Shaping the brain through experience: effects of stressful life events on hippocampal neurogenesis, morphology and function

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Spatial learning in the Morris water maze reduces newborn cell survival in the rostral hippocampus but does not affect neurogenesis; a discussion of the current state of confusion.

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Abstract
Neurogenesis was shown previously to be increased after hippocampal learning in the water maze. This task represents different challenges that can each influence neurogenesis differently; stress associated with forced swimming may reduce neurogenesis, whereas exercise during swimming may stimulate neurogenesis. To establish their contribution to different phases of neurogenesis, we here replicated an experimental design reported to increase neurogenesis, but separated the water maze components stress, learning and swimming. We further took into account potential differences between the main anatomical subregions of the hippocampus. Our results show that all animals readily learned the task. Despite the very similar design, we did not reproduce the previously reported increase, but rather found a decrease in BrdU number in the rostral hippocampus, that was paralleled by an increased proliferation in the infrapyramidal blade after learning, whereas neurogenesis was not altered. We discuss in detail possible explanations for our results and review the present confusion in literature on this topic. Although water maze training can increase neurogenesis, this occurs only under selective conditions. The phenomenon is not very stable and small adaptations to the design can already yield negative or even opposite results in terms of neurogenesis. Critical factors are training intensity, intertrial interval, the temporal BrdU design, a.o. In conclusion, water maze results should be interpreted carefully when it involves correlations with hippocampal neurogenesis. Clearly, a well standardized and/or more advanced approach is required before the precise relation between hippocampal learning and neurogenesis can be fully appreciated.
Chapter 7. Addendum

Introduction

The adult hippocampal dentate gyrus produces new neurons in substantial numbers, a phenomenon that occurs in various animal species (Amrein et al., 2004) and is extensively regulated. While stimulated by exercise and environment, neurogenesis is potently inhibited by stress e.g. (van Praag et al., 1999a; Heine et al., 2004b; Mayer et al., 2006). Given the well established role of the hippocampus in learning and memory (O’Keefe and Dostrovsky, 1971) the interaction between neurogenesis and hippocampal learning has attracted considerable attention (Leuner et al., 2006). Gould et al (1999) reported an increased survival of newborn neurons one week after hippocampus dependent learning. Others (van Praag et al., 1999a) failed to find such effects despite seemingly similar designs, or have reported contradictory data (Ambrogini et al., 2004; Olariu et al., 2005; Van der Borght et al., 2005; Ehninger and Kempermann, 2006; Leuner et al., 2006; Mohapel et al., 2006; Van der Borght et al., 2007).

A commonly used task to assess hippocampal function in these studies is the water maze (Morris, 1984), in which animals use spatial cues to find a submerged platform. Correlations have been established between neurogenesis and water maze performance (Kempermann and Gage, 2002; Drapeau et al., 2003). After water maze acquisition, newborn cell survival was reported to increase (Gould et al., 1999; Ambrogini et al., 2000; Kempermann and Gage, 2002; Dobrosy et al., 2003; Hairston et al., 2005; Leuner et al., 2006; Kee et al., 2007), remain unchanged (van Praag et al., 1999a; Van der Borght et al., 2005) or decrease (Ambrogini et al., 2004; Olariu et al., 2005; Ehninger and Kempermann, 2006; Mohapel et al., 2006; Azpiri et al., 2007).

It is important to realize that the MWM comprises different challenges for an animal that each may influence neurogenesis in opposite directions. First, the task involves learning under stress (Beiko et al., 2004) which may reduce neurogenesis (Gould et al., 1997; Heine et al., 2004b). In contrast, exercise associated with swimming can enhance neurogenesis (van Praag et al., 1999b; van Praag et al., 1999a; Ra et al., 2002), while learning itself can stimulate neurogenesis (Gould et al., 1999; Leuner et al., 2004; Azpiri et al., 2007; Dalla et al., 2007).

Given these potential confounders, we here separated the water maze components stress, learning and exercise and studied their contribution to different phases of neurogenesis. At 1-2 weeks after mitosis, adult-generated granule cells start to form connections with the CA3 area (Hastings and Gould, 1999; Markakis and Gage, 1999) and hippocampal dependent learning around that time may increase the survival of new cells incorporated into the hippocampal circuitry. From this, we hypothesized that newborn cells can be “rescued” when exposed to a learning task 7-10 days after mitosis. This idea has been supported by Gould et al, 1999, showing that learning increased the survival of 7 days old cells. Thus, we used a similar design in which rats were injected with BrdU 7 days before MWM exposure and quantified changes in
proliferation (Ki-67), newborn cell survival (BrdU) and neurogenesis (doublecortin) 14 days after mitosis, i.e. 3 days after the last training trial.

Since recent studies have shown anatomical differences along the hippocampal axis and between the two pyramidal blades in learning induced changes in cell survival, anxiety, synaptic plasticity and cognition, regional differences were taken into account as well (Scharfman et al., 2002; Silva et al., 2006).

**Materials and Methods**

*Animals and experimental design*

For this experiment twenty-four male Wistar rats (Harlan, Zeist, The Netherlands) were used, six weeks old at the start of the experiment, housed with three animals per cage and maintained on a regular 12h:12h light/dark cycle.

On day 0, all animals were injected intraperitoneally with a single dose of BrdU (Sigma; 200 mg/kg bodyweight). To separate the effects of stress, learning and exercise in the task, we randomly assigned animals on day 7 to one of the following groups: first, a regular Morris water maze training (MWM) group, which involves swim stress, exercise and learning. Second, a swim-yoked control group (Morimoto et al.); which involves elevated swim stress, exercise but no learning. Third, a corticosterone injected group (CORT), in which animals received corticosterone injections known to elevate plasma corticosterone levels (van Gemert et al., 2006) without involving exercise or learning. Fourth, a control group was used (CON) that, except handling, remained undisturbed in their home cages. On day 14, i.e. 7 days after the onset of the training trials (Fig. 1), all animals were sacrificed by transcardial perfusion. This would allow ample time for possible changes in the DCX population to become apparent.

![Diagram](image)

**Morris Water maze**

The MWM group was subjected to the Morris water maze, without previous habituation, in the morning for four days (day 7-10) during which the animals received three training trials per day. Trials had a intertrial interval (ITI) of 30
minutes apart and were ended when the animals had located and climbed on
the platform, or after a maximum of 120 seconds. The platform (diameter: 12
cm) was in the northeastern (NE) quadrant of a circular pool (150 cm in
diameter). Trials were started in each of the other quadrants (NW, SW or SE)
and starting points were alternated with each trial. Animals were placed in
the water facing the wall of the pool. When animals had climbed the platform, they
were left there for an additional ten seconds to establish a cognitive map. All
trials were analyzed (Ethovision; Noldus, Wageningen, The Netherlands) for
latency, swim distance and mean distance to platform. The MWM and the
YOK group were introduced into the Morris water maze in the same fashion
except that for the former, the platform was absent. For each trial the average
swim-time of the MWM group was used to determine the swim time of the
YOK group. The maze was situated in a room adjacent to the home cages.

Corticosterone injections
To simulate stress exposure associated with the MWM, animals in the CORT
group received a single daily corticosterone (40 mg/kg) injection in the morning
of the same days as when water maze training was performed. Corticosterone
(12.5 mg/ml in arachidic-oil) was injected subcutaneously (van Gemert et al.,
2006).

Brain tissue processing
Different stages of neurogenic were studied in perfusion-fixed brain tissue
according to protocols described previously (Heine et al., 2004b; Mayer et al.,
2006). Antibodies used were; murine anti-BrdU (Roche Diagnostics,
Netherlands, 1: 2000), polyclonal rabbit anti-Ki-67 (Novocastra, New Castle,
UK, 1:2000), and polyclonal goat anti-Doublecortin (DCX, Santa Cruz, 1:800).

Stereological Quantification
Because of their low occurrence, all BrdU+ and Ki-67+ cells were counted
manually at 200x magnification in both hemispheres. Quantification was done
in every 12th hippocampal section and the number of cells was multiplied by 12
to estimate total cell number. DCX+ cells were quantified stereologically by
systematic random sampling in a unilateral fashion using the MicroBrightfield
Stereoinvestigator system (Microbrightfield Inc, Germany). BrdU and Ki-67
cells were counted in the granular, subgranular and hilar region. Rostral-to-
caudal as well as supra- versus infrapyramidal location of the immunopositive
cells was compared separately. A division was made between the rostral
hippocampus (which contains the major part of the dorsal hippocampus) and
the caudal hippocampus (which contains a small part of the dorsal hippocampus
and the whole ventral hippocampus). All sections rostral to bregma -4.52
(Paxinos and Watson, 1986) where considered rostral and other sections were
considered caudal. For the whole hippocampus at least 10 sections per animal
were quantified, and for the rostral and caudal part minimal 5 sections were
used.
Statistics
Data are presented as mean ± SEM. Statistical analysis was done using SPSS 11. Behavioural data were analyzed by a general linear model for repeated measures. Immunohistochemical data are compared using a one-way ANOVA. Pair wise comparison was done with a Fisher post-hoc test. A p-value of <0.05 was considered significant.

Results
Morris water maze training
Comparison of the swimming distance and latency showed a significant decrease in both mean distance and mean latency over the subsequent training trials in the learning group (p<0.001 not shown). For distance, separate analysis of trials revealed p-values <0.05 comparing trials 6 through 12 to trial 1. For latency, separate analysis of trials revealed p-values of p<0.05 comparing trials 3 and 5 to trial 1 and p-values of p<0.01 comparing trials 6 through 12 to trial 1. Distance and speed was the same for the YOK and MWM group, indicating similar exercise levels.

Adult generated cell survival (BrdU)
The presence of BrdU+ cells in the hilus, SGZ and granular cell layer indicated that migration had occurred (example in fig 2A). Given their age of 14 days, cell numbers in the GCL were relatively low and SGZ and GCL numbers were therefore pooled. In the rostral part of the hippocampus, a significant reduction was found in BrdU+ cellnumber in MWM relative to CON. The CORT group did not differ from CON but showed significantly more BrdU+ cells compared to both the MWM and YOK groups (Figure 3C) (ANOVA, F<sub>3,19</sub> 4.44, p=0.016; posthoc comparison CON vs MWM p=0.03; CORT vs MWM p=0.003; CORT vs. YOK p = 0.03). BrdU+ numbers in the supra- and infra-pyramidal blade, in the caudal part of the hippocampus, or in the entire hippocampus did not differ between the groups (not shown).

Proliferation
Numbers of Ki-67+ cells (fig 2B) differed significantly in the infra-pyramidal blade of the rostral hippocampus. In the MWM group, a significant increase was found compared to the CON and YOK groups (ANOVA, F<sub>3,19</sub> 3.24, p= 0.043; posthoc comparison CON vs MWM p=0.01; MWM vs YOK p = 0.03) (fig 3B). Numbers in the CORT group did not differ from control. Numbers in suprapyramidal blade (not shown), in the caudal part per se (fig 2B), or in the entire hippocampus (fig 2A) did not differ between the groups.

Neuronal maturation (DCX)
DCX+ cells were present in large numbers with their somata generally located in the SGZ and their extensions penetrating the GCL reaching into the
molecular layer (Fig 2C). The number of DCX+ cells did not differ between the groups in any subregion. A non-significant trend was found towards a reduction in the learning (MWM) and swimming (Morimoto et al.) groups as opposed to control.

Figure 2. A. BrdU immunohistochemistry in the dentate gyrus of the hippocampus shows 1 week old cells present in the subgranular zone (sgz), shown by the arrowhead and granular cell layer (gcl), shown by the arrow. B. Ki-67 immunohistochemistry shows proliferating cells (arrowhead) mainly in the subgranular zone (sgz) of the dentate gyrus. C. Doublecortin immunohistochemistry shows large numbers of developing, young neurons in the subgranular zone. D. In the rostral hippocampus a significant reduction of BrdU+ cells in the MWM learning group as opposed to control animals was found. The CORT group did not differ from control animals but showed significantly more BrdU+ cells compared to both the MWM and YOK groups (ANOVA, F1,10 4.44, p=0.016; posthoc comparison CON vs MWM p=0.03; CORT vs MWM p=0.005; CORT vs YOK p = 0.03). BrdU+ numbers in suprag- and infrapyramidal blade in the caudal part of the hippocampus, or in the entire hippocampus, did not differ between the groups (not shown). E. The MWM group showed a significant increase in Ki-67+ cells compared to CON and YOK groups only in the infrahilar part of the rostral hippocampus (ANOVA, F1,10 3.24, p= 0.043; posthoc comparison CON vs MWM p=0.01; MWM vs YOK p = 0.03). Numbers in the suprapyramidal blade (not shown), in the caudal part (D), or in the entire hippocampus (A) did not differ between the groups.
Discussion
We have studied different components of the water maze, i.e.; stress, learning and exercise and addressed their separate contributions to the main phases of neurogenesis in different hippocampal subregions. We adopted a paradigm previously reported to increase neurogenesis, in which BrdU-labeled cells are 7 days old when the animal is subjected to the task. Despite the fact that all animals readily learned the task and despite our choice for the same species, gender, number per group, quantification method and the very comparable experimental design, we fail to reproduce the almost threefold increase in BrdU cell number as reported by Gould et al., (1999), but rather found a decrease in BrdU+ cell number in the rostral part in the MWM group. This was paralleled by increases in Ki-67+ cells in the infrapyramidal blade of the same area in the MWM group compared to the CON and YOK groups.

Since ours is not the first study that failed to reproduce an increase in neurogenesis after learning, we will discuss possible explanations in more detail. Even if memory training increases neurogenesis under selective conditions, we and others show that the phenomenon is not very stable and critically depends on various methodological issues. The critical importance of the temporal design regarding the BrdU injections e.g., has been emphasized previously (Greenough et al., 1999) and some studies may have failed to find effects because of this (van Praag et al., 1999a). However, also studies using similar designs have reported decreases in neurogenesis after learning (Dobrossy et al., 2003; Van der Borght et al., 2005; Mohapel et al., 2006; Aziiri et al., 2007), although minor differences in design were still present. Also, training frequency and intensity may determine whether neurogenesis is stimulated or decreased, as will be discussed below.

A general feature of the MWM task is that it represents learning under stress (Beiko et al., 2004; Engelmann et al., 2006) which may decrease neurogenesis. In agreement with Gould et al., 1999, we failed to find effects on neurogenesis in the YOK group, indicating that the exposure to swim stress and exercise has had no effect here. Of interest, the group injected with stress levels of corticosterone (vehicle 1.3 ± 0.1 μg/dl; cort 60.0 ± 11.3 μg/dl; P<0.001) of van Gemert et al., 2006 also failed to show decreases in neurogenesis compared to controls, whereas still significantly more BrdU+ cells were found as compared to the MWM and YOK groups. Since the effects of both corticosterone injections and swim stress alone on neurogenesis were no longer present when studied 3-7 days later, this indicates that the consequences of swim stress for newborn cell survival are modest and shortlasting. In this respect, the study by Ehninger and Kempermann (2005) is of interest as prior habituation to the water maze abolished the negative effects of learning on neurogenesis, suggesting that the initial rise in corticosterone level together with a novel learning context is instrumental.
Chapter 7. Addendum

If BrdU+ cell number would have been temporarily decreased as a result of swim stress or corticosterone, compensatory responses may have caused a normalization of their numbers, e.g. through additional proliferation of existing BrdU+ cells. Normalization after stress can indeed happen rapidly (Heine et al., 2004b; Mayer et al., 2006) and is consistent with the current increase we found in proliferation. An increase in overall hippocampal proliferation has also been found in another study (Lemaire et al., 2000) suggesting that after learning, more cells stay in a proliferative state rather than rapidly engaging in a neuronal phenotype. This is confirmed by our DCX data, that did not differ between the groups. Unlike BrdU that reflects a few hours of bioavailability, DCX is expressed in immature neurons of 4 to 14 days of age (Brown et al., 2003), which results in DCX+ cells to be present in relatively large numbers. Hence, despite reductions in BrdU number, the present lack of an effect in DCX could be explained by stochastic differences due to selective changes in e.g. only young DCX+ cells, that might have gone unnoticed when total DCX+ cell number is assessed. The increase reported in the plasticity marker PSA-NCAM (Van der Borght et al., 2005) indicates that at least some neuronal plasticity changes do occur after MWM training.

The present reduction in newborn cell survival after MWM training was found in the rostral hippocampus. Both anatomical and functional differences are present between hippocampal subregions (Colombo et al., 1998; Ambrogini et al., 2000; Bannerman et al., 2004; Poehlizen et al., 2004; Banasr et al., 2006; Silva et al., 2006; Maggio and Segal, 2007). Regional changes in neurogenesis have further been linked to effects of antidepressants, while subregion-specific changes in PSA-NCAM were implicated in memory consolidation (Lopez-Fernandez et al., 2007). A plausible hypothesis to explain differences between subregions in relation to stress and learning could be the distribution of MRs and GRs and their ratio within the hippocampus (de Kloet et al., 2005). Learning related stress could e.g. activate more easily GRs in the dorsal hippocampus. Given the different functionalities associated with different parts of the hippocampus, it will be of interest to address effects of learning on neurogenesis in future studies also in a region specific manner (Banasr et al., 2006; Lopez-Fernandez et al., 2007).

As the earlier mentioned compensatory mechanisms could be triggered by previous cell loss, a role for apoptosis is of considerable relevance, both regarding stress (Lucassen et al., 2006) and learning. Various studies have addressed cell selection during learning (Dobrossy et al., 2003; Olariu et al., 2005; Dupret et al., 2007). This concept assumes a sensitive period during development of the BrdU labeled cell regarding the time of learning (Leuner et al., 2006). During the approximately 4 weeks of their development, adult generated cells are highly susceptible to cell death and about 50% of them die within 14 days (Dayer et al., 2003). Acquisition of a hippocampal learning task during this period of vulnerability may force immature cells to decide whether to differentiate or die.

This selection of cells only occurs during a restricted time window of
neuronal development and during selective phases of the learning trial (Dobroisy et al., 2003; Drapeau et al., 2007; Dupret et al., 2007) and the optimal sensitivity to learning-related input appears to develop relatively late (Aimone et al., 2006). Dobroisy et al. (2003) elegantly demonstrated that the number of cells born in the second, asymptotic phase of learning are upregulated, whereas the second phase of learning appears to reduce the number of cells born during the first phase of learning. A recent study has shown that blocking apoptosis indeed impairs memory while inhibiting learning-induced cell survival (Dupret et al., 2007). This indicates that spatial learning involves a selective stabilization process through cumulative “waves” of increases in the production of new neurons, followed by a rapid and selective removal of others (Chambers and Conroy, 2007).

An other option is that the existing granule cells die only after they have been involved in an early phase of the learning process. Once a more durable memory is formed or information has been transferred into the cortex, these “initial” cells would become obsolete and can be discarded. However, this option would assume that young granule cells participate in memory processes, long before they e.g. develop mature electrophysiological properties, a concept which still has to be proven.

In the present design, MWM training may have had a dual effect, inducing survival of the immature (< 4 days after birth) and apoptosis of the more mature cells (between 9 and 11 days of age). Since cell number of the DG granular cell layer remains remarkably constant under these and other conditions (Heine et al., 2004a; Heine et al., 2004b), yet unknown control mechanisms must be involved, likely through signaling between dying and newborn cells. So far, methodological issues related to their loss of protein markers (Lucassen et al., 1995), have hampered identification of the phenotype of apoptotic cells. Although its involvement in spatial learning has been recently established (Dupret et al., 2007), it further remains unclear how their rapid removal, i.e. within hours, is linked to the much slower changes in neurogenesis. Clearly, the role of apoptosis in learning warrants further study.

As mentioned above, also the frequency and intensity of MWM training may influence whether neurogenesis is stimulated or decreased. The level of difficulty does e.g. depend on pool size and inter trial interval (ITI). In a comparable design, a single day of training e.g. increased the survival of 8-day old BrdU labeled cells studied one week later. In contrast, when animals were trained for 2 days, survival of these cells was decreased (Olariu et al., 2005). The direction of this effect is similar to that found in other studies where an even longer period of training resulted in fewer newborn hippocampal neurons (Mohapel et al., 2006). Fewer training trials were associated with enhanced cell survival whereas more trials were associated with no effect or even decreases in survival (Ambrogini et al., 2004; Olariu et al., 2005; Snyder et al., 2005; Van der Borght et al., 2005; Mohapel et al., 2006). Consequently, decreases and increases in cell birth may have interfered and overlapped in these designs, possibly resulting in the absence of a net effect at the time of sacrifice.
Chapter 7. Addendum

Another factor is training intensity. In both the Gould et al. (1999) and our present study, animals were trained for 4 days, but we subjected animals to 3 trials per day with 30 minutes of rest between trials. Gould et al. (1999) used 4 trials per day and a shorter ITI of 60 seconds only. Such a more intense training may have reached asymptotic levels earlier (Dalla et al., 2007; Dupret et al., 2007) and hence posed a stronger stimulus for the hippocampus, possibly resulting in stronger changes in neurogenesis. If the “demand” on the hippocampus has not been strong enough, recruitment of new cells into the hippocampal circuitry may not have happened to a significant extent. Taken together, a complex regulation of cell survival and death occurs during learning whereby training, hippocampal demand and stress may interfere with the survival of previously generated groups of cells.

In addition to effects of learning on neurogenesis, it is of interest to briefly mention whether, in turn, neurogenesis affects learning and memory. Following irradiation and/or genetic elimination of neurogenesis (Madsen et al., 2003; Rola et al., 2004), both a lack of effect (Shors et al., 2002), decreases (Snyder et al., 2005) and improvements in hippocampal dependent working memory have been reported (Winocur et al., 2006; Saxe et al., 2007). Ablating neurogenesis even enhanced working memory (Saxe et al., 2007), whereas Kec et al. (2007) showed a preferential incorporation of newborn neurons in spatial memory networks. Clearly, hippocampal neurogenesis is not an unequivocal link between structural and functional plasticity and also other substrates like apoptosis, synaptogenesis and spine formation are important in this respect.

In conclusion, consistent with results from other groups, we can not reproduce the stimulating properties of learning on neurogenesis, but find a reduced newborn cell survival in the rostral hippocampus and an increased proliferation in its infrapyramidal blade. Given the different functionalities of hippocampal subregions, it will be of interest to take into account such differences in future studies as well. Importantly, even if memory training can increase neurogenesis under specific conditions, the phenomenon is not very stable and critically depends on issues related to the frequency and intensity of MWM training, the temporal design of BrdU, the type of memory task and the demand it poses on the hippocampus, a.o. Given the present variation and confusion in literature, more sophisticated and better standardized experimental designs and approaches are expected to provide more insight (Dupret et al., 2007; Kec et al., 2007; Saxe et al., 2007) in the precise relationship between hippocampal learning and neurogenesis, before its functional relevance can be fully appreciated.

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Chapter 7. Addendum


Chapter 7. Addendum


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