Int. J. Dev. Biol. 39: 1005-1013 (1995)

Original Article

Expression of isoforms of the neural cell adhesion molecule (NCAM) and polysialic acid during the development of the *Bufo arenarum* olfactory system

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ABSTRACT The neural cell adhesion molecule (NCAM), a member of the immunoglobulin superfamily that promotes Ca^{2+}-independent cell-cell adhesion, is expressed as various isoforms generated by alternative splicing. In this study, the expression of the 180 kDa isoform (180-NCAM), total NCAM (180, 140 and 120 kDa isoforms) and the polysialic acid moiety of NCAM (PSA) were analyzed during the development of the olfactory system of the toad *Bufo arenarum* using specific antibodies and immunofluorescence light microscopy. NCAM and PSA were not found in the ectodermal thickening corresponding to the olfactory placode at early larval stage (stage 17), but by stage 19, total NCAM, 180-NCAM and PSA were all expressed in the invaginating olfactory placode at the sites of cell-cell contact and in the differentiating olfactory epithelium. Later, NCAM isoforms and PSA were found also in the primary fibers of the olfactory nerve and in the olfactory bulb. However, the expression of 180-NCAM decreased near the end of larval development and was absent in post-metamorphic and adult animals. In contrast, total NCAM (representing 140 and/or 120 kDa isoforms) and PSA continued to be expressed in olfactory tissues of post-metamorphic and adult animals, consistent with the persistent neural plasticity of this tissue. Because 180-NCAM has been associated with non-proliferating neurons, its down-regulation in post-metamorphic and adult olfactory system may be associated with the regenerative capability and continuous cell turnover documented for this region in adult animals.

KEY WORDS: *NCAM, PSA, amphibian olfactory system*

Introduction

The olfactory system is an excellent model for the study of neural proliferation, nerve growth and synaptogenesis because of the persisting turnover of neurons that occurs in adult tissues (Graziadei and Monti-Graziadei, 1978). In vertebrates, the olfactory system includes the olfactory nerve, made of fibers from the olfactory neurons originated in the nasal placode, and the olfactory epithelium, originated from the invaginated regions of the placode. Olfactory neurons differentiate into primary sensory neurons whose axons project into the forebrain (Webb and Noden, 1993).

In the central nervous system (CNS), the spatial relationships and reciprocal contacts between cells are regulated by the differential temporal and spatial expression of cell-cell and cell-matrix adhesion molecules (CAMs; see reviews by Edelman, 1984; Rutishauser, 1989; Edelman and Crossin, 1991). The neural cell adhesion molecule (NCAM) is a conserved membrane protein that is highly expressed in neural tissues, where it plays an important role in regulating the adhesiveness of neural cells during development (Hoffman *et al.*, 1984; Edelman and Crossin, 1991), including the olfactory system (Cremer *et al.*, 1994). NCAM is a member of the immunoglobulin superfamily (Cunningham *et al.*, 1987; Williams, 1987; Buck, 1992) that promotes Ca^{2+}-independent cell-cell adhesion interactions. Modifications in the transmembrane and extracellular regions of the NCAM molecule result in three isoforms of approximately 120, 140 and 180 kDa (Rutishauser and Jessel, 1988; Linnemann and Bock, 1989), generated by alternative mRNA splicing of a single gene copy (Murray *et al.*, 1986a,b). Also, the post-translational addition of homopolymers of α-2,8-linked polysialic acid (PSA) (Rothbard *et al.*, 1982; Finne *et al.*, 1983; Finne and Mäkelä, 1985) results in a form of NCAM expressed predominantly in embryonic tissues (embryonic NCAM). Removal of PSA with neu-

Abbreviations used in this paper: NCAM, neural cell adhesion molecule; PSA, polysialic acid; CNS, central nervous system.

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0214-6282/95/$03.00

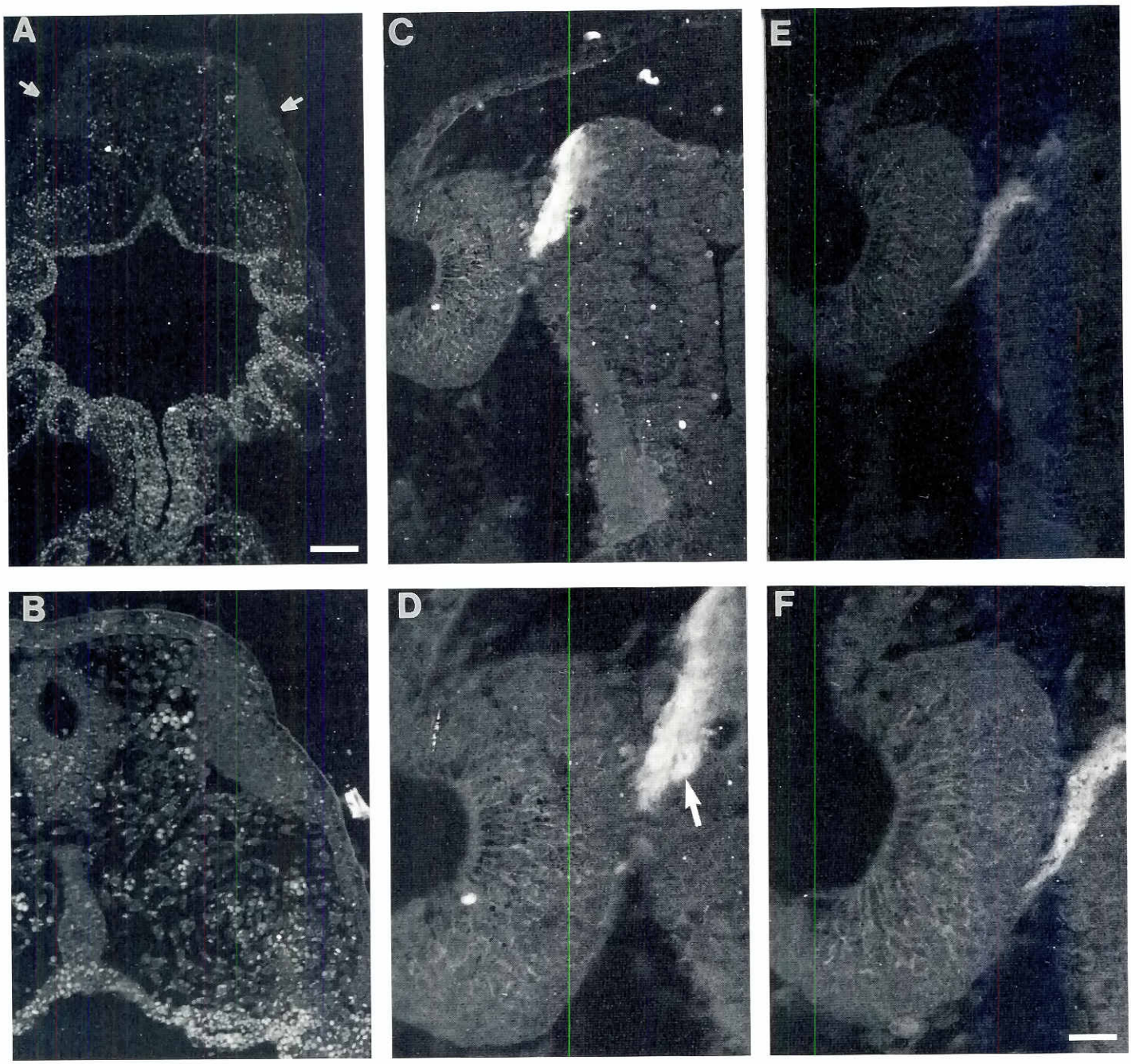
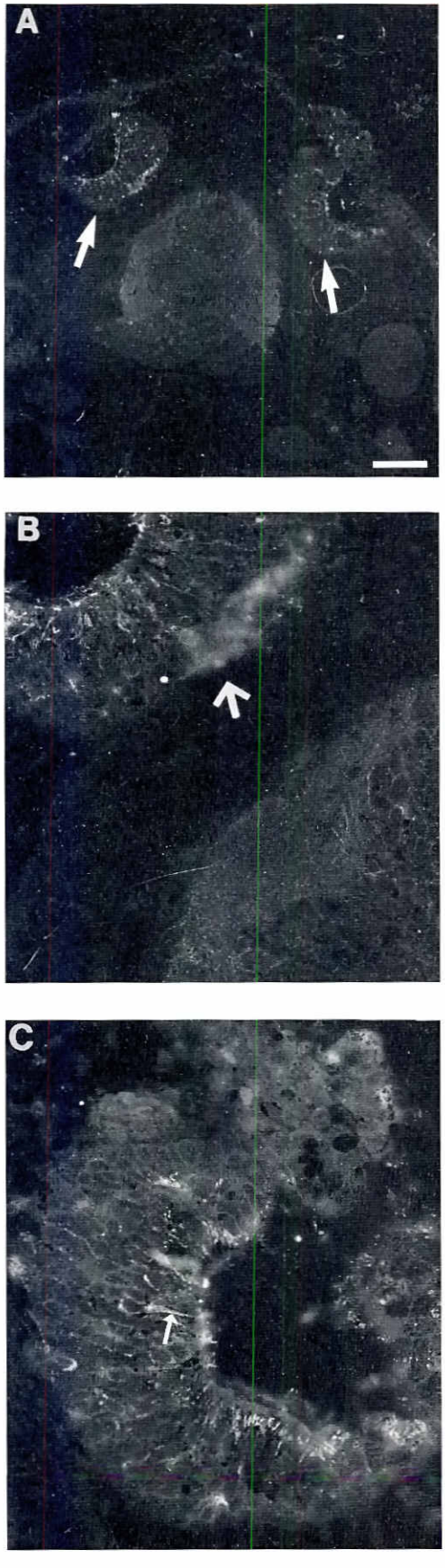


Fig. 1. Immunofluorescence detection of total NCAM (180, 140 and 120 kDa isoforms) and 180-NCAM in horizontal sections of the olfactory placode of early *Bufo arenarum* embryos. (A) *Stage 17 embryo. The olfactory placodes are seen as a thickened epithelium (arrows). No total NCAM immunoreactivity can be seen in this region at this stage.* **(B)** *Higher magnification micrograph showing detail of the placode of a section adjacent to the one depicted in A. The vitelline granules are distinguished as bright spots.* **(C)** *At stage 20, the olfactory placode is invaginating.* **(D)** *Higher magnification of the olfactory placode wing prominent NCAM expression at the sites of cell-cell contact. Intense total NCAM immunostaining is also detected at the placode-forebrain contacting area (arrow).* **(E)** *Section adjacent to C showing the expression of 180-NCAM, distributed in a similar pattern to NCAM.* **(F)** *At higher magnification the distribution of 180-NCAM is evident at the sites of cell-cell contact in the plasma cell membrane and at the placode-forebrain contacting area. Bar: A, 135 μm; F, 60 μm.*

roaminidase interferes with retinal histogenesis and motoneuron pathways *in vivo* (Rutishauser *et al.*, 1985; Rutishauser and Landmesser, 1991; Tang *et al.*, 1992). During development, changes in carbohydrate and polypeptide forms of NCAM have been associated with the acquisition of neural plasticity, proliferation and differentiation of the CNS in vertebrates (Linnemann and Bock, 1989). Because olfactory neurons are the only neurons known to maintain a continuous cell turnover



in adult vertebrates, the olfactory system constitutes a unique model in which to study the expression and modulation of PSA and NCAM isoforms. Previous observations showed that the expression of PSA is high in embryonic nervous tissues and low in adult nervous tissues (Seki and Arai, 1993). However, the expression of PSA is retained in the olfactory bulb of adult mammals (Miragall *et al.*, 1990), suggesting a critical role of this molecule in axonal growth of adult olfactory neurons. The expression of NCAM has been reported during the development of the olfactory nerve in the mouse (Miragall *et al.*, 1989), chicken (Murakami *et al.*, 1991) and rat (Seki and Arai, 1993). In adult mammals, the olfactory system still expresses the three major NCAM isoforms, including a highly sialylated form of the 180-NCAM (Miragall *et al.*, 1989). In amphibians, NCAM is expressed in the olfactory system (Key and Akeson, 1990a; Levi *et al.*, 1990), including a uniquely glycosylated 200 kDa form of NCAM found in adult frogs (Key and Akeson, 1990b, 1991). However, the pattern of expression of NCAM isoforms during the development of the olfactory system of amphibians is not known. Here we studied the expression of NCAM isoforms and PSA in developing and adult tissues of the olfactory system in the toad *Bufo arenarum* using specific antibodies and immunofluorescence.

Results and Discussion

Differences among vertebrate taxa in the expression of NCAM isoforms and PSA has complicated our understanding of the role that specific isoforms play during different developmental stages of the nervous system (Levi *et al.*, 1990; Becker *et al.*, 1993a,b; Seki and Arai, 1993). In mammals, the most prominent isoforms of NCAM expressed during the developmental phase of neurite outgrowth are 140-NCAM and 120-NCAM. After the formation of synapses, 180-NCAM becomes the major isoform expressed in the synaptic membranes, where it is thought to anchor the cells by providing a link between the cytoskeleton and the cell surface (Pollerberg *et al.*, 1985). Consistent with these observations, other studies have demonstrated that 180-NCAM is less able than 140-NCAM to act as a substrate for neurite outgrowth (Doherty *et al.*, 1992).

In contrast to mammals, in this study we found that 180-NCAM was expressed in toad larval stages but decreased near the end of larval development and it was absent in post-metamorphic and adult olfactory tissues. The 140 and/or 120 kDa NCAM isoforms and PSA were present in the olfactory system during metamorphosis, in post-metamorphic stages and in adults.

Fig. 2. Distribution of PSA in horizontal sections of the developing olfactory pit at stage 22. (A) *Low-power photomicrograph of a histological section immunostained with anti-PSA. The immunostaining is limited to the olfactory pits (arrow). No PSA immunoreactivity is observed at the forebrain level. Note that the developing olfactory nerve is not included in this section.* **(B)** *Higher magnification of the olfactory epithelium and a part of the forebrain showing PSA immunoreactivity (arrow).* **(C)** *In the epithelium, the PSA immunostaining distribution is more prominent at the areas of cell-cell contact and the apical surface of the epithelium. Some cells with dendritic processes show a strong immunoreactivity (arrow). Bar: A, 200 μm; B, 120 μm; C, 60 μm.*

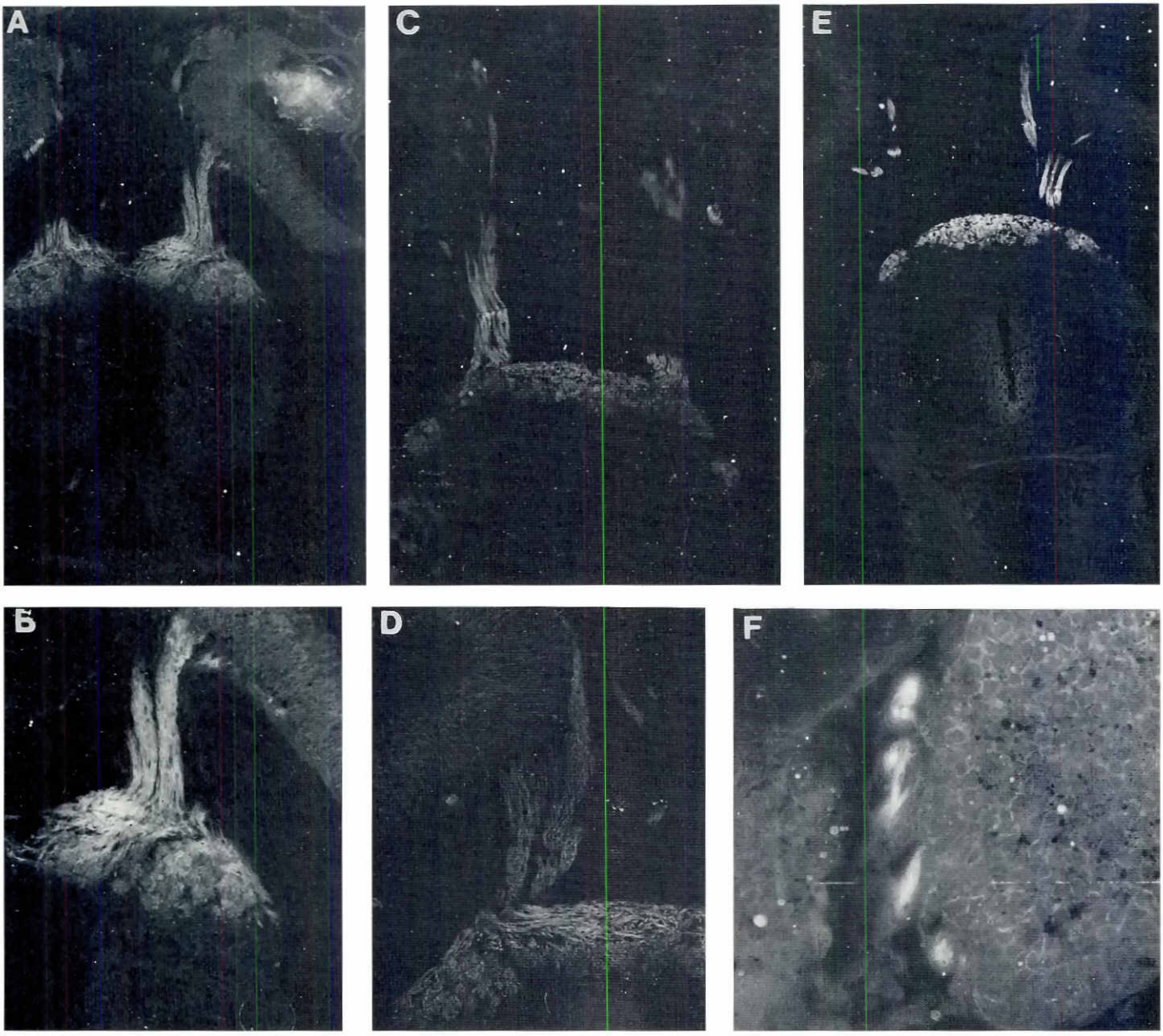


Fig. 3. PSA, total NCAM (180, 140 and 120 kDa isoforms) and 180-NCAM expression in horizontal sections of premetamorphic (stage II) tadpoles. (A) *Low magnification micrograph of the anterior area of a tadpole showing PSA distribution. The PSA is expressed in the olfactory nerve and developing olfactory bulb.* **(B)** *Detail of A showing the immunoreactivity in the olfactory nerve and bulb.* **(C)** *Total NCAM expression in the olfactory nerve and olfactory bulb area.* **(D)** *Detail showing NCAM distribution in olfactory nerve and bulb.* **(E)** *Adjacent section to C showing the distribution pattern of 180-NCAM, similar to that of total NCAM.* **(F)** *Detail of the olfactory epithelium showing intense expression of 180-NCAM in areas of epithelial cell-cell contact and in nerve bundles.*

At tail bud stage (stage 17) the olfactory placode could be distinguished as a thickening of the ectoderm at both sides of the embryonic head (Fig. 1A, arrows). No significant NCAM or PSA immunoreactivity was seen in this region at this stage. The small fluorescent points observed in the placode were probably due to the presence of vitelline granules (Fig. 1B).

The olfactory placodes began to invaginate at stage 19, coinciding with the initiation of cardiac beating. By this stage a small number of NCAM immunoreactive cells were seen between the thickened epithelium of the newly formed olfactory pit and the forebrain (not shown). A few hours later, at stage 20, the olfactory placode had invaginated and the nasal pit was clearly discernible. Co-localized distribution of total NCAM (Fig. 1C and D), 180-NCAM (Fig. 1E and F) and PSA (not shown) were observed in the epithelium. The staining was especially prominent in the plasma membrane at the sites of cell-cell contact (Fig. 1D and F). At this stage, the forebrain was in close contact with the olfactory pit and fiber bundles running through the basal region of the

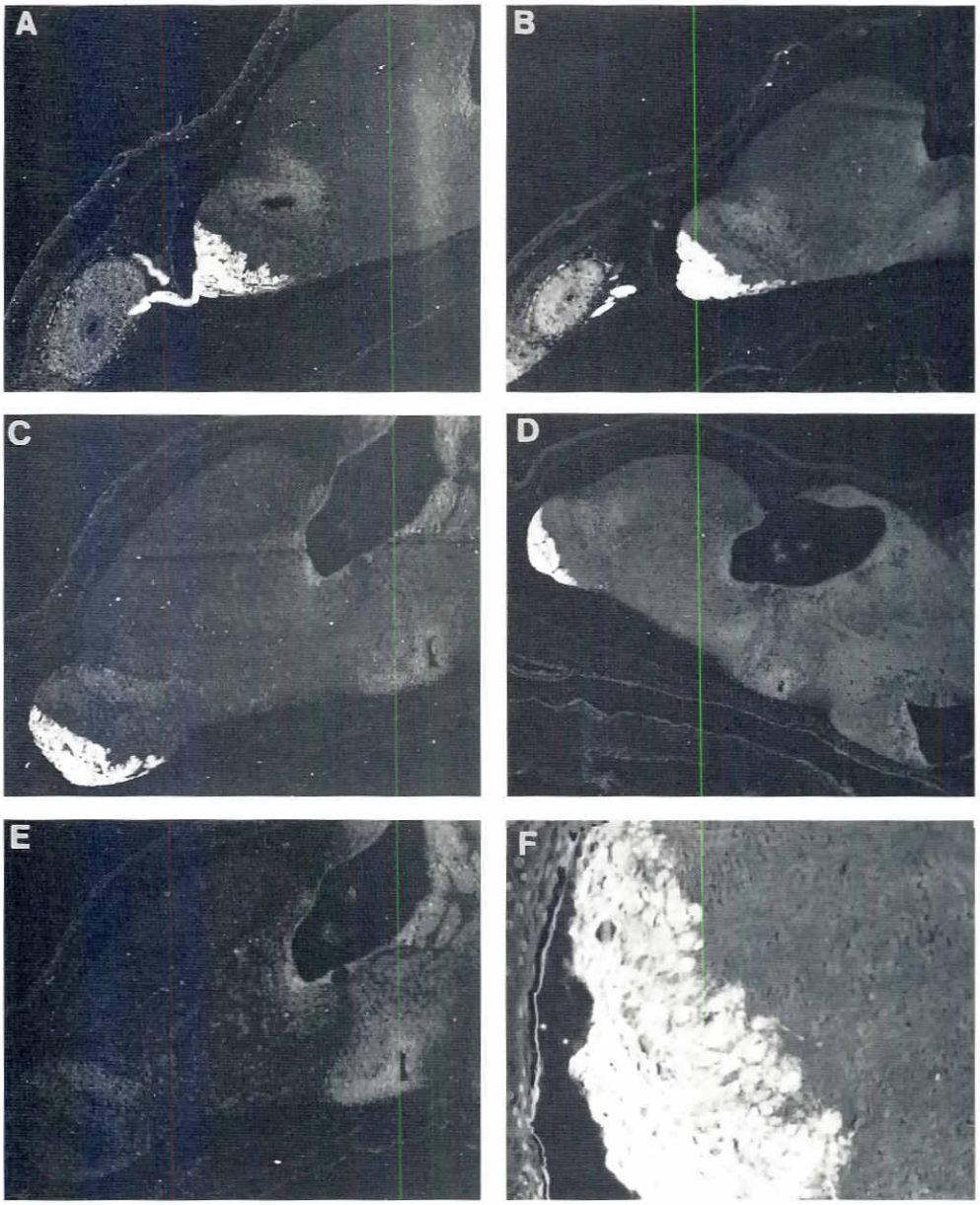


Fig. 4. Total NCAM, 180-NCAM and ß-catenin expression in sagittal sections of prometamorphic tadpoles. (A) *Total NCAM distribution in the olfactory pit, olfactory nerve and olfactory bulb at stage 35.* **(B)** *Adjacent section showing 180-NCAM.* **(C)** *At stage 37, NCAM distribution is more prominent in the olfactory bulb area.* **(D)** *Adjacent section to panel C, showing 180-NCAM distribution.* **(E)** *ß-catenin immunoreactivity in an adjacent section. Note the absence of immunostaining in the olfactory bulb area, but its presence in other NCAM-positive areas of the brain.* **(F)** *Higher magnification showing 180-NCAM expression in the glomeruli area of the olfactory bulb.*

olfactory epithelium showed intense immunostaining for total NCAM (Fig. 1D), 180-NCAM (Fig. 1F) and PSA (not shown).

During the development of the olfactory system in the mouse, NCAM appears first in the placode in its un-polysialylated form. Later, a highly sialylated form of the 180-NCAM persists in the olfactory system of adults (Miragall *et al.*, 1989). In contrast to mammals, we found that PSA was always co-expressed with NCAM through all the developmental stages of the toad olfactory system. In the olfactory epithelium, NCAM was homogeneously distributed in the different epithelial cell layers. In contrast, PSA expression was highest in the receptor cells, whereas more immature elements confined to the deepest epithelial layer showed only a moderate PSA reactivity. This distribution pattern of PSA is similar to that of neuron-specific enolase (NSE) during rat olfactory placode differentiation (Pellier and Astic, 1994) and luteinizing hormone-releasing hormone (LHRH)-positive neurons (Schwanzel-Fukuda and Pfaff, 1989; Schwanzel-Fukuda *et al.*, 1992). NCAM has been shown to be present specifically in LHRH-positive migratory neurons in the mouse (Schwanzel-Fukuda *et al.*, 1992) and chicken (Murakami *et al.*, 1991; Norgren and Brackenbury, 1993). At stage 22, PSA expression (Fig. 2A) was particularly prominent in bundles entering the forebrain at the site of the future olfactory bulb (Fig. 2B). Traced through serial, sagittal or transversal sections, aggregates of PSA-immunoreactive olfactory neurons and axons were seen in contact with the rostral tip of the forebrain forming a thin cap along its ventromedial surface. In the olfactory epithelium, PSA was expressed at sites of cell-cell contacts (Fig. 2C) and the sig-

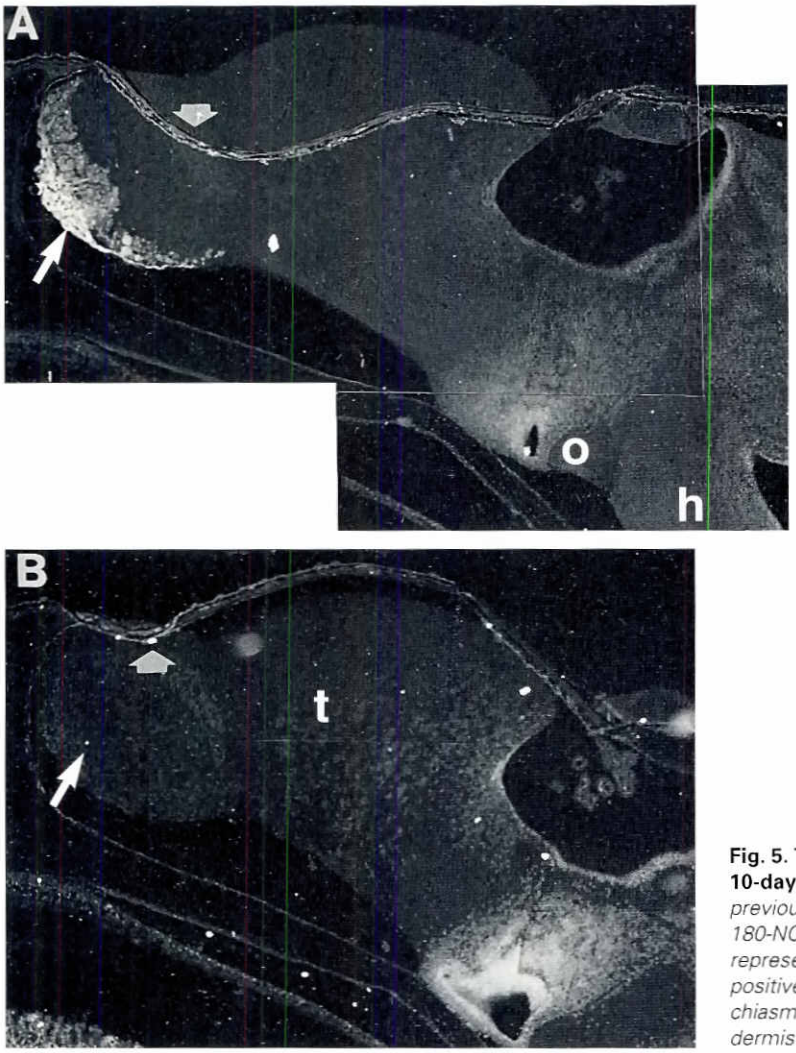


Fig. 5. Total NCAM and 180-NCAM expression in sagittal sections of 10-day-old toads. (A) *Total NCAM is observed in the olfactory bulb as previous stages (arrow).* **(B)** *Adjacent section showing absence of 180-NCAM in the olfactory bulb (arrow), indicating that total NCAM (A) represents 140 and/or 120 kDa isoforms. Note other areas of the brain positive for 180-NCAM. Areas labeled are: t is telencephalon, o is optic chiasm and h is hypothalamus. Short arrows in A and B indicate the epidermis.*

nal was distinctively strong in scattered cells that because of their apical dendrite-like processes resembled sensory neurons (Fig. 2C, arrow).

At premetamorphic larval stages, NCAM and PSA distribution in the olfactory epithelium did not differ from that observed in previous stages (Fig. 3). Multiple axons, which appeared to be the central processes of the developing nerves, were seen exiting the epithelium of the olfactory pit into the nasal mesenchyme and extending to the forebrain.

Those axons, which constitute the nerve fascicles that will end in the developing olfactory bulb, showed high expression of PSA (Fig. 3A and B), total NCAM (Fig. 3C and D) and 180-NCAM (Fig. 3E and F).

At prometamorphic stage, a long and distinct olfactory nerve showed high immunostaining of total NCAM (Fig. 4A and C), 180-NCAM (Fig. 4B and D) and PSA (not shown). The immunoreactivity was also very intense in the olfactory bulb, where total NCAM, 180-NCAM and PSA were detectable in the developing nerve fiber layer and in the glomeruli area (Fig. 4F). No immunoreactivity was detected, however, in the deeper layers of the bulb, indicating that N-CAM and PSA were selectively expressed by the primary sensory olfactory axons. At this stage, axons of the receptor cells can be seen projected also to the accessory olfactory bulb, a distinct structure dorsal and caudal to the main olfactory bulb, but the differentiation of this system occurs later (near metamorphic climax). The expression and function of cell adhesion molecules in the accessory olfactory system remains to be clarified.

The Ca^{2+}-dependent cell-cell adhesion molecule, N-cadherin, and ß-catenin, a cadherin-associated protein, were also studied in sequential sections at prometamorphic stage, using specific antibodies (Fig. 4E). N-cadherin and ß-catenin were found expressed in other areas of the brain, with a distribution comparable to NCAM, similarly to the co-localized expression of both types of molecules in other tissues (Peralta Soler and Knudsen, 1991). However, N-cadherin and ß-catenin were absent from the NCAM-positive regions of the olfactory nerve and bulb, providing more evidence for the uniqueness of the olfactory system within the CNS.

Near the metamorphic climax, the staining pattern of the different NCAM isoforms changed. At stage 42 (midclimax), the expression of 180-N-CAM decreased, compared to previ-

ous stages. In contrast, total N-CAM (140 and/or 120 kDa isoforms) and PSA retained similar immunoreactivity as in previous stages. After metamorphosis, in 10 day-old toads, 180-NCAM was not detected in the olfactory bulb (Fig. 5B), but the expression of total NCAM (Fig. 5A) and PSA (not shown) persisted. Because 180-NCAM was negative, the isoforms now recognized by the anti-total NCAM antibody were only the 140 and/or 120 kDa isoforms. In fully mature adult toads, the 140 and/or 120 NCAM isoforms and PSA continued to be expressed in the olfactory nerve and bulb (Fig. 6A and B), whereas no 180-NCAM was detected in those tissues (Fig. 6C). Because the expression of 180-NCAM has been associated with post-mitotic neurons in nonproliferative zones of the brain, it has been proposed as a marker for neuronal differentiation (Pollerberg *et al.*, 1985; Persohn and Schachner, 1990; Becker *et al.*, 1993a). The down-regulation of 180-NCAM in late amphibian larval stages and in adults is consistent with the proliferative activity found in olfactory neurons of both juvenile and adult animals (Graziadei and Monti-Graziadei, 1978). Our data are in contrast with the persistence of this isoform in the olfactory bulb of mice (Miragall *et al.*, 1989), suggesting a higher cell turnover of olfactory neurons in amphibians than in mammals. Because the metamorphic processes of amphibians occur in different environments (aquatic and terrestrial), one could speculate that the differences in developmentally-regulated expression of cell-cell adhesion molecules between mammals and amphibians may represent differences in adaptive mechanisms associated to the evolution of these species.

The persistence of PSA in the toad adult olfactory system is comparable to the findings of embryonic forms of NCAM in the olfactory system of other vertebrates (Miragall *et al.*, 1989). It also provides additional evidence on the importance of the expression of polysialylated forms of NCAM in areas of the adult brain that exhibit high neuronal plasticity (Seki and Arai, 1991; Theodosis *et al.*, 1991; Bonfanti *et al.*, 1992; Theodosis and Poulain, 1993; Alonso, 1994), including those of amphibians (Becker *et al.*, 1993a,b, 1994).

In summary, our data suggest that developmentally-controlled changes in the expression of NCAM isoforms may regulate the neuronal plasticity and proliferative characteristics of the olfactory system of amphibians.

Materials and Methods

Animals and tissue processing

Bufo arenarum embryos were obtained by *in vitro* fertilization according to described methods (Casco *et al.*, 1992). Embryos and larvae were maintained in 10% Holtfreter solution at 22°C and fed daily with boiled lettuce. Animals were staged according to Gossner (1963), sacrificed by immersion in tricaine methane sulfonate (MS 222; 1:200; Sigma, St Louis, USA) and fixed in Bouin or Zamboni fixative. Samples were then dehydrated and embedded in paraffin. Adults were euthanized with an overdose of anesthetic and perfused from the heart with amphibian Ringer's solution followed by Zamboni fixative. After vascular perfusion, the brain was extracted and post-fixed for 12 h in fresh fixative. Alternatively, embryonic, larval and adult brains were fixed in Bouin's solution overnight at 4°C and subsequently immersed in 0.1 M phosphate buffer (pH 7.0) containing 20% sucrose, and embedded in tissue tek (Miles Lab, West Haven, CT, USA) for frozen sectioning.

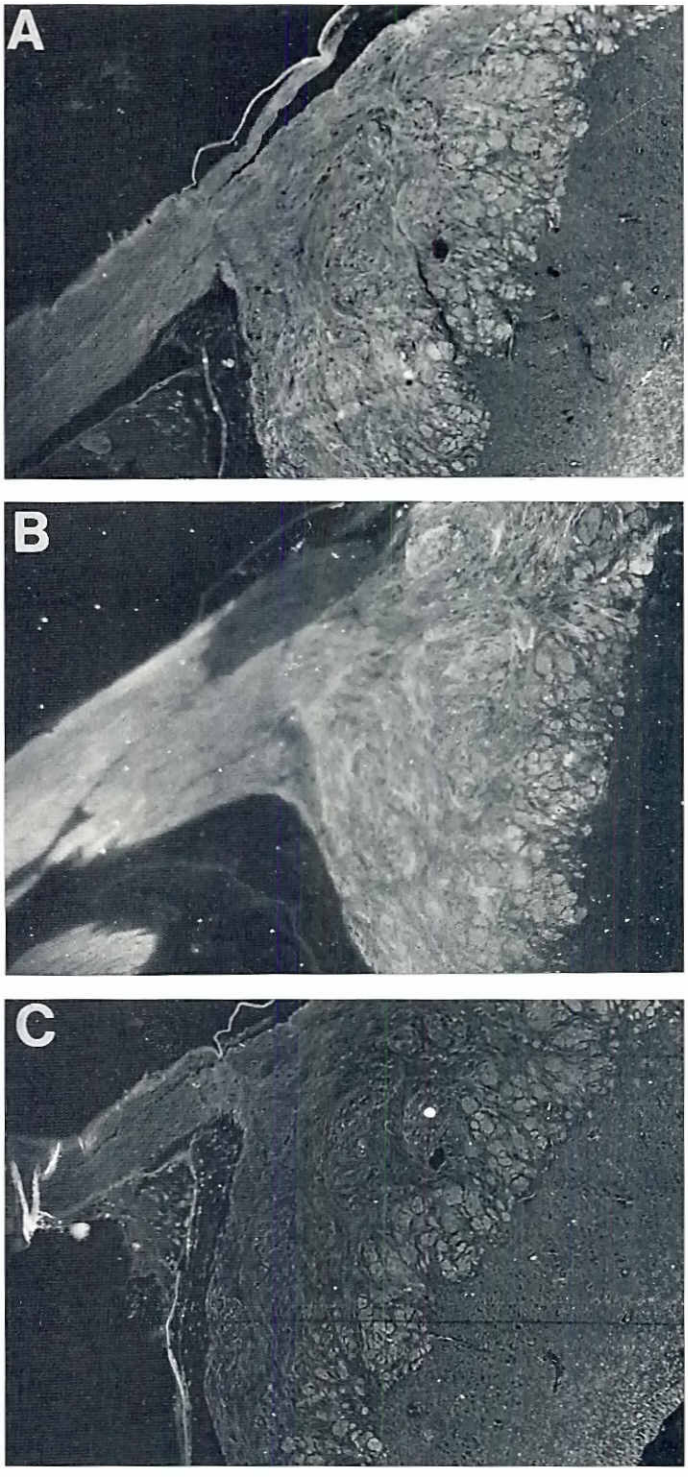


Fig. 6. Sagittal sections of adult olfactory bulb and nerve. (A) *Total NCAM.* **(B)** *PSA and* **(C)** *180-NCAM immunostaining. Note the absence of 180-NCAM* **(C).**

Antibodies

Mouse monoclonal anti-NCAM antibodies (supernatants) were developed in Dr. Rutishauser's lab (Dept. of Genetics, Case Western Reserve Univ., Cleveland, OH, USA) (Watanabe *et al.*, 1986), and were purchased from the Developmental Studies Hybridoma Bank, main-

tained by the Dept. of Pharmacology and Molecular Sciences, Johns Hopkins Univ. School of Medicine, Baltimore, MD and the Dept. of Biological Sciences, Univ. of Iowa, Iowa City, IA, USA. The anti-180 kDa NCAM polypeptide (4d MAb) is directed against the cytoplasmic domain, and the anti-total NCAM (5e MAb) recognizes a common extracellular domain of the three isoforms (180, 140 and 120 kDa isoforms).

The anti-PSA (mAb 735) was developed in Dr. R. Gerardy-Schahn's lab (Medical School, Hannover, Germany) (Fosch *et al.*, 1985) and was received as a kind gift from Dr. C.G. Becker (Dept. Neurobiology, Swiss Federal Inst. Technol., Zurich, Switzerland). Mouse monoclonal antibodies 12F7 anti-ß-catenin and 13A9 anti-N-cadherin have been described previously (Johnson *et al.*, 1993; Knudsen *at al.*, 1995).

Immunocytochemistry

Transversal, sagittal or horizontal 6 µm-thick sections were cut from frozen or paraffin-embedded tissues and mounted on gelatin-coated slides. Paraffin sections were deparaffinized, hydrated, washed in PBS and treated with 5% non-fat powdered milk and 0.2% triton X-100 in PBS. Sections were incubated for 16 h at 4°C with the primary antibodies, washed and incubated with goat anti-mouse-FITC (1:30, Sigma, St. Louis, USA) for 60 min, rinsed in PBS and mounted in 30% glycerol in PBS with 0.2% n-propylgalate. In control sections the primary antibody was replaced by a non-immune mouse serum.

Acknowledgments

We thank Dr. C.G. Becker for providing the anti-PSA antibody. This work was supported in part by the Argentina National Council for Research (CONICET; PID -3378900/92). Mrs. P. Roig and M.J. Morilla are gratefully acknowledged for their excellent technical assistance.

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Accepted for publication: November 1995