Histochem Cell Biol (2008) 129:513–523
DOI 10.1007/s00418-008-0387-x

ORIGINAL PAPER

Expression of glycine receptors and gephyrin in the rat cochlea

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Accepted: 11 January 2008 / Published online: 30 January 2008

Abstract The cochlear efferent feedback system exerts direct impact on cochlear nerve activity and balances interaural sensitivity. So far, acetylcholine, GABA and dopamine are known to be transmitters of the inhibitory efferent system. Despite the wealth of information about glycinergic neurotransmission in the central auditory system, the inhibitory glycine receptor (GlyR) has not yet been regarded as a target molecule of efferent transmission in the cochlea. Using RT-PCR, in situ hybridization and immunohistochemistry, we show that GlyRα3, GlyRβ and gephyrin are expressed in the organ of Corti and spiral ganglion neurons. Furthermore, two alternative splice variants of GlyRα3, corresponding to the long (α3_L) and short (α3_K) human isoforms, could be distinguished. The localization of glycine receptors below inner hair cells and in outer hair cells of the adult cochlea suggests that these inhibitory receptors may serve as target molecules of the efferent olivocochlear bundle.

Keywords GlyR · Transmitter · IHC · OHC · Inhibition

Abbreviations

ACh	Acetylcholine
CGRP	Calcitonin-gene related peptide
CN	Cochlear nucleus
EF-LOC	Efferent lateral olivocochlear (bundle)
EF-MOC	Efferent medial olivocochlear (bundle)
GLRA	Designation for human glycine receptor alpha genes
Glra	Designation for rodent glycine receptor alpha genes
Glra_rn	Designation for rat glycine receptor alpha genes
Glrb	Designation for rodent glycine receptor beta gene
GlyR	Glycine receptor
$GlyR_A$	Adult isoform of the glycine receptor
$GlyR_N$	Neonatal form of the glycine receptor
GlyRα3_K	Short splice variant of GlyRα3
GlyRα3_L	Long splice variant of GlyRα3
IHC	Inner hair cell
LOC	Lateral olivocochlear (system)
LSO	Lateral superior olive
MOC	Medial olivocochlear (system)
nAChR	Nicotinic acetylcholine receptor
NF200	Neurofilament 200
OHC	Outer hair cell
P	Postnatal day
sc	Spinal cord
SG	Spiral ganglion
SOC	Superior olivary complex

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Introduction

The inhibitory glycine receptor (GlyR) is a ligand-gated pentameric postsynaptic chloride channel expressed in the mammalian spinal cord and brainstem as well as in higher brain regions and the retina (Betz and Laube 2006). GlyRs are composed of developmentally and spatially regulated α subunits and a structurally homologous β polypeptide. To date, four different genes (*Glra1–4*) encoding ligand binding α subunits (α1–4) and one gene (*Glrb*) encoding the β subunit are known in vertebrates (Betz and Laube 2006). The β polypeptide contributes to postsynaptic anchoring of GlyRs by linking the receptor complex to gephyrin (Meyer et al. 1995). The adult isoform of the glycine receptor, $GlyR_A$, is a heteropentamer composed of two α1 and three β subunits (Grudzinska et al. 2005). $GlyR_A$ replaces the neonatal form of the glycine receptor, $GlyR_N$, which is presumed to be a homopentamer of five α2 subunits, at 2 weeks after birth (Becker et al. 1988). While the α3 subunit is predominantly found in higher brain regions associated with sensory integration in adult mammals (Malosio et al. 1991), GlyRα4 mRNA is transiently expressed in embryonal spinal cord and the developing male genital ridge (Harvey et al. 2000). GlyR heterogeneity is increased by alternative splicing. The long (GlyRα3_L) and short (GlyRα3_K) transcript variants of the human GlyRα3 receptor gene (*GLRA3*) are characterized by the presence or absence of exon 9 (45 bp), resulting in different receptor desensitization behavior (Nikolic et al. 1998).

In the auditory brainstem, GlyRα1, α3 and β transcripts have been detected in the cochlear nucleus (CN) and superior olivary complex (SOC) of adult rats (Sato et al. 2000). A developmentally regulated expression of GlyRα1 protein has been observed in all auditory brainstem nuclei (Friauf et al. 1997). Glycine receptors modulate neuronal activity in the auditory brainstem by lateral/sideband inhibition in the dorsal cochlear nucleus (Evans and Zhao 1993). Moreover, glycinergic and GABAergic inhibition sharpens the tuning for frequency modulated sounds in the inferior colliculus (Koch and Grothe 1998). On the level of the lateral superior olive (LSO), glycinergic interneurons of the medial nucleus of the trapezoid body are involved in localization of sound sources (Moore and Caspary 1983).

Inhibitory neurotransmission in the mammalian cochlea is mediated by the efferent medial (MOC) and lateral olivocochlear (LOC) bundle. Before the onset of hearing, the MOC bundle directly contacts inner hair cells (IHCs) by axosomatic synapses. Around the onset of hearing (~P12 in rats), these connections degenerate and are replaced by axodendritic synapses of the ipsilateral LOC bundle with the afferent auditory nerve terminals below IHCs (Guinan 1996; Simmons 2002). In adult animals, the medial olivocochlear (MOC) system predominantly contacts outer hair cells (OHCs) of the contralateral ear by axosomatic synapses. Recent findings suggesting a role of LOC efferents in the compensation of excitability changes of the auditory nerve after acoustic injury (Puel et al. 2002; Groff and Liberman 2003; Darrow et al. 2007) and in the balance of interaural sensitivity (Darrow et al. 2006) underscore the crucial function of the cochlear efferent feedback system for normal hearing. So far, acetylcholine (ACh), GABA, dopamine and calcitonin-gene related peptide (CGRP) have been identified as transmitters in efferent nerve terminals of the cochlea (Simmons 2002).

Here, we describe the detection of glycine receptors in the rat cochlea by RT-PCR, in situ hybridization and immunohistochemistry. The results are discussed in the context of a novel tool for modulation of hair cell function and auditory nerve excitability.

Materials and methods

Animals

For this study, Wistar rats (Charles River, Sulzfeld, Germany) were used. Care and use of the animals and the experimental protocol were reviewed and approved by the animal welfare commissioner and the regional board for scientific animal experiments in Tübingen.

Tissue preparation

For RNA isolation, cochleae were dissected and immediately frozen in liquid nitrogen. For in situ hybridization and immunohistochemistry, cochleae were isolated and prepared as previously described (Knipper et al. 2000). Briefly, cochleae were fixed by injection of 2% paraformaldehyde/2% sucrose (all chemicals from SIGMA-Aldrich, Munich, Germany, unless indicated otherwise) in 50 mM phosphate buffered saline (pH 7.4) into the round and oval window. In order to avoid decalcification in cochleae older than postnatal day 10 (>P10), the bony part of the lateral cochlear wall was removed. After incubation with 25% sucrose and 1 mM protease inhibitor (Pefabloc; Roche Diagnostics, Mannheim, Germany) in HEPES-Hanks solution, cochleae were embedded in O.C.T. compound (Miles Laboratories, Elkhart, IN, USA). Cryosections of 10 µm were made, mounted on SuperFrost*/plus microscope slides, dried for 1 h and stored at −20°C before use. Alternatively, whole-mount in situ hybridization and immunohistochemistry were used for animals >P10 as described in Engel et al. (2006). For each experiment at least three animals of the indicated age were used ($n = 3$).

RT-PCR

Total RNA was extracted from rat cochleae with the RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription (RT) into cDNA was performed using the Sensiscript Reverse Transcription Kit (Qiagen, Hilden, Germany) and oligo-dT_{15} primers (Roche, Penzberg, Germany) following the manufacturer's instructions. For the polymerase chain reaction (PCR), the following primers were used (size of the PCR product is given in brackets). *Glra1*, forward: 5′ CCTTCTGGATCAACATGGATGCT G 3′, reverse: 5′ CGCCTCTTCCTCTAAATCGAAGCA GT 3′ (243 bp); *Glra2*, forward: 5′ ATCCCTCGCAGACC CTATCT 3′, reverse: 5′ TAAACTGGGGCAAGGTGA GT 3′ (553 bp); *Glra3*, forward: 5′ GGCTGAAGGACTC ACTTTGC 3′, reverse: 5′ TGAATCGACTCTCCCTCAC C 3′ (*Glra3* primers were designed to detect possible splice variants corresponding to the human *GLRA3* splice variants α3_L and α3_K; α3_L: 512 bp, α3_K: 467 bp); *Glrb*, forward: 5′ CGGGATCCATTCAAGAGACA 3′, reverse: 5′ GCTCGAGCCACAC-ATCCAGTGCCTT 3′ (732 bp); *gephyrin*, forward: 5′ CAAGGTGGCTAGAAGACAT C 3′, reverse: 5′ ACCACTGGAAACTTATTAACTTC 3′ (573 bp); *β-actin*, forward: 5′ TGAGACCTTCAACACCC CAG 3′, reverse: 5′ CATCTGCTGGAAGGTGGACA 3′ (655 bp). Distilled water served as a negative control.

PCR was performed with PuReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Freiburg, Germany). The PCR program consisted of an initial denaturation phase of 3 min at 94°C, 35–40 cycles of denaturation at 94°C (30 s), annealing at 58°C (30 s) and extension at 72°C (90 s) and a final synthesis step of 10 min at 72°C. The resulting PCR products were separated on agarose gels and stained with ethidium bromide. PCR products of GlyRα3, GlyR*β* and gephyrin were sequenced and compared to the corresponding sequence data from GeneBank by BLAST (http://www.ncbi.nlm.nih.gov). Designation of GlyRα3 exons refers to the Ensembl automatic gene annotation system (http://www.ensembl.org; Curwen et al. 2004) and is distinct from the original description by Nikolic et al. (1998).

Riboprobe synthesis and in situ hybridization

For specific riboprobes, the PCR fragments of GlyRα3, GlyR*β* and gephyrin were cloned into the pCRII-TOPO Vector (Invitrogen, Karlsruhe, Germany), respectively and used for in vitro transcription. Complementary strands for sense and antisense probes were transcribed from either SP6 or T7 promoter sites in the presence of digoxigenin-labeling mix (DIG; Roche Diagnostics).

Whole mount in situ hybridizations with GlyRα3, GlyR*β* and gephyrin riboprobes were performed as described (Engel et al. 2006). Briefly, cochleae of indicated age were fixed with 2% paraformaldehyde for 30 min followed by dehydration in 100% methanol overnight at −20°C. After rehydration and digestion with proteinase K (2 μg/ml) at 37°C for 3 min, cochleae were post-fixed in 2% paraformaldehyde for 15 min. DIG-labeled antisense or sense probes were diluted in hybridization solution containing 25% microarray hybridization buffer (Amersham Biosciences, Freiburg, Germany), 25% nuclease free water and 50% formamide. Hybridization was carried out at 55°C overnight. Subsequent washing and detection steps were performed as described (Knipper et al. 1999, 2000). Each hybridization was done in at least three different animals at a given age.

Fluorescence immunohistochemistry

For immunohistochemistry, rat cochlear sections and whole mount preparations were stained and imaged as described (Knipper et al. 2000, 1998; Engel et al. 2006). The mouse monoclonal antibody mAb4a (Pfeiffer et al. 1984), which is directed against a common N-terminal epitope of GlyRα1–4 subunits, was obtained as hybridoma supernatant. For specific detection of the GlyRα3 subunit, a rabbit polyclonal antibody (SIGMA-Aldrich) was used. Mouse monoclonal antibodies against gephyrin (BD Transduction Laboratories, Heidelberg, Germany) and neurofilament 200 (NF200; The Binding Site, Heidelberg, Germany) were used. Primary antisera were visualized with Cy3- (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or Alexa488-conjugated secondary antibodies (Molecular Probes, Leiden, The Netherlands). Sections were mounted in Vectashield mounting medium containing DAPI nuclear staining (Vector Laboratories, Burlingame, CA, USA). Specimens were photographed using an Olympus AX70 microscope equipped with epifluorescence illumination and 40× (numerical aperture 1.0) or 100× oil immersion objectives (numerical aperture 1.35) (Ruttiger et al. 2004). Images were acquired using a CCD color view 12 camera and imaging system Analysis (SIS, Münster, Germany). Each staining was performed at least in triplicate in three animals of a given age and genotype. Immunohistochemical analyses were performed on postnatal day 8 (P8) or adult (>P21) rat cochleae.

Results

Amplification of GlyR and gephyrin cDNA in the mammalian cochlea

Using RT-PCR, GlyRα3 (512 bp), GlyR*β* (732 bp) and gephyrin (573 bp) transcripts were amplified from rat cochlea at P14 (Fig. 1, P14). *β*-Actin (655 bp) was used as

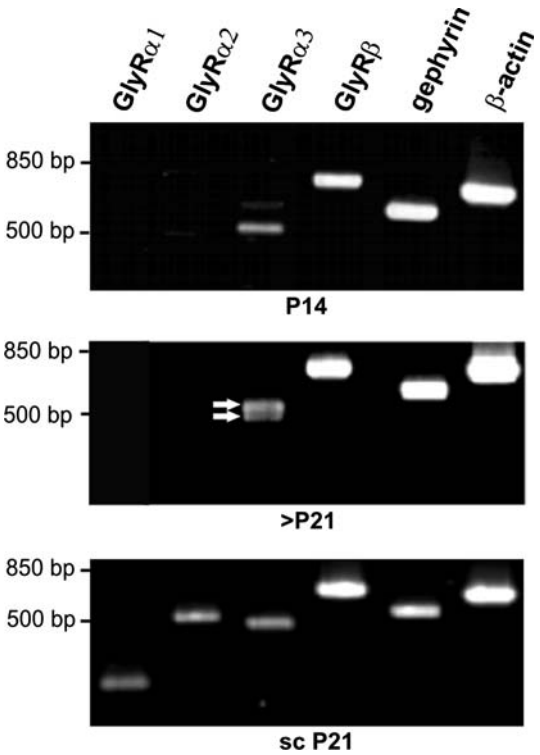

Fig. 1 Detection of GlyRα3, GlyR*β* and gephyrin transcripts in the rat cochlea. cDNA from rat cochleae at different postnatal stages (P14, >P21) was analyzed by RT-PCR. P14 and >P21: amplification of GlyRα3 (512 bp), GlyR*β* (732 bp) and gephyrin (573 bp) transcripts. Note the double band for GlyRα3 (467 bp/512 bp, indicated by *arrows*) in adult animals (>P21). GlyRα1 and GlyRα2 transcripts were not detected. sc P21: spinal cord from P21 animals was used as a positive control for GlyRα1–3, GlyR*β*, gephyrin and *β*-actin. *β*-Actin (655 bp) was used as a housekeeping gene

a housekeeping gene. GlyRα3 primers were designed to detect possible splice variants corresponding to the human *GLRA3* short (GlyRα3_K) and *GLRA3* long (GlyRα3_L) subunit isoforms. RT-PCR from adult cochlea (>P21) revealed a double band for GlyRα3 transcripts, indicated by arrows in Fig. 1 (>P21). GlyRα1 and α2 transcripts could not be amplified at any of the stages analyzed. Spinal cord cDNA (Fig. 1, sc P21) was used as a positive control for GlyRα1–3, GlyR*β*, gephyrin and *β*-actin.

The two PCR products of the GlyRα3 double band (Fig. 2a, arrows) were cloned into the pCRII-TOPO vector. Sequencing of the insert-PCR products revealed two GlyRα3 transcript variants (Fig. 2b). The longer GlyRα3 transcript variant consisting of 512 bp (Fig. 2c, *Glra3*_rn_L) showed 99% identity with the rat *Glra3* cDNA sequence (*Glra3*_rn) from GeneBank (accession number: NM_053724). The shorter transcript consisting of 467 bp (Fig. 2c, *Glra3*_rn_K) carried a 45 bp-deletion between nucleotides 1120 and 1164 of the coding sequence

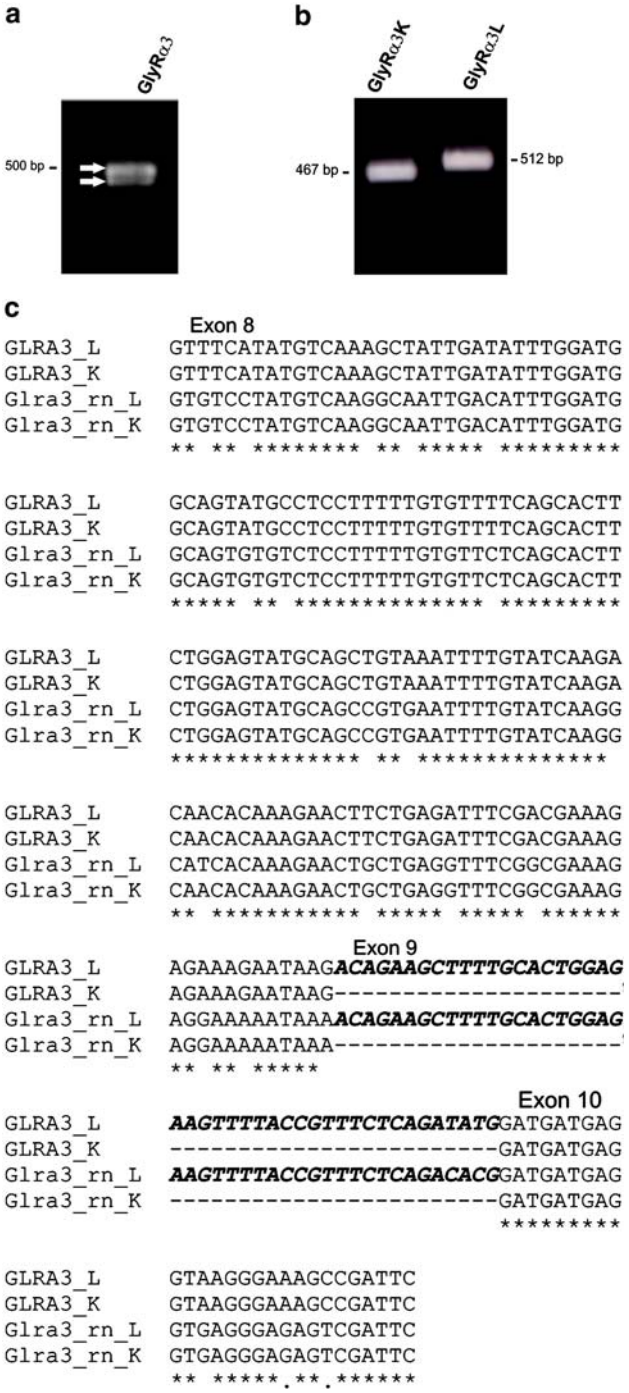

Fig. 2 Detection of GlyRα3_K and GlyRα3_L splice variants in the adult rat cochlea (>P21). **a** GlyRα3 transcripts were amplified from the adult rat cochlea by RT-PCR. Note the double band (indicated by *double arrow*). **b** GlyRα3_K (467 bp) and GlyRα3_L (512 bp) splice variants were detected by insert-PCR after cloning of PCR fragments into the pCRII-TOPO vector. **c** Alignment of long and short GlyRα3 human (*GLRA3*_L, *GLRA*_K) and rat (*Glra3*_rn_L, *Glra3*_rn_K) cDNA sequences (exon 8–10). Identical nucleotides in all four sequences are indicated by an *asterisk*. The 45 bp-stretch of exon 9 (in *bold italics*) is missing in the cDNA sequences of *GLRA3_K* and *Glra3_rn_K*

(Fig. 2c, in bold italics). Analysis of the rat *Glra3* and human *GLRA3* nucleotide sequences with the Ensembl automatic gene annotation system revealed highly conserved exon–intron borders. The 45 bp-deletion in *Glra3*_rn_K corresponds to the missing exon 9 (45 bp) of the human *GLRA3*_K cDNA sequence (Fig. 2c, exon 9). Thus, the two *Glra3* transcripts amplified from rat cochlea exhibit the features of the previously described human *GLRA3* short (α3_K) and long (α3_L) splice variants and are therefore referred to as GlyRα3_K and GlyRα3_L in the following text. Sequence data from the GlyR*β* and gephyrin PCR fragments confirmed identity of the amplified transcripts with the corresponding sequences from rat CNS in GeneBank (*Glrb*: NM_053296; *gephyrin*: NM_022865, data not shown). In summary, RT-PCR results indicate that GlyRα3, GlyR*β* and gephyrin transcripts are expressed in the rat cochlea. For the GlyRα3 subunit, two isoforms corresponding to the long and short human transcript variants were detected.

mRNA localization of glycine receptors and gephyrin by in situ hybridization

Aiming to gain insight into the localization of GlyR subunit and gephyrin mRNA in the rat cochlea, riboprobes directed against GlyRα3, GlyR*β* and gephyrin transcripts were produced and in situ hybridization was performed on adult (>P21) rat cochlea. As no sufficient signals were obtained on cryosections after decalcification (data not shown), whole mount in situ hybridization was employed. In Fig. 3, an overview of a single cochlear turn illustrates mRNA signals of GlyRα3 (Fig. 3a), GlyR*β* (Fig. 3c) and gephyrin transcripts (Fig. 3e) in spiral ganglia (SG). Higher magnification of the spiral ganglion neurons (boxes in Fig. 3a, c, e) indicated a cytoplasmic localization of GlyRα3 (Fig. 3b), GlyR*β* (Fig. 3d) and gephyrin (Fig. 3f) mRNA. The corresponding sense probes (Fig. 3b, d, f, insets) did not show any signal. In a next step, the area of hair cells was viewed with higher magnification in the adult rat cochlea (>P21). GlyRα3 (Fig. 4a), GlyR*β* (Fig. 4c) and gephyrin (Fig. 4e) transcripts were identified in OHCs (filled arrows). While gephyrin mRNA was also detected on the level of IHCs (Fig. 4e, open arrow), no hybridization signal was obtained for GlyRα3 and GlyR*β* mRNA (Fig. 4a, c, open arrow) in IHCs. The corresponding sense probes did not produce a signal (Fig. 4b, d, f).

Protein detection and localization of glycine receptors and gephyrin in the rat cochlea

We aimed to visualize GlyRα3 and gephyrin proteins in the organ of Corti and SG neurons using the monoclonal mAb4a antibody recognizing all GlyRα subunits, a GlyRα3-specific polyclonal antibody and a monoclonal antibody against the anchoring protein gephyrin. Beyond P10, rat cochleae must be decalcified before cutting cryosections. The available antibodies did not produce a sufficient signal on decalcified cryosections. Therefore, the lateral bony wall of the cochleae was removed before embedding and cryosectioning specimens >P21 in order to circumvent decalcification.

On the level of SG neurons, a dot-like staining pattern, which is typical of glycine receptors in CNS neurons (Friauf et al. 1997; Becker et al. 1991), was observed for GlyRα3 (Fig. 5a, open arrows) and gephyrin protein (Fig. 5b, open arrows) at >P21.

The localization of GlyRα3 and gephyrin protein in the organ of Corti before and after the onset of hearing is depicted in Fig. 6: prior to the onset of hearing, mAb4a, which is directed against a common N-terminal epitope of GlyRα1–4 subunits, detected weak dot-like signals characteristic for postsynaptic GlyRs at the base of IHCs, shown for the apical and midbasal cochlear turn in rat cryosections at P8 (Fig. 6a, b). At this age, no signal for the GlyRα protein could be observed on the level of OHCs (data not shown). After the onset of hearing, GlyRα/α3 protein was visualized with the monoclonal antibody mAb4a (Fig. 6c, green) and the GlyRα3-specific antibody (Fig. 6d, red) in the adult organ of Corti (>P21). For animals >P21 GlyR α/α3 clusters (Fig. 6c, d) were detected in the upper part of afferent fibers that project to IHCs (Fig. 6c, d, open arrowheads), but not within IHCs themselves (Fig. 6c, d, dotted outline). At this age, a dot-like staining for GlyRα and GlyRα3 protein was observed at the base of OHCs (Fig. 6c, d, closed arrowheads). Staining with a monoclonal antibody revealed a similar protein distribution for gephyrin (Fig. 6e, f, green) being expressed in the upper part of afferent fibers projecting to IHCs (Fig. 6e, open arrowheads) and at the base of OHCs (Fig. 6f, closed arrowheads) in cochleae >P21. In addition, a weak dot-like signal for gephyrin (Fig. 6e, open arrow) was detected in IHCs (dotted outline) at this age.

The localization of GlyRα3 protein in OHCs was further analyzed by whole mount immunohistochemistry (Fig. 7). A patchy signal for GlyRα3 polypeptide (red, asterisk) was detected in the three rows of OHCs (Fig. 7a, c, d, f, filled arrows). OHC nuclei were labeled with DAPI (Fig. 6b, c, e, f, blue) and the nerve fibers terminating at the OHCs were stained with an antibody against NF200 (Fig. 7a–f, green, filled arrowhead). Typical clusters of GlyRα3 protein were located to the cell membrane of OHCs (Fig. 7d, f). Omission of primary antibodies did not produce any signal (Fig. 5–7, data not shown).

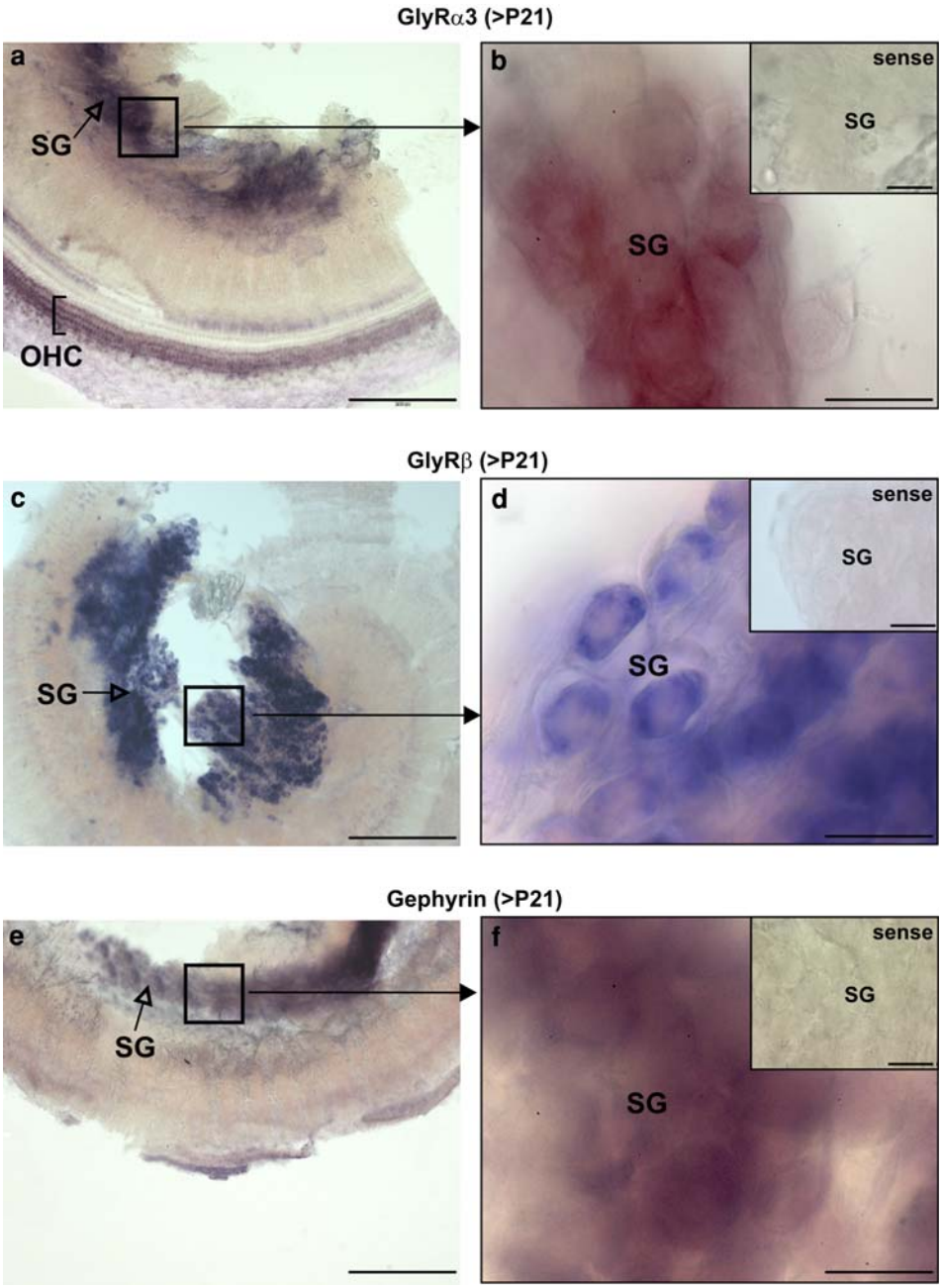

Fig. 3 GlyRα3, GlyRβ and gephyrin mRNA expression in spiral ganglion neurons of adult rat cochleae (>P21). The *boxes* in **a**, **c** and **e** indicate the areas, where **b**, **d** and **f** were taken from. **a**, **b** GlyRα3 mRNA expression in spiral ganglion neurons (*SG*, *open arrow*) at different magnifications using whole mount in situ hybridization of adult rat cochlea (>P21). The overview (**a**) displays also labeling of the outer hair cells (*OHC*, *bracket*). **c**, **d** GlyRβ mRNA expression in spiral ganglion neurons (*SG*, *open arrow*). **e**, **f** Gephyrin mRNA expression in spiral ganglion neurons (*SG*, *open arrow*). Higher magnifications indicate cytoplasmic staining of GlyRα3 mRNA (**b**), GlyRβ mRNA (**d**), and gephyrin mRNA (**f**) in SG. No signals were detected upon hybridization with the corresponding sense probes (*insets* in **b, d, f**). *Scale bars* in **a, c, e** 200 µm, in **b, d, f** 20 µm

Discussion

In the present study, we detected glycine receptors and gephyrin in the rat cochlea and analyzed their distribution by whole mount in situ hybridization and fluorescence immunohistochemistry.

Glycine receptor isoforms detected in the cochlea

The present study indicates an expression of GlyRα3, GlyRβ and gephyrin in the rat cochlea. In contrast, GlyRα1 and GlyRα2 transcripts were not detected at any postnatal or mature stage analyzed (see Fig. 1).

GlyRα3: the "sensory" subunit

Among the distinct GlyRα subunit variants, the role of the GlyRα3 subunit has long been elusive. GlyRα3 transcripts have been detected in the olfactory bulb and cerebellum (Malosio et al. 1991), the auditory brainstem (Sato et al. 2000) and the dorsal horn of the spinal cord in adult rodents (Harvey et al. 2004). Growing evidence for GlyRα3 expression in brain regions associated with sensory processing is indicative of a crucial role of GlyRα3 in sensory integration. This notion is supported by the detection of GlyRα3 in the dorsal horn of the spinal cord, where it has been identified as a key intermediate in the transmission of pain

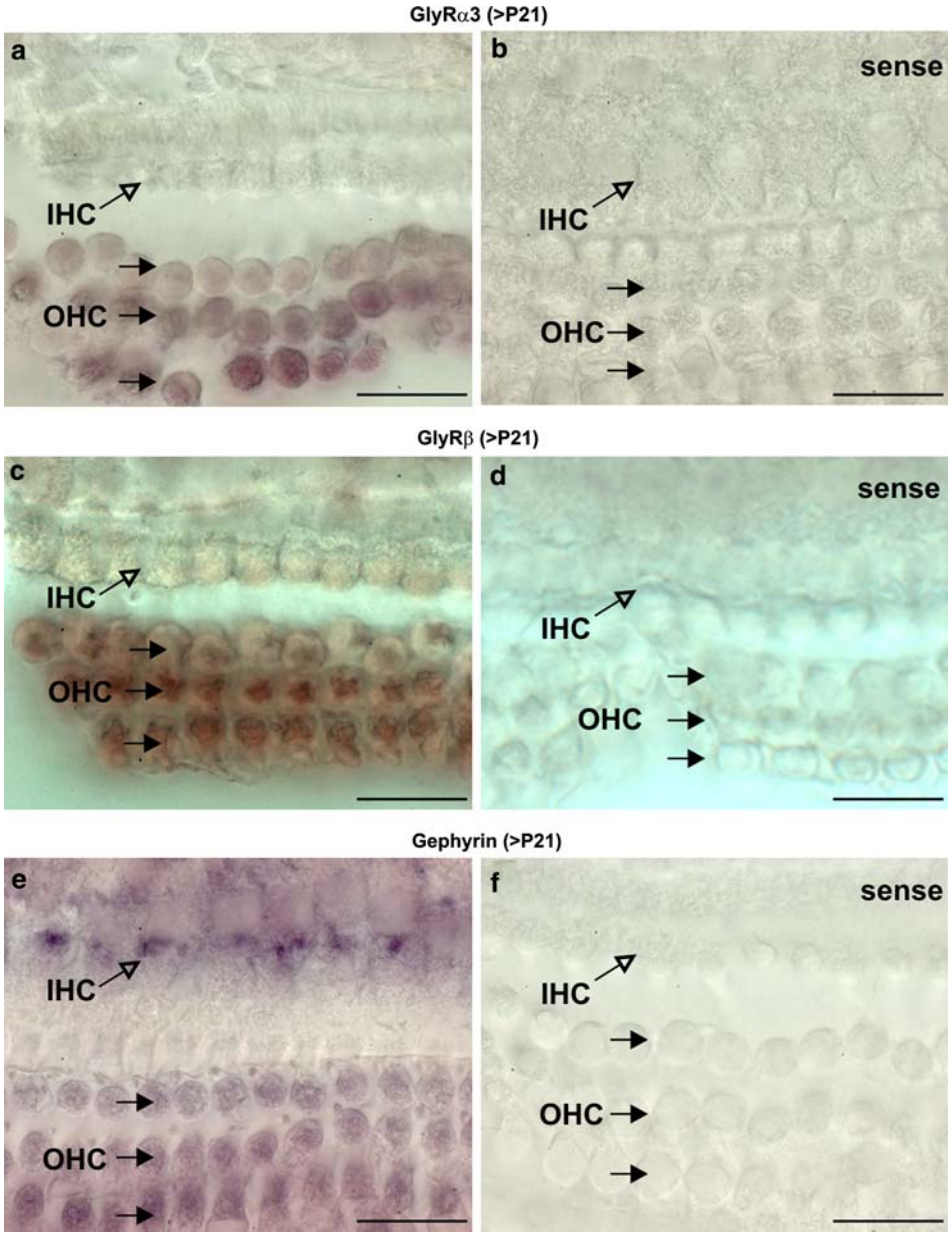

Fig. 4 GlyRα3, GlyR*β* and gephyrin mRNA expression in the adult organ of Corti (>P21). **a, c, e** antisense probes; **b, d, f** sense probes. **a** GlyRα3 transcripts were detected in outer hair cells (*OHC*, *filled arrows*) by whole mount in situ hybridization. No signal was obtained for IHCs (*open arrow*). **c** OHCs (*filled arrows*), but not IHCs (*open arrow*) revealed a signal for GlyR*β* transcripts in adult rat cochleae. **e** An intense hybridization signal for gephyrin was detected in IHCs (*open arrow*) and OHCs (*filled arrows*). **b, d, f** No signals were detected upon hybridization with the corresponding sense probes. *Scale bars* 20 µm

signals from the periphery to the brain (Harvey et al. 2004). The identification of GlyRα3 mRNA and protein in the rat cochlea further supports the concept of GlyRα3 as the "sensory" GlyRα subunit variant. To date, it is not understood, how the distinct kinetics of GlyRα3 relates to such a role. Recombinant GlyRα3 channels display fast kinetics, yet have a lower affinity for glycine than GlyRα1 channels (Kuhse et al. 1990).

We detected GlyRα3 splice variants corresponding to the human isoforms GlyRα3_K and GlyRα3_L in the rat cochlea (see Fig. 2). So far, alternative splicing of GlyRα3 mRNA has only been described in humans (Nikolic et al. 1998) and mice (Harvey et al. 2004). The short GlyRα3_K isoform lacks the 45 bp-stretch of exon 9, which corresponds to a loss of 15 amino acids in the channel segment. Recombinant ion channels of the two splice variants exhibit different channel kinetics. The long GlyRα3_L isoform displays a higher affinity for glycine and desensitizes more slowly and to a lesser extent than GlyRα3_K (Nikolic et al. 1998). Electrophysiological recordings from native glycine receptors in isolated hair cells and from recombinant cochlear GlyRα3_K and GlyRα3_L channels will help in the characterization of the channel properties of cochlear glycine receptor isoforms and in the understanding of their distinctive role for hearing.

GlyRβ and gephyrin

GlyR*β* and gephyrin transcripts were detected in the rat cochlea by RT-PCR and in situ hybridization. Gephyrin

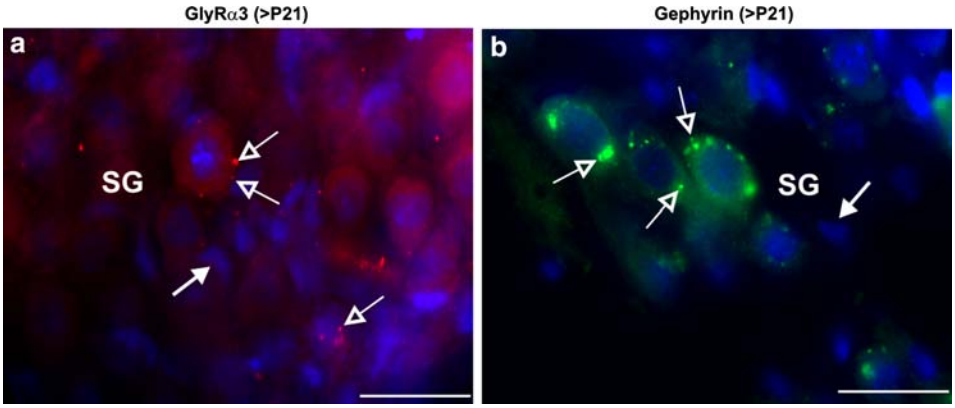

Fig. 5 Immunostaining of GlyRα3 and gephyrin protein in cryosections of SG neurons (>P21). **a** A dot-like staining pattern was observed for GlyRα3 protein (*open arrows*) in the cell membrane of SG neurons (*SG*, round nuclei) using the GlyRα3-specific polyclonal antibody. **b** Gephyrin protein (*open arrows*) was detected in SG neurons (*SG*) with the monoclonal antibody against gephyrin. **a**, **b** The focus was set on the cell surface of SG. The falciform nuclei of satellite cells (*closed arrows*) are also visible. Cell nuclei were counterstained with DAPI. *Scale bar*s: 50 μm

protein was also observed in immunohistochemical stainings.

To date, it is not known whether native GlyRα3 channels form α3 homopentamers or α3β heteropentamers. The equal distribution of GlyRα3, GlyRβ3 and gephyrin mRNA in OHCs and SG neurons documented here (Figs. 3, 4) supports the possibility of α3β heteropentamers in the rat cochlea. Further studies will be required to elucidate the molecular composition of cochlear glycine receptors. Presumably, gephyrin anchors the cochlear glycine receptors to the cytoskeleton via binding to the β subunit (Meyer et al. 1995) and is crucial for postsynaptic clustering of glycine receptors, as described for CNS and retina (Kneussel and Betz 2000). This is supported by the observation of characteristic GlyRα3 and gephyrin protein clusters below IHCs (Fig. 6c–e) and in OHCs (Fig. 6c, d, f) of the adult (>P21) rat cochlea, reminiscent of postsynaptic GlyR clusters in the cell membrane of CNS neurons (Friauf et al. 1997; Becker et al. 1991).

In IHCs of the mature inner ear however, only gephyrin mRNA was detected by whole mount in situ hybridization (Fig. 4c), whereas no signal for GlyRα3 (Fig. 4a) and GlyRβ mRNA (Fig. 4b) was seen. These findings were supported by immunohistochemical stainings, where only gephyrin (Fig. 6e), but not GlyRα3 protein (Fig. 6c, f) was observed in IHCs >P21. Expression of gephyrin in regions largely devoid of glycinergic synapses has already been described for rodent CNS and retina (Kneussel and Betz 2000). There is growing evidence for gephyrin being involved in postsynaptic clustering of $GABA_A$ receptors in these regions [for review see Kneussel and Betz (2000)]. While it is necessary to specify the expression of gephyrin transcripts and protein in IHCs in more detail, it is of extreme interest to consider a role of gephyrin as an anchor protein for IHC-specific ion channels other than glycine receptors.

Glycine receptors in the cochlea: target molecules of efferent innervation?

The distribution of GlyR and gephyrin mRNA and protein in the cochlea strongly suggests that the inhibitory glycine receptor is a target molecule of the efferent olivocochlear bundle.

Glycine receptors in OHCs

After the onset of hearing, nerve fibers of the MOC system contact the basolateral end of OHCs with axosomatic synapses (Simmons 2002). The MOC bundle works as a sound-evoked feedback loop, which reduces the contribution of OHCs to cochlear amplification and protects the inner ear against acoustic trauma (Guinan 1996). To date, ACh and GABA have been identified as inhibitory transmitters of the MOC system.

In the present study, GlyRα3, GlyRβ and gephyrin transcripts were localized to OHCs of adult animals (>P21) by whole mount in situ hybridization (see Fig. 4). Immunostaining of cryosections revealed GlyRα/α3 (Fig. 6c, d) and gephyrin protein (Fig. 6f) at the base of OHCs. Furthermore, GlyRα3 protein clusters were detected by whole mount immunohistochemistry in the cell membrane of all three rows of OHCs sparing the cytoplasm and the cell nucleus (Fig. 7). This pattern is characteristic for postsynaptic GlyR clusters in CNS neurons (Becker et al. 1991; Friauf et al. 1997). Taken together, the localization of GlyR clusters at the base of OHCs suggests that inhibitory glycine receptors may act as target molecules of the MOC

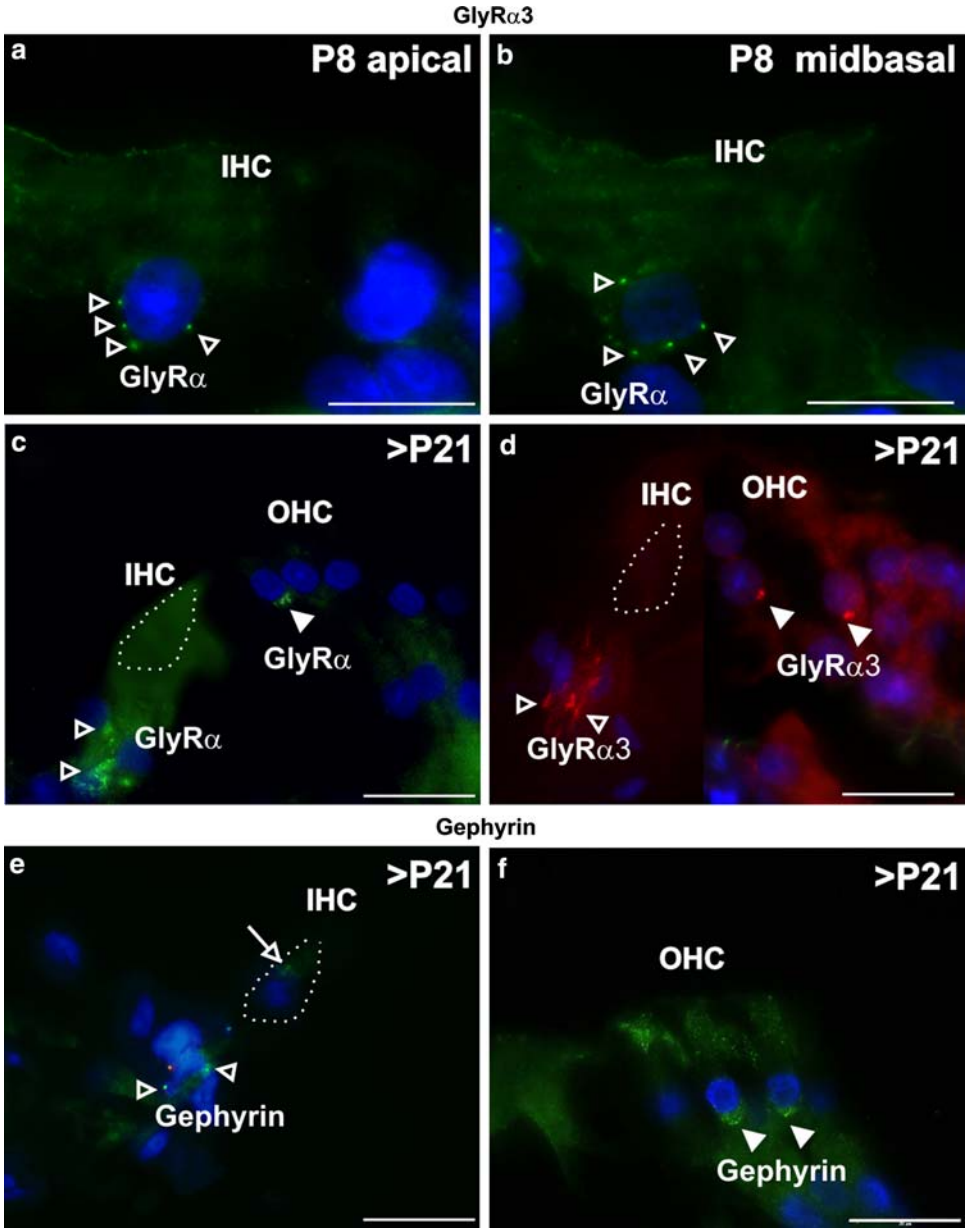

Fig. 6 GlyRα/GlyRα3 and gephyrin protein expression on the level of the organ of Corti. **a**, **b** Using the monoclonal antibody mAb4a, which detects all GlyRα subunits, GlyRα protein (*green*) was detected in cryosections of immature rat cochleae (P8) at the base of IHCs (*open arrowheads)*, shown for the apical as well as for the midbasal cochlear turn. **c** In the mature organ of Corti (>P21), a dot-like GlyRα protein staining (*green*, *open arrowheads*) was detected in the upper part of afferent fibers that project to the IHCs (*dotted line*) and at the base of OHCs (*green*, *closed arrowheads*) using the monoclonal antibody mAb4a. **d** Using the polyclonal GlyRα3-specific antibody, GlyRα3 protein (*red*, *open arrowheads*) was detected in adult rat cochleae (>P21) in the upper part of afferent fibers that project to the IHCs (*dotted line*) and at the base of OHCs (*red*, *closed arrowheads*). **e** Gephyrin protein (*green, open arrow*) was visualized in the IHCs (*dotted line*) and in the upper part of afferent fibers (*green*, *open arrowheads*) that project to the IHCs (*dotted line*). **f** Gephyrin protein was also detected at the base of OHCs (*closed arrowheads*). The specimens were counterstained with DAPI highlighting the cell nuclei (*blue*). **a**, **b** Cryosections, *scale bar*: 10 µm, **c**–**f** cryosections of non-decalcified tissue, *scale bar*: 20 µm

efferent system (Fig. 8, EF-MOC) and contribute to protection of the inner ear against acoustic overexposure. This is supported by the effects of strychnine, the competitive antagonist of the inhibitory glycine receptor. One of the prodromal symptoms in strychnine intoxication is hyperacusis (Becker 1992). It is not known, however, whether this phenomenon is due to central or peripheral disinhibition or both. Chronic local administration of strychnine to the inner ear of guinea pigs disrupts efferent activity (Dolan et al. 1999) and results in a permanent threshold shift for high frequencies after acoustic trauma (Yamasoba and Dolan 1997). Currently, these observations are attributed to strychnine acting as an antagonist on the α9 subunit of the nicotinic ACh receptor (nAChR) (Erostegui et al. 1994; Rothlin et al. 1999). It will be challenging to analyze these aspects in future studies, taking into consideration that GlyRs are expressed in OHCs.

Glycine receptors at the IHC synaptic complex

The LOC efferent system modulates auditory nerve excitability (Darrow et al. 2007) and balances interaural sensitivity (Darrow et al. 2006). ACh, GABA, dopamine and CGRP have been identified as transmitters of the LOC system so far (Simmons 2002; Maison et al. 2003). Around the onset of hearing (~P12 in rats), the axosomatic synaptic contacts of the MOC bundle with the IHCs are replaced by axodendritic synapses of the ipsilateral LOC bundle with the afferent auditory nerve terminal below the IHCs (Simmons et al. 1996). The switch from axosomatic to

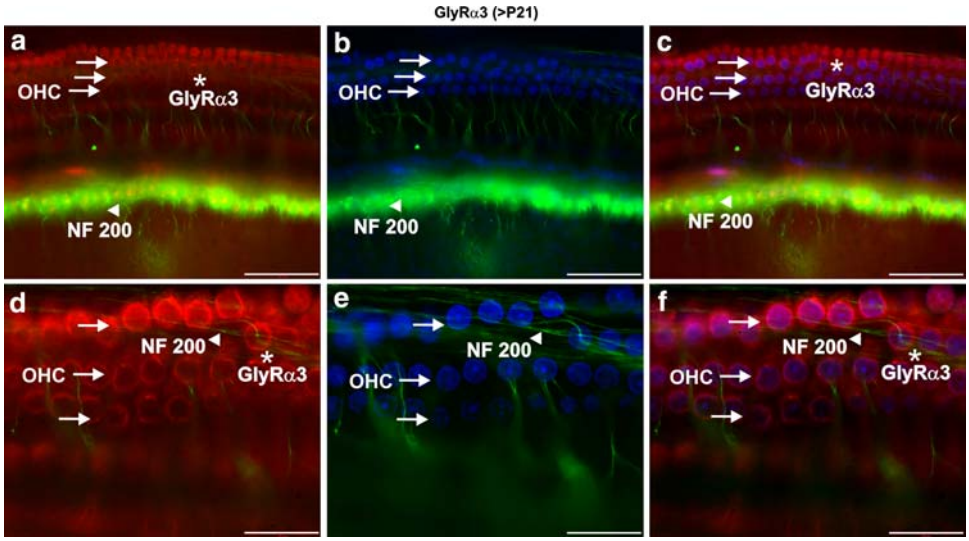

Fig. 7 GlyRα3 protein expression on the level of the OHCs in the adult organ of Corti (>P21). **a–c** GlyRα3 protein (*red*, *asterisk*) was detected in OHCs (*filled arrows*) by whole mount immunohistochemistry. Specimens were co-immunolabeled with anti-neurofilament 200 (NF200, *green*, *filled arrowhead*). **d–f** Higher magnifications of **a–c**. Note the localization of GlyRα3 protein (*red*, *asterisk*) in the cell membrane of OHCs (*filled arrows*). NF200-immunopositive nerve fibers (*green*, *filled arrowhead*) cross the tunnel of Corti terminating at the OHCs. Cell nuclei were counterstained with DAPI (*blue*). *Scale bars* in **a–c** 50 µm, in **d–f** 20 µm

axodendritic innervation of the IHC around the onset of hearing is mirrored by a change in the expression pattern of efferent receptor proteins/ion channels on the IHC level (Katz et al. 2004).

In this study, expression of GlyRα3 transcripts was observed in spiral ganglion neurons after the onset of hearing (Fig. 3a, b). No signal for GlyRα3 transcripts was detected within IHCs in adult animals (Fig. 4a). On the protein level, immunostaining of cryosections before the onset of hearing revealed the characteristic postsynaptic dot-like staining pattern for GlyRα clusters at the base of IHCs (Fig. 6a, b) in animals of P8. After the onset of hearing, GlyRα3 protein was no longer detected in IHCs, but in the upper part of afferent fibers projecting to IHCs (Fig. 6c, d) and in SG neurons (Fig. 5a).

Considering both mRNA and protein data, we conclude that GlyRα3 transcripts are transported from SG neurons to the afferent nerve terminals, where they form inhibitory glycine receptors, after the onset of hearing (Fig. 8, EF-LOC). The differential expression pattern of glycine receptors on the IHC level before and after the onset of hearing resembles the switch from axosomatic to axodendritic efferent innervation of the IHC. Therefore we suggest that GlyRs may act as target molecules of efferent innervation on the level of the afferent fibers under the IHC.

While further studies are required to specify the sub-cellular localization of GlyR protein, its presumed action as an additional inhibitory transmitter for an analogous modification of IHC signaling may be of striking clinical and scientific interest.

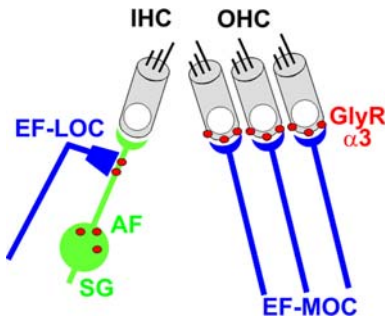

Fig. 8 Schematic diagram of the proposed glycinergic innervation of inner (*IHC*) and outer hair cells (*OHC*) after the onset of hearing. The efferent fibers of the LOC bundle (*EF-LOC*, *blue*) form axodendritic synapses with the afferent dendrites (*AF*, *green*) of the spiral ganglion neurons (*SG*) below the IHCs. GlyRα3 protein is expressed in SG neurons and afferent dendrites below the IHCs. Furthermore, GlyRα3 protein is detected at the basolateral end of OHCs (see Fig. 6), which are contacted by efferent fibers of the MOC bundle (*EF-MOC*, *blue*) via axosomatic synapses

Conclusion

In conclusion, GlyRα3, GlyRβ and gephyrin transcripts as well as GlyRα3 and gephyrin protein clusters were detected in the rat cochlea. The distinct localization of glycine receptors within the cochlea points to a possible modulatory role of glycinergic neurotransmission within the efferent olivocochlear system. Further immunohistochemical and electrophysiological studies will be necessary to elucidate the role of glycine receptors in inner ear signal transduction.

References

Becker CM (1992) Convulsants acting at the inhibitory glycine receptor. In: Herken H, Hucho F (eds) Handbook of experimental pharmacology. Selective neurotoxicity, vol 102. Springer, Berlin, pp 539–575

Becker CM, Hoch W, Betz H (1988) Glycine receptor heterogeneity in rat spinal cord during postnatal development. Embo J 7:3717–3726

Becker CM, Seitanidou T, Triller A (1991) Conservation of antigenic epitopes of the inhibitory glycine receptor in rodent and goldfish CNS. Brain Res Mol Brain Res 11:327–333

Betz H, Laube B (2006) Glycine receptors: recent insights into their structural organization and functional diversity. J Neurochem 97:1600–1610

Curwen V, Eyras E, Andrews TD, Clarke L, Mongin E, Searle SM, Clamp M (2004) The Ensembl automatic gene annotation system. Genome Res 14:942–950

Darrow KN, Maison SF, Liberman MC (2006) Cochlear efferent feedback balances interaural sensitivity. Nat Neurosci 9:1474–1476

Darrow KN, Maison SF, Liberman MC (2007) Selective removal of lateral olivocochlear efferents increases vulnerability to acute acoustic injury. J Neurophysiol 97:1775–1785

Dolan DF, Yamasoba T, Leonova E, Beyer LA, Raphael Y (1999) Morphological and physiological effects of long duration infusion of strychnine into the organ of Corti. J Neurocytol 28:197–206

Engel J, Braig C, Ruttiger L, Kuhn S, Zimmermann U, Blin N, Sausbier M, Kalbacher H, Munkner S, Rohbock K, Ruth P, Winter H, Knipper M (2006) Two classes of outer hair cells along the tonotopic axis of the cochlea. Neuroscience 143:837–849

Erostegui C, Norris CH, Bobbin RP (1994) In vitro pharmacologic characterization of a cholinergic receptor on outer hair cells. Hear Res 74:135–147

Evans EF, Zhao W (1993) Varieties of inhibition in the processing and control of processing in the mammalian cochlear nucleus. Prog Brain Res 97:117–126

Friauf E, Hammerschmidt B, Kirsch J (1997) Development of adult-type inhibitory glycine receptors in the central auditory system of rats. J Comp Neurol 385:117–134

Groff JA, Liberman MC (2003) Modulation of cochlear afferent response by the lateral olivocochlear system: activation via electrical stimulation of the inferior colliculus. J Neurophysiol 90:3178–3200

Grudzinska J, Schemm R, Haeger S, Nicke A, Schmalzing G, Betz H, Laube B (2005) The beta subunit determines the ligand binding properties of synaptic glycine receptors. Neuron 45:727–739

Guinan J (1996) Physiology of olivocochlear efferents. In: Dallos P, Popper N, Fay R (eds) The cochlea. Springer, New York, pp 435–502

Harvey RJ, Schmieden V, Von Holst A, Laube B, Rohrer H, Betz H (2000) Glycine receptors containing the alpha4 subunit in the embryonic sympathetic nervous system, spinal cord and male genital ridge. Eur J Neurosci 12:994–1001

Harvey RJ, Depner UB, Wassle H, Ahmadi S, Heindl C, Reinold H, Smart TG, Harvey K, Schutz B, Abo-Salem OM, Zimmer A, Poisbeau P, Welzl H, Wolfer DP, Betz H, Zeilhofer HU, Muller U (2004) GlyR alpha3: an essential target for spinal PGE2-mediated inflammatory pain sensitization. Science 304:884–887

Katz E, Elgoyhen AB, Gomez-Casati ME, Knipper M, Vetter DE, Fuchs PA, Glowatzki E (2004) Developmental regulation of nicotinic synapses on cochlear inner hair cells. J Neurosci 24:7814–7820

Kneussel M, Betz H (2000) Receptors, gephyrin and gephyrin-associated proteins: novel insights into the assembly of inhibitory postsynaptic membrane specializations. J Physiol 525(Pt 1):1–9

Knipper M, Bandtlow C, Gestwa L, Kopschall I, Rohbock K, Wiechers B, Zenner HP, Zimmermann U (1998) Thyroid hormone affects Schwann cell and oligodendrocyte gene expression at the glial transition zone of the VIIIth nerve prior to cochlea function. Development 125:3709–3718

Knipper M, Gestwa L, Ten Cate WJ, Lautermann J, Brugger H, Maier H, Zimmermann U, Rohbock K, Kopschall I, Wiechers B, Zenner HP (1999) Distinct thyroid hormone-dependent expression of TrKB and p75NGFR in nonneuronal cells during the critical TH-dependent period of the cochlea. J Neurobiol 38:338–356

Knipper M, Zinn C, Maier H, Praetorius M, Rohbock K, Kopschall I, Zimmermann U (2000) Thyroid hormone deficiency before the onset of hearing causes irreversible damage to peripheral and central auditory systems. J Neurophysiol 83:3101–3112

Koch U, Grothe B (1998) GABAergic and glycinergic inhibition sharpens tuning for frequency modulations in the inferior colliculus of the big brown bat. J Neurophysiol 80:71–82

Kuhse J, Schmieden V, Betz H (1990) Identification and functional expression of a novel ligand binding subunit of the inhibitory glycine receptor. J Biol Chem 265:22317–22320

Maison SF, Adams JC, Liberman MC (2003) Olivocochlear innervation in the mouse: immunocytochemical maps, crossed versus uncrossed contributions, and transmitter colocalization. J Comp Neurol 455:406–416

Malosio ML, Marqueze-Pouey B, Kuhse J, Betz H (1991) Widespread expression of glycine receptor subunit mRNAs in the adult and developing rat brain. Embo J 10:2401–2409

Meyer G, Kirsch J, Betz H, Langosch D (1995) Identification of a gephyrin binding motif on the glycine receptor beta subunit. Neuron 15:563–572

Moore MJ, Caspary DM (1983) Strychnine blocks binaural inhibition in lateral superior olivary neurons. J Neurosci 3:237–242

Nikolic Z, Laube B, Weber RG, Lichter P, Kioschis P, Poustka A, Mulhardt C, Becker CM (1998) The human glycine receptor subunit alpha3. Glra3 gene structure, chromosomal localization, and functional characterization of alternative transcripts. J Biol Chem 273:19708–19714

Pfeiffer F, Simler R, Grenningloh G, Betz H (1984) Monoclonal antibodies and peptide mapping reveal structural similarities between the subunits of the glycine receptor of rat spinal cord. Proc Natl Acad Sci USA 81:7224–7227

Puel JL, Ruel J, Guitton M, Wang J, Pujol R (2002) The inner hair cell synaptic complex: physiology, pharmacology and new therapeutic strategies. Audiol Neurootol 7:49–54

Rothlin CV, Katz E, Verbitsky M, Elgoyhen AB (1999) The alpha9 nicotinic acetylcholine receptor shares pharmacological properties with type A gamma-aminobutyric acid, glycine, and type 3 serotonin receptors. Mol Pharmacol 55:248–254

Ruttiger L, Sausbier M, Zimmermann U, Winter H, Braig C, Engel J, Knirsch M, Arntz C, Langer P, Hirt B, Muller M, Kopschall I, Pfister M, Munkner S, Rohbock K, Pfaff I, Rusch A, Ruth P, Knipper M (2004) Deletion of the Ca2+-activated potassium (BK) {alpha}-subunit but not the BK{beta}1-subunit leads to progressive hearing loss. Proc Natl Acad Sci USA 101:12922–12927

Sato K, Kuriyama H, Altschuler RA (2000) Expression of glycine receptor subunit mRNAs in the rat cochlear nucleus. Hear Res 144:47–52

Simmons DD (2002) Development of the inner ear efferent system across vertebrate species. J Neurobiol 53:228–250

Simmons DD, Mansdorf NB, Kim JH (1996) Olivocochlear innervation of inner and outer hair cells during postnatal maturation: evidence for a waiting period. J Comp Neurol 370:551–562

Yamasoba T, Dolan DF (1997) Chronic strychnine administration into the cochlea potentiates permanent threshold shift following noise exposure. Hear Res 112:13–20