Pattern of neurofilament expression in the developing cochleovestibular ganglia *in vivo,* and modulation by neurotrophin 3 *in vitro*

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SUMMARY

Neurofilaments are the cytoskeletal intermediate filaments in neurons. They consist of three proteins (NFPs) with estimated molecular masses of 68, 160 and 200 kDa. The expression of NFPs by cells is regarded as a hallmark of neuronal differentiation and evolution. In the present study we analyzed the pattern of NFP expression in the neurons of the chicken cochleo-vestibular ganglion (CVG) during development and early post-hatching stages. We also investigated the expression of NFPs "in vitro" and their regulation by the neurotrophic factor NT3 to assess the participation of NFPs in neurotrophin-induced effects. The occurrence of NFP immunoreactivity (IR) "in vivo" and "in vitro" was studied using both Western-blot and immunohistochemistry on whole mount embryos, tissue sections and primary cultures.

The 68 kDa NFP subunit was expressed as from stage E4 to E10, while the 160 kDa NFP subunit was found in all developmental stages and on the early post-hatching days (P10). The 200 kDa NFP subunit was observed at later developmental stages from E10 to P30. The immunoreactivity for each NFP subunit detected in inner ear sections was consistent with the data obtained by immunoblotting. NFPs were localized in the neuronal perikarya and their processes, allowing us to establish the temporal pattern of innervation of the sensory neuroepithelia located in the inner ear. Primary cultures with NT3 reproduced the "in vivo" pattern of NFP expression. The present results demonstrate that NFP subunits are developmentally regulated, suggesting that they may be specifically involved in the maturation of CVG neurons. Furthermore, the findings obtained in cultured neurons point to a regulation of NFP expression by neurotrophin-3.

Key Words: Inner ear development – Neuronal cytoskeleton – Neurofilament proteins – Neurotrophins

INTRODUCTION

In mammalian tissues, different cell types are characterized by their different contents and specific types of intermediate filament proteins, a major cytoskeletal component that can be distinguished by immunological and biochemical criteria (Virtanen et al., 1985). Among these proteins, neurofilament proteins (NFP) are intermediate filaments that are characteristic of neurons (Hoffman and Lasek, 1975; Lazarides, 1982). Neurofilaments are the main intermediate filaments forming the cytoskeleton of differentiated and mature neurons (Fusch and Weber, 1994). They consist of "triplets" of three related proteins (neurofilament proteins, NFPs) with estimated molecular masses of 68, 160 and 200 kDa (for references, see Xu et al., 1994). Precise

knowledge of the embryonic time at which neuronal differentiation takes place, as well as of the factors controlling it, might be of capital importance in experimental and/or pathologic studies in which NFPs are involved. The cochleovestibular ganglion (CVG), which contains the neurons whose processes innervate the hair cells of sensory epithelia of the inner ear (Pujol, 1980; White and Warr, 1983), provides a good model to approach these topics and has been used successfully by some authors (for references see Represa et al., 1992; Represa et al.,1993; Vázquez et al., 1994). The extracelular factors involved in inner ear development include several neurotrophic factors belonging to the family of neurotrophins (see Barbacid, 1995; Chao and Hempstead, 1995) and these are candidates for the regulation of the organization of inner ear innervation: neuronal differentiation, neurite outgrowth, and synaptogenesis (Anniko et al., 1983; Ard and Morest, 1984; Pirvola et al., 1992; Van De Water et al., 1992; Avila et al., 1993; Represa et al., 1993; Vázquez et al., 1994). The present study was undertaken to analyze the temporal-spatial pattern of NFP expression during CVG development as well as the mechanisms underlying the regulation of NFPs by the neurotrophin-3 (NT3).

MATERIALS AND METHODS

Isolation and Preparation of Tissues

Hen-fertilized eggs (Cross strain), which were incubated for different periods of time (from 3 to 20 days) at 38°C and 80% relative humidity were used in this study. After incubation, the embryos were extracted and placed in petri dishes containing Hank's balanced salt solution. Then, the embryonic age (E) was determined (Hamburger and Hamilton, 1951); here, the embryonic time is referred to in hours or days. The whole cephalic region, the temporal bone primordia or the CVG were isolated from E4 to hatching, as described previously (Represa and Bernd, 1989; Represa et al., 1991). In addition, the same pieces were obtained from 10 —and 30 days— posthatching (P) chickens. The organs or tissue samples obtained were processed for histology and immunocytochemistry, while isolated CVG were used for culture or Western blot techniques.

Immunohistochemistry

Frozen sections were obtained from chick embryos at the stages mentioned above, and neuron-enriched cell cultures were used for light microscopical immunohistochemistry. Whole embryos were fixed in 4% paraformaldehyde (0.1M phosphate buffer, 4h, 4°C), rised in 0.1 M phosphate buffer saline (PBS), and frozen. Specimens were sectioned 20 μm thick, placed on gelatin-coated microscope slides, stored at 0-4°C until use, and processed for peroxidase immunohistochemistry as follows. The sections were washed with 0.1 M PBS for 1 h, treated for 30 min with PBS solution containing 1% bovine serum albumin, and 1% fetal calf serum. Thereafter, they were incubated overnight at 4°C with primary antibodies. The following commercially available mouse monoclonal antibodies, were used: 1) anti 68 kDa NFP (clone NR-4, Sigma, used diluted at 5 μg/ml). 2) anti 160 kDa NFP (clone NN-18, Sigma, used diluted at 5 μg/ml). 3) anti 200 kDa NFP (clone NE-14, Sigma, used diluted at 5 μg/ml). 4) anti pan-NFP (clone N-52, Sigma, used diluted at 5 μg/ml). Additionally, a rabbit polyclonal antibody which recognizes the phosphorylated and non-phopshorylated isoforms of all three NFP subunits (ref. N-4142, Sigma, used diluted 1 μg/ml) was used as control. Sections were then incubated with the secondary antibody for 1 h at room temperature, and the reaction developed using a commercially available kit (Kit Vectorstain, Vector), and DAB as chromogen. For control purposes, representative sections were incubated with preimmune mouse serum instead of the primary antibody, or the primary antibody was omitted in the incubation medium, and then processed as described above. Under these conditions no specific immunostaining was observed.

Immunohistochemistry was also performed in cultures of dissociated CVG. After different periods in culture (see below), the CVG cells were fixed in 4% paraformaldehyde (0.1M PBS, 4h, 4°C), rinsed in 0.1M PBS, and processed for immunocytochemistry as described for tissue sections. The antibodies used were clones NR-4, NN-18 and NE14. Cell measurements and counting were carried out using a Quantimed Image Analysis System (Quantimed 500MC, Leica).

Western blotting

Protein lysates from dissected CVG at the developmental stages indicated above were obtained (for details, see Vázquez et al., 1994). Pellets from dissected CVG were transferred to a protein extraction lysis buffer (containing 50mM Tris-HCl, 150mM NaCl, 5mM EDTA, 1% Triton-X-100 and 0.25% Na deoxycholate). Once the proteins had been extracted, the lysates were processed (Smith et al., 1985) using a Micro-BCA kit to determine the final concentration of protein in the extraction buffer for each stage. All protein samples were loaded at the same concentration of 20 μg of protein/well on a 8% SDS electrophoresis gel (SDS-PAGE), after which they were removed and blotted onto a nitrocellulose filter. Immunodetection was performed using only the above-mentioned rabbit polyclonal antibody at a dilution of 1 μg/ml. Blots were developed using the ECL Western blotting

detection system (Amersham), and quantified densitometrically with a laser densitometer (Helena) (for details see Represa et al., 1993).

Cultures of dissociated CVG

CVG isolated from E4 chick embryos were dissociated in neuron-enriched cell cultures and grown in cultures for different periods of time (ranging between 48 hours and 14 days). The dissected CVG were incubated for 25 min at 37°C in Earle's salt solution (Ca^{2+} and Mg^{2+}-free) containing 0.1% trypsin and 0.1% dispase. Dissociated single cells were obtained by gentle passage through a flame-polished Pasteur pipette. The dissociated cell suspension was enriched for neurons to 90-95% or more by the preplating technique of McCarthy and Partlow (1976). Neuron-enriched cell suspensions were seeded at a density of 4,000 cells per well, in 16 mm PORN/laminin coated microwell culture dishes. The culture medium was M-199 with 6 g/l glucose and 1% (v/v) heat-inactivated horse serum. Control cultures were maintained in M-199 medium with 6 g/l glucose and 5% (v/v) heat-inactivated horse serum.

The culture medium was further supplemented with recombinant NT-3 (# N-260, Alomone Labs Ltd. Israel) at a concentration of 5 ng/ml; this has a differentiating effect on CVG neurons (Avila et al., 1993). The culture medium was changed every 48 hours. Neurite outgrowth and neuronal survival were estimated after culture, according to the criteria of Davies et al. (1986). After different periods in culture, the cells were processed for immunohistochemistry as described above.

Results

"In vivo" studies

Immunoblotting analyses revealed that in the developing chick CVG there is a well defined chronological expression of NFP subunits, with up-or down-regulation in the expression of some of them, and no coincidence in the expression of all three NFP subunits (Fig. 1). During the earliest developmental stages studied (4E and 7E), 68 kDa NFP was prominent, being present only at residual levels at E10. Interestingly, this protein was co-expressed with 160 kDa NFP, whose maximum expression was reached by E10, and remained unvaried at low levels in the CVG during E14 and E18. After hatching (P10), the 160 kDa NFP subunit was only detectable at residual levels. Regarding the 200 kDa NFP subunit, its expression started at E10 and progressively increased with development, a maximum being reached after hatching by P10 and it was also detectable in significant amounts on P30. Microdensitometric analyses of

the immunoblots showed that the 68 kDa NPF subunit corresponded to 40-50% of the total amount of NFP at E4; 30% at E7, and only 10% at E10. With respect to the 160 kDa NFP subunit, the relative amounts were 50-60% at E4, about 70% at E7, 95-100% at E10, between 30-40% at E-14/E-18, and 10% at P10. Finally, regarding the 200 kDa NFP subunit, the estimated relative amounts were about 5% at E10, 45% at E14, 60-65% at E18, and 85% and 70% at P10 and P30, respectively (Fig. 2). Taken together, the results of the Western-blots suggest that the only subunit of NFP present in posthatching CVG neurons is that of 200 kDa.

In agreement with the findings from the immunoblotting study, specific immunostaining for each NFP subunit was obtained in whole mount embryos and tissue sections, an almost identical pattern of temporal expression being observed. In whole mount embryos, the earliest NFP expressed in the CVG was the smaller isoform of 68 kDa, which was also present in other sensory cranial ganglia of E3 chick embryos (Fig. 3). Likewise, in sections from E3 embryos, the immunoreactivity (IR) for the 68 kDa NFP subunit was localized in the developing brain stem and in a subpopulation of CVG cells mainly localized in the medial part (cochlear portion). Nevertheless, a faint IR was also visualized in a subset of cells located in the lateral part (vestibular portion) of the ganglion (Fig. 4). As development progressed (E4 and E7) the IR decreased in the cochlear portions while it increased in the vestibular one, being completely absent at E10 and later (data not shown).

Regarding the distribution of IR for the 160 kDa NFP subunit, the data obtained at E4 were identical to those described for the low NFP subunit, small processes at the opposite poles of the cells being visible at this time, with a length between 5 and 25-30 µm and occasionally reaching the neural tube or the otic vesicle. In E7 embryos, the CVG cells displayed an intense immunostaining, were ovoid in shape, and some of the neuronal processes, branched or dichotomized, were seen to run beneath the neurosensorial epithelia before entering it (Figs. 5, 6). Interestingly, the density of innervation was higher in the vestibular than in the cochlear epithelium (Fig. 7). This innervation was seen in all parts of the neuroepithelia with the exception of the distal ending of the cochlear duct. At E10, the only noticeable change was an increase in the branching of nerve fibers within the neurosensorial epithelia, and the start of the innervation of the distal part of the cochlear duct (Fig. 8). By E14, no substantial changes were found but the ends of the intraepithelial nerve fibers were dilated. The pattern of immunostaining with the antibody directed against the 200 kDa NFP subunit basically matched that described for

the medium NFP subunit on E14. In CVG, positive IR was observed in the neuronal somata (Fig. 9) as well as in their peripheral and central processes. However, immunolabelling was absent or was weak in the intraepithelial nerve fibers.

On E18 both afferent and efferent fibers were clearly immunolabelled for the 160 and 200 kDa NFP subunits, although the afferent fibers were more strongly labeled in the case of the 200 kDa subunit. At this time, the pattern of innervation of the neurosensorial epithelia was characterized by a dilation of the external endings of the intraepithelial nerve fibers and a typical "caliciform" morphology (Figs. 10, 11). Finally, on P10 and P30 the pattern of innervation was identical to that reported for later embryonic stages (data not shown).

"In vitro" studies

Dissociated neurons of CVG obtained at E4 were maintained for different times, between 1 and 26 days, in a culture medium enriched with NT-3 at a concentration of 5 ng/ml. As can be seen, the 68 kDa NFP subunit was observed from 1 to 6 days in culture, but the density of the cells displaying IR progressively decreased (Figs. 12, 15). Regarding the 160 kDa NFP subunit, the density of immunoreactive neurons first increased, maximal values being reached at 6 days, and thereafter progressively decreased, although it was present in all samples analyzed (Figs. 13, 15). Finally, cells showing 200 kDa NFP were detectable only after 10 days in culture and their numbers increased with time (Figs. 14, 15).
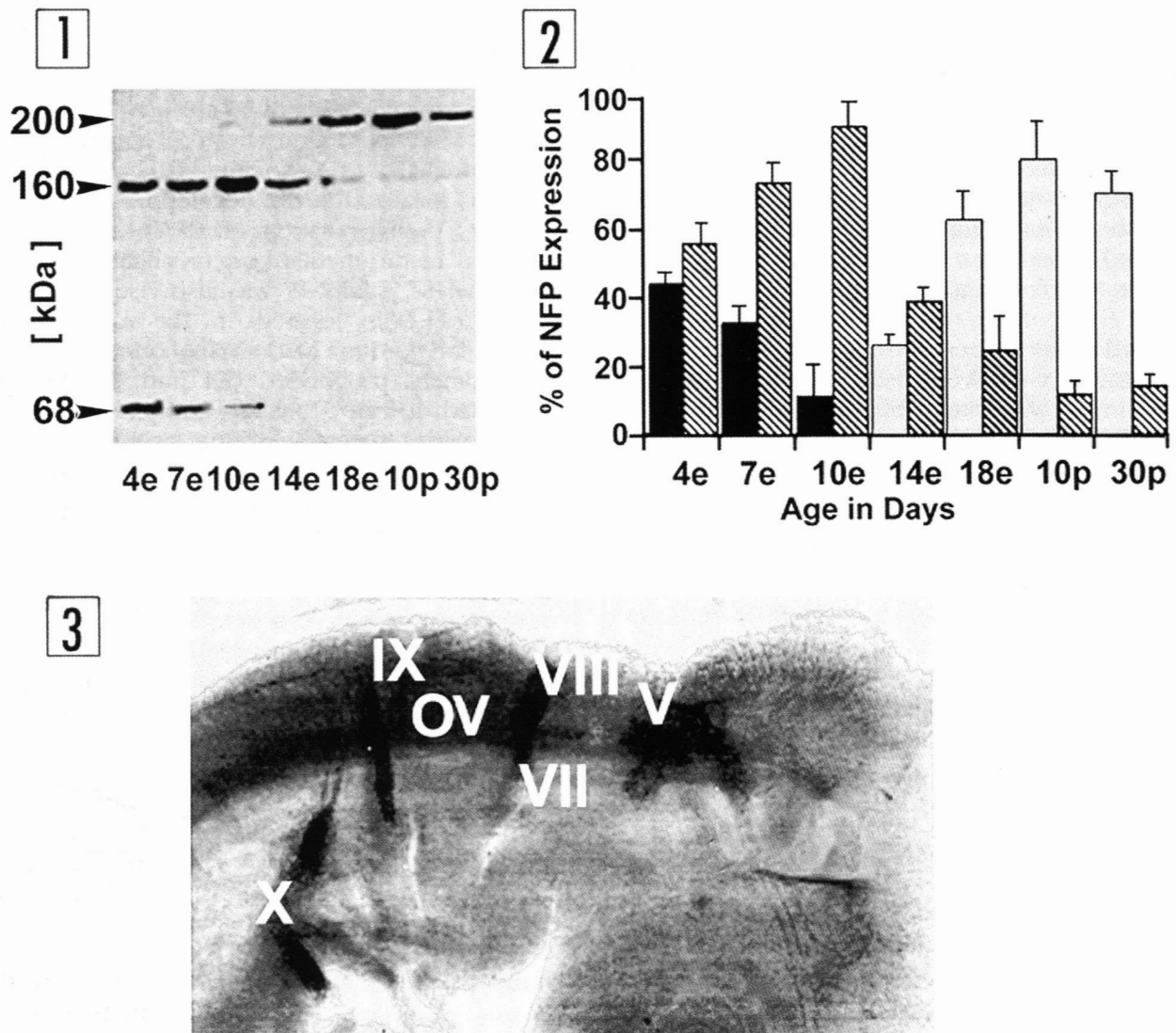

Fig. 1.- Western blot detection of NFP immunoreactivity in a developmental series of cochleovestibular ganglia (CVG) of embryonic (e) and post hatching (p) days. In the CVG protein extracts, the 68, 160 and 200 kDa molecular weight bands are stained. The immunoblotting analysis revealed a well defined chronological expression of NFPs subunits and no parallelism in their expression.

Fig. 2.- Densitometric analysis of the NFP detection in immunoblots showing the changes in relative immunoreactivity of NFP isoforms at different embryonic (e) and post-hatching (p) ages. The mean of each value was obtained from densitometric scanning of 3 Western blots. Changes of the distinct NFP isoforms are respectively represented by black bars (68 kDa), hatched bars (160 kDa) and gray bars (200 kDa).

Fig. 3.- Immunostaining for the 68 kDa NFP isoform in a 3-day chick embryo using the whole mount technique. The 68 kDa NFP is present in the cochleo-vestibular ganglion (VIII), otic vesicle (OV) and other sensory cranial ganglia (V, VII, IX and X). x15.

Fig. 4.- Light micrograph from a frontal section made through the head of 3-day chick embryo. Tissue sections were processed with a 68-kDa NFP antibody. The immunoreactivity (IR) appears localized in the developing neural tube (nt) and in a population of CVG cells (arrow). Scale Bar = 250 μm.
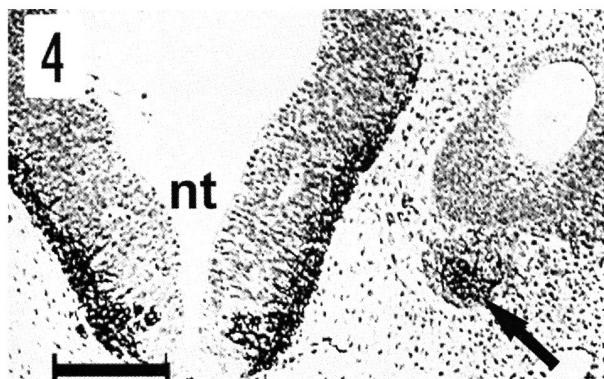
Fig. 5.- Light micrograph from a parasagittal section through the inner ear of a 7-day chick embryo. Tissue sections were processed with a 160-kDa NFP antibody. Neuronal processes running underneath the sensory epithelium (se) display intense NFP immunoreactivity. Scale Bar = 200 μm.
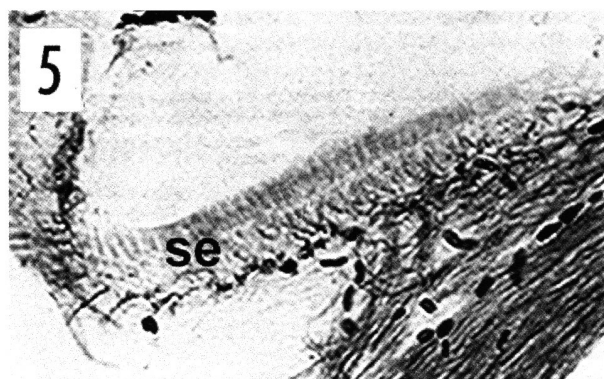
Fig. 6.- Light micrograph from a parasagittal section through the inner ear vestibule of a 7-day chick embryo. Tissue sections were processed with a 160-kDa NFP antibody. The NFP stained neuronal processes branches beneath the macular epithelium (me). Scale Bar = 125 μm.
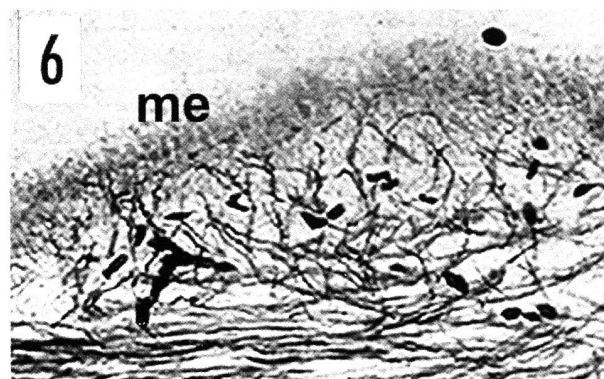
Fig. 7.- Light micrograph from a parasagittal section through the cochlear duct of 7-day chick embryo. Tissue sections were processed with a 160-kDa NFP antibody. NFP-stained neuronal processes can be observed entering the cochlear epithelium (ce). At this 7-day stage, the 160 kDa NFP-stained fibers innervate the entire cochlear epithelium with the exception of the distal end of the cochlear duct. Scale Bar = 75 μm.
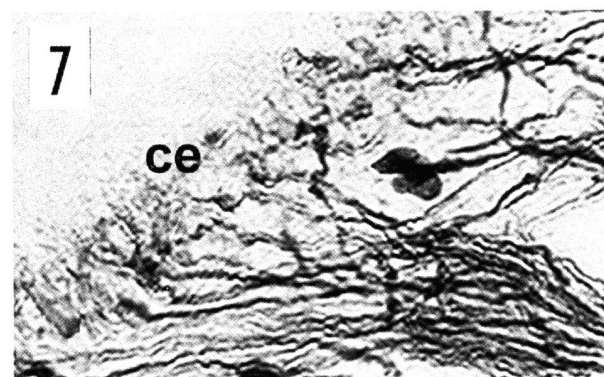
DISCUSSION

The present study was undertaken to analyze the developmental changes in NFPs during inner ear CVG development and to assess whether the ear regulatory factor NT3 has any effect in controlling the expression of neurofilaments. The occurrence of NFP immunoreactivity (IR) was studied "in vivo" using both Western-blot and immunohistochemistry on whole mount and tissue sections. The data obtained from these studies demonstrate that different isoforms of NFPs are present in the cochleovestibular neurons throughout development and postnatal stages. The 68 kDa NFP subunit was found during very early embryonic stages, from E4 to E10, being down-regulated later on. By contrats, the 160 kDa NFP isoform was found in most of the embryonic developmental stages being down-
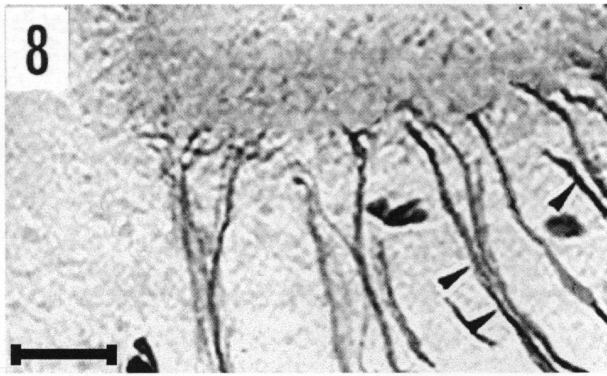
Fig. 8.- Light micrograph from a parasagittal section through the cochlear duct of 10-day chick embryo. Tissue sections were processed with a 160-kDa NFP antibody. In a 10-day chick embryo, NFP-stained fibres (arrowheads) begin to innervate the distal part of the cochlear duct sensory epithelium. Scale Bar = 75 µm.
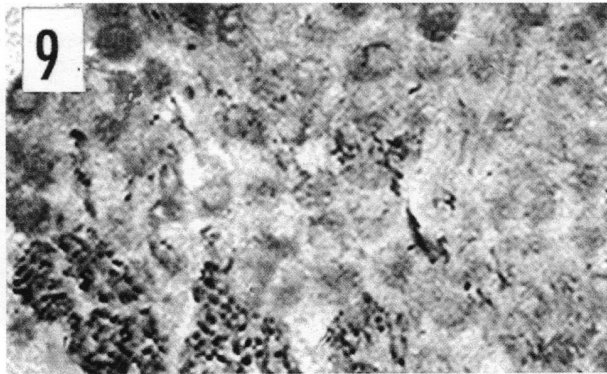

Fig. 9.- Light micrograph from a section through the cochleovestibular ganglion (CVG) of a 14-day chick embryo. Tissue sections were processed with a 200-kDa NFP antibody. In 14-day chick embryos, NFP immunoreactivity can be observed in the neuronal bodies within the ganglion. Scale Bar = 75 µm.
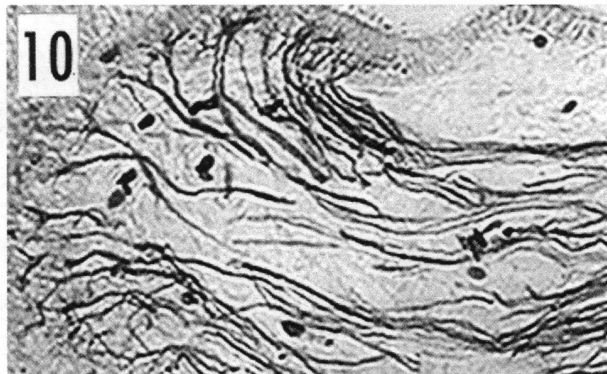

Fig. 10.- Light micrograph from a section through the vestibule of an 18-day chick embryo. Tissue sections were processed with a 200-kDa NFP antibody. In 18-day chick embryos, both afferent and efferent fibres are clearly immunolabelled for 200 kDa NFP. Scale Bar = 100 µm.
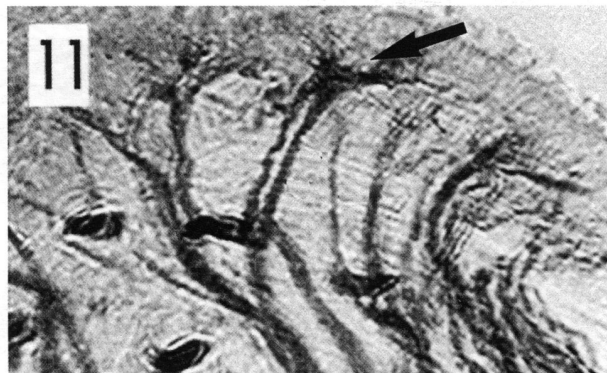

Fig. 11.- Higher magnification of figure 10. In 18-day chick embryos the ends of the intraepithelial nerve fibers are dilated, to give rise to a typical calyx-like morphology (arrow). Scale Bar = 50 µm.

regulated postnatally, while the 200 kDa NFP subunit was well expressed during later developmental stages and postnatal life up to P30. The present results demonstrate that NFP subunits are developmentally regulated "in vivo", suggesting that they may be specifically involved in the different maturation steps of CVG neurons, such the innervating of target cells.

These results can be correlated with the observed accumulations of NF immunoreactive material in the trigeminal, nodose, petrous and cervical dorsal root ganglia in mice, which starts on around gestation day 17 in the rat embryo and is limited to some neurons (Ayer-Lelievre et al., 1985). The increase in NF immunoreactivity has been tentatively attributed to specific cell types selected for their morphology or for their function or their physiological state (Philippe and Droz, 1988; Rambourg et al., 1983; Romand et al., 1988). In adult rats, these two neuronal
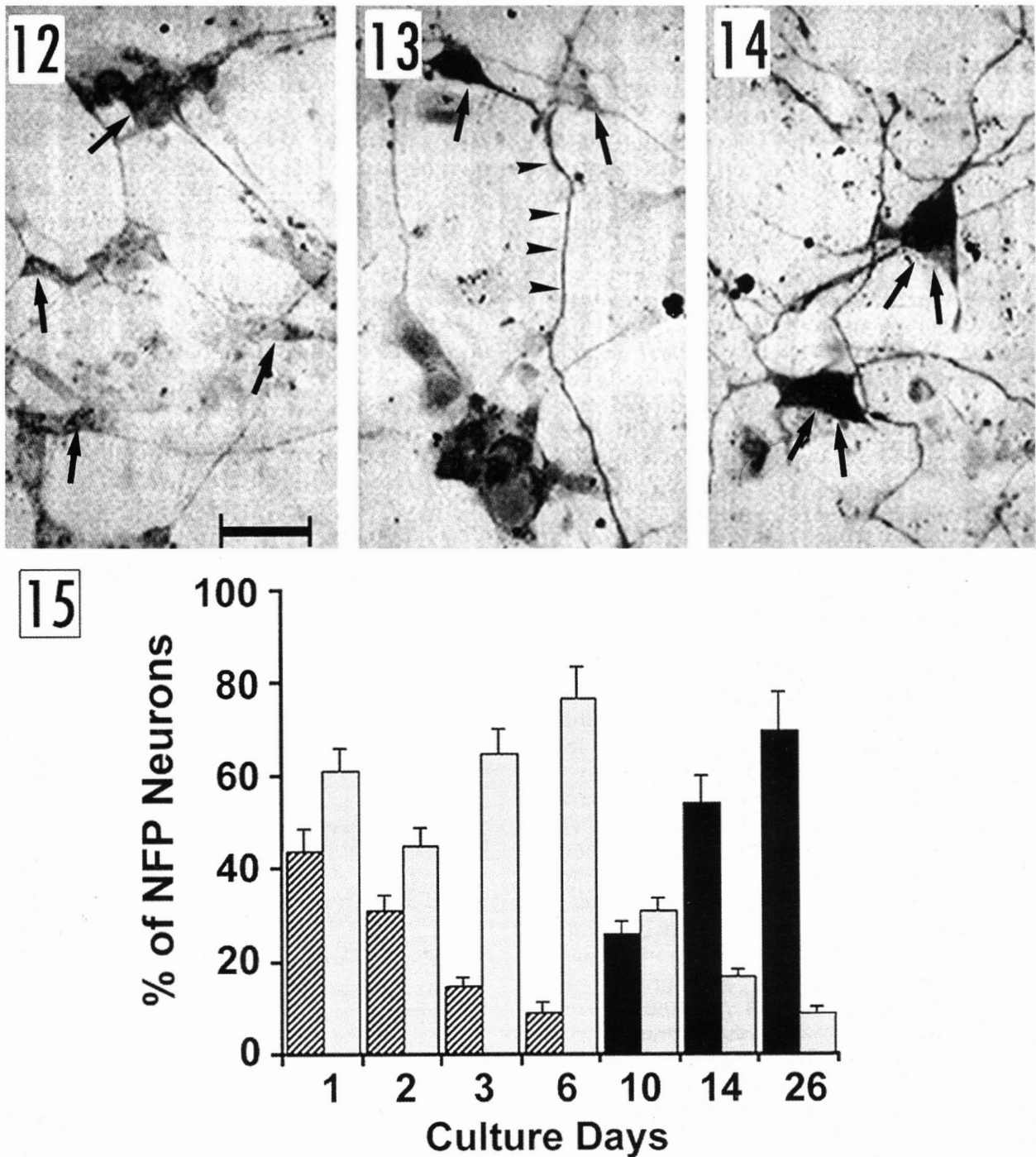
Fig. 12.- Light photomicrograph of neuron-enriched dissociated cell culture stained for the 68 kDa NFP subunit after 2 days "in vitro". CVG dissociated neurons were obtained from a 4-day chick embryo and maintained in culture medium with NT-3 (5 ng/ml). Neurons expressing different levels of 68 kDa NFP can be observed (arrows). Scale Bar = 75 μm.

Fig. 13.- Light photomicrograph of neuron-enriched dissociated cell culture stained for the 160 kDa NFP subunit after 6 days "in vitro". CVG dissociated neurons were obtained from a 4-day chick embryo and maintained for 6 days in culture medium with NT-3 (5 ng/ml). Neurons expressing different levels of 160 kDa NFP can be observed. NFP immunoreactivity appears located in the neuronal perikarya (arrows) and extend the entire length of the neurites (arrowheads). Scale Bar = 75 μm.

Fig. 14.- Light photomicrograph of neuron-enriched dissociated cell culture stained for the 200 kDa NFP subunit after 26 days "in vitro". CVG dissociated neurons were obtained from a 4-day chick embryo and maintained in culture medium with NT-3 (5 ng/ml). Surviving neurons grown for ten or more days in the presence of 5 ng/ml of NT3 showed heavy immunostaining (arrows) when labeled with 200 kDa NFP. Scale Bar = 75 μm.

Fig. 15.- Cell counting analysis showing the changes in the number of neurons expressing the different NFP subunits "in vitro". CVG dissociated neurons were obtained from 4-day chick embryo and maintained in culture for 1 to 26 days in the presence of 5 ng/ml of NT3. Cultures were processed for immunocytochemistry with specific antibodies against the 68, 160 and 200 kDa NFP (clones NR-4, NN-18 and NE14). Cell measurements and counting were carried out using a Quantimed Image Analysis System (Quantimed 500MC, Leica). Neurons expressing the 68 kDa NFP subunit were observed from 1 to 6 days (hatched bars). The 160 kDa NFP subunit (gray bars) was detected in neurons of all periods in culture analyzed. Expression of 200 kDa NFP (black bars) was detectable in neuron-enriched cell cultures from 10 days "in vitro" onwards.Expression of 200 kDa NFP (black bars) was detectable in neuron-enriched cell cultures from 10 days "in vitro" onwards.

sub-populations have also been observed in the vestibular ganglion (Hafidi and Romand, 1989); the intensely immunoreactive perikarya could correspond to larger neurons which display a large number of neurofilaments in their cell bodies when observed by electron microscopy (Rosenbluth, 1965). The present results provide a closer analysis and allow quantifying the onset and modification of the different neurofilament protein sub-units during maturation of the statoacoustic ganglion cells. Studies in other neuronal systems have shown that the immunoreactivity of neurofilament subunits can be altered in response to changes in the neuron, such as development, aging, degeneration and regeneration (Bignami et al., 1986; Drager and Hofbauer, 1984; Goldstein et al., 1987; Sternberger et al., 1985). In the mouse retina, for example, a population of ganglion cells, with low neurofilament immunoreactivity in their somas becomes intensely immunoreactive after axotomy. Such a response could result in modifications to the synthesis and degradation rates of neurofilament proteins (Goldstein et al., 1987). Differences in immunoreactivity could also arise from changes in the post-translational modification of neurofilament subunits, such as phosphorylation or dephosphorylation, which could affect the affinity of the antibody for the subunit (Drager and Hofbauer, 1984).

On the other hand, NFP expression observed "in vitro" demonstrated that NT3 is able to mimic the "in vivo" developmental regulation of these cytoskeletal proteins in parallel with a neurite-induced effect. NT3 is known to behave as a trophic factor for some neuronal populations, which is also the case for cochleo-vestibular neurons that are considered to be primarily of placodal origin (D'Amico-Martel and Noden, 1983). Dissociated CVG neuron-enriched cell cultures revealed neurite outgrowth and neuronal survival in the presence of exogenous NT3. The present results are in agreement with previous studies on CVG neurons, which in culture had been induced to form neurite-like processes by treatment with the neurotrophins of the NGF family, including NT3 (Represa et al., 1991, 1993; Avila et al., 1993; Vázquez et al., 1994). Experiments by Pirvola et al. (1992) using in situ hybridization have revealed the expression of NT3 mRNA in the developing sensory epithelium of the mouse inner ear. According to the analysis of null mutant mice, the NGF family of neurotrophins emerge as molecules that are required for inner ear innervation (Ernfors et al., 1995; Schimmang et al., 1995).

The present study shows that the cytoskeletal protein NFP is differentially affected "in vitro" by the presence of the growth factor NT3. We have demonstrated here that NFP increases its expression in cultured CVG neurons after the addition

of NT3, in parallel to NT3-induced neurite outgrowth. Although no previous relationship between NFPs and neurotrophic factors has been reported in developing sensory ganglia, Jacobs and Stevens (1986) have shown that after Nerve Growth Factor treatment PC12 cells become more resistant to microtubule depolarization. Furthermore, NGF can regulate NFP expression in PC12 cells in parallel to a neurite-induced effect (Greene et al., 1983; Brugg and Matus, 1989).

Taken together, the above observations suggest that NFP may be a key protein in inner ear development, which would be involved in neurotrophin-induced cytoskeletal arrangement during the neurite outgrowth of auditory neurons to reach their central targets. Therefore, neurofilament proteins are promising indicators for the study of changes that may occur under normal or pathological developmental conditions in the inner ear.

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