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PHYSICAL SKILL LEARNING INCREASES NEUROGENESIS THROUGH CELL SURVIVAL IN THE ADULT HIPPOCAMPUS

by

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ABSTRACT OF THE DISSERTATION

Physical skill learning increases neurogenesis through cell survival in the hippocampus

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The dentate gyrus is a major site of plasticity in the adult brain, giving rise to thousands of new neurons every day. While the majority of these cells die within two weeks of their birth, they can be rescued from death by various forms of learning. The successful acquisition of select types of associative and spatial memories can increase the number of these cells that survive. Here, we investigated the possibility that an entirely different form of learning, physical skill learning, could rescue these new neurons from death. To test this possibility, rats were trained with a physically-demanding and technically-difficult version of a rotarod procedure. Acquisition of the physical skill greatly increased the number of new hippocampal cells that survived. The number of surviving cells positively correlated with performance on the task. Only animals that successfully learned the task retained the cells that would have otherwise died. Animals that failed to learn, and those that did not learn well, did not retain any more cells than those that were untrained. Importantly, acute voluntary exercise in activity wheels did not increase the number of surviving cells. These data indicate that skill learning, and not physical activity *per se,* increased the number of surviving cells. Moreover, learning an easier version of the task did not increase cell survival. These data are consistent with previous studies revealing that learning rescues new neurons from death, but only when

acquisition is sufficiently difficult to achieve. Finally, complete hippocampal lesions did not disrupt acquisition of this physical skill. Therefore, learning this motor skill task does not depend on the hippocampus, even though it can increase the number of surviving cells in the structure. These data, and their implications, suggest that humans who learn new and complicated sports or other physical skills will retain more new neurons than humans that do not engage in effortful activities.

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GENERAL INTRODUCTION

The hippocampal formation is a medial temporal lobe structure that is required for many forms of novel learning. In animal models, selective lesioning or inactivation of the hippocampus results in performance deficits during training on a wide variety of associative and spatial learning tasks (Beylin et al., 2001; Kim, Rison, & Fanselow, 1993; Maren & Holt, 2000; Otto & Poon, 2006; Weiss, Bouwmeester, Power, & Disterhoft, 1999). In humans, damage to the hippocampal formation can result in similar learning impairments and anterograde amnesia (Richards, 1973; Scoville & Milner, 1957). However, the hippocampal formation is not required for all forms of learning. For example, the hippocampus is necessary for acquisition of a trace eyeblink conditioning response, but it is not needed to learn a highly similar delay eyeblink conditioning response (Beylin et al., 2001).

Eyeblink conditioning is a form of classical Pavlovian conditioning in which a previously neutral conditioned stimulus (CS; usually a white noise, or a tone) is paired with an aversive unconditioned stimulus (US). The US is one that normally elicits a blink from the animal, and in most cases it is a small corneal airpuff, or a low-intensity shock to the *orbicularis oculi*, the muscle that controls the closing of the eyelid (Christian & Thompson, 2003; Gormezano, Schneiderman, Deaux, & Fuentes, 1962; McCormick, Lavond, & Thompson, 1982; Thompson & Steinmetz, 2009). Through repeated pairings of the CS and the US the animal learns that the CS predicts the onset of the US, and it blinks in response to the CS. This blink to the CS is known as a conditioned response (CR), and the percentage of CRs emitted over the course of training is often used to

estimate performance during training (Christian & Thompson, 2003; Thompson & Steinmetz, 2009).

There are several different forms of eyeblink conditioning, with the two most common forms being delay conditioning and trace conditioning. During delay conditioning the CS is presented at the start of a trial, and it remains present for the full duration of the trial. Near the end of each trial the US is also presented and the CS and US briefly overlap before coterminating. Animals without an intact hippocampus can acquire this delay eyeblink response (Beylin et al., 2001; Schmaltz & Theios, 1972; Solomon & Moore, 1975; Solomon, Solomon, Schaaf, & Perry, 1983). In fact, very few regions of the brain are necessary for this form of learning. Circuits within the cerebellum are necessary, and even reportedly sufficient, for acquisition of this delay response (Thompson & Steinmetz, 2009).

Trace conditioning differs from delay conditioning, because during trace conditioning the CS and the US are separated in time by a stimulus free period known as a trace interval. Because the stimuli are separated in time during trace conditioning the animal must maintain a memory trace of the CS, to associate it with the US. Animals require more trials to learn this trace response, and they tend to reach lower levels of asymptotic performance than those that are trained to learn delay conditioning (Bangasser, Waxler, Santollo, & Shors, 2006; Beylin et al., 2001; Solomon & Groccia-Ellison, 1996; Waddell & Shors, 2008). Furthermore, the introduction of this trace period alters the brain circuitry necessary to learn the task. An intact cerebellum is necessary for this form of learning, but it is no longer sufficient. Instead, the hippocampus is also necessary for this form of learning (Beylin et al., 2001).

Why is the hippocampus necessary for trace eyeblink conditioning, when it is not required for delay eyeblink conditioning? Several studies have suggested that the hippocampus is necessary for associating stimuli that are temporally discontiguous. During trace conditioning the stimuli are separated in time, and the association between these two stimuli requires the hippocampus. However, during delay conditioning the stimuli are not separated in time, and this learning does not require the hippocampus. With that said, the hippocampus can also be required for learning even when the stimuli are temporally contiguous. During a procedure known as very-long delay conditioning the interstimulus interval (the time between the CS and the US) is extended. During normal delay conditioning the interstimulus interval is approximately 750ms. However, during very-long delay conditioning the interstimulus interval is nearly double that. This very-long delay conditioning procedure is much more difficult to learn, and critically acquisition of this task requires an intact hippocampal formation (Benedetta Leuner, Waddell, Gould, & Shors, 2006). Based on these findings, it has been proposed that the hippocampus becomes more involved as the task demands increase (Beylin et al., 2001).

The hippocampus is required for acquisition of trace eyeblink conditioning, but it is not required for the long-term retention of the trace memory. Lesions of the hippocampal formation before training prevent acquisition of the trace response. Lesions immediately after training prevent animals from performing the learned response. However, when the hippocampus is lesioned thirty days after the initial training experience the trace memory remains intact, and animals display no deficit in performing the previously learned response (Kim, Clark, & Thompson, 1995). These results suggest that the hippocampus is necessary for the acquisition and initial processing of a trace

memory, but that it is not the long-term storage site of that memory. Of course, the hippocampus is involved in more than just classical eyeblink conditioning, it is also necessary for spatial learning (Morris, Garrud, Rawlins, & O'Keefe, 1982; O'Keefe & Nadel, 1978) acquisition of fear conditioning (Anagnostaras, Maren, & Fanselow, 1999; Kim et al., 1993; Otto & Poon, 2006; Parsons & Otto, 2010; Phillips & LeDoux, 1992), and many other forms of learning.

The role of the hippocampus in learning and memory

Because the hippocampus is necessary for the formation of many new memories myriad theories have emerged regarding exactly how the hippocampus contributes to learning and memory. Several theories of hippocampal function suggest that the hippocampus is required for acquisition and the retrieval of memories that require conscious awareness, but not for those that can be learned without conscious awareness (Moscovitch, 1992, 2008; Squire, Stark, & Clark, 2004). These theories are based off of the results of studies conducted on patients with medial temporal lobe damage and amnesia. These patients typically display remarkable learning impairments, and an inability to form new memories that require conscious awareness. The most classic example of learning impairments following hippocampal damage come from studies conducted on now the famous patient H.M. (Henry Gustav Molaison). H.M. had his hippocampus (and extrahippocampal structures) bilaterally removed, to treat epileptic seizures. Following the removal of his hippocampus H.M. was unable to form many new explicit memories (Milner, Corkin, & Teuber, 1968; Richards, 1973; Scoville & Milner, 1957). For example, thirty minutes after returning from lunch H.M. could not recall what

he ate for lunch, nor could he recall even having gone to lunch. In general, H.M. was unable to form new semantic and episodic memories regarding his experiences. However, H.M. was capable of learning, and retaining, new physical skills and implicit memories. When trained to trace the lines of a drawing by using only the image of the drawing reflected in a mirror H.M. improved performance over the course of several days of training (Milner 1962, Milner, Squire & Kandel 1998). Despite successful acquisition of this physical skill, H.M. could not accurately describe how he learned the skill. These, and similar findings in amnesic patients (Reviewed in Hannula & Greene, 2012), led to the formation of declarative memory theory.

Declarative memory theory proposes that long-term memory can be separated into two distinct systems (Cohen & Squire, 1980). The first of these systems is the declarative memory system, which includes episodic and semantic memories. Traditionally, declarative memories are those that require conscious thought for recollection (Cohen 1984; Squire & Cohen 1984). The second memory system is the nondeclarative system. Nondeclarative memories include habituation, priming, simple classical conditioning, habits, and procedural learning. Unlike declarative memories, nondeclarative memories can be recalled without conscious thought (Squire et al., 2004). In amnesic patients, and patients with hippocampal damage, the declarative memory system is impaired. However, in these same subjects, the nondeclarative memory system typically remains intact. Therefore, the hippocampus has been selectively implicated in acquisition of new declarative memories. Because declarative memory is characterized by the ability to consciously recall previously learned information, it has been suggested that the hippocampus is necessary for acquisition of memories that require conscious awareness

(Squire et al., 2004). However, the hippocampus is not traditionally thought of as necessary for the formation of memories that do not require conscious awareness. With that said, recent research is beginning to challenge this view, by revealing that hippocampus may also contribute to several forms of unconscious learning (Hannula & Greene, 2012).

One widely used test of implicit learning is the contextual cueing task (Chun & Jiang, 1998). During this task subjects are presented with a series of images. Each image is composed of an array of distractors, and one target stimulus. The subject must press a button to indicate if the target stimulus is rotated to the left, or to the right. During training some of the distractor arrays repeat, whereas others are only presented once. Therefore, on some trials the subject is presented with novel arrays, and on other trials they experience the repeated arrays. The repeated arrays provide a stable configuration, which can be used to predict the location of the target stimulus. During training healthy subjects increase their reaction time to presentations of both the novel and the repeated arrays (Chun & Jiang, 1998). However, these subjects have a greater facilitation of reaction time during trials in which the repeated arrays are presented. The subjects use their prior knowledge of these repeated arrays to predict where the target stimulus will be located, allowing them to have a faster response time on the repeated trials. Importantly, the shorter reaction time during the presentation of the repeated arrays occurs even when subjects cannot explicitly recognize them. Because this facilitation occurs without explicit knowledge, it is considered to reflect implicit motor sequence learning.

Amnesic subjects with medial lobe damage can learn to perform this contextual cuing procedure (Chun & Phelps, 1999). Like healthy subjects, amnesic patients improve

their reaction time during training. They decrease their reaction time to both the novel and the repeated arrays. However, unlike healthy subjects, amnesic patients do not display a facilitation of reaction time during the presentation of repeated arrays. This suggests that amnesic subjects cannot form the implicit memories of the repeated arrays necessary to predict where the target stimulus will occur. Therefore, the hippocampus may be involved in this form of unconscious skill learning. However, in this study the amnesic patients had extensive medial temporal lobe damage, and the hippocampal formation was not the only brain structure that was impaired. Therefore, their implicit learning deficits cannot solely be attributed the effects of hippocampal damage. Furthermore, there has been some debate about the implicit nature of this task (Chun & Jiang, 2003). Even so, these results suggest that the hippocampus may be involved in some aspects of unconscious learning.

Additional evidence for the involvement of the hippocampus in unconscious learning comes from studies using the serial reaction time task (Reed & Johnson, 1994). During this task individuals are instructed to map the location of a visual stimulus presented on a screen to corresponding key presses. For example, if the visual stimulus can appear in one of four locations then the individual is given four different keys. Each key corresponds to one of the spatial locations on the screen. When the visual stimulus is presented the subject should press the key that corresponds to that spatial location of the stimulus. Unbeknownst to the subject some of the stimuli are presented in a predetermined sequence. This sequence occasionally repeats during training. On some trials the subject is presented the sequenced stimuli, and on other trials they are presented non-sequenced stimuli. During training the subject learns the repeated sequence. When

they are presented the sequenced stimuli their reaction time is faster than when they are presented the non-sequenced stimuli. This improved reaction time during the sequenced trials is observed even when the subject is not explicitly aware of the relationship between the repeating stimuli. Amnesic patients display deficits in this task (Curran, 1997; Shanks, Channon, Wilkinson, & Curran, 2006), suggesting that medial temporal damage can impair this form of implicit motor sequence learning. Using a slightly modified version of this task, Gheysen and colleagues (2010) used fMRI to record brain activity during the early and late stages of a serial color-matching task. An increase in hippocampal activity occurred during both the early and the late phases of this form of implicit motor learning. This hippocampal activity significantly correlated with the motor sequence learning, and when the motor sequence was altered this hippocampal activity was also affected. Overall, these and other results (Reviewed in Hannula & Greene, 2012), suggest that the hippocampal formation may be involved in more than just declarative learning, instead it may also contribute to acquisition of various unconscious memories.

A history of the discovery of adult neurogenesis

For more than a century it was believed that the adult brain was incapable of producing new neurons (Gross, 2000). We now know that this is not the case, and that new neurons are produced in the mammalian brain throughout the entire lifespan. Moreover, this adult neurogenesis occurs across a wide range of species including rats, mice, macaques, marmosets, songbirds, and even humans (Altman & Das, 1965; Eriksson et al., 1998; Goldman & Nottebohm, 1983; Gould, Reeves, et al., 1999; Kaplan & Hinds,

1977; Kornack & Rakic, 1999). Initially, evidence for adult neurogenesis came from studies conducted by Altman in the 1960s. Using [^{3}H]-thymidine autoradiography, Altman reported the presence of new neurons in the neocortex, the dentate gyrus of the hippocampal formation, and the olfactory bulb of adult rats (Altman, 1962; Altman 1969). However, at the time, Altman's discoveries were largely dismissed, in part because of technological limitations which prevented assessing the fate of these cells. As such, Altman's findings did little to alter the belief that no new neurons were produced in the adult brain (Gross, 2000).

Following Altman's work, another group of researchers led by Michael Kaplan also used [^{3}H]-thymidine autoradiography to reveal the presence of adult-born cells in the dentate gyrus and olfactory bulb of mice. Using electron microscopy they provided further support for the existence for adult neurogenesis by revealing that these cells displayed ultrastructural characteristics of neurons (Kaplan & Hinds, 1977). However, these findings were challenged by the work of Pasko Rakic, who failed to find evidence of neurogenesis in the primate brain (Eckenhoff & Rakic, 1988; Rakic, 1985). Rakic suggested that neurogenesis was limited to the brains of lower mammals. He proposed that while new neurons may be incorporated into the brains of rats and mice, they almost certainly were not produced in the higher mammals, such as monkeys and humans (Gross 2000). This became the prevailing belief until the early 1990s when various technological developments greatly facilitated the study of adult neurogenesis. One of the most important developments was the creation of a synthetic thymidine analogue known as bromodeoxyuridine (BrdU). Just like [^{3}H]-thymidine, BrdU is administered systemically and incorporates into the DNA of dividing cells. However, unlike [^{3}H]-thymidine, which

requires the use complex autoradiography, BrdU can be visualized with immunohistochemical techniques. These techniques allow for the possibility of labeling cells with not just BrdU, but also other cell-type specific markers. These markers, such as NeuN (neuron specific enolase), TOAD-64 (turned on after division 64), GFAP (glial fibriallary acidic protein), and O4 (oligodendrocyle cell surface marker number 4) are expressed predominantly in one type of cell. Therefore, double-labeling with BrdU and one of these markers allows researchers to determine the fate of the newly-generated cells. For the first time, laboratories were able to determine if these adult-born cells were actually neurons (Gross 2000). Using these techniques, several laboratories reported the presence of adult-born cells that incorporated neurons specific markers, conclusively revealing that new neurons are produced in the adult brain (Cameron & McKay, 2001; Gould, Beylin, Tanapat, Reeves, & Shors, 1999; Gould & Tanapat, 1999; Kempermann, Kuhn, & Gage, 1997; Kuhn, Dickinson-Anson, & Gage, 1996). Most astonishingly, these new neurons were also found to be produced in the brains of adult humans (Bhardwaj et al., 2006; Eriksson et al., 1998). Therefore, new neurons are produced in the adult brain each day, and this process occurs across many mammalian species, including rats, mice, and most importantly, humans.

New neurons are not generated throughout the entire adult brain. Instead, they have been primarily localized to two discrete regions. The first of these regions is the dentate gyrus of the hippocampal formation. Within the sub-granular zone of the dentate gyrus radial-like like adult stem cells continuously divide, and are thought to give rise to these new neurons (Seri et al., 2001; Seri et al., 2004). The newly-generated neuronal precursor cells migrate just a short distance, remaining in the granule cell layer of the

dentate gyrus zone as they mature. The second region where new neurons are produced in the adult brain is an area known as the sub-ventricular zone. In this region, which lines the walls of the lateral ventricles, new neurons are also continuously generated. These cells then migrate a long distance, along what is known as the rostral migratory stream, until they reach their destination of the olfactory bulb (Alvarez-Buylla & Garcia-Verdugo, 2002; Lois & Alvarez-Buylla, 1994).

Modulating adult neurogenesis

Following the rediscovery of adult neurogenesis, many laboratories began to examine how internal and external factors might modulate this neurogenic process. The process of adult neurogenesis involves several different stages, and manipulations and training procedures can influence the number of new neurons by altering some, or all, of these steps. This process is often times simplified into two distinct steps. The first step involves the division of these cells. Factors that increase, or decrease, the number of new cells produced are said to influence proliferation of these cells. To assess proliferation, a single injection of BrdU is typically given after a manipulation. For example, animals may be allowed to exercise for several weeks before receiving one injection of BrdU. These animals are typically sacrificed several hours, or one day later. If these manipulations influence the proliferation of these cells than more, or fewer, BrdU-labeled cells should be observed in the dentate gyrus following such manipulations.

We now know that many different factors can influence the proliferation of these cells. Aerobic exercise is one of the most well-characterized, because it causes a large increase in the number of cells that are produced. The first study to demonstrate this

effect was reported by van Praag and colleagues in 1999. In this study, two weeks of daily voluntary exercise resulted in an approximately fifty percent increase in the number of new cells generated in the dentate gyrus (van Praag, Kempermann, & Gage, 1999). Since then, numerous studies have revealed similar effects (Fabel et al., 2003; Kronenberg et al., 2006; Trejo, Carro, & Torres-Aleman, 2001), including a report that just one day of exercise can significantly increase the number of cells produced (Steiner, Zurborg, Hörster, Fabel, & Kempermann, 2008). Interestingly, both voluntary (van Praag et al., 1999) and forced (Lou, Liu, Chang, & Chen, 2008; Trejo et al., 2001) exercise increase cell proliferation. However, lower-intensity forced exercise has a greater effect than higher intensity exercise (Lou et al., 2008). This is likely because the higher intensity physical training procedures are more stressful to an animal, and stress has been shown to decrease the number of cells produced (Gould & Tanapat, 1999). Additionally, not all studies have reported positive effects of exercise on neurogenesis. Social isolation can prevent the exercise induced increase in neurogenesis in both adult male (Stranahan, Khalil & Gould 2006) and female rats (Leasures & Decker 2009). When adult rats were group housed and given voluntary access to activity wheels for twelve days this exercise greatly increased the number of new cells produced. However, when animals were single housed and allowed to exercise for the same twelve day period this exercise actually decreased the number of cells produced. This negative effect of exercise on proliferation in socially isolated rats was attributed to the effect of stress, as social isolation increased corticosterone levels in these animals. When corticosterone levels were reduced (via adrenalectomy and low-dose administrations of corticosterone) exercise increased the number of new cells produced in socially isolated animals. Therefore, while exercise can

increase the number of proliferating cells, under certain, particularly stressful, circumstances it can also decrease the number of cells that are produced. It should be noted that social isolation did not prevent the exercise induced increase in proliferation in adult mice (Kannagara et al., 2009), suggesting that species differences may underlie some of these effects. Regardless of potential species differences, numerous studies report that physical activity can increase the number of new hippocampal cells produced in both rats and mice.

The exact mechanism(s) by which exercise increases cell production is unknown; however, several key mediators have been identified. One of the most likely candidates for mediating the proneurogenic effect of exercise is insulin-like growth factor 1. The peripheral infusion of IGF-1 increases the proliferation of dentate gyrus granule neurons (Aberg, Aberg, Hedbäcker, Oscarsson, & Eriksson, 2000). Additionally, IGF-1 levels are increased following physical activity (Carro, Nuñez, Busiguina, & Torres-Aleman, 2000), and the blockade of peripheral IGF-1 prevents the exercise induced increase in neurogenesis (Trejo, Carro & Torres-Alemán 2001). Vascular endothelial growth factor (VEGF) has also been implicated in mediating the effect of exercise on the proliferation of these cells, as the peripheral blockade of VEGF prevents the exercise induced increase in neurogenesis, without altering baseline levels of neurogenesis in sedentary animals (Fabel et al., 2003).

In addition to exercise, other environmental factors can influence the production of these cells. Alcohol, nicotine, stress, and sleep deprivation all decrease the number of new cells produced (Abrous et al., 2002; Gould & Tanapat, 1999; Guzman-Marin et al., 2005; He, Nixon, Shetty, & Crews, 2005). For example, when adult rats self-administered

nicotine over the course of 42 days it significantly decreased the number of new neurons produced in the dentate gyrus (Abrous et al., 2002). Conversely, anti-depressants, blueberries (presumably through their anti-oxidant effects), and sexual-experiences all increase the number of cells produced (Creer, Romberg, Saksida, van Praag, & Bussey, 2010; Hodes, Yang, Van Kooy, Santollo, & Shors, 2009; Leuner, Glasper, & Gould, 2010; Leuner, Mendolia-Loffredo, & Shors, 2004; Marlatt, Potter, Lucassen, & van Praag, 2012; van Praag et al., 1999; van Praag, 2009) The number of new cells produced also varies as a function of age. Approximately 10,000 new cells are produced each day in the hippocampus of young-adult rats (Cameron & McKay, 2001). However, less than half of this number are produced in older rats (Rao & Shetty, 2004). These results suggest that a large number of factors can influence the number of proliferating cells in the adult dentate gyrus.

Regardless of the number of cells generated, approximately half undergo programmed cell death one-to-two weeks after their birth. In other words, even though many new cells might be produced in response to some environmental manipulations, a great number of those do not survive beyond a few weeks, and they typically die before they have become functionally connected with other neurons in the adult brain. This critical choice between undergoing apoptosis or surviving is the second of the two main steps in this neurogenic process. Environmental factors can also influence this step of adult neurogenesis. Some of the first studies suggesting that an animal's environment can influence the number of surviving adult-born neurons were performed not in mammals, but in songbirds. In these studies Nottebohm and colleagues were examining the neural mechanisms of avian song learning. Nottebohm noticed that new neurons were

incorporated into specific areas of the avian brain, including the hippocampus. Furthermore, the number of cells in an animal's hippocampus at a given time changed depending on the age of the animal, the season, and presumably the complexity of the animal's environment (Barnea, 1994, 1996). These results led Nottebohm to propose that learning could modulate the number of newborn hippocampal neurons. Since then numerous studies have reported that mental training can rescue these cells from death, thereby increasing the number of immature neurons that survive.

The term "mental training" has come to encompass a number of different learning phenomenon and/or procedures. We use it to refer to the direct manipulations of training procedures, the goal of which is to induce skill learning. In order to learn the skill, animals often require many trials each day, often over multiple days and they must exert sustained effort and/or concentration during the training manipulation. The types of training that we know to effectively increase the number of new neurons that survive involve processes related to associative learning and spatial learning (Gould, Beylin, Tanapat, Reeves, & Shors, 1999; Shors, Anderson, Curlik, & Nokia, 2012). After learning, the surviving neurons remain in the dentate gyrus for several months (Leuner et al., 2004). During this time the cells will form functional monosynaptic connections with area CA3 of the hippocampus and polysynaptic connections with efferent sites elsewhere (van Praag et al., 2002). Importantly, learning influences the number of cells that survive, however it does not seem to alter the number that are produced. When animals were trained to learn an associative or spatial learning task, that learning increased the number of one-to-two week old hippocampal neurons that survived. However, this learning did not alter the number of cells that were produced at the time of, or shortly

after, the training experience (Anderson, Sisti, Curlik, & Shors, 2011; Gould, Beylin, et al., 1999). Therefore, while exercise and many other factors can influence the proliferation of these cells, learning does not seem to have this effect. Instead, learning increases the number of neurons that survive to become fully functional mature neurons in the adult hippocampus.

With that said, rescuing new neurons from death is not so simple. Training will rescue these cells only when successful learning occurs during that training. Animals that fail to learn, or those that learn very poorly, do not retain any more of the new cells than animals that are not trained at all (Curlik & Shors 2011). These relationships produce strong positive correlations between how well an animal learns the skill and the number of surviving cells in that animal's dentate gyrus (Curlik & Shors, 2011; Dalla, Bangasser, Edgecomb, & Shors, 2007; Sisti, Glass, & Shors, 2007). Furthermore, when learning is pharmacologically prevented during training, the cells do not survive (Curlik & Shors, 2011). Thus, training by itself does not rescue these cells from death. Instead, learning must occur during the training process.

However, not all forms of learning rescue these cells. In general, learning rescues new neurons only when that learning is difficult to master. For example, learning delay eyeblink conditioning does not rescue new cells from death (Gould, Beylin, et al., 1999). This type of association is easy to learn and occurs with minimal if any conscious awareness (Beylin et al., 2001; Clark & Squire, 1998). When the associations are more difficult to acquire, the cells respond and survive. For example, learning to associate two stimuli that overlap but are separated by a long temporal window (very-long delay conditioning) requires many more trials to learn, and learning this association increases

the number of surviving neurons (Leuner, Waddell, Gould & Shors, 2006). Similarly, learning to associate two stimuli that do not occur together in time increases the number of surviving neurons -- but only if the temporal gap is sufficiently long (Waddell, Anderson, & Shors, 2011). This relationship between task difficulty and neuronal survival also extends into spatial learning. For example, learning to navigate to a visible platform in a water maze task does not increase the number of cells that survive, but learning to find the platform using only spatial cues outside the maze does rescue new neurons (Gould, Beylin, et al., 1999).

Of course, individual animals (including humans) tend to learn at different rates. To account for individual differences in learning ability, we typically assess how many trials are necessary for any given individual to learn, and have repeatedly observed strong positive correlations between the number of trials necessary for an individual animal to learn and the number of surviving cells in that animal's dentate gyrus (Curlik & Shors, 2011; Dalla et al., 2007; Waddell & Shors, 2008). In other words, animals that learn the skill but that require more trials of training to do so tend to retain more cells than animals that learn with less effort. Additionally, learning will rescue cells from death only when that learning is novel. Initial acquisition of a trace eyeblink response rescued cells from death. However, simply performing a previously leaned trace response did not (Anderson et al., 2011). Furthermore, when the trace response was extinguished reacquisition of this response also did not rescue cells from death. This reacquisition was easier than then initial acquisition of the task, which may explain why only the initial learning experience increased the number of surviving cells. Together, these results suggest that learning will have the greatest impact on neurogenesis when the training task itself is challenging, and

when the individual animal requires many trials and/or days of training to master the skill.

Summary and Aims

A large body of literature has revealed that either exercise or learning can increase the number of new neurons in the adult brain. However, exercise seems to have its greatest effect on the proliferation of these cells, whereas learning tends to influence the numbers that survive. In practice, exercise and learning often coexist and interact. For example, learning to perform a new sport or dance engages many different learning processes, which require some degree of cognitive effort. Here, we hypothesized that similar forms of physical skill learning would rescue new cells from death in the adult rat hippocampus. However, we predicted that the physical skill learning would rescue cells only when the skill was sufficiently difficult to master. To test this hypothesis, groups of rats were trained with a modified version of the classic rotarod procedure. Essentially, the animals had to acquire the physical skills necessary to remain on top of a rotating cylindrical rod. This study consisted of three separate experiments. In the first experiment we determined whether physical skill learning would rescue adult-born hippocampal cells from death. In the second experiment we investigated the possibility that physical skill learning would rescue these cells only when the skill was sufficiently difficult to master. Finally, in the third experiment we performed bilateral hippocampal lesions to determine whether acquisition of this physical skill required an intact hippocampal formation.

GENERAL METHODS FOR ALL EXPERIMENTS

Subjects

Adult male Sprague-Dawley rats, ranging from 60 to 90 days of age were individually housed and given access to food and water *ad libitum*. Animals were maintained on a constant 12 hour light/dark cycle. The light cycle began at 7 a.m. and ended at 7 p.m. All procedures were designed to full comply with the PHS Policy on Humane Care and use of Laboratory Animals, and the Guide for the Care and Use of Laboratory Animals.

Labeling new hippocampal cells

BrdU is a thymidine analog, which incorporates into the DNA of all cells currently in the S-phase of the cells cycle at the time of, and for several hours after, the BrdU injection. Once inside a cell the BrdU will remain there, and it will incorporate into the DNA of any of the progeny of those BrdU-labeled cells. Because BrdU crosses the blood brain barrier, one intraperitoneal (i.p). injection of a sufficient dose of BrdU will label nearly all of the cells in the dentate gyrus (and elsewhere) that are currently in the S-phase of the cell cycle. At the start of each experiment all animals received one single i.p. injection of BrdU (200mg/kg). Previous research has revealed that this is an optimal dose of BrdU, labeling all of the cells currently dividing in the dentate gyrus at the time or the injection (Cameron & McKay, 2001; Eadie, Redila, & Christie, 2005).

Within the adult rat hippocampus, new neurons are generated from neural precursor cells with a cell cycle of approximately 25hrs. Therefore, one day after the

BrdU injection the number of BrdU-labeled cells will nearly double, as many of the initial precursor cells will have divided. The number of BrdU-labeled cells continues to increase for several more days after the BrdU injection, as these precursor cells continue to divide. Between one and two weeks after the BrdU injection a large number of these new cells undergo apoptosis (Gould, Beylin, et al., 1999). Therefore, the total number of BrdU-labeled cells in the dentate gyrus drastically decreases during this time period (Cameron & McKay, 2001; Gould, Beylin, et al., 1999). This is also the period during which learning can rescue these cells from death. Therefore, we began the physical skill training exactly one-week after the BrdU injection. Previous research using associative and spatial learning has revealed that this is the optimal time to rescue these cells from death (Anderson et al., 2011; Döbrössy et al., 2003). If animals are trained immediately after labeling there is no increase in cell survival (Anderson et al., 2011). Essentially there is a critical period in the life of these cells where they can be rescued from death, and that time period corresponds includes one-to-two weeks after they are born. Therefore, all animals in the current experiments were trained one week after the BrdU injection.

Skill training

The rotarod (Med Associates Inc. Model #ENV575) is a cylindrical rod that was elevated 26.7cm above a platform. The rod was capable of accelerating, or maintaining a constant velocity, over a five minute period. Performance on a constantly accelerating version of the rotarod task has been reported to improve over several days of training (Buitrago, Schulz, Dichgans, & Luft, 2004). This increase in performance could not be

accounted for by an increase in physical fitness, but rather by the acquisition of a physical skill. Therefore, we chose the constantly accelerating rotarod procedure as our test of a difficult physical skill. Rats were placed on the rotarod, while it was stationary, facing away from the direction that the rod would begin rotating. In this position, the animal had to move forward to remain on the rod while it was rotating. Once all animals were placed on the stationary rod in the correct orientation, a trial began.

During each trial with the accelerating procedure the rotarod linearly accelerated from 1.47 cm/sec to 14.74 cm/sec during a five-minute period. After five minutes, the rod no longer accelerated, and it remained at the constant maximum velocity of 14.74 cm/sec. The behavioral measure was the latency to fall from the rod. Animals were allowed to remain on the rod until they fell off, or until ten minutes had passed. The time from the start of one trial to the start of the next was twenty minutes. Training with the slow velocity version of the task was similar. However, during this slow procedure the rotarod did not accelerate. Instead, it began and remained at the slow constant velocity of 2.94cm/sec.

Animals trained with the accelerating rotarod procedure were later parsed into "good learners" and "poor learners" based on their behavioral performance. The animals were divided into four quartiles, based on their average latency to fall from the rotarod during all sixteen trials of training. Good learners were those in the top quartile, with the greatest average latency, whereas poor learners were those in the bottom quartile, with the smallest average latency. When group sizes were not evenly divisible by four, we rounded down.

To calculate the distance that each animal traveled during each trial of the accelerating rotarod procedure we first calculated the angular velocity of the rotarod, per second, at the starting base velocity of 4RPM. This value was 24^0 per second (4RPM * 360^0=1440^0; 1440^0/60seconds=24^0/sec). Knowing that the rotarod linearly accelerated at 0.12RPM per second, it was determined that the rod accelerated 0.72^0 per second (0.12RPM*360^0/60seconds = 0.72^0/second). The angular velocity of the rotarod was then calculated at "t" seconds, where "t" was the time spent on the rotarod ($\omega=24^0 + 0.72t^0$). As the maximum angular velocity of the rod was reached after 300 seconds "t" could at most be 300 for the purposes of calculating the angular velocity.

The distance traveled during each trial, in degrees, was calculated using the piecewise function below. Briefly put, when an animal remained on the rod for fewer than 300 seconds the total distance travelled was calculated as the integral of the angular velocity at "t" seconds. When an animal remained on the rod for at least 300 seconds the total distance travelled was calculated as the sum of the integral of the angular velocity at 300 seconds and the distance travelled during each subsequent second, during which time the rotarod maintained its' maximum angular velocity of 240^0 per second. Note that, for this equation "t" represented the total time spent on the rod, and it could be any value between 1 and 600 seconds.

$$f(t) = \begin{cases} 0.36t^2 + 24t, & if\ 0 < t < 300 \\ (0.36 * 300^2) + (24 * 300) + ((t - 300) * 240), & if\ t \geq 300 \end{cases}$$

To calculate the distance travelled during training with the slow procedure, we first determined that the angular velocity of the rotarod to be 48^0/second. The latency to fall from the platform was then multiplied by this angular velocity to get the total

distance travelled in degrees. The number of degrees travelled during a trial was converted into centimeters by multiplying the value by $1/360^{th}$ of the circumference of the rotarod (total circumference of the rotarod was 21.98cm).

Immunohistochemistry for BrdU

Twenty-one days after the BrdU injection, animals were deeply anesthetized with sodium pentobarbital (100mg/kg) and transcardially perfused with 4% paraformaldehyde. Brains were extracted and post-fixed in 4% paraformaldehyde at 4 degrees Celsius for 24 hours before being transferred to phosphate buffered saline (PBS). Forty-micrometer coronal sections were taken through the entire rostral-caudal extent of the dentate gyrus of one hemisphere, and every twelfth section was mounted onto a glass slide. Tissue was then stained for the presence of BrdU using standard peroxidase methods. Briefly described, slides were pretreated by heating in 0.1 M citric acid (pH 6.0). The slides were then rinsed in PBS, incubated in trypsin for 10 minutes, denatured in 2N HCl for 30 minutes, rinsed and incubated overnight in primary anti-mouse BrdU (1:200, Becton Dickson) and 0.5% Tween 20. The next day the slides were incubated in biotinylated anti-mouse antibody (1:200, Vector Laboratories) for 60 minutes, before being placed in avidin-biotin complex (1:100, Vector Laboratories) for sixty minutes. The slides were then placed in diaminobenzidine for four minutes, counterstained with cresyl violet, and coverslipped with Permount glue (Fischer Scientific, Fair Lawn, NJ). All slides were coded so that the researcher was blind to the experimental condition of each slide. The number of BrdU-positive cells in the dentate gyrus of each slice (granule cell layer + hilus) was counted by hand. All cell counts were multiplied by 24 (2 hemispheres X

every 12th section), to estimate the total number of BrdU-labeled cells present in the entire dentate gyrus of both hemispheres.

EXPERIMENT #1

PHYSICAL SKILL LEARNING INCREASES THE NUMBER OF SURVIVING ADULT-BORN HIPPOCAMPAL CELLS

Introduction

Both associative and spatial learning can increase the number of surviving cells in the adult hippocampus (Ambrogini et al., 2000; Dalla, Papachristos, Whetstone, & Shors, 2009; Gould, Beylin, et al., 1999; Leuner et al., 2004; Waddell & Shors, 2008). In this first experiment, it was hypothesized that an entirely different form of learning, physical skill learning, would also rescue these cells. We chose physical skill learning because it is vastly different from associative and spatial learning, while still being analogous to forms of learning that humans engage in every day, such as learning a new dance, sport, or arrangement on an instrument. Furthermore, over the past decade, a large body of literature has revealed a positive relationship between physical activity and adult neurogenesis. As previously stated, exercise tends to increase the number of proliferating cells, whereas learning increases the numbers that survive. Therefore, we hypothesized that physical skill learning would rescue new hippocampal cells from death, while simple physical activity would not. We used a variation of the rotarod procedure for our test of physical skill learning, as animals acquire the physical skills necessary to remain on top of the rotarod during this form of training (Buitrago et al., 2004). To determine whether this form of skill learning increased the number of surviving cells we trained one group of animals with the accelerating rotarod procedure. To control for the possibility that exercise, and not learning, might rescue these cells we examined the number of surviving cells in an additional group of animals that were not trained with the rotarod. Instead,

these animals were allowed to exercise in activity wheels. The number of surviving BrdU-labeled cells in these groups was compared to the number of cells in an additional group, which remained experimentally naïve.

Methods

All groups received one single intraperitoneal injection of BrdU (200mg/kg) at the start of the experiment. Seven days after the injection, when many of these new cells begin to undergo apoptosis, and when learning can rescue them from death (Anderson et al., 2011), the first group was trained with an accelerating version of the rotarod procedure (Trained, *N*=13). Training consisted of four trials per day, for four consecutive days. The second group was not trained with the rotarod. Instead, seven days after the BrdU injection, these animals were placed in new cages, which allowed for free access to an attached activity wheel (Exercised, *N*=15; Med Associates Inc. Model #ENV046). The activity wheels were 35.6 cm in diameter (111.78 cm circumference). The wheels were connected to an automated computer system, which allowed for continuous recording of the number of quarter rotations made by each animal every hour. Animals were placed in these new cages at 4:30pm, and they remained in them for eighty-eight hours, before being returned to their home cages (which did not include activity wheels) at 8:30am, following four dark cycles. Therefore, this group was allowed to exercise during the same four days that the first group was trained with the rotarod. A third group of animals remained experimentally naïve. They were not trained, nor did they receive access to activity wheels (Naïve, *N*=18). All animals were perfused twenty-one days after the BrdU injection, because at this point cells that were not rescued from death will have

undergone apoptosis. The number of BrdU-labeled cells that remained in each animal's dentate gyrus provided an index of how many of these new cells were rescued from death as a result of physical skill learning and/or exercise.

Results

In this first experiment, we determined how well the animals learned the accelerating rotarod procedure, and whether that learning increased the number of remaining BrdU-labeled cells in the adult dentate gyrus. One group of rats was trained with an accelerating rotarod procedure. Performance during rotarod training was analyzed with a repeated measures analysis of variance, with trial as the repeated measure, and latency to fall as the dependent measure. This analysis revealed that the group trained with the rotarod procedure increased their latency to fall from the rotarod as training progressed ($F_{15,180}$=8.96, $p\leq$0.01; Fig. 1a), indicating that these animals acquired the physical skills necessary to remain on top of the rotarod as it accelerated. A second group was not trained with the rotarod. Instead, they were placed in new home cages, which allowed for free access to activity wheels for four consecutive days. A third group remained experimentally naïve. Animals given access to activity wheels ran almost twenty times further than animals did during rotarod training (t_{26}=7.56, $p\leq$0.01; Fig. 1b). The number of surviving BrdU-labeled cells differed between these three groups ($F_{2,43}$=4.96, $p\leq$0.05; Fig. 2). Tukey post-hoc comparisons revealed that the trained group retained more new cells then both the naïve group ($p\leq0.05$), and the exercised group ($p\leq0.05$). Despite engaging in a large amount of physical activity, the group that exercised in the wheels did not retain any more new cells than naïve animals ($p>0.05$).

These results suggest that skill learning, and not merely physical exercise, rescued hippocampal cells from death (Fig. 1c).

Previous studies have suggested that training on learning tasks rescues new cells from death, but only when that learning is successful (Curlik & Shors, 2011; Dalla et al., 2007). To examine this hypothesis in the current study, trained animals were separated into good learners (n=3) and poor learners (n=3), based on their average latency to fall from the rotarod across all sixteen trials of training. Differences in the performance of the rotarod task between these two groups was assessed with a repeated measures analysis of variance, with trial as the repeated measure, learning condition as the independent measure, and latency to fall as the dependent measure. The results revealed main effects of trial ($F_{15,60}$=7.02, $p\leq$0.01), and learning condition ($F_{1,4}$=28.87, $p\leq$0.01), and a significant trial by condition interaction ($F_{15,60}$=4.2, $p\leq$0.05), indicating that the good learners increased their performance over the four days of training, whereas the poor learners did not (Fig. 3a). A difference in the number of surviving BrdU-labeled cells was observed between the good learners, poor learners, and naïve animals ($F_{2,21}$=4.86, $p\leq$0.05). Post-hoc Tukey comparisons indicated that the good learners retained more new cells than naïve animals ($p\leq$0.05), whereas the poor learners did not ($p>$0.05; Fig. 3b). A correlation was also observed between the average latency to fall from the rotarod during the third day of training and the number of surviving cells in each animal's dentate gyrus (r=0.56, $p\leq$0.05; Fig 4a). The number of BrdU-labeled cells did not significantly correlate with the average latency to fall across all four days of training (r=0.35, $p>$0.05; Fig. 4b). Nor did the number of surviving cells correlate with each individual animal's greatest latency to fall from the rotarod during any one of the sixteen trials (r=0.20, $p>$0.05; Fig.

4c). Together, these results reveal that acquisition of a physical skill can increase the number of surviving new cells in the dentate gyrus. This increase in cell survival was due to learning, and not merely exercise, and it was only observed in those animals that mastered the task.

Discussion

The results from this first experiment revealed that animals improve their performance during training with a constantly accelerating rotarod procedure. Over the course of training animals increased the time they remained on the rotarod from an average of 25 seconds at the beginning of training to just over 200 seconds at the end of training. This increase in performance likely reflects acquisition of the gross motor skills necessary to stay on the rod as it accelerates (Buitrago et al., 2004). Animals trained to acquire this physical skill retained approximately 20% more BrdU-labeled cells than animals that were untrained. Others have reported that a skilled reaching response can likewise increase the number of surviving cells. In this previous study adult rats were food-restricted, and trained to use their dominant forelimb to retrieve food pellets through a small hole in a plexiglass partition (Wurm et al., 2007). The animals were trained with 50 or 100 trials per day, and over several days of training they increased the percentage of times they successfully retrieved the food pelltet. Acquisition of this skilled reaching response also increased the number of surviving hippocampal cells. Animals trained with this skilled reaching task retained over 2000 more cells than untrained animals.

Our present results expand on these general findings to indicate that the increase in cell survival is only observed in those animals that master the physical skill. No increase in cell number was observed in animals that did not acquire the skill. We separated animals into four quartiles, based on each animal's average latency to fall from the rotarod during all sixteen trials of training. The "good learners" were those in the top quartile, with the greatest average latency to fall from the rotarod, whereas the "poor learners" were those in the bottom quartile, with the shortest latency to fall. As one would expect, the good learners consistently outperformed poor learners during training. The good learner's greatest latency to fall from the rotarod during the last day of training was 400 seconds, whereas the poor learners remained on the rotarod for just under 100 seconds. The good learners retained nearly 1300 more cells (37%) than untrained animals. The poor learners did not retain significantly more cells than untrained animals. In fact, the poor learners only retained approximately 450 (13%) more cells than naïve animals. These results are consistent with those from studies using associative learning, which have found that learning must be successful in order to rescue cells from death (Dalla et al., 2007; Sisti et al., 2007; Curlik & Shors 2011). Additionally, we observed a positive correlation between each animal's average performance during the third day of training and the number of surviving cells in that animal's dentate gyrus. Similar correlations have been reported with associative learning (Dalla et al., 2007). When animals were trained with trace conditioning their average performance during the third day of training significantly correlated with the number of surviving new cells in that animal's dentate gyrus. Overall, our current results suggest that acquisition of the physical skills necessary to remain on top of the constantly accelerating rotarod will

increase the number of surviving adult-born hippocampal cells, provided that the skill is successfully acquired.

Importantly, exercise alone did not increase the number of surviving cells. Animals given access to activity wheels ran nearly twenty times as far as animals did during rotarod training. However, despite this increased physical activity, the animals that exercised in the activity wheels did not retain any more new cells than naïve animals. Animals that exercised in wheels retained just 2% more cells than naïve animals; however, those that were trained with the rotarod procedure retained 20% more cells than naïve animals. Because the animals given access to activity wheels engaged in so much more exercise than animals did during training with the rotarod, and this exercise did not rescue cells from death, it is unlikely that that the physical exercise that occurred during rotarod training increased the number of surviving cells in the current experiment. Instead, we attribute the increase in cell survival following rotarod training to acquisition of the physical skills necessary to remain on top of the rotarod during training.

In the current experiment we administered one single injection of BrdU one-week before the exercise or skill learning began. As a result, the new cells were one week old at the start of training. At this time point nearly all of the cells that were labeled with BrdU were no longer dividing. Instead they were making the critical choice between surviving, or undergoing apoptosis (Anderson et al., 2011). Therefore, the effect of rotarod training was limited to the survival, but not the proliferation, of these cells. This explains why exercise had no observable effect on neurogenesis in the current study – exercise exerts its greatest effect on the proliferation of these cells. Training with the rotarod task increased the number of surviving cells that were one-to-two weeks old at

the time of the training procedure. Because the animals also engaged in exercise during this rotarod training it is possible that this training also increased the number of cells produced during the training experience. However, because the BrdU was administered one week before rotarod training began, any effect of exercise on the proliferation of these cells would not have been observed in the current experiment. To determine whether rotarod training increases proliferation animals could receive one injection of BrdU before each day of training. The animals would then be sacrificed immediately at the end of the training experience, and the number of new cells that were produced during training would be assessed. Previous research has revealed that a combination of learning and exercise can result in an even greater increase in neurogenesis than either learning, or exercise, alone (Fabel et al., 2009). In this experiment animals were either given access to activity wheels, or housed in an enriched environment. The animals exercised in the activity wheels, and they learned about their enriched environment. Both manipulations increased the number of adult-born neurons; however a combination of exercise and environmental enrichment resulted in an even greater increase in neurogenesis than either manipulation alone. Therefore, learning and exercise can have additive effects on neurogenesis, likely because exercise increases the number of new cells produced, whereas learning increases the numbers that survive (Fig. 5). It is possible that training with the rotarod procedure also results in simultaneous increases in proliferation and cell survival; however future research is needed to explore that possibility.

Several studies have reported that exercise can increase the number of cells that survive following physical training (Wu et al., 2008; van Praag et al., 1999).Why would these studies report an increase in the number of surviving cells following exercise,

whereas we observed no such increase in our current experiment? These contradictory findings can be explained by differences in the experimental protocols used. The studies that reported an increase in the number of surviving BrdU-labeled cells following exercise used multiple injections of BrdU, which were spread out across multiple days. Importantly, these BrdU injections were performed while the animals were given access to activity wheels. This is in contrast to the present study, in which animals were injected once with BrdU one week before the exercise began. When BrdU is administered during, or shortly after, periods of physical activity it will label more cells than when it is administered in sedentary animals, simply because exercise increases the number of cells that are produced. Since more new cells are dividing in response to exercise, BrdU will incorporate into more of these new cells. These studies found that more new cells survived weeks after the BrdU-injections, following a prolonged period of physical activity. However, just because more of these BrdU-labeled cells remained does not mean that exercise influenced the ultimate fate of those cells, preventing those cells from undergoing apoptosis. Exercise may have influenced the fate of those cells, but it is also possible that more new cells survived simply because more were produced in the first place. Remember, when BrdU is administered during a period of physical activity more new cells will be labeled with BrdU. Since more new cells were labeled with the initial injections of BrdU, it makes sense that a greater number of cells survived. Therefore, the increased number of surviving cells observed in these experiments may not reflect a direct influence of exercise on the survival of these cells *per se*. Instead, it may simply reflect the fact that more cells survived simply because more cells were proliferating in response to exercise.

Outside of our current results we know of only one study which has directly examined the effect of exercise on the survival of adult-born hippocampal cells (Snyder, Glover, Sanzone, Kamhi, & Cameron, 2009). To determine whether exercise directly influenced cell survival, adult mice received two injections of BrdU, spaced eight hours apart. Two days later some of the mice were given unlimited access to activity wheels. After several weeks of exercise the number of surviving cells was estimated. Interestingly, this prolonged exercise regimen significantly increased the number of surviving cells. Therefore, it is possible that prolonged periods of physical activity will increase neuronal survival, whereas acute periods do not. Regardless, the present findings suggest that physical skill learning can increase the number of surviving cells in the adult hippocampus, because the skill learning began when the new neurons were about to undergo apoptosis, and when learning can rescue them from death (Anderson et al., 2011). Therefore, our current results reveal that physical skill learning can have a long-term impact on the structure of the adult hippocampal formation through increasing the number of surviving adult-born hippocampal cells in the dentate gyrus.

EXPERIMENT #2

ACQUISITION OF A DIFFICULT, BUT NOT AN EASIER, PHYSICAL SKILL INCREASES THE NUMBER OF SURVIVING CELLS

Introduction

The results from the first experiment indicate that physical skill learning can increase the number of surviving cells in the adult hippocampus. Previous research has suggested that associative and spatial learning will increase the number of surviving cells only when that that learning is sufficiently difficult to achieve. In these studies difficulty is traditionally operationalized as the number of trials and/or days required to learn a task. Acquisition of procedures that can be learned in comparatively few trials, such as delay eyeblink conditioning or the visible platform version of the Morris water maze does not increase the number of surviving cells. However, when these tasks are made more difficult, by altering the task parameters, this learning requires more trials, and it does rescue cells (Gould, Beylin, et al., 1999; Leuner, Waddell, Gould, & Shors, 2006). Furthermore, in multiple experiments and under multiple conditions, we have observed strong positive correlations between the number of trials necessary for an individual animal to learn and the number of surviving cells in that animal's dentate gyrus (Curlik & Shors, 2011; Waddell & Shors; 2008; Dalla et al., 2007). Specifically, when an animal requires many trials to learn a trace eyeblink conditioning task they retain more new cells than animals that learn the same task in comparatively fewer trials. Previously we have operationalized the successful acquisition of the conditioned response as the number of trials required to emit 60% conditioned responses out of any block of 100 trials. We have also examined other criteria, such as the number of trials required to emit eight out of

nine consecutive conditioned responses. Using both of these criteria we have observed that animals that take longer to reach criterion retain more new cells than those that do so with fewer trials. Regardless of how quickly these animals learn they still retain more new cells than animals that fail to learn the task. For example, when acquisition of trace eyeblink conditioning was facilitated via the administration of the cognitive enhancing compound D-cycloserine animals could learn the task in very few trials. Many of the animals reached our learning criterion on the first day of training. Because these animals learned the task they retained more new cells than untrained animals. However, of those animals that were trained, those that took longer to learn retained more new cells than those that quickly mastered the task (Curlik & Shors, 2011). Together, these results suggest that learning will have its greatest effect on neurogenesis when the training task itself is challenging, and when the individual animal requires many trials and/or days of training to master the task.

Therefore, we hypothesized that physical skill learning would increase cell survival only when the physical skill was sufficiently difficult to master. To test this hypothesis we trained two new groups of animals. One group was trained with the accelerating rotarod procedure as previously described. The second group was trained with a variation of the rotarod procedure, in which the rod did not accelerate during training. Instead, the rotarod began and remained at a slow constant velocity. We predicted that by removing the acceleration component the task would be less complex, and animals would require fewer trails to master it. Because this task should be mastered in fewer trials we predicted that acquisition of the slow rotarod procedure would not increase the number of surviving cells, whereas acquisition of the more complex

accelerating procedure would.

Methods

Three groups of animals were used in this experiment. One group was trained with the accelerating rotarod procedure (Accelerating, *N*=18). A second group was trained with a slow velocity version of the task (Slow, *N*=17). During each trial of slow rotarod training the rotarod began and remained at a slow constant velocity of 8RPM. The third group was experimentally naïve (Naïve, N=31). All animals received one single i.p. injection of BrdU (200mg/kg), and they began training one week later. The animals were perfused twenty-one days after the BrdU injection, and the number of surviving BrdU-labeled cells was assessed.

Results

Performance during training with the accelerating or the slow rotarod procedure was analyzed using a repeated measures analysis of variance, with trial as the repeated measure, training condition as the independent measure, and the latency to fall from the rotarod as the dependent measure. This analysis revealed main effects of trial ($F_{15,495}$=19.383, $p \leq 0.01$), and training condition ($F_{1,33}$=62.20, $p \leq 0.01$), and a significant trial by training condition interaction ($F_{15,495}$=9.66, $p \leq 0.01$), indicating that the group trained with the slow procedure outperformed the group trained with the accelerating procedure (Fig. 6a). Separate repeated measures ANOVAs were then conducted for each training condition, with trial as the repeated measure, and the latency to fall from the rotarod as the dependent measure. These analyses revealed a main effect of trial for both

the accelerating ($F_{15,255}$=5.37, p≤0.01), and the slow ($F_{15,240}$=15.43, p≤0.01) conditions, revealing that both groups increased their latency to fall from the rotarod as training progressed (Fig. 6a). The group trained with the slow rotarod procedure also travelled twice as far as the accelerating group did during training (t_{33}=4.65, p≤0.01; Fig. 6b).

The number of surviving BrdU-labeled cells was assessed using a univariate analysis of variance with the training condition as the independent measure, and the number of surviving BrdU+ cells as the dependent measure. This analysis revealed a significant main effect of training condition ($F_{2,63}$=4.72, p≤0.05). Post-hoc Tukey comparisons revealed that the group trained with the accelerating rotarod procedure retained more BrdU-labeled cells than naïve animals (p≤0.05), whereas the group trained with the slow speed procedure did not (p>0.05). These results suggest that acquisition of the accelerating rotarod procedure increased the number of surviving cells, whereas acquisition of the slow rotarod procedure did not.

As in the first experiment, rats trained with the accelerating procedure were separated into good learners (n=4) and poor learners (n=4). The behavioral performance of these animals was assessed using a repeated measures analysis of variance, with trial as the repeated measure, learning condition as the independent measure, and the latency to fall from the rotarod as the dependent measure. This analysis revealed a significant effect of trial ($F_{15,90}$=3.67, p≤0.01), and learning condition ($F_{1,6}$=649.46, p≤0.01), with a significant trial by condition interaction ($F_{15,90}$=2.96, p≤0.01), indicating that the good learners outperformed the poor learners (Fig. 7a). The number of BrdU-labeled cells differed between the good learners, poor learners, and naïve animals ($F_{2,36}$=5.42, p≤0.01).

Post-hoc Tukey comparisons revealed that the good learners retained more new cells than naïve animals ($p \leq 0.05$), whereas the poor learners did not ($p > 0.05$; Fig 7b).

Correlations were examined between each animal's performance during training with the accelerating rotarod procedure and the number of surviving BrdU-labeled cells in that animal's dentate gyrus. The average latency to fall during the third day of training ($r=0.38$, $p>0.05$; Fig. 8a), the average latency across all four days of training ($r=0.42$, $p>0.05$; Fig. 8b), and the greatest latency to fall during any one trial ($r=0.45$, $p>0.05$; Fig. 8c), did not significantly correlate with the number of surviving cells. However, across both Experiment #1 and Experiment #2 all of these correlations were the positive direction. Therefore, we combined the data from the first and second experiments, and examined correlations between behavioral performance and cell survival in this larger sample (N=31). When combining the subject pools from both experiments the average latency on the third day of training ($r=.44$, $p \leq 0.05$; Fig. 9a), the average latency across all four days of training ($r=.40$, $p \leq 0.05$; Fig. 9b), and the greatest latency during any one trial ($r=.36$, $p \leq 0.05$; Fig. 9c) all significantly correlated with the number of surviving cells. Therefore, physical skill learning increased the number of surviving cells provided that the skill was sufficiently complex, but successfully mastered.

Discussion

Rats trained with the accelerating or the slow rotarod procedures increased their latency to fall from the rotarod during training. Animals trained with the accelerating procedure began training with an average latency to fall from the rotarod of 45 seconds.

At the end the training, their latency to fall increased to almost 200 seconds. Animals trained with the slow rotarod procedure quickly increased their latency to fall from the rotarod, and they displayed much higher levels of performance during the last day of training. During the first trial of training with the slow procedure animals remained on the rotarod for approximately 60. However, these animals displayed a large increase in performance, and by the last day of training they remained on the rotarod for almost 480 seconds. It is unclear if this difference in performance between the accelerating and slow rotarod conditions was due to the accelerating procedure being more difficult to learn, or simply more difficult to perform. Regardless, it is clear that the accelerating rotarod procedure is a much more difficult task, requiring many more trials of training to master. Because the slow rotarod procedure could be acquired in very few trials, we predicted that acquisition of this task would not increase the number of surviving hippocampal cells. As in the first experiment, the group trained with the accelerating rotarod procedure retained nearly 900 more cells than naïve animals. However, consistent with our hypothesis, training with the slow velocity version of the task did not significantly increase the number of surviving cells. Animals trained with the slow rotarod procedure retained just 400 more cells than naive animals. These results suggest that physical skill learning will increase cell survival only when the skill is sufficiently complex.

Animals that mastered the accelerating rotarod procedure retained proportionally more new cells than naïve animals. Those that that successfully learned the accelerating task retained approximately 42% more cells than naïve animals, whereas those that failed to learn the accelerating procedure retained 26% more new cells than naïve animals. Although the poor learners retained more new cells than naïve animals this difference

was not statistically significant. Therefore, these results are consistent with those from Experiment #1, where we revealed an increase in cell survival in those animals that successfully mastered the accelerating rotarod task. When combining the data from the first two experiments we observed significant positive correlations between each animal's behavioral performance and the number of surviving cells in that animal's dentate gyrus. An animal's average performance across all four days of training correlated with the number of surviving cells. Their average performance during the third day of training also predicted the number of cells that survived. Previously, we have observed similar correlations using associative learning. For example, when training animals to learn a trace eyeblink response each animal's average performance across all four days of training positively correlated with the number of surviving cells in that animal's dentate gyrus (Curlik & Shors, 2011; Dalla et al., 2007; Nokia, Sisti, Choksi, & Shors, 2012). Likewise, their performance during the day of training correlated with the number of surviving cells (Dalla et al., 2007). In our current experiments a positive correlation was also observed between each animal's greatest latency to fall from the rotarod during any one of the sixteen trials, and the number of surviving cells in that animal's dentate gyrus. Together, these results indicate that individual differences in each animal's acquisition of a physical skill can influence the number of cells that survive after training.

The results from Experiment #1 suggested that learning, but not exercise, increased the number of surviving cells. Despite engaging in a large amount of physical activity animals given access to activity wheels did retain any more new cells than those that were experimentally naïve. However, animals trained with the accelerating rotarod procedure did rescue cells from death. It is possible that the exercise that occurred during

rotarod training may have been different than the exercise that occurred when using the activity wheels. Perhaps there is some aspect of the exercise that occurs during rotarod training that rescues cells from death, independent of the learning that also occurs during training. In the current experiment, we measured the distance that each group ran during training with both the slow and accelerating rotarod procedures. The group trained with the slow procedure ran twice as far as the group trained with the accelerating procedure. However, only the group trained with the accelerating procedure rescued cells from death. These results further suggest that skill learning, but not exercise, increased the number of surviving cells in these experiments. Therefore, consistent with previous results using associative learning, we report here that physical skill learning can increase the number of surviving hippocampal cells provided that the skill is sufficiently difficult, and successfully acquired.

EXPERIMENT #3

THE HIPPOCAMPUS IS NOT REQUIRED FOR ACQUISITION OF THIS PHYSICAL SKILL

Introduction

Previous research has suggested that learning will rescue new hippocampal cells from death only when that learning requires an intact hippocampal formation (Gould, Beylin, et al., 1999). For example, acquisition of trace eyeblink conditioning, very-long delay conditioning, or the hidden platform version of the Morris water maze requires an intact hippocampal formation, and learning these procedures increases the number of surviving adult-born hippocampal cells (Beylin et al., 2001; Gould, Beylin, et al., 1999; Leuner et al., 2006; Morris et al., 1982). Conversely, acquisition of delay eyeblink conditioning or the visible platform version of the Morris water maze does not require an intact hippocampus, and these forms of learning do not rescue new hippocampal cells from death (Beylin et al., 2001; Gould, Beylin, et al., 1999; Morris et al., 1982). In Experiment #1 and Experiment #2 we revealed that acquisition of a complex physical skill could increase the number of surviving adult-born hippocampal cells. Many forms of physical skill learning are thought to be hippocampal-independent. Instead, acquisition of these tasks is thought to rely on striatal, cerebellar, and cortical networks (Goddyn, Leo, Meert, & D'Hooge, 2006; Karni et al., 1995). For example, human subjects with hippocampal damage, and amnesic patients, can still acquire a variety of different physical skills. As previously mentioned, patient H.M. could learn a mirror tracing task even though he did not have an intact hippocampal formation. H.M. could also acquire other motor skills, such as rotary pursuit and bimanual tracking skills (Corkin 1968).

These, and similar findings of intact skill learning in amnesic patients, lead to the formation of declarative memory theory (Cohen & Squire, 1980). As discussed, this theory states that long-term memory can be divided into two main categories. The first of these categories is declarative memory. Declarative memories are those that can later be consciously recalled by an individual, and they include semantic and episodic memories. The second category of memory is non-declarative memory. These memories do not require conscious thought for recollection, and they include forms of learning such as priming, habituation, and procedural learning. Traditionally, the hippocampal formation is thought to be necessary for the formation of new declarative memories. However, it is not typically viewed as necessary for the formation of nondeclarative memories. Because most physical skills fall under the domain of procedural memory, an intact hippocampal formation is not traditionally thought of as necessary to learn these skills.

Results from animal studies suggest that the hippocampal formation is not necessary for acquisition of many new physical skills. For example, one study reported that hippocampal lesions did not impair the ability of rats to learn an acrobatic motor skill (Gould et al., 2002). In this study, animals were first trained to walk across a runway by stepping on regularly spaced aluminum rods. To ensure that the animals were motivated to perform this task the researchers restricted the amount of water that the animals received before training. Every time the animal successfully walked across the runway, by stepping on the aluminum rods, it was rewarded with a small amount of water. After this initial training experience the distance between the rods was altered, so that they were irregularly space. The animals were then tested for their ability to step across the aluminum rods at this new distance. Animals without an intact hippocampus acquired the

physical skills necessary to perform this peg-running task just as well as animals that had a functioning hippocampus, further suggesting that the hippocampal formation is not required for acquisition of new physical skills.

Several studies have examined how hippocampal lesions affect rotarod performance. In one such study adult rats without an intact hippocampal formation were trained with an accelerating rotarod procedure, which was very similar to our own (Goddyn et al., 2006). These animals were trained with four trials of rotarod training, over the course of one day. Like our procedure the rotarod accelerated from 4-40RPM over the course of five minutes. Hippocampal lesions did not alter the latency to fall from the rotarod during this form of training. Another study has reported that hippocampal lesions do not disrupt performance during rotarod training (Deacon, Croucher, & Rawlins, 2002). However, in this study the animals were trained with only one trial. These studies suggest that hippocampal lesions do not disrupt acquisition of the physical skills necessary to remain on top of the rotarod as it accelerates; however, both of these studies used very few trials, and in both studies the animals were trained for only one day. It is possible that hippocampal lesions may have a more pronounced effect on physical skill learning when that learning is spaced out across multiple days of training.

We conducted this third experiment, to determine whether acquisition of the physical skills associated with our rotarod procedure required an intact hippocampal formation. Based on the aforementioned studies revealing that hippocampal lesions do not disrupt rotarod performance, we predicted that the form of physical skill learning used in Experiment #1 and Experiment #2 would not require an intact hippocampal formation. If so, these results would reveal that hippocampal independent forms of

learning can also increase the number of surviving cells in this structure. To test this hypothesis we performed bilateral hippocampal lesions in one group of rats, and sham lesions in another group before training with the accelerating rotarod procedure.

Methods

Bilateral excitotoxic lesions of the entire hippocampal formation (Lesion, *N*=9) or sham lesions (Sham, *N*=19) were imposed on new animals before training with the accelerating rotarod procedure (Fig. 10a-c). All rats were anesthetized with sodium pentobarbital (60mg/kg, i.p.).The scalp was cleaned with Betadine before incisions were made. Rats received either sham, or hippocampal, lesions. Sham animals received bilateral infusions of 0.1% phosphate buffered saline (PBS). Rats with lesions of the hippocampus were bilaterally infused with the excitotoxin N-methyl-D-asparate (NMDA) at a dose of 20mg/ml. All infusions (volume: 0.35 µl; rate: 0.5 µl/minute) were made via a Stoelting microinfusion pump at 12 sites within the hippocampus (AP: –2.5 mm, ML: ±1.6, DV: –3.8 mm; AP: –4.2 mm, ML: ±2.6, DV: –3.1 mm; AP: –5.3 mm, ML: ±5.0 mm, DV: –5.9 mm; AP: –5.3 mm, ML: ±4.2 mm, DV: –3.4 mm; AP: –5.8 mm, ML: ±4.6 mm, DV: –6.1 mm; AP: –6.0 mm, ML: ±5.6 mm, DV: –4.1 mm). AP coordinates were measured relative to Bregma, ML coordinates from the midline, and DV coordinates from the surface of the brain (dura). Each infusion occurred two minutes after the insertion of the needle to the infusion site, to allow time for the tissue to settle. The infusions were separated from the end of one to the start of the next by five minutes. Following the completion of infusions, the holes in the skull were covered with bone wax

and the wound closed. All animals were singly housed after surgery. They were allowed at least one week to recover before the start of behavioral testing.

Approximately one week after behavioral testing, all rats were heavily anesthetized with a sodium pentobarbital solution (Sleepaway, Butler Schein) and perfused with 0.9% saline solution followed by 10% buffered formalin. Brains were removed and post-fixed in 10% formalin for at least 24h. They were then transferred to a 30% sucrose-formalin cryoprotectant solution, where they were allowed at least three days to become fully saturated with the sucrose-formalin. The brains were then sectioned into 50-µm coronal sections using a cryostat. The sections were mounted onto gelled slides and stained with 0.1% cresyl violet to verify the lesions. Rats were excluded from the study if the lesions were misplaced or incomplete. Lesions were identified by the location of the needle track, absence of nerve cell bodies, and gliosis, or the presence of darkly stained astrocytes (Bangasser, Santollo, & Shors, 2005). Animals, both sham and lesioned, were also excluded if there was extensive damage to extra-hippocampal areas, i.e. tissue damage due to needle placement.

Results

This third experiment was designed to determine whether an intact hippocampus is necessary for acquisition of the physical skills necessary to perform our accelerating rotarod procedure. Performance during accelerating training was assessed using a repeated measures analysis of variance, with trial as the repeated measure, lesion condition as the independent measure, and the latency to fall as the dependent measure. The results revealed a significant effect of trial ($F_{15,405}$=10.84, $p\leq$0.01), no effect of

lesion condition ($F_{1,26}$=2.27, p>0.05), and a trial by lesion condition interaction ($F_{15,390}$=2.42, p≤0.01). Separate repeated measures ANOVAs conducted for each condition revealed that both the sham ($F_{15,270}$=4.37, p≤0.01), and the lesioned ($F_{15,120}$=7.64, p≤0.01) groups increased their latency to fall from the rotarod during training, indicating that both groups acquired the task (Fig. 11a). Following training with the accelerating procedure both groups were then trained with the slow procedure. The latency to fall from the rotarod during slow training was examined using a repeated measures ANOVA, with trial as the repeated measure, lesion condition as the independent measure, and latency to fall as the dependent measure. This analysis revealed no main effect of trial ($F_{15,390}$=0.66, p>0.05), but a significant effect of lesion condition ($F_{1,26}$=16.04, p≤0.01), and a trial by lesion condition interaction ($F_{15,390}$=4.36, p≤0.01), indicating that lesioned group outperformed the sham group during this slow procedure (Fig. 11b). Together, these results reveal that an intact hippocampal formation is not necessary for acquisition of the physical skills necessary to perform the accelerating or the slow rotarod procedures.

Interestingly, animals without an intact hippocampus did not fully retain their performance between the end of each session and the beginning of the next. In other words, the lesioned animals consistently displayed a decrease in the latency to fall from the rotarod between the last trial of each daily session, and the first trial of the next. For example, during the last trial of the first session of accelerating training the lesioned group had a latency to fall from the rotarod of 103 seconds. However, during the first trial of the next day of training the lesioned group remained on the rotarod for only 57 seconds. This decrease in the latency to fall from the rotarod between sessions was not

observed in the sham group, or in any of the groups trained in the first two experiments, which did not receive any form of surgery. During training with the slow rotarod procedure the lesioned animals also displayed this inter-session decrease in the latency to fall from the rotarod. However, this was not as pronounced as it was during accelerating training. For example, during the last trial of the first day of slow rotarod training the lesioned group had a latency to fall from the rotarod of 537 seconds. However, on the first trial of the next day of training the lesioned group's latency was 477 seconds.

To determine whether acquisition of the physical skill occurred between, or within, sessions a repeated measures analysis of variance of the sham conditions performance during accelerating training was conducted, with trial as the repeated measure, session number as the independent measure, and latency to fall as the dependent measure. This analysis revealed a significant effect of session ($F_{3,72}$=3.05, $p\leq$0.05), with no effect of trial ($F_{3,216}$=0.79, p>0.05), and no trial by session interaction ($F_{9,216}$=1.29, p>0.05). These results indicate that the sham animals increased their latency to fall from the rotarod between sessions, but not within sessions, as no significant improvement in their latency to fall from the rotarod was observed within each session. This same analysis was then conducted using data from the lesioned group. The results from this analysis revealed significant main effects of session ($F_{3,32}$=8.66, $p\leq$0.01), and trial ($F_{3,96}$=11.24, $p\leq$0.01), with no session by trial interaction ($F_{9,96}$=0.37, p>0.05), indicating that the lesioned group increased their latency to fall from the rotarod both within, and between, each daily session. Therefore, the sham animals only made large improvements in their latency to fall from the accelerating rotarod between each daily session, whereas the lesioned animals increased their latency to fall both between and within sessions.

To determine whether acquisition of the slow rotarod procedure occurred within, or between, sessions a repeated measures ANOVA of the sham condition's latency to during slow rotarod training was conducted, with trial as the repeated measure, session number as the independent measure, and latency to fall as the dependent measure. This analysis revealed a significant effect of session ($F_{3,72}$=3.05, $p\leq0.05$), with no effect of trial($F_{3,216}$=0.79, $p>0.05$), and no trial by session interaction ($F_{9,216}$=1.29, $p>0.05$). Interestingly, this main effect of session reflected a decrease in their latency to fall from the rotarod across sessions, perhaps as a result of overtraining. This same analysis was then conducted using data from the lesion group. This analysis revealed a main effect of trial ($F_{3,96}$=11.24, $p\leq0.01$), and session ($F_{3,32}$=8.70, $p\leq0.01$), with no trial by session interaction ($F_{9,96}$=0.37, $p>0.05$), revealing that the lesioned group continued to increase its latency to fall to fall from the rotarod both within, and between, sessions, consistent with the results from the accelerating rotarod procedure. Why the lesioned animals continued to increase their latency to fall from the rotarod during slow training, whereas the sham animals did not is unclear (see discussion below). However, these results indicate that the hippocampal formation is not necessary for acquisition of the physical skills associated with the accelerating or the slow rotarod procedures. Thus, a hippocampal-independent form of physical skill learning can rescue new cells from death in the adult hippocampus.

Discussion

The current results reveal that the physical skills associated with our accelerating rotarod procedure can be learned without an intact hippocampal formation. Both

hippocampal-lesioned, and sham animals, improved their latency to fall from the rotarod over the four days of accelerating training. Sham animals began training with a latency to fall from the rotarod of just over 30 seconds; however, by the last day of training this increased to almost 140 seconds. Similarly, animals without an intact hippocampal formation began accelerating training with a short latency to fall from the rotarod, just over 30 seconds. However, and the end of accelerating training these animals remained on the rotarod for almost 240 seconds. During training with the slow rotarod procedure the sham animals actually decreased their latency to fall from the rotarod across trials. These animals began slow training with a latency to fall of 275 seconds. However, this consistently decreased during training, and these sham animals ended slow training with a latency to fall from the rotarod of 170 seconds. Whereas the sham animals decreased performance during slow training the lesioned animals greatly increased their latency to fall from the rotarod during this procedure. During the first trial of slow rotarod training the lesioned animals has a latency to fall from the rotarod of over 400 seconds; however, by the end of training all of these animals remained on the rotarod for the full 600 seconds. Because the hippocampal lesioned animals increased their latency to fall from the rotarod during training these results indicate that that the hippocampus is not necessary for acquisition of the physical skills used in Experiment #1 and Experiment #2. They are also are also consistent with the results from previous studies, which have reported that the hippocampus is not necessary for acquisition of similar forms of rotarod training (Deacon et al., 2002; Goddyn et al., 2006).

Hippocampal lesioned animals tended to remain on the rotarod for a longer period of time than their sham counterparts. This response is not easily explained, and was

unexpected. One possibility is that the hippocampal lesioned animals may have been hyperactive, as disruptions of the hippocampal formation can result in hyperactivity (Anagnostaras, Maren, & Fanselow, 1999; Teitelbaum & Milner, 1963). Therefore, the lesioned group may have outperformed the sham group simply because they were more active, and not because they were learning any better. It is also possible that the sham group experienced overtraining during this experiment. This would explain why the sham group's performance decreased during training with the slow rotarod procedure. Another possibility is that a damaged hippocampus may be more disruptive to acquisition of this task than no hippocampus at all. Similar findings have been observed with a hippocampal-independent form delay eyeblink conditioning. Bilateral lesions of the entire hippocampal formation did not disrupt acquisition of a delay eyeblink response. However, the administration of scopolamine directly into the hippocampal formation before training did impair this form of learning (Solomon et al., 1983). These results reveal that a disrupted hippocampus can be more detrimental to learning than no hippocampus at all. In the current study, it is possible that the sham surgery damaged to the hippocampal formation. This damage may have impaired acquisition of the physical skill. These results would explain why the sham condition displayed shorter latencies to fall from the rotarod in the experiment than animals did during Experiment #1 and Experiment #2 (when these animals did not receive any surgery). In the current experiment the sham condition's greatest latency to fall from the accelerating rotarod during one trial was 132 seconds. However, in Experiment #1 the animals remained on the accelerating rotarod for over 200 seconds. Similarly, in Experiment #2 animals trained with the accelerating rotarod procedure remained on the rotarod for over 190

seconds. The greater latencies to fall from the rotarod in animals that did not undergo surgery suggest that some aspect of the sham surgery may have impaired the animal's performance during rotarod training. Indeed, sham surgeries have been reported to impair both spatial and sequential learning in adult rats (Eckart, Huelse-Matia, & Schwarting, 2011). Importantly, in our current experiment lesions of the hippocampal formation did not result in these same deficits. Therefore, like specific forms of associative learning, a damaged hippocampus may be more disruptive to acquisition of this physical skill than no hippocampus at all.

Another possible explanation for the improved performance of the lesioned animals comes from one recent study, which examined the effect of hippocampal lesions on a serial reaction time task. As previously mentioned, the serial reaction time task is traditionally used as a test of implicit motor sequence learning, and therefore many learning theories predict that this form of learning will not require on an intact hippocampal formation (Moscovitch, 1992, 2008; Squire & Zola, 1996). Indeed, lesions of the dorsal hippocampal formation before training did not impair the acquisition of this serial reaction time task (Eckart et al., 2011). Instead, these hippocampal lesions actually facilitated acquisition of this procedure. Hippocampal lesioned animals outperformed sham animals, and animals that did not receive surgery. The lesioned animals displayed a decreased reaction time, and an increased accuracy, indicating that this improved performance was not due to an accuracy/reaction time trade off. The authors suggested that the dorsal hippocampal lesions may have facilitated performance by reducing interference. It is possible that the hippocampal lesions in our current experiment may have also facilitated learning by reducing interface, or distractions.

In our current experiment animals without an intact hippocampus did not fully retain their performance from the end of each session to the beginning of the next. For example, during the last trial of the first session of accelerating training the hippocampal lesioned animals displayed a latency to fall from the rotarod of 103 seconds. However, during the first trial of the next session of training these animals displayed a latency to fall of just 57 seconds. This between sessions decrease in the latency to fall from the rotarod was consistent, being present during training with both the accelerating and the slow procedures. Furthermore, this decrease in performance was not observed in the sham condition, or in any of the groups trained in Experiment #1 or Experiment #2. This decrease in the latency to fall from the rotarod between sessions suggests that the hippocampus may be involved in maintaining performance of the physical skill between sessions. Regardless of the interpretation, it is clear the hippocampal formation is not necessary for acquisition of the physicals skills associated with the accelerating rotarod procedure. Furthermore, the results form Experiment #1 and Experiment #2 reveal that this form of physical skill learning can increase the number of surviving cells in the adult hippocampal formation. Therefore, our current results reveal that hippocampal-independent forms of physical skill learning can rescue new ells from death in the adult dentate gyrus.

GENERAL DISCUSSION

The results from Experiment #1 revealed that physical skill learning could increase the number of surviving cells within the adult hippocampal formation. This effect was not due to an increase in exercise, because animals that exercised during the same time period did not retain any more cells than sedentary animals. The results from Experiment #2 revealed that physical skill learning would rescue cells from death only when the skill was sufficiently complex. During both of these experiments the increase in cell survival was observed only in those animals that mastered the task. Those that failed to learn, or those that learned poorly, did not retain any more cells than naïve animals. Significant correlations were observed between each individual animal's performance during accelerating training, and the number of surviving cells in that animal's dentate gyrus. The average latency to fall from the rotarod across all four days of training, the average latency to fall from the rotarod on the third day of training, and each individual animal's greatest latency to fall from the rotarod during any one trial, each correlated with the number of surviving hippocampal cells. Together, these results suggest that physical skill learning will increase the number of surviving hippocampal cells when only the skill is sufficiently complex, but successfully learned (Fig. 12).

Nearly all of the forms of learning that rescue these cells require an intact hippocampal formation. For example, acquisition of trace eyeblink conditioning, or the hidden platform version of the Morris-water maze procedure, requires an intact hippocampus, and this learning increases the number of surviving cells (Beylin et al., 2001; Gould, Beylin, et al., 1999; Hagan, Salamone, Simpson, Iversen, & Morris, 1988). Conversely, delay eyeblink conditioning, or the visible platform version of the Morris-

water maze procedure, can be learned without a hippocampus, and this learning does not increase cell survival (Beylin et al., 2001; Gould, Beylin, et al., 1999; Hagan et al., 1988). However, when acquisition of delay conditioning is made more difficult, by increasing the length of the inter-stimulus interval, it then requires the hippocampus, and this learning then rescues cells (Leuner et al., 2006). Together, these results suggest learning might rescue cells from death only when that learning requires an intact hippocampal formation. Indeed, this would provide a very parsimonious explanation for why some, but not all, forms of learning increase cell survival. However, our present results clearly refute this possibility. The physical skills associated with the accelerating rotarod procedure could be learned without an intact hippocampus; however, this learning still increased the number of surviving adult-born hippocampal cells. Therefore, hippocampal-independent forms of learning can rescue cells from death in this structure. This finding is consistent with the results of one previous experiment, where animals were trained with an associative learning task known as contiguous trace eyeblink conditioning. This form of learning increased cell survival, and is believed to be hippocampal-independent, as contiguous trace fear conditioning does not require the hippocampal formation (Bangasser et al., 2006). However, to date no laboratories have directly examined the hippocampal dependence of this form of eyeblink conditioning.

Our results reveal that learning can increase the number of surviving cells in the adult hippocampal formation even when the hippocampus is not necessary for that learning to occur. However, do all forms of learning that require the hippocampus rescue these cells from death? Recently, we have examined this question and revealed a hippocampal-dependent form of learning that does not rescue these cells. This task,

known as short trace conditioning, differs from standard trace eyeblink conditioning, because the duration of the inter-stimulus interval is reduced. The shorter inter-stimulus interval facilitates acquisition of the task; however, this learning still requires the hippocampal formation. Importantly, learning this short trace procedure did not increase the number of surviving cells (Waddell et al., 2011). Therefore, not all hippocampal-dependent forms of learning will rescue these cells. Together with our current results these findings reveal that the hippocampal dependence of a learning procedure is neither necessary, nor sufficient, to predict whether that learning will rescue new neurons from death.

Even though the hippocampus is not necessary for acquisition of the physical skills associated with our rotarod procedure, hippocampal activity may still contribute to that learning. Several theories of hippocampal function predict that the hippocampus is not necessary for the formation of new memories that do not require conscious awareness (Moscovitch, 1992, 2008). However, recent research has challenged this view, by suggesting that the hippocampus may also contribute to the formation of various implicit memories (Hannula & Greene, 2012). As previously mentioned, the hippocampus may be involved in acquisition of the implicit contextual cueing, and serial reaction time procedures (Chun & Phelps, 1999; Curran, 1997; Shanks et al., 2006). Furthermore, an increased in hippocampal activity has been observed during both the early and late stages of implicit motor sequence learning in humans (Gheysen, Van Opstal, Roggeman, Van Waelvelde, & Fias, 2010). This hippocampal activity correlated with the motor sequence learning, and when the motor sequence was altered the hippocampal activity was also affected. These results suggest that even though the hippocampus may not be necessary

for some forms of learning, it still may contribute to that learning. Exactly how the hippocampus would contribute to physical skill learning is unknown; however, one study has reported a role for the hippocampus in the consolidation of motor sequence learning in humans (Albouy et al., 2008). In this study subjects were trained with an implicit occulomotor sequence learning task. They were then tested for their performance 30min, 5hrs, or 24hrs after training. Hippocampal activity during the training experience did not correlate with performance of the learned sequence 30min or 5hrs later. However, this hippocampal activity did correlate with a gain in performance 24hrs after the training session. These results suggest that the hippocampus may be involved in the overnight consolidation of an implicit occulomotor skill. The hippocampus may also be involved in the consolidation of the physical skills necessary to remain on top of the rotarod in our current experiments. In Experiment #3 animals without an intact hippocampus displayed a decrease in their latency to fall from the rotarod between the last trial of each day of training and the first trial of the next. These intersession performance deficits suggest that the hippocampus may be involved in the consolidation of the physical skills necessary to perform the rotarod procedure. Of course, future research is necessary to explore this possibility. Minimally, we can predict that neurons within the hippocampus are at least engaged by this form of physical skill learning, because acquisition of this skill rescued new hippocampal cells from death.

Processes of learning that do not require the hippocampus still engage neurons within this structure (Christian & Thompson, 2003). For example, acquisition of the delay eyeblink response does not require an intact hippocampal formation (Beylin et al., 2001), however this form of learning does increase the firing frequency of neurons within

areas CA1, CA3, and CA4 of the hippocampus, as well as within the granule cell layer of the dentate gyrus (Berger, Alger, & Thompson, 1976). This increased hippocampal activity forms a "model" of the animal's conditioned response during training. The increased activity begins early in training, and it is present after just a few trials. On each trial this hippocampal activity typically parallels, and precedes, the conditioned response by 25-35ms. This neural activity increases progressively over the course of training. As animals learn the conditioned response this increased neural activity becomes more and more prevalent. Animals trained with delay eyeblink conditioning display this increase in hippocampal activity, however those trained with unpaired stimuli do not. Therefore, this increase in hippocampal activity does not simply result from the presentation of the conditioned or unconditioned stimulus. Nor does this increased neural activity result from the animal's performance of the unconditioned response. Instead, this activity reflects acquisition of the association between the conditioned and the unconditioned stimuli. Therefore, this hippocampal-independent form of delay conditioning can still alter neural activity within the hippocampal formation. It is possible that other forms of hippocampal-independent learning may also alter neural activity within the region, and this activity may influence the number of new hippocampal cells that survive. Exactly how other forms of learning would alter neural activity is unknown, however several studies have revealed that learning can influence both widespread rhythmic neuronal activity, and the intrinsic electrophysiological properties of individual neurons within the hippocampal formation. Either, or both, of these learning-induced electrophysiological changes may influence the number of new cells that survive in response to learning.

Electrophysiological changes in response to learning may influence the number of cells that survive following that learning

Many forms of learning are associated with changes in oscillatory neural activity. One of these most well characterized is the theta oscillation, which occurs at a frequency 3-12Hz (Buzsáki, 2002). This theta rhythm reflects the synchronous firing of thousands of neurons. Within the hippocampus, and it can be modulated by learning. For example, during acquisition of a hippocampal-independent delay eyeblink conditioning procedure theta activity in the hippocampus increases (Nokia, Penttonen, Korhonen, & Wikgren, 2009; Nokia & Wikgren, 2010). It is possible that this increase in theta activity during learning may engage immature hippocampal cells, promoting their survival (Shors et al., 2012). Recently, we tested this this hypothesis by training animals to learn a novel association. Following acquisition of this initial task the animals were then trained to learn a similar but different association. Animals acquired both associations, and acquisition of the first task greatly facilitated acquisition of the second, similar, task. Because of this facilitated acquisition during training with the second task, hippocampal theta activity increased during only acquisition of the first task. No increase in theta activity was observed during acquisition of the second task. Even though acquisition of the second task did not alter hippocampal theta activity, this learning still increased the number of surviving cells. Therefore, a learning induced increase in theta activity is not necessary for learning to rescue these new neurons from death. Of course, other forms of oscillatory neural activity also occur in the hippocampus. For example, low frequency rhythms (Sirota & Buzsáki, 2005), moderate frequency gamma rhythms (Lisman, 2005), and high frequency ‘ripples’ (Chrobak & Buzsáki, 1996; Chrobak, Lörincz, & Buzsáki,

2000) all occur in the hippocampal formation, and these neural rhythms are also associated with learning and memory. It is possible that learning may influence cell survival by altering these rhythms; however, figure research is necessary to explore this possibility.

Learning alters the oscillatory activity of cell assemblies within the hippocampal formation; however, it can also alter the electrophysiological properties of individual hippocampal neurons. These intrinsic electrophysiological changes may influence the number of hippocampal cells that survive following learning. Normally, following a sustained period of stimulation pyramidal neurons in area CA1 of the hippocampus will express a slow afterhyperpolarization (sAHP), which can last for up to two seconds. This sAHP makes these cells less excitable, and less likely to fire in response to additional stimulation. Essentially, the sAHP serves to limit the number of action potentials that a cell can discharge in response to prolonged stimulation (Madison & Nicoll, 1984). When the amplitude of the sAHP is large comparatively few action potentials can be discharged. However, when the amplitude is small more action potentials can be fired in response to the same stimulation. During acquisition of various associative (Moyer, Thompson, & Disterhoft, 1996), contextual (Kaczorowski & Disterhoft, 2009), and spatial memories (Oh, Kuo, Wu, Sametsky & Disterhoft 2003) the amplitude and the area of the sAHP in CA1 pyramidal neurons is reduced. Essentially, these cells become more excitable simply as a result of learning. For example, following successful acquisition of trace eyeblink conditioning the sAHP from CA1 pyramidal cells is reduced, resulting in these cells becoming more excitable. This increase in excitability is observed only in those animals that successfully learn the trace conditioning task. Hippocampal neurons

from animals that fail to learn, or those are trained with unpaired stimuli, do not display this increase in intrinsic excitability. Importantly, this increase in excitability is transient. It occurs only during acquisition of a memory, and it only persists for several days following acquisition (Disterhoft & Oh, 2006). These sustained increases in hippocampal excitability may influence the number of immature neurons that survive following learning. Because this increase in neuronal excitability is observed only in those animals that successfully learn, and because it is transient, this increase neural excitability may explain why learning has its greatest effect on cell survival when that learning requires many trials/and or days to master. The new hippocampal neurons in animals that require more trials (or days) to learn a task would presumably be exposed to a longer period of excitability than those in the hippocampus of animals that quickly learn, or that fail to learn. This would explain why we repeatedly observe strong positive correlations between the number trials required for an animal to learn a task, and the number of surviving cells in that animal's dentate gyrus. Exactly how this period of increased neural excitability may influence the survival of these cells is unknown, although various neurotransmitter and neurotrophin systems may mediate this effect.

Neurotransmitter systems implicated in mediating the effect of learning on neurogenesis

The mechanism(s) through which learning rescues new neurons is unknown, partly because we do not fully understand the mechanisms underlying learning itself. One potential mediator of the effect of learning on neurogenesis is gamma-aminobutyric acid (GABA). Activation of the GABA receptor typically has an inhibitory effect on neurons.

This GABAergic activity activates ligand-gated chloride channels, causing chloride ions to enter and hyperpolarize the cell. During the development of the hippocampal formation immature hippocampal neurons are actually depolarized by GABAergic stimulation (Owens & Kriegstein, 2002). This depolarization occurs because these immature hippocampal neurons have a much higher intracellular concentration of chloride than their mature counterparts. As such, when GABA binds to these immature cells it typically causes chloride ions to leave these cells, and the net effect is the depolarization of the cell. During development this GABAergic depolarization has a trophic effect on immature hippocampal cells, increasing the likelihood that they will survive (Owens & Kriegstein, 2002). Immature neurons generated in the adult-hippocampal formation also depolarize in response to GABAergic input. During the first two weeks of their development these adult-born hippocampal neurons receive depolarizing GABAergic input from local interneurons. This depolarization causes neuronal progenitor cells to adopt a neuronal fate (Tozuka, Fukuda, Namba, Seki, & Hisatsune, 2005). Instead of adopting glial or astrocytic fates more of these new cells become neurons. This GABAergic depolarization also facilitates the functional and morphological maturation of these immature neurons. Converting the GABA induced depolarization to a hyperpolarization impaired the synaptic and dendritic development of these new cells (Ge et al., 2006). Together, these results suggest that local GABAergic activity can promote the differentiation and development of these new neurons, and it is possible that learning may influence the survival of these cells by altering this GABAergic activity.

Activation of the *N*-methyl d-aspartate (NMDA) receptor can also modulate the effect of learning on neuronal survival. NMDA receptor activation is necessary for trace eyeblink conditioning (Curlik & Shors, 2011; Leuner, Falduto, & Shors, 2003), as well as most types of spatial navigation learning including the hidden platform version of the Morris water maze (Bannerman, Good, Butcher, Ramsay, & Morris, 1995). Moreover, the survival of two-week old hippocampal neurons requires activation of NMDA receptors (Tashiro, Sandler, Toni, Zhao, & Gage, 2006). We recently examined the effects of NMDA receptor antagonism on cell survival after learning. Antagonism of these receptors each day before training prevented learning, and thereby prevented the increase in cell survival. Antagonism each day after training did not prevent learning, but also did not prevent the increase in cell survival (Curlik & Shors, 2011). In line with our results, another recent study reported that infusions of an NMDA antagonist during the training period disrupted acquisition of a delayed matching-to-place task, and abolished learning-induced changes in neurogenesis (Tronel et al., 2010). We have also used a cognitive enhancing drug, d-cycloserine, to enhance activity of NMDA receptors during learning (Curlik & Shors, 2011). In this case, more animals learned, and as a consequence, more cells were rescued from death. Although these data once again associate the increase in cell survival with learning and not training, they do not indicate that NMDA receptor activation is *necessary* for the increase in cell survival. Clearly, it is important to identify the mechanisms that keep new neurons alive after learning. However, this is no easy task. Most of the likely candidates—GABA, NMDA, BDNF (Brain-derived neurotrophic factor), Ach (acetylcholine), etc.—are intimately tied into learning. As such, it is difficult to manipulate or eliminate one without affecting the

other. Of course, this is how a unified system works—one component cannot occur without the other. They are positively related to one another and oftentimes feedback on each other.

A functional role for adult born neurons

Over the past decade, there has been increasing interest in the role that new neurons actually play in the instantiation of learning. The first study to report such a relationship took advantage of an antimitotic agent known as MAM, which significantly reduces the number of new cells that are produced across time. After several weeks of treatment (but in the absence of the drug), we observed a select deficit in learning to associate events across time (Shors et al., 2001), an effect that has been replicated using another antimitotic known as TMZ (Nokia, Anderson, Shors, submitted). Since then, other techniques have been developed to reduce cell numbers, including focused irradiation or genetic manipulations (Reviewed in Shors et al., 2012). Of these treatments anti-mitotics tend to be the easiest to administer, being delivered via intraperitoneal injections, or osmotic pumps. As such, these compounds are not target specific, and can interfere with the proliferation of cells in other areas of the CNS and the periphery. Focused irradiation, on the other hand, can target precursor cells in the hippocampus, but it requires specialized equipment which is not readily available. Moreover, irradiation can lead to inflammation, especially in the immediate weeks after treatment. Genetic techniques can specifically target neuronal precursor cells, but they can have side effects, which impair aspects of performance unrelated to learning. Also, the genetic targets may have multiple functions, or be involved in other physiological processes, independent of

their role in regulating those involved in neurogenesis. Therefore, evaluating how these new neurons contribute to learning and memory is difficult, and is probably best done by examining the learned responses following several different ablation techniques, in independent groups of animals and laboratories. These converging operations would help to ensure that any behavioral changes are due to a reduction in hippocampal neurogenesis, and not merely side effects of the chosen ablation technique. These techniques have revealed that adult-born hippocampal cells are not only necessary for trace eyeblink conditioning. They also appear to be necessary for trace fear conditioning (Achanta, Fuss, & Martinez, 2009; Shors, Townsend, Zhao, Kozorovitskiy, & Gould, 2002), and for learning to distinguish patterns that are closely related to one another in space. (Clelland et al., 2009).

Although some forms of learning (such as trace conditioning and pattern separation) are especially sensitive to the loss of the new cells, others can be learned in their near absence. For example, when neurogenesis was disrupted, with either antimitotics or whole brain irradiation, animals could readily learn to associate stimuli that occur close together and/or overlap in time (delay conditioning). They could also learn to associate one stimulus with a context in which a fearful event occurs (contextual fear conditioning) (Achanta et al., 2009; Clark et al., 2008; Deng, Saxe, Gallina, & Gage, 2009; Kitamura et al., 2009; Shors et al., 2002). However, others have reported that disrupting neurogenesis impairs contextual learning (Hernández-Rabaza et al., 2009; Imayoshi et al., 2008; Winocur, Wojtowicz, Sekeres, Snyder, & Wang, 2006; Wojtowicz, Askew, & Winocur, 2008). These discrepancies may be due to the fear conditioning protocols used, or to species differences (Snyder, Choe, et al., 2009; See Shors et al.,

2012 for review). These new neurons also do not appear to be necessary for acquisition of the spatial Morris water maze procedure (Dupret et al., 2008; Goodman et al., 2010; Jaholkowski et al., 2009; Jessberger et al., 2009; Madsen, Kristjansen, Bolwig, & Wörtwein, 2003; Saxe et al., 2006; Shors et al., 2002; Snyder, Hong, McDonald, & Wojtowicz, 2005).Overall, the current data suggest that the new cells are most engaged when the task demands are high and mastering them depends on cognitive flexibility (Burghardt, Park, Hen, & Fenton, 2012; Shors et al., 2012)**.**

Are more new neurons better?

We often assume that more is better, and this includes new neurons. Even though manipulations that effectively disrupt neurogenesis can impair learning, the number of proliferating cells in healthy animals (i.e. those with "normal" levels of neurogenesis) does not necessarily relate to how well those animals learn. For example, the number of proliferating cells in an individual animal's dentate gyrus does not predict the amount of learning that will occur in the future in that animal (Bizon & Gallagher, 2005; Nokia, Sisti, Choksi, & Shors, 2012). Of course, it remains conceivable that some neurogenic manipulation could increase cell proliferation beyond the normal range of individual differences, and thereby influence the potential for learning in the future. Several experiments have examined this question. In one study, animals increased their cell numbers by nearly 70% by living in an enriched environment. Afterwards, they were able to learn and remember a novel object for longer periods of time. The increase in memory was reportedly related to the increase in cell number because it did not occur when the new cells were not produced (Bruel-Jungerman, Laroche & Rampon 2005). In another

study, neurogenesis was increased by allowing animals to exercise in activity wheels for approximately two months (Clark et al., 2008). Again, the animals learned a new task faster (the Morris water maze). However, when neurogenesis was disrupted the exercise no longer facilitated learning.

What if a multitude of new neurons were produced in an otherwise healthy individual -- would this increase learning? A recent study addressed this question directly. A genetic manipulation was developed, which nearly doubled the number of surviving neurons in the adult dentate gyrus. The animals performed within the normal range on tests of spatial navigation learning, contextual fear conditioning, and novel object recognition, but they were better able to distinguish between two highly similar overlapping contexts (Sahay, Scobie, et al., 2011). These data are consistent with the results of another study, which revealed that voluntary exercise could increase cell production, and also facilitate spatial pattern separation (Creer et al., 2010). Together, it appears that interventions that increase neurogenesis can facilitate important processes related to learning and memory, including pattern separation (Sahay, Wilson, & Hen, 2011), memory resolution (Aimone, Deng, & Gage, 2011), timing and/or cognitive flexibility (Burghardt et al., 2012; Shors et al., 2012). Again, it would appear that tasks that require some degree of cognitive effort and/or discriminatory skill are the most likely to be dependent on and even facilitated by the presence of these new neurons.

Importantly, not all cognitive enhancements that occur as a result of physical exercise or mental training are due to the production or presence of new neurons. For example, increases in proliferation as a result of exercise did not critically contribute to increases in motor skill learning or contextual fear conditioning (Clark et al., 2008). Nor

did the enhanced spatial learning after environmental enrichment depend on their presence (Meshi et al., 2006). More directly, and as noted, genetically increasing the number of cells did not noticeably alter simple spatial, contextual, or novel object recognition learning (Sahay, Scobie, et al., 2011). Overall, these results suggest that both mental and physical training may facilitate future learning, and some of these effects are potentially mediated by increases in adult neurogenesis. Because exercise and learning excerpt widespread changes on the brain's structure and function, it is highly unlikely that changes to this one process, or any process for that matter, account for all the observed changes in cognition following training.

Human implications

Whereas the majority of studies on adult hippocampal neurogenesis have been performed in rodents, several studies indicate the presence of new neurons in the human hippocampus. The first of these studies was performed by Eriksson and colleagues (1998). In this study researchers performed post-mortem examinations of human brain tissue from cancer patients that received BrdU to track the growth of their cancer. BrdU+ cells were observed in both the dentate gyrus and the olfactory bulb. Several of the BrdU+ cells in the dentate gyrus also expressed neuronal markers, indicating that new neurons are produced in the human hippocampus throughout life. A more recent study has confirmed these findings, using both BrdU labeling, and a novel carbon dating technique in post mortem tissue (Bhardwaj et al., 2006). These results clearly reveal that the human hippocampus incorporates new neurons throughout adult life. However, the development of these new cells in humans may differ from their development in rodents.

One recent study of macaque monkeys revealed that new hippocampal neurons take more than six months to fully mature (Kohler, Williams, Stanton, Cameron, & Greenough, 2011). For comparison, new neurons generated in the rat hippocampus fully mature after four weeks (Brown et al., 2003; Kempermann, Gast, Kronenberg, Yamaguchi, & Gage, 2003; McDonald & Wojtowicz, 2005). While this study was conducted in macaques it is likely that adult-born neurons in the human hippocampus display a similarly long maturation time.

To our knowledge, no studies have directly assessed the effects of learning on neuronal survival in adult humans. The problem here is that it is not yet possible to precisely assess the number of newborn cells as they are produced within the living human hippocampus. One recent study attempted to sidestep this technological limitation by examining cerebral blood volume, a neural correlate of neurogenesis, in mice and humans (Pereira et al., 2007). The study began by demonstrating a relationship between dentate gyrus cerebral blood volume and neurogenesis in mice, as well as an increase in both measures after exercise. When neurogenesis was impaired, via irradiation of the dentate gyrus, exercise no longer increased neurogenesis; nor did it increase cerebral dentate gyrus cerebral blood volume. Based on these data, the researchers suggested that exercise-induced increases in dentate gyrus cerebral blood volume can be considered a neural correlate of exercise-induced neurogenesis in humans. The researchers then went on to study these relationships in healthy humans, except for the fact that they were sedentary. The subjects engaged in twelve weeks of physical training, with four one-hour sessions of aerobic exercise per week. This exercise program greatly increased cerebral blood volume in the dentate gyrus, where the new neurons are generated. Furthermore,

each individual's change in dentate gyrus cerebral blood volume correlated with the change in their maximum oxygen consumption after training. The participants also expressed a facilitation in the initial acquisition of a declarative memory, suggesting that exercise improves declarative learning via an increase in neurogenesis in healthy human beings (Pereira et al., 2007). However, these results do not demonstrate a precise relationship between exercise and neurogenesis, and once it is possible to assess adult neurogenesis in human subjects future research will be necessary to confirm that manipulations that alter neurogenesis in rodent models also do so in humans.

Conclusions

Overall, our present results indicate that acquisition of a difficult physical skill can increase the number of surviving cells in the adult hippocampus, provided that the skill is successfully learned. Although technical challenges currently prevent the assessment of neurogenesis in humans while they are learning, our results suggest that learning a complex physical skill, such as a new dance, or sport, may increase the number of neurons in the adult hippocampus. An increase in neuronal content likely prepares the brain for future learning experiences, and may even reduce some of the cognitive decline that occurs during aging and/or the onset of Alzheimer's disease.

LIST OF ABBREVIATIONS

ACh……...……acetylcholine

BDNF…….……...brain-derived neurotrophic factor

BrdU……. bromodeoxyuridine

CNS……...……central nervous system

GABA……..…...*gamma*-Aminobutyric acid

GFAP……...…….. glial fibrillary acidic protein

IGF-1……..insulin-like growth factor 1

i.p…….……...……intraperitoneal

MAM…….… methylazoxymethanol acetate

NeuN……..……. neuron specific enolase

NMDA……..*N*-Methyl-D-aspartic acid

O4…….……oligodendrocyle cell surface marker number 4

RPM…….……..rotations per minute

sAHP…….…….slow afterhyperpolarization

s.e.m. ……...……....standard error of the mean

TOAD-64…… turned on after division 64

TMZ……...…….……temozolomide

VEGF……..vascular endothelial growth factor

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Figure 1

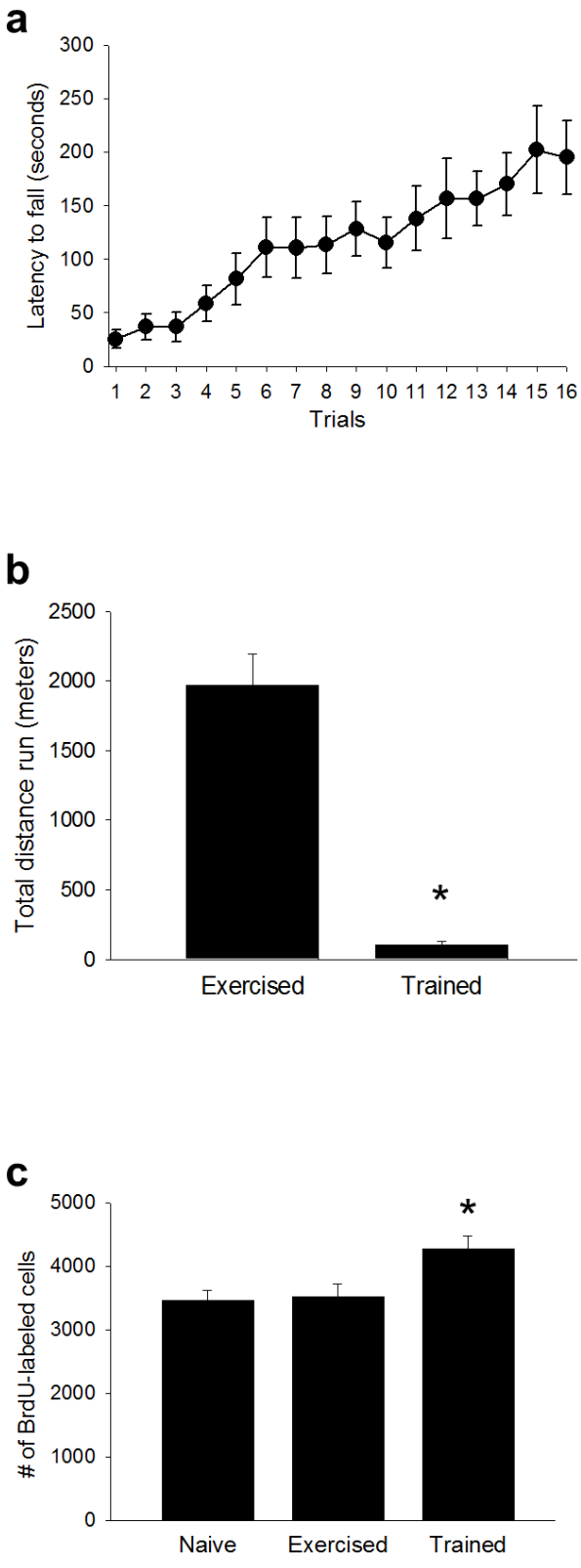
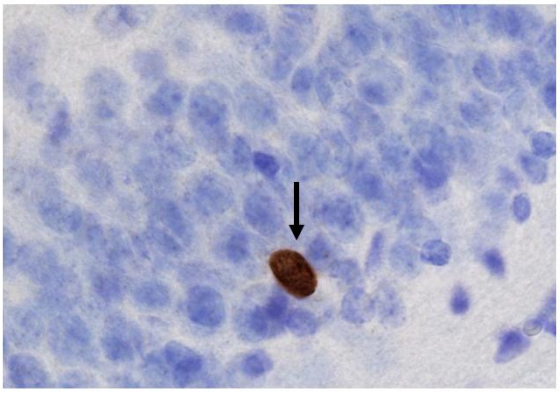

a
Latency to fall (seconds)
300
250
200
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Trials
b
Total distance run (meters)
2500
2000
1500
1000
500
0
*
Exercised
Trained
c
# of BrdU-labeled cells
5000
4000
3000
2000
1000
0
*
Naive
Exercised
Trained

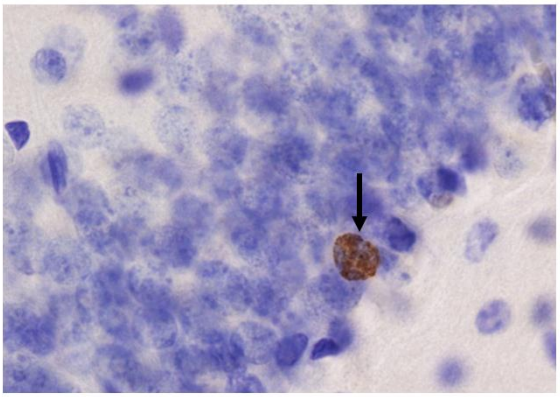
Figure 1: (A) Trained animals increased their latency to fall from the accelerating rotarod during training. (B) Skill learning required minimal physical activity. The group given access to activity wheels (exercised) ran nearly twice as far as animals did during accelerating rotarod training (trained). (C) Skill learning, but not exercise, rescued cells from death. The trained group retained more new cells than the naïve group, and the exercised group. Despite engaging in a large amount of physical activity the group given access to activity wheels did not retain any more cells than naïve animals. Data are from Experiment #1, and represent the mean ± s.e.m. Asterisks indicate $p \leq 0.05$.

Figure 2

Naive



Exercised



Trained

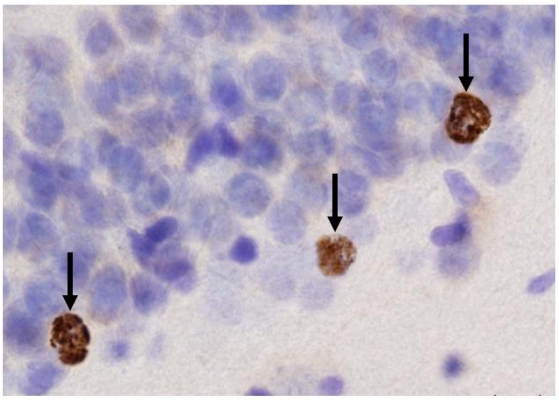


Figure 2: Representative BrdU-labeled cells from Experiment #1 from the naïve, exercised or trained conditions. Arrows represent BrdU-labeled cells.

Figure 3

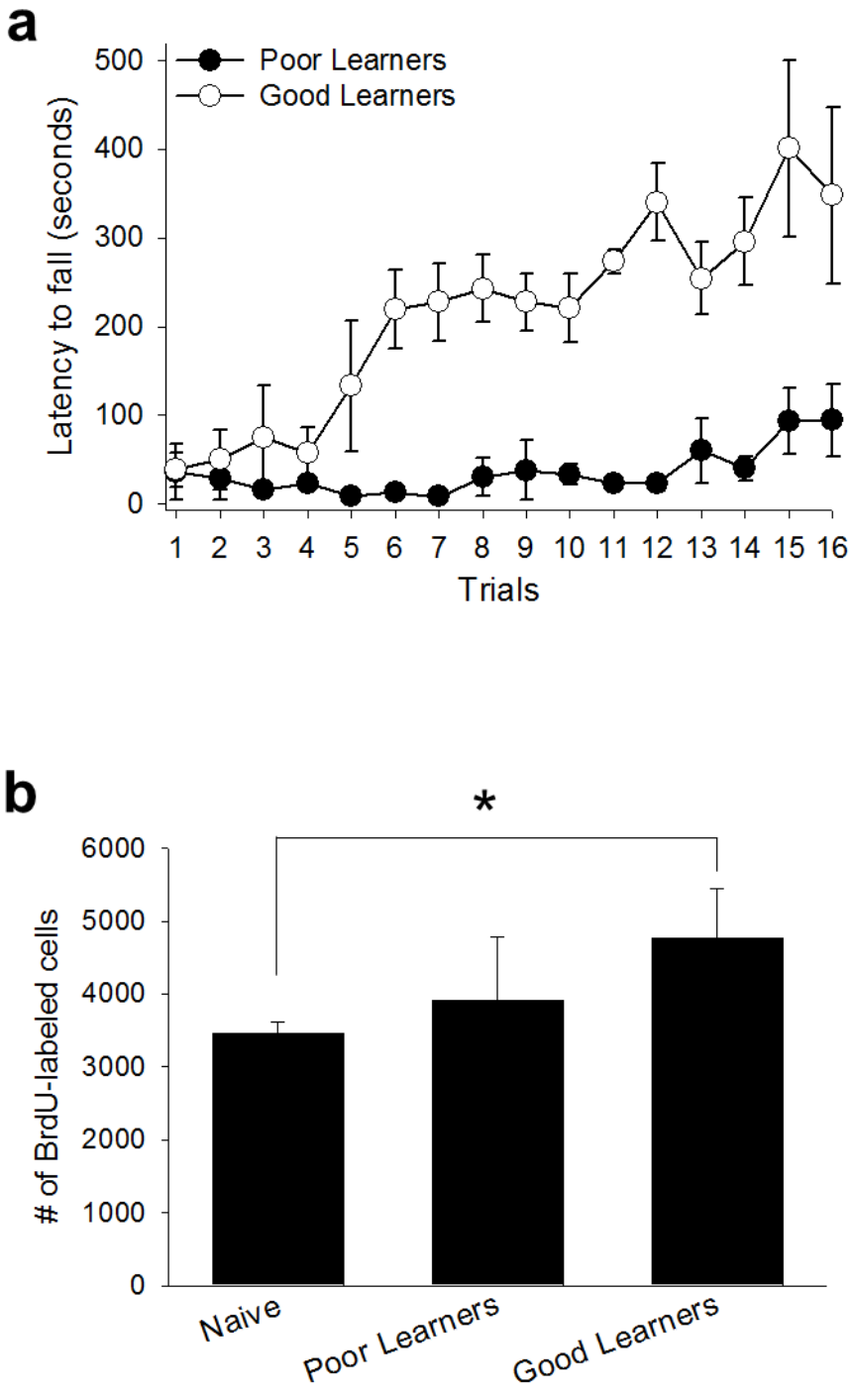

a
Poor Learners
Good Learners
Latency to fall (seconds)
0
100
200
300
400
500
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Trials
b
*
# of BrdU-labeled cells
0
1000
2000
3000
4000
5000
6000
Naive
Poor Learners
Good Learners

Figure 3: (A) The good learners consistently remained on the accelerating rotarod for a longer period of time than the poor learners. (B) The good learners retained more new cells than naïve animals, whereas the poor learners did not. Data are from Experiment #1, and represent the mean ± s.e.m. Asterisk indicates $p \leq 0.05$.

Figure 4

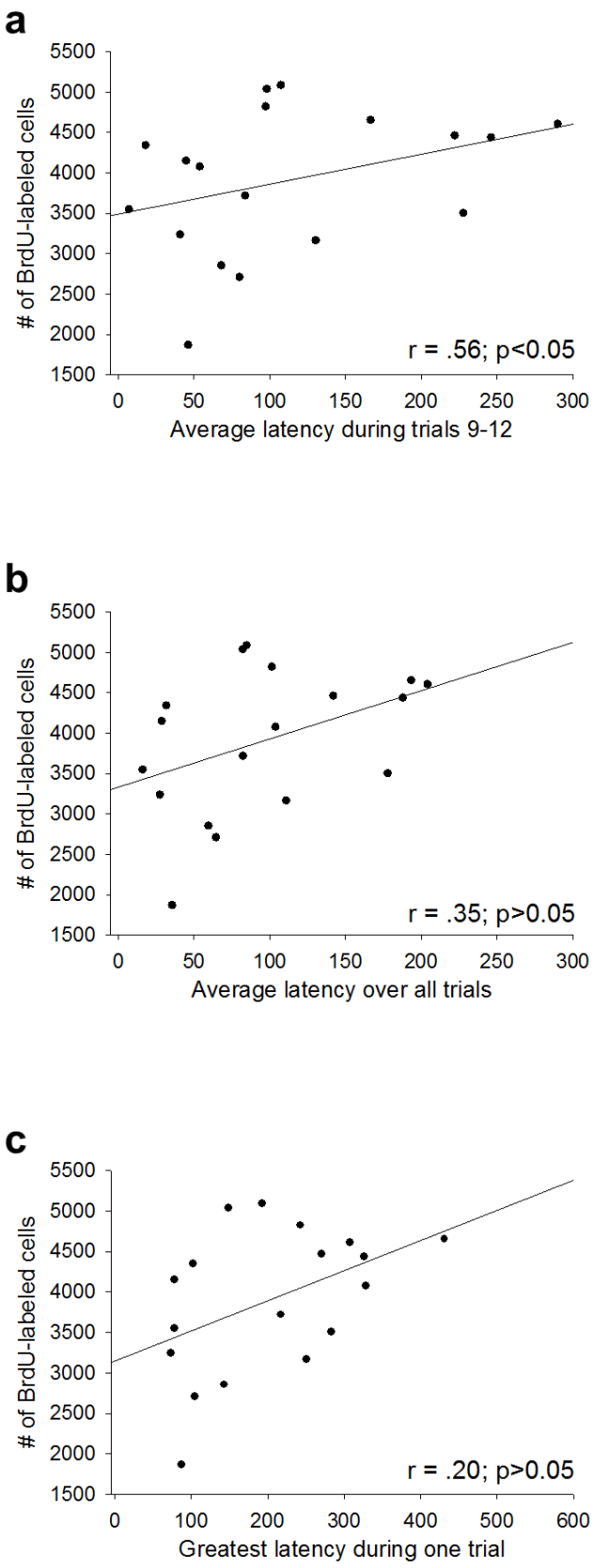

a
# of BrdU-labeled cells
5500
5000
4500
4000
3500
3000
2500
2000
1500
0
50
100
150
200
250
300
Average latency during trials 9-12
r = .56; p<0.05
b
# of BrdU-labeled cells
Average latency over all trials
r = .35; p>0.05
c
# of BrdU-labeled cells
0
100
200
300
400
500
600
Greatest latency during one trial
r = .20; p>0.05

Figure 4: Correlations between behavioral performance and the number of surviving BrdU+ cells in the group trained with the accelerating procedure. (A) A significant correlation was observed between performance on the third day of training and the number of surviving BrdU-labeled cells. (B) The average performance over all four days of training, and (C) the greatest latency to fall from the rotarod during any one trial did not significantly correlate with the number of surviving cells. Data are from Experiment #1.

Figure 5

Key:

○ = mature neuron

● = immature neuron

⊗ = dead neuron

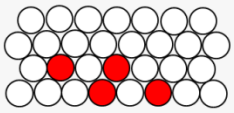
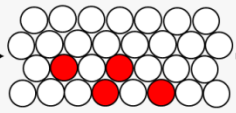
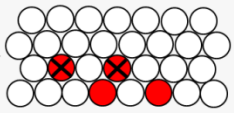
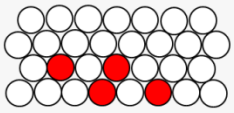
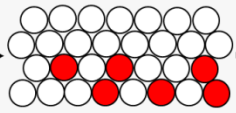
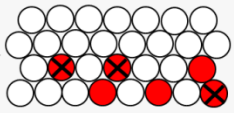
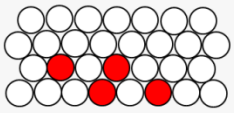
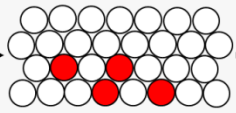
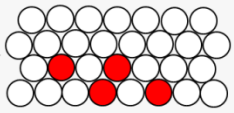
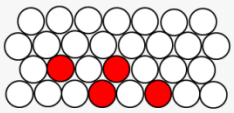
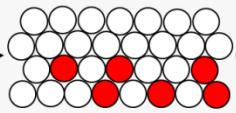
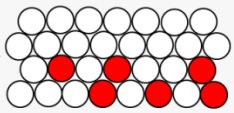
	Before	During	After
No training			
Physical exercise			
Mental training			
Mental and Physical (MAP) training			

Figure 5: Physical exercise greatly increases the number of new neurons produced during training (van Praag, et al., 1999; van Praag, 2009), and mental training increases the numbers that survive after training (Gould et al., 1999; Shors et al., 2012). In principle, a combination of both mental and physical training should be more effective than either training approach alone, increasing the overall number of neurons that survive to become mature functioning neurons in the adult brain (Fabel et al., 2009; Curlik & Shors 2012).

Figure 6

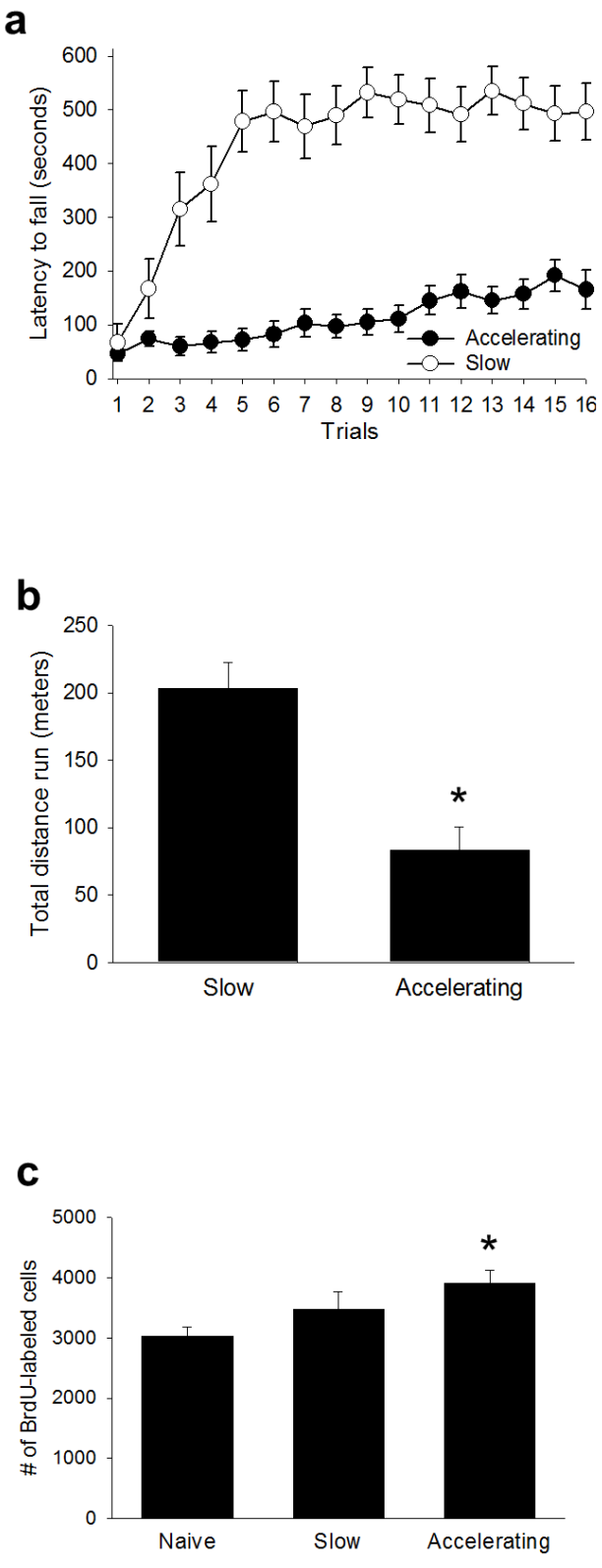

a
Latency to fall (seconds)
600
500
400
300
200
100
0
Accelerating
Slow
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Trials
b
Total distance run (meters)
250
200
150
100
50
0
*
Slow
Accelerating
c
# of BrdU-labeled cells
5000
4000
3000
2000
1000
0
*
Naive
Slow
Accelerating

Figure 6: (A) Animals trained with the accelerating, or the slow rotarod, procedure increased their latency to fall from the rotarod during training. However, the group trained with the slow procedure remained on the rotarod for a much longer period of time than the group trained with the accelerating procedure. (B) Animals traveled twice as far during training with the slow procedure than they did during training with the accelerating procedure. (C) The group trained with the accelerating rotarod task retained more new cells than naïve animals, whereas the group trained with the slow task did not. Data are from Experiment #2, and represent the mean ± s.e.m. Asterisks indicate $p \leq 0.05$.

Figure 7

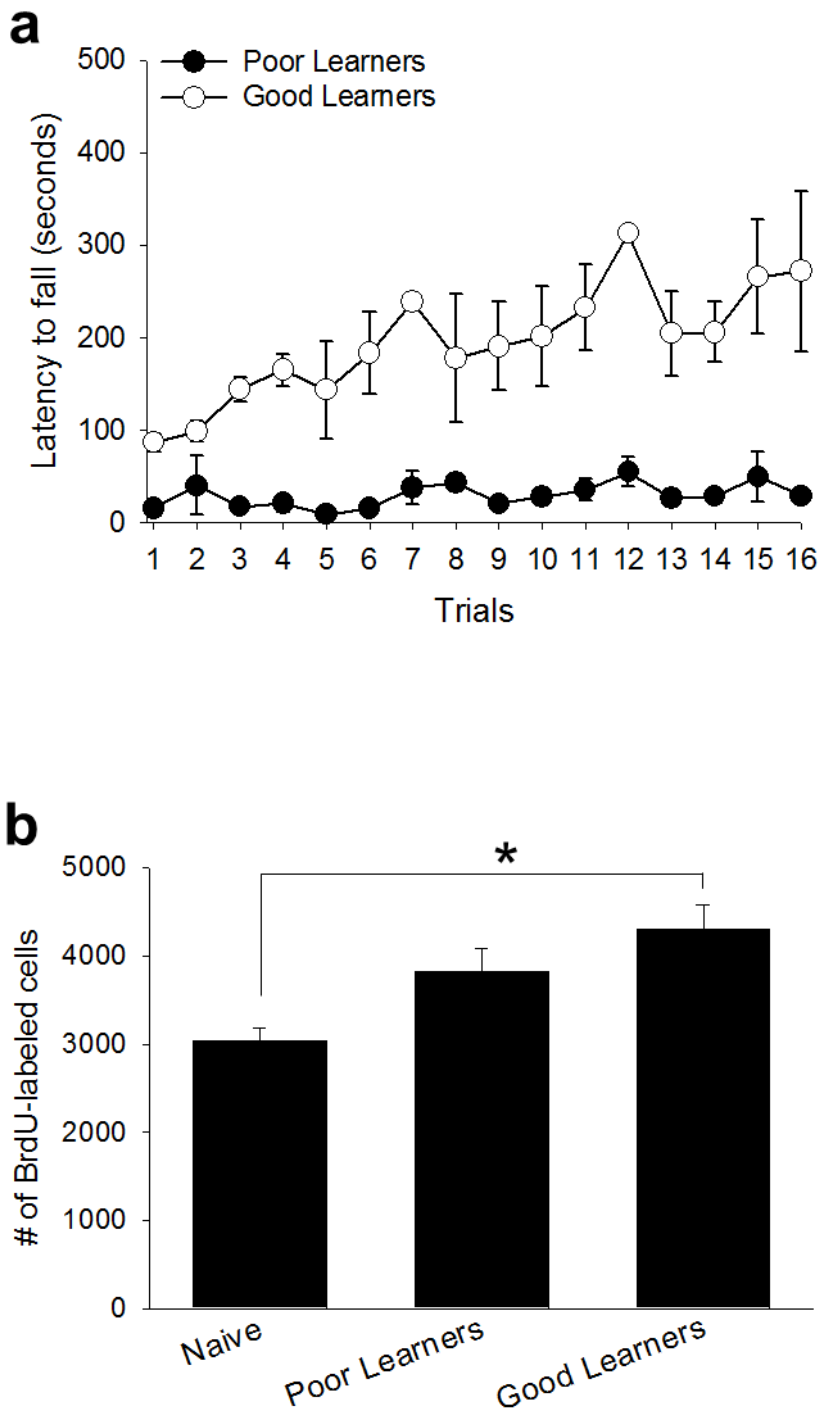
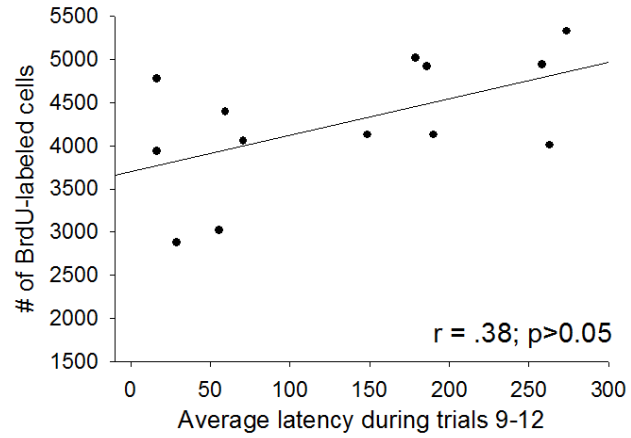
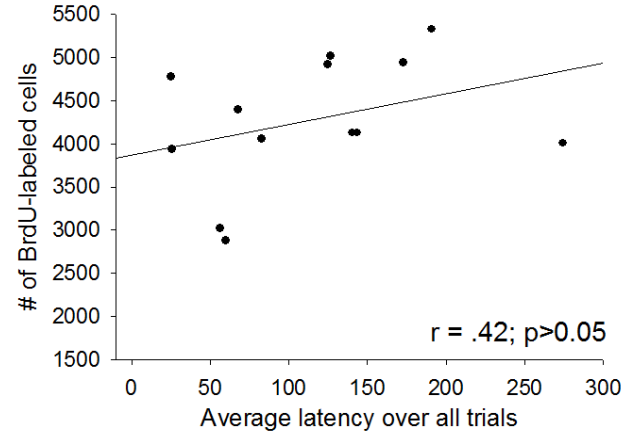

a
Poor Learners
Good Learners
Latency to fall (seconds)
500
400
300
200
100
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Trials
b
*
# of BrdU-labeled cells
5000
4000
3000
2000
1000
0
Naive
Poor Learners
Good Learners

Figure 7: (A) During training with the accelerating rotarod procedure the good learners outperformed the poor learners, (B) and the good learners retained more new cells than animals that were not trained. The poor learners displayed no such increase in cell survival. Data are from Experiment #2, and represent the mean ± s.e.m. Asterisk indicates $p \leq 0.05$.

Figure 8


# of BrdU-labeled cells
5500
5000
4500
4000
3500
3000
2500
2000
1500
0
50
100
150
200
250
300
Average latency during trials 9-12
r = .38; p>0.05

b


# of BrdU-labeled cells
5500
5000
4500
4000
3500
3000
2500
2000
1500
0
50
100
150
200
250
300
Average latency over all trials
r = .42; p>0.05

c

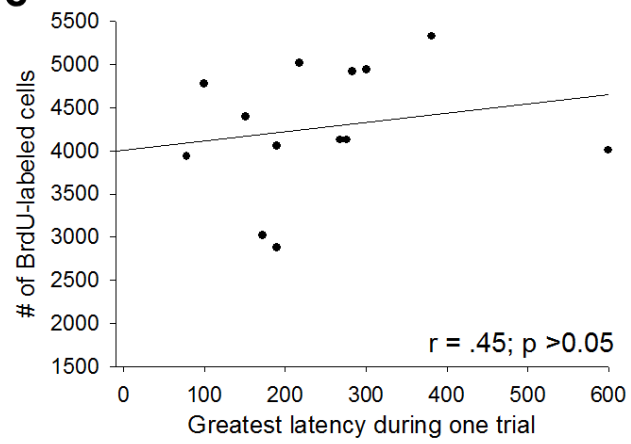
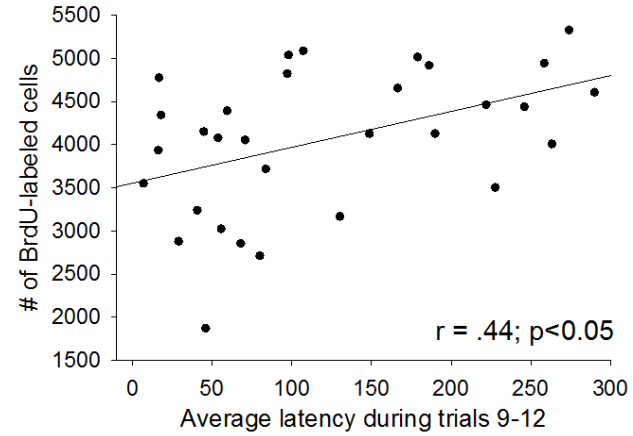
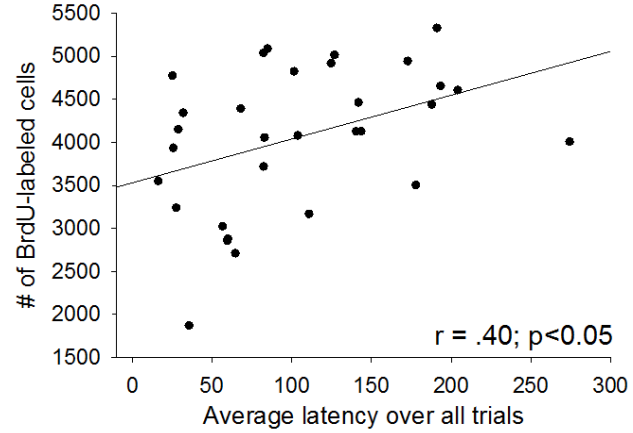

# of BrdU-labeled cells
5500
5000
4500
4000
3500
3000
2500
2000
1500
0
100
200
300
400
500
600
Greatest latency during one trial
r = .45; p >0.05

Figure 8: Correlations between behavioral performance and the number of surviving cells in the group trained with the accelerating rotarod procedure in Experiment #2. No significant correlations were observed. (A) The average latency to fall from the rotarod on the third day of training, (B) the average latency across all four days of training, and (C) each animal's greatest latency to fall from the rotarod during any one of the sixteen trials did not correlate with the number of surviving cells.

Figure 9


# of BrdU-labeled cells
5500
5000
4500
4000
3500
3000
2500
2000
1500
0
50
100
150
200
250
300
Average latency during trials 9-12
r = .44; p<0.05

b


# of BrdU-labeled cells
5500
5000
4500
4000
3500
3000
2500
2000
1500
0
50
100
150
200
250
300
Average latency over all trials
r = .40; p<0.05

c

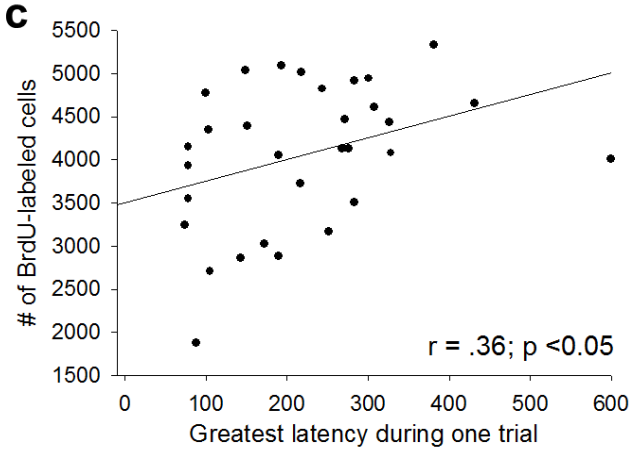

# of BrdU-labeled cells
5500
5000
4500
4000
3500
3000
2500
2000
1500
0
100
200
300
400
500
600
Greatest latency during one trial
r = .36; p <0.05

Figure 9: All of the correlations observed in Experiment #1 and Experiment #2 were in the positive direction. Therefore, we combined the data from both experiments and examined correlations between physical skill learning and cell survival in this larger sample. Significant correlations were observed between the number of surviving cells and (A) each animal's average performance on the third day of training, (B) their average performance across all four days of training, (C) and their greatest latency during any one of the sixteen trials.

Figure 10

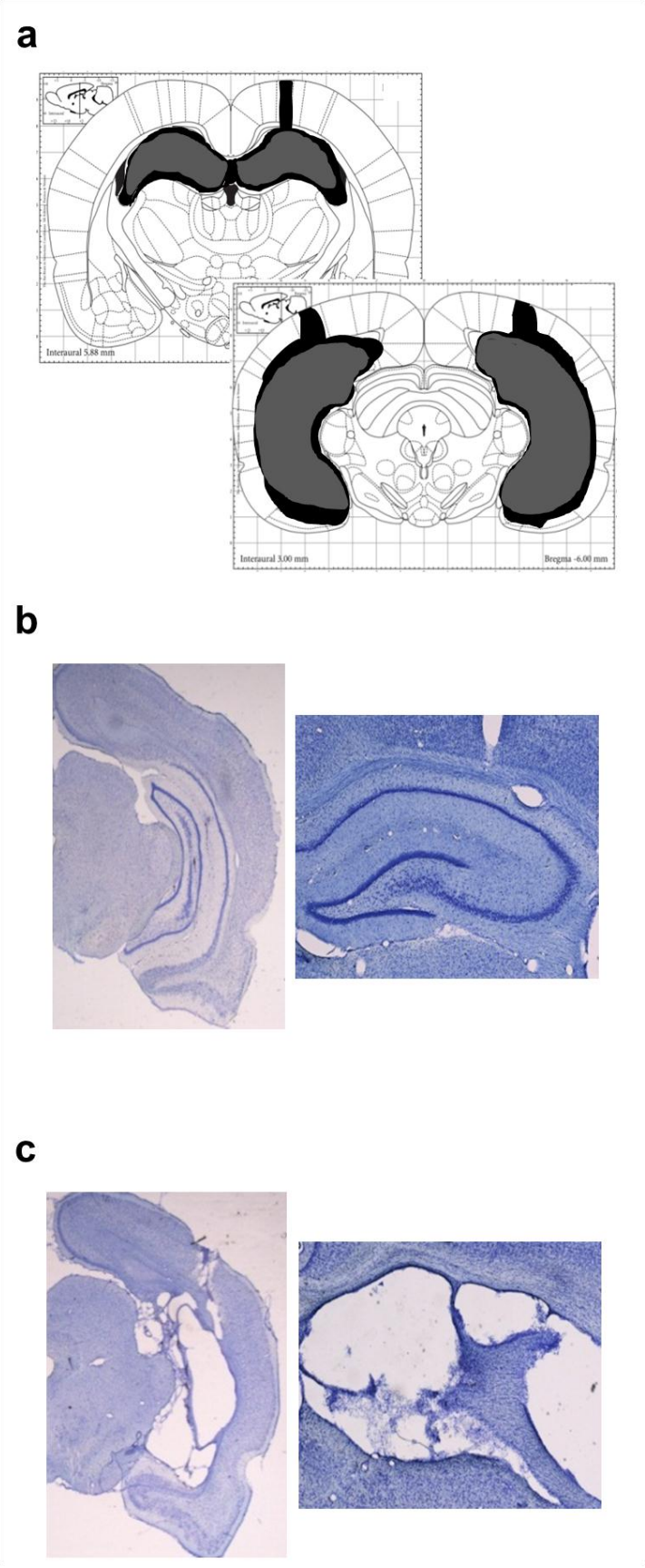


Figure 10: (A) Reconstructions of largest (black) and smallest (gray) lesions (Paxinos & Watson 2005). Representative histology from a (B) sham-operated animal and (C) a hippocampal lesioned animal from Experiment #3.

Figure 11

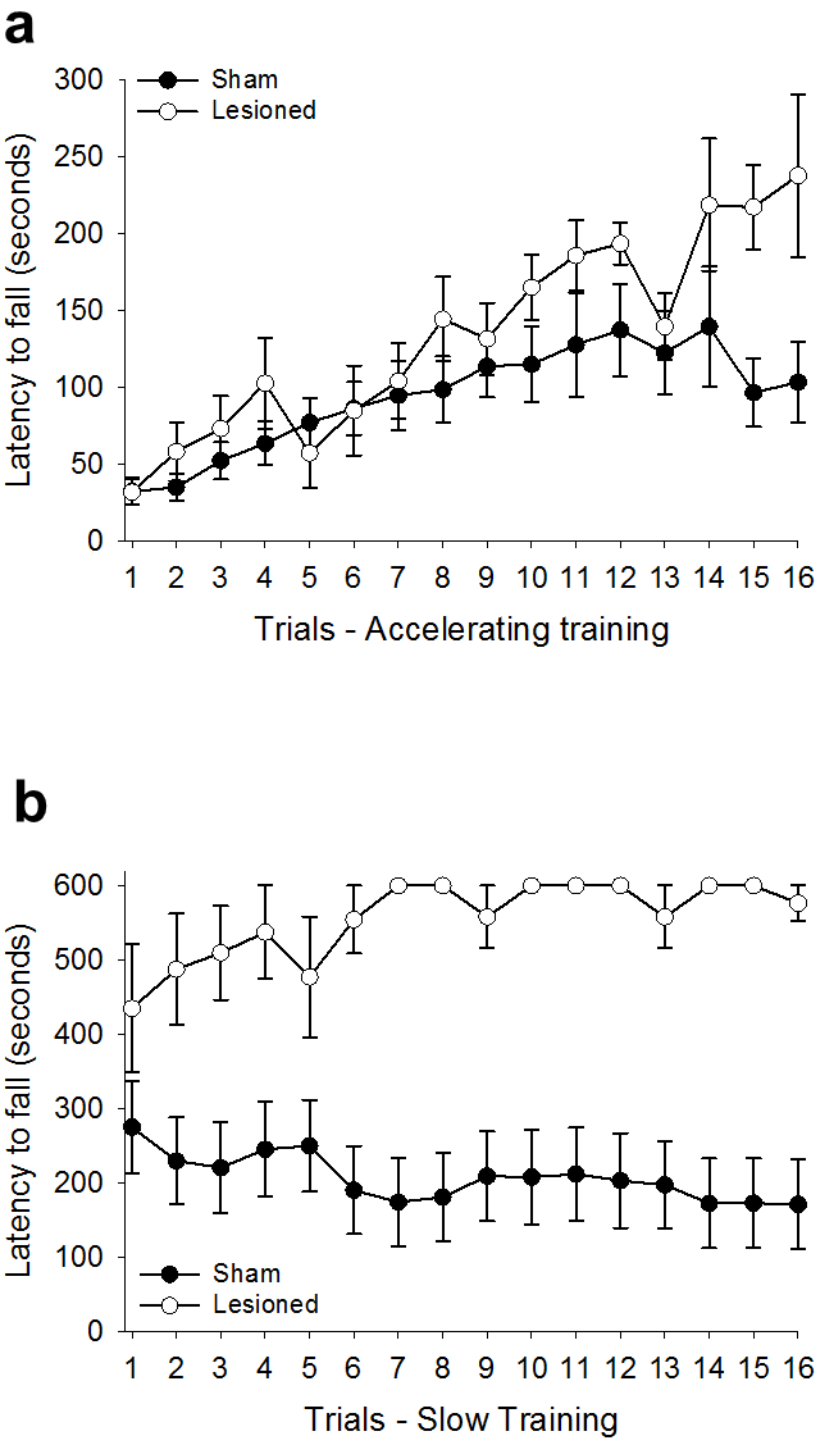

a
Sham
Lesioned
Latency to fall (seconds)
0
50
100
150
200
250
300
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Trials - Accelerating training
b
Latency to fall (seconds)
0
100
200
300
400
500
600
Sham
Lesioned
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Trials - Slow Training

Figure 11: (A) Animals with or without a functional hippocampus successfully learned the accelerating rotarod procedure. (B) During training with the slower version of the task the lesioned animals outperformed the sham animals. Data are from Experiment #3, and represent the mean ± s.e.m.

Figure 12

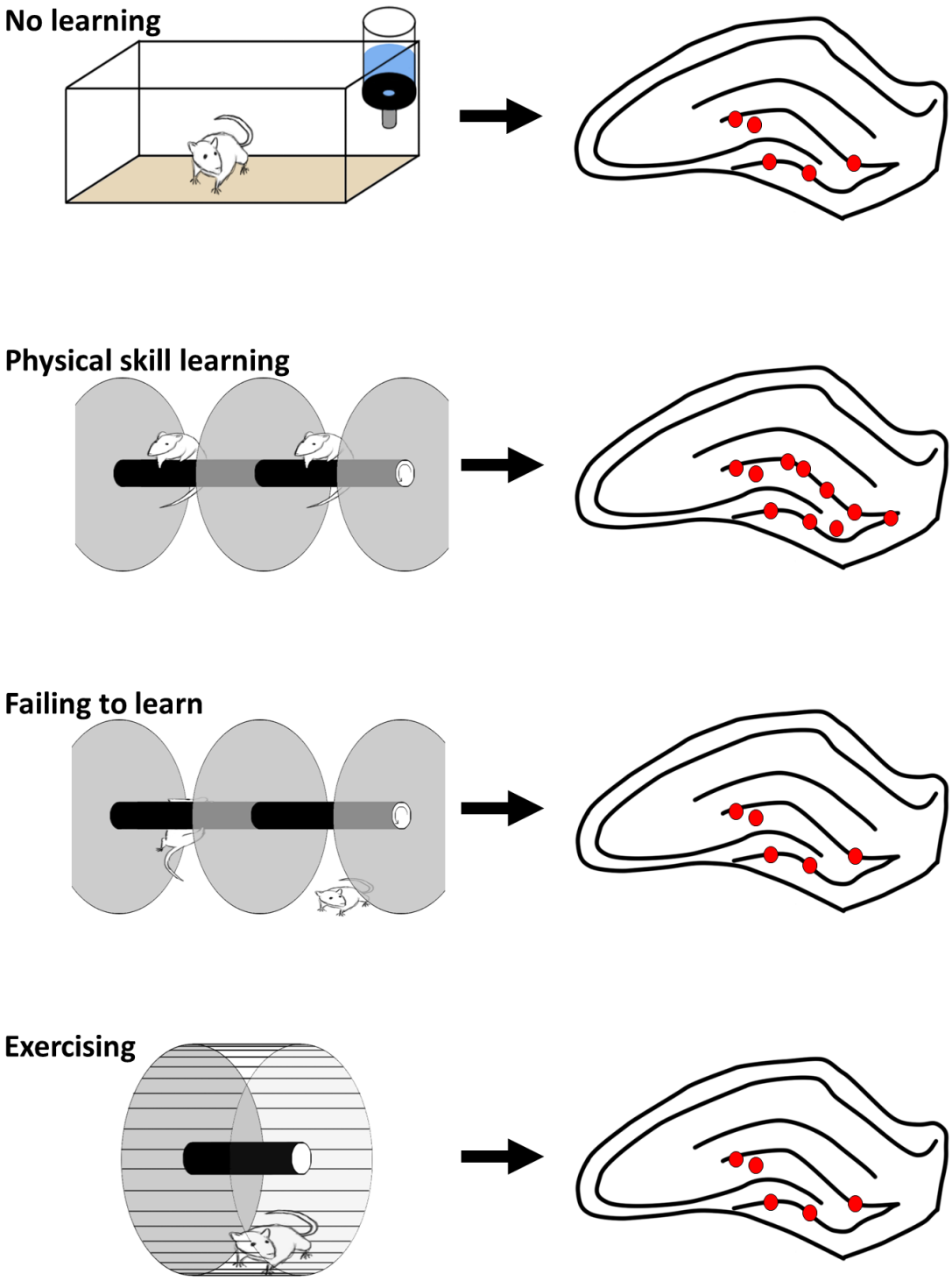

No learning
Physical skill learning
Failing to learn
Exercising

Figure 12: Animals that learned the physical skills necessary to remain on top of an accelerating rotarod displayed a large increase in the number of surviving hippocampal cells. This increase was observed only in animals that successfully acquired the skill. Those that failed to learn, or that learned poorly, displayed no such increase in cell survival. An additional group of animals were not trained on the rotarod. Instead, they were allowed to exercise in activity wheels. This voluntary exercise did not rescue cells from death. Together, these results suggest that skill learning, and not merely exercise, increases the number of surviving neurons in the adult hippocampus. (Red circles represent surviving immature hippocampal neurons).