"Replacement of diseased auditory neurons by cell transplantation"

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1. ABSTRACT

The auditory nerve is an important target in hearing restoration research along with the hair cells. Although there are several potentially useful therapeutic options to rebuild lost hearing, cell transplantation is a very realistic option. Cells can be infused into the auditory nerve without compromising the auditory brainstem responses and damaging the membranous labyrinth. The final fate of transplanted cells may be determined by the intrinsic molecular program and the extracellular guidance cues. The first factor may be largely decided by the type of donor cell used and the second factor can be modified by the application of various molecules. Our recent experiments using ontogenetic-stage/region-restricted precursors and embryonic stem cells suggest that donor cells at later development stages seemed to have more mature intrinsic molecular programs to guide them more precisely and efficiently to the final expected destination. We discuss the critical interactions between the extracellular molecules such as myelin-derived inhibitory molecules expressed after CNS injury and the intracellular actin dynamics regulated by Rho GTPases in relation to the regeneration of the auditory neurons.

2. INTRODUCTION

Hearing impairment is one of the common disabilities in the modern era and affects 250 million people worldwide (1). Most of hearing loss is so-called sensorineural hearing loss where the hair cells are initially damaged and the auditory ganglion cells are secondarily compromised (2) (Figure 1, A). In another type of hearing impairment, the initial insult to the auditory nervous system occurs as a lesion in the axons of the nerve and with time the degeneration proceeds toward the cochlea and the brainstem (2, 3) (Figure 1, B). Therefore, the auditory neurons are commonly injured or degenerate at the most advanced stage in either of these pathological processes. This indicates that replacement of morbid auditory neurons becomes an indispensable part in reviving lost hearing.

For the replacement of morbid auditory neurons, there is compelling evidence that cell transplantation provides a realistic opportunity. In this review we focus our discussion on the replacement of auditory neurons.

3. ANATOMY: THE HAIR CELLS AND AUDITORY NEURONS/COCHLEAR NUCLEUS

Recently, we described the anatomy of the peripheral auditory nervous system in the scope of hearing restoration elsewhere (2). Briefly, the auditory nerve is a bundle of auditory neurons spanned from the temporal bone to the brainstem through the cerebellopontine angle cistern (Figure 1). The portion of the auditory nerve observed in neurosurgical retromastoid approach is the cisternal portion of the auditory nerve that is proximal to the Schwann-glial transitional zone and therefore CNS in nature (2) (Figure 1). The auditory neurons have bipolar processes that connect the hair cells with cells from the cochlear nucleus. Transplanted cells should have the same bipolar morphology with extended axonal projections from the temporal bone.

The auditory neurons express trkB and trkC and their survival depends on various neurotrophins such as BDNF and NT-3 that are produce by the hair cells, the Schwann cells, and supporting cells (4, 5). Therefore, selective preservation of the hair cells with auditory neuronal degeneration occurs as exemplified clinically (see below). Experimentally we confirmed this condition using our rat auditory nerve injury model (6); selective auditory neuronal degeneration with total preservation of hair cells after auditory nerve compression was confirmed electrophysiologically (ABR and DPOAE) and histologically (immunohistochemical staining for hair cells in frozen sections and surface preparations) (unpublished data).

The embryogenesis and survival of cochlear nucleus cells intimately depend on the auditory neurons; innervation by auditory neuron and subsequent synaptic activity are essential for normal development of the cochlear nucleus cells (7).

4. AUDITORY NEURONS AS A TARGET OF HEARING RESTORATION

In various pathological conditions, the auditory neurons are a primary target for hearing restoration.

4.1. Vestibular schwannoma

Hearing preservation in vestibular schwannoma treatment is still remained as an unresolved problem in neurosurgery (8-10). Reported success rates of hearing preservation after microsurgical removal of vestibular schwannomas varies from less than 30% to more than 80% (11-13), although direct comparison of these data cannot be justified due to the difference of various factors such as patient selection criteria, methods of defining hearing preservation and length of follow up period (14, 15). Although gamma knife surgery (GKS) boasts higher rates of hearing preservation (16), this modality of treatment is also associated with an additional hearing loss (17). Rates of hearing decline after microsurgical/GKS treatments range from approximately 20 to 60% and this rate becomes higher the longer the postoperative follow up (17-22). These results indicate that some means other than

surgical/radiosurgical modalities should be sought to resolve the problem of hearing impairment in vestibular schwannoma patients along with those for hair cell replacement.

4.2. Cochlear nerve deficiency

With the progress of MR imaging techniques such as high-resolution T2-weighted fast spin-echo sequences or submillimetric 3-D constructive-interference in-steady-state (CISS) sequences, the four major nerves within the internal auditory canal, facial, cochlear, superior and inferior vestibular nerves, can be visualized (23, 24). The term "cochlear nerve deficiency" is used to refer to those cases in which the auditory nerve is either small (hypoplasia) or absent (aplasia) on MRI (24). This pathological state may occur developmentally or as the squealae of postnatal degeneration due to various causes. For these patients, building the auditory nerve may become a choice of potential treatment. As it was noted that even a minimal number of residual nerve fibers is sufficient to deliver some acoustic information to the higher auditory center (25), total replacement of auditory nerve fibers may not be necessary.

4.3. Cochlear implant and auditory brainstem implant

For successful performance of cochlear implants, the presence of an ample number of functional auditory neurons is mandatory (26). Auditory brainstem implants (ABI) are utilized in the patients with neurofibromatosis II or other pathological conditions where bilateral auditory nerves degenerate (27). Even in ABI it is worth while to preserve the auditory neurons as many as possible to prevent trans-synaptic (neuronal) degeneration of the cochlear nucleus cells and cells in the higher auditory nuclei (28).
Collectively, these clinical facts urge us to find some means to repair degenerated auditory neurons.

5. THERAPEUTIC MODALITIES FOR HEARING PRESERVATION/RESTORATION

Various therapeutic modalities come into consideration in order to prevent or ameliorate degeneration of the auditory neurons and foster its regeneration.

5.1. Prevention of progression of injuring process in the auditory nerve

In spinal cord injuries, mechanical disruption of axons in the white matter may primarily occur and a subsequent cascade of biochemical events may lead to delayed or secondary cell death that evolves over a period of days to weeks (29). The auditory nerve proximal to the TZ is composed of axons embedded in astrocytes like the white matter in the spinal cord and can be regarded as a part of the CNS (Figure 1). Thus it may be worth examining whether similar therapeutic strategies against acute spinal cord injury and cerebral trauma may also effective to halt or ameliorate the pathological processes in acute auditory nerve injury. However, in spite of an enormous compilation of clinical and experimental trials, there are no medical and pharmacologic strategies of
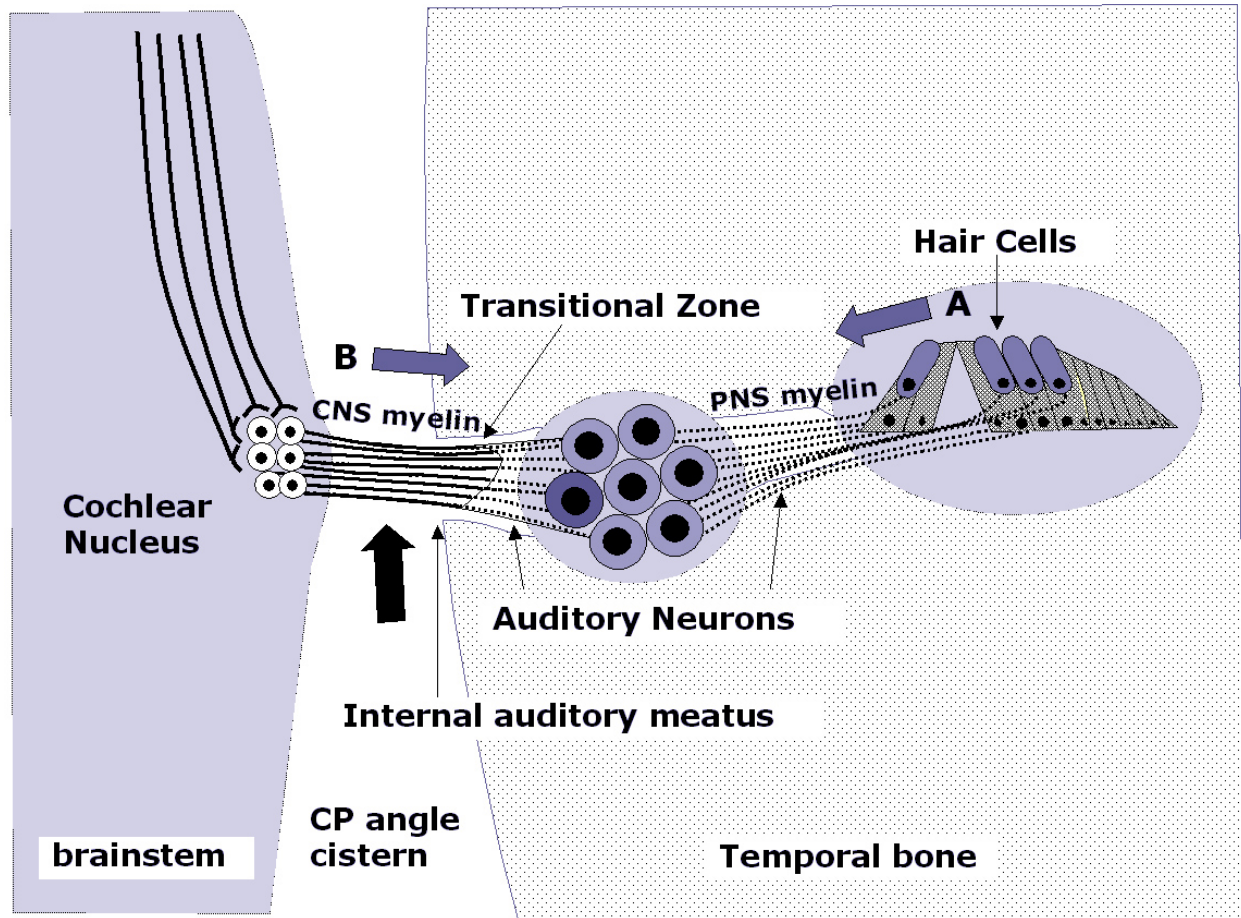
Figure 1. The auditory nerve is a bundle of auditory neurons connecting between the hair cells and the cochlear nucleus cells. The portion of the auditory nerve observed in neurosurgical retromastoid approach is the cisternal portion of the auditory nerve (large arrow) that is proximal to the Schwann-glial transitional zone and therefore CNS in nature. Regardless of the initial lesion site (arrows, A and B), the auditory neurons commonly degenerate in these pathologies at last.

proven benefit to prevent the progression of the secondary spinal cord injury except support of arterial oxygenation and spinal cord perfusion pressure (30, 31). Such hemodynamic approaches have been tried in acute auditory nerve injury; a tactic to maintain microcirculation of the auditory nerve using topical and systemic application of a calcium blocker and systemic use of hydroxyethyl starch, a plasma volume expander, ameliorated postoperative hearing disturbances after vestibular schwannoma removal (32). Another clinical study reported that trigeminocardiac reflex that causes bradycardia, asystole and/or arterial hypotension by intraoperative surgical manipulation of the trigeminal nerve is a negative prognostic factor for hearing preservation in patients undergoing vestibular schwannoma surgery (33).

These adjunctive interventions may play some roles in preventing progression of auditory neuronal degeneration more or less but cannot increase the number of viable auditory neurons in the patients with long-standing hearing disorders.

5.2. Encouraging neurite extension from damaged auditory neurons

In motor cranial nerves such as the facial nerve, axonal regeneration can be expected after axotomy. In the facial nerve, the neuronal cell bodies are spared even after the axons are transected (34). In fact, degeneration extends not only distally (Wallerian degeneration) but also proximally toward the cell body (retrograde degeneration). However, retrograde degeneration toward the cell body stopped at a point a few segments proximal from the severed site and then regeneration can start from this site. In contrast, however, this does not hold true in auditory nerve injury. In this sensory cranial nerve, when axons are severed, not only the axons themselves but also the cell bodies degenerate in a retrograde fashion (3). As the retrograde degeneration of damaged auditory neurons proceeds quite rapidly in acutely injured conditions (35), it is not realistic to expect neurite extension occur in such short time window.

Taken together, there is currently no evidence for axonal regeneration in auditory neurons.

5.3. Recruitment of endogenous cells

One possible way to re-install the auditory neurons may be the recruitment of endogenous progenitors/precursors. In animal models of stroke, the occurrence of ectopic neurogenesis has been reported (36-38). These newly born neurons are expected to compensate for the loss of neuronal function caused by strokes (39). However, one serious problem of this approach is the number of newborn neurons is too small for recovery of neurological functions (37). Thus, tactics to enhance the proliferation, survival, and/or neuronal maturation of these cells were tested; following transient ischemia, hippocampal CA1 pyramidal neurons underwent extensive degeneration and by infusion of FGF-2 and EGF endogenous progenitors were recruited in situ and massive regeneration of pyramidal neurons was induced and these newly regenerated neurons were integrated into the neural circuitry (38). In a focal demyelinating lesion in the dorsal spinal cord in the adult rats, injection of Shh resulted in a sustained burst of cell proliferation (40). Infusion of transforming growth factor-alpha in animals with a selective lesion of the dopaminergic nigrostriatal system induced massive proliferation of forebrain stem cells, followed by migration of both glial and neural progenitors toward injection side (41).

In the inner ear, hair cells and supporting cells share a population of common epithelial progenitors (42). In fact, supporting cells are able to change their phenotype and become new hair cells in birds following hair cell damage (43). In mammal, it was reported that the hair cells can be replaced by transfection of the deafened ear with the basic helix-loop-helix (bHLH) transcription factor *Atoh1;* this factor could stimulate to produce new hair cells probably from the surrounding supporting cells and lead to measurable functional recovery (44).

Although there is some evidence for neural progenitors in normal adult ears (45), there is no evidence for such cells in animals or patients with long-standing, profound hearing loss.

5.4. Cell transplantation

Cell transplantation is a direct way to rebuild lost hearing by replenishing viable cells into the host auditory nerve. To connect the hair cells and the cochlear nucleus cells, transplanted cells should extend their processes bipolarly. For this purpose, there are several candidate cells to be evaluated.

6. DONOR CELLS

6.1. ES cells

The pluripotent embryonic stem (ES) cells are derived from the inner cell mass of blastocysts, have the ability to give rise to all three embryonic germ layers (ectoderm, endoderm, and mesoderm), indicating an enormous potentials as the donor cells (46). On the other hand, some technologies for selection of pure cell populations are needed to obtain appropriate cells for transplantation as described below. Otherwise, they would differentiate into unexpected tissue.

Another problem is that it is a long and winding road for ES cells to reach the final expected destination because ES cells should be guided by various exogenous factors at each step of differentiation. In a previous study (47), we used a technique called SDIA (stromal-cell-derived inducing activity) to induce neural differentiation (48) and recently another technique to efficiently differentiate ES cells into neural precursors on the matrix components of the human amniotic membrane in serum-free medium (amniotic membrane matrix-based ES cell differentiation, AMED) was reported as an refinement of such technique (49). However, even with these technical progresses, tumorigenesis is still remained as an unresolved issue in ES cell application *in vivo* (50).

6.2. Somatic stem cells

After birth, adult stem cells reside in various microenvironments ("niches") (51). Adult neural stem cells were identified in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampus region in the adult brain (51-54). The SVZ astrocytes have stem cell features and give rise to transient amplifying cells, which further give rise to neuroblasts. Neuroblasts differentiate into neurons and oligodendrocytes (54, 55). As such, somatic adult cells tend to give rise to cells specific to the organ system in which they reside but can produce several types of cell.

The first report in which cell transplantation was applied to the inner ear was performed using one of such neural stem cells (56). Many studies on restoration of retinal ganglion cells, however, demonstrated that the mature mammalian organs rarely accept and incorporate stem cells or to promote further differentiation (57-59).

6.3. Ontogenetic-stage and region-restricted precursors

One hypothesis is that committed progenitor or precursor cells at later ontogenetic stages might have a higher probability of success upon transplantation than cells at earlier stage (59). In retinal regeneration studies, ES cells or brain- and retina-derived stem cells transplanted into adult retina have shown little evidence of being able to integrate into the host retina and differentiate into new photoreceptors (58, 60). This might be because the mature mammalian retina lacks the ability to accept and incorporate stem cells or to promote photoreceptor differentiation (59). In contrast, precursors taken from the developing retina at a time coincident with the peak of rod genesis (60) can integrate, differentiate into rod photoreceptors, form synaptic connections and improve visual function (59).

Following such a scenario, one particular cell line suitable for auditory neuronal replacement was established and its biological behavior was investigated not only *in vitro* but also *in vivo* (61, 62).

In the inner ear, at E10.5 neuroblasts delaminate from the ventral area of the otocyst to form the audio-vestibular ganglion but at E13.5 most sensory epithelial cells and neurons have exited the cell cycle and hair cells start to differentiate (2). Based on this embryological

knowledge, a group of the cells were excised from the ventral otocyst at embryonic day E10.5 from a transgenic mouse (Immortomouse®) (63) and a conditionally immortal cell line was established as US-VOT-N33 (64). This N33 cell line has been comprehensively characterized *in vitro* using a panoply of markers assessed by immunolabelling, RT-PCR and electrophysiology and was confirmed to be derived from auditory neuroblasts. It expresses the transcription factors GATA3, NeuroD, Brn3a and Islet-1, the cytoskeletal protein β3-tubulin and the potassium channel KCNQ3 (65). NeuroD is down-regulated during differentiation *in vitro*, as observed for cochlear neuroblasts *in vivo*, and the lack of expression of Pax2 and cytokeratin upon differentiation distinguishes N33 from ventral, otic epithelial cells.

The Immortomouse carries a temperature-sensitive variant of the T-antigen derived from the SV40 virus and regulated by a γ-interferon-inducible promoter (63). Therefore, the neuroblasts derived from this mouse proliferate infinitely under the proliferative condition *in vitro* (the presence of γ-interferonγ (IF) at 33°C). Prior to cell transplantation, γIF is removed and cells are transplanted to the auditory nerve (its temperature is about 39°C). These environmental changes induce the neuroblasts to differentiate into auditory neurons within the host auditory nerve.

N33 cells were transplanted into the intact auditory nerve to observe how they behave *in vivo* (62); the transplanted cells migrated peripherally and centrally and aggregated to form coherent groups of the cells simulating the spiral ganglion cells in the Rosenthal's canals that could be called "ectopic ganglia". Approximately 7 days after transplantation, the cells expressed beta3-tubulin and adopted a similar morphology to native spiral ganglion neurons. They developed bipolar projections aligned with the host nerves. There was no evidence for uncontrolled proliferation *in vivo* and up to 300 cells survived in an injected animal for at least 63 days, the longest observation period in this study. These results indicated that immortal cell lines can potentially be used in the mammalian ear and that significant numbers of cells differentiate within the auditory nerve tract.

7. TECHNICAL CONSIDERATIONS OF CELL TRANSPLANTATION TO THE AUDITORY NERVE

In our experimental studies, we transplanted the cells into the auditory nerve using a thin tube inserted into the auditory nerve trunk without touching to the membranous labyrinth, the membrane-sealed fluid containing spaces (the scala media and scala tympani) (2) (Figure 1). Breaching these membranes causes disturbance of the ionic balance among these fluid-containing chambers, resulting in hearing impairment (2).

There is some concern about the trauma of inserting a tube directly into the auditory nerve trunk even if its diameter is very thin. However, we found surprisingly little functional degradation as reflected in the recordings of

ABR (62). It was likely that the fused silica tube was prone to slip into the extracellular space between the axons rather damaging the axons themselves. The same condition may occur in a neurosurgical maneuver to insert a drainage tube with the diameter of two millimeters or so into the ventricle through the frontal cortex of the brain to alleviate the pressure derived from hydrocephalus. In this maneuver, the neurosurgeons do not experience any adverse side effects such as deterioration of neurological status of the patients.

In our technique in the rats, infused cells seemed to proceed in the space between the axons as the cells are gradually infused by an infusion pump. In a situation where the host auditory nerve is atrophic and totally non-functioning as experienced clinically, the insertion of a tube into such auditory nerves may be justified because additional functional deteriorations may not be incurred by this procedure.

Moreover, we can send cells to the region of the cochlear nucleus using the auditory nerve as a conduit, another advantage of this surgical approach.

8. SHAPING TRANSPLANTED CELLS

Two factors, internal molecular programs and extracellular guidance cues, may play crucial roles in deciding the final fate of transplanted cells.

8.1. Internal molecular program

After plating, cultured embryonic hippocampal neurons extend several short immature neurites at first but then one of these immature neurites elongates and acquires axonal characteristics. This occurs *in vitro* in a uniform and symmetric environment (66), indicating that internal molecular programs play an important role in axonal development (67). The instruction for such polarized growth of cells is formed immediately postmitotic stage; the centrosome, the Golgi apparatus and endosomes cluster together close to the area where the first neurite will form. This first neurite ultimately forms the axon and the other neurites become dendrites (68). In our study, transplanted N33 cells adopted a bipolar shape within 4 days after transplantation to the auditory nerve (62). In the experiments with ES cells under the same experimental conditions we did not observe the same response (47). It is likely that N33 are ontogenetic-stage/region-restricted and therefore some built-in internal molecular program seemed to dominate the initial phase in forming the cell morphology.

8.2. External environmental cues

Along with internal programs, various extrinsic factors around the cells decide cell behavior as typically exemplified in the growth cones behavior under repulsive and chemoattaractant cues (69). In order to enhance neurite outgrowth after cell transplantation, manipulating the environment around transplanted cells may become an important adjunct as we have demonstrated *in vitro*; N33 extended bipolar neuritic processes in response to FGF-1 and FGF-2 (61, 70) and proliferation increases in the
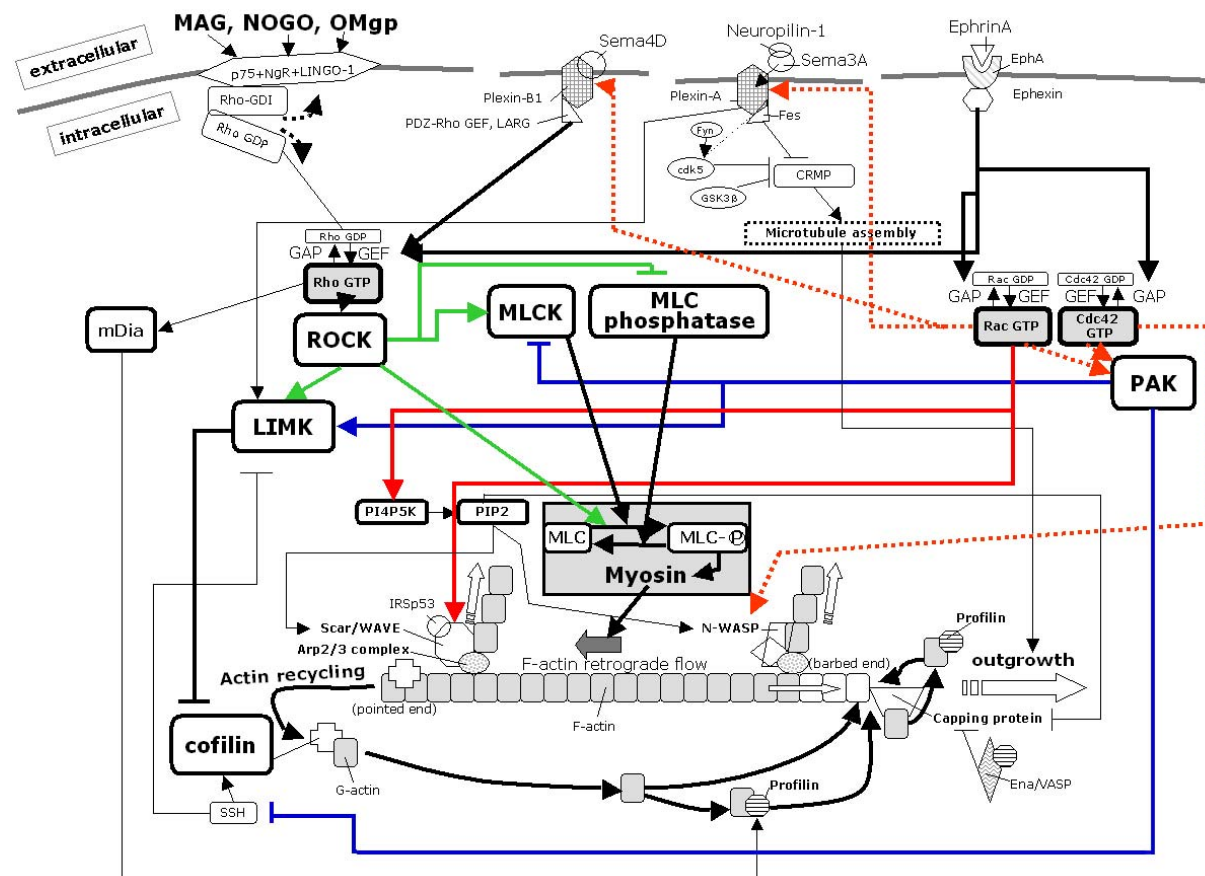
Figure 2. The dynamic movement of actin filaments in the leading edge of growth cone/neurite regulated by Rho GTPases-related enzymes. G-actin monomers bind to the extending edge of neurites and this polymerization is coupled with retrograde F-actin flow by activated myosin. The function of myosin is controlled by MLCK and MLC phosphatase that are positively and negatively regulated by PAK and ROCK, respectively, although they both inhibit cofilin, actin-depolymerizing factor, through LIMK. The extracellular ligands such as myelin-inhibitory molecules interact with the receptors in the plasma membrane and cause growth cone collapse or neurite retraction through Rho GTPases-regulated enzymes. Note that some of the inter-relationships have been established in non-neuronal cell lines and need confirmation in neurons. Also, in vitro and in vivo studies are combined in this figure. Pointed arrows represent activation and flat arrows represent inhibition (see the text).

presence of IGF-1 as observed in normal cochlear neuroblasts (71, 72).

8.2.1. Rho GTPases and actin reorganization in growth cone/neurite outgrowth

A growing evidence of literature implies that small GTPases play pivotal roles in the regulation of neuronal morphogenesis, including migration, polarity, axon growth and guidance, dendrite elaboration and plasticity, and synapse formation. There are many good reviews relevant to this topic (69, 73-79) and Figure 2 depicts some essential pathways that may be intimately related to growth cone/neurite outgrowth with a emphasis on several extracellular signals.

GTPases such as Rho, Rac, and Cdc42 work as molecular switches and this switching is accomplished by two proteins, guanine nucleotide exchange factors (GEFs)

and GTPase activating proteins (GAPs); GEF facilitates the exchange of GDP for GTP, thereby switching Rho GTPases on. Subsequently, activated Rho GTPases bind to downstream effectors and different biological activities are elicited (73, 79). In contrast, increased endogenous GTPase activity switches off activated Rho GTPases (80). Third protein, guanine dissociation inhibitors (GDI), regulate the nucleotide-binding state of the Rho family and regulates of both the GDP/GTP cycle and the membrane association/dissociation cycle (81).

In general, the activation of Rac and Cdc42 promotes growth cone/neurite outgrowth and growth cone motility, whereas the activation of Rho causes neurite outgrowth inhibition and a growth cone collapse, indicating an antagonism exists between Rac/Cdc42 and Rho GTPases, although some exceptions to this rule exist (73, 74, 76, 78, 82-86).

For example, Rac GTP and Cdc42 GTP not only stimulate PAK (a positive regulator for growth cone/neurite outgrowth) but also N-WASP and Scar/WAVE that are required for actin reorganization in neurite extension (87, 88). On the contrary, Rho-GTP activates ROCK that negatively regulates the outgrowth of growth cone/neurite (89, 90) (Figure 2).

The dynamic movement of actin filaments in the leading edge of growth cone is a vital part in neurite extension (91). Cofilin severs F-actin at the proximal end (depolymerization) and the resulting G-actin monomers bind to the extending barbed end of the neurites (polymerization); recycling of F-actin or tread milling takes place (91-95). Activation of cofilin may increase filopodial length by increasing the G-actin pool (92), thus enhancing polymerization at filopodial tips (96, 97) (Figure 2).

Actin polymerization at the leading edge is coupled with retrograde F-actin flow (91); after being assembled at the leading edge, these F-actin bundles and networks are translocated proximally by retrograde F-actin flow powered by myosin motor force and myosin contraction (91, 98, 99), resulting in growth cone collapse or neurite retraction (74). The function of myosin is positively regulated by phosphorylation of myosin regulatory light chain (MLC) by myosin light chain kinase (MLCK) (100). PAK inhibits MLCK (101), causing decreased phosphorylation of MLC and decreased actomyosin contractility (102). On the contrary, ROCK phosphorylates MLC phosphatase, as well as MLC itself, both of which lead to increased contraction of the actomyosin network (103, 104). Thus, ROCK and PAK have opposing effects on myosin contractility, although they both inhibit cofilin through LIMK (105-107).

8.2.2. MAG, NOGO, OMgp and other extracellular guidance cues

It is well known that after CNS injury, growth-inhibitory molecules, MAG, NOGO, and OMgp, are expressed in the myelin and they inhibit axonal regeneration (81, 108-110). This condition is observed in spinal cord injury and optic nerve injury where the oligodendrocytic CNS myelin is mechanically injured (109). The portion of the auditory neurons to which mechanical stress is imposed in usual retromastoid approach is wrapped by CNS myelin (111) and therefore myelin-derived growth inhibitory molecules may express when the CP angle portion of the auditory nerve is traumatized by surgical procedures.

These inhibitory molecules bind to NgR that interacts directly with p75, LIGO-1, and Rho-GDI (112, 113), resulting in the recruitment of Rho to the membrane (Figure 2). Once Rho is recruited to cell membrane and released from Rho-GDI, Rho-GEF activates it by dissociating GDP and binding GTP (113). Rho-GTP stimulates ROCK and in turn activates LIMK (106, 107), leading to the downregulation of cofilin activity. At the same time, ROCK induces contraction of actomyosin via the pathway mentioned above. Collectively, these events result in growth cone collapse or neurite retraction.

There have been many studies in spinal cord injury and optic nerve crush where Rho GTPases and downstream ROCK were manipulated (109). To name a few, C3 transferase that selectively inactivates Rho without affecting other GTPases, was applied to the site of over-hemisection injury in adult mice and some long distance regeneration through the lesion site and past the lesion was observed (114). In a experiment where the optic nerve of adult rat was crushed and treated with C3 transferase, numerous cut axons traversed the lesion to regrow in the distal white matter of the optic nerve (115).

As mentioned above, the retinal ganglion neurons are bipolar neurons like the auditory neurons and an *in vitro* study indicated that the growth cones of cultured retinal ganglion cells collapsed when treated with soluble Ephrin-A (89). Ephrin-A activates Rho and inhibits Rac/Cdc42 through Ephexin (116) (Figure 2). Therefore, specific inhibitors of Rho and ROCK can block the collapse of RGC growth cones (89).

The effectiveness of ROCK inhibitor in Semaphorin/Plexin system is also shown. Plexin-B1 binds to PDZ-RhoGEF and LARG and stimulates Rho activation leading to repulsive response (117, 118) (Figure 2). Growth cone collapse through this pathway is inhibited by an inhibitor of Rho GTPases (118).

Sema3A/neuropilin complex permits Fes/Fps to phosphorylates Plexin-A1 and CRMP, leading to growth cone collapse through the disturbance of microtubule assembly (Figure 2). CRMP inhibition causing growth cone collapse is induced also by Fyn/Cdk5 and GSK3β (74, 119, 120) (Figure 2). Actually, overexpression of CRMP-2 in injured rat hypoglossal motor neurons accelerates nerve regeneration (121).

So far, however, there has been no *in vivo* study to test the effectiveness to inhibit Rho or ROCK in attempting neuronal regeneration in the auditory neurons. For this purpose, the auditory nerve is a convenient target because its functional evaluation can be easily performed in a highly subjective manner using ABR without performing time-consuming behavior tests in SCI.

In conclusions, two approaches should be taken in concert in order to revive auditory nerve function; one is to select the optimal cells such as those that efficiently differentiate and take bipolarity and another is to apply exogenous factors that simulate neurite extension enough to reach the target structures at each side of the cell processes.

9. ACKNOWLEDGEMENTS

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Abbreviations: ABI; auditory brainstem implant, ABR; auditory brainstem response, Arp2/3; actin-related protein 2/3, Brn3a; POU-domain transcription factor Pou4f1, Cdc42; cell division cycle 42, Cdk; cyclin-dependent protein kinase, CNS; central nervous system, CP; cerebellopontine , CRMP; collapsin response mediator protein, DPOAE; distortion product otoacoustic emissions, EGF; epidermal growth factor, Ena/VASP; enabled/vasodilator-stimulated phosphoprotein , ES; embryonic stem, F-actin; filamentous actin, FGF; fibroblast

growth factor, G-actin; globular actin, GAP; GTPase-activating protein, GATA3; GATA binding protein 3, GEF; guanine nucleotide exchange factor, GKS; gamma knife surgery, GSK; Glycogen synthase kinase, GTP; guanosine triphosphate, IGF; insulin-like growth factor, IRSp53; insulin receptor substrate p53, KCNQ3; the potassium channel KCNQ3, LARG; leukemia-associated Rho GEF, LIMK; LIM-domain-containing protein kinase , LINGO1; leucine rich repeat and Ig domain containing 1, MAG; myelin-associated glycoprotein , mDia; mammalian homolog diaphanous, MLC; myosin regulatory light chain, MLCK; myosin light chain kinase, MR; magnetic resonance, NgR; Nogo-66 receptor , N-WASP; neuronal Wiskott-Aldrich Syndrome protein, OMgp; Oligodendrocyte-myelin glycoprotein , p75; low affinity NGF receptor, PAK; p21-activated kinase, PDZ; postsynaptic density protein of 95kDa, DLG, ZO-1 homology, PI4P5K; phosphatidylinositol-4-phosphate 5-kinase, PIP2; phosphatidylinositol-4,5-bisphosphate, Rac1; Ras-related C3 botulinum toxin substrate 1, Rho; Ras homologous member, ROCK; Rho-associated kinase, RT-PCR; reverse transcriptase-polymerase chain reaction, Scar; suppressor of cAMP receptor mutation, Sema; semaphorin, Shh; sonic hedge hog, SSH; slingshot, SGZ; subgranular zone, SVZ; subventricular zone, TZ; transitional zone, WAVE; WASP family verprolin-homologous protein

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