### Expression of the SK2 calcium-activated potassium channel is required for cholinergic function in mouse cochlear hair cells

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Efferent inhibition of cochlear hair cells is mediated by 'nicotinic' cholinergic receptors functionally coupled to calcium-activated, small conductance (SK2) potassium channels. We recorded from cochlear hair cells in SK2 knockout mice to evaluate further the role of this channel in efferent function. Since cholinergic inhibitory synapses can be found on inner or outer hair cells, depending on developmental age, both cell types were studied. To determine if SK channel activity was indeed eliminated, seconds-long voltage-gated calcium influx was used to activate slowly rising and falling calcium-dependent potassium currents. These were identified as SK currents by their time course, calcium dependence and sensitivity to block by apamin in wild-type IHCs. IHCs from knockout mice had no SK current by these same criteria. Thus, the SK2 gene is solely responsible for encoding the SK channels of inner hair cells. Other aspects of hair cell excitability remained relatively unaffected. Unexpectedly, cholinergic synaptic currents were entirely absent from both inner and outer SK2-knockout hair cells. Further, direct application of ACh caused no change in membrane current, implying absent or otherwise dysfunctional ACh receptors. Immunohistology of whole-mounts using the antibody to the synaptic vesicle protein 2 (SV2) revealed a pronounced reduction of efferent innervation to outer hair cells (OHCs) in the knockout cochleas. Quantitative RT-PCR analysis, however, showed no change in the mRNA levels of  $\alpha$ 9 and  $\alpha$ 10 nicotinic ACh receptor (nAChR) genes. Thus, some aspect of translation or subsequent protein processing leads to non-functional or absent ACh receptors. These results indicate that SK2 channels are required both for expression of functional nAChRs, and for establishment and/or maintenance of efferent terminals in the cochlea.

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Efferent inhibition of cochlear hair cells is mediated by the release of acetylcholine (ACh) from neurones originating in the superior olivary complex of the brainstem. In the mature cochlea, efferents synapse largely on to outer hair cells (OHCs) to regulate the voltage-dependent feedback process of electromotility by which OHCs amplify the cochlear vibration pattern. Before the onset of hearing (about neonatal day 12 in rats and mice) efferent fibres transiently make cholinergic synapses with inner hair cells (IHCs) (Simmons *et al.* 1996; Katz *et al.* 2004). Efferent synapses on both IHCs and OHCs are

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mediated by the  $\alpha 9\alpha 10$  'nicotinic' receptors that form Ca<sup>2+</sup>-permeable ligand-gated cation channels. Activation of these receptors briefly depolarizes the hair cell before associated calcium-activated SK channels open to cause a larger, longer-lasting hyperpolarization (Glowatzki & Fuchs, 2000; Oliver *et al.* 2000). Thus, hair cells uniquely employ what would ordinarily be an excitatory neuro-transmitter receptor to produce inhibition, but only with the collaboration of calcium-dependent SK channels. Presumably, in the absence of SK channels, cholinergic effects would be excitatory, as indeed has been shown in recordings from hair cells using potassium channel blockers (Marcotti *et al.* 2004). Of more interest would be

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such 'reversed' efferent effects in an intact animal model in which the consequences for auditory behaviour could be assessed. To that end we examined cholinergic inhibition of hair cells in young (1–3 weeks postnatal) SK2-knockout transgenic mice (Bond *et al.* 2004).

The calcium-activated potassium channels mediating cholinergic inhibition of hair cells are SK channels based upon their small conductance, judged by the low associated variance or noise (Fuchs & Murrow, 1992*a*,*b*) and their block by sub-micromolar levels of apamin (Nenov et al. 1996; Tucker & Fettiplace, 1996; Yamamoto et al. 1997; Yuhas & Fuchs, 1999; Dawkins et al. 2005), a peptide toxin from bee venom that is specific for SK-type calcium-activated potassium channels. Molecular identification based on RT-PCR, immunolabelling and the functional profile of cloned channels suggest that the SK2 gene encodes these channels in hair cells (Dulon et al. 1998; Oliver et al. 2000; Nie et al. 2004; Matthews et al. 2005). Thus, we have examined cholinergic inhibition in cochlear hair cells of SK2 knockout mice to answer two main questions. Is the SK2 gene solely responsible for encoding SK-type channels of cochlear hair cells? And, what are the functional consequences of SK2 loss for cholinergic inhibition in these cells? Here we establish that SK currents are indeed absent from cochlear hair cells in the knockout mouse, confirming recent reports (Johnson et al. 2007). However, also absent were any efferent synaptic effects. Further, direct application of ACh elicited no detectable membrane currents from the knockout hair cells, although it does so reliably in hair cells from wild-type mice. In addition, efferent innervation of outer hair cells was severely reduced in the 3-week-old knockout cochleas. Thus, as has been suggested previously (Walsh et al. 1998), efferent feedback may play a role in synaptic development and maturation of cochlear function, including organization of the cholinergic synapse itself.

#### Methods

#### Preparation of tissue and solutions

All experiments were performed with wild-type or homozygous SK2-knockout mice, as previously described (Bond *et al.* 2004). Thus, 'knockout' as used throughout refers to the homozygous knockout mouse. PCR analysis confirmed the different genotypes. The procedures for preparing and recording from the postnatal organ of Corti were essentially identical to those published previously (Glowatzki & Fuchs, 2000; Lioudyno *et al.* 2004). Animal protocols were approved by the Johns Hopkins University Animal Care and Use Committee. Mouse pups, 6–24 days old (6–13 for IHC recordings and 9–24 for OHC recordings) were anaesthetized using pentobarbital (45 mg kg<sup>-1</sup>, I.P.) and decapitated for all the experimental procedures. The organ of Corti was exposed and the apical or upper-middle turns removed for recording. Hair cells were visualized using an Axioscope (Zeiss, Oberkochen, Germany) with a 40× water immersion DIC objective,  $4\times$  magnification and a NC70 Newvicon camera (Dage MTI, Michigan City, IN, USA) for display. Whole-cell, tight-seal voltage-clamp recordings were made with 1 mm borosilicate glass micropipettes (WPI, Saratosa, FL, USA) ranging from 4 to 10 M $\Omega$  resistance. Electrodes were advanced through the tissue under positive pipette pressure to avoid extensive dissection. In this way it was possible to maintain the integrity of synaptic contacts on hair cells.

The standard pipette solution was (mM): 135 KCl, 3.5 MgCl<sub>2</sub>, 0.1 CaCl<sub>2</sub>, 5 EGTA, 5 Hepes, 2.5 Na<sub>2</sub>ATP, pH 7.2. The standard extracellular solution was (mM): 5.8 KCl, 144 NaCl, 1.3 CaCl<sub>2</sub>, 0.7 NaH<sub>2</sub>PO<sub>4</sub>, 5.6 glucose, 10 Hepes, pH 7.4. Mg<sup>2+</sup> was excluded from the extracellular solution to maximize current flow through the hair cell ACh receptor (Weisstaub et al. 2002; Gomez-Casati et al. 2005). In some experiments, ACh was applied in saline containing  $40 \text{ mM K}^+$  (substituted for Na<sup>+</sup>). This 'high potassium' condition was intended to maximize driving force on potassium currents at negative membrane potentials. Recordings were made at room temperature (22–25°C). For  $Ca^{2+}$  current measurements, the pipette solution was (mM): 120 CsMeSO<sub>3</sub>, 20 CsCl, 10 Na<sub>2</sub>-phosphocreatine, 4 MgCl<sub>2</sub>, 5 EGTA, 5 Hepes, 4 Na<sub>2</sub>ATP, pH 7.35, and extracellular solution was (mM): 5.8 KCl, 100 NaCl, 1 MgCl<sub>2</sub>, 0.7 NaH<sub>2</sub>PO<sub>4</sub>, 5.6 glucose, 10 Hepes, 35 TEA-Cl, 4 4-AP, 200 nM TTX, 10 BaCl<sub>2</sub> or 10 CaCl<sub>2</sub>, pH 7.35. Solutions, including experimental drugs, were applied by gravity flow into the bath chamber (1.5 ml volume) at a rate of 2-3 ml min<sup>-1</sup>.

#### **Electrical stimulation and recording**

Electrical stimulation experiments were performed for IHCs from wild-type and knockout mice as previously described (Goutman et al. 2005). Bipolar electrical stimulation of efferent axons was provided by a theta glass micropipette (20–30  $\mu$ m tip diameter) positioned about 20  $\mu$ m modiolar to the base of an IHC, near the course of the inner spiral bundle. The position of this pipette was adjusted until current passage through it caused synaptic currents in the IHC under study. Smaller tip diameters of the stimulation pipette were less successful. An electrically isolated constant current source (model DS3, Digitimer Ltd, Welwyn Garden City, UK) was triggered via the data-acquisition computer to generate pulses up to  $\sim$ 50  $\mu$ A, 50–500  $\mu$ s long to stimulate efferent axons. With some experience in positioning the stimulation pipette, 50  $\mu$ s long pulses were sufficient for stimulation. With this method we were able to stimulate IPSCs in every wild-type IHC recorded. Currents and

voltages were low-pass filtered at 2–10 kHz and digitized at 10–50 kHz. IPSCs were identified manually using a search routine for event detection with a threshold of 5 times the RMS noise.

# Immunostaining and confocal imaging of organ of Corti

Organ of Corti sections were derived from inner ear tissue dissected from postnatal day 21 (P21) wild-type and knockout mice. Isolated cochleas were perfused through the round window with ice-cold 4% PLP fixative (4% paraformaldehyde, buffered with 0.12 M monobasic sodium phosphate, pH 7.4 – '0.12 M PB'). After fixation at  $4^{\circ}$ C for 3 h, excess bone and connective tissue were dissected away in the 0.12 M phosphate buffer (PB), specimens rinsed 4 times for 20 min each in 0.12 M PB and individual turns microdissected for processing as whole-mounts.

For immunolabelling, individual turns were initially blocked overnight at 4°C in the blocking buffer (60 mM phosphate buffered saline - '60 mM PBS' at pH 7.4, supplemented with 10% normal donkey serum and 0.25% Triton X-100) to reduce non-specific labelling. The antibody against SV2, synaptic vesicle protein 2 (1:200, monoclonal, developed by Kathleen M. Buckley at Harvard Medical School, MA, USA) was applied to the individual turns for 3 h at 4°C. After rinsing 3 times for 20 min each in the blocking buffer, individual turns were treated with Alexa 488-conjugated secondary IgG (donkey, 1:1000, Molecular Probes, Invitrogen, CA, USA) for 2 h at room temperature. Then, the individual turns were rinsed 3 times for 20 min each in 60 mM PBS (pH 7.4) at room temperature and stained with DAPI (4',6-diamidine-2'-phenylindole dihydrochloride, Roche, Switzerland). Finally the turns underwent 5 washes of 10 min each in 60 mM PBS prior to mounting on glass slides in Fluor Save reagent (Calbiochem, Cat. No. 345789).

Whole mounts of cochlear turns were viewed on a Zeiss (Thornwood, NY, USA) LSM 510 META confocal microscope with a Zeiss 40× water immersion C-Apochromat objective (numerical aperture (NA), 1.2). Z-series were taken at 0.4  $\mu$ m intervals with 25–40 optical sections per sample. For quantification, confocal optical sections were built into z-axis reconstructions. SV2-positive efferent terminals (boutons) were counted in 3-D reconstructions to assure location near DAPI-labelled nuclei of OHCs.

#### **Quantitative RT-PCR**

For quantitative RT-PCR, whole cochleas were dissected from wild-type and SK2-knockout mice at P21. To conserve reagents for purification of total RNA (RNeasy Mini Kit Cat. No. 74104, Qiagen Inc.), three cochleas were processed in the same QIAshredder (Cat. No. 79654, Qiagen Inc.) according to the protocol of the manufacturer. The concentration of isolated RNA was measured using a NanoDrop (Thermo Fisher Scientific Inc. Waltham, MA, USA). Total RNA was reverse-transcribed (Omniscript RT Kit Cat. No. 205111, Qiagen Inc.) in the presence of random primers for 1 h at 37°C.

Real-time PCRs were performed in triplicate for each gene transcript in wild-type and knockout using a SYBR Green PCR Kit (Cat. No. 204143, Qiagen Inc.), and expression levels were determined by comparison with a housekeeping gene,  $\beta$ -actin. The amplicon for  $\beta$ -actin was 60 bp (primers: CCCTGAGGCTCTTTTCCAG, GTT-TCATGGATGCCACAGG); for  $\alpha 9$ , 78 bp (primers: CCATCACCAAAAGCTCCTGT, AACCAAAGGTCAGG-TTGCAC); for  $\alpha 10$ , 63 bp (primers: CTTGAGACC-AGTGGCAGACA, TGAGACAACGTCACCTCCAG); for SK2, 80 bp (primers: ACGATCATCCTGCTTGGTCT, TCATCTGCTCCATTGTCCAC). All primers were synthesized by Invitrogen. The cycle parameters used were 95°C for 15 min to denature the DNA templates and activate Taq polymerase, followed by 45 amplification cycles, each comprising denaturation (94°C for 15 s), annealing ( $60^{\circ}$ C for 30 s), and extension ( $72^{\circ}$ C for 30 s) in the LightCycler 1.2 instrument with LightCycler version 3 software (Roche, Indianapolis, IN, USA). The temperature transition rates were programmed at  $20^{\circ}C s^{-1}$ . Fluorescence was measured at the end of the annealing period of each cycle to monitor the amplification. The amplication efficiencies of the primers were tested in a validation experiment using serial dilutions of a wild-type cDNA. The amplification efficiencies of target and housekeeping genes were monitored to ensure that these differed by no more than  $\pm 0.05$  (data not shown). Immediately after the amplification, melting curves were recorded by cooling from 95°C to 65°C at 20°C s<sup>-1</sup>, and then heating slowly at 0.1°C s<sup>-1</sup> until 95°C. Melting curves were then converted to melting peaks by calculating the negative derivative of the fluorescence *versus* temperature relation (-dF/dT). A melting curve analysis verified that a single product was amplified in all reactions (Read et al. 2001; van der Velden et al. 2003). The qRT-PCR experiments were conducted 5 times and averaged.

#### Statistics

Mean values are presented  $\pm$  standard error (S.E.M.) unless stated otherwise. Statistical significance for pairwise comparisons was derived from two-tailed unpaired *t* tests. A value of P < 0.05 was considered statistically significant. For each PCR run, the relative mRNA level

For each PCR run, the relative mRNA level was determined by the expression  $2^{-\Delta\Delta Ct} (\Delta Ct =$ 

Ct<sub>target gene</sub> – Ct<sub>β-actin</sub>,  $\Delta\Delta$ Ct =  $\Delta$ Ct<sub>ko or wt</sub> –  $\Delta$ Ct<sub>wt</sub>, Livak & Schmittgen, 2001). Thus, this term expresses the level of the target transcript relative to that of β-actin in wild-type *versus* knockout. If the expression level of the target gene relative to that of β-actin is the same in wild-type and knockout,  $\Delta\Delta$ Ct = 0;  $2^{-\Delta\Delta$ Ct} = 2° = 1. Ct stands for 'cycle threshold', meaning the cycle number at which each product rose sufficiently above background. The mean and S.E.M. of the value  $2^{-\Delta\Delta$ Ct} for each mRNA in each target gene, across all runs, were plotted.

#### Results

#### SK currents produced by depolarizing voltage commands were absent in neonatal SK2-knockout IHCs

Before studying cholinergic function in hair cells from SK2-knockout mice, it was necessary first to confirm that the knocked out gene really encoded the SK current of cochlear hair cells. To do so requires an independent measure of the SK2 component other than activation by way of the usually associated ACh receptor. Therefore, SK currents were activated by prolonged calcium influx through voltage-gated Ca<sup>2+</sup> channels, as demonstrated previously in turtle (Tucker & Fettiplace, 1996) and mouse (Marcotti et al. 2004) hair cells. When these experiments were performed on OHCs, only quite small SK currents were found, presumably because OHCs have much smaller voltage-gated calcium currents to activate correspondingly smaller SK currents than in neonatal IHCs (Marcotti et al. 2004; Knirsch et al. 2007). Therefore, we used neonatal IHCs to determine whether the deleted SK2 gene is responsible for the SK current of cochlear hair cells.

Depolarizing voltage commands produced rapidly rising outward currents (see details below) that could reach several nanoamps peak amplitude at positive membrane potentials. When the depolarization continued for more than 1 s, a second component of outward current arose in wild-type IHCs over the course of 1–2 s, depending on membrane potential, and gave rise to very slowly decaying tail currents upon return to a negative membrane potential (Fig. 1Aa and Ba, black traces). This slowly rising and falling outward component was effectively eliminated by 300 nm apamin (Fig. 1*Aa*, grey traces), making it likely to flow through SK-type channels. In contrast, when knockout IHCs were run through the identical voltage protocol, only the rapidly rising, gradually inactivating component of outward current with small, rapid tail currents was seen (Fig. 1Ab and Bb, black traces). When 300 nM apamin was applied the overall profile of outward current was essentially unaffected (Fig. 1Ab, grey traces). The small drop in peak outward current reflects a gradual rundown that also occurred in control recordings (see Fig. 2*B*)

Further evidence for the presence of SK-type channels was obtained from use of the L-type voltage-gated calcium channel blocker, nifedipine, to prevent calcium influx. The voltage-gated calcium channels in cochlear hair cells are encoded by the CaV1.3 gene (Platzer et al. 2000; Michna et al. 2003), a dihydropyridine-sensitive channel related to that found in cardiac muscle. Consistent with the notion that voltage-gated calcium influx accumulated to produce the late current through SK channels, application of 50  $\mu$ M nifedipine caused an 'apamin-like' change in shape of outward currents produced by seconds-long depolarization. A slowly rising secondary component of outward current and accompanying slow tail currents in wild-type IHCs were selectively abolished by nifedipine, leaving behind the rapidly activating, slowly inactivating outward currents (Fig. 1Ba). When this same experiment was conducted on knockout hair cells, their outward currents were only slightly diminished at peak amplitude, leaving the overall shape essentially unchanged by nifedipine (Fig. 1Bb).

The slow tail currents proved to be a reliable measure of SK channel activity and sensitivity to pharmacological blockade (Fig. 2). These tail currents had half-times of decay that could exceed 2 s, easily distinguishing them from the more rapidly decaying tail currents through voltage-gated potassium channels. Both the peak amplitude and half-duration of the tail current depended on the size of the preceding voltage step, reaching a maximum for conditioning voltages between 0 and +24 mV. For successive depolarizations positive to 0 mV the peak amplitude of the tail current plateaued, then became smaller, consistent with its activation by calcium influx, reduced by the smaller driving force at positive membrane potentials. Slow tail currents, based on a criterion of at least 50 pA at 1 s after repolarization, were found in 55 out of 70 wild-type IHCs. In contrast, none of 40 IHCs from knockout cochleas had such current. The effects of apamin and nifedipine, as well as curare, used here as an alternative SK channel blocker (Ishii et al. 1997) were quantified by measuring the peak 'drug-sensitive' tail current amplitude 500 ms after the depolarizing voltage command in both wild-type and knockout IHCs (Fig. 2A). The apamin, curare and nifedipine-sensitive tail currents, obtained by subtraction, were many-fold larger in wild-type than in knockout IHCs. In contrast, 'drug-sensitive' tail currents in knockout IHCs were identical to 'rundown' measures made in the same genotype over equivalent time periods without drug exposure (Fig. 2B).

#### Voltage-gated calcium currents were unchanged in SK2-knockout IHCs

On the basis of these findings it appears that IHCs from knockout mice have no SK channels that can be

activated by prolonged opening of voltage-gated Ca<sup>2+</sup> channels, as can be accomplished reliably in wild-type IHCs. We next confirmed that this failure does not arise from an underlying loss of voltage-gated calcium channels. Calcium and barium currents were revealed by blockade of voltage-sensitive potassium channels in both wild-type and knockout hair cells (Fig. 3). As shown in previous studies (Platzer *et al.* 2000; Beutner & Moser, 2001; Marcotti *et al.* 2003*b*; Grant & Fuchs, 2008), calcium currents in mammalian cochlear hair cells were rapidly activating and minimally inactivating (over a time course of 100 ms at 20–22°C). Calcium channels in knockout IHCs were functionally indistinguishable from those in wild-type IHCs. For example, when 10 mM calcium was replaced by 10 mM barium, current amplitude

doubled in IHCs of both genotypes. Peak barium currents were 1.89-fold ( $\pm$  0.33, n = 5) larger than peak calcium currents in wild-type IHCs, and 2.03-fold larger ( $\pm$  0.45, n = 6) than peak calcium currents in knockout IHCs, showing similar permeability ratios of barium to calcium in both genotypes. Also, exposure to 10  $\mu$ M nifedipine reduced the barium current by 50–70% in both wild-type ( $62 \pm 8\%$ , n = 4) and knockout ( $52 \pm 4\%$ , n = 2) hair cells. Activation time-course as a function of membrane potential, based on single exponential fits, ranged between 1.0 and 0.3 ms at room temperature in IHCs from both genotypes. In particular, the activation time constant for calcium current at the peak of the *I*–*V* relation (~0 mV) averaged 0.40 ms ( $\pm$  0.12, n = 6) in wild-type, and 0.41 ms ( $\pm$  0.24, n = 6) in knockout IHCs. Finally, a slight decay





Membrane currents evoked during 3-s-long voltage steps between -88 and +24 mV in wild-type (WT) and SK2-knockout (KO) IHCs. *Aa*, at membrane potentials positive to -40 mV an initially rapid rise in outward current in a WT IHC (P7) was followed by a slowly rising component, accompanied by a slowly decaying tail current on repolarization to -48 mV. Exposure to 300 nm apamin (grey traces) eliminated the slow outward component and slow tails. *Ab*, the same protocol caused rapidly rising outward currents in a KO IHC (P10), but no later slow component. Apamin had little to no effect on membrane current. *Ba*, same protocol as in *A*. Slowly rising and decaying outward component in a WT IHC (P8) was eliminated by 50  $\mu$ M of the L-type calcium channel blocker nifedipine (residual currents in grey). *Bb*, in contrast, nifedipine exposure caused little to no change in rapid outward currents in a KO IHC (P9). Note that the voltage-dependent outward currents of the SK2-knockout IHCs are similar in overall shape and magnitude to those in wild-type IHCs after the SK2 component was blocked.

in current was observed for the largest calcium currents in some IHCs (Fig. 3). However, this behaviour was not found consistently, and was not specifically associated with IHCs of either genotype. The maximal percentage decay of calcium current at 60 ms among six wild-type IHCs was  $7 \pm 8\%$ , and  $11 \pm 9\%$  in six knockout IHCs. This slight drop in current may reflect calcium-dependent inactivation (Marcotti *et al.* 2003*b*; Grant & Fuchs, 2008), but was not investigated further in this work.

Thus, based on the similar kinetics, preferential barium permeability, and sensitivity to dihydropyridines, the same CaV1.3-type channel ( $\alpha$ 1D) is expressed in both wild-type and knockout IHCs. Most importantly, lack of the SK2 gene in IHCs had no bearing on calcium (or barium) current amplitude (Fig. 3*C*). Wild-type IHCs had average peak barium currents of 854.3 pA ( $\pm$  181.4, n = 7), while knockout IHCs had average peak barium currents of 714.4 pA ( $\pm$  87.64, n = 7; P = 0.50). Thus, the differential appearance of SK currents in wild-type and knockout IHCs did not result from the absence of activating calcium influx in the transgenic inner hair cells.

Other aspects of IHC excitability were essentially equivalent, though not identical, in wild-type and knockout hair cells. Neonatal IHCs (before the onset of hearing) from both genotypes had voltage-gated potassium currents that activated positive to -36 mV and, in the presence of apamin, curare or nifedipine, inactivated slightly during a 2-s-long depolarization (Fig. 1). These currents were approximately 12% smaller on average in knockout IHCs than in wild-type IHCs. The outward current amplitude (measured at 60 ms) produced by a voltage step to 0 mV averaged 1402 pA ( $\pm$  49.21) in 58

control IHCs, and 1223 pA ( $\pm$  60.79) in 60 SK2-knockout IHCs (difference significant at *P* = 0.02). Also, activation time constants at +28 mV (measured between 0 and 60 ms after the voltage transition) were slightly shorter in knockout (2.6 ms  $\pm$  0.1, *n* = 31) than in wild-type mice (3.3 ms  $\pm$  0.1, *n* = 49) and significantly different (*P* << 0.01).

In older IHCs (postnatal days 18–19), an additional component of outward current that activated rapidly even at negative membrane potentials was observed. This developmentally late component was iberiotoxin sensitive and so presumably reflects the expression of BK-type channels after the onset of hearing (Kros *et al.* 1998). The iberiotoxin-sensitive component (measured 1.5 ms after a step to +36 mV) averaged 920.0 pA ( $\pm$  99, n=7) in knockout, and 1263 pA ( $\pm$  283, n=3) in wild-type IHCs. This difference was not significant (P = 0.17).

### Synaptic currents were absent from SK2-knockout hair cells

Measurements from neonatal IHCs demonstrated that SK currents were totally eliminated, while other voltage-gated conductances, including calcium channels, were little changed. Next, we investigated synaptic activity during application of high potassium saline to evoke synaptic currents. Since IHCs are transiently subject to efferent innervations in the first two postnatal weeks (Glowatzki & Fuchs, 2000; Katz *et al.* 2004), and after the onset of hearing, these efferents preferentially innervate OHCs, it was possible also to examine cholinergic inhibition





*A*, apamin-sensitive tail currents in a wild-type (WT) IHC (P11) were obtained by subtraction of currents in the presence of apamin from those in control condition. Dotted vertical line indicates measurement point for quantitative comparison of wild-type and SK2-knockout hair cells. *B*, average magnitude of SK-like tail currents in wild-type (WT) and SK2-knockout (KO) IHCs. Tail currents were obtained by subtraction from control currents in each of the listed blockers: 300 nm apamin, 100  $\mu$ m curare (alternative SK channel blocker) and 50  $\mu$ m nifedipine (L-type calcium channel blocker). Holding voltage was -48 mV in all cases. In each condition the maximal drug-sensitive tail current (usually following an activating voltage step to +24 mV) in the KO IHCs was not different from the rundown effect over the time of recording. Mean and standard error indicated and number of cells tested listed above each bar. in both IHCs and OHCs. Whole-cell, ruptured-patch voltage-clamp recordings were collected from hair cells in the ex vivo organ of Corti preparation as previously described (Katz, 1979; Glowatzki & Fuchs, 2000; Katz et al. 2004; Lioudyno et al. 2004; Gomez-Casati et al. 2005; Goutman et al. 2005). In wild-type hair cells 'spontaneous' release of ACh from efferent endings was observed as inward current transients at -84 mV holding potential. These could be increased in frequency with elevated external potassium (40 mM) (the high potassium saline itself caused a slow inward current through the resting membrane conductance). At -84 mV, both the cation current through the AChR, and current through SK channels were inward-going. Positive to -60 mV in 5-6 mM external potassium, these synaptic currents were biphasic (Glowatzki & Fuchs, 2000; Lioudyno et al. 2004). High potassium-evoked synaptic currents were observed in 28/31 neonatal inner hair cells (IHCs) from wild-type mice (P6–P12, Fig. 4Aa). However, spontaneous synaptic currents were never seen under equivalent conditions in 20 IHCs from knockout mice (Fig. 4Ab). On a few occasions, electrical stimulation of efferent fibres was used to evoke synaptic release on to neonatal IHCs from wild-type mice (Fig. 4Ac), an alternative method for studying efferent effects (Goutman *et al.* 2005). Electrical stimulation failed to evoke synaptic currents in three IHCs from knockout mice (Fig. 4Ad). Likewise, potassium-evoked synaptic currents were observed in 19/26 OHCs from older wild-type mice (P9–P24, Fig. 4Ba). However, spontaneous synaptic currents were never seen under equivalent conditions in 12 OHCs from knockout mice (Fig. 4Bb).

Thus, cochlear hair cells from knockout mice appeared to have none of the readily detectable efferent synaptic currents observed in wild-type mice. Indeed, although only the SK2 gene is disrupted in the transgenic animal, the hair cells failed to show any detectable postsynaptic





Calcium (barium) currents were isolated with a combination of K<sup>+</sup> channel blockers, including 4-AP and TEA, as well as a Na<sup>+</sup> channel blocker, TTX. *A*, inward currents from a wild-type (WT) IHC (P8) recorded initially with 10 mM external calcium (black trace), then in 10 mM external barium (grey trace) which more than doubled the peak amplitude. Application of 10  $\mu$ M nifedipine caused a 50–70% reduction in barium current amplitude (light grey trace). *B*, same experiment carried out on a knockout (KO) IHC (P10) produced equivalent changes in current. *C*, average inward currents in 10 mM calcium (*n* = 3), 10 mM barium (*n* = 7) for wild-type (WT, black bars) and knockout (KO, grey bars) IHCs. Means not significantly different between cell types. Rise time for inward current at 0 mV from single exponential fit for WT (black) and KO (grey) IHCs. Right-hand *y*-axis applies only to time constant data.

activity, including that through the ligand-gated nAChRs themselves. This was an unexpected result and prompted investigation of other aspects of the cochlear efferents in the knockout mice. Thus, we examined the innervation pattern of the transgenic cochleas using antibodies that selectively labelled efferent terminals.

## Efferent innervation of OHCs from SK2-knockout mouse

Efferent contacts on OHCs were visualized with confocal microscopy using antibodies to the synaptic vesicle

associated protein SV2 (Buckley & Kelly, 1985) or to choline acetyltransferase (ChAT). After the onset of hearing ( $\sim$  postnatal day 12, P12), prominent SV2-positive synaptic endings can be found on the basal pole of most wild-type OHCs (Fig. 5*A*). Antibodies to SV2 provided better signal-to-noise than those to ChAT (Supplementary Fig. 1), although both show similar outcomes when quantifying presynaptic terminals (boutons) on OHCs from P21 wild-type and knockout cochleas (Fig. 5 and Supplementary Figs 1 and 2). There was no change in overall cochlear morphology or hair cell number. However, there was a marked





Aa, voltage-clamp recording from postnatal day 12 (P12) wild-type (WT) IHC. Exposure to high potassium saline caused a steady inward current at -84 mV, through its effect on resting conductance, and transient inward currents due to release of ACh from associated efferent endings depolarized by high potassium. Ab, high potassium saline caused steady inward current but no synaptic currents in a knockout (KO) IHC (P9). Ac and Ad, electrical stimulation-shocked efferent axons, producing synaptic currents in a WT IHC (P7), but not in a KO IHC (P6). Arrow indicates the electrical shock. Ba, application of 40 mm K<sup>+</sup> saline causes a slow inward current and numerous rapid inward transients (synaptic currents) at -84 mV on a wild-type (WT) neonatal OHC (P9). Bb, application of 40 mm K<sup>+</sup> caused slow inward current, but no synaptic currents in knockout (KO) OHCs (P12).

reduction in the number of efferent terminals (boutons) on knockout OHCs (Fig. 5). SV2-positive terminals (boutons) associated with DAPI-stained OHC nuclei were counted in cochlear segments from four wild-type and three knockout mice (P21). Each antibody-exposed cochlear segment was processed individually, resulting in nine wild-type and six knockout immunolabelling experiments. All mutant segments had many fewer SV2-positive terminals (boutons) per OHC nucleus than did normal cochlear segments (80% down). The average number of boutons per OHC was 1.93 ( $\pm$  0.07) for wild-type and 0.39 ( $\pm$  0.04) for knockout (P < 0.0001). Furthermore, it was observed (in confocal *z*-axis reconstructions) that the small number of remaining SV2positive 'boutons' in knockout cochlear segments tended to end among supporting cells, rather than on OHCs.





Compressed confocal z-stacks showing efferent nerve terminals (boutons) labelled by antibodies to the synaptic vesicle protein SV2. Green demarcates SV2-antibody label, blue shows the DAPI-labelled OHC nuclei (contrast and edge-enhanced in Photoshop). *A*, outer hair cell region, upper-middle turn, wild-type cochlea (WT experiment no. 1 in *C*). *B*, the number of labelled boutons was greatly reduced in the knockout (KO experiment no. 1 in *C*). Scale bar corresponds to 10  $\mu$ m. Imaged with a Zeiss 40X water immersion C-Apochromat objective (NA, 1.2). *C*, counts of SV2-positive boutons per OHC nucleus in 9 wild-type cochlear segments and 6 knockout cochlear segments (from 4 WT and 3 KO mice, P21). Numbers of OHCs counted indicated above each bar. Sum shows significant difference in mean and s.E.M. of boutons per OHC for 266 wild-type and 240 knockout OHCs (*P* < 0.0001). Boutons were counted in 3-D z-stacks to assure their location on OHCs.



**Figure 6. SK2-knockout hair cells lose cholinergic sensitivity** *Aa*, application of 1 mm ACh evoked inward current at -94 mV in a wild-type (WT) IHC (P10). *Ab*, 1 mm ACh caused no change in membrane current in a knockout (KO) IHC at -94 mV (P8). *Ac*, average membrane current produced by applied ACh at -94 mV in wild-type and knockout IHCs. *Ba*, application of 1 mm ACh caused inward

## Cholinergic sensitivity was absent in SK2-knockout hair cells

The marked reduction in efferent innervation eliminates synaptic activity per se as an indicator of cholinergic sensitivity in the SK2-knockout hair cells. Therefore, cholinergic sensitivity also was assessed by direct application of ACh on to hair cells from a nearby perfusion array to determine if nAChRs were functional in knockout hair cells. In IHCs and OHCs from wild-type cochleas, the application of  $100 \,\mu\text{M}$  or  $1 \,\text{mM}$ ACh in 5.8 mM external potassium saline gave rise to prominent inward currents at -94 mV (Fig. 6Aa and Ba). Under these recording conditions both the cation current through nAChRs, as well as associated potassium flux through SK channels, will be inward-going. Robust responses averaging nearly 200 pA in amplitude could be elicited repeatedly from 47/48 wild-type IHCs younger than P12 (Fig. 6Ac) and currents evoked by ACh averaged 140 pA in amplitude in 25/30 wild-type OHCs aged P10-P24 (Fig. 6Bc). In contrast, none of 27 IHCs and 17 OHCs from SK2-knockout mice produced detectable changes in membrane current when exposed to ACh (Fig. 6). To increase driving force on associated SK current at negative membrane potentials, ACh also was applied in 40 mM potassium saline (Fig. 6*Ca* and *Da*), but here too no membrane currents were evoked from knockout hair cells by even much longer-lasting application of ACh (Fig. 6*Cb* and *Db*). Sixteen of 17 KO OHCs (Fig. 6*Bc*) were tested in this condition. In contrast to these ligand-gated channels, voltage-dependent membrane conductances were not significantly different in wild-type and knockout OHCs. Outward membrane current (flowing through voltage-gated potassium channels) during a voltage step to -4 mV from a holding potential of -84 mV averaged 1788 pA  $(\pm 356)$  in 21 wild-type OHCs, and 1257 pA  $(\pm 141)$  in 14 knockout OHCs (P = 0.25).

# Gene expression associated with efferent inhibition in SK2-knockout cochleas

Since there was no detectable synaptic activity, nor response to direct application of ACh, we next asked whether AChR expression was disrupted in the knockout hair cells. Unfortunately, despite repeated efforts in this and other laboratories, we have yet to find generally available antibodies that reliably label nAChR subunits in cochlear tissue. Thus, we examined the mRNA expression level for the  $\alpha$ 9 and  $\alpha$ 10 subunits of the nicotinic ACh receptor (nAChR). We used quantitative RT-PCR on whole-cochlea tissue from postnatal day 21 wild-type and SK2-knockout mice. The Ct (threshold cycle number) values for each target gene ( $\alpha$ 9,  $\alpha$ 10 and SK2) and internal control gene ( $\beta$ -actin) were provided from real-time PCR as shown in Fig. 7A. Each reaction was run in triplicate and the illustrated experiment was repeated 5 times to provide the average values shown in Fig. 7B. As expected, mRNA for the housekeeping gene  $\beta$ -actin was present at higher levels than that of  $\alpha 9$ ,  $\alpha 10$  or SK2 (detected with fewer amplification cycles). By visual inspection it is clear that  $\beta$ -actin,  $\alpha 9$  and  $\alpha 10$  occurred at similar levels in wild-type (continuous lines) and SK2-knockout (continuous lines and symbols) tissues. Using the  $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), the data were quantified as a change in gene expression normalized to that of  $\beta$ -actin (see Methods). mRNA levels for  $\alpha$ 9 and  $\alpha$ 10 were not significantly changed in the knockout cochlear tissues:  $1.15 \pm 0.16$  ( $\alpha 9$ ) and  $1.14 \pm 0.15$  ( $\alpha 10$ ), relative to wild-type (Fig. 7B). As expected, gene expression of the SK2 channel was essentially zero in knockout tissues (Fig. 7B). The residual amount of SK2 transcript from the knockout was equal to or less than the amount from the untreated blank control (not shown).

#### Discussion

The present work investigates hair cells from the cochleas of SK2 knockout mice (Bond *et al.* 2004). These experiments provide compelling evidence that the SK2 gene is solely responsible for encoding this class of small conductance, calcium-activated potassium channel in cochlear hair cells, in agreement with a previous report (Johnson *et al.* 2007). Apparently, no other gene product can substitute for SK2, consistent with previous studies of the selective expression of the SK2 gene in vertebrate cochlea (Dulon *et al.* 1998; Matthews *et al.* 2005). Further, the deletion of SK2 function appeared not to disrupt other features of hair cell electrophysiology. Most voltage-gated ionic currents were essentially unchanged in wild-type and knockout hair cells. This includes the 'L-type' voltage-gated calcium currents that were used

current at -94 mV in a WT OHC (P14). *Bb*, 1 mm ACh had no effect on a KO OHC at -94 mV (P14). *Bc*, average change in membrane current produced by ACh at -94 mV in WT and KO OHCs. *Ca*, at -94 mV, 1 mm ACh was used in high potassium saline to increase driving force through AChR-associated SK channels in a WT IHC (P10). *Cb*, even under these conditions ACh produced no change in membrane current in a KO IHC (P10). *8/*27 knockout IHCs were tested with 1 mm ACh in 40 mm K<sup>+</sup> saline at -94 mV, the remainder were tested in 5.8 mm K<sup>+</sup> saline in *Ac*. *Da*, inward current at -94 mV produced by 1 mm ACh in 40 mm K<sup>+</sup> saline in a WT OHC (P9). *Db*, application of 40 mm K, but not ACh, could cause inward current in a KO OHC (P12) held at -94 mV. These conditions applied to 16/17 OHCs presented in *Bc*.

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Figure 7. Quantitative analysis of  $\alpha$ 9,  $\alpha$ 10 and SK2 mRNA levels in wild-type and SK2-knockout mice

Real-time PCR performed with whole-cochlear RNA from the wild-type and SK2-knockout mice was used to evaluate the levels of each of the  $\alpha$ 9,  $\alpha$ 10 subunits comprising the nicotinic ACh receptor (nAChR) and SK2 RNAs, normalized to the house keeping gene,  $\beta$ -actin (n = 5; see Methods). *Aa*, *Ab* and *Ac*, amplification plot showing the real-time RT-PCR SYBR Green I fluorescence history *versus* cycle number for target genes  $\alpha$ 9,  $\alpha$ 10 and SK2 (black), and house keeping gene,  $\beta$ -actin (grey) in wild-type (WT, continuous lines) cochlear cDNA and SK2-knockout (KO, continuous lines) cochlear cDNA. The horizontal dashed line indicates the threshold to determine Ct values. *B*, relative expression compared with wild-type was calculated as the mean of  $2^{-\Delta\Delta Ct} \pm$  s.E.M. and shows that  $\alpha$ 9 and  $\alpha$ 10 mRNA levels are not significantly altered in SK2-knockout mice compared to wild-type to activate SK currents in wild-type IHCs. A similar method was used previously to activate SK-type currents in hair cells of turtles (Tucker & Fettiplace, 1996) and mice (Marcotti et al. 2004; Johnson et al. 2007). Further, the later developmental arrival of rapidly activating, iberiotoxin-sensitive 'BK-type' potassium channels shown previously in mammals (Kros et al. 1998; Langer et al. 2003; Hafidi et al. 2005) and birds (Fuchs & Sokolowski, 1990) occurred on schedule in both wild-type and knockout IHCs. A previous study also found that voltage-gated potassium currents appeared normally in SK2-knockout IHCs (Johnson et al. 2007). However, in that work, voltage-gated calcium currents were significantly smaller in the P6-P8 knockout IHCs. It is not certain why the present study found no significant difference in calcium current amplitude, although the conditions of recording were substantially different in the two cases: 10 mM versus 1.3 mM Ca<sup>2+</sup>, room temperature *versus* body temperature, and differential degrees of potassium channel block.

Unexpectedly, unlike voltage-gated conductances in hair cells, the ionotropic AChRs were profoundly affected by deletion of the SK2 gene. SK2-knockout outer hair cells were completely insensitive to exogenous ACh, implying absent or otherwise dysfunctional ACh receptors. Likewise, spontaneous cholinergic synaptic currents were not seen in knockout outer hair cells. This last observation, however, results also from the greatly diminished cholinergic innervation of outer hair cells in the knockout cochleas. SV2-positive efferent terminals (boutons) were dramatically reduced in number under OHCs in the knockout cochleas. Because of the developmental and structural complexity of afferent and efferent innervation under IHCs, quantitative analysis of efferent boutons was not attempted there. However, presumptive efferent synapses also were reduced on the neonatal knockout IHCs, since finger-like extensions encircling wild-type IHCs were not observed in the knockout (Supplementary Fig. 3). Since spontaneous synaptic currents (even when efferents were depolarized by high potassium saline) also were not detected in knockout IHCs, it seems safe to conclude that functional cholinergic synapses on neonatal IHCs were indeed absent, as from OHCs. There is then a correspondence between a lack of functional cholinergic receptors, as determined by the failure of directly applied ACh to elicit membrane currents, and abnormal efferent innervation in the knockout hair cells.

Why would SK2 deletion disrupt efferent innervation of the cochlea? This is particularly surprising since other

mice. However, as expected, SK2 mRNA level decreased about 98% in the SK2-knockout mice. Statistically significant differences (P < 0.05) in expression levels compared with wild-type are indicated by an asterisk.

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genetic alterations at this synaptic contact do not have such an effect. In particular, knockout of the  $\alpha$ 9 receptors that also eliminates cholinergic sensitivity only slightly reduces, but does not eliminate efferent contacts on OHCs (Vetter et al. 1999). Recently, a similar effect was reported in a mouse lacking functional  $\alpha 10$  (Vetter *et al.* 2007), the other subunit that contributes to hair cell AChRs (Elgoyhen et al. 2001). Efferent innervation is maintained, despite the fact that hair cells become insensitive (the  $\alpha$ 9 knockout), or very poorly sensitive (the  $\alpha 10$  knockout), to exogenous ACh in these transgenic models (Vetter et al. 2007). Thus, among ion channel deletions examined to date, loss of SK2 causes the most severe disruption in efferent innervation. Even some of the few remaining SV2-positive boutons appeared to locate among Deiters cells, rather than on OHCs.

SK2 deletion could alter synaptic structure and function in two general ways: by somehow interrupting intrinsic regulation of postsynaptic maturation or by altering extrinsic activity-dependent signalling between hair cell and presumptive efferent terminal that is required for synapse formation and stabilization. Two arguments can be made against this extrinsic mechanism. First, as mentioned, loss of the hair cell AChR itself does not prevent efferent synapse formation. Second, we found no evidence for functional ACh receptors, as required if aberrant 'excitatory' signalling without SK channels were to repulse efferent contacts. A third possibility should be mentioned, that SK2 channels also are expressed in efferent neurones and their absence causes unknown presynaptic deficiencies. While this cannot be ruled out, the fact that efferent axons in the SK2 knockout reach the cochlea, shows that some aspects of pathfinding and axonal growth remain operational.

Another hint that hair cell activity per se regulates synaptic maturation is provided by genetic deletion of the voltage-gated calcium channels located at hair cell afferent synapses. In contrast to the foregoing manipulations that reduce efferent function, genetic deletion of the CaV1.3 voltage-gated calcium channels that serve afferent transmission from IHCs leads to prolonged maintenance of efferent synaptic contacts on IHCs that ordinarily are eliminated at the onset of hearing (Brandt et al. 2003; Glueckert et al. 2003; Nemzou et al. 2006). Finally, SK2 knockout IHCs have reduced afferent synaptic efficacy (Johnson et al. 2007). This change is attributed to altered excitability in the absence of SK channels, presumably due to greater calcium influx without the negative feedback of SK gating. Given that efferent contacts are sustained in IHCs with reduced calcium influx (CaV1.3 knockouts), it is reasonable to propose that efferent synapse formation is suppressed when neonatal calcium influx is no longer regulated by SK feedback.

The present studies show that functional AChRs are absent from the SK2-knockout hair cells. However,

messenger RNAs for  $\alpha$ 9 and  $\alpha$ 10 continue to be expressed in the SK2-knockout cochleas. It would be of interest to examine the localization of AChRs in SK2-knockout hair cells. However, reliable antibodies for the  $\alpha$ 9 and  $\alpha$ 10 subunits are still not generally available. In cochlear hair cells, it is thought that Ca2+-permeable ACh receptors and SK channels constitute a Ca<sup>2+</sup>-signalling microdomain in the postsynaptic membrane. It has been suggested that, through such close coupling, the gating kinetics of the SK channels determine the time course of synaptic action, outlasting the driving calcium signals (Oliver et al. 2000). Therefore, AChRs and SK channels may be components of a macromolecular synaptic complex whose assembly and proper trafficking can be disrupted by the loss of SK channels. It will be of interest to examine the ultrastructure of SK2 knockout hair cells to determine if other features, such as the synaptic cisterns, also depend on SK2 expression.

An original motivation for this work was the possibility of studying efferent regulation of the cochlea under conditions in which the sign of that regulation was reversed from inhibition to excitation. That is, in the absence of the associated SK2 channels, nAChRs should provide ligand-gated cationic flux that depolarizes the hair cells. The present experiments, however, found no evidence of residual excitation that could be mediated by ACh. Even seconds-long exposure to 1 mM ACh had no effect on the membrane conductance or excitability of SK2-knockout hair cells (Supplementary Fig. 4). Nonetheless, given that these recordings were restricted to the apical half of the cochlea, it is still possible that residual efferent effects persist in the basal turn.

While the SK2-knockout mouse may not advance studies of efferent synaptic electrophysiology *per se*, the intriguing effects on efferent innervation patterns motivate additional effort to understand the factors that regulate synapse formation and maturation during neonatal cochlear development.

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J.-H. Kong and others

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#### Supplemental material

Online supplemental material for this paper can be accessed at: http://jp.physoc.org/cgi/content/full/jphysiol.2008.160077/DC1