Research Report

The motirod: a novel physical skill task that enhances motivation to learn and thereby increases neurogenesis especially in the female hippocampus

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ABSTRACT

Males and females perform differently on a variety of training tasks. In the present study we examined performance of male and female rats while they were trained with a gross motor skill in which they learn to maintain their balance on an accelerating rotating rod (the accelerating rotarod). During training, many animals simply step off the rod, thus terminating the training. This problem was addressed by placing cold water below the rod. We termed the new training procedure “motirod” training because the trained animals were apparently motivated to remain on the rod for longer periods of time. Groups of male and female adult Sprague-Dawley rats were trained on either the standard accelerating rotarod or the motirod for four trials per day on four consecutive days. Latency to fall from the rod (in seconds) was recorded. The motivating feature increased performance especially in females (p = .001). As a consequence of enhanced performance, females retained significantly more new cells in the dentate gyrus of the hippocampus than those trained on the accelerating rotarod or those that received no training. In addition, individuals that learned well retained more new cells, irrespective of sex or task conditions. Previous studies have established that new cells rescued from death by learning remain in the hippocampus for months and mature into neurons (Leuner et al., 2004a; Shors, 2014). These data suggest that sex differences in physical skill learning can arise from sex differences in motivation, which thereby influence how many new neurons survive in the adult brain.

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1. Introduction

In partnership with the Office of Research on Women’s Health (ORWH) the NIH has developed a strategic plan entitled A Vision for 2020 for Women’s Health Research. These policies were implemented in phases beginning in October 2014. The primary objective of this strategic plan is to increase awareness and gain a better understanding of conditions and diseases that specifically affect women’s health and quality of life. The plan will also benefit men, by encouraging rigorous research into the

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roles that sex and gender play in disease risk. With this change in policy we, as scientists, will confront a number of striking sex differences in our research programs and these differences will surface in perhaps unexpected ways. Over the past 15 years, our laboratory has reported a number of rather dramatic stress differences in rodent models of learning. For example, we reported that female rats can learn a classically conditioned eyeblink response faster than males. However, exposure to a stressful event can suppress the same type of learning in females while actually enhancing performance in males (Wood and Shors, 1998; Wood et al., 2001; Shors, 2001). However, during operant conditioning tasks, females often outperform males and do not express the performance deficits typically observed in males, a phenomenon referred to as “learned helplessness,” a putative model of depression (Dalla et al., 2008; Shors et al., 2007). These sex differences in performance and learning have consequences for numerous measures of plasticity and brain function. In the case of classical eyeblink conditioning, females retained more new neurons in their hippocampus than did males, simply as a result of sex differences in learning (Dalla et al., 2008). In the present experiment, we report yet another example of a sex difference in learning and performance. In this particular instance, the sex difference only emerges as the task demands change, thereby illustrating the importance of attending to sex differences while remaining aware of their dynamic response to changing experimental conditions.

Learning increases the number of new neurons in the adult brain by rescuing them from death (Shors, 2014). These cells are generated in the hippocampus of the adult brain. Under “normal” conditions, most of the new cells die, even before they have fully matured into neurons. Recently, we reported that acquisition of new physical skills required to perform the accelerating rotarod procedure increases the number of these cells that survive (Curlik et al., 2013). Despite the positive effects of rotarod training on cell survival, there were performance outcomes that diminished our enthusiasm for using this particular task. Specifically, many rats would simply step off the rod once it began to accelerate. Since there was no consequence for this behavior we hypothesized that the rats were not motivated to perform the task. To address this problem we placed a shallow pool of cold water under the rod. It was hypothesized that training with the addition of this condition (no training, rotarod, motirod) as the independent measure, and the number of surviving BrdU+ cells in the total dentate gyrus as the dependent measure. Results indicated a significant interaction between the number of BrdU+ cells and training condition ($F_{(2,28)}=15.32$, $p<0.001$; Fig. 2A). Post-hoc Tukey comparisons revealed that females trained on the motirod retained significantly more cells than females that were not trained ($p<0.001$) and those trained on the rotarod ($p<0.05$). Females trained on the rotarod retained more BrdU+ cells than the untrained females ($p<0.05$). These differences among the three female training conditions were observed in both the GCL ($F_{(2,28)}=10.27$, $p<0.001$) and the hilus ($F_{(2,28)}=5.47$, $p<0.05$). Taken together, these results suggest that with a main effect of trial ($F_{(15,529)}=25.91$, $p<0.001$) and training condition ($F_{(2,35)}=12.76$, $p<0.01$). Separate repeated-measures ANOVA’s conducted for each sex revealed main effects of trial for both males ($F_{(15, 240)}=10.39$, $p<0.001$) and females ($F_{(15,285)}=17.5$, $p<0.001$). These results suggest that both sexes increased their latency to fall from the rod as training progressed. Separate repeated measures analysis of variance indicated that the latency to fall from the rod as training progressed over the 16 trials in all four trained groups: male rotarod ($F_{(15, 135)}=5.14$, $p<0.001$), male motirod ($F_{(15,105)}=5.56$, $p<0.001$), female rotarod ($F_{(15,150)}=1.81$, $p>0.05$) and female motirod ($F_{(15,135)}=21.22$, $p<0.001$). These results suggest that each of the four groups successfully learned the motor skill task.

To detect sex differences in learning, we compared the performance of males versus females during training on the two tasks. Separate repeated-measures ANOVA’s revealed a significant interaction between trial and rotarod condition for females ($F_{(15,285)}=5.68$, $p<0.001$; Fig. 1D), but not for males ($F_{(15,240)}=0.72$, $p>0.05$; Fig. 1E). These results indicate that females trained on the motirod outperformed females trained on the rotarod. However, there was no difference in performance in males trained on the rotarod compared to the motirod. Therefore, the addition of the motivating stimulus had a pronounced enhancing effect on learning in females.

Body weights differed significantly between male and female rodents. To determine whether these differences accounted for the performance differences during training, we conducted a Pearson correlation analysis between individual body weights and latency to fall from the rod. There was no correlation between average latency to fall from the rod and body weight in females ($r = -0.22$, $p > 0.05$), males ($r = -0.47$, $p > 0.05$), or in both sexes combined ($r = -0.29$, $p > 0.05$). These results suggest that individual differences in body weight did not account for individual differences in performance during training with the motor skill tasks.

2.2. Neurogenesis: effect of training on cell survival in females

Animals were sacrificed three weeks following the BrdU injection, a time point when most cells that would have died would have already done so. The remaining cells tend to survive for at least several months. Analysis of cell survival was performed separately for males and females due to differences in volume and density of their hippocampi. In the first analysis, we examined the effects of training versus no training on the number of surviving cells in either sex. We used a one-way analysis of variance, with training condition (no training, rotarod, motirod) as the independent measure, and the number of surviving BrdU+ cells in the total dentate gyrus as the dependent measure. Results indicated a significant interaction between the number of BrdU+ cells and training condition ($F_{(2,28)}=15.32$, $p<0.001$; Fig. 2A). Post-hoc Tukey comparisons revealed that females trained on the motirod retained significantly more cells than females that were not trained ($p<0.001$) and those trained on the rotarod ($p<0.05$). Females trained on the rotarod retained more BrdU+ cells than the untrained females ($p<0.05$). These differences among the three female training conditions were observed in both the GCL ($F_{(2,28)}=10.27$, $p<0.001$) and the hilus ($F_{(2,28)}=5.47$, $p<0.05$). Taken together, these results suggest that...
Fig. 2 – Neurogenesis: effect of motirod training on cell survival. (A) Females trained on the motirod \((n=10)\) retained significantly more newly generated cells than females that were not trained \((n=10; p<.001)\) and females trained on the rotarod \((n=11; p<.05)\). Females trained on the rotarod retained significantly more cells than untrained females \((p<.05)\). (B) In males a one-way ANOVA revealed no significant differences among the three groups \((p>0.05)\). However, an independent samples t-test revealed that male rats trained on the motirod \((n=8)\) retained significantly more BrdU+ cells than naïve animals \((n=9)\). Asterisks indicate \(p<0.05\).
females that were trained with an accelerating rotarod task rescued more cells than untrained animals, and that when this task was modified to include a motivating factor the animals retained even more cells in the dentate gyrus. These results are consistent with a previous study which reported that acquisition of a motor skill not dependent on the hippocampus will nonetheless increase the number of cells that survive in the adult dentate gyrus (Curlik et al., 2013), and go beyond these data to indicate a more stressful and motivating task can further enhance the number of surviving cells. These data likewise agree with reports that more effortful training conditions tend to rescue the greatest number of new neurons (Dalla et al., 2007; Curlik and Shors, 2011; Curlik et al., 2013, 2014, Waddell and Shors, 2008; Shors, 2014). Although both trained groups of females successfully learned the motor skill, the females trained on the motirod outperformed those on the rotarod, and thus retained a greater number of cells. The overall impact that training had on cell survival is displayed in Fig. 3A, where an independent samples t-test suggests that females trained in either task rescued a significantly greater number of BrdU-labeled cells than in those that were not trained (t(29)=28.25, p<0.001).

2.3. Neurogenesis: effect of training on cell survival in males

A one-way ANOVA, with training condition as the independent measure (no training, rotarod, motirod), and the number of surviving BrdU+ cells in the total dentate gyrus as the dependent measure, revealed no significant interaction between the number of BrdU+ cells and training condition in males (F(2,24)=2.58, p=0.10) (Fig. 2B). There was no interaction between the number of surviving cells and training condition in either the GCL (F(2,24)=3.11, p=0.06). However, an independent samples t-test between the number of cells in animals untrained versus trained on the motirod was significant (t(19)=11.84, p<0.05). We did not test separate untrained control groups for each training condition and therefore cannot conduct an ANOVA with independent variables according to conditions on the rotarod test. However, we were able to use a t-test to further assess the overall impact of training (irrespective of the type of training) on cell survival. The t-test was used to compare the number of BrdU-labeled cells in trained male rats (motirod and rotarod) to those that were present in males that were not trained. A t-test revealed that the males that were trained on either the rotarod or motirod retained significantly more cells in the dentate gyrus than males that were not trained (t(29)=23.11, p<0.05) (Fig. 3B). The trained males also retained more cells than untrained males in the GCL (t(29)=23.40, p<0.05) and the hilus (t(29)=23.62, p<0.05). These results are consistent with the previous findings reporting that successful acquisition of a motor skill will increase the number of surviving newly born cells in the dentate gyrus (Curlik et al., 2013).

2.4. Correlations between behavioral performance and cell survival

No significant correlations were observed between the average latency to fall from the rod across all four days of training and the number of surviving cells in the male dentate gyrus during rotarod (r=0.54, p=0.11) and motirod training (r=0.40, p=0.32) or in the female dentate gyrus; female rotarod (r=0.32, p=0.36) and female motirod (r=0.51, p=0.14). However, correlations were all in a positive direction. Therefore, all trained males were combined and all trained females were combined for correlation analyses to give a larger sample size and to get a more distinct effect of training in general. In all trained males there was a significant positive correlation between the average performance across the four days of training and the number of cells surviving in the dentate gyrus (r=0.49, p<0.05) (Fig. 3D). In trained males significant correlations were also observed between number of cells retained in the dentate gyrus and average performance on last day of training (r=0.57, p<0.05) and the last day average minus the first day average (r=0.56, p<0.05).

In females that were trained on either of the tasks, average performance across the four days of training correlated with the number of cells retained in the dentate gyrus (r=0.56, p<0.01; Fig. 3C). Significant correlations were also observed between the number of surviving cells in the dentate gyrus and the average performance on the first day of training (r=0.61, p<0.01), the third day of training (r=0.58, p<0.01), and the last day of training (r=0.50, p<0.05). The correlation between the number of cells rescued and the average performance across all training sessions was likely stronger in females due to the fact that the female motirod group outperformed all other groups, and therefore retained proportionately more cells.

3. Discussion

The present data indicate that sex differences in acquisition of a physical motor skill have consequences for neurogenesis in the adult brain, specifically increasing cell survival in the adult dentate gyrus. Groups of male and female rodents were trained on two different types of a physical skill learning task in order to determine how well they learned these tasks and whether these forms of learning were capable of preventing the death of new cells generated in the adult dentate gyrus. Males performed similarly on both the standard rotarod task as well as on the motirod, a modified version in which cold water was included at the bottom of the platform to make the task more motivating. These trained males successfully learned their respective tasks and retained significantly more new cells in the dentate gyrus than males that did not undergo any training. On the other hand, females performed better on the motirod than they did on the rotarod, and thus, females trained on the motirod task retained a significantly greater number of cells than both females trained on the rotarod and untrained females. These results are in agreement with existing literature reporting that cells are capable of being rescued from death by successful learning (Dalla et al., 2007; Curlik and Shors, 2011; Curlik et al., 2013; Shors, 2014). To our knowledge this was the first demonstration of sex differences in performance on the rotarod, a gross motor skill task (Buitrago et al., 2004). With respect to the motirod test, it was developed in our laboratory and therefore...
no other studies have been conducted using it to study learning, per se.

3.1. Sex differences in learning

Sex differences in learning have been well documented in the literature. It is widely reported that males outperform females in spatial learning tasks (Galea and Kimura, 1993; Galea et al., 1996). In humans, males made fewer errors and required fewer trials to learn while learning a new route on a map (Galea and Kimura, 1993). The authors suggested that this was likely due to sex differences in learning strategy, rather than learning ability. Similarly, male rodents often outperform females during training on the spatial navigation task using the Morris Water Maze (Galea et al., 1996; Beiko et al., 2004; Perrot-Sinal et al., 1996). During fear conditioning, an animal learns to associate a contextual cue with an aversive stimulus, typically a footshock. It is reported that females generally express less conditioned freezing behavior than males during both the acquisition and testing phases of the procedure (Maren et al., 1994; Pryce et al., 1999). Meanwhile, adult females tend to outperform males in associative learning tasks such as trace and delay eyeblink conditioning.
Most relevant are studies reporting that females outperform males during training on operant conditioning tasks requiring active avoidance (Dalla et al., 2008; Shors et al., 2007). The sex differences reported here are largely consistent with these findings because animals trained on the motirod are learning to avoid the aversive water condition through an operant response.

### 3.2. Sex differences in learning versus performance

Sex differences in learning are oftentimes influenced and sometimes misinterpreted because of sex differences in performance. For example, sex differences in fear itself can modify performance during fear conditioning. Also, female rats are more active than males, as indicated in running wheels and the open-field test (Beatty and Fessler, 1977; Hyde and Jerussi, 1983). These differences can contribute to sex differences in fear conditioning, especially when the absence of movement rather than freezing, per se, is used to assess performance. Performance effects play an even more significant role in operant conditioning, because the animal must initiate an overt motor response. A classic example is learned helplessness, during which animals are first subjected to a one-way avoidance task (fixed ratio 1 (FR1)). In this task, the animal must pass through the door of a shuttle box in order to avoid a footshock. Female rats tend to escape sooner than males during training with a FR1 task (Dalla et al., 2008; Shors et al., 2007). Their performance is facilitated by their increase in activity compared to males. During the test of helplessness, animals must now transverse the shuttle-box two times (fixed ratio 2 (FR2)). As noted, females are more active and thus are less likely to freeze and thus learn the correct response. In contrast, males are more likely to freeze and much less likely to enter the side of the shuttlebox in which the shock occurred. One could argue that males are “smarter” in that regard (Shors, 1998; van Haaren et al., 1990; Beatty and Beatty, 1970). Certainly, one could conclude that females do not demonstrate learned helplessness (Dalla et al., 2008; Dalla and Shors, 2009).

Sex differences in performance likely contribute to the sex differences in motirod behavior as well. As described above, the motirod differs from the standard accelerating rotarod in that animals are presumably “motivated” to remain on the rod and thus avoid dropping into cold water just under the rod. The addition of this motivating feature increased the performance in females, but not in males. There are several explanations for these differences. It could be because females are more active although there were no sex differences in the rotarod and it also depends on activity. It could also be that the fear of the cold water is more potent in females. More likely are sex differences in weight. Females weigh substantially less than do males and this weight difference may give them an advantage for remaining on the rod.

### 3.3. Hormonal regulation of sex differences in learning

Sex differences in learning are often influenced and sometimes mediated by sex hormones. For example, in classical eyeblink conditioning, a task in which females outperform males, the sex difference is most robust when females are trained in proestrus, a stage of the estrous cycle when estrogen levels are particularly high (Shors et al., 1998; Dalla et al., 2009). Females that are ovariectomized (without estrogen) perform similarly to males, suggesting the activational effects of estrogen and perhaps progesterone are important for enhancing performance in females (Wood and Shors, 1998). That said, estrogens do not appear to be acting alone to enhance performance (Leuner et al., 2004b). Ovariectomized females that were provided physiological replacement of estradiol performed similarly to males (Leuner et al., 2004b). Sex differences in eyeblink conditioning only emerge after puberty and are not observed during or before puberty (Hodes and Shors, 2005), suggesting that they are dependent on the emergence of a mature estrous cycle. Testosterone, which is present at higher concentrations in males, is not required to express sex differences in eyeblink conditioning but it does organize them in females. Females that are given testosterone at birth perform like males when they become adults (Shors and Miesegaes, 2002). It would be of interest to determine whether the sex differences in motirod training reported here are evident in pre-pubescent males and females, before they have sexually matured, and before large differences in gross body weight have manifested themselves.

### 3.4. Cellular proliferation versus survival

The present experiment focused on the effects of physical skill learning on cell survival and not on cell proliferation. To be specific, we examined the effects of physical skill training on new cells that were already present when training began. It is important to distinguish between these two aspects of neurogenesis because they are differentially regulated and influenced by different conditions such as growth factors (Aberg et al., 2000), learning (Gould et al., 1999), exercise (van Praag et al., 1999), and environmental enrichment (Kempermann et al., 1997). Sex differences in neurogenesis also have been reported, and these studies indicate that gonadal hormones influence cell proliferation (for review see Galea, 2008). For example, one study examined the rate of cell proliferation in the dentate gyrus of adult meadow moles as a function of sex and seasonal differences (Galea and McEwen, 1999). Both proliferation and survival were enhanced in males and correlated with concentrations of testosterone. On the other hand, large fluctuations in proliferation were observed in female voles across seasons, which also correlated with endogenous hormone concentrations. In laboratory rodents, however, females produced more new cells than did males with no apparent difference in cell survival (Tanapat et al., 1999). In addition, high levels of estrogen positively correlated with cell proliferation in the female rats. These and other studies indicate that gonadal hormones modulate aspects of neurogenesis including cell survival. However, these studies are assessing the effects of hormones on how many cells survive as a consequence of enhanced proliferation, not on the numbers that survive that are already present when the hormonal manipulation occurs. As is evident, these are highly complex and dynamic systems and making generalizations about them is not realistic.

Two studies have examined whether sex differences influence learning to then influence cell survival. In the first study, adult animals were injected with BrdU once and then trained one week later with trace eyeblink conditioning. Females
outperformed males during conditioning, which further increased the number of cells that survived (Dalla et al., 2009). In the second study, adult animals received a single BrdU injection and were then trained 6–10 days later on a spatial learning task. This study utilized the Morris Water Maze, which produced a different pattern of findings but a similar effect of learning; that is, males outperformed females and subsequently rescued a greater number of cells in the dentate gyrus (Chow et al., 2013). The present study is in agreement with these findings, such that females outperformed males on the motirod task and thus rescued more cells in the dentate gyrus as a result. Although these studies used different types of training tasks, the findings are consistent because the enhanced performance rescued more new cells from death, leading to an increase in cell survival.

It is well established that exercise increases neurogenesis in the adult hippocampus, primarily if not exclusively through an increase in cell proliferation. (van Praag et al., 1999; Kobilo et al., 2013). For example, we recently examined the effect of exercise alone on cell survival and noted no change in cell number (Curlik et al., 2013). As in the current experiment, BrdU was injected once in adult rats. One week later, one group was trained on the accelerating rotarod and another group was given free access to running wheels. Even though animals that had access to the running wheels traveled approximately twenty times farther than the animals trained on the rotarod, their number of surviving cells did not increase whereas numbers did increase in animals that were trained on the accelerating rotarod test. The cells were already present when the exercise and/or training began, thereby indicating that exercise alone does not rescue new neurons from death, although it would presumably increase the numbers that are generated. Based on these findings, we propose that the increase in cell number reported here in response to training on the motirod reflects an increase in cell survival and not in proliferation and that the increase only occurs in response to learning the new physical skill.

3.5. Fate of adult-born hippocampal cells

The results from the experiments presented here demonstrate that successful acquisition of these motor skills tasks rescues newly-born adult hippocampal cells from death. While it is beyond the scope of this study, previous research has provided evidence that these adult born cells in the dentate gyrus mature into functional neurons in the mammalian brain. Early studies implementing double labeling with neuronal markers reported that newly generated cells in the adult hippocampus have a similar morphology to mature hippocampal neurons and display similar properties to these neurons (Gould et al., 1999; van Praag et al., 2002). These adult born cells in the dentate gyrus are capable of generating action potentials and receive synaptic inputs from the perforant path (van Praag et al., 2002). Based on these and other studies it is widely accepted that new adult cells in the dentate gyrus become functional neurons that are integrated into the hippocampal circuitry (reviewed in Vivar and van Praag, 2013). Most studies have not targeted cells that are rescued from death by learning and it is possible that their fate could change as a result, although unlikely. After learning, more than 80% of the new cells in the GCL were double labeled with BrdU and neuron specific markers TuJ1 and NeuN. Moreover, these cells remain in the hippocampus more than two months after the learning experience, thereby indicating that they have been incorporated as part of the existing hippocampal circuitry (Leuner et al., 2004a).

3.6. Sex differences in mental illness and health

Women are especially vulnerable to mental illnesses that are induced or exaggerated by stressful life events. These illnesses include depression, post-traumatic stress disorder and social phobias (Kessler, 2003; Parker and Brodtie, 2010). However, most neuroscientific experiments have been conducted exclusively in males. Because of this historical behavior, we know relatively little about sex differences in the brain, especially as they relate to the high incidence of stress-related mental illness in women. This is changing, thanks to the Office of Research on Women's Health (ORWH) and the NIH strategic plan to include studies on sex and gender differences in biomedical and basic science research. With these new guidelines, many such reports will be produced and it will be up to us as scientists to fully understand and translate the meaning and the meaningfulness of sex differences in learning into practical applications for women and men. Indeed, the results presented here were recently translated into a clinical intervention known as MAP Training, for mental and physical skill training (Shors et al., 2014). The idea behind this intervention is to increase the number of new neurons with aerobic exercise and then keep them alive through mental training with focused attention meditation. In a proof-of-concept study, we provided MAP Training to young traumatized women in the local community who were recently homeless and taking care of their infants. To facilitate participation, we adopted an exercise routine similar to the Zumba dance program, which is very popular in women. Eight weeks of training (twice a week) significantly increased their overall physical health (oxygen consumption) as well as decreasing measures of anxiety and depression. This is just one example of how we can capitalize on sex differences in motivation to develop novel gender-considered interventions, which enhance mental and physical health, especially in women.

4. Experimental procedure

4.1. Subjects

Male and female Sprague-Dawley rats were bred at Rutgers University in the Department of Psychology. At 28 days after birth animals were weaned and housed in groups of 2–3 males and 2–4 females in standard plastic shoebox style cages (44.5 cm long by 21.59 cm wide by 23.32 cm high). Animals were given access to food and water ad libitum and were maintained on a 12:12 h light-dark cycle; the light cycle began at 7 a.m. and ended at 7 p.m. All handling and experiments were carried out in the light portion of the cycle. All experiments were conducted with full compliance with the rules and regulation specified by the PHC Policy on Human Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals. The Rutgers University Animal Care and Facilities Committee approved all procedures.
4.2. Experimental design

Four groups of animals were used to investigate whether sex differences were observed during training with a gross motor skill task (the “rotarod”, Fig. 1B) and whether there were differences among and between adult male and female rats when this task was modified by including water as a motivating factor (the “motirod”; Fig. 1C). These four groups consisted of males trained on the rotarod (n=10), males trained on the motirod (n=8), females trained on the rotarod (n=11), and females trained on the motirod (n=10). All groups received one single intraperitoneal injection of 5-bromo-2-deoxyuridine (BrdU; 200 mg/kg) at the start of the experiment when they were approximately 60 days of age (PND 60). An additional male and female group also received the BrdU injection at this time point and served as untrained controls, they did not receive any training; male “no training” (n=9) and female “no training” (n=10). See experimental timeline in Fig. 1A.

BrdU incorporates itself into replicating DNA during the S phase of the cell cycle and serves as a marker of cells that are actively proliferating around the time of the injection. Many newly born cells undergo apoptosis approximately seven days after the injection. This time point is optimal to rescue these cells from death (Anderson et al., 2011). Therefore, training on the rotarod and motirod began seven days following the injection of BrdU.

Rotarod and motirod training consisted of four trials per day over four consecutive days. The male and female “no training” groups remained in their home cages during this period and remained experimentally naïve. All six groups of animals were perfused exactly three weeks following the BrdU injection. Many cells that were not rescued by the training procedure would have undergone cell death at this time. Therefore the number of BrdU labeled cells observed at this time point is used to determine the number of cells that were rescued from death by learning.

4.3. BrdU immunohistochemistry and quantification

The majority of adult born hippocampal cells die within one and three weeks of birth, unless they are rescued from death through new and effortful learning (Anderson et al., 2011; Curlik and Shors, 2011; Curlik et al., 2013). Therefore, twenty-one days after the BrdU injection all animals were deeply anaesthetized with sodium pentobarbital (100 mg/kg) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were extracted and post-fixed in 4% paraformaldehyde at 4°C for 24-48 h to preserve the tissue structure before being transferred to phosphate buffered saline (PBS). A vibratome was used to obtain 40 μm coronal sections through the entire rostral-caudal extent of the dentate gyrus in one hemisphere. This is the standard practice in our laboratory, as no differences in neurogenesis have been observed in the dentate gyrus of the right or left hemisphere (Dalla et al., 2007; Anderson et al., 2011). Every twelfth slice was mounted onto a superfrost glass slide (Fisher, Suwanee, GA, USA) and allowed to air dry. Once dry, the tissue was then stained using standard peroxidase methods to visualize the cells that incorporated BrdU as described previously (Curlik and Shors, 2011). Briefly summarized, the tissue was pretreated with heated 0.1 M citric acid (pH 6.0). The tissue was then rinsed with 0.1 M PBS, incubated in trypsin for 10 min, and denatured in 2N HCl for 30 min with PBS rinses in between. The tissue was then incubated overnight in primary mouse anti-BrdU (1: 200, Becton-Dickinson, Franklin Lakes, NJ, USA) and 0.5% Tween-20 (Vector Laboratories, Burlingame, CA, USA). The next day, tissue was rinsed and incubated in biotinylated anti-mouse antibody (1: 200, Vector Laboratories) for 60 min and then placed in avidin-biotin-horseradish peroxidase (1: 100, Vectastain ABC Kit, Vector Laboratories) for 60 min. The tissue was then placed in diaminobenzidine (DAB SigmaFrost tablets, Sigma, Atlanta, GA, USA) for four minutes, rinsed, counterstained with 0.1% cresyl violet, dehydrated, cleared, and coverslipped with Permout glue (Fisher Scientific, Fair Lawn, NJ, USA).

Quantitative analysis was performed blind to the experimental condition by coding each slide. Estimates of the total number of BrdU-positive cells were determined using a modified unbiased stereology protocol (West et al., 1991; Gould et al., 1999). The number of BrdU-positive cells in the dentate gyrus of each slice (granule cell layer and hilus) was counted by hand at 1000X on a Nikon Eclipse 80i light microscope. The number of cells was multiplied by 24 to obtain an estimate of the total number of BrdU-positive cells in the entire dentate gyrus of both hemispheres.

4.4. Physical skill training

The rotarod (Four-station Rotarod for rat, Model #ENV575, MED Associates Inc., Georgia, VT, USA) is a cylindrical rod that is elevated 26.75 cm above a platform. The rod can either accelerate or maintain a constant velocity over a five-minute period. Previous studies have shown that performance on the accelerating rotarod improves over several days (Buitrago et al., 2004; Curlik et al., 2013), and we have previously demonstrated that adult male rats trained on the accelerating rotarod retain significantly more new hippocampal cells (Curlik et al., 2013). It is presumed that rodents acquire this task because of a natural inclination to run. In a recent study researchers set up exercise wheels in the wild and monitored the wheels with automated cameras and motion detectors in order to determine whether mice would voluntarily run on the wheels (Meijer and Robbers, 2014). This study reported that wild mice displayed similar running wheel behavior to mice captive in a laboratory setting. These results suggest that rodents have a natural inclination for running activity and that wheel running can be ruled out as pathological phenomenon developed only in animals housed in a captive laboratory setting. To further enhance their motivation to perform the task, we modified the standard version of the rotarod by placing cold water directly under the rod (approximately 55 mm deep). Animals were removed from the water as soon as they fell off the rod (within seconds) and immediately dried off with a towel. This modified version of the task is referred to as the “motirod.”

Groups of animals were trained on either the standard accelerating rotarod (“rotarod”) or the motirod task. Animals in both training conditions were placed on the rotarod while stationary, facing in the opposite direction that the rod began...
rotating. Thus, animals had to move forward in order to remain on the rotating rod. Each trial began once all animals were placed on the rod in the correct orientation. In all trials the rotarod linearly accelerated from 1.47 cm/sec to 14.74 cm/sec over a five-minute period. After the first five minutes of a trial, the rod no longer accelerated and remained at a constant maximum velocity of 14.74 cm/sec. The latency to fall from the rod (in seconds) was the recorded behavioral measure of the task. Animals were allowed to remain on the rod until they fell off or until 10 min had passed. The time from the start of one trial to the start of the next trial was twenty minutes.

4.5. Data analyses

We first determined how well both the males and females learned the rotarod and motirod tasks, and whether performance differed between the sexes and two training procedures. A repeated-measures analysis of variance (ANOVA) was used to analyze the behavioral data, with training condition (rotarod, motirod) and sex (male, female) as between-subject factors, and training trial (1–16) as the within-subject factor. We then examined whether the rotarod and motirod training increased the survival of newly born cells in the dentate gyrus, and whether more cells were rescued in the trained animals compared to those that were not trained. The number of surviving BrdU-positive cells was assessed using a univariate analysis of variance with the rotarod condition (no training, rotarod, motirod) as the independent measure, and the number of the surviving BrdU-positive cells in the dentate gyrus as the dependent measure. Because the volume and density of the dentate gyrus in males are significantly larger than females (Chow et al., 2013; Dalla et al., 2009), all analyses of cell survival were performed separately for each sex. Pearson correlations were calculated to examine the relationships between motor skill performance and the number of surviving BrdU-positive cells in the dentate gyrus. Post-hoc tests were performed using Tukey’s procedure.

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References


