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Histamine H_3 Receptor Activation Counteracts Adenosine A_{2A} Receptor-Mediated Enhancement of Depolarization-Evoked [^{3}H]-GABA Release from Rat Globus Pallidus Synaptosomes

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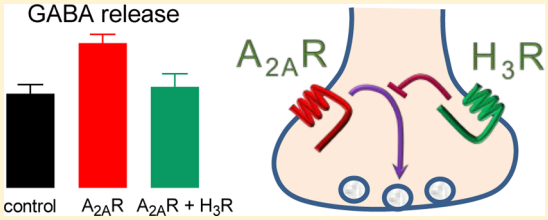
ABSTRACT: High levels of histamine H_3 receptors (H_3Rs) are found in the globus pallidus (GP), a neuronal nucleus in the basal ganglia involved in the control of motor behavior. By using rat GP isolated nerve terminals (synaptosomes), we studied whether H_3R activation modified the previously reported enhancing action of adenosine A_{2A} receptor (A_{2A}R) stimulation on depolarization-evoked [^{3}H]-GABA release. At 3 and 10 nM, the A_{2A}R agonist CGS-21680 enhanced [^{3}H]-GABA release induced by high K^+ (20 mM) and the effect of 3 nM CGS-21680 was prevented by the A_{2A}R antagonist ZM-241385 (100 nM). The presence of presynaptic H_3Rs was confirmed by the specific binding of *N*-α-[methyl-^{3}H]-histamine to membranes from GP synaptosomes (maximum binding, B_{max}, 1327 ± 79 fmol/mg protein; dissociation constant, K_d, 0.74 nM), which was inhibited by the H_3R ligands immepip, clobenpropit, and A-331440 (inhibition constants, K_i, 0.28, 8.53, and 316 nM, respectively). Perfusion of synaptosomes with the H_3R agonist immepip (100 nM) had no effect on K^+-evoked [^{3}H]-GABA release, but inhibited the stimulatory action of A_{2A}R activation. In turn, the effect of immepip was blocked by the H_3R antagonist clobenpropit, which had no significant effect of its own on K^+-induced [^{3}H]-GABA release. These data indicate that H_3R activation selectively counteracts the facilitatory action of A_{2A}R stimulation on GABA release from striato-pallidal projections.



KEYWORDS: *Adenosine A_{2A} receptor, histamine, histamine H_3 receptor, globus pallidus, basal ganglia, GABA release*

Histamine regulates a variety of mammalian brain functions including sleep-wake rhythmicity, motor activity, attention and learning as well as different aspects of body homeostasis.[1] Upon release from axon terminals of neurons located in the hypothalamic tuberomammillary nucleus the amine acts at three G protein-coupled receptors (H_1, H_2, and H_3) widely expressed in the brain.[1,2] Histamine H_3 receptors (H_3Rs) are mainly located on nerve terminals and control the release and synthesis of histamine and the release of several other neurotransmitters or neuromodulators namely acetylcholine, glutamate, noradrenaline, γ-aminobutyric acid (GABA), dopamine, serotonin (5-hydroxytryptamine, 5-HT), and substance P.[2,3]

The globus pallidus (GP) forms part of the basal ganglia, a group of subcortical neuronal nuclei involved in the control of motor behavior among other functions.[4] There exists strong evidence that the GP plays a critical role in basal ganglia physiology, and thus in the pathophysiology of motor disorders, including Parkinson's disease, in which alterations in the pattern and synchrony of discharge of pallidal neurons have been reported.[5] The neuronal population of the GP is mainly conformed by GABAergic neurons, divided into two groups according to the target of their axons, the subthalamic nucleus (STN) or the striatum.[4,5] In turn, the main synaptic afferents to the GP are striato-pallidal GABAergic axons,[4] with additional innervation by glutamatergic fibers originated primarily in the STN[6,7] and dopaminergic afferents from *substantia nigra pars compacta*.[8]

The rat GP is also innervated by histaminergic fibers[9] and possesses a high density of H_3Rs, mostly located on the axons of neurons projecting to the nucleus, as indicated by the very low levels of the corresponding mRNA found in the GP. Further, H_3R mRNA is expressed at high levels by the GABAergic striatal neurons whose axons conform the main synaptic input to the GP.[10]

Although little is still known about the function of pallidal H_3Rs, it is conceivable that these receptors regulate GP synaptic flow at the presynaptic level, as shown for other brain regions.[2] In a previous work, we showed that H_3R activation reduced the depolarization-evoked release of [^{3}H]-D-aspartate from rat GP slices and inhibited glutamatergic transmission in vivo,[11] but failed to detect any significant action on [^{3}H]-GABA release from slices. However, for the terminals of striato-nigral neurons the inhibitory action of H_3Rs on depolarization-evoked [^{3}H]-GABA release depends on the concomitant activation of dopamine D_1 receptors, which stimulate neurotransmitter release through a signaling pathway involving cAMP and protein kinase A (PKA).[12−14] Striatal neurons projecting to the GP do not express D_1 receptors but possess adenosine A_{2A}

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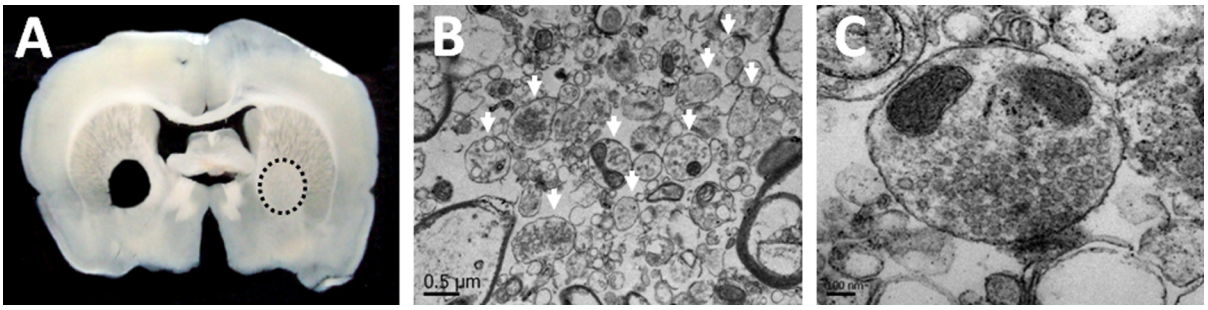

Figure 1. Rat globus pallidus synaptosomes. (A) Brain slice illustrating the dissection of the globus pallidus (dotted circle). (B) Micrograph of the synaptosomal preparation at ×20 000. There are numerous intact rounded structures (arrows) of a range of size bearing the characteristics of nerve terminals, namely, small and rounded vesicles, mitochondria, and electro-dense regions most likely corresponding to active zones. Synaptosomes constitute a significant proportion of the particulate matter present in the preparation. Myelin was identified by its multilamellar structure and mitochondria were identified by the presence of a double membrane and cristae. (C) Micrograph of the synaptosomal preparation at ×50 000 showing a single synaptosome.

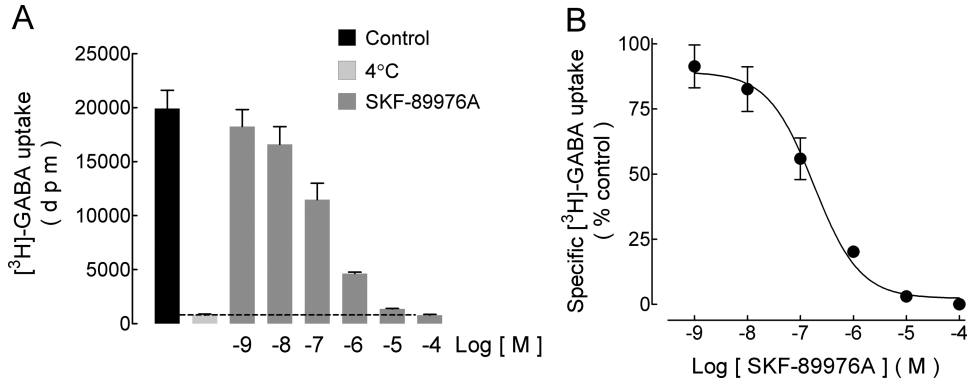

Figure 2. Inhibition by SKF-89976A of [^{3}H]-GABA uptake by rat GP synaptosomes. Synaptosomes were incubated for 4 min with [^{3}H]-GABA/GABA (50 nM/3 μM) in the absence and presence of the indicated concentrations of the inhibitor SKF-89976A, added 10 min before. (A) Representative experiment. Values are means ± SEM from three replicates. Nonspecific uptake was determined in parallel samples incubated at 4 °C. (B) Analysis of the concentration−response curve for SKF-89976A. [^{3}H]-GABA uptake is expressed as percentage of control values after subtraction of nonspecific uptake. Values are means ± SEM of three replicates from the experiment shown in panel (A). The curve drawn is the best-fit adjust to a logistic equation. The IC_{50} value is given in the text.

receptors (A_{2A}Rs), also coupled to the cAMP/PKA pathway,[15] and shown to modulate GABA release in rat GP.[16−19] Collaterals of intrinsic GP neurons also release GABA, but these cells do not express A_{2A}R mRNA.[20]

In the indirect pathway of the basal ganglia, striato-pallidal neurons synapse with GABAergic GP neurons that project to the STN. In turn, glutamatergic STN neurons innervate GABAergic neurons in the *substantia nigra pars reticulata* (SNr) which control the activity of thalamo-cortical neurons. Under resting conditions, the activity of striatal projection neurons is very low and the spontaneous firing of GP neurons exerts a tonic inhibition on the intrinsic activity of STN neurons.[4,21] Stimulation by cortical afferents activates striato-pallidal neurons and thus the indirect pathway making the regulation of GABA release by striato-pallidal terminals critical for basal ganglia function.

In this work, we therefore studied the effect of coactivating A_{2A} and H_3 receptors on the depolarization-evoked [^{3}H]-GABA release from rat GP isolated nerve terminals (synaptosomes), and our results showed that H_3R activation counteracted the stimulatory action of A_{2A}Rs.

RESULTS AND DISCUSSION

1. Morphological Characteristics of GP Synaptosomes. Figure 1A illustrates the dissection of the GP from coronal brain slices, and Figure 1B and C shows representative electron micrographs of the GP synaptosomal preparation. There were numerous rounded structures bearing the characteristics of nerve terminals, namely, small and rounded vesicles, mitochondria, and electro-dense regions most likely corresponding to active zones. Figure 1B and C indicates that our preparation was enriched in isolated nerve terminals.

2. [^{3}H]-GABA Uptake by Rat GP Synaptosomes. In the rat GP, the GABA transporters GAT-1 and GAT-3 are mainly expressed on axonal terminals and glial cells, respectively.[22] Figure 2A shows that SKF-89976A, a high-affinity inhibitor of the GAT-1 transporter, fully inhibited the specific [^{3}H]-GABA uptake by rat GP synaptosomes. The effect was concentration-dependent (Figure 2B) with IC_{50} 0.24 μM (pIC_{50} 6.62 ± 0.06, mean ± standard error, SEM, three experiments). A comparison with the IC_{50} values reported for cloned rat GABA transporters (GAT-1 0.53 μM, GAT-3 4 390 μM)[23] indicated that in our synaptosomal preparation GABA was taken up mostly, if not exclusively by the isolated nerve terminals.

3. [^{3}H]-NMHA Binding to Rat GP Membranes. Saturation [^{3}H]-NMHA binding to GP membranes yielded maximum specific binding (B_{max}) 1327 ± 79 fmol/mg protein (three experiments, Figure 3A) and equilibrium dissociation constant (K_d) 0.74 nM (pK_d 9.13 ± 0.05). Although the K_d value is similar to that found for membranes obtained from the whole

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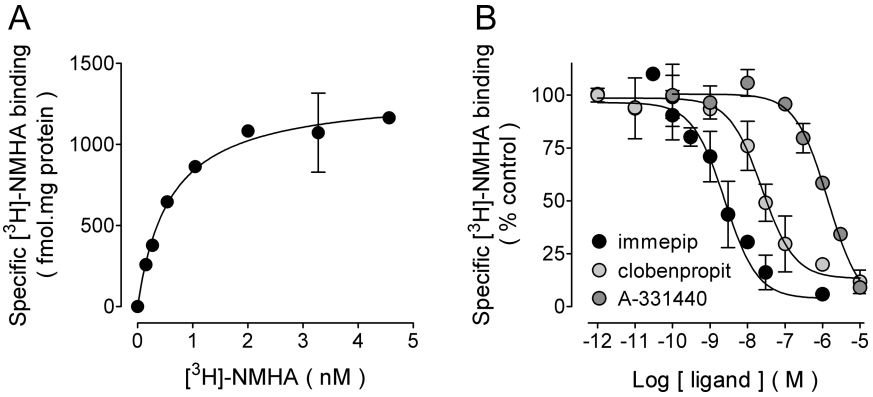

Figure 3. Binding of *N*-α-[methyl-^{3}H]-histamine ([^{3}H]-NMHA) to membranes from rat globus pallidus synaptosomes. (A) Saturation binding. Membranes were prepared as described in Methods and then incubated with the indicated concentrations of [^{3}H]-NMHA. Specific receptor binding was determined by subtracting the binding in the presence of 10 μM histamine from total binding. Points are means ± SEM from triplicate determinations from a single experiment, which was repeated a further twice. The line drawn is the best fit to a hyperbola. Best-fit values for the equilibrium dissociation constant (K_d) and maximum binding (B_{max}) are given in the text. (B) Inhibition by the H_3R agonist immepip and the antagonists/inverse agonists clobenpropit and A-331440. Membranes were incubated with ∼1.5 nM [^{3}H]-NMHA and the indicated drug concentrations. Values are expressed as the percentage of control specific binding and are means ± range from duplicate determinations from a representative experiment. The line drawn is the best fit to a logistic equation for a one-site model. pK_i values calculated from the best-fit IC_{50} estimates are given in the text.

rat GP (K_d 0.58 nM, B_{max} 162 ± 29 fmol/mg protein),[11] the density is 8-fold higher, indicating that H_3Rs were highly concentrated in the GP nerve terminals.

Specific [^{3}H]-NMHA binding was inhibited in a concentration-dependent manner by the H_3R agonist immepip and the antagonists/inverse agonists clobenpropit and A-331440 (Figure 3B). Values for the inhibition constants (−log K_i, pK_i) from three experiments were 9.55 ± 0.20 (K_i 0.28 nM) for immepip, 8.07 ± 0.21 (K_i 8.53 nM) for clobenpropit, and 6.50 ± 0.04 (K_i 316 nM) for A-331440. These values allowed for the calculation of receptor occupancy by H_3R ligands in functional experiments.

4. Characteristics of Depolarization-Evoked [^{3}H]-GABA Release from GP Synaptosomes. Changing the K^+ concentration in the superfusion medium from 4 to 20 mM increased [^{3}H]-GABA efflux from rat GP synaptosomes to 341 ± 15% and 278 ± 12% of basal at the peak of release in the first and second depolarizing stimuli, respectively (Figure 4A), with an overall S2/S1 ratio of 0.602 ± 0.021 (45 experiments).

In synaptosomes perfused with KRH solution without $CaCl_2$, K^+-evoked [^{3}H]-GABA release was reduced by 73.6 ± 10.1% (n = 3, P < 0.001 when compared with the release in the presence of 1.8 mM $CaCl_2$, Student's t test; data not illustrated), indicating that most of the depolarization-induced [^{3}H]-GABA release was due to exocytosis. Basal [^{3}H]-GABA efflux was modestly but significantly reduced when $CaCl_2$ was omitted from the perfusion medium (81.0 ± 1.5% of control values, P = 0.001, Student's t test).

Depolarization-evoked [^{3}H]-GABA release was not modified by the presence in the perfusion medium of the GAT-1 inhibitor SKF-89976A (3 μM, 103.1 ± 8.0% of control values, P = 0.717, Student's t test; data not illustrated), indicating that under our experimental conditions the perfusion prevented that the released mixture of [^{3}H]-GABA/GABA yielded the concentration required to significantly activate GABA transport by GP synaptosomes.

5. Effect of A_{2A}R Activation on Depolarization-Evoked [^{3}H]-GABA Release from GP Synaptosomes. The activation of A_{2A}Rs has been shown to modulate GABA release from rat GP slices. However, whereas Floran et al.[19] reported that the selective agonist CGS-21680[24] increased K^+-evoked [^{3}H]-GABA release in a wide concentration range (10 nM to 10 μM, EC_{50} 65 nM), Zahniser and co-workers[16,17] showed a dual action of the agonist on the release of endogenous GABA induced by electrical stimulation with a facilitatory effect at 1 and 10 nM and inhibition at higher concentrations (100 and 1000 nM).

We therefore tested four concentrations (3, 10, 30, and 300 nM) of the A_{2A}R agonist on K^+-evoked [^{3}H]-GABA release from rat GP synaptosomes. Figure 4A illustrates the experimental protocol and the effect of 3 and 300 nM CGS-21680. At 3 and 10 nM, CGS-21680 significantly enhanced K^+-evoked [^{3}H]-GABA release to 159.9 ± 20.3 and 143.3 ± 9.4% of control values (S2/S1 ratios: control 0.426 ± 0.029, 3 nM CGS-21680 0.699 ± 0.099, 10 nM CGS-21680 0.670 ± 0.051, three experiments, P < 0.05 for both concentrations when compared with control values, Figure 4B). These concentrations (3 and 10 nM) are below the K_i value reported for CGS-21680 (15 nM),[25] indicating an amplification effect in the signaling pathway underlying the receptor action.

The selective A_{2A}R antagonist ZM-241385[24] prevented the enhancing action of 3 nM CGS-21680 on K^+-evoked [^{3}H]-GABA release (Figure 4C; CGS-21680 135.2 ± 5.9% of controls, CGS-21680 and 100 nM ZM-241385 104.9 ± 10.0% of controls; S2/S1 ratios: control 0.535 ± 0.041; CGS-21680 0.750 ± 0.022, P < 0.05; CGS-21680 and ZM-241385 0.578 ± 0.047, P > 0.05, three experiments). This result confirmed that the stimulatory action of CGS-21680 was receptor-mediated.

In contrast to the effect at 3 and 10 nM, in a different series of experiments (not illustrated), 30 nM CGS-21680 had no significant effect on K^+-evoked [^{3}H]-GABA release (105.8 ± 7.7% of controls, S2/S1 ratios: control 0.656 ± 0.040, CGS-21680 0.662 ± 0.039; P > 0.05), whereas at 300 nM in six out of seven experiments there was a reduction in the release when compared with control values (mean 90.2 ± 7.1% of controls), but the analysis of the S2/S1 ratios did not yield statistical significance (S2/S1 ratio 0.601 ± 0.036; P > 0.05). Adenosine A_1 receptors are expressed at high density in the rat GP (475 ± 37 fmol/mg protein versus 270 ± 20 fmol/mg protein for A_{2A}Rs),[16] and electrophysiological evidence indicates

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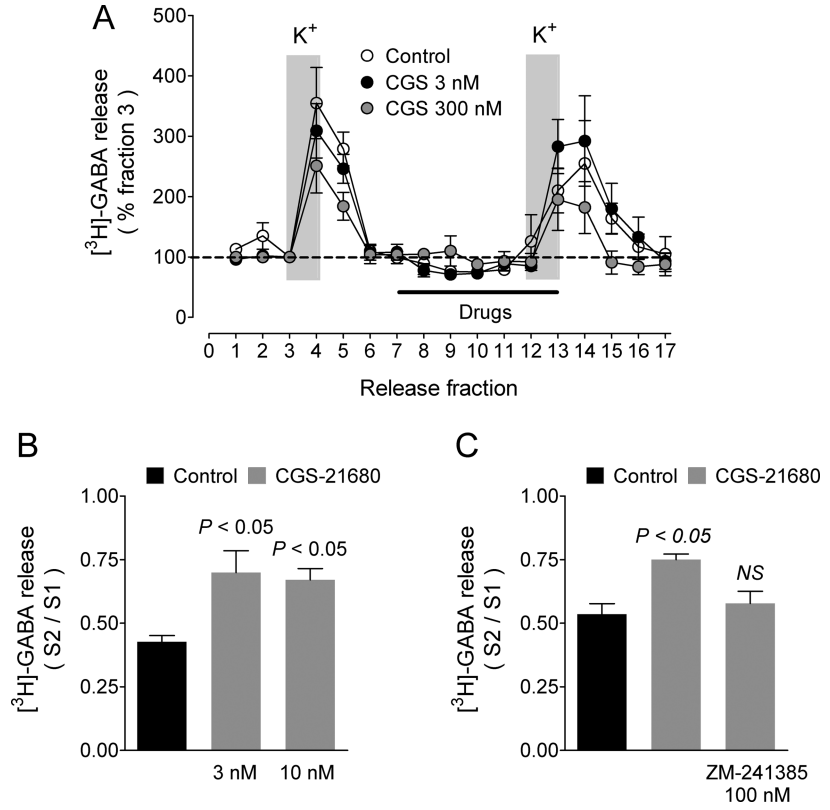

Figure 4. Effect of the adenosine A_{2A} receptor agonist CGS-21680 on depolarization-evoked [^{3}H]-GABA release from rat globus pallidus synaptosomes. (A) Representative experiments for 3 and 300 nM. Labeled synaptosomes were perfused with KRH solution (4 mM K^+), and neurotransmitter release was evoked by raising the K^+ concentration in the perfusion medium to 20 mM for the periods indicated by the vertical gray bars. Where required, drugs under test were present for the period indicated by the black bar. Values are expressed as a percentage of [^{3}H]-GABA in fraction 3 and represent the means ± SEM of 4–6 replicates. (B) Statistical analysis for 3 and 10 nM CGS-21680. Values are means ± SEM from three experiments. *P* values are for the comparison with control release (no drugs added). For panels (B) and (C), the statistical analysis was performed with ANOVA and Dunnett's post hoc test. (C) Blockade by the A_{2A} receptor antagonist ZM-241385 of the effect of 3 nM CGS-21680. Values are means ± SEM from three experiments. NS, not different from control release.

that they are also present in striato-pallidal neurons.[26] Therefore, and as suggested previously for GP slices,[16] one possibility is that at high concentrations CGS-21680 binds and activates a fraction of A_1 receptors (K_i for the cloned rat receptor 2600 nM)[25] which through their coupling to $G\alpha_{i/o}$ proteins inhibit both cAMP formation and voltage-activated Ca^{2+} channels[27] and can thus oppose the A_{2A}R-mediated stimulation of GABA release. However, a dual effect of CGS-21680 through different signaling pathways,[15] as shown for the A_{2A}R-mediated modulation of acetylcholine release,[28] cannot be excluded.

6. Effect of H_3R Activation on Depolarization-Evoked [^{3}H]-GABA Release from GP Synaptosomes. When added alone to the perfusion medium, neither the H_3R agonist immepip (100 nM) nor the antagonist/inverse agonist clobenpropit (3 μM) had significant effect on depolarization-evoked [^{3}H]-GABA release (111.3 ± 7.0% and 98.4 ± 5.6% of control values, respectively; S2/S1 ratios: control 0.565 ± 0.030, immepip 0.626 ± 0.029, clobenpropit 0.558 ± 0.058, n = 3, $P > 0.05$ for both drugs when compared with control values; Figure 5A).

However, and as shown in Figure 5B, immepip inhibited the stimulatory effect of A_{2A}R activation on K^+-evoked [^{3}H]-GABA release (3 nM CGS-21680, 150.1 ± 13.7% of control values; CGS-21680 and immepip, 102.5 ± 6.3% of controls; S2/S1 ratios: control 0.630 ± 0.036; 3 nM CGS-21680 0.832 ± 0.044; CGS-21680 and immepip 0.581 ± 0.065; $P < 0.05$ and $P > 0.05$ for CGS-21680, and CGS-21680 and immepip, respectively, when compared with controls, three experiments). The inhibitory effect of immepip on the A_{2A}R-mediated enhancement of K^+-evoked [^{3}H]-GABA release was concentration-dependent, with IC_{50} 5.2 nM (pIC_{50} 8.28 ± 0.27; Figure 5C).

Figure 5D illustrates a different series of experiments in which the effect of immepip (100 nM) was partially blocked by the H_3R antagonist/inverse agonist clobenpropit (3 nM CGS-21680, 148.7 ± 9.8% of controls; immepip 104.9 ± 7.5% of controls; CGS 21680 and immepip and 3 μM clobenpropit 125.1 ± 5.0% of controls; S2/S1 ratios: control 0.582 ± 0.054, 3 nM CGS-21680 0.832 ± 0.067, $P < 0.001$ when compared with control values; immepip 0.558 ± 0.040, $P > 0.05$ versus controls; CGS-21680 and immepip and clobenpropit 0.706 ± 0.043, $P < 0.01$ when compared with CGS-21680 and immepip and $P < 0.05$ when compared with CGS-21680 alone; four experiments). The extent of blockade by clobenpropit of the effect of immepip was 47.2 ± 4.1%, a value within the range calculated (46–54%) on the basis of the affinities of the H_3R for the ligands determined from the inhibition of [^{3}H]-NMHA binding to GP synaptosomal membranes (K_i values: immepip 0.28 nM and clobenpropit 8.53 nM). We did not attempt to test larger concentrations of clobenpropit because at micromolar concentrations imidazole-containing compounds can directly inhibit P- and N-type voltage-activated Ca^{2+} channels

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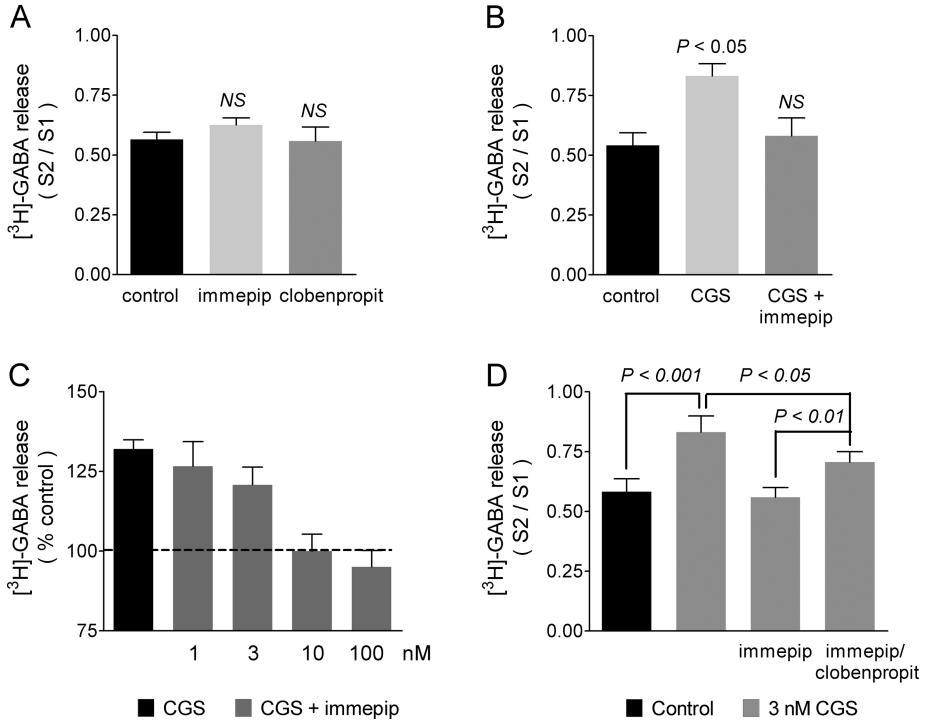

Figure 5. Effect of histamine H_3 receptor activation on the stimulatory action of the A_{2A} receptor agonist CGS-21680 on depolarization-evoked [^{3}H]-GABA release from rat globus pallidus synaptosomes. The experimental protocol was as described in the legend to Figure 4. Where required, drugs under test were present for 5 min (CGS-21680 and immepip) or 10 min (clobenpropit) before and during the second depolarizing stimulus. (A) Effect of the H_3 receptor agonist immepip (100 nM) or the antagonist/inverse agonist clobenpropit (3 μM) alone on depolarization-evoked [^{3}H]-GABA release. Values are means ± SEM from three experiments. NS, no statistically different from control release (no drugs added), ANOVA and Dunnett's post hoc test. (B) Inhibition by immepip (100 nM) of the effect of the A_{2A} receptor agonist CGS-21680 (3 nM). Values are means ± SEM from three experiments. The statistical analysis was performed with ANOVA and Dunnett's test. (C) Effect of different concentrations of immepip. To allow for the variability between experiments, values were expressed as percentage of control release. Values are means ± SEM from the combined data from five experiments with three to five determinations for each concentration of immepip. (D) Blockade by clobenpropit (3 μM) of the effect of immepip (100 nM). Values are means ± SEM from four experiments. The statistical analysis was performed with ANOVA and Tukey's post hoc test.

(IC_{50} values for antazoline 10.5 ± 1.1 and 109.5 ± 9.8 μM, respectively).[29] Further, the use of the nonimidazole antagonist/inverse agonist A-331440 was prevented by the low affinity of pallidal H_3Rs for this drug (*Ki* 316 nM; Results, section 3).

The partial inhibition by clobenpropit of the agonist effect could also be explained by the antagonist not yielding equilibrium with H_3Rs present in GP synaptosomes. However, the binding of 0.2 nM [^{3}H]-clobenpropit (21 °C) to guinea pig cerebro-cortical membranes yields its maximum at 10−13 min,[30] and pallidal synaptosomes were perfused with a 15 000-fold higher concentration (3 μM) for 10 min at 37 °C. Therefore, under our experimental conditions, drug affinities, and not an equilibrium issue, appear to account for the partial blockade by clobenpropit of the effect of immepip.

In a previous work, we failed to observe any significant effect of H_3R activation on depolarization-stimulated [^{3}H]-GABA release from rat GP slices,[11] in contrast to the action reported for slices from rat SNr and striatum.[12−14] The nerve terminals of GABAergic striato-nigral neurons are endowed with both dopamine D_1 and histamine H_3 receptors,[31] and in both nigral and striatal slices H_3R agonists had no effect when tested alone but markedly inhibited the enhancing effect of D_1 receptor stimulation on K^+-evoked [^{3}H]-GABA release.[12,13]

H_3Rs couple to $G\alpha_{i/o}$ proteins and thus their inhibitory effect on neurotransmitter release most likely involves the reduction in depolarization-induced calcium entry,[32,33] via the action of $G\beta\gamma$ complexes at the pore-forming α_1-subunit of N- and P/Q-type voltage-operated calcium channels.[34] In this regard, D_1 receptor-mediated facilitation and H_3R-mediated inhibition of GABA release from striatal terminals appear to converge at P/Q-type Ca^{2+} channels, with the facilitatory effect involving the cAMP/PKA pathway.[14] In line with an action of H_3Rs at P/Q-type channels, in dissociated hypothalamic neurons, the H_3R-mediated reduction in the frequency of spontaneous GABAergic inhibitory postsynaptic currents (sIPSCs) was occluded by blocking P/Q-type Ca^{2+} channels but not by N- or L-type Ca^{2+} channel blockers.[35] Further, A_{2A}R-mediated enhancement of acetylcholine release from striatal synaptosomes relies on two signaling pathways, one involving the cAMP/PKA pathway and P-type Ca^{2+} channels, and the other mediated by a cholera toxin-insensitive G protein, protein kinase C (PKC), and N-type calcium channels.[28] Because both D_1 and A_{2A} receptors stimulate adenylyl cyclase activity, one plausible explanation for the selective action of H_3R activation on A_{2A}R-mediated facilitation of [^{3}H]-GABA release reported herein is that the cAMP/PKA pathway facilitates the opening of P/Q-type Ca^{2+} channels present in the striato-pallidal axons and that are also controlled by H_3Rs.

Upon transfection, H_3Rs formed heteromers with dopamine D_1 and D_2 receptors,[36,37] and in striatal membranes H_3R activation decreased the affinity of D_2 receptors for their agonists.[36] Further, in SK-N-MC cells expressing H_3 and D_1 receptors, the coupling of the latter shifted from $G\alpha_s$ to $G\alpha_{i/o}$ proteins, and therefore, their activation no longer resulted in cAMP

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formation but in inhibition of forskolin-induced cAMP accumulation.[37] Thus, another and interesting possibility is that the functional interaction between H_3 and A_{2A} receptors reported herein for GABA release is underlain by the formation of H_3R–$A_{2A}R$ complexes. In regard to this point, preliminary experiments with rat GP synaptosomal membranes show that the H_3R agonist immepip increases in a modest (2-fold) but significant manner the K_i value for the $A_{2A}R$ agonist CGS-21680, suggesting that dimerization between H_3 and A_{2A} receptors does take place and reduces $A_{2A}R$ affinity for its agonists.

H_3Rs may have spontaneous or constitutive activity, which for cloned receptors depends on the expression level and for native receptors has been reported mainly for H_3 autoreceptors.[38] For H_3Rs expressed in CHO cells, constitutive activity was evident at levels greater than 300 fmol/mg protein[39] and the very high density (1327 ± 79 fmol/mg protein) of H_3Rs present in GP synaptosomal membranes raised thus the possibility that these receptors possessed constitutive activity. However, the lack of effect of the H_3R antagonist/inverse agonist clobenpropit on K^+-evoked [^{3}H]-GABA release from rat GP synaptosomes indicated that H_3Rs located on GABAergic afferents did not have constitutive activity nor were they significantly occupied by endogenous histamine, most likely removed by the perfusion procedure.

7. Possible Trans-Synaptic Effects of H_3R Activation on Depolarization-Evoked [^{3}H]-GABA Release. Given the complexity of the basal ganglia synaptic circuitry, our experimental approach bears intrinsic limitations that include, but are not restricted to, possible trans-synaptic effects of H_3R activation. In this regard, in the rat GP, the release of GABA is inhibited by dopamine D_2, serotonin 5-HT_{1B}, and group III metabotropic or kainate glutamate receptors,[17,40−42] and H_3Rs inhibit the release of dopamine, serotonin, and glutamate.[11,43,44] However, H_3R activation does not inhibit K^+-evoked [^{3}H]-dopamine release from GP slices,[11] and for glutamate and serotonin the predicted outcome of the activation of H_3Rs located on the corresponding nerve terminals would be the disinhibition of GABA release, opposite to the results presented herein. Because the activation of muscarinic receptors stimulate spontaneous [^{3}H]-GABA release from rat GP slices[45] and H_3R activation reduces acetylcholine release,[46] the H_3R-mediated reduction in [^{3}H]-GABA release reported herein could also involve an indirect action due to the inhibition of depolarization-induced acetylcholine release from GP synaptosomes.

8. Functional Implications. Out of the three types of GP neurons characterized on the basis of their electrophysiological and morphological properties, type A and B (GABAergic) neurons fire spontaneous action potentials and project mainly to the subthalamic nucleus (STN) although they also directly contact SNr neurons.[4,47] STN neurons also possess autonomous generation of action potentials, and their rate and pattern of activity is precisely regulated by GABAergic afferents from the GP and by glutamatergic afferents from the cerebral cortex.[48,49]

The axons of STN neurons release glutamate upon GABAergic neurons located in both SNr and GP, and according to the disinhibition model of the basal ganglia function[4] the H_3R-mediated inhibition of GABA release reported herein would translate to increased neuronal firing of GP neurons, leading to reduced firing of SNr neurons by two mechanisms. First, the increased GABAergic input would diminish the activity of STN neurons and hence their excitatory input onto SNr neurons. Second, the disinhibition of GP neurons will increase their direct inhibitory effect on SNr neurons. The reduced activity of SNr neurons will then lead to the disinhibition of thalamo-cortical neurons and thus in facilitation of motor behavior.

Of note, if analyzed separately, the H_3R-mediated inhibition of GABA release described herein would have functional actions on the firing of GP neurons opposite to the inhibition of glutamate release described previously.[11] In contrast to other neurons of the basal ganglia, striatal projection neurons are mostly silent and require cortical excitation for their activation. It is thus tempting to propose that under resting conditions H_3Rs control mainly the feedback circuit GP → STN → GP by reducing glutamate release onto GP neurons,[11] leading to increased activity of both STN and SNr neurons and thus to the inhibition of thalamo-cortical neurons. In turn, when motor programs are executed, H_3R-mediated inhibition of GABA release may control the circuit striatum → GP → STN → SNr, leading to reduced activity of STN and SNr neurons and disinhibition of thalamo-cortical neurons.

This proposal is highly speculative, and both in vitro and in vivo experiments are required for a better understanding of the function of pallidal H_3Rs and its consequences on basal ganglia physiology. One important point to consider is that H_3R-mediated inhibition of GABA release from striato-pallidal terminals proposed to result in increased activity of GP neurons and thus in enhanced GABA release onto STN neurons, can paradoxically increase the efficacy of cortical excitatory synaptic inputs to the STN through the activation of $GABA_A$ and/or $GABA_B$ receptors that inhibit/reset autonomous activity by deactivating postsynaptic voltage-dependent Na^+ channels and generate hyperpolarization-induced rebound burst firing through the deinactivation of postsynaptic voltage-dependent Ca^{2+} and Na^+ channels.[21]

Finally, in rat GP slices, histamine increases neuronal firing (EC_{50} ~10 μM) through a postsynaptic, H_2 receptor-mediated action.[50] This information and our results indicate that histamine controls at the pre- and postsynaptic levels the activity of GP neurons. However, the affinity of H_3 and H_2 receptors for histamine differs substantially (K_i 5 nM and 2 μM for human receptors, respectively),[51,52] and the effect of H_3Rs may therefore be exerted tonically, whereas H_2 receptor-mediated actions may predominantly take place during the arousal state, in which the activity of histaminergic neurons increases markedly, leading to enhanced transmitter release in the target nuclei.[1]

9. Conclusion. The GP has emerged as a key point in the control of the basal ganglia motor output,[53,54] and in this work we showed that histamine H_3 receptor activation counteracts the facilitatory action of adenosine A_{2A} receptors on GABA release from striato-pallidal afferents. Through this presynaptic effect, H_3 receptors could contribute to the regulation of basal ganglia function.

METHODS

1. Animals. Rats (males, Wistar strain, 250−300 g), bred in the Cinvestav facilities, were used throughout the experiments. All procedures were approved controlled by the Cinvestav Animal Care Committee and in accordance to the guidelines for the care and use of laboratory animals issued by the National Institutes of Health (NIH Publications No. 8023, revised 1978) and the Mexican Council for Animal Care. All efforts were made to minimize animal suffering and to use only as many animals were required for proper statistical analysis.

2. Synaptosome Preparation. After decapitation, the brain was quickly removed from the skull and the forebrain was cut and

immersed in ice-cold Krebs-Henseleit solution. Coronal slices (300 μm thick) were then obtained with a vibratome (World Precision Instruments, Sarasota, FL). The pallidal tissue was carefully dissected from the slices, avoiding the adjacent striatum which contains high levels of H_3Rs (Figure 1A). The composition of the Krebs-Henseleit solution was as follows (mM): NaCl 116, KCl 3, $MgSO_4$ 1, KH_2PO_4 1.2, $NaHCO_3$ 25, D-glucose 11; pH, 7.4 after saturation with O_2/CO_2, 95:5% v:v. To reduce excitoxicity, $CaCl_2$ was not added to the solution.

Synaptosomes were prepared essentially as described by Cristóvão-Ferreira et al.[55] Briefly, GP slices from five rats were placed in 10 mL of 0.32 M sucrose solution containing 10 mM Hepes, 1 mg/mL bovine serum albumin, and 1 mM EDTA (pH 7.4 with NaOH) and then homogenized using 10 strokes of a hand-held homogenizer. The suspension was centrifuged (1500*g*, 4 °C, 10 min), and the supernatant was collected and centrifuged (14 000*g*, 12 min, 4 °C). The resulting pellet was resuspended in 5 mL of Percoll (45%) in a modified Krebs-Hepes solution (in mM: NaCl 140, Hepes 10, D-glucose 5, KCl 4.7, EDTA 1, pH 7.3 with NaOH). After centrifugation (2 min, 14 000*g*, 4 °C), the upper phase was collected and brought up to 20 mL with Krebs-Ringer-Hepes (KRH) solution before further centrifugation (20 000*g*, 20 min). The supernantant was discarded, and the pellet (synaptosomes) was resuspended in the appropriate solution. The composition of the KRH buffer was as follows (mM): NaCl 113, $NaHCO_3$ 25, KCl 4.7, $MgCl_2$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.8, D-glucose 15, Hepes 20; pH 7.4 with NaOH.

3. Electron Microscopy of the GP Synaptosome Preparation. The synaptosomal preparation was washed twice in 1 mL phosphate-buffered saline solution (PBS) by centrifugation at 15 000*g* (3 min). The final pellet was fixed (1 h at room temperature) with glutaraldehyde (5% in PBS) followed by mixing by inversion for 10 min. After three washes with 1 mL of PBS, each of 5 min, samples were postfixed in PBS containing 2% osmium tetroxide for 30 min at room temperature. Samples were washed twice in 1 mL of PBS for 5 min and then four times (5 min each) with 1 mL of filtered distilled water before prestaining with uranile (1% in water) for 30 min. After two washes (5 min each) with 1 mL of filtered distilled water, samples were dehydrated in a graded series of alcohol (50, 60, 70 and 80% ethanol, 10 min each, then 90% ethanol for 15 min and finally three times in 100% ethanol for 15 min under stirring). Samples were then infiltrated in Spurr resin under mixing (50% resin/50% ethanol for 3 h, 75% resin/25% ethanol overnight and 100% resin for 6 h with changes of the solution each 2 h for the last step). Finally, samples were incubated for 72 h at 60 °C. Thin sections (~70 nm thick) were cut on a Reichert Ultracut instrument, stained with lead citrate, and examined in a JEM 1400 transmission electron microscope (Cinvestav Unit of Electron Microscopy). The preparations were analyzed by randomly selecting grid squares at low magnification (×20), at which details of the sample were not visible. Representative fields were then photographed at ×20 000 or ×50 000.

4. Binding of *N*-α-[Methyl-^{3}H]-histamine ([^{3}H]-NMHA) to Synaptosomal Membranes. The synaptosomal pellet was resuspended in 30 mL of 10 mM Tris-HCl solution (4 °C) containing 1 mM EGTA and lysed with a Polytron (3 cycles, 5 s each). The suspension was centrifuged at 32 000*g* (20 min, 4 °C), and the pellet (synaptosomal membranes) was resuspended in incubation buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4). Protein contents were determined via the bicinchoninic acid assay (BCA; Pierce, Rockford, IL).

Binding experiments (~10 μg protein aliquots) were carried out and analyzed as described in detail elsewhere.[11] Saturation binding data were fitted to a hyperbola and inhibition data to a logistic (Hill) equation (nonlinear regression with Prism 5, Graph Pad Software, San Diego, CA). Values for inhibition constants (K_i) were calculated according to the equation:[56] $K_i = IC_{50}/1 + \{[D]/K_d\}$, where [D] is the concentration of [^{3}H]-NMHA present in the assay and K_d is the mean value for the equilibrium dissociation constant estimated from saturation analysis.

5. Uptake of [^{3}H]-GABA by GP Synaptosomes. Synaptosomes were resuspended in KRH solution supplemented with 10 μM aminooxyacetic acid (to prevent degradation of [^{3}H]-GABA). Aliquots (140 μL) were placed into plastic tubes and incubated for 15 min at 37 °C before the addition of a mixture of [^{3}H]-GABA/GABA in a 50 μL volume to yield the required concentrations (50 nM/3 μM). After 5 min at 37 °C, incubations were filtered through Whatman GF/B glass fiber paper, presoaked in 0.3% polyethylenimine. Filters were washed three times with ice-cold KRH solution and soaked in 4 mL of scintillator, and then the tritium content was determined by scintillation counting. Nonspecific uptake was determined in samples incubated at 4 °C. Where required, the GABA uptake inhibitor SKF-89976A was added in a 10 μL volume 10 min before the labeled neurotransmitter.

6. Depolarization-Evoked [^{3}H]-GABA Release from GP Synaptosomes. Synaptosomes were suspended in KRH solution supplemented with 10 μM aminooxyacetic acid, 2 U/mL adenosine deaminase (to break down endogenous adenosine), and [^{3}H]-GABA/GABA (80 nM/3 μM). After incubation for 30 min at 37 °C, the synaptosomal suspension was apportioned randomly between the chambers of a superfusion apparatus (15 chambers in parallel; 100 μL per chamber) and superfused (1 mL/min) with KRH medium.

Synaptosomes were perfused for 20 min before the collection of 17 fractions of 1 mL (1 min) each. [^{3}H]-GABA release was stimulated by changing to a solution containing high K^+ (20 mM, KCl substituted for NaCl) for fractions 4 and 13, returning to normal KRH solution between these fractions and after the second K^+ stimulus. Drugs under test were present 5 min (CGS-21680 and immepip), 7 min (ZM-241385), or 10 min (clobenpropit) before and throughout the second K^+ stimulus (i.e., fractions 8−13 for CGS-21680 and immepip, fractions 6−13 for ZM-241385 and fractions 8−13 and a 5 min perfusion period between fractions 5 and 6 which was not collected for clobenpropit). The double-pulse protocol allows for the same synaptosomal sample being the control for the effect of drugs under test.

For experiments where the Ca^{2+}-dependence of [^{3}H]-GABA release was tested, synaptosomes were perfused for 15 min before and throughout the collection of fractions with KRH solution without $CaCl_2$ (normal or high K^+ as required).

The superfusate fractions were mixed with 4 mL of scintillation liquid, and the tritium content was determined by scintillation counting. The amount of tritium remaining in the synaptosomal tissue was determined by treating each chamber with 0.5 mL of HCl (1 M) for 30 min before addition of scintillator. Tritium efflux into the superfusate was calculated as a fraction of tritium present in the corresponding tissue at the onset of the respective collection period. To allow for variations between chambers, fractional values were transformed to a percentage of the fraction collected immediately before the first change to the high K^+ medium (i.e., the release in fraction 3 was set to 100%). To test for statistical differences between treatments, after subtraction of basal release, the area under the release curve for 6 fractions after the change to high K^+ (i.e., fractions 3−8 and 12−17) was determined for each individual chamber and the ratio of the second over the first K^+ stimuli (S2/S1) was calculated. Statistical comparisons were performed with Student's *t* test or one-way ANOVA and post hoc Dunnett's or Tukey's test (Graph Pad Prism 5.0) as appropriate.

7. Drugs. The following drugs were purchased from Sigma-Aldrich (Mexico City, Mexico): Adenosine deaminase, aminooxyacetic acid hemihydrochloride, CGS-21680 (2-*p*-(2-carboxyethyl)-phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride hydrate), clobenpropit dihydrobromide, histamine dihydrochloride, immepip dihydrobromide, SKF-89976A (1-(4,4-diphenyl-3-butenyl)-3-piperidinecarboxylic acid hydrochloride), ZM-241385 (4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a} {1,3,5}triazin-5-yl-amino]-ethyl)phenol). [2,3-^{3}H]-γ-Aminobutyric acid ([^{3}H]-GABA, 82 Ci/mmol) and *N*-α-[methyl-^{3}H]-histamine (85.4 Ci/mmol) were from PerkinElmer (Boston, MA).

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Author Contributions

G.-E.M.-F. and J.-A.A.-M. designed the study. G.-E.M.-F., J.E.-S., and R.G.-P. conducted experiments. G.-E.M.-F. and J.-A.A.-M. performed data analysis. R.M-G. helped conceive the study. G.-E.M.-F. and J.-A.A.-M. wrote the manuscript.

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Notes

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■ ABBREVIATIONS

$A_{2A}R$, adenosine A_{2A} receptor; GABA, γ-aminobutyric acid; GP, globus pallidus; [^{3}H]-NMHA, N-α-[methyl-^{3}H]histamine; H_3R, histamine H_3 receptor; SNr, *substantia nigra pars reticulata*; STN, subthalamic nucleus

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