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Salicylate Alters the Expression of Calcium Response Transcription Factor 1 in the Cochlea: Implications for Brain-Derived Neurotrophic Factor Transcriptional Regulation[S]

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) is a key neurotrophin whose expression is altered in response to neurological activity, influencing both short- and long-term synaptic changes. The BDNF gene consists of eight upstream exons (I-VII), each of which has a distinct promoter and can be independently spliced to the ninth coding exon (IX). We showed recently that the expression of BDNF exon IV in the cochlea is altered after exposure to salicylate, an ototoxic drug that in high doses is able to induce hearing loss and tinnitus. These changes were a crucial trigger for plasticity changes in the central auditory system. BDNF exon IV expression is regulated via interaction between calcium-response elements CaRE1, CaRE2, and CaRE3/Cre (CaREs) that are bound by the transcription factors CaRF1, upstream stimulatory factors 1 and 2 (USF1/2), and cAMP/Ca^{2+} response element-binding protein (CREB), respectively. To determine whether the salicylate-induced changes in cochlear BDNF exon IV expression include a differential use of the CaRE binding proteins, we studied the level of the corresponding binding proteins in the spiral ganglion neurons before and after systemic application of concentrated salicylate using in situ hybridization and RT-PCR. BDNF exon IV and CaRF1 expression were up-regulated after application of salicylate, whereas USF1/2 and CREB mRNA expression remained unaffected. The changes in BDNF exon IV and CaRF1 expression were also dose-dependent. The data show Ca^{2+} and CaRF1 as messengers of trauma (salicylate)-induced altered BDNF levels in the cochlea. Furthermore, they also provide the first evidence that a differential regulation of BDNF transcription factors might participate in BDNF-mediated plasticity changes.

Brain-derived neurotrophic factor (BDNF) plays an important role in neuronal survival, cortical development, and synaptic plasticity (Thoenen, 2000). BDNF transcription is stimulated dramatically by membrane depolarization (Ghosh et al., 1994b), neural activity during kindling, or long-term potentiation (Patterson et al., 1992). 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 (Rüttiger et al., 2007; Tan et al., 2007), the present work adopts the system of Aid et al. (2007).

The BDNF gene possesses eight 5′-noncoding exons (I-VIII), each of which is independently regulated and can be alternatively spliced to a common 3′-exon (IX), which contains the protein coding sequence (Timmusk et al., 1993; Aid et al., 2007). Each upstream exon is affected by neuron-specific signal transduction mechanisms (West et al., 2001; Aid et al., 2007). Although the individual function of the BDNF transcripts are not yet known, a differential role during plasticity-associated changes of synaptic efficacy has been suggested (Patterson et al., 1992; Thoenen, 2000; West et al., 2001).

We demonstrated recently that the application of the ototoxic drug salicylate results in the up-regulation of BDNF exon IV and VI (previously exons III and IV) in spiral gan-

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ABBREVIATIONS: BDNF, brain-derived neurotrophic factor; CaRF1, calcium response transcription factor 1; USF, upstream stimulatory factor; CREB, cAMP/Ca^{2+} response element binding protein; CaRE, calcium response element; PCR, polymerase chain reaction; RT-PCR, reverse-transcriptase polymerase chain reaction; NMDA, *N*-methyl-D-aspartate; L-VSCC, L-type voltage-sensitive Ca^{2+} channel; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair(s).

glion neurons of the cochlea, and that this up-regulation is associated with changes in cortical gene expression and tinnitus perception (R. Panford-Walsh, W. Singer, L. Rüttiger, S. Hadjab, J. Tan, M. Geisler, U. Zimmerman, I. Köpschall, K. Röhbock, A. Vieljans, et al., submitted). Trauma-induced up-regulation (R. Panford-Walsh, W. Singer, L. Rüttiger, S. Hadjab, J. Tan, M. Geisler, U. Zimmerman, I. Köpschall, K. Röhbock, A. Vieljans, et al., submitted; Tan et al., 2007) and age-related decrease (Rüttiger et al., 2007) of cochlear BDNF levels were discussed as a trigger for pathological plasticity responses in the central auditory system. As such, the elucidation of the mechanisms that participate in the trauma-induced alteration of cochlear BDNF levels may be crucial for future therapeutic designs.

The neuronal responses after systemic application of salicylate are contradictory. Salicylate either reduces (Wallhäusser-Franke et al., 2003) or increases neuronal firing rates (Evans and Borerwe, 1982; Wallhäusser-Franke et al., 2003) and exhibits dose-dependent effects (Stypulkowski, 1990). A dose-dependent effect of salicylate has been described to act by potentiating NMDA receptor currents in cortical or spiral ganglion neurons (Peng et al., 2003). Salicylate, however, may also influence cochlear neuron excitability through its inhibitory influence on outer hair cell motility (Grosh et al., 2004), thereby differentially facilitating the excitatory effects of glutamate (Müller et al., 2003). It is most interesting that NMDA receptors and L-type voltage-sensitive Ca^{2+} channels (L-VSCC), which mediate the calcium-dependent recruitment of distinct transcription factors on BDNF promoters, were both described as being expressed in cochlear neurons (Niedzielski and Wenthold, 1995; Waka et al., 2003).

To date, the promoter of BDNF exons I, IV, and VI have been studied in detail (Tabuchi et al., 2002; Takeuchi et al., 2002; Tao et al., 2002). Within the exon IV promoter, three calcium response elements (CaREs) have been identified (Tao et al., 2002), which are bound by cAMP/Ca^{2+} response element binding protein (CREB) (Montminy and Bilezikjian, 1987), upstream stimulatory factors 1 and 2 (USF1 and USF2) (Sawadogo and Roeder, 1985), and calcium-response transcription factor 1 (CaRF1) (Tao et al., 2002) in a cooperative manner.

CREB interacts with CaRE3 by binding to the consensus sequence known as cAMP response element (Kornhauser et al., 2002). CREB is prebound in an inactive form and activates the transcription of exon IV after phosphorylation through calmodulin kinase IV (Kornhauser et al., 2002). The heterodimer consisting of USF1 and USF2 (Vallet et al., 1998) binds to CaRE2 (Sawadogo and Roeder, 1985). Finally, CaRE1 is bound in a calcium- and neuron-selective manner by CaRF1, which is known to be expressed in distinct regions of the brain, with the highest expression in the hippocampus (Tao et al., 2002). It is assumed that for normal neuronal plasticity, these transcription factors are expressed at relatively stable levels (Chen et al., 2003b).

Here, we used RT-PCR and in situ hybridization to investigate the expression of CaRF1, USF1/2, and CREB in the cochlea after exposure to salicylate. We observed striking changes not only in BDNF exon IV expression but also in the expression of the transcription factor CaRF1; a dose-dependent effect on gene expression was also observed. Our data show that the expression (as opposed to the activity) of protein factors involved in the regulation of BDNF exon IV transcription is altered. Such a mechanism would dramatically alter the currently accepted view of the BDNF activation during plasticity responses.

Materials and Methods

Animals and Drug Application. Female adult Wistar rats weighing between 200 and 300 g were used in this study. Animal care and treatment was based on the institutional guidelines of the University of Tübingen Veterinary Care Unit.

For systemic application, animals received intraperitoneal injections of different concentration of salicylate (aspirin; Sigma, Munich, Germany) (350 and 500 mg/kg body weight) 3 h before sacrifice. Control animals received the corresponding volumes of saline solution.

Tissue Preparation. Before decapitation, animals were asphyxiated with carbon dioxide. Cochleae were then isolated and dissected as described in Knipper et al. (2000). For immunostaining, cochleae were dissected and fixed by injection with 2% paraformaldehyde for 30 min, and the bony part of the lateral wall of the cochlear turns was removed. All cochleae for in situ and for immunohistochemistry were embedded in O.C.T. compound (Miles Laboratories, Elkhart, IN), cryosectioned at 10 μm, and mounted on SuperFrost*/Plus microscope slides at −20°C.

Riboprobe Synthesis and in Situ Hybridization. Antisense and sense primers for the amplification of BDNF exons IV, VI, and IX from rat genomic DNA were constructed as described in Timmusk et al. (1993). For riboprobe synthesis of the CaRE binding proteins, mRNA was isolated from rat hippocampus with the Dynal mRNA Direct Kit (Invitrogen, Karlsruhe, Germany). Reverse transcription was done with Superscript II (Invitrogen). PCR was used to amplify the CaRE binding proteins.

The following primer pairs were used: 1) CREB: 5′ TTG ATT CAT GAC CAT GGA CTC TGG CAG 3′ and 5′ TTG AAT TCT TAA TCT GAC TTG TGG CAG TAA AG 3′, accession number NM_134443; 2) USF1: 5′ ATG AAG GGG CAG CAG AAA ACA G 3′ and 5′ ACG GCG GTT GTA CTC CCA GAT 3′, accession number NM_031777; 3) USF2: 5′ CAA TGA GCT CCT GAG GCA GCA GAT C 3′ and 5′ CAC ACA CAC ACG CGC ACA CAT ACA C 3′, accession number NM_031139; and 4) CaRF1: 5′ AAG TTG CAG CCA AGA CTC TCC TC 3′ and 5′ AGT CAA AAC TGC CCA TCA TCC AC 3′, accession number XM_217411. In the PCR reaction, cDNA was denatured for 3 min at 94°C followed by 33 cycles consisting of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. A final extension was done at 72°C for 10 min. Riboprobe synthesis was carried out as described in Rüttiger et al. (2007) and Tan et al. (2007).

In situ hybridization was performed as described previously (Wiechers et al., 1999). In brief, after incubating the slides with antidigoxigenin antibody conjugated to alkaline phosphatase (1:750; Roche, Mannheim, Germany), the sections were then allowed to develop in the substrate solution containing nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). The sections were observed at different time periods to monitor the development of the substrate into a colored product. Sections from controls and treated animals were stopped at the same time, mounted, and viewed using an Olympus AX70 microscope (Olympus, Tokyo, Japan). All experiments were done in duplicate with $n = 3$ for each group, control and treated animals.

Immunohistochemistry. For immunostaining, slices were washed two times in 50 mM phosphate-buffered saline (1× PBS). Sections were incubated for 30 min in NGS at room temperature. The primary antibody, phospho-CREB (Ser133) (Cell Signaling Technology, Danvers, MA), was diluted 1:50 and incubated on the slices overnight at 4°C. On the second day, sections were washed with 1× PBS three times. Primary antisera were visualized with Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA), diluted 1:1500, and incubated for 1 h at room temperature in the dark. Slices were washed again three times with 1× PBS. Sections were then mounted in Vectashield mounting medium containing 4,6-diamidino-2-phenylindole nuclear staining

(Vector Laboratories, Burlingame, CA) and viewed using an Olympus AX70 microscope. The experiments were done in duplicate with $n = 3$ for each group, control and treated animals.

Semiquantitative Reverse-Transcription PCR. Total RNA was isolated from cochleae using the Total RNeasy Kit from Qiagen (Hilden, Germany) and treated with DNase (Ambion, Huntingdon, Cambridgeshire, UK) to remove DNA contaminants. A duplex PCR reaction was performed as described in Zuccato et al. (2001). A reduced number of cycles (27) was initially tested to ensure nonsaturating amplification of GAPDH and cyclophilin and an equivalent usage of cDNAs in both control and treated samples. The PCR primer sequences for BDNF exon IV and GAPDH were as described in Zha et al. (2001). The calcium-response element binding proteins were amplified using the primer pairs described above. Amplification of cyclophilin was accomplished using the following primer sequences: 5′ CGT GCT AGC ACT GGG GAG AAA 3′ and 5′ CAT GCC TTC TTT CAC CTT CCC AAA GAC 3′, accession number NM_017101. The amplification conditions used for BDNF exon IV and GAPDH were as described in Tan et al. (2007). For CaRF1 and USF1, the amplification program consisted of an initial denaturation phase of 94°C for 3 min, 33 cycles of 1-min denaturation (94°C), 1 min of annealing (61°C), 1.5-min extension (72°C), and a final extension phase of 10 min at 72°C. For USF2 and CREB, an initial denaturation phase of 94°C for 3 min was used, followed by 33 cycles of 30-s denaturation (94°C), 30 s annealing (65°C), 40 s extension (72°C), and a final extension phase of 10 min at 72°C. The PCR products were cloned and sequenced. 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 GAPDH or cyclophilin level. Control and treated groups were then compared with each other, and the data were expressed as a mean percentage of control (set at 100%) and standard error of the mean (S.E.M.). Statistical analysis was done using the Student's t test ($*$, $p < 0.05$). The number of animals used for statistical analyses was the same within each pair of control and treated animals. For statistical analyses, 3 h after 350 mg/kg salicylate, cochleae from 10 rats were analyzed for BDNF exon IV and CaRF1. Ten rats were analyzed for USF1, USF2, and CREB. The number always indicates animals in each group (control and treated), not the sum of both. For BDNF, exon IV and CaRF1 analysis after dose-dependent application, 10 rats in each group were analyzed.

Results

Expression of BDNF and the CaRE Binding Proteins in Spiral Ganglion Neurons. BDNF exon IV is known to be expressed in the adult cochlea (Panford-Walsh et al., submitted; Schimmang et al., 2003; Tan et al., 2007). We show for the first time the detection of the four transcription factors of BDNF exon IV in spiral ganglion neurons of the mammalian cochlea using RT-PCR (Fig. 1A) and in situ hybridization (Fig. 1 B). Primers were designed for the CaRE binding proteins as described under *Materials and Methods*. The expected PCR products were amplified with a bp length of 594 bp for CaRF1, 416 bp for USF1, 599 bp for USF2, and 991 bp for CREB (arrows in Fig. 1A). For in situ hybridization, riboprobes were synthesized from the cloned PCR products. Antisense riboprobes specific for BDNF exon IV (data not shown) and the CaRE binding proteins produced positive hybridization signals in the spiral ganglion neurons of the cochlea (Fig. 1B shown for the midbasal turn). No hybridization signals were observed using sense probes (Fig. 1B, insets).

The cochlea is tonotopically organized; i.e., the sensory cells in the apical (top) turn respond specifically to low-frequency sound waves, whereas the sensory cells in the basal (bottom) turn respond to high frequency sound waves. Along the tonotopic axis, BDNF exon IV is expressed in a gradient, with increasing expression in the midbasal/basal turns of the cochlea (Schimmang et al., 2003; Rüttiger et al., 2007). Using sequence-specific riboprobes, we confirmed the tonotopic distribution of BDNF in spiral ganglion neurons of the cochlea (Fig. 2A). A strikingly similar pattern of expression was observed for CaRF1 (Fig. 2 B), with mRNA expression increasing in spiral ganglion neurons of the medial and midbasal/basal turns. In contrast, no significant changes were seen in the expression of CREB, USF1, or USF2 along the tonotopic axis (shown for USF2, Fig. 2C).

Systemic Injection of Concentrated Salicylate Alters CaRF1 Expression in the Cochlea. BDNF exon IV is up-regulated after induction of tinnitus by systemic injection of concentrated salicylate (Panford-Walsh et al., submitted). We thus proceeded to investigate the effect of salicylate on the expression of BDNF exon IV and its transcription factors CaRF1, USF1/2, and CREB in spiral ganglion neurons of the cochlea. Three hours after salicylate application, the cochleae of the individually treated animals were prepared and analyzed by RT-PCR. BDNF exon IV mRNA expression was significantly up-regulated in cochlear tissue after injection of 350 mg/kg salicylate (Fig. 3A). A similar increase in cochlear CaRF1 expression was observed upon salicylate injection (Fig. 3B), whereas the same treatment had no effect on the expression of USF1, USF2, and CREB (Fig. 3C).

The effect of salicylate on cochlear gene expression was further confirmed by in situ hybridization on cryosections of isolated cochleae (Fig. 4). As seen with RT-PCR, both BDNF

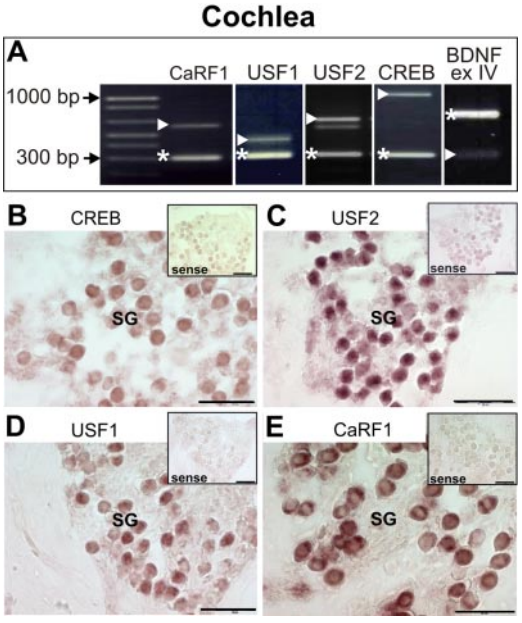

Fig. 1. Expression of CaRE binding proteins in the cochlea. Primers were designed for the transcription factors CaRF1, USF1, USF2 and CREB. A, the four transcription factors were amplified from the mammalian cochlea by RT-PCR. CaRF1 had a predicted size of 594 bp; USF1, 416 bp; USF2, 599 bp (a double band was consistently seen; however, sequence analysis confirmed the upper band as USF2); and CREB, 911 bp. Gene-specific bands are indicated by arrowheads. Internal control bands (cyclophilin for CaRF1, USF1/2, and CREB; GAPDH for BDNF exon IV) are indicated by asterisks. B, the expression of CaRF1, USF1/USF2, and CREB in spiral ganglion neurons was confirmed by in situ hybridization, shown here for the midbasal turn of the rat cochlea.

exon IV and CaRF1 were strongly up-regulated in the spiral ganglion neurons of the cochlea after injection of 350 mg/kg salicylate (Fig. 4, A and B; shown for the midbasal cochlear turns). Again, treatment with salicylate had no effect on the expression of USF1/2 (Fig. 4C, shown for USF2) or CREB (data not shown). Hybridization with sense riboprobes produced no detectable signal (insets in Fig. 4, A–C).

Dose-Dependent Alteration of Activity-Dependent Genes in the Cochlea. To assess in detail the dynamics of CaRF1 expression in spiral ganglion neurons of the cochlea induced by acute salicylate application (Figs. 3 and 4), we used in situ hybridization and RT-PCR to test the effects of 350 and 500 mg/kg salicylate injected intraperitoneally (Fig. 5). For BDNF exon IV, a peak at 350 mg/kg salicylate was observed followed by a decrease at higher salicylate doses shown in spiral ganglion neurons of the medial turn of the cochlea (Fig. 5, A and C). Likewise, CaRF1 expression was significantly up-regulated 3 h after injection of 350 mg/kg salicylate but was decreasing at higher doses of salicylate (Fig. 5, B and D). No changes in gene expression were observed for USF1, USF2, or CREB using various doses of salicylate (data not shown).

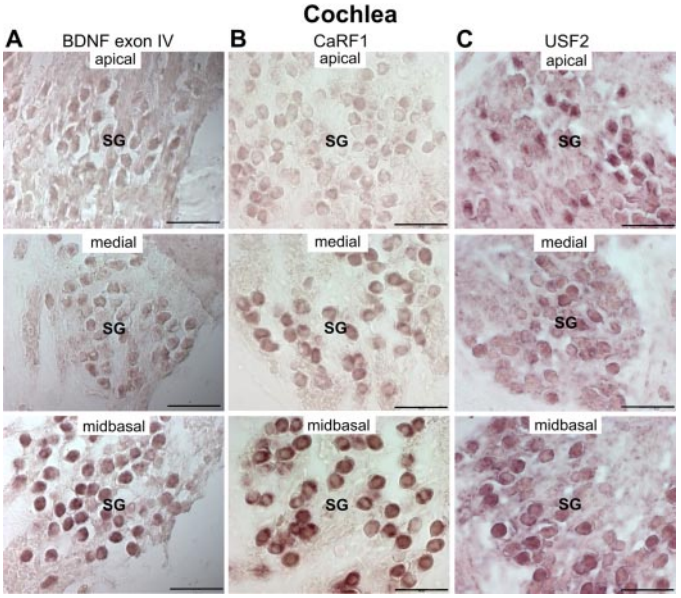

Fig. 2. Tonotopic distribution of the BDNF exon IV and the CaRE binding proteins. A, for BDNF exon IV, an increase in mRNA expression in spiral ganglion neurons can be seen from the apical toward the more basal cochlear turns. B, for the transcription factor CaRF1, a similar tonotopic distribution of the mRNA is seen, with an increase in expression from the apical toward the more basal turns of the cochlea. C, USF1, USF2, and CREB do not show changes in expression along the tonotopic axis, shown for USF2. SG, spiral ganglion. Scale bars, 50 μm; $n = 3$ for each group of control and treated animals.

Phosphorylation of CREB after Systemic Injection of Concentrated Salicylate. Because it is known that CREB is prebound to the promoter in an inactive form and is activated by phosphorylation through calmodulin kinase IV (Kornhauser et al., 2002), the impact of salicylate on the phosphorylation of CREB in spiral ganglion neurons was tested. In a preliminary experiment, a change in phosphorylation of CREB after salicylate injection (350 mg/kg) (Supplemental Fig. S1, red staining) is shown by immunohistochemistry. An antibody against phospho-CREB (Ser133) was used for these experiments. A tonotopic phospho-CREB expression gradient was observed in the nuclei of spiral ganglion neurons in control animals (Supplemental Fig. S1A, left column, red staining). The highest number of phospho-CREB immunopositive cells was noted in untreated animals in the most apical cochlear turn, which perceives low frequencies (Supplemental Fig. S1A, top left image, red staining). This gradient was surprisingly the reverse of that normally seen for BDNF exon IV and CaRF1 (Fig. 2, A and B). After salicylate treatment, the phosphorylation of CREB was particularly increased in the nuclei of spiral ganglion neurons of the most basal, high-frequency-generating cochlear turns in comparison with control animals (Supplemental Fig. S1A, bottom images, and B). Although more detailed and quantitative data are required, it seems that the number of phospho-CREB-positive cells in more apical cochlear turns remained unchanged (Supplemental Fig. S1A). Higher magnification of spiral ganglion neurons of the basal cochlear turn underlined the strong increase in CREB phosphorylation (Supplemental Fig. S1B, red staining, arrows). Up-regulation of phospho-CREB in the basal/midbasal cochlear turns is in accordance with the mRNA expression of BDNF exon IV and CaRF1 in the basal turn of the cochlea and the mRNA up-regulation after salicylate treatment in this area (Figs. 2, A and B, and 3, A and B).

Discussion

BDNF is known as an immediate early gene with mRNA transcription occurring rapidly without the need of new protein synthesis (Lauterborn et al., 1996). BDNF transcription is regulated by neuronal activity (Shieh and Ghosh, 1999); up-regulation of BDNF expression was first seen after exper-

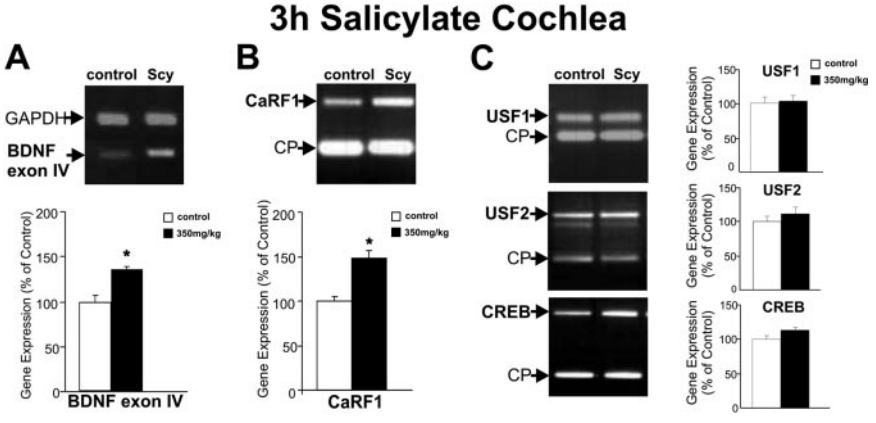

Fig. 3. RT-PCR analysis of BDNF exon IV and CaRE binding proteins after salicylate treatment. A, BDNF exon IV is significantly up-regulated 3 h after salicylate (Scy) injection (representative PCR and graph, $n = 10$ in each group, control and treated animals). B, CaRF1 is also significantly up-regulated 3 h after salicylate injection (representative PCR and graph, $n = 10$ in each group, control and treated animals). C, USF1 ($n = 10$ in each group, control and treated animals), USF2 ($n = 10$ in each group, control and treated animals), and CREB ($n = 10$ in each group, control and treated animals) expression are not changed after salicylate injection. GAPDH and cyclophilin (CP) are used as internal PCR controls. The values are expressed as the mean percentage of control. *, $p < 0.05$

imentally induced seizure (Isackson et al., 1991). An increase was also seen in vivo after sensory stimulation and in vitro after neuronal activity, suggesting that neuronal activity affects BDNF regulation (Ghosh and Greenberg, 1995). Furthermore, BDNF exon IV is expressed in the auditory system, and its expression is known to be affected by exposure to salicylate (Panford-Walsh et al., submitted).

Although the expression of the transcription factors of BDNF exon IV is presumed to be relatively stable (Chen et al., 2003a), we found here that the expression of the transactive elements of the BDNF exon IV promoter is regulated in an activity-dependent manner. The exact mechanism of regulation of these element is still unclear, although it is well known that depolarization and the subsequent Ca^{2+} influx induces BDNF exon IV transcription via pre-existing transcription factors (West et al., 2001).

In this study, we investigated the expression of BDNF exon IV and its transcription factors in the spiral ganglion neurons of the mammalian cochlea in response to injection of concentrated salicylate. We showed that salicylate is able to induce an alteration not only in BDNF exon IV expression but also in the expression of its transcription factors. Three hours after injection, an up-regulation of BDNF exon IV and CaRF1 was observed in spiral ganglion neurons (Figs. 3 and 4), suggesting that CaRF1 plays an important role in the regulation of BDNF exon IV expression in the cochlea. Furthermore, the changes in gene expression are dependent on the dose of salicylate given (Fig. 5).

A dose-dependent effect of salicylate has been described to act by potentiating NMDA receptor currents in cortical (Vane et al., 1998) or cochlear neurons (Peng et al., 2003). The experimental conditions used may thus act through changes in glutamate or NMDA receptors, which initiate the calcium-

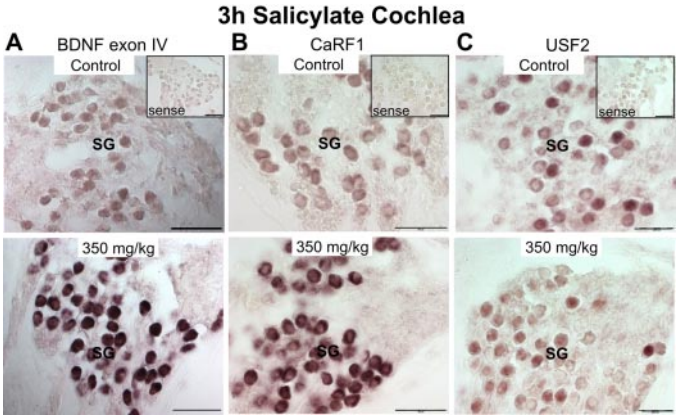

Fig. 4. In situ hybridization analysis of BDNF exon IV and CaRE binding proteins after salicylate treatment 3 h after salicylate. A, the mRNA expression of BDNF exon IV in spiral ganglion neurons is up-regulated 3 h after salicylate injection. B, CaRF1 expression is also up-regulated in spiral ganglion neurons 3 h after salicylate injection. C, the expression of USF1, USF2, and CREB, however, remained unchanged (shown for USF2). Images show the midbasal turn of the mammalian cochlea. SG, spiral ganglion. Scale bars, 50 μm; $n = 3$ for each group of control and treated animals.

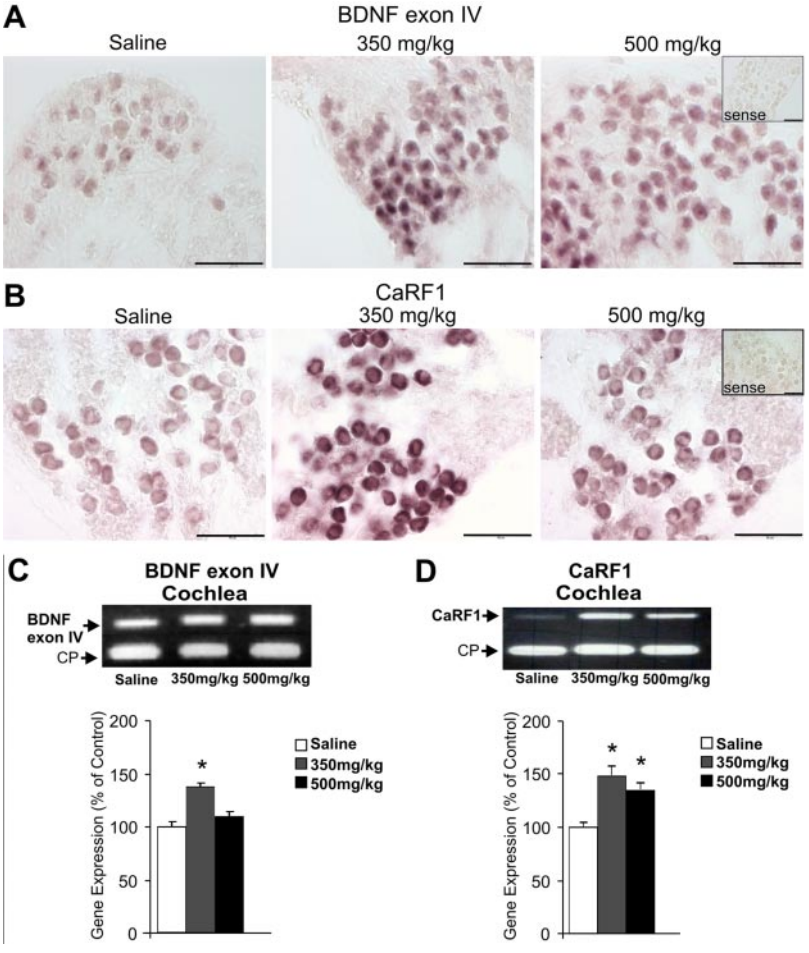

Fig. 5. Dose-dependent effect of salicylate application on BDNF exon IV and CaRF1 expression. A and C, for BDNF exon IV, dose-dependent effects are observed in spiral ganglion neurons 3 h after salicylate injection, shown by in situ hybridization (A, shown for the apical turn) and RT-PCR (C). A significant up-regulation is seen after injection of 350 mg/kg salicylate, which is decreasing after 500 mg/kg salicylate. Scale bars in A, 50 μm, $n = 3$ for each group control and treated animals. SG, spiral ganglion. Cyclophilin (CP) is used as an internal PCR control (C). Statistical analysis was done using the Student's t test. *, $p < 0.05$ (representative PCR and graph, $n = 10$ for each group, control and treated animals). B and D, in spiral ganglion neurons of the cochlea, a dose-dependent effect is also seen for CaRF1 expression after application of various concentrations of salicylate. CaRF1 expression is significantly increased at 350 mg/kg salicylate; this up-regulation is decreasing at 500 mg/kg salicylate, but the expression is still significantly higher than in control animals, shown by in situ hybridization (B, shown for the midbasal turn) and RT-PCR (D). Scale bars in B, 50 μm, $n = 3$ for each group, control and treated animals. CP is used as an internal PCR control (D). Statistical analysis was done using the Student's t test. *, $p < 0.05$. Representative PCR and graph, $n = 10$ for each group, control and treated animals.

dependent recruitment of transcription factors known to act on BDNF exon IV (Shieh and Ghosh, 1999).

Activity-dependent transcription subsequent to Ca^{2+} influx affects the different promoters of BDNF (Kim et al., 2001). There are different ways in which calcium can enter the cell, but not all lead to equivalent activation of downstream gene expression. Calcium entry via NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors, glutamate receptors, or release from intracellular Ca^{2+} stores are not sufficient to induce BDNF expression (Ghosh et al., 1994a). Only calcium influx through VSCCs increases BDNF expression and subsequent cell survival (Ghosh et al., 1994a; Tao et al., 2002).

In agreement with the current view that glutamate is the afferent neurotransmitter of hair cells, acting on defined glutamate receptors (Puel, 1995), salicylate was shown recently to potentiate glutamate-mediated currents in cochlear neurons (Peng et al., 2003). This would directly lead to a change in the opening kinetics of voltage-dependent L-type Ca^{2+} channels. Considering that up- or down-regulation of BDNF is dependent on the degree of stimulation (Tabuchi et al., 2000), we suggest that the observed dose-dependent effects of salicylate on CaRF1 described in the present study may result from an indirect dose-dependent effect of salicylate on L-type Ca^{2+} channels in cochlear neurons. These changing Ca^{2+}-signaling events can lead to activation of Ca^{2+}/calmodulin-dependent protein kinases, which cause an up-regulation of BDNF mRNA expression (Zafra et al., 1992). It is currently presumed that these in vivo Ca^{2+}-signaling cascades do not in parallel act on the expression of CaRE binding proteins because they are supposed to be expressed relatively stable in the cell. The changes in CaRF1 expression after salicylate application presented in this study, however, indicate that the stability of transacting factors may not be a general rule.

In line with the previous view, the current data indicate that the transcription factors CREB, USF1, and USF2 show no changes in gene expression after injection of concentrated salicylate. A possible explanation could be that they are prebound in an inactive state to the BDNF promoter. Upon a specific activation event, they would then presumably assist in the initiation of transcription. Indeed, it has been shown that USF1/2 are present in unstimulated and membrane-depolarized cells (Chen et al., 2003b) and that the DNA binding activity of the USFs to the BDNF exon IV promoter is not regulated by Ca^{2+} influx (Chen et al., 2003b).

CREB is known to be prebound to the BDNF exon IV promoter in an inactive form (West et al., 2001). Ca^{2+} influx leads to phosphorylation of CREB at Ser133, followed by recruitment of the CREB binding protein, which acts as a transcriptional coactivator (West et al., 2001). The localization of CREB is not influenced by the intracellular Ca^{2+} level (West et al., 2001). Thus, the nonresponsiveness of CREB mRNA in cochlear neurons to various concentrations of salicylate treatment documented in the present study is not surprising. It is also shown that an increase in the phosphorylation of CREB protein after salicylate injection (Supplemental Fig. S1) takes place specifically in the spiral ganglion neurons of those cochlear turns in which BDNF exon IV mRNA is up-regulated after salicylate treatment. This supports the notion that the phosphorylation of CREB, by either CaMKs or cAMP-dependent protein kinase (Zha et al., 2001), may be involved in the signaling pathway that is initiated in spiral ganglion neurons after salicylate treatment. In further studies, the phosphorylation mechanism of CREB and its involvement in transcriptional events of BDNF in spiral ganglion neurons should be analyzed in detail.

In vitro studies with embryonal cortical cells did already point to CaRF1 being regulated in a calcium- and neuron-selective manner similar to BDNF exon IV (Tao et al., 2002). It has been shown that CaRF1 expression is only affected by Ca^{2+} influx through L-VSCCs, consistent with the hypothesis that CaRF1 drives the calcium-dependent regulation of BDNF. CaRF1 expression is highest in the brain, although it is also expressed at low levels in non-neuronal tissue. CaRF1 expression in the brain is seen in areas in which activity-dependent BDNF exon IV transcripts occur. Thus, the expression pattern of CaRF1 is consistent with its role in regulation of BDNF exon IV transcription (Tao et al., 2002). Considering these observations, we may suggest a similar scenario for CaRF1 and BDNF exon IV in cochlear neurons subsequent to salicylate treatment.

Our hypothesis is that moderate degrees of injury lead to enhancement of Ca^{2+} levels in cochlear neurons (e.g., by lower and medium concentrations of salicylate or moderate noise trauma, $<$ 100 dB) and may induce a correspondent dose-dependent up-regulation of CaRF1, whereas higher degrees of injury lead to higher increases in Ca^{2+} levels in cochlear neurons (e.g., via salicylate $>$500 mg/kg of body weight or acoustic trauma $>$120 dB) and may induce an opposite (decline) effect on CaRF1. Ca^{2+}-imaging studies in cochlear neurons subsequent to various degree of acoustic trauma and various doses of salicylate may be required, however, to verify this concept.

The data in the present study show that injection of concentrated salicylate, which is known to induce tinnitus in humans and rodents, has an effect not only on the expression of BDNF exon IV but also on the expression of its transcription factors. Although CREB, USF1, and USF2 do not show any changes in their expression pattern after salicylate treatment, CaRF1 is clearly up-regulated in a dose-dependent manner. This suggests that CaRF1 is a crucial component of BDNF exon IV transcription. Bound as the last of the three transcription factors, CaRF1 could be responsible for the regulation of BDNF expression under different trauma conditions. Further studies must be done to distinguish between the degrees of injury, which cause effects in BDNF exon IV and CaRF1 expression.

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