GABAergic Modulation of the Activity of Globus Pallidus Neurons in Primates: In Vivo Analysis of the Functions of GABA Receptors and GABA Transporters


The external and internal segments of the globus pallidus (GPe and GPI, respectively) are components of the basal ganglia circuitry. GPe is part of the indirect pathway of the basal ganglia, whereas GPI is one of the output nuclei that project to the motor thalamus and brain stem (e.g., Wichmann and DeLong 2003). The inhibitory neurotransmitter GABA is ubiquitous in both segments of the globus pallidus (GP), being released primarily from striatal afferents and from local GABAergic axon collaterals (Smith et al. 1998).

GABAergic transmission is mediated by ionotropic GABA_A and metabotropic GABA_B receptors. Both types of receptors are expressed in pallidal neurons in nonhuman primates and humans (Billinton et al. 2000; Bowery et al. 1999; Charara et al. 2000, 2004, 2005; Kultas-Ilinsky et al. 1998; Waldvogel et al. 1998, 1999, 2004). Electron microscopic studies have shown that GABA_A receptors are clustered at GABAergic synapses, but are also abundant at nonsynaptic locations. GABA_B receptors are predominately found at extrasynaptic sites (Charara et al. 2005).

Functional studies in rats have shown that pallidal GABA_A receptors induce fast inhibitory postsynaptic potentials (Kita 2001; Kita and Kitai 1991; Nakamishi et al. 1985). In monkeys, local administration of GABA_A antagonists increases neuronal firing, suggesting that pallidal cells are under a constant GABAergic tone (Kita et al. 2004; Matsumura et al. 1995). Information available concerning the function of pallidal GABA_B receptors is more limited, and comes exclusively from rodents. Patch-clamp recordings have shown that activation of GABA_B receptors reduces the frequency of miniature excitatory postsynaptic currents (Chen et al. 2002).

Given the prominence of GABA receptors at extrasynaptic sites, many of the effects of GABA receptor activation in the pallidum may be mediated by the spillover of synaptic GABA, as has been described for other brain areas (Isaacson et al. 1993; Scanziani 2000). It is likely that the concentration of GABA at these extrasynaptic sites is primarily determined by the actions of plasma-membrane bound GABA transporters (GATs), a family of proteins with at least four distinct members, namely GAT-1, GAT-2, GAT-3, and B-GAT (Dalby 2003). Of these, only GAT-1 and GAT-3 mRNA or protein expression have been described in the rodent and primate pallidum (Durkin et al. 1995; Ikegaki et al. 1994; Ng et al. 2000; Wang and Ong 1999; Yasumi et al. 1997).

These studies investigated the contribution of GABA receptors and GATs to GABAergic transmission in the primate pallidum. We recorded the electrophysiological activity of single pallidal neurons before, during, and after local microinfusions of GABA_A and GABA_B agonists and antagonists, as well as GAT blockers. In addition, we explored the subcellular distribution of GAT-1 and GAT-3 using electron microscopic techniques.

METHODS

Animals

Five rhesus monkeys (Macaca mulatta, 3–5 kg) were used for these studies. The animals were housed under conditions of protected

INTRODUCTION

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contact housing, with ad libitum access to food and water. All experimental protocols were performed in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals and the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals (amended 2002). All studies were approved by the Institutional Animal Care and Use Committee of Emory University.

**General outline of procedures**

Two monkeys were used to examine changes in the neuronal discharge in GPe and GPi before, during, and after microinjections of GABAergic compounds into the pallidum. In these studies, muscimol and gabazine (SR-95531 hydrobromide) were used to activate or block GABA_\text{A} receptors, respectively; for GABA_\text{B} receptors, we used the agonist R(+-)-baclofen hydrochloride (baclofen) and the antagonist CGP-55845. To block GABA transporters, the selective GAT-1 inhibitor, SKF-89976A hydrochloride, and the semi-selective GAT-3 inhibitor, (S)-SNAP-5114, were used for GAT-3 with 80-fold higher affinity than to GAT-1 (Borden 1996; Dalby 2003; Dhar et al. 1994). In a third monkey, the effects of the two GAT blockers on GABA levels in GPe were studied with microdialysis.

For electron microscopic studies, immunocytochemical localization of GAT-1 and GAT-3 was performed in brain sections from two different drug-naïve monkeys.

**Surgical procedure and initial electrophysiological mapping**

The animals were first trained to sit in a primate chair, to adapt to the laboratory environment, and to permit handling by the experimenter. Under aseptic conditions and isoflurane anesthesia (1–3%), we implanted metal chambers for chronic recording (16 mm ID) over trephine holes in the skull. The chambers were affixed to the skull with dental acrylic. Two chambers, each stereotactically directed at the pallidum on either side of the brain, were placed at an angle of 50° from the vertical in the coronal plane. Along with the recording chambers, metal head holders were embedded into the acrylic cap to permit head stabilization during recording and microinfusion procedures. After surgery, the animals were allowed to recover for ≥1 wk.

During experimental sessions, the animals were awake and seated in a primate chair with their heads restrained, but free to move their body and limbs. Initial electrophysiological mapping served to outline the borders of the GPe and GPi. In all electrophysiological experiments, neuronal activity was recorded extracellularly with standard tungsten microelectrodes (Z = 0.5–1.0 MΩ at 1 kHz; FHC, Bowdoinham, ME). The electrical signals were amplified (DAM-80 amplifier; World Precision Instruments, Sarasota, FL), filtered (400–10,000 Hz; Krohn-Hite, Brockton, MA), displayed on a digital oscilloscope (DL1540; Yokogawa, Tokyo, Japan), and made audible with an audio amplifier. During the electrophysiological mapping sessions and the subsequent recording-injection sessions, neurons in the pallidum were identified by their characteristic high-frequency discharge, interspersed with pauses in the case of GPe cells (DeLong 1973; DeLong et al. 1985).

**Intracerebral injections**

The system used to perform intracerebral injections while recording extracellular activity has been described in detail elsewhere (Kliem and Wichmann 2004). The recording-injection device consisted of a combination of fused silica tubing (40 μm ID; 103 μm OD; Polyimide Technologies, Phoenix, AZ) and a tungsten microelectrode (Z = 0.5–1.0 MΩ; FHC), both placed inside a protective sleeve of polycarbonate tubing (0.5 mm OD; MicroLumen, Tampa, FL). The tip of the silica tubing was adjusted to project 1 mm from the polyimide sleeve, and the electrode tip extended 50–100 μm further. The silica tubing was connected to a liquid switch (CMA/110, CMA Microdialysis, Solna, Sweden) to permit exchange of solutions while maintaining a continuous flow of liquid. The ports of the liquid switch were connected to gas tight syringes (CMA), which were driven with a microinfusion pump (CMA/102, CMA).

The recording-injection system was lowered into the brain with a microdrive (MO-95B, Narishige, Tokyo, Japan), after perforating the dura with a 20-gauge guide tube. While being lowered in the brain, the injection tube was filled with artificial cerebrospinal fluid (ACSF) comprised of (in mM) 143 NaCl, 2.8 KCl, 1.2 CaCl_2, 1.2 MgCl_2, and 1 Na_HPO_4, pH 7.2–7.4. When the target structure (GPe or GPi) was reached (based on the previous electrophysiological mapping and the discharge characteristics of the neurons recorded during the injection session), the activity of single neurons was recorded before, during, and after injection of drugs. The neuronal signal was collected to computer disk using a data acquisition interface (Power1401,CED, Cambridge, UK) and commercial software (Spike2, CED) for later offline analysis.

Once a neuron was isolated with sufficient quality, and the recording microelectrode was stable for ≥60 s, the infusion pump was started to run at a rate of 0.3 μl/min. ACSF in the injection tubing was first flushed out of the system (≈0.5 μl). Because of the system’s dead space, this lasted about 100 s. Therefore the 60-s epoch immediately preceding the arrival of the drug at the tip of the injection system was considered to reflect the cell’s basal activity. Then, 1 μl of the drug solution under study was injected. The drug infusion was followed by injection of 1 μl of ACSF, to wash out the drug from the injection area. Pilot experiments showed that injecting a total volume of ≥2.5 μl of ACSF at 0.3 μl/min does not affect neuronal activity. Each neuron was recorded for ≥10 min after the end of the drug injection. Occasionally, more than one injection was done along the same tract; these injections were separated by ≥1 mm in depth. In some experiments, the combined effects of an agonist and antagonist were tested. In these cases, we first infused 1 μl of the antagonist, followed by 1 μl of a solution containing both the agonist and the antagonist. The basal activity was recorded ≥60 s before starting the first injection.

The drugs and doses injected were as follows: muscimol, 114 ng (Tocris Cookson, Ellisville, MO); baclofen, 213 ng (Sigma-Aldrich, St. Louis, MO); gabazine, 368 ng (Tocris Cookson); CGP-55845, 402 ng (Tocris Cookson); SKF-89976A, 720 ng (Tocris Cookson); SNAP-5114, 500 ng (Tocris Cookson). The drug doses were selected to be in the range of those used in previous studies using local administration of these compounds to the pallidum (Chen et al. 2002; Inase et al. 1996; Kita et al. 2004; Wenger et al. 1999). All drugs were dissolved in ACSF, and the pH was adjusted to 7.2–7.4. Before being loaded into the injection systems, all solutions were filtered with a 0.2 μm pore size nylon membrane (Fisher Scientific, Hampton, NH). For control injections, ACSF was injected.

**Analysis of injection data**

In the analysis, we only included cells that were confirmed to be in the target structure (GPe or GPi), based on the depth data gathered during recording and the histological analysis of the cresyl violet-stained sections (see Perfusion and tissue processing). Spike detection was accomplished using the Spike 2 program through a waveform-matching process, with subsequent principal component analysis. All the following steps of the analysis were done in Matlab (Mathworks, Natick, MA).

Interspike intervals (ISIs) were used to calculate second-by-second discharge rates, which were subsequently smoothed using a sliding 21-point moving average. The discharge rate in the 60-s segment of data before delivery of drugs was defined as the neuron’s basal activity. An injection was considered effective if the frequency of the recorded neuron differed from this baseline by >2 SD, 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 pilot experiments with our injection system. According to this (arbitrary) definition of
drug effect, the duration of the effects ranged from 140 to 600 s, based on experiments in which recovery of the basal firing rate was seen and which allowed a determination of the length of the drug’s action.

For individual experiments, the discharge rate during the maximal effect period after drug injection was compared with the basal firing rate (with the nonparametric Wilcoxon signed-rank test for paired data). We also expressed the discharge rate during the maximal effect period as a percentage of the basal firing, and compared that value to control experiments (with the nonparametric Mann-Whitney U test).

The relationship between the postinjection firing rate or burst index (as explained in the next paragraph) and the basal firing rate or the localization of neurons in the anterior-posterior, medio-lateral and dorso-ventral directions were examined using the Spearman rank test.

We also calculated a burst index for each drug injection. Bursts were detected with the “surprise” method (Legendy and Salcman 1985), using a Poisson surprise value of 10, and a minimal burst length of three spikes. We calculated a burst index as the proportion of spikes occurring within bursts compared with the total number of spikes. The Wilcoxon test was used to compare burst indices during the 60-s control epoch preceding the drug injection and the maximal effect period during drug injections. Strictly speaking, this was, of course, only possible in cells in which a drug effect was observed. In cases in which no effect occurred, the data segment between 200 and 260 s after the beginning of the drug injection was used instead of the maximal effect period. In addition to these paired observations, we used the Mann-Whitney test to compare changes in burst indices after each drug treatment against changes in burst indices after ACSF injections.

Microdialysis procedure and analysis of GABA in the dialysates

Microdialysis experiments were performed to measure GABA levels in GPe before and during blockade of the GABA transporters Gat-1 or Gat-3. We used custom-modified microdialysis probes (CMA, 2-mm cuprophone membrane, cut-off 6,000 Da). First, a 22-gauge guide cannula with a fitting stylet was lowered into the brain with the microdrive. The tip of the guide cannula was positioned 1 mm above the target in the GPe. The stylet was then removed and the microdialysis probe inserted. The probes extended 3 mm from the end of the guide cannula. To avoid excessive tissue damage from repeated probe insertions, the total number of microdialysis penetrations was kept to a minimum and tracts were separated by 60–240 s after the beginning of the drug injection was used instead of the maximal effect period. In addition to these paired observations, we used the Mann-Whitney test to compare changes in burst indices after each drug treatment against changes in burst indices after ACSF injections.

Analysis of microdialysis data

GABA concentrations in the dialysates were normalized to the mean of the first three samples of the respective experiment. Each drug or control experiment was run in triplicate, and the normalized values obtained at each sampling point were averaged across the three experiments. Each sample of the drug experiments was compared against the respective sample of the control experiments, with a nonparametric Mann-Whitney U test.

Perfusion and tissue processing

At the conclusion of the electrophysiology and microdialysis experiments, the animals were deeply anesthetized with an overdose of pentobarbital sodium (100 mg/kg, iv) and perfused transcardially with cold oxygenated Ringer solution, followed by 4% paraformaldehyde in phosphate buffer (PB; 0.1 M, pH 7.4). The brains were removed from the skull and cut into 10-mm-thick blocks in the frontal plane. Tissue sections (60 μm) were obtained with a vibratome and collected in cold phosphate-buffered saline (PBS; 0.01 M, pH 7.4). Sections of the pallidal area were stained with cresyl violet for light microscopic verification of the location of electrodes and microdialysis probes.

GAT immunocytochemistry

The brain tissue was prepared for electron microscopy, following a methodology detailed previously (Charara et al. 2004). Briefly, the monkeys were perfused as described above, using 4% paraformaldehyde and 0.1% glutaraldehyde as fixative, and the brains were cut in 60-μm sections. The tissue sections were treated with 1% sodium borohydride and placed in a cryoprotectant solution, frozen at −80°C, thawed, and washed in PBS. The immunocytochemical localization of the GAT-1 and GAT-3 transporters was performed using the avidin-biotin complex (ABC) method (Hsu et al. 1981). After blocking nonspecific binding with 10% normal goat serum (NGS) and 1% bovine serum albumin (BSA) in PBS, the sections were incubated for 2 days at 4°C in the primary antibody solution (anti-GAT-1 1:500 or anti-GAT-3 1:1000; both from Chemicon, Temecula, CA). The specificity of these antibodies has been established (Ikegaki et al. 1994), and they have been used extensively (Calcagnotto et al. 2002; Conti et al. 1998; Minelli et al. 1995, 1996; Ribak et al. 1996a,b; Shi et al. 1997). The sections were incubated in biotinylated goat anti-rabbit IgG (1:200; Vector Labs, Burlingame, CA) and incubated in the ABC solution (1:100; Vectastain Standard kit, Vector Labs). All immunoreagents were diluted in PBS containing 1% NGS and 1% BSA. Sections were rinsed in PBS and Tris buffer (0.05 M, pH 7.6) before being placed in a solution containing 0.025% 3–3’-diaminobenzidine tetrahydrochloride (Sigma-Aldrich), 0.01 M imidazole (Fisher Scientific), and 0.006% H2O2. The reaction was terminated by repeated washes in PBS.

The sections were washed in PB (0.1 M, pH 7.4) and postfixed in osmium tetroxide (1% in PB). This was followed by washings in PB and dehydration in a graded series of ethanol and propylene oxide. Uranyl acetate (1%) was added to the 70% ethanol to improve contrast in the electron microscopic analysis. The sections were embedded in resin (Durcupan ACM; Fluka, Ft. Washington, PA) on microscope slides and placed in the oven for 48 h at 60°C. Blocks from the GPe were cut out from the slides and glued on the top of resin blocks. Serial ultrathin sections were then cut on a ultramicrotome (Leica Ultracut T2), collected onto Pioloform-coated single copper grids, stained with lead citrate (Reynolds 1963), and analyzed with an electron microscope (Zeiss EM 10C).

Analysis of ultrastructural data

The ultrastructural analysis was carried out on ultrathin transverse sections collected from the surface of each block of pallidal tissue.
The sections were scanned at ×20,000–25,000, and randomly selected fields containing immunoreactive elements were photographed. The labeled elements were categorized into various groups based on ultrastructural features (Peters et al. 1991), and their relative proportion was calculated and expressed as a percent of total labeled elements for each transporter in the areas examined. Our group has previously used similar quantitative methods for various receptor subtypes (Charara et al. 2004; Hubert and Smith 2004).

RESULTS

Electrophysiologic studies

These experiments examined the effects of local infusions of GABAergic compounds on neuronal activity recorded in the immediate vicinity of the pallidal injection site. No systematic differences were observed between GPe and GPi cells, and therefore results from both pallidal segments were pooled. The lack of difference between the two structures is in agreement with the fact that the ultrastructural localization of GABA_A and GABA_B receptors is similar in both segments of the GP (Charara et al. 2005). Control injections of ACSF in GPe or GPi (n = 8) had no effect on the activity of recorded neurons (Fig. 4).

Activation and blockade of GABA_A receptors in the GP

In 8/10 cells tested, the GABA_A receptor agonist muscimol (114 ng/μl) strongly reduced the firing rate of neurons close to the injection site in either segment of the GP. The firing rate after muscimol was significantly different from the basal discharge (Fig. 1A; P = 0.0195, Wilcoxon test). On average, the firing rate decreased by 43% compared with the baseline period, and the change in firing rate was significantly different from ACSF injections (Fig. 4; P < 0.0001, Mann-Whitney test). Changes in bursting were not analyzed because the number of spikes occurring during the maximal effect period after muscimol injections was too low. The GABA_A receptor antagonist gabazine (368 ng/μl), given concurrently with muscimol (n = 4), reversed these changes (P = 0.63 compared with basal firing, Wilcoxon test; P = 0.80 compared with ACSF injections, Mann-Whitney test; Fig. 4).

To examine whether GABA_A receptors in GP are tonically activated by their endogenous ligand, the GABA_A receptor antagonist gabazine was administered alone. Gabazine (368 ng/μl) had no consistent effects on pallidal activity. As shown in Fig. 1B2, seven cells increased their firing rate, five decreased, and five showed no significant change. An example of a cell in which gabazine had no effect is presented in Fig. 1B1.

On average, gabazine increased pallidal firing by 6.65%. This change was not significantly different from the basal discharge (P = 0.99, Wilcoxon test) or from the firing rate after ACSF injections (Fig. 4; P = 0.66, Mann-Whitney test).

We also explored the possibility that the variability of responses after gabazine could be explained by the basal firing frequency or the localization of the tested neurons. We found that the changes in firing after gabazine were not dependent on the basal frequency (P = 0.11, Spearman test), the basal burst index (P = 0.52, Spearman test), or the location of neurons in dorso-ventral, medio-lateral, or anterior-posterior stereotactic coordinates (P = 0.96, 0.82, and 0.91, respectively, Spearman test).

Although gabazine induced an increase of the burst index in some cells (5/17), this was not significantly different from the basal burst index in a summary analysis of these responses in individual cells (P = 0.48, Wilcoxon test). The changes in burst index in drug injection experiments were also not different from burst index measurement obtained during control ACSF infusion studies (P = 0.83, Mann-Whitney test). However, there was a significant correlation between the basal burst

**FIG. 1.** Effects of muscimol and gabazine on pallidal discharge rate. A1: example of effect of muscimol on the discharge rate of an external segment of the globus pallidus (GPe) neuron. A2: discharge rate of neurons in GPe and the internal segment of the globus pallidus (GPi) during the baseline period and at the point of maximal effect of muscimol injections. B1: example of a gabazine injection that did not alter the discharge rate of a GPi neuron. B2: discharge rate of cells in GPe and GPi during the baseline period and at the point of maximal effect of gabazine injection. In A1 and B1, duration of muscimol or gabazine infusions is indicated by a horizontal bar. Dashed lines represent mean discharge rate ± 2 SD.
index and the burst index after gabazine ($P < 0.0001$, Spearman test), indicating that neurons with a higher tendency to discharge in bursts at baseline tended to react to gabazine application with an increase in burst firing.

**Activation or blockade of GABA$_B$ receptors in the GP**

The GABA$_B$ receptor agonist baclofen (213 ng/µl) decreased the discharge rate of eight of nine pallidal neurons (Fig. 2A). The change was significantly different from the basal firing ($P = 0.008$, Wilcoxon test). The firing rate decreased on average by 63%, a change that was significantly different from the control ACSF injections ($P = 0.001$, Mann-Whitney test; Fig. 4). The number of spikes during the maximal effect period after the administration of baclofen was too low to analyze changes in the index of bursting. The effect of baclofen on the after the administration of baclofen was too low to analyze changes in the index of bursting. The effect of baclofen on the discharge rate induced by CGP-55845 and the basal firing, Wilcoxon test; $P = 0.12$ compared against ACSF injections, Mann-Whitney test; Fig. 4).

Infusions of the GABA$_B$ antagonist CGP-55845 (402 ng/µl) evoked, in many cases (11 of 15), increases in the firing rate, which were significantly different from baseline ($P = 0.012$, Wilcoxon test; Fig. 2B). The average increase (31.58%) was marginally significantly different from ACSF injections ($P = 0.057$, Spearman test). There was no correlation between the changes in discharge rate induced by CGP-55845 and the basal firing frequency ($P = 0.15$, Spearman test), the basal burst index ($P = 0.08$, Spearman test), or the location of neurons ($P = 0.73$, 0.16, and 0.25 for dorso-ventral, anterior-posterior, and medio-lateral coordinates, respectively, Spearman test).

Interestingly, application of CGP-55845 induced an increase in the tendency of many pallidal cells (9/15) to fire in bursts. However, in a paired statistical analysis, these changes were not significantly different from the basal burst index ($P = 0.97$, Wilcoxon test) and only marginally significantly different from the pooled results from ACSF injections ($P = 0.059$, Mann-Whitney test). As was the case for the gabazine experiments, the basal burst index correlated significantly with the burst index after CGP-55845 ($P = 0.01$, Spearman test). The change in bursting was not correlated with the basal firing frequency ($P = 0.94$, Spearman test) or the location of the neurons ($P = 0.73$, 0.69, and 0.84 for dorso-ventral, anterior-posterior, and medio-lateral coordinates, respectively, Spearman test).

**Effects of blocking GABA uptake on the firing of GP cells**

Our observations indicated that GP firing was inhibited as a result of either GABA$_A$ or GABA$_B$ activation. A large proportion of these receptors is located extrasynaptically (Charara et al. 2005; Chen et al. 2004), and it is unclear under which circumstances they are activated. To test whether endogenous GABA could induce an inhibition similar to the one observed after the pharmacological manipulations, we administered blockers of the GABA uptake carriers GAT-1 and GAT-3.

Blockade of GAT-1 with SKF-89976A (720 ng/µl) decreased the firing of a majority of neurons (6/9; Fig. 3A), but this effect did not reach statistical significance ($P = 0.07$, compared with basal firing, Wilcoxon test, and $P = 0.16$ against ACSF injections, Mann-Whitney test; Fig. 4). The average reduction was $\sim 40\%$.

Injections of the GAT-3 blocker SNAP-5114 (500 ng/µl) inhibited the firing in seven of eight neurons (Fig. 3B). Compared with basal firing rate, this change had a marginal significant difference ($P = 0.054$, Wilcoxon test) and was significantly different from control injections ($P = 0.01$, Mann-Whitney test; Fig. 4).

![Fig. 2](http://jn.physiology.org/) Effects of baclofen and CGP-55845 on pallidal discharge rate. A1: example of the response of a GPe cell to baclofen exposure. A2: discharge rate of GPe and GPi cells during baseline period and at the point of baclofen’s maximal effect. B1: CGP-55845 induced a slight increase in firing rate of this GPe cell. B2: discharge rates of GPe and GPi cells during baseline period and at the point of maximal effect of CGP-55845. In A1 and B1, duration of drug infusions is indicated by horizontal bars. Dashed lines represent mean discharge rate $\pm 2$ SD.
Microdialysis studies

To test whether the inhibitory effects observed after blocking the GABA transporters could be due to increased GABA levels, microdialysis studies were performed to measure extracellular GABA in the GPe. In agreement with previous studies (Robertson et al. 1991; Tossman et al. 1986), the average basal level of GABA was 0.47 \( \pm \) 0.21 \( \mu \)M. Administration of the GAT-1 blocker, SKF-89976A (100 \( \mu \)M), caused a three-fold increase in GABA baseline levels (\( P < 0.03 \) compared with ACSF infusion, Mann-Whitney test; Fig. 5). This increase outlasted the administration of SKF-89976A for >30 min. The GAT-3 blocker SNAP-5114 (100 \( \mu \)M) caused a comparable increase in the levels of GABA (\( P = 0.04 \), Mann-Whitney test), but in this case, the extracellular GABA levels returned to baseline 10 min after infusion of ACSF (Fig. 5).

Subcellular distribution of GAT-1 and GAT-3

To further understand the mechanisms underlying the regulation of GABA levels by GAT-1 and GAT-3 in the monkey pallidum, we examined the ultrastructural localization of these transporters.

At the light microscopy level, strong labeling for GAT-3 and moderate labeling for GAT-1 was apparent in both pallidal segments (data not shown). The staining was associated with punctate elements in the neuropil, sometimes surrounding cell bodies, especially in the case of GAT-3.

Observations at the ultrastructural level were carried out in GPe. A total of 161 GAT-1–labeled elements and 184 GAT-3–immunoreactive structures were analyzed. These elements were found in a total surface of 640.7 and 776.4 \( \mu \)m\(^2\) of GAT-1 and GAT-3 immunostained tissue, respectively.

The majority of GAT-1–labeled elements (57.6%) were preterminal axonal segments (Fig. 6, A and C), whereas glial processes accounted for \( \sim \)30% of immunoreactive profiles (Fig. 6, B and C). These glial processes were, in most cases, dispersed in the neuropil (Fig. 6B) and occasionally apposed to terminals, forming symmetric synapses (Fig. 6C). On the other hand, GAT-3 labeling was found almost exclusively (90%) in glial processes. In many cases, the glial extensions surrounded “rosettes” of terminals around single dendrites (Fig. 6, D and E).
FIG. 5. SKF-89976A and SNAP-5114 (100 μM each) increase GABA levels in the monkey GPe. Dialysate samples were collected every 10 min. SKF-89976A or SNAP-5114 were administered during sample 4 (horizontal bar). Data area means ± SD from 3 experiments for each treatment in a single monkey. Difference from ACSF experiments (Mann-Whitney U test): *P < 0.05.

D I S C U S S I O N

We have presented evidence that activation of either GABA_\text{A} or GABA_\text{B} receptors induces strong inhibitory effects in GPe and GPi. On the other hand, application of GABA receptor antagonists resulted in both increased and decreased activity of pallidal neurons, suggesting that the basal GABAergic control of pallidal neurons might be more heterogeneous than previously thought. The electrophysiological and biochemical effects of the GAT blockers suggest that GATs significantly limit the ambient level of GABA in the extracellular space. Together with the electron microscopic finding that GAT-1 and GAT-3 are predominantly localized in glia, this observation favors the view that a substantial quantity of GABA escapes the synaptic cleft and may contribute to GABAergic transmission at extrasynaptic receptor sites.

Technical considerations

The main limitation of the combined recording-injection system used in this study is that the extent of diffusion of injected drugs and the drug concentration at the receptor cannot be assessed with certainty. Given the possibility of drug gradients within the tissue under study, somatic and dendritic binding sites may, in fact, be exposed to substantially different concentrations of drugs. This is perhaps a particular problem in the pallidum where individual neurons have large dendritic trees, extending ≈1 mm away from the soma (Sato et al. 2000; Yelnik et al. 1984, 1997). Some of our results differ from previous studies, perhaps due to differences in the geometric arrangements of the recording-injection assemblies. For instance, Kita et al. (2004) recently reported that injection of the GABA receptor antagonist gabazine disinhibits pallidal neurons more strongly than in our study. The recording-injection system employed by this group featured a 600- to 700-μm separation between the end of the injection tube and the tip of the electrode; this configuration may have increased the possibility that the injected drugs reached distal dendrites. In contrast, in our injection systems, the distance between the injection site and the tip of the electrode was only 50–100 μm.

A related technical limitation is that the injected compounds may have diffused to receptors on neurons in the vicinity of the one being recorded, subsequently influencing release from axon collaterals reaching the neuron under study. In general, our results argue against this possibility. For instance, if the GABA_\text{A} and GABA_\text{B} agonists had predominately inhibited neighboring neurons, one would expect a reduction of collateral inhibition and thus increased firing of the recorded neuron. Because most cells in our experiments were strongly inhibited by the GABA receptor agonists, we concluded that the effects observed were mainly due to direct activation of GABA receptors on the recorded neuron.

Similar to other studies (Kita et al. 2004; Matsumura et al. 1995), we found that the onset of drug effects and their duration was variable among injections. It is conceivable that differences in the microenvironment at the injection site may have affected the diffusion of the injected drugs (e.g., presence of myelinated axons or glial processes). In addition, subtle differences in receptor distribution or availability may also impact the duration, magnitude, and latency of drug effects. Because most of these factors cannot be controlled in the in vivo conditions, we did not attempt to analyze the duration of drug effects. In an effort to minimize the variability between experiments, the distance between the electrode tip and the end of the injection tube, the injection volume, and the injection rate were all kept constant.

GABA_\text{A} and GABA_\text{B} receptors modulate the activity of GPe and GPi cells

GABA_\text{A} receptor-mediated inhibition of pallidal activity has been described in previous studies in rodents and primates (Baron et al. 2002; Kita 1992, 2001; Kita and Kitai 1991; Nakanishi et al. 1985). Therefore the observed decrease on GP firing after muscimol injections was expected. These responses may be mediated by synaptic or extrasynaptic receptors, because both types of receptors have been described in the monkey GP (Charara et al. 2005). There is increasing evidence that extrasynaptic GABA_\text{A} receptors can be pharmacologically distinguished from synaptic receptors because of their unique subunit composition (for review, see Semyanov et al. 2004). In the hippocampus and cerebellum, extrasynaptic GABA_\text{A} receptors contain the benzodiazepine-insensitive δ subunit and the α4, α5, or α6 subunits (Semyanov et al. 2004). Interestingly, the mRNA for δ and α4 subunits is present at moderate to high levels in the monkey GPe and GPI (Kultas-Ilinsky et al. 1998), but it is not known whether the subunit composition could also be exploited in the pallidum to distinguish extrasynaptic from synaptic receptors.

Our findings also show strong GABA_\text{B} mediated inhibition of pallidal neurons. Based on ultrastructural studies in the monkey pallidum, the baclofen effects could be mediated by...
pre- or postsynaptic GABA_B receptors. While most of GABA_B receptors are located extrasynaptically, along the plasma membrane of pallidal dendrites, a small proportion of GABA_B receptors are presynaptic, found in putative glutamatergic boutons (Charara et al. 2000, 2005). Previous in vitro electrophysiological data from rat brain slices have indicated that baclofen has significant presynaptic effects, whereas it induces only weak postsynaptic responses in pallidal neurons (Chen et al. 2002), despite the fact that in the rat GP most of GABA_B receptors are localized postsynaptically (Chen et al. 2004). In our study, the in vivo condition used does not allow us to distinguish between pre- and postsynaptic effects. Nevertheless, the electrophysiological and immunocytochemical data presented in this and previous studies (Charara et al. 2005) indicate that GABA_B receptors are in position to modulate the activity of pallidal cells through complex mechanisms.
Activation of pallidal GABA<sub>A</sub> and GABA<sub>B</sub> receptors by endogenous transmitter

We investigated the presence of significant endogenous GABAergic inhibition of GPe and GPi cells by local injections of GABA<sub>A</sub> or GABA<sub>B</sub> receptor antagonists.

In some cells, gabazine caused a substantial increase in firing, whereas it failed to do so in others. When all recorded neurons were pooled, results for gabazine and control experiments did not differ significantly. In contrast, Kita et al. (2004) recently reported that injections of gabazine in the monkey GPe uniformly increased the firing of all of the neurons tested. The discrepancy between their study and ours is most likely due to differences in the design of injection devices used in the two studies. It is also important to consider that the observations made by Kita et al. (2004) may apply to a specific subset of pallidal cells, because the sampling of neurons in their study was restricted to those that responded to electrical stimulation of the motor cortex.

Injections of the GABA<sub>B</sub> antagonist, CGP-55845, evoked an increase in the firing rate in a larger proportion of GP neurons than the GABA<sub>A</sub> antagonist. However, in the aggregate, these changes were not significantly different from controls. We assessed whether the antagonist responses were correlated with the basal firing rate, the firing pattern, or the location of neurons in the GP, but no such correlations were identified. Thus the variable responses may indicate that the GABAergic inhibition is not uniform among pallidal cells, and the sources of this heterogeneity remain to be determined.

Our findings suggest that the state of activation of GABA receptors may also affect the firing pattern of pallidal neurons. We observed increased bursting in a small number of GP neurons after gabazine and in the majority of cells tested with CGP-55845. Analysis of correlations suggested that the antagonist-evoked changes in burstiness may be related to the basal burst index of the cell.

The results obtained with CGP-55845 suggest that endogenous GABA, through activation of GABA<sub>B</sub> receptors, dampens pallidal bursting under physiologic conditions. The increased burstiness seen in our experiments is reminiscent of that observed in animal models of Parkinson’s disease (PD), where GP neurons exhibit a higher degree of burst firing in the dopamine-depleted than in the normal state (Bevan et al. 2002; Filion and Tremblay 1991; Ni et al. 2000; Soares et al. 2004). It is not clear, however, how these observations would compare with the parkinsonian condition, because current models of the disease predict opposite changes in GABA levels in the two pallidal segments. In addition to changes in overall GABA levels, changes in the level of expression (Calon et al. 2000, 2003; Johnston and Duty 2003) or subcellular distribution of pallidal GABA<sub>B</sub> receptors could be related to the appearance of burstiness.

GAT and GAT-3 transporters regulate the ambient pallidal GABA level

Our results show that GATs strongly modulate the amount of GABA in pallidal extracellular space. Blockade of either of these transporters substantially increased GABA levels in the microdialysis experiments, and in most cases, decreased the activity of neurons in both pallidal segments.

Functional implications

In both pallidal segments, it appears that a substantial amount of synthetically released GABA could reach its receptor targets through diffusion from the synapse to extrasynaptic sites, perhaps increasing the diversity and complexity of GABAergic responses (Cherubini and Conti 2001; Mody and Pearce 2004; Mody et al. 1994). For instance, under conditions
of relatively low GABA release, the activation of synaptic GABA$_A$ receptors could be responsible for most of the neuronal responses to GABA, whereas increased GABA release may result in a more substantial spillover, resulting in activation of extrasynaptic receptors, particularly of the GABA$_B$ type.

These mechanisms may also affect some of the neuronal responses seen in diseases of basal ganglia origin. In PD, for instance, expression of GABA$_A$ and GABA$_B$ receptors is altered in both pallidal segments. In general, both types of GABA receptors are down-regulated in the GPe and up-regulated in GPi (Calon et al. 2000, 2001; Chadha et al. 2000; Johnston and Duty 2003; Pan et al. 1985; Robertson et al. 1990), but it is not known if there are changes in the subcellular and subsynaptic distribution of these receptors or if the distribution of GABA transporters is also disrupted.

In summary, our results indicate that activation of either GABA receptor subtype inhibits significantly the activity of pallidal neurons. Blockade of GAT-1 or GAT-3 increases the ambient GABA levels, as measured by microdialysis, concomitant with a decrease of neuronal firing rate, highlighting the importance of GABA clearance to maintain spontaneous activity in GPe and GPi. Together with the electron microscopic finding that the majority of GABA transporter molecules are located at extrasynaptic sites, these results suggest that a substantial proportion of released GABA diffuses away from the synapse to activate extrasynaptic GABA$_A$ and GABA$_B$ receptors in the monkey pallidum.

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