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Neuronally Modulated Transcription of a Glycine Transporter in Rat Dorsal Cochlear Nucleus and Nucleus of the Medial Trapezoid Body

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ABSTRACT

Neurotransmitter transporters limit transmitter concentration at the postsynaptic membrane by removing neurotransmitters from the synaptic cleft. Not only do neurotransmitter transporters contribute to the regulation of synaptic transmission, but they themselves might be dynamically regulated by neuronal activity of the neurons in which they are expressed. In this experiment, we investigated the question of whether the transcription of two different glycine transporters, Glyt1 and Glyt2, is influenced by neuronal activity. These transporters are found in the dorsal cochlear nucleus (DCN) and medial nucleus of the trapezoid body. Glyt1 and Glyt2 mRNA were measured by using hybridization histochemistry and a semiquantitative reverse transcription polymerase chain reaction. *Decreases* in auditory primary afferent activity, caused by either unilateral labyrinthectomy or disruption of the middle ear ossicles, caused a reduction in Glyt2, but not Glyt1 mRNA in the ipsilateral DCN and in the contralateral medial nucleus of the trapezoid body. Acoustic stimulation at either 10 kHz or 40 kHz was used to provide controlled *increases* in primary afferent activity, evoking localized increases in Glyt2 mRNA in clusters of neurons in the DCN. The location of these clusters corresponded to the regions of the auditory tonotopic map devoted to these frequencies. The duration of changes in Glyt2 mRNA evoked by unilateral labyrinthectomy, measured with the reverse transcription polymerase chain reaction, was 5–10 days. These data provide the first example of in vivo regulation of transporter transcription by neuronal activity. J. Comp. Neurol. 415:175–188, 1999.

Indexing terms: auditory system; Glyt1; Glyt2; hybridization histochemistry; polymerase chain reaction

Chemical synaptic transmission is the primary means of intercellular communication in most nervous systems. Neuronally triggered synaptic release changes the extracellular concentrations of specific transmitters, and this change is detected by specialized postsynaptic receptors. Activation of these specific receptors either increases (excitatory) or decreases (inhibitory) postsynaptic discharge. Synaptic transmission is influenced not only by the quantity of released transmitter and the density of postsynaptic receptors, but also by how rapidly the released transmitter diffuses from the postsynaptic cleft. Synaptic transmitter transporters, found in both neurons and neuroglia, provide an additional mechanism for the active removal (reuptake) of neurotransmitters from the synaptic cleft as well as the possible replenishment of transmitter in synaptic terminals (Attwell and Mobbs, 1994; Uhl and Johnson, 1994; Worrall and Williams, 1994; Lester et al., 1996; Otis et al., 1996; Diamond and Jahr, 1997).

Within the past decade, molecular studies have identified a "super family" of ion-coupled transporters that are responsible for the uptake of several neurotransmitters

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(Uhl and Johnson, 1994; Worrall and Williams, 1994; Lester et al., 1996). Although the molecular and short-term physiological properties of transporters are increasingly understood, the possible longer-term functions of transporters have not been examined.

Two transporters for the amino acid glycine are expressed in the central nervous system: Glyt1 and Glyt2 (Liu et al., 1993; Jursky et al., 1994; Jursky and Nelson, 1995; Luque et al., 1995; Zafra et al., 1995a,b; Goebel, 1996). Glyt1 is expressed primarily but not exclusively in neuroglia (Adams et al., 1995). Glyt2 is expressed in neurons in the caudal brainstem, spinal cord, and cerebellum (Zafra et al., 1995a,b; Goebel, 1996). In particular, two auditory brainstem nuclei, the DCN and MNTB, express Glyt2.

High- and low-affinity glycine uptake mechanisms are found in DCN neurons (Staatz-Benson and Potashner, 1987, 1988). In brain-slice preparations, glycine is released by electrical stimulation of the DCN (Staatz-Benson and Potashner, 1988). More than one subset of DCN neurons is both glycinergic and expresses the Glyt2 transporter (Wenthold et al., 1987; Zafra et al., 1995a; Gates et al., 1996; Romand and Avan, 1997). Cartwheel neurons, found in the molecular layer, express glycine, γ-aminobutyric acid (GABA), and Glyt2 (Wenthold et al., 1987; Kolston et al., 1995; Gates et al., 1996; Romand and Avan, 1997). Tuberculoventral neurons, found in the polymorphic cell layer, express glycine and Glyt2 (Wenthold et al., 1987; Kolston et al., 1995).

In this experiment, we asked, does auditory primary afferent activity influence the transcription of either Glyt1 or Glyt2 mRNA in glia and neurons of the DCN and MNTB? Controlled *decreases* in auditory primary afferent activity were effected by unilaterally damaging the labyrinth as well as by removing unilaterally one of the middle ear ossicles. Controlled *increases* in auditory primary afferent activity were effected by pure tone acoustic stimulation at either high or low frequencies (40 kHz or 10 kHz). Both increases and decreases in auditory primary afferent activity caused corresponding changes in Glyt2 in the ipsilateral DCN and contralateral MNTB. These data support a possible role for Glyt2 in adaptive changes in the neural circuitry of the auditory system.

Abbreviations

asc7	ascending branch of facial nerve
DCN	dorsal cochlear nucleus
DVN	descending vestibular nucleus
ECu	external cuneate nucleus
GiA	nucleus reticularis gigantocellularis α
GrC	granule cell layer
g7	genu of the facial nerve
icp	inferior cerebellar peduncle
LVN	lateral vestibular nucleus
MNTB	medial nucleus of the trapezoid body
MVN	medial vestibular nucleus
Pr	nucleus prepositus hypoglossi
PnV	ventral pontine reticular nucleus
Py	pyramidal tract
scp	superior cerebellar peduncle
SG	Scarpa's ganglion
SO	superior olive
sol	tract of the solitary nucleus
sp5	spinal trigeminal tract
Sp5	spinal trigeminal nucleus
SVN	superior vestibular nucleus
VCP	ventral cochlear nucleus posterior
VCN	ventral cochlear nucleus
7	facial nucleus
8n	eighth nerve
9	lobule 9 (uvula)
10	lobule 10 (nodulus)

MATERIALS AND METHODS

Fifty-two Sprague Dawley rats of both sexes (160–320 g) served as subjects in different aspects of this experiment. The rats were housed and handled according to the guidelines of the National Institutes of Health on use of experimental animals. During surgical procedures, the rats were anesthetized with ketamine hydrochloride (50 mg/kg), xylazine (6 mg/kg), and acepromazine maleate (1.2 mg/kg). The nomenclature of the rat atlas of Paxinos and Watson (Paxinos and Watson, 1986) was used in the illustrations.

Unilateral labyrinthectomy

The labyrinth was surgically destroyed unilaterally in 10 rats. A retroauricular 5-mm incision was made to reach the middle ear through the tympanic membrane. The stapes was disinserted from the oval window. A fine dental drill was used to open and destroy the cochlea as well as the vestibular labyrinth.

Surgical disruption of the ossicular chain

In five rats, an asymmetry in auditory input was created by unilaterally removing the incus, one of the middle ear bones. The tympanic membrane was slit, and the incus was withdrawn with fine forceps through the incision, leaving the oval window intact. This operation caused no central damage to either the auditory or vestibular systems. Disruption of the ossicular chain causes a reduction of 40–60 dB in acoustic stimulation without damaging the auditory-vestibular nerve (Schuller and Schleuning, 1994).

Acoustic stimulation

Ten rats were stimulated with pure tones (10–40 kHz; 60–80 dB) continuously for 24–72 hours. The speaker used for auditory stimulation was suspended 20 cm above a $30 \times 30 \times 20$ cm enclosure in which rats were housed and sound pressure level measurements were made at the center of the enclosure.

Hybridization histochemistry

Hybridization histochemistry was done in 12 rats at different times after a unilateral labyrinthectomy. The rats were anesthetized; the brainstems were removed, fresh frozen, cut in 14-μm sections, and thaw-mounted on slides. Two 35-mer oligonucleotide sequences were used to construct probes for Glyt2 mRNA. One probe, Glyt2a, was inverse complementary to cDNA sequence, 2185–2219, near the 3′ end of rat Glyt2 cDNA (5′-ATCATCATCTCGATGTCTTCACAGAACCTCTGCAA-3′). A second probe, Glyt2b, was inverse complementary to cDNA sequence, 67–101, near the 5′ end of Glyt2 cDNA (5′-TTTGCGTCAGTGGGAAGCAAGGTGCCCTTCGTTTG-3′).

An oligonucleotide probe for the glycine transporter, Glyt1, was used to compare the distribution of this transporter with that of Glyt2. The Glyt1 probe included the rat cDNA sequence 452–486 (5′-CATCATCTTCCGCTACGTCTACTGGTGCAACATCA-3′). The probes were end-labeled with dATP (α^{33}P) (NEN #NEG-312H) by using a DuPont/

NEN oligonucleotide 3′ end-labeling system (#NEP-100) and purified with an NENSORB 20 column and had activities of 40–60,000 cpm/μl. After hybridization, tissue sections were exposed to an x-ray film for 24 hours before dipping and exposure to NTB2 (Kodak #165433) nuclear emulsion for 6–9 days. Sections were counterstained through the photographic emulsion with neutral red before cover-slipping.

Optical measurements of hybridization signals

Hybridization signals in DCN and MNTB were photographed under both lightfield and darkfield illumination by using a digital camera (Apogee Instruments) with a resolution of 1,500 × 1,000. Photographed images were analyzed by using a computer-based image analysis system (Image Pro Plus™). The contrast and intensity of the photographs were edited with Corel Photo-Paint™. Figures were composed with Corel Draw™ and printed with a resolution of 1,440 × 720 dpi.

Measurement of the hybridization signals from the DCN was done from images photographed at 40× magnification so that an entire transverse section through a DCN could be captured in a single image. Objects composed of single or multiple silver grains were counted for equal areas within both the superficial and deep layers of the DCN. Objects were counted from a minimum of four transverse sections taken at different rostro-caudal distances within the DCN. This method of analysis excluded differences that might be attributed to variations in tissue thickness and the angle at which the histologic sections were cut.

In the MNTB, hybridization signals of individual neurons were quantified at higher magnification, 400×. A square template (32 × 32 μm) was superimposed over labeled neurons within the MNTB and objects within its bounds were counted. Within each histologic section through the MNTB, 10–25 labeled neurons could be identified. Objects were counted from a minimum of four transverse sections, taken at different rostrocaudal distances within the MNTB.

Statistical methods

A multivariate analysis of variance (MANOVA) was used to test for differences between left and right hybridization signals (object counts) in the DCN and MNTB. Specifically, a mixed model with nested effects was used. For the analysis of DCN, the model terms were: (1) rat (random), (2) side (fixed, left or right), (3) rat × side interaction (random), and (4) histologic section nested within rat. For the analysis of MNTB, the model terms were (1) rat (random), (2) side (fixed, left or right), (3) rat × side interaction (random), (4) histologic section nested within rat (random), (5) side × histologic section nested within rat interaction (random), and (6) cell nested within histologic section nested within rat (random).

For the analysis of DCN, object counts in the superficial and deep layers were summed together to form dependent variables. Subsequently, they were analyzed separately as multivariate vectors. For the analysis of MNTB, there was only one count and this was analyzed as a univariate dependent variable. All data were analyzed by using SAS PROC GLM, version 6.12 for Windows.

Semiquantitative reverse transcription polymerase chain reaction

A semiquantitative reverse transcription polymerase chain reaction technique (RT-PCR) was used to measure the time course of decreased Glyt2 mRNA transcription in 15 rats after a unilateral labyrinthectomy (Eastwood et al., 1997). Three rats were euthanized at 6, 24, 72, 120, and 240 hours after a unilateral labyrinthectomy. In each rat, the two DCNs were removed and total RNA was isolated with TRIzol™ (Gibco). RT was done with an oligo(dT) 3′ primer. For PCR, oligonucleotide primers were selected to amplify a 400-bp gene product of Glyt2 spanning the region of 2001–2400. The forward and reverse primers were 5′-GTATCTGCGCACACACAAG-3′ and 5′-GATCCAGATCACGGAGCA G-3′, respectively. These primers bracketed the region identified by the 35-mer oligonucleotide probe for Glyt2 that was inverse complementary to the cDNA sequence, 2185–2219, of rat Glyt2.

As a control for ensuring that equal amounts of total RNA were derived from each pair of tissue samples, primers that amplified a 589-bp gene product of the "housekeeping gene" cyclophilin, were included in each RT-PCR reaction (5′-CACCGTGTTCTTCGACATC-3′ and 5′-GTGAGAGCAGAGATTACAG-3′). The primers used for the amplification of Glyt2 cDNA had a concentration of 1.5 ng/μl. The primers used for the coamplification of cyclophilin had a concentration of 0.2–0.4 ng/μl. The number of PCR cycles (25 cycles), were chosen to remain within linear regions of cDNA amplification. PCR gene products were separated on a 1.5% agarose gel, stained with ethidium bromide, and photographed with a digital camera with a 900 × 1,200 pixel resolution. The intensity of individual bands on the gel was quantified with a densitometric scanning program (SigmaGel™).

RESULTS

Glyt1 and Glyt2 mRNA hybridization signals in dorsal and ventral cochlear nuclei

Oligonucleotide probes for Glyt1 and Glyt2 had different patterns of hybridization in neurons in the dorsal and ventral cochlear nuclei. Glyt1 labeling was more diffuse. It extended throughout the brainstem and included white matter and neurons (Fig. 1B). The white matter labeling was most apparent by examination of the inferior cerebellar peduncle (icp), spinal tract of V (sp5), and pyramidal tract (Py), all of which hybridized the Glyt1 probe. Glyt1 hybridization signals were present in neurons in the ventral cochlear nucleus (VCN) and in glial cells throughout the brainstem.

The pattern of hybridization for the Glyt2 probe was restricted to neurons. The probe labeled neurons in the DCN, the lateral and superior vestibular nuclei (LVN, SVN), ventral aspect of the medial vestibular nucleus (MVN), nucleus reticularis gigantocellularis α (GiA), and the ventral aspect of the spinal trigeminal nucleus (Sp5). The Glyt2 hybridization signal from VCN neurons was weaker than for neurons in the DCN (Fig. 1C,D). In the DCN, the Glyt2 probe labeled two bands of neurons. One band was in the molecular layer, corresponding to the location of glycinergic cartwheel neurons. The other band was in the deeper polymorphic cell layer, corresponding to the location of glycinergic tuberculoventral neurons.

Two different oligonucleotide probes were constructed from opposite ends of the cDNA sequence as a test for

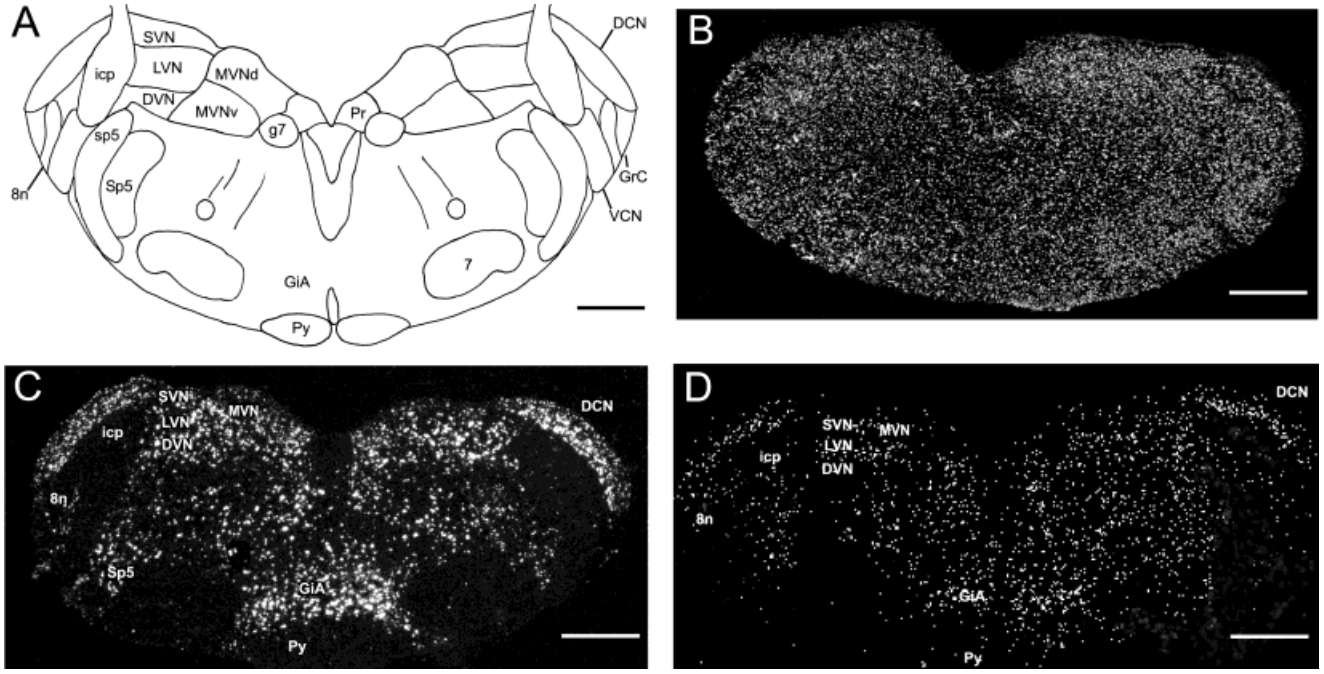

Fig. 1. Patterns of hybridization of Glyt1 and Glyt2 oligonucleotide probes in the brainstem of rats. **A:** Schematic illustration of the rat brainstem at the level at which the transverse sections in B,C, and D were taken. Oligonucleotide probes for Glyt1 (B), Glyt2a (C), and Glyt2b (D) were hybridized with transverse sections through the brainstem at the level of the DCN of rats that had previously received a unilateral labyrinthectomy. **B:** Glyt1 hybridization in a transverse section of a rat that had received a left unilateral labyrinthectomy 48 hours before sacrifice. **C:** Glyt2a hybridization in a transverse brainstem section of a rat that had received a left unilateral labyrinthectomy 72 hours before killing. **D:** Glyt2b hybridization in a transverse brainstem section of a rat that had received a left unilateral labyrinthectomy 48 hours before killing. In C and D, note the relative decrease in Glyt2 hybridization in the neurons of the deeper layer of the left DCN compared with the right DCN. For abbreviations, see list. Scale bars = 1 mm in B–D.

specificity for the hybridization of Glyt2. Both probes hybridized with the same subset of neurons within the brainstem and specifically within the DCN. Hybridization of the Glyt2a probe is illustrated in Figure 1C, and hybridization of the Glyt2b probe is illustrated in Figure 1D. Differences in the apparent intensity of hybridization reflect mainly different durations of exposure to the photographic emulsion before it was developed.

The patterns of hybridization of the two Glyt2 probes were analyzed in greater detail in the DCN. Figure 2 compares this pattern of hybridization in the DCN and at higher magnification at the level of individual neurons. Both probes labeled more cells in the polymorphic cell layer of the DCN than in the molecular layer (Fig. $2A_1$,B_1). Both probes did not label the small granule cells with cell body diameters that were less than 5 μm (Figure $2A_3$,B_3), distributed throughout the DCN. Neither did they label the infrequent large neurons with cell body diameters exceeding 18 μm (see neurons indicated by black arrowheads in Figure $2A_3$,B_3). Rather, they identified neurons whose soma diameters were 10–16 μm. These neurons probably correspond to stellate cells that are found throughout the DCN figure.

Unilateral labyrinthectomy reduces Glyt2 mRNA in the ipsilateral DCN

Because the transcription of Glyt2 mRNA is neuronally specific and the transcription of Glyt1 mRNA is not, we used the oligonucleotide probe for Glyt2 for further testing of the idea that neuronal activity alters the transcription of the Glyt2 transporter. For rats that received a unilateral labyrinthectomy, a clear difference in the hybridization patterns for Glyt2 mRNA within the DCN, emerged 12–144 hours after the operation. The hybridization signal for Glyt2 in neurons in the DCN ipsilateral to the labyrinthectomy decreased (Figs. 1C,D, 3). This reduction was evident throughout the rostral-caudal extent of the DCN, but most pronounced in the central regions (Fig. 3). In contrast, the almost uniform hybridization pattern for Glyt1 was unaffected in a rat that had been unilaterally labyrinthectomized with a postoperative survival time of 3 days (Fig. 1B). In two unoperated "control" rats, no differences between the hybridization signals from either the polymorphic cell layers or molecular layers were detected.

Unilateral disarticulation of the ossicular chain reduces Glyt2 mRNA in the ipsilateral DCN

It is possible that a unilateral labyrinthectomy could confound trophic influences caused by damaging the auditory-vestibular nerve with differences in neuronal activity caused by the consequent reduction in primary acoustic inputs. As an alternative to reducing primary acoustic activity by means of a unilateral labyrinthectomy, we disrupted the ossicular chain in the middle ear by removing one of the middle ear ossicles, the incus. Unlike a labyrinthectomy, this procedure causes a unilateral conductive hearing loss of 40–60 dB without damaging the auditory-vestibular nerve (Schuller and Schleuning, 1994).

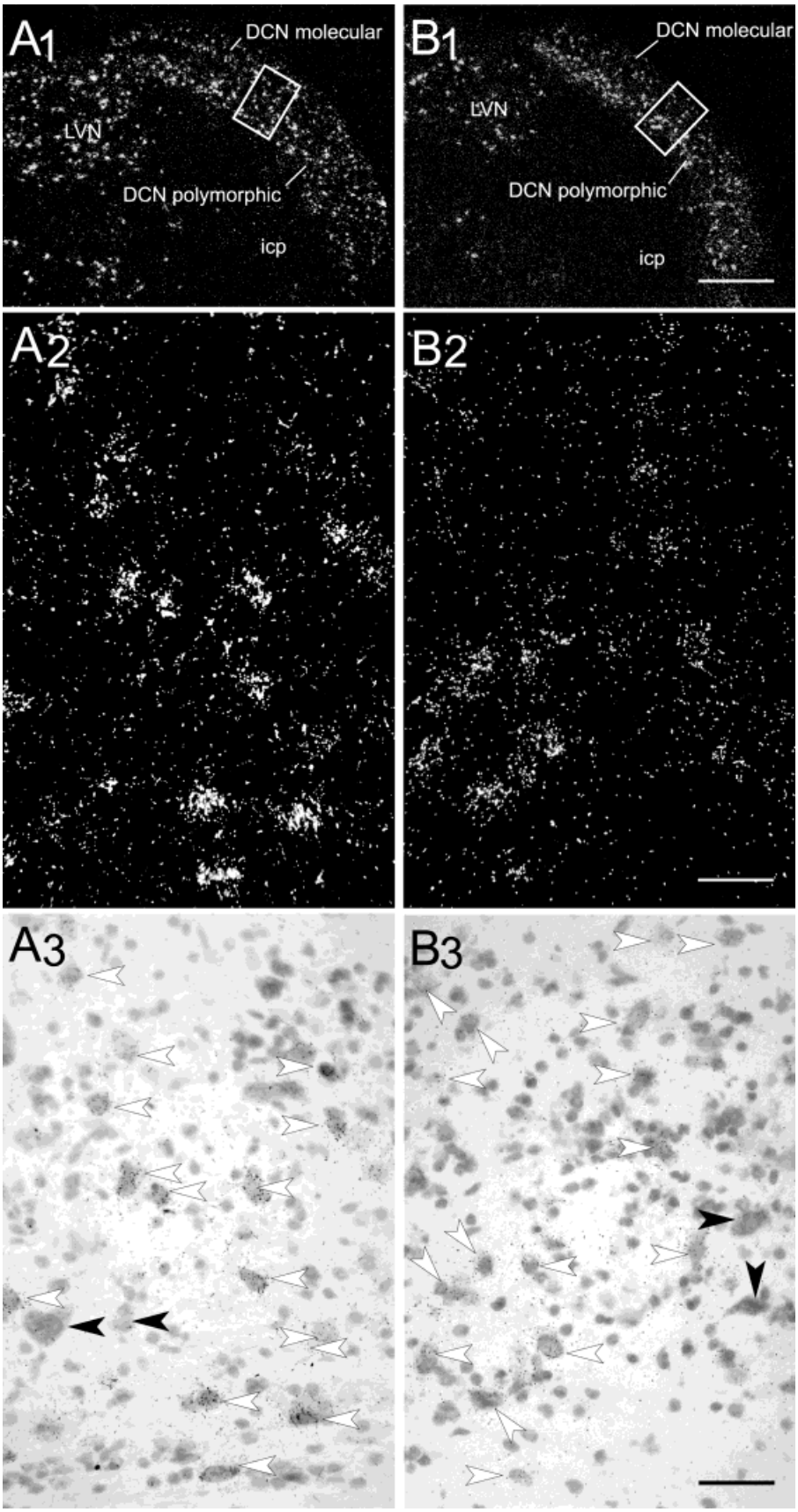

Fig. 2. Comparison of hybridization signals from Glyt2a and Glyt2b oligonucleotide probes in the DCN. Hybridization signals from neurons in the right DCN of two rats by using two different oligonucleotide probes are illustrated. **A_1:** A transverse section through the right DCN hybridized with the Glyt2a probe. **B_1:** A transverse section through the right DCN hybridized with the Glyt2b probe. The white rectangles in A_1 and B_1 are shown at higher magnification in both darkfield and lightfield photomicrographs in **A_{2-3}**and **B_{2-3}**, respectively. The white arrowheads in A_3 and B_3 indicate medium neurons in both the polymorphic and molecular layers that hybridized with the Glyt2 probes. The black arrowheads indicate large cells in the polymorphic layers that did not hybridize with either probe. For abbreviations, see list. Scale bars = 500 μm in B_1 (applies to A_1,B_1), 50 μm in B_2 and B_3 (applies to A_2,B_2, A_3, B_3).

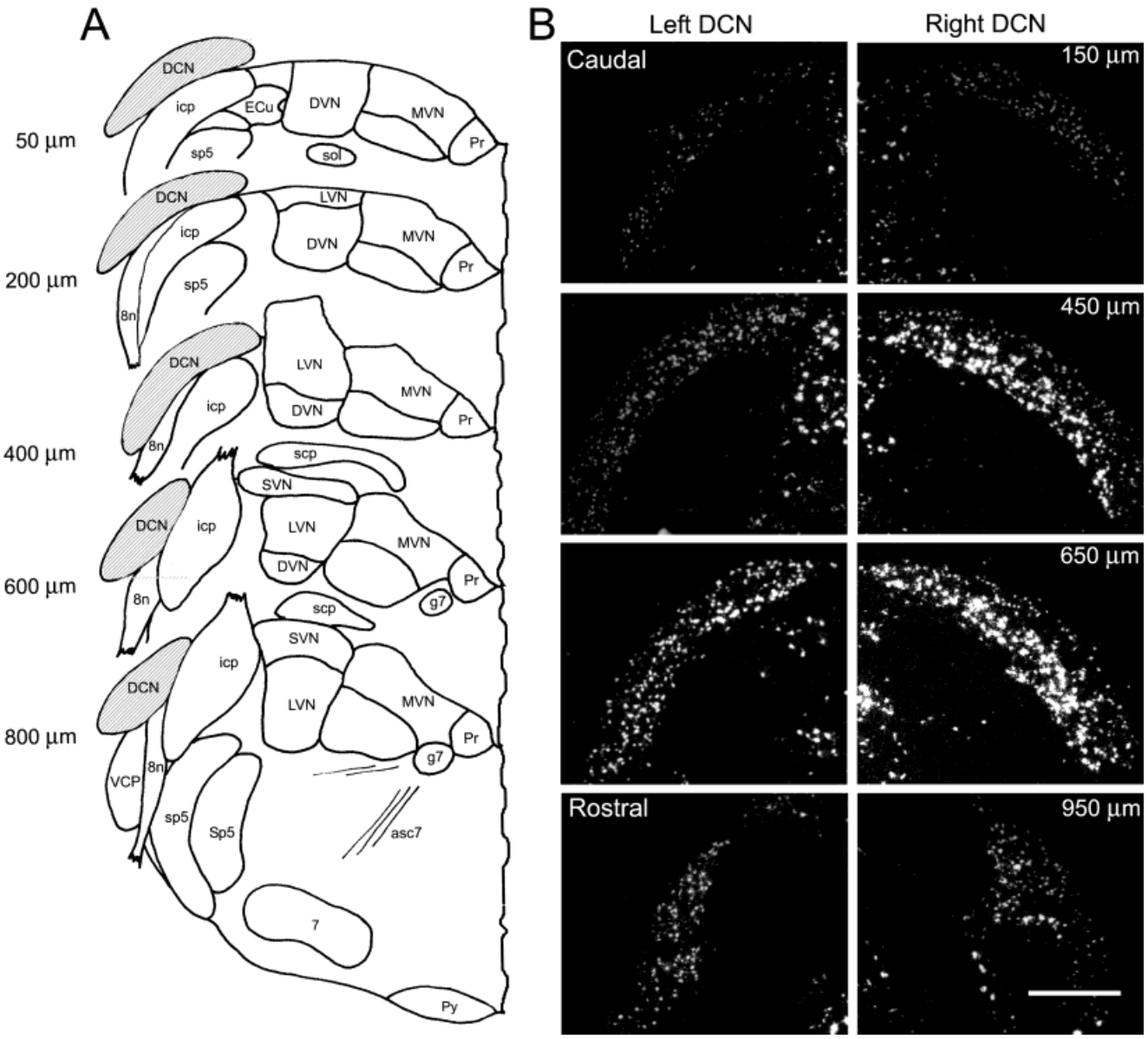

Fig. 3. Decrease in Glyt2a in the left DCN after a left labyrinthectomy. **A:** Schematic of the location of the DCN (shaded area) in the rat brainstem. The column of numbers on the left refers to distances from the caudal pole of the DCN. **B:** Comparison of the left and right DCN of a rat whose left labyrinth was surgically destroyed 48 hours before killing. Transverse sections through the DCN, under dark field illumination show a stronger hybridization signal for Glyt2 mRNA in neurons in the deeper, polymorphic cell layer of the right DCN. The numbers in the upper right-hand corner of each panel indicate distance from the caudal pole of the DCN. For abbreviations, see list. Scale bar = 500 µm.

After the operation, rats were exposed to ambient noise of the animal vivarium for 48 hours. The conductive hearing loss caused a reduction in Glyt2 mRNA in DCN ipsilateral to the operated ear (Fig. 4).

Quantification of reduced Glyt2 mRNA in the ipsilateral DCN after either a unilateral labyrinthectomy or unilateral ossicular chain disruption

Measurements were made of the Glyt2 hybridization signals in separate layers of the DCN after either unilateral labyrinthectomy (n = 5) or ossicular chain disruption (n = 5). The average postoperative survival time of the labyrinthectomized rats was 52 hours, whereas the average postoperative survival time of the rats with ossicular disruption was 56 hours. Each DCN was divided into halves of equal area. The deeper layer corresponded to the polymorphic cell layer. The more superficial layer corresponded to the combined pyramidal layer and molecular layer. Single and multiple grains ("objects") were counted within these bounds (Fig. 5A,B).

By using a MANOVA statistical analysis (see Materials and Methods section), the relative Glyt2 hybridization signals from each of these layers were compared. The signals from the layers ipsilateral to either the labyrinthec-

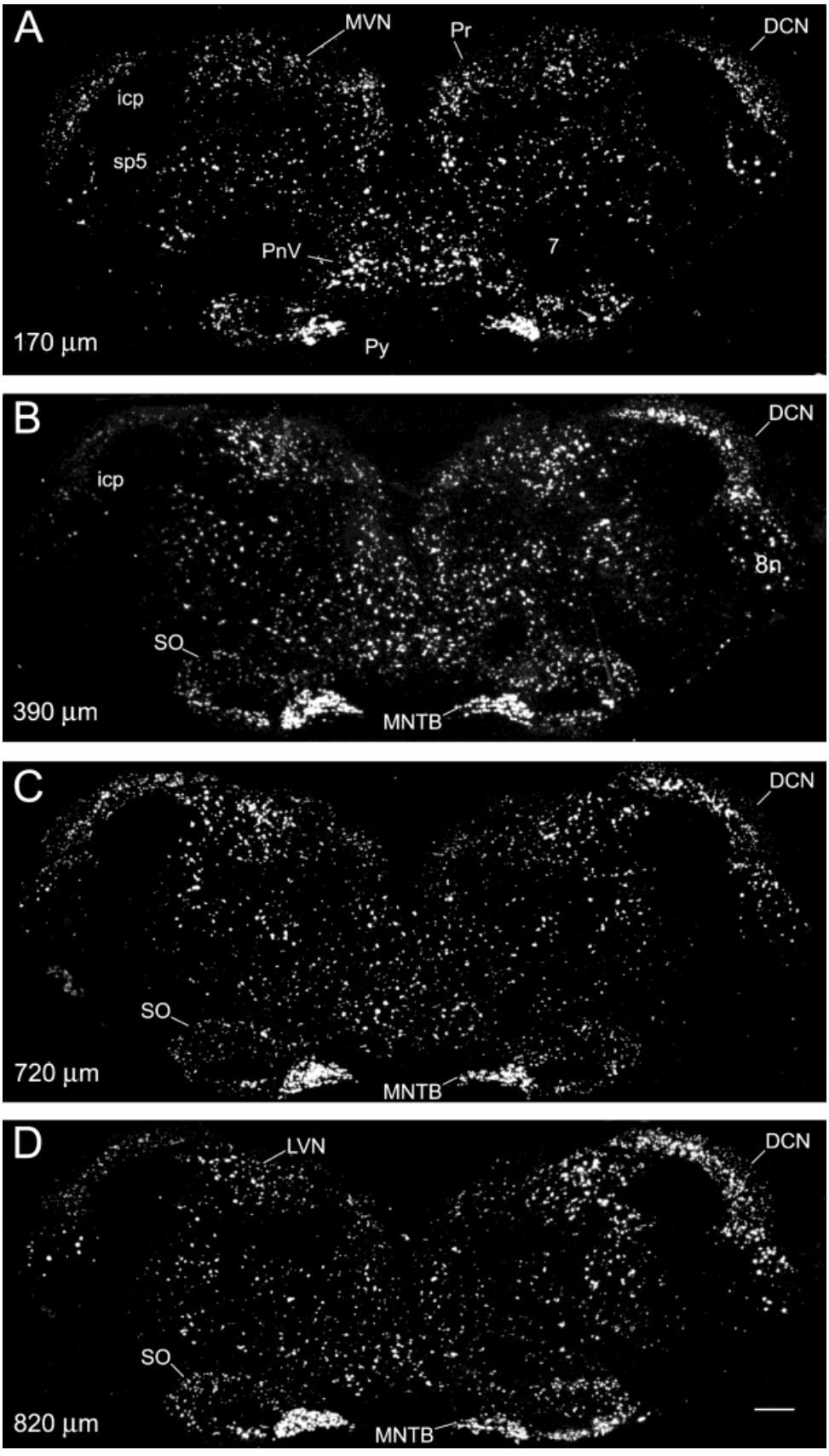

Fig. 4. Disarticulation of the left middle ear ossicles causes a decrease in Glyt2 mRNA in neurons in the left DCN. Glyt2 hybridization was decreased in the left DCN of a rat in which two of the three middle ear ossicles (incus and malleus) were removed from the left middle ear 50 hours before killing. **A–D:**Transverse sections through the DCN, under darkfield illumination show that neurons in the polymorphic cell layer of the right DCN, contain greater concentrations of Glyt2 mRNA than do neurons of the left DCN. **C,D:** The most intense labeling for Glyt2 mRNA is observed in the MNTB. The left and right MNTB are shown at higher magnification in Figure 6. The numbers in the lower left corner of each panel indicate the distance from the caudal pole of the DCN. For abbreviations, see list. Scale bar = 500 µm in D (applies to A–D).

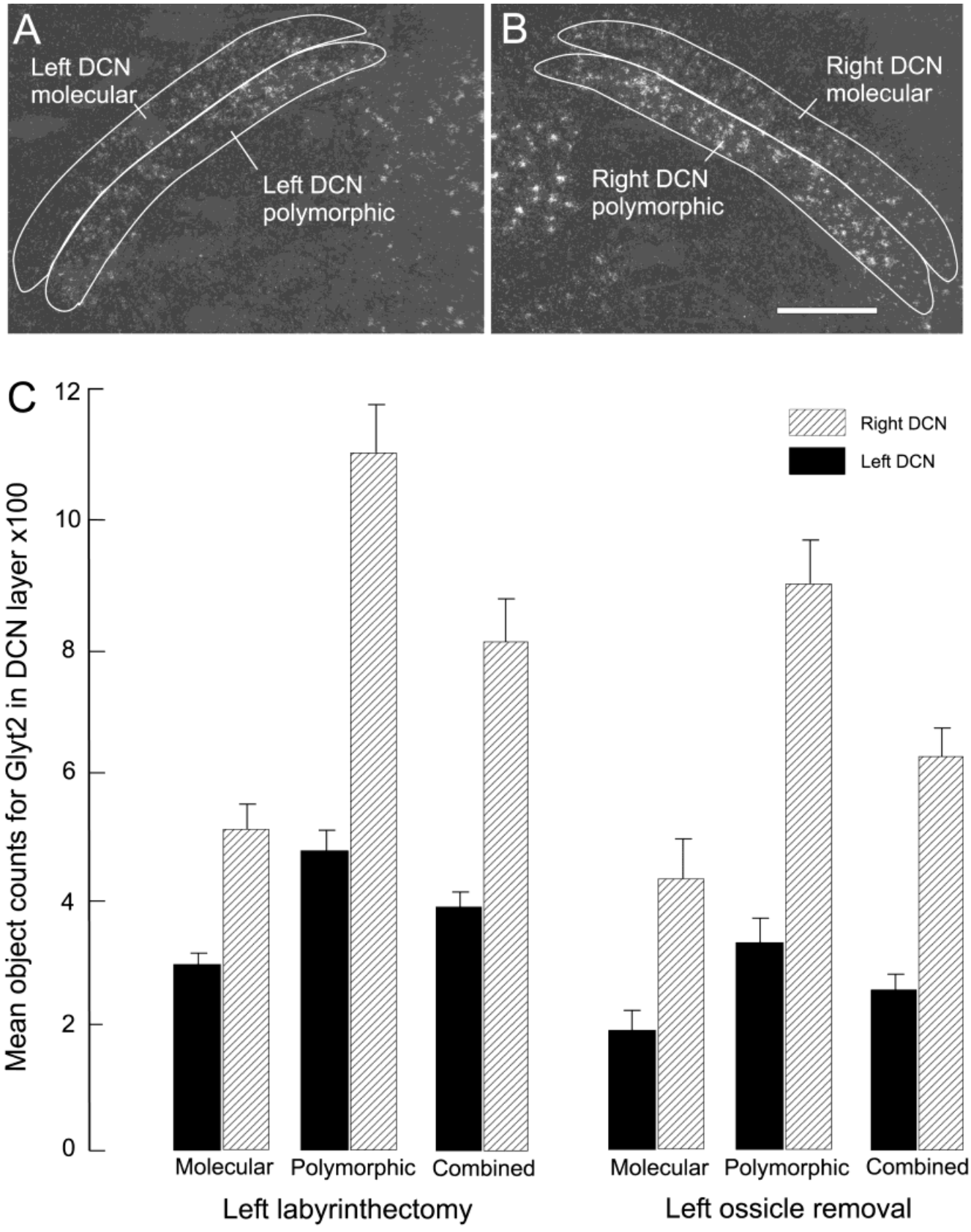

Fig. 5. Measurement of Glyt2a hybridization in different layers of the DCN after unilateral labyrinthectomy or unilateral removal of middle ear ossicles. The Glyt2a hybridization signals from the molecular and polymorphic layers of the left (**A**) and right (**B**) DCN were analyzed after a unilateral labyrinthectomy. The hand-drawn boundaries in A and B compose equal areas from which "objects" (multiple silver grains) were counted automatically for the molecular and polymorphic layers. Note the relatively greater hybridization signals from the layers in the right DCN. **C:** Histograms were constructed from measurements of hybridization signals (see Materials and Methods section). Separate measurements were made for signals derived from the molecular (superficial) and polymorphic (deep) layers of the DCN after either a unilateral labyrinthectomy (n = 5) or a unilateral removal of the middle ear ossicles (n = 5). Error bars represent standard errors of the mean. By using a multivariate analysis of variance (see Materials and Methods section), each of the differences between left and right layers was significant (P less than or equal to 0.001). For abbreviations, see list. Scale bar = 500 mm in B (applies to A,B).

tomy or the ossicular chain disruption were reduced compared with the signals from the layers of the contralateral DCN (Fig. 5C). These differences were significant (P less than or equal to 0.001).

Reduction in Glyt2 mRNA in the MNTB contralateral to the unilateral labyrinthectomy and ossicular chain disruption

The DCN was not the only auditory nucleus in which Glyt2 mRNA was found in abundance. Neurons in the MNTB were even more heavily labeled by oligonucleotide probes for Glyt2 (Figs. 4, 6). In contrast to the relative decrease in Glyt2 mRNA in the DCN caused by ipsilateral labyrinthectomies or ossicular disruption, cells in the contralateral MNTB had reduced hybridization signals than did cells in the ipsilateral MNTB. This pattern of labeling is consistent with the crossed descending projection to the MNTB from "globular-bushy" cells in the anteroventral cochlear nucleus (Warr, 1972; Tolbert et al., 1982; Smith et al., 1991; Kuwabara et al., 1991).

Measurements of relative hybridization signals from the left and right MNTB were made in four rats after unilateral labyrinthectomy and in four rats after unilateral ossicular disruption (see Materials and Methods section). In each rat, silver grains were counted from at least 100 neurons in both the left and right MNTB at five different rostrocaudal levels. A 32-μm square was positioned over each neuron, and all objects within the square were counted. This process was repeated for each labeled MNTB neuron within a histologic section. These measurements showed a reduction in Glyt2 mRNA contralateral to either the unilateral labyrinthectomy or ossicular disruption (P less than or equal to 0.001, Fig. 6B). Our statistical analysis undoubtedly underestimates the differences between MNTB neurons on the operated and unoperated sides because the photographic emulsions were often saturated by β-particle emissions from neurons that had a higher expression of Glyt2 mRNA.

Acoustic stimulation at low and high frequencies causes regional increases in Glyt2 mRNA within the DCN corresponding to a tonotopic map

If the transcription of Glyt2 mRNA in DCN neurons is related to eighth nerve activity, it should not only *decrease* when neuronal activity falls below spontaneous levels, it should also *increase* when neuronal activity exceeds spontaneous levels. Pure-tone acoustic stimuli (10 kHz or 40 kHz, 60–80dB) were used selectively to stimulate eighth nerve activity. Because acoustic frequencies are mapped on the DCN in a tonotopic manner, a pure-tone stimulus activates a discrete region of the DCN, corresponding to a frequency-specific location with respect to the tonotopic map (Ryan et al., 1988; Yajima and Hayashi, 1989). This method allows a comparison of "stimulated" and "unstimulated" neurons within the same DCN. Stimulation at 40 kHz caused an increase in Glyt2 mRNA in neurons in the dorsomedial aspect of the DCN (Fig. 7B). Stimulation at 10 kHz caused an increase Glyt2 mRNA in neurons in the ventrolateral aspect of the DCN (Fig. 7C). These spatially discrete increases in Glyt2 mRNA occurred in locations within the DCN predicted, based on the physiologically defined tonotopic map of the DCN (Yajima and Hayashi, 1989). As a control, the middle ear bones were removed unilaterally from two rats and these animals were subsequently exposed to a 40-kHz stimulus for 48 hours. Increased Glyt2 mRNA was detected in the dorsomedial aspect of the DCN with an intact acoustic input, whereas the hybridization signal from the DCN on the operated side was uniformly weaker (data not shown).

Change in Glyt2 mRNA in the DCN after a unilateral labyrinthectomy measured with quantitative RT-PCR

A modification of a semiquantitative RTPCR was used to measure the time course of the decrease and subsequent recovery of Glyt2 mRNA in the DCN after a unilateral labyrinthectomy. A neuronal-specific, constitutively expressed mRNA for cyclophilin was used as a control (Goldner and Patrick, 1996). The identity of the PCR-amplified 400-bp Glyt2 gene product was confirmed by sequencing the fragment amplified by PCR. In this fragment, 350/400 bp were recovered and corresponded to the exact inverse complement of rat cDNA sequence (2001–2350) for Glyt2. A Southern blot of the PCR-amplified gene product by using a ^{32}P–end-labeled 35-mer Glyt2 oligonucleotide probe, previously used hybridization histochemistry, demonstrated that the amplified gene fragment hybridized with this probe (Fig. 8A,B).

A one-way factorial ANOVA was also used to evaluate possible differences in the transcription of a neuronally expressed "housekeeping" gene, cyclophilin, in the left and right DCN after a unilateral labyrinthectomy. No difference between the two DCNs in cyclophilin transcription was detected (Fig. 8C,D). This control further emphasizes that not all gene transcriptions are similarly affected by cellular activity. A one-way factorial ANOVA was used to compare the ratios of Glyt2/cyclophilin gene fragments for each of three rats at five different postlabyrinthectomy intervals. This analysis demonstrated that the Glyt2/cyclophilin ratios were time-dependent (P less than or equal to 0.02) (Fig. 8D).

Differences between Glyt2 cDNA for the deafferented and intact DCNs were evaluated for each postoperative period with one-way t-tests with Scheffé's correction. The difference between the ratios of Glyt2/cyclophilin at 72 hours after the unilateral labyrinthectomy reached significance (P less than or equal to 0.02). Differences at either 24 or 120 hours postoperatively fell short of significance (P less than or equal to 0.10). These data indicate that changes in Glyt2 transcription in the DCN induced by the unilateral labyrinthectomy are "compensated" approximately 5–10 days after the operation.

DISCUSSION

Identification of DCN neurons containing glycine transporter

Glyt2 was localized to neurons in several brainstem nuclei, including the DCN. Within the DCN, Glyt2 mRNA was localized to neurons, whereas Glyt1 mRNA was localized primarily to white matter and glial cells. These findings agree with previous studies of the tissue distribution of these two glycine transporters (Adams et al., 1995; Zafra et al., 1995a,b; Jursky and Nelson, 1996). The Glyt1 oligonucleotide probe used in this experiment identified a sequence of the Glyt1 mRNA that is common to the three different isoforms (Glyt1a, b, c) (Adams et al., 1995). It is possible that an oligonucleotide probe directed against a sequence unique to Glyt1b, an isoform expressed more

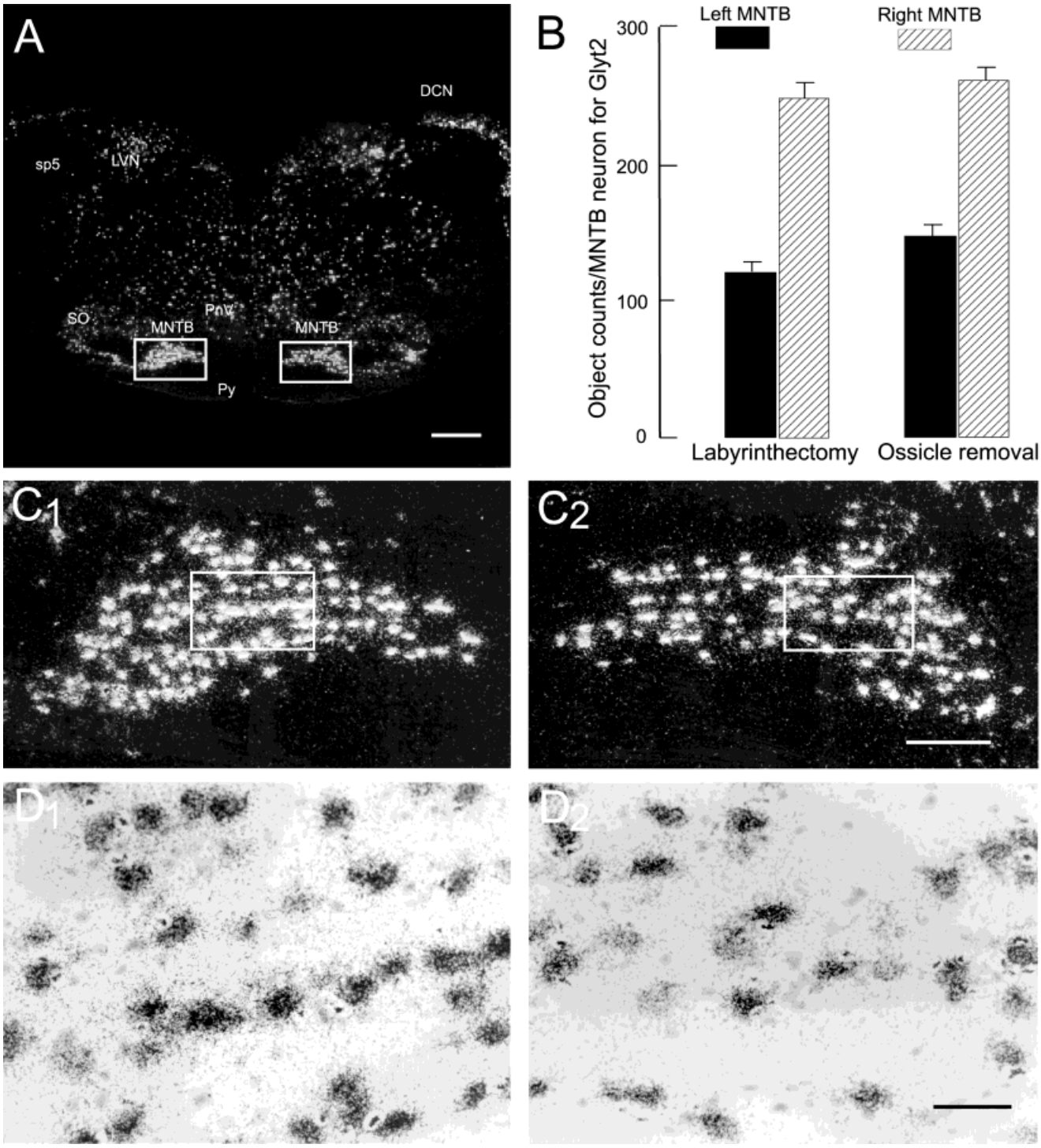

Fig. 6. Disarticulation of middle ear ossicles causes asymmetric Glyt2 hybridization in the MNTB. **A:** Darkfield photomicrograph of a transverse section through the rostral DCN of a rat 2 days after the left ear drum was punctured and the incus was removed. Note the greater hybridization signals from the right DCN and left MNTB. **B:** Multiple silver grain "objects" were counted in 20–90 neurons within the left and right MNTB in each of four rats with left labyrinthectomies and in four rats with removal of the left ossicular chain. The error bars represent standard errors of the mean. The left-right differences in "object" counts/neuron were significant (P less than or equal to 0.001). The areas delineated by the white rectangles in A are shown at higher magnification in $\mathbf{C}_{1,2}$, darkfield photomicrographs of left and right MNTB. Note the relatively greater hybridization signal in neurons in the left MNTB. The areas delineated by white rectangles are shown at higher magnification in $\mathbf{D}_{1,2}$, higher magnification brightfield photomicrographs of neurons in the left and right MNTB. For abbreviations, see list. Scale bars = 1 mm in A; 250 μm in C_2 (applies to C_1,C_2); 50 μm in D_2 (applies to D_1,D_2).

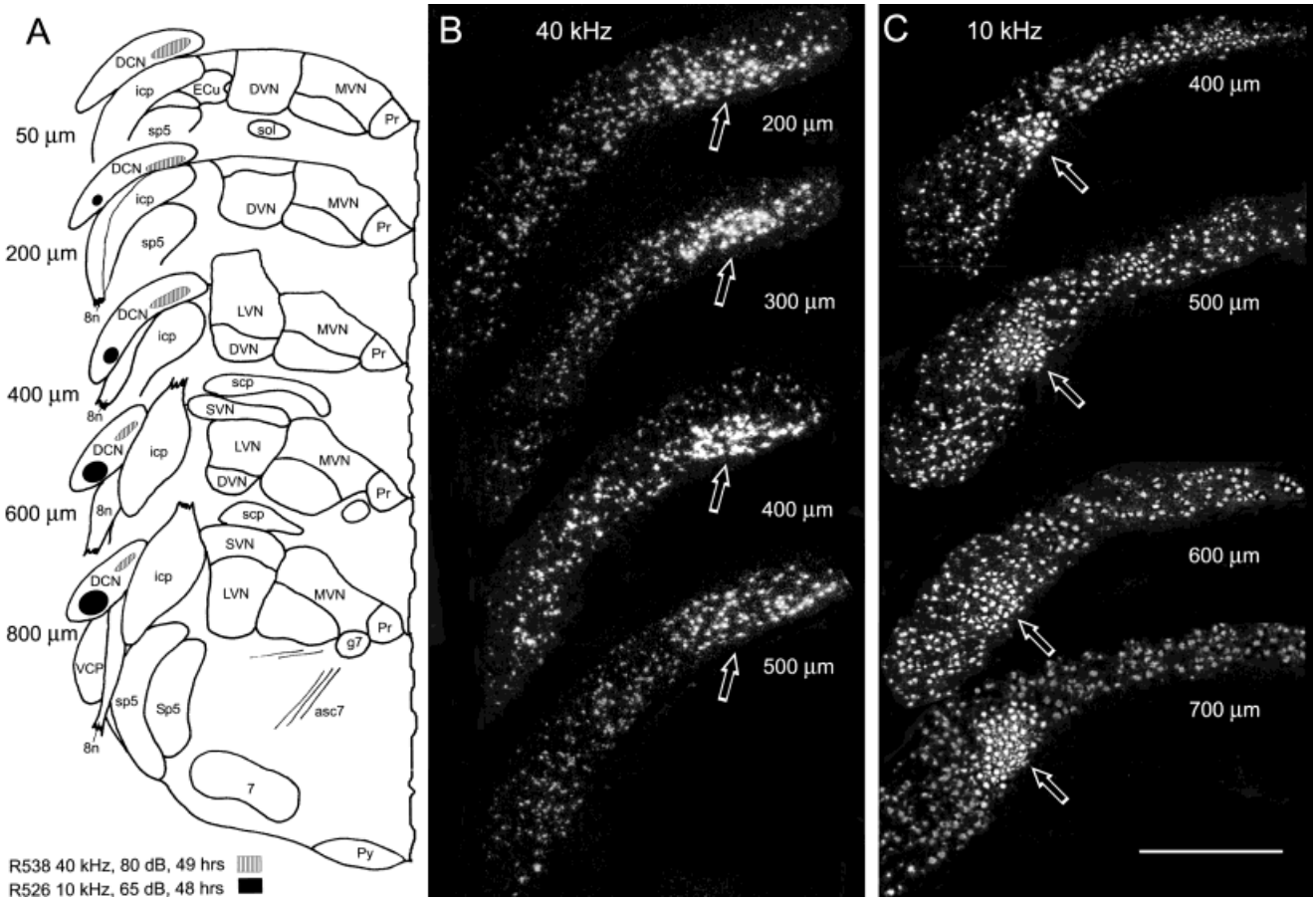

Fig. 7. Increase in Glyt2 mRNA in dorsal cochlear neurons evoked by acoustic stimulation. Acoustic stimulation of either 10 kHz or 40 kHz was delivered for approximately 48 hours. This stimulation evoked increases in Glyt2 transcription in neurons in different regions of the DCN. **A:** Illustration of regions of the DCN activated by the acoustic stimulation. The region activated by 40 kHz stimulation is illustrated by vertical lines. The region activated by 10 kHz stimulation is demarcated by the black-filled form. **B:** Photomontage of four serial sections taken through the DCN of a rat stimulated for 49 hours at 40 kHz, 80 dB. The arrows identify regions of greater hybridization to the probe for Glyt2. **C:** Photomontage of four serial sections taken through the DCN of a rat stimulated for 48 hours at 10 kHz, 65 dB. Numbers on right in B and C refer to distance from the caudal pole of the DCN. For abbreviations, see list. Scale bar = 500 µm in C (applies to B,C).

specifically in glia and neurons, may have shown a pattern of labeling in the DCN influenced by cellular activity.

Two layers within the DCN contained neurons that hybridized with the Glyt2 probe. The superficial molecular layer contains glycinergic cartwheel neurons (Wenthold et al., 1987; Kolston et al., 1995; Gates et al., 1996; Golding and Oertel, 1996, 1997; Romand and Avan, 1997). The deeper polymorphic cell layer contains more densely packed glycinergic, strychnine-sensitive, tuberculoventral neurons (Wenthold et al., 1987; Wickesberg and Oertel, 1990; Kolston et al., 1995). The transcription of Glyt2 mRNA in both layers was influenced by experimentally controlled increases and decreases in primary afferent activity.

Cartwheel neurons can be identified physiologically by their characteristic "complex spike," which is similar in configuration to the "complex spike" of cerebellar Purkinje neurons (Davis and Young, 1997; Golding and Oertel, 1997). These complex spikes can be driven only modestly by acoustic stimuli. By contrast, tuberculoventral neurons are more directly coupled to auditory primary afferent activity. In immunohistochemical investigations, tuberculoventral neurons, but not cartwheel neurons, retain their immunoreactivity to an antibody to glycine if they are freshly fixed or if continuous electrical stimulation of the eighth nerve is used to maintain their activity in vitro before fixation. The immunoreactivity of tuberculoventral neurons, but not cartwheel neurons, is decreased if the tissue slice is maintained without electrical stimulation before fixation (Wickesberg et al., 1994). Our data agree with these immunohistochemical results showing that alterations in auditory primary afferent activity influenced the transcription in tuberculoventral neurons in the polymorphic cell layer. However, our data also show that differences in evoked activity contribute to differences in Glyt2 mRNA transcription in cartwheel neurons in the molecular layer.

Superior olivary neurons expressing Glyt2 and responding to changes in auditory primary afferent activity: MNTB

Neurons throughout the superior olive are glycinergic (Helfert et al., 1989; Adams and Mugnaini, 1990) and were

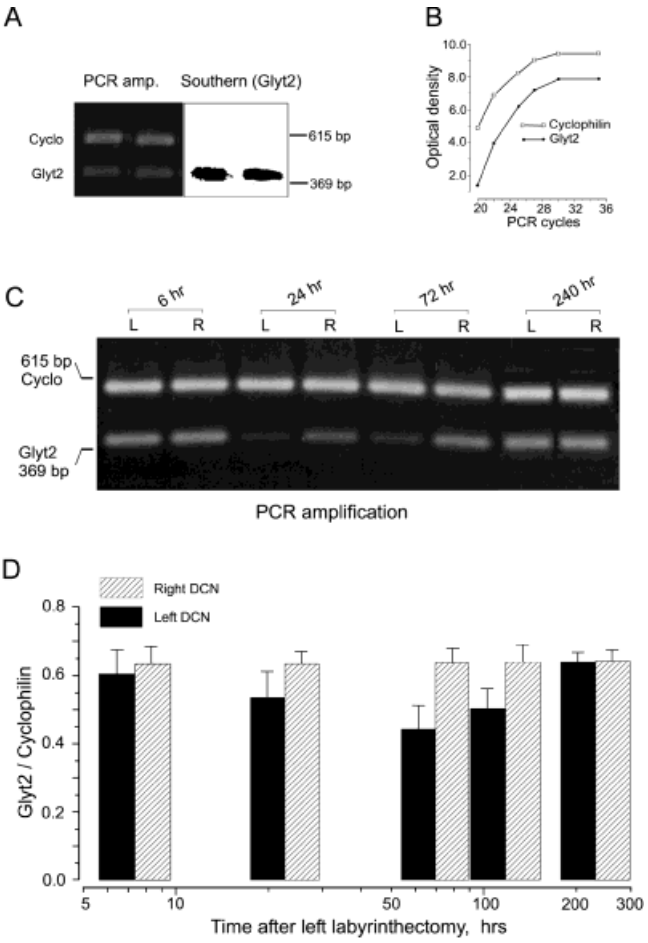

Fig. 8. Time course of decrease and recovery of Glyt2 mRNA in the DCN as indicated by quantitative reverse transcription-polymerase chain reaction (RT-PCR). Quantitative RTPCR was used to measure the time course of decrease in Glyt2 mRNA and its recovery after a unilateral labyrinthectomy (see Materials and Methods section). Twenty-five cycles of PCR were performed by using two sets of primers. One set amplified a 400-bp gene product of Glyt2. A second primer pair amplified a 589-bp gene product for cyclophilin, a "housekeeping gene." **A:** Southern blot of these two gene products, by using an oligonucleotide probe for Glyt2 end-labeled with ^{32}P, showed the specificity of the hybridization of the Glyt2 probe in hybridizing with the PCR-amplified Glyt2 gene product, but not the cyclophilin gene product. **B:** The linear range of PCR cycles in amplifying gene products for cyclophilin (open squares) and Glyt2 (filled circles) extended from 20–30 cycles. **C:** RT-PCR (25 cycles) was used to coamplify cDNAs for Glyt2 and cyclophilin from the left and right DCNs of four rats euthanized at 6, 24, 72, and 240 hours after the labyrinth on the left side was destroyed. Note that the cyclophilin (cyclo) bands, controls for RNA loading, were equal. The bands representing PCR-amplified Glyt2 gene product were differentially amplified in a time-dependent manner. Specifically, after 24 and 72 hours, the left band was decreased relative to the right band. Markers of 369 and 615 bp are listed to the left for calibration. **D:** Semilogarithmic plot of the influence of a unilateral labyrinthectomy on the relative concentration of Glyt2 cDNA, coamplified and corrected for cyclophilin cDNA concentration. Each bar represents the mean plus 1 SEM for measurements of Glyt2/cyclophilin ratios of three rats at each of five postlabyrinthectomy intervals. The ratios of Glyt2/cyclophilin from gels for each of three rats at five different postlabyrinthectomy intervals were compared with a one-way factorial analysis of variance and were significant (P less than or equal to 0.02). The difference at 72 hours was statistically significant (t-test, P less than or equal to 0.02, with Scheffé's correction).

labeled by the Glyt2 oligonucleotide probe in the present experiment. However, only in MNTB neurons was the transcription of Glyt2 mRNA modified by unilateral labyrinthectomy or by unilateral disruption of the ossicular chain. The axons of globular busy cells in the ventral cochlear nucleus (VCN) compose a unique synaptic innervation of the contralateral MNTB where the soma of each principal neuron is encapsulated by the large calyces of Held (Kuwabara et al., 1991). Recordings from globular busy cells show that these neurons evince "primary-like activity" with sustained discharges at higher intensities of stimulation (Smith et al., 1991).

Besides receiving a strong auditory primary afferent input, globular busy cells of the VCN receive a direct glycinergic projection from ipsilateral glycinergic tuberculoventral neurons in the DCN (Wickesberg et al., 1991). This inhibitory projection accounts for as much as 40% of the synaptic terminals found on the dendrites of globular busy cells (Ostapoff and Morest, 1991). Thus, globular busy cells receive tonically "mixed messages" from vestibular primary afferents (excitatory) and tuberculoventral neurons (inhibitory).

On balance, the tonic activity of the auditory primary afferent projection to globular bushy cells appears to prevail over the tonic inhibitory input from the glycinergic tuberculoventral neurons. In rats with unilateral labyrinthectomies, the decreased tonic activity ipsilateral to the labyrinthectomy, causes decreased Glyt2 transcription in the contralateral MNTB.

Functional role for changes in Glyt2 mRNA transcription

Although the activity-dependent regulation of Glyt2 mRNA transcription in DCN and MNTB neurons is easily observed under both conditions of increased and decreased auditory primary afferent activity, the functional consequences of this regulation remain speculative. Neuronally regulated transporter transcription might prevent depletion of synaptic transmitter from synaptic terminals. Alternatively, neural regulation of transporter expression might provide a negative feedback regulation for transmitter release.

If the transmitter transporter protein Glyt2 expressed in synaptic terminals were proportional to the concentration of Glyt2 mRNA found in cell bodies, then an increase in Glyt2 might be evoked at higher discharge frequencies to prevent local depletion of glycine. Without activity-dependent regulation of the Glyt2 transporter, the synaptic release of glycine could "run down" at higher frequencies of discharge because of synaptic depletion. Conversely, at lower frequencies of discharge, a lower expression of Glyt2 would be needed to replenish the presynaptic pools of glycine. Presently, despite the classic work on the release of acetylcholine (Birks and MacIntosh, 1961; Potter, 1970), little is known about how cytoplasmic pools of synaptic transmitters in presynaptic terminals are influenced by changes in neuronal activity. Also little is known about the concentration-dependence of nonvesicular transmitters release.

The concentration of synaptic transmitter transporter at a particular synaptic site could be determined by the recent discharge history of the neuron. When activity in auditory primary afferents increases, the activity of neurons within the DCN also increases, leading to increased transcription of Glyt2 mRNA and increased expression of

Glyt2 in synaptic terminals. The consequent increased reuptake of glycine would decrease the synaptic efficacy of tuberculoventral neurons on their target cells, multipolar neurons, and bushy cells, in the posteroventral (Wickesberg et al., 1991) and anteroventral cochlear nucleus (Wickesberg and Oertel, 1990). Conversely, decreased auditory primary afferent activity would decrease the transcription of Glyt2 mRNA and reduce the reuptake of glycine in the synaptic terminals. This process would result in a relative increase in the glycine reaching postsynaptic receptors on multipolar neurons and bushy cells. Thus, activity-dependent expression of Glyt2 could act as a negative feedback mechanism, limiting long-term deviations in the mean discharge frequency of postsynaptic neurons with glycine receptors.

Functionally, the result of either the "depletion prevention" or "negative feedback" views of transporter function would be equivalent. Increases or decreases in presynaptic neuronal discharge would either (1) affect the concentration of glycine in the synaptic cleft by modulating transcription and expression of Glyt2 transporter, or (2) affect the concentration of glycine available for release by modulating the transcription and expression of Glyt2 transporter accordingly. Either of these mechanisms could alter the dynamics of postsynaptic discharge based on the history of the presynaptic discharge.

These longer-term actions of transmitter transporters could have greater functional significance than their modest role in shaping the decay of individual synaptic currents (Attwell and Mobbs, 1994; Worrall and Williams, 1994; Uhl and Johnson, 1994; Lester et al., 1996; Otis et al., 1996; Diamond and Jahr, 1997). Our data provide the first unequivocal evidence for neuronal regulation of transcription of a transmitter transporter. In the specific case of the auditory system, regulation of the Glyt2 transporter in DCN and MNTB neurons could contribute to frequency-specific central adaptation. This kind of long-term auditory adaptation has been observed in human subjects (Elliott and Fraser, 1970). More generally, activity-dependent regulation of neuronally expressed transporters may provide an adaptive mechanism common to many neural systems.

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