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PROTECTIVE EFFECT OF RASAGILINE IN AMINOGLYCOSIDE OTOTOXICITY

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Abstract—Sensorineural hearing losses (SNHLs; e.g., ototoxicant- and noise-induced hearing loss or presbycusis) are among the most frequent sensory deficits, but they lack effective drug therapies. The majority of recent therapeutic approaches focused on the trials of antioxidants and reactive oxygen species (ROS) scavengers in SNHLs. The rationale for these studies was the prominent role of disturbed redox homeostasis and the consequent ROS elevation. Although the antioxidant therapies in several animal studies seemed to be promising, clinical trials have failed to fulfill expectations. We investigated the potential of rasagiline, an FDA-approved monomanine oxidase type B inhibitor (MAO-B) inhibitor type anti-parkinsonian drug, as an otoprotectant. We showed a dose-dependent alleviation of the kanamycin-induced threshold shifts measured by auditory brainstem response (ABR) in an ototoxicant aminoglycoside antibiotic-based hearing loss model in mice. This effect proved to be statistically significant at a 6-mg/kg (s.c.) dose. The most prominent effect appeared at 16 kHz, which is the hearing sensitivity optimum for mice. The neuroprotective, antiapoptotic and antioxidant effects of rasagiline in animal models, all targeting a specific mechanism of aminoglycoside injury, may explain this otoprotection. The dopaminergic neurotransmission enhancer effect of rasagiline might also contribute to the protection. Dopamine (DA), released from lateral olivocochlear (LOC) fibers, was shown to exert a protective action against excitotoxicity, a pathological factor in the aminoglycoside-induced SNHL. We have shown that rasagiline enhanced the electric stimulation-evoked release of DA from an acute mouse cochlea preparation in a dose-dependent manner. Using inhibitors of voltage-gated Na^+-, Ca^{2+} channels and DA transporters, we revealed that rasagiline potentiated the action potential-evoked release of DA by inhibiting the reuptake. The complex, multifactorial pathomechanism of SNHLs most likely requires drugs acting on multiple targets for effective therapy. Rasagiline, with its multi-target action and favorable adverse effects profile, might be a good candidate for a clinical trial testing the otoprotective indication.

Key words: sensorineural hearing loss, kanamycin, auditory brainstem response, lateral olivocochlear efferents, dopamine, rasagiline.

INTRODUCTION

SNHLs and the lack of their effective pharmacological treatment

Hearing loss (HL) is the most frequent human sensory deficit. In contrast to its conductive forms, there is no specific drug therapy for sensorineural hearing losses (SNHLs; e.g., ototoxicant drug- and noise-induced HL or presbycusis), except for symptomatic approaches with moderate efficacy. One of the main reasons for the absence of specific tools to prevent and cure SNHLs is the insufficient knowledge of the basic molecular mechanisms of normal and impaired adult hearing and of the endogenous protective factors.

A consensus is evolving that the imbalance of the redox homeostasis and the consequent increase in reactive oxygen and nitrogen species (ROS, RNS) is a common pathological basis in all the acquired forms of SNHLs (Mukherjea et al., 2011), as well as in the many inherited forms (Noben-Trauth and Johnson, 2009). This knowledge initiated testing of different antioxidants and ROS scavengers (Tabuchi et al., 2010; Mukherjea et al., 2011) for the protection of the cells of the organ of Corti and auditory neurons, which are primary targets in SNHLs.

Rasagiline

Rasagiline, a selective propargylamine inhibitor of monoamine oxidase inhibitor (MAO) type B, has been applied to Parkinson's disease in clinical practice (Finberg, 2010). In addition to selectively inhibiting the

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Abbreviations: ABR, auditory brainstem response; ANOVA, analysis of variance; DA, dopamine; EM, electron microscopy; FR, fractional release; Glu, glutamate; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; HL, hearing loss; IHCs, inner hair cells; LOC, lateral olivocochlear; MAO, monoamine oxidase inhibitor; RNS, reactive nitrogen species; ROS, reactive oxygen species; SNHLs, sensorineural hearing losses; VGCC, voltage-gated calcium channel; VGSC, voltage-gated sodium channel.

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dopamine (DA) metabolizing enzyme MAO-B, it also has a cell protective action. It has been shown to protect against neural degeneration (Huang et al., 1999; Speiser et al., 1999; Youdim et al., 2006), oxidative damage and apoptosis (Tabakman et al., 2004; Siderowf and Stern, 2006). These protective effects provide a rational to test its effect in different forms of SNHLs. Furthermore, as an enhancer of DAergic neurotransmission (Weinreb et al., 2010) in the central nervous system, it may also potentiate the release of DA from the lateral olivocochlear (LOC) efferents, which is considered to be a protective feedback pathway of the cochlea (Pujol et al., 1993; Pujol, 1994; Lendvai et al., 2011; Maison et al., 2013).

The cochleoprotective role of DA released from LOC efferent fibers

It has been shown that the excessive release of glutamate (Glu) from inner hair cells (IHCs) in noise-induced HL, presbycusis, cochlear ischemia or aminoglycoside-induced ototoxicity results in the excitotoxic damage of the primary auditory neurons (Duan et al., 2000; Ruel et al., 2007; Tabuchi et al., 2010; Bernarding et al., 2013). LOC efferents, forming axodendritic synapses with the auditory neurons, serve as the effector arm of the auditory neurons – cochlear nucleus – lateral superior olivary complex – cochlea short-loop feedback and provide protection to the auditory neurons against excitotoxicity by releasing DA. DA inhibits the postsynaptic effects of Glu and protects the IHC-afferent nerve synapse (Halmos et al., 2005, 2008; Ruel et al., 2007; Lendvai et al., 2011). Intracochlear application of the D_2/D_3 dopamine receptor agonist piribedil reduced the characteristic electrophysiological and structural changes evoked by acoustic trauma and ischemia (Pujol et al., 1993; d'Aldin et al., 1995a,b; Gil-Loyzaga, 1995), and D_1, D_2 receptor agonists were shown to inhibit the NMDA- and AMPA-induced firing of the primary afferent nerve (Oestreicher et al., 1997). Although drugs acting on the DAergic system have not yet been tested thoroughly, theoretically, any drug able to boost the function of this system could hold preventive or curative promises for SNHLs (Halmos et al., 2005; Lendvai et al., 2011).

Aminoglycoside ototoxicity and its use as a SNHL model

Aminoglycoside antibiotics, which still need to be used in the treatment of certain serious infections caused by aerobic gram-negative bacteria, can induce irreversible HL (Xie et al., 2011). Hair cells, especially the outer hair cells and the IHC ribbon synapse, together with the auditory neurons, are very vulnerable to the administration of aminoglycosides (Ylikoski et al., 1974; Dodson, 1997; Duan et al., 2000; Maruyama et al., 2008; Fransson et al., 2010; Liu et al., 2013). The pivotal role of normal redox state disturbances, generation of ROS and excitotoxic damage of the auditory neurons in the pathomechanism has been shown in several studies (Basile et al., 1996; Sha and Schacht, 1999; Duan et al., 2000; Poirrier et al., 2010; Huth et al., 2011). This serious side effect is the basis of a well-established animal model used in hearing research (Wu et al., 2001). As the aminoglycoside induced HL involves oxidative stress, ROS generation and excitotoxic neuronal damage, we tested the effect of rasagiline in the kanamycin-induced hearing loss model.

EXPERIMENTAL PROCEDURES

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

General experimental paradigm of kanamycin-induced ototoxicity and application of rasagiline. All animal care and experimental procedures were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Procedures were approved by the Animal Use Committee of the Institute of Experimental Medicine, Hungarian Academy of Sciences. Selections of the mouse strain and the type and concentration of aminoglycoside antibiotic were based on data from the literature (Wu et al., 2001). Our preliminary experiments (data not shown) 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. Male BALB/c mice, age 4 weeks, were purchased from Charles River, Germany.

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 (physiological saline), (2) Kanamycin, 800 mg/kg, (3) Rasagiline, 3 mg/kg, and (4) Kanamycin, 800 mg/kg + Rasagiline, 3 mg/kg. Treatment groups contained eight mice each. (One mouse in group 4 died during the auditory brainstem response (ABR) measurement under anesthesia.) Kanamycin sulfate (USB Corporation, Cleveland, OH) was injected s.c. twice daily (8–9 a.m. and 6–7 p.m.) for 2 weeks. The first dose of the antibiotic was administered on the day of the first ABR measurement (6–7 p.m.) after all the measurements had been performed. Doses of rasagiline mesylate (3 mg/kg, s.c.; TEVA) were given once daily at the same time as the morning dose of kanamycin, but the injections were separate. In this way, the first dose of rasagiline was delivered 14 h after the first kanamycin dose. Rasagiline treatments lasted 5 weeks. Mice in the Control group were injected s.c. by an equivalent amount of physiological saline. In the kanamycin treatment group, after the 2nd week, the kanamycin injections were replaced by injections of physiological saline till the end of the 5th week.

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 (start-up 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 other 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 (n = 20 in each treatment group), which were calculated based on the first set of experiments. Two mice in the Control group, one in the kanamycin group and two in the kanamycin + rasagiline, 6 mg/kg treatment group died during the ABR measurement under anesthesia. The kanamycin dose and the treatment protocols were the same as before.

In vivo recordings of ABRs. Mice were anesthetized by i.p. injections of ketamine (100 mg/kg) and xylazine (10 mg/kg). Body temperature was maintained by a feedback-controlled heating pad. The auditory thresholds were determined by an ABR workstation (Tucker-Davis Technologies, Alachua, FL). Click (0.4-ms duration) and tone burst (3-ms duration, 0.2-ms rise/decay) stimuli were generated by the SigGen software package and delivered in a closed acoustic system to the external auditory meatus through a plastic tube connected to an EC1 electrostatic speaker. ABRs were recorded with subdermal needle electrodes as the potential difference between an electrode on the vertex and an electrode behind the left or right pinna. The rear leg served as a ground. The evoked responses were amplified, and 800 sweeps were averaged in real time. The intensity was increased in 10-dB steps from 0 to 80-dB in 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 in both studies were analyzed using a linear mixed statistical model (to take into account the fact that every animal was measured on each frequency, the ''nlme'' package of the R statistical program was used (Pinheiro et al., 2013; R Core Team, 2013), followed by pairwise comparisons of the treatments, calculated using contrasts (Warnes, 2011). Left and right ear values were averaged in the first set of experiments. Model effects were tested together based on their F values. 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 release from the LOC terminals

Measuring the release of DA from mouse and guinea-pig cochlea. CD-1 male mice, weighing 20–35 g, were used. Procedures were approved by the Animal Use Committee of the Institute of Experimental Medicine, Hungarian Academy of Sciences. We used the microvolume superfusion method as described earlier (Gáborján et al., 1999; Halmos et al., 2005, 2008). Briefly, the bulla tympani was opened. 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 ganglion spirale, the afferent auditory fibers, the axons and axon terminals of the efferent bundles and both the inner and outer hair cells. All experiments were carried out in a perilymph-like solution (Ikeda et al., 1991), which contained 150 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2.75 mM HEPES and 2.25 mM Tris at 37 °C. The pH was adjusted to 7.4. The osmolarity was set by D-glucose, and the solution was gassed continuously with 100% O_2.

The cochleae were incubated with 0.2 μM [^{3}H]dopamine (specific activity: 31.0–59.3 Ci/mmol; [7,8-^{3}H]DA, Amersham, UK) for 35 min, placed in a microvolume plexi chamber (three cochleae per chamber) and then superfused with a perilymph-like solution (3 ml/min). After one hour pre-perfusion, the outflow was collected in 3-min fractions. The released radioactivity, indicating the release of DA from the LOC terminals, was determined by assaying 500 μl aliquots of each sample with a liquid scintillation counter (Packard Tri-Carb 1900TR). After collecting the samples for 57 min (19 fractions), each cochlea was transferred from the microchambers to 500 μl of 10% trichloroacetic acid for one day; 100 μl was then used to measure the tissue content of the radioactivity. Earlier HPLC measurements in our laboratory showed that 91–95% of the released radioactivity was attributable to [^{3}H]DA and its metabolites DOPAC and HVA (Gáborján and Vizi, 1999).

Electrical field stimulation, evoking action potentials in the LOC efferents, was applied for one collection period (3 min) at 30-V, 5-Hz and 0.5-ms impulse duration at the 3rd (S_1) and 13th (S_2) fractions. The pulses were delivered by a Grass S88 stimulator (West Warwick, USA) through platinum electrodes at the top and bottom of the tissue chamber. 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 $CdCl_2$ 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.

In addition to the reversibility and reproducibility of DA release and its inhibition by voltage-gated sodium channel (VGSC) or voltage-gated calcium channel (VGCC) blockade (indications of neuronal exocytosis; see Gáborján and Vizi, 1999; Gáborján et al., 1999; Halmos et al., 2008), the viability of the cochlear preparation was also shown by light- and electron microscopy (EM) performed immediately before and after the experiments (Halmos et al., 2008).

Data analysis and statistics. 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 (S_1 and S_2) 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 (Halmos et al., 2000, 2005). The effects of drugs on the field stimulation-evoked [^{3}H]DA release were expressed by the calculated ratio of FR S_2 over FR S_1 (FRS_2/FRS_1). Data are expressed as the means ± SEM. Analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference method for multiple comparisons was used to compare the treatment groups with the R 14.1 program. Levels of significance were as follows: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

RESULTS

In vivo effect of rasagiline on aminoglycoside-induced hearing impairment

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.

First, a five-week-long study was started with eight mice in each treatment group (Fig. 1) to explore the time dependency of the threshold changes. Kanamycin (800 mg/kg, s.c.), administered for 2 weeks twice daily impaired the hearing of BALB/c mice. 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, see the legend of Fig. 1). After 3 weeks, a plateau in impairment was reached (Fig. 1). Administration of rasagiline showed a clear tendency of attenuation of the kanamycin-induced threshold elevation. This is clearly seen at all four frequencies at any time point measured, although the difference was not statistically significant (Fig. 1). Contrary, the trace of rasagiline administration alone (3 mg/kg) was sometimes below, sometimes above the control trace (physiological saline) at all four frequencies during the 5-week-long experiment. This is in accordance with the lack of significant effect of rasagiline on the 'control' threshold (Fig. 1).

The kanamycin-induced hearing loss developed thoroughly up to the 3rd week, and the influence of rasagiline on kanamycin action did not change during the 5 weeks. Therefore, in a second set of experiments, we tested the effect of rasagiline on threshold shifts in the 3rd week at 0.5 and 6 mg/kg (s.c.) doses. Administration of kanamycin caused a significant shift in the auditory thresholds both in click ($p < 0.01$) and tone burst stimulation modes (4 kHz, $p < 0.05$; 8 kHz, $p < 0.001$; 16 kHz, $p < 0.001$; 24 kHz, $p < 0.001$). The effect was more robust at the higher frequencies (Fig. 2). 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 dose-dependency of the rasagiline effect was more prominent when its action in 3 mg/kg dose was included in the plotting (Fig. 2). The most pronounced protection appeared at 16 kHz (Fig. 2).

We showed in a separate experiment that rasagiline alone did not influence significantly the auditory thresholds during the 3-week-long treatment even in the highest dose (6 mg/kg). The estimated overall difference was 0.24 ± 0.928 dB ($p = 0.798$, $n = 7$).

Effect and mode of action of rasagiline on the release of DA from mouse cochlea

Rasagiline enhanced the electrical field stimulation-evoked release of DA from isolated mouse cochlea preparations (Fig. 3). The effect was concentration-dependent and reached a plateau at 100 μM (Fig. 3, inset). The resting release of DA was not affected in any concentration applied (Fig. 3).

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 Cd^{2+} (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 Ca^{2+} influx. Under these conditions, rasagiline failed to increase the release of DA (Fig. 4).

Blocking the reuptake of DA into the nerve terminals is a known way of potentiation of DAergic neurotransmission. 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. Cooling down the temperature to 17 °C before S_2, but after S_1, approximately doubled the FRS_2/FRS_1 ratio (2.52 ± 0.4, $n = 4$), confirming its efficacy in inhibition of the uptake, similar to what we have shown in brain slices (Vizi, 1998; Vizi et al., 2004). The inhibitory effect of 10 μM nomifensine on mouse cochlear DA reuptake has already been demonstrated in our previous work (Halmos et al., 2008). During inhibition of DA uptake by either nomifensine (10 μM) or low temperature (17 °C), the potentiating effect of rasagiline 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 (Fig. 5).

DISCUSSION

Current therapeutic regimen and potential new drugs in SNHLs

Contrary to the conductive HLs, there are no specific pharmaceuticals for the sensorineural forms in the treatment of hearing deficits. Various hearing aids and cochlear implants have been proven to be effective therapies in appropriate clinical cases; however, a specific drug therapy is still missing. In current clinical

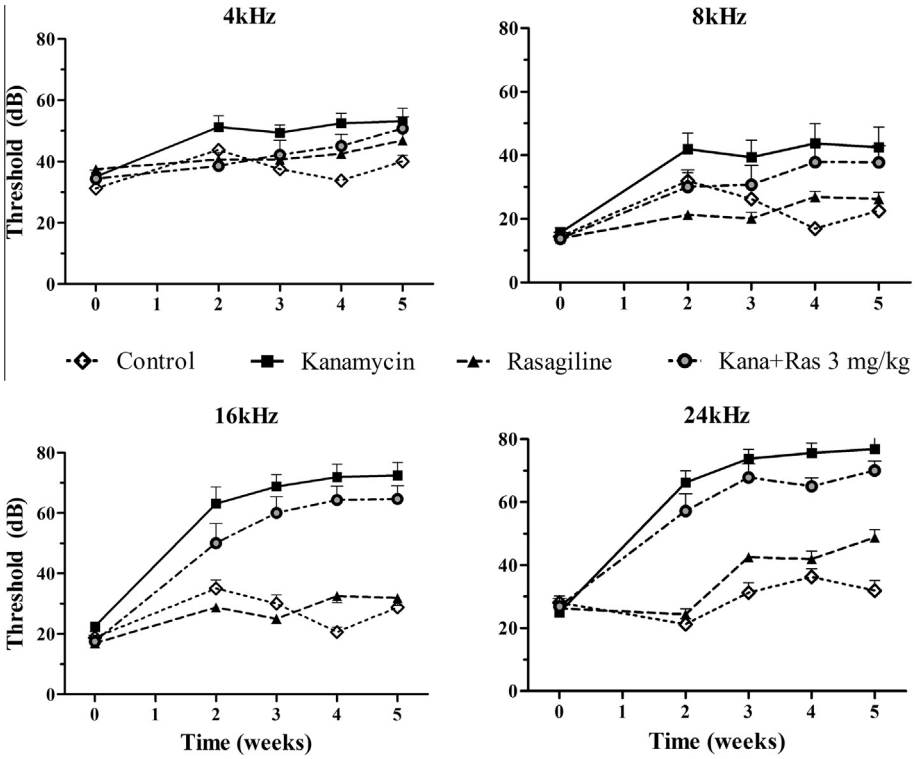

Fig. 1. 5-Week-long follow-up of *in vivo* rasagiline effect in an aminoglycoside-induced SNHL model in mice. Kanamycin (800 mg/kg, s.c., twice daily) was administered for 2 weeks, and it induced an elevation in hearing thresholds, especially at higher frequencies (compared to Control; p values were 0.017, 0.066, < 0.001 and < 0.001 at 4, 8, 16 and 24 kHz, respectively). Rasagiline treatments (3 mg/kg, s.c., once daily) were started 14 h after the first dose of kanamycin, and they lasted 5 weeks. Although rasagiline showed a tendency to decrease the kanamycin-induced threshold elevation at all measured time points and frequencies, these effects were not statistically significant. Mice in the Control group received physiological saline s.c. twice daily for 5 weeks. The effect of rasagiline alone did not differ from the Control. ABRs were recorded in BALB/c mice at four frequencies, as described in the Methods. Data are the mean ± SEM; n = 8, except in Kanamycin + Rasagiline (n = 7). A linear mixed model, followed by pairwise comparisons, was used for the statistical analysis (see Methods).

practice, steroids, thrombolytics, vasodilators and nootropic drugs are administered.

Potentially new therapeutic approaches in SNHLs based on animal studies, including antioxidants and ROS/RNS scavengers, apoptosis inhibitors, neuroprotective compounds, anti-inflammatory drugs (such as steroids, aspirin or TNF-α inhibitors), neurotrophic factors or different gene therapeutic approaches (Atar and Avraham, 2005; Rybak and Whitworth, 2005; Maruyama et al., 2008; Fransson et al., 2010; Mukherjea et al., 2011; Rudnicki and Avraham, 2012; Kohrman and Raphael, 2013), have been applied, but they have failed to fulfil expectations. Although several animal studies have shown significant effects of antioxidant therapy, clinical studies have not yet reached a conclusive result (Tabuchi et al., 2010; Mukherjea et al., 2011). Therefore, we considered it relevant to test whether rasagiline, a registered drug with a complex neuroprotective, antiapoptotic and antioxidant effect, possessed any otoprotective action.

Testing the potential otoprotective action of rasagiline *in vivo* in an aminoglycoside-induced form of SNHL

Compounds showing a potential to prevent or cure hearing impairments in *in vitro* experiments need reliable *in vivo* testing to support their applicability in therapy. The otoprotective effects of a compound can be tested *in vivo* by measuring its effect on an auditory threshold elevated by a pathological insult. The use of aminoglycoside antibiotics, which have a well-known ototoxic side effect in medical practice, is widely accepted for evoking hearing impairment and testing potentially otoprotective compounds (Basile et al., 1996; Song et al., 1997; Duan et al., 2000; Nekrassov and Sitges, 2000; Wu et al., 2001). The mechanism of aminoglycosides-induced toxicity involves excitotoxicity (Basile et al., 1996; Duan et al., 2000) and the pivotal role of oxidative stress and ROS (Basile et al., 1996; Sha and Schacht, 1999; Poirrier et al., 2010; Huth et al., 2011). To determine the threshold *in vivo*, the recording of the ABR is a method of choice to obtain objective audiograms. The mouse is a well-established experimental model for human audition as it possesses a similar cochlear anatomy, physiology and pattern of ototoxicity-related hearing loss (Wu et al., 2001; Fernandez et al., 2010).

Based on the literature (Wu et al., 2001) and preliminary experiments, we used 800 mg/kg kanamycin (s.c.) for 2 weeks in our aminoglycoside-induced SNHL model to test the otoprotective potential of rasagiline *in vivo*. The kanamycin-evoked shift in the auditory thresholds was more pronounced at higher frequencies,

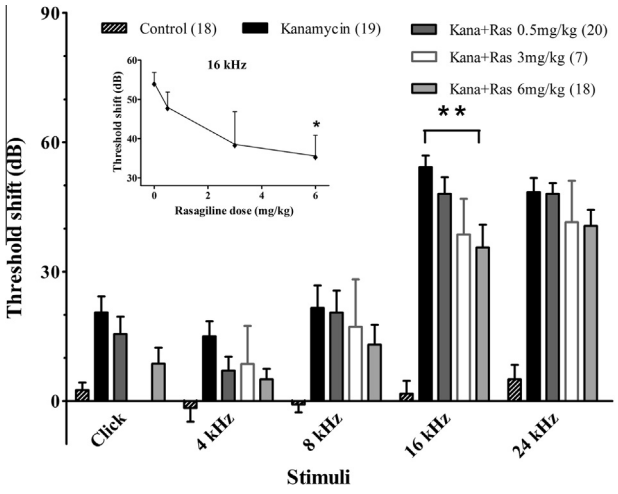

Fig. 2. Rasagiline attenuated the kanamycin-induced hearing impairment in BALB/c mice. ABRs were recorded right before drug administration (start-up threshold) and 3 weeks later as described in the Methods. Threshold shifts were calculated as the difference between the two measurements. Kanamycin (800 mg/kg, s.c., twice daily) was administered for 2 weeks, and it induced a significant loss of hearing in both the click and frequency selective tone burst stimulations. Rasagiline treatments (0.5 and 6 mg/kg, s.c., once daily) were started 14 h after the first dose of kanamycin and lasted till the second threshold measurement in the 3rd week. Mice in the Control group received physiological saline s.c. Respective data of the Kanamycin + Rasagiline, 3 mg/kg treatment ($n = 7$; no click measurements) were included in the figure (empty bars) to help demonstrate the dose-dependent effect of rasagiline. The inset emphasizes this dose-dependent effect at 16 kHz, which is in the highest sensitivity frequency range of hearing in mice. Data are the mean ± SEM; the number of experiments is given in parentheses. A linear mixed model, followed by pairwise comparisons, was used for the statistical analysis (see Methods; $^{**}p < 0.01$).

which was in perfect accordance with the observations of other studies in both human clinical practice and in laboratory animals. Aminoglycoside ototoxicity appears as a high-frequency SNHL (Wu et al., 2001; Guthrie, 2008). Plotting the auditory thresholds as a function of time, measured at different frequencies, demonstrated that the plateau in the effect of kanamycin was reached after 3 weeks. This result was in good agreement with prior clinical observations that the ototoxic effect of the aminoglycosides might start after the cessation of treatment, develop slowly and ultimately become irreversible (Xie et al., 2011). In our experiments, the kanamycin-induced hearing loss had a tendency to be attenuated by the concomitant application of a single dose per day of rasagiline (3 mg/kg), and this beneficial tendency was maintained at multiple frequencies during the experiments that lasted for 5 weeks. The effect of rasagiline on the auditory thresholds showed dose-dependency. The most pronounced effect was exerted at 16 kHz. This frequency is right in the range of the hearing sensitivity optimum (15–20 kHz) of the mouse (Ehret, 1976) and is the equivalent of the human 1–4 kHz optimum. With these findings, it is tempting to hypothesize that the otoprotection by rasagiline could be predominantly exerted in the frequency range most relevant to speech acquisition.

The question arises regarding the potential mechanism of the otoprotective action of rasagiline. Rasagiline, indicated for the treatment of idiopathic Parkinson's disease by the FDA, possesses neuroprotective, anti-apoptotic and antioxidant properties all in one. It upregulates the synthesis of anti-apoptotic members of the Bcl-2 family and of the neurotrophic factors BDNF and GDNF, while it

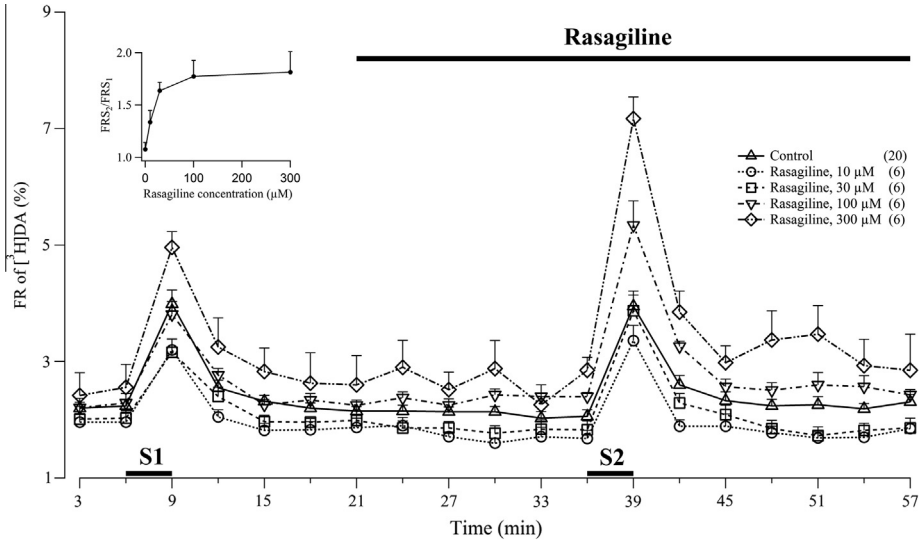

Fig. 3. Rasagiline increased the electric field stimulation-evoked release of DA in a dose-dependent manner in the mouse cochlea. Rasagiline was added to the perfusion from the 21st min and maintained till the end of the experiment (horizontal line). S1 and S2 bars show the electrical field stimulations (5 Hz, 0.5 ms, 900 shocks). Rasagiline was applied in the 10–300 μM concentration range (Ras 10, Ras 30, Ras 100 and Ras 300). The inset indicates the dose-dependent rasagiline effect on the electrical stimulation-evoked fractional release (FR) of DA, which is expressed as the FRS_2/FRS_1 value (ratio of the effect of stimulation in the presence compared to the absence of rasagiline). Data presented are means ± SEM; the number of experiments is given in parentheses.

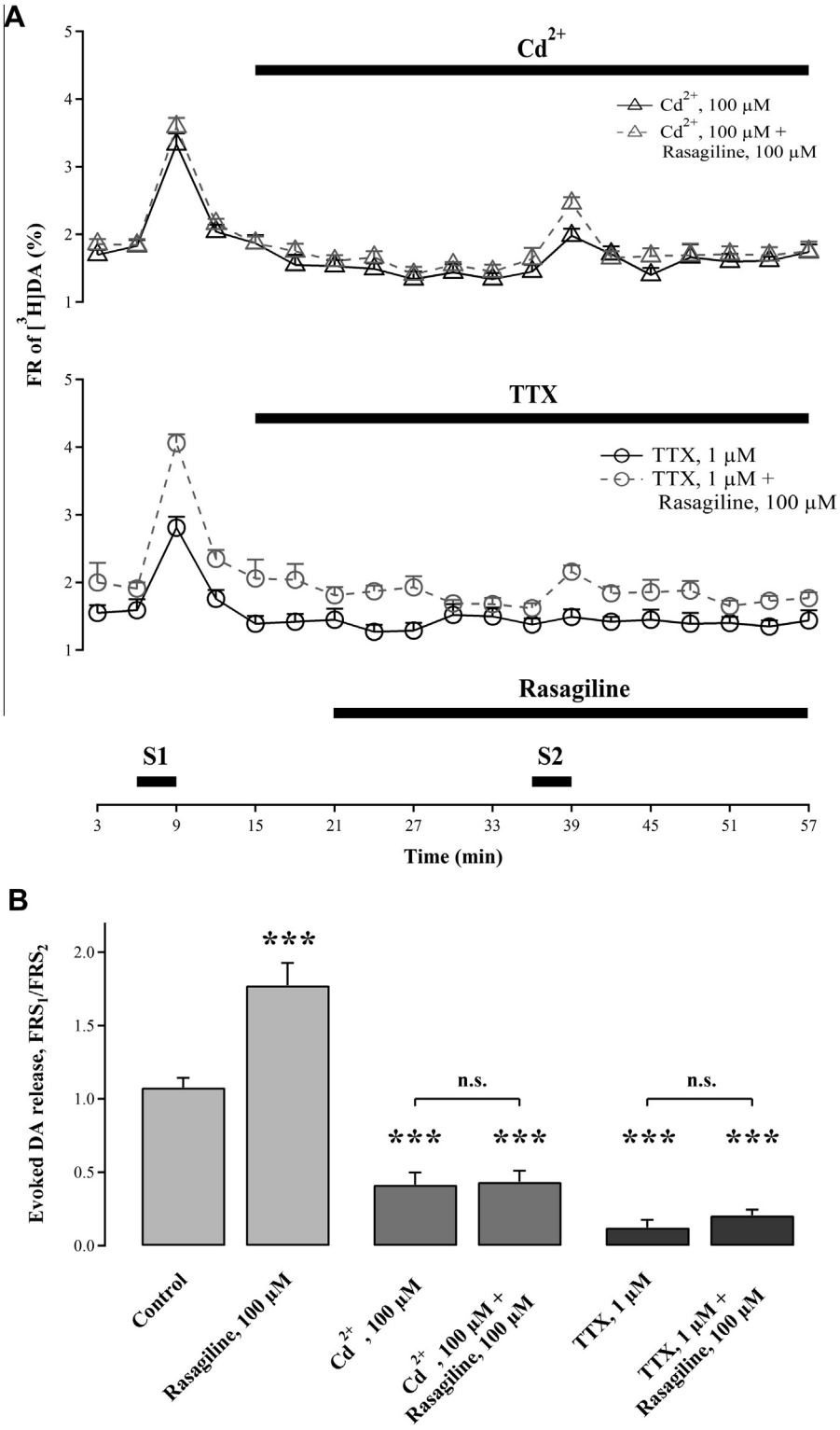

Fig. 4. Rasagiline (100 μM) did not have any effect on electrical field stimulation-evoked DA release during inhibition of VGCCs or VGSCs. A) Blocking VGCCs (Cd^{2+}, 100 μM) and VGSCs (TTX, 1 μM) hindered the effect of electric stimulation on the fractional release (FR) of DA, and the potentiating effect of rasagiline was also lost. Drug application is indicated by the respective horizontal lines. B) Summary and statistical analysis of the effect of Cd^{2+} (100 μM), TTX (1 μM), rasagiline (100 μM; Ras 100) and their combined application on electrical field stimulation-evoked DA release (FRS_2/FRS_1). The asterisks indicate that all treatment resulted in a significant effect compared to the Control. Rasagiline lost its potentiating effect in the presence of VGCC and VGSC inhibition (n.s., not significant). Data are presented as means ± SEM. The number of experiments was 6–6 in each treatment groups, except for in the Control ($n = 20$). ANOVA followed by Tukey's multiple comparisons; $^{***}p < 0.001$.

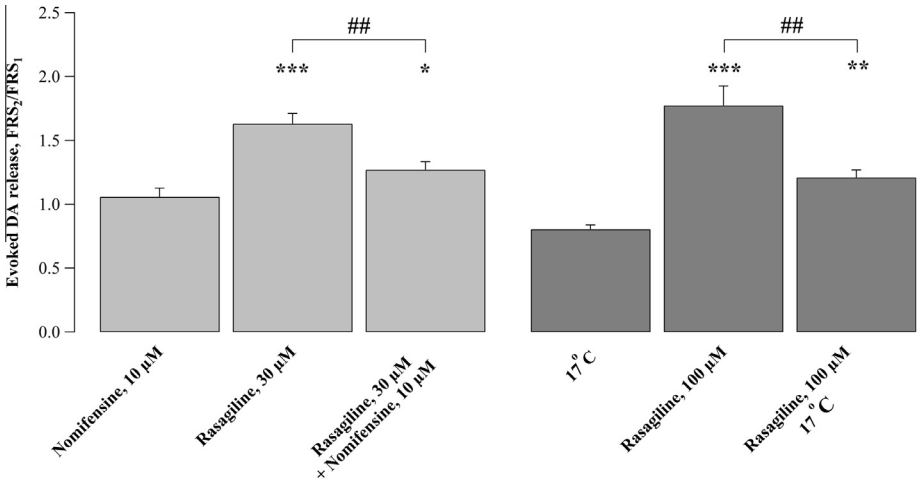

Fig. 5. Inhibition of DA uptake carriers by nomifensine or low temperature inhibited the rasagiline-induced potentiation of the electrical field stimulation-evoked DA release in the mouse cochlea. Application of nomifensine (10 μM) or cooling down the perfusion buffer to 17 °C was started 15 min before the beginning of the measurement of DA release (i.e., in the pre-perfusion) and was maintained till the end of the experiment. Rasagiline was administered before S_2, as in all the other experiments. Asterisks show the comparisons to Nomif 10 and 17 °C, respectively. Further comparisons are indicated with the hashmarks. Data are presented as means ± SEM; $n = 6$ in all treatment groups. ANOVA followed by Tukey's multiple comparisons; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Nomif 10, nomifensine, 10 μM; Ras 30, rasagiline, 30 μM; Ras 100, rasagiline, 100 μM.

downregulates the pro-apoptotic Bad and Bax proteins (Bar-Am et al., 2005; Weinreb et al., 2005; Youdim et al., 2006). It also increases antioxidant enzyme (glutathione peroxidase and catalase) activities (Kitani et al., 2000) and inhibits mPTP opening, mitochondrial swelling and cytochrome c release (Youdim et al., n.d.; Maruyama et al., 2001; Akao et al., 2002) and caspase 3 activation (Bar-Am et al., 2005). A decrease in the synaptic density of NMDA- and AMPA receptors, responsible for initiating excitotoxicity, has also been reported with rasagiline treatment (Gardoni et al., 2011). These cellular mechanisms are considered responsible for the positive *in vivo* effects of rasagiline. In addition, rasagiline has provided protection in closed head injury (Huang et al., 1999) and in experimental focal ischemia (Speiser et al., 1999), and it was also supposed to slow the progression of Parkinson's disease (Hoy and Keating, 2012). Furthermore, its neuroprotective effect has also been demonstrated in the peripheral nervous system, i.e., in the retina (Eigeldinger-Berthou et al., 2012).

These effects of rasagiline may counteract the damages that aminoglycosides cause by disturbing redox homeostasis, producing ROS (Basile et al., 1996; Sha and Schacht, 1999; Poirrier et al., 2010; Huth et al., 2011), and by impairing the function of auditory neurons via excitotoxicity (Ruel et al., 2007; Tabuchi et al., 2010) and depletion of the essential neurotrophic factors (Poirrier et al., 2010).

In addition to these well-characterized actions, rasagiline also potentiates DAergic neurotransmission in the brain (Weinreb et al., 2010), and DA has an important role in the feedback loop providing endogenous protection against SNHLs (Lendvai et al., 2011). Moreover, a recent study based on screening a library of FDA-approved pharmaceuticals consisting of 640 compounds found that DA-modulating drugs bear protective effects against ototoxic aminoglycosides and cisplatin (Vlasits et al., 2012).

Endogenous protective pathway in the cochlea – boosting effect of rasagiline on LOC terminals to increase DA release

In our *in vitro* experiments, we investigated the potential of rasagiline to enhance the release of DA from the LOC terminals. DA-containing LOC fibers compose the efferent part of the cochlea-brainstem short-loop feedback, which plays an important role in inhibiting the harmful overactivation of the auditory neurons (Pujol, 1994; Ruel et al., 2007; Lendvai et al., 2011). The overactivation of the Glu receptors is the consequence of the excessive release of Glu from hair cells, occurring in different types of SNHLs (Lendvai et al., 2011), and this excitotoxicity leads to neuronal damage, like in ischemic brain injury (Vizi et al., 2013). Considering the protective actions of cochlear DA, several target sites have appeared as candidates for increasing the endogenous DAergic protection. We have already shown that 5-$HT_{6/7}$ antagonists (Doleviczényi et al., 2008), group II mGluR ligands (Doleviczényi et al., 2005), selective NMDA receptor agonists (Halmos et al., 2008) and D_2 DA receptor antagonists (Halmos et al., 2005) provide new possibilities for the enhancement of DA release from the LOC terminals in the cochlea (Lendvai et al., 2011).

Boosting of protective LOC feedback in synchrony with the endogenous, action potential-evoked release of DA seems to be superior to simply evoking DA release from the terminal independently of the on-going axonal activity of the LOC efferents or to directly activating the postsynaptic DA receptors by the administration of appropriate receptor ligands. It can be hypothesized that rasagiline, registered as a selective MAO-B inhibitor

type anti-parkinsonian drug, would meet this requirement by inhibiting the metabolism of DA and loading up its stores (Hársing and Vizi, 1984) in the LOC terminals. Indeed, rasagiline enhanced the action potential-evoked release of DA in the cochlea in a dose-dependent manner and did not influence the resting release. The relatively higher concentrations needed for its action might be due to the predominantly MAO-A-dependent deamination of DA in mice (Garrick and Murphy, 1980; Fornai et al., 1999). At higher concentration rasagiline loses its MAO-B selectivity and inhibits MAO-A, as well (Youdim et al., 2006).

Properly functioning VGSCs and VGCCs are necessary prerequisites for the classical exocytotic release of neurotransmitters. The dependence of the potentiating effect of rasagiline on the proper functioning of VGSCs and VGCCs confirmed that its action was connected to the on-going axonal activity of the LOC efferents. In contrast to indirect acting sympathomimetics, such as amphetamine, which induce the release of DA independently of action-potential-dependent vesicular release (Fleckenstein et al., 2007).

In previous reports inhibition of DA reuptake by rasagiline was found in the central nervous system (Lamensdorf et al., 1996; Jankovic and Stacy, 2007). The role of the inhibition of DA reuptake into the LOC efferent terminals in the action of rasagiline was supported by the loss of the potentiating effect of the drug during the pre-inhibition of DA uptake by the selective DA uptake inhibitor nomifensine and by a low temperature.

Rasagiline did not enhance the resting release, being in line with the therapeutic aim of boosting the action potential based LOC feedback response without causing a continuous and endogenous protection independent elevation of DA level. Continuously enhanced level of DA could also be resulted in desensitization of DA receptors attenuating the protective effect of the firing LOC terminals.

The question arises regarding how the doses used *in vivo* relate to the concentrations used *in vitro* and whether the otoprotective concentration of rasagiline could be reached in humans. A simplified calculation, presuming 60% water content of body mass and perfect absorption of rasagiline and its distribution in body water suggested that the *in vivo* doses and the *in vitro* concentrations we used were approximately the same order of magnitude. Considering the general experience that the effective human doses are usually lower by an order of magnitude than those used in mice and that rasagiline is very well tolerated, its use in SNHLs is a reliable possibility. The preferentially MAO-B-dependent deamination of DA in human, contrary to the mouse, where MAO-A is predominant (Garrick and Murphy, 1980; Fornai et al., 1999), might further support the feasibility of a lower dose of the MAO-B inhibitor rasagiline for otoprotection in human.

An otoprotective therapy might be delivered in the form of prevention, intervention or regeneration. Theoretically, the preventive therapy holds the highest chance of curative action. In our case administration of rasagiline started 14 h after the first injection of kanamycin and still it attenuated the threshold shift significantly in 6 mg/kg dose.

Direct translation of our results to clinical application would suggest the use of rasagiline in prevention or intervention of acute trauma caused by an aminoglycoside antibiotic. However, the spectrum of possible therapeutical indications is wider, because of the strong similarities in the patomechamism of the different SNHLs (Hawkins, 1973; Poirrier et al., 2010; Mukherjea et al., 2011). Oxidative stress and the consequent elevation in ROS level is a key factor in presbycusis (Yamasoba et al., 2013), platinum-based anticancer drugs- (Kopke et al., 1997; Schacht et al., 2012) and noise exposure-induced HLs (Henderson et al., 2006), as well. Degeneration of the auditory nerves is also playing an important role in all of these SNHLs (Ylikoski et al., 1974; van Ruijven et al., 2005; Makary et al., 2011; Maison et al., 2013; Yamasoba et al., 2013). Therefore rasagiline, having antioxidant, neuroprotective and antiapoptotic effect, is predisposed for being also a promising choice of therapeutic tool for treating SNHLs other than the aminoglycoside induced one. In case of antitumor therapy by cisplatin and related compounds the concomitant administration of rasagiline to prevent or attenuate the side effects, similarly to its acute use in aminoglycoside therapy, might be a feasible way of application. On the other hand, chronic treatment with rasagiline seems to be the reasonable therapy in presbycusis and persistent, moderate-level noise exposure induced HLs.

The complex pathomechanism of SNHLs, structured rather like a network than like a linear cascade, together with the failure to find the breakthrough in therapy till now, suggests that single-target interventions hold less promise in the therapy of SNHLs. Based on the significant overlaps in the pathomechanism of SNHLs, rasagiline, with its multi-target action, might be effective in treating not only the aminoglycoside-induced HL but other forms of SNHLs as well. Its good tolerability, proven since its introduction to human therapy in 2006, also supports the applicability of this new therapeutic indication.

CONFLICT OF INTEREST

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