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# NEWLY-GENERATED CELLS FROM THE ROSTRAL MIGRATORY STREAM IN THE ACCESSORY OLFACTORY BULB OF THE ADULT RAT

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**Abstract**--Cell proliferation in the accessory olfactory bulb of the adult rat was analysed after systemic injection of 5-bromo-2'-deoxyuridine, detected immunocytochemically at different survival times and compared with proliferating cell nuclear antigen immunostaining. As previously described in the main olfactory bulb, local cell proliferation was absent or very limited. By contrast, starting from 15 days after bromodeoxyuridine administration, many immunoreactive nuclei were present in the granular layer, and to a lesser extent, in other layers of the accessory olfactory bulb. This suggests that the newly-generated cells are migrating elements of the rostral migratory stream which are known to reach the olfactory bulb in 15 days.<sup>21</sup> By immunocytochemical detection of the polysialylated isoform of the neural cell adhesion molecule, a weakly-adhesive cell-surface molecule expressed by newly-generated/migrating cells of the rostral migratory stream,<sup>5,33</sup> we found a high number of immunoreactive cells in the different layers of the accessory olfactory bulb. Most of these cells were observed in the granular layer and showed the morphology of migrating neuroblasts. Some immunoreactive cells displaying neuronal morphology were also detected in the external plexiform and glomerular layers. Double labelling experiments demonstrated that these cells are newly-generated cells.

These results demonstrate the occurrence of newly-added cells in the accessory olfactory bulb of the adult rat, which likely correspond to the neuronal precursors originating from the rostral migratory stream. This could be relevant since the accessory olfactory bulb of rodents plays an important role in the hard wiring of a simple olfactory memory system for sexual pheromones.

*Key words:* brain, plasticity, cell migration, polysialic acid, bromodeoxyuridine, pheromones.

Neurogenesis in the olfactory bulb of rodents persists far beyond the post-natal period.<sup>1,3</sup> Recently, this neurogenesis has been related to the migration of cells generated in the subependymal layer of the lateral ventricle (SEL, according to the Boulder Committee terminology<sup>6</sup>) that subsequently move towards the main olfactory bulb (MOB) to differentiate into granule and periglomerular cells.<sup>21,23</sup> Interestingly, these cells, as well as other newly-generated cells in the adult brain,<sup>37</sup> express the highly sialylated, weakly

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*Abbreviations:* AOB, accessory olfactory bulb; BrdU, 5-bromo-2'-deoxyuridine; BSA, bovine serum albumin; DAB, 3-3'-diaminobenzidine; EPL, external plexiform layer; GL, glomerular layer; GrL, granular layer; HRP, horseradish peroxidase; LOT, lateral olfactory tract; MOB, main olfactory bulb; N-CAM, neural cell adhesion molecule; PCNA, proliferating cell nuclear antigen; PSA, polysialic acid; PSA-N-CAM, polysialylated isoform of N-CAM; SEL, subependymal layer; TBS, Tris-buffered saline.

adhesive isoform of the neural cell adhesion molecule (N-CAM) i.e. PSA-

NCAM, which is usually abundant during development, and acts as a permissive factor in neural morphogenesis (see for example Refs 13, 31). In particular, PSA-NCAM is expressed by proliferating and migrating cells (also referred to as the rostral migratory stream) in the SEL of the lateral ventricle and in its rostral extension to the olfactory bulb, and by subpopulations of the granule and periglomerular cells in the MOB.<sup>5,33</sup> These studies demonstrated that, in the forebrain, PSA-NCAM represents a cell surface molecule specifically associated to cells of the rostral migratory stream, that can be used to identify the newly-generated elements moving to the olfactory bulb (see also Refs 22, 29).

By contrast, cell proliferation in the accessory olfactory bulb (AOB) is thought to be restricted to the early post-natal period.<sup>3</sup> The AOB is a layered structure located in the dorsal and medial portion of the olfactory bulb and innervated by fibres originating in the vomeronasal organ,<sup>10,20</sup> which are renewed, as in the MOB, throughout life. The vomeronasal system is present in most terrestrial vertebrates, including man.<sup>27</sup> In rodents, it plays an important role in modulating behavioural responses elicited by pheromones. In particular, female mice form an olfactory memory of male pheromones at mating, and the synaptic changes underlying this memory occur in the first relay of the system, the AOB.<sup>2,8</sup>

In the present study, we have analysed cell proliferation in the AOB by intraperitoneal injection of 5-bromo-2'-deoxyuridine (BrdU), detected immunocytochemically at different survival times to identify the migrating cells into the migration pathway and the olfactory bulb. Moreover, we detected the proliferating cell nuclear antigen (PCNA), a peptide functioning both in DNA replication and repair as a subunit of DNA polymerase, which can be used as a specific marker for entry into cell division (see for example Ref. 44). Finally, using a monoclonal antibody that specifically recognizes polysialic acid (PSA) on N-CAM,<sup>32</sup> we investigated the presence of PSA-NCAM within the AOB, and in double labellings its co-occurrence with BrdU positivity.

## EXPERIMENTAL PROCEDURES

### *Tissue preparation*

Tissues were derived from 12 female adult Wistar rats (three- to six-months-old) purchased from Stefano Morini (Italy) and maintained in a controlled environment (14 h light/10 h dark) with food and water *ad libitum*. All experiments were performed in accordance with current EU and Italian law, under authorization of the Italian Ministry of Health, n. 600.8/24433/82.20/AG1826. Animals were deeply anaesthetized with intraperitoneal sodium pentobarbital (Pentothal Sodium, Gellini, Italy; 60 mg/100 g i.p.) and then perfused intracardially first with an heparinized saline solution (25 IU/ml in 0.9% NaCl, for 2–3 min) followed by a freshly prepared solution of 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, or the same fixative with the addition of 0.8% picric acid. After dissection, brains fixed in paraformaldehyde only were post-fixed overnight in the same fixative, cryoprotected in ascending sucrose solutions, frozen in liquid nitrogen-cooled isopentane at -70 C, and cryostat sectioned in series. Brains fixed in paraformaldehyde and picric acid were post-fixed in the same fixative for two

additional hours at room temperature and cut in series on a Vibratome, or dehydrated and embedded in paraffin wax. Coronal, parasagittal and horizontal Vibratome sections (75  $\mu$ m) were collected in 0.1 M Tris-buffered saline (TBS) to be processed as free-floating sections. Cryostat and paraffin sections (10  $\mu$ m) were collected onto poly-L-lysine (Sigma, St Louis, U.S.A.)-coated slides.

### *Bromodeoxyuridine labelling*

Ten rats were injected intraperitoneally with 2 mg BrdU/100 g body weight in 0.1 M Tris, pH 7.4. Animals underwent one or two subsequent BrdU administrations, separated by an interval of 24 h; then were perfused intracardially, after 1 h, six, 15 or 30 days survival.

### *Antibodies*

The following primary antibodies were used:

- (1) anti-PSA-NCAM, a monoclonal mouse immunoglobulin M (IgM) raised against the capsular polysaccharides of meningococcus group B that share  $\alpha$ -2,8-PSA residues with PSA-N-CAM (for further details on production and specificity, see Ref. 32); it was used at a dilution of 1/4000;
- (2) anti-bromodeoxyuridine (anti-BrdU, Boehringer, Germany), a monoclonal mouse immunoglobulin G; it was used on cryostat sections at a dilution of 1/100;
- (3) anti-PCNA, a monoclonal mouse immunoglobulin G (Dako, DK; for further details on its production and specificity, see Refs 14,24); it was used on paraffin sections at a dilution of 1/200.
- (4) anti-glial fibrillary acidic protein (GFAP, polyclonal, Dako, DK 1/500) All antibodies were diluted in TBS containing 0.25% bovine serum albumin (BSA). For primary antibodies used on Vibratome sections, 1% Triton X-100 was added in the diluent.

### *Immunocytochemistry*

Single immunostaining for PSA-NCAM was carried out on freely-floating Vibratome sections first incubated in 1% BSA in TBS for 1 h to block non-specific binding sites, followed by incubation in the anti-PSA-NCAM antibody for 48 h at 4° C. To reveal immunoreactivity, affinity purified anti-IgM immunoglobulins coupled to horseradish peroxidase (IgG-HRP, Sigma, U.S.A.) were used, at a dilution of 1/50.

Single immunostaining for BrdU was carried out on cryostat sections. These were treated with 2 M HCl for 1 h at 37° C, neutralized and subsequently incubated overnight at 4° C with the primary anti-BrdU antibody, followed by a biotinylated anti-mouse IgG (Vector, U.K.), diluted 1/200.

Single immunostaining for PCNA was carried out on paraffin sections. These were incubated overnight at room temperature in the anti-PCNA antibody, followed by a biotinylated anti-mouse IgG (SPA, Italy), diluted 1/200.

To reveal immunoreactivity, we used either 0.1% 3,3'-diaminobenzidine (DAB) and 0.01% H<sub>2</sub>O<sub>2</sub> or glucose oxidase-nickel-DAB<sup>38</sup> as substrates.

Double labellings were carried out on cryostat sections

using a sequential staining procedure. The sections were first incubated with the anti-BrdU antibody overnight at 4°C, followed by a biotinylated anti-mouse IgG. The reaction was revealed with DAB and H<sub>2</sub>O<sub>2</sub>. After thorough rinsing, slides were incubated in the anti-PSA-NCAM antibody 24 h at 4°C, followed by IgG-HRP. This immunoreaction was revealed using the glucose oxidase-nickel-DAB method. For observation, all sections were dehydrated and mounted in DPX (Raymond A. Lamb, U.K.).

Immunocytochemical controls included (i) incubation of sections in mouse ascites fluid containing IgM irrelevant antibodies recognizing a proteic epitope of Men B bacteria (at the same dilution as the PSA-NCAM antibody); (ii) incubation omitting the primary antibodies; and (iii) incubation employing inappropriate secondary antibodies. All these control tests showed the specificity of single and double immunostainings.

#### *Quantification of bromodeoxyuridine-immunoreactive nuclei*

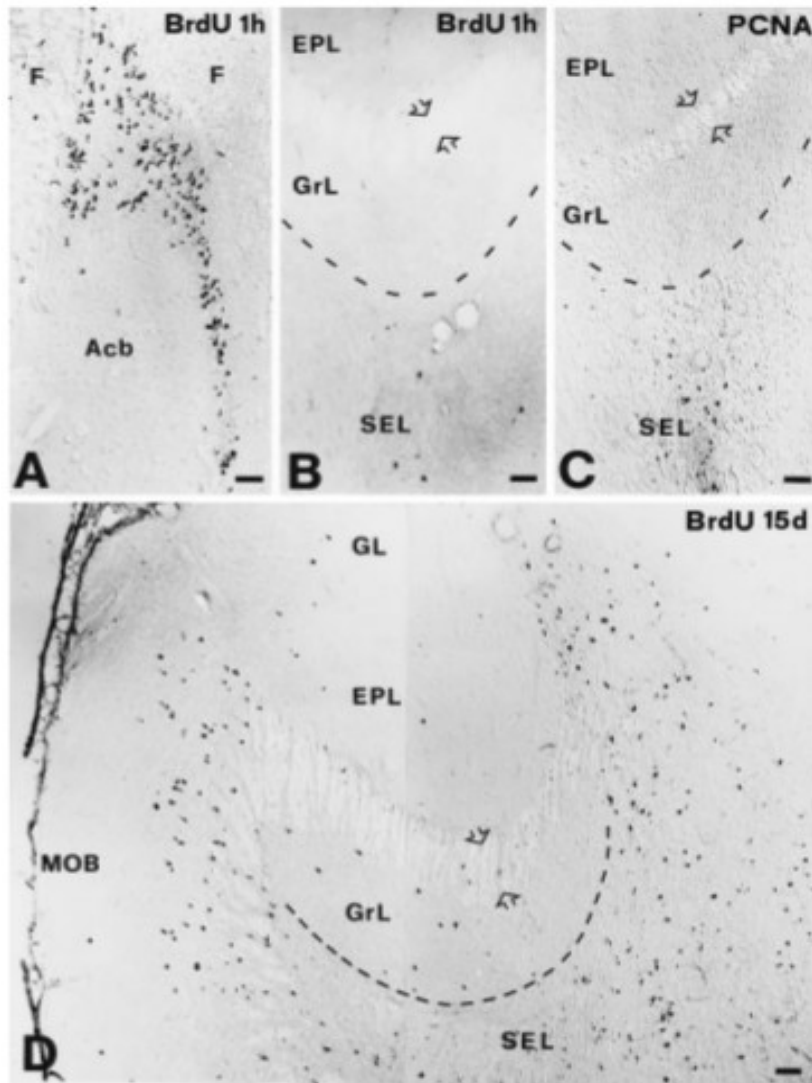
BrdU-positive nuclear profiles were counted on sections of the olfactory bulb cut at the level of the AOB, from four animals which underwent BrdU injections 15 days before. To obtain an estimate of the amount of newly-generated cells in the AOB and MOB granular layers (GrLs), respectively, the BrdU-positive nuclear profiles were counted within a reference area corresponding to a 40x magnification, rectangular microscopic field, placed in the centre of the AOB GrL and in the MOB GrL, on the same section. The rectangle was always positioned with its base at the limit of the SEL, orthogonally-oriented with respect to the direction of cell migration. A total number of 1598 nuclei were counted.

## **RESULTS**

The AOB is a lens-shaped, layered structure located in the dorsal part of the olfactory bulb.<sup>10,39</sup> The sequence of layers in the AOB is similar to that of the MOB, including a glomerular layer (GL), an external plexiform layer (EPL), and a GrL. However, notable differences in their organization are present. In the AOB, the glomeruli are smaller and less distinct. The output neurons, analogous of the mitral cells, are located in the EPL and are not organized in a monolayer as in the MOB. The GrL and the EPL are separated by fascicles of white matter forming the lateral olfactory tract (LOT). In coronal sections, the GrL has a V-shaped aspect, the more ventral part being directed towards the underlying SEL of the olfactory bulb.

#### *Detection of bromodeoxyuridine-immunoreactive cells*

Proliferating cells were identified immunocytochemically after BrdU-incorporation in the nucleus. Their distribution in the SEL and in the AOB was evaluated in animals killed at different survival times post-injection, from 1 h to 30 days. At 1 h, a great number of BrdU-positive nuclei were present in the SEL of the lateral ventricle and in its rostral extension (Fig. 1A). By contrast, a few immunoreactive nuclei (about 5–8/section) were detectable in coronal sections of the olfactory bulb, where they appeared concentrated in the SEL (Fig. 1B). At this survival time, only few scattered BrdU-immunoreactive nuclei could be detected in the GrL and



in the GL of the MOB, and occasionally in the AOB. At six days post-injection, the pattern of BrdU immunoreactivity in the olfactory bulb was similar to that observed after 1 h, although the number of immunoreactive nuclei was highly increased in the SEL (not shown). On the other hand, 15 days after the BrdU injection, a great number of immunoreactive nuclei were detected throughout the olfactory bulb, the majority of them being localized in the GrL of both the MOB and AOB (Fig. 1D). By contrast, in the SEL of the olfactory bulb the immunoreactive nuclei were very scarce. In the AOB, the BrdU-positive nuclei were also abundant at the level of the LOT, where they frequently appeared localized in the narrow bands of gray matter intercalated between the fibre fascicles (Fig. 1D). Isolated nuclei were also detected in the EPL and GL (Fig. 1D). In order to establish a comparison between the number of newly-generated cells in the AOB and MOB, we counted BrdU-positive nuclear profiles within a reference area in the respective GrLs. The GrL was chosen since, both in the AOB and MOB, it is the region wherein the majority of newly-generated cells were found. Moreover, the AOB GrL is easily recognizable and its boundaries are sharp enough to establish a defined area for counting. Our counts indicated that BrdU-positive nuclei in the AOB GrL correspond to about 44% ( $\pm 2,25\%$ ) of those found in the same reference area within the MOB GrL.

The distribution of the BrdU-positive nuclei at 30 days p.i. (not shown), was similar to that observed at 15 days p.i. However, some of these nuclei in the

GrL showed a more punctate, weaker staining, in comparison with that observed in earlier survival times, which is probably due to subsequent cell divisions (see Refs 12, 29).

#### *Detection of proliferating cell nuclear antigen immunoreactive cells*

In sections cut across different levels of the olfactory bulb, nuclei immunoreactive for PCNA appeared more numerous in comparison with those observed after 1 h BrdU-injection. They were consistently observed in the SEL area and, to a lesser extent, in the close adjacent tissue (Fig. 1C). In the AOB, some of these nuclei were detected in the inner part of the GrL, adjacent to the dorsal part of the SEL. Isolated PCNA-positive nuclei could be only occasionally observed in the remaining portions of the olfactory bulb. In accord with the observations carried out after 1 h BrdU administration, the PCNA staining revealed a great number of proliferating cells in the SEL of the lateral ventricle and in its rostral extension.

#### *Polysialylated neural cell adhesion molecule immunoreactivity in the accessory olfactory bulb*

Cells immunoreactive for PSA-NCAM were observed within all the layers of the AOB, although the majority of them were found in the GrL (Figs 2-4). Their distribution matched that of the BrdU-positive nuclei. As previously described, the immunocytochemical detection of PSA-NCAM in thick Vibratome sections of the olfactory bulb allowed the visualization of cells in a Golgi-like manner.<sup>5</sup>

In parasagittal sections of the SEL rostral extension, corresponding to the

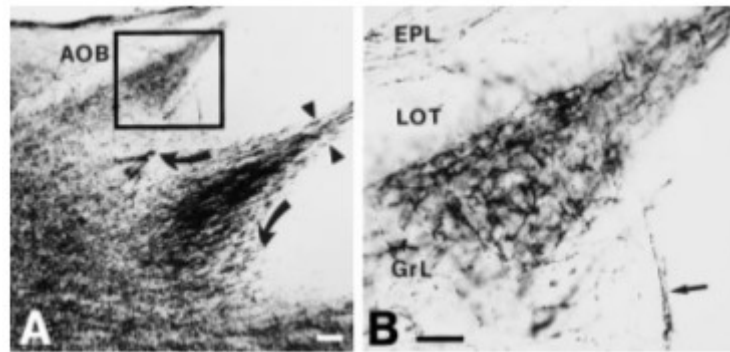
**Fig. 1.** Cell proliferation visualized after systemic injection of BrdU, revealed immunocytochemically at different survival times (A,B,D), and by direct immunostaining for PCNA (C). One hour after the BrdU treatment, a high number of immunoreactive nuclei are visible in the SEL rostral extension (A), but not in the olfactory bulb (B). In coronal sections of the olfactory bulb, cut at the level of the AOB (B), only few BrdU-positive nuclei are localized in the SEL, below the ventral limit of the AOB (indicated by the dotted line). A similar distribution can be observed for PCNA-positive nuclei (C): in the olfactory bulb, they appear more numerous in comparison with BrdU-positive nuclei at 1 h, but always restricted to the SEL area. By contrast, 15 days after the BrdU injection (D), a great number of immunoreactive nuclei are visible throughout the AOB and MOB. They appear more concentrated in the respective granular layers (GrL, indicated in the AOB). Some BrdU-positive nuclei are also visible in the external plexiform (EPL) and glomerular (GL) layers of the AOB. Note several nuclei among the LOT fibre fascicles (indicated also in B and C by the open arrows). F, anterior forceps of the corpus callosum; Acb, nucleus accumbens; MOB, main olfactory bulb; SEL, subependymal layer of the olfactory bulb. Scale bars=50 µm.

tangentially-oriented portion of the rostral migratory stream, as already reported in the rat<sup>5</sup> and mouse,<sup>33</sup> PSA-NCAM staining revealed a great number of tightly-packed, heavily-stained cells (Fig. 2A). In these sections, at the level of the AOB, some bipolar-shaped PSA-NCAM-immunoreactive cells were consistently detected outside the migratory stream, in the tissue comprised between the SEL (corresponding to the migration pathway) and the GrL of the AOB (Fig. 2A,B). These cells were orthogonally-oriented with respect to the migratory pathway.

The shape and orientation of PSA-NCAM-positive cells in the AOB were different within each layer. The morphology and laminar distribution of the



immunoreactive cell types are summarized in Fig. 5. Immunoreactive cells in the GrL and in the LOT were prevalently small and spindle-shaped (Fig. 3A). Most of them showed the bipolar morphology characteristic of migrating neuroblasts, with an oval/elongated cell body (5 x 6-10  $\mu\text{m}$ ). In the GrL, PSA-NCAM-immunoreactive cells with round to piriform cell bodies (4-8  $\mu\text{m}$ ) and short side branches to their processes, reminiscent of a very simple dendritic tree, were also observed. The overall distribution of the

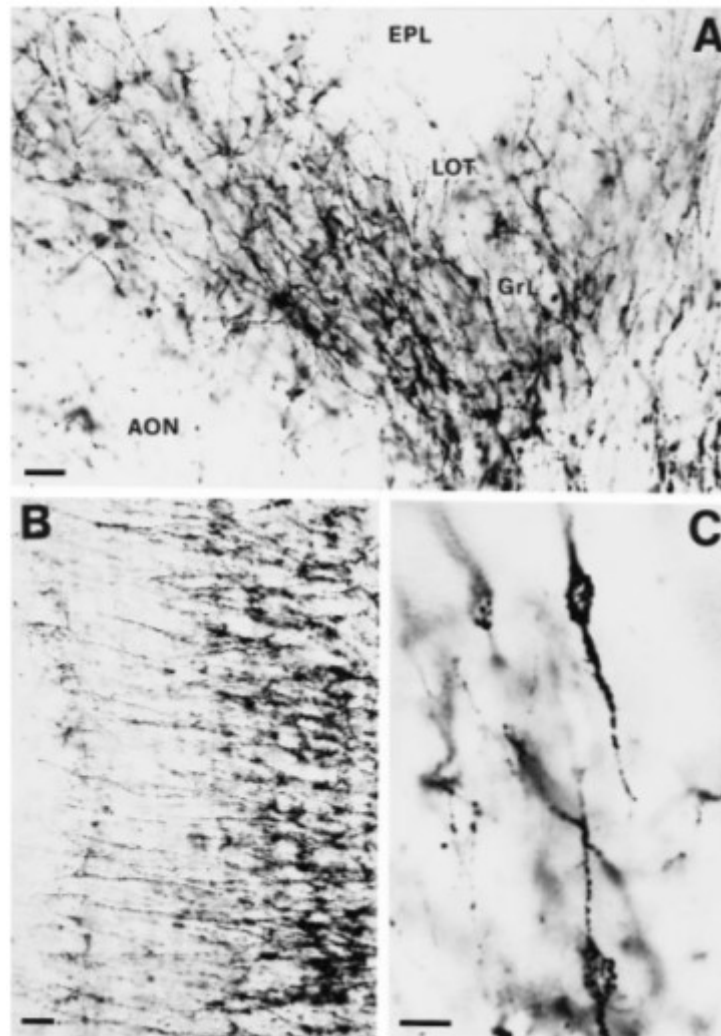


**Fig. 2.** Immunoreactivity for PSA-NCAM in a parasagittal section of the adult rat forebrain at the level of the accessory olfactory bulb (AOB). A) The rostral migratory stream (arrowheads), is recognizable by the high number of tangentially-oriented, PSA-NCAM-immunoreactive cells, most of which spread in a fan-shaped manner towards the main olfactory bulb (arrows). PSA-NCAM immunoreactivity is also present in the AOB, where it appears particularly concentrated in the granular layer (GrL, panel, showed at higher magnification in B). Two elongated, radially-oriented cells, which appear strongly immunoreactive for PSA-NCAM on their processes (arrow), are visible outside the rostral migratory stream, between this latter and the AOB granular layer. Note the small, unipolar/bipolar PSA-NCAM-immunoreactive cells in the GrL and tangentially-oriented processes in the external plexiform layer (EPL). LOT, lateral olfactory tract. Scale bars: A=100  $\mu\text{m}$ ; B=50  $\mu\text{m}$ .

immunoreactive cells in the GrL was characterized by a fan-like, radial arrangement, from the ventrally located SEL of the olfactory bulb (Fig. 3A). Such an arrangement was less regular than that previously observed in the correspondent layer of the MOB (Fig. 3B; for further details see Ref. 5). In the LOT, the majority of the PSA-NCAM-immunoreactive cells were bipolar and radially aligned across the fibre tract (Figs 3C, 6E). They were frequently detected within those portions of the gray matter intermingled with the fascicles of LOT fibres, and spanning between the GrL and the EPL. In the EPL and in the GL, bipolar shaped cells were rare, the majority of the PSA-NCAM-immunoreactive cells displaying a neuronal morphology (Fig. 4). These cells were characterized by a piriform/triangular cell body (6-8 x 8-10  $\mu\text{m}$ ), recognizable dendritic branches and, sometimes, an axonal-like process (Fig. 4A). As shown by parasagittal and horizontal sections, cells in the EPL had long, tangentially-oriented processes (Fig. 4A), in sharp contrast with the radially-oriented cells of the deeper layers. On the other hand, cells in the GL displayed shorter processes, frequently surrounding the glomeruli (see Fig. 4B,C).

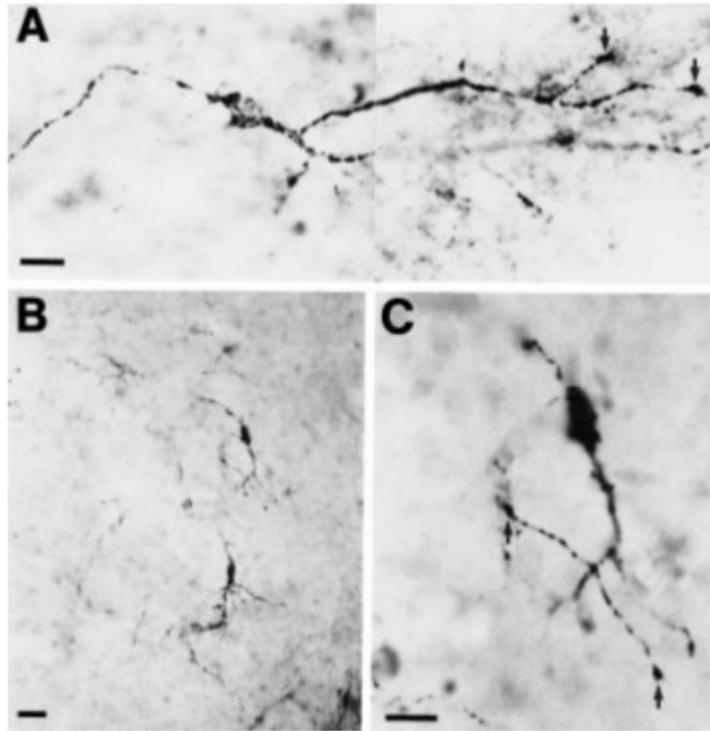
The PSA-NCAM-positive cells throughout the AOB consistently showed a strong punctate labelling on their cell body and processes (Figs 3, 4), similar to that previously described in neurons of the MOB<sup>5</sup> and other brain areas.<sup>4</sup> Some of the PSA-NCAM-positive cells in the AOB, belonging to all the morphological types described above, were characterized by processes

bearing at their tip strongly immunoreactive swellings, reminiscent of growth cones (Fig. 4).

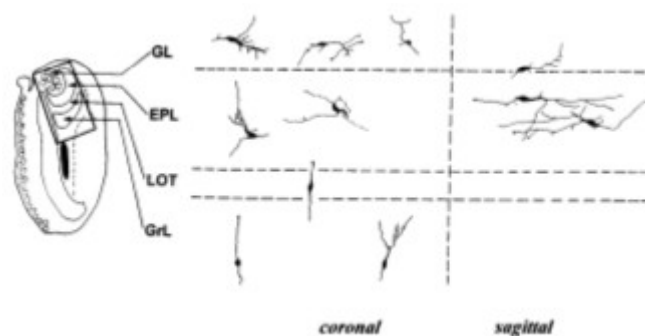


**Fig. 3.** PSA-NCAM immunoreactivity in the granular layer of the AOB (A) and MOB (B), and in the lateral olfactory tract (C). Coronal views. A high number of unipolar/bipolar immunoreactive cells are visible in the V-shaped AOB granular layer (GrL). These cells are radially-oriented, but their arrangement appears less regular and more fan-shaped in comparison with those detected in the correspondent layer of the MOB (B). C) Two spindle-shaped, PSA-NCAM-positive cells across the lateral olfactory tract (LOT). Note their radial arrangement and characteristic punctate reaction on the cell body and processes. AON, anterior olfactory nucleus; EPL, external plexiform layer. Scale bars: A,B=30  $\mu$ m; C=10  $\mu$ m.

It is worth noting that no immunoreactive cells showing an astrocytic morphology were detected in the AOB, or elsewhere in the GrL and GL of the olfactory bulb.



**Fig. 4.** PSA-NCAM-immunoreactive cells in the external plexiform layer (A) and in the glomerular layer (B,C) of the AOB. A) Parasagittal section showing an immunoreactive cell with neuronal morphology and characteristic punctate reaction on the cell body and processes. These latter appear tangentially-oriented. An axonal-like process is visible on the left. The dendritic process on the right has two main branches, one of which is out of focus; the other displays two highly immunoreactive enlargements at the tip (arrows). B) Coronal section showing two small, neuronal like cells in the area occupied by the glomeruli. One of these cells, showed at higher magnification in C, displays characteristic, highly PSA-NCAM-positive enlargements (arrows). Scale bars: A,C=10  $\mu$ m; B=20  $\mu$ m.



**Fig. 5.** Camera lucida drawings depicting the morphology and laminar distribution of representative PSA-NCAM-immunoreactive cells in the AOB, from coronal and sagittal thick Vibratome sections. Note the prevalent bipolar shape and radial orientation of the cells observed in the lower layers of the AOB, which are localized close to the SEL area (indicated in black). Cells in the more superficial layers show a neuronal morphology. Immunoreactive cells in the EPL are frequently characterized by processes oriented in the horizontal plane. EPL, external plexiform layer; GL, glomerular layer; GrL, granular layer; LOT, lateral olfactory tract.

### *Polysialylated neural cell adhesion molecule/bromodeoxyuridine double labellings*

In sections of the olfactory bulb double labelled for BrdU and PSA-NCAM, 15 or 30 days after the BrdU injections, the cells showing a nuclear staining for BrdU in the AOB displayed PSA-NCAM immunoreactivity on their cell body and processes (Fig. 6B-E). In favourable sections, the double-labelled cells appeared unipolar/bipolar-shaped and always showed the characteristic PSA-NCAM punctate staining on their cell body and processes. The BrdU-positive nuclei usually appeared elongated along the main axis of the cell, protruding on one side. Although the majority of cells were double-labelled, some PSA-NCAM-positive cells which did not show BrdU positivity in their nucleus were also observed. For technical reasons, it was difficult to obtain a high number of specimens suitable for an exact quantitative evaluation of those cells which were only positive for PSA-NCAM.

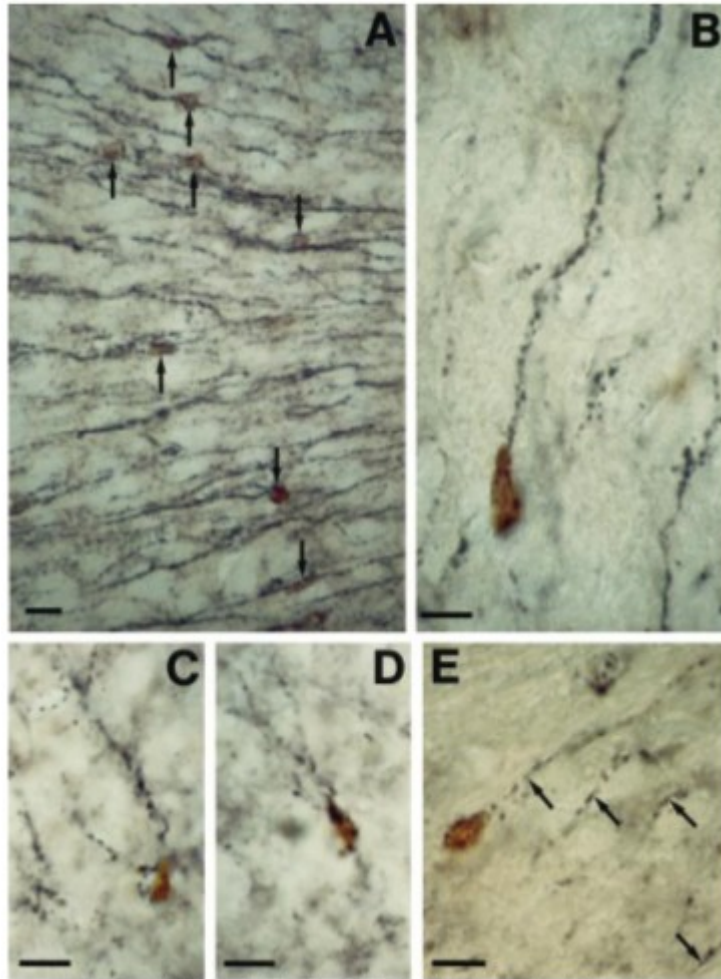
The co-occurrence of the two antigens was observed both in the AOB and MOB (Fig. 6A), showing a very similar type of distribution. As expected, the majority of the double-labelled cells were localized within the respective GrL, namely the areas enriched in BrdU-positive nuclei at the survival times examined (see above).

### *Glial fibrillary acidic protein*

In the AOB and MOB, the GFAP immunostaining revealed a widespread distribution of common, stellate astrocytes. In GFAP/PSA-NCAM double labellings, no overlapping between the GFAP-positive astrocytes and the PSA-NCAM-positive cells was detected (data not shown).

## **DISCUSSION**

Newly-generated cells in the accessory olfactory bulb of the adult rat: cell proliferation or migration? The olfactory bulb of rodents displays considerable cell proliferation and plasticity during the postnatal/juvenile life and even beyond sexual maturity. Progressive addition of glomeruli takes place postnatally in the MOB, which implies a continued proliferation and growth of neural elements with an ongoing organization of these elements into new complex circuits.<sup>19,30</sup> Moreover, an increase in the number of synapses and in the volume of the external plexiform layer have also been described. Both cell proliferation and construction of neural circuits have been observed beyond the postnatal and juvenile life, until the animals reach their full adult size (up to six months in rats and 10-12 weeks in mice). Furthermore, in recent studies, using retroviral lineage tracing in postnatal rats<sup>23</sup> and vital dye injection in adult mice,<sup>21</sup> it has been demonstrated that progenitor cells in a discrete region of the SEL of the lateral ventricle give rise to cells that migrate to the olfactory bulb, where they possibly differentiate into granule and periglomerular cells.



**Fig. 6.** Double labelling for BrdU (brown) and PSA-NCAM (black) in the MOB (A) and AOB (B-E) of the adult rat, 15 days after the BrdU injection. A) In the GrL of the MOB, virtually all the BrdU-positive cells (arrows) also display PSA-NCAM staining on their cell bodies and processes. Note the bipolar shape and radial arrangement of most of the double-labelled cells. B-E) Double-labelled cells in the AOB GrL (B-D) and LOT (E), characterized by an elongated, BrdU-positive nucleus and PSA-NCAM-immunoreactive processes. In C and D, these processes are reminiscent of a simple dendritic tree. In the LOT (E), the processes (arrows) are radially-arranged between the fibre fascicles. Scale bars: A=40  $\mu\text{m}$ ; B-E=10  $\mu\text{m}$ .

However, most of the studies concerning the post-natal construction of neural circuitry and cell proliferation/migration to the olfactory bulb were referred to the MOB.<sup>1,9,21,23,30</sup> Previous studies<sup>3</sup> reported that postnatal neurogenesis in the rat AOB is detectable only during the first two months of life, in comparison with the MOB where these phenomena were described up to six months of age. Bayer<sup>3</sup> described the newly-generated cells in the AOB as extremely sparse and raised the possibility that most of them were glia. In the present study, we show the occurrence of newly-generated cells in the AOB of the adult rat. Interestingly, these cells could be only detected in animals which underwent systemic injection of BrdU and killed after 15 or 30 days survival. By contrast, in the whole olfactory bulb of animals killed 1 h and six days after BrdU injection, or treated immunocytochemically to reveal PCNA staining, most of the dividing cells were concentrated in the SEL. At 1 h, the BrdU-immunoreactive nuclei appeared very scarce in the SEL of the olfactory bulb, whereas they were abundant in more posterior parts. This is in

agreement with a previously described caudorostral gradient in the rate of cell proliferation all along the SEL.<sup>1</sup> A similar pattern of distribution was also observed for the PCNA-positive nuclei, although they were slightly more numerous and more scattered in the SEL area in comparison with those observed 1 h after the BrdU injection. This could be expected since the peptide PCNA, which is expressed in the nucleus of dividing cells during the G1 and S-phase,<sup>11,24</sup> is believed to persist in the nucleus of the newly-generated cells for at least 20 h, as estimated from in vitro studies.<sup>7</sup> Finally, six days after the BrdU treatment, although the number of immunoreactive nuclei in the olfactory bulb was evidently increased, they were still concentrated in the SEL.

Taken together, these results confirm that in the AOB of the adult rat, local cell proliferation is absent or very limited (see also Ref. 3). Moreover, our findings about cell proliferation all along the SEL appear in agreement with the recent demonstration that a migratory stream occurs from the lateral ventricle, subserving the displacement of a high number of neuronal precursors towards the olfactory bulb.<sup>21,23</sup> These studies indicated that 15 days is the average time spent by newly-produced cells to reach their final destination in the olfactory bulb. In particular, during the first week cells migrate tangentially along the SEL rostral extension to reach the centre of the olfactory bulb, and only during the second week do they spread radially towards the superficial layers. For these reasons, our results strongly suggest that

the newly-generated cells identified in the AOB, starting from 15 days after BrdU treatment, correspond to migrating elements from the rostral migratory stream.

The occasional occurrence of few labelled nuclei outside the SEL, 1 h after BrdU injection or immunolabelled for PCNA, suggests that a small number of cells can proliferate in situ. However, although a low rate of local cell proliferation can not be excluded, the occasional labelled nuclei could also indicate a further division of migratory elements, which have been recently demonstrated to occur along the migration pathway.<sup>25</sup> In keeping with this hypothesis is also the more punctate reaction and fragmentation of the BrdU-positive nuclei, observed in the AOB of animals which underwent long survival times (see Ref. 12).

### *Expression of polysialylated neural cell adhesion molecule*

In the present study, by using an antibody that specifically recognizes PSA-NCAM, we found a strong immunoreactivity associated with certain cell populations in the different layers of the adult AOB. PSA-NCAM immunoreactivity in the AOB of the adult mouse was previously described,<sup>33</sup> but only a few positive cells were reported to be detectable in the GrL. The PSA-NCAM-positive cells described here were characterized by a typical morphology and orientation in each layer. Bipolar cells characterized by a small, elongated cell body and showing the typical morphology of the undifferentiated, migrating neuroblasts<sup>18,46</sup> were abundant in the GrL and in the LOT. These cells were frequently radially-oriented. Their morphology and spatial distribution were very similar to that displayed by the PSA-NCAM-positive elements previously described in the GrL of the MOB.<sup>5</sup> The remaining cells in the GrL were also characterized by small-sized cell bodies, but were

provided with a simple dendritic arborization. For this reason and for their radial orientation, they likely correspond to undifferentiated/differentiating granule cells, which represent the main cellular type of this layer.<sup>10</sup> The PSA-NCAM-positive cells in the EPL and in the GL exhibited an evident neuronal morphology, with slightly larger cell bodies in respect to those in the GrL. In the EPL, their dendrites were tangentially-oriented. Such results suggest that these cells may correspond to differentiating interneurons, and not to the projecting neurons which reside in this layer (mitral/tufted cells). These latter, in fact, are usually characterized by larger cell bodies (7-14 x 22-34  $\mu\text{m}$ ) and vertically-oriented dendrites terminating with characteristic "glomerular arbors".<sup>40</sup> However, since the diameter of mitral/tufted cells perikarya vary in a relatively wide range and some of their dendrites lack a terminal glomerular arbor<sup>40</sup> we cannot rule out the possibility that some of the PSA-NCAM-positive cells in the EPL correspond to mitral/tufted cells. Moreover, if polysialylation in the olfactory bulb is associated with newly-generated/undifferentiated cells, this implies that such cells did not yet display their final size and dendritic morphology. The possibility that such cells correspond to not fully differentiated elements is strengthened by the high expression of PSA-NCAM on their whole surface, along with the observation of highly-immunoreactive swellings at the tip of certain processes. The PSA-NCAM-positive cells observed within the GL did not show a particular orientation of the dendritic arborization and were distributed randomly around the glomeruli. For these reasons, we think they may correspond to periglomerular cells.

On the whole, the pattern of distribution of PSA-NCAM-positive cells in the AOB was similar to that previously observed in the MOB, that is a great number of small bipolar cells, radially-arranged in the granular layer, and only scattered neuronal-like cells in the EPL and GL (see Ref. 5). The slight difference observed in the orientation of the immunoreactive cells in the GrL of these two regions (parallel and regular in the MOB, convergent in a fan-shaped manner and more random in the AOB) is due to their different anatomical organization.<sup>10</sup>

The newly-generated cells expressing PSA-NCAM in the AOB displayed the morphological features of either migrating cells or interneurons characteristic of the different layers. These observations are in keeping with results obtained in the MOB, where the neuronal commitment in cells of the rostral migratory stream was primarily inferred on a morphological basis.<sup>21,23</sup> The prevalent neuronal commitment of cells of the rostral migratory stream is also confirmed by the expression of a neuron-specific, class III  $\beta$ -tubulin, both in the rat<sup>29</sup> and mouse.<sup>16,42</sup> In transgenic models, this assumption was further supported by transplantation of cells carrying the reporter gene  $\beta$ -galactosidase attached to the promoter of the neuron-specific enolase gene, in the SEL of adult host animals.<sup>21</sup> It is highly unlikely that PSA-NCAM positive cells are astrocytes, since they always showed a morphology very different from that of the stellate astrocytes which are the main glial type in the AOB, and there is no co-localization with GFAP staining in double labelling experiments. In addition, as described in previous reports<sup>16,22,29</sup> no GFAP immunoreactivity could be detected in the migrating cells of the SEL, even in the more anterior part of the rostral migratory stream.

*Structural plasticity in the accessory olfactory bulb.*

N-CAM is a cell surface glycoprotein that participates in many cell-cell and cell-substrate interactions (for review, see Ref. 13). The post-translational addition to N-CAM of high amounts of the polymer,  $\alpha$ -2,8-linked polysialic acid, or PSA, greatly reduces its degree of adhesivity.<sup>15,35,45</sup> This isoform of N-CAM, commonly known as the “embryonic” or polysialylated N-CAM (PSA-NCAM) is abundant during development, being involved in most dynamic events of neural morphogenesis, including neuronal migration (for review see Refs 31, 34). PSA-NCAM continues to be expressed in discrete areas of the adult CNS capable of undergoing structural remodelling.<sup>4,26,36,37,41</sup> In the olfactory bulb of adult rodents the persistence of PSA-NCAM has been linked to the well known neuronal plasticity occurring in this brain region.<sup>4,26</sup> Such a plasticity involves two main types of structural remodelling, both associated with the expression of the molecule: i) the continuous renewal of the olfactory nerve fibres occurring in the most external layer of the bulb;<sup>9,26</sup> and ii) the long distance migration of newly-generated, undifferentiated cells from the SEL rostral extension towards the granule and glomerular layers of the MOB. Indeed, in the adult rodent forebrain PSA-NCAM is specifically expressed by proliferating and migrating cells of the rostral migratory stream,<sup>5,33</sup> as well as by subpopulations of the granule and periglomerular cells of the MOB.<sup>5</sup> Further evidence for a permissive role of PSA-NCAM in the rostral migratory stream has been recently provided with either genetic deletion of an highly polysialylated N-CAM isoform,<sup>43</sup> or the enzymatic removal of polysialic acid,<sup>28</sup> both the experiments leading to an inhibition of tangential migration in this system.

The pattern of distribution of PSA-NCAM-positive cells in the AOB matched that of the BrdU-positive nuclei after 15 days survival. Double labellings revealed a high co-expression of the two antigens in the same cells, indicating that the PSA-NCAM-positive neurons in the AOB actually correspond to the newly-generated cells which appear in this region after such survival time. It is worth noting that cells identified by PSA-NCAM staining in the olfactory bulb likely correspond to the entire mass of migrating/differentiating elements, whereas double-labelled elements represent a fraction which has incorporated the BrdU within the temporal interval of its availability after injections. This can explain the presence of cells which are immunostained only for PSA-NCAM in PSA-NCAM/BrdU double labellings (see also Ref. 33). Thus, our results strongly suggest that part of the newly-generated cells in the rostral migratory stream leave their migration pathway in the rostral extension of the SEL to penetrate into the AOB, at the level of its GrL. This hypothesis is strengthened by the occurrence of bipolar-shaped PSA-NCAM-positive cells between the SEL and the AOB, which are oriented orthogonally with respect to the migratory stream. In this context, it is worth noting that the tangential migration of neuronal precursors of the rostral migratory stream has been recently demonstrated to occur within “glial tubes” formed by the astrocytic glia in the SEL rostral extension.<sup>22,29</sup> The glial tubes become ill-defined at the level of the AOB,<sup>29</sup> namely the area where tangential migration turns into radial migration.

As previously described in the MOB,<sup>5</sup> the majority of the newly-generated cells detected in the AOB 15 days after BrdU injection were localized in the GrL. Since the GrL contains most of the interneurons of the olfactory bulb, it might represent the prevailing target of an hypothetical cell renewal. A difference



was observed in the density of newly-generated cells within the AOB and MOB. From qualitative observations it appeared evident that the amount of PSA-NCAM-immunoreactive cells in the AOB was lower than that detected in the MOB. Our quantitative data confirmed that the density of newly-generated cells in the AOB GrL correspond to about 44% of that observed in the MOB GrL. Taken together, these data suggest that newly-generated cells of the rostral migratory stream are directed both in the MOB and AOB. However, apart from the rate of cell addition in these two regions of the olfactory bulb, it is worth noting that the newly-generated cells display the same type of laminar distribution as well as a very similar morphology and orientation, suggesting the occurrence of a common pattern of structural plasticity both in the adult AOB and MOB.

## CONCLUSIONS

Our findings show that cell proliferation persists into the accessory olfactory bulb of an adult mammal, demonstrating that cell renewal during adult-hood is not restricted to the MOB. Moreover, the present data strongly suggest that the newly-generated cells in the AOB correspond to migratory elements from the SEL that can be identified by PSA-NCAM staining. Although, at present, we are not able to assess if the newly-added cells in the AOB (and elsewhere in the olfactory bulb) actually undergo long-time survival and differentiation, the neuronal-like morphology displayed by a subset of the PSA-NCAM-positive cells, corresponding to themorphological types of the two main classes of interneurons in the AOB, strongly suggests that a cell renewal can also occur. Evidence for such a structural plasticity in the adult AOB gives rise to several speculations, considering the involvement of this region in the modulation of behavioral responses elicited by pheromones. Behaviour studies have shown that female mice form an olfactory memory of the pheromones of the male with which they mate (for review see Ref. 8). This simple memory system occurs at the dendrodendritic synapses between granules and mitral/tufted cells in the AOB and can be experimentally induced by concomitant infusion of glutamate receptor agonists and pheromone exposure.<sup>17</sup> Moreover, fading of this olfactory memory takes place with a defined decay rate (at about 40-50 days in the mouse). We have demonstrated here that most of the newly-generated cells described in the AOB by the present study reach the GrL and likely correspond to granule neurons. Thus, a de novo addition of cells within a region of the adult brain wherein a modulation of firm-wired memories does occur, could represent an interesting model for further investigations on the ultimate role played by a population of migrating cells from the rostral migratory stream.

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