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MODULATION OF GABA RELEASE FROM THE THALAMIC RETICULAR NUCLEUS BY COCAINE AND CAFFEINE: ROLE OF SEROTONIN RECEPTORS

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Abstract

Serotonin receptors are targets of drug therapies for a variety of neuropsychiatric and neurodegenerative disorders. Cocaine inhibits the re-uptake of serotonin (5-HT), dopamine, and noradrenaline while caffeine blocks adenosine receptors and opens ryanodine receptors in the endoplasmic reticulum. We studied how 5-HT and adenosine affected spontaneous GABAergic transmission from thalamic reticular nucleus (TRN). We combined whole-cell patch clamp recordings of miniature inhibitory post-synaptic currents (mIPSCs) in ventrobasal (VB) thalamic neurons during local (*puff*) application of 5-HT in wild type (WT) or knockout mice lacking 5-HT_{2A} receptors (5-HT_{2A} −/−). Inhibition of mIPSCs frequency by low (10 μM) and high (100 μM) 5-HT concentrations was observed in VB neurons from 5-HT_{2A}−/− mice. In WT mice, only 100 μM 5-HT significantly reduced mIPSCs frequency. In 5-HT_{2A}−/− mice, NAN-190, a specific 5-HT_{1A} antagonist, prevented the 100 μM 5-HT inhibition while blocking H-currents that prolonged inhibition during post-puff periods. The inhibitory effects of 100 μM 5-HT were enhanced in cocaine binge-treated 5-HT_{2A} −/−. Caffeine binge treatment did not affect 5-HT-mediated inhibition. Our findings suggest that both 5-HT_{1A} and 5-HT_{2A} receptors are present in presynaptic

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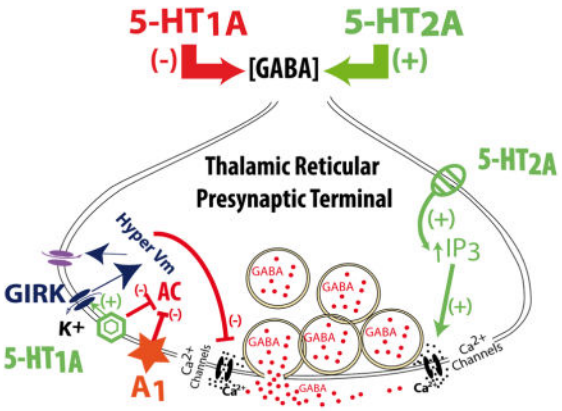
TRN terminals. Serotonergic-mediated inhibition of GABA release could underlie aberrant thalamocortical physiology described after repetitive consumption of cocaine.

Graphical Abstract



Keywords

cocaine; caffeine; thalamic reticular nucleus; GABA; serotonin

Introduction

Serotonin receptors and their associated intracellular pathways are conserved through evolution and have been described as targets of drug therapies for a variety of neuropsychiatric and neurodegenerative disorders (Nichols & Nichols, 2008), and alterations in 5-HT receptor levels have been demonstrated in human patients suffering from several psychiatric disorders (Lopez-Figueroa et al., 2004). Some of these neuropsychiatric disorders involve dysregulation (i.e., altered inhibitory processing) of cortical afferents from the ventrobasal thalamus (VB), which is normally regulated by inhibitory input from the thalamic reticular nucleus (TRN) (Llinas et al., 2002).

Hyperpolarization-activated cyclic nucleotide-gated H-currents have been described to be activated in response to membrane hyperpolarization and contribute to the pacemaker depolarization that generates rhythmic activity of thalamcortical neurons (McCormick & Pape, 1990a). In VB neurons, 5-HT depolarized the membrane potential (Varela & Sherman, 2009) after changing the voltage-dependence of H-currents (Lee & McCormick, 1996) while increasing their amplitude (McCormick & Pape, 1990b). Serotonin can also depolarize (Sanchez-Vives et al., 1996; Monckton & McCormick, 2002) without affecting high-frequency (40Hz) action potential firing of GABAergic TRN neurons (Pinault & Deschenes, 1992). However, other authors showed that bath-applied 5-HT hyperpolarized VB neurons (Monckton & McCormick, 2002). TRN neurons express both 5-HT_{1A} and 5-HT_{2A} receptors in their somas and dendrites (Cornea-Hébert et al., 1999; Rodriguez et al., 2011), but the role of serotonergic receptors at presynaptic GABAergic terminals of TRN are still unclear.

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Caffeine (a well-known antagonist of IP_3 receptors and an agonist of ryanodine receptors; McPherson et al., 1991) induces Ca^{2+} release from neuronal ryanodine intracellular stores (Garaschuk et al., 1997; Rankovic et al., 2010), and blocks adenosine receptors (Fredholm, 1995; Fredholm et al., 1999). Caffeine-induced Ca^{2+} release from the endoplasmic reticulum is known to trigger spontaneous GABA release in retinal amacrine cells (Warrier et al., 2005), as well as spontaneous Glutamate release in rat barrel cortex (Simkus & Stricker, 2002). Adenosine inhibits thalamocortical glutamate efferents reportedly activating presynaptic A_1-type receptors (Fontanez & Porter, 2006), and has anti-oscillatory effects by blocking GABAergic transmission between TRN and VB neurons (Ulrich & Huguenard, 1995). The activation of presynaptic A_1-receptors strongly suppressed 5-HT_{2A}-mediated increases in spontaneous excitatory minis in cortical layer V pyramidal neurons (Stutzmann et al., 2001). However, little is known about the role of serotonergic 5-HT_{2A} receptors on GABAergic transmission at a thalamic level.

In a previous study (Goitia et al., 2013), our group showed that cocaine *binge* administration led to considerable disinhibition of thalamic GABAergic transmission, while methylphenidate (MPH) did not induced such alterations. Given that MPH has no effect on serotonin transporters (Glowinski & Axelrod, 1966; Ross & Renyi, 1969; Ritz et al., 1987; Wise & Bozarth, 1987; Pan et al., 1994; Kuczenski & Segal, 1997; Segal & Kuczenski, 1999; Howes et al., 2000), we hypothesized that differences observed between these psychostimulants could be due to cocaine-dependent prolonged activation of serotonergic receptors expressed at the TRN nucleus level (Rodriguez et al., 2011).

Here we studied the presynaptic role of 5-HT on spontaneous GABAergic release from the TRN to the VB nucleus. We used focal (*puff*) application of 5-HT in slices from mice (WT or 5-HT_{2A} −/−) treated with either cocaine or caffeine *binge*. Our results described for the first time a strong inhibition of miniature inhibitory post-synaptic current (mIPSC) frequency by 5-HT *puff* application onto VB neurons from WT or 5-HT_{2A} −/− mice. Further characterization of local 5-HT effects suggests that cocaine-induced effects on thalamic GABAergic transmission are mediated by enhancing presynaptic 5-HT_{1A} inhibitory action on the terminals of TRN neurons.

Our results suggest that inhibition of GABA release by 5-HT_{1A} receptors is counteracted by the activation of 5-HT_{2A} receptors. 5-HT_{1A} receptors, through the activation of $G_{i/o}$ protein, would inhibit adenylate cyclase while increasing the probability of the opening of *G-protein-activated inwardly rectifying K^+ channels* (GIRK), which would hyperpolarize the membrane potential, facilitating the opening of H-channels (Millan et al., 2008; Luscher et al., 1997). However, 5-HT_{2A}-mediated activation of PLC and IP_3, increasing intracellular [Ca^{2+}], would facilitate GABA release (Millan et al., 2008). In addition, caffeine treatment would exert a blocking effect on A1 receptors (Fredholm, 1995; Fredholm et al., 1999), and remove the down-regulation of adenylate cyclase, partially compensating for the inhibitory effects of 5-HT_{1A} receptors on GABA release.

Results presented here could help understand the role of serotonin receptors in multiple neuropsychiatric and neurodegenerative disorders.

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Materials and Methods

Animals

We used male 129Sv/Ev mice (18–23 days old), either WT (from the Central Animal Facility at University of Buenos Aires) or knockout for the 5-HT_{2A} receptor (Weisstaub et al., 2006). Principles of animal care were in accordance with the ARRIVE guidelines and CONICET (2003), and approved by its authorities using OLAW/ARENA directives (NIH, Bethesda, MD, USA).

Psychostimulant administration

Cocaine (15 mg/kg) or caffeine (5 mg/kg) were administered using 'binge-like' protocols (3 *i.p.* injections, 1 h apart; Spangler et al., 1993; Urbano et al., 2009; Bisagno et al., 2010; Goitia et al., 2013), and control animals received saline injections equally timed. The binge-like administration pattern has been designed to mimic compulsive human cocaine abuse (Spangler et al., 1993).

Thalamocortical slices

Slices were obtained as previously described (Urbano et al., 2009; Bisagno et al., 2010; Goitia et al., 2013), 1 hr after the administration of the *binge* protocol. Mice were deeply anesthetized with tribromoethanol (250 mg/Kg; *i.p.*) followed by transcardial perfusion with ice-cold low sodium/antioxidants solution (composition in mM: 200 sucrose, 2.5 KCl, 3 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 20 D-glucose, 0.4 ascorbic acid, 2 pyruvic acid, 1 kynurenic acid, 1 $CaCl_2$, and aerated with 95% O_2/5% CO_2, pH 7.4), and then decapitated. Thalamocortical brain slices (300 μm) were obtained gluing both hemispheres onto a vibrotome aluminum stage (Integraslicer 7550 PSDS, Campden Instruments, UK), submerged in a chamber containing chilled low-sodium/high-sucrose solution (composition in mM: 250 sucrose, 2.5 KCl, 3 $MgSO_4$, 0.1 $CaCl_2$, 1.25 NaH_2PO_4, 0.4 ascorbic acid, 3 myo-inositol, 2 pyruvic acid, 25 D-glucose, and 25 $NaHCO_3$). Slices were cut sequentially and transferred to an incubation chamber at 37°C for 30 min containing a stimulant-free, low Ca^{2+}/high Mg^{2+} normal ACSF (composition in mM: 125 NaCl, 2.5 KCl, 3 $MgSO_4$, 0.1 $CaCl_2$, 1.25 NaH_2PO_4, 0.4 ascorbic acid, 3 myo-inositol, 2 pyruvic acid, 25 d-glucose, and 25 $NaHCO_3$ and aerated with 95% O_2/5% CO_2, pH 7.4; Urbano et al., 2009, Bisagno et al., 2010).

Whole-cell patch-clamp recordings

Whole-cell patch clamp recordings were made at room temperature (20–24°C) in normal ACSF with $MgCl_2$ (1 mM) and $CaCl_2$ (2 mM). Patch electrodes were made from borosilicate glass (2–3 MΩ) filled with a voltage-clamp high Cl^-, high Cs^+/QX314 solution (composition in mM: 110 CsCl, 40 HEPES, 10 TEA-Cl, 12 Na_2phosphocreatine, 0.5 EGTA, 2 Mg-ATP, 0.5 Li-GTP, and 1 $MgCl_2$. pH was adjusted to 7.3 with CsOH). To block Na^+ currents and avoid postsynaptic action potentials, 10 mM *N*-(2,6-diethylphenylcarbamoylmethyl) triethylammonium chloride (QX-314) was added to the pipette solution (Urbano et al., 2009, Bisagno et al., 2010). Signals were recorded using a MultiClamp 700 amplifier commanded by pCLAMP 10.0 software (Molecular Devices, CA,

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USA). Data were filtered at 5 kHz, digitized and stored for off-line analysis. Capacitance and leak-currents were electronically subtracted using a standard pCLAMP P/N subtraction protocol. Spontaneous (non-electrically evoked) mIPSCs were recorded from VB neurons in the presence of tetrodotoxin (TTX, 3 μM), DL-2-Amino-5-phosphonopentanoic acid sodium salt (DL-AP5, 50 μM) and 6-Cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX, 20 μM), and analyzed using Mini Analysis (Synaptosoft, Fort Lee, NJ, USA). Cumulative probability amplitude and inter-event interval curves were fitted to a single exponential equation: $y = y_0 + a*\exp(-b*time)$; and mIPSC **median** amplitude and intervals (i.e., frequency^{-1}) were compared across groups.

We used focal, *puff* application of 5-HT to minimize internalization of its receptors, which has been previously described by other authors using both agonists and antagonists (Gray & Roth, 2001). During *puff* experiments, 5-HT (10 or 100 μM) was focally applied, either alone or together with 1-(2-Methoxyphenyl)-4-(4-phthalimidobutyl) piperazine hydrobromide (NAN-190, 5-HT_{1A} antagonist) or 4-Ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium chloride (ZD-7288, a H-current, I_h inhibitor) through a patch pipette filled up with the same ACSF recording solution and connected to a Picospritzer II (General Valve Corporation, Fairfield, NJ) at ~50μm from the cell being patched. In each recording (2min. 30s long), mIPSC frequency was calculated in 15s time bins. The *puff* was applied at 1:00 to 1:30 min of recording, allowing us to determine *pre-puff* (0–1:00), *puff* (1:00–1:30), and *post-puff* (1:30–2:30) frequencies. *Puff* and *post-puff* frequencies are shown as normalized to the *pre-puff* frequency from each recorded VB neuron.

To test the effectiveness of *puff* applications on GABAergic synapses, we applied a VB holding potential between −70 and −90 mV (to enhance mIPSC amplitude by over 2 fold peak-to-peak noise amplitude during quantification), and applied ACSF containing $CdCl_2$ (1mM), a Ca^{2+} channel blocker. This blocked mIPSCs and calcium currents recorded from postsynaptic VB neurons (Fig. 1A, B, respectively). We also confirmed that the mIPSCs being recorded were GABAergic through the *puff* application of picrotoxin, a GABA-A receptor inhibitor (Fig. 1C).

Statistical analysis

InfoStat software (Univ. Nacional de Córdoba, Argentina) was used for statistical comparisons. Statistics were performed using Student's t-test or ANOVA and Tukey-Kramer or LSD Fisher multiple comparisons post hoc tests when applicable. Differences were considered significant if $p < 0.05$. Whenever the data did not comply with assumptions of the parametric tests, non-parametric Wilcoxon-Mann-Whitney or Kruskal-Wallis tests were performed followed by paired comparisons. Data is presented as mean ± standard error of the mean.

Materials

Cocaine-HCl, Caffeine, DL-AP5, CNQX, 5-HT, picrotoxin, and NAN-190 were purchased from Sigma-Aldrich (Argentina), TTX from Alomone labs. (Israel) and ZD 2788 from Tocris (USA).

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Results

Local application of 5-HT inhibits GABA release from presynaptic reticular thalamic terminals from WT and 5-HT$_{2A}$ −/− mice

We studied the role of 5-HT on the frequency and amplitude of spontaneous GABAergic release from the TRN onto VB neurons. We used focal (*puff*) application of 5-HT in thalamocortical slices from mice (WT or 5-HT$_{2A}$ −/−) in the presence of DL-AP5 (NMDA receptor antagonist), CNQX (AMPA receptor antagonist), and TTX (voltage-gated sodium channel blocker). The *puff* pipette was located near the soma of VB neurons that were recorded in voltage-clamp whole-cell configuration, allowing us to study presynaptic terminals (Fig. 1). GABA mIPSC frequency was reduced during puff application of 5-HT compared to the *pre-puff* period, recovering during *post-puff* period (Fig. 2A; ACSF *vs.* 5-HT 100 μM). Although an apparent reduction in GABA mIPSC amplitude was observed during *puff* application of 5-HT, no significance was observed comparing median amplitudes before and during the *puff* (6.5%±0.8 reduction in median amplitudes *puff* vs. *pre-puff*; n=12 VB neurons; Student's *t*-test, $p>0.05$).

Low (10μM) and high (100μM) 5-HT concentration reduced GABA mIPSC frequency during *puff* (Fig. 2B, filled bars) in a larger percent in VB neurons from 5-HT$_{2A}$ −/− mice compared to WT (two-way ANOVA, p=0.0048). During the 5-HT 10μM *puff,* percent inhibition was significant only in VB neurons from 5-HT$_{2A}$ −/− mice (Student's t-test; p<0.0001). During *post-puff* (Fig. 2B; dashed bars), there were also differences between WT and 5-HT$_{2A}$ −/− (two-way ANOVA, simple effects; p=0.0006). For 5-HT at 10μM, the percent inhibition during *post-puff* periods was significantly higher for the 5-HT$_{2A}$ −/− group (Student's t-test; p=0.0006), while for 5-HT 100μM *post-puff* percent inhibition was significant only for the WT group (Student's t-test; p=0.0379).

5-HT-dependent inhibition of GABA release in reticular thalamic terminals from 5-HT$_{2A}$ −/− mice was mediated by 5-HT$_{1A}$ receptors and H-currents

We then determined which 5-HT receptor mediated the 5-HT *puff* inhibitory effects on GABA release in slices from 5-HT$_{2A}$ −/− mice. We included the specific 5-HT$_{1A}$ antagonist NAN-190 (100 μM) in both the puff pipette and bath extracellular solutions, and observed no significant reduction in GABAergic mIPSC frequency during high concentration 5-HT (100 μM) *puff* application in 5-HT$_{2A}$ −/− mice (Fig. 3A, B). Applying the H-current blocker ZD-7288 (10 μM) significantly reduced the inhibitory effects of 5-HT 100 μM *puff* (Fig. 3A, B; One way-ANOVA; p=0.03 comparing 5-HT 100 μM vs. 5-HT 100 μM+ZD-7288 10 μM in VB neurons from 5-HT$_{2A}$ −/− mice). The *post-puff* effect of 5-HT 100 μM was not significantly affected by either NAN-190 or ZD-7288 application (One way-ANOVA; p>0.05).

Cocaine and caffeine binge treatments altered the inhibitory effects of 5-HT 100 μM puff application on GABA release

We repeated the same experimental approach using slices from WT and 5-HT$_{2A}$ −/− mice treated with either a cocaine *binge* (3×15 mg/kg, 1 hour apart; *i.p.*), or a caffeine *binge* (3×5 mg/kg, 1 hour apart; *i.p.*) (Fig. 4). Although percent inhibition was significant during the

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puff for cells from both cocaine-treated WT and 5-HT_{2A} −/− mice (Student's t-test: $p<0.05$), cells from cocaine-binge treated 5-HT_{2A} −/− mice manifested a significantly larger inhibition than WT (Fig. 4, left plot; Student's t-test, $p<0.001$, cocaine *binge* treated WT vs. 5-HT_{2A} −/−). Importantly, cocaine binge induced a greater *post-puff* inhibition only in 5-HT_{2A} −/− mice (One-way ANOVA: $p=0.015$; comparing *post-puff* in 5-HT_{2A} −/− *vs. post-puff* from cocaine binge treated 5-HT_{2A} −/−).

On the other hand, caffeine *binge* treated WT and 5-HT_{2A} −/− showed significant inhibition in GABA mIPSC frequency only during 5-HT 100μM *puff* (Student's t-test: $p<0.05$), but not during *post-puff* periods (Student's t-test: $p>0.05$) (Fig. 4, right plot). Caffeine reduced 5-HT-mediated inhibition of GABA mIPSC frequency in 5-HT_{2A}−/− mice (Fig. 4, right plot; One-way ANOVA: $p<0.001$; comparing *puff* in 5-HT_{2A} −/− *vs. puff* from caffeine binge treated 5-HT_{2A} −/−).

Discussion

Results presented here demonstrate that both 5-HT_{1A} and 5-HT_{2A} receptors are located in the presynaptic terminals of TRN neurons (Fig. 5). According to the canonical intracellular pathways of these receptors (Millan et al., 2008), the inhibitory role of 5-HT_{1A} receptors on synaptic GABA release could be mediated by their inhibition of adenylyl cyclase and the activation of H-currents. Adenosine receptor type 1 was previously described in these neurons (Ulrich & Huguenard, 1995; Dixon et al., 1996), and would also use this intracellular pathway, occluding the activation of 5-HT_{1A} receptors. On the other hand, 5-HT_{2A} receptors would activate phospholipase C (PLC) pathways, increasing intracellular [Ca^{2+}] and its concomitant augmentation of GABA release (Fig. 5).

Here, we used focally applied 5-HT onto reticular afferents and VB neurons. This experimental approach minimized desensitization followed by internalization of 5-HT receptors during minutes to hour-long bath application of agonists/antagonists (Gray & Roth, 2001). Inhibitory effects of 5-HT on GABA mIPSC frequency suggested that 5-HT receptors were acting presynaptically. Combining pharmacological tools with mice lacking 5-HT_{2A} receptors (5-HT_{2A}−/−; Weisstaub et al., 2006), we described inhibitory 5-HT_{1A} receptors at presynaptic terminals from 5-HT_{2A}−/− mice. The lower inhibitory effects of 5-HT 100 μM *puff* in VB neurons from WT mice suggested that 5-HT_{2A} receptors could counteract the inhibitory actions of 5-$HT_{1A.}$ Only 5-HT_{1A} and 5-HT_{2A} receptors were involved in the modulation of GABA release from presynaptic terminals of TRNs (Fig. 5), since applying the 5-HT_{1A} receptor specific antagonist NAN-190 in slices from 5-HT_{2A}−/− mice yielded no effect of a 5-HT 100 μM *puff*.

These results are consistent with previous immunohistochemical reports describing the expression of both 5-HT_{1A} and 5-HT_{2A} receptors and 5-HT transporter (SERT)-containing afferent fibers at the somas of TRNs (Rodriguez et al., 2011). At the somatic level of these neurons, it has been reported that bath applied 5-HT depolarized VB cells (Sanchez-Vives et al. 1996; Monckton & McCormick 2002) without affecting high frequency (40Hz) action potential firing (Pinault & Deschenes, 1992).

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In other brain structures, 5-HT_{1A} inhibitory and 5-HT_{2A} excitatory effects on synaptic release have also been reported. In rat pyriform cortex, bath applied 5-HT (100 μM) and 4-Iodo-2,5-dimethoxy-α-methylbenzeneethanamine hydrochloride (DOI, a 5-HT_{2A} specific agonist; 10 μM) increased GABAergic mIPSC frequency in pyramidal neurons trough activation of 5-HT_{2A} receptors (Marek & Aghajanian, 1996). In prefrontal cortical slices from rats in the third postnatal week (age range similar to the one used in this study), bath-applied 5-HT initially elicited a depolarization (pharmacologically determined to be mediated by 5HT_{2A} receptors) of layer V pyramidal neurons then gradually shifted to a long duration hyperpolarization period mediated by 5-HT_{1A} receptors (Béïque et al., 2004). 5-HT_2 receptor activation has been shown to facilitate GABA release, while 5-HT_1 receptors were involved in preventing such activation (Fink & Göthert, 2007). Bath-applied 5-HT inhibited Ca^{2+} currents of caudal Raphe neurons through the activation of 5-HT_{1A} receptors (Bayliss et al., 1997). Therefore, 5-HT would inhibit action potential afterhyperpolarization, inducing an increase in action potential frequency in these cells. The inhibitory effects of 5-HT_{1A} are mediated by both neuronal hyperpolarization and inhibition of adenylate cyclase (De Vivo & Maayani, 1986).

Presynaptic actions of 5-HT_{2A} receptors described in our study confirm previously accepted paradigms in which only 5-HT_{1A} receptors were considered to be located at presynaptic sites, while 5-HT_{2A} receptors are thought to be localized in postsynaptic structures (Nichols & Nichols, 2008). Nevertheless, understanding the role of 5-HT_{1A} receptors modulating GABAergic transmission is relevant to their role in multiple psychiatric and neurological disorders. In postmortem schizophrenia patients an increase in 5-HT_{1A} receptor density in prefrontal cortex has been reported (Bantick et al., 2001). Activation of 5-HT_{1A} receptors produced antidepressant-like effects in animal models (Lucki, 1991), and in knockout mice lacking 5-HT_{1A} receptors have been previously used as genetic models of anxiety disorder (Toth, 2003).

Our results also described a key role for H-currents on the presynaptic inhibitory effects mediated by a 5-HT puff. Using 5-$HT_{2A}{}^{-/-}$ animals, we observed significantly lower inhibitory effects during 100 μM 5-HT *puff* application after blocking H-currents with ZD-7288. Basal activation of H-currents would normally inhibit GABA release, probably by shunting membrane resistance as described in distal-apical dendritic compartments of cortical pyramidal neurons (Berger et al., 2003). Morphological and functional experiments have confirmed the presence of H-channels negatively influencing GABA release from rodent globus pallidus neurons (Boyes et al., 2007). Furthermore, H-currents can be tonically activated in presynaptic terminals, reducing the spontaneous release of mIPSCs while inactivating Ca^{2+}-gated channels (Huang et al., 2011). 5-HT_2 receptors reduced H-currents and influenced the number of those channels (Liu et al., 2003).

The inhibitory 5-HT puff effects were altered after cocaine or caffeine *binge* treatments. We observed that a cocaine *binge* prolonged 5-HT-mediated inhibition during *post-puff* periods in 5-$HT_{2A}{}^{-/-}$ mice, suggesting an enhancement of 5-HT_{1A}-mediated inhibitory effects. Repetitive cocaine administration attenuated the ability of 5-HT to enhance spontaneous excitatory postsynaptic currents in the medial prefrontal cortex through impairment of 5-HT_{2A} coupling to its intracellular pathways (Huang et al., 2009), which would potentiate a

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5-HT_{1A}-mediated inhibition like the one presented here. The fact that a cocaine *binge* did not affect 5-HT *puff*-mediated inhibition during GABA release from cells in WT mice may be due to internalization/downregulation processes of 5-HT_{1A} receptors, previously described in rats (Perret et al., 1998). Cocaine-induced desensitization of 5-HT_{1A} autoreceptors was observed in Raphe nucleus after chronic fluoxetine treatment (an antagonist of SERT) (Le Poul et al., 2000). 5-HT_{1A} desensitization is known to occur after the prolonged activation of G protein intracellular pathways (Castro et al., 2003; Shi et al., 2007), which eventually can result in the internalization of those receptors (Gray & Roth, 2001).

Results presented here described a novel mechanism showing that GABA release can indeed be modulated by the interaction between 5-HT_{1A} and 5-HT_{2A} receptors, which supports our previous hypothesis underlying differential effects of cocaine and methylphenidate on TRN synaptic terminals (Goitia et al., 2013). Furthermore, the prolonged inhibition of GABA release described here after cocaine *binge* treatment may desensitize 5-HT_{1A} receptors, allowing 5-HT_{2A} to play a greater role, resulting in higher GABAergic mIPSC frequencies as described by our group (Urbano et al., 2009; Bisagno et al., 2010; Goitia et al., 2013). Homeostatic compensatory mechanisms (Mee et al., 2004; Baines, 2005) of GABAergic transmission would also explain the previously observed prolonged activation of this inhibitory synapse after cocaine *binge* treatment (Urbano et al., 2009; Bisagno et al., 2010; Goitia et al., 2013). Serotonergic mediated inhibition in mice treated with cocaine may trigger compensation at the somatic TRN level, reducing the expression of inhibitory 5-HT_{1A} receptors. Further experiments are needed to support this hypothesis.

Adenosine type 1 receptors are present in the somatosensory thalamocortical system (Fontanez & Porter, 2006), that exert robust antioscillatory effects by simultaneously decreasing excitatory and inhibitory synaptic transmission (Ulrich & Huguenard, 1995). The present study involved a *binge* treatment with a low caffeine dose (5 mg/kg). A previous study that measured brain concentration of caffeine after an intraperitoneal administration (20mg/kg) reported that caffeine brain levels reached up to 100μM (Hepper & Davies, 1999). Therefore it might be estimated that our protocol using 3× 5mg/kg of caffeine might have produced caffeine brain levels in the tens of micromolar range. Such caffeine concentration levels have been ascribed to adenosine receptor blockage (Fredholm, 1995; Fredholm et al., 1999), rather than acting through the inhibition of phosphodiesterases (Aoyama et al., 2011), or the opening of IP_3 receptors expressed in TRN (Garaschuk et al., 1997; Rankovic et al., 2010). Furthermore, ryanodine receptors expressed in both TRN (Budde et al., 2000) and VB neurons (Coulon et al., 2009) might also be involved in caffeine modulation of GABA release shown in this study.

At the postsynaptic level, adenosine receptors have been described to inhibit H-currents in relay thalamic neurons (Pape, 1992). Also, adenosine and serotonin receptors enhanced leak potassium currents (Pape, 1992; Coulon et al., 2010). Further experiments are still needed to clarify 5-HT and caffeine role of the intrinsic properties of postsynaptic thalamocortical neurons.

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In cortical pyramidal neurons, adenosine A1 receptors preferentially affect 5-HT_{2A}-mediated enhancement of spontaneous postsynaptic excitatory synaptic events (Stutzmann et al., 2001), contrary to what we report here. In our hands, caffeine-mediated inhibition of adenosine receptors located in presynaptic TRN terminals prevented the inhibitory effects of 5-HT_{1A} receptors during 100 μM puffs in slices from 5-HT_{2A}−/− mice. Therefore, only in the absence of 5-HT_{2A} receptors was caffeine able to affect 5-HT mediated control of GABA release. One likely mechanism for these results could be due to blockade of adenosine A1 receptors after caffeine treatment (Fredholm, 1995; Fredholm et al., 1999), releasing their down-regulation of adenylate cyclase. An increase in the abundance of available adenylate cyclase might partially compensate for the previously observed inhibitory effects of 5-HT_{1A} receptors on GABA release at 5-HT_{2A} −/− reticular synaptic terminals.

The present work highlights the role of 5-HT in modulating GABA release at VB thalamic nucleus during normal physiological activity. In addition, novel 5-HT-mediated mechanisms described here might help explain the long-lasting, detrimental effects of cocaine and caffeine dysregulation of thalamic GABAergic transmission. Such mechanisms could induce permanent changes in sensory thalamic processing.

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Abbreviations

AC	adenylyl cyclase
ACSF	artificial; cerebrospinal fluid
ADHD	attention deficit/hyperactivity disorder
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione disodium salt hydrate
DAT	dopamine transporter
DL-AP5	DL-2-amino-5-phosphonovaleric acid
GIRK	G-protein-activated inwardly rectifying K^+ channels
5-HT	serotonin
mIPSC	spontaneous miniature inhibitory post-synaptic current

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MPH	methylphenidate-HCl
NET	norepinephrine transporter
PLC	phospholipase C
SERT	serotonin transporter
TRN	thalamic reticular nucleus
TTX	tetrodotoxin
VB	ventrobasal nucleus

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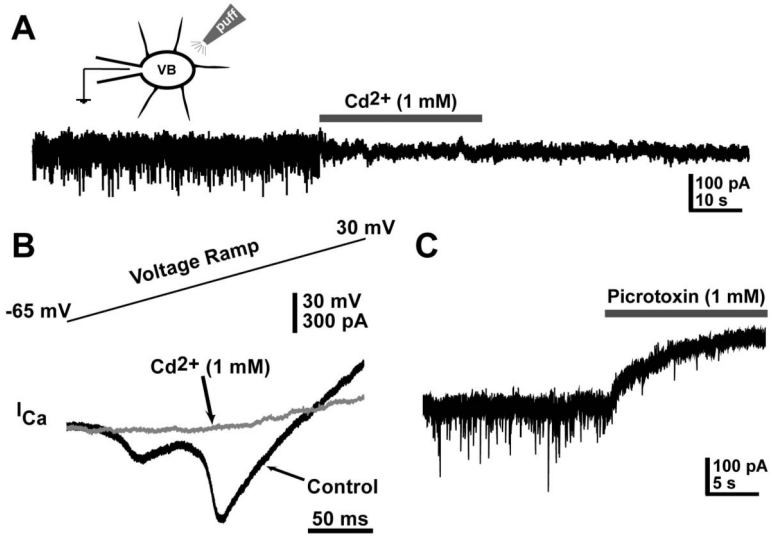

Figure 1. Focal *puff* application of drugs can affect both ore- and postsynaptic components of GABA release from thalamic reticular nucleus

(A) Whole-cell patch clamp recording of inhibitory post-synaptic miniatures currents (mIPSCs) in a VB neuron on which ACSF containing Cd^{2+} (1mM) was *puffed*, showing that blockade of presynaptic calcium channels (and therefore blockade of GABA release from TRN terminals) results in the lack of mIPSCs in the VB neuron. (B) Recording of a VB neuron before (black record) and during (gray record) a Cd^{2+} *puff* (1mM). Before the puff was applied, the voltage ramp triggered Ca^{2+} currents, both low-voltage and high-voltage activated, but these currents were not triggered when Cd^{2+} was applied, since it blocks Ca^{2+} channels in the postsynaptic neuron (VB neuron being patched). (C) Recording of a VB neuron on which ACSF containing picrotoxin (1mM) was *puffed*, showing that the mIPSCs recorded were GABAergic.

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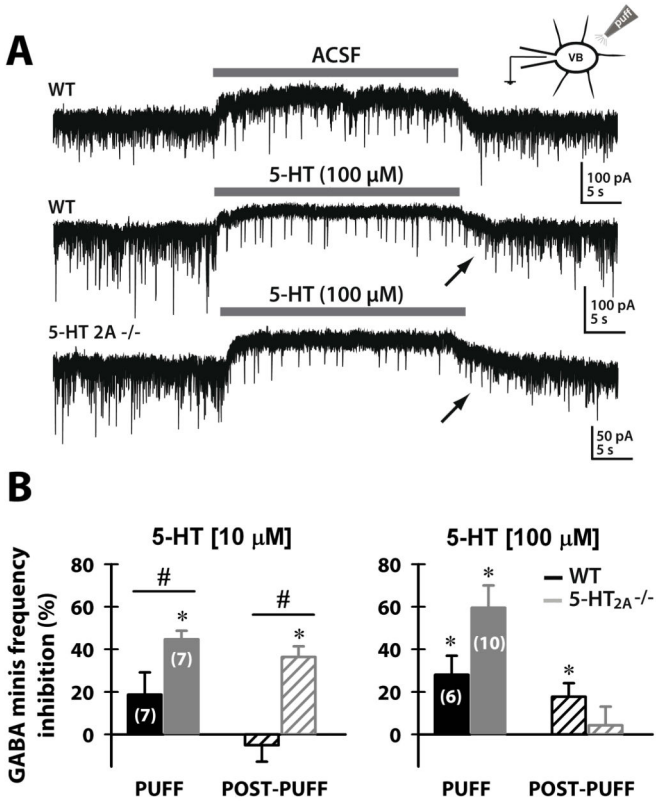

Figure 2. GABA release from the TRN is reduced by focal 5-HT *puff* application in a dose-dependent manner

(A) Representative whole-cell recordings showing mIPSCs from VB neurons from WT and 5-HT_{2A} −/− mice. The uppermost record shows that an ACSF puff does not modify the frequency of mIPSCs in a VB neuron from a WT mouse, while in the middle and lower records ACSF with 5-HT 100µM reduced the appearance of mIPSCs in VB neurons from WT or 5-HT_{2A} −/− mice. This effect was reversible, and soon after the puff ended, the frequency began to recover towards initial values (arrows). (B) Percent inhibition (relative to the pre-puff mIPSC frequency for each neuron) of mIPSCs during a 5-HT 10µM (left graph) or 100µM (right graph) 30 sec puff and during 1 min post-puff in VB neurons from WT (black bars) and 5-HT_{2A} −/− mice (grey bars). During the *puff* (filled bars), the percent inhibition was higher for 5-HT_{2A} −/− neurons at both 5-HT concentrations used (two-way ANOVA, p=0.0048). The percent inhibition was significant during the 5-HT 100µM puff both in cells from 5-HT_{2A} −/− (Student's t-test; p=0.0003) and WT (Student's t-test; p=0.024) mice, but during the 5-HT 10µM puff, only in 5-HT_{2A} −/− mice was the percent inhibition significantly different (Student's t-test; p<0.0001). Post-puff (dashed bars): there were differences between WT and 5-HT_{2A} −/− only for the lower 5-HT concentration tested (two-way ANOVA, simple effects; p=0.0006), that is, the percent inhibition was significant only for the 5-HT_{2A} −/− mice (Student's t-test; p=0.0006). After the 5-HT 100µM puff, the percent inhibition was significant only for the WT group (Student's t-test; p=0.0379). *p < 0.05 compared to pre-puff frequency. # p < 0.05, WT vs. 5-HT_{2A} −/−.

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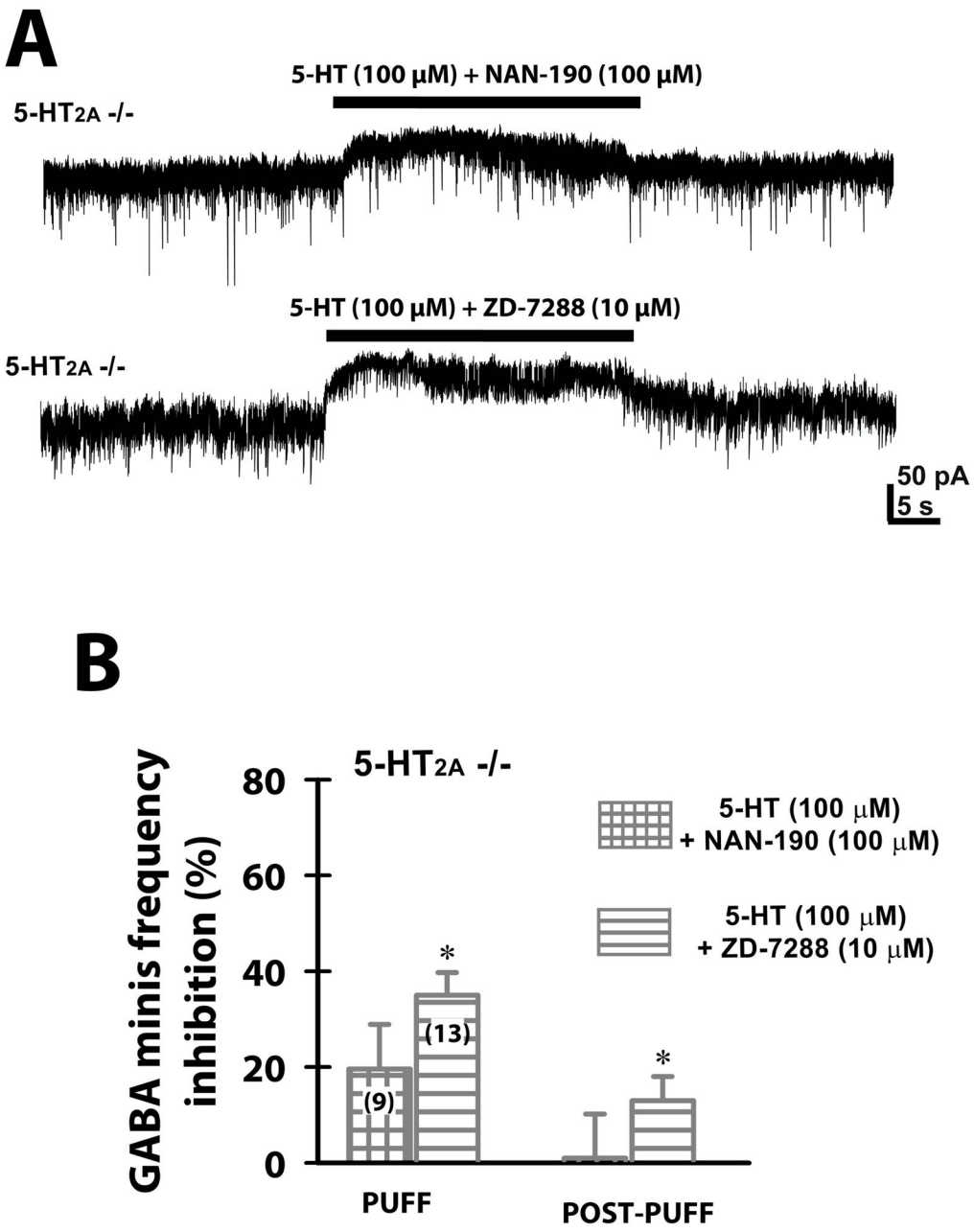

Figure 3. Inhibitory effects of a local *puff* 5-HT on GABA release in TRN terminals from 5-HT$_{2A}$ −/− mice were mediated by 5-HT$_{1A}$ receptors and H-currents

(A) Representative whole-cell recordings showing mIPSCs from VB neurons obtained from 5-HT$_{2A}$ −/− mice during 30 sec puff application of 5-HT 100μM+NAN-190 100μM, or 5-HT 100μM+ZD-7288 10 μM. (B) Percent inhibition (relative to the pre-puff mIPSC frequency for each neuron) of mIPSCs during a 5-HT 100μM (in presence or absence of NAN-190 100μM or ZD-7288 10μM) 30 sec puff and during 1min post-puff in VB neurons from 5-HT$_{2A}$ −/− mice. The percent inhibition was not significant during the 5-HT puff in the presence of NAN-190 (Student's t-test; $p>0.05$). In the presence of ZD-7288, percent

inhibition was significant during both puff (Student's t-test; p=0.0105), and post-puff (Student's t-test; p=0.0017 for 5-HT+ZD) periods. *p< 0.05 compared to pre-puff frequency.

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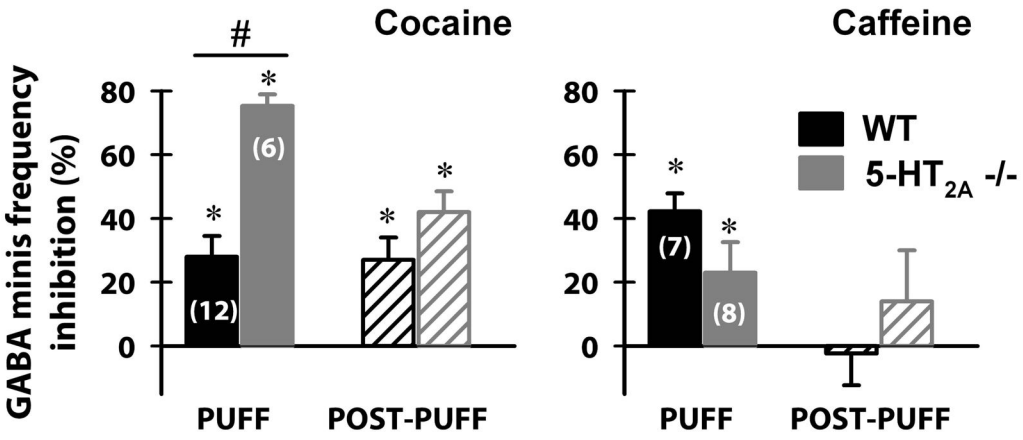

Figure 4. Cocaine and caffeine *binge* treatments altered the inhibitory effects of 5-HT 100 μM *puff* application on GABA release from TRN terminals in cells from 5-HT$_{2A}$ −/− mice

Percent inhibition (relative to the *pre-puff* mIPSC frequency for each neuron) of mIPSCs during a 5-HT 100μM 30 sec *puff* and during 1min *post-puff* in VB neurons from WT (black bars) and 5-HT$_{2A}$ −/− (grey bars) mice treated with cocaine *binge* (3×15 mg/kg, 1 hour apart; *i.p.*) or caffeine binge (3×5 mg/kg, 1 hour apart; *i.p.*), and sacrificed 1 hr after receiving the last injection. The percent inhibition was significantly different during the *puff* for both cocaine-treated WT and 5-HT$_{2A}$ −/− mice (Student's t-test: $p<0.05$), with significantly higher inhibition in cells recorded in VB neurons from cocaine *binge* treated 5-HT$_{2A}$ −/− mice (Student's t-test, $p<0.001$, cocaine *binge* treated WT vs. 5-HT$_{2A}$ −/−). *Post-puff* inhibition levels were greater in VB neurons from cocaine-treated 5-HT$_{2A}$ −/− mice (One-way ANOVA: $p=0.015$; comparing *post-puff* in 5-HT$_{2A}$ −/− *vs. post-puff* from cocaine binge treated 5-HT$_{2A}$ −/−). Caffeine *binge* treated WT and 5-HT$_{2A}$ −/− showed significant inhibition in GABA mIPSC frequency only during the 5-HT 100 μM puff (Student's t-test: $p<0.05$), but not during post-puff periods (Student's t-test: $p>0.05$). Mean inhibition during 5-HT 100 μM *puff* was reduced in VB neurons from caffeine binge treated 5-HT$_{2A}$−/− mice (One-way ANOVA: $p<0.001$; comparing *puff* in 5-HT$_{2A}$ −/− *vs. puff* from caffeine binge treated 5-HT$_{2A}$ −/−). *$p<0.05$ compared to pre-puff frequency. # $p<0.001$ comparing WT *vs.* 5-HT$_{2A}$ −/− groups.

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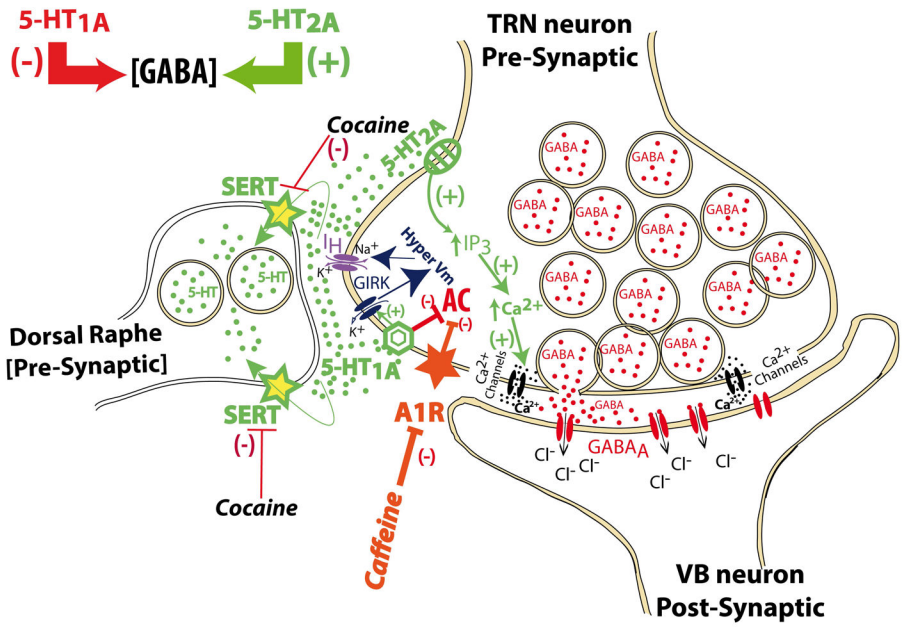

Figure 5. Representative cartoon of the probable intracellular pathways underlying the results found in this study

Serotonin release from dorsal Raphe nucleus afferents (Rodríguez et al., 2011) are known to act on presynaptic **5-HT$_{1A}$** receptors activating a $G_{i/o}$ protein that would inhibit adenylate cyclase while increasing the probability of the opening of *G-protein-activated inwardly rectifying K^+ channels* (GIRK) (Millan et al., 2008; Luscher et al., 1997). In addition, type 1 adenosine receptors (A_1) that are highly expressed in thalamic neurons (Ulrich & Huguenard, 1995; Dixon et al., 1996) have been also described to activate GIRK channels (Luscher et al., 1997). The prolonged activation of GIRK currents would hyperpolarize the membrane potential, facilitating the opening of H-channels. The opening of additional H-channels would have a shunting effect, reducing Ca^{2+} spike summation (Berger et al., 2003), ultimately resulting in a reduction of GABA release. On the other hand, **5-HT$_{2A}$**-mediated activation of PLC and IP_3, increasing intracellular [Ca^{2+}], would facilitate GABA release (Millan et al., 2008). Caffeine treatment would exert a blocking effect on A1 receptors (Fredholm, 1995; Fredholm et al., 1999), and remove the down-regulation of adenylate cyclase, partially compensating for the inhibitory effects of 5-HT_{1A} receptors on GABA release.