Electrophysiological properties of neurosensory progenitors derived from human embryonic stem cells

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Abbreviations: 4-AP, 4-aminopyridine; AN, auditory neuron; AP, action potential; AP\textsubscript{Max}, maximum number of action potentials; BDNF, brain derived neurotrophic factor; bFGF, basic fibroblast growth factor; cDNA, complementary deoxyribonucleic acid; DIV, days \textit{in vitro}; EGF, epidermal growth factor; HCN, hyperpolarization-activated cyclic nucleotide-gated channels; hESC, human embryonic stem cell; HFFs, human foreskin fibroblast feeders; \(I_h\), hyperpolarization-activated current; \(I_K\), potassium current; \(I_Na\), sodium current; \(K\), slope factor; NBM, neurobasal media; NFM, neurofilament; NS, neurosphere; NT3, neurotrophin 3; NTs, neurotrophins; pps, pulses per second; qRT-PCR, quantitative real time polymerase chain reaction; \(R_{in}\), input resistance; \(R_s\), series resistance; RMP, resting membrane potential; RNA, ribonucleic acid; SEM, standard error of the mean; TEA, tetraethylammonium; TTX, tetrodotoxin; \(V_{th}\), half-activation voltage; Y27, small peptide Y27632; ZD7288, 4-ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium chloride
Abstract

In severe cases of sensorineural hearing loss where the numbers of auditory neurons are significantly depleted, stem cell-derived neurons may provide a potential source of replacement cells. The success of such a therapy relies upon producing a population of functional neurons from stem cells, to enable precise encoding of sound information to the brainstem. Using our established differentiation assay to produce sensory neurons from human stem cells, patch-clamp recordings indicated that all neurons examined generated action potentials and displayed both transient sodium and sustained potassium currents. Stem cell-derived neurons reliably entrained to stimuli up to 20 pulses per second (pps), with 50% entrainment at 50 pps. A comparison with cultured primary auditory neurons indicated similar firing precision during low-frequency stimuli, but significant differences after 50 pps due to differences in action potential latency and width. The firing properties of stem cell-derived neurons were also considered relative to time in culture (31-56 days) and revealed no change in resting membrane potential, threshold or firing latency over time. Thus, whilst stem cell-derived neurons did not entrain to high frequency stimulation as effectively as mammalian auditory neurons, their electrical phenotype was stable in culture and consistent with that reported for embryonic auditory neurons.

Introduction

Sensorineural hearing loss occurs when the delicate sensory hair cells of the inner ear are injured by factors such as loud noise, trauma, and exposure to ototoxic compounds or simply ageing. Currently, the principal treatment for sensorineural hearing loss is a cochlear implant. This device reinstates the transmission of sound information to the central auditory pathway by providing direct electrical stimulation to the primary auditory neurons (in the absence of hair cells; Seligman and Shepherd 2004). This neural population provides the critical link between the peripheral cochlea and the central auditory system, and auditory neurons are capable of responding to high stimulation rates with temporal acuity (Kiang et al. 1965; Javel and Viemeister 2000). Importantly, whilst the cochlear implant relies upon a functional population of primary auditory neurons to convey auditory input to higher neural centers, these auditory neurons themselves are often vulnerable to degeneration after hearing loss, or may even be the site of primary damage. An extensive loss of primary auditory neurons is assumed to significantly reduce the effectiveness of a cochlear implant.

Stem cells offer an opportunity to restore auditory function by replacing lost auditory neurons in cases of severe depletion. A number of studies have now demonstrated the potential of stem cells to differentiate into appropriate neurosensory progenitors, including those of human origin (Shi et al. 2007; Chen et al. 2009; Chen et al. 2012; Nayagam et al. 2013). The expression of key developmental markers in the differentiation of human stem cells toward an auditory neural lineage has recently been documented (Chen et al. 2012; and reviewed by Gunewardene et al. 2012) and includes the expression of key proteins and transcription factors Sox 2, Pax2/8, FoxG1, Six1, Nestin and Brn3a (Chen et al. 2012), Brn3a, GATA3 and peripherin (Shi et al. 2007), Pax 2, Brn3a, peripherin, and neurofilament (Nayagam et al. 2013) and NeuroD1, Brn3a, GATA3, Islet1, peripherin, and neurofilament (Gunewardene et al. 2013).
The method used to generate sensory neurons in the present study is based on previously published protocols from our laboratories for deriving neural crest progenitors (Holt et al. 2006; Denham and Dottori 2011; Liu et al. 2011; Nayagam et al. 2013). Recent literature supports the use of neural crest progenitors in a cell replacement therapy for deafness, given the molecular similarity of this population to placode-derived sensory neurons (Huisman and Rivolta 2012; Nayagam et al. 2013). In addition, we have recently demonstrated that neurons derived from this induction protocol express key auditory neural proteins including NeuroD1, Brn3a, GATA3, Islet1 and neurofilament (Fig 1; Gunewardene et al. 2013) and are capable of making synapses on developing mammalian hair cells in vitro (Nayagam et al. 2013). Given that neural crest progenitors can also be readily obtained from adults (Yang and Xu 2013), they have the potential to facilitate the development of patient-matched cell transplants in the future (Huisman and Rivolta 2012; Yang and Xu 2013).

An important challenge to overcome in developing a cell replacement therapy for hearing loss is the development of a functionally stable stem cell-derived neural population (Needham et al. 2013). This entails both the development of electrically active neurons and their functional integration including formation of synapses with target neurons in the cochlear nucleus. We and others have previously shown that human stem cell-derived neurons can fire action potentials, and possess the core currents and channel families necessary for this task (Chen et al. 2009; Nayagam et al. 2013). Amongst these are the inward Na+ currents (I_{Na}) and sustained outward K+ currents (I_{K}). These are arguably the most basic currents necessary to instigate action potentials, and therefore communicate meaningful signals to their target/s. The next milestone in our experimentation is to develop neurons with an electrical phenotype capable of processing information in a similar manner to the primary auditory neurons. Most notably, the glutamatergic primary auditory neurons possess a large complement of ion channels that enable them to respond to complex signals with temporal precision (reviewed Needham et al. 2013). A key feature of this neural phenotype is the ability to reliably follow high frequency stimulation since this is a hallmark of acoustic stimuli, as well as the electrically encoded input from a cochlear implant. Interestingly, little is known about the definitive firing rates of auditory neurons in response to electrical stimulation in humans. However, what is clear from clinical studies, is that pitch discrimination deteriorates as stimulation levels approach 300 pulses per second (Shannon 1983; Zeng 2002; Vandali et al. 2013). Thus, based upon these data, it seems reasonable to expect that replacement neurons be capable of firing at similar rates to endogenous auditory neurons as a reduction in firing entrainment would likely affect the amount of information encoded in the signal relayed to the brain, and therefore the accurate perception of sound.

Here we examine the electrical profile of human embryonic stem cell (hESC) derived neurosensory progenitors over time in vitro, and compare their responses to high frequency stimulation with that of the primary auditory neuron population.
Materials and Methods

Stem cell culture

The following procedures are based on protocols described previously by Dotti and Pera (2008), Hotta and colleagues (2009), and Nayagam and colleagues (2013). The H9 human embryonic stem cell line (WA-09, WiCell) was cultured in 20% serum-containing medium on mitomycin-C-treated human foreskin fibroblast feeders (HFFs) and passaged weekly by mechanical dissociation. For neural induction, mechanically dissociated hESCs were placed on a fresh HFF feeder layer in organ culture dishes with Neurobasal media (NBM) supplemented with 500 ng/ml recombinant noggin (R&D Systems). Neurobasal media contained Neurobasal A medium supplemented with 1% N2, 2% B27, 2 mM L-glutamine and 0.5% Penicillin/Streptomycin (all sourced from Invitrogen). Cells were cultured for 14 days without passing, and the media was replaced every second day. To make neurospheres, fragments of noggin-treated colonies were mechanically dissected and transferred to individual wells of a low-attachment 96-well plate in NBM supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) at 20 ng/mL each (Peprotech). After 4 days, neurospheres were plated onto HFF feeders grown on gelatin-coated glass coverslips (HFF density of 4.7 x 10⁴ cells/coverslip) and cultured in NBM supplemented with EGF and bFGF (at 20 ng/ml each) for the first 24 hrs, then replaced with media containing EGF, bFGF and 25 µM Y27632 (Sigma Aldrich) for a further 48 hrs. Following Y27632 treatment, cultures were maintained at 37°C (10% CO₂) in NBM supplemented with brain derived neurotrophic factor (BDNF) and neurotrophin3 (NT3) at 10 ng/ml each (Chemicon) until required for electrophysiological recordings. Media was replaced every 2-3 days. The timeline of neural induction and electrophysiological recordings are illustrated in Figure 1.

Quantitative real time polymerase chain reaction (qRT-PCR)

For qRT-PCR, hESCs were grown as described above with the exception that BDNF and NT3 were not added after 24 days in vitro (24 DIV). Cells were collected at five time points: 19, 21, 24, 28 and 35 DIV. RNA was extracted from samples using the SV Total RNA Isolation Kit (Promega Corporations) according to the manufacturer’s instructions and quantified using a NanoDrop spectrophotometer (Thermoscientific). Equal amounts of total RNA (60ng) per sample were reverse transcribed using the High Capacity RNA-to-cDNA kit (Life Technologies). The cDNA products were transferred into the qRT-PCR reaction using Taq man gene expression master mix (Life technologies). Commercially available probes obtained from Life technologies included NeuroD1 (Hs01922995_s1: FAM dye), SLC17A7 (VGLUT1; Hs00220404_m1: FAM dye), and β-actin as the housekeeper reference gene (4326315E: VIC dye, primer limited). Standard curves were generated for the probes by a 1:10 serial dilution of control cDNA sample and the reaction efficiencies were >95% for both probes. The qRT-PCR reactions were run in duplex with both the gene of interest probe and the housekeeping probe present. Negative controls included a no template control (water only) and a reverse transcriptase negative sample (without cDNA). qRT-PCR reactions were run using a RotorGene Q instrument (Qiagen, Hilden, Germany) and the Rotor-gene 6000 software (Version 1.7, Corbitt Research) was used to analyse the results. The 2^(-ΔΔCt) delta-delta analysis algorithm was used to determine the relative quantification of each sample at the time points analyzed (Livak et al. 2001). The threshold values generated from each probe's standard
curves were used to determine the CT values of the experimental samples. The expression levels of the experimental samples were measured in triplicate and normalized against the endogenous control, β-Actin, to obtain the ΔCt value. The ΔCt values were standardized against the calibrator’s ΔCt (VGLUT1-undifferentiated stem cells; NeuroD1-19 DIV stem cells) to determine the ΔΔCt value. The relative quantification was then calculated as 2^-ΔΔCt. Statistical analyses of the qRT-PCR data were performed using GraphPad Prism. The relative quantification data was analyzed using a two-tailed Student's t-test, with assumed equal variance for comparison between the fold changes of the undifferentiated cells to differentiating cells at each time point noted (n=3, per time point). Values of p<0.05 were considered statistically significant. Data are presented as the mean ± standard error of the mean (SEM).

**Immunocytochemistry**

Stem cell cultures were fixed after 28 DIV, in a solution of 4% paraformaldehyde for 10 min. Cells were then rinsed three times for 10 min in phosphate buffered saline and immunostained for the neurosensory markers Brn3a (Millipore MAB1585; 1:1000) and Neurofilament medium subunit (NFM; Millipore AB5735; 1:1000), using identical methods to those previously described (Nayagam et al., 2013). Alexafluor secondary antibodies (Molecular Probes) were applied at a concentration of 1:500 and preparations mounted in ProLong Gold (Molecular Probes).

**Auditory neuron culture**

As described in detail previously (Needham et al. 2012; Brown et al. 2013), cultures of auditory neurons were prepared from postnatal day four to seven (P4-P7) Wistar rat pups. Dissociated cells were resuspended in NBM then plated onto circular glass coverslips (10 mm diameter; Menzel-Glaser) pre-coated for 1 day with poly-ornithine (500 µg/ml; Sigma) and mouse laminin (0.01 mg/ml; Invitrogen). Cultures were maintained at 37°C (10% CO2) for up to 48 hrs in NBM supplemented with BDNF and NT3 (media replenished daily). Primary auditory neurons were visually identified as round, phase-bright cells with ~15-20 µm soma diameter and a prominent eccentric nucleus (Needham et al., 2012).

**Electrophysiology**

Coverslips were transferred to a recording chamber fitted to an AxioExaminer D1 microscope (Carl Zeiss) for electrophysiological recordings. As previously detailed (Needham et al. 2012; Nayagam et al. 2013), cells were superfused at 1-2 ml/min with solution of the following composition (in mM): 137 NaCl, 5 KCl, 10 HEPES, 1 MgCl2, 2 CaCl2, 10 glucose (pH 7.35; 300-305 mOsmol/kg). Neurons were visually identified by a round-to-oval soma (diameter of ~10 µm) and patent bipolar processes. Whole-cell patch clamp recordings were made at room temperature using borosilicate microelectrodes (2-6 MΩ; 1.0 mm O.D.; 0.58 mm I.D., Sutter) filled with an internal solution containing (in mM): 115 K-gluconate, 10 HEPES, 7 KCl, 0.05 EGTA, 2 Na2ATP, 2 MgATP, 0.5 Na2GTP (pH 7.3; 290-295 mOsmol/kg). All chemicals were purchased from Sigma-Aldrich (Sydney, Australia) unless otherwise indicated.

Signals were recorded with a MultiClamp 700B amplifier (Molecular Devices), data acquisition system (Digidata 1440A, Molecular Devices), and AxoGraph X analysis software (AxoGraph Scientific). Records were digitized at 50 kHz and filtered at 10 kHz. Series resistance (R_s) was monitored in response to a 10
mV voltage step (mean 16 ± 0.6 MΩ; cells with Rs greater than 30 MΩ were excluded). Rs was routinely compensated online (up to 70%). No corrections were made for voltage errors for uncompensated Rs. In current clamp, pipette capacitance neutralization was applied and bridge balance utilized to compensate errors due to Rs. All recordings were made from a holding potential of -73 mV, and corrections for liquid junction potential (12.8 mV; JPCalcW, Prof P H Barry, Sydney) were made offline.

The normalized current-voltage relationship for instantaneous tail currents was fitted with the following Boltzmann equation $I - I_{\text{min}} / I_{\text{max}} - I_{\text{min}} = (1 + \exp ((V - V_h) / K))^{-1}$, where V is membrane potential, $V_n$ the half-activation voltage, and K the slope factor. Statistical significance was determined by student t-tests, or ANOVA with Tukey method for pairwise comparisons (MiniTab 16); data were considered significant where $p < 0.05$. Results are presented as mean values ± SEM; n denoting the number of cells in which measurements were made.

**Results**

**Stem cell-derived neurons are physiologically active**

Whole-cell patch clamp recordings were made from cells displaying a bipolar neuronal morphology (n=86; Fig 2A). The mean resting membrane potential (RMP) of the population was -49.2 ± 0.8 mV, whilst the mean input resistance ($R_i$) was 1.4 ± 0.1 GΩ, and membrane capacitance 7.7 ± 0.3 pF. This population of neurons did not display spontaneous firing, but were all capable of generating an action potential in response to membrane depolarization (Fig 2B; examples shown for neurons at -73 mV holding potential or RMP [inset]). The majority of cells (68%) fired a single action potential at stimulus onset. Of the remaining cells, 26% fired a maximum of between 2 and 6 action potentials, and 6% displayed tonic firing (up to 13 action potentials). The mean maximum number of action potentials fired (AP$_{\text{Max}}$) across the population was 2.2 ± 0.2. Firing threshold was -39.3 ± 0.6 mV, with an average action potential amplitude of 91.6 ± 1.8 mV at threshold, or 100.3 ± 1.4 mV during supra-threshold input (+190 pA). The mean latency of action potentials was 40.2 ± 2.3 ms at threshold and 4.7 ± 0.2 ms with supra-threshold stimulation.

Voltage-clamp recordings were used to identify the key currents underlying the firing properties of stem cell-derived neurons. All cells examined in voltage-clamp displayed a fast-activating, rapidly-inactivating inward current in response to increasing membrane depolarization (Fig 2 C-D). This transient inward current was abolished in the presence of 1 μM tetrodotoxin (TTX), a selective voltage-gated Na$^+$ channel antagonist (Fig 2 D inset). An I-V curve recorded in the presence of K$^+$ channel blockers 10 mM tetraethylammonium (TEA) and 1 mM 4-aminopyridine (4-AP) indicated activation of $I_{\text{Na}}$ occurred at potentials positive to -40 mV, with peak current at -13 mV (mean -106 ± 41 pA/pF; n=4). To determine the voltage dependence of $I_{\text{Na}}$, normalized tail currents (measured at 0 mV following a -113 mV prepulse and voltage steps from -133 to -3 mV; data not shown) were fitted with a Boltzmann function to reveal the mean half-activation voltage ($V_n$) of -55.4 ± 2.8 mV and slope factor (K) of 6.3 ± 1.1 (n=4). The TTX-sensitive Na$^+$ current was followed by a sustained outward current (Fig 2C), which could be attenuated
by combined application of TEA and 4-AP as illustrated previously (Nayagam et al. 2013). The sustained portion of the outward current (denoted as \( I_{K-sustained} \)) increased steadily with increasing depolarization. When converted to current density (to account for possible variations in cell size), \( I_{K-sustained} \) was smaller than that observed in the population of primary auditory neurons (Fig 2E). The difference between the populations was significant at +30 mV (p<0.001; ANOVA).

In current-clamp, hyperpolarization of the cells evoked a steady change in membrane potential but did not evoke the classic voltage ‘sag’ response seen in primary auditory neurons (Fig2F). In addition, in voltage-clamp membrane hyperpolarization initiated a small, steady inward current (Fig2G). Unlike the primary auditory neurons, a large, slowly-activating, non-inactivating inward current indicative of hyperpolarization-activated currents (\( I_h \)) was not observed (Fig 2G). Instead, the current induced in hESCs closely matched that seen in auditory neurons in the presence of 50 \( \mu \)M ZD7288 (the \( I_h \)-selective antagonist), confirming the absence of the inward current mediated by hyperpolarization-activated cyclic nucleotide-gated (HCN) channels.

**Firing properties with time in culture**

The electrophysiological properties described to this point reflect the mean response across the entire population. To assess the stability or maturation of this response over time, firing properties were also considered relative to the period of time in culture. Recordings were made between 31 and 56 days \textit{in vitro} (DIV), and grouped into the following clusters on the basis of mean time in culture; 31 DIV (n=10), 35 DIV (n=29), 42 DIV (n=18), 48 DIV (n=15), and 53 DIV (n=14). Assessment of the passive membrane properties revealed no difference in RMP (Fig 3A; p = 0.28; ANOVA), or change in \( R_{in} \) with culture duration (Fig 3B; \( p = 0.23 \); ANOVA). Firing threshold (Fig 3C) was elevated at 35 DIV relative to the other time points, but this difference was only significant when compared to 48 DIV (p=0.004; ANOVA). There was no significant difference in threshold between the other clusters, including between 31 DIV and 53 DIV. The maximum number of action potentials fired (\( AP_{max} \)) showed increased variability after 31 DIV, with a significant change seen in mean \( AP_{max} \) between groups (Fig 3D; p=0.045; ANOVA). Significant differences were evident between 31 DIV and 42 DIV (p=0.049; \textit{t}-test), 48 DIV (p=0.018; \textit{t}-test) and 53 DIV (p=0.028; \textit{t}-test). Firing latency at threshold (Fig 3E) and in response to supra-threshold stimulation (Fig 3G) were consistent with time in culture (p=0.29 and p=0.34 respectively; ANOVA). Further, no significant difference was seen in action potential amplitude (Fig 3F; p=0.94; ANOVA) or half-width (Fig 3H; p=0.33; ANOVA) measured during supra-threshold stimulation.

**Firing entrainment to high frequency stimulation**

To determine the ability of hESC-derived neurons to entrain to high frequency stimulation, firing activity was examined during a train of pulses. Supra-threshold pulses (1.0 - 2.0 nA; 0.3 ms duration) were presented from 2.5 to 1000 pulses per second (pps), and outcomes assessed as the probability of an action potential firing in response to each pulse (averaged over a train of 50 pulses, and multiple presentations). As demonstrated in Figure 4A, our population of stem cell-derived neurons (n=43) displayed good firing entrainment (defined as a firing probability greater than 0.9) to stimuli presented between 2.5 and 20 pps. The probability fell to 0.5 at 50 pps (i.e. a 50% chance of firing to each pulse),
and 0.05 by 200 pps. For comparison with the population of primary auditory neurons, we recorded firing activity of auditory neurons (n=17) under the same conditions (Fig 4A). Whilst this population revealed a similar entrainment in response to stimuli presented from 2.5 to 20 pps, the reduction in firing probability with increasing stimulus rate was less pronounced. Instead, the reduction in firing entrainment to 0.5 was not observed until pulse trains of 100 pps. Firing probability was reduced to 0.06 at 500 pps, and 0.02 at 1000 pps. Differences between the groups were statistically significant at 67 pps (p=0.025; ANOVA), 100 pps (p<0.001; ANOVA) and 200 pps (p=0.048; ANOVA).

Underlying the variance in firing entrainment between the stem cell-derived population and native auditory neurons was a significant difference in action potential latency and width (Fig 4B-C). The latency of the first spike averaged 1.82 ± 0.15 ms in stem cell-derived neurons, as compared to 1.10 ± 0.09 ms in auditory neurons (p=0.002; ANOVA), whilst half-width was 2.12 ± 0.11 ms in the stem cell population versus 0.93 ± 0.07 ms in auditory neurons (p<0.001; ANOVA). Further, there were significant differences in the descending slope of the action potential (p<0.001; hESC -27.37 ± 2.23 mV/ms; auditory neurons -84.79 ± 9.57 mV/ms; ANOVA) and the time constant of the slow decay (p=0.01; hESC 18.62 ± 2.51 ms; auditory neurons 6.32 ± 1.51 ms; ANOVA).

**Discussion**

This study describes the activity of human stem cell-derived neurons after extended time in culture, and in response to high frequency stimulation. The findings reported herein, confirm that our differentiation assay consistently produces a population of electrically active neurons which possess Na⁺ and K⁺ currents.

We have observed that the described population of stem cell-derived neurons predominantly displays a rapidly-adapting phenotype. Classified in accordance with the previously defined convention for primary auditory neurons (Mo and Davis 1997a; Adamson et al. 2002b), rapidly-adapting cells fire between one and six action potentials during a 300 ms stimulus, whilst slowly-adapting cells fire seven or more action potentials over the same period. Interestingly, the predominance of this phenotype is comparable with recordings from early postnatal primary auditory neurons in vitro, where the majority displayed a rapidly-adapting profile (Needham et al. 2012). In addition, murine stem cell-derived neurons uniformly show rapidly-adapting responses over a period of 12 days in vitro (Tong et al. 2010). The rapidly-adapting phenotype identified in the current study was stable for five weeks in culture, supporting the use of our excitable population of cells as replacement cells for auditory neurons.

The firing profile and physiological properties recorded from this population of stem cell-derived neurons nicely match those reported for embryonic day 14-15 (E14-15) mammalian auditory neurons *in situ* (Marrs and Spirou 2012). In particular, both populations exhibit similar RMP, R_{in}, and firing threshold, and display a broader action potential when compared to postnatal auditory neurons. Of additional interest is the absence of a voltage ‘sag’ in both E14-15 auditory neurons and our population of stem cell-derived neurons. The presence of a ‘sag’, and the hyperpolarization-activated current (I_{h}) which underlies this activity, is consistently reported in postnatal primary auditory neurons *in vitro* (Mo
and Davis 1997b; Szabo et al. 2002; Zhou et al. 2005; Needham et al. 2012), and has also been noted in later embryonic development of both primary auditory neurons and auditory brainstem neurons (Marrs and Spirou 2012). This hints at $I_h$ and voltage ‘sag’ as key markers of auditory neuron maturity. Notably, $I_h$, which regulates neuronal excitability in part through control of $R_{\text{in}}$, can be influenced by the application of neurotrophins (Needham et al. 2012). Likewise, a lower (i.e. more hyperpolarized) RMP, which in our stem cell-derived neurons remained in the order of -50 mV across the five weeks in vitro, is also suggestive of a maturing neural phenotype and may also be assisted by the presence of BDNF in the culture media (Purcell et al. 2013). As recently demonstrated by Purcell and colleagues (2013), expression of the K⁺ channel KCNQ4 dramatically increased in stem cell-derived neurons following BDNF exposure. These authors also reported a concomitant reduction in RMP, thereby highlighting the potential to produce specific neuronal subtypes using appropriate soluble molecules.

Physiological properties such as RMP and $R_{\text{in}}$ are of interest in terms of cochlear cell-based therapy, because these properties contribute to the overall responsiveness of replacement neurons to stimulation from a cochlear implant. Promisingly, our population of stem cell-derived neurons responded to low frequency pulsatile stimulation with high precision, however they were not able to match the efficiency of postnatal primary auditory neurons to entrain to high frequency input. Despite an extended period in culture, which we hypothesized may be sufficient to induce more electrically mature phenotypes, the present data suggests that other factors are required to reach this stage of maturation. Spontaneous activity and synapse formation are possible key steps in the production of mature electrical phenotypes from stem cell-derived neurons.

As indicated by the work of Marrs and Spirou (2012), one of the key factors in the maturation of auditory neurons is the formation of synapses with hair cells. Moreover, spontaneous firing may assist in directing the formation of specific synaptic connections and in activating auditory neurons during embryogenesis (Lippe 1994). Spontaneous action potentials generated by immature inner hair cells are primarily mediated by Ca⁺⁺ channels, which disappear around the onset of hearing (Kros et al. 1998). The release of neurotransmitter at the basal surface of the inner hair cell is likely to modulate the activity of the developing auditory nerve (Beutner and Moser 2001; Beutner et al. 2001). In a similar way, electrical stimulation has been shown to promote survival of auditory neurons in vitro (Hegarty et al. 1997; Hansen et al. 2001) and in vivo (Shepherd et al. 2005), and normal synaptic activity can be recovered following chronic electrical stimulation in congenitally deaf cats (Ryugo et al. 2005). Taken collectively, these data support the idea that spontaneous activity or electrical depolarization is an important feature in the terminal differentiation of auditory neurons, and this may be essential in order for them to reach their mature electrophysiological phenotype.

Whilst the described population of stem cell-derived neurons was not observed to achieve a mature electrical phenotype in vitro, they may be capable of doing so once transplanted in vivo. Recent evidence suggests that stem cell-derived neurons can incorporate into the deaf gerbil cochleae, and improve auditory-evoked response thresholds after 10 weeks (Chen et al. 2012). Interestingly, the stem cell-derived neurons used by Chen (2012) displayed similar basic firing properties prior to their transplantation, to the stem cell-derived neurons described in the present study. Moreover, the voltage-gated ion channels that control neural activity are sensitive to changes in their environment, including
concentration of neurotrophic factors (Adamson et al. 2002a; Zhou et al. 2005; Needham et al. 2012; Purcell et al. 2013) and excitatory input (Leao et al. 2005; Holt et al. 2006; Hassfurth et al. 2009), both of which are likely to play a role following their transplantation into the deaf cochlea. Determining how to harness and balance both in vitro and in vivo factors to derive functionally appropriate stem cell-derived neurons will be an important task in developing a stem cell therapy for the deaf cochlea.

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Figure Legends

Figure 1. Timeline of hESC differentiation, electrophysiological recordings and neurosensory protein expression in vitro. (A) Neurosphere (NS) formation (stage 1) was followed by treatment with ROCK/Rho kinase inhibitor Y27632 (Y27; Stage 2) and neurotrophins (NTs; Stage 3). Electrophysiological recordings were made between 31 and 56 days in vitro (DIV); recording clusters denoted by arrows. (B) Immunocytochemical assessment of cells at 28 DIV revealed expression of key markers of auditory neural lineage, Brn3a and neurofilament (NFM). Scale bar = 100 µm (relative to all images). (C-D) Expression of NeuroD1 (C) and VGLUT1 (D) at 19, 21, 24, 28 and 35 DIV as measured by qRT-PCR. Results presented as fold changes relative to the endogenous control, ß-Actin. Significance: *=p<0.05; **=p<0.01; ***=p<0.001. DIV - days in vitro; hESC – human embryonic stem cell; HFFs – human foreskin fibroblast feeders; UD – undifferentiated.

Figure 2. Electrophysiological properties of hESC-derived neurons. A. A photomicrograph of a differentiated stem cell with bipolar morphology and recording electrode attached [scale bar – 10 µm]. The typical profile observed in current-clamp (B) in response to current injections (-60 to 200 pA; holding potential of -73 mV) included a single action potential followed by a large, sustained depolarization during depolarizing current, and a large membrane hyperpolarization during hyperpolarizing current. Inset depicts activation of action potentials at the cell’s resting membrane potential (-51 mV). In voltage-clamp (C-D), membrane depolarization evoked a fast inward sodium current (D) followed by a sustained outward K⁺ current (C). Inset in D shows removal of the inward sodium current in the presence of 1 µM TTX (gray). A comparison with cultured primary auditory neurons (ANs; gray) revealed that the sustained portion of the outward K⁺ current (Iₖ-sustained; measured over 50 ms period shown by bar in C) as displayed in current density (E), was smaller in stem cell-derived neurons (black). F. Current-clamp recordings showed that hyperpolarization-evoked voltage ‘sag’ [denoted by arrow] observed in auditory neurons (gray) was absent in hESC-derived neurons (black). Likewise, in voltage-clamp recordings (G) stem cells (black) demonstrated a small inward current during hyperpolarization, whereas auditory neurons (gray) produced a large, slowly-activating current that was abolished by the Iₖ-specific antagonist ZD7288 (light gray).

Figure 3. Firing properties of hESC-derived neurons with time in culture. The mean measure at each time point (black square) and individual responses (gray circles) are shown for resting membrane potential (RMP; A), input resistance (B), firing threshold (C), maximum number of action potentials fired during a 300 ms depolarization (D), latency of the first action potential (AP) at firing threshold (E) or supra-threshold (+190 pA; G), action potential amplitude (F), and action potential duration at 50% of its amplitude (half-width; H).

Figure 4. Firing activity in response to pulse trains. A. Firing probability calculated as the number of action potentials fired per pulse, averaged over 50 presentations. The hESC neurons (black) did not entrain to stimuli as consistently as the primary auditory neurons (gray), as also shown by examples of firing responses during 67 pps stimuli (B) for a typical auditory neuron (AN; left) and hESC-neuron (right). Action potentials were distinguished from stimulus artifact as events >50 mV in amplitude; dashed lines illustrate relative amplitude of stimulus artifact in each example. C. The longer latency and broader
action potentials of the hESC-derived neurons (black) compared to auditory neurons (gray) contributed to their ability to reliably follow high frequency stimuli.
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Figure 1
Figure 2
Figure 3
Figure 4
Highlights:

1. Neurosensory progenitors derived from human stem cells are electrically active
2. The activity of stem cell-derived neurons resembles embryonic auditory neurons
3. The electrical profile of neurosensory progenitors is stable over 5 weeks *in vitro*
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