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Short communication

Training memory without aversion: appetitive hole-board spatial learning increases adult hippocampal neurogenesis

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Abstract

Learning experiences are potent modulators of adult hippocampal neurogenesis (AHN). However, the vast majority of findings on the learning-induced regulation of AHN derive from aversively-motivated tasks, mainly the water maze paradigm, in which stress is a confounding factor that affects the AHN outcome. Currently, little is known regarding the effect of appetitively-motivated training on AHN. Hence we studied how spatial learning to find food rewards in a hole-board maze modulates AHN (cell proliferation and immature neurons) and AHN-related hippocampal neuroplasticity markers (BDNF, IGF-II and CREB phosphorylation) in mice. The 'Trained' mice were tested for both spatial reference and working memory and compared to 'Pseudotrained' mice (exposed to different baited holes in each session, thus avoiding the reference memory component of the task) and 'Control' mice (exposed to the maze without rewards). In contrast to Pseudotrained and Control mice, the number of proliferating hippocampal cells were reduced in Trained mice, but they notably increased their population of immature neurons assessed by immunohistochemistry. This evidence shows that hole-board spatial reference learning diminishes cell proliferation in favor of enhancing young neurons' survival. Interestingly, the enhanced AHN in the Trained mice (specifically in the suprapyramidal blade) positively correlated with their reference memory performance, but not with their working memory. Furthermore, the Trained animals increased the hippocampal protein expression of all the neuroplasticity markers analyzed by western blot. Results show that the appetitively-motivated hole-board task is a useful paradigm to potentiate and/or investigate AHN and hippocampal plasticity minimizing aversive variables such as fear or stress.

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1. Introduction

Neurogenesis is the process of producing new neurons for their integration into the brain circuitry, and the dentate gyrus (DG) of the hippocampus is a main adult neurogenic niche (Altman and Das, 1965). It is widely accepted nowadays that a reduction of AHN leads to impoverished hippocampal-dependent function, while enhanced neurogenesis improves cognition and emotional regulation in both healthy and pathological subjects (Castilla-Ortega et al., 2011; Shors et al., 2014). Therefore, clinical therapies that potentiate AHN could ameliorate hippocampal malfunction in a certain number of diseases (Shors et al., 2014).

Importantly, AHN is extensively modulated by environmental stimuli. The environmental-induced modulation of AHN occurs by means of internal mediators such as neurotrophic factors, growth factors or stress hormones that impact the hippocampus at different stages of AHN, especially cell proliferation (i.e. the generation of the new neurons) and the survival/maturation of the immature neurons (from birth up to ~3-4 weeks old) that usually die unless they are exposed to relevant stimulation (Balu and Lucki, 2009; Castilla-Ortega et al., 2011). At this young age, neurons experience a *critical period* of enhanced plasticity in which they respond to GABA-signaling with depolarization and they express endogenous immature neuron markers (Castilla-Ortega et al., 2011; Tashiro et al., 2007). Interestingly, the environment-dependent regulation of the cell proliferation and survival processes occurs independently. For example, environmental enrichment mainly increases hippocampal cell survival while physical exercise preferentially increases cell proliferation in rodents (Fabel et al., 2009).

Learning experiences also greatly influence AHN. In general terms, engaging in hippocampal-dependent learning potentiates survival of immature hippocampal neurons that were born before learning rescuing them from death. Besides, significant direct correlations between AHN and memory performance measures are usually reported (Castilla-Ortega et al., 2011; Epp et al., 2013; Shors, 2014). There is not yet a consensus, however, regarding cell proliferation. Proliferating cell numbers have been shown to be either unaffected, increased or decreased by cognitive training (Epp et al., 2013; Prickaerts et al., 2004). Intriguingly, most of the results on the learning-induced modulation of AHN derive from studies employing aversive training paradigms (**Supplementary material**), such as the very widely used water maze -in which the rodent must find a hidden platform to escape from a water tank. Training in aversively-motivated tasks such as the water maze is stressful for animals (Harrison et al., 2009) and, compared to appetitive or neutral memories, aversive or stressful memories may engage particular neurobiological mechanisms involved in emotional memory regulation (Knapska et al., 2006; Korz and Frey, 2004; Payne et al., 2006). Stress is also a potent modulator of AHN. It usually decreases cell proliferation and survival of the adult-born hippocampal neurons (Schoenfeld and Gould, 2013), although moderate forms of stress may be pro-neurogenic instead (Kirby et al., 2013). Accordingly, it is suggested that the level of stress experienced during training influences the AHN outcome reported in water maze studies, being a main confounding factor (Aztiria et al., 2007; Ehninger and Kempermann, 2006; Mohapel et al., 2006; Prickaerts et al., 2004).

Taking this into account, both AHN and memory should also be investigated in appetitive training paradigms (e.g. motivated by finding a food or water reward) that minimize stress during testing (Henrich-Noack, 2014). Interestingly, positively rewarding experiences may potentiate AHN even in spite of elevated levels of glucocorticoids (Schoenfeld and Gould, 2013). Nevertheless, the effect of appetitively-motivated training on AHN has been barely investigated (**Supplementary material**) and the few available studies have mostly failed to find pro-AHN effects [i.e. increased AHN: (Yagi et al., 2016); mixed effects: (Bardi et al., 2013; Kumazawa-Manita et al.,

2013; Olariu et al., 2005; Schaefers, 2015)]. This study investigates AHN (immature neurons and cell proliferation) and hippocampal neuroplasticity markers related to AHN regulation [brain-derived neurotrophic factor (BDNF), insulin like growth factor-2 (IGF-II) and cAMP response element-binding protein (CREB) phosphorylation] in mice trained in an appetitively-motivated hole-board spatial navigation task. This paradigm allows training simultaneously of two different hippocampal-dependent cognitive components, spatial reference and working memory (Oades, 1981), and rewards are placed in four spatial locations which may increase the cognitive challenge compared to learning a single one (Castilla-Ortega et al., 2010; Sampedro-Piquero et al., 2014). Although hole-board training modulates another aspects of hippocampal neuroplasticity such as long-term potentiation (Uzakov et al., 2005), to date there is no information about the effect of hole-board training on AHN (**Supplementary material**).

2. Materials and methods

2.1. Animals

Twenty-six 3 month-old male C57BL/6J mice were acquired from Janvier (Le Genest-Saint-Isle, France) and were single-housed in standard conditions (12 h light-dark cycle, lights on at 8:00 a.m.). Mice were randomly assigned to three experimental groups*:* Control (n=8), Pseudotrained (n=10) or Trained (n=8).

The experimental procedures were performed in accordance with the European (Directive 2010/63/UE) and Spanish regulations (Real Decreto 53/2013 and Ley 32/2007) for animal research.

2.2. Food deprivation

Four days prior to the hole-board training (Days 1-4, **Fig. 1A**), all mice were handled daily for 5 min and they were fed with 2.5 gr/day of their habitual pellet diet (A04, SAFE DIETS, Paris, France) plus two pieces (~0.06 gr each) of chocolate cereal (Choco Krispies®, Kellogs), placed in their home-cage. At the beginning of the behavioral testing (Day 5, **Fig. 1A**), animals had reached ~85% of their free-feeding body weight. On Days 5-11, mice were feed with 2.5 or 3 gr/day of their pellet diet to maintain their weight stable during the experiment.

2.3. Hole-board training

2.3.1. Apparatus

We used an automatized version of a hole-board apparatus (Cibertec, Madrid, Spain; described in **Fig. 1B**) equipped with an infrared system to detect head dips and with a custom computer software for experiment configuration and data collection.

2.3.2. Training procedure

Behavioral assessment was carried out between 8:30 a.m. and 2 p.m. Daily, a false bottom of the maze was filled with chocolate cereal to avoid discrimination of the baited holes based on olfaction, and the apparatus was rotated 90º to prevent orientation by intra-maze cues. The apparatus was cleaned with 30% ethanol after each session.

The hole-board protocol was based on our previous methods (Castilla-Ortega et al., 2010). Mice from the Pseudotrained and Trained groups were habituated to eat from the holes in one daily 6 min habituation session (i.e. a 'shaping' session) performed on Days 5-6 with all 16 holes baited with 0.03 g of chocolate cereal (**Fig. 1A, C**).

Subsequently, training was conducted on four consecutive days (Days 7-10, **Fig. 1A**). Each training day comprised four sessions distributed in blocks of two consecutive sessions each; with a 90 min interval separating the training blocks (**Fig. 1C**). Four holes were baited in each training session and the mouse was placed randomly in one of the four arena corners as the starting position. The session stopped when the animal ate all the four rewards or 4 min had expired. The location of the four rewards was fixed through the experiment for the Trained mice, while the Pseudotrained mice were exposed to a different pattern of baited holes in each session (**Fig. 1C**). After completing training, one reversal trial was performed to study the effect of changing rewards' locations on memory performance in the Trained animals (**Fig. 1C**). Spatial reference and working memory performance was assessed in each session by means of the following ratios: Reference Memory Ratio (RM-ratio): total number of visits to the baited holes divided by the total number of hole visits; Working Memory Ratio (WM-ratio): number of food-rewarded visits divided by the total number of visits to the baited holes (Castilla-Ortega et al., 2010).

Regarding the Control mice, they performed the same number of sessions and spent a similar time in the maze than the Pseudotrained and Trained groups. However, none of the holes were ever baited (**Fig. 1C**) so they ate the corresponding chocolate cereal in their home-cage.

2.4. Brain analyses

2.4.1. Hippocampal tissue collection

On day 11 (**Fig. 1A**), mice were deeply anesthetized and briefly intracardially perfused with 0.1 M phosphate-buffered saline (pH 7.4). Brains were dissected out and cut through the interhemispheric fissure. The left brain hemisphere was post-fixed for 48 h in a 4 % paraformaldehyde solution at 4ºC and cut into coronal (35 µm) vibratome sections for immunohistochemistry. The right brain hemisphere was immediately frozen at -80ºC in dry ice for protein analysis.

2.4.2. Immunohistochemistry and cell quantification

Immunohistochemistry for AHN was performed in free-floating sections using extravidin-conjugated peroxidase and diaminobenzidine as the chromogen (Castilla-Ortega et al., 2016; Ladron de Guevara-Miranda et al., 2017). The primary antibodies used were mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) (diluted 1:1000, P8825, Sigma-Aldrich, Madrid, Spain) to detect cells undergoing division; and goat polyclonal anti-doublecortin (DCX) (1:200, sc-8066, Santa Cruz Biotechnology, Heidelberg, Germany) that labels immature neurons up to ~3-4 weeks old (Brown et al., 2003). According to their morphology, the DCX+ neurons were classified as Type-1 (with absent or short dendritic processes) or as Type-2 (with at least one radial apical dendrite penetrating the granule cell layer) (**Fig. 2B**) (Castilla-Ortega et al., 2016; Ladron de Guevara-Miranda et al., 2017).

Cell counting was carried out in the DG granule cell layer in one every four sections of the dorsal hippocampus (between -1.22 and -2.54 mm from bregma) (Paxinos and Franklin, 2001) using the software ImageJ (US National Institutes of Health, Maryland, USA) as previously described (Ladron de Guevara-Miranda et al., 2017). The suprapyramidal (SupraDG) and infrapyramidal (InfraDG) blades of the DG were analyzed separately. Results were expressed as the number of positive cells per mm^2.

2.4.3. Western Blot

Protein extraction and western blot analysis were performed as described elsewhere (Serrano et al., 2012; Vida et al., 2013). The primary antibodies used were rabbit anti-BDNF (diluted 1:200, SAB2108004, Sigma-Aldrich), mouse anti-CREB-1 (1:1000, sc-377154, Santa Cruz Biotechnology), goat anti-pCREB1 (1:100, Ser133; sc-7978, Santa Cruz Biotechnology), rabbit anti-IGF-II (1:250, sc-5622, Santa Cruz Biotechnology) and mouse anti-vinculin (1:100, sc-25336, Santa Cruz Biotechnology) as a housekeeping protein. The images were analyzed by densitometry using the ImageJ software.

2.5. Statistical analyses

One-way ANOVA tests [independent variable: 'group' (Control, Pseudotrained or Trained)] were used to analyze the neurogenesis-related markers in the whole DG and the hippocampal plasticity-related markers studied by western blot. Repeated measures ANOVA tests were used to analyze the memory measures across training and the neurogenesis-related markers per DG blade [for behavioral measures: 'group' (Pseudotrained or Trained) as independent variable and 'training session' or 'training day' as dependent variable with repeated measures; for histological measures: 'group' (Control, Pseudotrained or Trained) as independent variable and the 'DG blade' (SupraDG or InfraDG) as dependent variable with repeated measures]. Post-hoc Fisher's least significant differences (LSD) analysis were used to study group differences. Correlations were Pearsons'. Only significant results ($p < 0.05$) are reported.

3. Results

3.1. Hole-board training

Mice from the Trained group notably increased their RM-ratio (mean ± S.E.M. on Day 4 = 0.717 ± 0.05) compared to the Pseudotrained mice (mean ± S.E.M. on Day 4 = 0.315 ± 0.01) (repeated measures ANOVA per training session: 'group': $F(1,6) = 63.456$, $p < 0.001$; 'session': $F(16,256) = 6.425$, $p < 0.001$; 'group x session': $F(16,256) = 11.569$, $p < 0.001$; per training day: 'group': $F(1,16) = 65.694$, $p < 0.001$; 'day': $F(3,48) = 15.797$, $p < 0.001$; 'group x day': $F(3,48) = 29.488$, $p < 0.001$; post hoc analysis is shown in **Fig. 1D,E**). On the other hand, there were no between-groups differences in the WM-ratio, as both the Trained and the Pseudotrained mice progressively improved their working memory performance across training (repeated measures ANOVA per training session: 'session': $F(16,256) = 2.651$, $p < 0.001$; per training day: 'day': $F(3,48) = 12.420$, $p < 0.001$; **Fig. 1F,G**). Interestingly, both groups significantly improved (post hoc: $p < 0.05$) their WM-Ratio on the fourth training day compared to the first and second days [mean ± S.E.M. of the Pseudotrained mice: Day 1 = 0.489 ± 0.03; Day 2 = 0.461 ± 0.02; Day 3 = 0.540 ± 0.04; Day 4 = 0.624 ± 0.04; mean ± S.E.M. of the Trained mice: Day 1 = 0.407 ± 0.03; Day 2 = 0.485 ± 0.02; Day 3 = 0.579 ± 0.04; Day 4 = 0.612 ± 0.04; **(Fig. 1G)**]. These results show that, by the end of training, both the Trained and Pseudotrained mice displayed a working memory performance over chance.

3.2. AHN and correlation with learning

Compared to the Control and Pseudotrained groups, the number of proliferating PCNA+ cells in the DG were reduced in Trained mice (mean ± S.E.M. of PCNA+ cells/mm^2 in the total DG: Control = 59.244 ± 5.44; Pseudotrained = 51.558 ± 1.87; Trained = 49.937 ± 2.99) (repeated measures ANOVA per DG blade: 'group': $F(2,23) = 6.532$, $p < 0.05$; 'DG blade': $F(1,23) = 20.045$, $p < 0.001$; one-way ANOVA for the total DG: 'group': $F(2,23) = 6.294$, $p < 0.05$; post hoc analysis is shown in **Fig. 2A,C**). While the Trained mice did not show changes in the Type-1 DCX+ neurons (**Fig. 2B,D**), they

notably increased the number of Type-2 DCX+ neurons (repeated measures ANOVA per DG blade: 'group': $F(2,23) = 16.284$, $p < 0.001$; 'DG blade': $F(1,23) = 80.883$, $p < 0.001$; 'group x DG blade': $F(2,23) = 3.490$, $p < 0.05$; one-way ANOVA for the total DG: 'group': $F(2,23) = 16.236$, $p < 0.001$; post hoc analysis is shown in **Fig. 2B,E**); resulting in an enhanced total number of DCX+ cells compared with the other treatments (mean ± S.E.M. of DCX+ cells/mm^2 in the total DG: Control = 134.219 ± 5.99; Pseudotrained = 133.717 ± 5.65; Trained = 185.709 ± 11.68) (repeated measures ANOVA per DG blade: 'group': $F(2,23) = 14.034$, $p < 0.001$; 'DG blade': $F(1,23) = 75.341$, $p < 0.001$; 'group x DG blade': $F(2,23) = 4.600$, $p < 0.05$; one-way ANOVA for the total DG: 'group': $F(2,23) = 13.639$, $p < 0.001$; post hoc analysis is shown in **Fig. 2B,F**).

Interestingly, increased AHN was postively correlated with learning in the Trained mice. Specifically, the Type-2 DCX+ neurons in the supraDG correlated with their RM-Ratio on training Day 2 (i.e. Day 8 of the experiment in **Fig. 1A**) ($r = 0.734$, $p < 0.05$); particularly on sessions 1-3 (**Fig. 2G**). On the contrary, no correlation was found between the Type-2 DCX+ neurons with the WM-Ratio on any training day or session (**Fig. 2H**).

3.3. Plasticity-related proteins in the hippocampus

The hippocampal protein levels of BDNF and IGF-II were increased in Trained mice as well as CREB phosphorylation (one-way ANOVA: BDNF: $F(2,23) = 3.866$, $p < 0.05$; IGF-II: $F(2,23) = 4.689$, $p < 0.05$; pCREB/CREB1: $F(2,23) = 4.907$, $p < 0.05$; post hoc analyses are shown in **Fig. 3A,B,C**). Groups were similar in the expression of CREB1 and the housekeeping protein vinculin (data not shown).

4. Discussion

This study used three experimental groups that differed in their cognitive training demands. The Control mice merely explored the empty apparatus, while the Pseudotrained mice learned to obtain cereal randomly located in the maze and, similar to the Trained mice, they progressively improved their spatial working memory performance (i.e. the short-term memory for the spatial locations already visited in a particular session). However, the Trained mice also received spatial reference memory training, acquiring a long-term memory for a fixed set of spatial locations that were baited in every session.

Since only the Trained mice showed AHN-related changes compared to Controls, these must be driven by reference memory learning. Both spatial reference and working memory performance in the hole-board depend on the hippocampus, but they involve different information processing mechanisms (Oades, 1981). Numerous studies have demonstrated the role of AHN for spatial reference memory (Castilla-Ortega et al., 2011), while the link between working memory and the adult-born hippocampal neurons remains ambiguous (Hernandez-Rabaza et al., 2009; Saxe et al., 2007). It should be noted that reference memory as assessed in this study may also involve some working memory components (i.e. the RM-Ratio considers total hole explorations including re-visits), augmenting cognitive demands. Nevertheless, the increased number of immature hippocampal neurons in the Trained mice correlated with their RM-Ratio, but not with their WM-Ratio. Specifically, animals that performed better at an early acquisition phase (i.e. second day of training) showed more new neurons. However, AHN did not correlate with the reference memory in the late phase of acquisition (asymptotic performance) contrary to what it is reported in the water maze (Sisti et al., 2007). Because all Trained animals had successfully learned the hole-

board task by the end of training (i.e. RM-Ratio ≥ ~0.6), performance at the initial acquisition days could be more effective to discriminate the best learners in this study. Another explanation is the different learning nature of the water maze and the hole-board paradigms. The former may involve a faster or less effortful early task acquisition, since only one spatial location should be learned and memory formation is strengthened by the stressful swimming experience (Korz and Frey, 2004). In relation to this, it is also possible that the concomitant processing of both reference and working memories in the hole-board may make this task more cognitively demanding that simple reference memory learning. Finally, it is worth mentioning that the correlation reported in our study for AHN and reference memory was mainly found for AHN in the SupraDG blade. This functional and anatomical DG division shows different AHN dynamics than the InfraDG blade (Jinno, 2011) and it is the most responsive to spatial tasks (Gallitano et al., 2016).

Interestingly, the learning-induced potentiation of AHN in the Trained mice specifically involved an augmented population of immature neurons whose morphology was typical of post-mitotic neurons aged at least one week (Plumpe et al., 2006). However, the population of immature neurons with an underdeveloped-like morphology -that may still retain their mitotic capacity (Plumpe et al., 2006)- was unchanged by learning, and the PCNA+ cells were reduced. Therefore, hole-board reference memory training increased the number of immature neurons but decreased cell proliferation in the DG. Additional experiments using a birthdating marker are necessary to confirm the specific age of the young neurons that were potentiated by the hole-board learning and whether the observed effects should be attributed to increased survival and/or maturation or differentiation.

The learning-induced survival and differentiation of young neurons is a common outcome after training in aversively-motivated hippocampal tasks (Castilla-Ortega et al., 2011; Epp et al., 2013; Shors, 2014). These surviving neurons may contribute to memory acquisition, consolidation or updating, facilitating task performance (Castilla-Ortega et al., 2010; Deng et al., 2009; Trouche et al., 2009); or they could entail a neurobiological reserve to overcome future new learnings. Regarding cell proliferation, some studies have reported its reduction after water maze spatial reference memory training, but this phenomenon was partially attributed to a stress effect (Aztiria et al., 2007; Ehninger and Kempermann, 2006). Because here we employed an appetitive, low stress-inducing training spatial task, and all the experimental groups were equally exposed to the potentially stressful factors present in the behavioral protocol (i.e. mild-food deprivation, handling or maze exposure), the reduced cell proliferation reported here can only be attributed to learning. On the other hand, the key study of Dobrossy et al. (2003) reported that mastering (i.e. reaching asymptotic performance) a water maze spatial navigation task potentiated both proliferation and survival of hippocampal neurons generated during the late phase of learning. But neurons generated during the early acquisition phase were reduced, and such reduction predicted better reference memory performance. This outcome apparently differs from our study, because we report reduced proliferating cells by the end of training instead. Once again, this discrepancy may be attributed to the different learning nature of the task employed here. It is clearly demonstrated, in any case, that hippocampal neurons of different ages compete for inputs (Borgmann et al., 2016; McAvoy et al., 2016), reducing the amount of proliferating cells or even removing certain neuron populations unable to establish suitable synaptic connections (Dobrossy et al., 2003; Dupret et al., 2007) could favor the functional integration and survival of immature neurons and strengthen memory.

In addition to AHN, hippocampal expression of BDNF, IGF-II and pCREB was enhanced in the Trained mice. These factors are usually upregulated after hippocampal-dependent learning contributing to memory formation and synaptic plasticity (Cunha et al., 2010; Chen et al., 2011; Silva et al., 1998). But, to the best of our knowledge, they have not yet been investigated after hole-board spatial training. The upregulation of these plasticity-related proteins in the hippocampus may contribute to the AHN result, because BDNF, IGF-II and CREB-mediated signaling enhance survival of the developing hippocampal neurons, also promoting their maturation and functional integration into the DG circuits (Agis-Balboa et al., 2011; Merz et al., 2011; Waterhouse et al., 2012). The hippocampal neurons undergoing maturation express receptors for both BDNF and IGF-II (Agis-Balboa et al., 2011; Donovan et al., 2008) as well as high levels of pCREB that persist until the neuron matures (Merz et al., 2011).

In conclusion, both AHN and hippocampal neuroplasticity factors are potentiated by reference memory learning in a hole-board spatial navigation task. In despite of the popularity of the cognitive hole-board type tasks (van der Staay et al., 2012), this is the first evidence linking this paradigm to AHN, which has been scarcely investigated after appetitively-motivated training. Future studies may use the hole-board paradigm to corroborate AHN results obtained in aversively-motivated spatial tasks, or as a low-stress cognitive training to stimulate AHN in rodents (Henrich-Noack, 2014).

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FIGURE LEGENDS

Fig. 1. Hole-board training. (A) Experimental design. (B) Hole-board apparatus. The arena (45 x 45 cm) contained 16 equidistant holes in its central zone (5.5 cm apart, 2.5

cm diameter, 3 cm depth) and it was surrounded with transparent (25 cm high) clear Plexiglas walls. The testing room was illuminated 46 lux and several spatial cues (black cards in different geometric shapes) were placed on the walls to facilitate spatial orientation. (C) Example of an habituation session ('shaping' session), a training day schedule (including four training sessions) and the reversal trial for each experimental condition. The reversal trial was performed 45 min after the last training session on Day 10. (D,E) Reference memory ratio expressed per session and per day. (F,G) Working memory ratio expressed per session and per day. (D,F) The reversal trial disrupted reference memory performance (post hoc: $p < 0.001$ vs the last training session) but not working memory performance in the Trained mice, supporting the independence between these memory components. Post hoc LSD difference of the Trained group vs the Pseudo group: *$p < 0.05$; **$p < 0.001$. Data are expressed as mean ± S.E.M.

Fig. 2. Hole-board training increases adult hippocampal neurogenesis. (A) Representative photographs of PCNA expression. (B) Representative photographs of DCX expression. The lower portion of B shows a more highly magnified section of the above DCX expression with a black arrow that points to a Type-1 DCX+ neuron that shows a rounded, underdeveloped-like morphology with short dendritic processes. The black arrow points a Type-1 DCX+ neuron that shows a rounded, underdeveloped-like morphology with short dendritic processes. The white arrow shows a Type-2 DCX+ with a radial dendrite penetrating the granule cell layer. (C) The number of PCNA+ proliferating cells was decreased in the Trained mice. (D) There were no differences among groups in the number of the Type-1 DCX+ neurons. (E, F) The Type-2 DCX+ neurons and the total number of DCX+ cell was notably increased in the Trained mice. (G) The density of Type-2 DCX+ neurons in the SupraDG correlated with the reference memory ratio of the Trained mice on training Day 2 (that corresponded to Day 8 of experiment in **Fig. 1A**). (H) Absent correlation was found between the working memory ratio and the Type-2 DCX+ neurons in the Trained mice. Training sessions of Day 2 are shown as an example. Post hoc LSD difference of the Trained vs the Control and Pseudo groups: *$p < 0.05$; **$p < 0.001$. Data are expressed as mean ± S.E.M.

Fig. 3. Hole-board training increases hippocampal protein expression of plasticity-related markers (A) BDNF, (B) IGF-II and (C) CREB phosphorylation. Post hoc LSD difference of the Trained vs the Control and Pseudo groups: *$p < 0.05$. Data are expressed as mean ± S.E.M.

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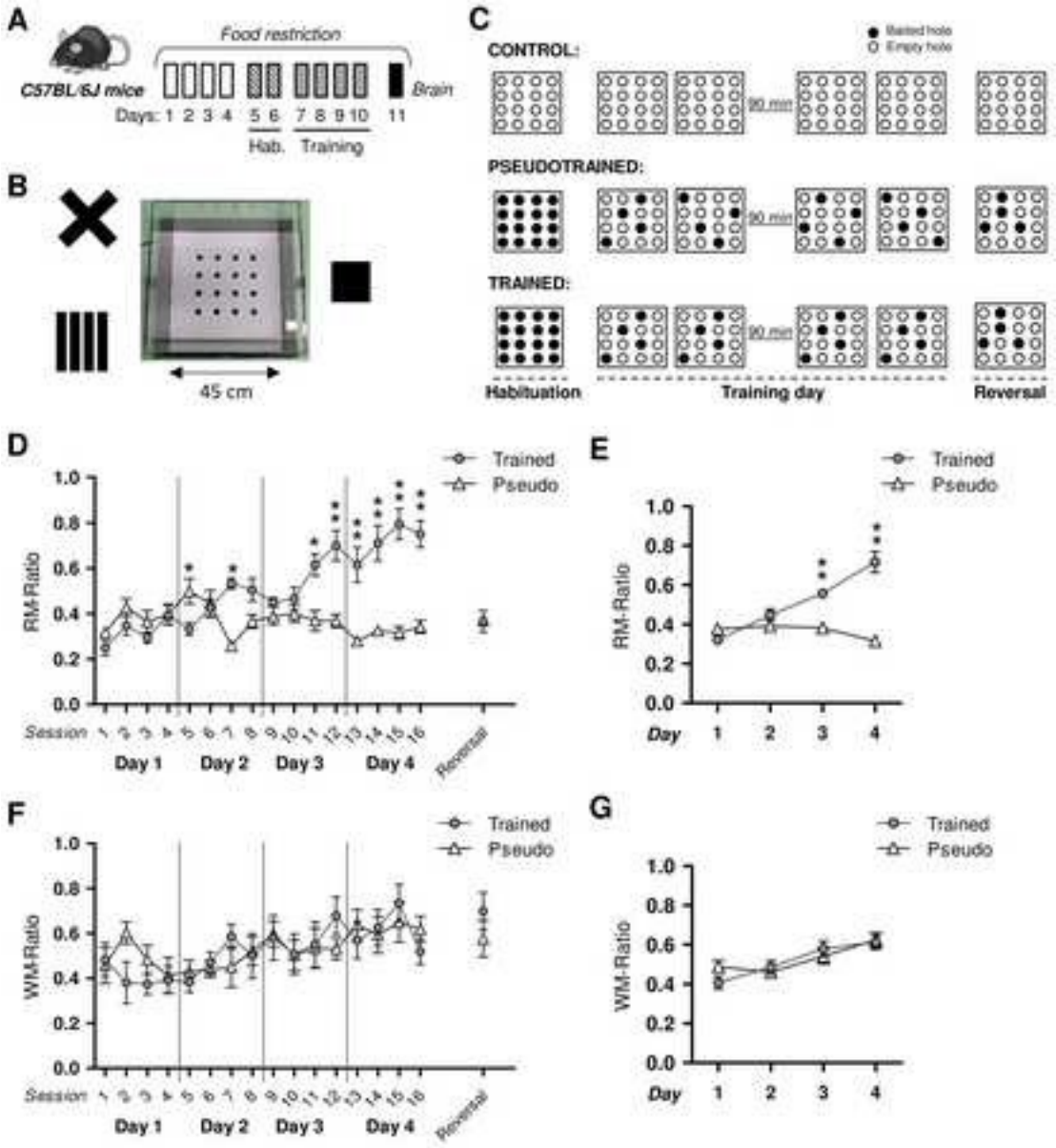

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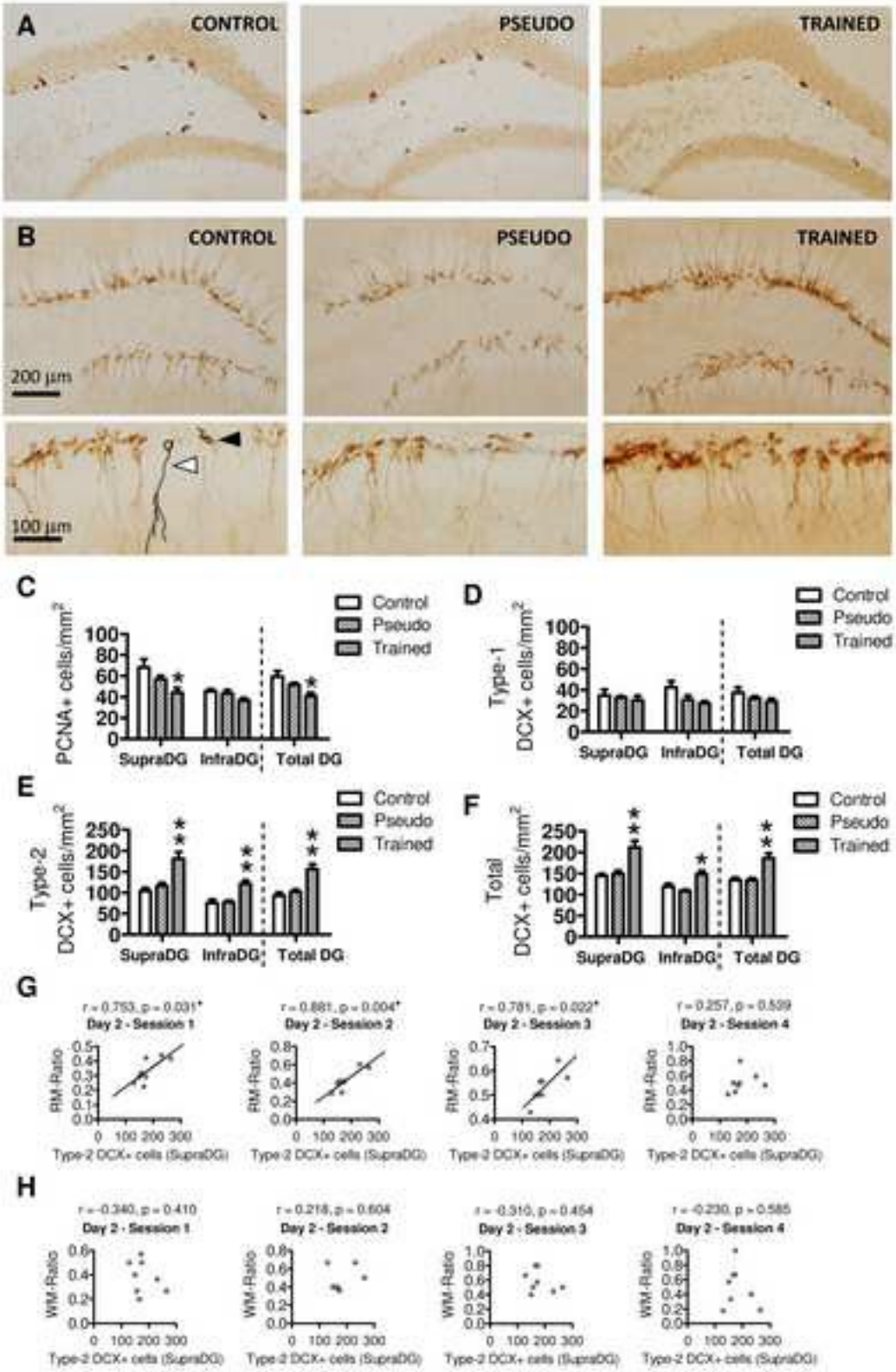

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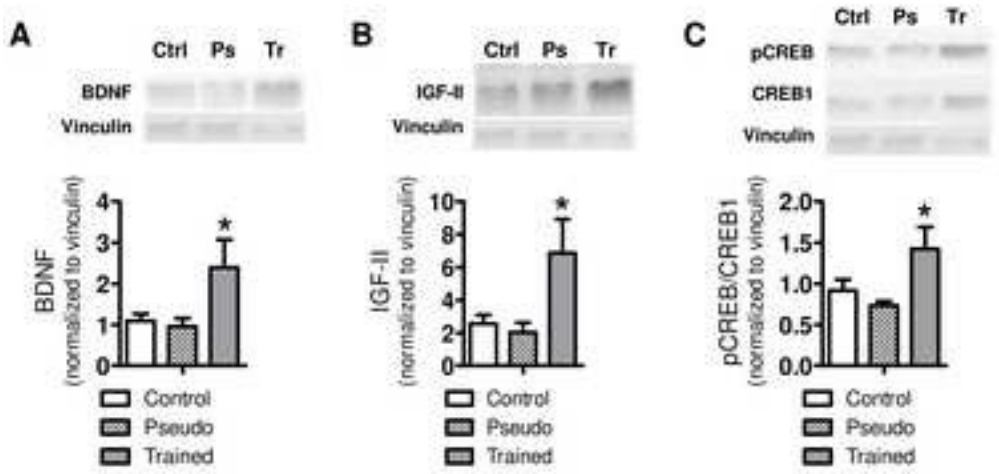

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Fear conditioning	Agis-Balboa, R.C., Arcos-Diaz, D., Wittnam, J., Govindarajan, N., Blom, K., Burkhardt, S., Haladyniak, U., Agbemenyah, H.Y., Zovoilis, A. Salinas- Riester, G., Opitz, L., Sananbenesi, F., & Fischer, A. (2011). A hippocampal insulin-growth factor 2 pathway regulates the extinction of fear memories. *The EMBO Journal, 30,* 4071-4083. Kirby, E.D., Friedman, A.R., Covarrubias, D., Ying, C., Sun, W.G., Goosens, K.A., Sapolsky, R.M., & Kaufer, D. (2012). Basolateral amygdala regulation of adult hippocampal neurogenesis and fear-related activation of newborn neurons. *Molecular Psychiatry, 17,* 527-536. Petsophonsakul, P., Richetin, K., Andraini, T., Roybon, L., & Rampon, C. (2017). Memory formation orchestrates the wiring of adult-born hippocampal neurons into brain circuits. *Brain, structure & function, 222,* 2585-2601. Pham, K., McEwen, B.S., Ledoux, J.E., & Nader, K. (2005). Fear learning transiently impairs hippocampal cell proliferation. *Neuroscience, 130,* 17-24. Restivo, L., Niibori, Y., Mercaldo, V., Josselyn, S.A., & Frankland, P.W. (2015). Development of adult-generated cell connectivity with excitatory and inhibitory cell populations in the hippocampus. *The Journal of Neuroscience: the official journal of the Society for Neuroscience, 35,* 10600-10612. Snyder, J.S., Choe, J.S., Clifford, M.A., Jeurling, S.I., Hurley, P., Brown, A., Kamhi, J.F., & Cameron, H.A. (2009). Adult-born hippocampal neurons are more numerous, faster maturing, and more involved in behavior in rats than in mice. *The Journal of Neuroscience: the official journal of the Society for Neuroscience, 29,* 14484-14495.
Active avoidance	Van der Borght, K., Meerlo, P., Luiten, P.G., Eggen, B.J., & Van der Zee, E.A. (2005). Effects of active shock avoidance learning on hipocampal neurogenesis and plasma levels of corticosterone. *Behavioural Brain Research, 157,* 23-30.
Passive avoidance	Nikolakopoulou, A.M., Dermon, C.R., Panagis, L., Pavlidis, M., & Stewart, M.G. (2006). Passive avoidance training is correlated with decreased cell proliferation in the chick hippocampus. *The European Journal of Neuroscience, 24,* 2631-2642.

Appetitive training paradigms	Manuscripts investigating the effect of learning on AHN
Dry radial 8 arm maze	Kumazawa-Manita, N., Hama, H., Miyawaki, A., & Iriki, A. (2013). Tool use specific adult neurogenesis and synaptogenesis in rodent (Octodon degus) hippocampus. *PLoS ONE, 8*, e58649. Yagi, S., Chow, C., Lieblich, S.E., & Galea, L.A. (2016). Sex and strategy use matters for pattern separation, adult neurogenesis, and immediate early gene expression in the hippocampus. *Hippocampus, 26*, 87-101.
Dry land maze	Bardi, M., True, M., Franssen, C.L., Kaufman, C., Rzucidlo, A., & Lambert, K.G. (2013). Effort-Based Reward (EBR) training enhances neurobiological efficiency in a problem-solving task: Insights for depression therapies. *Brain Research, 15,* 101-110.
T maze	Schaefers, A.T. (2015). Environmental enrichment and working memory tasks decrease hippocampal cell proliferation after wheel running – A role for the prefrontal cortex in hippocampal plasticity? *Brain Research, 22*, 125-133.
Conditioned food preference	Olariu, A., Cleaver, K.M., Shore, L.E., Brewer, M.D., & Cameron, H.A. (2015). A natural form of learning can increase and decrease the survival of new neurons in the dentate gyrus. *Hippocampus, 15,* 750-762.
Hole-Board	-

SUPPLEMENTARY TABLE 1. Table shows published manuscripts regarding the effect of learning on adult hippocampal neurogenesis in both aversive and appetitive training paradigms. Papers were searched on PubMed by the terms "(((training) OR memory) OR learning)" AND "adult hippocampal neurogenesis" and additionally, we thoroughly analysed reviews on this matter.

Aversive training paradigms		Appetitive training paradigms	
Water maze	34	Dry radial 8 arm maze	2
Eyeblink conditioning	9	Dry land maze navigation	1
Fear conditioning	6	Delayed T-Maze alternation	1
Active avoidance	1	Conditioned food preference	1
Passive avoidance	1	Hole-Board	0
TOTAL	**51 (91 %)**	**TOTAL**	**5 (9%)**

SUPPLEMENTARY TABLE 2. Manuscripts investigating the effect of learning on AHN (including proliferation, survival, maturation and/or functional integration of the adult-born hippocampal neurons). While this review does not pretend to be exhaustive, it shows a clear preference for the use of aversive learning paradigms (i.e. where the animal has to escape a stressful environment or it has to experience unpleasant stimuli such as air puffs applied to the cornea or electric shocks) over appetitively-motivated tasks (i.e. where the animal searches for natural rewards such as food or water). Methods employed for this review and the references of the manuscripts included in this table are shown in the Supplementary Table 1.