INTRODUCTION

In the central nervous system, the neurotransmitter glutamate activates ion-channel associated (ionotropic) and G protein–coupled (metabotropic) glutamate receptors. The family of metabotropic glutamate receptors (mGluRs) contains mGluR1 and mGluR5 (group I), mGluR2 and mGluR3 (group II), and the group III receptors mGluR4, mGluR6, mGluR7, and mGluR8. Receptor diversity is further increased by alternative splicing and dimerization.
formation: A prominent region for alternative splicing is the intracellular C-terminals of the receptors, resulting, for example, in mGluR7a and mGluR7b isoforms that contain intracellular isoform-specific binding surfaces for regulatory proteins. In contrast, dimer formation is a general principle of G protein–coupled receptors, resulting in the formation of homo- and heterodimeric mGluRs with distinct functional properties.

Group I receptors are generally expressed at the postsynapse where they increase the release of neurotransmitters of the postsynaptic neuron. In contrast, group II receptors were found at both sides of the synaptic cleft and preferentially reduce neurotransmitter release. Except for mGluR6, group III mGluR types are localized at the presynaptic terminal of glutamatergic neurons where they function as autoreceptors. There, presynaptic autoreceptors limit the release of glutamate into the synaptic cleft, thereby potentially protecting sensory tissue, such as the cochlea, against noxious stimuli and excitotoxicity.

The mechanosensory neurons in the cochlea are divided in inner hair cells (IHCs) and outer hair cells (OHCs) that both release glutamate from a highly specialized presynaptic structure, termed ribbon synapse. While OHCs amplify both release glutamate from a highly specialized presynaptic terminals of cochlear fibers that inhibit efferent signaling onto IHCs. The expression and localization of group I mGluRs in the cochlea is well described (see eg. ). In contrast, just a few studies concentrated on group II and III receptors. Group II mGluRs were detected in efferent lateral olivocochlear fibers that inhibit efferent signaling onto IHCs. Polymorphisms that correlate with age-related hearing deficits or noise-induced hearing loss were described for the group III receptor mGluR7. A general expression of mGluR7 and mGluR8 was observed in IHCs and OHCs. Recently, we described in detail the localization of group II and group III mGluR types at the IHC ribbon synapse in cochlear wholemounts of gerbils and mice. While mGluR4 and mGluR8b were observed at the presynaptic terminal of IHC ribbon synapses, we found mGluR2 to be expressed at the postsynaptic site. MGLuR3, MGLuR6 and MGLuR8a could not be detected at IHC ribbon synapses.

Previously, two studies reported a role of mGluR7 in age-related and noise-induced hearing impairment as well as in the severity of tinnitus. However, a detailed investigation analyzing the exact synaptic position of mGluR7 in IHCs of the cochlea is missing. In addition, individual analysis of the two prominent C-terminal splice variants mGluR7a and mGluR7b was not performed. Both would be essential to understand the function of this receptor and to design new pharmacological concepts that might protect the cochlea against noxious stimuli and excitotoxicity. Here, we elucidated the localization of mGluR7a and mGluR7b at the IHC ribbon synapse in detail in cochlear wholemounts of the mouse, using newly generated and characterized immunosera. Our results describe the exact localization of potential drug targets for possible treatments of hearing impairment.

### 2 | MATERIALS AND METHODS

Experiments were carried out in accordance with the guidelines of the University Erlangen-Nürnberg and the Deutsche Forschungsgemeinschaft. The extraction of cochlea and retina was approved by the local authorities (Amt für Veterinärwesen der Stadt Erlangen, AZ TS-3/2021) and conducted in accordance with the European Communities Council Directive (2010/63/EU).

#### 2.1 | Generation of mGluR7b-specific antibodies

While antibodies recognizing mGluR7a are commercially available (Table 1), immunosera specific for mGluR7b were newly generated by Synaptic Systems (Göttingen, Germany). In brief, a synthetic peptide corresponding to the very C-terminal and isoform-specific amino acids 900

| Protein | Epitope | Dilution | Serum type | Reference |
|---------|---------|----------|------------|-----------|
| CtBP2   | mouse aa 361-445 | 1: 300   | Mouse monoclonal | BD Bioscience (612044) |
| Ribeye  | rat aa 95-207  | 1:5000   | Guinea pig polyclonal | Synaptic systems (192104) |
| mGluR4  | rat aa 834-912  | 1: 150   | Rabbit polyclonal | 57 |
| mGluR7a | human aa 900-912 | 1:1000   | Rabbit polyclonal | Sigma-Aldrich (07-239) |
| mGluR7b | human aa 900-922 | 1:1000   | Rabbit polyclonal | Made by synaptic systems |
| mGluR7b | human aa 900-922 | 1: 500   | Guinea pig polyclonal | Made by synaptic systems |
| mGluR8  | human aa 34-514  | 1: 50    | Mouse monoclonal | R&D systems (MAB5277) |
| PSD95   | mouse aa 64-247  | 1: 250   | Mouse monoclonal | Synaptic systems (124011) |

Note: Overview of primary antibodies used in this study. CtBP2 is identical to the C-terminal part (B-domain) of Ribeye. The epitope is characterized by species and amino acid (aa) region.
to 922 of mGluR7b (NCIPPVRKSVQKSVTWTIPPTV—identical between human, mouse, rat, bovine and other species) was coupled to keyhole limpet hemocyanin to enhance the immune response. New Zealand rabbits and Hartley guinea pigs were immunized subcutaneously with 1 mg peptide/animal using standard protocols. To purify mGluR7b-specific antibodies from the collected sera by affinity chromatography, the mGluR7b peptide was covalently attached to modified agarose beads (SulfoLink) via thioether bonds.

2.2 Validation of antibodies recognizing C-terminal sequences in mGluR7a or mGluR7b

The specificity of mGluR7a and mGluR7b antibodies used throughout this study was tested in cell culture (Figures 1A and S1). Human embryonic kidney cells (HEK-293 cells, ATCC no. CRL 1573) were grown in 6-well-plates at 37°C and 5% CO₂ on glass coverslips coated with poly(l-lysine) in minimum...
The synaptic localization of mGluR7a has been described in detail in the mammalian retina. Thus, we used this tissue to test if mGluR7a- and mGluR7b-specific antibodies would recognize the epitopes of endogenously expressed receptors. Following a previous protocol, vertical cryostat sections of adult mouse retina were incubated with mGluR7a- or mGluR7b-specific antibodies (Table 1). To identify synapses, sections were co-stained for the presynaptic marker protein Ribeye (Table 1). After washing, the secondary antibodies (AlexaTM 488, 568, or 647-conjugated anti-mouse, -rabbit or -guinea pig) were applied together with DAPI to stain cellular nuclei for 45 minutes at 25°C.

2.3 Immunocytochemistry with cochlear wholemount preparations

Mice of different ages (C57BL/6; P12 to 12 months) were obtained from the animal facility of our university. The preparation and immunostaining of cochlear wholemounts was described before. In brief, the cochlea were fixed in 4% paraformaldehyde (PFA) solved in PBS for 15 minutes and decalcified in 140 mM EDTA at 4°C for 18 hours (except P12). Prior to antibody staining, the cochlea were blocked in 5% goat serum and 0.3% Triton X-100 in PBS for 1 hours at 25°C. Primary antibodies directed against mGluR7a, mGluR7b, Ribeye, or PSD95 were diluted in 0.1% goat serum, 0.1% Triton X-100 in PBS as specified in Table 1 and applied overnight at 4°C. The binding sites of primary antibodies were visualized by the secondary antibodies goat anti-rabbit AlexaTM 488 or 568, goat anti-mouse AlexaTM 647, or goat anti-guinea pig AlexaTM 488 or 568 (1:500-1:1000; Thermo Fisher Scientific, Darmstadt, Germany). Stained wholemounts were mounted with Aqua Poly/Mount (Polysciences Inc., Warrington, PA). Fluorescent signals were detected by confocal microscopy as described in the next paragraph. In all stainings, we analyzed apical/middle turns of cochlear wholemounts prepared from 6- to 8-week old mice, except for the developmental studies presented in Figure 5D, E.

2.4 Data analysis

The antibody stainings of transfected HEK-293 cells, vertical cryostat sections of mouse retina and cochlear wholemounts were repeated between three and six times. For controls, only secondary antibodies were used which showed no detectable signals. Fluorescent signals were recorded using a 20× or 63× objective (1.4 oil, Apochromat, Zeiss, Oberkochen, Germany) using ZEN blue 2012 software and a Zeiss Axio Imager Z2, equipped with an ApoTome. Alternatively, signals were recorded...
using a Laser Scanning Microscope 710 (Zeiss) and the ZEN black 2012 software with corresponding imaging modules, a stack interval of 0.9 μm and a pinhole of 1 airy unit. Fluorescence profiles recorded in confocal images were measured with the ImageJ tool “Plot Profile” (ImageJ version 1.49 g; U.S. National Institutes of Health, Bethesda, Maryland, USA). Imaris 8 (Bitplane) was used to reconstruct 3D images from recorded z-stacks and the measurement of distances and angles between calculated centers of gravity. To localize specific cochlear frequency regions, each organ of corti was recorded in low magnification (20x) using tile scans and stitched together using ZEN blue 2012. Thereafter, cochlear frequency maps were created for each organ of corti using a freely available Image J plug-in from the Eaton-Peabody Laboratories and the previously published place-frequency map.26

To analyze the possible changes of mGluR7a or mGluR7b at IHC ribbon synapses along the tonotopic axis and during development (Figure 5), we counted the number of fluorescent signals representing Ribeye, PSD95, mGluR7a, or mGluR7b at IHCs using the graphic elements arrow tool implemented in the ZEN black 2012 software (Zeiss). The area containing IHCs was enlarged to reliably assign fluorescent signals manually with arrows that were counted by the software. All obtained values were normalized to the number of counted Ribeye puncta. The same software was also used to count superimposed and/or apposed signal pairs for the combinations Ribeye/PSD95, Ribeye/mGluR7a, Ribeye/mGluR7b, PSD95/mGluR7a, PSD95/mGluR7b, and mGluR7a/mGluR7b at Ribeye-positive IHC synapses of our stainings shown in Figures 2-4. In this case, arrows were placed manually at superimposed and/or apposed pairs of puncta that were counted by the software.

Figure panels were arranged in Adobe Photoshop (CS5; Adobe Systems Inc., San Jose, CA) and fluorescent signals were slightly contrast enhanced and enlarged, if needed for better visualization. Statistical significance was evaluated with the one-way ANOVA test using the Microcal Origin Software (Microcal Software, Northhampton, MA). Errors are expressed as ±SD.

3 | RESULTS

3.1 | Generation and validation of mGluR7a- and mGluR7b-specific antibodies

Previously, we detected the transcripts of mGluR7a and mGluR7b in the mouse cochlea.22 So far, the localization of both receptors at IHC ribbon synapses has not been studied in detail. The immunoserum originally used to describe the localization of mGluR7a at ribbon synapses of the retina24 is not available anymore. Recently, we identified an immunoserum directed against mGluR7a (Table 1) that was used before by others to stain this receptor in the retina.27 In addition, we obtained antibodies newly generated in rabbit or guinea pig recognizing the mGluR7b isoform (Table 1). Importantly, these antibodies bind to and thus label isoform-specific domains of mGluR7a or mGluR7b located at the intracellular side of the plasma membrane. This results in the maximal possible distance between potential pre- or postsynaptic signals, which is a prerequisite to reliably distinguish pre- from postsynaptic receptor localization in the following.

Throughout this study, all immunosera were used to stain receptor epitopes in cochlear wholemounts. Thus, we tested if the three mGluR7 immunosera mentioned above would bind to their antigens in HEK-293 cells and in tissue sections. While mGluR7a antibodies stained cells expressing mGluR7a, cells expressing the mGluR7b splice variant were not labeled (Figure 1A). Likewise, mGluR7b sera generated in rabbit or guinea pig specifically recognized the mGluR7b isoform, but not mGluR7a. Importantly, none of the three mGluR7 immunosera bound to other group III mGluRs (mGluR4, mGluR6, mGluR8a, or mGluR8b, Figure S1).

Next, we tested if the mGluR7 sera would bind to the native receptor epitope in neuronal tissue. Incubating vertical cryostat sections of the mouse retina for mGluR7a reproduced the originally described staining pattern for this receptor isoform24. Clearly visible are two prominent immunoreactive strata in the outer half of the inner plexiform layer (white arrowheads in Figure 1B) and a more diffuse staining toward the inner half of this layer. As reported previously, negligible mGluR7a signals could be detected in the outer plexiform layer. To our knowledge, the distribution of the mGluR7b variant was not analyzed in the retina before. Nevertheless, we tested both newly generated mGluR7b sera but could not detect any significant staining (data not shown). In contrast, both mGluR7b sera labeled structures at IHC ribbon synapses in cochlear wholemounts, as shown in Figures 3 and 4. From these data, we conclude that (i) all three mGluR7 sera tested do not cross-react with other group III mGluRs and (ii) that they recognize their native epitopes in neuronal tissue.

3.2 | Localization of mGluR7a at presynaptic specializations of inner hair cell (IHC) ribbon synapses

Next, we incubated cochlear wholemounts of adult mice with the validated mGluR7a immunoserum. To define the exact position of the receptors at IHC ribbon synapses, the tissue was co-stained with antibodies directed against the presynaptic marker Ribeye and the postsynaptic marker PSD95.
The analyses of fluorescent signals by confocal microscopy revealed a punctate pattern for mGluR7a, indicating a synaptic localization of this receptor in the cochlea (Figure 2B). Closer inspection revealed a perfect colocalization of the receptor with Ribeye (Figure 2C), while co-localization with PSD95 was only partial (Figure 2D). This difference in co-localization between pre- and postsynaptic markers is also evident in the fluorescent profiles that show the fluorescence intensities of the encircled synapses along the dashed arrows (lower panels of Figure 2C, D).

To analyze our data in more detail, we used black and white mirror images that show symmetrical patterns relative to a defined midline if the localization of two labels coincides in the cochlear wholemount (Figure S2). In these mirror images, all strong and well-defined mGluR7a clusters coincide with Ribeye signals. However, some mGluR7a/Ribeye-positive presynapses lack any detectable postsynaptic structure (arrowheads in Figure S2). In addition, we observed some faint and more fuzzy signals for mGluR7a that do not coincide with Ribeye (circles in Figure S2). To quantify these observations, we counted strong and clearly detectable pairs of signals for the combinations Ribeye/PSD95, Ribeye/mGluR7a, or PSD95/mGluR7a that were superimposed and/or apposed at individual synapses of our wholemount preparations. The highest number of fluorescent puncta were counted for Ribeye: of 566 Ribeye-positive synapses. Of these, 20 synapses lacked any detectable staining for mGluR7a (3.35%), while 58 showed no PSD95 label (10.25%). In addition, we observed four mGluR7a-positive puncta without detectable signals for Ribeye or PSD95 (0.73%). It needs to be determined if these mGluR7a puncta are indeed localized at other structures than IHC synapses, or if our observations are (at least partially) caused by technical limitations. Given the small number of four mGluR7a puncta not co-localized with Ribeye, we did not analyze this topic further.

Next, we 3D reconstructed the fluorescent signals from confocal images (Figure 2E). The centers of gravity were calculated for each color of individual triple-labeled synapses (Figure 2F, white dots), and distances and angles were measured. Our analysis revealed a mean distance between Ribeye (presynapse) and PSD95 (postsynapse) of 276 ± 74.2 nm (Figure 2G). This value is similar to the mean distance between mGluR7a and PSD95 (223 ± 76.7 nm). In contrast, the distance between mGluR7a and Ribeye is significantly shorter (90.1 ± 25.7 nm). A presynaptic localization of mGluR7a is also consistent with the measured angles between the centers of gravity that were larger for alpha (42.1 ± 23.3°) and beta (122 ± 28.5°), and smallest for gamma (15.4 ± 9.01°; Figure 2H). Figure 2I shows more examples of reconstructed synapses. Based on our data, we conclude that mGluR7a is localized at the presynapse of IHCs close to the synaptic ribbon.

3.3 MGlur7b is located presynaptically at IHC ribbon synapses

Since we observed a presynaptic localization of mGluR7a at IHC ribbon synapses, we also analyzed the distribution of the mGluR7b isoform. To this end, we incubated cochlear wholemounts of adult mice with the mGluR7b immunoserosum generated in rabbit, characterized in Figures 1A and S1. The tissue was co-labeled for Ribeye and PSD95, as before. Similar to the distribution of mGluR7a (Figure 2B), also mGluR7b showed a punctate staining pattern close to the presynaptic ribbon (Figure 3A). Higher magnifications of the fluorescent signals revealed a perfect superimposition between mGluR7b and Ribeye, while PSD95 was slightly displaced (Figure 3B, C). This can be best seen in the fluorescent profiles that compare fluorescence intensities of the two encircled synapses along the dashed arrows (lower panels of Figure 3C). As before, individual signals were compared in mirror images (Figure S3). There, most strong and well-defined mGluR7b labels coincide with Ribeye signals, while 1 mGluR7b/Ribeye-positive presynapse lacks PSD95 (arrowhead in Figure S3). Fainter mGluR7b signals that do not coincide with Ribeye are encircled (Figure S3). Again, we quantified all well-defined signal pairs for the combinations Ribeye/PSD95, Ribeye/mGluR7b, or PSD95/mGluR7b in our wholemount preparations. The highest number of fluorescent puncta was counted for Ribeye: of 295 synapses stained for Ribeye, four lacked mGluR7b staining (1.36%), while 16 synapses were not labeled for PSD95 (5.42%). We did not detect any strong and well-defined mGluR7b cluster without a Ribeye signal.

As shown in Figure 2, we reconstructed the obtained signals from confocal images (Figure 3D), calculated the centers of gravity for the individual colors (Figure 3E, white dots), and measured distances and angles. The shortest distance was calculated between mGluR7b and Ribeye (105 ± 30.1 nm), while distances between mGluR7b and PSD95 (202 ± 58.5 nm), or between Ribeye and PSD95 (282 ± 68.7 nm) were significantly longer (Figure 3F). The smallest angle was measured for gamma (15.5 ± 7.33°), while alpha (31.3 ± 15.5°) and beta (133 ± 20.4°) were significantly flatter (Figure 3G). More examples of reconstructed synapses are shown in Figure 3H. We conclude that mGluR7b is a presynaptic receptor at IHC ribbon synapses.

3.4 Both mGluR7 isoforms are co-localized at IHC ribbon synapses

Given that both mGluR isoforms were detected close to the presynaptic ribbon of IHCs, we next co-incubated cochlear wholemounts with immunoserosa specific for the two
receptor splice variants. To visualize presynaptic ribbons, wholemounts were co-stained with antibodies specific for CtBP2, which is identical to the C-terminal B-domain of Ribeye (13; Table 1). Confocal images showed a perfect superimposition of presynaptic ribbons with mGluR7a and mGluR7b (Figure 4A-D). The co-localization of both mGluR7 isoforms with presynaptic ribbons can be compared best in the intensity profiles of the two encircled synapses that is shown in the lower panels of Figure 4D along the dashed arrows.

As before, we aimed to analyze our stainings in more detail by 3D reconstructing the fluorescent signals. To co-stain both mGluR7 isoforms on the same wholemount, the mGluR7a immunoserum generated in rabbit that was already used in Figure 2 was applied together with a mGluR7b serum generated in guinea pig (Table 1). Unfortunately, unlike the rabbit mGluR7 sera used in Figures 2 and 3, this guinea pig serum repeatedly produced background signals that prevented a reliably 3D reconstruction of mGluR7b signals compared to background noise. To allow a more detailed analysis of our stainings—at least to some extent—we compared individual fluorescence in mirror images (Figure S4). Nearly all mGluR7a signals coincide with mGluR7b, and vice versa. The same holds true when comparing mGluR7a or mGluR7b signals with CtBP2, representing the localization of presynaptic ribbons. Faint mGluR7a signals that do not coincide with CtBP2 and/or mGluR7a are encircled in Figure S4. We also observed weak mGluR7b staining that lacks nearby mGluR7a and CtBP2 (arrowheads in Figure S4). As before, we quantified strong and well-defined signal pairs for the combinations CtBP2/mGluR7a, CtBP2/mGluR7b, or mGluR7a/mGluR7b. We counted 198 fluorescent puncta for CtBP2. Of these, 13 CtBP2 puncta were

FIGURE 3. mGluR7b is a presynaptic receptor at inner hair cell ribbon synapses. A, Confocal micrograph of a mouse cochlear wholemount that was stained with antibodies directed against mGluR7b (green), Ribeye (red), postsynaptic density protein 95 (PSD95) (magenta), and DAPI, as in Figure 2B. Enlargements of the boxed region show a perfect superimposition of mGluR7b with Ribeye (B), but not with PSD95 (C). To visualize the position of mGluR7b in respect of the pre- and postsynaptic marker proteins, fluorescent profiles were measured for the two encircled synapses in the lower panels (b’ + c’ or b” + c”), as described in Figure 2C, D, D, E, 3D reconstruction of fluorescent signals. Centers of gravity (white balls) were calculated and connected by white dashed lines, as in Figure 2F. F, G, Calculated mean distances and angles between the centers of gravity are compared in the bar diagrams, calculated from 93 synapses from three animals. Errors are ±SD, P-values < .00001 are indicated by three asterisks. H, More 3D reconstructed synapses labeled as in (D).
not superimposed with mGluR7a (6.57%) and 15 lacked mGluR7b (7.58%). Two synapses were labeled for CtBP2 and mGluR7a, but not for mGluR7b (1.08%). In addition, we observed three mGluR7a and 2 mGluR7b-positive puncta without associated signals for CtBP2 (mGluR7a: 1.60%; mGluR7b: 1.08%). Due to the small number of isolated mGluR7a and mGluR7b signals, we did not analyze their location further.

3.5 The presence of mGluR7a and mGluR7b at IHC ribbon synapses changes along the tonotopic axis and during development

Frequency is encoded by the position of the IHCs within the cochlea. Along this tonotopic axis, IHCs form varying numbers of ribbon synapses with less ribbons present in apical and basal relative to middle regions. Thus, we analyzed if the expression of mGluR7a and mGluR7b in respect of the number of synapses present along the tonotopic axis would change. To this end, we stained cochlear wholemounts of adult mice as in Figures 2 and 3, and counted the number of fluorescent puncta in three different regions representing apical, middle, and basal turns (Figure 5A). The number of PSD95 clusters was normalized by the number of Ribeye signals which yielded relations between 93% and 111% in the areas analyzed, ensuring the robustness of our experimental procedure (Figure 5B, C). Next, the number of mGluR7a or mGluR7b signals was normalized by the number of stained ribbons. Compared to the frequencies encoded in the apical and middle turns, we observed a significant reduction at high frequencies of about 28% for mGluR7a (Figure 5B), and of about 15% or 18% for mGluR7b (Figure 5C).

The expression of neurotransmitter receptors at the IHC ribbon synapse are regulated during development. Since it is unknown if these regulations involve mGluRs as well, here we analyzed the expression of mGluR7a and mGluR7b at IHC ribbon synapse in mice of different ages. Cochlear wholemounts were stained, and recorded fluorescent puncta were counted as described above. Again, for controlling our study design, we calculated the ratio between the synaptic markers, which resulted in ratios between 90% and 100% (Figure 5D, E). Comparing the ratio of mGluR7a signals in regard to stained ribbons showed a significant reduction of receptors between P12 and 6 months of about 18% and between P12 and 12 months of about 17%. Furthermore, between 8 weeks and 12 months we found a reduction of about 14% (Figure 5D). For mGluR7b, we observed a significant reduction between P12 and 8 weeks compared to 6 and 12 months of about 20% to 23% (Figure 5E).
FIGURE 5  The number of mGluR7a and mGluR7b clusters at inner hair cell (IHC) ribbons is reduced at higher frequencies and in older animals. A, Sketch of the cochlea indicating the approximate position of analyzed frequency regions at apical, middle, and basal turns. Red dots symbolize the ribbon synapses of IHC along the tonotopic axis. Cochlear wholemounts were co-stained for mGluR7a, Ribeye, and postsynaptic density protein 95 (PSD95) (B), or for mGluR7b, Ribeye, and PSD95 (C), as in Figures 2 and 3. Fluorescent signals were recorded and counted in the regions indicated in (A). To normalize our stainings, all values were divided by the numbers of counted Ribeye clusters. At all three regions of all wholemounts analyzed, the ratio between the two synaptic markers, Ribeye and PSD95, was calculated to be around 100% (93% to 111%), indicating that we can reliably stain, record, and count fluorescent signals along the tonotopic axis. Analyzing the ratio between mGluR7a-positive puncta and stained ribbons showed a significant reduction at high frequencies in respect of apical and middle turns by about 28% (B). A slightly lower reduction at high frequencies was observed for mGluR7b versus apical (15%) and versus middle regions (18%); C. For each receptor, we analyzed three or four animals and counted 394 ribbons (mGluR7a) and 480 ribbons (mGluR7b) at 4-8 kHz, 645 ribbons (mGluR7a) and 483 ribbons (mGluR7b) at 12-32 kHz, and 456 ribbons (mGluR7a) and 322 ribbons (mGluR7b). D, E, Cochlear wholemounts of adult mice of different ages were co-stained with antibodies as in Figures 2 and 3 and fluorescent signals were analyzed in apical/middle turns. As in (B, C) the ratio between synaptic markers remained about constant at all ages analyzed (90% to 100%). The ratio between mGluR7a specific puncta and stained ribbons was significantly reduced in animals of 6 and 12 months by about 14% to 18% (mGluR7a, D), or by about 20% to 23% (mGluR7b, E). Numbers of animals and stained ribbons analyzed for mGluR7a were: four animals at P12-921 ribbons, three animals at 8 weeks-565 ribbons, four animals at 6 months-1244 ribbons, and three animals at 12 months-393 ribbons. For mGluR7b, we analyzed four animals for each age and counted 376 ribbons (P12), 454 ribbons (8 weeks), 342 ribbons (6 months), 421 ribbons (12 months). Errors are ±SD, P-values < .05 or .005 are indicated by one or two asterisks, respectively.
4 | DISCUSSION

This study describes a co-localization of the C-terminal isoforms mGluR7a and mGluR7b at presynaptic specializations of IHC ribbon synapses in the cochlea. The World Health Organization counts 466 million people (about 6% of the world’s population) who suffer from different forms of hearing impairment (www.who.int/health-topics/hearing-loss). Hearing impairment is often preceded by neurodegeneration in the cochlea, which can result from excessive glutamate concentration in the synaptic cleft, for example at the IHC ribbon synapse. Indeed, it has been suggested that the primary cause of synapse loss during noise and aging in the cochlea is a consequence of glutamate overexposure, a concept that was phrased “glutamate excitotoxicity.” Presynaptically, localized mGluR types are well suited to protect glutamatergic neurons from glutamate excitotoxicity, since they can couple to inhibitory signal pathways that limit presynaptic glutamate release into the synaptic cleft.9-12

The presynaptic localization of mGluR7a and mGluR7b at IHC ribbon synapses observed in this study is consistent with previous data that report a preferential presynaptic localization of both receptor isoforms in various brain regions, although in some instances a postsynaptic mGluR7a was observed. At presynapses of glutamatergic neurons, the receptors are suggested to function as autoreceptors that regulate the glutamate concentration in the synaptic cleft and thus can protect neurons from noxious stimuli, glutamate excitotoxicity, and cell death.9-12 This idea is supported by the relative low affinity of the mGluR7 agonist binding site to glutamate close to the millimolar range, which enables the receptor to serve as a low-pass filter in neurotransmission, functioning as synaptic block for excessive glutamate release.37 Based on the described presynaptic localization of mGluR7a and mGluR7b at IHC ribbon synapses in this study, and since the N-terminal agonist binding site between both mGluR7 isoforms is identical, we propose a similar function for these receptors in the cochlea.

At low and middle frequencies and in younger animals, about every IHC ribbon synapse expresses mGluR7a and mGluR7b. In contrast, at high frequencies, or in 6- and 12-month-old animals, we observed a significant amount of ribbon synapses that were not stained for mGluR7a and mGluR7b. Interestingly, since more than 10 years, mGluR7 is the only mGluR type clearly associated with age-related or noise-induced hearing deficits.17-20 The observed absence of mGluR7 isoforms at some IHC ribbon synapses encoding high frequencies and in older animals might offer a molecular explanation. Sound waves first enter the base of the cochlea via the oval window, where the higher frequencies are detected. Thereafter, the traveling wave proceeds along the cochlea to middle and apical regions, where middle and lower frequencies are represented. As a consequence, the entire traveling wave always passes through the basal region, even by lower frequencies that are not encoded there. This in turn results in a constant deflection of stereocilia of IHCs located at the basal region, as long as any sound is present. As a consequence, stereocilia of high-frequency encoding IHCs are constantly oscillating, which is not the case for IHCs encoding lower frequencies. Thus, IHCs located near the base of the cochlea might be more susceptible for stress factors such as noise trauma or aging than other IHCs along the tonotopic axis. As explained in the previous paragraph, based on the observed presynaptic localization of mGluR7a and mGluR7b, we suggest a protective role for the receptors at IHC synapses. Thus, it is tempting to speculate that high-frequency encoding IHCs are more susceptible for alterations in mGluR7 expression than IHCs encoding lower frequencies. Our speculation is supported by a recent study by Yu et al showing that a single nucleotide polymorphisms in the mGluR7 gene is that is likely to upregulate receptor expression results in individuals that are less susceptible to noise-induced hearing loss.18

mGluRs were described as “constitutive dimers,” and this dimerization seems to be a prerequisite for proper function.4,6,38 The observed co-localization of mGluR7a and mGluR7b at IHC ribbon synapses offers the possibility of heterodimer formation between both mGluR7 isoforms with distinct functional properties. To our knowledge, a physiological relevance of homo- versus heterodimer formation (e.g., different ligand affinity, G-protein coupling, cellular trafficking) has not been investigated for mGluR7 isoforms. We found just one recent study reporting that mGluR2/mGluR7 heterodimers showed a cooperative effect resulting in a faster kinetics combined with a higher glutamate affinity and efficacy, when compared to mGluR2 homodimers in HEK-293 cells. Recently, molecular structures of the mGluR2/mGluR7 were solved that might explain the above described physiological findings.7

Distinct properties of homo- versus heteromeric receptors might be caused by regulatory proteins that selectively interact with the isoform-specific intracellular C-terminals of mGluR7a and mGluR7b. Indeed, different binding profiles for intracellular proteins were described for the two mGluR7 isoforms. For example, the glutamate receptor-interacting protein GRIP1, protein kinases A or C interact with mGluR7a, but not with mGluR7b.39-41 Vice versa, filamin-A and protein phosphatase 1 bind only to the C-terminus of mGluR7b, but not to mGluR7a.42,43 The functions of most of the abovementioned protein interactions were not investigated, yet. However, recently, it has been shown that the surface expression of mGluR7b
is regulated by protein phosphatase 1 in primary cortical neurons.\textsuperscript{44} Besides receptor trafficking, interacting proteins can regulate other receptor characteristics in neurons, for example agonist sensitivity, receptor desensitization, and the activity of G protein–coupled signal pathways. Therefore, it is possible that mGluR7a/mGluR7b heterodimers might be regulated differently compared to their homodimeric counterparts.

In a previous study, we described a presynaptic localization of mGluR4 and mGluR8b at IHC ribbon synapses.\textsuperscript{22} As mGluR7a and mGluR7b, also mGluR4 and mGluR8b belong to group III of the mGluR family classification. Previously, it was shown that receptors of this group form heterodimers among each other.\textsuperscript{6} Thus, in principle, mGluR7a and mGluR7b could form heterodimers with mGluR4 and mGluR8b, increasing the diversity of mGluR signaling at IHC ribbon synapses even further. Higher order heteromers were reported for group I G protein–coupled receptors, including receptors for adenosine, dopamine, and endocannabinoids. For example, the adenosine receptors A1 and A2A were described as "rhombus-shaped heterotetramer" formed by a dimer of A1 and A2A dimers.\textsuperscript{45} Recently, tetramers composed of adenosine A2A receptor and the dopamine D2 receptor, or of the adenosine A2A receptor and the cannabinoid CB1 receptor were reported.\textsuperscript{46,47} In contrast to the above described examples, mGluRs were not observed to form higher order heteromers using time-resolved FRET techniques in cell culture.\textsuperscript{6} Nevertheless, given the presence of mGluR4, mGluR7a, mGluR7b, and mGluR8b at IHC ribbon synapses (\textsuperscript{22} and this study), in principle a formation of heterotetramers seems to be possible. Obviously, more studies are needed to elucidate the composition and function of possible mGluR dimers and/or tetramers at IHC ribbon synapses.

Ribbon synapses are not only present in hair cells of the cochlea, but also formed by neurons in the retina.\textsuperscript{13} There, presynaptic endings of cone and rod photoreceptors and of cone and rod connecting bipolar cell types contain this synapse type.\textsuperscript{48} Ribbon synapses of the sensory neurons in both sensory tissues (IHCs and photoreceptors) contain mGluR8a or mGluR8b.\textsuperscript{22,49,50} Here, we identified mGluR7a and mGluR7b to be present at ribbon synapses of IHCs, while these receptors were not detected in photoreceptors.\textsuperscript{24} Instead, in the retina, mGluR7a was described at ribbon synapses of cone connecting bipolar cells (\textsuperscript{\textsuperscript{24}}and Figure 1C of this study).

The synaptic ribbon divides the presynaptic terminal into two symmetrical halves. Bipolar cells of the retina contact two postsynaptic elements that are opposed to these two presynaptic halves. There, mGluR7a was detected in pre- or postsynaptic membranes on just one side of the synaptic ribbon, indicating a distinct glutamatergic signal transduction between the bipolar cell presynapse and the two postsynaptic structures.\textsuperscript{24} In contrast to bipolar cells, IHC ribbon synapses contact one postsynaptic structure of spiral ganglion neurons, only.\textsuperscript{14} Our 3D reconstructions of confocal images suggest that both mGluR7 splice variants are localized in the presynaptic membrane close to the synaptic ribbon. Indeed, in our 3D reconstructions, the largest numbers were calculated for the angle beta (mGluR7a-122° and mGluR7b-133°), being consistent with a location of the receptors in the presynaptic membrane between the ribbon and the postsynaptic density. Indeed, given the relative low affinity of the mGluR7 agonist binding site close to the millimolar range, it was proposed that this glutamate receptor might function best if located close to the release site of glutamate.\textsuperscript{4}

The 2D representation of our co-stainings has some limitations. This is most evident in Figure 3C, where signals for mGluR7b (green) and PSD95 (magenta) show various degrees of overlap, resulting in white. At first glance, this would imply a localization of mGluR7b at or close to postsynaptic structures. However, one has to keep in mind that our panels show 2D representations 3D synapses. Depending on the orientation of a given synapse in space, pre- and postsynaptic structures might be located one in front of the other. Therefore, depending on the localization of both labels relative to the observer, any degree of overlap is possible. To circumvent this limitation, we performed 3D reconstructions which enabled us to measure appropriate distances in space.

In addition to visualize recorded fluorescent signals in 2D overlays and 3D reconstructions (Figures 2-4), individual colors are compared in b/w mirror images in the supplementary Figures S2-S4. This presentation shows symmetrical patterns relative to a vertical midline if the localization of two fluorescent colors coincides in the cochlear wholemount. In these mirror images, it becomes evident that some recorded signals for mGluR7a and mGluR7b do not coincide with pre- and/or postsynaptic markers (please see encircled signals in Figures S2-S4). We observed that signals present at IHC synapses are generally strong and well-defined. In contrast, most signals highlighted in the supplementary figures that do not coincide with other colors are rather faint and diffuse. The following explanations seem possible: The observation that most of these signals lack a nearby presynaptic ribbon might indicate that other neurons than IHCs express mGluR7 isoforms, suggesting multiple functions for these receptors in the cochlea. Alternatively, because most of these mGluR7a or mGluR7b signals have a rather fuzzy and faint appearance, they could be localized at synapses that are slightly out of focus and therefore, nearby pre- and/or postsynaptic markers might not have been recorded.
However, some signals recorded in our wholemount preparations could simply originate from a certain degree of unspecific binding of the antibodies.

In addition to age-related hearing deficits and noise-induced hearing loss in humans, several neurodevelopmental disorders are associated with single nucleotide polymorphisms and mutations in the GRM7 gene, including autism spectrum disorders, decreased muscle tone, leukodystrophies, and seizures.51 Moreover, mGluR7-deficient mice showed alterations in associative learning, depression, epilepsy, fear responses, motor function, social behavior, and sleep.52-55 One recent publication speculates that the reported alterations in fear responses could originate from possible hearing deficits in mGluR7 ko mice.55 However, to date, hearing impairments were not explicitly studied in mGluR7-deficient mice. Given the described function of mGluR7 as a low-pass filter in glutamatergic neurotransmission in the central nervous system,37 mGluR7a and mGluR7b might not be essential to transmit acoustic information to higher brain areas. Rather, these receptors might modulate long-term properties of IHCs, for example synaptic sensitivity. Because mGluR7 isoforms can invert the exciting effect of the neurotransmitter glutamate into neuronal inhibition via intracellular G protein-coupled signal cascades, the presynaptic localization of mGluR7a and mGluR7b is well suited to protect IHC ribbon synapses from overstimulation and excitotoxic effects.

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CONFLICT OF INTEREST
Both authors confirm that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS
L. Klotz prepared and stained cochlear wholemounts, transfected and stained HEK-293 cells, and performed microscopy analysis, 3D reconstructions, and quantifications, as well as data evaluation and interpretation. R. Enz designed experiments, evaluated and interpreted the data, and wrote the paper. Both authors read, revised, and approved the manuscript.

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SUPPORTING INFORMATION
Additional Supporting Information may be found online in the Supporting Information section.

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