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MYELIN-INDUCED INHIBITION IN A SPIRAL GANGLION ORGAN CULTURE – APPROACHING A NATURAL ENVIRONMENT *IN VITRO*

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Abstract—The performance of a cochlear implant depends on the defined interaction between afferent neurons of the spiral ganglion and the inserted electrode. Neurite outgrowth can be induced by neurotrophins such as brain-derived neurotrophic factor (BDNF) via tropomyosin kinase receptor B (TrkB). However, neurotrophin signaling through the p75 neurotrophin receptor (p75) inhibits neurite outgrowth in the presence of myelin. Organotypic cultures derived from postnatal (P3-5) mice were used to study myelin-induced inhibition in the cochlear spiral ganglion. Neurite outgrowth was analyzed and quantified utilizing an adapted Sholl analysis. Stimulation of neurite outgrowth was quantified after application of BDNF, the selective TrkB agonist 7,8-dihydroxyflavone (7,8-DHF) and a selective inhibitor of the Rho-associated kinase (Y27632), which inhibits the p75 pathway. Myelin-induced inhibition was assessed by application of myelin-associated glycoprotein (MAG-Fc) to stimulate the inhibitory p75 pathway. Inhibition of neurite outgrowth was achieved by the selective TrkB inhibitor K252a. Stimulation of neurite outgrowth was observed after treatment with BDNF, 7,8 DHF and a combination of BDNF and Y27632. The 7,8-DHF-induced growth effects could be inhibited by K252a. Furthermore, inhibition of neurite outgrowth was observed after supplementation with MAG-Fc. Myelin-induced inhibition could be overcome by 7,8-DHF and the combination of BDNF and Y27632. In this study, myelin-induced inhibition of neurite outgrowth was established in a spiral ganglion model. We reveal that 7,8-DHF is a viable novel compound for the stimulation of neurite outgrowth in a myelin-induced inhibitory environment. The combination of TrkB stimulation and ROCK inhibition can be used to overcome myelin inhibition.

Key words: BDNF, 7,8-DHF, Y27632, neurotrophin, spiral ganglion, cochlear implant.

INTRODUCTION

Despite recent innovative technical improvements, the performance of cochlear implants (CI) is still variable, and complex sound perception, such as listening to music, is limited (Gfeller et al., 2007). One major reason behind the limited tonal sound perception is unselective neuronal stimulation by electrode array (Wilson and Dorman, 2008). After the insertion of the CI into the scala tympani (ST), the electrode contacts of straight electrode type of CI may reside against the lateral wall of the ST (Gstoettner et al., 1999), while spiral ganglion neurons (SGNs) are located in the modiolus, which is adjacent to the medial wall of the ST. The performance of a CI is dependent on the defined interaction between the electrode contacts and the SGNs (O'Leary et al., 2009). The spatial gap between the stimulating electrode contact and the receiving neuronal tissue plays a crucial role regarding the amount of energy that is needed to stimulate the afferent neurons of the spiral ganglion (Hahnewald et al., 2016). The interference of adjacent electrode contacts leads to unspecific stimulation with a high degree of spread of the signaling current and high-energy consumption (Fu et al., 2005; Hughes and Stille, 2010). This current spread represents a major limitation that prevents the incorporation of more effective independent channels in CIs, consequently limiting their performance. One goal in the development of CIs is reducing the spatial distance between the electrode contacts and neurites (Shibata et al., 2011). A possible biological approach is to stimulate the outgrowth of spiral ganglion neurites onto the electrodes (Leake et al., 2011; Landry et al., 2013; Wise et al., 2016).

Development, growth and survival of sensory neurons in the inner ear rely on neurotrophins such as

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Abbreviations: 7,8-DHF, 7,8-dihydroxyflavone; ATP, Adenosine triphosphate; BDNF, Brain-derived neurotrophic factor; B27, B27 supplement; BSA, Bovine serum albumin; cAMP, Cyclic adenosine monophosphate; CI, Cochlear implant; CREB, cAMP response element-binding protein; DAPI, 4′,6-diamidino-2-phenylindol; GFAP, Glial fibrillary acidic protein; K252a, Tyrosine kinase inhibitor k252a; MAG, Myelin-associated glycoprotein; MAPK, Mitogen-activated protein kinase; MLCK, myosin light chain kinase; NC, Negative control; NDS, Normal donkey serum; NF-200, Neurofilament 200; NgR, Nogo receptor; NMRI, Naval Medical Research Institute; NOGO, Neurite outgrowth inhibitor; P75, P75 neurotrophin receptor; PFA, Paraformaldehyde; PBS, Phosphate-buffered saline; PI3K, Phosphatidylinositol 3-kinase; RhoA, Ras homolog member A; ROCK, Rho-activating kinase; SGN, Spiral ganglion neuron; ST, Scala tympani; THRC, Tuebingen Hearing Research Center; TRK, Tyrosine kinase receptor.

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brain-derived neurotrophic factor (BDNF) and its receptor (Rubel and Fritzsch, 2002; Fritzsch et al., 2004, 2006; Wise et al., 2005, 2016; Euteneuer et al., 2013; Kondo et al., 2013; Xie et al., 2013). BDNF acts via tropomyosin kinase receptor B (TrkB), triggering intracellular signaling cascades including the PI3K-Akt and MAPK pathways, to induce neurite growth and cell survival (Brunet et al., 2001; Reichardt, 2006; Minichiello, 2009; Green et al., 2012). The p75 neurotrophin receptor (p75) also plays a role in BDNF-mediated signaling (Williams et al., 2005). The expression of p75 in the adult human cochlea was localized in the glial cells, including Schwann cells and satellite glial cells in the Rosenthal canal, in the central nerve bundles within the modiolus, and in the osseous spiral lamina of the human cochleae (Liu et al., 2012a). In the developing rodent cochlea, p75 expression was described in primary auditory neurons, Pillar cells in the organ of Corti (Sato et al., 2006) and the inner sulcus of the organ of Corti (Gestwa et al., 1999). In the mature rodent cochlea, p75 is expressed at low levels in primary auditory neurons and cochlear Schwann cells (Tan and Shepherd, 2006; Tan et al., 2010). BDNF-induced activation of p75 leads to a modulation of the affinity and specificity of neurotrophins, such as BDNF and Trk receptors (He and Garcia, 2004). P75 binds BDNF with low affinity, however, in a complex with ubiquitous protein Sortilin, it changes into a high-affinity receptor for proneurotrophins, such as proBDNF (Teng et al., 2010). The response on p75 to neurotrophins can occur either independently or by modulating the affinity and specificity of the Trk receptors for neurotrophins (Huang and Reichardt, 2003). The modulation of Trk receptor function by p75 has various effects including the promotion of ligand binding and endocytosis as well as the promotion of axonal growth (Reichardt, 2006). When Trk receptors are absent, activated p75 by neurotrophins has been shown to induce neuronal apoptosis and regulate cell survival (He and Garcia, 2004). Therefore, BDNF activation through p75 has a negative effect on neurite outgrowth (Park et al., 2010). However, the role of p75 is not only restricted to spiral ganglion neurons but is also dependent on various other factors. P75 is also expressed on Schwann cells after axotomy (Johnson et al., 1986). In an axotomy organ culture non-myelinating Schwann cells can be found. The role of TrkB/p75 signaling interference is very delicate and the neurotrophin mediated effects as neurite growth can also be affected by this fluctuating TrkB/p75 balance. Moreover, the inhibitory activity of myelin affects neurotrophin-induced stimulation of neurite growth and is therefore involved in the inhibition of neurite outgrowth (Cai et al., 1999). An up-regulation of p75 expression was reported after noise (Tan et al., 2010) and aminoglycoside (Tan and Shepherd, 2006) damage. A protective role of p75 on cochlear spiral ganglion cells was suggested by these authors. Myelin also interferes in p75 signaling, and myelin-associated glycoprotein (MAG) acts through a co-receptor of p75, the Nogo receptor (NgR) (He and Koprivica, 2004). This leads to an activation of Rho-activated kinase (ROCK) via Ras homologue member A (RhoA) and results in inhibition of neuronal growth (Yamashita and Tohyama, 2003). In p75-knockout models, neurotrophin-induced neurite outgrowth is increased, suggesting that p75 plays a central role in myelin-associated inhibition of neurotrophin-induced growth (Yamashita et al., 2005). The existence of myelin has also been reported in SGNs, which indicates the possibility of myelin-induced inhibition of neurite outgrowth in SGNs (Martini, 1994). In the CNS, myelination and remyelination has been the detected when myelin inhibiting proteins such as Nogo and other myelin dependent proteins are absent although the expression of Nogo and other myelin inhibiting proteins is variable (Chong et al., 2012). Axonal regeneration is more promising in the peripheral nervous system. Schwann cells are important for the regeneration and remyelination by providing neurotrophic support and axon guidance channels (Radtke and Kocsis, 2012). Up to date, the exact mechanisms on how exactly myelination and remyelination of peripheral axons is regulated are still debatable. However, the effects of myelin inhibition may be of central interest for further studies on neurotrophin-induced effects in SGNs, such as neuronal survival or neurite outgrowth.

The current study therefore utilized an approach to approximate natural conditions in a subadult axotomy *in vitro* model by using an organotypic culture model of spiral ganglions. Myelination was introduced by a controlled addition of myelin associated glycoprotein. The selective stimulation of TrkB was achieved using a natural flavonoid derivative, 7,8-dihydroxyflavone (7,8-DHF). Finally, selective intervention within the inhibitory myelin-activated p75 signaling cascade on neurite outgrowth was investigated by applying the selective inhibitor of ROCK, Y27632.

EXPERIMENTAL PROCEDURES

Animals and preparation

Postnatal Naval Medical Research Institute (NMRI) mice (Charles River, Sulzfeld, Germany) were used to prepare the spiral ganglion. Animals were bred in an in-house animal facility. All animals received care in accordance with the standards described by the German ‘Law on Protecting Animals’ (Tierschutzgesetz) and with the European Directive 2010/63/EU for the protection of animals used for experimental purposes. Harvesting of the cochleae was approved by local authorities (Regierungspräsidium Tübingen, Application dated 04. June 2010) in accordance with the guidelines regarding the care and use of animals for experimental procedures. The explantation of the cochlea ganglion was performed according to the method described by Sobkowicz and colleagues (Sobkowicz et al., 1993). Mice aged postnatal day 3–5 (p3-p5) were decapitated. Dissection of the complete bony labyrinth capsules from the skull base was conducted in 4% paraformaldehyde (PFA) (4% PFA, Carl Roth GmbH, Karlsruhe, Germany) in phosphate-buffered solution (PBS). The specimen was then relocated in PBS, and the following preparation was performed under sterile conditions. Bony portions of the cochlea, the Stria vascularis, the Reissner’s