eNeuro

Research Article: New Research | Cognition and Behavior

Genetic ablation of neural progenitor cells impairs acquisition of trace eyeblink conditioning

https://doi.org/10.1523/ENEURO.0251-19.2019

Cite as: eNeuro 2019; 10.1523/ENEURO.0251-19.2019

Received: 28 June 2019
Revised: 9 August 2019
Accepted: 23 August 2019

Manuscript Title: Genetic ablation of neural progenitor cells impairs acquisition of trace eyeblink conditioning

Abbreviated Title: Ablating neural progenitors slows tEBC acquisition

Author Names and Affiliations:

Lisa N. Miller[1], Craig Weiss[1], & John F. Disterhoft[1]

[1]*Department of Physiology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois 60611*

Author Contributions: LNM and JFD designed research; LNM and CW developed the methodology; LNM performed research and analyzed data; LNM, CW, and JFD wrote the paper.

Correspondence should be addressed to: Lisa Miller at lisamiller2021@u.northwestern.edu and/or John Disterhoft at jdisterfhoft@northwestern.edu

Number of Figures: 3
Number of Tables: 1
Number of Multimedia: 0

Number of words for Abstract: 247
Number of words for Significance Statement: 87
Number of words for Introduction: 656
Number of words for Discussion: 1113

Acknowledgments: This study was supported by the NIH though grants RF1 AG017139 and R37 AG008796 to JFD. Imaging work was performed at the Northwestern University Center for Advanced Microscopy generously supported by NCI CCSG P30 CA060553 awarded to the Robert H Lurie Comprehensive Cancer Center. The authors would like to thank Amy Rapp, Mike McCarthy, and Dr. Matthew Oh for technical support and general feedback.

Conflict of Interest: Authors report no conflict of interest.

Funding sources: This study was supported by the NIH though grants RF1 AG017139 and R37 AG008796 to JFD. Imaging work was performed at the Northwestern University Center for Advanced Microscopy generously supported by NCI CCSG P30 CA060553 awarded to the Robert H Lurie Comprehensive Cancer Center.

Genetic ablation of neural progenitor cells impairs acquisition of trace eyeblink conditioning

Abstract

Adult-born neurons are believed to play a role in memory formation by providing enhanced plasticity to the hippocampus. Past studies have demonstrated that reduction of neurogenesis impairs associative learning, but these experiments used irradiation or neurotoxic substances, which may have had unintended off-target effects. Therefore, to investigate the role of these adult-born neurons more precisely, we utilized C57BL/6-Tg(Nes-TK*,-EGFP)145Sker transgenic mice (Nes-TK) to selectively ablate newborn neurons. Nes-TK mice were fed a chow infused with valganciclovir to induce the ablation of neural progenitor cells. After being on this diet for four weeks, subjects were trained on trace eyeblink conditioning (tEBC), a hippocampus-dependent temporal associative memory task. Following the completion of training, brain sections from these animals were stained for doublecortin, a marker for immature neurons, to quantify levels of neurogenesis. We found that male transgenic mice on valganciclovir had significantly decreased amounts of doublecortin relative to male control animals, indicating a successful reduction in levels of neurogenesis. In conjunction with this reduction in neurogenesis, the male transgenic mice on valganciclovir learned at a significantly slower rate than male control mice. The female Nes-TK mice on valganciclovir showed no significant decrease in neurogenesis and no behavioral impairment relative to female control mice. Ultimately, the results are consistent with, and expand upon, prior studies that demonstrated that adult-born neurons are involved in the formation of associative memories. This study also provides a foundation to continue to explore the physiological role of newborn neurons with *in vivo* recordings during behavioral training.

Significance Statement

Newborn neurons in the adult brain have been shown to be involved in associative learning, but many prior studies illustrating this point used neurotoxins or irradiation to ablate newborn neurons, which may have had unintended off-target effects. Therefore, we utilized a transgenic mouse model to eliminate adult-born neurons in a more controlled, precise manner. Ultimately, we demonstrate that reduction of neurogenesis leads to an impairment in learning in males, and that levels of neurogenesis are associated with rate of learning and overall performance on trace eyeblink conditioning.

1. Introduction

Neurogenesis in the adult brain occurs in the dentate gyrus (DG) and produces new neurons that mature into granule cells and integrate into existing circuitry (Altman & Das, 1965; Dayer et al., 2003). These highly excitable neural progenitor cells are believed to play a role in memory formation by providing enhanced plasticity to the hippocampus (Snyder et al., 2001; Mongiat et al., 2009; Suter et al., 2018). Indeed, there are some studies that have found that reducing the number of newborn neurons impairs memory acquisition on different associative memory tasks. Specifically, ablation of newborn neurons through systemic administration of an antimitotic agent prevented male rats from learning trace eyeblink conditioning (Shors et al., 2001). Additionally, elimination of adult-born neurons in male rats through fractionated irradiation led to an impairment in the hippocampal-dependent place-recognition test, but had no effect on the hippocampal-independent object-recognition task (M'Harzi et al., 1991; Steckler et al., 1998; Madsen et al., 2003). However, there have also been conflicting results depending on the species and methodology used to reduce neurogenesis. Snyder et al. (2005) observed that eliminating newborn neurons through irradiation did not impact acquisition on the hippocampal-dependent Morris water maze (MWM), but did impair long term memory on this task. However, a study in male mice failed to see an impact of ablating newborn neurons on MWM performance (Saxe et al., 2006). Furthermore, while systemic administration of an antimitotic agent reduced

freezing in male rats during trace, but not contextual, fear conditioning (Shors et al., 2002), elimination of newborn neurons through irradiation and genetic manipulations in male mice led to a reduction in freezing behavior during contextual, but not trace, fear conditioning (Saxe et al., 2006). These inconsistencies therefore warrant further investigation into the involvement of adult-born neurons in associative learning.

A limitation of these past studies is that the large majority of them made use of irradiation or neurotoxic substances that may have had unintended off-target effects. For example, the antimitotic agent methylazoxymethanol acetate (MAM) used in some studies has been shown to impact the overall health of an animal and to induce hypo-activity (Dupret et al., 2005). With newer genetic techniques, however, we can investigate whether adult-born neurons are necessary for acquisition of associative learning with greater precision and fewer potential confounds. Specifically, we utilized C57BL/6-Tg(Nes-TK*,-EGFP)145Sker transgenic mice (Nes-TK) to selectively reduce the number of newborn neurons to investigate the role of these neural progenitor cells (Yu et al., 2008). These mice express a modified herpes simplex virus thymidine kinase driven by a nestin promoter and its second intron regulatory element, which allows for temporally regulated induced ablation of dividing neural progenitors through systemic administration of ganciclovir or its prodrug, valganciclovir (Yu et al., 2008, Mustroph et al., 2015).

To study whether adult-born neurons are necessary for learning, we trained animals on trace eyeblink conditioning (tEBC), a hippocampal-dependent temporal associative memory task in which an otherwise neutral conditioning stimulus (CS) is paired with an aversive unconditioned stimulus (US) that causes a reflexive eyeblink response. The two stimuli are separated in time by a stimulus-free trace interval, and subjects learn to associate the two stimuli over many trials. Upon learning this association, subjects start to close their eye during the trace interval in anticipation of the US, which is known as the conditioned response (CR). The advantages of tEBC are that it takes many trials for subjects to learn, which allows for

comparisons of the rate of learning, as well as the ability to look at changes in cellular activity over the course of learning through *in vivo* recording methods.

The goal of this study was to address whether genetic ablation of neural progenitor cells affects acquisition of tEBC, using both male and female mice. Ultimately, we found that a reduction in the number of newborn neurons impairs acquisition of this hippocampus-dependent temporal learning task, and that levels of neurogenesis are correlated with overall performance and rate of learning in male mice.

2. Materials and Methods

2.1. Animals

Animal care procedures were conducted in accordance with NIH guidelines and as approved by the Northwestern University Institutional Animal Care and Use Committee. The Nes-TK transgenic mouse line (JAX stock #029671; RRID: IMSR_JAX:029671) was originally developed in the laboratory of S.G. Kernie (Columbia University) (Yu et al., 2008). Mice were bred in Northwestern University's animal facility and the genotype of each animal was determined by tail snip sent to Transnetyx. Both male and female mice were used in this study, and estrous cycle was not monitored (Prendergast et al., 2014; Fritz et al., 2017).

Four weeks prior to behavioral training, at approximately 8-14 weeks of age, mice were singly housed and provided *ad libidum* access to either regular chow or chow infused with valganciclovir (Val) (1350 mg/kg; Custom Animal Diets), a valine ester pro-drug of ganciclovir. This schedule was based on the findings from Shors et al. (2001) that demonstrated that newborn neurons in rats are about 1-2 weeks old when they become involved in learning tEBC. However, there is a 1-2 week delay in the maturation of young granule cells in mice as compared to rats (Snyder et al., 2009), which is why mice were started on their given diet four weeks prior to tEBC. Assigned diets were maintained until the animals were euthanized. The experimental group consisted of Nes-TK mice on Val-chow, and the three control groups

included Nes-TK mice on regular chow, wild type (WT) mice on Val-chow, and WT mice on regular chow. These control groups were used to investigate whether the drug or genotype alone would have an effect on learning. Ultimately, after there was no observed difference in learning among the three groups, they were combined into one control group in order to avoid using animals unnecessarily. Val-chow was weighed weekly to monitor food intake in order to calculate average Val dosage.

2.2. Trace Eyeblink Conditioning

Two weeks prior to the start of behavioral training, mice underwent headbolt implantation surgery, during which subdermal wires were placed around the orbicularis oculi muscle to measure eyeblink response via electromyography (EMG) activity. After one week of recovery, mice were handled for 5 minutes a day for three days and then habituated to the training chamber for two days, for approximately 45-60 minutes per day. Finally, subjects were trained two at a time on tEBC for ten days.

During tEBC, mice were head-fixed atop a freely rotating cylinder (Heiney et al., 2014; Lin et al., 2016). Each session was comprised of 40 trials, each consisting of a 250-ms whisker displacement (CS; ±50 μm at 62 Hz, delivered by a comb attached to a Piezo actuator) paired with a 30-ms air puff to the cornea (US; 3.5 psi, delivered by a blunted 16 gauge needle pointed at the eye). Presenting the CS and US to different sensory modalities prompts learning that requires integration of sensory information at higher level cortical structures. This is in contrast to other trace conditioning paradigms where the CS and US are both applied to the whisker pad (Troncoso et al., 2004), which may be mediated by motor neurons or the brainstem. The two stimuli were separated by a 250-ms stimulus-free trace interval, and the mean intertrial interval was 45 s (range of 30-60 s). White noise (78-80 dB) was played for the duration of each session. Air pressure and vibration intensity were calibrated between each set of animals using a manometer (Fisher Scientific) and a displacement sensor (optoNCDT 1320, Micro-Epsilon),

173 respectively. The displacement sensor also provided real-time confirmation that the whisker
174 vibration was delivered during each trial. Custom LabView software was used to control stimuli
175 presentation, data collection, and data analysis. Conditioned responses (CRs) were defined as
176 EMG activity during the 200-ms before US presentation that was >4 standard deviations above
177 baseline for >15-ms, with the baseline being defined as the 250-ms prior to CS onset. An animal
178 was considered to reach learning criterion when it demonstrated CRs for at least 60% of trials
179 within a session.
180
181 *2.3. Immunohistochemistry*
182 Two weeks after the completion of training, mice underwent an intracardial perfusion
183 with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA). The brain
184 was removed and stored in 4% PFA overnight at 4°C. The following day the brains were rinsed
185 with PBS and stored in 30% sucrose in PBS for cryoprotection. Brains were sliced on a freezing
186 microtome into 40 µm coronal sections, and a 1 in 6 series of the dorsal hippocampus
187 (approximately -1.5 mm to -2.7 mm posterior to bregma; a total of six sections) was selected
188 from each brain for immunofluorescent staining.
189 Immunofluorescent staining was performed as per the protocol from the laboratory of
190 J.S. Rhodes (University of Illinois) (Mustroph et al., 2015). Free-floating sections were first
191 washed with tris-buffered saline (TBS). To denature DNA, the sections were then treated with a
192 solution of 50% deionized formamide and 2X saline-sodium citrate for two hours at 65°C,
193 washed in 2X saline-sodium citrate for 15 min, treated with 2M hydrochloric acid for 30 min at
194 37°C, and washed with 0.1M borate buffer for 10 min. After a rinse in TBS, sections were
195 blocked in a solution of TBS with 3% normal goat serum and 0.3% Triton X-100 (TBS-X) for 30
196 min before incubating in a primary antibody dilution in TBS-X at 4°C for 48 hours. Sections were
197 then washed with TBS, blocked in TBS-X for 30 min, and incubated in a secondary antibody
198 dilution in TBS-X for three hours. Primary antibodies used were rabbit anti-doublecortin (DCX)

(1:250, Abcam; RRID:AB_732011) and mouse anti-neuronal nuclear protein (NeuN) (1:500, Abcam; RRID:AB_10711040). Secondary antibodies used were donkey anti-rabbit, and goat anti-mouse (all 1:250, Invitrogen; RRID:AB_141637 and AB_2535804) and were conjugated to AlexaFluor 594 and 647, respectively.

2.4. Quantification and Statistical Analysis

All sections were imaged on a confocal microscope with a 20X objective in order to visualize the entire granule cell layer of dentate gyrus in each of the six sections per animal. A z-stack of images was produced to encompass the complete thickness of the granule cell layer. Using Nikon's NIS Elements software, a maximum intensity projection was created in the z-plane to use for cell counting. The area of the granule cell layer and the number of DCX-positive cells within the granule cell layer were calculated using a custom analysis in NIS Elements. The volume of each dentate gyrus section was calculated by multiplying this calculated area with the section thickness so that the average number of DCX-positive cells could be expressed per μm^3 of dentate gyrus.

All statistical analyses were done with StatView, with $p < 0.05$ considered statistically significant. Repeated measures ANOVA was used to compare learning curves, with different groups as the independent variable and training day as the repeated measure. Unpaired t-tests were used to compare groups on each day of training, as well as the number of DCX+ cells between the experimental and the combined control groups. Pearson's correlation coefficient was used to examine the relationship between the number of DCX+ cells and various measures of learning. Outliers were removed if they exceeded two standard deviations beyond the mean; based on this criterion, one male animal was excluded from the experimental group. Data are expressed as mean ± SEM. A complete list of statistical tests and results can be found in Table 1.

3. Results

To ablate neural progenitor cells, Nes-TK mice were placed on a Val-infused diet four weeks prior to behavioral training. These mice were considered the experimental group, and both male and female mice exceeded the desired Val dosage of 200 mg/kg/d (215 ± 8.2 mg/kg/d and 235 ± 9.3 mg/kd/d, respectively) (Yu et al., 2008). The control groups consisted of Nes-TK mice on regular chow, WT mice on Val-chow, and WT mice on regular chow.

All mice were trained on tEBC for ten days, with 40 trials per session. Subjects' EMG activity was monitored for CRs preceding US presentation, and an animal was considered to have reached learning criterion when it showed CRs for at least 60% of trials within any session. An example EMG trace of a well-timed CR is shown in Figure 1A. There was no significant difference in learning among the three types of male controls ($F(2,8) = 3.84$, $p = 0.0677$), so they were combined into one control group, which is depicted in Figure 1B. The same was done for the female control group ($F(2,3) = 0.51$, $p = 0.6454$), shown in Figure 1C. Additionally, there was no significant difference in learning between the male and female control groups ($F(1,15) = 0.63$, $p = 0.6346$). These two groups learned at approximately the same rate, reaching learning criterion on days 4 and 3 of training, respectively (Fig. 1B and 1C).

The male experimental group learned at a significantly slower rate than the male control animals ($F(1,22) = 15.2$, $p = 0.0008$), reaching learning criterion on day 10 of training (Fig. 1B). Additionally, there was a significant interaction between group and training day for male subjects ($F(11,242) = 1.837$, $p = 0.0487$). Unpaired t-tests revealed that there was a significant difference between the experimental and control groups starting on the first day of training ($p = 0.022$), but this difference was no longer present by the final day of training ($p = 0.062$). Training days two through nine were also significantly different ($p < 0.05$), and a complete list of p-values can be found in Table 1. Female Nes-TK mice on the Val diet showed no difference in learning relative to the female control group ($F(1,9) = 0.89$, $p = 0.3696$) (Fig. 1C).

Two weeks after the completion of training on tEBC, the number of DCX+ cells in DG was quantified to validate the effect of valganciclovir on neurogenesis in Nes-TK mice (Fig. 2). DCX is a microtubule-associated protein expressed by immature neurons that is used as a marker for neurogenesis (Brown et al., 2003; Couillard-Despres et al., 2005). The male experimental mice had significantly fewer DCX+ cells relative to the male control group (7054 ± 1529 cells/μm^3 and 16754 ± 2165 cells/μm^3, respectively), showing a 58% decrease in the number of immature neurons (p = 0.0011). Female experimental mice, however, only showed a nonsignificant 38% decrease relative to the female control group (10487 ± 4120 cells/μm^3 and 16886 ± 2304 cells/μm^3, respectively) (p = 0.0947) (Fig. 2D).

A significant positive correlation was observed between the number of DCX+ cells and the average percent CRs across all ten days of training for male subjects (r = 0.574, p = 0.0027), indicating that male animals that had higher levels of neurogenesis performed better overall (Fig. 3A). Similarly, there was a significant negative correlation between the number of DCX+ cells and the number of trials it took for a subject to display 6 CRs within a sliding 10-trial block for male animals (r = -0.519, p = 0.0121), suggesting that male animals that had more newborn neurons learned at a faster rate (Fig. 3B). Female subjects, however, showed no significant correlation between the number of DCX+ cells and either of these measures of learning (r = -0.025, p = 0.943 and r = 0.102, p = 0.77, respectively) (Fig. 3C and 3D), although the Val diet may not have been as effective in our female mice as it was in our male mice.

4. Discussion

This study used genetic ablation of neural progenitor cells to explore whether adult neurogenesis is necessary for associative learning in mice. Ultimately we found that decreasing neurogenesis led to an impairment in acquisition of tEBC. Male Nes-TK mice on valganciclovir showed a nearly 60% reduction in the number of DCX+ cells and learned at a significantly slower rate than male control animals. Thus, newborn neurons are indeed involved in temporal

associative learning, a finding that is in accordance with a previous study that used MAM to diminish the number of adult-born neurons in rats (Shors et al., 2001). Following this neurotoxic ablation of newborn neurons, Shors et al. (2001) observed a significant impairment in rats' ability to learn tEBC. This previous study only eliminated newborn neurons born 1-2 weeks prior to training, while our study inhibited neurogenesis continuously before and during tEBC. However, our subjects were able to eventually reach learning criterion by the end of training, unlike the results reported in the previous study where rats injected with MAM failed to reach criterion. Our findings indicate that adult-born neurons contribute to learning this temporal association but are not the only dentate gyrus neurons that contribute. These other neurons are likely other dentate gyrus neuron types or existing mature granule cells, neither of which was affected by our manipulation. The fact that the rats injected with MAM were unable to reach learning criterion was likely due to non-specific side-effects of MAM, or possibly due to a species difference. It should be stressed that we observed impairment in learning starting on the very first training day while the previous study did not (Shors et al., 2001), which further emphasizes the important role of adult-born neurons in learning.

In addition to an impairment in the rate of learning, we found that neurogenesis was correlated with various measures of learning in male mice. We observed a positive correlation between the number of DCX+ cells and the average percent CRs across all ten days of training, which suggests that male animals with more newborn neurons tended to perform better overall. This result is consistent with previous reports that demonstrated the same positive correlation between number of newborn neurons and average percent CRs in rats trained on tEBC (Curlik & Shors, 2011). We also observed a significant negative correlation between the number of DCX+ cells and the number of trials it took for male subjects to display 6 CRs within a moving block of 10 trials, indicating that male animals that had higher levels of neurogenesis learned faster. This is the opposite of the correlation reported by Curlik & Shors (2011), who found that rats that took longer to learn tEBC showed a higher retention of neurons born prior to the

beginning of training. These findings are not mutually exclusive, as Curlik & Shors (2011) injected bromodeoxyuridine prior to behavioral training in order to examine the survival of newborn neurons, while our study compared overall production of adult-born neurons. Thus, while slower learning may be associated with increased survival of new neurons, our data suggests that subjects with higher levels of neurogenesis learn at a faster rate. Interestingly, we observed no significant correlations between the number of DCX+ cells and either measure of learning for female subjects. This would suggest that females make use of alternative cell types or mechanisms in order to acquire tEBC and/or that females are less affected by the Val diet. Prior studies have demonstrated that not only do females use different strategies for spatial navigation, but that neurogenesis is differentially correlated with performance on a radial arm maze task depending on what strategy is employed (Yagi et al., 2015).

Interestingly, we observed no behavioral effect in the Nes-TK female mice on valganciclovir. This is likely due to the fact that there was no significant reduction in neurogenesis relative to the female control group. It is possible that this manipulation was not effective in the females due to some degree of genetic drift during colony creation or maintenance, as other studies have utilized female Nes-TK mice with clear success (Hollands et al., 2017). This could also explain why we did not see as large a decrease in neurogenesis in the male subjects as was observed in the initial study that used this transgenic line (Yu et al., 2008). Additionally, the male and female control groups learned at the same rate, unlike previous reports that found that female rats learned faster than males (Dalla et al., 2009). A possible reason for this disparity could be the difference in species, as the current study used mice while Dalla et al (2009) used rats. Another possibility is the difference in the behavioral task, as the CS and US in the previous study were white noise and a shock to the eyelid, respectively, with a trace period of 500-ms (Dalla et al., 2009), while we used a 250-ms trace interval in the current study, with a whisker vibration CS and air puff US. With a longer trace period, and more trials needed to reach learning criterion, the previous behavioral paradigm

may have been more difficult to learn than the paradigm used in the current study. Therefore, it is also possible that we could observe a difference in the rate of learning between male and female mice if we used a longer trace interval. Regardless of these differences, our results clearly show in male mice that reduced neurogenesis in the dentate gyrus impairs the rate of hippocampal-dependent eyeblink conditioning.

Recent work by Suter et al. (2018) discovered cells within dentate gyrus that showed changes in firing rate that started at CS onset and persisted through the trace period during tEBC. This finding suggests that there are cells within DG that are bridging the temporal gap between stimuli, which would play a vital role in associative learning. Because the cells that showed this persistent firing were highly excitable, the cell type was hypothesized to be either newborn neurons or mossy cells, as both are more excitable than granule cells (Mongiat et al., 2009; GoodSmith et al., 2017; Senzai & Buzsaki, 2017). Additionally, recent work by Madroñal et al. (2016) has demonstrated that while mature granule cells within DG are not involved in retrieval of tEBC memory, transient inhibition of these cells led to a rapid, transient decrease in conditioned responses. This suggests that mature dentate granule cells are involved in maintenance of associative memory. In the present study we have demonstrated that adult-born neurons are indeed involved in acquiring tEBC, which provides a foundation to further explore exactly how newborn neurons and other cell types in dentate gyrus contribute to associative learning through *in vivo* recording techniques during behavioral training.

References

Altman J & Das GD. (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *Journal of Comparative Neurology,* 124(3), 319-335. doi:10.1002/cne.901240303.

Brown JP, Couillard-Després S, Cooper-Kuhn CM, Winkler J, Aigner L, & Kuhn HG. (2003). Transient expression of doublecortin during adult neurogenesis. *Journal of Comparative Neurology,* 467(1), 1-10. doi:10.1002/cne.10874.

Couillard-Després S, Winner B, Schaubeck S, Aigner R, Vroemen M, Weidner N, Bogdahn U, Winkler J, Kuhn HG, & Aigner L. (2005). Doublecortin expression levels in adult brain reflect neurogenesis. *European Journal of Neuroscience*, 21(1), 1-14. doi:10.1111/j.1460-9568.2004.03813.x

Curlik DM & Shors TJ. (2011). Learning increases the survival of newborn neurons provided that learning is difficult to achieve and successful. *Journal of Cognitive Neuroscience,* 23(9), 2159-2170. doi:10.1162/jocn.2010.21597.

Dalla C, Papachristos EB, Whetstone AS, & Shors TJ. (2009). Female rats learn trace memories better than male rats and consequently retain a greater proportion of new neurons in their hippocampi. *Proc. Natl Acad. Sci. USA,* 106(8), 2927-2932. doi:10.1073/pnas.0809650106.

Dayer AG, Ford AA, Cleaver KM, Yassaee M, & Cameron HA. (2003). Short-term and long-term survival of new neurons in the rat dentate gyrus. *Journal of Comparative Neurology,* 460(4), 563-572. doi:10.1002/cne.10675.

Dupret D, Montaron MF, Drapeau E, Aurousseau C, Le Moal M, Piazza PV, & Abrous DN. (2005). Methylazoxymethanol acetate does not fully block cell genesis in the young and aged dentate gyrus. *European Journal of Neuroscience,* 22, 778-783. doi:10.1111/j.1460-9568.2005.04262.x.

Fritz AK, Amrein I, & Wolfer DP. (2017). Similar reliability and equivalent performance of female and male mice in the open field and water-maze place navigation task. *American Journal of Medical Genetics,* 175(3), 380-391. doi:10.1002/ajmg.c.31565.

GoodSmith D, Chen X, Wang C, Kim SH, Song H, Burgalossi A, Christian KM, & Knierim JJ. (2017). Spatial representations of granule cells and mossy cells of the dentate gyrus. *Neuron,* 93(3), 677-690. doi:10.1016/j.neuron.2016.12.026.

Heiney SA, Wohl MP, Chettih SN, Ruffolo LI, & Medina JF. (2014). Cerebellar-dependent expression of motor learning during eyeblink conditioning in head-fixed mice. *Journal of Neuroscience,* 34(45), 14845-14853. doi:10.1523/JNEUROSCI.2820-14.2014.

Hollands C, Tobin MK, Hsu M, Musaraca K, Yu TS, Mishra R, Kernie SG, & Lazarov O. (2017). Depletion of adult neurogenesis exacerbates cognitive deficits in Alzheimer's disease by comprising hippocampal inhibition. *Molecular Neurodegeneration,* 12, 64. doi:10.1186/s13024-017-0207-7.

Lin C, Disterhoft J, & Weiss C. (2016). Whisker-signaled eyeblink classical conditioning in head-fixed mice. *Journal of Visualized Experiments,* 109, e53310. doi:10.3791/53310.

399 Madroñal N, Delgado-García JM, Fernández-Guizán A, Chatterjee J, Köhn M, Mattucci C, Jain
400 A, Tsetsenis T, Illarionova A, Grinevich V, Gross CT, & Gruart A. (2016). Rapid erasure of
401 hippocampal memory following inhibition of dentate gyrus granule cells. *Nature*
402 *Communications,* 7:10923. doi:10.1038/ncomms10923.
403
404 Madsen TM., Kristjansen PE., Bolwig TG., & Wortwein G. (2003). Arrested neuronal
405 proliferation and impaired hippocampal function following fractionated brain irradiation in the
406 adult rat. *Neuroscience, 119*(3), 635-642. doi:10.1016/S0306-4522(03)00199-4
407
408 M'Harzi M, Jarrad LE, Willig F, Palacios A, & Delacour J. (1991). Selective fimbria and thalamic
409 lesions differentially impair forms of working memory in rats. *Behavioral and Neural Biology,*
410 56(3), 221-239. doi:10.1016/0163-1047(91)90364-V.
411
412 Mongiat LA, Espósito MS, Lombardi G, & Schinder AF. (2009). Reliable activation of immature
413 neurons in the adult hippocampus. *PLoS One,* 4(4), e5320. doi:10.1371/journal.pone.0005320.
414
415 Mustroph ML., Merritt JR., Holloway AL., Pinardo H., Miller DS., Kilby CN., Bucko P., Wyer A., &
416 Rhodes JS. (2015). Increased adult hippocampal neurogenesis is not necessary for wheel
417 running to abolish conditioned place preference for cocaine in mice. *European Journal of*
418 *Neuroscience, 41,* 216-226. doi:10.1111/ejn.12782.
419
420 Prendergast BJ, Onishi KG, & Zucker I. (2014). Female mice liberated for inclusion in
421 neuroscience and biomedical research. *Neuroscience & Biobehavioral Reviews,* 40, 1-5.
422 doi:10.1016/j.neubiorev.2014.01.001.
423

Saxe MD, Battaglia F, Wang JW, Malleret G, David DJ, Monckton JE, Garcia ADR, Sofroniew ER, Santarelli L, Hen R, & Drew MR. (2006) Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. *Proc. Natl Acad. Sci. USA,* 103(46), 17501-17506. doi:10.1073/pnas.0607207103.

Senzai Y & Buzsáki G. (2017). Physiological properties and behavioral correlates of hippocampal granule cells and mossy cells. *Neuron,* 93(3), 691-704. doi:10.1016/j.neuron.2016.12.011.

Shors TJ, Miesegaes G, Beylin A, Zhao M, Rydel T, & Gould E. (2001). Neurogenesis in the adult is involved in the formation of trace memories. *Nature,* 410, 372-376. doi:10.1038/35066584.

Shors TJ, Townsend DA, Zhao M., Kozorovitskiy Y, & Gould E. (2002). Neurogenesis may relate to some but not all types of hippocampal-dependent learning. *Hippocampus,* 12(5), 578-584. doi:10.1002/hipo.10103.

Snyder JS, Kee N, & Wojtowicz JM. (2001). Effects of adult neurogenesis on synaptic plasticity in the rat dentate gyrus. *Journal of Neurophysiology,* 85(6), 2423-2431. doi:10.1152/jn.2001.85.6.2423.

Snyder JS, Hong NS, McDonald RJ, & Wojtowicz JM. (2005). A role for adult neurogenesis in spatial long-term memory. *Neuroscience,* 130(4), 843-852. doi:10.1016/j.neuroscience.2004.10.009.

449 Snyder JS, Choe JS, Clifford MA, Jeurling SI, Hurley P, Brown A, Kamhi JF, & Cameron HA.
450 (2009). Adult-born hippocampal neurons are more numerous, faster-maturing and more
451 involved in behavior in rats than in mice. *Journal of Neuroscience,* 29(46), 14484-14495.
452 doi:10.1523/JNEUROSCI.1768-09.2009.
453
454 Steckler T, Drinkenburg WHIM, Sahgal A, & Aggleton JP. (1998). Recognition memory in rats –
455 II. Neuroanatomical substrates. *Progress in Neurobiology,* 54(3), 313-332. doi:10.1016/S0301-
456 0082(97)00061-0.
457
458 Suter EE, Weiss C, & Disterhoft JF. (2018). Differential responsivity of neurons in perirhinal
459 cortex, lateral entorhinal cortex, and dentate gyrus during time-bridging learning. *Hippocampus,*
460 29(6), 511-526. doi:10.1002/hipo.23041.
461
462 Troncoso J, Múnera A, & Delgado-García JM. (2004). Classical conditioning of eyelid and
463 mystacial vibrissae responses in conscious mice. *Learning and Memory,* 11, 724-726.
464 doi:10.1101/lm.81204.
465
466 Yagi S, Chow C, Lieblich SE, & Galea LAM. (2015) Sex and strategy use matters for pattern
467 separation, adult neurogenesis, and immediate early gene expression in the hippocampus.
468 *Hippocampus,* 26(1), 87-101. doi:10.1002/hipo.22493.
469
470 Yu TS, Zhang G, Liebl DJ, & Kernie SG. (2008). Traumatic brain injury-induced hippocampal
471 neurogenesis requires activation of early nestin-expressing progenitors. *Journal of*
472 *Neuroscience*, 28(48), 12901–12912. doi:10.1523/JNEUROSCI.4629-08.2008.

Legends

Table 1. Statistics Table. All data sets are assumed normal distribution.

Figure 1. Trace eyeblink conditioning in mice. **A.** EMG activity from an animal trained on tEBC (lower trace), depicting a well-timed conditioned response (CR). The timing of the conditioned stimulus (CS; whisker vibration) and unconditioned stimulus (US; air puff) presentation are shown at the top of the panel. **B-C.** Learning curves for the male (B) and female (C) control and experimental groups. Average percent CRs are shown for each day, where "H" refers to days of habituation and "T" refers to days of training. Error bars represent SEM. Post-hoc t-tests were used to test statistical differences for each day of training (* $p < 0.05$; ** $p < 0.01$).

Figure 2. Measuring neurogenesis in the adult brain. **A-B.** Sample images of DCX expression in DG in sections from a male control animal (A) and a male experimental animal (B). **C.** Zoomed in views of DCX+ cells from panels A and B (left and right, respectively). **D.** Quantification of the number of DCX+ cells within the granule cell layer, expressed as number of cells per μm^3 of DG. The male experimental group (n = 13) showed a significant decrease relative to the male control group (n = 11) (** $p < 0.01$). There was no significant difference between the female experimental group (n = 5) and the female control group (n = 6) ($p > 0.05$). Error bars represent SEM. Scale bars: (B) 100 μm (C) 50 μm.

Figure 3. Amount of neurogenesis is correlated with learning in male mice. **A, C.** The number of DCX+ cells per μm^3 of DG is positively correlated with the average percentage of CRs across all ten days of training for males (A) ($r = 0.574$, $p = 0.0027$), but not females (C) ($r = -0.025$, $p = 0.943$). **B, D.** The number of DCX+ cells per μm^3 of DG is negatively correlated with the number

498 of trials it took to show 6 CRs within a sliding block of 10 trials for males (B) (r = -0.519, p =
499 0.0121), but not for females (D) (r = 0.102, p = 0.77).
500

Table 1: Statistics table.

Description	Type of test	Sample size	Statistical data
Figure 1: Comparison of learning curves			
Male Control Groups	RM ANOVA	WT/Reg: n = 3 WT/Val: n = 3 Nes-TK/Reg: n = 5	*Group*: $F = 3.84$ $p = 0.0677$
Female Control Groups	RM ANOVA	WT/Reg: n = 3 WT/Val: n = 1 Nes-TK/Reg: n = 2	*Group*: $F = 0.51$ $p = 0.6454$
Male vs Female Controls	RM ANOVA	Male: n = 11 Female: n = 6	*Group*: $F = 0.63$ $p = 0.6346$
Male; Con vs Exp	RM ANOVA	Con: n = 11 Exp: n = 13	*Group*: $F = 15.2$ $p = 0.0008$ *Interaction*: $F = 1.837$ $p = 0.0487$
Male; Con vs Exp All sessions	Unpaired t-tests	Con: n = 11 Exp: n = 13	H1: $p = 0.06$ H2: $p = 0.24$ T1: $p = 0.022$ T2: $p = 0.0071$ T3: $p = 0.0011$ T4: $p = 0.029$ T5: $p = 0.018$ T6: $p = 0.0017$ T7: $p = 0.0087$

			T8: $p = 0.0045$ T9: $p = 0.034$ T10: $p = 0.062$
Female; Con vs Exp	RM ANOVA	Con: n = 6 Exp: n = 5	*Group*: $F = 0.89$ $p = 0.3696$
Figure 2: Comparing number of DCX+ cells			
Male; Con vs Exp	Unpaired t-test	Con: n = 11 Exp: n = 13	$p = 0.0011$
Female; Con vs Exp	Unpaired t-test	Con: n = 6 Exp: n = 5	$p = 0.0947$
Figure 3: Correlation between learning and number of DCX+ cells			
Male; DCX vs Average CRs	Pearson's Correlation	n = 24	$r = 0.574$ $p = 0.0027$
Male; DCX vs Trials to 6 CRs of 10 trials	Pearson's Correlation	n = 24	$r = -0.519$ $p = 0.0121$
Male and Female; DCX vs Average CRs	Pearson's Correlation	n = 35	$r = 0.38$ $p = 0.0285$
Male and Female; DCX vs Trials to 6 CRs of 10 trials	Pearson's Correlation	n = 35	$r = -0.394$ $p = 0.0227$