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Role of Islet 1, BDNF and nanoparticles in development, function and regeneration of the auditory system.

Úloha Islet1, BDNF a nanočástic ve vývoji, funkci a regeneraci sluchového systému.

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ABSTRACT

A successful regenerative therapy of the inner ear cannot be developed without a detailed knowledge of the role that particular genes and factors play during the development and in the normal function of the auditory system. Islet1 transcription factor and brain derived neurothrophic factor (BDNF) have great potential to play an important role in regenerative inner ear therapy as both have been shown to be sufficient for self-repair regeneration in cochlea in animal studies. In this study we investigated the roles these two factors play in the development and function of the auditory system.

In the transgenic mice used in the study, overexpression of Islet1 affected cell specification during embryonic development, leading to enlargement of the cochleovestibular ganglion and accelerated nerve fiber extension and branching in mutant embryos. The hearing of young transgenic mice was not affected. However, it started to decline in 1-month-old animals. This early onset of age-related hearing loss was found to be a consequence of the neurodegeneration of the olivocochlear system caused by Pax2-driven Islet1 misexpression in the hindbrain. Our data provide the first evidence that the alteration of the olivocochlear efferent system accelerates the age-related functional decline of hearing without the loss of OHCs.

The functional role of BDNF in the mature auditory system was studied using mutant mice with conditional deletion of BDNF in the lower parts of the auditory pathway. The deletion of BDNF impaired frequency and intensity coding, and affected the inhibitory circuitry in the inferior colliculus, suggesting the importance of BDNF for normal sound processing in the ascending auditory pathway.

Another important step in regenerative therapy is the atraumatic delivery of an active agent to the specific cochlear tissue for targeted action. We tested three types of nanoparticles (NPs; liposomes, polymersomes and polylysine) as a potential tool for minimally invasive intracochlear drug delivery after middle ear application in adult animals. All NPs penetrated the round window membrane and were identified in the spiral ganglion, the organ of Corti and the lateral wall, producing no distinct morphological or functional damage to the inner ear. Using a model neurotoxic drug, liposomes and polymersomes were shown to be capable of carrying into the inner ear an active drug that elicits a detectable biological effect.

Results presented in this thesis contribute to knowledge about the role of Islet1 and BDNF in the development and function of the auditory system. Together with results of nanoparticle testing these data may contribute to the development of regenerative therapy for the inner ear.

ABSTRAKT

Podmínkou úspěšného vývoje regenerační terapie ztráty sluchu je detailní znalost funkce jednotlivých genů a faktorů uplatňujících se během vývoje sluchového systému. Mezi faktory důležité pro vyvolání procesů regenerace ve vnitřním uchu patří transkripční faktor Islet1 a mozkový neurotrofní faktor BDNF. V předkládané dizertační práci je studována role obou faktorů ve vývoji a funkci sluchového systému a také možnosti využití nanočástic jako možného bezpečného prostředku pro jejich cílené doručení do kochley.

U embryí transgenních myší se zvýšenou expresí Islet1 (s Pax2 promotorem) bylo pozorováno větší kochleovestibulární ganglion a indukoval se zrychlený růst a větvení nervových vláken u embryí. Funkční testy měření kmenových potenciálů a otoakustických emisí ukázaly, že u mladých transgenních myší byla sluchová funkce na úrovni kontrolních myší, ale byl pozorován brzký nástup sluchové ztráty způsobené stárnutím. Tato sluchová ztráta souvisela s degenerací zakončení eferentních kochleárních vláken mediálního olivokochleárního systému, která byla způsobena misexpresí Islet1 v zadním mozku. Výsledky poprvé ukázaly, že poškození mediálního olivokochleárního systému může urychlit vznik sluchové ztráty během stárnutí bez ztráty vnějších vláskových buněk.

Úloha BDNF ve sluchovém systému byla studována na modelu mutantních myších s podmíněnou delecí BDNF v nižších oddílech sluchové dráhy. Měření odpovědi neuronů v colliculus inferior ukázalo, že u myší s delecí BDNF došlo k narušení kódování intenzitních a frekvenčních vlastností zvukových stimulů. Výsledky tak ukazují, že přítomnost BDNF v nižších částech sluchové dráhy je nezbytná pro správnou funkci celého sluchového systému při zpracování behaviorálně významných zvuků.

Důležitým předpokladem úspěšné regenerativní léčby je atraumatické a cílené dodání aktivní látky specificky do příslušné části vnitřního ucha. Testovali jsme 3 typy nanočástic (NP) jako možného prostředku doručení aktivní látky: liposomy, polymersomy a polylysiny. NP byly aplikovány u dospělých myší na membránu okrouhlého okénka a byla analyzována jejich distribuce ve vnitřním uchu. Všechny tři typy NP úspěšně pronikaly do vnitřního ucha a byly nalezeny ve spirálním gangliu, Cortiho orgánu i laterální stěně a to bez morfologického nebo funkčního poškození vnitřního ucha. Na příkladu NP plněných neurotoxickou látkou disulfiramem byla ukázána možnost využít liposomů a polymersomů jako nosičů aktivní látky, která vyvolá ve vnitřním uchu biologický efekt měřitelný funkčním testem.

Předkládaná práce přináší nové poznatky o funkci transkripčního faktoru Islet1 a mozkového neurotrofního faktoru BDNF ve sluchovém systému. Spolu s diskutovanými možnostmi cíleného doručení (targeted drug delivery) mohou výsledky přispět k vývoji regenerativní léčby senzorineurálních poruch sluchu.

1.   INTRODUCTION

Over 5% of the world's population has disabling hearing loss. Appetite to recreational noise among the young people and demographic aging of the society will lead to growth of the population suffering from hearing loss increasing social and economic demand to develop regenerative treatment for the cochlea.

Most often hearing loss is caused by damage to the inner ear or the VIII cerebral nerve (auditory nerve). At present, no remedy is available and people, suffering from hearing loss can only benefit from hearing aids and cochlear implants. The ideal scenario, however, would be a regeneration of damaged parts of the cochlea with full restoration of their function.

At present no practical methods for cochlear regeneration have been developed, but major approaches are outlined. These include self-repair, direct trans-differentiation, mitotic proliferation (dedifferentiation) and cell transplantation (Nakagawa, 2014). These approaches in turn can employ a variety of methods such as gene therapy, molecular therapy and stem-cell therapy. First experimental attempts to initiate inner ear regeneration via activation of genes having protective and regenerative effect or deactivation of proapoptotic or damaging genes in the inner ear have been successful. However, to coordinate regenerative processes resulting in creation of morphologically and functionally mature cell a detailed understanding of mechanisms of inner ear development, maturation and degeneration is required.

Islet 1 belongs to LIM-homeodomain (LIM-HD) family of transcription factors that plays fundamental roles in development. During normal ear development, ISL1 appears first in the otic placode and it is then upregulated in the cells that will form the cochleovestibular ganglion during placode invagination (Li et al., 2004). Upon initiation of hair cell differentiation, the expression of ISL1 is downregulated in cochlear epithelium in vertebrates (Radde-Gallwitz et al., 2004) and is not expressed in mature inner ear. It is not the case for chick inner ear that possess robust expression of Isl1 in adult auditory and vestibular supporting cells (Li et al., 2004), which can contribute to regeneration potential of chick sensory epithelium. Based on the idea that expression of developmental genes in mature hair cells may lead to reprogramming and rejuvenation of damaged hair cells, Huang et al. forced *Isl1* expression in mature outer and inner hair cells in mice, by this way protecting their hearing from age-related and noise-induced hearing loss (Huang et al., 2013).

Neurotrophic factors are proteins responsible for the growth and surviving of developing neurons, and maintenance of mature neurons in the central and peripheral nervous

system (Maness et al., 1994, Terenghi, 1999). During inner ear development brain derived neurotrophic factor (BDNF) is expressed in the otic vesicle (E11–E18) when the formation of the cochlear duct occur and cochlear neurites reach presumptive sensory epithelium (Pirvola et al., 1992, Pirvola et al., 1994, Schecterson and Bothwell, 1994). Positive effects of BDNF in supporting of neuronal surviving and synaptic plasticity made it a great candidate for drug development for the treatment of many neuodegenerative disesases (Levivier et al., 1995, Schabitz et al., 1997, Bemelmans et al., 1999, Kalra et al., 2003, Hoshaw et al., 2005, Makar et al., 2009, Nagahara et al., 2009) as well as for prevention of spiral ganglion degeneration after hair cell loss (Ramekers et al., 2012, Khalin et al., 2015). The role of BDNF in the mature cochlea, however, is still elusive.

Another important step in the regenerative therapy represents delivery of an active agent to the specific cochlear tissue for targeted action. As direct drug delivery to the cochlea is associated with a risk of irreversible damage to the ear, a minimally invasive targeted delivery of therapeutic agent is needed. Nanoparticles (NPs) are among the recently developed drug delivery technologies and are proposed as a suitable tool for cochlear regenerative therapy (Jero et al., 2001, Tamura et al., 2005, Ge et al., 2007, Maeda et al., 2007, Praetorius et al., 2007, Zou et al., 2008, Zhang et al., 2010).

2.   AIMS OF WORK AND HYPOTHESES

In the present study we aimed to gain an insight into the role of Islet1 transcription factor and brain derived neurotrophic factor in development and function of the auditory system using two transgenic mice models. Besides that we aimed to test three types of nanoparticles for their ability to safely penetrate inside the cochlea and deliver there sufficient amount of an active agent.

2.1.   Role of ISL1 in development and function of the auditory system

Hypothesis: ISL1-expressing cells are the common precursors for both sensory epithelia and neurons suggesting its potential role for regeneration of these types of inner ear cells. By modulating the expression of Isl1, we expected to change the fate specification of cells in the inner ear.

Specific aims:

- To test whether overexpression of Pax2Isl1 affects embryonic development of the inner ear and increases the sensory and neural domains in developed cochlea.
- To test whether Pax2Isl1 overexpression leads to functional and/or morphological changes in hearing in adult mutant mice compared to wild type animals.
- To analyze the process of aging in the auditory system in mutant and wild type animals.

2.2. Role of "peripheral" BDNF in the auditory system

Hypothesis: BDNF in the lower parts of the auditory pathway or within the cochlea is necessary for the maturation of auditory nerve activity after onset of hearing.

Specific aims:

- To study a role of BDNF in the lower part of the mature auditory pathway in mouse using extracellular recording of neuronal activity in the inferior colliculus (IC) of BDNF^{Pax2} knock-out mice.
- To understand the physiology behind the reduced range of suprathreshold ABR responses generated at the level of the IC in BDNF^{Pax2} knock-out mice.

2.3. Use of nanoparticles as a potential drug delivery tool

Hypothesis: Nanoparticles are a suitable tool for delivery of active agents to the cochlea during regenerative therapy.

Specific aims:

- To study the ability of nanoparticles to penetrate the round window membrane and their distribution inside cochlea.
- To compare the pattern of cochlear distribution of different types of nanoparticles.
- To determine whether an active agent can be delivered to the inner ear using nanoparticles in sufficient amount and condition to produce a detectable effect.

3. MATERIALS AND METHODS

3.1. Animals

The studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). The experimental protocols were approved by the Expert Commission of the Academy of Sciences and followed the guidelines of the EU directive 2010/63/EU. Mice were kept under standard laboratory conditions in a constant environment (12/12 h light/dark cycle), with food and water available ad libitum. All experimental procedures were conducted during the light phase of the cycle.

To study the role of transcription factor Isl1 in the auditory system wild-type (WT, n = 38) or heterozygous Pax2-Isl1 transgenic mice ($Tg^{+/-}$, n = 50) generated on an FVB background (strain code 207, Charles River) were used.

Wild type (WT, n = 4) and $BDNF^{Pax2}$-KO mice (KO, n = 4), obtained by mating Pax2-Cre (Ohyama and Groves, 2004) with BDNFlox/lox mice (Rios et al., 2001) were used to study the role of BDNF in the lower auditory brain regions.

Nanoparticle transport in the inner ear and their ability to deliver there an active agent was tested using 40 2–3 months old C3H mice. In individual animals, either polylysine (n = 5), polylysine-plasmid complex (n = 5), liposomes (n = 8), disulfiram-loaded liposomes (n = 5), polymersomes (n = 7) or disulfiram-loaded polymersomes (n = 6) were applied unilaterally to the middle ear. In controls, saline (n = 2), or disulfiram solution (n = 2) was applied instead of nanoparticles.

For the hearing measurements, surgical procedures and extracellular measurements of neuronal activity mice were anaesthetized by intraperitoneal injection of 35 mg/kg of ketamine (Calypsol 5%, Gedeon Richter Ltd., Budapest, Hungary or Narkamon 5%; Spofa, Prague, Czech Republic) and 6 mg/kg of xylazine (Xylapan 2%, Vetoquinol, Gorzów Wielkopolski, Poland or Sedazine 2%; Fort Dodge, Animal Health, Fort Dodge, Iowa) in saline via intraperitoneal injection.

All measurements were performed in a sound-attenuating and anechoic chamber in anesthetized mice maintained on a temperature - regulated blanket.

3.2. Hearing measurements

The hearing function of mice was studied using recording of Auditory Brainstem Responses (ABR) and Distortion Product Otoacoustic Emission (DPOAE). ABRs were evoked

using short tone bursts (3 ms duration, 1 ms rise/fall times, frequencies of 2, 4, 8, 16, 32 and 40 kHz) presented with decreasing stimulus level (from 100 to 0 dB SPL) by 5dB with each step. DPOAEs over a F2 frequency range from 4 to 38 kHz were recorded.

Average for all animals in the group audiograms and DPOAE-grams were produced to compare results between experimental and control groups.

3.3. Extracellular recording of the neuronal activity in the inferior colliculus

A small hole in the animal's skull was drilled to access the IC. IC neuronal activity was recorded extracellularly using either 16-channel single shank probe (NeuroNexus Technologies) or single parylene-coated tungsten electrode (Bionic Technologies Inc). Responses evoked by single tones, two-tone stimuli and broad band noise were recorded. Using custom made Matlab software obtained results were analyzed and following parameters were calculated: the excitatory response threshold, the characteristic frequency, the quality factor Q10 (a measure of frequency selectivity), the inhibitory strength, the dynamic range and the minimum first-spike latency (mFSL).

3.4. Round window membrane application

Cochlear RW niche was approached through the hole in the acoustic bulla. Nanoparticle suspension was injected into the gelfoam placed in the RW niche. Three types of nanoparticles were used: liposomes, polymersomes and hyperbranched polylysine. Nanoparticles were either labeled with fluorescent dye to enable their detection in the cochlear structures or loaded with a model active agent to evaluate carrying ability of the nanoparticles. As an active agent either DNA plasmid (polylysine) or neurotoxic drug disulfiram (liposomes, polymersomes) was used.

3.5. Evaluation of gene expression and protein levels

Gene expression was analysed using RT-qPCR (Myo7a, prestin, IsI1) or qPCR (Isl1). Levels of parvalbumin, GAD67 and GAPDH were assessed using Western Blot.

3.6. Morphological analysis

In the end of the experiment animals were sacrificed and tissues were taken for histological analysis. Embryos, brain and cochlea sections as well as whole mount cochlear preparation were stained using appropriate primary (anti-ISL1, anti-PAX2, anti-parvalbumin,

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anti-GAD67, anti-prestin, anti-200-kDa neurofilament protein, anti-choline acetyltransferase, anti-myosin 7a, anti- active caspase-3) and secondary (IgG-peroxidase, Cy3-conjugated, AlexaFluor 488-conjugated) antibodies. Sections then were examined under confocal microscope. Quantitative analysis was performed using ImageJ and optical dissector software Ellipse®. For the dye-tracing experiment a two-color tracing system was applied.

3.7. Statistical Analysis

Data are presented either as the mean ± standard deviation (SD) or standard error of the mean (SEM) for values with normal distributions or the median (Mdn) for values with non-normal distributions. For statistical analysis, the GraphPad Prism software (San Diego, USA) was used. To assess differences in the mean or median values between groups Student's t tests, one-way or two-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test, Kruskal-Wallis tests with Dunn's multiple comparison test, or chi-square tests were employed. Significance was assigned at the $P<0.05$ level.

4.   RESULTS

4.1. Role of ISL1 in development and function of the auditory system

The Pax2-Isl1 transgenic mice [Tg(Pax2-Isl1)] were generated using a transgenic construct, containing Isl1 gene insert and the 8.5-kb Pax2 regulatory sequences with regulatory activities in the otic vesicle, where it closely mimic the activities of the endogenous Pax2 gene at E9.5 and midbrain–hindbrain region. $Tg^{+/-}$ mice exhibited increased levels of motor hyperactivity (2.8 folds higher), including augmented locomotion and circling behavior compared to WT littermates. The average number of surviving transgenic heterozygous offspring per litter (1.926 ± 0.2871, n = 27) was significantly lower compared to their WT littermates (3.296 ± 0.3988).

The ISL1+ expression domain was enlarged in the $Tg^{+/-}$ otocysts. The size of the cochleovestibular ganglion delaminating from the otic epithelium and differentiating into ganglion neurons was increased in $Tg^{+/-}$ embryos compared to WT littermates. Increased expression levels of Isl1 in mutant embryos were also associated with more rapid differentiation of afferent fibers.

After birth hearing of $Tg^{+/-}$ and WT animals mice did not differ. However, starting from 1 month of age hearing of $Tg^{+/-}$ mice began to deteriorate with primary changes in

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amplitude of distortion product otoacoustic emissions (DPOAEs). Decline of DPOAE amplitudes started from the high frequency region in 1–2 months old $Tg^{+/-}$ mice progressing rapidly and disappearing completely by the age of 6–9 months. In *WT* animals DPOAE amplitude decreased with age as well, however, in much slower pace still being present in 10–15 months old animals. Hearing thresholds changed according to otoacoustic emission decline suggesting primary inefficiency of the outer hair cells. Analysis of OHC number, spatial organization, and expression of prestin, F-actin, and myosin 7a did not reveal any significant abnormalities that could explain the extent of the functional decline. Analysis of outer hair cell innervation by descending efferent system (medial olivocochlear system, MOC), however, provided the explanation. Choline acetyl transferase staining revealed 20% MOC terminals volume reduction in 2 months old and 34% volume reduction in 10 to 15-month-old $Tg^{+/-}$ animals compared to *WT*. Postnatal expression of the Isl1 transgene detected in MOC region of the brainstem suggests that the misexpression of ISL1 could negatively affect the development and function of MOC neurons.

4.2. Role of "peripheral" BDNF in the auditory system

Knock-out mice with deletion of BDNF under Pax2 promoter (BDNFPax2KO) used in this study were generated by mating Pax2-Cre mice (Ohyama and Groves, 2004) with BDNFlox/lox mice. Zuccotti et al. (2012) showed that in obtained knock-out mice BDNF is deleted in the cochlear epithelium and spiral ganglion, DCN, and IC. Lack of BDNF in these mice is accompanied by slight elevated ABR thresholds to click and to most pure tone frequencies and decreased amplitude of the first ABR wave, corresponding to cochlear nerve activity. These functional changes were shown to be caused by altered IHC exocytosis resulting from loss of ribbon synapses, particularly in high frequency cochlear region. Interestingly, this deficit shows up upon hearing onset at P12. Another finding worth paying attention is decreased amplitude of reflecting IC activity ABR waves IV–V compared to previous waves corresponding to lower brain structures (Zuccotti et al., 2012).

To obtain more insight into the role of peripheral BDNF on basic sound processing and understand the physiology behind the reduced range of suprathreshold ABR responses generated at the level of the IC in knock-out mice, electrophysiological response behavior of IC neurons in BDNFPax2 knock-out (KO) and wild type (WT) mice was assessed in the present work.

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Extracellular recording of neuronal activity in the IC in 3–4 -month-old WT and KO mice was performed. The responses to BBN and pure tones of a total of 469 IC units were recorded, comprising 264 units from WT (n=4) and 232 from KO (n=4). The whole sample of units was divided into three main non-overlapping frequency bands according to their characteristic frequency (CF): 4–9 kHz (low), 10–15 kHz (middle), and 16–30 kHz (high).

The thresholds of IC neurons were significantly higher in KO than in WT mice. Besides that, the parameters of the RIFs such as dynamic range (WT 52.37 ± 11; KO 43.23 ± 11, ****p<0.0001) and slope (WT 0.01424 ± 0.006; KO 0.01919 ± 0.007, ****p<0.0001), but not maximum response magnitude (WT 20.52 ± 12.48; KO 21.83 ± 15.56, n.s. p>0.05) were significantly reduced in KO mice suggesting impaired intensity coding in the IC of these mice. Impaired sensitivity of high-frequency neurons manifested as prolonged mFSL (WT: Mdn = 6.8; KO: Mdn = 7.5, ****p<0.0001) and lack of sharply tuned high frequency neurons in KO mice suggest importance of BDNF particularly for proper function of behaviorally relevant high frequency IC part.

In both groups of mice almost 80% of neurons displayed low-frequency and/or high-frequency lateral inhibition. The strength of firing rate suppression by lateral inhibition was calculated as a percentage of rate suppression separately for low- and high-frequency sideband inhibition. Low CF neurons did not exhibit altered sideband inhibitory strengths in the absence of BDNF. Likewise, in the neurons with a CF of 11–30 kHz, the low-frequency sideband was similar in WT and KO animals. However, high-frequency sideband inhibition strength was significantly reduced in KO mice (**p<0.01). This indicates that after the onset of hearing, BDNF in lower brain parts increases the inhibitory strength of IC neurons.

Our finding strengthens the conclusion that BDNF may improve signal-to-noise ratio by increasing the inhibitory strength of neurons. In many projection neurons, changes in inhibitory conductances are assumed to require specialized GABA (γ- aminobutyric acid) A receptors. Particularly, the differentiation of PV basket cells, a subpopulation of GABAergic neurons may play a role during the alteration of inhibitory strength in KOs. To investigate a role of PV for the observed changes in inhibitory strength in KO mice, we used antibodies for PV and antibodies for the 67-kDa isoform of GABAergic interneurons. We found that the intensity and number of PV immuno-positive puncta, but not the number of PV immuno-positive somata or GAD67-immuno-positive puncta, were decreased in the IC and AC of BDNF[Pax2]-KO mice. Reduced levels of PV expression were also confirmed by Western blots.

4.3. Nanoparticles as a potential drug carrier for regenerative inner ear therapy

NP distribution in the inner ear after RWM application was evaluated in the RWM- the site of most probable NP entrance into the cochlea, and cochlear tissues disposed to irreversible damage and cell loss in SNHL: SG, organ of Corti and the lateral wall.

All three types of nanoparticles tagged with a fluorescent marker penetrated through the RWM and were detected in cells in all layers of the RWM one day after NP application. In case of polylysine strong affinity of NPs to cochlear bone was noticed. This let polylysine penetration also into the scala vestibuli through the bone surrounding round window and through the oval window (stapes and annular ligament).

After passing the RWM, the NPs spread into the perilymph of the ST and penetrated individual cochlear structures.

Spiral ganglion. One day after application NPs were found in SG of all cochlear turns. Nanoparticles of all types were found in the cytoplasm of spiral ganglion cells, polylysine was detected also extracellularly. When visualized using confocal microscopy, the NPs are visible as red or green fluorescent dots that in case of intracellular localization are thought to be an accumulations of NPs in endosomes and lysosomes. Nanoparticles of neither type were detected in the nuclei of the SG cells.

Organ of Corti. A small amount of NPs were found in the organ of Corti in all cochlear turns. Liposomes and polymersomes were diffusely distributed in the cytoplasm of hair cells (inner and outer hair cell), between hair cells and in supporting cells while polylysines created rather small clusters between and inside cells often near cell nucleus. Strong specific fluorescent signal suggested high polylysine saturation of basilar membrane of the organ of Corti and bonny wall of the modiolus.

Lateral wall. Liposomes and polymersomes were mainly detected between or within the fibroblasts of the spiral ligament and sparsely in all three layers of the stria vascularis in the basal and middle turns of the cochlea. In contrast, polylysine was found more often dispersed or forming small clusters in the extracellular matrix of the lower part of the spiral ligament and less often as intracellular clusters. When specific polylysine fluorescence was found in the stria vascularis it was usually related to capillary walls.

Application of neither type of NPs had any overt impact on the hearing thresholds 2 weeks after the treatment.

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Ability of nanoparticles to deliver intracochlearly an active substance was tested using either DNA plasmid in case of polylysine or disulfiram in case of liposomes and polymersomes.

Unfortunately, polylysine failed to deliver DNA plasmid inside cochlea suggesting that certain modification to the complex polylysine-plasmid should be made for successful delivery.

In contrast, application of disulfiram-loaded liposomes and polymersomes evoked pronounced biological effect. As disulfiram is a neurotoxic drug, by its application we expected to produce a negative effect on cochlear structure and/or function that would be detectable using functional and morphological evaluation. Two weeks after the treatment with disulfiram-loaded NPs significant threshold shifts (20–35 dB) was found in both treated groups. According to the results of DPOAE measurement the function of outer hair cells was not altered significantly. Morphological evaluation revealed pronounced damage to the spiral ganglion. Signs of morphological damage were evident in the SG cells of all cochlear turns as early as 2 days after NP application. Cytoplasmic localization of caspase-3 immunoreactivity in SG neurons detected 7 days after treatment suggested apoptotic cell death after disulfiram-loaded NP administration. During one week after NP application number of survived neurons reached in average values of 31–47%. No differences were found between the signs of apoptosis induced by disulfiram delivered by liposomes or polymersomes.

No changes were detected after application of solution of disulfiram in saline which indicated that morphological alterations and the pronounced hearing loss observed in the mice treated with disulfiram-loaded NPs must be produced by the disulfiram released from NPs once within the cochlea.

5. DISCUSSION

5.1. Pax2 in development and function of the auditory system

In this study for gene manipulation in mutant mice Pax2 regulatory sequence was used. Pax2 is one of the earliest genes to be expressed in the pre-otic region (Hans et al., 2004) in developing inner ear. PAX2 is a key regulator of otic cell identity and shape in chick (Christophorou et al., 2010) and PAX2 combined with PAX8 is needed for mouse ear development (Bouchard et al., 2010). In the mouse, PAX2 is expressed in all sensory and some non-sensory epithelia, but within sensory epithelia at E16.5 it is restricted to hair cells (Lawoko-Kerali et al., 2004). Although PAX2 is expressed in differentiating hair cells, mature murine

hair cells do not express PAX2 protein. Pax2 affects the regional patterning of the early otocyst and cellular patterning within the sensory epithelia of the inner ear.

At E7.5 Pax2 expression is initiated also in the neural plate, particularly in the midbrain-hindbrain boundary (Puschel et al., 1992, Rowitch and Mcmahon, 1995). This crucial developmental signaling center is responsible for patterning mesencephalic and metencephalic regions of the vertebrate brain. (Dworkin et al., 2012, Fritzsch et al., 2015). Pax2 expression in these regions is extensive at E8.0, becomes limited to isthmus area by E9 (Rowitch and Mcmahon, 1995) and completely ceases after E11 (Puschel et al., 1992).

5.2.   Role of ISL1 during embryonic development

We examined the functional role of ISL1 in the development and maintenance of auditory sensory cells and neurons in the inner ear. In compliance with our hypothesis Pax2-regulated Isl1 overexpression increases the embryonic ISL1+ domain and the size of the cochleovestibular ganglion in the inner ear at E10.5. Isl1 overexpression is also accompanied by premature afferent fibers differentiation- rapid extension and branching of fibers connecting developing brainstem and inner ear in E12.5 embryos. Our findings confirm that Isl1 as a developmental gene plays an important role in the inner ear cell fate specification in early stages of the inner ear development. These stages have been recently shown to be crucial in production of a sufficient amount of functional mechanosensory cells from the pluripotent stem cells *in vitro* (Koehler et al., 2013). More than that, a protective role of Isl1 in adult hair cells against noise induced and age-related hearing loss has been recently reported (Huang et al., 2013). All together this makes Isl1 a promising candidate for use in gene or stem cell therapy to regenerate damaged inner ear epithelium.

5.3.   Detrimental effect of ISL1 brain misexpression in adult animals

Pax2-Isl1 transgenic mice were created on the basis of FVB strain, which is a mouse strain with a normal pattern of age related hearing deterioration. Our results of hearing evaluation in the FVB strain are comparable to previous reports (Jones et al., 2006, Martin et al., 2007) that show completely preserved DPOAEs up to at least 5 months of age and the minor ABR threshold shift of 20 dB in 14-month-old animals compared to 1-month-old mice. In contrast, $Tg^{+/-}$ mice displayed a significant functional hearing deficit already at the youngest analyzed group (1–5 months). This was conditioned primarily by the premature decline in DPOAEs found in the youngest $Tg^{+/-}$ group. $Tg^{+/-}$ mice lost DPOAEs completely by the age of

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6–9 months, whereas 10–15-month-old *WT* animals still had apparent otoacoustic emissions in the middle frequency region. The pattern of hearing loss in $Tg^{+/-}$ mice conformed to the pattern of age related hearing loss associated with the decrease of DPOAE amplitudes starting from the high frequency cochlear region (Schuknecht, 1964).

DPOAEs reflect the cochlear amplification facilitated by OHCs (Trautwein et al., 1996, Hofstetter et al., 1997). Usually, a decrease of DPOAE amplitudes relates to the loss of OHCs (Trautwein et al., 1996, Hofstetter et al., 1997) or the decline in their function (Li et al., 1999, Popelar et al., 2006). Accordingly, we detected a higher OHC loss in the high frequency region of the cochlea in 1 to 5-month-old $Tg^{+/-}$ mice compared to *WT*. The higher loss of OHCs in $Tg^{+/-}$ animals could be associated only with the loss of DPOAEs at high frequencies (32–40 kHz), whilst the number of OHCs in the middle frequency region of $Tg^{+/-}$ cochleae did not differ significantly from that in *WT* animals. Since the complete absence of DPOAEs could not be explained by the loss of OHCs, another possibility for the loss of cochlear amplification in $Tg^{+/-}$ might be an impaired function of OHCs.

Cochlear amplification is thought to be of two main sources: somatic, caused by prestin motility (Liberman et al., 2002, Dallos et al., 2008) and streocilia-based (Chan and Hudspeth, 2005, Kennedy et al., 2005). Prestin is a motor protein located in the OHC lateral wall and it is responsible for the OHC motility (Liberman et al., 2002). We noticed no obvious difference in the prestin distribution and prestin mRNA expression in the OHCs between *WT* and $Tg^{+/-}$ animals. The analysis of stereocilia of the OHCs neither revealed stereocilia abnormalities in $Tg^{+/-}$ animals. Thus, the premature and severe DPOAE loss in $Tg^{+/-}$ animals was unlikely due to prestin abnormalities or the deterioration of stereocilia.

The maintenance and function of OHCs is influenced by the MOC efferent innervation. The activation of the MOC system enhances auditory processing of distracting noise (Winslow and Sachs, 1987, Kawase and Liberman, 1993, May and McQuone, 1995) and protects the inner ear from acoustic trauma (Liberman, 1991, Maison et al., 2002, Lauer and May, 2011). MOC terminals are the endings of the efferent fibers, which make OHCs hyperpolarized when activated. We detected ISL1 misexpression in the neurons in the region of the superior olivary complex where MOC efferents originate of $Tg^{+/-}$ mice, which may affect the formation of MOC efferent neurons. The reduced or altered MOC $Tg^{+/-}$ efferents may contribute to the reduction of MOC terminals and accelerate the deterioration of OHC function in mutant mice. Similarly, a surgical de-efferentation accelerates the age-related deterioration of DPOAEs without an extensive loss of OHCs (Liberman et al., 2014). The reduction in the

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MOC-negative-feedback-gain-control may also explain the particular deterioration in the hearing thresholds of $Tg^{+/-}$ animals at 16 kHz since this region has the maximal density of MOC terminals (Maison et al., 2003).

Our data provide the first evidence that the alternation of MOC efferent system accelerates the age-related functional decline of hearing without the loss of OHCs.

5.4.   Role of "peripheral" BDNF in the auditory system

$BDNF^{Pax2}$ deletion was shown to result in altered IHC exocytosis and decreased sensitivity of the auditory nerve, particularly in the high frequency region (Zuccotti et al., 2012). These changes become apparent upon hearing onset at P12. It has been shown more than once in previous studies that peripheral deafferentation and decreased peripheral input shown up as decreased amplitude of ABR wave I more centrally located auditory nuclei tend to compensate the decreased input by hyperactivity expressed in normal or less decreased amplitude of later ABR waves especially corresponding to the IC waves IV–V (Salvi et al., 1990, Schaette and McAlpine, 2011, Konrad-Martin et al., 2012, Sergeyenko et al., 2013). This is called "central gain" and has been implicated in the generation of tinnitus (Lockwood et al., 2002, Knipper et al., 2013). This is, however, not the case for $BDNF^{Pax2}$KO (Zuccotti et al., 2012, Chumak et al., 2015) in which suprathreshold amplitude of waves IV–V decrease compared to the previous waves suggesting a role of "peripheral" BDNF in activity of the inferior colliculus.

Reduced exocytosis in otherwise mature IHCs in high-frequency cochlear turns of 2–3 -week-old $BDNF^{Pax2}$KO mice (Zuccotti et al., 2012) pointed out a critical role of BDNF for pre- and postsynaptic maturation of IHCs. The alterations in ABR waves I and IV found in $BDNF^{Pax2}$KO (Zuccotti et al., 2012) but not $BDNF^{TrkC}$-KO mice (Chumak et al., 2015) indicate that BDNF activities that are carried out independently of higher auditory brain regions improve sound sensitivity at least up to the level of the IC.

The current findings suggest that BDNF-dependent effects on auditory nerve activity are responsible not only for improving the ABR amplitude size of high-frequency sound responses spreading along the ascending pathway, but also for expanding the range of CF thresholds in these high frequency CF regions. The affected CF regions >10 kHz span areas (15–20 kHz) where murine behavioral auditory thresholds are at their lowest level (Hage and Ehret, 2003). We thus have to consider the improved CF thresholds, short latencies, and the increased strength of inhibitory sidebands of sharply tuned IC neurons in WT versus $BDNF^{Pax2}$KO animals in the context of an enhanced auditory fidelity in behaviorally relevant

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frequency regions. The IC neurons that show altered tuning characteristics depending on the presence of BDNF are reminiscent of type II (Hage and Ehret, 2003) and type I (Hernandez et al., 2005) class IC neurons. Both IC cell types predominate in high-frequency regions and are characterized by their short latency, narrow and sharp tuning, low-frequency border with a shallow slope, and high frequency border with a steep slope (Hage and Ehret, 2003, Hernandez et al., 2005). It thus may be considered that these neurons are shaped under the control of BDNF activities at hearing onset.

PV expressing interneurons play a crucial role for hippocampal microcircuit formation and plasticity changes (Jonas P, 2007). The significant loss of density of PV-immunopositive puncta in the IC and AC may suggest that a BDNF-modified driving force for auditory processing improves the baseline for mature circuit formation and plasticity changes along the entire ascending auditory network. This finding, moreover, suggests that the altered brain network activity shaped under the influence of BDNF in the lower parts of the auditory CNS or within the cochlea is a prerequisite for cortical maturation processes that occur with sensory experience (Kilgard et al., 2002, Han et al., 2007, Carcea and Froemke, 2013).

5.5. Nanoparticles as a potential drug carrier for regenerative inner ear therapy

The results of this study show that all three types of NPs, although composed of different materials and have different structure, can penetrate inside the cochlea and enter identical cochlear structures (i.e., the SG, the organ of Corti and the lateral wall). Besides RWM polylysine was able to permeate inside cochlea through the cochlear and stapedial bone and annular ligament surrounding footplate of the stapes (oval window). It increased permeation of polylysine into the scala vestibuli and, possibly into the tissues of interest such as SG, OC and lateral wall. Inner ear entry of substances through the oval window (Salt et al., 2012, King et al., 2013) and cohlear bone (Mikulec et al., 2009) has been previously shown, but considered less effective compared to RWM permeation (Salt et al., 2012, Zou et al., 2012a, Zou et al., 2012b).

Within the cochlear tissues liposomes and polymersomes were localized mainly intracellularly and only rarely in the intercellular space (organ of Corti), while polylysines were detected both, intra- and extracellularly in all analysed structures. The cochlear distribution of different types of NPs applied on the RWM has been previously demonstrated for poly(lactic/glycolic acid) NPs (Tamura et al., 2005, Ge et al., 2007), lipid nanocapsules (Zou et al., 2008), hyperbranched polylysines (Zhang et al., 2011) and silica NPs (Praetorius et al., 2007).

To determine the efficiency of drug delivery to the inner ear we introduced experiments using nanoparticles loaded with an active agent.

Polylysine have been proposed as effective polymeric carrier for gene delivery *in vitro* (Zauner et al., 1998, Vanderkerken et al., 2000, Putnam et al., 2001, Zhang et al., 2010, Kim, 2012, Zhao et al., 2016). To test transfection efficiency of polylysine-plasmid complex *in vivo* we used two plasmids: polylysine-plFL was tagged with fluorescent marker and was supposed to be used as a positive control of polylysine-plasmid intracochlear permeation, and polylysine-eGFP, contained plasmid, that in case of its intracellular delivery and successful transfection would be detected as green fluorescence. Unfortunately, we were not able to detect any specific fluorescence inside cochlea after RWM application of either polylysine-plasmid complex 1 and 3 days after injection. Experiments on intracochlear application of polylysine-plasmid complex would be needed to find out if failure in transfection was due to permeability issue.

As a payload for polymersomes and liposomes, neurotoxic drug disulfiram was selected. Several neurotoxic drugs such as ouabain (Schmiedt et al., 2002) or b-bungarotoxin (Palmgren et al., 2010) have been used in the previous studies to induce selective damage of neurons in the cochlea. However, in contrast to disulfiram, these drugs can transit alone through the RWM due to different physical parameters, which makes them unsuitable for testing a NP-based drug delivery system. When used clinically, disulfiram has several negative side effects including induction of a peripheral neuropathy (Tonkin et al., 2000, Bevilacqua et al., 2002, Tonkin et al., 2004). Recent *in vitro* studies demonstrated that disulfiram-induced apoptotic cell death is due to proteosomal inhibition (Chen et al., 2001, Chen et al., 2006, Wickstrom et al., 2007). The results of our study demonstrate that the amount of disulfiram transported by NPs into the cochlea was sufficient to produce a toxic effect in the SG, which corresponds to the site where the largest amount of NPs was detected. In other cochlear tissues including the lateral wall or the organ of Corti, the pathological changes were negligible.

The results of the present study suggests that polymersome and liposome NPs are capable of carrying a payload into the inner ear that elicits a biological effect, with consequences measurable by a functional readout.

6.   CONCLUSIONS

Role of ISL1 in development and function of the auditory system

- The transgenic expression of Pax2-Isl1 results in an enlargement of ISL1+ expression domain with an increased number of ISL1+ cells in the otic epithelium and delaminating neuroblasts of the ganglion in the E10.5 mouse otocyst.
- The transgenic expression of Pax2-Isl1 is accompanied by rapid extension and branching of fibers connecting developing brainstem and inner ear in E12.5 embryos.
- The misexpression of Pax2-Isl1 in the MOC area is most likely the reason for MOC efferent system degeneration in $Tg^{+/-}$ mice that resulted in accelerated age-related hearing loss

Role of "peripheral" BDNF in the auditory system

- Pax2-BDNF deletion increases thresholds and constricts dynamic range of IC neurons
- Pax2-BDNF deletion alters latency and frequency selectivity of IC neurons
  suggesting that after the onset of hearing, BDNF in lower brain parts sharpens a very narrow tuning characteristic of IC neurons.
- Deletion of BDNF affects the inhibitory strength of IC neurons and reduces the density of PV-immunopositivity in IC and AC projecting neurons which may affect temporal processing of complex sound
- The source of BDNF that sharpens IC neuronal responses is not fully clear, however, it is surely not auditory cortex or hippocampus.

Use of nanoparticles as a potential drug delivery tool

- All three tested types of nanoparticles (liposomes, polymersomes and polylysine) penetrate through the RWM and enter cells within the cochlea after middle ear in mice
- All types of nanoparticles are found in the spiral ganglion, the organ of Corti and spiral ligament suggesting unsatisfied targeting of unlabeled nanoparticles
- Unloaded nanoparticles are not overtly toxic to cochlear structure or function.
- Although polylysine is claimed to be a suitable tool for gene delivery, in our experiment transfection was unsuccessful.
- Liposome and polymersome NPs loaded with neurotoxic drug are capable to deliver it inside cochlea in sufficient amount to produce an effect detectable functionally and morphologically.

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8. LIST OF OWN PUBLICATIONS

Publications related to the thesis

1. **Chumak T**, Bohuslavová R, Macová I, Dodd N, Buckiová D, Fritzsch B, Syka J, Pavlínková G: Deterioration of the Medial Olivocochlear Efferent System Accelerates Age-Related Hearing Loss in Pax2-Isl1 Transgenic Mice. *Mol. Neurobiol.*, 2016; 53(4): 2368-2383
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2. **Chumak T**, Rüttiger L, Lee SC, Campanelli D, Zuccotti A, Singer W, Popelář J, Gutsche K, Geisler HS, Schraven SP, Jaumann M, Panford-Walsh R, Hu J, Schimmang T, Zimmermann U, Syka J, Knipper M: BDNF in Lower Brain Parts Modifies Auditory Fiber Activity to Gain Fidelity but Increases the Risk for Generation of Central Noise After Injury. *Mol. Neurobiol.*, 2015 [Epub ahead of print]
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Bohuslavová R, Dodd N, Macová I, **Chumak T**, Horak M, Syka J, Fritzsch B, Pavlínková G: Pax2-Islet1 Transgenic Mice Are Hyperactive and Have Altered Cerebellar Foliation.
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