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### New neurons in the adult brain

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*Document Version*

Publisher's PDF, also known as Version of record

*Publication date:*

2006

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*Citation for published version (APA):*

van der Borght, K. (2006). *New neurons in the adult brain: A study on the regulation and function of neurogenesis in the adult rodent hippocampus*. s.n.

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# Chapter 6

## Morris water maze learning in two rat strains increases PSA-NCAM expression in the dentate gyrus, but has no effect on hippocampal neurogenesis

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*Behavioral Neuroscience* (2005), 119(4): 926-932

## Abstract

*The present study investigated whether Morris water maze learning induces alterations in hippocampal neurogenesis or NCAM polysialylation in the dentate gyrus. Two frequently used rat strains, Wistar and Sprague-Dawley, were trained in the spatial or the non-spatial version of the water maze. Both training paradigms had neither an effect on survival of newly formed cells that had been labeled seven to nine days prior to the training, nor on progenitor proliferation in the subgranular zone. However, the granule cell layer of the spatially trained animals contained significantly more PSA-NCAM positive cells. These data demonstrate that Morris water maze learning causes plastic change in the dentate gyrus, without affecting hippocampal neurogenesis.*

## Introduction

The hippocampal dentate gyrus has, together with the olfactory bulb, the unique feature that it continues to produce new neurons during adult life (Altman, 1969; Altman and Das, 1965; Alvarez-Buylla and Garcia-Verdugo, 2002; Gross, 2000). The newly formed hippocampal neurons originate from undifferentiated progenitors that reside in the subgranular zone (SGZ) of the dentate gyrus (DG). Upon migration into the granule cell layer (GCL) they differentiate and become mature, functional granule cells (Cameron and McKay, 2001; Dayer et al., 2003; Hastings and Gould, 1999; Markakis and Gage, 1999; Van Praag et al., 2002).

The regulation of adult hippocampal neurogenesis appears to be activity-dependent. Epileptic seizures in the dentate gyrus (Parent et al., 1997), amygdala kindling (Scott et al., 1998) or long-term potentiation in the mossy fibers (Derrick et al., 2000) enhance proliferation of hippocampal progenitors in the subgranular zone. Increased behavioral activity, such as wheel running (Trejo et al., 2001; Van Praag et al., 1999b) and enriched housing (Kempermann et al., 1997; Nilsson et al., 1999), also stimulates hippocampal neurogenesis. Moreover, it has been reported that hippocampus-dependent learning tasks, such as the Morris water maze or trace eyeblink conditioning, have a positive effect on the formation of new neurons (Gould et al., 1999a). This effect seem to be specific for hippocampus-dependent learning tasks, since hippocampus-independent tasks, such as delay eyeblink conditioning or active shock avoidance learning, did not cause any changes in neurogenesis (Gould et al., 1999a; van der Borght et al., 2005a). It could be hypothesized that the activation of the hippocampal formation by certain types of learning can, at least partly, prevent the high level of cell death that normally occurs within two weeks after the generation of hippocampal granule neurons (Cameron and McKay, 2001; Dayer et al., 2003). However, using a somewhat different protocol, other researchers were not able to replicate these data for the Morris maze task in mice (Van Praag et al., 1999a), or they even found a decreased cell survival after spatial learning (Ambrogini et al., 2004b). Thus, spatial learning may affect hippocampal neurogenesis, but conflicting reports exist in the literature.

Newly formed, immature hippocampal granule neurons express the polysialylated form of the neural cell adhesion molecule (PSA-NCAM) (Nakagawa et al., 2002; Seki and Arai, 1993). The presence of PSA-NCAM is generally associated with plastic changes in the central nervous system. It is abundantly expressed during development, where it mediates cell migration, neurite outgrowth and synaptogenesis (Edelman, 1986; Seki and Rutishauser, 1998). In adulthood, NCAM polysialylation is strongly reduced, but it appears to be upregulated in circumstances requiring structural remodeling (Ronn et al., 2000). Demyelination of the spinal cord, for instance, or hippocampal damage caused by epileptic seizures increase PSA-NCAM expression in the lesioned area (Dominguez et al., 2003; Oumesmar et al., 1995). PSA-NCAM has also been shown to be involved in learning as was shown by experiments in which PSA groups were removed from the NCAM molecule by

treating rats with the enzyme endoneuraminidase NE (endo-N). This treatment resulted in impaired Morris water maze acquisition and retention (Becker et al., 1996). Moreover, different types of learning, like passive shock avoidance learning, Morris water maze training and contextual fear conditioning have been reported to stimulate NCAM polysialylation (Fox et al., 1995; Murphy et al., 1996; Sandi et al., 2003).

The current study was aimed to investigate spatial learning-induced plastic changes in the dentate gyrus in relation to neurogenesis. Since the potential effect of hippocampus-dependent learning on adult neurogenesis is still debated, we investigated whether Morris water maze learning in rats affects survival of newly formed cells and proliferation of hippocampal progenitors in the dentate gyrus. We also analyzed PSA-NCAM expression in the dentate gyrus to relate learning-induced changes in NCAM polysialylation to potential alterations in hippocampal neurogenesis. Because it is known that learning performance and hippocampal neurogenesis differ significantly between inbred laboratory mouse strains (Kempermann and Gage, 2002a; Kempermann and Gage, 2002b), we decided to compare learning capacity, baseline neurogenesis and learning-induced changes in plasticity in the dentate gyrus between two widely used rat strains, Wistar and Sprague-Dawley.

## Materials and Methods

### *Animals and housing*

Twenty-four male Wistar rats ( $338 \pm 24$  g, bred in our own facilities) and twenty-one male Sprague-Dawley rats ( $336 \pm 30$  g, Harlan, Horst, The Netherlands) were individually housed. The animals had free access to water and food and were kept under a 12:12h light:dark cycle, lights on at 7:00 a.m. All procedures concerning animal care and treatment were in accordance with the regulations of the ethical committee for the use of experimental animals of the University of Groningen (DEC number 2719).

### *Morris water maze training and BrdU injections*

The Morris water maze consisted of a black pool (diameter 140 cm) filled with water ( $26 \pm 1^\circ\text{C}$ ). A small, black platform (diameter 9 cm) was placed at 23 cm from the border of the pool and 2.5 cm under the water surface in order to make it invisible to the animals. The behavior of the animals in the pool could be tracked with a camera connected to a computer. Specialized software (Ethovision, Noldus, Wageningen, The Netherlands) allowed us to measure various parameters, such as swim speed, the distance moved and the latency to find the platform.

Place learners (Wistar:  $n=8$ , Sprague-Dawley:  $n=7$ ) were trained using a protocol of five trials per day, with an intertrial interval of twenty minutes, for five consecutive days. Animals were allowed to swim for maximally 60 seconds per trial. The first trial of the first day was performed without a platform, in order to give the animals the opportunity to habituate to the swimming procedure. In the second trial of the first day, the platform was

present in the maze. If the animals had not been able to find the platform within 60 seconds, they were guided there by the experimenter. After having reached the platform, animals were kept there for 10 seconds to give them the opportunity to orientate themselves to the spatial cues that were present in the experimental room. The platform was kept in the same position for three days, though the starting position of the animals was changed between trials. After three days, the platform position was changed and the animals had to learn the new position of the platform on training days 4 and 5.

Two control groups were included in the experiment: home cage controls (Wistar: n=8, Sprague-Dawley: n=7) and cue learners (Wistar: n=8, Sprague-Dawley: n=7). The cue learners underwent the same procedure as the place learners, except for the fact that the spatial learning component was lacking. The platform was made visible to the animals by placing it 1 cm above the water surface, by making it white-colored and by placing a flag on it. In every trial, the platform was placed in a different position. Home cage controls remained undisturbed throughout the experiment.

Seven to nine days before the start of the training, all animals were intraperitoneally injected with 100 mg/kg BrdU (Sigma, St. Louis, USA) dissolved in saline (20 mg/ml) once a day for three consecutive days.

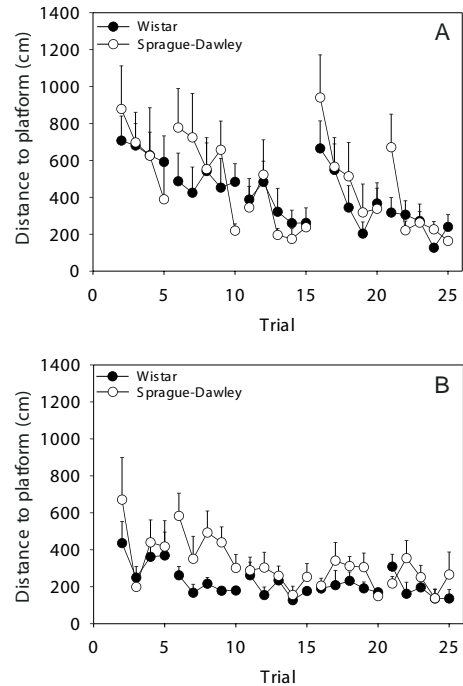
#### *Brain processing and immunocytochemistry*

One day after training, approximately 18-20 hours after the last training session, animals were sacrificed by transcardial perfusion with heparinized saline, followed 2.5% paraformaldehyde and 0.05% glutardialdehyde in 0.1 M phosphate buffer (PB). After dehydration in 30% sucrose, 40  $\mu$ m coronal sections were cut on a cryostat microtome. Twelve series spanning the entire hippocampus (Bregma -2.12 to Bregma -6.30) were collected in cryoprotectant (0.05 M PB, 25% glycerol and 25% ethylene glycol) and stored at -20°C until they were used for immunocytochemistry.

BrdU and Ki-67 immunocytochemistry were performed on every twelfth section of the hippocampus, using a protocol as described earlier (van der Borgh et al., 2005a). In brief, sections for the BrdU staining underwent some extra steps for DNA denaturation. For this purpose, they were exposed to 50% formamide in 2XSSC at 65°C and 0.2 M HCl at 37°C. The primary antibodies that were applied were rat-anti-BrdU (1:800, Oxford Biotechnology, Oxfordshire, UK) and mouse-anti-Ki-67 (1:200, Novocastra, Newcastle upon Tyne, UK). As secondary antibodies, biotinylated donkey-anti-rat and biotinylated sheep-anti-mouse (both 1:200, Jackson, West Grove, USA) were used. Staining was visualized with diaminobenzidine (20 mg/100 ml, DAB) as chromogen.

For the PSA-NCAM staining, five to six representative sections from the dorsal hippocampus were selected. After preincubation with 3% normal rabbit serum and 0.5% triton-X100 they were incubated with the primary antibody (1:1000, mouse-anti PSA-NCAM IgM, Chemicon, Temecula, USA) for 96h. As a secondary antibody, rabbit-anti-mouse IgM (1:200, Jackson) was used. After incubation with the ABC kit (Vector, Burlingame, UK), staining was visualized with DAB.

Figure 1: Learning curves of Wistar ( $n=8$ ) and Sprague-Dawley ( $n=7$ ) rats in the place (A) or cue (B) version of the Morris water maze. Training consisted of five trials per day for five consecutive days. The first trial on day 1 was performed without a platform. In the group of place learners, the platform was relocated after three days of training. Both rat strains performed equally well in the place learning task, but Wistar performed significantly better than Sprague-Dawleys during cue learning ( $P=0.001$ ). Data are expressed as mean distance moved before reaching the platform  $\pm$  S.E.M.

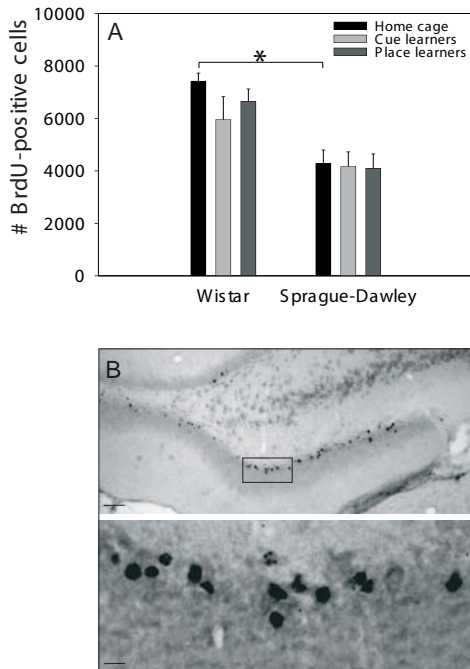


### Quantification

During the analysis of the brain material, the experimenter was blind to the treatment of the animals. BrdU and Ki-67 immunopositive cells were counted in every twelfth section of the hippocampal formation with a 40x objective. Only cells that were in the subgranular zone or one cell diameter deviating from this region were included. BrdU-positive cells that were lying in the granule cell layer were counted as well. The number of counted cells was multiplied by twelve to get an estimation of the total number of positive cells per dentate gyrus. For the PSA-NCAM staining, all cells in the subgranular and granular layer were counted in five to six sections that were randomly chosen to be representative for the dorsal hippocampus. The average cell number per section was calculated.

### Statistics

Morris Water maze behavioral data were analyzed with a repeated measures ANOVA. When three experimental groups were compared, BrdU, Ki-67 and PSA-NCAM cell counts were statistically tested with a one-way ANOVA. If this revealed a significant outcome, a Bonferroni test was applied for post hoc testing. Comparison between two groups was performed using an independent-samples t-test.



*Figure 2: The number of BrdU-positive cells per dentate gyrus. A) Neither in Wistars ( $n=8$  per group) nor in Sprague-Dawleys ( $n=7$  per group) any difference was observed in BrdU-positive cell number between home cage controls, cue learners or place learners. However, Wistar home cage controls had significantly more BrdU-positive cells than the Sprague-Dawley home cage control animals ( $* P<0.01$ ). Data are expressed as mean  $\pm$  S.E.M. B) Representative photomicrographs of BrdU-immunocytochemistry. Scale bar = 50  $\mu$ m in the upper panel. A magnification of the selected region is shown in the lower panel (scale bar = 10  $\mu$ m).*

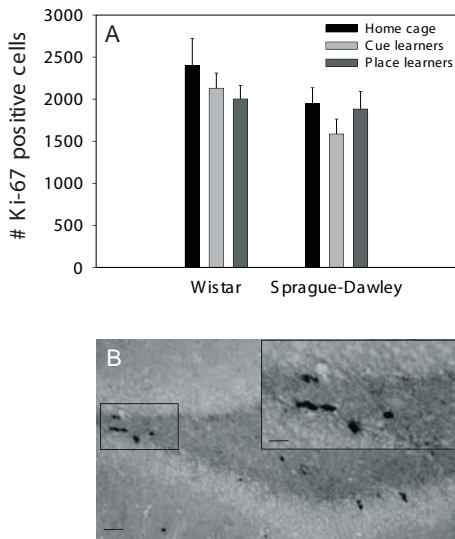
## Results

### *Behavioral testing*

The two rat strains performed equally well in the spatial version of the Morris water maze, in which they had to find the hidden platform (Fig. 1A, Between strains:  $F(1,13)=0.10$ ,  $P=0.75$ , strain  $\times$  trial:  $F(13,169)=0.70$ ,  $P=0.76$ ). After relocation of the platform on day 4, the animals quickly learned to find the new position of the platform. Also in this reversal learning paradigm, no differences were observed between Wistar and Sprague-Dawley (Fig. 1A, Between strains:  $F(1,13)=1.53$ ,  $P=0.24$ , strain  $\times$  trial:  $F(9,117)=0.86$ ,  $P=0.57$ ). As expected, the rats showed a decrease in the distance they needed to swim to find the platform ( $P<0.001$  for both the first fifteen trials and the last ten trials). The latency to find the platform could not be used as an indicator of learning performance, since the two strains significantly differed in swim speed (Wistar:  $18.5\pm 0.6$  cm/s, Sprague-Dawley:  $23.2\pm 0.4$  cm/s,  $F(1,29)=32.99$ ,  $P<0.001$ ). Therefore, the distance swum by the rats until they reached the platform was taken.

In the cued version of the Morris water maze, the rats acquired the task rapidly (Fig. 1B,  $P<0.001$ ). Sprague-Dawley rats swam a greater distance before reaching the platform than Wistars (Fig. 1B, Between strains:  $F(1,13)=20.27$ ,  $P=0.001$ ), but both strains managed to acquire the task. Moreover, there was no significant interaction between strain and trial ( $F(23,299)=0.93$ ,  $P=0.56$ ). Learning speed differed significantly between place learners and rats that were trained with the visible platform (Wistar:  $P<0.001$ , Sprague-Dawley:  $P<0.01$ ).





*Figure 3: Ki-67 expression in the hippocampal subgranular zone. A) Neither hippocampus-independent nor hippocampus-dependent learning in the water maze caused a change in hippocampal cell proliferation. This was the case for both rat strains (Wistar  $n=8$  for all groups, Sprague-Dawley:  $n=7$  for all groups). Also, no strain differences were observed in Ki-67 expression. Data are expressed as mean  $\pm$  S.E.M. B) Example of Ki-67 immunocytochemistry in the hippocampus (scale bar = 50  $\mu\text{m}$ ). The insert shows an enlargement of the selected region (scale bar = 10  $\mu\text{m}$ ).*

### *BrdU*

In order to investigate the effects of the learning task on the survival of newly formed hippocampal cells, rats were injected with the thymidine analogue BrdU seven to nine days before the start of the training. One day after the last training, animals were sacrificed and brains were processed for immunocytochemistry. Quantification of the number of BrdU-positive cells in the dentate gyrus did not reveal any differences between home cage controls, cue learners and place learners (Fig. 2). This was the case for both rat strains (Wistar:  $F(2,23)=1.49$ ,  $P=0.25$ ; Sprague-Dawley:  $F(2,20)=0.032$ ,  $P=0.97$ ). These data indicate that Morris water maze learning did not promote survival of newly generated cells in the hippocampus. However, a significant difference was observed in the number of BrdU-positive cells between home cage controls of the two rats strains, with Sprague-Dawleys having 42% less positive cells than Wistars ( $F(1,14)=28.94$ ,  $P<0.01$ ).

### *Ki-67*

The Ki-67 protein is expressed in all cells during all phases of the cell cycle, except G0 (Scholzen and Gerdes, 2000) and can therefore be considered as a good indicator for the number of proliferating cells that were present at the moment of perfusion. Quantification of the number of Ki-67 positive cells in the subgranular zone showed that neither place learning nor cue learning caused a change in hippocampal cell proliferation (Fig. 3, Wistar:  $F(2,23)=0.78$ ,  $P=0.47$ ; Sprague-Dawley:  $F(2,20)=0.99$ ,  $P=0.39$ ). Also, Ki-67 expression did not differ between the home cage controls of both strains ( $F(1,14)=1.40$ ,  $P=0.26$ ), indicating that baseline hippocampal cell proliferation is similar for Wistars and Sprague-Dawleys.

### PSA-NCAM

The binding of  $\alpha 2,8$ -linked polysialic acid homopolymers to the neural cell adhesion molecule (PSA-NCAM) has been associated with plastic changes in the brain. Moreover, PSA-NCAM is expressed by immature neurons in the adult hippocampus. Analysis of PSA-NCAM immunocytochemistry showed a significant effect of place learning. In the Wistar rats, place learners had 19% more PSA-NCAM positive cells than home cage controls (Fig. 4,  $P < 0.05$ ). Also in the Sprague-Dawleys a learning effect was observed. Place learners had 31% more immunoreactive cells compared to home cage controls ( $P < 0.001$ ). Moreover, comparison between the two strains, with regard to baseline PSA-NCAM expression in home cage controls, showed that Wistar rats had 40% more PSA-NCAM positive cells in the dentate gyrus than Sprague-Dawleys ( $P < 0.001$ ).

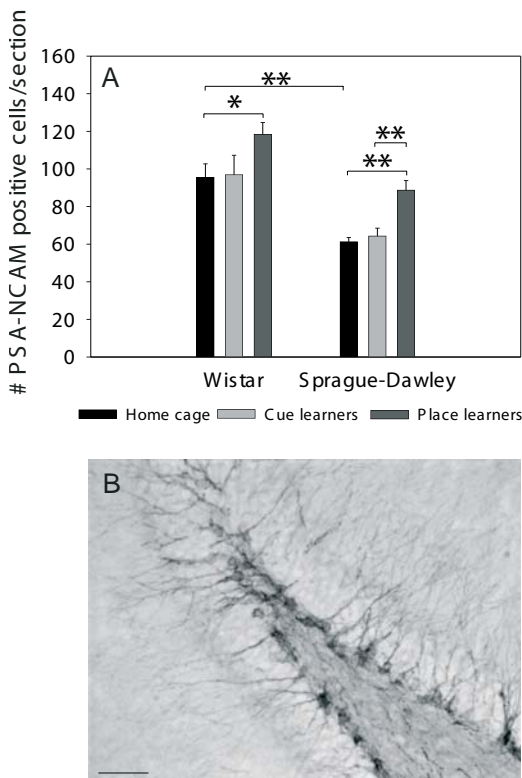


Figure 4: PSA-NCAM expression in the hippocampal subgranular zone. A) Both in Wistars and Sprague-Dawleys a learning-induced increase in PSA-NCAM immunoreactivity in the subgranular zone was observed. In Sprague-Dawleys, place learners also had significantly more PSA-NCAM positive cells than cue learners. Moreover, when comparing home cage control animals, a significant difference was observed between Wistars and Sprague-Dawleys, with Wistars having more PSA-NCAM immunopositive cells than Sprague-Dawleys. \*  $P < 0.05$ , \*\*  $P < 0.001$ . Data are expressed as mean  $\pm$  S.E.M. B) Photomicrograph of PSA-NCAM immunocytochemistry in the dentate gyrus. Scale bar = 50  $\mu$ m.

## Discussion

The present study investigated the occurrence of plastic changes in relation to neurogenesis in the hippocampal dentate gyrus following training in a spatial learning task, the Morris water maze. The data show that place learning in the water maze induced an increased expression of PSA-NCAM. Hippocampal progenitor proliferation and survival of newly formed cells were not altered by the spatial learning task.

The literature on spatial learning-induced changes in newly formed hippocampal cell survival is not entirely consistent. Between one and three weeks after their formation, a large part of the newly formed granule cells die (Cameron and McKay, 2001; Dayer et al., 2003; Hastings and Gould, 1999). Gould and colleagues (1999) reported that training rats in a spatial learning task within this critical period, that is, starting seven days after injection with BrdU, could prevent many newly formed cells from undergoing apoptosis. In contrast, others observed a negative effect on survival of newly generated hippocampal cells, when starting Morris water maze training eight to ten days after BrdU administration (Ambrogini et al., 2004b). In the present study, in which water maze training was started seven to nine days after BrdU injections, no effects on survival of BrdU-labeled cells could be demonstrated. Possibly, the time window in which the effects of learning on newly formed cell survival are investigated is very narrow.

At the age of 10 days, only 9% of the cells has formed axons towards the CA3 region (Hastings and Gould, 1999), which reduces the possibility that the BrdU-labeled cells in the present study actively participated in the learning process and that this participation could rescue them from going into apoptosis. Moreover, other experimental approaches in which neurogenesis was partially ablated by treatment with antimetabolic drugs (Shors et al., 2001; Shors et al., 2002) or by cranial irradiation (Madsen et al., 2003; Snyder et al., 2005) did not result in an impairment in Morris water maze learning. These studies suggest that hippocampal neurogenesis is not required for Morris water maze learning, which minimizes the likelihood that water maze learning stimulates hippocampal neurogenesis.

Our data also indicated that Morris water maze learning had no effect on cell proliferation in the hippocampal subgranular zone. This fits with other reports (Gould et al., 1999a; Van Praag et al., 1999b), although there is also evidence for an increase in hippocampal cell proliferation after Morris water maze learning (Lemaire et al., 2000). A recent study by Dobrossy and colleagues (Dobrossy et al., 2003) demonstrated that Morris water maze learning does not result in a net change in cell proliferation, but that cell proliferation is increased during the initial phase of the learning process, and that these newly formed cells die during the late phase of the learning process. In the current study, cell proliferation was determined on the basis of Ki-67 expression, which only provides information about the number of proliferating cells at the moment of perfusion, which was one day after the last training. Dynamic changes during the learning process can therefore not be excluded. Yet, our data suggest that hippocampus-dependent learning does not cause long-term changes in hippocampal cell proliferation.

The increase in the number of cells that express the polysialylated form of NCAM eighteen hours after training is in line with earlier studies (Fox et al., 1995; Murphy et al., 1996; Sandi et al., 2003) and it indicates that the learning task induced plastic changes in the dentate gyrus. PSA-NCAM is mainly observed in the subgranular zone of the dentate gyrus, the site of hippocampal neurogenesis and it is also expressed by newly formed cells that are one to three weeks old (Nakagawa et al., 2002; Seki, 2002a; Seki, 2002b; Seki and Arai, 1993; Seki and Arai, 1999). However, since our data show did not show any changes in BrdU-positive cell number after learning, the learning-induced increase in NCAM polysialylation is probably associated with plastic changes in the dentate gyrus, such as neurite outgrowth, dendritic branching or modification of intracellular signaling cascades (Muller et al., 1996; Rutishauser et al., 1988), but not with alterations in hippocampal neurogenesis.

Finally, under baseline conditions, *i.e.* in home cage control animals, Wistar rats had significantly more BrdU-positive cells in the granule cell layer than Sprague-Dawleys. Because the Ki-67 staining showed that the production of new cells was similar for the two strains, it can be suggested that, within the time window that was investigated, a higher percentage of newly formed cells had died in Sprague-Dawley rats. The strain difference in BrdU-positive cell number was reflected in PSA-NCAM expression, which is expressed by immature neurons. The strain-dependent difference in hippocampal neurogenesis had no impact on performance in the Morris water maze, which reduces the probability of a direct relation between the formation of new granule neurons and hippocampus-dependent learning.

In summary, we demonstrate a spatial learning-induced increase in NCAM polysialylation in the dentate gyrus, but no effect on hippocampal neurogenesis. These data show that behavioral interventions that induce plastic changes in the hippocampal formation are not sufficient for inducing alterations in hippocampal neurogenesis.

