

# Activity-Dependent Regulation of the Potassium Channel Subunits Kv1.1 and Kv3.1

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## ABSTRACT

Afferent activity, especially in young animals, can have profound influences on postsynaptic neuronal structure, function and metabolic processes. Most studies evaluating activity regulation of cellular components have examined the expression of ubiquitous cellular proteins as opposed to molecules that are specialized in the neurons of interest. Here we consider the regulation of two proteins (voltage-gated potassium channel subunits Kv1.1 and Kv3.1) that auditory brainstem neurons in birds and mammals express at uniquely high levels. Unilateral removal of the avian cochlea leads to rapid and dramatic reduction in the expression of both proteins in the nucleus magnocellularis (NM; a division of the avian cochlear nucleus) neurons as detected by immunocytochemistry. Uniform downregulation of Kv1.1 was reliable by 3 hours after cochlea removal, was sustained through 96 hours, and returned to control levels in the surviving neurons by 2 weeks. The activity-dependent changes in Kv3.1 appear to be bimodal and are more transient, being observed at 3 hours after cochlea removal and recovering to control levels within 24 hours. We also explored the functional properties of Kv1.1 in NM neurons deprived of auditory input for 24 hours by whole-cell recordings. Low-threshold potassium currents in deprived NM neurons were not significantly different from control neurons in their amplitude or sensitivity to dendrotoxin-I, a selective K<sup>+</sup> channel antagonist. We conclude that the highly specialized abundant expression of Kv1.1 and 3.1 channel subunits is not permanently regulated by synaptic activity and that changes in overall protein levels do not predict membrane pools. *J. Comp. Neurol.* 470: 93–106, 2004. © 2004 Wiley-Liss, Inc.

**Indexing terms:** cochlea removal; immunohistochemistry; voltage-gated potassium channel

Neurons and muscle depend on afferent innervation for maintenance of their normal anatomical and chemical properties (for reviews, see Globus, 1975; Sherrard and Bower, 1998; Altschuler et al., 1999). Lesion of afferent axons or dramatic changes in the amount or pattern of electrical activity often lead to alterations in neuron size, shape, RNA and protein synthesis, oxidative enzyme activity, and various electrophysiological properties in postsynaptic cells (Peterson and Kernell, 1970; Redfern and Thesleff, 1971; Pappone, 1980; Escobar et al., 1993; Taxi and Eugene, 1995; Desai et al., 1999; Francis and Manis, 2000; Abdulla and Smith, 2001; Astic and Saucier, 2001; Him and Dutia, 2001; Rubel and Fritzsche, 2002; but see Lo and Erzurumlu, 2001). It is noteworthy that the vast majority of studies that have examined the influence of afferent input on the intrinsic cellular or molecular properties of postsynaptic neurons have studied properties common to most or all neurons, e.g., protein and RNA

synthesis, transmitter regulation, general synaptic proteins, ubiquitous transcription factors, or cytoskeletal proteins. In the past decade, however, it has become evident that the intrinsic electrical properties as well as the anat-

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omy of individual neurons can be highly specialized for their specific function.

We have used the brainstem auditory pathways of the chick, gerbil, and mouse to understand further some of the cellular and molecular events that occur following deprivation of excitatory input (reviewed in Rubel et al., 1990; Rubel and Fritzsche, 2002). Again, however, we have concentrated on the general cellular characteristics and largely ignored the unique specializations of these neurons. In the study reported here, we carefully examine the two  $K^+$  channel proteins that are expressed at uniquely high levels in neurons specialized for processing temporal information with millisecond-range accuracy. Our goal was to determine whether this unique phenotype is temporarily or permanently regulated by afferent input in the nucleus magnocellularis (NM) neurons of the chick brainstem.

Neurons of the auditory brainstem nuclei exhibit specialized electrical properties that are likely to have an important role in preserving the temporal information in acoustic signals while responding to high rates of synaptic inputs (for reviews, see Oertel, 1999; Trussell, 1999). Exemplifying this is the remarkably robust expression of low- and high-voltage-activated (LVA and HVA, respectively) outward  $K^+$  currents (Manis and Marx, 1991; Reyes et al., 1994; Brew and Forsythe, 1995; Koyano et al., 1996; Rathouz and Trussell, 1998; Wang et al., 1998; Golding et al., 1999; Brew et al., 2003). The LVA  $K^+$  currents rapidly activate in response to small depolarization, thereby speeding the membrane time constant and minimizing temporal summation of synaptic inputs, which is critical for timing coding function of these neurons.

HVA  $K^+$  channels are expressed abundantly in neurons that fire at high rates (for reviews, see Gan and Kaczmarek, 1998; Wang et al., 1998; Rudy and McBain, 2001), including NM neurons (Parameshwaran et al., 2001). The HVA  $K^+$  currents activate at more depolarized membrane potentials, also inactivate very slowly, and deactivate rapidly. Activation of the HVA currents rapidly repolarizes the cell and shortens the duration of action potentials, enabling neurons to fire at high frequency in response to synaptic inputs.

At least 23 different alpha subunits of the voltage-gated potassium (Kv) family (Kv1.1–1.8, Kv2.1–2.2, Kv3.1–3.4, Kv4.1–4.3, Kv5.1, Kv6.1, Kv8.1, and Kv9.1–9.3) have been cloned (Coetzee et al., 1999). The likely molecular candidates for the LVA and HVA  $K^+$  currents in brainstem auditory neurons are Kv1.1 and Kv3.1, respectively, encoded by the genes *Kcna1* and *Kcnc1*, respectively (Trussell, 1999; Brew et al., 2003; Macica et al., 2003). Expression of both channel genes (*Kcna1* and *Kcnc1*) has been described in rodent brainstem auditory nuclei (Perny et al., 1992; Grigg et al., 2000; Rudy and McBain, 2001), and expression of both *Kcnc1* and its product (Kv3.1) has been described in detail in avian auditory brainstem neurons (Parameshwaran et al., 2001; Parameshwaran-Iyer et al., 2003). Anatomical data on the expression of Kv1.1 in avian brainstem auditory neurons are still lacking, and little is known about the regulation of these proteins in postnatal birds or mammals.

We use immunohistochemical methods to show that neurons of chicken NM express Kv1.1 at high levels, relative to other auditory and nonauditory areas of the brain-

stem. We demonstrate that this robust normal level of expression can be transiently modified by excitatory afferent input. Deprivation of auditory nerve activity by unilateral cochlea removal leads to a rapid and uniform reduction of Kv1.1 labeling that subsequently recovers by 2 weeks. Afferent regulation of Kv3.1 protein is more complex and highly transient; the initial effect of deprivation appears to be bimodal, and normal protein levels re-emerge by 24 hours following cochlea removal. Surprisingly, the reductions in Kv1.1 expression 24 hours after activity deprivation were not paralleled by changes in the amplitude or pharmacology of low-threshold outward  $K^+$  currents, as we show by a series of in vitro, whole-cell patch-clamp experiments. One electrophysiological feature that was influenced by our manipulation was the threshold of action potential generation. Deprived NM neurons had a significantly higher spike threshold and reduced spike amplitude relative to their nondeprived counterparts in the same brain slices.

## MATERIALS AND METHODS

### Animals and surgery

More than 70 white leghorn chickens were used for data collection. Chickens were hatched from eggs (supplied by H & N, Redmond, WA) incubated in our facility. Unilateral surgical removal of the cochlea (basilar papilla; BP) was performed on 5–10-day-old hatchlings for the anatomical studies (or on day 1 or 2 for the electrophysiological studies), as described previously (Born and Rubel, 1985; Garden et al., 1994). Briefly, animals were anesthetized by the inhalation anesthetic methoxyflurane (Metofane; Pitman-Moore Pharmaceuticals, Bray, Ireland). The tympanic membrane was punctured by using a #30 needle, and the columella was removed, exposing the oval window. A pair of fine forceps was used to remove the BP. The excised BP was floated in a Petri dish and examined to ensure complete removal. The ear canal was then closed with cyanoacrylic glue. This procedure leaves the cochlear ganglion neuronal cell bodies intact but immediately eliminates all action potentials in the auditory portion of the eighth nerve and NM neurons (Born and Rubel, 1985; Born et al., 1991). Previous research has shown that the changes in NM neuron number and size as well as several other intrinsic properties are independent of age for at least the first several postnatal weeks (Born and Rubel, 1985; Rubel and Fritzsche, 2002).

Survival times ranged from 1 hour to 2 weeks (see Results for numbers at each survival time). At the appropriate survival time, animals were deeply anesthetized with an overdose of pentobarbital (Pentosol; Delmarva Laboratories, Midlothian, VA) and perfused transcardially with either 4% paraformaldehyde or 10% formalin. The two fixatives yielded similar results. Vibratome sections (40  $\mu$ m thick) were collected in phosphate-buffered saline (PBS; pH 7.3). All brains were sectioned in the transverse plane, and care was taken to orient each brain such that the sections were bilaterally symmetrical. All immunohistochemistry steps were performed on free-floating sections. Experimental procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

### Kv1.1 immunohistochemistry

Affinity-purified, polyclonal Kv1.1 antibodies were made as described previously (Wang et al., 1993). Biochemically and in mammalian tissue, anti-Kv1.1 does not cross-react with other *Shaker*-family K<sup>+</sup> channels or with K<sup>+</sup> channels from other families (Wang et al., 1993).

Tissue sections were rinsed in PBS three times at 10 minutes per rinse between steps. One hour preblock of tissue in PBS containing 4% normal goat serum (NGS), 0.1% gelatin, and 0.02% Triton X-100 preceded incubation in primary antibody. Sera were diluted at 1:500, 1:100, or 1:50 in 0.1% gelatin/PBS with 0.02% Triton-X 100. Because several batches of antibodies were used, different dilutions for each batch were examined to make sure that changes in expression were not masked by floor effects or antibody saturation. Incubation with the primary antibody lasted for 50 hours at 4°C. Endogenous peroxidase activity was quenched in 3% H<sub>2</sub>O<sub>2</sub> for 10 minutes. Biotinylated goat anti-rabbit secondary antibody was diluted at 1:400 in 0.1% gelatin and 0.02% Triton X-100 for 1 hour at room temperature, followed by avidin-biotin conjugation (ABC). Sections were kept overnight in PBS at 4°C. For amplification, the secondary antibody and ABC steps were repeated, and then the sections were reacted with a nickel-intensified 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) reaction. Negative controls were processed as above but incubated with 4% NGS instead of primary antibody. Sections were mounted onto gelatin-coated slides, kept overnight in a 37°C oven, dehydrated, cleared, and coverslipped with DPX (Electron Microscopy Sciences, Fort Washington, PA).

To confirm the specificity of Kv1.1 labeling in chick brain, some sections received solutions of anti-Kv1.1 preincubated with the fusion protein GST 1.1, which contains the specific carboxy-terminal sequence of mouse Kv1.1. The primary solution for anti-Kv1.1 was prepared as above, and fusion protein was added at a ratio of 1 µg protein/µl of primary serum. This solution was incubated for 2 hours at room temperature before it was added to tissue. In addition, control sections were processed without primary antibody with each group of sections.

### Kv3.1 immunohistochemistry

Affinity-purified, polyclonal antibodies recognizing mouse Kv3.1b were obtained from Sigma (St. Louis, MO). The specificity of this antibody in the chick has been described (Parameshwaran et al., 2001). Tissue sections were rinsed in PBS three times at 10 minutes per rinse between steps. Endogenous peroxidase activity was quenched in 1% H<sub>2</sub>O<sub>2</sub> and 50% methanol PBS for 10 minutes. Before incubation in primary antibody, the tissues were preblocked for 1 hour in 10% fetal bovine serum (FBS), 1% Triton X-100, and 0.02% Na<sup>+</sup> azide in Dulbecco's modified Eagle's medium (DMEM). Sera were diluted at 1:5,000 in 2% bovine serum albumin. (Sections from one animal were processed at a dilution of 1:2,000.) Sections were incubated with primary antibody overnight at 4°C. Biotinylated goat anti-rabbit secondary antibody was diluted at 1:400 in 0.1% gelatin and 0.02% Triton X-100 for 1 hour at room temperature. After ABC incubation for 1 hour, tissue sections were kept overnight in PBS at 4°C and then processed with a nickel-intensified DAB reaction. Negative controls were processed without primary

antibody. Sections were mounted, dehydrated, cleared, and coverslipped as described above.

### Densitometry

To quantify the differences in expression level of Kv1.1 and Kv3.1 proteins present in afferent-deprived NM neurons versus neurons with intact afferents on the other side of the same tissue section, the optical density (OD) of the DAB reaction product in individual cells was analyzed by using NIH Object Image. Care was taken to cut sections symmetrically so that the same tonotopic region was analyzed on each side of the brain. One section from the brain of each animal that contained at least 40 NM neurons on each side and was from the middle one-third of the rostrocaudal axis of the nucleus was selected for this analysis. Cells were visualized under a 63× oil objective (N.A. 1.4) on a Leitz (Wetzlar, Germany) Aristoplan microscope. A digital camera (Sony DXC-960) was used to transfer the image to the computer for digitizing and to a video monitor. A field outside the NM was imaged and used for background subtraction. NM neurons were outlined, and the average OD of immunoreactivity in the neurons was measured, yielding OD measurements of a minimum of 40 NM neurons on each side of the brain of each animal. Overlapping cells were not included. Nuclear regions were not excluded because clear boundaries of the nuclei could not always be detected due to the thickness of the sections. In all cases, the two sides of the same tissue section were analyzed in one sitting without any changes in the microscope settings. Any small changes in current affecting image intensity would then be randomized between sides of the brain.

### Statistical analysis

The quantitative and statistical procedures are similar to those described in detail in earlier publications (Born and Rubel, 1988; Hyde and Durham, 1990; Garden et al., 1994). Absolute ODs of labeled neurons vary among brain sections and among the brains of different animals because of a host of factors, such as perfusion, rinsing, age of antibody, and variability of the chemical reaction that produces the colored precipitate. Thus, in our opinion, raw data cannot be meaningfully combined across tissue sections or across subjects. For statistical analyses of grouped data, we used the following normalization procedure. We calculated a standard score (Z-score) for each NM neuron by normalizing all the OD scores obtained from a given animal to the mean and standard deviation of ODs for the NM neurons on the side of the section opposite the manipulation (control side of the brain). The formula [(OD - mean of the control ODs)/standard deviation of the control ODs] was used to determine a Z-score for each neuron. This formula provides a Z-score that is equal to the number of standard deviations a particular neuron's OD varies from the mean OD of NM neurons on the side of the tissue section contralateral to the manipulation. By normalizing the OD of each neuron to the mean OD of control NM neurons in the same tissue section, comparisons across animals and survival times are possible.

To determine whether there is a statistically significant effect of afferent deprivation on Kv immunoreactivity at various times following cochlea removal, the mean Z-score for afferent-deprived neurons from each animal was treated as a single observation and combined with the

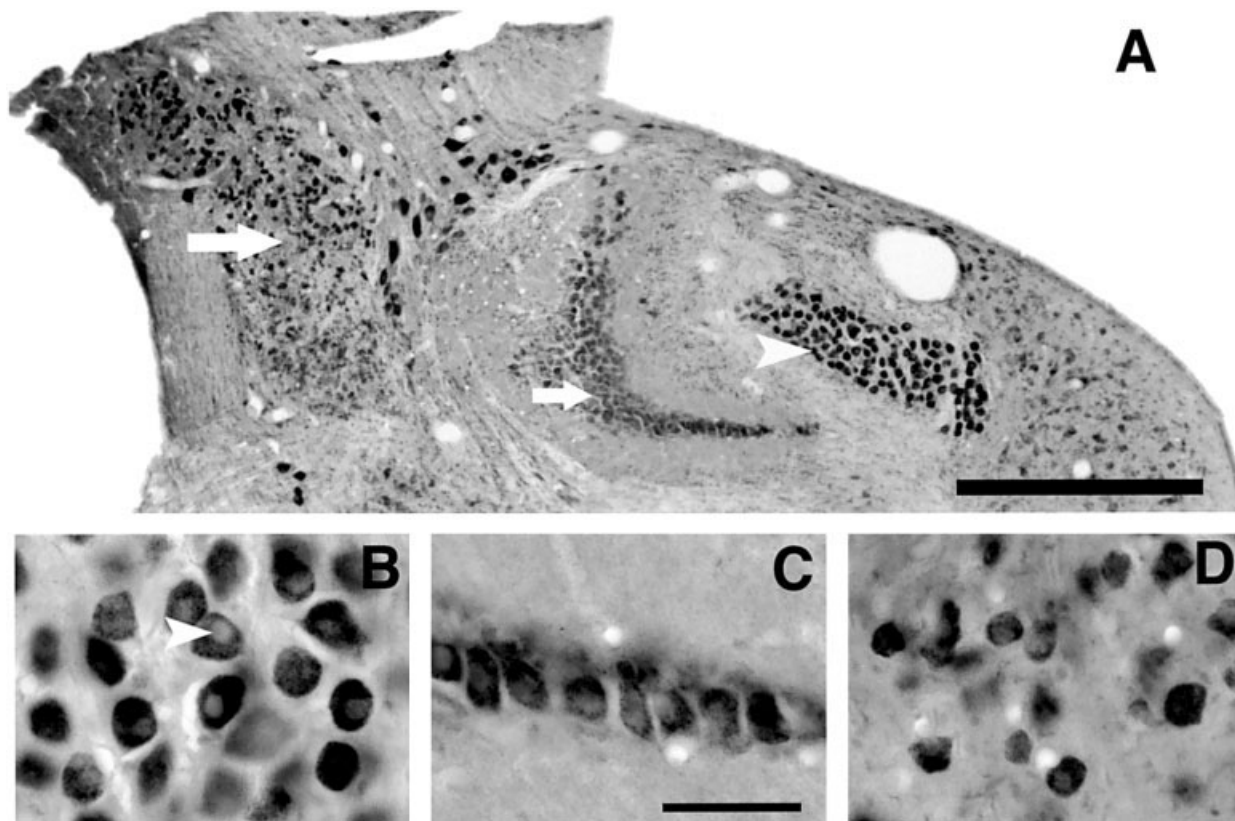


Fig. 1. Normal Kv1.1 labeling of auditory nuclei and nearby structures in the posthatch chick brainstem. **A:** Dorsal quadrant of a coronal section at low power shows intense immunoreactivity by neurons in the nucleus magnocellularis (NM; indicated by arrowhead) and the nucleus laminaris (NL; indicated by small arrow) and heterogeneous labeling of the nucleus angularis (NA; indicated by large arrow) compared with other areas of the brainstem. **B:** High-power

view of the NM shows that neuronal labeling is throughout the volume of the somatic compartment but is generally excluded from the nuclei (arrowhead) and processes. **C:** High-power view of the NL shows intense and homogeneous labeling of NL neurons, with little staining of the neuropil regions above and below the NL. **D:** High-power view of the NA shows heterogeneous labeling of neuronal somata. Scale bar = 500  $\mu\text{m}$  in A; 50  $\mu\text{m}$  in C (applies to B–D).

mean afferent-deprived Z-scores for all animals in that survival-time group. The mean Z-score for all contralateral neurons from all animals served as the control data in statistical analysis. Significant differences between the mean afferent-deprived Z-scores of different groups and the mean contralateral Z-score were detected by a one-way ANOVA. Significant differences between the control group and any of the experimental groups were detected by a Scheffe's post hoc test. For averaged Z-scores, means and standard deviations are reported.

#### Slice preparation and in vitro whole-cell recording

Brainstem slices (150–200  $\mu\text{m}$  thick) were prepared from 2- or 3-day-old hatchlings (for 24-hour survival time), as described previously (Reyes et al., 1994; Monsivais et al., 2000). For recording, slices were transferred to a 0.5-ml chamber mounted on a Zeiss (Oberkochen, Germany) Axioskop FS with a 40 $\times$  water-immersion objective and infrared differential interference contrast (DIC) optics; the slices were continuously superfused with artificial cerebrospinal fluid (ACSF) at a rate of 2–3 ml/min. ACSF contained (in mM): 130 NaCl, 26  $\text{NaH}_2\text{CO}_3$ , 2.5 KCl, 2

$\text{CaCl}_2$ , 1  $\text{MgCl}_2$ , 1.25  $\text{NaH}_2\text{PO}_4$ , and 10 dextrose; it was constantly gassed with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  and had a pH of 7.4. Unless otherwise noted, all reagents were obtained from Sigma.

Voltage clamp experiments were performed with an Axopatch 200B amplifier, and current clamp experiments were performed with an Axoclamp 2B amplifier (Axon Instruments, Union City, CA). During current clamp recordings, a slice was perfused with normal ACSF. All voltage clamp recordings were performed in the presence of 1.0  $\mu\text{M}$  tetrodotoxin (TTX) dissolved in ACSF; in some experiments, dendrotoxin-I (DTX<sub>I</sub>, Alomone Labs, Jerusalem, Israel) was used at a concentration of 0.1  $\mu\text{M}$ . All recordings were performed at room temperature (22–23°C).

Patch pipettes were drawn to 1–2  $\mu\text{m}$  tip diameter and had resistances between 3 and 6 M $\Omega$ . Pipettes were filled with a solution containing (in mM): 135 K-gluconate, 5 KCl, 5 EGTA, 10 HEPES, and 1  $\text{MgCl}_2$ ; the pH was adjusted to 7.2 with KOH, and osmolarity was between 280 and 290 mOsm. The liquid junction potential was 10 mV, and all data are presented without correction for the junction potentials. Data were low-pass-filtered at 3 or 5 kHz

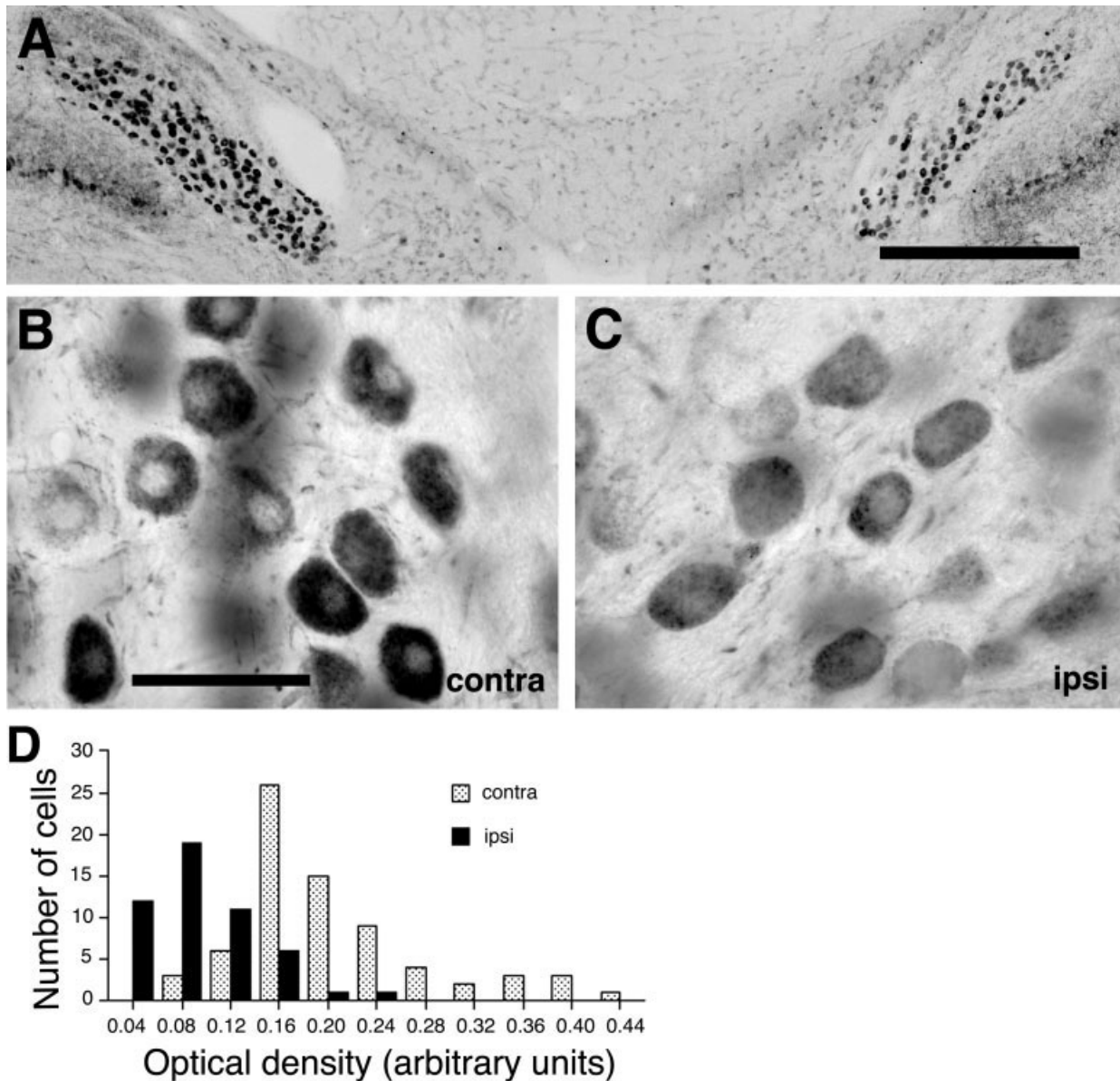


Fig. 2. Unilateral activity deprivation of the auditory system leads to a reduction of Kv1.1 immunoreactivity in the ipsilateral NM. **A:** In a transverse section of a brainstem that had been deprived for 24 hours prior to tissue preparation, the labeling of the deprived NM (right) is weaker than on the contralateral side. **B,C:** Labeling in the

NM on both sides of the brain at higher magnification. **D:** Quantification of optical density (OD) in NM neurons of this brain section shows that distribution of ODs in the ipsilateral side differs from that in the contralateral side. Scale bar = 500  $\mu\text{m}$  in A; 50  $\mu\text{m}$  in B (applies to B, C).

and digitized with an ITC-16 (Instrutech, Great Neck, NY) at 20 kHz for both on- and off-line analysis. All recording protocols were written and run by using the acquisition and analysis software Axograph, version 4.5 (Axon Instruments).

In each voltage clamp recording, series resistance (4–8  $\text{M}\Omega$ ) was compensated for by 80–90%, and cells were clamped to a potential of  $-70$  mV. Hyperpolarizing and depolarizing voltage steps (5-mV increment, 100-ms duration) were presented, and I-V relations were made by measuring the current amplitude at 5 ms before the end of

each step. We compared the LVA  $\text{K}^+$  current between the neurons ipsilateral and contralateral to the manipulation, by measuring the current amplitude in response to a step to  $-50$  mV. This command potential provided substantial activation of the LVA current while minimally activating a higher threshold  $\text{K}^+$  conductance (Rathouz and Trussell, 1998).

Input resistance ( $R_{\text{in}}$ ) measured under current clamp conditions was determined from the slope (S) of the regression lines fit through V-I curves. Because  $R_{\text{in}}$  is substantially lower at depolarized potentials relative to hy-

perpolarized potentials (Reyes et al., 1994), we also quantified outward rectification in each neuron, as the ratio of S above and below rest ( $S_{\text{depol}}/S_{\text{hyperpol}}$ ). Spike threshold was measured in response to the minimal current that was required to elicit an action potential. We defined the threshold as the inflection point on the rising

phase of the action potential, which we identified from the first derivative of the voltage waveform. Means and standard deviations are reported.

**Photomicrograph production**

Photomicrographs were taken with a CoolSnap HQ digital camera (Roper Scientific, Tucson, AZ) attached to a Zeiss Axioplan 2ie microscope. Images were opened in Adobe Photoshop (v. 7), and contrast was enhanced by adjusting levels and increasing the gamma.

**RESULTS**

**Expression of Kv1.1 protein in brainstem auditory neurons**

The normative characteristics of Kv1.1 immunolabeling were examined in the auditory brainstem nuclei, including the NM, the nucleus angularis (NA), the nucleus laminaris (NL), and the superior olivary nucleus (SON), of 5–10-day-old unoperated animals (n = 5). All neuronal cell bodies in both the NM and NL were intensely immunoreactive (Fig. 1A–C). In general, the labeling appeared slightly more intense in NM neurons than in NL neurons, but both nuclei were remarkably homogeneous in their labeling density. The labeling appeared throughout the neuronal soma cytoplasm but did not appear to invade dendritic or axonal processes to any great extent. Labeling was generally excluded from the nucleus, but this was not always evident because of the thickness of the tissue sections. NA neurons showed heterogeneous labeling (Fig. 1D). Some neuronal cell bodies and processes close to the soma were strongly labeled, whereas other neurons exhibited only weak immunoreactivity and no labeling of processes. SON neurons (not shown) were uniformly weakly labeled or unlabeled. Glial cells and processes did not appear to be immunoreactive anywhere in the brainstem. Although the region surrounding the NM and NL contains a small number of  $\gamma$ -aminobutyric acid (GABA)ergic interneurons (von Bartheld et al., 1989), these could not be identified in immunoreacted sections.

Because our Kv1.1 antibody was raised against mouse protein, we tested the specificity of our labeling in a preincubation experiment. Preincubation with the mouse fusion protein to which the antibody was raised completely eliminated specific labeling of our tissue sections (data not

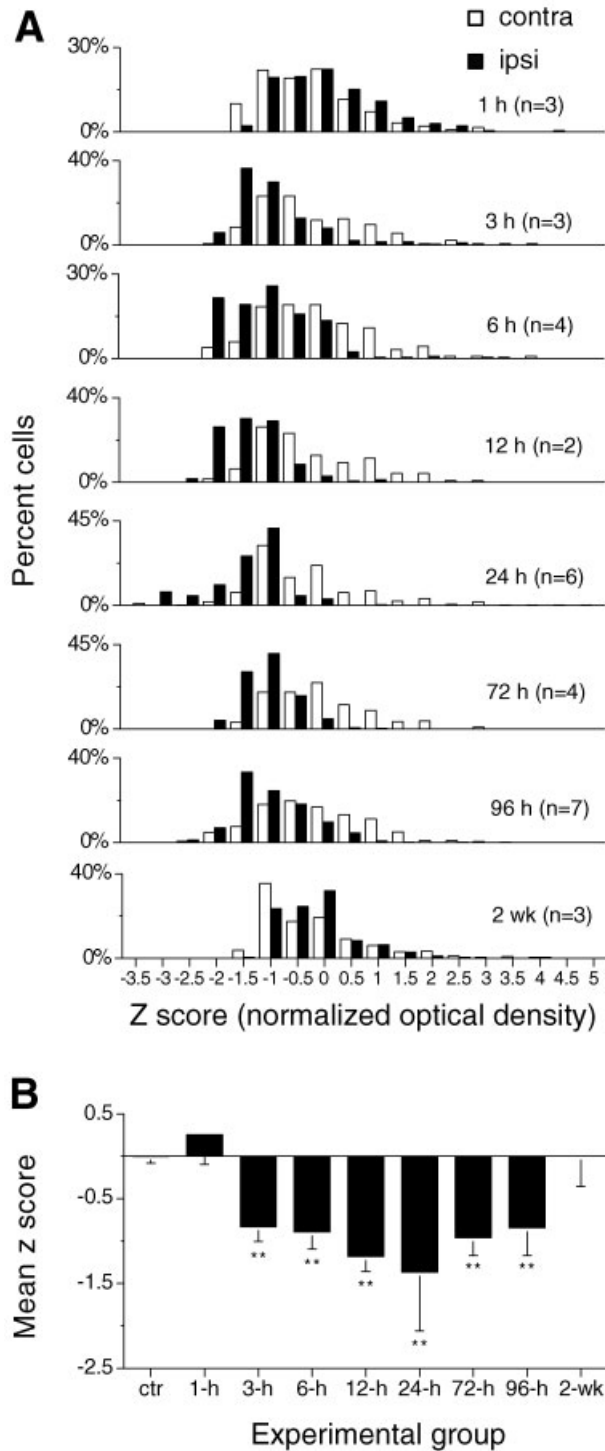


Fig. 3. The decline in Kv1.1 immunoreactivity develops rapidly and persists through 4 days. **A:** The time period following cochlea removal is indicated in the upper right of each panel, followed by the number of animals. OD measurements from several brains are normalized and combined at each of eight time points from 1 hour to 2 weeks. Percentage of cells is plotted against Z-scores binned at a bin width of 0.5 for each survival time group. The distribution of ipsilateral Z-scores at 3, 6, 12, 24, 72, and 96 hours showed a significant decrease (more negative Z-scores or less immunoreactivity) compared with the contralateral Z-scores. Significant shifts were not observed in the 1-hour and 2-week groups. **B:** The change in mean Z-score over time after deprivation. The mean Z-scores from control and experimental groups (with the standard deviations of each group) are shown. Differences between the mean Z-scores of different afferent-deprived groups and the control group were detected by a one-way ANOVA. \*\*,  $P < 0.01$ , significant difference between experimental groups and the control group detected by Scheffe's post hoc test.

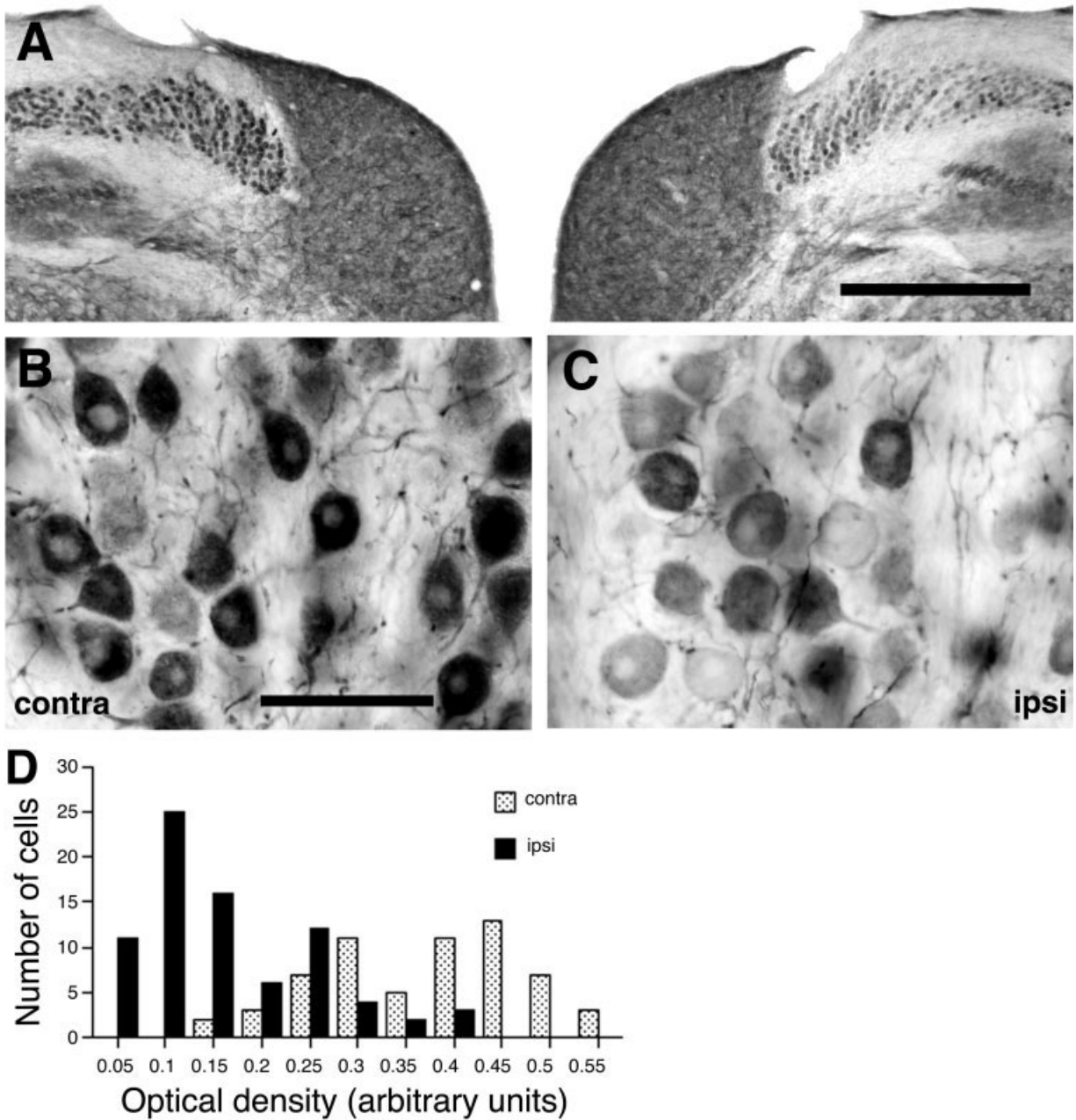


Fig. 4. Unilateral activity deprivation of the auditory system leads to a reduction of Kv3.1 immunoreactivity in the ipsilateral NM. **A:** In a transverse section of a brainstem that had been deprived for 6 hours prior to tissue preparation, the labeling of the deprived NM (right) is noticeably weaker than on the contralateral side. **B,C:** Labeling of NM

neurons on both sides of the brain at higher magnification. **D:** Quantification of OD in NM neurons from this brain section shows that distribution of ODs in the ipsilateral side differs from that in the contralateral side. Scale bar = 500  $\mu$ m in A; 50  $\mu$ m in B (applies to B, C).

shown). As a negative control, we incubated tissue sections without the primary antibody and found that all specific labeling was eliminated as well (data not shown).

**Dependence of Kv1.1 labeling on afferent input**

Figure 2 shows a representative transverse section from a chick allowed to survive for 24 hours after unilateral

cochlea removal. When we examined Kv1.1 labeling in NM neurons that had been deprived of afferent excitatory input by unilateral cochlea removal and compared the labeling intensity of these neurons with that of NM neurons contralateral to the manipulation at this time, a dramatic decrease in the density of reaction product was readily apparent. The low-power photomicrograph of the brainstem section in Figure 2A shows that Kv1.1 immu-

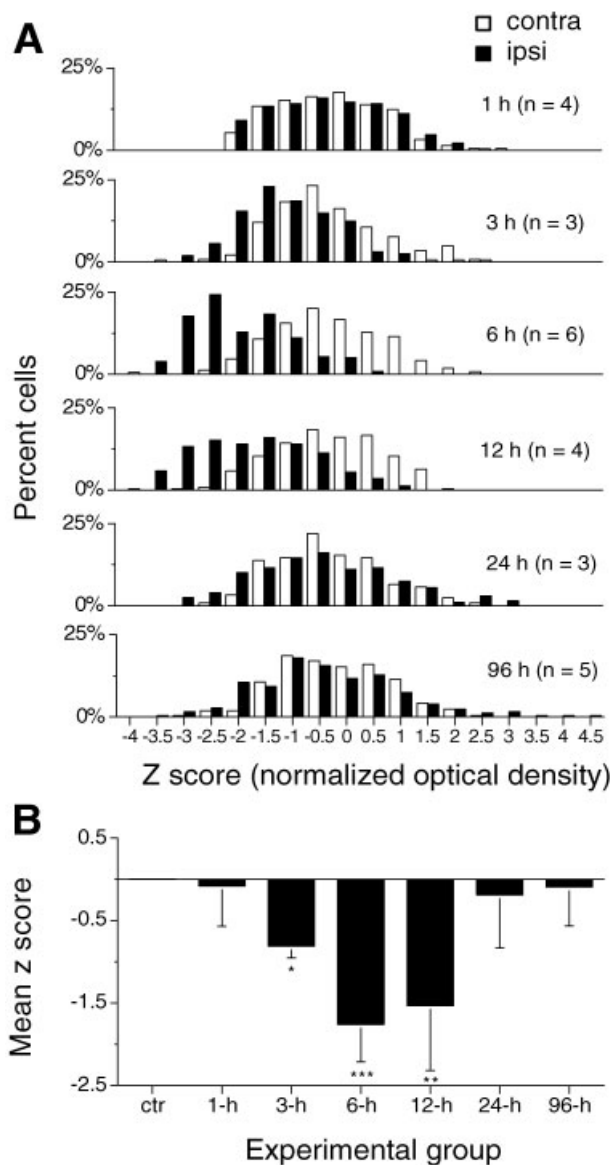


Fig. 5. The decline in Kv3.1 immunoreactivity develops rapidly and persists transiently through 12 hours. **A:** The time period following cochlea removal is indicated on the upper right of each panel, followed by the number of animals. The distribution of ipsilateral Z-scores at 3, 6, and 12 hours showed a significant decrease (more negative Z-scores or less immunoreactivity) compared with the contralateral Z-scores. Significant differences in immunoreactivity were not observed in 1-, 24-, and 96-hour groups. **B:** The change in mean Z-score over time after deprivation. The mean Z-scores from the control and experimental groups (with standard deviations of each group) are shown. Differences between the mean Z-scores were analyzed by a one-way ANOVA. \*, \*\*, \*\*\*,  $P < 0.05$ , 0.01, and 0.0001, respectively, significant differences between experimental groups and the control group detected by Scheffe's post hoc test.

nonreactivity in the NM ipsilateral (ipsi) to the cochlea removal was reduced relative to the immunoreactivity of the contralateral (contra) NM. The difference in labeling intensity between the ipsilateral and contralateral nuclei can be more easily appreciated at higher magnification

(Fig. 2B,C). Analyses of the OD of the reaction product of individual NM neurons on the two sides of this brainstem section yielded the data shown in Figure 2D. At 24 hours, in this animal and in all animals at this survival time, the ipsilateral distribution of ODs was shifted to smaller values relative to the contralateral distribution.

### Time course of changes in Kv1.1 immunolabeling

We compared the ipsilateral and contralateral densities of the reaction product at various times following unilateral cochlea removal. The decline in Kv1.1 labeling developed rapidly after removal of the cochlea. Kv1.1 labeling in the ipsilateral NM was significantly reduced by 3 hours following the manipulation.

Figure 3A illustrates the distribution of Z-scores at eight time points following cochlea removal. Percentage of cells is plotted against Z-scores binned at the bin width of 0.5 for each survival time. The same X-axis scale is used for all groups, with the Z-scores ranging from  $-3.5$  to  $5.0$ . The time period following cochlea removal is indicated, followed by the number of animals. The mean Z-scores (standard deviations) of the scores computed for the deprived side of the brain at 1, 3, 6, 12, 24, 72, and 96 hours and 2 weeks following cochlea removal were  $0.253 (\pm 0.349)$ ,  $-0.830 (\pm 0.174)$ ,  $-0.893 (\pm 0.202)$ ,  $1.183 (\pm 0.178)$ ,  $-1.367 (\pm 0.695)$ ,  $-0.960 (\pm 0.210)$ ,  $-0.843 (\pm 0.328)$ , and  $0.000 (\pm 0.357)$ , respectively (Fig. 3B). Significant differences among the mean Z-scores of different survival time groups and the control group were found by a one-way ANOVA [ $F = 29.903$ ,  $DF (8, 55)$ ,  $P < 0.001$ ].

Scheffe's post hoc tests revealed that the distributions of ipsilateral Z-scores at 3, 6, 12, 24, 72, and 96 hours was significantly shifted to the left, with more negative Z-scores (less reaction product) compared with the contralateral Z-scores. Significant changes were not observed in 1 hour or 2 weeks following cochlea removal. In addition, each of these six experimental groups (3, 6, 12, 24, 72, and 96 hours) showed significantly lower mean Z-scores ( $P < 0.01$ ), compared with the control neurons on the contralateral side of the same brains. Examination of the grouped data in Figure 3A and examination of the raw data at each survival time (not shown) did not reveal any trend toward bimodality in ODs from the experimental side of the brain. This is in contrast to our earlier studies of protein synthesis, RNA synthesis, and ribosomal integrity at survival times from 6 to 48 hours (Steward and Rubel, 1985; Born and Rubel, 1988; Rubel et al., 1990; Garden et al., 1994, 1995a,b; Hartlage-Rübsamen and Rubel, 1996).

### Dependence of Kv3.1 labeling on afferent input and time course of changes in Kv3.1 immunolabeling

NM neurons highly express the Kv3.1 subunit, confirming the report of Parameshwaran et al. (2001). The level of Kv3.1 expression in NM neurons is also dependent on afferent activity. After unilateral deprivation, Kv3.1 immunoreactivity in the ipsilateral NM decreased compared with the contralateral nucleus (Fig. 4A–C). For example, at 6 hours, the ipsilateral OD distribution was shifted to smaller values relative to the contralateral distribution (Fig. 4D).

The time course and pattern of changes in Kv3.1 labeling following unilateral cochlea removal differed from that



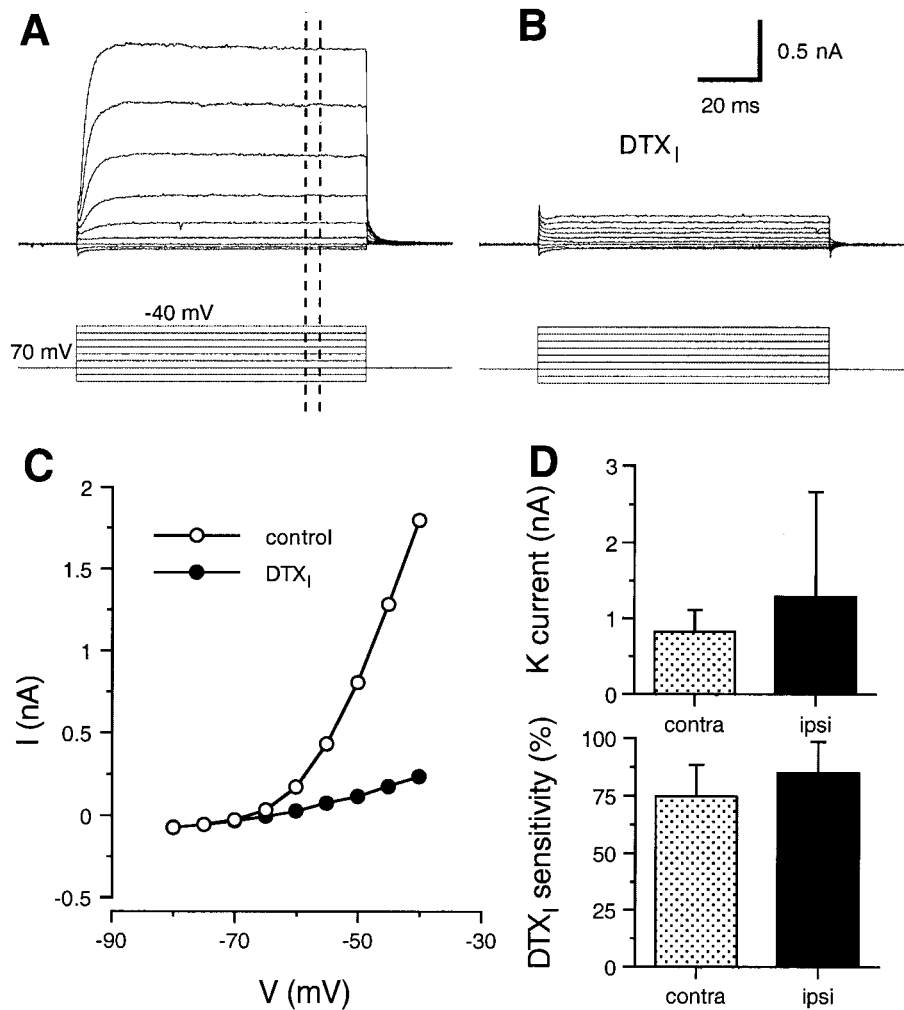


Fig. 6. Low-threshold outward currents do not differ in amplitude or sensitivity to dendrotoxin-I (DTX<sub>1</sub>). **A:** Voltage clamp recording of an NM neuron contralateral to the deprivation side illustrates low-threshold outward currents ( $V_{\text{HOLD}} = -70$  mV,  $V_{\text{STEP}} = -80$  to  $-40$  mV, 100-ms step duration). **B:** DTX<sub>1</sub> (0.1  $\mu$ M) abolishes most of the outward currents. **C:** The I-V relation of these recordings shows that outward currents begin to develop at  $-60$  mV (open circles). In the

presence of DTX<sub>1</sub>, outward currents evoked by  $-60$  mV and higher potentials are largely reduced. **D:** The average outward current evoked at  $-50$  mV is not different in amplitude between contralateral and ipsilateral neurons (upper panel). Also, DTX<sub>1</sub> blocks a similar proportion of the outward current evoked at  $-50$  mV on both sides of the brain (lower panel). Means and SDs are shown.

seen with Kv1.1 labeling. The first time we observed a significant difference (3 hours) was similar, but the recovery was much more rapid. Figure 5A illustrates the distribution of Z-scores ranging from  $-4.0$  to  $4.5$  at six survival times. The distribution of ipsilateral Z-scores at 3, 6, and 12 hours was significantly shifted to the left, with more negative Z-scores compared with the contralateral Z-scores. Significant decreases in reaction product were not observed at 1, 24, and 96 hours. The mean Z-scores (standard deviations) of the experimental sides of the brains at 1, 3, 6, 12, 24, and 96 hours were  $-0.083 (\pm 0.490)$ ,  $-0.810 (\pm 0.144)$ ,  $-1.760 (\pm 0.456)$ ,  $-1.532 (\pm 0.788)$ ,  $-0.190 (\pm 0.646)$ , and  $-0.096 (\pm 0.471)$ , respectively (Fig. 5B). The mean Z-scores of different afferent-deprived groups were significantly different from that of the control group, detected by a one-way ANOVA [ $F = 28.908$ ,  $DF (6, 43)$ ,  $P < 0.0001$ ].

Scheffe's post hoc test showed that three experimental groups (3, 6, and 12 hours) had significant differences in mean Z-scores compared with the control group. There were no significant differences between either of the 1-, 24-, or 96-hour groups and the control group ( $P > 0.9$ ). At 6 or 12 hours after cochlea removal, the pattern of Kv3.1 labeling on the afferent-deprived side appeared to be bimodal. One group of deprived neurons exhibited decreased labeling by 2–4 standard deviations, and the remainder exhibited near-normal labeling.

### Outward currents under voltage clamp

We made whole-cell recordings *in vitro* to examine the LVA component of outward K<sup>+</sup> currents. Under voltage clamp, we held neurons at a membrane potential of  $-70$  mV and applied hyperpolarizing and depolarizing voltage commands in 5-mV increments. A time-dependent outward cur-

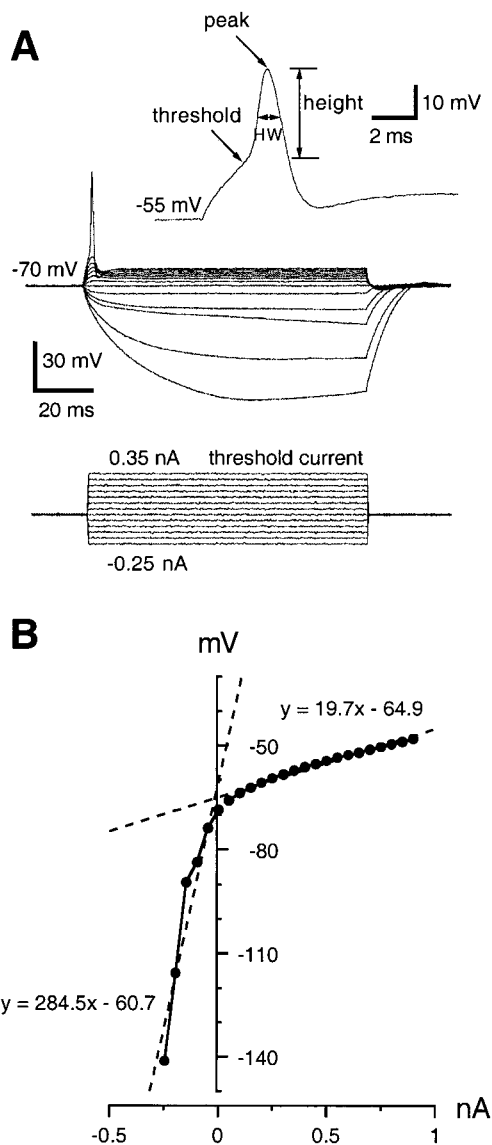


Fig. 7. Responses of an NM neuron contralateral to the deprived side to injected currents illustrate basic electrical properties and the measurements we made. **A:** NM neurons do not fire repetitively with strong depolarization, and the steady-state responses to depolarizing stimuli are smaller than responses to hyperpolarizing stimuli. Threshold current is defined as the minimum current required to elicit an action potential. **B:** I-V function derived from this recording shows the outward rectification apparent in the shallow slope of the function at depolarized potentials. Regression lines (dashed) provide a measure of input resistance, which is read as the slope in the regression line equations.

rent developed at steps to  $-60$  mV and more positive voltages and reached more than  $1$  nA at  $-40$  mV (Fig. 6A). We measured the current at  $10$ – $15$  ms before the end of the voltage step and assessed the I-V relationship by plotting this amplitude as a function of step potential. In the presence of DTX<sub>I</sub>, most of the outward current was blocked and the I-V relation became more linear (Fig. 6B,C), indicating that Kv1.1 is a major contributor to these currents.

TABLE 1. Effect of 24-Hour Deafferentation on NM Electrophysiology<sup>1</sup>

	Control (n = 13)	Deafferented (n = 15)	P value by t-test
RMP (mV)	$-60.3 \pm 3.5$	$-60.3 \pm 5.5$	$>0.10$
Rectification <sup>2</sup>	$0.13 \pm 0.07$ (n = 12)	$0.15 \pm 0.10$ (n = 13)	$>0.10$
Threshold I (nA)	$0.32 \pm 0.18$	$0.50 \pm 0.20$	$<0.05^*$
AP threshold (mV)	$-42.3 \pm 6.0$	$-34.6 \pm 7.4$	$<0.01^{**}$
AP peak (mV)	$-3.6 \pm 10.0$	$-5.7 \pm 11.7$	$>0.10$
AP height (mV)	$38.7 \pm 11.0$	$29.0 \pm 9.3$	$<0.05^*$
AP half-width (ms)	$0.77 \pm 0.12$	$0.71 \pm 0.15$	$>0.10$

<sup>1</sup>Means and standard deviations are shown. AP, action potential; NM, nucleus magnocellularis; RMP, resting membrane potential.

<sup>2</sup>Rectification =  $R_{in}$  depolarized to rest/ $R_{in}$  hyperpolarized to rest.

\* $P < 0.05$ , significant difference.

\*\* $P < 0.01$ , very significant difference.

Twenty-four hours after unilateral cochlea removal, we compared the amount of LVA K<sup>+</sup> current between NM neurons ipsilateral and contralateral to the manipulation. The amount of outward current evoked by a step from  $-70$  to  $-50$  mV did not differ significantly between neurons ipsilateral ( $1.28 \pm 1.39$  nA,  $n = 6$  cells) and contralateral ( $0.82 \pm 0.23$  nA,  $n = 10$  cells) to the manipulation (Fig. 6D), suggesting that the total number of K<sup>+</sup> channels open during this stimulus did not differ between the groups. As an additional means of testing for differences in the functional expression of Kv1.1, we compared the sensitivity of the LVA currents with DTX<sub>I</sub>. Ipsilateral ( $85 \pm 14\%$  blocking,  $n = 6$  cells) and contralateral neurons ( $75 \pm 14\%$  blocking,  $n = 7$  cells) had similar sensitivity to DTX<sub>I</sub>, suggesting that the molecular composition of the open K<sup>+</sup> channels was not different between the ipsilateral and contralateral neurons.

### Electrical properties under current clamp

We next examined the response of NM neurons to current injection under current clamp. This approach gave us a way of confirming our voltage clamp results and allowed us to measure basic electrophysiological properties of neurons ipsilateral and contralateral to the manipulation. Figure 7A illustrates a family of voltage responses to 100-ms steps of direct current in a representative NM neuron. We measured the amplitude of voltage responses 5 ms before the end of each stimulus in order to construct V-I curves. Outward rectification in these neurons is apparent around resting membrane potential (RMP), giving rise to V-I curves that are shallow at depolarized potentials and steep at hyperpolarized potentials (Fig. 7B).

The slope of linear regression lines through the V-I data points provided us with measures of input resistance ( $R_{in}$ ). Making a ratio of the  $R_{in}$  above and below RMP ( $R_{in}$ -depol/ $R_{in}$ -hyperpol) gave us a measure of each neuron's outward rectification and minimized the contribution of non-voltage-dependent changes in  $R_{in}$ , as would be expected if the neurons had changed size. The responses to current injection also provided two measures of excitability. We defined threshold current as the minimum amount of current that was required for eliciting an action potential. For the near threshold-action potentials evoked from a membrane potential of  $-55$  mV, we measured threshold, voltage peak, spike half-width, and spike height (Fig. 7A, inset).

Table 1 summarizes the electrophysiological properties of NM neurons ipsilateral and contralateral to the cochlea

removal, measured under current clamp. Again, all measurements were made approximately 24 hours after the cochlea removal. Deprived neurons were not significantly different from their nondeprived counterparts in RMP. Furthermore, outward rectification was not significantly different between the two populations of neurons, which is consistent with the voltage clamp studies showing no statistically significant difference in the amount of low-threshold  $K^+$  currents. However, the excitability of deprived neurons was depressed. Threshold current was significantly higher in deprived neurons, which coincided with a large increase in the voltage at which spikes initiated and resulted in the reduced action potential height. The peak amplitude and half-width did not significantly differ in spikes generated by deprived or nondeprived neurons.

## DISCUSSION

NM neurons express extremely high and uniform levels of Kv1.1 and Kv3.1 protein compared with other regions of the brainstem and midbrain. Depriving NM neurons of afferent activity by surgical removal of the cochlea leads to a rapid and uniform reduction in the Kv1.1 immunoreactivity that recovers by 2 weeks. The deprivation also initiates the downregulation of Kv3.1 at about the same time, but the reduction in Kv3.1 may be bimodal and is more transient than that seen for Kv1.1, recovering at 24 hours post surgery. The time course of the decreases observed in Kv channel proteins correlates with other early changes after cochlea removal in chickens.

### Possible mechanisms for activity-dependent regulation of ion channels

The question of how activity (or activity-related factors) regulates identified voltage-gated  $K^+$  and  $Na^+$  channels has been examined in various cell types including endocrine (Levitan et al., 1995), muscle (Offord and Catterall, 1989), and neural (Allen et al., 1998; Liu and Kaczmarek, 1998; Ishikawa et al., 1999). At least part of the cascade of events regulating the levels of these channels has been identified. Calcium influx through plasma membrane  $Ca^{2+}$  channels is a crucial factor coupling neuronal electrical activity with the intracellular biochemical processes that influence gene expression (for review, see Finkbeiner and Greenberg, 1998). Calcium influx is necessary for the regulation of  $Na^+$  and  $K^+$  channel abundance (Sherman and Catterall, 1984; Offord and Catterall, 1989; Chiamvimonvat et al., 1995; Liu and Kaczmarek, 1998). However, electrical activity can also work through calcium-independent pathways (Levitan et al., 1995).

Intracellular levels of cyclic AMP (cAMP), whose synthesis by adenylyl cyclase can be driven by activity or neurotransmitter activation (Offord and Catterall, 1989; Zirpel et al., 1998), can regulate voltage-gated  $K^+$  and  $Na^+$  currents in a variety of cell types (Offord and Catterall, 1989; Smith and Goldin, 1992; Bosma et al., 1993; McAnelly and Zakon, 1996; Yuhi et al., 1996; Allen et al., 1998; Golowasch et al., 1999), and cAMP can regulate the expression of  $K^+$  channels at the transcriptional level, via cAMP-responsive elements (CREs) within ion channel genes (Mori et al., 1993; Gan et al., 1996). Heterologous expression of Kv1.1 subunits in frog oocytes and mamma-

lian cell lines has shown that changes in intracellular [cAMP] trigger changes in Kv1.1 protein and the ionic currents mediated by these channels (Bosma et al., 1993; Levin et al., 1995). In a glioma cell line that expresses Kv1.1, Allen et al. (1998) found that regulation of this gene's expression by cAMP is primarily post-transcriptional, involving changes in mRNA stability. In transformed HEK293 cells, elevation of cAMP levels caused phosphorylation of Kv1.1, its translocation to the membrane, increases in current amplitude, and a negative shift in current activation thresholds (Winkhofer et al., 2003).

It will be of considerable interest to evaluate the relationships between the changes in immunolabeling for protein that we have described here with changes in expression of the *Kcna1* and *Kcnc1* genes and the time courses of post-translational changes following deafferentation. At first glance, we would hypothesize that the early reduction of Kv1.1 and 3.1 protein is independent of gene expression, reflecting translational rate or protein stability. Two previous papers that have examined the regulation of K-channels in somewhat related ways have noted that whereas the time course of gene expression changes is 2–3 hours, the concomitant changes in protein were not seen until 8–12 hours after the manipulations (Levitan et al., 1995; Allen et al., 1998). Interestingly, these studies in pituitary and glial cell lines found that elevations of cAMP and KCl caused decreases in gene and protein expression. On the other hand, as noted above, both Kv1.1 and 3.1 protein recover to apparently normal levels in deafferented NM neurons. We might expect that the recovery to normative protein expression levels would be driven by concomitant recovery in gene expression. Experiments are under way to examine these hypotheses.

### Time course of changes in Kv channels compared with other changes after activity deprivation and functional significance

Both voltage-dependent and second messenger-mediated mechanisms may contribute to changes in ion channel levels in NM neurons after deprivation. Deprivation of the synaptic activity to the avian cochlear nucleus leads to a cascade of events in the postsynaptic elements (for review, see Rubel and Fritzsche, 2002). One of the earliest and most dramatic changes in NM is an abrupt cessation of action potential activity, due to the silencing of auditory nerve fibers that normally drive activity via glutamate receptors on NM neurons (Born and Rubel, 1988; Born et al., 1991; Canady et al., 1994). Within 30 minutes after cochlea removal, protein synthesis decreases by about 50% (Steward and Rubel, 1985), and glucose uptake also decreases dramatically (Lippe et al., 1980). Furthermore, deprivation leads to a disruption of both cAMP and inositol triphosphate ( $IP_3$ )-mediated cellular processes (Zirpel et al., 1995, 1998; Zirpel and Rubel, 1996), including the maintenance of normal intracellular calcium concentration ( $[Ca^{2+}]_i$ ). By 1 hour after cochlea removal, the  $[Ca^{2+}]_i$  increased more than twofold (Zirpel et al., 1995). Reduction in Kv channel proteins was not observed at 1 hour after cochlea removal (Figs. 3, 5), but was initially observed at the 3-hour survival time. Therefore, of the changes we have studied, the increase in  $[Ca^{2+}]_i$ , as well as decreases in protein and RNA synthesis

and glucose uptake, preceded our ability to observe changes in Kv channel proteins. Any or all of these preceding alterations may underlie the subsequent changes in Kv1.1 and Kv3.1 expression levels we observed after removing afferent activity.

Time courses of other changes in NM neurons following activity deprivation appear to parallel that of Kv channels. Cytoskeletal proteins decrease by 3 hours (Kelley et al., 1997). Ribosome integrity decreases, and oxidative metabolism and mitochondria proliferation increase by 6 hours after cochlea removal (Durham and Rubel, 1985; Garden et al., 1994; Hartlage-Rübsamen and Rubel, 1996). Enhancement of bcl-2 mRNA was observed in a subpopulation of NM neurons at 6 and 12 hours following deafferentation (Wilkinson et al., 2002).

It is also of interest to note the shapes of the OD distribution in comparison with other changes we have observed. Careful examination of the individual animal OD distribution or the group data reveals similar normal distribution for Kv1.1 and Kv3.1 antibody labeling on the control side of the brain. On the other hand, the patterns appear quite different for the two channel proteins following ipsilateral cochlea removal. ODs for Kv1.1 appear to decrease uniformly across the entire group of NM neurons by 3 hours. This is evidenced by the fact that the individual animal and group distributions are unimodal at each survival time. The ODs of the surviving deafferented neurons do not return to the level seen on the contralateral side of the brain until somewhere between 4 days and 2 weeks after the surgery. The pattern seen for Kv3.1 is distinctly different. At 6 or 12 hours after cochlea removal, the pattern of labeling appears to be bimodal. One group of neurons shows decreased labeling by 2–4 standard deviations, while the remainder shows near-normal labeling. Furthermore, by 24 hours the density of reaction product bound to the Kv3.1 epitope did not differ from that on the normal side of the brain.

This pattern of change is reminiscent of the changes observed in protein synthesis, RNA synthesis, and ribosomal integrity (Steward and Rubel, 1985; Garden et al., 1994, 1995b; Hartlage-Rübsamen and Rubel, 1996), and is consistent with the hypothesis that the cells showing quite dramatic changes in labeling for Kv3.1 at 6 or 12 hours following cochlea removal are the subpopulation that go on to degenerate. A direct test of this hypothesis will require future double-labeling studies similar to those of Garden et al. (1994).

It is important to re-emphasize that this study differs from previous studies by our group or by others in that both of the ion channel proteins we have examined here are unusually highly expressed in NM neurons (as in the mammalian cochlear nucleus) and are thought to be important for phase locking (Manis and Marx, 1991; Reyes et al., 1994; Oertel, 1999; Trussell, 1999; Parameshwaran et al., 2001). In previous studies, we and others have focused on general metabolic properties and ubiquitous proteins such as cytoskeletal proteins and calcium binding proteins (Rubel and Fritsch, 2002). The observation that cellular levels of Kv1.1 and Kv3.1 appear to return to normal within a day or a few weeks in the NM neurons in the complete absence of eighth nerve excitatory input is important in that it shows these unique phenotypic characteristics of NM neurons do not require normal input to be expressed. Deprivation causes a transient decrease, but

protein levels return to appropriate homeostatic levels independent of excitatory activity from the eighth nerve. The molecular signals responsible for this biphasic plasticity will be interesting to discover.

Finally, we recognize that it is important to determine whether the histological changes we observe are reflected in the cellular physiology of NM neurons. However, the reductions in Kv1.1 protein expression at 24 hours after cochlea removal were not paralleled by observable changes in the amplitude or pharmacology of LVA  $K^+$  currents revealed by whole-cell recordings from NM neurons in a slice preparation. The lack of a correlation between the cellular physiology and the immunocytochemical results might be due to differential turnover times of cytosolic versus plasma membrane-localized Kv proteins. Several studies suggest that channel protein levels and trafficking to the membrane are differentially regulated by activity-modulated signaling pathways (reviewed in West et al., 2002; Ma and Jan, 2002). Although a significant change in LVA  $K^+$  currents was not seen, deprived neurons exhibited decreased neuronal excitability. Because neuronal excitability is primarily determined by the interaction between  $Na^+$  and  $K^+$  conductance, our data imply that activity deprivation disturbs this balance, perhaps by altering the density, spatial distribution, or biophysical properties of  $Na^+$  channels.

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