# Effects of spaced learning in the water maze on development of dentate granule cells generated in adult mice

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**Running title:** Learning-induced adult hippocampal neurogenesis in mice

Number of pages: 24

Number of Figures 8

Number of table 3

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**Keywords**: adult neurogenesis, hippocampus, spatial memory, mice, water maze

## ABSTRACT

New dentate granule cells (GCs) are generated in the hippocampus throughout life. These adult-born neurons are required for spatial learning in the Morris water maze (MWM). In rats, spatial learning shapes the network by regulating their number and dendritic development. Here we explored whether such modulatory effects exist in mice. New GCs were tagged using thymidine analogs or a GFP-expressing retrovirus. Animals were exposed to a reference memory protocol for 10 to 14 days (spaced training) at different times after newborn cells labeling. Cell proliferation, cell survival, cell death, neuronal phenotype and dendritic and spine development were examined using immunohistochemistry. Surprisingly, spatial learning did not modify any of the parameters under scrutiny including cell number and dendritic morphology. These results suggest that although new GCs are required in mice for spatial learning in the MWM, they are, at least for the developmental intervals analyzed here, refractory to behavioral stimuli generated in the course of learning in the MWM.

## INTRODUCTION

One of the major challenges in neurobiology is to understand the cellular mechanisms underlying long–term memory. The hippocampal formation (HF) is a major structure involved in spatial learning that produces long-term memory traces. Altering the integrity of the HF function by different means impairs learning and vice versa, structural and functional changes occurring in the hippocampus in the course of learning, such as synaptic remodeling and long-term potentiation, are key signatures of long-term memory processes (Bailey and Kandel, 1993;Moser, 1999). These adjustments have an adaptive value as they enlarge our behavioral repertoire by adding new skills, or altering previously acquired ones, and thus allow us to adapt to any new life situation.

The discovery of a *de novo* hippocampal production of neurons in the adult brain has raised the fascinating hypothesis that the new generated neurons are involved in hippocampal-dependent memory (Altman, 1962;Gross, 2000). In support of this hypothesis there is abundant literature showing that new neurons are recruited by spatial learning in the Morris water maze (MWM) (Kee et al., 2007) and that ablating new neurons impairs spatial learning (Dupret et al., 2008;Garthe et al., 2009). Moreover, spatial learning itself regulates adult neurogenesis in complex ways (see table 1). Indeed, it promotes the survival of adult-born neurons that are relatively mature (generated one week before training) (Drapeau et al., 2007;Dupret et al., 2007;Epp et al., 2011;Gould et al., 1999;Hairston et al., 2005;Sisti et al., 2007), induces the death of cells (Ambrogini et al., 2004) that are more immature (Dupret et al., 2007), and stimulates proliferation of precursors (Dupret et al., 2007). In addition spatial learning promotes the dendritic development of the surviving neurons in a homeostatic manner; these effects persist for several months, are specific to neurons born during adulthood and depend on the cognitive demand (Tronel et al., 2010). All these learning-evoked changes in cell number and dendritic arbors rely on the activation of NMDA receptors (Curlik and Shors, 2011;Tronel et al., 2010). One question that remains to be answered is how learning-induced activity modulates adult neurogenesis.

To answer this question and motivated by the thought that genetically-engineered mice will be used, we verified in a first step whether similar regulation exists in mice, the work cited above being carried out exclusively in rats. Using validated protocols in rats, we

therefore studied the effect of spatial learning on cell survival, cell death, cell proliferation and dendritic development in mice.

Insert Here Table 1

## MATERIALS AND METHODS

*Animals.*

We used three-month-old male C57BL/6J mice (n=101, batch 1-3, Charles River France), three-month-old female C57BL/6J mice (n=36, batch 5, Charles River France) or CD1 male mice (n=19, batch 4, Charles River France). An additional group of 7 week-old females (n=16, batch 6, Leloir institute) was used (see table 2). Males individually housed and female housed in groups of 4 per cage were maintained under a 12 h light/12 h dark cycle (lights on from 8:00 A.M. to 8:00 P.M.) in a temperature- (22 $\pm$ 3°C) and humidity-controlled facility. Animals had *ad libitum* access to food and water for 2 weeks after arrival. Experiments were performed in accordance with the European Union (2010/63/UE), the French National Committee local recommendations (Batches 1-5$^{th}$ ), and the Institutional Animal Care and Use Committee of the Fundación Instituto Leloir (Batch 6$^{th}$ ).

Insert Here Table 2

*Thymidine analog injections.*

Newly born cells were labeled by the incorporation of synthetic thymidine analogs (XdU [where X represents Cl, or I]). The learning groups received a single injection of IdU and of CldU at different time points before the onset of training (see table 2), both at equimolar doses of 50 mg BrdU/kg (CldU: 42.76 mg/kg/10mL; IdU: 57.65 mg/kg/10mL, ip). The different control groups were injected with XdU within the same period. CldU (Sigma) was dissolved in NaCl (0.9%) and IdU (Sigma) was dissolved in 400µl 1N NH4OH and 9.6 ml of NaCl.

*Production of viral vectors.*

Retroviral particles based on the Moloney murine leukemia virus were assembled using three plasmids containing the envelope (CMV-vsvg), viral proteins (CMV-gag/pol), and GFP as in previous work (Laplagne et al., 2006). Plasmids were transfected into 293T

cells using deacylated polyethylenimine (PEI). Virus-containing supernatant was harvested 48 h after transfection and concentrated by two rounds of ultracentrifugation. Virus titer was typically ~$10^5$ particles/µl.

*Stereotaxic surgery for retroviral delivery.*

Seven or seventeen days before behavioral training (table 2), mice were anesthetized (150 µg ketamine + 15 µg xylazine in 10µl of saline / g) and virus (1 µl at 0.15 µl/min) was infused into the right DG using sterile microcapillary calibrated pipettes (Drummond Scientific) at the following coordinates: AP: -2 mm, L: -1.5 mm lateral, V:-1.9 mm from Bregma.

*Morris water maze training.*

Mice were tested in a Morris water maze (MWM) as described previously (Dupret et al., 2008). The MWM consisted of a circular tank built of white plastic (150 cm diameter, 60 cm height) filled with water (20 ± 1 °C) that had been made opaque by the addition of a non-toxic white cosmetic adjuvant. During the pretraining session, mice were allowed to swim for 60 sec in the water maze without a platform. Then, they were placed upon the platform raised at the surface of the water where they were required to stay at least for 15 sec. Finally, they were allowed to swim for a 30 sec period that was ended by a climbing trial onto the hidden platform. The platform was never localized in the quadrant used for the training sessions. At the end of the pre-training, all mice swam actively and were able to climb onto the platform and stay on it for 15 sec. During training, mice in the Learning group (L) were required to locate the hidden platform (1.5 cm under the water in a fixed location) from variable random start positions. They received 3 daily trials separated by a 5 minute inter-trial interval. A trial terminated when the animal climbed onto the platform. Mice that failed to find the platform within a 60 second cut-off time were placed onto the platform by the experimenter and had to stay there for 15 sec before being placed back in their home cage for the 5 min inter-trial interval. The releasing point (starting point) differed for each trial and different sequences of releasing points were used day to day. Twenty four hours after completion of training, the hidden platform was removed and memory for the platform location was assessed during a probe test. During this test mice

were allowed to freely swim in the water maze for 60 seconds and performances were assessed by time spent in the target quadrant where the platform was located, and by total number of crossings in the exact platform location. In the first experiments (Table 2), one control group was used (C) consisting of animals that were transferred to the testing room at the same time and with the same procedures as the learning group but that were not exposed to the water maze. In experiment 5-6$^{Th}$, an additional group of mice that was trained to find a visible platform (VP) in a fixed location was used.

*Immunohistochemistry*

One day after the probe test, trained animals were perfused transcardially with 30 ml PBS, pH 7.3, containing heparin ($5.10^4$ IU/ml), followed by 30 ml of 4% paraformaldehyde in 0.1 M of phosphate buffer, pH 7.3. The different age-matched control groups were killed the same day. After 1 week of post-fixation in PAF, brains were sliced using a vibratome (batchs 1-5$^{th}$) or a cryostat (batch 6$^{th}$). Sections were conserved at -20°C in cryoprotectant solution until processing. Free-floating coronal sections (40 µm for batches 1-5 and 60 µm for batch 6) were processed using a standard immunohistochemical procedure (Dupret et al., 2007;Dupret et al., 2008) to visualize the thymidine analogs (CldU, IdU) in alternating one-in ten sections using different anti-BrdU antibodies from different vendors (For CldU: rat primary at 1/1000, Accurate Chemical and Scientific Corporation; For IdU: mouse primary at 2000, BD Biosciences). Bound antibodies were visualized with biotin-labeled donkey anti-rat antibodies (1/1000, Jackson for CldU) or biotin-labeled horse-antibodies (1/200, Abcys for IdU). Cell proliferation and cell death were studied using rabbit antibodies directed against the phosphorylated form of histone3 (pH3; 1/500, Upstate, Lake Placid, NY, USA) or against the activated-caspase3 (1/400, Cell signaling). Bound antibodies were visualized with biotin-labeled goat anti-rabbit antibodies (1/200, DAKO). For each antibody, sections from all animals were processed in parallel, and immunoreactivities were visualized by the biotin–streptavidin technique (ABC kit; Dako) using 3,3-diaminobenzidine as chromogen.

*Analysis of cell numbers on DAB stained sections*

Cell counting was performed on coded sections when the experimenter performed the behavioral training, and on non-coded sections when the experimenter was naïve to the training procedure. The number of X-immunoreactive (IR) cells in the supragranular and infragranular blades of the left DG was estimated on counts made by systematic random sampling of every tenth section along the rostrocaudal axis of the hippocampal formation using a modified version of the optical fractionator. All of the X-IR cells were counted on each section and the resulting numbers were tallied and multiplied by the inverse of the sections sampling fraction (1/ssf = 20). When counting was performed in both sides the numbers were tallied and multiplied by 1/10.

*Analysis of the dendritic arbor on DAB stained sections*

The dendritic arbor was first analyzed in batch 1 by analyzing IdU-Doublecortin (DCX) double labeled cells. Briefly, sections were first incubated for 48h with a rabbit anti-DCX primary antibody (1/2000, Abcam); upon incubation with a biotinylated goat anti-rabbit secondary antibody, immunoreactivity was visualized with DAB as chromogen, leading to brown staining of immunoreactive cells. Sections were then treated with 2N HCl 37°C for 30 min and incubated for 48h with a rat anti-BrdU (1/500, BD Biosciences) antibody. Biotinylated secondary antibodies (goat anti-rat; 1/1000, Jackson) were revealed with Vector SG as chromogen, leading to a blue staining.

Retrovirally-labeled cells (batch 5) were visualized on 40 µm-thick sections using the standard immunohistochemical procedure described above using an eGFP antibody (1/500; BD PharMingen).

Morphometric analysis of IdU-DCX and virus-labeled neurons were performed with a x100 objective, using a semi-automatic neuron-tracing system (Neurolucida; Microbrightfield, Colchester, VT, USA). Briefly, neurons were selected based on the following criteria: (i) neurons exhibited vertically-orientated dendrites that extended into the dentate molecular layer and (ii) dendrites of selected neurons had minimal overlap with the dendrites of adjacent cells in order to unambiguously trace the dendritic tree. Data for various metric measurements were calculated, including the cell body area and total dendritic length.

*Analysis of the dendritic arbor and spines using Immunofluorescence.*

For batch 6, one-in-six sections (thickness 60 µm) were incubated with rabbit polyclonal antibodies directed against GFP (1/500; Invitrogen) and bound antibodies were visualized with a donkey anti-rabbit Cy3 (1/250; Jackson ImmunoResearch). Dendritic length and spine density were analyzed using confocal microscopy (Zeiss LSM 510). For dendritic length measurements, images were acquired (40X; NA, 1.3; oil-immersion) taking z-series including 35 – 50 optical slices, airy unit = 1 at 0.8 µm intervals. Dendritic length was measured from projections of three-dimensional reconstructions onto a single plane using Zeiss LSM image Browser software. For spine counts, selected GFP-IR dendritic segments were located in the middle third of the molecular layer. High-resolution images were acquired using a 63X objective (NA, 1.4; oil-immersion) and taking z-series of 40 – 200 optical slices of airy unit = 1 at 0.1 µm intervals as previously described (Morgenstern et al., 2008). All dendritic spines within 2 µm from the shaft were counted on projections onto a single plane. Four to 9 dendritic segments (40 –50 µm in length) were imaged per DG region per mouse (with 6 to 10 mice per experiment), choosing fragments at a distance ≥150 µm from each other to avoid sampling multiple dendrites from single neurons. A similar number of fragments were chosen from the infrapyramidal and suprapyramidal blades.

*Analysis of cellular phenotypes*

To examine the phenotype of XdU-IR cells, one-in-ten sections were incubated with BrdU antibodies from different vendors (For IdU: mice antibodies from Becton Dickinson at 1/2000 ; For CldU : rat antibodies from Accurate at 1/11000), which were revealed using CY3-anti-mice or CY3–anti-rat antibodies (1/1000; Jackson Immunoresearch). Sections were then incubated with goat anti-DCX antibodies (1/1000; Santa Cruz Biotechnology), which were visualized with an Alexa-488 anti-goat IgG (1/1000; Jackson). The percentage of XdU-labeled cells expressing DCX was determined throughout the DG using a confocal microscope with helium–neon and argon lasers (DMR TCSSP2AOBS; Leica).

*General procedures*

In the first batch of animals (first experiment), we examined whether spatial learning (L) influences the survival of neurons born 7 or 3 days before training, cell death and cell proliferation in male C57BL/6J mice. Animals were sacrificed one day after a probe test performed at the end of the learning phase. The Control group (C), which was not exposed to the task, was killed at the same time.

In the second batch of animals (second experiment), we examined whether spatial learning (L) influences the survival of neurons born 15 or 10 days before training, as well as cell death and cell proliferation in male C57BL/6J mice. Animals (L, C) were sacrificed as previously described.

In the third batch of animals (third experiment), we examined whether spatial learning (L) influences the survival of neurons born 28 or 21 days before training in male C57BL/6J mice. Animals (C, VP, L) were sacrificed as previously described.

In the fourth batch of animals (fourth experiment), we examined whether spatial learning (L) influences the survival of neurons born 15 or 3 days before training in CD1 outbred mice. Animals and their controls (C) were sacrificed as previously described.

In the fifth batch of animals (fifth experiment), we examined whether spatial learning (L) influences the dendritic development of genetically-labeled neurons born 7 days before training in female C57BL/6J mice. Animals (C,VP,L) were sacrificed as previously described.

In the sixth batch of animals (sixth experiment), the influence of spatial learning (L) on the dendritic development of genetically labeled neurons born 17 days before training in female C57BL/6J mice. Animals (C,VP,L) were sacrificed as previously described.

*Statistical analysis*

Differences between groups were analyzed with an ANOVA or a Student *t* test. Relationships between behavioral scores and cell numbers were evaluated using the Pearson correlation test.

## RESULTS

### Spatial learning does not affect survival of neurons born one week before training

In the first experiment (Table 2, batch 1) newborn GCs were labeled with two different thymidine analogs according to a protocol established in rats (Dupret et al., 2007): male C57BL/6J mice were injected with IdU and CldU 7 days and 3 days before training, respectively.

Insert Here Figures 1,2

Animals learned the task as shown by the decrease in latency (Fig. 1A, $F(9,144)=9.84, p<0.001$) or distance travelled (data not shown, $F(9,144)=7.29$, $p<0.001$) to find the platform. Cells labeled with IdU and CldU were counted to monitor changes in survival induced by learning (Fig. 2 A,B). No differences were found in the number of IdU-IR (Fig. 1B, $t_{28}=1.25$, $p>0.05$) and of CldU-IR cells (Fig. 1C, $t_{28}=0.78$, $p>0.05$) between the learning and the control groups. As a consequence the number of apoptotic cells expressing activated-caspase3 (Fig. 2C) was similar between groups (Fig. 1D, $t_{28}=1.28$, $p>0.05$). In addition, the number of proliferating cells revealed by the number of cells expressing $pH_3$ (Fig. 2D) was not influenced either by spatial learning (Fig. 1E; $t_{28}=0.31$, $p>0.05$). Finally, we examined the phenotype of IdU and CldU cells and found that more than 90 % of these cells colocalized with the immature neuronal marker doublecortin (DCX; Fig. 2 E,F; Table 3).

Insert Here Table 3

This observation indicated that thymidine analog labeled-cells were mostly neurons and that learning did not influence neuronal differentiation. Given the existence of large inter-individual differences in the rate of learning we searched for correlations between behavioral scores and cell numbers. Independently of the way the behavioral scores were calculated (mean of the latency or mean of the distance over the entire training period or

over the last three days of training) no correlation was found with IdU or CldU-labeled cells (Fig. 3) or the number of apoptotic or proliferating cells (data not shown).

Insert Here Figure 3

The hypothesis behind this study is that learning would influence development and survival of newborn cells through activation of inputs and/or outputs that might influence their integration within the network. In the experiment described above, none of the parameters used to study different aspects of neurogenesis was influenced by learning. Given that adult-born neurons are slower maturing in mice than rats (Snyder et al., 2009), we modified our protocol in order to tag older cells at the time of training (Table 2, batch 2). Then, a similar experiment was carried out, now with GCs labeled 15 or 10 days before training (Fig. 4). Although animals learned the task (*latency*: $F(9,153)=9.84$, $p<0.001$; *distance*: $F(9,153)=7.94$), no differences were found between control and trained groups in the number of neurons labeled at any age (CldU: $t_{29}=0.08$, $p>0.05$; IdU: $t_{29}=1.24$, $p>0.05$), or the level of apoptosis ($t_{29}=0.51$, $p>0.05$) or proliferation ($t_{29}=1.07$, $p>0.05$). There was no correlation either between behavioral scores and cell numbers (data not shown).

Insert Here Figure 4

In a third experiment (Table 2, batch 3), we again modified the protocol of injection of IdU and CldU (respectively 28 and 21 days before MWM training) and included a visible platform (VP) group, where mice were allowed to swim towards a visible platform. We found no effect on cell survival (Fig. 5, CldU: $F(2,37)=0.19$, $p>0.05$; IdU: $F(2,37)=0.097$ $p=0.91$). Apoptosis and cell proliferation were not measured, since in the previous experiment these parameters were not influenced by learning.

Insert Here Figure 5

We finally investigated whether the effect of spatial learning was dependent on the mouse strain. In particular, we used an outbred strain (CD1) given that all experiments in rats were done with outbreds. In this case, IdU and CldU were injected 15 or 3 days before training (Table 2, Fig. 6A). No effect was found in the number of IdU-IR cells (Fig. 6B, $t_{17}$=0.53, p>0.05), CldU-IR cells (Fig. 6C, $t_{17}$=0.72, p>0.05), apoptotic cells (Fig. 6D, $t_{17}$=0.53, p>0.05) and proliferating cells (Fig. 6E, $t_{17}$=1.74, p>0.05).

Insert Here Figure 6

In summary, these four experiments showed that spatial learning did not modify the survival of cells generated within one month before training, did not eliminate new cells from the dentate network, and did not induce a compensatory increase in cell proliferation. Given that we have shown in rats that spatial learning can regulate neuronal dendritic development without influencing cell survival (Lemaire et al., 2012), we then focused on dendritic arbors.

**Dendritic morphology of new GCs is not modified by spatial learning**

In rats we have shown that the dendritic arbor of cell generated one week before training is increased by spatial learning (Tronel et al., 2010). So in a first step, we analyzed on representative animals of the first experiment (Table 2, Batch 1), the dendritic arbors of IdU-labeled cells expressing DCX (Fig 2G) according to a previously described method (Tronel et al., 2010). A quantitative analysis using Neurolucida revealed that dendritic length (C: 137.2 ± 11.4; L: 156.3± 10.8 µm, $t_{12}$=1.21, p>0.05), and area of the cell body (C: 44 ± 1.7; L: 43.2 ± 1.4 µm², $t_{12}$=0.33, p>0.05) were not influenced by spatial learning.

We next performed a similar experiment but in this case the cells generated one week before training were labeled using a GFP-expressing retrovirus (Table 2, Batch 5, Fig. 2H). Given that the effect of learning on cell survival has been shown to be more pronounced in female rats compared to male (Dalla et al., 2009) and that individual housing (Martin and Brown, 2010) may reduce the ability of the brain to respond to environmental challenges, female C57BL/6J mice housed in groups of 4 were used. Although female mice learned the task (Fig. 7A), dendritic tree measurements revealed no differences between

groups, confirming IdU-DCX results and indicating that dendritic length is not modified by learning in the MWM (Fig. 7B, $F(2,23)=0.006$, $p>0.05$).

Insert Here Figure 7

In the last experiment also performed in group-housed female mice GCs were labeled 17 days before training in the MWM (Table 2, Batch 6, Fig. 8A). Thus, morphological analysis was performed in four-week-old GCs (Fig. 8B). We found a similar dendritic length for all three groups (Fig. 8C, $F(2,10)=0.55$, $p>0.05$). Dendritic spine density, the morphological correlate of glutamatergic synapses impinging onto GCs, was also analyzed in this experimental group (Fig. 2J). Learning did not modulate the number of input connections in a global manner (Fig. 8D, $F(2,10)=1$, $p>0.05$).

Overall these results strengthen the notion that learning does not influence the maturation of neurons generated within one month before training in adult mice.

Insert Here Figure 8

## DISCUSSION

In this work we have observed that the MWM learning paradigm produces no effects on the proliferation, survival/death and morphology of newborn GCs of the adult mouse dentate gyrus. To study the influence of spatial learning on the different steps of neurogenesis, systemic injection of thymidine analogs followed by exposure to the MWM was used and combined to specific markers of cell proliferation or death. To investigate whether spatial learning modulates more subtle neuronal characteristics that could be revealed by morphology, we either analyzed the morphology of IdU-DCX labeled cells or performed retroviral labeling of GCs to expose them to the MWM at different ages. In both cases, dendritic arborization was similar for all neurons of a given age, whether or not exposed to the MWM. In addition spine density was not either influenced by training in the water maze. So, independently of the method used, no differences were found between mice that learned spatial information and those that did not.

These results are surprising given the previous observations that learning the water maze regulates cell numbers in a complex way (see new table I). These effects were not observed when animals were exposed to the pool without a platform or when they were trained to find a visible platform, a task that does not require the integrity of the hippocampus indicating that stress or a "light" exercise (swimming for a few minutes) are not involved in the learning-induced regulation of cell birth/cell death and cell proliferation. Spatial learning also promotes an increase in dendritic tree length and spine density in immature and mature adult-born neurons. All these studies were done in rats (male outbred SD singly housed in our experiments or housed in groups of 5-6 (Gould et al., 1999;Shors et al., 2012) which are not averse to aquatic environments (Finlay and Sengelaub, 1981). It is to note that in female SD (Chow et al., 2013) and in male Long-Evans rats the most frequently used strain for memory studies (Epp et al., 2011;Snyder et al., 2005), spatial learning did not influence cell survival. In another study, it was suggested that such lack of effect may be due to the regiment of BrdU labeling (Mohapel et al., 2006).

One possible explanation for the discrepancy observed between rats and mice is linked to the task that we used, which may have induced a greater stress response in mice and thus mask a potential pro-surviving effect. Indeed, mice are primarily terrestrial

(Whishaw and Tomie, 1996) and display poorer performance than rats in wet mazes that are more stressful for them (Lipp et al., 1987;Whishaw and Tomie, 1996). Supporting this view it has been shown that learning-induced increase in corticosterone levels is higher in the MWM compared to the dry Barnes maze (Harrison et al., 2009). In fact the Barnes maze induced a modest increase in corticosterone that facilitated proficient learning. In contrast, the MWM induced a modest increase in corticosterone in some mice but a larger increase in others that were less efficient to find the hidden platform; this observation suggests that the elevated corticosterone impaired learning in mice with a greater stress response (Harrison et al., 2009). In addition to the task itself, the water temperature that we used (20°C) might be stressful for mice (in contrast to what has been observed in rats) given their small body size. As a consequence, the stressing conditions (and the more prolonged release of corticosterone) induced by the wet maze (in our experimental conditions) might have counterbalanced an accelerated maturation induced by learning in mice.

Another confounding variable could be the influence of physical exercise (or fatigue). Indeed, an increase in the survival of cells generated 8 days before exposure to the task was reported using a mass training protocol (Trouche et al., 2009). These effects were, however, significantly different from those observed in the swim control group only one month after learning (Trouche et al., 2009). More recently, an increase in cell survival was observed using a delayed matching to place protocol in the water maze that is more cognitively demanding (Haditsch et al., 2013), but it is unclear whether this effect is specific since control groups for stress or physical exercise were lacking.

Another explanation may be linked to differences in the strength of learning that may be weaker in mice, as reflected by the long training they have to go through to reach performances similar to those observed in rats. Indeed, we and others have shown that learning increases cell survival (or cell death depending of the age of the cell at the time of training) only when that learning is successful. This conclusion was reached either by using group level analysis that consists in categorizing animals either as good learners or poor learners (Döbrössy et al., 2003;Drapeau et al., 2003;Drapeau et al., 2007) or by relating individual differences in learning group (only) to the number of proliferating or surviving

cells (Drapeau et al., 2003). As discussed recently by Lazic and collaborators (Lazic et al., 2014), group level associations cannot be used to infer individual level association and individual level analysis should not be performed through all of the data, without regard for the experimental groups. Using an individual level analysis (in the learning groups for each experiment separately), we were unable to find any correlation (positive or negative) between the behavioral scores and cell numbers.

Furthermore, it has been shown that learning a conditioned response will preferentially rescue new neurons from death when learning is difficult to achieve (Curlik and Shors, 2011). Our results do not fit with these observations. Indeed, when compared to our own data obtained in rats the MWM is considerably more difficult to learn for mice, as a much longer interval is required for mastering the task. Even under those circumstances we did not observe modulatory effects of learning on cell survival. To strengthen this notion, rats trained in the water maze with spaced trials –that tend to learn faster and remember longer- did not possess significantly more cells than animals exposed to the same amount of massed trials (Sisti et al., 2007).

Alternatively, adult-generated neurons may be less important for navigation through space in mice compared to rats. This hypothesis is supported by the observation that spatial learning in the water maze causes a greater increase in the proportion of newborn neurons expressing Zif268 in rats compared to mice (Snyder et al., 2009) and that impaired adult neurogenesis in mice has rendered highly variable outcomes in the water maze (Koehl and Abrous, 2011;Marin-Burgin and Schinder, 2012). In contrast, so far all studies performed in mice have shown that ablating adult neurogenesis impairs behavioral spatial pattern separation (Clelland et al., 2009;Sahay et al., 2011;Tronel et al., 2012). These data suggest that adult generated neurons in mice may be more specialized in paradigms related to spatial pattern separation. Clearly, studies directly comparing the relationship between neurogenesis and learning processes in rats and mice are missing,

It is important to note that developing GCs of the adult mouse hippocampus are, under specific conditions, highly sensitive to network activity. In fact, there are physiological and pathological conditions that modulate the development of adult born GCs. Voluntary running, seizures and chronic antidepressants are all conditions that

accelerate neuronal maturation (Overstreet-Wadiche et al., 2006;Piatti et al., 2011;Santarelli et al., 2003;Steib et al., 2014;van Praag et al., 1999), in a manner that is strongly dependent of the electrical activity of the network (Ge et al., 2006;Piatti et al., 2011). However, given that adult-generated neurons in mice mature slower than in rats, we cannot exclude that the critical period within which cell survival might be influenced by learning extends beyond 4 weeks or/and the effects of learning are delayed in time, i.e. appeared several weeks after the end of training. In support of this, a specific pro-surviving effect of massed training in the water maze has been observed one month after training (Trouche et al., 2009).

The difference illustrated here between mice and rats is not unprecedented. For example, learning to find a hidden platform using a protocol similar to that used in the present study (and aimed to measure reference memory) induces growth of the mossy fiber system in rats (Ramirez-Amaya et al., 2001), but not in mice (Rekart et al., 2007). Interestingly, mice and rats rely on different strategies to solve the water maze, which could be linked to different implementation of the hippocampal circuitry. Thus mice were described to primarily use egocentric non spatial cues to solve the maze (Whishaw, 1995). Other differences in their hippocampal circuitry have been reported, and for instance the entorhinal cortex of rats project contra-and ipsi-laterally to the hippocampus whereas that of mice projects only ipsi-laterally (van Groen T. et al., 2002). Evolutionary divergence between mice and rats has also been reported for the olfactory bulb (Hendriksen et al., 2014). These differences could be due to the evolutionary distance between mice and rats, only 90% of the rat genome having its counterpart in the mouse genome (Gibbs et al., 2004). The adage that “a mouse is not a small rat” when studying memory is also relevant in the search of models and treatments in psychiatry disorders such as depression (Cryan and Mombereau, 2004).

In conclusion, these data reveal that the structural plasticity of adult-born dentate neurons in response to learning differs dramatically between mice and rats. These findings, together with previous observations (Snyder et al., 2009), emphasize the need to carefully consider species differences when studying adult neurogenesis.

**Acknowledgments**

The microscopy was done in the Bordeaux Imaging Center a service unit of the CNRS-INSERM and Bordeaux University, member of the national infrastructure France BioImaging. The help of P Legros is acknowledged. A.F.S. is an investigator of the Argentine Research Council (CONICET), and M.F.T. was supported by a CONICET fellowship. This work was supported by grants from the Argentine Agency for the Promotion of Science and Technology (PICT2010-1798 to AFS), from Inserm, Région Aquitaine, Agence Nationale pour la Recherche (MemoNeuro ANR2010-BLAN-1408-01 to D.N.A.) and from CONICET and ECOS SUD-Argentina (to A.F.S. and D.N.A.).

## FIGURE LEGENDS

**Table 1. Review of experiments performed in rats to study the influence of learning on the development of new dentate neurons.** Day: D. Month: M. Training: T. The sign "-" refers to "before training". If not indicated, animals received a spaced training.

**Table 2. Summary of the different experiments.** Male or female mice (C57BL/6J or CD1) housed individually or in groups were injected with IdU (7, 15 or 28 days) or CldU (3,10 or 21 days) or GFP-expressing retrovirus (7 or 17 days) before learning. Two days after completion of training, animals were sacrificed for immunohistochemistry. Days: D, Training=T. The sign "-" refers to "before training".

**Table 3: Spatial learning does not influence neuronal differentiation.** Animals of the first batch were injected with IdU and CldU seven or three days before training respectively. Most of the cells expressed DCX, a marker of immature neurons, and the percentage of XdU+ cells expressing DCX was similar in both Control and Learner group.

**Figure 1. Survival of cells generated 7 or 3 days before training is not affected by spatial learning.** A. Schematic representation of the experiment (upper panel) and latency to find the escape platform (bottom panel). B. Number of IdU-IR cells (born 7 days before training). C. Number of CldU-IR cells (born 3 days before training). D. Apoptotic cell death measured by the number of activated-caspase3-IR cells. E. Cell proliferation measured by pH3-IR cells. C: control animals not exposed to the pool. L: animals trained to find a hidden platform.

**Figure 2. Examples of adult-born neurons in the mouse dentate gyrus.** A. IdU-labeled cells. B. CldU-labeled cells. C. Dying cells expressing the activated caspase 3 in a thionin counterstaining. D. Dividing cellsvisualized by pH3 staining. E. IdU-labeled cells (in red) expressing DCX (in green). F. CldU-labeled cells (in red) expressing DCX (in green). G. The arrow points to an IdU-labeled cell stained in blue (vector sg) and expressing DCX in brown (DAB). H. Neuron labeled with a GFP-expressing retrovirus. Scale bar: A-G10 μm. H: 20 μm.

**Figure 3. Correlation between the number of IdU-IR cells (left panel) and CldU-IR cells (right panel) and behavioral scores (**mean of the latencies (a,b,e,f) or mean of the distances (c,d,g,h) necessary to find the hidden platform over the entire training period (a,c,e,g) or over the last three days of training (b,d,f,h).

**Figure 4. Survival of cells generated 15 or 10 days before training is not affected by spatial learning.** A. Schematic representation of the experiment (upper panel) and latency to find the escape platform (bottom panel). B. Number of IdU-IR cells (born 15 days before training). C. Number of CldU-IR cells (born 10 days before training). D. Apoptotic cell death measured by the number of activated-caspase3-IR cells in a thionin counterstaining. E. Cell proliferation measured by pH3-IR cells. C: control animals not exposed to the pool. L: animals trained to find a hidden platform.

**Figure 5. Survival of cells generated 28 or 21 days before training is not affected by spatial learning.** A. Schematic representation of the experiment (upper panel) and latency to find the escape platform (bottom panel). B. Number of IdU-IR cells (born 28 days before training). C. Number of CldU-IR cells (born 21 days before training. C: control animals not exposed to the pool. VP: animals trained to find a visible-platform. L: animals trained to find a hidden platform.

**Figure 6. The survival of immature neurons in CD1 mice is not influenced by spatial learning.** A. Schematic representation of the experiment (upper panel) and Latency to find the escape platform (bottom panel). B. Number of IdU-IR cells (born 15 days before training). C. Number of CldU-IR cells (born 3 days before training). D. Apoptotic cell death measured by the number of activated-caspase3-IR cells. D. Cell proliferation measured by pH3-IR cells. C: control animals not exposed to the pool. L: animals trained to find a hidden platform.

**Figure 7. Development of the dendritic arbor of neurons born one week before training is not influenced by spatial learning.** A. Schematic representation of the experiment (upper panel) and latency to find the escape platform (bottom panel). B. Dendritic tree length of GFP-labeled neurons. C: control animals not exposed to the pool. VP: animals trained to find a visible-platform. L: animals trained to find a hidden platform.

**Figure 8. Development of dendritic arborisation of neurons born seventeen days before training is not influenced by spatial learning.** A. Schematic representation of the experiment (upper panel) and latency to find the escape platform (bottom panel). B. Illustration of GFP-labeled neurons (scale = 20 μm) and their spines (scale = 2 μm) (lower panel). C. Dendritic tree length quantification of GFP-labeled neurons. D. Spine density quantification of GFP-labeled neurons. C: control animals not exposed to the pool. VP: animals trained to find a visible platform. L: animals trained to find a hidden platform.

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| Experiment title | Strain | Sex | Rat's age | Dose | Days of labeling | Duration of training | Time of killing post-T | Results | Reference |
|---|---|---|---|---|---|---|---|---|---|
| ***Spatial learning increases cell survival*** | | | | | | | | | |
| Cells born **1 week before** training | SD | ♂ | 300–350 g | 200 | BrdU -D7 | 4 | D 5 | L>HC=C=Y | (Gould et al., 1999) |
| | SD | | | 2x100 | BrdU -D7 | 2 x 4 trials/D for 4D | D5 | L>C | (Hairston et al., 2005) |
| | SD | ♂ | 2M | 50 | IdU -D7 | 6 | D7 | L>HC=C=Y | (Dupret et al., 2007) |
| | SD | ♂ | 70 D | 200 | BrdU -D7 | 4D with spaced or 4D with massed T pooled | D6<br>D14 | GL>BL=HC<br>GL>BL | (Sisti et al., 2007) |
| **Sex** effect of training | SD | ♂♀ | | 200 | BrdU -D6 | 5 | D16 | ♂ L>HC; ♀ L=HC | (Chow et al., 2013) |
| **Strain** effect of training | SD/LE | ♂ | 50-75 | 200 | BrdU -D7 | 5 | D16 | SD: L>C ; LE: L=C | (Epp et al., 2011) |
| Effects in **old** rats | SD | ♂ | 21 M | 50 | BrdU -D13-D9 | 8 | D9 | GL>BL=HC | (Drapeau et al., 2007) |
| Effects on **mature** new neurons | SD | ♂ | 2M | 3x100 (1/D) | BrdU -2M, -4M | 6 | D7 | L=HC | (Lemaire et al., 2012) |
| ***Spatial learning increases cell death*** | | | | | | | | | |
| Cells produced during **the learning phase** | SD | ♂ | 2M | 50 | BrdU D1-D4 | Complete: 8<br>Short training : 4<br>Partial training : 4 | D9<br>D5<br>D9 | L>HC=Y<br>L=HC=Y<br>L=HC=Y | (Döbrössy et al., 2003) |
| Effect **in old rats** | SD | ♂ | 21 M | 50 | BrdU D1-D5 | 12 | D13 | GL<BL=HC | (Drapeau et al., 2007) |
| Cells produced **less than 5D** before learning | | | | 50 | BrdU -D3, -D4<br>CldU -D3L | 5<br>6 | D5<br>D7 | L<HC<br>L<HC | (Dupret et al., 2007) |
| Time course of **apoptosis** | SD | ♂ | 2M | | | D3, D4, D5, D6, D8 | 1D after T | L>HC from D4 | (Dupret et al., 2007) |
| | SD | ♂ | 5M | | | 2 x5 trials for 5D | D8 | L>HC | (Ambrogini et al., 2004) |
| ***Spatial learning increases cell proliferation*** | | | | | | | | | |
| Cells produced during **the asymptotic phase** of learning | SD | ♂ | 2M | 50 | BrdU D5-D8 | 8 | D9, D35 | L>HC=Y | (Döbrössy et al., 2003) |
| | SD | ♂ | 2M | 50 | BrdU D3-D5 | 5 | D6 | L>HC=Y | (Lemaire et al., 2012) |
| Time course (Ki67) | SD | ♂ | 2M | | | D3, D4, D5, D6, D8 | 1D after T | L>HC from D5 | (Dupret et al., 2007) |
| ***Spatial learning increases dendritic development*** | | | | | | | | | |
| Neurons immature at the time of T | SD | ♂ | 2M | 50 | XdU –D7, -D3<br>RetroV -D7 | 6<br>6 | D7, D36, D66 | XdU-Dcx cells: L>HC<br>GFP cells: L>HC | (Tronel et al., 2010) |
| Neurons produced during T | SD | ♂ | 2M | 50 | BrdU D1-D4 | 8 | D9 | BrdU-Dcx cells: L>HC | (Tronel et al., 2010) |
| Neurons mature at the time of T | SD | ♂ | 2M | | RetroV -2M,4M | 6 | 1D after T | L>HC | (Lemaire et al., 2012) |

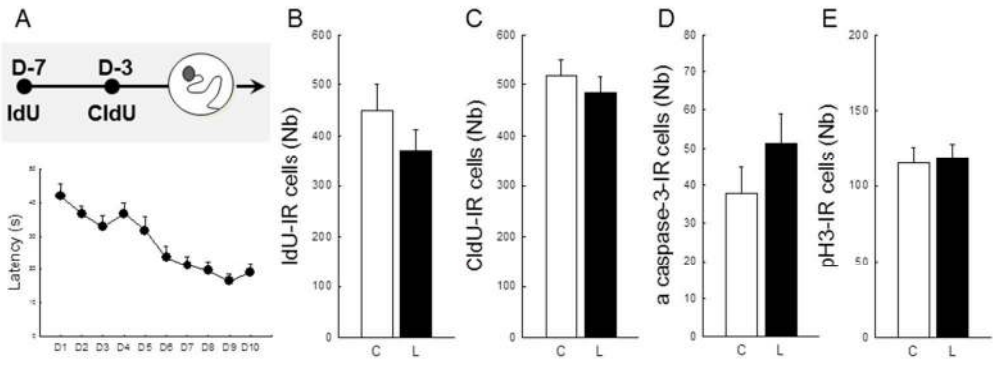
**Table 1. Review of experiments performed in rats to study the influence of learning on the development of new dentate neurons.**

| Batchs | Strain Age | Sex | Housing | Effectif | IdU Injection | CldU Injection | Retrovirus labeling | Length of training | Time of Killing | Cell age at the time of killing |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | C57BL/6J 13 W | M | Single | C=13 L=17 | -D7 | -D3 | | 10 D | 2D after T | IdU:19D CldU:15D |
| 2 | C57BL6J 13 W | M | Single | C=13 L=18 | -D15 | -D10 | | 10 D | 2D after T | IdU:27D CldU:22D |
| 3 | C57BL/6J 13 W | M | Single | C=14 VP=10 L=16 | -D28 | -D21 | | 14 D | 2D after T | IdU:40D CldU:37D |
| 4 | CD1 13 W | M | Single | C=9 L=10 | -D15 | -D3 | | 10 D | 2D after T | IdU:27D CldU:15D |
| 5 | C57BL/6J 13W | F | Grouped (by 4) | C=8 VP=6 L=12 | | | -D7 | 10 D | 2D after T | 19D |
| 6 | C57BL6 6-7W | F | Grouped (by 4) | C=6 VP=4 L=6 | | | -D17 | 10 D | 2D after T | 29D |

**Table 2. Summary of the different experiments**.

| | IdU-DCX-IR cells(%) | CldU-DCX-IR cells(%) |
|---|---|---|
| Control | 91.1 ± 1.7 | 90.0 ± 2.2 |
| Learner | 90.3 ± 2 | 92.0 ± 1.1 |
| Significance | $T_{11}$=0.30, p>0.05 | $T_{11}$=0.77, p>0.05 |

Table 3: **Lack of effect of spatial learning on neuronal differentiation.**




Trinchero et al., Figure 1

Figure 1
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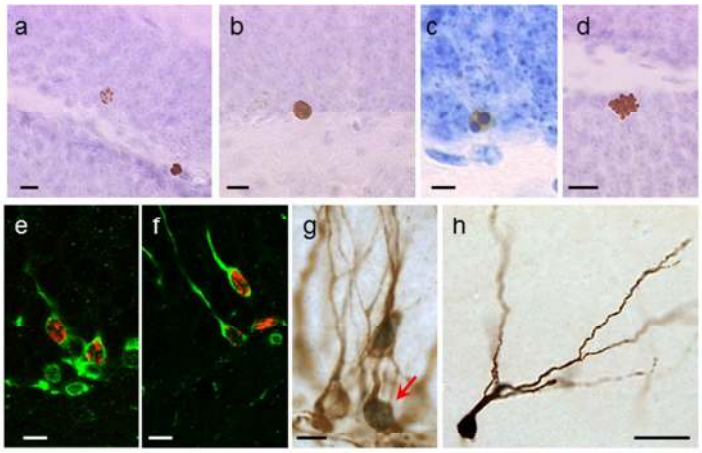
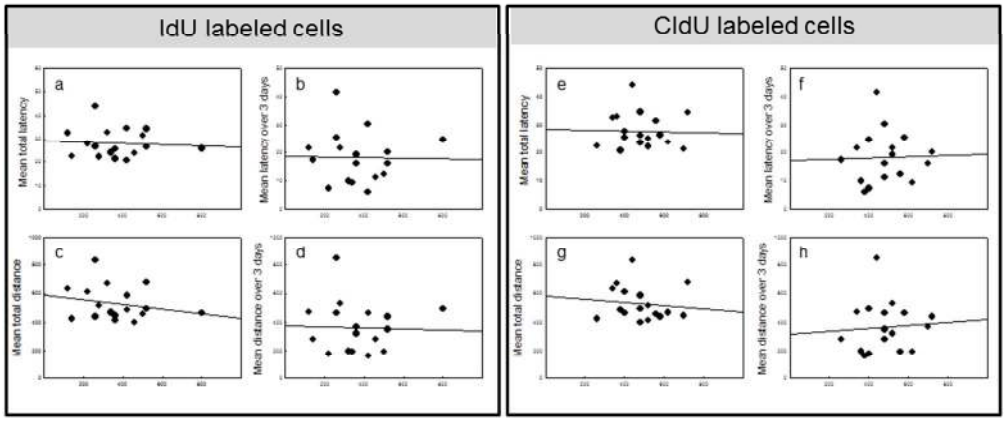



Trinchero et al.,Figure 2

Figure 2
254x190mm (96 x 96 DPI)




Trinchero et al., Figure 3

Figure 3
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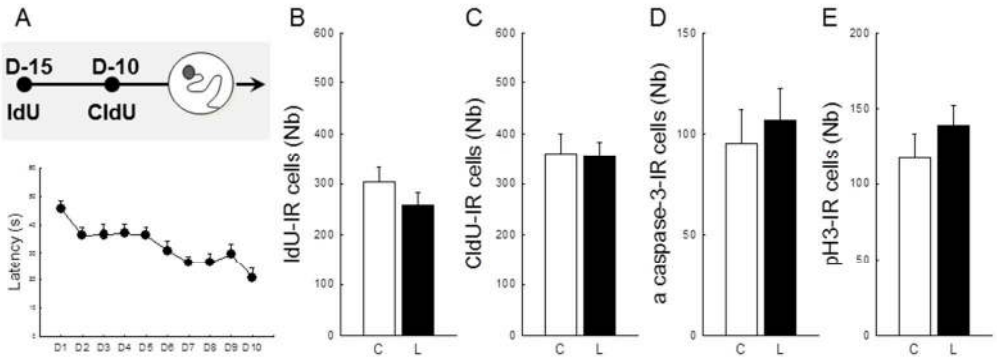


Trinchero et al., Figure 4

Figure 4
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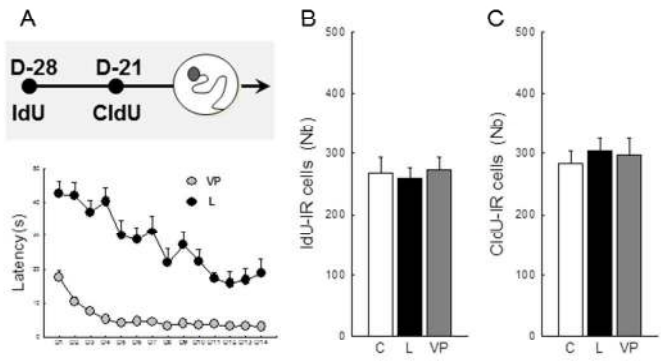


Trinchero et al., Figure 5

Figure 5
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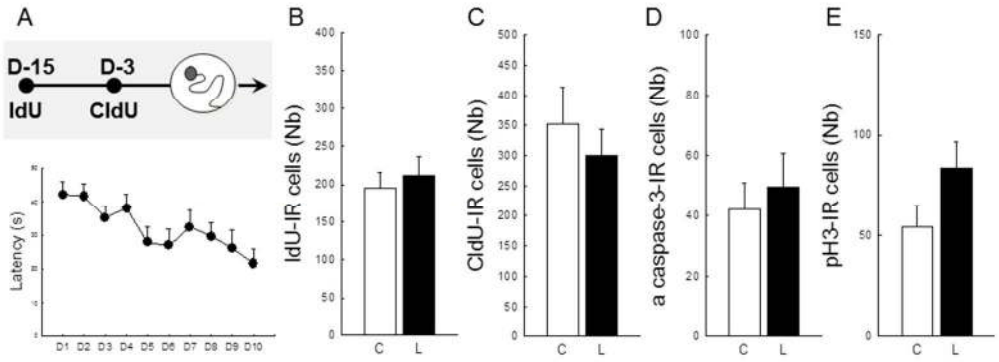
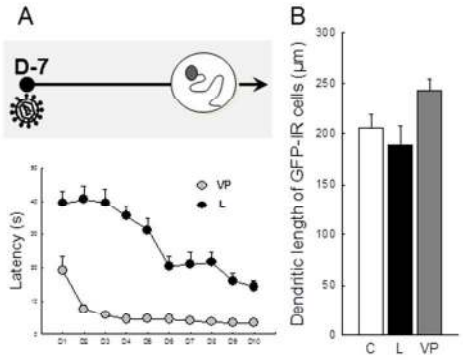


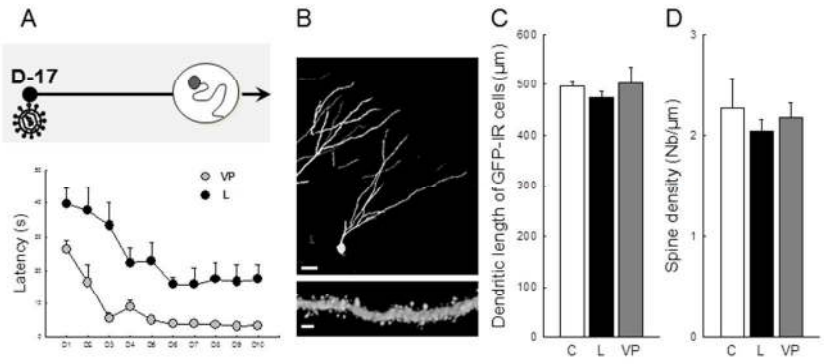

Trinchero et al., Figure 6

Figure 6
254x190mm (96 x 96 DPI)




Trinchero et al., Figure 7

Figure 7
254x190mm (96 x 96 DPI)




Trinchero et al., Figure 8

Figure 8
254x190mm (96 x 96 DPI)