Research Report

Slow age-dependent decline of doublecortin expression and BrdU labeling in the forebrain from lesser hedgehog tenrecs

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ABSTRACT

In addition to synaptic remodeling, formation of new neurons is increasingly acknowledged as an important cue for plastic changes in the central nervous system. Whereas all vertebrates retain a moderate neuroproliferative capacity, phylogenetically younger mammals become dramatically impaired in this potential during aging. The present study shows that the lesser hedgehog tenrec, an insectivore with a low encephalization index, preserves its neurogenic potential surprisingly well during aging. This was shown by quantitative analysis of 5-bromo-2′-deoxyuridine (BrdU) immunolabeling in the olfactory bulb, paleo-, archi-, and neocortices from 2- to 7-year-old animals. In addition to these newly born cells, a large number of previously formed immature neurons are present throughout adulthood as shown by doublecortin (DCX) immunostaining in various forebrain regions including archicortex, paleocortex, nucleus accumbens, and amygdala. Several ventricle-associated cells in olfactory bulb and hippocampus were double-labeled by BrdU and DCX immunoreactivity. However, most DCX cells in the paleocortex can be considered as persisting immature neurons that obviously do not enter a differentiation program since double fluorescence labeling does not reveal their co-occurrence with numerous neuronal markers, whereas only a small portion coexpresses the pan-neuronal marker HuC/D. Finally, the present study reveals tenrecs as suitable laboratory animals to study age-dependent brain alterations (e.g., of neurogenesis) or slow degenerative processes, particularly due to the at least doubled longevity of tenrecs in comparison to mice and rats.

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Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; Calb, calbindin; CR, calretinin; Cy, carbocyanine; DCX, doublecortin; GAD, glutamate decarboxylase; -ir, -immunoreactivity; NDS–TBS-T, 5% normal donkey serum in TBS, containing 0.3% Triton X-100; Parv, parvalbumin; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TBS–BSA, TBS, containing 2% bovine serum albumin; βIIIITub, βIII-tubulin

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1. Introduction

Neuronal proliferation in the developing central nervous system (CNS) is known to rapidly decline in the postnatal period. However, neurogenesis persists in the adulthood (for review, see Rakic, 2002; Taupin and Gage, 2002; Kempermann, 2006; Taupin, 2006). Neurogenesis was primarily shown for the granule cells of rodent dentate gyrus (Altman and Das, 1965; Gage et al., 1998; Ehninger and Kempermann, 2008) and olfactory bulb (Altman 1969; Graziaidei and Graziaidei, 1979; Gritti et al., 2002) that contains numerous neurons originating in the subventricular zone and migrating rostralwards along the rostral migratory stream (Lois and Alvarez-Buylla, 1994; Doetsch et al. 1997).

All major vertebrate taxa display neurogenesis in adulthood (García-Verdugo et al., 2002; Gould, 2007; Ihnwo and Pillay, 2007), which had been additionally shown for reptiles and in classical studies for birds, e.g., canaries repeatedly learning new songs (reviewed by Nottebohm, 2008). Remarkably, adult neurogenesis was also reported for nonhuman primates (Kornack and Rakic, 1999, 2001; Pencea et al., 2001, Bernier et al., 2002; Rakic, 2002) and man (Eriksson et al., 1998; Johansson et al. 1999).

Currently, the preferred method for the detection of neurogenesis is the incorporation of the exogenously applied thymidine analogue 5-bromo-2′-deoxyuridine (BrdU) during the S-phase of the cell cycle into newly synthesized DNA and its subsequent immunohistochemical detection. The microtubule-associated protein doublecortin (DCX; Gleeson et al., 1999) was reported to indicate neurogenesis (Brown et al., 2003; Couillard-Després et al. 2005), and it is a widely accepted marker for the detection of immature neurons (Ehninger and Kempermann, 2008; von Bohlen und Halbach, 2007). Such DCX-immunopositive, immature neurons were described for several brain regions from various mammalian species (see, e.g., Gómez-Climent et al., 2008; Luzzati et al., 2009; Zhang et al., 2009).

However, there are only restricted data on the time course of DCX-immunoreactivity (ir) in forebrain neurons during aging (Siwak-Tapp et al., 2007). In addition, to the best of our knowledge, comparable data on DCX-immunolabeling of immature neurons and neurogenesis are still lacking for mammals with poorly differentiated brains, particularly tenrecs (see also Lindsey and Tropepe, 2006). The lesser hedgehog tenrec (Echinops telfairi) classically considered an insectivore, but more recently grouped among the superorder Afrotheria (Murphy et al., 2001), does not display the same patterns as described for other mammals (Kornack and Rakic, 1999, 2001; Pencea et al., 2001, Bernier et al., 2002; Rakic, 2002) and man (Eriksson et al., 1998; Johansson et al. 1999).

In parallel, the whole neocortex was also devoid of labeled cells. Independent of their age, all tenrecs contained DCX-immunopositive cells and fibers in the nucleus accumbens (Figs. 2D and 3D) and in the amygdala (Figs. 2E, F, and 3F). In the paleocortex (Künzle and Radtke-Schuller, 2001), DCX-immunoreactive cells were detectable only in the granular layer of dentate gyrus, particularly in its subgranular zone, whereas none was found in the indusium griseum and the neocortex (Figs. 2G and 3G).

In the olfactory bulb (for morphological details, see Radtke-Schuller and Künzle, 2000), DCX-immunoreactive cells at the ventricular wall were densely arranged, showing a darkly stained cell body but sparsely visible processes (Figs. 2A and B, 3A and B). More distant from the ventricular wall, cells were less densely arranged, but displayed numerous long, mostly radially aligned, processes. No labeled cells were found in the outer fourth of the olfactory bulb. In the hippocampus (Künzle and Radtke-Schuller, 2001), DCX-immunoreactive cells were detectable only in the granular layer of the dentate gyrus, particularly in its subgranular zone, whereas none was found in the indusium griseum and the neocortex (Figs. 2G and 3G).

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2. Results

2.1. Qualitative findings of BrdU and DCX immunoperoxidase labeling

BrdU labeling of cell nuclei occurred throughout all layers and regions of the selected brain fields, i.e., olfactory bulb, paleo-, archi-, and neocortices, and was observed in animals from all age groups investigated. Labeled profiles lined the ventricular wall both in the olfactory bulb (Fig. 1A) and, more caudally, of the lateral ventricle (Fig. 1B). In the hippocampus, immunoreactive cells were typically found in the subgranular zone of the dentate gyrus in younger animals (Fig. 1C) as well as in old ones (Fig. 1D).

DCX immunostaining also revealed periventricular (and other) cells in the selected brain areas; this label was cytoplasmic and, thus, provided an impression of the morphology of the cells as exemplified for 2- and 7-year-old tenrecs (Figs. 2 and 3). While the distribution patterns of BrdU- and DCX-immunoreactive cells appeared similar, e.g., in the dentate gyrus, it differed in the paleocortex and neocortex.

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2.2. Age-dependent decrease but persisting BrdU-labeled profiles

In all four fields investigated, the number of BrdU-labeled profiles (as calculated for 1 mm³) was significantly higher in younger animals, i.e., at 2 or 3 years of age, than in older animals (Figs. 4A–F). No statistically significant differences in BrdU cell densities were found between animals older than 3 years of age, although a gradual decrease with the aging of the animals was evident. Similarly, the ratio of immunoreactive cells detected at the ventricular wall to all labeled cells in the selected area was
higher in younger than in older animals but showed no further significant decrease after the age of 4 years.

2.3. The number of doublecortin-immunoreactive cells decreases with age

The number of DCX-immunoreactive cells was significantly higher in younger (2- and 3-year-old) than in older animals in the investigated brain areas, including the olfactory bulb, paleocortex, and archicortex (Figs. 5A, B, C, and C). This age-dependent decrease was observed both for the total cell numbers and for the cells in the most densely packed fields, i.e., the densocellular layer and the subgranular zone, respectively (Figs. 5D and E). Noteworthy, DCX-immunoreactive cells were completely missing in the neocortex at any age.

2.4. Neurons coexpressing doublecortin- and BrdU-immunoreactivity

Double immunofluorescence labeling revealed that, within the rostral migratory stream, a large number of cells located near the ependyma displayed both BrdU- and DCX-ir as exemplified

Fig. 1 – Immunoperoxidase staining of BrdU in several regions of the tenrec forebrain exemplified for differently aged animals. (A) Strong, ventricle-associated labeling in the bulbous olfactorius (OB) and some sparsely scattered BrdU-immunoreactive cells in adjacent bulbar regions from a 2-year-old animal. (B) More caudally located sections from the same animal display an immunolabeled rim delineating the second ventricle. (C) Numerous cells in the hippocampal dentate gyrus are immunopositive in a 3-year-old animal, whereas in (D), apparently less BrdU-containing cells are visible in the hippocampus from a 7-year-old animal. Scale bar = 100 μm.

Fig. 2 – Immunoperoxidase labeling of doublecortin (DCX) in the forebrain from 2-year-old tenrecs. (A) In the olfactory bulb, a strong DCX-staining is found around the ventricle and in the granule cell layer. (B) The subventricular zone around the second ventricle is DCX-immunopositive, whereas the remaining tissue appears immunonegative. (C) In the hippocampus, heavily stained cells are revealed predominantly in the subgranular zone of dentate gyrus; also note the numerous immunoreactive fibers ascending toward the molecular layer and those in the dentate hilus. Other areas of the hippocampal formation such as subiculum and CA1-3 are devoid of DCX-ir. (D) In the nucleus accumbens, DCX-ir is detected in numerous small cells and fine fibers. (E) Immunoreactive perikarya and fibers in the paleocortex (to the left) and the amygdala. The latter region is shown at higher magnification in (F). Note the long fibers and small cells which are, however, more weakly stained than the allocated rim ensheathing the ventricle. Scale bars: in F (also valid for A–D): 100 μm, in E = 200 μm.
for a 3-year-old tenrec (Figs. 6A–A”). In the dentate gyrus, only a minor subpopulation of DCX-stained cells showed a BrdU immunosignal (Figs. 6B–B”).

2.5. Missing coexpression of doublecortin and various markers of adult neurons

Selected forebrain sections from 2- and 5-year-old tenrecs were subjected to double fluorescence labeling of DCX and each one of various neuronal markers. Fig. 7 summarizes the data obtained from the younger animals; largely identical findings were obtained on the older animals (not shown). Whereas the pan-neuronal marker HuC/D was only observed in DCX-immunonegative cells in the dentate gyrus (Fig. 7A), the paleocortex contained a small subpopulation of neurons coexpressing DCX and HuC/D (Fig. 7B). DCX-immunopositive cells were devoid of immunoreactivities for the pan-neuronal markers βIII-tubulin (Figs. 7C and D) and SMI 311 (Figs. 7E and F), for glutamate decarboxylase (GAD)67 (Fig. 7G), and for the calcium-binding proteins parvalbumin (Figs. 7H), calbindin (Figs. 7I and K), and calretinin (Figs. 7L and M). The lack of coexpression of these markers by DCX-immunopositive cells was found both in dentate gyrus (Figs. 7A, C, E, H, I, and L) and in paleocortex (Figs. 7B, D, F, G, K, and M).

3. Discussion

The main finding of the present study is that the lesser hedgehog tenrec, an animal with a poorly differentiated cortex (Rehkämper, 1981), preserves its capability to produce new cells throughout adult life.

3.1. Technical considerations

The DCX-immunolabeling had not been applied to tenrecs before. However, the specificity of the elected antibody for the detection of DCX has been demonstrated by Western blot analysis (Brown et al., 2003) and was verified by data from a variety of animal species (Gómez-Climent et al., 2008; Xiong et al., 2008; Cai et al., 2009; Luzzati et al., 2009).

As there are virtually no published data on the assessment of cytogenesis in the tenrec brain, the applied labeling protocols had to be developed specifically for the present study. BrdU was used to visualize mitotically active cells because this is the current standard method although BrdU immunolabeling does not allow to distinguish between cell proliferation, cell cycle re-entry, and DNA repair as summarized by Taupin (2007) discussing its pitfalls, limitations, and validation. It might also be that the protocol for the intraperitoneal BrdU application (four times each second day) could be further optimized in future studies, e.g., by considering different modes of pulse labeling (Striedter and Keefer, 2000). However, higher doses of BrdU, e.g., the saturating concentration of 200 mg/kg body weight (often used for studies in rodents), might be toxic (Taupin, 2007). Applying a lower dose of BrdU (e.g., 50 mg/kg instead of 100 mg/kg body weight) may reduce the risk that some of the obtained BrdU immunosignals are due to detection of DNA repair during apoptosis (Cooper-Kuhn and Kuhn, 2002) but also may cause insufficient labeling of mitotically active cells.
Assuming that the BrdU-labeled cells indeed represent (the descendants of) mitotically active cells (Taupin, 2007), there remains the question whether in our case, this cell proliferation generates neurons or glial cells, or both. As a low rate of gliogenesis has been reported in the adult and even senile rodent brain (Korr, 1982) and progenitor cells in the dentate gyrus of mice express glial fibrillary acidic protein (GFAP; Seri et al., 2001), we performed double immunostaining for BrdU and two glial marker proteins. Preliminary data show that BrdU immunopositive cells failed to coexpress the glial markers, GFAP and S100β, although many glial cells were labeled for GFAP and/or S100β (data not shown). The lack of double labeling for glial markers in BrdU-containing cells especially in the dentate gyrus cannot be fully explained presently. It might be that in tenrecs (in contrast to mice), the neuronal progenitor cells downregulate the expression of glial markers before entering the mitotic cycle. Likewise, newly generated glial cells may be very few and/or may rapidly die and thus could not be discovered in our study. Further research is required to elucidate this problem.

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### 3.2. Neurogenic potential of tenrecs

At the age of 2 or even 3 years, tenrecs show a large neuroproliferative potential, especially when compared to age-matched mammals of similar weight but higher encephalization index, i.e., to rats. Forebrain lateral ventricles can be considered as the richest source of stem cells in rodents (see, e.g., Doetsch et al., 1997; Xu et al., 2005) as well as in tenrecs (as indicated by BrdU label; cf. our Fig. 1). Unlike in rats, however, newly generated neurons/immature neurons (indicated by DCX expression) can be identified in considerable numbers even in old tenrecs, although the rate of neurogenesis evidently drops in older animals. Obviously, a large pool of newly born neurons is being generated in the olfactory bulb, hippocampus, and paleocortex.

In contrast to archi- and paleocortex, the tenrec’s neocortex was found to be devoid of neurons immunoreactive for the immaturity marker DCX. This finding is in apparent contrast to the presence of BrdU cells in the neocortex, since DCX expression is thought to be specific for early stages of neuronal

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**Fig. 4** — Quantification of age-dependent BrdU immunolabeling. Although BrdU-labeled profiles were observed even in older animals, a significant reduction was found between 3- and 4-year-old animals in the olfactory bulb (A), paleocortex (B), hippocampus (C), dentate gyrus of hippocampus (C′), and neocortex (D). The proportion of cells near the ventricular wall showed a similar decrease during aging both in olfactory bulb (E) and neocortex (F). In (A–D), percentages indicate the decrease of cell numbers with age, related to 2-year-old animals. Data are reported as mean±SD, *P<0.05.
differentiation following proliferation. However, newly generated neurons often seem to lack DCX expression in the neocortex of other mammals (Dayer et al., 2005); thus, our data do not exclude a plasticity potential of the tenrec neocortex associated with cell proliferation.

The present study reveals the strongest DCX-ir in the rostral migratory stream, dentate gyrus, and paleocortex in all age groups. These regions are known to contain DCX-immunoreactive cells in all mammals studied so far, e.g., in the rat (Nacher et al., 2001; Kempermann, 2006). Furthermore, the tenrec’s nucleus accumbens and amygdala contain DCX-immunopositive fibers and cells, which is in line with previous data on the nucleus accumbens in mice (Yang et al., 2004) and the amygdala in mice (Shapiro et al., 2008) and monkeys (Bernier et al., 2002; Zhang et al., 2009).

3.3. DCX-expressing cells in the tenrec’s forebrain

Whereas both BrdU incorporation and DCX expression are putatively associated with adult neurogenesis, our study revealed an imperfect correlation between the two markers. The BrdU-immunoreactive (i.e., supposedly newly-born) cells (i) were much less in number than the DCX expressing cells (i.e., supposed immature neurons), (ii) rather rarely coexpressed DCX, and (iii) were primarily found at the ventricular surface, whereas the DCX-expressing cells occupied distinct layers within the tissue proper. Nonetheless, both cell populations appear to be related to neurogenesis. First, the BrdU-immunopositive cells apparently failed to coexpress the glial markers glial fibrillary acidic protein (GFAP) and S100β (data not shown) such that they apparently did not represent mere glial cell proliferation. Second, DCX-ir reflects cytoskeletal dynamics typical for immature neurons (Gleeson et al., 1999). Moreover, as mentioned above, the DCX-expressing cells occurred predominantly in brain areas known to host adult neurogenesis in other mammals.

Hippocampal DCX-immunopositive cells are solely restricted to the dentate gyrus, which has been repeatedly emphasized as a brain field of neurogenic and plastic potentials (Kempermann, 2006; Ehninger and Kempermann, 2008). In comparison to the adult dentate gyrus of higher mammals (Nacher et al., 2001; Jin et al., 2004; von Bohlen und Halbach, 2007), the tenrecs appear to exhibit a much larger number of strongly DCX-immunoreactive cells.

The observation that there was only a partial colocalization of DCX with BrdU might be explained by the assumptions that cells (i) start to express DCX not before the end of the S-phase and (ii) continue to express DCX for a rather long period, during their migration into nonependymal layers and (partial) morphological differentiation (i.e., probably more than the 8–10 days over which BrdU was applied). Furthermore, the observation that the DCX-immunoreactive cells rarely coexpress mature neuronal markers suggests that newborn cells hardly and/or slowly differentiate into mature neurons.

The majority of DCX-immunoreactive cells are concentrated in layer II of the paleocortex. Very similarly shaped cells, called tangled cells with semilunar–pyramidal transition type, were recently found in the rat paleocortex (Gómez-Climent et al., 2008). Such immature neurons with a plastic phenotype were supposed to enter a dormant stage but not

![Fig. 5](image-url)
the final phase of their neuronal differentiation program in the same layer. In tenrecs, paleocortical DCX-immunopositive cells are not labeled with BrdU which suggest that they have been formed many days or even weeks ago (at least before application of BrdU) but resided in layer II to serve as a potential reservoir which, on demand, may complete their differentiation program. Whereas a minority of these cells indeed appeared to differentiate further—a few cells coexpressed DCX and the pan-neuronal marker HuC/D—most DCX-immunoreactive cells were devoid of other neuronal markers (SMI311 and βIII-tubulin) or markers indicating a GABAergic phenotype. Such DCX-expressing cells may represent ‘dormant immature neurons’ in tenrec brain, similar as suggested for the rat paleocortex (Gómez-Climent et al., 2008).

4. Experimental procedures

4.1. Animals

This study was performed with 22 lesser hedgehog tenrecs (Echinops telfari) of both genders from the breeding colony at the University of Munich. Usually, two to five animals were found that diminished adult neurogenesis in the common marmoset precedes old age (Leuner et al., 2007). Recently, three studies reported a drastic reduction of hippocampal DCX-ir in dogs (Siwak-Tapp et al., 2007; Pekcec et al., 2008; Hwang et al., 2009). Diminished neurogenesis and age-associated cognitive deficits seem to be correlated (Jessberger and Gage, 2008; Kempermann, 2006). By contrast, increased neurogenesis was reported for Alzheimer’s disease (Jin et al., 2004). Impaired adult neurogenesis was observed in triple transgenic mice with age-dependent β-amyloidosis and tau hyperphosphorylation, but the data on altered neurogenesis in animal models for aspects of Alzheimer’s disease remain conflicting (Rodriguez et al., 2008).

The observed age-dependent decline of BrdU-immunolabeling in tenrecs appears to occur slower and to be less dramatic than in most rodents. Rather, it resembles the recently observed delayed reduction of neurogenesis in long-lived Ames dwarf mice (Sun and Bartke, 2007). Neurogenesis in tenrecs, as possible laboratory animals, might be a valuable topic of future investigations especially of slow and long-lasting alterations, particularly if considering the at least doubled longevity of tenrecs in comparison to mice and rats.
housed in an aluminum cage containing at least one wooden nestbox (for details about the animals and their environment, see Künzle et al., 2007). The groups of 2-, 3-, 4-, and 5-year-old tenrecs comprised five animals each and two tenrecs were 7 years old. All animals were treated according to the European Communities Council Directive (86/609/EEC) and following the ethical guidelines of the laboratory animal care and use committee at the University of Munich. This project was approved by the administration of Upper Bavaria (license no. 55.2-1-54-2531-85-05).

Fig. 7 – Immunofluorescence double labeling of doublecortin (DCX and Cy2; green) and various neuronal markers (Cy3; red) in the subgranular zone of the dentate gyrus (A, C, E, I, and L) and layer II of the paleocortex (B, D, F, G, K, and M) from 2-year-old tenrecs. The pan-neuronal marker HuC/D is not visible in DCX-containing neurons in the dentate gyrus (A), whereas a few cells coexpress both markers in the paleocortex as indicated by their yellow color (B). On the other hand, neither in the dentate gyrus nor in the paleocortex DCX was coexpressed in cells immunoreactive for βIII-tubulin (βIIIITub; C and D), the neurofilament marker SMI311 (E and F), glutamate decarboxylase 67 (GAD67; G), parvalbumin (Parv; H), calbindin (Calb; I and K), and calretinin (CR; L and M). Scale bar = 50 µm.
4.2. BrdU injections

Prior injection of BrdU, the tenrecs were anaesthetized with intraperitoneally applied tribromoethanol (in pentanal 76948; both from Sigma, Taufkirchen, Germany; 1.0 mL/100 mg body weight). BrdU solution for the intraperitoneal injection was freshly prepared by dissolving BrdU (B5002; Sigma) in 0.9% saline. All tenrecs received four injections of BrdU (100 mg/kg body weight) each second day, e.g., on days 1, 3, 5, and 7 of the experiment.

4.3. Tissue preparation

Nine days after the first BrdU injection, animals were anaesthetized again intraperitoneally with tribromoethanol and perfused transcardially with saline followed by a fixative containing 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed immediately and immersed in the same fixative for 24 h, which was followed by immersion in sucrose (30%) for cryoprotection. Thirty-micrometer-thick serial sections were cut through the whole forebrain in the coronal plane on a frozen microtome. Sections from the obtained 10 series per animal were collected in 0.1 M Tris-buffered saline, pH 7.4 (TBS), containing sodium azide.

4.4. Histochemistry for light microscopy

A first series comprising each tenth section from all 22 tenrec forebrains used in this study was Nissl-stained with cresyl violet. Forebrains used in this study was Nissl-stained with cresyl violet. The omission of primary antibodies resulted in the expected absence of cellular staining. Double immunofluorescence labeling with switched fluorophores related to the relevant markers produced unaltered staining patterns.

4.5. Immunofluorescence labeling

Concomitant labeling of BrdU and DCX on selected sections from tenrec forebrains of differently aged groups was initiated by the pre-treatment of sections with 4 N HCl for 5 min. Next, the sections were blocked with NDS–TBS-T and incubated with a mixture of rat-anti-BrdU (1:1000 in NDS–TBS-T) and goat-anti-DCX (1:500) overnight. Rinsed tissue was then reacted with a cocktail of carboxyamine (Cy3)-conjugated donkey-anti-rat IgG and Cy2-tagged donkey-anti-goat IgG (both from Dianova; 20 µg/mL TBS–BSA) for 1 h.

Sections from two 2- and 5-year-old tenrecs were applied to the simultaneous detection of DCX and pan-neuronal markers (Hu/C/D, βIII-tubulin, and SM1 311, revealing non-phosphorylated neurofilaments), glutamate decarboxylase (GAD) as a marker enzyme of GABAergic neurons or calcium-binding proteins revealing neuronal subpopulations. Therefore, goat-anti-DCX (1:500 in NDS–TBS-T) was applied in mixtures with mouse antibodies directed against Hu/C/D (Invitrogen, Karlsruhe, Germany; 1:100), βIII-tubulin (Chemicon, Temecula, CA, USA, now available from Millipore, Billerica, MD, USA; MAB 1637; 1:150), or SM1 311 (Covance, Emeryville, CA; 1:500) and one of the following rabbit antibodies: anti-parvalbumin (Swant, Bellinzona, Switzerland; 1:500), anti-calbindin (Synaptic Systems, Göttingen, Germany), anti-calretilnin (Swant; 1:800), and anti-GAD67 (Chemicon; AB108; 1:500). The sections were then treated with mixtures of Cy2–donkey-anti-goat IgG and Cy3-conjugated antibodies directed mouse or rabbit, respectively (all from Dianova; 20 µg/mL TBS–BSA) for 1 h. Finally, most sections were rinsed, mounted, air-dried, and coverslipped with Entellan as described above. To quench the moderate autofluorescence, some sections were treated with Sudan Black B (according to Schnell et al., 1999).

All stained sections were extensively rinsed, mounted onto slides, air-dried, and coverslipped with Entellan in toluene (Merck, Darmstadt, Germany).

4.6. Microscopy and imaging

A conventional fluorescence microscope (Axioplan, Zeiss, Oberkochen, Germany) was used for the rough inspection of stained sections. Images from daiminobenzidine-stained sections were captured with a digital microscope camera Axio-Cam HCR running on Axiolab 3.1 software (both Carl Zeiss Vision, Jena, Germany) and adjusted in Adobe Photoshop 7.0 (Adobe Systems Inc., Mountain View, CA, USA). Selected, fluorescently labeled tissues were analyzed more detailed with a confocal laser scanning microscope LSM 510 Meta from Zeiss. The brightness of confocal laser scanning micrographs was occasionally slightly changed by Adobe Photoshop, but otherwise not altered.

4.7. Quantitative analyses

The densities of BrdU and DCX-immunoreactive cells in the olfactory bulb, paleocortex, hippocampus, and neocortex were calculated and compared.
quantified on immunoperoxidase-stained serial sections by means of Neurulucida™ system (MicroBrightField, Inc., Colchester, VT).

For each animal, six sections of the olfactory bulb, six sections of the neocortex starting cranially at the level of the anterior commissure, and five sections containing the hippocampus and paleocortex starting with the appearance of the granular layer were chosen for analyses. For all four fields of interest, the complete regions were outlined and labeled cells were counted using Neurulucida™ software. With respect to the section thickness, the number of profiles was calculated onto 1 mm³. Since cell numbers did not differ within age groups (P > 0.05, one-way ANOVA), data were pooled for each group. Subsequent two-sample comparisons were assessed using Student’s t-test or the Mann–Whitney U-test. Significance level was set at P < 0.05. Computations were performed using the SPSS 10.0 statistical package.

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