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

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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

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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.

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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

in the absence (CM controls) or presence of the inhibitors in the medium collected from the auditory brainstem slices were included to evaluate off-target effects of the inhibitors. Investigating the primary target of the inhibitors was outside of the scope of this study.

EXPERIMENTAL PROCEDURES

Animals

Postnatal Sprague–Dawley rat pups (P12–15, $n = 35$) were obtained from the Harlan Laboratories, B.V., the Netherlands, and used for the preparation of auditory brainstem slices. Animals were maintained under standard conditions with food and water available *ad libitum*. All animal-related procedures were conducted in accordance with local ethical guidelines and approved animal care protocol (approval N329/07 and N3/11; Stockholms norra djurförsöksetiska nämnd).

Interface organotypic brainstem slice culture

Following animals decapitation after pentobarbital sodium overdose the skulls were opened longitudinally along the midline and the excised brains were placed in ice-cold dissecting medium (Hank's balanced salt solution, HBSS), supplemented with 20% glucose and 1% antibiotic/antimycotic (penicillin/streptomycin/amphotericin B). Transverse sections of the brainstem (300-μm-thick) were obtained using a tissue-chopping device. Slices encompassing the proximal part of the cochlear nerve and the CN according to their local anatomical landmarks (2–3 slices per brain harvested) were propagated as interface cultures (Stoppini technique (Stoppini et al., 1991) on sterile polyester membranes (24-mm diameter inserts with 0.4-μm pore size; Corning), coated with 10 μg/ml poly-D-lysine (PDL) (1 ml applied for 20 min, removed, membranes air dried overnight under sterile conditions) and 10 μg/ml laminin afterward (200 μl applied for 15 min, removed, membranes air dried overnight under sterile conditions). Coated membrane inserts were placed in a six well-plate containing 1 ml of the brainstem slice culture medium per well. Prior to placing brainstem explants on each insert (one explant per insert) the inserts were rinsed on the inside with 1 ml of the brainstem slice culture medium. The medium consisted of Dulbecco's modified Eagle's medium (DMEM; high glucose, L-glutamine, pyruvate) supplemented with 30% HBSS, 10% fetal bovine serum, 6.5 g/l glucose, 25 mM Hepes and 1% antibiotic/antimycotic (penicillin/streptomycin/amphotericin B). In these conditions the slices could be maintained in culture (37 °C, 5% CO_2) for up to 4 weeks. The following day after the preparation, brainstem slices were thoroughly washed in culture medium to remove cell debris. The medium was changed every other day.

HNPC line

The HNPC line used for this study was originally established (Carpenter et al., 1999) by L. Wahlberg, Å. Seiger, and colleagues at the Karolinska University Hospital and was kindly provided to us via Prof. A. Björklund (Dept. Exp. Med. Sci., Lund University, Sweden). The cell line was derived from a forebrain tissue of a 9-week-old (post conception) human embryo. The HNPCs were cultured in DMEM-F12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2.0 mM L-glutamine (Sigma, St. Louis, MO, USA), 0.6% glucose (Sigma), N2 supplement (Invitrogen) and 2.0 μg/ml heparin (Sigma) and grown as free-floating clusters (neurospheres). The growth factors human basic fibroblast growth factor (hbFGF, 20 ng/ml; R&D Systems, Minneapolis, MN, USA), human epidermal growth factor (hEGF, 20 ng/ml; Invitrogen) and human leukemia inhibitory factor (hLIF, 20 ng/ml; Sigma) were added every 3–5 days to the culture. Neurospheres were passaged by mechanical dissociation every 7–10 days and reseeded as single cells at a density of 1×10^5 cells/ml. Cells used in the study were passaged 11–13 times. A fraction of the HNPCs expressed the reporter gene green fluorescent protein (GFP) that had been transduced to the cells using a lentiviral infection (Englund et al., 2002a) at multiplicity of infection (MOI) of 0.1 rendering 10% of the cells GFP positive. MOI of 0.1 was used as it had been shown not to affect HNPCs growth characteristics, maintain the ability of GFP-expressing cells to form both neurons and glia upon differentiation *in vitro* and *in vivo* and not to be transferred to host cells after *in vivo* transplantation (Englund et al., 2000, 2002b). For co-cultures and CM monocultures HNPCs were seeded as spheres with a diameter of 0.25–0.45 mm. An estimate of a total number of cells in neurospheres of different sizes was done using a geometrical concept formula for a sphere volume calculation $V = 4\prod R^3/3$ (V-volume; $\prod$-constant, 3.14159; R-radius), where a counted number of cells constituting a sphere radius in a two dimensional plane was used as R resulting in an approximate total cell number per sphere V.

HNPCs and brainstem explant in co-culture

We deposited GFP-HNPC neurospheres adjacent to the CN of the brainstem slices according to the local anatomical landmark at a distance of 0.5 mm and kept the co-culture for 2 weeks (described as specimen in the text). The deposition distance chosen was set as comparable to the sizes of seeded neurospheres. Single neurospheres of appropriate size were collected with a p200 pipette, transferred to membranes with explants and immediately delivered in a close vicinity to CN by ejection. The sphere deposition was monitored by placing a grid ruler under a well with a membrane to have the distance equal for all conditions enabling comparison between groups in a reliable manner. Pharmacological treatment with inhibitors was performed from the very beginning of the co-culture. During the entire culture period a Nikon inverted microscope TS100 (Nikon, Kanagawa, Japan) was used to monitor cell morphology, survival and distribution. The number of co-cultures used per treatment conditions is presented in Table 1.

CM experiments

CM experiments where HNPCs were grown in monoculture in the absence (CM controls) or presence

Table 1. Statistical output data (mean ± S.E.M.) and significance test results. Data are shown as mean ± S.E.M. Asterisks indicate significant difference. Number of co-culture and CM monoculture repetitions for each condition indicated in cell survival raw is the same for the rest of the studied criteria

	Control	PD168393	Y27632	DMSO	Non-parametric ANOVA
Cell survival (%)					
Monoculture in CM	13.2 ± 1.5 (n = 4)	9.6 ± 1.1 (n = 6)	12.1 ± 2.3 (n = 4)	10.3 ± 1.0 (n = 4)	
Co-culture	11.9 ± 2.2 (n = 7)	10.1 ± 1.0 (n = 6)	7.4 ± 0.8 (n = 11)		
Cells migrating inside the explant (%)					
Co-culture	0.11 ± 0.05	0.05 ± 0.02	0.36 ± 0.04		***P = 0.0008
Distance traveled (μm)					
Co-culture	86.3 ± 26.3	90.1 ± 35.9	258.8 ± 39.1		**P = 0.0047
Neurite length (μm)					
Monoculture in CM	218.9 ± 22.3	209.1 ± 8.7	189 ± 22.3	195.3 ± 14.9	
Co-culture	193.2 ± 12.9	278.4 ± 18.8	261.3 ± 11.4		**P = 0.0051
Cell differentiation (%)					
Monoculture in CM					
TUJ1+	47.9 ± 9.5	25.9 ± 4.3	37.1 ± 8.1	32.6 ± 1.4	
GFAP+	24.2 ± 2.6	22.3 ± 3.3	20.8 ± 8.2	17.8 ± 3.6	
TUJ1+/GFAP+	27.9 ± 8.4	51.8 ± 3.3	42.2 ± 7.3	49.7 ± 4.2	
Co-culture					
TUJ1+	59.5 ± 10.3	36.2 ± 3.4	69.8 ± 6.5		*P = 0.0146
GFAP+	9.1 ± 3.2	14.4 ± 3.4	7.7 ± 2.3		
TUJ1+/GFAP+	31.4 ± 7.4	49.4 ± 1.8	22.5 ± 5.2		**P = 0.0046

of the inhibitors in the medium collected from the auditory brainstem slices were included to evaluate off-target effects of the inhibitors. CM was collected from P12 to P15 rat postnatal organotypic auditory slices grown for 2 weeks as previously described (see Interface organotypic brainstem slice culture section of Experimental procedures). The medium was routinely changed every other day and collected. Medium from different collection time points was not mixed, but used to emulate the fresh medium change schedule of co-cultures in HNPCs monoculture with or without the inhibitors added. The inhibitors were added to the collected CM prior to seeding the HNPCs. CM was diluted with fresh brainstem slice medium at a 5:1 ratio (5 parts CM:1 part brainstem slice medium) prior to feeding the monocultures. The number of GFP-HNPCs neurospheres per treatment conditions is presented in Table 1.

Chemicals

(R)-(+)-*trans*-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexane-carboxamide, 2HCl (Y27632; Calbiochem; Merck Chemicals; solution formulation in H_2O) was used at 5 μM (Lingor et al., 2007). The blocker 4-[(3-bromophenyl)amino]-6-acrylamidoquinazoline (PD168393) (Calbiochem; Merck Chemicals; solution formulation in dimethyl sulfoxide, DMSO) was used at 100 nM (He and Koprivica, 2012).

Vehicle controls

For PD168393-treated cultures vehicle (DMSO) controls were done. In the co-culture and CM monoculture experiments PD168393 formulation was diluted in the culture medium resulting in 0.001% DMSO content which previously was not shown to be toxic to cells (Qi et al., 2008). Vehicle controls were done on HNPCs grown in monoculture in CM (see Experimental procedures: CM experiments) with DMSO added to the culture medium to the final content of 0.001%. The number of GFP-HNPCs neurospheres is presented in Table 1.

Immunocytochemistry and immunohistochemistry

Cell morphology and differentiation of the brainstem slice and the GFP-HNPCs were studied in co-culture after fixation with 4% paraformaldehyde (PFA) followed by immunohistochemistry. The cultures were fixed in 4% PFA in a phosphate-buffered saline (PBS, pH 7.4) for 1 h at room temperature (RT). After 5× wash in PBS the cultures were treated with ice-cold 20% methanol in PBS for 5 min at RT. After 3× rinsing in PBS the tissues were allowed for permeabilization using 0.5% Triton-X in PBS overnight at 4 °C. After the final wash 3× with PBS the preparations were incubated with a 20% bovine serum albumin (BSA; Sigma) blocking solution for 12 h at 4 °C prior to incubation with primary antibodies. The co-cultures were incubated with different primary antibodies diluted in 5% BSA in PBS overnight. The following primary antibodies in specific dilutions were used: monoclonal mouse (Covance; MMS-435P) and polyclonal rabbit (Covance; PRB-435P) anti-neuronal class III β-Tubulin 1:500; polyclonal rabbit anti-glial fibrillary acidic protein 1:500 (GFAP; DAKO; z0334); goat polyclonal to GFP 1:200 (GFP-FITC; Abcam, Cambridge, UK; ab6662); mouse monoclonal to chondroitin sulfate proteoglycan 1:200 (Abcam; ab78689). After 4× wash with PBS for 5 min the primary antibody–antigen complexes were visualized with the secondary

antibodies Cy-3, Cy-5 and Alexa 488 (Jackson ImmunoResearch, West Grove, PA, USA) by incubation for 4 h at RT followed by 4× was with PBS for 5 min. Staining specificity was confirmed by omission of the primary antibody. 4′,6-Diamidino-2-phenylindole (DAPI) nuclear stain (10 μg/ml DAPI for 10 min) was done before the final 4× PBS washout of the membranes with co-cultured brainstem slices and GFP-HNPCs. Membranes were cut out and mounted on glass slides with Prolong Gold mounting medium (Invitrogen) and examined using a fluorescence microscope (Axio Observer Z1 and LSM 700, Zeiss). The brightness and contrast of the presented images were adjusted to aid visualization (Adobe Photoshop CS5 12.0, Adobe Systems Inc., San Jose, CA, USA).

Cell quantification and statistical analysis

All of the quantification, except cell differentiation counts, was done on digital 20× field (Axio Observer Z1 and LSM 700, Zeiss) collages (Adobe Photoshop CS5) of each PFA fixed specimen (for co-cultures) or of each neurosphere (for CM monocultures). Statistical output data and significance tests were done using GraphPad Prism 5 (GraphPad Prism Software, Inc., San Diego, CA, USA). Graphically the data are presented as bars showing mean ± S.E.M. of the output data for each of the studied criteria.

HNPCs survival was calculated as a percentage of a number of detectable DAPI fluorescent stain- or/and GFP-positive cells after PFA fixation, counted in cell counter plugin in ImageJ, of an estimated total number of cells in neurospheres before seeding.

Number of cells invading the explant was calculated as a percentage of a total number of GFP- and GFP/neuronal class III β-tubulin (TUJ1)-positive cells which have invaded the slice of an estimated total number of cells in neurospheres before seeding per specimen in each treatment group (output data are presented as mean ± S.E.M. per treatment).

Migration distance (d_1, d_2, …, d_n) of HNPCs within the explant was calculated as a length of a line perpendicular to the tangent line on the explant circumference containing the CN at the tangent point *T*, connecting the cell soma inside the explant and the tangent point *T* on the explant circumference (Fig. 2). GFP+/TUJ1+ process length was determined by measuring its entire length using free-hand measurement tool in ImageJ. Length was defined as the distance along a process from the cell soma to the process end or to the end of the longest branch of branched processes. Length of up to 150 randomly selected processes in each specimen was measured regardless of their location (e.g. inside or outside of the explant). Processes length output data for each treatment are presented as mean ± S.E.M of the average processes length values per specimen in each treatment group.

Differentiation markers expression cell counting was done on real-time images acquired using 63× oil objective on LSM 700 laser scanning microscope (Zeiss) and ZEN 2010 imaging software (Carl Zeiss MicroImaging GmbH; release version 6.0). For each co-culture specimen and neurospheres in CM monoculture 30 randomly selected 63× acquisition frames were taken, a total number of nuclei and a number of either nuclei or nuclei with cytoplasm positive for TUJ1, GFAP or TUJ1/GFAP per frame counted. The output is presented as percentage.

Significance for statistical output differences in co-culture and CM monoculture different compound groups was done using non-parametric Kruskal–Wallis one-way analysis of variance (ANOVA). Dunn's multiple comparison post-test was performed to investigate sources of variance.

RESULTS

Characterization of the rat postnatal organotypic auditory brainstem

We cultured P12–15 rat postnatal auditory brainstem explants containing the CN and a part of the AN for 2 weeks as an interface culture on polyester membranes coated with PDL and laminin. Whole-mounts showed that the slices maintained a well-preserved organotypic organization and flattened with time. Glial cells with different morphology were identified using immunostaining for GFAP and the neurons were immunostained with TUJ1 marker (Fig. 1). Neither glial sprouting with typical stellate morphology nor neuronal sprouting could be observed along the border of acute *in vitro* brainstem slices. On day one following explant preparation glial processes emerged from the outside of the explant with few neurons spreading out the extensions (Fig. 1a). Explant glial cells with stellate morphology vastly proliferated during the course of 2 weeks in culture forming glial patches along the slice border (Fig. 1b). Explanted neurons also tended to spread along the slice border with time (Fig. 1c). In some locations at a 2-week time point the glial cells still appeared to extend their processes exceeding the neuronal outgrowths. The neurons tended to spread along the border of the slice with very few cells migrating out of the slice margins while glia could also fasciculate further out of the slice vicinity. Glia-associated marker anti-chondroitin sulfate proteoglycan antibody in conjunction with either TUJ1 or GFAP antibody visualized CSPG distribution on glial cells surface and on intercellular space substrate associated with glia fasciculation (Fig. 1b, c).

Integration of HNPC with the auditory explant after Y27632 and PD168393 treatment

The auditory brainstem explant culture system was used as a screening platform (Fig. 2) for evaluation of the integration potential of GFP-expressing HNPCs. For co-cultures and CM monocultures HNPCs were seeded as spheres with a diameter of 0.25–0.45 mm with approximate total cell number estimated for different sphere diameters (Fig. 3a). Prior to a deposition of the spheres the fraction of HNPCs expressing GFP was estimated to be approximately 10% (lentiviral infection with MOI of 0.1). In the co-cultures GFP-HNPCs

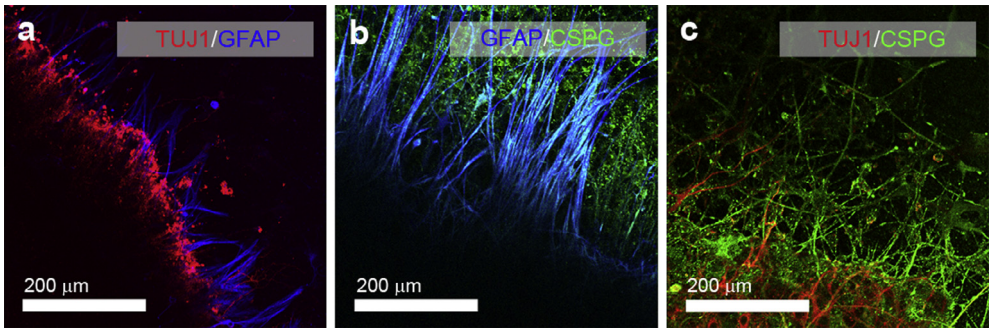

Fig. 1. Illustration of the sprouting process and CSPGs/glia/neurons spatial relationship that occur in auditory brainstem slice culture. PFA fixed, fluorescence microscopy images of the rat postnatal auditory brainstem on the following day after isolation (a) and in monoculture on polyester membranes on day 14 (b and c). Cell morphology and a relationship between a neuronal marker TUJ1 and glia-associated markers GFAP and CSPG expression are shown. (a) Following day after isolation glial processes emerge from the outside of the explant border with neurons not spreading out the extensions. (b and c) After 2 weeks in culture explant glial cells with stellate morphology vastly proliferate outside of the slice border with CSPGs distributed on glial cells surface and on intercellular space substrate associated with glia fasciculation. Neurons sprout out without migrating outside of the slice margins while glia can also form bundles and patches which can be seen further out.

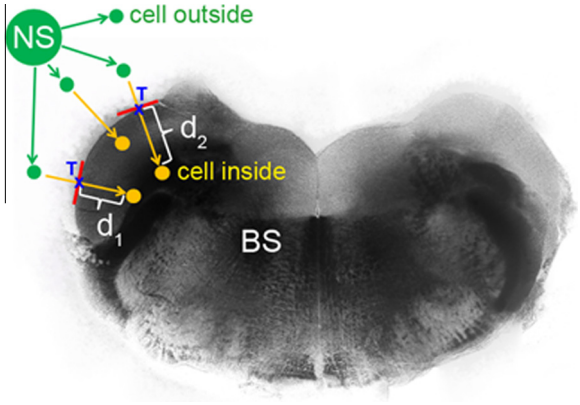

Fig. 2. A schematic diagram (not representative of relative size) of HNPCs neurosphere deposition to auditory brainstem explant with an illustration of a principle to determine the distance of explant penetration by HNPCs. Using the Stoppini method (Stoppini et al., 1991) 300-μm-thick brainstem slice, *BS*, encompassing the CN and a part of the auditory nerve was used to test transplanted GFP-HNPCs potential to advance into auditory brainstem tissue (live-cell, bright field, day 0 *in vitro* image). A GFP-HNPCs neurosphere (a schematic diagram), *NS*, was immediately deposited at a 0.5-mm distance from the CN of the freshly isolated explant placed at a fluid/air interface on a polyester membrane. The co-culture (described as a specimen in the text) was maintained for 2 weeks. The distance HNPCs migrated within the explant after crossing the explant border (d_1, d_2, …, d_n) was calculated as a length of a line perpendicular to the tangent line (red) on the explant circumference containing the CN at the tangent point *T*, connecting the cell soma inside the explant (yellow) and the point *T* on the explant circumference.

neurospheres were deposited adjacent to the CN according to the local anatomical landmark at a distance of 0.5 mm. The deposition distance chosen was set as comparable to the sizes of seeded neurospheres. GFP-HNPCs extensively migrated toward the explant, suggesting that human cells can respond to migration-promoting cues exerted from the rat tissue. However, the migration and in-growth of the GFP-HNPCs processes into the explant rarely extended past explant neuronal outgrowth exceeding glial processes at the slice border preventing HNPC-derived processes from contact with auditory neurons. Pharmacological agents ROCK inhibitor Y27632 and EGFR blocker PD168393 were thus tested at concentrations of 5 μM (Lingor et al., 2007) and 100 nM (He and Koprivica, 2012) respectively. Effect of the treatment on explant penetration by GFP-HNPCs was evaluated quantitatively based on the following criteria: (i) number of HNPCs located within the explant, (ii) distance migrated by the HNPCs deep into the explant, (iii) length of the GFP+/TUJ1+ processes developed was measured and (iv) the morphology and phenotypes displayed based on GFP-expression. Estimates of the surviving HNPCs number in co-cultures and in CM monocultures were included for comparison. Survival of HNPCs was calculated as a number of detectable DAPI fluorescent stain- or/and GFP-positive cells.

Survival. We estimated mean percentage of surviving HNPCs per explant in control and treated groups (Table 1, Fig. 3b) in co-culture. HNPCs survival in CM monoculture was done to evaluate possible off-target effects of the inhibitors or vehicle (Table 1, Fig. 3c). No significant difference in cell survival was detected in co-cultures, CM monoculture groups and in vehicle controls (Kruskal–Wallis non-parametric ANOVA).

HNPC located inside the explant. After seeding on membranes GFP-HNPCs neurospheres flattened as the cells began migrating and extending processes in the surroundings. Directed migration toward the explant was observed in both treated and control groups as judged by the GFP immunoreactivity gradient showing accumulation of GFP-expressing HNPCs at the explant border. However, as compared with the control group (Fig. 4a) and PD168393-treated explants (Fig. 4b) only Y27632 appeared to significantly influence the migratory and outgrowth behavior of the transplanted GFP-HNPCs into the explant (Fig. 4c). Y27632-treated explants showed improved growth of the GFP-HNPCs into the brainstem slice tissue. There GFP-HNPCs somata with extended processes were observed both in the explant as well as nearby as the cells have migrated away from the sphere. Kruskal–Wallis non-parametric ANOVA (***$P < 0.005$) and following Dunn's multiple

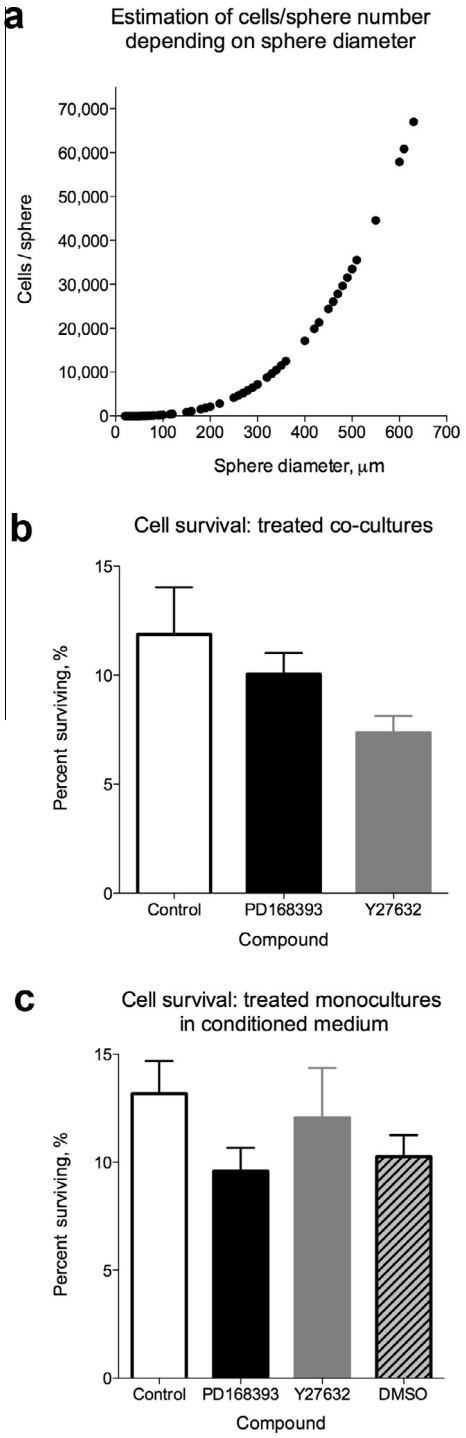

Fig. 3. Assessment of HNPCs survival. (a) Estimation of cells/sphere number depending on sphere diameter. (b) No statistically significant difference (Kruskal–Wallis non-parametric ANOVA) in HNPCs survival was detected in HNPCs 2-week co-culture groups. (c) HNPCs grown in monoculture in CM groups and in vehicle control group did not differ in cell survival after 2 weeks in culture (Kruskal–Wallis non-parametric ANOVA). Data are presented as bars showing mean ± S.E.M. of the output data.

comparison post-test ($^{**}P < 0.05$) showed significant difference in the number of GFP-HNPCs that migrated deep into the explant in Y27632-treated co-cultures as compared to PD168393-treated and control co-cultures (Table 1, Fig. 5a).

Migration distance of HNPCs within explant. Distance traveled by GFP-HNPCs deep into the explant from the explant border, morphologically delineated laterally on both sides of the auditory brainstem slice by the CN, was measured (Table 1, Figs. 2 and 5b). Y27632 enhanced the migratory behavior of GFP-HNPCs into the explant which increased more than double fold as compared with controls and PD168393-treated co-cultures ($^{**}P < 0.005$ non-parametric ANOVA, $^{*}P < 0.05$ Dunn's post-test).

Length of GFP-HNPCs process outgrowth. In co-culture both compounds tested resulted in GFP-HNPCs developing relatively long GFP+/TUJ1+ processes (both outside and inside the explant) as compared with the control co-culture group (Table 1, Fig. 5c, $^{**}P < 0.05$ non-parametric ANOVA, $^{*}P < 0.05$ Dunn's post-test). No significant difference in GFP+/TUJ1+ processes length was found in CM monoculture group and in vehicle control group (Table 1, Fig. 5d, Kruskal–Wallis non-parametric ANOVA).

GFP-HNPCs morphologies and phenotypes. Morphology and differentiation profile of GFP-HNPCs in the co-cultures and CM monocultures subjected to pharmacological treatments and in their respective controls were evaluated using immunohistochemistry. In both treatment groups in CM monocultures and in co-cultures GFP-HNPCs had a distinctly bipolar appearance with elongated mostly unbranched processes with few small branches at the end. GFP-HNPCs in co-culture control group had shorter processes and mostly multipolar somata (Fig. 4d). In the presence of the EGFR blocker PD168393 HNPCs cell migration in co-culture was not alleviated which was reflected in non-elongated cell somata while processes elongation was still observed (Fig. 4e). GFP-HNPCs co-cultured in the presence of ROCK inhibitor Y27632 displayed elongated processes and elongated cell somata characteristic of migrating cells with targeted direction toward the explant (Fig. 4f). Neuronal and glial differentiation was detected in both co-culture (Table 1, Fig. 6a) and CM monoculture (Table 1, Fig. 6b) groups according to TUJ1 and GFAP staining. Double-labeled cells were also detected. No significant difference in the cell fate acquired by HNPCs in CM monocultures and in vehicle control group was observed (Kruskal–Wallis non-parametric ANOVA), while in co-cultures there was a significant difference between Y27632- and PD168393-treated HNPCs acquiring neuronal fate ($^{*}P < 0.05$ non-parametric ANOVA, $^{*}P < 0.05$ Dunn's post-test) and double-labeled TUJ1+/GFAP+ cells ($^{**}P < 0.005$ non-parametric ANOVA, $^{**}P < 0.05$ Dunn's post-test), though not different from the fates acquired in control co-culture.

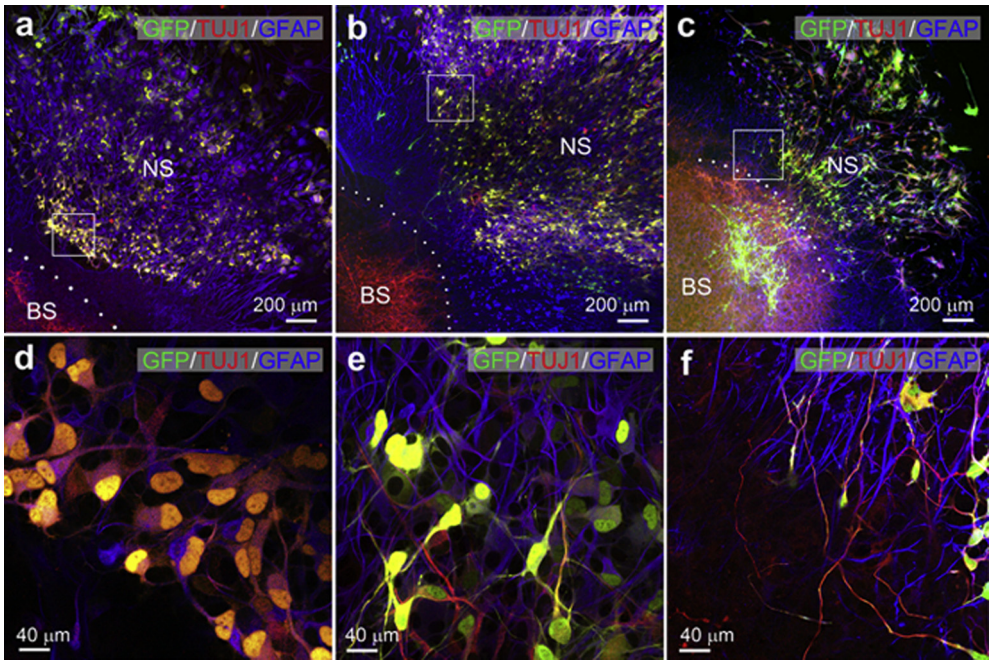

Fig. 4. Both ROCK inhibitor Y27632 and EGFR blocker PD168393 enhance elongation of GFP-HNPCs processes, while only Y27632 stimulates the migratory and outgrowth behavior of the transplanted cells into the explant. (a) Control brainstem slice, *BS*, co-culture with GFP-HNPC showing that cells from transplanted neurosphere, *NS*, do not invade the slice core area beyond the margins (•••••) of the organotypic slice preparation. (b) PD168393 treated co-cultures did not display enhanced GFP-HNPCs migration into the explant similar to control co-cultures. (c) GFP-HNPCs advanced migration and extension of processes into the brainstem slice further beyond its margin are observed in the presence of the ROCK inhibitor Y27632. (d) Higher magnification image of the boxed area shown in (a) HNPCs grown in untreated co-cultures developed relatively short processes and had non-elongated cell somata. (e) Higher magnification image of the boxed area shown in (b) morphology observed in GFP-HNPCs co-cultured with EGFR blocker PD168393. The migration was not alleviated in the presence of the drug which is reflected in non-elongated cell somata while processes elongation was still present. (f) Higher magnification image of the boxed area shown in (c) GFP-HNPCs co-cultured in the presence of ROCK inhibitor Y27632 displayed elongated processes and elongated cell somata characteristic of migrating cells with targeted direction toward the explant. PFA fixed, fluorescence microscopy.

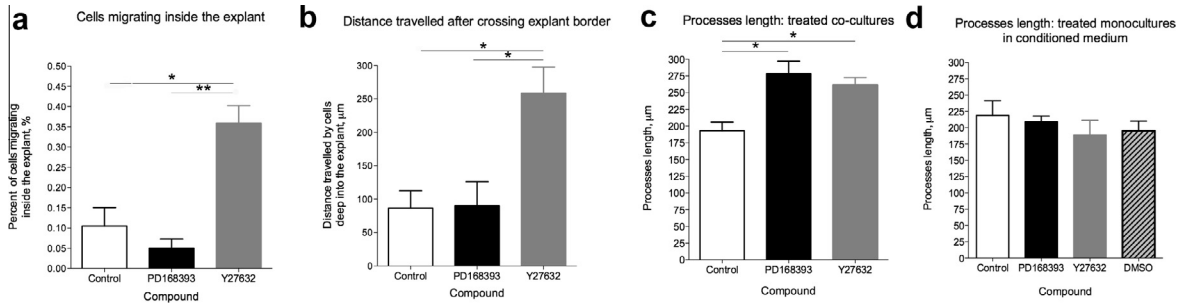

Fig. 5. Only Y27632 stimulates the migratory and outgrowth behavior of the co-transplanted HNPCs. (a) Only Y27632 increased HNPCs motility in co-culture resulting in significantly larger number of HNPC migrating inside the explant (Kruskal–Wallis ANOVA, $^{**}P < 0.05$; Dunn's post-test; $^{*}P < 0.05$). (b) Increased motility led to a longer distance traveled by HNPCs within the explant in Y27632-treated co-cultures (Kruskal–Wallis ANOVA, $^{**}P < 0.005$; Dunn's post-test, $^{*}P < 0.05$). (c) Both Y27632 and PD168393 increased GFP-HNPCs processes length as compared to controls (Kruskal–Wallis ANOVA, $^{**}P < 0.05$; Dunn's post-test, $^{*}P < 0.05$). (d) CM monoculture treatment with either of the inhibitors and in the vehicle-treated group did not result is significant processes elongation (Kruskal–Wallis ANOVA). Data are presented as bars showing mean ± S.E.M. of the output data. Asterisks indicate significant difference between compared groups.

DISCUSSION

There is a great interest in identifying factors and therapeutic strategies to preserve SGNs from further deterioration including SGNs regrowth with their fiber projections via implanted stem and precursor cells conversion. Recently Jeon et al. (2011) showed that SGNs explant neurites are inhibited by central glia *in vitro* and that this effect is not attributed to glia-secreted factors like semaphorins (Pasterkamp and Verhaagen, 2006) or glial cell surface proteins like ephrins (Sandvig et al., 2004), but rather extracellular matrix (ECM) proteins such as CSPGs. CSPG-induced growth inhibition as well as inhibition posed by many other central nervous system (CNS)-derived inhibitory substrates has been shown to signal via ROCK and EGFR pathways (Snow et al., 1994; Lingor et al., 2007; He and Koprivica,

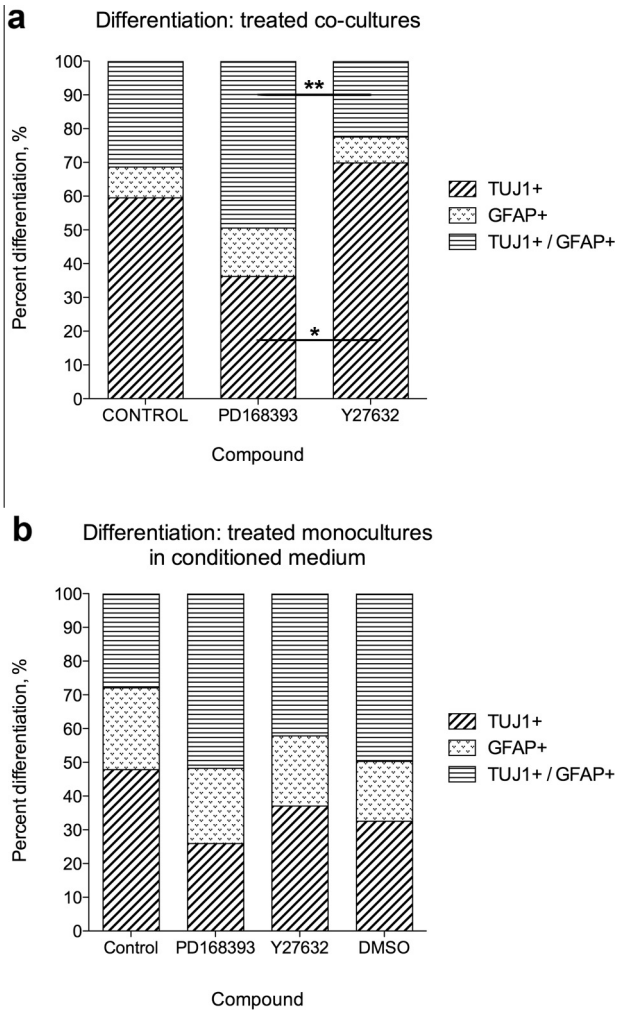

Fig. 6. GFP-HNPCs neuronal and glial differentiation in treated co-cultures and in treated CM monocultures. (a) Co-culture treatment with Y27632 resulted in a more pronounced neuronal maturation of HNPCs according to significantly larger number of cells with neuronal TUJ1 expression (Kruskal–Wallis ANOVA, $^{*}P < 0.05$; Dunn's post-test, $^{*}P < 0.05$) and significantly less number of cells expressing both neuronal marker TUJ1 and glial marker GFAP as compared to PD168393-treated co-cultures (Kruskal–Wallis ANOVA, $^{**}P < 0.005$; Dunn's post-test, $^{**}P < 0.05$). (b) No significant difference in the cell fate acquired by HNPCs in CM monocultures and in vehicle controls was observed (Kruskal–Wallis ANOVA). Data are presented as bars showing mean ± S.E.M. of the output data. Asterisks indicate significant difference between compared groups.

2012). In the present study we used human GFP-HNPCs as donor cells for migration and phenotypic differentiation assessment in co-culture with rat auditory explant *in vitro* model to investigate the effects of pharmacological ROCK and EGFR inhibition. In untreated co-cultures the GFP-HNPCs were observed to be migrating in the direction of the explant illustrating the presence of undefined guiding cues for targeted migration. However, the majority of the transplanted GFP-HNPCs did not extend processes or migrate deep into the slice as would be necessary for the formation of functional contacts between the GFP-HNPCs and the CN. We show that modulation of ROCK activity by Y27632 inhibitor and EGFR activity by PD168393 inhibitor increases the length of HNPC-derived GFP + /TUJ1 + processes outgrowth in the presence of auditory explant central glia *in vitro*. Further, application of Y27632 resulted in HNPCs enhanced motility inside the explant and pronounced bipolar morphologies as compared to control co-cultures. Our data suggest that the guidance and attraction cues for targeted GFP-HNPCs migration produced by the auditory explants work in synergy with the inhibitors, promoting HNPC-derived neurite outgrowth. Therefore the co-culture system not only recapitulates complex events pertaining to the AN milieu *in vitro*, but also represents a tool for testing the effects induced by inhibition of numerous signaling pathways triggered by CNS inhibitory substrates. The Rho/ROCK signaling cascade is involved in intracellular regulation of the cell's response to external cues, including regulation of actin dynamics (Lie et al., 2010), important for directed cell migration and other cellular behaviors. The cascade is also a convergence point for signaling of multiple inhibitory substrates (Lingor et al., 2007). Previous *in vitro* reports have demonstrated Y27632 inhibitor increases/stimulates neurite outgrowth of: DRG on inhibitory aggrecan substrates (Borisoff et al., 2003), human Ntera-2 neurons on CSPGs substrates (Lingor et al., 2007) and retinal axons on a complex CSPGs mixtures (Monnier et al., 2003). Other ROCK inhibitors e.g. H-1152, dimethylfasudil and fasudil have also been shown to increase neurite length *in vitro* (Lingor et al., 2007; Lie et al., 2010). *In vivo* studies on optic nerve crush in the adult rat showed an increase in axonal regeneration in agreement with the *in vitro* data (Lingor et al., 2007). However besides alleviative effects, Y27632 has been reported to be detrimental on neurite outgrowth of dissociated cortical neurons grown in co-culture with astrocytes due to alterations in CSPGs astrocytic processing resulting in increased CSPGs presence within the ECM *in vitro* (Chan et al., 2007). Thus increased expression and deposition of CSPGs by extremely plastic astrocytes (Lau et al., 2011) counteract stimulatory effect of Y27632 on cut and developing axons limiting sizable regeneration over the CSPG-rich sites. However since CSPGs buildup takes time stimulation of axonal growth under Y27632 treatment is observed until the lesion site has substantially developed (Chan et al., 2007). It can be speculated then that these contravening effects depend on time delay since lesion, temporal pattern in which individual CSPGs are produced and their location in the lesion milieu (Siebert et al., 2014). In our co-culture system application of Y27632 at 5 μM concentration for a short two-week period resulted beneficially in HNPCs developing significantly elongated GFP + /TUJ1 + processes as compared to controls. The concentration used has been shown to be the lowest at which a significant increase in neurite length on CSPGs substrates is observed *in vitro* (Lingor et al., 2007). In agreement with ROCK involvement in regulating actin dynamics, HNPCs grown in Y27632 treated co-culture showed an enhanced migration into and within the explant in addition to more pronounced bipolar cell morphologies. No detectable effect of Y27632 on co-cultured HNPCs survival was found. CM Y27632 treated monocultures did not develop processes significantly different in length from CM monoculture controls. This implicated

the presence of a non-soluble factor affecting HNPCs neurite length in the presence of the explant in untreated co-cultures consistent with the factor distribution on cell surface or/and intercellular space substrate. Because brainstem explant confines different cell types that interact closely, explant-derived CM and ECM factors result from a complex cellular cross-talk which accounts considerably for the discrepancy observed with a single cell-type culture-derived CM and ECM cf. Chan et al. (2007).

Improvement in neurite outgrowth on myelin substrates and CSPGs neurite outgrowth inhibitory activity neutralization by EGFR inhibitors has been evidenced in e.g. DRG, cerebellar granule cells, retinal ganglion cells (RGCs) *in vitro* and *in vivo* in lesioned optic nerve fibers in adult mice and sensory and bladder recovery after spinal cord injury in rats (Koprivica et al., 2005; Erschbamer et al., 2007; Douglas et al., 2009; He and Koprivica, 2012). However the mechanism by which EGFR antagonists exert their action is still under debate. In case of disinhibited RGC, neurite growth in the presence of EGFR inhibitors, including PD168393 and AG1478, Koprivica et al. (2005) attributed it to inactivation of axonal EGFR while Douglas et al. (2009) claim that it is a result off-target effect on RGC/retinal glia through the induced secretion of neurotrophins. We tested PD168393 at 100 nM concentration, which was shown to be effective at neutralizing CSPGs inhibitory activity on retinal explants grown in collagen gels *in vitro* (He and Koprivica, 2012). No detectable effect of PD168393 on co-cultured HNPCs survival was found. While in Y27632-treated co-cultures HNPCs developed mostly fusiform-shaped morphologies of migrating cells with elongated cell body, in PD168393 co-cultures these traits were less pronounced with a cell body having a more round shape. As with Y27632-treated co-cultures HNPCs in PD168393-treated co-cultures developed significantly longer GFP+/TUJ1+ processes than in untreated co-cultures, however no significant effect of PD168393 on cell motility was detected as opposite to HNPCs in Y27632-treated co-cultures. Here we did not address the primary target of PD168393 in our HNPCs-auditory explant co-culture. No significant GFP+/TUJ1+ processes elongation observed in PD168393-treated CM monocultures could be indicative of the absence of either a soluble inhibitory ligand to be counteracted by the inhibitor on HNPCs or glia cells to act upon. In addition we did not detect any significant differences in the cell fate HNPCs acquired (e.g. proportion of TUJ1+, GFAP+ and TUJ1+/GFAP+ labeled cells) in either PD168393- or Y27632-treated monocultures as compared to CM monoculture controls allowing to exclude the off-target effects of the inhibitors on a cell fate. However, in the co-cultures treated with the inhibitors a significantly different ratio in acquired cell fate by HNPCs was detected between Y-27632 and PD168393 treated co-cultures, though not significant when compared to control co-culture HNPCs acquired fate. More HNPCs with neuronal fate (TUJ1+) and less double-labeled TUJ1+/GFAP+ cells were observed in the Y27632-treated co-cultures, while PD168393-treated co-cultures resulted in less HNPCs having neuronal fate (TUJ1+) and more double-labeled cells. GFAP was previously described as a marker for immature neurons in similar cell lines (Skogh et al., 2010) as well as for neuronal progenitors in neurogenic regions in the CNS (Alvarez-Buylla et al., 2000). Therefore it appears that the tested inhibitors have different influence on HNPCs maturation into a later stage neuronal phenotype in the presence of factors produced by the auditory explant as judged by the amount of double-labeled cells and cell morphology, while initial glial and neuronal formation is observed for both. Increased ratio of TUJ1+ cells in Y27632-treated co-cultures suggests similarities to the neuronal induction observed in human embryonic stem cells (hESC) into neural-crest (NC) like cells grown on mouse embryonic fibroblast (MEF) feeder layer in the presence of Y27632 (Hotta et al., 2009). There NC markers were not expressed in the presence of Y27632 in hESC grown on fibronectin substrate, which suggested that manipulating of signaling pathways by exposure to Y27632 was not sufficient to induce neuronal phenotype, but required priming by MEF feeders-secreted factors to initially set hESC to differentiate toward NC. The induction factors have not been studied in full and require further studies (Hotta et al., 2009).

In this paper we show that targeted reduction of signaling cascades triggered by the inhibitory factors of glial origin can improve migration of donor cells transplanted to an auditory brainstem explant. Pharmacological inhibition in the co-culture system provides an insight into inhibitory signaling mechanisms that will be beneficial for future *in vivo* studies. Since multiple signals converge to regulate developing and regenerating axon growth, other signaling cascades than Rho/ROCK and EGFR may be involved in inhibition of neurite outgrowth. Further, balancing negative growth cues with growth-promoting factors is a critical determinant for successful regeneration. In our experimental setup we can explore the efficacy of such a combination with e.g. neurotrophic factors (BDNF, NT-3). Considering that therapeutic window varies with chemical compounds their potency to induce maximal neurite outgrowth will be different and therefore the validity to be a promising target for pharmacological intervention in regenerative paradigms will range.

AUTHOR CONTRIBUTION STATEMENT

Conceived and designed the experiments: EN PO. Performed the experiments: EN AK YJ. Analyzed the data: EN UEJ PO. Wrote the paper: EN PO. Contributed reagents/materials/analysis tools: UEJ PO. All authors have approved the final article.

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