Muscarinic presynaptic modulation in GABAergic pallidal synapses of the rat

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Hernández-Martínez R, Aceves JJ, Rueda-Orozco PE, Hernández-Flores T, Hernández-González O, Tapia D, Galarraga E, Bargas J. Muscarinic presynaptic modulation in GABAergic pallidal synapses of the rat. J Neurophysiol 113: 796 –807, 2015. First published November 12, 2014; doi:10.1152/jn.00385.2014.—The external globus pallidus (GPe) is central for basal ganglia processing. It expresses muscarinic cholinergic receptors and receives cholinergic afferents from the pedunculopontine nuclei (PPN) and other regions. The role of these receptors and afferents is unknown. Muscarinic M1-type receptors are expressed by synapses from striatal projection neurons (SPNs). Because axons from SPNs project to the GPe, one hypothesis is that striatopallidal GABAergic terminals may be modulated by M1 receptors. Alternatively, some M1 receptors may be postsynaptic in some pallidal neurons. Evidence of muscarinic modulation in any of these elements would suggest that cholinergic afferents from the PPN, or other sources, could modulate the function of the GPe. In this study, we show this evidence using striatopallidal slice preparations: after field stimulation in the striatum, the cholinergic muscarinic receptor agonist muscarine significantly reduced the amplitude of inhibitory postsynaptic currents (IPSCs) from synapses that exhibited short-term synaptic facilitation. This inhibition was associated with significant increases in paired-pulse facilitation, and quantal content was proportional to IPSC amplitude. These actions were blocked by atropine, pirenzepine, and mamba toxin-7, suggesting that receptors involved were M1. In addition, we found that some pallidal neurons have functional postsynaptic M1 receptors. Moreover, some evoked IPSCs exhibited short-term depression and a different kind of modulation: they were indirectly modulated by muscarine via the activation of presynaptic cannabinoid CB1 receptors. Thus pallidal synapses presenting distinct forms of short-term plasticity were modulated differently.

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Finally, because some GABAergic synapses in the GPe arise from pallidopallidal axon collaterals (Jaeger and Kita 2011; Miguez et al. 2012; Sims et al. 2008), it was important to observe if they are also modulated. Importantly, electrophysiological and optogenetic techniques have already demonstrated that a main difference between striatopallidal and pallidopallidal synapses is their short-term synaptic plasticity: striatopallidal connections display short-term facilitation (STF), whereas terminals from pallidal neurons exhibit short-term depression (STD) (Miguez et al. 2012; Sims et al. 2008). Striatopallidal synapses have a lower release probability than pallidopallidal synapses. In agreement with these previous studies, we divided the GABAergic synapses studied in this work according to the type of short-term plasticity that they exhibit. We then asked whether both of them can be modulated by muscarinic receptors and, if that is true, whether they are modulated in the same way.

MATERIALS AND METHODS

Animals. All procedures were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (1996 revision) and followed the guidelines from the Institutional Committee for the Production, Care and Use of Laboratory Animals (NOM-062-ZOO–1999) of the Instituto de Fisiología Celular from the Universidad Nacional Autónoma de México. Because the scientific questions of the present investigation were to disclose cholinergic muscarinic actions in the GPe, a precise identification of the origin of synaptic inputs or neurons was not required. Therefore, it was decided that there was no need to use transgenic animals at this stage. In addition, every effort was made to minimize the number of animals needed to attain statistical significance. To our knowledge, our procedures do not induce animal suffering of any type.

Slice preparation. The experiments were performed on brain slices obtained from Wistar rats. Briefly, the rats (postnatal day 15–30) were anesthetized and perfused intracardially with a choline-Cl solution containing (in mM) 124 (CH₃)₂NCl/Cl·CH₂CH₂OH, 2.5 KCl, 1.3 MgCl₂, 2.4 CaCl₂, 1.2 NaH₂PO₄, 26 NaHCO₃, and 10 glucose (saturated with 95% O₂ and 5% CO₂). Thereafter, animals were decapitated, and their brains obtained. Each hemisphere was cut using the Vibratome (1000 Classic; Warner Instruments, Hamden, CT). Sagittal slices (350 μm thick) were cut at an angle of 10° to the midline using the Vibratome (Beurrier et al. 2006). Slices contained both the Str and the GPe. Slices were then transferred to saline containing (in mM) 0.001 tetrodotoxin, 140 NaCl, 3 KCl, 10 CaCl₂, 2 MgCl₂, 10 HEPES, 1 CaCl₂, 2.5 MgCl₂, 2.4 Mg⁶⁺-ATP, 0.4 Na⁺-GTP, 5 lidocaine N-ethyl bromide (QX-314), and 1% bicytion (pH 7.2, 282 mosmol/l) and left at 25–27°C, saturated with 95% O₂ and 5% CO₂, 298 mosmol/l) and left with oxygenated saline solution (4–5 ml/min).

Whole cell recordings. Recordings were made at room temperature (~25°C). Neurons were visualized using infrared differential interference microscopy (Nikon Instruments, Melville, NY). Micropipettes for whole cell recordings were pulled (Sutter Instrument, Novato, CA) from borosilicate glass tubes (1.5-mm outer diameter; WPI, Sarasota, FL) for a final DC resistance of 4–6 MΩ when filled with internal saline containing high Cl⁻ and the following composition (in mM): 72 K₃H₂PO₄, 36 KCl, 10 NaCl, 10 EGTA, 10 HEPES, 1 CaCl₂, 2.5 MgCl₂, 2.4 Mg⁶⁺-ATP, 0.4 Na⁺-GTP, 5 lidocaine N-ethyl bromide (QX-314), and 1% bicytion (pH 7.2, 282 mosmol/l). In these conditions, IPSCs are inward currents when recorded at a holding potential of −80 mV, where intrinsic currents are either small or not available (Perez-Rosello et al. 2005; Tecuapetla et al. 2007).

IPSCs were evoked by field stimulation in the Str (≥500 μm outside the GPe border) and recorded in GPe neurons. We used concentric bipolar tungsten electrodes with a 12.5-μm diameter at the tip (FHC, Bowdoinham, ME). Recordings were obtained with an Axopatch 200B electrometer (Axon Instruments, Foster City, CA) and monitored with an oscilloscope (Tektronix, Beaverton, OR). Series resistance ranged from 5 to 20 MΩ and was commonly compensated up to 80%. Input and access resistance were continuously monitored during the experiment with a voltage command, and experiments were discarded if changes >20% in the evoked transmembrane current were observed. Recordings were done in the presence of 6-cyano-2,3-dihydroxy-7-nitroquinoxaline disodium salt (CNQX; 10 μM) and dl-2-amino-5-phosphonopentanoic acid (APV; 50 μM), antagonists of glutamate receptors. IPSCs could be totally blocked by 10 μM bicuculline or gabazine (Perez-Rosello et al. 2005; Tecuapetla et al. 2007). Stimulation consisted of either stimulus trains of 10 shocks at 20 Hz every 20 s to assess short-term synaptic plasticity (STSP) or, alternatively, 2 shocks (paired pulse) with a 50-ms interpulse interval delivered at 0.1 Hz. Stimulation parameters were adjusted to obtain synaptic currents of 100–500 pA during the control (about half the maximal amplitude). These responses were achieved with stimulus strengths from 1 to 20 V. IPSC amplitudes were measured from basal line to peak for the first response in a train (IPSC₁). For the subsequent responses (IPSC₂,…, IPSCₙ), the basal line remaining from the previous response was subtracted. We measured the STSP ratio as IPSCₙ/IPSC₁.

Amplitudes of IPSCs after trains of stimuli that evoked STD were normalized and fitted ad hoc with a single exponential decay to approximate a global time constant for depression:

\[ IPSC(t) = y_0 + A_1 e^{-\tau t}, \]

where \( y_0 \) is the asymptotic maximal value, \( A_1 \) is the exponential coefficient, and \( \tau \) is the time constant (in ms).

For IPSCs trains that exhibited STF, a good fit was also obtained with a single inverted exponential function with one time constant:

\[ IPSC(t) = y_0 - A_1 e^{-\tau t}, \]

where \( y_0 \) is the asymptotic maximal value, \( A_1 \) is the exponential coefficient, and \( \tau \) is the time constant. Ten pulses separated by 50-ms intervals were given to induce the trains of IPSCs to see if STSP was depressing (STD) or facilitating (STF), so time constants ± estimation errors are reported in milliseconds. The paired-pulse ratio (PPR) is the amplitude ratio between the second IPSC and the first IPSC (IPSC₂/IPSC₁).

Immunocytochemical procedures. After recordings were made, neurons were injected with 1% biocytin to identify the cells and then processed for immunocytochemistry as previously described (Ibáñez-Sandoval et al. 2006). The slices were incubated in streptavidin conjugated with Cy3 (1:200 dissolved in PBS; Zymed Laboratories, San Francisco, CA). This allowed us to visualize the recorded neuron. Thereafter, slices were incubated for 30 min with 1% bovine albumin to block nonspecific binding sites. Slices were then incubated for 36 h with a rabbit antibody against parvalbumin (anti-PV; Abcam; 1:2,000, dissolved in PBS containing 0.25% Triton-X). The slices were then rinsed three times with PBS and incubated with a goat vs. rabbit secondary antibody for 1 h. This antibody was conjugated with FITC (Vector Laboratories, Burlingame, CA). Samples were mounted with Vectashield (Vector Laboratories) and observed in a confocal microscope (Olympus FV-1000). Most neurons recorded were PV positive.

Dissociated neurons. In the present work the GPe was dissected from 300-μm thick brain slices incubated for 20 min with 1 mg/ml papain (Calbiochem, Billerica, MA) at 34°C. Slices were transferred to a low-Ca²⁺ (0.4 mM CaCl₂) saline solution to obtain isolated GPe neurons by mechanical dissociation. The suspension was plated into a petri dish mounted on the stage of an inverted microscope (Nikon Instruments) with saline containing (in mM) 0.001 tetrodotoxin, 140 NaCl, 3 KCl, 5 BaCl₂, 2 MgCl₂, 10 HEPES, and 10 glucose (pH 7.4 with NaOH, 300 ± 5 mosmol/l with glucose). Voltage-clamp record-
ings were performed on GPe neurons of 13- to 30-μm main diameter (capacitance 12–13 pF). Internal saline contained (in mM) 180 N-methyl-D-glucamine, 40 HEPES, 10 EGTA, 2 MgCl₂, 2 ATP, 0.4 GTP, and 0.1 leupeptin (pH 7.2 with H₂SO₄, 280 ± 5 mosmol/l). Whole cell recordings were obtained with an Axopatch 200B electrometer (Axon Instruments) and monitored with pClamp (version 8) and a 125-kHz DMA interface (both from Axon Instruments). Series resistance (<10 MΩ) was compensated (70–80%). Ba²⁺ (5 mM) currents were recorded while Na⁺ channels were blocked (1 μM tetrodotoxin). Current-voltage relationships (I–V plots) of Ba²⁺ currents were built before and after drug applications with current responses to either voltage-step commands (20 ms) from −80 to 50 mV (in 10-mV steps) or with 200-ms voltage-ramp commands (0.7 mV/ms) from −90 to 50 mV. Both methods yielded equal results (Perez-Burgos et al. 2008). The present article shows representative responses to ramp commands where peak evoked currents correspond to the minimum of the I–V plot.

**Drugs.** Drugs were prepared before each experiment and added to the superfusion saline in the final concentration indicated. The AMPA/kainic acid-selective antagonist CNQX (10 μM), NMDA antagonist APV (50 μM), tetrahydro-4-hydroxy-N,N,N,N-tetramethyl-2-furanmethanammonium chloride (±)-muscarnine chloride; 1 μM, tropine tropate, α-hydroxymethyl)benzeneacetic acid 8-methyl-8-azabicyclo[3.2.1]oct-3-yl ester (atropine sulfate salt monohydrate), 5,11-dihydroxy-11-[4-methyl-1-piperazinyl]acetyl]-6H-pyrido[2,3-b]1,4-benzodiazepin-6-one dihydrochloride (prezepine dihydrochloride), (5S)-5-[6(8)-dihydro-8-oxofoxo[3,4-e]-1,3-benzodioxol-6-yl]-5,6,7,8-tetrahydro-6.6-dimethyl-1.3-dioxolo[4,5-g]isiquinolinium iodide [1(S),9(R)(−)-bicuculline methodidate], and N-ethyl-2-phenyl-N-(4-pyridylmethyl)hydrocycylamide (tropicamid) were obtained from Sigma RBI (St. Louis, MO). The cannabinoid CB₁ receptor antagonist N-(piperidin-1-yl)-5-(4-isodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM 251) and the cannabinoid agonist R-(−)-[2,3-dihydo-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenemethanone mesylate (WIN 55,212-2 mesylate) were obtained from Tocris (Ellisville, MO). Mamba toxins 7 and 3 (MT-7 and MT-3) were obtained from Alomone Labs (Jerusalem, Israel).

**Statistics.** In many cases, distributions of measurements of small samples were not normal. Therefore, distribution-free statistics were used. Unless stated otherwise, in cases where recordings from the same neurons were compared before and after treatment, Wilcoxon t-tests were used. Unpaired samples were compared with the Mann-Whitney U-test. In the same way, multiple treatments were compared with the either Friedman ANOVA or Kruskal-Wallis ANOVA and post hoc Tukey or Dunn statistics. P < 0.05 was used as significance level. Statistical analysis of synaptic variability was assessed with the coefficient of variation (CV = standard deviation of IPSC peak amplitude/mean IPSC peak amplitude). The mean quantal content (CV⁻²) was approximated as is customary: (mean IPSC amplitude)²/(IPSC amplitude variance)² (Bekkers and Stevens 1990; Lupica et al. 1992; Rodriguez-Moreno et al. 1997; Sims et al. 2008). A relation with positive slope between CV⁻² and IPSC amplitude is expected only when the change of presynaptic

**RESULTS**

There are different types of short-term synaptic plasticity in GABAergic synapses of the GPe. A field stimulus delivered within the Str will activate axons whose terminals may make synapses with postsynaptic GPe neurons (Figs. 1, A–C) (Cooper and Stanford 2001). This finding has been corroborated in many instances and was determined using mainly electrophysiological methods in vitro and in vivo (reviewed in Jaeger and Kita 2011). However, electrical stimulation of the Str might activate both striatopallidal and pallidostriatal axons (Goldberg and Bergman 2011; Kita 2007; Mallet et al. 2012; Schwab et al. 2013). The terminals they activate onto GPe neurons are

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**Fig. 1.** Short-term synaptic plasticity in external globus pallidus (GPe) inhibitory synapses. A 20-Hz stimulus train with a field electrode located in the striatum (Str) in slightly inclined parasagittal slices that include the Str and the GPe evoked inhibitory postsynaptic currents (IPSCs) in postsynaptic GPe neurons in all cases. A: most IPSCs recorded exhibited short-term facilitation (STF synapses) when field stimulus was delivered inside the Str. Average (n = 15 traces) is denoted by a thick black trace, and variation of responses in different trials is denoted by thin gray traces in A–C. B: in one-third of cases, evoked IPSCs exhibited short-term depression (STD synapses). C: when the stimulus was given inside the GPe while a GPe neuron was recorded in coronal slices. STD synapses were also recorded. D: a single exponential function was reasonably well fitted to STF. E and F: a single exponential function was reasonably well fitted to STD. IPSC₁, first response in a stimulus train; IPSCₙ, subsequent responses. Time constants (τ) obtained with exponential fits in STD synapses recorded in sagittal or coronal slices were not significantly different. STF synapses were never recorded when the stimulating electrode was in the GPe.

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distinguished by the type of short-term synaptic plasticity that they exhibit: striatopallidal terminals exhibit facilitation (STF), whereas pallidopallidal synapses show depression (STD) (Migueléz et al. 2012; Sims et al. 2008). When strong enough stimuli are applied, the latency cannot (Ogura and Kita 2000) distinguish between them. Therefore, because we used electrophysiological methods for this investigation and did not need to precisely isolate the origin of the synapses studied to reach our research goals, we functionally designed the evoked inputs by the class of short-term synaptic plasticity that they exhibited.

Thus, in one of our samples, a thin field electrode (see MATERIALS AND METHODS) located in the Str will evoke IPSCs exhibiting short-term facilitation (STF synapses; Fig. 1A) in n = 16 of 22 trials. In the rest, n = 6 of 22 trials (about one-third), we evoked IPSCs that exhibited short-term depression (STD synapses; Fig. 1B) (Beurrier et al. 2006). In this article, however, these synapses are referred to as STF or STD synapses because of possible contamination from the field stimulus. In addition, perhaps not all sources of GABA that target GPe neurons have been identified (reviewed in Menasé-Segovia et al. 2004). The STF ratio, calculated as the mean amplitude of the last 7–10 IPSCs divided by the mean amplitude of the first IPSC, was 1.72 ± 0.023 (mean ± SE; n = 10; median 1.59; Fig. 1A). An ad hoc fitting of a single inverted exponential function (see MATERIALS AND METHODS) to facilitating synapses yielded a time constant (τ; fitted value ± estimation error) of 150 ± 30 ms (r = 0.97; Fig. 1D).

In contrast, STD synapses (Fig. 1, B and C) had an STF ratio of 0.60 ± 0.01 (n = 6; median 0.60), and a simple exponential decay yielded τ = 83 ± 11 ms (r = 0.98; Fig. 1E). When IPSCs from STD synapses were activated by intrapallidal stimuli in coronal slices (Fig. 1C), the STF ratio was 0.45 ± 0.01 (n = 4; median 0.46), and a simple exponential decay yielded a τ = 67 ± 12 ms (r = 0.97; Fig. 1F; not significantly different from that obtained in sagittal slices). These results corroborated that striatal field stimulation can evoke IPSCs in postsynaptic pallidal neurons with two different types of short-term plasticity: STF and STD. The larger proportion corresponded to STF synapses when field stimulus was delivered inside the Str. When field stimulus was delivered within the GPe, only STD synapses could be recorded. In the following section, we show the muscarinic modulation of STF and STD synapses.

**Activation of cholinergic muscarinic receptors presynaptically inhibits STF and STD synapses.** The actions of muscarinic receptor activation were examined in STF synapses while IPSCs were evoked in postsynaptic GPe neurons (only paired responses are shown; Cooper and Stanford 2001). Figure 2A shows a representative time course of IPSC amplitude (each symbol represents the average of 12 individual events ± SE, in absolute amplitude) in two different conditions: control (1) and during the addition of 1 μM muscarine (2) into the bath saline (horizontal bar). Muscarine reduced IPSC amplitude (first IPSC of the pair) in all cases. Insets at right show traces taken at different times during the time course (as indicated by numbers). This connection exhibited paired-pulse facilitation in control (1), and after IPSC reduction by muscarine (2) paired-pulse facilitation was enhanced, as shown with normalized and superimposed traces.

Figure 2B shows a similar experiment in a STD synapse. There was paired-pulse depression in the control (paired IPSCs are shown). In this synapse, muscarine also decreased IPSC amplitude in all experiments. IPSC reduction by muscarine converted paired-pulse depression into paired-pulse facilitation, as shown in the insets at right.

Muscarinic actions looked similar in both STF and STD synapses. A sample from both synapses showed that the PPR increased in all connections tested (Fig. 2C; n = 9; P < 0.01), suggesting a presynaptic site of action. Muscarine also induced IPSC decrease in all synapses tested either with STD or STF in control conditions (Fig. 2D; in percentage, a 56 ± 5% decrease; n = 22; P < 0.001). The change in IPSC amplitude was accompanied by an increase in the CV (Fig. 2E; n = 19; P < 0.001), again suggesting a presynaptic origin.

A comparison of CV−2 (see MATERIALS AND METHODS) as a function of IPSC amplitude before and during muscarine disclosed a relation between CV−2 and IPSC amplitudes in both facilitating and depressing synapses (Fig. 2F; Bekkers and Stevens 1990; Rodríguez-Moreno et al. 1997; Salgado et al. 2005; Sims et al. 2008; Tecuapetla et al. 2007), suggesting that muscarine preserved this relation. That is, reduction in amplitude signified less quanta of released transmitter in both STF and STD synapses.

Taking these results together, the following partial conclusion can be reached: muscarine decreases IPSC amplitude in inhibitory pallidal synapses. These synapses may exhibit either facilitation or depression, suggesting that one role of cholinergic inputs in the GPe is to regulate GABA release at the presynapse, as previously shown for the terminals of local axon collaterals interconnecting SPNs (Perez-Rosello et al. 2005). This conclusion is supported by an increase in the PPR and CV as well as a dependency of CV−2 on IPSC amplitude (Rodríguez-Moreno et al. 1997; Salgado et al. 2005).

**Muscarinic modulation of STF synapses is mediated by M1-class receptors.** Figure 3A shows that muscarinic actions are specific and reversible in IPSCs evoked from STF synapses. A time course similar to that shown in Fig. 2A (each symbol in the time course is the average of 12 individual events ± SE) shows that 1 μM muscarine decreased IPSC amplitude in all evaluated neurons and that addition of 10 μM atropine to the bath saline induced a recovery of IPSC amplitude, suggesting that muscarine actions were blocked at its receptor. Atropine blocked muscarinic actions in all synapses tested. Insets at right are representative traces taken from the time course, as indicated by numbers. The paired line graph in Fig. 3B shows a summary of results for the corresponding sample, in absolute IPSC amplitude (before and during muscarine: n = 6; P < 0.01), and after addition of atropine in the presence of muscarine (n = 6; P < 0.03).

Figure 3C describes an alternative protocol showing the time course of the actions of 1 μM pirenzepine, an M1-class receptor-prefering antagonist, in a STF synapse. Pirenzepine had no significant actions by itself. However, the actions of 1 μM muscarine added in the presence of pirenzepine were blocked completely (the same result as in Fig. 3A was obtained when pirenzepine was administered after muscarine; not shown). Inset at right shows paired responses taken at different times during the time course, as denoted by numbers.

The paired line graph in Fig. 3D shows no significant changes in IPSC amplitude during addition of muscarine in the presence of pirenzepine. The same results were obtained when MT-7 (500 nM) was used instead of pirenzepine (n = 3; not
In contrast, 1 μM tropicamide, an M₄-type receptor-prefering antagonist (Betz et al. 2007), could not block the actions of muscarine (n = 3; not shown), and MT-3 (500 nM), a specific M₄-type receptor antagonist, was also used with negative results (n = 3; not shown).

These results suggest that STF inhibitory synapses in the GPe are regulated presynaptically by M₁-type receptors (Perez-Rosello et al. 2005). Pirenzepine also blocked muscarinic actions on STD synapses (n = 4; not shown, but see below).

Fig. 2. Presynaptic muscarinic modulation of STF and STD synapses. A: time course of muscarinic action on IPSCs evoked by stimulating a STF synapse [in the presence of 10 μM 6-cyano-2,3-dihydroxy-7-nitroquinoxaline disodium salt (CNQX) and 50 μM dl-2-amino-5-phosphonopentanoic acid (APV)]. IPSCs are reduced in amplitude after 1 μM muscarine. Averaged absolute amplitude ± SE of first IPSC of the pair is plotted in the time course. Horizontal bar indicates time of muscarine application. Representative traces in inset at right are pairs of responses evoked at different moments during the time course in A as indicated by numbers: control (1) and after 1 μM muscarine (2). Superimposed traces normalized to the first IPSC amplitude are also shown for comparison. B: time course of muscarinic action on IPSCs evoked in an STD synapse: IPSCs are also reduced in absolute amplitude by 1 μM muscarine. Traces in inset at right are representative IPSCs taken at different moments during the time course in B as indicated by numbers. Superimposed traces normalized to the first IPSC show that synaptic depression turned to facilitation. C: paired line plot of paired-pulse ratio (PPR = second IPSC/first IPSC). PPR was increased significantly (**P < 0.01). D: IPSC absolute amplitudes were decreased in all cases of a sample of inhibitory synapses (**P < 0.001). E: the coefficient of variation (CV) was increased in most cases (**P < 0.001). F: quanlal content (CV⁻²) as a function of IPSC amplitude was similar before and after muscarine (NS, not significantly different).
Mediation of muscarinic actions differs in STF compared with STD synapses. To see whether muscarinic actions were mediated directly or indirectly by presynaptic cannabinoids in STF or STD synapses, we employed a selective antagonist of CB₁ receptors (Fukudome et al. 2004; Lau and Vaughan 2008). It is known that striatopallidal terminals possess presynaptic cannabinoid CB₁-type receptors that decrease GABA release (Chen et al. 2011; Engler et al. 2006; Ergetová and Elphick 2000; Szabo et al. 1998).

First, we examined whether CB₁-type receptors antagonists could block muscarinic modulation in STF synapses. Figure 4A shows the time course of IPSC amplitude before and during the addition of 1 μM AM 251, a CB₁-class receptor antagonist. The cannabinoid antagonist had no actions by itself, suggesting that endogenous cannabinoids are at very low concentrations in the extracellular fluid of this preparation. In the continuous presence of AM 251, a subsequent addition of 1 μM muscarine had its usual action: it decreased IPSC amplitude. Inset at right shows IPSC traces acquired during the time course, as denoted by numbers. There is paired-pulse facilitation. This result suggests that the action of muscarine on STF synapses was independent of and not mediated via cannabinoids. In this particular experiment, the GABA_A receptor antagonist gabazine (10 μM) blocked all the remaining current, suggesting that all IPSC was mediated by GABA and therefore that these inhibitory pallidal STF synapses activated in the Str are GABAergic. The paired line graph in Fig. 4B shows the individual behavior of STF synapses: muscarine acted in the presence of AM 251 in 12 of 18 GPe neurons recorded during intrastriatal field stimulation for a 49 ± 7% decrease in IPSC amplitude (P < 0.001).

A similar experiment was performed in STD synapses (Fig. 4C). As in STF synapses, the amplitude of IPSCs recorded on a GPe cell had no significant changes during the time of addition of 1 μM AM 251. However, in contrast to STF synapses, the actions of 1 μM muscarine were blocked by AM 251 in STD synapses. Inset shows IPSC traces acquired during the time course, as denoted by numbers (see paired-pulse depression). The paired line graph in Fig. 4D shows the individual behavior of a sample of STD synapses: muscarine actions were antagonized in the presence of AM 251 (n = 6). Again, gabazine blocked all synaptic current, showing that STD synapses in the GPe are also GABAergic. When the action of muscarine in the presence of AM 251 was compared in STF vs. STD connections, differences were significant (P < 0.001). The results thus suggest that cannabinoids released to
the extracellular space and acting as retrograde transmitter (Engler et al. 2006) mediate the actions of muscarine in STD synapses. Retrograde actions of cannabinoids have been described in many brain synapses, and their synthesis is commonly associated with metabotropic receptors that are present in the striatopallidal complex (e.g., Chen et al. 2011; Poisik et al. 2003).

However, if the above conclusion is true, then STD synapses should express presynaptic cannabinoid receptors. Moreover, muscarinic signaling may also induce the synthesis of cannabinoids; that is, M1-type receptor signaling may occur through a Gq/11 protein that produces changes in intracellular calcium (e.g., Perez-Burgos et al. 2008), and therefore, we should observe postsynaptic actions in some GPe neurons and presynaptic modulation of STD synapses. To test this hypothesis, we recorded the postsynaptic actions of muscarine in acutely dissociated GPe neurons. Figure 6A shows the time course of the action of 1 μM muscarine in whole cell Ca2+ current amplitudes evoked in a GPe neuron with ramp-like voltage commands (Perez-Burgos et al. 2008; Perez-Rosello et al. 2005). Clearly and reversibly, muscarine reduced the Ca2+ current (shaded areas) in 6 of 16 neurons from the GPe (37% in this sample of recorded neurons dissociated from the GPe center; Fig. 6C). Figure 6D shows that MT-7 blocked the actions of muscarine when administered together, and I–V plots in Fig. 6E show that administration of muscarine plus MT-7 does not differ from the control conditions. It was only

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Fig. 4. Muscarinic presynaptic modulation differs in STF and STD synapses. A: time course of IPSC amplitude before (1) and during (2) addition of the CB1-class receptor antagonist AM 251 (1 μM). The antagonist had no action by itself. Subsequent addition of 1 μM muscarine (3) in the presence of AM 251 had its usual action: reduction in IPSC amplitude. Thus blockade of CB1 receptors did not reduce muscarinic actions in STF synapses. Inset at right shows representative paired responses taken from the time course in A as denoted by numbers. B: summary of sample statistics (n = 10; ***P < 0.001, AM 251 vs. AM 251 + muscarine). C: time course of IPSC amplitude from an STD synapse before (1) and during (2) addition of 1 μM AM 251. The antagonist had no action by itself. However, the action of 1 μM muscarine (3) was blocked in the presence of AM 251 (cf., Fig. 2B). Inset at right shows representative recordings from the time course in C as denoted by numbers. D: sample statistics of STD synapses show no significant muscarinic actions in the presence of AM 251.

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when MT-7 was washed out that the actions of muscarine were revealed. This is clearly shown in a sample of neurons (Fig. 6F; n = 9; P < 0.001 with Friedman ANOVA and P < 0.05 for the post hoc test between muscarine + MT-7 vs. muscarine alone). This result suggests that postsynaptic receptors in GPe neurons that express muscarinic receptors are M1 class, a reason why both STF and STD synapses could be blocked by both pirenzepine and MT-7. This action could also be by atropine (n = 3; not shown).

Nevertheless, the Ca2+ current of most GPe neurons did not respond to muscarine (n = 10/16, or 63%; Fig. 6, G–I). Probably, the cell population in the GPe is heterogeneous (Goldberg and Bergman 2011; Hoover and Marshall 2002; Mallet et al. 2012; Poisik et al. 2003). These results imply that although all pallidal inhibitory synapses may be modulated, only a subset of postsynaptic neurons expresses muscarinic receptors and may produce cannabinergic mediators upon their activation.

**DISCUSSION**

Facilitating synapses are modulated by muscarinic receptors. Field stimulation inside the Str in sagittal slices (Beurrier et al. 2006) evokes IPSCs in postsynaptic GPe neurons (Cooper and Stanford 2001) in the presence of CNQX plus APV (see MATERIALS AND METHODS). These IPSCs are GABAergic because they can be completely blocked by the actions of gabazine or bicuculline (Goldberg and Bergman 2011; Jaeger and Kita 2011). These IPSCs were significantly reduced in amplitude after activation of muscarinic receptors in a way similar to that observed for the IPSCs recorded between SPNs inside the Str (Perez-Rosello et al. 2005). Muscarinic action is most probably presynaptic because it was accompanied by increases in both PPR and CV as well as a relation between CV−2 and IPSC amplitude (Bekkers and Stevens 1990; Cooper and Stanford 2001; Rodriguez-Moreno et al. 1997; Salgado et al. 2005; Sims et al. 2008; Tecuapetla et al. 2007). These responses could be recorded in both STF synapses and STD synapses. Thus a main purpose of the present work, to demonstrate the functionality of cholinergic muscarinic receptors within the GPe, was fulfilled.

A correlation between the type of short-term synaptic plasticity and the origin of the synapses has been previously shown (Miguelez et al. 2012): STF synapses are mostly striatopallidal synapses, whereas STD synapses are mostly pallidopallidal synapses, and in fact, we could only record from STF synapses when field stimulation was given inside the Str and not inside the GPe. Moreover, most evoked IPSCs after field stimulation inside the Str followed STF plasticity. When field stimulation was given inside the GPe, we could only record STD synapses. However, because a field stimulus cannot precisely isolate the origin of the IPSCs, and because other sources of GABA may be present (Mena-Segovia et al. 2004), in the present article we have referred to the synapses recorded as either facilitating (STF synapses) or depressing (STD synapses) during intrastriatal stimulation, as have most previous electrophysiological studies (Jaeger and Kita 2011; Sims et al. 2008).

Previous studies show that about two-thirds of GPe neurons are immunoreactive to parvalbumin (PV+). These neurons mainly project to STN, GPi, and SNr. On the other hand, about one-third of GPe neurons are PV− (Kita 2007). The latter may express preproenkephalin (Mallet et al. 2012) and project to the Str. However, other types of neurons are not discarded (Cooper and Stanford 2000; Nambu and Linhas 1994; Rodrigo et al. 1998). Available knowledge points toward a predominant type of neuron receiving STF synapses (reviewed in Jaeger and Kita 2011). In the present study, we show that in a small sample of PV+ neurons that were recorded and labeled, the synapses recorded were STF synapses. Because terminals from the same axons have been shown to express and are responsive to M1-receptor activation inside the Str (Perez-Rosello et al. 2005), we conclude that these synapses may be modulated directly by ACh. However, all possible retrograde messengers have not been discarded.

Muscarine actions were specific because they were blocked by muscarinic receptor antagonists of the M1-class receptors such as pirenzepine and MT-7 but not by the M4-class antagonists such as tropicamide or MT-3. Although M1 and M4 receptors are the functional muscarinic receptors in SPNs, only M1 receptors are expressed in both classes of SPNs. M4 receptors are preferentially expressed in dSPNs. Therefore, M1
receptors are virtually the only functional muscarinic receptors of iSPNs (Yan et al. 2001), and these neurons configure a most important projection to the GPe (Schwab et al. 2013). Thus the present results demonstrate that M1 receptors presynaptically regulate STF synapses (most probably striatopallidal synapses). These results may have been expected given the previous knowledge mentioned above, but they had not been observed experimentally. Thus STF synapses constitute a first target for cholinergic modulation in the GPe. A possible contamination of other inputs due to field stimulation runs against obtaining this robust result. Blockade by the selective CB1-receptor antagonist AM 251 did not affect muscarinic modulation of STF synapses (Engler et al. 2006; Fukudome et al. 2004; Lau and Vaughan 2008); therefore, we discarded the notion that muscarinic presynaptic modulation of STF synapses was mediated indirectly via cannabinoid synthesis and CB1 presynaptic receptors known to be present in striatopallidal terminals (Chen et al. 2011; Engler et al. 2006; Ergetová

**Fig. 6.** Postsynaptic action of muscarine in some GPe neurons. **A:** time course of Ca$^{2+}$ current ($I_{Ca}$) modulation by 1 μM muscarine in an acutely dissociated GPe neuron. Modulation was reversible. **B:** current-voltage relationships before and during addition of muscarine (obtained with ramp commands; see Perez-Burgos et al. 2008). **C:** paired-line graph shows a sample of responsive cells ($n = 6$; *$P < 0.05$). **D:** mamba toxin-7 (MT-7; 50 nM) blocked muscarinic action (1 μM). However, when MT-7 was washed off, the action of muscarine was readily revealed. **E:** current-voltage relationships before and during addition of muscarine in the presence or absence of MT-7. **F:** a sample of neurons treated with MT-7 and muscarine shows muscarinic actions only after MT-7 was washed off ($n = 9$; ***$P < 0.001$ for Friedman ANOVA; *$P < 0.05$ for the pair treated with MT-7 + muscarine vs. muscarine alone after post hoc test). **G:** most GPe neurons (about 2/3) did not respond to muscarine. **H:** current-voltage relationships of nonresponsive cells before and after muscarine application. **I:** sample of nonresponsive cells.
Sources of ACh to exert this modulation are the PPN (Charara and Parent 1994; Mena-Segovia et al. 2004; Woolf and Butcher 1986) and some cholinergic neurons within or in the vicinity of the GPe (Bengtson and Osborne 2000; Rodrigo et al. 1998). PPN afferents also reach the Str (Dautan et al. 2014; Mena-Segovia et al. 2004; Woolf and Butcher 1986), although a main cholinergic source in this nucleus comes from interneurons (Wilson et al. 1990).

Depressing synapses are modulated by muscarinic receptors in an indirect way. IPSCs from STD synapses decreased their amplitude and increased both their PPR and CV during activation of muscarinic receptors, again supporting a presynaptic mechanism. However, the behavior of STD synapses was different: a previous application of AM 251 blocked all muscarinic modulation. This result is consistent with an indirect modulation mediated by cannabinoids. In support of this inference, application of the selective CB1-receptor agonist WIN 55212-2 reduced IPSCs from STD synapses. The interpretation of these results is as follows: first, CB1 receptors are present and functional in the synaptic terminals of STD synapses, and second, muscarinic actions are mediated indirectly by CB1 receptors. These results on STD synapses were observed despite possible contamination due to field electrode stimulation, suggesting that they are robust.

Consistent with these results, we showed that chelation of intracellular calcium in recorded postsynaptic GPe neurons abolished muscarinic modulation of STD synapses. This result shows that mediation of a postsynaptic component was a necessary step (Engler et al. 2006) for presynaptic inhibition to occur. Moreover, during recordings of whole cell Ca2+ currents in a sample of dissociated GPe neurons, we found that about one-third of them were responsive to muscarine. The receptor involved in these cases is the M1 type. Not only does the cholinergic system innervate most basal ganglia nuclei (Mena-Segovia et al. 2004), but the dopaminergic system accomplishes a similar role (Benazzouz et al. 2014). Therefore, when it is said that there is a balance between these two systems in the basal ganglia, how is the balance reached in each of these nuclei simultaneously? Is the imbalance in any of them a source of disorder?

One partial answer was to show the role of ACh in the GPe. Interestingly, it had diverse roles: pre- and postsynaptic. In vivo, GPe neurons commonly exhibit tonic firing with pauses (Bugaysen et al. 2010; Goldberg and Bergman 2011). Excitatory inputs from the STN or inhibitory inputs from the Str or GPe axon collaterals increase or decrease their basal firing, respectively, perhaps generating a temporal code that carries striatal population-coded activity based on neuronal ensembles (Carrillo-Reid et al. 2008; Goldberg and Bergman 2011). When GABAergic transmission is blocked, firing rate is increased and becomes more regular (Goldberg and Bergman 2011; Jaeger and Kita 2011; Schwab et al. 2013). In the present study, we have shown that one physiological way to decrease inhibitory inputs to the GPe is the activation of muscarinic receptors: a suppression of inhibition will make GPe neurons less sensitive to incoming inputs. Some GPe neurons express muscarinic M1 receptors postsynaptically, and the balance between pre- and postsynaptic actions is at the moment unknown.

Consistent with the above results, there is a heterogeneous population of GPe neurons (Cooper and Stanford 2000; Mallet et al. 2012; Nambu and Llinas 1994), although most are immunoreactive to PV (Kita 2007; Mallet et al. 2012). A single class of GPe neuron may display a whole variety of firing patterns (Deister et al. 2013; Goldberg and Bergmann 2011;
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