LEARNING & NEUROGENESIS:
ARE NEW NEURONS RESCUED FROM DEATH
WITH EACH NEW LEARNING EXPERIENCE?

by
HELENE M. SISTI

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And approved by

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ABSTRACT OF THE DISSERTATION

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by HELENE M. SISTI
Dissertation Director:
Dr. Tracey J. Shors

New cells in the adult hippocampus become apoptotic, e.g. begin programmed cell death, about one week after they are generated. If animals begin to learn a hippocampal-dependent task, at precisely the time when these cells would normally begin apoptosis, then the fate of these cells is altered. Instead of dying, the newly generated cells survive. These new cells differentiate into mature neurons and become fully integrated into the hippocampal network. Thus far, much of this work has focused on a single training experience. An important question has yet to be addressed: are new cells rescued from death with each new learning experience? In the present series of experiments, animals were trained with two phases of eyeblink conditioning. During the first phase, animals either learned the same task, a different one, or remained in their home cage. A single injection of BrdU was given after the first training experience had been completed, and one week before the start of the second training experience. Animals that were trained with a single phase of eyeblink conditioning retained more BrdU-labeled cells than those trained with two phases or no training at all. When animals were re-categorized based on learning during the second phase, instead of by training condition, there was a significant positive correlation between improvement and number of new neurons. Animals that demonstrated a larger degree of improvement retained more one week old neurons than animals that did not learn very well, regardless of previous experience. Overall, these data suggest that even during a second training experience, learning can rescue one week old neurons from death.
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GENERAL INTRODUCTION

The function of the hippocampus, which lies within the medial temporal lobe, became clear after a now famous patient (H.M.) had his removed (Scoville and Milner, 1957). Due to debilitating seizures, the hippocampus was surgically ‘lesioned’ which led to specific types of learning impairment and memory loss (Corkin, 2002). What H.M. can and can’t learn, what he does and does not remember has been extensively investigated over the last 50 years (Corkin, 2002). A key finding that emerged from this work is that the hippocampus plays a role in the formation of declarative memories – those memories that are directly accessible to conscious recollection (Squire, 1987). They are most often reflected in the ‘awareness’ of an event and can be assessed with numerous tasks, including the mirror-drawing task. In this task, the subject is asked to draw an object based on the reflected image in the mirror. Improvement in the accuracy and quality of the drawing over training sessions is an indicator of learning. The ability to recall the occurrence of the training session is an example of declarative memory. H.M.’s performance on the task improved across days (i.e. procedural learning), yet he had no memory of ever being trained on the task. Therefore, hippocampal damage led to a deficit in declarative memory but not procedural learning. This phenomenon is observed in the amnesic population at large (Manns and Squire, 2001). This dissociation between procedural learning and declarative memory includes not only perceptual-motor tasks, but cognitive tasks as well (Squire, 1987). An overwhelming body of evidence has established the critical role of the hippocampus in learning processes beyond that of declarative memory (O’Keefe and Nadel, 1979).

**Importance of hippocampus in temporal associations**

The hippocampus is particularly important in associating stimuli that are separated in time (McGlinchey-Berroth et al., 1997). To investigate its role in forming temporal associations, an eyeblink classical conditioning task, where one stimulus, an auditory cue, predicts the occurrence of another, airpuff to the eye, is often used (Pavlov 1927, Smith et al., 1969). The former is the conditioned stimulus (CS) and the latter is the
unconditioned stimulus (US) (Pavlov, 1927). Learning is measured by evaluating how well the animal learns to predict and accurately time the occurrence of the US. That is, after repeated presentations of the CS and US pairing, the animal learns to close its eyelid just before onset of the US; this accurately timed eyeblink is considered a conditioned response (CR). When the temporal relationship between the CS and US is changed, the brain regions necessary for learning are also changed. To examine this, two versions of the task have been used, delay and trace conditioning. In delay conditioning, the CS overlaps and co-terminates with the US. Thus, the two stimuli are contiguous in time. In the trace version, the CS and US are separated by a stimulus-free interval – the stimuli are temporally discontiguous. According to Pavlov, a neuronal representation, i.e. the trace, must exist to link the CS with the US (Pavlov, 1927; Solomon et al., 1986). In one study, both hippocampal lesions and multi-unit activity recordings of the hippocampus were examined during variations of delay and trace eyeblink conditioning (Solomon et al., 1986). Animals with hippocampal lesions learned delay conditioning; however, during training with the trace task, they had difficulty accurately timing the conditioned response. In a second experiment, they recorded hippocampal activity during acquisition of either a 500 ms trace, a 2000 ms trace (which animals are unable to learn), or explicitly unpaired presentations of the CS and US. Early in training, at the onset of the CS and throughout the trace interval, hippocampal activity increased. As training progressed, hippocampal activity shifted to just before the onset of the US, coinciding with the emergence of the conditioned response. In a subsequent study, the duration of the trace interval was manipulated (Moyer et al., 1990). When a short (300 ms) trace was used, neither hippocampal nor neocortical lesions before training had any effect on acquisition of CRs; animals can still learn this task and do so as well as sham controls (Moyer et al., 1990). However, increasing the trace by only 200 ms prevented acquisition entirely, but only in the rabbits with hippocampal lesions. Even after 25 days of training, rabbits failed to express conditioned responses. Neocortical lesions had no affect on acquisition of CRs at either interval. They performed as well as sham controls, and emitted CRs on 80% of trials by 16 days of training. Thus, the hippocampus is critical in linking temporally discontiguous events (Wallenstein et al., 1998). Not only does the presence of the trace interval determine conditioned responding, its duration does as well.
The hippocampus plays a critical role in associating stimuli that are discontiguous in time and this process is conserved across species, including rabbits (Solomon et al., 1986), rats (Beylin et al., 2001), mice (Tseng et al., 2004) and humans (McGlinchey-Berroth et al., 1997). Tseng et al. (2004) compared acquisition of the conditioned response using delay or a trace task that had a 250 ms interval. During the last 3 days of training, both groups were expressing the learned response on 60% of the trials. However, after hippocampal lesions with ibotenic acid, mice could only accurately express the conditioned response in the delay version. In the trace version, conditioned responses were significantly reduced. In humans with bilateral medial-temporal lobe amnesia, acquisition of trace conditioning was impaired at all 3 trace intervals that were tested, 500, 750, and 1000 ms (McGlinchey-Berroth et al., 1997), whereas delay conditioning proceeded as normal (Gabrieli et al., 1995). Given the obvious neuroanatomical differences between the rat, mouse and human brain, it is not surprising that the precise temporal parameters may differ between species; nevertheless, the involvement of the hippocampus in forming temporal associations is conserved.

**Memories of trace and delay tasks**

Animals with hippocampal lesions at the time of training can not learn the trace task but can learn the delay task (Solomon et al., 1986; Moyer et al., 1990). Therefore, the hippocampus is necessary for learning the CR; but what about memory? To test the role of the hippocampus in the memory of the trace task, hippocampal lesions were imposed either one day or one month after training (Kim et al., 1995). Memory of the conditioned response was then tested. Animals that had lesions one day after training did not express CRs, even when training continued for an additional 10 days. On the contrary, when lesions were not imposed until one month after training, animals could accurately time and predict the CR- thus, their memory was intact even though they did not have a functional hippocampus. In a similar study, animals received lesions of the hippocampus or medial prefrontal cortex 1 day, 1 week, or 4 weeks after training (Takehara et al., 2003). One day later, cortical lesioned animals expressed the same number of CRs as they did before conditioning, however, hippocampal lesioned animals produced virtually
no responses, reaching about 30% after three days of training. One week later, cortical lesioned animals accurately predicted the CS. Hippocampal animals tended to express fewer CRs than cortical animals, but their performance was similar to their pre-lesion levels. By two and four weeks later, the hippocampal lesion had no effect on CRs and animals express memory of the CR as well as cortical animals. Therefore, the hippocampus has a time-limited role in trace conditioning; it is needed for very recent (less than a week), but not remote memories (Takehara et al., 2003).

**Delay and trace differ in task difficulty**

Trace and delay conditioning procedures differ not only in their dependence on the hippocampus, but also in the number of trials required to learn the task (Solomon et al., 1996). The delay task is acquired more quickly than the trace task even when the duration and magnitude of the CS and US are similar (Beylin et al., 2001; Pavlov, 1927; Bangasser et al., 2006). In addition, asymptotic performance is higher in the delay task than the trace task. The function of the hippocampus may not necessarily be to associate temporally discontiguous stimuli. It may participate in the acquisition of difficult associations. Shors and colleagues hypothesized that the ‘role of the hippocampus may not be limited to learning about discontiguous events in time and space, rather the structure can become engaged simply as a function of task difficulty’ (Beylin et al., 2001).

To test this, animals were trained on a version of the delay task referred to as the very long delay task (VLD). In this task, the interstimulus interval (ISI), time between CS onset and US onset, was increased. Generally, optimal conditioning occurs with interstimulus intervals that are 200 to 400 ms, and decreased acquisition is observed with shorter or longer intervals. In this experiment, the ISI was increased by extending the duration of the CS to 1,500 ms. It still overlapped and co-terminated with the US, but it was much longer in duration than in standard delay; it was 6 times longer. Sham and hippocampal-lesioned animals were trained with 1,200 trials of the very long delay task and then 300 trials of trace, or 1,2000 trials of the trace task and then 300 trials of the very long delay task. Hippocampal lesions led to impaired early acquisition for both the trace and the very long delay task. Thus, temporal discontiguity is not essential to engage
the hippocampus. Very long delay conditioning also engages the hippocampus. Using this task, it was discovered that the hippocampus also functions as result of task difficulty (Beylin et al., 2001). As expected, hippocampal lesions did not impair delay conditioning with a short interstimulus interval. The delay task is a relatively easy task for the animals to learn. This is consistent with the idea that the hippocampus becomes engaged as task difficulty increases. Hippocampal-lesioned animals did eventually learn the very long delay task, though they could not learn the trace task. Thus, the hippocampus becomes engaged and perhaps even critically so if the task is sufficiently difficult to learn, as in very long delay conditioning.

Hippocampal neurogenesis in the adult brain

Newly generated neurons in the adult brain have been identified in several brain regions including the endriti, hypothalamus, and neocortex, with much larger populations along the wall of the lateral ventricle (subventricular zone), the olfactory bulbs, and the hippocampus (Gould, 2007). The hippocampus, specifically the dentate gyrus, is among the most active neurogenic regions (Gould, 2007). These neurons arise from the subgranular zone of the dentate gyrus, the border between the granule cell layer and the hilus. The precise cell lineage of hippocampal cells is not entirely clear, however evidence suggests that these neurons arise from a constitutively active population of radial glial cells (Seri et al., 2001). Radial glial cells have been identified along the inner granule cell layer and develop into mature neurons (Seri et al., 2004). Data are derived from a combination of techniques including retroviral labeling and immunohistochemistry to selectively label newly generated cells, and laser and electron microscopy to visualize morphological changes across development. In one study, when proliferating hippocampal cells were selectively destroyed, the first new cell type to arise was GFAP expressing cells which later expressed neuron-specific proteins (Seri et al., 2001). GFAP, glial fibrillary acidic protein, is used to identify glial cells. Even without targeted ablation, new hippocampal cells express GFAP early in their development (Garcia et al., 2004). Although GFAP is widely used to identify glial cells, it is found in
diverse cell types outside of the central nervous system, including liver, kidney and gut (Garcia et al., 2004). As such, there remains some uncertainty regarding the source of these cells—that is, do astrocytes really give rise to new neurons? Some have simply described these cells as GFAP-expressing neural progenitor cells (Garcia et al., 2004, Emsley et al., 2005). While glial cells, or GFAP-expressing progenitors, represent one putative source of new neurons, other possibilities exist. For instance, when a cluster of new neurons is identified, there is often an increased density of blood vessels nearby (Palmer et al., 2000). A positive correlation between neurogenesis and angiogenesis, the birth of new vasculature, occurs in both mice and humans (Pereira et al., 2007). Furthermore, running, which increases cerebral blood flow, increases cell proliferation quite dramatically (van Praag et al., 1999; Pereira et al., 2007). An intriguing possibility is that bi-directional communication between blood and nerve cells leads to the synthesis of neural progenitor cells de novo. Indeed, the angiogenic protein, vascular endothelial growth factor (VEGF), promotes the growth of vascular endothelial cells and also stimulates the growth of neuronal precursors both in vitro and in vivo (Jin et al., 2002). Although speculative, perhaps the delivery of lipids, proteins and carbohydrates across blood and nerve cell membranes contribute to the generation of new cells in the hippocampus. Regardless of their mechanism for synthesis, newly generated cells that give rise to neurons in the adult hippocampus are found along the inner granule cell layer of the dentate gyrus (Palmer et al., 1997).

**Cell cycle**

As new hippocampal cells are generated, they progress through the characteristic four phases of the cell cycle. The cell grows (G1), DNA is synthesized (S), the cell prepares for division (G2) and finally, the cell completes division during mitosis (M). Just as in development, cells in the G1 phase do not always progress towards mitosis and sometimes exit the cell cycle and enter the rest phase, G0 (Alberts et al., 1983). The duration of the cell cycle is species and tissue dependent. In the young adult rat hippocampus, the duration of the cell cycle has been estimated in vivo to be 25 hours (Cameron and McKay, 2001). In young adult mice, it is approximately half the time, 13 hours (Christie and Cameron, 2006). The study of neurogenesis in vivo has been
investigated using primarily two approaches: tritiated thymidine combined with autoradiography and bromo-deoxyuridine injection combined with immunohistochemistry. The latter technique has several advantages over the former, and is currently the most widely used method for studying adult neurogenesis. It eliminates the need for radioactive material and can be used with thicker tissue sections, which provide a better estimate of cell counts using stereological procedures (West et al., 1991). It has been widely adapted and has facilitated progress in this field since the initial discovery of adult neurogenesis using tritiated thymidine (Altman and Das, 1965).

**Labeling new cells**

The ability to label newly dividing cells and track their survival *in vivo* is critical to understanding their function in the hippocampus, particularly with regards to learning. The most widely used technique to do this is BrdU immunohistochemistry (Taupin, 2006). Bromo-deoxyuridine (BrdU) is a thymidine analog. It is incorporated into DNA during the S-phase of the cell cycle, during which DNA is single stranded and BrdU can bind to the thymidine base pair, adenosine. Because this only occurs during a fraction of the cell cycle, BrdU only labels a fraction of mitotic cells. BrdU is dissolved in saline, is injected into the intraperitoneal cavity (i.p.), crosses the blood brain barrier, and is available for up to 2 hours after injection. Thus, BrdU is incorporated into mitotic cells that are in the S-phase within two hours after injection. Once incorporated inside these cells, it remains there throughout any future cell divisions, without substantial dilution (Cameron and McKay, 2001; Taupin, 2006). After animals are sacrificed, brain tissue is fixed with paraformaldehyde and BrdU is visualized using standard immunohistochemical techniques. Essentially, the tissue is treated with an antibody that was created to bind specifically to BrdU. The antibody complex is then reacted with a peroxidase which gives a cell containing BrdU a distinct brown color. Some issues have been raised regarding potential problems with this method, such as the potential of BrdU to label not only mitotic cells but also cells that are undergoing DNA repair; toxicity of BrdU in high doses; and dilution of label (Taupin, 2006). Relative to the advantage of tracking the survival of new cells over time, continued investigation has demonstrated that these technical concerns are fairly negligible (Taupin, 2006). DNA repair represents
a trivial proportion of cells that would potentially incorporate BrdU; dose response curves have been done to determine non-toxic doses that still label new cells; and the label is not significantly diluted over repeated cell divisions (Cameron and McKay, 2001). The use of BrdU immunohistochemical techniques has been instrumental in tracking the fate of newly generated cells in the hippocampus (Gould, 2007). For instance, animals may be injected with BrdU and then exposed to a broad range of experimental manipulations, such as stress, exercise or of particular interest here, different types of learning (Kempermann, 2006). After the brain is removed and the tissue is treated with immunohistochemical procedures, the number of surviving BrdU-labeled cells is counted and compared with controls. In so doing, the effect of different types of learning, as well as other types of experimental manipulations, on the fate of new neurons in the hippocampus can be tracked with relative ease.

**New neurons in the hippocampus**

Newly generated hippocampal cells possess properties of mature granule cells as early as four weeks (Christie and Cameron, 2006). They continue to exhibit significant growth between four weeks and four months (van Praag et al., 2002). Throughout this period, the size of the soma, total dendritic length, dendritic branching, and spine density all increase by about 60% (van Praag et al., 2002). During the first 3 days after mitosis, apical dendrites extend through the granule cell layer at a rate of 10 µm per day (Shapiro et al., 2007). When the cells are 4 and 5 days old, the dendrites double in length each day as they continue to extend towards the molecular layer. Between six and ten days after mitosis, new granule cells extend axons to the CA3 layer of the hippocampal formation; just as mature dentate granule cells do (Hastings and Gould, 1999). Interestingly, basal dendrites appear early in cell development but are no longer apparent as the cell matures, a feature also observed in the young, developing brain (Ribak et al., 2004). Another property of new cells in the adult brain which is also observed in the young brain is the manner in which they respond to the amino acid neurotransmitter, GABA (gamma amino butyric acid) (Ribak and Shapiro, 2007, Ge et al., 2006). Initially, diffuse, tonic GABA surrounding the cell is adequate to induce intracellular changes in the new cell. As the cell develops, it is no longer responsive to ambient GABA, rather it requires synaptic-
mediated input. It depolarizes in response to the binding of GABA, then as the cell matures, it hyperpolarizes in response to the binding of GABA (Ge et al., 2006). This is the same stereotypical pattern observed in development, and in both cases, it is attributed to the sequential expression of chloride transporters (Ge et al., 2006). Ultimately, newly generated cells in the dentate gyrus of the adult brain are completely integrated into the hippocampal network (Figure 1). They form functional synapses and are capable of firing action potentials (van Praag et al., 2002; Toni et al., 2007).

**Factors that influence cell proliferation and survival**

In general, the term ‘adult neurogenesis’ refers to the ‘entire set of events leading to the production of new neurons in the adult brain, from precursor cell division to functionally integrated survival’ (Lledo et al., 2006). However in the broadest and simplest sense, adult neurogenesis includes two processes: (1) proliferation, where the number of new cells increases as they undergo repeated cell divisions, and (2) survival, the fate of a single cell to either develop into a mature neuron or instead to become apoptotic and die. Not surprisingly, each of these processes is affected by both exogenous and endogenous factors. For instance, exercise, environmental enrichment, group housing, stress, ischemia and a long list of neurotransmitters and growth factors alter the proliferation of new neurons in the adult brain (van Praag et al., 1999; Gould et al., 1999; Cameron et al., 1998). However, fewer environmental events have been demonstrated to regulate their survival; one factor which increases the probability that a new neuron will survive is learning (Shors, 2004).

**Neurogenesis and learning**

*Are new neurons necessary for learning?*

Given the importance of the hippocampus in learning and its propensity to produce new neurons, Shors and colleagues hypothesized that new neurons were involved in learning. They further hypothesized that it would be involved in the trace conditioning task, because this type of learning requires the hippocampus (Shors et al., 2001). To determine whether new neurons were necessary for learning, they blocked proliferating cells using a cancer treatment drug, the DNA methylating agent methylazoxymethanol acetate
(MAM). Animals were injected with either MAM or saline and trained on either delay or trace conditioning. Both groups of animals were tested for pain sensitivity, motor activity, stress hormone levels, hippocampal volume, and excitatory post synaptic potentials in CA1. On all measures, MAM-treated animals were similar to saline-treated controls. Both groups could learn the delay task. However, when number of learned responses on the trace task was compared, animals with relatively few new cells could not learn the trace task. The MAM-treated animals emitted few CRs compared to saline controls. When MAM treatment was stopped, the ability to learn trace conditioning was recovered (Shors et al., 2001).

Additional studies have tested whether the new neurons are necessary for learning. These data are somewhat inconsistent, which may be in part due to the technical challenges of this approach. To test if new neurons are necessary for a given task, these new cells must be selectively destroyed. It is somewhat difficult to prevent new cell proliferation in vivo without any other detrimental consequences. Focused irradiation has been successfully used. Focused irradiation selectively reduces ongoing neurogenesis in the dentate gyrus without any detectable damage to mature neurons or reduced cell proliferation in the subventricular zone (Wojtowicz, 2006; Winocur et al., 2006). Snyder et al (2005) used low dose irradiation to inhibit hippocampal cell proliferation of rats then tested them on learning and memory using the Morris water maze. This is a hippocampal-dependent spatial task in which animals use environmental cues to locate a hidden platform. Training began 4 weeks after irradiation treatment, so that new neurons 4 weeks or younger were not available during training. Animals could learn the task and even remember it when tested one week later. However, when tested two and four weeks later, irradiated treated animals had no memory of the platform location, whereas controls did (Snyder et al., 2005). Therefore, new neurons were not necessary in spatial learning or short-term memory of the platform location, however new neurons were necessary for long term retrieval of the memory. This is consistent with previous experiments in which MAM-treated rats could learn the location of a hidden platform in the Morris water maze as well as controls (Shors et al., 2002). These data highlight the importance of
distinguishing between different learning processes, specifically acquisition versus retention.

In other hippocampal-dependent tasks, such as fear conditioning, the role of new neurons is also not entirely clear. In one study, preventing new cell proliferation with MAM had no effect on contextual fear conditioning (Shors et al., 2001). However, in another study, irradiation prevented contextual fear conditioning, which relies on the hippocampus, but left cued fear conditioning in tact (Saxe et al., 2006). In this study, irradiated animals were also trained with the Morris water maze. Long term spatial memory was tested. Contrary to the report by Snyder et al. (2005), they found no long term deficit in spatial memory (Saxe et al., 2007). Thus, new neurons are necessary in the formation of associative trace memories, however they are not necessary to learn spatial tasks. Whether or not they are necessary in retention and/or memory retrieval is unclear.

**How does learning affect newly generated cells?**

**Hippocampal-dependence**

The current data suggest that eliminating new neurons induced deficits in some types of learning tasks while leaving most types of learning intact. Learning engages different brain regions depending on the type of task. Do learning tasks that require the hippocampus affect cells in the same manner as those that do not? Shors and colleagues hypothesized that learning which depends on the hippocampus would enhance the survival of new neurons (Gould et al., 1999). They evaluated two types of learning: spatial learning using the Morris water maze and associative learning using eyeblink conditioning. For each task, a hippocampal-dependent and hippocampal-*independent* version was used. In the Morris water maze, when the platform is hidden just below the surface of the water, the hippocampus is needed. However, when the platform is made visible, the hippocampus is not needed. For eyeblink conditioning, both trace (hippocampal-dependent) and delay (hippocampal-*independent*) tasks were used. They discovered that learning which requires the hippocampus, regardless of whether it was spatial or associative, enhanced the probability that new neurons would survive. That is, animals trained using the hippocampal-dependent version of either the Morris water maze
or eyeblink conditioning had higher numbers of BrdU-labeled cells compared to hippocampal-independent versions of both tasks. Animals trained with the visible platform or with delay conditioning had similar number of new neurons as animals that received no training.

**Proliferation vs. survival**

A critical distinction in the study of learning and neurogenesis is distinguishing between cell proliferation and cell survival. That is, does learning regulate neurogenesis by changing the rate of cell production or by affecting the rate of cell survival? Under standard laboratory conditions, new cells begin to diminish seven days after mitosis (Cameron et al., 1993; Dayer et al., 2003). Animals were injected with tritiated thymidine and sacrificed at different time points after injection. When the number of new cells was counted, there was a peak one week after thymidine injection. After this point, the number of cells dropped dramatically. That is, these cells begin to die. Shors and colleagues tested if learning can rescue these cells from death (Gould et al., 1999). As such, animals were injected with BrdU and one week later, when the number of cells normally diminishes, animals started to learn a hippocampal-dependent or hippocampal-independent task. Importantly, only animals that successfully learned the task, e.g. reached asymptotic levels of conditioned responding, were included for analysis. As described above, learning indeed rescued one week old neurons from death. In the same study, the effect of learning on cell proliferation was also tested. Animals were injected with several doses of BrdU, not one week prior, but instead throughout the four day training experience. There was no difference in the number of surviving BrdU-labeled cells between these animals and controls. Therefore, learning enhances the survival of one week old neurons, while its effects on proliferation are less clear.

**Learning-sensitive period of cell development**

The age of the cell at the start of the training experience is a critical factor in rescuing cells from death. In a recent study, the effect of environmental enrichment on different populations of new cells was examined (Tashiro et al., 2007). Environmental enrichment is not a hippocampal-dependent learning task *per se*. However, it provides the animal
with a stimulating environment such as toys, running wheels, and littermates in which some degree of learning occurs. BrdU was injected at different time points before environmental enrichment; immediately before enrichment, or one, two or three weeks prior to enrichment. The largest increase in BrdU-labeled cells occurred in cells that were one week old at the time of the enrichment. The number of cells that were three weeks of age at time of enrichment was not increased nor decreased. The authors conclude that indeed one week is in fact a critical sensitive period in the cell’s development during which it is highly responsive to environmental cues (Tashiro et al., 2007). The effect of spatial learning on different aged cells has also been examined (Drapeau et al., 2003). Animals were injected with BrdU within one to four days before spatial learning. In this case, the number of surviving cells was actually decreased (Dobrossy et al., 2003). Therefore, learning-related stimuli have differential effects on cell proliferation and survival. The effect depends in part on the age of the cell. Recall that newly generated hippocampal cells begin to extend axons between six and ten days of age (Hastings and Gould, 1999). Furthermore, cells respond differently to major neurotransmitters implicated in learning depending on their stage of development (Ge et al., 2006). Therefore, this time point may be critical due to the unique physiological properties of a cell at this stage (Figure 2). Studies are currently underway in this laboratory to compare the effects of trace conditioning on different aged populations of cells.

**Learning or acquisition of a new task**

In the broadest sense, the term ‘learning’ is used to imply a change in behavior as a result of acquiring new information. When animals are trained with trace eyeblink conditioning, they learn that the presence of a tone predicts the occurrence of a shock. As a result of this new experience, they begin to close their eye just before the onset of the US. Thus, the presence of the conditioned response emerges and increases over time. This increase in conditioned responses over time is often defined more specifically as the rate of acquisition. Several experiments have demonstrated a positive correlation between the rate of acquisition and BrdU-labeled cells (Leuner et al., 2006; Dalla et al., 2007). For
instance, learning using two versions of the trace task was compared to determine the effect on one week old cells (Dalla et al., 2007). Trace conditioning, which enhances cell survival, was compared with another task, contiguous trace conditioning (CTC). This task is similar to trace except that the CS is presented a second time and coincides with the US. Thus, unlike trace, the CS and US are presented contiguously. Animals show very low numbers of conditioned responses when the CS and US are only presented simultaneously (Smith et al., 1969). However, in the contiguous trace conditioning task, the CS is presented both during the US, as well as 500 ms before the US (Bangasser et al., 2006). When conditioned responses were compared between the trace and the contiguous trace task, animals that learned well had high numbers of cells, regardless of the training condition (Dalla et al., 2007). A similar phenomenon was observed using the Morris water maze (Sisti et al., 2007). In this study, all animals were trained to find a hidden platform, except the manner in which they were trained differed. One group was exposed to massed training, where all training trials occurred in one day. A second group was exposed to spaced training, in which the same number of trials was distributed across four days. Again, BrdU was injected one week before start of training to determine whether training would rescue one week old cells from death. Learning was assessed by using their performance on the last four trials of training. Learning increased the number of surviving cells, regardless of whether animals were trained in the spaced or massed condition. Good learners had more BrdU-labeled cells than poor learners (Figure 3; Sisti et al., 2007).

The effect of training was also examined using trace conditioning (Leuner et al., 2006). Animals were exposed to only 200 trials of the trace task one week after BrdU injection. Because this time point is critical in rescuing cells from death, the question was: is this single session alone at this time point adequate to rescue a new population of cells? To reach learning criterion, animals require more than 200 trials; 60% conditioned responding is typically observed on the third and fourth day of training. Despite not yet reaching 60% conditioned responding, a significant positive correlation was observed between number of CRs and cells generated one week before training. Thus, animals in this condition were not yet reliably predicting the onset of the US, yet those that
expressed higher numbers of conditioned responses retained more BrdU-labeled cells (Leuner et al., 2006). In another study, the rate of acquisition was explicitly manipulated by including trials in which only the CS was presented before paired training (Waddell and Shors, 2008). By presenting CS alone trials, in combination with paired trials of the CS and US, more trials are required to reach an asymptotic level of performance. This delay in reaching criterion induced by CS alone presentations has been described as latent inhibition (Lubow and Moore, 1959). Animals that required more trials to reach criterion had more BrdU-labeled cells than those that required fewer trials.

Thus far, several learning-related parameters have been correlated with the survival of one week old neurons. For instance, asymptotic performance, e.g. mean number of learned responses at the end of training, correlates positively with cell survival (Dalla et al., 2007). Animals that express more learned responses at the end of training retain more BrdU-labeled cells. Similarly, asymptotic performance in a spatial learning task also correlated positively with cell survival (Sisti et al., 2007). Animals were categorized as good or poor learners based on asymptotic performance. Consistent with the previous study, good learners retained more cells than poor learners. Both of these studies compared learning at the end of training. Number of learned responses at the beginning of training also correlates positively with cell survival (Leuner et al., 2004). In that study, animals had not yet reached asymptotic performance, yet those that expressed more CRs retained more cells. In the following series of experiments, the extent to which a second training experience affects the survival of one week old neurons will be examined.
EXPERIMENT 1
Performing a task that has previously been learned is not sufficient to rescue new neurons from death

INTRODUCTION

To date, all of the studies on trace eyeblink conditioning and neurogenesis have focused on a single training experience (Gould et al. 1999; Shors et al. 2001, 2002; Leuner et al., 2004, 2006; Dalla et al., 2007; Waddell and Shors, 2008). No studies to date have examined the effects of subsequent learning experiences on new population of new cells. Typically, animals are naïve when they first receive a BrdU injection. One week after the injection, they are removed from their home cages and for the first time, they are exposed to the entire set of stimuli associated with the training experience. As such, a persistent question regarding this phenomenon is what happens after the task has already been learned; does continued training on the same hippocampal-dependent task recruit an entirely new population of cells for survival? Or once a task is learned, does the increase in cell survival to occur?

What happens after learning?
Once an animal has learned the temporal association between the CS and the US, several learning-related events may occur. Perhaps the most intuitive learning process that occurs is that the association tends to weaken with time; this is simply referred to as forgetting (Pavlov, 1927). Forgetting may be measured by the decrease in the number of conditioned responses. However, the number of conditioned responses may decrease for other reasons as well. For example, if an animal is repeatedly presented with the conditioned stimulus in the absence of the unconditioned stimulus, it will begin to change its behavior. Specifically, the animal learns that the CS no longer predicts the US and hence, the number of conditioned responses decreases. Although on the surface, the behavior may look like forgetting – in both cases a decrease in the number of conditioned responses is observed – it is actually quite different. This decrease in CRs as a result of
CS alone trials is known as extinction and was first described by Pavlov (1927). Extinction is an active learning process in which the animal learns a new association, namely that the CS does not predict the US. In extinction, the absence of a conditioned response indicates learning. The degree to which the CR is extinguished depends on features of the training experience (Kehoe, 2006). After extinction training, the number of conditioned responses tends to drop quite dramatically and in some cases, it may actually inhibit subsequent learning (Bouton, 1989; Bouton and Swartzberger, 1989).

**Does extinction affect subsequent learning and cell survival?**

While extinction has been extensively investigated in the context of learning theory, its neural basis is poorly understood (Bouton and Moody, 2004; Myers and Davis, 2002). In the context of neurogenesis, extinction has received little attention. In order to understand how learning-related processes may affect new neurons, it is important to tease apart the underlying learning processes that lead to the expression of the conditioned response. As noted, the absence of a conditioned response does not indicate the absence of learning. After extinction training, the animal learns to suppress its previously conditioned response when the conditioned stimulus is presented. Although the animal does not express any overt behavior, learning indeed occurs. In most cases, when the animal is re-exposed to the training apparatus and a paired set of stimuli, conditioned responding to the CS re-emerges quite rapidly, a phenomenon called facilitated re-acquisition (Kehoe, 2006). Under some circumstances, prolonged extinction training has been demonstrated to slow subsequent learning (Bouton, 1989; Bouton and Swartzentruber, 1989). Generally, in order to retard subsequent acquisition, the extinction training must be more extensive than the initial acquisition training. Furthermore, this phenomenon has been demonstrated using animal models of fear behavior, with less evidence for it using trace eyeblink conditioning procedures (Bouton, 1989).

The first question we asked here was whether a new population of cells would be rescued from death in animals that had already learned the trace response and were subsequently exposed to yet more trace conditioning. Animals were exposed to two phases of trace
conditioning. During the first phase, animals are removed from their home cages and trained for 800 trials across four days with trace eyeblink conditioning, a hippocampal-dependent task that rescues one week old neurons from death (Gould et al., 1999; Leuner et al., 2004; Dalla et al., 2007). After Phase 1 training was complete, animals were injected for the first time with a single dose of BrdU. One week later, animals are re-exposed to the same training procedures as in the first phase – 800 trials across four days. The performance in these animals was compared to that in another group of animals which remained in their home cages during phase 1, received a BrdU injection when they were naïve, and one week later trained with only one phase of trace conditioning – 800 trials across four days. If performing the same hippocampal-dependent task for a second time rescues a new population of cells, then animals that received two phases of trace conditioning should have a similar number of BrdU-labeled cells as those that were trained with only one phase. Both of these groups should have more BrdU-labeled cells than untrained animals.

The second question asked here was whether extinction training, immediately following the first phase of trace conditioning, would make subsequent performance during a second training experience more difficult. That is, would previous extinction training interfere with subsequent memory retrieval and would this, in turn, increase the number of cells that would be rescued?

A second group of animals was trained with two phases of trace conditioning in a similar manner. However in this group, at the end of the Phase 1, animals were trained with CS alone trials until the conditioned response was extinguished (400 trials across two days). When the CS alone is presented, animals learn to suppress the conditioned response. Animals trained with extinction trials learn that the CS does not always predict the occurrence of the US, whereas animals that were never trained with extinction trials learned that the CS always predicts the occurrence of the US. One day later, BrdU was injected and one week later, animals were again trained with 800 trials of trace conditioning. Number of learned responses at the start of Phase 2 was compared between animals that received two phases of trace conditioning with or without extinction training. Number of BrdU-labeled cells was compared with animals that received two phases of
trace conditioning with or without extinction, only one phase of trace conditioning, or no training at all. If extinction interferes with subsequent learning or expression of the conditioned response, then it is possible that the number of surviving new neurons may differ between groups. It is possible that the second training experience could be more difficult for the animals because they would have learned conflicting pieces of information regarding the CS. Initially the CS predicts the US and later it as no consequence. This type of learning may rescue more new neurons from death.

METHODS

Subjects
Adult male Sprague-Dawley rats at least 60 days of age were used for these experiments. Animals were singly housed in a temperature and humidity controlled vivarium with free access to food and water. Light-dark cycle was 12 hours with lights on at 7:00 a.m. and off at 7:00 p.m. There were four groups of animals in this experiment: animals trained with two phases of trace eyeblink conditioning (Trace Trace; n=8); animals trained with two phases of trace eyeblink conditioning followed by two days of extinction training at the end of phase 1 (Trace Extinction Trace; n=6); animals trained with only one phase of trace eyeblink conditioning (Trace Alone, n=5); naive animals that received no training and remained in their home cage throughout the duration of the experiment (Naïve; n=6).

Eyeblink conditioning: surgeries
For eyeblink conditioning, electrodes with four ultra thin wires (0.001mm) were made in the laboratory. The tips of the wires were stripped, crimped, and placed in a small, plastic connector. Two wires are used to pass mild electric current (0.65 mA) to the eyelid and the other two are used to record the eyelid muscle activity (endritic graphy, EMG). For surgeries, rats were anesthetized with sodium pentobarbital and isoflurane. The fur around the animal’s eyelid and on top of the animal’s head was shaved with an electric razor and Betadyne™ was applied to the area. A small incision was made on the rat’s head to expose the surface of the skull. Hydrogen peroxide and saline were applied
to stop bleeding and dry the exposed skull. Each head stage was placed on the surface of the rat’s skull and held in place using dental acrylic and four small screws, partially screwed into the skull. A thin needle is used to pierce the flesh around the eye and thread the wires through the skin. Three wires were placed equidistant around the rat’s right eyelid, and the fourth wire was equally spaced caudal to the eyelid. Wires are partially stripped, clipped and coiled so the end of the wire sits against the rats’ flesh. Dental acrylic was re-applied around the head stage and antibiotic ointment was placed around wound after surgery had been completed. During surgical recovery, rats were placed under a heat lamp and upon awakening, were returned to their home cages. A minimum of 3 days was allowed for recovery before the start of training.

Eyeblink conditioning: apparatus and training

The eyeblink chamber was a sound-attenuated Plexiglas box (10 x 12 x 10 in) with metal bars as the floor. Each chamber houses one animal where the animal can ambulate freely. A speaker was placed on top of the chamber to deliver the auditory cue. To deliver electrical impulses and record eyelid muscle activity, connectors were plugged into the head stage of each animal. The connectors attached to electronics that both delivered electrical impulses and recorded muscle activity that was visualized using an oscilloscope (Lafayette Inc; Coulbourn Instruments). These were attached to desktop computers which displayed the electromyographs (EMG) of eyeblink muscle activity (Viewdac™). For eyeblink conditioning, standard laboratory procedures were followed. On the first day rats were exposed to the chamber for one habituation session. During the habituation session, no tone or shock was administered and spontaneous blinks were recorded; animals were acclimated to the experimental procedures and apparatus. The next day, spontaneous blinks were again recorded for approximately 15 min. Animals are then exposed to 10 trials of the conditioned stimulus, a burst of white noise (83 dB). These CS alone presentations were done to habituate the animals to the sound so that the stimulus was not novel once training begins. The first day of training began immediately after CS habituation. For trace conditioning, a paired trial included a 250 ms conditioned stimulus (83 dB, 5 ms rise/fall time), a 500 ms stimulus-free interval (trace), and a 100 ms unconditioned stimulus (0.65 mA electrical pulse). The intertrial interval was
randomized between 20 and 30 seconds. Once the animal learned to predict the occurrence of the US, it closed its eyelid just before the onset of the US. In order to be counted as a conditioned response (CR), the EMG signal of the eyeblink must meet the following criteria: more than 4 standard deviations above baseline and occur within 500 ms before onset of the US. Baseline was defined as the 250 ms interval preceding onset of the CS.

Animals were trained with two consecutive 100 trial blocks were per day (200 trials per day). One phase of training continued for four days for a total of 800 trials, or eight 100 trial blocks. Phase 1 and phase 2 were done in a similar manner, except habituation procedures were only done before the start of phase 1. By phase 2, animals had already been well acclimated to the training apparatus and procedures. For animals that were trained with extinction, two consecutive 100 trial blocks were done on day 5, 24 hr after the end of acquisition. Another two 100 trial blocks were done on day 6. Therefore, animals received 400 CS alone trials across the two days that follow acquisition training (800 paired trials across four days). Before and after training, animals remained singly housed in their home cages. See Figure 4 for schematic of experimental design.

**BrdU-injections**

Bromodeoxyuridine (BrdU) was mixed at 15 mg/ml in 0.9% saline, pH = 7.4. A single dose (200 mg/kg) was injected into the intraperitoneal cavity (i.p.) at least two days after phase 1 training had been completed. Animals that received only one phase of training remained in their home cages during the first phase. One week after BrdU injection, animals were exposed to another four days of trace conditioning. The habituation procedures were not repeated, before the start of the second phase.

All animals were sacrificed 21 days after BrdU injection. At this time point, the majority of BrdU-labeled cells in the granule cell layer have differentiated into neurons (Christie and Cameron, 2006). The cell population of interest is those cells that were one week old at the start of the second phase of training. By using only a single dose of BrdU one week before phase 2, only the cells that are one week old at the start of phase 2 are
labeled. By counting the number of these BrdU-labeled cells, one can determine how the second phase of training affected these cells. While multiple labeling may lead to a larger sample of the population being rescued, it may also increase variability due to differences in cell cycle kinetics. If an injection was given before both phase 1 and phase 2, cells of different ages would be labeled. Using peroxidase methods, all cells would appear similar, with a distinct brown color. It is currently not technically feasible to distinguish between two cell populations of different ages. Attempts are being made to accomplish this, i.e. the use of other thymidine analogs such as iododeoxyuridine (IdU) along with BrdU. However, differences in antigenicity of respective antibodies for IdU and BrdU are not always equivalent. That is, BrdU and IdU do not bind to their respective labels with equal affinity (unpublished data; Leuner et al., *SfN Abstract* 2007). IdU has a higher affinity for its antibody than BrdU, thus leading to higher numbers of labeled cells in this population. Furthermore, in a small percentage of animals, BrdU sometimes goes undetected (unpublished data; Cameron and McKay, 2001). The randomness and unpredictability of this event provided a final reason why a single BrdU injection was favored for these experiments. If two phases of training (with or without extinction) rescues an entirely new population of cells, then these animals should have approximately as many BrdU-labeled cells as animals trained with only one phase. Minimally, if a second training experience enhances cell survival then animals trained with two phases should have more BrdU-labeled cells than untrained animals.

**Perfusions and histology**

All animals were sacrificed 21 days after BrdU injection, more than one week after Phase 2 training was complete. This rest interval after the end of training allows time for cells not rescued by learning to complete apoptosis, i.e. programmed cell death. It also allows time for the cells that are rescued by learning to differentiate into neurons. Animals were deeply anesthetized with sodium pentobarbital and transcardially perfused with 4% paraformaldehyde in PBS. Brains were stored in 4% paraformaldehyde at 4°C for at least two days, then transferred to endriti-buffered saline (0.1M PBS, pH=7.4) before cutting. The right hemisphere was mounted onto an oscillating tissue slicer and sections were cut at 40 μm. Every 12th section of the hippocampus was mounted onto slides and used for
BrdU-immunohistochemistry using peroxidase methods. Briefly, citrate buffer, pH 6.0, was microwaved until boiling. Sections were placed into citrate buffer, reheated for another 5 minutes, allowed to cool at room temperature for 15 minutes, and rinsed with 0.1M PBS. Trypsin was used to permeabilize cell membranes; 2N hydrochloric acid in PBS was used to denature DNA. Sections were incubated in primary antibody, mouse anti-BrdU, (1:100, Becton Dickinson, San Jose, CA) overnight at 4°C. The next day sections were incubated in secondary biotinylated anti-mouse (1:200, Vector) followed by avidin-biotin complex (Vectastain ABC Kit, Vector, Burlingame, CA), then 3-3’-diaminobenzidene (DAB SigmaFast tablets, Sigma-Aldrich). Mounted sections were stained with 0.1% cresyl violet in order to visualize the entire granule cell layer. Sections were then dehydrated in progressively increasing concentrations of ethanol, and finally in xylene. Slides were coverslipped with Permount™. BrdU-labeled cells were counted using a 100x oil-immersion objective on a light microscope with the experimenter blind to condition. The ocular piece was 10x so therefore, cells were magnified 1,000 times. To estimate the total number of BrdU-labeled cells in the dentate gyrus, cell counts were multiplied by a factor of 24 (2 hemispheres x 12 serial sections).

**Statistical Analysis**

Performance during classical eyeblink conditioning was assessed by comparing the mean number of conditioned responses during each of the eight 100 trial blocks. To compare the effects of extinction training on subsequent learning, the first 100 trial block of phase 2 was analyzed in smaller units – five 20 trial blocks. Mean conditioned responses across phase 1, across phase 2 and between end of phase 1 and start of phase 2 were compared using one-way ANOVA with repeated measures followed by Tukey post hoc test, as well as independent or paired samples t-test. Mean BrdU-labeled cells in the dentate gyrus were also compared using one-way ANOVA followed by Tukey post hoc test.
RESULTS

**Learning during the first phase**
In trace eyeblink conditioning, learning is defined by how accurately the animal can predict the onset of the unconditioned stimulus by the end of training. For any single trial, when the animal closes its eyelid immediately before the occurrence of the US, a conditioned response is recorded.

As training trials progress, an increase in the frequency of conditioned responses indicates learning. To measure learning, the number of conditioned responses across trials was compared. On any given trial, the occurrence of a conditioned response was recorded as a 1 and the absence of the CR was recorded as a 0. Therefore, on any single block of 100 trials, the sum, the mean, and the percentage all have the same value. For each animal, the percentage of conditioned responses for every block of 100 trials was calculated. The mean and standard error of the mean for each training group of animals was calculated. Animals in the Trace Trace (n=8) condition expressed 36 ± 7 % conditioned responses during the first trial block and increased to 67 ± 8 % conditioned responses during the last trial block of phase 1 indicating that they learned to predict the US \[F(7,42)=3.25; p<0.01\] (Table 1, Figure 5). Animals in the Trace Extinction Trace condition (n=6) expressed 46 ± 8 % during the first trial block and reached a similar level of conditioned responding 67 ± 8 % during the last trial block of phase 1 thereby also indicating learning \[F(7,35)=3.4; p<0.01\] (Table 2, Figure 5). For the animals that only received a single phase of trace conditioning one week after the BrdU injection (Trace Alone), mean conditioned responses increased from 42 ± 10 % to 79 ± 2 %; therefore, these animals learned that the CS predicts the occurrence of the US \[F(3,12) = 3.80; p<0.05\]. Rate of acquisition and conditioned responding by the end of initial training was similar across groups of animals \(p > 0.05\).

**Extinction trials**
After day 4 of phase 1, animals in the Trace Extinction Trace group were returned to the conditioning chambers on days 5 and 6 for extinction training. An extinction trial was defined as the presentation of the conditioned stimulus without the unconditioned
stimulus, or simply CS alone. Procedures for extinction trials were similar to paired CS-US trials. However, animals were trained 400 extinction trials across two days (200 trials per day). They were trained until mean conditioned responses was below 20%, which is lower than that observed during the first trial block of acquisition. Indeed, animals expressed fewer conditioned responses after the first trial block of extinction; they decreased from $67 \pm 8\%$ during the end of acquisition to $29 \pm 3\%$ [$F_{(1,5)}=19.2; p < 0.01$]. By the end of the two days of extinction training, the expression of conditioned responses decreased even further to $15 \pm 3\%$ [$F_{(3,15)}=8.50; p<0.01$ (Figure 5)]. Animals almost completely suppressed the previously acquired conditioned response. Therefore, animals in the Trace Extinction Trace group learned that the conditioned stimulus is no longer predictive of the unconditioned stimulus.

**Learned responses at the end of phase 1 compared with start of phase 2**

During the time between the end of phase 1 and the start of phase 2, animals remained in their home cages. At least 48 hrs after the end of phase 1 training, animals from both groups received a single BrdU injection. For all animals, this was one week after the first day of training (day 8). Animals remained in their home cages until the start of phase 2. One week after the BrdU injection, they began training for the second phase of trace conditioning which was similar to the first phase – 800 paired CS-US trials across four days. To determine how well the animals remembered the task, the last trial block of Phase 1 was compared to the first trial block of Phase 2. For animals in the Trace Trace group, conditioned responding remained high. Although there was a slight decrease from $67\%$ to $50\%$, it was non-significant [$F_{(1,7)}=3.81; p > 0.05$]. With the conditioning parameters used here, an 83dB burst of white noise as the CS, a 500 ms stimulus-free trace, and a 0.65 mA electrical impulse, male rats typically reach asymptotic performance at about 60% (Gould et al., 1999; Shors et al., 2001; Leuner et al., 2004; Dalla et al., 2007). By the end of the first day, mean percentage of conditioned responses was close to 60 indicating a memory of the CS-US association. In the Trace Extinction group, mean number of conditioned responses was also compared between the last trial block of extinction training and the first trial block of phase 2. Although the conditioned response was extinguished at the end of phase 1, at the start of phase 2 animals were performing as
well as those that received no extinction training. Compared to the last trial block of extinction, number of conditioned responses increased dramatically \( [F_{(1,5)} = 21.19; p < 0.01] \). On the first trial block, mean CRs were 50.8 ± 7.7 %. By the end of the first day of the second phase, mean CRs for this group of animals was 65 ± 6 %. Thus, animals remembered the task despite suppression of the conditioned response at the end of phase 1. More than one week after acquisition and extinction training, animals could still predict the occurrence of the US, and did so as well as animals with no extinction training.

In order to determine whether there was any difference in re-emergence of the conditioned response between groups of animals with or without extinction training, the first 100 trial blocks of phase 2 was analyzed in smaller units – five blocks of 20 trials were compared. For this analysis, all six of the Trace Extinction Trace animals were included. In the Trace Trace group, six of the eight animals were included. This was because for two of the animals, the smallest complete unit of data available was as a 100 trial block due to technical difficulty. Somewhat surprisingly, there was no difference between mean conditioned responses of Trace Trace and Trace Extinction Trace animals for any of the trial blocks examined, even the first 20 trials. Mean conditioned responses across the first 20 trials for animals that received extinction training was 24 ± 7 %, which was similar to animals that did not receive extinction training, 26 ± 11 % \( [F_{(1, 10)} = 0.016; p > 0.05] \). Of the first five 20 trial blocks of phase 2, the largest difference occurred during trials 21 – 40, although mean conditioned responses was still very similar; animals with no extinction training expressed 60 ± 11 % CRs and animals with extinction training expressed 47 ± 8 % \( [F_{(1, 10)} = 1.00; p > 0.05] \). In the animals that received extinction training, this rapid re-emergence of the conditioned response provides evidence that the extinction trials did not “erase” the original CS-US association. Extinction trials did not inhibit or retard subsequent re-acquisition of the CS-US association. Therefore, learning that the CS does not always predict the US did not interfere with the ability to remember the CS-US pairing.
When mean number of CRs at the end of phase 1 was compared with mean number of CRs during the first 20 trial blocks of phase 2 using a paired samples t-test, there was a significant decrease in conditioned responding in both Trace Trace \( t(5)=3.30 \ p<0.05 \) and Trace Extinction Trace groups \( t(5)=5.96, \ p <0.01 \). However, as previously described, by the end of the first day of phase 2, both groups of animals are performing close to learning criterion, 60%. After more than one week in their home cages, upon returning to the conditioning procedures the conditioned response re-emerged rapidly in both groups of animals. Task acquisition during the second phase is facilitated by having already learned the task during the first phase; this phenomenon has been described as facilitated re-acquisition (Estes, 1950).

**Learning during the second phase**

In animals in the Trace Trace group, the percentage of conditioned responses continued to increase across the eight 100 trial blocks from 50.6 ± 8.5 to 76 ± 4.0 \( F(7,49)=3.16; \ p <0.01 \). Therefore during the second phase, the accuracy and reliability with which the animals could predict the US improved. Although the frequency of conditioned responses continued to increase during this phase, animals had already exceeded learning criterion (60%) by the end of the first day. In animals in the Trace Extinction Trace group, mean conditioned responses did not significantly increase across trial blocks, however there was a trend; conditioned responses at the start were 50.8 ± 7.7 % and rose to 68.8 ± 9.6 % on the last trial block \( F(7,28)=2.2; \ p=0.06 \). Similar to the animals with no extinction training, these animals were above learning criterion by the end of the first day (65 ± 6 %). During the second phase, in both groups of animals the eyeblinks become more accurate and also more discrete. That is, the EMG responses of the eyeblinks appeared stronger and contained less noise. It appeared that the animals became more skilled at anticipating the occurrence of the US. Thus for animals that received two phases of training, the second phase may indicate an ability to master the task.
Newly dividing cells labeled after phase 1

In this experiment, the cell population of interest is the cells that are dividing after animals have already learned the hippocampal-dependent trace conditioning task. Therefore, BrdU was given after animals had completed phase 1 training. With the conditioning parameters used here, four days of trace conditioning has been repeatedly demonstrated to rescue new neurons from death, and therefore, these animals would presumably have rescued new one week old neurons from death (Gould et al., 1999; Shors et al., 2001; Leuner et al., 2004, 2006; Dalla et al., 2007). A single BrdU injection one week before the start of the second phase was preferred for this experiment for reasons already described in the methods. Some animals remained in their home cage during phase 1; therefore, they were naïve at the start of phase 2. These animals received only a single phase of trace conditioning (Trace Alone). The number of BrdU-labeled cells in this group was compared to animals that received two phases of trace conditioning, either with or without extinction training, as well as naïve untrained animals that remained in their home cage throughout the duration of the experiment. All animals were sacrificed 21 days after a single BrdU injection; therefore, the same cell population was compared across all groups. Animals that were trained for two phases of trace conditioning and were never exposed to extinction trials had 3588 ± 271 BrdU-labeled cells in the dentate gyrus (n=8). This was similar to the animals that did receive extinction training at the end of phase 1; mean number of BrdU-labeled cells in the dentate gyrus for this group was 3031 ± 146 (n=6). Mean number of BrdU-labeled cells of naïve untrained animals was 3595 ± 438 (n=6). Animals that received only a single phase of trace eyeblink conditioning had significantly higher number of cells than all other groups, 5531 ± 450 [F(3,21) = 9.14; p< 0.01 (Figure 6)]. Therefore, performing a task that has already been learned does not recruit an entirely new population of cells. Importantly, animals that were trained for two phases were already performing above learning criterion (60% CRs) on the first day of phase 2 (second trial block). Although these animals continued to improve their performance during phase 2, the temporal association between the CS and the US had already been formed more than one week earlier.
DISCUSSION

This experiment was designed to address a persistent question regarding learning-induced cell survival; namely, is a second population of newly generated cells recruited for survival if the task has already been learned? In order to answer this question, animals were trained with two phases of a hippocampal-dependent task. Animals were trained with four days of trace eyeblink conditioning and then injected with a single dose of BrdU; one week later, they were re-trained using the same conditioning procedures. Some of the animals were trained with extinction trials immediately after acquisition; these animals trained with the unpaired conditioned stimulus learned that the conditioned stimulus does not always predict the CS. Furthermore, they learned to suppress the conditioned response when the CS was presented. Regardless of extinction training, animals remembered the task quite well, as indicated by their performance on the first day of the second phase. During the last 100 trials on the first day of phase 2, mean conditioned responses for animals in the Trace Trace group was close to their level of conditioned responding at the end of acquisition training (phase 1). Upon initial re-exposure to the training procedures, they were already performing close to learning criterion typically observed with this task. Similarly, animals in the Trace Extinction Trace group were also performing near criterion early in phase 2. Therefore, more than one week after first learning the task, animals with or without extinction training remembered and could accurately predict the occurrence of the US.

Once the trace task has already been learned, there is a decreased dependency on the hippocampus. During phase 2 when the animals perform the trace task for the second time, there is already a memory of the CS-US association in place. Although the hippocampus was critical during the first phase of trace conditioning, it may not have been as important during the second phase. As time progresses, there is a decreased involvement of the hippocampus for retrieving the trace memory (Kim et al., 1995; Takehara et al., 2003). One day after training, hippocampal lesions dramatically impair performance of a previously learned response; however, if the hippocampal lesion is not imposed until one
week after acquisition, then animals express a similar number of conditioned responses as they did before the lesion (Kim et al., 1995; Takehara et al., 2003). Therefore, the ability to retrieve the memory and express the learned response more than one week after it is learned does not depend on the hippocampus. In this experiment, the numbers of new cells in the Trace Trace and Trace Extinction Trace group were similar to the number of new cells in untrained animals. Only animals with a single phase of trace conditioning had elevated numbers of BrdU-labeled cells. Perhaps no new neurons were rescued during the second phase because animals had already learned the CS-US association more than one week prior; hence, during the second phase the hippocampus was not even necessary for expressing the memory of the task. Thus, although they performed the trace task for 800 trials during phase 2, they had already learned the CS-US association during phase 1.

A fundamental distinction in the study of learning and memory is distinguishing between effects due to learning versus those due to performance (Tolman, 1954). In determining the function of neural progenitor cells in learning in vivo, this distinction becomes increasingly important. For instance at the end of phase 1, animals in the Trace Trace group are expressing a high number of learned responses whereas animals in the Trace Extinction Trace group are expressing very few. Upon considering only the change in behavior between the end of phase 1 and the start of phase 2, one can see that animals in the Trace Extinction Trace group express a dramatic increase in the number of conditioned responses. They express about 15% CRs at the end of phase 1 and increase four-fold to about 60% on the first day of phase 2. However in the Trace Trace animals, there is very little change in the number of conditioned responses. Obviously, this difference in behavior between groups is due to the fact that the unconditioned stimulus is not presented to the Trace Extinction Trace animals at the end of phase 1. The training conditions were changed (400 CS alone trials were given) so that these animals would learn to suppress the expression of the learned response. Despite the rapid re-emergence of the conditioned response at the start of phase 2, new neurons were not recruited in this group. Despite a change in performance, there was no real change in learning; at least
with regards to acquisition of the CS-US association. Perhaps this explains why new neurons were not recruited.

These data are consistent with previous studies from our laboratory which demonstrate that learning *per se*, not mere exposure to training, is an important factor in rescuing new neurons from death (Dalla et al., 2007; Sisti et al., 2007). In one study, animals were trained with either standard trace conditioning, or contiguous trace conditioning, in which the CS is presented twice during a trial (Bangasser et al., 2006). Regardless of which task animals were trained with, those that learned well had more BrdU-labeled cells than those that learned poorly (Dalla et al., 2007).

In the present experiment, during the second phase of trace conditioning animals are merely performing what they have already learned during the first phase. Animals with or without extinction training have already learned that the conditioned stimulus predicts the occurrence of the unconditioned stimulus. Therefore these data provide further evidence that learning *per se* is a critical factor in rescuing new neurons from death.

The data from this experiment also speaks to the role of the hippocampus in learning-induced cell survival. Once animals have learned the trace task, they become progressively less dependent on the hippocampus as time progresses (Kim et al., 1995; Takehara et al., 2003). More than one week after trace conditioning, animals with a hippocampal lesion can still accurately retrieve the trace memory that was acquired before hippocampal-damage (Takehara et al., 2003). In a recent study, the role of hippocampal-dependence in learning-induced cell survival was tested (Leuner et al., 2006). To do this, animals were trained with two days of delay conditioning immediately followed by four days of trace conditioning. In naïve animals, the hippocampus is required to learn the trace task (Solomon et al., 1986). When the hippocampus is lesioned, animals cannot learn this version of the task. However, this deficit can be reversed if animals learn the delay version of the task before the hippocampus is damaged. That is, animals that learn delay conditioning do not need an intact hippocampus to subsequently learn the trace task (Beylin et al., 2001). Therefore, animals were trained
with four days of trace conditioning one week after BrdU injection with one critical exception – during the two days preceding trace conditioning, animals were trained with the delay version of the task (Leuner et al., 2006). Interestingly, when animals learned the delay task prior to the trace task, the number of surviving one week old neurons was similar to naïve animals. By attenuating the role of the hippocampus in learning, the number of cells that were rescued from death was reduced. The number was reduced so much that the cell counts were similar to animals that received no training at all (Leuner et al., 2006). Therefore, it appears that a minimal degree of hippocampal activation may be necessary for learning to rescue hippocampal cells from death.

It is important to note that during phase 2, the number of conditioned responses continued to increase across trials of training. However, animals were already close to learning criterion on the first day of the second training experience. With the conditioned and unconditioned stimuli that were used here, an 83 db auditory cue followed 0.5 sec later with a 0.65 mA pulse to the eyelid, male rats typically reach asymptotic responding at or near 60% (Gould et al., 1999; Shors et al., 2001; Leuner et al., 2004). In this experiment, animals in both Trace Trace and Trace Extinction Trace groups were near 70% by the end of the second day. In addition, the eyeblinks, as indicated by electromyography signals, became more discrete and precisely timed during this phase. Thus, accuracy and reliability of the precisely timed learned response continued to improve, although knowledge of the CS-US pairing had already been acquired. In a sense, during the second training experience animals began to master performance of a task that they had already learned. This concept will be explored more rigorously in the general discussion.

In this experiment, animals are re-exposed to a task that they have already learned. It is possible that neurons presumably rescued by the first training experience are re-activated during the second training experience. The re-activation of new neurons was not explicitly tested. However, others have examined re-activation of new neurons when animals are re-exposed to a similar environment (Tashiro et al., 2007). Animals were exposed to an enriched environment at different time points after BrdU injections; they were later re-exposed to the same environment just before perfusions (Tashiro et al.,
In standard laboratory conditions, rats and mice have free access to food and water, but not much else. In the enriched environment, animals are provided with running wheels, toys, and are housed with their litter mates. Although enriched environment does not specifically include hippocampal-dependent learning tasks, it provides a stimulating environment in which some degree of learning occurs and furthermore, it enhances the survival of one week old neurons (van Praag et al., 1999; Tashiro et al., 2007). Following perfusions, hippocampal sections were labeled for the neuronal nucleus protein NeuN, and the immediate early gene c-fos, along with BrdU. The immediate early gene, c-fos, is widely used as an indicator of cellular activity (Tashiro et al., 2007). Thus, cells that contain all three labels, BrdU, NeuN and c-fos, represent activated new neurons. They found that cells which were one week old at the start of the first exposure to the enriched environment were significantly higher in number than mitotic cells labeled either earlier or later; in addition, animals in which one week old cells were labeled had the highest number of activated new neurons. This effect appears to be specific to re-exposure to the same environment. In a subsequent experiment, animals were either re-exposed to the same enriched environment or exposed to a new experience, the Morris water maze, just before perfusions. In this case, a different immediate early gene was used to measure activation of new cells, Zif268 (Tashiro et al., 2007). Again, re-exposure to the same environment increased the number of activated new neurons compared to controls – only in the animals exposed to the same environment; animals exposed to the water maze training immediately before perfusions did not have elevated numbers of activated new neurons. Interestingly, in trace eyeblink conditioning cells generated one week before training are not only rescued from death, but they remain in the granule cell layer one and two months after the learning experience (Leuner et al., 2004). Activation of these cells was not tested, but it certainly remains possible that these new neurons are involved in subsequent performance of the task.

In the present experiment, newly dividing cells were labeled after a hippocampal-dependent task had already been learned. When animals were re-exposed to the training procedures more than one week later, all animals remembered the task quite well regardless of extinction training. Although animals from both groups continued to
improve during the second phase, they were already performing near learning criterion on the first day. Because the animals had already learned the CS-US association, perhaps it was not necessary to recruit an entirely new population of cells to perform the task. In fact, when the survival of new neurons is enhanced by trace conditioning, they remain in the hippocampus for at least two months after learning (Leuner et al., 2004); from an evolutionary perspective, it seems to make poor biological sense to recruit an entirely new population of cells for performance of a task that has recently been learned. Indeed, new neurons in the hippocampus are activated when re-exposed to the same environment (Tashiro et al., 2007). Therefore, perhaps they are also activated when performing a previously learned response. Overall, these data provide another example of the importance of learning, not merely performance, in rescuing one week old neurons from death.
EXPERIMENT 2

New learning predicts cell survival

INTRODUCTION

In the previous experiment, performing a hippocampal-dependent task that had already been learned did not rescue one week old neurons from death. To determine this, it was important to distinguish between learning versus performance. In the next experiment, I investigate whether learning a new and different task would enhance the survival of one week old neurons. Here again, this fundamental distinction in the study of learning and memory becomes evident; tasks must be similar enough so that the effect on cell survival can be attributed to learning per se and not performance or other confounding variables. For instance, environmental cues, physical activity, stress, and type of learning have all been demonstrated to regulate neurogenesis (van Praag et al., 1999; Pham et al., 2003; Gould et al., 1999; Shors et al., 2001). If the new learning experience is different in regards to one or more of these properties, an interaction between or among them may occur, thereby also affecting the fate of new cells. By changing only the temporal relationship between two similar learning tasks, new information is provided to the animal so that learning per se and its effects on the survival of one week old neurons can be evaluated. Using two versions of an eyeblink-conditioning task, we asked the following question: is a new population of cells rescued from death with each new learning experience?

Two associative learning models which share several important features are the trace and very long delay (VLD) eyeblink conditioning tasks. In both tasks, animals learn to associate an auditory cue with a mild electrical pulse to the eyelid. The qualities of the conditioned stimulus and unconditioned stimulus are similar; therefore, animals in both groups experience similar sensory cues. The conditioned response is also similar. In both tasks, animals learn to close their eyelid immediately before the onset of the unconditioned stimulus; therefore, all animals learn to express the same motor response. However, animals learn a different temporal relationship depending on the task. During
training with the very long delay task, the conditioned stimulus is temporally contiguous with the unconditioned stimulus. That is, the CS overlaps and co-terminates with the offset of the unconditioned stimulus (Figure 7). In addition, the duration of the conditioned stimulus in VLD is substantially longer; the CS is presented for 1500 ms, six times longer than in the trace conditioning task. In trace conditioning, the CS is presented for 250 ms, followed by a 500 ms stimulus free interval, and finally the unconditioned stimulus. On the surface, this difference may appear to be minor and inconsequential. On the contrary, many investigations have been focused on understanding the function of the hippocampus in learning and remembering temporal relationships; data across laboratories and animal models have established that even a 200 millisecond change in stimulus can alter the neural circuitry that makes learning and memory possible (Moyer et al., 1990). Given that the central nervous system operates on the order of milliseconds, i.e. firing of action potentials, this is not so surprising. In this experiment, the use of two similar, but distinctly different, tasks allows learning variables to be carefully isolated and evaluated, and in turn, those factors which affect the fate of newly dividing cells may be identified.

In addition to using the same conditioned stimulus and unconditioned stimulus for both tasks, they share several other important properties. For instance, when animals are trained on either trace or VLD, the overall rates of acquisition are similar. Because rates of acquisition have been demonstrated to play a role in determining the fate of one week old neurons, it is important that rates of acquisition are similar (Waddell and Shors, 2008). A naïve animal will learn the trace conditioning task at about the same rate as it will learn very long delay conditioning. Although early in the training number of conditioned responses tends to differ, the overall rates of acquisition are comparable (Beylin et al., 2001; Leuner et al., 2006). Secondly, both tasks must engage the hippocampus. In a recent study, the role of the hippocampus in learning the very long delay task was tested. In animals with hippocampal lesions, learning very long delay was significantly impaired compared to animals with sham lesions (Beylin et al., 2001). After extensive training (>1,000 trials), lesioned animals did begin to learn the very long delay task; regardless, the involvement of the hippocampus was indeed evident. Because hippocampal
activation is a critical factor in rescuing new neurons from death, both tasks should involve the hippocampus (Gould et al., 1999; Leuner et al., 2006). Finally, each task, when learned in isolation, must be sufficient to rescue one week old neurons from death. In a recent study, animals were trained in a similar manner, 800 trials of the same conditioned stimulus and unconditioned stimulus across four days. One group of animals was trained with very long delay conditioning and a second group was trained with trace conditioning (Leuner et al., 2006). BrdU was injected one week before the start of training. In animals that learned either the trace or the very long delay task, the number of surviving new neurons was higher compared with the animals trained with standard delay conditioning or animals that received no training. Number of new neurons that were rescued was not significantly different in animals trained with either trace or VLD (Leuner et al., 2006). To date, trace and very long delay are the only two associative learning models that rescue one week old neurons from death. With each task, animals learn a different temporal relationship between stimuli, yet they are exposed to similar environmental and sensorimotor experiences. Therefore, trace and very long delay conditioning are particularly well suited to address the question: is a new population of cells rescued from death with each new learning experience?

The experimental design is similar to the previous experiment in that animals are trained with two phases of eyeblink conditioning separated by more than one week in their home cage. As before, animals are injected with BrdU after the first training experience is complete, one week before the start of the second training experience. However, in this experiment, one group of animals is trained with trace conditioning during phase 1 and then they are trained with a different task, very long delay conditioning (VLD) during phase 2. The second group of animals is also trained with a new task during the second phase, however the sequence of tasks is presented in the reverse order – animals are trained with very long delay conditioning during phase 1 followed by trace conditioning during phase 2. Learning and number of new neurons are evaluated.
METHODS

Subjects
Adult male Sprague-Dawley rats at least 60 days of age were used for these experiments. Training conditions included trace conditioning during phase 1 and very long delay conditioning during phase 2 (n=7) and another group of animals trained with very long delay conditioning during phase 1 followed by trace conditioning during phase 2 (n=11). Housing conditions were similar to the previous experiment.

Eyeblink conditioning procedures
Surgeries were done as described in the previous experiment. Apparatus and training procedures were also similar. For trace conditioning, the conditioned stimulus was a burst of white noise (82-83 dB) lasting 250 ms. It was followed by a 500 ms stimulus-free interval and a mild electrical pulse to the eyelid (0.65 mA) lasting 100 ms. For very long delay conditioning, the CS was a burst of white noise (82-83 dB) which remained on for six times as long, 1,500 ms. The conditioned stimulus and unconditioned stimulus overlapped and co-terminated. The unconditioned stimulus was an electrical pulse to the eyelid (0.65 mA) lasting 100 ms. The US was presented during the last 100 ms of the CS (after the auditory cue had been on for 1,400 ms). One group of animals was trained with eight 100 trial blocks (200 trials per day) across four days of the trace eyeblink conditioning task. Three days after they had learned the trace task during phase 1, animals were injected with a single dose of BrdU (200 mg/kg). One week after BrdU, these animals were trained with a different task, very long delay conditioning. During the second phase, training procedures were similar to the first phase, however, no habituation procedures were done. During phase 2, these animals were trained with very long delay conditioning for eight 100 trial blocks (200 trials per day) across four days (Trace then VLD, n=7). As before, when animals were not being trained, they remained in their home cages. A second group of animals was exposed to the same training procedures and BrdU injection schedule; however, in this group, animals learned the very long delay
conditioning task (VLD) first during phase 1, and more than one week later they were trained with the trace conditioning task (VLD then Trace, n=11).

**BrdU injections and histology**

BrdU was mixed as previously described. In order to track the fate of a single cell population, i.e. cells generated *after* learning one task but one week *before* learning a different one, a single injection of BrdU is used. Just as before, all animals were sacrificed 21 days after BrdU injection. Transcardial perfusions, brain sectioning and cell counts were done as previously described.

**Statistical analysis**

On any given trial, a conditioned response is counted as a 1 and the absence of a conditioned response is counted as a 0. As such, when the number of CRs is added across a 100 trial block, the value of the sum is equal to both the value of the mean and the value of the percentage. For example, if an animal expresses a CR on 60 out of 100 trials for any given 100 trial block, the sum is 60, the mean is 60, and the percentage is 60.

To evaluate learning, mean conditioned responses were compared across trial blocks (100 trials per block) using one-way ANOVA. In some cases individual trial blocks were compared using an independent or paired samples t-test. Mean BrdU-labeled cells were compared using one-way ANOVA. During phase 2 for all animals, the individual learning curve was determined by finding the best-fit straight line across the eight 100 trial blocks using least squares linear regression. These calculations were done in Sigma Plot (version 8.0) which fits the straight line in matrix terms and uses Cholesky decomposition to invert the matrix and produce the regression curve (Draper and Smith, 1981). The dependent variable was the sum of conditioned responses for each of the eight 100 trial blocks and the independent variable was the trial block number, i.e. 1 – 8. The $b_1$ value from the regression equation is the change in the y-axis (slope). As such, it represents the increase/rise in conditioned responses, or simply, how much the animal improved its overall performance across trial blocks. A steep slope would indicate a large improvement whereas a shallow slope would indicate a small improvement; no inferences are made regarding the speed at which this may occur. Because the objective
of these experiments is to determine the fate of one week old neurons after learning (i.e. after phase 1), $b_1$ reported here always refers to Phase 2. Pearson’s product moment correlation was calculated to evaluate the relationship between $b_1$ and number of BrdU-labeled cells. That is, does overall improvement during the second training experience predict the number of surviving one week old neurons?

**RESULTS**

*Learning during first phase*

Animals that were trained with either trace or very long delay during phase 1 successfully learned the task (Figure 8). For animals that learned the trace task first, the mean number of conditioned responses on the first block of 100 trials was $22 \pm 4 \%$. By the last trial block of phase 1, mean number of conditioned responses had increased to $60 \pm 7 \% \left[F(7,42)=3.71; p<0.05\right]$. Similarly, animals that were trained with the very long delay task during phase 1 also expressed an increase of conditioned responses across trial blocks. On the first trial block, animals expressed $33 \pm 4 \%$ conditioned responses and increased to $70 \pm 7 \%$ by the last trial block $\left[F(7,63)=5.89; p<0.01\right]$. Although animals trained with very long delay task tended to express a slightly higher number of conditioned responses, there was no significant differences on any of the trial blocks ($p>0.05$). Therefore, regardless of the temporal discontiguity between the conditioned stimulus and unconditioned stimulus, animals in both groups learned to accurately predict the occurrence of the US.

*Learning at the end of phase 1 compared with start of phase 2*

During the end of phase 1, both groups of animals were performing at or above 60%; this is the point at which asymptotic performance is often observed in male rats using these training conditions (Gould et al, 1999, Beylin et al, 2001, Leuner et al 2006, Shors et al 2001). After the end of training, all animals were injected with a single dose of BrdU (200 mg/kg) and one week later they were trained with a different task. To determine whether animals could use the information they had learned during phase 1 in order to
learn the new temporal relationship in phase 2, mean conditioned responses between the last trial block of phase 1 and the first trial block of phase 2 were compared. Animals that learned the trace task during phase 1 expressed a similar number of conditioned responses across the end of phase 1 (60 ± 7 %) and the start of phase 2 (56 ± 7 %) \([t_{(6)}=0.34; p >0.05]\). However, animals that learned the very long delay task during phase 1 had a dramatic drop in mean conditioned responses at the start of phase 2 \([t_{(10)}=6.60; p <0.01]\). At the end of phase 1 VLD training, animals were expressing 70 ± 7 % responses. During phase 2, animals were trained with the trace task in which the CS is only 250 ms and is followed by a 0.5 sec stimulus free interval and finally the US. On the first trial block of trace conditioning, they only expressed 16 ± 4 % conditioned responses. Mean CRs only increased slightly on the second trial block (22 ± 5 %). Because animals in either condition expressed a similar number of conditioned responses at the end of phase 1, differences in learning during phase 2 can not be attributed to differences in the previously learned association. These data suggest that animals could not easily adapt their responses for the trace task when they had previously learned the very long delay task. Therefore, training animals with the same two tasks resulted in very different patterns of learning depending only on the order in which they were presented. As previously described, the conditioned stimulus and unconditioned stimulus were qualitatively similar; therefore animals were exposed to similar sensory-perceptual experiences. The same training procedures and apparatus were used. Even the learned response was similar – closing the eyelid before onset of the US. The primary difference between these two tasks was the temporal relationship between the two. In one version, the CS and US are temporally contiguous (VLD), in another version they are not (Trace). With respect to phase 2, learning very long delay then trace is more difficult than learning trace and then very long delay. Despite similar levels of acquisition of both tasks during the first phase, a dramatic difference occurs during the second phase. Therefore, regarding phase 2, the very long delay then trace task sequence is more difficult for the animals than the reverse.

**Learning during the second phase**
In animals that learned the trace task during the first phase, and the very long delay task during the second phase, the mean number of conditioned responses remained close to 60% throughout all 800 trials of very long delay conditioning, i.e. asymptotic performance. On the first trial block, the mean number of conditioned responses was 56 ± 7 % and on the last trial block, mean number of CRs was 67 ± 11 %. This was a slight increase but was not significant ($F_{(7,42)} = 0.816; p > 0.05$). There appears to be ‘transfer of learning’ in the animals that learn the trace task first and then the very long delay task (Thorndike and Woodworth, 1901). That is, animals use information about the temporal relationship they learned during the trace task in order to adapt their conditioned responses to accurately predict the unconditioned stimulus during the very long delay task in phase 2. They appear to use what they have learned in phase 1 about the CS-US association and apply it to a different temporal relationship in phase 2. This is quite different from the pattern of conditioned responses that is observed in the very long delay then trace group. In these animals, there appears to be no immediate transfer of learning. On the contrary, learned responses are inhibited; CRs are not reliably expressed until later in phase 2. That is, at the start of phase 2 in which animals are trained with the trace task, mean number of CRs is below 20%. On the first trial block, they only express 16 ± 4 % conditioned responses (Figure 8). This is less than they expressed on the first trial block of phase 1; it is also less than the conditioned responding that is observed in naïve animals exposed to the trace task for the first time (Table 4). Interestingly, they express a steady and gradual increase over the course of the 800 trials. While they only express 16 ± 4 % CRs on the first trial block of phase 1, by the last trial block they express 57 ± 6 % conditioned responses [$F_{(7,70)} = 12.32; p < .01$]. Although it takes more trials for these animals to learn, they do indeed learn the different temporal relationship in trace conditioning. By the end of phase 2, the animals learn to accurately predict the occurrence of the US.

**Newly dividing cells labeled after phase 1 and before phase 2**

Similar to the previous experiment, BrdU was injected one week before the start of phase 2. Again, all animals were perfused 21 days after BrdU injection. Therefore, the cell population is similar across groups. In animals trained with trace then very long delay,
the mean number of BrdU-labeled cells in the dentate gyrus was 4092 ± 282 (n=7). This was not different from animals trained with very long delay then trace (p>0.05). In this group, the mean number of BrdU-labeled cells in the dentate gyrus was 3,333 ± 181 (n=11). These cell counts were compared to the animals that were trained with only a single phase of training (5531 ± 450) or no training (3596 ± 438) reported in the previous experiment. Animals exposed to a single phase of training (Trace Alone) had a higher number of BrdU-labeled cells than animals in either the Trace → VLD or the VLD → Trace condition or untrained animals [F(3,25)=8.74; p<0.01].

New neurons and individual learning
Although there was no overall group effect of the number of BrdU-labeled cells and phase 2 learning, there was an obvious difference in learning between animals that learned the Trace → VLD sequence and animals that learned the VLD → Trace sequence. The VLD → Trace sequence was clearly a more difficult task sequence, as indicated by the initially low number of conditioned responses. Animals trained with the Trace → VLD sequence were already performing at asymptote and did not express any increase in the number of learned responses (p>0.05). However, animals trained with the VLD → Trace task sequence did demonstrate new learning. There was a significant increase in the number of conditioned responses across 800 trials [F(7,70) 12.32; p < .01]. Therefore, it was of interest to characterize the individual learning curves of the animals that demonstrated new learning and then determine if learning would be correlated with the number of surviving new neurons. In order to do this, the sum of the conditioned responses of each of the eight 100 trial blocks was determined for each animal. (The presence of a conditioned response is recorded as a 1 and the absence is recorded as a 0; therefore, the sum of CRs for a 100 trial block has the same value as the percentage or mean). The eight values were plotted so that trial block (or time) is on the x-axis and number of conditioned responses was on the y-axis. The best fit straight line was determined for each animal using the regression function in Sigma Plot (version 8.0). Sigma Plot fits the straight line in matrix terms and uses Cholesky decomposition to invert the matrix and produce the regression curve (Draper and Smith, 1981). For each animal trained with the most difficult task sequence (very long delay then trace), the least
squares method was used and the slope of the best-fit line was calculated. As previously described, because $b_1$ from the regression equation is the rise in the y-axis (slope), in this experiment $b_1$ is the overall increase in CRs across trial blocks. A steep slope indicates a large change in learned responses across training, whereas a shallow slope indicates only a slight change in learned responses across training. Best, average and worst learners are graphed in Figure 9. For each training condition, the $b_1$ value from the regression equation was compared to the number of BrdU-labeled cells using Pearson’s product moment correlation. There was a significant positive correlation between overall improvement ($b_1$) and number of BrdU-labeled cells in animals trained with very long delay then trace ($R=0.75$, $p<0.05$; Figure 10). Although there was no group effect of cell survival, animals that demonstrated a large degree of improvement had a high number of BrdU-labeled cells. For animals in the trace then very long delay group, individual learning curves during phase 2 were then also calculated. In the trace then very long delay group, there was no correlation between $b_1$ and BrdU-labeled cells ($R=0.57$, $p>0.05$). As a group, these animals did not express a significant increase in number of learned responses during the second training experience. In other words, in the group that demonstrated no change in learning, there was no correlation between improvement and number of new neurons (trace then very long delay). On the contrary, in the group that demonstrated a dramatic change in learning, there was a significant positive correlation between overall improvement across the second training experience and the number of new neurons (very long delay then trace).

**DISCUSSION**

The purpose of this experiment was to determine whether a new population of hippocampal cells is rescued from death with each new learning experience. In order to test this, it was important to match several attributes of the two tasks so that changes in learning could be isolated and evaluated. The importance of controlling for confounding variables becomes particularly clear when one considers the numerous factors that affect neurogenesis. For instance, environmental enrichment, physical activity, stress, and type
of learning all regulate neurogenesis (van Praag et al., 1999; Pham et al., 2003; Shors et al., 2002). If animals are trained with two tasks that differ on one or more of these attributes, it becomes more challenging to isolate those effects which are due to new learning versus those that may be due to a change in physical activity or stress. Ideally, the tasks would be similar in terms of sensory and perceptual information, environmental cues, and motor patterns that the animal must perform, yet each task would provide new and unique information to the animal. In addition, it is also important that (1) each task is equally difficult or easy to learn (2) the hippocampus is involved (3) each task when learned individually rescues one week old neurons from death. Both the trace task and the very long delay task meet each of these criteria.

In this experiment, animals were trained with two different tasks, trace and very long delay eyeblink conditioning. The design of this experiment was similar to the previous one in that each animal was trained with eyeblink conditioning during two phases which occurred about a week apart. As before, animals were injected with BrdU after phase 1 had been completed and one week before the start of phase 2. In so doing, the fate of cells generated after one learning experience, but one week before another, could be determined. However, there are important differences between the design of experiment 1 and that used here for experiment 2. In the trace eyeblink conditioning task, the conditioned stimulus and the unconditioned stimulus are temporally discontiguous. That is, there is a 0.5 sec time interval between the offset of the CS and the onset of the US during which no stimuli are presented. In a second task, very long delay eyeblink conditioning, the conditioned stimulus and the unconditioned stimulus are temporally contiguous. There is no stimulus free gap. Instead, the CS overlaps and co-terminates with the offset of the US. In addition, in the very long delay task, the conditioned stimulus is presented for a longer period of time; the auditory cue remains on six-times longer than the auditory cue in trace conditioning. Thus, despite sharing several properties, each task has a unique temporal contingency.

Each group of animals was trained sequentially with both tasks; only the sequence in which they were trained differed. One group learned trace (phase 1) then very long delay
(phase 2); in the other group, the sequence was reversed. The six major findings were: (1) when animals are trained with the very long delay task, after already having learned the trace task, they immediately express a high number of learned responses. The number of learned responses remains stable, i.e. animals perform at asymptote, throughout the entire training period of phase 2 (2) the number of BrdU-labeled cells in the trace then very long delay group was similar to untrained animals. That is, the number of surviving cells generated one week before the second training experience was similar to animals with no training (3) in this group, which demonstrated no significant change in learning during phase 2, there was no correlation between phase 2 learning and BrdU-labeled cells (4) when animals were trained with the trace task, after already having learned the very long delay task, they initially expressed a very low number of learned responses. However, as training progressed, there was a significant increase in conditioned responding and animals eventually learned the trace task (5) the total number of BrdU-labeled cells in the very long delay then trace group was similar to untrained animals (6) in this group, there was a significant positive correlation between overall improvement and number of BrdU-labeled cells. Animals that had a larger increase in conditioned responses had more BrdU-labeled cells than those with a small increase in CRs. Each of these findings raises several important issues regarding the relationship between learning and neurogenesis.

Behaviorally, there is an effect of task sequence. Reversing only the order in which the animals were trained resulted in a dramatic difference in learning during the second phase. In neither group of animals was a new population of cells rescued from death. However, in the group that demonstrated a significant change in learning during phase 2, a significant positive correlation occurred between overall improvement (b₁) and how many one-week old neurons survived. Each of these findings is discussed in turn.

How easily or quickly a new task is learned is determined in part by previous learning experiences; this is a fundamental learning phenomenon that is highly conserved across species and tasks. In classical conditioning, one striking example of this phenomenon is referred to as the CS pre-exposure effect, or latent inhibition (Solomon and Moore, 1975; Domjan, 2003). In this procedure, repeated presentations of the conditioned stimulus alone induce a slower rate of acquisition in subsequent pairings of that same conditioned
stimulus with an unconditioned stimulus. Although the conditioned stimulus was never presented alone in these experiments, animals began phase 2 with very different amounts of CS pre-exposure depending on which task sequence they were trained. In very long delay conditioning, on a single trial the CS is on for six times longer than a single trial of trace conditioning, 1.5 sec compared with 0.25 sec respectively. Thus, animals trained with the very long delay task during the first phase have been exposed to the CS for a total of 20 min (800 trials x 1.5 sec / 60 sec). On the contrary, animals trained with trace task during phase 1 are only exposed to the CS for barely more than 3 min (800 trials x 0.25 sec / 60 sec). Although the CS and US are always paired, there is still a large difference in CS pre-exposure between groups. Animals trained with very long delay first have been exposed to the CS for 17 minutes longer than the other group. A learned response for either task must be accurately timed on the order of milliseconds. It is possible that a difference of nearly 20 min might alter subsequent learning, as demonstrated by the animals trained with very long delay conditioning and then trace.

The effects of latent inhibition of trace conditioning have recently been examined (Waddell and Shors, 2008). In that study, conventional latent inhibition procedures were used. That is, the unpaired conditioned stimulus (CS alone) was presented for several trials before animals were trained with paired CS-US trials. Therefore, animals first learn that the conditioned stimulus has no consequence, but later they learn that the CS actually predicts the occurrence of a mildly aversive stimulus. Specifically, animals were trained for 600 trials across three days (200 trials per day) with only the conditioned stimulus (Waddell and Shors, 2008). The CS was an auditory cue (burst of white noise) lasting 250 ms. The same conditioned stimulus was then paired with an unconditioned stimulus for trace conditioning. One day after CS pre-exposure, animals were trained with 800 trials across four days of trace conditioning. The rate of acquisition was then compared with animals trained with only four days of trace conditioning (no CS pre-exposure). Indeed, extended exposure to the conditioned stimulus inhibited subsequent trace conditioning. That is, animals with the CS pre-exposure required more trials to reach the same level of conditioned responding as animals with no CS pre-exposure (Waddell and Shors, 2008). This bears some resemblance to the phenomenon reported here; extended
CS pre-exposure retards subsequent acquisition of the CS-US association, despite the fact that in this experiment the conditioned stimulus was always paired with an unconditioned stimulus. Thus, the slow rate of acquisition observed during phase 2 of the very long delay then trace group might be explained in part by the CS pre-exposure effect or latent inhibition. However, this explanation is speculative and would require further experiments.

The degree to which CS pre-exposure interferes with subsequent trace conditioning is probably less important than the role of temporal discontinuity. A second, perhaps more compelling explanation is the shift in temporal contiguity. The hippocampus is important in associating stimuli that are discontinuous in time (Wallenstein et al., 1998). In the present experiment, animals learn two temporal contingencies. In the trace task, the CS and US are discontinuous whereas in the very long delay task, the CS and US are contiguous. When the discontinuous relation is learned first, the animals can readily learn the contiguous relation. For example, animals learn trace then they easily adapt their conditioned responses to learn the contiguous association during very long delay training. However, the reverse is not true. When animals first learn that the CS and the US are contiguous in time, they have difficulty learning during the second phase, that the CS is now temporally discontinuous with the US. A somewhat similar phenomenon was observed using the standard delay task (Leuner et al., 2006). Animals were trained with two days of delay conditioning immediately followed by trace conditioning. When animals started training with the trace task, there was a significant drop in number of conditioned responses. This occurred despite the fact that the animals had previously learned during delay training that the CS predicts the occurrence of the US. They eventually learn the trace task, however, animals required more than 800 trials (Leuner et al., 2006). One possibility is that when the temporal gap is inserted, the animals initially behave as if it was an extinction trial. That is, in the trace task the CS is off, and it is immediately followed by a 0.5 sec stimulus free interval. After this temporal gap, or trace, only then is the US presented. They previously learned that the CS overlaps and co-terminates with the US. Therefore, the animals expect that the end of the CS is the end of the trial. However, this is not the case. They eventually learn that 500 ms after
the end of the CS, the US is presented. They eventually learn this new temporal contingency, despite requiring more trials. To understand precisely why one task sequence is more difficult than the other, additional experiments that are designed to explicitly test the role of temporal discontiguity are needed. Apart from why this phenomenon occurs, one can see that what is learned in the future is affected by what was learned in the past, which may in turn affect the fate of newly generated cells in the hippocampus.

Regardless of how learning during the first phase affected learning during the second phase, it is clear that in regards to phase 2 one task sequence is much more difficult than the other. In animals that were first trained with the very long delay task, there was a dramatic decrease in the number of conditioned responses at the start of phase 2 when a different task was presented. When animals were first exposed to the trace task, they were unable to accurately predict the occurrence of the unconditioned stimulus. However, as training trials progressed, their performance improved. It continued to improve over the entire training period. By the end of phase 2, they were performing at approximately 60%, indicating that they had successfully learned the new temporal relationship (Figure 8). Only the animals in this group demonstrated new learning. Learning is an important predictor of cell survival (Leuner et al., 2004; Sisti et al., 2007; Dalla et al., 2007). As such, it was of interest to further characterize the individual learning curves for each animal and determine whether there was a relationship between learning and number of surviving BrdU-labeled cells. To do this, the total number of conditioned responses during each of the eight 100 trial blocks of phase 2 was plotted for each animal. The best fit straight line was determined using the regression function in Sigma Plot (version 8.0); the dependent variable was the total number of conditioned responses on a given trial block and the independent variable was the trial block number, i.e. 1-8. The slope ($b_1$) was used as a measure of learning (Figure 9). A large $b_1$ value indicates a steep slope and hence represents a large change in the number of CRs. When these values were correlated with the number of BrdU-labeled cells, there was a significant, positive correlation (Figure 10). Therefore, how much an animal improves its performance over time predicts how many of the newly generated neurons survive.
In the present experiment, there was a significant positive correlation between learning and surviving new cells. It occurred only in the group trained with the very long delay task first and then the trace task. The second training experience did not rescue an entirely new population of cells from death. However, the absence of an overall group effect does not preclude the existence of a systematic relationship between learning and cell survival. There are at least three critical factors that may explain why an entirely new population of one week old neurons was not rescued. These factors include: (1) decreased involvement of the hippocampus during the second training experience (2) a reduced cell population available for rescue after the first training experience (3) cells from the first training experience may have been re-activated and re-used during the second training experience. Evidence for each of these explanations is discussed in turn.

Activation of the hippocampus seems to be an important property in rescuing one week old neurons from death (Gould et al., 1999; Shors et al., 2002; Leuner et al., 2006). As time progresses, memory of the CS-trace-US association becomes progressively less dependent on the hippocampus (Kim et al., 1995; Takehara et al., 2003). When the hippocampus is lesioned one week after training with the trace task, hippocampal-lesioned animals did not express as many conditioned responses as animals with sham or prefrontal lesions. However, there was some preservation of the trace memory. When the hippocampus was lesioned four weeks after training, the number of conditioned responses remained high. Hippocampal-lesioned animals could recall the trace memory as well as controls (Takehara et al., 2003). Thus, in regards to the trace conditioning task, hippocampal-dependence has a time limited role. In these experiments, animals start the second phase of training ten days after the end of phase 1. This coincides with a decreased dependence on the hippocampus. In another study, trace conditioning was rendered hippocampal-independent by training animals with the delay task immediately before exposure to the trace task (Leuner et al., 2006). Animals trained with two days of delay conditioning immediately followed by four days of trace conditioning had a similar number of BrdU-labeled cells as untrained controls (Leuner et al., 2006). That is, attenuating hippocampal-dependence attenuated the effect on cell survival. Therefore, in
the present experiment the number of one week old neurons that were rescued from death may have been reduced due to decreased hippocampal-involvement during the second training experience.

Secondly, it is also important to consider the impact that different stages of the learning process have on cell survival. In a recent study, the impact of different stages of learning on cell death was examined (Dupret et al., 2007). Using a spatial learning task, the Morris water maze, two stages of learning were described. The early stage was defined as the period during which acquisition of the task occurs and a dramatic change in the behavioral response occurs. The late stage was defined as the period during which asymptotic performance occurs and a behavioral change is no longer observed. To measure the extent of cell death in the hippocampus, two specific apoptotic markers were used, caspase-3 and fractin (Dupret et al., 2007). When animals that learned the spatial task were compared to control and yoked animals, a significant increase in both apoptotic makers occurred, representing an increase in hippocampal cell death. To determine which stage of learning led to increased cell death, animals were sacrificed at different time points during training. The increase in cell death occurred once the animals had reached asymptotic performance, but not before this point (Dupret et al., 2007). Furthermore, this effect was specific to the dentate gyrus; no changed occurred in CA1 or CA3. It was also specific to hippocampal-dependent learning; there was no change when animals were trained on the visible platform, which is the hippocampal-independent version of the task (Dupret et al., 2007). In the present experiment, animals reach asymptotic performance by the end of phase 1 training, regardless of task sequence. It is possible, then, that this asymptotic learning may have induced apoptosis in the hippocampus, thereby reducing the number of cells available for rescue during the second phase.

Despite the unique information provided by each task, these tasks are indeed similar. As was described previously, a recent study demonstrated that new neurons are activated when animals are re-exposed to the same environment (Tashiro et al., 2007). Data from this laboratory has demonstrated that nearly all, if not all, of the mitotic cells (S-phase)
available for rescue at the start of training are indeed rescued (Waddell and Shors, 2008). To test this, two groups of untrained animals were compared to one group of trained animals. All three groups were given a single BrdU injection. One week later, on Day 7, trained animals began their first day of training with the trace task and one group of untrained animals was perfused. Number of BrdU-labeled cells in this group indicates the number of one week old neurons available for rescue at the start of training. Twenty-four hours after the last day of training, Day 12 after BrdU injection, trained animals were sacrificed. The third group of untrained animals was sacrificed at the same time. Number of BrdU-labeled cells in all three groups were compared. Trained animals had significantly more BrdU-labeled cells than untrained animals sacrificed on Day 12. However, trained animals had a similar number of BrdU-labeled cells as those sacrificed on Day 7. Therefore, four days of trace conditioning rescued all the cells that were available for rescue at the start of training – at least those mitotic cells that were labeled and in the S-phase at the start of training (Waddell and Shors, 2008). Furthermore, these cells remain in the hippocampus one and two months after training, long after the hippocampus is even needed (Leuner et al., 2004). From an evolutionary perspective, it does not make biological sense to rescue an entirely new population of neurons for a recently acquired task. Therefore, it is possible that cells presumably rescued during the first training experience would be used during the second training experience.

In this experiment, animals were trained with two different tasks. The only difference between groups was the order in which animals were trained. By reversing only the task sequence, a very different pattern of learning was observed, as well as a different effect on cell number. Animals trained with very long delay then trace had difficulty accurately predicting the occurrence of the unconditioned stimulus. Over the course of training, these animals eventually learned the task. In this group that demonstrated new learning, there was a significant positive correlation between the amount of improvement and the number of surviving one week old neurons. It seems that in order for learning to enhance the survival of newly generated cells with each subsequent learning experience, the new task must be sufficiently difficult or challenging so that new learning will occur.
COMBINED ANALYSIS

New learning rescues new neurons from death, regardless of previous experience with the same or a different task

INTRODUCTION

A subtle but critical distinction in the study of learning and memory is distinguishing between effects due to learning versus those due to performance (Tolman, 1955). When the behavior of animals is averaged across a group, effects due to learning may be difficult to decipher (Gallistel et al., 2004). It may even leave important psychological phenomena undetected (Gallistel et al., 2004; Papachristos and Gallistel, 2006). This issue becomes increasingly important when investigating the relationship between neural precursor cells in vivo and psychological phenomena. Therefore in this section, the behavior of each individual animal is further examined. The goal of these experiments was to determine how a second training experience affects the fate of one week old neurons. As such, particular attention is given to performance throughout the second training experience. Emphasis is placed on learning parameters that have already been shown to predict the survival of new cells (Leuner et al., 2004; Dalla et al., 2007; Sisti et al., 2007).

Learning per se is a critical factor in rescuing new neurons from death (Leuner et al., 2004, Dalla et al., 2007; Sisti et al., 2007). Animals must not only be exposed to the training procedures and experience the same stimuli, they must demonstrate that they have adequately learned the temporal contingencies or spatial relations of a given hippocampal-dependent task. How well the animal learns a task can be measured in more than one way. For instance, both asymptotic performance and improvement across training can predict the survival of one week old neurons (Drapeau et al., 2003; Dalla et al., 2007; Sisti et al., 2007; Sisti and Shors, present data). In the initial report that learning rescues cells from death, animals trained with trace conditioning needed to meet a learning criterion of 60% (Gould et al., 1999). Animals that failed to express
conditioned responses on 60% of the trials were considered poor learners and were not included in the analysis. More recently, animals were trained with two versions of trace conditioning and were categorized as good or poor learners again using this 60% threshold (Dalla et al., 2007). Importantly, the training parameters were similar across studies, e.g. 82-83 dB white noise for the conditioned stimulus (250 ms), 500 ms trace, 0.65 mA current (100 ms). Animals at or above 60% conditioned responding retained more one week old neurons than those below this level of responding (Dalla et al., 2007). It is proposed here that the asymptotic level of conditioned responding (60% with these training procedures) is a critical learning threshold and that this threshold may be important in determining cell survival for a second training experience. Secondly, overall improvement across training was also evaluated. In the second experiment, linear regression was used to evaluate learning during the second training experience. The value of $b_1$ from the regression equation indicates the change in conditioned responses across trial blocks (the slope). When $b_1$ was correlated with the number of surviving BrdU-labeled cells, a significant, positive correlation was detected ($R=0.75$; $p<0.05$; Figure 10). Thus far, this was only detected in the animals trained with very long delay and then trace. Here, initial level of performance at the start of phase 2 and the improvement throughout the second training experience are compared with number of BrdU-labeled cells in animals across all training conditions.

**METHODS**

*Subjects*

Animals from both experiments were combined for further analysis. All animals were always perfused 21 days after a single BrdU injection. BrdU was always injected one week before the start of phase 2 so that number of surviving cells can be compared across groups. Animals with only a single phase of trace conditioning remained in their home cages during the first phase.
Defining learning parameters

The least squares method was applied to animals in the first experiment as in the second experiment. That is, the best-fit straight line across the eight 100 trial blocks of phase 2 was determined. These calculations were done in Sigma Plot (version 8.0) which fits the straight line in matrix terms and uses Cholesky decomposition to invert the matrix and produce the regression curve (Draper and Smith, 1981). As previously described, the dependent variable was the sum of conditioned responses for each of the eight 100 trial blocks and the independent variable was the trial block number, i.e. 1 – 8. The $b_1$ value from the regression equation is the change in the y-axis (slope). As such, it represents the increase/rise in conditioned responses, or simply, how much the animal improved its performance across trial blocks – the steeper the slope, the greater the degree of improvement. To evaluate performance at the start of phase 2, the mean number of conditioned responses across day 1 was calculated for all animals.

Evaluating new learning

Animals across all 5 training conditions were collapsed, and the correlation between $b_1$ and BrdU-labeled cells was tested using Pearson’s product moment correlation. Animals from the five groups were exposed to different training procedures. Some animals were naïve at the start of phase 2 (Trace Alone), some animals already had experience with the task (Trace Trace and Trace Extinction Trace), and others learned a different task that was either easy or difficult to acquire (Trace then very long delay or Very long delay then Trace). Collapsing animals in this manner ignores the training procedures that underlie performance. The subtle distinction between learning versus performance becomes increasingly important when testing how psychological phenomena affect the fate of neural progenitor cells in vivo. Therefore, to determine if other correlations existed, specific criteria were set so that only the animals that met a given set of criteria would be included in the statistical test of Pearson’s product moment correlation. Criteria were based on factors that already seemed to be important in rescuing cells from death, such as improvement and 60% conditioned responding. Animals that met criteria would qualify
for Pearson’s product moment correlation test in which $b_1$ and BrdU-labeled cells were compared.

Improvement ($b_1$) is a good predictor of cell survival. In animals trained with very long delay than trace, there was a significant correlation between improvement and one week old neurons. When animals started the second phase, many of them had already experienced the training procedures, and in some cases, the very same task. Here it is proposed that a minimum degree of improvement may be necessary to rescue new neurons from death during a second training experience. In this case, individual animals are compared to the rest of the sample; the median value of $b_1$ was determined across animals. If the value of $b_1$ for a given animal was above or equal to the median value ($\mu_{b_1}$), then they qualified for the Pearson’s product moment correlation test. If the value of $b_1$ for an animal was less than the median value ($b_1 < \mu_{b_1}$), then the animal did not meet this criterion and was not included in Pearson’s test.

In addition, the number of conditioned responses that each animal expressed on the first day of the second learning experience was determined. The mean of the first and second trial block of phase 2 were calculated for each animal. Several animals had not yet surpassed 60% conditioned responding at the start of the second phase, regardless of training condition. As previously described, using these training parameters 60% conditioned responding is often used to indicate whether or not an animal has learned the task (Gould et al., 1999; Dalla et al., 2007). If the animal is above 60%, we consider that it has learned the task, if it is not then more training may be needed. Therefore, a second criterion was set for animals to be included in Pearson’s product moment correlation test. Animals needed to be below 60% on the first day of Phase 2. What is of interest here is determining what is happening to cells when new learning during the second phase occurs. If an animal is not yet at 60%, then some new learning may be occurring, regardless of previous experience with the same or different task. Therefore, in order for an animal to be included in the Pearson’s test, an animal must meet two criteria, first, it must be below learning threshold at the start of phase 2 and second, it must express a minimal amount of improvement ($b_1 \geq \mu_{b_1}$). Also, to determine the flexibility of 60% as
a learning threshold, a lower threshold of learning was also tested. Instead of 60%, the
criterion was adjusted in a subsequent iteration so that in order to qualify for Pearson’s
test, animals needed to be below 50%.

To summarize, an animal must meet each of the following two criterion to be included in
Pearson’s product moment correlation test: the animal must be both below learning
threshold at the start of the second training experience (in this experiment, less than 60%
conditioned responding on day 1 of phase 2) and the animal must demonstrate a minimal
degree of improvement ($b_1 \geq \mu_{b_1}$). In essence, the animal must show evidence of new
learning during the second training experience, despite previous experience with the same
task, a different task, or no task at all. An important distinction is that animals that meet
criteria simply qualify for Pearson’s test of $b_1$ and BrdU-labeled cells. It does not
determine whether cells generated one week before phase 2 will be high or low. This
number is predicted by the value of $b_1$ (overall improvement).

**RESULTS**

When animals across all 5 training conditions were collapsed without regard to training
conditions, there was no significant relationship between $b_1$ and BrdU-labeled cells
($p>0.05$). Given the importance of learning *per se* in rescuing new neurons from death,
the first criterion to be set was performance at the start of the second training experience.
If animals were still below an asymptotic level of conditioned responding (60% with
these procedures), then the animal may still be learning the temporal contingency
between the conditioned stimulus and the unconditioned stimulus. Therefore, despite any
previous training, if animals are below this threshold at the start of the second phase, then
it is possible that some new learning may still be occurring. If new learning is still
occurring, e.g. if animals have not yet reached an asymptotic level of conditioned
responding, then the fate of one week old neurons may be affected.
The mean number of conditioned responses across the two trial blocks on day 1 of phase 2 was calculated. The performance of an animal at the start of phase 2 depended in part on its training condition. For instance, an animal that was trained with trace then very long delay could easily adapt its conditioned response and expressed a high number of CRs, i.e. 60%, very early in training. In this training condition, animals first learn that the conditioned stimulus and the unconditioned stimulus are temporally discontiguous. During trace, the CS and US are separated by a brief stimulus-free interval of 0.5 sec. After learning that the CS and the US are temporally discontiguous, they are then trained with the very long delay task. During this task, they learn that the conditioned stimulus and unconditioned stimulus are no longer separated by a temporal gap. They learn that the CS overlaps and co-terminates with the US. As such, the temporal contingency has changed and the stimuli are no longer discontiguous. The animals easily adapt to this new temporal relationship. Early in training, the group is performing near 60%. They remain at a remarkably stable level of asymptotic performance throughout the entire second training period (Figure 8). When considering all training conditions, many animals drop below 60% at the start of the second training experience. The decrease was most obvious in the group of animals trained with very long delay first then trace. Acquisition of the trace task is initially impaired when animals first learn a task in which the stimuli are contiguous, then subsequently learn a task in which the CS and US are discontiguous. Over the course of the second training experience, the animals learn to predict the US as indicated by the steady increase in the number of CRs across phase 2.

In this combined analysis, what is of interest is how well the animals express the learned response on the first day of the second training experience, regardless of previous experience. When an animal fails to express 60% CRs on a trial block, it has not yet adequately learned the CS-US association and some new learning was necessary. In looking at the performance at the start of the second training experience, some animals that were previously trained with the same task actually fell below 60% on the first day of phase 2. This occurred in animals trained with or without extinction trials. Recall that animals remained in their home cages for approximately one week between the two training experiences. It is possible that the CS-US association weakened during the rest
interval and some new learning was indeed necessary, even though the association had previously been learned. For each animal the question was asked: is this animal below 60% CRs on day 1 of phase 2?

Secondly, the performance throughout the rest of the second training experience was determined. It was hypothesized that because most animals were previously trained, in these experiments a minimal degree of improvement would be necessary to determine if learning predicts cell survival. As such, the median of \( b_1 \) was determined. The median value of \( b_1 \) was 3.6726 (\( \mu_{b_1}=3.6726 \)). For all animals, values of \( b_1 \) were always carried out to four decimal places.

When only these two criteria were used, e.g. animals were both below learning threshold at the start of the second training experience and the animals demonstrated a minimal degree of improvement during phase 2 (\( b_1 \) value for the animal was greater than the median value), 18 animals met the criteria. Animals from all training conditions were represented. Nine out of 11 animals were from the very long delay then trace group, 3 were from trace then very long delay, 2 were from trace extinction trace, 2 were from trace trace, and 2 were from trace alone. Thus, half of the animals that met criteria were from the training condition with the most difficult task sequence, e.g. during the first phase they learned the very long delay task with contiguous stimuli followed by the trace task in which the stimuli are now discontiguous. Using Pearson’s product moment correlation, the correlation between \( b_1 \) and BrdU-labeled cells was significant (\( R=0.81; p<0.05 \)). That is, if an animal had trouble predicting the occurrence of the unconditioned stimulus at the start of the second phase, but then demonstrated a minimal degree of improvement, then learning will predict cell survival. Specifically, an animal with a high \( b_1 \) (steep slope / large increase in CRs) would have a high number of BrdU-labeled cells and an animal with a lower \( b_1 \), closer to the median, would have a low number of BrdU-labeled cells. This occurred despite previous training on the same or different task, as indicated by representation of animals from all groups. Four of the animals had been trained with the same task, e.g. two with extinction training at the end of phase 1 and two with no extinction training at the end of phase 1. Three of the animals were from the
trace then very long delay group. When the overall mean of the trace then very long delay group was determined, they were above 60% very early in training. However, not every single animal could immediately predict the occurrence of the unconditioned stimulus with the change in temporal parameters. In this experiment, three of these animals were below learning threshold at the start of the second training experience. As training progressed, they improved. The $b_1$ values for each of these animals was $TV606_{b_1} = 3.8452$, $TV604_{b_1} = 7.8330$, and $TV508_{b_1} = 6.0000$. Therefore, learning threshold at the start of the second training experience and overall improvement ($b_1$) combine to determine whether learning predicts cell survival with a second learning experience ($R=0.818; p<0.05$).

To determine if 60% was indeed a critical threshold at the start of the second phase, the learning threshold was reduced to 50%. That is, animals that were below 50% at the start of phase 2 were included in Pearson’s test. Using this criterion, three animals from above were eliminated, one from trace then very long delay group, one from very long delay then trace and one from trace extinction trace. The total number of animals that met criteria was now reduced from 18 to 15, however, the correlation was still significant, ($R=0.810; p<0.05$). It was reduced very slightly by only 0.008. Therefore, 60% is not a ‘magic number’ in determining whether learning would predict cell survival. The learning threshold may be slightly lower. The criterion was set to 60% because this is the point at which robust conditioned responding tends to occur with the training procedures used here (Gould et al., 1999; Shors et al., 2002; Leuner et al., 2004; Dalla et al., 2007).

**DISCUSSION**

The objective of these experiments was to determine how a second learning experience would affect the survival of one week old neurons. Animals entered the second phase of training with different types of experiences depending on the training condition to which they were assigned. Some animals had previous exposure with the same task. Some animals had previously learned a different task. Other animals were completely naïve.
Within each training condition, there were additional differences as well. In animals trained with two different tasks, the second task was either easy or difficult to learn depending on which task was presented first. That is, the data suggest that animals trained with trace then very long delay could readily adapt their timing of the conditioned response when the temporal parameters were shifted. However, animals trained with the reverse sequence, very long delay then trace, had difficulty adapting the learned response and required nearly the entire training session to again accurately predict the occurrence of the unconditioned stimulus. Interestingly, animals trained with the same task twice had a slightly lower level of conditioned responding at the start of the second training experience compared with the end of the first training experience. That is, despite training on the same exact task twice, animals required several trials before they reached the level of asymptotic performance typically observed under these conditions. In order to discover if learning during a second training experience would rescue one week old neurons from death, it was necessary to evaluate learning for each individual animal during the second phase. It was discovered that if the task had not yet been mastered (conditioned responding was below asymptotic levels) and if a certain degree of improvement occurred, then learning will predict the number of new neurons in the hippocampus regardless of previous experience. Animals that demonstrate a large increase in conditioned responses across the second training experience retain more one week old neurons than those that demonstrate a small increase in learned responses across phase 2.

**Learning threshold**

Asymptotic performance of eyeblink conditioning has been demonstrated to predict the survival of one week old neurons in the hippocampus (Dalla et al., 2007). This relationship was discovered by comparing good versus poor learners based on their level of conditioned responding. Animals that reached a criterion of 60% CRs during training were categorized as good learners and animals that failed to reach this criterion were categorized as poor learners, regardless of which version of trace conditioning they had learned. Animals were trained with either 800 trials of trace conditioning, using similar procedures as those used here, or with 800 trials of contiguous trace conditioning. When
animals were categorized as good or poor learners based on a 60% criterion and regardless of which task the animals were trained, the good learners had more surviving BrdU-labeled cells than poor learners (Dalla et al., 2007). Using this same criterion, in the present series of experiments some animals had not yet learned the CS-US association as well as other animals at the start of the second training session. Despite previous experience with the same task or a different one, animals had not yet reached 60% by the end of the first day. Even though some animals were trained with a similar task more than one week prior, they still required more than one day of training to reach the level of asymptotic performance that is typically observed with these training conditions (60%). As such, new learning was still occurring; this was important in determining the conditions for which learning during a second training experience predicts survival of cells generated one week prior.

Learning is a dynamic process that can be categorized into different stages. This concept has been explored by Abrous and colleagues where two stages of learning were compared based on performance of animals in the Morris water maze (Dobrossy et al., 2003; Drapeau et al., 2003, Dupret et al., 2007). A sharp decrease in the time to reach the platform was defined as the early stage of learning. The point at which asymptotic performance occurs, i.e. no further decrease in escape latency, was defined as the late stage. Each of these stages affects cell proliferation and survival differently (Drapeau et al., 2003; Dupret et al., 2007). For example, BrdU was injected during the first four days of water maze training (the early stage of learning). Animals were trained for another four days and sacrificed at different time points. The late stage of learning actually decreased the number of surviving cells generated during the early stage. These cells were immature, they were only between 1 and 4 days old (Dobrossy et al., 2003). It is important to note that as immature cells, they are from a different population of cells than those examined here; they are not yet one week of age. Their cellular properties at this immature stage may result in them responding differently to learning-related events (Figure 2; Tashiro et al., 2007). In another study, the effect of early and late stage learning on cell proliferation and survival was evaluated in aged rats. When comparing cells generated about one week before the start of the early stage of learning, cell survival
was enhanced. Furthermore, in aged rats those that learned well had more surviving cells than those rats that learned poorly (Drapeau et al., 2003). The authors concluded that the cognitive status, e.g. how well animals learned the task, of aged rats predicted number of surviving cells. In the present experiment, the cognitive status at the start of the second training experience was defined as the mean number of learned responses on the first day of the second training phase. It was then compared to the asymptotic level of conditioned responding that typically occurs with these training procedures. If the cognitive status at the start of the second training experience indicates that the response had not yet been adequately acquired, then as long as the animal demonstrates an improvement in performance, the degree of improvement will predict the number of cells that survive.

**Improvement**

Improvement across training blocks is a good predictor of cell survival. When animals in just one training condition were analyzed, those trained with very long delay then trace, a positive, significant correlation was detected. Individual learning during the second training experience was mathematically described by finding the best-fit straight line for each animal (the least squares method). Here it was hypothesized that in order for learning to predict cell survival in a second training experience, a minimal degree of improvement is necessary. The minimum degree of improvement was arbitrarily set at the median value. If animals were in the top half of improvers, they met one of the necessary conditions for Pearson’s test. Therefore, if animals had not yet adequately acquired the CS-US association (below 60%), but demonstrated a minimal degree of improvement, then learning predicted cell survival, regardless of previous training with the same or different task. For these animals, those with a large change in CRs as indicated by its $b_1$ value (steep slope) had a high number of BrdU-labeled cells. Those with a small change in CRs as indicated by the $b_1$ value (shallow slope) had a low number of BrdU-labeled cells. In some instances, animals that were already performing above asymptote, or only demonstrated a small improvement had high cell counts. However, because these animals fail to meet the criteria, they do not qualify for Pearson’s test. These criteria are not intended to explain the behavioral and cellular processes of every single animal. They are merely used to determine which factors of those that were
tested will best determine when learning predicts cell survival. In these experiments, if new learning occurred during the second training experience, then despite previous experience, a larger improvement indicated more new cells would survive (R=0.81; p <0.05).

When animals were categorized based strictly on learning during the second training experience, without regard to training condition, a significant positive correlation between improvement and BrdU-labeled cells occurred. There were animals from all of the five training conditions that met learning criteria – they were below 60% at the start of phase 2 and expressed a minimal degree of improvement (b₁ ≥ µb₁). Of the 18 animals that met these criteria, there were 4 animals that had previously learned the same task, 3 animals had learned the trace then very long delay task sequence, and 3 had no prior experience at the start of the second phase. Nine of the 18 animals that met criteria were from animals trained with very long delay then trace. When the animals were categorized based on training condition, this group of animals was the only group in which a positive correlation between improvement and cell survival occurred. Animals trained with very long delay first followed by the trace task also expressed the most dramatic change in performance during the second training experience. Thus, it seems important to consider the training conditions which led to this remarkable change in learning and in turn, its effects on one week old neurons.

**Task difficulty or task demands**

Rates of acquisition have been used as a measure of task difficulty (Beylin et al., 2001). In general, tasks that take a longer amount of time to learn tend to be tasks that are more difficult, or have higher task demands, than those that are quickly learned. In human learning, this is somewhat intuitive. This occurs is animal learning as well. When the conditioned response appears early in training, the task is interpreted as being relatively easy to learn. On the contrary, when the conditioned response emerges late in training, the task is more difficult for the animal to learn. An example in eyeblink conditioning is the standard delay and trace task, where the former is easier than the latter. Task difficulty may also be increased when the animal is presented with conflicting pieces of
information. One example is latent inhibition or CS pre-exposure (Lubow and Moore, 1959). Using latent inhibition training, the animal first learns that the conditioned stimulus has no consequence, but later learns that it predicts the occurrence of a mildly aversive event. They learn two different associations about the same CS; hence the task demands are higher compared with animals that only learn a single association. In a recent study, CS pre-exposure was used to slow acquisition of the trace task (Waddell and Shors, 2008). Animals were first trained with several days of the conditioned stimulus alone, followed by four days of trace conditioning. When animals were trained with CS alone trials immediately before trace conditioning, acquisition of the trace task was impaired; that is, decremented acquisition was observed (Waddell and Shors, 2008). Animals trained with CS pre-exposure required more training trials to reach 60% conditioned responding than those trained with no CS pre-exposure. Because the task demands were increased, more trials were necessary to reach asymptotic responding. Interestingly, these animals had the highest number of surviving one week old neurons compared with easier versions of the trace task (Waddell and Shors, 2008). In a related study, the rates of acquisition were facilitated, or in other words, the task demands were reduced (Shors et al., SfN Abstract 2007). The trace interval was reduced from 500 ms to only 250 ms. When the trace task was made less difficult using a shorter trace interval, 250 ms, compared with a trace task using a longer temporal gap (500 ms), these animals required the fewest number of trials to reach 60% conditioned responding. For these animals trained with an easier version of trace conditioning, the number of BrdU-labeled cells was similar to those that had received no training at all (Shors et al., SfN Abstract 2007). Thus, reducing the task demands not only made the task easier to learn, it also reduced the likelihood of rescuing one week old neurons. In the second experiment presented here, animals trained with the very long delay task during the first training experience followed by the trace task were the only animals that demonstrated a dramatic drop in learned responses at the start of the second training experience. Thus, the very long delay then trace sequence was interpreted as the most difficult task sequence based on the animals’ acquisition during the second training experience. The positive correlation between improvement and cell survival that was detected in animals trained with this task sequence may have been due to the fact that this training condition had the
highest task demands. Furthermore, when animals were collapsed across training conditions and analyzed based on learning during phase 2, half of the animals that met criteria were from the group of animals that were trained with the most difficult task sequence. Therefore, task difficulty may be a critical factor in rescuing neurons from death with a second learning experience.

The objective of these experiments was to determine how a second training experience would affect the fate of one week old neurons. Essentially, if new learning was demonstrated during the second phase, then new cells were rescued. As previously described, task difficulty also seems to play a role. This concept is graphed in three dimensions in Figure 12. Along the y-axis is the number of BrdU-labeled cells generated after one learning experience and before the second learning experience. Along the z-axis, which runs perpendicular to the page, is the degree of overall improvement. It is the value of $b_1$ from the least squares regression equation that was calculated for each animal. Both the y- and z-axis are direct representations of the data. The x-axis is a categorical learning variable and is conceptual. It represents three levels of task difficulty – easy, moderate, or difficult. Because half of the animals that met criteria were from the group trained with the most difficult task sequence, it is proposed that task difficulty is important in rescuing cells from death with subsequent learning experiences. When animals were trained with the very long delay task first followed by the trace task, there was a dramatic change in learning. These animals required nearly the entire 800 trials to approach 60% conditioned responding. This is in direct contrast to all other training conditions. Other groups of animals were performing near 60% by the second day of the second phase. When analyzed by training condition, only animals trained with the most difficult task sequence had a positive correlation between improvement and BrdU-labeled cells. Thus, it seems that increasing task demands increases the likelihood that new learning will occur and hence, new neurons will be rescued.

**Conclusions**

In these experiments, there were two significant, positive correlations. When animals were analyzed based on training condition, there was a significant positive correlation
between improvement and number of BrdU-labeled cells in animals trained with the most
difficult task sequence (R=0.75; p<0.05). When animals across all five training
conditions were compared, there was also a significant correlation in the animals that
demonstrated new learning during the second training experience. Animals that were not
yet performing above an asymptotic level of conditioned responding at the start of the
second training experience and demonstrated a minimal degree of improvement were
evaluated. In these animals, greater improvement indicated more surviving BrdU-labeled
cells (R=0.818; p<0.05). This relationship was significant when the learning threshold
was set at 60% or even when it was only 50% (R=0.810; p<0.05). When the mean
number of BrdU-labeled cells was compared across all groups, only animals trained with
a single phase of trace conditioning retained more cells. However, the absence of an
overall group effect does not preclude the existence of a systematic relationship between
learning and cell survival. When animals were categorized based on learning during the
second training experience, those that demonstrated a greater degree of improvement
retained more one week old neurons than those with a smaller change in learned
responses.

By combining animals across all training conditions, the relationship between learning
and neurogenesis was further characterized. The objective of these experiments was to
determine how a second learning experience would affect the survival of one week old
neurons. Animals entered the second phase with different types of experiences. Some
had already learned the same task; some had learned the same task but subsequently had
that response extinguished. Others had learned a different task, and still others had no
previous experience at the start of phase 2. In essence, it was discovered that regardless
of previous experience, if new learning occurred, more cells would be retained in the
adult hippocampus, the vast majority of which differentiate into neurons (Christie and
Cameron, 2006).
GENERAL DISCUSSION

Major findings
The goal of the first experiment was to determine whether performing a task that had already been learned would rescue a new population of cells from death. Previously, it has been demonstrated that learning, not simply exposure to training, is a critical component in enhancing cell survival (Sisti et al., 2007). This occurs with different types of learning, both spatial and associative (Dalla et al., 2007). Based on these experiments, it was expected that re-training on the same task would not be sufficient to rescue new neurons from death. Indeed, it was not. Regardless of extinction training, animals re-train on a task that they had already learned had similar number of BrdU-labeled cells as animals that received no training. These findings provide further evidence that learning *per se* is critical in enhancing cell survival. In addition, these data demonstrate that 2 days of extinction training immediately after 4 days of trace conditioning will suppress the conditioned response, but it is not sufficient to retard subsequent re-acquisition. Both groups, regardless of extinction training, performed similarly when re-exposed to the learning task approximately one week later. In addition, overall cell counts between groups were similar. It is possible that cells presumably rescued from the first task were re-activated and re-used during the second training experience (Tashiro et al., 2007).

The goal of the second experiment was to determine whether learning a different task would rescue a new population of one week old neurons. In the second experiment, animals were trained with two tasks, trace and very long delay. One group of animals was trained with trace then very long delay, and the other group was trained with the reverse sequence. In one sequence, trace followed by very long delay, animals expressed a high number of conditioned responses early in training and maintained asymptotic performance throughout the entire training period. In the other sequence, very long delay followed by trace, a very different pattern of behavior was observed. When animals were trained with trace conditioning after previously learning very long delay, there was a sharp drop in the number of conditioned responses at the start of phase 2. These data
suggest that acquisition of the trace task was more difficult when it was preceded by the very long delay task. However, these animals demonstrated a gradual and steady increase in the number of learned responses across training. By the end of the second training experience, the mean number of conditioned responses was near 60%, the approximate percentage point at which asymptotic learning usually occurs with these training conditions (Gould et al. 1999; Shors et al., 2001; Leuner et al., 2004, 2006). For animals with the more difficult task sequence, a significant, positive correlation occurred between extent of improvement during training and the number of BrdU-labeled cells that survived. Those that demonstrated the largest change in conditioned response during the second training experience retained the highest number of BrdU-labeled cells.

Learning *per se* is a critical factor in rescuing one week old neurons from death (Dalla et al., 2007; Sisti et al., 2007). Therefore, rather than categorize animals based on training condition, they were re-categorized based on learning during the second phase. If an animal had failed to reach 60% conditioned responding on the first day of the second training phase and demonstrated a minimal degree of improvement over the course of training, then new learning occurred. For these animals that demonstrated new learning during the second phase, the number of BrdU-labeled cells was compared with the overall improvement during phase 2 (the \( b_1 \) value, or slope, from the regression equation). There was a significant, positive correlation between improvement and number of surviving neurons (\( R=0.81; \ p<0.05 \)). Thus, the degree of improvement predicted the number of one week old neurons rescued by the second learning experience, regardless of previous training. Many of the animals that demonstrated new learning were trained with the very long delay task first, followed by the trace task. Training with this task sequence resulted in the most remarkable change in learning during phase 2 compared with all other training conditions. It appeared that this task sequence was the most difficult. Indeed, when the number of BrdU-labeled cells was compared with overall improvement in this training group alone, there was a significant positive correlation (\( R=0.75; \ p<0.05 \)). Task difficulty as well as overall improvement may be critical in rescuing new neurons from death during a second training experience (Figure 12).
**Post-learning processes**

To determine how newly generated hippocampal cells are affected after a task has already been learned, one must first describe the possible learning-related events that may occur. Upon considering various post-learning processes, many questions arise. For instance, does the CS-US association weaken with time, as in forgetting (Pavlov, 1927)? Does the memory of the temporal relationship decay to the same extent for every single animal; what factors might determine this rate of decay? Furthermore, how does the second learning experience interact, if at all, with the previously learned association? Will learning be facilitated or impeded by previously acquired information? Finally, what role might the hippocampus, and in particular newly generated neurons, have in these learning processes? These questions engender several learning-related topics, such as forgetting, transfer of learning and proactive interference (Pavlov, 1927). Each of these learning processes, as well as the potential role of the hippocampus and new neurons, will be discussed in turn.

**Forgetting**

Once an association between a conditioned stimulus and an unconditioned stimulus has been formed, there are several possible scenarios with which the animal may be faced. Perhaps the simplest scenario is that once training has been completed on a given task, the animal is simply returned to its home cage for an indefinite period of time. In studying learning and memory processes, the absence of an event may be equally as important as the occurrence of an event. In this example, when an animal is simply returned to its home cage, the animal is no longer experiencing the CS and the US. Nor is it exposed to the training apparatus and the associated contextual stimuli. As a result, the association between the conditioned stimulus and the unconditioned stimulus begins to weaken (Pavlov, 1927). In other words, as time progresses the memory of the temporal contingency between the CS and the US begins to decay. This is intuitively understood in human learning and is commonly referred to as forgetting. Despite the intuitive nature of this process, the weakening of an association between a conditioned and an unconditioned stimulus is relevant, quantifiable, and is dependent on several factors. Each of these factors can be categorized into two broad classes: (1) the training
experience (2) the retention interval, or the time between the end of training and the point at which memory of the association is tested. Properties of the training experience that will affect subsequent retrieval of the memory include the number of training trials, the distribution of trials, the magnitude and duration of the CS and the US, and the temporal contingency of the CS and the US (Kehoe, 2006). Properties of the retention interval that will affect subsequent memory retrieval include the duration of the retention interval, as well as the contextual stimuli and experiences to which the animal is exposed during this period. In these experiments, animals were trained with 800 trials of an eyeblink conditioning task and then were returned to their home cages. They remained in their home cages for more than one week before re-exposure to the training apparatus and a similar or different task. During this retention interval, some weakening of the CS-US association occurred. This is indicated by the slight decrease in the number of conditioned responses expressed at the start of the second training experience in animals that were exposed to the same task twice. Individual differences in the rate and extent of CS-US decay occur just as individual differences in the rate of acquisition and asymptotic performance of the CS-US occur. Despite similar training procedures and similar durations of retention intervals, all animals will not retrieve the CS-US memory to the same degree. This factor was considered in defining the learning threshold criterion across all training conditions. If at the start of the second training experience an animal was not performing at the asymptotic level that occurs with these training conditions, then it was presumed that the CS-US association had weakened. If it was below 60%, it had fallen below a critical learning threshold and some new learning was necessary. Some animals had maintained a strong CS-US association and immediately expressed a high number of conditioned responses at the start of phase 2. However, just as in humans, all animals do not learn and forget at the same rate. Analyses of these individual differences in learning and performance were critical in determining the relationship between learning and cell survival during a second learning experience. In animals that continued to learn during the second training experience, the degree of improvement predicted the survival of new neurons. Animals that expressed a larger increase in learned responses retained more new cells in the hippocampus than those that expressed a
smaller increase in learned responses, regardless of previous experience with the same or a different task.

Forgetting, or decay of CS-US association, is one of many processes that may occur after learning. In these experiments, the animals did not remain in their home cages indefinitely. Rather, animals were exposed to a second training experience. Several animals were trained with a different task during the second phase (Experiment 2). Animals were trained with one of two possible sequences: trace during phase 1 followed by very long delay during phase 2, or the reverse. Interestingly, the order in which the animals learned these tasks had significant consequences on learning during the second training experience. When animals were trained with the trace task during the first phase, then later were trained with the very long delay task, these animals expressed a high number of learned responses very early in the second training experience. This occurred despite the fact that the very long delay task had never been presented to the animal. They performed at 60% conditioned responding on the first day and remained at an asymptotic level of performance throughout the entire duration of the second training experience. On the contrary, in animals trained with the very long delay task first and then the trace task, acquisition was severely impaired. In this sequence, learning the very long delay task interfered with subsequent learning of the trace task. The former phenomenon is an example of transfer of learning while the latter is an example of proactive interference (Thorndike and Woodworth, 1901). Each of these is described in more detail below.

**Transfer of learning and proactive interference**
What is learned in the past affects what is learned in the future (Shors, 2006). Previous information may either retard or facilitate the acquisition of a subsequent task. Whether subsequent learning is facilitated or impeded depends on several factors including the quality of the information. In the second experiment, an example of both of these instances was observed. In animals trained with trace during phase 1 followed by very long delay, animals immediately expressed a high number of learned responses, despite never having been
exposed to the very long delay task. This is a good example of transfer of learning, first formally described by Thorndike and Woodworth (1901). When the temporal relationship was changed, these animals were able to use their previously acquired knowledge regarding the relationship between the CS and the US to accurately predict the occurrence of the unconditioned stimulus. During the second training experience, the duration of the CS was increased and its contingency with the US was altered. During very long delay, the CS overlapped and co-terminated with the US. With these alterations, the animals quickly adapted their learned responses accordingly. Despite a change in the temporal relationship between the CS and US, they continue to close their eyelid just before the onset of the unconditioned stimulus. In fact, animals that learn the very long delay task after having learned trace, express an even higher initial number of CRs than naïve animals that have no prior experience. In other words, learning the very long delay task is actually facilitated if it is preceded by trace conditioning. This is quite different than the behavior of animals trained with the reverse sequence. In animals trained with very long delay first followed by trace conditioning, knowledge of the CS-US association that was acquired initially actually made learning the CS-US association during the trace task more difficult. At the start of training with the trace task, these animals were performing below the level at which naïve animals perform. This is an example of proactive interference. It is difficult to explain precisely why this phenomenon occurred. It may have been due to some degree of latent inhibition or to the unique change in temporal contingencies. Latent inhibition is a learning phenomenon in which exposure of the unpaired conditioned stimulus before training will impede subsequent acquisition of the paired CS and US association (Lubow and Moore, 1959). In this experiment, even though the conditioned stimulus was still paired with the unconditioned stimulus, the duration of the CS was substantially longer in the very long delay task. It is possible that the extensive CS pre-exposure during the first phase made the subsequent CS-US association more difficult to acquire. However, it is also possible that learning a relationship that is temporally discontiguous becomes more difficult once the animal has already learned that these stimuli are temporally contiguous. Further experiments would be needed to explicitly test this.
Hippocampus and new learning

In the present series of experiments, the potential of a second learning experience to enhance the survival of one week old neurons was tested. Animals were trained with two phases of a hippocampal-dependent task. It was discovered that regardless of prior experience, new learning rescued one week old neurons from death. In animals that learned a different task during the second phase, some could easily transfer information regarding the CS-US relationship to the new task (trace then very long delay). For other animals, this task was much more difficult (very long delay then trace). Previously, hippocampal-dependence has been demonstrated to be an important factor in rescuing one week old neurons from death (Gould et al., 1999). Therefore, the unique role of the hippocampus during the second training experience with a different task is given further consideration here.

Role of the hippocampus in transfer of learning

In some cases learning during the second training experience was facilitated and in others it was impeded. How might the hippocampus be involved in these training situations? Acquisition of new information that is congruent with previous information (trace then very long delay) or incongruent with previous information (very long delay then trace) may require different degrees of hippocampal activity. The role of the hippocampus in the transfer of learning has been explicitly tested (Winocur and Salzen, 1968). The effect of hippocampal lesions in rats on each of three learning processes was tested: acquisition, retention, and transfer. The training apparatus was a straight runway with a start box on one end and a goal box on the opposite end. Animals were mildly food deprived so that a food pellet served as a reinforcer. When animals reached the goal box, they had to choose one of two cues to receive the food reward. The cues were various sized circles depending on the condition. Combined across conditions, 3 different sized circles were used: large, intermediate, and small. On a given trial, two different sized circles were presented; one was paired with a food reinforcer and the other was not. The experiment consisted of two phases: training before surgery and training after surgery. Post-surgery training included the presentation of a novel stimulus. Transfer of learning occurred when the animal could successfully apply pre-operative training rules to the novel
stimulus presented during the second phase. For instance, in one condition, a large circle was paired with a reinforcer and an intermediate circle was unpaired. Animals were trained until they could accurately predict the reinforcer. Then hippocampal, cortical or sham lesions were imposed. Following surgeries, these animals were now presented with an intermediate circle that was paired, and a new, small circle that was unpaired. Successful transfer of learning occurred when the animal learned that the previously unpaired stimulus (intermediate circle with no food reinforcer) now contained the food reinforcer. The experimental design was counter-balanced so that all combinations and sequences of paired and unpaired circles were tested. It was discovered that the hippocampus was necessary for animals to accurately transfer pre-operative learning to post-operative training conditions. Sham and cortical lesioned animals had no trouble accurately predicting that a new, novel stimulus now indicated presence of a food reward. However, animals with hippocampal lesions failed to make this critical transference (Winocur and Salzen, 1968).

The discovery that the hippocampus is critical in transfer of learning has important implications for the data presented here. Hippocampal-dependence is an important feature in rescuing one week old neurons from death; trace but not delay enhances cell survival, similarly place learning but not cued learning enhances cell survival (Gould et al., 1999). In these experiments, while there may be decreased hippocampal-dependence in the retrieval of the same previously acquired trace CS-US association, as demonstrated by others (Kim et al., 1995; Takehara et al., 2003); the hippocampus may function in other ways that enable the animal to transfer what it has learned from one task to the rapid acquisition of a different task. Animals that successfully demonstrated transfer of learning (trace then very long delay condition) were included in the overall positive correlation, provided they demonstrated a minimum degree of improvement and were below asymptotic performance when first presented with the new task. That is, as long as new learning occurred, new cells were rescued. Perhaps the hippocampus is critically engaged in the transfer of learning, thereby rescuing new cells from death.
Role of the hippocampus in proactive interference

While the hippocampus may be important in the transfer of learning, what about the training condition in which learning was impaired? That is, when previous training with very long delay induced impaired learning of the trace task, was the hippocampus involved? As previously described, it is possible that the change in acquisition in the very long delay then trace group may have been due in part to extensive CS pre-exposure. CS pre-exposure is essentially a type of proactive interference by which exposure to the CS before training interferes with subsequent acquisition of a CS-US association. In a recent study, the effects of hippocampal, cortical and sham lesions on latent inhibition were compared (Schmajuk et al., 1994). The delay eyeblink conditioning task was used in which the conditioned stimulus was a 500 ms tone (85 dB) and the unconditioned stimulus was a 150 ms corneal air puff. Some animals were pre-exposed to the conditioned stimulus alone for 400 trials across four consecutive days, 100 trials per day. Other animals remained in their home cages during this period. Subsequently, all animals were trained for five days with 100 CS-US paired trials per day. Animals that remained in their home cages before CS-US training, i.e. had no prior CS exposure, expressed a gradual and steady increase in the number of conditioned responses over the course of training as expected. Animals that were pre-exposed to the conditioned stimulus and received sham or cortical lesions never expressed more than 10% conditioned responses throughout the entire course of the five day training period. That is, subsequent acquisition of the CS-US association was severely impaired – latent inhibition was observed. However, animals that received hippocampal lesions performed just as well as those that were never exposed to the conditioned stimulus. That is, when the hippocampus was lesioned, CS pre-exposure had no effect on subsequent acquisition (Schmajuk et al., 1994). Therefore, just as the hippocampus is important in facilitating subsequent learning (transfer of learning), it also plays a role in impeding subsequent learning (proactive interference / latent inhibition). This may in turn affect the survival of one week old neurons.

The previous studies on transfer of learning and latent inhibition suggest that rather than acting to specifically facilitate or impede acquisition of subsequent information, the
hippocampus may be critical in the general process of information integration (Winocur and Salzen 1968; Schmajuk et al., 1994). These learning processes may be important, and to different degrees, in rescuing one week old neurons from death. In the very long delay then trace group, despite the pairing of the conditioned stimulus with the unconditioned stimulus, the duration of the CS during the very long delay task was six times the duration of that during the trace task. This difference may have induced some degree of latent inhibition during the second training experience. Alternatively, the order in which contiguous stimuli were presented may also have been important. Future experiments would be needed to test these explanations. Regardless of why learning was initially impaired in this training condition, these animals needed the full training session to adapt the previously acquired association and correctly predict the occurrence of the US with the new parameters. Essentially, they had to re-learn the CS-US temporal association. When the surviving cells in this group alone were analyzed, a greater degree of improvement correlated with a higher number of surviving cells. When animals that demonstrated transfer of learning were added (trace then very long delay), the correlation was further strengthened. In essence, new learning correlated with the survival of one week old neurons, regardless of previous experience.

Previously, the importance of hippocampal-dependence in rescuing new neurons from death has been demonstrated (Gould et al., 1999). When considering more than one training experience, the hippocampus may still be involved but to a different degree and in a slightly different manner. The hippocampus may be important in integrating new information, regardless of whether the new information is congruent or incongruent with existing knowledge. Although the hippocampus is critical in the acquisition of certain fundamental associations, its role in learning and memory processes changes with time. That is, the hippocampus may be necessary in the acquisition of a given task, but not necessarily in the retrieval of it (Kim et al., 1995; Takehara et al., 2003). This dissociation between learning and memory has been integral in understanding the function of the hippocampus. Similarly, the distinction between different types of learning and memory processes will undoubtedly continue to be important in understanding how learning enhances the survival of newly generated neurons.
Evaluating learning and its impact on cell survival

As studies on the topic of learning and neurogenesis increase, so too are the various methods that are used to evaluate learning (Leuner et al., 2004; Dalla et al., 2007; Sisti et al., 2007; Dupret et al., 2007; Waddell and Shors, 2008; Dalla et al., in preparation). Various methods have all detected a significant positive correlation between overall learning and the number of surviving one week old neurons in the hippocampus (Leuner et al., 2004; Dalla et al., 2007; Sisti et al., 2007; Dupret et al., 2007; Waddell and Shors 2008, Dalla et al., in preparation). This illustrates both the robustness and the complexity of the relationship between learning and cell survival. Some of these learning indices include asymptotic performance, trials-to-criterion, a change-point algorithm, and in these experiments, linear regression (Dalla et al., 2007; Waddell and Shors, 2008; Gallistel et al., 2004; Dalla et al., in preparation, Sisti and Shors, unpublished data).

Each of these is discussed in turn.

Asymptotic performance

Thus far, perhaps the most widely used index of learning in the study of adult hippocampal neurogenesis has been asymptotic performance. Using both associative and spatial learning models, asymptotic performance predicts the survival of one week old neurons (Dalla et al., 2007, Sisti et al., 2007; Drapeau et al., 2003). For instance, in comparing two versions of the trace conditioning task, asymptotic performance correlated with surviving BrdU-labeled cells (Dalla et al., 2007). Animals that expressed a higher number of learned responses by the end of training retained more hippocampal neurons generated one week before training. Using a spatial learning task, asymptotic performance was also used to categorize good and poor learners (Sisti et al., 2007). In this experiment, animals were trained with either massed or spaced trials on the Morris water maze. By the end of training, those animals with a lower asymptote retained more newly generated cells than animals that ended training with a higher asymptote. In this case, a lower asymptote indicated superior learning as determined by time to reach the platform. In other studies that used the Morris water maze, learning and performance was further defined by comparing the early stage of learning with the late stage, during which asymptotic performance is observed (Dupret et al., 2007).
one week old neurons was evaluated, those animals that had a lower asymptotic performance (learned better) retained more new cells than those with a higher asymptotic performance (Dupret et al., 2007). In the present series of experiments, asymptotic performance was used to determine the critical learning threshold at the start of the second training experience. That is, if an animal had not yet surpassed a level of asymptotic performance that typically occurs with these training conditions (60% conditioned responding), then new learning regarding the CS-US association may have still been occurring. Thus, using several different training conditions, asymptotic performance has been successfully used to predict the survival of one week old neurons.

**Trials-to-criterion**

Recently, trials-to-criterion has been demonstrated to be a good predictor of cell survival (Waddell and Shors, 2008). In that experiment, the rate of acquisition was intentionally altered by changing the training conditions. Animals were exposed to several days of training trials with the conditioned stimulus alone before they started training with the paired conditioned stimulus and unconditioned stimulus. As predicted, animals with CS pre-exposure required more training trials with the CS and US to reach the same level of conditioned responding as those with no CS pre-exposure (Waddell and Shors, 2008). When the number of trials to reach criterion was correlated with the number of surviving one week old neurons, a significant correlation was observed. Those animals that required more trials to reach criterion had more cells than those that required fewer trials. It was suggested that the degree of effort of each animal was critical in rescuing hippocampal cells from death (Waddell and Shors, 2008). When animals needed to ‘work harder’ to reach an asymptotic level of conditioned responding then more cells were rescued. Consistent with these data, in a recent experiment the number of trials-to-reach criterion was quantified in a more statistically rigorous way using a change-point algorithm for each individual animal (Gallistel et al., 2004). The change-point algorithm assumes that learning is not a gradual process but rather it occurs in step-like manner and can be characterized by an abrupt increase in learned responses (Gallistel et al., 2004). This abrupt increase in conditioned responses has been colloquially referred to as the ‘aha’ moment and is unique for each animal. Similarly, the point at which asymptotic
performance occurs for each animal is also unique, and is derived statistically from the change-point algorithm (Papachristos and Gallistel, 2006). When this algorithm was used to define the point at which learning occurs for each animal, e.g. the number of trials required for a given animal to reach its own asymptote, a significant positive correlation occurred between asymptotic trial and BrdU-labeled cells (Dalla et al., in preparation). Combined these data suggest that when a task is sufficiently difficult, either due to an animal’s innate ability or due to the training conditions, then more new neurons are rescued from death (Waddell and Shors, 2008, Dalla et al, in preparation).

**Linear regression**

In the present series of experiments, individual learning during the second training experience was quantified for each animal using linear regression. The total number of learned responses across blocks of trials was plotted. Using the least squares method the best fit straight line was determined. From the regression equation, values of \( b_1 \) were compared. Because \( b_1 \) represents the slope, or the rise in learned responses across trial blocks, it was interpreted as overall improvement. A steeper slope indicated a larger improvement compared with a shallow slope. In animals trained with very long delay then trace, \( b_1 \) values correlated with the number of surviving one week old neurons. Animals that demonstrated a greater amount of improvement retained a higher number of one week old neurons.

Subsequently, rather than categorize animals based on training condition, they were re-categorized based on learning. Learning during the second training experience was evaluated for animals across all training conditions. When animals were re-categorized based on learning, instead of training, a second positive, significant correlation was observed (\( R=0.81; \ p<0.05 \)). New learning was evaluated by measuring the number of learned responses on day 1 of phase 2, as well as the degree of improvement throughout the course of this training phase. To meet learning criterion, animals had to be below an asymptotic level of performance (60%) at the start of the second phase and had to have a \( b_1 \) value that was higher than the median. For these animals that met criteria, the number of BrdU-labeled cells was correlated with \( b_1 \) from the regression equation (slope). When
new learning occurred during the second training experience, despite previous training
with the same or different task, then improvement (slope from the regression equation)
predicted cell survival. Those animals that expressed greater overall improvement
retained more one week old neurons in the hippocampus.

Just as in humans, learning in animals can be measured in more than one way. In these
experiments, learning was evaluated by applying a widely used mathematical approach –
linear regression. The total number of learned responses was summed for each of the
eight blocks of trials. A straight line was then fit using the least squares method (Sigma
Plot; Draper and Smith, 1966). A steep slope indicated a large change in learned
responses between the first and last trial block; a shallow slope indicated a small change
in learned responses across trial blocks. Regardless of training condition, a steep slope
indicated a high number of new neurons; a shallow slope indicated a low number of new
neurons. These data demonstrate that even with a second learning experience, new
learning will rescue new cells from death.

**Animal model of declarative memory**

These experiments were designed to further characterize the relationship between
learning and neurogenesis. By using two tasks which engage the hippocampus, trace and
very long delay, the role of subsequent learning in neurogenesis was tested. However,
training animals with these two tasks also uncovered a unique pattern of learning.
Learning during the second phase was significantly different depending only on the order
in which animals learned the tasks. Animals that learned trace during the first training
experience could easily learn very long delay during the second training experience. On
the contrary, animals that learned very long delay during the first training experience had
difficulty learning the trace task. As model of learning, the trace conditioning task has
been widely used in both animals and humans. It has even been used as a model of
declarative (or conscious) memory. Finally, the implications of trace as an animal model
of awareness are discussed in light of the data presented here.
Trace eyeblink conditioning as an animal model of awareness

The trace eyeblink conditioning task has been used as a model of declarative memory or awareness (Clark and Squire, 1998). In a creative series of experiments using human volunteers, delay and trace tasks were presented to the subjects. In both versions, combinations of paired and unpaired trials were presented. In a paired trial, the conditioned stimulus predicts the unconditioned stimulus. In an unpaired trial, the conditioned stimulus is presented alone. The presentations of paired and unpaired trials were randomized. After a string of non-reinforced trials, a reinforced trial is expected, despite the fact that the events are independent of one another and that the probability of a reinforced trial has not changed. This phenomenon is a well established psychological phenomenon known as the ‘gambler’s fallacy’ (Clark and Squire, 1998; Shors, 2004). For example, if four consecutive unpaired trials were presented, the subject would come to expect that the next trial would be a paired trial, and the probability of a CR might increase or decrease depending on the task (Clark et al., 2001). In both delay and trace tasks, human subjects developed expectancies consistent with the gambler’s fallacy but only in one task did these expectancies actually influence their performance. During delay conditioning, conditioned responses were not influenced by expectancy, but rather by associative strength (Clark et al., 2001). That is, the probability of a CR was high following a string of CS-US trials, but the probability of a CR was low following a string of CS alone trials. Interestingly, this was contrasted with humans’ performance on the trace conditioning task. In this case, when expectancy was high so was the probability of a conditioned response; however when expectancy of a CS-US trial was low so was the probability of a CR (Clark et al., 2001). The authors note that this finding highlights a fundamental difference between the delay and trace tasks, namely that in trace conditioning subjects may develop declarative (conscious) knowledge of the stimulus contingencies which in turn may influence performance. In a similar study, versions of the trace and delay task were compared in humans that were amnesic with hippocampal damage, or normal volunteers (Clark and Squire, 1998). Tasks were varied in terms of quality and duration of the conditioned stimulus and its temporal contingency with the unconditioned stimulus. Again, the awareness or knowledge of the CS-US event was compared with performance on either the trace or delay task. Knowledge or awareness of
the event was assessed with a variety of questions regarding the stimulus parameters. Normal volunteers acquired the delay task regardless of their knowledge about the CS-US contingencies. This is contrasted with the trace task. Only individuals who developed knowledge of the CS-US contingencies successfully acquired the task (Clark and Squire, 1998). None of the amnesic patients demonstrated awareness of the CS-US association in the trace task and none of them successfully learned this task; however, the delay task was acquired at a normal rate and was not correlated with knowledge of the CS-US contingencies (Clark and Squire, 1998). Therefore, in humans, the trace task has been used as a model of awareness or declarative memory (Clark and Squire, 1998; Shors, 2004).

In the present experiments, when animals learned the trace task before the very long delay task, animals expressed an asymptotic level of performance very early in training. As a group, they expressed close to 60% conditioned responding on the first day despite the fact that the task was novel. Animals trained with the trace task not only learn about the temporal relationship between the CS and the US, they also may develop a declarative knowledge regarding the stimulus contingency, as suggested by Clark and Squire (1998). Considering that the trace task has been used as model of declarative memory, whereby an awareness of the stimulus contingencies is acquired, perhaps this rapid and immediate transfer of knowledge is not surprising. Although speculative, perhaps animals immediately expressed a high number of CRs because they had not only acquired the CS-US association while learning the trace task, they had also acquired knowledge regarding the existence of the CS-US contingency. When the tasks were reversed, animals had difficulty adapting the learned response because the quality of the information learned was different. That is when animals learned the very long delay task first then the trace task, the learned responses dropped dramatically and the new temporal contingencies were essentially re-learned. If the delay task does not require declarative memory, perhaps the animal lacks an awareness of the temporal contingency in this case. Although trace and very long delay conditioning may be similar in terms of task difficulty, as indicated by rates of acquisition (Beylin et al., 2001), they may not be similar with regards to awareness of the event or the ability to retrieve the declarative
memory. In these experiments, an awareness of the event may have facilitated subsequent learning. That is, awareness of the trace CS-US contingency may have facilitated acquisition of the very long delay CS-US contingency. If no awareness of the event was ever acquired, subsequent learning was more difficult.

**Conclusions**

As learning occurs, familiar concepts are retrieved from memory and new information is encoded. Of the incoming new information, some fits easily with what is already known whereas other concepts can be quite difficult to grasp. In these experiments, a reductionist learning model, eyeblink conditioning, was used to determine whether a second learning experience rescues one week old neurons from death. At the start of the second learning experience, animals had been exposed to different types of training. Some had already learned the association; others had learned a different version of it. Still other animals had no prior experience. When animals were categorized based on learning, rather than training condition, a significant positive correlation occurred between improvement and number of surviving new neurons (Figures 11 and 12). Thus, it was discovered that new learning enhances the probability that a one week old neuron will survive, regardless of previous experience.
REFERENCES


Shors, T.J. Significant life events and the shape of memories to come: a hypothesis. *Neurobiology of Learning and Memory* 85, 103-115. 2006.


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CR</td>
<td>Conditioned response</td>
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<tr>
<td>CS</td>
<td>Conditioned stimulus</td>
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<tr>
<td>DCX</td>
<td>Doublecortin</td>
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<tr>
<td>GABA</td>
<td>gamma amino butyric acid</td>
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<tr>
<td>ITI</td>
<td>Intertrial interval</td>
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<tr>
<td>US</td>
<td>Unconditioned stimulus</td>
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FIGURE LEGENDS

Figure 1. A) The hippocampus of an adult rat is shown. The laminar structure of the hippocampus can be seen using Timm’s stain and a light microscope. B) Neural progenitor cells line the inner granule cell layer and develop into mature neurons. Four BrdU-labeled cells are visualized using immunohistochemistry and a fluorescent microscope.

Figure 2. Learning affects cells during a critical period. New hippocampal cells normally become apoptotic, or begin programmed cell death, at approximately one week after mitosis. When cells are one week old, hippocampal-dependent learning can rescue these new neurons from death. This sensitive period may be a result of changes in cellular properties that occur throughout their development. Time of BrdU injection is used to mark the ‘birthday’ of new cells.

Figure 3. Good learners have more BrdU-labeled cells than poor learners, regardless of training condition. Animals were trained on a spatial task with either massed or spaced trials. In massed training, 16 trials occurred in one day. In spaced training, 16 trials were distributed across four days. Regardless of massed or spaced training, animals that performed better by the end of training retained more new cells than those that did not perform as well (Sisti et al., 2007). A) An animal with massed trials that learned poorly retained few surviving cells. B) An animal with massed trials that learned well retained many new cells. C) An animal with spaced trials that learned poorly retained few new cells. D) An animal with spaced trials that learned well retained many new cells. (BrdU-labeled cells are brown. Mature granule cells are purple.)

Figure 4. A) Schematic of experimental design for Experiment 1 (same task). Animals were trained with two phases of the trace eyeblink conditioning task. Animals in the Trace Extinction Trace condition received an additional two days of training with extinction trials (conditioned stimulus alone). Animals in Trace Alone condition remained in their home cages during the first phase. One week before the start of the
second phase, all animals received a single BrdU injection. All animals were sacrificed 21 days after BrdU injection. B) Schematic of experimental design for Experiment 2 (different task). Animals were trained with two phases of different eyeblink conditioning tasks. Some animals were trained with the trace task during phase 1 followed by the very long delay task during phase 2. Other animals were trained with the reverse sequence: very long delay during phase 1 followed by the trace task during phase 2. As before, one week before the start of the second phase, animals received a single BrdU injection. All animals were sacrificed 21 days after BrdU injection.

Figure 5. Animals remembered the trace conditioning task more than one week after the end of training, regardless of extinction training. During the first phase, both groups of animals learned the trace task as indicated by an increase in the number of learned responses. Two days of extinction training resulted in suppression of the learned response (x1 – x4). Animals with or without extinction training were performing close to 60% at the start of phase 2. Animals trained only during the second phase (Trace Alone) increased the number of learned responses across trial blocks indicating that they learned to predict the unconditioned stimulus.

Figure 6. Animals trained with only a single phase had more new neurons than animals trained with two phases, regardless of extinction. Mean number of BrdU-labeled cells in the Trace Alone group was higher than in those animals that received two phases of training or no training at all.

Figure 7. Two associative learning tasks were used to determine whether learning a different task would rescue new neurons from death. In the trace task, the conditioned stimulus (250 ms) was a burst of white noise. It was followed by a stimulus-free interval, the trace (500 ms), and then an unconditioned stimulus (100 ms, 0.65 mA electrical pulse to eyelid). In the very long delay task, the conditioned stimulus was also a white noise. However, the duration of the CS was increased by a factor of six (1,500 ms). There was no stimulus-free interval. Instead, the CS overlapped and co-terminated with the unconditioned stimulus.
Figure 8. **There was a significant interaction of task sequence.** All animals learned during the first training experience, regardless of the task. Animals that learned the trace task first readily learned the very long delay task during phase 2. However, animals that learned the very long delay task first had a significant decrease in conditioned responding at the start of phase 2. As training progressed, the number of learned responses steadily increased indicating that they eventually learned the task.

Figure 9. **Learning during the second training experience was evaluated using linear regression.** Best, average and worst learners from very long delay then trace group are shown. A steep slope (large $b_1$ value) indicated a large improvement across trial blocks. A shallow slope (small $b_1$ value) indicated a small improvement across trial blocks.

Figure 10. **In animals trained with very long delay then trace, there was a significant positive correlation between improvement ($b_1$) and number of BrdU-labeled cells.**

Figure 11. **When all animals were re-categorized based on learning during phase two, not by training condition, a significant positive correlation was observed.** There was a significant positive correlation between improvement ($b_1$) and number of BrdU-labeled cells, regardless of previous experience. Animals that demonstrated greater improvement retained more new cells than animals that only demonstrated a small improvement.

Figure 12. **Task difficulty and overall improvement may both be critical in rescuing new neurons from death during a second training experience.** The y-axis is the number of BrdU-labeled cells. The z-axis, which runs perpendicular to the page, is the value of $b_1$ (improvement during phase 2). These data points are the same as those in Figure 11 (the y-axis is the same; the x-axis of Figure 11 is the z-axis in this figure). The x-axis represents a categorical learning variable, task difficulty. As tasks become
increasingly difficult, and improvement continues to occur, the survival of new neurons may be enhanced, regardless of previous experience.
Table 1.

Mean conditioned responses ± standard error of the mean for each block of 100 trials

**Phase 1: Trace**

<table>
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<tr>
<th></th>
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<tr>
<td></td>
<td>36 ± 7</td>
<td>37 ± 10</td>
<td>39 ± 9</td>
<td>48 ± 10</td>
<td>53 ± 8</td>
<td>59 ± 7</td>
<td>62 ± 5</td>
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**Phase 2: Trace**

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<tr>
<td></td>
<td>50 ± 6</td>
<td>57 ± 11</td>
<td>63 ± 7</td>
<td>75 ± 4</td>
<td>64 ± 5</td>
<td>66 ± 5</td>
<td>69 ± 4</td>
<td>76 ± 4</td>
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Table 2.

Mean conditioned responses ± standard error of the mean for each block of 100 trials

**Phase 1: Trace and Extinction**

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<td>46 ± 8</td>
<td>43 ± 7</td>
<td>47 ± 7</td>
<td>54 ± 8</td>
<td>55 ± 9</td>
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<td>67 ± 8</td>
<td>29 ± 3</td>
<td>22 ± 3</td>
<td>23 ± 2</td>
<td>15 ± 3</td>
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**Phase 2: Trace**

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<tbody>
<tr>
<td>Mean</td>
<td>50 ± 7</td>
<td>65 ± 6</td>
<td>57 ± 8</td>
<td>69 ± 6</td>
<td>69 ± 5</td>
<td>77 ± 7</td>
<td>61 ± 11</td>
<td>68 ± 9</td>
</tr>
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</table>
Table 3.

Mean conditioned responses ± standard error of the mean for each block of 100 trials

**Phase 1: Trace**

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<tr>
<td>31±10</td>
<td>40±9</td>
<td>41±5</td>
<td>51±6</td>
<td>58±9</td>
<td>58±9</td>
<td>56±8</td>
<td>60±7</td>
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**Phase 2: Very Long Delay**

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<td>74±9</td>
<td>67±11</td>
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Table 4.

Mean conditioned responses ± standard error of the mean for each block of 100 trials

**Phase 1: Very Long Delay**

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<td>34 ± 4</td>
<td>56 ± 4</td>
<td>47 ± 3</td>
<td>59 ± 6</td>
<td>66 ± 7</td>
<td>69 ± 6</td>
<td>72 ± 7</td>
<td>71 ± 8</td>
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**Phase 2: Trace**

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<tr>
<td>16 ± 4</td>
<td>22 ± 5</td>
<td>29 ± 7</td>
<td>37 ± 6</td>
<td>51 ± 8</td>
<td>57 ± 9</td>
<td>48 ± 6</td>
<td>57 ± 6</td>
</tr>
</tbody>
</table>
Figure 1.

A)

B)
Growth cones responsive to incoming stimuli

Past critical period
Mature connections established.

No dendritic growth cones

Time of BrdU Injection

< 5 days

6–10 days

> 10 days

Cellular properties

Growth cones responsive to incoming stimuli

Past critical period
Mature connections established.
Figure 3.

A. B. C. D.

Poor learners Good learners

Massed training Spaced training
Figure 4.

A) Experiment 1: **SAME Task**

- **Trace**
  - 4 days of trace conditioning
  - 4 days of trace conditioning (one week later)

- **Trace Extinction Trace**
  - 4 days of trace conditioning
  - 2 days of extinction
  - 4 days of trace conditioning

- **Trace Alone**
  - NO TRAINING
  - 4 days of trace conditioning

B) Experiment 2: **DIFFERENT Task**

- **Trace VLD**
  - 4 days of trace conditioning
  - 4 days of VLD conditioning (one week later)

- **VLD Trace**
  - 4 days of VLD conditioning
  - 4 days of trace conditioning

BrDU Injection (200 mg/kg)
Figure 5.
Figure 6.

![Bar graph showing BrdU-labeled cells for different conditions: Naive, Trace Alone, Trace Trace, and Trace EXT Trace. The Trace Alone condition has a significantly higher number of labeled cells compared to the other conditions, indicated by an asterisk (*) symbol.]
Figure 7.

TRACE

VLD

\(time\)
Figure 8.
Figure 9.

Best learner

Average learner

Worst learner

\[ b_1 = 10.6310 \]

\[ b_1 = 6.5238 \]

\[ b_1 = 1.4047 \]
Figure 10.

Animals trained with very long delay then trace task

R = 0.75
p < 0.05
n = 11
Animals that demonstrate new learning during a second training experience regardless of previous experiences
Figure 12.

New neurons

DIFFICULT      MODERATE      EASY

Overall improvement (b1)

Task difficulty

2000  4000  5000  6000
CURRICULUM VITAE

Helene M. Sisti
152 Frelinghuysen Road
Piscataway, NJ 08854
hsisti@rutgers.edu
(732) 445-2842

EDUCATION

2003 - 2008 Rutgers University, Ph.D.  Behavioral Neuroscience
2000 Temple University, M.Ed.  Psychology / Kinesiology
1996 Dartmouth College, B.A.  Psychological & Brain Sciences

THESES

Master’s Thesis: Naloxone suppression and morphine enhancement of voluntary wheel running activity in rats. Temple University

Doctoral Dissertation: Learning and neurogenesis: Effects of training and re-training on the survival of new neurons. Rutgers University

RESEARCH & TEACHING EXPERIENCE

2003 – Present Teaching Assistant
Rutgers University, Department of Psychology
Taught Conditioning and Learning Laboratory
Assisted in administration of large lecture courses including Physiological Psychology, Cognition, and Conditioning and Learning

2001 - 2003 Laboratory Technician
(reporting primarily to the Principal Investigator)
The Rockefeller University, Dr. Bruce S. McEwen
Research projects included effects of stress on adult neurogenesis in the hippocampus. I co-authored 5 manuscripts and learned multiple-labeling immunohistochemistry and confocal microscopy. Worked with many visiting scientists from local and international institutes.

1999 - 2001 Teaching & Research Assistant
Temple University, Department of Psychology
Research Methods in Behavioral Neuroscience • Led undergraduates in behavioral neuroscience experiments
Independent Research in Behavioral Neuroscience • Mentor for undergraduate students in psychology

1998 - 2000  
**Graduate Assistant**  
*Temple University, Department of Psychology*  
Using DSM-IV criteria, I assessed the impact of cognitive processes on female adolescent behavior. This clinical study was designed to measure the effect of attitudes on the behavior of youth, specifically in an urban environment.

1993  
**Laboratory Technician**  
*Columbia University, College of Physicians and Surgeons*  
Assisted in researching the effects of hypothermia on glutamate excitotoxicity as a result of focal ischemia. I performed surgeries for carotid artery occlusion in rats.

**HONORS & PROFESSIONAL ORGANIZATIONS**

- Sigma Xi, The Scientific Research Society  *2004 - Present*
- Travel Award to attend 2006 Forum of European Neuroscience Societies (FENS) Conference in Vienna, Rutgers University - Graduate School of New Brunswick  
- Society for Neuroscience  *2000 - Present*
- Pavlovian Society  *2004 - Present*

**LABORATORY SKILLS & EXPERIENCE**

**Behavioral preparations**  
Classical conditioning models including eyeblink and fear conditioning  
- Electrode construction for electromyography  
- Computer programming in Viewdac™

**Operant conditioning**  
Spatial learning models including water maze  
Learned helplessness  
Social transmission of food preference

**Laboratory procedures**  
Stereotaxic surgeries  
Mixing drugs with a broad range of solubility  
Brain sectioning using vibratome, cryostat, and freezing microtome  
Animal breeding (rats and mice)  
Lavage and cytology of estrous cycle

**Neuroscience techniques**  
Multiple labeling immunohistochemistry  
Confocal microscopy
Electron microscopy (took a course for one semester)
Radioimmunoassay
Timm silver-sulfide stain
In situ hybridization

PUBLICATIONS

Sisti, HM, Glass AL, Shors TJ. Neurogenesis and the spacing effect: Trials distributed over time enhance memory and cell survival. Learning and Memory, May 10;14(5):368-75, 2007. (Selected for cover article of the June 2007 issue.)


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ABSTRACTS & PRESENTATIONS


Sisti HM, Role of NMDA2B receptor in adult neurogenesis, The Rockefeller University, McEwen Laboratory Retreat, October 2002


Sisti HM, The role of opioid systems in voluntary wheel running activity, Temple Neuroscience Program Conference, February 2000

Sisti HM, Exercise and physical activity: The role of endogenous opioids. Northeast Sport Psychology Conference, April 1999

PRESS RELEASE