THE ROLE OF ADULT-DERIVED NEURONS

IN LEARNING AND MEMORY

by

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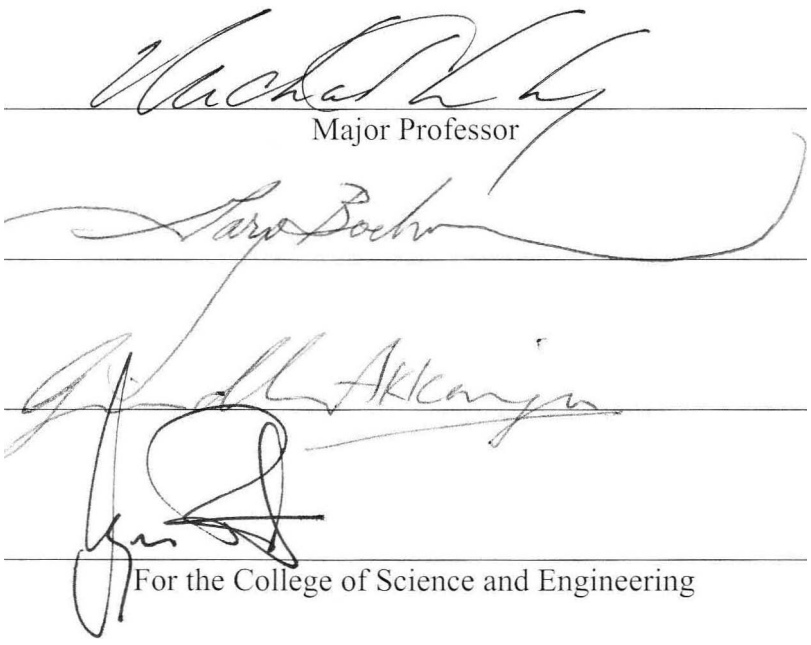

Major Professor

For the College of Science and Engineering

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I. INTRODUCTION:

The hippocampus is an important anatomical structure in the brain and is located in the medial temporal lobe. The hippocampus plays an important role in learning (Gould et al., 1999), as well as spatial and recognition memory, particularly declarative memory – the memory reserved for facts and events (Broadbent et al., 2004; Dupret et al., 2007; Squire et al., 2004). The study of the famous patient H.M., who had bilateral surgical removal of the medial temporal lobe, revealed how necessary the hippocampus is to acquire new memories (Squire and Zola-Morgan, 1991). In order for cortical activity to be converted into stable long-term memory, the hippocampus must be activated at the time of learning. The hippocampus is important for the acquisition of new facts and events, which can then be recalled later (Squire and Zola-Morgan, 1991).

The hippocampal formation is made up of two types of cells, pyramidal neurons and granule cells, which form layers that wrap around each other. The pyramidal neurons originate in the ventricular germinal layers located below the ventricular wall along the CA1 area, and migrate a short distance directly from here to their final target region. These neurons are generated between days E10 and E18 of the mouse. At birth, the pyramidal cell layer of the mouse hippocampus is a 6 to 10 cell body thick region with little organization. Postnatally-generated glial cells contribute to both an increase in hippocampal volume and a decrease in the thickness of this region as the pyramidal layer begins to contract.

Granule cells appear at approximately the same time in development, but take about three times longer than pyramidal neurons to generate. It has been hypothesized that the germinal layer that gives rise to the dentate gyrus (DG) is different from that which forms the pyramidal neurons. While this has not been proven, it is well known that at birth in the

rodent, the hilus is populated by a large number of postmitotic cells and these cells quickly migrate a short distance and compact to form the dentate gyrus. During this time, ventricular neural cells are thought to migrate to the hilar region and remain there into adulthood, allowing for the generation of new granule cells throughout the animal's life in a process known as adult neurogenesis. When the hippocampus begins making its connections, three processes occur: axonal pathfinding, target recognition, and synapse formation. In axonal pathfinding, an axon navigates to its target region by following pioneer neurons that provide a template. In target recognition, the axon must recognize the correct target region and cell, before the synapse formation occurs (Amaral et al., 2006). Inputs to the hippocampus follow the perforant pathway (see Figure 1): cells from the entorhinal cortex provide the input to the

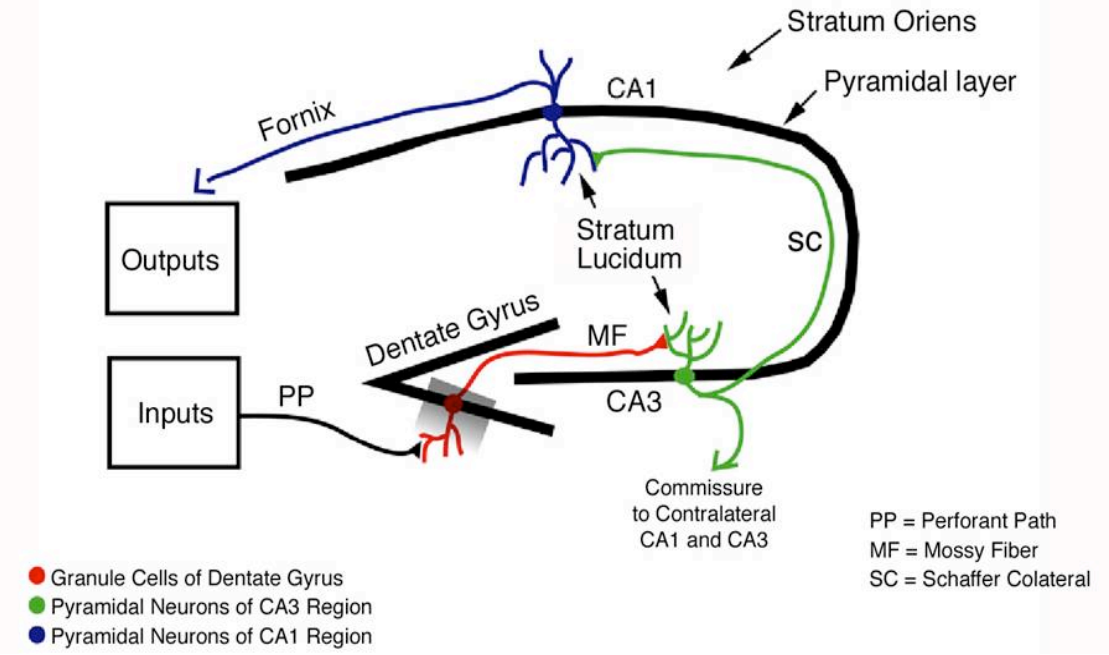

Figure 1. Pathways of the hippocampus. Inputs to the hippocampus arrive from the entorhinal cortex via the perforant pathway, and synapse on the granule cells of the dentate gyrus. These neurons, in turn, synapse on the pyramidal cells of the CA3 region, which follow the Schaffer Collateral to the pyramidal neurons of the CA1 region. These cells then send their outputs through the fornix.

granule cells. Mossy fibers, axons of the granule cells, synapse on the CA3 pyramidal cells, which in turn synapse onto CA1 pyramidal cells via the Schaffer collateral (Christie and Cameron, 2006).

In the adult hippocampus, neurogenesis continues throughout adulthood (Wiskott et al., 2006; Zhao et al., 2008). Neural stem cells can be consistently isolated from the subgranular zone of the dentate gyrus in the hippocampus. These adult neural stem cells can differentiate *in vivo* into all different types of neural cells and glial cells, including neurons, astrocytes, and oligodendrocytes (Zhao et al., 2008). During neurogenesis, different stages of progenitor cells can be identified by their morphologies and by the expression of specific molecular markers (see Figure 2) (Zhao et al., 2008). As the neural stem cells are proliferating in the subgranular zone, they express several markers, including nestin, a transiently expressed intermediate filament protein. The daughter cells that are born from this proliferation begin to differentiate into mature neurons. As the new, immature neuron is differentiating, it also begins to migrate a short distance into the granule cell layer of the dentate gyrus (Guo-li Ming and Song, 2005; Zhao et al., 2008) and send dendrites up through the granule cell layer into the molecular layer. It also sends an axon into the CA3 region (Christie and Cameron, 2006). At this point, the cells are no longer expressing nestin, but have begun to express doublecortin, the microtubule-associated protein. While continuing their journey into the granule cell layer, doublecortin expression will discontinue, and a mature neuronal marker protein, neuronal nuclei (NeuN), will begin to be expressed. At this point, the cells have become mature neurons (Abrous et al., 2005; Guo-li Ming and Song, 2005; Zhao et al., 2008). During the course of adult neurogenesis, most of these young neurons will die, however some will integrate into the circuitry of the hippocampus (Christie

and Cameron, 2006). The journey from newborn cell in the subgranular zone to integration into the hippocampus takes approximately 21 days in the rodent.

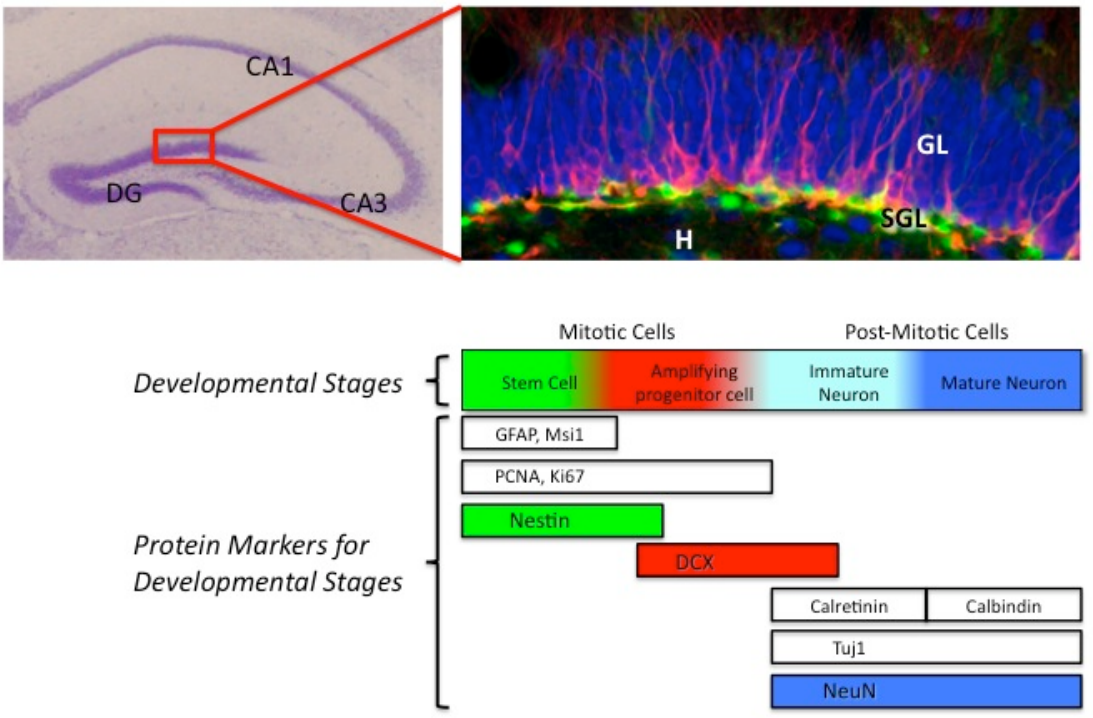

Figure 2. Adult neurogenesis in the adult hippocampus. A blow-up of the boxed region of the dentate gyrus (DG) is shown to the right. The DG is made up of a densely organized granular layer (GL) of mature neuronal cell bodies. At the base of the GL lies the sub-granular layer (SGL) of neural precursors. The stage of neuronal development and the marker genes expressed in these various stages are shown below (adapted from Abrous, D. N. et al. Physiol. Rev. 85: 523-569 2005).

Environmental factors have major impacts on neurogenesis. Exercise, such as voluntary running, is well-known to increase neurogenesis and protect the brain from neurodegeneration, in addition to increasing synaptic plasticity, a hallmark of learning (Cotman et al., 2007; Stranahan et al., 2007; Trejo et al., 2008; van Praag et al., 1999). Synaptic plasticity is the molecular mechanism by which the brain is able to alter the strength

and number of synapses. Synapses in the hippocampus either increase or decrease in strength according to the information it is given (O'Carroll et al., 2006). Providing an enriched environment to lab animals, such as toys and playmates, has also been shown to increase neurogenesis (Williams et al., 2001). In contrast, aging and stress are known to decrease levels of neurogenesis (Aimone et al., 2009; Zhao et al., 2008). Interestingly, chronic antidepressants have also been shown to increase neurogenesis, and can even reverse the depressing effects caused by stress (Malberg, 2004; Santarelli et al., 2003). Neurogenesis may even be a requirement to achieve the behavioral effects of antidepressants, however the exact mechanism with which this occurs is not well understood (Santarelli et al., 2003). While these mechanisms can be used to manipulate the level of neurogenesis, the actual function of these new neurons is still not known.

Learning itself may actually induce the proliferation, migration, and differentiation of new neurons, but this regulation is complex. Learning, as opposed to simple training, activates the new neuron to integrate and survive (Zhao et al., 2008). One study showed that animals retained more neurons if they had several opportunities spaced over time to learn a task versus a group that learned the task in one massed session (Sisti et al., 2007). Additionally, apoptosis of newborn cells is a component of the selective integration of new cells into the hippocampal circuitry, and when it is blocked in rats, they exhibit impaired performance in spatial learning tasks (Dobrossy et al., 2003). Also, different phases of learning have different impacts on hippocampal neurogenesis. The early phase of the Morris water maze has no effect on cell proliferation, compared to the late phase that exhibits increased cell proliferation as well as increased pruning of new synapses, which are all markers of enhanced memory acquisition (Dobrossy et al., 2003).

Degeneration of the neurons in the hippocampus can lead to various dementias such as Alzheimer's Disease and Parkinson's Disease (Zhao et al., 2008). One can actually track the progression of Alzheimer's Disease by the rate of hippocampal atrophy (Barnes et al., 2009). Furthermore, the rate of neurogenesis decreases with age concurrently with age-related memory decline. Interestingly, these effects may be improved when neural stem cells are transplanted into the brain in rodents (Grigoryan et al., 2000). However, it is still unknown whether these new neurons in the hippocampus serve any functional purpose in learning and memory. Therefore, it is important to understand the process of adult neurogenesis, especially given the prevalence of neurologic disease within our aging population. Approximately 10% of adults above 65 years and nearly half of adults above 85 years suffer from dementia (Thompson et al., 2008). Neurogenesis is a clear example of the plastic qualities of the brain, and learning how to manipulate these cells could lead to an effective treatment or cure for these dementias (Guo-li Ming and Song, 2005).

In order to understand the role of adult-derived hippocampal neurons, adult neurogenesis has been blocked by lesioning (Broadbent et al., 2004), irradiation (Winocur et al., 2006), and transgenes that promote apoptosis in neural precursors (Dupret et al., 2007; Yu et al., 2005; Yu et al., 2008). Unfortunately, some of these treatments, such as lesioning and irradiation, may also affect the functions of other cell populations, which could contribute to the measured dysfunctional hippocampus-dependent task. The results from these studies have been confusing, as memory deficits were reported in some studies (Broadbent et al., 2004; Fan et al., 2007; Snyder et al., 2005), but not others (Madsen et al., 2003; Meshi et al., 2006; Rola et al., 2004; Saxe et al., 2006). The reasons for this discrepancy may be due to the lack of specificity for the ablation of the hippocampal

neurons, increased inflammation due to lesioning or because there is no standard protocol for the behavioral tests used to evaluate hippocampus-dependent learning (Dupret et al., 2007).

When addressing hippocampal neurogenesis, it is important to be able to recognize any new cells that are produced during neurogenesis. One way to do this is to introduce bromodeoxyuridine (BrdU), which is a thymidine analogue that incorporates into DNA during the S-phase of the cell cycle (Guo-li Ming and Song, 2005). Other options include infecting cells with viral vectors that express a live reporter, or using various endogenous markers present at different stages of neuron development (Christie and Cameron, 2006; Guo-li Ming and Song, 2005). As previously mentioned, the expression of several gene products can be used to determine the stage of development for proliferating neural precursors.

The difficulty in measuring learning and memory in any animal model is the absence of recognizable speech. Therefore, behavioral tests, or paradigms, have been developed to measure different aspects of central nervous system function. These tests measure behaviors that relate to functions as simple as locomotor activity, to more complex interactions that correlate with anxiety, helplessness, and learning and memory (Crusio et al., 1989a; Crusio et al., 1989b; Eichenbaum, 1996).

The open field test is used as a simple measure of motor activity. It measures the behavior of the animal upon removal from its familiar environment and preventing its escape by a wall. The test looks at the body movement, and where the mouse spends most of its time. Emotional or anxious animals will spend more time hugging the walls, where a less anxious animal will spend more time exploring the center of the chamber (Walsh and Cummins, 1976). Lack of activity is also measured and can be used as an indication of

stress. The elevated zero maze is used to measure anxiety or emotionality. A circular platform is raised off of the floor. Half of the circle has walls surrounding it, and the other half has no walls. The amount of time the mouse spends exploring the areas without walls is compared to the time the mouse stays hidden within the walls to measure anxiety-related behaviors (Shors et al., 2002).

Contextual fear conditioning is used as a hippocampus-dependent learning and memory task. An unconditioned stimulus, a light turning on in a dark chamber, is paired with an unconditioned stimulus, a footshock (Misane et al., 2005). The mouse is conditioned to expect a shock when the light turns on. When a mouse learns to associate the shock with a specific context, it will freeze in anticipation of the shock that is also associated with the context. On the first day of the test, the mouse is conditioned to associate the light stimulus with the unconditioned shock. Following a rest day which helps to form a link between the two time-lagged stimuli (Misane et al., 2005), the mouse is placed back into the chamber under the same contextual cues, but this time, no footshock follows the light stimulus. If the mouse learns to associate the light with the footshock, it should initially freeze. After several applications of an unpaired light stimulus with no footshock, the animal will begin to move more as fear is extinguished. This learning process is controlled by NMDA receptors in the hippocampus and has been shown to be mandatory for contextual fear conditioning (Misane et al., 2005).

The Morris water maze is used as a hippocampus-dependent spatial learning task. The hippocampus is necessary for acquisition and retrieval of spatial information, and also for consolidation and storage of this information (D'Hooge and De Deyn, 2001). A mouse is placed in a tub full of water with a hidden platform, and learns to escape to the platform

using extramaze spatial cues on the adjacent walls (Morris, 1984). The test is repeated over several days, and the time it takes for the mouse to reach the platform should decrease over the course of these days because the mouse learns where the platform is located. This test is sensitive to hippocampal damage (Broadbent et al., 2004).

II. HYPOTHESIS:

We believe that adult-derived neurons play an additive role in learning and memory. To test this hypothesis, we used a transgenic mouse designed to inhibit neurogenesis when given the antiviral drug ganciclovir (Yu et al., 2008). In this mouse, a nestin promoter drives a herpes simplex virus thymidine kinase transgene. Expression of this viral gene has no effect on the stem cells or the generation of new neurons from these stem cells in the normal adult mouse (Yu et al., 2008). However, upon administration of the antiviral drug ganciclovir, the herpes simplex virus thymidine kinase will phosphorylate ganciclovir, which can then be incorporated into the DNA as a thymidine analogue. This disrupts DNA synthesis, and any dividing cells are killed. With this model, it is possible to block the generation of adult neurons without potential effects on bystander cells. Interestingly, the blockade is both inducible and reversible. Terminating drug administration allows for neurogenesis to resume normally. This transgene also expresses a bacterial green fluorescent protein (GFP) under the control of the same nestin promoter and its second intron regulatory element (Yu et al., 2008). The second intron has *cis* regulatory elements that are important in certain cell populations. These elements drive expression in neural stem cells, but not in other cell populations. Thus, early neural progenitor cells expressing nestin will also express GFP.

We used a two-by-two experimental design to test the effects not only of the ganciclovir treatment versus a saline control treatment, but also the effects of an enriched environment versus a normal environment on adult neurogenesis-dependent learning. Half of the mice received a subcutaneous osmotic pump that delivered either ganciclovir or saline. Additionally, in each of those two groups, half of the mice were housed in a standard normal

cage, and the other half were placed in an enriched environment. Because mice in a standard cage with little to engage their minds may have very low levels of neurogenesis, we used an enriched environment to increase the level of neurogenesis to ensure that we did not see a basement effect when we added ganciclovir to block neurogenesis. In addition to examining the hippocampi of these mice to determine the treatment effects on adult neurogenesis, we also utilized the above mentioned behavioral paradigms to test locomotor activity, anxiety, spatial navigation, and learning and memory.

III. METHODS:

Animals

Animals were housed and cared for in the vivarium at Texas Christian University. All experiments were approved through Texas Christian University's Institutional Animal Care and Use Committee. At the time of weaning, a small (<0.5cm) piece of the tip of the animal's tail was removed and DNA extracted to determine if the animal carries the correct genetic components. Only mice carrying the transgene were included in the study. After weaning, male and female mice were separated and were housed in groups of four. All mice were given food and water freely and were on a 12-hour light/dark cycle in the vivarium. Mice were checked daily to ensure that they were healthy, and all cages were changed out with clean cages on a weekly basis. When mice reached twelve weeks of age, transgenic mice were moved to new cages in groups of two to three (male mice were kept with their original cagemates). Half of these mice were placed in standard cages, while the other half were placed in a larger enrichment cage with a running wheel, hut, tunneling pipe, and ball toy.

Surgery

An osmotic pump (Alzet 2002, Durect Corp. Cupertino, CA) containing either ganciclovir, kindly provided by Dr. Steve Kernie (UT Southwestern Medical Center, Dallas, TX), or saline was surgically implanted subcutaneously in each mouse. The pumps delivered 100ng/kg/day, for two weeks. The mouse was first anesthetized by isoflurane inhalation. An incision was made between the scapulae, and the connective tissue was spread apart to make a pocket. The pump was inserted into the pocket with the opening of the pump

pointing away from the incision. The incision was then closed with staples. At the end of two weeks, the mice underwent another surgery to replace the used pump with a new one loaded with the same amount of ganciclovir or saline.

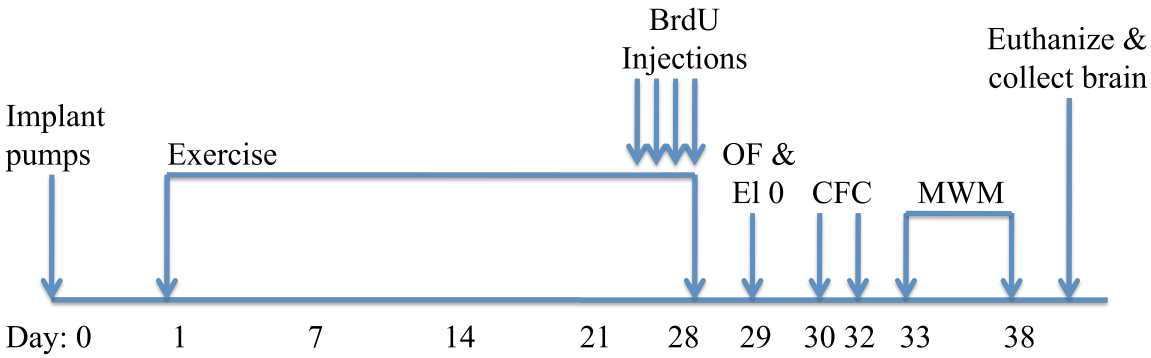

Figure 3. Treatment and testing schedule.

Treatment – Days 1-28

Animals remained in their cages, either the standard sedentary cage or enriched environment cage, for four weeks (28 days) with food and water given freely (Figure 3). Mice were checked daily for health and any post-operative adverse effects with the osmotic pump, and cages were changed out with clean cages every week. Running wheels were checked to ensure that they were in proper functioning order.

BrdU Injections – Days 25-28

The thymide analogue BrdU was utilized to label dividing cells. It incorporates into the genetic material during the S phase of the cell cycle (Dupret et al., 2007). 100mg/kg of BrdU was injected i.p. into each mouse daily for four days, starting four days before the start of behavioral testing (Figure 3).

Behavioral Testing – Days 29-38

Open Field Test (Day 29): This is a simple test of motor activity (and emotionality to a lesser extent), in which one places each individual animal into a small plexiglass box surrounded by an array of infrared sensors encased within an outer box complete with a house light and a fan. Motion in the X, Y, and Z plane are recorded, along with the amount of time spent in the open center of the box versus against the walls or corners. Often, more emotional or anxious animals spend more of their time stationary and against the walls, while less anxious animals spend more time exploring the center.

The recording program (Activity Monitor, St. Albans, VT) was used to record movement inside the boxes (Med Associates, Inc., St. Albans, VT). The computer program monitored the movement of each mouse. The mice were allowed to roam freely in the boxes for twenty minutes. Each mouse was tested only once. After each trial, all boxes were thoroughly cleaned with Odormute.

Elevated Zero Maze (Day 29): This test occurred immediately following the open field test, and is designed to measure anxiety or emotionality in the animal. A circular plastic platform, 63cm in diameter, is lifted off the floor 50cm. Half of the circle is enclosed with tall plastic walls, while the other half has no walls. This test relies on the inherent conflict between exploration of a novel area and avoidance or its aversive features (being off the ground and having no walls on either side). Each animal is tested only once.

A ceiling-mounted camera was used to track and analyze the animals' movements via the EZ Video software (Accuscan Instruments, Columbus, OH). The mice were all placed in the same location (in the middle of a part of the circle with no walls), facing west. The mice

were free to move around the circle for five minutes. After each trial, the ring was thoroughly cleaned with Odormute.

Contextual Fear Conditioning (Days 30 and 32): This task is a well-established hippocampus-dependent learning and memory task that is excellent for measuring memory consolidation. The animal is placed into a small plexiglass chamber (Freeze Monitor System Unit, San Diego Instruments, San Diego, CA) with an electrified grid floor that can deliver a mild shock (0.7mA). The chamber has a design around it of black dots on a white background. A 5 milliliter solution of a 1:10 dilution of peppermint oil was placed in the bottom of each chamber. When the chamber light turns on, a single brief, mild foot shock was administered. Three foot shocks were given over the course of six minutes on Day 1 of testing (Figure 4). Animals were returned to their home cages at the conclusion of their trials. Forty-eight hours later, animals were returned to the chambers. The chamber light turned on again at predetermined intervals, but no foot shock was administered. The testing period on this day lasted twelve minutes per trial. Freezing or movement was monitored via infrared beams, and the information sent to a computer. Freeze Monitor System software (San Diego Instruments, San Diego, CA) was used to monitor the chambers. An animal that remembers the context accurately will show exaggerated freezing behavior versus the first day.

Day 1 (Training)			
Time (seconds)	**Action**	**Cumulative Time (seconds)**	**Cumulative Freezing Time Measured (seconds)**
0	Light ON	0	
0 – 90	Acclimation	90	
90 – 92	Shock ON	92	
92 – 182	Measure Freezing	182	90
182 – 184	Shock ON	184	
184 – 274	Measure Freezing	274	180
274 – 276	Shock ON	276	
276 –366	Measure Freezing	366	270
366	Light OFF	366	

Day 3 (Testing)			
Time (seconds)	**Action**	**Cumulative Time (seconds)**	**Cumulative Freezing Time Measured (seconds)**
0	Light ON	0	
0 – 720	Measure Freezing	720	720
720	Light OFF	720	

Figure 4. Schedule of light stimuli and shock stimuli on Day 1 and Day 3 of contextual fear conditioning.

Morris water maze (Days 33-38): To perform this frequently used hippocampus-dependent spatial learning task, a mouse was placed into a tub of water from each of four locations every day, and allowed to find a submerged platform, which remained in a fixed location. Extramaze cues were provided. The mice were allowed to find the hidden platform during a six-day testing period. A white tub, 130cm in diameter and 55cm in height, was situated directly underneath a ceiling-mounted camera. The camera was connected to computer software (AccuTrak, Accuscan Instruments) that monitored the movement of the

mouse in the water. The tub was filled with enough water to cover the platform by 2cm. Non-toxic, washable white paint was added to the tub to make the water opaque, and to provide color contrast between the black mouse and white background and thus enhance the software recognition sensitivity. At the beginning of each day of testing, the temperature of the water was adjusted if needed to 19-21 degrees Celsius.

Before the first trial on the first day of testing, the mouse was placed on the platform for 10 seconds. Then the mouse was placed semi-randomly at one of the four locations to begin the trial. Each trial lasted 60 seconds. If the mouse found the platform, it was removed to its cage with a heat lamp. If the mouse failed to find the platform, it was gently led or placed on the platform for 10 seconds before being removed to its heated cage. Mice were run in groups of four to six. After each mouse swam once, the first mouse started its second trial. There were four trials each day to ensure that each mouse was able to start from each of the four locations. The trials were run the same on days 1 through 5 of this behavioral test.

On day 6 of this maze, the platform was removed for the probe trial. Each mouse only swam two trials on this day, starting south, then west (the two locations farthest from where the platform was located). The computer software monitored how much time the mouse swam in the area where the platform used to be.

Euthanization and Brain Harvesting – Day 39

The day after the last behavioral test, the animals were anesthetized with ketamine (100mg/kg) and xylazine (5mg/kg), and then transcardially perfused with 1X phosphate

buffered saline (PBS), followed by 4% paraformaldehyde (PFA). The brains were harvested and placed in 4% PFA.

Sectioning and Immunohistochemistry

After allowing the brains to fix overnight in 4% PFA, they were set in 3% agarose/ 1X PBS cubes. The brains were then sectioned coronally on a vibratome (LeicaVT100S, Leica Microsystems, Bannockburn, IL) in 50 micrometer sections. The sections were placed in wells with 1% PFA.

BrdU immunohistochemistry: Six sections were taken from each brain and stained for BrdU integration. First, the sections were washed three times in 1X PBS for ten minutes each. Second, the sections were denatured with 1N HCl for 30 minutes. The sections were then washed in 1X PBS three times for two minutes each. Next, the sections were neutralized with 0.1M Borax for 10 minutes. The sections were then washed again in 1X PBS six times for ten minutes each. Then the primary antibody, rat anti-BrdU (Adcam, Inc. Cambridge, MA), was applied along with PBS plus Triton (PBST) and 5% donkey serum. The sections were left at 4 degrees Celsius overnight. The next day, the sections were washed in PBST six times for ten minutes each before the secondary antibody was applied. The secondary antibody used was donkey anti-rat HRP for BrdU (Jackson Immunoresearch, West Grove, PA), and PBST and 5% donkey serum were simultaneously applied. The sections were left at 4 degrees Celsius overnight. The next day, the sections were washed in PBST five times for ten minutes each, followed by one ten-minute wash with plain PBS. A liquid DAB substrate (Invitrogen, Carlsbad, CA) was mixed and applied to the sections for approximately 20 minutes. After the stain darkened the nuclei to a satisfactory level, the

DAB stain was rinsed off with 1X PBS three times for ten minutes each. The sections were placed on slides coated with gelatin and allowed to dry overnight. The next day, the slides were dehydrated with ethanol and xylene, and then covered with coverslips.

Neurogenesis was also assessed by immunohistochemistry for expression of DCX. Similar to BrdU immunohistochemistry, sections were incubated in goat anti-DCX (Santa Cruz Biotechnology, Santa Cruz, CA) followed by an HRP conjugated donkey anti-goat antibody (Jackson Immunoresearch) and labeled using the DAB substrate.

Immunofluoresence staining: Some sections were also stained with immunofluorescent labeled antibodies. Six sections were taken from each brain, and washed in 1X PBS over two hours, changing the wash every twenty minutes. The primary antibodies, rabbit anti-GFP (Invitrogen), goat anti-doublecortin (Santa Cruz Biotechnology), and mouse anti-NeuN (Millipore, Billerica, MA), were applied with PBST and 5% donkey serum and remained at 4 degrees Celsius overnight. The next day, the sections were washed for two hours in PBST, changing the wash every twenty minutes. The secondary antibodies were then applied: donkey anti-rabbit Cy2, donkey anti-goat Cy3, and donkey anti-mouse Cy5 (all from Jackson Immunoresearch), along with PBS plus Triton and 5% donkey serum simultaneously, and remained at 4 degrees Celsius overnight. The following day, sections were washed in PBST for two hours, changing washes every twenty minutes, then applied to slides. The slides were immediately covered with coverslips.

IV. RESULTS:

Open Field Test

Total distance traveled, distance traveled in the center of the maze, total ambulatory time and ambulatory episodes (Figure 5) were examined between all groups with a one-way analysis of variance (Figure 5). A significant difference was found in center distance traveled (Figure 5B) between the mice housed in a normal environment with saline and mice housed in a normal environment with ganciclovir in a Bonferroni post hoc test ($p=0.028$). No other significant differences were found.

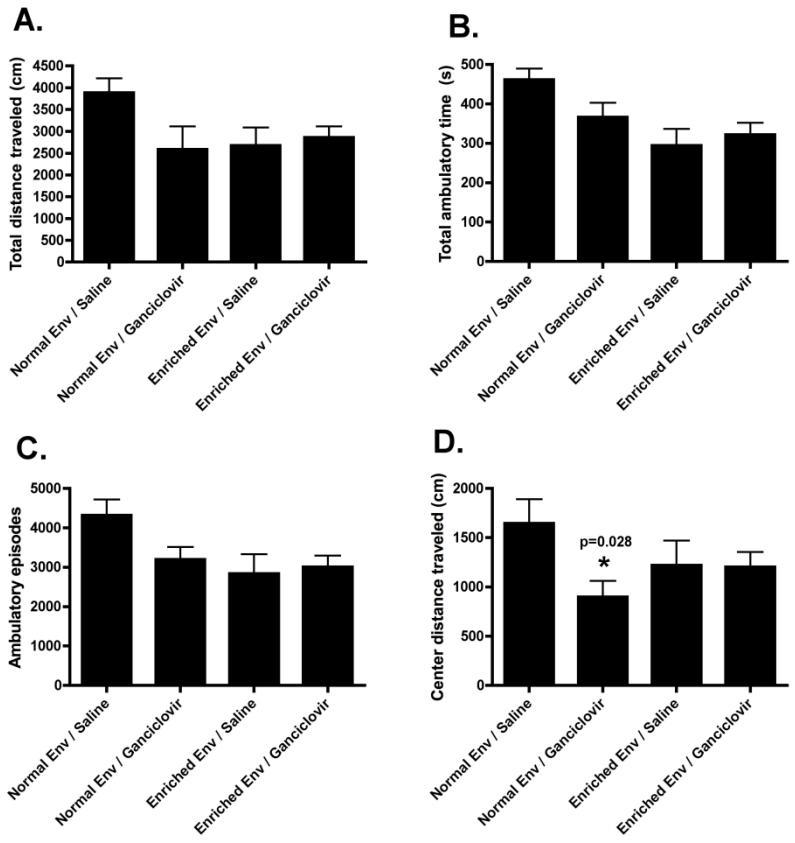

Figure 5. Results of Open field testing. (A)Total distance traveled, (B) center distance traveled, (C) total ambulatory time, and (D) ambulatory episodes in the open field test. All groups were analyzed using a one-way analysis of variance for each variable. Error bars represent standard error.

Elevated Zero Maze

Results were analyzed using a one-way analysis of variance. Percentage of time spent in the open area and percentage of time spent in the closed area were compared between all groups (Figure 6). Neither the treatment given to the mice nor the environment that they were housed in had a significant effect on percentage of time spent in either area of the elevated zero maze ($p=0.84$).

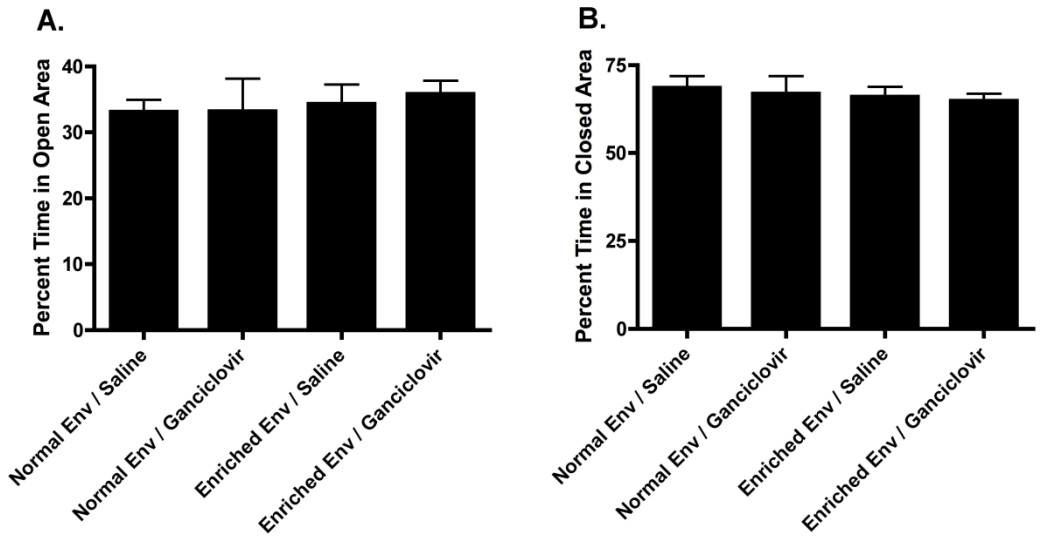

Figure 6. Results of elevated zero testing. (A)Percentage of time spent in the open area in the elevated zero maze. (B) Percentage of time spent in the closed area of the elevated zero maze. Error bars represent standard error.

Contextual Fear Conditioning

Mice were placed in the contextual fear chamber for six minutes on Day 1. During these six minutes, three mild footshocks were administered. Mice in an enriched environment who were given saline froze more than mice in sedentary cages given ganciclovir, but there is no statistical difference between the groups (Figure 7). On Day 3 of

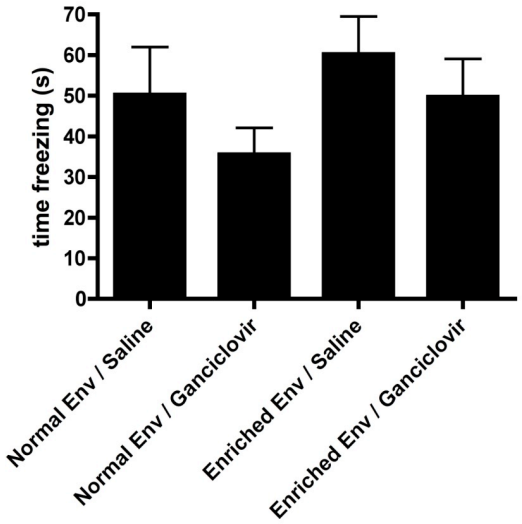

Figure 7. Mean freezing time during day one of contextual fear conditioning. Mice were placed in the contextual fear chamber for six minutes on day 1. Mice in an enriched environment who were given saline froze more than mice in sedentary cages given ganciclovir, but there is no statistical difference between the groups. Error bars show standard errors.

	gan/run	gan/sed	sal/run	sal/sed
Mean	49.74	35.54	60.22	50.26
Standard deviation	40.55	21.73	38.22	43.73
Standard error	9.303	6.55	9.269	11.69
Minimum	2.2	7.2	14.6	2.1
Median	45.7	31.7	48.2	36.15
Maximum	129.1	82	142.1	141
Lower 95% CI of mean	30.19	20.94	40.57	25.02
Upper 95% CI of mean	69.28	50.13	79.87	75.51

Table 1. Descriptive statistics for mean time frozen during day one of contextual fear conditioning.

testing, each mouse remained in the chamber for 12 minutes, but no footshock was administered. Time freezing at four equally-spaced timepoints was compared across all groups with a two-way analysis of variance with repeated measures ($p=0.32$; Figure 8A). As expected, mice given ganciclovir and kept in standard housing froze less than the other groups, but the difference was not significant. All groups exhibited fear extinction – less freezing time at the end of the trial than at the beginning. Mean freezing times were also measured with a one-way analysis of variance. Mice given ganciclovir and housed in a

normal environment again had the lowest time freezing, while mice given saline in an enriched environment had the highest, but the differences were not significant (p=0.49;

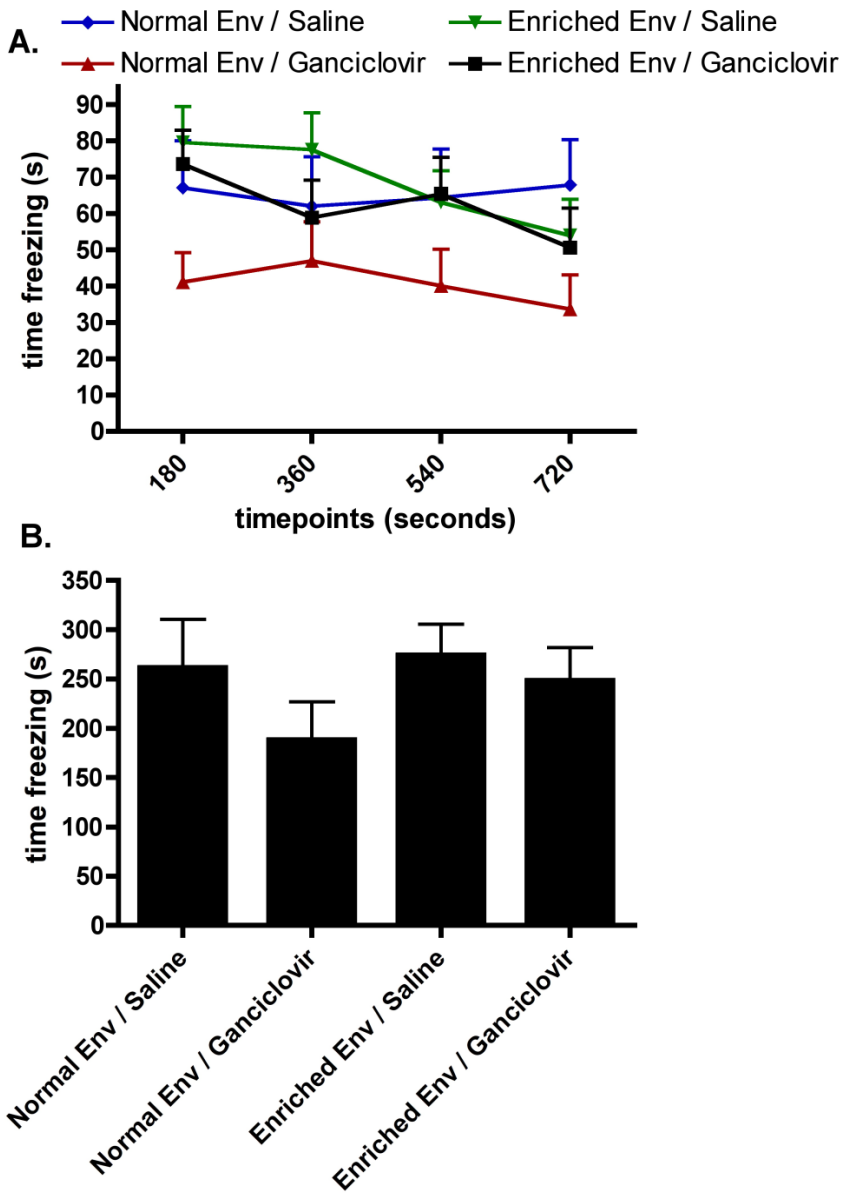

Figure 8. Freezing time on day 3 of contextual fear conditioning. Mice were placed in the chamber for 12 minutes on this day, but no footshocks were administered. Bullets represent mean freezing time for all mice in that group. Amount of time frozen decreased for nearly all groups. (A) Freezing time at four equally-spaced timepoints. No significant difference was found between any two groups after performing a two-way repeated measures analysis of variance. (B) Mean freezing times for each group. Error bars represent standard error.

Figure 8B). Time freezing on Day 3 was higher than on Day 1 of testing, in all mice except the group given ganciclovir in a normal environment. This suggests that a trend exists in which all but this group learned the conditioned response in the context of the novel environment.

Morris Water Maze

Latency to escape to the hidden platform and distance swam to reach the platform was measured and compared across all groups using a two-way analysis of variance with repeated measures. Time to reach the platform decreased with time across all groups ($p=0.87$; Figure 9A), but there was no significant difference between any two groups. Distance swam to reach the platform also decreased across all groups with time ($p=0.946$; Figure 9B), but again, no significant difference was found.

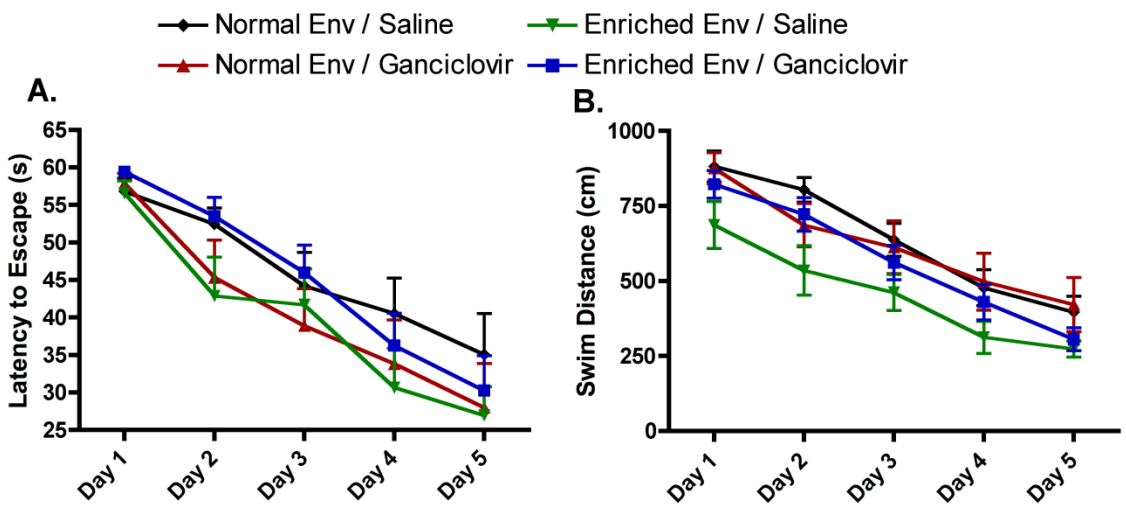

Figure 9. Results of Morris Water Maze testing. (A)Latency in the Morris water maze. Time to reach the platform decreased in all groups over time. No significant difference between any two groups was found after performing a two-way analysis of variance with repeated measures. (B) Distance swam to reach the platform in the Morris water maze. Distance decreased in all groups over time. No significant difference between any two groups was found after performing a two-way analysis of variance with repeated measures.

Effect of Ganciclovir on Adult Neurogenesis

Because our goal was to determine the role of adult-derived neurons on the measures of learning listed, we processed the mouse brains to determine the efficacy of using ganciclovir to block adult neurogenesis. Saline treated animals display a substantial number of DCX expressing precursors in the DG (Figure 10A), while 28 days of ganciclovir treatment is sufficient to block proliferation of the neural precursors as assessed by DCX expression (Figure 10B). While some DCX expressing cells can be found in the DG of animals treated with ganciclovir, they usually have an immature morphology in which they do not extend cellular processes and do not migrate into the granule cell layer (Figure 10D). This again suggests that the ganciclovir treatment is arresting development of this population of cells. Unfortunately, we have found that ganciclovir treatment may not always work.

Some animals display varying ranges of adult neurogenesis, from a few DCX positive cells in the granular layer, up to a level similar to that of the saline treated control (Figure 10E).

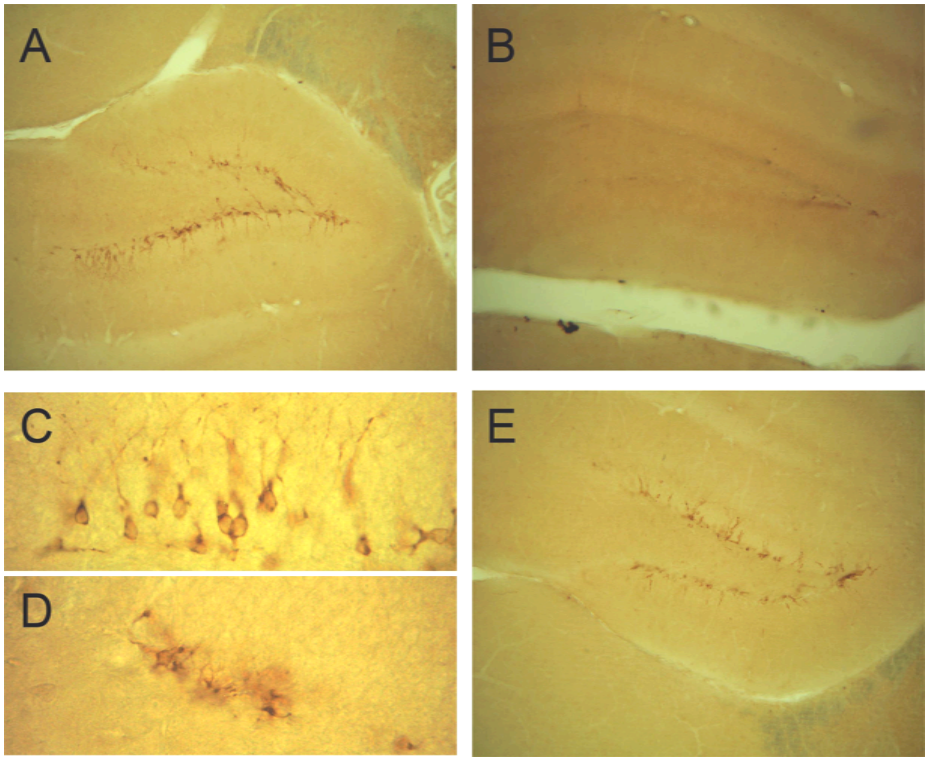

Figure 10. Doublecortin (DCX) expression in the dentate gyrus. (A) Representative DCX immunohistochemistry in the dentate gyrus of an animal in normal housing and receiving saline for 28 days. (B) Treatment of normal housed animals with ganciclovir for 28 days blocks the generation of the DCX-expressing neural progenitor population in the dentate gyrus. (C and D) Representative high-magnification images of the dentate gyrus from animals treated with saline (C) or ganciclovir (D). (E) Ganciclovir did not appear to block proliferation of neural precursor cells in some animals.

V. DISCUSSION:

Our experiments were designed to measure the contribution of adult-derived hippocampal neurons on learning and behavior. We hypothesized that the new neurons created from adult neurogenesis may augment learning, which could be measured with various behavioral paradigms. Previous research has been inconclusive in defining the function of these new neurons.

The results from our research have only partially supported our hypothesis. As expected, the animals given ganciclovir had some decreased performance in behavioral tests compared to other groups, but the differences were not significant. While we had expected to see differences in spatial learning and memory as assessed by Morris water maze, this was not the case. Only in the contextual fear conditioning paradigm did we see a trend that suggests that blocking adult-neurogenesis might negatively impact a hippocampus-dependent learning task.

Tasks that measure hippocampus-independent functions were not expected to demonstrate differences between treatments. This was generally the case. However normal housed animals receiving ganciclovir traveled significantly less distance within the center segment of the open field test when compared to control animals (Figure 5B). This might indicate that the ganciclovir-treated animals display thigmotaxis, or a proclivity to remain along the outer walls of the apparatus, and thus indicate an increase in anxiety. Center distance traveled in ganciclovir-treated mice is approximately half of the total center distance traveled in the control animal. A similar decrease was seen in total distance traveled (Figure 5A) however the difference was not significant. It is likely that the significant difference in center distance traveled is an indication of a lower activity level in the ganciclovir-treated

animals, and not representative of an increase in anxiety. Interestingly, enriched environmental housing also produces a decrease in total distance and total center distance traveled, along with decreased ambulatory time and episodes. While none of these changes are significant, it is worth noting that the change is not additive with respect to ganciclovir administration. It is important to note that a number of studies have shown that voluntary wheel running can cause a reduction in exploratory behavior and locomotor activity when measured by the open field test (Burghardt et al., 2004; Fuss et al., 2009; Leasure and Jones, 2008).

With respect to hippocampus-dependent tasks, no significant differences were seen between treatment groups or housing groups in contextual fear conditioning. This was unexpected and disheartening. While a trend did exist in a decrease in freezing time for normally housed animals treated with ganciclovir, it was not significant. And, in fact, this trend existed on day one of training (Figure 7), and thus could implicate an effect of the drug treatment that is irrespective of hippocampal function. Unfortunately, we are not sure if the testing apparatus itself is actually functioning properly. Reports from Dr. Boehm's lab here at TCU also suggest that in many cases, the testing apparatus may be too sensitive to minor animal movements and that visual scoring may be more appropriate. This may be the case in our study as there was little extinction of fear on day three, when a footshock did not follow the conditioned stimulus. And, in fact, the control animals did not display a great deal of freezing behavior on day three which could indicate they either did not learn, or that the apparatus did not work properly. Similar studies from the Boehm lab have produced freezing times in excess of 100 seconds following the first stimulus, which far exceeds the mean freezing time of 68 seconds demonstrated by our animals.

We had also hoped to see differences in spatial learning as assessed by Morris Water Maze. This was not the case. All treatment and housing groups learned to escape the water by swimming quickly to the platform. However, we did experience difficulty in properly assessing many animals in this task. First, several animals were either unable to swim, or uninterested in swimming. One likely explanation is that the final position of the osmotic pump may hinder their ability to swim. Over the 28 days of this study, the osmotic pump placed subcutaneously along one side of the spine was able to shift positions. In some cases, the pump migrated to the lateral aspect of the mouse, resting between the front and hind leg. In these animals, swimming was disrupted. The animals attempted to swim, but their hind legs tended to sink and the animal was unable to move toward the platform.

It is interesting to speculate that because all animals, regardless of treatment or housing condition, were able to learn to locate the hidden escape platform, perhaps the task was too easy to learn. This is an attractive hypothesis to explain our results in that the contribution of new neurons to the adult hippocampus is minimal in the standard housing condition. This makes it less likely that a reduction in such a small contribution of adult neurogenesis would be measurable in a learning task that is too easy. Instead, a spatial learning task that could increase in difficulty to the point that all animals would eventually fail to learn might detect the small contribution of adult-derived hippocampal neurons to spatial memory. Such a model has been explored by Bimonte-Nelson and colleagues and has been shown to test both working and reference memory (Bimonte-Nelson et al., 2003).

We are also now beginning to demonstrate that the degree of adult-neurogenesis blockade by ganciclovir is quite variable. Figure 10 provides representative brain sections in which normal neurogenesis occurs, and in which ganciclovir is able to block this

neurogenesis (Figure 10A and 10B). Unfortunately, we are also seeing examples in which the degree of blockade varies from complete to negligible, as shown by Figure 10E. The primary reason for this variability is likely that the concentration of ganciclovir used in the pump is close to the solubility of the drug in water, that the drug precipitates out of water and clogs the release tube in the osmotic pump. We assumed that such a high concentration was necessary in order to deliver enough drug throughout the study to block neurogenesis (Dr. S. Kernie, UT Southwestern Medical Center, Dallas, personal communication). However, this may not be the case. Reports from Dr. Kernie's lab suggest that a lower concentration delivered for a longer period may provide more reproducible targeted disruption of proliferation of the neural precursors. It will be important in the future to test a range of ganciclovir dosages in order to find one that provides consistent disruption of neurogenesis.

Finally, it is important to mention that as we were completing this study, a manuscript published by Clelland et al demonstrated that adult neurons may play a role as pattern separators for stimuli that are presented with little spatial separation (Clelland et al., 2009). In this study, two forms of ablation of neurogenesis were used. The first involved low-dose focal irradiation. Animals receiving irradiation were tested for the degree of microglial activation, a measure of central inflammation, and shown to have negligible inflammation. Neurogenesis was completely ablated in these animals. Further, animals receiving irradiation were selectively impaired in their ability to differentiate locations in a maze that had a low degree of separation, but not in their ability to differentiate high separation distances. In addition, these mice were deficient in their ability in a pattern separation task using a mouse touch screen. Similar results were found in animals that received a dominant-negative Wnt (dnWnt) expressing lentivirus injected into the DG. This viral construct was shown to

disrupt Wnt signaling locally in the DG, a process that is important in progenitor cell proliferation. In this second, independent method to knock-down neurogenesis, animals receiving the dnWnt construct displayed similar deficiencies in pattern separation to the irradiated animals. These results demonstrate that adult-derived DG neurons are important for the ability of the DG to perform pattern separation tasks properly.

Conclusion

The first step to being able to use adult-derived stem cells as a therapy is to define their functional role in any tissue. Our research has shown trends that new neurons in the hippocampus are involved in learning and memory, but more work needs to be done to determine the extent of the help these neurons provide. If these neural precursors were to be used in therapy, would they make a difference? It is critical to fully understand the functional role of these adult-derived neurons in order to work with them to find a cure for dementias such as Alzheimer's Disease and Parkinson's Disease.

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ABSTRACT

THE ROLE OF ADULT-DERIVED NEURONS IN LEARNING AND MEMORY

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Neurogenesis is well known to occur in the dentate gyrus of the hippocampus throughout adulthood, but the function of these new neurons is not fully understood. We performed a two-by-two experiment to test if these new neurons play a role in learning and memory. The mice were placed in either an enriched environment cage to increase neurogenesis, or a normal cage. We used a transgenic mouse with the ability to knock out neurogenesis using the antiviral drug ganciclovir, and half these mice were given ganciclovir while the other half were given saline. We tested the relationship between the number of new neurons in the dentate gyrus and with performance on behavioral and learning tasks. These behavioral tasks tested motor ability, anxiousness, spatial memory and hippocampus-dependent learning ability. We then compared the performance on these tests across all groups. Our findings show that there is a trend between number of new neurons and hippocampus learning ability.