

D₂ AUTORECEPTOR INHIBITION REVEALS OXYGEN–GLUCOSE DEPRIVATION-INDUCED RELEASE OF DOPAMINE IN GUINEA-PIG COCHLEA

G. HALMOS,^{a,b} Z. DOLEVICZÉNYI,^{a,b} G. RÉPÁSSY,^b Á. KITTEL,^a E. S. VIZI,^{a*} B. LENDVAI^a AND T. ZELLES^a

^aInstitute of Experimental Medicine, Hungarian Academy of Sciences, Szigony u. 43, H-1083 Budapest, Hungary

^bDepartment of Otolaryngology, Head and Neck Surgery, Semmelweis University, School of Medicine, Budapest, Hungary

Abstract—Dopamine (DA), released from the lateral olivocochlear (LOC) efferent terminals, the efferent arm of the short-loop feedback in the cochlea, is considered as a protective factor in the inner ear since it inhibits auditory nerve dendrite firing in ischemia- or noise-induced excitotoxicity leading to sensorineural hearing loss (SNHL). In the present study we investigated the effect of oxygen–glucose deprivation (OGD), an *in vitro* ischemia model, on guinea-pig cochlear [³H]DA release in a microvolume superfusion system. We found that OGD alone failed to induce a detectable elevation of [³H]DA level, but in the presence of specific D₂ receptor antagonists, sulpiride and L-741,626, it evoked a significant increase in the extracellular concentration of [³H]DA. D₂ negative feedback receptors are involved not exclusively in the regulation of synthesis and vesicular release of DA, but also in the activation of its reuptake. Thus, D₂ receptor antagonism interferes with the powerful reuptake of DA from the extracellular space. To explore the underlying mechanism of this DA-releasing effect we applied nomifensine and found that the effect of OGD on cochlear DA release in the presence of D₂ antagonists could be inhibited by this selective DA uptake inhibitor. This finding indicates that the OGD-evoked DA release was mainly mediated through the reverse operation of the DA transporter. The two structurally different D₂ antagonists also augmented the electrical field stimulation-evoked release of DA proving the presence of D₂ autoreceptors on dopaminergic LOC terminals. Our results confirm the presence and role of D₂ DA autoreceptors in the regulation of DA release from LOC efferents, and suggest a protective local mechanism during ischemia which involves the direct transporter-mediated release of DA. Increasing the release of the protective transmitter DA locally in the inner ear may form the basis of future new therapeutic strategies in patients suffering from SNHL. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: cochlea, dopamine release, oxygen-glucose deprivation, ischemia, transporter, D₂ autoreceptor.

*Corresponding author. Tel: +36-1-210-9421; fax: +36-1-210-9423. E-mail address: esvizi@koki.hu (E. S. Vizi).

Abbreviation: DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; EM, electron microscopy; FR, fractional release; Glu, glutamate; HVA, homovanillic acid; IHC, inner hair cells; LOC, lateral olivocochlear efferent; OGD, oxygen–glucose deprivation; SNHL, sensorineural hearing loss; TTX, tetrodotoxin.

0306-4522/05/\$30.00+0.00 © 2005 Published by Elsevier Ltd on behalf of IBRO. doi:10.1016/j.neuroscience.2005.01.023

The activity of the synapse between the inner hair cells (IHCs) and the type I afferent dendrites of the auditory nerve is modulated by the lateral olivocochlear efferent (LOC) fibers. The LOC efferents arise from the lateral cells of the superior olivary complex and radiate to the ipsilateral cochlea terminating beneath the IHCs forming en passant contacts at the dendrites of the type I afferent neurons (Spendlin, 1973; Warr and Guinan, 1979; Eybalin and Pujol, 1989; Eybalin, 1993; Puel, 1995; Puel et al., 2002). Several transmitters including acetylcholine, GABA (Eybalin and Altschuler, 1990; Felix and Ehrenberger, 1992), serotonin (Gil-Loyzaga et al., 1997; Vicente-Torres et al., 1998; Gil-Loyzaga et al., 2000), different neuropeptides (Fex and Altschuler, 1981; Altschuler et al., 1985; Takeda et al., 1986; Safieddine et al., 1997) and dopamine (DA; Eybalin et al., 1993) were identified as possible modulators of the IHC-auditory nerve synapse (Safieddine et al., 1997).

The cochlea is very vulnerable to a drop in blood supply. Not only the dysfunction of the supplier arteries but other pathological noxae (e.g. endolymphatic hydrops or noise trauma) can cause ischemia in the organ of Corti (Okamoto et al., 1990; Thorne and Nuttall, 1987, 1989; Vass et al., 1995). Ischemic insult of the cochlea results in an excessive release of glutamate (Glu) from IHCs and probably also from supporting cells, adjacent to the hair cells (Matsubara et al., 1998) and the increased level of Glu can be measured in the perilymph (Hakuba et al., 1997, 2000). Overstimulation of Glu receptors leads to extreme Na⁺ and Ca²⁺ influx and constant depolarization of the cells. Increase in intracellular sodium concentration is followed by water entry, then acute swelling of the afferent nerve (Billett et al., 1989; Pujol et al., 1990). The high intracellular Ca²⁺ level causes free radical production and activation of different enzymes that degrade cellular components (Pujol et al., 1990; Lipton, 1999; Pujol and Puel, 1999). Reduction of Glu efflux by hypothermia (Hyodo et al., 2001) or inhibition of AMPA/kainite-, but not the NMDA receptors (Hakuba et al., 2000) prevents hearing loss and neurite degeneration due to *in vivo* ischemic insults. Excitotoxicity, the excitatory amino acid produced neurodegeneration, also plays a role in the pathomechanisms of sudden sensorineural hearing loss (SNHL; Pujol and Puel, 1999), presbycusis (Seidman et al., 1999), aminoglycoside-induced ototoxicity (Duan et al., 2000) and tinnitus (Sahley and Nodar, 2001).

Excitotoxicity in the cochlea activates the auditory nerve–cochlear nucleus–lateral superior olivary complex–cochlea short-loop. The activity of this feedback leads to release of transmitters from the LOC terminals and neuro-

protection in the inner ear (Pujol, 1994). DA has been identified as a transmitter of this projection (Eybalin et al., 1993) raising the idea to study the neuroprotective effects of DA. In line with the theory of the cochleo-protective role of DA, released from LOC efferents, noise stimulation resulted in a decrease of DA and an increase of 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) content in the cochlea (Gil-Loyzaga et al., 1993, 1994). Several other studies also indicated the possible protective role of DA in noise- or ischemia-induced cochlear damage (Puel et al., 1988; Pujol et al., 1993; d'Aldin et al., 1995a,b; Gil-Loyzaga, 1995; Oestreicher et al., 1997).

Using RT-PCR, $D_{2(\text{long})}$ and D_3 receptors were suggested to transmit the action of DA in the mouse cochlea (Karadaghy et al., 1997). In accordance with these findings, functional evidence indicated that the protective effect of DA is mediated via D_2 and D_3 receptors (Gil-Loyzaga, 1995; Puel, 1995). The type and function of DA autoreceptors are, however, less well understood. While Gáborján et al. (1999) found a primarily D_1 receptor-mediated facilitation of cochlear DA release, Halmos et al. (2002) suggested the involvement of D_2 autoreceptors. The role of D_2 autoreceptors on dopaminergic nerve terminals in the brain is to provide a negative feedback control on the amount of DA in the synaptic or extracellular space (Langer, 1997).

Because of the neuroprotective role of cochlear DA, we studied the direct effect of oxygen–glucose deprivation (OGD), as an *in vitro* ischemia model, on the release of DA in the guinea-pig cochlea. To facilitate the detection of the released DA, presynaptic autoreceptors were inhibited concomitantly. The question of the autoreceptor type was addressed by the use of two structurally different and selective D_2 receptor antagonists.

EXPERIMENTAL PROCEDURES

Animals and tissue preparation

We used male guinea-pigs, weighing 250–350 g. We minimized animal suffering and the number of animals used, in accordance with the National Institutes 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. We used the microvolume superfusion method as described by Gáborján et al. (1999) with some minor modifications. The bulla tympani were 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 osmolality was set by D-glucose and the solution was gassed continuously with 100% O_2 . In a set of experiments the perfusion buffer was gassed with 100% N_2 and contained saccharose instead of glucose from the 24th minute of the experiments OGD.

The cochleae were incubated with 0.2 μM [^3H]DA (specific activity: 31.0 Ci/mmol) for 35 min. Each cochlea was then placed in a microvolume plexi chamber (one cochlea per chamber) and

superfused with perilymph-like solution. To collect the released [^3H]DA in a shorter time window and to try to increase the difference between the O_2 content of perilymph-like solution during control and OGD conditions (O_2 partial pressure of the room and the buffer tends to equilibrate through the silicone tube used) we applied a higher perfusion rate than before (Gáborján and Vizi, 1999; Gáborján et al., 1999, 2001; Halmos et al., 2000, 2002; see comparison in Fig. 1A). After a 1-h pre-perfusion the outflow was collected in 3-min fractions. The released radioactivity was determined by assaying 500 μl aliquots of each sample with a liquid scintillation counter (Packard Tri-Carb 1900TR, Meriden, CT, USA). After collecting the samples for 57 min (19 fractions) each cochlea was transferred from the microchambers to 500 μl of 10% trichloroacetic acid for 1 day; then 100 μl was used to measure the tissue content of radioactivity. Earlier HPLC measurements in our laboratory showed that 91–95% of the released radioactivity was attributable to [^3H]DA and its metabolites DOPAC and HVA and was of neuronal origin (Gáborján et al., 1999). Electrical field stimulation was applied for one collection period (3 min, 360 pulses) at 60 V, 2 Hz and 0.5 ms impulse duration at the 3rd and 13th fractions. Electrical field stimulation depolarizes all the excitable elements of the perfused tissue preparation including both the type I and II afferents the lateral and medial efferents and the outer and IHCs. As only the LOC release DA of this tissue, we can assume that these fibers are the source of the measured radioactivity. The most probable mechanism of the action of field stimulation is direct depolarization of these fibers and consequent exocytotic release of DA. In some experiments we did not apply the 2nd electrical stimulation and tested the effect of OGD ($n=8$), 10 μM sulpiride ($n=6$) and OGD+10 μM sulpiride ($n=6$) purely on the resting efflux of DA through 12 collection periods (8th to 19th fractions). OGD alone had no detectable effect during the 36 min. In the presence of sulpiride the DA-releasing effect of OGD evolved in 6 min; therefore, the 2nd stimulation was always used in the rest of the experiments. The pulses were delivered by a Grass S88 stimulator (West Warwick, USA) through platinum electrodes at the top and bottom of the tissue chamber. Drugs were added to the perfusion solution at the beginning of the 8th fraction (24th min) and were maintained till the end of the experiment. OGD was applied from the 24th min.

Additionally to the reversibility and reproducibility of DA release and its inhibition by VDSC (tetrodotoxin [TTX]; Fig. 1) or VDCC blockade (see Gáborján and Vizi, 1999; Gáborján et al., 1999), the viability of our preparation was also shown by light and electron microscopy (EM) performed right before and after the experiments with 3 ml/min perfusion speed shown in Fig. 1A. Both differential interference contrast (Olympus BX50WI microscope; LUMPLanFI 40 \times /0.80 w objective; data not shown) and EM images have proved the structural integrity of the organ of Corti in our preparation in the beginning and at the end of the perfusion. Fig. 1D shows the preserved neural elements underneath an IHC in a preparation which was placed into the fixative right after the perfusion. The fixative contained 4% paraformaldehyde, 0.5% glutaraldehyde in PB, pH 7.4. The solution was changed several times for 5 h and the organ remained in a fresh portion of the fixative overnight. After washing with phosphate buffer several times, the preparations were postfixed in 1% OsO_4 for 30 min. Following washing with distilled water, the tissue blocks were dehydrated in graded ethanol, block-stained with 2% uranyl acetate in 70% ethanol for 1 h and embedded in Taab 812 (Taab; Aldermaston, Berks, UK). Ultrathin sections were examined in a Hitachi 7100 transmission electron microscope (Hitachi Corporation, Japan).

Data analysis, statistics and chemicals used

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 total radioactivity present in the tissue at the

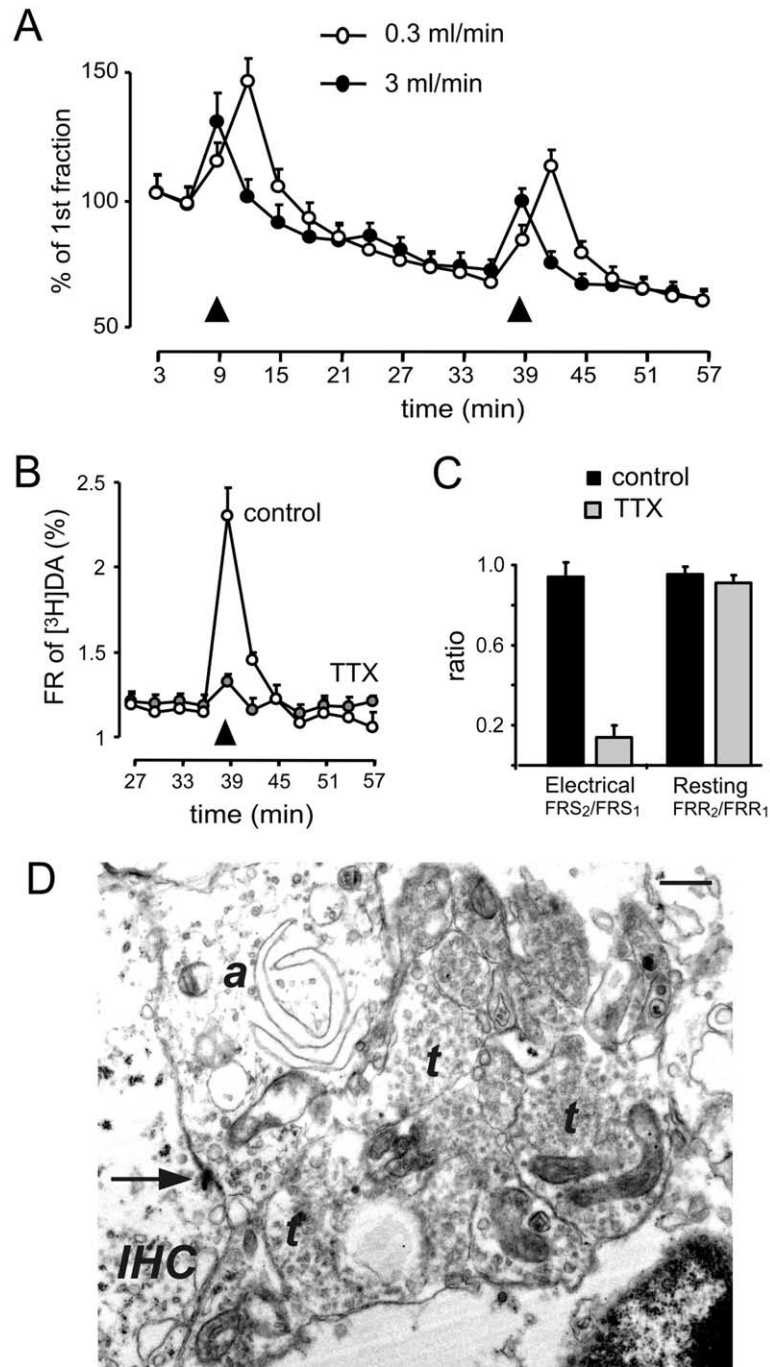


Fig. 1. Measurement of [³H]DA from nerve elements in the isolated guinea-pig cochleae. (A) Low (open circles) and high (dark circle) perfusion rates caused different temporal dynamics of DA release from the cochlea. Electric stimulation-evoked release of DA took place in a shorter time window at higher perfusion speed (3 ml/min). Release of DA is shown in the ratio of the 1st fraction. Electrical field stimulations (black triangles) evoked reversible and reproducible increase in the FR. (B) TTX applied in 1 μ M concentration from the 8th fraction inhibited the electrical stimulation-evoked release of DA. (C) Bar chart shows that the effect of TTX on DA release was selective on the electrical stimulation-evoked release (FRS₂/FRS₁), and that the resting efflux was unaffected (FRR₂/FRR₁). Data presented are means \pm S.E.M. (D) EM image of neural elements underneath an IHC in a cochlea preparation placed into the fixative right after a 3 ml/min perfusion experiment shown in 1A. The arrow indicates the ribbon synapse between the IHC and the afferent dendrite (a). t, axon terminal; scale bar=1 μ m.

time of sample collection. The FR by 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 (Halmos et al., 2000). 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). The effect of the drugs on the resting outflow of tritium was determined as the ratio of the sum of the two highest consecutive resting FR values in the presence of the drug (FRR₂) and the sum of the two consecutive

FR values before the drug reached the cochleae (FRR_1 ; FRR_2/FRR_1). The two cochleae of a guinea-pig were used in parallel, each one in a separate tissue chamber with a different treatment protocol. Number of experiments (n) shows the number of individual cochleae used in the treatment group, each one from a different animal. Data are expressed as the means \pm S.E.M. ANOVA followed by the Dunnett post hoc test was used to determine the statistical significance using the Statistica 6.0 program (Statsoft Inc., USA). TTX, (\pm)sulpiride, L-741,626 ((\pm)-3-[4-(4-chlorophenyl)-4-hydroxypiperidinyl]methylindole) and nomifensine maleate were from Sigma (St. Louis, MO, USA). [7,8- 3H]DA (specific activity 31.0 Ci/mmol) was purchased from Amersham (UK).

RESULTS

Release of [3H]DA from isolated cochleae

After the cochleae had been loaded with the [3H]DA and preperfused for 1 h, the resting efflux of radioactivity was 9829 ± 345 Bq/g ($FR = 1.70 \pm 0.04\%$; $n = 102$) in the 1st collection period and it showed a stable decrease over the experiments. By the 7th collection period when perfusions

of D_2 antagonists and OGD were started (see Experimental Procedures) the released radioactivity was reduced to 7810 ± 282 Bq/g ($n = 85$, 80% of the initial value). Electrical field stimulation released additional radioactivity from the tissue ($S_1 = 6675 \pm 271$ Bq/g, $FRS_1 = 1.25 \pm 0.05$; $n = 102$). In control experiments, after 30 min the stimulation was repeated (S_2) and resulted in a mean FRS_2/FRS_1 ratio of 0.94 ± 0.07 ($n = 10$). The field stimulation-evoked release of DA was sensitive to TTX ($1 \mu M$) treatment, suggesting that [3H]DA was released from neural elements by vesicular exocytosis (Fig. 1B).

Effect of oxygen and glucose deprivation on the release of [3H]DA

OGD caused no detectable change in the resting efflux of [3H]DA (Fig. 2A and Fig. 3D) and did not influence the field stimulation-evoked release of DA (Fig. 2). To determine whether this apparent lack of effect can be attributable to the sensitivity of our method, we enhanced the extracellu-

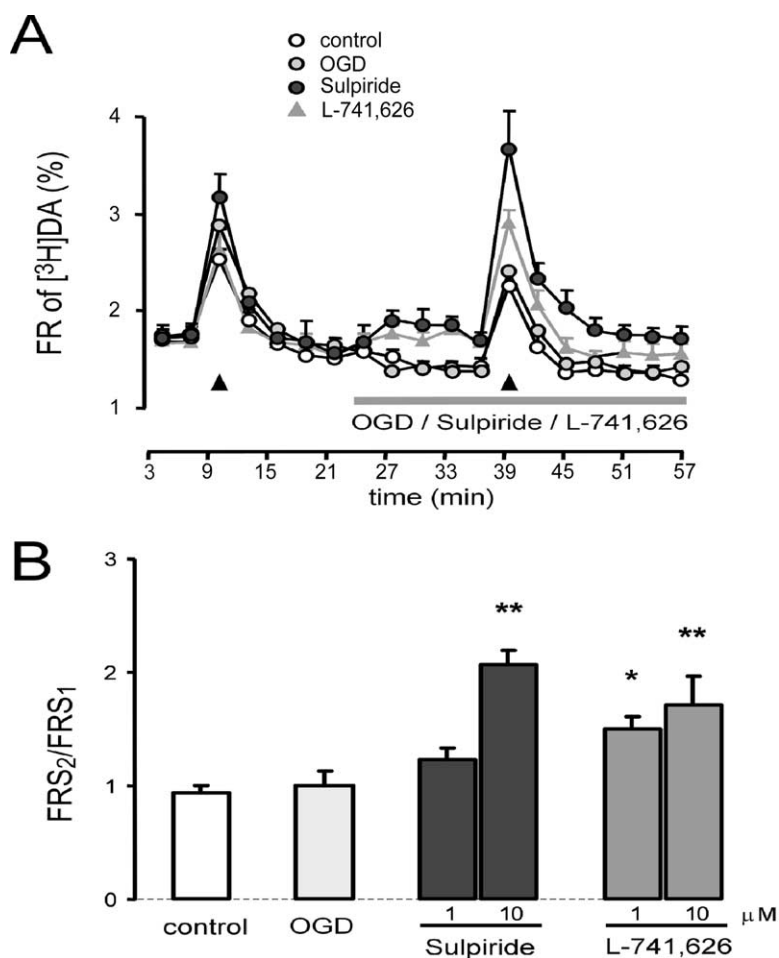


Fig. 2. Dopaminergic terminals of the lateral olivocochlear efferents are equipped with functional D_2 autoreceptors in the cochlea. (A) Sulpiride (dark gray circle) and L-741,626 (light gray triangle) significantly increased the electrical field stimulation-evoked release of [3H]DA from isolated cochlea in $10 \mu M$ concentration. OGD (light gray circle) did not influence the release. D_2 antagonists and OGD were applied from the 8th fraction as indicated by the horizontal gray line. Field stimulations are indicated by the black triangles. The symbols represent the mean values and the vertical bars show the S.E.M. (B) Summary bar chart of the effect of OGD ($n = 8$), sulpiride ($n = 6$ each) and L-741,626 ($n = 5$ and 6) on the electrically evoked DA release as revealed by the FRS_2/FRS_1 values. Data presented are mean \pm S.E.M.; asterisks indicate significant differences from control ($n = 10$; * $P < 0.05$, ** $P < 0.01$).

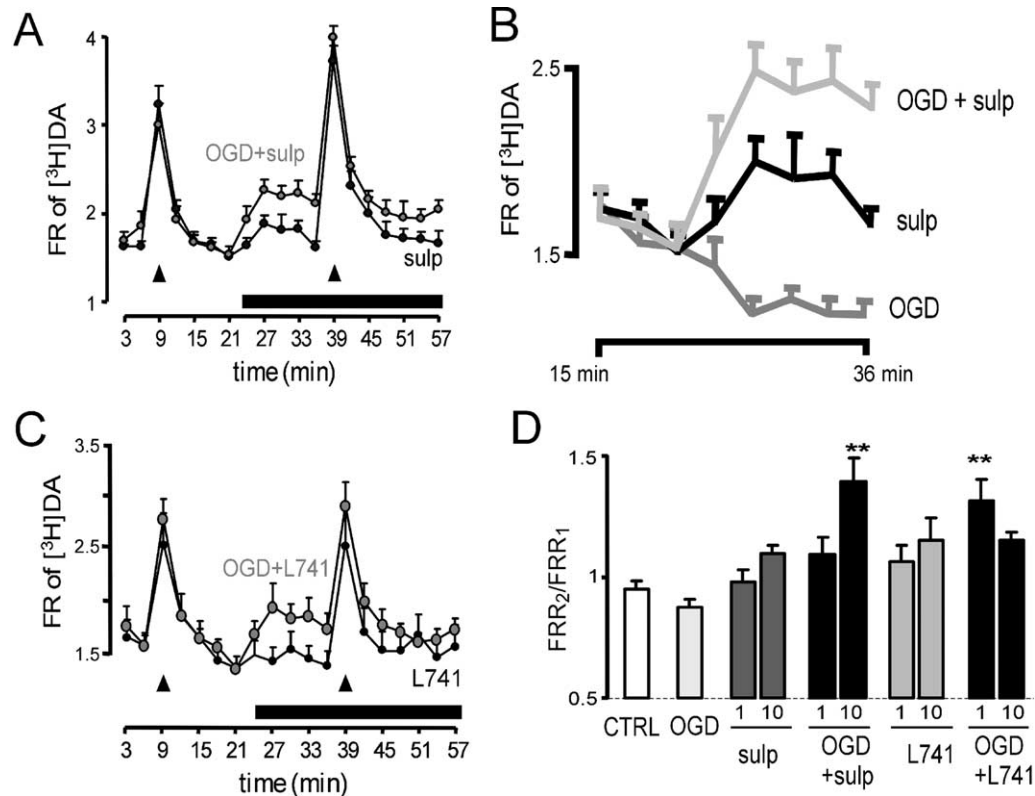


Fig. 3. OGD induced the release of DA in resting conditions when the D₂ receptors were blocked. (A) Effect of 10 μM sulpiride alone (dark circle) and in combination with OGD (light circle) on the release of [³H]DA from guinea-pig cochlear preparation. Field stimulations are indicated by the black triangles. (B) Higher magnification of the release time course between the 15th and the 36th minutes. Sulpiride in 10 μM concentration caused a slight, not significant, effect on the resting DA outflow (black line). OGD-induced release of DA in the presence of 10 μM sulpiride (light gray line) reached a plateau within 6 min. OGD alone had no effect (dark gray line). (C) Effect of 1 μM L-741,626 alone (black dot) and in combination with OGD (gray circle). Field stimulations are indicated by the black triangles. (D) Summary bar chart shows the effect of OGD, sulpiride, L-741,626 and OGD in the presence of the D₂ antagonists on the resting outflow of DA (FRR₂/FRR₁). In the case of OGD, 10 μM sulpiride and OGD+10 μM sulpiride FRR₂/FRR₁ values from experiments lacking the 2nd field stimulation (see Experimental Procedures) were included in the statistical analysis. Data presented are means±S.E.M. (*n*=10, 16, 6, 12, 6, 12, 5, 6, 6, 6 from left to right, respectively; asterisks indicate significant differences from control, * *P*<0.05, ** *P*<0.01).

lar concentration of the released DA by adding sulpiride, an antagonist of the D₂ inhibitory autoreceptor. In the presence of 10 μM sulpiride, OGD induced an elevation of the basal DA level (Fig. 3A, B, D). Although sulpiride at higher concentration exhibited a tendency toward increasing the resting DA outflow (Fig. 3B), this effect was not significant (Fig. 3D). To confirm the data obtained with the use of sulpiride, we tested another, structurally different D₂ DA receptor antagonist, L-741,626 (Kulagowski et al., 1996) on the release of DA from isolated guinea-pig cochleae. In accordance with the data obtained with sulpiride, L-741,626 also tended to increase the resting DA outflow alone and boosted OGD to significantly increase the basal release of DA at 1 μM concentration (Fig. 3C, D). Interestingly, the release of DA was not increased significantly in response to OGD when 10 μM L-741,626 was used (Fig. 3D). L-741,626 at 10 μM did not increase further significantly the field stimulation-evoked DA release either compared to its effect at 1 μM (Fig. 2B). The loss of D₂ selectivity of the potent antagonist L-741,626 at higher concentration (Bowery et al., 1996) might explain this finding. Unwanted D₁ antagonism could be an example.

Gáborján et al. (1999) showed a primarily D₁ receptor-mediated facilitation of cochlear DA release.

Effect of D₂ antagonists on field stimulation-evoked release of [³H]DA

Because the effect of OGD was visible only in the presence of D₂ antagonists, we further analyzed the effect of the D₂ autoreceptor. In contrast to the effect on the resting DA efflux, perfusion of sulpiride (10 μM) and L-741,626 (1 and 10 μM) significantly increased the electrical field stimulation-evoked release of DA from the isolated cochleae (Fig. 2). The effect of the D₂ antagonists on the FRR₂/FRR₁ value was not influenced by OGD (Fig. 3A, C).

Effect of nomifensine on the release of [³H]DA

The DA uptake blocker nomifensine (10 μM) alone significantly increased the electrical field stimulation-evoked release of DA (Fig. 4A) and failed to influence the resting DA outflow (Fig. 4B). In the presence of 10 μM nomifensine, OGD plus sulpiride (10 μM) failed to induce release of cochlear DA (Fig. 4B). When nomifensine was present in

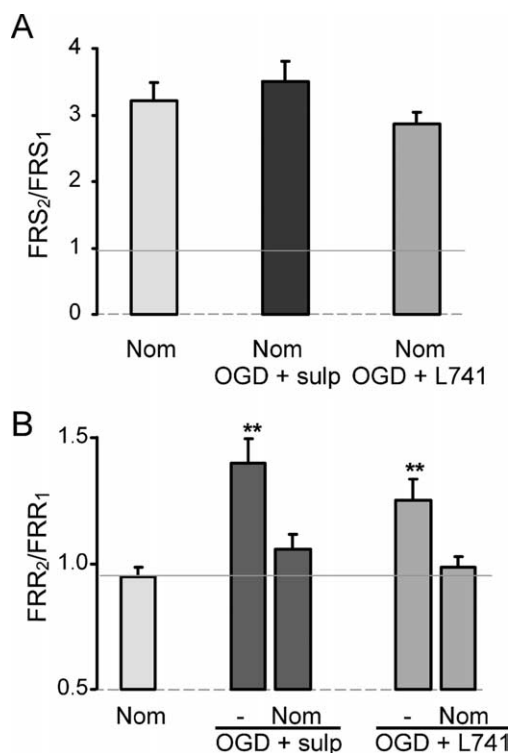


Fig. 4. Effect of the DA uptake inhibitor nomifensine on the OGD-evoked release of DA. (A) The effect of nomifensine (10 μ M; $n=4$) on field stimulation-evoked release of DA was not influenced by OGD applied in the presence of the D_2 receptor antagonists sulpiride ($n=7$) and L-741,626 ($n=6$). Gray line indicates the mean of FRS_2/FRS_1 values of the control experiments. Data presented are means \pm S.E.M. (B) Summary bar chart shows the inhibitory effect of nomifensine on the resting release of DA evoked by OGD+sulpiride and OGD+L-741,626. Gray line indicates the mean of FRR_2/FRR_1 values of the control experiments. Data presented are means \pm S.E.M. ($n=4, 12, 7, 6$, and 6 from left to right, respectively; ** $P<0.01$).

the perfusate L-741,626 (1 μ M) was also unable to facilitate the release of DA during OGD (Fig. 4B). The increase of field stimulation-evoked DA release in the presence of nomifensine was not facilitated further by the combined application of OGD and any of the D_2 antagonists (Fig. 4A).

DISCUSSION

Our experiments revealed that OGD, which can be taken as an *in vitro* model of ischemia, was able to evoke the release of DA from the terminals of LOC fibers in the guinea-pig cochlea preparation. OGD itself did not cause any measurable change either in the resting or field stimulation-evoked release of tritiated DA. However, mimicking ischemic insult by OGD in the presence of D_2 receptor inhibitors resulted in detectable elevation of DA release. It seems that blockade of D_2 autoreceptors is an amplifying factor to increase the concentration of OGD-released DA in the extracellular space, from which our superfusion method samples. Feedback inhibition of the exocytotic, vesicular release of neurotransmitters by presynaptic autoreceptors, such as D_2 receptors on dopaminergic terminals, has been known for a long time (Farnebo and

Hamberger, 1971; Vizi, 1979). D_2 autoreceptor-mediated downregulation of DA synthesis has also been shown (Kehr et al., 1972; Lindgren et al., 2001). Recently, there is growing evidence for the role of presynaptic auto- and heteroreceptors in the regulation of uptake carriers (Gulley and Zahniser, 2003). Strong evidence indicates that D_2 receptor stimulation facilitates the carrier-mediated reuptake of DA (Meiergerd et al., 1993; Cass and Gerhardt, 1994; Dickinson et al., 1999; Mayfield and Zahniser, 2001; Wu et al., 2002). All three types of the D_2 -mediated mechanisms work to reduce the extracellular concentration of DA, although their control may be independent (Wu et al., 2002). Blockade of D_2 receptors suspends these actions.

The underlying mechanism of the cochlear DA-releasing effect of OGD in our experiments involves action on the DA transporter, because nomifensine, a selective DA uptake inhibitor, blocked the OGD-induced increase in DA level in the presence of D_2 antagonists. It is known that nomifensine blocks not only the uptake of DA, but also the reverse operation of the DA transporter (Lonart and Zigmond, 1991; Kim et al., 1995; Zelles et al., 1995; Buyukuysal and Mete, 1999). Since the prevention of reuptake by inhibition of the transporter itself would increase the extracellular level of DA released in any non-transport-dependent way, we should assume the reverse operation of the transporter to explain inhibition of OGD-evoked release. In this sense, OGD would induce the uptake carrier to transport in the opposite direction, which leads to the release of DA. The inhibition of the transporter by nomifensine abolishes this releasing action of OGD. There are several examples in the CNS for the reverse operation of neurotransmitter transporters during ischemia. The carrier-mediated release of Glu due to ischemic insult is a well known phenomenon (Lipton, 1999). The biogenic amine serotonin (Nagao et al., 1995), noradrenaline (Schomig, 1988; Milusheva et al., 2003) or DA (Kim et al., 1995; Leviel, 2001) is also released in this way by ischemia or high Glu (Lonart and Zigmond, 1991). Reversal of these sodium-dependent transporters is based on the increase of intracellular sodium concentration that is always the case in ischemic neurons (Lipton, 1999). In the cochlea, research was focused on the excitotoxicity due to ischemia or noise-induced excessive Glu release from the IHCS (Pujol et al., 1993; Puel et al., 1994; Pujol and Puel, 1999) and on the protective role of DA released from the LOC terminals during the insults (Puel et al., 1988; Pujol et al., 1993; d'Aldin et al., 1995a,b; Gil-Loyzaga, 1995; Oestreicher et al., 1997). The precise mechanism of the release under pathologic conditions has not been explored in either case yet. Our paper shows for the first time that the reverse operation of the DA uptake system participates in the OGD-evoked release of DA in the organ of Corti. The level of DA in the extracellular space and in the perfusion buffer remains low and undetectable with our method because the released DA, through D_2 autoreceptors, facilitates the uptake carrier to transport in the forward mode. Blockade of D_2 receptors by selective antagonists eliminates this counteraction and reveals the release of DA from LOC terminals due to the ischemic insult. We can

speculate that the extracellular concentration of DA *in vivo* can be kept in a level sufficient for neuroprotection, but the negative feedback helps to avoid the extreme DA levels that could produce harmful levels of free radicals by the metabolism of DA (Fahn and Cohen, 1992).

The D₂ receptor antagonists also facilitated the electrical field stimulation-evoked DA release. These results, by using two structurally different and selective antagonists in more reliable concentrations, confirm our previous neurochemical finding (Halmos et al., 2002) that has already raised the possibility of the presence of functional D₂ negative feedback receptors on the LOC terminals in the cochlea. Their significant effect on field stimulation-evoked exocytotic release is probably due to disinhibition of the multiple feedback action of D₂ autoreceptors.

Glu excitotoxicity that is due to ischemic insult is an important component in the pathomechanisms of several diseases, like sudden SNHL (Pujol and Puel, 1999), and presbycusis (Seidman et al., 1999). The same process seems to be partially responsible for aminoglycoside-induced hearing loss (Duan et al., 2000), noise trauma (Okamoto et al., 1990) and tinnitus (Sahley and Nodar, 2001). Glu and DA seem to have opposite effects at the IHC-afferent dendrite-LOC axon terminal synaptic complex: Glu activates while DA inhibits primary auditory nerve fibers. Stimulation of the LOC efferent fibers decreases the amplitude of the intensive sound stimulation-evoked cochlear compound action potential, which shows the protection of the overstimulated auditory nerve fibers (Puel et al., 1988). DA has an effect on the spontaneous firing rate of the afferent fibers and the AMPA- and NMDA-induced activity can be dose-dependently inhibited by the application of DA D₁ and D₂ agonists (Oestreicher et al., 1997; Puel et al., 2002). This finding has been further confirmed: the intracochlear application of piribedil, a D₂/D₃ agonistic drug, given prior to noise exposure or during ischemia, can decrease the change of cochlear potentials. The morphological findings, i.e. the swelling of the afferent dendrites, can also be prevented by the application of piribedil (Pujol et al., 1993; d'Aldin et al., 1995a,b; Gil-Loyzaga, 1995). These experimental results suggest that Glu receptor antagonists or certain DA receptor agonists can be effective against different diseases. In practice, only the NMDA antagonist memantine and the NMDA/AMPA antagonist caroverine have been tested for clinical use and found effective (Denk et al., 1997; Ehrenberger, 2002). Drugs acting on DA receptors have not been tested yet thoroughly.

In conclusion, our data indicate the presence of functional D₂ autoreceptors on LOC efferents in the guinea-pig cochlea. Blockade of these autoreceptors inhibits released DA-induced facilitation of reuptake and therefore reveals the release of DA from LOC terminals that is due to OGD-induced reverse transport. Since DA released from LOC terminals protects primary auditory nerve fibers against abnormal cytodestructive firing, our new finding demonstrates the presence of a local protective mechanism in the inner ear that may effectively supplement the protective short-loop feedback circle between

the brainstem and the cochlea during ischemia or any excitotoxic insult. Strengthening of the short-loop feedback mechanism at the LOC efferent terminal level in the organ of Corti may open a new direction for drug therapy of SNHL.

Acknowledgments—This study was supported in part by the Hungarian Research Fund (OTKA T 034622, T 034722, T 037459 and Ts 040736) and the Hungarian Medical Research Foundation (470/2003 and 123/2003). The authors wish to thank Erika Tischler for excellent technical assistance.

REFERENCES

- Altschuler RA, Hoffman DW, Reeks KA, Fex J (1985) Localization of dynorphin B-like and alpha-neoendorphin-like immunoreactivities in the guinea pig organ of Corti. *Hear Res* 17:249–258.
- Billett TE, Thorne PR, Gavin JB (1989) The nature and progression of injury in the organ of Corti during ischemia. *Hear Res* 41:189–197.
- Bowery BJ, Razaque Z, Emms F, Patel S, Freedman S, Bristow L, Kulagowski J, Seabrook GR (1996) Antagonism of the effects of (+)-PD 128907 on midbrain dopamine neurons in rat brain slices by a selective D₂ receptor antagonist L-741,626. *Br J Pharmacol* 119:1491–1497.
- Buyukuysal RL, Mete B (1999) Anoxia-induced dopamine release from rat striatal slices: involvement of reverse transport mechanism. *J Neurochem* 72:1507–1515.
- Cass WA, Gerhardt GA (1994) Direct *in vivo* evidence that D₂ dopamine receptors can modulate dopamine uptake. *Neurosci Lett* 176:259–263.
- d'Aldin C, Eybalin M, Puel J-L, Characon G, Ladrech S, Renard N, Pujol R (1995a) Synaptic connections and putative functions of the dopaminergic innervation of the guinea pig cochlea. *Eur Arch Otorhinolaryngol* 252:270–274.
- d'Aldin C, Puel J-L, Leducq R, Crambes O, Eybalin M, Pujol R (1995b) Effect of a dopaminergic agonist in the guinea pig cochlea. *Hear Res* 90:202–211.
- Denk DM, Heinzl H, Franz P, Ehrenberger K (1997) Caroverine in tinnitus treatment: a placebo-controlled blind study. *Acta Otolaryngol* 117:825–830.
- Dickinson SD, Sabeti J, Larson GA, Giardina K, Rubinstein M, Kelly MA, Grandy DK, Low MJ, Gerhardt GA, Zahniser NR (1999) Dopamine D₂ receptor-deficient mice exhibit decreased dopamine transporter function but no changes in dopamine release in dorsal striatum. *J Neurochem* 72:148–156.
- Duan M, Agerman K, Ernfors P, Carlén B (2000) Complementary roles of neurotrophin 3 and a *N*-methyl-D-aspartate antagonist in the protection of noise and aminoglycoside-induced ototoxicity. *Proc Natl Acad Sci USA* 97:7597–7602.
- Ehrenberger K (2002) Clinical experience with caroverine in inner ear disease. *Adv Otorhinolaryngol* 59:156–162.
- Eybalin M (1993) Neurotransmitters and neuromodulators of the mammalian cochlea. *Physiol Rev* 73:309–373.
- Eybalin M, Altschuler RA (1990) Immunoelectron microscopic localization of neurotransmitters in the cochlea. *J Electron Microscop Tech* 15:209–224.
- Eybalin M, Charachon G, Renard N (1993) Dopaminergic lateral efferent innervation of the guinea-pig cochlea: immunoelectron microscopy of catecholamine-synthesizing enzymes and effect of 6-hydroxydopamine. *Neuroscience* 54:133–142.
- Eybalin M, Pujol R (1989) Cochlear neuroactive substances. *Arch Otorhinolaryngol* 246:228–234.
- Fahn S, Cohen G (1992) The oxidant stress hypothesis in Parkinson's disease: evidence supporting it. *Ann Neurol* 32:804–812.
- Farnebo LO, Hamberger B (1971) Drug-induced changes in the release of ³H-monoamines from field stimulated rat brain slices. *Acta Physiol Scand Suppl* 371:35–44.

- Felix D, Ehrenberger K (1992) The efferent modulation of mammalian inner hair cell afferents. *Hear Res* 64:1–5.
- Fex J, Altschuler RA (1981) Enkephalin-like immunoreactivity of olivocochlear nerve fibers in cochlea of guinea pig and cat. *Proc Natl Acad Sci USA* 78:1255–1259.
- Gáborján A, Halmos G, Répássy G, Vizi ES (2001) A new aspect of aminoglycoside ototoxicity: impairment of cochlear dopamine release. *Neuroreport* 12:3327–3330.
- Gáborján A, Lendvai B, Vizi ES (1999) Neurochemical evidence of dopamine release by lateral olivocochlear efferents and its presynaptic modulation in guinea-pig cochlea. *Neuroscience* 90:131–138.
- Gáborján A, Vizi ES (1999) Characterization of voltage dependent calcium channels on the lateral olivocochlear efferent fibers of guinea pig. *Neurosci Lett* 269:49–51.
- Gil-Loyzaga P (1995) Neurotransmitters of the olivocochlear lateral efferent system: with an emphasis on dopamine. *Acta Otolaryngol* 115:222–226.
- Gil-Loyzaga P, Bartolome MV, Vicente-Torres MA (1997) Serotonergic innervation of the organ of Corti of the cat cochlea. *Neuroreport* 8:3519–3522.
- Gil-Loyzaga P, Bartolome V, Vicente-Torres A, Carricondo F (2000) Serotonergic innervation of the organ of Corti. *Acta Otolaryngol* 120:128–132.
- Gil-Loyzaga P, Fernández-Matheos P, Vicente-Torres MA, Remezal M, Cousillas H, Arce A, Esquifino A (1993) Effect of noise stimulation on cochlear dopamine metabolism. *Brain Res* 623:177–180.
- Gil-Loyzaga P, Vicente-Torres MA, Fernandez-Mateos P, Arce A, Esquifino A (1994) Piribedil affects dopamine turnover in cochleas stimulated by white noise. *Hear Res* 79:178–182.
- Gulley JM, Zahniser NR (2003) Rapid regulation of dopamine transporter function by substrates, blockers and presynaptic receptor ligands. *Eur J Pharmacol* 479:139–152.
- Hakuba N, Gyo K, Yanagihara N, Mitani A, Kataoka K (1997) Efflux of glutamate into the perilymph of the cochlea following transient ischemia in the gerbil. *Neurosci Lett* 230:69–71.
- Hakuba N, Koga K, Shudou M, Watanabe F, Mitani A, Gyo K (2000) Hearing loss and glutamate efflux in the perilymph following transient hindbrain ischemia in gerbils. *J Comp Neurol* 418:217–226.
- Halmos G, Gáborján A, Lendvai B, Répássy G, Z Szabó L, Vizi ES (2000) Veratridine-evoked release of dopamine from guinea pig isolated cochlea. *Hear Res* 144:89–96.
- Halmos G, Lendvai B, Gáborján A, Baranyi M, Z Szabó L, Csokonai Vitéz L (2002) Simultaneous measurement of glutamate and dopamine release from isolated guinea pig cochlea. *Neurochem Int* 40:243–248.
- Hyodo J, Hakuba N, Koga K, Watanabe F, Shudou M, Taniguchi M, Gyo K (2001) Hypothermia reduces glutamate efflux in perilymph following transient cochlear ischemia. *Neuroreport* 12:1983–1987.
- Ikeda K, Saito Y, Nishiyama A, Takasaka T (1991) Effects of pH on intracellular calcium levels in isolated cochlear outer hair cells of guinea pigs. *Am J Physiol* 261:C231–C236.
- Karadaghy AA, Lasak JM, Chomchai JS, Khan KM, Drescher MJ, Drescher DG (1997) Quantitative analysis of dopamine receptor messages in the mouse cochlea. *Mol Brain Res* 44:151–156.
- Kehr W, Carlsson A, Lindqvist M, Magnusson T, Atack C (1972) Evidence for a receptor-mediated feedback control of striatal tyrosine hydroxylase activity. *J Pharm Pharmacol* 24:744–747.
- Kim KW, Kim DC, Kim YH, Eun YA, Kim HI, Cho KP (1995) Ca^{2+} -dependent and -independent mechanisms of ischaemia-evoked release of [3H]-dopamine from rat striatal slices. *Clin Exp Pharmacol Physiol* 22:301–302.
- Kulagowski JJ, Broughton HB, Curtis NR, Mawer IM, Ridgill MP, Baker R, Emms F, Freedman SB, Marwood R, Patel S, Patel S, Ragan CI, Leeson PD (1996) 3-((4-(4-Chlorophenyl)piperazin-1-yl)-methyl)-1*H*-pyrrolo-2,3-b-pyridine: an antagonist with high affinity and selectivity for the human dopamine D4 receptor. *J Med Chem* 39:1941–1942.
- Langer SZ (1997) 25 Years since the discovery of presynaptic receptors: present knowledge and future perspectives. *Trends Pharmacol Sci* 18:95–99.
- Leviel V (2001) The reverse transport of DA, what physiological significance? *Neurochem Int* 38:83–106.
- Lindgren N, Xu ZQ, Herrera-Marschitz M, Haycock J, Hokfelt T, Fisone G (2001) Dopamine D_2 receptors regulate tyrosine hydroxylase activity and phosphorylation at Ser40 in rat striatum. *Eur J Neurosci* 13:773–780.
- Lipton P (1999) Ischemic cell death in brain neurons. *Physiol Rev* 79:1431–1568.
- Lonart G, Zigmond MJ (1991) High glutamate concentrations evoke Ca^{2+} -independent dopamine release from striatal slices: a possible role of reverse dopamine transport. *J Pharmacol Exp Ther* 256:1132–1138.
- Matsubara A, Kawabata Y, Takumi Y, Usami S, Shinkawa H, Haruta A, Matsuda K, Tono T (1998) Quantitative immunogold cytochemistry reveals sources of glutamate release in inner ear ischemia. *Acta Otolaryngol Suppl* 539:48–51.
- Mayfield RD, Zahniser NR (2001) Dopamine D_2 receptor regulation of the dopamine transporter expressed in *Xenopus laevis* oocytes is voltage-independent. *Mol Pharmacol* 59:113–121.
- Meiergerd SM, Patterson TA, Schenk JO (1993) D_2 receptors may modulate the function of the striatal transporter for dopamine: kinetic evidence from studies in vitro and in vivo. *J Neurochem* 61:764–767.
- Milusheva E, Sperlagh B, Shikova L, Baranyi M, Tretter L, Adam-Vizi V, Vizi ES (2003) Non-synaptic release of [3H]noradrenaline in response to oxidative stress combined with mitochondrial dysfunction in rat hippocampal slices. *Neuroscience* 120:771–781.
- Nagao T, Ibayashi S, Sadoshima S, Izumi J, Fujishima M (1995) Citalopram, a serotonin reuptake inhibitor, and brain ischemia in SHR. *Brain Res Bull* 38:49–52.
- Oestreicher E, Arnold W, Ehrenberger K, Felix D (1997) Dopamine regulates the glutamatergic inner hair cell activity in guinea pigs. *Hear Res* 107:46–52.
- Okamoto A, Tamura T, Yokoyama K, Kobayashi N, Hasegawa M (1990) Effect of loud sound exposure on cochlear blood flow. *Acta Otolaryngol* 109:378–382.
- Puel J-L (1995) Chemical synaptic transmission in the cochlea. *Prog Neurobiol* 47:449–476.
- Puel J-L, Bobbin RP, Fallon M (1988) An ipsilateral cochlear efferent loop protects the cochlea during intense sound exposure. *Hear Res* 37:65–70.
- Puel J-L, Pujol R, Tribillac F, Ladrech S, Eybalin M (1994) Excitatory amino acid antagonists protect cochlear auditory neurons from excitotoxicity. *J Comp Neurol* 341:241–256.
- Puel J-L, Ruel J, Guitton M, Wang J, Pujol R (2002) The inner hair cell synaptic complex: physiology, pharmacology and new therapeutic strategies. *Audiol Neurootol* 7:49–54.
- Pujol R (1994) Lateral and medial efferents: a double neurochemical mechanism to protect and regulate inner and outer hair cell function in the cochlea. *Br J Audiol* 28:185–191.
- Pujol R, Puel J-L (1999) Excitotoxicity, synaptic repair, and functional recovery in the mammalian cochlea: a review of recent findings. *Ann NY Acad Sci* 884:249–254.
- Pujol R, Puel J-L, d'Aldin CG, Eybalin M (1993) Pathophysiology of the glutamergic synapses in the cochlea. *Acta Otolaryngol* 113:330–334.
- Pujol R, Rebillard G, Puel J-L, Lenoir M, Eybalin M, Decasens M (1990) Glutamate neurotoxicity in the cochlea: a possible consequence of ischaemic or anoxic conditions occurring in ageing. *Acta Otolaryngol Suppl* 476:32–36.
- Safieddine S, Prior AM, Eybalin M (1997) Choline acetyltransferase, glutamate decarboxylase tyrosine hydroxylase, calcitonin gene-related peptide and opioid peptides coexist in lateral efferent neurons of rat and guinea-pig. *Eur J Neurosci* 9:356–367.

- Sahley TL, Nodar RH (2001) A biochemical model of peripheral tinnitus. *Hear Res* 152:43–54.
- Schomig A (1988) Adrenergic mechanisms in myocardial infarction: cardiac and systemic catecholamine release. *J Cardiovasc Pharmacol Suppl* 1:S1–S7.
- Seidman MD, Quirk WS, Shirwany NA (1999) Mechanisms of alterations in the microcirculation of the cochlea. *Ann NY Acad Sci* 884:226–232.
- Spoendlin H (1973) Innervation of the cochlear receptor. In: *Basic mechanisms in hearing* (Moller A, ed), pp 185–230. New York: Academic Press.
- Takeda N, Kitajiri M, Girgis S, Hillyard CJ, MacIntyre I, Emson PC, Shiosaka S, Tohyama M, Matsunaga T (1986) The presence of a calcitonin gene-related peptide in the olivocochlear bundle in rat. *Exp Brain Res* 61:575–578.
- Thorne PR, Nuttall AL (1987) Laser Doppler measurements of cochlear blood flow during loud sound exposure in the guinea pig. *Hear Res* 27:1–10.
- Thorne PR, Nuttall AL (1989) Alterations in oxygenation of cochlear endolymph during loud sound exposure. *Acta Otolaryngol* 107:71–79.
- Vass Z, Brechtelsbauer PB, Nuttall AL, Miller JM (1995) Effect of endolymphatic hydrops on capsaicin evoked increase in cochlear blood flow. *Acta Otolaryngol* 115:754–758.
- Vicente-Torres A, Bartolome MV, Carricondo F, Esquifino A, Gil-Loyzaga P (1998) HPLC detection of serotonin within the rat cochlea. *Neuroreport* 9:3699–3701.
- Vizi ES (1979) Presynaptic modulation of neurochemical transmission. *Prog Neurobiol* 12:181–290.
- Warr WB, Guinan JJ (1979) Efferent innervation of the organ of Corti: two separate systems. *Brain Res* 173:152–155.
- Wu Q, Reith ME, Walker QD, Kuhn CM, Carroll FI, Garris PA (2002) Concurrent autoreceptor-mediated control of dopamine release and uptake during neurotransmission: an in vivo voltammetric study. *J Neurosci* 22:6272–6281.
- Zelles T, Chernaeva L, Baranyi M, Deri Z, Adam-Vizi V, Vizi ES (1995) Transmitter release by non-receptor activation of the alpha-subunit of guanine nucleotide regulatory protein in rat striatal slices. *J Neurosci Res* 42:242–251.

(Accepted 13 January 2005)
(Available online 24 March 2005)