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Morris Water Maze Learning in Two Rat Strains Increases the Expression of the Polysialylated Form of the Neural Cell Adhesion Molecule in the Dentate Gyrus But Has No Effect on Hippocampal Neurogenesis

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In the current study, the authors investigated whether Morris water maze learning induces alterations in hippocampal neurogenesis or neural cell adhesion molecule (NCAM) polysialylation in the dentate gyrus. Two frequently used rat strains, Wistar and Sprague–Dawley, were trained in the spatial or the nonspatial version of the water maze. Both training paradigms did not have an effect on survival of newly formed cells that were labeled 7–9 days prior to the training or on progenitor proliferation in the subgranular zone. However, the granule cell layer of the spatially trained rats contained significantly more positive cells of the polysialylated form of the NCAM. These data demonstrate that Morris water maze learning causes plastic change in the dentate gyrus without affecting hippocampal neurogenesis.

Keywords: Wistar, Sprague–Dawley, spatial learning, plasticity, adhesion molecules

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

The regulation of adult hippocampal neurogenesis appears to be activity dependent. Epileptic seizures in the DG (Parent et al., 1997), amygdala kindling (Scott, Wang, Burnham, De Boni, & Wojtowicz, 1998), or long-term potentiation in the mossy fibers (Derrick, York, & Martinez, 2000) enhance proliferation of hippocampal progenitors in the SGZ. Increased behavioral activity, such as wheel running (Trejo, Carro, & Torres-Aleman, 2001; Van Praag, Kempermann, & Gage, 1999) and enriched housing (Kempermann, Kuhn, & Gage, 1997; Nilsson, Perfilieva, Johansson, Orwar, & Eriksson, 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, Beylin, Tanapat, Reeves, & Shors, 1999). This effect seems to be specific for hippocampus-dependent learning tasks, because hippocampus-independent tasks, such as delay eyeblink conditioning or active shock avoidance learning, did not cause any changes in neurogenesis (Gould et al., 1999; Van der Borght, Meerlo, Luiten, Eggen, & Van der Zee, 2005). 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 2 weeks after the generation of hippocampal granule neurons (Cameron & 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, Christie, Sejnowski, & Gage, 1999), or they even found a decreased cell survival after spatial learning (Ambrogini et al., 2004). 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 & 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 & Rutishauser, 1998). In adulthood, NCAM polysialylation is strongly reduced, but it appears to be upregulated in circumstances requiring structural remodeling (Ronn, Berezin, & Bock, 2000). Demyelination of the spinal cord, for instance, or hippocampal damage caused by epileptic seizures increase PSA-NCAM expression in the lesioned area (Dominguez, Blasco-Ibanez, Crespo, Marques-Mari, & Martinez-Guijarro, 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—such as passive shock avoidance learning,

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Morris water maze training, and contextual fear conditioning—have been reported to stimulate NCAM polysialylation (Fox, O'Connell, Murphy, & Regan, 1995; Murphy, O'Connell, & Regan, 1996; Sandi et al., 2003).

In the current study, we aimed to investigate spatial learning-induced plastic changes in the DG in relation to neurogenesis. Because 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 DG. We also analyzed PSA-NCAM expression in the DG 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 & Gage, 2002a, 2002b), we decided to compare learning capacity, baseline neurogenesis, and learning-induced changes in plasticity in the DG between two widely used rat strains, Wistar and Sprague–Dawley.

Method

Rats and Housing

A total of 24 male Wistar rats (338 ± 24 g, bred in our own facilities) and 21 male Sprague–Dawley rats (336 ± 30 g, Harlan, Horst, the Netherlands) were individually housed. The rats had free access to water and food and were kept under a 12:12-hr light–dark cycle, lights on at 7:00 a.m. All procedures concerning care and treatment of the rats were in accordance with the regulations of the ethical committee for the use of experimental animals of the University of Groningen (DEC No. 2719).

Morris Water Maze Training and Bromodeoxyuridine (BrdU) Injections

The Morris water maze consisted of a black pool (diameter: 140 cm) filled with water (26 ± 1 °C). A small, black platform (diameter: 9 cm) was placed 23 cm from the border of the pool and 2.5 cm under the water surface to make it invisible to the rats. The behavior of the rats 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 with a protocol of five trials per day, with an intertrial interval of 20 min, for 5 consecutive days. Rats were allowed to swim for maximally 60 s per trial. The first trial of the 1st day was performed without a platform to give the rats the opportunity to habituate to the swimming procedure. In the second trial of the 1st day, the platform was present in the maze. If the rats had not been able to find the platform within 60 s, they were guided there by the experimenter. After having reached the platform, rats were kept there for 10 s 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 3 days, though the starting position of the rats was changed between trials. After 3 days, the platform position was changed, and the rats had to learn the new position of the platform on Days 4 and 5 of training.

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 rats 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 9 days prior to the start of the training, all rats were intraperitoneally injected with 100 mg/kg BrdU (Sigma, St. Louis, Missouri) dissolved in saline (20 mg/mL) once a day for 3 consecutive days.

Brain Processing and Immunocytochemistry

One day after training, approximately 18–20 hr after the last training session, rats were sacrificed by transcardial perfusion with heparinized saline, followed by 2.5% paraformaldeyde and 0.05% glutardialdehyde in 0.1 M phosphate buffer. After dehydration in 30% sucrose, 40 μm 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 phosphate buffer, 25% glycerol, and 25% ethylene glycol) and stored at −20 °C until they were used for immunocytochemistry.

We performed BrdU and Ki-67 immunocytochemistry on every 12th section of the hippocampus, using a protocol as described earlier (Van der Borght et al., 2005). 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; Biotechnology, Oxford, Oxfordshire, England) and mouse-anti-Ki-67 (1:200; Novocastra, Newcastle upon Tyne, United Kingdom). As secondary antibodies, biotinylated donkey-anti-rat and biotinylated sheep-anti-mouse (both 1:200; Jackson ImmunoResearch, West Grove, Pennsylvania) were used. Staining was visualized with diaminobenzidine (20 mg/100 mL, 3,3′-diaminobenzidine tetrahydrochloride [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, California) for 96 hr. As a secondary antibody, rabbit-anti-mouse IgM (1:200, Jackson ImmunoResearch) was used. After incubation with the ABC kit (Vector, Burlingame, United Kingdom), staining was visualized with DAB.

Quantification

During the analysis of the brain material, the experimenter was blind to the treatment of the rats. BrdU and Ki-67 immunopositive cells were counted in every 12th section of the hippocampal formation with a 40× objective. Only cells that were in the SGZ or one cell diameter deviating from this region were included. BrdU-positive cells that were lying in the GCL were counted as well. The number of counted cells was multiplied by 12 to get an estimation of the total number of positive cells per DG. 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 analysis of variance. When three experimental groups were compared, BrdU, Ki-67, and PSA-NCAM cell counts were statistically tested with a one-way analysis of variance. If this revealed a significant outcome, then a Bonferroni test was applied for post hoc testing. We performed comparison between the two groups using an independent-samples t test.

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—see Figure 1A, between strains: $F(1, 13) = 0.10$, $p = .75$; Strain × Trial: $F(13, 169) = 0.70$, $p = .76$. After relocation of the platform on Day 4, the rats 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 rats—see Figure 1A, between strains: $F(1, 13) = 1.53$, $p = .24$; Strain × Trial: $F(9, 117) = 0.86$, $p = .57$. As expected, the rats showed a decrease in the distance they needed to swim to find the platform ($p < .001$ for both the first 15 trials and the last 10 trials). The latency to find the platform could not be used as an indicator of learning performance, because the two strains significantly differed in swim speed (Wistar: 18.5 ± 0.6 cm/s, Sprague–Dawley: 23.2 ± 0.4 cm/s), $F(1, 29) = 32.99$, $p < .001$. 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 (see Figure 1B, $p < .001$). Sprague–Dawley rats swam a greater distance before reaching the platform than Wistar rats—see Figure 1B, between strains: $F(1, 13) = 20.27$, $p = .001$. However, both strains managed to acquire the task. Moreover, there was no significant interaction between strain and trial, $F(23, 299) = 0.93$, $p = .56$. Learning speed differed significantly between place learners and rats that were trained with the visible platform (Wistar: $p < .001$; Sprague–Dawley: $p < .01$).

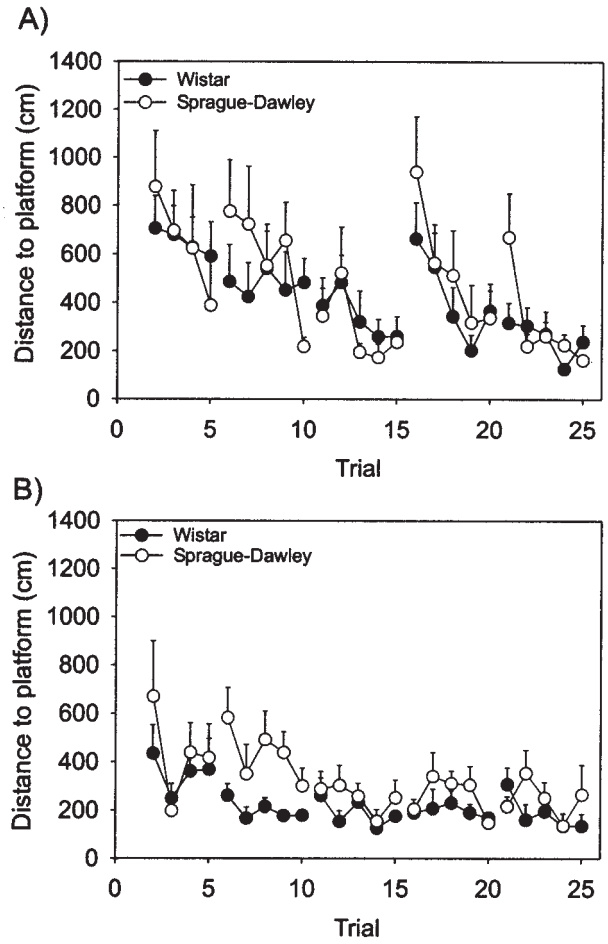

Figure 1. Learning curves of Wistar ($n = 8$) and Sprague–Dawley ($n = 7$) rats in the place (A) or cue (B) version of the Morris water maze. Training consisted of five trials per day for 5 consecutive days. The first trial on Day 1 was performed without a platform. In the group of place learners, the platform was relocated after 3 days of training. Both rat strains performed equally well in the place-learning task, but Wistar rats performed significantly better than Sprague–Dawley rats during cue learning ($p = .001$). Data are expressed as mean distance moved before reaching the platform plus or minus standard error of the mean.

BrdU

To investigate the effects of the learning task on the survival of newly formed hippocampal cells, we injected rats with the thymidine analog BrdU 7–9 days before the start of the training. One day after the last training, rats were sacrificed and brains were processed for immunocytochemistry. Quantification of the number of BrdU-positive cells in the DG did not reveal any differences between home-cage controls, cue learners, and place learners (see Figure 2). This was the case for both rat strains—Wistar: $F(2, 23) = 1.49$, $p = .25$; Sprague–Dawley: $F(2, 20) = 0.03$, $p = .97$. 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–Dawley rat strains having 42% less positive cells than Wistar rat strains, $F(1, 14) = 28.94$, $p < .01$.

Ki-67

The Ki-67 protein is expressed in all cells during all phases of the cell cycle, except G0 (Scholzen & 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 SGZ showed that neither place learning nor cue learning caused a change in hippocampal cell proliferation—see Figure 3, Wistar: $F(2, 23) = 0.78$, $p = .47$; Sprague–Dawley: $F(2, 20) = 0.99$, $p = .39$. Also, Ki-67 expression did not differ between the home-cage controls of both strains, $F(1, 14) = 1.40$, $p = .26$, indicating that baseline hippocampal cell proliferation is similar for Wistar and Sprague–Dawley rat strains.

PSA-NCAM

The binding of α2,8-linked polysialic acid homopolymers to the 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 (see Figure 4, $p < .05$). Also in the Sprague–Dawley rats, a learning effect was observed. Place learners had 31% more immunoreactive cells compared with home-cage con-

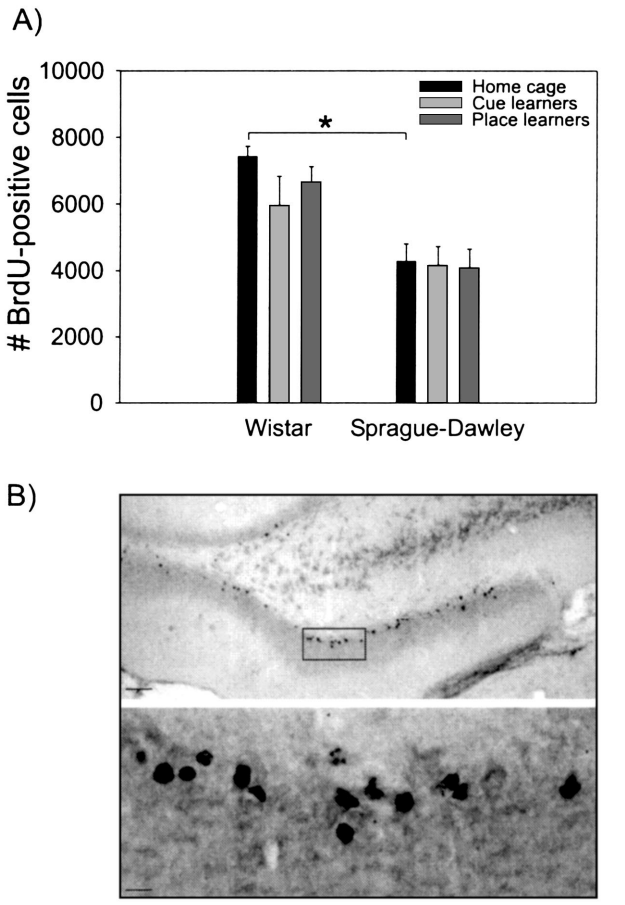

Figure 2. The number of bromodeoxyuridine (BrdU)-positive cells per dentate gyrus. (A) Neither in Wistar rats ($n = 8$ per group) nor in Sprague–Dawley rats ($n = 7$ per group) was any difference observed in BrdU-positive cell number between home-cage controls, cue learners, or place learners. However, Wistar home-cage controls had significantly more BrdU-positive cells than the Sprague–Dawley home-cage control rats (*$p < .01$). Data are expressed as mean plus or minus standard error of the mean. (B) Representative photomicrographs of BrdU-immunocytochemistry. Scale bar = 50 μm in the upper panel. A magnification of the selected region is shown in the lower panel (scale bar = 10 μm).

trols ($p < .001$). 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 DG than Sprague–Dawley rats ($p < .001$).

Discussion

In the current study, we investigated the occurrence of plastic changes in relation to neurogenesis in the hippocampal DG 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 1 and 3 weeks after their formation, a large part of the newly formed granule cells die (Cameron & McKay, 2001; Dayer et al., 2003; Hastings & Gould, 1999). Gould et al. (1999) reported that training rats in a spatial learning task within this critical period, that is, starting 7 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 8–10 days after BrdU administration (Ambrogini et al., 2004). In the current study, in which water maze training was started 7–9 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 toward the CA3 region (Hastings & Gould, 1999), which reduces the possibility that the BrdU-labeled cells in the current 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 antimitotic drugs (Shors et al., 2001; Shors, Townsend, Zhao, Kozorovitskiy, & Gould, 2002) or by

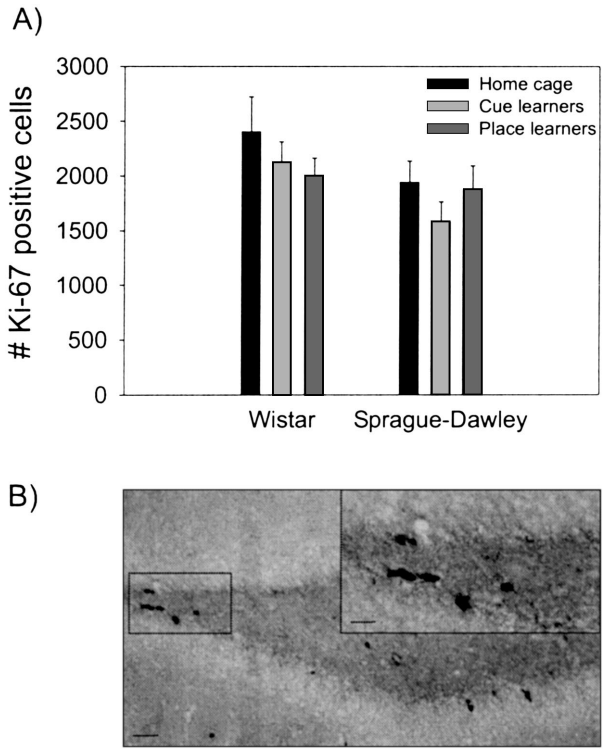

Figure 3. Ki-67 expression in the hippocampal subgranular zone. (A) Neither hippocampus-independent nor hippocampus-dependent learning in the water maze caused a change in hippocampal cell proliferation. This was the case for both rat strains (Wistar, $n = 8$ for all groups; Sprague–Dawley, $n = 7$ for all groups). Also, no strain differences were observed in Ki-67 expression. Data are expressed as mean plus or minus standard error of the mean. (B) Example of Ki-67 immunocytochemistry in the hippocampus (scale bar = 50 μm). The insert shows an enlargement of the selected region (scale bar = 10 μm).

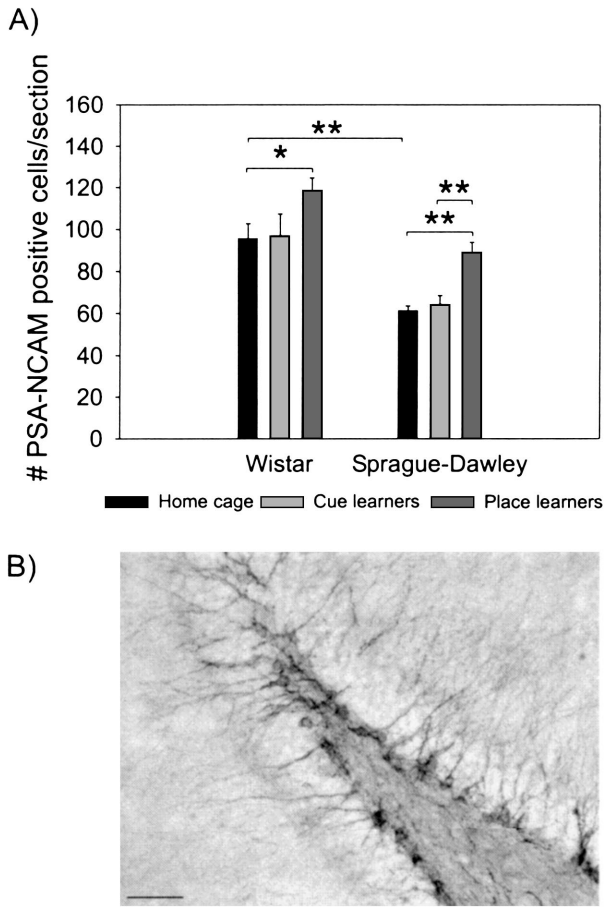

Figure 4. The polysialylated form of the neural cell adhesion molecule (PSA-NCAM) expression in the hippocampal subgranular zone. (A) Both in Wistar rats and Sprague–Dawley rats, a learning-induced increase in PSA-NCAM immunoreactivity in the subgranular zone was observed. In Sprague–Dawley rats, place learners also had significantly more PSA-NCAM positive cells than cue learners. Moreover, when comparing home-cage control rats, we observed a significant difference between Wistar rats and Sprague–Dawley rats, with Wistar rats having more PSA-NCAM immunopositive cells than Sprague–Dawley rats (*$p < .05$, **$p < .001$). Data are expressed as mean plus or minus standard error of the mean. (B) Photomicrograph of PSA-NCAM immunocytochemistry in the dentate gyrus. Scale bar = 50 μm.

cranial irradiation (Madsen, Kristjansen, Bolwig, & Wortwein, 2003; Snyder, Hong, McDonald, & Wojtowicz, 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 SGZ. This fits with other reports (Gould et al., 1999; Van Praag, Kempermann, & Gage, 1999), although there is also evidence for an increase in hippocampal cell proliferation after Morris water maze learning (Lemaire, Koehl, Le Moal, & Abrous, 2000). A recent study by 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 1 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 expressed the polysialylated form of NCAM 18 hr 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 DG. PSA-NCAM is mainly observed in the SGZ of the DG, the site of hippocampal neurogenesis, and it is also expressed by newly formed cells that are 1–3 weeks old (Nakagawa et al., 2002; Seki, 2002a, 2002b; Seki & Arai, 1993, 1999). However, because our data 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 DG, such as neurite outgrowth, dendritic branching, or modification of intracellular signaling cascades (Muller et al., 1996; Rutishauser, Acheson, Hall, Mann, & Sunshine, 1988), but not with alterations in hippocampal neurogenesis.

Finally, under baseline conditions, that is, in home-cage control rats, Wistar rats had significantly more BrdU-positive cells in the GCL than Sprague–Dawley rats. 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 DG without affecting 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.

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