NEUROGENESIS DEPENDENT LEARNING & IMPLICATIONS FOR EPISODIC MEMORY IN RODENT MODELS.

A Thesis

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by

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ABSTRACT

This thesis reviews research into the hippocampus as it relates to episodic memory, neurogenesis, and their potential functional intersection. Chapter 2 presents a novel model for episodic-like memory in rats, and relates it to previous models and its potential application for human episodic memory. In chapter 3, we present research comparing adult neurogenesis between spatial and non-spatial hippocampal dependent tasks. The results presented in chapter 3 suggest that neurogenesis may only pay a role in hippocampal tasks that are encoded alone across several days, and thus correspond to the time scale in which new neurons become integrated into the dentate gyrus.

BIOGRAPHICAL SKETCH

Orriana Sill graduated from Eastern Michigan University in 2007 with a degree in Psychology, and minors in mathematics and human biology. She continued her education in the Behavioral and Evolutionary Neuroscience program through the Cornell Psychology Department starting in 2007. Her research and subsequent papers and master's thesis were supervised by Dr. David Smith.

"To Bryce Cartmill: Among other reasons, for blocking reddit on my computer so I would get this done."

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CHAPTER 1

INTRODUCTION

Overview of Hippocampal Function

The peculiar shape of the hippocampus has made its function a subject of scientific speculation since the renaissance (Lewis, 1922-3), and for less traceable reasons, since classical times. The 2nd century physician Galen described the psaliodes or "arched structure" of the fornix in great detail, although his views on functionality have been lost to history (Mayne, 1860; Singer, 1999). Galen's contemporary Herophilus, the first known researcher to correctly link the brain to both sensory and motor processing, also spent a lot of time studying the brain areas surrounding the third ventricle, as he believed these structures functioned as the supreme command center of the central nervous system (Von Staden, pp. 316). The hippocampus has remained one of the more popular areas of study, as well as having become one of the most useful. The single layered sea-horse shaped structure has mostly unilateral connections to and from the cortex, making it one of the easier regions for which connectivity could be mapped and computational models proposed. Early in vivo electrophysiology studies led to the discovery of oscillations such as the theta rhythm (Green & Arduini, 1954), inhibitory and excitatory signaling (Kandel et al., 1961), as well as discoveries of several of the major neurotransmitters (Biscoe & Straughan, 1966; Curtis et al., 1970). It's cells are also one of the easiest to keep alive and functional in lab culture (Banker and Gosselin, 1991), which allowed for hippocampal cells to be of use for the discovery of long term potentiation and depression (Schwatzkroin & Wester, 1975), as well as seminal research into limiting or treating damage due to hypoxia or stroke (Fowler, 1989).

Yet even so, the general function of the hippocampus remains a point of discussion, does the degree of transfer between human and non-human hippocampal research data. This introduction will provide a brief overview of the current understanding of the role of the mammalian hippocampus, particularly by comparing functional and anatomical differences

between the rodent and human hippocampus. We will then briefly examine how these differences relate to episodic memory, and adult hippocampal neurogenesis, the main topics of the papers to follow.

The hippocampus proper is made up of 3 regions: CA1, CA2 & CA3 (and formerly CA4), where 'CA' is the abbreviation for the original latin name for the region cornu ammonis, meaning horn of the ram (Mayne, 1860). The relative size of these regions to each other and the rest of the brain are fairly conserved between mammals with the exception of cetaceans, although smaller mammals tend to have slightly larger hippocampi (Manger, 2006). Overall, each mammalian species tends to develop a hippocampus which corresponds closely to the natural log ratio of the animal's body size.

In all mammalian species, the main input of the hippocampus is in the medial temporal region known as the entorhinal cortex. The entorhinal cortex receives input from all higher order cortical sensory areas and consolidates a signal into the dentate gyrus of the hippocampus. This is the first projection in what is known as the trisynaptic circuit. The dentate gyrus, in turn, projects to CA3 (synapse 2) which then projects to CA1 (synapse 3), which feeds back to the entorhinal cortex and CA3 and other areas (Amaral & Witter, 1995).

The main variation between hippocampal function of mammalian species may not be entirely rooted in anatomical differences of the hippocampus itself, but rather in the type of input sent from the entorhinal cortex. The entorhinal cortex does in fact show a great deal of variation between species. In rats, the entorhinal cortex is a relatively simple structure with only 2 cytoarchitecturally distinct subregions. In humans, at least 8 such subregions have been identified (Krimer et al., 1997). Although there is evidence that both species possess a map of spatially specific cells known as "grid cells" (Hafting et al., 2005; Doeller et al., 2010) there is evidence that entorhinal cortex of humans possesses cells which respond to higher order path integration functions as well, such as clockwise or counterclockwise movement within this grid (Jacobs et al., 2010). Such cells have not yet been observed in rats.

The hippocampus of both rats and humans can be demonstrated to be involved in path integration, or the process of integrating direction of movement from external cues and internal cues of movement and distance to determine the route traveled. However, there are strong differences in terms of which modalities and methods employ to make this calculation.

In electrophysiology experiments, the human and primate hippocampal cells can most often be found to respond to specific visually perceived objects or visual views independent of the primate's actual position in space. These cells are thus more aptly called spatial view cells (Rolls, 1999; Ekstrom, 2003). The 'place cells' discovered in rodents respond to the current physical location in space and are less sensitive to the rat's orientation (O'keefe & Dostrovsky, 1971). In fact, place cells often stay intact if the rat is in the dark (Quirk et. al., 1990). This suggests the rat may be using haptic sensory or otherwise vestibular input to encode its location in space as opposed to or in addition to vision.

Place cells in the hippocampus (and by extension, primate spatial view cells) play a central role in spatial orientation and navigation (O'Keefe & Nadel, 1971). However not all of the early ideas about the mechanism of these cells were correct, as some of the same navigational tasks given to rats can be managed by species with much simpler neuronal arrangements than that of the mammalian hippocampus, most notably the honey bee.

Many insects are also capable of complex path integration to calculate "dead-reckoning" computations, in which the insect will calculate distance and angle on the way to a destination to return to its starting point. Yet if an ant is displaced a few meters at the start if its journey from its nest, it is unable to deduce the direction towards home. This is true even if the landmarks are those it has traversed by before, and even if the ant has foraged much further from that point to find food in the past (Gallistel, 1990).

The hippocampus is thought to enable navigation via "cognitive mapping". The cognitive mapping hypothesis states that hippocampus enables us to form a relational map between objects and their position in space, and thus enable mammals to take shortcuts

through space or adapt to changes in that environment (O'keefe & Nadel, 1978). It I worthwhile to note that honeybees seem to be able to form a sparse cognitive map according to this original definition and they can learn to adapt their behavior to obtain a reward if the changes in their environment are small (Pahl et al., 2007).

It is clear that for rats as well as humans this is not the only function of the hippocampus, as it is required in many non-navigational tasks. In rodents, these include memory for temporal sequences (Fortin et al., 2002, Eichenbaum, 2004, Lehn et al. 2009) as well as a general spatiotemporal context in which memories take place (Cohen & Eichenbaum, 1993, Eichenbaum, 1999).

Episodic memory across species

In humans, the hippocampus was first and foremost implicated in its role in the formation of new autobiographical memories (Scoville & Milner, 1957). Some prominent researchers still maintain that the memory which enables autobiographical memory is a function limited only to humans. This human-specific memory was defined more tightly as "episodic memory," or the memory of unique episodes incorporating "what" took place "where" and "when". The explanation is that mammalian hippocampus evolved first in the mammals for cognitive mapping or other basic relational processing, and then this brain region was retrofitted to enable episodic memory within the human line (Tulving, 2002; Feeney and Roberts, 2012).

It's more commonly accepted that the hippocampus evolved to play a general role which cognitive mapping and episodic memory either both have in common or both derived from(Cohen & Eichenbaum, 1993). It is clear that any memory of a life episode will must necessarily contain a representation of setting, the "what" and "where" of cognitive mapping. It is also increasing understood that our phylogenetically distant cousins are still capable of some very "episodic-like" functions, even if they may lack the full integration of the human episodic experience. The rodent hippocampus, at the very least, supports a

spatio-temporal context which links memories with those that followed close in time and/or space (Good & Honey, 1993; Smith & Mizumori 2006a, Alvarez et al., 2012).

Would demonstrating the rodent hippocampus is necessary for "what", "where" and "when" dimensions all at once be enough to irrefutably demonstrate than this non-human hippocampus is also encoding episodic memory? It shouldn't be. Even insects are capable of "what", "where" and "when" memory if these factors are defined loosely enough.

The smallest of insect brains is capable of maintaining a circadian rhythm to tell time (Wiener, 2000). Insects are generally able to behave specifically within events that can be predicted by time of day, such as returning to a specific food source at a location that is available in the morning but not later on in the day (Gallistel, 1990). Bees can demonstrate memory of the components of episodic memory as well as long as the 'when' part of episodic memory can be interpreted as a circadian-based time of day. In Pahl et al. (2007), bees were able to locate a sugar water reward at different times of day, (when) if they chose the right box in a larger enclosure (where) and entered the correct arm within a maze-like box labeled with the appropriate visual cues(what). The bees did much better if only 1 of these 3 factors were altered a time between multi-trial sets, and the bee learned could this task across many days of exposure. For this reason, it is important to stress that some of the more basic rodent episodic-like memory models (Griffin et al., 2007, Manns et al., 2007) might not be entirely rigid. Other than independent manipulation and a non-circadian representation of time, the most crucial factor should be that the memory being demonstrated is indeed a unique episode, a memory of an event with a well defined beginning and end rather than a memory of conditioned behavior in a series of similar repeated episodes, as is the case with Pahl et al., 2007. There is no evidence that any insect is capable of encoding a memory for an episode to the detail required by this more stringent definition, in which long-term retrieval is based on linear rather than a circadian understanding of time.

Olfactory sequence learning has been put forth as a model for episodic memory in rodents because it involves the memory for specific, albeit non-spatial, events in time (Fortin et al., 2002). Furthermore, a far greater area of the rat cortex is devoted to olfaction than that of vision compared to primates, so olfaction-based tasks might be more useful in demonstrating rat's full episodic abilities. Thus, the episodic memory model put forth in this thesis builds on an olfactory model, requires an integration of all 3 episodic dimensions of what, where, and when in unique episodes. This study also closes some potential loopholes in previously used designs that might enable the rat to potentially reach criterion using non-episodic strategies.

Adult neuorgenesis across species

The second paper of thesis deals with one last difference not yet mentioned between rat and human hippocampal circuitry, that of adult neurogenesis in the dentate gyrus. The dentate gyrus is a region of the hippocampal formation which varies between species in the number of newborn cells which continue to be regenerate in adulthood. Rodents generate and integrate many more cells into their dentate gyri than humans or other primates do at similar developmental periods, so any practical benefit or function of neurogenesis should be much more apparent in rodent models (Eriksson, 1998, Kornack & Rakic, 1999).

In both rats and humans, these cells undergo differentiation in the subventricular zone. The immature cells begin to form dendrites and migrate toward the subgranular zone of the dentate gyrus after about a week (Pencea et al., 2001). In rodents, the immature granule cells have reached the dentate gyrus and begin forming axons by 2 weeks. By about the 17th day of development, the new axons (mossy fibers) will form connections to CA3 pyramidal cells and hilar neurons. The new cells are fully integrated into the dentate gyrus by the third week, and continue maturing until they are indistinguishable from fully mature neurons. This process has been argued to take from 2 months (Zhao et al., 2008), to four months (van Praag et al., 2002). There is only slight modification to this timescale in

primates. Migration is slightly more time consuming, as the cells due to the greater distance the cells have to travel within a larger brain. Even so, half of the newborn cells have migrated by the time they age 14 days. But it also takes primate granular cells about 6 months to reach full maturity (Kohler et al., 2011).

It was observed very early in adult neurogenesis studies that many more cells are born in the subgranular layer than survive, and that death rate increases rapidly after birth. In rats, new subgranular cells begin to die off after about 6 days of life, and show only about a 10% survival rate by day 15 (Altman & Das, 1966). Even so, young adult rats have about 9000 cells incorporated into their hippocampus each day (Cameron & McCay, 2001). Adult Macaques were estimated to have only about 1000 new cells added per day, despite the differences in brain size (Kornack & Rakic, 1999). In both rats and primates, hippocampal adult neurogenesis peaks by puberty and then progressively declines with age (Kuhn et al., 1996; Gould et al., 1999). The spike at puberty has been demonstrated to not be a factor of a spike in gonadal hormones, as neutered or ovariectomized rats show similar rates of neurogenesis in the dentate gyrus (Ho et al., 2012).

We also know that the total number of granular cells in the dentate gyrus increases slightly with age, even if there is little room for expansion. The dentate gyrus will contain more numerous but smaller cells, with cell counts still increasing after a year of a rat's life (Bayer, 1982). Macaque monkeys show a small increase in dentate gyrus cell count as they age as well (Jabes, 2011). Some mathematical models which seek to explain the functional significance of neurogenesis depend on this not being the case. Or at least, that the total growth of this region in adulthood is so small as to be discounted (Becker, 2005). Other mathematical models better account for this influx, and also better predict the results of the neurogenesis research presented in this thesis (Aimone et al., 2006).

Once newborn cells become incorporated into the dentate gyrus, it's important to note that these cells are much more excitable than those around them. The neuron has a reduced threshhold for long-term potentiation yet is insensitive to GABAergic inhibition

(Snyder et al., 2001, Ambrogini et al., 2004, Epositio et al., 2005). The lack of inhibition suggests that granule cells should be expected to show a non-differential firing pattern for a critical period of indeterminate length rather than show the contextual firing preferences observed in place cells (Smith & Mizumori, 2006a). This is verified by electrophysiological data. Although one cannot know the age of the cells one is recording from, we do know that granule cells will form place cells, yet are much more likely than CA3 place cells to be active if moved into new environments on the same recording day (Leutgeb et al., 2007). We also know that a much smaller percentage of cells in the dentate gyrus can be recorded via electrophysiology than we know to be present, suggesting that most granule cells do not remain highly excitable for long (Alme et al., 2010).

So what are these new neurons up to? It's worth noting that some would argue that adult neurogenesis may not have much if any functional significance at all, and that this is merely a byproduct of evolutionary development. In general, the more alar and anterior the origin of a brain region is on the embryonic neural tube, the longer neurogenesis continues into development. Both the dentate gyrus and the olfactory cortex are accepted to have adult neurogenesis, and they are both stem from alar and anterior developmental tissue. It is possible that although other alar/anterior regions such as layers 2-6 of the cortex perform their functions with no observable adult neurogenesis, the energy an animal might waste to generate a few extra cells in the dentate gyrus do not constitute any practical evolutionary disadvantage (Finlay et al., 2001).

But these neurons might play a role in behavior and learning after all, as life experiences modify neurogenesis rates in a seemingly telling way. Voluntary exercise increases neurogenesis and the survival of new neurons in the dentate gyrus (Kee et al., 2007), as does exposure to an enriched environment (Tashiro et al., 2007). Both of these factors have been demonstrated to improve cognitive performance in rats(Hilman et al., 2008; van Praag et al., 1999).

Depression and stress have a known negative effect on cognitive performance (Mendl, 1999). Psychosocial stress will inhibit neurogenesis in both rodents and primates (Gould et al., 1997; Gould et al., 1998). Fluoxetine, the active ingredient in the antidepressant drug Prozac, has been demonstrated to increase neurogenesis (Malberg et al., 2000) and most tellingly, this drug cannot be effective if neurogenesis is blocked due to irradiation (Santarelli et al., 2001).

There also seems to be a link between neurogenesis and hippocampal-based memory. Training on hippocampal-dependent tasks have been demonstrated to increase neurogenesis rates, and the animals that have learned the most during that training tend to be capable of the most neurogenesis. This is true for the Morris water maze (Epp et al., 2007, Gould et al., 1999), and trace eyeblink conditioning (Leuner et al., 2004). In both of these studies, non-hippocampal versions of these tasks did not have the same effect on neurogenesis. Dampening neurogenesis through irradiation or drugs will impair performance on both of these tasks, which demonstrates the correlation between neurogenesis and these tasks is not coincidental (Raber et al., 2004; Shors et al., 2001).

It is possible though to find studies which find no link between neurogenesis and performance on these hippocampal dependent tasks. However, a pattern quickly emerges if one analyzes training time scales for the different experimental methods more carefully. Aimone et al., (2006) proposes that the new neurons could be useful to "temporally stamp" memories which took place during its critical period of high activity. By assigning new neurons to new memories, it should provide a unique index to locate memories that will never overlap with other neurons. If we assume that the critical period of a new granule cell lasts for several days, much of the contradictory results in the literature can be reconciled. Neurogenesis plays a role and contextual fear conditioning if the task is presented over multiple days (Anderson et al.,. 2011), but not if the design is set up so the learning and final testing are only 24 hours apart (Dupret, 2004; Shors et al., 2001). The same pattern holds for the Morris Water maze: impaired neurogenesis affects Morris water maze performance

only across several intermediate days, not a single day (Madsen et al. 2003; a Snyder et al. 2005).

Rabaza et al. (2009) failed to find a result on hippocampal dependent spatial alteration tasks across multiple testing days, but the rats were exposed to both contexts during training sessions (and thus new granule cells would be expected equally active in both contexts, and an impairment of neurogenesis should not impair performance). In all positive relationships I examined, as well as the non-spatial task in my own neurogenesis study, tasks were separated across two days or more.

The second paper enclosed in this thesis sought evidence that focal irradiation of adult hippocampal neurogenesis would impair performance on a hippocampal-dependent spatial task and a hippocampal-dependent olfactory association task. Aimone et al. (2006)'s temporal stamp hypothesis predicted the pattern of results better than other models, as we failed to find a relationship between irradiation and task performance when contexts to be differentiated were separated by mere minutes in the training phase, despite continuing for up to 15 days during the testing phase. We did find a relationship between impaired neurogenesis and performance when the contexts and learning were separated by several days in the olfactory task.

To date, there has been no published research directly investigating the relationship between adult hippocampal neurogenesis and the temporal component of episodic memory. Further research should be done to explore this, and the model I present in this thesis could be modified to make this possible. Once the link between neurogenesis and performance on an episodic memory task is established, it would be possible to estimate- or, if other methods make measurement possible, behaviorally confirm the length of the critical period in granule cells development.

CHAPTER 2

A COMPARISON OF THE EFFECTS OF TEMPORARY HIPPOCAMPAL LESIONS ON SINGLE AND DOUBLE CONTEXT VERSIONS OF THE OLFACTORY SEQUENCE MEMORY TASK

Introduction

In recent years, much research has been focused on episodic memory in animals (Babb & Crystal, 2006; Clayton & Dickinson, 1998; Eacott & Norman, 2004; Ergorul & Eichenbaum, 2004; Kart-Teke, De Souza Silva, Huston, & Dere, 2006). By definition, episodic memories include memory for the individuals, objects and events that were part of the episode (what), as well as the place where the events occurred (where) and the time of their occurrence (when). The odor sequence memory task requires subjects to remember individual odors and their position in the temporal sequence of events (Fortin, Agster, & Eichenbaum, 2002; Kesner, Gilbert, & Barua, 2002). Thus, this task has become an important model for studying memory for individual events (what) and the temporal sequence in which they occur (when).

Various authors have suggested that hippocampal encoding of the spatial context, as exemplified by place cell firing, reflects the 'where' component of episodic memory (Anderson & Jeffery, 2003; Nadel, Willner, & Kurz, 1985; Smith & Mizumori, 2006a) and this is consistent with the well-known role of the hippocampus in processing contextual information (e.g. Hirsh, 1974). Requiring the rats to perform the odor sequence task in a context dependent manner would incorporate a key component of episodic memory. In the present study, we have modified the odor sequence task by training rats to choose the earlier

odor in one context (a white box) and to choose the later odor in another context (a black box) and we compared the effects of temporary inactivation of the dorsal hippocampus in the new dual-context task and the original single-context task.

Previous studies have used lists containing 5 odors (Fortin *et al.*, 2002; Kesner *et al.*, 2002) or 6 odors (Wolff, Gibb, & Dalrymple-Alford, 2006). In the present study, we used 7-item lists so that a greater variety of probes could be constructed for each lag size, which refers to the number of intervening odors during the sequence presentation. One problem with shorter lists is that most of the possible probes contain one (or both) of the first and last items from the list. These items may be easier to remember (e.g. due to recency and primacy effects) and rats could exhibit moderately good performance by remembering the first and last items, even without maintaining memory for the items of the middle of the list. The use of longer lists mitigates this problem by allowing for the construction of many probes that do not contain the first or last odor from the sequence.

Methods

The subjects were eight adult male Long-Evans rats that were food deprived to approximately 85% of their free feeding weight. All of the rats were first trained to a criterion on the single context task, followed by surgery to implant guide cannula for intrahippocampal infusions. All procedures complied with guidelines established by the Cornell University Animal Care and Use Committee. After recovery, the rats were re-trained to the criterion and then tested with saline and muscimol using a within subjects design. The rats were then trained on the dual context version of the task. After reaching the behavioral

criterion in this task, the rats were again tested in each of the contexts with saline and muscimol infusions.

Details of the odorants, apparatus and general training procedures have been published elsewhere (Butterly, Petroccione, & Smith, 2011). Briefly, trials consisted of the presentation of a sequence of odor cues, presented one at a time mixed into cups of digging medium with a buried sucrose pellet reward (45 mg, Bioserve, Frenchtown, NJ) in each cup. This was immediately followed by a memory probe which consisted of the simultaneous presentation of two cups containing odor cues from the sequence, but only the cup containing the earlier odor from the sequence had a buried reward (100 mg sucrose pellet). Digging responses in the later odor were not rewarded. The odors for each trial were randomly selected from a set of 20 pure odorants (for details see Butterly *et al.*, 2011). Probes included odors selected from each of the odor positions within the sequence and each of 3 different lag sizes. There were 4 different probes of lag sizes 1 and 2, and 3 probes of lag size 3 (i.e. odors with 1, 2 or 3 intervening odors in the sequence).

All of the rats were first trained to a behavioral criterion of 80% correct over 30 trials on the single context task. This ensured that all of the rats were performing the task equivalently well (84.03±3.39% correct, mean ± SEM) and only rats that reached the criterion were included in the experiment. Various training methods were used to bring the rats to this level of performance and the duration of training varied considerably (70-270 trials, mean=146.13±25.21). The best results were achieved by gradually shaping the rats to select the earlier odor from sequences of increasing length (3 odors, 4 odors, etc.) until they

were able to perform with 7 item sequences. For each sequence length, the rats were trained until they got 5 consecutive correct choices before advancing to the next longer sequence. After reaching the criterion, the rats underwent stereotaxic surgery to implant bilateral guide cannulae for the infusion of muscimol (0.6µl of a solution containing 1 µg/µl of muscimol) or saline solution into the dorsal hippocampus (one infusion site per hemisphere in dorsal CA1, 3.6 mm posterior and 2.6 mm lateral to Bregma, 2.2 mm ventral to the cortical surface, Fig 1).

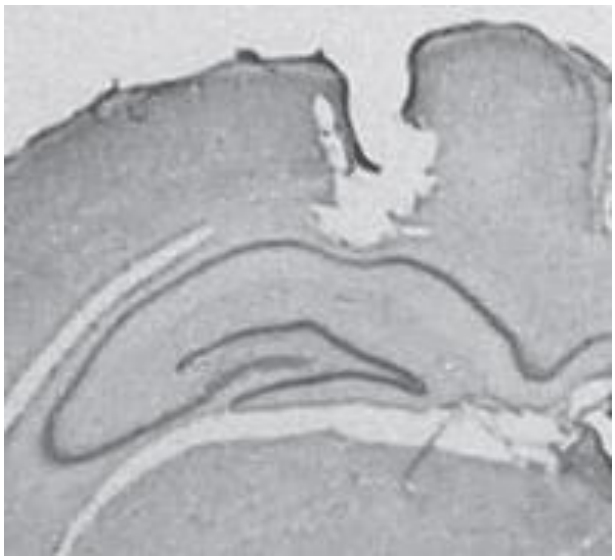

Figure 1: *A representative section with the location of the infusion cannula in the dorsal hippocampus.*

All procedures complied with guidelines established by the Cornell University Animal Care and Use Committee. After recovery, the rats were retrained to the criterion and then given test sessions (9 trials per session) with saline or muscimol infusions given 30 min prior to starting the session. Each rat was given two saline control sessions, followed by two muscimol and then two additional saline control sessions in a within-subjects design. Performance did not differ across the two muscimol sessions ($t_{(7)}$=1.49, p=.18) so the percent correct data were combined across the two sessions of each condition and submitted

to a repeated measures ANOVA. One rat died after the test sessions for the what-when task, leaving 7 subjects for the second (what-where-when) experiment.

After completing the test sessions for the single task, the rats were trained on the dual-context version of the task. All previous training for the single-context task took place in a white chamber. For the dual context task, the same white chamber was used and a second black chamber was introduced. The two contexts also differed in terms of the color of the surrounding area (black walls or white curtains), the substrate in the chamber (uncovered Plexiglass floor or a black rubber mat), the 65 dB continuous background masking noise (white noise or pink noise) and the ambient odor left by wiping out the chamber with baby wipes prior to each training session (unscented or scented, Rite Aid, Inc).

For the dual context task, the rats were given training trials as described above, except that they took place in the black box and the rats were required to select the odor that had been presented *later* on the list during the probe. In order to ensure that performance on the first task remained high, continuing trials in the original (white box) context with the 'select the earlier odor' rule were interleaved with training in the new context. After reaching the criterion on the dual context task (80% correct over 30 trials in each context), the rats were given 2 saline and 2 muscimol test sessions in an ABAB design. The four sessions were needed to give an adequate number of test trials in each of the two contexts and for counterbalancing. Each session included trials in each of the two contexts (9 trials of each injection condition and each context for a total of 36 trials).

Results

Muscimol infusions significantly impaired task performance in the single context version of the task (repeated measures ANOVA of the three conditions: saline 1, muscimol and saline 2, $F[2,14]=16.89$, $p<.001$, Fig 2). The temporary lesions impaired performance on all probes, regardless of lag size. A repeated measures ANOVA with lesion condition and lag size as within subjects factors confirmed a main effect of the temporary lesions ($F[1,14]=62.26$, $p<.001$), but no effect of lag size ($F[2,14]=0.80$, $p=.45$) and no interaction of lag size and lesion condition ($F[2,14]=0.91$, $p=.42$). Interestingly, performance during the muscimol session remained significantly above chance (65.97±3.30% compared to chance performance of 50% correct, $t_{(7)}=6.00$, $p<.005$).

We compared performance on probe trials that did and did not contain either the first or last odor from the list. For example, the two kinds of probe trials did not differ during the saline sessions ($t_{(7)}=0.06$, $p=.95$) or during the muscimol sessions ($t_{(7)}=0.21$, $p=.84$) described above. Indeed, the average percent correct for both kinds of probes was nearly identical. The equivalent performance on the two kinds of probes confirms that with our training procedures, the rats did not adopt a strategy of remembering the first or last odors without attending to those in the middle of the list. The same pattern of results was seen in the following dual context experiment.

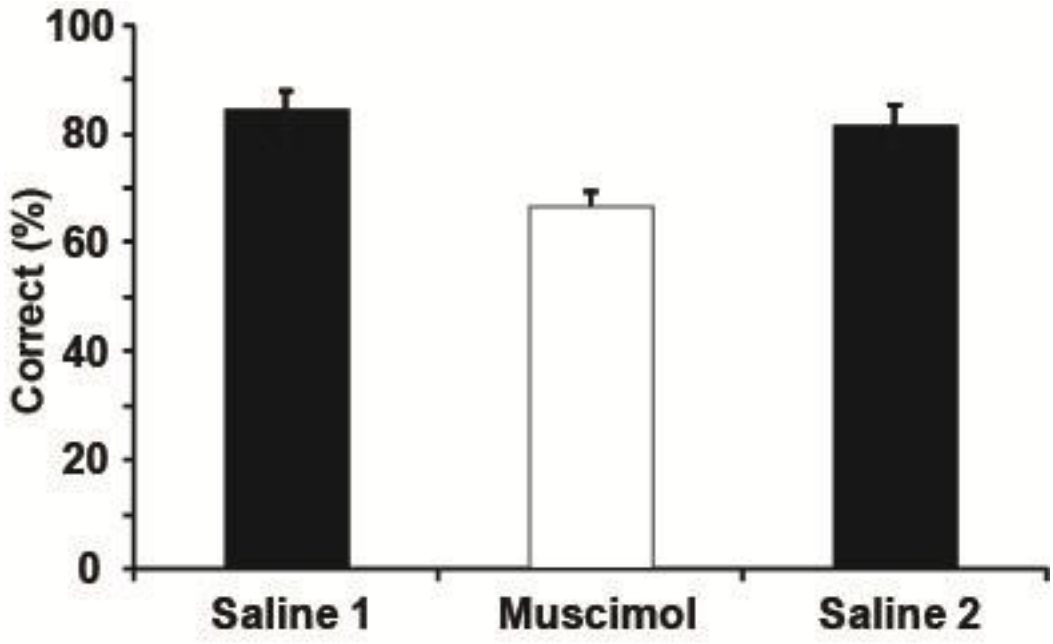

Figure 2: *the percentage of trials with a correct choice on the probe in the single context task during saline and muscimol sessions*

During testing in the dual context task, there were no differences in performance across the two contexts ($t_{(6)}$=0.46, p=.66) or across the two muscimol sessions ($t_{(6)}$=0.44, p=.67), so the percent correct data were combined to form saline and muscimol conditions which were compared with a paired samples t-test. The average data for each test session are shown in figure 3. Muscimol infusions significantly impaired performance on the dual context task ($t_{(6)}$=6.06, p<.001). In contrast to the single context task, performance on the dual context task dropped all the way to chance levels during the muscimol sessions (50.79±4.27% correct, which did not differ from chance, $t_{(6)}$=0.19, p=.86), suggesting that the temporary muscimol lesions caused a greater impairment than in the previous single context task. This was confirmed by a significantly greater lesion-induced decrement in performance in the dual context task than in the single context task (comparison of

difference scores computed for each subject by subtracting performance during the muscimol sessions from performance during the saline sessions, for the two tasks, $t_{(6)}=2.89$, $p<.05$).

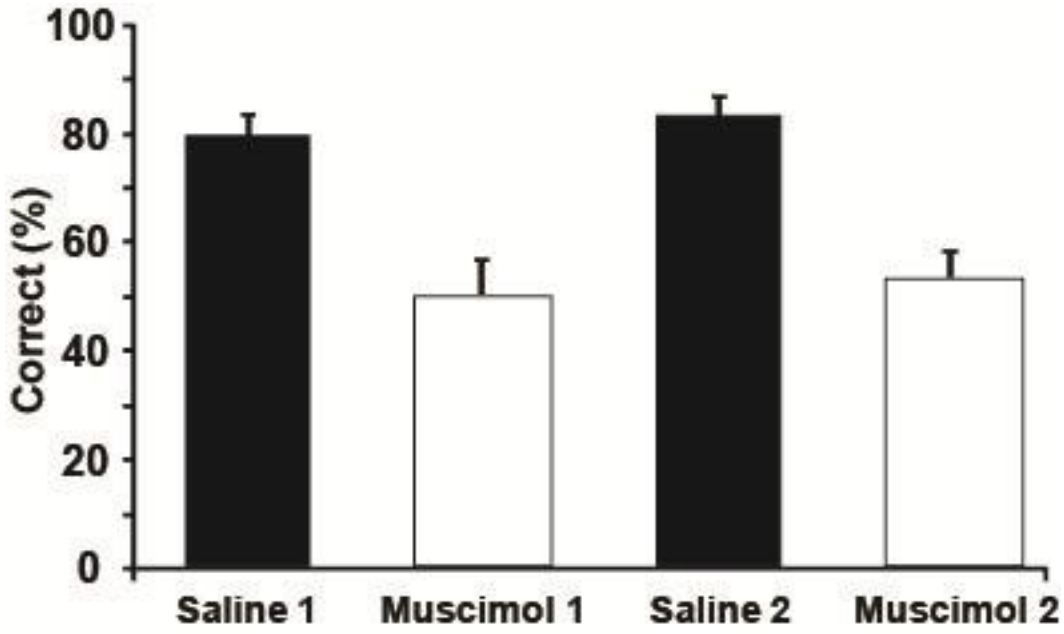

FIGURE 3: *The percent correct during saline and muscimol sessions of the dual context task. Note that each session (i.e. each bar in the plot) includes trials from each of the two contexts.*

Although previous studies with these procedures have shown that rats can't directly detect the buried reward (Butterly *et al.*, 2011), the rats of the present study were tested after the completion of training by presenting them 20 trials involving two randomly selected odors from the training list, but with only one of the cups baited. If the rats could detect the buried reward, they would be expected to choose the baited cup at a rate that was greater than chance. The rats chose the baited cup 49.0±4.19% of the time, which did not differ significantly from chance performance ($t_{(4)}=0.54$, $p=.62$).

Discussion

These results demonstrate that rats can learn a dual context version of the odor sequence task which involved learning a 7-item odor list and learning to follow different rules (pick the earlier or later odor) in separate contexts, within the same testing session. Thus, this task adds a context processing requirement to the well-known odor sequence learning task (Fortin *et al.*, 2002; Kesner *et al.*, 2002). Since episodic memory involves memory for the spatial context in which events occurred, these results are relevant to animal models of episodic memory and they join a growing literature indicating that the component memory processes that contribute to episodic memory are present in a variety of species (Babb & Crystal, 2006; Clayton & Dickinson, 1998; Eacott & Norman, 2004; Ergorul & Eichenbaum, 2004).

Consistent with previous studies that used permanent lesions (Fortin *et al.*, 2002; Kesner *et al.*, 2002), temporary inactivation of the dorsal hippocampus with muscimol caused a significant impairment in the single context version of the task. Interestingly, the rats with temporary lesions performed significantly above chance levels in the single context task, but the lesions completely abolished performance of the dual context version of the task in the same subjects. These results suggest that hippocampal lesions may cause significant deficits in tasks that require some of the components of episodic memory (e.g. what and where in the odor sequence task). However, the additional requirement of context-dependent expression of the 'what-when' memory made the task fully dependent on the hippocampus. The increased hippocampal role with the addition of episodic memory components supports

the well documented hippocampal role in episodic memory (e.g. Rosenbaum *et al.*, 2005; Vargha-Khadem *et al.*, 1997).

These results are also consistent with accounts of hippocampal function that emphasize its role in processing contextual information (e.g. Smith, 2008). As mentioned, episodic memories involve memory for the spatial context in which events occurred (e.g. at the office, in a restaurant, etc.) even when the details of the spatial geometry and the precise locations of events are lost. Context is therefore a natural way to construe the 'where' component of what-where-when models of episodic memory. However, we are cautious about suggesting that the present task constitutes a clear case of "what-where-when" memory. Because the contextual information was present at the time of the probe trials, the rats were not explicitly required to remember the context. Instead, the context may have served as a discriminative cue which was used to retrieve the appropriate rule (i.e. pick the earlier or later odor). Nevertheless, the rats did have to process and encode the context sufficiently for recognition and discrimination. Thus, the task involves the encoding and discrimination, if not the un-cued recall, of the spatial context component of episodic memory in addition to the 'what' and 'when' components. The use of different contexts as a component of episodic memory models is advantageous because associating specific memories with different contexts provides a means for subjects to minimize interference (Butterly *et al.*, 2011) and may therefore be a more manageable way for rodents to associate items and temporal aspects of experience with the location where they occurred.

This task can also be thought of as a special kind of conditional discrimination task, in which the predictive value of discriminative cues depends on the presence of another cue

(e.g. in the presence of X: A+/B-, in the presence of Y: B+/A-). The conditional cues (X and Y) can be individual stimuli, locations within an environment or different contexts. Interestingly, the role of the hippocampal system in these tasks has not been entirely clear, with some studies finding a lesion induced impairment (Smith, Wakeman, Patel, & Gabriel, 2004) (Lee & Solivan, 2010; Rajji, Chapman, Eichenbaum, & Greene, 2006) and others finding mild impairment or none at all (McDonald *et al.*, 1997; Sanderson, Pearce, Kyd, & Aggleton, 2006). Most of these tasks involve a single pair of discriminative cues (A and B) which have reversed predictive values depending on the conditional cue. In contrast, the present task requires that the rats use the conditional cue (the context) in order to retrieve the correct rule (pick the earlier or later odor) and apply it to the probe odors drawn from a sequence of seven odors. This added complexity, with the requirement to hold the 'what' and 'when' information in memory, in addition to using the context as a conditional cue, may account for the fact that performance dropped all the way to chance in the dual context task.

The critical role of the hippocampus in memory for individual items and events, when they occurred and the context in which they occurred is supported by neurophysiological data showing that hippocampal neurons respond to each of these components. Hippocampal neurons fire in response to a variety of task relevant events, including responses to various kinds of cues and reinforcers (e.g. Kang & Gabriel, 1998; Smith & Mizumori, 2006b; Solomon, Vander Schaaf, Thompson, & Weisz, 1986; Wood, Dudchenko, & Eichenbaum, 1999). Spatially localized firing patterns (i.e. place fields) are well known and, as discussed above, constitute a neural representation of the context

(Anderson & Jeffery, 2003; Nadel *et al.*, 1985; Smith & Mizumori, 2006a). Finally, recent data suggest that hippocampal firing is also sensitive to temporal aspects of experience, since hippocampal neurons fire in a temporally determined pattern (Gill, Mizumori, & Smith, 2011; Macdonald, Lepage, Eden, & Eichenbaum, 2011; Pastalkova, Itskov, Amarasingham, & Buzsaki, 2008) and hippocampal neuronal population responses evolve over time in a manner that could encode the temporal aspects of memory (Manns, Howard, & Eichenbaum, 2007). The present results suggest that better than chance performance can be maintained in the absence of hippocampal coding of some components (e.g. what and when) but that hippocampal processing is critical when the additional requirement of contextual discrimination is added.

CHAPTER 3

THE ROLE OF ADULT HIPPOCAMPAL NEUROGENESIS IN REDUCING INTERFERENCE

Introduction

Adult neurogenesis has emerged as an integral and crucial process within the hippocampus. Due to their strategic location in the dentate gyrus, new neurons are thought to play a key role in learning and memory as well as in some related hippocampal functions such as the regulation of drug addiction, emotions, and stress (Becker & Wojtowicz, 2007; Deng, Aimone, & Gage, 2010; Jacobs, van Praag, & Gage, 2000; Noonan, Bulin, Fuller, & Eisch, 2010; Schoenfeld & Gould, 2011). Computational studies predict a role for new neurons in pattern separation and interference reduction on the basis of unique properties of these cells (Becker, 2005). One such property is the ability of new neurons to undergo a complete turnover while they grow and become transformed from one developmental stage to another during the course of several days to weeks (Deng *et al.*, 2010). Behavioral tasks that included pattern separation have already been exploited in experiments (Clelland *et al.*, 2009; Creer, Romberg, Saksida, van Praag, & Bussey, 2010). These studies have shown that mice with reduced neurogenesis are impaired on such tasks. Paradoxically, Saxe et al (2007) observed improvement in a working memory task involving pattern separation. The discrepancy may be accounted for by the reliance upon different memory systems in these tasks, with the former (Clelland *et al.*, 2009; Creer *et al.*, 2010) being hippocampal-dependent, whereas the latter task (Saxe *et al.*, 2007) may rely more on extra-hippocampal working memory circuits in the prefrontal cortex. Nonetheless, all these tasks require the animal to

represent and separate events occurring almost simultaneously, or interleaved within a single experimental test session.

In contrast, Aimone et al (2006) and Becker and Wojtowicz (Becker, 2005; Becker & Wojtowicz, 2007) proposed another form of pattern separation, for events separated by days, specifically related to turnover of new cells. Our model predicts that hippocampal neurogenesis should be critical when subjects must form two distinct memories for highly interfering items as long as the two learning experiences occur at different times so that distinct populations of new neurons are available for the encoding of each item. In the present study, we directly test this prediction using a recently developed task that has those specific features (Butterly, Petroccione, & Smith, 2011). In this task, rats learn two highly interfering lists of odor pairs, one after the other in different contexts. For comparison, we also examined the role of neurogenesis in another hippocampal dependent task that we have used previously (blocked spatial alternation, Smith & Mizumori, 2006b) and that also involved learning interfering responses. Thus, the present study examined the role of adult neurogenesis in pattern separation and memory interference within and across experimental sessions. In addition, recognizing a dynamic, reciprocal relationship between learning and neurogenesis, we deployed a battery of tests to estimate the number of young neurons and their rate of proliferation and survival in relation to behavioral performance.

Materials and Methods: Animals and Time Line

Fifty four male Long-Evans rats were obtained from Charles River (Quebec) in 5 batches, at approximately every two months. The animals were three months old on arrival

and were kept in the animal facility at Guelph University (Ontario Canada) one week prior to any procedures. Rats in the irradiated group were anesthetized and underwent procedures for cranial irradiation at the Guelph Veterinary Clinic adjacent to the animal facility, as described previously (Winocur, Wojtowicz, Sekeres, Snyder, & Wang, 2006). Control rats were anesthetized but were not exposed to irradiation.

Ten rats (6 controls and 4 irradiated) served as untrained cage controls, and were kept in small animal cages at the University of Toronto for the duration of the experiment. They received injections of BrdU at 4 weeks prior to the perfusion in synchrony with the trained rats. The remaining 44 irradiated and non-irradiated animals were transported by air to Cornell University (Ithaca, NY, USA) for all behavioral tests and kept there until the experiments were completed. Behavioral training began 5 weeks after the irradiation. The rats (n=44) were first trained on the olfactory discrimination task, which lasted approximately 3 weeks. After completing the olfactory discrimination task, 10 of the rats (5 controls, 5 irradiated) were given injections of bromodeoxyuridine (BrdU) at 200 mg/kg (i.p.) and perfused 1 week later in order to determine whether olfactory training affects the subsequent production of new neurons.

One week after completing the olfactory task, 32 of the rats (8 rats in each of 4 groups, described below) began training on the plus maze task, which took up to 15 days to complete. One week prior to the plus maze task, the rats were injected with a single dose of bromodeoxyuridine (BrdU) at 200 mg/kg (i.p.) in order to determine whether plus maze training affects the survival of new neurons. The timing of the BrdU injection was planned specifically to detect changes in neuronal survival of 1-2 week old neurons during the plus

maze task as described previously for other hippocampal dependent tasks (Sisti, Glass, & Shors, 2007; Tronel *et al.*, 2010). Because the olfactory discrimination task occurred prior to the BrdU injection we did not expect the neuronal survival of the BrdU-positive cells to be affected by the olfactory training. The animal's health was monitored throughout the duration of the study. The weights of the control and irradiated rats were monitored and did not differ (control = 544.77±11.76, mean ± SEM; irradiated = 550.3±13.6 at the outset of training, $t_{(24)}$=0.31, p=.41). The animals were perfused exactly 25 days after BrdU injection, regardless of how quickly they reached the criterion in the plus maze task.

Perfusion, sectioning and sampling

Each animal was deeply anaesthetized with isofluorane and then intracardially perfused with 300ml 0.1M phosphate buffered saline (PBS, pH 7.4) followed by 200ml cold 4% paraformaldehyde (PFA, pH 7.4, 4 °C) in PBS. The brain was carefully removed from the skull and placed in 4% PFA at 4 °C for 24 hours. Later, the brain was stored in 0.1% sodium azide in PBS until sectioning. The right hippocampus from each animal was carefully isolated and coronally sectioned at 30μm thickness using a vibratome. Twelve sections were sampled evenly across the whole length of the hippocampus and stained for several markers of neurogenesis as described below.

In a subset of animals (5 controls, 5 irradiated) the left hemisphere was sectioned coronally at 40μm. Two sections from several regions of the brain were sampled representing the olfactory bulb (OB), the rostral migratory stream (RMS) and the subventricular zone (SVZ). Stereotaxic coordinates were +6.7mm, +4.2mm, +3.2mm, +0.7mm and –0.92mm (Paxinos & Watson, 1998).

CaBP/BrdU Immunohistochemistry.

Double labeling of BrdU positive cells with calcium binding protein (CaBP) was used to identify newly-born dentate granule cells. The hippocampal sections were incubated with 1N hydrochloric acid for 30 minutes at 45°C followed by three 5-minute washes. The sections were incubated with anti-BrdU primary antibody (rat, 1:200 in 0.3% Triton-X PBS, Serotec) for 24 hours at 4°C followed by three 5-minute washes. The sections were incubated with the secondary antibody (rabbit anti-rat IgG Alexa Fluor 488, 1:200 in 0.3% Triton-X PBS, Molecular Probes) for 2 hours at room temperature followed by three 5-min washes. Then, the sections were incubated with anti-calbindin primary antibody (rabbit, 1:200 in 0.3% Triton-X PBS, Chemicon) for 72 hours at 4°C followed by three 5-min washes. The sections were incubated with the secondary antibody (goat anti-rabbit IgG Alexa Fluor 568, 1:200 in 0.3% Triton-X PBS, Molecular Probes) for 2 hours at room temperature. Finally, the sections were washed three times and mounted on slides with mounting medium (Fluoromount, Sigma).

Doublecortin Immunohistochemistry.

Doublecortin (DCX) labeling was used to identify recently born neurons. Free-floating sections were incubated with a primary goat anti-DCX antibody (1:200, Santa Cruz Biotechnology, 24 hours at 4ºC), followed by Alexa488 donkey anti-goat secondary antibody (1:200; Invitrogen; 2 hours at RT). All antibodies were diluted in phosphate-buffered saline containing 0.03% Triton X-100.

Ki67 Immunohistochemistry.

Ki67 labeling was used to observe cell proliferation at the time of perfusion. Sections were incubated with anti-Ki67 primary antibody (rabbit, 1:200 in 0.3% Triton-X PBS, Vector Laboratories) for 18 hours at room temperature followed by three 5-minute washes. Then, the sections were incubated with secondary antibody (goat anti-rabbit IgG Alexa Fluor 568, 1:200 in 0.3% Triton-X PBS, Molecular Probes) for 2 hours at room temperature followed by three 5-min washes. Finally, the sections were washed three times and mounted on slides with mounting medium (Fluoromount, Sigma).

Cell counting.

In hippocampal sections the single-labeled cells were counted under a fluorescent microscope (40X) in the subgranular zone, excluding the upper and lower edges of the sections. The double-labeled cells (CaBP and BrdU) were counted using a confocal microscope (Leica). The total number of cells (per dentate gyrus) was obtained by multiplying the average number of cells per section by the total number of sections, in each animal.

In coronal sections used for estimates of cell number in the SVZ and RMS, cells expressing BrdU were counted within the region covered by DCX-positive cells. In the OB, BrdU positive cells were counted within 4 square visual fields (500x500 μm) located within the area covered by NeuN-positive cells. All counts were done on a fluorescent microscope. The cells numbers are given as densities (per mm^2 of area examined).

General Behavioral Methods and Rationale.

We trained rats on two different behavioral tasks that we have used previously and that have been shown to be hippocampal dependent in our laboratory (Butterly *et al.*, 2011; Smith & Mizumori, 2006a). In experiment 1, rats learned two lists of interfering odor pairs. They learned the first list over the course of several daily training sessions, followed by training on the second list during subsequent training days. In experiment 2, the rats were trained to remember and approach two different reward locations on a plus maze. Rewards were placed on the east arm for the first half of each training session and on the west arm for the second half, so the two reward locations were learned concurrently.

Methods for Experiment 1: Olfactory Discrimination Task

Subjects were 44 adult male Long-Evans rats. Prior to training, the rats were placed on a restricted feeding regimen (80-85% of free feeding weight). The rats were trained to dig in cups of odorized bedding material to retrieve buried food rewards (45 mg sucrose pellets, Bioserve, Inc., Frenchtown, NJ). All of the rats were first trained on one list of odor pairs. They were then given training on a second list of odor pairs either in the same context or a different context, yielding a 2X2 design with irradiation condition (control or irradiated) and context condition (same or different) as factors. One rat was excluded due to experimenter error in the training procedure, resulting in 11 rats in each group, except the control-different condition which had 10 rats.

The two contexts differed along the following dimensions: color of the chamber

(white or black), color of surrounding area (either an open experimental room with black painted walls or a 6x8 feet area enclosed by white plastic blinds), substrate in the chamber (uncovered Plexiglass floor or a black rubber mat), the 65 dB continuous background masking noise (white noise or pink noise) and the ambient odor left by wiping out the chamber with baby wipes prior to each training session (unscented or scented, Rite Aid, Inc.).

The rats were trained in Plexiglas chambers (45cm wide X 60cm long X 40cm deep) equipped with a removable divider, which separated the odor presentation area from an area where the rats waited during the intertrial interval. Odor cues were presented in ceramic cups (8.25cm in diameter, 4.5cm deep). The digging cups fit into circular cutouts cemented to the floor of the chamber to discourage the rats from moving the cups or tipping them over. Twenty-four pure odorants served as cues (for details see Butterly *et al.*, 2011). Briefly, the amount of each odorant was calculated so that it produced an equivalent vapor phase partial pressure when mixed with 50 ml of mineral oil (Cleland et al. 2002). 10 ml of each odorant solution was then mixed with 2 liters of corncob bedding material and stored in covered containers.

Prior to training, the rats were acclimated to each of the two contexts for two ten minute sessions in order to control for possible effects of novelty on neurogenesis. The rats were then shaped to dig in cups of bedding to retrieve buried rewards. After the rats had learned to reliably retrieve the rewards from the bottom of the cups, they began training on the first of two lists of odor pairs. Each list contained 8 odor pairs (16 individual odors). The two odors comprising each pair were always presented together, in separate cups. Within

each odor pair, one odor always contained a buried reward and the other did not. The predictive value of the odors (rewarded or non-rewarded) was counterbalanced across subjects and their presentation locations for each trial (left or right side of the chamber) were randomized. The daily training sessions consisted of 64 trials (8 trials with each odor pair, presented in an unpredictable sequence).

At the start of each trial, the experimenter placed the two cups containing the odorized bedding in the assigned locations (left or right) and removed the divider so that the rat could approach the cups. The rat was allowed to dig until he retrieved the reward. A digging response was recorded if the rat displaced any of the bedding, except when stepping into the cup without investigating. After consuming the reward, the rat was returned to the waiting area and the divider was replaced. During an inter-trial interval of approximately 10 seconds, the experimenter prepared the cups for the next trial. The rats were given daily training sessions on list 1 until they reached a behavioral criterion of 90% correct choices on two consecutive sessions.

After reaching the criterion, the rats were given 5 training sessions on a second list of 8 odor pairs. The rats were trained in either the same context where they learned the first list or in a different context. The training sessions for list 2 were carried out in the same manner as the list 1 training sessions, except that the second list contained 8 new odor pairs. In order to induce high levels of interference between the two lists, each of the new odor pairs for list 2 consisted of a novel odor and an odor which had previously been presented in list 1. Of the 8 odors taken from list 1, half had been rewarded previously and half had not. For example, if the first two odor pairs on list 1 were A+/B- and C+/D-, the first two odor

pairs on list 2 would be X+/A- and D+/Y-. This ensured that the rats could not adopt a strategy of simply approaching the novel odor (or avoiding the familiar odor) within each new odor pair.

Previous studies indicated that the rats could not smell the buried rewards (Butterly *et al.*, 2011). Nevertheless, a subset of the rats (n=18) were tested to ensure that the rats were not able to directly detect the pellets. After the completion of training, the rats were given a session consisting of 24 trials (3 trials with each of the 8 rewarded odors from list 2). On each trial, the rats were presented with two cups containing the same odor. However, only one of the cups was baited. If the rats could directly detect the pellets, they would be expected to perform better than chance (50%). The rats chose the baited cup 49.53% of the time, which did not differ from chance ($t_{(17)}$= .265, p=.80). The long-term effects of the olfactory task on neurogenesis were measured by estimating the number of proliferating, Ki-67-positive progenitors and immature, DCX-positive neurons.

Methods for Experiment 2: Plus Maze Task.

The subjects were 32 adult male Long-Evans rats which had previously been trained in the olfactory discrimination task for experiment 1. The rats were trained to approach the east arm of a plus maze for reward during the first half of each training session and to approach the west arm during the second half. In this experiment we sought to determine whether suppression of neurogenesis would impair learning in this task and whether training on this task would increase the survival of new neurons, as has been reported in other hippocampal dependent tasks (Epp, Haack, & Galea, 2011; Sisti *et al.*, 2007; Tronel *et al.*,

2010). To this end, all of the rats were given a single injection of BrdU (200 mg per kg i.p.) 7 days prior to beginning training. In order to control for exercise, handling and exposure to the rewards, the rats were divided into two groups. One group received regular training sessions and the other served as a yoked control group in which each rat was given the same number of sessions as a trained rat, but they were given control sessions which did not permit learning about predictable reward locations (described below). If learning to discriminate the go east and go west trials induced neurogenesis that was greater than that seen in the yoked controls, then the neurogenesis could not be attributed to factors other than learning.

The rats were trained on a plus maze (102 cm across) that occupied a circular area enclosed by curtains (3 m in diameter). Distinctive objects were attached to the curtain to serve as distal visual cues. Prior to training, they were given several sessions in which they were acclimated to the plus maze and trained to retrieve chocolate milk rewards (0.2 ml Nestle's Quik) from cups at the ends of the maze arms. The rats in the trained condition were then given daily training trials consisting of 2 blocks of 15 trials each. During the first block of every training session, the reward was always placed at the end of the east arm. During the second block, the reward was always placed at the end of the west arm. Trials began when the rat was placed on the maze facing outward at the end of an arm and ended when the rat arrived at the reward. During an intertrial interval (ITI) of approximately 20 seconds, the rats were placed on a platform adjacent to the maze. The position of the ITI platform was constant throughout training. The start positions for each trial were randomly designated from among the 3 non-reward arms. Training continued with the same two

reward locations presented each day until the rats attained a behavioral criterion of at least 75% correct choices on two consecutive sessions. Training was discontinued if the rat did not achieve this criterion in 15 days.

Each rat in the yoked control condition was given the same number of sessions as his trained counterpart. The training trials for the yoked control rats were identical to those described above, except that instead of placing the rewards in predictable locations (i.e. the east and west arms, as above), the rewards were placed on randomly designated arms. Control rats choose the rewarded arm on 64.3% of the trials, on average, over all training sessions. The training procedures for the yoked controls were designed to approximate this as closely as possible. On each trial, two randomly designated arms were baited (although the rat was only allowed to run until he found one of the rewards). With 2 of the 3 non-start arms baited, the rats pick a rewarded arm 66.6% of the time by chance. Additionally, analysis of the total number of arm entries confirmed that trained rats and yoked controls ran similar distances on the maze ($F[1,28]=0.71$, $p=.41$). Thus, the yoked control rats were given the same number of training trials and rewards, but they could not learn to remember and approach two different predictable reward locations.

Results: Effects of Irradiation on Neurogenesis.

Neurogenesis was selectively reduced in the dentate gyrus (DG) but not in the olfactory tract at 9 weeks and 14 weeks after irradiation (end of the study). A comparison of cell densities at 9 weeks (n=5 per group) and at 14 weeks (n=16 per group) showed a significant effect of irradiation (DCX, $F[1,41]=45.936$, $p<0.001$) but no main effect of time

(F[1,41]=3.82, p=0.058). The average reduction of neurogenesis after 14 weeks, as measured by the number of DCX+ new neurons, was 85% ($t_{(30)}$=9.290, p<0.001). In contrast, there was no effect of irradiation on neurogenesis in the olfactory tract. The density of BrdU+ cells counted in 5 regions of the olfactory system was not affected by the irradiation procedures (ANOVA with irradiation condition and location showed no effect of irradiation, F[1,110]=0.331, p=0.556, and no interactions of the irradiation and location factors, Fig. 4).

Figure 4: (

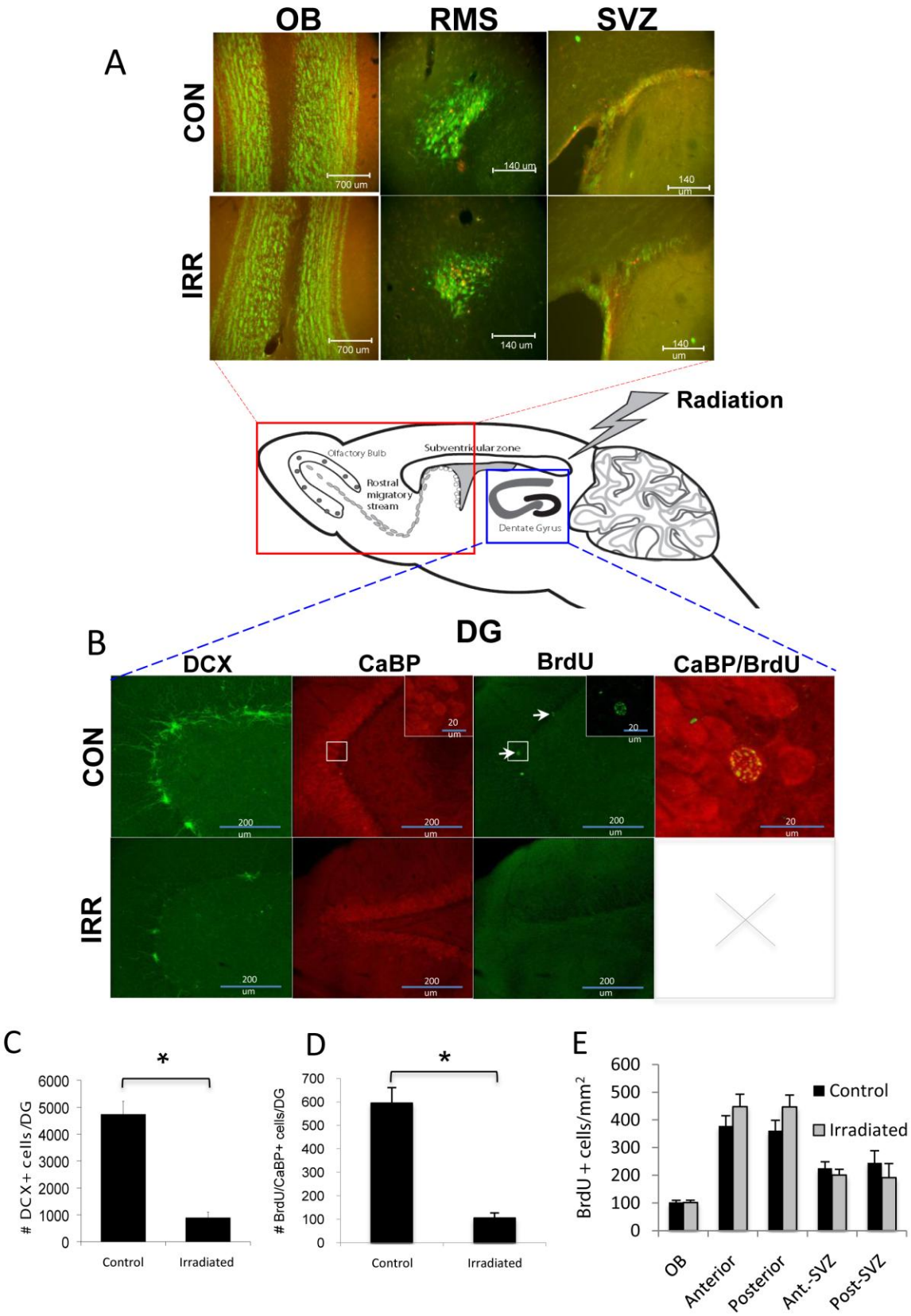
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Figure 4: *Two neurogenic regions in the rat brain. Images in A show staining for NeuN (green) and BrdU (red) in the olfactory bulb (OB), and DCX (green) and BrdU (red) in the rostral migratory stream (RMS) and the subventricular zone (SVZ). Images in B show staining for DCX, CaBP, and BrdU as indicated in the dentate gyrus (DG). Irradiation was applied selectively to the rear of the brain in order to reduce neurogenesis in the DG but not in the olfactory system. There was too little CaBP/BrdU to image in irradiated rats. Irradiation caused an 85% reduction in neurogenesis in the dentate gyrus (plot C, D), but had no effect on any of the olfactory regions (plot E).*

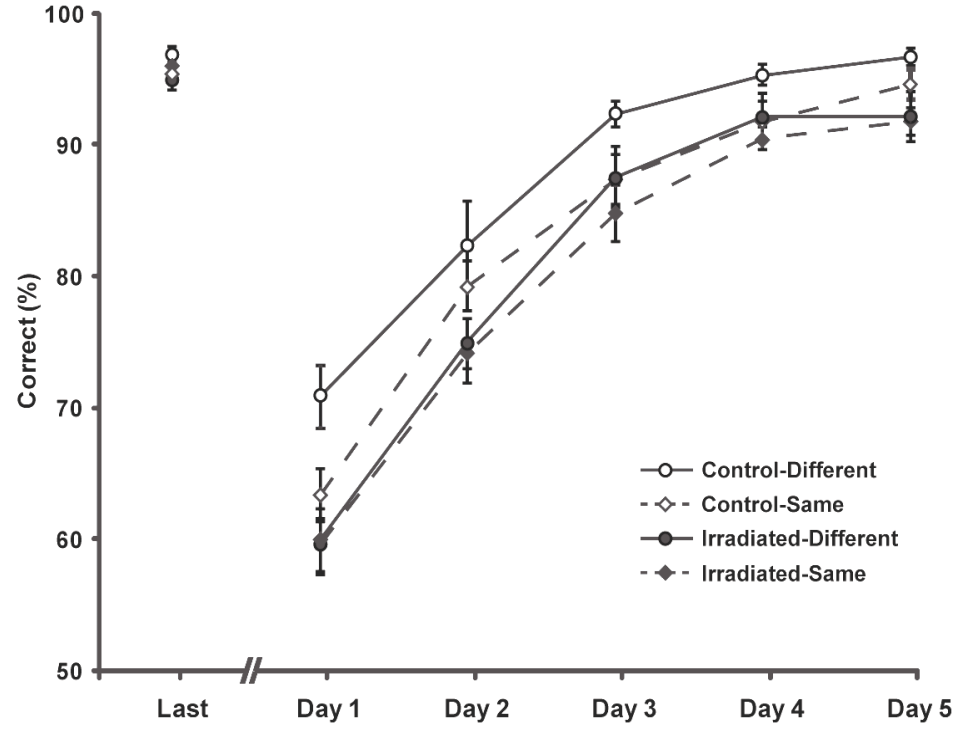
Results for Neurogenesis Experiment 1: Olfactory Discrimination Task

As expected, suppression of neurogenesis did not impair learning of the first list of odors. Control and irradiated rats did not differ in the number of training sessions needed to reach the criterion (irradiated mean = 4.41 sessions, control mean = 4.14 sessions, $t_{(41)}=1.06$, p=.30) and there were no differences between control and irradiated rats in terms of their performance on the final training session of list 1 (F[1,39]=2.05, p=.45). Importantly, these results indicate that the irradiated rats did not have a general impairment in olfactory sensory processing or olfactory learning.

However, suppression of neurogenesis did cause a significant impairment in performance on the second list. The percent correct on each day of training were submitted to a repeated measures ANOVA with training session (5 days of training on list 2) as the within subject factor and irradiation condition (Control or Irradiated) and context condition (Same or Different) as between subjects factors (Fig. 5). This analysis revealed a significant main effect of training session (F[1,39]=12.76, p<.001), a significant main effect of irradiation (F[1,39]=12.76, p<.001), with controls performing significantly better than irradiated subjects, and a main effect of context (F[1,39]=4.09, p<.05), with subjects in the

different context condition performing significantly better than subjects in the same context condition. However, there was no significant interaction of the context and irradiation conditions ($F[1,39]=1.52$, $p=.23$). This result suggests that the suppression of neurogenesis impaired performance regardless of the context condition, in contrast to our previous findings that muscimol lesions of the dorsal hippocampus selectively impaired performance in the different context condition but not in the same context condition (Butterly *et al.*, 2011). Whereas the muscimol lesions had no impact on performance in the same context condition, the suppression of neurogenesis may have had more widespread effect on performance, including impairment in the same context condition. This may have occurred because neurogenesis was suppressed throughout the hippocampus, whereas the muscimol lesions were specific to the dorsal hippocampus. We revisit this issue in the general discussion.

Figure 5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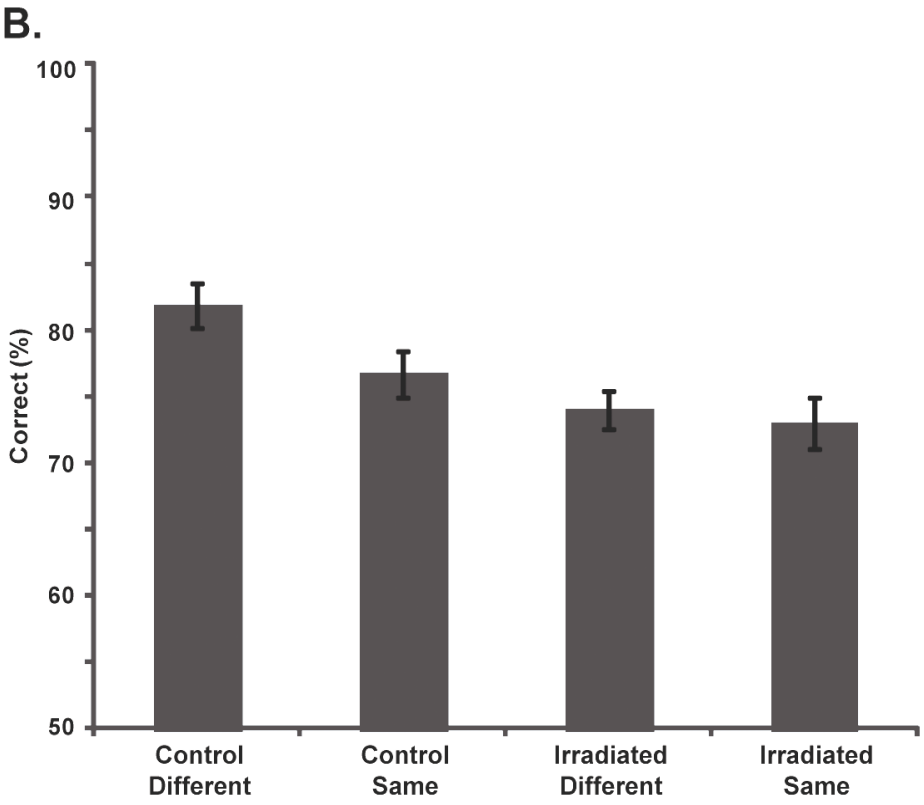
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Figure 5: *Olfactory discrimination performance. In panel A, the average percent correct choices are shown for control (open symbols) and irradiated rats (filled symbols) and for the different context (solid lines) and same context conditions (dashed lines). Performance data are shown for the final session of list 1 training (Last) and the five training sessions of list 2. In panel B, the same data are shown averaged across all five days of training on list 2.*

Assessment of Interference in Each Condition

Our hypothesis was that new neurons play a beneficial role in learning because they provide a means of overcoming interference for items learned at different times. The effects of interference can be assessed by comparing performance on the two lists. When there is little opportunity for interference (e.g. when learning non-interfering material), performance on the second list is facilitated by prior experience on the first list (Butterly *et al.*, 2011). However, if proactive interference occurs, performance on the second list will not be facilitated and can even be impaired by prior learning on the first list. Since interference is typically most pronounced during the initial stages of learning, we compared performance during the first three sessions of list 2 to performance during the same sessions of list 1 (Fig.6). A similar pattern of results was seen when all five sessions of list 2 were analyzed.

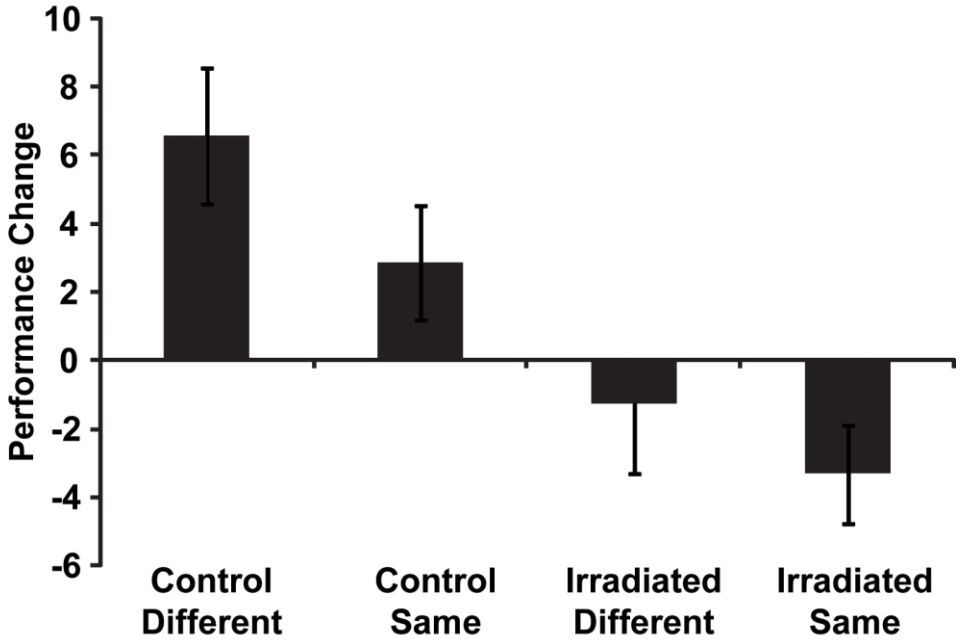

Figure 6: *Neurogenesis and Interference. Change in performance from list 1 to list 2, computed as the average percent correct during the first three sessions of list 2 minus the average percent correct during the first three sessions of list 1, is shown for each of the experimental groups. Facilitation is indicated by better performance on list 2 than on list 1 (positive values) while interference is indicated by worse performance on list 2 (negative values).*

Control rats that learned the two lists in different contexts performed significantly better on the second list than on the first (paired samples t-test: $t_{(9)}$=-3.96, p<.005). That is, when contextual information was available to disambiguate the two conflicting lists, control rats did not experience interference and performance was facilitated on the second list. In contrast, control rats that learned the two lists in the same context showed no such facilitation (i.e. no significant change in performance from list 1 to list 2, $t_{(10)}$=1.70, p=.12). Irradiated rats were not able to use contextual information to disambiguate the two lists and performance was not facilitated on list 2 ($t_{(10)}$=0.61, p=.56). Interestingly, irradiated rats in

the same context condition showed an even greater evidence of interference, as performance was significantly worse on list 2 than list 1 ($t_{(10)}$=2.33, p<.05).

Measures of neurogenesis for the rats in the olfactory discrimination experiment were subjected to an ANOVA with irradiation condition and training condition (trained or untrained cage controls) as factors (Fig. 7). Rats in the same context and different context training groups did not differ in terms of neurogenesis (e.g. DCX, F[1,37] = 0.72, p =0.40) so these groups were combined for these analyses. Although there was a main effect of irradiation (DCX labeled cells, F[1,39] =65.22, p<0.0001), there was no effect of training condition (DCX, F[1,37] = 0.06, p = 0.82) nor was there an interaction of the irradiation and training condition factors (F[1,37] = 2.846, p = 0.10). Analysis of the Ki67-positive cells revealed similar results. Thus, the olfactory learning did not have long-term effects on levels of neuronal production in terms of proliferation or neuronal differentiation.

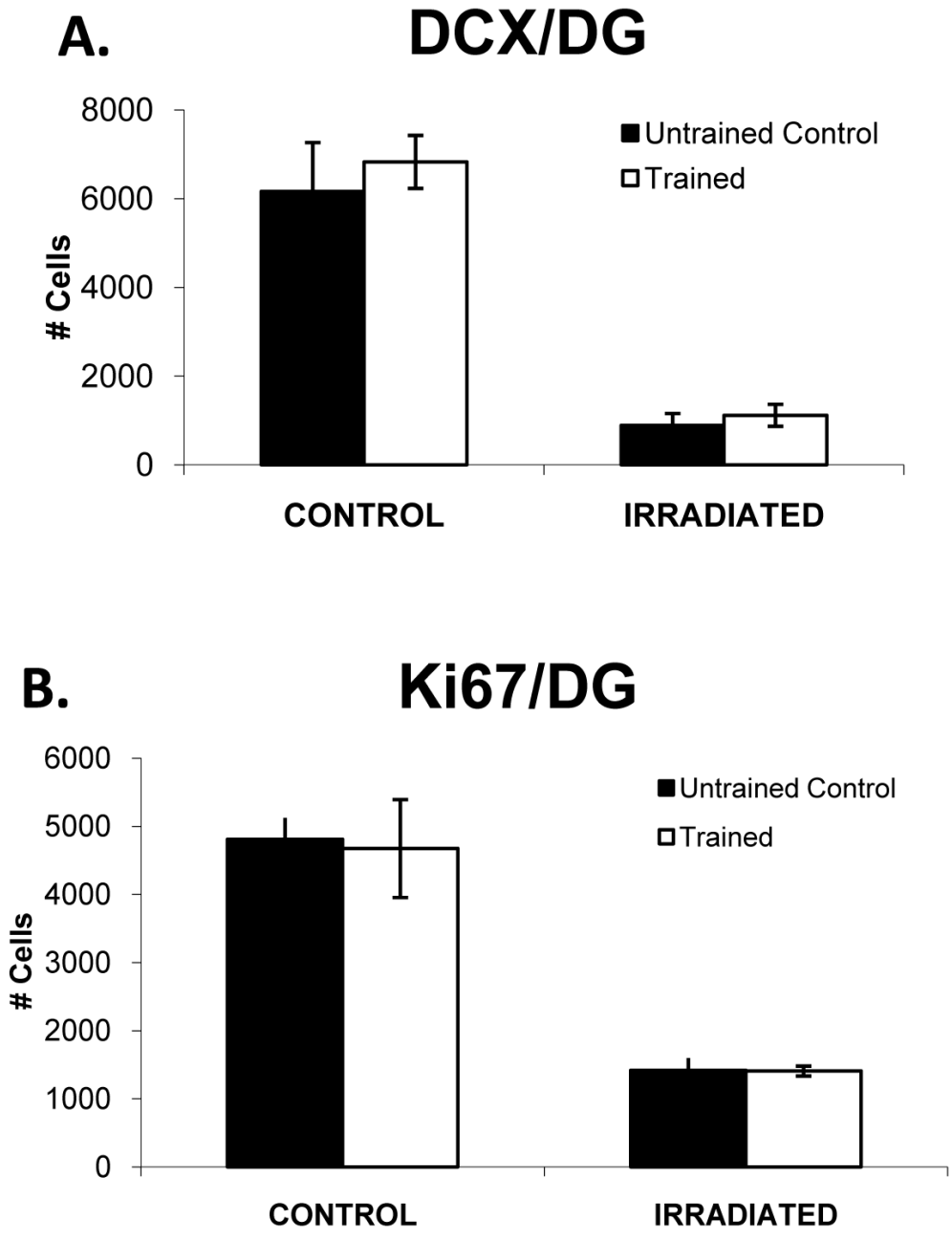

Figure 7: *Olfactory discrimination training and neurogenesis. Estimates of neurogenesis in rats trained in the olfactory discrimination task and untrained control rats are shown for irradiated and control subjects. The number of DCX+ cells per dentate gyrus is shown in A and the number of Ki67+ cells is shown in B. The trained group includes rats from the different context and same context training conditions since these groups did not differ.*

Results for Neurogenesis Experiment 2: Plus Maze Task Behavior.

In contrast to the odor task, irradiated rats showed no evidence of impairment on the plus maze task. Control and irradiated rats did not differ in terms of the number of sessions needed to reach the behavioral criterion (control mean = 7.5 sessions, irradiated mean = 9.0 sessions, $t_{(16)}$=.614, p=.55, Fig. 8a). Two control rats and 3 irradiated rats failed to reach the criterion within the 15 sessions that were given. Control and irradiated rats also exhibited similar levels of performance throughout training. To assess this, the percentage of trials with a correct response were submitted to a repeated measures ANOVA with group (control and irradiated) as a between subjects factor and training stage as a within subjects factor (3 stages, including the first, middle and last training sessions). The rats took a variable number of training sessions to reach the criterion, so the middle training session was simply the session that was half way between the first and last session. For those rats that received an even number of sessions, the average of the two middle-most sessions was used. For example, for a rat that required 12 sessions to reach the criterion, the average performance on sessions 6 and 7 was used as the middle session. This analysis showed no difference between groups (F[1,14]=0.16, p=.69) and no interaction of the group and training stage variables (F[1,28]=0.75, p=.48, Fig. 8b). The latency to reach the reward and various measures of inflexible behavioral responding (e.g. right or left turn biases) were also assessed and no group differences were found.

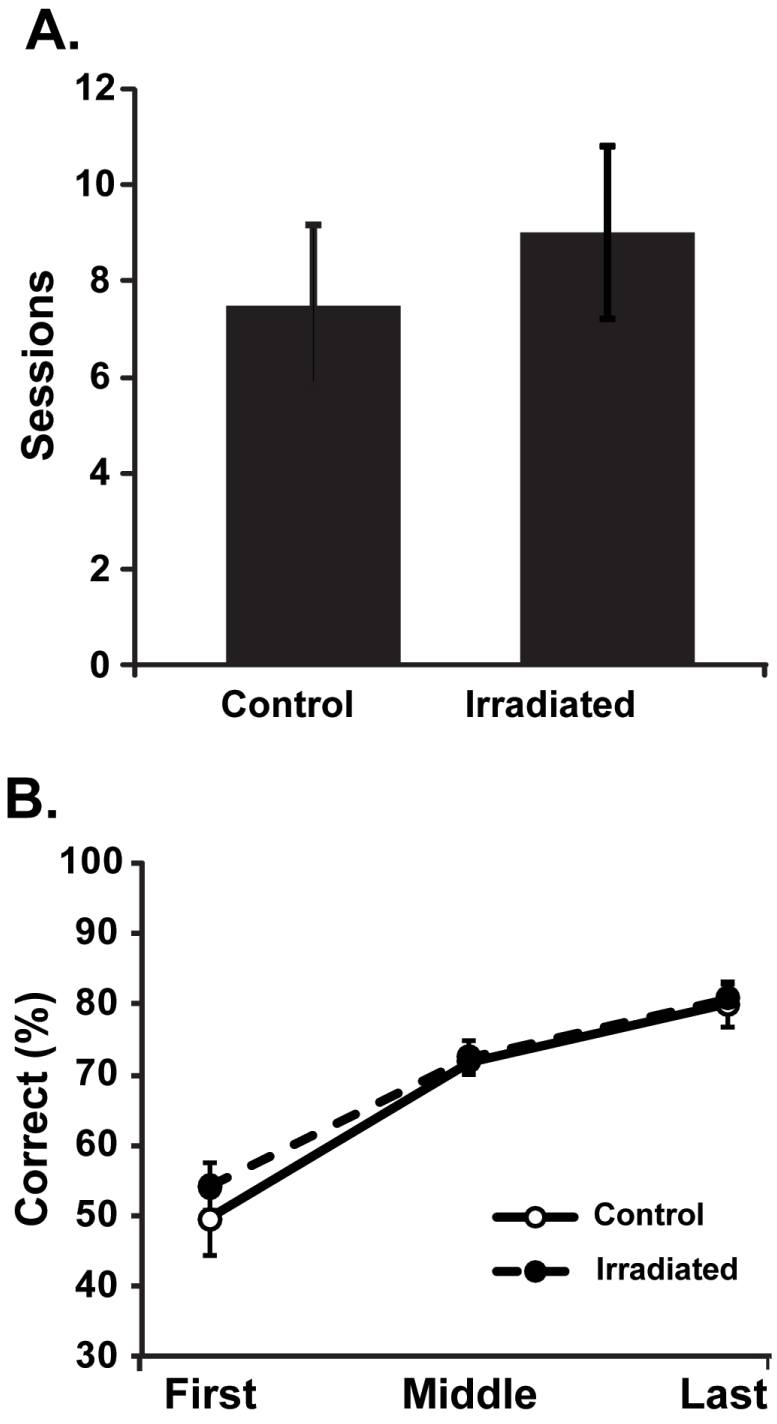

Figure 8: *Plus maze performance. The number of sessions needed to reach the behavioral criterion in the plus maze task are shown in A. Plot B illustrates the percentage of trials with a correct response for control (open) and irradiated rats (filled). Data are shown for the first training session, the session midway through training and the final training session.*

Effects of Maze Training on Neurogenesis and Survival.

Neurogenesis was compared among all animals participating in the plus maze during weeks 9-14 of the study. There was a clear effect of irradiation but none of the markers (BrdU, DCX, Ki67) revealed any differences between trained rats, yoked controls, and cage controls. This lack of differences held for both non-irradiated and irradiated animals (Fig. 9).

Measures of neurogenesis (BrdU, DCX, Ki67) for the rats trained in the plus maze task were subjected to an ANOVA with irradiation condition and training condition (3 groups: trained, yoked controls and untrained caged controls) as factors. One irradiated and 3 non-irradiated animals were excluded from immunohistochemical analysis due to poor fixation of the tissue. There was a significant main effect of irradiation on the survival of BrdU+ cells born 1 week prior to the beginning of learning ($F[1,40]=40.354$, $p<0.0001$), but there was no effect of training condition ($F[1,40]=0.311$, $p=0.51$) nor was there an interaction of irradiation condition and training condition ($F[1,40]=0.162$, $p=0.162$). Similar results were seen in the rate of maturation, as indicated by CaBP/BrdU labeling (main effect of irradiation: $F[1,37]=26.253$, $p<0.0001$, all others n.s.) and in DCX labeled cells (main effect of irradiation: $F[1,39]=96.986$, $p<0.0001$, all others n.s.).

Figure 9

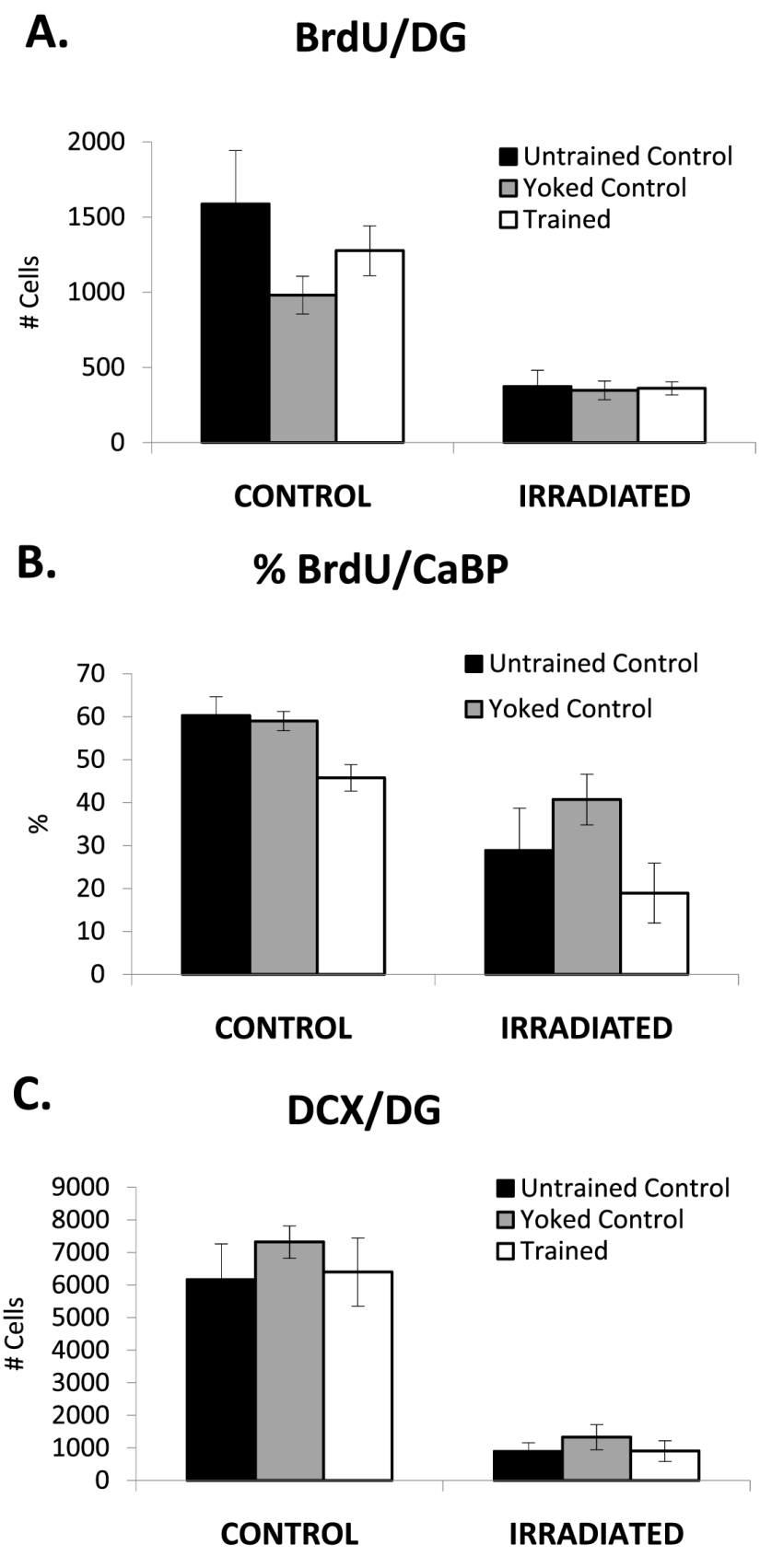
A.
BrdU/DG
2000
1500
1000
500
0
Cells
Untrained Control
Yoked Control
Trained
CONTROL
IRRADIATED
B.
% BrdU/CaBP
70
60
50
40
30
20
10
0
%
Untrained Control
Yoked Control
CONTROL
IRRADIATED
C.
DCX/DG
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Cells
Untrained Control
Yoked Control
Trained
CONTROL
IRRADIATED

Figure 9: *Plus maze training and neurogenesis. Effects of spatial learning on neurogenesis are shown for rats trained in the plus maze task, for yoked controls (see Methods for details) and for untrained, cage controls. The survival of BrdU+ cells born 1 week before the start of training was not affected by learning (trained group) nor was it affect by handling or exposure to the training apparatus (yoked controls), compared to the untrained controls (plot A). Similarly, the rate of maturation as indicated by CaBP/BrdU labeling was the same in trained rats, yoked controls and untrained controls (plot B). The density of DCX+ cells was also the same in trained rats, yoked controls and untrained controls (plot C).*

Discussion

Suppression of hippocampal neurogenesis significantly impaired performance on the olfactory discrimination task. Although performance on the first list was entirely unaffected by the loss of neurogenesis, the rats performed significantly worse than controls when they were confronted with a second list of interfering items and irradiated rats experienced significantly more interference than controls. These results therefore support accounts which suggest that hippocampal neurogenesis plays a critical role in mitigating interference (Aimone *et al.*, 2006; Becker & Wojtowicz, 2007).

Interestingly, irradiation produced no impairment in the plus maze task. Although the olfactory discrimination task and the maze task differ in a number of ways, both tasks are impaired by temporary inactivation of the hippocampus (Butterly *et al.*, 2011; Smith & Mizumori, 2006a) and both tasks induce interference. One potentially important difference between the two tasks is the different time courses for learning the interfering items. In the odor discrimination task, the rats learned the two lists of interfering items sequentially, over the course of several days. In contrast, the competing responses of the maze task were trained concurrently with both responses rewarded within each training session. Thus, the plus maze places a high demand on spatial working memory, requiring the animal to

remember the current reward location and to ignore the other location. The lack of an impairment in the irradiated animals on this task is consistent with the finding that rodents with reduced neurogenesis actually outperform controls on a working memory version of the 8-arm radial maze (Saxe *et al.*, 2007). On the other hand, the impairment seen in the sequentially learned olfactory task supports the hypothesis that the gradual addition of new neurons is an important mechanism for differentially encoding potentially interfering memories more widely separated in time (Becker & Wojtowicz, 2007).

Specifically, we proposed the cohorts of newly-born neurons to be selectively sensitive to the incoming perforant path synaptic inputs and that they transmit the signals encoding common experiences to CA3. Following a relatively brief sensitive period of reduced firing thresholds and heightened plasticity, the cohort would progress to a further state of maturation wherein neurons are less responsive to afferent stimulation, permitting the next wave of young adult neurons to be preferentially recruited. The synaptic mechanisms responsible for the sensitive period include reduced GABA-ergic inhibition and enhanced NMDA-dependent plasticity (Becker & Wojtowicz, 2007; Deng *et al.*, 2010; Snyder, Kee, & Wojtowicz, 2001). This putative mechanism causes similar events spaced across several days to be encoded by distinct populations of young dentate gyrus neurons. The young neurons in turn contribute to distinct memory traces being formed in downstream regions. In contrast, the maze task may not benefit from neurogenesis because concurrently learning the competing responses does not allow distinct neural populations to differentially encode them.

The odor task was specifically designed to induce interference through the use of overlapping odors on the two lists. Nevertheless, interference is also an important aspect of the maze task. It involves serial reversals of the 'go east' and 'go west' rules, which produce strong interference, and the choice point presents the rat with an array of cues that have been associated with both reward locations. This likely leads to intrusions of the memory for the incorrect reward location and most errors consisted of entries into the incorrect reward arm, rather than random entries into arms that were never rewarded (data not shown). Thus, both tasks involve interference.

Importantly, however, the type of interference differs in the two tasks. In the odor task, similar patterns (overlapping odor pairs) must be mapped to different responses. If the rat can encode the odor pairs learned in the two lists as separate events, using new neurons to generate distinctive memory traces for the overlapping inputs, the task of learning the correct response to an overlapping odor pair becomes greatly simplified as the overlap has been reduced. On the other hand, for the spatial reversal learning task, a single spatial location (the choice point) must be associated with multiple competing responses. The interference cannot be resolved by separating similar inputs, but requires learning the reward value of alternative responses to a given input. These observations suggest that the presence of interference, by itself, is not sufficient to engage neurogenesis dependent pattern separation processes. Instead, neurogenesis may be specifically beneficial for resolving the interference arising from overlapping inputs, particularly when the memories are acquired over the course of a sufficiently long timeframe.

Of course, the olfactory task and the maze task differed in other ways. For example the two tasks differ in terms of modality (visuospatial versus olfactory). However, the impairment seen in the olfactory task was not likely due to general olfactory processing deficits, since irradiation did not cause damage to the rostral migratory stream leading to the olfactory bulb, and the irradiated rats were entirely unimpaired in learning the first list of eight odor pairs. The spatial component of the maze task is probably not an important factor in the differential effects of the irradiation on the two tasks since neurogenesis has been shown to be important for some spatial tasks (Clelland *et al.*, 2009). The observation that olfactory tasks are not consistently impaired and spatial tasks are not consistently spared, suggests that modality is not the critical factor. Moreover, the fact that the two tasks are hippocampal dependent indicates that they both engage general hippocampal mechanisms despite the modality differences.

The two tasks of the present study also differed in terms of contextual manipulations. The olfactory discrimination task involved an explicit manipulation of the environmental context whereas the maze task did not. However, our results suggested that irradiation impaired performance regardless of the contextual manipulation. This result stands in contrast to our previous finding that temporary muscimol lesions of the dorsal hippocampus selectively impaired performance in the different context condition but not in the same context condition (Butterly *et al.*, 2011). In that study, the muscimol lesions had no discernible effect on performance in the same context condition, suggesting a highly specific deficit in the ability to use contextual information to resolve interference. The present results

suggest that hippocampal neurogenesis may play a more general role in resolving interference regardless of whether there is an environmental contextual component.

The present results also raise the possibility that hippocampal neurogenesis may play an important role in a different kind of context. Ongoing neurogenesis may provide an internal context that gradually varies over time, allowing overlapping events to be separated into distinct memory traces when they are well separated in time, even in the absence of differentiating environmental contexts. This is consistent with the idea that ongoing neural processes form an ever changing temporal context and that individual events are embedded within this temporal context in a manner that allows for distinct representations for similar events that occur at different times (Manns, Howard, & Eichenbaum, 2007; Polyn & Kahana, 2008). As mentioned above, the recruitment of new neurons may serve to tag each memory trace with its own unique temporal context, thereby reducing interference (Aimone *et al.*, 2006; Becker, 2005; Becker, Macqueen, & Wojtowicz, 2009).

In the present study, irradiated rats exhibited proactive interference from previously learned odor pairs. Interestingly, Winocur and colleagues (2012) demonstrated retroactive interference in irradiated animals. Specifically, after animals learned distinct stimulus-response associations based on black versus white visual cues, only those subsequently given a "confusing" interfering event, a grey cue that had no predictive value for reward, later showed impaired performance on the original black-white discrimination. Their findings were interpreted as supporting the notion that intact animals used new hippocampal neurons to form separate contextualized memories for the original and interfering events, whereas irradiated animals may have relied upon a striatal stimulus-response strategy. However, they

did not exclude the possibility that the irradiated animals showed stimulus generalization after exposure to the grey cue and were no longer able to maintain the distinction between the original black and white cues. Such generalization could have accounted for their results. Our present experiments deal specifically with this possibility by showing that neurogenesis was critical for mitigating interference between overlapping associations learned at different times, and was not due to a basic deficit in olfactory stimulus discrimination.

As mentioned above, learning in the plus maze task was not impaired by irradiation. In addition, our experiments failed to show any reciprocal effect of learning on neurogenesis. There were no differences in the numbers of new neurons between rats trained in the olfactory task and untrained controls. However, since the measurements were performed at the end of the study, after the olfactory and plus maze tasks were completed, any effect of olfactory training may have been obscured by the subsequent maze training. Nevertheless, olfactory training did not produce an effect on neurogenesis that was so large that it could be detected even after maze training. However, recent studies (Dupret *et al.*, 2007; Epp, Spritzer, & Galea, 2007) suggest that the effects of training on neuronal survival may be subtle, with training-induced neuronal survival being restricted to specific phases of learning. One week old cells may survive at a higher rate in trained rats, while other cells born during later phases of training may show reduced survival, resulting in no net effect.

In summary, the results of this study confirm that new neurons are involved in hippocampal dependent processes that resolve memory interference. At the mechanistic level, interference between successive learning episodes may be related to the mechanism of

pattern separation as proposed by theoretical models (Aimone, Wiles, & Gage, 2009; Becker & Wojtowicz, 2007). Further experimentation exploring these ideas seems warranted.

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