Chapter 15

Detection of Excitatory and Inhibitory Synapses in the Auditory System Using Fluorescence Immunohistochemistry and High-Resolution Fluorescence Microscopy

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Abstract

In sensory systems, a balanced excitatory and inhibitory circuit along the ascending pathway is not only important for the establishment of topographically ordered connections from the periphery to the cortex but also for temporal precision of signal processing. The accomplishment of spatial and temporal cortical resolution in the central nervous system is a process that is likely initiated by the first sensory experiences that drive a period of increased intracortical inhibition. In the auditory system, the time of first sensory experience is also the period in which a reorganization of cochlear efferent and afferent fibers occurs leading to the mature innervation of inner and outer hair cells. This mature hair cell innervation is the basis of accurate sound processing along the ascending pathway up to the auditory cortex. We describe here, a protocol for detecting excitatory and inhibitory marker proteins along the ascending auditory pathway, which could be a useful tool for detecting changes in auditory signal processing during various forms of hearing disorders. Our protocol uses fluorescence immunohistochemistry in combination with high-resolution fluorescence microscopy in cochlear and brain tissue.

Key words Protein, Antibody, Immunohistochemistry, Fluorescence, Microscopy

1 Introduction

In the visual, somatosensory, olfactory, and auditory system, detection and identification of environmental stimuli depends on the developmental induction of intracortical inhibition (for review *see* [1]). Intracortical inhibition is partly induced by brain-derived neurotrophic factor (BDNF), which is released from pyramidal neurons and initiates the formation of inhibitory contacts of parvalbumin-expressing interneurons (basket cells), onto the somata of excitatory neurons (for review *see* [2–5]). This sharpening of spatial and temporal cortical resolution is likely initiated by the first

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sensory experience, a commonality among sensory organs. In the auditory system, sensory input starts with hearing onset, which is around postnatal day 12 in rodents [6]. At this age, functionally relevant maturation steps occur in the cochlea, including the maturation of cholinergic and dopaminergic feedback from the olivary complex to inner and outer hair cells (IHCs, OHCs). In the immature state, spontaneous Ca^{2+} action potentials (APs) of IHCs are modified by olivocochlear efferent fibers, projecting from the medial portion of the superior olivary complex to the cochlea (for review *see* [7]). They remain in axosomatic contact with IHCs, until they are eliminated and IHCs are innervated mainly by afferent fibers, having few, if any, remaining efferent contacts [8]. One major modulator of these developmental changes in the organ of Corti is thyroid hormone (TH). TH drives the termination of Ca^{2+} APs, including the elimination of cholinergic axosomatic efferent contacts [9]. The mature cochlear afferent fibers are innervated by efferent axodendritic input, which can suppress or facilitate sound-evoked and spontaneous afferent activity through the release of inhibitory (GABA, glycine, dopamine) or excitatory (acetylcholine) transmitters [10–16]. Another TH dependent step is the upregulation of otoferlin, a C2-domain protein essential for IHC exocytosis by acting as a Ca^{2+} sensor in vesicle fusion [17–20]. The presynaptic vesicles of the IHC are tethered to so-called ribbons [21–24]. The presynaptic ribbon's role is proposed to support the continuous release of a high number of vesicles [25–27], enabling an extremely high rate of exocytosis through maintenance of a large readily releasable pool. This ensures the coding of sound intensity in postsynaptic neurons over a wide dynamic range [28, 29] and enables precise sound processing along the ascending auditory pathway [30]. For this reason, peripheral cochlear changes in signal transmission can lead to central compensation mechanisms, which might be related to an imbalance of excitation and inhibition, resulting in changes of central auditory plasticity (for review *see* [31–34]). These changes can be analyzed at the molecular level by studying the expression of marker proteins for excitatory or inhibitory neurons (e.g. glutamate transporters, parvalbumin) and markers for plasticity changes (e.g. activity-regulated cytoskeletal protein Arg3.1/arc).

We describe here our methodology of fluorescence immunohistochemistry including preparation of cochlear and brain tissue to visualize different marker proteins in the auditory system that can give insight into excitatory and inhibitory processes along the ascending auditory pathway [1, 32, 35]. At the level of the IHC, as shown in Fig. 1, the analyses of presynaptic ribbon structures (Fig. 1b, c) in combination with the postsynaptic marker parvalbumin (PV) (Fig. 1b), which labels postsynaptic swellings [36, 37], might give insight into a possible loss of synaptic contacts that are associated with hearing disorders [36]. Analyzing IHC ribbon

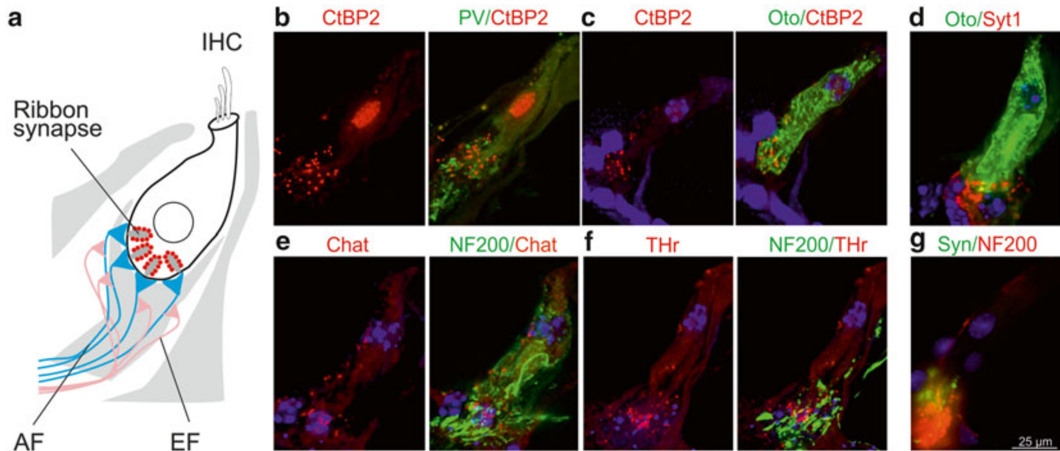

Fig. 1 Immunohistochemistry of the mouse inner ear. All images show inner hair cells (IHCs). (**a**) Cartoon of the IHC, including ribbon synapses (*red dots*) and afferent (AF, *blue*) and efferent (EF, *pink*) innervation. The supporting cells are delineated in *grey*. (**b**) CtBP2/RIBEYE immunostaining (*red*) labels presynaptic structures in the IHC, while parvalbumin staining (PV, *green*) labels postsynaptic regions. (**c**) CtBP2/RIBEYE immunostaining (*red*) labels presynaptic structures in the IHC, whereas staining for otoferlin (Oto, *green*) labels the IHC. (**d**) Immunostaining for otoferlin (Oto, *green*) labels the IHC and staining for synaptotagmin 1 (*red*) labels a presynaptic marker protein. (**e**) Immunostaining for NF200 (*green*) labels afferent fibers, while staining for cholinacetlytransferase (Chat, *red*) labels mature cholinergic efferent contacts. (**f**) Immunostaining for NF200 (*green*) labels afferent fibers and staining for tyrosinhydoxylase (THr, *red*) labels mature dopaminergic contacts. (**g**) Immunostaining for synaptophysin (Syn, *green*) labels efferent fibers, whereas staining NF200 (*red*) labels afferent fibers. Cell nuclei are counterstained with DAPI, scale bar = 25 μm

structures in combination with otoferlin (Fig. 1c), which serves as a Ca^{2+} sensor for synaptic vesicle release at hair cell ribbon synapses [17, 18, 38], might give insight into the functionality of presynaptic structures [39]. Changes in postsynaptic structures (e.g., a reduced number of afferent fibers) can indicate alterations in hearing function. Therefore, neurofilament NF200 (Fig. 1e, f), a marker for afferent fibers [36], can be studied in combination with cholinacetyltransferase (Chat) (Fig. 1e), a marker for mature cholinergic input [7], tyrosinhydroxylase (THr) (Fig. 1f), a marker for dopaminergic nerve terminals [10], or synaptophysin (Fig. 1g), a marker for efferent fibers [40]. Alterations in these markers might point to changed cholinergic and dopaminergic feedback from the olivary complex or to developmental deficits which could lead to changed signal transmission [41].

In higher order auditory centers, such as the inferior colliculus (IC) (Fig. 2) or the auditory cortex (AC) (Fig. 3), neuronal markers for excitation and inhibition can be used to analyze probable imbalances of excitation and inhibition occurring, for example, during hearing disorders. In the IC, we show in Fig. 2a the combination of glutamate-decarboxylase (GAD67), a marker for

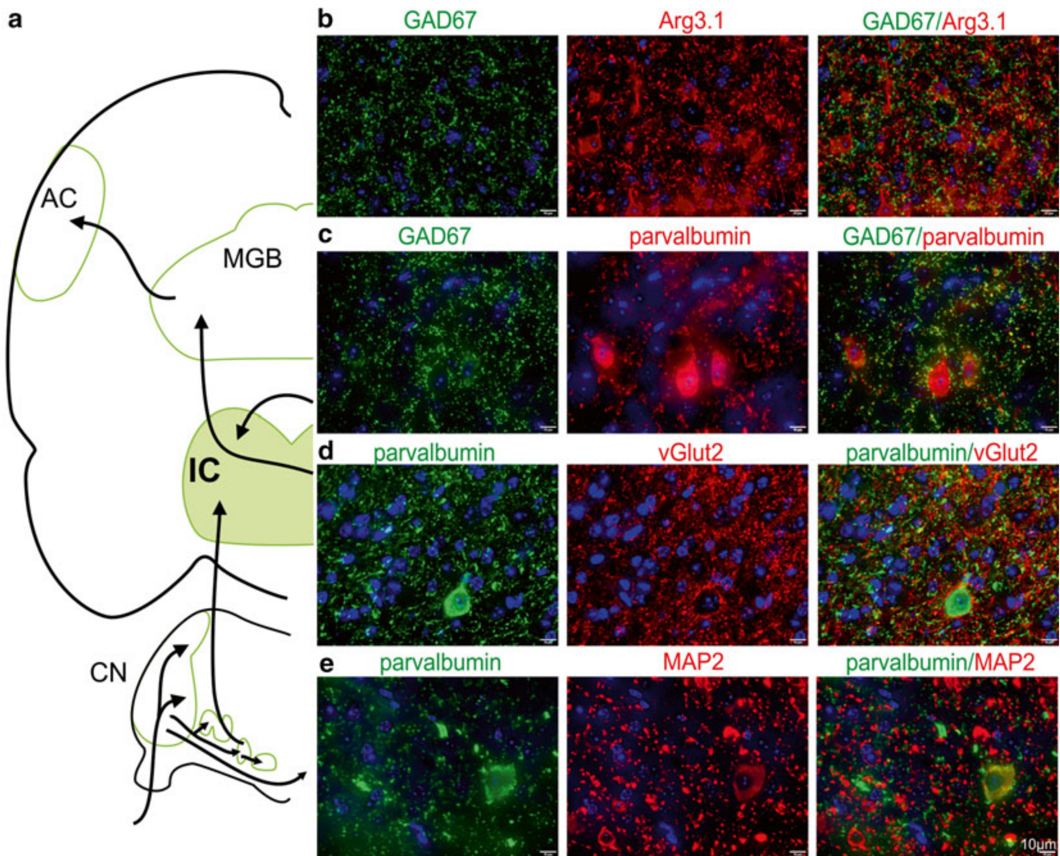

Fig. 2 Immunohistochemistry of the mouse inferior colliculus. (**a**) Cartoon of the ascending auditory pathway (*black arrows*) showing the cochlear nucleus (CN), the inferior colliculus (IC), the medial geniculate body (MGB), and the auditory cortex (AC). The IC is highlighted in *green*. (**b**) Immunostaining for GAD67 (*green*) labels GABAergic inhibitory neurons, whereas staining Arg3.1/arc (*red*) labels excitatory glutamatergic neurons. (**c**) Immunostaining for GAD67 (*green*) labels GABAergic inhibitory neurons, while staining parvalbumin (*red*) labels inhibitory interneurons. (**d**) Immunostaining for parvalbumin (*green*) labels inhibitory interneurons and vGlut2 (*red*) staining localizes to excitatory axon terminals. (**e**) Immunostaining for parvalbumin (*green*) labels inhibitory interneurons, whereas MAP2 (*red*) staining labels a neuronal marker protein. Cell nuclei are counter-stained with DAPI. Scale bar = 10 μm

inhibitory neurons [42], in combination with the activity regulated gene Arg3.1/arc, a marker for excitatory neurons [43] that was correlated with changes in central auditory activity [44–47]. GAD67 can also be co-stained with other inhibitory marker proteins, such as PV, shown in the IC (Fig. 2b) and AC (Fig. 3a), which is a marker for inhibitory interneurons [48] and is associated with perisomatic inhibition [49] and plasticity [50]. Another possibility is the costaining of GAD67 (Fig. 3c) with vGAT (Fig. 3c), a marker for GABAergic nerve endings [51].

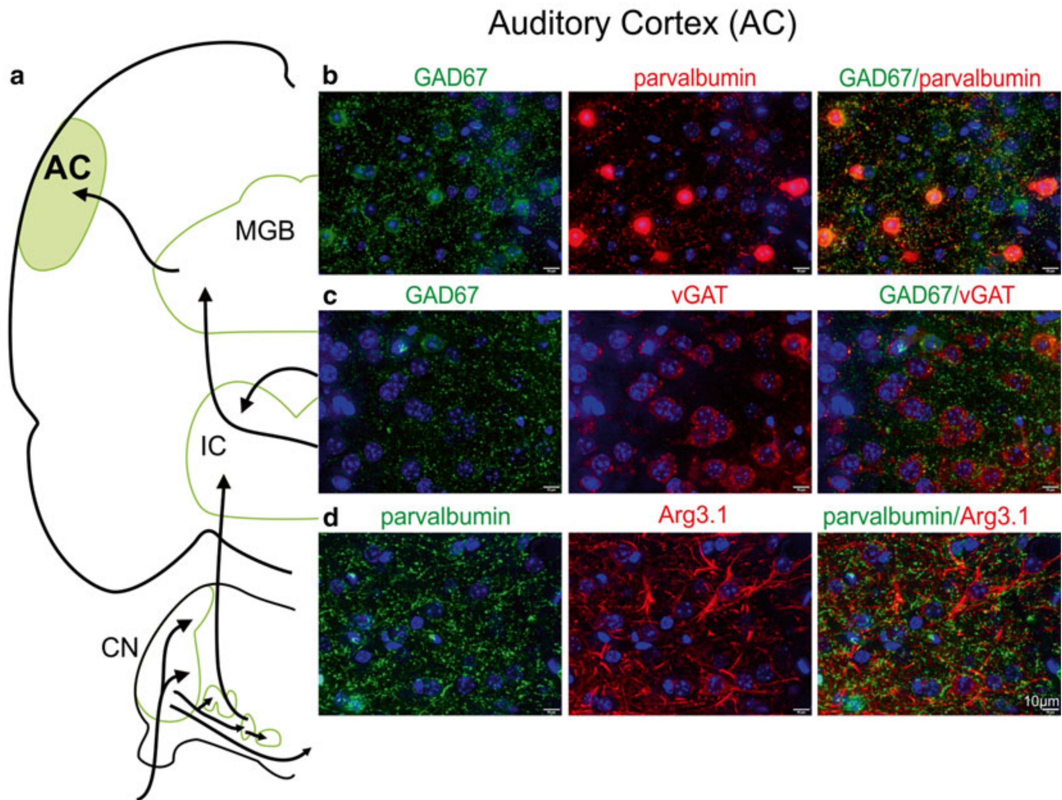

Fig. 3 Immunohistochemistry of the mouse auditory cortex. (**a**) Cartoon of the ascending auditory pathway (*black arrows*), showing the cochlear nucleus (CN), the inferior colliculus (IC), the medial geniculate body (MGB), and the auditory cortex (AC). The AC is highlighted in *green*. (**b**) Immunostaining for GAD67 (*green*) labels GABAergic inhibitory neurons, while parvalbumin (*red*) staining labels inhibitory interneurons. (**c**) Immunostaining for GAD67 (*green*) labels GABAergic inhibitory neurons, while vGAT (*red*) staining labels GABAergic nerve endings. (**d**) Immunostaining for GAD67 (*green*) labels GABAergic inhibitory neurons and Arg3.1/arc (*red*) staining labels excitatory glutamatergic neurons. Cell nuclei were counterstained with DAPI, scale bar = 10 μm

PV (Fig. 2d) can be combined with markers for glutamatergic excitatory neurons such as vGlut2 (Fig. 2d), which is localized in excitatory axon terminals [52], and either Arg3.1/arc (Fig. 3c) or the neuronal marker protein MAP2 (Fig. 2d) [53]. All of these antibody combinations can illustrate alterations in excitation or inhibition on different cellular levels that in turn are likely associated with changes in signal transmission along the ascending auditory pathway. The pictures shown in Figs. 1, 2, and 3 show examples of antibody combinations in different tissues along the ascending auditory pathway. Readers may of course feel free to use other antibodies, to study other proteins and tissues of interest.

2 Materials

Prepare all solutions using ultrapure water, which is prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C. Prepare and store all reagents at room temperature (RT) (unless otherwise indicated). Diligently follow all waste disposal regulations when disposing waste materials. All solutions should be autoclaved unless otherwise indicated. In addition, make sure that all experiments involving animals are in accordance with relevant guidelines and regulations.

2.1 Tissue Preparation (See Note 1)

All protocols using animals should be approved by the institutional animal care and use committee.

1. Tools for tissue preparation: Sharp scissors for decapitation, stable forceps to remove the skull, bended forceps to remove the whole brain, actuated tweezers to open the bulla and remove the cochlea.
2. Binocular microscope to visualize removing the cochlea from the bulla.

2.2 Tissue Fixation

1. 10× Phosphate-Buffered Saline (PBS): Mix 0.5 M Na_2HPO_4 ($2H_2O$), 1.5 M NaCl in 900 mL ultrapure water and adjust to pH 7.0 with HCl, then top-off to 1 L. For 1× PBS dilute 10× PBS in water.
2. 2 %/4 % Paraformaldehyde (PFA): Mix 20 g/40 g PFA in 900 mL 1× PBS. Leave at 54 °C in a water bath until the powder is completely dissolved (*see* **Note 2**). Adjust the volume to 1 L with 1× PBS, prepare 50 mL aliquots and store at −20 °C. Do not autoclave.
3. Rotating mixer.

2.3 Embedding Solution for the Cochleae

1. RDO: Rapid decalcifier (e.g., Apex Engineering Product Corporation, Aurora, IL).
2. 25 % Sucrose-Hanks solution: Dilute 10× Hanks' balanced salt solution (HBSS), which is stored at 4 °C, with ddH_2O, to a 1:10 solution. Add 250 g sucrose to 1 L of 1× HBSS , and store in aliquots of 15 or 50 mL at −20 °C. Do not autoclave.
3. Optimal Cutting Temperature (O.C.T.) compound.

2.4 Embedding Solutions for Brain Tissue

1. 1× PBS.
2. 4 % Agarose: Mix 4 g agarose in 100 mL 1× PBS + 0.4 % PFA (*see* **Note 3**). Heat agarose in microwave until it melts (~55 °C). Prepare fresh every time and do not autoclave.
3. Cryoprotectant: Mix 150 g of sucrose in 200 mL 1× PBS and 150 mL Ethylene glycol. Adjust the volume to 500 mL with 1× PBS. Store at −20 °C.

4. Vibratome to section tissue (e.g., Leica VT 1000S (Leica Biosystems, Wetzlar, Germany)).

2.5 Immunhisto-chemistry

2.5.1 Reagents for Both Cochlea and Brain Immunohistochemistry

1. Washing buffer for all specimens: 1× PBS.
2. Primary antibodies (*see* **Note 4**).
3. Secondary antibodies: Use a fluorescence-conjugated antibody against the species that generated the primary antibody. Dilute the secondary antibody in the same buffer as the primary antibody.
4. Size four art paintbrush.
5. Glass cuvettes for washing or transferring brain sections to microscope slides (*see* Subheading 3.3, **step 10**).
6. In the present description, we used a Cy3-conjugated anti-rabbit antibody (Jackson Immuno Research, West Grove, PA) diluted 1:1500 and an Alexa488-conjugated anti-mouse antibody (Life Technologies, Darmstadt, Germany) diluted 1:500. Store according to the manufacturer's instructions.

2.5.2 Reagents Only for Immunohistochemistry on Cochlear Tissue

1. Permeabilization buffer for cochlear sections: 10 mL 1× PBS, 50 μL Triton-X 100. Prepare 1× PBS + 0.5 % Triton. Store at 4 °C.
2. Blocking solution for cochlear sections: 0.4 g Normal Goat Serum (NGS), 10 mL 1× PBS. Prepare 4 % NGS (*see* **Note 5**).
3. Reaction buffer for antibody dilution on cochlear sections: Prepare by mixing 2 g (2 %) NaCl, 100 μL Triton X-100, 1 mL NGS in 100 mL 1× PBS (*see* **Note 6**).

2.5.3 Reagents Only for Immunohistochemistry on Brain Sections

1. Blocking and permeabilization solution: Prepare by mixing 0.3 g bovine serum albumin (BSA) and 20 μL Triton-X 100 in 10 mL of 1× PBS (*see* **Note 7**). Store at 4 °C.
2. Buffer for antibody dilution: Prepare BSA in a range of 0.5–1.5 % in 10 mL of 1× PBS and 10 μL Triton-X 100 (*see* **Note 8**).
3. Mounting and nuclear staining (can be used on all different types of tissue sections): Vectashield mounting medium with or without DAPI (Vector Laboratories, Burlingame, CA, USA) (*see* **Note 9**).

3 Methods

Carry out all steps at room temperature (RT) unless otherwise indicated.

3.1 Tissue Preparation (See Note 1)

1. After decapitation open the skull along the lateral ridges (*see* **Note 10**), remove the complete brain in one piece after cutting the cranial nerves and transfer immediately to 50 mL of 2 % or 4 % PFA for mice and rats, respectively. If you only use brain tissue, *see* Subheading 3.1, **step 8**.

2. Take the residual skull and cut it in half. Use a dissecting microscope to remove the cochlea. Use actuated tweezers, to open the bulla and remove the cochlea by breaking out the attached part of the cochlea to the temporal bone (*see* **Note 11**).
3. Transfer the cochleae to 2 % PFA (*see* **Note 12**) and fix for 2 h at 4 °C on a rotating wheel.
4. Before decalcification remove the PFA and rinse the tissue in ddH_2O. Transfer the tissue to filtered R.D.O. (*see* **Note 13**) until the tissue is soft (*see* **Note 14**).
5. Transfer the decalcified cochlea to Sucrose-Hank's solution and keep rotating the tissue at 4 °C overnight.
6. Embed the cochleae in Tissue-Tek, freeze and store at −80 °C (*see* **Note 15**).
7. Cochleae are cut in 10 μm sections using a Cryostat (Leica Cryostat 1720 Digital Leica, Wetzlar, Germany) and transferred to microscope slides (Superfrost Plus). Store slides at −20 °C until use.
8. Fix brain tissue for 48 h at 4 °C on a shaker. After 24 h exchange the PFA.
9. Take the tissue out of the PFA and rinse with ddH_2O.
10. Embed the tissue in 4 % agarose (*see* **Note 16**) and store in 0.4 % PFA in 1× PBS at 4 °C.
11. Brains are sectioned at 40–60 μm on a vibratome (e.g., VT1000S; Leica, Wetzlar, Germany).
12. Slices are stored in a 24-well plate in Cryoprotectant at −20 °C.

3.2 Immunhistochemistry for Cochlear Tissue (See Note 17)

1. Take the microscope slides with the cochlear sections out of the −20 °C freezer and allow them to thaw at RT for ~30 min.
2. Add 1 mL of permeabilization buffer to each slide and incubate for 10 min.
3. Rinse the slides with 1× PBS.
4. Pipette 100 μL of blocking solution on each slide and incubate for 30 min in a humidified chamber (*see* **Note 18**).
5. Dilute the primary antibodies in reaction buffer to the desired concentration (*see* **Note 4**).
6. After removing the reaction buffer apply 100 μL of antibody solution to each slide. Incubate at 4 °C overnight in a humidified chamber.
7. Rinse slides with 1× PBS.
8. Wash 3× for 30 min in 1× PBS using a glass cuvette.
9. Dilute secondary antibody in reaction buffer.

10. Take the slides out of the glass cuvette, apply 100 μL of secondary antibody to each slide, and incubate in a humidified chamber for 1 h at RT.
11. Rinse the slides with 1× PBS.
12. Wash 3× for 30 min in 1× PBS using a glass cuvette.
13. Mount the tissue slices using Vectashield mounting medium with or without DAPI (*see* **Note 9**).

3.3 Immunhistochemistry for Free-Floating Brain Slices (See Notes 19 and 20)

1. Take the slices out of the cryosolution using a paintbrush and transfer to a fresh 24-well plate containing 1× PBS.
2. Wash 2× for 15 min in 1× PBS (*see* **Note 21**).
3. Permeabilization and blocking step: After removing PBS, incubate slices for 30 min in 3 % BSA containing 0.2 % Triton-X 100.
4. Dilute primary antibodies in 0.5–1.5 % BSA, containing 0.1 % Triton-X 100, to the desired concentration (*see* **Notes 4** and **8**).
5. After removing the permeabilization/blocking buffer, cover the sections with antibody solution and incubate at 4 °C overnight in a humidified chamber (*see* **Note 22**).
6. After removing the antibody solution, wash the slices 3× for 15 min in 1× PBS.
7. Dilute secondary antibodies in 0.5–1.5 % BSA, containing 0.1 % Triton-X 100, to the desired concentration (*see* **Note 8**).
8. After removing PBS, cover sections with antibody solution and incubate for 1 h at RT in a humidified chamber (*see* **Note 22**).
9. After removing the antibody solution, wash 3× for 15 min in 1× PBS.
10. To transfer the brain sections onto microscope slides, place them one at a time in the glass cuvette filled with water and use a paintbrush to transfer them to microscope slides.
11. Mount the slices with Vectashield mounting medium with or without DAPI (*see* **Note 9**)

3.4 Microscopy

1. Sections shown here were viewed using an Olympus BX61 microscope (Olympus, Center Valley, PA, USA) equipped with an X-Cite Lamp (Olympus). Images were acquired using an Olympus XM10 CCD monochrome camera and analyzed with cellSens Dimension software (OSIS).
2. To increase spatial resolution, slices were imaged over a distance of 10–60 μm (*see* **Note 23**) in steps of 0.49 μm within an image-stack along the *z*-axis (*z*-stack) followed by 3-dimen-

sional deconvolution, using a cellSens Dimension built-in algorithm. Typically *z*-stacks consisted of 5–30 layers (*see* **Note 23**), for each layer one image was acquired per fluorochrome (*see* **Note 24**).

4 Notes

1. Animal care procedures and treatments were performed in accordance with institutional and national guidelines, following approval by the University of Tübingen, Veterinary Care Unit, and the Animal Care and Ethics Committee of the regional board of the Federal State Government of Baden-Württemberg, Germany.
2. Shake the bottles in between to help the powder to dissolve. Work under a hood as the PFA powder is toxic.
3. The amount of agarose depends on the number of samples to be embedded.
4. The primary antibody depends on the gene of interest. Follow the manufacturer's instructions for storage and dilution. The following antibodies were used as seen in Figs. 1, 2, and 3: anti-CtBP2/RIBEYE antibody (Cell Applications, San Diego, CA, USA) diluted 1:100; mouse anti-parvalbumin antibody (Sigma-Aldrich, Munich, Germany) diluted 1:100 for cochlear tissue or 1:500 for brain tissue; mouse anti-otoferlin antibody (Life Spam, Echingen, Germany) diluted 1:100; rabbit anti-synaptotagmin 1 antibody (Synaptic Systems, Göttingen, Germany) diluted 1:50; mouse anti-NF200 antibody (Sigma) diluted 1:8000 and a rabbit NF200 antibody (Sigma) diluted 1:6000; rabbit anti-cholinacetyltransferase antibody (Merck Millipore, Darmstadt, Germany) diluted 1:150; rabbit anti-tyrosinhydroxylase antibody (Merck Millipore) diluted 1:100; goat anti-synaptophysin antibody (Santa Cruz, Heidelberg, Germany) diluted 1:50; mouse anti-GAD67 antibody (Merck Millipore) diluted 1:300; rabbit anti-Arg3.1/arc antibody (Synaptic Systems) diluted 1:500; rabbit anti-parvalbumin (Merck Millipore) diluted 1:3000; rabbit anti-vGlut2 antibody (Synaptic Systems) diluted 1:100; rabbit anti-MAP-2 antibody (Synaptic Systems) diluted 1:500; rabbit anti-vGAT antibody (Merck Millipore) diluted 1:100.
5. Blocking solution is prepared freshly for each immunostaining procedure.
6. Store the remaining reaction buffer at 4 °C, to dilute the secondary antibody the next day. Do not store longer.
7. Use a magnetic stir bar.

8. The amount of BSA depends on the antibody. The investigator evaluates final concentrations.
9. Nuclear staining is not obligatory. If you do not want a nuclear stain use mounting medium without DAPI. Here, we used Vectashield.
10. To open the skull, cut along the lateral ridges on the upper sides of the skull. Do not destroy the bulla containing the cochlea.
11. Cochlea dissections require practice, so start with young adult animals around P20, as the bone is not too hard yet.
12. Use approximately 10 mL of PFA for two cochleae.
13. RDO is a black liquid and that is filtered before use. Use a 0.22 μm sterile filter attached to a syringe. A yellow clear solution is obtained after pressing the RDO through the filter.
14. The time for decalcification depends on the age of the animal. Decalcification is unnecessary for mice and rats up to P7. Generally do not decalcify with RDO longer than 90 min. For animals older than P7, perform the decalcification in a small Petri dish and check every few minutes with tweezers, to determine if the tissue is soft. The investigator determines the final time for different ages and species.
15. Make small pots of aluminum foil to embed the cochleae in Tissue Tek.
16. For embedding use a 12-well plate. Apply 4 % agarose at ~55 °C to the well and place the whole brain into the agarose, leaving a little space between the bottom of the well and the tissue. When the agarose is hard, remove the brain with the agarose from the well, using a spatula, and place in a 50 mL tube containing 0.4 % PFA in 1× PBS. Store at 4 °C.
17. Do not allow tissue slices to dry out during the immunohistochemistry procedure as drying will stop the reaction. Take care that slices are always covered with enough solution. For longer incubation steps use a humidified chamber.
18. Place paper tissue soaked with ddH_2O on the bottom of the humidified chamber. Place little wood sticks or part of plastic pipettes (2 mL) on the moistened tissue. The chamber for cochlear slides should offer the possibility of setting the microscope slides horizontally next to each other on the wood sticks/plastic pipettes. Close the lid without touching the slides.
19. Remove and apply solutions by pipetting. Be careful not to aspirate brain slices into the tip of the pipette.
20. Gently shake the 24-well plate for all steps.

21. Choose volumes so that tissue slices are covered and the liquid does not evaporate. We suggest 300 μL per well.
22. For the humidified chamber, use a box that will accommodate a 24-well plate. Cover the bottom with tissue paper soaked in ddH_2O and close with a lid.
23. The distance of imaging for a *z*-stack, and thereby, the number of layers, depends on the thickness of the tissue and the staining and has to be determined by the investigator.
24. The theoretical resolution (Abbe's law of diffraction of monochromatic light) of this system along the x- and y-axes was estimated at 203 nm for Cy3 (emission maximum $E_{max} = 570$ nm) and 185 nm for Alexa488 ($E_{max} = 517$ nm), respectively. The deconvolution process in the *z*-axis is likely to improve the resolution further, since it reduces the object size in the *x*- and *y*-axes for a projected *z*-stack, due to a reduction of blur in both axes.

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