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Midazolam Reverses Salicylate-Induced Changes in Brain-Derived Neurotrophic Factor and Arg3.1 Expression: Implications for Tinnitus Perception and Auditory Plasticity

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ABSTRACT

Tinnitus is a phantom auditory perception, which can be induced via application of concentrated sodium salicylate, and is known to be associated with hearing loss and altered neuronal excitability in peripheral and central auditory neurons. The molecular features of this excitability, however, has been poorly characterized to date. Brain-derived neurotrophic factor (BDNF), the activity-dependent cytoskeletal protein (Arg3.1, also known as Arc), and c-Fos are known to be affected by changes in excitability and plasticity. Using reverse transcription-polymerase chain reaction, in situ hybridization, and immunohistochemistry, the expression of these genes was monitored in the rat auditory system after local (cochlear) and systemic application of salicylate. Induction of tinnitus and hearing loss was verified in a behavioral model. Regardless of the mode of salicylate application, a common pattern became evident: 1) BDNF mRNA expression was increased in the spiral ganglion neurons of the cochlea; and 2) Arg3.1 expression was significantly reduced in the auditory cortex. Local application of the $GABA_A$ receptor modulator midazolam resulted in the reversal not only of salicylate-induced changes in cochlear BDNF expression, but also in cortical Arg3.1 expression, indicating that the tinnitus-associated changes in cochlear BDNF expression trigger the decline of cortical Arg3.1 expression. Furthermore, local midazolam application reduced tinnitus perception in the animal model. These findings support Arg3.1 and BDNF as markers for activity changes in the auditory system and suggest a role of GABAergic inhibition of cochlear neurons in the modulation of Arg3.1 plasticity changes in the auditory cortex and tinnitus perception.

Tinnitus is primarily caused by acoustic or chemical trauma and is usually associated with hearing loss (Eggermont and Roberts, 2004). Concentrated salicylate, the active component of aspirin, is known to induce hearing loss and tinnitus (Boettcher and Salvi, 1991). Salicylate also induces abnormal neuronal excitability in central and peripheral auditory neurons (Boettcher and Salvi, 1991; Cazals, 2000; Eggermont and Roberts, 2004). Using a rat behavioral model of tinnitus (Rüttiger et al., 2003), which is conceptually different from other models (Jastreboff and Brennan, 1994; Bauer et al., 2000), we studied the effects of systemic and local application of salicylate on BDNF and Arg3.1 gene expression in the spiral ganglion neurons of the cochlea and in the auditory cortex.

Brain-derived neurotrophic factor (BDNF) is a key neurotrophin involved in neuronal survival and differentiation. BDNF has a complex gene structure, and the nomenclature has been altered recently (Aid et al., 2007). Although we published previous work using the old nomenclature system (Tan et al., 2007), the present work adopts the system of Aid et al. (2007). Neuronal activity influences the distinct promoters of BDNF exons I to VIII (Timmusk et al., 1993;

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ABBREVIATIONS: BDNF, brain-derived neurotrophic factor; Arg3.1/Arc, activity-regulated cytoskeletal protein; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; AC, auditory cortex; LOC, lateral olivocochlear; MDZ, midazolam; PBS, phosphate-buffered saline; Scy, salicylate; PRh, perirhinal cortex; art.P., artificial perilymph; NMDA, *N*-methyl-D-aspartate; SPL, sound pressure level.

Lauterborn et al., 1996) and BDNF exons IV and VI (former exons III and IV) are both expressed in the auditory system (Tan et al., 2007). c-Fos expression is also linked to neuronal excitability (Zhang et al., 2002), and in the auditory system, c-Fos expression is up-regulated after salicylate treatment (Wallhausser-Franke et al., 2003).

Arg3.1 (Lyford et al., 1995) expression is postulated to provide homeostatic compensation for short-term changes in synaptic strength (Shepherd et al., 2006), and recent data indicate that Arg3.1 expression is enhanced during sensory experiences, including nontraumatic sound (Mahlke and Wallhausser-Franke, 2004), smell (Zou and Buck, 2006), and during formation of activity-driven neuronal networks (Ramirez-Amaya et al., 2005). Furthermore, Arg3.1 expression is correlated with BDNF-induced plasticity changes (Ying et al., 2002).

We observed striking changes in BDNF and Arg3.1 expression subsequent to the induction of tinnitus. The gene expression changes are comparable with the changes seen after noise-induced tinnitus (Tan et al., 2007), in which BDNF expression in the cochlea and Arg3.1 expression in the auditory cortex were dramatically altered.

Furthermore, in light of the known role of inhibitory efferent lateral olivocochlear (LOC) fibers in changes of cochlear nerve excitability and protection from excitotoxic effects (Darrow et al., 2007), we tested the effect of an efferent agonist, the benzodiazepine receptor agonist midazolam, on BDNF and Arg3.1 gene expression and on tinnitus behavior. Both gene expression and tinnitus behavior could be reversed upon application of midazolam, suggesting that disinhibition of cochlear activity triggers the alteration of plasticity genes in both the cochlea and auditory cortex.

Materials and Methods

Animals and Drug Application. Adult female Wistar rats weighing 200 to 300 g were studied. Animal care and treatment was based on the institutional guidelines of the University of Tübingen Veterinary Care Unit (Tübingen, Germany).

Salicylate was administered either locally or systemically. For local application, the rats were anesthetized with an intraperitoneal injection of ketamine hydrochloride (75 mg/kg; Ketavet, Pharmacia, Erlangen, Germany) and xylazine hydrochloride (5 mg/kg, Rompun; Bayer AG, Leverkusen, Germany). The bony bulla of the rats was exposed retroaurically through a 1-cm surgical cut in the skin and a 1-mm hole drilled through the bone at the top end of the bulla, close to the ear canal. The mucosa within the hole was removed, and the round window niche was visualized, just above the arteria stapedialis. During surgery, one of two methods was used: 1) gel foam pellets (Gelita Tampon; Braun, Melsungen, Germany) were placed through the hole in the bulla into the round window niche and supplied with different volumes (5, 10, and 20 μl) of artificial perilymph or salicylate solution (sodium salicylate, 70 mg/ml supplied concentration; Sigma, München, Germany). Twenty hours after surgery, animals were sacrificed; 2) in animals who were trained previously according to the behavioral paradigm (see below), a second 1-mm hole was drilled adjacent to the first, allowing the introduction of a metal canula (26 gauge, diameter 0.45 mm) attached to a rubber tube. The hole above the round window niche allowed improved visualization of the positioning process. The canula was positioned just before the round window and fixed with epoxy glue (Histoacryl; Braun Aesculap, Tuttlingen, Germany) and dental cement (Paladur; Kulzer Laboratory Product Division, Hanau, Germany). A mini osmotic pump (Alzet, 1.0 μl/h for 7 days; DUREC Cooperation, Cupertino, CA) filled with either 100 μl of midazolam (Dormicum 1 mg/ml; Roche, Grenzach-Wyhlen, Germany) or artificial perilymph as control was then attached to the rubber tube implanted under the skin. The animals were allowed to recover for 6 days. Salicylate was then administered systemically (350 mg/kg, see below), and the behavior was tested after 3 h. Equal volumes of systemically administered saline were used as control. For systemic application, the animals received intraperitoneal salicylate injections (350 mg/kg of body weight). In conjunction with the salicylate injections, some animals also received midazolam (0.5 mg/kg). Animals were first injected with salicylate, and 2.5 h later, animals were injected with either midazolam or the corresponding volume of saline solution. Animals were sacrificed 3 h after the initial salicylate injection. Systemic kainate injections (12 mg/kg; Sigma-Aldrich, Steinheim, Germany) were carried out in the same manner.

Salicylate levels in both the serum and cochlear fluids for selected animals were measured. A single systemic injection of 350 mg/kg (3 h) salicylate led to similar levels of salicylate in the cochlear fluid compared with local application of 5 μl of salicylate solution (see *Results*).

Auditory Brainstem Response Measurements. Anesthesia of animals and auditory brainstem response measurements were performed as described previously (Tan et al., 2007). Hearing measurements were performed for saline controls and for salicylate-treated rats before local application of salicylate using gel foam pellets and after operation.

Animal Behavioral Model. Tinnitus perception in rats was tested using a behavioral model (Rüttiger et al., 2003). Animals were trained to seek a reward upon the presentation of an external sound and to remain still in the absence of sound (silence). After 4 to 8 weeks of training, the animals had learned to discriminate silence and sound. When animals were suitably trained, salicylate was administered (as above), and their behavior was monitored in the presence and absence of an external sound. The specificity of salicylate trauma for hearing loss and tinnitus induction was evaluated by a number of control behavioral experiments using a variety of sound levels, frequency tones, and visual stimulations. For initial behavior experiments (Fig. 1), $n = 55$ rats were studied. For Fig. 11, $n = 2$ animals for each treatment were studied [one animal for the midazolam (MDZ)/saline group did not perform in the final behavior test].

Analysis of Salicylate Concentrations in Blood and Cochlea Fluid. Blood samples were taken from single rats directly after decapitation and serum-frozen for later analysis with high-performance liquid chromatography. For sampling cochlear fluid, the cochleae were separated from the skull, and small amounts of fluid (1–2 μl) were taken through the round and oval window by means of glass capillaries (2 μl; Drummond Scientific Company, Broomall, PA).

Tissue Preparation. Cochleae were isolated and dissected as described previously (Knipper et al., 2000). Before use, tissue samples were cryosectioned at 10 μm thickness for in situ hybridization, mounted on SuperFrost/Plus microscope slides (R. Langenbrinck Medizin-und-Labortechnik, Teningen, Germany) and stored at −20°C.

Auditory cortices were identified in accordance with Paxinos and Watson (1998). Tissues within the anterior and posterior ectosylvian sulci (roughly delineating A1) were dissected. For RNA isolation, tissues were treated as described previously (Tan et al., 2007). For the detection of mRNA and protein in brain sections, brains were removed and immediately frozen in ice-cold isopentane over an ethanol/dry-ice bath. Frozen brains were then stored for 3 to 5 days at −20°C and embedded in O.C.T. compound (Miles Laboratories, Elkhart, IN). The brains were cryosectioned at 10 μm thickness and mounted on SuperFrost/Plus microscope slides. Brain sections were post-fixed for 10 min in 2% phosphonoformic acid before use.

On the other hand, brains were fixed for 48 h in 4% paraformaldehyde, embedded in 4% agarose, and stored in PBS + 0.4% paraformaldehyde at 4°C. The tissue was sectioned at 60 μm with the VT

1000S vibrating microtome (Leica, Wetzlar, Germany). Slices were kept in 1× PBS in a 24-well plate.

Riboprobe Synthesis. Antisense and sense primers for the amplification of BDNF exons I, II , IV , VI, and IX were constructed as described in Timmusk et al. (1993). To amplify c-Fos and Arg3.1, primers were designed as described in Tan et al. (2007). Riboprobes were synthesized as described previously (Tan et al., 2007).

In Situ Hybridization of Cochlear Sections. In situ hybridization was performed as described previously (Wiechers et al., 1999). The sections (cryosections) were allowed to develop in the substrate solution containing nitro-blue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). Sections were viewed using an Olympus AX70 microscope (Olympus, Hamburg, Germany). The number of animals was the same within each group of control and treated animals ($n = 3$ animals each). In situ hybridization was repeated for each member of the control and treatment groups (e.g., for each of the three animals in the control group, in situ hybridization was performed twice, for a total of $n = 6$ in situ hybridizations).

In Situ Hybridization of Brain Sections. The location and organization of the rat auditory cortex is well described (Paxinos and Watson, 1998; Doron et al., 2002). Coronal sections were chosen to detect mRNA or protein signals within the area of the primary auditory cortex (AC). For both detection of mRNA and protein levels, we selected an area of the primary auditory cortex between 4.2 and 5.2 mm posterior to Bregma (Doron et al., 2002). The number of animals was the same within each group of control and treated animals ($n = 3$ animals each). In situ hybridization was repeated for each member of the control and treatment groups.

Colocalization of Arg3.1 mRNA and Protein in Brain Sections. For localization of Arg3.1 mRNA and protein, rat brain sections (60 μm) cut with a vibratom were prepared as described above. Colocalization was carried out on free-floating sections. After the PBS wash, sections were incubated in freshly prepared 0.25% acetic anhydride in Tris-HCl (10 mM), pH 8. Sections were then washed again in PBS and dehydrated with an ethanol series, incubated in chloroform, and rehydrated again. The sections were washed twice in 2× standard saline citrate (300 mM NaCl, and 30 mM sodium citrate, pH 7) for 5 min each and prehybridized for 1 h at 37°C. Hybridization with the Arg3.1 riboprobe was carried out as described in Tan et al. (2007). For protein detection on these slices, sections were washed briefly in PBS + 0.05% Tween 20, and endogenous peroxidases were blocked in 3% H_2O_2. After a brief wash in PBS, sections were blocked using the Avidin-Biotin blocking kit (Vector Labs, Peterborough, UK) according to the manufacturer's instructions. After a PBS wash, the sections were incubated at 4°C overnight with a primary Arg3.1 antibody (Santa Cruz, Heidelberg, Germany; Synaptic Systems, Goettingen, Germany). After incubation with the secondary antibody (biotinylated goat antirabbit; Vector Labs), the sections were washed in PBS, and chromogenic detection was carried out (3-amino-9-ethylcarbazole). Nuclei were counterstained with Methyl green (Vector Labs), and sections were viewed using an Olympus AX70 microscope. The number of animals was the same within each group of control and treated animals ($n = 3$ animals each). Colocalization of Arg3.1 mRNA and protein was repeated for each member of the control and treatment groups.

Quantification of Arg3.1 Protein in the Auditory Cortex. Arg3.1 immunoreactive cells were counted in the AC of brains using an integrated microscopic counting chamber to fix the area of interest delineated by a square of 2450 μm^2. The number of Arg3.1 immunopositive cells from eight 2450-μm^2 squares on four rat brain sections for each treatment group (between 4.2 and 5.2 mm posterior to Bregma) (Doron et al., 2002) including the auditory cortex were counted, and the average was taken. These squares were placed with respect to the cellular anatomy of the AC: they covered cortical layers II to VI and spanned the bulk of the area designated as the primary AC according to Doron et al. (2002). Two rats were used for each treatment group (control, salicylate, and salicylate plus midazolam), and two brain sections from each rat (i.e., $n = 4$ brain sections for each treatment group) were analyzed for Arg3.1 immunoreactivity. Data are expressed as mean cell count ± S.E.M.. Statistical analysis was performed using the two-tailed Student's t test.

Semiquantitative Reverse-Transcription PCR. Isolation of total RNA from both cochlear and brain tissues and PCR primer sequences and amplification conditions were performed as described previously in Tan et al. (2007). The number of cycles and annealing temperature were optimized so that the signals obtained for Arg3.1, BDNF, c-Fos, glyceraldehyde-3-phosphate dehydrogenase, and cyclophilin were not saturated. The number of animals used for PCR was the same within each group of control and treated animals ($n = 3$ animals each). For statistical analyses of the PCR results, animals were pooled. Densitometric analysis was performed using the Alpha Imager 2200 from Biometra (Göttingen, Germany). The intensity of the amplified band produced for each gene was normalized for each reaction to the coamplified glyceraldehyde-3-phosphate dehydrogenase or cyclophilin level. Control and treated groups were then compared with each other, and the data are expressed as mean percentage of control (set at 100%) and S.E.M. Statistical analysis was done using Student's t test: *, $p < 0.05$. For local application of salicylate c-Fos, BDNF exon IV and Arg3.1 PCRs were performed two times each (Figs. 2, 5, and 6). For statistical analyses 3 h after 350 mg/kg salicylate injection, BDNF exon IV PCR was performed 22 times for cochlear tissue and 16 times for auditory cortex. c-Fos PCR was performed 16 times and Arg3.1 PCR, 10 times (Fig. 5). For statistical analyses of systemic application of salicylate and midazo-

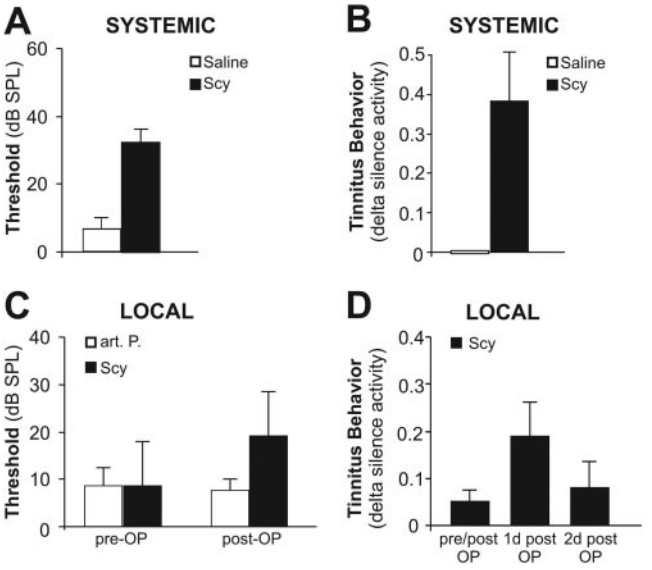

Fig. 1. Local and systemic application of salicylate leads to hearing loss and tinnitus. A, a shift in the hearing threshold of adult Wistar rats is observed 3 h after a single systemic injection of high doses of salicylate (350 mg/kg) (saline: 6.8 ± 3.2 dB SPL, $n = 23$ ears/12 rats; salicylate: 32.0 ± 4.2 dB SPL, $n = 5$ ears/5 rats). B, a significant increase in tinnitus behavior was observed subsequent to high doses of salicylate (350 mg/kg, $n = 55$) that was not observed after injection of saline. C, local, cochlear application of salicylate leads to elevated hearing thresholds after application of the gel foam to the round window. Hearing thresholds were measured before the operation (pre-OP) to insert the gel foam in control ($n = 10$ ears/5 rats) and treated ($n = 30$ ears/15 rats). Threshold was measured again after the operation (post-OP). Hearing thresholds in control animals (artificial perilymph) remained unchanged post-OP (8.6 ± 3.9 dB SPL versus 7.6 ± 2.45 db SPL, $n = 10$ ears/5 rats), whereas rats receiving locally applied salicylate had significantly increased hearing thresholds post-OP (8.6 ± 9.3 dB SPL versus 19.2 ± 9.3 dB SPL, $n = 28$ ears/14 rats); $p < 0.001$ D, analyzing phantom noise perception using our animal behavior model, hearing loss was associated with a significant increase in tinnitus behavior (delta silence activity) shown for the first and second day after operation. The peak in tinnitus behavior occurred within 1 day (~20 h) of the operation.

lam BDNF exon IV, PCR was performed 7 times and Arg3.1 PCR, 13 times (Fig. 7). For statistical analyses of the local application of midazolam and systemic application of salicylate BDNF exon IV PCR was performed 10 times and Arg3.1 PCR, 13 times (Fig. 8).

Results

Local and Systemic Application of Salicylate Induces Tinnitus in a Rat Behavioral Model. Salicylate is known to induce tinnitus (phantom auditory sensation) and hearing loss in animal behavioral models (Cazals, 2000). In a first step, we assessed the impact of our systemic and local salicylate application on hearing function and tinnitus behavior in adult rats. We observed that a systemic injection of 350 mg/kg salicylate (Scy) causes a significant increase in the hearing threshold (Fig. 1A) as determined by measuring click auditory brainstem responses. Hearing loss was associated with a significant increase in tinnitus behavior (Fig. 1B relative to saline control). Furthermore, we were able to show for the first time that local round window application of salicylate is also able to induce hearing loss and tinnitus behavior in rats. In contrast to the application of 5 μl of control solution (artificial perilymph) to the round window niche, the application of the same volume of salicylate (70 mg/ml) resulted in a significantly enhanced threshold, measured after operation (Fig. 1C; compare artificial perilymph to Scy). The associated increase in tinnitus behavior 1 day after operation is shown in Fig. 1D. A comparable change in hearing threshold and tinnitus behavior was also seen in animals with noise-induced tinnitus (Tan et al., 2007).

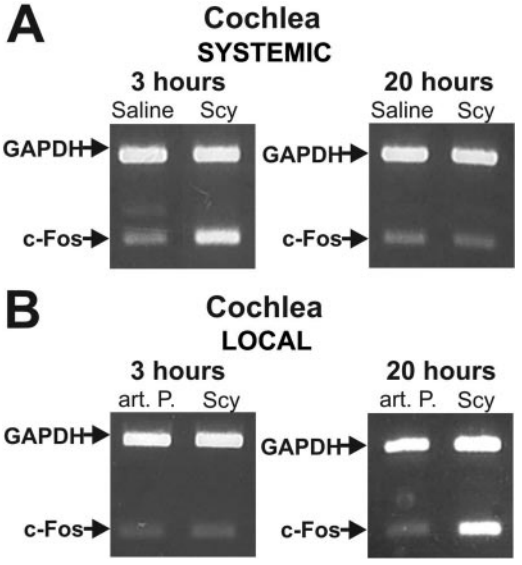

Fig. 2. c-Fos expression is up-regulated in the cochlea after systemic and local application of salicylate. A, 3 h after a single systemic injection of salicylate, c-Fos is dramatically up-regulated in the cochlea compared with saline-injected controls. Twenty hours after the original injection, the level of gene expression has returned to control levels. B, 3 h after local application of salicylate, no change in c-Fos expression is seen compared with controls (artificial perilymph, 5 μl on gel foam). A strong up-regulation is, however, seen 20 h after application of the salicylate-soaked (5 μl, 70 mg/ml) gel foam to the round window of the cochlea.

To assess the degree to which local and systemic application of salicylate reaches the cochlea and thus affects cochlear nerve activity, we analyzed c-Fos expression in cochlear tissue at various points in time after local or systemic administration of salicylate. Systemically injected salicylate induced an up-regulation of c-Fos expression after 3 h (Fig. 2A, left), whereas after 20 h, the level of c-Fos expression had returned to the values observed in the controls (Fig. 2A, right). Local application of salicylate, however, did not affect c-Fos expression at 3 h (Fig. 2B, left) but at 20 h (Fig. 2B, right), corresponding to the peak in tinnitus behavior seen after the implantation of the gel foam (Fig. 1D). This delay is probably due to a slow release of the fluid from the gel foam. To quantify the effect, the level of salicylate in the cochlea was measured. The concentration of salicylate in the cochlear fluid was measured 20 h after local application of 5 μl of salicylate (70 mg/ml) and 3 h after injection of 350 mg of salicylate/kg of body weight, confirming that although the treatment methods differ, comparable levels of salicylate were present in the cochlear fluid (data not shown). In the following experiments, we therefore examined short-term effects 20 h after local application of salicylate and 3 h after systemic application of salicylate.

Activity-Dependent Gene Expression in the Peripheral and Central Auditory Pathway. The BDNF gene is structured such that eight upstream untranslated exons (I-VIII) can all be alternatively spliced to the common protein coding exon (IX) to produce an identical protein product. BDNF exons IV and VI are expressed in the spiral ganglion neurons of the cochlea, as shown by in situ hybridization (Fig. 3, A and B). Hybridization using sense probes produced no detectable signal (Fig. 3, A and B, insets). BDNF exon IX (as part of the same mRNA) is always detectable (data not shown), whereas exons I and II and Arg3.1 are not expressed in the cochlea (data not shown). c-Fos transcripts were also readily detectable in the spiral ganglion neurons of the cochlea (Fig. 3C). The specificity of detection was again shown

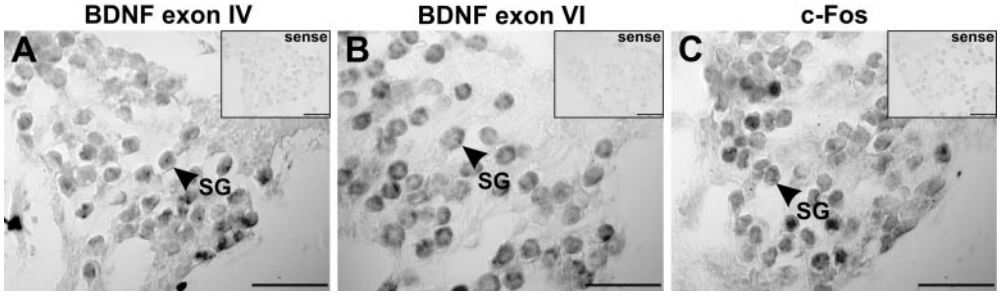

Fig. 3. Expression of c-Fos and BDNF transcripts in cochlear spiral ganglion neurons. A and B, BDNF exon IV and VI mRNA were detected in spiral ganglion neurons. mRNA is localized to the nuclei of the spiral ganglion neurons (SG, arrowheads). Hybridization using sense riboprobes produced no chromogenic signal (insets). C, c-Fos mRNA expression was detected using sequence-specific antisense riboprobes. mRNA is localized to the nuclei of the spiral ganglion neurons (SG, arrowheads). Scale bars, 50 μm.

by a lack of signal after hybridization with a sense riboprobe (Fig. 3C, inset).

There is no evidence for Arg3.1 expression in the cochlea (unpublished observations). Arg3.1 is known to be expressed in the brain and is strongly up-regulated in the molecular and granular layers of the dentate gyrus after neural excitation (e.g., kainate injection) (Lyford et al., 1995). To date, most studies have been performed in the mouse and in brain areas not including the auditory cortex. Furthermore, recent evidence shows that the expression of Arg3.1 is not identical in rats and mice (D. Kuhl, personal communication). To confirm the specificity of our antibodies in rats, we used a dual mRNA/protein detection method to evaluate the induction of Arg3.1 expression in the rat dentate gyrus after kainate injection (compare Fig. 4A, left and right). A combination of sense riboprobe and the absence of primary antibody confirm the specificity of the method (Fig. 4A, inset). At higher magnification (Fig. 4B, left and right), Arg3.1 mRNA signals (violet) are visible in the vicinity of cell nuclei (blue, asterisks), whereas the protein signals appear at a greater distance (orange dots, arrows). Upon kainate injection, Arg3.1 mRNA and protein is significantly increased (compare Fig. 4B, left and right).

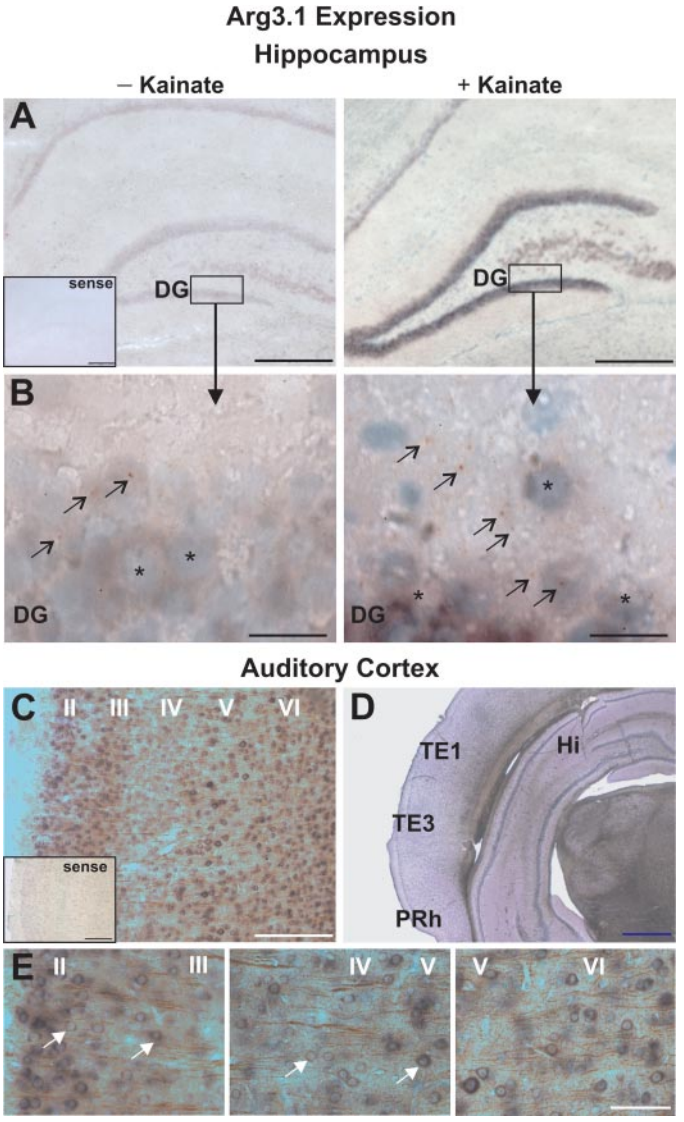

Fig. 4. Expression of Arg3.1 mRNA and protein in the rat brain. A and B, using a combined in situ hybridization and immunohistochemistry method, both Arg3.1 mRNA (violet) and protein (orange, arrows) were shown to be up-regulated in the granular layer of the dentate gyrus (DG) after kainate injection (A and B, compare left and right). Hybridization with sense riboprobes plus omission of the primary antibody produced no signals (inset in A, left). Arg3.1 mRNA was found mainly in the vicinity of cell nuclei (asterisks), whereas punctate protein signals were observed at a greater distance (arrows). Nuclei are stained with Methyl Green; scale bars, 200 μm in A, 20 μm in B. C, Arg3.1 protein is expressed in the auditory cortex. The cortical layers from the temporal cortex area 3 (TE3) are shown to express Arg3.1 mRNA and protein. Arg3.1-specific antibodies detect Arg3.1-expressing cells within TE3. The staining is abolished in the absence of the primary antibody (inset). Scale bars, 500 μm. D, borders of the hippocampus (Hi), TE1, TE3, and PRh were identified according to Paxinos and Watson (1998) and Doron et al. (2002). A representative image of the area is shown. Scale bars, 1 mm. E, Arg3.1-positive cells are seen in both the external (II and III) and internal (IV and V) granular and pyramidal cell layers (arrows). Arg3.1 mRNA (violet) appears in the perinuclear space, whereas Arg3.1 protein (orange, arrows) appears in cellular projections. Scale bars, 50 μm.

Confident of the specificity of our detection methods, we proceeded to assess the degree to which Arg3.1 protein is expressed in the rat auditory cortex. Arg3.1 signals were localized within the hippocampus (Fig. 4, A and B) and within the entire temporal (TE1, TE3) and perirhinal cortex (PRh), as shown for TE3 (Fig. 4C). The borders of TE1, TE3, and PRh were determined according to Doron et al. (2002) and Paxinos and Watson (1998) (Fig. 4D). At higher magnification, it became evident that Arg3.1-positive cells were present in all layers of the cortex (Fig. 4, C and E). Furthermore, analogous to the hippocampus, in the auditory cortex, Arg3.1 mRNA seems to be concentrated in the perinuclear space (Fig. 4E, purple staining, arrows), whereas the protein is seen in cellular projections (Fig. 4E, orange staining).

Alteration of Activity-Dependent Genes in the Cochlea and Auditory Cortex. Having established the expression of BDNF, c-Fos, and Arg3.1 in the cochlea and auditory cortex, we proceeded to examine the changes in gene expression associated with salicylate-induced tinnitus in rats. The cochleae and auditory cortices of the individually treated animals were prepared and analyzed using RT-PCR. Figure 5 illustrates the RT-PCR analysis of mRNA expression changes in BDNF exon VI, Arg3.1, and c-Fos in the cochlea (Fig. 5A) and the auditory cortex (Fig. 5C). Densitometric analysis (Fig. 5, B and D) was used to quantify mRNA expression changes. For each treatment, a representative picture for BDNF exon VI, Arg3.1, and c-Fos is shown. After a short-term (local or systemic) treatment of salicylate application, we observed a significant enhancement of c-Fos, BDNF exon IV (data not shown), and BDNF exon VI in the cochlea (Fig. 5, A and B). In the auditory cortices of the same animals, a significant reduction in Arg3.1 mRNA in response to salicylate injection (Fig. 5, C and D) was seen, whereas BDNF exon VI (Fig. 5, C and D), c-Fos (data not shown), and BDNF exon IV (data not shown) expression remained unchanged. Each experiment was repeated.

Concentration-Dependent Changes in BDNF and Arg3.1 Expression. The temporal activation of c-Fos in the auditory system seems to be affected by the mode of salicylate application (Fig. 3), and we observe a temporal difference in tinnitus behavior after either systemic or local application of salicylate (Fig. 1). Furthermore, different doses of salicylate have been reported to differentially affect neuronal excitability (Kumagai, 1992). We therefore proceeded to investigate the influence of different concentrations of salicylate

on gene expression. To examine whether the changes in gene expression in the cochlea induced by short-term salicylate application (Fig. 5, A and B) are dose-dependent and in parallel to observe the effect of salicylate without generalized effects on the central nervous system, we tested the effect of 5, 10, or 20 μl of 70 mg/ml salicylate applied to the round window niche. Using RT-PCR, a concentration-dependent enhancement of BDNF exon VI was seen for the cochlea (Fig. 6A), which could be directly linked to a dose-dependent decrease of Arg3.1 in the auditory cortex (Fig. 6B). This observation strengthens the notion of an inverse relationship between BDNF exon IV/VI expression in the peripheral and Arg3.1 in the central auditory pathway.

Salicylate-Induced Changes in Gene Expression are Reversed by the GABA Receptor Modulator Midazolam. It is currently hypothesized that the pathologically enhanced activity occurring during phantom sensation is due to a loss of GABA-mediated surround inhibition in subcortical and cortical areas (Eggermont and Roberts, 2004). The concept of disinhibition during tinnitus has not been extended to the cochlea despite several studies underscoring the inhibitory potential of the LOC efferent system on the modulation of cochlear nerve activity (Darrow et al., 2007).

Presuming that the reciprocal changes in BDNF and Arg3.1 expression (Figs. 5 and 6) may be due to a loss of efferent inhibition, we investigated whether the salicylate-induced changes in BDNF and Arg3.1 expression in the cochlea and auditory cortex may be altered by the application of the benzodiazepine ($GABA_A$) receptor agonist midazolam. In a first approach and similar to recent studies (Murashita et al., 2007), systemic application of midazolam was used. Figure 7 illustrates the RT-PCR analysis of BDNF exon VI and Arg3.1 expression changes after systemic injection of Scy and MDZ. When 0.5 mg of midazolam/kg of body weight was injected 2.5 h after the initial injection of salicylate (350 mg/kg of body weight), the enhancement of BDNF transcripts in the cochlea was completely reversed; moreover, it was reduced below the level observed in control animals (shown for BDNF exon VI; Fig. 7A). Likewise, the reduction of Arg3.1 expression in the auditory cortex caused by short-term salicylate injections (Fig. 5, C and D) was also reversed by midazolam, causing Arg3.1 expression to approach levels observed in saline-injected animals (Fig. 7B). Densitometric analysis was used to quantify expression changes.

Systemically applied midazolam presumably stimulates GABA receptors in the entire brain, possibly resulting in gene expression changes. As expected, we did observe an effect of systemic midazolam application on cortical gene expression (a slight down-regulation of Arg3.1 that was not statistically significant; data not shown). To thus remove a possible central effect caused by systemic midazolam application and to verify participation of the LOC system, we combined systemic application of salicylate with round window application of midazolam (Fig. 8). The significant increase in BDNF expression (shown for BDNF exon VI) after systemic injection of salicylate is completely reversed by local application of midazolam (Fig. 8A, Scy/MDZ). Cochlear application of midazolam had no effect on gene expression when applied to control animals, which had received previously an injection of physiological saline solution (Fig. 8A, saline/MDZ). To our surprise, round window application of midazolam also reversed the strong reduction of Arg3.1 expression in the AC, which occurs after systemic injection of salicylate (Fig. 8B). Again, midazolam had no effect on gene expression in control animals (saline/MDZ). These results strongly suggest that a reduction of GABA-mediated inhibition at the level of the auditory nerve plays a central role not only for the tinnitus-associated enhancement of BDNF expression in spiral ganglion neurons but also the associated decline of Arg3.1 in the auditory cortex.

To add an increased measure of reliability and to visualize gene expression changes in a more natural context, we analyzed tissue sections of the cochlea and auditory cortex. We were able to further confirm the effect of midazolam on gene

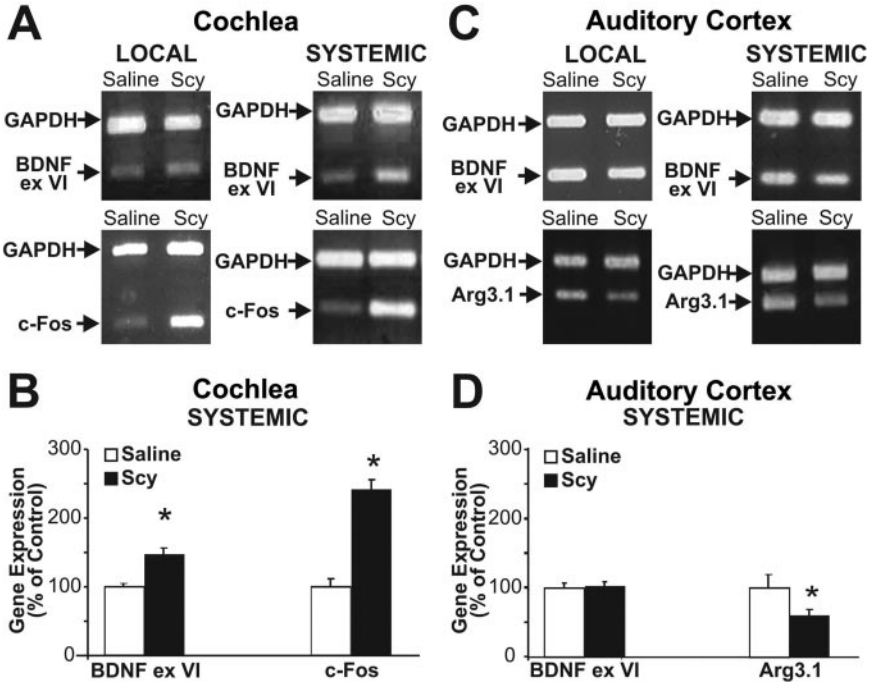

Fig. 5. Local and systemic application of salicylate leads to changes in activity-dependent gene expression in the cochlea and auditory cortex. A and B, 3 h after either systemic injection or 20 h after local application of salicylate, BDNF expression (shown for exon VI) in the cochlea is significantly increased. c-Fos expression is also increased under these conditions. C and D, in the auditory cortex, BDNF exon VI expression remains unchanged, whereas Arg3.1 expression is significantly reduced. Expression is shown as a percentage of control gene expression ± S.E.M., t test; *, $p < 0.05$.

expression using in situ hybridization in the cochlea (Fig. 9) and both in situ hybridization and immunohistochemistry in the auditory cortex (Fig. 10). Systemic injection of salicylate increased BDNF exon IV (Fig. 9, A–D) and exon VI (Fig. 9, E–G) expression in cochlear spiral ganglion neurons (shown for the midbasal cochlear turn) compared with saline-injected controls (compare Fig. 9, A with C, and 7E with 9G). Midazolam produced no changes when injected into saline-treated control rats (Fig. 9, B and F) but was again able to completely reverse elevated BDNF transcript levels when combined with salicylate injection (Fig. 9, D and H). The experiment was repeated with similar results.

In the auditory cortex, we again looked at Arg3.1 expression after systemic injection of salicylate and local round window application of midazolam (Fig. 10). A slight decrease in Arg3.1 mRNA (blue) and protein (red) was observed in all layers of the cortex after systemic injection of salicylate (Fig. 10, A and B). This was again reversed by the subsequent injection of midazolam (Fig. 10C). As in the cochlea, midazolam injected into control rats produced no changes in gene expression (data not shown). To quantify the observed changes, we counted the number of Arg3.1 immunoreactive neurons in a fixed area of the auditory cortex, the amygdala, piriform cortex, and thalamus. Although no significant difference in the number of Arg3.1-immunopositive cells could be seen between control and salicylate-treated groups in the thalamus or piriform cortex (data not shown), counting of immunopositive neurons in the auditory cortex (Fig. 10A, closed arrow) confirmed the dramatic reduction of Arg3.1 expression after systemic injection of salicylate at the protein level (Fig. 10, B and D: 166 ± 35 and 43 ± 20, respectively). The number of Arg3.1-immunopositive cells was restored to control levels, however, when a subsequent dose of midazolam was administered (Fig. 10, C and D: 166 ± 35 and 161 ± 36, respectively). Arg3.1 mRNA was also observed within the Arg3.1-immunopositive cells (Fig. 10A, C, open arrows). It is interesting that in the amygdala, although injection of salicylate also resulted in reduced Arg3.1 expression, this change in gene expression was not influenced by subsequent midazolam application to the cochlea (Fig. 10E). This finding highlights the specificity of the cochlear efferent system in exclusively balancing gene expression patterns within the central auditory pathway but not other brain areas.

Midazolam Reduces the Perception of Salicylate-Induced Tinnitus in a Rat Behavioral Model. To gain a first indication of whether the midazolam mediated the reversal of the salicylate-induced BDNF and Arg3.1 expression changes is related to tinnitus sensation, we studied the behavior of animals after local application of midazolam and systemic application of salicylate (Figs. 8 and 11). In control animals, systemic injection of physiological saline solution, combined with cochlear application of either artificial perilymph solution (art.P./Saline) or midazolam (MDZ/Saline) resulted in no tinnitus behavior. After systemic application of salicylate and local application of artificial perilymph solution (art.P./Scy), a significant increase in tinnitus behavior was seen, consistent with previous results (Fig. 1). In contrast, the local application of midazolam in combination with the systemic application of salicylate (MDZ/Scy) resulted in a remarkable reduction in tinnitus behavior. These preliminary data support the concept of a loss of GABA-mediated cochlear inhibition in the regulation not only of cochlear and cortical gene expression but also in the perception of tinnitus.

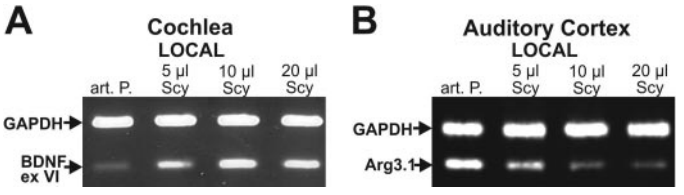

Fig. 6. Local application of salicylate affects BDNF and Arg3.1 expression in a dose-dependent manner. A, in the cochlea, a dose-dependent effect is seen for BDNF expression (shown for exon VI) after the application of various concentrations of salicylate to the round window niche, increasing with higher doses of salicylate. B, in contrast, in the auditory cortices of the same animals, Arg3.1 is down-regulated with increasing doses of salicylate. Representative PCR pictures are shown in each case.

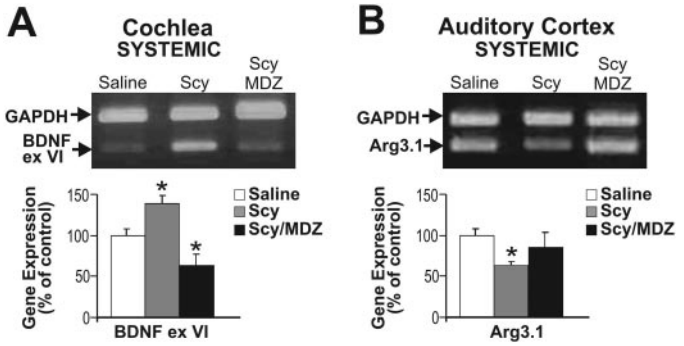

Fig. 7. Systemic injection of midazolam reverses the salicylate-induced changes in BDNF and Arg3.1 expression in the cochlea and auditory cortex. A, 3 h after the systemic application of salicylate, BDNF expression (shown for exon VI) is increased in the cochlea. The injection of midazolam 0.5 h before sacrifice results in a significant reduction in BDNF exon VI expression in the cochlea to below control (saline-injected animals) expression. B, in the brains of the same animals, Arg3.1 expression is reduced in the auditory cortex after systemic injection of salicylate. The subsequent injection of midazolam restores gene expression to control levels. Representative PCR pictures are shown in each case. Expression is shown as a percentage of control gene expression ± S.E.M., *t* test; *, $p < 0.05$.

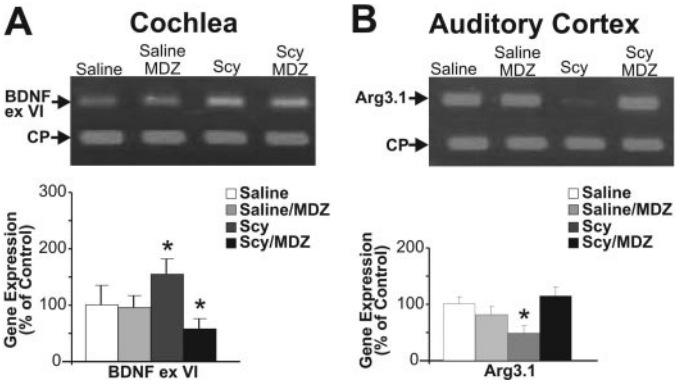

Fig. 8. Local application of midazolam alters gene expression in the cochlea and auditory cortex. A, 3 h after systemic injection of salicylate, BDNF expression (shown for BDNF exon VI) is increased in the cochlea compared with saline-injected controls. The subsequent local application of midazolam restores gene expression to the control levels (compare Scy and Scy/MDZ). Local application of midazolam to the cochleae of saline-injected controls had no effect on gene expression. B, in the auditory cortices of the same animals, Arg3.1 expression was dramatically reduced after injection of salicylate. This was reversed by the subsequent injection of midazolam (compare Scy and Scy/MDZ). Again, midazolam had no effect on gene expression when applied to the cochleae of saline-injected control animals. *, $p < 0.05$.

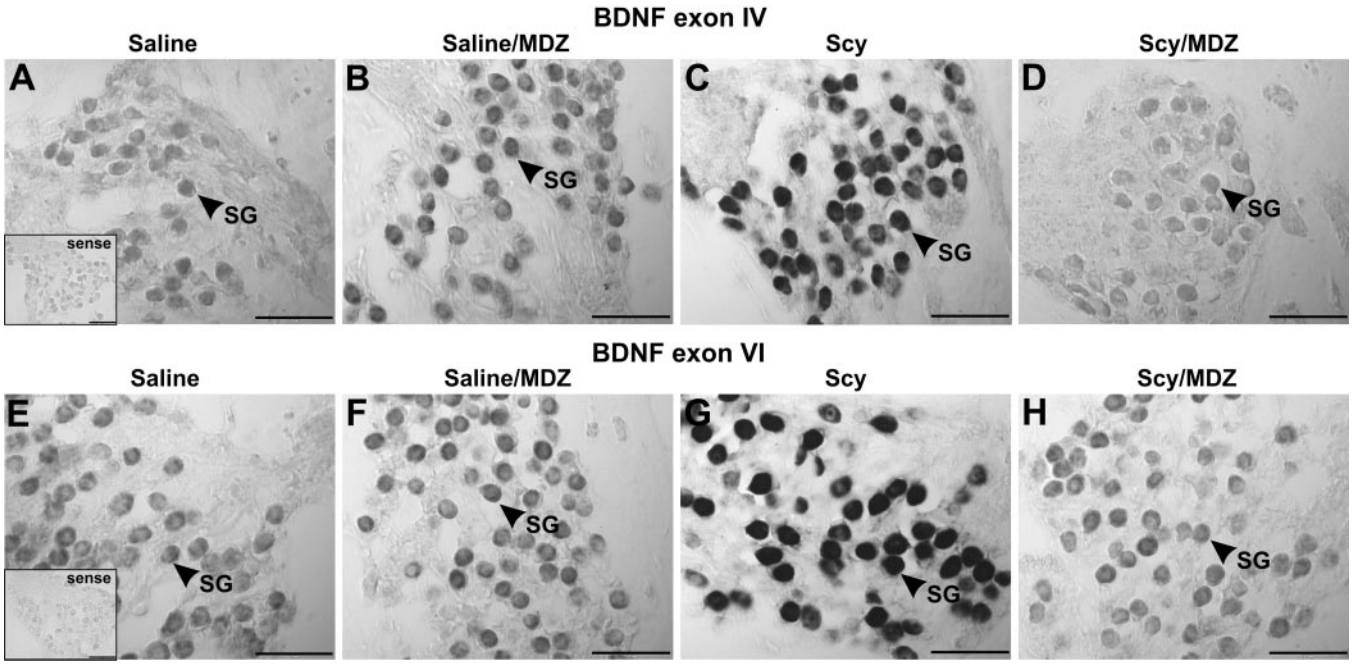

Fig. 9. Systemic injection of midazolam reverses salicylate-induced gene expression changes in spiral ganglion neurons. Using in situ hybridization, BDNF expression in the cochlea was analyzed after systemic injection of salicylate and local application of midazolam. A to D, BDNF exon IV expression is up-regulated in spiral ganglion neurons of the cochlea after systemic injection of salicylate (compare A and C). Local application of midazolam to the cochleae of saline injected controls had no effect on gene expression in the cochlea (compare A and B). Local application of midazolam to the round window of the cochlea returns gene expression to control levels (compare A and D). E to H, BDNF exon VI expression is also up-regulated in spiral ganglion neurons of the cochlea after systemic injection of salicylate (compare E and G). No effect of midazolam on gene expression is seen in saline-injected controls (compare E and F), whereas local application of midazolam returns gene expression to control levels (compare E and H). Scale bars, 50 μm.

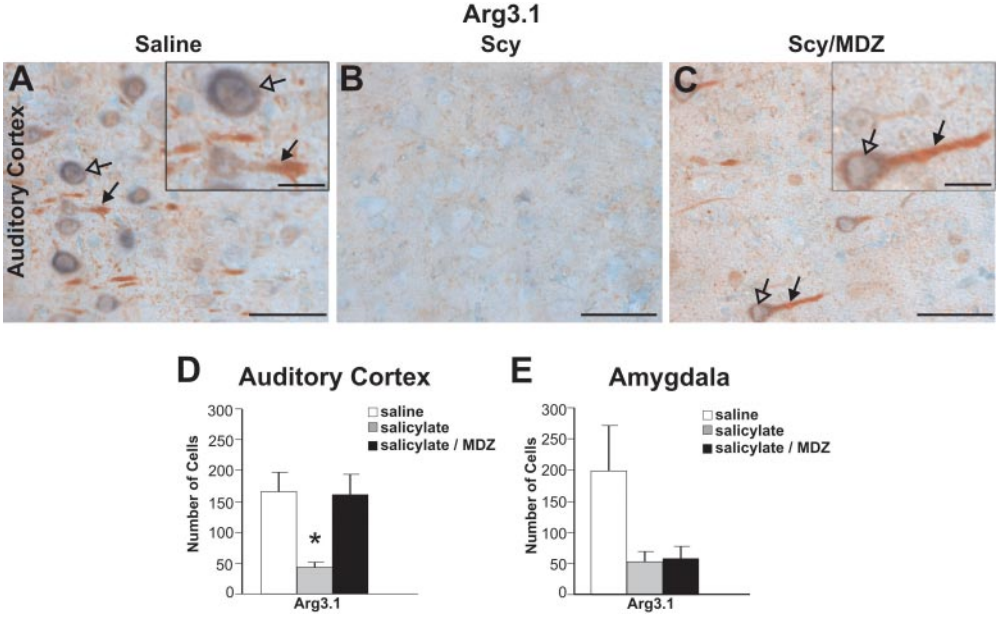

Fig. 10. Local application of midazolam alters gene expression in cortical neurons. A to C, using a combined in situ hybridization and immunohistochemistry, Arg3.1 expression in the auditory cortex (shown here for layer V) was analyzed after systemic injection of salicylate and local application of midazolam. A, Arg3.1 is normally expressed in cortical neurons [mRNA (blue), open arrow; protein (red), closed arrow]. mRNA and protein expression is dramatically reduced after the systemic injection of salicylate (B) and is restored by a subsequent local application of midazolam [mRNA (blue), open arrow; protein (red), closed arrow] (C). Insets in A and C show a higher magnification of the double staining. D and E, the number of Arg3.1 protein-positive cells was counted in brain sections after each treatment. D, in the auditory cortex, quantitative analysis confirmed the immunohistochemistry results. E, other brain areas also showed a decrease in BDNF-expressing cells after injection of salicylate (shown for the amygdala); however, the recovery of gene expression in response to midazolam was specific to the AC. $*$, $p < 0.005$. Scale bars, 50 μm; insets, 20 μm.

Discussion

Using an animal behavioral model (Rüttiger et al., 2003) we confirmed the induction of tinnitus after both systemic and cochlear application of salicylate (Fig. 1). Both treatments result in an enhancement of BDNF expression in the cochlea and a decrease in Arg3.1 expression in the auditory cortex (Figs. 5, 7, 8, and 10). To reverse these changes in gene expression and to investigate the possible role of inhibitory signaling in the cochlea, we analyzed the effects of the $GABA_A$ receptor modulator midazolam. After tinnitus induction with salicylate, the additional application of midazolam was shown to reverse the BDNF and Arg3.1 expression changes and tinnitus behavior (Fig. 11). Our results suggest that BDNF and Arg3.1 are key players in the underlying mechanism involved in the onset and persistence of salicylate-induced tinnitus.

The expression of BDNF exons IV, VI, and c-Fos requires the calcium-dependent recruitment of transcription factors (Tabuchi et al., 2000; Takeuchi et al., 2002; Tao et al., 2002) This may be mediated by activated NMDA receptors. Recently, salicylate was described to act by potentiating NMDA receptor currents in cortical (Vane et al., 1998) or spiral ganglion neurons in a dose-dependent manner (Peng et al., 2003). A salicylate-induced increase in Ca^{2+} through NMDA receptors may not only participate in increased BDNF expression but may also alter spontaneous auditory nerve activity (Puel et al., 2002; Muller et al., 2003). Indeed, salicylate-induced tinnitus has been shown to be associated with an increase in spontaneous activity in the cochlear nerve (Cazals, 2000). Thus, the increase in BDNF expression, which we observed in the cochlea, seems to be a reliable marker for the altered neuronal activity, which accompanies tinnitus.

Our data further show that the changes in BDNF expression, and most likely the associated enhanced auditory nerve activity, are influenced by a loss of $GABA_A$ receptor-mediated inhibition at the level of cochlear neurons. In line with this view is the observed impact of salicylate on cochlear and cortical gene expression (Figs. 5 and 6) and the reversal of BDNF up-regulation by locally applied midazolam (Fig. 8). These findings suggest a mechanism whereby the removal of inhibitory signals in the cochlea leads to an enhancement of BDNF expression. This could finally lead to altered central auditory effects, including changes in cortical gene expression and auditory perception.

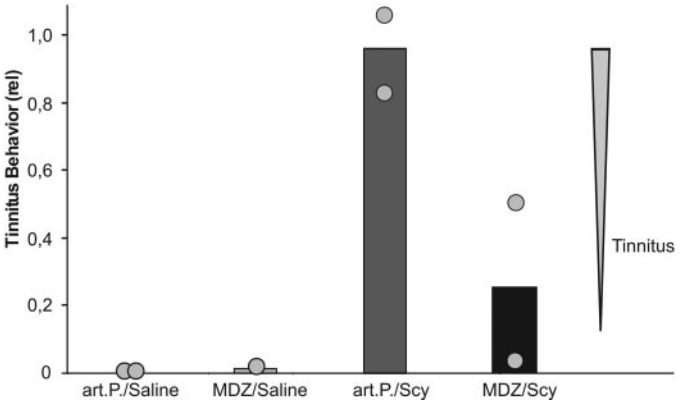

Fig. 11. Local application of midazolam counteracts salicylate-induced tinnitus behavior in the rat behavioral model. Local application of art.P. or MDZ (1 mg/ml) to the round window niche for 1 week (with miniosmotic pumps and ear cannulas, flow 1 μl/h) did not lead to tinnitus behavior in rats after control treatment (systemic injection of saline, art.P./Saline, and MDZ/Saline). Systemic injection of salicylate leads to pronounced tinnitus behavior (art.P./SCY). Locally applied midazolam could partly counteract salicylate-induced tinnitus behavior (MDZ/Scy). Symbols show tinnitus behavior of individual rats, and bars represent the mean tinnitus behavior.

Although one might argue that salicylate-induced tinnitus is completely unrelated to the more common noise-induced tinnitus, we have evidence that the molecular events are highly similar in each case. We have recently shown that after noise-induced tinnitus, cochlear BDNF expression was increased, whereas Arg3.1 expression in the auditory cortex was reduced (Tan et al., 2007). This process was also associated with enhanced expression of BDNF and GABA in the inferior colliculus, as well as a reduction in local field potentials in the auditory cortex (Tan et al., 2007). We discussed the possibility that hyperactivity of the auditory nerve and brainstem nuclei up to the level of the inferior colliculus induces the decrease of thalamocortical input through enhanced subcortical GABA expression. Likewise, Brozoski et al. (2007) observed elevated brainstem activity and decreased forebrain activity in rats with evidence of tinnitus using manganese-enhanced magnetic resonance imaging (Brozoski et al., 2007). Furthermore, high doses of salicylate have been shown to lead to a mean reduction in maximal field potential amplitudes in AC (Eggermont and Kenmochi, 1998), supporting the model of reduced thalamocortical input as a correlate of tinnitus.

How could a reduced thalamocortical input or forebrain activity be causally linked to the cortical hyperexcitability and correlated plasticity (Cazals, 2000; Kaltenbach and Afman, 2000; Eggermont and Roberts, 2004), which occurs during tinnitus perception? Unrestrained potentiation can result in saturation of a neuron's ability to encode information (Moser et al., 1998). A homeostatic compensation for these acute changes in synaptic strength is therefore required to maintain neuronal output in the normal range (Turrigiano and Nelson, 2004). One candidate for this homeostatic maintenance is Arg3.1 (Shepherd et al., 2006), whose role in plasticity changes is beginning to be understood in more detail (Tzingounis and Nicoll, 2006). Assuming that Arg3.1 controls synaptic homeostasis and thereby synaptic plasticity, a decrease of Arg3.1, as shown in the present study, would have a detrimental influence on cortical plasticity. The decrease in Arg3.1 expression seen here (Figs. 5–8 and 10) and previously (Tan et al., 2007) suggests that during tinnitus, the neurons of the auditory cortex can no longer accurately process the signals coming from the periphery. This could be due to the associated change in spontaneous auditory nerve activity, which could be perceived as a "nonsense" signal in higher brain areas. It will be of immense interest to analyze whether the reduced Arg3.1 levels in the auditory cortex are associated with the observed hyperexcitability in the auditory cortex during tinnitus.

Of further interest is the precise location of injured peripheral neurons and cortical neurons with reduced Arg3.1 expression, because altered cortical Arg3.1 expression and tinnitus behavior seem to be triggered by the periphery (Figs. 6, 8, and 10). We would therefore expect that those cortical neurons with reduced Arg3.1 levels would correspond to the frequency region of injured peripheral neurons in patients

with tinnitus. Indeed, patients with tinnitus identify a range of frequencies spanning the region of their hearing loss as resembling their tinnitus sensation (Eggermont and Roberts, 2004).

Recent findings show that the activation of $GABA_A$ receptors reduces cochlear injury after acoustic trauma (Murashita et al., 2007). The most prominent inhibitory signaling pathway in the cochlea is composed of the LOC and the medial olivocochlear efferents. Cochlear efferent feedback has not only been shown to balance interaural sensitivity (Darrow et al., 2007), but removal of LOC neurons also increases the vulnerability to acute acoustic injury (Darrow et al., 2007), emphasizing the crucial role of LOC efferents for the control of afferent auditory nerve activity. It is possible, therefore, that induction of tinnitus by salicylate results in a loss of GABAergic inhibition at the level of the cochlea. This may be due to a direct effect of salicylate on GABAergic fibers, as suggested for the hippocampus (Gong et al., 2008), leading ultimately to an increase in BDNF expression in spiral ganglion neurons and an increased spontaneous activity in the cochlear nerve and in the dorsal cochlear nucleus (Brozoski et al., 2002). The final result is altered cortical gene expression, altered neuronal plasticity, and altered auditory perception.

Our novel results regarding plasticity changes after cochlear trauma lead to the following postulations: 1) the salicylate-induced up-regulation of BDNF in spiral ganglion neurons may induce an imbalance of central auditory neuronal activity associated with tinnitus; 2) loss of peripheral $GABA_A$ receptor-mediated inhibition may cause aberrant plasticity changes in the auditory cortex; 3) tinnitus-associated changes in cochlear BDNF expression seem to trigger altered Arg3.1 expression in the auditory cortex; and 4) the influence of an inhibitory efferent feedback on peripheral neurons and cortical plasticity should be considered when designing tinnitus therapies.

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