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ORIGINAL ARTICLE

Origin, migration and fate of newly generated neurons in the adult rodent piriform cortex

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Abstract Newly generated neurons are continuously added to the olfactory epithelium and olfactory bulbs of adult mammals. Studies also report newly generated neurons in the piriform cortex, the primary cortical projection site of the olfactory bulbs. The current study used BrdU-injection paradigms, and in vivo and in vitro DiI tracing methods to address three fundamental issues of these cells: their origin, migratory route and fate. The results show that 1 day after a BrdU-injection, BrdU/DCX double-labeled cells appear deep to the ventricular subependyma, within the white matter. Such cells appear further ventral and caudal in the ensuing days, first appearing in the rostral piriform cortex of mice at 2 days after the BrdU-injection, and at 4 days in the rat. In the caudal piriform cortex, BrdU/DCX labeled cells first appear at 4 days after the injection in mice and 7 days in rats. The time it takes for these cells to appear in the piriform cortex and the temporal distribution pattern suggest that they migrate from outside this region. DiI tracing methods confirmed a migratory route to the piriform cortex from the ventricular subependyma. The presence of BrdU/NeuN labeled cells as early as 7 days after a BrdU injection in mice and 10 days in the rat and lasting as long as 41 days indicates that some of these cells have extended survival durations in the adult piriform cortex.

Keywords Neurogenesis · Olfactory system · Doublecortin · Bromodeoxyuridine · DiI tracing

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Introduction

The olfactory system is one of the oldest and most well preserved mammalian sensory systems. Within the olfactory system, there are two regions where newly generated neurons continue to be added throughout adulthood, the olfactory epithelium and the olfactory bulbs. In the olfactory epithelium, neurons are generated locally from progenitor cells (Altman and Das 1966), and the mechanisms of proliferation have been well described (Beites et al. 2005). In contrast to these locally generated neurons, newly born neurons in the olfactory bulbs are generated in the subventricular zone and must migrate a relatively long distance along the rostral migratory stream (RMS) (Altman 1969; Bayer 1983; Lois et al. 1996). It should be noted that newly generated neurons in the olfactory bulbs of adults are found in many mammals (Luskin 1993; Lois and Alvarez-Buylla 1994), including non-human primates

(Pencea et al. 2001; Bedard et al. 2002), and in humans (Bedard and Parent 2004; Curtis et al. 2007).

A third region of the olfactory system, the piriform cortex, also displays newly generated neurons in the adult brain of several mammalian species, including rodents, rabbits and non-human primates (Bernier et al. 2002; Luzzati et al. 2003; Pekcec et al. 2006; Shapiro et al. 2007b). The piriform cortex makes up a majority of the olfactory cortex, receives a major portion of the olfactory bulb projection axons (Price 1973; Haberly and Price 1977; Carlen et al. 2002) and odors are transformed into odor maps in distinct regions of the piriform cortex (Zou et al. 2005). Moreover, olfactory enrichment in adult mice has been shown to enhance the survival and differentiation of newly generated neurons in the piriform cortex (Shapiro et al. 2007b), and also causes increased neurogenesis in the olfactory bulbs (Rochefort et al. 2002; Shapiro et al. 2007b). Adult neurogenesis in the olfactory bulbs is important in odor discrimination, indicating that the newborn neurons become integrated into functional circuitry (Gheusi et al. 2002). Although it is not known whether functional integration occurs for the newly generated neurons in the adult piriform cortex, it is known that in adult mice, they can survive for at least 21 days and display a mature neuronal phenotype (Shapiro et al. 2007b).

In neonatal mice, a ventrocaudal migratory stream was described as a branch of the RMS where cells migrate to the most rostral portion of the piriform cortex (De Marchis et al. 2004). In adult mice, this pathway persists to a lesser extent relative to the RMS and newly generated neurons are observed in several structures adjacent to the rostral portion of the piriform cortex (De Marchis et al. 2004). In contrast, a pathway that is separate from the RMS is present in adult monkeys and rabbits for migrating neurons generated in the subventricular zone to reach the piriform cortex (Bernier et al. 2002; Luzzati et al. 2003). It is possible that a similar pathway exists in adult rodents for newly generated neurons to migrate from the subventricular zone of the lateral ventricle to the caudal portion of the piriform cortex and its adjacent structures.

The purpose of the current study is to address three issues regarding newly generated neurons in the adult rodent piriform cortex. First, a time-course BrdU-analysis was combined with double-immunolabeling techniques to determine how long it takes for newly generated neurons to appear in the piriform cortex of adult rats and mice. This experiment addresses the issue of local vs. distant origin because cells generated from local progenitors will appear at earlier timepoints compared to those that must migrate into the piriform cortex. Then, in vivo and in vitro tracing techniques were used to map putative migratory pathways of the newly generated neurons in the rat piriform cortex. Finally, because most of the neurons generated along the rodent RMS degenerate and are not functionally integrated (Biebl et al. 2000; Winner et al. 2002), this experiment assayed the percent of newly generated neurons that survive and differentiate in the adult rat piriform cortex at 10,15, 28 and 41 days after the first of four daily BrdU-injections. Together, these studies provide the first comprehensive analysis of the origin, route of migration and fate of newly generated neurons in the adult rodent piriform cortex.

Materials and methods

Animals

Adult male, CD-1 mice (30–50 g; single BrdU-injection) and adult male, Sprague-Dawley rats (300–450 g; Simonsen, Gilroy, CA single and multiple BrdU-injection paradigms and in vivo DiI microinjections) and neonatal (P5–10; in vitro DiI) Sprague-Dawley rats were used. All rodent protocols were approved in advance by the Institutional Animal Care and Use Committees at the University of California at Irvine.

BrdU experiments to determine whether the newly generated neurons in the adult piriform cortex are born locally

Pulse-chase BrdU injection

A single BrdU injection (100 mg/kg; i.p.) was given to rats ($N = 4$ per timepoint) and mice ($N = 4$ per timepoint) at 1 day ($N = 4$), 2 days ($N = 4$), 3 days ($N = 4$), 4 days ($N = 4$), 5 days ($N = 4$), 6 days ($N = 4$) and 7 days ($N = 4$) prior to perfusions. The analysis of BrdU-labeled cells has been described previously for newborn neurons in the hippocampus (Parent et al. 2006). This approach was used to determine how long after a single injection of BrdU, cells double-labeled with BrdU/DCX or BrdU/NeuN, could be found in the piriform cortex. This analysis used both adult rats and mice to determine if species differences occur. Analysis of the piriform cortex was divided between the rostral and caudal portions of the piriform cortex, as described below.

In vivo and in vitro tracing studies to determine the migratory pathway of newly generated neurons from the subventricular zone

1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)

DiI was microinjected into the lateral ventricles of adult male, Sprague-Dawley rats (300–450 g; Simonsen). These

injections were performed to label putative precursor cells within the ventricular subependyma, and to trace their labeled offspring that emanate from the ventricles. Hamilton syringes were filled with the fluorescent lipophilic tracer DiI (Invitrogen, Carlsbad, CA) and the needle was placed into the wall of the lateral ventricle at the coordinates of −3.8 mm from bregma, 5.8 mm lateral from midline and 5 mm deep obtained from the atlas by Paxinos and Watson (1998). The rats were then perfused transcardially at 1–7 days following the microinjection surgery: 1 day (N = 3), 2 days (N = 3), 3 days (N = 3), 4 days (N = 4), 5 days (N = 4), 6 days (N = 4) and 7 days (N = 4). For each group of rats, sham animals that had saline injected into the same stereological coordinates were used. When a verifiable bolus of the tracer was not observed within, or along the walls of the lateral ventricle, the animal did not display any labeled cells in the migratory route to, and/or within, the piriform cortex. In addition, Cell Tracker Green (Chemicon, Burlingame, CA) was used (De Marchis et al. 2001) to confirm the DiI findings.

To confirm data on the ventrocaudal migratory stream in developing rodents (De Marchis et al. 2004), an in vitro DiI analysis was used in neonatal rats to visualize the migratory stream using real-time video microscopy. Coronal sections of postnatal rat brains (P5–10; N = 3–4 rat pups per timepoint) at a thickness of 350 μm were cut with a Vibratome. Slices were collected and transferred onto a piece of Whatman filter paper (0.45 μm pore size; 13 mm diameter). A small piece of DiI crystal (Invitrogen) was inserted into areas subjacent to the lateral ventricle. The slices were cultured with DMEM containing 10% FCS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in an incubator with 5% CO_2 for 1–2 h and then replaced with Neurobasal A (Invitrogen) supplemented with B-27 (Invitrogen), 2 mM L-glutamine, 10 mM glucose, 100 U/ml of penicillin and 100 μg/ml of streptomycin and further incubated. Slices were examined daily for cell movement from 1 to 7 days.

BrdU-injections to determine the fate of newly generated neurons in the adult piriform cortex

Five groups of rats (N = 4 per group) were given four daily injections of BrdU (50 mg/kg, i.p.) and allowed to survive for 10,15, 28 and 41 days after the first of the four BrdU injections. In addition, a control group (N = 5) was used that received four daily injections and were sacrificed 2 h after the last injection. The 10-day timepoint was chosen because single pulse BrdU data show that BrdU/DCX labeled cells do not appear in the caudal piriform cortex until 7 days. Therefore, we chose a timepoint after 7 days. Also, several BrdU injections were necessary to label a greater number of progenitor cells because (1) BrdU may only be bioavailable for 2 h after the injection (Cameron and McKay 2001), (2) the population of progenitor cells that we are examining appears to have a slow rate of proliferation (Belvindrah et al. 2002), and (3) the basal levels of neurogenesis in the region that we are examining appears to be very low (Nowakowski and Hayes 2000; Bauer and Patterson 2005; Elder et al. 2006). At the appropriate timepoints, rats were anesthetized with an overdose of Nembutal (50 mg/kg; i.p.) and then perfused intracardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffer. Brains remained intact within the cranium for 48 h at 4°C prior to removal, to allow for better fixation for electron microscopy (Shapiro et al. 2005). The number of BrdU-labeled cells was counted from every sixth coronal section containing rostral or caudal portions of the piriform cortex. This was done by randomly placing a 137 um^2 grid squares (N = 20 counts per animal) on sections containing the piriform cortex and counting the number of BrdU-labeled cells that fell within the counting frame. In addition, the number of BrdU-labeled cells that double-labeled for either DCX or NeuN was counted within the frame. The results are reported as total number of BrdU-labeled cells per 1,000 um^2, as well as the percent of BrdU-labeled cells that double-label for DCX or NeuN.

In addition, serial sections to those used for the above analysis were used for double-labeling with DCX and NG-2 to determine if any of the DCX-labeled cells expressed markers of an oligodendrocyte lineage, as has been shown for newly born neurons in the adult mouse neocortex (Dayer et al. 2005). Within the counting frame placed over these sections, the number of DCX and DCX/NG-2 double-labeled cells was determined.

Immunocytochemistry

Immunohistochemistry with BrdU and DCX antibodies was used as previously described (Shapiro et al. 2007a, b). Briefly, every sixth section in the coronal plane was chosen for DCX immunocytochemistry. The sections were processed using a standard avidin-biotin peroxidase technique and DCX antibodies (C & N termini: 1:200, Santa Cruz Labs, Santa Cruz, CA) as previously described (Rao and Shetty 2004; Ribak et al. 2004; Shapiro et al. 2007a,b). In addition, control sections were reacted without the primary DCX antibody to verify antibody specificity. No reaction product was observed at either the light or electron microscopic level in these control sections. Sections were mounted onto glass slides, dehydrated in graded alcohol baths and then coverslips were applied. In addition, adjacent sections were used for double-immunolabeling with anti-BrdU (Roche, Indianapolis, IN) employing a method previously described (Shapiro et al. 2007a). Because DCX clearly labels the perikaryal cytoplasm of immature

neurons and does not interfere with the imaging of the BrdU label found in the nuclei of cells, it was used in combination with BrdU immunolabeling for the quantitative analysis.

Polysialylated neural cell adhesion molecule (PSA-NCAM)

To further confirm the immature neuronal phenotype of the DCX-labeled cells, antibodies to PSA-NCAM (Chemicon, Temecula, CA) were used and double-labeling with BrdU was carried out as above, substituting the PSA-NCAM antibody for the DCX. This labeling method was the same as that described above except that an anti-rat IgG was used for the BrdU labeling and a fluorescent anti-mouse IgG antibody was used to label the PSA-NCAM.

Neuronal nuclei (NeuN; Chemicon, Temecula, CA)—Immunohistochemical examination for BrdU and the mature neuronal marker NeuN was performed to determine whether any of the BrdU-expressing cells had obtained a mature neuronal phenotype. For these studies, NeuN (1:2,000) and anti-rat BrdU (AbD Serotec, Raleigh, NC, 1:200) were used as the primary antibodies. For the secondary reaction, fluorescent anti-rat and anti-mouse IgG antibodies were used to label the BrdU and NeuN expressing cells, respectively as previously described (Shapiro et al. 2007b).

NG-2 Because Dayer et al. (2005) suggested that NG-2-labeled cells in the cortex might be generating newly born neurons locally, double-labeling with DCX and NG2 (Upstate USA, Chicago, IL) was carried out as above, with the anti-NG-2 incubation occurring simultaneous with the incubation for DCX. Fluorescent anti-rabbit IgG (Invitrogen, Carlsbad, CA) was used to label the NG-2 antibody.

Ki-67 To determine whether any of the DCX-labeled cells within the subcortical white matter, subjacent gray matter, or within the piriform cortex were mitotically active, Ki-67 (Vector Labs, Burlingame, CA) double-labeling was performed. Sections processed for either the NG-2 or Ki-67 antibodies, were examined at all of the timepoints (1–7 days for single BrdU injections; 10, 15, 28 and 41 days after four BrdU injections).

Immuno-electron microscopy

To determine the ultrastructural characteristics of DCX-labeled cells along this migratory stream, several 50 μm sections containing DCX-positive cells in the piriform cortex and SVZ of the caudal lateral ventricle were selected. These tissue sections were processed into plastic blocks for thin sectioning using a routine embedding procedure (Shapiro et al. 2005). A short series of ultrathin (60–80 nm) sections containing these two areas was cut with an ultramicrotome (Reichert-Jung, Ultracut E), and sequential sections were collected on mesh and formvar-coated slot grids and stained for contrast with uranyl acetate and lead citrate. Images of DCX-labeled cells were captured with a Gatan (Pleasanton, CA) UltraScan digital camera attached to a Philips CM10 transmission electron microscope.

Laser-scanning confocal microscopy

Fluorescent-tagged secondary antibodies (Alexaflour 488, 555 and 647; 1:200, Invitrogen, Carlsbad, CA) were used to allow simultaneous visualization of immunoreaction product using Argon and Helium/Neon laser lines. This method was employed to verify that the BrdU-labeled nucleus was surrounded by perikaryal cytoplasmic labeling for DCX, or within the NeuN-labeling, and not that BrdU was localized within a satellite cell as has been previously documented for neocortex (Kornack and Rakic 1999; Koketsu et al. 2003). Images were captured with a BioRad (Hercules, CA) Radiance 2100 laser scanning confocal microscope using lambda-strobing to avoid nonspecific cross-excitation or detection. Confocal Z-stack images were obtained and orthogonal images were used to confirm that BrdU-labeled nuclei were contained within DCX- or NeuN-labeled cells.

Distribution of labeled cells at 1–7 days following a single BrdU pulse

BrdU/DCX and BrdU/NeuN At 1–7 days after a single BrdU-injection, BrdU/DCX and BrdU/NeuN double-labeled cells were examined in the rostral portions of the piriform cortex [Mice = 2.34 to -0.94 mm from bregma, in Paxinos and Franklin (2000); Rat = 3.70 to −3.30 mm from bregma, in Paxinos and Watson (1998)] and in the caudal portions of the piriform cortex (Mice = −0.95 to −2.700 mm; Rat = −3.31 to −4.67 mm from bregma). For this analysis, the distribution of these double-labeled cells was plotted onto schematic diagrams of the brain. Quantitative analysis was not performed on these preparations because of the relatively small number of double-labeled cells observed after only 1 injection of BrdU. This same method was used to map the distribution of DiI-labeled cells in the rat caudal portions (−3.31 mm to −4.67 mm from bregma) of the adult rat brain.

Quantitative analysis

BrdU quantification To determine the numbers of BrdU/DCX cells relative to those cells that only label for BrdU, both types of cells were counted from every sixth 50 μm thick section containing the piriform cortex. The timepoints selected for this analysis were at 10, 15, 28 and 41 days after the first of four BrdU injections ($N = 4$ for each timepoint). A modified stereological procedure was used to count these cells. Images of the piriform cortex were first captured from every sixth 50 μm thick section. A 137 μm^2 counting frame was placed over the images at 20 random areas per animal, and all of the BrdU-labeled cells, BrdU/DCX and BrdU/NeuN double-labeled cells within each counting frame were counted.

Results

The results of this study suggest that the newly generated neurons of the adult piriform cortex arise from the subventricular zone of the lateral ventricle, migrate through subcortical white and gray matter structures to reach the piriform cortex, and some of these cells differentiate and survive in the piriform cortex for the 41-day duration of the study.

Temporal appearance of BrdU/DCX double-labeled cells in the adult piriform cortex to determine whether their origin is local or distant

BrdU/DCX

Rostral piriform cortex At this level through the rodent brain, BrdU/DCX double-labeled cells are observed ventral to the RMS at the short timepoints (1–3 days after a single BrdU injection) after a BrdU injection. At the longer timepoints analyzed (4–7 days after a single BrdU injection), these double-labeled cells were found in the piriform cortex, anterior olfactory nuclei, accessory olfactory bulb, olfactory tubercle and islands of Calleja (Fig. 1). It should be noted that in one section from a mouse at the 2 day survival timepoint (out of 72 sections examined from 4 animals), one example of a BrdU/DCX double-labeled cell was found at the base of layer iii of the piriform cortex at this rostral level. These data indicate that newly generated neurons (BrdU/DCX double-labeled cells) that migrate along the RMS exit via a ventral extension prior to reaching the olfactory bulbs.

Caudal piriform cortex In adult mice, BrdU/DCX double-labeled cells were first observed in the caudal piriform cortex beginning at 4 days after a single BrdU-injection. Beginning 2 days after the BrdU-injection, BrdU/DCX double-labeled cells first appear in the gray matter adjacent to the subcortical white matter, and within the deep endopiriform nuclei. The number of these double-labeled cells observed in both the deep endopiriform nucleus and piriform cortex increased with each successive day examined after a single-BrdU-injection (mean # of cells per section: 2 days = 2.4, 3 days = 3.7, 4 days = 4.1, 5 days = 4.9, 6 days = 5.5 and 7 days = 7.6 cells; $N = 6$ sections/animal).

In adult rats, BrdU/DCX double-labeled cells were first detected in the caudal piriform cortex at 7 days after a single BrdU injection. The number of these double-labeled cells at this timepoint was very small (less than 1 per section). Beginning 3 days after the BrdU-injection, BrdU/DCX double-labeled cells first appear in the gray matter adjacent to the sub-cortical white matter, and at 4 days after a single BrdU-injection, such cells are observed within the deep endopiriform nuclei (mean # of cells per section: 4 days = 3.2, 5 days = 4.5, 6 days = 6.1 and 7 days = 8.4 cells; $N = 6$ sections per animal). The number of these cells observed in this region increased with each successive day examined. Three-dimensional reconstructions of stacked images through the *Z*-axis of such cells in the deep endopiriform nuclei and the piriform cortex, as well as orthogonal images were used to confirm that the BrdU-labeled nucleus was contained within a DCX-labeled perikaryal cytoplasm, and not within satellite cells (Fig. 2). These findings were confirmed using BrdU and PSA-NCAM double-labeling.

BrdU/NeuN

Rostral piriform cortex In adult mice, BrdU/NeuN double-labeled cell types first appear in the rostral portion of the piriform cortex by 7 days after a single BrdU-injection. However, at this timepoint, there were very few (mean # of cells ≤ 1 per section) of these cell types observed. In adult rats, no BrdU/NeuN double-labeled cells were found in the rostral piriform cortex at 1–7 days after a single BrdU injection.

Caudal piriform cortex In adult mice, BrdU/NeuN double-labeled cell types first appear in the deep endopiriform nuclei beginning 5 days after a single BrdU-injection, and are observed in the piriform cortex beginning at 7 days after a single BrdU-injection. However, at these timepoints, there were very few (mean # of cells ≤ 3 per section) of these cell types observed. In adult rats, no BrdU/NeuN double labeled cells were found in the piriform cortex at 1–7 days after a single BrdU injection. These data show that newly generated neurons are found in the adult piriform cortex of mice before that of rats, and are

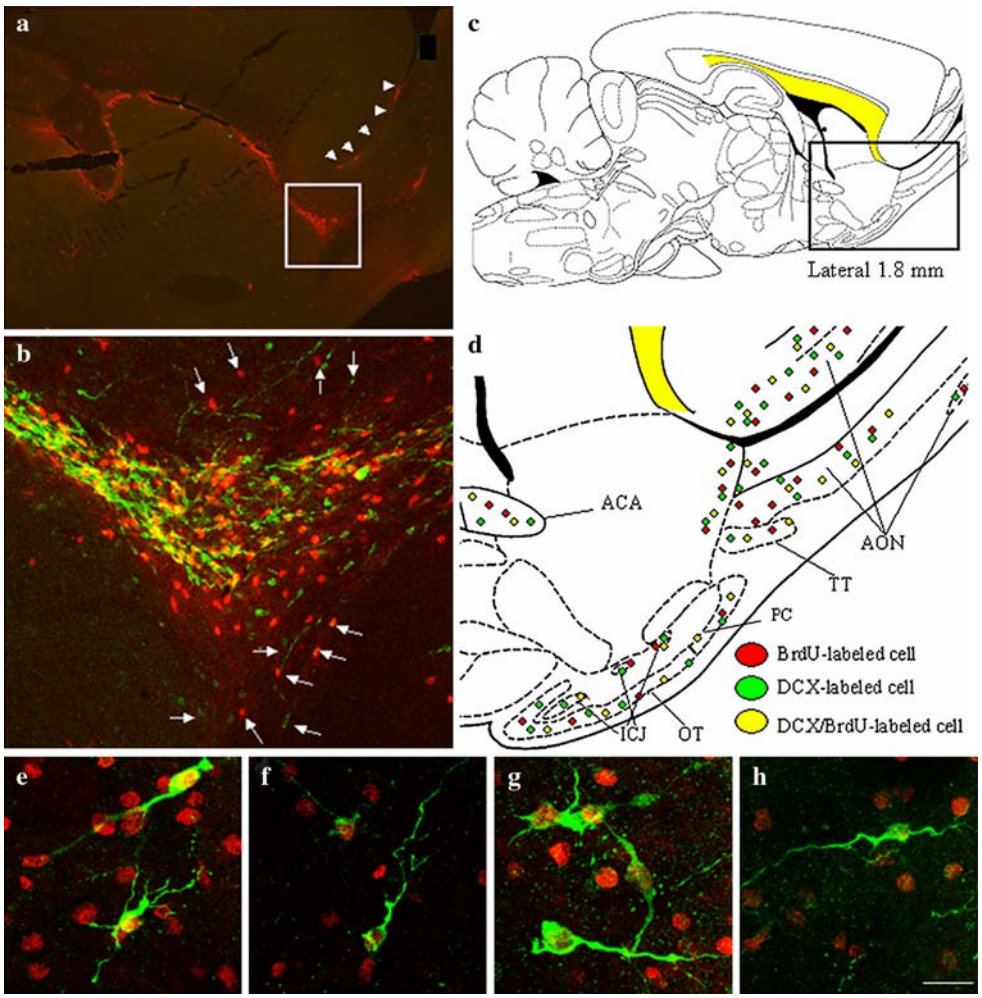

Fig. 1 The ventrocaudal migratory stream at the elbow of the adult rodent RMS. **a** Sagittal section showing BrdU-labeling throughout most of the extent of the RMS, originating at the lateral ventricle and extending into the olfactory ventricle. Note that the elbow of the RMS is highlighted by the *boxed area* and the *arrowheads* depict the pathway to the accessory olfactory bulb that also contains BrdU-labeled cells. **b** Shows the elbow of the RMS at higher magnification to illustrate the DCX, BrdU and DCX/BrdU double-labeled cells at this site. Note that some of these cells can be seen to exit in both dorsal and ventral (*arrows*) directions. **c** A schematic diagram adapted from Paxinos and Watson (1998) to show the location of the elbow (*boxed area*) of the RMS and the orientation of the section in **a**. **d** An enlargement of the boxed area from **c** showing the locations of BrdU, DCX, and BrdU/DCX-labeled cells in several anterior olfactory structures (*ACA* anterior part of the anterior commissure, *AON* anterior olfactory nuclei, *ICJ* islands of Calleja, *OT* olfactory tubercle, *TT* tenia tecta). The appearance of these newly generated cells in this migratory stream and destination sites extend the previous findings of De Marchis et al. (2004). **e–h** Confocal images of the BrdU/DCX double-labeled cells ventral to the RMS and within the ventrocaudal migratory stream. *Scale bar* in **h** = **a** 300 μm; **b** 60 μm; **e** and **g** 10 μm; **f** and **h** 15 μm

consistent with the longer time it takes for BrdU/DCX double-labeled cells to reach the piriform cortex in rats.

DCX/Ki-67

In the subventricular zone of the lateral ventricles there were clusters of DCX-labeled cells that also labeled for the mitotic marker Ki-67. This labeling pattern suggested that these double-labeled cells were neuroblasts, and several of them appeared together in clusters. Such clusters of neuroblasts were not found outside of the subventricular zone. However, individual and chains (continuous and non-continuous) of cells including those double-labeled for DCX/Ki-67 were found both, in the subventricular zone and in the subcortical white matter. Outside of these two areas none of the DCX-labeled cells were co-labeled with Ki-67. Therefore, these data indicate that neuronal precursors for the newly generated neurons in the caudal piriform cortex are found in the subventricular zone and its contiguous subcortical white matter.

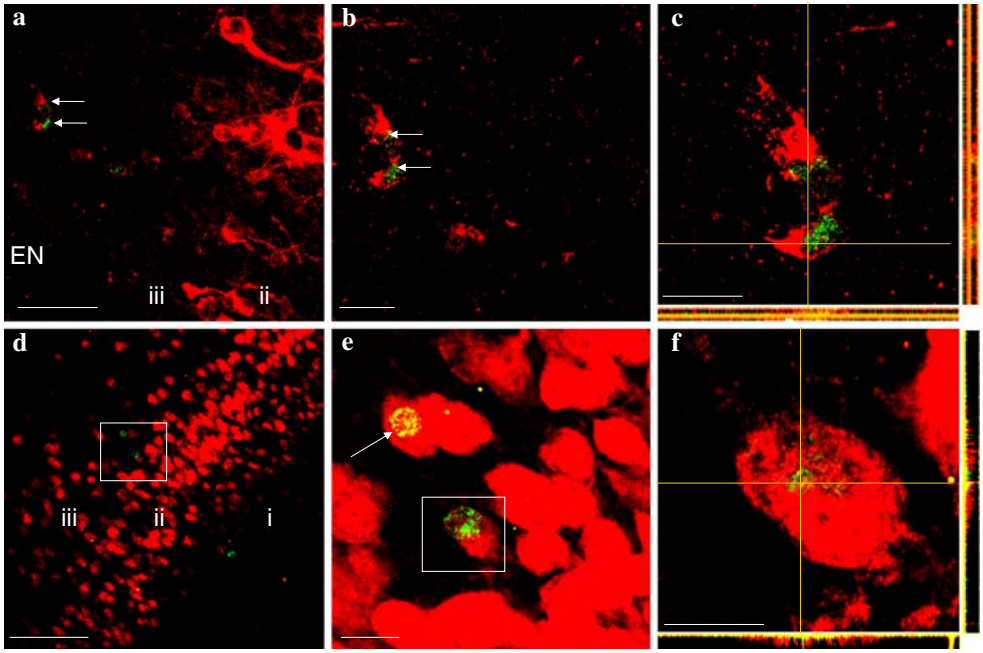

Fig. 2 Confocal Z-stack images show BrdU/DCX and BrdU/NeuN double-labeled cells in the deep endopiriform nucleus and the piriform cortex at 15 days after the first of four BrdU-injections in the adult rat. This timepoint was selected because the double-labeled cells were most abundant then. In **a**, a low magnification image shows DCX-labeled cells in layers ii and iii of the piriform cortex, as well as two BrdU/DCX-labeled cells (*arrows)* in the deep endopiriform nucleus (*EN*). These cells are enlarged in **b** and orthogonal views are presented in **c** to demonstrate that the BrdU-labeled nucleus is contained within the DCX-labeled perikaryal cytoplasm. In **d**, NeuN-labeled cells are shown in layers ii and iii of the piriform cortex. Note that in layer iii (*boxed region*) there are 2 BrdU-labeled cells. These 2 BrdU-labeled cells are enlarged in **e** to demonstrate that one of these BrdU-labeled cells is a satellite cell (*arrow*) and the other is a BrdU/NeuN double-labeled cell (*boxed cell*). The *boxed area* is enlarged in **f** and orthogonal views are presented to demonstrate that the BrdU-labeled nucleus is contained within the NeuN-labeled cell. *Scale bars* = 100 μm in **d**, 50 μm in **a**, 15 μm in **b** and **e**, and 10 μm in **c** and **f**

Tracer studies to determine whether a migratory stream exists between the SVZ and the caudal piriform cortex

In vivo DiI, adult rats

To test the hypothesis that DCX-labeled newborn neurons migrate from the caudal portions of the lateral ventricle, to the caudal piriform cortex, tracer studies were conducted. DiI was microinjected into the caudal portion of the lateral ventricle and allowed to survive for various durations. The results show that a stream of DiI-labeled cells was found in the subcortical white matter lateral, caudal and ventral from the injection site at 1 day after the injection (Fig. 3). At longer survival durations after the injection, the DiI-labeled cells were found further away from the injection site (Fig. 3). The labeled cells at these later timepoints formed a continuous and non-continuous stream of cells directed laterally and caudally. Some labeled cells left the stream to enter the adjacent striatum and layer VI of parietal and temporal cortex. Injection of DiI in sites that were outside of the lateral ventricle did not label any cells in this stream. Also, few, if any labeled cells were found in the contralateral hemisphere after an injection of DiI into the lateral ventricle.

In vitro DiI, P5–P10

To confirm the presence of a migratory route for cells generated in the caudal portion of the SVZ and migrating to the piriform cortex, an experiment using in vitro slices and time-lapse video and fluorescent microscopy was performed in early postnatal slices. The rationale for this is similar to the findings in the rostral piriform cortex where De Marchis et al. (2004) showed a robust migration along the ventrocaudal migratory stream at early postnatal ages and much less in the adult. The results show that a robust migratory route exists from the caudal portion of the lateral ventricle to the caudal piriform cortex in early postnatal rats (Fig. 4). Analysis of the furthest distance migrated for each successive day following DiI placement showed that labeled cells migrated as far as 200 μm from the lateral ventricle by 1 day and were located within the subcortical white matter. At 2 days, the labeled cells were observed to

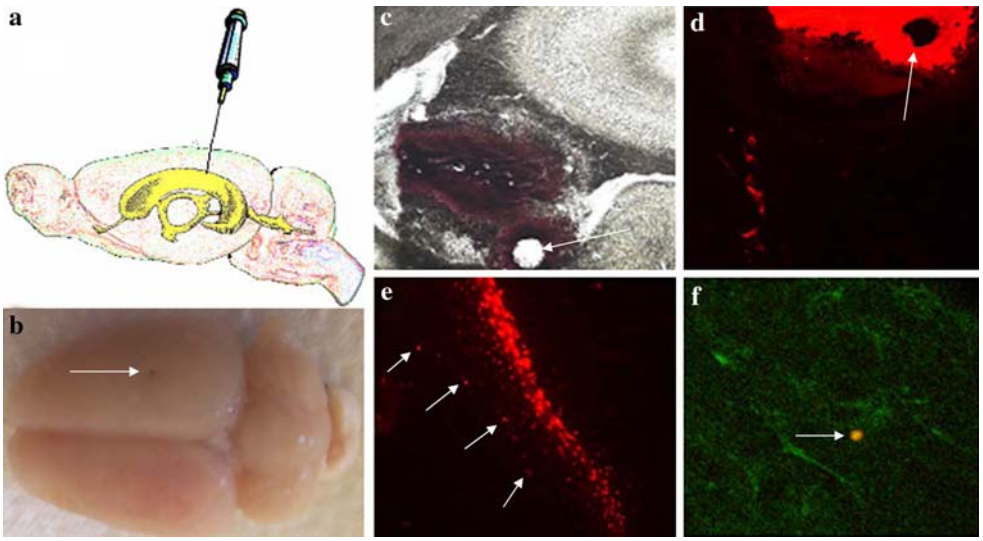

Fig. 3 Summary of the in vivo DiI labeling experiments. **a** Lateral view of the rat brain depicting the location where DiI was injected into the caudal portion of the lateral ventricle. **b** A view of the dorsal surface of a rat brain showing the actual site of DiI-injection (*arrow*). **c** The injection site is shown in a low magnification, brightfield, unstained section. The bolus of DiI is located in the fimbria lateral to the dorsal hippocampus and indicates that DiI went into the lateral ventricle. **d** Fluorescent image 3 days after DiI implantation showing DiI labeled cells that likely originated in the subventricular zone and are found ventral to the lateral ventricle. *Arrows* in **c** and **d** indicate the same needle hole in the fimbria. **e** At 5 days after the DiI infusion, DiI labeled cells form a stream of cells that is further away from the lateral ventricle and within the subcortical white matter. Note some DiI labeled cells (*arrows*) have left the white matter tract and are located in the adjacent gray matter. **f** At Day 7, a DiI-labeled cell (*arrow*) is first observed in layer ii of the adult, rat piriform cortex. DCX-labeled processes (*arrow*) are also shown in this high magnification image

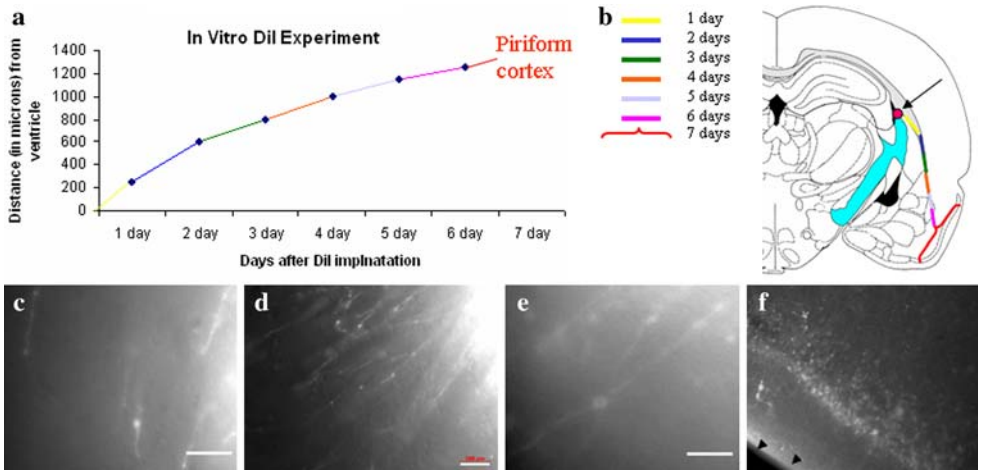

Fig. 4 Summary of the in vitro DiI tracing of the ventral migration of cells emanating from the subventricular zone of the lateral ventricle and migrating to the piriform cortex. **a** Line graph showing the distance that DiI labeled cells are found from the site of DiI placement in the lateral ventricle over a 7-day period. **b** Schematic diagram shows the location of the DiI placement (*arrow*) on the lateral wall of the lateral ventricle. The color lines (*see key*) represent the temporal migration of the DiI-labeled cells emanating from the lateral ventricle. **c** Fluorescent image obtained 1 day after the DiI insertion showing bipolar cells (*arrows*) that are about 200 μm from the lateral ventricle. **d** DiI-labeled bipolar cells are shown at 3 days after DiI placement. At this timepoint the DiI-labeled cells are found as far as 800 μm from the lateral ventricle. **e** High-magnification micrograph showing a DiI labeled bipolar cell that is located 1,000 μm from the lateral ventricle at 4 days after DiI placement. **f** The piriform cortex is shown at 7 days after DiI placement. Note the numerous DiI–labeled cells that are located in layers ii and iii of piriform cortex. The pial surface (*arrowheads*) is located at the lower left of the figure. *Scale bars* = **c** 125 μm, **d** 100 μm **e** 62.5 μm; **f** 250 μm

be as far as 600 μm from the lateral ventricle. Most of them were still in the ventral extent of the subcortical white matter, but some of these cells were located in the gray matter ventral to the subcortical white matter. At 3 days, the stream of DiI-labeled cells through the subcortical white matter was extremely dense and many cells were located in the gray matter ventral to the subcortical white matter at 800 μm from the lateral ventricle. At 4 days, DiI-labeled cells were seen as far as 1,000 μm from the lateral ventricle. Beginning at 5 days after the DiI implantation, some DiI-labeled cells were found at the border of layer iii of the piriform cortex. At 6 days, the first DiI-labeled cells were observed in the piriform cortex, and there was an increased population of such cells in the gray matter regions ventral and caudal to the lateral ventricles. By 7 days, many DiI labeled cells were found in the piriform cortex. These data are summarized in Fig. 4. Thus, both in vitro and in vivo DiI experiments reveal a similar route for newly generated cells to migrate from the caudal lateral ventricle to the caudal piriform cortex. This route will be referred to as the caudoventral migratory stream because it arises from the caudal portion of the lateral ventricle and extends ventrally.

Morphology of labeled cells along the caudoventral migratory stream

Large DCX-labeled cells with processes up to 1,000 μm long and spanning most of the width between the external capsule and the piriform cortex were observed in the endopiriform nucleus (Fig. 5). These cells are consistent with the previous description by Nacher et al. (2001). The cell bodies of these DCX-labeled cells were relatively large (20–50 μm) in diameter, and situated along the radial axis of the processes of these relatively larger DCX-labeled cells, were smaller DCX/BrdU-labeled cells (Fig. 5). The double-labeled cells in the endopiriform nucleus were mostly bipolar or multi-polar (occasionally unipolar cells were observed) and usually had at least one dendritic process oriented toward the piriform cortex (Fig. 5). Furthermore, 10 days after the first of four daily BrdU injections, BrdU/NeuN double-labeled cells were found throughout the endopiriform nucleus.

White matter regions also displayed BrdU/DCX double-labeled cells. These regions included the internal capsule, external capsule and the corpus callosum (Fig. 6). The distribution of BrdU/DCX-labeled cells in the subcortical white matter appeared along the lateral walls of the lateral ventricle (Fig. 6), extending to the most caudal and inferior regions of this structure (Fig. 6) as well as to the most rostral and ventral parts. Within these white matter and subcortical white matter regions, individual and chains of DCX, BrdU, and DCX/BrdU labeled cells were found (Fig. 6). These cells were similar in size to similarly aged, newborn neurons in the dentate gyrus (Fig. 6). The double-immunolabeled cells in the regions by the white matter had the morphology of migrating immature neurons in that leading and/or trailing processes were observed for many of them (Fig. 6).

Electron microscopic analysis was performed on DCX-labeled cells in the wall of the caudal lateral ventricle. The data for these cells revealed that they had the ultrastructural features of immature migrating cells (Alvarez-Buylla and Garcia-Verdugo 2002). For example, the perikaryal cytoplasm was labeled with electron dense immunoreaction product, and the short processes that emanated from the two poles ran parallel to the wall of the lateral ventricle (Fig. 6). The nuclei of these cells were ovoid and lacked immunolabeling (Fig. 6). The DCX-labeled cells lined up as continuous or non-continuous chains of cells between the white matter tract and the lumen of the lateral ventricle. Unlabeled cells were clearly distinguishable by their ultrastructural features and were identified as either ependymal cells or type B astrocytes (Alvarez-Buylla and Garcia-Verdugo 2002). The ependymal cells lined the lateral ventricles and were identified by their characteristic cilia and microvilli (Fig. 6). Astrocytes were identified by bundles of intermediate filaments within their translucent cytoplasm, and the cell bodies of these astrocytes were sometimes observed to be less than 1 μm from the cell bodies of DCX-labeled cells (Fig. 6). Consistent with previous studies, astrocytic processes were frequently surrounding both DCX-labeled and unlabeled cells (Doetsch et al. 1997, 1999; Yang et al. 2004). These observations are similar to those made by others for radial glial cells and their progeny in the rostral portion of the lateral ventricle (Alvarez-Buylla and Garcia-Verdugo 2002).

The piriform cortex of both rats and mice displayed DCX-labeled unipolar, bipolar and multipolar cells in layers ii and iii with labeled dendrites extending into layer i. DCX-labeled cells had immunolabeled dendritic processes that displayed growth cones with lamellipodia and filopodia indicating their immaturity as neurons (Fig. 7). DCX-labeled cells in layer iii had a bipolar morphology, were oriented perpendicular to the pial surface, and appeared to have trailing and lead processes, as well as elongated nuclei (Fig. 7). Electron microscopy of DCX-labeled cells in layer ii confirmed the presence of a thin shell of perikaryal cytoplasm that contained DCX-immunoreaction product (Fig. 7). No axosomatic synapses were observed for these DCX-labeled cells, and

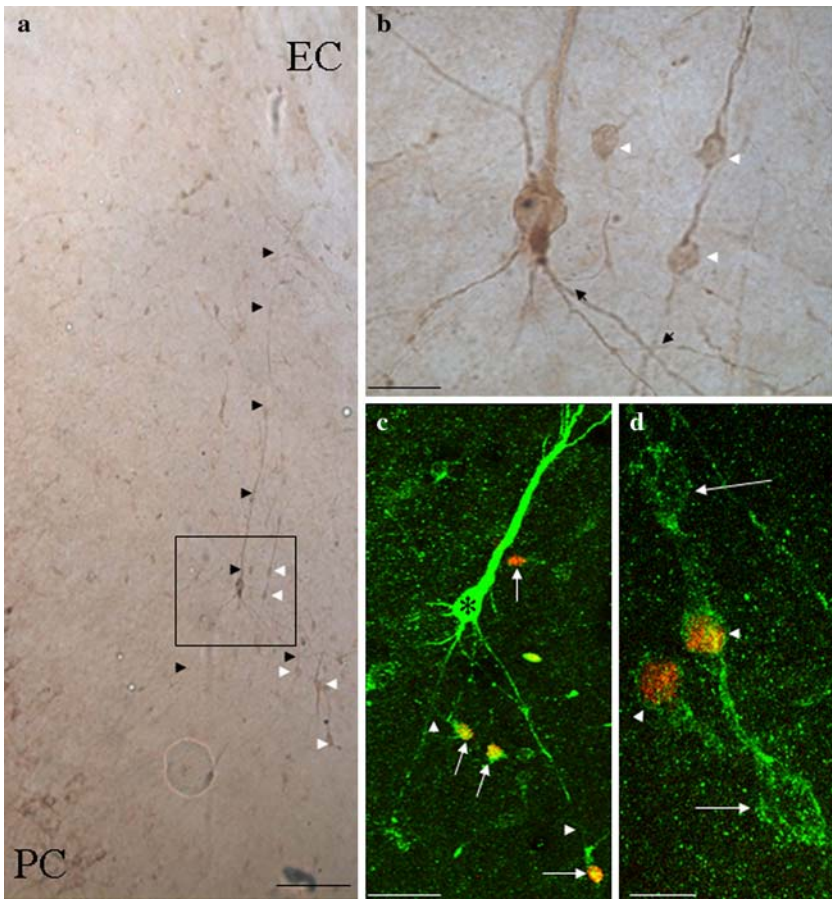

Fig. 5 Light and confocal micrographs of DCX and DCX/BrdU-labeled cells in the endopiriform nucleus and piriform cortex of adult rodents. **a** DCX-labeled cells with long (>1,000 μm) processes (*arrowheads*) spanning much of the distance between the subcortical white matter (*EC*) and the piriform cortex (*PC*). **b** shows the *boxed area* in **a** at higher magnification to reveal that smaller DCX-labeled bipolar cells (*arrows*) have one or both of their processes adjacent (*arrowheads*) to the large DCX-labeled cell. **c** Confocal microscopy confirms that some of the smaller DCX-labeled cells are double-labeled for BrdU (*arrows*). *Green* indicates DCX in dendritic and perikaryal cytoplasm while red indicates BrdU within nuclei. Note that the fine DCX-labeled processes of the BrdU/DCX double-labeled cells (*arrows*) are adjacent to the processes of the large DCX-labeled cell (*arrowheads*). **d** A chain of DCX and BrdU/DCX double-labeled cells in the endopiriform nucleus. Note that the middle 2 DCX-labeled cells have BrdU-labeling within their nuclei (*arrowheads*), while the DCX-labeled cells (*arrows*) located above and below these double-labeled cells do not have BrdU-labeled nuclei. *Scale bars* = **a** 75 μm; **b** 15 μm; **c** 60 μm; **d** 10 μm

processes of astrocytes apposed their somal surfaces (Fig. 7).

Fate of newly generated neurons in the piriform cortex

Analysis of the number of BrdU-labeled cells that double-label for DCX in rats showed that in the rostral piriform cortex at 10 days after the first of four daily BrdU injections, 44% of the BrdU-labeled cells were double-labeled for DCX (Fig. 8). At this timepoint, less than 1% of the BrdU-labeled cells were double-labeled for NeuN. At 15 days, 29.2% of the BrdU-labeled cells were double-labeled for DCX, and 8.2% were double-labeled for NeuN. At 28 days, 2.7% of the BrdU-labeled cells were double-labeled for DCX, and 5.5% were double-labeled for NeuN. At 41 days, 0.5% of the BrdU-labeled cells were double-labeled for DCX, and 0.7% were double-labeled for NeuN.

In the caudal piriform cortex, 47% of the BrdU-labeled cells were double-labeled for DCX at the 10 day timepoint (Fig. 8). At this timepoint, 1.3% of the BrdU-labeled cells were double-labeled for NeuN. Fifteen days after the first of four daily BrdU injections, 33.3% of the BrdU-labeled cells were double-labeled for DCX. At this timepoint, 13.1% of the BrdU-labeled cells were double-labeled for NeuN. At 28 days, 6.0% of the BrdU-labeled cells were double-labeled for DCX and 9.7% of the BrdU-labeled cells were double-labeled for NeuN. By 41 days, 3.8% of the BrdU-labeled cells were labeled for DCX and 6.4% of the BrdU-labeled cells were double-labeled for NeuN (Fig. 8). It is pertinent to note that at the 10-day timepoint,

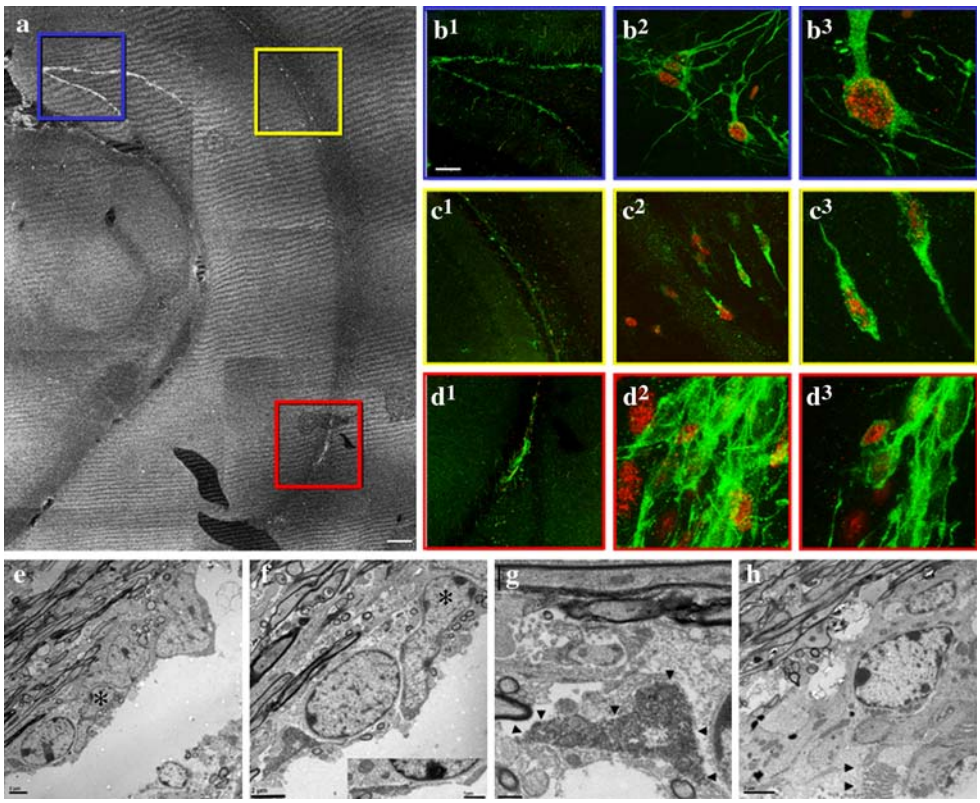

Fig. 6 Caudal and ventral portions of the caudoventral migratory stream. **a** Low magnification confocal photomontage showing DCX/BrdU double-labeled cells in the caudal portion of the adult rodent hippocampus and lateral ventricles. The hippocampus is used as a reference point as well as to compare the newly generated neurons. **b**^{1} Low magnification of the hippocampus showing DCX (*green*) and BrdU (*red*) labeled cells. **b**^{2} Higher magnification of the hippocampus showing the DCX-labeled cells (*green*) with BrdU-labeling (*red*) in their nuclei. **b**^{3} Enlargement of a single BrdU/DCX double-labeled cell in the hippocampal dentate gyrus showing its apical dendrite. **c**^{1} Enlargement of the upper *yellow box* showing DCX (*green*) and BrdU (*red*) labeled cells in the subcortical white matter of the caudal portion of the brain. **c**^{2} Shows that these cells are double-labeled with BrdU/DCX and have a lead and/or a trailing process. **c**^{3} Shows these cells at higher magnification to illustrate their morphology. Note that these elongated BrdU/DCX double-labeled cells have morphological features of migrating immature neurons. **d**^{1} The lower *red panel* in **a** shows DCX (*green*) and BrdU (*red*) labeling in the ventral portion of this caudal section of adult rat brain. **d**^{2} This region is shown at higher magnification to illustrate a cluster of cells with many BrdU and DCX-labeled cells. **d**^{3} shows that one of the cells in the cluster is a BrdU/DCX double-labeled cell. **e** Electron micrographs of a DCX-labeled cell (*asterisk*) from the same region of the caudal portion of the subcortical white matter depicted by the *yellow box* in **a**. Note the large numbers of myelinated axons (indicating the position in subcortical white matter) and DCX-labeling in the thin shell of perikaryal cytoplasm and its leading/trailing process. **f** An enlargement of the DCX-labeled cell (*asterisk*) and its leading/trailing process. The inset shows a portion of this process adjacent to an astrocyte. **g** An enlargement of the leading/trailing process (*arrowheads*) labeled with DCX-electron dense reaction product. **h** A low magnification view of the same region as **e–g** but only 50 μm away to show the microvilli of the ependymal cell (*arrowheads*). This cell type lines the lateral ventricles and confirms the location of this DCX-labeled cell. *Scale bar* in **a** = 160 μm; *Scale bar* in **b**^{1} = 80 μm for **b**^{1}, **c**^{1} and **d**^{1}; 16 μm for **b**^{2} and **c**^{2}; and 6 μm for **a**^{3}, **b**^{3}, **d**^{2} and **d**^{3}; *Scale bars* in **e**, **f** and **h** = 2 μm, in the *inset* of **f** = 1 μm, and in **g** = 0.5 μm

18% of the DCX-labeled cells were also labeled for NG2, compared to 12% at 15 days, 7.0% at 28 days and less than 1.0% at 41 days (Fig. 8).

Discussion

The experiments reported in this paper addressed the origin, migratory route and survival duration of newly generated neurons in the adult rodent piriform cortex. The data obtained address these issues and provide further information about this unique population of neurons.

Origin of newly generated neurons in the adult piriform cortex

The time-course BrdU analysis determined the time for newly generated neurons to appear in the rostral and caudal piriform cortex of adult rats and mice. The relative time (2 and 4 days for mice and 4 and 7 days for rats, respectively, see Table 1) it takes for them to appear there suggests that they migrate from outside of this region. Evidence in support of this hypothesis can be derived from adult neurogenesis studies in the dentate gyrus, an area where local progenitor cells are present. Several groups have

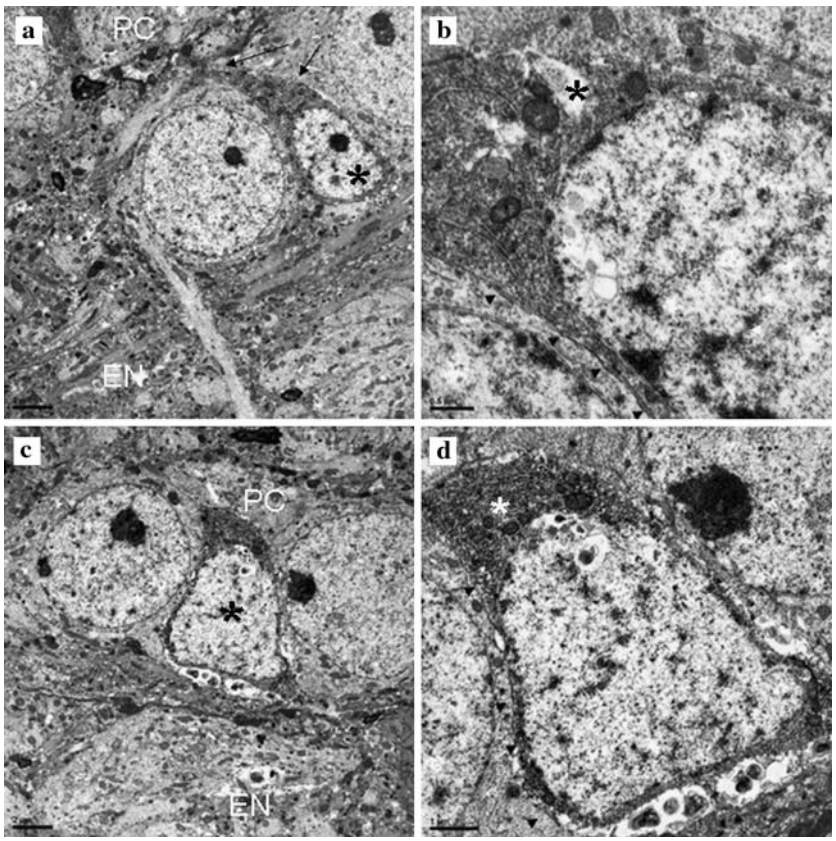

Fig. 7 Electron micrographs of DCX-labeled cells in the piriform cortex (*PC*) of adult mice. **a** shows a small DCX-labeled cell (*asterisk*) in between two larger unlabeled somata in the piriform cortex. Note that its DCX-labeled apical process (*arrows*) is oriented toward layer i. The apical portion of this cell is enlarged in **b** to show DCX-labeling in the soma (*asterisk*) and a thin glial process (*arrowheads*) separates the soma from the adjacent unlabeled cell body. **c** An example of a DCX-labeled (*asterisk*) bi-polar cell at the base of layer iii of piriform cortex (*PC*). This DCX-labeled cell is also sandwiched between 2 unlabeled cells. This cell is enlarged in **d** to demonstrate the DCX-labeling (*asterisk*) within its thin shell of perikaryal cytoplasm of both the apical and basal processes. Note the thin glial slip (*arrowheads*) that is adjacent to this DCX-labeled (*asterisk*) cell. *Scale bars* = 2 μm for **a** and **c**; 0.5 μm for **b**; 1 μm for **d**

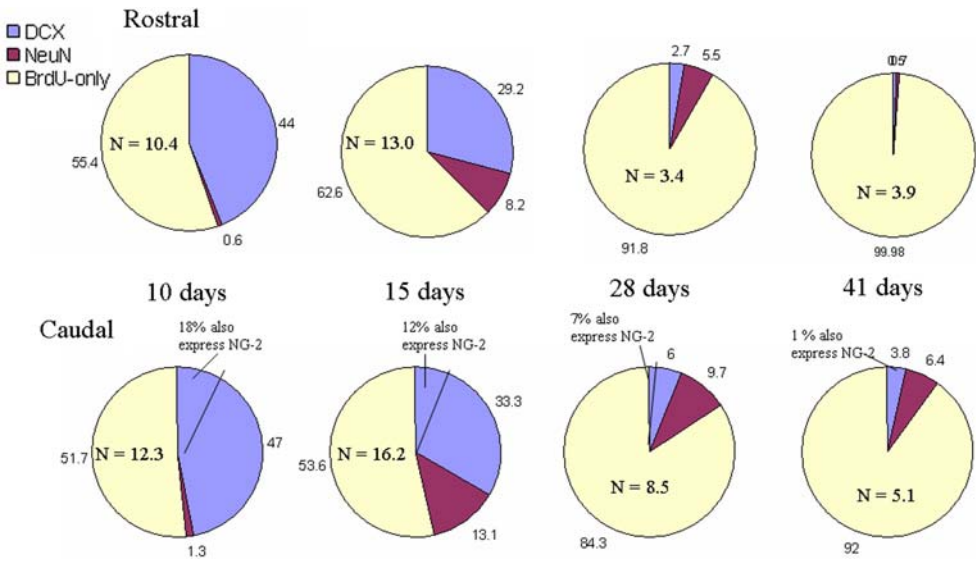

Fig. 8 Pie charts showing the percent of BrdU-labeled cells that also label for DCX or NeuN for both the rostral and caudal portions of the piriform cortex at 10, 15, 28 and 41 days after the first of four daily BrdU injections. Note that the number of BrdU-labeled cells per 2,500 um^2 is given in the area representing the BrdU-only labeled cells. The numbers outside of the pie charts are the percentages reported in the "Results" section of the text. Note, that a small percent of the DCX-labeled cells also express NG-2 at some of the timepoints examined

Table 1 Appearance of neurons in piriform cortex after a single BrdU-injection

	Mouse		Rat	
	Rostral	Caudal	Rostral	Caudal
Days to appear in piriform cortex				
BrdU/DCX	2	4	4	7
BrdU/NeuN	7	7	10	10

demonstrated DCX-labeled cells in the dentate gyrus after only 4 h following a BrdU injection (Kempermann et al. 2003; Shapiro et al. 2007a). If newly born neurons in the piriform cortex were generated locally, they would presumably express DCX prior to the times in which they do so in the present study. Further evidence for migration as opposed to local generation of newly generated neurons can be derived from the Ki-67 data. Ki-67 was used because it can detect local division and no DCX/Ki-67 labeled cells are found outside of the subcortical white matter. However, it needs to be emphasized that some of the DCX-labeled cells within the white matter are double-labeled for DCX and Ki-67 (See Supplemental data). This suggests that once the DCX-labeled cells exit the white matter, that they are no longer proliferative. In addition, the fact that no Ki-67 labeled cells are found in the piriform cortex suggests that progenitor cells are not present in the piriform cortex. Thus, these two results are extremely relevant to the question of local versus distant generation of neurons and provide further support for the migration of newly generated neurons into the piriform cortex from distant regions.

Of further interest regarding local generation as opposed to migration of newly generated neurons in the piriform cortex is the NG-2 data. NG-2 was used because Dayer et al. (2005) show that a sub-population of neurons expressing the immature neuronal marker CRMP4 in neocortex were double-labeled with NG-2, but none of the DCX-labeled cells were double-labeled with NG-2 in neocortex. They suggested that because NG-2 can label oligodendrocyte precursors that perhaps such cells were generating the newly born neurons locally. Alternatively, our analysis shows that a small population of the DCX-labeled cells in the endopirform nucleus double-label for NG-2. However, the lack of Ki-67 labeling for any of the DCX-labeled cells in this region suggests that these cells are not proliferative, and are instead probably newly generated cells that have not fully differentiated along a neuronal or oligodendrocyte lineage.

It is pertinent to note that Nacher et al. (2002) reported the appearance of a similar population of "small cells resembling migrating neuroblasts" that are labeled for PSA-NCAM in the piriform cortex, endopiriform nucleus and ventral end of the corpus callosum. They also showed that these smaller PSA-NCAM-labeled cells were situated adjacent to long PSA-NCAM-labeled processes. This is similar to the current study showing small DCX/BrdU double-labeled cells, situated along large DCX-labeled cells with processes spanning the distance between the subcortical white matter and the piriform cortex (Fig. 5). The discontinuous chains of cells in the migratory stream to the caudal piriform cortex contrasts with the continuous chains of cells described for the RMS (Wichterle et al. 1997), and this difference may provide a basis for the relative differences in the rate of neurogenesis and migration of newly generated neurons in these pathways. It should be noted that although Nacher et al. (2002) allude to the possibility that some of the PSA-NCAM-labeled cells in the piriform cortex might be newly generated, their study focused on the effects of an NMDA antagonist on PSA-NCAM expression in the piriform cortex and did not directly address the issues of origin and migration of these cells.

Migratory route for newly generated neurons to reach the rostral piriform cortex

Data from this and several other studies (De Marchis et al. 2004; Bernier et al. 2002; Luzzati et al. 2003) indicate that the progenitor cells for the newly generated neurons in the adult piriform cortex arise from the subventricular zone. Whereas the data from rabbits and monkeys are conclusive that the newly generated neurons of the adult piriform cortex arise from the subventricular zone of the lateral ventricle, it is less clear for rodents. The current dogma is that all of the cells generated in the rodent subventricular zone migrate rostrally to enter the RMS en route to the olfactory bulbs (see Sawamoto et al. 2006). However, a previous study using Cell Tracker Green in neonatal and adult mice showed that newly generated cells departed from the RMS at the "elbow" and coursed in a ventro-caudal migratory stream to enter the rostral piriform cortex (De Marchis et al. 2004). Data from the current study using DiI and BrdU/DCX double-labeling are consistent with the notion of a ventral migratory stream to the rostral piriform cortex.

Migratory route for newly generated neurons to reach the caudal piriform cortex

As indicated in the Results section, newly generated neurons appear in the rostral and caudal piriform cortex at different times after BrdU injections (appearing first in the rostral part). These data suggested that either a second migratory route exists for those newly generated neurons

destined for the caudal piriform cortex, or that the newly generated neurons in the rostral piriform cortex migrate to the caudal piriform cortex. Data from both in vivo and in vitro studies provide support for the first possibility and the proposed route is shown in a schematic diagram (Fig. 9). Because the origin (caudal portion of the lateral ventricles) and direction of this migratory stream is different than the ventrocaudal migratory stream to the rostral piriform cortex, another name was given to refer to it, the caudoventral migratory stream. It should be noted that a homologous migratory route has been reported in adult primates (Bedard et al. 2002; Bernier et al. 2002) where newly born neurons emanate from the temporal horn of the lateral ventricle to the deep portion of the temporal lobe in a migratory stream referred to as the "temporal stream" to reach the amygdala, piriform and inferior temporal cortices of monkeys (In primates, the piriform cortex becomes a temporal lobe structure). It is pertinent to note that in the current study, DCX/BrdU-labeled cells were observed in several amygdaloid nuclei that are adjacent to portions of the caudoventral migratory stream (data not shown).

Another important caveat of the current findings is that in vitro studies were performed on brain slices from neonatal brains and not on those from adult brains because the latter do not survive in culture long enough for the cells to reach the piriform cortex. The fact that both in vivo and in vitro DiI tracer studies show a similar migratory stream indicates that newly generated neurons in the adult rat migrate along an established migratory route found already in the neonatal brain. In this sense, the current data for the caudoventral migratory stream are similar to those for the ventrocaudal migratory stream described by De Marchis et al. (2004) in that it exists in both neonatal and adult mice.

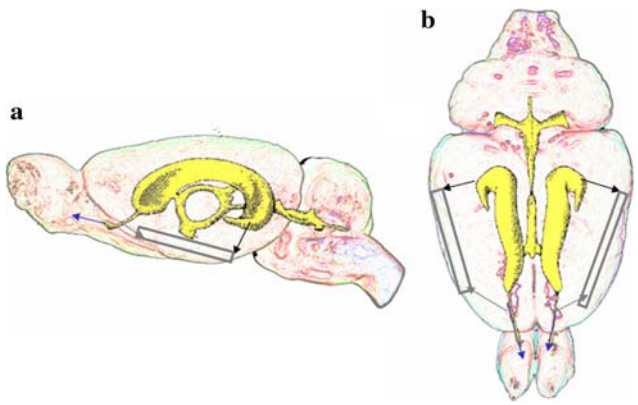

Fig. 9 Schematic diagram of the rodent brain showing the RMS (*blue arrow*), ventrocaudal migratory stream (*gray arrow*) and the caudoventral migratory stream (*black arrow*). **a** Lateral view showing the rostral migratory stream (*blue arrow*) heading toward the olfactory bulb. Extending from this stream is a smaller stream, the ventrocaudal migratory stream (*gray arrow*), that goes to the rostral portion of the piriform cortex (*gray rectangle*). In contrast, the caudal portion of the lateral ventricle has a migratory route to the caudal portion of the piriform cortex (*black arrow*), the so-called caudoventral migratory stream. **b** Dorsal view of the rodent brain to illustrate these same migratory streams using the same color coding

Survival and differentiation of newly generated neurons in the adult piriform cortex

Newly generated neurons were examined at 4 timepoints (10, 14, 28 and 41 days) after a series of 4 daily BrdU injections. The fact that by 41 days, almost no newly born neurons (those with NeuN) persist in the rostral portions of the piriform cortex, but some of these cells do survive up to 41 days in the caudal piriform cortex is an interesting finding. Shapiro et al. (2007b) have shown that olfactory enrichment enhances the survival of newly generated neurons in the adult mouse piriform cortex. Thus, it is possible that novel olfactory stimuli are necessary for the long-term survival and integration of newly generated neurons in the adult piriform cortex. Evidence in support of this hypothesis can be seen in the hippocampus, where learning and memory, as well as other novel stimuli such as exercise and environmental enrichment can enhance the differentiation and survival of newly generated neurons (Kempermann et al. 1997, 2003; Cameron and McKay 2001).

It is also possible that functional differences exist for the rostral and caudal portions of the piriform cortex. Evidence supporting this idea can be derived from the data showing that the newly generated neurons take two separate routes to the rostral and caudal portions of the piriform cortex, and the survival and differentiation of the two populations of neurons are different. Several other studies support the assumption of a rostro-caudal heterogeneity in the piriform cortex which could sustain differential information processing and underlie selective degeneration following pilocarpine-induced seizures (Datiche et al. 2001; Chen et al. 2007; Sugai et al. 2005). Therefore, the differences involving newly generated neurons in the rostral and caudal portions of the piriform cortex may be linked to these other findings involving odor processing, rostro-caudal heterogeneity, or both.

Another interesting observation is the persistence of small numbers of BrdU/DCX double-labeled cells in the caudal piriform cortex at the 41-day timepoint indicating that not all of the cells have differentiated into mature neurons (express NeuN) by this time. DCX is expressed in immature, migrating neurons (Francis et al. 1999; Brown et al. 2003; Rao and Shetty 2004). However, there is a relatively large population of DCX-labeled cells in the piriform cortex that do not label with BrdU, even after several consecutive injections with BrdU (Nacher et al.

2001; Shapiro et al. 2007b). One explanation for this low level of BrdU/DCX double-labeling is that the rate of neurogenesis in the caudal portions of the ventricular subependyma may be low. If these progenitor cells divided only once every two days, daughter cells that are born 10 days after the first BrdU injection would still be labeled with BrdU (assuming the progenitor cell is labeled on the 4th day of BrdU injections). Considering a 10 day migration to the piriform cortex in rats, such cells would not appear in the piriform cortex before 20 days after the first BrdU injection. In the hippocampal dentate gyrus, DCX-labeled cells persist for up to 3 weeks after they are generated (Kempermann et al. 2003). Thus, the presence of DCX-labeling for 21 days after newly generated neurons reach their destination in the caudal piriform cortex suggests that process outgrowth and differentiation are still occurring for this population of cells. The extended period of time for the full development and differentiation of the newly generated neurons in the adult piriform cortex may explain why previous studies did not consider these cells as newly generated.

In conclusion, BrdU/NeuN double-labeled cells in the adult piriform cortex are observed at later timepoints than the initial appearance of BrdU/DCX double-labeled cells (see Table 1). The fact that no BrdU/NeuN double-labeled cells are found at earlier timepoints suggests that the newly generated neurons in the adult piriform cortex differentiate into mature neurons in this brain region. These findings are consistent with the results from two previous studies in adult rodents (Pekcec et al. 2006; Shapiro et al. 2007b). Therefore, the origin of these cells is in the ventricular subependyma, and they migrate either via a ventrocaudal or caudoventral route to reach the piriform cortex.

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