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Localization and pharmacological modulation of GABA-B receptors in the globus pallidus of parkinsonian monkeys

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Abstract

Changes in GABAergic transmission in the external and internal segments of the globus pallidus (GPe and GPi) contribute to the pathophysiology of the basal ganglia network in Parkinson's disease. Because GABA-B receptors are involved in the modulation of GABAergic transmission in GPe and GPi, it is possible that changes in the functions or localization of these receptors contribute to the changes in GABAergic transmission. To further examine this question, we investigated the anatomical localization of GABA-B receptors and the electrophysiologic effects of microinjections of GABA-B receptor ligands in GPe and GPi of MPTP-treated (parkinsonian) monkeys. We found that the pattern of cellular and ultrastructural localization of the GABA-BR1 subunit of the GABA-B receptor in GPe and GPi was not significantly altered in parkinsonian monkeys. However, the magnitude of reduction in firing rate of GPe and GPi neurons produced by microinjections of the GABA-B receptor agonist baclofen was larger in MPTP-treated animals than in normal monkeys. Injections of the GABA-B receptor antagonist CGP55845A were more effective in reducing the firing rate of GPi neurons in parkinsonian monkeys than in normal animals. In addition, the injections of baclofen in GPe and GPi, or of CGP55845A in GPi lead to a significant increase in the proportion of spikes in rebound bursts in parkinsonian animals, but not in normal monkeys. Thus, despite the lack of changes in the localization of GABA-BR1 subunits in the pallidum, GABA-B receptor-mediated effects are altered in the GPe and GPi of parkinsonian monkeys. These changes in GABA-B receptors function may contribute to bursting activities in the parkinsonian state.

Keywords

External segment of the globus pallidus; internal segment of the globus pallidus; Parkinson's disease; GABAergic transmission; ultrastructural localization; nonhuman primate; in vivo electrophysiology

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INTRODUCTION

The activity of neurons in the external and internal segments of the globus pallidus (GPe and GPi, respectively) is regulated by GABAergic afferents that originate from the striatum and from local collaterals of GPe neurons (Kita, 2007; Nambu, 2007). The fast inhibitory actions of GABA are mediated by the activation of the ionotropic GABA-A receptors (Rudolph and Mohler, 2004; Sieghart and Sperk, 2002), whereas slow and prolonged inhibitory effects are mediated by the G-protein-coupled GABA-B receptors (Bettler and Tiao, 2006; Couve et al., 2000). Functional GABA-B receptors are heterodimers, formed by GABA-BR1 (GABA-BR1a or GABA-BR1b) and GABA-BR2 subunits (Pin et al., 2009). Through the regulation of voltage-activated Ca^{2+} channels, presynaptic GABA-B receptors inhibit the release of GABA and other neurotransmitters, while postsynaptic GABA-B receptors may inhibit neuronal excitability by activating rectifying K^{+} (GIRK) channels (Bowery, 2002; Ulrich and Bettler, 2007).

In both GPe and GPi, postsynaptic GABA-B receptors are abundantly expressed in dendrites, while presynaptic receptors are found in GABAergic and glutamatergic axons and terminals (Charara et al., 2004; Charara et al., 2000; Chen et al., 2004). In a previous study we showed that pharmacological activation of local GABA-B receptors inhibits the firing activity of GPe and GPi neurons of normal monkeys, while injections of a GABA-B receptor antagonist have little effects on spontaneous pallidal activity (Galvan et al., 2005). An explanation for the apparent lack of a functional GABAergic tone at GABA-B receptors may be that in the pallidum (Charara et al., 2005; Chen et al., 2004), as in other brain areas (e.g., Kulik et al., 2003; Lacey et al., 2005), GABA-B receptors are found mostly at extrasynaptic sites, suggesting that diffusion of GABA from the synaptic cleft is required for receptor activation. Such diffusion may occur under conditions that favor increase in GABA release, such as synchronous bursting activities of GABAergic inputs (Scanziani, 2000). In vitro studies, indeed, have demonstrated that GABA-B receptors in the rat GP are only activated during episodes of repetitive stimulation of GABAergic afferents (Kaneda and Kita, 2005). Studies of pallidal responses to putamenal stimulation in awake monkeys have also shown that GABA-B receptors are predominately engaged when putamenofugal fibers are activated with burst stimulation paradigms, while pallidal inhibition by single stimulation of the striatum does not involve GABA-B receptors (Kita et al., 2006).

In Parkinson's disease (PD), the increased synchronization and bursting activity of pallidal neurons (Nini et al., 1995; Soares et al., 2004; Wichmann and Soares, 2006) may favor the activation of pallidal GABA-B receptors. Several previous anatomical reports have begun to address this issue. Thus, in PD patients and in animal models of the disease, the levels of protein or mRNA for GABA-B receptors were found to be lower than normal in GPe, and up-regulated in GPi (Calon et al., 2000; Calon et al., 2003; Johnston and Duty, 2003). Knowing that the magnitude of GABA-B receptor-mediated effects relies partly on the localization and relative abundance of functional receptor proteins, the goals of the present study were to assess potential changes in the ultrastructural distribution of pre- and post-synaptic pallidal GABA-B receptors, and determine the effects of GABA-B receptor ligands on GPe and GPi neuronal activity in monkeys rendered parkinsonian by systemic injections of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

MATERIALS AND METHODS

Animals

Twelve drug-naïve adult rhesus monkeys (*Macaca mulatta*, 4–7 kg) were used in these studies. All animals were housed with *ad libitum* access to food and water. At the beginning of the experiments, the monkeys were acclimated to the laboratory, and trained to permit

handling by the experimenter and to sit in a primate chair. All experiments were performed in accordance with the National Institutes of Health's "Guide for the Care and Use of Laboratory Animals" (Garber et al., 2010) and the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals (amended 2002), and were approved by the Chemical Safety Committee and the Animal Care and Use Committee of Emory University.

MPTP administration

To induce nigrostriatal dopamine depletion, 7 animals (three used in the anatomical studies and 4 used in the physiological experiments) received weekly injections of the dopaminergic neurotoxin MPTP (0.2–0.8 mg/kg, i.m., Sigma-Aldrich, St. Louis, MO) until moderate parkinsonism developed. The severity of parkinsonian motor symptoms was assessed weekly using rating scales for parkinsonism and computer-assisted behavioral scoring methods as described in detail in our previous studies (Galvan et al., 2010; Kliem et al., 2010; Wichmann et al., 2001). The total amount of MPTP administered ranged from 3.2 to 14.1 mg/kg and the treatment lasted from 1 to 18 months. All MPTP-treated monkeys were stably parkinsonian for at least 6 weeks before the start of the electrophysiological or anatomical studies.

Immunohistochemical localization of GABA-B R1

Three MPTP-treated and four age-matched control animals were deeply anesthetized with an overdose of pentobarbital (100 mg/kg, i.v.), and then perfused transcardially with cold oxygenated Ringer's solution, followed by 4% paraformaldehyde and 2% acrolein in phosphate buffered saline (PBS, 0.01 M, pH 7.4). The brains were removed from the skull and frontal sections (60 μ m) were obtained with a vibratome and collected in cold PBS (0.01M, pH 7.4). The sections were then subjected to the immunohistochemical procedures mentioned below.

Specificity of antibodies—To confirm the specificity of the GABA-B R1 antibodies, we used brain sections obtained from two wild-type and two GABA-B R1 knockout mice (Schuler et al., 2001), generously provided by Dr. Bernhard Bettler (University of Basel, Basel Switzerland). The mice were perfused using the same protocol described above for monkeys, and 60 μ m-thick brain sections were incubated with GABA-B R1 antibodies and processed for light microscopy (LM) immunocytochemistry as described immediately below.

Processing of tissue for light microscopy—To assess potential changes in the expression level of GABA-B R1 immunoreactivity between the control and parkinsonian states, sections containing GPe and GPi from approximately the same antero-posterior levels of the brain (14.70 to 12.45 mm from the interaural line, according to Paxinos et al., 2000) were selected in normal and MPTP-treated parkinsonian monkeys. To control for variability in staining intensity, sections from the seven animals were processed simultaneously. After pretreatment with 10% normal goat serum and 1% bovine serum albumin in PBS, the sections were incubated for two days at 4°C in the primary antibody solution (anti-GABA-B R1, 1:1,000 cat no. sc-7338 Santa Cruz Biotechnology, Santa Cruz, CA). This antibody was raised towards an epitope at the C-terminus of GABA-B R1 of rat origin, and binds similarly to GABA-B R1a and R1b isoforms. To reveal the primary antibodies, the sections were incubated in biotinylated goat anti-rabbit IgG (1:200; Vector labs, Burlingame, CA) for 2 hours, and in avidin-biotin complex (ABC) solution (1:100; Vectastain Standard kit, Vector labs) for 90 min. The sections were then rinsed in PBS and TRIS buffer (0.05 M, pH 7.6) and placed in a solution containing 0.025% 3-3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich), 0.01M Imidazole (Fisher Scientific) and 0.006% H₂O₂. The reaction was

terminated by repeated washes in PBS. All immunoreagents were diluted in PBS containing 1% normal goat serum, 1% bovine serum albumin and 0.1% Triton X-100.

For the parkinsonian monkeys, the extent of the MPTP-induced damage to the dopaminergic nigrostriatal system was assessed by staining sections at the level of the striatum and the substantia nigra with mouse anti-tyrosine hydroxylase (TH) antibodies (1:1000, Millipore). The TH immunoreactivity was revealed using biotinylated goat anti-mouse IgG (1:200, Vector labs) and ABC solution.

After revealing the GABA-B R1 or TH antibodies binding sites, the sections were mounted on gelatin-coated slides, dehydrated in alcohol, immersed in toluene and a coverslip was applied with Cytoseal XYL (Richard-Allan Scientific, Kalamazoo, MI). The slides were digitized with an Aperio Scanscope CS system (Aperio Technologies, Vista, CA).

Processing of tissue for electron microscopy—Sections from the 3 MPTP-treated and 3 of the control animals were processed and stained as described above. The ultrastructural preservation of the tissue from the fourth untreated animal was suboptimal, and was not used for the electron microscopy analysis. The sections were first treated with 1% sodium borohydride, placed in a cryoprotectant solution, frozen at -80°C , and then thawed and washed in PBS. Triton X-100 was omitted from all incubations. After revealing GABA-B R1 antibody labeling with diaminobenzidine, the sections were washed in phosphate buffer (PB, 0.1M, pH 7.4) and postfixed in osmium tetroxide (1% in PB). This was followed by further rinses in PB, and dehydration by exposure to increasing concentrations of ethanol and propylene oxide. Uranyl acetate (1%) was added to the 70% ethanol solution. The sections were then embedded in resin (Durcupan ACM; Fluka, Ft. Washington, PA) on microscope slides and placed in the oven for 48 hours at 60°C . Blocks from GPe or GPi were cut out from the slides and glued onto resin blocks. Sixty nanometer thick sections were cut from the surface of the blocks with an ultramicrotome (Leica Ultracut T2) and collected on Pioloform-coated copper grids, stained with lead citrate for 5 min to enhance tissue contrast, and examined with an electron microscope (Zeiss EM-10C or JEOL model 1011) at 20,000–25,000X. Electron micrographs of fields containing immunoreactive elements were acquired using a digital camera (DualView 300W; Gatan, Inc., Pleasanton, CA) controlled by DigitalMicrograph software (version 3.10.1; Gatan).

Data analysis—Using Imagescope viewer software (Aperio), the digital images of the stained tissue slides were examined, and a 0.7x magnification image containing the putamen, GPe and GPi was obtained. The images were then imported into ImageJ (National Institutes of Health, Rasband, 1997–2009) for additional processing.

For optical density measurements, the images were converted into 16-bit grayscale format and inverted. GPe or GPi were outlined as regions of interest, and the integrated optical density in these regions determined. To control for differences in background staining, the optical density measurement in the internal capsule immediately medial to GPe and GPi on the same section was subtracted from that obtained in GPe or GPi. For statistical analysis, density measurements were averaged from three sections per animal from GPe or GPi. The resulting mean values for GPe and GPi from normal and MPTP-treated animals were then compared with Mann-Whitney tests.

To determine the extent of nigrostriatal dopaminergic denervation, we measured the optical density in TH-immunostained striatal sections of MPTP-treated monkeys. The optical density measurement in the internal capsule on the same section was considered to reflect background staining and was subtracted from that obtained in the striatum. These values

were compared against measurements made from striatal sections at the same rostrocaudal level in normal monkeys.

For the electron microscopic analysis of GABA-B R1 localization, randomly encountered immunoreactive elements were photographed from a total surface of at least 1000 μm^2 of immunostained tissue for GPe or GPi in each monkey. Two different blocks of tissue were used per structure for each monkey. The labeled elements were categorized based on ultrastructural features (Peters et al., 1991). GABA-B R1-immunopositive axon terminals were classified as symmetric, asymmetric or unidentified terminals based on the type of synaptic contacts they established.

In each animal, the number of labeled elements in GPe or GPi was quantified and the relative proportion of each category of elements was calculated and expressed as percent of total labeled elements in the areas examined, as previously reported in other studies (Charara et al., 2004; Galvan et al., 2004; Galvan et al., 2010). These values were used for the statistical comparisons between normal and MPTP-treated monkeys.

Electrophysiological recordings and local drug administration

Surgical procedure and initial electrophysiological mapping—Four MPTP-treated, stably parkinsonian monkeys, and one untreated animal received metal chambers for chronic recordings. Under aseptic conditions and isoflurane anesthesia (1–3%), a stainless steel recording chamber (inner diameter 16 mm, Crist Instrument Co., Hagerstown, MD), stereotactically directed at the left pallidum with a 36° angle from the vertical in the coronal plane, was affixed to the skull with dental acrylic, along with metal holders (Crist Instrument) for head stabilization. Metal screws were used to anchor the acrylic to the bone. After surgery, the animals were allowed to recover for at least 1 week.

During experimental sessions, the animals were seated in a primate chair with their heads restrained, but free to move their body and limbs. All recordings were conducted while the animal was fully awake, judged by maintained eye opening and occasional body and face movements. In each session, the dura was pierced with a 20-gauge guide tube, and a tungsten microelectrode ($Z = 0.5\text{--}1.0\text{ M}\Omega$ at 1 kHz; FHC, Bowdoinham, ME) was lowered into the brain with a microdrive (MO-95B; Narishige, Tokyo, Japan). Extracellular neuronal electrical signals were recorded, amplified (DAM-80 amplifier; WPI, Sarasota, FL), filtered (400–10,000 Hz; Krohn-Hite, Brockton, MA), displayed on a digital oscilloscope (DL1540; Yokogawa, Tokyo, Japan), and made audible via an audio amplifier. The neuronal signals were digitized (sampling rate 25 kHz) and stored onto computer disk using a data acquisition interface (Power1401; CED, Cambridge, UK) and commercial software (Spike2, CED) for off-line analysis.

GPe neurons were identified according to their location ventromedial to the putamen, and by their characteristic high frequency discharge, interspersed with pauses (DeLong, 1971; Elias et al., 2007). GPi cells were identified based on the depth of the electrode (at least 2 mm ventral to the first GPe unit), the identification of ‘border’ cells between the GPe and GPi (DeLong, 1971), and their high-frequency firing rates (DeLong, 1971).

Intracerebral injections—Microinjections were performed using a device that combines a tungsten microelectrode with a section of fused silica injection tubing, as described previously (Galvan et al., 2010; Galvan et al., 2005; Kliem and Wichmann, 2004). The injection tubing was connected to a 1 ml syringe (CMA Microdialysis, Solna, Sweden), driven by a remotely controlled infusion pump (Model 102, CMA). The recording-injection system was lowered into the brain using a microdrive and positioned in GPe or GPi. Once neuronal spiking was isolated with sufficient quality, the spontaneous activity of the cell was

recorded for at least 90 s. The recording continued during the subsequent delivery of the drug under study (1 μ l, delivered at 0.3 μ l/min), and for at least 300 s after the end of the infusion. In some cases, more than one injection was performed along the same track. In these cases, the injections were separated by at least 1 mm, and with a time lapse of at least 20 min between injections.

Drugs—Baclofen (213 ng/ μ l, Tocris Bioscience, Ellisville, MO) and CGP55845A (402 ng/ μ l, Tocris) were dissolved in artificial cerebrospinal fluid (aCSF, comprised of (in mM) 143 NaCl, 2.8 KCl, 1.2 CaCl₂, 1.2 MgCl₂, 1 Na₂HPO₄), and the pH adjusted to 7.2–7.4. Before being loaded into the injection systems, all solutions were filtered with a 0.2 μ m pore size nylon membrane (Fisher Scientific, Hampton, NH). Artificial CSF was used for control injections.

Perfusion and tissue processing—At the conclusion of the electrophysiology experiments, the animals were deeply anesthetized and perfused as described above, using 4% paraformaldehyde and 0.1% glutaraldehyde as fixative, and the brains were sectioned as described above. A series of sections containing GPe and GPi was stained for Nissl substance to visualize electrode penetrations, while alternate sections were immunolabeled for the neuronal marker microtubule associated protein 2 (mouse anti-MAP2, 1:1000, Millipore) to assess the extent of neuronal damage induced by the electrode tracks. In MPTP-treated monkeys, TH immunostaining was done to evaluate the degree of nigrostriatal dopaminergic denervation. The labeling for MAP2 and TH staining was visualized using the ABC method as described above.

Analysis of electrophysiologic data—The analysis was conducted only on recordings of cells confirmed to be in the GPe or GPi, based on depth readings during the recording sessions and on the results of the histological analysis. Spike sorting was performed off-line with a waveform matching algorithm, followed by principal component analysis (Spike2). Inter-spike interval (ISI) distribution histograms were constructed to verify the recording quality for each spike train. All subsequent steps of the analysis were done in Matlab (Mathworks, Natick, MA).

ISI data were used to calculate second-by-second readouts of firing rates which were subsequently smoothed using a sliding 20-point moving average. These readouts were used to select a ‘control’ (baseline) segment (60 s of data preceding the drug infusions) and a ‘drug effect’ segment. A drug injection was considered to have a significant effect if the firing rate remained continuously either above the 90th percentile, or below the 10th percentile of the pre-injection baseline activity, for at least 60 s, with an effect onset no longer than 200 s after the beginning of the drug injection. The 200 s latency parameter was chosen based on previous experiments (Galvan et al., 2005). The ‘effect’ segment was defined as the 60 s period centered on the highest or lowest point of the firing rate after the drug application, unless the neuron’s firing rate remained at zero spikes/s for 60 s. In these cases, the drug effect was chosen to include the decline of the firing rate, to increase the number of ISIs for computations. In cases in which no effect was observed (firing rate did not change above or below the 90–10th percentile window after the injection within 200 s of the start of the injection), a 60 s data segment centered on a randomly chosen time point within the 200 s period after the start of the injection was used as the ‘effect segment’. These segments of recordings, selected on the basis of changes in firing rates, were used to classify the effect periods of drugs into cells showing decreases, increases or no effect.

To calculate the magnitude of the drug-induced effects in discharge rates, the mean firing rate during the effect period was expressed as a percent change from baseline. In some subgroups of neurons, GABA-B receptor ligands induced both increases and decreases of

firing. Therefore, cases with positive and negative changes were compared independently between normal and MPTP-treated cases.

We also calculated second-by-second readouts of coefficients of variation (CV, smoothed using a sliding moving average as described above). These readouts were used to select 'effect' segments with the same criteria as described above for the firing rate readouts. The control segments were the same as those selected on the basis of firing rates. As changes in CV are more likely to reflect changes in firing patterns, these CV-based effect segments were used in subsequent steps of the analysis to examine descriptors of firing patterns, including descriptors of burst discharges or brief decelerations of neuronal firing and power spectral analyses.

Burst indices were calculated using the Legendy and Salzman burst detection method (Legendy and Salzman, 1985; Wichmann and Soares, 2006). Deceleration of neuronal firing was determined with the method described by Elias et al (Elias et al., 2007). For bursting or deceleration events, a 'surprise' value of 3 was used. Burst and deceleration indices were calculated as the proportion of spikes in the bursts or decelerations (compared to the total number of spikes), or based on the proportion of time spent by the cell in either bursts or decelerations. A burst was further classified as a 'rebound burst' if the ISI immediately before the burst was at least three times longer than the mean ISI of the neuron.

A power spectral analysis was performed to examine oscillatory properties of neuronal discharge in the segments selected. This analysis was done with the Neurospec 2.0 Matlab functions for frequency domain analyses (written by David Halliday, Halliday et al., 1995; Nielsen et al., 2005). For each neuron, the raw spectra were integrated in the 1–3 Hz, 3–8 Hz, 8–13 Hz, 13–30 Hz, and 30–100 Hz ranges, and the resultant values expressed as a fraction of the power in the entire 1–100 Hz band (for a similar approach see Soares et al., 2004).

Data from normal animals—For a comparison between MPTP-treated and normal monkeys, we used the data generated here as well as similarly collected data from normal animals that were obtained previously (Galvan et al., 2005), further supplemented with recordings done in an additional control monkey.

Statistics—Statistical comparisons were performed with SPSS (SPSS Inc, Chicago, Ill), using Mann-Whitney test for independent samples, and the Wilcoxon signed-rank test for related samples. $P < 0.05$ was accepted as indicating significant differences. Differences between normal and MPTP-treated monkeys in proportions of responses of pallidal neurons to GABA-B receptor ligands were analyzed with Chi-square tests. A two-tailed Pearson test was used to assess the correlation between the percentage change in firing rate and the difference in proportion of spikes in rebound bursts from baseline to drug effect.

RESULTS

All MPTP-treated monkeys were moderately and stably parkinsonian. Their scores ranged from 16 to 26 on our rating scale of parkinsonian motor signs (maximum 27 points, Wichmann et al., 2001). The intensity of TH immunostaining in the putamen of all MPTP-treated animals was below 10% of that found in control monkeys (as shown in Galvan et al. 2010).

GABA-B receptor immunohistochemistry

Light microscopy—Brain sections from GABA-BR1 receptor knock-out mice (Schuler et al., 2001) showed no detectable immunolabeling, while striatopallidal tissue from wild type

animals displayed strong GABA-BR1 immunoreactivity (Fig. 1A), confirming the specificity of antibodies used in our study to detect GABA-BR1.

At the light microscopic level, the pattern of GABA-BR1 immunostaining in the globus pallidus of normal and parkinsonian monkeys was similar to that found in our previous study (Charara et al., 2000), i.e., both pallidal segments showed strong labeling, mostly on cell bodies and neuropil elements (Fig. 1B,C). No significant difference (Mann-Whitney test, $p > 0.05$) was found in the intensity of GABA-BR1 immunostaining in GPe and GPi between normal and MPTP-treated monkeys as revealed with optical density measurements of immunoreactivity (Fig. 1D).

Electron microscopy—In agreement with our previous data (Charara et al., 2000), the most commonly observed GABA-BR1-positive elements in both pallidal segments of normal and parkinsonian monkeys were unmyelinated axons, followed by dendrites and axon terminals (Fig. 2). The proportion of labeled unmyelinated axons was about three times that of immunoreactive dendrites and terminals in GPe and GPi (Fig. 2F). The relative abundance of the different GABA-BR1 positive structures in MPTP-treated monkeys was not significantly different from that in the normal animals ($p > 0.05$, Mann-Whitney test).

In both normal and MPTP-treated monkeys, most GABA-BR1-positive terminals with clear synaptic junctions formed symmetric synapses (proportion of symmetric synapses among all labeled boutons: GPe, $72 \pm 5\%$ and $86 \pm 3\%$; GPi, $78 \pm 5\%$ and $79 \pm 11\%$, in normal and MPTP-treated animals respectively). In all experimental groups, GABA-BR1-positive terminals that formed asymmetric synapses accounted for 2–6% of total labeled boutons.

Effects of GABA-B receptor activation or inactivation on the firing of pallidal neurons

Basic firing characteristics of pallidal neurons—Our results are based on recordings obtained from 33 GPe and 25 GPi neurons in which the stability and isolation of single units was maintained before, during and after microinjections of aCSF or drugs in the vicinity of the recorded cells. These cells were recorded in four MPTP-treated monkeys. Table 1 provides descriptors of neuronal firing recorded during the pre-injection period in parkinsonian animals and compares them with those obtained under similar conditions in normal monkeys (Galvan et al, 2005, supplemented with recordings from one additional normal animal, see Methods).

Several firing pattern parameters were significantly different between the normal and parkinsonian monkeys. In GPi, the burst and deceleration indices (based on the proportion of spikes or the proportion of time within bursts or decelerations) were significantly higher in MPTP-treated monkeys than in controls. In addition, we found higher oscillatory power in the 8–13 Hz band in both pallidal segments of MPTP-treated monkeys than in controls. Oscillatory power in the 1–3 Hz range and 13–30 Hz range also increased in GPi and GPe, respectively (all differences assessed with Mann-Whitney test, $p < 0.05$).

Control injections, behavioral effects—No new control experiments were carried out in the present study in order to minimize tissue damage. However, results from our previous studies indicated that control injections of aCSF into either pallidal segment did not result in significant changes of the parameters examined here (Galvan et al., 2010). Injections of aCSF, baclofen or CGP55845A in GPe or GPi did not induce behavioral changes.

Effects of baclofen and CGP55845A on GPe neurons—The GABA-B receptor agonist baclofen decreased the firing of 9/10 GPe neurons in MPTP-treated monkeys (Figs. 3A). The proportion of responding cells was not different from that found in normal monkeys (Fig. 4A, $p > 0.05$, Pearson Chi-square test), but the magnitude of the decreases in

firing compared to baseline, was larger in MPTP-treated monkeys (Fig. 4B, $p = 0.031$ Mann-Whitney test). Baclofen also altered the patterns of firing in GPe neurons (see example traces in Fig. 3A). Most prominently, the proportion of spikes in rebound bursts was significantly increased in MPTP-treated monkeys (from $3.8 \pm 6\%$ to $66.2 \pm 41\%$, $p = 0.02$, Wilcoxon test), but not in normal animals (Fig. 5A).

Injections of the GABA-B receptor antagonist CGP55845A led to a decrease in firing in 6/17 neurons, and an increase in 4/17 GPe neurons in MPTP-treated monkeys. This distribution of responses was not different from that observed in GPe neurons of normal monkeys ($p > 0.05$, Pearson Chi-square test, Fig. 4A). We also did not find significant differences between normal and MPTP-treated animals in terms of magnitude of changes in firing rates after CGP55845A injections ($p > 0.05$, Mann-Whitney test, Fig. 4B). CGP55845A treatment did not have significant effects in any of the other parameters of firing that were examined here (Fig. 5A).

Effects of baclofen and CGP55845A on GPi neurons—Similar to the effects observed in GPe, the most common effect of baclofen on GPi cells was a reduction in firing rate. Five of seven GPi neurons tested in the MPTP-treated monkeys showed a decrease in firing in response to local baclofen application. This proportion of responses did not differ from that documented in normal monkeys ($p > 0.05$, Pearson chi-square test, Fig. 4A). The effects of baclofen on the magnitude of changes in firing rate were not different between the two groups ($p > 0.05$, Mann-Whitney test, Fig. 4B). As in GPe, baclofen treatment induced an increase in the proportion of spikes in rebound bursts in GPi cells of parkinsonian monkeys (from 2.8 ± 5 to $28.3 \pm 41\%$, $p = 0.04$, Wilcoxon test, Fig. 5B). This effect was not seen in normal monkeys. Baclofen also induced significant changes in the power spectral distribution, with increases in the 8–13 Hz range (from $2.8 \pm 1\%$ to $3.5 \pm 1\%$; $p = 0.04$, Wilcoxon test) and a reduction in the 30–100 Hz range (from $79 \pm 6\%$ to $75 \pm 5\%$, $p = 0.04$, Wilcoxon test). In GPi neurons of normal monkeys, no baclofen-induced changes were seen in these or other parameters of firing pattern.

Injection of the GABA-B receptor antagonist CGP55845A resulted in a reduction of firing in 9/14 GPi neurons in MPTP-treated animals (Fig. 3B). This proportion of responses was significantly different from normal monkeys, in which this drug had no effect on most neurons (4/5, $p = 0.016$, Pearson chi-square test, Fig. 4A). Also, decreases in firing rate were larger in magnitude after MPTP treatment, without reaching statistical significance when compared to normals ($p = 0.053$, Mann-Whitney test, Fig. 4B). CGP55845A injections in GPi increased the proportion of spikes in rebound bursts (from 5 ± 10 to $27 \pm 39\%$, $p = 0.05$, Wilcoxon test, Fig. 5B and example traces in Fig. 3B). This effect was not seen in normal animals. A regression analysis showed that the increases in the proportion of spikes in rebound bursts were not consistently related to corresponding decreases in firing rate.

DISCUSSION

We found that the general pattern of cellular and ultrastructural distribution of GABA-BR1 subunits in GPe and GPi was similar between parkinsonian and normal monkeys. In both conditions, labeling was mostly found in unmyelinated axons, dendrites and putative GABAergic terminals. Despite the similar pattern of distribution, the effects of local microinjections of drugs acting at GABA-B receptors differed significantly between normal and parkinsonian animals. Thus, the baclofen-induced reduction in firing was more pronounced in the GPe of MPTP-treated animals than in normal monkeys, while injections of the GABA-B receptor antagonist CGP55845A which did not significantly affect the activity of GPi neurons in normal animals, reduced the firing rates of GPi neurons in MPTP-treated monkeys. Finally, the reductions in firing observed with baclofen in GPe and GPi,

and with CGP55845A in GPi were accompanied by an increase in the proportion of spikes in rebound bursts in parkinsonian animals, but not in normal monkeys. Our results suggest that GABA-B receptor-mediated effects on synaptic transmission are altered in the MPTP-treated monkeys, and that these alterations may contribute to changes in firing activities of GPe and GPi neurons in the parkinsonian state.

Localization of GABA-BR1 subunits in normal versus parkinsonian conditions

There were no differences in the intensity of GABA-BR1 immunolabeling in GPe or GPi between normal and parkinsonian monkeys. While measurements of optical density at low magnification are related to stereological counts of labeled elements at higher magnifications (Scorcioni et al., 2008), the sensitivity of this method to detect changes in receptor levels expression may be less than that of other techniques. This could explain the discrepancy between our data and autoradiography binding studies that suggested a downregulation of GABA-B receptors in GPe, and an upregulation in GPi, in parkinsonian patients and MPTP-treated monkeys (Calon et al., 2000; Calon et al., 2003). It is noteworthy that our data are solely focused on the GABA-BR1 subunit, thereby cannot rule out that changes in GABA-B receptor binding for their ligands may occur despite the lack of significant changes in protein expression described in our study.

Our ultrastructural observations showed that unmyelinated axons accounted for most of the GABA-BR1 labeled elements in both pallidal segments of MPTP-treated monkeys. We did not establish the sources of these axons, but it is reasonable to assume that most of them originated from the striatum (Smith et al., 1998). In fact, most labeled terminals found in this and our previous studies of normal monkeys (Charara et al., 2000; Charara et al., 2005) displayed the ultrastructural features of GABAergic striatal boutons (Smith et al., 1998). These data suggest that activation of presynaptic GABA-B receptors may act to attenuate striatopallidal GABAergic transmission in the monkey pallidum, as previously demonstrated in slices of rodent brains (Kaneda and Kita, 2005). The activation of GABA-B receptors also modulates glutamatergic transmission in rat brain slices, most likely through presynaptic inhibition of transmission at subthalamopallidal synapses (Chen et al., 2002). In agreement with these data, our immunocytochemical data showed that some of the presynaptic labeling in GPe and GPi was associated with terminals forming asymmetric, presumably glutamatergic synapses.

The presence of GABA-BR1 in dendrites of GPe and GPi neurons indicates that these receptors may also mediate slow postsynaptic inhibitory effects in these regions. In vitro and in vivo data have, indeed, demonstrated that GABA-B receptor activation inhibits the activity of GP neurons, and that some of this inhibition relies on postsynaptic GABA-B receptors in normal rats and monkeys (Galvan et al., 2005; Kaneda and Kita, 2005).

The distribution of GABA-BR1-labeled elements was similar in normal and MPTP-treated monkeys, suggesting that the anatomical substrates for pre- and postsynaptic GABA-B receptormediated effects are not strongly altered in the parkinsonian state. However, the immunoperoxidase technique that was used in this study is highly sensitive but has low spatial resolution (Galvan et al., 2006), so that differences in levels of internalization, plasma membrane expression and subsynaptic localization of GABA-BR1 in the parkinsonian condition cannot be assessed with this method. Thus, despite the apparent similarity in the overall distribution of GABA-BR1 subunits in normal and parkinsonian monkeys, our anatomic data do not rule out that changes in trafficking or turnover of GABA-B receptors may underlie some of the functional differences between normal and MPTP-treated monkeys described in our electrophysiological studies. Future studies using immunogold particles will provide further insight into possible MPTP-induced changes in the subcellular and subsynaptic localization of these receptors. For instance, such studies

will help us to delineate more definitively whether there are changes in the proportion of receptors expressed in the membrane (as opposed to the cytoplasmic compartment), or in the subsynaptic area (as opposed to extrasynaptic locations).

Effects of GABA-B receptor activation or inactivation on firing rates

The activation of GABA-B receptors with baclofen resulted in strong decreases of firing rates in GPe and GPi of both normal and parkinsonian animals, but the decreases in firing produced by the same amount of infused baclofen were more pronounced in GPe neurons of MPTP-treated monkeys than in controls. While an increase in the expression of GABA-B receptors in the parkinsonian state could account for this difference, the available evidence indicates either no change (current report, and de Groote et al., 1999) or even a downregulation of GPe GABA-B receptors binding (Calon et al., 2003) in parkinsonism. Alternatively, the coupling of these receptors to G-protein effectors or their assembly to auxiliary units (Schwenk et al., 2010) could be potentiated after dopamine depletion, resulting in enhanced responses to activation.

The firing rate decreases observed with baclofen probably result from postsynaptic GABA-B-mediated hyperpolarization (Kaneda and Kita, 2005). However, these decreases in firing could also be mediated by presynaptic inhibitory control of glutamatergic transmission via GABA-B heteroreceptors expressed in glutamatergic terminals (Chen et al., 2002).

Administration of the GABA-B receptor antagonist CGP55845A decreased the firing rate of most GPi neurons in MPTP-treated monkeys, in contrast to the lack of a net effect of this drug on the firing rate of normal GPi neurons. It is possible that CGP55845A-induced decreases in firing are mediated via blockade of GABA-B autoreceptors, leading to enhanced GABA release which may then act via postsynaptic GABA-A and GABA-B receptors. This suggestion is supported by our anatomical data showing GABA-B immunolabeling in putative GABAergic terminals.

In some cases, administration of CGP55845A resulted in increased firing. This effect could be the result of disinhibition, mediated by blockade of postsynaptic GABA-B receptors, or of reduced excitation via blockade of presynaptic receptors in glutamatergic terminals. Future experiments in which glutamatergic compounds are infused in combination with GABA-B receptor ligands will help elucidate the extent of *in vivo* modulation of GABA-B receptors on glutamatergic transmission.

The similarity of the effects of GABA-B receptor agonists and antagonists on neuronal recordings in parkinsonian animals is paradoxical. A possible explanation may be that the postsynaptic effects of the GABA-B receptor agonist strongly outweighed any presynaptic effects this agent may have had, but that presynaptic GABA-B receptors were subject to a more substantial tone of endogenous GABA than postsynaptic receptors. Indeed, it has been suggested that pharmacological differences between pre- and post-synaptic GABA-B receptors occur because they may be exposed to different concentrations of ambient or released GABA (Laviv et al., 2010). In the monkey pallidum, terminals and unmyelinated axons tend to be surrounded by glial processes enriched in GABA transporters which, under normal conditions, may effectively remove GABA from the extracellular space. Our previous studies have suggested that the effects of GABA transporters towards extracellular GABA clearance in the GPi is less effective in parkinsonian monkeys (Galvan et al. 2010) potentially providing a mechanism to promote higher concentrations of extracellular GABA (and increased tonic activation of GABA-B receptors) in specific microdomains in GPi. Another possibility is that glial processes, which most likely contribute for the bulk of GABA reuptake in the monkey pallidum (Galvan et al., 2010), also release GABA through anion channels, as recently shown in cerebellar slices (Lee et al., 2010).

GABA-B Receptor Effects on Bursting Activity

Baclofen in GPe and GPi and CGP55845A in GPi also increased the proportion of spikes in rebound bursts in MPTP-treated monkeys. These effects, which were not seen in normal monkeys, appear to be a prominent feature of GABA-B receptors activation in the parkinsonian state.

A rebound burst is defined here as a burst preceded by a prolonged pause in firing (Wichmann and Soares, 2006). In in vitro studies, rebound bursts occur in pallidal neurons after strong hyperpolarization (Cooper and Stanford, 2000; Nakanishi et al., 1990; Nambu and Llinas, 1994; Stanford, 2003), and it has been proposed that this type of firing contributes to oscillatory activity in the 'GPe-STN pacemaker' (Plenz and Kital, 1999). In the parkinsonian state, neurons in both pallidal segments show distinct changes in burst firing (Boraud et al., 1998; Fillion and Tremblay, 1991; Hutchison et al., 1994; Wichmann and Soares, 2006), but in most of these studies, it was not determined if these bursts could be categorized as rebound bursts.

Our results indicate that activation of postsynaptic GABA-B receptors (by baclofen or by endogenous GABA, presumably released after blockade of autoreceptors) promotes rebound bursting. Importantly, the decreases in firing rate *per se* were not solely responsible for the appearance of these bursts. The fact that this phenomenon was more frequent in pallidal neurons of MPTP-treated monkeys indicates that dopamine depletion may increase the tendency of these cells to fire in rebound bursts.

Previous studies have shown that activation of GABA-B receptors is required to induce rebound bursts in STN neurons (Bevan et al., 2007) and that activation of GABA-B receptors in GPe and GPi is dependent on repetitive (burst) firing of striatal afferents (Kita et al., 2005). In this way, GABA-B receptor activity in the GPe-STN loop may participate in the self-sustaining burst cycle (Plenz and Kital, 1999). Our results suggest that this phenomenon may be facilitated in parkinsonian conditions.

Conclusion

The results of our study suggest that a specific antagonist for postsynaptic GABA-B receptors in GPe or GPi could reduce the incidence of rebound bursts in parkinsonism, and may, thus, have antiparkinsonian efficacy. Currently, the use of GABA-B receptor antagonists in parkinsonian individuals remains speculative, since none of the available GABA-B receptor antagonists would clearly distinguish between pre- or postsynaptic GABA-B receptors. In addition, given the widespread distribution of GABA-B receptors in the CNS, a pharmacological agent would need to rely on functional or pharmacological distinctiveness of pallidal GABA-B receptors, which has not been demonstrated.

However, several recent studies have shown a divergence in the potency of GABA-B receptor agonists and antagonists at pre- or postsynaptic locations (Pinard et al., 2010). A recent report has shown that the pharmacology and kinetics of GABA-B receptors is determined by auxiliary proteins (Schwenk et al., 2010), a finding that may promote the development of pharmacological or molecular tools to dissect pre and postsynaptic GABA-B receptor subtypes, and enhance their local specificity.

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ABBREVIATIONS

ABC	Avidin-biotin complex
aCSF	Artificial cerebro-spinal fluid
CV	Coefficient of variation
GABA-BR1	GABA-B receptor R1 subunit
GP	Globus pallidus
GPe	External segment of globus pallidus
GPi	Internal segment of globus pallidus
MAP2	Microtubule associated protein -2
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PB	phosphate buffer
PBS	phosphate buffered saline
TH	Tyrosine hydroxylase
SNc	Substantia nigra pars compacta

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Fig. 1. GABA-B R1 immunolabeling in GPe and GPi displays no discernable differences between normal and parkinsonian monkeys at the light microscopic level

A. Test of antibody specificity: Representative brain sections processed for GABA-B R1 immunolabeling at anterior (**A1, A3**) and posterior (**A2, A4**) levels of the striatopallidal complex in wild type (WT) and GABA-B R1 knockout mice. **B, C.** Low and high power images of GABA-BR1 immunostaining in the striatopallidal complex of a normal (**B**) and an MPTP-treated (**C**) monkey. **D.** Values of optical densities measured in basal ganglia for GABA-B R1 immunostaining. Abbreviations: Str, Striatum; GP, globus pallidus; GPe, external globus pallidus; GPi, internal globus pallidus. Scale bars: A4, 0.5 mm (applies to all A panels); C, 1 mm (applies to B); C', 0.05 mm (applies to B', B'', C').

Fig. 2. Ultrastructural localization of GABA-B R1 immunoreactivity in GPe and GPi of normal and MPTP-treated monkeys

A–E. Examples of GABA-BR1-labeled elements. Arrows point to labeled unmyelinated axons, arrowheads indicate aggregates of labeling in axon terminals making symmetric contacts, while double arrowheads indicate labeling in a terminal forming an asymmetric contact. **F.** Percentages of immunoreactive elements, where 100% is the total number of GABA-B R1 labeled elements. Columns represent means + SD. Abbreviations: d, dendrites; t, terminals. Scale bars: 1 μ m in A, B, C, D; 0.5 μ m in E.

Fig. 3. Effects of GABA-B receptor compounds on GPe and GPi firing in MPTP-treated monkeys

A. Example of decreased firing in a GPe neuron of a parkinsonian monkey, after local administration of the GABA-B receptor agonist baclofen. During the injection of baclofen, this neuron showed brief increases in firing that were not long enough to be considered as drug-induced effects. **B.** Example of decreased firing in a GPi neuron of a parkinsonian monkey after local administration of the GABA-B receptor antagonist CGP55845A. The horizontal thick line indicates the period of drug injection. The solid thin line indicates the median firing rate at baseline, and the dashed lines show the 90th and 10th percentiles. The right side of the figure shows original spike traces obtained at the time points marked in A and B. The isolation of the unit was preserved during and after the injections. In B a second smaller unit is also apparent in the second time point recording trace. The arrow indicates a rebound burst.

Fig. 4. Types and magnitudes of changes in firing rate after activation or blockade of GABA-B receptors in GPe and GPi

A. Percentages of GPe (left) and GPi (right) neurons that decreased, increased or did not show any significant change in firing rate in response to baclofen or CGP55845A in normal and parkinsonian monkeys. *, different from normal animals ($p < 0.05$, Chi-square test). **B.** Changes in firing rate expressed as a percent change from baseline. Circles indicate individual neurons. For each Normal and MPTP group, cases with positive changes (light gray columns) and those with negative changes (dark gray columns) were compared separately. Each column represents means + SD. *, different from normal group, $p < 0.05$ Mann-Whitney test.

Fig. 5. The proportion of rebound bursts increases after modulation of GABA-B receptors in pallidal neurons of MPTP-treated monkeys

A, B. Proportion of spikes in rebound bursts during the baseline recording and during the effect period of baclofen or CGP55845A in GPe (A) and GPi (B). Each column represents means + SD *, value obtained from effect period different from baseline, $p < 0.05$ Wilcoxon test.

Table 1

Firing characteristics of GPe and GPi neurons at baseline.

	GPe		GPi	
	Normal	MPTP	Normal	MPTP
Number of neurons	24	33	16	25
Firing rate (spikes/s)	65.1 ± 34.1	53.8 ± 25.8	67 ± 32	63.8 ± 33
Burst index (spikes, %)	26 ± 22	29 ± 17	17 ± 13	29 ± 15*
Burst index (time, %)	8 ± 6	9 ± 5	6 ± 4	9 ± 4*
Rebound bursts (%)	6 ± 13	4 ± 9	2 ± 4	7 ± 2
Deceleration index (spikes, %)	3 ± 2	4 ± 2	2.4 ± 2	4 ± 2*
Deceleration index (time, %)	14 ± 14	14 ± 9	7 ± 7	13 ± 9*
Integrated power spectrum (%)	1–3 Hz	5 ± 4	3 ± 2	2 ± 3
	3–8 Hz	6 ± 4	6 ± 4	6 ± 5
	8–13 Hz	3.3 ± 2	4 ± 2*	3.6 ± 1
	13–30 Hz	9.8 ± 4	12 ± 3*	11 ± 4
	30–100 Hz	74 ± 10	72 ± 11	76 ± 8

Data are means ± SD.

* p < 0.05, Mann-Whitney test of differences between parkinsonian and normal monkeys