Investigation of drug therapy in sensorineural hearing losses

PhD Thesis

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Budapest
2014
**Introduction**

**General Considerations**

Several sensory organs support adequate orientation in everyday life. One of the most important ones is hearing, which is a perception that results from a series of extremely complex mechanical and electrophysiological processes. During our life several external and internal stress factors impact our hearing organ that could lead to damages in this extremely finely regulated system. These stress factors accumulate due to increasing life expectancy and impact us for a longer and longer period of time. In most cases similar pathophysiological mechanisms are found in the background of these processes, like e.g. noise load, increasing perfusion disturbances, interactions emerging as a result of the effects and side effects of pharmaceutical products, and treatment procedures developed for various diseases, as well as several diseases evoked by a pathogen impairing the sensorineural part of hearing. A fundamental aspect of these processes is glutamate mediated excitotoxicity developing as a result of various noxae, as well as imbalances of redox processes that lead to the development of reactive free radicals and consequently to severe cell injury. (Bondy and Lee 1993; Reynolds and Hastings 1995).

From among the endogenous protective mechanisms dopamine (DA) released from the neurons of the lateral olivocochlear efferent bundle (LOC) and inhibiting abnormal overactivation of primary auditory neurons exerts a protective effect and plays a significant role against excitotoxicity. It is possible to facilitate prevention of excitotoxic harmful effects both by increasing DA release and re-uptake to storages and by inhibiting DA degradation.

In view of the fact that no verified effective therapy is available for the case of acute or chronic sensorineural hearing loss (SNHL), the possibility of favorably influencing the metabolism of DA that has a protective effect against excitotoxicity arises to facilitate mitigation or potentially prevention of hearing loss.
**Rasagiline**

In clinical practice rasagiline is a selective MAO-B inhibitor administered for the treatment of Parkinson disease. It elevates the DA level by inhibiting DA degradation, and it also exerts direct neuroprotective effects. It provides protection against glutamate induced excitotoxicity, inhibits neurodegeneration, and also protects against apoptotic processes through various mechanisms.

**Protective role of DA released from the LOC**

A majority of stressful effects damaging the inner ear evokes a high amount of glutamate (Glu) release from the inner hair cells (IHC) that results in an excitotoxic impairment of the neurons in the primary auditory nerve. LOC efferents form part of the primary auditory neuron - cochlear nucleus - lateral superior olive - cohele short loop feedback circle through axodendritic synapses on peripheral terminals of primary auditory neurons, and they inhibit overactivation of auditory neurons via DA release, thus providing protection against their excitotoxic injury. DA inhibits the postsynaptic effect of Glu and protects the IHC - afferent auditory neuron synapse.

**Ototoxic side effects of aminoglycoside antibiotics**

Aminoglycoside antibiotics are indispensable pharmacetic products in case of infections caused by several types of Gram negative aerob bacteria. Members of this active agent group may provoke inner ear damages to various extent. The damages are usually irreversible, bilateral and initially exert their damaging effect at higher frequencies. Both apoptosis and necrosis play a role in the cell injury process caused by aminoglycosides. Several studies demonstrated that normal redox state disturbances, increased ROS formation, and excitotoxic injury of auditory nerve neurons play a significant role in the pathomechanism (Basile et al., 1996; Sha and Schacht, 1999; Duan et al., 2000; Poirrier et al., 2010; Huth et al., 2011).
Animal experimental model to study aminoglycoside induced hearing loss

No prolonged experiments could be performed in mice as it seemed adult animals were resistant to ototoxic side effects of aminoglycosides. The animal model developed by Wu and coworkers, however, allowed conducting the adequate studies (Wu et al., 2001). Based on measurements performed using brainstem evoked response audiometry (BERA) hearing loss developing in animals first develops at higher frequencies, then progresses towards deeper sounds. Hearing loss evoked this way in mice greatly resembles processes observed in humans, thus the model is also suitable for testing otoprotective active agents. The significant similarity observed in the pathological background of the various forms of sensorineural hearing loss raises the possibility that agents demonstrating otoprotection in the aminoglycoside model may be effective in other forms of sensorineural hearing loss, as well.

Objectives

An important reason why pharmaceutical product development for the treatment of sensorineural hearing losses of various origin has not been successful so far is our lack of comprehensive knowledge of the potential pharmaceutical targets. Another important reason most probably is the multicomponent, complex, network-like pathomechanism, the process of which cannot be interrupted by inhibiting one element of the network.

Our aim was to look for a pharmacological approach or drug molecule that effectively mitigates sensorineural hearing loss by exerting its action on several target points. We wished to study the otoprotective efficacy of inhibiting the excitotoxicity / oxidative stress / abnormal ROS level increase all considered as most important causes in the background of various forms of SNHL, as well as the targeted utilization of modulating the endogenous protective mechanism (LOC-DA).
This is why we wished to study the protective effect of the DA neurotransmission stimulator, antioxidant and neuroprotective rasagiline against SNHL, and to analyze the possibility and the mechanism of increasing protective DA release from LOC efferents using rasagiline.

Main objectives of my experimental work were the following:

1.) To set up both an objective audiometric method that can be used in mice, and the mouse model of hearing loss evoked by aminoglycoside antibiotics in Hungary, which could help routine examination of the protective effect of molecules with suspected otoprotective effect against sensorineural hearing loss.

2.) To determine whether rasagiline influences normal, intact hearing, and whether it has any otoprotective effect in a type of SNHL evoked by aminoglycoside antibiotics; and if so, at what dose and frequencies.

3.) To work out the measurement of DA released from the LOC efferents on mice specimen. Literature shows that DA release was measured only from guinea pig's coelia previously.

4.) To study in vitro whether rasagiline showing otoprotective effect increases DA release in mouse cochlea and if so, what the dose-effect relationship is; to explore the role of the voltage gated Sodium- and Calcium channels (VGSC and VGCC retrospectively) and that of DA re-uptake in physiological and rasagiline induced cohelear DA release.

Methods

In vivo measurement of the rasagiline effect in the aminoglycoside-induced ototoxicity model

Selections of the mouse strain and the type and concentration of aminoglycoside antibiotics were based on literature data (Wu et al., 2001). Our preliminary experiments testing different mouse strains,
aminoglycoside antibiotics and concentrations of kanamycin, confirmed that the most pronounced and reliable aminoglycoside-induced hearing loss, suitable for testing otoprotection, could be produced in BALB/c mice by administering kanamycin in an 800 mg/kg s.c. dose.

First, a set of experiments exploring also the dynamics of the effect of kanamycin and rasagiline was carried out. Mice were assigned to one of the following four experimental groups:
1. control group (physiological saline),
2. Kanamycin 800 mg/kg,
3. Rasagiline 3 mg/kg,
4. Kanamycin 800 mg/kg + rasagiline 3 mg/kg.

Auditory thresholds were determined in both ears from the ABRs. Thresholds were taken from each animal prior to the start of the drug treatments on the 1st week (startup threshold), 2 weeks after the start of drug treatment, and then weekly up to 5 weeks (5 measurements in sum). The threshold shift gives the difference of an actual threshold value and the threshold measured in the same mouse before any treatment (start-up threshold).

Based on the time-dependent threshold changes measured in the first set of experiments, a 3-week-long experiment was performed, and two additional doses of rasagiline were tested
1. Control,
2. Kanamycin, 800 mg/kg,
3. Kanamycin, 800 mg/kg + rasagiline 0.5 mg/kg.
4. Kanamycin 800 mg/kg + rasagiline 6 mg/kg.

The ABR was measured in the left ear exclusively. The experiment was carried out with larger sample sizes.
In vivo recordings of ABRs.

Mice were anesthetized by i.p. injections of ketamine (100 mg/kg) and xylazine (10 mg/kg). We generated click and tone burst stimuli in a closed acoustic system, in which we connected the electrostatic speaker through a plastic tube into the external ear canal of the animals. ABR waives were captured with subcutan needle electrodes. Evoked responses were amplified, and 800 sweeps were averaged in real time. Acoustic intensity was increased in 10 dB steps from 0 dB to 80 dB in the click stimulation mode. To obtain auditory thresholds at different frequencies, the sound intensity of the tone burst stimuli were attenuated in 10-dB steps. Threshold was defined as the lowest intensity at which a visible ABR wave was seen.

Statistical analysis

Threshold data of both studies were analyzed in a linear mixed statistical model (taking into account that we measured every animal at each frequency, we used the "nlme" package of the R statistical software). All factors and potential interactions were evaluated with the cut-off for inclusion of P < 0.05. The Tukey–Kramer corrections of p-values and confidence limits were applied.

In vitro measurement of DA released from LOC terminals

After decapitation of the mice the bony tympanic bulla was removed. The bony capsule of the cochlea was removed under stereomicroscopic guidance, the stria vascularis was stripped, and the cochlea was fractured at the basis of the modiolus. Our preparation contained the spiral ganglion, the afferent auditory fibers, axons and axon terminals of the efferent bundles, as well as inner and external hair cells.
All experiments were carried out in a perilymph-like solution, and the solution was continuously gassed with 100% O₂. The cochleae were incubated with 0.2 µM [³H]dopamine for 35 minutes, placed in a microvolume plexi chamber (3 cochleae per chamber) and superfused them with a perilymph-like solution (0.3 ml/min). After an hour of pre-perfusion the outflow was collected in 3-min fractions. Electrical field stimulation, evoking action potentials in the LOC efferents, was applied for one collection period (3 min) at the 3rd (S₁) and 13th (S₂) fractions. Rasagiline was added to the perfusion solution at the beginning of the 8th fraction (21th min) and was maintained till the end of the experiment. Perfusion of CdCl₂ and TTX was started 6 min earlier (from the 15th min). The application of nomifensine and a decrease in the temperature to 17 °C were started in the 45th min of pre-perfusion and were maintained till the end of the experiment.

**Data and statistical analysis**

To best describe the release of DA during one collecting period, the fractional release (FR) of the tritium outflow was determined as the percentage of the total radioactivity present in the tissue at the time of sample collection. The FR due to the field stimulations (S₁ and S₂) was calculated by the area under-the-curve, i.e., by subtracting the mean of the basal release, determined from FR values before and after the stimulation, from the total FR during the electrical stimulation. The effects of drugs on the field stimulation-evoked [³H]DA release were expressed by the calculated ratio of FR S₂ over FR S₁ (FRS₂/FRS₁). Data are expressed as the means ± S.E.M. We used variance analysis (ANOVA) and Tukey’s Honest Significant Difference multiple comparisons to compare the various treatment groups (R 14.1 software). Applied levels of significance: *p < 0.05; **p < 0.01 and ***p < 0.001.
Results

*In vivo effect of rasagiline on aminoglycoside induced hearing loss*

The effect of rasagiline on SNHL was tested in the kanamycin-induced hearing loss model in mice (Wu et al., 2001). Auditory thresholds were measured at four different frequencies (4kHz, 8kHz, 16kHz and 24kHz). The shift of the auditory thresholds was highly significant (p<0.001) at higher frequencies (16 and 24 kHz), while the ototoxic effect was less pronounced at lower frequencies (not even significant at 8 kHz).

Rasagiline mitigated the kanamycin-evoked hearing impairment by 0.5–8 and 8–19 dB when applied in 0.5 and 6 mg/kg dose, respectively. The most pronounced protection appeared at 16 kHz.

*Measurement of DA release from LOC efferents in mice cochleae. The role of Ca$^{2+}$ and Na$^+$ channels, and DA uptake, in the release of DA from the cochlea*

Under control circumstances, the electrical field stimulation of isolated cochleae produced the stable and reproducible release of DA. Omission of Ca$^{2+}$ from the 24th minute until the end of the experiment reduced the evoked release by 60%. Surprisingly, the resting release increased during the perfusion of the Ca$^{2+}$-free solution, which might be partly attributable to a compensatory Ca$^{2+}$ outflow from the bone.

Perfusion of the non-selective voltage-sensitive Ca$^{2+}$ channel blocker Cd$^{2+}$ (100 µM) caused a 41% inhibition in the second electrical stimulation-evoked DA release, while The voltagesensitive Na$^+$ channel blocker TTX (1 µM) decreased the evoked release by 88%.

The DA uptake inhibitor nomifensine dose-dependently increased the electrical stimulation-evoked release of DA in a concentration range of 10–100 µM. Nomifensine did not change the basal DA release.
Rasagiline enhanced the electrical field stimulation evoked release of DA from isolated mouse cochlea preparations. The effect was concentration dependent and reached a plateau at 100 µM. The resting release of DA was not affected in any concentration applied. To explore the possible molecular mechanism of the action underlying the effect of rasagiline on the DA release evoked by the field stimulation, we tested the effect of 100 µM rasagiline during the inhibition of VGCCs and VGSCs. In the presence of Cd²⁺ (100 µM) and TTX (1 µM), respectively, the stimulation-evoked release was completely inhibited, providing evidence that the release of DA was due to axonal activity and Ca²⁺ influx. Under these conditions, rasagiline failed to increase the release of DA.

In order to test whether the uptake inhibition is a possible mechanism in rasagiline action on cochlear DA release, we measured the effect of rasagiline in the presence of uptake inhibition by low temperature or nomifensine. During inhibition of DA uptake by either nomifensine (10 µM) or low temperature (17 °C), the potentiating effect of rasagiline on electric stimulation induced DA release was hampered significantly. These findings indicate that rasagiline inhibits DA uptake in isolated in vitro cochlea preparations, thereby potentiating DA’s release from the LOC in response to axonal activity.

Conclusions

In my PhD thesis related work I studied the otoprotective effect of rasagiline - a pharmaceutical product already introduced in the therapy of Parkinson disease - in sensorineural hearing loss induced by aminoglycoside antibiotics and the potential influence of rasagiline on the endogenous DA-erg protective mechanism playing a potential role
in this otoprotective effect, as well as its mechanism. The following conclusions can be drawn based on our associated results:

1.) During our work we worked out and set (and filled up a gap in Hungary):
- the method of measuring the brainstem evoked response potential (ABR) in vivo in mice, performed at various frequencies (objective audiometry)
- the mouse model of hearing loss induced by aminoglycoside antibiotics (kanamycin model), which is a type of sensorineural hearing loss.
When combined with the objective audiometric method, the model is suitable for testing the otoprotective effect of various substances, i.e. drug target molecules in a frequency dependent manner.

2.) We determined that rasagiline decreased sensorineural hearing loss induced by kanamycin in mice (hearing threshold shift) in a dose dependent manner (0,5 - 6 mg/kg, s.c.). This effect proved to be most prominent at 16 kHz, which is right in the range of the hearing sensitivity optimum of the mouse. Rasagiline itself did not influence normal hearing even at highest doses applied.

3.) We set and used for the first time in literature the measurement of DA from LOC efferents. This allowed us to perform both our in vivo and in vitro experiments in the same species of experimental animals.

4.) We demonstrated that rasagiline increases electric stimulation induced DA release in mouse cochlea preparations in a dose dependent manner (10-300 µM). By inhibiting voltage-gated Ca²⁺- and Na⁺-channels, and by inhibiting DA re-uptake we verified that rasagiline induced potentiation of action potential evoked DA release by inhibiting the transmitter re-uptake process. DA released from the LOC efferents is considered to have a protective effect against sensorineural...
hearing loss. The action potential dependent DA release increasing effect of rasagiline may contribute to the protective effect against hearing loss induced by kanamycin, an aminoglycoside antibiotic.

**List of the author's own publications**

*Publications used in the PhD thesis*


*Other publications related to the Phd thesis*


*Other publications not related to the Phd thesis*


