Development of an Embryonic Stem Cell-Based Therapy for Replacement of Spiral Ganglion Neurons

by

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For my parents, Greg and Sachiko. For my little sister, Sophia. For everybunny who made me smile throughout this process.

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Introduction

1.1 The cell biological basis of hearing

We perceive the world around us in multiple, profound ways, engaging our highly evolved senses to shape our existences. As social creatures, humans rely on their sense of hearing for communication and survival. The noise level on planet Earth seems to increase everyday, with human populations exploding in growth, new construction projects cropping up to accommodate our growth, and the constant blaring of the latest soundtrack to our human progress. The subtleties of noise are what enable us to read and convey specific emotions, discuss with others our perceptions of the world, and create and appreciate music. Our hearing helps us react to oncoming hostile or meteorological threats, locate our loved ones, or hone in on prey. In many ways, our sense of hearing is both refined and instinctual, with an underlying cellular and neurological basis that provides us with significant insights into our perception.

On the cellular level, specialized sound receptors in our peripheral nervous systems communicate frequency information to "first order" spiral ganglion neurons that synapse with "second-order" neurons in the brain's lower auditory centers. When any of these cells in the auditory pathway is damaged in mammals, permanent dysfunction can arise due to the inability of these cells to regenerate, resulting in permanent hearing loss. Hearing loss (HL) has different forms, causes, and implications

in human health; it is currently the most prevalent sensory impairment in the world with nearly 30 million affected individuals in North America alone (*Hildebrand et al.*, 2008). Beyond treatment and intervention costs, HL poses other significant public health concerns. Adults who acquire HL later in life are at an increased risk of developing anxiety and depression, likely from being cut off from such a fundamental mode of communication (*Kvam et al.*, 2007; *de Graaf and Bijl*, 2002). Ludwig van Beethoven, arguably the greatest musician and composer of all time, tragically suffered from this isolating condition (*Huxtable*, 2001). And in young children where auditory circuits in the brain are deprived of stimuli, significant degeneration and cortical rearrangements within the auditory cortex can have detrimental effects on cognitive development (*Finney et al.*, 2001; *Sharma et al.*, 2002).

If we are to restore and treat HL, we need to first understand the individual components that give rise to our sense of hearing. Sounds are produced by a coincident series of vibrations: the most simple to envision is a tuning fork producing a single sine wave. One can imagine, however, that the spectrum of complex frequencies layered upon one another giving rise to unique noises, words, and music stretches *ad infinitum*. Pitch, intensity, and other mechanical and temporal information in the "acoustic waveform" allow us distinguish subtle variations in sound quality, for example that the trombone's middle C sounds distinct from that of the piano (*Yost*, 2007). Our ability to distinguish this so-called musical coloring, or "timbre," plays a critical role in human communication, as subtle fluctuations in a word's pronunciation could imply elation, stress, or anger.

Like all mammals we normally hear with two ears, or binaurally. The relative incidence of a sound wave reaching each ear allows us to localize the source, even in environments saturated with noise, like parties or restaurants. Acoustic waveforms in the environment are captured by our outer ear pinnae and funneled into our heads through the external auditory meatus (Figure 1.1(1)). One of the marvels of our sense

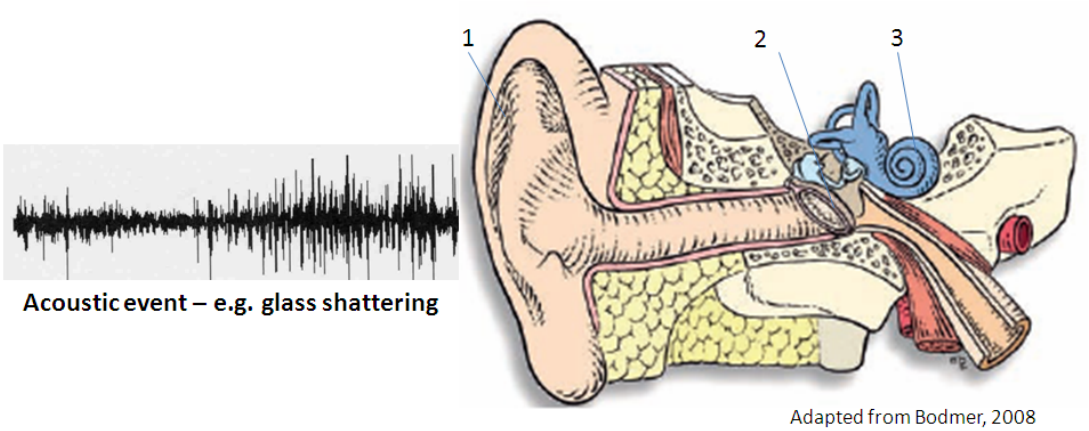

Figure 1.1: Acoustic waveforms transform as they pass from the air to the cochlea.

of hearing is the incredible amount of frequency information selectively preserved from noisy environments in the transfer of waveforms from air into biological tissue, in other words low to high impedence environments (*Yost*, 2007). When waveforms reach the middle ear, tiny bones there (Figure 1.1(2)) convert the mechanical frequency energy into a vibrational energy, which is transferred into fluid-filled chambers within the tiny, snail-shaped hearing organ, the cochlea (Figure 1.1(3)). In response, a flexible basilar membrane (BM), which divides the cochlear duct into scala media (SM) and scala tympani (ST) chambers, will vibrate and activate fluids within the chamber. These modified cerebral spinal fluids within are calibrated to optimize the response of hair cells within the organ of Corti (OoC) to sound (Figure 1.2).

Inner and outer hair cells and the BM are graded in morphology and function along the cochlear spiral to maximally decompose high frequencies at the base and low frequencies at the apex into neurochemical impulses. BM displacements shift the electric potential of IHCs and OHCs, activating intracellular motors to convert mechanical energy into an electrochemical signal that conveys specific frequency information to innervating first-order afferent spiral ganglion neurons (SGN). This process is known

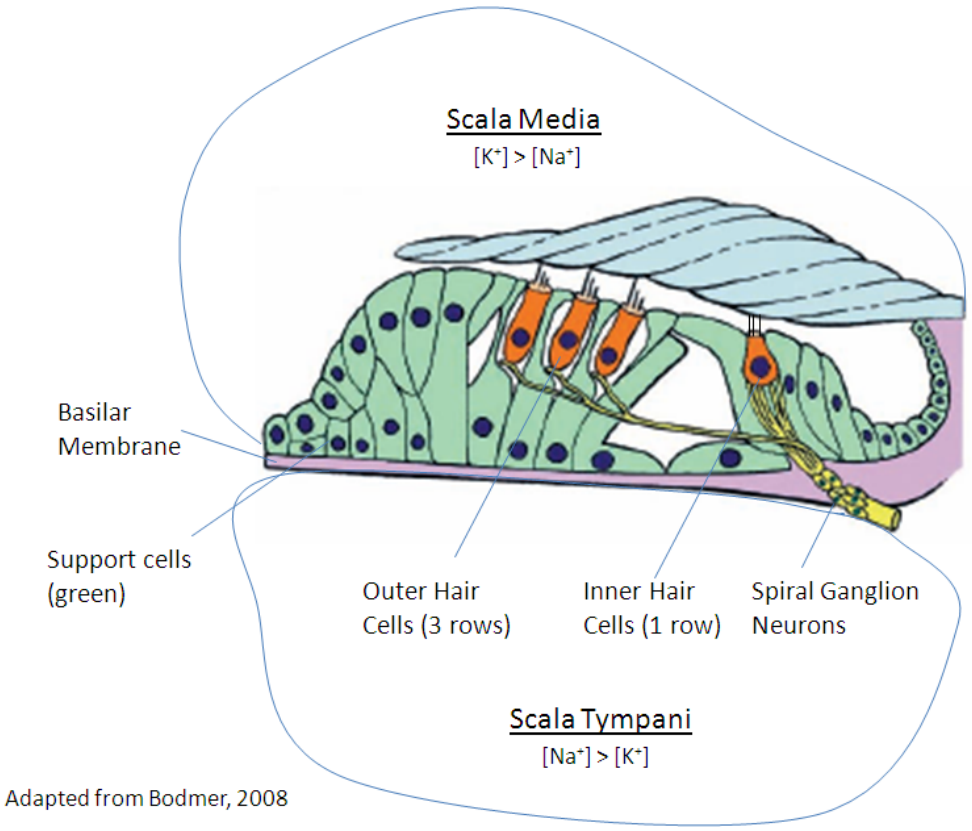

Figure 1.2: The organ of Corti contains hair cells that deliver neurotransmitter messages to the auditory nerve.

as "mechanotransduction" (*Grillet et al.*, 2009). To encode complex acoustic waveforms into their component frequencies, a gradation of BM and OoC specializations evolved along the cochlear spiral from base to apex. This "tonotopic" organization, or correlation between function and cochlear location, is conserved in every neural element of the auditory system, from SGN afferents in the auditory periphery to frequency maps in Heschl's gyrus in the primary auditory cortex (*Lauter et al.*, 1985; *Bertrand et al.*, 1991; *Davis*, 2003; *Raphael and Altschuler*, 2003). See Figure 1.3.

Individually depolarized IHCs (with feedback from the OHCs and auditory nerve efferents) convert frequency information into the release of neurotransmitters, released into the synaptic cleft at auditory nerve terminals (*Dallos*, 1992). The primary neurotransmitter utilized in this communication is the ubiquitous excitatory amino acid glutamate (*Littman et al.*, 1989; *Eybalin*, 1993; *Puel*, 1995), though other dynamic

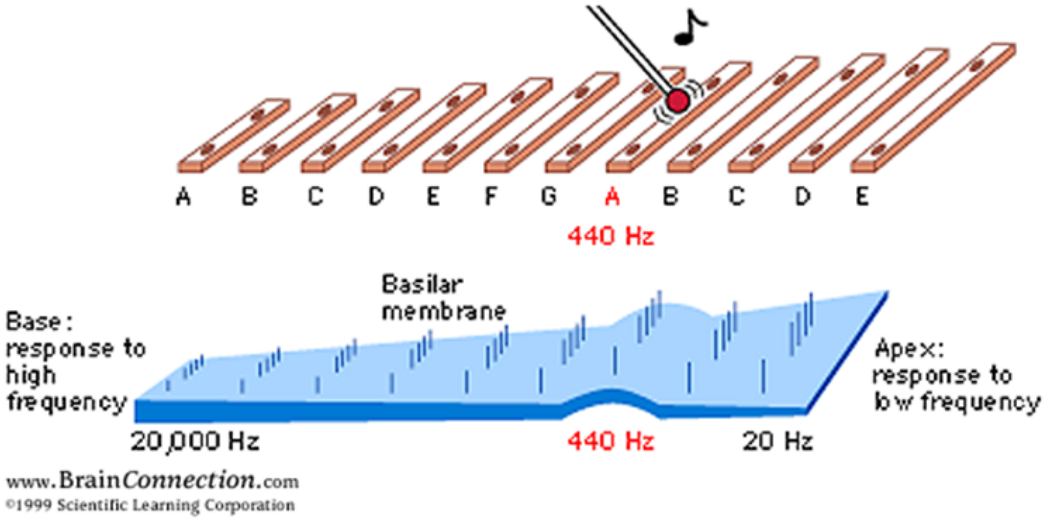

Figure 1.3: Basilar membrane uncoiled: at the base, cochlear elements maximally respond to high frequency sounds; at the apex, low frequency sounds.

inputs from glycine, for example, may also contribute. The release of transmitter into the synaptic cleft depolarizes SGN, which also possess distinct morphological properties and electrochemical potentials from base to apex that precisely convert glutamatergic signals into electrical impulses, or action potentials (AP) (*Nadol et al.*, 1990; *Adamson et al.*, 2002a; *Rusznak and Szucs*, 2009; *Zhou et al.*, 2005). Specific AP patterns generated by SGN encode information regarding the acoustic waveform - intensity, frequency, and timing - and are transmitted along the SGN central projection to the CNS, where higher brain structures in the auditory cortex allow for perception.

1.2 Sensorineural hearing loss

1.2.1 Causes and effects

Sustaining a healthy cochlea requires a delicate homeostasis. The cochlea is sensitive to many inputs. For example, the hearing loss suffered by Beethoven was almost as legendary as his genius, yet the etiology of his impairment is still a mystery. The element lead was found in tufts of his hair suggesting a mechanism of hair cell damage (Argon National Lab study), but possibly more compelling was the exceptional bone growth around his skull, suggesting Paget's disease and resultant pinching of auditory nerve fibers in the temporal bone (*Wolf*, 2001). Further speculation brings up the possibility of an auto-immune disease of the intestine based on his medical records, with the toxic-byproducts circulating into the ear (*Karmody and Bachor*, 2005). Whatever the reason, Beethoven's pathology likely began with damaged cellular components in the cochlea, leading to or accompanied by deterioration of the auditory nerve. This irreversible condition is known as sensorineural hearing loss (SNHL). See Figure 1.4.

IHCs of the cochlea, named for the distinct stereocilia or "hair" bundles projecting from their apical domains, are the auditory system's sensory receptors. Their intricate morphology enables exquisite decomposition of complex frequency information, but their position in the periphery leaves them in close proximity to the hazards of daily life. We are born with only a few thousand IHCs, yet have very limited capacity to regenerate these cells so prone to damage. Overexposure to loud noise produces mechanical trauma at the roots of the stereocilia, damaging the cell membrane (*Hu and Zheng*, 2008) and precipitating stress, inflammation, or apoptotic pathways that, depending on the decibels and duration, can damage IHCs or completely ablate the organ of Corti. For review, see *Le Prell et al.* (2007); *Bodmer* (2008); *Dinh and Van De Water* (2009). Certain aminoglycoside drugs, such as neomycin and kanamycin,

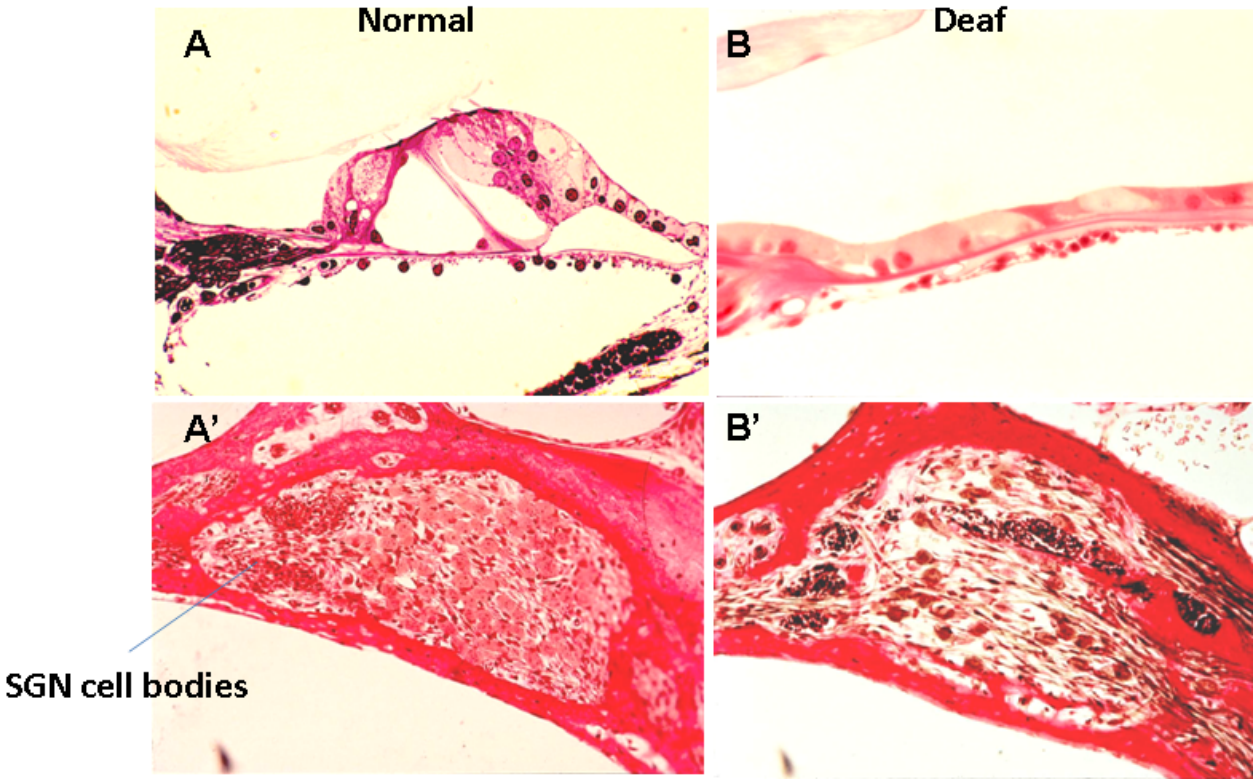

Figure 1.4: Sensorineural hearing loss: spiral Ganglion Neurons (SGN) degenerate damage to cochlear hair cells.

were once used to treat bacterial infections but are now prescribed with great caution as they are known to be ototoxic, specifically killing hair cells (*Forge and Schacht*, 2000). Additionally, given the myriad of genes known to contribute to the cytoskeletal and intracellular motors that then create IHC and OHC cell surface specializations, it is not hard to imagine any mutations in these genes could result in hearing defects, such as those seen in Usher syndrome, deafness caused by a mutation in a scaffolding protein, for example (*Frolenkov et al.*, 2004; *Denoyelle et al.*, 1999).

Bipolar SGN rely on trophic support from the IHCs innervating their dendrites and from the first neurons they synapse with at the level of the cochlear nucleus. The general consensus in the auditory field is that cochlear damage leads to a secondary, progressive degeneration in auditory nerve afferents, beginning at the peripheral nerve terminals (*Otte and Kerr*, 1978; *Webster and Webster*, 1981). This likely involves a loss in the activity-dependent survival cues upon which SGN rely: IHCs provide trophic support to innervating fibers through the release of neurotrophic factors (NTFs) such as glial cell-line derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF) and Neurotrophin-3 (NT-3) (*Ylikoski et al.*, 1993; *Schecterson and Bothwell*, 1994; *Staecker et al.*, 1996a; *Tan and Shepherd*, 2006). To be discussed in later sections, NTFs play a major role in neuron survival. SGN survival is also dependent on depolarizing electrical activity at the synapse (*Hansen et al.*, 2001; *Shepherd et al.*, 2005) created by neurotransmitter binding receptors on the SGN terminals. One study found electrical stimulation to be synergistic with NTF treatment to promote the survival of cultured SGN (*Hegarty et al.*, 1997). Conversely, when innervation from central neurons is lost as in acoustic neuroma in the cochlear nucleus, degeneration of SGN central processes can result (*Moore*, 1994; *Prasher et al.*, 1995; *Maricich et al.*, 2009).

1.2.2 Intervention strategies

Preventing damage to the cochlea can be as simple as inserting ear plugs before a rock concert to block intense mechanical bandwidths of noise from stressing delicate HC structures. Pharmacological intervention, however, is standard for targeting the molecular mechanisms that lead to hearing loss, largely based on new technology enabling us to screen for detectable shifts in cochlear gene expression that arise in aging, cancer, or after trauma. As previously discussed, HC damage usually precipitates into cell death due to the inability of these cells to withstand the intrinsic stress and inflammation cascades upregulated with trauma. One of the major pro-inflammatory cytokines released by cochlear support cells is Tumor Necrosis Factor-α (TNFα) (*Satoh et al.*, 2003), which utilizes a positive feedback mechanism with other inflammation-associated cytokines to further-promote hair cell damage. Corticosteroids are known to interfere with this feedback process and have been utilized for several decades to treat acute forms of hearing loss, but only chronic perfusion with these drugs can prevent further loss (*Dinh and Van De Water*, 2009). Similar strategies look to block HC stress and apoptosis cascades as a way to prevent death, for example the use of antioxidants such as vitamin C to block the accumulation of reactive oxygen species that contribute to apoptosis induction (*Kawamoto et al.*, 2004; *LePrell et al.*, 2010).

As described above, the spiral ganglion neurons are poised to degenerate after hair cell loss. Depending on the species and etiology, this secondary degeneration occurs more or less rapidly, but swift intervention is always necessary to prevent further degeneration. In the case of noise-induced hearing loss, an overabundance of glutamate released by IHCs at the SGN synapse can lead to excitotoxicity in the neurons, though some mechanisms of repair are in place for such trauma (*Pujol and Puel*, 1999). Attenuating glutamatergic transmission in auditory nerve after noise enhanced SGN survival (*Ruel et al.*, 2005; *Chen et al.*, 2009b). Cochlear infusion of

NTFs, which promote neuronal differentiation and survival in general, contributed to SGN maintenance in the adult, also increased SGN survival after trauma (*Miller et al.*, 1997; *Yamasoba et al.*, 1999; *Gillespie and Shepherd*, 2005; *Agterberg et al.*, 2009). When combined with electrical stimulation by implanted electrode, auditory neurons showed enhanced survival following cochlear trauma (*Kanzaki et al.*, 2002; *Chikar et al.*, 2008).

Many strategies have been suggested to prevent or reverse damage to the cochlea's cellular components after trauma. While these pharmacological approaches provide some alleviation in progressive hearing loss, none can reverse the condition and restore the sensation of sound. Hearing aids amplify noise and rely on substantial survival of these cells to restore hearing, but loss of first-order SGN is currently one of the biggest hurdles to overcome in developing an effective treatment for this sensory impairment.

1.2.3 Cochlear implants: hearing restoration with limitations

In 1957 French physicians postulated that they could create representations of sounds in the brain by activating auditory nerve fibers with pulses of current, therefore bypassing damaged cochlear elements (*Seitz*, 2002). The profoundly deaf human subject could perceive vague sounds upon current stimulation, but with little resolution (*Djourno and Eyries*, 1957). These pioneering experiments in large part laid the groundwork for the creation of a cochlear prosthesis, or a device that could reproduce the function of cochlear components to restore hearing. Through inter-disciplinary collaborations by innovators like William House, significant improvements in surgical techniques and a better understanding of cochlear mechanotransduction have made the CI more reliable in simulating sound, making the device more accessible. Cochlear implants have proven to be the most successful prosthesis for restoring any sensory impairment; on the order of 100,000 patients have been implanted, but with a high degree of variability in performance.

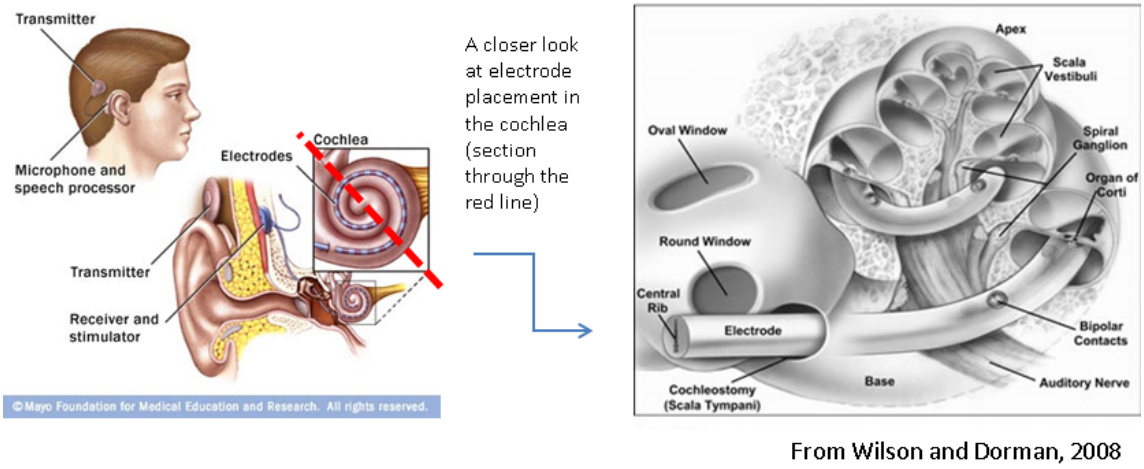

Figure 1.5: Cochlear implant overview.

The premise behind the modern day CI is to recreate peripheral auditory function by acquiring frequency information from acoustic waveforms in the air, and relaying this information to a speech processor, where signals are reduced to electrical impulses. These pulses of current are transmitted through a multiple electrode array that is inserted into the cochlea's scala tympani from base to apex (*Cooper and Craddock*, 2006). Electrical currents emanating from individual electrodes make their way through cochlear tissue toward remaining auditory nerve peripheral processes (*Miller and Spelman*, 1990). With today's CI, many patients can understand upwards of 80% of speech but pitch discrimination is still poor (*Wilson and Dorman*, 2008). See Figure 1.5. An algorithm that can encode the "temporal fine structure" (frequency, timing, and intensity) of acoustic waveforms in the environment must be developed to improve speech and music perception for CI users (*Stickney et al.*, 2004; *Pfingst and Xu*, 2005; *Xu and Pfingst*, 2008).

The benefit gained from CI depends on a significant number of patient-specific factors. As a result there is a wide range of clinical outcomes. One fundamental variable is the number of auditory nerve fibers present at the time of implantation (*Pfingst*, 1990; *Kileny et al.*, 1991; *Blamey et al.*, 1996; *Skinner et al.*, 1997; *Incesulu and Nadol*, 1998). When the electrode array is implanted into the scala tympani, the

hope is that electrical impulses will penetrate the tissue and reach any SGN peripheral nerve terminals remaining. In cases of profound sensorineural hearing loss, very few SGN fibers remain for CI electrodes to stimulate (*Sly et al.*, 2007). Often peripheral nerve terminals deteriorate into Rosenthal's canal and are too far from the site of stimulation (*Snyder et al.*, 2008; *Wilson and Dorman*, 2008), making it impossible to convey frequency information from electrodes to the CNS. Currently efforts are being made to reconfigure the electrodes to allow for improved selectivity of stimulation, and to grow degenerated fibers toward the electrode (*Evans et al.*, 2009; *O'Leary et al.*, 2009; *Shibata et al.*, 2010). When too few SGN survive, however, these clever strategies are pointless. For these reasons, there is a critical need to replace the SGN to increase the utility, applicability, and clinical success of this device.

1.3 Improving CI efficacy using stem cells to replace SGN

1.3.1 Mobilizing endogenous stem cells to replace lost tissue

Recent discoveries in the stem cell field the past decade have revealed that a quiescent population of stem cells exist in nearly every adult tissue. These so-called adult stem cells have a "multipotent" capacity to differentiate into phenotypes within their tissue of origin (For review see *Conti et al.* (2005) and *Raff* (2003)). While no definitive inner ear stem cell has been identified, some evidence suggests they do exist in the adult and early post-natal cochlea (*Rivolta et al.*, 1998; *Li et al.*, 2003; *Rask-Andersen et al.*, 2005; *Nicholl et al.*, 2005; *Oshima et al.*, 2007; *Martinez-Monedero et al.*, 2008). Tissue from these regions can be harvested and then dissociated into passageable cultures *in vitro*, where cells with proliferative, self-renewing properties form aggregates of precursors that, upon exposure to differentiation cues, can potentially generate hair cell-like and SGN-like cells. In these studies, greater success was obtained when "spheres" were derived from younger animals, suggesting that

if there is a progenitor population in this structure, its activity decreases with age (*Oshima et al.*, 2007; *Chen et al.*, 2009a). This decline in regenerative capacity with age is a common theme in stem cell populations within nearly every organ of the body and arises from a combination of factors affecting telomere length after stem cell divisions and a decline in maintenance signals within the stem cell niche (*Ferron et al.*, 2009; *Sahin and Depinho*, 2010), and in part explains why hearing loss only gets progressively worse and never improves.

Multipotent spheres generated from heterogeneous tissues consist of many cell types, and therefore do not provide us with information as to the exact cellular origin of the stem cells, leaving us with little idea about their developmental origin and the cues we can utilize to drive their differentiation. Immortalized cell lines have been derived from inner ear tissue (*Bianchi et al.*, 2005), but apparently bias toward support cell differentiation, secreting factors important in developing SGN neurite outgrowth. An auditory "neuroblast" line has been derived from early-stage embryonic statoacoustic ganglion precursors, expressing the neuronal marker β-III tubulin *in vitro*, but fewer than 30% formed neurons in the *in vivo* model (*Nicholl et al.*, 2005).

Rather than harvest and then expand cells from one animal for transplantation into another, it is possible to target inner ear supporting cells (putative stem cells) for transformation with cell-cycle regulators or transgenes to mobilize them for transdifferentiation into specialized IHCs or SGN (*Kawamoto et al.*, 2003; *Izumikawa et al.*, 2005; *Minoda et al.*, 2007). In these studies, genes known to regulate hair cell differentiation and mitosis were delivered to deafened sensory epithelium using viral vectors in guinea pig, with hair cell regeneration and restoration of function as detected by auditory brainstem response (ABR). The SGN supporting cell, the Schwann cell, does undergo functional changes after cochlear damage (*Hurley et al.*, 2007) and can be transduced to release neurotrophic factors that promote SGN survival after trauma

(*Pettingill et al.*, 2008). Transdifferentiation of Schwann into neurons has not been attempted, likely because these cells originate from a transient population of cells known as the neural crest, rather than the otocyst (*Jessen and Mirsky*, 2005).

In 2006, researchers in Japan identified "pluripotency factors" that when introduced at specific combinations into adult fibroblasts, could transform adult tissue into embryonic stem cell-like cells with a similar open chromatin configuration and capacity to differentiate into any cell of the body (*Takahashi and Yamanaka*, 2006). Since the initial report on these so-called induced pluripotent stem (iPS) cells created a flood of attempts to guide their differentiation into specialized cell types; these cells hold incredible potential for therapeutic use because they can be derived directly from the patient, eliminating the need for allogenic donor tissue and the associated risk of immunogenic responses that complicate engraftment.

Recent reports found that the level of pluripotency varies between iPS cell lines, with some having a greater bias toward post-mitotic differentiation, and others preferring to stay proliferative, creating a greater risk for tumor formation (*Nishimura et al.*, 2009; *Hu et al.*, 2010). The fact that these cells are transformed through a series of genetic manipulations delivered through viral vectors may also activate oncogenic cascades leading to cancer (*Nair*, 2008). Further research into these interesting cells must be done before therapeutic utility can be definitively established.

1.3.2 Embryonic stem cells to replace lost tissue

Stem cells exist in many different forms and have variable potential to generate new tissues. The methods used to transduce them into new phenotypes differ between cell types and produce variable results. As discussed above, our ability to convert adult stem cells into specialized cells is limited by our knowledge of the signals involved in their mobilization to differentiate, our access to these cells, and by our ability to maintain these cells over time in culture for developmental studies. Often the pursuit

of functional cell replacement strategies leads us back to pluripotent embryonic (ES) cells. Life begins as a single "totipotent" stem cell - a fusion of haploid egg and sperm cells containing the genetic blueprints for building the organism. As the single cell divides, new cells eventually differentiate into more and more specialized ones along defined axes, secreting inductive signals that transduce lineage-specific genetic cascades.

Cells derived from the inner cell mass (ICM) of pre-implantation blastocysts are ES cells. ES cells have high levels of the enzyme telomerase, which redistributes nucleotides to the ends of chromosomes after cell divisions (*Thomson et al.*, 1998; *Amit et al.*, 2000), an activity linked to apparent limitless replicative lifespan. This self-renewing capacity potentially allows for the production of large numbers of neuronal subtypes under controlled conditions *in vitro*, making these cells ideal for treating neurodegenerative disorders (*Lindvall et al.*, 2004). When reintroduced into developing blastocysts, ES cells contribute to all cell types in all three germ layers (*Bradley et al.*), and advances in the field of developmental biology toward identifying the inductive signals involved in generating these tissues has led to improved creation of more specific cell types *in vitro*. Exposure to select combinations of such signals, genetic manipulations, or co-culture with cells that are present in the microenvironment of the desired phenotype during development, are all effective ways to produce neurons capable of generating action potentials (*Strubing et al.*, 1995; *Bain et al.*, 1995; *Johnson et al.*, 2007; *Robertson et al.*, 2008). If as many aspects of the target cell's development can be replicated in ES cells, the latter may acquire more specific morphological and biochemical features of the target cell for improved functional replacement. For review, see (*Keller*, 2005).

The derivation of ES cells from the ICM, which gives rise to the fetus, poses several valid ethical concerns. For example, is the derivation of immortalized cell lines destruction of a human life, or do we fulfill society's greater need for disease

cures by salvaging to-be-discarded embryos from fertility clinics? Since the first ES cell lines were established (*Thomson et al.*, 1998), this debate has raged on, and the research on existing human lines was tightly restricted under federal mandate (*Scott and Reijo Pera*, 2008), until President Obama lifted these restrictions in early 2009. In spite of a decade of limited research, a myriad of protocols now exist for promoting differentiation of ES cells into distinct neuronal subtypes *in vitro*, an overwhelming majority of studies being conducted in mice with the hope that techniques can be translated into human (*O'Shea*, 2001). Technical challenges remain, however, in obtaining similar differentiation after transplantation *in vivo*. ES cell therapeutics *in vivo* must overcome the risk of teratoma formation by implanted cells if they are still undifferentiated, but inflammatory responses may arise if cells are differentiated prior to placement. Additionally, further elucidation of the developmental cues that give rise to neuronal subtypes within the nervous system is crucial to driving ES cell differentiation toward these specialized phenotypes.

1.3.3 Tracking cells after implantation: the problem with gene transfer and epigenetics

One of the biggest challenges facing stem cell (SC)-based cell replacement therapies is the ability to track the progress of implanted cells. Preliminary studies must demonstrate the efficacy of SC implants to endure by providing compelling data on cell survival, differentiation, migration, and integration into the host tissue. In the case of nerve replacement, visualization of dynamic process outgrowth toward targets must be clearly shown, with ES cell-derived neurites clearly distinguishable from those of host cells neurites. Effective labels will also aid in assessing immunogenic and tumorigenic events after transplantation. Currently, techniques to noninvasively track cells in live tissue are being developed by delivering magnetic nanoparticles across the cell membrane and subsequently visualize cells with magnetic resonance

imaging (MRI) (*Bulte et al.*, 2001; *Bos et al.*, 2004).

In the stem cell field a great deal of energy is spent on developing these elusive labels to enable all of the above. While the concept of identifying implanted cells seems straightforward, in practice there are several technical challenges that have hindered the progress of ES cell-based therapeutics. Advancements in image processing and genetic engineering have improved our ability to track implanted cells, for example the use of confocal microscopy to detect with great resolution the progress of implanted cells expressing fluorescent proteins originally isolated in jellyfish. Despite these applications, labeling of ES cells remains problematic. Genetic manipulations that allow us to express these fluorescent proteins in undifferentiated ES cells can only take place within the transcriptionally active euchromatin that maintains self-renewing and pluripotency factors at high levels in the genome. ES cells readily integrate and transcribe foreign DNA, or transgenes, within this active region of their genome (*Meshorer and Misteli*, 2006). If the transgene encodes a "developmentally neutral" fluorescent label rather than a lineage determination gene, individual ES cells that highly express the label can be clonally expanded in an undifferentiated state to create stable cell lines (*Vallier et al.*, 2007).

Once ES cells differentiate, the hope is for their continued, uniform expression of fluorescent proteins such as eGFP or mCherry, enabling us to visualize new and changing cell structure and integration into host tissue. The problem in differentiating ES cells is, in having undergone extreme changes in membrane and cytoskeletal properties, transcriptional machinery is now re-dedicated to the expression of differentiation genes, rather than the previously-mentioned "stem" genes such as Oct3/4 or SSEA. The major chromatin rearrangements accompanying these changes often silence formerly active regions of the genome (*Meshorer et al.*, 2006; *Wu and Sun*, 2006; *Efroni et al.*, 2008), including the expression of transgenes. Unless the transgene is inserted into a constitutively active locus, differentiated cells rarely maintain

fluorescent reporter expression once they have acquired new and distinct genetic profiles both *in vitro* and *in vivo*, making it difficult to prove cells are of ES cell origin, as opposed to host tissue with fluorescent accumulations of superimposed apoptotic debris. Constitutively-active promoters, bioluminescent fusion proteins, and lipophilic dyes are some of the methods attempted to overcome this challenge, as described in Appendix A.

1.3.4 SGN replacement strategies

Several groups throughout the world are pursuing cell replacement in the inner ear to replace lost hair cells and SGN. For replacement of the SGN, the strategy has generally been to develop a population of cells *ex vivo* with the potential to differentiate after implantation *in vivo*. Stem cell-derived neurons must demonstrate several features of the SGN phenotype in order to prove success of the therapy: the cells must be electrically excitable and possess a bipolar morphology to receive stimulation at one end, and synapse with lower auditory centers at the other. The cells must also relay between these structures the same neurochemical messages utilized by endogenous SGN, primarily in the form of the neurotransmitter glutamate. These features can be identified most easily *in vitro*; *in vivo* demonstration of stem cell differentiation must utilize an effective cell label for tracking after implantation.

Previous work investigating the potential utility of stem cells to replace auditory nerve has encountered limited success. *Tamura et al.* (2004) found a majority of fetal neural stem cells (NSCs) implanted into deafened cochleae differentiated into glia (80%) rather than neurons (less than 10%). *Coleman et al.* (2006); *Corrales et al.* (2006); *Parker et al.* (2007); *Shi et al.* (2007) induced NSCs or ES cells to form neural progenitors before implantation into scala tympani and found both survival and differentiation of neuronal cells to be low. The poor survival and differentiation into the target neuronal phenotype could be due to many of the pro-inflammatory

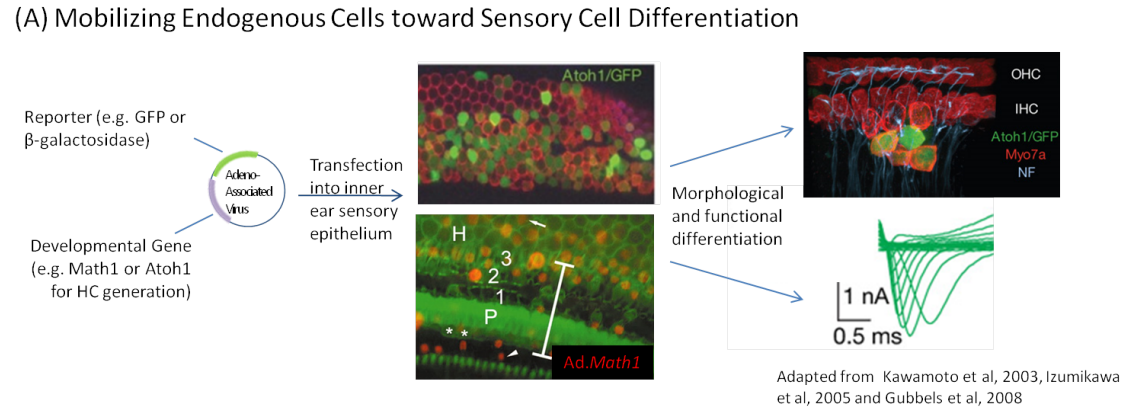

Figure 1.6: Differentiation paradigms for cell replacement in the inner ear

factors released by cochlear structures after damage, as discussed in a previous section. The adult microenvironment in which stem cells must differentiate *in vivo* would therefore contain requisite developmental signals to promote neuronal differentiation; a co-transplant of stem cells and/or neural tissue may provide the appropriate cues for neuronal survival and differentiation (*Hu et al.*, 2004, 2005a; *Coleman et al.*, 2007). Studies based on this premise found increased numbers of neurons both *in vitro* and *in vivo*, but the differentiation was sparse.

In this dissertation, I begin to address the fundamental problems surrounding differentiation and survival of stem cell-derived neurons in the inner ear. First, little attention has been previously given to a possible "bystander" effect, i.e. whether the implanted cells or the act of implanting cells stimulates endogenous cells to alter their behaviors and subsequently affect differentiation of implanted cells. Controls must be presented in any study to account for aforementioned inflammation mediators or trophic factors present in the traumatized cochlea that may also influence differentiation. Such factors can significantly affect survival and differentiation, making it impossible to attribute positive results to the methodology used, or the influence of these factors. Additionally, the implantation of ES cells, with their incredible capacities for proliferation and self-renewal, create a great risk for the formation of tumors, if their differentiation is not induced before or immediately after implantation. Implantation of stem cells or progenitor cells requires some cues for guiding differentiation after implantation. Also, many cell replacement studies have examined the effect of inter-species donor and host for better identification of implanted cells (*Corrales et al.*, 2006; *Hu et al.*, 2005b).

1.4 Directed differentiation of ES cells into SGN

One of the most effective ways to guide ES cell differentiation toward a phenotype of interest is to first have an understanding of the molecular and genetic mechanisms

that define the phenotype during development. These mechanisms may then be reproduced in differentiating ES cells toward generating a specific phenotype. A common theme in neuronal development is that transient, non-neural structures shape neuronal differentiation through the secretion of inductive factors. The positional and temporal actions of these factors spur cell-cell communication to first establish the neuronal phenotype, with glial supporting cells appearing later. Varied exposure to secreted factors selectively activates differentiation cascades to produce unique specializations in neuronal form and function.

Understanding the specific genes and inductive signals in the cochlear microenvironment that contribute to the development and maintenance of sensory spiral ganglia may give us the information necessary to recapitulate this developmental progression in differentiating ES cells. The first manifestation of the mammalian cochlea is the otic placode, a slight invagination of surface ectoderm around the hindbrain region of the developing nervous system. An otic "vesicle" emerges and pinches off into the "otocyst", a spherical structure of multi-potent progenitor cells (*Barald and Kelley*, 2004; *Noramly and Grainger*, 2002). Combinations of inductive signals from the surrounding mesodermal and neuroectodermal tissue pattern lineage differentiation of otocyst progenitors into either patches of sensory epithelium or neural competent cells. For example, specific members of the fibroblast growth factor (FGF) are critical in otic induction (*Leon et al.*, 1995; *Ladher et al.*, 2000; *Brumwell et al.*, 2000; *Alvarez et al.*, 2003; *Wright et al.*, 2004; *Zelarayan et al.*, 2007), while others promote migration and differentiation of developing SGN (*Hossain et al.*, 1996; *Adamska et al.*). Wnts, bone morphogenetic proteins, Hedgehog (Hh) signals, insulin-like growth factors, and NTFs also contribute to the regional and cellular specializations seen along the cochlear spiral (*Ladher et al.*, 2000; *Riccomagno et al.*, 2005; *Driver et al.*, 2008; *Puligilla and Kelley*, 2009).

Establishment of neural progenitors occurs early in inner ear morphogenesis and

is initiated and maintained in part by the interaction of all the secreted factors mentioned above. Individual cells within the developing otocyst receive heterogeneous exposure to factors, generating "stochastic variations" in gene expression (*Kageyama et al.*, 2008) within an otherwise uniform population of cells. In particular, the transient expression of basic helix-loop-helix (bHLH) transcription factors commit cells to either a sensory or neuronal lineage. *Blader et al.* (1997) found disruption of Hh signaling in developing zebrafish expanded the domain of Neurog1-positive cells and produced ectopic neurons, suggesting this pathway regulates Neurog1 and therefore neuronal commitment. Apparently multiple inputs influence neuronal commitment via Neurog1, however, as effectors in the β-catenin pathway were found to bind the Neurog1 promoter (*Israsena et al.*, 2004), and mutations in the genes encoding Eya1 and Tbx1 transcription factors cause severe defects in SGN development and inner ear morphogenesis (*Friedman et al.*, 2005; *Raft et al.*, 2004).

Neuroblasts transiently express Neurog1 as they delaminate from the anteroventral portion of the otocyst into the surrounding mesoderm (*Fekete and Wu*, 2002; *Fritzsch*, 2003). In the developing otocyst, cells adjacent to Neurog1-positive neuroblasts are induced to express an alternative bHLH factor, Math1, which commits cells to the sensory hair cell lineage (*Bermingham et al.*, 1999; *Raft et al.*, 2007). This mechanism of "lateral inhibition", whereby Neurog1-expressing cells de-activate neuronal gene expression in adjacent cells through the Notch pathway, generates the ratio of neurons to hair cells. The importance of Neurog1 in establishing neuronal identity in the inner ear was underscored in mice lacking the gene, which developed smaller cochleae and lacked both innervation of radial fibers to hair cells and SGN cell bodies in the cochleae of these mutants (*Ma et al.*, 2000), see Figure 1.7.

Neurog1 signal transduction in developing SGN is now known to activate a combination of sensory neuron-associated factors including Brn3a, NeuroD1, and Gata3 to further-establish SGN specializations (*Huang et al.*, 2001; *Kim et al.*, 2001; *Karis*

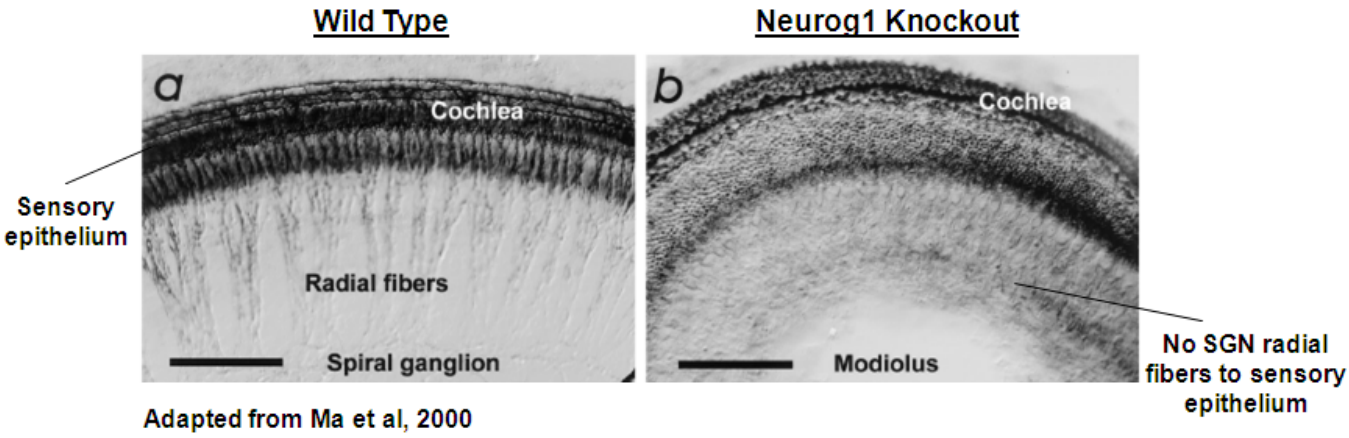

Figure 1.7: Neurog1 null mutants completely lack SGN innervation to sensory epithelium.

et al., 2001; *Fritzsch*, 2003). These transcription factors regulate cell body size (with a functional correlation, see *Davis* (2003)), and upregulate the machinery needed for the critical neuronal responses to NTFs: the neurotrophic factor receptors (NTFRs). Activation of NTFRs by corresponding gradients of neurotrophic factors corresponds with neurite outgrowth, appropriate target innervation, and ion channel composition to confer specific functions (*Ernfors et al.*, 1995; *Farinas et al.*, 2001; *Adamson et al.*, 2002a).

Beyond its effects in the ear, Neurog1 initiates an early wave of neurogenesis to establish neuronal identities in the developing PNS and central nervous systems (CNS) (*Sommer et al.*, 1996; *Lee*, 1997; *Ma et al.*, 2000; *Cau et al.*, 1997). Deletion of the gene in mice results in failure to form subsets of peripheral ganglia and establish glutamatergic pathways (*Ma et al.*, 1996, 1998; *Schuurmans et al.*, 2004). These

interesting functions in directing neuronal differentiation in the inner ear suggest that Neurog1 could be an interesting regulatory candidate to assess for its ability to direct SGN differentiation from undifferentiated cells. Overexpression studies of Neurog1 have revealed the power of this factor to reproduce similar neuronal identities *in vitro* and *in vivo*, while inhibiting gliogenesis (*Sun et al.*, 2001; *Kim et al.*, 2008).

Neurotrophic factors (NTFs) are indispensable components in the maturation of delaminated neuroblasts into mature SGN. Neurog1-positive neuroblasts upregulate specific combinations of NTF receptors shortly after delamination (*Staecker et al.*, 1996b; *Farinas et al.*, 1998; *Brumwell et al.*, 2000; *Hossain et al.*, 2008; *Sun and Salvi*, 2009). A major focus of inner ear developmental studies is to identify temporal changes in the sensitivities of SGN to particular NTFs under normal and pathological physiological conditions for better intervention strategies to preserve SGN. A yet-to-be-determined number of NTFs play a role here, but I will largely focus on the effects of GDNF, BDNF and NT-3 (*Qun et al.*, 1999). NT-3 is critical for both the early survival of nascent SGN and precise innervation of neurons to the sensory epithelium, while a later BDNF gradient confers functional specializations along the cochlear spiral (*Ernfors et al.*, 1995; *Fritzsch et al.*, 1997; *Adamson et al.*, 2002a; *Tessarollo et al.*, 2004; *Sun and Salvi*, 2009). SGN sensitivity to GDNF also increases as these neurons mature, likely to serve a neuroprotective role through activation of the GDNF receptor (*Hashino et al.*, 1999; *Wei et al.*, 2007).

1.4.1 Goal: Reproduce SGN development in ES cells

In this dissertation I outline new strategies to derive cells capable of functional SGN replacement. ES cells, with their incredible pluripotential for differentiating into any cell in the body, provide hope toward this end. Previous work has corroborated this potential, but many technical challenges remain. In their undifferentiated state, ES cells express fewer major histocompatibility complexes (MHCs) that may tag

more differentiated cells for an immunogenic response after implantation (*Fairchild et al.*, 2004), may therefore ideal for transplantation if the risk of teratoma formation can be reduced. At the same time, the unlimited capacity of ES cells to self-renew poses the risk for tumor formation. The most challenging aspects of ES cell-based therapeutics is our incomplete knowledge of the positional and temporal cues present in the developing embryo that reinforce specific phenotypes from undefined precursors.

In previous sections I identified some of the genetic and extracellular cues that are critical to SGN development: activation and transient expression of the proneural transcription factor Neurog1 commit otic neuroblasts to the neuronal lineage, and subsequent upregulation of NTFRs confers SGN sensitivity to trophic factors in the microenvironment. This dissertation is based on the hypothesis that transient activation of Neurog1 with exogenous activation of NTFRs in differentiating ES cells would augment differentiation toward an SGN-like phenotype. A transgenic Neurog1-inducible ES cell line allowed us to reproduce the temporal expression of this transcription factor (*Velkey*, 2005). The cell line enabled implantation of undifferentiated cells into a deafened guinea pig model with immediate induction of neuronal differentiation. Delivery of NTFs to implanted cells may transduce intracellular events that further-hone the SGN phenotype on the morphological, neurochemical, and functional levels. Here I present *in vitro* and *in vivo* differentiation studies seeking to identify the neuronal subtype conferred by transient Neurog1 activation, and assess the ability of NTFs to modulate the resultant phenotype. Ultimately, the cells show great potential to participate as an interface between a CI electrode and the CNS.

CHAPTER II

Glutamatergic, Neuronal Differentiation of Mouse Embryonic Stem Cells After Transient Expression of Neurog1 and Treatment with BDNF and GDNF: *in vitro* and *in vivo* studies

2.1 Introduction

Embryonic stem (ES) cells have the potential to replace tissues lost by injury, disease, or normal aging if lineage differentiation can be controlled. Replicating the intrinsic and extrinsic factors influencing fate choice provides one possible method toward obtaining the specific phenotype of interest for stem cell therapies Delivery of such cues to ES cells in vivo can be particularly challenging, and transplantation studies face the additional obstacle of immune rejection, which may be circumvented by implantation of undifferentiated ES cells (*Drukker et al.*, 2006; *Robertson et al.*, 2007), followed by differentiation *in vivo*.

Glutamatergic spiral ganglion neurons (SGN) that make up the auditory nerve convey sensory information from inner hair cells (IHCs) to the central nervous system, and are particularly susceptible to death secondary to IHC loss. Regeneration of SGN does not occur endogenously. The advent of new technologies such as cochlear

implants has accelerated the need to replace SGN in order to improve efficacy of these devices, which rely on surviving neurons to restore hearing. Previous reports have found that placement of ES cells or tissue-derived stem cells into the relatively immunoprivileged cochlea eventually yielded a small percentage of neurons while a majority of implanted cells differentiated into glia (*Hu et al.*, 2004; *Tamura et al.*, 2004; *Hu et al.*, 2005a,b; *Nicholl et al.*, 2005; *Coleman et al.*, 2006; *Corrales et al.*, 2006; *Coleman et al.*, 2007; *Parker et al.*, 2007; *Ulfendahl et al.*, 2007). Chronic intrascalar application of neurotrophic factors (NTFs) increased the number of stem cells found displaying a neuronal phenotype, but the percentage was still under 25% (*Altschuler et al.*, 2008).

Neurogenin 1 (Neurog1) is a proneural bHLH transcription factor that activates a downstream cascade of NeuroD1, Brn3a, Gata3, and NTF receptors necessary for normal SGN differentiation, migration, and survival (*Huang et al.*, 2001; *Karis et al.*, 2001; *Fritzsch*, 2003). Mice deficient in Neurog1, NeuroD1, or Brn3a display varying degrees of SGN disruption, with SGNs completely absent in Neurog1 null mice (*Ma et al.*, 2000; *Huang et al.*, 2001; *Kim et al.*, 2001). Overexpression of Neurog1 in neural progenitors promotes neuronal differentiation while inhibiting gliogenesis, even in the presence of glial-inducing factors (*Sun et al.*, 2001).

In the present study we develop a more efficient approach that allows us to guide ES cell differentiation in vivo. We hypothesized that ES cells could be differentiated toward a glutamatergic neuronal phenotype by inducing pan-neural gene expression with Neurog1, followed by providing endogenous cues to further hone the target phenotype and/or promote survival of the desired phenotype. Neurog1 is expressed for 48h in the developing embryonic otocyst (*McCormick et al.*, 1996; *Fritzsch*, 2003). We tested whether 48h transient Neurog1 expression in pluripotent mouse ES cells, combined with brain derived neurotrophic factor (BDNF) and glial cell line derived neurotrophic factor (GDNF) treatment could promote ES cell differentiation toward

a SGN-like phenotype for auditory nerve replacement.

2.2 Materials and Methods

2.2.1 Materials and Methods - *in vitro*

Inducible Neurog1 embryonic stem cell line. ES cells were derived from a parent mouse ES cell line (N7) (*Velkey*, 2005) containing a tet-inducible Neurog1. The N7 cell line contains a reverse tet transactivator (rtTA) upstream of the constitutively active ROSA26 locus that is puromycin selectable (*Kyba et al.*, 2002). To derive the N7 line, a plasmid containing the Neurog1 cDNA and an ATG start codon was integrated into the ES cell genome upstream of a tet operon (tetOp). Downstream of the integration site, a truncated neomycin resistance cassette had been placed into the line (*Velkey*, 2005), making it G418 selectable.

Cell culture and transfection. To track implanted cells, the N7 cell line was transfected with a plasmid containing eGFP driven by the human ubiquitin ligase C (UbC) promoter (*Wulff et al.*, 1990; *Schorpp et al.*, 1996) (Figure 2.1). The cell line had been previously modified with eGFP driven by the CMV promoter, but we found this construct to be particularly susceptible to methylation upon differentiation. $2.75x10^6$ N7-Neurog1 cells were plated in a 6cm dish and allowed to grow to 60-70% confluency. The cells were transfected with 2μg of the UbC-eGFP-HygR DNA using lipofectamine and PLUS reagent (Invitrogen). Selection with hygromycin for cells that integrated the new UbC construct was followed by identification of individual colonies with well-defined edges uniformly expressing eGFP. Colonies were transferred to a 96-well plate and dissociated with trypsin and EDTA. Colonies that survived additional selection in puromycin (2 μg/mL) and hygromycin (300 μg/mL) were expanded to $75cm^2$ flasks in ES cell maintenance media consisting of sterile-filtered DMEM (Invitrogen) with 10% ES cell-tested fetal bovine serum (Atlanta Biologi-

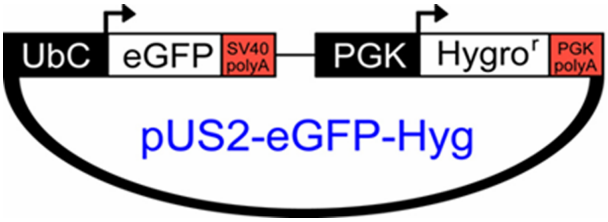

Figure 2.1: Diagram of Promoter.

cals), 5% embryonic stem cell supplement (DMEM with 24 mg/mL HEPES buffer, 4 mg/mL L-glutamine, and 70 ng/mL β-mercaptoethanol) and 0.5 μg/mL leukemia inhibitory factor (LIF, Chemicon International). One subclone, K9, was chosen based on continuous, homogenous bright eGFP expression 24-48h after Doxycycline-induced (Dox) differentiation of cells in vitro.

Differentiation *in vitro.* K9 ES cells were expanded in $75cm^2$ flasks to 80% confluency, and then plated for differentiation. Cells were washed with 1X Hanks Balanced Salt Solution (HBSS, Invitrogen) to remove growth media and cellular debris, and then dissociated using 0.25% trypsin and 1mM EDTA in 1X HBSS for 2 minutes at 37C. Dissociation was blocked using complete media without LIF and cell clumps were triturated to a single-cell suspension. After centrifugation for 2 min, cells were resuspended in serum-free differentiation medium (80% F12/DMEM (Invitrogen), 20% Neurobasal medium (Invitrogen), with B27 and N2 supplements, and 10 mM sodium pyruvate (Gibco)). $5x10^5$ cells per well were plated in 6-well plates. Four sterile, 13mm diameter Thermanox cell culture coverslips were placed at the bottom of each well and coated with 0.1% gelatin (Sigma).

Expression of the Neurog1 transgene was induced by exposure to 1g/mL Dox (Sigma) for 48h. After Dox was removed from the culture, Neurog1 expression decreased after 24h. See Figure 2.2. Cells were grown with Dox alone, or with Dox and BDNF (20 ng/mL, QED Bioscience) and GDNF (100 ng/mL, Amgen). After three days in culture with medium changes every 24hr, the serum-free medium was replaced with DMEM containing 5% knockout serum replacement (Gibco) and 5%

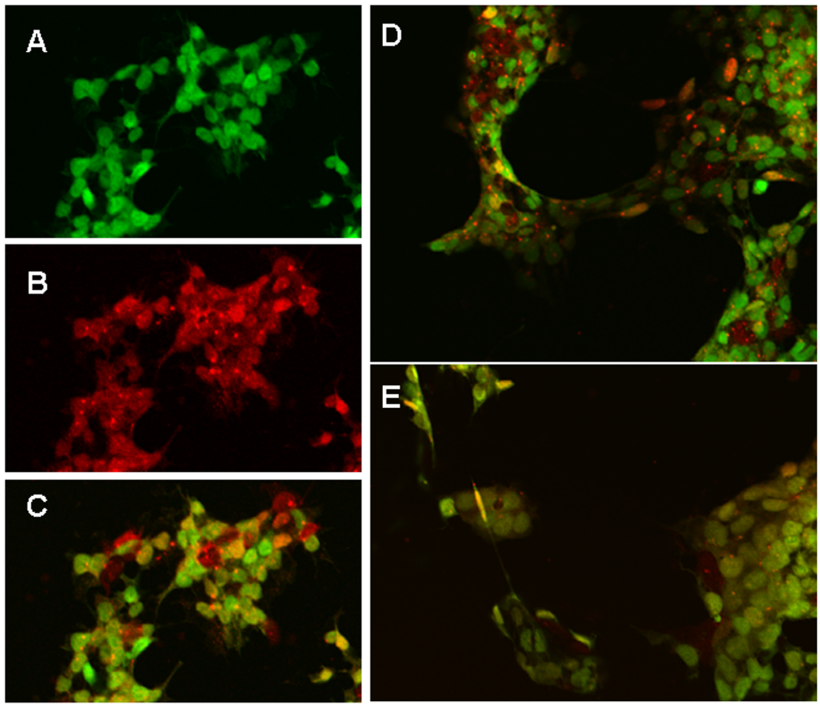

Figure 2.2: Doxycycline exposure activates Neurog1.

ES cell supplement without Dox to promote long-term survival.

***In vitro* Assessment - Immunostaining.** Cells were fixed in 4% paraformaldehyde for 15 minutes at 24h, 72h, and 5D. Coverslips from three replicate cultures were washed in phosphate buffered saline (Dulbecco's PBS, Gibco) and incubated overnight at 4C with the antibodies to Neurog1 (1:100, Chemicon), the neuronal marker TUJ1 (class III β-tubulin, 1:300, Covance), VGLUT1 and VGLUT2 (both 1:500, Synaptic Systems), and to the glial fibrillary acidic protein (GFAP, 1:1500, Advance Immunochemical) diluted in 0.1% Triton-X in PBS. The coverslips were washed with D-PBS and TUJ1 binding was visualized using an Alexa 633 secondary (1:500, Molecular Probes, Carlsbad, CA). VGLUT1 and VGLUT2 were visualized using Alexa 594nm (1:500, Molecular Probes). GFAP was visualized with Alexa 568 (1:300, Molecular Probes).

In each treatment group, three coverslips from each of the three replicate cultures were quantified (n = 9 coverslips/group). Nine images were taken from each coverslip

using an Olympus FluoView 500 Confocal microscope (n = 81 images/treatment group). The total number of neurons present was estimated as the number of cells positive for TUJ1 only, TUJ1 and eGFP, and TUJ1 and VGLUT1/2. The number of cells co-labelled for TUJ1 and VGLUT1/2 was taken to be the total number of neuronal, glutamatergic cells. The total number of cells was estimated as the total number of neuronal cells plus the number of cells positive for eGFP only, VGLUT1/2 only, GFAP and TUJ1, and GFAP only. Hoechst 33 stain revealed normal nuclei as well as sparse, tiny puncta of nuclei in dead cells that did not co-express any of these markers.

***In Vitro* Assessment - qRT-PCR.** Stem cells from three treatment groups (uninduced control at 0h, 72h Dox with NTFs, and 5D Dox with NTFs) were examined using qRT-PCR for the expression of NTF receptors Ntrk1, Ntrk2, Ntrk3 and the α subunit of the GDNF receptor. Additionally, qRT-PCR was performed for Neurog1 and three of its downstream targets up-regulated during SGN differentiation: Brn3a, GATA3 and NeuroD1. All TaqMan Gene Expression Assays were purchased from Applied Biosystems.

$5x10^5$ cells were plated on 0.1% gel-coated 6-well dishes in 80:20 differentiation media containing Dox and NTFs. Three experimental replicates were performed. At 0h, 72h and 5D, cells were washed with 1X HBSS to remove media and RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA integrity and concentrations were obtained using the Agilent Bioanalyzer 2100. Only RNA with an integrity number above 9.0 was used. 1g of RNA from each treatment group was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Each experimental replicate was assessed in triplicate using the ABI PRISM 7900HT Sequence Detection System analyzer for Real Time PCR, which gives a threshold cycle (CT) number. CT was normalized to the GAPDH housekeeper *Murphy and Polak* (2002), and then used to

calculate fold changes in gene expression using the $2^{-\delta\delta}$CT method (**?**). Fold changes for the 72h and 5D treatment groups were taken as any increase in gene expression compared to uninduced controls at 0h and were plotted on a log-scale.

Preparation of mouse ES cells for *in vivo* placement. The K9 cell line was grown to 80% confluency in ES cell maintenance medium as described above, and dissociated using Hanks-based enzyme-free cell dissociation buffer (GIBCO) with 1mM EDTA into a single-cell suspension immediately before implantation. Cells were resuspended in serum-free differentiation media without Dox and diluted to a concentration of $5x10^4$ cells per microliter immediately before implantation. Cell viability was assessed by staining a small sample (<5%) with Trypan Blue prior to and after the surgeries were completed.

2.2.2 Materials and Methods - *in vivo*

Guinea pig deafening. NIH strain guinea pigs (Elm Hill Breeding Lab), weighing 275 to 315 grams, were systemically deafened. Administration of 450-mg/kg kanamycin, s.c. was followed by anesthesia with ketamine/xylazine then 60 mg/kg ethacrynic acid was administered i.v. (jugular vein) 2 hours later. This results in nearly complete loss of inner and outer hair cells and subsequent loss of SGN. Auditory brainstem responses were taken to confirm deafening.

***In vivo* placement of mouse ES cells and neurotrophic factor infusion.** ES cells were slowly aspirated into a 30g needle and 1mL syringe. The needle was introduced into the lumen of a PE10 cannula, the cannula filled and the needle removed. A glass microliter syringe and 30g needle filled with saline was then introduced into the PE cannula. The opposite end of the cannula (a modified 33g needle) was inserted into scala tympani through a cochleostomy in the base of the cochlea and into the osseous spiral lamina at a ventral angle with a small puncture of the modiolar wall. Following a slow bolus injection of 250,000 cells in 5L, the cannula was left

in place for 10 minutes. It was then replaced by a cannula into scala tympani filled with 25 L of Doxycycline (Bedford Laboratories, Bedford, OH) at a concentration of 10 g/mL, sufficient for two days of Dox infusion. The cannula was attached to an osmotic pump (Alzet, model 2002, 14 day reservoir, flow rate 0.5 l/hr) containing BDNF 100 g/mL and GDNF 10 g/mL for 14 days of delivery and the pump was then implanted subcutaneously on the back (*Prieskorn and Miller*, 2000). The pump was changed at 3 days, to allow a total of 27 days of NTF infusion (2 days Dox, 25 days BDNF/GDNF) into the scala tympani. As controls for the influence of forced expression of Neurog1, three animals (GP 6, 7 and 8) received stem cells, but were only infused with BDNF/GDNF without the prior 2 days of Dox to induce Neurog1.

Fixation and sectioning. Four weeks following intrascalar placement of ES cells (8-9 weeks following deafening), animals were heavily anesthetized with 0.5 mL of sodium pentobarbital i.p. (FatalPlus, Vortech, Dearborn, MI) followed by transcardiac perfusion with phosphate buffered saline, then 4% paraformaldehyde in phosphate buffer. The cochleae were then carefully removed, membranes of the round and oval windows were opened with a fine tipped forceps, an opening made in the otic capsule at the apex and the cochlea placed into the same fixative for 4 hours. Cochleae were then decalcified in 5% EDTA for 5-10 days at 4o C. Cochleae were cryoprotected in increasing concentrations of sucrose in PBS (5%-20%) over 2 days followed by PBS/20% sucrose/OCT mixture for 15 minutes under vacuum pressure. Finally, the cochleae were flash frozen in 20% sucrose/OCT in isopentane cooled in liquid nitrogen. Six micron cryostat sections were cut in a paramodiolar plane.

Immunocytochemistry and *In Situ* Hybridization. Mid-modiolar sections were identified based on gross visual assessment, generally twenty-five to thirty such sections were obtained from each cochlea. Three sections were assessed for eGFP staining only. Three sections (sections 11, 16 and 21) were used for co-immunolabeling with mouse monoclonal antibody to TUJ1 and polyclonal antibodies to vesicular glu-

tamate transporter 1 and vesicular glutamate transporter 2 (both at 1:500). The three adjacent sections were immunostained with polyclonal rabbit antibody to GFAP. Fluorescent in situ hybridization (FISH) localization of mouse genomic DNA and immunostaining for TUJ1 was carried out on sequential sections. Semi-adjacent sections were also immunostained with polycolonal rabbit antibody to GATA3 or to NeuroD1.

For immunohistochemistry, sections were pre-treated with Universal Blocking Reagent (BioGenex, San Ramon, CA) for 10 minutes at room temperature and then processed for immunocytochemistry as described above. Sections were coverslipped using FluoroMount G (SouthernBiotech, Birmingham, AL) mounting media. FISH was performed using mouse COT-1 DNA (Invitrogen Corporation, Carlsbad, CA) to identify mouse genomic DNA. The probe was labeled using digoxigenin-16-dUTP and a nick translation kit (both from Roche Applied Science, Indianapolis, IN) following the manufacturer's instructions. To visualize the hybridization signal, slides were incubated with rhodamine labeled anti-digoxigenin (1:200, Roche Applied Science, Indianapolis, IN) for 1 hour at room temperature in the dark. Sections were co-labeled using antibody to TUJ1 (as above) visualized with an anti-mouse antibody conjugated to Alexa 594.

Quantitative Assessments of Stem Cells. Three sections (sections 11, 16 and 21 from each of the five cochleae) were used for quantitative assessment of co-labeling with eGFP, TUJ1 and VGLUT1/2 and adjacent sections (12, 17, 22) for GFAP immunolabeling. Digital images were acquired on an Olympus FV-500 or Zeiss LSM510 confocal microscope and imported into Metamorph Image Analysis (Universal Imaging) for quantitative assessment. All cells in the profile through the scala tympani in the basal turn that were labeled at least 3X over background were counted as positive. The number of cells immunolabeled for TUJ1 (including those co-labeling with eGFP), those labeled with eGFP only plus the number of GFAP immunolabeled cells from the adjacent section was determined for the basal most profile through

scala tympani. The percentage of TUJ1 cells with a glutamatergic phenotype was estimated by dividing the number of cells co-immunolabeling with VGLUT1/2 and TUJ1 by the total number of TUJ1 immunolabeled cells. The number of cells that co-expressed eGFP with TUJ1, VGLUT1/2, and GFAP was also determined.

Quantitative Assessments of Spiral Ganglion Neurons. The density of remaining spiral ganglion neurons was assessed in the three TUJ1 immunostained sections from guinea pigs 1-5 as well as from three deafened guinea pigs without placement of stem cells, assessed four weeks following deafening. The number of TUJ1 immunostained neurons with a profile through the nucleus was determined in the two basal most profiles through Rosenthal's canal and divided by the area of Rosenthals canal to give a measure of SGN density.

2.3 Results

2.3.1 Results - *in vitro* differentiation

24h *in vitro* differentiation. After 24h in vitro K9 ES cells growing in neuronal differentiation medium displayed a typical ES cell morphology except that many displayed small neurites (Figure 2.3) in both the Dox only and in the Dox plus NTF treatment groups. The concentration of Dox used in these studies was able to induce a majority of cells to express the tet-inducible Neurog1 transgene based on immunostaining (Supplemental Materials). The neuronal differentiation observed is consistent with previous reports on the effects of forced Neurog1 expression (*Sun et al.*, 2001; *Velkey*, 2005). At this time, 54 ± 8% of ES cells exposed to Dox alone expressed TUJ1, compared to 56 ± 5% of ES cells treated with Dox and NTFs (Figure 2.3). There was nearly complete overlap of TUJ1 and vesicular glutamate transporters VGLUT1 and VGLUT2 immunostaining. 50 ± 7% of TUJ1 positive ES cells (with or without NTFs) expressed VGLUT1 and VGLUT2. The ES cells

that did not receive Dox did not express TUJ1, VGLUT1 or VGLUT2, nor did they show neurite outgrowth even when treated for 24h with BDNF and GDNF. No GFAP immunostaining reactivity was observed in any of the four treatment groups at this time. See Figure 2.5 for summary.

72h *in vitro* differentiation. fter 72h *in vitro*, there was robust neuronal differentiation in Neurog1-induced ES cells, regardless of NTF supplementation. There was a significant increase in the percentage of induced cells expressing neuronal markers when exposed to BDNF and GDNF (69 ± 7%) compared to unexposed Neurog1-expressing cells (54 ± 10%) ($p < 0.0073$) (Figure 2). Substantial neuritic networks formed in the Dox treated groups, but at this stage there was no apparent difference in neurite density between Dox only and Dox plus NTFs. At 72h the percentage of TUJ1 positive ES cells that co-labeled with VGLUT1/2 had increased in both Dox only (65 ± 5%) and Dox plus NTF (69 ± 7%) groups ($p < 0.2493$) (Figure 2). A very small number of cells expressed GFAP at this point (<5%). ES cells in differentiation media without Dox continued to lack evidence of neuronal or glial cell differentiation, and remained morphologically ES-cell like in shape and size.

Day 5 *in vitro* differentiation. After 5D *in vitro*, the number of GFAP positive cells increased dramatically, with 30% of cells in both Neurog1-induced groups differentiating into a glial phenotype. At day 5 in culture, 56 3% of cells in the Dox plus NTF group formed neurons based on TUJ1 staining, compared with 45 ± 6% in the Dox-only group. The difference was significant after adjusting for GFAP positive cells that co-labelled for GFAP ($p < 0.001$) (Figure 2.4 and Figure 2.5). 75 ± 11% of the ES cell-derived neurons were glutamatergic (based on co-labeling for VGLUT1/2) in the Dox-treated group receiving NTFs, while 67 ± 11% expressed VGLUT1/2 in the Dox only group ($p < 0.1649$) (Figure 2.3 and Figure 2.5. Unlike 72h cultures, there was also an apparent increase in the density of neuritic networks in the Dox plus NTF condition compared with Dox only (unquantified). eGFP downregulation

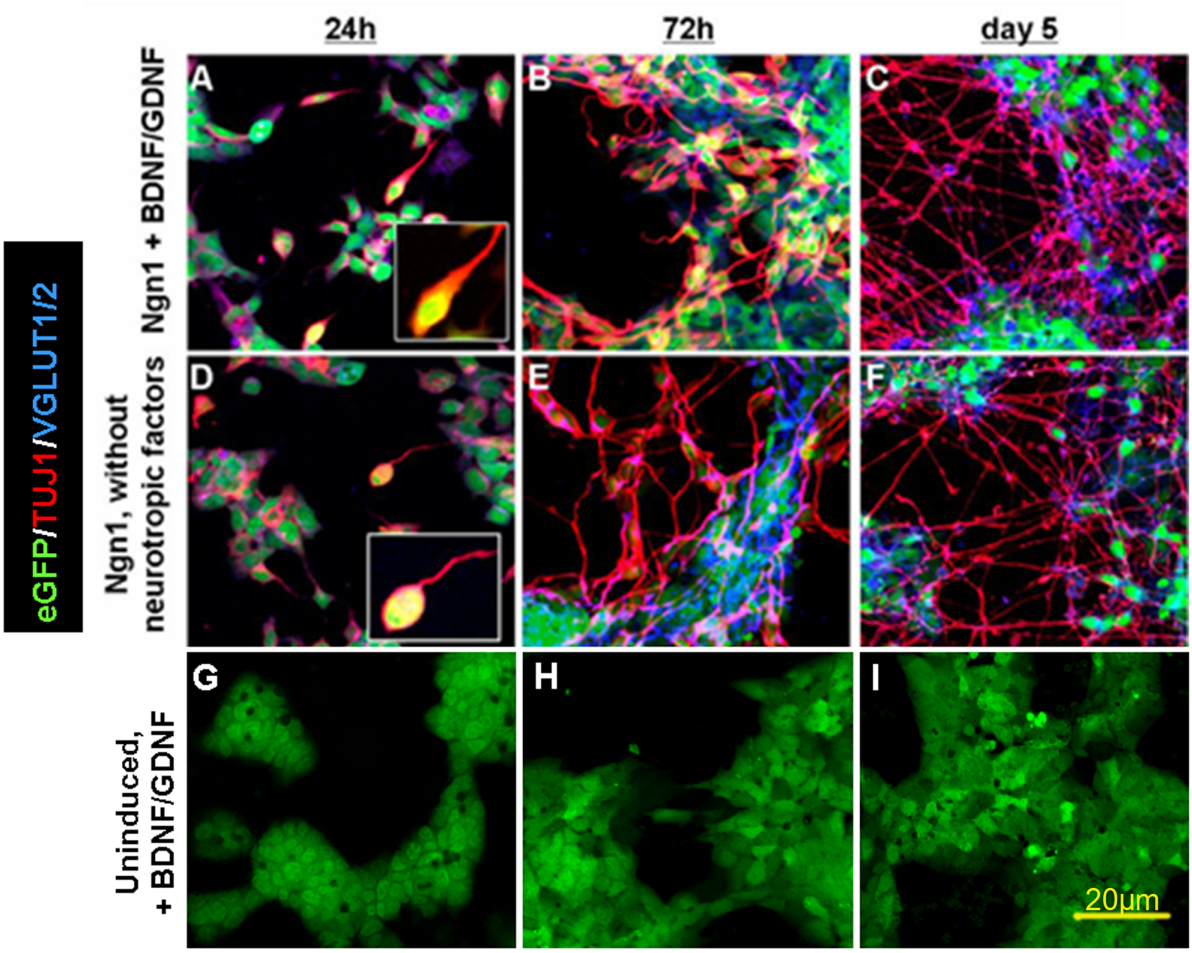

Figure 2.3: Differentiation of ES cells at 24h, 72h, and 5D *in vitro*.

increased between 72h and 5D, while un-induced cells continued to fluoresce and did not express TUJ1, VGLUT1/2, or GFAP, regardless of treatment with NTFs.

Expression of sensory-related genes. Expression of Neurog1 was upregulated after Dox treatment, remaining elevated after 72h but decreasing at 5D. Large upregulation of Ntrk2 and Ntrk3 (formerly TrkB and TrkC) as well as the alpha 1 subunit of the GDNF receptor was observed at both 72h and 5D following treatment with Dox and NTFs (Figure 5). Ntrk1 (formerly TrkA) showed only a small upregulation at 72h that was no longer observed at 5D. A large up-regulation of Brn3a, GATA3 and NeuroD1, which are all downstream targets of Neurog1 during normal SGN de-

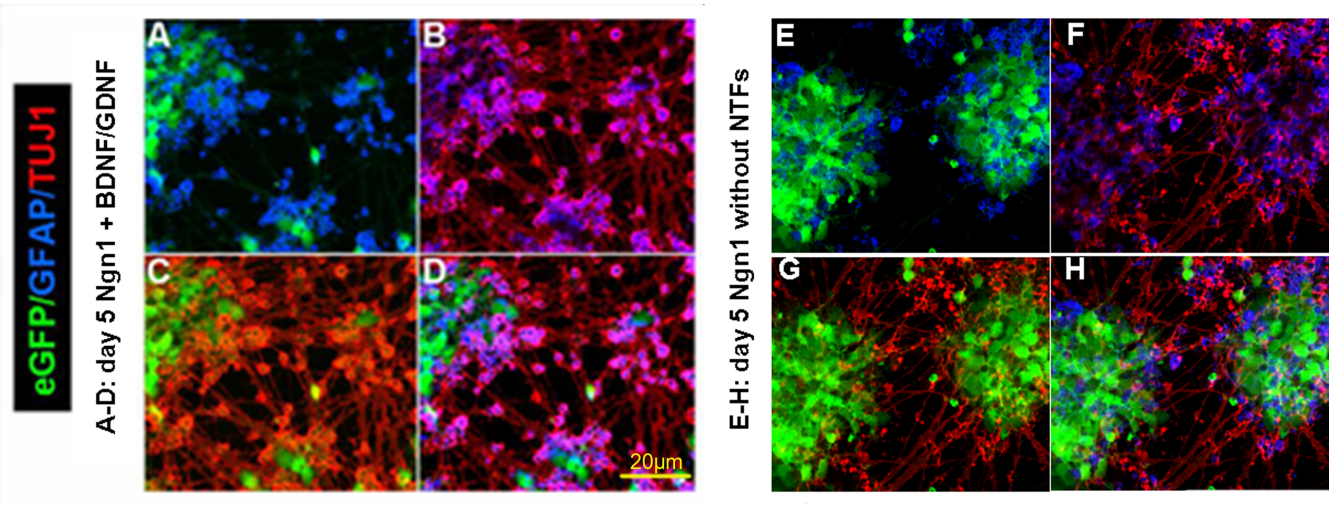

Figure 2.4: GFAP positive cells appear by 5 days *in vitro*.

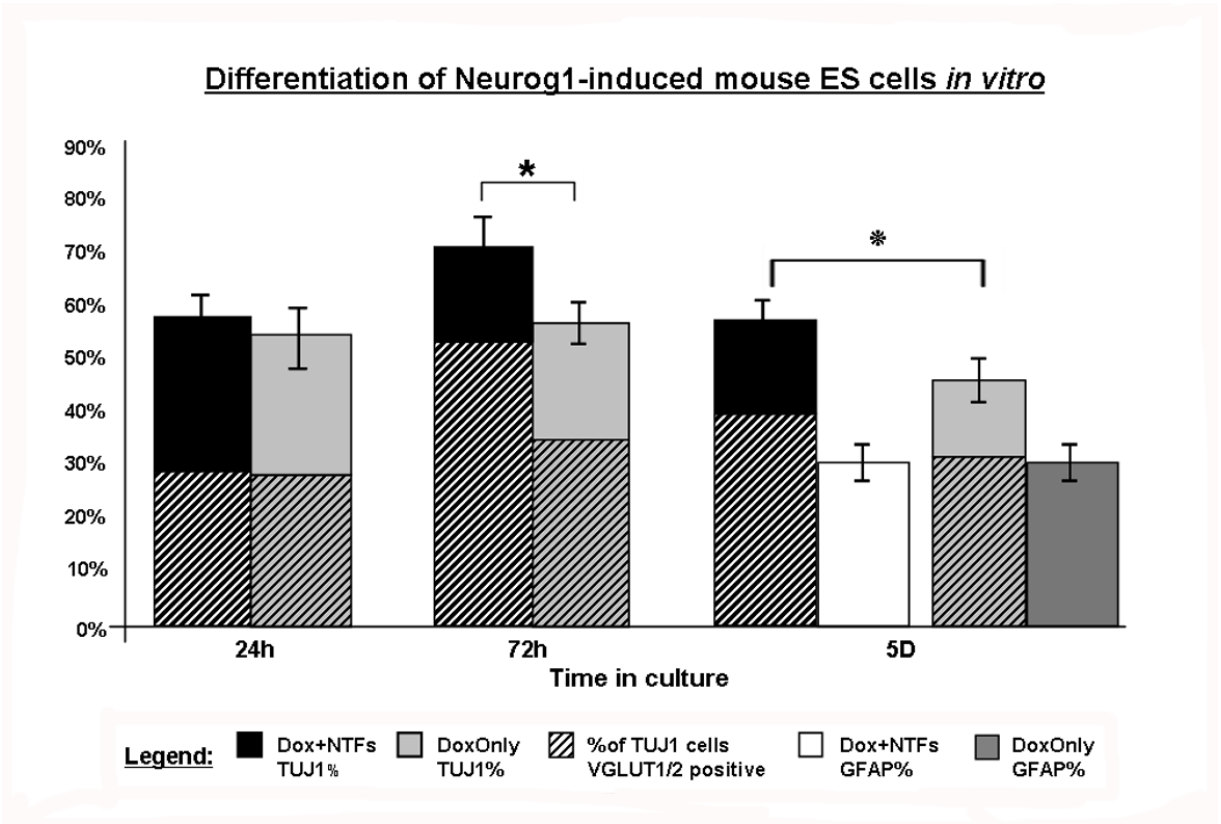

Figure 2.5: Glutamatergic neuronal and glial differentiation *in vitro*.

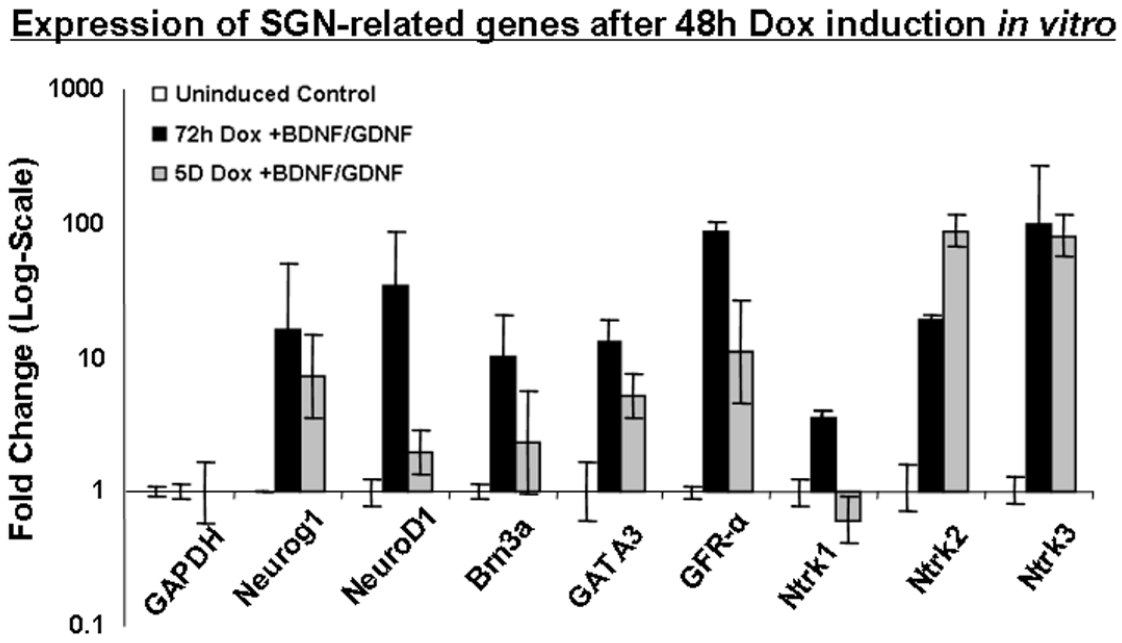

Figure 2.6: Gene expression at 72h and 5D *in vitro*.

velopment (*Huang et al.*, 2001; *Karis et al.*, 2001), occurred at 72h. Expression of Brn3a and NeuroD1 had decreased between 72h and 5D, suggesting that cells express these transcription factors only transiently, which is consistent with expression profiles during normal SGN development (Figure 2.6).

2.3.2 Results - *in vivo* differentiation

The *in vivo* studies employed the treatment combination determined *in vitro* to give the highest percentage of neuronal phenotype in vivo: 48h of Dox exposure plus BDNF and GDNF supplementation. Since NTFs also act as survival factors for SGN (*Miller et al.*, 1997), BDNF and GDNF treatment were expected to further enhance survival of implanted ES cells reaching a SGN-like phenotype (*Ulfendahl et al.*, 2007). This was supported by our recent study showing intrascalar GDNF promoted survival of B5 ES cells placed into scala tympani (*Altschuler et al.*, 2008). Therefore BDNF and GDNF supplementation continued for the entire 4 week survival period.

ES cells four weeks after placement. Numerous ES cells were present in scala tympani and modiolus of the guinea pig cochlea four weeks following implantation. A mid-modiolar section typically contained more than 500 ES cells, often near 1000, while a typical profile through the scala tympani at the basal turn of the cochlear spiral contained 200-400 ES cells (TABLE 1), with the greatest number present in the two basal-most profiles through scala tympani, although ES cells were also observed in more apical profiles through the scala tympani and vestibuli (Figure 2.7). Cells were also present among remaining SGN with peripheral processes in Rosenthal's canal and their central processes in the modiolus (Figure 2.8).

Additional ES cells were present in small numbers around the region of the scar where hair cells formerly resided (Figure 2.8D), as well as in the spiral ligament of the lateral wall. ES cells were identified as eGFP positive and were typically small (10-12 microns), round and most commonly present throughout scala tympani, often in small clusters (Figure 2.7). Although many cells in the scala tympani were positive for TUJ1, only a few co-labelled with eGFP. Very few GFAP positive cells were identified in most animals. Cells co-expressing TUJ1 and eGFP were usually larger than the eGFP-only cells, often with a stellate or fusiform shape (Figure 2.8). Undifferentiated ES cellss, identified as small, round, positive for eGFP only, were commonly found among SGN and among peripheral processes sitting in Rosenthal's canal, and infrequently found in the modiolus. A few small round eGFP positive cells were occasionally found in the scar region, where supporting cells had replaced inner and outer hair cells in the deafened organ of Corti, and others had invaded the spiral ligament/lateral wall as well as scala media (Figure 2.8).

***In situ* hybridization of cochlear sections.** While immunostaining for TUJ1 identified hundreds of immunopositive cells in profiles through scala tympani in mid-modiolar sections most of these did not express eGFP (Table 1, Figures 6 and 7). This is consistent with previous studies when stem cells placed into the cochlea downregu-

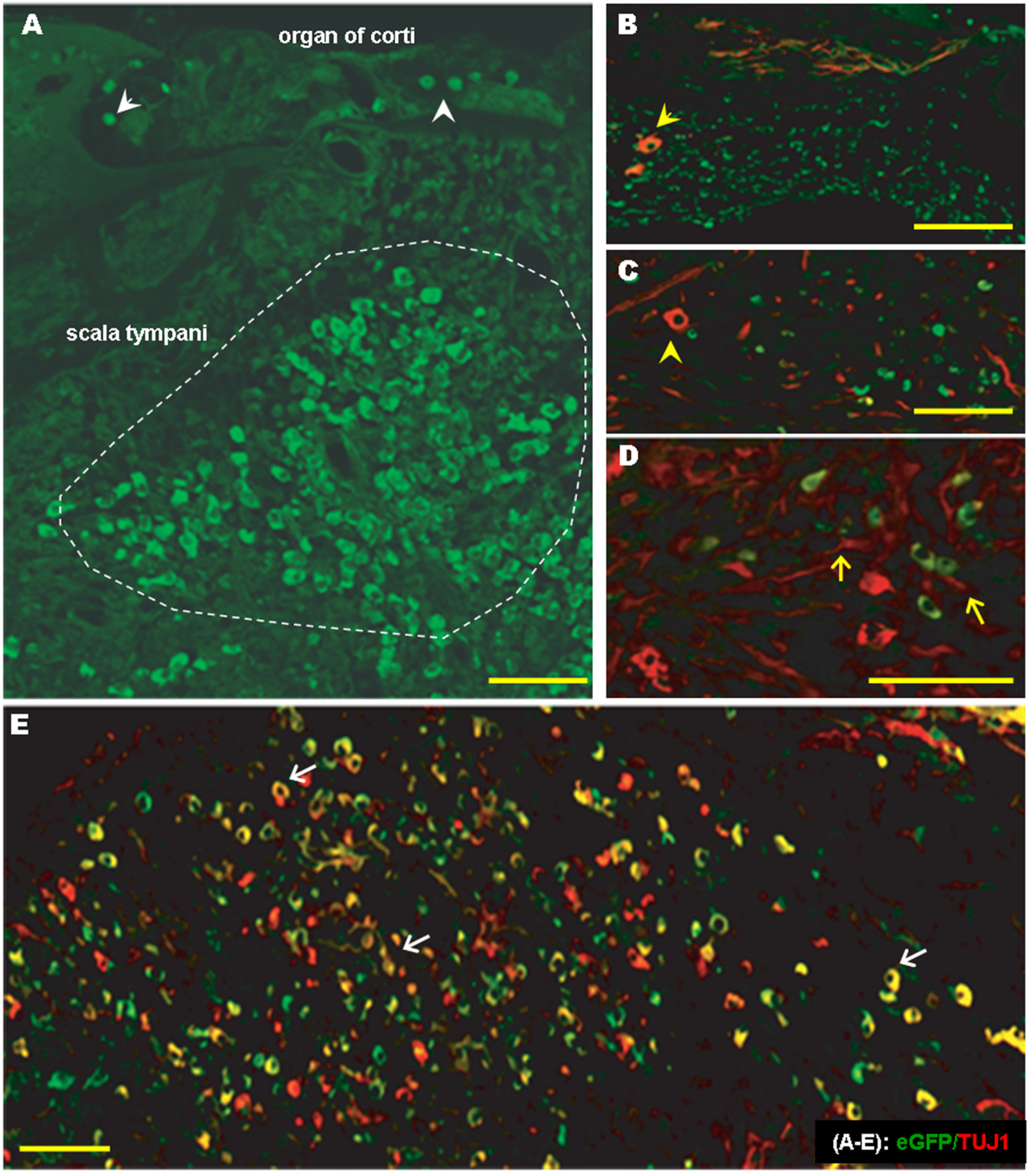

Figure 2.7: eGFP and TUJ1 expression in a cryostat section through the basal-most profile of scala tympani (ST).

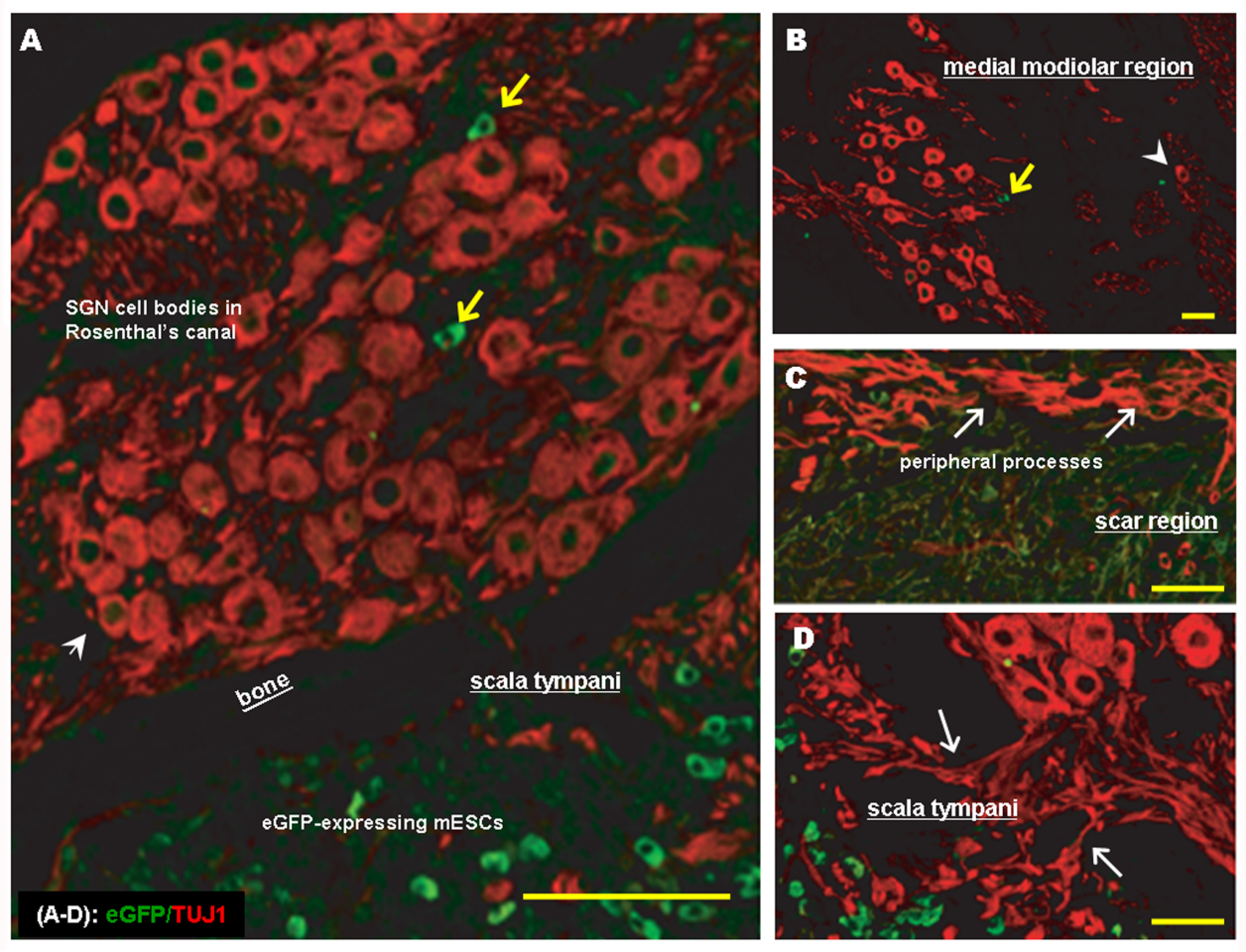

Figure 2.8: ES cells in the region of spiral ganglion.

lated both β-galactosidase and GFP several weeks following placement *Parker et al.* (2007). This also raises the question of whether eGFP was downregulated with differentiation or the less likely alternative that endogenous neurons or neuronal precursors of guinea pig origin migrated into scala tympani. One approach used XY fluorescent in situ hybridization (FISH) to distinguish stem cells in the cochlea (*Parker et al.*, 2007). We employed a comparable approach, by combining immunocytochemistry for TUJ1 and FISH labeling for mouse genomic DNA. The FISH punctuate nuclear labeling of over 90% of TUJ1 expressing cells in scala tympani (Figure 2.9), indicated that most if not all TUJ1 immunolabeled cells in scala tympani originated from the ES cells. FISH labeling of TUJ1 positive cells was most common in scala tympani and scala vestibuli and less common in Rosenthal's canal or other compartments of the cochlea.

Assessment of Phenotype *in vivo*.

GFAP immunostaining (astrocyte differentiation): In 4 of the 5 Neurog1 induced animals (GP2-5), fewer than 8% of the ES cells in profiles through basal scala tympani were immunopositive for GFAP, an astrocyte-restricted intermediate filament and glial phenotype marker. In one animal (GP 1), however, 39% of the cells in profiles through basal scala tympani were GFAP immunolabeled (Figure 2.10 and Figure 2.11). Most of the GFAP positive cells were not-co-labeled with eGFP, suggesting this expression was downregulated upon differentiation into a glial phenotype, as was seen in vitro. The number GFAP immunostained stem cells in the 3 animals without Dox (GP 6-8) was also below 8%, very comparable to GP2-5 with Neurog1 induction.

TUJ1 Immunostaining (neuronal differentiation). Numerous TUJ1 positive cells were seen in profiles through the scala tympani and scala vestibuli (Figures 6, 7). Cells that were TUJ1 positive and eGFP negative were more likely to have a neuronal appearance with a large spherical or fusiform shape and processes (Fig-

ure 2.7) than those that were co-labeled with eGFP, supporting the conclusion that the UbC promoter was methylated with differentiation. Cells expressing TUJ1 were considered to be of K9 ES cell origin when located in scala tympani or scala vestibuli (based on the high percentage co-labeled with FISH for mouse genomic DNA). It was more difficult to identify K9-derived TUJ1 positive cells in compartments that contained endogenous TUJ1 immunolabeled neurons, such as the SGN in Rosenthal's canal. Some large spindle-shaped TUJ1 positive cells were present among SGN that co-labeled with eGFP, indicating their ES cell origin and TUJ1 immunolabeled cells with a typical neuronal appearance were often present in positions in the modiolus that would not be typical for SGN or cochlear root neurons, suggesting a K9 ES cell origin (Figure 2.8). Co-labeling for TUJ1 and VGLUT1/2 was present in cells across the range of TUJ1 size and shapes (Figure 2.9).

Sections that were co-labeled for VGLUT1/2 and TUJ1 with eGFP were used for quantitative evaluation. An estimate was made of the number of ES cells in the most basal scala tympani profile reaching a neuronal phenotype based on TUJ1 immunostaining by dividing the number of TUJ1 positive neurons in the profile by the total of TUJ1 and eGFP only positive neurons plus the number of GFAP positive cells (taken from the adjacent section). Results are shown in Figure 9 and Table 1. The percentage of TUJ1 positive cells ranged from a low of 52% (GP 1) to a high of 78% (GP 4), with a mean across the five experimental guinea pigs of 66%. This compares to 56% of stem cells reaching a neuronal phenotype with comparable treatments by 5D in vitro (Figure 4).

The percentage of stem cells reaching a neuronal phenotype based on TUJ1 immunostaining was lower in the three animals (GP 6-8) that did not receive 2 days of Dox to force Neurog1 induction. On average, 27% of cells were positive for TUJ1, which is comparable to what we observed in a recent study of B5 stem cells lacking the Neurog1 transgene. These B5 cells were placed into scala tympani and received

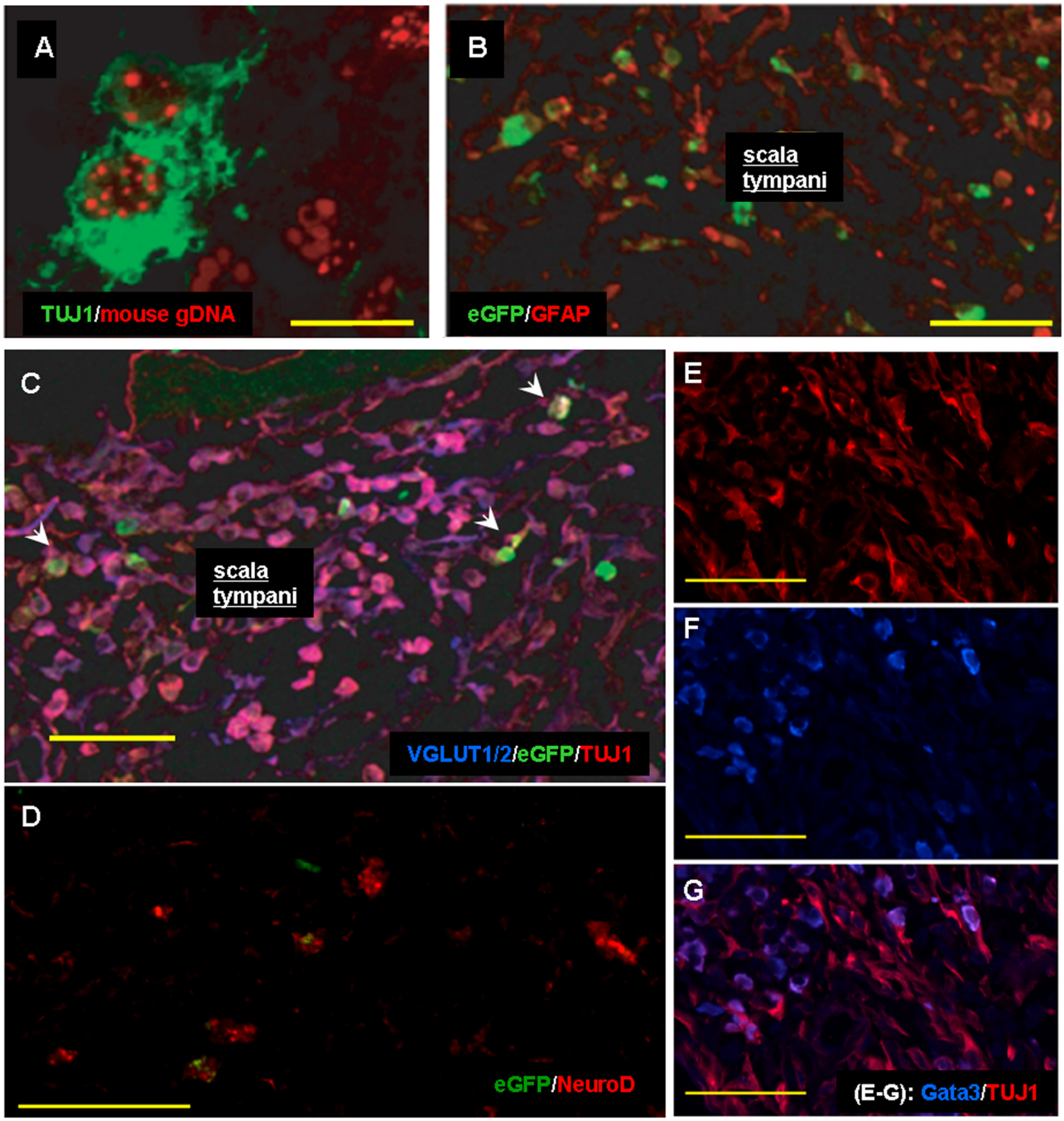

Figure 2.9: Phenotype differentiation of ES cells *in vivo*.

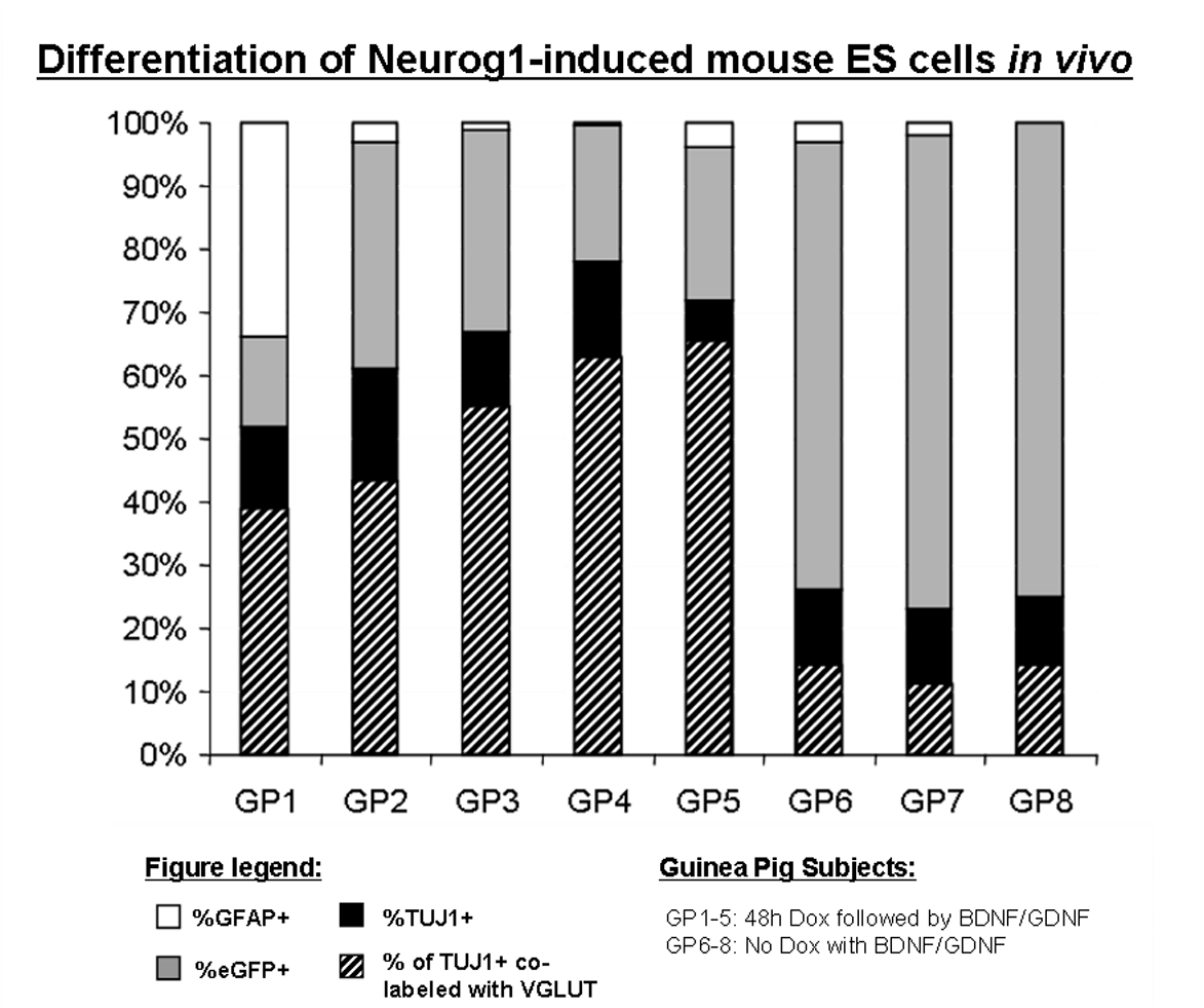

Figure 2.10: Differentiation of ES cells four weeks after implantation into deafened guinea pig cochlea.

instrascalar GDNF and the percentage reaching a neuronal phenotype was well below what was observed in GP 1-5 with forced Neurog1 expression (*Altschuler et al.*, 2008).

VGLUT1/2 Immunostaining (glutamatergic differentiation). Co-labeling for TUJ1 and VGLUT1 and VGLUT2 (Figure 2.9) was used to determine the percentage of ES cells with a glutamatergic phenotype in the basal-most profile of scala tympani. The percentage of ES cells in profiles through the basal scala tympani that reached a neuronal phenotype (based on TUJ1 immunolabeling) and were glutamatergic ranged from 72% (GP 2) to 89% (GP 5), with a mean across the five guinea pigs

Subject	TUJ1+	eGFP+ only	GFAP+	total	mean %TUJ1+	mean %eGFP+	mean %GFAP+
GP1	144	70	120	334	52%	14%	34%
	253	41	101	395			
	201	49	162	412			
GP2	234	95	2	331	61%	36%	3%
	146	115	10	271			
	168	109	10	287			
GP3	183	124	2	309	67%	32%	1%
	259	71	9	339			
	193	106	1	300			
GP4	324	108	2	434	78%	21.75%	0.25%
	242	64	1	308			
	320	78	0	398			
GP5	164	46	3	213	72%	24%	4%
	100	42	5	147			
	129	42	13	184			
Mean across Dox-treated subjects:					66%	26%	8%
GP6	96	232	12	340	26%	71%	3%
	74	201	10	285			
	83	258	7	348			
GP7	52	228	5	285	23%	75%	2%
	83	210	8	301			
	71	236	4	311			
GP8	92	233	1	326	25%	75%	0%
	64	241	1	305			
	81	252	0	333			
Mean across No Dox subjects:					24.6%	74%	2%

Figure 2.11: Glutamatergic, neuronal differentiation of ES cells four weeks after implantation into deafened guinea pig cochlea.

of 79%. This compares very closely to the 75% of ES cell-derived neurons considered glutamatergic based on VGLUT1/2 co-labeling following comparable treatments *in vitro*.

Immunostaining for downstream targets of Neurog1. Large numbers of GATA3 immunopositive cells were found in profiles through scala tympani. Many but not all of the TUJ1 positive stem cells in basal scala tympani co-immunolabeled for GATA3 (Figure 2.9). There were also a few GATA3 immunopositive cells that did not co-label with TUJ1, while co-labeling of GATA3 with eGFP was rare. Many fewer stem cells were immunopositive for NeuroD1 and these were more likely to co-label

with eGFP than TUJ1.

Spiral Ganglion Neuron Assessment. The remaining SGN at 4 weeks following deafening had a mean density of 6.7 SGN per $10,000 mm^2 \pm 1.0$ (standard deviation) compared to the normal density of 9.5 per 10,000 mm2 $\pm$ 0.6 from our normative data base (e.g. Miller et al, 2007). The density was further reduced to a mean of 4.8 per $10,000 mm^2 \pm 1.8$ in GPs 1-5, suggesting a continued loss of SGN over the longer period following deafening, however this decrease in density from 4 weeks was not significant. There was also not a significant difference between the right (no ES cells implanted, only contralateral effect of NTFs) and left (ES cells implanted, dox plus NTFs) cochleae from GP 1-5, where the mean SGN density was only slightly lower at 4.0 per $10,000 mm^2 \pm 1.2$ in the right, non-treated, ears.

2.4 Discussion

The proneural bHLH transcription factor Neurog1 has previously been shown to strongly promote neuronal differentiation in precursors of various germ layers (*Sun et al.*, 2001; *Kim et al.*, 2004, 2008). Neurog1 is also critical for SGN differentiation during development, but the only experimental evidence linking it to glutamatergic specification has come from knockout models, where Neurog1 was found to play a role in glutamatergic vs. gabaergic fate choice (*Schuurmans et al.*, 2004). In the current investigation, differentiation was controlled in vivo and in vitro using a stepwise protocol in which the Neurog1 transgene was induced, followed by application of neurotrophic factors known to act on receptors downstream of Neurog1. This enabled implantation of undifferentiated ES cells with differentiation induced subsequent to placement with the hope that implanting undifferentiated cells would bypass the immune response.

We show that physiologically-relevant 48h expression of Neurog1 efficiently promotes ES cell differentiation into a glutamatergic, neuronal phenotype (56% in vitro

and 66% in vivo). This is a basic requirement for auditory nerve replacement, since SGN primarily rely on glutamate for transmission of sensory information to the CNS (*Eybalin*, 1993).

Inclusion of NTFs may have mostly provided additional support for SGN-like ES cell survival (*Mou et al.*, 1997; *Shepherd et al.*, 2008), although others have shown that they can also function to enhance differentiation. For review see *Huang and Reichardt* (2001). It is known that cochlear electrical stimulation can also enhance SGN survival in synergy with NTF application. For review see *Green et al.* (2008), and stimulation may promote neuronal differentiation in ES cells in vitro (*Yamada et al.*, 2007). We found that *in vitro* application of BDNF and GDNF resulted in a slight increase in neuronal differentiation after Neurog1 expression (56% compared to 45% without NTFs at 5D) and caused a notable increase in neurite outgrowth, though the latter was not quantified.

The mechanism of BDNF and GDNF action is likely by upregulating NTF receptors by SGN-specific transcription factors Brn3a, NeuroD1, and Gata3. This process has been documented in development (*Huang et al.*, 2001; *Calella et al.*, 2007), and we observed that with differentiation, ES cells expressed these markers both in vitro and in vivo. The expression of the GDNF receptor, along with BDNF receptor Ntrk2 and NT-3 receptor Ntrk3, remained elevated by 5D, while Ntrk1 was lost at 5D after a slight upregulation at 72h. This provides further evidence that Neurog1-induced ES cells are SGN-like, as a majority of vertebrate VIIIth nerve ganglia express Ntrk2 and Ntrk3 and to a lesser extent Ntrk1 depending on the species (*Don et al.*, 1997; *von Bartheld and Fritzsch*, 2006; *Catania et al.*, 2007).

Previous studies aimed at inducing differentiation of uncommitted precursors in the cochlea found that a majority of cells became glia rather than the target neuronal phenotype (*Hu et al.*, 2004; *Tamura et al.*, 2004; *Hu et al.*, 2005a,b; *Nicholl et al.*, 2005; *Coleman et al.*, 2006; *Corrales et al.*, 2006; *Coleman et al.*, 2007; *Parker et al.*,

2007; *Ulfendahl et al.*, 2007). In our studies, we found only one of the five experimental animals had a significant percentage of GFAP positive cells, with 30% reaching a glial phenotype, while the other four animals had fewer than 1%. In the Dox-treated groups at 72h in vitro, various glial-like derivatives began populating the dish at percentages similar to the one in vivo outlier (approximately 30% of cells under both conditions were GFAP positive, indicating BDNF and GDNF did not influence gliogenesis). Glial cells of the inner ear, however, derive from neural crest cells (*Jessen and Mirsky*, 2005), a different subpopulation of neural precursors than those that form the spiral ganglion.

The introduction of factors to prolong ES cell survival at this time point likely influenced the presence of additional neural phenotypes in the dish. The knockout serum replacement added to the media contains bone morphogenetic proteins, which may have influenced astrocyte differentiation in the present study (*Mabie et al.*, 1997; *Gossrau et al.*, 2007; *Imura et al.*, 2008). The emergence of glia at this time is consistent with the role of proneural bHLH factors in promoting neurogenesis while inhibiting gliogenesis, the latter proceeding once bHLH inhibition has been lifted *Ma et al.* (1998); *Nieto et al.* (2001); *Sun et al.* (2001). It is clear from our studies that a subpopulation of ES cells does not terminally differentiate by 72h and can be influenced by components in the medium to differentiate into an astrocyte phenotype.

Random integration of the eGFP transgene resulted in downregulation with differentiation, likely via normal epigenetic mechanisms that silence foreign DNA. This problem might be alleviated by using stronger promoters (*Chung et al.*, 2002; *Hong et al.*, 2007), but whether these will remain constitutively active upon differentiation with Neurog1 is not likely. More promising approaches would be to use an insulator, a special class of DNA placed upstream of a promoter to prevent heterochromatin-mediated downregulation of the transgene (*Geyer and Clark*, 2002), or targeted expression in a constitutively active site in the mouse ES cell genome. Downregulation

of eGFP complicated identification of implanted ES cells following differentiation, particularly when the cells become integrated into native tissue containing neurons and glia that are positive for the same neural markers. ES cells could be identified using a Y chromosome marker (*Parker et al.*, 2007), since this cell line was derived from a male mouse blastocyst, but this would only enable identification of nuclei, not neurites emanating from cells of ES origin. One potential approach to visualizing fine cell processes would be to employ a construct in which the microtubule-associated protein Tau is fused with a fluorescent reporter, thereby remaining anchored to the cytoskeleton of ES cells as they differentiate (*Pratt et al.*, 2000).

Degeneration of the auditory nerve occurs subsequent to the loss of inner hair cells (*Webster and Webster*, 1981), and neither cell type is normally replaced by any endogenous mechanisms. Some restoration of hearing can be obtained from a cochlear implant (also known as cochlear prosthesis), which bypasses IHC function through coding acoustic stimuli into an electrical impulses delivered directly to remaining auditory nerve. Cochlear implant performance, however, depends on the amount of auditory nerve remaining (*Pfingst*, 1990; *Kileny et al.*, 1991; *Blamey et al.*, 1996; *Skinner et al.*, 1997; *Incesulu and Nadol*, 1998; *Rubinstein et al.*, 1999; *Miller et al.*, 2000). At the same time, a more substantial loss of SGN experimentally may enhance migration and survival of implanted ES cells, possibly due to the elevated expression of trophic factors in the damaged cochlea (*Corrales et al.*, 2006; *Sekiya et al.*, 2007; *Ulfendahl et al.*, 2007). Replacement of auditory nerve using an approach similar to the one we have outlined could therefore be of great value to those with little or no nerve remaining. Our results demonstrate that recapitulating developmental cues can promote differentiation of ES cells toward a phenotype expressing markers of SGN development. We show this differentiation occurs at high percentages both in vitro and without inducing an immune response in vivo. Future in vivo and in vitro studies are necessary to determine if these ES cell derived neurons are capable of functional

integration to replace auditory nerve and improve the efficacy of cochlear prostheses.

CHAPTER III

A Model for Neuronal Maturation: Development of Electrochemical Properties in Neurog1-induced Mouse Embryonic Stem Cells

3.1 Introduction

Nascent neurons undergo significant modifications in membrane properties as they mature into actively-communicating, functional members within a defined network. Many of these modifications - enhanced expression of specific ion channels to increase or decrease thresholds, or alterations in cell contacts and process outgrowth to provide more refined inputs - are thought to occur through activity-dependent mechanisms and subsequent to exposure to tissue-derived factors, after the neuronal fate has been established (*Spitzer*, 2006). These inductive signals, often released by non-neural tissues, selectively activate pathways for neuronal subtype specification; varied exposure creates functional diversity within sensory ganglia to enable the encoding of a broad spectrum of stimuli information (*Adamson et al.*, 2002a).

Hierarchies of genetic networks regulate the transformation of ES cells into immature neurons, and these into specialized neuronal subtypes (*Ibanez and Ernfors*, 2007; *Marmigere and Ernfors*, 2007). Our ability to drive ES cell differentiation toward these subtypes for cell replacement therapies is only limited by our knowledge

of the interaction of genes within these networks. Neurogenin-1 is a key regulator of the neuronal fate choice: expressing a basic helix-loop-helix (bHLH) domain, this transcription factor recognizes a highly conserved consensus sequence present in the promoters of many sensory neuron-specific genes, activating downstream effectors to further-specify the sensory neuron lineage (*Huang et al.*, 2001; *Bertrand et al.*, 2002; *Fritzsch*, 2003; *Seo et al.*, 2007). Once a cell expresses Neurog1, it is committed to the neuronal lineage, co-incidentally upregulating cell-surface ligands that inhibit neuronal differentiation in neighboring cells (*Raft et al.*, 2007). This "lateral inhibition" mechanism has been characterized in great detail for the inner ear sensory structures: Neurog1-negative cells eventually give rise to sensory epithelia "patches", with Neurog1-positive cells eventually innervating these patches.

We previously demonstrated that transient overexpression of Neurog1 in mouse ES cells generated a high percentage of neurons with neurochemical and morphological features similar to spiral ganglion neurons (SGN) of the auditory nerve (*Reyes et al.*, 2008). While these cells hold promise for the treatment of irreversible nerve degeneration, ES cells with such features may not differentiate into functional neurons, often having neuronal characteristics but not behaving like one by generating action potentials (AP) for active cell-cell communication (*Balasubramaniyan et al.*, 2004; *Strubing et al.*, 1995; *Donato et al.*, 2007). Researchers must give attention to the genetic (intrinsic) and epigenetic (extrinsic) factors that promote neuronal maturation beyond acquisition of the neuronal fate, and assess the real promise of such cells by characterizing emergent electrochemical properties that confer function.

In this study we used whole-cell electrophysiology to determine whether induction of Neurog1 was consistent with our goal to create an excitable population of neurons capable of encoding frequency information from a CI electrode to the central nervous system. Our hope is for these cells to improve the efficacy and accessibility of these CI devices, which have proven widely successful in the restoration of hearing if any SGN

have survived after the onset of hearing loss *Wilson and Dorman* (2008). We were also interested in whether Neurog1 induction was sufficient to activate intrinsic pathways toward functionality *in vitro* without the inductive signals that may be present in development. Our results indicate that Neurog1 overexpression is an efficient and expedient method to produce functional neurons with rapid adaptation to stimuli *in vitro*, and the cells we generated provide an interesting model for the study of sensory neuron development.

3.2 The intrinsic electrophysiological properties of neurons derived from mouse embryonic stem cells overexpressing Neurogenin-1

The following subsections are from an article submitted by M. Tong, J. Hernandez, R. A. Altschuler, and R. K. Duncan

My specific contributions to this article aside from intellectual input and commenting on the manuscript were providing differentiated ES cells for electrophysiological assessment and performing immunocytochemistry for neuronal differentiation and select ion channel expression.

3.2.1 Introduction

The engineering of embryonic stem (ES) cells holds great promise for the treatment of neurodegenerative disease and neurological trauma, but many challenges remain. Major advancements will require new strategies for guiding lineage determination, differentiation, and integration into a highly organized, heterogeneous environment. One exciting approach involves the ex vivo induction of ES cells into neural progenitors or mature neuronal subtypes prior to implantation (*Rossi and Cattaneo*, 2002). By first directing differentiation to a neuronal lineage, the risk of ongoing ES cell

proliferation after transplantation is reduced. Pre-differentiation also allows control over the exogenous and endogenous factors influencing the development of mature phenotypic traits. However, the ability to specify distinct axodendritic morphology, discharge patterns, synaptic structure, and neurotransmitter systems remains a distant goal. Therefore, an essential step toward developing viable stem cell therapies is a greater understanding of the proneural signaling cascades that end in a desired structure and function.

Neural commitment can be induced *in vitro* by exposure to media supplements or co-culture with feeder cell layers (for review, see *Robertson et al.* (2008)). Alternatively, a neural fate can be forced by overexpression of proneural genes (*Reyes et al.*, 2008; *Sugimoto et al.*, 2009). In a previous study, forced expression of the proneural gene neurogenin-1 (Neurog1) in mouse ES cells rapidly and efficiently produced β-III tubulin positive neurons with markers for glutamatergic neurochemistry (*Reyes et al.*, 2008). Neurogenin-1 (Neurog1) is a basic helix-loop-helix transcription factor involved in sensory system development (for review, see *Korzh and Strhle* (2002)). Expression of Neurog1 promotes determination of a neuronal lineage and inhibits gliogenesis (*Sun et al.*, 2001). Deletion of this gene leads to the loss of progenitor cells in the trigeminal and otic placodes (*Ma et al.*, 1998) and contributes to the loss of sensory neurons of the dorsal root ganglia (*Ma et al.*, 1999). Interestingly, peripheral auditory nerve is absent in Neurog1 null mutants, but sensory hair cells within the cochlea develop normally (*Ma et al.*, 2000).

Thus, the sensory neurons derived from Neurog1-induced ES cells could potentially be used to replace or regenerate sensory cranial nerves such as the vestibule-cochlear ganglion. However, progression toward function requires events far downstream of Neurog1, since neither this gene nor its immediate effector NeuroD directly induces genes encoding neurotransmitters, neuroreceptors, or ion channels (*Seo et al.*, 2007). It remains unknown whether Neurog1-induction alone is sufficient to drive matu-

ration of ES cells into a functional neuronal phenotype. Therefore, in the present study, we used whole-cell electrophysiology to characterize the discharge pattern and voltage-sensitive conductances in Neurog1-induced ES cells. Electrical properties were monitored in culture for up to 12 days to determine the rapidity of acquiring a stable pattern of excitability and to determine whether Neurog1 induction further specifies a unique, homogeneous subtype. Our results indicated that Neurog1 overexpression efficiently and rapidly produced functional neurons expressing voltage-gated Na^+, Ca^{2+}, and K^+ channels within four days after induction. The majority of cells adopting a neuronal morphology were also capable of firing sodium-based action potentials, making them attractive candidates for therapies requiring regeneration of glutamatergic sensory neurons.

3.2.2 Materials and Methods

Cell culture and in vitro differentiation

Neurons were derived from an ES cell line containing a tetracycline-inducible Neurog1 transgene and an eGFP cassette driven by the constitutive UbC promoter, as described previously (*Reyes et al.*, 2008). Undifferentiated ES cells of this line were maintained in sterile-filtered DMEM (Invitrogen, Carlsbad, CA) with 10% stem cell-compatible fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 5% embryonic stem cell supplement (DMEM with 21.65 mg/ml HEPES buffer, 3.63 mg/ml L-glutamine, and 63.63 ng/ml β-mercaptoethanol) and 0.5 μg/ml leukemia inhibitory factor (recombinant human LIF, Millipore, Bedford, MA). ES cells were plated for in vitro differentiation in serum-free media consisting of 80% DMEM/F12, 20% Neurobasal and 10 mM sodium pyruvate (Invitrogen) supplemented with B27 and N2 (1X; Invitrogen). Cells were plated at a density of 5 x 10^5 cells per well in 6-well dishes filled with 13 mm diameter Thermanox cell culture coverslips (Nunc, Naperville, IL) coated with 0.1% gelatin (Sigma). Doxycycline (Dox) (1 μg/ml; Sigma, St Louis,

MO) was added to the differentiation media for 72h in experimental groups. After 72h, Dox was washed off with 1X Hanks' balanced salt solution (HBSS, Gibco) and exchanged for normal differentiation media without Dox. Control cells were simultaneously grown in differentiation media without the addition of Dox. All cultures were maintained in a humidity controlled incubator at 37 °C in the presence of 5% CO_2.

Immunocytochemistry

Cells were prepared for immunocytochemistry by fixing with 4% paraformaldehyde for 10 minutes at room temperature. After blocking with Universal Blocking Reagent (Bio Genex, San Ramon, USA), the cells were incubated overnight at 4 °C with antibodies to NaV1.6 (1:150; NeuroMab, Davis, CA) and the neuronal marker TUJ1 (class III-tubulin, 1:300; Covance, Madison, WI). Primary antibodies were diluted in 0.1% Triton-X100 in phosphate buffered saline. AlexaFluor secondaries (1:500; Invitrogen) were applied for 2 hours at room temperature to visualize primary labeling. Control preparations were treated in parallel, except for the exclusion of primary antibodies.

Electrophysiology

Electrophysiological studies were performed on cells with multipolar or monopolar morphology (Fig 1A) at 2-12 days *in vitro* (DIV). Whole-cell current- and voltage-clamp recordings were performed at room temperature ($21-23$ °C) using an Axopatch 200B amplifier with a Digidata 1200 or 1322A digitizer (Molecular Devices, Sunnyvale, CA). Data were acquired using pCLAMP 9 software (Molecular Devices). Glass electrodes were pulled from borosilicate capillaries (1B100F-4, World Precision Instruments, Sarasota, FL) to achieve an electrical resistance of 3-6 Mω. Electrodes were coated with R6101 (Dow Corning, Midland, MI) to reduce stray capacitance. Electrodes were filled with an internal solution containing (in mM): 112 KCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 11 EGTA, 10 HEPES, 5 Na_2ATP, and buffered to pH 7.2 with KOH. The external solution contained (in mM): 137 NaCl, 5 KCl, 1.7 $CaCl_2$ 1 $MgCl_2$, 10 HEPES,

16 glucose and buffered to pH 7.2 with NaOH. Voltage-dependent properties of Na^+, Ca^{2+} and K^+ channels were measured under voltage-clamp mode. To record Na^+ or Ca^{2+} currents, KCl in the internal solution was replaced by CsCl (120 mM). In addition, 20 mM tetraethylammonium-Cl (TEA-Cl) was added in both internal and external solutions to completely block K^+ currents. To further isolate Na+ or Ca2+ currents, the external solution contained 0.5 mM NiCl2 and 0.1 mM $CdCl_2$ to block Ca^{2+} channels or 100 nM TTX to block Na^+ channels, respectively. To record K+ current, 100 nM TTX, 0.5 mM $NiCl_2$, and 0.1 mM $CdCl_2$ were added to the external solution. All chemicals used in the electrophysiology studies were purchased from Sigma. In all configurations, data were sampled at 10-20 kHz and low-pass filtered with a 4-pole Bessel filter at a 5 kHz cut-off frequency. Leak currents and capacitative transients were subtracted on-line. A liquid junction potential of -4 mV was corrected in all figures. Uncompensated series resistance was not corrected. Averaged results were obtained by pooling cells collected over consecutive days (e.g. 4.5 DIV from cells recorded at days 4 and 5 after plating). Three different culture durations were tested: 4.5, 8.5, and 11.5 DIV.

Data analysis

Peak and steady-state currents were analyzed in Clampfit, part of the pCLAMP 9 software suite. For Na^+ and Ca^{2+} currents, the voltage-sensitivity of activation was determined from conductance-voltage (G-V) curves. In these instances, conductance was determined from peak currents and estimates of the reversal potential for each current type. For K^+ currents, activation curves were constructed from tail-currents elicited by a final step to -24 mV. Normalized activation curves were fit to a single-order Boltzmann equation, G/Gmax or I/Imax = $1/(1 + \exp((V_{1/2} - V)/k))$, where $V_{1/2}$ is the half-activation voltage, V is the voltage command, and k is the Boltzmann slope. The voltage-sensitivity of inactivation was determined from tail-currents at -24 mV following 4-second activation steps. Normalized curves were fit to a single-order

Boltzmann equation. Averages are presented as means + one standard error of the mean. When comparing changes in various parameters across DIV groups, a one-way analysis of variance (ANOVA) was conducted with a Tukey-Kramer post-hoc test for significant differences between the 4.5 DIV group and each of the older cultures. The criterion for a statistically reliable difference in any comparison was $p < 0.05$.

3.2.3 Results

Excitability of Neurog1 induced cells.

Induction of Neurog1 for 72 hours produced a high percentage of mouse ES cells acquiring a neuron-like morphology (Fig 1A), as described previously (*Reyes et al.*, 2008). Current injection in those cells extending at least one long, thin process elicited fast action potentials as early as 4 DIV, and their excitability could be maintained for at least 12 DIV (Fig 1B). Exposure to TTX eliminated these spikes, indicating that evoked action potentials were due to the activity of voltage-gated sodium channels (Fig 1C). The passive and active electrical properties of induced cells were assessed at three time points during culture (4.5, 8.5, and 11.5 DIV). These results are summarized in Table 1. Cell size, estimated from membrane capacitance (C_m), was relatively constant throughout this time period ($p > 0.05$). On average, tested neurons had resting potentials of -40 mV with no statistically reliable changes over time in culture. At early points in the culture, shortly after Neurog1 induction, 88% of the targeted cells were capable of generating action potentials. By 11.5 DIV, action potentials could be evoked from 100% of the tested cells. At 4.5 and 11.5 DIV, 90% of the neurons fired only one action potential at any current step. There was a small increase in the average maximum number of action potentials at 8.5 DIV with over 70% of the neurons in this group firing multiple action potentials. Although these differences in spike number were statistically reliable ($p < 0.05$), the maximum number of spikes remained, on average, between 1 and 3. Therefore, in all culture groups,

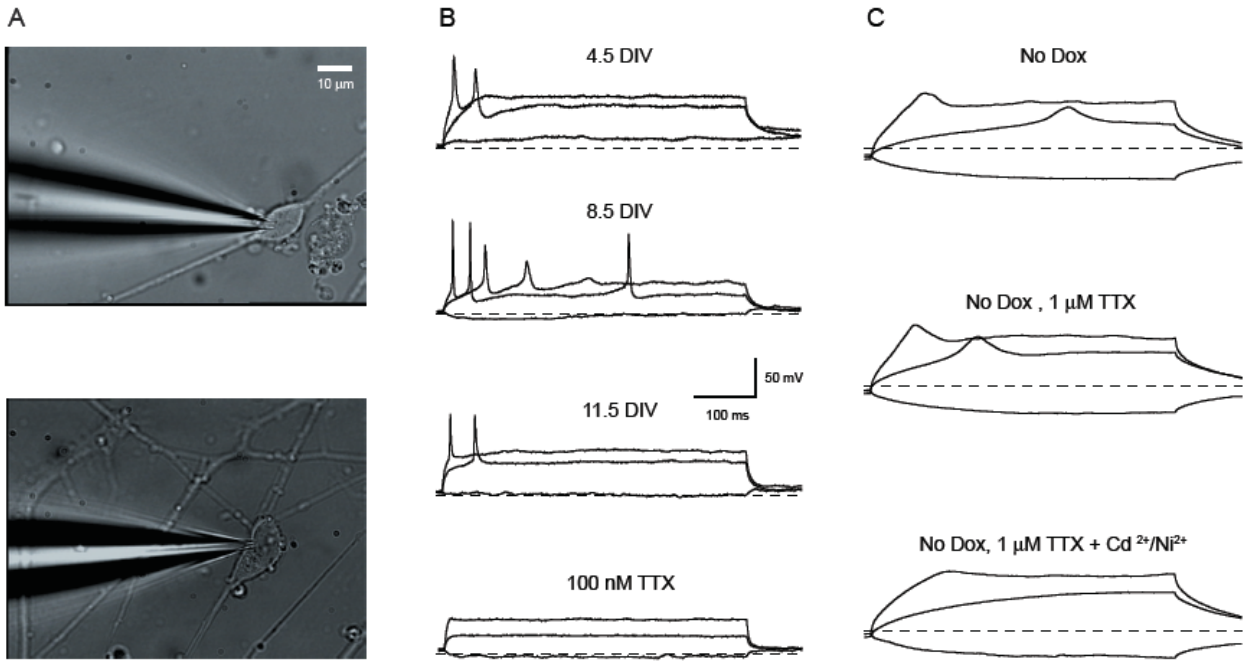

Figure 3.1: Functional maturation of Neurog1-induced mES cells *in vitro*

firing patterns were rapidly adapting (i.e. few spikes, occurring only at the onset of the current-clamp stimulus). In addition to time-dependent changes in maximum spike number, action potential threshold varied with DIV, becoming more positive with increasing time in culture ($p < 0.05$). Although the resting ionic currents appeared to be homogeneous and mature by 4 DIV, the conductances responsible for spike threshold and frequency were modulated over time.

In contrast, uninduced control cells rarely differentiated into neural-like cells with active voltage responses. A previous report noted that this ES cell line did not spontaneously differentiate into TUJ1-positive neurons within 5 DIV (*Reyes et al.*, 2008). Our cells were cultured for longer periods of time, so it was important to determine if uninduced cells adopted neuronal traits after 5 DIV. The number of TUJ1 positive cells was assessed for control and induced cultures at 10 DIV. Similar to the selection rubric for electrophysiological assays, we counted only those cells with clearly defined neurites. Based on these data, only 1-5% of the neurons in induced cultures could be attributed to spontaneous differentiation of ES cells. The majority

of control cells at 11-12 DIV exhibited passive responses to current steps (7 of 12 cells, 58%). Several others responded with small, broad depolarizations superimposed on passive membrane responses (4 of 12 cells, 33%; Fig 1D). Half-widths for the active phase of these depolarizations exceeded 15 ms. External application of TTX (1 μM) had no effect, but Ca^{2+} channel antagonists (0.1 mM Cd^{2+} and 0.5 mM Ni^{2+}) eliminated these depolarizations. Therefore, the active responses in these control cells were carried by Ca^{2+} current rather than Na^{+}. Only one cell in the control group fired brief action potentials with half-widths less than 4 ms (data not shown). The shape, frequency, and latency associated with the response of this cell were consistent with those data from the TTX-sensitive Neurog1-induced cells, indicating that a small percentage of the control cells adopt a functional phenotype by 12 DIV (1 of 12, 8%). Combined with the lower frequency of spontaneous differentiation, these data imply that firing properties recorded from Dox-induced cells could be associated with Neurog1 overexpression with confidence.

Properties of Na^{+} currents

Under voltage-clamp conditions, fast-activating and inactivating inward currents in Neurog1 induced cells were completely blocked by the external application of 100 nM TTX (Fig 2A; 5 of 5 cells at 4 DIV), indicating the dominance of TTX-sensitive sodium channel subtypes in these cells. Some preparations were stained with an antibody to NaV1.6, a sodium channel α subunit common in neurons and responsible for fast-inactivating, TTX-sensitive currents (Fig 2B). The presence of NaV1.6 in TUJ1 positive cells suggests that this channel contributes to the excitability of Neurog1-induced neurons.

Sodium currents were isolated with potassium and calcium channel blockers and elicited from a pre-conditioning step of -124 mV (Fig 2C). Current-voltage (I-V) relationships were obtained from peak Na^{+} currents and averaged for cells at 4.5, 8.5, and 11.5 DIV (Fig. 2D). Sodium currents activated near -50 mV and peaked at

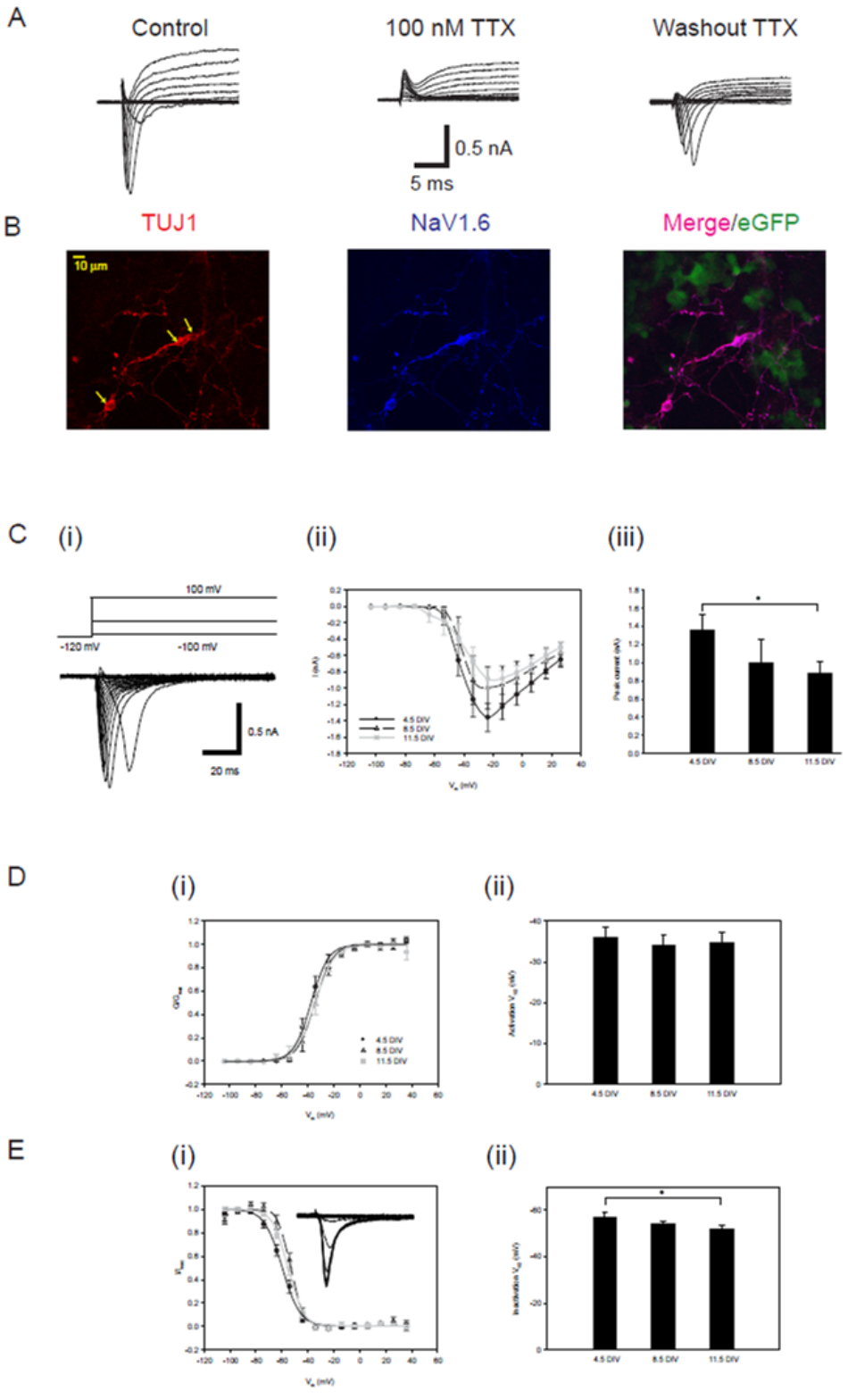

Figure 3.2: Na^+ channels in Neurog1-induced mouse ES cells

about -20 mV, regardless of time after differentiation. Average peak current decreased over time in culture. A paired t-test between peak current at 4.5 and 11.5 DIV was statistically significant ($p < 0.05$), but the one-way ANOVA across all three culture groups did no reach the significance criterion. Therefore, it is difficult to conclude that sodium channel density systematically decreased with increasing time in culture. The voltage dependence of Na^+ channel activation (Fig. 2D) and inactivation (Fig. 2E) were relatively constant over DIV. Differences in half-activation and half-inactivation voltage across culture duration were statistically insignificant ($p > 0.05$).

Properties of Ca^{2+} currents

A slowly inactivating inward current was revealed by blocking both K^+ channels and TTX sensitive Na^+ currents (Fig. 3A). This current was eliminated by removing external Ca^{2+} (5 of 5 cells) but was augmented when Ba^2+ was used as the charge carrier. Thus, this residual inward current was attributed to voltage-gated Ca^{2+} channel activity. Peak Ba^{2+} current changed non-monotonically during the 12 day culture period ($p < 0.05$; Fig. 3B). Post-hoc tests verified that the increase in current at 8.5 DIV compared with 4.5 was reliable ($p < 0.05$). Voltage-dependent activation curves shifted progressively to more negative values with time in culture ($p < 0.05$; Fig 3C and D). In contrast, inactivation $V_{1/2}$ was unchanged during the culture period ($p > 0.05$; Fig 3E and F).

Properties of non-inactivating K^+ currents

To characterize the properties of K^+ currents, recordings were made in the presence of Na^+ and Ca^{2+} channel blockers. Non-inactivating K+ currents were recorded by stepping to command potentials from a holding of -40 mV. An example from a cell at 9 DIV is shown in Fig. 4A. Developmental changes in steady-state I-V curves revealed a time-dependent change in current response, which reached a maximum at 8.5 DIV ($p < 0.05$ for one-way ANOVA across DIV using I_{ss} at 56 mV; Fig. 4B). These currents were activated by steps above -40 mV, regardless of DIV. Half-activation

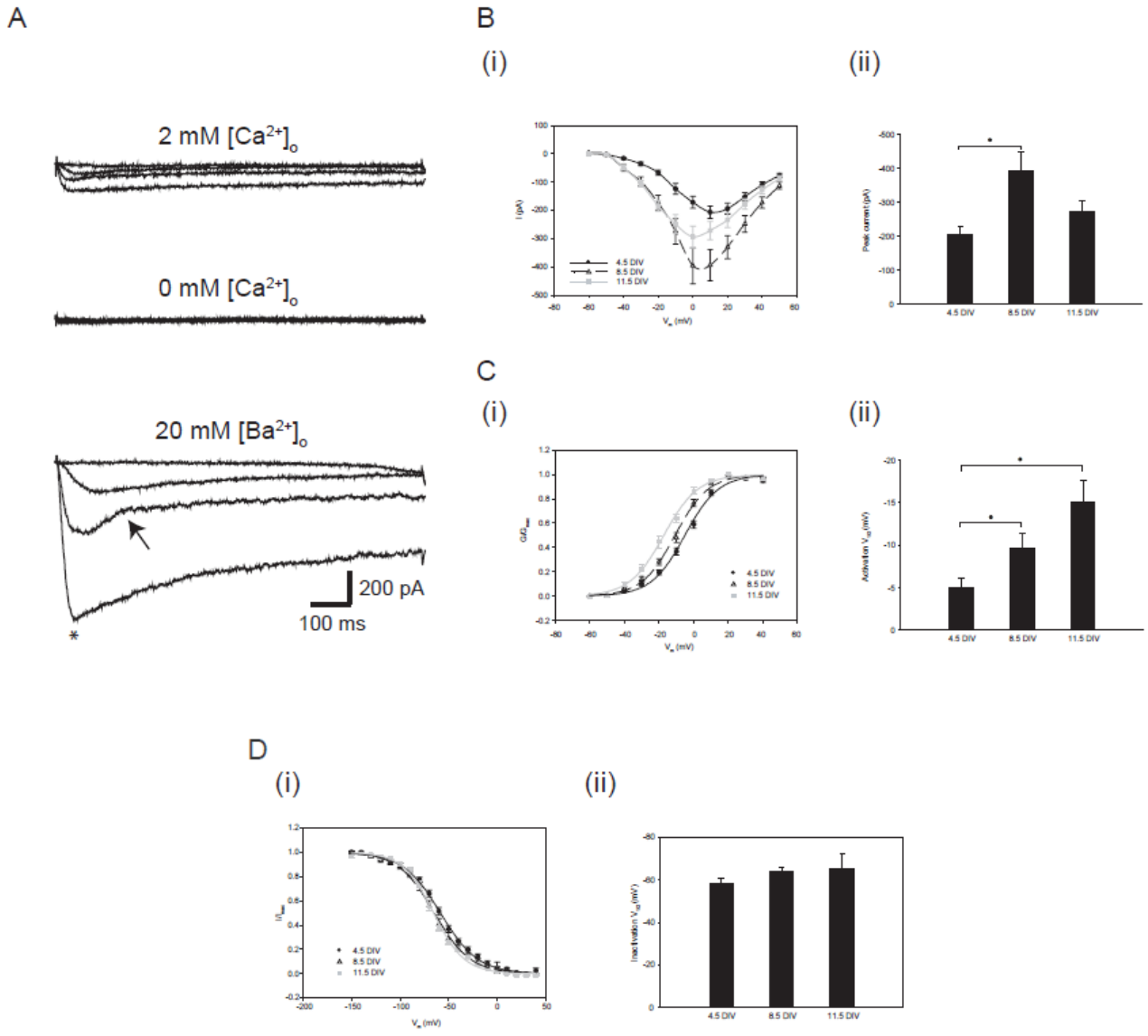

Figure 3.3: Calcium currents contributing to Neurog1 induced mESCs.

voltage, measured from fits to tail currents, shifted to more positive potentials with longer culture times (Fig. 4D). This shift was small-only about 7 mV-but reliable (p ¡ 0.05). According to post-hoc tests, differences between 4.5 DIV and each of the other longer time points were statistically significant, indicating a sudden shift in $V_{1/2}$ after 4.5 DIV ($p < 0.05$; Fig. 4D).

Non-inactivating K^+ currents were sensitive to external TEA (Fig. 4E). Steady-state currents at maximum conductance levels were 70% blocked by 10 mM TEA (Fig 4F). Although the current amplitude was affected by TEA, the voltage-response of the I-V curves was similar between control and TEA solutions.

Properties of inactivating K^+ currents

Inactivating outward currents were isolated by subtracting currents elicited from a holding at -80 mV from those using a holding of -40 mV (Fig. 5A). Similar to non-inactivating K^+ currents, the peak amplitudes of the A-type currents were maximal at 8.5 DIV ($p < 0.05$, post-hoc test between 4.5 and 8.5 DIV at 56 mV; Fig 5B). The voltage-dependence of channel inactivation was tested with hyperpolarizing pre-conditioning steps. Despite a slight rightward shift in voltage-dependence (Fig 5C), half-inactivation voltage was unchanged over the culture period ($p > 0.05$; Fig 5D). Inactivation rates were best fit with a double exponential curve. For currents elicited by the step to 100 mV, there were no statistically reliable differences in the fast or slow time constants across DIV ($p > 0.05$). When the data were pooled together, the average fast time constant was 14.6 $\pm$ 2.3 ms and the slow was 100.6 $\pm$ 13.5 ms. Unlike the non-inactivating K^+ currents, inactivating currents were insensitive to 10 mM external TEA (Fig. 5E-F).

3.2.4 Discussion

The results of this study show that transient, forced overexpression of Neurog1 is sufficient to rapidly drive ES cells from pluripotency to fate determination to function.

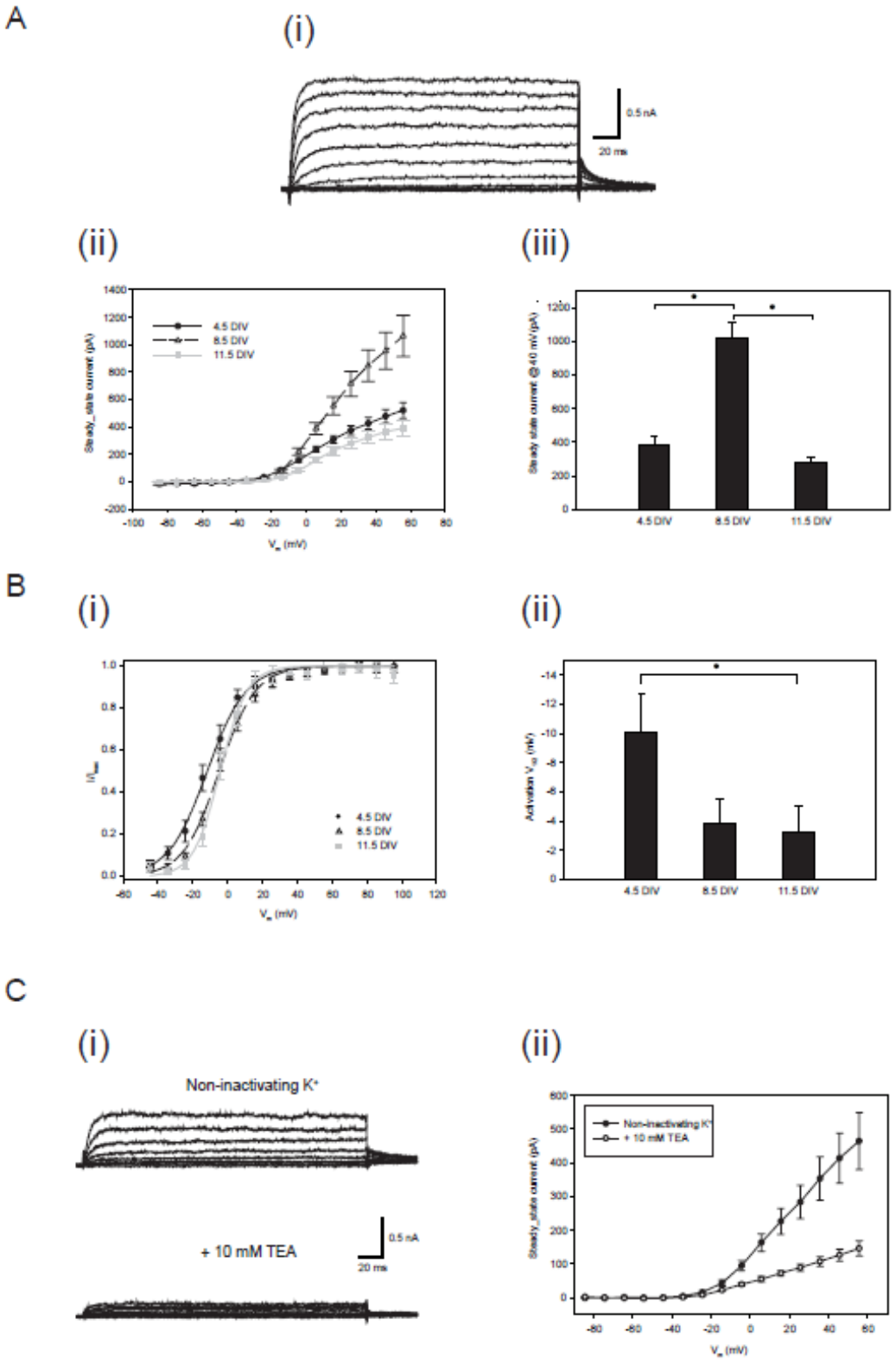

Figure 3.4: Non-inactivating K^+ channels in Neurog1 induced cells.

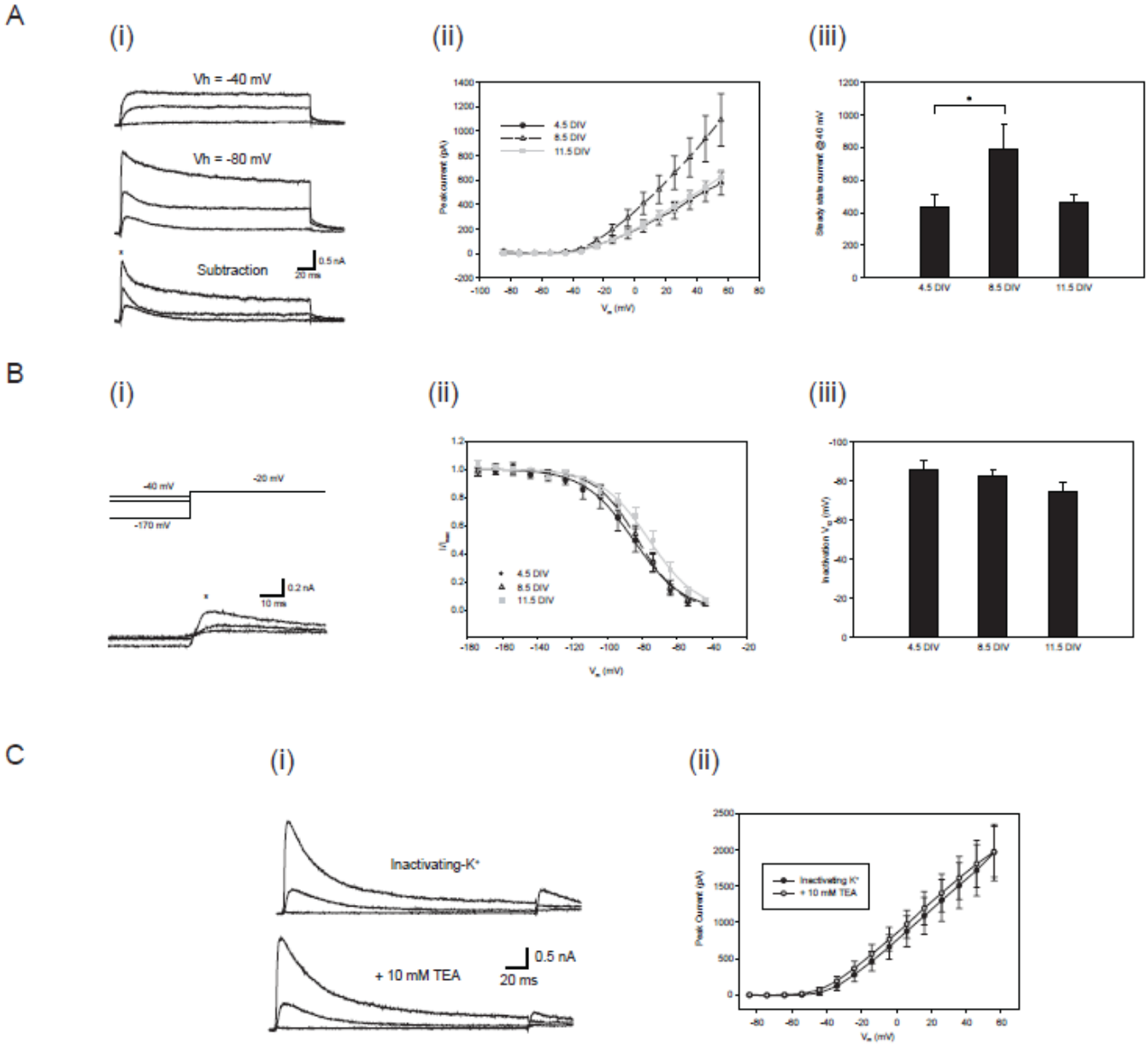

Figure 3.5: Inactivating K^+ currents in Neurog1-induced mouse ES cells.

By four DIV, all induced neurons acquired voltage-gated conductances and over 80% were capable of firing fast Na^+ spikes. Excitability was maintained for at least 12 days in culture, and the overall electrical properties of the cells were relatively homogeneous up to this time point.

In general, mature neurons are differentiated from ES cells by first coaxing these cells to adopt a neuronal lineage (*Abranches et al.*, 2009; *Bibel et al.*, 2004; *Zhang et al.*, 2001) followed by differentiation using exogenous factors or removal of mitogenic cues (*Robertson et al.*, 2008). Numerous studies have demonstrated the production of neuron-like cells using these methodologies, but assessment of a neuronal phenotype is typically limited to morphological and histological descriptions. When assessed for functionality, the outcome is highly variable. One study reported the absence of voltage-gated sodium channel activity in morphologically and biochemically mature "neurons" (*Balasubramaniyan et al.*, 2004). In other reports, Na^+ action potentials were elicited from a moderate to high percentage of stem cell-derived neurons (30 - 100%) (*Bain et al.*, 1995; *Carpenter et al.*, 2001; *Li et al.*, 2009). Although Neurog1 does not directly regulate the transcription of genes involved in neuronal excitability (*Seo et al.*, 2007), overexpression of Neurog1 of ES cells can initiate a transcriptional program that ultimately leads to a functional neuronal phenotype without intermediate culture steps or specialized media.

Forced expression of Neurog1 also appeared to bypass some transitional steps in neurogenesis. In many neurons, early phases of development are characterized by spontaneous or evoked Ca^{2+} action potentials that guide maturation, synaptogenesis, and network formation (*Moe et al.*, 2005; *Spitzer*, 2006). Spontaneous differentiation of ES cells in the uninduced cultures produced neural-like cells that may have been following this maturation program, since many of these cells exhibited broad, slow, Ca^{2+}-based responses by 12 DIV. In contrast to uninduced cells, Na^+ action potentials appeared shortly after Neurog1 induction, and Ca^{2+} action potentials were

absent from Neurog1-induced neurons. Recordings from neurons during the 72 hour induction phase showed initial acquisition of outward potassium currents followed by inward sodium currents and possibly small levels of calcium currents (data not shown). Although intrinsic excitability may be required for development of neuronal circuits, these transitional phases may not be necessary in applications that require mature phenotypes.

For future therapeutic strategies, it will be important to associate phenotypic traits with the transcriptional signaling cascades initiated by proneural genes. Therefore, we must identify the relevant ion channel genes induced by Neurog1 signaling. Ion channel gene families are large, and their functional properties are diverse. The biophysical properties of many of these channels are cataloged by the International Union of Pharmacology in a series of compendia (for overview, see *Yu et al.*). Data from the compendia were compared with that reported in the present study to identify classes of channels most likely involved in the excitability of our derived neurons. Conclusions drawn from voltage-clamp data focused on broad classes of ion channel types rather than specific configurations, since errors could be introduced by poor spatial control of the cell membrane potential. However, we never observed notches or bumps in the time course of any currents. Therefore, more subtle space-clamp effects on channel kinetics, current amplitudes, and voltage-sensitivity are expected to be small (*Hartline and Castelfranco*, 2003).

Voltage-gated Na^+ channels can be divided into two categories based on their sensitivity or resistance to TTX. In our study, Na^+ current was eliminated by TTX, indicating that resistant isoforms NaV1.5, NaV1.8, and NaV1.9 played no role in Neurog1-induced excitability. Many of the remaining TTX-sensitive subunits are found in sensory neurons of the dorsal root (*Black et al.*, 1996; *Djouhri et al.*, 2003), trigeminal (*Tsai et al.*, 2001), and vestibulocochlear ganglia (*Fryatt et al.*, 2009; *Hossain et al.*, 2005). NaV1.6 is common to each of these neural populations and appears

to play an important role in Neurog1-induced ES cells as well (Fig. 2B). Further study is required to determine if other NaV subunits are expressed and if these also contribute to the excitability of our cells.

The non-inactivating, or delayed rectifier, K^+ currents were sensitive to high voltages with a $V_{1/2}$ near -3 mV in later cultures. Typically, high voltage-activated K^+ channels shape the action potential waveform but play less of a role in governing spike threshold and latency. In our cells, the voltage activation range for these currents was unchanged after block with TEA, suggesting the presence of a single delayed-rectifier with partial sensitivity to 10 mM TEA. Channels with these traits include the Kv2 family as well as Kv3.1 and Kv3.2. Most of these channels can be found in sensory neurons (*Bocksteins et al.*, 2009; *Gravagna et al.*, 2008; *Ozaita et al.*, 2002); and Kv2.1 and Kv3.1 are specifically expressed in the inner ear (*Adamson et al.*, 2002b; *Varela-Ramirez et al.*, 1998). Therefore, Kv2 and Kv3 delayed rectifiers are strong candidates for governing the excitability of Neurog1-induced ES cells.

Our data also revealed inactivating, or A-type, K^+ currents. In general, A-currents control neuronal excitability by adjusting spike rate and by altering spike duration during a sustained stimulus. In our study, these currents were insensitive to TEA, downplaying a role for the TEA-sensitive Kv3.3 and Kv3.4 subunits. Instead, the insensitivity to TEA and the kinetics of inactivation point to the involvement of Kv1.4 or channels in the Kv4 family, which also are found in nociceptive ganglia (*Djouhri et al.*, 2003; *Liu and Simon*, 2003), olfactory sensory neurons (*Han and Lucero*, 2006), and primary auditory neurons (*Adamson et al.*, 2002b).

In addition to voltage-sensitive Na^+ and K^+ currents, induced neurons expressed a small voltage-gated Ca^{2+} current that could limit firing frequency by activating Ca^{2+}-sensitive potassium channels or guide other processes including neurite growth, cell motility, and neurotransmitter release (*Neher and Sakaba*, 2008; *Zheng and Poo*, 2007). The high activation range of this current is inconsistent with the induction of

T-type calcium channels. Other varieties (i.e. L, N, P, Q, or R) are all commonly found in sensory neurons, often segregating by type into different subcellular domains. To determine the putative role(s) of this current, it will be critical to localize contributing Ca^{2+} channels to specific axosomatic compartments.

Although we found at least some varieties of Na^{+}, Ca^{2+} and K^{+} channels in differentiated cells, other key voltage-dependent ion channels were absent, including several classes that play important roles in regulating cell resting potential. For example, we found no evidence for inward rectifiers, low-voltage-activated K+ channels, and HCN channels responsible for so-called H-currents. Each of these channel classes could impact resting potential as well as contribute to spike threshold, shape, and frequency. In our cells, the resting potential was near -40 mV, regardless of culture duration (Table 1). Since the threshold for Na^{+} channel activation was also about -40 mV, again regardless of culture duration, many of these channels were inactivated under resting conditions. As a result, induced neurons infrequently spiked from rest in response to injected currents. Spikes were apparent only after first hyperpolarizing the cell with a conditioning pre-step. This pre-hyperpolarization is a common requirement in stem cell-derived neurons (*Carpenter et al.*, 2001; *Li et al.*, 2009; *Martinez-Monedero et al.*, 2008). It is possible that a mature phenotype has not been reached, a required co-factor is missing, or the derived neurons are not in fact normal. Neurotrophic factors or further genetic manipulation could be used to drive resting to more negative potentials. These strategies also may allow customization of transmitter and firing subtypes, enabling a wide range of phenotypic traits. Alternatively, if ES cell-derived neurons are used in conjunction with biohybrid implant devices, stimulation paradigms could be adjusted to release Na+ channel inactivation and enable spike propagation.

Sensory neurons in the auditory periphery, trigeminal system, and dorsal root ganglia exhibit a variety of firing patterns, including bursting and repetitive firing, but

strongly adapting responses are the most commonly reported discharge pattern (*Brocard et al.*, 2006; *Catacuzzeno et al.*, 2008; *Rusznak and Szucs*, 2009; *Zhang et al.*, 2004). orced expression of Neurog1 in mouse ES cells resulted in neurons with a rapidly adapting discharge rate or a single spike at the onset of the stimulus. Therefore, these cells are excellent candidates for replacement of degenerating sensory neurons. In addition, the model system described in this paper is ideal for exploring the influences of trophic factors, topology, and host tissues. By understanding the transcriptional events leading from induction to neural specification, stem cell therapy can begin to reach its full potential.

3.3 Significance of this work

ES cells hold incredible promise for the treatment of neurodegenerative diseases. Given the thousands of neuronal subtypes that inhabit the PNS and CNS, there is still a great need to further characterize the phenotype of ES cell-derived neurons beyond their expression of molecular markers to determine whether they are responsive to the appropriate inputs. Toward the end of creating excitable sensory neurons, we can use electrophysiology to directly assess the potential of sensory-like ES cell-derived neurons to transform stimulus information into specific action potential (AP) patterns for active cell-cell communication (*Miles et al.*, 2004).

In the study presented here, we showed that neuronal, glutamatergic neurons generated after transient Neurog1 induction in mES cells (*Reyes et al.*, 2008) additionally undergo rapid maturation of membrane properties over time in culture. Only after transgene induction do we observe mature sodium currents within 4 DIV differentiation; without induction, rare, spontaneously-arising neurons appeared beyond 11 DIV, but showed only slow, Ca^{2+}-driven spikes. During *in vivo* development, new neurons generally utilize Ca^{2+} currents to propagate neuronal maturation cues and communicate within dynamic, developing networks in the nervous system, and over

time Na^+ channels emerge to generate faster responses to stimuli (*Spitzer et al.*, 1994). Ca^{2+} also plays a crucial role as a second-messenger in various aspects of neuronal differentiation, from proliferation to neurotransmitter specification (*Gu and Spitzer*, 1995). In our ES cell differentiation paradigm, Neurog1 induction so rapidly confers functionality that it bypasses the developmental stage where Ca^{2+} currents drive depolarization.

Closer investigation into the precise mechanisms of the mature firing properties that emerged later in our cultures revealed interesting correlations with our target neuron, the spiral ganglion (SGN). The SGN are a heterogeneous population of first-order sensory neurons that relay neurochemical messages from the auditory sensory receptor cell, the inner hair cell (IHC), to the CNS. A rich literature exists on the electrophysiological responses of SGN to stimuli, with a strong correlation between their firing patterns and the specific stimuli they encode, high to low frequencies, being well established (*Mo and Davis*, 1997; *Jagger and Housley*, 2003; *Adamson et al.*, 2002b; *Zhou et al.*, 2005; *Bakondi et al.*, 2008; *Fryatt et al.*, 2009; *Rusznak and Szucs*, 2009). In general our cells showed a "rapid adaptation" under current clamp, much like the SGN innervating IHC at the base of the cochlea where high frequency acoustic information is encoded (*Adamson et al.*, 2002a).

Previous work seeking to derive functional neurons from ES cells utilized protocols that required several weeks of culture at minimum before detecting functionality (*Strubing et al.*, 1995; *Balasubramaniyan et al.*, 2004; *Biella et al.*, 2007; *Donato et al.*, 2007; *Johnson et al.*, 2007; *Cho et al.*, 2008). Our ability to produce functional neurons within a few days of *in vitro* differentiation may help deter the high cost and elevated risk of contamination associated with long-term cultures. Furthermore, given the fact that Neurog1 is required in the development of trigeminal and vestibular sensory ganglia (*Fode et al.*, 1998; *Huang et al.*, 2001), the cells we generated may overlap in function with these other sensory neurons, since we did not provide additional

cues to further-hone the SGN phenotype in this study. The properties reported here can possibly be further-modulated using neurotrophic factors such as BDNF, as has been previously demonstrated (*Lesser and Lo*, 1995; *Cummins et al.*, 2000; *Adamson et al.*, 2002a; *Ahn et al.*, 2007; *Colley et al.*, 2009), to customize the composition of ion channels and therefore alter resting membrane potential, thresholds, and AP firing patterns. The transient expression of Neurog1 in development likely acts upstream of tissue-specific transcriptional cascades that further distinguish sensory subtypes (*Fritzsch*, 2003; *Marmigere and Ernfors*, 2007), and have a wide range of therapeutic uses with few side effects when locally applied to the cite of pathology (for review, see *Lanni et al.* (2010)). This possibility will be explored further in Chapter IV.

CHAPTER IV

Modulating the Neuronal Phenotype with Target-Derived Neurotrophic Factors

4.1 Introduction

Developing sensory neurons in the peripheral nervous system (PNS) rely on dynamic, context-dependent interactions with neurotrophic factors (NTFs) for survival, differentiation, function, and accurate target innervation. The diversity of sensory neurons and the information they convey from peripheral stimuli receptors to the central nervous system (CNS) is in part due to the developmental influences that NTFs exert through receptor tyrosine kinase pathways (*Cordon-Cardo et al.*, 1991; *Klein et al.*, 1991; *Huang and Reichardt*, 2001). These pathways regulate various aspects of the neuronal phenotype, from membrane properties that confer unique physiological functions to influencing neurotransmitter identity and neurite outgrowth. Loss of specialized sensory neurons can permanently disconnect us from the reality around us, the "harsh decree" of nature that disabled spontaneous regeneration of nerves in increasingly complex organisms (*Ramon y Cajal*, 1928).

Embryonic stem (ES) cell-derived neurons may provide the ability to replace the function of lost neurons, as they have been demonstrated to acquire morphological characteristics and express tissue-specific markers of neurons of the sensory lineage

(*Pomp et al.*, 2005; *Shi et al.*, 2007; *Kondo et al.*, 2008; *Martinez-Monedero et al.*, 2008). For replacement of sensory nerves in the periphery, ES cell-derived neurons must express relevant functional markers, such as glutamate transporters or receptors, which would suggest glutamatergic neurotransmission (*Reyes et al.*, 2008), but not reveal whether the cells can provide the most basic role of the neuron, the action potential (AP), to propagate the glutamatergic message. In Chapter III we established a role for the proneural transcription factor Neurog1 in conferring electrochemical properties to differentiating mES cells where previous differentiation protocols failed to correlate morphological and neurochemical markers with functionality (*Biella et al.*, 2007; *Balasubramaniyan et al.*, 2004; *Strubing et al.*, 1995).

A useful assessment of phenotype is the presence of neurotransmitter-related markers and the expression of functional ion channel combinations that enable delivery of unique sensory information from the periphery to the CNS. In the auditory system, afferent neurons of the spiral ganglion express combinations of channels that confer specificresting potentials, thresholds, and firing patterns correlated with maximal processing of high or low frequency stimuli (*Mo and Davis*, 1997; *Zhou et al.*, 2001; *Adamson et al.*, 2002b; *Davis*, 2003; *Sun and Salvi*, 2009). This information is relayed to the CNS by releasing the excitatory neurotransmitter glutamate (but potentially dopamine as well) onto target neurons in the cochlear nucleus (*Eybalin*, 1993; *Inoue et al.*, 2006; *Ruel et al.*, 2006). Our goal is to recreate the relevant and requisite physiological profiles of mature sensory neurons in ES cell-derived neurons to enhance the efficacy of cell replacement therapies.

In the present study we sought to characterize the potential of Neurog1-induced ES cells to functionally replace spiral ganglion neurons (SGN) of the auditory nerve. We hypothesized that two of the NTFs involved in SGN development, BDNF and NT-3, acting on cognate receptors upregulated following transient Neurog1 expression (*Reyes et al.*, 2008), could refine the neuronal subtype as they do during develop-

ment, selectively activating pathways to confer SGN functionality. Our rationale was the temporal gene expression cascade would more closely match the development of delaminated otocyst neuroblasts fated to become SGN. This was tested by building a transmitter and ion channel expression profile using quantitative Real Time PCR and immunocytochemistry, and comparing neurons differentiated under our paradigm with other sensory neurons based on previous reports on their transmitter and channel expression. We show that at the molecular level, neurotrophic factors modulate very specific channels at the concentrations tested, and these changes likely have implications on the functional phenotype (*Lesser et al.*, 1997; *Adamson et al.*, 2002a; *Ahn et al.*, 2007; *Colley et al.*, 2007). We also provide evidence that supports the ability of NTFs to promote neurite outgrowth, but has little influence on neurotransmitter phenotype.

4.2 Materials and Methods

Cell culture and *in vitro* differentiation

A mouse ES cell line containing a tetracycline-inducible Neurog1 transgene and eGFP driven by the constitutive UbC promoter were used in this study (*Velkey*, 2005; *Reyes et al.*, 2008). Undifferentiated ES cells of this line were grown in standard conditions (37 °C, 5% CO_2) and maintained in sterile-filtered DMEM (Invitrogen, Carlsbad, CA) with 10% ES cell-tested fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 5% embryonic stem cell supplement (DMEM with 21.65 mg/mL HEPES buffer, 3.63 mg/mL L-glutamine, and 63.63 ng/mL β-mercaptoethanol) and 0.5 g/mL leukemia inhibitory factor (recombinant human LIF, Millipore, Bedford, MA). ES cells were dissociated using Hanks'-based enzyme free cell dissociation buffer (Invitrogen) and re-plated for *in vitro* differentiation in serum-free medium consisting of 80% F12/DMEM, 20% Neurobasal medium, with B27 and N2 supplements (Invitrogen), 10 mM sodium pyruvate (Gibco/Invitrogen) (80:20 medium), and Doxy-

cycline (Dox) (Sigma, St Louis, MO) at 1 g/mL. $5x10^5$ cells per well were plated in 6-well dishes containing 13 mm diameter Thermanox cell culture coverslips (Nunc, Naperville, IL) previously coated with 0.1% gelatin (Sigma). At three days *in vitro* differentiation (DIV), Dox was removed by washing in 1x Hanks' Balanced Salt Solution (HBSS, Gibco). Control cells were simultaneously grown in differentiation media without the addition of Dox.

We tested the effects of 20 ng/mL BDNF or 20 ng/mL NT-3 on neurite outgrowth and the expression of ion channel and neurotransmitter-associated markers after neuronal differentiation with Neurog1. We assessed cells at two, three, and five DIV for modulations in gene expression using Quantitative Real Time PCR (qRT-PCR) and corresponding changes in the presence of related proteins using immunocytochemistry (described below).

Immunocytochemistry (ICC), Image Acquisition, Quantification

At sequential time points, coverslips containing differentiating mES cells were retrieved from wells and washed with 1x HBSS. The cells on the coverslips were fixed with 4% paraformaldehyde for ten minutes at room temperature, followed by 3 x 10 minute washes in D-PBS with calcium and magnesium (Invitrogen), blocking with Power Block Universal Blocking Reagent (Bio Genex, San Ramon, USA), and three additional ten minute washes in D-PBS. Coverslips were then incubated overnight at 4 °C with antibodies diluted in 0.1% Triton-X in PBS. ICC localization of the following antibodies were carried out: the neuronal marker β-III tubulin (TUJ1, 1:300; Covance, Madison, WI), Kv1.4, Kv4.2 and Nav1.6 (all 1:150; NeuroMab, Davis, CA), VGLUT1 and tyrosine hydroxylase (TH) (all 1:500, Synaptic Systems, Germany). The next day, coverslips were washed with D-PBS and binding was visualized with Alexa secondary antibodies from Invitrogen.

Each TUJ1 and phenotype marker (i.e. specific channel or neurotransmitter) combination was tested on three replicate coverslips from each experimental condition.

Four images were taken at 20x or 40x from the center of each triplicate coverslip, generating 12 images per condition. The number of neurons per image was counted based on TUJ1-positive cell bodies, and the number cells that co-labelled with a phenotype marker was quantified to generate the percentage of neurons positive for the phenotype marker. All images were acquired on an Olympus Fluo-View 500 Confocal Microscope and then imported into MetaMorph software for analysis.

To quantify neurite length, we calibrated MetaMorph software to measure pixel area at (20x or 40x). We used the available tracing tool to track long, TUJ1-positive processes ("neurites") emanating from cell clusters and individual cell bodies, and quantified the number of pixels traced. The total number of pixels was assumed to be correlated with neurite length, and was averaged across 12 images per condition. Higher pixel totals would indicate increased neurite growth since neurite diameter did not appear to change. Each pixel corresponded to .26μm. Each image was approximately 650μm x 650μm.

Quantitative real-time PCR assessment of differentiating ES cells. We selected a panel of auditory nerve-related channel genes that contribute to resting potential, AP firing properties, and thresholds (*Beisel et al.*, 2000; *Adamson et al.*, 2002b; *Bakondi et al.*, 2008; *Fryatt et al.*, 2009; *Rusznak et al.*, 2008; *Rusznak and Szucs*, 2009). We also included genes indicative of sensory neuron neurotransmitter identity in sensory neurons in general, such as tyrosine hydroxylase and VGLUTs 1 and 2, which indicate dopaminergic and glutamatergic differentiation, respectively. All TaqMan Gene Expression Assays were purchased from Applied Biosystems. $5x10^5$ cells were plated in each condition on 0.1% gelatin-coated six-well dishes in 80:20 differentiation medium containing Dox and NTFs. For each treatment group three experimental replicates were performed. RNA was collected from Neurog1-induced ES cells ± NTF combinations, and uninduced cells.

At 0 h, two, three, and five DIV, cells were washed with 1x HBSS to remove

medium and RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol with the exception of homogenization of the cell lysate for 30 seconds immediately after addition of ethanol. RNA integrity and concentrations were obtained using the Agilent Bioanalyzer 2100. Only RNA with an integrity number above 9.0 was used. 1 μg of RNA from each treatment group was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Each experimental replicate was assessed in triplicate using the ABI PRISM 7900HT Sequence Detection System analyzer for Real Time PCR, which gives a threshold cycle (CT) number. CT was normalized to the GAPDH housekeeping gene (*Murphy and Polak*, 2002), and then used to calculate fold changes in gene expression using the $2^{-\Delta\Delta CT}$ method (*Livak and Schmittgen*, 2001). Fold changes for the two, three, and 5 DIV treatment groups were taken as any increase in gene expression compared with uninduced controls at 0 h and were plotted on a log scale. A paired two sample t-test was performed on the ΔCT values of genes between treatment groups and un-induced controls, and significance was considered at $p \leq 0.05$.

4.3 Results

Neurogenin-1 induction generates an ion channel expression profile that correlates with those present in SGNs. Two days induction of the Neurog1 transgene was not sufficient to elevate the expression of any voltage-gated potassium or sodium channels assessed, but did upregulate the L-type, voltage-gated calcium channel Cav2.2 when compared to the reference control ES cells where the transgene was not induced. Cav2.2 may contribute to neurite outgrowth, synaptic transmission, and axon guidance by providing calcium influx during sensory neuron development (*Chi et al.*, 2009). This is consistent with the tendency of developing sensory neurons is to first utilize calcium as a second messenger in neuronal maturation, with cell communication arising through slow-inactivating calcium-driven spikes rather than

the later-arising, fast-inactivating sodium spikes (*Spitzer*, 2006). With the exception of Kv7.2 of the KCNQ4 family and Nav1.3, which were both significantly upregulated at all time points assessed when compared to uninduced ES cells, the other voltage-gated potassium and sodium channels assessed (Cav2.1, Kv1.1-6, BK, Nav1.2) showed no elevation in expression at two DIV (Fig 4.1), and this corroborated electrophysiological testing of ES cell-derived neurons, where little or no electrical activity was detected under current-clamp (Chapter III).

An additional day of transgene induction resulted in large increases in the expression of channel genes, and many of the channels correlated with functionality detected by both current and voltage clamps (Chapter III). The potassium channel blocker 4-aminopyridine (4-AP) selectively antagonizes Kv1 channel subunits, and when applied to Neurog1-induced cells after three DIV under patch-clamp resulted in significantly decreased K^+ currents. Kv1.1 through Kv1.6 all showed some upregulation in expression compared to the expression level at the previous day, with Kv1.6 being significantly upregulated ($p \leq 0.05$). Additionally, many of these channels, such as the BK channel in the large-conductance calcium-activated potassium channel family, likely play a role in AP firing and voltage-dependent current relationships unveiled in electrophysiological characterization of SGN (*Beisel et al.*, 2000; *Adamson et al.*, 2002b; *Bakondi et al.*, 2008; *Fryatt et al.*, 2009; *Rusznak and Szucs*, 2009). Interestingly, only the Kv1.4 subunit of the KCNA family was not upregulated after Neurog1 expression. Kv1.4 plays an important role in fast-inactivation and therefore dampens neuronal excitability, making it a target for neuropathic pain in dorsal root ganglion (*Rasband et al.*, 2001; *Rusznak and Szucs*, 2009). See Fig 4.1 for detailed fold changes after Neurog1 induction.

BDNF and NT-3 selectively affected expression of channel genes. At two DIV neuronal differentiation, activation of Ntrk receptors following BDNF treatment accelerated the transcription of the sodium channel genes encoding Nav1.6 and

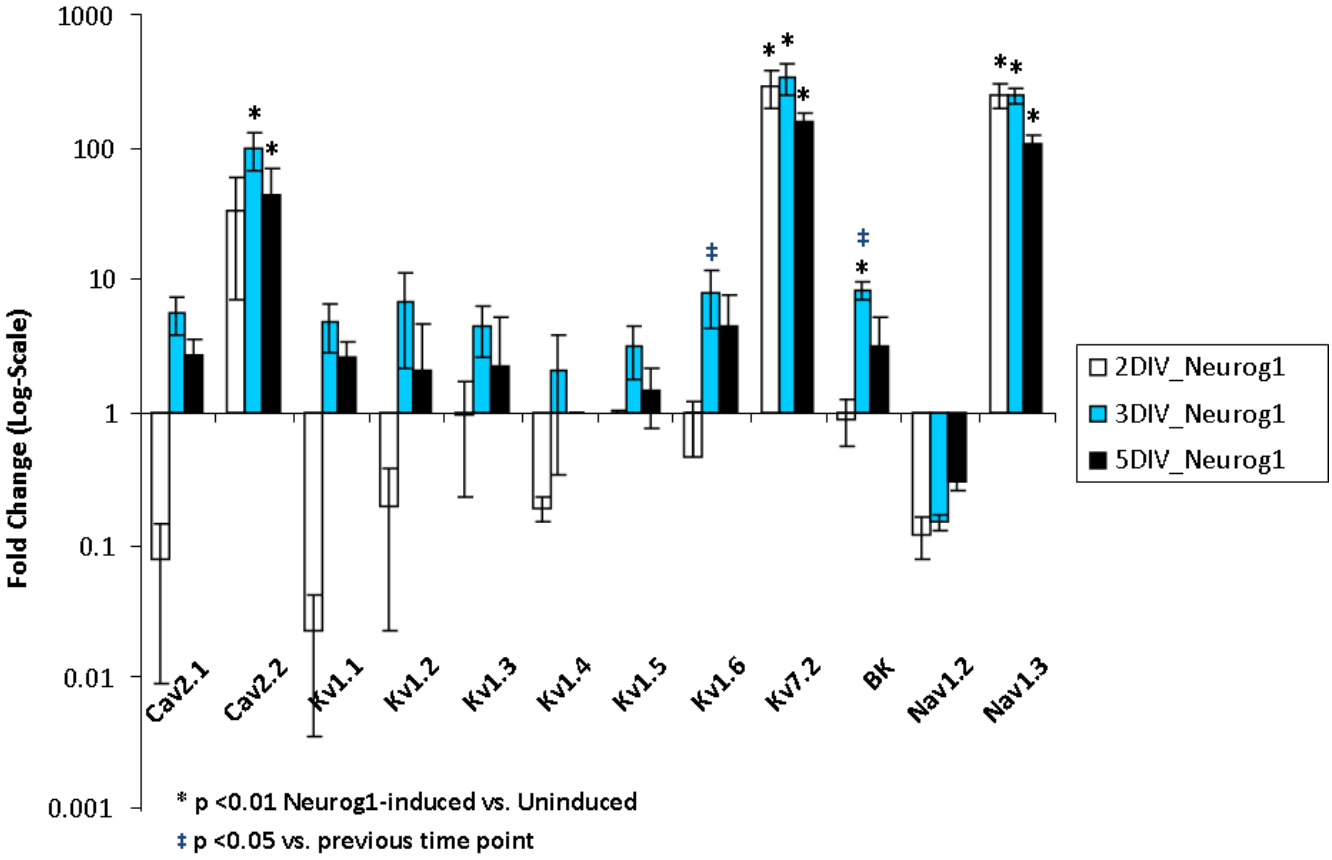

Figure 4.1: Neurog-1 induction alone upregulates channels correlated with SGN functionality in ES cell-derived neurons within 3 DIV differentiation

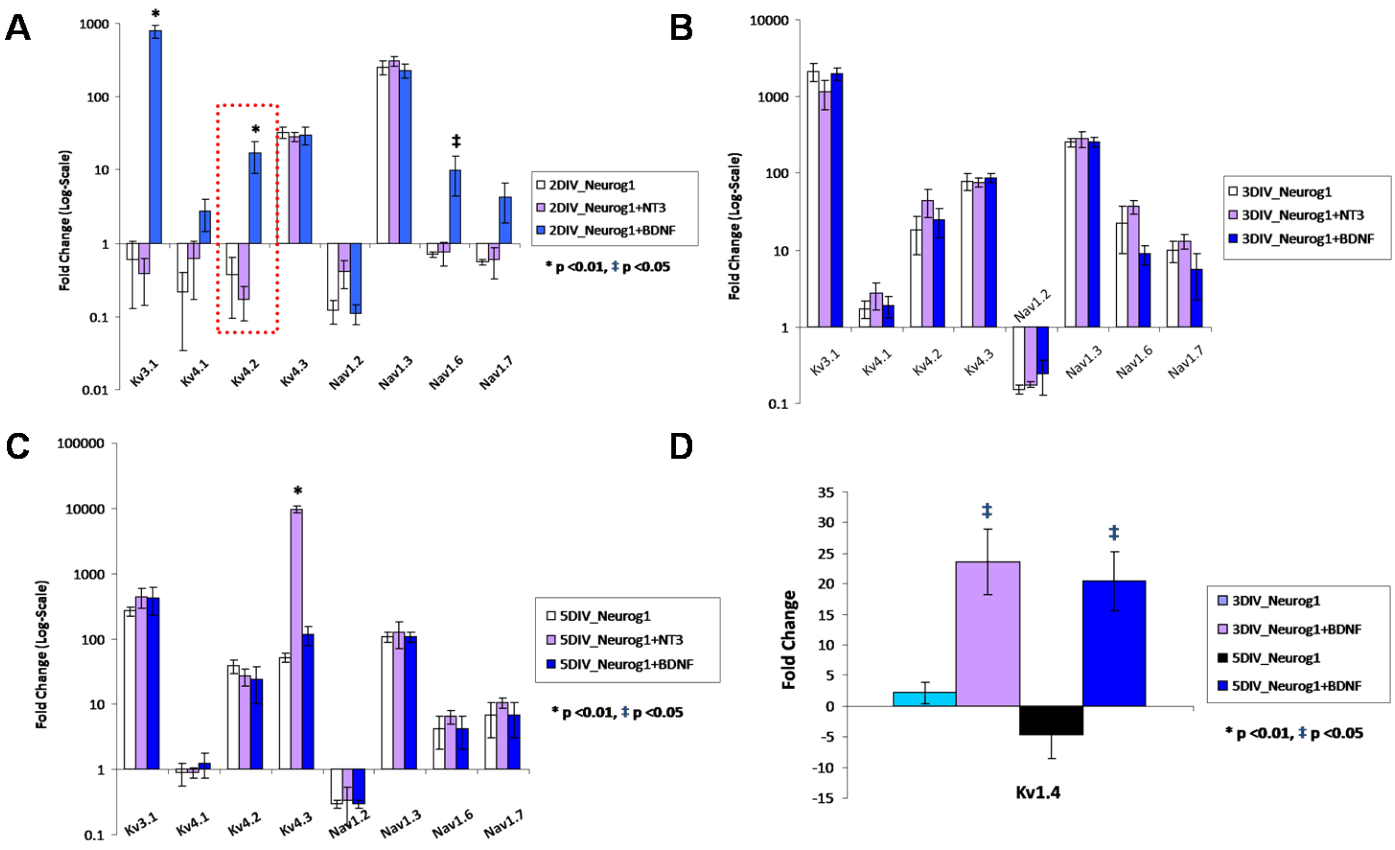

Figure 4.2: BDNF and NT-3 selectively modulate SGN-related channels at varied time points

Nav1.7, as well as the SGN-related channel genes Kv3.1 and Kv4.2 (Figure 4.2), suggesting that BDNF could accelerate excitability in differentiating cells. To assess potential modulation at the protein level, we employed Kv4.2 to corroborate gene expression and channel assembly using a reliable mouse antibody against the Kv4.2 subunit (1:100, NeuroMab Davis, CA). Following two days of transgene expression, Kv4.2 expression was significantly upregulated from a low base line, and correlative changes in protein expression could be detected using ICC. We found without addition of BDNF, fewer than 2% of cells were faintly positive for Kv4.2. Application of BDNF had a dramatic effect on the percentage of cells in culture that expressed Kv4.2 protein (Figure 4.3).

Elevated levels of specific transcripts were only sustained when BDNF exposure

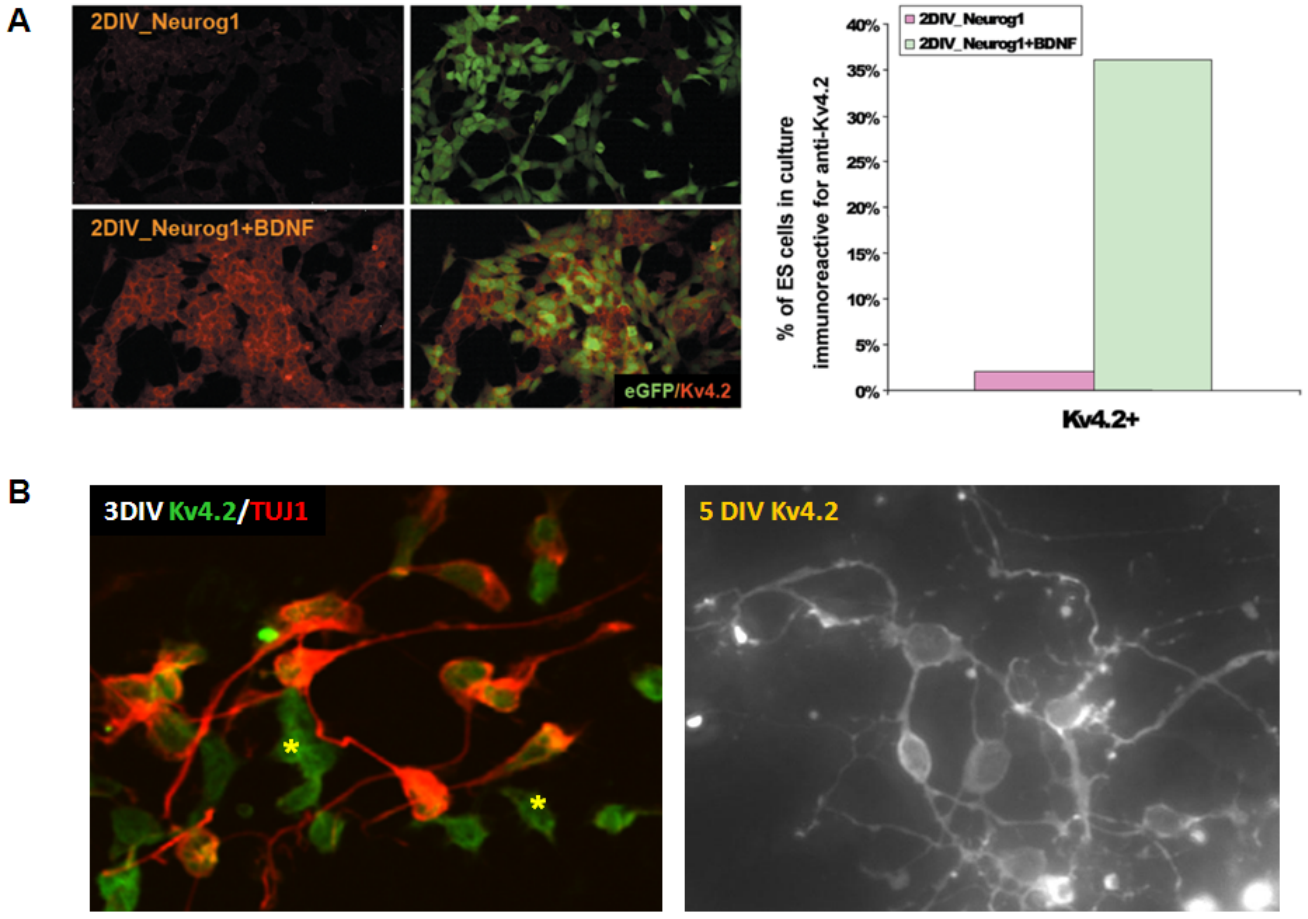

Figure 4.3: BDNF rapidly accelerates Kv4.2 expression

occurred at the early two DIV time point: by three and five DIV, Nav1.6, Nav1.7, Kv3.1 and Kv4.2 expression was roughly equivalent across Neurog1-induced ES cells, regardless of exposure to NTFs. This suggested that BDNF had transient effects on recently assembled Ntrk2 receptors. Alternatively, BDNF may have activated Kv1.4 expression at three and five DIV while Neurog1 alone failed to induce expression of Kv1.4 (Figure 4.2). At the concentration tested, NT-3 modulated expression of the Kv4.3 subunit only, which underwent a nearly 10,000-fold increase in expression at five DIV. Kv4.3 may contribute to transient K^+ currents that create a rapidly-adapting response in central auditory processing (*Rusznak et al.*, 2008). These studies suggest that through selective activation of neurotrophic factor pathways, variable expression of ionic currents can be obtained to recreate very specific phenotypes.

Neurotrophic factors did not affect neurotransmitter expression by ES cell-derived neurons. qRT-PCR assessment revealed Neurog1 induction significantly upregulated the genes involved in excitatory neurotransmission in the cochlea: glutamate (VGLUT1) and dopamine (tyrosine hydroxylase, TH). A marker for the inhibitory neurotransmitter GABA (vesicular GABA transporter, VGAT), was not significantly altered, suggesting that these cells are likely excitatory, as predicted based on Neurog1 knockout models (*Schuurmans et al.*, 2004); nor did exposure of differentiating Neurog1-induced cells to 20ng/mL of BDNF or NT-3 affect the expression of these genes. See Fig 4.4. These results suggested that Neurog1 produces a multitransmitter identity that confers excitatory neurotransmission, and this aspect of the phenotype cannot be further modulated by NTFs.

Immunocytochemistry was used to assess the presence of markers involved in excitatory neurotransmission, VGLUT1 and TH. The number of neurons within a field was difficult to ascertain, as no definitive cell body could be conclusively identified within cell clusters. Therefore, only distinguishable neuronal cell bodies were assessed for co-labeling with neurotransmitter markers. Of the cells expressing the neuronal

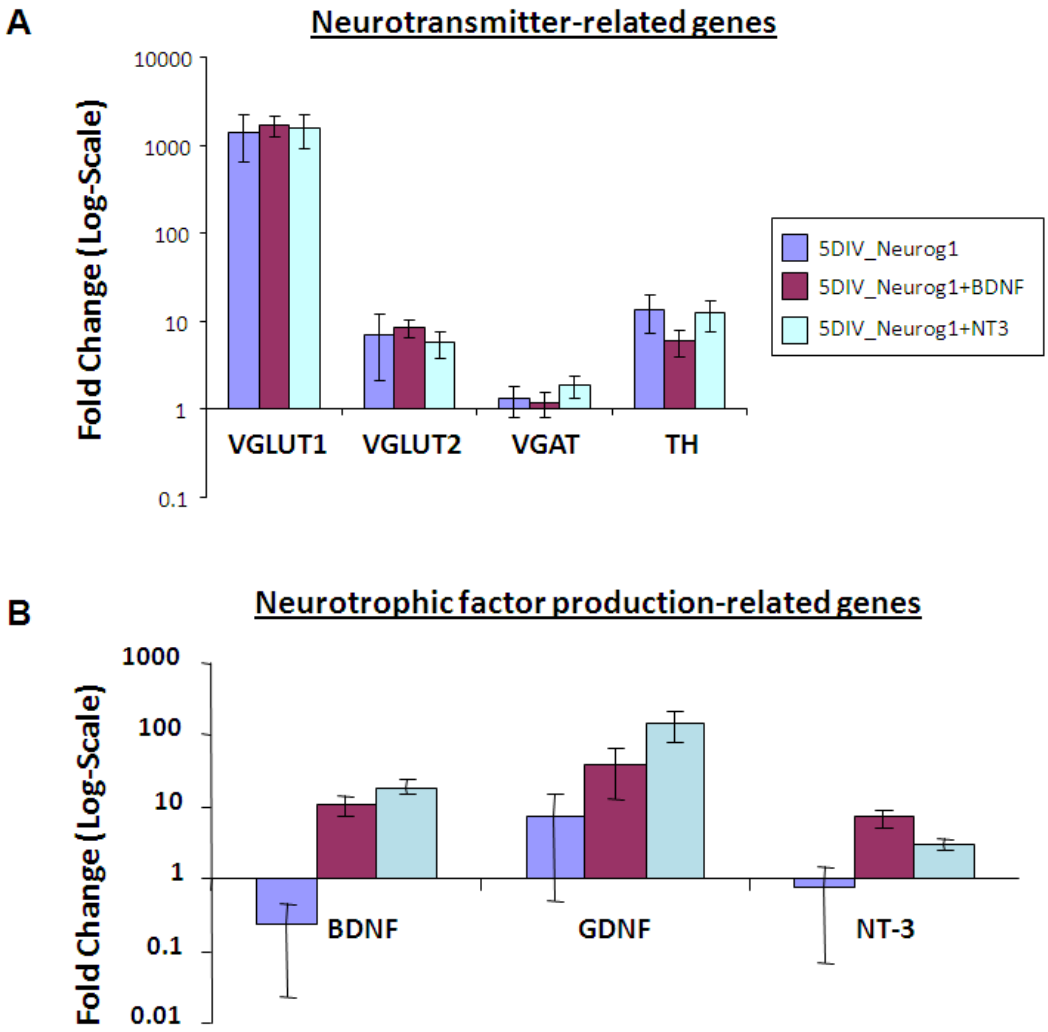

Figure 4.4: Neurotransmitter and neurotrophic factor-related gene expression

marker β-III tubulin, the number co-labelled for a neurotransmitter marker was calculated at five DIV. Application of neither BDNF nor NT-3 affected the percentage of neurons co-labeled for TH or VGLUT1. In Neurog1-induced cells, and Neurog1-induced cells with NTF treatment, 90-100% of TUJ1-positive cells were also positive for the neurotransmitters assessed. This confirms that the neurons may have a multi-transmitter identity, and Fig 4.5 demonstrates the cells may indeed express multiple transmitters.

Quantification of neurite length after NTF exposure. Neurite length was determined by with the number of pixels traced over neurites at five DIV. Quantifi-

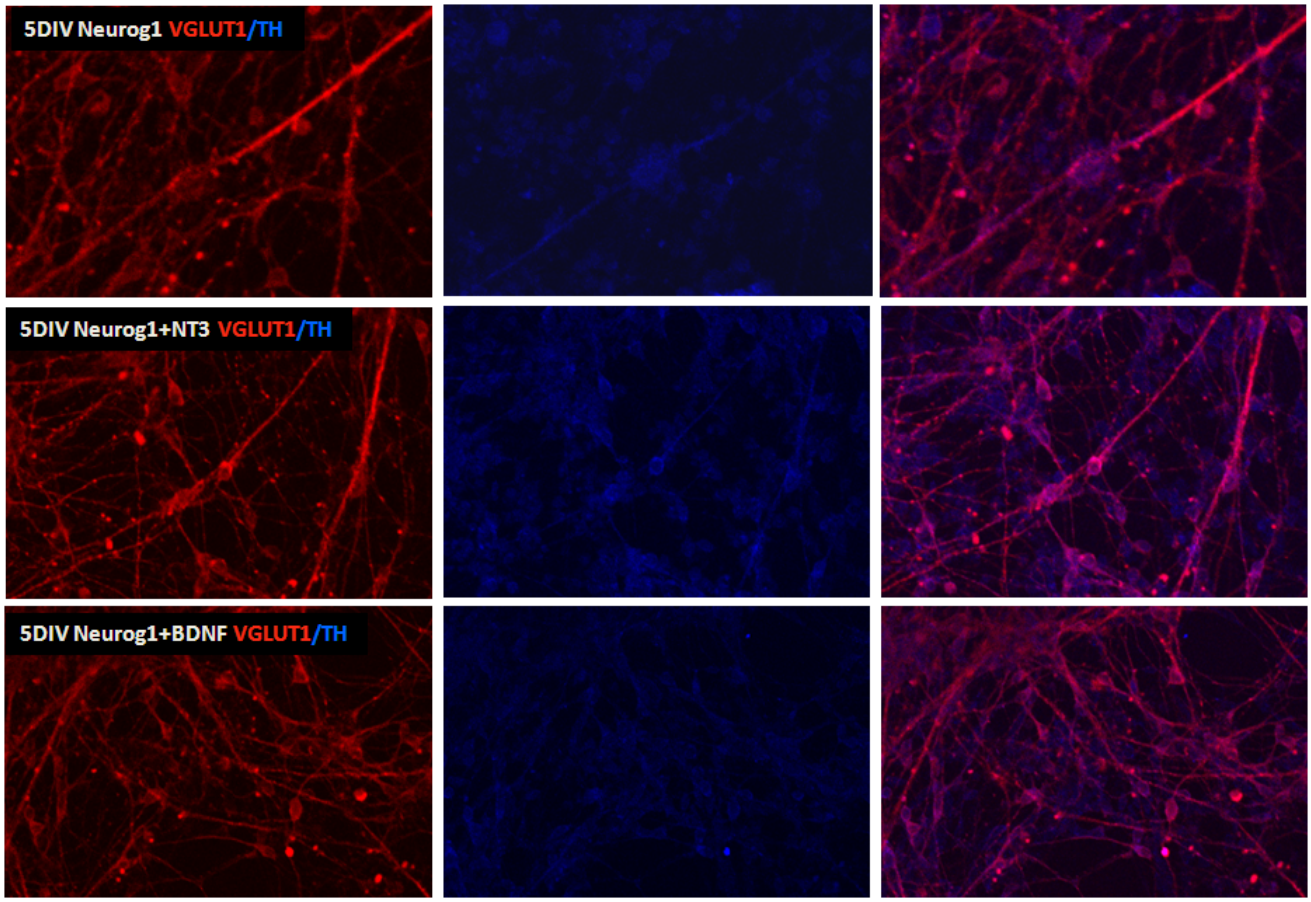

Figure 4.5: Neurotrophic factors did not affect neurotransmitter identity in Neurog1-induced neurons *in vitro*

cations are provided in Figure 4.6. Using this approach we found Neurog1 induction alone generated on average 2390μm of neurites per image, and exposure to 20ng/mL NT-3 slightly affected this number, albeit at a level that did not reach traditional statistical significance (2860μm/image, $p \leq 0.06$). BDNF application at 20ng/mL had a far more pronounced effect, increasing the number of microns to 4800μm/image ($p \leq 0.0005$). This change may be due to a specific action of BDNF on the extension and branching of developing neurites, or may be a non-specific effect emerging from increased neuronal survival after BDNF exposure rather than neuritogenesis per se. (The number of nuclei, based on Hoechst staining, was divided into total neurite length to obtain an estimate number of neurites per cell). Additionally, clusterings of undifferentiated or non-neuronal cells may produce trophic factors that affect neuritogenesis and outgrowth, and the distribution of these clusters throughout the culture well can also affect neurite lengths. In the Neurog1 and Neurog1+NT3 cultures, larger clusters of mostly non-neuronal (non-TUJ1 positive) cells were more numerous, while in the Neurog1+BDNF condition, the clusters were more compact and expressed β-III tubulin, see Figure 4.6.

4.4 Discussion

The derivation of functional neuronal subtypes from embryonic stem cells would significantly advance the field of stem cell-based replacement strategies, allowing for a closer recreation of the required phenotype. The temporal and positional cues that activate developmental pathways toward precise phenotypes must be replicated in differentiating cells in order to enhance eventual integration and functional restoration. These factors could be delivered to implanted stem cells via cannula or other forms of drug delivery to guide differentiation within desired target tissues (*Prieskorn and Miller*, 2000; *Swan et al.*, 2008). We have previously shown that this approach has great potential to reproduce specific phenotypes such as the spiral ganglion neurons

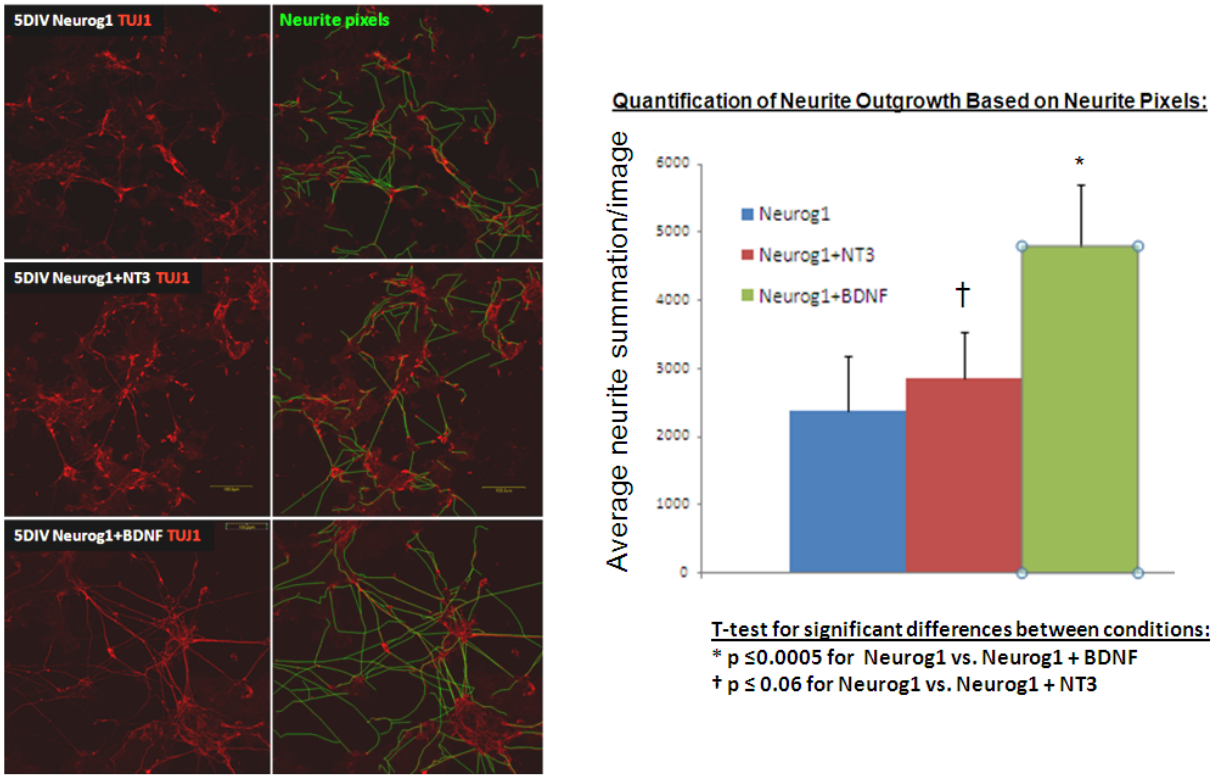

Figure 4.6: Quantification of neurites based on pixels

of the auditory nerve, as 75% of cells implanted into the scala tympani differentiated into neuronal cells expressing markers suggestive of glutamatergic transmission (*Reyes et al.*, 2008).

When grown in defined medium including factors known to further-influence the electrical phenotype, we found that transient expression of the proneural transcription factor Neurog1 rapidly elicited firing patterns and passive electrical properties that closely match the development of sensory neurons. Neurog1 is required for neurogenesis of sensory trigeminal (TGN), vestibular (VGN), and auditory (SGN) peripheral ganglia (*Huang et al.*, 2001), and we can draw comparisons between five DIV Neurog1-induced ES cell-derived neurons and the physiological profiles of other sensory ganglia. Each ganglion is a collection of heterogeneously-functioning neurons uniquely suited to convey specific sensory information to the CNS, and as such express multiple neurotransmitters and ion channel combinations. Like SGN, TGN and VGN

also utilize glutamate in neurotransmission and Kv1 subunits to set electrochemical potentials (*Yamashita et al.*, 1991; *Bilsland et al.*, 1999; *Andrews and Kunze*, 2001; *Liu and Simon*, 2003; *Hotchkiss et al.*, 2005; *Rocha-Sanchez et al.*, 2007; *Long et al.*, 2008; *Iwasaki et al.*, 2008). We observed that after transient, three days induction of the Neurog1 transgene, the cells highly expressed Kv1 subunits as well as Kv3.1, Kv4.2 and voltage-gated sodium channels, which are all known to contribute to SGN function (*Zhou et al.*, 2001; *Adamson et al.*, 2002a; *Bakondi et al.*, 2008; *Rusznak and Szucs*, 2009), but this was not observed at two DIV. Several of these channels overlap in expression with TG and VG, however, indicating that Neurog1 induction alone may not be sufficient to specify the SGN subtype (Fig 4.7).

Further modulation of Neurog1's downstream effectors is likely necessary to more closely create the desired functional phenotype. Toward this end, neurotrophic factors (NTFs) provide an exciting avenue through which to modify the phenotype of differentiating ES cell-derived neurons (*Adamson et al.*, 2002a; *Ahn et al.*, 2007; *Colley et al.*, 2007; *Silva et al.*, 2009), and are noteworthy for their therapeutic effects in neuronal injury (*Abe*, 2000; *Lanni et al.*, 2010). Additional exposure to Neurog1-induced cells to BDNF accelerated the expression of Kv3.1, Kv4.2, and Nav1.6-7 (Fig 4.2) at the early two DIV time point, but elevated levels of these transcripts were not observed at later time points. At three and five DIV we found BDNF selectively and significantly upregulated expression of the Kv1.4 subunit while expression of other Kv1 subunits remained unchanged after exposure to BDNF. Interestingly, NT-3 only affected Kv4.3 expression at 5 DIV. (Fig 4.7). This may be due to the fact that these cells produce proteins required for neurotransmitter production, and the level of NT-3 we tested did not affect the concentration of NT-3 already present in the culture Figure 4.4. An ELISA assay could measure the amount of NT-3 present before and after addition of various concentrations of NTF to determine if this is the case, otherwise the NT-3 receptor Ntrk3 may not be as highly expressed as originally

presumed relative to the BDNF receptor Ntrk2. Additionally, the gene encoding glial cell-line derived neurotrophic factor (GDNF) significantly increased by 5 DIV, i.e. only after removal of Neurog1 expression enabled gliogenesis, and the presence of this factor in the culture may also influence subtype-specific properties.

NTF exposure can have permanent effects on neuronal differentiation, as well (*Agterberg et al.*, 2009); ES cell-derived neurons need not receive continuous infusion of NTFs after implantation. For example, a relatively low concentration of BDNF (20 ng/mL) specifically upregulated Kv1.4 subunit expression in neurons approaching a mature phenotype (Fig 4.2). Kv1.4 regulates neuronal excitability and is decreased in overactive sensory neurons that create neuropathic pain (*Rasband et al.*, 2001; *Wells et al.*, 2007), so enhanced expression of this subunit may provide a way of decreasing excitability, making the cells more rapidly adapting to stimuli. Conversely, blockade of BDNF signaling may downregulate Kv1.4 expression to decrease activation thresholds and therefore increase excitability. Interestingly, however, electrophysiological characterization of Neurog1-induced ES cell-derived neurons revealed an insensitivity to tetraethylammonium (TEA) (Chapter III), potentiating a role of Kv1.4 in our cells without NTF exposure. Kv4.3, significantly upregulated at 5 DIV by NT-3, plays a role in transiently activating K^+ currents, and its modulation may affect action potential duration (*Rusznak et al.*, 2008). While we observed modulations of subtype markers with NTF exposure, additional application of other secreted factors present in SGN development, such as FGFs (*Hossain et al.*, 1996; *Adamska et al.*) or BMPs (*Shi et al.*, 2007) may reinforce sensory neuron-specific differentiation pathways.

These preliminary data support reports in the literature where specific channel combinations can arise in ES cell-derived neurons via selective activation of NTF receptors (*Lesser et al.*, 1997; *Ahn et al.*, 2007). For example *Colley et al.* (2007) found activation of the BDNF receptor increased surface expression of the Kv1.3 subunit in neurons in the olfactory bulb, while *Adamson et al.* (2002a) found BDNF and NT-3

exposure altered the firing properties of neonatal SGN likely through modulating the expression levels of Kv3.1 and Kv4.2. Importantly, if these channels can be selectively activated in ES cell-derived neurons, we may obtain a wide range of phenotypes for cell replacement in heterogeneously-functioning ganglia. Further differentiation may occur with increased concentrations of neurotrophic factors; conversely, these channels might also be preferentially modulated by blocking NTF signaling (*Lu et al.*, 2003). qRT-PCR analysis indicated that these cells express genes related to BDNF, NT3, and GDNF production. Additionally, a majority of the non-neuronal cells in these cultures were positive for both Kv4.2 (Fig 4.3, yellow asterisks examples) and neurotransmitter markers (Fig 4.5). This is a major limitation in using RNA analysis from entire cultures as a measure of phenotypic and functional changes within relatively few neurons. Molecular techniques must always be carefully weighed against the histological for a better idea of whether the target cells have truly increased their expression of a particular marker. The approach does have it strengths in earlier cultures, however, where a greater proportion of cells are neuronal.

Neurog1-induced cells acquired an excitatory, glutamatergic identity regardless of exposure to NTFs, expressing VGLUTs 1 and 2 and also expressing the dopaminergic marker tyrosine hydroxylase (TH) at high levels reflected in both RNA and cytochemical assays. VGAT was not detected in the qRT-PCR analysis. Neurog1 may establish a firm neurotransmitter identity that NTFs cannot modulate through receptor activation, although evidence suggests that Neurog1 itself does not directly regulate genes related to neurotransmitter phenotype (*Seo et al.*, 2007). More likely, downstream effectors of Neurog1 must somehow promote the excitatory phenotype. Since glutamate is among the most abundantly-utilized neurotransmitters in the nervous system, it is so fundamental in cell-cell communication and information processing that ES cells may already have a natural bias toward glutamatergic differentiation (*Wu et al.*, 2007; *Silva et al.*, 2009). Additionally, glutamate may not be a specific marker, as

virtually every cell in our body utilizes this amino acid, however small the amount. Furthermore, the presence of tyrosine hydroxylase implies dopaminergic transmission, but does not indicate through which receptor the message is mediated, and therefore does not indicate whether the cells are excitatory or inhibitory (*Berry and Cottrell*, 1975). The possible multi-transmitter identity predicted by co-labeling of TH and VGLUT1 (Figure 4.5) is not uncommon in the brain (*Chuhma et al.*, 2009).

BDNF-treated cultures had significantly more neurites compared to NT-3 treated cultures, the latter showing no difference with Neurog1-induced cells without NTF exposure. While these results do not isolate a specific response of neurites to NTF application - presumably the presence of neurites would be correlated with the number of neurons present, which is increased after exposure to NTFs (*Reyes et al.*, 2008) - BDNF has been previously implicated in promoting neurite outgrowth from cultured SGN and cortical neurons (*Kimpinski et al.*, 1997; *Labelle and Leclerc*, 2000; *Gillespie et al.*, 2001; *Evans et al.*, 2009). The elevated presence neurites as measured by our approach alone will therefore not identify a neurite outgrowth mechanism over a neurotrophic mechanism. Additionally, the many cell clusters that form in all the cultures must be systematically characterized in more detail in order to tease out the underlying mechanisms potentially affecting neuritogenesis: are cells within the clusters predominantly glial-like, undifferentiated stem cells, or immature neurons? What potential guidance or trophic factors might these clusters secrete into the microenvironment, and do the amounts vary across experimental conditions?

The new area of functional cell replacement using stem cell-derived neurons is filled with challenges and questions intriguing to both developmental biologists and neuroscientists. Testing the specific transcriptional events that direct functional differentiation is a critical part of creating desired phenotypes. Consideration must be given to the neurotransmitter and electrochemical identity: should ES cell-derived neurons achieve a specific functional profile before implantation (*ex vivo* manipula-

tion), or will the microenvironment of damaged tissue sufficiently promote differentiation of cells into functional neurons? Functional recovery may be reached sooner if differentiating cells *in vivo* receive cues that accelerate the expression of certain channels, such as those that modulate resting membrane potential or action potential patterns.

Sensory neuron	Neurotransmitter expression	Electrophysiological characteristics	Voltage-sensitive channels identified as contributing to function	Channel expression profiles detected by...	References
Spiral ganglion	Glutamate, Dopamine, Glycine, Serotonin	Rapid to fast adapting for processing high to low frequency acoustic stimuli	Kv1.1, Kv1.2, Kv1.4, Kv3.1, Kv4.2, Kv7.2-7.5, Nav1.2, Nav1.6, Nav1.7	RT-PCR, IHC, electrophysiology	Eybalin 85, Adamson et al 2002, Biesel et al 2000, Hossain et al 2005, Ruel et al 2006, Long et al 2008, Fryatt et al 2009
Vestibular ganglion	Glutamate, Aspartate, Acetylcholine	Detection of linear and angular acceleration, spatial information	Kv7.4, Kv1.2, Kv1.4, TTX-sensitive sodium currents	Quantitative and RT-PCR, IHC	Yamashita et al, 1991; Rocha-Sanchez et al 2007; Iwasaki et al 2008; Hotchkiss et al 2005
Trigeminal ganglion	Glutamate, Serotonin, Substance P	Tactile, nociceptive sensory information from facial areas	Kv1.5 , Kv2.1, Kv4.3	Electrophysiology, Quantitative PCR	Yamashita et al 2001, Andrews et al 2001, Liu et al 2003
Dorsal Root Ganglion	Glutamate, Acetylcholine, Substance P, Monoamines	Tactile, nociceptive, proprioceptive sensory information from the body	Kv1.1, Kv1.2, Kv1.4, Nav1.2, Nav1.6, Nav1.8, Nav1.9	IHC, Quantitative PCR	Price & Mudge 1983, Rasband et al 2001, Bilsland & Hayter 2007, Djouhri et al 2003
Neurog1-induced differentiated ES cell	Glutamate, Tyrosine Hydroxylase (Dopaminergic)	All of the above?	Kv1.1, Kv1.2, Kv1.3, Kv1.6, Kv3.1, Kv4.2, Kv4.3, Kv7.2, Nav1.3, Nav1.6, Nav1.7	Quantitative PCR, immunocytochemistry	Hernandez et al, 2010 *in preparation*
Neurog1-induced differentiated ES cell +BDNF or +NT-3	Glutamate, Tyrosine Hydroxylase (Dopaminergic)	All of the above?	Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.6, Kv3.1, Kv4.2, Kv4.3, Kv7.2, Nav1.3, Nav1.6, Nav1.7	Quantitative PCR, immunocytochemistry	Hernandez et al, 2010 *in preparation*

Figure 4.7: Comparison of the functional phenotypes of sensory neurons and ES cell-derived neurons

CHAPTER V

Conclusions and Future Directions

5.1 Significance

This work details progress toward overcoming challenges in embryonic stem (ES) cell-based therapeutics. If the steps in their differentiation could be precisely controlled and manipulated, ES cells offer incredible potential for replacing cells lost due to injury, disease, or aging. One of the biggest hurdles toward this end is our limited knowledge of the many factors involved in directing differentiation *in vivo*. The "century of biology" is upon us, and new technologies have given us novel, significant insights into both the developmental origins of specific phenotypes and the bases of diseases that arise in certain cell populations. With this information we can attempt to recreate a microenvironment that may bring ES cells closer to mimicking functional phenotypes *in vivo*. Conversely, if we pinpoint the precise genetic mutations or protein modifications that gave rise to a certain pathology, we may find more effective ways to treat it pharmacologically or with gene therapy.

The loss of specialized tissues is currently the main limiting factor in the advancement of new technologies to restore sensations. Electronic devices can only compensate for a fraction of the biological functions evolved over thousands of years. The cochlear implant (CI) is one of the best examples of such a technology, as its ability to restore hearing apparently plateaus as the number of specialized neurons

available for stimulation decreases. The CI attempts to simulate the cochlea's natural ability to transduce mechanical sound waves into electrical messages delivered to the auditory nerve, and has been able to provide some restoration of hearing to many patients. Several limitations remain, however, before this device can benefit the estimated 30 million Americans who suffer from some form of hearing loss. On the engineering side, algorithms to better decompose the "temporal fine structure" of acoustic waveforms in the environment are necessary to improve speech perception and music appreciation. On the biological side, irreversible loss of the spiral ganglion neurons constituting auditory nerve prevents stimulation from CI electrodes from reaching the CNS and restoring hearing.

In characterizing mouse ES cell-derived neurons, we were not necessarily seeking a population with the full, identical complement of unique markers as individual SGN. In fact, given the heterogeneity in electrochemical properties and morphologies, it would be difficult to identify exactly what the subtype requires: at the very least, differentiated cells must show a bipolar morphology capable of receiving inputs at their dendrites and conveying that message within endogenous glutamatergic networks linking the cochlea to the brain. ES cell-derived neurons must additionally react to depolarizing electrical signals emanating from CI electrodes; the ability of these cells to open specific combinations of ion channels to permit electrochemical flux across the membrane is what enables neuron-to-neuron communication. Many of these features may be ascertained using gene transfer and application of inductive signals, which can then be assessed using histological and electrophysiological techniques.

5.1.1 Translating *in vitro* findings to *in vivo* therapies

In this research, ES cells were induced to differentiate into neurons under the premise that overexpression of the proneural bHLH transcription factor Neurog1

would initiate a series of orchestrated events leading to the emergence of an SGN-like phenotype (*Ma et al.*, 1996, 2000; *Fode et al.*, 1998; *Sun et al.*, 2001; *Fritzsch*, 2003). Relatively recent inducible transgene technology (*Kyba et al.*, 2002) allowed us to more closely recapitulate SGN developmental cues, reproducing the in the transient nature of Neurog1 expression in the ear. We obtained widespread differentiation of ES cells into neurons after only two to three days of forced Neurog1 expression and *in vitro* differentiation on a monolayer. These cells co-expressed markers for early neuronal differentiation (e.g. β-III tubulin) and glutamatergic neurotransmission (e.g. VGLUTs 1 and 2), and for the large part appeared to have a bipolar morphology extending processes in opposite directions from its cell body.

We assessed the precise mechanism of the specific downstream effectors mediating this differentiation after Neurog1 expression and found our cells transiently expressed other transcription factors, specifically NeuroD1, Brn3a, and Gata3, all akin to an established sensory neuron developmental pathway (*McEvilly et al.*, 1996; *Kim et al.*, 2001; *Huang et al.*, 2001; *Karis et al.*, 2001). This result may not be particularly surprising, given Neurog1 has been found to selectively activate the expression of genes that encode not only these transcription factors but also genes for neurotransmission and neurotrophic factors (*Schuurmans et al.*, 2004). Interestingly, after Neurog1 induction the cells do show plasticity after exposure to growth factors (*Velkey*, 2005). bHLH transcription factors are quite ancient as they retain structural similarities across all vertebrate species (*Fritzsch and Beisel*), suggesting a critical, early role in the neurogenesis process.

Neurog1 is not exclusive to the SGN phenotype, as it is also critical to the development of other sensory neurons derived from neurogenic placodes in the epidermal ectoderm (*Fode et al.*, 1998; *Ma et al.*, 1998, 1999; *Begbie et al.*, 2002). Additional modulation of Neurog1's initial pan-neuronal activation may help guide differentiating ES cells toward a more specific phenotype. This may be achieved by exposing

cells to NTFs (examined in this dissertation), or possibly (in addition) FGFs, Wnts, BMPs, Hhs, other small molecules that modulate gene expression. The fact that Neurog1-positive cells alone have electrically excitable membrane properties implies that these cells may be capable of replacing other neurons derived from the sensory placode, such as the trigeminal and vestibular ganglion (*Huang et al.*, 2001). We therefore can not limit our comparison of these cells with SGN alone: further modulation of firing properties and morphology using NTFs may create neurons with the potential to replace the range of Neurog1-dependent sensory neurons.

Neuronal differentiation following transient Neurog1 expression selectively upregulated expression of the BDNF and NT-3 receptors Ntrk2 and Ntrk3, but did not significantly upregulate or affect the NGF receptor Ntrk1. This expression profile closely matched that of auditory nerve (*Don et al.*, 1997; *Catania et al.*, 2007), suggesting that Neurog1 positions cells to maximally respond to these two NTFs, not unlike the spiral ganglion in development (*Ernfors et al.*, 1995; *Farinas et al.*, 1998, 2001; *Agerman et al.*, 2003). Upon further activation of the Ntrk2 receptor with BDNF, Neurog1 ES cell-derived neurons appeared to be enriched for the SGN phenotype based on elevated levels of Kv1.4, Kv3.1, Kv4.2 mRNA transcripts. These channels likely contribute to rapid adaptation properties identified in a majority of SGN (*Mo and Davis*, 1997; *Adamson et al.*, 2002b; *Zhou et al.*, 2005), and may have a role in the fast inactivation recorded in Neurog1-induced cells using electrophysiology (Chapter III). Modulating channel expression via NTF exposure may therefore enrich ES cell differentiation toward a different firing pattern.

Toward the end of spiral ganglion neuron replacement, we developed a novel technique that allowed for implantation of undifferentiated Neurog1-inducible ES cells into the basal scala tympani compartment within the relatively immunoprivileged, deafened guinea pig cochlea. SGN density is drastically reduced five weeks after administration of aminoglycoside drugs, creating an animal model of sensorineural

hearing loss. Osmotic pumps implanted subdermally on the guinea pigs' backs allowed for continuous delivery of a tetracycline analogue (Doxycycline, Dox) into the base of the cochlea. Once bound to the reverse tet-transactivator in this stem cell line, Dox initiates transcription of the Neurog1 transgene within the HPRT locus (*Kyba et al.*, 2002; *Velkey*, 2005). Two days delivery of Dox to implanted cells transiently activated Neurog1 transcription, and was followed by four weeks delivery of BDNF and GDNF to the cells. We controlled for bystander effects by implanting cells with BDNF and GDNF infusion but without induction of the Neurog1 transgene to determine if differentiation was due to induction into the neuronal lineage via Neurog1 rather than physiological effects stimulated by the act of implanting stem cells.

Four weeks after implantation animal subjects were sacrificed and their implanted cochleae were assessed for stem cell differentiation. We found differentiation *in vivo* closely matched that observed *in vitro*, with a majority of Neurog1-induced ES cells taking on a neuronal, glutamatergic phenotype. The cells also expressed NeuroD and Gata3, transcription factors downstream of Neurog1 that further-hone the SGN phenotype in development and upregulate NTF receptors that allow for the transduction of the physiological response to NTF exposure. These studies demonstrated that recapitulation of developmental factors that augment differentiation of ES cells into the target phenotype *in vitro* can be successfully recapitulated *in vivo* using various drug delivery methods.

5.2 Future directions

5.2.1 Neurite outgrowth and guidance

In patients with sensorineural hearing loss, stem cells implanted into the scala tympani and/or modiolar region must migrate or extend neuronal processes into Rosenthal's canal where endogenous SGN once resided. Further characterization of

the extracellular matrices present in the internal. A technique to attract process outgrowth toward the cochlear nucleus would provide significant advancements toward the end of connecting CI electrode in the scala tympani with the CNS. Potentially, a simultaneous delivery of stem cells with promoters of neurite outgrowth may enhance that extension, but the directionality may be lost. A new study in spinal cord regeneration demonstrated lentiviral delivery of the gene encoding BDNF to cells rostral to the site of stem cell transplantation created a gradient of BDNF that attracted stem cell-derived neurites toward the gradient (*Bonner et al.*, 2010). In the auditory system it may be possible to transfect fibroblasts producing ECM within Rosenthal's canal or the internal auditory meatus to generate a similar gradient to attract implanted stem cells toward the CNS.

A rigorous assessment of neurite outgrowth in cultures of heterogeneously differentiating ES cells is one of the more difficult aspects of research of this type. While it is crucial to determine what factors could potentially attract neurite sprouting toward the site of CI electrode, creating a controlled culture in which to systematically test and analyze varying concentrations is quite challenging. Comparmentalized cultures (*Campenot*, 1979; *Kimpinski et al.*, 1997; *Pazyra-Murphy and Segal*, 2008) provide one avenue to study this question. In these so-called "Campenot chambers", putative guidance factors penetrate through a seal between compartments as a gradient into the chamber containing neurons. If the neurons show a positive response in the presence of that factor, then they will project neurites toward that compartment. This approach would also allow us to test for the ability of nanofiber material to orient neurites and affect polarity. Additionally, cochlear nucleus (CN) tissue with and without attractant or repellant molecules may be cultured in one of these compartments, giving us a great amount of power to test the potential for our cells to synapse with second order neurons in CN. As discussed in Chapter IV, neurotrophic factors may potentially serve as guidance cues for target finding in developing neurons.

The mechanisms that promote neurite outgrowth following differentiation of ES cells into neurons using Neurog1 must be carefully understood if we are to guide implanted cells toward the CNS. Given the role of NTFs in promoting neurite outgrowth in a number of different neuronal subtypes assessed (*Labelle and Leclerc*, 2000; *Gillespie et al.*, 2001; *Deumens et al.*, 2006; *Evans et al.*, 2009), and the enhanced SGN-like differentiation observed after application with BDNF and NT-3, these molecules are excellent candidates to test in the study of neurite guidance *in vivo*. Gelatin beads can be soaked in NTF solutions, and then implanted into tissue to the effect of redirecting developing neurites (*Mistretta and Liu*, 2006). Once the proper molecules have been identified, beads may be treated in a solution of these molecules and then implanted within the modiolus to both redirect peripheral process outgrowth back toward scala tympani, or along the central processes to guide implanted cells toward cochlear nucleus.

5.2.2 Replacement SGN and CI electrodes: what's feasible?

One of the exciting projects that has arose from this work is the search for an effective coating for the CI electrode that will enable ES cell-derived neurons to both adhere and differentiate. One collaborator has found that the glycosaminoglycan hyaluronan can create a three-dimensional matrix over the electrode, and Doxycycline can be infused into the matrix. Cells implanted into the Dox-infused hyaluronan showed increased neuronal differentiation immediately surrounding the electrode. Fig 5.1 demonstrates the electrode (yellow outline) surrounded by differentiating cells (red). Further investigation into the ability of this material to conduct electrical stimulation from the CI electrode, and optimization of the matrices used to adhere cells to the electrode, are currently required. Systematic testing of other extracellular matrix (ECM)-based polymers must also be assessed to gain maximum differentiation, neurite outgrowth, adhesion, and electric conductance. Eventually, ES cell-derived

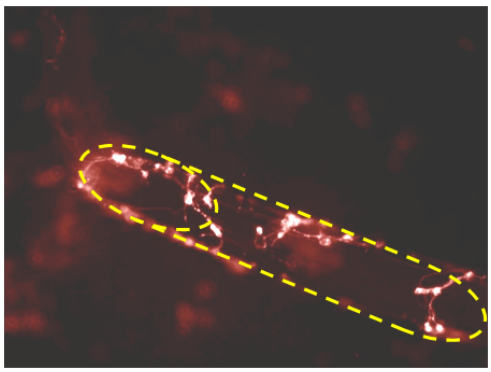

Figure 5.1: Neurog1-induced stem cells differentiate in 3D hyaluronon around CI electrode.

neurons treated to generate maximal responses to low or high frequency stimuli will need to be targeted to sites of stimulation along the implant electrode to enhance the encoding of these stimuli.

5.3 Final thoughts

The road toward effective embryonic stem cell-based therapies to replace the function of lost neurons is fraught with technical challenges, and the ethical debate will provide conversation for years to come. The incredible potential within a flask of ES cells to differentiate into any tissue of the body is well-documented, but this characteristic is a double-edged sword. Given the greater than 40,000 genes in the human genome, major regulatory pathways, such as those activated by Neurog1 must be identified, to enable increasingly specific recapitulation of the developmental cues that produce desired phenotypes. Should activation of and exposure to such effectors occur *ex vivo* immediately prior to implantation, or would more differentiated cells elevate the risk of immunogenic reaction from the host? Should subjects in the studies receive drugs for immune suppression to reduce the risk of inflammation? Conversely, is it safe to implant undifferentiated cells with immediate, localized delivery of such effectors, and if so how long should delivery be continued?

This work is based on the premise that modern day technologies have aided the human race toward understanding what weakens us, and preventing, dampening, or reversing the effects. The cochlear implant simulates the function of lost auditory hair cells by decomposing acoustic waveforms into individual frequencies converted into electric impulses, which then spread from electrodes implanted into the scala tympani. Electric current representing sound emanates to remaining auditory nerve fibers, restoring hearing in the brain. New biological interfaces between damaged tissues and prosthesis may restore sensation but the core limiting factor is our inability to accurately recreate the specialized functions of lost cells - whether biologically or electronically. Interestingly, new technologies have increased the noise level on planet Earth, and human progress is now inextricably tied to solving the problems that accompany human progress.

APPENDICES

APPENDIX A

Identification of Implanted Embryonic Stem Cells

A.1 Assessment of current methods for visualization

ES cells can be efficiently transfected and cloned to create stable cell lines. Once ES cells differentiate, maintained expression of transferred genes, or transgenes, is often difficult, as chromatin remodeling often silences once-active regions of the genome (*Efroni et al.*, 2008). In creating a cell line suitable for transplantation, ES cells must continue to express preferably a bioluminescent reporter for tracking. One method to circumvent differentiation-mediated transgene silencing would be to use a powerful, constitutively active promoter like the elongation factor-1α (EF1α) (*Wang et al.*, 2008) or the virally-derived cytomegalovirus (CMV) promoter to drive reporter expression (*Ward and Stern*, 2002; *Bagchi et al.*, 2006). This approach has not been particularly successful in ES cells: fluorescent proteins such as eGFP or mCherry must continue to fluoresce in these structures to allow unequivocal identification. Previous work in our collaborator's lab found neither EF1α, CMV, nor PGK promoters could not maintain transgene expression following differentiation (unpublished data), so we turned to the UbC promoter, for which there was strong evidence to support its abil-

ity to maintain widespread transgene expression in mice (*Schorpp et al.*, 1996; *Wulff et al.*, 1990).

Transfer of genes encoding fluorescent markers has limitations in differentiating ES cells. To visualize dynamic neurite process outgrowth, *Pratt et al.* (2000) engineered a transgenic ES cell line in which GFP was inserted into the locus encoding neuronal microtubule-associated protein Tau was expressed in differentiating cells. Once cells differentiated into the neuronal lineage and expressed Tau, GFP fluorescence occurred, providing a powerful in which to visualized dynamic process outgrowth (*Tucker et al.*, 2001; *Vierbuchen et al.*, 2010). Fluorescent lipophilic dyes have also been extensively studied for their retention in the membranes of transplanted cells, but these had major limitations in hematopoietic studies (*Wang et al.*, 2005; *Lassailly et al.*, 2010) because stem cells tend to shed their membranes as they differentiate, allowing for diffusion of dye particles into endogenous tissue. Gender matching female or male donor cells with male or female host animals may also allow for identification of transplanted cell using chromosomal markers for X or Y (*Parker et al.*, 2007).

The goal of this Appendix section is to underscore the importance of continuing efforts to overcome the many challenges in ES cell visualization after transplantation. Attempts from our lab to create effective ES cell labels will be described. We employed: (1) transfer of genes encoding bioluminescent proteins and (2) the application of membrane-bound fluorescent lipophilic labels.

A.2 Methods and Methods

ES cell maintenance. All cells were grown at 37 °C in 5% CO_2. Undifferentiated Neurog1-inducible ES cells were maintained on 0.1% gelatin-coated tissue culture flasks in sterile-filtered DMEM (Invitrogen 11965) with 10% ES cell-tested fetal bovine serum (Atlanta Biologicals), 5% embryonic stem cell supplement (DMEM with 21.65 mg/mL HEPES buffer, 3.63 mg/mL L-glutamine, and 63.63 ng/mL β-

mercaptoethanol). Leukemia inhibitory factor (recombinant human LIF, Millipore, Bedford, MA) was added to the ES cell growth medium at 500 ng/mL to retain ES cell pluripotency.

Neuronal Differentiation. ES cells were washed free of serum proteins in Ca^{2+}/Mg^{2+}-free HBSS followed by dissociation from maintenance medium using Hanks'-based enzyme free cell dissociation buffer (Invitrogen) with 1mM EDTA and re-plated for differentiation in serum-free medium consisting of 80% F12/DMEM, 20% Neurobasal medium, with B27 and N2 supplements (Invitrogen), 10 mM sodium pyruvate (Gibco/Invitrogen), termed "80:20" defined medium. Doxycycline (Dox) (Sigma, St Louis, MO) was added at 1 μg/mL to induced the Neurog1 transgene. $5x10^5$ cells per well were plated in six-well dishes or 35mm dishes, with 13 mm diameter Thermanox cell culture coverslips (Nunc, Naperville, IL) placed at the bottom of each well and coated with 0.1% gelatin (Sigma). To induce expression of the Neurog1 transgene, Dox was added to differentiation medium from 0-72h (or zero to three days *in vitro*, DIV) in experimental groups. At three DIV, Dox was washed off with 1x Hanks' Balanced Salt Solution (HBSS, Gibco). Control cells were simultaneously grown in differentiation media without the addition of Dox.

ES cell line stably expressing eGFP. $2.75x10^6$ N7-Neurog1 cells were plated in a 6cm dish and allowed to grow to 60-70% confluency. To create a stable ES cell line expressing eGFP, confluent cells were transfected with 2μg of the DNA encoding UbC promoter driven-eGFP and hygromycin resistance. Twenty-five to thirty percent transfection efficiency was achieved using Lipofectamine and PLUS reagent (Invitrogen). Selection with hygromycin for cells that integrated the UbC construct was followed by identification of individual colonies with well-defined edges that uniformly expressed eGFP. Colonies were transferred to a 96-well plate and dissociated with .25% trypsin and EDTA. Colonies that survived additional selection in puromycin (2 g/mL) and hygromycin (300 g/mL) were expanded to $75cm^2$ flasks in ES cell

maintenance medium with 500 ng/mL LIF. One subclone, K9, was chosen based on continuous, homogenous bright eGFP expression 24-48h after Doxycycline-induced (Dox) differentiation of cells in vitro.

Tau-GFP cell line to visualize neuronal cytoarchitecture. The same transfection protocol described above was employed to derive clones from the N7 line that expressed the pTP6 vector, a generous gift from Dr. Thomas Pratt at the University of Edinburgh (*Pratt et al.*, 2000). The vector described in the Pratt article has been widely used in investigations of dynamic neurite outgrowth in regeneration studies both *in vitro* and *in vivo* (*Wianny et al.*, 2008; *Li et al.*, 2009; *del Rio and Soriano*, 2010). Tau-GFP expression was driven by the chick β-actin (CBA) promoter in 20-25% of undifferentiated ES cells at high confluency. Cells were then dissociated with Hanks'-based dissociation buffer with 1mM EDTA and centrifuged at 3000 rpm for 2 minutes before being resuspended at a concentration of 200,000 cells/mL in DMEM without phenol red (Invitrogen 21063) on ice. Cells were transported to the Fluorescence-Activated Cell Sorting (FACS) facility at the University of Michigan Cancer Center and low stringency selection for cells that expressed GFP was performed by the facility operator. From the $25x10^6$ cells taken for sorting, we obtained approximately $12x10^6$ cells that were re-plated in ES cell maintenance medium at 500,000 cells per well in a 0.1% gelatin-coated 24-well cell culture dish.

Carboxyfluorescein succinimidyl ester (CFSE) Fluorescent Dye. The CFSE dye was purchased from Invitrogen and assessed *in vitro* for its potential to label undifferentiated cells *ex vivo* before implantation and *in vivo* differentiation. A 5mM stock solution of CFSE was prepared by adding 18 L of high grade DMSO to one vial of dye. CFSE was applied to undifferentiated cells to assess dye retainment from 0h, i.e. before initiation of differentiation. 4 L CFSE stock was added to 2mL HBSS to create a 10 M solution, and this was added to a flask of undifferentiated cells for 15 minutes at 37 °C. ES cell maintenance medium was reintroduced to the cells

for 1 hour to allow incorporation and ensure complete modification of the CFSE dye. Cells were washed once with 1x HBSS and then dissociated and plated at densities as described above, and then assessed at time points of interest after differentiation for dye retainment. To assess the ability of the dye to label already differentiating ES cells, dye was applied after differentiation to assess the efficacy of labeling. Adherent cells were washed once with 1x HBSS and 2 L of CFSE stock was added to 1mL 1x HBSS to create a 10 M solution, and this solution was added to the 35mm dish. Cells incubated in the dye for 15 min at 37C in 5% CO2 before another wash with 1x HBSS. Fresh differentiation medium was added back to the cells and incubated 1h. Cells either continued to grow for assessment at later time points, or were fixed with 4% PFA.

Immunocytochemistry methods. Coverslips containing differentiated cells were fixed in 4% paraformaldehyde and washed in phosphate buffered saline (Dulbecco's PBS, Gibco) 3 times before overnight incubation at 4C with the antibodies to the neuronal marker TUJ1 (class III β-tubulin, 1:300, Covance), diluted in 0.1% Triton-X in PBS. The coverslips were washed with D-PBS and TUJ1 binding was visualized using an Alexa 633 secondary (1:500, Molecular Probes, Carlsbad, CA).

A.3 Results

Ubiquitin Ligase C promoter. Neurog1-inducible ES cells (line N7) had been previously transfected with plasmid DNA encoding eGFP driven by the CMV promoter (Figure **??**A). Cells that integrated the DNA were resistant to the antibiotic hygromycin, and individual cells that showed bright eGFP expression and hygromycin resistance were selected for clonal expansion into a stable ES cell line uniformly expressing eGFP. Upon differentiation, very few (5%) of cells showed continue expression of eGFP after differentiation (Figure 1Aii). CMV was replaced with DNA encoding the UbC promoter, and N7 cells were transfected with the plasmid (Fig-

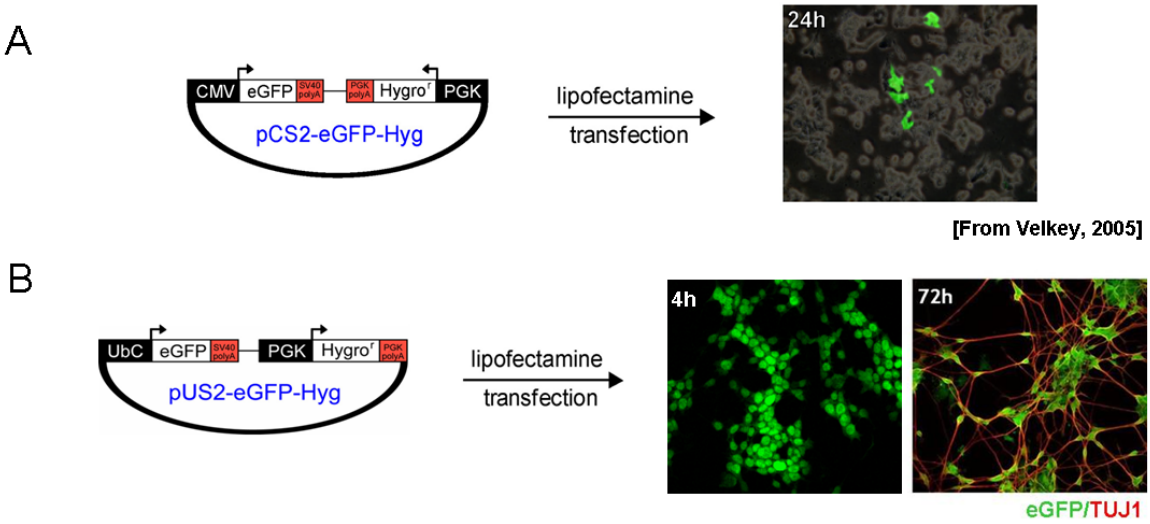

Figure A.1: UbC maintains transgene expression better than CMV, but methylation still occurs upon differentiation both *in vitro*

ure A.1B). Hygromycin resistant, eGFP positive cells were expanded into cell lines and the brightest line (K9) was used for differentiation studies. While we found a higher percentage of cells maintained eGFP expression immediately after initiation of differentiation, a majority of differentiated cells downregulated the transgene by 3 DIV (72h), and we could not detect any eGFP expression in the extensive neurites *in vitro*.

Tau-GFP fusion protein. Tau (also known as MAPT) is a microtubule-associated protein transported from the neuronal cell body down axons during nerve cell development (*Binder et al.*, 1985). The Tau gene can be fused to a reporter such as β-galactosidase or eGFP to visualize dynamic neurite outgrowth in developing neurons (*Callahan and Thomas*, 1994; *Pratt et al.*, 2000; *Tucker et al.*, 2001). In these contexts, the fluorophores are fused to the C-terminus of the Tau protein, revealing fluorescent cytoplasm in neurons that are ideal for visualizing axons and dendrites. We hypothesized that we could create a cell line without antibiotic selection for integrated transgenes through sequential sorting experiments using FACS. We predicted

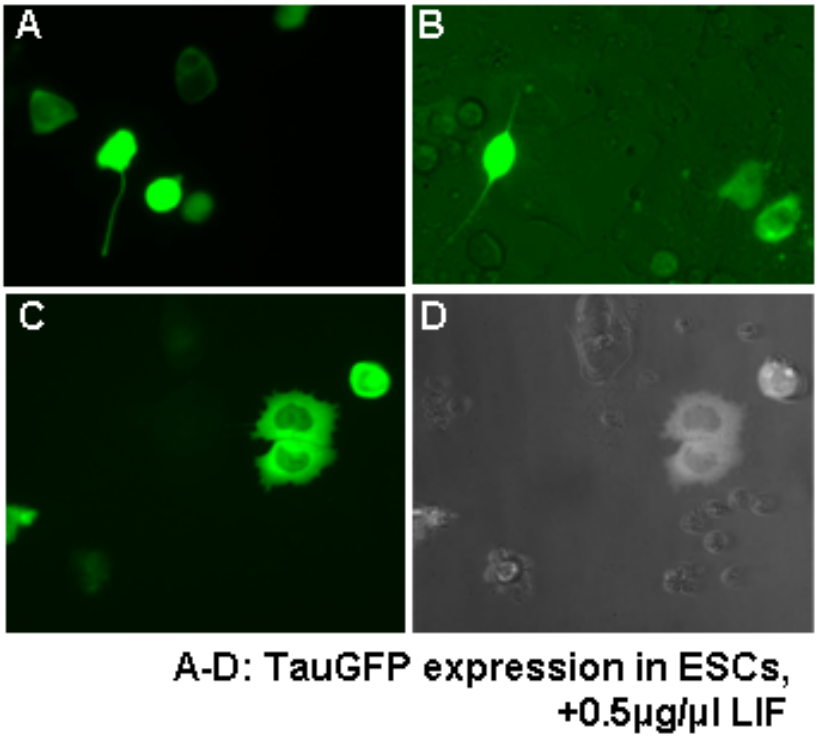

Figure A.2: Tau induces undefined differentiation in ES cells

that undifferentiated cells of our inducible Neurog1 line would integrate the Tau-eGFP fusion protein in maintenance medium, and only upon Dox-induced differentiation would we be able to visualize the extensive network of neurites that develop in vitro without resorting to the aid of immunocytochemical techniques.

More CFSE. The fluorochrome dye carboxyfluorescein succinimidyl ester (CFSE) was tested for its retainment in cells as they transform from undifferentiated to differentiated states. The dye was applied to undifferentiated cells in maintenance medium and allowed to incorporate before being dissociated and resuspended in differentiation medium containing Dox to induce Neurog1 expression. We found that the dye was retained in fewer than 20% of cells after 48h differentiation, and continued to dilute throughout the developing neurons as they underwent extreme morphological and neurochemical changes over time. The dye will likely not serve as an effective way to visualization the progress of undifferentiated ES cells that are induced to differentiate

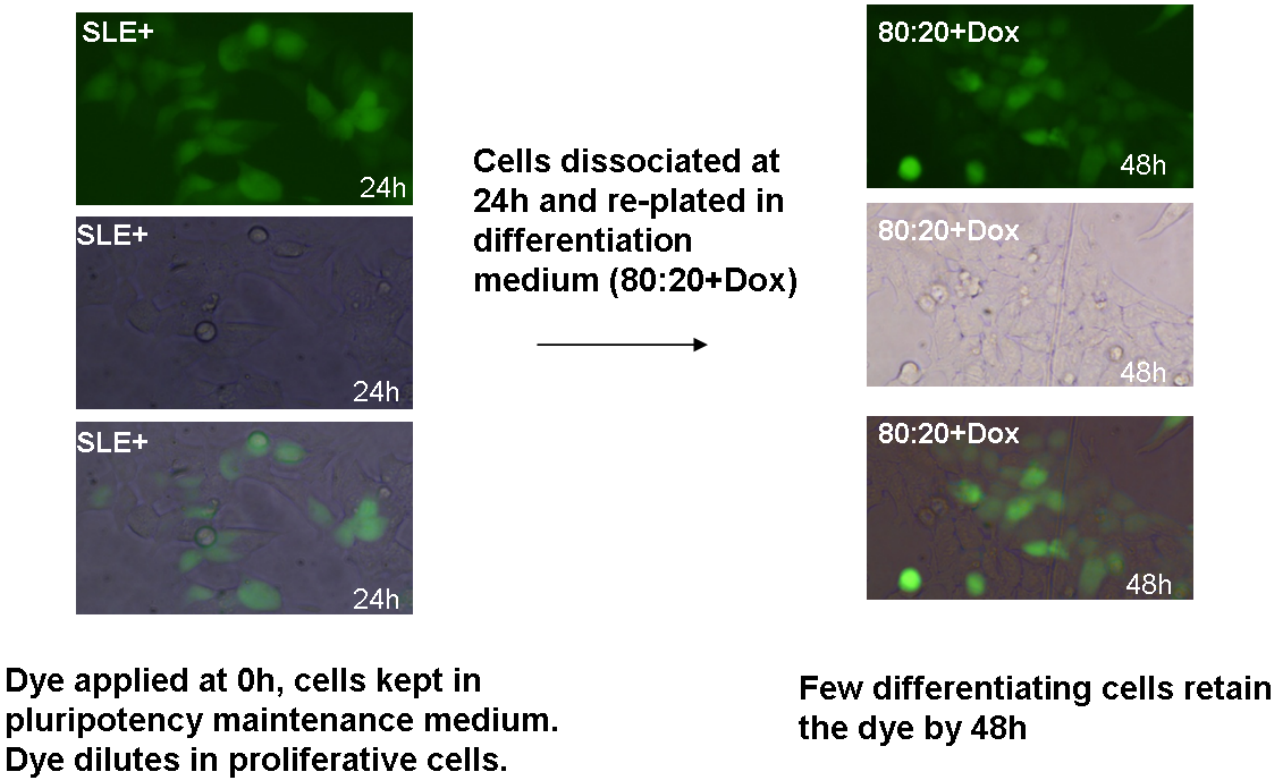

Figure A.3: The fluorochrome CFSE shows some degree of retainment depending on the differentiating phase of ES cells at the time of application

in vivo. Figure A.3 (*Wang et al.*, 2005)

A.4 Discussion

In this Appendix I outlined various attempts used in generating ES cell lines that would allow for effective *in vivo* tracking after implantation. The ideal label would remain active after differentiation, and allow for both clear distinction of implanted cells from donor tissues and visualization of phenotypic structural components such as neurite outgrowth. As detailed in previous chapters, the major goal of our studies is to connect ES cell-derived neurons implanted in the periphery to midbrain structures in the CNS. The most common technique utilizes gene transfer, with constitutive promoters driving expression of transgene, but as detailed above, this technique is

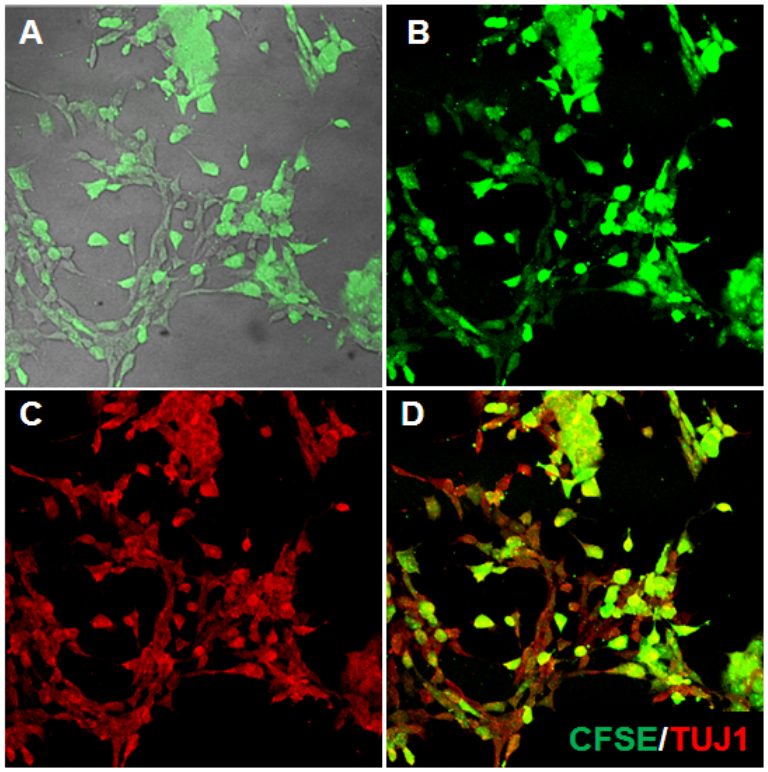

Figure A.4: Upon differentiation, cells do not efficiently retain the fluorescent label CFSE

not a reliable way of tracking differentiating ES cells. This problem may potentially be circumvented through more effective integration of transgenes into the genome using other delivery methods (*Liew et al.*, 2007; *Zaragosi et al.*, 2007; *Davidson et al.*, 2009). New avenues of cell labeling involve the use of magnetic nanoparticles that diffuse throughout the cytoplasm and allow for visualization of the entire cell using magnetic resonance imaging (MRI) (*Bulte et al.*, 2001; *Bos et al.*, 2004; *Thorek et al.*, 2006). It may also be necessary to target a transgene encoding fluorescent proteins directly to a locus containing a neuronal promoter, so once the cells differentiate into neurons, they express the marker.

Downregulation of transgenes in differentiating cells could also be caused by the random integration into a region of the stem cell genome inactivated subsequent to differentiation. One way to protect against these possible effects is with insulators, a special class of DNA that can block heterochromatin-mediated silencing, thereby preventing downregulation of the gene *Geyer and Clark* (2002). The β-globin intron is one such insulator and has been shown to generate uniform transgene expression in xenopus embryos (*Sekkali et al.*, 2008) and reduce variability in transgenic mice (*Potts et al.*, 2000). Insertion of the β-globin insulator sequences flanking eGFP may prevent downregulation of the transgene upon ES cell differentiation.

BIBLIOGRAPHY

BIBLIOGRAPHY

Abe, K. (2000), Therapeutic potential of neurotrophic factors and neural stem cells against ischemic brain injury.

Abranches, E., M. Silva, L. Pradier, H. Schulz, O. Hummel, D. Henrique, and E. Bekman (2009), Neural differentiation of embryonic stem cells in vitro: a road map to neurogenesis in the embryo, *PLoS One*, *4*(7), e6286.

Adamska, M., H. Herbrand, M. Adamski, M. Krger, T. Braun, and E. Bober (), Fgfs control the patterning of the inner ear but are not able to induce the full ear program, *Mech Dev*, *109*(2), 303–13.

Adamson, C. L., M. A. Reid, and R. L. Davis (2002a), Opposite actions of brain-derived neurotrophic factor and neurotrophin-3 on firing features and ion channel composition of murine spiral ganglion neurons, *J Neurosci*, *22*(4), 1385–96.

Adamson, C. L., M. A. Reid, Z. L. Mo, J. Bowne-English, and R. L. Davis (2002b), Firing features and potassium channel content of murine spiral ganglion neurons vary with cochlear location, *J Comp Neurol*, *447*(4), 331–50.

Agerman, K., J. Hjerling-Leffler, M. P. Blanchard, E. Scarfone, B. Canlon, C. Nosrat, and P. Ernfors (2003), Bdnf gene replacement reveals multiple mechanisms for establishing neurotrophin specificity during sensory nervous system development, *Development*, *130*(8), 1479–91.

Agterberg, M. J., H. Versnel, L. M. van Dijk, J. C. de Groot, and S. F. Klis (2009), Enhanced survival of spiral ganglion cells after cessation of treatment with brain-derived neurotrophic factor in deafened guinea pigs, *J Assoc Res Otolaryngol*, *10*(3), 355–67.

Ahn, M., D. Beacham, R. E. Westenbroek, T. Scheuer, and W. A. Catterall (2007), Regulation of na(v)1.2 channels by brain-derived neurotrophic factor, trkb, and associated fyn kinase, *J Neurosci*, *27*(43), 11,533–42.

Altschuler, R. A., K. S. O'Shea, and J. M. Miller (2008), Stem cell transplantation for auditory nerve replacement, *Hear Res*, *242*(1-2), 110–6.

Alvarez, Y., et al. (2003), Requirements for fgf3 and fgf10 during inner ear formation, *Development*, *130*(25), 6329–38.

Amit, M., M. Carpenter, M. Inokuma, C. P. Chiu, C. P. Harris, M. Waknitz, J. Itskovitz-Eldor, and J. thomson (2000), Clonally derived human embryonic stem cell lines maintain pluripotency and proliferativep otential for prolonged periods of culture, *Dev Biol*, *227*, 271–8.

Andrews, E. M., and D. L. Kunze (2001), Voltage-gated k+ channels in chemoreceptor sensory neurons of rat petrosal ganglion, *Brain Res*, *897*(1-2), 199–203.

Bagchi, B., M. Kumar, and S. Mani (2006), Cmv promotor activity during es cell differentiation: potential insight into embryonic stem cell differentiation, *Cell Biol Int*, *30*(6), 505–13.

Bain, G., D. Kitchens, M. Yao, J. E. Huettner, and D. I. Gottlieb (1995), Embryonic stem cells express neuronal properties in vitro, *Dev Biol*, *168*(2), 342–57.

Bakondi, G., A. Por, I. Kovacs, G. Szucs, and Z. Rusznak (2008), Voltage-gated k+ channel (kv) subunit expression of the guinea pig spiral ganglion cells studied in a newly developed cochlear free-floating preparation, *Brain Res*, *1210*, 148–62.

Balasubramaniyan, V., A. H. de Haas, R. Bakels, A. Koper, H. W. Boddeke, and J. C. Copray (2004), Functionally deficient neuronal differentiation of mouse embryonic neural stem cells in vitro, *Neurosci Res*, *49*(2), 261–5.

Barald, K. F., and M. W. Kelley (2004), From placode to polarization: new tunes in inner ear development, *Development*, *131*(17), 4119–30.

Begbie, J., M. Ballivet, and A. Graham (2002), Early steps in the production of sensory neurons by the neurogenic placodes, *Mol Cell Neurosci*, *21*(3), 502–11.

Beisel, K. W., N. C. Nelson, D. C. Delimont, and B. Fritzsch (2000), Longitudinal gradients of kcnq4 expression in spiral ganglion and cochlear hair cells correlate with progressive hearing loss in dfna2, *Brain Res Mol Brain Res*, *82*(1-2), 137–49.

Bermingham, N. A., B. A. Hassan, S. D. Price, M. A. Vollrath, N. Ben-Arie, R. A. Eatock, H. J. Bellen, A. Lysakowski, and H. Y. Zoghbi (1999), Math1: an essential gene for the generation of inner ear hair cells, *Science*, *284*(5421), 1837–41.

Berry, M. S., and G. A. Cottrell (1975), Excitatory, inhibitory and biphasic synaptic potentials mediated by an identified dopamine-containing neurone, *Journal of Physiology*, *244*, 589–612.

Bertrand, N., D. S. Castro, and F. Guillemot (2002), Proneural genes and the specification of neural cell types, *Nat Rev Neurosci*, *3*(7), 517–30.

Bertrand, O., P. F., and P. J. (1991), Evidence for a tonotopic organization of the auditory cortex observed with auditory evoked potentials, *Acta Oto-laryngologica*, *111*(491), 116–123.

Bianchi, L. M., Z. Daruwalla, T. M. Roth, N. P. Attia, N. W. Lukacs, A. L. Richards, I. O. White, S. J. Allen, and K. F. Barald (2005), Immortalized mouse inner ear cell lines demonstrate a role for chemokines in promoting the growth of developing statoacoustic ganglion neurons, *J Assoc Res Otolaryngol*, *6*(4), 355–67.

Bibel, M., J. Richter, K. Schrenk, K. L. Tucker, V. Staiger, M. Korte, M. Goetz, and Y. A. Barde (2004), Differentiation of mouse embryonic stem cells into a defined neuronal lineage, *Nat Neurosci*, *7*(9), 1003–9.

Biella, G., F. Di Febo, D. Goffredo, A. Moiana, V. Taglietti, L. Conti, E. Cattaneo, and M. Toselli (2007), Differentiating embryonic stem-derived neural stem cells show a maturation-dependent pattern of voltage-gated sodium current expression and graded action potentials, *Neuroscience*, *149*(1), 38–52.

Bilsland, J., M. Rigby, L. Young, and S. Harper (1999), A rapid method for semi-quantitative analysis of neurite outgrowth from chick drg explants using image analysis, *J Neurosci Methods*, *92*(1-2), 75–85.

Binder, L. I., A. Frankfurter, and L. I. Rebhun (1985), The distribution of tau in the mammalian central nervous system, *J Cell Biol*, *101*(4), 1371–8.

Black, J. A., S. Dib-Hajj, K. McNabola, S. Jeste, M. A. Rizzo, J. D. Kocsis, and S. G. Waxman (1996), Spinal sensory neurons express multiple sodium channel alpha-subunit mrnas, *Brain Res Mol Brain Res*, *43*(1-2), 117–31.

Blader, P., N. Fischer, G. Gradwohl, F. Guillemont, and U. Strahle (1997), The activity of neurogenin1 is controlled by local cues in the zebrafish embryo, *Development*, *124*, 4557–4569.

Blamey, P., et al. (1996), Factors affecting auditory performance of postlinguistically deaf adults using cochlear implants, *Audiol Neurootol*, *1*(5), 293–306.

Bocksteins, E., A. L. Raes, G. Van de Vijver, T. Bruyns, P. P. Van Bogaert, and D. J. Snyders (2009), Kv2.1 and silent kv subunits underlie the delayed rectifier k+ current in cultured small mouse drg neurons, *Am J Physiol Cell Physiol*, *296*(6), C1271–8.

Bodmer, D. (2008), Protection, regeneration and replacement of hair cells in the cochlea: implications for the future treatment of sensorineural hearing loss, *Swiss Med Wkly*, *138*(47-48), 708–12.

Bonner, J. F., A. Blesch, B. Neuhuber, and I. Fischer (2010), Promoting directional axon growth from neural progenitors grafted into the injured spinal cord, *J Neurosci Res*, *88*(6), 1182–92.

Bos, C., et al. (2004), In vivo mr imaging of intravascularly injected magnetically labeled mesenchymal stem cells in rat kidney and liver, *Radiology*, *233*(3), 781–9.

Bradley, A., M. Evans, M. H. Kaufman, and E. Robertson ().

Brocard, F., D. Verdier, I. Arsenault, J. P. Lund, and A. Kolta (2006), Emergence of intrinsic bursting in trigeminal sensory neurons parallels the acquisition of mastication in weanling rats, *J Neurophysiol*, *96*(5), 2410–24.

Brumwell, W. A., C. L. Hossain, D. K. Morest, and P. Bernd (2000), Role for basic fibroblast growth factor (fgf-2) in tyrosine kinase (trkb) expression in the early development and innervation of the auditory receptor: in vitro and in situ studies, *Exp Neurol*, *162*(1), 121–45.

Bulte, J. W., et al. (2001), Magnetodendrimers allow endosomal magnetic labeling and in vivo tracking of stem cells, *Nat Biotechnol*, *19*(12), 1141–7.

Calella, A. M., C. Nerlov, R. G. Lopez, C. Sciarretta, O. von Bohlen und Halbach, O. Bereshchenko, and L. Minichiello (2007), Neurotrophin/trk receptor signaling mediates c/ebpalpha, -beta and neurod recruitment to immediate-early gene promoters in neuronal cells and requires c/ebps to induce immediate-early gene transcription, *Neural Dev*, *2*, 4.

Callahan, C. A., and J. B. Thomas (1994), Tau-beta-galactosidase, an axon-targeted fusion protein, *Proc Natl Acad Sci U S A*, *91*(13), 5972–6.

Campenot, R. B. (1979), Independent control of the local environment of somas and neurites, *Methods Enzymol*, *58*, 302–7.

Carpenter, M. K., M. S. Inokuma, J. Denham, T. Mujtaba, C. P. Chiu, and M. S. Rao (2001), Enrichment of neurons and neural precursors from human embryonic stem cells, *Exp Neurol*, *172*(2), 383–97.

Catacuzzeno, L., B. Fioretti, D. Pietrobon, and F. Franciolini (2008), The differential expression of low-threshold k+ currents generates distinct firing patterns in different subtypes of adult mouse trigeminal ganglion neurones, *J Physiol*, *586*(Pt 21).

Catania, S., A. Germana, R. Cabo, F. J. Ochoa-Erena, M. C. Guerrera, J. Hannestad, J. Represa, and J. A. Vega (2007), Neurotrophin and trk neurotrophin receptors in the inner ear of salmo salar and salmo trutta, *J Anat*, *210*(1), 78–88.

Cau, E., G. Gradwohl, C. Fode, and F. Guillemot (1997), Mash1 activates a cascade of bhlh regulators in olfactory neuron progenitors, *Development*, *124*(8), 1611–21.

Chen, W., S. L. Johnson, W. Marcotti, P. W. Andrews, H. D. Moore, and M. N. Rivolta (2009a), Human fetal auditory stem cells can be expanded in vitro and differentiate into functional auditory neurons and hair cell-like cells, *Stem Cells*, *27*(5), 1196–204.

Chen, Z., M. Peppi, S. G. Kujawa, and W. F. Sewell (2009b), Regulated expression of surface ampa receptors reduces excitotoxicity in auditory neurons, *J Neurophysiol*, *102*(2), 1152–9.

Chi, X. X., B. S. Schmutzler, J. M. Brittain, Y. Wang, C. M. Hingtgen, G. D. Nicol, and R. Khanna (2009), Regulation of n-type voltage-gated calcium channels (cav2.2) and transmitter release by collapsin response mediator protein-2 (crmp-2) in sensory neurons, *J Cell Sci*, *122*(23), 4351–62.

Chikar, J. A., D. J. Colesa, D. L. Swiderski, A. Di Polo, Y. Raphael, and B. E. Pfingst (2008), Over-expression of bdnf by adenovirus with concurrent electrical stimulation improves cochlear implant thresholds and survival of auditory neurons, *Hear Res*, *245*(1-2), 24–34.

Cho, M. S., et al. (2008), Highly efficient and large-scale generation of functional dopamine neurons from human embryonic stem cells, *Proc Natl Acad Sci U S A*, *105*(9), 3392–7.

Chuhma, N., W. Y. Choi, S. Mingote, and S. Rayport (2009), Dopamine neuron glutamate cotransmission: frequency-dependent modulation in the mesoventromedial projection, *Neuroscience*, *164*(3), 1068–83.

Chung, S., T. Andersson, K. C. Sonntag, L. Bjorklund, O. Isacson, and K. S. Kim (2002), Analysis of different promoter systems for efficient transgene expression in mouse embryonic stem cell lines, *Stem Cells*, *20*, 139 145.

Coleman, B., J. Hardman, A. Coco, S. Epp, M. de Silva, J. Crook, and R. Shepherd (2006), Fate of embryonic stem cells transplanted into the deafened mammalian cochlea, *Cell Transplant*, *15*(5), 369–80.

Coleman, B., J. B. Fallon, L. N. Pettingill, M. G. de Silva, and R. K. Shepherd (2007), Auditory hair cell explant co-cultures promote the differentiation of stem cells into bipolar neurons, *Exp Cell Res*, *313*(2), 232–43.

Colley, B. S., K. C. Biju, A. Visegrady, S. Campbell, and D. A. Fadool (2007), Neurotrophin b receptor kinase increases kv subfamily member 1.3 (kv1.3) ion channel half-life and surface expression, *Neuroscience*, *144*(2), 531–46.

Colley, B. S., M. A. Cavallin, K. Biju, D. R. Marks, and D. A. Fadool (2009), Brain-derived neurotrophic factor modulation of kv1.3 channel is disregulated by adaptor proteins grb10 and nshc, *BMC Neurosci*, *10*, 8.

Conti, L., et al. (2005), Niche-independent symmetrical self-renewal of a mammalian tissue stem cell, *PLoS Biol*, *3*(9), e283.

Cooper, H., and L. Craddock (2006), *Cochlear Implants: A Practical Guide*, second ed., 425 pp., Whurr, West Sussex PO19 8SQ, England.

Cordon-Cardo, C., et al. (1991), The trk tyrosine protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3, *Cell*, *66*(1), 173–83.

Corrales, C. E., L. Pan, H. Li, M. C. Liberman, S. Heller, and A. S. Edge (2006), Engraftment and differentiation of embryonic stem cell-derived neural progenitor cells in the cochlear nerve trunk: growth of processes into the organ of corti, *J Neurobiol*, *66*(13), 1489–500.

Cummins, T. R., J. A. Black, D. D. Sulayman, and S. G. Waxman (2000), Glial-derived neurotrophic factor upregulates expression of functional sns and nan sodium channels and their currents in axotomized dorsal root ganglion neurons, *The Journal of Neuroscience*, *20*(23), 8754–8761.

Dallos, P. (1992), The active cochlea, *J Neurosci*, *12*(12), 4575–85.

Davidson, A. E., T. E. Gratsch, M. H. Morell, K. S. O'Shea, and C. E. Krull (2009), Use of the sleeping beauty transposon system for stable gene expression in mouse embryonic stem cells, *CSH Protoc*, *2009*(8), pdb prot5270.

Davis, R. L. (2003), Gradients of neurotrophins, ion channels, and tuning in the cochlea, *Neuroscientist*, *9*(5), 311–6.

de Graaf, R., and R. Bijl (2002), Determinants of mental distress in adults with a severe auditory impairment: differences between prelingual and postlingual deafness, *Psychosomatic Medicine*, *64*(1), 61–70.

del Rio, J. A., and E. Soriano (2010), Regenerating cortical connections in a dish: the entorhino-hippocampal organotypic slice co-culture as tool for pharmacological screening of molecules promoting axon regeneration, *Nat Protoc*, *5*(2), 217–26.

Denoyelle, F., S. Marlin, D. Weil, L. Moatti, P. Chauvin, E. N. Garabedian, and C. Petit (1999), Clinical features of the prevalent form of childhood deafness, dfnb1, due to a connexin-26 gene defect: implications for genetic counselling, *Lancet*, *353*(9161), 1298–303.

Deumens, R., G. C. Koopmans, R. J. Jaken, K. Morren, T. Comhair, S. Kosar, H. W. Steinbusch, C. G. Den Bakker, and E. A. Joosten (2006), Stimulation of neurite outgrowth on neonatal cerebral astrocytes is enhanced in the presence of bdnf, *Neurosci Lett*, *407*(3), 268–73.

Dinh, C. T., and T. R. Van De Water (2009), Blocking pro-cell-death signal pathways to conserve hearing, *Audiol Neurootol*, *14*(6), 383–92.

Djouhri, L., X. Fang, K. Okuse, J. N. Wood, C. M. Berry, and S. N. Lawson (2003), The ttx-resistant sodium channel nav1.8 (sns/pn3): expression and correlation with membrane properties in rat nociceptive primary afferent neurons, *J Physiol*, *550*(Pt 3), 739–52.

Djourno, A., and C. Eyries (1957), [auditory prosthesis by means of a distant electrical stimulation of the sensory nerve with the use of an indwelt coiling.], *Presse Med*, *65*(63), 1417.

Don, D. M., A. N. Newman, P. E. Micevych, and P. Popper (1997), Expression of brain-derived neurotrophic factor and its receptor mrna in the vestibuloauditory system of the bullfrog, *Hear Res*, *114*(1-2), 10–20.

Donato, R., E. A. Miljan, S. J. Hines, S. Aouabdi, K. Pollock, S. Patel, F. A. Edwards, and J. D. Sinden (2007), Differential development of neuronal physiological responsiveness in two human neural stem cell lines, *BMC Neurosci*, *8*, 36.

Driver, E. C., S. P. Pryor, P. Hill, J. Turner, U. Rther, L. G. Biesecker, A. J. Griffith, and M. W. Kelley (2008), Hedgehog signaling regulates sensory cell formation and auditory function in mice and humans, *J Neurosci*, *28*(29), 7350–8.

Drukker, M., H. Katchman, G. Katz, S. Even-Tov Friedman, E. Shezen, E. Hornstein, O. Mandelboim, Y. Reisner, and N. Benvenisty (2006), Human embryonic stem cells and their differentiated derivatives are less susceptible to immune rejection than adult cells, *Stem Cells*, *24*(2), 221–9.

Efroni, S., et al. (2008), Global transcription in pluripotent embryonic stem cells, *Cell Stem Cell*, *2*(5), 437–47.

Ernfors, P., T. Van De Water, J. Loring, and R. Jaenisch (1995), Complementary roles of bdnf and nt-3 in vestibular and auditory development, *Neuron*, *14*(6), 1153–64.

Evans, A. J., B. C. Thompson, G. G. Wallace, R. Millard, S. J. O'Leary, G. M. Clark, R. K. Shepherd, and R. T. Richardson (2009), Promoting neurite outgrowth from spiral ganglion neuron explants using polypyrrole/bdnf-coated electrodes, *J Biomed Mater Res A*, *91*(1), 241–50.

Eybalin, M. (1993), Neurotransmitters and neuromodulators of the mammalian cochlea, *Physiol Rev*, *73*(2), 309–73.

Fairchild, P. J., S. Cartland, K. F. Nolan, and H. Waldmann (2004), Embryonic stem cells and the challenge of transplantation tolerance, *Trends Immunol*, *25*(9), 465–70.

Farinas, I., G. A. Wilkinson, C. Backus, L. F. Reichardt, and A. Patapoutian (1998), Characterization of neurotrophin and trk receptor functions in developing sensory ganglia: direct nt-3 activation of trkb neurons in vivo, *Neuron*, *21*(2), 325–34.

Farinas, I., et al. (2001), Spatial shaping of cochlear innervation by temporally regulated neurotrophin expression, *J Neurosci*, *21*(16), 6170–80.

Fekete, D. M., and D. K. Wu (2002), Revisiting cell fate specification in the inner ear, *Curr Opin Neurobiol*, *12*(1), 35–42.

Ferron, S. R., M. A. Marques-Torrejon, H. Mira, I. Flores, K. Taylor, M. A. Blasco, and I. Farinas (2009), Telomere shortening in neural stem cells disrupts neuronal differentiation and neuritogenesis, *J Neurosci*, *29*(46), 14,394–407.

Finney, E. M., I. Fine, and K. R. Dobkins (2001), Visual stimuli activate auditory cortex in the deaf, *Nat Neurosci*, *4*(12), 1171–3.

Fode, C., G. Gradwohl, X. Morin, A. Dierich, M. LeMeur, C. Goridis, and F. Guillemot (1998), The bhlh protein neurogenin 2 is a determination factor for epibranchial placode-derived sensory neurons, *Neuron*, *20*(3), 483–494.

Forge, A., and J. Schacht (2000), Aminoglycoside antibiotics, *Audiol Neurootol*, *5*, 3–22.

Friedman, R. A., L. Makmura, E. Biesiada, X. Wang, and E. M. Keithley (2005), Eya1 acts upstream of tbx1, neurogenin 1, neurod and the neurotrophins bdnf and nt-3 during inner ear development, *Mech Dev*, *122*(5), 625–34.

Fritzsch, B. (2003), Development of inner ear afferent connections: forming primary neurons and connecting them to the developing sensory epithelia, *Brain Res Bull*, *60*(5-6), 423–33.

Fritzsch, B., and K. Beisel (), Evolution and development of the vertebrate ear, *Brain Res Bulletin*, *55*(6), 711–721.

Fritzsch, B., I. Farinas, and L. F. Reichardt (1997), Lack of neurotrophin 3 causes losses of both classes of spiral ganglion neurons in the cochlea in a region-specific fashion, *J Neurosci*, *17*(16), 6213–25.

Frolenkov, G. I., I. A. Belyantseva, T. B. Friedman, and A. J. Griffith (2004), Genetic insights into the morphogenesis of inner ear hair cells, *Nat Rev Genet*, *5*(7), 489–98.

Fryatt, A. G., C. Vial, M. Mulheran, M. J. Gunthorpe, and B. D. Grubb (2009), Voltage-gated sodium channel expression in rat spiral ganglion neurons, *Mol Cell Neurosci*, *42*(4), 399–407.

Geyer, P. K., and I. Clark (2002), Protecting against promiscuity: the regulatory role of insulators, *Cell Mol Life Sci*, *59*(12), 2112–27.

Gillespie, L. N., and R. K. Shepherd (2005), Clinical application of neurotrophic factors: the potential for primary auditory neuron protection, *Eur J Neurosci*, *22*(9), 2123–33.

Gillespie, L. N., G. M. Clark, P. F. Bartlett, and P. L. Marzella (2001), Lif is more potent than bdnf in promoting neurite outgrowth of mammalian auditory neurons in vitro, *Neuroreport*, *12*(2), 275–9.

Gossrau, G., J. Thiele, R. Konang, T. Schmandt, and O. Brustle (2007), Bone morphogenetic protein-mediated modulation of lineage diversification during neural differentiation of embryonic stem cells, *Stem Cells*, *25*(4), 939–49.

Gravagna, N. G., C. S. Knoeckel, A. D. Taylor, B. A. Hultgren, and A. B. Ribera (2008), Localization of kv2.2 protein in xenopus laevis embryos and tadpoles, *J Comp Neurol*, *510*(5), 508–24.

Green, S. H., R. A. Altschuler, and J. M. Miller (2008), *Cell death and cochlea protection*, 1 ed., 275-320 pp., Springer, New York, NY, USA.

Grillet, N., P. Kazmierczak, W. Xiong, M. Schwander, A. Reynolds, H. Sakaguchi, J. Tokita, B. Kachar, and U. Muller (2009), The mechanotransduction machinery of hair cells, *Sci Signal*, *2*(85), pt5.

Gu, X., and N. C. Spitzer (1995), Distinct aspects of neuronal differentiation encoded by frequency of spontaneous ca2+ transients, *Nature*, *29*(6534), 784–7.

Han, P., and M. T. Lucero (2006), Pituitary adenylate cyclase activating polypeptide reduces expression of kv1.4 and kv4.2 subunits underlying a-type k(+) current in adult mouse olfactory neuroepithelia, *Neuroscience*, *138*(2), 411–9.

Hansen, M. R., X. M. Zha, J. Bok, and S. H. Green (2001), Multiple distinct signal pathways, including an autocrine neurotrophic mechanism, contribute to the survival-promoting effect of depolarization on spiral ganglion neurons in vitro, *J Neurosci*, *21*(7), 2256–67.

Hartline, D. K., and A. M. Castelfranco (2003), Simulations of voltage clamping poorly space-clamped voltage-dependent conductances in a uniform cylindrical neurite, *J Comput Neurosci*, *14*(3), 253–69.

Hashino, E., R. Y. Dolnick, and C. S. Cohan (1999), Developing vestibular ganglion neurons switch trophic sensitivity from bdnf to gdnf after target innervation, *J Neurobiol*, *38*(3), 414–27.

Hegarty, J. L., A. R. Kay, and S. H. Green (1997), Trophic support of cultured spiral ganglion neurons by depolarization exceeds and is additive with that by neurotrophins or camp and requires elevation of [ca2+]i within a set range, *J Neurosci*, *17*(6), 1959–70.

Hildebrand, M. S., et al. (2008), Advances in molecular and cellular therapies for hearing loss, *Mol Ther*, *16*(2), 224–36.

Hong, S., D. Y. Hwang, S. Yoon, O. Isacson, A. Ramezani, R. G. Hawley, and K. S. Kim (2007), Functional analysis of various promoters in lentiviral vectors at different stages of in vitro differentiation of mouse embryonic stem cells, *Mol Ther*, *15*, 1630 1639.

Hossain, W. A., X. Zhou, A. Rutledge, C. Baier, and D. K. Morest (1996), Fibroblast growth factors (fgf-1 and fgf-2) promote migration and neurite growth of mouse cochlear ganglion cells in vitro: immunohistochemistry and antibody perturbation, *Exp Neurol*, *138*, 121–43.

Hossain, W. A., S. D. Antic, Y. Yang, M. N. Rasband, and D. K. Morest (2005), Where is the spike generator of the cochlear nerve? voltage-gated sodium channels in the mouse cochlea, *J Neurosci*, *25*(29), 6857–68.

Hossain, W. A., C. D'Sa, and D. K. Morest (2008), Interactive roles of fibroblast growth factor 2 and neurotrophin 3 in the sequence of migration, process outgrowth, and axonal differentiation of mouse cochlear ganglion cells, *J Neurosci Res*, *86*(11), 2376–91.

Hotchkiss, K., M. Harvey, M. Pacheco, and B. Sokolowski (2005), Ion channel proteins in mouse and human vestibular tissue, *Otolaryngol Head Neck Surg*, *132*(6), 916–23.

Hu, B., J. Weick, J. Yu, L. X. Ma, X. Q. Zhang, J. A. Thomson, and Z. S. C. (2010), Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency, *Proc Natl Acad Sci U S A*, *107*(9), 4335–40.

Hu, B. H., and G. L. Zheng (2008), Membrane disruption: an early event of hair cell apoptosis induced by exposure to intense noise, *Brain Res*, *6*(1239), 107–18.

Hu, Z., M. Ulfendahl, and N. P. Olivius (2004), Central migration of neuronal tissue and embryonic stem cells following transplantation along the adult auditory nerve, *Brain Res*, *1026*(1), 68–73.

Hu, Z., M. Andang, D. Ni, and M. Ulfendahl (2005a), Neural cograft stimulates the survival and differentiation of embryonic stem cells in the adult mammalian auditory system, *Brain Res*, *1051*(1-2), 137–44.

Hu, Z., D. Wei, C. B. Johansson, N. Holmstrom, M. Duan, J. Frisen, and M. Ulfendahl (2005b), Survival and neural differentiation of adult neural stem cells transplanted into the mature inner ear, *Exp Cell Res*, *302*(1).

Huang, E. J., and L. F. Reichardt (2001), Neurotrophins: roles in neuronal development and function, *Annu Rev Neurosci*, *24*, 677–736.

Huang, E. J., W. Liu, B. Fritzsch, L. M. Bianchi, L. F. Reichardt, and M. Xiang (2001), Brn3a is a transcriptional regulator of soma size, target field innervation and axon pathfinding of inner ear sensory neurons, *Development*, *128*(13), 2421–32.

Hurley, P. A., J. M. Crook, and R. K. Shepherd (2007), Schwann cells revert to non-myelinating phenotypes in the deafened rat cochlea, *Eur J Neurosci*, *26*(7), 1813–21.

Huxtable, R. J. (2001), Beethoven: a life of sound and silence, *Mol Interv*, *1*(1), 8–12.

Ibanez, C. F., and P. Ernfors (2007), Hierarchical control of sensory neuron development by neurotrophic factors, *Neuron*, *54*(5), 673–5.

Imura, T., K. Tane, N. Toyoda, and S. Fushiki (2008), Endothelial cell-derived bone morphogenetic proteins regulate glial differentiation of cortical progenitors, *Eur J Neurosci*, *27*(7), 1596–606.

Incesulu, A., and J. Nadol, J. B. (1998), Correlation of acoustic threshold measures and spiral ganglion cell survival in severe to profound sensorineural hearing loss: implications for cochlear implantation, *Ann Otol Rhinol Laryngol*, *107*(11 Pt 1), 906–11.

Inoue, T., A. Matsubara, S. Maruya, Y. Yamamoto, A. Namba, A. Sasaki, and H. Shinkawa (2006), Localization of dopamine receptor subtypes in the rat spiral ganglion, *Neurosci Lett*, *399*(3), 226–9.

Israsena, N., M. Hu, W. Fu, L. Kan, and J. A. Kessler (2004), The presence of fgf2 signaling determines whether beta-catenin exerts effects on proliferation or neuronal differentiation of neural stem cells, *Dev Biol*, *268*(1), 220–31.

Iwasaki, S., Y. Chihara, Y. Komuta, K. Ito, and Y. Sahara (2008), Low-voltage-activated potassium channels underlie the regulation of intrinsic firing properties of rat vestibular ganglion cells, *J Neurophysiol*, *100*(4), 2192–204.

Izumikawa, M., R. Minoda, K. Kawamoto, K. A. Abrashkin, D. L. Swiderski, D. F. Dolan, D. E. Brough, and Y. Raphael (2005), Auditory hair cell replacement and hearing improvement by atoh1 gene therapy in deaf mammals, *Nat Med*, *11*(3), 271–6.

Jagger, D. J., and G. D. Housley (2003), Membrane properties of type ii spiral ganglion neurones identified in a neonatal rat cochlear slice, *J Physiol*, *552*(Pt 2), 525–33.

Jessen, K. R., and R. Mirsky (2005), The origin and development of glial cells in peripheral nerves, *Nat Rev Neurosci*, *6*(9), 671–82.

Johnson, M. A., J. P. Weick, R. A. Pearce, and S. C. Zhang (2007), Functional neural development from human embryonic stem cells: accelerated synaptic activity via astrocyte coculture, *J Neurosci*, *27*(12), 3069–77.

Kageyama, R., T. Ohtsuka, H. Shimojo, and I. Imayoshi (2008), Dynamic notch signaling in neural progenitor cells and a revised view of lateral inhibition, *Nat Neurosci*, *11*(11), 1247–51.

Kanzaki, S., T. Stover, K. Kawamoto, D. M. Prieskorn, R. A. Altschuler, J. M. Miller, and Y. Raphael (2002), Glial cell line-derived neurotrophic factor and chronic electrical stimulation prevent viii cranial nerve degeneration following denervation, *J Comp Neurol*, *454*(3), 350–60.

Karis, A., I. Pata, J. H. van Doorninck, F. Grosveld, C. I. de Zeeuw, D. de Caprona, and B. Fritzsch (2001), Transcription factor gata-3 alters pathway selection of olivocochlear neurons and affects morphogenesis of the ear, *J Comp Neurol*, *429*(4), 615–30.

Karmody, C. S., and E. S. Bachor (2005), The deafness of ludwig van beethoven: an immunopathy, *Otol Neurotol*, *26*(4), 809–14.

Kawamoto, K., S. Ishimoto, R. Minoda, D. E. Brough, and Y. Raphael (2003), Math1 gene transfer generates new cochlear hair cells in mature guinea pigs in vivo, *J Neurosci*, *23*(11), 4395–400.

Kawamoto, K., S. H. Sha, R. Minoda, M. Izumikawa, H. Kuriyama, J. Schacht, and Y. Raphael (2004), Antioxidant gene therapy can protect hearing and hair cells from ototoxicity, *Mol Ther*, *9*(2), 173–181.

Keller, G. (2005), Embryonic stem cell differentiation: emergence of a new era in biology and medicine, *Genes and Development*, *19*(10), 1129–1155.

Kileny, P. R., S. Zimmerman-Phillips, J. L. Kemink, and S. P. Schmaltz (1991), Effects of preoperative electrical stimulability and historical factors on performance with multichannel cochlear implant, *Ann Otol Rhinol Laryngol*, *100*(7), 563–8.

Kim, S., Y. S. Yoon, J. W. Kim, M. Jung, S. U. Kim, Y. D. Lee, and H. Suh-Kim (2004), Neurogenin1 is sufficient to induce neuronal differentiation of embryonal carcinoma p19 cells in the absence of retinoic acid, *Cell Mol Neurobiol*, *24*(3), 343–56.

Kim, S. S., et al. (2008), Neural induction with neurogenin1 increases the therapeutic effects of mesenchymal stem cells in the ischemic brain, *Stem Cells*, *26*(9), 2217–2228.

Kim, W. Y., B. Fritzsch, A. Serls, L. A. Bakel, E. J. Huang, L. F. Reichardt, D. S. Barth, and J. E. Lee (2001), Neurod-null mice are deaf due to a severe loss of the inner ear sensory neurons during development, *Development*, *128*(3), 417–26.

Kimpinski, K., R. B. Campenot, and K. Mearow (1997), Effects of the neurotrophins nerve growth factor, neurotrophin-3, and brain-derived neurotrophic factor (bdnf) on neurite growth from adult sensory neurons in compartmented cultures, *J Neurobiol*, *33*(4), 395–410.

Klein, R., et al. (1991), The trkb tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3, *Cell*, *66*(2), 395–403.

Kondo, T., P. L. Sheets, D. A. Zopf, H. L. Aloor, T. R. Cummins, R. J. Chan, and E. Hashino (2008), Tlx3 exerts context-dependent transcriptional regulation and promotes neuronal differentiation from embryonic stem cells, *Proc Natl Acad Sci U S A*, *105*(15), 5780–5.

Korzh, V., and U. Strhle (2002), Proneural, prosensory, antiglial: the many faces of neurogenins, *Trends Neurosci*, *25*(12), 603–5.

Kvam, M. H., M. Loeb, and K. Tambs (2007), Mental health in deaf adults: symptoms of anxiety and depression among hearing and deaf individuals, *J Deaf Stud Deaf Educ*, *12*(1), 1–7.

Kyba, M., R. C. Perlingeiro, and G. Q. Daley (2002), Hoxb4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors, *Cell*, *109*(1), 29–37.

Labelle, C., and N. Leclerc (2000), Exogenous bdnf, nt-3 and nt-4 differentially regulate neurite outgrowth in cultured hippocampal neurons, *Brain Res Dev Brain Res*, *123*(1), 1–11.

Ladher, R. K., K. U. Anakwe, A. L. Gurney, G. C. Schoenwolf, and P. H. Francis-West (2000), Identification of synergistic signals initiating inner ear development, *Science*, *290*(5498), 1965–7.

Lanni, C., S. Stanga, M. Racchi, and S. Govoni (2010), The expanding universe of neurotrophic factors: therapeutic potential in aging and age-associated disorders, *Curr Pharm Des*, *16*(6), 698–717.

Lassailly, F., E. Griessinger, and D. Bonnet (2010), Microenvironmental contaminations induced by fluorescent lipophilic dyes used for non-invasive in vitro and in vivo cell tracking, *Blood*, *PMID 20215639*, journal article.

Lauter, J. L., P. Herscovitch, C. Formby, and M. E. Raichle (1985), Tonotopic organization in human auditory cortex revealed by positron emission tomography, *Hear Res*, *20*, 199205.

Le Prell, C. G., D. Yamashita, S. B. Minami, T. Yamasoba, and J. M. Miller (2007), Mechanisms of noise-induced hearing loss indicate multiple methods of prevention, *Hear Res*, *226*(1-2), 2243.

Lee, J. E. (1997), Basic helix-loop-helix genes in neural development, *Curr Opin Neurobiol*, *7*(1), 13–20.

Leon, Y., E. Vazquez, C. Sanz, J. A. Vega, J. M. Mato, F. Giraldez, J. Represa, and I. Varela-Nieto (1995), Insulin-like growth factor-i regulates cell proliferation in the developing inner ear, activating glycosyl-phosphatidylinositol hydrolysis and fos expression, *Endocrinology*, *136*(8), 3494–503.

LePrell, C. G., K. Ohlemiller, D. Dolan, and J. M. Miller (2010), Development of therapeutics to protect the inner ear: Support from animal models, *Acoustical Society of America 159th Meeting Lay Language Papers*.

Lesser, S. S., and D. C. Lo (1995), Regulation of voltage-gated ion channels by ngf and ciliary neurotrophic factor in sk-n-sh neuroblastoma cells, *J Neurosci*, *15*(1 Pt 1), 253–61.

Lesser, S. S., N. T. Sherwood, and D. C. Lo (1997), Neurotrophins differentially regulate voltage-gated ion channels, *Mol Cell Neurosci*, *10*(3-4), 173–83.

Li, H., H. Liu, and S. Heller (2003), Pluripotent stem cells from the adult mouse inner ear, *Nat Med*, *9*(10), 1293–9.

Li, H., H. Liu, C. E. Corrales, J. R. Risner, J. Forrester, J. R. Holt, S. Heller, and A. S. Edge (2009), Differentiation of neurons from neural precursors generated in floating spheres from embryonic stem cells, *BMC Neurosci*, *10*, 122.

Liew, C. G., J. S. Draper, J. Walsh, H. Moore, and P. W. Andrews (2007), Transient and stable transgene expression in human embryonic stem cells, *Stem Cells*, *25*(6), 1521–8.

Lindvall, O., Z. Kokaia, and A. Martinez-Serrano (2004), Stem cell therapy for human neurodegenerative disorders-how to make it work, *Nat Med*, *10 Suppl*, S42–50.

Littman, T., R. P. Bobbin, M. Fallon, and J. L. Puel (1989), The quinoxalinediones dnox, cnox and two related congeners suppress hair cell-to-auditory nerve transmission, *Hear Res*, *40*(1-2), 45–53.

Liu, L., and S. A. Simon (2003), Modulation of ia currents by capsaicin in rat trigeminal ganglion neurons, *J Neurophysiol*, *89*(3), 1387–401.

Livak, K. J., and T. D. Schmittgen (2001), Analysis of relative gene expression data using real-time quantitative pcr and the 2(-delta delta c(t)) method, *Methods*, *25*(4), 402–8.

Long, L., G. Li, W. Chen, J. Liu, and Y. Tang (2008), Distribution of serotonin immunoreactivity in the spiral ganglion neurons of mouse cochlea, *Int J Pediatr Otorhinolaryngol*, *72*(7), 1003–6.

Lu, P., L. L. Jones, E. Y. Snyder, and M. H. Tuszynski (2003), Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury, *Exp Neurol*, *181*(2), 115–29.

Ma, Q., C. Kintner, and D. J. Anderson (1996), Identification of neurogenin, a vertebrate neuronal determination gene, *Cell*, *87*(1), 43–52.

Ma, Q., C. Fode, F. Guillemot, and D. J. Anderson (1999), Neurogenin1 and neurogenin2 control two distinct waves of neurogenesis in developing dorsal root ganglia, *Genes Dev*, *13*(13), 1717–28.

Ma, Q., D. J. Anderson, and B. Fritzsch (2000), Neurogenin 1 null mutant ears develop fewer, morphologically normal hair cells in smaller sensory epithelia devoid of innervation, *J Assoc Res Otolaryngol*, *1*(2), 129–43.

Ma, Q. F., Z. F. Chen, I. D. Barrantes, J. L. de la Pompa, and D. J. Anderson (1998), neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia, *Neuron*, *20*(3), 469–482.

Mabie, P. C., M. F. Mehler, R. Marmur, S. Q. Papavasiliou, A. and, and J. A. Kessler (1997), Bone morphogenetic proteins induce astroglial differentiation of oligodendroglial-astroglial progenitor cells, *J Neurosci*, *17*, 4112 4120.

Maricich, S. M., A. Xia, E. L. Mathes, V. Y. Wang, J. S. Oghalai, B. Fritzsch, and H. Y. Zoghbi (2009), Atoh1-lineal neurons are required for hearing and for the survival of neurons in the spiral ganglion and brainstem accessory auditory nuclei, *J Neurosci*, *29*(36), 11,123–33.

Marmigere, F., and P. Ernfors (2007), Specification and connectivity of neuronal subtypes in the sensory lineage, *Nat Rev Neurosci*, *8*(2), 114–27.

Martinez-Monedero, R., E. Yi, K. Oshima, E. Glowatzki, and A. S. Edge (2008), Differentiation of inner ear stem cells to functional sensory neurons, *Dev Neurobiol*, *68*(5), 669–84.

McCormick, M. B., R. M. Tamimi, L. Snider, A. Asakura, D. Bergstrom, and S. J. Tapscott (1996), Neurod2 and neurod3: distinct expression patterns and transcriptional activation potentials within the neurod gene family, *Mol Cell Biol*, *16*(10), 5792–800.

McEvilly, R. J., L. Erkman, L. Luo, P. E. Sawchenko, A. F. Ryan, and M. G. Rosenfeld (1996), Requirement for brn-3.0 in differentiation and survival of sensory and motor neurons, *Nature*, *384*(6609), 574–7.

Meshorer, E., and T. Misteli (2006), Chromatin in pluripotent embryonic stem cells and differentiation, *Nat Rev Mol Cell Biol*, *7*(7), 540–6.

Meshorer, E., D. Yellajoshula, E. George, P. J. Scambler, D. T. Brown, and T. Misteli (2006), Hyperdynamic plasticity of chromatin proteins in pluripotent embryonic stem cells, *Dev Cell*, *10*(1), 105–16.

Miles, G. B., D. C. Yohn, H. Wichterle, T. M. Jessell, V. F. Rafuse, and R. M. Brownstone (2004), Functional properties of motoneurons derived from mouse embryonic stem cells, *J Neurosci*, *24*(36), 7848–58.

Miller, A. L., D. J. Morris, and B. E. Pfingst (2000), Effects of time after deafening and implantation on guinea pig electrical detection thresholds, *Hear Res*, *144*(1-2), 175–86.

Miller, J., and F. Spelman (1990), *Cochlear Implants: Models of the Electrically Stimulated Ear*, first ed., 422 pp., Springer-Verlag, New York, NY, USA.

Miller, J. M., D. H. Chi, L. J. O'Keeffe, P. Kruszka, Y. Raphael, and R. A. Altschuler (1997), Neurotrophins can enhance spiral ganglion cell survival after inner hair cell loss, *Int J Dev Neurosci*, *15*(4-5), 631–43.

Minoda, R., M. Izumikawa, K. Kawamoto, H. Zhang, and Y. Raphael (2007), Manipulating cell cycle regulation in the mature cochlea, *Hear Res*, *232*(1-2), 44–51.

Mistretta, C. M., and H. X. Liu (2006), Development of fungiform papillae: patterned lingual gustatory organs, *Arch Histol Cytol*, *69*(4), 199–208.

Mo, Z. L., and R. L. Davis (1997), Endogenous firing patterns of murine spiral ganglion neurons, *J Neurophysiol*, *77*(3), 1294–305.

Moe, M. C., M. Varghese, A. I. Danilov, U. Westerlund, J. Ramm-Pettersen, L. Brundin, M. Svensson, J. Berg-Johnsen, and I. A. Langmoen (2005), Multipotent progenitor cells from the adult human brain: neurophysiological differentiation to mature neurons, *Brain*, *128*(9), 2189–99.

Moore, D. R. (1994), Auditory brainstem of the ferret: long survival following cochlear removal progressively changes projections from the cochlear nucleus to the inferior colliculus, *J Comp Neurol*, *339*(2), 301–10.

Mou, K., C. L. Hunsberger, J. M. Cleary, and R. L. Davis (1997), Synergistic effects of bdnf and nt-3 on postnatal spiral ganglion neurons, *J Comp Neurol*, *386*(4), 529–39.

Murphy, C. L., and J. M. Polak (2002), Differentiating embryonic stem cells: Gapdh, but neither hprt nor beta-tubulin is suitable as an internal standard for measuring rna levels, *Tissue Eng*, *8*(4), 551–9.

Nadol, J., J. B., B. J. Burgess, and C. Reisser (1990), Morphometric analysis of normal human spiral ganglion cells, *Ann Otol Rhinol Laryngol*, *99*(5 Pt 1), 340–8.

Nair, V. (2008), Retrovirus-induced oncogenesis and safety of retroviral vectors, *Curr Opin Mol Ther*, *10*(5), 431–8.

Neher, E., and T. Sakaba (2008), Multiple roles of calcium ions in the regulation of neurotransmitter release, *Neuron*, *59*(6), 861–72.

Nicholl, A. J., A. Kneebone, D. Davies, D. I. Cacciabue-Rivolta, M. N. Rivolta, P. Coffey, and M. C. Holley (2005), Differentiation of an auditory neuronal cell line suitable for cell transplantation, *Eur J Neurosci*, *22*(2), 343–53.

Nieto, M., C. Schuurmans, O. Britz, and F. Guillemot (2001), Neural bhlh genes control the neuronal versus glial fate decision in cortical progenitors, *Neuron*, *29*(2), 401–13.

Nishimura, K., T. Nakagawa, K. Ono, H. Ogita, T. Sakamoto, N. Yamamoto, K. Okita, S. Yamanaka, and J. Ito (2009), Transplantation of mouse induced pluripotent stem cells into the cochlea, *Neuroreport*, *20*(14), 1250–4.

Noramly, S., and R. M. Grainger (2002), Determination of the embryonic inner ear, *J Neurobiol*, *53*(2), 100–28.

O'Leary, S. J., R. R. Richardson, and H. J. McDermott (2009), Principles of design and biological approaches for improving the selectivity of cochlear implant electrodes, *J Neural Eng*, *6*(5), 055,002.

O'Shea, K. S. (2001), Directed differentiation of embryonic stem cells: genetic and epigenetic methods, *Wound Repair Regen*, *9*(6), 443–59.

Oshima, K., C. M. Grimm, C. E. Corrales, P. Senn, R. Martinez Monedero, G. S. Geleoc, A. Edge, J. R. Holt, and S. Heller (2007), Differential distribution of stem cells in the auditory and vestibular organs of the inner ear, *J Assoc Res Otolaryngol*, *8*(1), 18–31.

Otte, H., J. Schuknecht, and A. Kerr (1978), Ganglion cell population in normal and pathological human cochlea, *Laryngoscope*, *88*, 1231–1246.

Ozaita, A., M. E. Martone, M. H. Ellisman, and B. Rudy (2002), Differential subcellular localization of the two alternatively spliced isoforms of the kv3.1 potassium channel subunit in brain, *J Neurophysiol*, *88*(1), 394–408.

Parker, M. A., D. A. Corliss, B. Gray, J. K. Anderson, R. P. Bobbin, E. Y. Snyder, and D. A. Cotanche (2007), Neural stem cells injected into the sound-damaged cochlea migrate throughout the cochlea and express markers of hair cells, supporting cells, and spiral ganglion cells, *Hear Res*, *232*(1-2), 29–43.

Pazyra-Murphy, M. F., and R. A. Segal (2008), Preparation and maintenance of dorsal root ganglia neurons in compartmented cultures, *J Vis Exp*, *20*.

Pettingill, L. N., R. L. Minter, and R. K. Shepherd (2008), Schwann cells genetically modified to express neurotrophins promote spiral ganglion neuron survival in vitro, *Neuroscience*, *152*(3), 1813–21.

Pfingst, B. E. (1990), Changes over time in thresholds for electrical stimulation of the cochlea, *Hear Res*, *50*(1-2), 225–36.

Pfingst, B. E., and L. Xu (2005), Psychophysical metrics and speech recognition in cochlear implant users, *Audiol Neurootol*, *10*(6), 331–41.

Pomp, O., I. Brokhman, I. Ben-Dor, B. Reubinoff, and R. S. Goldstein (2005), Generation of peripheral sensory and sympathetic neurons and neural crest cells from human embryonic stem cells, *Stem Cells*, *23*(7), 923–30.

Potts, W., D. Tucker, H. Wood, and C. Martin (2000), Chicken beta-globin 5'hs4 insulators function to reduce variability in transgenic founder mice, *Biochem Biophys Res Commun*, *273*(3), 1015–8.

Prasher, D. K., T. Tun, G. B. Brookes, and L. M. Luxon (1995), Mechanisms of hearing loss in acoustic neuroma: an otoacoustic emission study, *Acta Otolaryngol*, *115*(3), 375–81.

Pratt, T., L. Sharp, J. Nichols, D. J. Price, and J. O. Mason (2000), Embryonic stem cells and transgenic mice ubiquitously expressing a tau-tagged green fluorescent protein, *Dev Biol*, *228*(1), 19–28.

Prieskorn, D. M., and J. M. Miller (2000), Technical report: chronic and acute intracochlear infusion in rodents, *Hear Res*, *140*(1-2), 212–5.

Puel, J. L. (1995), Chemical synaptic transmission in the cochlea, *Prog Neurobiol*, *47*(6), 449–76.

Pujol, R., and J. L. Puel (1999), Excitotoxicity, synaptic repair, and functional recovery in the mammalian cochlea: a review of recent findings, *Ann N Y Acad Sci*, *884*, 249–54.

Puligilla, C., and M. W. Kelley (2009), Building the world's best hearing aid; regulation of cell fate in the cochlea, *Curr Opin Genet Dev*, *19*(4), 368–73.

Qun, L. X., U. Pirvola, M. Saarma, and J. Ylikoski (1999), Neurotrophic factors in the auditory periphery, *Ann N Y Acad Sci*, *28*(884), 292–304.

Raff, M. (2003), Adult stem cell plasticity: fact or artifact?, *Annu Rev Cell Dev Biol*, *19*, 1–22.

Raft, S., S. Nowotschin, J. Liao, and B. E. Morrow (2004), Suppression of neural fate and control of inner ear morphogenesis by tbx1, *Development*, *131*(8), 1801–12.

Raft, S., E. J. Koundakjian, H. Quinones, C. S. Jayasena, L. V. Goodrich, J. E. Johnson, N. Segil, and A. K. Groves (2007), Cross-regulation of ngn1 and math1 coordinates the production of neurons and sensory hair cells during inner ear development, *Development*, *134*(24), 4405–15.

Ramon y Cajal, S. (1928), Degeneration and regeneration of the nervous system. translated by raoul m. may, p. 769.

Raphael, Y., and R. A. Altschuler (2003), Structure and innervation of the cochlea, *Brain Res Bull*, *60*(5-6), 397–422.

Rasband, M. N., E. W. Park, T. W. Vanderah, J. Lai, F. Porreca, and J. S. Trimmer (2001), Distinct potassium channels on pain-sensing neurons, *Proc Natl Acad Sci U S A*, *98*(23), 13,373–8.

Rask-Andersen, H., M. Bostrom, B. Gerdin, A. Kinnefors, G. Nyberg, T. Engstrand, J. M. Miller, and D. Lindholm (2005), Regeneration of human auditory nerve. in vitro/in video demonstration of neural progenitor cells in adult human and guinea pig spiral ganglion, *Hear Res*, *203*(1-2), 180–91.

Reyes, J. H., K. S. O'Shea, N. L. Wys, J. M. Velkey, D. M. Prieskorn, K. Wesolowski, J. M. Miller, and R. A. Altschuler (2008), Glutamatergic neuronal differentiation of mouse embryonic stem cells after transient expression of neurogenin 1 and treatment with bdnf and gdnf: in vitro and in vivo studies, *J Neurosci*, *28*(48), 12,622–31.

Riccomagno, M. M., S. Takada, and D. J. Epstein (2005), Wnt-dependent regulation of inner ear morphogenesis is balanced by the opposing and supporting roles of shh, *Genes Dev*, *19*(13), 1612–23.

Rivolta, M. N., N. Grix, P. Lawlor, J. F. Ashmore, D. J. Jagger, and M. C. Holley (1998), Auditory hair cell precursors immortalized from the mammalian inner ear, *Proc Biol Sci*, *265*(1406), 1595–603.

Robertson, M. J., G. Phung, and D. V. Schaffer (2008), Neural stem cell engineering: directed differentiation of adult and embryonic stem cells into neurons, *Frontiers in Bioscience*, *13*, 21–50.

Robertson, N. J., F. A. Brook, R. L. Gardner, S. P. Cobbold, H. Waldmann, and P. J. Fairchild (2007), Embryonic stem cell-derived tissues are immunogenic but their inherent immune privilege promotes the induction of tolerance, *Proc Natl Acad Sci U S A*, *104*(52), 20,920–5.

Rocha-Sanchez, S. M., K. A. Morris, B. Kachar, D. Nichols, B. Fritzsch, and K. W. Beisel (2007), Developmental expression of kcnq4 in vestibular neurons and neurosensory epithelia, *Brain Res*, *1139*, 117–25.

Rossi, F., and E. Cattaneo (2002), Opinion: neural stem cell therapy for neurological diseases: dreams and reality, *Nat Rev Neurosci*, *3*(5), 401–9.

Rubinstein, J. T., W. S. Parkinson, R. S. Tyler, and B. J. Gantz (1999), Residual speech recognition and cochlear implant performance: effects of implantation criteria, *Am J Otol*, *20*(4), 445–52.

Ruel, J., J. Wang, R. Pujol, A. Hameg, M. Dib, and J. L. Puel (2005), Neuroprotective effect of riluzole in acute noise-induced hearing loss, *Neuroreport*, *16*(10), 1087–90.

Ruel, J., J. Wang, D. Demmes, S. Gobaille, J. L. Puel, and G. Rebillard (2006), Dopamine transporter is essential for the maintenance of spontaneous activity of auditory nerve neurones and their responsiveness to sound stimulation, *J Neurochem*, *97*(1), 190–200.

Rusznak, Z., and G. Szucs (2009), Spiral ganglion neurones: an overview of morphology, firing behaviour, ionic channels and function, *Pflugers Arch*, *457*(6), 1303–25.

Rusznak, Z., G. Bakondi, K. Pocsai, A. Por, L. Kosztka, B. Pal, D. Nagy, and G. Szucs (2008), Voltage-gated potassium channel (kv) subunits expressed in the rat cochlear nucleus, *J Histochem Cytochem*, *56*(5), 443–65.

Sahin, E., and R. A. Depinho (2010), Linking functional decline of telomeres, mitochondria and stem cells during ageing, *Nature*, *464*(7288), 520–8.

Satoh, H., G. S. Firestein, P. B. Billings, J. P. Harris, and E. M. Keithley (2003), Proinflammatory cytokine expression in the endolymphatic sac during inner ear inflammation, *J Assoc Res Otolaryngol*, *4*, 139–147.

Schecterson, L. C., and M. Bothwell (1994), Neurotrophin and neurotrophin receptor mrna expression in developing inner ear, *Hear Res*, *73*(1), 92–100.

Schorpp, M., R. Jager, K. Schellander, J. Schenkel, E. F. Wagner, H. Weiher, and P. Angel (1996), The human ubiquitin c promoter directs high ubiquitous expression of transgenes in mice, *Nucleic Acids Res*, *24*(9), 1787–8.

Schuurmans, C., et al. (2004), Sequential phases of cortical specification involve neurogenin-dependent and -independent pathways, *Embo J*, *23*(14), 2892–902.

Scott, C., and R. A. Reijo Pera (2008), The road to pluripotence: the research response to the embryonic stem cell debate, *Hum Mol Genet*, *15*(17), R3–9.

Seitz, P. R. (2002), French origins of the cochlear implant, *Cochlear Implants Int*, *3*(2), 77–86.

Sekiya, T., M. C. Holley, K. Kojima, M. Matsumoto, R. Helyer, and J. Ito (2007), Transplantation of conditionally immortal auditory neuroblasts to the auditory nerve, *Eur J Neurosci*, *25*(8), 2307–18.

Sekkali, B., H. Thi Tran, E. Crabbe, C. De Beule, F. Van Roy, and K. Vleminckx (2008), Chicken beta-globin insulator overcomes variegation of transgenes in xenopus embryos, *Faseb J*, *22*(7), 2534–2540.

Seo, S., J. W. Lim, D. Yellajoshyula, L. W. Chang, and K. L. Kroll (2007), Neurogenin and neurod direct transcriptional targets and their regulatory enhancers, *Embo J*, *26*(24), 5093–108.

Sharma, A., M. F. Dorman, and A. J. Spahr (2002), A sensitive period for the development of the central auditory system in children with cochlear implants: implications for age of implantation, *Ear Hear*, *23*(6), 532–9.

Shepherd, R. K., A. Coco, S. B. Epp, and J. M. Crook (2005), Chronic depolarization enhances the trophic effects of brain-derived neurotrophic factor in rescuing auditory neurons following a sensorineural hearing loss, *J Comp Neurol*, *486*(2), 145–58.

Shepherd, R. K., A. Coco, and S. B. Epp (2008), Neurotrophins and electrical stimulation for protection and repair of spiral ganglion neurons following sensorineural hearing loss, *Hear Res*, *242*(1-2), 100–9.

Shi, F., C. E. Corrales, M. C. Liberman, and A. S. Edge (2007), Bmp4 induction of sensory neurons from human embryonic stem cells and reinnervation of sensory epithelium, *Eur J Neurosci*, *26*(11), 3016–23.

Shibata, S. B., S. R. Cortez, L. A. Beyer, J. A. Wiler, A. Di Polo, B. E. Pfingst, and Y. Raphael (2010), Transgenic bdnf induces nerve fiber regrowth into the auditory epithelium in deaf cochleae, *Exp Neurol*, *doi:10.1016/j.expneurol.2010.01.011*.

Silva, A., J. Pereira, C. R. Oliveira, J. B. Relvas, and A. C. Rego (2009), Bdnf and extracellular matrix regulate differentiation of mice neurosphere-derived cells into a gabaergic neuronal phenotype, *J Neurosci Res*, *87*(9), 1986–96.

Skinner, M. W., L. K. Holden, and T. A. Holden (1997), Parameter selection to optimize speech recognition with the nucleus implant, *Otolaryngol Head Neck Surg*, *117*(3 Pt 1), 188–95.

Sly, D. J., L. F. Heffer, M. W. White, R. K. Shepherd, M. G. Birch, R. L. Minter, N. E. Nelson, A. K. Wise, and S. J. O'Leary (2007), Deafness alters auditory nerve fibre responses to cochlear implant stimulation, *Eur J Neurosci*, *26*(2), 510–22.

Snyder, R. L., J. C. Middlebrooks, and B. H. Bonham (2008), Cochlear implant electrode configuration effects on activation threshold and tonotopic selectivity, *Hear Res*, *235*, 23–38.

Sommer, L., Q. Ma, and D. J. Anderson (1996), neurogenins, a novel family of atonal-related bhlh transcription factors, are putative mammalian neuronal determination genes that reveal progenitor cell heterogeneity in the developing cns and pns, *Mol Cell Neurosci*, *8*(4), 221–41.

Spitzer, N. C. (2006), Electrical activity in neuronal development, *Nature*, *444*(7), 707–712.

Spitzer, N. C., X. Gu, and E. Olson (1994), Action potentials, calcium transients and the control of differentiation of excitable cells, *Curr Opin Neurobiol*, *4*(1), 70–7.

Staecker, H., R. Kopke, B. Malgrange, P. Lefebvre, and T. R. Van de Water (1996a), Nt-3 and/or bdnf therapy prevents loss of auditory neurons following loss of hair cells, *Neuroreport*, *7*(4), 889–94.

Staecker, H., T. R. Van De Water, P. P. Lefebvre, W. Liu, M. Moghadassi, V. Galinovic-Schwartz, B. Malgrange, and G. Moonen (1996b), Ngf, bdnf and nt-3 play unique roles in the in vitro development and patterning of innervation of the mammalian inner ear, *Brain Res Dev Brain Res*, *92*(1), 49–60.

Stickney, G. A., K. Nie, Y. Y. Kong, H. Chen, and F. G. Zeng (2004), Temporal fine structure: the missing component in speech processing algorithms, *Intnl Congress Series*, *1273*, 23–26.

Strubing, C., G. Ahnert-Hilger, J. Shan, B. Wiedenmann, J. Hescheler, and A. M. Wobus (1995), Differentiation of pluripotent embryonic stem cells into the neuronal lineage in vitro gives rise to mature inhibitory and excitatory neurons, *Mech Dev*, *53*(2), 275–87.

Sugimoto, Y., T. Furuno, and M. Nakanishi (2009), Effect of neurod2 expression on neuronal differentiation in mouse embryonic stem cells, *Cell Biol Int*, *33*(2), 174–9.

Sun, W., and R. J. Salvi (2009), Brain derived neurotrophic factor and neurotrophic factor 3 modulate neurotransmitter receptor expressions on developing spiral ganglion neurons, *Neuroscience*, *164*(4), 1854–66.

Sun, Y., M. Nadal-Vicens, S. Misono, M. Z. Lin, A. Zubiaga, X. Hua, G. Fan, and M. E. Greenberg (2001), Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms, *Cell*, *104*(3), 365–76.

Swan, E. E., M. J. Mescher, W. F. Sewell, S. L. Tao, and J. T. Borenstein (2008), Inner ear drug delivery for auditory applications, *Adv Drug Deliv Rev*, *60*, 1583–1599.

Takahashi, K., and S. Yamanaka (2006), Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, *Cell*, *126*(4), 663–676.

Tamura, T., et al. (2004), Transplantation of neural stem cells into the modiolus of mouse cochleae injured by cisplatin, *Acta Otolaryngol Suppl*, (551), 65–8.

Tan, J., and R. K. Shepherd (2006), Aminoglycoside-induced degeneration of adult spiral ganglion neurons involves differential modulation of tyrosine kinase b and p75 neurotrophin receptor signaling, *Am J Pathol*, *169*(2), 528–543.

Tessarollo, L., V. Coppola, and B. Fritzsch (2004), Nt-3 replacement with brain-derived neurotrophic factor redirects vestibular nerve fibers to the cochlea, *J Neurosci*, *24*(10), 2575–84.

Thomson, J., J. Itskovitz-Eldor, S. Shapiro, M. Waknitz, J. Swiergiel, V. Marshall, and J. Jones (1998), Embryonic stem cell lines derived from human blastocysts, *Science*, *282*(5391), 11451147.

Thorek, D. L. J., A. K. Cheny, J. Czuprynaz, and A. Tsourka (2006), Superparamagnetic iron oxide nanoparticle probes for molecular imaging, *Annals of Biomedical Engineering*, *34*(1), 23–38.

Tsai, C. W., J. J. Tseng, S. C. Lin, C. Y. Chang, J. L. Wu, J. F. Horng, and H. J. Tsay (2001), Primary structure and developmental expression of zebrafish sodium channel na(v)1.6 during neurogenesis, *DNA Cell Biol*, *20*(5), 249–55.

Tucker, K. L., M. Meyer, and Y. A. Barde (2001), Neurotrophins are required for nerve growth during development, *Nat Neurosci*, *4*(1), 29–37.

Ulfendahl, M., Z. Hu, P. Olivius, M. Duan, and D. Wei (2007), A cell therapy approach to substitute neural elements in the inner ear, *Physiol Behav*, *92*(1-2), 75–9.

Vallier, L., M. Alexander, and R. Pedersen (2007), Conditional gene expression in human embryonic stem cells, *Stem Cells*, *25*(6), 1490–7.

Varela-Ramirez, A., C. Trujillo-Provencio, and E. E. Serrano (1998), Detection of transcripts for delayed rectifier potassium channels in the xenopus laevis inner ear, *Hear Res*, *119*(1-2), 125–34.

Velkey, J. M. (2005), Lineage differentiation of mouse embryonic stem cells, *University of Michigan Dissertations*.

Vierbuchen, T., A. Ostermeier, Z. P. Pang, Y. Kokubu, T. C. Sudhof, and M. Wernig (2010), Direct conversion of fibroblasts to functional neurons by defined factors, *Nature*, *463*(7284), 1035–41.

von Bartheld, C. S., and B. Fritzsch (2006), Comparative analysis of neurotrophin receptors and ligands in vertebrate neurons: tools for evolutionary stability or changes in neural circuits?, *Brain Behav Evol*, *68*(3), 157–72.

Wang, R., J. Liang, H. Jiang, L. J. Qin, and H. T. Yang (2008), Promoter-dependent egfp expression during embryonic stem cell propagation and differentiation, *Stem Cells Dev*, *17*(2), 279–89.

Wang, X. Q., X. M. Duan, L. H. Liu, Y. Q. Fang, and Y. Tan (2005), Carboxyfluorescein diacetate succinimidyl ester fluorescent dye for cell labeling, *Acta Biochim Biophys Sin (Shanghai)*, *37*(6), 379–85.

Ward, C. M., and P. L. Stern (2002), The human cytomegalovirus immediate-early promoter is transcriptionally active in undifferentiated mouse embryonic stem cells, *Stem Cells*, *20*(5), 472–5.

Webster, M., and D. Webster (1981), Spiral ganglion neuron loss following organ of corti loss: a quantitative study, *Brain Res*, *212*, 1730.

Wei, D., Z. Jin, L. Jrlebark, E. Scarfone, and M. Ulfendahl (2007), Survival, synaptogenesis, and regeneration of adult mouse spiral ganglion neurons in vitro, *Dev Neurobiol*, *67*(1), 108–22.

Wells, J. E., E. T. Rose, K. C. Rowland, and J. F. Hatton (2007), Kv1.4 subunit expression is decreased in neurons of painful human pulp, *Journal of Endodontics*, *33*(7), 827–829.

Wianny, F., et al. (2008), Derivation and cloning of a novel rhesus embryonic stem cell line stably expressing tau-green fluorescent protein, *Stem Cells*, *26*(6), 1444–53.

Wilson, B. S., and M. F. Dorman (2008), Cochlear implants: current designs and future possibilities, *J Rehabil Res Dev*, *45*(5), 695–730.

Wolf, P. (2001), Creativity and chronic disease. ludwig van beethoven (1770-1827), *West J Med*, *175*(5), 298.

Wright, T. J., R. Ladher, J. McWhirter, C. Murre, G. C. Schoenwolf, and S. L. Mansour (2004), Mouse fgf15 is the ortholog of human and chick fgf19, but is not uniquely required for otic induction, *Dev Biol*, *269*(1), 264–75.

Wu, H., and Y. E. Sun (2006), Epigenetic regulation of stem cell differentiation, *Pediatric Research*, *59*(4), 21R–25R.

Wu, H., J. Xu, Z. P. Pang, W. Ge, K. J. Kim, B. Blanchi, C. Chen, T. C. Sudhof, and Y. E. Sun (2007), Integrative genomic and functional analyses reveal neuronal subtype differentiation bias in human embryonic stem cell lines, *Proc Natl Acad Sci U S A*, *104*(34), 13,821–6.

Wulff, B. S., M. M. O'Hare, E. Boel, L. E. Theill, and T. W. Schwartz (1990), Partial processing of the neuropeptide y precursor in transfected cho cells, *FEBS Lett*, *261*(1), 101–5.

Xu, L., and B. E. Pfingst (2008), Spectral and temporal cues for speech recognition: implications for auditory prostheses., *Hear Res*, *242*(1-2), 132–40.

Yamada, M., et al. (2007), Electrical stimulation modulates fate determination of differentiating embryonic stem cells, *Stem Cells*, *25*, 562570.

Yamashita, H., T. Sekitani, Y. Okinaka, T. Inokuma, H. Shimogori, K. Moriya, and H. Hara (1991), Cell culture study of the vestibular ganglion cells. morphology and immunohistochemical activity, *Acta Otolaryngol Suppl*, *481*, 153–7.

Yamasoba, T., J. Schacht, F. Shoji, and J. M. Miller (1999), Attenuation of cochlear damage from noise trauma by an iron chelator, a free radical scavenger and glial cell line-derived neurotrophic factor in vivo, *Brain Res*, *815*(2), 317–25.

Ylikoski, J., U. Pirvola, M. Moshnyakov, J. Palgi, U. Arumae, and M. Saarma (1993), Expression patterns of neurotrophin and their receptor mrnas in the rat inner ear, *Hear Res*, *65*(1-2), 69–78.

Yost, W. (2007), *Fundamentals of Hearing: an Introduction*, fifth ed., 338 pp., Academic Press Elsevier, Burlington, MA, USA.

Yu, F. H., V. Yarov-Yarovoy, G. A. Gutman, and W. A. Catterall (), Overview of molecular relationships in the voltage-gated ion channel superfamily, *Pharmacol Rev*, *57*(4), 387–95.

Zaragosi, L. E., N. Billon, G. Ailhaud, and C. Dani (2007), Nucleofection is a valuable transfection method for transient and stable transgene expression in adipose tissue-derived stem cells, *Stem Cells*, *25*(3), 790–7.

Zelarayan, L. C., V. Vendrell, Y. Alvarez, E. Dominguez-Frutos, T. Theil, M. T. Alonso, M. Maconochie, and T. Schimmang (2007), Differential requirements for fgf3, fgf8 and fgf10 during inner ear development, *Dev Biol*, *308*(2), 379–91.

Zhang, S. C., M. Wernig, I. D. Duncan, O. Brustle, and J. A. Thomson (2001), In vitro differentiation of transplantable neural precursors from human embryonic stem cells, *Nat Biotechnol*, *19*(12), 1129–33.

Zhang, X. F., et al. (2004), Differential action potentials and firing patterns in injured and uninjured small dorsal root ganglion neurons after nerve injury, *Brain Res*, *1009*(1-2), 147–58.

Zheng, J. Q., and M. M. Poo (2007), Calcium signaling in neuronal motility, *Annu Rev Cell Dev Biol*, *23*, 375–404.

Zhou, X., C. Baier, W. A. Hossain, M. Goldenson, and D. K. Morest (2001), Expression of a voltage-dependent potassium channel protein (kv3.1) in the embryonic development of the auditory system, *J Neurosci Res*, *65*(1), 24–37.

Zhou, Z., Q. Liu, and R. L. Davis (2005), Complex regulation of spiral ganglion neuron firing patterns by neurotrophin-3, *J Neurosci*, *25*(33), 7558–66.