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Original Article

Differential expression of microtubule associated protein MAP-2 in developing cochleovestibular neurons and its modulation by neurotrophin-3

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ABSTRACT Microtubule associated proteins (MAPs) are essential cytoskeletal proteins in developing neurons. The present study was undertaken to analyze the expression of MAP2 and its isoforms (a,b,c) during the embryonal and early post-hatching development of chicken cochleovestibular ganglion (CVG) neurons. Moreover, we have investigated MAP2 expression in primary cultures of CVG neurons, and whether it is regulated by neurotrophin-3 (NT3). The expression of MAP2 immunoreactivity (IR) was studied using both Western blot and immunohistochemistry on tissue sections and primary cultures. *In vivo* MAP2c was expressed from incubation day 4 (E4) to E10, and MAP2b was found in all embryonal stages studied and at post-hatching day 10 (P10), whereas MAP2a was restricted to the post-hatching periods. The cellular localization of IR was in the neuronal perikarya and their peripheral processes (dendrites) but not in axons. Primary cultures matched the *in vivo* pattern of MAP2 expression, and IR was localized in neuronal cell bodies and the initial segment of the neuronal processes. Exogenous NT3 regulated the expression of MAP2 isoforms in a dose dependent manner. At the survival dose of 0.5 ng/ml NT3, the main MAP2 expression was MAP2c. Conversely, at the neuritogenic dose of 5 ng/ml NT3 increased MAP2b and MAP2a expression, but not MAP2c. The present results demonstrate that MAP2 isoforms are developmentally regulated, thus suggesting that each isoform is specifically involved in CVG neuron maturation. Furthermore, we provide evidence of MAP2 regulation in culture by the neurotrophic factor NT3.

KEY WORDS: *inner ear development, cochleovestibular ganglion, microtubule associated proteins, neurotrophin-3, chick embryo*

Introduction

Microtubule associated proteins (MAPs) play important roles in the development and maintenance of the nervous system (Matus, 1988, 1991; Tucker, 1990; Johnson and Jobe, 1992). Among these, MAP2 is the most abundant in the brain. It consists of a pair of proteins with approximate molecular masses of 280 and 260 kDa, denominated MAP2a and MAP2b, respectively. In addition, MAP2c has been isolated in the embryonic brain with an estimated molecular weight of 68-70 kDa (Riederer and Matus, 1985). Furthermore, it was recently demonstrated that this low molecular MAP2 is composed of two forms, one neuronal (MAP2c properly) and the other glial (MAP2d) (Doll *et al.*, 1993).

During development of the mammalian central nervous system (CNS) MAP2c is expressed in early embryogenesis and gradually disappears around the time of synaptic maturation, when neurite outgrowth declines and the cytoskeleton becomes more stable (Riederer and Matus, 1985; Doll *et al.*, 1993). Concurrently, the levels of the other MAP2 isoforms increase and remain stable in mature neurons (Riederer and Matus, 1985; Tucker *et al.*, 1988), as sometimes occurs in MAP2d levels too (Doll *et al.*, 1993). In fact, MAP2a appears at the end of the differentiation process, while MAP2b is found rather early and increases in concentration during maturation (Riederer and Matus, 1985). Regarding cellular localization of these proteins, MAP2a and MAP2b are localized in dendrites and absent in axons (Bernhardt and Matus, 1984), whereas MAP2c is present in developing axons (Tucker *et al.*, 1988). The cell distribution of the corresponding MAP2a and MAP2b mRNAs is consistent with that of

Abbreviations used in this paper: CVG, cochleovestibular ganglion; NT3, neurotrophin-3; MAP2, microtubule associated protein type 2; IR, immunoreactivity.

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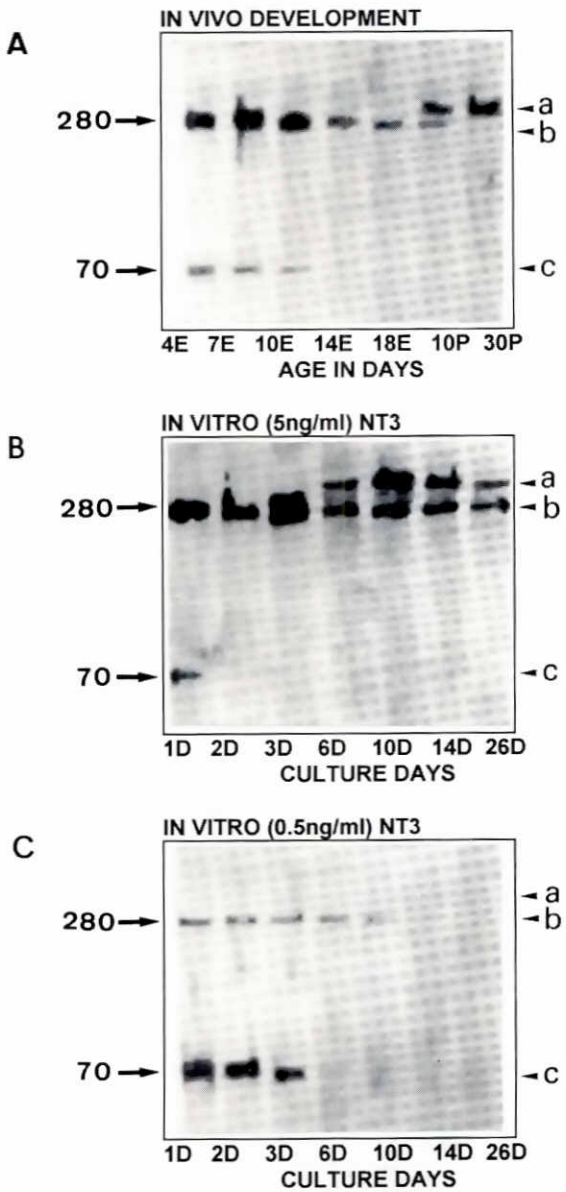

Fig. 1. MAP2-like protein immunoreactivity during normal development of cochleovestibular ganglia (CVG) and in cultured cochleovestibular neurons. *SDS-polyacrylamide gradient electrophoresis of CVG and cells transferred to nitrocellulose and immunostained with anti-MAP2a,b,c monoclonal antibody (clone HM-2, Sigma). The developmental stages are indicated as embryonic (E) or posthatching (P) days, whereas time in vitro is represented as culture days (D). Two MAP2 isoforms with close M_r of 280 kDa (arrow) are indicated by "a" and "b", while the lower Mr band of 70 kDa corresponds to the "c" isoform.* **(A)** *MAP2 expression in Western blot of an E and P day series of CVG during normal in vivo development. 280 kDa and 70 kDa molecular weight protein bands stained CVG samples with α MAP2 antibody. MAP2 isoform expression was stage-dependent; MAP2b was expressed throughout development, while MAP2c and MAP2a were restricted to an early and posthatching stages, respectively.* **(B and C)** *Western blot of the expression of MAP2 isoforms in cochleovestibular neurons throughout various periods in culture from 1 (1D) to 26 (26D) culture days. Immunoblots from CVG neuron-enriched cultures supplemented with 5* **(B)** *or 0.5 ng/ml* **(C)** *of exogenous NT3 show differences in MAP2 "a", "b" and "c" isoforms expression which were related to time in culture and NT3 concentration.*

the proteins (Garner *et al.*, 1988b; Landry *et al.*, 1994), whereas that of MAP2c is found in the neuronal perykarya but not in the axons (Papandrikopoulou *et al.*, 1986).

The majority of the above data were obtained from mammalian CNS, while little is known about the expression and distribution of MAP2 isoforms in the peripheral nervous system, including the cochleovestibular ganglion (CVG), especially during development (Hafidi *et al.*, 1992; Riederer and Barakat-Walter, 1992). Nevertheless, the ability of MAPs to bind and assemble elements of the cytoskeleton and to form cross-links between them is fundamental to neuronal morphogenesis (Tucker, 1990; Matus, 1991; Johnson and Jobe, 1992).

This study was undertaken to analyze the role of MAP2 isoforms during the development of chicken CVG neurons. We document the temporal and spatial patterns of MAP2 isoform expression and localization *in vivo* as well as *in vitro*. Moreover, we show evidence for a modulatory effect of NT3 (Barbacid, 1994, 1995) in regulating MAP2 expression in cultured peripheral neurons. This model provides an excellent means for the study of the role of neurotrophic factors in the regulation of neuronal cytoskeletal proteins.

Results

Expression of MAP2 isoforms in vivo

Western blot

In the homogenates of chicken CVG obtained at different embryonal (E) and posthatching (P) ages, immunolabeling with the monoclonal antibody HM-2 showed developmental changes in the expression of MAP2 isoforms (Fig. 1A). Detectable, but low levels of MAP2c, regarded as the embryonic isoform of this protein (estimated molecular mass of 70 kDa) were found exclusively between E4 and E10. Concomitantly, there was also MAP2b expression (estimated molecular mass of 280 kDa). The amount of MAP2b reached a maximum level at E7, and then progressively decreased from E7 to E18, also being detectable in the early posthatching period (E10) (Fig. 1A). MAP2a (estimated molecular weight of 280 kDa) was exclusively found in posthatching animals, being the only MAP2 isoform present in the CVG of 30 day (P30) animals (Fig. 1A).

The densitometric analysis of three Western blots (Fig. 2A) revealed: a) a decrease of MAP2c between E4 and E7; b) an increase in the amount of detectable MAP2b between E4 and E7, and progressive after-hatching decrease; c) MAP2a detection only in the posthatching period, and increasing with age.

Immunohistochemistry

Specific immunoreactivity (IR) for MAP2 was found in CVG sections in all examined embryos and posthatching animals. Nevertheless, IR localization depended on the antibody used and the developmental stage. At E4 MAP2 IR was found segregated to neurons localized in the ventro-lateral portion of the ganglion (Fig. 3A,B). IR was found exclusively in the neuronal perykarya, whereas both the central and peripheral processes of these neurons were unlabeled (Fig. 3B,C). The labeled cells were the large ones which develop from the otic vesicle and correspond to differentiating neuroblasts (Fig. 3C). On the contrary, the neural crest-derived small cells were unreactive. This early IR pattern was observed with all antibodies used, including that which recognizes only MAP2c. These observations suggest that IR in this developmental stage is due, at least in part, to MAP2c.

At E7 (Fig. 3D,E,F) there was an increase in the number of immunoreactive cells with antibodies recognizing MAP2a and MAP2b, and there was no segregation in the distribution of the immunoreactive neurons. Moreover, positive IR was also found in the peripheral processes of the labeled neurons, whereas those projecting to CNS remained unreactive. The pattern of immunostaining observed with the monoclonal antibody against MAP2c was almost identical, suggesting again that at least a part of IR can be due to this MAP2 isoform.

Figure 4 illustrates MAP2 immunoreactivity when development increased. At E10, MAP2c immunoreactivity highly decreased in the neuronal perykarya, whereas that for MAP2a and MAP2b remained strong in both neuronal somata and peripheral (dendrite) cell processes but totally absent in the central (axonal) process. In the analyses at later stages of development, this cellular distribution pattern remained basically unchanged. However, after E14 there was a generalized decrease in the number of immunolabeled neuronal somata as well as in the intensity of immunostaining, without variations in the immunostaining intensity within the dendritic arbor. Finally, at E18 IR was low and the number of perykarya displaying MAP2 was very scarce. A decrease in the labeling of dendrites was also noticeable.

After hatching (P10 and P30) the percentage of immunoreactive somata was low but the IR was strong in the distal tip (intraepithelial fibers) of the dendritic processes. Furthermore, as in the embryonal period, no IR for any MAP2 isoform was observed in the central processes of the CVG neurons.

Interestingly, during a part of the embryonal period studied MAP2 IR was also found in an area of the brainstem which will develop as the nuclei tangenzialis and magnocellularis, which are targets of the central processes of the CVG neurons (Fig. 4D,E). In older embryos (E18) the pattern of immunostaining was consistent with axonal labeling. However, since we have never found MAP2 immunoreactivity in the central processes of CVG neurons they cannot be regarded as afferents from CVG. Probably the MAP2 IR fibers found in these nuclei come from CNS structures and not from the periphery.

Expression of MAP2 isoforms in vitro

The expression of MAP2 immunoreactivity was also investigated in primary 12 h cultures of CVG neurons (supplemented just with fetal bovine serum), which were obtained at different periods of incubation or postnatal life, ranging from E4 to P30. In these conditions approximately 90% of dissociated cells were identified as neuron cells. In E4 cultured neurons 38.8% of the cells expressed MAP2c and 58% displayed MAP2b. In explants from E7, only 18% of the nerve cells displayed MAP2c and 79% showed MAP2b. When the time increased until E10, MAP2c expression disappeared and 58.7% showed MAP2b. Finally, at E14 and E18 only MAP2b was detected, the percentage of immunoreactive cells being about 26.4% and 21.6%, respectively. No IR was obtained for MAP2a. In the post-hatching period, no MAP2c or MAP2b was found at P10 or P30. In contrast, there was a strong expression of MAP2a, which labeled 46% and 82% of the cells at P10 and P30, respectively (Table 1).

Regarding the cellular localization of IR, this was exclusively found in the somata at E4 and corresponded to MAP2c and MAP2b isoforms. At E7 IR was observed in addition to the somata in the initial segments of the cell processes. This pattern was identical for E10 and E14, but at times the distal tip of the cell

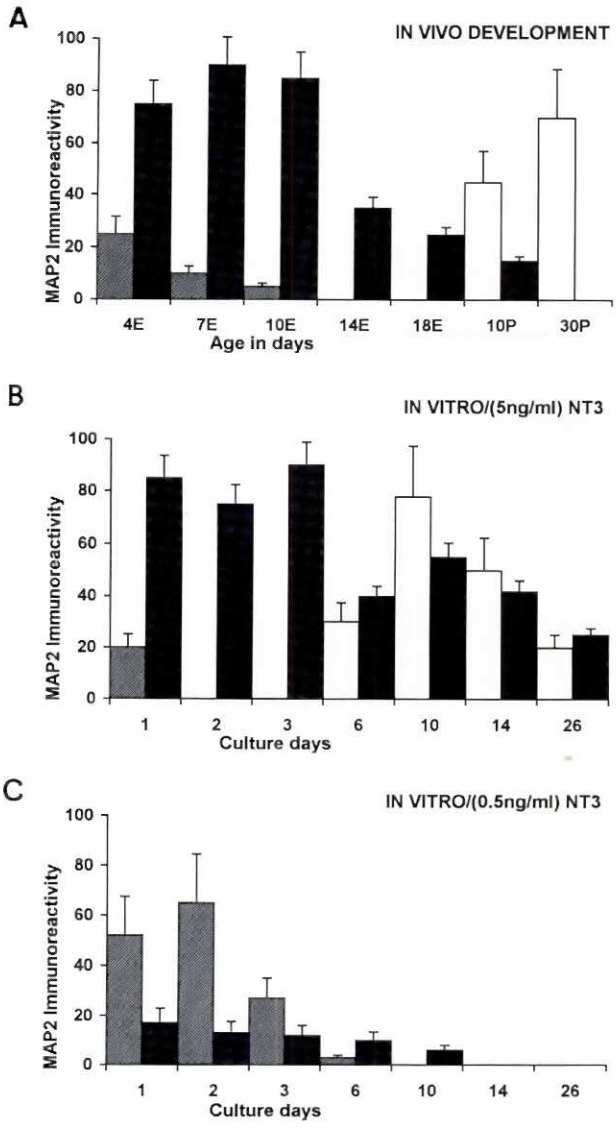

Fig. 2. Quantitative changes in relative immunoreactivity of MAP2 isoforms in chick CVG at different periods of time *in vivo* (A) and *in vitro*(B,C). *Densitometric values were obtained from scanning of 3 Western blots as those shown in Figure 1. The amount of changes of the isoforms "2a" (white bars), "2b" (black bars) and "2c" (hatched bars) are expressed as the mean ±SEM, of triplicate determination at each 3 different Western blots.* **(A)** *MAP2b expression in vivo was detected in most of the stages studied from 4E to 10P day samples, but no at 30P. Moderate or little MAP2c expression can be observed at 4E, 7E and 10E days, respectively, while samples from older stages were negative. The CVG samples from 10P and 10ED showed MAP2 immunoreactivity corresponding to the "a" isoform, which was restricted to these posthatching stages.* **(B)** *MAP2 expression in vitro at doses of 5 ng/ml NT3: MAP2c was detectable just in 1D cultures, whereas the predominant isoform from 1D to 6D was MAP2b. MAP2a expression was detected in 6-day cultured cells and thereafter their expression reached maximum values by 10 days in culture, which corresponds with E14 in vivo. Notice that NT3-induced MAP2a expression in vitro earlier than in vivo.* **(C)** *MAP2 expression in vitro at a dose of 0.5 ng/ml: cultured neurons expressed significant MAP2c for a 3-day period, whereas MAP2b was present at residual levels between 1D and 10D and MAP2a was undetectable. Note the correlation between MAP2 expression and time in culture with the presence of exogenous NT3.*

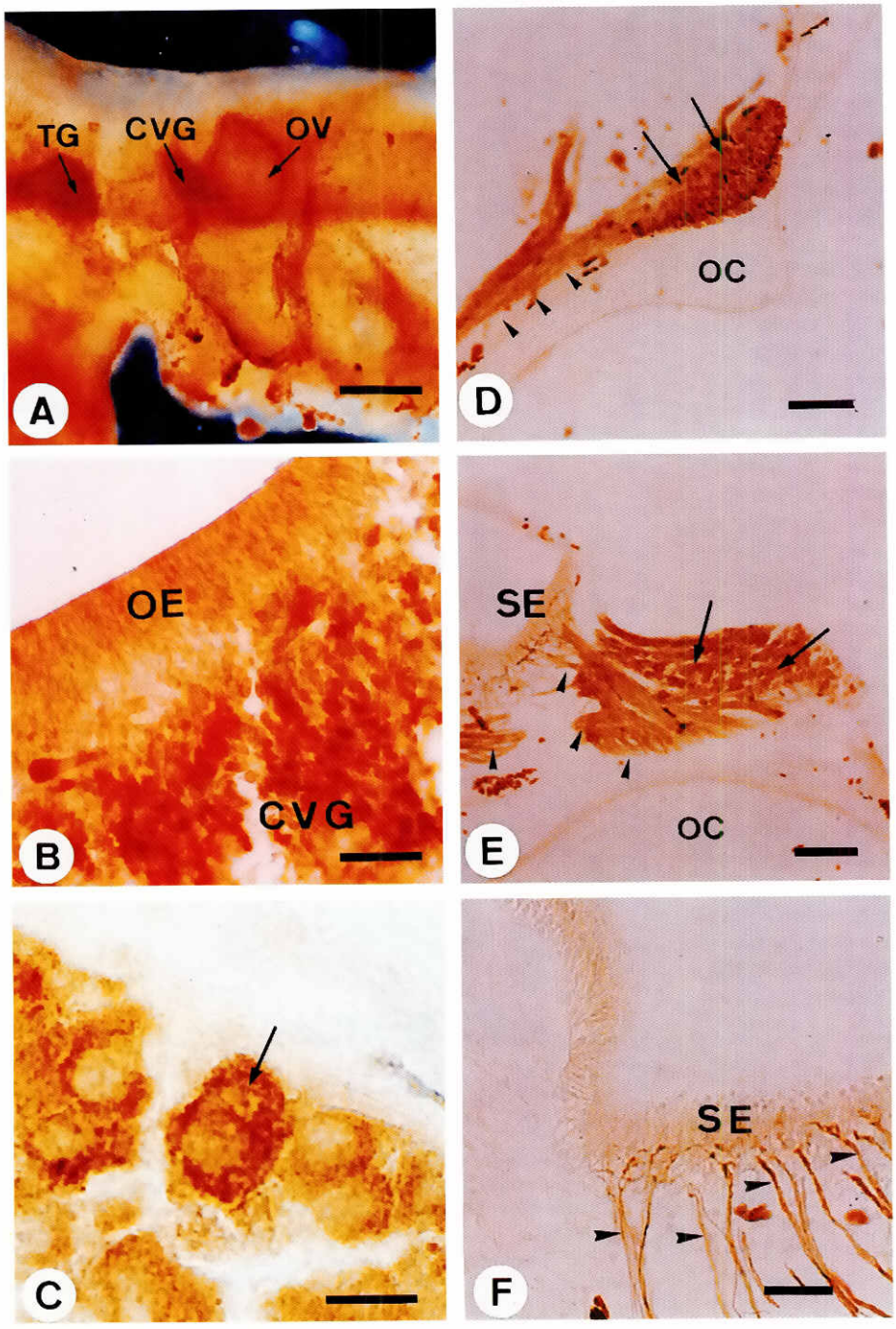

Fig. 3. MAP2 immunoreactivity *in vivo*. **(A)** *Whole-mount of a 4-day chick embryo processed with an α MAP2c antibody (clone B62). Trigeminal (TG) Cochleovestibular Ganglion (CVG) and Otic Vesicle (OV) in the cephalic region are shown at low magnifications. TG and CVG displayed positive MAP2 immunoreactivity.* **(B)** *Light micrograph of 25 μm parasagittal section through the CVG and otic epithelium (OE) of a 4-day chick embryo. Tissue sections were processed with α MAP2c antibody (clone B62). Note MAP2 immunoreactivity in the CVG.* **(C)** *The high magnification photomicrograph represents an area of the CVG, where immunoreactivity appeared in cell bodies of large neuroblast (arrow) but not in emerging neurites.* **(D,E,F)** *Light micrographs of 25 μm transversal section through the otic region of a 7-day chick embryo. Tissue sections were processed with α MAP2a,b,c antibody (clone HM-2, Sigma). Immunocytochemical localization of MAP2 in chick vestibular* **(D)** *and cochlear* **(E)** *ganglion showed strong immunoreactivity in cell bodies within the ganglia (arrows), and moderate immunoreactivity in dendrites projecting from the ganglion to the inner ear sensory epithelium (SE). No stained axonal projections can be observed between the CVG and brainstem throughout the Otic Capsulae (OC).* **(F)** *In higher magnification dendrites entering the sensory epithelium also displayed faint immunoreactivity (arrowheads). Bars: A, 300 μm; B, 100 μm; C, 25 μm; D,E, 200 μm; F, 125 μm.*

processes also resulted immunolabeled. This labeling was consistent with the expression of MAP2c and MAP2b since no labeling was found when MAP2a predominated (Table 1).

Regulation of MAP2 isoforms by NT3 in vitro

NT3 shows neurotrophic effects in CVG neuron-enriched cell cultures

The neurotrophic effects of NT3 were studied on dissociated, neuron-enriched cultures of cochleovestibular ganglia. The CVGs were isolated from E4, E7 and E9 chick embryos, dissociated and plated as neuron-enriched cell culture for various periods of time, ranging from 48 hours to 26 days. In these cultures neurons (seen as refringent cells) were intermingled with non-neuronal cells consisting of spindle-shaped Schwann cells and a few flattened fibroblasts.

In contrast to the lack of significant survival promoting effects of neurotrophin-free chemically defined medium (less than 2%), a vigorous response was observed upon addition of either 0.5 ng/ml or 5 ng/ml of exogenous NT3 to these neuron-enriched cultures of varying periods *in vitro* up to 26 days. The number of cochleovestibular neurons bearing processes in culture with NT3 was both

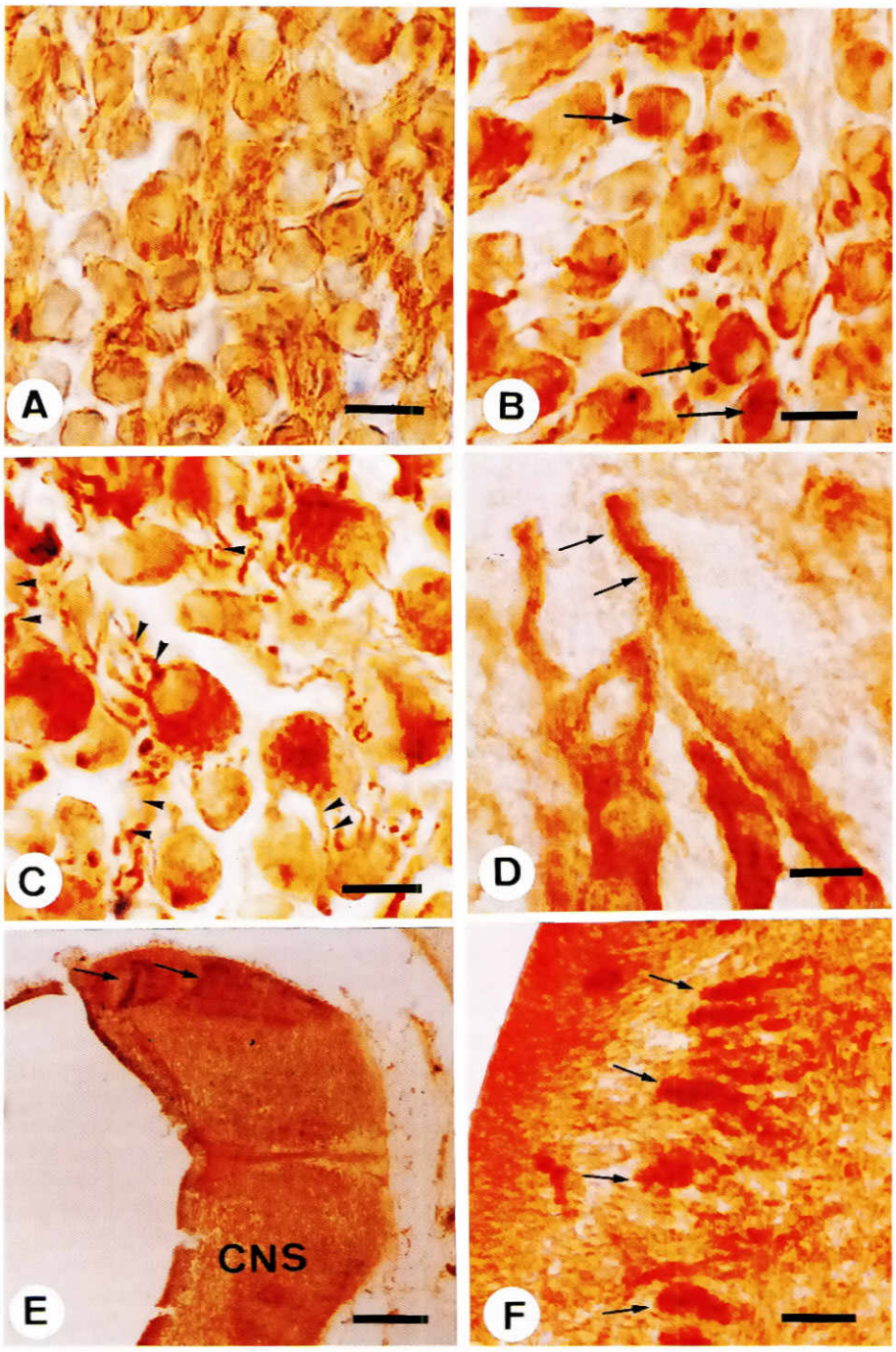

Fig. 4. MAP2 immunoreactivity *in vivo*. **(A,B,C,D)** *Light micrographs of 25 μm parasagittal section through the CVG of a 4-, 7-, 10- and 18-day chick embryo. Tissue sections were processed with α MAP2 antibody (clone AP-20, Sigma) which recognizes just the "a" and "b" isoforms but not the "c" isoform.* **(A)** *No cells displaying MAP2a and b immunoreactivity can be observed at 4 days.* **(B)** *Specific immunostaining appeared in the ganglion by 7E and MAP2 immunoreactivity was expressed at different levels in the neuronal perykarya (arrows).* **(C)** *At E10 MAP2 immunostaining was increased and nearly all cell bodies exhibited MAP2 immunostaining. Emerging neurites also displayed strong immunostaining (arrowheads).* **(D)** *From E18 and later stages MAP2 immunoreactivity as well as the number of positive cell bodies gradually decreased. Strong immunostaining was localized in dendrites (arrows), while neuronal bodies exhibit only moderate or low Immunoreactivity.* **(E)** *Light micrograph of 25 μm transversal section through the central nervous system (CNS) of an 18-day chick embryo at the level of cochlear and vestibular areas (arrows).* **(F)** *Immunocytochemical localization of MAP2 in the medulla oblongata of CNS at 18E is shown in higher magnification from "E". MAP2 protein immunoreactivity appeared on the cochlear nuclei (arrows). Bars: A,B,C,D, 30 μm; E, 200 μm; F, 75 μm.*

stage- and dose-dependent. The percentage of cells surviving and also extending long neurites in the presence of 5 ng/ml NT3 throughout development is presented in Figure 5 (A,B).

The developmental pattern of responsiveness of neuron enriched CVG dissociated cell cultures to NT3 closely parallels that observed in previous work (Avila *et al.*, 1993), with maximal effects between E7 and E14 (data not shown).

To examine the dose-response relationships of NT3 survival effects on CVG neurons, ganglia were isolated from E7 and E9 chick embryos, which are the stages of maximal response to NT3. Dissociated ganglion cell cultures were grown in the presence of NT3 at concentrations ranging from 0.1 to 10 ng/ml, and neuronal survival was assayed. A significant increase in neuronal survival was achieved at NT3 levels of 0.5 ng/ml. In the presence of 0.5 ng/ml NT3 many refringent cell bodies displayed the morphological features characteristic of neurons, and exhibited a spherical, ovoid or bipolar shape, from which small neurites extended and arborized. Additionally, an increase in surviving neurons and an active neuritogenesis response were observed upon addition of 5 ng/ml NT3 to cell cultures. The neurotrophic response of CVG neurons to exogenous NT3 reached saturation at 5 ng/ml (Fig. 5B).

TABLE 1

EXPRESSION OF MAP2 ISOFORMS IN CVG DISSOCIATED CELLS

	4E	7E	10E	14E	18E	10P	30P
Isoforms							
MAP2a						46%	82%
MAP2b	58%	79%	58.7%	26.4%	21.6%		
MAP2c	38.8%	18%					
Cell localization							
Perikarya	+	+	+	+	+	+	+
Processes	-	+	+	+	+	-	-

Expression of MAP2 immunoreactivity in primary newly dissociated CVG explanted for 12 h with fetal bovine serum. Each CVG was obtained at different embryonal or posthatching periods, ranging from 4E to 30P. Total CVG dissociated neurons were counted in fresh cultures as birefringent cell bodies; this total number has been taken as 100% of CVG neurons for each stage. Numbers in the table indicate the percentage of MAP2 immunoreactive neurons with respect to the 100% total neurons (previously counted in fresh cultures), while (-) or (+) indicate presence of absence of MAP2 immunoreactivity respectively within a specific cellular

MAP2 expression in CVG neuron-enriched cell cultures was culture time- and dose-dependent

NT3 was able to influence the expression of MAP2 isoforms in CVG cultures (Figs. 1B,C and 2B,C). At a dose of 0.5 ng/ml the cultured neurons mainly expressed MAP2c for a short time (72 h), MAP2b being present at residual levels for a long time (10 days). Immunostaining of the cultured neurons showed IR mainly in the neuronal somata and, occasionally, in the initial segment of their processes. Moreover, in comparing cultured cells of the same age it was observed that in young cultures almost all cells displayed MAP2c IR, and only a low percentage also immunolabeled with the monoclonal antibody against MAP2a and MAP2b.

At higher doses of NT3, 5 ng/ml, MAP2c was detectable only in 24-hour cultures, whereas the predominant isoform at 24 and 48 h was MAP2b. This isoform reached the maximum levels at 72 h, then remaining unchanged (26 days). Moreover, MAP2a expression was detected in 6-day cultured cells and thereafter, their expression reached maximum values at 10 days in culture, which corresponds with E14 *in vivo*. Thus NT3-induced MAP2a expression is present *in vitro* before than *in vivo*. Results from immunohistochemical analysis were consistent with those of immunoblotting (Fig. 6). In fact, in young cultures enriched with NT3 the monoclonal antibody against MAP2c labeled only a very small neuronal population, whereas that against MAP2a and MAP2b isoforms labeled most, if not all, nerve cell profiles. Thus, NT3 at doses of 5 ng/ml (considered to be neuritogenic; Represa *et al.*, 1993) accelerates the temporal expression of mature MAP2 isoforms. The cell localization was primarily perikaryal and only the proximal segments of the neuronal processes were immunolabeled.

Discussion

In this study we have demonstrated variations in the MAP2 isoforms expression during avian CVG development. Furthermore, our results suggest that these changes may be mimicked *in vitro* and that MAP2 expression may be modulated by NT3.

As reported in mammalian developing and adult CNS (for references see Przyborsky and Cambray-Deakin, 1995), MAP2 isoforms were developmentally regulated in the pre- and post-hatching periods analyzed (from E4 to P30). In fact, MAP2c was the predominant isoform in early developmental stages, MAP2b was found throughout embryonic development, and MAP2a was only detected in the postnatal period and was the only MAP2 isoform present in later post-hatching period. Immunoblotting techniques have previously been employed to determine the profile of MAP2 protein levels during neuronal maturation (Francon *et al.*, 1982; Binder *et al.*, 1984; Riederer and Matus, 1985; Garner *et al.*, 1988a; Tucker *et al.*, 1988). Each of these studies report that MAP2c is present at high concentrations during early stages of neuronal differentiation, and that it decreases as development progresses. They also report a progressive upregulation and maintenance of MAP2a and MAP2b protein variants during neuronal differentiation (Riederer and Matus, 1985; Garner *et al.*, 1988b; Tucker *et al.*, 1988; Charriere-Bertrand *et al.*, 1991). In other sensory neurons MAP2a cannot be detected at any developmental stage, not even after hatching, appearing only rather late in neuronal maturation (Riederer and Matus, 1985). Thus, our findings basically agree with these reports, and the slight differences in the chronology of MAP2 isoform expression might be attributed to tissue- or species-specific peculiarities (Riederer, 1990; Duc *et al.*, 1991; Cohen and Cotanche, 1992).

In the chicken embryos the CVG neurons neuritogenesis starts at E4-E5, and the peripheral processes (dendrites) reach their target (the sensory neuroepithelia of the inner ear) at E6-E7. Thereafter, sensory cell innervation takes place, being completed at E14-E15 (Avila *et al.*, 1993; Represa *et al.*, 1993). All these phenomena coincide with an increase in neuronal somata size and in out-growth and sprouting of the dendritic arbor (Riederer and Matus, 1985; Cohen and Cotanche, 1992; Avila *et al.*, 1993; Represa *et al.*, 1993). In other peripheral ganglia, such as dorsal root ganglia, it has been observed that these changes course with an increase of MAP2b expression (Riederer and Matus, 1985; Riederer, 1990; Duc *et al.*, 1991). Since no data are available on MAP2 isoforms in the development of avian CVG, based just on developmental chronology of CVG neurons present findings suggest that MAP2c could be involved in the early phases of neuronal cytoskeleton arrangement and in the start of neuritogenesis; MAP2b in the outgrowth of the dendritic processes, and their peripheral distribution forming synaptic contacts; and MAP2a in stabilizing neuronal cytoskeleton thus also peripheral synaptic contacts.

Results from the immunohistochemical study *in vivo* demonstrated that MAP2 isoforms, independently of developmental stage, are restricted to neuronal perikarya and peripheral processes (dendrites) and are never found in the central (axons) ones. These findings cannot be attributed to technical deficiencies since the antibodies used regularly recognized MAP2 isoforms in both Western blot and immunohistochemistry. Furthermore, these localizations are consistent with previous results in embryonal and adult neurons (Alaimo-Beuret and Matus, 1985; Matus *et al.*, 1986; Hernández *et al.*, 1989; Riederer and Barakat-Walter, 1992; Naves *et al.*, 1996). However, our results contrast with observations in the developing rat spiral (cochlear) ganglion where MAP2 labeling was present in both nerve cell bodies and their central processes. Species-specific differences, microheterogeneity in MAP2 isoform composition or different sensitivity of the antibodies used, might account for these discrepancies.

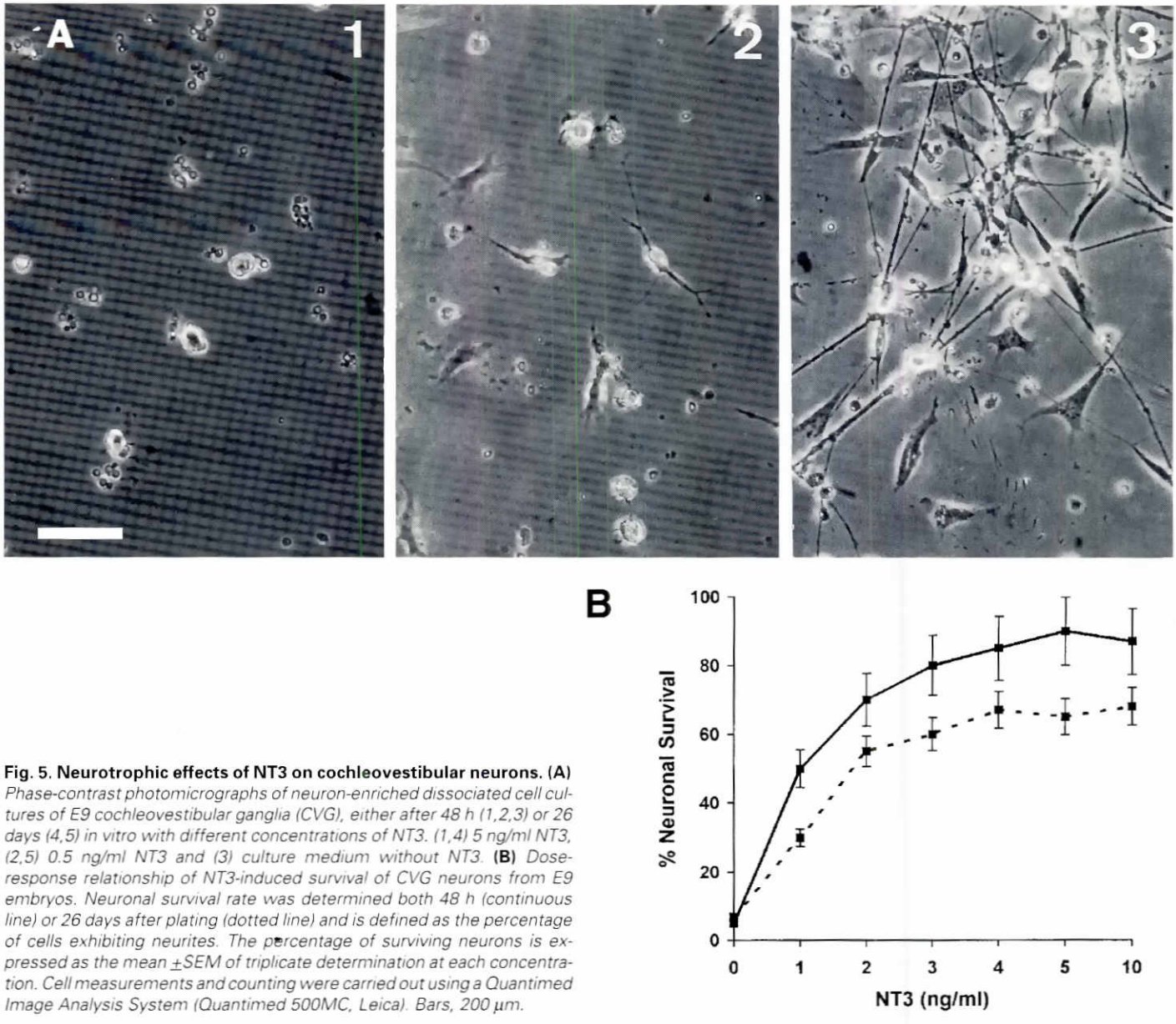

Fig. 5. Neurotrophic effects of NT3 on cochleovestibular neurons. (A) *Phase-contrast photomicrographs of neuron-enriched dissociated cell cultures of E9 cochleovestibular ganglia (CVG), either after 48 h (1,2,3) or 26 days (4,5) in vitro with different concentrations of NT3. (1,4) 5 ng/ml NT3, (2,5) 0.5 ng/ml NT3 and (3) culture medium without NT3.* **(B)** *Dose-response relationship of NT3-induced survival of CVG neurons from E9 embryos. Neuronal survival rate was determined both 48 h (continuous line) or 26 days after plating (dotted line) and is defined as the percentage of cells exhibiting neurites. The percentage of surviving neurons is expressed as the mean ±SEM of triplicate determination at each concentration. Cell measurements and counting were carried out using a Quantimed Image Analysis System (Quantimed 500MC, Leica). Bars, 200 μm.*

MAP2 isoforms were also expressed in primary CVG cultures obtained at different incubation times. Nevertheless, differences were found between young and old cultures. In the first case, MAP2 expression matched that observed *in vivo*, and was consistent with those by Hernández *et al.* (1989) and Riederer (1990) in cultured dorsal root ganglia neurons. The cell localization of MAP2 immunoreactivity *in vitro* was mainly in the neuronal perykarya and in the initial segment of the neuronal processes, as in cultured dorsal root ganglia neurons (Hernandez *et al.*, 1989). On the other hand, we have shown in CVG neurons that high levels of MAP2 protein are found in older cultures, as has been observed in other neuronal cultures (Przyborski and Cambray-Deakin, 1995). In cultured cells MAP2 seems to be required for neurite extension (Dinsmore and Solomon, 1991), but from the present results MAP2 may also be important in the stabilization of mature neuronal morphology *in vitro*.

One of the main goals of this study was to assess whether the expression of MAP2 isoforms in developing CVG is modulated by Neurotrophin-3. MAP2 is involved in nervous system development, which in turn is under the control of neurotrophic factors (see Klein, 1994; Snider, 1994). Developing CVG express TrkC (Pirvola *et al.*, 1994), which is regarded as the high-affinity receptor for NT3 (see Barbacid, 1995). Sensory epithelia innervated by CVG neurons are a source of NT3 (Schecterson and Bothwell, 1994). Furthermore, it has been shown that neurotrophins influence CVG development (Avila *et al.*, 1993; Represa *et al.*, 1993; Ylikoski *et al.*, 1993). Present results demonstrate that NT3 can modulate MAP2 isoform expression *in vitro*. Thus, the role of neurotrophins in developing CVG might be accomplished, at least in part, by their modulation in the expression of these cytoskeletal proteins. At a dose of neuronal survival NT3 (0.5 ng/ml; Represa *et al.*, 1993) MAP2c was the isoform most strongly expressed, the expression

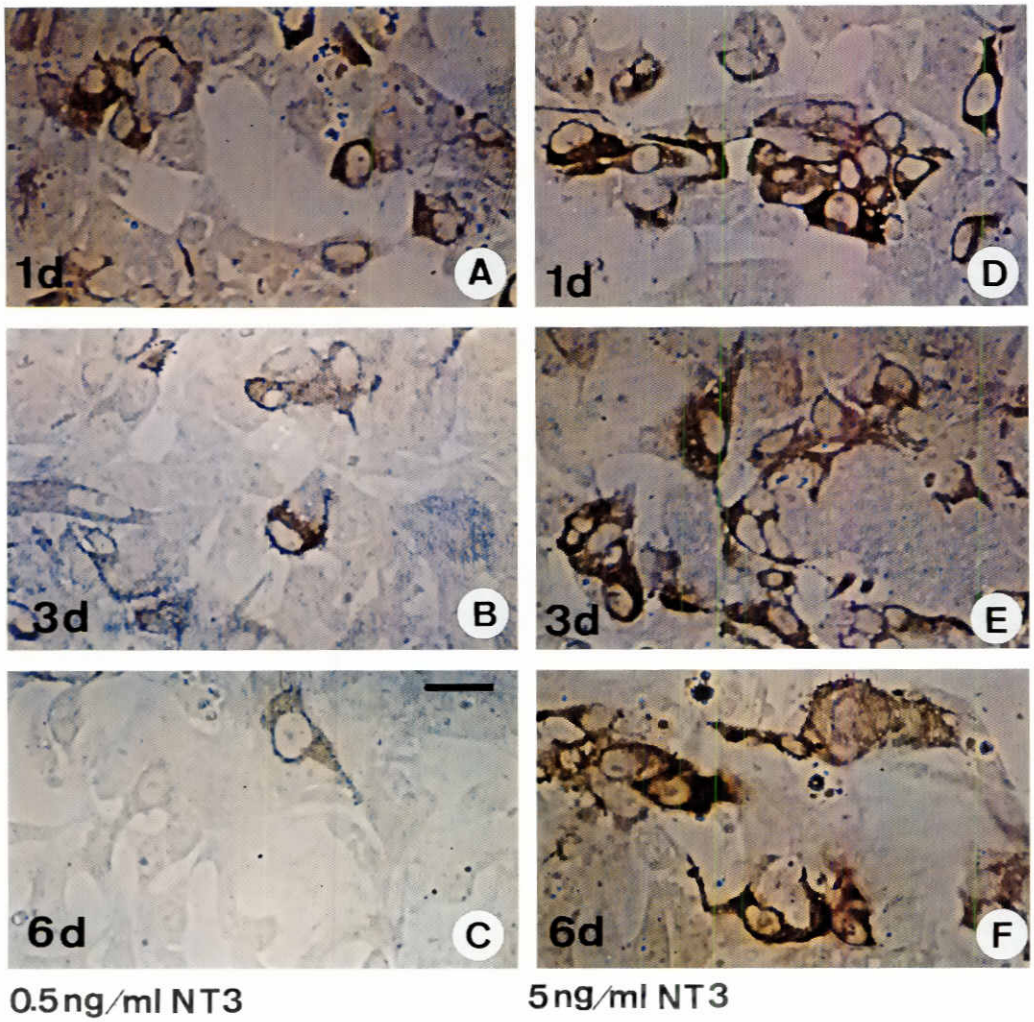

Fig. 6. MAP2 immunoreactivity *in vitro*, effect of NT3 on MAP2 isoforms expression. *Phase-contrast photomicrographs of neuron-enriched dissociated cell cultures of E9 cochleovestibular ganglia (CVG), after 1, 3 and 6 days in vitro (1d,3d,6d), with different concentrations of NT3. Cultures were processed with α MAP2a,b,c antibody (clone HM-2 , Sigma) which recognizes the three isoforms (a,b and c). Surviving neurons grown in presence of 5 ng/ml of NT3 showed heavy immunostaining when labeled with MAP2* **(D,E,F)**, *while little immunoreactivity for MAP2 could be observed in neurons cultured with 0.5 ng/ml of NT3 for 3 or 6 days* **(A,B,C)**. *The cell localization of MAP2 immunoreactivity was primarily perikaryal and neuronal processes were little or not immunolabeled. Bar in C: 30 μm in all panels.*

of MAP2b was discrete, and MAP2c was undetectable. Conversely, at a dose which induces neuritogenesis (5 ng/ml; Represa *et al.*, 1993) MAP2a and MAP2b were upregulated, whereas MAP2c expression was restricted at the earliest developmental stage. Taken together, our findings suggest that MAP2c is related to neuronal survival, while MAP2a and MAP2b could be involved in the outgrowth and stabilization of neuronal processes. NT3 probably regulates MAP2 synthesis at the transcriptional or post-transcriptional levels, being involved in the phosphorylation of cytoskeletal proteins. MAP2 can be extensively phosphorylated (García-Rocha and Avila, 1995) which may be important in regulating MAP2 binding to microtubules or other proteins (Brugg and Matus, 1991). Several studies have suggested that phosphorylation regulates MAP2 function because phosphorylated MAP2 is less effective in promoting microtubule polymerization, and phosphorylation of MAP2 to various degrees may affect MAP2 function in different ways. MAPs phosphorylation could also play a role in changing its binding to other structural components (for references see García-Rocha and Avila, 1995; Riederer *et al.*, 1995).

The role of MAPs in the peripheral nervous system has not been completely elucidated, but they probably act in the central nervous system promoting microtubule polymerization and stabilization (Tanaka and Sabry, 1995), and probably also maintaining the cylindrical form of neuronal processes (Weisshaar *et al.*, 1992; Weisshaar and Matus, 1993). In particular, MAP2 reduces the critical concentration of tubulin required to polymerize microtubules and to maintain neuronal morphology by regulating the microtubule spacing (Cáceres *et al.*, 1992; Chen *et al.*, 1992; Takemura *et al.*, 1992; Sharma *et al.*, 1994). During the early stages of CVG development MAP2c was observed in neuronal somata and in the initial segment of both *in vivo* and *in vitro* CVG neurons. MAP2c is a poor promoter of microtubule assembly (Mareck *et al.*, 1980) since it lacks the sequence which may serve to stabilize the cytoskeleton, although it probably stimulates tubulin polymerization without complete stabilization of the cytoskeleton (Papandrikopoulou *et al.*, 1986). Thus, MAP2c might in fact, at this time, maintain neuronal cytoskeleton plasticity. Conversely, the maintenance of MAP2b, and the developmental upregulation of MAP2a suggest they are involved in the maturation and stabilization of neuronal processes. It is now well established that MAPs, including MAP2, bind to microtubules and suppress their intrinsic instability, rendering more stable (Drechsel *et al.*, 1992). MAP2a

and MAP2b proteins are associated with the stabilization of mature dendritic processes (Bernhardt and Matus, 1984; Ferreira *et al.*, 1987; Tucker *et al.*, 1988).

Taken together, the present results suggest that MAP2 and their isoforms may be key proteins in inner ear development that would be involved in neurotrophin-induced effects as intracellular targets and we hypothesize that NT3 may be involved, via Trak-C binding, in microtubule bundling and stability during neurite outgrowth of inner ear neurons to reach the hair cells in the sensory epithelium.

Materials and Methods

Preparation of tissues

Hen fertilized eggs (Straing Cross), incubated for different periods of time at 38°C and with 80% relative humidity, were used throughout this study. The embryos were extracted, killed and placed in Petri dishes containing Hank's balanced salt solution. Embryonic age was determined by staging according to Hamburger and Hamilton (1951) and expressed as embryonic time (E-days). Either the whole cephalic region, the temporal bone primordia or CVG were isolated from E4 to hatching embryos and post-hatching (P-days), including E4, E7, E10, E14, E18, P10 and P30 animals. The methodology was described previously (Represa and Bernd, 1989; Represa *et al.*, 1991; Vázquez *et al.*, 1994). Dissected cephalic regions and temporal bones were processed for histology and immunocytochemistry, while isolated CVG were used for culture or Western blot techniques.

Immunohistochemistry

Immunohistochemistry was carried out on frozen sections obtained from chick embryos at the stages mentioned above, as well as on neuron-enriched cell cultures. Whole embryos were fixed in 4% buffered paraformaldehyde (0.1 M phosphate buffered saline, PBS) for 4 h at 4°C, then rinsed in 0.1 M PBS, and frozen in 2-methylbutane (Aldrich) which had been chilled in liquid nitrogen. Embryos were coated with Tissue Teck (Miles Laboratories), cut in 20 μm sections, and the sections placed on gelatin coated-slides and stored at 0-4°C until use (within 72 h). Thereafter sections were washed with 0.1 M PBS for 1 h, treated for 30 min with a PBS solution containing 5% bovine serum albumin and 5% fetal calf serum, and incubated with the primary antibodies diluted in the same PBS containing 0.2% Triton X-100. Incubation was carried out overnight in a humid chamber at 4°C with the following mouse monoclonal antibodies: a) clone HM-2 (Sigma, diluted 1:500) which recognizes all MAP2 isoforms; b) clone AP-20 (Sigma, diluted 1:500) which recognizes MAP2a and MAP2b; clone B62, diluted 1:250 against MAP2c. This MAP 2c antibody was raised against a 18-amino acid synthetic peptide of the COOH-terminus (VTSKCGSLGNIHHPGGG). After washing, sections were incubated with a sheep anti-mouse IgG for 1 h at room temperature (Vector, purchased prediluted) and the reaction developed with a commercially available commercial kit (Vectorstain, Vector) using DAB (Sigma) as a chromogen.

Immunohistochemistry was also performed on cultures of dissociated GVG neurons and on neuron-enriched CVG cell cultures. After different periods in culture (see below), the CVG cells were fixed in 4% paraformaldehyde (0.1 M PBS, 4 h, 4°C), rinsed in 0.1 M PBS and processed for immunocytochemistry as described for tissue sections. Cell measurements and counting were carried out using a Quantimed Image Analysis System (Quantimed 500MC, Leica) (for details see Vázquez *et al.*, 1994).

For control purposes, representative sections or cultures were processed in the same way and incubated with a pre-immune mouse serum instead of the primary antibody, or omitting primary antibody. Under these conditions no specific immunoreactivity was observed.

Cultures of dissociated CVG

CVG isolated from E4 chick embryos were dissected, dissociated in neuron-enriched cell culture and grown *in vitro* for various periods of time, ranging from 48 h to 14 days. Dissected CVG were incubated for 25 min at 37°C in Ca^{2+} and Mg^{2+}-free Earle's salts solution containing 0.1% trypsin and 0.1% dispase. Dissociated single cells were obtained by gentle passing through a flame-polished Pasteur pipette. The dissociated cell suspension was enriched for neurons 90-95% or greater 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 (Nunc). Culture medium was M-199 with 6 g/l glucose and 1% (v/v) heat-inactivated horse serum (GIBCO). Control cultures were maintained in just M-199 medium with 6 g/l glucose and 5% (v/v) heat-inactivated horse serum (GIBCO). Culture medium was further supplemented, where indicated, with recombinant NT3 (# N-260, Alomone Labs Ltd., Israel) at concentrations of 0.5 and 5 ng/ml. These concentrations were chosen because they had been previously determined to have surviving and differentiating effects, respectively, on CVG neurons (Avila *et al.*, 1993). Culture medium was changed every 48 h. Neurite outgrowth and neuronal survival were estimated after culture, being defined as the fraction of cells exhibiting neurites of length greater than two soma diameters (Davies *et al.*, 1986). After different periods in culture the cells were processed for Western blot or immunocytochemistry.

Western blotting

In three experiments, protein lysates from dissected CVG or from neuron-enriched cell cultures of chick embryos at the stages described above, were obtained. Since dissected CVG size increases during development, the number of ganglia needed for the lysate at each stage was estimated from the total amount of protein/ganglion as previously determined (Vazquez *et al.*, 1994). Neuron enriched cell cultures were washed in HBSS, harvested, and collected and then centrifuged (1000g, 5 min). In both cases pellets were transferred to a protein extraction lysis buffer containing 50 mM Tris-HC1, 150 mM NaC1, 5 mM EDTA, 1% Triton-X-100 and 0.25% Na deoxycholate. Once the proteins were extracted, the lysates were processed according to the method of Smith *et al.* (1985) using a Micro-BCA kit (Pierce) to determine the final protein concentration in the extraction buffer. All protein samples were loaded at the same concentration of 20 μg of protein/well on a 8% SDS electrophoresis gel (SDS-PAGE), then removed and blotted onto a nitrocellulose filter. Immunodetection was carried out using a mouse monoclonal antibody (clone HM-2, Sigma) diluted 1:500, which recognizes all MAP2 isoforms. Blots were developed using ECL Western blotting detection system (Amersham), and quantified with the aid of a laser densitometer (Helena).

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