Neuroscience 287 (2015) 43–54

EFFECTS OF ROCK INHIBITOR Y27632 AND EGFR INHIBITOR PD168393 ON HUMAN NEURAL PRECURSORS CO-CULTURED WITH RAT AUDITORY BRAINSTEM EXPLANT

E. NOVOZHILOVA, [a*] **U. ENGLUND-JOHANSSON,** [b] **A. KALE,** [a] **Y. JIAO** [a,c] **AND P. OLIVIUS** [a,d*]

[a] *Center for Hearing and Communication Research and the Department of Clinical Sciences, Intervention and Technology (CLINTEC), Karolinska Institute, Karolinska University Hospital, Stockholm 17176, Sweden*

[b] *Department of Ophthalmology, Institution of Clinical Sciences in Lund, Lund University, 221 84 Lund, Sweden*

[c] *Department of Otolaryngology, Head and Neck Surgery, Beijing Tongren Hospital, Capital Medical University, 100730 Beijing, China*

[d] *Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, SE-58183, Sweden*

Abstract—Hearing function lost by degeneration of inner ear spiral ganglion neurons (SGNs) in the auditory nervous system could potentially be compensated by cellular replacement using suitable donor cells. Donor cell-derived neuronal development with functional synaptic formation with auditory neurons of the cochlear nucleus (CN) in the brainstem is a prerequisite for a successful transplantation. Here a rat auditory brainstem explant culture system was used as a screening platform for donor cells. The explants were co-cultured with human neural precursor cells (HNPCs) to determine HNPCs developmental potential in the presence of environmental cues characteristic for the auditory brainstem region *in vitro*. We explored effects of pharmacological inhibition of GTPase Rho with its effector Rho-associated kinase (ROCK) and epidermal growth factor receptor (EGFR) signaling on the co-cultures. Pharmacological agents ROCK inhibitor Y27632 and EGFR blocker PD168393 were tested. Effect of the treatment on explant penetration by green fluorescent protein (GFP)-labeled HNPCs was evaluated based on the following criteria: number of GFP-HNPCs located within the explant; distance migrated by the GFP-HNPCs deep into the explant; length of the GFP+/neuronal class III β-tubulin (TUJ1)+ processes developed and phenotypes displayed. In a short 2-week co-culture both inhibitors had growth-promoting effects on HNPCs, prominent in neurite extension elongation. Significant enhancement of migration and in-growth of HNPCs into the brain slice tissue was only observed in Y27632-treated co-cultures. Difference between Y27632- and PD168393-treated HNPCs acquiring neuronal fate was significant, though not different from the fates acquired in control co-culture. Our data suggest the presence of inhibitory mechanisms in the graft–host environment of the auditory brainstem slice co-culture system with neurite growth arresting properties which can be modulated by administration of signaling pathways antagonists. Therefore the co-culture system can be utilized for screens of donor cells and compounds regulating neuronal fate determination.

Key words: hearing, organotypic culture, neuronal restoration, Y27632, PD168393.

INTRODUCTION

Therapeutic strategies to protect hearing

Mechanisms contributing to sensorineural hearing impairment resulting in a slow but progressive loss of spiral ganglion neurons (SGNs) are still under debate (Van de Heyning and Kleine Punte, 2010). SGNs degeneration arises from the damage to either their peripheral (dendrites) or central (axons forming the auditory nerve, AN) processes (Spoendlin, 1987). Recovering damaged neuronal circuitry is therefore crucial in developing therapeutics for people with sensorineural hearing loss. Attempts to preserve SGNs from further deterioration have been made (Shibata et al., 2011). Mostly they are confined to the introduction of neurotrophic factors. The factors can be overexpressed (Husseman and Raphael, 2009) using a viral vector to transduce cells (e.g. brain-derived neurotrophic factor (Rejali et al., 2007; Shibata et al., 2010); glial cell-derived neurotrophic factor (Kanzaki et al., 2002) or introduced via a passive release matrix (e.g. neurotrophin-3 (Richardson et al., 2007). Stimulating residual SGNs electrically directly (Buchman

*Corresponding authors. Address: Center for Hearing and Communication Research, Karolinska Universitetssjukhuset, Solna, Byggnad M1, 17176 Stockholm, Sweden. Tel: +46-8-51-773833; fax: +46-8-348546.
E-mail addresses: 1035bms@gmail.com (E. Novozhilova), petri.olivius@ki.se (P. Olivius).

Abbreviations: AN, auditory nerve; ANOVA, analysis of variance; BSA, bovine serum albumin; CI, cochlear implant; CM, conditioned medium; CN, cochlear nucleus; CSPGs, chondroitin sulfate proteoglycans; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DRG, dorsal root ganglion; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; HBSS, Hank's balanced salt solution; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hESC, human embryonic stem cells; HNPCs, human neural precursor cells; MEF, mouse embryonic fibroblast; MOI, multiplicity of infection; NC, neural-crest; PBS, phosphate-buffered saline; PDL, poly-D-lysine; PFA, paraformaldehyde; RGC, retinal ganglion cell; ROCK, Rho-associated kinase; RT, room temperature; SGNs, spiral ganglion neurons; TUJ1, neuronal class III β-tubulin.

http://dx.doi.org/10.1016/j.neuroscience.2014.12.009
0306-4522/

et al., 1999) with a cochlear implant (CI) can functionally replace damaged parts of the inner ear (Buchman et al., 1999), unlike hearing aids which make sounds louder by amplification. Regardless of the CI surgery success even when the SGNs population is relatively low (Clark et al., 1988), still insufficient number or poor condition of residual SGNs undermines CI performance (Bradley et al., 2008). Auditory brainstem implants, which stimulate electrically directly the CN in the brainstem bypassing the AN, have not yet proved to be satisfactory replacements for the AN, as the recipients do not achieve a considerable improvement in hearing (Otto et al., 2002). Nonetheless preservation of the central processes of the AN is still beneficial in brainstem implants since transneuronal degeneration of CN neurons and cells in the higher auditory nuclei can happen due to lack of input produced by the reduced population of SGNs (Morest et al., 1997).

Stem and precursor cell-based therapies

Regrowing SGNs with their fiber projections via implanted stem and precursor cell conversion would be a way to improve hearing function in patients who receive CI or have severe AN malfunctions. More than a decade of intensive pre-clinical studies evaluating potential stem cell types, ranging from embryonic stem cells to inner ear precursor cells, has proven that both hair cells and SGNs can to some extent be replaced (Li et al., 2003; Olivius et al., 2003; Rask-Andersen et al., 2005; Regala et al., 2005; Corrales et al., 2006; Rivolta et al., 2006; Coleman et al., 2007; Martinez-Monedero et al., 2008; Nishimura et al., 2009). However donor cells proliferative and differentiating potential varies depending on environmental cues and the developmental context in which the cues are encountered. Therefore donor cells preparation and conditioning to produce sensory neurons, ways of delivery, supplementary treatment with growth factors, removal of inhibitory signals from the donor/host tissue and use of biocompatible materials to aid cell delivery and growth need scrutiny.

Organotypic auditory brainstem slice culture system

Organotypic slice cultures of different brain regions have been used as a 3D model system to study a variety of developmental and disease-related processes (Gahwiler, 1981). Due to the preserved tissue cytoarchitecture, an organotypic brain slice culture is suitable to study cell-to-cell interactions including incorporation and differentiation of neural stem cells in host tissues (Scheffler et al., 2003). We have established conditions for the propagation of rat auditory brainstem slice cultures containing the CN and a part of the AN (Thonabulsombat et al., 2007; Glavaski-Joksimovic et al., 2008; Novozhilova et al., 2013). Slice cultures with the auditory brainstem region contain the CN, the first relay station in the auditory system that receives input from the AN. By exposing stem and precursor cells to intrinsic and extrinsic factors, pertaining to the AN milieu, temporal and spatial cues related to SGNs development are recapitulated. Evaluation of interaction with the host tissue of donor cells of human origin, e.g. human neural precursor cells (HNPCs) used in the current study is important since human cells are likely to be required in a future clinical setting.

Screening for factors modulating neurite outgrowth

In spite of a greater ability of HNPCs to survive and differentiate into neurons when cultured in the presence of an organotypic culture rather than in monoculture (Novozhilova et al., 2013), the low efficiency of co-cultured cell integration remains a problem. Lack of growth-promoting properties by the graft–host interface environment is to a large extent attributed to the accumulation of glia-associated molecules such as proteoglycans, galactolipids and myelin and the complex network of downstream signals they trigger (Yiu and He, 2006). Manipulating astroglial environment of the host will to a certain extent help dissect molecular events regulating efficient generation of neurons from grafted donor cells. Acutely dissected brainstem explants with preserved organotypic organization and lesion-induced neuronal and glial sprouting offer a convenient model system that facilitates manipulation and characterization of inhibitory signaling events hampering donor cells efficiency at producing functionally integrated neurons *in vitro*.

Many of the inhibitory molecules found at the lesion site activate the GTPase Rho with its effector Rho-associated kinase (ROCK) (Sivasankaran et al., 2004; Yiu and He, 2006) and epidermal growth factor receptor (EGFR) (Yiu and He, 2006), providing a strong rationale to target them following injury. These signaling mediators transduce inhibitory signals that trigger specific cytoskeletal rearrangements in the developing neurite growth cone. Proteoglycans consisting of a protein core and a chondroitin sulfate side chain (CSPGs) are considered to have a major growth inhibitory effect on developing and regenerating neurons (Rolls et al., 2006). GTPase Rho, which is a key intracellular regulator of cytoskeletal dynamics (Hall, 1998) has been implicated in growth cone collapse and retraction of neurites while activated (Lehmann et al., 1999; Wahl et al., 2000; Dergham et al., 2002).

Pharmacological inhibition of Rho and its downstream effector ROCK with Y27632 inhibitor has been shown to attenuate the arrest of neurite outgrowth on inhibitory substrates *in vitro* (Lingor et al., 2007) and improved functional recovery *in vivo* (Dergham et al., 2002; Fournier et al., 2003; Lingor et al., 2007). Likewise, EGFR activation induces neurite projections growth inhibition to occur. Administration of EGFR inhibitors e.g. PD168393 has been shown to enhance neurite extensions from dorsal root ganglion (DRG) grown on inhibitory substrates and to significantly promote regeneration of the crushed optic nerve in adult mice (Koprivica et al., 2005).

Our study

Here we sought to determine if pharmacological attenuation of Rho/ROCK and EGFR signaling with their respective inhibitors Y27632 and PD168393 in our HNPCs-auditory explant co-culture would facilitate neurite outgrowth of HNPCs in the presence of the explant inhibitory central glia. Conditioned medium (CM) experiments where HNPCs were grown in monoculture