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SHORT REPORT

Electrophysiological properties of neurosensory progenitors derived from human embryonic stem cells

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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

Abbreviations: 4-AP, 4-aminopyridine; AN, auditory neuron; AP, action potential; AP_{Max}, maximum number of action potentials; BDNF, brain derived neurotrophic factor; bFGF, basic fibroblast growth factor; cDNA, complementary deoxyribonucleic acid; DIV, days *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_h, half-activation voltage; Y27, small peptide Y27632; ZD7288, 4-ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium chloride

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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, while 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.
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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, while 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, 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), Pax2, 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). Among 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.

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