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Expression of N-CAM-180 and N-Cadherin during development in two south-american anuran species (*Bufo arenarum* and *Hyla nana*)

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SUMMARY

Cadherins and N-CAM are Ca^{++}-dependent and Ca^{++}-independent cell adhesion molecules respectively. These molecules play a key role in morphogenesis and histogenesis. We determined the spatiotemporal pattern of N-cadherin and N-CAM-180 kDa expression by immunohistochemistry during development in two South-American anuran species (*Bufo arenarum*, toad and *Hyla nana*, frog).

Both N-cadherin and N-CAM were not detectable during early developmental stages. Expression of N-cadherin appeared between the inner and the outer ectoderm layers at stages 19-20. At stages 24 -25, N-cadherin was expressed in the neural tube and the heart. In early tadpoles, N-cadherin expression increased along with the central nervous system (CNS) morphogenesis, and reached its maximum level at metamorphic climax stage. N-Cadherin expression was not uniformly distributed. At stage 42, olfactory placodes and retina expressed N-cadherin. Contrary to N-CAM, the strongly myelinated cranial nerves were not labeled. N-Cadherin was present in several mesoderm derivatives such as the notochord, heart and skeletal muscle. The non-neural ectoderm and the endoderm were always negative.

Expression of N-CAM appeared first in the neural tube at stages 24-25 and the level of expression became uniform from pre-metamorphic to metamorphic climax tadpoles. At this latter stage, a clear N-CAM immunolabeling appeared in the nerve terminals of pharynx and heart. N-Cadherin and N-CAM were found mainly co-expressed in the CNS from early tadpole to metamorphic climax tadpole.

Our results show that the expression of N-CAM and N-cadherin is evolutionary conserved. Their increased expression during late developmental stages suggests that N-CAM and N-cadherin are involved in cell contact stabilization during tissue formation.

INTRODUCTION

Cell adhesion is a multifactorial mechanism that depends on the expression of tissue-specific isoforms of cell adhesion proteins, surface density of individual molecules and regulatory factors (Edelman, 1988). Cell and substrate adhesion molecules (CAMs and SAMs, respectively) act as morphogenetic regulators during development (Edelman, 1984; Edelman, 1992; Takeichi, 1991; Albelda and Buck, 1990).

Cadherins are a family of cell surface molecules regulating morphogenesis by a calcium-dependent

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adhesion mechanism during the development of various tissues (Takeichi, 1988, 1991). The most extensively studied member of the cadherin family in the nervous system is N-cadherin (Hatta *et al.*, 1987). N-cadherin plays a key role in neurulation (Fujimori *et al.*, 1990; Hatta and Takeichi, 1986), neuroepithelial morphogenesis (Matsunaga *et al.*, 1988b; Takeichi *et al.*, 1991), axon fasciculation (Drazba and Lemmon, 1990), and axon elongation (Bixby and Zhang, 1990; Bixby et al, 1987; Doherty *et al.*, 1991; Letourneau *et al.*, 1990; Matsunaga *et al.*, 1988a; Neugebauer *et al.*, 1988; Tomaselli *et al.*, 1988). N- and R-cadherin are transiently and differentially expressed in specific fiber tracts of the pons and spinal cord, suggesting that cadherins may provide a homophilic cue for guiding axon outgrowth (Redies *et al.*, 1992).

In the neuromuscular system, several cell adhesion molecules contribute to the regulation of nerve-muscle interactions, including N-CAM, N-cadherin, fibronectin, laminin, s-laminin, and heparan sulfate proteoglycans (Chiu *et al.*, 1986; Sanes *et al.*, 1986; Hatta *et al.*, 1987; Hunter *et al.*, 1989). In chick embryos, N-Cadherin is expressed at high levels by newly formed myotubes and is then downregulated on myofibers to very low levels prior to hatching (Hatta *et al.*, 1987).

N-CAM is a homophilic binding transmembrane protein that belongs to the immunoglobulin superfamily. N-CAM is involved in the histogenesis of nervous tissues and is an early marker of neural tissues. There are three major isoforms of N-CAM: the 120, 140 and 180 kDa (Rutishauser, 1984; Cunningham *et al.*, 1987).

N-CAM is expressed by developing muscle fibers during their innervation period but is downregulated later in development (Moore and Walsh, 1985; Covault and Sanes, 1986; Tosney *et al.*, 1986). In the adult, denervation leads to a re-expression of muscle fiber N-CAM (Covault and Sanes, 1985; Rieger *et al.*, 1985; Moore and Walsh, 1986; Cashman *et al.*, 1987). Parallels between the expression of muscle N-CAM and the susceptibility of muscle fibers to innervation suggest that N-CAM is one of the molecular markers used by muscle fibers to promote neurite growth and innervation (Rutishauser *et al.*, 1983; Bixby *et al.*, 1987; Landmesser *et al.*, 1988; Booth *et al.*, 1990).

The expression of N-cadherin and N-CAM has been studied during the development of several species. An evolutionary conserved expression of cell adhesion molecules was suggested by earlier studies of N-CAM in different species, including amphibians (Hoffman *et al.*, 1984). However, later studies showed differences in the expression of N-CAM isoforms during the development of different types of amphibians (Saint-Jeannet *et al.*, 1989). These differences may have evolutionary significance for the development of the CNS and muscle during amphibian metamorphosis, since the expression of cell adhesion molecules often precedes the differentiation of cells into nervous and muscle tissues (Saint-Jeannet *et al.*, 1989; Levi, 1993). In this paper we describe the expression patterns of N-CAM-180 and N-cadherin during the development of two South-American anuran species (*Bufo arenarum* and *Hyla nana)*.

MATERIALS AND METHODS

Animals

Sexually mature *Bufo arenarum* toads were caught during the month of June in Paraná, Entre Ríos. Embryos and tadpoles were obtained by *in vitro* fertilization. Males and females were kept in water, at 20ºC during 24 hr. The females were then injected with 2,500 IU of human chorionic gonadotrophin hormone (hCGH) (Endocorion, Elea®, Buenos Aires). Twelve hours later, eggs were fertilized with testis extracts as described (Rengel *et al.*, 1988).

The developmental stages were determined according to Gosner (1960). We studied embryos at stages 3, 7, 13-14, 17, 19-20, 24-25, and tadpoles at stages 28 and 41.

Hyla nana larvae frogs from 31 to 42 stages, were collected in a semi-permanent pond, near the city of Concepción (23º 22' S; 57º 18' W), Paraguay.

Histology

Bufo arenarum embryos and tadpoles were fixed with Carnoy solution and *Hyla nana* tadpoles were fixed in 10% formaldehyde in 0.1 M phosphate buffer, pH 7.4.

The specimens were dehydrated in ethanol, embedded in paraffin (Cicarelli®, Buenos Aires). Five microns-thick cross and sagital sections were obtained with a Reichert Jung Hn 40 microtome. Sections were dried at room temperature onto 1% gelatin-coated glass slides.

Reagents

The monoclonal antibody 4d is a mouse IgG1 (chicken, frog) directed to 180 kDa N-CAM polypeptide (cytoplasmic domain). It was developed in Dr. Rutishauser's lab (Dept. of Genetics, Case Western Reserve Univ., Cleveland OH, USA), and was purchased from the Developmental Studies Hybridoma Bank (maintained by the Dept. of Pharmacology and Molecular Sciences, Johns Hopkins Univ. School of Medicine, Baltimore, MD and the Dept. of Biological Sciences, Univ. Iowa, Iowa City IA, USA). The monoclonal antibody 13A9, a mouse IgG1 that recognizes N-cadherin from different species, including chicken and *Xenopus*, has been previously described (Knudsen *et al.*, 1995). The monoclonal antibody GC-4, a mouse IgG1 to chicken N-cadherin was purchased from Sigma Chemical Co. (St. Louis, MO). All monoclonal antibodies were used at a dilution of 1:50. As a control we used mouse normal serum from Sigma Chemical Co. (St. Louis, MO).

For detection of primary antibody binding, we used a VECTASTIN® Elite ABC kit containing biotin-conjugated anti-mouse/rabbit IgG, avidin DH and biotinylated horseradish peroxidase H, and a VECTOR® DAB substrate kit (3,3' diaminobenzidine tetrahydrochloride and H_2O_2), according to specification sheets.

Immunohistochemistry

Sections of *Bufo arenarum* and *Hyla nana* embryos and tadpoles were deparaffinized, hydrated, permeabilized with 0.1% Triton X100 in phosphate buffer saline (PBS) for 15 min, and treated with normal horse serum for 30 min. Normal serum was replaced by primary antibodies, and the sections were incubated overnight at 4ºC. The primary antibodies were removed followed by two rinses in PBS of 5 min each. Secondary antibodies were incubated for 30 min followed by a 5 min rinse in PBS. Sections were treated with 0.3% H_2O_2-methanol to block endogenous peroxidase for 1 hr and washed in PBS for 15 min. The sections were incubated in ABC for 30 min, and rinsed in PBS for 5 min. Color developed with DAB substrate in 30 seconds. Slides were mounted in Canada balsam (Biopack®, Bs. As). Controls were carried out by replacing the primary antibody with mouse non-immune serum. Samples were examined and photographed in a BX50 Olympus Microscope.

RESULTS

Expression of N-CAM during the development of *Bufo arenarum*

N-CAM was first detected at the stage 24-25 (operculum closed or complete, respectively). Figs. 1a and b show a strongly positive neural tube, at IV ventricle level, and the VII cranial ganglia, with negative heart, gills, notochord, otic vesicles and epidermal ectoderm. Similar immunolabeling was detected at stages 28 and 41, but at stage 41 a positive signal was also observed in the nerve terminals of the pharynx and heart (Figs. 1c, d and e).

Expression of N-Cadherin during the development of *Bufo arenarum*

N-Cadherin was not detected with the monoclonal antibody 13A9 from stage 3 to the opercular fold stage. At stages 24-25 and 28, N-cadherin was observed with similar distribution, but weaker signal than N-CAM (not shown). Additionally, at these stages, N-cadherin was found on the heart both on atrium septum and ventricle trabeculae (Fig. 2d). Tadpoles of stage 41 exhibited N-cadherin expression on neural tube, cranial ganglia, heart, and nerve terminals of the pharynx (Figs. 2a, b and c).

The GC-4 antibody gave a weak signal at the interface between the inner and the outer layer of the ectoderm at stage 19-20 (Fig. 3a), and in the immature skeletal muscle and pharynx nervous terminals at stage 41 (Figs. 3c, d and e).

We observed differences in the patterns of developmental expression of N-CAM and N-cadherin in neural cells. At stage 24-25, N-CAM (Fig. 1b) and N-cadherin (not shown) were localized on basolateral and upper membrane of the cells behind of ventricular epithelium. At stage 41, N-cadherin expression was strongest at the cellular tip close to the ventricular epithelium (Fig. 3b). N-CAM, while maintaining a cell surface distribution, also showed an incipient expression in ventricular cells (not shown).

Expression of N-CAM 180 during the development of *Hyla nana*

At stage 31, N-CAM immunoreactivity was not detected. At stage 41-42, the CNS was uniformly positive for N-CAM (Fig. 4). Immunolabeling

Fig. 1 - Cross sections of *Bufo arenarum*. **(a)** Expression of 180 kDa N-CAM in a stage 24-25 embryo. Observe the specific signal in the neural tube, at the IV ventricle level, and in the VII cranial ganglia. (X 13). **(b)** Detail of neural tube and VII cranial ganglia (arrow) at 24-25 stage. The immunolabeling is negative in the ventricular layer (X 66). **(c)** Expression of 180 kDa N-CAM in the neural tube at larval stage 41. Note also the incipient expression in nerve terminals of the pharynx and heart (small arrows) (X 13). **(d)** Detail of pharynx (X 66) and **(e)** detail of ventricle of larval stage 41 with positive signals present in the neuromuscular terminals (arrow) but absent in red blood cells (X 330). **nt:** neural tube; **ph:** pharynx; **h:** heart; **g:** gills; **nc:** notochord; **r:** red blood cells; **m:** myofibers.

Fig. 2 - Cross sections of *Bufo arenarum*. **(a)** N-cadherin expression at stage 24-25 in the heart (X 66). **(b)** Expression of N-cadherin in larval stage 41. Similar to N-CAM, N-cadherin immunolabeling is present in the neural tube, at IV ventricle level, in the VII cranial ganglia (arrow), nerve terminals of pharynx (small arrows) and myocardium (X 13). **(c)** Detail of N-cadherin expression on heart stage 41 larvae. Note its distribution on inter - atrial septum (arrows) and ventricle trabeculae (head arrows). The N-cadherin expression is maintained constant from stage 24-25 to 41 (X 66). **(d)** Control section of stage 41 larvae (X 13). **nt:** neural tube; **ph:** pharynx; **a:** atrium; **v:** ventricle; **r:** red blood cells.

Fig. 3 - Cross sections of *Bufo arenarum*. **(a)** Expression of N-cadherin in stage 19-20 embryo. Observe the weak signal in the interface between the inner and the outer layer of the ectoderm (small arrows). (X 133). **(b)** Detail of neural tube at stage 41 larvae. N-Cadherin expression changes from stage 19-20 to stage 41. At stage 41 N-cadherin is located at ventricular layer level (small arrows). (X 133). **(c)** Expression of N-cadherin in the pharynx of larvae at stage 41. Note that only nerve terminals have positive immuno-reaction but with different distribution of 180 kDa N-CAM at the same stage of development (thick arrows) (X 133). **(d)** Detail of skeletal muscle in stage 41 larvae. Observe N-cadherin down-regulation during skeletal myofiber differentiation (X 133). **(e)** Detail of skeletal muscle at stage 41 larvae. Note that the immunoreactivity is localized along the Z lines. (X 133). **nt:** neural tube; **mm:** mature myofibers; **im:** immature myofibers; **c:** cartilage.

was also found in the nares, eyes, nervous bundles, and the neuromuscular junction (Fig. 4 and 6a).

N-CAM expression was homogeneous in most regions of the CNS, including the telencephalon, diencephalon, rhomboencephalon and spinal cord (Fig. 4). There was a localized immunoreactivity in the ganglion and inner nuclear cell layers of the eye (Fig. 6a). A strong signal was detected in the sensorial cells around the olfactory pit (Fig. 6a).

Expression of N-Cadherin during the development of *Hyla nana*

Sagital sections of *Hyla nana* tadpoles were done through the medial pallium, the medial part of the diencephalon, rhomboencephalon and spinal cord. Contrary to N-CAM, N-cadherin expression was not uniform within the CNS. N-Cadherin was present on the olfactory pit and the olfactory bulb. At the telencephalon level, the immunolabeling in ventricular pallium was higher than in the medial and lateral pallium. Strong labeling was observed on the ventricular layer of the 3rd ventricle (Fig. 5). In the diencephalon habenular region, an immunopositive cell mass of the sub-habenular area was observed, lying ventral and lateral to the habenular nucleus proper (Fig. 5). Additionally, most of the thalamic neurons as well as the optic chiasma area showed N-cadherin expression. In the optic tectum, the three cellular layers had strong immunoreactivity. In the cerebellum, a strong signal was detected in the Purkinje cell layer. Rhomboencephalon and spinal cord were homogeneously immunolabeled (Fig. 5).

N-Cadherin was also expressed in the cell junctions of the remaining tail notochord, but not in

Fig. 4 - Sagital section of *Hyla nana* tadpole at stage 41-42. N-CAM is present in the nares, nerve terminals and in the CNS. **n:** nares; **on:** olfactory nerve; **gn:** gill nerve; **ot:** optic tectum; **cb:** cerebellum; **rb:** rhomboencephalon; **tl:** telencephalon; **sc:** spinal cord; **IV:** IV ventricle.

Fig. 5 - Sagital section of *Hyla nana* tadpole at stage 41-42. N-Cadherin is present in the nares and in the CNS. High expression is observed in the olfactory bulb, the optic tectum and Purkinje cells. **n:** nares; **ot:** optic tectum; **cb:** cerebellum; **rb:** rhomboencephalon; **tl:** telencephalon; **sc:** spinal cord; **IV:** IV ventricle; **ob:** olfactory bulb; **tha:** thalamus; **och:** optic chiasma; **nc:** notochord; **arrowheads:** presumptive vertebrae, **l:** liver.

the presumptive vertebrae (Fig. 5). A weak N-cadherin expression was observed in the esophagus and liver (Fig. 5).

At stage 42, a clear expression of N-cadherin was observed in the retina, particularly in the outer nuclear and ganglion cell layers (Fig. 6b). The myelinated nervous tracts were not labeled.

DISCUSSION

Studies on the expression of N-CAM and N-cadherin during the development of amphibians have been limited to *Xenopus* and Urodeles (Balak *et al.*, 1987; Levi *et al.*, 1987; Kitner, 1988; Choi *et al.*, 1990; Detrick *et al.*, 1990; Fujimori *et al.*, 1990; Geiger *et al.*, 1990a; Ginsberg *et al.*, 1991; Simonneau *et al.*, 1992; Becker *et al.*, 1993; Becker *et al.*, 1993). The development of nervous and muscle tissues depends mainly on the developmentally regulated expression of N-CAM-180 and N-cadherin. Therefore, we investigated the expression patterns of these two cell adhesion molecules during the development of two unrelated South-American anuran species, *Bufo arenarum* and *Hyla nana*. We found some similarities and differences with previous studies in amphibians in the developmental temporo-spatial expression of N-cadherin and N-CAM.

Our major findings are: 1) N-Cadherin, which is the most abundant neural cadherin, is not detectable in the earliest stages of *Bufo arenarum* development. It first appears at stage 19 (heart beat) - 20 (gill circulation) in the interface between the inner and the outer layer of the ectoderm, as detected with the GC-4 monoclonal antibody. Comparing our results with Simonneau *et al.* (1992), there seems to be a delay between the transcription and translation processes of N-Cadherin in the anuran brain. However, we cannot exclude that the differences may be due to differential developmental timing of the species. Our results confirm previous data showing that N-cadherin mRNA is not detectable in the absence of neural

induction. In experiments of ectoderm exposed to a heterologous neural inducer, it was found that this precedes the morphogenetic events associated with early neural development (Detrick *et al.*, 1990); 2) expression of N-cadherin in *Bufo arenarum* is restricted to the neural tube at stage 24 (operculum closed) - 25 (operculum complete), and continuously increases up to larval stage 41. However, we cannot exclude the existence of different N-cadherin isoforms of other cadherin not detectable with the antibodies used in this study. N-Cadherin expression in *Xenopus laevis* (Simonneau *et al.*, 1992) is high in the developing nervous system up to stage 42, and gradually diminishes, so at pre-metamorphic stage 50 (corresponding to stage 27 of *Bufo arenarum*), only a very low level of expression remains in the central nervous system. In contrast, in *Bufo arenarum*, N-Cadherin expression gradually increases from stage 24 - 25 up to stage 41. The different distribution pattern found with 13A9 and GC-4 anti-N-cadherin antibodies may be due to the presence of different isoforms of N-cadherin. Consistent with this, Tashiro *et al.* (1996) have recently obtained from a *Xenopus* tailbud cDNA library, a cDNA for a novel N-cadherin, named XmN-Cadherin (*Xenopus* maternally expressed neural cadherin). The authors postulated a dynamic role for XmN-Cadherin during development. Dissection experiments during the metamorphic and adult stages of *Xenopus* revealed that maternally inherited mRNA was relatively uniformly distributed within the embryo, but the zygotically expressed mRNA was present almost exclusively in neural tissues, including brain, the anterior part of the spinal cord, and the optic and otic vesicles; 3) as in *Xenopus laevis*, in *Bufo arenarum* and *Hyla nana*, N-cadherin is present in mesoderm derivatives such as the notochord (*Hyla nana*) and the heart (*Bufo arenarum*). However, N-cadherin was absent in the pronephros and the myotomes. In skeletal muscle, N-cadherin appears only during late differentiation stages. Additionally, in *Bufo arenarum*, the GC-4 antibody detected differential signals depending on the stage of dif-

Fig. 6 - Sagital section of *Hyla nana* tadpole at stage 41-42, at eye level. **(a)** N-CAM expression, **(b)** N-Cadherin expression **(c)** Control. **n:** nares; **small arrow head:** maxillary branch of the v cranial nerve; **g:** ganglionar layer; **in:** inner nuclear layer; **on:** outer nuclear layer; **small arrows:** sensorial cells.

ferentiation of skeletal muscle fiber bundles. Our data show that there are differences in N-cadherin expression patterns between anuran species, suggesting different roles in the development of the nervous system of non-related amphibians; 4) N-CAM-180, which has been associated with non-proliferating neurons, is not detectable in the first stages of *Bufo arenarum* development (Paz *et al.*, 1995), and first appears in the stages 24-25. N-CAM is uniformly expressed in the neural tube from stage 24 to stage 41, and at stage 41 is also expressed in the nerve terminals of pharynx, and heart. In contrast, N-CAM expression is variable in the olfactory system which exhibits a high cell turnover during adulthood (Becker *et al.*, 1993; Paz *et al.*, 1995). 5) N-CAM is absent in mesoderm derivatives of *Bufo arenarum* and *Hyla nana*, but is expressed in the neural tube and in the nervous terminals of pharynx, and heart. The high expression at cell contact sites supports the role of N-CAM-180 in cell contact stabilization (Becker *et al.*, 1993).

In summary, our results show the developing expression pattern of N-CAM and N-cadherin in two South-American amphibian species. The differences and similarities in the expression patterns of these two cell adhesion molecules during the development of unrelated amphibians suggest that they have still undefined specific developmental roles.

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