Deafness Disrupts Chloride Transporter Function and Inhibitory Synaptic Transmission

Carmen Vale,1 Jon Schoorlemmer,2 and Dan H. Sanes3
1School of Medicine and Centro Regional de Investigaciones Biomedicas, University of Castilla-La Mancha, Albacete 02071, Spain, 2Department of Biochemistry and Molecular Biology, Mount Sinai School of Medicine, New York, New York 10029, and 3Center for Neural Science and Department of Biology, New York University, New York, New York 10003

Loss of sensory function leads to atrophy or death within the developing CNS, yet little is known about the physiology of remaining synapses. After bilateral deafening, gramicidin-perforated-patch recordings were obtained from gerbil inferior colliculus neurons in a brain slice preparation. Afferent-evoked IPSPs had a diminished ability to block current-evoked action potentials in deafened neurons. This change could be attributed, in part, to a loss of potassium-dependent chloride transport function, with little change in K–Cl cotransporter expression. Treatments that suppressed chloride cotransport (bumetanide, cesium, and genistein) had little or no effect on neurons from deafened animals. These same treatments depolarized the $E_{\text{IPSC}}$ of control neurons. Semiquantitative RT-PCR and immunohistochemical staining indicated no change in the expression of chloride cotransporter mRNA or protein after deafness. Therefore, profound hearing loss leads rapidly to the disruption of chloride homeostasis, which is likely attributable to the dysfunction of the potassium-dependent chloride cotransport mechanism, rather than a downregulation of its expression. This results in inhibitory synapses that are less able to block excitatory events.

Key words: inferior colliculus; gerbil; auditory; inhibition; development; KCC2; plasticity

Introduction

The function of central synapses after blindness, deafness, or comparable forms of deafferentation is mostly unknown. However, in vivo results suggest that synaptic strength is adversely affected (Sherman and Spear, 1982; Klinke et al., 1999). An understanding of these changes seems particularly important in light of the compensatory changes that accompany peripheral injury (Pallas et al., 1999; Gold and Knudsen, 2000; Syka, 2002) and the potential to restore stimulation with prosthetic devices (Klinke et al., 1999; Král et al., 2002). One crucial question is whether the balance between inhibitory and excitatory synaptic drive is altered by hearing loss. For example, some in vivo studies show an apparent decrease of inhibition in the adult inferior colliculus (IC) or cortex after partial hearing loss (Kitzes and Semple, 1985; Rajan, 1998; Mossop et al., 2000). Until recently, there was no direct evidence that inhibitory synapses themselves undergo a change in function after deafness (Vale and Sanes, 2000, 2002), and the molecular loci of these changes remain unexplored. The goal of the present study was to measure inhibitory synaptic strength after deafness and determine whether any loss of strength was attributable to a decline in chloride cotransporter expression or loss of transport function.

Synaptic inhibition mediated by GABA_A or glycine receptors involves activation of a Cl⁻ conductance (Bormann et al., 1987). Intracellular chloride ([Cl⁻]) is regulated primarily by two cation–chloride cotransporter family members: an Na–K–2Cl cotransporter (NKCC1) leads to cytoplasmic accumulation of chloride, and a K–Cl cotransporter (KCC2) extrudes chloride (Delpire et al., 1994; Payne et al., 1996, 2003; Payne, 1997). During early development, [Cl⁻], is relatively high because of NKCC1 activity (Plotkin et al., 1997; Clayton et al., 1998; Kanaka et al., 2001). As KCC2 expression increases, [Cl⁻], drops below the electrochemical equilibrium (Lu et al., 1999; DeFazio et al., 2000; Hübner et al., 2001), leading to a transition from inhibitory synapse-evoked depolarizations to hyperpolarizations (Wang et al., 1994; Owens et al., 1996; Ehrlich et al., 1999; Kakazu et al., 1999; Rivera et al., 1999).

Two recent studies demonstrate that synaptic or electrical activity can influence chloride homeostatic mechanisms. GABA_A receptor activation facilitates KCC2 expression and the appearance of GABA_A-mediated hyperpolarizations in hippocampal cultures (Ganguly et al., 2001). In contrast, axonal injury to motor neurons decreases KCC2 expression, resulting in GABA_A-mediated depolarizing responses (Nabekura et al., 2002). There is some indication that epilepsy is also associated with improper chloride balance (Cohen et al., 2002).

Because deafness leads to a depolarization of the inhibitory synaptic reversal potential (Kotak and Sanes, 1996; Vale and Sanes, 2000, 2002), we now examine both the expression and functional status of chloride transport mechanisms in control and deafened gerbils. In control neurons, chloride transport could be blocked by three different pharmacological treatments, whereas the weak inhibitory synapses from deaf animals were unaffected. RT-PCR and immunohistochemical analyses both
showed that chloride cotransporters were well expressed in deaf animals, leading to the conclusion that chloride extrusion mechanisms are functionally disrupted. These results suggest that normal auditory activity regulates inhibitory synaptic strength through the functional status of a chloride cotransporter.

Materials and Methods
Cochlear ablation. All of the protocols were reviewed and approved by the New York University Institutional Animal Care and Use Committee. Both cochleas were removed in postnatal day 7 (P7) gerbils (Meriones unguiculatus), using a method described previously (Sanes et al., 1992; Vale and Sanes, 2000). Briefly, P7 pups were anesthetized with hypothermia until respiration ceased and animals did not respond to nociceptive stimuli. A small hole was made in the cochlear wall, and the contents were removed with a forceps. A piece of Gelfoam was placed in the cavity, and the wound was closed. After surgery, animals were warmed on a heating pad and returned to the litter when respiration and motor activity recovered. Successful ablations were confirmed before each brain slice experiment. Experiments were performed 1–7 d after surgery.

Brain slice preparation. Control and bilaterally ablated P8–P14 gerbils were anesthetized with chloral hydrate (350 mg/kg). After decapitation, coronal slices of 300 μm containing the rostral IC were obtained as described previously (Vale and Sanes, 2000). The tissue was cut in cold oxygenated artificial CSF (ACSF) containing (in mM): 123 NaCl, 4 KCl, 1.2 KH2PO4, 1.3 MgSO4, 28 NaHCO3, 15 glucose, 2.4 CaCl2, and 0.4 μl−ascorbic acid, pH 7.3 when oxygenated with 95% O2–5% CO2. After 2 hr, slices containing the rostral IC were placed in a recording chamber and superfused with oxygenated ACSF (7 ml/min) at room temperature (22–24°C).

Electrophysiology. Gammicidin-perforated-patch recordings in voltage- and current-clamp mode (PC-501A; Warner Instruments, Hamden, CT) were obtained as described previously (Rhee et al., 1994; Vale and Sanes, 2000, 2002). Recording electrodes were fabricated from borosilicate glass microcapillaries (outer diameter, 1.5 mm), and the tip resistance was 5–10 MΩ. Gammicidin (Sigma, St. Louis, MO) was used as the membrane-perforating agent to allow recording without influencing the [Cl]i. The internal pipette solution contained (in mM): 132.5 KCl, 0.6 EGTA, 10 HEPES, 2 MgCl2, 2 ATP, 0.3 GTP, and 5 N-(2,6-dimethyl-phenylcarbamoylmethyl)riethylammonium bromide (QX-314), pH 7.2. The integrity of the perforation was verified by adding QX-314 (Alamone, Jerusalem, Israel) to the intracellular pipette solution. The presence of depolarization-evoked breakaway action potentials (APs) from linear fits of the current–voltage curves plotting IPSC amplitude versus membrane holding potential.

RT-PCR to determine NKCC1 and KCC2 expression. Slices of 600 μm were obtained from the IC of P9 and P14 control and BCA gerbils as described for brain slice preparation, using RNase-free medium. The central nucleus of the IC was dissected under the microscope, and the tissue was immediately frozen in dry ice and kept at −80°C. RNA was isolated from two slices per experimental sample using TRIzol reagent (Invitrogen, San Diego, CA) and treated with RNase-free DNase and phenol extracted twice. RT-PCR assays were performed using the One-Step RT-PCR kit (Qiagen, Hilden, Germany). In all of the cases, successful amplification was dependent on reverse transcription demonstrating amplification of mRNA templates. Reactions contained test mRNA (−5 μl), RNAsin (0.5 μl), and 0.8 μM each primer. Reactions were mixed on ice and cycled as follows: reverse transcription for 30 min at 50°C, heat inactivation for 15 min at 90°C, and 30 PCR cycles (1 min at 94°C; 2 min at 58°C, and 1 min at 72°C). Aliquots (10 μl) were removed at several time points during an extended 5 min incubation at 72°C to monitor exponential amplification. Samples were analyzed by ethidium bromide staining after separation in a 2% agarose–Tris-borate-EDTA gel. Primer pairs were chosen to reside in different exons and within regions of sequence identity between different species, based on the following GenBank entries (accession number in parentheses): human NKCC1 cDNA (U30246), mouse NKCC1 cDNA (U13174), rat NKCC1 cDNA (AF 015161), rat KCC2 cDNA (U55816), and mouse genomic KCC2 sequences (A011033), conceptually translated in comparison with the rat cDNA.

Two different primer pairs were used for both NKCC1 (NK5–NK6 and NK7–NK8) and KCC2 (KCC21–KCC22 and KCC21–KCC23). Sizes of amplified DNA fragments were as predicted. The primers used were the following: GPDH1, ctaacaggccctctcatgctgc; GPDH2, staacctgaccttgagccgtc; KCC21, gacaccaccggcggccgccg; KCC22, acacaagagtgtctgagcgc; KCC23, ggcgtaccacagcggaaatgtagta; NK5, gaagttgggggaagaaccagc; NK6, tctctggttgggtctgctgt; NK7, ggtgctttggtgagggtttg; and NK8, aagatgacgctctgtgaccc.

The intensity of each PCR band was measured with a fixed-size sampling area, using the image analysis program Quantity One (Bio-Rad Laboratories, Hercules, CA). Values were expressed as the ratio of mean intensity for each cotransporter band divided by the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) band for each experimental condition.

Immunohistochemical localization of KCC2 protein. Immunohistochemical localization of KCC2 protein was performed with an affinity-purified rabbit anti-KCC2 polyclonal antibody (Upstate Biotechnology, Lake Placid, NY). P14 control and BCA gerbils were deeply anesthetized with sodium pentobarbital (80 mg/kg). Their vasculature was immediately flushed with 0.9% saline, followed by 4% paraformaldehyde in PBS. Brains were removed, postfixed in 4% paraformaldehyde for 2 hr, frozen in 2-methyl butane on dry ice, embedded in mounting media, and stored at −80°C. A Leica (Nussloch, Germany) cryostat produced 25 μm frozen coronal sections that were mounted on slides.

Immunoperoxidase labeling was performed as described previously (Williams et al., 1999). IC sections from control and BCA animals were washed three times in PBS and then placed in 2% goat serum (G5)–PBS containing 0.2% Triton X-100 for 2 hr at room temperature. Sections were incubated at 4°C for 48 hr with anti-KCC2 primary antibody diluted 1:100 in G5–PBS. After three PBS washes, sections were incubated for 2 hr at room temperature with a biotin-conjugated goat anti-rabbit
IgG (1:200 dilution; Vector Laboratories, Burlingame, CA). Sections were washed three times in PBS and incubated for 3 hr in a avidin–horseradish peroxidase solution prepared from an ABC kit (Vector Laboratories). After three washes in PBS, the horseradish peroxidase reaction was performed using diaminobenzidine (DAB) (0.015% in PBS; Sigma) and 0.001% hydrogen peroxide. Sections were washed three times in PBS, taken through an ethanol–xylene dehydration series, and coverslipped. Photographs of DAB-stained sections were taken with a Leica microscope. Controls for the immunohistochemistry experiments were obtained by incubating one of each four consecutive slides without primary antibody. None of the slides that were run without primary antibody showed staining.

To quantify the immunohistochemical staining pattern, a second set of sections were performed with a biotinylated secondary antibody anti-rabbit IgG (Vector Laboratories) and streptavidin (Alexa Fluor 488 conjugate; Molecular Probes, Eugene, OR). The sections were then examined with a confocal microscope (Leica TCS SP2; 40× objective), and intensity measures were acquired (Leica PowerScan software). An area of 94 μm² was imaged in the central nucleus from control and BCA tissue sections, and the mean pixel intensity above background was obtained.

**Results**

**Inhibitory synaptic strength was reduced after complete deafness**

Previous reports have demonstrated a deafness-induced change in $E_{\text{IPSC}}$ and inhibitory conductance (Vale and Sanes, 2000, 2002). To examine the effective strength of inhibitory synapses after deafness, we tested the ability of IPSPs to block current-evoked APs in control and BCA animals. As shown in Figure 1A, LL-evoked IPSPs and current-evoked APs were first elicited independently. The LL-evoked IPSPs were then presented concurrently or at a fixed latency to the current-evoked action potentials. In control neurons, the evoked IPSPs suppressed APs in 97 ± 2% (n = 15) of the trials (Fig. 1B). In BCA neurons, the IPSPs were able to block APs in only 43 ± 11% (n = 14) of the trials. Thus, the ability of LL-evoked IPSPs to inhibit current-evoked APs was reduced considerably in BCA neurons compared with controls ($p < 0.0001$; $t = -4.859$; df = 27). The functional duration of inhibition was also tested by evoking APs at fixed latencies to the IPSPs (see Materials and Methods). Figure 1C shows the mean duration of inhibition in control and BCA animals. The duration of inhibition was significantly longer in control neurons (81 ± 13 msec; $n = 10$) than in BCA neurons (27 ± 10 msec; $n = 13$; $p < 0.005$; $t = -3.245$; df = 21). Therefore, both measures of synaptic strength indicate that inhibitory connections are less able to suppress suprathreshold excitatory events after deafness.

An additional set of experiments were performed in whole-cell current-clamp mode to assess neuron excitability. Although resting membrane potential did not differ significantly between groups (control, −53.8 ± 0.8 mV, $n = 16$; BCA, −52.3 ± 0.8 mV, $n = 14$), it was possible that action potential threshold changed after deafness. To evaluate this possibility, the action potential threshold was subtracted from resting membrane potential as an indicator of neuron excitability. Action potential threshold was 16 ± 3 mV above rest (n = 15) for control neurons and 18 ± 2 mV above rest (n = 16) for BCA neurons, and there was not a significant difference between groups ($p = 0.417$; $t = -0.824$; df = 29).

**Blockade of chloride transporters had a smaller effect on deafened neurons**

If chloride homeostasis was compromised in deaf animals, then one would expect the loop diuretic bumetanide (Russell, 2000) to have a relatively modest effect on BCA neuron synaptic physiol-
In control neurons, 100 μM bumetanide depolarized the $E_{\text{IPSC}}$ in all of the neurons tested (12 ± 2 mV; n = 21). In contrast, bumetanide produced only a small depolarization in BCA neurons (4 ± 2 mV; n = 13). As shown in Table 1, the bumetanide-induced change was significantly smaller in BCA neurons compared with controls ($p < 0.03; t = 2.375; df = 32$). This experiment indicates that bumetanide-sensitive cotransporters, such as KCC2 and NKCC1, participate in the $[\text{Cl}^-]$ regulation of control neurons but are less active after deafness. Because bumetanide at 20 μM is a selective antagonist of NKCC (Gillen et al., 1996; Payne, 1997), we examined whether this concentration would cause a negative shift in the $E_{\text{IPSC}}$ of BCA neurons. Although the average shift of −5 mV ($n = 6$) suggested such a trend, there was not a significant difference ($p > 0.3; t = 1.07; df = 10$), indicating that NKCC does not contribute significantly to $\text{Cl}^- \text{I}$ regulation in IC neurons at the ages studied.

**Table 1. Effect of bumetanide on $E_{\text{IPSC}}$**

<table>
<thead>
<tr>
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<th>KYN</th>
<th>100 μM Bumetanide</th>
<th>20 μM Bumetanide</th>
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<tr>
<td>Control</td>
<td>−73.1 ± 2.1</td>
<td>−60.7 ± 2.9*</td>
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<tr>
<td>BCA</td>
<td>−54.0 ± 2.5</td>
<td>−49.6 ± 1.8</td>
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<td>BCA</td>
<td>−55.3 ± 3.3</td>
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*p < 0.005 versus $E_{\text{IPSC}}$ in KYN.

Potassium-dependent chloride extrusion was decreased in deafened neurons

To examine the functional role of KCC2 in control and BCA neurons, we tested the effect of intracellular $K^+$ concentration ($[K^+]_i$) on $E_{\text{IPSC}}$. Because activation of KCC2 requires the simultaneous presence of $K^+$ and $\text{Cl}^-$, we examined the effect of a $Cs^+$-containing pipette solution using the gramicidin-perforated-patch configuration. Figure 2A shows IPSCs from control and BCA neurons under both recording conditions. The control neuron recorded with $Cs^+$ exhibited a more depolarized $E_{\text{IPSC}}$ than that recorded with $K^+$. In contrast, the BCA neuron recorded with $Cs^+$ displayed a similar $E_{\text{IPSC}}$ to that recorded with $K^+$. For control neurons, the average $E_{\text{IPSC}}$ obtained with $K^+$ was $-74 \pm 1.8$ mV ($n = 45$), and that obtained with $Cs^+$ was $-49.5 \pm 2.8$ mV ($n = 19$). In BCA neurons, the average $E_{\text{IPSC}}$ obtained with $K^+$ was $-49.7 \pm 2.2$ mV ($n = 33$), and that obtained with $Cs^+$ was $-42.7 \pm 2.9$ mV ($n = 18$). An ANOVA indicated that there was a main effect ($p < 0.0001; F = 41.943; df = 3$), and pairwise comparisons showed that there was a large effect of $Cs^+$ on $E_{\text{IPSC}}$ in control neurons ($p < 0.001; t = 7.601; df = 62$), but not in BCA neurons ($p > 0.05; t = 1.815; df = 49$). Control and BCA neurons recorded with $Cs^+$-containing pipette solution did not differ significantly from each other ($p > 0.1; t = 1.671; df = 35$). Figure 2B summarizes these observations, which indicate that chloride regulation depends on $[K^+]_i$, in control neurons, but that this mechanism is nearly absent after deafness. The effect of cesium on $E_{\text{IPSC}}$ in BCA neurons is similar to that previously reported in immature auditory neurons (Kakazu et al., 1999).

Phosphorylation-dependent chloride extrusion was decreased in deafened neurons

KCC2 has a C-terminal protein tyrosine kinase (PTK) consensus site, and outward transport of chloride requires PTK activity (Payne, 1997; Kelsch et al., 2001). If PTK-dependent chloride transport is compromised in deaf animals, then a membrane-permeable inhibitor of PTK that reduces KCC2-mediated transport (genistein) should have larger effect on control neurons, compared with BCA neurons (Akiyama et al., 1987; Kelsch et al., 2001).

Figure 3A shows representative IPSCs from control and BCA neurons, and demonstrates the effect of genistein exposure. In the control neuron, a 10 min perfusion of 50 μM genistein caused a significant depolarization of the $E_{\text{IPSC}}$, whereas the same treatment had no effect on the BCA neuron. As shown in Figure 3B, the average $E_{\text{IPSC}}$ depolarization for control neurons was $15 \pm 4$ mV ($n = 10$), and this was a significant effect ($p < 0.006; t = 3.165; df = 18$). In contrast, genistein led to an average $E_{\text{IPSC}}$ depolarization of only $2 \pm 1$ mV ($n = 6$) in BCA neurons, and this was not a significant change ($p > 0.7; t = 0.273; df = 10$). The average genistein-induced $E_{\text{IPSC}}$ depolarization was significantly smaller for BCA neurons compared with controls ($p = 0.040; t = 2.266; df = 14$). Increasing the genistein concentration to 100 μM and perfusion of genistein for 30 min did not heighten the effect on $E_{\text{IPSC}}$ in BCA neurons (data not shown).

Chloride cotransporter expression in control and deafened animals

The $E_{\text{IPSC}}$ depolarization observed in BCA neurons could be caused by reduced expression of an outward chloride cotransporter, such as KCC2, or increased expression of an inward chloride cotransporter, such as NKCC1. To examine this hypothesis, the level of mRNAs encoding both chloride cotransporters in the ICs of control and BCA animals was measured by RT-PCR. In the absence of published DNA sequences for KCC2 and NKCC1 in gerbil, primers were designed in regions of rodent (and human) sequence identity, assuming cross-species sequence identity in those same regions. Indeed, NKCC1, KCC2, and GAPDH sequences were readily amplified after reverse transcription of gerbil mRNA (Fig. 4A). This was true using two different primer pairs for both NKCC1 (either NK5–NK6 or NK7–NK8) and KCC2 (either KCC21–KCC22 or KCC21–KCC23). The gerbil template yielded bands identical in size to their rat counterparts (Fig. 4A), as expected in case of rodent sequence identity.

The levels of mRNA encoding either NKCC1 (Fig. 4B) or KCC2 (C) were unchanged in BCA animals compared with controls. This was true at both survival ages examined, P9 and P14. Expression levels of GAPDH showed that mRNA input levels from P9 and P14 samples taken from control or BCA animals were nearly identical (Fig. 3 B, C, lanes marked GAPDH). Moreover, the exponential increase in DNA content with cycle number suggested amplification was semiquantitative, satisfying a condition to reliably measure differences in mRNA levels. The ratio of cotransporter band intensity to GAPDH was assessed after 30 cycles for each animal, and this ratio did not exhibit a significant difference among groups for any of the four primers (KCC21–KCC22, $p > 0.9$, $F = 0.103$, df = 3; KCC21–KCC23, $p > 0.88$, $F = 0.226$, df = 3; NK5–NK6, $p > 0.9$, $F = 0.053$, df = 3; and NK7–NK8, $p > 0.9$, $F = 0.007$, df = 3). Nor were significant differences observed after 22 or 25–26 cycles. These results indicate that expression of the mRNAs encoding two major chloride cotransporters in the IC is not affected by deafening.

KCC2 protein is similarly localized in control and deafened animals

To determine whether the $E_{\text{IPSC}}$ depolarization observed in deaf animals was attributable to reduced translation of the KCC2 pro-
tein, the immunohistochemical staining pattern was assessed. Figure 5 shows images of a control section (top), KCC2 staining in the gerbil IC of control (middle), and BCA (bottom) inferior colliculus. At the level of light microscopy, the anti-KCC2 antibody appeared to label the plasma membrane of neuronal somata and neuropil throughout the gerbil IC of both control and BCA animals. There was no apparent difference in the distribution or intensity of staining between control and experimental animals.

In confirmation of the staining pattern obtained for the anti-KCC2 antibody in gerbil IC, the immunohistochemical pattern was also observed for adult rat and gerbil cerebellum (data not shown), and this conformed to the pattern described previously (Williams et al., 1999). These results suggest that protein translation of KCC2 cannot account for the deafness-induced change in chloride homeostasis.

The immunohistochemical findings were quantified in a separate set of experiments on fluorescently labeled tissue, using a confocal microscope. The mean pixel intensity from control sections (123 ± 7) and from BCA sections (115 ± 7) did not differ significantly (p > 0.3; t = −0.957; df = 18).
However, Deitch and Rubel, 1984; Durham and Rubel, 1985; Born and Montalcini, 1949; Webster and Webster, 1979; Parks, 1981; altered metabolism in the central auditory nervous system (Levi-Montalcini, 1949). Many anatomical studies have shown that reduced neural activity decreases inhibitory postsynaptic strength 

Chloride transport in the central auditory system without a decrease in the expression of cotransporter mRNA or protein. 

**Discussion**

The proper balance of excitatory and inhibitory drive is an essential feature of all of the neuronal computational circuits. The auditory coding properties that are presumed to underlie sound localization and spectrotemporal discrimination are each sensitive to manipulations that block inhibition (Grothe, 1994; Suga et al., 1999; Carney, 1999; Brand et al., 2002; Klug et al., 2002; Pollak et al., 2002; Syka, 2002). Therefore, regulation of the molecular mechanisms that control inhibitory synaptic strength, such as chloride homeostasis, is a fundamental component of most auditory coding properties. The present study examined the basis for decreased inhibitory synaptic strength after bilateral deafness. Our results show that deafening leads to a functional decrease of chloride transport in the central auditory system without a decrease in the expression of cotransporter mRNA or protein.

**BCA decreases inhibitory postsynaptic strength**

Many anatomical studies have shown that reduced neural activity or deafferentation lead to postsynaptic cell death, atrophy, and altered metabolism in the central auditory nervous system (Levi-Montalcini, 1949; Webster and Webster, 1979; Parks, 1981; Deitch and Rubel, 1984; Durham and Rubel, 1985; Born and Rubel, 1988; McMullen et al., 1988; Hashisaki and Rubel, 1989; Hyson and Rubel, 1989). However, in vivo electrophysiology studies show that the remaining synapses do function and may undergo physiological alterations. After unilateral deafness, the remaining ear elicits greater-than-normal excitatory responses in the ipsilateral IC (Kitzes and Semple, 1985; McAlpine et al., 1997), and GAD levels decline rapidly (Mossop et al., 2000). In each of three different experimental manipulations, the neurons from deafened animals were less sensitive to blockade of the chloride homeostatic mechanism. First, the loop diuretic bumetanide at 100 µM shifted $E_{\text{IPSC}}$ to more positive values in control animals and did not alter $E_{\text{IPSC}}$ in BCA neurons (Table 1). Both NKCC1 and KCC2 cotransporters are sensitive to the loop diuretics (Payne, 1997; Isenring et al., 1998; Russell, 2000), and the net depolarization of $E_{\text{IPSC}}$ in control neurons suggests that KCC2 activity was attenuated. Second, substitution of Cs⁺ for K⁺ in the internal pipette solution depolarized $E_{\text{IPSC}}$ by 25 mV in control neurons, but did not modify $E_{\text{IPSC}}$ in BCA neurons (Fig. 2). A lack of effect of cesium on intracellular chloride concentration has been reported previously on immature lateral superior olivary neurons (Kakazu et al., 1999). Third, the PTK inhibitor that blocks KCC2 activity, genistein, depolarized $E_{\text{IPSC}}$ in control neurons, but did not alter $E_{\text{IPSC}}$ in BCA neurons (Fig. 3). These experiments left open the possibility that either KCC2 expression or function had declined.

A second nonexclusive hypothesis was that NKCC activity was upregulated after deafness. NKCC transports Cl⁻ into the cell, particularly in immature neurons (Plotkin et al., 1997; Kakazu et al., 1999). However, 20 µM bumetanide did not significantly affect $E_{\text{IPSC}}$ in BCA neurons, suggesting that NKCC activity is low in IC neurons at the ages studied (Gillen et al., 1996; Payne, 1997; Kakazu et al., 2000). Because other chloride transport mechanisms do not maintain low [Cl⁻] in the absence of KCC2 (Rivera et al., 2002), we did not assess their function in deafened animals.

**Alteration of intracellular chloride regulators after deafness**

In the present study, the depolarization of $E_{\text{IPSC}}$ was attributable to a positive shift in $E_{\text{Cl}^-}$, because the resting membrane potential was unaltered and extracellular Cl⁻ was constant. In control conditions, intracellular chloride is generally lower than expected from passive distribution (Thompson et al., 1988; Thompson and Gahwiler, 1989), and several proteins are involved, including KCC2, NKCC1, the Cl⁻–HCO₃⁻ exchanger, and the Na⁺- dependent Cl⁻–HCO₃⁻ exchanger (Payne et al., 2003). Of these, KCC2 is the primary contributor to maintaining low [Cl⁻] (Thompson et al., 1988; Payne et al., 1996; Payne, 1997; Plotkin et al., 1997; Kakazu et al., 1999; Lu et al., 1999; Rivera et al., 1999).

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Deafness does not affect KCC2 or NKCC1 expression

Our initial hypothesis was that the expression of KCC2 was decreased by deafening. This seemed reasonable, because the manipulation was occurring during early postnatal development, and increased KCC2 expression is commonly observed during this period with an attendant switch to hyperpolarizing response (Ehrlich et al., 1999; Kakazu et al., 1999; Rivera et al., 1999).

Changes in chloride cotransporter expression are observed during normal development and also after injury. For example, a decrease in KCC2 expression occurs after axotomy in vagus motor neurons (Nabekura et al., 2002), and an increase or decrease of KCC2 expression can be elicited in hippocampal cultures by exposure to a GABA_A agonist or antagonist, respectively (Ganguly et al., 2001). In fact, depolarizing GABAergic synaptic responses have recently been observed in temporal lobe tissue obtained from human epilepsy patients (Cohen et al., 2002).

Because chloride extrusion was clearly diminished in neurons from deaf animals (Figs. 2 and 3, Table 1), we examined cotransporter expression in two ways. Bilateral deafening did not alter the mRNA levels of KCC2 or NKCC1 in IC neurons as assessed by semiquantitative RT-PCR (Fig. 4B, C). Furthermore, immunohistochemical visualization of the KCC2 protein revealed that it was abundant in the ICs of both control and BCA animals.

Brain-derived neurotrophin (BDNF) signaling has been shown to downregulate KCC2 expression by 50% in hippocampal slices, and this is associated with a depolarization of \( E_{\text{IPSP}} \) (Rivera et al., 2002). Compared with these results, the present findings differ in an interesting way: the \( E_{\text{IPSP}} \) is depolarized to a similar degree, but there is no indication that KCC2 expression has changed, and certainly not by 50% (Fig. 4). Interestingly, BDNF-dependent expression of KCC2 has also been reported in mice that overexpress the BDNF, but the effect is opposite to that reported in vitro (Aguado et al., 2003). Therefore, the depolarization of \( E_{\text{IPSP}} \) and loss of inhibitory synaptic strength in deaf animals is attributable to a change in the functional status of potassium-dependent chloride extrusion.

Hypothetical basis for loss of K–Cl cotransport function

Because the present results indicate that KCC2 and NKCC1 expression were unaltered by deafness (Figs. 4 and 5), we propose that the functional status of K–Cl cotransporter(s) was affected. This is somewhat surprising in that published reports on developing systems, as well as models of injury, have associated a depolarized \( E_{\text{IPSP}} \) with KCC2 expression levels only (Ehrlich et al., 1999; Kakazu et al., 1999; Rivera et al., 1999, 2002; Ganguly et al., 2001; Nabekura et al., 2002). Although there is little information on the regulation of KCC2 in neural tissue, there is a broad literature on the functional modulation of other cation–chloride cotransporter family members. In particular, the normal physiology of KCC1 is closely linked to the cytoplasmic domains; removal of 8 C-terminal or 89 N-terminal amino acids can abolish function without affecting surface expression of the protein in Xenopus oocytes (Casula et al., 2001). In erythrocytes, KCC activation involves a dephosphorylation event, and Src family kinases have been shown to negatively regulate K–Cl cotransporter-activating phosphatases (Jennings and Schulz, 1991; Lauf et al., 1992; Flattman et al., 1996; De Franceschi et al., 1997). In a related manner, potassium–chloride cotransport is regulated by protein kinase G in vascular smooth muscle cells, although mRNA expression is not affected (Adraga et al., 2002).

In contrast to the ubiquitously expressed KCC1, neuron-specific KCC2 has a tyrosine phosphorylation consensus site (Payne, 1997), and a single amino acid substitution is sufficient to reduce transporter activity in oocytes (Strange et al., 2000). Furthermore, KCC2 function can be deactivated by membrane-permeable protein tyrosine kinase inhibitors in cultured hippocampal neurons (Kelsch et al., 2001). It has been suggested that PTK phosphorylation of KCC2 may be part of the overall maturation of GABAergic synapses (Kelsch et al., 2001).

Together, the findings from other systems suggest that deafness leads to an alteration in the KCC2 phosphorylation state,
possibly at the tyrosine residue. This is supported by the finding that genistein can depolarize $E_{\text{tpsc}}$ in control, but not deaf, neurons (Fig. 3). Auditory-evoked synaptic transmission in the IC could modulate KCC2 phosphorylation via many receptor systems. One viable possibility is that afferent terminals contain and release neurotrophins that elicit postsynaptic KCC2 phosphorylation. Neurtrophin expression is observed in the developing rat IC, and the cognate receptors for BDNF and neurotrophin-3 are expressed at the postsynaptic junctions in gerbil (Hafidi et al., 1996; Hafidi, 1999). Such a modulatory role would complement that long-lasting effect of neurotrophins on KCC2 expression (Rivera et al., 2002; Agudo et al., 2003).

In summary, our results suggest that modulation of KCC2 physiology may help to explain certain CNS pathologies, as well as the physiological set point of normal inhibitory synapses (Golding and Oertel, 1996; Vardi et al., 2000, Kanaka et al., 2001; Ueno et al., 2002). This mechanism differs from the many cases in which KCC2 expression is modified. Although the activity-dependent modulation of KCC2 has yet to be studied, it is known that NMDA receptor activation can stimulate an NKCC2 cotransporter (Sun and Muriali, 1998). Therefore, it may be possible to restore inhibitory synaptic strength by examining how KCC2 depends on ionicotropic and metabotropic signaling at the synapse.

References


Kotak VC, Sanes DH (1996) Developmental influence of glycinergic}