

## ORIGINAL ARTICLE

# Synaptic Adhesion Molecules Regulate the Integration of New Granule Neurons in the Postnatal Mouse Hippocampus and their Impact on Spatial Memory

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## Abstract

Postnatal hippocampal neurogenesis induces network remodeling and may participate to mechanisms of learning. In turn, the maturation and survival of newborn neurons is regulated by their activity. Here, we tested the effect of a cell-autonomous overexpression of synaptic adhesion molecules on the maturation and survival of neurons born postnatally and on hippocampal-dependent memory performances. Families of adhesion molecules are known to induce pre- and post-synaptic assembly. Using viral targeting, we overexpressed three different synaptic adhesion molecules, SynCAM1, Neuroligin-1B and Neuroligin-2A in newborn neurons in the dentate gyrus of 7- to 9-week-old mice. We found that SynCAM1 increased the morphological maturation of dendritic spines and mossy fiber terminals while Neuroligin-1B increased spine density. In contrast, Neuroligin-2A increased both spine density and size as well as GABAergic innervation and resulted in a drastic increase of neuronal survival. Surprisingly, despite increased neurogenesis, mice overexpressing Neuroligin-2A in new neurons showed decreased memory performances in a Morris water maze task. These results indicate that the cell-autonomous overexpression of synaptic adhesion molecules can enhance different aspects of synapse formation on new neurons and increase their survival. Furthermore, they suggest that the mechanisms by which new neurons integrate in the postnatal hippocampus conditions their functional implication in learning and memory.

**Key words:** adult neurogenesis, hippocampus, synaptic integration, plasticity

## Introduction

In the mammalian brain, neurogenesis stops at birth except in the dentate gyrus of the hippocampus and the subventricular zone, where lifelong neurogenesis is commonly referred to as adult neurogenesis (Altman and Das 1965; Eriksson et al. 1998). Although the function of adult neurogenesis in the hippocampus is debated (Hennand Vollmayr 2004; Jaholkowski et al. 2009; Groves et al. 2013; Lazic et al. 2014), several lines of evidence indicate that the addition of new neurons enhances synaptic plasticity of the hippocampal network. Indeed, immature

neurons display enhanced excitability and plasticity between 3 and 7 weeks after division (Schmidt-Hieber et al. 2004; Ge et al. 2007; Tronel et al. 2010; Marin-Burgin et al. 2012) and the experimental reduction of hippocampal adult neurogenesis in rodents results in learning and memory impairments (Saxe et al. 2006; Deng et al. 2009). Notably, the optogenetic inhibition of new, still immature hippocampal granule neurons during a spatial memory recall task decreased performance (Gu et al. 2012), indicating that new neurons are directly involved in memory retrieval (Zhao et al. 2008). Inversely, several studies

show that improved learning and memory performances can be induced by increasing adult neurogenesis with enriched environment or voluntary exercise (Kempermann et al. 1997; van Praag et al. 1999) (but see Hauser et al. 2009; Lazic et al. 2014), by the cell-autonomous inhibition of apoptosis in newly-formed neurons (Sahay et al. 2011) or by the enhancement of neuronal fate differentiation (Richetin et al. 2015).

The process of maturation and synaptic integration of new granule neurons varies greatly across species and lasts from about a month in rodents to several months in primates (Kohler et al. 2011; Brus et al. 2013). It follows distinct steps which are strongly activity-dependent: quickly after their differentiation, newborn neurons extend their axons to the CA3 region and their dendrites towards the molecular layer of the dentate gyrus. During this period, they receive functional GABAergic input (Song et al. 2013) which, owing to a high chloride intracellular concentration, is depolarizing (Tozuka et al. 2005; Ge et al. 2006). They also express N-methyl-D-aspartate receptors (NMDAR), but no  $\alpha$ -amino-3 hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) and are therefore synaptically silent (Chancey et al. 2013). The concurrent GABA<sub>A</sub> receptor-mediated depolarization and glutamate-mediated NMDAR activation lead to the expression of AMPAR at the post-synaptic membrane, thereby unsilencing the activated neurons (Chancey et al. 2013). This stage is required for the formation of dendritic spines, since interfering with GABA<sub>A</sub>-mediated depolarization or D-serine availability, a NMDAR co-agonist, result in impaired dendritic development and synaptic integration (Ge et al. 2006; Sultan et al. 2015). Upon formation, immature dendritic spines preferentially contact pre-existing axon terminals already synapsing with one or several postsynaptic granule neurons and thereby form multiple synapse boutons (MSB); (Toni et al. 2007). The consequent transformation of MSB into single synapse boutons suggests that dendritic spines from new neurons compete with spines from more mature neurons (Toni et al. 2007; Toni and Sultan 2011; Bergami and Berninger 2012). Concomitantly, the majority of postnatally-born neurons die during the first month of maturation (Kempermann et al. 2003) and this drastic elimination of new neurons occurs during the first steps of glutamatergic synaptogenesis, during the third week of maturation (Tashiro et al. 2007). Furthermore, knocking out the essential NMDAR subunit NR1 specifically in newborn neurons drastically reduces their survival. This effect can be mitigated by the inhibition of overall NMDAR activity with the NMDAR selective antagonist 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) during the critical phase of maturation (Tashiro et al. 2006). Together, these results suggest that the competitive process of synapse formation and stability plays a crucial role in the survival of newborn neurons. It is therefore possible that manipulations aimed at increasing synapse formation specifically on new neurons may improve their survival during this critical phase. In turn, these manipulations may result in improved hippocampal-dependent memory performances. To directly test this possibility, we used a viral-mediated gene transfer approach to increase the expression of synaptic adhesion molecules specifically in new granule, hippocampal neurons, in 7- to 9-week-old mice. In the context of synaptic competition between immature and mature neurons, the use of a retroviral approach is crucial, since it enables to test the cell-autonomous effect of an increase in synapse formation on the integration of a subset of newborn neurons, without interfering with the mature neuronal network into which they integrate.

Several adhesion molecules enable pre- and post-synaptic assembly. These proteins include neurexins/neuroligins and

the SynCAM proteins of the immunoglobulin superfamily (Biederer and Stagi 2008; Jin and Garner 2008; Giagtzoglou et al. 2009). Interestingly, the expression of these molecules in non-neuronal cells enables synapse formation with co-cultured neurons, indicating that they have strong synaptogenic properties (Scheiffele et al. 2000; Biederer et al. 2002; Graf et al. 2004). We overexpressed three different synaptic adhesion molecules in new granule neurons: SynCAM1, Neuroigin-1B (NL1B) and Neuroigin-2A (NL2A). These molecules were chosen for their distinct and complementary roles: SynCAM1 is a transmembrane protein located pre- and post-synaptically that engages in hetero- and homophilic adhesive interactions. In neurons, SynCAM1 overexpression increases excitatory synapse number, whereas the loss of SynCAM1 or the overexpression of its cytosolic domain (ctSynCAM1), acting as a dominant negative form of SynCAM1, result in fewer synapses (Biederer et al. 2002). Neuroligins are transmembrane proteins located post-synaptically, and act as ligands for presynaptic neurexins (Ichtchenko et al. 1995; Nguyen and Sudhof 1997; Boucard et al. 2005; Comoletti et al. 2006; Craig and Kang 2007). Similar to SynCAM, Neuroligins mediate the formation and maintenance of synapses between neurons *in vitro* and *in vivo* (Scheiffele et al. 2000; Graf et al. 2004; Prange et al. 2004; Kwon et al. 2012; Zeidan and Ziv 2012) and are involved in the formation of excitatory synapses (Budreck et al. 2013). There are several isoforms and splice variants of Neuroligins with different roles in specific synapse formation (Nguyen and Sudhof 1997; Chih et al. 2005; Varoqueaux et al. 2006) and Neuroigin-1B (NL1B) overexpression increases glutamatergic synaptogenesis in cultured hippocampal neurons (Chih et al. 2005). NL1 is only present at excitatory synapses (Song et al. 1999) whereas Neuroigin-2 (NL2) is present at inhibitory synapses (Varoqueaux et al. 2004; Hoon et al. 2011). NL2A overexpression in hippocampal neurons induces both glutamatergic and GABAergic synapse formation (Chih et al. 2006).

These molecules were co-expressed with a fluorescent reporter to enable the identification of the targeted neurons and enable the assessment of their maturation and survival. We found that SynCAM1 and Neuroigin-1B overexpression had significant but subtle effects on the synaptogenesis of new neurons and did not modify their survival. In contrast, NL2A overexpression significantly increased both the glutamatergic and GABAergic inputs on new neurons and increased their survival. However, despite increased neurogenesis, mice with NL2A overexpressing new hippocampal neurons displayed impaired spatial memory performances.

## Materials and Methods

### Experimental Animals

The animals used in this study were 7- to 9-week-old C57BL/6-J male mice. Mice were group-housed in standard cages under light- (12 h light/dark cycle) and temperature-controlled (22 °C) conditions. The maximal number of mice per cage was five. Food and water were available *ad libitum*. Every effort was made to minimize the number of animals used and their suffering. Experimental protocols were approved by the Swiss animal experimentation authorities.

### Virus-mediated Gene Expression

To selectively express the genes of interest in new hippocampal neurons, we used Moloney murine leukemia viruses (MoMuLV) as previously described (Zhao et al. 2006). Final virus

titers were  $10^7$ – $10^8$  particles forming unit/milliliter (pfu/mL) and 2  $\mu$ L of the mix were injected into each of the two dentate gyri at the following coordinates from the Bregma: anteroposterior –2 mm, lateral +1.75 or –1.75 mm and dorso-ventral –2.25 mm. After every injection and throughout the experiment, animals were regularly monitored for their physical recovery.

To test proper expression and localization of the gene of interest, Human embryonic kidney (HEK) 293T cells were infected with  $10^4$  pfu of retrovirus carrying each construct (1  $\mu$ L), and immunohistochemistry and immunoblotting against the corresponding adhesion molecule were performed. Expression of the proteins was effective and localized at the cell plasma membrane (SynCAM1, NL1B, NL2A) or in the cytosol (SynCAM1 cytosolic tail), showing proper addressing of the proteins after synthesis (Supplementary Fig. 1).

### Brain Slice Preparation and Immunohistochemistry

Mice were perfused with 4% paraformaldehyde in phosphate buffered saline, their brains were cryoprotected and sectioned at a thickness of 60  $\mu$ m. RFP signal was amplified using rabbit anti-RFP IgG (600-401-379 Rockland Immunochemicals, Gilbertsville, Pennsylvania, USA; 1:1000) and Hylite 594 goat anti-rabbit IgG secondary antibody (61056-1-H594 Anawa trading SA, Wangen, Switzerland; 1:500). GFP signal was amplified using Chicken anti-GFP IgG (GFP-1020 Aves Labs, Tigard, Oregon, USA; 1:1000) and Dylight 488 goat anti-chicken IgY (603-141-126 Anawa Trading SA; 1:500). Vesicular GABA Transporter (VGAT) was detected using mouse anti-VGAT IgG (131011 Synaptic Systems GmbH, Goettingen, Germany; 1:1000) and Hylite 594 goat anti-mouse IgG secondary antibody (61057-05-H594 Anawa trading SA; 1:500). GABA<sub>A</sub> receptors were detected using rabbit anti-GABA<sub>A</sub> receptor IgG (AGA-001 Alomone Labs, Jerusalem, Israel; 1:500) and donkey Dylight 649 anti-rabbit IgG secondary antibody (611-743-127 Anawa trading SA; 1:500). Arc was detected using rabbit anti-Arc IgG (156003 Synaptic systems; 1:500) and donkey Dylight 649 anti-rabbit IgG secondary antibody (611-743-127 Anawa trading SA; 1:500).

In HEK 293T cells cultures infected with MoMuLVs, SynCAM1 was detected using chicken anti-SynCAM1 IgG (CM004-3 MBL International, Woburn, MA, USA; 1:1000) and Dylight 488 goat anti-chicken IgY (603-141-126 Anawa Trading SA; 1:500). ctSynCAM1 was detected using rabbit anti-SynCAM1 IgG (S 4945, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland; 1:5000) and Cy3 goat anti-rabbit IgG secondary antibody (111-165-144, Jackson ImmunoResearch Laboratories, Westgrove, PA, USA; 1:500). NL1B was detected using goat anti-Neurologin-1 IgG (ANR-036; Alomone labs; 1:25) and donkey Alexa 555 anti-goat IgG secondary antibody (A21432 Invitrogen, Lucerne, Switzerland; 1:250). NL2A was detected using rabbit anti-Neurologin-2 IgG (sc-50394 Santa Cruz biotechnologies; 1:100) and goat Hylite 594 anti-rabbit IgG secondary antibody (61056-1-H594 Anawa trading SA; 1:500). 4,6 Diamidino-2-phenylindole (DAPI) was used to reveal nuclei.

### Confocal Microscopy and Image Analyses

Hippocampal sections were imaged using a Zeiss LSM 710 confocal microscope (Carl Zeiss, Feldbach, Switzerland). Global views of neurons were imaged with a  $\times 40$  oil lens and a z-step of 2  $\mu$ m, and dendrites or mossy fiber terminals (MFT) were imaged with a  $\times 63$  oil lens and a z-step of 0.38  $\mu$ m. To assess GABAergic synapse density, dendrites were imaged with a  $\times 40$  oil lens and a z-step of 0.45  $\mu$ m. To measure the differences in

axonal length between ctSynCAM1-expressing and control neurons, the beginning and the end of the CA3 area were imaged using a  $\times 40$  oil lens and a z-step of 2  $\mu$ m. All analyses were performed using Fiji software (freely available at <http://fiji.sc/>).

Dendritic spine density was defined as the number of spines divided by the length of the dendritic segment. Dendritic spine density and diameter were measured on maximal intensity projections. Protrusions with a diameter inferior to 0.3  $\mu$ m were considered as filopodia, with a diameter comprised between 0.30  $\mu$ m and 0.55  $\mu$ m as thin spines and protrusions with a diameter greater than 0.55  $\mu$ m as mushroom spines. GABAergic synapses were defined as appositions of vesicular GABA transporter (VGAT)-positive and GABA<sub>A</sub> receptor (GABA<sub>A</sub>R)-positive puncta after immunohistochemistry against VGAT and GABA<sub>A</sub>R. GABAergic synapse density was analyzed on individual optical sections of confocal z-stacks and measured as the number of GABAergic synapses divided by the length of the dendritic segment.

The maturation of MFT was evaluated by measuring their area, perimeter, number of extensions and circularity, defined as  $4 \times \pi \times \text{area} / (\text{perimeter})^2$ . The area and perimeter of MFT were measured on maximal intensity projections by tracing the contour of the MFT, excluding the satellites and filopodia. The number of extensions of the MFT was defined as the number of filopodia or satellites starting at the core of the MFT. Branching of satellites was not included in the counting (Fig. 1J).

Sholl analysis was performed on maximal intensity projections. Since neurons from different parts of the dentate gyrus had a different maximal length, the space between each radius was set up at 10% of the maximal length of the neuron (Krzisch et al. 2013).

Maximal dendritic extension was measured on maximal intensity projections and was defined as the ratio between the distance from the soma to the tip of its longest dendrite and the distance from the soma to the end of the molecular layer of the dentate gyrus (Krzisch et al. 2013).

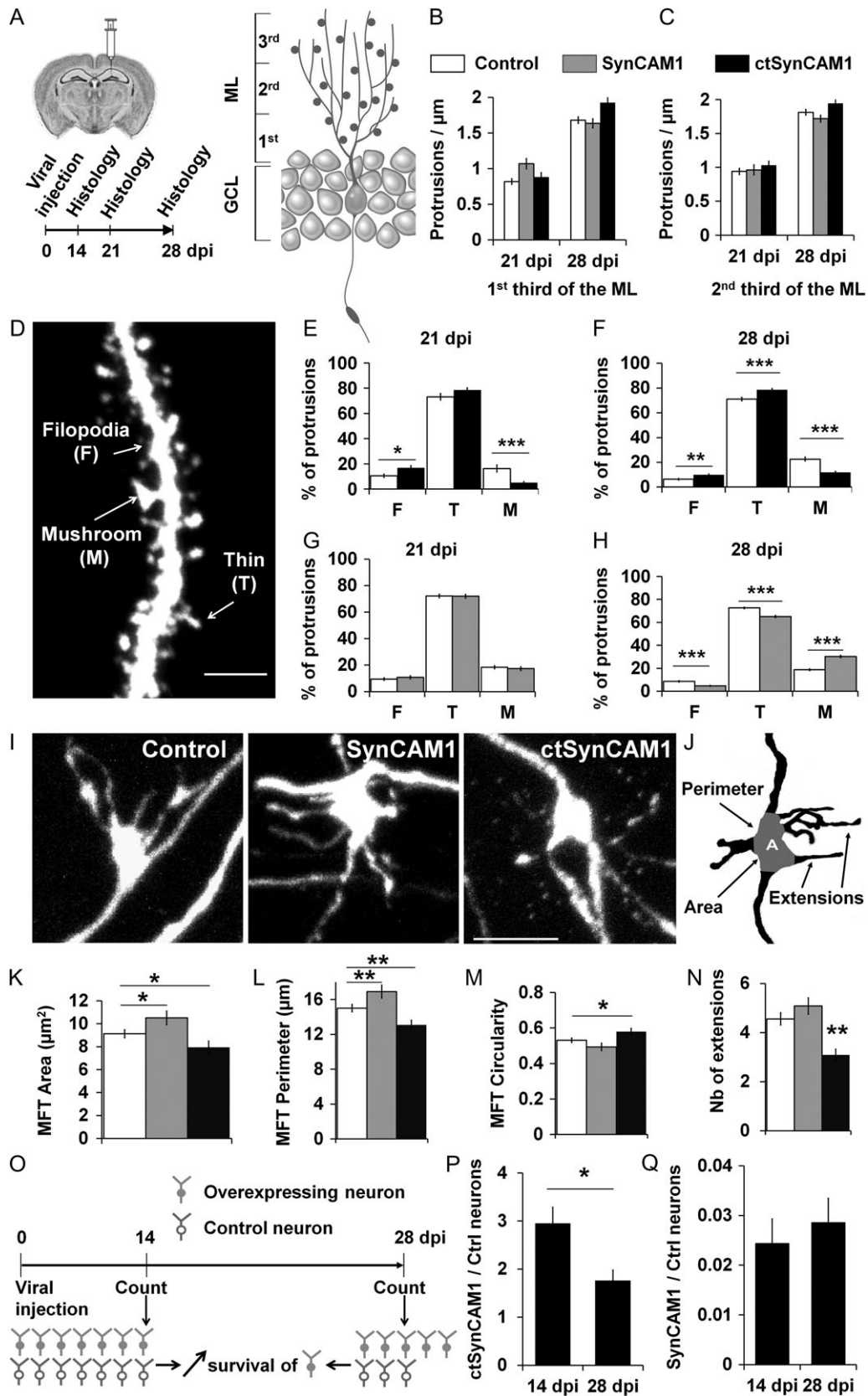
The survival of new neurons was assessed by injecting eight mice with the same mixture of retroviruses, and analyzing four mice at 14 days post injection (dpi) and four mice at 28 dpi. For each mouse, the ratio between the total number of overexpressing neurons and the total number of control neurons was calculated at the two timepoints. An increased ratio indicated increased survival of neurons overexpressing the protein of interest (Fig. 1O); (Tashiro et al. 2006).

To compare the neuronal activity of control and overexpressing neurons, retrovirus injected mice were placed in environmental enrichment for 10 min. After enrichment for 30–90 min mice were sacrificed. One hundred neurons of each group were analyzed per mouse, and the proportion of new neurons that expressed the immediate-early gene Arc was calculated for control and overexpressing neurons in each mouse.

### Behavioral Tests

#### Elevated Plus Maze

Anxiety-like behavior was assessed with the elevated plus maze (EPM). The EPM consists of two open and two enclosed arms ( $30.5 \times 5.5 \text{ cm}^2$ ) emanating from a central platform ( $5.5 \times 5.5 \text{ cm}^2$ ). Mice were placed in the center of the maze facing a closed arm and allowed to freely explore the maze for 5 min. Animals' behavior was recorded and analyzed using ANY-maze software (Stoelting).



**Figure 1.** Effect of ctSynCAM1 and SynCAM1 overexpression on newborn neurons maturation and survival. (A). Left panel: experimental timeline and schematic procedure for in vivo gene delivery: The dentate gyrus of 7- to 9-week-old mice was injected with a mix of two retroviruses, one carrying the expression cassette of the

### Morris Water Maze

Spatial learning and memory was assessed using the Morris water maze at 28 dpi. Behavioral testing was conducted in a dimly lit room (30 lx at the rim of the water). The water maze pool ( $\varnothing$  140 cm) was filled with 20 cm water (25 °C) made opaque by adding 200 ml of milk. An escape platform ( $\varnothing$  12 cm) was submerged 1 cm below the water surface in the east quadrant (target quadrant) of the pool. To facilitate the animals' orientation five distinct cues were glued inside of the pool approximately 20 cm above water level.

Water maze training took place across seven consecutive days for four trials/day per animal with an inter trial interval of 15 s. A trial was completed when the animal climbed on the platform or 40 s had elapsed. Upon failure to find the target platform during training, animals were guided to the target platform and left there for 10 s. Following training, spatial memory was assessed by two probe trials, conducted at 24 h and 120 h after last training session. Opposite starting positions were used for each probe trial. During probe trial sessions, the platform was removed from the pool and animals were left to swim for 40 s. The latency to find the platform during training sessions as well as the time animals spend in each quadrant during the probe test was analyzed using ANY-maze software (Stoelting).

### Immunoblotting

Transfected HEK293T cells were homogenized in ice-cold lysis buffer (15 mM Tris-HCl, pH 7.6, 320 mM sucrose, 1 mM DTT, 1 mM MgCl<sub>2</sub>, 3 mM EDTA-K, 0.5% protease inhibitor cocktail (Sigma, P8340), 1% phosphatase inhibitor cocktail 1 (Sigma, P2850), and 1% phosphatase inhibitor cocktail 2 (Sigma, P5726). Protein samples were sonicated and frozen at -80 °C. Protein extracts (10 mg) were run on 10% SDS-PAGE by application of 110 V for 90 min. After running, proteins were transferred on nitrocellulose membranes (84261514; Whatman) by application of 375 mA for 90 min at 4 °C. Then, membranes were incubated in the Odyssey blocking buffer (927-40000; LI-COR) at room temperature for 1 h and then incubated overnight at 4 °C with the following primary antibodies: mouse anti-RFP (RF5F, ThermoFisher scientific, 1/1000), rabbit anti-GFP (A-11122, ThermoFisher scientific, 1/1000), rabbit anti-SynCAM1 (S 4945, Sigma-Aldrich, 1:1000), goat anti-Neuroigin-1 (Alomone labs; 1:250), rabbit anti-Neuroigin-2 (Santa Cruz biotechnologies; 1/500). After washes in Phosphate Buffer Saline (PBS), secondary antibodies anti-mouse IgGIRDye 800 (1:10 000, 610-132-121; Bioconcept), anti-rabbit IgG Alexafluor 680 (1:10 000, Juro, Lucerne, Switzerland) and anti-goat IgG Alexafluor 680 (1:10 000, Juro, Lucerne, Switzerland) were applied for 1 h at room temperature and protected from light. After washing with PBS, membranes were scanned with the ODYSSEY Infrared Imaging System (LI-COR Biosciences).

### Statistical Analyses

Hypothesis testing was two-tailed. All analyses were performed using GraphPad Prism 6 (Graphpad Software, Inc., La Jolla, California, USA). First, Shapiro-Wilk tests were performed on each group of data to test for distribution normality. When the distribution was not normal, the non-parametric Mann-Whitney test was applied. When the distribution was normal, the equality of variances of the groups was tested and the adequate unpaired t-test was used. Data are presented as mean  $\pm$  SEM. For Sholl analyses, the total dendritic length of control and over-expressing neurons was calculated and used for statistical analyses. Two-way repeated measure ANOVA was used to compare treatment groups in the Morris water maze and one sample t-test was applied to estimate whether the percent time spent in the target quadrant during the probe trial was significantly different from 25 %.

## Results

### ctSynCAM1 Decreases the Synaptic Maturation and Survival of New Neurons

To investigate whether SynCAM1 is involved in the synaptic integration of new neurons, we overexpressed ctSynCAM1 and the green fluorescent protein (GFP) in new hippocampal neurons using a retroviral vector derived from the Moloney murine leukemia virus (MoMuLV, Zhao et al. 2006). ctSynCAM1 contains the full length cytoplasmic tail of SynCAM1, so cannot engage in adhesive interactions. It therefore acts as a competitive inhibitor of endogenous SynCAM1. The same viral vector enabling the expression of the red fluorescent protein (mRFP1) alone was co-injected as a control, to enable the identification of wild-type new neurons in the same mice (Supplementary Fig. 1A). In vitro infection of HEK 293T cells with the vector encoding ctSynCAM1 resulted in strong cytoplasmic expression of GFP and membrane expression of ctSynCAM1, as observed by immunohistochemistry and western blot (Supplementary Fig. 1B and C).

Both viral vectors were then injected in the dentate gyrus of 7- to 9-week-old mice and, 14, 21 or 28 days later (days post-injection, dpi), labeled new neurons were observed using confocal microscopy (Fig. 1A). We first assessed the development of their dendritic branching using Sholl analysis. The number of branches was transiently increased by ctSynCAM1 expression at 21 dpi, but not at 14 or 28 dpi (Mann-Whitney test on the total dendritic length, 14 dpi:  $p > 0.05$ ; 21 dpi:  $p = 0.002$ ; 28 dpi:  $p > 0.05$ ; Supplementary Fig. 2A), suggesting a role of SynCAM1 in dendritic maturation.

The vast majority of dendritic protrusions bears glutamatergic synapses and appears during the third week after cell division (Zhao et al. 2006; Toni et al. 2007). To assess the development of glutamatergic input on new neurons, we measured the density of dendritic protrusions at 21 and 28 dpi.

gene of interest, and the other one carrying the control construct. Animals were sacrificed at 14, 21 or 28 dpi and new neurons' morphology and survival was analyzed. Right panel: Illustration of a granule neuron in the granule cell layer (GCL), projecting dendrites in the ML. (B, C) Density of dendritic protrusions of control (white), SynCAM1-overexpressing (grey) and ctSynCAM1-overexpressing (black) newborn neurons in the first third (B) and second third (C) of the ML. (D) Confocal maximal intensity projection of a dendrite, illustrating the three classes of dendritic protrusions: filopodia (F), thin (T) and mushroom spines (M). (E, F) Proportion of protrusions with filopodia, thin or mushroom morphologies in ctSynCAM1-expressing neurons at 21 (E) and 28 dpi (F). (G, H) Proportion of filopodia, thin and mushroom spines in control and SynCAM1-overexpressing neurons at 21 (G) and 28 dpi (H). (I) Confocal maximal intensity projections of representative MFT for each group at 28 dpi. (J) Schematic illustration of the perimeter, projection area, circularity and number of extensions on MFT. (K-N) Mean area (K), perimeter (L), circularity (M) and number of extensions (N) on MFT. (O) Schematic illustration of the relative quantification of survival of transgenic and control neurons. (P) Average ratio of the total number of ctSynCAM1-expressing neurons and the total number of control neurons per mouse. (Q) Average ratio of the total number of SynCAM1-overexpressing neurons and the total number of control neurons per mouse. dpi: days post-injection; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . Scale bars: 5  $\mu$ m (D) and 10 m (I).

The density of dendritic protrusions was not modified at 21 and 28 dpi in both the first third of the molecular layer (ML, principally innervated by commissural/associative afferences, 21 dpi:  $p > 0.05$ ; 28 dpi:  $p > 0.05$ ; Fig. 1A and B) and the middle third of the ML (innervated by perforant path afferences from the entorhinal cortex, 21 and 28 dpi:  $p > 0.05$ ; Fig. 1A and C).

The maturation of dendritic protrusions is characterized by an increase in diameter of the protrusions' tip (Zhao et al. 2006; Toni et al. 2007), reflecting the expression of AMPA receptors and synapse unsilencing (Takumi et al. 1999; Matsuzaki et al. 2001). To examine whether ctSynCAM1 interfered with the maturation of dendritic protrusions, we measured the diameter of the tip of dendritic protrusions. Protrusions with a diameter inferior to  $0.3\ \mu\text{m}$  were considered as filopodia, with a diameter comprised between  $0.30\ \mu\text{m}$  and  $0.55\ \mu\text{m}$  as thin spines and protrusions with a diameter greater than  $0.55\ \mu\text{m}$  as mushroom spines (Fig. 1D); (Sultan et al. 2013). At 21 and 28 dpi, the proportion of mushroom spines was decreased and the proportion of filopodia and thin spines was increased by ctSynCAM1 expression (filopodia: 21 dpi:  $p = 0.017$ ; 28 dpi:  $p = 0.001$ ; thin: 21 dpi:  $p > 0.05$ ; 28 dpi:  $p = 0.0001$ ; mushroom: 21 dpi:  $p = 0.0003$ ; 28 dpi:  $p < 0.0001$ ; Fig. 1E and F), indicating that interfering with SynCAM1 altered the maturation of dendritic protrusions on new neurons.

We next examined the effect of ctSynCAM1 expression on the axonal maturation of new neurons. The main efferences from new granule neurons are mossy fibers, with terminals (MFT) synapsing on dendrites of CA3 area pyramidal neurons. With maturation, there is an increase in the length of mossy fiber axons and of MFT surface area, perimeter and number of extensions protruding from MFT and a decrease in circularity (Zhao et al. 2006; Toni et al. 2008). New neurons expressing ctSynCAM1 displayed reduced morphological maturation of MFT at 28 dpi (area:  $p = 0.02$ ; perimeter:  $p = 0.007$ ; circularity:  $p = 0.04$ ; number of extensions:  $p = 0.003$ ; Fig. 1I–N). Thus ctSynCAM1 expression also reduced the axonal maturation of new neurons.

Finally, we examined whether the decreased maturation of dendritic protrusions and MFT was associated with changes in neuronal survival. Survival between 14 and 28 dpi was assessed by injecting mice with the same mixture of ctSynCAM1 and control virus and comparing the ratio of ctSynCAM1-expressing over control neurons at both time points (Fig. 1O); (Tashiro et al. 2006). The ratio between ctSynCAM1-expressing and control new neurons decreased between 14 and 28 dpi, indicating that ctSynCAM1 expression decreased new neuron survival ( $p = 0.04$ ; Fig. 1P).

Together, these results indicate that ctSynCAM1 interferes with the pre- and post-synaptic maturation as well as with the survival of newborn neurons. We therefore assessed whether synCAM1 overexpression in new neurons was sufficient to enhance their synaptic integration and survival.

### SynCAM1 Overexpression Increases Presynaptic Maturation but not the Survival of New Neurons

To enable the overexpression of SynCAM1 in a cohort of new neurons, we engineered the MoMuLV retroviral vector to express SynCAM1 and mRFP1. A retroviral vector encoding for GFP was used as a control. Membrane expression of SynCAM1 on HEK 293T cells was confirmed by immunohistochemistry and Western blot (Supplementary Fig. 1B and C). Both viral vectors were then injected in the dentate gyrus and new neurons were examined using confocal microscopy. At 21 and 28 dpi,

SynCAM1 overexpression did not affect dendritic tree complexity (21 dpi:  $p > 0.05$ ; 28 dpi:  $p > 0.05$ ; Supplementary Fig. 2B) or spine density (first third of the ML: 21 dpi:  $p > 0.05$ ; 28 dpi:  $p > 0.05$ ; second third of the ML: 21 dpi:  $p > 0.05$ ; 28 dpi:  $p > 0.05$ ; Fig. 1B and C). At 28 dpi however, SynCAM1 overexpression increased the proportion of mushroom spines and decreased the proportion of thin spines and filopodia (filopodia:  $p < 0.0001$ ; thin:  $p < 0.0001$ ; mushroom:  $p = 0.003$ ; Fig. 1G–H). SynCAM1 overexpression also increased MFT area and perimeter at 28 dpi (area:  $p = 0.01$ ; perimeter:  $p = 0.007$ ; Fig. 1I–N). Thus SynCAM1 overexpression increased the presynaptic and postsynaptic maturation of new neurons.

We next assessed whether SynCAM1 overexpression increased the survival of new neurons by co-injecting SynCAM1-GFP and RFP control viruses. The ratio of SynCAM1-overexpressing and control neurons was similar at 14 dpi and 28 dpi ( $p > 0.05$ ; Fig. 1Q), indicating that SynCAM1 overexpression did not modify the survival of new neurons.

Together, these results indicate that SynCAM1 plays a role in the pre- and post-synaptic maturation of new neurons as well as in their survival. However, the overexpression of SynCAM1 was not sufficient to increase new neurons' survival.

### Neuroigin-1B Overexpression Increases Dendritic Spine Density but not the Survival of New Neurons

We next assessed the effect of NL1B overexpression on the synaptic integration of new neurons. To this aim, we engineered a MoMuLV viral vector to enable the co-expression of NL1B and GFP and a virus enabling the expression of mRFP1 was used as a control. The specificity and efficiency of transgene expression was controlled on HEK 293T cells by immunohistochemistry and western blot (Supplementary Fig. 1B and C).

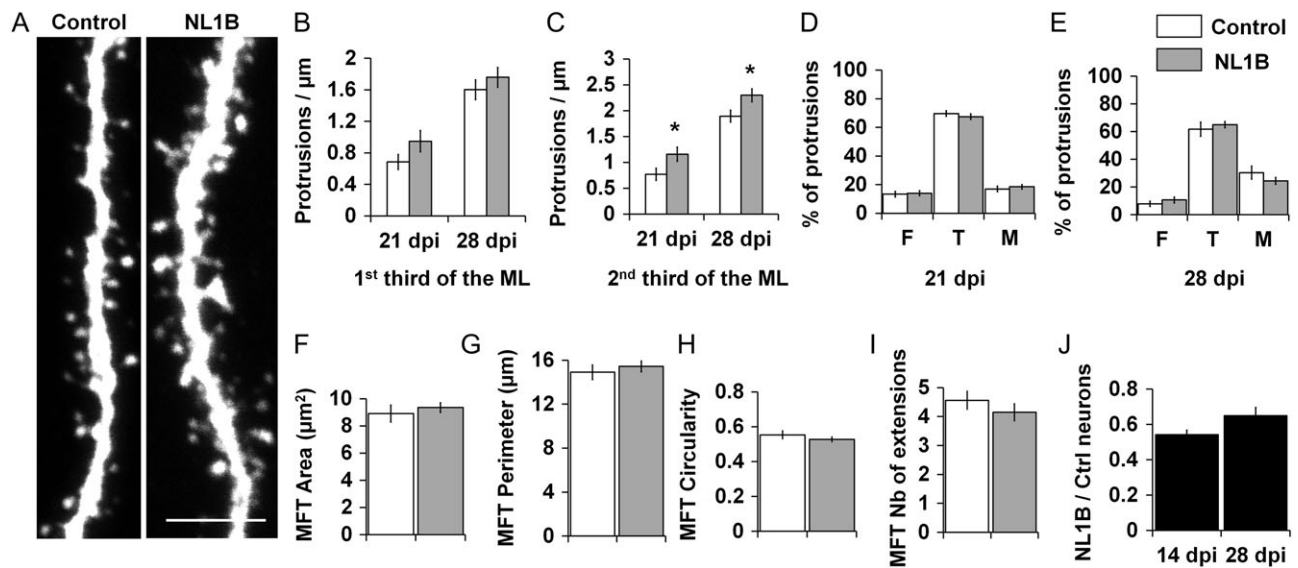
On new granule neurons, NL1B overexpression did not modify dendritic tree complexity (14 dpi:  $p > 0.05$ ; 21 dpi:  $p > 0.05$ ; 28 dpi:  $p > 0.05$ ; Supplementary Fig. 2C), but increased the density of dendritic protrusions in the second third of the ML at 21 and 28 dpi, but not in the first third of the ML (first third of the ML: 21 and 28 dpi:  $p > 0.05$ ; second third of the ML: 21 dpi:  $p = 0.04$ ; 28 dpi:  $p = 0.02$ ; Fig. 2A–C).

In contrast to SynCAM1, NL1B overexpression did not affect the diameter of dendritic protrusions ( $p > 0.05$ ; Fig. 2D and E) or the MFT area, perimeter, circularity, or number of extensions at 28 dpi ( $p > 0.05$ ; Fig. 2F–I). Finally, we measured the survival of NL1B-overexpressing new neurons between 14 and 28 dpi. The proportion of NL1B-overexpressing relative to mRFP1-expressing control newborn neurons did not change between 14 and 28 dpi ( $p > 0.05$ ; Fig. 2J), indicating that survival was not modified by NL1B overexpression.

Together, these results indicate that NL1B overexpression in newborn neurons slightly increased the density of dendritic protrusions but did not modify their maturation, the maturation of MFT, nor the survival of newborn neurons.

### Neuroigin-2A Overexpression Increases Glutamatergic and GABAergic Synapses on Newborn Neurons, as well as their Survival

Given the critical role of GABAergic input on the development of new neurons (Tozuka et al. 2005; Ge et al. 2006), we hypothesized that NL2A overexpression may have a greater effect on new neuron maturation than NL1B or SynCAM1. To test this possibility, we injected mice with a MoMuLV viral vector encoding GFP and NL2A and a control vector encoding mRFP1



**Figure 2.** Effect of NL1B overexpression on new neurons. (A) Confocal maximal projections of dendrites from control or NL1B-overexpressing newborn neurons at 28 dpi. Scale bar: 2  $\mu$ m. (B, C) Density of dendritic protrusions of control and NL1B-overexpressing newborn neurons in the first third (B) and the second third of the ML (C). (D, E) Percentage of filopodia, thin and mushroom spines in control and NL1B-overexpressing neurons at 21 (D) and 28 dpi (E). (F–I) Mean area (F), perimeter (G), circularity (H) and number of extensions (I) on MFT at 28 dpi. (J) Average ratio of the total number of NL1B-overexpressing neurons over the total number of control neurons per mouse at 14 and 28 dpi. \* $p < 0.05$ .

(Supplementary Fig. 1). NL2A overexpression transiently increased dendritic tree complexity at 14 dpi, but not at later time points (14 dpi:  $p = 0.009$ ; 21 and 28 dpi:  $p > 0.05$ ; Supplementary Fig. 2). Furthermore, NL2A increased the density of dendritic protrusions in the first and second third of the ML at 21 dpi and in the first third of the ML at 28 dpi (first third: 21 dpi:  $p < 0.0001$ ; 28 dpi:  $p = 0.001$ ; second third: 21 dpi:  $p = 0.02$ ; 28 dpi:  $p > 0.05$ ; Fig. 3A and B). Furthermore, in the second third of the ML the proportion of filopodia was reduced at 28 dpi (21 dpi:  $p > 0.05$ ; 28 dpi:  $p = 0.009$ ; Fig. 3C–E). These results indicate that NL2A increased the number and maturation of glutamatergic synaptic inputs on new neurons. We next assessed the effect of NL2A overexpression on the maturation of MFT. At 28 dpi, NL2A overexpression increased MFT area, but did not modify MFT perimeter, number of extensions and circularity (area:  $p = 0.005$ ; perimeter:  $p > 0.05$ ; circularity:  $p > 0.05$ ; number of extensions:  $p > 0.05$ ; Fig. 3F–I). We then assessed the effect of NL2A overexpression on the density of inhibitory synapses on new neurons, using VGAT- and GABA<sub>A</sub> receptor immunostaining. We quantified the density of appositions of VGAT-positive and GABA<sub>A</sub>R-positive puncta on the soma and proximal dendrites of new neurons. GABAergic synapse density was increased on NL2A-overexpressing neurons (soma:  $p = 0.009$ ; proximal dendrite:  $p = 0.03$ ; Fig. 3J–L). Thus, consistent with a role of NL2A in both glutamatergic and GABAergic synapse formation, the overexpression of this adhesion molecule on newborn neurons increased the density of glutamatergic and GABAergic synaptic inputs, without modifying their output synapses.

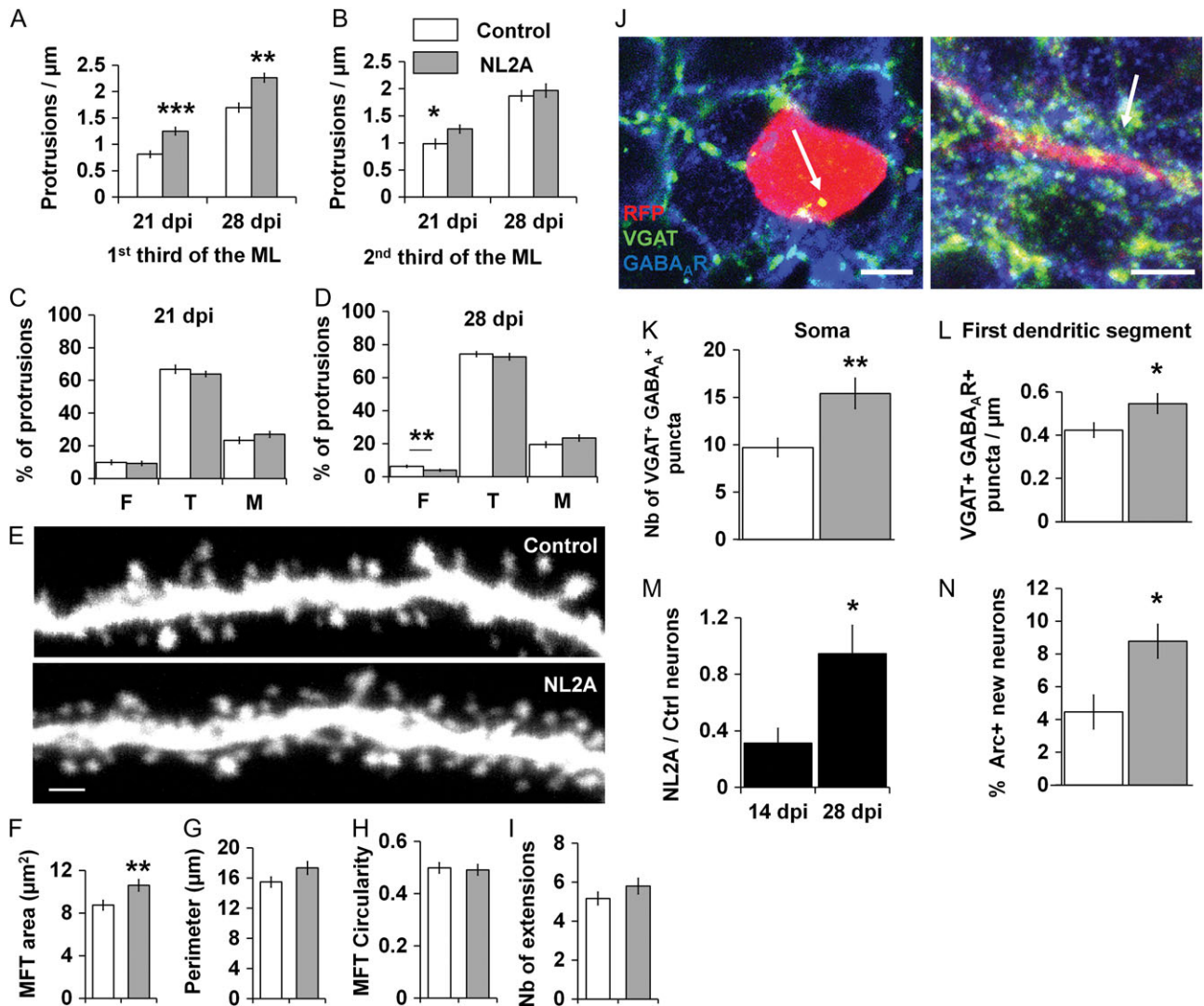
We then assessed the effect of NL2A overexpression on the survival of new neurons. NL2A overexpression increased survival between 14 and 28 dpi by 3 fold ( $p = 0.03$ ; Fig. 3M). The increased number of glutamatergic and GABAergic synapses on new neurons may result in changes in the activity of the targeted new neurons. To assess the recruitment of NL2A-overexpressing new neurons in network activity, we examined the expression of the immediate-early gene activity-regulated

cytoskeleton-associated protein (Arc) in mice living in an enriched environment. Mice were virally injected and, 28 days later, were placed into an enriched environment for 10 min and sacrificed 30–90 min later. The ratio between the number of newborn neurons expressing Arc and the total number of newborn neurons was measured for NL2A-overexpressing and control newborn neurons. NL2A-overexpressing new neurons expressed Arc significantly more frequently than control new neurons ( $p = 0.02$ ; Fig. 3N).

Together, these results indicate that NL2A overexpression in new neurons increased their synaptic integration and survival and enhanced their recruitment in network activity.

### Neuroigin-2A Overexpression in Newborn Neurons Decreases Spatial Learning Performances

Between 3 and 7 weeks after division, newborn neurons display increased plasticity and are preferentially recruited in learning tasks (Schmidt-Hieber et al. 2004; Ge et al. 2007; Gu et al. 2012). Since NL2A induced an increase in newborn neurons integration and survival during this period, we hypothesized that NL2A overexpression in newborn neurons may modify the animals' performances in spatial learning and memory tasks. To test this possibility, we assessed the behavior of mice injected with NL2A-overexpressing or GFP-expressing MoMuV (Fig. 4A). At 24 dpi, mice were first tested on an elevated plus maze, to assess anxiety. There was no intergroup difference in time spent in the open arm maze ( $p > 0.05$ , data not shown), indicating that anxiety level was similar between groups. At 28 dpi, mice were trained in a Morris water maze, in a fixed platform task for 7 days. There was no inter-group difference in swim speed ( $p > 0.05$ , data not shown) and distance traveled ( $p > 0.05$ , data not shown), indicating no difference in locomotion. During the training sessions, escape latencies were not significantly different between groups (two-way repeated measure ANOVA; main effect of time  $F(6162) = 22.53$ ,  $p < 0.0001$ ; main effect of treatment  $F(6162) = 3.67$ ,  $p > 0.5$ ; Fig. 4B), indicating that



**Figure 3.** Effect of NL2A overexpression on new neurons. (A, B) Density of dendritic protrusions of control and NL2A-overexpressing newborn neurons in the first third (A) and the second third of the ML (B). (C, D) Percentage of filopodia, thin and mushroom spines in control and NL2A-overexpressing neurons at 21 (C) and 28 dpi (D). (E) Representative confocal maximal intensity projections of dendritic segments of control and NL2A-overexpressing neurons in the first third of the ML at 28 dpi. Scale bar: 1 μm. (F–I) Mean area (F), perimeter (G), circularity (H) and number of extensions (I) on MFTs at 28 dpi. (J) Representative confocal plans of new neurons at 21 dpi (red) in slices immunostained for VGAT (green) and GABA<sub>A</sub> receptors (blue). Arrows point to GABAergic synapses, defined as appositions of GABAAR+ and VGAT+ puncta in soma (left panel) and dendritic segment (right panel). Scale bars: 5 μm. (K, L) Number of VGAT-positive and GABA<sub>A</sub> receptor-positive puncta per soma (K) and primary dendritic segment (L) in control and NL2A-overexpressing neurons at 21 dpi. (M) Average ratio between the total number of NL2A-overexpressing neurons and the total number of control neurons per mouse at 14 and 28 dpi. (N) Percentage of cells expressing Arc in control and NL2A-overexpressing newborn neurons after environmental enrichment. \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001.

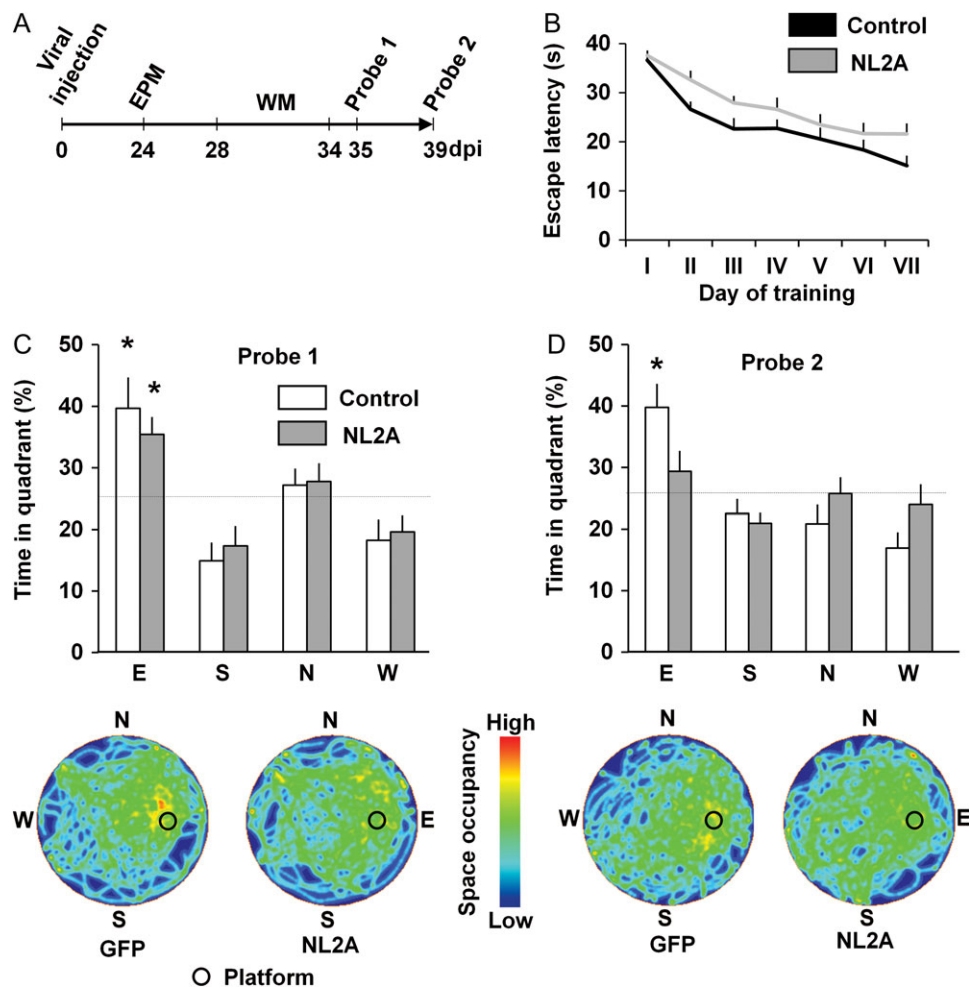
learning performances were similar between groups. Twenty-four and 120 hours after the last training session (i.e. at 35 and 39 dpi), memory was tested with a probe trial (Fig. 4A). Twenty-four hours after the last training session (probe 1), both groups of mice displayed a preference for the target quadrant (two-way repeated measure ANOVA, main effect of % time in quadrant  $F(3,81) = 31.27$ ,  $p < 0.0001$ , Bonferroni post-hoc tests target quadrant vs. opposite for both groups  $p < 0.01$ , target vs. left quadrant  $p < 0.01$ , target vs. right quadrant for GFP  $p < 0.001$  NL2A  $p > 0.05$ ) and the proportion of total time spent in the target quadrant was significantly above 25% chance level (one sample t-test for % time in target quadrant vs. chance level  $p < 0.01$  for both groups; Fig. 4C). At 120 h of delay (probe 2), however, the preference for the target quadrant was intact for mice injected with control MoMuLV, but was lost for mice

injected with NL2A-MoMuLV (two-way repeated measure ANOVA, main effect of % time in quadrant  $F(3,81) = 9.97$ ,  $p < 0.0001$ , Bonferroni post-tests target quadrant vs. opposite GFP  $p < 0.01$  NL2A  $p > 0.05$ , target vs. left quadrant GFP  $p < 0.001$  NL2A  $p > 0.05$ ; one sample t-test for % time in target quadrant vs. chance level GFP  $p < 0.01$ , NL2A  $p > 0.05$ ; Fig. 4D). Thus, NL2A overexpression in newborn neurons impaired the retention of spatial memory at 120 h delay.

## Discussion

Here, we examined the effects of the cell-autonomous overexpression of different synaptic adhesion molecules on the development, synaptic integration and survival of newborn neurons





**Figure 4.** NL2A overexpression in new neurons decreases memory performances at 40 dpi. (A) Experimental timeline: Mice were injected with virus expressing NL2A or GFP (as control). At 24 dpi, mice were tested on an elevated plus maze. At 28 dpi, mice were trained on a water maze for 7 days (4 trials per day, MWM1). A first probe trial was run 24 h later, followed by another trial at 120 h. (B) Escape latency time during the water maze training. (C) Time spent in each quadrant during the probe trial 24 h after the last training session. Bottom panels are heat maps of all animals' trajectories during the probe trial. (D) Time spent in each quadrant during the probe trial 120 h after the last training session. Bottom panels are heat maps of all animals' trajectories during the probe trial. \* $p < 0.05$ .

in the hippocampus of 7- to 9-week-old mice. We found that adhesion molecules enhanced distinct aspects of new neurons' integration into the neuronal network. In particular, SynCAM1 increased the size and thus the morphological maturation of dendritic spines and MFT, while NL1B increased spine density. Although significant, the effects of SynCAM1 and NL1B overexpression on the synaptogenesis of newborn neurons were small in amplitude and did not increase neuronal survival. In contrast, NL2A overexpression increased both dendritic spine maturation and density, as well as GABAergic innervation and led to a robust increase in newborn neuron survival. These effects of synaptic adhesion molecules on new neurons support the idea of a competitive integration of newborn neurons in the neuronal network. Surprisingly, despite enhanced new neuron survival and integration, mice with newborn neurons overexpressing NL2A showed impaired memory performances in a Morris Water maze task. Together, these results indicate that overexpression of synaptic adhesion molecules can enhance the synaptic integration of new neurons in pre-existing neuronal networks, as well as their survival, but interfere with their functional integration. This is the first report showing that an increase in adult neurogenesis results in the impairment of

memory retention of events that occurred subsequently to the manipulations of adult neurogenesis.

The effects of SynCAM1, NL1B, or NL2A on synapse formation on new granule neurons are consistent with previously-described roles in other models of synaptogenesis. Indeed, in a comparative study, Sara et al. 2005 showed that SynCAM1 but neither its cytosolic tail, nor NL1 increased synaptic efficacy in cultured hippocampal neurons, whereas only NL1 increased synapse number (Kwon et al. 2012). In vivo, the overexpression of SynCAM1 in forebrain neurons increased the number of dendritic spines, and especially of mushroom spines (Robbins et al. 2010) and also increased presynaptic terminal formation (Biederer et al. 2002; Fogel et al. 2007; Robbins et al. 2010). The splice variants of neuroligin demonstrate distinct properties and whereas NL1B overexpression increases glutamatergic puncta, NL2A overexpression increases the number of both glutamatergic and GABAergic puncta (Chih et al. 2006). On new neurons in the adult hippocampus, the cell-autonomous overexpression of the splice variant NL1 increases spine density and the synaptic AMPA/NMDAR excitatory post-synaptic current ratio (Schnell et al. 2012). Similarly, the global overexpression of SynCAM1 in tetracycline-controlled transgenic mice increased

spine density and volume of dendritic spines, as well as the survival of newborn neurons (Doengi et al. 2015). Thus, our results are consistent with the notion that, in vivo, adhesion molecules play a role in synaptogenesis in the adult brain and contribute to the regulation of synaptic integration of new neurons. Furthermore, they support the hypothesis that the formation of synapses on new hippocampal neurons regulates their survival.

Although the effect of a decrease in adult neurogenesis does not necessarily lead to a decrease in memory performances (Jaholkowski et al. 2009; Groves et al. 2013; Lazic et al. 2014), several lines of evidence indicate that an increase in adult neurogenesis results in improved performances in spatial memory tasks, such as in the Morris water maze (reviewed in Zhao et al. 2008; Deng et al. 2010; Ming and Song 2011). Notably, directing fate progression of hippocampal progenitors towards neuronal progeny by the cell-specific overexpression of the pro-neurogenic transcription factor Neuro-D1, restored the delayed development of dendritic spines and the reduced survival of newborn neurons mouse model of Alzheimer's disease and resulted in increased performances in a spatial memory test (Richetin et al. 2015). The role of new neurons in the formation of memory traces was further substantiated by the optogenetic silencing of their activity in mice undergoing a water maze test, which resulted in impaired platform location (Gu et al. 2012). Recent results however challenged the notion that increased neurogenesis results in improved memory performance (Akers et al. 2014). Indeed, in their study, Akers et al. showed that an increase in adult neurogenesis occurring after a learned event induces its forgetting. However, the inverted experimental design of Akers et al. tested the persistence of memories acquired before the increase in neurogenesis and is therefore not comparable to our experimental design. In view of these results, we therefore expected that mice with NL2A-induced increase in neurogenesis would express enhanced memory performances. The apparent discrepancy between these previous results and our observations may be viewed in the light of the mechanisms of synaptic integration of newborn neurons. In particular, we consider two possibilities.

The first possibility is that synaptic adhesion molecules may interfere with the synaptic plasticity of newborn neurons. Indeed, immature neurons display increased LTP expression at around 4 weeks after division, a developmental stage at which their involvement in mechanisms of learning is the greatest (Schmidt-Hieber et al. 2004; Gu et al. 2012). LTP requires structural plasticity of synapses, a mechanism that may be hindered by inter-cellular adhesion. Indeed, the overexpression of proteases increases LTP expression and memory performances (Madani et al. 1999). Inversely, the overexpression of NL1 (Dahlhaus et al. 2010), SynCAM1 (Robbins et al. 2010) or the manipulation of alternative splicing of Neurexin 3 (Aoto et al. 2013) reduce synaptic plasticity. In view of these recent observations, it is possible that NL2A overexpression may inhibit the synaptic remodeling necessary for the expression of LTP or LTD and therefore reduce the plasticity of newborn neurons. However, the increased Arc expression in NL2A-overexpressing new neurons argues against impaired synaptic plasticity in these cells.

The second possibility is that NL2A overexpression in new neurons induces synapse formation with inappropriate pre-synaptic partners, which would otherwise not have been contacted. The expression of the immediate-early gene Arc upon exposure to enriched environment in NL2A-overexpressing newborn neurons indicates that these cells are functionally integrated into the hippocampal network. However, input specificity may be impaired. Indeed, we have recently observed

that any given nascent dendritic spine from a new neuron is surrounded, and touches, on average five presynaptic boutons with which a synaptic contact can potentially be formed (Krzisch et al. 2015). It is believed that the connectivity between a given spine and its presynaptic bouton follows specific cues that are relevant to network activity occurring during the establishment of this connectivity (Bergami and Berninger 2012). Although not tested here, it is possible that such cues may be overridden by the over-expression of synaptic adhesion molecules and lead to lower input specificity of NL2A-overexpressing neurons. This possibility is consistent with the increased Arc expression in NL2A-overexpressing newborn neurons as compared with wild-type newborn neurons, indicating that a given stimulus will preferentially recruit NL2A-overexpressing new neurons. Recent work suggested that the alternative splicing of synaptic adhesion molecules may be a key mechanism for regulating neuronal recognition (Aoto et al. 2013; Takahashi and Craig 2013; Lah et al. 2014). In particular, alternative splicing of neurexins, the major ligand for neuroligins, produce over 1000 isoforms, some of which are restricted to specific subtypes of neurons (Schreiner et al. 2014). In this conceptual framework, the overexpression of NL2A in new neurons may interfere with synaptic identity and result in the formation of non-coherent networks, thereby disturbing the normal function of hippocampal network and its plasticity.

The synaptic integration of new neurons occurs simultaneously to the activity-dependent elimination of the majority of these cells. Although the mechanisms involved in the regulation, and the role of this selective elimination, remain unknown, the results presented here suggest that the specificity of connectivity is required for the proper function of new neurons. Since several adhesion molecules are mutated in psychiatric disorders, the subsequent impaired functional integration of new neurons may contribute to the impaired cognitive function associated with these pathologies.

## Supplementary Material

Supplementary material can be found at: <http://www.cercor.oxfordjournals.org/>

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