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Development of Cat-301 Immunoreactivity in Auditory Brainstem Nuclei of the Gerbil

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ABSTRACT

The developing brainstem auditory system has been studied in detail by using anatomical and physiological techniques. However, it is not known whether immature auditory neurons exhibit different molecular characteristics than those of physiologically mature neurons. To address this issue, we examined the distribution of Cat-301 immunoreactivity in the developing auditory brainstem of gerbils. Cat-301 is a monoclonal antibody that recognizes a 680-kD chondroitin sulfate proteoglycan similar to aggrecan, a high-molecular-weight chondroitin sulfate proteoglycan found in cartilage. In the central nervous system, Cat-301 immunoreactivity is localized to the extrasynaptic surface of neurons. It has been hypothesized by Hockfield and co-workers (Hockfield et al. [1990a]Cold Spring Harbor Symp. Quart. Biol. *55:*504–514) that the Cat-301 proteoglycan is a molecular marker indicating that a neuron has acquired mature neuronal properties.

In the current study, Cat-301 staining is first seen at 7 days after birth in the anterior ventral cochlear nucleus (AVCN), the posterior VCN (PVCN), and the medial nucleus of the trapezoid body (MNTB) shortly before the onset of sound-evoked activity. By 21 days after birth, neurons in the AVCN, the PVCN, and the lateral and medial superior olive have attained adult-like distributions of Cat-301 staining concomitant with the physiological maturation of these neurons. Neurons in MNTB attain adult-like distributions of Cat-301 immunoreactivity at 1 year. The maturation of Cat-301 immunoreactivity parallels the physiological maturation of gerbil auditory neurons, and the Cat-301 proteoglycan may play a role in the formation and/or stabilization of auditory synapses. J. Comp. Neurol. 380:319–334, 1997.

Indexing terms: proteoglycan; immunocytochemistry; neuron; hearing; rodent

In the Mongolian gerbil, auditory system development continues well after birth, and this system has been studied in considerable detail both anatomically and physiologically. For example, in the ear, the development of innervation and function has been described (Arjamand et al., 1988; Echteler et al., 1989; Harris and Dallos, 1984; Mills et al., 1994; Norton et al., 1991; Ryan and Woolf, 1992; Ryan et al., 1987, 1990; Woolf and Ryan, 1984, 1988; Woolf et al., 1986) as well as the development of single-unit responses and brainstem evoked potentials in the auditory brainstem (Donaldson and Rubel, 1990; Sanes and Rubel, 1988; Sanes et al., 1989; Smith and Kraus, 1987; Woolf and Ryan, 1985). In the cochlear nucleus, the developmental sequence of synaptogenesis (Schwartz and Ryan, 1985)

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and γ-aminobutric acid (GABA) immunoreactivity have been elucidated (Yu and Schwartz, 1987), and, recently, Kitzes and colleagues have described the postnatal ontogeny of connectivity in the superior olivary complex (Kil et al., 1995). Although the developing gerbil auditory system has been examined extensively by using anatomical and physiological techniques, less is known regarding the molecular development of this system. In particular, it is not known whether physiologically immature auditory neurons exhibit different molecular characteristics than those of physiologically mature neurons. To address this issue, we examined the distribution of the proteoglycan Cat-301 in the developing gerbil auditory brainstem.

Cat-301 is a monoclonal antibody generated against homogenized cat spinal cord (Hockfield and McKay, 1983) that recognizes subsets of neurons in the central nervous system (Hendry et al., 1984; Hockfield and Sur, 1990; Hockfield et al., 1990b; Kalb and Hockfield, 1988; McGuire et al., 1989; Sahin and Hockfield, 1990). Cat-301 immunoreactivity is localized to the extrasynaptic surface of neurons, and the Cat-301 antibody recognizes a 680-kD chondroitin sulfate proteoglycan similar to aggrecan, a high-molecular-weight chondroitin sulfate proteoglycan found in cartilage (Fryer et al., 1992).

Development of Cat-301 immunoreactivity on neurons in both the lateral geniculate nucleus (LGN) of the cat and the sciatic motor neurons of the hamster spinal cord has been correlated with the end of the period in which neuronal activity is thought to dramatically alter development (Kalb and Hockfield, 1988, 1990). In addition, if these structures are denervated before the appearance of Cat-301 immunoreactivity, then the neurons fail to develop expression of the Cat-301 antigen (Guimaraes et al., 1990; Kalb and Hockfield, 1992; Sur et al., 1988). It has been hypothesized that the Cat-301 proteoglycan is a molecular marker that indicates that a neuron has acquired mature neuronal properties (Hockfield et al., 1990b). In addition, the Cat-301 protein may play a role in the maintenance of these properties (Hockfield et al., 1990a).

The current study was undertaken to determine whether Cat-301 immunoreactivity is found in the gerbil auditory brainstem and, if so, the relationship between expression of Cat-301 immunoreactivity and functional measures of maturity. We found that a subset of neurons in the auditory brainstem of mature gerbils is immunopositive for Cat-301, corroborating the findings from previous studies (Schwartz and Hockfield, 1989). Interestingly, the onset of Cat-301 staining around neurons in several auditory brainstem nuclei occurs after the onset of spontaneous activity and shortly before the onset of sound-evoked activity. The maturation of Cat-301 staining occurs in parallel with the acquisition of mature physiological properties.

MATERIALS AND METHODS

Subjects

Mongolian gerbils (*Meriones unguiculatus*) were obtained from a commercial supplier (Tumblebrook Farms, West Brookfield, MA) or from the University of Washington breeding colony, which was established from the supplier. The animals used in this study were 5, 7, 9, 11, 14, 21, 31, 40, 65, and 365 days old. Three to five gerbils were examined at each time period. All manipulations followed established guidelines of animal care.

Immunohistochemistry

At the appropriate ages, animals were deeply anesthetized with ketamine (75 mg/kg) and Xylazine (5 mg/kg) and transcardially perfused with Ringer's solution (150 mM NaCl, 13.4 mM KCl, 4.9 mM $MgCl_2$) and 1 mM EDTA. The brains were quickly dissected free and postfixed for 6 hours in a modified Carnoy's solution (6 parts ethanol, 2 parts Chloroform, 1 part glacial acetic acid, and 1 part 10× Ringer's). The tissue was then rinsed overnight in 70% ethanol, blocked, and embedded in paraffin. A one-in-four series of 10 mm-thick transverse sections was mounted onto polylysine-coated slides and processed for Cat-301 immunohistochemistry.

Sections were deparaffinized through a series of graded xylenes and alcohols followed by rinses in 0.1 M Tris buffer. The tissue sections were incubated in 4% normal horse serum for 20 minutes. All immunocytochemical reagents were prepared in 1% bovine serum albumin (BSA), 0.1% sodium azide [except for the avidin biotin complex (ABC) reagent]. The sections were then incubated in the monoclonal antibody Cat-301 (provided by Dr. Hockfield) at a 1:20 dilution overnight at 4°C. Control sections were incubated overnight in normal mouse serum. Following the overnight incubation, all sections underwent one 5-minute wash in 0.1 M Tris and one 5-minute wash in 0.1 M Tris 1%, BSA pH 7.4, after incubation in the primary antisera and all subsequent reagents except the ABC reagent. The sections were then incubated in biotinylated horse anti-mouse serum for 1 hour, diluted 1:400, washed, and then incubated in ABC at a 1:6 dilution (Vectastain Elite ABC kit; Vector Labs, Burlingame, CA). Tissue sections were rinsed for 10 minutes in Tris and developed with diaminobenzidine as the chromagen (0.5 mg/ml; Sigma, St. Louis, MO) with 0.1% H_2O_2 and 1 mM Imidazole. Biochemical studies demonstrate that Cat-301 recognizes a chondroitin sulfate proteoglycan in the gerbil with characteristics identical to those observed for the Cat-301 antigen in other mammalian species (Guimaraes et al., 1990; Hendry et al., 1984; Hockfield and Sur, 1990; Hockfield et al., 1983, 1990a,b; Kalb and Hockfield, 1988; McGuire et al., 1989).

One brain from each time period was lightly counterstained with thionin following the immunohistochemistry. All sections were then dehydrated and coverslipped with DPX (BDH Limited, Poole, England).

RESULTS

Cat-301 immunoreactivity can first be observed in the gerbil brainstem by 7 days after birth (DAB) and, by 9 DAB, is distributed around neurons in many auditory nuclei. Figure 1A is a low-power micrograph of a thionin-stained section through the brainstem at 9 DAB showing the location of the ventral cochlear nucleus (VCN), dorsal cochlear nucleus (DCN), lateral superior olivary nucleus (LSO), medial superior olivary nucleus (MSO), and medial nucleus of the trapezoid body (MNTB). Note the very faint staining surrounding the neurons within the MNTB and VCN (Fig. 1B). By 11 DAB, this faint staining has intensified slightly, and, now, neurons in the LSO and MSO are also faintly surrounded by Cat-301 immunoreactivity (Fig. 2).

Cat-301 immunoreactivity continues to increase throughout the brainstem and reaches an adult-like pattern and intensity by 31 DAB. Note the intense staining within

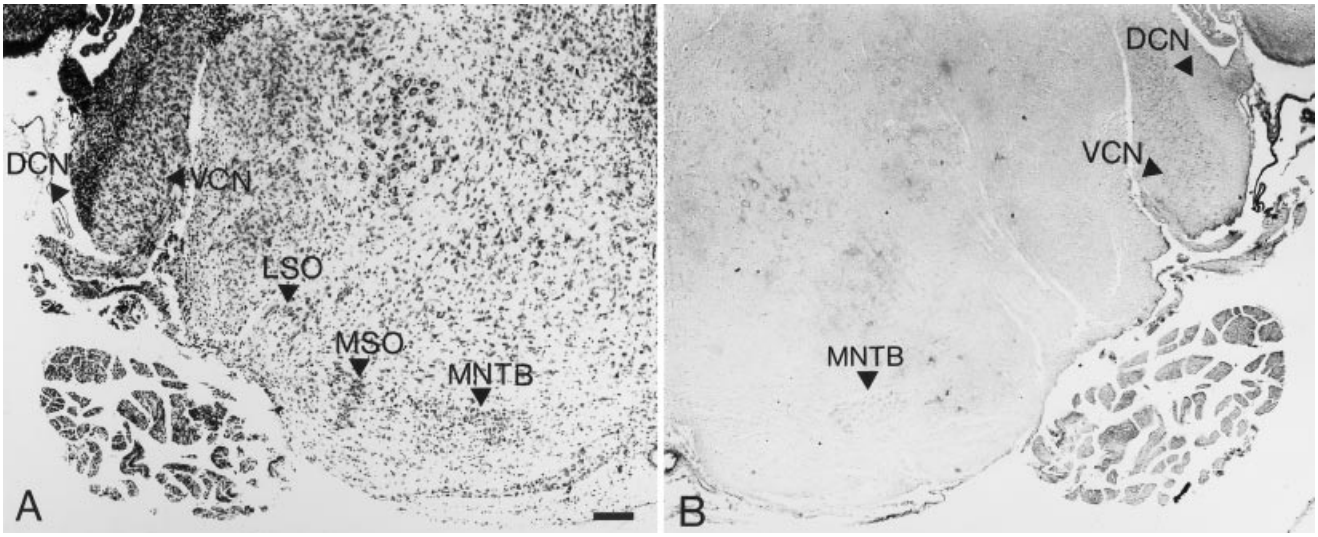

Fig. 1. Low-power micrograph of the gerbil brainstem at 9 days after birth (DAB). **A:** Thionin-stained tissue section demonstrates the locations of the ventral cochlear nucleus (VCN), the dorsal cochlear nucleus (DCN), the medial superior olivary nucleus (MSO), the lateral superior olivary nucleus (LSO), and the medial nucleus of the trapezoid body (MNTB). **B:** Alternate tissue sections stained with Cat-301. Note the very faint immunoreactivity within the VCN and MNTB. Scale bar = 100 μm.

VCN, MNTB, MSO, and LSO (Fig. 3). The onset and distribution of Cat-301 immunoreactivity within each auditory nucleus was carefully examined and the results are summarized below.

AVCN

The AVCN contains spherical bushy cells, globular bushy cells, and multipolar stellate cells. These cell types can be distinguished by their morphological and physiological characteristics (Cant, 1992; Rhode, 1991). Spherical bushy cells have bilateral projections to the MSO and LSO (Irvine, 1986; Kil et al., 1995): Globular bushy cells project to principal cells of the contralateral MNTB. Spherical and globular bushy cells receive excitatory input from auditory nerve fibers and can be identified consistently by their location within the AVCN.

Cat-301 immunoreactivity is first seen at 7 DAB in the AVCN. At this time, staining is limited to tufts or nests of neuropil (Fig. 4A). Staining is fibrillar and appears to abut, but not to surround, neuronal somata.

Animals at 11 and 14 DAB show increased immunostaining of AVCN neuronal cell somata. Although some neurons throughout the AVCN are positive, spherical bushy cells, which can be identified consistently based on their anterior location within the nucleus (Cant, 1992), show the greatest density of labeling. Approximately 30–50% of the cells are Cat-301 positive. Staining is limited to the edge of the cytoplasmic membrane and, in many cases, does not include the entire cell circumference (Fig. 4B).

Animals at 21 and 31 DAB show increasingly intense Cat-301 staining. Approximately 75% of the spherical cells are positive for Cat-301 immunoreactivity (Fig. 4C). Each cell shows immunoreactivity along the entire cell circumference, and the width of the band of stain is increased over that seen at earlier times. Immunoreactivity is localized to the neuronal soma and occasionally to a proximal dendrite. Granule cells in the small cell cap region of the AVCN are negative.

AT 65 DAB, staining is similar to that seen at 21 and 31 DAB (Fig. 4D). There appears to be a slight increase in staining in the neuropil between cells, which persists through 1 year of age (data not shown). The staining observed in the AVCN using the Cat 301 antibody is specific for the Cat 301 antigen. Figure 5 demonstrates that, when normal mouse serum is substituted for the primary antibody, there is no specific staining in the 65 DAB gerbil brainstem.

PVCN

Neurons in the PVCN include multipolar cells, octopus cells, and a small number of globular bushy cells. In the adult gerbil, Cat-301 immunoreactivity is found throughout the PVCN, but only octopus cells in our tissue can be identified due to their unique morphology.

At 7 DAB, the punctate pattern observed in the AVCN is also found in the neuropil of the PVCN. However, unlike the AVCN, occasional neural somata are also labeled with the Cat-301 antibody (Fig. 6A). These cells are fairly darkly stained around the entire circumference of the cell body and appear randomly distributed throughout the nucleus. By 11 DAB, the tufted pattern has disappeared completely, and a few large, very darkly labeled cell bodies with lightly stained dendrites can be found throughout the nucleus (Fig. 6B). Staining increases until 21 DAB, when the majority of cells in the PVCN are stained and this staining has assumed an adult-like pattern (Fig. 6C). Octopus cells stain very darkly with the Cat-301 antibody. Nonoctopus cells have a similar intensity of staining to that seen in the AVCN (not shown). In general, the dendritic staining of all the cells in the PVCN covers a long extent of the dendritic processes. At 1 year, there appears to be a slight loss of Cat-301 immunoreactivity in the PVCN, presumably due to the degeneration seen in this region (Ostapoff and Morest, 1989). The band of staining around the neurons is not as distinct as that seen at earlier times (Fig. 6D).

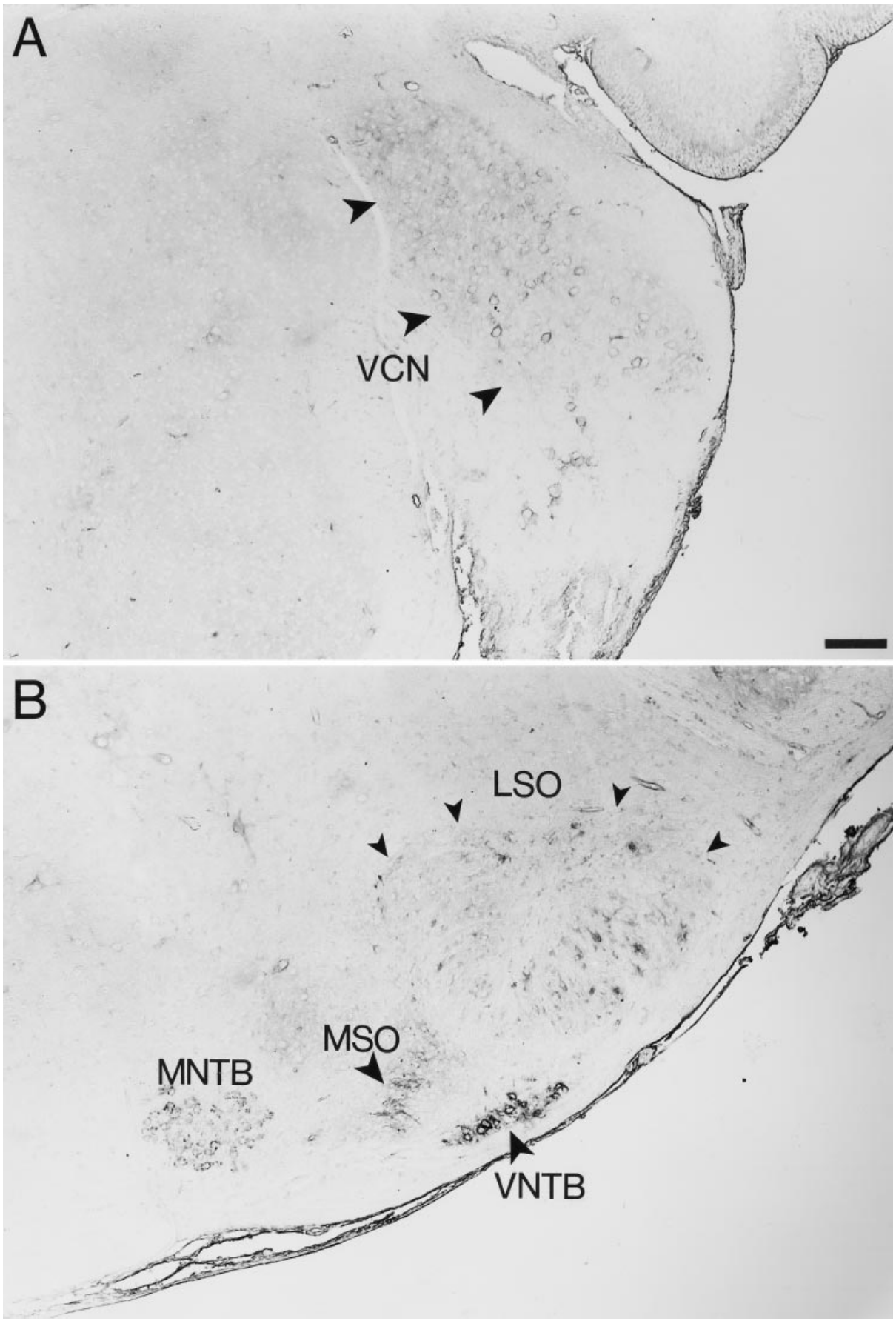

Fig. 2. Cat-301 immunoreactivity in the gerbil brainstem at 11 DAB. **A:** By 11 DAB, many neurons in VCN are immunopositive for Cat-301 (arrowheads). **B:** Neurons in MNTB, MSO, LSO, and ventral nucleus of the trapezoid body (VNTB) are also immunopositive (arrowheads). For abbreviations, see Figure 1. Scale bar = 100 µm.

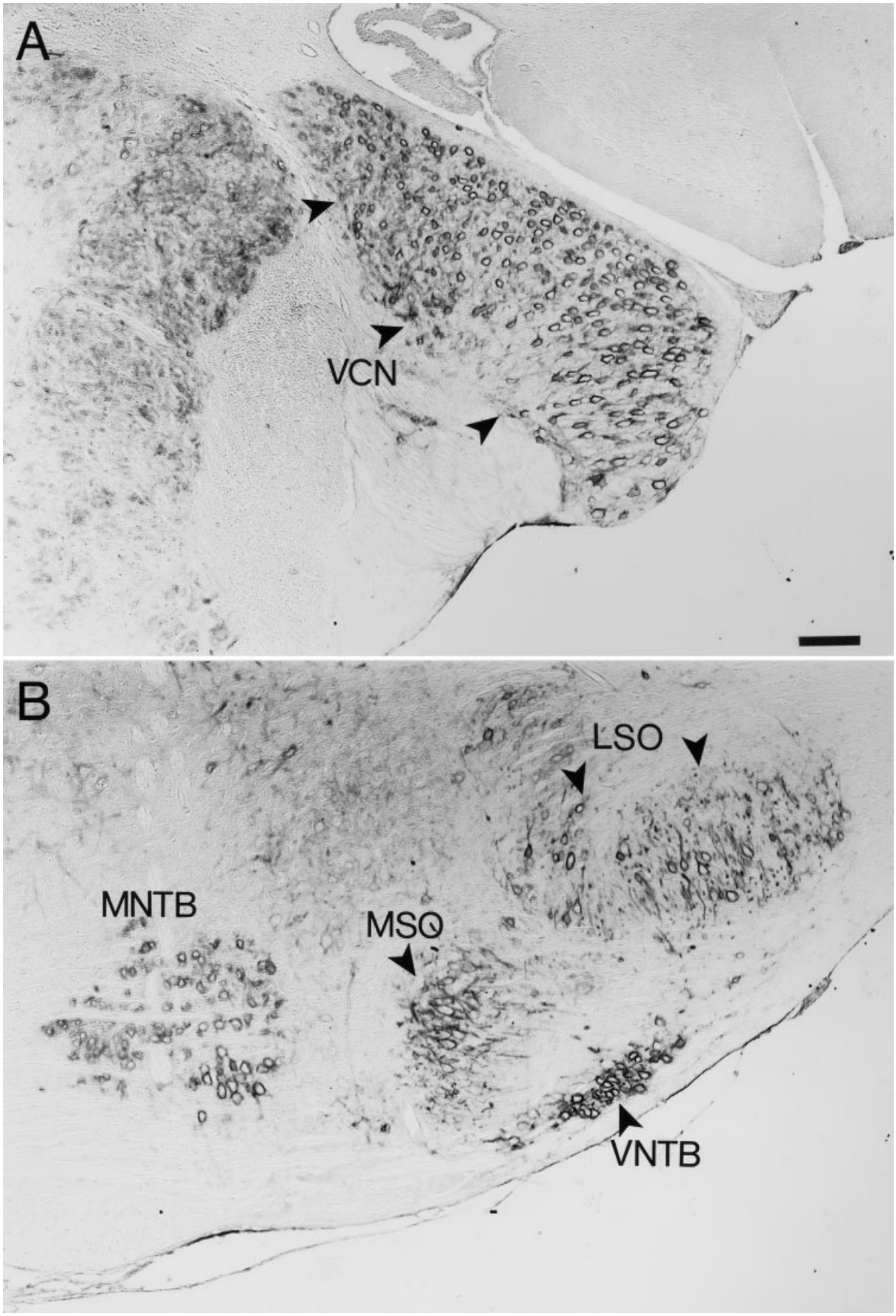

Fig. 3. Cat-301 immunoreactivity in the gerbil brainstem at 31 DAB. By 31 DAB, Cat-301 immunoreactivity has reached an adult-like pattern and intensity in the VCN (arrowheads in **A**) and the MNTB, VNTB, LSO, and MSO in **B** (some marked with arrowheads). For abbreviations, see Figures 1 and 2. Scale bar = 100 µm.

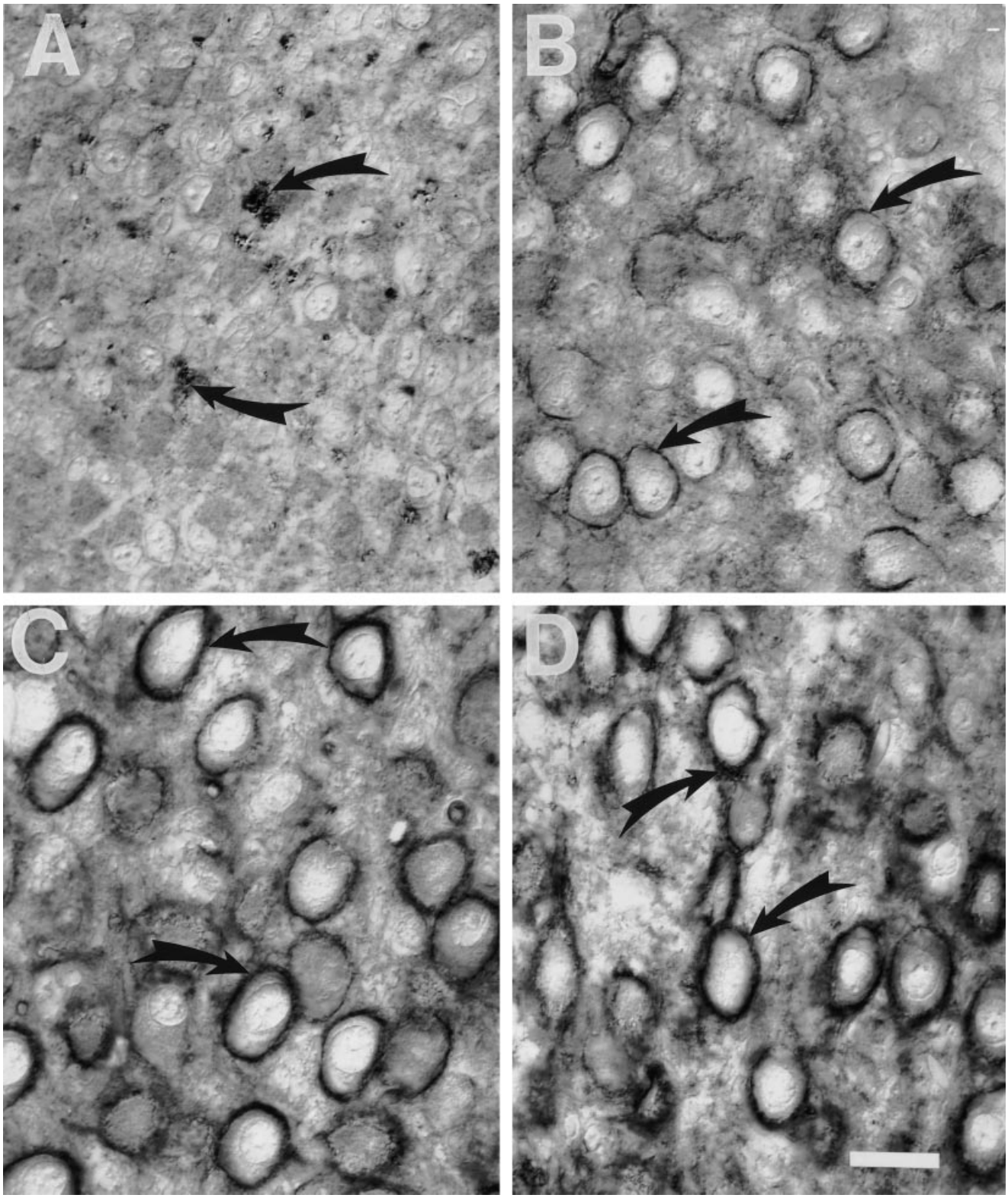

Fig. 4. Cat-301 immunoreactivity in the anterior ventral cochlear nucleus (AVCN). **A:** At 7 DAB, Cat-301 immunoreactivity is limited to tufts of neuropil (arrows). No staining around neuronal cell bodies can be observed. **B:** By 11 DAB, many neurons throughout the anterior VCN (AVCN) are Cat-301 positive. Staining is limited to the edge of the cytoplasmic membrane and does not include the entire cell surface in many cells (arrows). **C:** Adult-like staining patterns of Cat-301 can be observed at 21 DAB. Neurons are stained along their entire cell surface, and the width of staining has increased over earlier times (arrows). **D:** At 65 DAB, Cat-301 staining around neuronal cell surfaces is similar to that seen at 21 DAB (arrows). Scale bar = 20 μm.

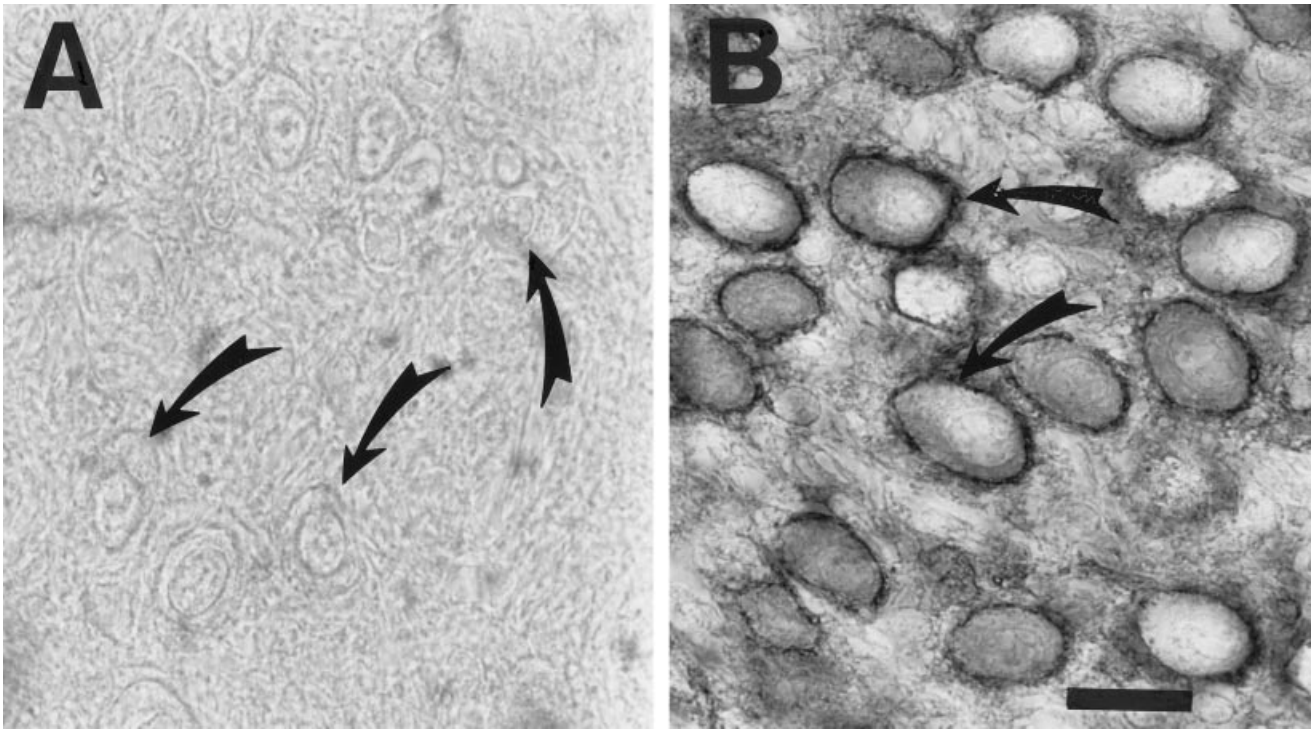

Fig. 5. Control for Cat-301 immunoreactivity. **A:** Sixty-five DAB AVCN-stained with normal mouse serum (1:10,000). There is no neuronal staining (arrows) or staining within the neuropil. The speckled appearance of the tissue is due to dust within our photographic system that is prominent at the settings used to visualize the unlabeled AVCN. **B:** Sixty-five DAB AVCN stained with the Cat-301 antisera. Note the heavily stained neurons (arrows). For abbreviations, see Figures 1 and 4. Scale bar = 20 μm.

DCN

The DCN can be divided into three layers: molecular, fusiform, and deep. The molecular layer, which is relatively acellular, contains stellate cells and cartwheel cells. The cell-dense fusiform layer contains fusiform cells, granule cells, stellate cells, and cartwheel cells. The deep layer contains giant cells and stellate cells.

No immunostaining is evident until 31 DAB in neurons of the DCN. At this time, a very small number of cells are faintly stained with the Cat-301 antibody (Fig. 7A). These cells appear to be located in the fusiform and deep layers of the DCN. No staining is apparent in the molecular layer. At 40 and 65 DAB, staining of these few cells resembles the pattern seen at 31 DAB. By 1 year, however, the staining has become much darker around those cells that are labeled. In addition, more cells appear to be immunolabeled for Cat-301 (Fig. 7B).

MNTB

There are three neuronal cell types in the MNTB; principle cells, elongate cells, and stellate cells. Principle cells comprise the vast majority of cells in this nucleus and receive large calyceal endings from globular bushy cells in the contralateral posterior division of the AVCN. The axons between the AVCN and the MNTB are large and myelinated. The combination of large synapses, large axons, and a one-to-one relationship between the AVCN and the MNTB neurons is thought to provide a secure and fast relay of auditory information to the contralateral side of the brainstem. Physiological response properties of MNTB principle cells mirror those of AVCN spherical cells. In addition, principle cells stain for glycine and are a major source of inhibitory input to the principal neurons of the ipsilateral LSO.

Cat-301 immunoreactivity is first observed in MNTB at 7 DAB. Unlike the tufted clumps of staining observed in the AVCN at this age, principal cells in the MNTB show a punctate pattern of staining along the somal surface (Fig. 8A). This staining progresses to include the entire circumference of the cell surface by 14 DAB (Fig. 8B). By 21 DAB, the staining pattern has become very intense around the principal cells (Fig. 8C). Staining intensity continues to increase through 1 year of age.

LSO

Neurons in the LSO receive bilateral input. Principal neurons of the LSO receive excitatory inputs from spherical bushy cells in the ipsilateral AVCN (Cant, 1992). They also receive inhibitory input from the ipsilateral MNTB, which, in turn, receives excitatory input from globular bushy cells in the contralateral AVCN. In addition, there appears to be a direct projection of the AVCN onto the ventral limb of the contralateral LSO (Kil et al., 1995). There is a matching of tonotopic maps between the inhibitory and excitatory inputs to LSO neurons (Sanes and Rubel, 1988; Tsuchitani and Boudreau, 1966).

Cat-301 immunoreactivity is weak in LSO neurons in all age groups when compared with the AVCN, PVCN, and MNTB. Very faint staining is seen in the neuropil at 9 DAB (Fig. 9A). Specific staining around the neuronal membrane can first be recognized at 11 DAB (Fig. 9B). Staining at this time includes the cell body and proximal dendrites in the

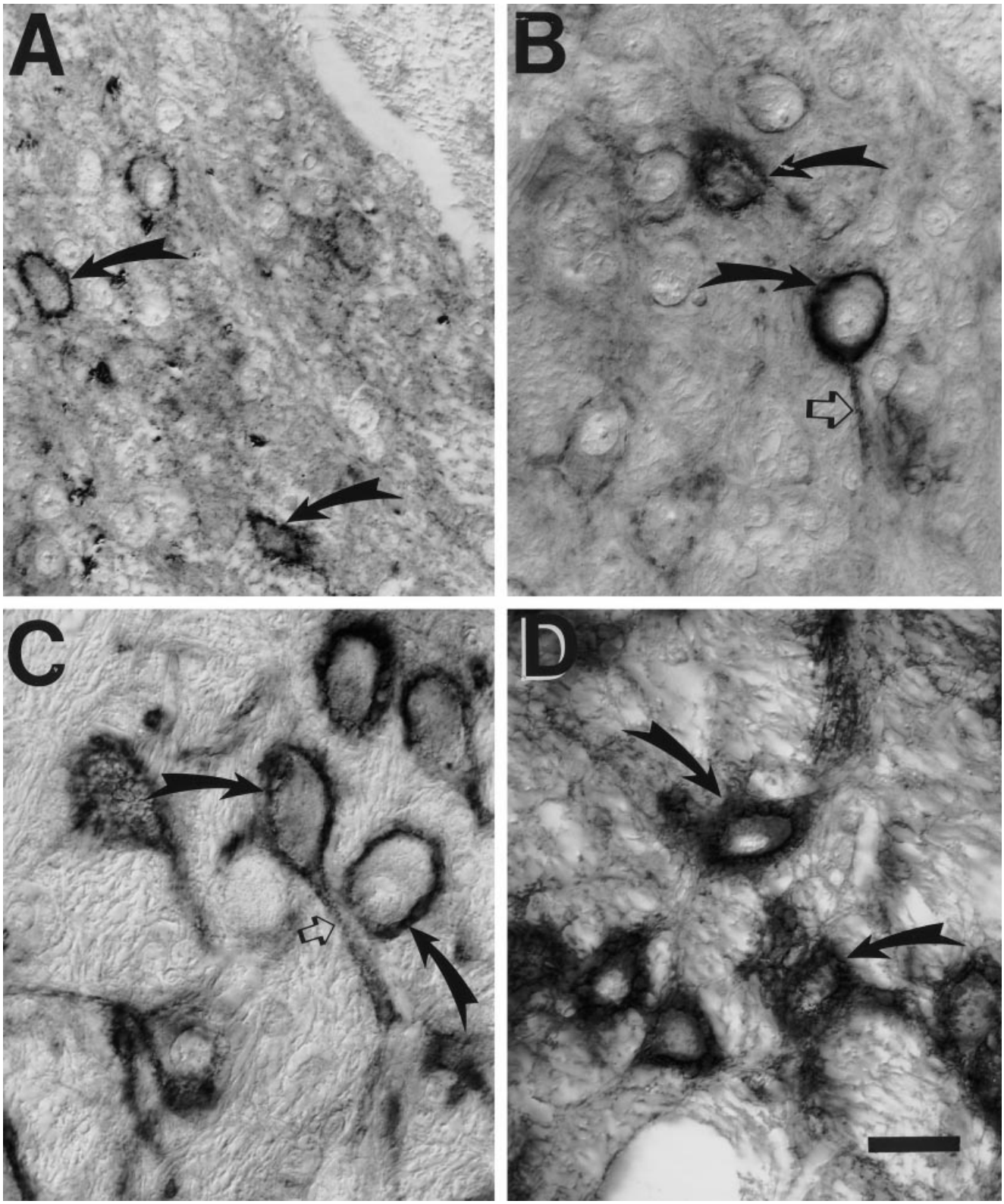

Fig. 6. Cat-301 immunoreactivity in the posterior VCN (PVCN). **A:** At 7 DAB, a punctate pattern of Cat-301 staining is seen in the neuropil of the PVCN. Occasional neural somata are also labeled (arrows). **B:** A few large, darkly labeled cell bodies (solid arrows) with lightly stained dendrites (open arrow) can be found throughout the nucleus at 11 DAB. **C:** At 21 DAB, the majority of neurons in the PVCN are stained with the Cat-301 antisera (solid arrows). The dendritic staining of the cells in the PVCN covers a long extent of the dendritic processes (open arrow). **D:** By 1 year, the band of staining around neurons is not as distinct as that seen earlier (arrows). Scale bar = 20 μm.

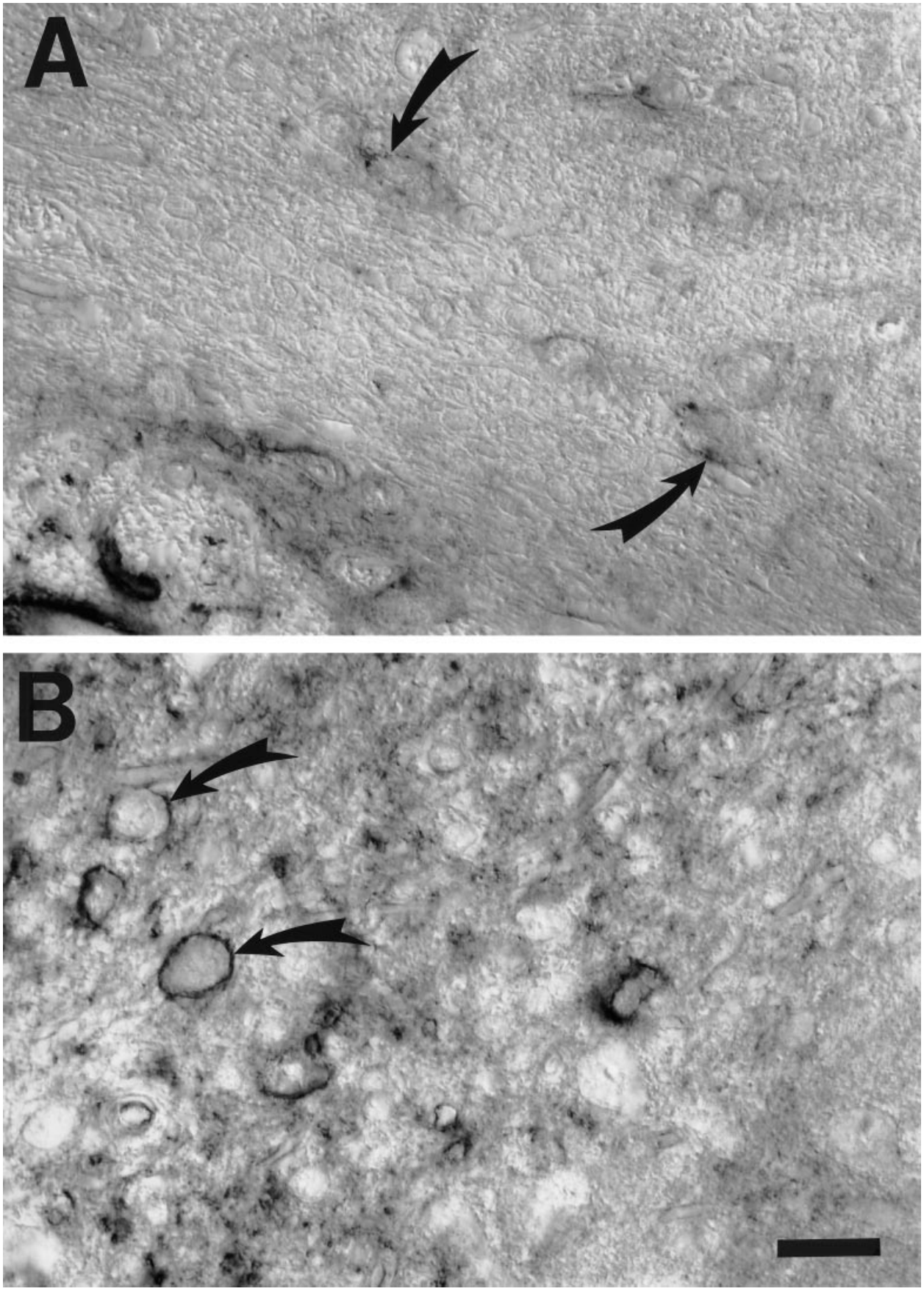

Fig. 7. Cat-301 immunoreactivity in the DCN. **A:** At 31 DAB, small numbers of cells located in the fusiform and deep layers of DCN are immunostained (arrows). **B:** By 1 year, Cat-301 staining has become much darker around those cells that are labeled, and more cells appear to be immunolabeled (arrows). Scale bar = 20 µm.

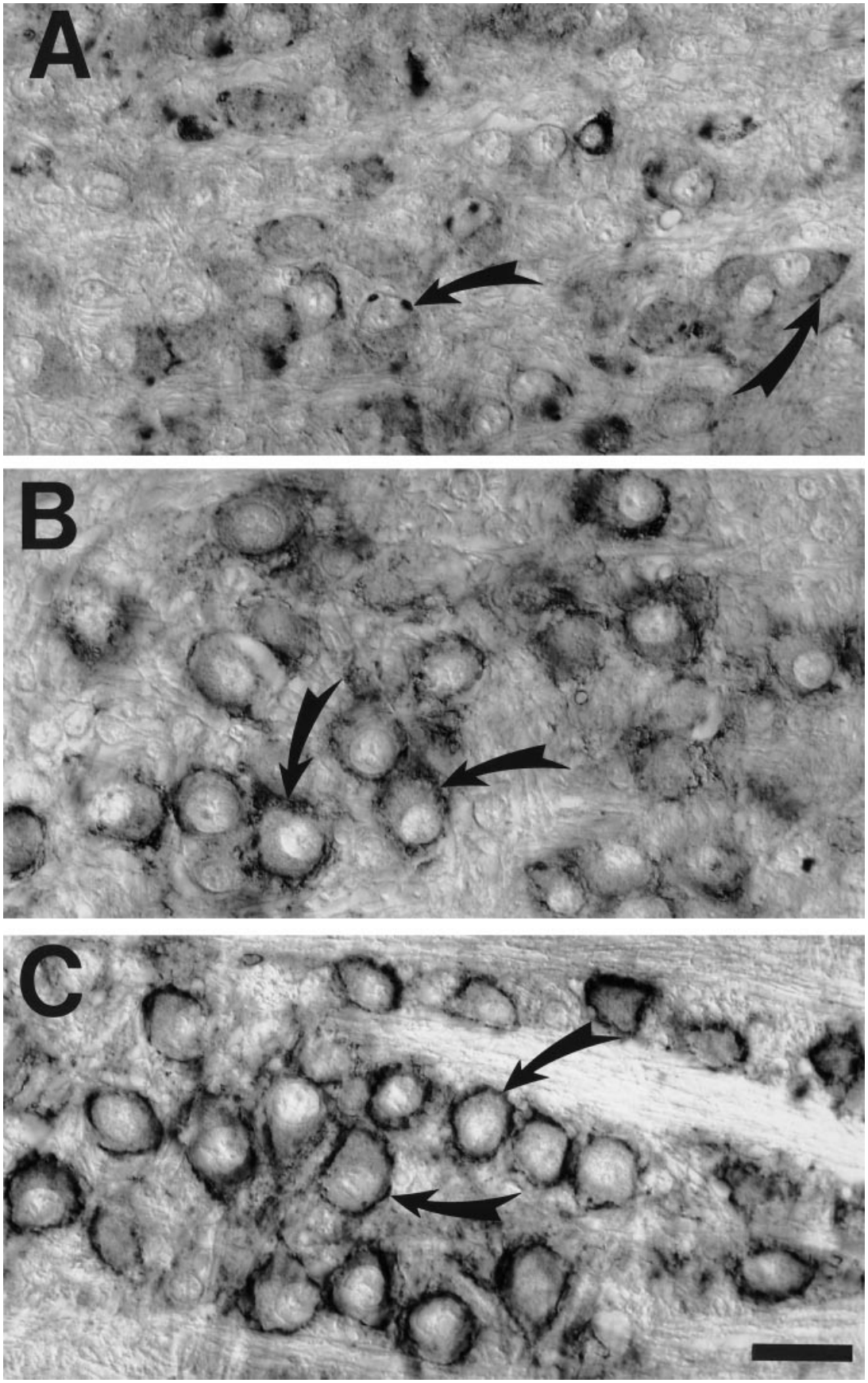

Fig. 8. Cat-301 immunoreactivity in the MNTB. **A:** Principal cells in the MNTB show a punctate pattern of Cat-301 immunostaining along the somal surface at 7 DAB (arrows). **B:** By 14 DAB, the entire neuronal surface is stained (arrows). **C:** The staining pattern increases in intensity by 21 DAB (arrows). Scale bar = 20 µm.

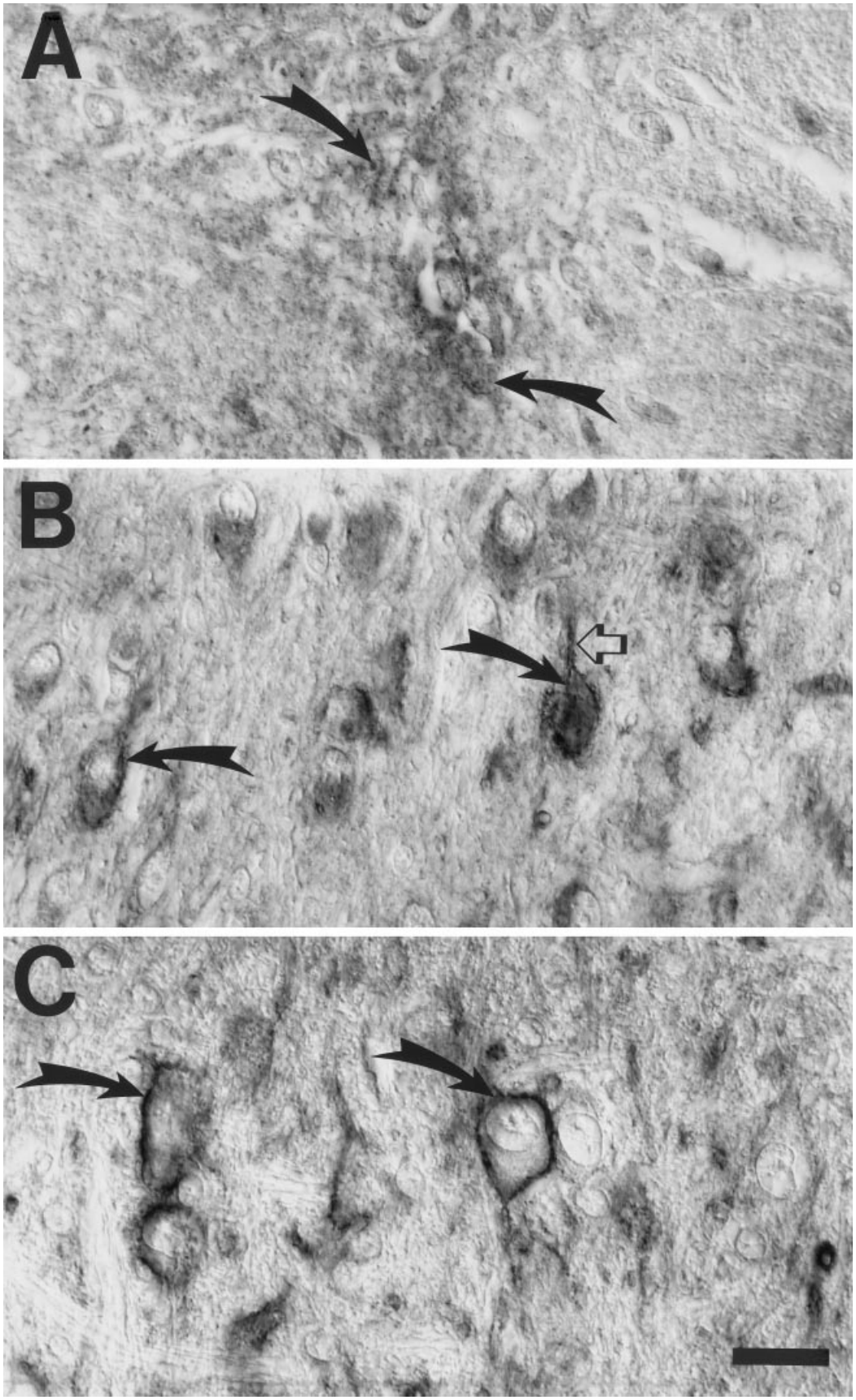

Fig. 9. Cat-301 immunoreactivity in the LSO. **A:** Very faint immunoreactivity is seen in the neuropil at 9 DAB (arrows). **B:** By 11 DAB, specific staining around the neuronal membrane can be observed (solid arrows). The proximal dendrites are also labeled (open arrow). **C:** Staining reaches an adult-like pattern and intensity by 21 DAB (arrows). Scale bar = 20 µm.

medial and lateral limbs of the LSO. The intensity of staining increases along the neuronal surface with age and reaches the adult-like pattern by 21 DAB (Fig. 9C). Staining intensity does not change significantly at older ages.

MSO

The principal neurons in the MSO receive bilateral, spatially segregated excitatory input from spherical bushy cells in the AVCN. The input is oriented such that ipsilateral spherical bushy cells project to the lateral dendritic field of MSO neurons, and contralateral spherical bushy cells project to the medial dendritic field (Cant, 1992).

Cat-301 immunostaining is present along cell bodies and both medial and lateral dendritic fields of mature MSO neurons. Staining is not present at 7 DAB. Immunostaining is first evident at 11 DAB (Fig. 10A) and increases until 21 DAB, when an adult-like pattern is achieved (Fig. 10B). Staining intensity continues to increase slightly during the period from 21 DAB to 1 year. Medial and lateral dendrites in the MSO are immunostained along a greater extent of their surface than dendrites in the other auditory nuclei (Fig. 10B).

DISCUSSION

Cat-301 immunoreactivity is first seen in the gerbil auditory brainstem nuclei at 7 DAB. The punctate pattern seen in the AVCN and PVCN at this early time is transient, and, by 11 DAB, staining is found around the cell soma. In contrast, neurons in the MNTB, LSO, and MSO show staining around the neuronal soma at the earliest times that Cat-301 immunoreactivity is seen in these nuclei (MNTB, 7 DAB; LSO, 9 DAB; MSO, 11 DAB). The density of stain around the neurons and proximal dendrites as well as the number of stained neurons increases over time until adult-like distributions are reached (AVCN, 21 DAB; PVCN, 21 DAB; MSO, 21 DAB; LSO, 21 DAB; MNTB, 1 year). The increased Cat-301 immunoreactivity in these auditory brainstem nuclei is correlated with an increase in Cat-301 protein in the entire gerbil brainstem. Cat-301 immunoreactivity increases in all auditory nuclei examined as the age of the animal increases, except within the PVCN. There appears to be a slight loss of Cat-301 immunoreactivity in the PVCN at 1 year. Although the cause of this decrease is not known, it is likely related to the degeneration seen in this nucleus. Degenerative lesions have been observed in the PVCN as early as 6 weeks of age, and these lesions include the formation of microcycts and degeneration of neuronal perikarya and axons (Ostapoff and Morest, 1989). The cause of this degeneration is not known, nor is it known whether these lesions result in any auditory deficits (Ostapoff and Morrest, 1989).

The development of Cat-301 immunoreactivity in the gerbil auditory brainstem nuclei appears to parallel many aspects of physiological and morphological development in these structures. An exception to this pattern is found in the DCN. Interestingly, the DCN shows only faint immunoreactivity for Cat-301 at 31 DAB, and, by 1 year, only moderate staining of a small group of neurons is observed. The lack of immunostaining in the DCN shows that the development of the proteoglycan recognized by Cat-301 is not necessary for the onset or maintenance of information coding throughout the central auditory system. Clearly, the DCN neurons are able to carry out their functions without this molecule. On the other hand, the AVCN, PVCN, and olivary nuclei express this epitope in a pattern that closely parallels the ontogeny of function, suggesting some fundamental role of Cat-301. For most of the remainder of this discussion, we shall focus on these parallels and return at the end to speculate on what may be relevant *differences* in information processing between the DCN and other brainstem auditory regions.

Development of Cat-301 staining and onset of neural activity

At 7 DAB, staining for Cat-301 is limited to a tufted pattern in both the AVCN and the PVCN and early somatic staining in the PVCN and MNTB. The significance of the tufted pattern is not clear. It may be that the Cat-301 protein is being transported through the eighth nerve into the AVCN and PVCN at this time and is just beginning to be deposited within the extracellular space of the nuclei. However, we have not observed Cat-301 immunoreactivity in auditory nerve fibers at this time. Alternatively, cells within these nuclei, which have yet to be identified, may be starting production of the Cat-301 protein. Finally, the protein itself may be modified during development in such a way that the Cat-301 antibody does not recognize it before 7 DAB. The latter case appears to be unlikely, because both the punctate staining and the staining around the neuronal somata can be observed in the PVCN at 7 DAB. In addition, neurons in the MNTB stain for Cat-301 along their somal surface at the earliest times at which they were examined (7 DAB).

The onset of staining around the neuronal membrane of many cells in the AVCN, PVCN, LSO, MSO, and MNTB occurs between 9 and 11 DAB (the DCN is the exception and will be discussed later). An adult-like pattern of Cat-301 immunoreactivity within the gerbil auditory brainstem is not related to the ingrowth of axons into their appropriate nuclei but, instead, appears to occur approximately 1 day before the neurons have been found to respond to acoustic stimuli. Spontaneous activity (although not evoked activity) can be recorded from auditory nerve fibers (Woolf and Ryan, 1986, 1988) by 10 DAB. Centrally, auditory brainstem responses (ABRs) can first be evoked by click stimulation at 12 DAB (Smith and Kraus, 1987; Woolf et al., 1988), although thresholds are very high (Donaldson and Rubel, 1990). Previous studies have found that, at 10 DAB, VCN neurons are unresponsive to sound, although they are spontaneously active (Woolf and Ryan, 1985). By 12 DAB, approximately 15% of neurons are responsive to auditory stimuli, and, at 14 DAB or older, the majority of VCN neurons respond to sound.

The increase in Cat-301 immunoreactivity to adult-like distributions between 11 and 21 DAB parallels the maturation of many physiological response properties of auditory neurons during this same time period. For example, physiological responses, including tuning of single unit responses, maximum discharge rates (Woolf and Ryan, 1985) and the latency and amplitude of the ABR (Woolf et al., 1988), is mature between 18 and 25 DAB. In addition, many parameters of VCN neurons improve between days 12 and 18: mean spontaneous discharge rate increases, neural thresholds improve, and the dynamic range of neurons increases. Most of these parameters exhibit adult characteristics by 18–22 DAB. Auditory coding properties in single neurons in the LSO have also been examined;

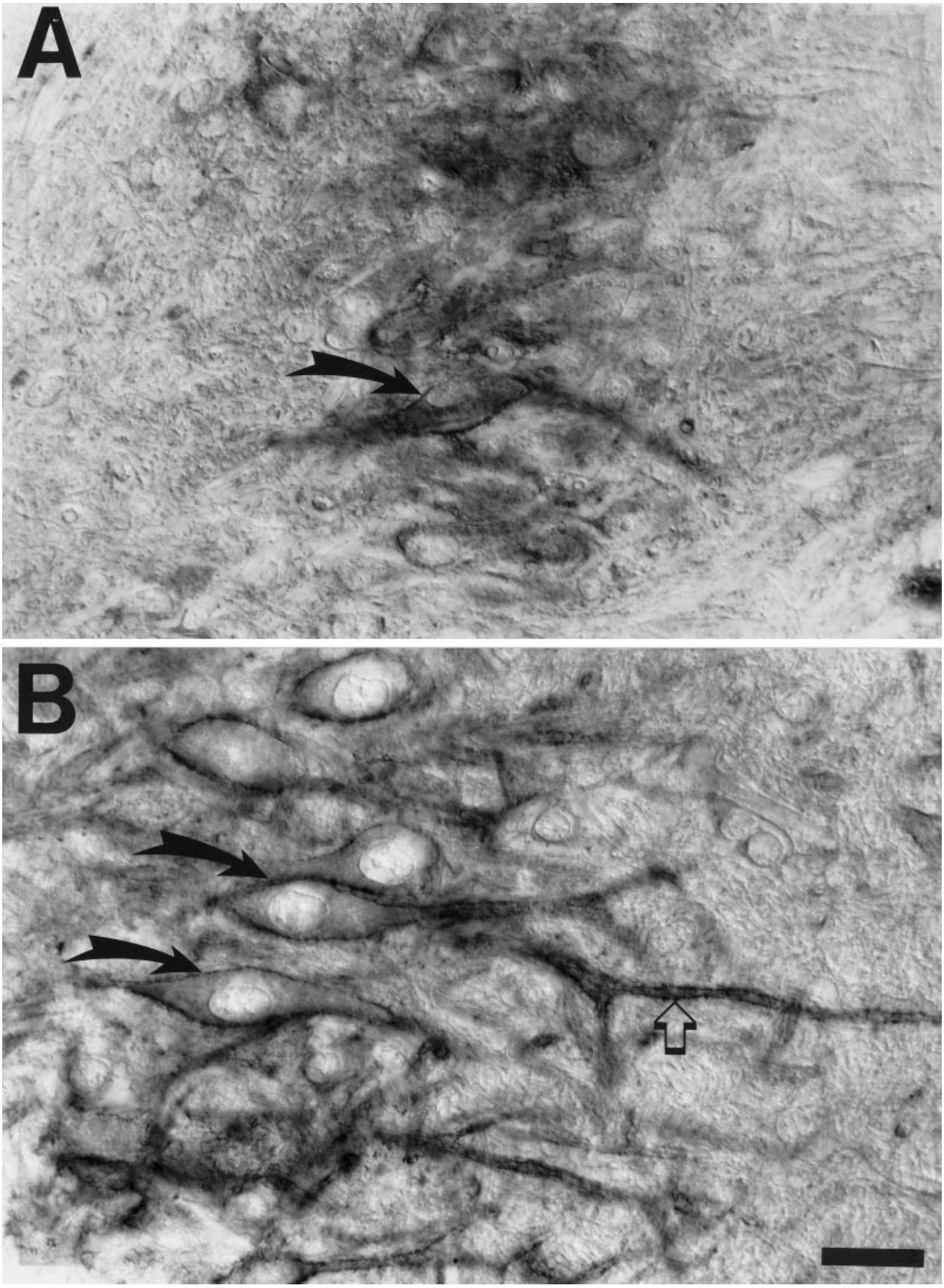

Fig. 10. Cat-301 immunoreactivity in the MSO. **A:** Cat-301 immunoreactivity is first seen at 11 DAB, when some neurons are stained along the somal surface (arrow). **B:** Immunostaining increases until 21 DAB, when an adult-like pattern is achieved (solid arrows). Medial and lateral dendrites in the MSO are immunostained along a greater extent of their surface than dendrites in other auditory nuclei (open arrow). Scale bar = 20 µm.

improvement in frequency selectivity, dynamic range, tonotopic alignment, and resolution have been observed during the first 3 postnatal weeks (Sanes and Rubel, 1988). The emergence of adult-like Cat-301 immunostaining patterns between 11 and 21 DAB is concomitant with the development of adult-like physiological responses of the auditory brainstem neurons.

Development of Cat-301 staining and synaptogenesis

At birth, VCN axons have already established ordered pathways to the contralateral MNTB, ipsilateral LSO, and both ipsilateral and contralateral MSO (Kil et al., 1995). Interestingly, developing VCN axons that innervate MNTB begin to exhibit terminal morphological characteristics of the calyx of Held at 5 DAB. At this time, Cat-301 immunoreactivity is distributed in a punctate pattern around MNTB neurons. By 14 DAB, the entire circumferences of the MNTB neurons are covered by Cat-301 immunoreactivity. This coincides with the appearance of the mature calyx, which occurs 14–16 DAB (Kil et al., 1995). In fact, cells in the brainstem auditory nuclei of the gerbil are surrounded by Cat-301 immunoreactivity by 11 DAB, and it is during the first 12–13 postnatal days that refinement of terminal processes occurs. An intriguing hypothesis is that Cat-301 may be involved in the formation of permanent and stable synaptic connections.

Role of Cat-301

The Cat-301 protein found in brain is structurally related to aggrecan, a high-molecular-weight, cell surface-associated sulfate chondroitin proteoglycan from cartilage (Fryer et al., 1992). Both aggrecan and the Cat-301 protein from brain form aggregates with hyaluronic acid. It has been postulated that the Cat-301 antigen may associate with neuronal surfaces by binding to hyaluronic acid, which surrounds many cells (Fryer et al., 1992). Electron microscopic studies have demonstrated that the Cat-301 protein is localized along neuronal surfaces but is excluded from synaptic regions (Hockfield and McKay, 1983; Hockfield et al., 1990a).

The Cat-301 antibody recognizes a cell surface antigen on subsets of neurons in the mammalian central nervous system, including central visual areas of the cat, monkey, and human (Deyoe et al., 1990; Hockfield and Sur, 1990; Hockfield et al., 1990a; Mize and Hockfield, 1989; Rausell and Jones, 1991), cat cerebellum (Sahin and Hockfield, 1990), cat primary auditory cortex (Wallace et al., 1991), frontal and parietal monkey cortex (McGuire et al., 1989), and hamster motor neurons (Kalb and Hockfield, 1988). Cat-301 labels defined neuron classes in the cat and primate visual system. In the LGN of these animals, the magnocellular or Y-cells (the neurons that process the motion component of a visual stimulus) label with the Cat-301 antibody (Hendry et al., 1984; Hockfield and Sur, 1990; Hockfield et al., 1983; Sur et al., 1988). In contrast, neurons involved in the processing of form and color express lower levels or completely lack Cat-301 immunoreactivity.

In addition, Cat-301 immunoreactivity has been found to be regulated by neuronal activity. Cats deprived of patterned visual input (i.e., monocular lid suture) during the critical period for maturation of physiological properties of Y-cells in the LGN show a decreased number of cells with response properties of the Y-cell class in the LGN (Sherman and Spear, 1982). This result is not found in adult cats that undergo visual deprivation (Sur et al., 1988). Monocular lid suture of animals during the critical period for maturation of Y-cells (before Cat-301 is expressed on these cells) reduces the development of Cat-301 immunoreactivity in the LGN (Sur et al., 1988), and this is correlated with the decrease in the number of Y-cells that can be identified physiologically.

A parallel regulation of Cat-301 immunoreactivity is also observed in the hamster spinal cord. A variety of pharmacological and surgical manipulations that alter the input to sciatic motor neurons in the hamster lead to a decrease in Cat-301 expression if they are performed before the onset of the Cat-301 immunoreactivity, between postnatal days 7 and 14 (Kalb and Hockfield, 1992). These lesions have no effect on Cat-301 immunostaining of hamster sciatic motor neurons when performed in adult animals after the onset of the normal Cat-301 development (Kalb and Hockfield, 1988, 1990).

From the findings noted above, Hockfield and coworkers have concluded that the onset of Cat-301 activity marks a point when neurons acquire their mature properties and that its expression appears to be regulated by neuronal activity during development (Hockfield et al., 1990a). The biochemical properties of Cat-301 indicate that it is a component of the extracellular matrix. The extracellular matrix has been hypothesized to stabilize synapses once the period of synaptic modifications has ended, and the Cat-301 antigen may be involved in this process (Hockfield et al., 1990a).

The onset of Cat-301 immunoreactivity around neurons in many nuclei of the gerbil auditory brainstem occurs after the onset of spontaneous activity and shortly before the onset of sound-evoked activity. The maturation of Cat-301 immunostaining between days 11 and 21 parallels the maturation of physiological properties of neurons in these nuclei. Once established, Cat-301 immunoreactivity remains present and essentially undiminished in the auditory nuclei (except for the PVCN) through 1 year.

It is not known whether afferent deprivation of the gerbil auditory brainstem before the onset of Cat-301 immunoreactivity prevents the expression of Cat-301. However, afferent deprivation of the VCN and MNTB in the gerbil results in either cell death with atrophy of the remaining cells or atrophy alone. Afferent deprivation or removal of the cochlea results in decreased cross-sectional area of neurons within these nuclei in adolescent gerbils (Hashisaki and Rubel, 1989; Pasic and Rubel, 1989, 1991; Pasic et al., 1994), and these decreases in cell size are fully reversible if eighth nerve activity is restored (Pasic and Rubel, 1989, 1991; Pasic et al., 1994). In contrast, animals that receive a cochlear ablation at or before 1 one week (before the onset of Cat-30 immunoreactivity around neurons) show a 59% reduction in neuron number in the AVCN. Animals that receive the ablation at 20 weeks (well after the onset of Cat-301 staining) show no cell loss after 3 months of recovery (Hashisaki and Rubel, 1989). Recent experiments by Moore and colleagues have shown that these age constraints are remarkably restricted; cochlear ablation at 5 DAB results in 76% cell loss in the AVCN, whereas cochlear ablation at 9 DAB results in only 5% cell loss in the AVCN (Tierney et al., 1995). Based on these findings, it appears that AVCN neurons are susceptible to afferent deprivation-induced cell death until only about 7–8 days of age. This correlates with the onset of Cat-301

immunoreactivity in the AVCN. It is possible that, once AVCN neurons are surrounded by Cat-301, they become protected from the intracellular events causing cell death following cochlear ablation. It will be of great interest to determine, first, whether early afferent deprivation reduces Cat-301 expression in the auditory system and, second, whether there is a causal relationship between the development of Cat-301 immunoreactivity and protection from cell death.

The role that the Cat-301 proteoglycan plays in the gerbil auditory system remains to be elucidated; however, our results suggest that Cat-301 may be involved in the development of appropriate synapses within the auditory brainstem. Neurons in AVCN receive large calyceal endings (called end bulbs of Held) from the eighth nerve. These large endbulbs provide extremely secure synaptic transmission and faithful preservation of the temporal properties of the eighth nerve spike train. These "primary like" units project via the trapezoid body to the major nuclei of the superior olivary complex, which includes the MSO and LSO. The basic processing of interaural time and intensity differences for spatial localization is thought to occur in these nuclei. It is along this pathway that Cat-301 staining is observed.

In contrast, there is little Cat-301 staining in the dorsal cochlear nucleus. The dorsal pathway from this nucleus has been proposed to be the first stage in pattern processing (Irvine, 1986). Neurons from this nucleus project mainly to the nucleus of the lateral lemniscus and the inferior colliculus. It is intriguing to speculate about the similarities in information processing of the parts of the motor, visual, and auditory pathways that show high levels of Cat-301 immunoreactivity. In all three cases, the temporal processing properties are of paramount importance, and, in these systems, the formation of aberrant synaptic connections could severely degrade the precision of this processing. It may be that, in the DCN, secure synapses are not required, because pattern processing rather than temporal processing is thought to occur. Thus, one might speculate that the positioning of the Cat-301 molecule is perfectly situated to *prevent* the formation of extraneous synaptic connections by inhibiting contact with potential postsynaptic sites. During development, when synaptic connections are being formed (and often pruned and modified), the presence of the Cat-301 molecule would be detrimental. When synaptic connections have been formed in their appropriate locations and are functioning correctly, the Cat-301 molecule may then be positioned to prevent the occurrence of aberrant synaptic connections.

We have demonstrated that physiologically immature auditory neurons in the gerbil brainstem exhibit different molecular characteristics than physiologically mature neurons. Specifically, physiologically immature neurons are surrounded by less Cat-301 staining than functionally mature neurons. The location and distribution of the Cat-301 protein suggests that it may be playing a role in maintaining appropriate response properties of these neurons by regulating the formation of their synaptic contacts.

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