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Prospects for Replacement of Auditory Neurons by Stem Cells

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Abstract

Sensorineural hearing loss is caused by degeneration of hair cells or auditory neurons. Spiral ganglion cells, the primary afferent neurons of the auditory system, are patterned during development and send out projections to hair cells and to the brainstem under the control of largely unknown guidance molecules. The neurons do not regenerate after loss and even damage to their projections tends to be permanent. The genesis of spiral ganglion neurons and their synapses forms a basis for regenerative approaches. In this review we critically present the current experimental findings on auditory neuron replacement. We discuss the latest advances with a focus on (a) exogenous stem cell transplantation into the cochlea for neural replacement, (b) expression of local guidance signals in the cochlea after loss of auditory neurons, (c) the possibility of neural replacement from an endogenous cell source, and (d) functional changes from cell engraftment.

1.1 Lack of spontaneous regeneration of the auditory nerve

The auditory nerve does not regenerate once damaged. There have been some reports that peripheral fibers could be repaired when the synapses had been destroyed by a mechanism identified as excess glutamate at the afferent endings in adult animals (Lerner-Natoli et al., 1997; Puel et al., 1997), but other mechanisms such as partial repair of damaged hair cells could account for recovery. There was no evidence of regenerated fibers or synapses in careful studies that quantified synaptic contacts after the fibers had retracted in models of noise damage (Kujawa and Liberman, 2009; Lin et al., 2011), and thus synaptogenesis with hair cells does not appear to be a mechanism for recovery, and after loss of fibers or of the neurons themselves, spontaneous regenerative mechanisms are lacking. Only in an *in vitro* model of newborn rat cochlear explants has there been any evidence that peripheral fibers could spontaneously regrow to hair cells (Wang and Green, 2011).

In our work using embryonic stem (ES) cells and spiral ganglion neurons (SGNs) from newborn mice we have conclusively established growth of fibers that make synapses with hair cells and contact neurons in the brainstem (Corrales et al., 2006; Martinez-Monedero et

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al., 2006; Shi et al., 2007; Martinez-Monedero et al., 2008; Brugeaud and Edge, 2009; Tong et al., 2011).

1.2. Characteristics of the auditory nerve

The auditory nerve conveys sound information rapidly to the brain and covers a wide range of sound levels and frequencies. Spontaneous firing of afferent neurons is thought to set a baseline that can then be perturbed to obtain an accurate estimate of onset of a sound (Liberman, 1982; Kawase and Liberman, 1992). Firing in response to single quanta from hair cells is thought to make the nerve particularly sensitive (Rutherford et al., 2012). A wide dynamic range is achieved by the sensitivity of hair cells combined with the frequency coding of single fibers in the auditory nerve (Liberman, 1982).

The timing, frequency and intensity of sounds are coded and transmitted from hair cells to SGNs via the ribbon synapse which promotes rapid, precise and sustained neurotransmitter release and signal transmission through multivesicular release and calcium channel positioning (Moser et al., 2006). These unique features are critical for transmitting the full complexity of sound. The inner hair cells transmit information to the brain through the primary afferent synapse while outer hair cells increase sensitivity of sound detection by amplifying the signal.

1.3. Development of afferent innervation

Understanding the genesis of SGNs and their afferent synapses is crucial for the development of regenerative approaches. SGNs are among the first neurons to be specified during development. As early as mouse embryonic day 9 (E9) neuroblasts delaminate from the otocyst to form the cochleovestibular ganglion (Carney and Silver, 1983). Initially, the delaminated neuroblasts consist entirely of proliferating progenitor cells, and the onset of neurogenesis is marked by the appearance of the first post-mitotic vestibular and spiral ganglion cells. Both the spiral ganglion, which coils along the length of the cochlear duct, and the vestibular ganglion are formed from the cochleovestibular ganglion. The proliferation phase ends at approximately E9.5 to E13.5 in the mouse and is followed by differentiation in a direction from base at E9.5 to E10.5 to apex at E12.5 to E13.5 (Matei et al., 2005; Koundakjian et al., 2007). SGNs are born 4 days earlier than hair cells at the base of the cochlea. Most SGNs generate action potentials by E14, 2 days after completion of neurogenesis (Marrs and Spirou, 2012). The SGNs form synapses throughout development continuing up to postnatal day 12 (P12).

The molecular cues that control the specification of auditory neurons have been largely attributed to a network of basic helix-loop-helix (bHLH) transcription factors. Neurogenin 1 (Ngn1) has a governing role in defining the early neural precursors within the Sox2 expressing sensory domain in the otocyst (Ma et al., 1998; Ma et al., 2000). Subsequently, NeuroD is activated in post-mitotic progenitors to promote neuronal differentiation by inhibiting non- neuronal fate (Liu et al., 2000; Kim et al., 2001; Lawoko-Kerali et al., 2004). Intriguingly, spiral ganglion cell fate is dependent as well on GATA3 which acts upstream of NeuroD (Karis et al., 2001; Lawoko-Kerali et al., 2004). The choice between sensory hair cell and neuronal fate by inner ear stem cells is also dependent on Notch signaling, and Sox2 acts upstream of Ngn1 (Jeon et al., 2011).

1.4. Potential for regeneration by manipulation of axonal guidance

After their genesis, SGNs project to hair cells and to cells in the cochlear nucleus. Their growth is guided by molecules such as netrins, semaphorins, slits and ephrins that exert repulsive and attractive forces on growth cones at the tips of axons and dendrites and are

important for the development of the auditory circuitry. Axonal growth can also be guided by permissive substrates in the matrix and by soluble trophic factors. Soon after SGNs are born, they become bipolar and are guided to their targets through the mesenchyme and Kolliker's organ. Thus, guidance information likely comes from surrounding tissues, such as Kolliker's organ, mesenchyme, and glia. Indeed, the repulsive slit and ephrin ligands are expressed in non-sensory tissues of the ear and function to keep axonal growth within the spiral lamina (Bianchi and Liu, 1999; Pickles et al., 2002; Battisti and Fekete, 2008). Extracellular matrix components within the mesenchyme provide permissive substrates, such as, integrins, laminin, and tenascin-C, to guide axons through the spiral lamina (Whitlon et al., 1999; Rodgers et al., 2001; Davies, 2007) before reaching hair cells. Moreover, netrin1 promotes neurite outgrowth of cultured SGNs, while ephrinB1 inhibits outgrowth (Bianchi and Gray, 2002; Lee and Warchol, 2008). Despite the presence of many host molecules in the inner ear, specific functions remain unclear. BDNF and NT3, neurotrophins expressed in the ear, may have some guidance activity, but that role is hard to dissect from the overall role in neuronal growth, survival and innervation (Fritzsch et al., 2004). Neurotrophins may control pathfinding by regulating the expression of other guidance cues.

We have initiated studies to evaluate the role of axonal guidance molecules in regeneration, and have found a negative influence of some axonal guidance molecules on SGN regeneration (Brugeaud and Edge, 2009). Manipulation of developmentally relevant guidance molecules will be important for rewiring of auditory circuitry with *in vitro*-derived neurons. There is some precedent for the inhibition of neuronal regeneration in the nervous system of adults by axonal guidance molecules that guide correct innervation in the embryo (Pasterkamp et al., 1998; Harel and Strittmatter, 2006). Repulsive guidance molecules do not recapitulate their role in embryonic guidance but, rather, may act aberrantly to inhibit regeneration at glial scars (Pasterkamp et al., 1998; Hata et al., 2006; Pasterkamp and Verhaagen, 2006). The neuropilin 1/semaphorin 3a and neogenin 1/RGMa receptors and ligands have been shown to prevent regeneration of axons in peripheral neurites (Kyoto et al., 2007; Tang et al., 2007; Tannemaat et al., 2007). Expression of these signaling pathways in the tissue of the adult after damage may inhibit SGN regeneration.

1.5. Regeneration of afferent synapses

The formation of a synapse requires initial contact followed by stabilization and acquisition of appropriate pre-and postsynaptic elements. The onset of synaptogenesis occurs prior to birth in mice, and is not dependent on synaptic activity. Action potentials can be triggered in the most immature SGNs by activation of hair cells at the apex of the cochlea in newborn rats (Tritsch and Bergles, 2010). Non-sensory cells play an essential role in the development of central auditory pathways. Supporting cells in organ of Corti initiate electrical activity in auditory nerves before hearing through spontaneous ATP release. This experience-independent activity subsides after the onset of hearing, preventing interference with accurate encoding of sound (Tritsch et al., 2007). Afferent synaptic-like contacts were described at E18 in rats (Huang et al., 2005) and ribbon synapses with apposed postsynaptic densities were present at birth in mice (Shnerson et al., 1981; Sobkowicz et al., 1982). Maturation is not complete until P12 at the onset of hearing (Grant et al., 2010). At the morphological level, ribbon synapses are made in two steps, formation of the presynaptic complex and assembly of the postsynaptic density (Sobkowicz et al., 1986). This sequence of events is typical for establishment of a synapse, where postsynaptic specializations are later to develop (Goda and Davis, 2003). Although there is a prepatterning of rudimentary postsynaptic organization prior to the arrival of the nerve, differentiation of the postsynaptic specialization is induced upon contact with the presynaptic ending. The postsynaptic specialization then influences assembly of the presynaptic active zone and its alignment with

the postsynaptic specialization. Similarly, after afferent nerve fibers reach the hair cells, synaptogenesis begins with accumulation of dense-core and sometimes clear vesicles at the hair cell membrane apposed to the nerve fiber. These vesicles are probably active zone precursors, which are found in other systems and deliver presynaptic proteins to the nascent synapses. Neurotransmitters, secreted signaling proteins such as Wnts, neurotrophins, and adhesion molecules provide the anterograde signals in the CNS (Goda and Davis, 2003). We have developed a system for studying the mechanisms of synaptogenesis in the postnatal animal (Tong et al., 2011), and the elucidation of factors affecting postnatal synaptogenesis can be informed by developmental mechanisms.

Type I SGNs in the mouse comprise 90% of the neurons in the ganglion and convey most of the sound information. Each type I neuron contacts only one hair cell, while each inner hair cell is connected with 10–20 type I neurons (Liberman, 1980; Meyer et al., 2009). The smaller type II neurons, which make up less that 10% of SGNs (Romand and Romand, 1987), contact multiple outer hair cells after passing the inner hair cells and turning towards the base of the cochlea. SGNs are pruned, resulting in type I contacts with inner, and type II contacts with outer hair cells (Echteler, 1992). At E12.5, peripheral projections of SGNs are branched and tangled, and pruning is not apparent until E15.5 when single unbranched fibers are seen in the base of the cochlea (Koundakjian et al., 2007). The mechanisms for pruning are not clear. The first contact of a projection with a hair cell may inhibit the progress of other branches and, in turn, induce their retraction. Our in vitro models of neural replacement provide systems for investigating mechanisms that determine synaptogenesis and pruning of type I and II SGNs.

1.6. Central connections: brainstem

Information is conveyed to the cochlear nucleus by axons of the SGNs, which bundle together to form the eighth nerve. The central projections of SGNs first reach the hindbrain around E12.5 in the mouse, as the SGNs are born, and precede their peripheral projections (Karis et al., 2001; Maklad et al., 2010; Lu et al., 2011). This process does not appear to depend on the presence of target neurons in the cochlear nucleus, as SGNs project into the hindbrain and bifurcate even in Atoh1 knockout animals, in which the majority of target neurons are never formed (Maricich et al., 2009). SGNs exhibit spontaneous activity by E14 and are able to drive third-order auditory brainstem neurons by E17 (Marrs and Spirou, 2012). SGNs acquire action potential-generating capacity at E14 prior to neurons of the ventral and medial nucleus of the trapezoid body (VCN and MNTB). Accordingly, auditory nerve synapses in the VCN are functional at E15, prior to VCN connectivity with the MNTB, which occurs at least 1 day later. This activity precedes cochlear-generated wave activity by 4 days and ear canal opening by at least 2 weeks (Marrs and Spirou, 2012).

Upon entering the brainstem, individual axons bifurcate and form two branches, one to the VCN and the other to the dorsal cochlear nucleus (DCN) (Fekete et al., 1984; Ryugo, 2008). Each branch independently seeks tonotopically appropriate postsynaptic targets and elaborates synapses, forming the large endbulbs of Held with spherical bushy cells located in the most anterior portion of the VCN and smaller, modified endbulbs with globular bushy cells (Ryugo and Parks, 2003; Ryugo et al., 2006) and boutons in the DCN. Type II neurons project to the small cell cap surrounding the cochlear nucleus. Endbulbs have a calyx-like appearance in which the main axon elaborates several branches that arborize repeatedly to enclose the postsynaptic cell in a nest of *en passant* swellings and terminals (Ryugo and Fekete, 1982). High frequency information from the base of the cochlea is sent dorsally while low frequency information is processed ventrally. Although each of these stages has been documented at the cellular level, our knowledge of the underlying mechanisms remains limited. The unusual size and shape of the endbulb of Held is essential for rapid and secure

synaptic transmission that allows detection of timing differences in auditory stimuli. The central auditory synapses display greater plasticity than the periphery, as shown by rescue of endbulbs in deaf cats given cochlear implants (Ryugo et al., 2005).

The progress from homogeneous neuroblasts to fully functional bipolar adult SGNs involves cell fate determination, pathfinding and synaptogenesis. Regeneration of these neurons and synapses will require that this complex series of steps be recapitulated, including the choice of type I or II; synapse formation with IHC or OHC; bifurcation in the cochlear nucleus; and synaptogenesis with target neurons in the brainstem. A detailed understanding of the molecular cues for differentiation and pathfinding will help to improve the efficiency of transplantation *in vivo*.

2.1. Loss of neurons (auditory neuropathy)

Neural degeneration has been thought to occur as a secondary consequence of hair cell loss but can also result from primary damage (Spoendlin, 1971; Liberman and M.J., 1982; Robertson, 1983; Puel et al., 1998; Hakuba et al., 2000; Wang et al., 2003). Damage to SGNs is an important clinical issue that has received increased attention because of recent work in which noise exposure caused primary afferent degeneration (Kujawa and Liberman, 2009). Loss of afferent synapses and retraction of fibers was seen after a 105 dB exposure for 2 hours and ultimately resulted in loss of neurons. No loss of hair cells was seen in this model. The low spontaneous rate, high-threshold neurons were more susceptible to degeneration; their loss resulted in a profound reduction in the threshold and wave 1-amplitude of the ABR. Accelerated loss of primary afferents was seen with age despite the recovery of threshold. Slow primary loss of neurons after recovery from the initial damage overturns a long-held view that recovery from a temporary threshold shift signals a restoration of normal cochlear function after noise-induced hearing loss. Temporary threshold shifts can be a harbinger of future problems due to neural degeneration, and neural loss without hair cell loss has been observed in human autopsy specimens (Makary et al., 2011).

A second model of auditory neuropathy is the ouabain-treated gerbil, in which the type I afferents are removed by round window administration of ouabain, a Na-K ATPase binding toxin (Schmiedt et al., 2002; Lang et al., 2005). The model is largely devoid of type I neurons in our hands, although there are occasional neurons surviving (Corrales et al., 2006). The ABR reflects this loss with thresholds above system output at most frequencies and hair cells are intact as measured by DPOAE (Lang et al., 2005).

2.2. Replacement of afferent neurons from resident cells

One route towards functional recovery is the replacement of SGNs from endogenous cell sources. Replacement of the neurons by regeneration as opposed to replacement by cell transplantation has the advantage that it would use normal mechanisms of peripheral regeneration and avoids the risk of teratomas, but it will require a better knowledge of the cells and signaling pathways that induce synaptogenesis. Unlike evolutionarily older animals, such as fish and birds, mammalian hair cells and SGNs do not exhibit significant regenerative capacity. However, several groups have demonstrated that proliferative cells, which display some of the hallmarks associated with neural stem cells, can be isolated from the organ of Corti. Most recently, we identified cochlear stem cells as Lgr5-expressing supporting cells (Shi et al., 2012). Cells isolated from Rosenthal's canal also showed stem cell properties and gave rise to neurons (Martinez-Monedero et al., 2008; Kempfle et al., 2010) with electrophysiological properties of SGNs. BMP signaling increased sensory neuronal differentiation from ES cell-derived neural progenitors (Shi et al., 2007) and also

influenced neural differentiation from neurospheres obtained from spiral ganglion (Kempfle et al., 2010).

Regrowth of peripheral fibers and contacts with hair cells after glutamate damage to primary afferent neurons have been demonstrated *in vitro* (Wang and Green, 2011). Hair cells in organ of Corti explants were de-afferented over the course of several hours but fibers regrew and contacted hair cells. Neurotrophins had a marked effect on fiber regrowth.

One approach would be to stimulate expansion and differentiation of endogenous progenitor cells with a drug or paracrine factors. Neural differentiation might also be achieved by factors that induce neural fates from terminally differentiated cells (Vierbuchen et al., 2010; Caiazzo et al., 2011). Among the factors that have been used to induce a neural phenotype are bHLH transcription factors, including Ascl1 (Vierbuchen et al., 2010; Caiazzo et al., 2011), and conversion to neurons has been demonstrated for fibroblasts as well as glial cells (Heinrich et al., 2011).

The spiral ganglion contains myelinating Schwann cells and satellite cells as well as fibroblasts that might be a source of new neurons for repair (Eybalin et al., 1996). The peripheral nervous system utilizes Schwann cells, myelinating glial cells and macrophages for regenerative support and clearance of extracellular matrix debris, creating an environment conducive to axonal regrowth at a rate of 1.5–2.0 mm/day (Buchthal and Kuhl, 1979; Rotshenker, 2011). Schwann cells proliferate after loss of SGNs, but don't spontaneously differentiate into neurons (Lang et al., 2011).

2.3. Replacement of afferent neurons from exogenous stem cells

Exogenous replacement of SGNs is a second approach to cell replacement after SGN death. This approach has been widely investigated in CNS diseases, such as Huntington's and Parkinson's and spinal cord injury (Lunn et al., 2011). Cell transplantation in the CNS is made easier by a low rate of immunological rejection. The inner ear has a degree of immune privilege that may help to reduce the rejection of transplanted cells (Harris and Ryan, 1995; Bodmer et al., 2002). Another advantage of the auditory system for the study of neuronal replacement is that a single primary afferent neuron connects the sensory receptor cell to the brain and that we have physiological metrics that can be applied in conjunction with histological tools for determination of cochlear structure and function.

The environment influences the function of transplanted cells, and one issue to be overcome in cell transplantation is the difference in environment compared to that during development. Newborn neurons in adult hippocampus and olfactory bulb exhibit hyperexcitability and enhanced synaptic plasticity at glutamatergic inputs during a critical period of maturation (Tashiro et al., 2006). One of the best-studied mediators of stem cell tropism is the G-protein coupled receptor CXCR4 and its only known ligand SDF-1, which promote chain migration, the principal mode of stem cell movement in the mature brain (Imitola et al., 2004). Stem cells may undergo tropism specific for pathology in several animal models of neurologic disease. Extrinsic factors drive transplanted neural stem cells to differentiate into neurons or glia (Gage, 2000). Both endogenous cells in the dentate gyrus and subventricular zone and cells engrafted into the brain have been observed to preferentially undergo neurogenesis when subjected to ischemia (Zhang et al., 2001; Chu et al., 2003), thus replacing the cells most susceptible to ischemic insult. Previous experiments have shown that the environment in the host cochlea is critical for transplanted stem cell survival, with greater engraftment seen at 1-3 days than at 7 days post-injury (Lang et al., 2008).

There are several sources available for exogenous replacement of SGNs. The adult inner ear stem cells are potentially a source of cells for transplantation. The identity of stem cells

within the ganglion would be valuable information because these cells could then be enriched prior to use in cell replacement strategies. In addition, ES cells can be differentiated into neurons. ES cell-derived neural progenitors were induced to adopt a sensory fate by culture in BMP4 (Shi et al., 2007). ES cells expressing Ngn1 adopted an SGN-like fate after culture in BDNF and GDNF (Reyes et al., 2008). Induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006) have been used to generate auditory neural lineage by co-culturing with mouse cochlear explants (Nishimura et al., 2009). Mesenchymal stem cells (MSCs), adult stem cells of mesodermal derivation, can home to the deafened cochlea, although no spontaneous transdifferentiation to cochlear cell types occurred after acoustic trauma or bone marrow mobilization (Lang et al., 2006; Tan et al., 2008). MSCs are reported to elicit protective effects via secretion of neurotrophic factors (Crigler et al., 2006) and inflammatory modulators (Pluchino et al., 2005). Adipose-derived stem cells have similar traits as MSCs (Zhou et al., 2011).

ES cells could theoretically be transplanted at any stage of differentiation to neurons. In our work we have found that neural progenitors derived from ES cells could be obtained *in vitro*, and comparison to neural transplantation revealed that this was likely to be the optimal stage of transplantation to avoid apoptosis that is prevalent when differentiated neurons are transplanted in a suspension (Shi et al., 2007; Yuan et al., 2012). The same phenomenon has been observed in previous CNS work (Bjorklund et al., 2003). Although these neurons were at an intermediate stage at which they could become any type of neuron they were beyond differentiation stages at which endoderm or mesoderm would be generated, thus minimizing teratoma formation. A combination of *in vitro* methods to stimulate ectoderm and neural fate commitment combined with the local cues provided by the inner ear environment were found to be sufficient to convert the cells to differentiated neurons that responded to cues for neurite growth and synaptogenesis and were functionally active (Tokano et al., 2012; Yuan et al., 2012).

We have shown a regenerative capacity in neurons placed in proximity to hair cells in culture. Isolated SGNs from neonatal mice formed synaptic contacts with hair cells in organotypic cultures of organ of Corti (Martinez-Monedero et al., 2006). This was also seen with neurons differentiated from mouse (Corrales et al., 2006; Nayagam et al., 2012) or human (Shi et al., 2007) ES cells. SGNs contacted more than one hair cell in the initial cultures and then underwent pruning of branches (Martinez-Monedero et al., 2006; Martinez-Monedero and Edge, 2007). We also found that postsynaptic densities were induced in the fibers that contacted hair cells and were closely apposed to the points of contact with presynaptic ribbons (Tong et al., 2011). Since the efficiency of reinnervation in these cultures was low, we used the system as an *in vitro* synaptogenesis assay to assess the importance of various molecules for regeneration. The low efficiency could be overcome in part by inhibition of one of the molecules expressed in sensory epithelium, repulsive guidance molecule, RGMa (Brugeaud and Edge, 2009). RGMa inhibition increased sprouting and axonal growth and accelerated synaptic pruning (Brugeaud and Edge, 2009). This suggested that axonal growth and sprouting were intrinsic but could be influenced by guidance molecules from the surrounding cells. Neurotrophins, moreover, increased synaptogenesis of afferent neurons with hair cells in organ of Corti explant cultures, and glutamate played an important role in establishing the connections (Tong et al., 2012).

A number of groups have tested ES cells for SGN replacement *in vivo* (Okano et al., 2005; Coleman et al., 2006; Corrales et al., 2006; Sekiya et al., 2006; Shi et al., 2007; Lang et al., 2008; Praetorius et al., 2008; Reyes et al., 2008). In our studies ES cell-derived mouse neural progenitor cells transplanted into the cochlear nerve trunk sent out abundant processes that occupied a significant portion of the space formerly occupied by the cochlear nerve. A portion of the neurites grew in fasciculating bundles projecting through Rosenthal's

canal into the osseous spiral lamina and ultimately into the organ of Corti, where they contacted hair cells (Corrales et al., 2006). Reinnervation in the base of the cochlea led to partial restoration of ABR amplitude in gerbils (Tokano et al., 2012) and improved thresholds in the mouse (Yuan et al., 2012). The areas of synaptogenesis as assessed by histology correlated with the frequencies where improved function was apparent by measurement of ABR, confirming functional synaptogenesis.

To restore auditory circuitry, functional connections to hair cells and cochlear nucleus must be made. Reporters for tracking the donor cells are required to visualize the new synapses. Optimizing the surgical approach increases the chance of measuring an improvement in ABR. The placement of the transplanted cells is important for their projection (Coleman et al., 2006; Martinez-Monedero and Edge, 2007). Cells transplanted into Rosenthal's canal and the auditory nerve trunk had more abundant projections to hair cells and cochlear nucleus than those placed in the scala tympani or scala media (Corrales et al., 2006; Shi et al., 2007; Yuan et al., 2012). The bony wall of the osseous spiral lamina can be a barrier to engraftment. The initial site for cell placement may also be critical, because neurites can be blocked when they encounter the Schwann-glial transitional zone (Sekiya et al., 2007). Growing axons from the peripheral nerve towards the spinal cord or brainstem abruptly stop when they encounter astrocytes in the CNS. Thus, neurites growing from cells transplanted to the cochlea may be inhibited when they reach the Schwann-glial transitional zone (Aldskogius and Kozlova, 2002).

ES cell-derived neural progenitors made contacts with vestibular hair cells (Kim et al., 2005), and ES cell transplantation into guinea pig cochlea resulted in growth of processes, although the processes did not appear to grow toward the organ of Corti (Okano et al., 2005). ES cells transplanted into the cochlear modiolus of animals with damaged SGNs grew fibers along the remaining neurons and to the organ of Corti (Sekiya et al., 2006). Neural stem cells have been used for transplantation into the cochlea of a mouse that had been treated with cisplatin to remove endogenous neurons, and the cells were present in the cochlea two weeks after grafting (Tamura et al., 2004).

The implanted cells would not have much clinical significance unless central connections could be established. In a co-culture system with the brainstem, ES cells survived, underwent neuronal differentiation, and migrated towards the cochlear nucleus (Thonabulsombat et al., 2007). We have found the neural progenitors derived from ES cells transplanted into the auditory nerve trunk sent extensive process towards the cochlear nucleus (Shi et al., 2007; Yuan et al., 2012).

The differentiation of ES cell-derived progenitors to neurons with the properties of SGNs, and our ability to transplant cells into the deafened cochlea followed by restoration of the neural circuitry is an exciting and significant step toward the ultimate goal of improved hearing. Whether the transplanted cells integrate is dependent upon the cell source, the site of engraftment, and the differentiation to physiologically functional neurons. Testing of molecules in the *in vitro* system provides a route to new methods to increase synaptogenesis.

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