

Agrin-Signaling Is Necessary for the Integration of Newly Generated Neurons in the Adult Olfactory Bulb

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In the adult forebrain, new interneurons are continuously generated and integrated into the existing circuitry of the olfactory bulb (OB). In an attempt to identify signals that regulate this synaptic integration process, we found strong expression of agrin in adult generated neuronal precursors that arrive in the olfactory bulb after their generation in the subventricular zone. While the agrin receptor components MuSK and Lrp4 were below detection level in neuron populations that represent synaptic targets for the new interneurons, the alternative receptor $\alpha 3\text{-Na}^+\text{K}^+\text{-ATPase}$ was strongly expressed in mitral cells. Using a transplantation approach, we demonstrate that agrin-deficient interneuron precursors migrate correctly into the OB. However, in contrast to wild-type neurons, which form synapses and survive for prolonged periods, mutant neurons do not mature and are rapidly eliminated. Using *in vivo* brain electroporation of the olfactory system, we show that the transmembrane form of agrin alone is sufficient to mediate integration and demonstrate that excess transmembrane agrin increases the number of dendritic spines. Last, we provide *in vivo* evidence that an interaction between agrin and $\alpha 3\text{-Na}^+\text{K}^+\text{-ATPase}$ is of functional importance in this system.

Introduction

In the adult mammalian forebrain, new neurons are generated throughout life by stem cell populations localized in the periventricular region. After their amplification, these cells undertake long distance migration into the olfactory bulb (OB), where they differentiate into various types of interneurons using GABA, dopamine, or glutamate as their neurotransmitters (Lledo et al., 2008). Intense investigations of adult neurogenesis over the past decades led to the description of adult neural stem cells, their stepwise transformation into neurons, and the sequence of their synaptic integration (Lledo et al., 2008).

Currently, little is known about the molecular signals that regulate the synaptic integration of neurons into existing neuronal networks. In an attempt to identify such signals, we isolated migratory neuronal precursors from the subventricular zone (SVZ) and the rostral migratory stream (RMS) and analyzed their gene expression by Serial Analysis of Gene Expression (SAGE)

(Pennartz et al., 2004) and microarray analysis (Boutin et al., 2010). One of the genes that showed strong and dynamic expression in this cell population was agrin.

Agrin, a proteoglycan existing in a variety of alternative splice forms, represents probably the best characterized synapse-inducing factor and has been particularly investigated at the neuromuscular junction (Song and Balice-Gordon, 2008; Williams et al., 2008). Here, agrin and its receptor complex, comprising the low-density lipoprotein receptor Lrp4 and the muscle-specific kinase (MuSK), are essential for the formation and stabilization of this particular synapse (Gautam et al., 1996; Kim et al., 2008; Song and Balice-Gordon, 2008; Zhang et al., 2008). Agrin is widely expressed in the developing and adult brain where it has been shown to interact with the LRP4/MuSK complex (Ksiazek et al., 2007) as well as with the alternative receptor $\alpha 3\text{-Na}^+\text{K}^+\text{-ATPase}$ ($\alpha 3\text{NKA}$) (Hilgenberg et al., 2006), suggesting that it might also play a role in neuron-to-neuron synapse formation (Hoch et al., 1993; O'Connor et al., 1994). Such a function is also supported by considerable amounts of *in vitro* data implicating agrin in processes like filopodia extension and spine formation (Ferreira, 1999; Maletic-Savatic et al., 1999; Bose et al., 2000; Annies et al., 2006; McCroskery et al., 2006; Matsumoto-Miyai et al., 2009; Ramseger et al., 2009). *In vivo*, analysis of agrin function in brain synapse formation has been difficult, due to the perinatal lethality of constitutive mouse mutants (Gautam et al., 1996; Lin et al., 2001). However, a lower amount of excitatory synapses in the postnatal cortex has been observed in agrin-deficient mice that were transgenically rescued (Ksiazek et al., 2007).

Here, we investigate the function of the agrin signaling pathway in adult neurogenesis. We demonstrate that agrin is necessary for

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morphological differentiation and survival of new OB interneurons and show that the transmembrane form of agrin (TM-agrin) is responsible for this function. Gain-of-function of TM-agrin induced excess synapses of new neurons in the OB. Finally, we provide evidence that $\alpha 3$ NKA is an active agrin receptor in this system.

Materials and Methods

Mice. Animals were treated according to guideline approved by the French Ethical Committee. *Agrin* mutants (Lin et al., 2001) (provided by P. Caroni, Friedrich Miescher Institute, Basel, Switzerland) were crossed with *actin-EGFP* transgenic mice (Hadjantonakis et al., 1998; Lin et al., 2001) in our animal facilities to generate donor mice. C57BL6 females (Charles River) aged from 7 to 10 weeks were used as host in the transplantation experiment.

RNA probes and in situ hybridization. cDNA fragments from mouse *Lrp4* (772 bp; position 6323–7095), mouse *MuSK* (909bp; position 2210–3119), mouse $\alpha 3$ NKA (*Atp1a3*; 744bp; position 3112–3856), and mouse *agrin* (320bp; 5160–5479) were cloned in pGEMT vector (Promega) and used as described (Tiveron et al., 2006).

Transplantations. E18.5 embryos were analyzed for GFP fluorescence; agrin expression status was determined by PCR [primers for the wild-type (WT) allele: forward 5'-CAGGGATAGTTGAGAAG-3', reverse 5'-GCTGGGATCTCATTGGTC-3'; for the mutant allele: forward 5'-TCGCAAGTTCTAATTC CA-3', reverse 5'-GGGCAGGGCTAACACCAA-3']. SVZ tissue from the lateral wall of the anterior lateral ventricle was transplanted in host animals (1.5 mm posterior to bregma, 0.8 mm lateral, and 2.4 mm deep) as described by Seidenfaden et al. (2006).

Matrigel assay. Cultures and analyses of SVZ explants were performed as previously described (Hack et al., 2002).

Postnatal electroporation. (Boutin et al., 2008). Mouse agrin-specific siRNA (siRNA3) and mock siRNA vectors were previously described (McCroskery et al., 2006). Both vectors were coelectroporated with a pCAGGS_EGFP vector for detection and morphological analyses. A chicken 6 kb TM-agrin fragment was cloned in the appropriate orientation (TM-agrin) or in the reverse orientation (TM-agrinREV) into the bicistronic expression vector pCAGGS_IRES_EGFP, which allows doubtless identification of transfected cells. This fragment encodes the active form of agrin, the y4z8 variant that additionally contains 4 aa at the y site and 8 aa at the z site (O'Connor et al., 1994; Neumann et al., 2001). For electroporation, mock siRNA was coelectroporated with TM-agrinREV to serve as control, and siRNA3 was coelectroporated with either TM-agrinREV or TM-agrin. C-Ag15 was fused to the murine Ig κ chain leader sequence and cloned into pCAGGS_IRES_EGFP.

Quantification and statistical analysis. Categorization and spine count were performed blind to experimental condition. Data are presented as mean $\pm$ SEM. The designation *n* represents the number of animals analyzed, except in Figures 3d and 4c, where it represents the number of dendrites counted in 6–12 animals. Student's unpaired *t* test was used to assess differences between data groups using Instat software (GraphPad Software). Differences were considered statistically significant when *p* < 0.05.

Results

In a SAGE-based study (Pennartz et al., 2004) we found a strong over-representation of agrin transcripts in purified adult olfactory interneuron precursors (Pennartz et al., 2004) in comparison to total brain tissue. In agreement, *in situ* hybridization and immunohistochemistry showed strong signals in the RMS (Fig. 1a) as well as in the granule cell layer (GCL), the mitral cell layer (MCL),

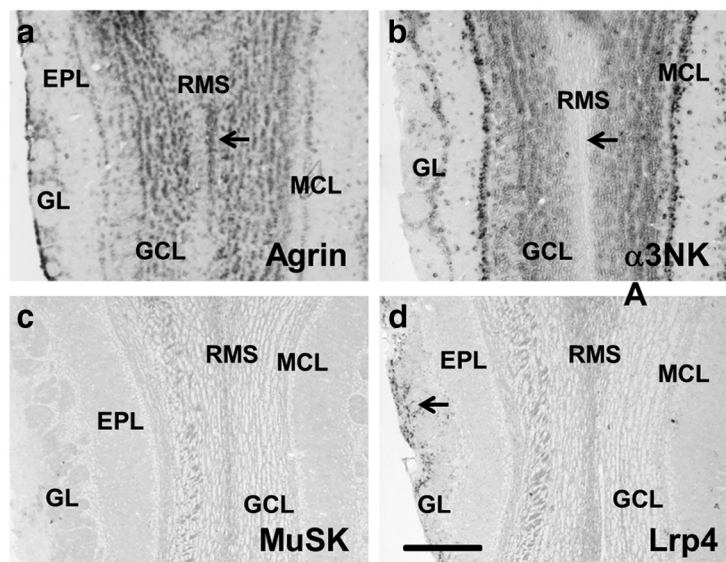


Figure 1. Expression of the agrin signaling system in the olfactory bulb. *a*, *In situ* hybridization for agrin shows expression in the RMS and in individual cells in the GCL, EPL, and GL (arrow). *b*, $\alpha 3$ NKA transcripts are absent from the RMS but strongly present in MCL, while expression in GCL and GL was weaker. *c*, *d*, MuSK mRNA was not expressed in the OB (*c*), and *Lrp4* was confined to the GL (*d*, arrow). EPL, external plexiform layer. Scale bar, 200 μ m.

and the glomerular layer (GL) (compare Fig. 1a, arrow, <http://mouse.brain-map.org/gene/show/11390>).

We investigated the expression components of the well described agrin signaling pathway that is functional at the neuromuscular synapse (Song and Balice-Gordon, 2008). In the OB, expression of MuSK was below detection level (compare Fig. 1c, <http://mouse.brain-map.org/gene/show/17965>), while *Lrp4* was restricted to the GL but was undetectable in the MCL, containing the main target population for newly arriving interneurons (compare Fig. 1d, <http://mouse.brain-map.org/gene/show/86700>). In contrast, mRNA for the alternative agrin receptor in the CNS, $\alpha 3$ NKA (Hilgenberg et al., 2006), was strongly expressed in the MCL, more weakly in the GCL and GL, and was absent from the RMS (compare Fig. 1b, <http://mouse.brain-map.org/gene/show/87535>).

Thus, $\alpha 3$ NKA was the only known agrin receptor for which we could detect an expression in mitral cells, the cell population that represents the main synaptic targets for new interneurons.

Next, we investigated the function of agrin in adult olfactory neurogenesis. Given that *agrin*-deficient mice die perinatally (Lin et al., 2001), precluding the analysis of postnatal events like olfactory bulb synaptogenesis, we used a transplantation approach. Mice deficient for all agrin isoforms (Lin et al., 2001) were crossed with an *actin-GFP* transgenic line to allow recognition of grafted-derived neurons. (Hadjantonakis et al., 1998). SVZ tissue from mutant embryos or littermate controls at the latest possible time point, E18.5, was transplanted into the SVZ of adult WT host mice (Fig. 2a) (Seidenfaden et al., 2006). Ten days postgrafting (dpg), both control and agrin-deficient transplanted cells were found in the host RMS (Fig. 2b). In the center of the OB, transplanted cells separated from the main stream, switching from tangential chain migration to individual radial migration (Fig. 2b). No difference was obvious between genotypes. We used Matrigel culture of SVZ explants to confirm these observations in a more controlled and quantifiable system (Hack et al., 2002). Here, WT and *agrin*-deficient explants showed the same typical migratory behavior, with cells exiting from the core-aggregate in

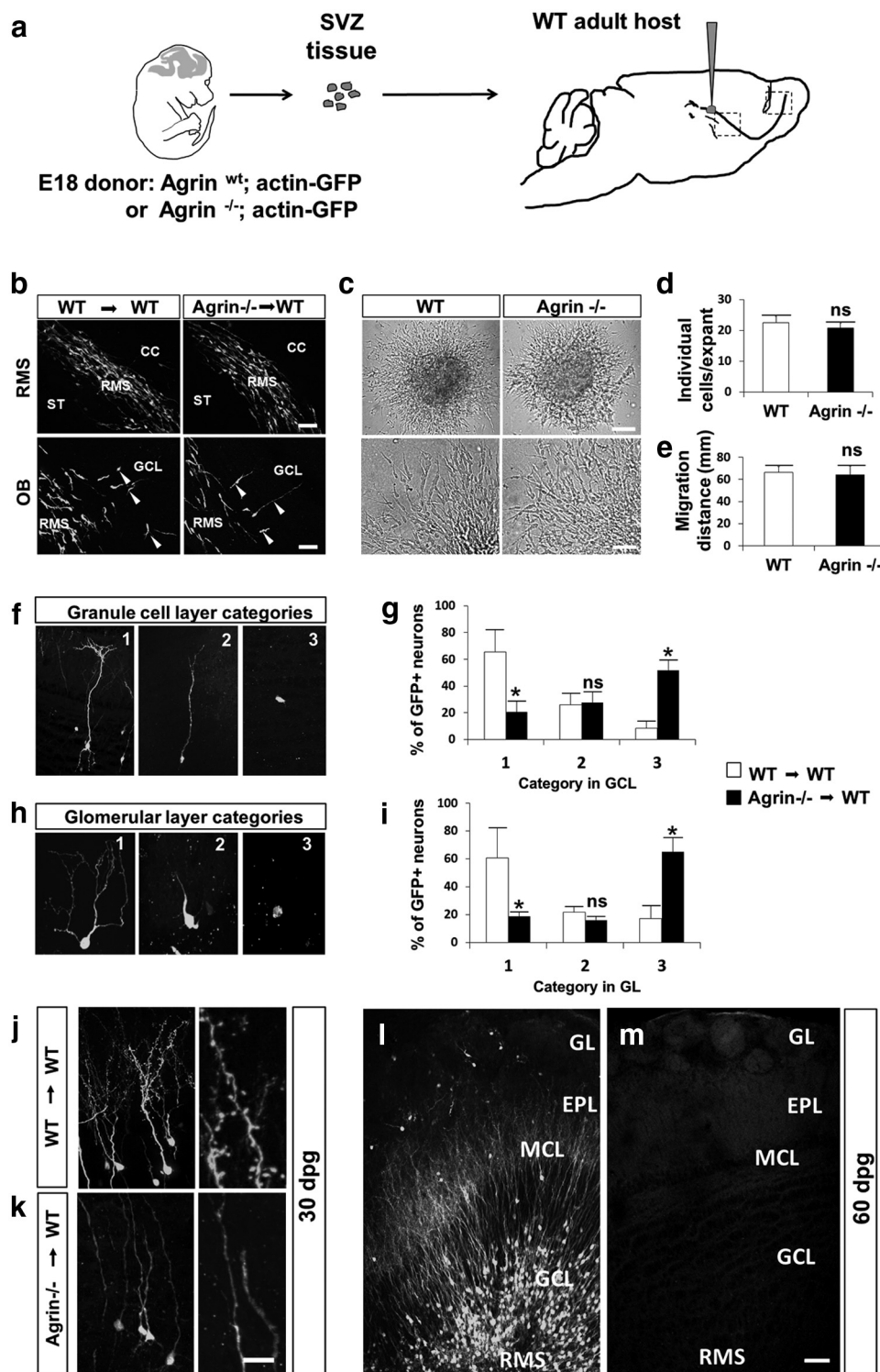


Figure 2. *Agrin*-deficient neuronal precursors do not integrate in the OB. **a**, Schematic representation of the experimental protocol. GFP-labeled WT or *agrin*-deficient precursors isolated at E18.5 were transplanted into the anterior SVZ of adult WT hosts. **b**, In the RMS, organization and distribution of mutant precursors were indistinguishable from controls (top panels). In the center of the OB, WT and *agrin*-deficient precursors showed the typical switch from tangential chain to radial individual migration (bottom panels, arrowheads). **c**, Matrigel culture of WT and *agrin*-deficient explants isolated at E18.5. Bottom panels are high-magnification images of the explants presented in the top panels. **d, e**, In both situations, explants showed the typical chain migration with no differences in the number of individual cells surrounding the explant (**d**) or migration distance (**e**). **f, h**, Examples for morphological categories of neurons in the GCL (**f**) and in the GL (**h**) as used for quantification. **g, i**, At 17 dpf, cells with mature morphologies (category 1) were significantly reduced when *agrin*-deficient tissue was transplanted ($n = 6$; control: $n = 5$; $*p < 0.05$), while simple morphologies (category 3) were over-represented in both the GCL (**g**) and the GL (**i**). **j, k**, At 30 dpf, integrated WT granule cells show mature morphology and dense coverage in the external plexiform layer (EPL) with dendritic spines (**j**). Right, High-magnification images of dendrites presented in left panels. At this time point, surviving *agrin*-deficient cells are rare. The few remaining cells show few signs of branching or protrusions (**k**). **l, m**, At 60 dpf, animals transplanted with WT SVZ tissue show generally large amounts of fully mature neurons in the OB (**l**). At this time point, mice grafted with mutant tissue were always devoid of GFP-positive cells (**m**). Scale bars: **b**, 20 μ m; **c**, top panels, 100 μ m; **c**, bottom panels, 40 μ m; **j, k**, left panels, 15 μ m; **j, k**, right panels, 5 μ m; **l, m**, 100 μ m. CC, corpus callosum; ST, striatum; ns, non-significant.

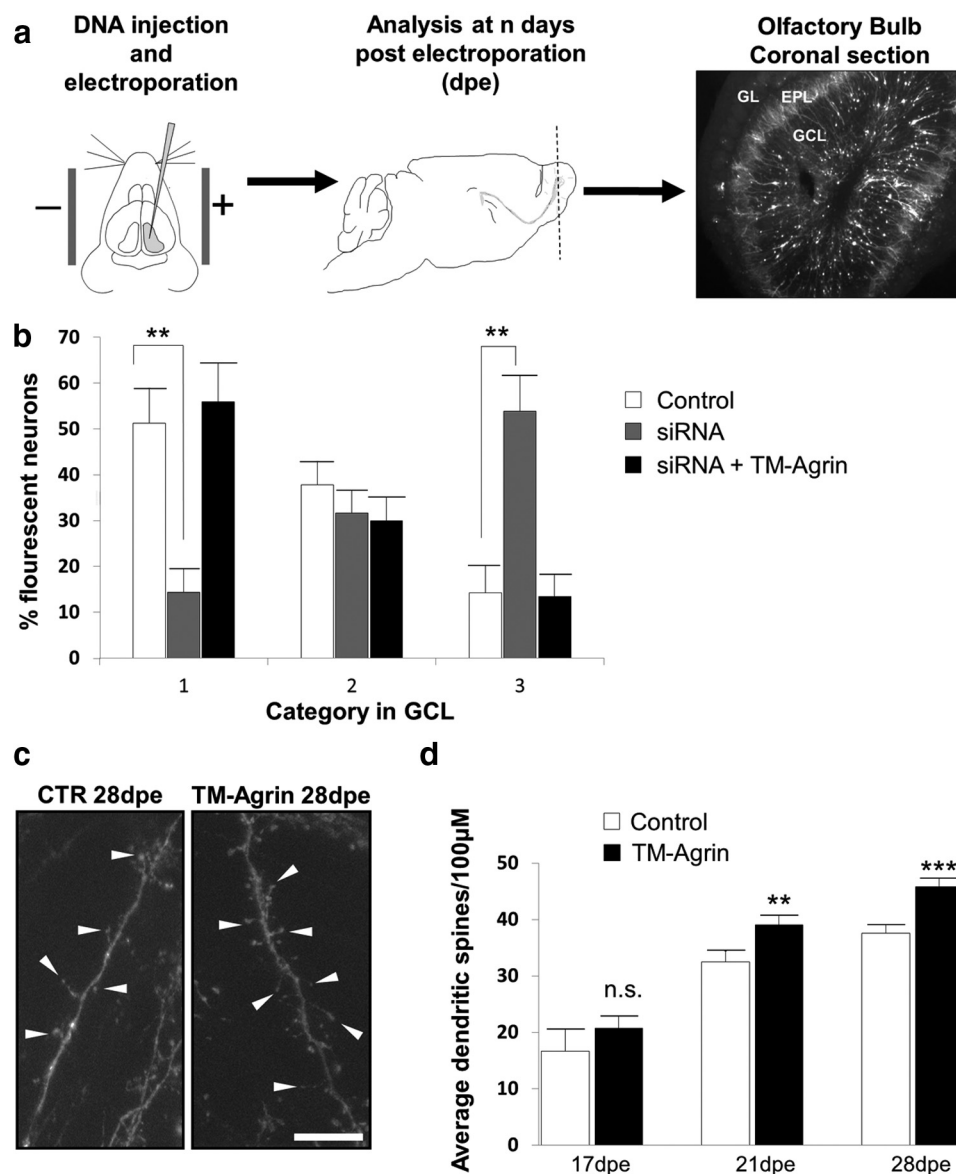


Figure 3. TM agrin is the active agrin isoform. *a*, Representation of the electroporation protocol. *b*, Morphological distribution of the electroporated cells in the GCL at 21 dpe. siRNA-induced knockdown of TM-agrin leads to the loss of cells with mature neuronal morphology in the GCL (category 1), while simple cells (category 3) are over-represented. This effect is entirely rescued by coelectroporation of a TM-agrin expression construct ($n = 6$ for control; $n = 10$ for siRNA; $n = 10$ for TM-agrin rescue). *c, d*, Gain-of-function of TM-agrin leads to an increasing number of dendritic spines in the EPL (17 dpe, $n = 20$ and 32 dendrites for control and TM; 21 dpe, $n = 26$ and 55 dendrites for control and TM; 28 dpe, $n = 91$ and 100 for control and TM. $**p < 0.01$, $***p < 0.001$, n.s. non significant, Student's *t* test). Scale bar, 30 μm.

a chain-like organization (Fig. 2*c*). The numbers of individual cells surrounding the explants (Fig. 2*d*) or migration distance (Fig. 2*e*) were indistinguishable. We conclude that the migratory behavior of agrin mutant neuronal precursors was normal.

Next, we investigated the fate of RMS-transplanted neuronal precursors after their arrival in the OB. For quantification of the differentiation state, we defined the following three categories of cellular morphologies in the GCL (Fig. 2*f*) and GL (Fig. 2*h*): first, multibranched cells with the morphology of mature neurons (category 1); second, cells with one main process and little or no secondary branching (category 2); and third, cells showing no signs of process outgrowth or branching (category 3).

Ten days postgrafting, the category distribution of cells in the OB after grafting of control or mutant tissue was comparable (data not shown). However, at 17 dpg both GCL and GL of hosts transplanted with mutant grafts contained a significantly lower percentage of

complex cell types of category 1, while cells that showed no signs of neuronal morphology (category 3) were strongly over-represented (Fig. 2*g,i*). At 30 dpg, transplantation of WT precursors led to integration of large numbers of cells with mature neuronal morphology and covered with dendritic spines in the OB (Fig. 2*j*). At this time point, *agrin*-deficient transplanted cells were rare. The few surviving cells that were observed in the GCL showed simple morphologies and lacked dendritic spines (Fig. 2*k*). At 60 dpg, hosts transplanted with WT tissue showed generally large amounts of fully mature neurons integrated in the OB (Fig. 2*l*), while animals with mutant grafts were always devoid of GFP-positive cells (Fig. 2*m*). Thus, agrin deficiency compromised integration and survival of new interneurons in the OB.

Alternative splicing leads to the generation of secreted and membrane-anchored forms of agrin (Burgess et al., 2000; Neumann et al., 2001). The TM form is the main form expressed in

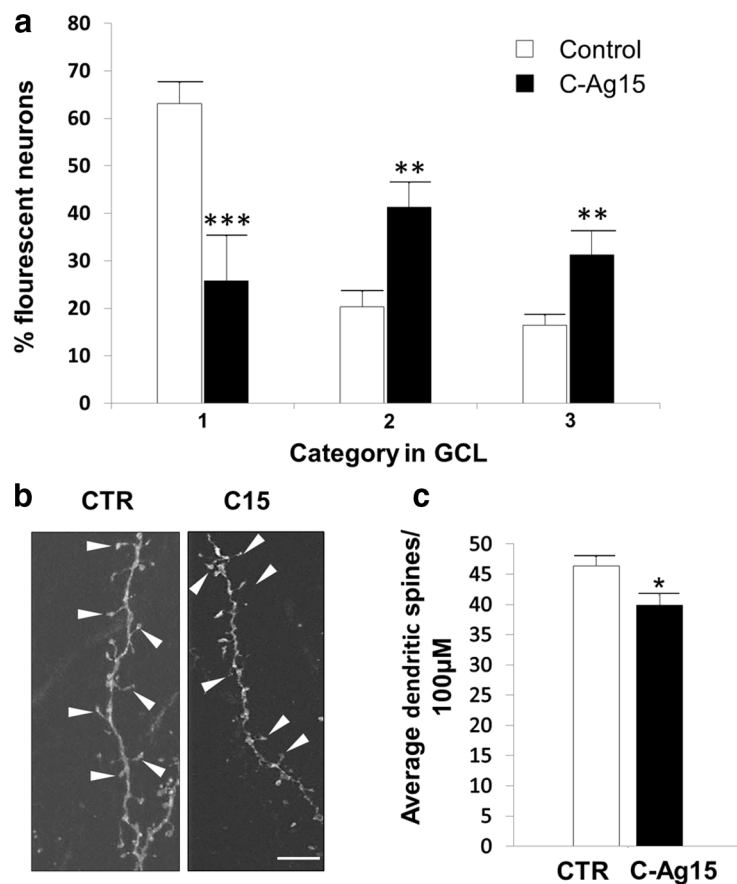


Figure 4. Expression of the competitive agrin antagonist C-Ag15 interferes with differentiation and spine formation in the OB. **a**, Electroporation of a C-Ag15 expression construct leads to the loss of cells with mature neuronal morphology and the overrepresentation of category 2 and 3 cells in the GCL ($n = 8$ for C-15Ag and for control; $**p < 0.01$, $***p < 0.001$, Student's t test). **b**, **c**, In parallel to the shift in categories, spine density is significantly reduced in the category 1 cells that show extensions into the EPL (control: $n = 69$, C-Ag15: $n = 59$; $*p < 0.05$, Student's t test). Scale bar, 10 μ m.

the CNS and has been implicated in process formation and synaptogenesis (Annie et al., 2006; McCroskery et al., 2006). We asked whether transmembrane agrin was the functional isoform in the OB. Postnatal forebrain electroporation allows the efficient manipulation of new neurons that invade the OB via the RMS (Fig. 3a) (Boutin et al., 2010). Transfection of a knock-down vector expressing a well characterized siRNA that specifically targeted agrin mRNA (McCroskery et al., 2006), together with EGFP for detection, induced the expected overrepresentation of cells with simple morphologies at the expense of mature phenotypes at 21 d postelectroporation (dpe) (Fig. 3b). This effect was entirely rescued by coelectroporation of the anti-agrin siRNA together with a TM-agrin expression vector (Fig. 3b). Next, we investigated the consequences of TM-agrin gain-of-function. Electroporation of the TM-agrin expression vector in wild-type mice had no influence on the morphological maturation of new neurons at 21 dpe (data not shown). However, average spine density in the external plexiform layer (EPL) was significantly increased at this time point and even higher at 28 dpe (Fig. 3c,d). Altogether, these data demonstrate that the TM form of agrin is sufficient for the correct morphological differentiation of new OB interneurons and suggest that this effect is due to formation and/or stabilization of their synapses.

The above expression data suggest that α 3NKA rather than MuSK/Lrp4 is involved in mediating agrin signaling in the OB. We aimed at providing functional evidence for possible agrin–

α 3NKA interactions. A C-terminal fragment of agrin, C-Ag15, has been shown to competitively antagonize interaction between endogenous agrin and the α 3NKA (Hilgenberg et al., 2006; Tidow et al., 2010). We expressed C-Ag15, fused to the murine Ig κ chain leader sequence for secretion, together with EGFP using postnatal brain electroporation and categorized the morphology of new interneurons at 21 dpe. These analyses revealed that expression of C-Ag15 induced a loss of mature phenotypes in the GCL and an increase of category 3 cell types, comparable to the results obtained by siRNA-mediated knockdown (compare Figs. 4a, 3b) or the transplantation studies (Fig. 2g). Furthermore, the small number of type 1 granule neurons that extended branched processes into the EPL showed a significant decrease in dendritic spine number compared with controls (Fig. 4b,c). These results are in agreement with an antagonistic function of C-Ag15 in agrin signaling and suggest that α 3NKA is a functional receptor.

Discussion

Unlike neurons that are born in the embryo, adult neuronal precursors of the OB have to induce and stabilize synapses in a mature and fully functional circuitry. The mechanisms underlying this integration are beginning to be elucidated. For example, it is now evident that neuronal activity is a key factor in the process regulating both survival and synaptic integration (Kelsch et al., 2010). In contrast, nothing is known about the actual molecular cross

talk that mediates the formation and stabilization of new synapses in the adult brain. Our work strongly suggests that agrin is an essential mediator of this interaction. Agrin is strongly expressed in neuronal precursors in the RMS and OB (O'Connor et al., 1994; Cohen et al., 1997). We find that agrin-deficient neurons transplanted into a wild-type forebrain show normal migration in the RMS and OB. However, at later stages mutant neurons show few dendritic spines and are eliminated, pointing to a function of the proteoglycan in synaptic integration. This is further supported by our analysis of the TM isoform of agrin, which we find to be necessary for morphological differentiation and sufficient for the induction of additional dendritic spines in the EPL.

In our expression studies, we cannot detect MuSK expression in the OB and find that LRP4 is present in the periglomerular layer and in proximal regions of the RMS, but not in the major target population for new neurons in the OB, the mitral cells. Although these findings are in agreement with data in the *Allen Brain Atlas* (<http://mouse.brain-map.org/welcome.do>), expression of Lrp4 in different layers of the OB, including mitral cells, has been found by others (Tian et al., 2006). We are left with this discrepancy, which awaits clarification.

However, we find that the ion-pump α 3NKA, which has been identified as an alternative agrin receptor (Hilgenberg et al., 2006), is particularly strongly expressed in the OB. It has been shown that an interaction between agrin and the competitive

agrin inhibitory fragment C-Ag15 produces an *agrin* mutant-like phenotype. Thus, the ion transporter might be the functional receptor in this system.

Interestingly, another brain region in which agrin is particularly strongly expressed and in which α 3NKA reaches comparably high levels as in the OB is the hippocampal formation, which also shows adult neurogenesis and therefore permanent synapse formation. It appears conceivable that, like in the OB, the controlled integration of new neurons in hippocampal neurogenesis depends on agrin signaling.

So far, the only *in vivo* study that approached agrin function in the CNS was based on agrin-deficient mice in which perinatal lethality was rescued by transgenic expression of the proteoglycan in motoneurons (Ksiazek et al., 2007). These animals survive to adulthood and show largely normal brain and neuron morphology except for an $\sim$ 30% reduction in the number of presynaptic and postsynaptic excitatory specialization. The fact that a functional and largely normal brain can develop in the absence of agrin is, at least at first glance, at odds with our finding of a total inability of new interneurons to maintain synapses and survive in the OB. However, in our transplantation and RNAi experiments agrin-deficient precursors have to compete for synaptic integration with coincidentally arriving cells that express normal amounts of agrin, while in the transgenic rescue situation all cortical neurons permanently lack the proteoglycan. This indicates that agrin is not essential for synapse formation in the CNS, but that it provides merely a selective advantage. Interestingly, our expression data suggest that in the OB MuSK/Lrp4, which mediates neuromuscular junction formation in an all-or-nothing fashion, might not be the main functional receptor complex. Instead, the alternative receptor α 3NKA is strongly expressed by the cell populations that represent synaptic targets for new neurons in the OB. Inhibition of this ion transporter by agrin induces a reduction in membrane potential and increases neuron excitability (Hilgenberg et al., 2006). In this scenario, agrin-deficient neurons would be selectively disadvantaged, in agreement with their loss after transplantation in WT hosts, further supporting the notion that neuronal activity is a key factor for the integration of neurons in the OB.

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