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Relationships between neuronal cell adhesion molecule and LHRH neurons in the urodele brain: a developmental immunohistochemical study

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SUMMARY

Polysialic acid (PSA), a homopolymer attached to neural cell adhesion molecule (NCAM) is considered a major hallmark of vertebrate cell migration. We studied the distribution of PSA-NCAM by immunohistochemistry, during brain development, in two urodele amphibians, *Pleurodeles waltl* and the neotenic newt *Ambystoma mexicanum*. In both species a gradual increase of immunolabelling was observed throughout the brain from developmental stage 30 to stage 52. At the onset of metamorphosis, some differences became evident: in *Pleurodeles* immunostaining was gradually restricted to the olfactory system while in *Ambystoma*, PSA-NCAM maintained a more extended distribution (for example throughout the telencephalic walls) suggesting, for the brain of this latter species, a rather preserved neuronal plasticity. The aim of the present work was to correlate the above described PSA-NCAM-immunoreactivity (IR) with the distribution of luteinizing hormone-releasing hormone (LH-RH) containing neurons, which represent a well known example of neural elements migrating from the olfactory placode. LHRH-IR, undetectable till stage 30, was later found together with PSA-NCAM-IR in both the olfactory system and septo-hypothalamic areas. Such observations further support a role of PSA in providing a migration route toward the establishment of a part, at least, of the urodele LHRH system. The possible functional meaning of the LHRH-containing neurons localized between dorsal and ventral thalamus of *Ambystoma*, never reported before in this area, almost devoid of PSA-NCAM-IR, is discussed.

INTRODUCTION

The brain of Urodeles has often been considered as primitive when compared with that of Anurans, being characterized by a compact lamina of periventricular gray matter and few clusters of migrated cells or nuclei (Becker *et al.*, 1993). Such primitivity was correlated with a reduced cellular migration during embryonic development, leading to a secondary simplification or retention of larval traits in the adult brain, also called paedomorphosis (Roth *et al.*, 1993). Recently, emphasis has been placed on one type of embryonic neuronal molecule involved

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in the cellular migration process: the neural cell adhesion molecule or NCAM, which is a phylogenetically conserved membrane protein occurring in the nervous tissues of all groups of vertebrates (Hoffman *et al.*, 1984; Becker *et al.*, 1993). The polysialylated NCAM (PSA-NCAM) represents an important form of post-translational modification of NCAM, involving glycosylation, and characterized by a strong reduction in binding rates (Hoffman and Edelman, 1983). The higher percentage of this isoform during the early period of cell migration is consistent with a role in promoting plasticity. In spinal cord motor neurons, for example, the highest level of PSA-NCAM is detected at the time axons are sorting toward the muscles (Fields and Itoh, 1996) while a decrease in PSA correlates with areas of increased morphological stability (Sunshine *et al.*, 1987, Fields and Itoh, 1996). Therefore, the length of the chain of sialic acid residues is developmentally regulated: during late embryonic life and early postnatal periods, PSA-NCAM is abundant throughout the nervous system and then rapidly decreases with the establishment of neuronal connections (Sunshine *et al.*, 1987; Shults and Kimber, 1992). Nevertheless, PSA-NCAM was found to persist in some regions of the adult rodent brain characterized by neuronal plasticity, such as the hypothalamo-neurohypophysial system (Theodosis *et al.*, 1991; Soares *et al.*, 2000) and the subependymal layer (Bonfanti and Theodosis, 1994).

Data concerning spatial and temporal variations of CAMs (total CAM, NCAM-180 and PSA-NCAM) during ontogeny of the amphibian brain are scarce with the exception of *Xenopus* brain (Sunshine *et al.*, 1987) and the optic tectum of both salamanders and frogs (Becker *et al.*, 1993) where NCAM expression patterns do not substantially differ.

In the present paper, the distribution of PSA-NCAM-IR was studied during brain development in two salamanders, the Spanish newt *Pleurodeles waltl* and the axolotl *Ambystoma mexicanum*. This latter is an aquatic species characterized by reproduction during the larval stages (neoteny). Our analysis was performed from early developmental stages till metamorphosis as well as in some juvenile and adult specimens.

LHRH containing neurons are a well known example of migrating cells which have been exhaustively investigated in both larval and adult Anurans (see for example King *et al.*, 1994; Northcutt and Muske, 1994; Hayes *et al.*, 1994; D'Aniello *et al.*, 1995). In these amphibians, developmental studies (Muske, 1993) have shown that LHRH neurons have at least two origins: the olfactory placode, which gives rise to the terminal nerve-septo-preoptic LHRH system, and a nonplacodal precursor giving rise to a second LHRH neuron subset localized in the caudal diencephalon and midbrain. Moreover, two different molecular forms of LHRH (mammalian-like and chicken II-LHRHs) were, respectively, expressed by the preoptic area and the caudal diencephalon/mesencephalon of both urodeles (Muske *et al.*, 1994) and anurans (D'Aniello *et al.*, 1995; Collin *et al.*, 1995).

By contrast, the only data concerning development of urodele LHRH neurons were reported by Murakami *et al.* (1992), in the red spotted newt *Cynops pyrrhogaster*) and by Northcutt and Muske (1994, in *Ambystoma mexicanum*).

Therefore the study of correlations between NCAM-PSA- and LHRH-IRs during development, in both the olfactory system and brain proper, might provide further contributions to better understanding the role of PSA-NCAM in promoting LHRH neuron settlement.

MATERIALS AND METHODS

Animals

The study protocol complied with Italian legislation on the care and use of animals in research.

Larvae, juvenile and adult specimens of *Ambystoma mexicanum* (adult n=1) and *Pleurodeles waltl* (adult n=1) were taken from breeding colonies reared in the laboratory. Developmental stages (from 22 through 52) were established according to Gallien and Durocher (1957), with some adaptations for *Ambystoma*. Juvenile and adult specimens were 9 months to 3 years old. All animals were anaesthetized with 1‰ methanesulfonate of tricaine in t.w. (MS222, Sandoz, Switzerland). Brains of the adult and juvenile specimens were removed and quickly fixed in Bouin's or Susa's solutions overnight at room temperature. The larvae (n= 40 of *Ambystoma*, from stage 22 through stage 52 and n= 45 of *Pleurodeles*, from stage 28 through stage 52), were immersed in the above fixatives (2 hours-overnight). Tissues were then washed, dehydrated through graded alcohols and n-butanol and embed-

ded in paraffin. Two series of 12 µm thick sections, following coronal and sagittal planes, were cut on a rotary microtome, mounted on glass slides and stored in boxes at room temperature.

Immunohistochemistry

Sections were deparaffinated, washed in phosphate buffered saline (PBS) and incubated in normal goat serum (1:50 in PBS) for 30 min to reduce non specific staining. After a rapid wash in PBS, sections were incubated, in a moist chamber in the dark at 4°C for 18-24 hours, in one of the following primary antibodies:

1. mouse IgM monoclonal anti PSA-NCAM, raised against the capsular polysaccharides of meningococcus group B that share α-2,8-PSA residues with PSA-N-CAM (Rougon *et al.*, 1986), 1:1000 dilution (courtesy of Dr. G. Rougon, Marseille, France);
2. rabbit polyclonal anti mammalian-LHRH (m-LHRH), 1:5000 dilution (courtesy of Dr R. Benoit, Montreal, Canada);
3. rabbit polyclonal anti chicken II-LHRH (c II-LHRH), 1:1000 dilution (courtesy of Dr F. Vandesande, Leuven, Belgium).

Immunoreaction was revealed with the avidin-biotin-horseradish peroxidase procedure (Vectastain ABC kit, Vector Labs, CA, USA) by using the diaminobenzidine (DAB, Sigma Chemical Co.) as the chromogen.

Specificity of the primary antibodies was tested in previous investigations (for the anti PSA-NCAM see Rougon *et al.*, 1986; for the anti m-LHRH see Schwanzel-Fukuda *et al.*, 1992 and for the anti c II-LHRH see van Gils *et al.*, 1993).

The reactions were considered to be specific when the omission of the primary antibodies totally abolished immunostaining.

RESULTS

In the urodele larvae, the closure of the neural tube occurred around stage 21. In the same period the optic vesicles became enlarged and nasal pits, ear vesicles together with lateral line placodes began to be observable. By stage 23, the prosencephalic vesicle has been subdivided into telencephalon and diencephalon by a deep cephalic flessure. By stage 27, the principal encephalic compartments were well recognizable in the brain of the two species under study.

The following observations refer to both *Ambystoma* and *Pleurodeles*; nevertheless the discrepancies observed in the two species are properly reported.

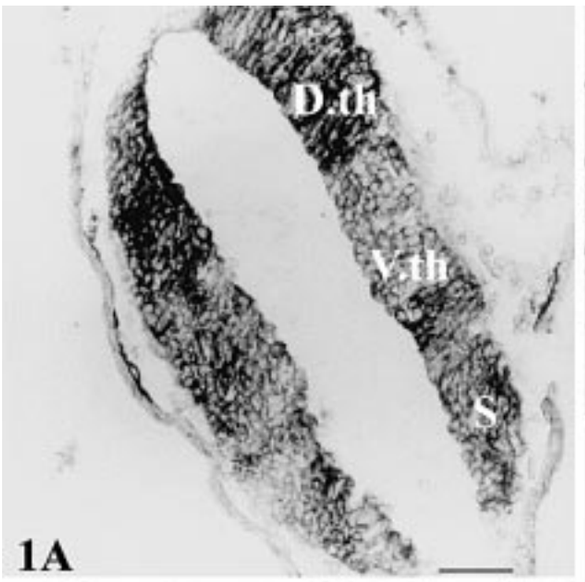
From developmental stage 22 through stage 27, both LHRH- and PSA-NCAM-IRs were absent in the epithelium of the olfactory pits and any area of the brain in both species.

PSA-NCAM-IR

The first appearance of PSA-NCAM-IR could be coincident to stage 28 (a bit later in the Axolotl) when patches of very intense immunolabelling were seen within the prosencephalon (Fig. 1A, stage 30) and the hindbrain. Cells of the olfactory pit were also stained. From stages 31/32 PSA-NCAM-IR became very abundant throughout the brain. A bit later (stage 36) the PSA-NCAM-IR began to assume its peculiar staining pattern in outlining the plasma membranes of the olfactory epithelium and of some cell bodies in the adjacent olfactory bulb (Fig. 1B). By stage 38, a number of cells showing the characteristic PSA-NCAM staining was localized in some selected prosencephalic areas such as the dorsomedial pallium of the telencephalon, plus some centers mainly related to olfactory function, such as olfactory bulbs, olfactory tracts and nuclei, amygdala, habenulae and habenular commissure of the diencephalon (Fig. 1C,E). The same type of immunolabelling was shown by the gray matter of the optic tectum and tegmentum of the mesencephalon (Fig. 1D).

Up to developmental 50-52 stages, the intensity and extension of the PSA-NCAM-IR were gradually increasing throughout the brain of both species. In particular, a number of distinct fiber bundles became heavily immunostained, such as the *stria medullaris* (well appreciable in the neuropil of the habenular nuclei, see for example Fig. 1E) and the nerve terminal roots which were intermingled with clusters of immunopositive cells in the septal area of the basal telencephalon (Fig. 1F). Immunopositive bundles were also seen in the preoptic area and the infundibular wall of the caudal hypothalamus (Fig. 3F).

At the onset of metamorphosis (stage 56), some differences became relevant between the two species: in *Pleurodeles* immunostaining was pro-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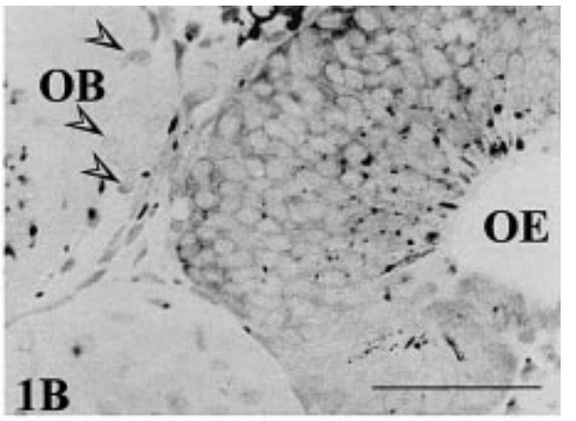
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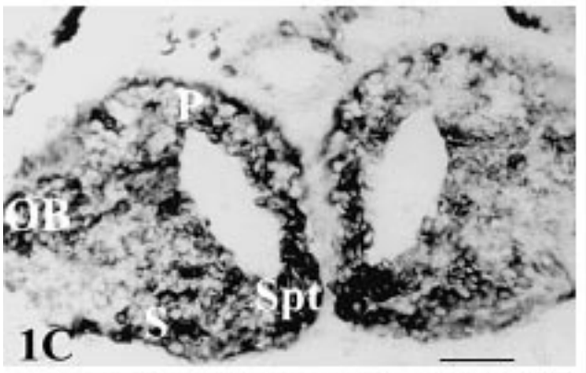
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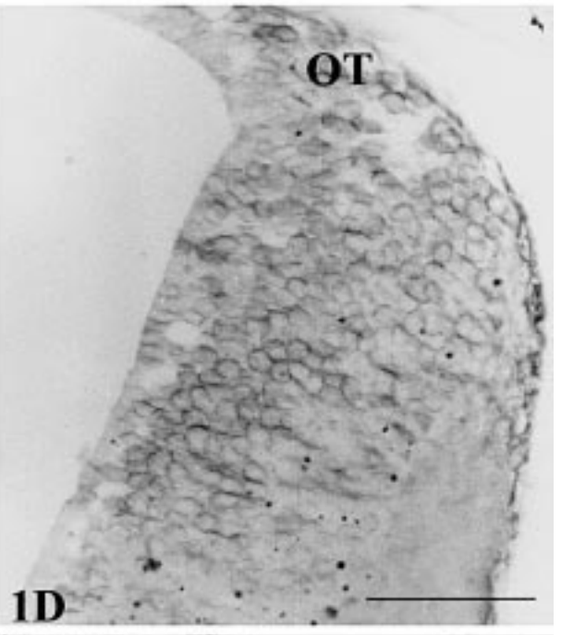
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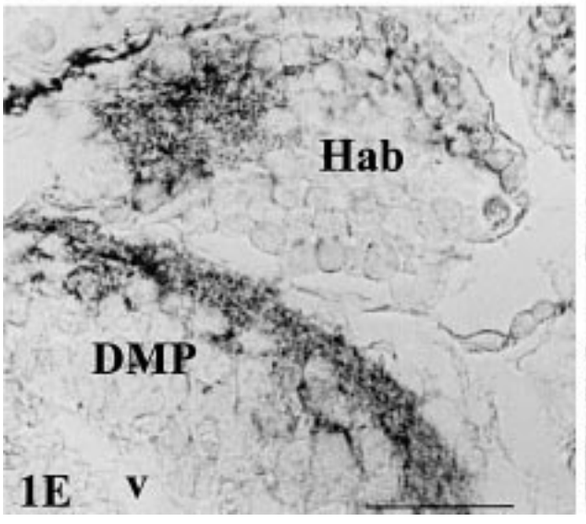
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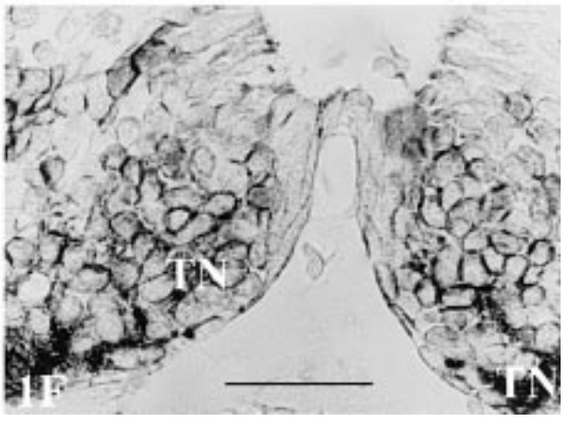
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gressively restricted to the olfactory system. By contrast, in *Ambystoma*, PSA-NCAM-IR persisted in the fiber bundles forming the terminal nerve (Fig. 2A), in several cells in the dorsal pallium (Fig. 2B) and in the amygdala of the telencephalon, and in the periventricular gray of the preoptic recess. A number of stained cell bodies was also detected in the ependymal and subependymal layers (Fig. 2B,C) of the thalamus and suprachiasmatic hypothalamus. Interestingly, some immunopositive cells were found within the paraventricular organ (PVO, Fig. 2C) of the hypothalamus.

LHRH-IR

Since in the adult brain of the salamander *Taricha granulosa* were reported (Muske *et al.*, 1994) two different forms of LHRH (mammalian-like and chicken II-LHRHs) expressed by different cell populations and axonal pathways, we have used two anti LHRH antibodies, one raised against the mammalian LHRH and the other one against the chicken II-LHRH, to evaluate possible differences in the expression of the two forms during development.

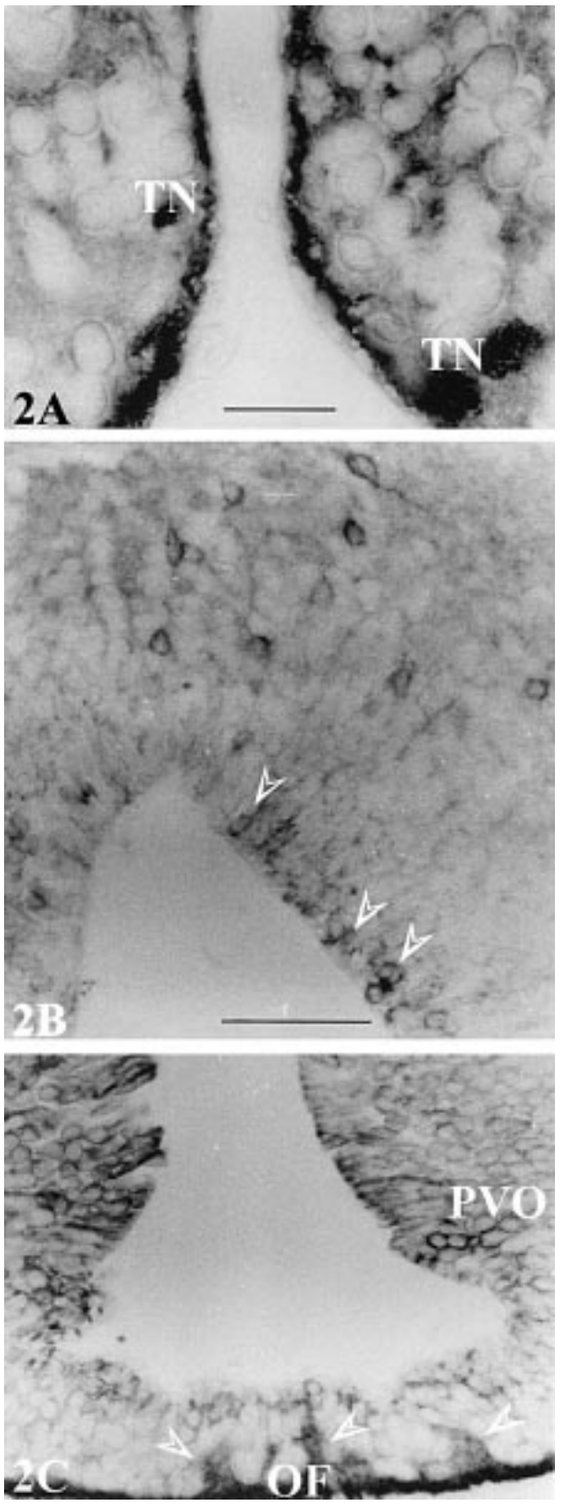
At stage 30, only m-LHRH-immunostaining was detected in the olfactory system (olfactory epithelium, fiber bundles in the olfactory nerve, cells and fibers in both the main and accessory olfactory bulbs) and hypothalamus. By stage 38 (after incubation with both anti m-LHRH and anti cII-LHRH antibodies) LHRH immunopositive neurons and fibers were observed in the posterior tubercle, mesencephalic tegmentum, periventricular gray of the optic tectum and pretectum (Fig. 3A), PVO and dorsal and ventral infundibular nuclei (NID and NIV). Only in *Ambystoma*, and for a limited period (stage 39), a bilateral small group of m-LHRH immunopositive tufted neurons sending their axons in the ventrolateral direction were found in the deepest layers of the periventricular gray, at the boundary between dorsal and ventral thalamus (Figs. 3B,C). Clusters of strongly labelled cells and terminals were observed in the dorsomedial (anterior olfactory nucleus) and ventromedial wall of the telencephalic hemispheres (Fig. 3D), septum and basal preoptic area. After immunostaining of serial sections, PSA-NCAM and m-LHRH-IRs were closely distributed in the ventral wall of the tuberal hypothalamus (Figs. 3E, 3F).

In juvenile and adult specimens, immunostaining was detected in numerous cells of the olfactory mucosa, in the glomerular layer and in a few cells of the adjacent olfactory bulb (Fig. 4A). Some positive cells were observed in the accessory bulb (Fig. 4B). An intense cII-LHRH-IR was shown in the basal telencephalon, preoptic area, tuberal hypothalamus, posterior tuberculum and ventral tegmentum of the mesencephalon (Fig. 4C).

DISCUSSION

In previous immunohistochemical studies, the description of PSA-NCAM-IR in developing amphibian brain was limited to the *Xenopus* brain (Sunshine *et al.*, 1987) and to the mesencephalic optic tectum of both *Discoglossus* and *Pleurodeles* (Becker *et al.*, 1993). Comparing the PSA-NCAM immunolabelling reported in the present research with that obtained by Becker *et al.* (1993) in *Pleurodeles* by using a rat monoclonal anti PSA-NCAM antibody, similarities either in localizations or in type of labelling were so close as to lead us to envisage the antibody used in our study (a mouse monoclonal anti PSA-NCAM antibody,

Fig. 1 - A) *Pleurodeles*, stage 30. A patching, strong PSA-NCAM immunolabelling was shown by the wall of the prosencephalon. In this brain coronal section, the deep cephalic flessure, occurring between telencephalon and diencephalon, caused the thalamus and caudal portion of the striatum to be seen in the same section level. D.th.=dorsal thalamus; V.th.=ventral thalamus; S= striatum, scale bar=100 μm. **B)** *Ambystoma*, stage 36. PSA-NCAM immunopositive cells in the olfactory epithelium (OE). Some faintly stained cells (arrowheads) were also seen in the adjacent olfactory bulb (OB). Black pigment dots were seen throughout the section, scale bar=10 μm. **C)** *Pleurodeles*, stage 38. A coronal section of the telencephalic hemispheres showing an intense PSA-NCAM-IR mainly localized in the septum (Spt), striatum (S) and olfactory bulb (OB). P=pallium, scale bar=100 μm. **D)** *Ambystoma*, stage 40. The characteristic PSA-NCAM-IR outlining the periventricular neuron plasma membranes in the optic tectum (OT) and tegmentum of the mesencephalon. Black pigment dots were seen throughout the section, scale bar=10 μm. **E)** *Pleurodeles*, stage 47. A well distinct PSA-NCAM-IR shown by fiber systems in the posterior pole of the telencephalic hemisphere (DMP=dorsomedial pallium). Positive nerve fibers, likely belonging to the *stria medullaris*, occurred in the neuropil of the habenular (Hab) nucleus. v=ventricle, scale bar=50 μm. **F)** *Ambystoma*, stage 52. A bilateral cluster of PSA-NCAM immunopositive cells in the basal telencephalon (septum). The heavily immunostained fiber forming the terminal nerve (NT) are also well detectable, scale bar=50 μm.



Rougon *et al.*, 1986) as a good marker for urodele developing brain. Moreover, all the controls carried out on brain sections indicate the specificity of the immunolabellings observed.

In the urodele developing brain, the PSA-NCAM-IR was actually undetectable till stage 28. By stage 30 the immunolabelling was observed and progressively increased throughout the brain, as reported in other vertebrate groups (see for example, Seki and Arai, 1993). By contrast, close to metamorphosis, the PSA labelling decreased to become restricted to selective areas with remarkable differences between the two species. In *Pleurodeles*, immunostaining was in fact progressively reduced to the olfactory system in comparison with *Ambystoma* brain where the PSA-NCAM-IR maintained a more extended distribution (for example through the dorsal pallium and amygdala of the telencephalon) possibly corresponding to a persistence of a widespread neuronal plasticity.

In our study, the distribution of LHRH immunolabelling was almost consistent with that reported in other amphibian species (see, for all, the exhaustive review by Muske, 1993). The earliest m-LHRH-IR detection (stage 30) is contemporary, at least in the olfactory system, with the appearance of PSA-NCAM immunolabelling. A bit later (stage 38), a well developed neuronal system labelled by both anti LHRH antibodies was seen to extend from the forebrain to the mesencephalic tegmentum in the two species. Numerous LHRH-immunoreactive cells were observed, from early development stages to adult specimens, in the hypothalamus. In particular, by comparing PSA- and LHRH-IRs in serial sections, the two markers were closely localized in the infundibular wall, especially at the middle of metamorphosis (stages 44/45).

Fig. 2 - A) *Ambystoma*, adult specimen. Tightly assembled PSA-NCAM immunopositive fiber bundles, forming the roots of the terminal nerve (TN), were seen in the septal area of the telencephalon, scale bar=2,5 μm. **B)** *Ambystoma*, adult specimen. Numerous PSA-NCAM immunopositive polymorphic cells were observed throughout the dorsal pallium of the telencephalon. Positive cells (arrowheads) were also seen in the ependymal and subependymal layers, scale bar =100 μm. ***C)*** *Ambystoma*, adult specimen. Suprachiasmatic hypothalamus. PSA-NCAM-IR in the periventricular grey (included the ependymal layer) of the preoptic recess. Some cells of the paraventricular organ (PVO) were immunopositive as well the some bundles of optic fibers (OF, arrowheads), scale bar =100 μm.

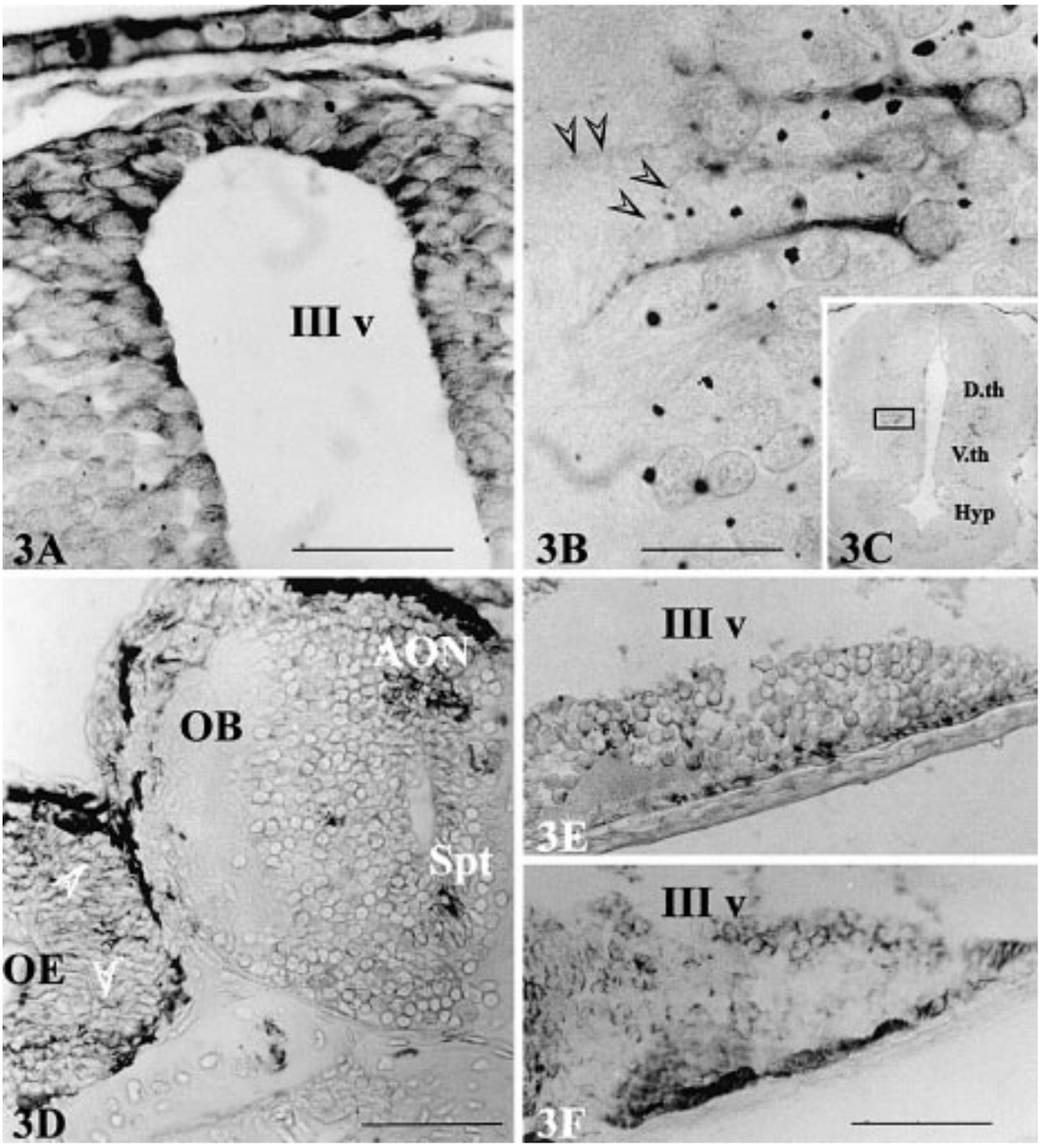
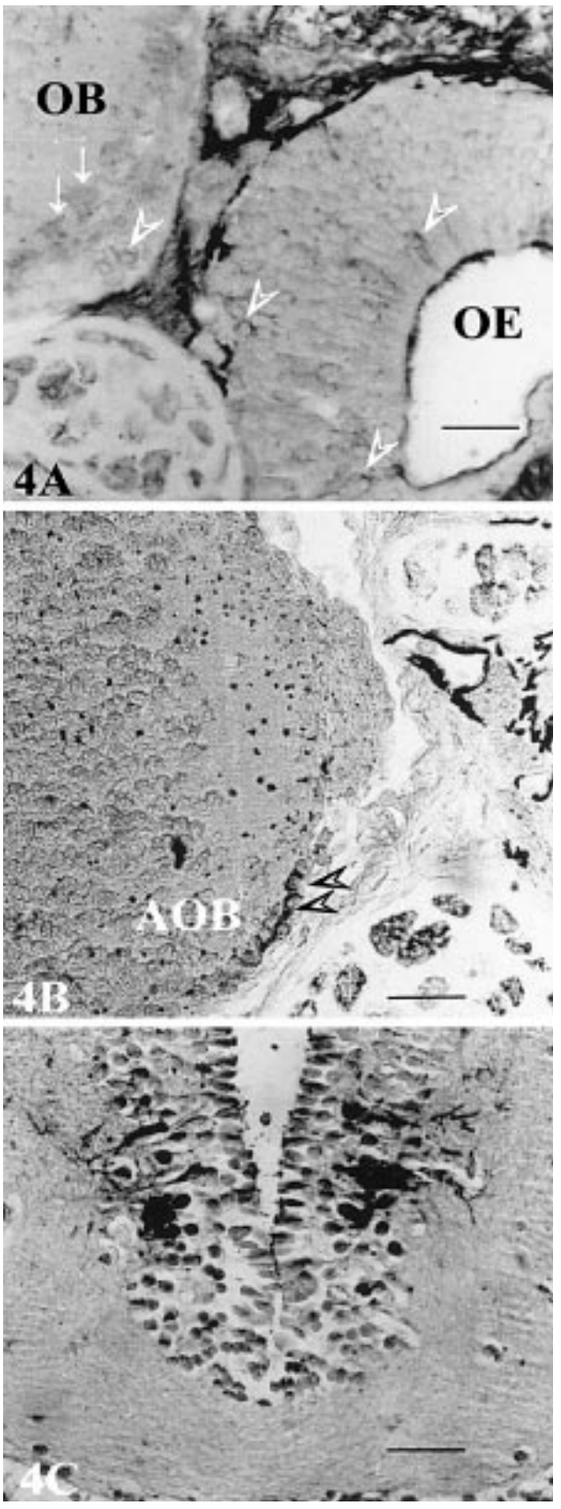


Fig. 3 - A) *Ambystoma*, stage 38. An abundant m-LHRH-IR in the pretectal area. (III v= third ventricle), scale bar=10 μm. **B,C)** *Ambystoma*, stage 39. A coronal section of the diencephalon (**C**, at lower magnification) showing a m-LHRH immunolabelled cluster of tufted neurons placed (in the inset in ***C***) at the boundary between dorsal (D.th) and ventral thalamus (V.th) and sending their processes (arrowheads) in a ventrolateral direction. Abundant pigment dots were seen throughout the section (Hyp= hypothalamus). Scale bar=10 μm. **D)** *Pleurodeles*, stage 43. A rostral coronal section of a telencephalic hemisphere showing m-LHRH immunopositive cells in the anterior olfactory nucleus (AON) and septum (Spt). Some labelled cells (arrowheads) were also seen in the olfactory epithelium (OE). Abundant melanophores were outlining both the structures. OB= olfactory bulb, scale bar=10 μm. **E,F)** *Pleurodeles*, stage 44. In two sagittal, serial sections of the posterior hypothalamus, the m-LHRH- ***(E)*** and the PSA-NCAM-**(F)** IRs were closely distributed in the ventral wall of the third ventricle (=III v), scale bar=10 μm.



Some peculiarities observed during *Ambystoma* brain development seem to deserve some comments.

Only in *Ambystoma*, stage 39, at the boundary between the dorsal and ventral thalamus, have we observed a small group of m-LHRH immunopositive neurons which were not seen later. Since occurrence of transient nervous structures is not unusual during ontogenesis, a transient phenotype may be hypothesized for such m-LHRH containing tufted neurons (although different mechanisms acting in the regulation of neuron number, such as the programmed cell death, can be also envisaged). On the other hand, a direct pathway from the hypothalamus to the dorsal pallium, through the amygdala, was shown in *Pleurodeles* during the first half of metamorphosis. The posterior part of this tract vanished during the second part of metamorphosis, remaining, in the adult, only its anterior (amygdalo-pallial) portion (Clairambault and Timmel, 1990). Another case of restriction was reported in the olfactory system of the same species (Clairambault and Timmel, 1990). Moreover, a possible transient expression of the caudal LHRH system, lacking in the adult, was also postulated in mammals (Muske, 1993).

In general, our results are basically in agreement with those reported for the developing mouse brain (Schwanzel-Fukuda *et al.*, 1992) and remarkably show close functional relationships between the two markers studied in the present work. It seems likely that, as in rodents, a PSA-NCAM scaffold formed by fiber systems of, respectively, terminal nerve and optic nerve and tract (which are characterized by an intense labelling long after metamorphosis) plays a crucial role in guiding LHRH containing structures toward their final location in the basal telencephalon and hypothalamic area, respectively.

Fig. 4 - A) *Pleurodeles*, adult specimen. m-LHRH immunopositive cells (arrowheads) were seen in the olfactory epithelium (OE) as well as, together with nerve fibers (arrows), in the olfactory bulb (OB), scale bar=50 μm. **B)** *Pleurodeles*, adult specimen. m-LHRH immunopositive cells (arrowheads) in the accessory olfactory bulb (AOB). Pigment dots are abundantly distributed throughout the olfactory bulb, scale bar=50 μm. ***C)*** *Ambystoma*, adult specimen. A coronal section of the caudal diencephalon showing a bilateral cluster of intensely cII-LHRH immunopositive neurons in the posterior tuberculum, scale bar=100 μm.

Of special interest seem to be the immunostained neurons found in the paraventricular organ or PVO, a circumventricular organ of non-mammals lining the third ventricle. This small nucleus plays a crucial role, in both Anura and Urodela, in courtship behavior in so far as it is directly implicated in the control of neuroendocrine events (Dubé *et al*, 1990) by way of hormonal synthesis (for example LHRH, Muske *et al.*, 1994 in *Taricha granulosa*) or monoamine (dopamine and serotonin) release (Beltramo *et al.*, 1998 in *Ambystoma*). Taken together, the results outline an interesting point. In mammals, a noticeable NCAM PSA-IR has been shown in some hypothalamic regions (Theodosis *et al.*, 1991; Bonfanti *et al.*, 1992) as well as a parallel between the appearance of PSA and dopamine (Schultz and Kimher, 1992). In *Ambystoma*, the PVO was characterized by PSA-NCAM-IR, LHRH immunopositive cells (present work) as well as a population of dopaminergic neurons (Beltramo *et al.*, 1998). Therefore multiple neurochemical events converging in the PVO suggest a pivotal role for this nucleus in urodele hypothalamic function, and leads us to envisage for PSA-NCAM a facilitating action in the development of the endocrine reproductive system.

In conclusion, the differences found in PSA-NCAM-IR during development of the two urodele species will be commented on in the light of some evolutionary considerations. As reported above, the progressive decreasing of PSA-NCAM-IR in *Pleurodeles* at the end of metamorphosis might correlate with an increasing morphological stability of the nervous tissue (Fields and Itoh, 1996), in contrast to the more extended plasticity conserved by the juvenile and adult *Ambystoma* brain. Paedomorphosis is considered as a state in which an adult of a taxon exhibits morphological characteristics displayed by juvenile forms of related groups (outgroups) as the result of heterochronic developmental processes (Alberch *et al.*, 1979). The Urodele perennibranchiate species (Proteidea, Sirenidea, Necturidea and Ambystomidea) are paedomorphic, and among Ambystomatidea only *Ambystoma* is paedomorphic. This species is commonly considered as exhibiting a "light" paedomorphosis with a fully developed nervous system (Roth *et al.*, 1993). Nevertheless, in the light of the present results, by comparing *Ambystoma mexicanum* and *Pleurodeles waltl*, we cannot agree with this statement. Our observations have, in fact, shown that at stages 55/56 (and later on) PSA-NCAM immunopositive cells and fibers were retained only in the olfactory system of *Pleurodeles*, while in *Ambystoma* numerous cells were immunoreactive throughout the telencephalon, preoptic area and PVO. Hence, at the end of metamorphosis, only a few adult traits (e.g. the anatomical organization) of the *Ambystoma* brain, when compared to that of *Pleurodeles*, are fully achieved. Other characteristics, such as the neuronal patterns, still show a high degree of phenotypic plasticity. Therefore, at least for the brain, *Ambystoma mexicanum* can be considered as a complete paedomorphic species.

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