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Changes in Glycine Immunoreactivity in the Rat Superior Olivary Complex Following Deafness

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Abstract

The balance between inhibitory and excitatory amino acid neurotransmitters contributes to the control of normal functioning of the auditory brainstem. Changes in the level of neuronal activity within the auditory brainstem pathways influence the balance between inhibition and excitation. Activity-dependent plasticity in the auditory pathways can be studied by creating a large decrease in activity through peripheral deafening. Deafness-related decreases in GABA have previously been shown in the inferior colliculus. However, glycine is a more prevalent inhibitory transmitter in the mature superior olivary complex (SOC). The present study therefore examined if there were deafness-related changes in glycine in the SOC using postembedding immunocytochemistry. Animals were bilaterally deafened by an intrascalar injection of neomycin. Five nuclei in the SOC, the lateral superior olive (LSO), superior paraolivary nucleus (SPoN), and the medial, lateral, and ventral nuclei of the trapezoid body (MNTB, LNTB, and VNTB) were examined 14 days following the deafening and compared to normal hearing age-matched controls. The LSO and SPoN were divided into high and low frequency regions. The number of glycine immunoreactive puncta on the somata of principal cells showed significant decreases in all regions assessed, with changes ranging from 50% in the VNTB to 23% in the LSO. J. Comp. Neurol. 494:179–189, 2006.

Indexing terms

auditory; brainstem; neomycin; superior olivary complex

Increases or decreases in activity induce plastic changes in the mature auditory pathways (recently reviewed by Syka, 2002; and more generally by Møller 2001, 2005). There is deafness-associated plasticity at the synaptic level in the mature auditory brainstem, with

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changes in amino acid neurotransmitters and receptors (Bledsoe et al., 1995; Potashner et al., 1997, 2000; Milbrandt et al., 1997; Benson et al., 1997; Ryugo et al., 1998; Caspary et al., 1999; Helfert et al., 1999; Mossop et al., 2000; Nakagawa et al., 2000; Sato et al., 2000a,b; Willott et al., 2000; Holt et al., 2005), in ion channels (Macica et al., 2003; Lu et al., 2004; von Hehn et al., 2004), as well as in synapse-related proteins such as protein kinases, Gap43, calbindin, ERK, SAPK, NT3 and BDNF (Idrizbegovic et al., 1998; Garcia et al., 2000; Illing and Michler, 2001; Suneja and Potashner, 2003b). Thus, there is a resulting influence on the balance between excitation and inhibition reflected in changes in neuronal response profiles (Bledsoe et al., 1995; Caspary et al., 1995; Francis and Manis, 2000; Kaltenbach and Afman, 2000; Mossop et al., 2000; Salvi et al., 2000; Syka et al., 2000; Syka and Rybalko, 2000) as well as in changes in tonotopic maps (Willott et al., 1982, 1993; Robertson and Irvine, 1989; Schwartz et al., 1993; Rajan and Irvine, 1998; Salvi et al., 1999; Nagase et al., 2000). A shift towards decreased inhibition and increased excitation has been reported in the CIC (Vale and Sanes, 2002) associated with decreases in GABA release (Bledsoe et al., 1995) and decreases in the GABA synthesizing enzyme GAD (Mossop et al., 2000). Glycine is a major inhibitory transmitter in the superior olivary complex (SOC) (Caspary et al., 1987, 1994; Wickesberg and Oertel, 1990; Grothe and Sanes, 1993; Wu and Kelly, 1994) and decreases in glycine release have been reported in specific SOC nuclei following deafening (Suneja et al., 1998). This would suggest that there may be an activity-dependent, deafness-associated decrease in glycine in the SOC. The present study, therefore, employed quantitative immunocytochemistry to compare the number of glycine-immunoreactive (-IR) axo-somatic puncta on neurons in five SOC regions: the lateral superior olive (LSO), medial, lateral, and ventral nuclei of the trapezoid body (MNTB, LNTB, and VNTB), and superior paraolivary nucleus (SPoN) in normal hearing and 14-day bilaterally deafened rats.

Materials and Methods

Adult, female Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were used in this study. Rats weighed between 200–300 g at the time of surgery and were at least 2 months old. The experimental (deafened) group (n = 5) was assessed 14 days following bilateral deafening via perfusion of 30 μl of 30% neomycin through the round window of the cochlea. The animals in the control group were age-matched and had normal hearing (n = 5).

Surgery

Surgical procedures were performed under aseptic conditions and normal body temperature was maintained throughout the procedure. Animals were anesthetized intramuscularly (i.m.) with a mixture of Ana Sed (8 mg/kg xylazine sterile solution 20 mg/ml; Ben Venue Laboratories, Bedford, OH) and Ketaset (75 mg/kg ketamine-HCl sterile solution 100 mg/ml; Fort Dodge Animal Health, Fort Dodge, IA). Local injections of 1% lidocaine-HCl solution (Abbott Laboratories, North Chicago, IL) were made at the site of each surgical incision. The lateral wall of each bulla was opened and 30 μL of 30% neomycin solution was slowly injected through the cochlear round window using a 50-μl Hamilton syringe. The total injection time was ~2 minutes per ear. Following surgery, animals were injected subcutaneously with 1 ml sterile 0.9% sodium chloride-HCl solution (Abbott Laboratories) and allowed to recover under a heating lamp. These and other procedures performed on

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animals were in accordance with the requirements of the University Committee on the Use and Care of Animals and in accordance with all federal and state laws.

Auditory brainstem responses

Hearing was assessed by ABR measures at 2, 10, and 20 kHz in all animals at the beginning of the study and only those with hearing in the normal range (2 kHz –15 dB, 10 kHz –15 dB, and 20 kHz –16 dB) were included in the study. Animals were anesthetized (8 mg/kg xylazine sterile solution 20 mg/ml; and 75 mg/kg ketamine-HCl sterile solution 100 mg/ml) and a transducer was placed in the ear canal that delivered a tone at varying intensities. The neurologic response from the auditory system was recorded from subdermal needle electrodes placed on top of the head, nose, and thigh. A second measure of hearing was done just prior to perfusion for animals in the deafness groups. An 80 dB shift from the normal control threshold averaged across the frequencies was considered necessary for continued inclusion in the study. At each frequency tested deafened animals were nonresponsive up to 105 dB.

Fixation and tissue processing

Animals were anesthetized by subcutaneous (s.c.) injection of chloral hydrate and perfused transcardially with a rinse of Ca^{2+}-free Ringer's variant followed by a fixative containing 2% paraformaldehyde and 1.25% glutaraldehyde in 0.12 M sodium phosphate buffer.

After perfusion, brains were removed and postfixed for 90 minutes in the same solution. The tissue was cut coronally into 150-μm sections using a vibrating microtome (Vibratome, St. Louis, MO). The sections were then incubated in 1% osmium tetroxide (OsO_4) on ice for 45 minutes. This and subsequent steps were carried out in the dark. The sections were then incubated in 2% uranyl acetate and dehydrated through a series of graded ethanol washes. Following dehydration, sections were incubated in a 1:1 mixture of EPON 812 and propylene oxide on a vertical rotator. The sections were next incubated in 100% EPON 812 overnight. Following this incubation, sections were placed between sheets of Mylar and incubated for 48 hours at 60°C. Following polymerization, all sections containing the SOC were examined. A comparable section (based on anatomical markers) from mid-rostrocaudal SOC containing all of the primary SOC nuclei was selected from each animal for further processing. The ventral portion of this section containing all five nuclei was dissected out of the Mylar with a scalpel and affixed to an EPON 812 blank. One-μm sections were cut using an ultramicrotome and systematic sampling was followed as described below. Five 1-μm sections (separated by 12 μm; the 1st, 13th, 25th, 37th, and 49th in the series) were analyzed from each rat. The 12-μm interval was chosen so that the space between sections would be larger than the size of an SOC principal cell nucleus. With the criteria that the profile of a neuron needed to contain a nucleus to be assessed, this ensured that any given neuron would only be analyzed once. Five adjacent 1-μm sections (the 2nd, 14th, 26th, 39th, and 50th in the series) received a Nissl stain (Toluidine blue) for anatomical reference.

Immunocytochemistry

The five sections selected for immunocytochemistry were rehydrated in solutions of decreasing ethanol concentration (100%, 70%, 50%, 35%). The sections were then treated

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for 40 minutes in sodium ethoxide for "etching," rehydrated, and then treated in 1% sodium periodate for 10 min for "bleaching." The standard protocol for immunocytochemistry using the ABC Elite Kit (Vector Laboratories, Burlingame, CA) was then followed. Briefly, the sections were blocked for 20 minutes at room temperature in a solution containing 5% normal goat serum (NGS) and 0.5 M Tris. Primary antibody incubation proceeded overnight in a 1:1,200 dilution of polyclonal rabbit antisera against bovine serum albumin–glutaraldehyde conjugates of glycine (antibody #139 Chemicon International, Temecula, CA) at 4°C. The specificity of this antibody has been previously verified in the retina (Kalloniatis and Fletcher, 1993) and in the auditory brainstem (Spirou and Berrebi, 1997). In the auditory system, sections containing the glycine-rich MNTB were incubated with glycine antibody preadsorbed overnight with 2–20 mM of glutaraldehyde-conjugated glycine or GABA antigen (Storm-Mathisen and Otterson, 1990). The glycine antibody did not show any cross-reactivity with GABA and glycine labeling was eliminated with an excess of the antigen. Sections were rinsed in 0.5 M Tris and incubated in goat antirabbit secondary antibody diluted 1:100. Following rinses in 0.5 M Tris, the tissue was incubated for 1 hour in the ABC solution (Vector). Sections were incubated with 0.05% diaminobenzidine (DAB) in 80 ml PBS solution containing 200 μl of 30% hydrogen peroxide. Sections were incubated in DAB for 3 minutes. Usually, by the 3-minute time point the neurons in the MNTB of normal hearing animals could be clearly distinguished as being labeled above background. The DAB reaction was stopped by removing the DAB solution from the slide holder and rinsing several times with ddH_2O. Following these rinses, sections were dried with compressed air and dehydrated in solutions of increasing ethanol concentration: 50%, 70%, 80%, 95%, 100%. Finally, sections were incubated in a xylene solution and covered using a Permount xylene solution.

Image acquisition and analysis

The middle three 1-μm sections (the 13th, 25th, and 37th) from the set of five immunostained sections (the 1st, 13th, 25th, 37th, and 49th) were used for quantitative analysis for each of the five rats in each group. If methodological damage to the section prevented accurate assessment of any SOC nuclei in one of these sections, the better-preserved of the remaining two immunostained sections (the 1st or 49th) was substituted for the analysis. Images were acquired on a Zeiss Axioscope (Thornwood, NY) photomicroscope with a Spot Camera (Diagnostic Instruments, Sterling Heights, MI). Images of the LSO, MNTB, LNTB, VNTB, and SPoN were taken at low magnification using Toluidine blue-stained sections. MetaMorph Imaging software (Universal Imaging, Westchester, PA) was used to designate seven regions of interest (ROIs) across these five nuclei. Rectangular regions were defined in the low-frequency (lateral) and high-frequency (medial) limb or portion of the LSO and SPoN and in the central portion of the MNTB, LNTB, and VNTB. We have previously observed that a large percentage of Gly-IR terminals in the rat MSO are axo-dendritic (Helfert et al., 1989), so this region was not assessed.

Images were then acquired from each individual ROI from the three chosen immunostained sections from each of the five rats in the two groups. All neurons containing nuclei within every ROI on each section were quantitatively assessed using MetaMorph software. Gly-IR

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puncta were defined by size, 0.05 μm^2 to 4.5 μm^2, shape (ovoidround), intensity of staining (at least 7× over background), and a location adjacent to the somatic profile. All such Gly-IR puncta immediately adjacent to neuronal so-mata (thus defined as axo-somatic) were individually counted for every neuron containing a nucleus within the ROI. Each individual neuron was also circled to generate a measure of cross-sectional area and perimeter. StatView software (Cary, NC) was used to perform an analysis of variance (ANOVA) on all axo-somatic puncta and somal size information from each of the 15 sections obtained from the normal group compared to all of the same data obtained from the 15 sections from the deafened group. For each variable measured (somal size and number of puncta), the average was calculated per animal and in those brain regions where high and low frequency were considered, two corresponding averages were calculated per animal. Therefore, the degrees of freedom were equal to 1 in the numerator (deaf vs. normal or high vs. low) and 8 in the denominator (five animals per group, n – 1 for each group, normal vs. deaf). A confidence interval of 95% was used to determine whether observed differences were significant. Post-hoc comparisons were performed using a Scheffe test.

Photomicrographs for illustration of results were obtained using a Spot Camera (Diagnostic Instruments) mounted on a Zeiss Axioscope. MetaMorph Imaging software (Universal Imaging) was used to add scale bars to the appropriate images. Each plate of photomicrographs was then assembled in Adobe PhotoShop CS (v. 8, Adobe Systems, San Jose, CA). Brightness and contrast were adjusted without other enhancements to the images.

Results

Glycine immunoreactivity (Gly-IR): general

Glycine immunostaining in the SOC of normal hearing animals was consistent with previous studies (Peyret et al., 1987; Helfert et al., 1989; Adams and Mugnaini, 1990; Henkel and Brunso-Bechtold, 1995; Vater, 1995; Rampon et al., 1996; Ostapoff et al., 1997; Spirou and Berrebi, 1997) (Figs. 1–5). Gly-IR neurons were most numerous in the MNTB, where practically all neurons were immunopositive (Fig. 1A). Numerous Gly-IR neurons were also observed in the VNTB (Fig. 2A), with fewer in the LSO (Fig. 3A), and still fewer in other SOC regions such as SPoN (Fig. 4A) and LNTB (Fig. 5A). The prevalence of Gly-IR axo-somatic puncta varied among the five subregions of the SOC assessed (Fig. 6). The highest number of Gly-IR axo-somatic puncta was found in the LSO and SPoN, with an intermediate number in the LNTB and VNTB, and the lowest number in the MNTB (Fig. 6). Small differences were found in the number of Gly-IR axo-somatic puncta per 100 μm of somatic perimeter between high- and low-frequency regions in the LSO of normal hearing rats, but not between high- and low-frequency regions in the SPoN (Fig. 6B,C).

Gly-IR axo-somatic puncta in normal hearing versus deafened rats

LSO—For LSO principal cells the number of Gly-IR puncta per 100 μm somatic perimeter was significantly (*P* 0.0001) lower (18.8 ± 0.6) in the high-frequency region compared to the low-frequency region (22.0 ± 0.5; Fig. 6B) of normal hearing animals. A significant decrease in Gly-IR puncta at 14 days following deafening was seen in both regions (Fig. 7A). In the high-frequency region there was a significant (*P* 0.0001) 25% decrease from

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18.8 ± 0.6 in normal hearing to 14.1 ± 0.7. In the low-frequency region there was a significant (*P* 0.05) 23% decrease from 22.2 ± 0.5 to 17.2 ± 1.0 labeled puncta. The decrease was 24% when averaged across both regions.

SPoN—In the SPoN there was no statistical difference in the number of Gly-IR puncta between the high- and low-frequency regions (Fig. 6C). The number of Gly-IR axo-somatic puncta showed a significant (*P* 0.0001) change in deafened rats, decreasing 36% from 19.3 ± 0.9 per 100 μm perimeter to 12.1 ± 0.8 in deafened animals in high-frequency regions with a comparable change (*P* 0.0001) from 18.1 ± 1.1 per 100 μm perimeter to 12.7 ± 0.9 in the low-frequency area (Fig. 7B).

MNTB—MNTB neurons had by far the fewest number Gly-IR axo-somatic puncta (Fig. 6A) in normal hearing and deafened animals. In normal hearing animals the number of puncta averaged 6.6 ± 0.3 per 100 μm perimeter, 35% of that found for LSO neurons. The number of Gly-IR puncta per cell profile decreased (*P* 0.001) 35% following deafness, to 4.3 ± 0.2 Gly-IR axo-somatic puncta per 100 μm perimeter (Fig. 7C).

VNTB—The VNTB of normal hearing rats had 13.8 ± 1.7 Gly-IR axo-somatic puncta per 100 μm perimeter (Fig. 6A). There was a significant (*P* 0.01) almost 50% decrease following deafness, with the number of puncta per 100 μm perimeter decreasing to 7.4 ± 0.7 (Fig. 7D).

LNTB—LNTB neurons had 15.4 ± 0.8 Gly-IR axosomatic puncta per 100 μm perimeter (Fig. 6A). There was a significant (*P* 0.01) 29% decrease, to 11.0 ± 0.8 Gly-IR axo-somatic puncta per 100 μm perimeter in the LNTB of deafened rats (Fig. 7E).

Cell size

Cell size of principal cells in each region was compared between normal hearing and deafened animals. While a decrease in the size of MNTB principal cells has been reported previously (Pasic et al., 1994), no significant change was found in the present study, although there was a trend towards a decrease (9%) from 255.9 ± 12.2 μm^2 for MNTB principal cells in normal hearing animals to 233.0 ± 6.1 μm^2 following deafness. In the LSO of normal hearing animals, the principal cells in the high-frequency region were significantly larger ($P = 0.05$) than neurons in the low-frequency region of normal animals (high frequency = 58.8 ± 0.96 μm^2, low frequency = 54.7 ± 1.3 μm^2), as described previously (Rietzel and Friauf, 1998; Sanes et al., 1992). LSO neurons, however, did not show any significant change in size following deafness, nor were any significant changes found in principal cells of SPoN, VNTB, and LNTB.

Discussion

The present study showed a deafness-related decrease in the number of Gly-IR puncta on the somata of principal cells in all five SOC nuclei evaluated. This is consistent with previous reports of deafness-associated decreases in Gly-IR cells and terminals in the cochlear nucleus (CN) (Willott and Turner, 1999, 2000). The decrease in labeled puncta could be a consequence of a decrease in glycine levels within some terminals, bringing them below the

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level of immunocytochemical detection, but still present. On the other hand, it could also reflect a physical reduction in the number of glycine-containing terminals making contact with somata. Such changes in terminals have been reported in the cochlear nucleus following noise-related hearing loss (Kim et al., 2004a–c). During development there is experience-dependent plasticity that involves extensive “pruning” or “trimming” of glycinergic terminals in both the MSO and LSO (Sanes and Takacs, 1993; Kandler and Friauf, 1995; Sanes and Friauf, 2000; Kapfer et al., 2002; Kim and Kandler, 2003). There could also be an “activity-dependent” decrease following deafness, associated with decreased activity. It is also possible that there is even a readjustment in the location of glycine-immunoreactive terminals, with terminals moving from somata to dendrites, similar to the experience-dependent changes Kapfer et al. (2002) reported in the medial superior olive during development. While we did not quantitatively assess glycine-immunolabeled puncta in the neuropil (corresponding to axo-dendritic terminals and cross-cut axons) in the present study, qualitatively they appeared to decrease in a comparable fashion as axo-somatic puncta rather than increase. Postembedding immunocytochemistry for glycine characterized by electron microscopy and adding flattened vesicles as a second metric for glycine containing terminals would help to resolve these issues.

Suneja et al. (1998) found that unilateral deafening, by cochlear ablation, produced decreases in glycine release in the cochlear nucleus but not in the LSO or MNTB. Since the SOC receives input from both ears, a bilateral deafening (as we used in the present study) may be necessary to produce changes in glycine levels in these regions. It is also possible that the 25% decrease in axo-somatic puncta we observed in the LSO may not be resolved when measuring release across a whole LSO preparation, as in Suneja et al. (1998), although it could still impact release at the level of the individual synapse. Also, differences in the method of deafening (cochlear ablation vs. intrascalar neomycin) or differences in the assessment times following deafness cannot be ruled out as contributing factors to the differences found when comparing these two results. Recent results from Suneja and Potashner (2003a) suggest that deafness may influence the intracellular pathways that modulate neurotransmitters and their release and, perhaps, a more effective release mechanism could compensate for less neurotransmitter available for release.

Our results showed differences in the percent decrease of Gly-IR axo-somatic puncta across SOC nuclei, ranging from a decrease of just under 50% in the VNTB to ~20% in the LNTB. Glycine is a major inhibitory influence in the SOC, with much of the glycinergic pathway originating in the MNTB, with a smaller contribution from the LNTB (Cant and Hyson, 1992; Kuwabara and Zook, 1992; Wu and Kelly, 1994; Spirou and Berrebi, 1997). SOC neurons receive both ipsilateral and contralateral glycinergic input, with ipsilateral input predominating (Wu and Kelly, 1991, 1992, 1994; Cant and Hyson, 1992; Grothe and Sanes, 1993; Smith et al., 2000; Brand et al., 2002; Srinivasan et al., 2004). In the LSO the balance between glutamatergic excitation from the cochlear nucleus and glycinergic inhibition is important for detection of interaural level differences (ILD) (Moore and Caspary, 1983; Finlayson and Adam, 1997; Irvine et al., 2001, for reviews). The response of LSO neurons to glycine can be biphasic. Green et al. (2003) reported glycine produced an initial hyperpolarization of LSO neurons followed by depolarization that could be related to an influence on their intracellular pH. An important role has also recently emerged for the

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glycinergic input onto MSO, where the interaural time difference (ITD) tuning is dependent not only on the timing of excitatory glutamatergic input from contra- and ipsilateral CN, but also on the precise timing of glycinergic inhibitory inputs which also function to adjust the slope of ITD functions (Grothe, 1994, 2003; Grothe and Sanes, 1994; Brand et al., 2002). With distinctly different functions contributing to ITD discrimination in MSO and ILD discrimination in LSO and still additional functions suggested in the SPoN (Behrend et al., 2002; Dehmel et al., 2002; Kulesza et al., 2003), as well as at least two different sources, we expect that glycinergic terminals would have different changes following deafness in different SOC nuclei. Lim et al. (2003) reported site-to-site variability in glycinergic synapses in the MNTB based on glycinergic mIPSC amplitudes, which they suggested could be due to presynaptic differences (increased rate of release) as well as postsynaptic receptor clustering. Determining whether these or other synaptic differences correlate with the remaining detectable glycinergic terminals following deafness would be of great interest.

Brand et al. (2002) suggested that age-related loss of inhibitory transmitters in the SOC, such as reported by Willot and colleagues (Willott and Turner, 1999; Willott et al., 2000) in the CN, could explain age-related hearing deficits such as a decreased ability to segregate sounds. Decreases in glycine levels following profound deafness, as found in the present study, could have a comparable effect if and when hearing is returned via a cochlear prosthesis. A reduced ability to segregate sounds through binaural interaction in the SOC would probably not have an impact on the monaural hearing that is produced when the profoundly deaf receive a unilateral cochlear prosthesis, which is currently the most common situation. On the other hand, more patients are now receiving bilateral cochlear prostheses and in this case deafness-induced decreases in glycine terminals, such as we found in the present study, could result in a reduced capacity for segregating sounds. Another conceivable outcome is that a return of activity could reverse the decreases in glycine induced by the loss of activity due to deafness, resulting in a return to normal processing. Future studies to examine the reversal of deafness-induced decreases in glycine following return of activity and comparing monaural versus binaural cochlear electrical stimulation could help to resolve this issue and increase our understanding of the impact that changes in glycine levels have on processing.

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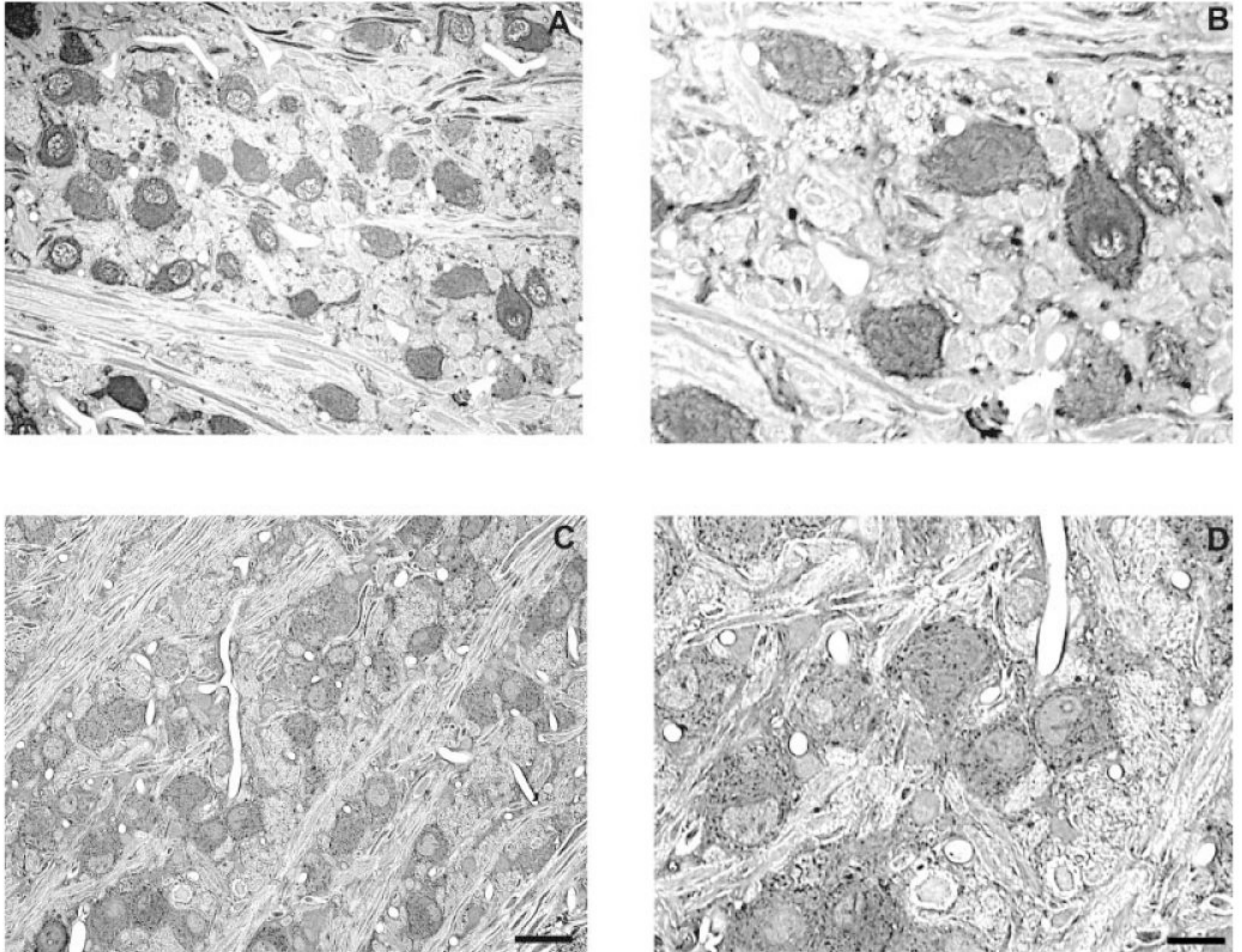

Fig. 1.

Representative photomicrographs of Gly-IR staining in the MNTB from normal hearing (**A,B**) and 14-day deaf (**C,D**) rats. B and D are enlargements of regions from A and C. Many of the glycine-immunolabeled principal cells of the MNTB from normal haring animals (A,B) are surrounded by Gly-IR puncta. Following 14 days of deafness the number of glycine immunoreactive axosomatic puncta significantly decreases. Scale bars = 30 μm in C (applies to A,C); 15 μm in D (applies to B,D).

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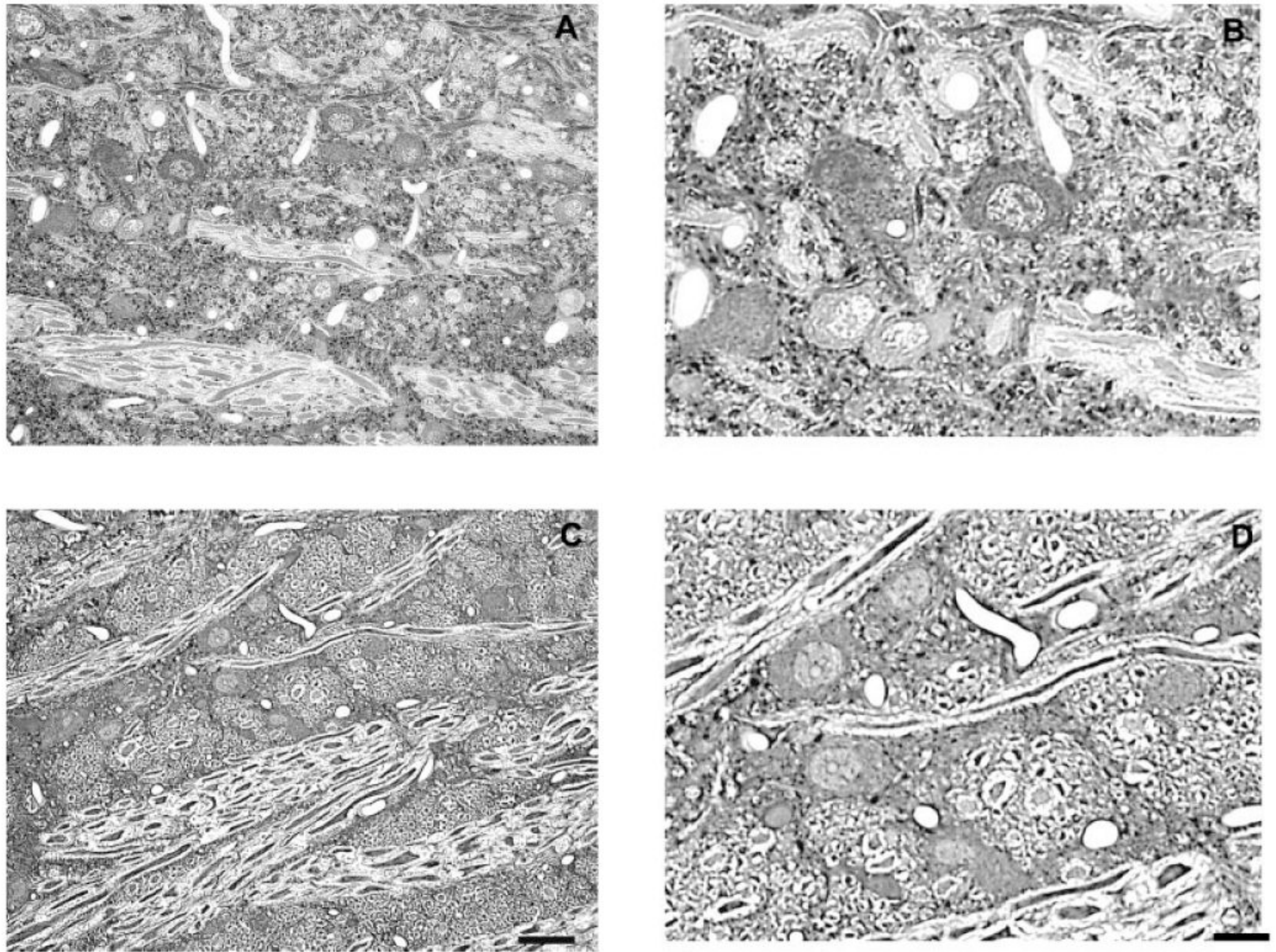

Fig. 2.

Representative photomicrographs of Gly-IR staining in the VNTB from normal hearing (**A,B**) and 14-day deaf (**C,D**) rats. B and D are enlargements of regions from A and C. In the VNTB from normal animals there is a mixture of Gly-IR and Gly-immunonegative neurons with several Gly-IR puncta surrounding each neuron. Following 14 days of deafness the number of glycine immunoreactive axosomatic puncta significantly decreases. Scale bars = 30 μm in C (applies to A,C); 15 μm in D (applies to B,D).

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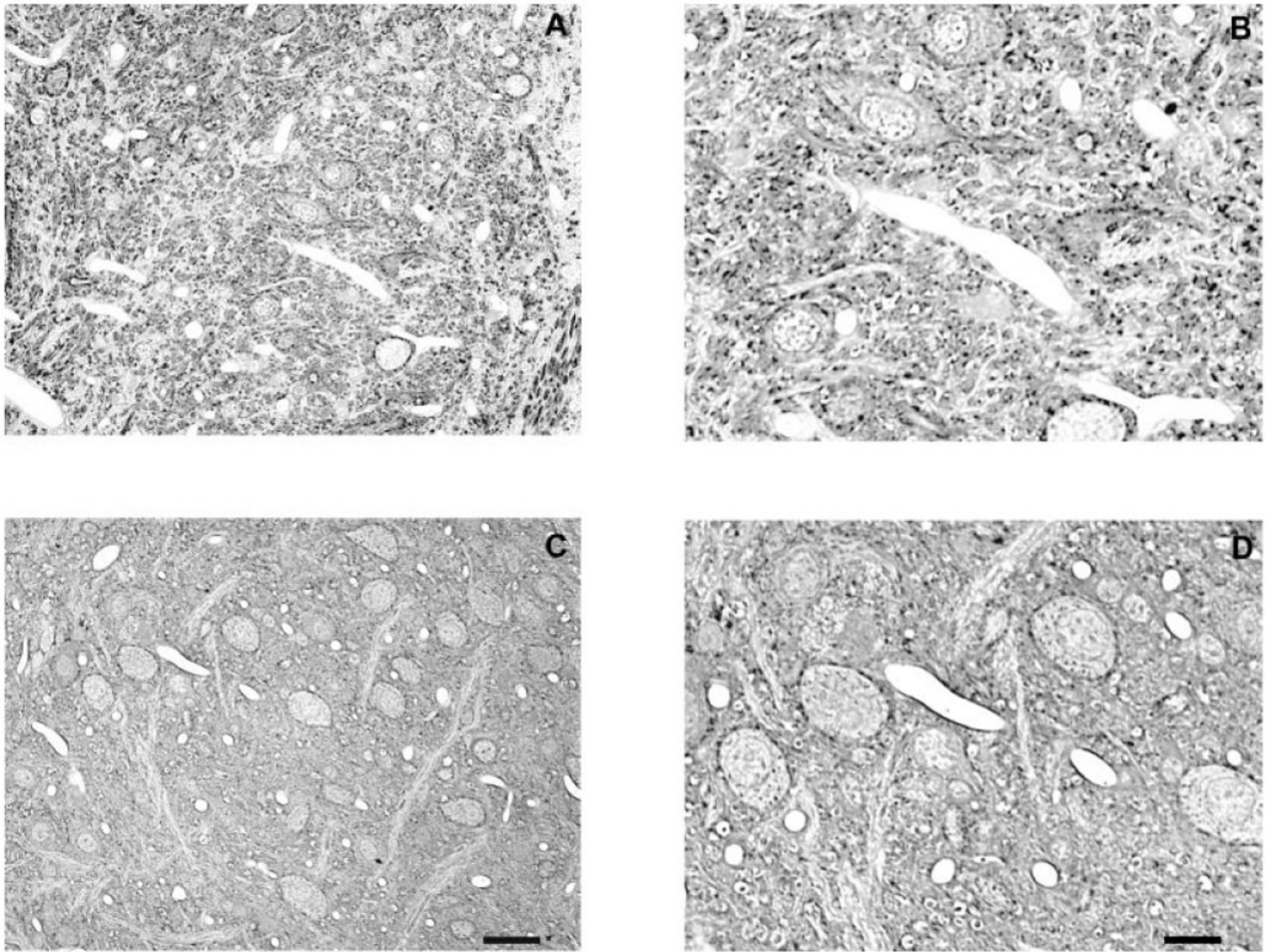

Fig. 3.

Representative photomicrographs of Gly-IR staining in the LSO from normal hearing (**A,B**) and 14-day deaf (**C,D**) rats. B and D are enlargements of regions from A and C. In the LSO from normal animals there are both Gly-IR and Gly-immunonegative neurons with several Gly-IR puncta surrounding each neuron. Following 14 days of deafness the number of glycine immunoreactive axosomatic puncta significantly decreases. Scale bars = 30 μm in C (applies to A,C); 15 μm in D (applies to B,D).

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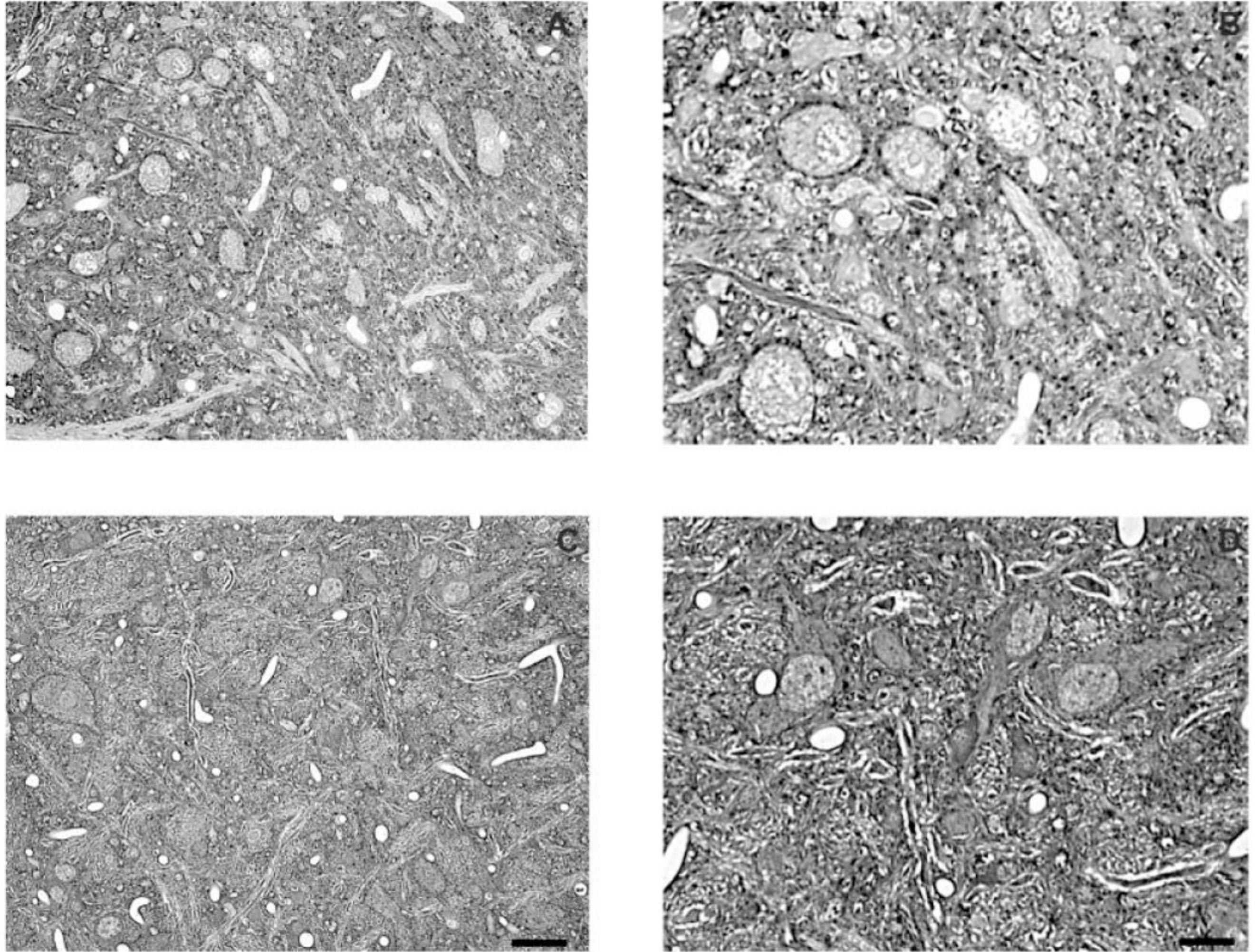

Fig. 4.

Representative photomicrographs of Gly-IR staining in the SPoN from normal hearing (**A,B**) and 14-day deaf (**C,D**) rats. B and D are enlargements of regions from A and C. In the SPoN from normal animals the neurons are primarily Gly-immunonegative with several Gly-IR puncta surrounding each neuron. Following 14 days of deafness the number of glycine immunoreactive axosomatic puncta significantly decreases. Scale bars = 30 μm in C (applies to A,C); 15 μm in D (applies to B,D).

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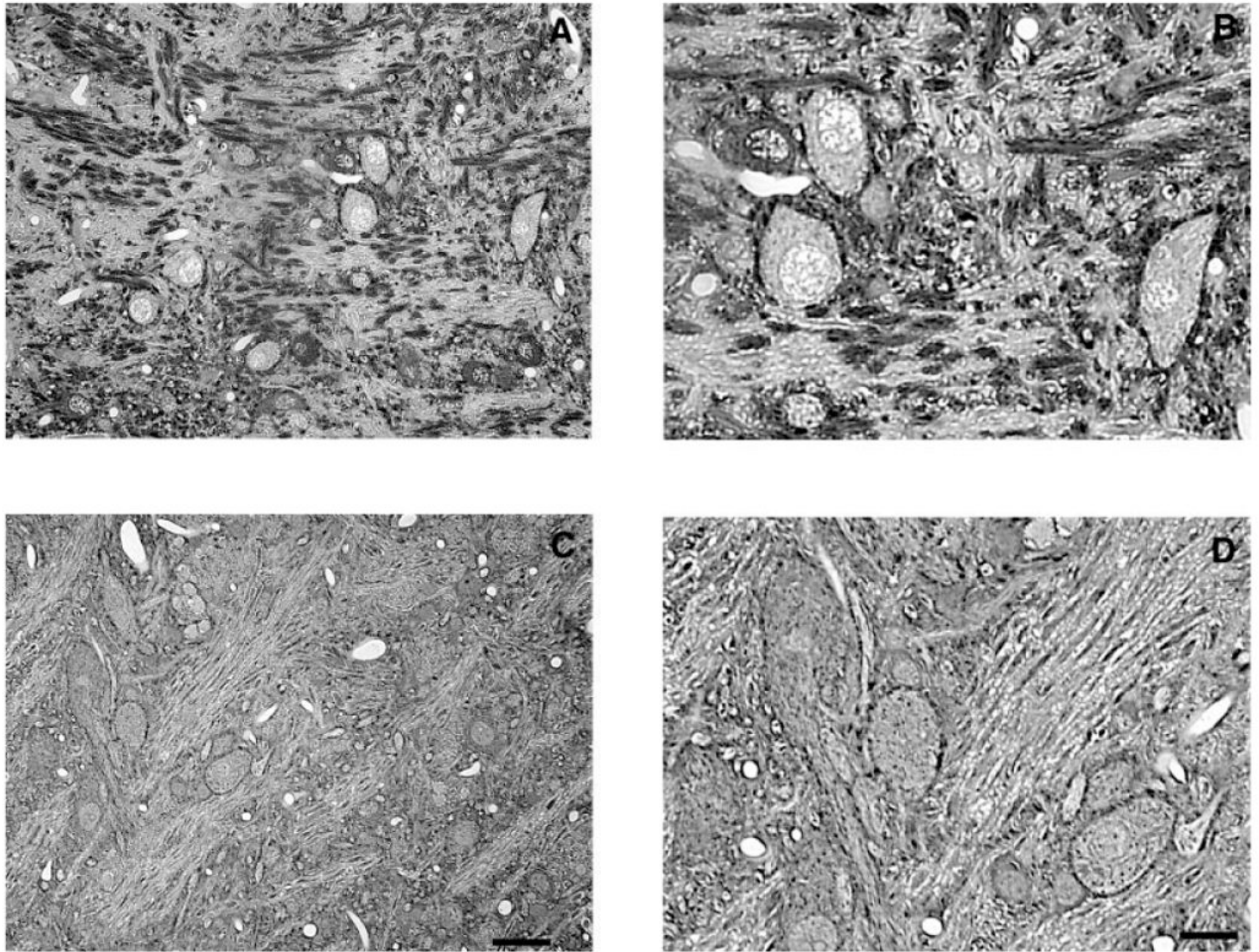

Fig. 5.

Representative photomicrographs of Gly-IR staining in the LNTB from normal hearing (**A,B**) and 14-day deaf (**C,D**) rats. B and D are enlargements of regions from A and C. In the LNTB from normal animals there is a mixture of Gly-IR and Gly-immunonegative neurons with several Gly-IR puncta surrounding each neuron. Following 14 days of deafness the number of glycine immunoreactive axosomatic puncta significantly decreases. Scale bars = 30 μm in C (applies to A,C); 15 μm in D (applies to B,D).

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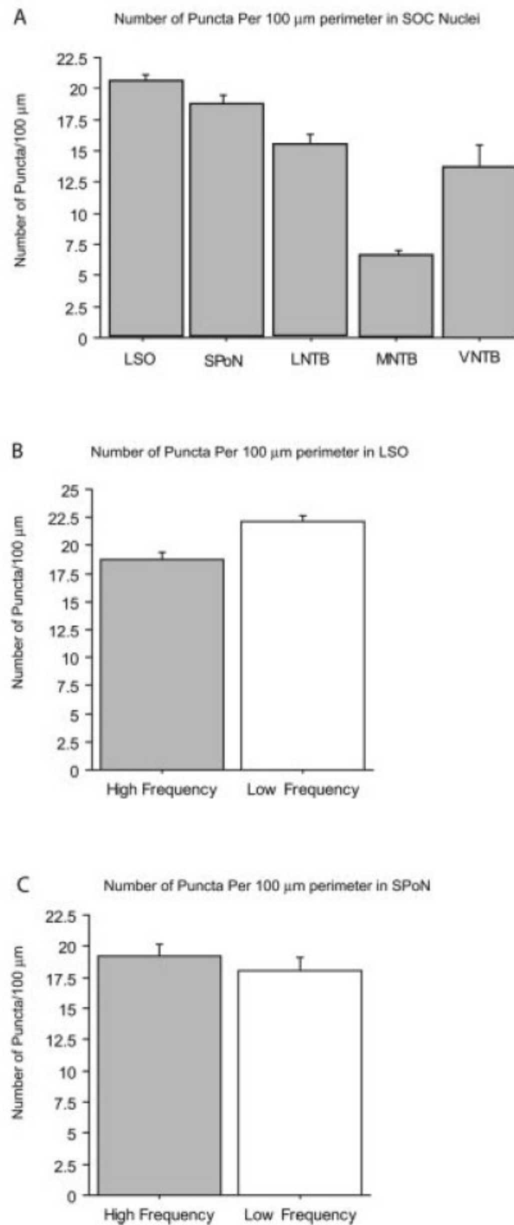

Fig. 6.
Average number of axo-somatic Gly-IR puncta per 100 µm of cell perimeter in SOC nuclei of normal hearing animals. In the five animals assessed, the LSO and SPoN were divided into high- and low-frequency regions. Of the neurons examined in each of the five nuclei the LSO (n = 255 high-frequency compared to 223 low-frequency neurons) and SPoN (n = 125 high-frequency compared to 93 low-frequency neurons) had the largest average number of glycine immunoreactive puncta, followed by neurons in the LNTB (n = 198 neurons), MNTB (n = 315), and VNTB (n = 143 neurons). Error bars are SEM.

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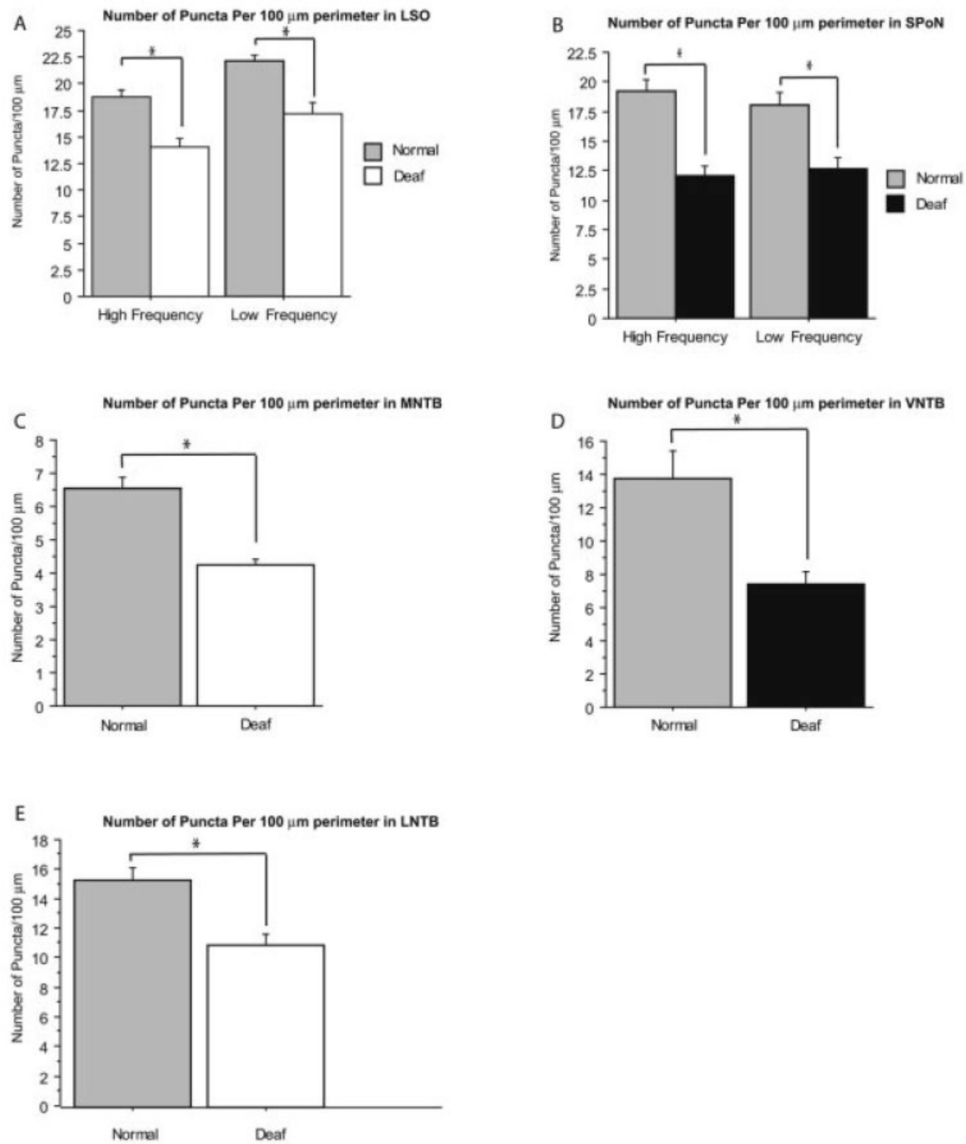

Fig. 7.

Comparison of the average number of axo-somatic Gly-IR puncta per 100 µm of cell perimeter in normal hearing and 14-day deaf animals in the different SOC nuclei. For each nucleus a comparable number of neurons was examined in the LSO (n = 223 low-frequency neurons in normal, 222 low-frequency neurons in deaf, 255 high-frequency neurons in normal, and 248 high-frequency neurons in deaf), SPoN (n = 93 low-frequency neurons in normal, 131 low-frequency neurons in deaf, 126 high-frequency neurons in normal, and 106 high-frequency neurons in deaf), LNTB (n = 198 neurons in normal and 127 neurons in deaf), MNTB (n = 315 neurons in normal and 282 neurons in deaf), and VNTB (n = 143 neurons in normal and 135 neurons in deaf). Error bars are SEM and asterisks denote significance of *P* 0.05.