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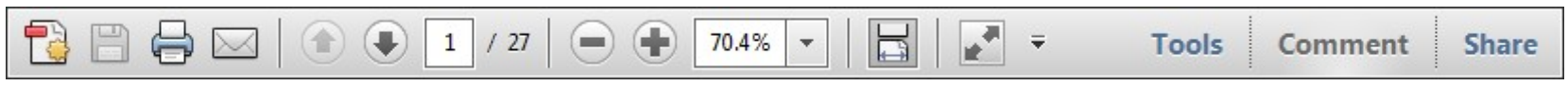

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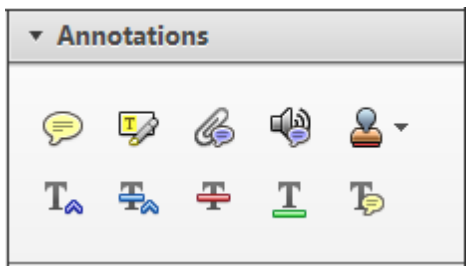

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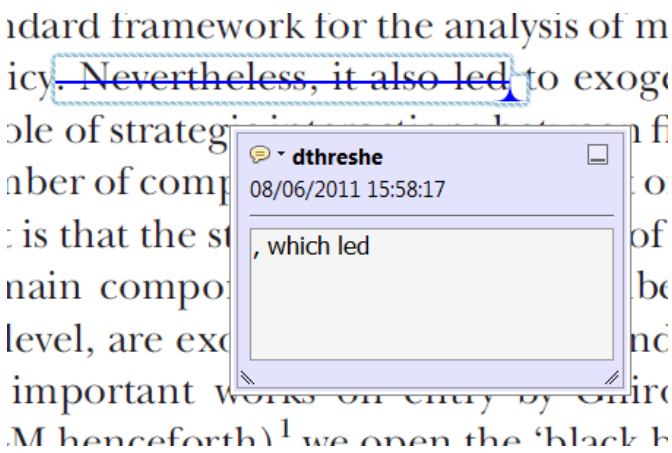

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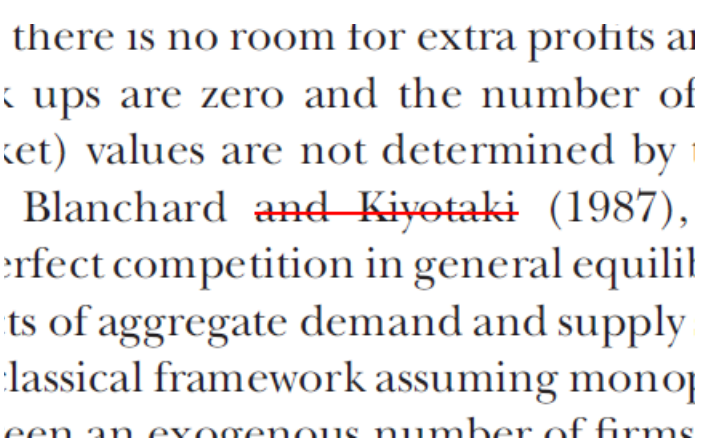

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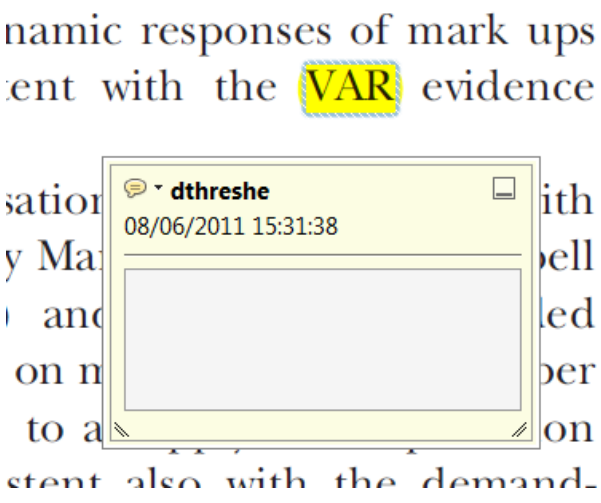

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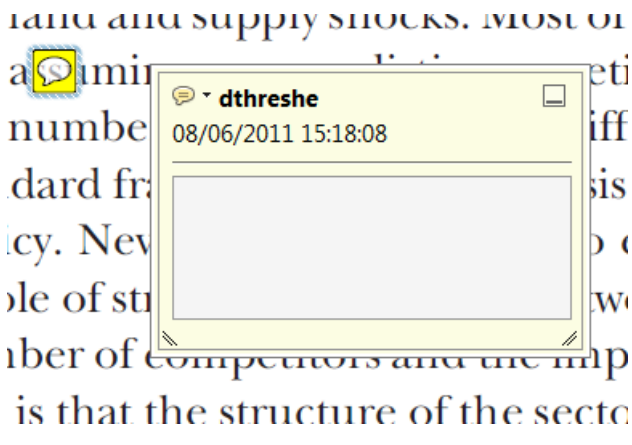

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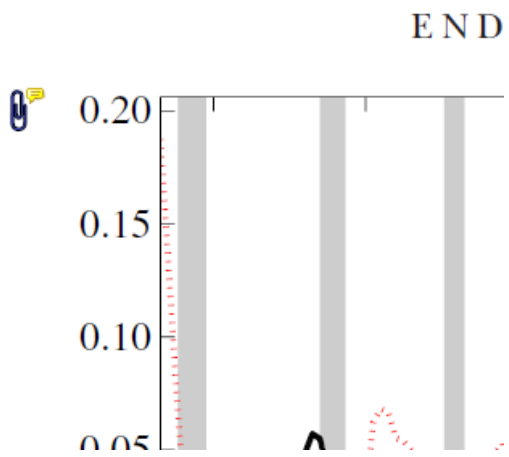

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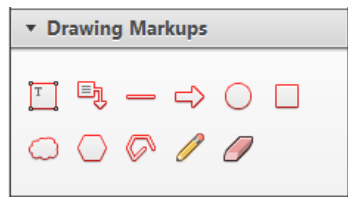

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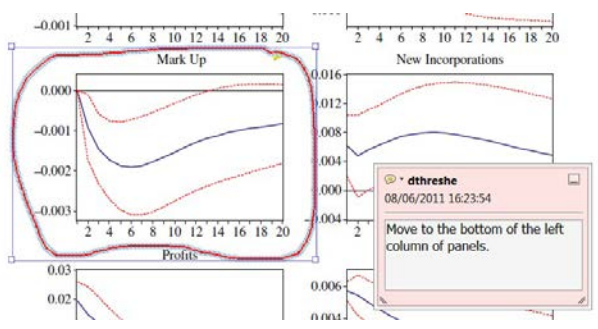

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Activation of 5-HT Receptors Inhibits GABAergic Transmission by Pre-and Post-Synaptic Mechanisms in Layer II/III of the Juvenile Rat Auditory Cortex

AQ4

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AQ3 AQ2

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KEY WORDS GABA$_A$ receptors; 5-HT receptors; GABA release; auditory cortex; cortical circuitry; whole-cell

ABSTRACT The specific mechanisms by which serotonin (5-HT) modulates synaptic transmission in the auditory cortex are still unknown. In this work, we used whole-cell recordings from layer II/III of pyramidal neurons in rat brain slices to characterize the influence of 5-HT on inhibitory synaptic activity in the auditory cortex after pharmacological blockade of excitatory glutamatergic transmission. We found that bath application of 5-HT (5 μM) reduced the frequency and amplitude of both spontaneous and miniature inhibitory postsynaptic currents (IPSCs), reduced the amplitude of evoked IPSCs, and enhanced facilitation of paired pulse ratio (PPR), suggesting presynaptic inhibition. To determine whether the serotonin receptors were involved in this effect, we studied the influence of specific 5-HT receptor agonists and antagonists on ɣ-aminobutyric acid (GABA)ergic synaptic transmission. The inhibiting influence of 5-HT in the GABAergic synaptic activity was mimicked by using the selective agonists of the 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors, 8(OH)-DPAT (10 μM) and DOI (10 μM), respectively; and it was prevented by their respective antagonists NAN-190 (1 μM) and ritanserin (1 μM). Furthermore, the application of the selective agonist of 5-HT$_{1A}$ receptors, 8-(OH)-DPAT (10 μM), produced PPR facilitation, while DOI application (5-HT$_{2A}$ agonist) did not change the PPR. Moreover, the 5-HT$_{2A}$ agonist reduced the amplitude of the IPSCs evoked by application of the selective GABA agonist, muscimol. These results suggest a presynaptic and postsynaptic reduction of GABAergic transmission mediated by 5-HT$_{1A}$ and 5-HT$_{2A}$ serotonergic receptors, respectively. **Synapse 00:000–000, 2014.** © 2014 Wiley Periodicals, Inc.

INTRODUCTION

The auditory cortex, like other cortical areas, receives an intense axonal projection from the serotonergic raphe nuclei neurons (Lidov et al., 1980). However, the exact role of the 5-HT projections and their receptors in the auditory cortex is not clear. Studies using the loudness dependence of auditory evoked potentials have suggested that 5-HT is involved in auditory processing (Juckel et al., 2003), in the integration of electrical signals in neurons, and in the processing of sensory signals (Ji and Suga, 2007; Jitsuki et al., 2011; Juckel et al., 2003). In fact, a recent *in vitro* study showed that 5-HT modulates intrinsic cellular excitability in pyramidal cells in the auditory

F.G.O. and O.T.R. contributed equally to this study.

Author contributions: FGO, OTR performed sIPSCs, mIPSCs and evoked IPSCs experiments. LD and LGC performed muscimol IPSCs currents. EAPP performed sIPSCs and muscimol IPSCs currents. FGO, OTR and HS performed data analysis. JCP and MA gave intellectual contributions and reviewed the manuscript. HS developed the ideas of the project and the experimental plan, interpreted the results and wrote the manuscript.

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cortex via multiple receptor subtypes, with opposite actions (Rao et al., 2010), and producing different types of long term plasticity (Dringenberg et al., 2014).

The existence of the serotonergic projections in the auditory cortex suggests the involvement of 5-HT in the modulation of functions related to attention, task-related processing of auditory information, the reorganization of the cortical auditory map, and the formation of acoustic signal memories relevant for behavior (Buonomano and Merzenich, 1998; Chowdhury and Suga, 2000; Dahmen et al., 2010; Jääskeläinen and Ahveninen, 2014; Ma and Suga, 2001; Scheich et al 2011).

Serotonin dysfunction is involved in the pathogenesis of numerous illnesses such as depression, attention deficit hyperactivity disorder (ADHD), schizophrenia, panic disorder and anxiety (Blier and Ward, 2003; Juckel et al., 2003; Kang et al., 2009; Williams et al., 2003). Many of the drugs commonly prescribed to treat these among other psychiatric conditions, such as psychostimulants, are selective serotonin reuptake inhibitors (SSRIs) designed to increase the extracellular levels of 5-HT in the brain (Andrews and Lavin, 2006; Dringenberg et al., 2014; Maya-Vetencourt et al., 2008). Studies analyzing the effects of serotonergic agonists on neuronal network activity in the auditory cortex have demonstrated several findings. For example, the fluoxetine reduces the long-term potentiation (LTP) recorded *in vivo* in the primary auditory cortex (A1) (Dringenberg et al., 2014), 5-HT modulates excitability of pyramidal neurons in the auditory cortex in rats (Rao et al., 2010) and mice (Xia et al., 2003) and 5-HT is involved in auditory learning in gerbils (Stark and Scheich, 1997). These studies, however, do not clarify the direct impact of the 5-HT on synaptic transmission. The determination of the mechanisms of action of the 5-HT on synaptic transmission in the auditory cortex is critical for understanding the regulation of the auditory functions associated with the balance of excitation and inhibition in auditory cortex circuits.

Interestingly, different studies suggest that inhibitory cortical interneurons are a principal target of the 5-HT system and they may promote sensory representation, attention, working memory and other executive functions (Bacci et al., 2005; DeFelipe et al., 1991; Paspalas and Papadopolus., 2001; Smiley and Goldman-Racik, 1996).

In the neocortex and other areas, the 5-HT exerts excitatory and inhibitory effects on GABAergic transmission (Deng and Lei, 2008; Jang et al., 2012; Lee et al., 2010; Rav-Acha et al., 2008; Saitow et al., 2009; Yan, 2002; Zhong and Yan, 2004; Zhou and Hablitz, 1999; for a review see Celada et al., 2013). These differential actions may be attributed to the participation of different receptor subtypes. For instance, 5-HT produces an increase of GABA release in prefrontal and entorhinal cortex, as well as in the hippocampus by 5-HT_3 receptor activation (Choi et al., 2007; Deng and Lei, 2008; Zhou and Hablitz, 1999; for a review see Celada et al., 2013), whereas activation of 5-HT_{2A} receptors increases GABA synaptic transmission in the prefrontal and visual cortex (Abi-Saab et al., 1999; Jang et al., 2012). In contrary, 5-HT_{2A} receptors decrease $GABA_A$ currents in prefrontal cortical neurons (Feng et al., 2001).

However, there has not been a detailed assessment on the effects of 5-HT on the GABAergic synaptic transmission in auditory cortex. In the present study the effects of 5-HT on GABAergic transmission were analyzed in layer II/III pyramidal neurons using whole-cell configuration in voltage clamp mode. Our results suggest that 5-HT produces a reduction of GABA release via activation of presynaptic 5-HT_{1A} receptors, and through the postsynaptic modulation of the $GABA_A$ currents by activation of 5-HT_{2A} receptors.

MATERIAL AND METHODS

Preparation

We used a coronal auditory cortex slice preparation similar to the one previously described (Atzori et al., 2001; Salgado et al., 2011). Sprague-Dawley rats, 25- to 30-days old (Charles River, Wilmington, MA) were anesthetized with 1 ml of isoflurane (99.9%, Baxter, Round Lake, IL) in an acrylic box, mounted in a laminar flow hood until the rats were areflexics. Posteriorly we cut the rat neck with a guillotine and the brain was extracted, according to the National Institutes of Health Guidelines (UTD IACUC number 04-04 and by the Ethics Committee at the Universidad Autonoma de Yucatan), and their brains sliced with a vibratome (VT1000, Leica, Germany) in a cold solution (0–4°C) containing (in mM): 126 NaCl, 3.5 KCl, 10 Glucose, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 1.5 $CaCl_2$ and 1.5 $MgCl_2$, pH 7.4 and was saturated with a mixture of 95% O_2 and 5% CO_2 (ACSF). Coronal slices (270-μm thick) from the most caudal fourth of the brain were retained after removing the occipital convexity, and subsequently incubated in ACSF at 32°C before being placed in the recording chamber. The recording area was selected dorsally to the rhinal fissure corresponding to the auditory cortex (Rutkowski et al., 2003, Fig. 1A). The extracellular solution also contained 6,7-Dinitroquinoxaline-2,3-dione (DNQX, 10 μM) and kynurenate (2 mM) to block alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)- and N-methyl-D-aspartate receptor (NMDAR)-mediated currents, respectively. F1

Electrophysiology

Slices were placed in an immersion chamber, where cells with a prominent apical dendrite, suggestive of pyramidal morphology, were visually selected using a

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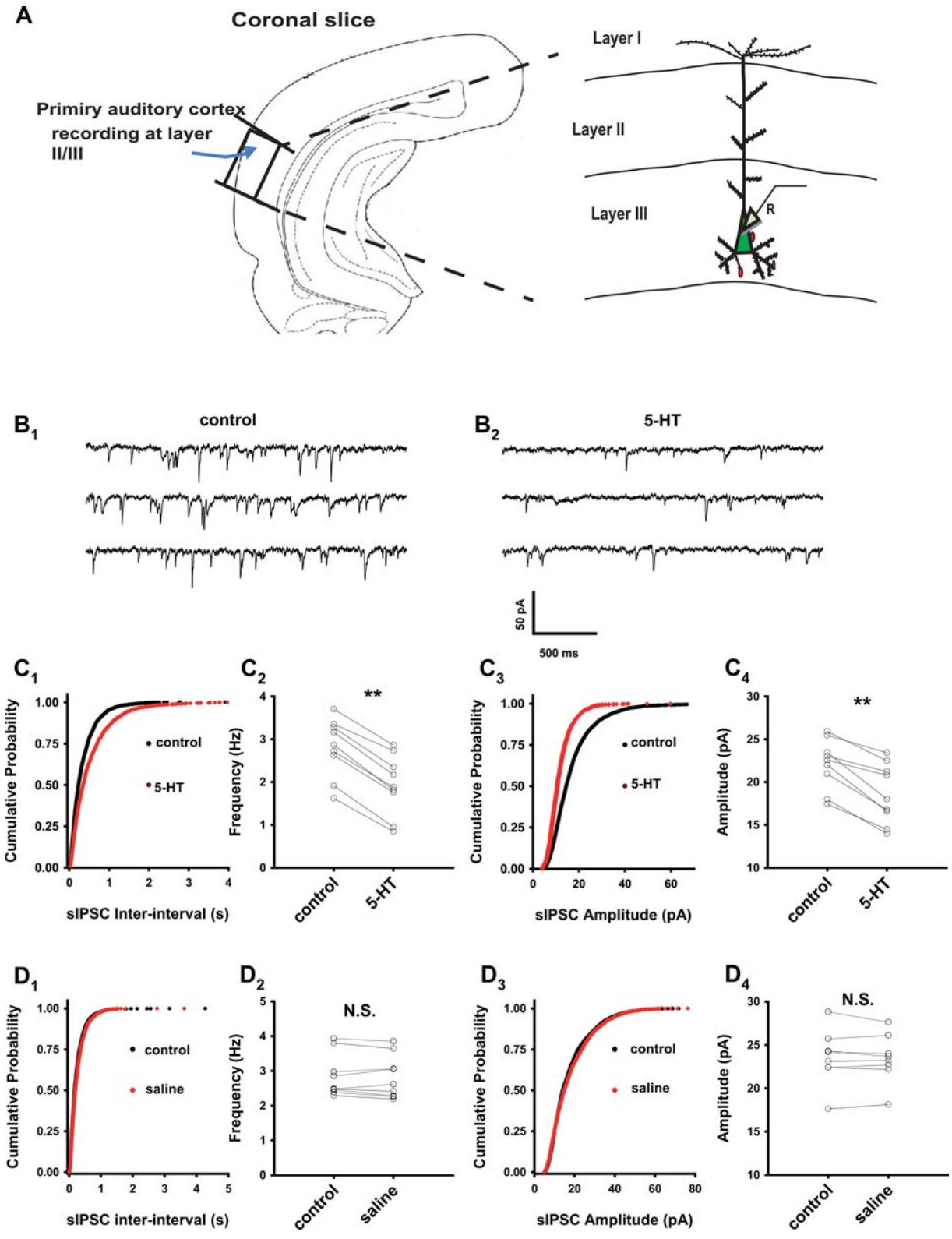

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Fig. 1. The 5-HT decreases the frequency and amplitude of sIPSCs. **A**. Diagram showing the area of recording in the auditory cortex. $\mathbf{B_{1-2}}$: Representative traces illustrating sIPSCs obtained in the absence (B_1) or in the presence (B_2) of 5-HT (5 μM). The 5-HT decreases the sIPSCs frequency and amplitude. **C**: Cumulative probability distribution of sIPSCs inter-event interval ($\mathbf{C_1}$) and amplitude, ($\mathbf{C_3}$) before and during application of 5-HT. $\mathbf{C_2}$ and $\mathbf{C_4}$: summary of 5-HT effect on sIPSC frequency ($n = 9$, $P = 0.001$, paired t test) and amplitude ($n = 9$, $P = 0.001$, paired t test), respectively. **D**: Cumulative probability distribution of sIPSCs inter-event interval ($\mathbf{D_1}$) and amplitude ($\mathbf{D_3}$) before and during application of saline solution. $\mathbf{D_2}$ and $\mathbf{D_4}$: summary of saline solution effect on sIPSC frequency ($n = 9$, $P = 0.48$, paired t test), and amplitude ($n = 9$, $P = 0.99$, paired t test), respectively. Double asterisk (**) indicate $P < 0.01$, (N.S.) indicate no significant differences. All recordings were done in presence of DNQX and kynurenic acid.

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BX 51 microscope (Olympus, Japan) with an infrared camera system (DAGE-MTI, Michigan City, IN). Inhibitory postsynaptic currents (IPSCs) were recorded in the whole-cell configuration, in voltage clamp mode, holding potential at Vh = −60 mV, with 3–5 M∧ electrodes filled with a solution containing (in mM): 100 $CsCl_2$, 5 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid K (BAPTA-K), 1 lidocaine *N*-ethyl bromide (QX314), 1 $MgCl_2$, 10 *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), 4 glutathione, 3 $ATPMg_2$, 0.3 $GTPNa_2$ and 20 phosphocreatine. The intracellular recording solution was titrated at pH 7.2 and osmolarity of 275 mOsm. The holding voltage was not corrected for the junction potential (<4 mV).

Electrically evoked IPSCs (eIPSCs) were measured by delivering two electric stimuli (90–180 μs, 10–50 μA), 50-ms-apart every 10 s with an isolation unit (A365, World Precision Instruments, Sarasota, FL), through a glass stimulation monopolar electrode filled with ACSF and placed at about 150–200 μm from the recording electrode. Before the start of an experiment, we set the duration and the amplitude of the stimulation in order to obtain an optimal response. Once these parameters were established for a particular record, they were maintained unchanged for the duration of the experiment. A 2-mV voltage step was applied at the beginning of every episode in order to monitor the quality of the recording. Access resistance (10–20 MΩ) was constantly monitored and remained stable during all the experiments (<20%). All signals were filtered at 2 KHz and sampled at 10 KHz. Muscimol was applied by puffer system through borosilicate microelectrodes placed in the vicinity of the proximal dendrites of postsynaptic currents (PCs) from which recordings were obtained. All experiments were performed at room temperature (25–28°C).

Drugs and solutions

All drugs were purchased from Sigma (St. Louis, MO) and TOCRIS (Ellisville, MO). In some experiments, pulses of $GABA_A$ agonist muscimol (100 μM) were applied at 100–200 μm from the recording areas, every 1 min. A stock solution of muscimol was diluted 10-fold in ACSF before being back-filled to a glass pipette similar to the one used for recording. Muscimol application was performed with a pressure system (picospritzer III, General Valve, Fairfield, NJ) through a glass pipette (≅ 25 psi, 3–12 ms). After recording an initial baseline for 10–15 min, the drugs were bath-applied for 20 min, until reaching a stable condition (as defined below in Statistical Analysis section). All of the drugs were prepared immediately before experiments and their exposure to light was avoided to prevent oxidation. DOI and ritanserin were diluted in methanol; NAN-190 and 8-OH-DPAT were diluted in DMSO.

Data analysis and statistics

We defined a statistically stable period as a time interval (10 min) in which the IPSC amplitude measured during 1-min blocks did not vary, according to RM ANOVA. All data are expressed as mean ± SE. Pair pulse ratio (PPR) was calculated as the mean of the second response divided by the mean of the first response, according to Harris and Cotman (1983, 1985). Only well-isolated IPSCs were considered for spontaneous and miniature postsynaptic currents (sIPSCs and mIPSCs), which were prefiltered off-line at 2 KHz. Spontaneous and miniature events were then analyzed with the MiniAnalysis program (Synaptosoft, Decatur, GA) using a semiautomatic procedure. Each detected event was inspected visually to exclude obvious artifacts before analysis. For cumulative probability plots of the sIPSCs or mIPSCs, the events were selected 10 min prior to and 10 min after reaching the maximal effect of 5-HT. Amplitude threshold was set as 2 × σ of the baseline noise, where σ noise was the standard deviation measured during periods of no visually detectable events and was usually <3 pA. Signals <5 pA were excluded from the measurements.

The effects of drug application on the IPSC amplitude changes are reported as: $R \equiv$ (1 - Atreat/Actrl), where Atreat and Actrl were respectively the mean IPSC amplitude of the first current in the PPF protocol in treatment or in control ($R = 1$ corresponded to no change whereas $R = 0$ corresponded to total inhibition. For example $R = 0.5$ corresponded to 50% of inhibition). Drug effects were assessed by measuring and comparing the different parameters (R, IPSC mean amplitude) between baseline (control) vs. treatment, with paired Student's *t* test. ANOVA with post hoc Tukey's test was used for comparisons between different groups of cells. Data were reported as different only if $P < 0.05$ % unless indicated otherwise. Single asterisks (*) indicate $P < 0.05$, double asterisk (**) indicate $P < 0.01$.

RESULTS

Effect of 5-HT on spontaneous and miniature IPSCs in layer II/III pyramidal cells of auditory cortex

All the experiments were performed in the presence of the glutamate channel blockers, kynurenic acid (2 mM) and 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10 μM). Spontaneous IPSCs (sIPSCs) were recorded in pyramidal cells at a holding potential of −60 mV. As illustrated in Figures $1B_1$ and $1B_2$, and summarized in Figures $1C_1$ and $1C_2$, bath application of 5-HT (5μM) to layer II/III pyramidal cells induced a large decrease in the frequency of sIPSCs ($n = 9$). The cumulative probability curve of sIPSCs frequency exhibited a significant right shift during 5-HT application (Fig. $1C_1$).

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Analysis of recordings from nine neurons showed that the sIPSC frequency was decreased from 2.8 ± 0.3 Hz during control condition, to 1.8 ± 0.3 Hz during 5-HT application, (35.7% decrease; Fig. $1C_2$, $n = 9$, $P = 0.001$, paired t test). A representative cumulative probability plot using data taken from the cell in Figures $1B_1$ and $1B_2$, shows that 5-HT also induced a decrease in the amplitude of sIPSCs (Fig. $1C_3$ and $1C_4$; this decrease was observed in all cells tested, p=0.001, paired t-Test); this effect was reversible in four of nine cells tested (data not shown). The sIPSC peak amplitude was 22.3 ± 1 pA in control condition and 18 ± 1.2 pA in presence of 5-HT, (Fig. $1C_4$, $n = 9$, $P = 0.001$; paired t test). In addition, we tested the effect of saline solution application in nine cells, and we found that neither frequency (2.9 ± 0.6 Hz in control vs. 2.8 ± 0.6 Hz in saline solution, $n = 9$, $P = 0.48$; paired t test) nor amplitude (24.3 ± 1.3 pA in control vs. 23.9 ± 1.2 pA in saline solution $n = 9$, $P = 0.99$; paired t test) changed under this condition (Fig. $1D_{1-4}$). Additionally, we compared changes in frequency between groups to show that 5-HT produced a large effect again the baseline conditions ($F = 4{,}324$, $P = 0.04$, ANOVA with post hoc Tukey's test, $n = 9$) and also if we compared with saline solution ($P = 0.029$, ANOVA with post hoc Tukey's test, $n = 9$). We also compared changes in amplitude between 5-HT and control conditions ($F = 6.77$; $P = 0.042$, ANOVA with post hoc Tukey's test, $n = 9$) and in presence of saline solution ($P = 0.003$, ANOVA with post hoc Tukey's test, $n = 9$).

Next, we eliminated the dependency on the action potential, assessing the effect of 5-HT on miniature IPSCs (mIPSCs), recorded in the presence of TTX (1 μM). As shown in Figures 2A and 2B, the frequency and amplitude of the mIPSCs were significantly affected by 5-HT application. The plots in Figures 2C and 2D, show the cumulative frequency and the amplitude distribution of the mIPSCs, in the presence or absence of 5-HT (5 μM). As shown, 5-HT caused a shift toward the right of the inter-event interval distribution, indicating a reduction in their frequency of occurrence. The frequency of the mIPSCs decreased from 2.04 ± 0.18 Hz in control condition, to 1.39 ± 0.2 Hz in the presence of 5-HT ($38.8\% \pm 5\%$ of reduction, $n = 10$; $P = 0.001$; paired t test, Fig. 2E). 5-HT also and significantly reduced the amplitude of mIPSCs from 17.9 ± 1.2 pA to 14.8 ± 1.1 pA (13.2% in reduction, $n = 10$; $P = 0.004$; paired t test, Fig. 2F).

Overall, the IPSC frequency results suggest a presynaptic component of 5-HT modulation of GABAergic transmission in layer II/III of auditory cortex, without ruling out the possibility that changes in the amplitude may additionally represent a postsynaptic modulation.

Effect of 5-HT on evoked IPSCs in layer II/III pyramidal cells of auditory cortex

To evaluate the effects of 5-HT application upon inhibitory postsynaptic currents evoked electrically (eIPSCs) on pyramidal neurons in layer II/III of the rat auditory cortex, the inhibitory postsynaptic currents (IPSCs) were recorded in the presence of nonselective glutamate receptor antagonists, DNQX (10 μM) with kynurenic acid (2 mM) or APV (100 μM). The identity of the GABAergic IPSCs were confirmed by their complete blockage by bicuculline ($n = 10$, 10 μM, data not shown).

The application of 5-HT (5 μM) reduced the amplitude of the eIPSCs (Fig. 3A); this effect was reversed in a period of 20–30 min (25 ± 5 min, Fig. 3A), representative traces are shown in Figure 3B. On average, the eIPSCs amplitude changed from 188 ± 26 pA in control conditions, to 100 ± 16 pA after 5-HT application ($46.7\% \pm 4.5\%$ of reduction with respect to control conditions, $P = 0.004$; paired t test, $n = 8$, Fig. $3C_1$).

When interneurons were stimulated using the paired pulse protocol (see Methods section), paired pulse facilitation of the IPSCs was observed in pyramidal neurons. It is expected that if a neurotransmitter (or agonist) decreases the release probability, the paired pulse ratio (PPR) would increase (Harris and Cotman, 1983, 1985). We measured the peak amplitude of the IPSCs evoked by a pair of pulses with a stimuli interval of 50 ms, and calculated the amplitude ratio of the second to the first eIPSC. Figure $3C_2$ shows all of these experiments. In the absence of 5-HT, the average amplitude ratio of the second to the first IPSC (PPR, A_2/A_1) was 1.1 ± 0.08. When 5-HT (5 μM) was applied to the external solution, the PPR was increased to 1.5 ± 0.1 (Fig. $3C_2$; $P = 0.021$; paired t test). Moreover, the analysis of coefficient of variation (CV) also was increased in presence of 5-HT (0.23 ± 0.02 in control to 0.32 ± 0.03, $n = 8$, $P = 0.012$, paired t test, $n = 8$, Fig. $3C_3$).

Together, changes in PPR facilitation and in the CV, added to the decrease in the frequency of mIPSCs, suggest that the effect of the 5-HT is a direct action on the presynaptic terminal which modifies the release of GABA. In addition, to validate this suggestion, we tested the effect of the low Ca^{2+} concentration (0.5 mM) on the paired pulse protocol. The perfusion of low Ca^{2+} ASCF reduced the amplitude of the eIPSCs from 173 ± 29 pA in control conditions, to 98 ± 15 pA (Fig. $3D_1$, $n = 5$, $P = 0.009$, paired t test), and increased the PPR ratio from 0.81 ± 0.06 in control conditions, to 1.32 ± 0.15 (Fig. $3D_2$, $P = 0.007$, paired t test, $n = 5$). The CV was also increased from 0.13 ± 0.03 in control conditions, to 0.35 ± 0.06 (Fig. $3D_3$, $P = 0.002$, paired t test, $n = 5$). All of these data suggest that 5-HT acted at the presynaptic terminal.

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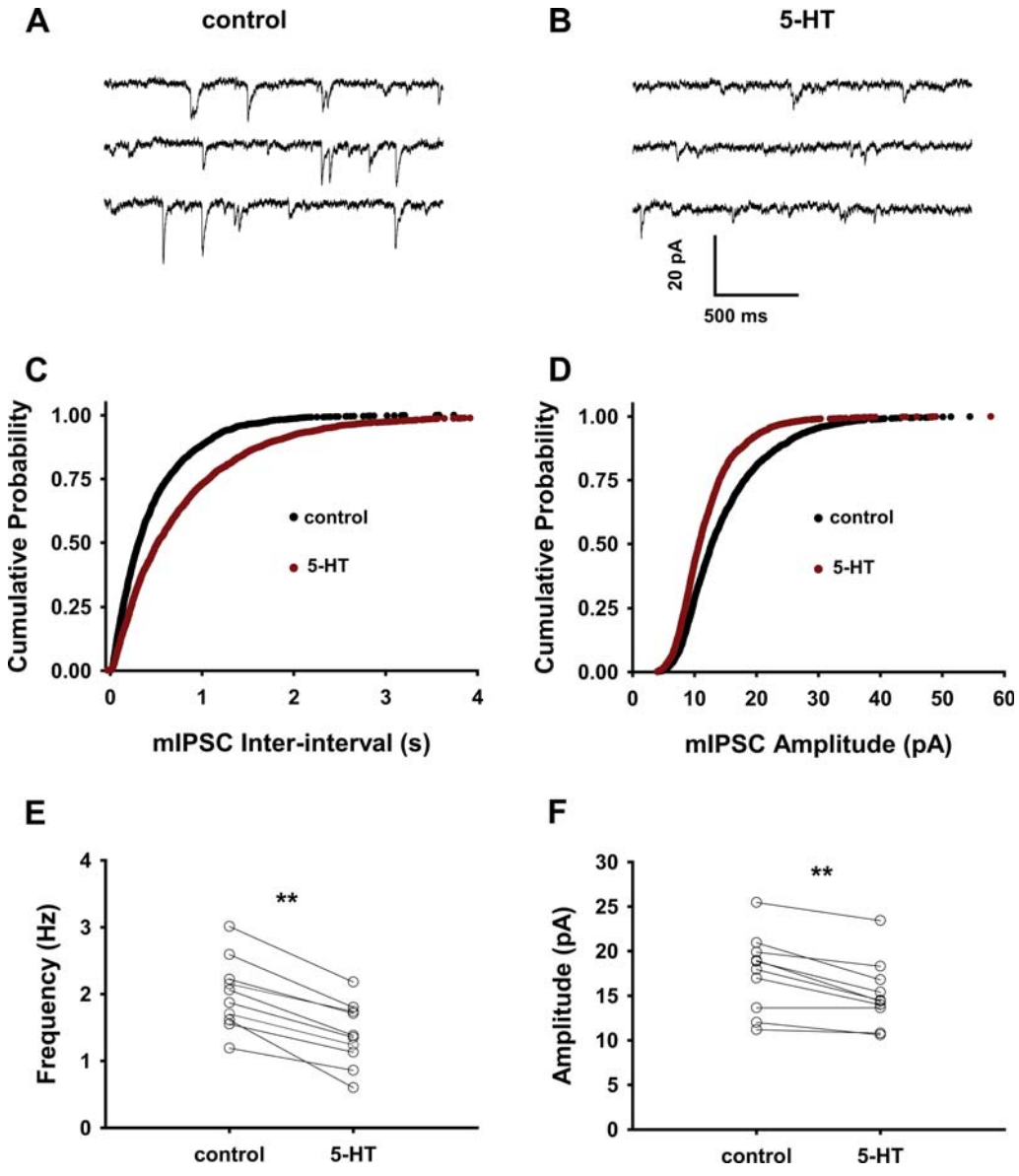

Fig. 2. The 5-HT decreases the frequency and amplitude of mIPSCs. **A** and **B**: Representative traces illustrating mIPSCs recorded in the presence of TTX (1 μM), in the absence (A) or in the presence (B) of 5-HT (5 μM). The 5-HT decreases the mIPSC frequency and amplitude. **C** and **D**: Cumulative probability distribution of mIPSCs inter-event interval (C) and amplitude (D) before and during application of 5-HT. **E** and **F**: summary of 5-HT effect on mIPSC frequency ($n = 10$, $P = 0.001$, paired t test) and amplitude ($n = 10$, $P = 0.004$, paired t test), respectively. The asterisk (**) indicates $P < 0.01$.

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Involvement of 5-HT_{1A} and 5-HT_{2A} receptors

Next, we sought to determine the subtype of 5-HT receptors mediating the inhibitory influence on the eIPSCs in pyramidal neurons, using selective agonists and antagonists of different subtypes of 5-HT receptors.

Because the 5-HT_{1A} and 5-HT_{2A} are the receptor subtypes which are expressed more frequently in the neocortex (Blue et al., 1998), we first investigated the possibility that 5-HT_{1A} receptors modulate eIPSCs. To test this hypothesis, we measured the effect of the selective agonist for the receptor 5-$HT_{1A/7}$ (8-OH-DPAT, 10 μM) on the eIPSC amplitude. The 8-OH-DPAT decreased eIPSC amplitude albeit to a lesser extent (the total decrease was 34% ± 4%, from 182 ± 16 pA in control to 121 ± 12 pA in 8-OH-DPAT; $P = 0.001$, paired t test, Fig. 4$A_{1–2}$, $n = 19$) with respect to the depression elicited by 5-HT (46.7% ± 4.5%, $n = 8$). These results suggest that 5-$HT_{1A/7}$ receptors are responsible for a great part of the modulation of the GABAergic transmission. To determine whether the site action of the 5-$HT_{1A/7}$ receptor activation was presynaptic or postsynaptic, changes in PPR were measured before and after 8-OH-DPAT inhibition. As shown in Figure 4A_3,

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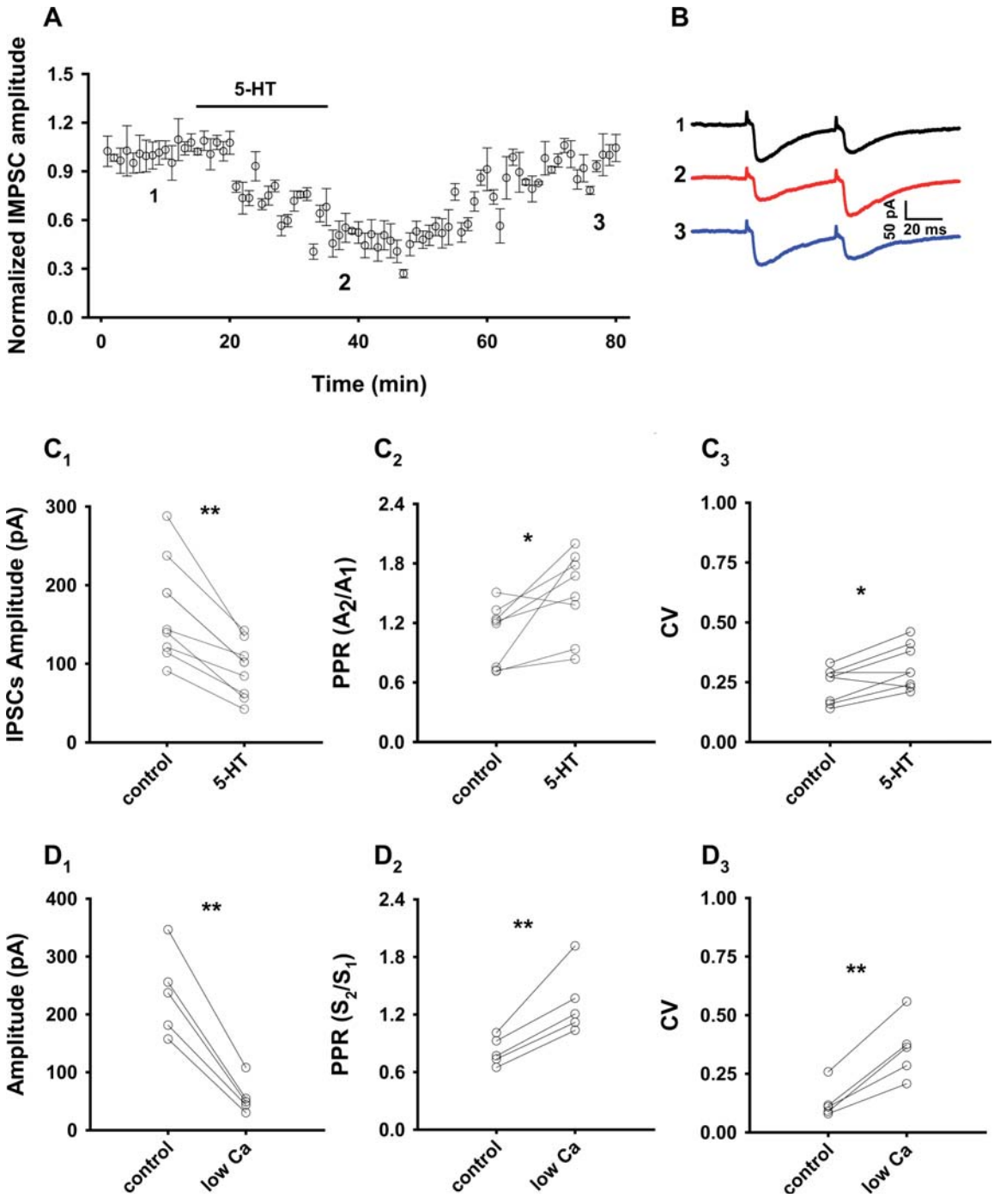

Fig. 3. The 5-HT decreases GABA release in layer II/III of auditory cortex. **A**: Temporal course showing that 5-HT (5 μM) reduces eIPSCs amplitude. The plotted data points were collected from the first electrical pulse. **B**: representative traces showing the effect of 5-HT application. **C1**: Summary of the reduction by bath application of 5-HT in all cells tested ($n = 8$, $P = 0.004$, paired t test) **C2**: Effects of 5-HT application upon paired pulse ratio (PPR), for individual cells. The 5-HT induced a significant change in PPR ($n = 8$, $P = 0.021$, paired t test). **C3**: Effects of 5-HT on coefficient of variation obtained for the amplitude of the first IPSCs ($n = 8$, $P = 0.012$, paired t test). **D1**: Low Ca2+ (0.5 mM) reduced the amplitude of IPSCS ($n = 5$, $P = 0.009$, paired t test). **D2**: Effects of low Ca2+ application, upon paired pulse ratio (PPR), for individual cells. Low Ca2+ induce changes in PPR ($n = 5$, $P = 0.007$, paired t test). **D3**: Effects of low Ca2+ on coefficient of variation obtained for the amplitude of the first IPSCs ($n = 5$, $P = 0.002$, paired t test). Single asterisks (*) indicate $P < 0.05$, double asterisk (**) indicate $P < 0.01$.

8-OH-DPAT significantly increased the PPR of the eIPSCs. PPR was 1.08 ± 0.07 in control condition, and 1.6 ± 0.18 after application of the agonist (Fig. 4A_3; $P = 0.016$, paired t test, $n = 19$), suggesting a presynaptic mechanism of the 5-$HT_{1A/7}$ receptor action on the eIPSCs.

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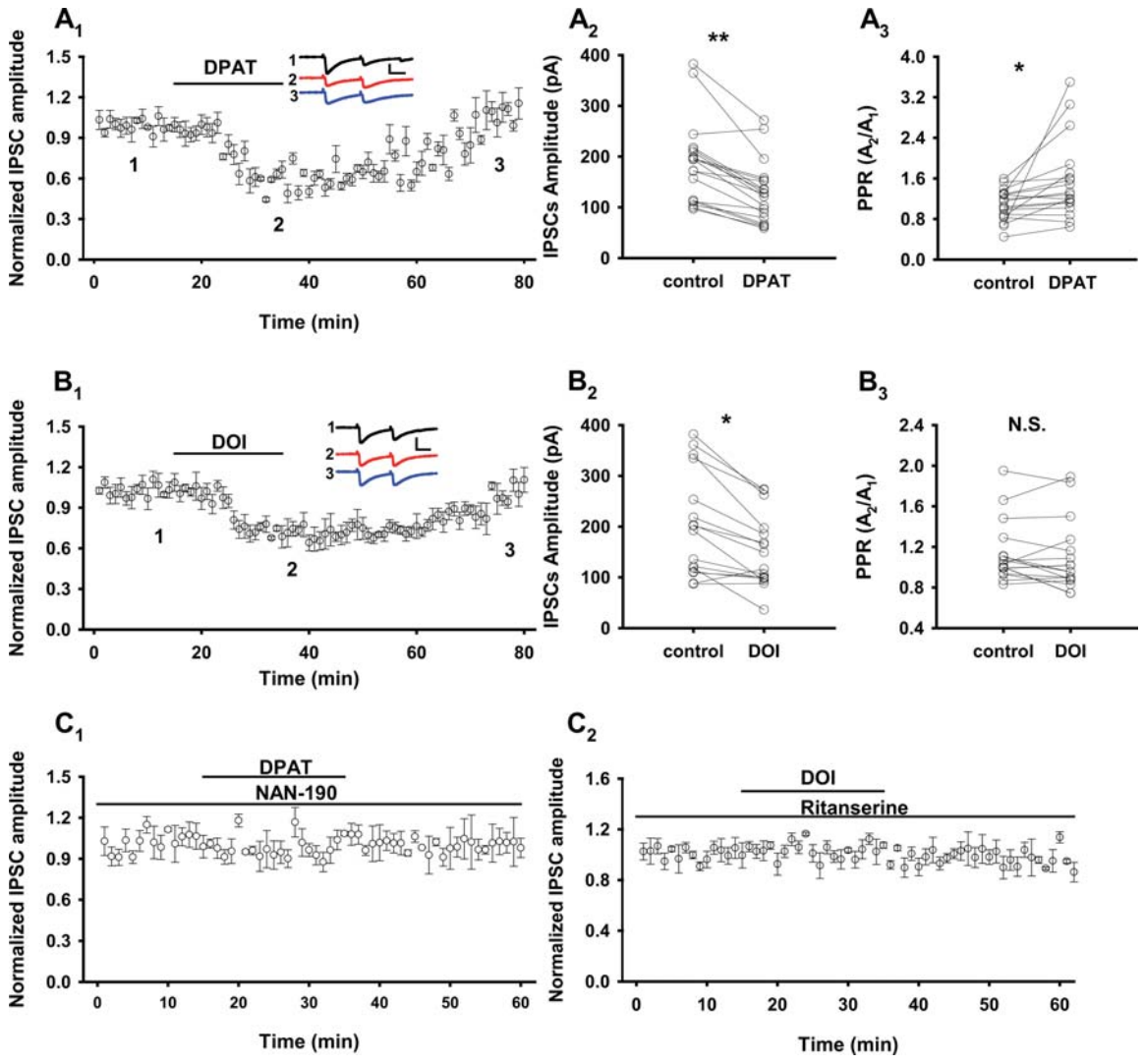

Fig. 4. Activation of the receptors 5-HT1A and 5-HT2A produced a decrease on GABAergic synaptic transmission in layer II/III. **A1**: Temporal course and representative traces showing the effect of 8-OH-DPAT (10 μM) on eIPSCs amplitude. **A2**: Summary of the reduction of the IPSCs amplitude by bath application of 8-OH-DPAT in all cells tested ($n = 19$, $P = 0.001$, paired t test). **A3**: Effects of 8-OH-DPAT application on the paired pulse ratio (PPR), for individual cells ($n = 19$, $P = 0.016$, paired t test). **B1**: Temporal course and representative traces showing the effect of DOI (10 μM) on the eIPSCs amplitude. **B2**: Summary of the reduction of IPSCs amplitude by application of DOI in the bath, in all cells tested ($n = 16$, $P = 0.01$, paired t test). A3: Effects of DOI application on paired pulse ratio (PPR) for individual cells ($n = 16$, $P = 0.58$, paired t test). **C1**: The antagonist for 5-HT1A receptors (NAN-190) blocked the reduction of the IPSCs amplitude by 8-OH-DPAT ($n = 10$, $P = 0.79$, paired t test). **C2**: The antagonist for 5-HT2A receptors (ritanserine) blocked the reduction of the eIPSCs amplitude by DOI ($n = 10$, $P = 0.86$, paired t test). Single asterisks (*) indicate $P < 0.05$, double asterisk (**) indicate $P < 0.01$, (N.S.) indicate no significant differences.

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Consistent with the latter results, the effect of 8-OH-DPAT in presence of the specific blocker for 5-HT_{1A} receptor (NAN-190 1 μM) was occluded. The reduction of eIPSCs amplitude by 8-OH-DPAT was only $3\% \pm 2.7\%$ in presence of the antagonist for 5-HT_{1A} receptors (Fig. $4C1_1$, $n = 10$; $P = 0.79$, paired t test). These results indicate that only 5-HT_{1A} receptors were activated after application of 8-OH-DPAT to modulate GABA release inhibition and discarding 5-HT_7 receptor participation.

To evaluate the contribution of 5-HT_{2A} receptors on the 5-HT-induced inhibition of the amplitude of the eIPSCs, we tested the action of the selective agonist for 5-HT_{2A} serotonergic receptors (DOI, Fig. $4B_{1-3}$). On average, DOI (10 μM) reduced the amplitude of the eIPSCs by $27\% \pm 4\%$, from 203 ± 26 pA in control, to 149 ± 20 pA in DOI (Fig. $4B_{1-2}$, $P = 0.01$, paired t test, $n = 16$). To determine whether the site of the inhibitory action of 5-HT_{2A} receptors on the eIPSCs was presynaptic or postsynaptic, changes in PPR were measured before and after DOI application. As shown in Figure $4B_3$, DOI application (10 μM) did not produce statistically significant changes in PPR. The PPR was 1.17 ± 0.07 in control condition and

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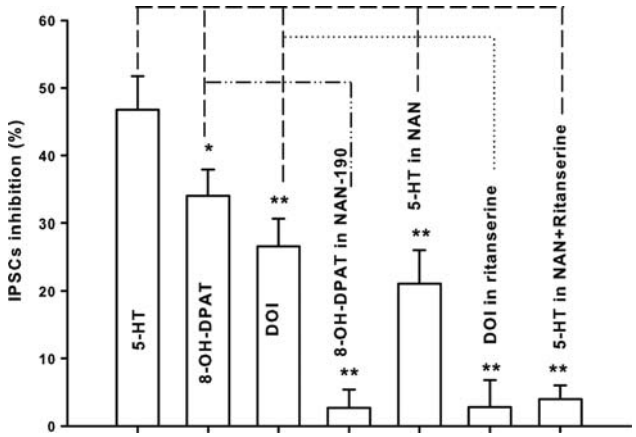

Fig. 5. Summary of the pharmacology experiments with agonists and antagonists of 5-HT on the eIPSCs from layer II/III of auditory cortex. **A**: The bar plot shows the percent of inhibition of IPSCs amplitude by 5-HT alone, or in the presence of the 5-HT1A and 5-HT2A antagonists. Note that 5-HT1A and 5-HT2A antagonists block the influence of 5-HT on the evoked IPSCs in layer II/III of auditory cortex (for 5-HT vs. 8-OH-DPAT, $P = 0.003$, 5-HT vs. DOI, $P = 0001$, 5-HT vs. 8-OH-DPAT in presence of NAN-190, $P = 0.0001$ 5-HT vs. 5-HT in NAN-190, $P = 0.019$; 5-HT vs. DOI in Ritanserin, $P = 0.0001$; 5-HT vs. 5-HT in NAN-190 and ritanserin, $P = 0.0001$, $F = 17.39$, ANOVA with post hoc Tukey's was used in all cases).

1.15 ± 0.12 after the agonist application (Fig. $4B_3$; $P = 0.58$, paired t test, $n = 16$). Consistent with the latter results, the effect of DOI in the presence of the specific antagonist for 5-HT_{2A} receptor (Ritanserin, 200 nM) was blocked. The reduction of eIPSCs amplitude by DOI was only $1\% \pm 2.4\%$ in presence of the antagonist for 5-HT_{2A} receptors (Fig. $4C_2$, $n = 10$; $P = 0.86$, paired t test).

We also tested the effect of 5-HT (5 μM) in the presence of the specific 5-HT_{1A} receptor antagonist (NAN-190). We observed that 5-HT produces a $20\% \pm 3\%$ decrease on the evoked IPSC amplitude (Fig. 5A, $n = 10$; $P = 0.019$, $F = 17.39$, ANOVA with post hoc Tukey's test). Additionally, we compared the effect of 5-HT in the presence of NAN-190 versus the effect produced by DOI. The comparison yielded no significant differences ($P = 0.78$, ANOVA with post hoc Tukey's test, Fig. 5A), suggesting that only 5-HT_{1A} and 5-HT_{2A} receptors produced the inhibition of GABAergic synaptic transmission. To test the last assumption, the effect of 5-HT was tested in presence of both antagonists (NAN-190 and ritanserin). We found that under this condition, all of the 5-HT effect was prevented ($P = 0.0001$ ANOVA with post hoc Tukey's test, $n = 5$, Fig. 5A).

The 5-HT-induced attenuation of postsynaptic GABAergic currents is mediated by 5-HT_{2A} receptors

The PPR data suggest: (1) a presynaptic action of 5-HT_{1A} receptors, (2) Lack of PPR change in presence of DOI suggested a postsynaptic action of 5-HT_{2A} receptors. (3) As a further test of pre- vs. postsynaptic actions, we tested the effects of specific agonists for 5-HT_{1A} and 5-HT_{2A} receptors on currents evoked by direct application of the $GABA_A$ agonist, Muscimol (100 μM).

Muscimol-induced IPSCs resulted in prolonged inward currents decaying in several seconds. As shown in Figures 6A–6C, application of 8-OH-DPAT did not produce any significant reduction on the $GABA_A$ current amplitude ($n = 5$; $P = 0.21$; paired t test, Figs. 6A–6C). However, the application of 8-OH-DPAT produced a slightly increase of current amplitude ($6\% \pm 4\%$) in two of five cells tested. In these two cells, the increase in the amplitude of muscimol induced IPSCs suggest a putative opposing postsynaptic effect of 5-HT_{1A} receptor activation on $GABA_A$ receptors. Taken together, our results showed that 5-HT_{1A} receptors did not modulate $GABA_A$ currents at the postsynaptic site, and were most consistent with a presynaptic mechanism for the 5-HT_{1A} receptor inhibition of GABA release.

On the other hand, application of DOI in the bath caused a significant reduction of the muscimol-induced IPSCs in five cells. The reduction was $28\% \pm 5\%$ ($n = 5$; $P = 0.009$; paired t test, Figs. 6D–6F). These results indicate that 5-HT acts postsynaptically to decrease peak amplitude of IPSCs via activation of 5-HT_{2A} receptors, either the perfusion of saline solution did not alter the amplitude of the currents evoked by muscimol ($5\% \pm 3\%$ of reduction, $n = 5$, $P = 0.9$, paired t test, Figs. 6G–6I).

DISCUSSION

Here, we provided evidence for a pre and postsynaptic modulation of inhibitory transmission in layer II/III of the juvenile rat auditory cortex.

Synaptic localization of the 5-HT modulation

The present study characterized the action of 5-HT in GABAergic synaptic transmission in layer II/III of the auditory cortex in rat juvenile (PD25–30). We found that 5-HT selectively depresses GABAergic synaptic currents by activation of 5-HT_{1A} and 5-HT_{2A} receptors. Our results suggest that 5-HT_{1A} and 5-HT_{2A} receptors exert an influence at two levels, presynaptically and postsynaptically, respectively. The evidence supporting these notions are as follows: (i) The frequency and the amplitude in sIPSCs were reduced by the application of 5-HT. (ii) A similar influence of 5-HT was found in the mIPSCs when the amine was tested in the presence of TTX (1 μM). (iii) Changes in coefficient of variation by 5-HT or by low Ca^{2+} concentration. (iv) The 5-HT_{1AR} agonist (8-OH-DPAT, 10 μM) reduced eIPSCs amplitude and increased paired pulse facilitation of the eIPSCs, whereas the 5-HT_{2A} agonist (DOI, 10 μM) reduced the eIPSCs amplitude without changes in paired

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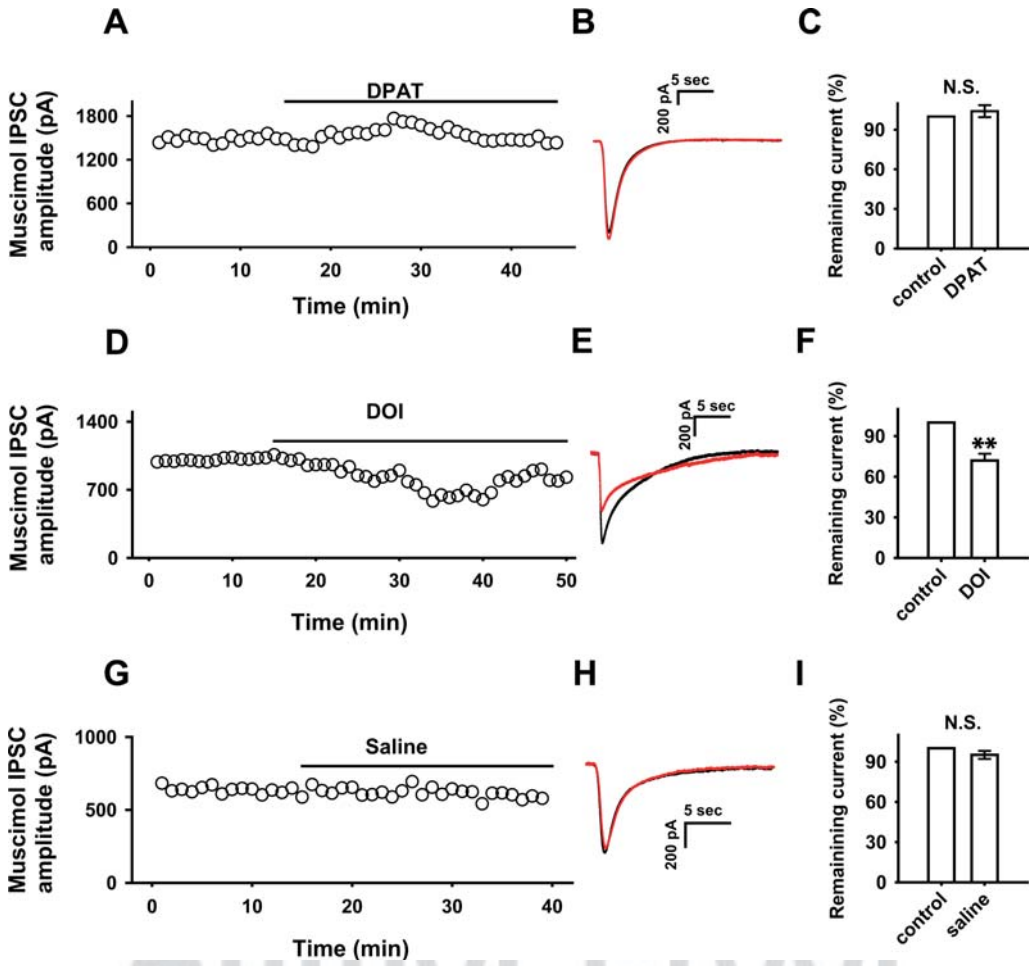

Fig. 6. The agonist of 5-HT2A receptor decreased muscimol-evoked currents but not the 5-HT1A receptor agonist. **A**: Example of the time course for the effect of 8-OH-DPAT (10 μM) on the inward currents produced by the application of the $GABA_A$ agonist, muscimol (100 μM). **B**: Representative traces illustrating the currents evoked by muscimol, in control condition and after 8-OH-DPAT application. **C**: Summary showing the effect of 8-OH-DPAT on induced GABAergic currents by muscimol $n = 5$, $P = 0.21$, paired t test. **D**: Example of the time course of the effect of DOI (10 μM) on the inward currents produced by the application of the $GABA_A$ agonist muscimol (100 μM). **E**: Representative traces illustrating currents induced by muscimol in control condition and after DOI application. **F**: Summary showing the effect of DOI on the GABAergic currents $n = 5$, $P = 0.009$, paired t test. **G**: Example of the time course of the effect of saline solution on the inward currents produced by the application of the $GABA_A$ agonist muscimol (100 μM). **H**: Representative traces illustrating currents induced by muscimol in control condition and after saline solution application. **I**: Summary showing the effect of saline solution on the GABAergic currents $n = 5$, $P = 0.9$, paired t test.

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pulse ratio at layer II/III of the auditory cortex. These effects were blocked in the presence of the selective antagonists for these receptors, NAN-190 or ritanserine, respectively. Finally, (v) the 5-HT postsynaptic action by a local muscimol application was mimicked by the 5-HT_{2A} agonist (DOI) but not for the 5-HT_{1A} agonist (8-OH-DPAT). These results indicated that the effects of 5-HT are specific and suggested that the actions of GABAergic axons on pyramidal neurons can be selectively regulated by 5-HT_{1A} receptors, whereas a postsynaptic effect is regulated by 5-HT_{2A} receptors (at least in the range of 5-HT concentration and age tested).

Our results demonstrated that 5-HT-mediated decrease in GABA release is action potential-independent, because application of 5-HT decreased the frequency and amplitude of mIPSCs. The 5-HT_{1A}-mediated GABA release inhibition might require the inhibition of presynaptic Ca^{2+} influx, or possibly $G_{\beta\gamma}$ complex interaction with SNARE complex at GABAergic terminals (Hamid et al., 2014). Notably, the mechanism underlying the 5-HT_{1A} receptor-mediated inhibition of GABA release from GABAergic terminals has been investigated in a basolateral amygdala preparation with functional GABAergic nerve terminals (Koyama et al., 1999). This study reports that the 5-HT-induced inhibition on the mIPSC frequency was not affected by either K^+ or Ca^{2+}-free external solution, but was abolished by a GTP-binding protein inhibitor, *N*-ethylmaleimide. Even more, 5-HT inhibits the frequency of miniature IPSCs by inactivating the adenylyl cyclase and cAMP

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signal transduction pathway via a G-protein-coupled 5-HT_{1A} receptor (Koyama et al., 1999). A similar process might be involved in the inhibition of GABA release induced by 5-HT_{1A} receptors in the auditory cortex. Even in the absence of direct ultra-structural evidence that the 5-HT_{1A} receptors are present at the axon terminals in interneurons in ventrolateral orbital cortex or in amigdala (Huo et al., 2010; Koyama et al., 1999), our data indicate that interneurons in the auditory cortex express these receptors.

This conclusion, coupled with the fact that the auditory cortex has a dense 5-HT innervation (Lidov, 1980), leads us to speculate that in intact animals, the endogenous 5-HT in the layer II/III of auditory cortex affects GABAergic synaptic transmission predominantly by directly inhibiting GABA release at the axon terminal. In fact, previous studies have shown a reduction of GABA release induced by 5-HT_{1A} receptors in the ventrolateral orbital cortex, amygdala and dentate gyrus (Huo et al., 2010; Koyama et al., 1999; Matsuyama et al., 1997). Moreover, our results showed that the application of 5-HT_{1A} receptor agonist has no effect on postsynaptic $GABA_A$ receptor currents in pyramidal neurons. Similar results were found in the prefrontal cortex (Feng et al., 2001) and in the hippocampus (Schmitz et al., 1995). The amount of depression in the evoked IPSCs produced by the 5-HT_{1A} specific receptor agonist which is prevented by the 5-HT_{1A} antagonist), indicated that about two thirds of the total depression is associated with the activation of 5-HT_{1A} receptors (see Figs. 3 and 4).

On the other hand, the application of the 5-HT_{2A} receptor agonist DOI produced a decrease in amplitude of the eIPSCs without modifying the PPR, and this effect was blocked by a 5-HT_{2A} antagonist. In addition, DOI caused an amplitude reduction of IPSCs evoked by muscimol. Together, these data indicated a postsynaptic locus for this modulation. This observation, and its interpretation, is fully consistent with anatomical studies indicating the predominant expression of 5-HT_{2A} receptors on somas and dendrites of pyramidal neurons in auditory cortex (Basura et al., 2008). The 5-HT_{2A} receptors are predominantly expressed in layer II/III, whereas a lower expression was reported for layer I, IV, and VI in the auditory cortex (Basura et al., 2008).

Electrophysiological evidence in prefrontal cortex indicates the inhibition of $GABA_A$ currents by 5-HT_{2A} receptors is mediated through a mechanism involving stimulation of PLCβ, phospholipid hydrolysis and activation of PKC-RACK1 complex (Feng et al., 2001). In agreement with this observation, PKC phosphorylation causes a reduction in the amplitude of $GABA_A$-activated currents (Krisheck et al., 1994). A similar process might be involved in the 5-HT_{2A} induced inhibition of postsynaptic $GABA_A$ currents in auditory cortex. In contrast, evidence suggests that 5-HT_{2A} receptor increases GABA release in entorhinal cortex (Deng and Lei, 2008). Also an increase in IPSCs amplitude, in a pre and postsynaptic locus was reported for the visual cortex (Jang et al., 2012). Regional differences in the composition of $GABA_A$ receptors and/or intracellular molecular mechanisms might account for differences between the visual cortex and the auditory cortex. In accordance with this assumption, modulation of $GABA_A$ currents depends on the subunit composition of α, β and γ-subunits to be phosphorylated by various kinases (Bright and Smart, 2013, Jang et al., 2012; Yan, 2002). In fact, phosphorylation of Ser409 in β_1 subunit by PKC decreases $GABA_A$ currents (Krishek et al., 1994). A similar reduction was previously reported for the 5-HT modulation of GABA synaptic transmission in prefrontal cortex involving $GABA_A$ receptor expression with γ2 subunits by PKC-RACK1 complex (Feng et al., 2001). Remarkably, 5-HT modulation in visual cortex involved the expression of $GABA_A$ receptors containing β2 subunits and phosphorylation by CaMKII. Indeed, CaMKII phosphorylation leads to increases in $GABA_A$ currents by phosphorylation of Ser327 (Jang et al., 2012). This suggests that 5-HT_{2A} receptor activation might selectively increase or decrease perisomatic inhibitory inputs, depending of the $GABA_A$ subunit composition and the intracellular pathway activated. Although the main finding of our study clearly indicates that GABAergic synaptic transmission is an important target of 5-HT in the auditory cortex, the physiological significance of the phenomenon is still elusive. We may speculate, however, the functional importance for the serotonergic modulation.

In general, given the known importance of the auditory cortex in attention, in task-related processing of auditory information, and in formation of memories about the behavioral relevance of acoustic signals (Dahmen et al., 2010; Jääskeläinen and Ahvenine, 2014; Scheich et al 2011), the inhibition of the GABAergic transmission by 5-HT at the auditory cortex described here, may have critical functional implications: First, our findings provide a cellular substrate to the hypothesis that a key function of the 5-HT system is to facilitate auditory cortex activity. Second, 5-HT modulation of GABAergic transmission has been proposed to alter synaptic plasticity and neuronal development (Dringenberg et al., 2014; Sale et al., 2010). A potential limitation exists in the interpretation of the presented results. More specifically, the auditory cortex is a complex circuit, and the effects on parts of this circuit, including the properties in excitability of the interneurons and the modulation of release of glutamate in excitatory synapses, have not yet been studied.

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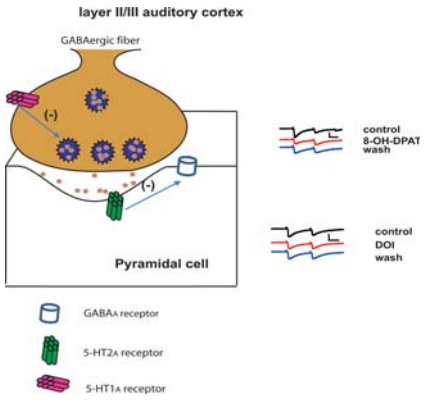

The 5-HT reduces GABAergic synaptic transmission throughout both the 5-HT1A and 5-HT2A receptors activation at two levels, presynaptically and postsynaptically, respectively, in layer II/III of the auditory cortex.

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