post-IR, and brains were rapidly extracted. Brains were cut on the midsagittal sulcus and immersion-fixed in 4% paraformaldehyde (PFA). PFA was changed twice daily for 2 d before brains were placed in cryoprotectant (30% sucrose in 0.1 M PBS and 0.1% sodium azide, [NaN3]). After brains sank – an indicator of cryoprotection – brain hemispheres were sectioned coronally on a freezing microtome (Leica) at a thickness of 30μm through the entire longitudinal extent of the hippocampus plus immediately anterior and posterior regions (distance from Bregma -0.82 to -4.24) in a 1:9 series and stored in 0.1% NaN3 in 1XPBS at 4°C until processed (Arguello et al., 2008).

Immunohistochemistry (IHC)

IHC was performed generally as previously described (Arguello et al., 2008; Lagace et al., 2007). For staining, one hemisphere from the series of 1:9 sections was mounted onto glass slides (Superfrost/Plus, Fisher) in rostral to caudal order and allowed to dry for 2h. For IHC to visualize BrdU-immunoreactive (BrdU+) cells, pretreatment consisted of antigen retrieval (0.01M citric acid, pH 6.0, 95°C, 15min), membrane permeabilization (0.1% trypsin in 0.1M Tris and 0.1% CaCl2, 10min), DNA denaturation (2N hydrochloric acid in 1×PBS, 30min), and quenching of endogenous peroxidases (0.3% hydrogen peroxide in 1×PBS, 30min). For IHC for visualization of doublecortin (DCX)+ and yellow fluorescent protein (YFP)+ cells, pretreatment consisted of only antigen retrieval. For all IHC, nonspecific staining was blocked by incubation with 3% normal donkey serum (NDS) in 0.1% TritonX-100 in 1×PBS for 60min. Sections were incubated in their respective primary antibody (rat-α-BrdU, 1:400, Accurate; goat-α-DCX, 1:500, Santa Cruz; rabbit-α-GFP [used to detect YFP],1:3000, Invitrogen) overnight at room temperature in 3% NDS, 0.1% Tween-20 in 1×PBS. The following day, sections were incubated in their respective biotinylated antibody (donkey anti-rat, donkey anti-goat, or donkey anti-rabbit, 1:200 in 1.5% NDS in 1×PBS, Jackson ImmunoResearch) for 60 min followed by rinses and then avidin-biotin complex (ABC, 1:50, Vector Laboratories) for 60min. Staining of BrdU+ and DCX+ cells was visualized using 3,3-Diaminobenzidine (DAB, Thermo Scientific Pierce), and counterstained with Nuclear Fast Red (Vector Laboratories).

Staining of YFP+ cells was visualized with CY3-conjugated Tyramide Signal Amplification Systems (TSA, Perkin-Elmer) and counterstained with DAPI (1:5000, Roche Applied Science). Based on parameter optimization, the quenching (H2O2 step) was performed for BrdU staining after Trypsin and HCl steps; for DCX prior to secondary incubation; and for YFP between the secondary and ABC incubations. Tissue for all staining was then dehydrated and cover slipped (VWR, No.1.5, 24x60mm) with DPX Mountant (Sigma-Aldrich).

Cell Quantification and Subregional Volume Determination

Using an Olympus BX-51 microscope (Tokyo, Japan), BrdU+, YFP+, and DCX+ cells were quantified using stereological principles. BrdU+ and YFP+ cells are rare populations, and therefore were assessed stereologically via exhaustive counting of cells in the subgranular zone (SGZ) of the hippocampus at 400X (NA=0.90), as previously described (Lagace et al., 2010; Lagace et al., 2007). Briefly, stereological parameters of BrdU+ and YFP+ cells used a section sampling fraction of 1/9, an area sampling fraction set to 1 to account for rare populations of cells, and a height sampling fraction of 1 (Decarolis et al., 2013; Lagace et al., 2010). The raw counts were multiplied by 18 to estimate the total number of BrdU+ or YFP+ cells in the entire SGZ of both hemispheres. The SGZ was defined as the inner half of the dentate gyrus granule cell layer (GCL) and two cell-widths into the hilus (Eisch and Harburg, 2006). BrdU+ cells presented darkly-stained or punctated irregular shaped

nuclei (Fig. 2A). Total YFP+ cells were quantified, but YFP+ Type-1 cells were also quantified based on their irregularly shaped soma and a characteristic central meandering projection with many branching processes, and fine filigree-tufted ends of the molecular-layer-leading process (Fig. 4A, boxed (Ables et al., 2010; Decarolis et al., 2013)). DCX+ cells were quantified via Stereo-Investigator software (MBF Bioscience, Williston, VT) at 400X (NA=0.70) using the optical fractionator. The section sampling fraction was 1/18 (grid size: 100 x 100, frame size: 80 x 80, estimated mean Schmitz-Hoff CE values were between 0.09 and 0.16 with an averaged group mean of 0.12 (Peterson, 2010), modified from (Klempin et al., 2012). Raw DCX+ counts were multiplied by 2 to give the total number of DCX+ cells in the SGZ.

Subregional volume determination was made for three discrete regions of the dentate gyrus (DG): Hilus, GCL, and Molecular Layer (Mol). The volume of each was calculated using Cavallieri's principle within Bregma -0.82 to -4.24 using Stereo-Investigator software (Gundersen and Jensen, 1987; Lagace et al., 2007). The Hilus was defined as the region within the infra- and suprapyramidal blades of the GCL and enclosed at the caudal ends of the pyramidal blades (Fig. 5A). The Mol was defined as the area between the GCL and the hippocampal fissure. Pilot experiments revealed that reliable and repeatable Mol measurements were achieved by enclosing the Mol dorsal to the suprapyramidal blade via connecting the most

caudal point of the suprapyramidal blade to the hippocampal fissure (Fig. 5A). After collecting Hilus, GCL, Mol volumes, the volumes were summed to generate the DG volume. Images were imported into Photoshop (Adobe Systems) and adjustments were made using the “level” function, in accordance with published guidelines for image presentation (Rossner and Yamada, 2004).

Statistical Analyses

Data are reported as mean±SEM for Sham, Acute, and Fractionated groups. Statistical analyses were performed using a two-way analysis of variance (ANOVA) followed by a Bonferroni’s post-hoc (brain region volume) or one-way ANOVA followed by a Tukey’s multiple comparison post-hoc test (neurogenesis measures). All statistical analyses were performed using Prism GraphPad (version 5.0d) software. Statistical significance was defined as $p<0.05$, but values of $p<0.01$ and $p<0.001$ are shown as well to indicate level of confidence.

Results

Acute and Fractionated Irradiation both Result in Fewer Proliferating (BrdU+) Cells 24 h and 3 Months after Exposure

Nestin-CreERT2/R26R-YFP mice in the Sham, Acute, and Fractionated groups were in good health throughout the experiment, gaining weight at a normal rate at the short time point (24 h) post-IR. Relative to Acute and Sham, Fractionated mice

gained more weight at the long time point (3 mo) post-IR, but neither Acute or Fractionated were significantly different from Sham when expressed as percent Sham (data not shown). To examine the influence of 56Fe particle irradiation on the number of proliferating hippocampal cells, mice received BrdU 2 h prior to sacrifice 24 h and 3 mo post-IR (Fig. 1)(Kee et al., 2002). BrdU+ cells were easily identified in the hippocampal SGZ of all mice, presenting typical darkly-stained nuclei with immunoreactive puncta (Fig. 2A inset). At both the 24 h and 3 mo time points, there was a significant effect of treatment on BrdU+ SGZ cell number (24 h: $F(2,29)=103.6$, $p<0.001$; 3 mo: $F(2,27)=13.49$, $p<0.001$). At the 24 h time point post-IR (Fig. 2B), post-hoc analysis revealed that mice in both Acute and Fractionated groups had fewer BrdU+ SGZ cells when compared to Sham (Acute vs. Sham: M=3118, 95% CI [2559, 3678], $p<0.001$; Fractionated vs. Sham: M=2455, 95% CI [1866, 3043], $p<0.001$). Acute and Fractionated groups were also significantly different from each other (M=-663.8, 95% CI [-1239, -88.22], $p<0.05$), with a greater decrease seen in Acute (74%) than in the Fractionated group (58%) when compared to Sham. At the 3 mo time point (Fig. 2C), post-hoc analysis revealed that mice in both Acute and Fractionated groups had fewer total BrdU+ cells when compared to mice in the Sham group (Acute vs. Sham: M=730.0, 95% CI [375.0, 1085], $p<0.001$; Fractionated vs. Sham: M=570.2, 95% CI [161.3, 979.1], $p<0.01$). At the 3 mo time point, there were moderately fewer BrdU+ cells in the Acute (46%) group than the Fractionate group (36%) compared to Sham; however, Acute and Fractionated

groups were not significantly different from each other (M=-159.8, 95% CI [-530.2, 210.6], p>0.05). Therefore, relative to Sham, both Acute and Fractionated 56Fe result in immediate and long-term deficits in the number of proliferating cells in the hippocampal SGZ.

Acute and Fractionated 56Fe-Particle Exposures both Result in Fewer Neuroblasts/Immature Neurons (DCX+) Cells 24 h and 3 Months Post-irradiation

While a decrease of proliferating SGZ cells is often reflective of a decrease in adult-generated neurons, the processes of proliferation and neurogenesis/survival can also be differentially regulated (Thomas et al., 2007). Therefore, we also quantified the number of DCX+ cells (Fig. 3), a widely used measure of neurogenesis levels (Couillard-Despres et al., 2005). DCX+ cells were evident in the SGZ and GCL of all mice, presenting their characteristic range of morphologies indicative of mitotic neuroblasts and post-mitotic immature neurons (Fig. 3A (Plumpe et al., 2006)). Similar to the BrdU results, at both 24 h and 3 mo post-IR there was a significant effect of treatment on the number of DCX+ GCL cells (24 h: Fig. 3B, $F(2,23)=29.54$, $p<0.001$; 3 mo: Fig. 3C, $F(2,26)=12.43$, $p<0.001$). At the 24 h time point post-IR (Fig. 3B), post-hoc analysis revealed that mice in both Acute and Fractionated groups had fewer DCX+ GCL cells when compared to Sham (Acute vs. Sham: M=23113, 95% CI [13661, 32566], $p<0.001$; Fractionated vs. Sham: M=29271, 95% CI [19018, 39523], $p<0.001$). Further post-hoc analysis revealed that while there appeared to

be a greater decrease in Fractionated (54%) than in Acute group (43%) when compared to Sham, Acute and Fractionated groups were not significantly different from each other (M=6157, 95% CI [-3295, 15609], p>0.05). At the 3 mo time point post-IR (Fig. 3C), post-hoc analysis revealed that mice in both Acute and Fractionated groups had fewer DCX+ cells when compared to Sham (Acute vs. Sham: M=6970, 95% CI [3275, 10664], p<0.001; Fractionated vs. Sham: M=6190, 95% CI [2048, 10333], p<0.01). While there appeared to be a slightly greater decrease in Fractionated (39%) than in Acute group (44%) when compared to Sham, further post-hoc analysis revealed that Acute and Fractionated groups were not significantly different from each other (M=-779.5, 95% CI [-4754, 3195], p>0.05). Therefore, relative to Sham, both Acute and Fractionated 56Fe particle IR result in immediate and long-term deficits in the number of immature neurons in the hippocampal GCL.

Acute and Fractionated 56Fe-Particle Exposures both Result in Fewer YFP+ Cells but Not Type-1 YFP+ Cells 24 h and 3 Months Post-irradiation

The decreases in BrdU+ and DCX+ cell number (Figs. 2,3) suggested an IR-induced decrease in adult hippocampal neurogenesis. However, BrdU and DCX IHC analyses alone do not allow assessment of the dynamic process of neurogenesis. In addition, neither BrdU nor DCX label the putative local source of adult-generated progenitors and neurons, the Type-1 neural stem cell (Lagace et al., 2007).

Therefore, to gain more clarity on how 56Fe particle irradiation influences neurogenesis, we examined the number of YFP+ cells in our TAM-injected Nestin-CreERT2/R26R-YFP mice.

As previously shown (Lagace et al., 2007), TAM induced YFP labeling of Type-1 cells with radial glial morphology in the SGZ as well as the derivative progenitors and mature granule neurons (Fig. 4A). Stereological quantification of total YFP+ cell number revealed that, as with the BrdU+ and DCX+ cell counts, there was a significant effect of treatment on the number of total YFP+ cells (24 h: Fig. 4B, $F(2,25)=5.712$, $p<0.01$; 3 mo: Fig. 4C, $F(2,30)=4.213$, $p<0.05$). At the 24 h time point post-IR (Fig. 4B), post-hoc analysis revealed that mice in both Acute and Fractionated groups had fewer total YFP+ cells in the SGZ when compared to Sham (Acute vs. Sham: M=4385, 95% CI [585, 8185], $p<0.05$; Fractionated vs. Sham: M=4800, 95% CI [914.2, 8686], $p<0.05$), with a similar level of decrease seen in both Acute (47%) and Fractionated groups (52%) compared to Sham (M=415.2, 95% CI [-3128, 3958], $p>0.05$). At the 3 mo time point post-IR (Fig. 4C), Acute and Fractionated groups also had fewer total YFP+ cells in the SGZ when compared to Sham (Acute vs. Sham: M=3580, 95% CI [138.6, 7020], $p<0.05$; Fractionated vs. Sham: M=3667, 95% CI [81.43, 7252], $p<0.05$), with a similar level of decrease seen in both Acute (40%) and Fractionated groups (41%) compared to Sham (M=87.30, 95% CI [-3254, 3428], $p>0.05$).

Total YFP+ cell number in the Nestin-CreERT2/R26R-YFP mouse is often used as the initial indicator of the influence of a manipulation on neurogenesis (Ables et al., 2010). Further determination of Type-1 cell number directly via stereology allows direct assessment of the influence of 56Fe particles on the nestin-lineage neural stem cells (Lagace et al., 2007). The YFP+ Type-1 cells can be identified via their characteristic irregularly-shaped soma, distinctive meandering and branching process, and fine filigree-tufted ends of the molecular-layer-leading process (Fig. 4A, boxed). There was no significant effect of IR on Type-1 YFP+ cell number at either the 24 h (Fig. 4D) or 3 mo time points post-IR (Fig. 4E; 24 h: $F(2,23)=2.829$, $p>0.05$; 3 mo: $F(2,30)=0.529$, $p>0.05$). Thus, relative to Sham, both Acute and Fractionated 56Fe particle IR results in immediate and long-term deficits in the total number of YFP+ cells, but not the Type-1 YFP+ cells in the hippocampal SGZ.

Neither Acute nor Fractionated 56Fe-Particle Exposures Result in Changes in Dentate Gyrus or Hippocampal Volume 3 Months Post-irradiation

One potential reason for the significantly decreased indices of neurogenesis seen in Acute and Fractionated mice is that IR might decrease the overall size of the hippocampus. To address this, we stereologically quantified the volume of hippocampal subregions (Mol, GCL, H, and DG; Fig. 5A) at the 3 mo time point post-IR (Fig. 5B). As expected given the different volumes of these subregions (Fig. 5A),

there was a main effect of subregion ($F(3,55)=2220.89$, $p<0.001$), with the DG and Mol being larger than the GCL and H. However, there was no effect of treatment ($F(2,55)=2.16$, $p>0.05$) or interaction of treatment and subregion ($F(6,55)=0.58$, $p>0.05$). These data show that Acute and Fractionated IR had no effect on hippocampal subregional volume.

Discussion

Ground-based studies widely support that acute HZE particle exposure damages the CNS, leading to cellular and structural deficits (e.g. decreased adult hippocampal neurogenesis) and functional deficits (e.g. diminished performance on hippocampal-dependent tasks). These changes underscore the potential risk of HZE particle exposure to mission success (Cucinotta et al., 2009; Rola et al., 2004; Shukitt-Hale et al., 2000). However, prior to our study presented here, it was unclear if fractionated and acute exposure to HZE particles had similar deleterious effects on adult hippocampal neurogenesis. Fractionated exposure is presumed to more closely mimic the chronic low exposure that astronauts receive in space (Schimmerling, 2010). Therefore, this present study was designed to directly compare acute and fractionated HZE particle exposure in an effort to clarify the risk of short- and long-term space flight in regards to adult hippocampal neurogenesis. Using an inducible transgenic mouse model to label nestin-expressing stem cells and their progeny, we demonstrate that both acute (e.g. non-fractionated) and

fractionated 56Fe particle exposure produce similar negative effects on adult hippocampal neurogenesis. Specifically, both acute and fractionated HZE particle exposure decreased the number of progenitors and neuroblast/immature neurons in the adult mouse hippocampus. In contrast to the negative effects on these later stages of adult hippocampal neurogenesis, neither acute nor fractionated HZE particle exposure changed the number of putative hippocampal neural stem cells. As discussed below, our acute findings are generally consistent with prior work, with the intriguing difference that here we show no decrease in the number of adult neural stem cells. In addition, our fractionated findings are highly novel, showing robust short- and long-term decreases in adult hippocampal neurogenesis. Taken together, these data provide evidence that ground-based studies in space radiation using acute and fractionated HZE particle exposure are similarly detrimental, at least in regards to adult hippocampal neurogenesis.

Decreased Adult Hippocampal Neurogenesis after Acute or Fractionated 56Fe-Particle Exposure

Our data on acute 56Fe particle exposure have similarities to previously published data. For example, our data are consistent with the literature that 56Fe particle exposure decreases adult hippocampal neurogenesis (Encinas et al., 2008; Manda et al., 2008; Rola et al., 2008; Rola et al., 2004; Rola et al., 2005; Rosi et al., 2012). Methodological differences make it difficult to make precise comparisons in the

magnitude of the effects presented here versus those in prior results. However, even with these differences it is useful to note that both our stereological assessment here (seen after exposure to 100 cGy, 300 MeV/n 56Fe, LET=238 keV/μM) and prior results with quantitative but non-stereological assessment (1000 MeV/n 56Fe, LET=148 keV/μM (Rola et al., 2004; Rola et al., 2005)) show ~40% decrease in DCX+ cells in the SGZ 3 mo post-IR. Therefore, a central consistency of our work with prior work is confirmation that acute exposure to 56Fe results in relatively long term decreases in adult hippocampal neurogenesis. These may indeed contribute to the hippocampal functional deficits seen in various models (Britten et al., 2012; Cherry et al., 2012; Higuchi et al., 2002; Manda et al., 2008; Shukitt-Hale et al., 2003; Shukitt-Hale et al., 2000).

In addition to this confirmation that acute 56Fe exposure decreases adult hippocampal neurogenesis, our data also provide several novel insights into the effects of fractionated 56Fe particle exposure. Specifically, our work shows that both acute and fractionated 56Fe exposure result in 40-50% in DCX+ cell number 24 h and 3 mo post-IR. It is interesting to consider our results using a Poisson distribution, which gives the probability a cell will be hit at a given fluence (Nelson, 2003). For example, the estimated average hits a neuron (10μm diameter) would receive in our Acute and Fractionated groups are 2.0 and 0.4 per exposure, respectively. This can be interpreted to mean that a neuron on average is hit twice

with one exposure (Acute) or a neuron is hit 0.4 times per day over the course of 5 days of exposure (Fractionated). Although the overall total number of HZE particles that hit a neuron is predicted to be the same (2 hits per neuron after 5 days) in both Acute and Fractioned groups, in the Fractioned 56Fe group there may be a time interval between 2 different hits in the same neuron. The interval between fractions presumably allows DNA repair and possible recovery from radiation-induced damage, as observed after low LET radiation exposure. Here our results demonstrate that 56Fe particle exposure – irrespective of acute or protracted fractionated exposure – results in a long lasting decrease in the process of adult hippocampal neurogenesis (Figs. 2, 3). Hence, fractioned 56Fe particle exposure is likely equally damaging as acute 56Fe particle exposure, suggesting minimum repair activity could be carried out during the short interval between fractions. It agrees with the general notion that HZE particle induced DNA lesions are difficult or impossible to be repaired properly (Asaithamby and Chen, 2011; Asaithamby et al., 2008; Groesser et al., 2011).

Neural Stem Cell Number Is Unchanged after Acute or Fractionated 56Fe-Particle Exposure

While there is an equivalent loss of immature neurons and neuroblasts after both acute and fractionated irradiation (Fig. 3B, 3C), there is no change in stem cell number after either acute or fractioned irradiation (Fig. 4D, 4E). This is intriguing, as

an intact neural stem cell pool is thought to be an ongoing source of new neurons throughout life (Ables et al., 2010; Bonaguidi et al., 2011; Kempermann et al., 2004b). Therefore, the maintenance of the stem cell pool we show here suggests that the irradiated brain still maintains the potential to regenerate the lost adult-generated neurons.

An important difference between our work and the published literature is that we report no decrease in the number of neural stem cells in the SGZ after acute or fractionated exposure. This differs from published work showing that acute 100 cGy 56Fe particle radiation leads to a 45% reduction in the number of the quiescent neural stem cells in the SGZ (Encinas et al., 2008). Both our work and this prior work share similar design, including the irradiation of 2-mon old mice, and both use transgenic mice to label nestin-expressing stem cells in DG. However, there are key differences. For example, Encinas et al. used head-only exposure, while we used whole-body exposure. It is possible that head-only exposure induces a distinct physiological response than whole-body exposure, leading to an immune response that diminishes the adult neural stem cell population (Pecaut and Gridley, 2011; Rola et al., 2008; Ziv et al., 2006), a concept that warrants future study.

There are other important differences between Encinas et al. and our study. For example, Encinas et al. used a lower LET 56Fe particle (1000 MeV/n, LET=148 keV/μM), examined only a short 24 h time point, used a constitutive nestin “reporter”

mouse coupled with immunostaining to identify and quantify certain cell types (e.g. neural stem cells and transiently amplifying progenitor cells), and employed a 24 h delay between BrdU and kill. In contrast, our study used a higher LET 56Fe particle (300 MeV/n, LET=238 keV/μM), evaluated both 24 h and 3 mo time points post-IR, used an inducible nestin transgenic mouse to examine stem cells and their progeny using YFP+ immunostaining and morphological analysis, used immunostaining for markers of stages of neurogenesis (e.g. DCX to infer neurogenesis), and employed a 2 h delay between BrdU and kill. These differences make it challenging to directly compare the studies. Our study assesses a broader span of the dynamic process of neurogenesis, since we assess DCX+ cell number (Couillard-Despres et al., 2005) and proliferating cells by exogenous S-phase marker BrdU, as well as the number of YFP+ stem cells and their YFP+ progeny. However, our results may be swayed by the fact that although mice were “adult” at time of irradiation, our mice were young adult (5-6 weeks of age) at the time that recombination was induced. Regardless, a point of agreement in both studies is that 56Fe particle exposure decreases the number of dividing SGZ cells. Aside from this common result, additional studies are needed to assess which of these individual or combined experimental and methodological differences contribute to the maintenance of adult neural stem cell number reported here.

Conclusions, Limitations and Future Directions

Our data show robust short- and long-term decrease in adult hippocampal neurogenesis after acute or fractionated 56Fe HZE particle exposure. Thus, these data provide a direct comparison of how acute vs. fractionated exposure influence a cellular measure linked to hippocampal function. As such, these cellular data support the appropriateness of using ground-based acute-only exposure to assess CNS risk in astronauts during long-term interplanetary space travel. These data are also important as they represent the first step towards modeling the influence of a very low dose rate on hippocampal neurogenesis. However they are only the first step. Below we discuss the limitations and future directions of this work in the hope that they will be considered in regards to future experiments.

For example, one limitation of our study is the dose rate used and duration of exposure. Our dose rate (20 cGy/day) and duration (5 days) are 100X higher and 40X shorter than what is estimated for a mission to Mars (~0.2 cGy/day, 200 days) (Zeitlin et al., 2013). While our dose rate and duration were close to the limits currently feasible with ground-based simulation of GCR, future studies should utilize an even lower dose rate and even longer duration when possible. It is difficult to predict what influence lower dose rates would have on the CNS, but published works using low acute doses provide some insight. For example, some work already shows a negative influence of acute 20 cGy exposure on CNS function (Britten et al., 2012;

Lonart et al., 2012), suggesting even low acute doses can be damaging. However, other work comparing acute exposure of 10 vs. 100 cGy (Cherry et al., 2012) or 5 vs. 50 and 100 cGy (Poulose et al., 2011) reveal mixed results; low and high doses cause equal deficits in some behavioral and/or cellular assays, but the low doses do not cause deficits in other measures (Cherry et al., 2012; Poulose et al., 2011). Based on these CNS data and other non-CNS data showing a sparing effect after fractionation (Chang et al., 2007; Goldstein et al., 1981), one might hypothesize that low dose rates may not be as detrimental as higher dose rates. However, it is also possible that low dose rates over a longer period would be more detrimental as the cells have less time to recover between exposures. Finally, it is possible that the repeated exposure at a low dose rate has some stimulatory influence, resulting in a positive influence on some cellular or behavioral measures (Amundson et al., 2003). Clearly more work with low dose rates (0.2 cGy/day) and longer durations (minimum 5 days) is warranted.

Another limitation of the present study – and many other studies of “adult” neurogenesis – is the age of the mice at the time of recombination (5-6 weeks of age) and irradiation (~9-11 weeks of age). Our study spans a dynamic period of hippocampal neurogenesis, when levels of neurogenesis are decreasing rapidly to reach a lower steady state by 2 months of age (Amrein et al., 2011; Gilley et al., 2011). Focus on this developmental period was useful for this first study, as it

matched the age of HZE particle irradiation in other studies (Encinas et al., 2008), thus allowing comparison across publications. However, it would be more relevant for spaceflight to use older mice whose age is a rough equivalent of that of astronauts (6 mo old mouse is roughly equivalent to a 30 year old human (Flurkey et al., 2007)). This is particularly pertinent in regards to our data showing maintenance of adult neural stem cell number after 56Fe particle exposure (i.e. no change in adult neural stem cell number between Sham and IR groups was observed 24 h and 3 mo post-IR). The persistence of the Type-1 stem cell pool suggests that the irradiated hippocampus may have the potential for regeneration of adult neurogenesis (Bonaguidi et al., 2012). However, as stem cell characteristics change with age (Encinas et al., 2011; Encinas and Sierra, 2012), additional studies are warranted to assess the “functionality” of the stem cell pool at even longer time points post-IR. In addition, given the existence of numerous sources of neural stem cells in the adult mouse SGZ (Decarolis et al., 2013), future studies would also ideally utilize different inducible transgenic models to label and track stem cells arising from discrete sources to assess their contribution to adult neurogenesis in both young and older animals. The influence of age and time post-IR is particularly important to assess in future studies exploring the mechanisms underlying decreased neurogenesis, as the influence of irradiation on the neurogenic milieu, including the vasculature, likely changes over time (Mao et al., 2010; Rola et al., 2005).

Our findings show that a single exposure, as well as fractionated exposure, of particle radiation are both similarly detrimental to the dynamic process of adult hippocampal neurogenesis. Loss of neurogenesis is often linked to deficits in learning, memory, and mood regulation (Zhao et al., 2008). However, the influence of altered neurogenesis on hippocampal function is also state-dependent (Eisch and Petrik, 2012); for example, the requirement of neurogenesis for mood regulation is only seen under conditions of stress. Therefore, it would be of great utility to assess whether the loss of adult neurogenesis reported here results in functional deficits in hippocampal-dependent tasks, and to further assess whether the state-dependency of hippocampal function on neurogenesis is influenced by particle radiation.

There are two final points to make in regards to future directions. First, while the hippocampus is a brain structure controlling such mission-critical functions such as contextual memory and mood control, the hippocampus is less involved in other cognitive domains like executive function. Thus, another future direction is to place our work simulating the influence of GCR on hippocampal adult neurogenesis into a broader context of how the GCR influences the function of the cortex and other brain regions. Second, GCR is only one of the potentially detrimental stimuli evident during spaceflight that we can mimic in ground-based studies. Thus, when it is feasible, it would be compelling to assess the influence of the totality of the spaceflight experience or minimally combined factors (like GCR, microgravity, and

stress) on CNS structure and function. Such work will greatly help NASA plan for and achieve successful completion of future deep space missions.

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Figures

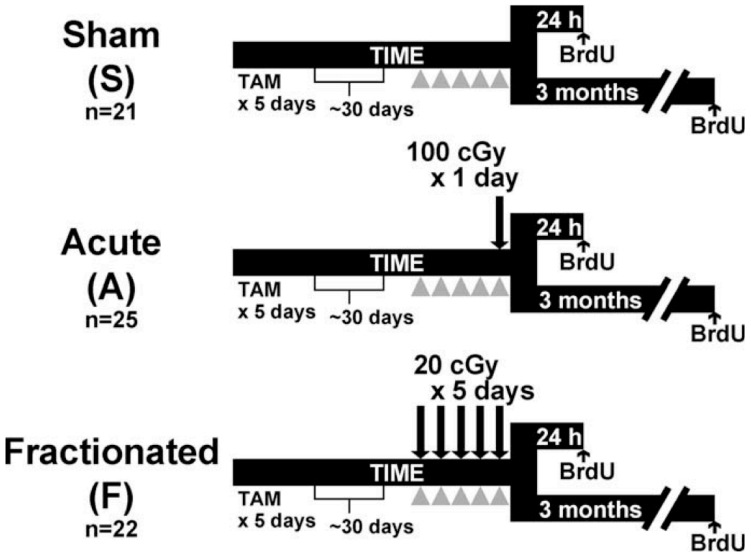

FIGURE 4-1. Time line of experiment and schematic of experimental groups. Nestin-CreERT2/R26R-YFP mice received Tamoxifen (TAM) for 5d at ~5-6 weeks of age, ~30d prior irradiation (IR). During the 5d experiment at Brookhaven National Laboratories/NSRL, mice in the Sham (S, n=21), Acute (A, n=25), and Fractionated (F, n=22) groups were placed in IR chambers for equivalent periods of time (grey arrowheads). Sham mice did not receive IR while in IR chambers. Mice in the Acute group received one exposure of 100 cGy on day 5 (single black arrow), and mice in the Fractionated group received 20 cGy on days 1-5 (five black arrows). All groups received BrdU 2 h prior to sacrifice, either at 24 h or 3 mo time points post-IR.

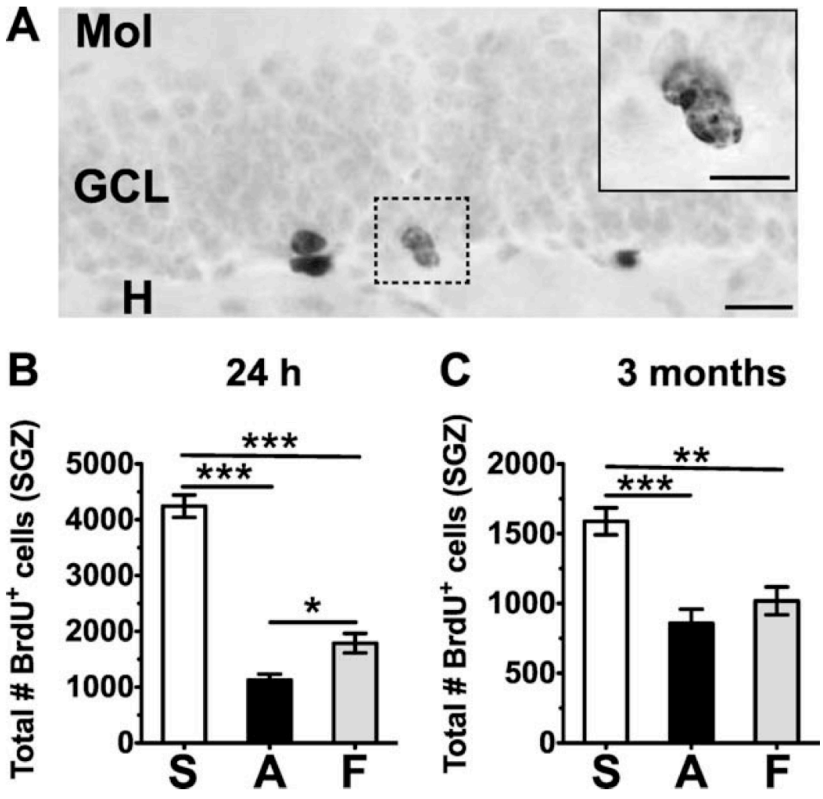

FIGURE 4-2. Acute and fractionated 56Fe-particle exposure result in fewer proliferating (BrdU+) cells at 24 h and 3 months postirradiation compared to sham exposure.

(A) Photomicrograph of BrdU+ cells in the SGZ (400X). Inset: cluster of BrdU+ cells (1000X) seen at lower power in dotted box (400X). Scale bar: (A) 20 μm; inset 10 μm. (B-C) Stereological quantification of BrdU+ cells at 24 h (B) or 3 mo (C) post-IR. GCL: Granule cell layer, H: Hilus, Mol: Molecular layer, SGZ: Subgranular zone. *p<0.05, **p<0.01, ***p<0.001; n=7-13/group.

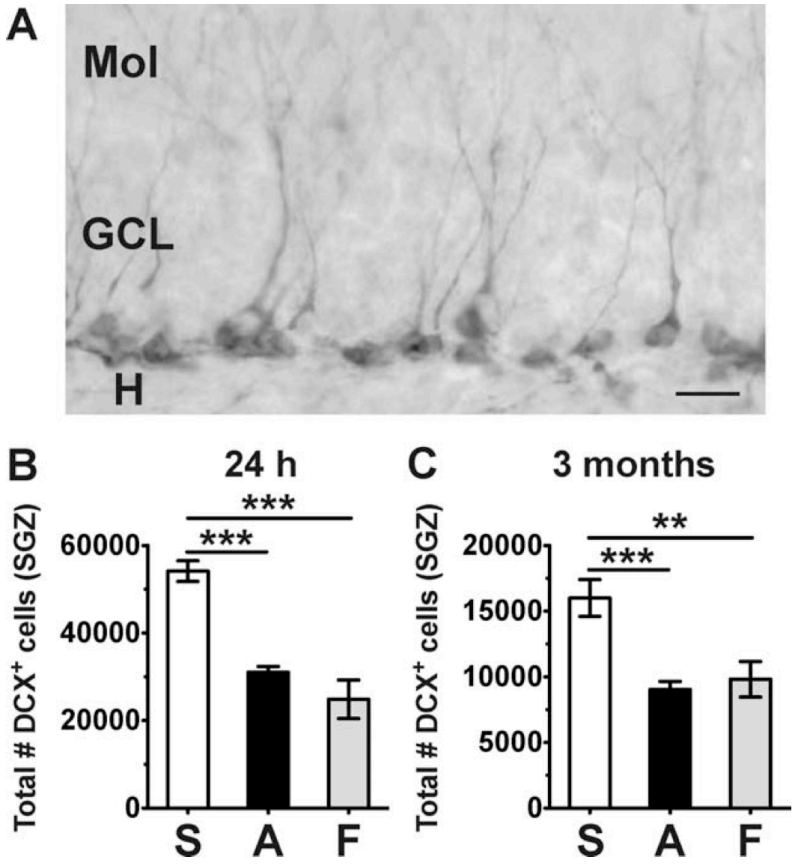

FIGURE 4-3. Acute and fractionated 56Fe-particle exposure result in fewer neuroblasts/immature neurons (DCX+) cells at 24 h and 3 months postirradiation compared to sham exposure.

(A) Photomicrograph of DCX+ cells in the SGZ extending their processes through the SGZ into the Mol (400X). Scale bar: (A) 20μm. (B-C) Stereological quantification of DCX+ cells at short (24 h, B) or long (3 mo, C) time points post-IR. GCL: Granule cell layer, H: Hilus, Mol: Molecular layer, SGZ: Subgranular zone. *p<0.05, **p<0.01, ***p<0.001; n=6-12/group.

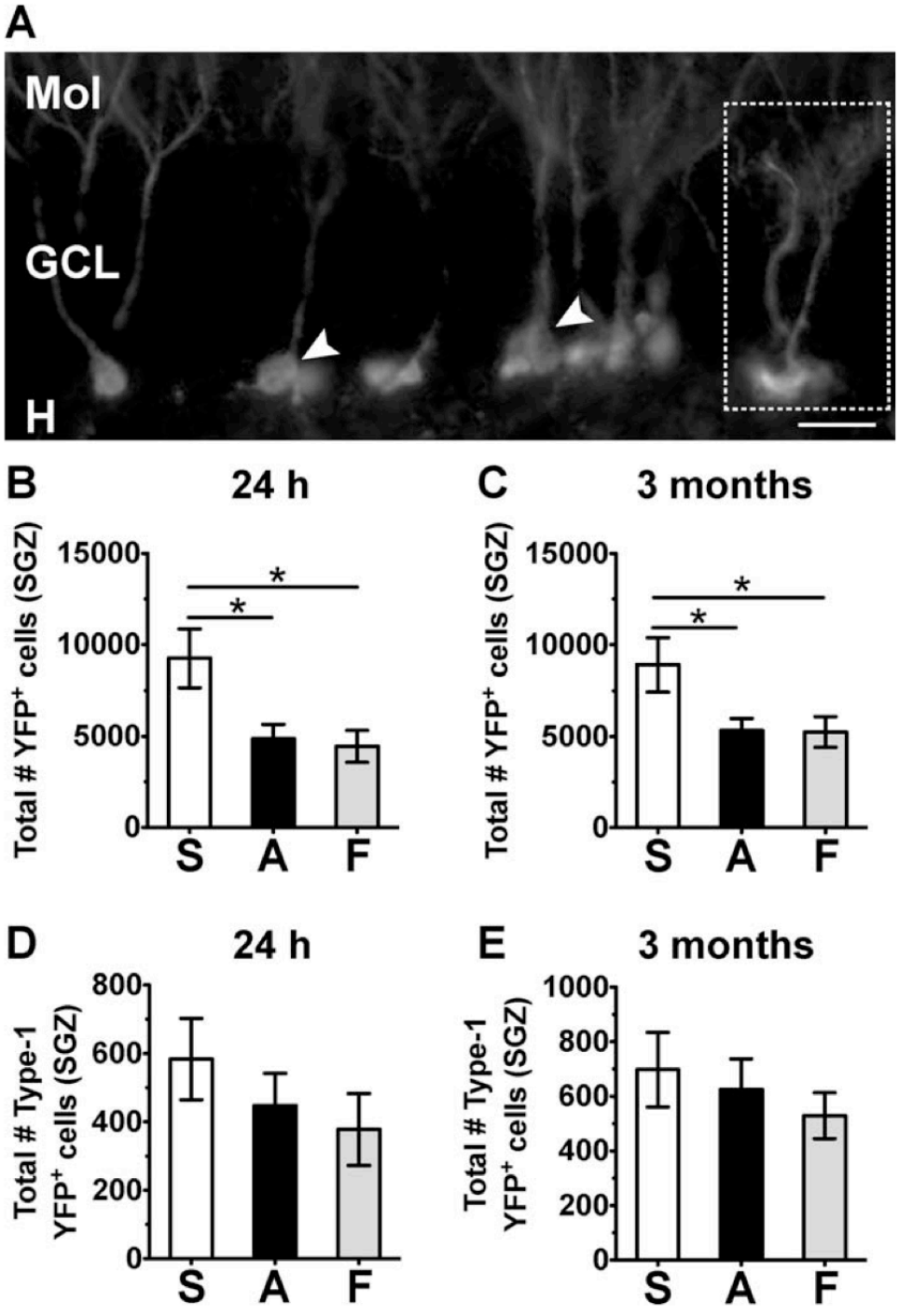
A
Mol
GCL
H
B
24 h
Total # YFP+ cells (SGZ)
15000
10000
5000
0
S
A
F
*
*
C
3 months
Total # YFP+ cells (SGZ)
15000
10000
5000
0
S
A
F
*
*
D
24 h
Total # Type-1 YFP+ cells (SGZ)
800
600
400
200
0
S
A
F
E
3 months
Total # Type-1 YFP+ cells (SGZ)
1000
800
600
400
200
0
S
A
F

FIGURE 4-4. Acute and fractionated 56Fe-particle exposure result in fewer YFP+ cells but not Type-1 YFP+ cells.

(A) Photomicrograph of YFP+ cells in the GCL of a Sham mouse 24 h post-IR (400X). White arrowheads: YFP+ cells with the morphology of mature GCL neurons. Boxed: Two YFP+ cells with the morphology of a Type-1 (1000X). Scale bar: (A) 50 μm. (B-D) Stereological quantification of YFP+ cells (B-C) and YFP+ Type-1 cells (D-E) at 24 h (B, D) and 3 mo (C, E) time points post-IR. GCL: Granule cell layer, H: Hilus, Mol: Molecular layer, SGZ: Subgranular zone. *$p<0.05$; n=7-12/group.

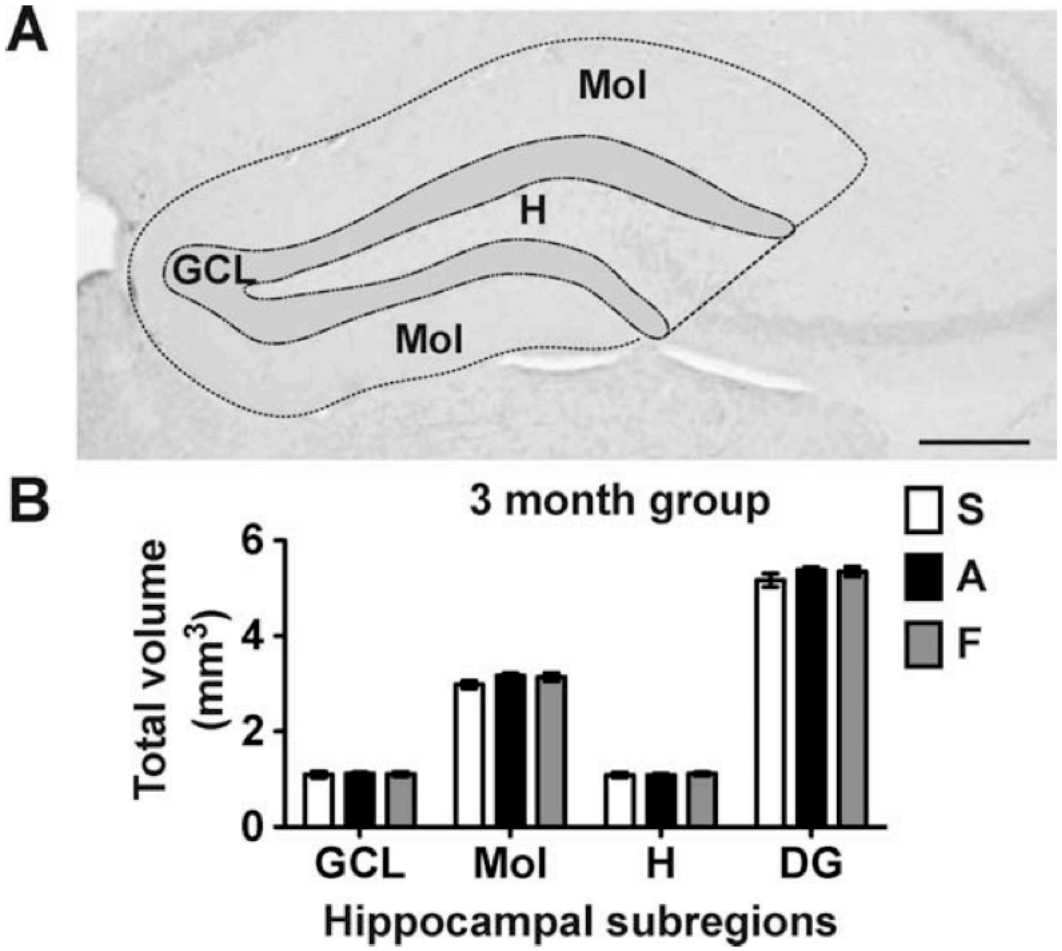

FIGURE 4-5. 56Fe-particle irradiation has no effect on hippocampal volume 3 months after exposure compared to Sham exposure.

(A) Photomicrograph of the hippocampus (400X) depicting the dentate gyrus subregional analysis used for volume analysis via stereology. (B) Stereological analysis of volume in hippocampal dentate gyrus subregions at 3mo (n=4-6/experimental condition). GCL: Granule cell layer, H: Hilus, Mol: Molecular layer.

CHAPTER FIVE:

CONCLUSIONS AND FUTURE DIRECTIONS

Overview

The hippocampal DG and SGZ regions are believed to contribute to learning and memory mechanisms. Understanding the neural basis of learning and memory could help in a wide range of situations, from helping addicts break the cycle of substance abuse to ensuring appropriate astronaut action during spaceflight missions. In regards to the field of addiction, this dissertation expands on previous work showing the role of the DG in learning and memory mechanisms of drug-associated reward memories, which is possibly mediated by adult neurogenesis. We show that the DG can become activated after re-exposure to a drug-associated context. We are the first to show that cranial irradiation diminishes extinction of young drug-associated reward memories and the reinstatement of older memories compared to sham controls. In regards to field of space radiation, this dissertation expands on how ground-based simulated space radiation effects adult neurogenesis. We are the first to report a fractionated study of HZE particle radiation in the CNS and found that both acute and fractionated ground-based HZE particle irradiation result in similar long-term deficits in adult hippocampal neurogenesis. Taken together, the data from this dissertation suggest DG neurocircuitry is mediated by adult neurogenesis which

may have a novel role in learning and memory mechanisms of reward memory and that a new exposure paradigm can decrease adult neurogenesis. We will review the major points, discuss the implications, and provide future direction experiments for each chapter.

Chapter 2. Retrieval of Drug/Context Association After Sequestration to Drug Context Activates Dentate Gyrus Granule Cell Neurons.

Research has used the behavioral task conditioned place preference (CPP) to examine the learning and memory mechanisms of forming and maintaining drug/context associations. However, to date, it has only been presumed that CPP makes this association. Therefore, to determine if CPP enables drug/context associations, we developed our morphine CPP experiments to examine the influence of 1) full access of the CPP box and 2) sequestration to the saline- or drug-paired context on DG cellular activation. We show data that suggest that drug/context associations are made within the DG using both full access or sequestration paradigms of morphine CPP. Further more, in the sequestration CPP paradigm, only mice that received morphine and were sequestered to the drug-paired context showed increased activation of DG neurons, compared to saline controls. While this work correlatively shows that CPP can establish drug/context associations in the DG, future causative experiments are needed

and are discussed below.

Future Directions, Chapter 2

The Role of Adult Neurogenesis in Drug/Context Associations: What is the contribution of adult neurogenesis on the establishing a drug-associated context reward memory? There are several experiments described below that correlatively address the aforementioned question, as well as correlative and causative future directions that should be considered. Previously published data suggest that both adult- and embryonic-generated DG neurons can become activated after learning and memory behaviors such as MWM and CFC (Ge et al., 2008; Kee et al., 2007; Stone et al., 2011). Therefore, we examined if adult-born granule cells (ABGCs) contributed to the DG activation during retrieval of reward memory. We used two well-known strategies to examine ABGCs: administration of 5-bromo-2'-deoxyuridine (BrdU) and our inducible transgenic mouse model (i.e. nestinCreERT2 x R26R-YFP). First for BrdU (150 mg/kg, i.p.), using a similar behavioral strategy used by Kee and colleagues, BrdU was administered to mice 2, 4, and 6 weeks prior to morphine CPP training (Fig. 5-1). Mice were then trained on morphine CPP (7 mg/kg, s.c.), tested ~24 h after the last training session, and killed 90 min after test. We found that all groups established place preference for morphine, had cFos expression, and had normal BrdU counts across the 2, 4, and 6-week BrdU-CPP interval groups (Fig. 5-1).

However, proportional analysis of the number of activated ABGCs (i.e. cFos+ and BrdU+ cell determined by confocal analysis) showed no significant difference across BrdU-CPP interval groups. This worked suggested to me that the proportion of ABGCs (determined by BrdU) that were activated (cFos) ~24 h after training were not immediately involved in retrieval of reward memory. We believed it was possible that our method for labeling ABGCs (i.e. BrdU) was not the most efficient (i.e. one injection of BrdU [150 mg/kg, i.p.] would label a sparse population of ABGCs). Therefore, we also performed this experiment using our nestinCreERT2 x R26R-YFP reporter mouse to inducibly label all of the putative nestin expressing adult-neural stem cells and their progeny with YFP (Lagace et al., 2007).

Using the nestinCreERT2 x R26R-YFP reporter mouse, we asked the question whether ABGCs could be activated after immediate retrieval of a previously learned drug-associated context reward memory. Similar to our BrdU approach, the nestinCreERT2 x R26R-YFP reporter mice (6-8 weeks old) received TAM to allow for recombination and expression of YFP to occur, or were administered vehicle. Six-to-eight weeks post-TAM administration, mice were trained on morphine CPP, tested ~24 h after training, and killed 90 min after test (Fig. 5-2). Both TAM and vehicle groups established morphine place preference, had a trend for increased cFos, and had similar total YFP numbers of ABGCs in the DG

(Fig. 5-2). While this study is ongoing, it appears that the proportion of ABGCs (YFP) that is activated (cFos) is low and similar between TAM and vehicle groups. Taking together our BrdU and nestinCreERT2 x R26R-YFP reporter mouse experiments; it appears that ABGCs may not be necessarily activated after an immediate retrieval of reward memory. However, based on evidence from Chapter 3 of my dissertation, ABGCs may be involved in the consolidation/reconsolidation process of reward memory. Therefore, future experiments examining the contribution of ABGCs to the DG circuit should verify that cFos cells are activated after retrieval and extinction of a previously learned reward memory (see Chapter 3 future directions, as the tissue from this experiment has been perfused and can be used to address this question).

As mentioned in the discussion of Chapter 2, one possible treatment avenue for addiction consists of increasing new DG neuron number or survival in the DG (Clelland et al., 2009). Supporting this notion, increased neurogenesis via environmental enrichment (EE), after cocaine CPP training, showed an elimination of cocaine CPP retrieval and extinction (Solinas et al., 2008). Therefore, to determine if increased neurogenesis can differentially alter rewarding memories, an experimental design that increases ABGCs (i.e. either by neuroregenerative drugs [P7C3 or Isoxazole 9] or behavioral strategies [EE]) prior to training of morphine CPP should be performed (Petrik et al., 2012; Pieper

et al., 2010; Solinas et al., 2008). However, it is possible that aside from DG neurogenesis, DG interneurons (Ikrar et al., 2013) may play a role in modulating DG output during retrieval of a drug/associated context reward memory.

Tag-and-Track a Rewarding Memory: How dose a rewarding memory progress through the brain? It appears that ABGCs play a role during different ages of a memory, which can be easily examined with tissue already available from Chapter 3 experiments (Kee et al., 2004). However, it is not known what role other brain regions have on the progression of a rewarding memory. Therefore, one future experiments should examine how a memory trace is created and if it travels to various brain regions after being acquired, retrieved, and extinguished. This can easily be done with a tag-and-track strategy developed for transgenic mice (ArcCreERT2 x R26R- STOP-floxed-EYFP, (Denny et al., 2014)), where Arc+ cells (another IEG) can be tagged with GFP after TAM administration. TAM would be given during training of morphine CPP. Once tagged, the retrieval of young, intermediate, and old reward memories should be examined and mice should be killed an hour post-test. IHC for GFP+ and Arc+ cells will reveal how engrams overtime change with the age of the a rewarding memory. In addition, brain regions involved in reward memory (i.e. NAc, VTA, amygdala, hippocampus, and PFC) should be examined and correlations between GFP+Arc+ cells could be made to examine the memory across time.

State Dependence or Novel Context-Association: Our data correlatively show that DG cFos activity coincides with re-exposure to a drug-context. We interpret that to mean that the increase in DG activity is related to reward memory retrieval. However, it may be possible that the observed increase in DG cFos is a consequence of changing states and contextual features between pairing and test days (Cunningham et al., 2006; Deng et al., 2013; VanElzakker et al., 2008). To account for this possibility, train 2 groups of mice on morphine CPP and on test day administer morphine to all animals immediately prior to the test on day 5. One group of animals will be sequestered to their previous morphine-paired context, and the results will be compared to the saline-paired mice. I hypothesize that mice sequestered to the morphine-paired context will have more DG cFos than the saline-paired controls. An additional control experiment should also use the same pairing strategy, but on test day a novel context (i.e. dotted walls with a smooth flooring) would be used instead of the previous morphine-paired context. However, it is likely that other brain regions, such as the amygdala, are also contributing to the reward memory (Redondo et al., 2014).

Necessity of IEG Activity In Altering Reward Memory: It has been suggested that the specific engrams of DG IEGs are involved in the contextual representation of a memory during retrieval (Denny et al., 2014; Goshen et al., 2011; Kheirbek et

al., 2013; Kheirbek et al., 2012; Smith and Bulkin, 2014; Takehara-Nishiuchi, 2014). In other words, the ability for specific engrams of IEGs to alter the contextual information or valence of a memory is possible (Cruz et al., 2013; Goshen et al., 2011; Redondo et al., 2014). In line with this logic, several studies have found that altering activation of DG IEGs, previously associated with a memory, can disambiguate or erase a memory (Garner et al., 2012; Liu et al., 2012; Ramirez et al., 2013). However, this strategy has never been tested using a reward behavior paradigm such as CPP. Therefore, using these transgenic strategies (i.e. activation/inactivation of context-specific IEGs in the DG during behavior), one future experiment should activate/inactivate DG IEGs during (acquisition) and after (consolidation) the pairing sessions of morphine CPP and during retrieval (reconsolidation) of reward memory. This type of experiment could be performed with the use of designer receptors exclusively activated by designer drugs (DREADDs). Briefly, these engineered G-protein coupled receptors are activated by the small molecule clozapine-N-oxide (CNO), that can either induce neuronal firing (hM3Dq), silencing (hM4Di), or cAMP (Gs-D). Viral DREADD constructs that are Cre dependent (AAV-Flex-DREADDs, Cassataro et al., 2014) are available and could stereotaxically be injected into the DG 2-3 weeks prior to morphine CPP. To test the necessity of IEG activation on reward memory, CNO and vehicle could be administered prior and through training days (acquisition), after each training session (consolidation), immediately prior or

after testing (retrieval or reconsolidation, respectively), or immediately before extinction training (extinction). I would hypothesize the activation/inactivation of DG IEGs associated with morphine CPP would cause deficits in acquisition, consolidation, reconsolidation, and extinction, but not retrieval, compared to vehicle controls.

Chapter 3. Image-Guided Cranial Irradiation-Induced Ablation of Dentate Gyrus Neurogenesis Diminishes Extinction of Young, but not Old, Extinction of Morphine Reward Memories.

In order to determine if adult neurogenesis had a role in learning and memory mechanisms involved in reward memory, we used image-guided cranial irradiation (IG-IR) to ablate neurogenesis and examined the retrieval and extinction after morphine CPP. No difference in retrieval of young and old reward memories was found. However, we are the first to show that after IG-IR caused major deficits in the extinction of young, but not old, morphine-associated context reward memories. This suggested to us, that adult neurogenesis might play a role in the extinction of young reward memory, similar to a fear memory study (Deng et al., 2009). These data open the field of adult neurogenesis to a potential treatment for altering memories. However, additional control experiments should be performed to make sure that our interpretation of our data is correct. These experiments and others are discussed below.

Future Directions, Chapter 3

Differential neuronal activation during retrieval and extinction: It is currently not known how neuronal activation differs in brain regions associated with learning and addiction. The lowest hanging fruit from this thesis work could use the tissue collected from the extinction experiments to analyze cFos in the PFC, amygdala, and NAc. Correlation analysis of cFos in various brain regions can provide a hint of how young and old reward memories move from the hippocampus. For example, if more PFC cFos is observed in mice that only experienced retrieval of a old, but not young memory, this suggests that the memory has become hippocampal independent and may not rely on the hippocampus for retrieval. It may be that the amygdala has more cFos as well. Therefore, an in depth analysis of cFos should be performed throughout the brain.

Necessity of ABGCs in Reinstatement: One of the questions we aimed to address in this experiment was if adult neurogenesis played a role in the reinstatement after extinction. To examine this I performed reinstatement on both the young and old memory extinction groups after each group extinguished the morphine-associated context reward memory. Using a priming dose of morphine (s.c., 7 mg/kg) I found no significant difference in the amount of time spent in the previous drug-context across time in the reinstatement of a young reward

memory. However, the reinstatement of an older memory showed that Sham mice spent more time in DC after reinstatement, whereas IG-IR mice had no significant difference (Fig. 5-3). These data suggest that an intact DG was capable of reinstatement of a previously extinguished old morphine-associated context reward memory. In addition, it appears that cranial irradiation has a beneficial role in preventing reinstatement of an older reward memory, whereas an intact DG is detrimental. One explanation is that adult neurogenesis is important in contextual representations of a memory, and the intact DG enabled the appropriate reinstatement. In addition, it is possible that a damaged DG may not be able to correctly associate the drug with the previously learned morphine-associated context, leading to no reinstatement. This suggests that a decrease in adult neurogenesis after extinction of a drug-associated context reward memory may be beneficial. However, it is still unclear what role, if any, adult neurogenesis plays in the reinstatement process. A summary of these data can be found in Figure 5-4.

Causative Study for ABGCs: It is possible that cranial irradiation induced alterations to the DG neurocircuitry (possibly by the ablation of adult generated neurons) are responsible for the diminished extinction of young reward memories. Therefore, it is important to perform a more causative study examining adult neurogenesis that would allow for a clearer interpretation of the

results. Cranial irradiation has previously been shown in the hippocampus to decrease cFos, reduce dendritic complexity, impair LTP, alter cytokines/chemokines/growth factors, and result in a complex gene profile that is time and dose dependent (Achanta et al., 2007; Mahmoud-Ahmed et al., 2006; Morganti et al., 2014; Parihar and Limoli, 2013; Son et al., 2014). A decrease in DG spine density has also been shown to persist for up to 1 month post-irradiation (Chakraborti et al., 2012). All of the aforementioned changes caused by cranial irradiation can possibly lead to changes in the DG neurocircuitry. Therefore a more specific ablation strategy of adult generated neurons should be used. This specific ablation strategy can be performed using GFAP-TK mice that will selectively ablate GFAP+ cells after ganciclovir administration. Ganciclovir can be chronically administered prior and during morphine CPP training and extinction. I hypothesize that ABGCs are necessary for extinction learning and therefore a similar deficit in extinction will be observed.

ABGCs are already known to have functions in pattern separation, which are considered to be more difficult behavioral tasks (Leutgeb et al., 2007; Myers and Scharfman, 2009, 2011; Lee and Kesner, 2004). However, it is not known if a more difficult reward task also involves ABGCs. To address this question, a difficult CPP paradigm (e.g. lower dose, one pairing, or similar contexts) should be performed after cranial irradiation or specific ablation (e.g. GFAP-TK) of

ABGCs. It would be particularly interesting to see if a more difficult CPP paradigm also has an effect on extinction learning and reinstatement, since potential treatments could be developed from those findings. I would hypothesize that an ablation of ABGCs prior to training would impair the more difficult rewarding behavioral tasks.

Chapter 4. Acute and Fractionated Exposure to High-LET 56Fe HZE-Particle Radiation Both Result in Similar Long-Term Deficits in Adult Hippocampal Neurogenesis.

Research has shown that ground-based HZE particle irradiation can be damaging to the CNS. However, until work from this dissertation was published, no studies had addressed the major issue of a chronic low dose of GCR astronauts would receive in out space. Using ground-based strategies to mimic galactic cosmic radiation, we are the first to show that mice irradiated with the iron HZE particle had similar deficits in adult-generated cells as well as immature neurons after a fractionated and acute exposure. Interestingly, no change in the putative adult-neural stem cell was observed after either exposure treatment. Taken together, these data suggest that acute and fractionated irradiation strategies can be used to appropriately assess risk to an astronaut during space flight missions. In addition, the data from Chapter 4 also suggest that there is the potential for a recovery of adult neurogenesis. These data aid the field of space

radiation in understanding the unique characteristics of HZE particles and how best to mimic them on the ground to simulate GCR. However, additional control experiments examining HZE particle radiation are necessary to fully understand the effects of GCR on the CNS and assess the risk astronauts will experience during space flight missions. It should be noted that other future directions mentioned from data Chapter 4 including lower dose-rates, aged mice, and behaviors of learning and memory and stress are currently being explored in the Eisch lab. Other future directions not currently being explored are discussed below.

Future Directions, Chapter 4

Functional Type-1 Cells: Based on our results with no deficit in the Type-1 cell population (by phenotypic analysis of YFP), it may be possible for adult neurogenesis to recover. In a separate manuscript by Rivera, DeCarolis, et al., 2014, we showed that acute exposure of 56Fe HZE particles to mice had a transient decrease in suppression of adult neurogenesis. Taken together these data suggest that there is the potential for the Type-1 cells to contribute to regeneration of adult neurogenesis. However, this potential regenerative capacity was only observed after one exposure to HZE particles. Therefore, future experiments should consider a fractionated exposure paradigm and thoroughly assess the Type-1 cell population as well as hippocampal-dependent behavior.

As mentioned in the introduction, there also appears to be heterogeneous lineages of adult-generated cells (Brunner et al., 2014; DeCarolis et al., 2013). It would be interesting to determine if HZE particle radiation differentially affects these lineages by comparing GLAST- and nestin-CreERT2 x R26R-YFP reporter mice 3 months post-HZE radiation (DeCarolis et al., 2013).

Chronic Exposure to Mixed-Field Space Radiation: One major setback with simulating GCR using ground-based HZE particle irradiation is the acute and mono-field radiation that is currently being used for animal models. Recently, NASA has asked researchers to try and account for these variables to more accurately assess risk for astronauts. Within the past few years, NASA Space Radiation Laboratory (NSRL) at Brookhaven National Laboratory (BNL) has developed a method to use a mixed-field of HZE particle radiation. This is important since GCR is comprised of more than one particle that can damage the CNS and body (Cucinotta, 2014). Studies using this mixed-field radiation are just starting to come out, but they do not account for the chronic exposure of GCR that would be experienced during a space flight mission. Therefore, future experiments should consider using a chronic exposure to a mixed-field of HZE particles. While this type of study is practically difficult (given the technical limitations at NSRL, researchers only have a limited amount of “beam time” to

perform these experiments), it would provide a better understanding of GCR on the CNS and would allow for appropriate risk to be determined.

Conclusion

This dissertation provides a foundation for other studies to examine the role of adult neurogenesis on reward learning and how different exposure methods of HZE particles can affect adult neurogenesis. First, we find that adult neurogenesis plays a role in extinction of young reward memory, but may be detrimental when drugs of abuse are reintroduced after extinction learning. This feature highlights the complexity involved in the learning and memory of reward memory and may possibly extend to fear memory (i.e. PTSD) as well. Second, ground-based simulated GCR using HZE particle irradiation to assess risk in astronauts is difficult given the constraints by the NSRL facility, however our work suggests that acute exposure HZE particle may reflect a chronic exposure of GCR. However, more work is necessary to determine if that remains true for lower doses of HZE particle radiation found in deep space. Overall, the DG and adult neurogenesis play a role in cognition. Therefore, understanding how the process of addiction and space radiation alters the DG is necessary for treatment for human addicts and appropriate countermeasures for astronauts, respectively.

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Figures

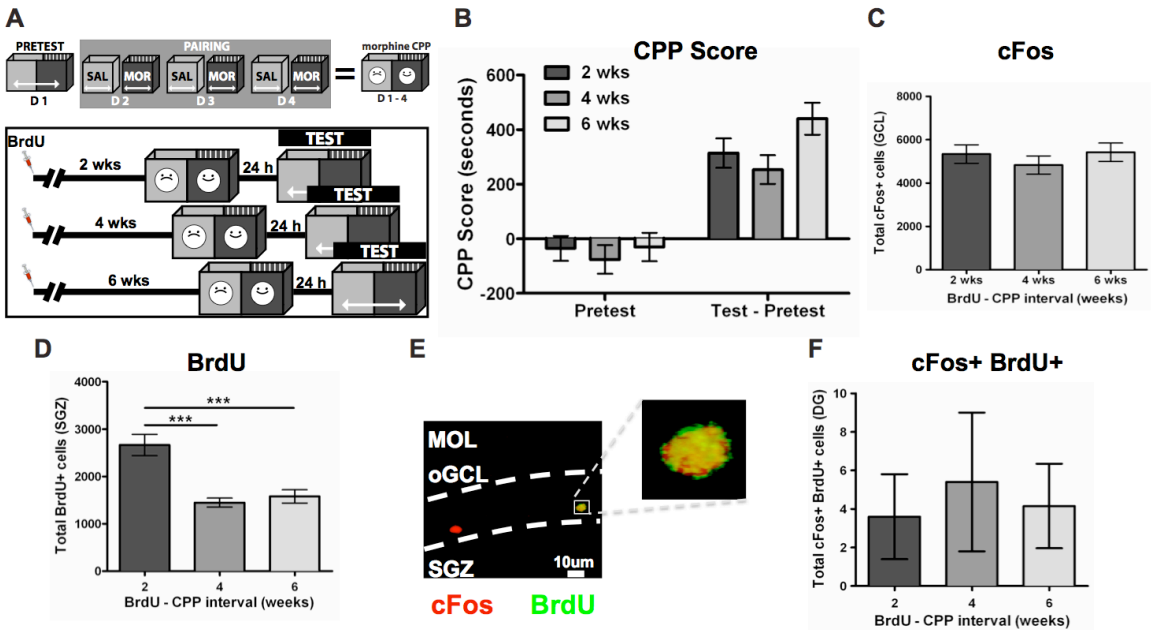

FIGURE 5-1. Experiment to determine if BrdU labeled adult-generated neurons are activated by retrieval of young morphine-context reward memory.

(A) Timeline of experiment. Briefly, BrdU was administered 2, 4, or 6 weeks prior to morphine CPP training and testing began ~24 h post-training for each group. (B) CPP scores for all groups. (C) cFos quantification was performed in the DG GCL. (D) BrdU was quantified for all groups. (E) Image of a double labeled BrdU+ cFos+ cell in the DG GCL. (F) The total number of double positive BrdU+cFos+ cells quantified throughout the DG. CPP, conditioned place preference; DG, dentate gyrus; GCL, granule cell layer.

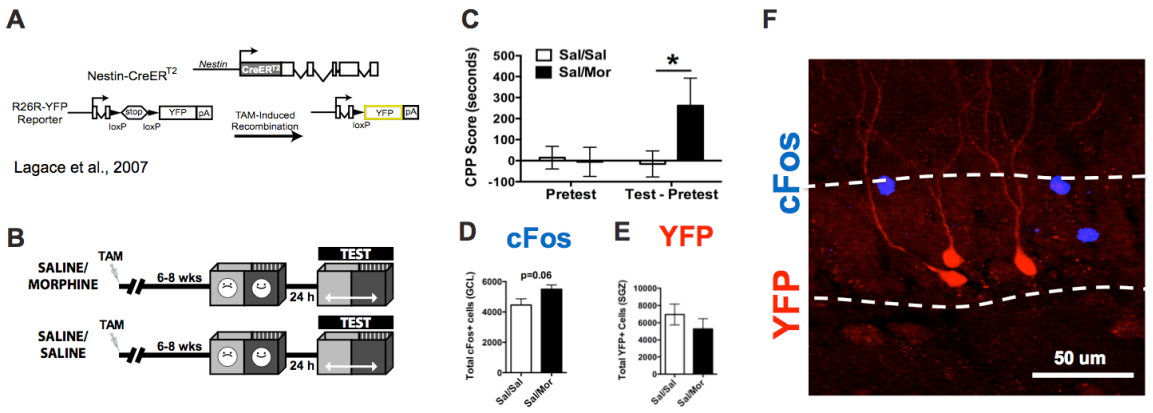

FIGURE 5-2. Experiment to determine if nestin-lineage cells are activated by retrieval of young morphine-context reward memory.

(A) The transgenic nestinCreERT2 x Rosa26R-YFP mouse used as a reporter for adult neurogenesis. Briefly, administration of TAM induces recombination and allows for translation of YFP in the nestin expressing cells and their subsequent progeny (Lagace et al., 2007). (B) The timeline of the experiment where TAM was administered to mice and 6-8 wks later morphine CPP training was performed. ~24 h after training the test was performed. (C) CPP scores were determined for both TAM and vehicle treated mice. (D-E) Total (D) CFos and (E) YFP was quantified in the DG GCL for both TAM and vehicle groups. (F) Image from confocal analysis of YFP+ and cFos+ cells.

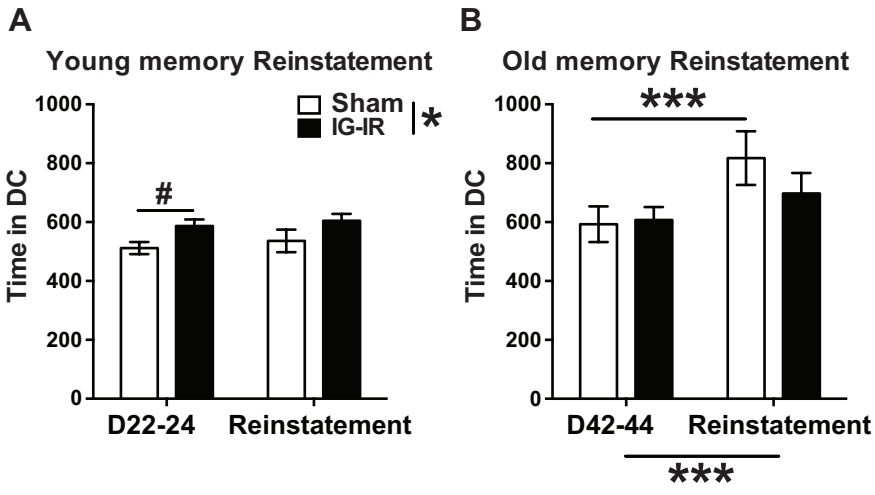

FIGURE 5-3. IG-IR mice showed no effect on reinstatement of an older reward memory, but Sham mice did reinstate.

(A-B) Time in drug context (DC) after priming dose (7 mg/kg) reinstatement of a (A) young and (B) old reward memory. Students *t*-test, $^{\#}P<0.05$; Two-way ANOVA, ***P<0.001.

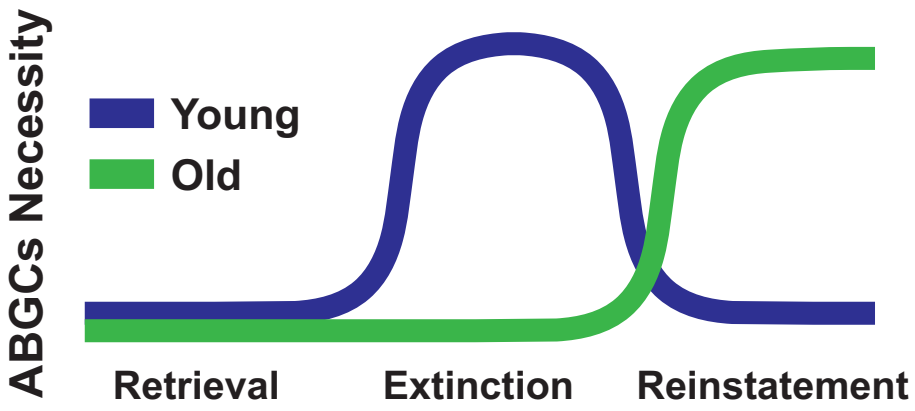

FIGURE 5-4. Summary of cranial irradiation experiment (Chapter 4).

ABGCs appear to not be necessary for retrieval of young and old reward memories. However, ABGC appear to be necessary for extinction of young, but not old, reward memories. In addition, ABGC appears to be necessary for reinstatement of an old, but not young, reward memory.