# Presynaptic maturation in auditory hair cells requires a critical period of sensory-independent spiking activity

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**The development of neural circuits relies on spontaneous electrical activity that occurs during immature stages of development. In the developing mammalian auditory system, spontaneous calcium action potentials are generated by inner hair cells (IHCs), which form the primary sensory synapse. It remains unknown whether this electrical activity is required for the functional maturation of the auditory system. We found that sensory-independent electrical activity controls synaptic maturation in IHCs. We used a mouse model in which the potassium channel SK2 is normally overexpressed, but can be modulated in vivo using doxycycline. SK2 overexpression affected the frequency and duration of spontaneous action potentials, which prevented the development of the $Ca^{2+}$-sensitivity of vesicle fusion at IHC ribbon synapses, without affecting their morphology or general cell development. By manipulating the in vivo expression of SK2 channels, we identified the "critical period" during which spiking activity influences IHC synaptic maturation. Here we provide direct evidence that IHC development depends upon a specific temporal pattern of calcium spikes before sound-driven neuronal activity.**

exocytosis | cochlea | calcium current | kcnn2

The development of neural circuits, including those of the sensory systems, relies on a combination of intrinsic genetic programs, as exemplified by the regulation of auditory hair cell development by the microRNA miR-96 (1), and experience-independent or spontaneous, structured patterns of electrical activity that occur during a critical period (2–4). Increased levels of intracellular $Ca^{2+}$ that result from spontaneous electrical activity have been shown to regulate a variety of cellular responses (5) that are thought to control, for example, synaptic remodelling (4). The primary sensory receptors in the adult mammalian cochlea, the inner hair cells (IHCs), respond to sound stimuli with small, sustained and graded receptor potentials (6). By contrast, immature IHCs fire spontaneous $Ca^{2+}$ action potentials for about 2 wk before the onset of hearing (7–11), which occurs at around postnatal day 12 (P12) in altricial rodents. By analogy with the visual system (2–4), action potentials in immature IHCs have been proposed to drive the development of the mammalian cochlea (7–12). However, this hypothesis has not been tested. In this study, we show that subtle changes in the pattern of action potential activity in IHCs in vivo prevent key developmental changes in the $Ca^{2+}$ dependence of neurotransmitter release at the cell's ribbon synapses that normally occur before the onset of hearing (13, 14). Moreover, we found that this dependence was restricted to a "critical period" during the second postnatal week of development, which is just before the onset of sound-induced responses.

## Results

In the mammalian auditory organ, the primary sensory IHCs elicit spontaneous spiking activity before the onset of hearing (7–11)). Action potentials in IHCs are generated primarily through the interplay between a depolarizing $Ca_V1.3$ $Ca^{2+}$ current (Cacna1d) and a repolarizing, delayed rectifier $K^+$ current (15, 16). However, the ability to sustain repetitive firing activity depends largely upon the activation of the transiently expressed small conductance $Ca^{2+}$-activated $K^+$ current SK2 (Kcnn2) (8, 17–19). Using near-physiological experimental conditions (35–37 °C and 1.3 mM extracellular $Ca^{2+}$) (10) and an electrophysiological approach applied to a transgenic mouse that overexpresses the SK2 channel (20), we investigated whether the spiking activity in vivo was required for IHC synaptic maturation.

**Effect of SK2 Overexpression in Immature IHCs.** Overexpression (OE) of SK2 channels in transgenic mice was evident from an increased SK2 immunofluorescence in the basolateral membrane of immature IHCs (Fig. 1*A*). This was associated with a significantly larger apamin-sensitive SK2 current (Fig. 1*B*) (17, 19) compared with control cells (two-way ANOVA $P < 0.0001$; Fig. 1*C*). Despite the larger size of the SK2 current in OE IHCs, its developmental time course was similar to that in control cells (8, 17, 21), being completely down-regulated by P16–P18 (Fig. 1*C*). Apart from the OE of SK2, all other currents expressed in immature OE IHCs were normal (Table 1). In prehearing IHCs, SK2 channels are also involved in action potential inhibition when activated by $Ca^{2+}$ entering hair cells through α9α10-nicotinic acetylcholine (ACh) receptors (8, 17, 18). These receptors are activated by the neurotransmitter ACh, which is released by the efferent fibers that make transient axosomatic synaptic contacts with IHCs (22). The expression of functional nicotinic receptors and the establishment and/or maintenance of efferent terminals requires the presence of SK2 channels (23, 24). We found that immature IHCs from SK2 OE mice showed a normal sensitivity to the extracellular application of ACh (Fig. S1 *A–D*). Moreover, the ACh-sensitive current in OE mice followed the same developmental time course to that of control cells (8, 17, 25) (Fig. S1*E*), indicating that the additional SK2 channels in OE mice are not coupled to α9α10 ACh receptors (17, 19). In addition, the

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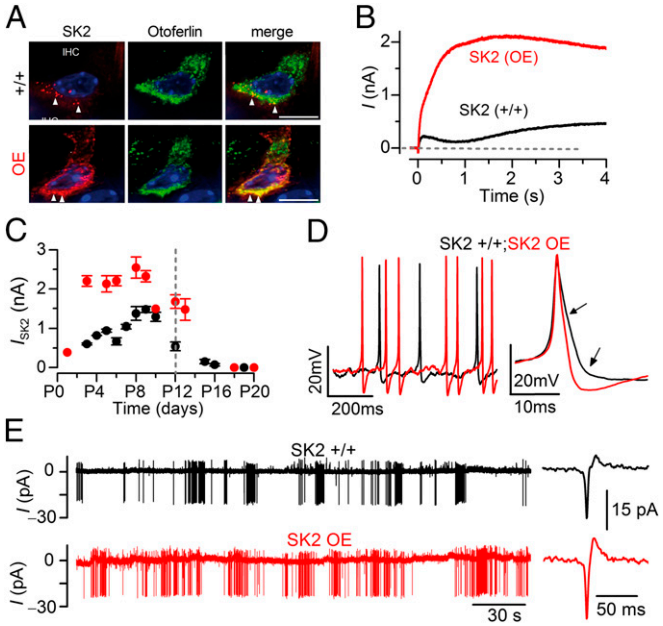


**Fig. 1.** SK2 channel OE alters action potential activity in immature IHCs. (*A*) Immature IHCs at P4 immunolabeled for SK2 channels (red) and the IHC marker otoferlin (green) in control ($^{+/+}$) and OE mice. Nuclei were stained with DAPI (blue). (Scale bar, 10 μM.) (*B*) Size of the outward $K^+$ current, which also contains the SK2 current ($I_{SK2}$), in control and immature OE IHCs recorded by applying 4 s voltage steps to –30 mV from a holding potential of –84 mV. (*C*) Size of the isolated $I_{SK2}$ as a function of development, which was obtained by subtracting the current during the superfusion of a $Ca^{2+}$-free solution from the total outward current (17, 19). Number of IHCs tested: controls 4, 2, 5, 7, 7, 2, 7, 6, 4, 3, 7, 2, 3, 1; SK2 OE 1, 6, 3, 3, 3, 3, 1, 9, 2, 2, 3. (*D*) Spontaneous action potentials recorded in whole-cell current clamp mode control (black) and SK2 OE (red) immature IHCs. Right panel shows an action potential on an expanded time scale. Note the faster repolarization phase of the spike in the SK2 OE IHC compared with the control cell. (*E*) Cell-attached recordings of spontaneous spikes from immature control and OE IHCs. Right panels show an expanded version of a single spike. All recordings were from apical IHCs at 37 °C and using physiological 1.3 mM $Ca^{2+}$.

efferent system seems unaffected by the OE of the SK2 channels since inhibitory postsynaptic potentials (IPSPs) and currents (IPSCs), which are mediated by the spontaneous or evoked release of ACh onto IHCs, were not significantly different between control and SK2 OE mice (Fig. S1 *F–I*).

**Spontaneous Action Potentials Promote IHC Development.** Control and SK2 OE IHCs fired spontaneous action potential activity during immature stages of development (Fig. 1*D*). However, we found that the larger SK2 current in immature SK2 OE IHCs caused a more robust repolarization of action potentials (Fig. 1*D*), which significantly decreased their width (control, 3.8 ± 0.2 ms, $n$ = 14; OE, 3.3 ± 0.1 ms, $n$ = 16; $P < 0.02$, P4–P7) and increased their frequency (control, 2.4 ± 0.4 Hz; OE, 8.4 ± 0.6 Hz; $P < 0.0001$, P4–P7). Action potential activity was further investigated using the cell-attached recording configuration (Fig. 1*E*) (10), which has the advantage over whole-cell recordings (Fig. 1*D*) of preserving the intracellular milieu, including the endogenous $Ca^{2+}$ buffers. In cell-attached mode, the spontaneous action potentials are manifest as currents (10). The mean spike frequency was again significantly higher in OE cells (control, 1.02 ± 0.14 Hz, $n$ = 20; OE, 1.86 ± 0.22 Hz, $n$ = 17; $P < 0.002$, P4–P7) and the spike peak-to-peak width (Fig. 1*E*, right panels) was significantly shorter (control, 4.4 ± 0.2 ms, 60 spikes from 10 cells; OE, 3.1 ± 0.1 ms, 72 spikes from 11 cells, $P < 0.0001$). The coefficient of variation of interspike intervals, which provides a quantitative measure of regularity in spike firing activity (26, 27), indicates that IHCs from both genotypes showed a burst-like firing pattern (Fig. 1*E*) as previously reported in immature apical IHCs from wild-type rodents (10). However, we found a significantly reduced coefficient of variation in SK2 OE IHCs (control, 3.7 ± 0.2, $n$ = 20; OE, 2.7 ± 0.2, $n$ = 17, $P < 0.0004$), which is likely to be a consequence of their higher action potential frequency.

We then investigated whether the altered action potential activity in immature IHCs overexpressing SK2 channels had any effect on their normal functional maturation (28) by performing electrophysiological recordings from IHCs of mature mice. The presynaptic function of IHCs was assessed by measuring the change in cell membrane capacitance ($\Delta C_m$) with membrane depolarization, which gives an indication of synaptic vesicle fusion to the cell membrane. The release of docked vesicles at functionally mature IHC ribbon synapses is linearly dependent on $Ca^{2+}$ influx via $Ca_V1.3$ channels (13, 14), which is thought to be suited for translating graded receptor potentials in response to sound. However, before the onset of hearing, the synaptic machinery of IHCs shows a steep, nonlinear $Ca^{2+}$ dependence, which is better suited for encoding spontaneous action potential activity (13, 14). We found that in SK2 OE IHCs, which had an abnormally high frequency of action potential activity, the linearization of the $Ca^{2+}$ dependence failed to develop (Fig. 2). In mature SK2 OE IHCs (P26–P32), the maximal size of the $Ca^{2+}$ current and the corresponding $\Delta C_m$ was similar to that of control littermates (Fig. 2 *A* and *B*). However, the exocytotic $Ca^{2+}$ dependence, defined as the variation in $\Delta C_m$ as a function of the $Ca^{2+}$ current and measured using the synaptic transfer function (14), was significantly ($P <$ 0.0001) less linear in the mature SK2 OE (power of 2.2 ± 0.2, $n$ = 9, average from fits to individual cells) than in control mature IHCs (power of 1.1 ± 0.1, $n$ = 8; Fig. 2*C*). Despite the biophysical differences, the number (Fig. S2 *A–C*) (14, 29) and the typical

**Table 1. Properties of immature and adult IHCs from SK2 OE mice**

| | Immature | | Adult | |
|---|---|---|---|---|
| | P3–P12 | | P17–P26 | |
| Biophysical properties | $^{+/+}$ | OE | $^{+/+}$ | OE |
| Resting potential (mV) | −55.1 ± 1.8, 9 | −54.0 ± 1.3, 15 | −68.5 ± 2.4, 4 | −71.9 ± 1.7, 5 |
| $I_{K1}$ at –124 mV (pA) | −182 ± 15, 42 | −183 ± 16, 29 | — | — |
| $I_K$ at 0 mV (nA) | 5.0 ± 0.2, 46 | 5.9 ± 0.4, 32* | 11.1 ± 0.5, 14 | 10.3 ± 1.7, 5 |
| $I_{Ca}$ at –11 mV (pA) | −480 ± 40, 4 | −543 ± 37, 3 | −118 ± 6, 8 | −146 ± 14, 9 |
| $I_{K,n}$ at –124 mV (pA) | — | — | −371 ± 29, 11 | −349 ± 29, 5 |
| $I_{K,f}$ at –25 mV (nA) | — | — | 3.0 ± 0.4, 3 | 2.3 ± 0.2, 4 |

Values are means ± SEM and number of hair cells. Not present (–). $I_K$, which represents the total outward $K^+$ current in IHCs (i.e., delayed rectifier + SK2) (15, 17), was the only current found to be significantly different between control and OE cells (*). $I_{K1}$, inward rectifier $K^+$ current (52); $I_{Ca}$, calcium current (16); $I_{K,f}$, $Ca^{2+}$-activated $K^+$ current (7).

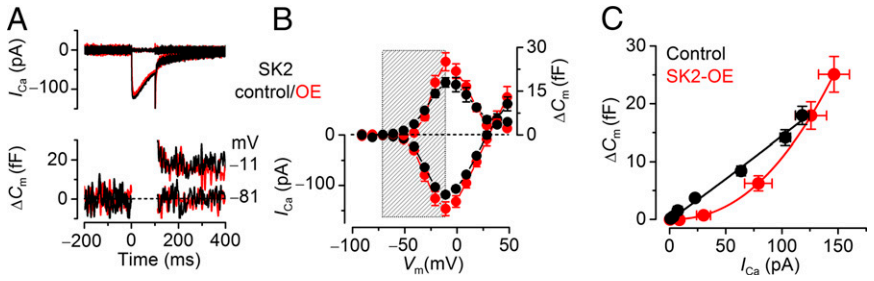


**Fig. 2.** Exocytotic $Ca^{2+}$ dependence in SK2 OE mature IHCs. Data are from apical coil control ($^{+/+}$: black) and SK2 OE (red) mature mouse IHCs (P23–P27). (*A*) $I_{Ca}$ and corresponding $\Delta C_m$ recordings in response to 100 ms voltage steps (10 mV increments) from −81 mV. For clarity, only the peak responses and those at −81 mV are shown. (*B*) Average $I_{Ca}$-voltage (*Lower*) and $\Delta C_m$-voltage (*Upper*) curves in control and OE IHCs. Maximal $I_{Ca}$ and $\Delta C_m$ were −118 ± 6 pA and 18.0 ± 1.6 fF (control $n = 8$); −146 ± 14 pA and 25.1 ± 3.1 fF (OE $n = 9$). (*C*) Synaptic transfer curves obtained by plotting the average $\Delta C_m$ against the corresponding $I_{Ca}$ for membrane potentials between −71 mV and −11 mV (shaded area in *B*). Fits are according to a power function $\Delta C_m = cI_{Ca}^N$ [Eq. 1] where $c$ is a scaling coefficient and the power is $N$.

ellipsoid shape (Fig. S2*D*) (30) of synaptic ribbons (14, 29) in mature SK2 OE IHCs were normal. The failure in the maturation of the exocytotic $Ca^{2+}$ sensitivity was not due to a more general developmental defect in SK2 OE IHCs since all other biophysical properties characteristic of mature cells were normal (Table 1 and Fig. S3). We would predict that in SK2 OE mice, the higher IHC exocytosis $Ca^{2+}$ dependence would lead to a narrowing of their dynamic range, causing a reduced sensitivity to low intensity sounds and possibly an overstimulation to those of high intensity. However, we found that auditory brainstem responses (ABRs) in SK2 OE mice, which reflect the activity of the auditory nerve, were normal in agreement with previous observations (31). It is possible that ABRs are not sensitive enough to resolve the reduced exocytotic $Ca^{2+}$ dependence, or alternatively, some compensatory mechanism within the auditory pathway may have occurred during the development of OE mice (14). Direct recordings from the afferent fibers are likely to be required to elucidate how exocytotic $Ca^{2+}$ dependence affects the transfer of auditory information at IHC ribbon synapses.

**Action Potentials Exert Their Developmental Role During a Critical Period.** We tested whether action potential–driven IHC maturation occurred during a specific time window or "critical period" of prehearing development. SK2 OE mice have a tetracycline-based genetic switch inserted into the 5′ untranslated region of the *Kcnn2* gene that allows channel expression to be lowered in vivo by dietary doxycycline (dox) administration, leaving expression only from the normal wild-type allele of *Kcnn2* (20, 32). For these experiments, SK2 OE and control pregnant or feeding females were given 0.5 mg/mL of dox daily in their drinking water. We found that in the continuous presence of dox, the frequency of spontaneous action potentials recorded in "whole-cell" from IHCs (3.1 ± 0.4 Hz, $n = 4$, P5) was not significantly different to that of control cells (2.4 ± 0.4 Hz, $n = 14$, P4–P7), thus fully restoring the normal spiking activity in SK2 OE cells. Moreover, the size of the $K^+$ currents characteristic of mature IHCs recorded in dox ($I_{K,f}$, 2.1 ± 0.2 nA, $n = 3$; $I_{K,n}$, −337 ± 42 pA, $n = 4$, P17–P21) were similar to those in control cells (Table 1). These findings, together with the fact that dox did not alter the exocytotic $Ca^{2+}$ dependence in control IHCs, indicate that dox treatment had no direct effect on the general function and development of cochlear IHCs.

We then investigated the exocytotic $Ca^{2+}$ dependence in mature IHCs when dox had been given to those mice from different developmental time points [embryonic day 0.5 (E0.5), E18.5, P3, P6, and P9]. We found that when the OE of SK2 channels in OE mice was reversed with dox during embryonic and early postnatal ages, the synaptic machinery of mature IHCs (P18–P21) had a normal linear exocytotic $Ca^{2+}$ dependence (Fig. 3 *A* and *B*). When dox was given from or after P6, it was unable to restore normal synaptic development (Fig. 3*B*), indicating that at least part of the "critical period" had been disrupted. Therefore, the lower boundary of the critical period is defined as the last time point at which dox administration was able to restore normal synaptic maturation (Fig. 3*B*). We then investigated the upper limit of the critical period by administering dox for more than 10 d, and removing it at different time points (E11.5, E15.5, P0, P3, and P6) to allow the OE of SK2 channels (Fig. 3 *C* and *D*). In this situation, the upper boundary would be just before the point at which dox removal does not interfere with normal synaptic development (Fig. 3*D*), such that normal spiking activity has been present for the whole critical period. We found that a normal near-linear exocytotic $Ca^{2+}$ dependence in mature IHCs (P17–P20) was only observed when dox was removed from P3 (Fig. 3 *C* and *D*). To convert the critical period boundaries defined above into an exact time window over which action potentials are able to influence the maturation of the synaptic machinery, we tested the time required for dox to reduce the OE phenotype following its application (used to define the critical period lower boundary) or for it to be increased after dox withdrawal (upper boundary). Three days after beginning dox administration, the size of the SK2 current in OE IHCs was reduced to values (1.51 ± 0.06 nA, $n = 4$) not significantly different to those in control cells (1.39 ± 0.14 nA, $n = 7$). The recovery from dox was slower, although after 9 d the SK2 current in OE IHCs was about 30% larger than that of control cells. After adjusting the time points shown in Fig. 3 *A–D* by the above delays (dox from, 3 d; dox removed, 9 or more days), the "critical period" over which spontaneous $Ca^{2+}$-induced electrical activity is required for IHC development was confined to the second postnatal week (Fig. 3*E*). Note that the upper limit of the critical period is defined by the cessation of IHC spiking activity at P12.

## Discussion

We provide direct evidence that sensory-independent action potential activity present in the developing auditory receptor IHCs is crucial for the maturation of their ribbon synapses. Furthermore, we show that this intrinsic regulation occurs during a critical time window that spans the second postnatal week of development.

Sensory receptors, such as those in the retina and auditory system, respond to external stimuli with graded receptor potentials that drive neurotransmitter release at their ribbon synapses (33, 34). Fusion of docked vesicles to the plasma membrane of functionally mature IHCs is linearly dependent on $Ca^{2+}$ influx via $Ca_V1.3$ channels (13, 14), which is believed to confer an ability to encode continuous and finely graded signals at sensory synapses (14, 35, 36). This linearization replaces the high-order exocytotic $Ca^{2+}$-dependence present in immature spiking IHCs at around the onset of hearing (13, 14). While otoferlin seems to be the main $Ca^{2+}$ sensor for IHC neurotransmission (37, 38), the acquisition of such linearity requires synaptotagmin IV (14).

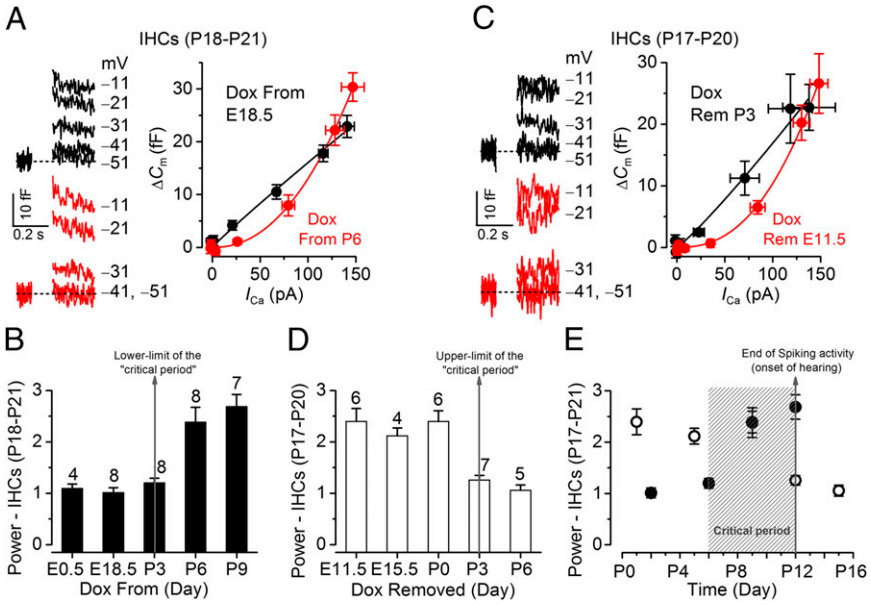


**Fig. 3.** The maturation of the exocytotic $Ca^{2+}$-dependence is determined over a critical period of development. (*A* and *B*) Exocytotic $Ca^{2+}$-dependence in mature IHCs from SK2 OE mice from different stages of development that have been treated in vivo with dox. The synaptic transfer curves (*A*, *Right*) were obtained as described in Fig. 2. Fits are according to Eq. **1**. Left panels (*A*) show average $\Delta C_m$ traces from 8 IHCs for both dox from E18.5 and P6 (recordings performed at P18–P21). The power (*N*) values from synaptic transfer curves obtained in mature IHCs, following dox application from different immature stages including those in *A*, are shown in *B* (data points are average *N* values from fits to single cells obtained using 50 and 100 ms duration voltage steps). Number of IHCs tested is shown above the columns. The lower limit of the critical period is indicated by the gray line. (*C* and *D*) Exocytotic $Ca^{2+}$-dependence in mature IHCs from SK2 OE mice in which dox was applied for at least 10 d before its removal (Rem; *C*) at different immature stages of development (recordings performed at P17–P20). Traces and curves were obtained as in *A*. The power values measured in mature IHCs when dox was removed are shown in *D* and the upper limit of the critical period is indicated. (*E*) Power values from *B* and *D* but corrected for the time delay in dox action as a function of age. Note that the linear exocytotic $Ca^{2+}$-dependence in mature IHCs is controlled by action potentials occurring over a "critical period" of development (gray shaded area), which corresponds to the second postnatal week.

Although the molecular composition of hair cell ribbon synapses has partially been elucidated (35, 39), the mechanisms controlling their development are poorly understood.

The coarse wiring of neural circuits during development is determined by a predetermined genetic program, which is then refined by experience-dependent neuronal activity (4, 40). A large body of evidence, especially from the visual system, indicates that spontaneous or experience-independent $Ca^{2+}$ action potentials are able to refine neuronal development by influencing the genetic program (41). This early spontaneous electrical activity has been shown to regulate a variety of cellular responses (5), including the remodeling of synaptic connections (4, 42) and the expression of ion channels (43). Spontaneous spiking activity in IHCs was initially described more than 10 y ago (7) and hypothesized to be required for normal development of several aspects of the auditory system (7, 8–12). However, a direct link between spontaneous electrical activity in IHCs and the refinement of the immature auditory pathway, including its possible role in synaptic ribbon development, has not previously been demonstrated.

The mouse model that we have used in this study (SK2 OE) has enabled us to make subtle, reversible modifications to spontaneous electrical activity in developing IHCs in vivo (using dox) without the more widespread effects observed when genes, including that encoding SK2 channels, are deleted (e.g., refs. 19, 23). We found that SK2 channel OE affected only the frequency and duration of spontaneous action potentials in immature IHCs and that this was sufficient to prevent the linearization in the exocytotic $Ca^{2+}$ dependence at the onset of hearing. Since SK2 channels are not directly linked to exocytosis and are only transiently expressed during immature IHC development (8, 17), our findings indicate a direct role for spontaneous electrical activity on the normal maturation of the IHC ribbon synapse. We found that such control is only established during a very narrow time window just before the onset of sound-induced electrical activity. Calcium transients associated with spontaneous spiking activity in IHCs increase the level of cytoplasmic $Ca^{2+}$ (11, 43), which could activate specific transcription factors and other regulatory molecules, as shown in B-lymphocytes (44). Our results suggest that in IHCs, the precise timing of $Ca^{2+}$ elevations during spontaneous electrical activity (11) is tightly regulated to ensure measured levels of intracellular $Ca^{2+}$ throughout a specific time window. This tight regulation is also emphasized by the fact that a similar failure in the development of the synaptic machinery has been observed in SK2 knockout mice, which showed long-lasting $Ca^{2+}$ action potentials (19). The overall picture is further complicated when considering that spontaneous activity in IHCs also varies with frequency location along the developing cochlea (10). Bursting activity is more prevalent in the apical low-frequency regions, and more regular spiking activity is observed at the basal high-frequency end (10). However, because spontaneous firing during the second postnatal week is driven by depolarization from the resting mechanotransducer current (11), differences in the pattern of activity are likely to be less significant or even absent. This is also suggested by the observation that the specific firing pattern is unlikely to influence the maturation of the synaptic machinery since both linear (mouse) (14) and high-order relations (gerbil) (13) can develop from immature apical coil IHCs that show a similar bursting firing pattern (10). Instead, tonotopic gradients in the spiking activity during the first postnatal week could influence additional aspects of auditory development, such as promoting the tonotopic differentiation of auditory neural circuits to and from the brain, which are known to be mainly established during this early period (12, 45, 46). The persistence of spontaneous activity in the second week, with a tightly regulated waveform and frequency, seems to drive the intrinsic functional maturation of

IHC ribbon synapses in preparation for their transition into graded sensory receptors at P12. Evidence for this hypothesis comes from the observation that disruption of the mechanotransducer apparatus [e.g., myosin 6 (Myo6) and epidermal growth factor receptor pathway substrate 8 (Eps8)] (47–49), which is required for spontaneous activity only during the second postnatal week (11), causes abnormal development of the IHC synaptic machinery (49, 50). A future challenge will be to establish how spontaneous electrical activity regulates the concentration and distribution of intracellular $Ca^{2+}$, and how that in turn leads to the changes in gene expression and protein function that are required to ensure the development of the auditory system with such exquisite sensitivity to stimulus frequency and intensity.

## Materials and Methods

**Electrophysiology.** Apical IHCs from mice overexpressing SK2 channels (SK2 +/T: OE) and their control littermates (+/+) were studied in acutely dissected organs of Corti from P1 to P27. The day of birth (P0) corresponds to E19.5. Animal studies were licensed by the Home Office under the Animals (Scientific Procedures) Act 1986 and were approved by the University of Sheffield Ethical Review Committee. Mice were genotyped as previously described (20). Note that homozygous SK2 OE (T/T) mice are embryonic lethal (20). Experiments were performed close to body temperature (35–37 °C) and with 1.3 mM $Ca^{2+}$ and 5.8 mM $K^+$ in the extracellular solution, similar to those of perilymph (51), to try and approach normal physiological recording conditions. A detailed description of voltage and current recordings and real-time changes in membrane capacitance ($\Delta C_m$) is available in *SI Materials and Methods*. Statistical comparisons of means were made by Student's two-tailed *t* test or one-way ANOVA. Unless otherwise specified, mean values are quoted ± SEM, where $P < 0.05$ indicates statistical significance.

**Immunostaining.** Cochleae from mature SK2 OE mice were fixed with 2% (wt/vol) paraformaldehyde for 2 h. The primary antibody directed against SK2 channels, otoferlin, $Ca_V1.3$, and CtBP2/Ribeye were detected with Cy3-conjugated or Alexa Fluor 488–conjugated secondary antibodies. The tissue was then imaged using a CCD camera and analyzed with cellSens Dimension software (Olympus soft imaging solutions, OSIS). See *SI Materials and Methods* for more details.

**Transmission Electron Microscopy.** Cochleae were fixed for 2 h with 2.5% (vol/vol) glutaraldehyde. Radial and horizontal ultrathin sections were examined in a transmission electron microscope. See *SI Materials and Methods* for more details.

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