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Establishment of a long-term spiral ganglion neuron culture with reduced glial cell number: Effects of AraC on cell composition and neurons

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HIGHLIGHTS

- Long-term culture of neonatal rat SGN with mitotic inhibition by AraC is possible.
- AraC is suitable for inhibition of uncontrolled proliferation of non-neuronal cells.
- AraC influences neither neuronal survival nor neurite outgrowth or soma diameter.
- By AraC-treatment it is possible to raise the neuronal ratio in cultured SGC.
- Neurites remain partly free of non-neuronal cells after AraC-treatment.

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ABSTRACT

Background: Sensorineural deafness is mainly caused by damage to hair cells and degeneration of the spiral ganglion neurons (SGN). Cochlear implants can functionally replace lost hair cells and stimulate the SGN electrically. The benefit from cochlear implantation depends on the number and excitability of these neurons. To identify potential therapies for SGN protection, *in vitro* tests are carried out on spiral ganglion cells (SGC).
New method: A glial cell-reduced and neuron-enhanced culture of neonatal rat SGC under mitotic inhibition (cytarabine (AraC)) for up to seven days is presented. Serum containing and neurotrophin-enriched cultures with and without AraC-addition were analyzed after 4 and 7 days.
Results: The total number of cells was significantly reduced, while the proportion of neurons was greatly increased by AraC-treatment. Cell type-specific labeling demonstrated that nearly all fibroblasts and most of the glial cells were removed. Neither the neuronal survival, nor the neurite outgrowth or soma diameter were negatively affected. Additionally neurites remain partly free of surrounding non-neuronal cells.
Comparison with existing method: Recent culture conditions allow only for short-term cultivation of neonatal SGC and lack information on the influence of non-neuronal cells on SGN and of direct contact of neurites with test-materials.
Conclusions: AraC-addition reduces the number of non-neuronal cells and increases the ratio of SGN in culture, without negative impact on neuronal viability. This treatment allows longer-term cultivation of SGC and provides deeper insight into SGN-glial cell interaction and the attachment of neurites on test-material surfaces.
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1. Introduction

About 5% of the world's population, over 360 million people, are affected by disabling hearing loss (WHO, 2015). Most of these patients suffer from sensorineural hearing loss that is

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characterized by irreversible damage of the sensory epithelium (hair cells) and partial degeneration of the auditory nerve, involving the spiral ganglion neurons (SGN). To date, cochlear implants (CIs) are the sole option for restoring hearing in patients affected by sensorineural hearing loss or deafness. The intracochlear electrode array of the CI enables the electrical stimulation of the SGN. Progressive development of the CI in recent years by means of technical innovations such as novel speech-processing strategies has improved the benefits of these implants for patients. Today, therefore, most implanted patients obtain open speech recognition (Battmer et al., 1999). However, technical improvements are limited by anatomical conditions in patients, which influence the important nerve–electrode interface. Further improvements to the CI outcome for patients with long-time deafness are, *inter alia*, affected by retraction of peripheral nerve fibers after hair cell loss and subsequent degeneration of the SGN. This neuronal degeneration occurs much more slowly in humans than in animals (Altschuler et al., 1999; Gillespie and Shepherd 2005; Glueckert et al., 2005; Alam et al., 2007; Liu et al., 2015). However, the preservation and regeneration of the SGN and their nerve fibers are assumed to be important aspects for further implant improvement.

Previous *in vivo* studies have detected the potential of simultaneous electrical stimulation and neurotrophic factor (NTF) treatment to increase SGN survival (Lousteau 1987; Hartshorn et al., 1991; Leake et al., 1991, 1999, 2011; Mitchell et al., 1997; Shinohara et al., 2002; Yamagata et al., 2004; Roehm and Hansen 2005; Scheper et al., 2009; Shibata et al., 2010). Additionally, neurite outgrowth of the peripheral spiral ganglion fibers from the modiolus into the scala tympani is reported (Wise et al., 2005, 2010; Glueckert et al., 2008; Shibata et al., 2010; Landry et al., 2013). These *in vivo* studies are based on cell culture experiments. Explanted primary spiral ganglion cells (SGCs), including the auditory neurons, are a well-established model for exploring their biological response to new therapy strategies for CI improvement. The composition of the cultured cells is influenced by the anatomical conditions of the inner ear. The cell bodies of the bipolar SGN are situated within Rosenthal's canal in the cochlea. Peripheral processes, the dendrites, project from the soma of the SGN through the osseous spiral lamina to the hair cells in the organ of Corti. Together, the central processes – the axons – form the auditory nerve, which terminates in the cochlear nucleus of the brain. Two types of SGN can be distinguished on the basis of factors including their morphology and peripheral target. The large type I SGN project to the inner hair cells – these account for 90–95% of the SGN population – whereas the remaining 5–10% are type II SGN that have a small cell body and connect to the outer hair cells (Spoendlin 1985; Rosbe et al., 1996; Nayagam et al., 2011; Green et al., 2012; Locher et al., 2014). The perikaryons of the type I and type II SGN are enveloped by satellite glial cells. Myelination of the neuronal cell bodies by this type of glial cell differs between mammalian species. For example, the neuronal somata in the human cochlea are unmyelinated, whereas rat SGN cell bodies are surrounded by thin myelin (Rosenbluth 1962; Romand and Romand 1990; Toesca 1996; Hibino et al., 1999; Liu et al., 2012; Locher et al., 2014). In contrast to unmyelinated SGN type II nerve fibers, the fibers of type I SGN are sheathed with typical compact myelin, produced by the associated spiral ganglion Schwann cells (SGSCs) (Rosenbluth 1962; Toesca 1996; Wang et al., 2009), in order to insulate individual ganglion neurons from neighboring neurons and thus prevent electrical interference (Hibino et al., 1999). SGSCs and satellite glial cells are located in the osseous spiral lamina, Rosenthal's canal and the modiolus (Hurley et al., 2007; Jeon et al., 2011). Myelination with peripheral myelin synthesized by SGSCs extends from the first node of Ranvier peripherally to the habenula perforata, bordering the organ of Corti, where the dendrites continue unmyelinated.

Therefore, the primary cultures derived from cells of Rosenthal's canal contain not only SGNs but also the above-mentioned non-neuronal cells, SGSCs and satellite glial cells (positive for S100 immunolabeling). The ratio of SGSCs and satellite glial cells to SGNs varies widely, between 1:1 (Vieira et al., 2007), 12:1 (Mattotti et al., 2015) and about 7500:1 (30,000:4) (Hansen et al., 2001). A small proportion of non-neuronal cells in the SGC culture is accounted for fibroblasts (3%; immunolabeled by vimentin), astrocytes (2%; GFAP-positive) and oligodendrocytes (<0.5%; RIP-positive (Hansen et al., 2001; Furze et al., 2008; Jeon et al., 2011)).

Glial cells play an important role in neuronal development, survival, regeneration and axonal guidance both *in vivo* and *in vitro*. This involves their expressing trophic factors such as brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and ciliary neurotrophic factor (CNTF), and their elaborating extracellular matrix and cell surface proteins for neurite promotion, such as laminin-1, which induces the close association of the neurons and glial cells (Bunge, 1994; Aletsee et al., 2001; Hansen et al., 2001; Boström et al., 2007; Vieira et al., 2007; Whitlon et al., 2009; Clarke et al., 2011; Jeon et al., 2011; Green et al., 2012; He et al., 2014; Sun et al., 2014; Mattotti et al., 2015).

In dissociated cell culture *in vitro*, there is a notable increase of the non-neuronal cell number detectable (Hansen et al., 2001; Boström et al., 2007; Whitlon et al., 2009), whereas proliferation due to reentry into the cell cycle of glial cells after deafening is considerably limited *in vivo* (Provenzano et al., 2011). This proliferation of the non-neuronal cells, especially of neonatal cells, leads to difficulties with long-term cultivation due to overgrowth of the SGN. A more severe problem is that the inevitable presence of other cell types than the SGN introduces an uncontrolled variable in cell culture experiments. Therefore, the effect of treatment strategies – such as surface coatings/structures or exogenous growth factor application on neuronal survival rate or neurite outgrowth and guidance in culture – could be mediated or augmented by the secretion of endogenous factors or molecules from the non-neuronal cells (Staecker et al., 1995; Marzella et al., 1997, 1999; Aletsee et al., 2001; Vieira et al., 2007; Whitlon et al., 2009).

Addition of the mitotic inhibitor cytarabine (AraC), a selective DNA synthesis inhibitor, is an established method of inhibiting fibroblast and glial cell proliferation in neuronal cultures, leading to purification of the Schwann cells and/or neurons (Brockes et al., 1979; Mao and Wang 2001; Negishi et al., 2003; Pettingill et al., 2008; Xiao et al., 2010; Jeon et al., 2011; Provenzano et al., 2011).

The intention of the present study was to establish a neuron-enhanced dissociated SGC culture of neonatal rats cultured for up to seven days, while inhibiting the proliferation of the non-neuronal cells by AraC. Dissociated neonatal SGC-cultures for testing of treatment strategies are commonly performed for two to five days. Therefore the cultured cells were analyzed after four days and the cultivation time of the neonatal cells was prolonged to one week. For a general maintenance of cells, serum (FCS) was added to the culture medium. Compared to that the neurotrophins BDNF and NT3 were chosen as additives for a specific neuronal support and to test these defined and well investigated growth factors in the mitotic inhibited SGC-culture. An additional reason to investigate BDNF and NT3 was that they are produced in vitro by the spiral ganglion Schwann cells. Since the Schwann cells were potentially reduced by mitotic inhibition, we aimed to mimic the production of these NTFs. To estimate the influence of mitotic inhibition, cell composition, neuronal viability and appearance in culture with and without mitotic inhibition were analyzed in order to consider suitability for *in vitro* testing in auditory research.

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