Mitotic activity, modulation of DNA processing, and purinergic signalling in the adult rat auditory brainstem following sensory deafferentation

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Abstract
A complex scenario of cellular network reorganization is caused by unilateral sensory deafferentation (USD) in the adult rat central auditory system. We asked whether this plasticity response involves mitosis. Immunohistochemistry was applied to brainstem sections for the detection and localization of mitotic markers Ki67 and PCNA, the growth-associated protein Gap43 and purine receptor P2X4. Fluorescent double staining was done for Ki67:PCNA and for both of them with HuC/HuD (neurons), S100 (astrocytes), Iba1 (microglia) and P2X4. Inquiring 1–7 days after USD, we found Ki67 expression to be changed in cellular profiles of cochlear nucleus (CN) with a significant increase in number by 1–3 days, followed by reset to control level within 1 week. USD-induced mitosis exclusively occurred in microglia and was absent elsewhere in the auditory brainstem. PCNA staining of small cellular profiles increased similarly but remained elevated. PCNA staining intensity also changed in CN, superior olive and inferior colliculus in neuronal nuclei, suggesting shifts in DNA processing. No apoptotic cell death was detected in any region of the adult auditory brainstem after USD. A comparison of anterograde and retrograde effects of nerve damage revealed proliferating microglia expressing P2X4 receptors in CN upon USD, but not in the facial nucleus after facial nerve transection. In conclusion, the deafferentation model studied here permits insight into the capacity of the adult mammalian brain to invoke mitosis among glia cells, adjustment of gene processing in neurons and purinergic signalling between them, jointly accounting for a multilayered neuro- and glioplastic response.

Abbreviations: BDNF, brain-derived neurotrophic factor; CaMKIIp, calcium–calmodulin-dependent protein kinase II, phosphorylated form; CIC, central inferior colliculus; CN, cochlear nucleus; DCN, dorsal cochlear nucleus; dl, deep layers of DCN; Gad67, glutamic acid decarboxylase of 67 kDa; Gap43, growth-associated protein 43; GFAP, glial fibrillary acidic protein; HuC/HuD, RNA-binding proteins characterizing neurons; i/c or c/i, ipsilateral-to-contralateral or contralateral-to-ipsilateral ratio of item counts; Iba1, ionized calcium-binding adaptor molecule present in brain microglia; Ki67, nuclear protein associated with cellular proliferation; LSO, lateral superior olive; MSO, medial superior olive; n7, facial nerve; N7, facial nucleus; n8, vestibulocochlear nerve; P2X4, ionotopic purine receptor; P2Y1, metabotropic purine receptor; PCNA, proliferating cell nuclear antigen; POD, postoperative day; r/l or l/r, right and left side of control brains considered symmetrical; S100, protein characterizing brain astrocytes; sl, superficial layers of DCN; tb, trapezoid body; USD, unilateral sensory deafferentation; VCN, ventral cochlear nucleus.

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1 | INTRODUCTION

Since the account of Windle (1956), initially cautious but then accelerating progress has been made to identify true structural plasticity in the adult mammalian brain. One contribution to this progress was the demonstration that cochlear ablation and the entailing sensory deafferentation of the cochlear nucleus (CN) cause cellular and molecular changes along the central auditory pathway of the adult rat brain. Degeneration of spiral ganglion afferents into CN is followed within days by the formation of maturing presynaptic boutons containing the growth-associated protein Gap43 (Hildebrandt, Hoffmann, & Illing, 2011; Illing, Kraus, & Meidinger, 2005). Origin of this re-innervation is cholinergic projections from neurons localized in the superior olivary complex of the brainstem (Kraus & Illing, 2004; Meidinger, Hildebrandt-Schoenfeld, & Illing, 2006). Importantly, moulding of neuronal networks following cochlear damage is not restricted to the directly affected primary auditory brainstem regions (Campos Torres, Vidal, & de Waele, 1999; Förster & Illing, 1998; Hildebrandt et al., 2011; Illing & Horváth, 1995), but was also reported in secondary regions including the central nucleus of the inferior colliculus (CIC; Argence et al., 2006; Milbrandt, Holder, Wilson, Salvi, & Caspary, 2000). While these studies provide strong evidence for deafferentation-induced reorganization of auditory brainstem circuitry, our understanding of the cellular and molecular events that regulate these changes is still incomplete. Attempting to acquire a deeper understanding of their dynamics, we addressed the basic question whether they comprise changes in the number of cells involved.

Antigen Ki67, also known as MKI67, is a nuclear protein that is strictly associated with cellular proliferation and may be an essential actor for it to occur. In continuously dividing cells, Ki67 is expressed in all phases of the cell cycle (Gerdes et al., 1984). Upon exit from the cell cycle, Ki67 has a short half-life of 1–1.5 hr (Bruno & Darzykiewicz, 1992; Heidebrecht, Buck, Haas, Wacker, & Parwaresch, 1996). Ki67 levels increase from S phase to G2 and M phases (Scholzen & Gerdes, 2000). It is located in the cellular nucleus during the interphase and markedly increases during the S phase of the cell cycle. Ki67 is absent from resting cells.

The proliferating cell nuclear antigen (PCNA) is a DNA clamping protein that acts as a processivity factor for DNA polymerase epsilon in eukaryotic cells and is essential for DNA replication. As DNA polymerase epsilon is also involved in re-synthesis of excised damaged DNA strands during DNA repair, PCNA is important for both DNA synthesis and DNA repair. Like Ki67, PCNA is expressed throughout the cell cycle, rising gradually during G1, peaking in S phase and declining in G2 and M (Zeng, Hao, Jiang, & Lee, 1994). With its half-life of about 20 hr (Bravo & Macdonald-Bravo, 1987), the PCNA protein is detectable in all cell cycle phases (Zeng et al., 1994) and may also occur at lower levels in non-dividing cells due to its involvement in epigenetic activities (Balajee & Geard, 2001; Bravo & Macdonald-Bravo, 1987; Zeng et al., 1994).

The number of cells within a given region of the brain may change by proliferation or cell death. A distinctive feature of apoptotic cell death is fragmentation of the genomic DNA into short sequences. These fragments accumulate in the nuclei of apoptotic cells, and their presence can be detected by TUNEL staining (or terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) in situ (Gavrieli, Sherman, & Ben-Sasson, 1992). This indicator was previously used to demonstrate that neurons in the early postnatal mouse auditory brainstem die as a consequence of their deafferentation but that by postnatal day 21 or later neurons remain viable despite a loss of cochlear input (Mostafapour, Cochran, Del Puerto, & Rubel, 2000).

To understand the cellular context of Ki67 and PCNA expression that we here report, their local presence was co-visualized with other molecular markers. The Hu proteins HuC and HuD are mRNA-binding proteins resident in neuronal cells that have left the mitotic cycle (Perrone-Bizzozero & Bird, 2013). The glial fibrillary acidic protein (GFAP) is an intermediate filament protein expressed by astrocytes and endothelial cells in the central nervous system. While GFAP is largely absent from astrocytic cell bodies, S100 stains their cell bodies and also those of oligodendrocytes (Richter-Landsberg & Heinrich, 1995). The ionized calcium-binding adaptor molecule 1 (Iba1) is a 17-kDa protein restricted to microglia both in vitro and in vivo. The Iba1 protein plays a role in regulating the function of microglia, especially of ramified and activated microglia (Ito et al., 1998). The purinoceptor family P2X opens ion channels for cations in response to binding extracellular ATP. These receptors may be expressed by neurons and glia cells depending on their state and have been implicated in various functions including those related to learning, memory and neuron-glia interactions (Burnstock, 2013). Specifically, P2X4 receptor activation may lead to release of brain-derived neurotrophic factor (BDNF) from...
microglial cells to act on TrkB receptors expressed on the soma of neurons, their processes and their presynaptic endings (Burnstock, 2008).

2 | MATERIALS AND METHODS

2.1 | Animals

This study is based on the brains from 50 adult female Wistar rats (for their superior physiological stability under anaesthesia) aged 7–20 weeks. Care and use of the animals followed the Council Directive 2010/63EU of the European Parliament and were approved by the appropriate local agency (Regierungspräsidium Freiburg, Germany, permission number 35/9185.81/G-07/83 and G16/168).

2.2 | Sensory (or primary) deafferentation by cochlear ablation

For surgery, rats were deeply anaesthetized by breathing 2% isoflurane (Forane; AbbVie) in pure oxygen. Analgesia was achieved with an initial dose of carprofen (Carprieve 50 mg/ml; Norbrook, 20 µl/200 g body weight) and daily oral application of carprofen (RIMADYL, Zoetis, 5 mg/200 g body weight). Following anaesthesia, tympanic membranes of both ears were checked for integrity and transparency. Unilateral cochlear ablation was performed on the left side using a retrotympanic approach (Illing, Horváth, & Laszig, 1997). The facial nerve was transected at its exit from the stylomastoid foramen. Tympanic membrane and ossicles were removed, and the bulla opening was widened to gain access to the cochlea. The bony wall of the cochlea was perforated with a spherical drill head and the interior of the cochlea, including the spiral ganglion, was thoroughly cleared. Subsequently, cochlea and bulla were filled with gel foam and the wound was surgically closed. This procedure entails total deafness of the affected ear (Michler & Illing, 2002). In the present study, the expression of Gap43 following cochlear ablation was used as its performance control (Illing et al., 2005). Throughout this report, the side of skull and brain on which facial nerve transection and cochlear ablation were done is referred to as ipsilateral.

2.3 | Brain preparation

Without surgery or after postoperative survival of specified duration, animals (controls: n = 9; POD1: n = 12; POD2: n = 1; POD3: n = 16; POD4: n = 1; POD7: n = 11) received lethal doses of sodium thiopental (Thiopental Inresa, Freiburg, Germany, 50 mg/ml per 200 g body weight i.p.) and were transcardially perfused with a fixation solution containing 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) at pH 7.4. Brains were carefully positioned to cut them bilaterally symmetrical.

2.4 | Immunohistochemistry

For visualizing the binding sites of antibodies (Table 1), free-floating brain sections were pre-incubated with 0.1% Triton, 0.045% H2O2, or, for nuclear proteins Ki67 and PCNA, with 1.2% H2O2 in 100% methanol, 1% milk powder (except for PCNA) and 5% normal serum, all in 0.02 M phosphate-buffered saline (PBS) at pH 7.4 for 30 min at room temperature. Following this pre-incubation, sections were incubated with primary antibodies (Table 1) for 36 hr at 4°C. Incubation with matching secondary antibodies (Table 1) and formation of an avidin–biotin complex (Elite ABC; Vector Laboratories) took 30 min each, followed by staining with 0.05% 3,3-di-aminobenzidine tetrahydrochloride (DAB; Sigma), 0.3% ammonium nickel sulphate and 0.0015% H2O2 in 50 mM Tris buffer at pH 7.2. Stained sections were mounted on gelatinesubbed slides, air-dried, dehydrated in increasing grades of alcohol and coverslipped with Entellan (Merck, Darmstadt, Germany). Antibodies PCNA-PC10 and PCNA-19F4 (Table 1) produced overall comparable staining patterns that are not differentially presented here (but see Ino & Chiba, 2000). We verified their specific binding on tissue of kidney, liver and intestine (Akerman et al., 1992; Fiore et al., 1998).

In order to identify specific spatial associations among the binding sites of different antibodies, Ki67, PCNA, Iba1, S100, HuC/HuD, glutamic acid decarboxylase Gad67, phosphorylated calcium–calmodulin-dependent protein kinase CaMKIIp and P2X4 were pairwise used for double immunofluorescence staining on free-floating sections, with 1.2% H2O2 in 100% methanol pretreatment only for Ki67 and PCNA, and incubated with primary antibodies (Table 1) for 36 hr at 4°C. Subsequently, incubation of matching secondary antibodies (Table 1) for 1 hr was performed serially, preceded by pre-incubation with 5% serum each lasting for 30 min. Co-visualizing antibodies directed against antibodies both raised in rabbit was accomplished by using anti-rabbit Fab fragments of goat origin, capping the first primary antibody prior to incubation with the second primary antibody. Media for all antibodies and sera contained 0.05% Triton X-100. Stained sections were exposed to a Sudan Black B incubation (Oliveira et al., 2010) and mounted on gelatine-subbed glass slides, air-dried overnight and coverslipped with Mowiol 4-88 (Carl Roth).

2.5 | Data acquisition

Photographs from ventral and dorsal CN, LSO, CIC and facial nucleus were taken with a digital camera (Axiocam;
Zeiss) connected to a light and fluorescence microscope (Axiophot run by AxioVision 9.4.1.0 and Axio Imager.M2 run by ZEN2.3, both Zeiss). If required, photographs were assembled with Photoshop (CS6 Ver. 13; Adobe) to produce multicolour overlays.

Evaluating staining for Ki67 quantitatively in VCN and CIC, light microscopic photographs were taken of VCN stained by DAB with a ×10 objective, photographs of CIC were taken with a ×5 objective, both with standard settings of the light source and at 8 bit grey tone depth. Photographic fields were positioned centrally at the widest mediolateral breadth of VCN and centred in CIC on both sides of the brainstem, moving marginally if major blood vessels crossed the field. From each brain, equally spaced sections were used for quantitative assessment: 4–7 of VCN and 3–4 of CIC. The resulting images were globally edited using Photoshop to set background brightness to 205. Counts were obtained from the entire cross sections of anterior VCN and of CIC, respectively, excluding their outermost borders. For evaluating small PCNA(+) profiles, photographs of sections stained with DAB were taken with a ×40 objective of VCN and with a ×100 objective of CIC. The resulting images were globally edited using Photoshop to set background brightness to 180. Counts were obtained per microscopic field. These procedures were identical for control and experimental brains.

The numbers of Ki67(+) and PCNA(+) profiles were determined by aid of image analysis software iTEM (Olympus soft imaging solutions 2010), setting detection parameters

| Table 1 | Antibodies |
|---------|------------|
| **Primary antibodies** | | |
| Gap43  | mo | Millipore; MAB347 | 1:5000/– | Merck Chemicals GmbH, Darmstadt, Germany |
| GFAP   | mo | Sigma; G3893 | 1:5000/– | Merck Chemicals GmbH, Darmstadt, Germany |
| HuC/HuD | mo | Invitrogen; A21271 | 1:250/1:125 | Thermo Fisher Scientific Inc., Waltham, MA, USA |
| HuC/HuD | rb | Abcam; ab184267 | –/1:125 | Abcam, Berlin, Germany |
| Iba1   | rb | Wako; 019-19741 | 1:1000/1:500 | Wako Pure Chemical Ind. Ltd, Osaka, Japan |
| Ki67   | rb | Abcam; ab15580 | 1:5000/1:500 | Abcam, Berlin, Germany |
| PCNA-PC10 | mo | Millipore; MAB424 | 1:5000/1:500 | Merck Chemicals GmbH, Darmstadt, Germany |
| PCNA-19F4 | mo | GeneTex; GTX39739 | 1:100/– | GeneTex, Inc. Irvine, CA, USA |
| P2X4   | rb | Alomone Labs; APR002 | 1:500/1:200 | Alomone Labs, Ltd., Jerusalem. Israel |
| P2Y1   | rb | Alomone Labs; APR009 | –/1:100 | Alomone Labs, Ltd., Jerusalem. Israel |
| S100   | rb | Dako; Z0311 | –/1:400 | DAKO, Agilent, Santa Clara, CA, USA |
| CaMKIIp | rb | Thermo Fisher; 702357 | –/1:2,000 | Thermo Fisher Scientific Inc., Waltham, MA, USA |
| Gad67  | mo | Millipore; MAB5406 | –/1:1,000 | Merck Chemicals GmbH, Darmstadt, Germany |

| **Secondary antibodies for DAB staining** | | |
| mo IgG | ho/Biotin | Vector; BA-2001 | 1:200 | Vector Laboratories, Burlingame, CA, USA |
| rb IgG | gt/Biotin | Vector; BA-1000 | 1:200 | Vector Laboratories, Burlingame, CA, USA |

| **Secondary antibodies for immunofluorescence staining** | | |
| gt IgG | dk/Alexa488 | Invitrogen; A11055 | 1:200 | Thermo Fisher Scientific Inc., Waltham, MA, USA |
| gt IgG | dk/Cy3 | Millipore; AP180C | 1:200 | Merck Chemicals GmbH, Darmstadt, Germany |
| mo IgG | dk/Alexa488 | Invitrogen; A21202 | 1:200 | Thermo Fisher Scientific Inc., Waltham, MA, USA |
| mo IgG | dk/Cy3 | Millipore; AP192C | 1:200 | Merck Chemicals GmbH, Darmstadt, Germany |
| rb IgG | dk/Alexa488 | Invitrogen; A21206 | 1:200 | Thermo Fisher Scientific Inc., Waltham, MA, USA |
| rb IgG | dk/Cy3 | Millipore; AP182C | 1:200 | Merck Chemicals GmbH, Darmstadt, Germany |
| rb Fab | gt | Jackson; 111-007-003 | 1:100 | Dianova, Hamburg, Germany |

| **Sera for immunohistochemical staining** | | |
| Normal horse serum | Vector; S-2000 | 1:20 | Vector Laboratories, Burlingame, CA, USA |
| Normal goat serum | Vector; S-1000 | 1:20 | Vector Laboratories, Burlingame, CA, USA |
| Normal donkey serum | Millipore; S30 | 1:20 | Merck Chemicals GmbH, Darmstadt, Germany |

Abbreviations: dk, donkey; gt, goat; ho, horse; mo, mouse; rb, rabbit.

for quantitative assessment: 4–7 of VCN and 3–4 of CIC. The resulting images were globally edited using Photoshop to set background brightness to 205. Counts were obtained from the entire cross sections of anterior VCN and of CIC, respectively, excluding their outermost borders. For evaluating small PCNA(+) profiles, photographs of sections stained with DAB were taken with a ×40 objective of VCN and with a ×100 objective of CIC. The resulting images were globally edited using Photoshop to set background brightness to 180. Counts were obtained per microscopic field. These procedures were identical for control and experimental brains.

The numbers of Ki67(+) and PCNA(+) profiles were determined by aid of image analysis software iTEM (Olympus soft imaging solutions 2010), setting detection parameters
for area, aspect ratio, diameter and grey value for selecting profiles to be counted for Ki67 and PCNA, respectively. For Ki67, profiles of 45 grey tone steps or more darkwards from the brightest pixels were selected. Parameters were specified for selectively detecting small PCNA(+) profiles (under 8 μm maximal diameter, roundness 0.1–1.0, convexity 0.8–1.0, 100 grey tone steps or more darkwards from brightest pixels), essentially belonging to microglia (see below), and darkly stained large round PCNA(+) nuclei (over 8 μm maximal diameter, roundness 0.3–1.0, convexity 0.9–1.0; 90 grey tone steps or more darkwards from brightest pixels), supposedly belonging to neurons. As the counted items were small against section thickness in all cases and only ratios of item counts were determined, no stereological corrections were applied.

### 2.6 | TUNEL staining

Nuclear DNA fragmentation in apoptotic cells was detected on cryo-cut brain sections using the TACS-XL DAB in situ apoptosis detection kit (Cat# 4828-30-DK; Trevigen). BrdU incorporation by terminal deoxynucleotidyl transferase at the site of DNA fragmentation was detected by a specific and sensitive biotinylated anti-BrdU antibody and visualized by a streptavidin-horseradish peroxidase conjugate. These brain sections were counterstained with methyl green.

### 2.7 | Confocal and ApoTome microscopy

In selected cases, the spatial affiliations between cell types and nuclear staining were explored in Z-stacks of focus levels (3 ± 1 stacks per section, 33 ± 5 images, z-steps of 0.3 μm), using a confocal Leica TSC SP8 (Objective, ×63 Oil/NA 1.4; acquisition software Leica Application Suite X; Leica) or an Axio Imager.M2 (Objectives × 40/NA 0.95, and × 100 NA 1.4). ApoTome2 (Zeiss) settings were 3 ± 1 stacks per section, 20 ± 5 images and z-steps of 0.275 μm. Red and green fluorescence was stimulated with a He-Ne and an argon laser, respectively, or appropriate LED-UV illumination with Colibri 7 (Zeiss). These pictures were generated with a 2 × 2 binning and show single layers of z-stacks. Co-localization by double immunofluorescence labelling was investigated qualitatively.

### 2.8 | Statistics

Using Prism software (GraphPad Software), we tested our data for normal distributions (using the Kolmogorov–Smirnov test) and equal variances (using Browne–Forsythe and Bartlett’s test). As our data did not consistently meet both criteria, we turned to the non-parametric Kruskal–Wallis H test for quantitative comparisons of multiple groups that need not be of equal size (Lowry, 1998). Dunn’s multiple comparisons test was used to specify p values for each pair comparison. All H and p values so obtained are reported in the results section. Significance levels were differentiated into (*** for p ≤ .001, (**) for p ≤ .01 and (*) for p ≤ .05. Data are presented as box-whisker plots to show median and distribution-based metric of the 10th and 90th percentile.

Although staining conditions were controlled with greatest care, subtle variations in tissue processing across experiments were unavoidable. Two measures were taken to compensate for variations superposing the experimental design. First, in all photographic field used for quantification, the brightness of background staining was normalized to a fixed grey tone value (see above). Second, instead of comparing absolute counts, group comparisons were done on left-to-right ratios for controls and on ipsilateral-to-contralateral (VCN) or contralateral-to-ipsilateral (CIC) ratios of counts within each section analysed. In addition, we had to make across-brain comparisons to determine whether ratio shifts are due to increased counts on one or decreased counts on the other side of the brainstem. For this test, counts obtained in control brains were compared with the supposedly USD-unaffected sides, that is contralateral VCN and, due to the crossing of the ascending auditory pathway, ipsilateral CIC.

### 3 | RESULTS

#### 3.1 | Ki67

Using Ki67 as a molecular marker for cells under division, we found that such cells are rare in the adult rat auditory brainstem. Unilateral sensory deafferentation (USD) caused a surge of reactive mitotic activity among cells of the ipsilateral VCN (Figure 1a), beginning within 1 day. Staining on the non-operated side remained largely unaffected (Figure 1b).

Inquiring into the cell type identity of Ki67(+) nuclei emerging after USD, Ki67 was never found in neurons. Neuronal cell bodies were identified by their HuC/HuD immunoreactivity that was never co-localized with Ki67(+) (Figure 2a). By contrast, Ki67 immunoreactivity was co-localized with Iba1, a marker identifying microglia, in ipsilateral VCN (Figure 2b,c). This was valid despite marked morphological variation among Ki67(+) profiles. Small microglia containing Ki67 were also found in ipsilateral (but not contralateral) DCN, predominantly in its deep layers (Figure 2d). It was particularly revealing to find incidences in which a microglial cell body contained two nuclei so as if it were caught on the verge of completed cellular division (Figure 2e,f). Almost all of the few Ki67(+) profiles found in VCN contralateral to USD also resided in Iba1(+) cell bodies (Figure 2g). Astrocytic cell bodies were identified by their S100 immunoreactivity. Almost all S100(+) profiles failed to show Ki67 (Figure 2h), but rare exceptions were observed
(Figure 2i). These supposedly mitotic astrocytes showed a smooth round cell body and appeared bilaterally in VCN as well as in VCN of control brains at the same low frequency.

Neither in LSO nor in CIC nor elsewhere in the auditory brainstem was there any indication of Ki67 immunoreactivity above control level at any time following USD.

### 3.2 Cellular and molecular context

Mitotic activity following USD occurred in a neuronal environment that did not reveal changes in cellular texture (Figure 3a,b), consistent with earlier studies showing that cochlear damage does not reduce the number of neurons in VCN of the mature rodent (Mostafapour et al., 2000). By sharp contrast, massive USD-dependent responses occurred among the populations of microglia identified by staining for Iba1 (Figure 3c,d) and of astrocytes stained for GFAP (Figure 3e,f), as reported earlier (de Waele, Campos Torres, Josset, & Vidal, 1996; Janz & Illing, 2014). Together with the lesion-induced increase in the number of Ki67(+) cells (Figure 3g,h), the density of cells staining for PCNA also rose (Figure 3i,j). As a marked difference to Ki67, PCNA was present even in control brains and contralateral to USD. This staining was particularly prominent in large nuclei (Figure 3j, inset). Following USD, many smaller nuclei turned PCNA(+) ipsilaterally (Figure 3i, inset), while staining in larger cell bodies appeared to lose definition compared with the staining of the opposite side. These changes took place at the time period when axons and presynaptic endings containing Gap43 emerged and climaxed (Figure 3k,l), indicating a scenario of substantial USD-induced nerve growth and synaptogenesis (Hildebrandt et al., 2011; Illing et al., 1997).

### 3.3 Ki67 (+) profile quantification

For a quantitative assessment of the increasing and decreasing Ki67 expression, stained profiles were counted by computer-aided analysis in sections midway through anterior VCN. We found a significant increase in the number of Ki67(+) nuclei against the contralateral side within 1 day after USD. This ipsilateral-to-contralateral ratio of Ki67(+) profiles remained high the following days but returned to bilateral balance by 7 days (Figure 4a; $H = 60.01, p < .0001$; control [median 1.16] vs. POD1 [8.82]; $p < .0001$, control vs. POD2-3 [12.02]; $p < .0001$; S control vs. POD7 [1.85]; $p > .99$, POD1 vs. POD2-3: $p > .99$; POD1 vs. POD7: $p < .0001$; POD2-3 vs. POD7: $p < .0001$).

Across-brain analysis revealed that measurements in control brains did not significantly differ from measurements in contralateral VCN in all survival groups, with one exception. By POD2-3, Ki67(+) profiles in the contralateral VCN were slightly but significantly more numerous than in controls or contralaterally at any other time after USD (Figure 4b; $H = 23.98, p < .0001$; control [median 12.97] vs. POD1 [4.81]; $p = .09$, control vs. POD2-3 [21.84]; $p = .10$, control vs. POD7 [13.10]; $p > .99$, POD1 vs. POD2-3: $p > .99$, POD1 vs. POD7: $p = .76$, POD2-3 vs. POD7: $p = .02$).

An equivalent analysis was made of the central inferior colliculus (CIC) of the same brains. In CIC, no USD-dependent changes were detected for Ki67 at any time (Figure 4c; $H = 2.13, p > .05$; control [median 0.99] vs. POD1 [1.18]; $p > .99$, control vs. POD2-3 [0.91]; $p > .99$, control vs. POD7 [1.21]; $p > .99$, POD1 vs. POD2-3: $p > .99$, POD1 vs. POD7: $p > .99$, POD2-3 vs. POD7: $p > .99$), and no differences were
found in ipsilateral CIC compared with controls by across-brain analysis ($H = 5.58$, $p = .13$; control [median 4.61] vs. POD1 [3.17]: $p = .183$; control vs. POD2-3 [4.93]: $p > .99$; control vs. POD7 [4.68]: $p > .99$; POD1 vs. POD7: $p = .27$; POD2-3 vs. POD7: $p > .99$).

3.4 PCNA

In VCN, PCNA was resident in microglia (Figure 5a) but not in astrocytes (Figure 5b). All Ki67(+) cells were always PCNA(+), while the converse did not apply as PCNA often resided in cells that did not contain Ki67 (Figure 5c,d). The different roles of Ki67 and PCNA in the dynamics of mitotic microglia could readily be verified under the microscope when a dividing cell body showed two separate Ki67(+) nuclei in a shared PCNA(+) cytoplasm (Figure 5d).

PCNA was also prominent in neuronal nuclei (Figure 5e). Specifically, it was localized in presumably inhibitory neurons characterized by Gad67 staining (Figure 5e’) as well as in presumably excitatory neurons stained for CaMKIIp (Figure 5e”). Ipsilateral to USD, neuronal PCNA decreased in intensity while PCNA in extra-neuronal cells grew more frequent (Figure 5f). Contralaterally and in unlesioned adult brains, many neurons but fewer microglia contained PCNA (Figure 5g).

3.5 Small PCNA(+) profile quantification

Quantifying the frequency of PCNA stained small cells by introducing an upper limit of 8 μm profile diameter in iTEM filters, an increase on the side of deafferentation was seen in VCN by POD 2-3 that was maintained at least until POD7, suggesting that PCNA remains in postmitotic cell bodies longer than Ki67 (Figure 4d; $H = 48.00$, $p < .0001$; control [median 1.21] vs. POD1 [1.41]: $p > .99$; control vs. POD2-3 [4.05]: $p < .0001$; control vs. POD7 [3.71]: $p < .0001$; POD1 vs. POD2-3: $p < .0001$; POD1 vs. POD7: $p < .0001$; POD2-3 vs. POD7: $p > .99$). Across-brain analysis revealed an increase
in the number of small profiles contralateral to USD by POD1 and POD2-3 (Figure 4e; $H = 15.02, p = .002$; control [median 80.00] vs. POD1 [160.00]: $p = .004$; control vs. POD2-3 [145.00]: $p = .007$; control vs. POD7 [120.00]: $p = .33$; POD1 vs. POD2-3: $p > .99$; POD1 vs. POD7: $p = .59$; POD2-3 vs. POD7: $p > .99$), confirming results obtained by Ki67 that USD has limited but quick and distinct effects on mitotic activity of the auditory pathway on the contralateral side.

As for Ki67, no deafferentation-dependent effect was seen for PCNA in CIC (Figure 4f; $H = 3.41, p = .33$; control [median 1.00] vs. POD1 [0.91]: $p > .99$; control vs. POD2-3 [1.20]: $p > .99$; control vs. POD7 [1.00]: $p > .99$; POD1 vs. POD2-3: $p = .62$; POD1 vs. POD7: $p > .99$; POD2-3 vs. POD7: $p > .99$). Similarly, across-brain analysis failed to show any USD-related changes in ipsilateral CIC compared with controls ($H = 7.90, p = .048$; control
3.6 | Cell death

Observing microglial cells proliferating in VCN as a consequence of USD (Figure 3c), an obvious suspicion was that this response might be related to cellular decay and the requirement to remove cellular debris. To investigate this issue, sections through the auditory brainstem were treated for staining of apoptotic cells by the TUNEL procedure at various points in time post-USD. In control brains, cells with a TUNEL signal were distinctly rare. Early after USD (POD1), when astrocytic growth just begins to show (Figure 6a), TUNEL(+) profiles failed to show an increase in frequency in VCN (Figure 6b) or elsewhere. When the astrocytic response has fully developed by POD7 (Figure 6c) and Gap43 expression indicates that synaptogenesis is maximally active (Figure 3k, cp. Illing et al., 1997; Meidinger et al., 2006), the cochlear nuclei were still devoid of TUNEL staining (Figure 6d). By that time, axons in the white matter underlying the cochlear nuclei showed several dying cells (Figure 6d), supposedly oligodendrocytes that were associated with cochlear nerve fibres now decaying. Apparently, mitotic activity within VCN as here described is not associated with local cell death in any grey matter region of the auditory brainstem. Rare TUNEL(+) nuclei, serving as positive controls for the staining procedure, were found across the brainstem (Figure 6b, insets), failing to show regional preferences.

3.7 | Neuronal PCNA

Deafferentation-dependent changes in PCNA residency in neuronal nuclei were noted (Figure 5f,g) and quantified. Large round nuclei intensely stained for PCNA were identified as belonging to neurons (Figure 7a). As already noted, neuronal PCNA was seen in both Gad67(+) and CaMKIIp(+) cell bodies, supposedly inhibitory and excitatory neurons, respectively (Figure 5e′,e″). Using automated image analysis setting a lower limit for profile size, a roundness criterion for profile shape and a lower level for staining intensity to deselect small or faintly stained profiles, the number of large, round, darkly stained profile was determined in
DAB-stained sections within microscopy fields of constant size (15.250 μm²) and symmetric position the ventral half of anterior VCN (Figure 7b). Ipsilateral-to-contralateral ratios were determined and submitted to statistical analysis (Figure 7c; H = 24.67, p < .0001, control [1.01] vs. POD3 [0.61]; p = .001; control vs. POD7 [1.00]; p > .99; POD3 vs. POD7: p = .0001). This result confirms that expression of neuronal PCNA in VCN is affected by USD.

### 3.8 Modulation of DNA processing in DCN, LSO and CIC

PCNA expression was also modulated in DCN and LSO, and perhaps also in CIC, by USD. In DCN, an elevated density of small profiles was seen on the lesioned side within days after USD (Figure 8a,b), particularly in its deeper layers where thin spiral ganglion axons are known to terminate. There were few small Ki67(+) profiles in DCN corresponding in size to the much more abundant small nuclei seen to be PCNA(+).

In LSO, a notable reduction in darkly stained large nuclei occurred on the lesioned side (Figure 8c,d), reminiscent of the effect reported for VCN (Figures 3i,j, 5f,g). A marginal reduction in PCNA staining intensity in large cell bodies on the lesion-affected contralateral side of CIC cannot be excluded (Figure 8e,f). USD-dependent shifts of PCNA expression in LSO and CIC would indicate transsynaptic effects as cochlear axons strictly end in the cochlear nucleus, so that this shift cannot be attributed to microglial responses toward decaying nerve fibres. Instead, it appears to be triggered by changes in electrical or trophic signalling as a consequence of sudden unilateral deafness.

### 3.9 Facial nucleus

As a consequence of unilateral facial nerve transection during surgery for USD, a massive increase in Iba1 immunoreactivity occurred in the facial nucleus on the lesioned side (Figure 9a,b). This increase, accompanied by an increase in the number of PCNA-staining profiles (Figure 9c,d),
was partly due to cellular growth, but mitotic activity also occurred on the side where the facial nerve had been cut (Figure 9e,f). As in VCN, we identified dividing cells as microglia based on Ki67:Iba1 co-localization (Figure 9g). Neither S100 (Figure 9h) nor HuC/HuD (not illustrated) was found to be co-localized with Ki67 in the facial nucleus of the adult rat brain, confirming earlier observations (Graeber, Tetzlaff, Streit, & Kreutzberg, 1988; Graeber et al., 1998). Additionally, as for VCN after USD, we verified that no TUNEL signal emerged after facial nerve transection (Figure 9i). Unlike VCN, there was no indication that Ki67 or PCNA expression is modulated in the contralateral facial nucleus. Specifically, Ki67 expression was zero on the non-transected side (Figure 9f).

At first sight, a rather similar pattern of cellular response appeared to develop in VCN and facial nucleus, although the signalling cascade that triggers them ran in opposite directions. Cutting the facial nerve sends a retrograde signal along the axons to their cell bodies within the facial nucleus. These neurons are able to re-invoker growth processes, regenerate their axons and, in rodents, re-innervate whiskers (Vaughan, 1990). By contrast, removing the spiral ganglion by cochleostomy deprives auditory nerve axons of their cell bodies, leading to their irrevocable anterograde (Wallerian) degeneration. Apart from similarities, important molecular differences between these different settings can be expected.

3.10 | P2X4

Following USD, there was a striking redistribution of P2X4 receptor immunocytochemistry in VCN, producing a pattern of staining that, for reasons of size and shape, suggested the emergence of P2X4(+) microglia on the lesioned side (Figure 10a).
as compared to b). This was confirmed by double staining for P2X4 receptors with Iba1 (Figure 10c,d), where receptor aggregations in ‘hot spots’ were suggested. P2X4 immunoreactivity also appeared to be expressed by neurons across the brainstem, but we failed to identify USD-dependent changes or to locate them on or in astrocytes anywhere in the auditory brainstem.

The circumstances under which microglial cells grow and divide are distinctly different in cochlear nucleus and facial nucleus, suggesting that their response might differ in mode. Searching for a distinguishing feature of microglial cells in these unlike environments, we co-stained sections after USD for Iba1 and P2X4 receptors also in facial nucleus. Remarkably, activated microglia in ipsilateral VCN (Figure 11a,b) expressed this purine receptor, whereas microglia in the facial nucleus did not (Figure 11c,d). We could also show that P2X4 was expressed by newly generated microglia. When cells are strongly Ki67(+), they co-stained for P2X4 in the cytoplasm (Figure 11a′), when the Ki67 signal was less intense P2X4 is seen enriched on their protoplasma membrane (Figure 11a″). The modulated expression of the ionotropic P2X4 receptor was not accompanied by a modulation of the metabotropic purine receptor P2Y1 after USD in cochlear nucleus or after facial nerve transection in facial nuclei (not illustrated).

4 | DISCUSSION

Five major results were obtained in this study. First, a unilateral cochlear ablation causes mitosis in the cochlear nucleus of the affected side confined to microglia (microgliosis), beginning by 1 day postlesion and ending within a week, resulting in a substantially densified microglial population. Second, few but distinct mitotic cells were also detected in VCN contralateral to USD. Third, the absence of dying cells at any postlesional time indicates a stable number of neurons and astrocytes through the period of USD-induced tissue reorganization. Fourth, although neurons neither die nor proliferate, they change processivity of DNA polymerases, indicating their plastic responsiveness on the level of gene management. Finally, many microglia of the proliferating subpopulation in the cochlear nucleus start expressing purinoceptor P2X4, suggesting a recruitment of purinergic signalling, a molecular response that appears to remain unused in the population of proliferating microglia of the facial nucleus after facial nerve transection.
4.1 Detection of mitosis

The study of mitotic activity in the adult mammalian brain has greatly benefited from the use of BrdU to permanently label proliferating cells. However, the requirement that it has to be administered exogenously raises issues related to the effects of timing and dosage of injections and possible variability in uptake. These concerns have stimulated interest in identifying and using intrinsic, potentially more reliable markers for proliferating cells (Junek, Rusak, & Semba, 2010). Also, BrdU labels cells in mitotic S phase lasting under 10 hr but then stays in the cells instead of being degraded. To study occurrence and time course of deprivation-dependent mitosis, we needed to use markers identifying cells undergoing a cell cycle lasting about 25 hr but then disappearing from the cells to visualize a discontinuation. Therefore, molecular markers Ki67 and PCNA were chosen over BrdU to perform this study.

We observed mitotic microglia between days 1 and 7 after USD in the cochlear nucleus on the affected side (Figures 1a, 2b–f, 4a). This period conspicuously corresponds to two landmarks of tissue dynamics induced by USD. First, fibre degeneration, loss of primary synapses and debris digestion are complete within 9 days of cochlear ablation (Gentschev & Sotelo, 1973). Second, the buildup of Gap43 expression indicating synaptogenesis reaches a maximum by about 7 days (Hildebrandt et al., 2011; Illing et al., 1997).

Following USD, PCNA(+) microglia also increased in number in the ipsilateral VCN (Figure 4d). This increase was late compared to Ki67 (Figure 4a) but persisted when Ki67 expression was down again by POD7 (Figure 4d). This difference permits two conclusions. Having shown that all Ki67(+) profiles also revealed PCNA immunoreactivity (Figure 5c,d), the number of microglia expressing PCNA must have increased at least by the same number as Ki67(+) microglia. As in Figure 4 ipsilateral-to-contralateral ratios are plotted, newly generated PCNA(+) microglia only contribute as a surplus to the PCNA(+) microglial population permanently present. Thus, the USD-dependent rise of PCNA(+) cells appears much less prominent than that of Ki67(+) cells. The increased frequency of PCNA(+) cells beyond POD2-3 indicates that the newly formed cells keep expressing PCNA when entering a postmitotic state while Ki67 was lost from their nucleus. These data suggest that PCNA is involved in mitosis but that it also assumes other functions of nuclear management.

Both Ki67(+) and PCNA(+) profiles were also found to increase in number in VCN contralateral to USD (Figure 4b,e).
Although the absolute numbers of profiles involved were much lower than on the lesioned side, this finding is remarkable for two reasons. First, decay of axons and synapses does not appear to be the only possible initiator for microglial proliferation. Second, it indicates that microglial mitosis also affects the contralateral, healthy auditory pathway, potentially aiding in adaptive responses.

4.2 Mitosis only among microglia

Microgliosis is a common response to brain injury or disease. A straightforward result of our studies is that USD-induced mitosis occurs exclusively among microglia. In sections double-stained for Ki67 and Iba1, we occasionally found strongly fluorescent Ki67(+) nuclei with no indication of accompanying Iba1. This might suggest location in another cell type. Indeed, we found rare instances of Ki67 in cell bodies containing S100 (astrocytes or oligodendrocytes, Figure 2i), but these were of the same very low frequency as in control brains. Alternatively, the appropriate conclusion might be that microglial cells responding to deafferentation may be met in a moment of their cell cycle when no Iba1 is expressed.

Our data do not allow a definite answer to the question whether the microglial population of brain parenchyma is
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self-sustained, deriving when required in cases of denervation or degeneration from mature resident cells, or whether USD also invokes a contribution by the hematopoietic system to increase the number of microglia in VCN. However, evidence from elsewhere renders the latter option unlikely (Ajami, Bennett, Krieger, Tetzlaff, & Rossi, 2007). The detection of abundant mitotic microglia is compatible with this view.

Since the studies of Altman (Altman & Das, 1965), it has been debated whether there is neuronal mitosis in the adult mammalian brain. Since then, many studies present confirming results (Ernst & Frisén, 2015), but, consistent with the regional specificity of neuronal mitosis, our data provide no hint that it occurs in the brainstem of the adult rat after sensory deafferentation. We conclude that the only cell type contributing to deafferentation-dependent plasticity by cell division is microglia, while the constancy of the number of neurons and essentially also astrocytes supporting tissue stability.

**FIGURE 10** Expression of the P2X4 receptor and localization in Iba1(+) profiles in VCN after USD. (a, b) Following USD, small P2X4(+) cell bodies (arrows) emerged on the lesioned side (shown at POD4). (c) P2X4 immunoreactivity tended to be patchy and was specifically localized with Iba1(+) profiles (arrowheads, shown at POD4; c′ and c″ show separated colour channels for control). (d) Localization of P2X4 on microglia in ‘hot spots’ (arrowheads) as seen at POD3 under the confocal microscope. Scale bar in b for a and b: 20 μm; for c: 20 μm; for d: 5 μm

**FIGURE 11** Expression of P2X4 receptors by microglia in VCN but not in facial nucleus. (a, b) P2X4 immunoreactivity (arrowheads) was spatiotemporally associated with Iba1(+) profiles on the side of afferent nerve degeneration, but not contralaterally (b). Insets a′ and a″ show association of P2X4 (arrowheads) with mitotic microglia as indicated by Ki67 immunoreactivity in VCN ipsilateral to USD (shown by POD2). (c, d) Upon unilateral facial nerve transection, no P2X4 immunoreactivity showed up on microglia in facial nucleus after transection of its efferent nerve on either side. Micrographs taken under the ApoTome. Scale bar: 20 μm
4.3 Microglial function

These observations do not oppose viewing microglial activity as responding to inflammation and decay of cellular structures (Ajami et al., 2007). However, beyond their macrophage activity, they have been shown to be related to constructive processes including the formation of new synapses (Bessis, Béchade, Bernard, & Roumier, 2007; Jung et al., 2009; Paolicelli et al., 2011; Svahn, Becker, & Graeber, 2014). Comparing different settings for cochlear and facial nuclei, microglia differed by expressing P2X4 receptors in an environment of synapse formation and maturation (Sominsky, De Luca, & Spencer, 2018) which we observed in VCN by the emergence of Gap43(+) presynaptic endings (Hildebrandt et al., 2011). The fact that microglial mitotic activity is significantly modulated in VCN contralateral to USD (Figure 4b,e) is additional support for their inflammation-independent functional competence.

P2X4 receptors may be expressed in central and peripheral neurons and in microglia and can also occur in epithelial tissues and endothelial cells, both in the plasma membrane and in intracellular compartments (Suurvilli, Boudinot, Kanellopoulos, & Rüütel Boudinot, 2017). When microglia divide as a consequence of USD, they initially contain the receptor protein in the cellular nucleus but then appear to move it near or into the cellular membrane (Figure 11a′,a″). This, then, puts them into a position to translate extracellular signals carried by purines into intracellular MAPK cascade activation (Janz & Illing, 2014) that eventually leads to the synthesis of neurotrophins such as BDNF (Coull et al., 2005; Trang, Beggs, Wan, & Salter, 2009). When BDNF is locally released from microglia, it can foster nerve fibres to grow and synapses to mature (Suzuki et al., 2007). Indeed, fibre growth and synaptogenesis were identified under the conditions and in the time frame here considered (Hildebrandt et al., 2011). Upon USD and the approach of Gap43 containing nerve fibres, astrocytes begin expressing PSA-NCAM and the matrix metalloprotease MMP2 (Fredrich, Zeber, Hildebrandt, & Illing, 2013). Both contribute to increase fluidity of surrounding nerve tissue, granting motility for growing structures. Release of MMP2 contributes to a disintegration of extracellular matrix including perineuronal nets, an effect that we discovered to take place in VCN upon USD, indicated by a massive loss of the extracellular matrix component neurocan when synaptogenesis surges (Heusinger et al., 2019).

The cooperation of astrocytes and microglia appears to be a requisite of nerve growth and synaptogenesis on the secondary sensory neurons of CN as we observed it in the adult auditory brainstem upon sensory deafferentation. The absence of microglia carrying P2X4 receptors and the lack of PCNA-related DNA reorganization in neurons of the facial nucleus upon nerve transection appear to reflect the fact that no comparable demand is posed on these to reorganize their local signalling network.

4.4 Neuronal PCNA

A previously little considered mode of epigenetic neuronal plasticity was observed and quantified in VCN. Involved in the regulation of gene activity, the homotrimer PCNA was seen to partly redistribute within, or disappear from, neuronal nuclei (Figures 3i,j; 5f,g; 7c, 8c–f). This was most obvious by POD3. Whereas the emergence of PCNA in microglia in VCN upon USD is in line with a role of PCNA in mitosis (Choe & Moldovan, 2017; Leonardi et al., 1992), its redistribution in postmitotic neurons must be related to other requirements. Even though we might not have captured all neuronal subtypes for quantification as, for instance, GABAergic neurons in VCN are rather small (Fredrich, Reisch, & Illing, 2009), the outcome was as surprising as significant (Figure 7c). Roles of PCNA in DNA repair and epigenetic modulation have been reported (Miller et al., 2010; Moldovan, Pfander, & Jentsch, 2007; Zhang, Shibahara, & Stillman, 2000). An epigenetic mode of plasticity on the level of chromatin structure has been suggested for hippocampal neurons after male tree shrews were exposed to social stress (Fuchs & Flügge, 2014). It seems inescapable to conclude that something important is going on in neurons affected by sensory deafferentation. Among the yet unknown scope of genes that change their activity upon sensory deafferentation appears to be those for insulin-like growth factor 1, interleukin-1ß and tumour necrosis factor α (Fuentes-Santamaria, Alvarado, Gabaldón-Ull, & Juiz, 2013; Fuentes-Santamaria et al., 2017). If a comparable neuronal response also occurs in transsynaptically affected LSO and CIC as qualitatively suggested by their patterns of PCNA staining (Figure 8c–f) remains to be quantitatively confirmed.

4.5 Facial nucleus

Our experimental model includes a dual opportunity to evaluate the findings. In addition to study consequences of anterograde axonal degeneration in the cochlear nucleus caused by severance of a sensory nerve, we searched for effects of retrograde axonal signalling in the facial nucleus due to transection of its motor nerve. Consequences of facial nerve transection have been studied before (Graeber et al., 1988, 1998; Ito et al., 1998), revealing that microglia are dividing upon transection-triggered retrograde signals into the brainstem, and that astrocytes show hypertrophy but no hyperplasia when nerve transection is done in the adult rodents (Tyzack et al., 2014). Based on a modified set of experimental evidence, we confirm these conclusions. Apoptosis as a cause for microcytosis can be excluded for both facial nucleus (Figure 9i) and cochlear nucleus (Figure 6).
However, we noticed that facial nucleus microglia, although proliferating, do not change their trimming of P2X4 purinergic receptors and that facial motor neurons, although initiating expressing regeneration-related genes (Schmitt et al., 2003), do not obviously alter their intracellular configuration of PCNA. Moreover, failure of P2X4 expression goes along with a lack of Gap43 modulation and a stable extracellular matrix, at least as neurocan, is concerned. By sharp contrast, Gap43 (Illing et al., 1997) and neurocan (Heusinger, Hildebrandt, & Illing, 2019) are massively modulated in VCN after USD.

5 CONCLUSIONS

The comparison between cellular responses in the cochlear nucleus due to destruction of the spiral ganglion and hence the eighth cranial nerve serving sensory function and in the facial nuclei due to transection of the seventh cranial nerve serving motor function reveals similarities and differences. The cochlear nucleus has to adjust to a permanent loss of ear-born nerve signals, while neurons in the facial nucleus prepare to regrow the motor nerve. Both cases involve apoptosis exclusively among microglia, growth but no proliferation of astrocytes, with a lack of apoptotic cell death indicating a stable population of neurons. At the same time, important differences among their neuro- and glioplastic response became apparent. In the cochlear nucleus, but not in the facial nucleus, expression of purinoceptors in microglia, changes in DNA polymerase processivity of neurons and growing-in of external nerve processes along with the generation and maturation of synapses take place, all on the side of deafferentation. The obvious conclusion is that these specific responses to deafferentation of the adult central nervous system are mutually supportive to transform abruptly deprived neuronal networks into an adaptive mode of functioning. Apparently, such requirements for a reorganization of intercellular signalling do not rest on the facial nucleus upon nerve transection. The piling of complexity levels involved in the regulation of adult brain plasticity and stability is even more appreciable when stimulating in addition to silencing the auditory pathway is taken into consideration (Rosskothten-Kuhl, Hildebrandt, Birkenhäuser, & Illing, 2018).

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CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA ACCESSIBILITY

The materials used in this study are made fully explicit and are available from public suppliers. Brain sections, protocols and measurement data on which this study is based are fully archived and accessible in the laboratory library of our department.

AUTHOR CONTRIBUTIONS

RBI designed the study. AT and HB did the experiments. AT, HB and RBI evaluated the data. RBI devised the figures and wrote the article. All authors approved the final manuscript.

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