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## Original Paper

# 5-HT<sub>6/7</sub> Receptor Antagonists Facilitate Dopamine Release in the Cochlea via a GABAergic Disinhibitory Mechanism

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**Abstract** In humans, serotonin (5-HT) has been implicated in numerous physiological and pathological processes in the peripheral auditory system. Dopamine (DA), another transmitter of the lateral olivocochlear (LOC) efferents making synapses on cochlear nerve dendrites, controls auditory nerve activation and protects the sensory nerve against overactivation. Using in vitro microvolume superfusion techniques we tested 5-HT<sub>6</sub> and 5-HT<sub>7</sub> receptor antagonists whether they can influence dopamine (DA) release from the guinea-pig cochlea in control and in ischemic conditions using currently available and new 5-HT<sub>6</sub> and 5-HT<sub>7</sub> antagonists and mixed antagonists, which were synthesized and characterized for the current study. While the 5-HT<sub>7</sub> antagonist SB-258719 was ineffective, SB-271046, which blocks the 5-HT<sub>6</sub> receptor, caused a significant increase in cochlear DA release what is contradictory with the excitatory nature of this type of receptor. Moreover, the mixed 5-HT<sub>6/7</sub> antagonist EGIS-12233 induced an even more pronounced increase in the resting DA release. To understand why the block of an excitatory receptor results in an increase instead of a decrease in function, we investigated the possible involvement of an indirect neural mechanism through an inhibitory system. In the presence of the GABA<sub>A</sub> receptor blocker bicuculline, EGIS-12233 failed to increase the release of DA, suggesting that the serotonin receptor modulation of DA release from the lateral olivocochlear efferents in the cochlea was produced indirectly by decreasing the GABAergic inhibitory tone on dopaminergic nerve endings. The mixed 5-HT<sub>7</sub>/D<sub>4</sub> receptor antagonist EGIS-11983 significantly increased both the stimulation-evoked and the resting DA release, while the selective D<sub>4</sub> blocker L-741,741 alone had no significant effect. Ischemia, simulated by oxygen and glucose deprivation from the perfusion solution had no action on the effect of the drugs. Drugs that can increase the release of DA from LOC terminals in the cochlea may have a role in the treatment of sensorineural hearing loss.

**Keywords** Cochlea - Dopamine release - Serotonin - 5-HT<sub>6</sub> - 5-HT<sub>7</sub> - Neuroprotection - GABAergic innervation

## Introduction

Serotonin (5-HT) receptors are divided into seven distinct classes (5-HT<sub>1</sub> to 5-HT<sub>7</sub>) largely on the basis of their structure and functions. While 5-HT<sub>1</sub> receptors couple preferentially to G<sub>i/o</sub> proteins and inhibit cAMP formation, 5-HT<sub>2</sub> receptors induce hydrolysis of inositol phosphates and the subsequent elevation of cytosolic [Ca<sup>2+</sup>]<sub>i</sub> via G<sub>q/11</sub> proteins. 5-HT<sub>3</sub> receptors are ligand-gated ion channels; 5-HT<sub>4</sub>, 5-HT<sub>5</sub>, 5-HT<sub>6</sub>, and 5-HT<sub>7</sub> receptors all couple to G<sub>s</sub> proteins and promote cAMP formation [1–4]. In humans, 5-HT has been implicated in the regulation of eating, sleeping, sexual behaviors, circadian rhythm, neuroendocrine function, vascular constriction and in the etiology of numerous disease states, including depression, anxiety, social phobia, schizophrenia, and obsessive–compulsive and panic disorders. However, the functional role of serotonin and 5-HT receptors is much less understood in the operation of the cochlear neural network.

Serotonergic fibers have recently been identified by immunocytochemistry in the cochlea [5, 6]. These fibers innervate regions below both the inner and the outer hair cells. Their cochlear distribution suggests that they belong to the lateral olivocochlear efferent system [5, 6]. The 5-HT<sub>1/2</sub> antagonist methysergide reduces the compound action potential of the auditory nerve in the guinea pig [7]. Measurement of 5-HT metabolites [8, 9], together with the identification of several 5-HT receptor mRNAs in the adult mammalian cochlea [10] revealed serotonergic activity in this organ. In the central auditory system, serotonergic fibers are involved in various modulatory functions [11, 12]. It has been suggested that serotonergic innervation of the organ of Corti could also modulate of the auditory process [6, 13]. 5-HT transporters are present in the cochlear serotonergic fibers. Application of 6-nitroquipazine, a selective 5-HT reuptake inhibitor, increases 5-HT levels and reduces the main metabolite 5-HIAA [13]. Beside its physiological role, 5-HT seems to play an important role in tinnitus; disrupted or modified 5-HT function initiate plastic changes, which strongly influence tinnitus [14]. Among 5-HT receptors, the role of 5-HT<sub>6</sub> and 5-HT<sub>7</sub> receptors is less understood in the brain. It has been demonstrated that local application of the 5-HT<sub>6</sub> receptor antagonist SB-271046 [15] dose dependently increased dopamine (DA) release in the frontal cortex [16] whereas the selective 5-HT<sub>7</sub> receptor antagonist SB-258719 [17] influenced glutamate and 5-HT release in rat raphe nuclei [18]. In the cochlea, several lines of evidence suggest that DA, released from lateral olivocochlear (LOC) fibers, may have a protective effect on cochlear function by inhibiting the overstimulation of auditory nerve endings through synaptic contacts. DA postsynaptically inhibits the toxic effect of extreme glutamate

levels [19–25].

As presynaptic release-modulating heteroreceptors represent suitable targets for pharmacological intervention [26], the aim of the present study was to uncover the role of 5-HT<sub>6</sub> and 5-HT<sub>7</sub> receptors in the modulation of the cochleoprotective DA release. We tested the effect of known, selective 5-HT<sub>6/7</sub> receptor antagonists and that of new substances, produced by EGIS Pharmaceuticals Plc., whether they can influence DA release from the guinea-pig cochlea in control and ischemic conditions. Our data indicated a possible indirect mechanism for the stimulatory action of the antagonists.

## Materials and Methods

### Animals and Tissue Preparation

We used male guinea pigs, weighing 250–350 g and minimized animal suffering, 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. We used the method as described before [27, 28]. 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 and removed.

### In Vitro Microvolume Superfusion

All experiments were carried out in a perilymph-like solution, which contained 150 mM NaCl, 3.5 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 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<sub>2</sub>. In a set of experiments the perfusion buffer was gassed with 100% N<sub>2</sub> and contained saccharose instead of glucose from the 24th min of the experiments (oxygen and glucose deprivation, OGD; [28]). The isolated cochleae were incubated for 35 min at 37°C in 1 ml of the perilymph-like solution containing 0.2 μM [<sup>3</sup>H]DA (specific activity: 31.0–59.3 Ci/mmol). Cochleae were then placed in plexi chambers (100 μl inside volume, one cochlea per chamber) and perfused with the perilymph-like solution at 3 ml/min rate. After 1 h preperfusion the outflow was collected in 3-min fractions for 57 min (19 fractions) and their DA content was determined by measuring the radioactivity of each sample. 0.5 ml aliquots of each sample were assayed with a liquid scintillation counter (Packard Tri-Carb 1900TR, Meriden, CT, USA). After the collection period, cochleae were transferred to 0.5 ml of 10% trichloroacetic acid for 24 h then 0.1 ml was used to measure the radioactivity of the tissue. Earlier HPLC measurements in our laboratory showed that 91–95% of the released radioactivity was attributable to [<sup>3</sup>H]DA and its metabolites DOPAC and HVA and was of neuronal origin [29]. The DA content of each fraction was determined as a percentage of radioactivity of the whole tissue during one collecting period (fractional release, FR). Electrical field stimulation was applied through platinum electrodes, placed on top and bottom of the chambers with a Grass S88 stimulator (West Warwick, RI, USA) during the 3rd (S<sub>1</sub>, 360 pulses) and the 13th fractions (S<sub>2</sub>, 360 pulses) at 60 V, 2 Hz, 0.5 ms duration. Drugs were added to the perfusion solution at the 24th min of the collecting period (8th fraction) and maintained till the end of the experiments. The FR values during S<sub>1</sub> and S<sub>2</sub> were calculated by subtracting the mean of the basal release (FR before and after the S) from the total FR during stimulation. The ratio of FR values of the second stimulation over the first (FRS<sub>2</sub>/FRS<sub>1</sub>) expresses the effect of the applied drug on the stimulation-evoked release. The effect of drugs on resting release was calculated by the ratio of the average of two resting FR values of the 9–10th fractions, in the presence of the drug (R<sub>2</sub>), over the 6–7th fractions (R<sub>1</sub>), when the drug has not reached the cochlea yet (FRR<sub>2</sub>/FRR<sub>1</sub>). Effect of bicuculline on the EGIS-12233-evoked release was tested in separate experiments with no electric stimulation. Bicuculline was present during the whole experiment. Additionally to the reproducibility of DA release and its inhibition by voltage-dependent sodium [28] or voltage-dependent calcium channel blockade [27, 29], the viability of our preparation was also shown by light- and electron microscopy (EM) performed right before and after the experiments [28, 30].

### Receptor Binding Studies

Competition binding studies for drugs acting as ligands on 5-HT<sub>6</sub> and 5-HT<sub>7</sub> receptors were performed at multiple serotonergic and dopaminergic receptors and adrenoceptors. The protocols employed for examination of drug affinities at specific receptor types are summarized in Table 1.

**Table 1** Conditions for competition binding at monoaminergic receptors, summarized experimental conditions

Receptor	Rat 5-HT <sub>1A</sub>	Human 5-HT <sub>1D</sub>	Rat 5-HT <sub>2A</sub>	Human 5-HT <sub>6</sub>	Human 5-HT <sub>7</sub>	Rat α <sub>1</sub>	Rat α <sub>2</sub>	Rat D <sub>1</sub>	Rat D <sub>2</sub>	Human D <sub>4</sub>
Tissue	Frontal cortex	BHK cells	Frontal cortex	HEK 293 cells	CHO cells	Frontal cortex	Frontal cortex	Striatum	Striatum	CHO cells
Radioligand (nM)	<sup>3</sup> H 8-OH-DPAT (0.8)	<sup>3</sup> H GR125743 (5.0)	<sup>3</sup> H Ketanserin (1.0)	<sup>3</sup> H LSD (3.0)	<sup>3</sup> H LSD (5.5)	<sup>3</sup> H Prazosin (0.3)	<sup>3</sup> H Idazoxan (8.9)	<sup>3</sup> H SCH 23390 (2.8)	<sup>3</sup> H Spiperone (0.6)	<sup>3</sup> H Spiperone (0.7)
Non-specific ligand (μM)	5-HT (10)	Yohimbine (10)	Cyproheptadine (10)	5-HT (100)	Clozapine (25)	Prazosin (1)	Phentolamine (10)	(+) SCH23390 (1)	(+) Butaclamol (1)	Haloperidol (25)
	Tris (50)	Tris (50)							Tris (50)	Tris (50)

Buffer components and concentrations (mM)	CaCl <sub>2</sub> (6.66) Pargyline (0.01) Ascorbic acid (0.16%)	CaCl <sub>2</sub> (4) Pargyline (0.01) Ascorbic acid (0.1%)	Tris (50)	Tris (50) MgCl <sub>2</sub> (10) EDTA (0.5)	Tris (50) MgSO <sub>4</sub> (10) EDTA (0.5)	Tris (50)	Tris (50)	Tris (50) MgSO <sub>4</sub> (5) EGTA (1)	NaCl (120) KCl (5) CaCl <sub>2</sub> (2) MgCl <sub>2</sub> (1) Ascorbic acid (0.1%)	NaCl (120) KCl (5) MgCl <sub>2</sub> (5) EDTA (1)
Incubation	30 min 25°C	60 min 25°C	15 min 37°C	60 min 37°C	120 min 27°C	45 min 25°C	30 min 25°C	30 min 37°C	15 min 37°C	120 min 27°C
pH	7.7	7.7	7.7	7.4	7.4	7.7	7.7	7.4	7.4	7.4
Literature reference	[31]	[32]	[33]			[34, 35]	[34]	[36, 37]	[38]	[39]

## Drugs Used in this Study

The 5-HT<sub>6</sub> receptor antagonist SB-271046 was synthesized by Dr. P. Matyus, Department of Organic Chemistry, Semmelweis University, Budapest, Hungary. The 5-HT<sub>7</sub> receptor ligands SB-258719 and EGIS-11983 (3-{4-[4-(4-chlorophenyl)-piperazine-1-yl]-butyl}-3-ethyl-1,3-dihydro-indole-2-on) and the mixed 5-HT<sub>6</sub>-HT<sub>7</sub> receptor ligand EGIS-12233 (5,7-dichloro-3-{4-[4-(4-chlorophenyl)-piperazine-1-yl]-butyl}-3-ethyl-1,3-dihydro-2H-indole-2-on) were synthesized by Dr. B. Volk with the technical collaboration of Drs. J. Barkoczy and G. Simig, Division of Chemical Research, EGIS Pharmaceuticals Plc [40] (Fig. 1). The D<sub>4</sub> receptor antagonist L-741,741 was purchased from Tocris, Northpoint, UK. Serotonin creatinine sulfate, yohimbine, cyproheptadine, clozapine, prazosin, phentolamine, (+)SCH-23390, (+) butaclamol, and bicuculline were purchased from Sigma (St. Louis, MO, USA). [<sup>3</sup>H]8-OH-DPAT, [<sup>3</sup>H]GR125743, [<sup>3</sup>H]ketanserin, [<sup>3</sup>H]LSD, [<sup>3</sup>H]prazosin, [<sup>3</sup>H]idazoxan, [<sup>3</sup>H]SCH-23390, [<sup>3</sup>H]spiperone, [<sup>3</sup>H]YM-09151-2 and [<sup>3</sup>H]dopamine HCl were obtained from New England Nuclear Life Science Products (Boston, MA, USA) and Amersham Life Sciences (Buckinghamshire, UK), respectively. All other chemicals were of analytical grade.

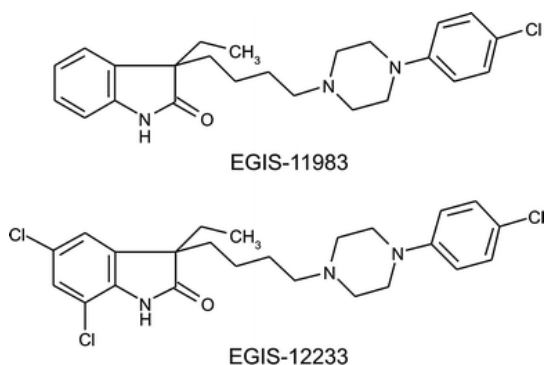


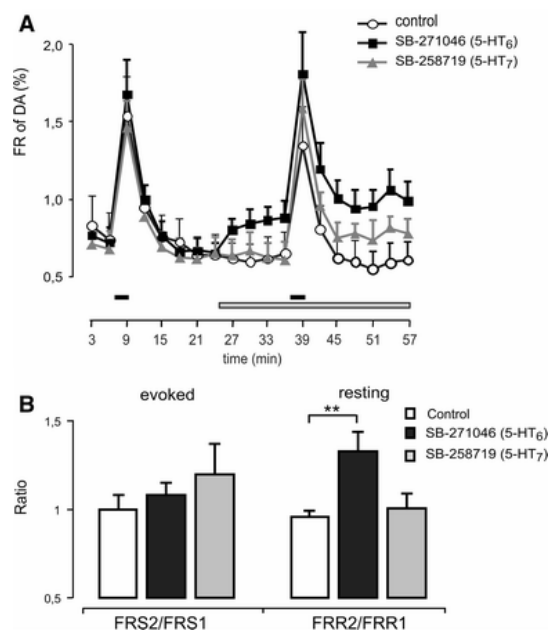
Fig. 1 The chemical structures of EGIS-11983 and EGIS-12233

## Statistical Analysis

Number of experiments (*n*) corresponds the number of microvolume superfusion experiments (each with a single cochlea). Experiments were performed in two microchambers parallel and each one treated with different drugs. Data are expressed as means ± SEM. ANOVA followed by Tukey HSD post hoc comparisons was used to determine the statistical significance.

## Results

Under control circumstances, electrical field stimulations of isolated cochleae produced stable and reproducible release of DA (Fig. 2a); the mean ratio of evoked DA release ( $FRS_2/FRS_1$ ) was close to one ( $0.94 \pm 0.1$ ,  $n = 7$ ). Between the electrical stimulations, the resting release, i.e., the DA outflow was also stable during the control experiments as revealed by the  $FRR_2/FRR_1$  ratio ( $0.93 \pm 0.04$ ,  $n = 7$ ). In the next experiments, we have tested the effect of 5-HT<sub>6</sub> and 5-HT<sub>7</sub> receptor antagonists on cochlear DA release: SB-258719 and SB-271046 are highly selective antagonists for the 5-HT<sub>7</sub> and 5-HT<sub>6</sub> receptors, respectively. We also tested the effect of EGIS-11983, a 5-HT<sub>7</sub> + D<sub>4</sub> antagonist; and EGIS-12233 a newly developed 5-HT<sub>6/7</sub> antagonist. Binding experiments confirmed the selectivity of SB-258719 and SB-271046 for their receptor subtypes (Table 2). EGIS-11983 preferentially binds to 5-HT<sub>7</sub> receptors versus 5-HT<sub>6</sub> receptors. However, EGIS-11983 also showed affinity to D<sub>4</sub> and other 5-HT receptors, such as 5-HT<sub>1D</sub> and 5-HT<sub>2A</sub>. EGIS-12233 was nearly equally bound to 5-HT<sub>6</sub> and 5-HT<sub>7</sub> receptors, but it showed affinity to 5-HT<sub>1D</sub>, as well (Table 2).



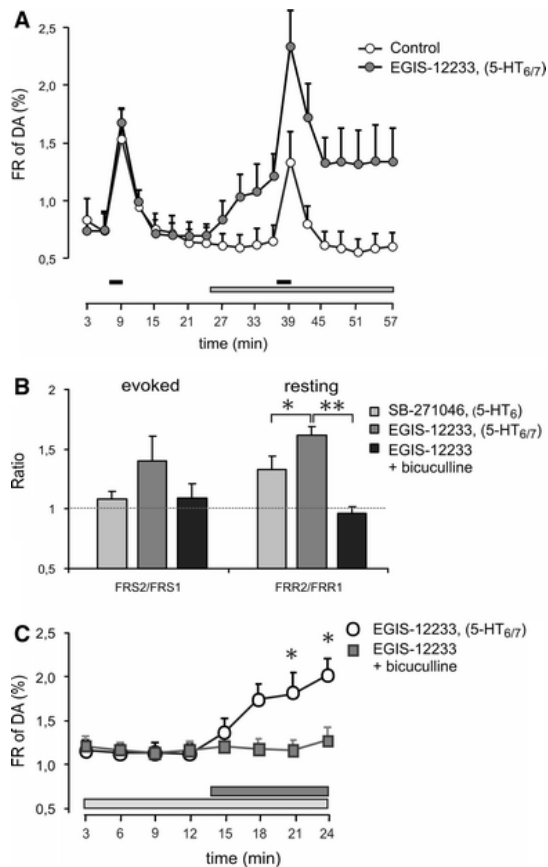
**Fig. 2** 5-HT<sub>6</sub> and 5-HT<sub>7</sub> antagonists differently influence DA release from isolated cochleae. **(a)** Release of DA in control experiments (*open circle*); in the presence of 10  $\mu$ M SB-271046, a selective 5-HT<sub>6</sub> receptor antagonist, (*black rectangle*), and 10  $\mu$ M SB-258719, a 5-HT<sub>7</sub> receptor antagonist (*gray triangle*). 5-HT receptor antagonists were applied from the 24th min as indicated by the horizontal gray bar. Electrical field stimulations ( $S_1$  and  $S_2$ ) are indicated by horizontal black bars. **(b)** Summary bar chart of the effect of SB-258719 and SB-271046 (10–10  $\mu$ M,  $n = 6$ –6) on the resting (FRR<sub>2</sub>/FRR<sub>1</sub>) and evoked (FRS<sub>2</sub>/FRS<sub>1</sub>) release of DA. Data presented are means  $\pm$  SEM; asterisks indicate significant difference from control ( $n = 7$ ; \*\*  $P < 0.01$ )

**Table 2** Binding characteristics of SB-258719, SB-271046, EGIS-11983 and EGIS-12233

Receptor	SB-258719	SB-271046	EGIS-11983	EGIS-12233
5-HT <sub>1A</sub>	0%	9%	11%	7%
5-HT <sub>1D</sub>	–	20%	36 nM	1 nM
5-HT <sub>2A</sub>	2%	0%	17 nM	60 nM
5-HT <sub>6</sub>	7%	0.15 nM	12%	13 nM
5-HT <sub>7</sub>	1.5 nM	0%	2.1 nM	9 nM
$\alpha_1$	8%	19%	93 nM	110 nM
$\alpha_2$	0%	22%	17%	20%
D1	0%	0%	1%	2%
D2	5%	4%	12%	6%
D4	0%	0%	13 nM	42 nM

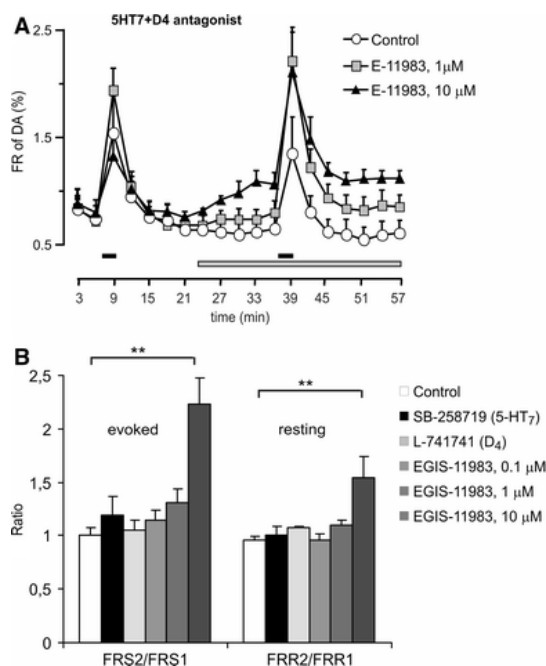
The displacement of the drugs are given in  $K_i$  values (nM) or in percentage at 100 nM concentration

The 5-HT<sub>7</sub> antagonist SB-258719 (10  $\mu$ M) was unable to modulate the release of DA. In contrast, the block of 5-HT<sub>6</sub> receptors by SB-271046 (10  $\mu$ M) significantly elevated the resting outflow of DA in isolated cochleae ( $P < 0.01$ ,  $n = 6$ , Fig. 2a, b). The electrical field stimulation-evoked release of DA did not change in the presence of SB-271046 (Fig. 2b). When both 5-HT<sub>6</sub> and 5-HT<sub>7</sub> receptors were blocked by EGIS-12233 ( $n = 7$ ) the increase in the resting DA release was larger than in the case of selective block of 5-HT<sub>6</sub> receptors ( $P < 0.05$ ; Fig. 3a, b). In the presence of EGIS-12233 the increase in the electrical stimulation-evoked release was nearly significant ( $P = 0.06$ , Fig. 3b). Because both 5-HT<sub>6</sub> and 5-HT<sub>7</sub> receptors produce stimulatory effects on target cells, the antagonists of these receptors are expected to have either an inhibitory effect (in case of tonic endogenous stimulation of the receptor) or no effect. Our finding that the inhibition of 5-HT<sub>6</sub>/5-HT<sub>7</sub> receptors caused excitation instead of depression in the release of DA from the cochlea raised the possibility of an indirect, disinhibitory mechanism. To address this question, we blocked the local GABAergic input by bicuculline, a selective GABA<sub>A</sub> receptor antagonist. In the presence of bicuculline (20  $\mu$ M) EGIS-12233 was not able to increase the resting release of DA in the cochlea (Fig. 3b, c).



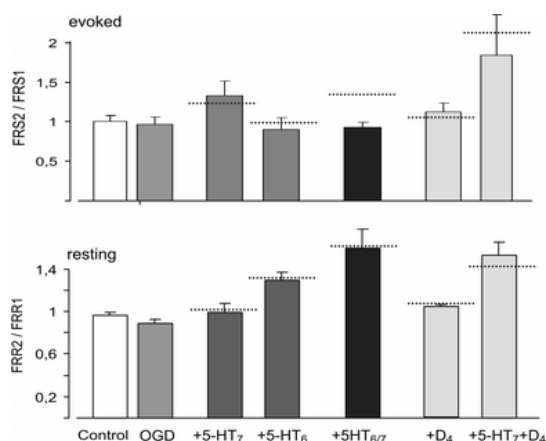
**Fig. 3** The effect of the mixed 5-HT<sub>6/7</sub> antagonist EGIS-12233 on cochlear DA release. **(a)** Release of DA in control experiments (*open circle*) and in the presence of 10  $\mu$ M EGIS-12233, a newly developed 5-HT<sub>6/7</sub> receptor antagonist (*gray circle*). The antagonist was applied from the 24th min as indicated by the horizontal gray bar. Electrical field stimulations ( $S_1$  and  $S_2$ ) are indicated by horizontal black bars. **(b)** Summary bar chart of the effect of EGIS-12233 (10  $\mu$ M,  $n = 7$ ) on the resting ( $FRR_2/FRR_1$ ) and evoked ( $FRS_2/FRS_1$ ) DA release alone and in the presence of 20  $\mu$ M bicuculline, a GABA<sub>A</sub> antagonist ( $n = 7$ ). Dotted line represents the unchanged release (ratio of 1). Asterisk indicates the significantly different effect of EGIS-12233 on resting release compared to SB-271046 (10  $\mu$ M,  $n = 6$ , \*  $P < 0.05$ ). **(c)** Resting release of DA over time; EGIS-12233 (*open circle*) failed to increase the resting DA outflow in the presence of bicuculline (*gray rectangle*). EGIS-12233 (10  $\mu$ M) was perfused from the 14th min (*dark gray bar*); bicuculline was present in the bath during the whole length of the experiment (*light gray bar*). ( $n = 6$ , \*  $P < 0.01$ ). Data presented are means  $\pm$  SEM

Next, we tested whether the inhibition of 5-HT<sub>7</sub> receptors could also facilitate drug action on non-serotonergic receptors, e.g. dopaminergic receptors, which are also involved in the modulation of DA release in the cochlea [28]. To test this assumption D<sub>4</sub> receptors were investigated whether 5-HT<sub>7</sub> antagonism can amplify the effect of their inhibition. The D<sub>4</sub> receptor antagonist L-741,741, at 1  $\mu$ M caused no effect on the resting or evoked release of cochlear DA (Fig. 4b). When the mixed 5-HT<sub>7</sub>/D<sub>4</sub> antagonist EGIS-11983 was used, not only the resting DA outflow ( $FRR_2/FRR_1$ ) was enhanced by the antagonist but the stimulation-evoked DA release ( $FRS_2/FRS_1$ ) increased significantly at 10  $\mu$ M concentration. At lower concentrations (0.1 and 1  $\mu$ M) there was no significant effect. Thus, the simultaneous block of both receptors uncovered the function of these receptors that was hidden when they were blocked separately.



**Fig. 4** The effect of the mixed 5-HT<sub>7</sub>/D<sub>4</sub> antagonist EGIS-11983 on cochlear DA release. **(a)** DA release over time under control conditions (*open circle*) and in the presence of 1  $\mu\text{M}$  (*gray rectangle*) and 10  $\mu\text{M}$  (*black triangle*) of EGIS-11983 applied from the 24th min (indicated by the horizontal gray bar). S<sub>1</sub> and S<sub>2</sub> are indicated by horizontal black bars. **(b)** Summary bar chart of the effect of mixed 5-HT<sub>7</sub>/D<sub>4</sub> (EGIS-11983) and D<sub>4</sub> (L-741,741, 1  $\mu\text{M}$ ,  $n = 6$ ) antagonists on the resting (FRR<sub>2</sub>/FRR<sub>1</sub>) and evoked (FRS<sub>2</sub>/FRS<sub>1</sub>) DA release. EGIS-11983 was applied at 0.1, 1, and 10  $\mu\text{M}$  concentration ( $n = 7$ –7 and 6, respectively). For comparison, the effect of SB-258719, a selective 5-HT<sub>7</sub> antagonists, on DA release is also shown (10  $\mu\text{M}$ ,  $n = 6$ ). Control,  $n = 7$ . Data presented are means  $\pm$  SEM

Oxygen-glucose deprivation (OGD) is thought to mimic the ischemic insult of the cochlea. In agreement with our earlier report [28], OGD did not produce elevation in the basal release of DA in the absence of D<sub>2</sub> dopamine receptor antagonists (Fig. 5). Repeating the experiments under OGD, the effect of receptor antagonists produced a similar pattern to that without OGD (Fig. 5). 5-HT<sub>7</sub> and 5-HT<sub>6</sub> antagonists produced essentially the same effect during OGD (Fig. 5). The mixed 5-HT<sub>6/7</sub> antagonist EGIS-12233 seemed to have smaller effect on the evoked DA release in OGD but this difference was not significant. The mixed 5-HT<sub>7</sub>/D<sub>4</sub> antagonist EGIS-11983 and the selective D<sub>4</sub> antagonist L-741,741 did not cause significantly different effect on resting or evoked DA outflow during OGD as compared to their effects without OGD (Fig. 5).



**Fig. 5** The effect of the 5-HT<sub>7</sub>, 5-HT<sub>6</sub> and D<sub>4</sub> antagonists on cochlear DA release during oxygen/glucose deprivation (OGD). The resting (FRR<sub>2</sub>/FRR<sub>1</sub>, *lower panel*) and the evoked (FRS<sub>2</sub>/FRS<sub>1</sub>, *upper panel*) release of DA was not statistically different between control and OGD-treated cochleae. During OGD the effects of the 5-HT<sub>7</sub> antagonist SB-258719 (+5-HT<sub>7</sub>, 10  $\mu\text{M}$ ,  $n = 6$ ), the 5-HT<sub>6</sub> antagonist SB-271046 (+5-HT<sub>6</sub>, 10  $\mu\text{M}$ ,  $n = 9$ ), the 5-HT<sub>6/7</sub> antagonist EGIS-12233 (+5-HT<sub>6-7</sub>, 10  $\mu\text{M}$ ,  $n = 8$ ), the D<sub>4</sub> antagonist L-741,741 (+D<sub>4</sub>, 1  $\mu\text{M}$ ,  $n = 5$ ), and the 5-HT<sub>7</sub>/D<sub>4</sub> antagonist EGIS-11983 (+5-HT<sub>7</sub> + D<sub>4</sub>, 10  $\mu\text{M}$ ,  $n = 5$ ) on DA release were not statistically different from their corresponding effects on DA release without OGD (indicated by dotted lines). Data presented are means  $\pm$  SEM

## Discussion

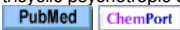



In order to explain the increase in the resting release of DA by antagonists of 5-HT<sub>6</sub> and 5-HT<sub>7</sub> receptors, which are coupled to the stimulatory G proteins and thus activate adenylate cyclase, we assume that 5-HT<sub>6</sub> and 5-HT<sub>7</sub> receptors are localized on an inhibitory element. In our previous study, we found that the block of GABA<sub>A</sub> receptors could prevent the excitatory action of 2R,4R-APDC, a group II metabotropic glutamate receptor agonist, on DA release [41]. This result suggested that most likely GABAergic elements of the cochlea contain the mGluRs, which turn off GABA release, and results in a decreased inhibition (disinhibition) on the dopaminergic terminals. Our previous experiments already indicated that spontaneous GABA release keeps the cochlear dopaminergic nerve endings under tonic inhibition [41]. In the present study, we showed that the 5-HT<sub>6</sub> and 5-HT<sub>7</sub> receptors also have an indirect mechanism of action on DA release from LOC terminals, i.e., located on the GABAergic neural elements they are most likely to induce GABA release which results in the inhibition of DA release. Prevention of the stimulatory action of serotonin by 5-HT<sub>6</sub> and 5-HT<sub>7</sub> antagonists, on the contrary, resulted in an elevation of DA release. It has to be mentioned that the mixed 5-HT<sub>6/7</sub> antagonist EGIS-12233 also shows significant affinity to 5-HT<sub>1D</sub> receptors (see Table 2). However, we found that the block of the GABAergic function (by bicuculline) abolished the enhancing action of EGIS-12233 (Fig. 3) suggesting that the serotonin receptors involved are located on GABAergic terminals in the cochlea. Assuming 5-HT<sub>1D</sub> receptors on GABAergic boutons, the inhibition of these receptors by EGIS-12233 would cause an opposite effect on DA release: an inhibition of release instead of the observed increase in DA release.


















There is accumulating evidence that manipulation of 5-HT<sub>6</sub> receptor function influences GABA activity in the nervous system. The strict connectivity between 5-HT<sub>6</sub> receptors and the GABAergic system is supported by the observation that 5-HT<sub>6</sub> receptor messenger RNA is primarily expressed in GABAergic neurons of the striatum [42]. In addition, the synthesizing enzyme for GABA, glutamic acid decarboxylase (GAD), and 5-HT<sub>6</sub> receptors are colocalized in the rat cerebral cortex and hippocampus [43]. The 5-HT<sub>6</sub> agonist, WAY-466, elevates GABA levels in multiple brain regions associated with anxiety, including frontal cortex and amygdala [44]. Another agonist of the 5-HT<sub>6</sub> receptor, compound 11q, has also been shown to increase GABA levels in the rat frontal cortex [45].



































The present results suggest that GABAergic fibers express functional 5-HT<sub>6/7</sub> receptors and their antagonists are able to decrease the release of GABA, which leads to the disinhibition of DA containing LOC terminals. Although the selective 5-HT<sub>7</sub> antagonist was ineffective to modulate DA release (with an insignificant tendency to increase the evoked release, see Fig. 2), functional 5-HT<sub>7</sub> receptors likely exist in the cochlea as (i) the mixed 5-HT<sub>6/7</sub> antagonist was more effective than the selective 5-HT<sub>6</sub>, (ii) the mixed 5-HT<sub>7</sub>/D<sub>4</sub> receptor antagonist produced a marked enhancement of cochlear DA release, i.e., it could increase both the resting and the electrical stimulus-evoked outflow, despite the lack of effect of selective D<sub>4</sub> ligand. Nevertheless, the activity the 5-HT<sub>6</sub> receptors dominates the DA release response to serotonergic influence. D<sub>4</sub> receptors may also participate in the modulation of DA release in the cochlea, similarly to D<sub>2</sub> receptors which have a role in the negative feedback modulation of DA release from the dopaminergic LOC terminals [28]. The finding that the mixed 5-HT<sub>7</sub>/D<sub>4</sub> was highly effective also indicates the supporting role of D<sub>4</sub> receptors, which may be visualized at higher concentration of the D<sub>4</sub> specific antagonist. The released DA can inhibit both natural sound stimulation- and pathological overstimulation-evoked activity of the afferent dendrites [20–25]. Our findings thus support the assumption that cochlear serotonergic fibers could be implicated in the complex modulatory processes of the auditory function, as other neuroactive substances of the olivocochlear system. Given the well-based theory of the protective action of cochlear DA (see Introduction), the 5-HT<sub>6/7</sub> antagonist-mediated enhancement of DA release from LOC terminals may serve as a fast-acting local mechanism in the cochlea by which these drugs can protect cochlear cells against harmful noxae during various physiological and pathophysiological processes. An augmented, multiple-target DA release action may form the basis of a potentially more effective therapy of sensorineural hearing losses.

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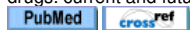
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