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Development of Adult-Type Inhibitory Glycine Receptors in the Central Auditory System of Rats

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ABSTRACT

Inhibitory synaptic activity is crucial for many aspects of acoustic information processing and mainly mediated by glycine and γ-aminobutyric acid, the two principal inhibitory neurotransmitters in the auditory system. Glycine exerts its inhibitory action via binding to postsynaptic receptors existing in various isoforms. Here we have investigated the spatiotemporal distribution of adult-type, strychnine-sensitive glycine receptors (GlyRs) in the rat auditory system by using a specific antibody against the ligand-binding α1 GlyR subunit. In adults, α1 GlyRs were found at all relay stations of the auditory pathway except for the medial geniculate body and the auditory cortex. In most brainstem nuclei, labeling was characterized by dense clusters of heavily immunoreactive puncta outlining the somata and proximal dendrites, indicative of a powerful glycinergic inhibition. No α1 immunoreactivity was seen in the auditory system of fetal rats, consistent with results obtained by others in the spinal cord. At birth, labeling was weak and restricted to defined nuclei of the cochlear nuclear complex and the superior olivary complex. By postnatal day 8, labeling was seen in all brainstem nuclei. At the first appearance of immunoreactivity, α1 GlyRs were diffusely distributed on the neuronal surface, yet they became clustered with age, finally densely incrusting the somata and proximal dendrites between the 3rd and 4th postnatal week, when the mature pattern of immunoreactivity was established. We never observed an overexpression of α1 GlyRs or a transient appearance in areas that are devoid of the receptor in adults. The late formation of glycinergic synapses harboring the adult-type GlyRs in the auditory system, at a time when internuclear connections have already formed, indicates that α1 GlyRs do not participate in early synaptogenesis. J. Comp. Neurol. 385:117–134, 1997.

Indexing terms: cochlear nucleus; superior olivary complex; glycine receptor isoform; strychnine sensitive; ontogeny; immunocytochemistry

Inhibitory neurotransmission in the mammalian spinal cord and brainstem is predominantly mediated by the amino acid glycine via binding to postsynaptic, strychnine-sensitive glycine receptors (GlyRs; Curtis et al., 1971; Young and Snyder, 1973). Within the brainstem, glycinergic neurons are particularly abundant in the auditory nuclei (Wenthold et al., 1987; van den Pol and Gorcs, 1988; Kolston et al., 1992; for review see Wenthold, 1991), where glycinergic neurotransmission is involved in basic functional aspects such as sound localization or lateral inhibition (e.g., Moore and Caspary, 1983; Glendenning et al., 1992; Vater et al., 1992). Glycine receptors are pentameric protein complexes composed of two transmembrane polypeptides of ca. 48 kDa (α subunit) and 58 kDa (β subunit), constituting a ligand-gated ion channel permeable for chloride ions (Langosch et al., 1990). The α subunits carry the binding sites for glycine and the specific antagonist strychnine (e.g., Ruiz-Gómez et al., 1990) and exist in at least four different isoforms (α1–α4), whose expression is spatially and developmentally regulated. The α2-contain-

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TABLE 1. Age and Number of Animals Used[1]

Age	E17/18	E20/21	P0	P4	P7/8	P10	P12	P16	P21	P29	P40	Adult
No.	3	2	3	3	4	2	3	2	3	2	2	4

[1]E, embryonic; P, postnatal.

ing GlyR is prevalent in fetuses and neonates and has a low strychnine-binding affinity, whereas the α1-containing isoform predominates in adults and is associated with the classic, strychnine-sensitive GlyR (for reviews see Betz, 1991; Betz et al., 1994; Becker, 1995; Kuhse et al., 1995). Moreover, the open time of the α2-containing GlyR isoform is significantly longer than that of adult-type GlyR (Takahashi et al., 1992) which may be related to specific developmental demands. In addition to the α and β subunits, the peripheral membrane polypeptide gephyrin is found at the cytoplasmic face of glycinergic synapses and plays a crucial role in the clustering of GlyRs (Triller et al., 1985; Kirsch et al., 1993). There is increasing evidence, however, that gephyrin can also be associated with GABAergic synapses (Cabot et al., 1995; Sassoé-Pognetto et al., 1995; Todd et al., 1995) and, therefore, antibodies against gephyrin are not a valid marker for GlyRs.

Within the auditory system of adult mammals, monoclonal antibodies against different GlyR subunits have been used to identify glycinergic inputs (Altschuler et al., 1986; Wenthold et al., 1988), yet most studies were restricted to the cochlear nuclear complex (CN) and have employed an antiserum against gephyrin as an indicator of GlyRs. In the present light-microscopical study, we have used monoclonal antibodies against the α1 subunit to determine the spatial distribution of GlyRs throughout all levels of the rat's central auditory system, i.e., from the CN to the auditory cortex. Our data indicate that GlyRs are abundant in the auditory brainstem nuclei and generally clustered on neuronal somata and proximal dendrites. As ontogenetic data on the protein level about the α1 GlyR isoform in the auditory system were still lacking, we have also analyzed the time course and expression level of the α1 subunit during ontogeny. We demonstrate that the α1 subunit is not present in the prenatal auditory system and that the expression level is steadily increased, reaching the mature distribution pattern before the end of the 4th postnatal week.

MATERIALS AND METHODS

A total of 33 albino Sprague-Dawley rats were used. Their ages and numbers are listed in Table 1. All protocols comply with the current German Animal Protection Law and were approved by the local animal care and use committee. Postnatal animals were overdosed with chloral hydrate (700 mg/kg body weight i.p.) and decapitated after respiratory arrest. Fetal animals were delivered via Cesarean section from deeply anesthetized dams and decapitated. Brains were quickly removed from the skull, blocked, frozen in liquid nitrogen, and stored at −80°C until further processing. Sixteen micrometer coronal sections of the brainstem were cut in a cryostat, thaw-mounted on gelatin-coated slides, and fixed in cold 5% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 5 minutes. After thorough rinsing, sections were dipped into PBS containing 0.5% Triton X-100 for 5 minutes and then preincubated for 30 minutes in a blocker solution containing 5% bovine serum albumin in PBS to prevent nonspecific binding of the antibodies. Incubation with the primary and secondary antibodies was performed on the slide in a wet chamber and at room temperature for 1.5 hours each. Processing for immunofluorescence was basically done as described previously (Kirsch et al., 1993). Briefly, the primary antibody against the α1 GlyR subunit (mAb 2b mouse monoclonal antibody; characterized by Pfeiffer et al., 1984; Schröder et al., 1991) was obtained as hybridoma supernatant and diluted 1:100 in PBS. The polyclonal secondary antiserum had been prepared in goat against mouse IgG's and conjugated to the fluorophore carboxymethylindocyanine 3 (Cy3; Dianova, Hamburg, Germany; diluted 1:500). After antibody treatment, sections were washed in PBS and either glass-covered without dehydration in glycerine-Mowiol jelly or dehydrated in graded ethanol and glass-covered with DPX. Controls, in which the primary antibodies were omitted, confirmed the specificity of the immunolabeling.

Labeled sections were analyzed with a microscope equipped with Plan-Neofluar lenses and with fluorescence illumination, employing a rhodamine filter set, and photomicrographs were taken on Kodak TMax100 or TMax400 film.

In order to test whether α1 GlyR subunits are unevenly distributed along the medio-lateral axis of the lateral superior olive (LSO; Sanes et al., 1987), a quantitative analysis of the immunofluorescent signals was performed. Twelve LSO nuclei from adults were analyzed at mid-rostrocaudal levels with the help of a laser scanning confocal microscope (Sarastro 2000, Molecular Dynamics, Sunnyvale, CA) and appropriate software (ImageSpace, Molecular Dynamics). The intensity of the Cy3 signal in individual sections was determined within circular areas of about 60,000 μm^2 (corresponding to about 600,000 pixels) centered over lateral, central, and medial aspects of the LSO (Fig. 3) and at a linear scale from 0–255 relative intensity values per pixel. Measurements were performed in the central part of the optic field to avoid distortions due to the nonuniformity of the field and data represent relative fluorescent units. Arithmetic averages were calculated for the three aspects of the LSO and a statistical

Fig. 1. Fluorescence photomicrographs of coronal sections through the cochlear nuclear complex (CN) of an adult rat, immunostained for the α1 subunit of the inhibitory glycine receptor (α1 GlyR). **A:** Posteroventral CN (PVCN) and dorsal CN (DCN). Note the difference in the distribution of labeling in the DCN layers as well as in central aspects (octopus cell region) vs. the ventral tip region of the PVCN. **B:** Rostral aspects of anteroventral CN (AVCN), containing the spherical cell area. α1 GlyR immunoreactivity (ir) is present throughout the nucleus. **C:** High-power view of the octopus cell region shown in A to illustrate paucity of α1 GlyR-ir in this area. **D:** High-power view of layers 1 and 2 of the DCN (i.e., the molecular and the granule-pyramidal cell layer), showing a high density of labeling in the neuropil. **E:** High-power view of the AVCN, illustrating that the α1 GlyR subunits are concentrated on the somata and proximal dendrites. In this and all subsequent figures, dorsal is toward the top and lateral to the right. Scale bar = 300 μm in A, 250 μm in B, 75 μm in C, 50 μm in D, 100 μm in E.

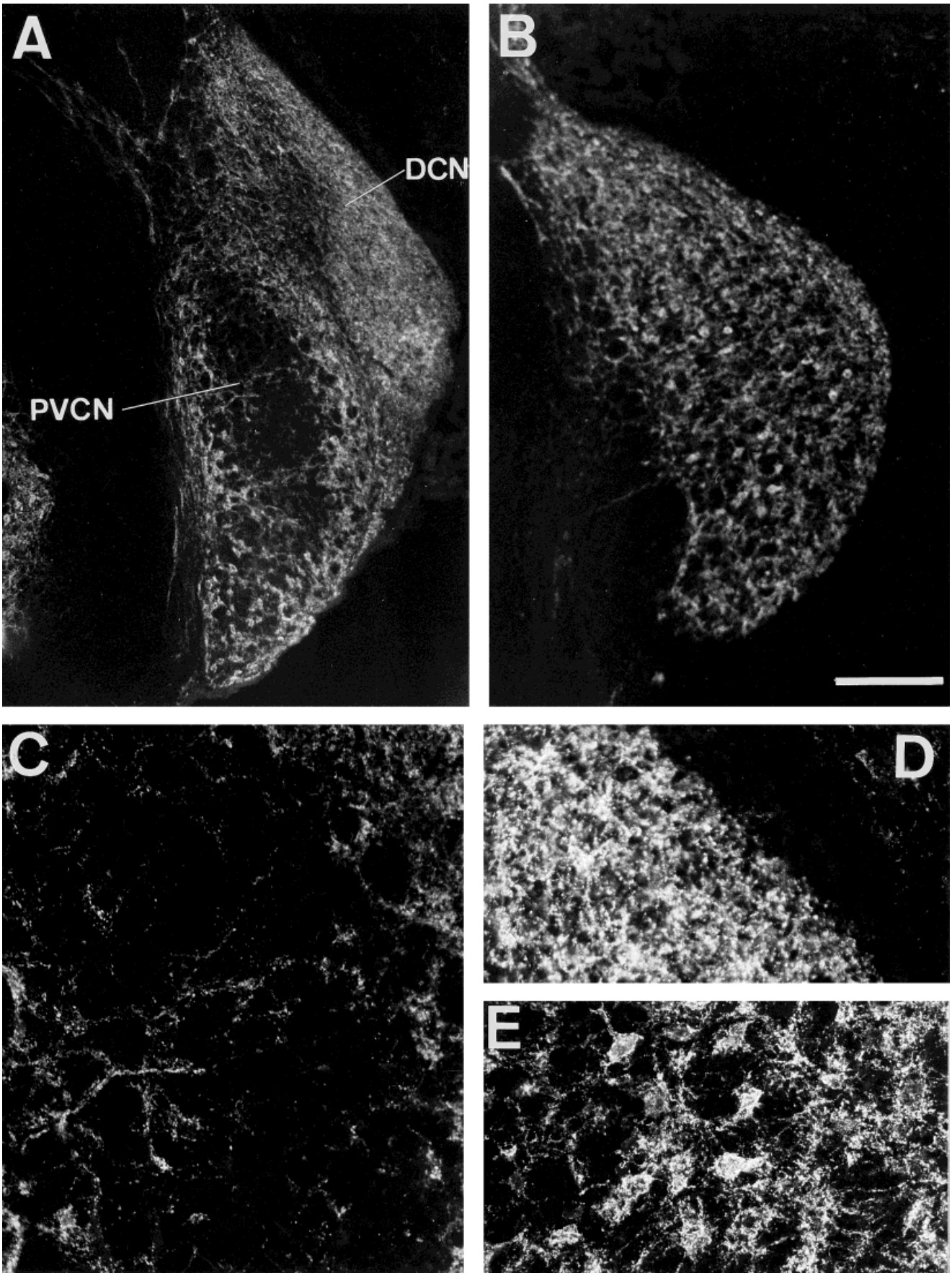


Figure 1

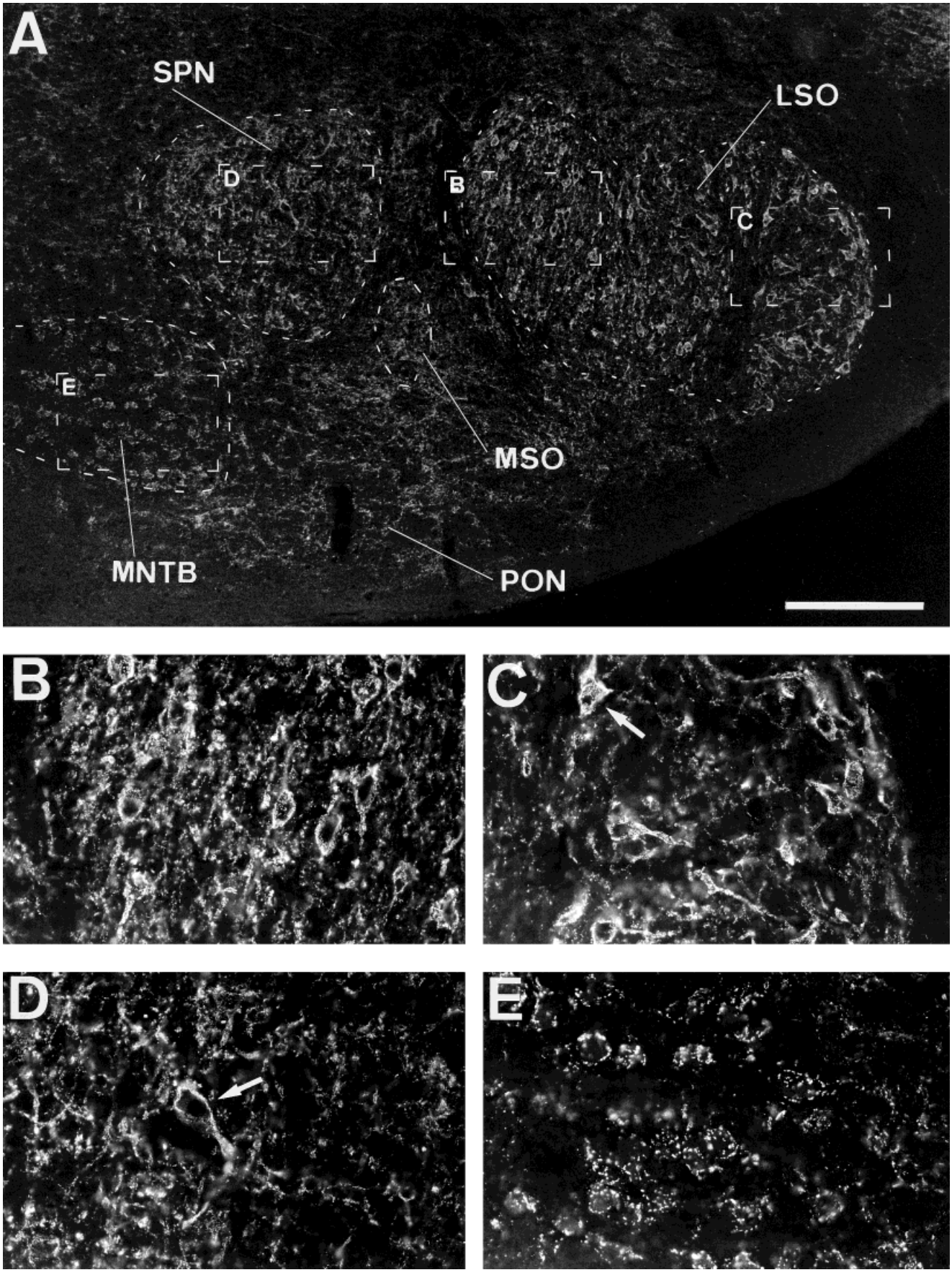


Figure 2

analysis was finally performed by using Systat software and the Wilcoxon signed rank test.

RESULTS

α1 GlyR immunoreactivity in the central auditory system of adult rats

In adult rats, immunoreactivity (ir) for the α1 GlyR subunit was present at virtually all levels of the caudal auditory brainstem, i.e., from the CN up to the inferior colliculus (IC). This indicates that the classic, strychnine-sensitive GlyR is expressed in these nuclei. Both the intensity and the pattern of labeling differed between the nuclei: strong labeling was found throughout most aspects of the superior olivary complex (SOC) and the CN, whereas a relatively weak signal density was seen in the IC. Intermediate levels occurred in the nuclei of the lateral lemniscus (NLL). In contrast to the high level of immunoreactivity in the auditory brainstem, we observed virtually no labeling above background in the medial geniculate body or the auditory cortex.

Cochlear nuclear complex. α1 GlyR-ir was found throughout all subdivisions of the CN, yet there were obvious differences in the pattern of labeling. In the posteroventral cochlear nucleus (PVCN), most neuronal somata and their proximal dendrites, particularly those located in the ventral tip region and, therefore, and because of their ellipsoid shape, presumably representing spherical globular cells (Perry and Webster, 1981; Hackney et al., 1990; Fleckeisen et al., 1991), were densely incrusted by immunoreactive puncta, denoting presumptive synaptic sites (Fig. 1A). Only little α1 GlyR-ir was found in the octopus cell region of the PVCN (Fig. 1A,C), consistent with previous immunocytochemical findings that octopus cells receive only a very small number of glycinergic boutons (Kolston et al., 1992; Wickesberg et al., 1994). In the anteroventral cochlear nucleus (AVCN; Fig. 1B,E), immunoreactive puncta outlined the cell bodies and proximal dendrites (Fig. 1E). Compared with the AVCN and PVCN, α1 GlyR-ir in the dorsal cochlear nucleus (DCN) was seen throughout all subdivisions and a high labeling intensity was observed, consistent with data obtained with in situ hybridization (Malosio et al., 1991). Labeling had a punctate appearance and was very prominent in the neuropil (Fig. 1D), where it was more extensive than in the ventral CN (VCN).

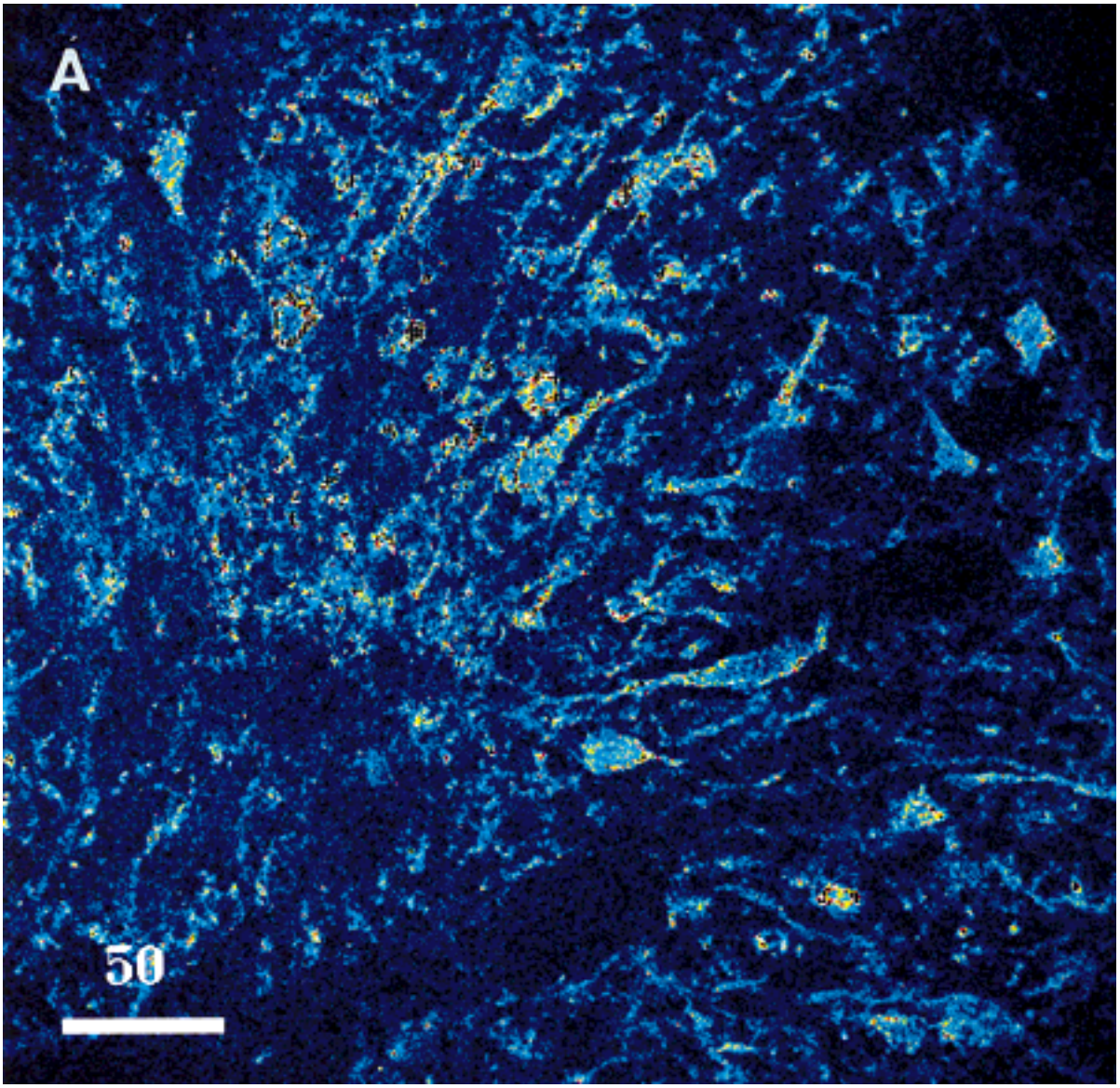
Superior olivary complex. α1 GlyR-ir was seen in all four major nuclei of the rat's SOC, i.e., the lateral superior olive (LSO), the superior paraolivary nucleus (SPN), the medial nucleus of the trapezoid body (MNTB), and the medial superior olive (MSO; Fig. 2A). In addition, labeling was also present in the periolivary nuclei (PON). The LSO and the SPN contained the highest α1 GlyR-ir of all auditory brainstem nuclei. The somata of these neurons were surrounded by intensely labeled immunopositive puncta, forming a halo around the somata while leaving the perikarya unlabeled (Fig. 2B–D). Most LSO neurons that were covered by α1 GlyR-ir puncta appeared to be bipolar cells, particularly those being located in central and medial aspects of the LSO (Figs. 2B, 3A). On the other hand, immunopositive neurons in the lateral limb of the LSO often resembled multipolar cells (Fig. 2C). In the MNTB, the labeling pattern differed from the other SOC nuclei in that the puncta were much more restricted to the somata (presumably of principal cells) and not associated with the proximal dendrites or the neuropil, and the density of the puncta was lower, resulting in a scattered, rather than a ring-like appearance (Fig. 2E). Nevertheless, it was obvious that virtually every MNTB principal cell was studded with a significant number of α1 GlyR clusters.

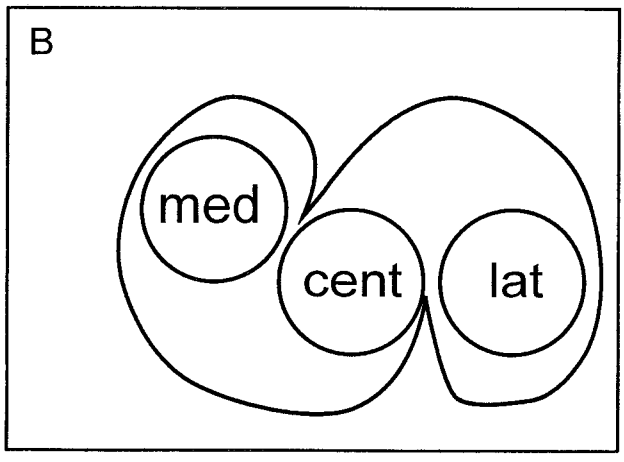
In order to investigate whether there is a non-uniform distribution of GlyRs and a gradient of α1 GlyR-ir in the LSO along the medio-lateral axis, we performed a quantitative analysis and determined the labeling intensity in the lateral, central, and medial aspect using laser scanning microscopy (Fig. 3). Based on the analysis of 12 LSO sections, α1 GlyR labeling intensity in the medial aspect averaged 5100 ± 1090 relative fluorescence units (mean ± standard deviation), in the central aspect 4,110 ± 1,110, and in the lateral aspect 3,870 ± 670 (Fig. 3C). The intensity in the medial aspect was significantly higher than in the central ($P = 0.019$) or the lateral aspect ($P = 0.004$; Wilcoxon signed rank test). By contrast, there was no statistically significant difference between the central and the lateral aspect ($P = 0.308$). These data are indicative of a higher concentration of GlyRs in the medial region of the LSO, coding for high frequencies, than in regions coding for lower frequencies. Furthermore, they are consistent with previous results obtained by ^{3}H-strychnine binding and quantitative autoradiography in the gerbil's LSO (Sanes et al., 1987).

Nuclei of the lateral lemniscus. All three subdivisions of the NLL, the ventral, intermediate, and dorsal (VNLL, INLL, and DNLL, respectively), contained a detectable amount of α1 GlyR-ir (Figs. 4,5). At a low magnification, dorso-ventral columns of labeling were striking, predominantly in the INLL and the DNLL (Fig. 4). The unstained gaps between these columns most likely were the fascicles in the fiber tract of the lateral lemniscus. The intensity of labeling was highest in the VNLL, where neuropil as well as some decorated somata were found (Fig. 5D). Both neuropil and somata were also labeled in the INLL (Fig. 5B,C), yet here decorated somata appeared to be smaller (soma diameters were less than 20 μm) than in the VNLL (some soma diameters exceeded 30 μm). In the DNLL, only very few somata were incrusted with α1 GlyR-ir puncta, whereas the neuropil was still heavily labeled (Fig. 5A).

Inferior colliculus. Labeling in the IC was analyzed only for the whole nucleus and not for individual subdivisions. Nevertheless, it was clearly resolved that α1 GlyR-ir throughout the IC was less intense than in the other auditory brainstem nuclei. While many immunoreactive puncta were seen in central aspects of the IC (Fig. 4), much

Fig. 2. Coronal sections through the superior olivary complex (SOC) of an adult rat. **A:** Overview of the SOC at low magnification, showing α1 GlyR immunoreactivity in every major nucleus, i.e., the lateral superior olive (LSO), the medial superior olive (MSO), the superior olivary nucleus (SPN), the medial nucleus of the trapezoid body (MNTB), and periolivary regions (PON). **B:** Medial limb of the LSO at higher magnification (cf. boxed area in A). Note immunoreactive puncta incrusting somata and proximal dendrites of bipolar cells. **C:** Lateral limb of the LSO at higher magnification (cf. boxed area in A). Multipolar neurons (arrow) are decorated with α1 GlyR subunits. **D:** Detail of the SPN (boxed area shown in A). Note intense immunoreactivity forming a halo around the soma of a neuron while the cell body is not stained (arrow). **E:** MNTB (boxed area shown in A) at high magnification. α1 GlyR-ir has a punctate appearance. Scale bar = 300 μm in A, 100 μm in B–E.





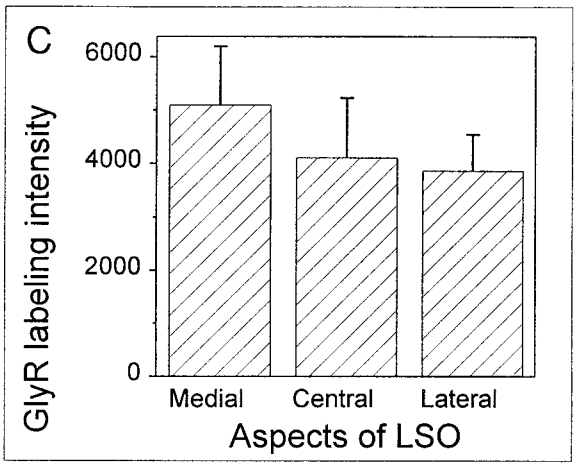


Fig. 3. Quantitative analysis of the density distribution of α1 GlyR subunits in medial, central, and lateral aspects of the adult LSO. **A:** Lateral and central aspect of the LSO at a mid-rostrocaudal level, imaged by using confocal microscopy and pseudocolor coding. White and black illustrate maximal and minimal signal intensity, respectively. Note fusiform, bipolar cells in the central aspect that are densely incrusted with immunopositive puncta. **B:** Schematic view of the LSO illustrating the position and size of the fields from which labeling intensity was determined. **C:** GlyR labeling intensities in medial, central, and lateral aspects of the LSO. Data are relative intensity values, and each bar represents the mean ± standard deviation of measurements from 12 LSOs. Labeling intensity in medial aspects is significantly higher than in central ($P = 0.019$) or lateral aspects ($P = 0.004$), indicating that there is an uneven distribution of the α1 GlyR subunits along the lateromedial frequency axis of the LSO. Scale bar = 50 μm.

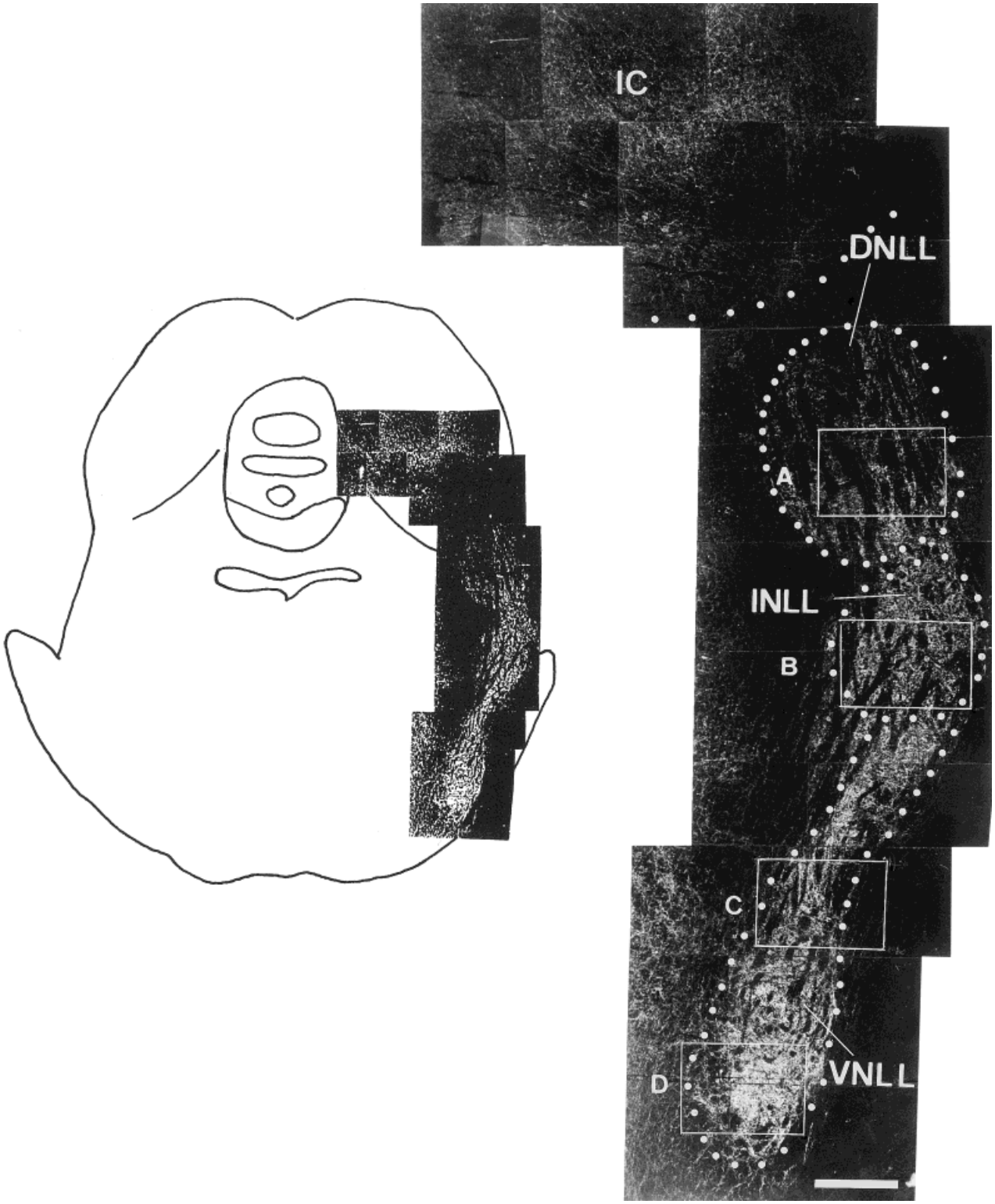


Fig. 4. Montage of photomicrographs from a coronal section through the lateral lemniscus of an adult rat. This low power image shows α1 GlyR immunoreactivity in all subdivisions of the nuclei of the lateral lemniscus (NLL), i.e., the dorsal NLL (DNLL), intermediate NLL (INLL), and the ventral NLL (VNLL). Areas in rectangles are shown enlarged in Figure 5. The intensity of labeling is particularly high in the VNLL. Thick fascicles within the lateral lemniscus appear to be devoid of labeling. Note that some immunoreactivity is also present in the inferior colliculus (IC). Scale bar = 250 μm.

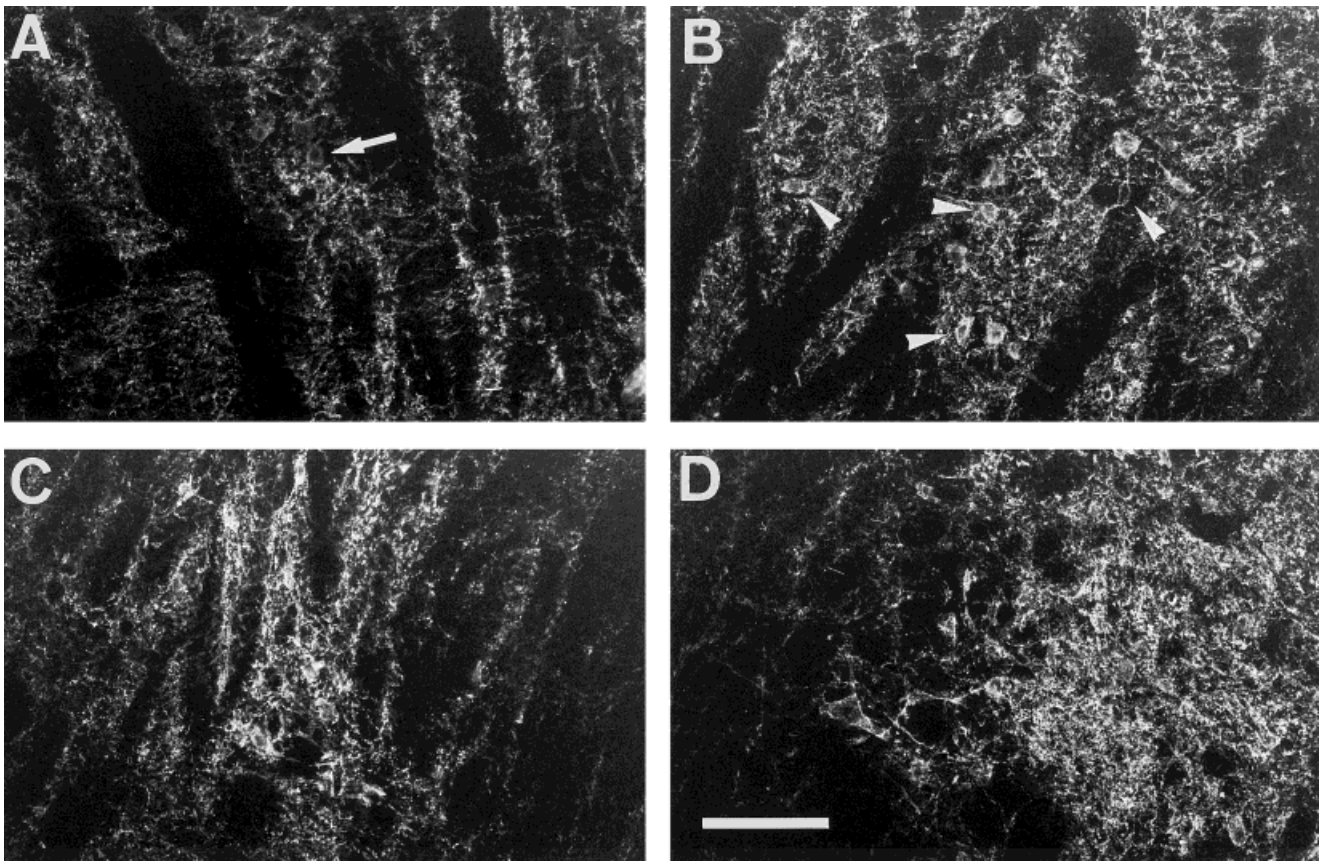


Fig. 5. High power views of the NLL regions indicated by the boxes in Figure 4. **A:** DNLL. α1 GlyR immunoreactivity is mainly seen in the neuropil, but few somata are also decorated (arrow). **B:** Ventral aspects of INLL and dorsal aspects of VNLL. Note higher number of decorated somata (arrowheads) as compared with the DNLL. **C:** Center of VNLL. Labeling is predominant in the neuropil. **D:** Ventral tip of VNLL. Somata as well as neuropil are heavily labeled. Scale bar = 100 μm in A–D.

less labeling could be detected in the periphery. Occasionally, single neurons were seen in central aspects, whose soma and proximal dendrites were densely covered with α1 GlyR-ir puncta (see Fig. 11D). These neurons had multipolar dendritic trees extending in all directions; they were consequently classified as multipolar cells (Oliver and Morest, 1984; Meininger et al., 1986). Compared to the CN, the SOC, and the NLL, the number of immunoreactive IC neurons was much lower. Moreover, we did not observe a spatial gradient of α1 GlyRs in the IC.

Medial geniculate body and auditory cortex. We did not find α1 GlyR-ir in the medial geniculate body or the auditory cortex in either adult or developing rats. Therefore, the developmental analysis described below was performed only in the medulla and the mesencephalon.

Development of α1 GlyR immunoreactivity in auditory brainstem nuclei of rats

During prenatal development, α1 GlyR-ir was not detectable in auditory brainstem nuclei. Immunoreactivity was first present at postnatal day (P) 0, yet not in all auditory nuclei. The spatial distribution and the intensity of the labeling signal increased considerably after P0, reaching adult-like patterns around P21 in most nuclei. The development of α1 GlyR-ir followed a spatio-temporal gradient, which runs from the high frequency region to the low frequency region. Unlike in cultivated spinal cord neurons, where a clustered appearance was detected from early on (St. John and Stephens, 1993), α1 GlyR-ir appeared to be originally diffusely distributed on the cell surface of auditory brainstem neurons (Fig. 6A,B). Only upon differentiation did the GlyRs cluster, resulting in a immunoreactivity that was seen as puncta on the cell bodies and proximal dendrites in juvenile and adult animals (Fig. 6C,D). We did not observe a differential time course of cluster formation for different nuclei. Likewise, cell body and dendritic clusters appeared to form at the same time in all neurons.

Cochlear nuclear complex. In the PVCN and the AVCN, α1 GlyR-ir was undetectable prior to P4 (Fig. 7A), and then it was still weak and restricted to the dorsal aspects of the nuclei, where high frequencies are represented in the adult. Immunoreactivity increased with age and by P8, it was seen in all aspects of the VCN except the most ventral tip (Fig. 7B). Around P21, the octopus cell region in the PVCN could be recognized because of its relative lack of signal (Fig. 7C). Immunoreactivity in the DCN was found earlier than in the VCN. It was barely visible at P0, when a very weak signal was found in dorso-medial aspects of the DCN (Fig. 7D). As these dorso-medial aspects are coding for high frequencies in the adult, this is indicative of high frequency regions maturing prior to those encoding low frequencies. Around P21, all

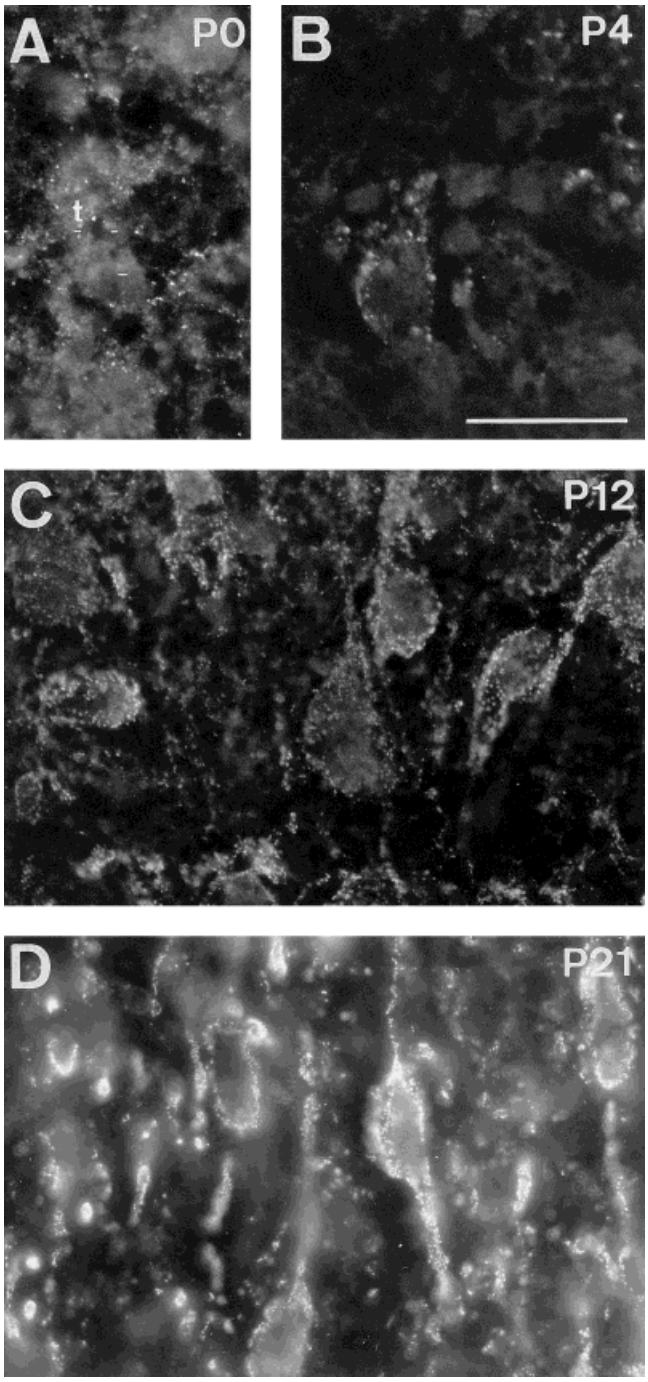


Fig. 6. High power views of LSO neurons in developing rats, illustrating the clustering of α1 GlyR subunits with age. **A,B:** In neonates, α1 GlyRs are diffusely distributed. **C,D:** Focal clusters form later and can be seen around the somata and proximal dendrites by P12. Scale bar = 15 μm in A–D.

layers of the DCN contained immunoreactivity (Fig. 7E). Taken together, the spatial distribution of labeling in all subdivisions of the CN was indistinguishable from the adult pattern after the end of the 3rd postnatal week.

Superior olivary complex. The time course of α1 GlyR-ir development in the SOC was similar to that in the CN. At P0, only a faint signal could be seen and it appeared to be restricted to medial parts of the developing LSO (Fig. 8A). During the 1st postnatal week, labeling became stronger but it was still restricted to the medial and central aspects of the LSO (Fig. 8B,C). By P10, all aspects of the LSO were labeled and neuronal somata had become outlined by dense clusters of immunopositive puncta (Fig. 8D). These clusters subsequently became more focal (Fig. 8E) and by P21, the adult pattern was reached (Fig. 8F; cf. Fig. 1A,C).

In the MNTB, the development of α1 GlyR-ir lagged behind that in the LSO. Whereas LSO neurons of the medial limb were already clearly labeled at P4, MNTB neurons were still almost devoid of labeling (Fig. 9A). Around P8, few puncta could be identified on most somata (Fig. 9B) and by P12, the number of puncta had increased considerably (Fig. 9C). As in the other nuclei, the adult-like pattern of labeling was reached at P21, when virtually every neuronal cell body was surrounded by immunopositive aggregates (Fig. 9D).

Neurons in the SPN displayed immunoreactivity to α1 GlyR subunits in a fashion similar to LSO neurons. There was only a weak signal at the day of birth (not shown) which increased during the first postnatal days and could be seen in all aspects of the SPN at P4 (Figs. 9A, 10A). Immunoreactive puncta on the somata, originally being diffusely arranged, became clustered during further development (Fig. 10B–D), and after the 1st postnatal week, many neurons were densely covered with immunofluorescent puncta on their soma and proximal dendrites (Fig. 10C).

The MSO was the only SOC nucleus in which α1 GlyR-ir appeared later than P8. Prior to P8, the MSO was identified as a dark, unstained area situated between the SPN, the LSO, and the PON, each found to be intensely labeled after P4 (Fig. 10A,B). MSO somata were never incrusted with immunoreactive puncta, but immunoreactivity was prominent in the neuropil. The adult-like labeling pattern in the MSO became visible by P21 (not shown).

Nuclei of the lateral lemniscus. α1 GlyR-ir in the NLL appeared between P4 and P7 and increased markedly with age in all three subdivisions. A quick and considerable raise in signal intensity occurred between P10 and P12, after which intense labeling was observed in the neuropil of the DNLL, the INLL, and the VNLL as well as around neuronal somata in the VNLL (Fig. 11A) and the INLL. The spatial pattern of immunoreactivity did not change after P12, but the number of puncta gradually increased until P21 (Fig. 11B), with the increase being more prominent in the neuropil than in pericellular structures. Like in the other auditory nuclei of the lower brainstem, the mature pattern of immunoreactivity in the NLL became visible after the end of the 3rd postnatal week.

Inferior colliculus. Like in the MSO, immunohistochemical detection of α1 GlyR subunits in the IC could not be achieved prior to P7, indicating that gene expression begins relatively late in this rostral brainstem nucleus. At P8, a relatively low number of puncta could be seen in the neuropil and around neurons (Fig. 12A). The number of puncta increased with age, both in the neuropil and on neurons, with clusters developing on somata and dendrites (Fig. 12B,C). The mature pattern was established between P21 and P28 (Fig. 12C,D). In some cases, peripheral dendrites became also incrusted with puncta and could be

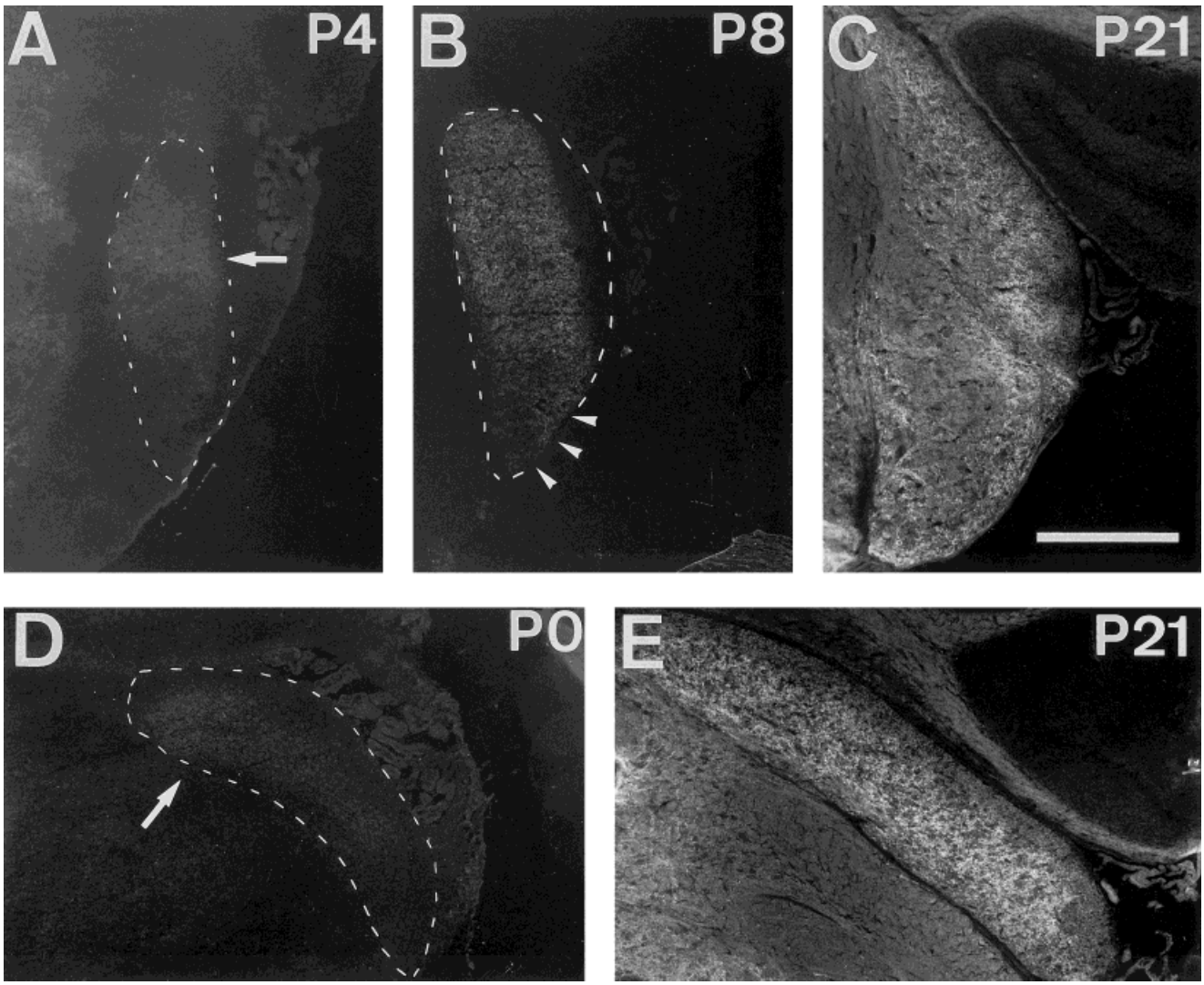


Fig. 7. Coronal sections through the CN of developing rats. **A:** Ventral CN (VCN) at postnatal day (P) 4. Immunoreactivity is still weak and restricted to the dorsal half of the VCN (arrow). **B:** AVCN at P8. Immunoreactivity has increased, but the ventral tip (arrowheads) is still less immunoreactive than the dorsal areas. **C:** PVCN and DCN at P21. The labeling pattern is indistinguishable from that of the adult (cf. Fig. 1A). **D:** DCN at P0. Only a very weak immunopositive signal can be seen in the dorsomedial aspect (arrow). **E:** DCN at P21. All DCN areas are filled with an immunofluorescent signal. Scale bar = 500 μm in A–E.

followed up to a distance of more than 100 μm (e.g., at P16, Fig. 12B). Such a distal location of α1 GlyR subunits was not observed in the other auditory nuclei. At all ages, the number of immunopositive IC neurons was much lower than in the more caudal brainstem nuclei. Nevertheless, if IC neurons were labeled, they were generally densely decorated with puncta (Fig. 12C,D). Thus, the IC was unique among the auditory brainstem nuclei because of its delayed development, the low density of labeled neurons, and the location of immunoreactivity on distal dendritic branches.

In summary, the developmental results showed that adult-type GlyRs were not expressed in the auditory brainstem of fetal rats (Fig. 13). Neurons began to express such receptors by P0, yet only in the DCN, LSO, and the SPN. Originally, α1 GlyRs appeared to be diffusely distributed on the cell surface, yet they became successively localized in focal clusters, or aggregates, on the cell bodies and proximal dendrites in most brainstem nuclei. There appeared to be a temporal pattern along the caudo-rostral axis of the central auditory system, since CN (at P0) and SOC nuclei (P0–P4) generally showed immunoreactivity earlier than the NLL (P4–P7) and the IC (P8). Furthermore, the CN and SOC also displayed the adult pattern prior to these rostral regions. The number of adult-type GlyRs increased abruptly after the initial expression, finally reaching mature concentrations and distribution patterns around P21 in most nuclei.

DISCUSSION

The two main results of this study are the presence of adult-type, α1 GlyR subunits in all auditory brainstem nuclei from the cochlear nuclear complex to the inferior colliculus and their exclusively postnatal development, reaching maturity before P21 in most nuclei. As α1 sub-

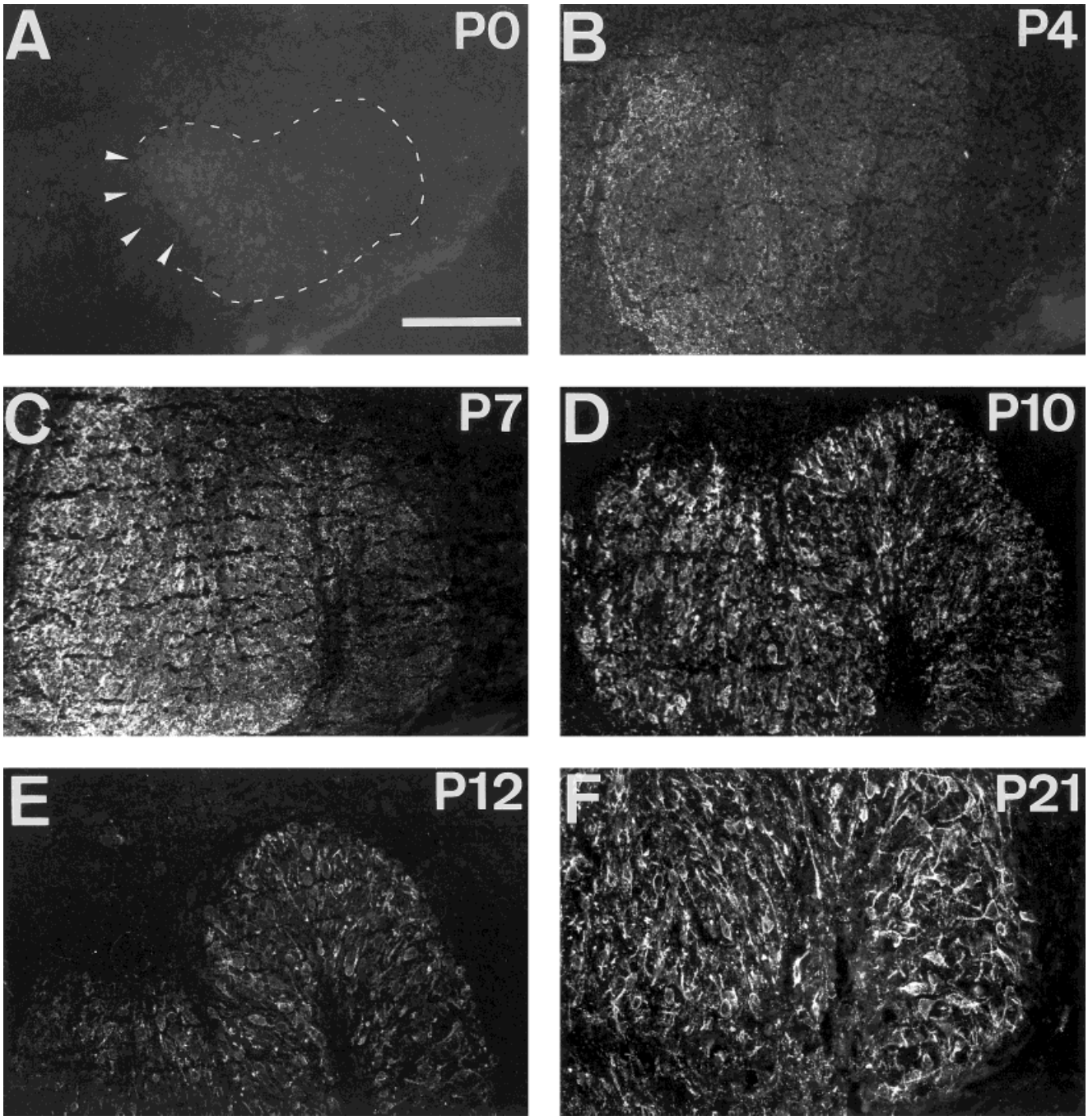


Fig. 8. Coronal sections through the LSO of developing rats. **A:** P0. α1 GlyR-ir can barely be seen in the anlage of the LSO (arrowheads). **B:** P4. Labeling in medial aspects of the nucleus is more prominent than in the lateral limb. **C:** P7. Signal intensity has increased, but the lateral limb is still almost devoid of labeling. **D,E:** P10 and P12. Immunoreactivity is now seen along the whole lateromedial axis of the LSO and most bipolar neurons appear to be decorated with immunopositive puncta. **F:** P21. The adult pattern has been achieved and many neuronal somata plus their proximal dendrites can clearly be seen. Scale bar = 200 μm in A–F.

units harbor the ligand-binding site of the GlyR, our data indicate that glycinergic neurotransmission is abundant at all levels of the central auditory system caudal to the diencephalon and predominantly in the LSO.

Technical considerations

For several reasons, the detection of glycinergic synapses with antibodies against the α1 GlyR subunit appears to be the best of all available techniques. First, it enables the identification of the ligand-binding polypeptides and not of associated polypeptides (gephyrin) that do not unambiguously colocalize with GlyRs (Cabot et al., 1995; Sassoé-Pognetto et al., 1995). In previous studies, gephyrin immunocytochemistry has been used to identify GlyRs in the auditory system (Altschuler et al., 1986; Wenthold et al., 1988), yet recently it became clear that

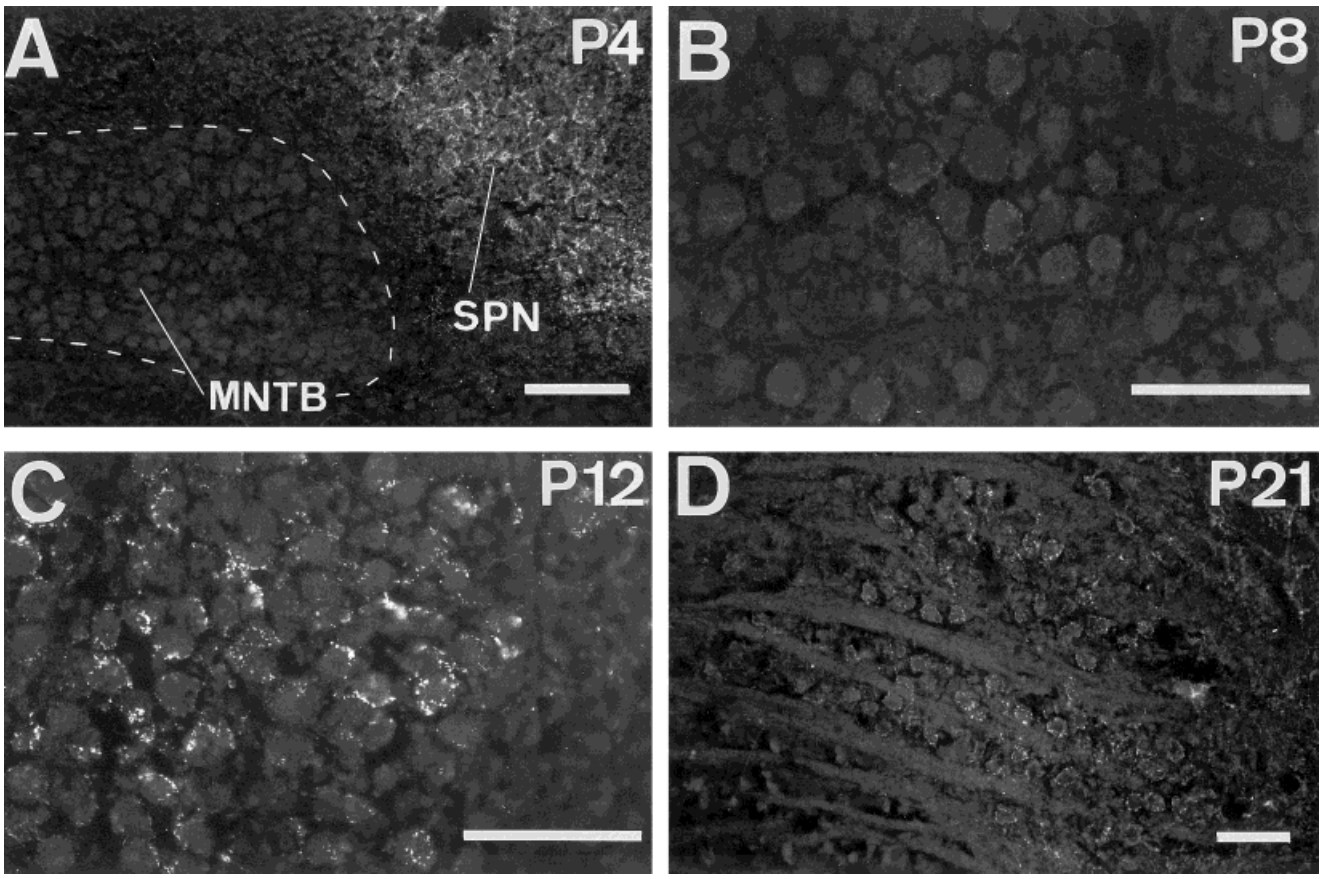


Fig. 9. Coronal sections through the MNTB of developing rats. **A:** P4. The MNTB is practically devoid of labeling, whereas the SPN already contains a high amount of α1 GlyR immunoreactivity. **B:** P8. Few puncta are seen on the somata of MNTB principal cells. **C:** P12. The number and intensity of puncta have clearly increased. **D:** P21. The adult pattern is seen, with virtually every neuron being surrounded by immunopositive puncta. Scale bars = 100 μm.

gephyrin is also present at synapses that do not contain GlyRs (Todd et al., 1995; Cabot et al., 1995; Sassoé-Pognetto et al., 1995). Second, although strychnine-binding combined with autoradiography is a selective technique for detecting GlyRs (on auditory neurons see Sanes and Wooten, 1987; Sanes et al., 1987; White et al., 1990; Fubara et al., 1996), its spatial resolution is inferior to immunocytochemistry. Moreover, different isoforms of GlyRs cannot be distinguished. Finally, labeling of neurons with antibodies against glycine, most frequently employed in the auditory system (Peyret et al., 1987; Wenthold et al., 1987; Aoki et al., 1988; Helfert et al., 1989; Adams and Mugnaini, 1990; Osen et al., 1990; Kolston et al., 1992; Pourcho et al., 1992), may be a misleading marking technique for inhibitory glycinergic input neurons (Wenthold and Hunter, 1990; Winer et al., 1995), since the amino acid glycine is also involved in metabolic aspects other than neurotransmission (Daly, 1990; Daly and Aprison, 1982) and acts as a coagonist of NMDA receptors (for review see Ascher and Johnson, 1994).

α1 GlyRs in the adult auditory brainstem

The regional distribution of GlyRs in the adult rat described here matches to a large extent with that revealed by different techniques in other species (bats: Vater, 1995; Winer et al., 1995; Fubara et al., 1996; baboon: Moore et al., 1996; cat: Glendenning and Baker, 1988; ferret: Henkel and Brunso-Bechtold, 1995; gerbil: Sanes et al., 1987; guinea pig: Wenthold et al., 1988; Kolston et al., 1992; mouse: Wickesberg et al., 1994). With the exception of Wenthold et al. (1988), who employed antibodies both against gephyrin and the α1 subunit, none of these studies was aimed at identifying α1 subunits. Therefore, the similarity of the results indicates that the different techniques for detecting GlyRs, despite their above-mentioned limitations, appear to be generally compatible in the auditory brainstem (see also [Sato et al., 1991] for an in situ hybridization study on α1 GlyR subunits in the rat brain). Some differences in the localization of GlyRs, however, did become apparent. For example, Wenthold et al. (1988, p. 427) described that all regions of the VCN contain heavy GlyR-ir. By contrast, we found only little labeling in the octopus cell area of the PVCN, which is consistent with reports of little or no ^{3}H-strychnine binding in the PVCN (Fubara et al., 1996, p. 89), of only a few glycine-ir puncta on octopus cells (Kolston et al., 1992, p. 445), of far less immunoreactive neuropil in the octopus cell area than in the remaining VCN (Moore et al., 1996, p. 509), or of the complete absence of puncta (Wickesberg et al., 1994, p. 315). In line with this, tuberculoventral cells, which are glycinergic and provide the glycinergic input to the PVCN (Oertel and Wickesberg, 1993), do not project to

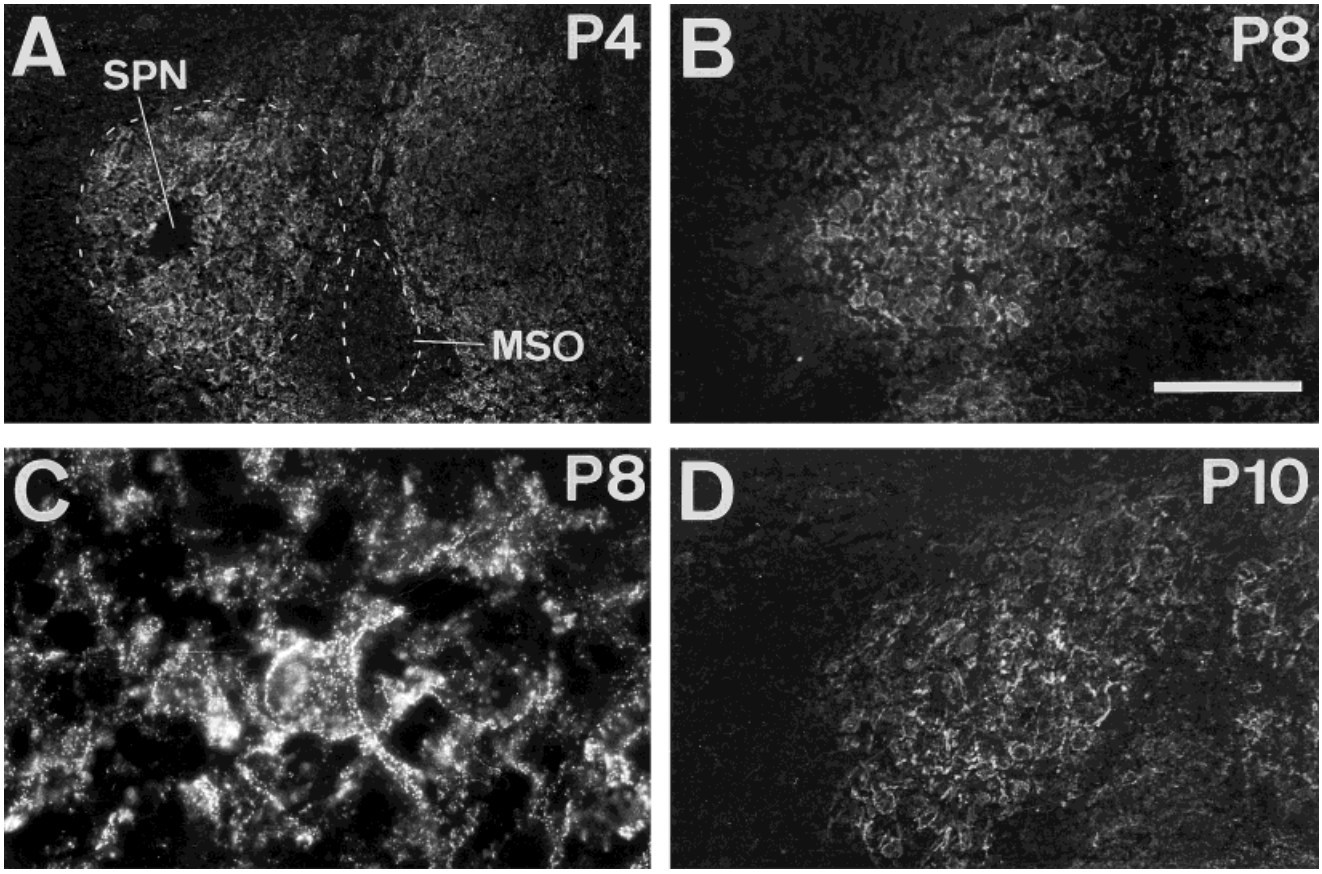


Fig. 10. Coronal sections through the SPN of developing rats. **A:** P4. Immunoreactivity is seen in all aspects of the nucleus, and neuronal somata are outlined. Note relative paucity of labeling in the MSO (see also B). **B,C:** P8. Labeling has become more clustered such that punctate labeling is associated with somata and proximal dendrites (C). **D:** P10. Note extensive perisomatic punctate α1 GlyR-ir around most SPN neurons. Scale bar = 200 μm for A, B, D, 40 μm for C.

the octopus cell area (Wickesberg et al., 1991). Taken together, there is more evidence in favor of a paucity or even a lack of GlyRs on PVCN octopus cells than against it.

Another discrepancy concerns the question of whether GlyRs are unevenly distributed along the frequency axis in several nuclei. Graded distributions have been described in the LSO of gerbils (Sanes et al., 1987) and in the IC of bats (Fubara et al., 1996), whereas a homogeneous distribution was seen in the cat's IC (Glendenning and Baker, 1988). Our results also show a non-uniform distribution of GlyRs in the LSO, with a gradient running from medial to lateral, i.e., along the high-to-low frequency axis. Since this orientation is consistent with the results of the aforementioned studies, the data indicate that high frequency hearing may be more heavily influenced by glycinergic inhibition than low frequency hearing.

As to the relative amount of GlyRs within the central auditory system, there is a good correspondence between the findings obtained in bats (Fubara et al., 1996) and rats (this article). In both species, the LSO is likely to contain the highest amount of GlyRs, whereas the medial geniculate body and the auditory cortex appear to be devoid of GlyRs. Results from studies in which glycinergic boutons, presumably representing axon terminals, have been analyzed, are in accord with this finding (Henkel and Brunso-Bechtold, 1995; Winer et al., 1995). MNTB neurons, which provide the major synaptic input to LSO neurons, form the most prominent glycine-immunoreactive cell group in the SOC (Aoki et al., 1988; Henkel and Brunso-Bechtold, 1995) and thus it is not surprising that very high amounts of GlyRs are found in the LSO. Virtually every LSO bipolar neuron appeared to have a significant supply of GlyRs which formed dense focal clusters, or aggregates, on the soma and proximal dendrites, thus incrusting the neuronal surface. The perisomatic and peridendritic clustering of the α1 subunits on LSO neurons indicates that these cells receive a strong glycinergic input which should be very effective in reliably shunting excitatory input, due to the soma-near location. Electrophysiological recording in the LSO has shown that this is indeed the case (Moore and Caspary, 1983). Moreover, this study has demonstrated that glycine is effectively involved in the subtracting process of detecting interaural intensity differences. By contrast, the finding that in the DCN and the NLL, α1 subunits are mainly located in the neuropil, rather than at pericellular or peridendritic sites, indicates that glycine exerts its influence at a greater distance from the somata and may have modulatory effects in these nuclei. Interestingly, spike recordings from DCN neurons have shown that glycine is resonsible for lateral/sideband inhibition and for the narrowing of the response area (Evans and Zhao, 1993), i.e., for modulatory activity. Moreover, glycine

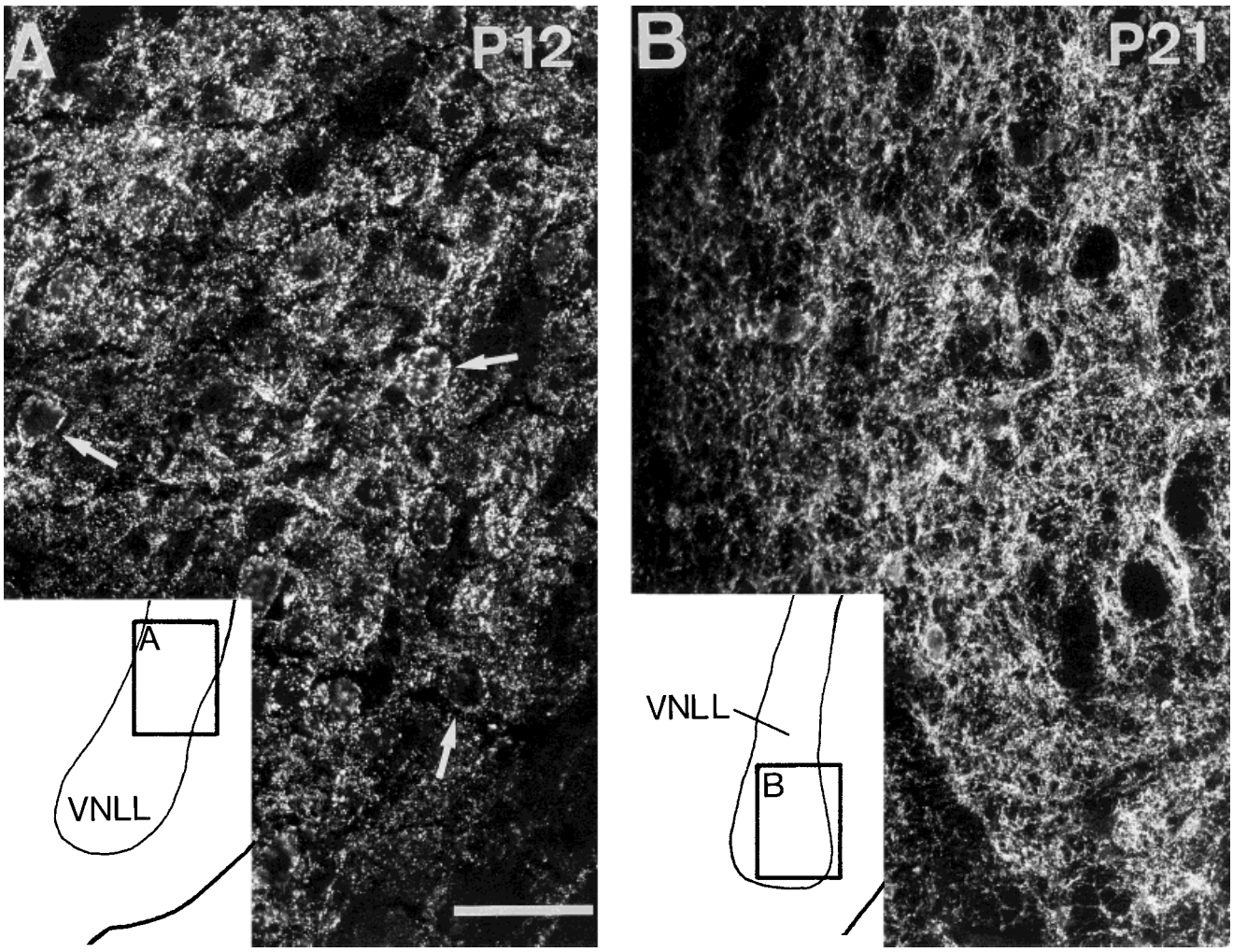


Fig. 11. Coronal sections through the NLL of developing rats. Insets show location of high magnification photomicrographs. **A:** VNLL at P12. Immunoreactivity is already strong and α1 GlyR-covered somata (arrows) can be detected within labeled neuropil. **B:** P21. Labeling has mainly increased within the neuropil and the adult pattern has been obtained. Scale bar = 40 μm in A, 80 μm in B.

also mediates contralateral inhibition in the DCN which probably arises from the commissural cells in the contralateral CN (Cant and Gaston, 1982; Wenthold, 1987).

Development of α1 GlyRs in the auditory brainstem

Our results on the time course of development indicate that the adult-type GlyR is not expressed in the auditory system of fetal rats and that only very little amount of the protein is present at the day of birth, being restricted to some nuclei (DCN, LSO, SPN). The development of immunoreactivity is gradual and follows a spatio-temporal gradient, which runs from the high frequency region to the low frequency region in the relay stations, in agreement with results from strychnine-binding in gerbils (Sanes and Wooten, 1987). There is an early developmental stage at which GlyRs are diffusely distributed, and focal clusters of GlyRs on the neuronal surfaces are formed only subsequently, yet relatively rapidly, during the 1st postnatal week. By the time of hearing onset around P12, those clusters are well-developed and aggregated around the somata, indicating that glycinergic neurotransmission is precise and efficient at a time when the ability to localize sound sources becomes important.

The mature pattern (subcellular and supracellular) is acquired during the 3rd postnatal week in all nuclei except the IC in which α1 GlyR development lasts almost until P28. We did not see any evidence for a temporary overexpression of α1 GlyRs, and we did not find a transient labeling in areas that are not equipped with GlyRs in the adult, i.e., the medial geniculate body and the auditory cortex. Thus it appears that the adult-type GlyRs are localized in the 'correct' auditory nuclei from the earliest stages of expression, yet that significant reorganization takes place at the subcellular level.

Comparing the expression of GlyRs with other developmental events of synaptogenesis in the rat's auditory brainstem, one must conclude that the α1-containing GlyR isoform appears relatively late in ontogeny. Many important steps of neural maturation are accomplished prenatally, such as the period of mitotic activity (Altman and Bayer, 1980, 1981; Weber et al., 1991), the process of

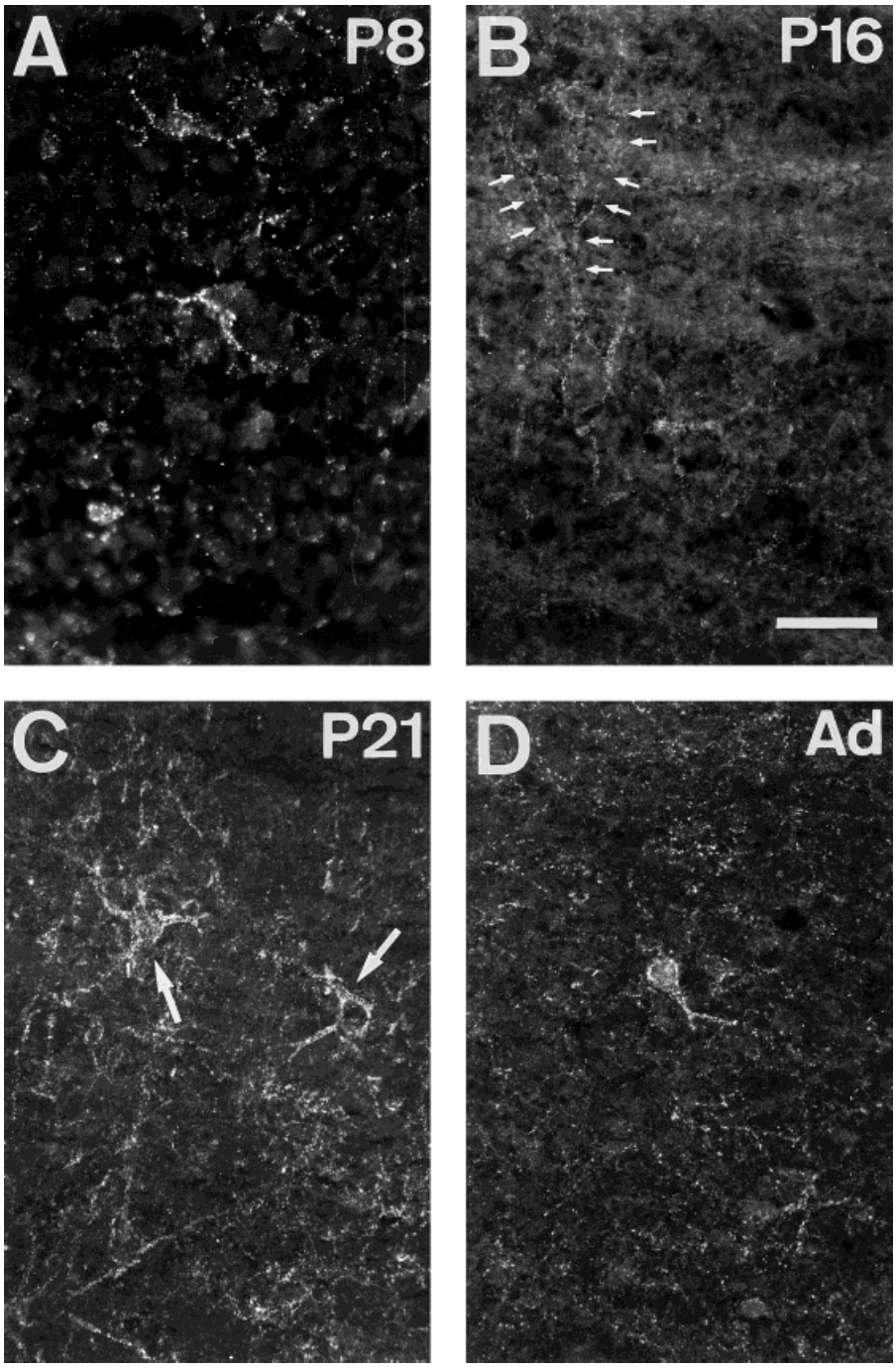


Fig. 12. Coronal sections through central aspects of the IC of developing rats. **A:** P8. Weak labeling can be seen around some neuronal somata and proximal dendrites. **B:** P16. Labeling has become more dense, and in a few neurons dendrites of first and higher order (arrows) are clearly decorated with immunoreactive aggregates. **C:** P21. α1 GlyR subunits are now densely incrusting some somata and proximal dendrites, enabling the morphological identification of the neurons (in this case multipolar cells, arrows). **D:** The pattern of immunoreactivity is basically adult-like after P28. Scale bar = 100 μm for A, C, D, 50 μm for B.

naturally occurring cell death (Harvey et al., 1990), and the period of axon ingrowth and early synapse formation (Mattox et al., 1982; Friauf and Kandler, 1990; Kandler and Friauf, 1993). Thus it appears that adult-type GlyRs do not participate in these crucial developmental events. Nevertheless, it does not imply that glycine does not contribute to the acquisition of the principal elements of the final neural assemblies in the auditory brainstem. At present, no study has investigated the development of glycine-immunopositive neurons and axonal endings in the auditory system of rats, but data obtained in the SOC of ferrets show that glycine appears by P7, i.e., long before

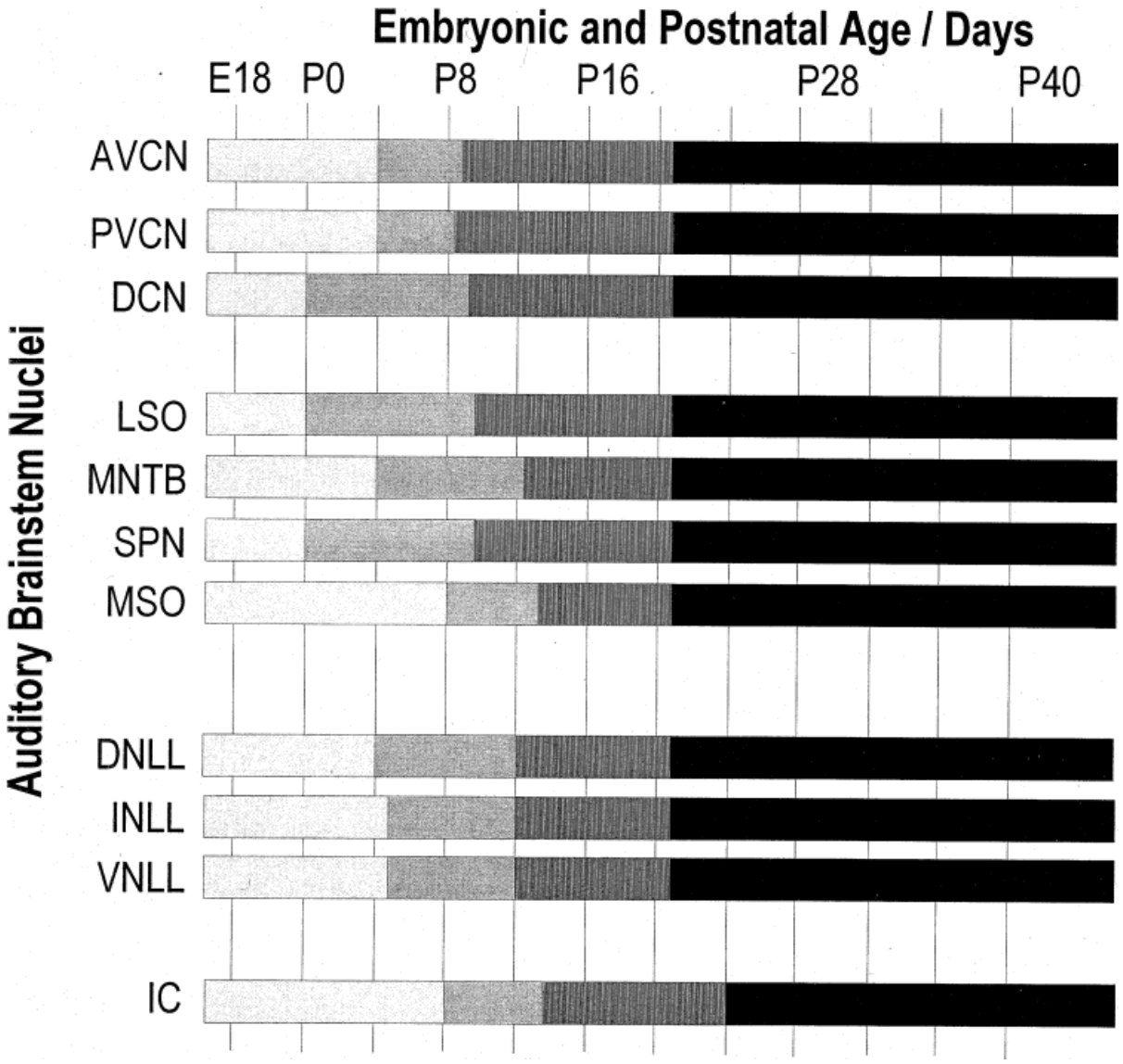


Fig. 13. Schematic representation of the appearance of α1 GlyR-ir in individual auditory brainstem nuclei of the embryonic and postnatal rat brain. White: undetectable signal; light grey: weak and evenly distributed signal on somata and dendrites; dark grey: stronger signal intensity and clustering of immunopositive puncta; black: adult-like distribution with immunopositive puncta surrounding most somata. The medial geniculate body and the auditory cortex have been omitted from this figure because of the absence of α1 GlyR-ir at every age.

hearing onset (around P28; Moore, 1982) and during the period of synaptogenesis (Brunso-Bechtold et al., 1992). Moreover, electrophysiological studies in fetal rats have identified functional glycinergic neurotransmission in the SOC as early as embryonic day 18, i.e., 4 days before birth and, therefore, in the absence of α1-containing GlyRs (Kandler and Friauf, 1995). Taken together, it is well conceivable that glycine is involved in early, prenatal synaptogenic processes in the rat auditory brainstem circuitries. If this is indeed the case, glycine must act via GlyRs other than those harboring α1 subunits, for example via the α2 subunit-containing GlyRs. The latter isoform is heavily expressed in the spinal cord of fetal and neonatal rats (Becker et al., 1988), yet there are no comparable data on the protein level in the auditory brainstem (for an in situ hybridization study at P7, see Sato et al., 1992). Preliminary results from our laboratory, however, indicate that α2 GlyR subunits are also present in the auditory brainstem nuclei of fetal and newborn rats and down-regulated during early postnatal life (Friauf et al., 1994, and unpublished observations). Thus it is likely that early glycinergic neurotransmission and potential maturational effects are mediated via GlyRs harboring α2 subunits.

Glycinergic contribution to synapse maturation has been investigated in the SOC of postnatal gerbils, and it was shown that abolishing glycinergic input between P7 and P21 impairs the morphological refinement of axons and dendrites which normally occurs during that period (Sanes et al., 1992a,b; Sanes and Takacs, 1993). These data suggest that glycine also participates in developmental processes later than those mentioned above. However, the manipulations were performed at a time when α1 and

α2 GlyR subunits probably co-exist and, therefore, it is unclear which GlyR isoform has contributed to the effects observed by Sanes and collaborators. In conclusion, the question of whether α1 GlyRs in the mammalian auditory system simply play a role in mediating classical inhibitory neurotransmission during acoustic information processing or whether they also participate in neuronal maturation awaits further analysis.

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