

Delayed onset of odor detection in neonatal mice lacking tenascin-C

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The olfactory bulb is one of the few regions in the adult mammalian forebrain in which neurons are constitutively replaced throughout life. New neurons generated in the subventricular zone migrate long distances along the rostral migratory stream to the olfactory bulb where they differentiate into interneurons. Neuronal precursor generation, migration and incorporation into the bulbar network occur in an environment rich in extracellular matrix molecules. We investigated the potential role of one of the constituents of the extracellular matrix, tenascin-C (TNC), in bulbar neurogenesis and olfactory performance using TNC-deficient mice. We found that TNC deficiency resulted in a delayed onset of olfactory responses in neonatal animals. This delay normalized at around postnatal day 10. Interestingly, this delay in early olfactory performance was not due to impaired bulbar neurogenesis as proliferation, migration, incorporation and fate determination of newborn bulbar interneurons were normal in TNC-deficient animals. Thus, we conclude that a constitutive lack of TNC does not affect bulbar neurogenesis, but instead leads to ontogenetically early impairments in olfactory detection.

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Introduction

Two regions in the postnatal brain have a continuous supply of new neurons. These are the dentate gyrus of the hippocampus (reviewed by Ming and Song, 2005) and the olfactory bulb (reviewed by Alvarez-Buylla and Lim, 2004; Lledo et al., 2005). There are four major processes in neurogenesis in the olfactory system. First, neural stem cells with self-renewing and pluripotential properties proliferate in the subventricular zone (SVZ, Doetsch et al., 1999). The neural stem cells, called type B cells, are ultrastructurally characteristic of astrocytes and express glial fibrillary acidic protein (GFAP, Doetsch et al., 1999). These cells

give rise to type C cells, which are rapidly dividing precursors that appear immature (Doetsch et al., 1997). These type C cells then produce neuroblasts (type A cells) that express neuron-specific β -tubulin and the polysialylated form of the neural cell adhesion molecule (NCAM). The second process is the migration of those neuroblasts through the rostral migratory stream (RMS) to the olfactory bulb (Saghatelian et al., 2004; Peretto et al., 2005). The third process is neuroblast maturation and fate specification into inhibitory interneurons of the olfactory bulb (Belluzzi et al., 2003; Carleton et al., 2003). The fourth and final process is an early wave of newborn neuron cell death that is activity-dependently regulated (Petreanu and Alvarez-Buylla, 2002; Yamaguchi and Mori, 2005).

Although the functional significance of bulbar neurogenesis is still poorly understood, accumulating evidence suggests that it may play a role in olfactory performances (reviewed by Lledo and Gheusi, 2003). Indeed, olfactory enrichment, which increases the generation of new bulbar neurons, improves short-term olfactory memory of mice (Rochefort et al., 2002). In contrast, transgenic mice with fewer new bulbar neurons (Gheusi et al., 2000; Enwere et al., 2004) are instead impaired in discriminating between two odorants, suggesting a functional link between the numbers of newborn neurons and olfactory abilities. At birth, the rodent olfactory bulb is anatomically immature and considerable neuronal development occurs after birth (Hinds and Kinds, 1976; Brunjes and Fraizer, 1986). In particular, neurogenesis of granule cells, the major type of interneuron in the bulb, begins after birth and continues into adulthood (Kaplan and Hinds, 1977).

The understanding of genetic and epigenetic factors regulating neurogenesis has increased in recent years (reviewed in Lledo et al., 2006). Specifically, several extracellular matrix molecules have been shown to play crucial functions in different aspects of bulbar neurogenesis. Slit repels neuroblasts from the SVZ (Wu et al., 1999; Mason et al., 2001; Nguyen-Ba-Charvet et al., 2004), whereas netrin secreted from the olfactory bulb attracts tangentially migrating neuroblasts (Murase and Horwitz, 2002), reelin detaches cells from the chains in the olfactory bulb core (Hack et al., 2002) and tenascin-R (TNR) facilitates the recruitment of neuroblasts to the olfactory bulb (Saghatelian et al., 2004).

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Throughout postnatal life, tenascin-C (TNC), another member of the tenascin family, is strongly expressed by GFAP⁺ astrocytes within the SVZ, RMS and olfactory bulb (Miragall et al., 1990; Gates et al., 1995; Jankovski and Sotelo, 1996; Thomas et al., 1996) and its restricted expression pattern suggests that it participates in olfactory bulb neurogenesis, either directly by interacting with specific cell surface receptors (Jones and Jones, 2000) or indirectly by binding to other matrix components (for reviews, see Crossin, 1996; Dityatev and Schachner, 2003). Recently, Garcion et al. (2004) reported that TNC regulates precursor cells responses to growth factors during perinatal life in the SVZ. They had previously shown that cell proliferation in the SVZ was reduced in TNC-deficient mice from postnatal day 0 (P0) to P17, although not at later stages of development (Garcion et al., 2001). However, in these studies, TNC-deficient mice from Saga et al. (1992) were used, which have been shown to express detectable levels of a truncated form of TNC (see Mitrovic and Schachner, 1995; Steindler et al., 1995; Settles et al., 1997). Expression of TNC by astrocytes along the entire glial tube surrounding the tangentially migrating neuroblasts in the RMS suggests that TNC plays a role in tangential migration of neuroblasts (Gates et al., 1995; Jankovski and Sotelo, 1996; Thomas et al., 1996). In particular, TNC may restrict neuroblast migration within the RMS as TNC repels other neural cell types (Husmann et al., 1995). Expression of TNC within the different layers of the olfactory bulb suggests that it may control fate and/or survival of newborn bulbar neurons. The widespread expression pattern of TNC in the SVZ–olfactory bulb pathway suggests that this molecule is implicated in different processes of adult neurogenesis and consequently in the olfactory performances.

To test this hypothesis, we used TNC-deficient mice where the entire TNC coding region is ablated (Evers et al., 2002). We found that TNC is required for the normal onset of olfactory responses in neonatal but is not the predominantly functional molecule in adult mice. We showed that the delayed development of olfactory abilities seen in the absence of TNC is not due to deficiencies in bulbar neurogenesis. Indeed, the proliferation of precursor cells, their migration, neuronal fate and survival were normal in the absence of TNC. Therefore, our results suggest a new function of TNC in early postnatal development of the murine olfactory system.

Results

TNC expression pattern in the forebrain of neonatal and young adult mice

Two ages were assessed for TNC expression in the olfactory system: P10 and 2–3 months. At P10, we found that TNC was expressed in both the SVZ (Figs. 1a, b) and RMS (Fig. 1d). We also confirmed previous data (Gates et al., 1995) showing that TNC staining in the SVZ at P10 was relatively weak, and staining in the RMS was mostly confined to the borders of the RMS, with its center being faintly stained (data not shown). In the olfactory bulb, TNC staining was weak but specific in the granule cell layer and strong in the glomerular layer (Fig. 1c), with TNC being expressed most prominently inside glomeruli at P10 (see also Gonzalez Mde et al., 1993; Gonzalez and Silver, 1994; see also Supplementary Fig. 1). In young adult mice, TNC is expressed by GFAP⁺ astrocytes in the SVZ, along the entire RMS, and in the

granule cell and glomerular layers (Gates et al., 1995; Jankovski and Sotelo, 1996; Thomas et al., 1996). We confirmed this restricted expression pattern (Fig. 1e) and showed by triple immunohistochemistry that TNC was found in the extracellular space, intermixed between GFAP⁺ astrocytes and PSA-NCAM⁺ cells, presumably neuroblasts (Figs. 1f–h). Furthermore, TNC labeling was weaker in the olfactory bulb than in the SVZ and RMS (Fig. 1e).

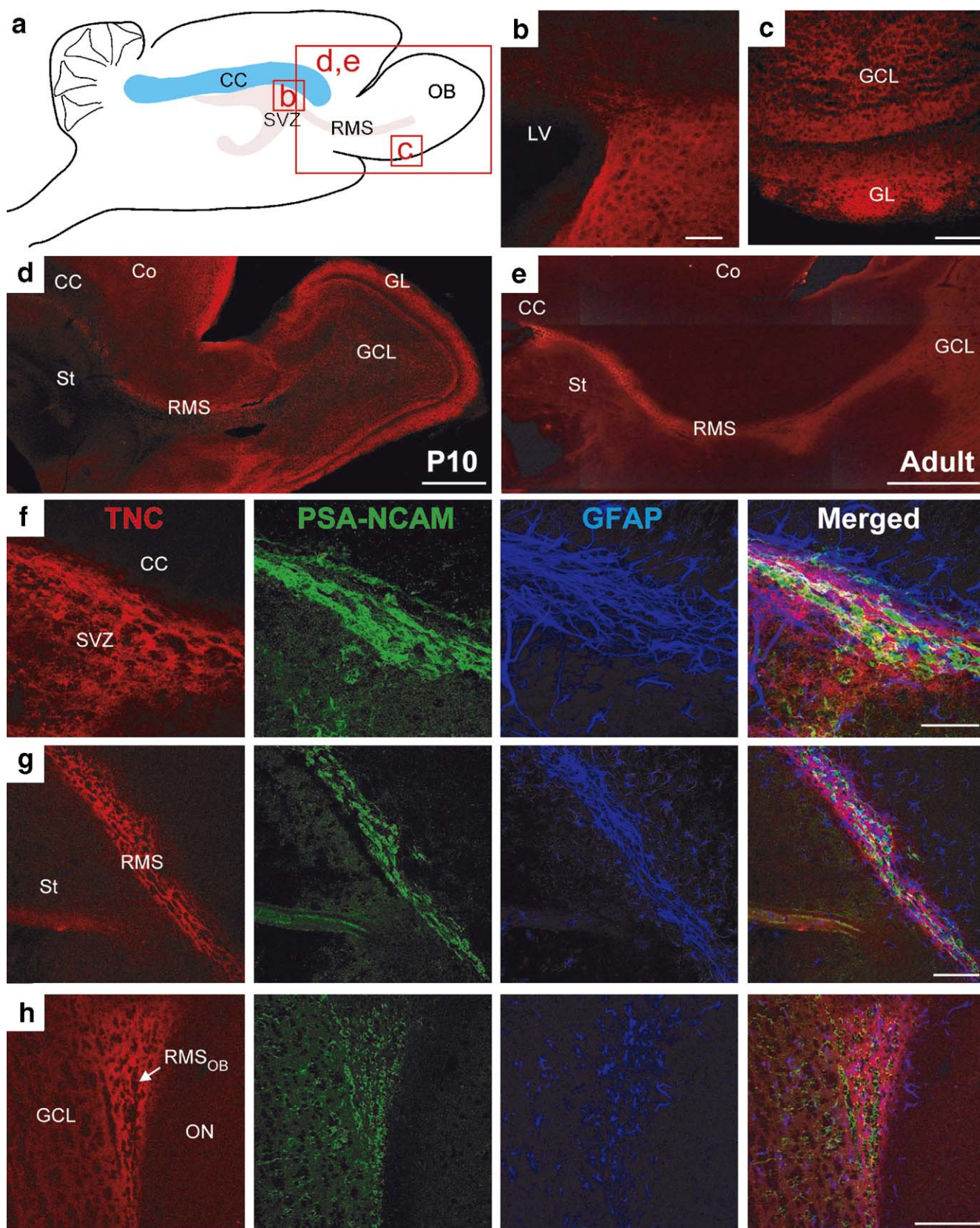
To further investigate the functional role of TNC in the adult olfactory neurogenic system (SVZ–RMS–olfactory bulb), we first analyzed its histoarchitecture in TNC-deficient mice. By DAPI and cresyl violet staining of cell nuclei at the level of SVZ, RMS and olfactory bulb, we found that the overall organization of these structures was qualitatively similar in wild-type and TNC mutant mice (data not shown). We then measured the mean areas of the anterior SVZ and the RMS (the two “germinative” regions), as well as those of the granule cell and glomerular layers of the olfactory bulb (the two “target” regions). Results show no differences between wild-type and mutant mice in all areas measured (P10 SVZ and RMS coronal sections: 0.108 ± 0.017 versus 0.116 ± 0.01 mm² for wild-type and TNC-deficient mice, respectively; $n = 4$, $P > 0.05$; P10 granule cell layer coronal sections: 0.995 ± 0.06 versus 0.938 ± 0.03 mm² for wild-type and TNC-deficient mice, respectively; $n = 4$, $P > 0.05$; P10 glomerular layer coronal sections: 0.42 ± 0.03 versus 0.48 ± 0.01 mm² for wild-type and TNC-deficient mice, respectively; $n = 4$, $P > 0.05$; adult SVZ sagittal sections: 0.034 ± 0.005 versus 0.046 ± 0.01 mm² for wild-type and TNC-deficient mice, respectively; $n = 4$, $P > 0.05$; adult RMS sagittal sections: 0.08 ± 0.007 versus 0.08 ± 0.06 mm² for wild-type and TNC-deficient mice, respectively; $n = 4$, $P > 0.05$; adult granule cell layer coronal sections: 0.99 ± 0.06 versus 0.94 ± 0.03 mm² for wild-type and TNC-deficient mice, respectively; $n = 4$, $P > 0.05$; adult glomerular layer coronal sections: 0.44 ± 0.09 versus 0.41 ± 0.03 mm² for wild-type and TNC-deficient P10 mice, respectively; $n = 4$, $P > 0.05$). Therefore, we conclude that TNC deficiency does not modify the mean area of the source and target structures of bulbar neurogenesis, in both neonatal and young adult TNC-deficient mice.

TNC-deficient mice display a delayed onset in early olfactory responses

Garcion et al. reported that in TNC-deficient mice SVZ cell proliferation was reduced in early postnatal life (Garcion et al., 2001). Thus, we studied olfactory performance of our TNC-deficient mice at this developmental stage. We used two different behavioral tests (see Experimental methods for details): a preference test and a measurement of ultrasound calls in response to olfactory cues. The preference test (Fig. 2a) showed that wild-type P2 pups had no preference for either compartment containing male-scented or clean shavings (Fig. 2b). From P3 to P6, pups spent more time in the compartment containing the male shavings (this response peaked at P5, when the pups stayed three times longer in the compartment with male shavings than clean shavings, Fig. 2b). Between P8 and P12, the pups either spent significantly more time in the compartment containing the clean shavings or showed no preference for either compartment. TNC-deficient mice showed a similar behavior, although development of this behavior occurred 24 h later than in the control wild-type littermates (t test, $P < 0.05$

between TNC-deficient and wild-type from P3 to P8, Fig. 2b). As the midline of the test box was crossed the same number of times by both groups of animals at all the ages examined (data

not shown), we assume that the delayed onset of olfactory responses in TNC-deficient mice is not due to impaired locomotion, but to a retarded development of olfactory abilities.



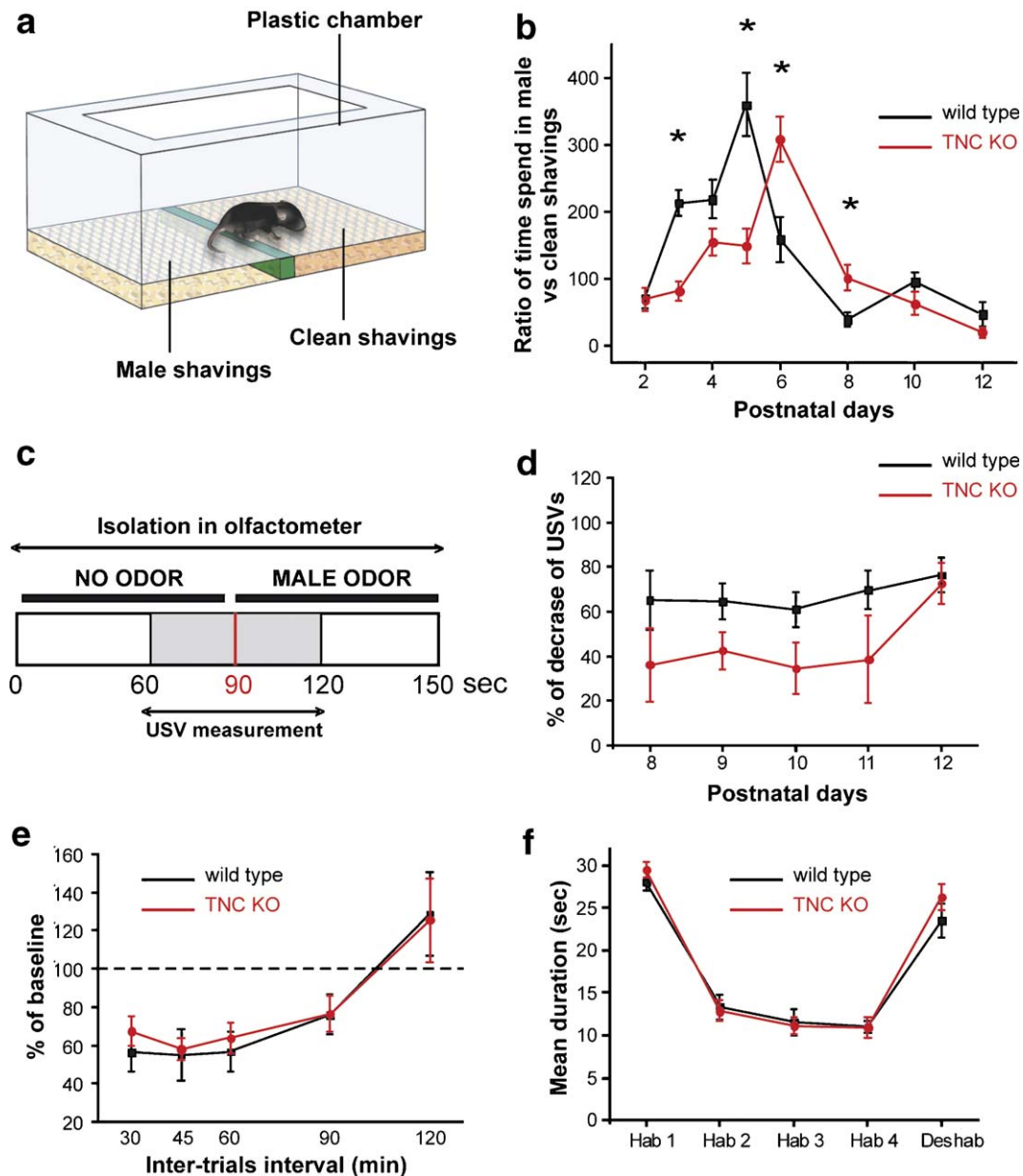


Fig. 2. Altered olfactory functions in neonatal but not adult TNC-deficient mice. (a) Scheme describing the experiment for the preference test. (b) Twenty-four-hour delayed choice curve in the preference test when pups can choose between non-familiar male and clean shavings. Note the statistically significant difference between the two genotypes in occupation of space between P3 and P8. (c) Scheme describing the protocol used for the USV test. (d) Reduction of USV emission rate when a non-familiar male odor replaces the odor-free isolation period. From P8 to P11, the reduction of USV is slightly weaker (although not significantly) in mutant mice, suggesting that they have altered olfactory detection abilities. (e) Adult mutant mice have normal olfactory memory. Effect of different inter-trial intervals on odor recognition in standard ($n = 9$) and TNC-deficient ($n = 11$) mice. Each bar represents the mean percentage of time spent investigating a given odor on the second exposure as compared to the first exposure. For both strains of mice, the time of investigation is reduced on the second exposure up to an inter-trial interval of 90 min. (f) Adult mutant mice have normal olfactory acuity. TNC-deficient and control mice were both habituated to one odor with four consecutive presentations, and the subsequent presentation of a different odor increased the investigation time to the level of the first odor presentation. Three different pairs of odors were used. Data are represented as mean \pm SEM.

Fig. 1. TNC expression pattern in P10 (b, c, d) and adult (e, f, g, h) mice. (a) Schematic drawing of a sagittal section of an adult mouse brain depicting the localization of the micrographs in panels b, c, d and e. (b) In P10 mice, TNC immunolabeling was observed in the SVZ, especially in its lateral part, shown here in the coronal plane. (c) Immunostaining of the granule cell and glomerular layers of the olfactory bulb. Note the very strong labeling in the glomerular layer. (d) Low magnification of a P10 sagittal forebrain section stained with anti-TNC antibody. (e) Parasagittal view of the SVZ–olfactory bulb pathway of an adult mouse after TNC immunohistochemistry. (f) Three-dimensional confocal reconstruction of an adult SVZ (in a sagittal plan) after immunostaining for TNC (red), PSA-NCAM (green) and GFAP (blue). Similar confocal photomicrographs are shown for the RMS (g) and the olfactory bulb (h, here in the coronal plane). Note that immunoreactivity is clearly weaker in the granule cell layer as compared to the RMS_{OB}. CC, corpus callosum; Co: cerebral cortex; GL: glomerular layer; GCL: granule cell layer; LV, lateral ventricle; ON: olfactory nucleus; RMS: rostral migratory stream; RMS_{OB}: rostral migratory stream of the olfactory bulb; St, striatum; SVZ: subventricular zone. Scale bars: b, 200 μ m; c, h, 100 μ m; d, e, 500 μ m; f, g, 50 μ m.

After P8, we observed no difference in olfactory responses between TNC-deficient mice and wild-type littermates. This may be partly due to the preference test not being sensitive enough to detect subtle modifications in olfactory responses 1 week after birth. Therefore, we used a second test based on ultrasound vocalization (USV) emission that we have recently shown to be more sensitive for newborn pups (Lemasson et al., 2005). We measured the decrease in USV emission by pups when a non-familiar male odor replaced an odor-free isolation period (Fig. 2c). From P8 to P11, the mean USV frequency emitted by wild-type pups decreased by about 60% in the presence of the male odor compared to the odor-free period (Fig. 2d). In P8 to P11 TNC-deficient mice, the mean USV frequency decreased to a lower level, although not significantly (reduction of $65 \pm 13\%$ versus $36 \pm 16\%$ at P8; $64 \pm 8\%$ versus $42 \pm 8\%$ at P9; $61 \pm 8\%$ versus $35 \pm 11\%$ at P10; $70 \pm 9\%$ versus $39 \pm 20\%$ at P11 for wild-type versus TNC-deficient mice). This means that the detection of a non-familiar male odor may be subtly different in the mutant pups between P8 and P11. From P12, the decrease in USV emission after presentation of a male odor was similar for both genotypes (Fig. 2d).

We then checked whether this subtle difference detected in TNC-deficient mice may entail consequences in adulthood. Therefore, we tested mice in an odor memory (Fig. 2e) and odor discrimination task (Fig. 2f) to examine olfactory performance in the adult. We first checked whether the two genotypes had similar degrees of olfactory exploration before performing these tasks. During the first 5 min of odor presentation, wild-type ($n = 9$) and TNC-deficient ($n = 11$) mice spent the same amount of time exploring the new odor (first exposure for the 30 min interval test, $t_{18} = 1.90$, $P > 0.05$; first exposure for the 120 min interval test, $t_{18} = 0.53$, $P > 0.05$). These data show that wild-type and TNC-deficient mice have the same levels of motivation and curiosity under our test conditions.

In the odor memory test (Fig. 2e), both wild-type and TNC-deficient mice showed a significant decrease in odor investigation during the second exposure of a given odor at 30, 45, 60 and 90 min (for wild-type mice: $t_{18} = 4.65$, $P < 0.001$; $t_{18} = 3.39$, $P < 0.01$, $t_{18} = 4.58$, $P < 0.001$; $t_{18} = 2.34$, $P < 0.05$ respectively; for mutant mice: $t_{18} = 4.10$, $P < 0.001$; $t_{18} = 7.26$, $P < 0.001$, $t_{18} = 4.50$, $P < 0.001$; $t_{18} = 2.48$, $P < 0.05$ respectively), but not at 120 min (for wild-type mice $t_{18} = 1.30$, $P > 0.05$; for mutant mice $t_{18} = 1.16$, $P > 0.05$; Fig. 2e). Thus, like normal mice, TNC-deficient mice are able to retain the trace of an odor for a time period shorter than 120 min (Bluthé et al., 1993). We then used a habituation–dishabituation test to assess olfactory discrimination. Both wild-type and TNC-deficient mice spent significantly less time investigating the first odor after the second, third and fourth exposure, as expected for habituation (Fig. 2f). When the second odor was then exposed, both groups of mice investigated the new odor pipette for as long as for the first exposure of the first odor. Therefore, odor discrimination was identical in both wild-type and TNC-deficient mice (Fig. 2f). Thus, we have shown that early

olfactory behavior is subtly altered in TNC-deficient mice, particularly from P3 to P8, while olfactory performance in adult TNC-deficient mice is normal.

As TNC is strongly expressed along the SVZ–olfactory bulb pathway in young and adult mice (Miragall et al., 1990; Gates et al., 1995; Jankovski and Sotelo, 1996; Thomas et al., 1996; see also Figs. 1d, e), we wondered whether the difference in olfactory behavior in neonatal TNC-deficient mice was due to specific changes in neonatal bulbar neurogenesis. Garcion et al. previously reported a reduced proliferation of neuronal precursors in the neonatal SVZ of TNC-deficient mice (Garcion et al., 2001), supporting our hypothesis. Therefore, we evaluated neurogenesis in both neonatal and adult TNC-deficient mice by assessing neuronal precursor proliferation in the SVZ and RMS, their tangential migration in the RMS and their survival and fate in the olfactory bulb.

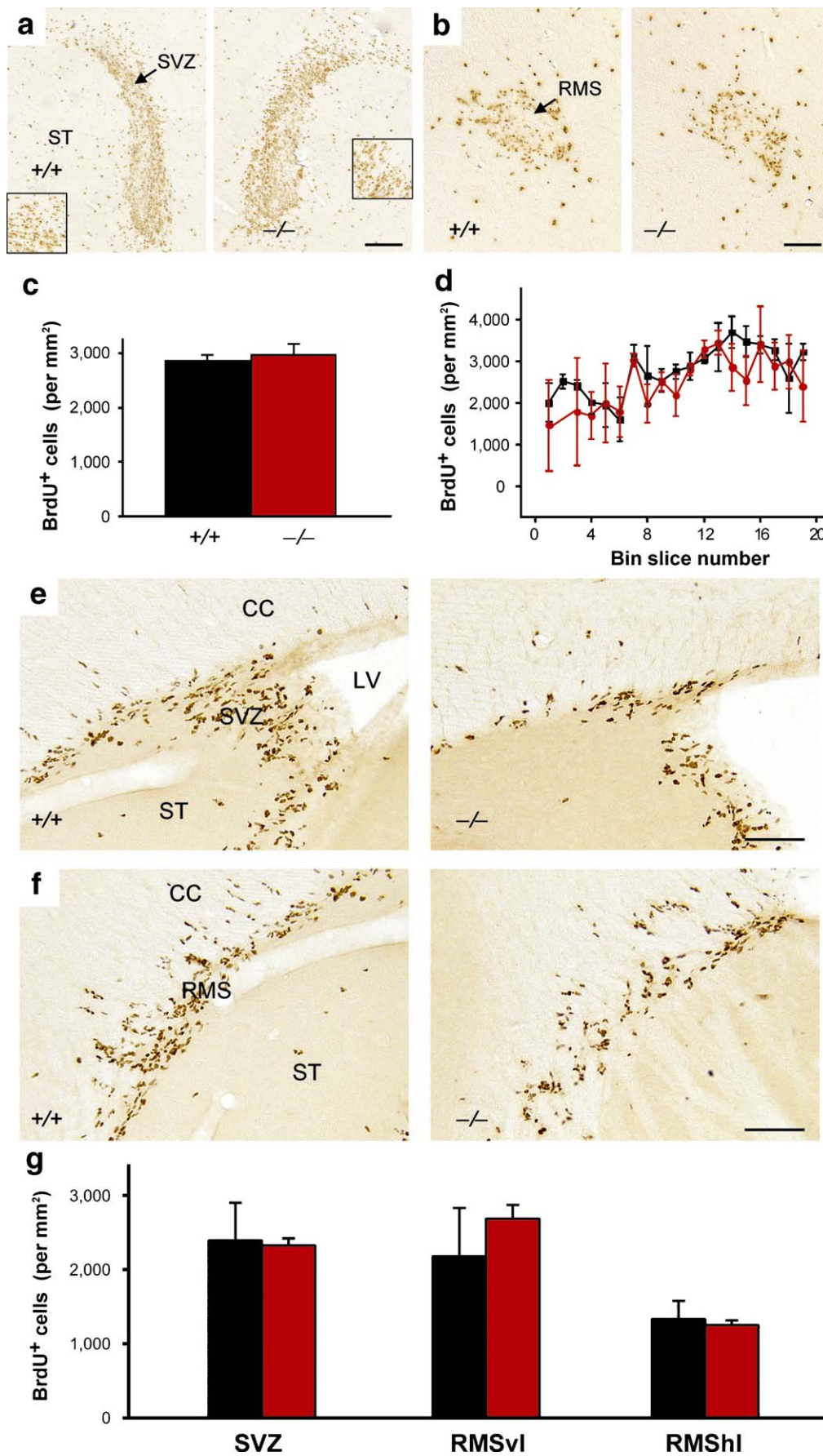
Proliferation of precursor cells in the SVZ and RMS

We measured the proliferation rate of SVZ and RMS precursor cells in either P10 or 2- to 3-month-old mice by injecting them with a single dose of BrdU, a marker of DNA synthesis, and using immunohistochemistry to look for BrdU in the brain 4 h later. We found no difference in the distribution of BrdU⁺ cells in the SVZ and RMS between TNC-deficient and wild-type mice at both ages (Figs. 3a, b, e, f). The densities of BrdU⁺ cells in the SVZ and RMS were identical for both groups at both ages (Fig. 3d) (2773 ± 117 versus 2823 ± 290 cells/mm² for wild-type and TNC-deficient P10 mice, respectively; $n = 4$, $P > 0.05$; 2132 ± 335 versus 2243 ± 259 cells/mm² for wild-type and TNC-deficient adult mice, respectively; $n = 4$, $P > 0.05$). Surprisingly, normal neural precursor proliferation was found in the neonatal SVZ of TNC-deficient mice because Garcion et al. (2001) reported altered precursor cell proliferation in the early postnatal SVZ of the TNC-deficient mice generated by Saga et al. (1992). We wondered whether the differences between their study and ours could be due to differences in the rostro-caudal level of the SVZ used for the analysis. Therefore, we counted the number of BrdU⁺ cells throughout the entire RMS and SVZ at P10. Again, we observed no difference in the number (data not shown) and density of newborn cells between the two groups of mice (Fig. 3c). Thus, neural precursor proliferation is normal in the SVZ and RMS of both neonatal and adult TNC-deficient mice.

Tangential migration of neuroblasts

As TNC is strongly expressed in the RMS, we tested whether its absence alters the tangential migration of PSA-NCAM⁺ neuroblasts. We first examined the general chain organization in the SVZ and RMS. The chains of neuroblasts seen by PSA-NCAM immunostaining in whole-mount preparations of the SVZ were similar in both two groups (Fig. 4a). An extensive network of

Fig. 3. Normal proliferation in the SVZ and RMS of P10 and adult TNC-deficient mice. (a, b) Photomicrographs at the level of the SVZ (a) and RMS (b) of P10 control (left panel) and TNC-deficient (right panel) mice, showing the distribution of BrdU⁺ nuclei. (c) Quantification of the distribution of BrdU⁺ nuclei along the rostro-caudal axis of the SVZ–RMS pathway. Values for TNC-deficient mice are shown in red; those for wild-type mice in black. At no level of the SVZ–RMS pathway is there a significant difference in mean BrdU⁺ cell density. (d) Quantification of the mean BrdU⁺ cell density in both the SVZ and RMS in P10 control (+/+) and TNC-deficient (−/−) mice. (e, f) BrdU immunostaining in sagittal sections through the forebrain of adult control (+/+, left) and TNC-deficient mice (−/−, right) showing the distribution of BrdU⁺ cells in the SVZ (e) and RMS (f). (g) Quantification of mean BrdU⁺ cell density in both the SVZ and RMS in adult control (+/+) and TNC-deficient (−/−) mice, showing no statistical difference between the two groups. CC, corpus callosum; LV, lateral ventricle; ST, striatum. Data are represented as mean \pm SEM. Scale bars: a, 200 μ m; b, e, f, 100 μ m.



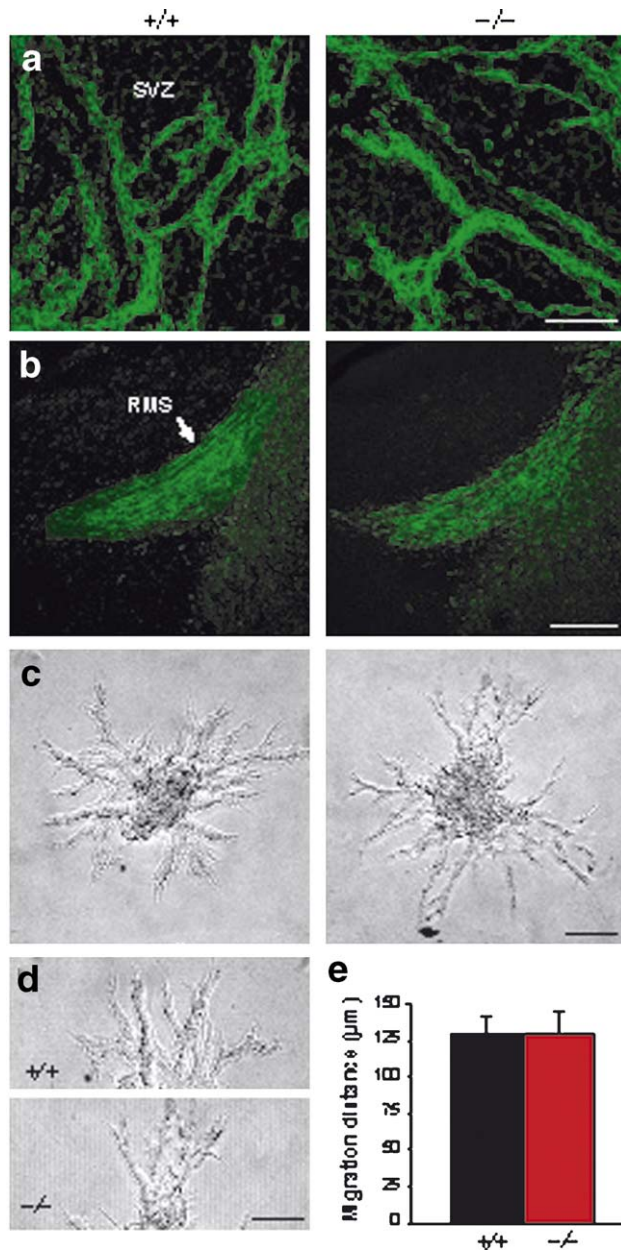


Fig. 4. Normal chain organization and migration of neuroblasts in TNC-deficient mice. (a) High magnification of whole-mount immunostaining of PSA-NCAM⁺ chains in the SVZ of control (+/+, left) and TNC-deficient mice (-/-, right). (b) PSA-NCAM immunostaining in the RMS of control (left) and mutant mice (right). (c) Phase-contrast images of SVZ explants of control (left) and TNC-deficient mice. (d) Higher view of SVZ explants showing normal formation of chain migration. (e) Quantification of migration distance, showing no difference between control (+/+) and TNC-deficient explants (-/-). Data are represented as mean \pm SEM. Scale bars: a, d, 50 μ m; b, 300 μ m; c, 100 μ m.

tangential pathways was clearly visible. Most of the chains in this network formed a longitudinal array that was unaltered in TNC-deficient mice. Furthermore, the organization of the PSA-NCAM⁺ chains along the migratory pathway to the olfactory bulb was similar in both genotypes (Fig. 4b).

We then assessed the rate of neuroblast migration by culturing SVZ explants on Matrigel (Wichterle et al., 1997). After 20 h of

culture, an extensive network of chains formed around the explants from P8 wild-type and TNC-deficient mice (Figs. 4c, d). Again, we observed no detectable difference between wild-type and TNC-deficient mice. The general organization of the network of chains and their length were identical (130 ± 11 μ m versus 130 ± 15 μ m for wild-type and TNC-deficient mice, respectively; 7 explants from two wild-type mice and 14 explants from four TNC-deficient mice, $P > 0.05$; Fig. 4e). Together, these results indicate that tangential migration remains normal in the absence of TNC.

Density of newborn cells in the olfactory bulb of neonatal and adult mice

We evaluated the number of newborn cells that integrate into the olfactory bulb circuitry by injecting either P10 or adult mice with four doses of BrdU in 1 day and using immunohistochemistry to look for BrdU in the brains 21 days later. Most BrdU⁺ cells were scattered throughout the granule cell layer at both ages (Figs. 5a, e). We observed no difference in the density of BrdU⁺ cells between the two groups at both ages in the granule cell layer (1636 ± 152 versus 1607 ± 70 cells/mm² for P10 wild-type and TNC-deficient mice, respectively, $n = 4$, $P > 0.05$, Fig. 5b; 240 ± 31 versus 218 ± 29 cells/mm² for adult wild-type and TNC-deficient mice, respectively, $n = 4$, $P > 0.05$, Fig. 5f) and the glomerular layer (1058 ± 65 versus 1105 ± 50 cells/mm² for P10 wild-type and TNC-deficient mice, respectively, $n = 4$, $P > 0.05$, Figs. 5c, d; 87 ± 9 versus 87 ± 9 cells/mm² for adult wild-type and TNC-deficient mice, respectively, $n = 4$, $P > 0.05$, Figs. 5g, h). Therefore, the density of newborn cells surviving 21 days was unaltered in TNC-deficient mice.

Fate of newborn cells in the olfactory bulb of neonatal and adult mice

Finally, we tested whether the fate of the newborn cells in the olfactory bulb was affected by measuring the proportion of cells double-labeled for BrdU⁺ and the neuronal marker NeuN (animals killed 21 days after BrdU injection; Fig. 6a). Orthogonal projections through three-dimensionally reconstructed BrdU⁺ cells showed that in both genotypes about 80% of the newborn cells were neurons ($n = 89$ and 93 cells from three wild-type and TNC-deficient mice, respectively; Fig. 6b). Therefore, considering that neuronal precursor proliferation, tangential migration of neuroblasts and the number of newborn neurons in the olfactory bulb are unchanged, we conclude that global bulbar neurogenesis remains unaltered in both P10 and adult TNC-deficient mice.

Discussion

Here, we have shown that mice constitutively deficient in TNC display a delayed onset of olfactory abilities during the first week after birth. However, unlike previous reports, we found no difference in bulbar neurogenesis between TNC-deficient and wild-type animals. Young adult TNC-deficient mice had normal olfactory functions and bulbar neurogenesis.

TNC and postnatal neurogenesis

Our comprehensive investigation of bulbar neurogenesis demonstrates that neural precursor proliferation and migration

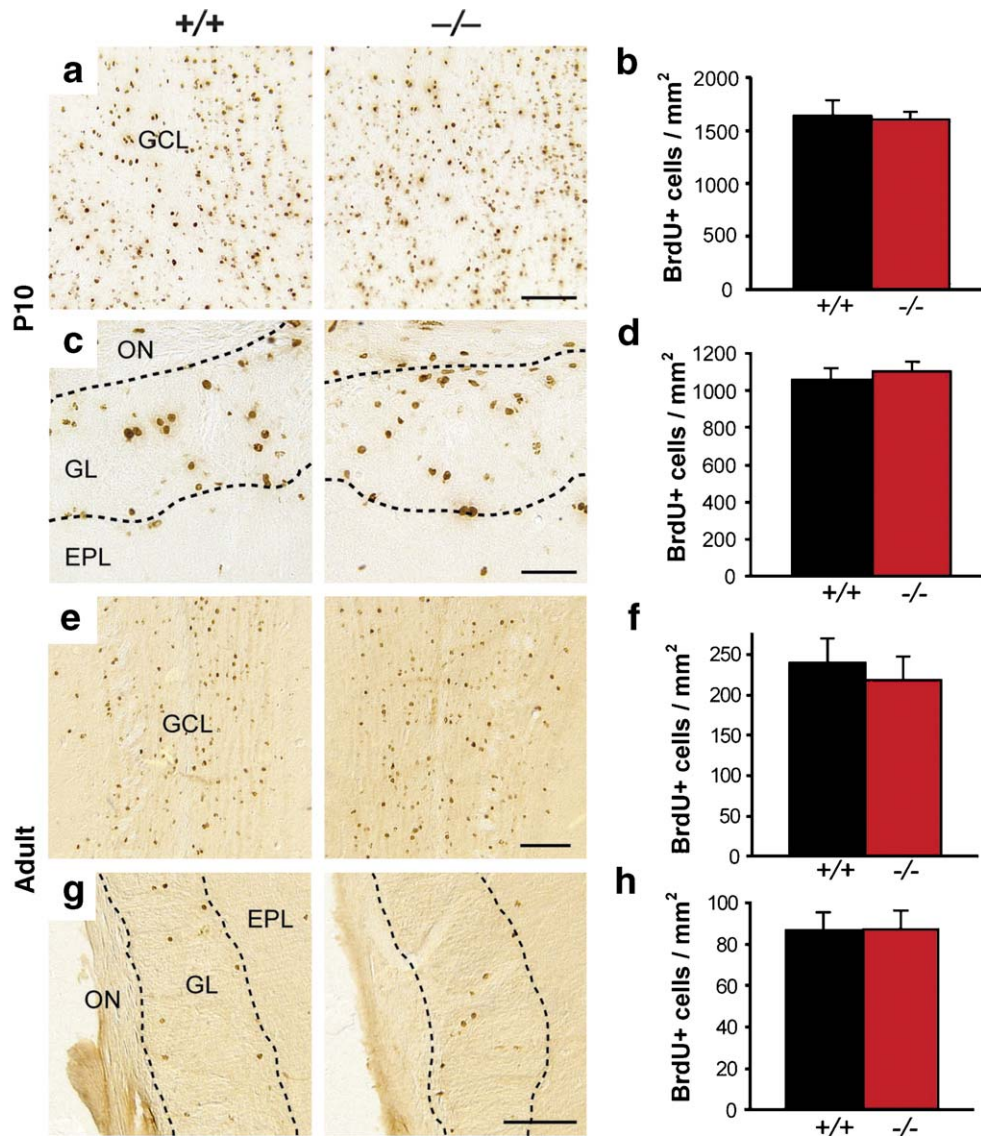


Fig. 5. Normal density of newborn cells in the olfactory bulb of P10 and adult TNC-deficient mice at 21 days after BrdU injections. (a) BrdU⁺ nuclei in the granule cell layer of P10 control (left) and TNC-deficient mice (right). No obvious difference in the number of newborn cells is observable. (b) Mean density of newborn granule cells in P10 control (+/+) and TNC-deficient mice (-/-). (c) BrdU⁺ nuclei in the glomerular layer of P10 control (left) and TNC-deficient mice (right). (d) Mean density of newborn periglomerular cells in P10 control (+/+) and TNC-deficient mice (-/-). (e) BrdU⁺ nuclei in the granule cell layer of adult control (left) and TNC-deficient mice (right). (f) Mean density of newborn granule cells in adult control (+/+) and TNC-deficient mice (-/-). (g) BrdU⁺ nuclei in the glomerular layer of adult control (left) and TNC-deficient mice (right). (h) Mean density of newborn periglomerular cells in adult control (+/+) and TNC-deficient mice (-/-). EPL, external plexiform layer; GCL, granule cell layer; GL, glomerular layer; ON, olfactory nerve. Data are represented as mean \pm SEM. Scale bars: a, e, g, 100 μ m; c, 40 μ m.

and the cellular density of newborn neurons in the olfactory bulb were normal in the absence of TNC. Within 21 days of BrdU injection, dividing cells that had incorporated BrdU migrated tangentially from the SVZ and RMS towards the olfactory bulb, migrated radially to their target regions (i.e., granule cell and glomerular layers) and then differentiated into neurons and integrated into the existing network (reviewed in Lledo and Saghatelian, 2005). Therefore, our experiments take into account most cellular histological processes characterizing bulbar neurogenesis. However, other parameters would need to be more carefully analyzed in TNC-deficient mice. For example, it is possible that these animals may communicate with each other abnormally, as could be probed for by electrophysiological

measurements in the olfactory bulb, or that newborn neurons may not differentiate into morphologically normal cells. Finally, since massive cell death of newborn granule cells occurs between 14 and 28 days after birth (e.g., Yamaguchi and Mori, 2005), we cannot rule out that survival of newborn granule cells may be altered after our investigated time point (e.g., 21 days).

There may be several reasons why we did not see the reduction of cell proliferation in the SVZ at P10 in TNC-deficient mice as reported by Garcion et al. (2001). The genotypes of the two TNC-deficient mice are different: in the TNC-deficient mice used in our study, the entire TNC coding region is ablated (Evers et al., 2002), whereas the mice used by Garcion et al. express a truncated fragment or fragments of the TNC molecule, although in reduced

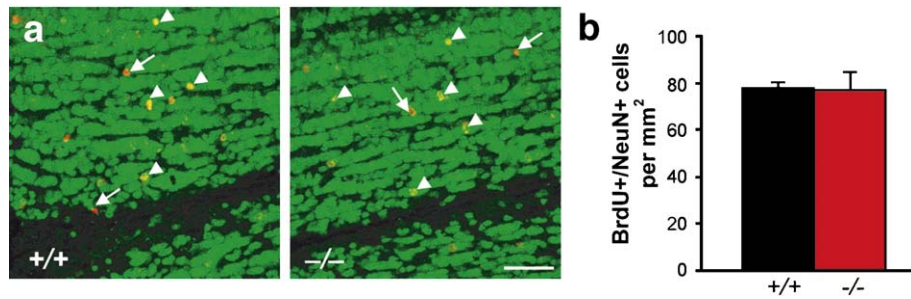


Fig. 6. Normal fate determination of newborn cells in the olfactory bulb of adult TNC-deficient mice. (a) Confocal 3D reconstruction of BrdU⁺ cells (red) in control (left) and TNC-deficient mice (right) stained for the neuronal marker NeuN (green). Reconstructed orthogonal projections are presented as viewed in the *x*-*z* (top) and *y*-*z* (right) planes. (b) Percentage of BrdU⁺ cells double-labeled with NeuN in control (+/+) and TNC-deficient mice (-/-). GCL: granule cell layer. Values in histogram are mean \pm SEM. Scale bar: 100 μ m.

amounts when compared to the wild-type mice (Saga et al., 1992; Mitrovic and Schachner, 1995; Steindler et al., 1995; Settles et al., 1997). It is known that different TNC fragments have different effects on cell behavior (Jones and Jones, 2000) and that TNC changes the proliferation rate of some neural cells in vitro (Nishio et al., 2003; Nishio et al., 2005). We thus propose that truncated TNC fragments reduced cell proliferation as observed by Garcion et al. (2001). Alternatively or in addition, the differences seen in cell proliferation in the SVZ may be due to different quantification methods: Garcion et al. counted BrdU⁺ cells in randomly defined regions (500 μ m² of size) in the SVZ on seven brain slices per animal (Garcion et al., 2001), whereas we quantified labeled cells throughout the entire SVZ–RMS pathway (corresponding to 17 slices per animal; for details, see Experimental methods), which is a more exhaustive and reliable quantification method.

During embryogenesis, TNC functions in generating the SVZ neural stem cell “niche” by coordinating growth factor signaling to accelerate neural stem cell development (Garcion et al., 2004). Although the present study was done on postnatal animals, we were unable to detect any function of TNC in neurogenesis of the olfactory bulb in our TNC-deficient mice. Therefore, one or several other extracellular matrix proteins may compensate for this TNC deficiency in mice with total ablation of the molecule as opposed to mice with only partial ablation (Faissner, 1997; Jones and Jones, 2000). In particular, chondroitin-sulfate-containing proteoglycans (CSPGs) are widely expressed in the extracellular matrix surrounding the SVZ and RMS (Thomas et al., 1996) – as is TNC – and have been shown to play a similar role as TNC, particularly in neurite outgrowth (Snow et al., 1990; Brittis et al., 1992). Therefore, it would be informative to study the expression pattern of CSPGs in the SVZ–olfactory bulb pathway of TNC-deficient mice (Thomas et al., 1996) and the role of this family of extracellular matrix molecules in postnatal neurogenesis by inactivating TNC at specific times and in specific regions to remove any compensatory mechanisms occurring in constitutively TNC-deficient mice. Alternatively, as other proteins may function in a similar manner as TNC, the role of TNC in olfactory bulb neurogenesis should be studied in mice simultaneously deficient for TNC and other extracellular matrix molecules. Finally, TNC that remains in germinal “niches” after birth may prove to be important only after injury or cell loss by providing an environment allowing the production and differentiation of precursor cells, as occurs during embryonic development (Garcion et al., 2004).

Astrocytes in the SVZ and RMS have been reported to secrete as yet unknown signaling factors for tangential migration (Mason

et al., 2001). As TNC is confined to neuroblasts in the entire tangential migratory route, TNC may be one of these factors. However, since compensatory mechanisms may have led to the apparently normal tangential migration in the TNC-deficient mice that we observed, the effect of acutely adding TNC onto wild-type neuroblasts in vitro (Mason et al., 2001) should be measured.

Finally, it is worth noting that TNC deficiency reduces L-type voltage-dependent Ca²⁺ channels (VDCCs)-mediated signaling in the adult hippocampus, resulting in deficits of synaptic plasticity (Evers et al., 2002). VDCCs expressed by dentate gyrus precursor cells are involved in excitation–neuronal fate choice coupling (Deisseroth et al., 2004). Thus, deficiency in TNC might have altered the fate of SVZ precursors via a VDCC-dependent signaling pathway. Our analysis of neuronal fate determination in the olfactory bulb neurogenesis revealed, however, no differences between wild-type and TNC mutant mice.

TNC and early olfactory abilities

In species that require parental care, evolution has ensured that newborns quickly learn and express robust olfactory responses to their surroundings (Sullivan, 2003). For example, newborn rats form early memories of odors associated with both pleasant (milk or warmth) and unpleasant stimuli (tail-pinch or shock) (McLean et al., 1993; Sullivan et al., 2000). These memories are expressed as odor preferences or aversions. Pups orient themselves toward the odor, even climbing obstacles to approach the odor (Sullivan et al., 2000). The unique neural circuitry responsible for this early olfactory learning is currently being investigated and has been shown to include the olfactory bulb among other brain structures (Sullivan, 2003). In the present study, we found that the onset of olfactory preference in TNC-deficient mice aged from P3 to P8 was delayed by 24 h. Normal olfactory bulb neurogenesis in P10 TNC-deficient mice despite altered olfactory behavior may have several explanations. It was recently shown that TNC repels postnatal olfactory sensory axons and inhibits their growth in vitro (Treloar and Greer, 2004). Since TNC forms a ring pattern around glomeruli during postnatal development at around P6 and then becomes homogeneously distributed in and around glomeruli at P10 (Gonzalez Mde et al., 1993; Gonzalez and Silver, 1994; see also Supplementary Fig. 1), TNC may act as a molecular boundary that stops the olfactory axons from extending, thus allowing precise formation of glomeruli at around P6. Then, at P10, the homogenous distribution inside glomeruli creates a permissive microenvironment that allows olfactory sensory axons and mitral cell primary dendrites to grow towards and connect to each other.

Indeed, TNC is generally conducive and promotes neurite outgrowth when expressed as a uniform substrate, while it is inhibitory for the advancing growth cones when expressed as a sharp boundary (Lochter et al., 1991, 1995; Husmann et al., 1992; Taylor et al., 1993). Therefore, it is conceivable that the targeting of olfactory axons to their corresponding glomeruli and the establishment of synaptic connections with mitral/tufted cells may be altered and/or delayed in TNC-deficient mice. This notion may explain the delayed olfactory response observed in early postnatal mice in our study. Indeed, olfactory functions are delayed in TNC-deficient mice between P3 and P8, a time period that closely corresponds to the period of glomerular formation (Gonzalez Mde et al., 1993; Gonzalez and Silver, 1994; Conzelmann et al., 2001; Kim and Greer, 2000).

Together, our findings suggest that there is a sensitive early period in the onset of olfactory responses during which TNC is involved, being independent of bulbar neurogenesis. This so far unknown function of TNC may be linked to ontogenetically early processes that take place during postnatal development of olfactory glomeruli. TNC thus may be involved in the fasciculation and/or sorting of outgrowing axons from olfactory sensory neurons. Both of these developmental events are fundamental to initiating and establishing the precise wiring of the olfactory system during a short time period after birth, a hypothesis that still remains to be addressed.

Experimental methods

Animals

Transgenic mice with undetectable levels of TNC expression (Evers et al., 2002) and age-matched wild-type littermates were derived from heterozygous parents with a mixed C57BL/6J \times 129Ola background. Mice between 2 and 4 months of age were referred to as “young adults”. Animals were kept at a constant temperature (22°C) on a 12 h light–dark cycle with free access to food and water. All experimental procedures were carried out according to Society for Neuroscience and European Union guidelines and were approved by our institutional committees for animal care and use.

Behavior

Eleven male TNC-deficient mice and nine wild-type littermates for the behavioral experiments were used. The olfactory performance of each animal was tested at early (from P2 to P12) and late (P60) stages of development.

Test of neonatal mice

Two odor-choice task. This test was conducted in a transparent plastic chamber divided by a solid separator into two olfactory stimulus environments. One of the chambers contained clean shavings, and the other contained bedding from unfamiliar adult mice. A stainless steel grate with a plastic mesh that allowed only olfactory cues to pass from the shavings, and on which the pups roamed freely during the test session covered the chamber containing the clean shavings. The chamber was not sealed, having a large hole in the lid to minimize stagnant odors. The pups were familiarized with the apparatus 1 day before the tests were run by placing only clean shavings in the two olfactory compartments. At the start of the testing session, the mouse was introduced to the center of the grate in the neutral zone midway between the two olfactory stimuli environments and the cover replaced. The test began and the mouse started exploring both chambers. During a 6-min session, we recorded the time spent in one half of the apparatus. We only scored for odor

investigation if the anterior snout of the animal was in that half of the apparatus. We changed the orientation of the animals between tests to control for any turning bias. The test chamber was cleaned and dried before testing each new mouse. After testing, the pups were returned to their mother.

Male odor-induced ultrasonic vocalization (USV) response test. The olfactory detection task has been previously described in detail (Lemasson et al., 2005). We randomly chose each pup, removed it from its home cage and placed it in the transparent chamber of an eight-channel olfactometer (Slotnick and Bodyak, 2002). A test session consisted of an initial isolation period of 90 s for baseline USV recording followed by 60 s of odor exposure. The odor consisted of shavings taken from the cage of unfamiliar male mice. We began USV recordings 10 s after placing the pups into the test chamber. During the test session, USV was detected using an ultrasonic microphone (SM2 Microphone, Ultra Sound Advice) placed 10 cm above each pup and connected to a bat detector (S25 Bat Detector, Ultrasound advice; frequency range 10–180 kHz). Ultrasound vocalizations were then extracted and automatically counted using in-house software (Lemasson et al., 2005). We compared the rate of vocalizations emitted during the final 30 s of baseline recording with the rate emitted during the first 30 s of odor exposure. We considered that the animals had detected the olfactory cues when the rate of USV emission decreased significantly during male odor exposure compared to the baseline period (i.e., no odor in the olfactometer). After each testing period, the pups were returned to their nest.

Test of young adult mice

Animals were exposed to odors by placing a glass Pasteur pipette containing a filter paper impregnated with an odor through the center of one side of the mesh top covering the animal's home cage. In all experiments, we recorded the time that the animals spent sniffing or investigating the odor. Four days before the tests were run, the animals were familiarized with the procedure by exposing them to mineral oil and then to odor stimuli different from those used in the test session.

Olfactory memory. Mice were exposed twice (5 min each time) to the same odorant for 30, 45, 60, 90 and 120 min intervals (Rochefort et al., 2002). The intervals were randomized in separate sessions 24 h apart, and different odors were used. We considered that animals were able to recognize an olfactory stimulus when they spent significantly less time investigating the odor at the second time of exposure.

Olfactory discrimination. For the olfactory discrimination task, we used a slightly modified version of the habituation–dishabituation test described previously (Gheusi et al., 2000). Mice were exposed to the first odor (habituation odor) successively for four times and then exposed once to a second odor (dishabituation odor). Each exposure lasted for 5 min. The inter-trial interval was 15 min. We prevented any odorant-specific effects by carrying out the test with three pairs of odors: octanal (Sigma) and (+)-terpinen (Interchim, Montluçon, France), (+)- and (–)-carvon (Fluka, Buchs Switzerland), butyraldehyde (Fluka) and isoamyl acetate (Fluka). The different odorant pairs were tested randomly in separate sessions at least 24 h apart. We considered that animals were able to discriminate between odors when they spent significantly more time exploring the new olfactory stimulus (dishabituation odor) during the fifth exposure than the familiar olfactory stimulus (habituation odor) during the fourth exposure.

BrdU injections

The DNA synthesis marker 5-bromo-2'-deoxyuridine (BrdU; Sigma) was dissolved in a sterile solution of 0.9% NaCl and 1.75% (0.4 N) NaOH that was injected intraperitoneally at a concentration of 50 mg/kg body weight. BrdU-containing cells were detected by immunohistochemistry after different survival times. A single dose of BrdU was given 4 h before killing the animal to assess proliferation. The animals were injected every 2 h four times before they were killed 21 days later to evaluate proliferation, migration and survival of newborn cells.

Immunohistochemistry

For all histological analyses that required tissue fixation by perfusion, the mice were deeply anesthetized with an overdose of sodium pentobarbital (100 mg/kg; Sanofi, Gentilly, France) and perfused transcardially with saline solution (0.9% NaCl) containing heparin (5×10^3 units/ml) at 37°C followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). Brains were removed and immersed overnight in the same fixative at 4°C. Immunohistochemistry was carried out on 40- μ m-thick free-floating coronal or sagittal sections cut using a vibrating microtome (VT1000S, Leica) and collected in PBS. Sections were first incubated at 4°C overnight with the following polyclonal or monoclonal antibodies: rabbit or mouse anti-TNC (clone pK7 or Chemicon, 1:200), mouse anti-PSA (AbCys, 1:200), mouse anti-NeuN (Chemicon, 1:200), rabbit anti-GFAP (Dako, Trappes, France, 1:500), mouse anti-GFAP (Chemicon, 1:1000) and rat anti-BrdU (Accurate Scientific, Harlan Sera-Lab, Loughborough, England, 1:200) antibodies. For BrdU immunostaining, the sections were pretreated with 0.2% Triton X-100 for 2 h, and DNA was denatured by incubation at 37°C for 30 min with 2 N HCl. Sections were then incubated at 4°C overnight with the anti-BrdU antibody, incubated at room temperature for 3 h with biotinylated donkey anti-rat IgG antibodies and then for 1 h in the avidin–biotin complex (ABC Kit, Vectastain Elite, Vector Laboratories, Burlingame, CA) followed by development with diaminobenzidine (DAB, 0.05%), to which 0.005% H_2O_2 had been added. Double and triple labeling immunofluorescence was carried out using the following fluorescent secondary antibodies: Alexa-568-labeled goat anti-rat IgG, Alexa-488-labeled goat anti-mouse IgG or IgM and Alexa 633 goat anti-mouse IgG1 (1:500, Molecular Probes, Poortgebouw, Netherlands). Sections were analyzed using either a standard microscope (BX51; Olympus, Hamburg, Germany) for peroxidase staining or a Zeiss confocal microscope (Carl Zeiss S. A. S., Le Pecq, France) equipped with Ar 488 nm, HeNe1 543 nm and HeNe2 633 nm lasers, using the LSM-510 software package for image acquisition and data analysis of fluorescently labeled tissue.

SVZ explant cultures

Cultures of SVZ explants were prepared as previously described (Wichterle et al., 1997). Briefly, brains from P8–P9 mice were removed and immersed in ice-cold HBSS medium (Gibco, Cergy Pontoise, France). The brains were cut in 200- μ m-thick coronal sections, and those containing the SVZ were selected for further preparation. Under a surgical microscope, the SVZ was dissected along the lateral wall of lateral ventricles, cut into small pieces (100 to 200 μ m in diameter) and then transferred to 70% Matrigel (BD Bioscience, CA, USA). After polymerization of the Matrigel by heating at 37°C for 10 min, we added neurobasal medium containing B27 supplement, L-glutamine (0.5 mM) and penicillin/streptomycin (1:1000) (all from Gibco). Cultures were maintained in a humidified, 5% CO_2 atmosphere incubator at 37°C.

Quantification and statistical analyses

All quantification was carried out blind to the mouse genotypes. For in vitro cell migration distance, the explants were examined using phase-contrast microscopy after 20 h of culture. Migration distance was determined as the maximum distance that cells had moved away from the perimeter of each explant. We counted BrdU-immunostained nuclei in every third 40 μ m section along the entire SVZ–olfactory bulb pathway. The number of newborn neurons in the olfactory bulb was assessed by counting the BrdU⁺ nuclei observed with 20 \times and 40 \times objectives for the entire granule cell and glomerular layers. We then related the number of BrdU⁺ nuclei to the area of the granule cell (including mitral cell and internal plexiform layers) and glomerular layers. For the RMS and SVZ, the density of BrdU⁺ cells was evaluated by relating the number of BrdU⁺ cells to the measured olfactory bulb surfaces occupied by these cells. The percentages of BrdU/NeuN double immunostained cells were obtained by analyzing 3D-reconstructed BrdU⁺ nuclei in the x – z and y – z orthogonal projections for the presence or absence of NeuN. Statistical analysis was by unpaired or paired Student's t tests.

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version at, doi:10.1016/j.mcn.2006.04.002.

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