Presynaptic modulation by somatostatin in the rat neostriatum is altered in a model of Parkinsonism

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Running head: SST modulation after dopamine denervation

Abstract: 250 w. Introduction = 442 w. Discussion = 1268 w.

VGL-H made and designed most experiments and help in writing the paper. EB-H helped in PCR experiments and discussion. JB helped in experimental design and writing. EG helped designing experiments and wrote the paper, a continuation of her published research.

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Abstract

Somatostatin (SST) is a peptide synthesized and released by a class of neostriatal local GABAergic interneurons which, to some extent, are in charge of the feedforward inhibitory circuit. Spiny projection neurons (SPNs) make synapses with each other via their local axon collaterals shaping the feedback inhibitory circuit. Both inhibitory circuits, feedforward and feedback, are related through SST which being released by interneurons, presynaptically inhibits connections among SPNs. Here we studied SST presynaptic modulation of synapses among SPNs in the 6-OHDA rodent model of Parkinsonism. We performed antidromic field stimulation from the external globus pallidus (GPe) and whole cell voltage clamp recordings of antidromically evoked inhibitory postsynaptic currents (IPSCs) among SPNs. SST presynaptically reduced IPSCs by about 34% in all control synapses tested. But after striatal dopamine (DA) deprivation three changes became evident: First, it was harder to evoke feedback inhibition. Second, presynaptic inhibition of some SPNs connections was larger than in controls: 57% reduction in about 53% of evoked IPSCs. Presynaptic inhibition was recorded from direct pathway neurons (dSPNs). Third, SST also induced presynaptic facilitation in some SPNs connections with 82% enhancement in about 43% of evoked IPSCs. Presynaptic facilitation was recorded from indirect pathway neurons (iSPNs). Both inhibition and facilitation were accompanied by corresponding changes in the paired pulse ratio. It is demonstrated that after DA deprivation SST modulation is altered in surviving feedback inhibitory synapses. It may underlie a homeostatic mechanism trying to compensate for the excitability imbalance between direct and indirect basal ganglia pathways found during Parkinsonism.
**Key words:** neostriatum, somatostatin, spiny projection neurons, feedback inhibition, presynaptic modulation, 6-hydroxy-dopamine, Parkinsonism.

**INTRODUCTION**

The striatal network plays a key role in action selection and learning by processing and integrating cortical and thalamic inputs (Barnes et al. 2005; Flores-Barrera et al. 2010; Wilson, 2004). The principal neurons supporting these functions are the spiny projection neurons (SPNs) which connect with each other via local axon collaterals shaping what has been called the feedback inhibition circuit (Bolam, 2010; Czubayko and Plenz 2002; Guzman et al., 2003; Kita, 1993; Koos et a. 2004; Surmeier et al. 2011; Taverna et al., 2008; Tecuapetla et al. 2007; 2009; Tunstall et al. 2002). Important striatal transmitters modulate feedback inhibition, as for example dopamine (DA) (Guzman et al. 2003; Taverna et al., 2008; Tecuapetla et al. 2007) and acetylcholine (ACh) (Perez-Rosello et al., 2005). Much less is known about peptidic modulators. They are co-released with GABA from local interneurons and SPNs. For instance, somatostatin (SST) has important actions on SPNs postsynaptic intrinsic properties (Galarraga et al. 2007; Vilchis et al. 2002) and on the presynaptic modulation of feedback inhibition (Lopez-Huerta et al. 2008). Interactions between DA and SST are known from behavioral correlates (Lu and Stoessl, 2002). And in the long term, SST mRNA synthesis is decreased in the striatum of Parkinsonian subjects (Asanuma et al. 1990; Augood et al. 1991; Espino et al. 1995; Soghomonian and Chesselet, 1991). Nevertheless, the fate of SST presynaptic modulation when striatal circuits are altered, for example, during Parkinsonism, is unknown.
Because during experimental Parkinsonism some local interneurons alter their output (Dehorter et al. 2009; Flores-Barrera et al. 2011; Sánchez et al. 2011), the main goals of the present work are to know whether SST actions are altered during DA deprivation and to figure out whether altered actions contribute or oppose the pathological unbalance between the direct and indirect pathways of the basal ganglia (BG) during Parkinsonism (Albin et al., 1989; Day et al. 2006; DeLong and Wichmann, 2007; Javoy et al. 1974). An additional goal would be to begin exploring what are the receptors involved.

In contrast to increased activity found in some interneurons (Dehorter et al. 2009), DA deprivation causes decreased inhibition among SPNs, more significantly, on direct pathway neurons (Taverna et al. 2008). However, there is insufficient evidence linking both phenomena, and probably, SST actions is one such link.

Therefore, the aim of the present study was to investigate striatal SST modulation of synaptic connections among SPNs in the rodent 6-hydroxy-dopamine (6-OHDA) model of Parkinsonism. We found that SST modulation of feedback inhibition is greatly altered in 6-OHDA treated rats as compared to the controls (López-Huerta et al., 2008). We also found evidence of some SST receptors that participate in part of this transmission.

Materials and Methods

Animals

Male Wistar rats were housed in clear plastic cages and maintained on a stable 12:12 hours dark/light cycle at room temperature (22 °C), with food and water ad libitum in our Animal House. The number of animals used in the
experimental samples was the minimal possible to attain statistical significance. All the procedures followed the National University of Mexico guidelines and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

6-OHDA lesions

Animals postnatal day (PD) 15 were deeply anesthetized with a mixture of ketamine (85 mg/kg, i.p.) and xylazine (15 mg/kg, i.p) and immobilized on a stereotactic frame (Fig. 1). Each animal received a unilateral injection of 6-OHDA (8 µg in 0.2 µl with 0.2 mg/ml of ascorbic acid) 0.1 µl/min in the substantia nigra pars compacta (SNc: coordinates 3.8 mm caudal, 1.8 mm lateral to bregma, and 7.1 mm ventral to the skull surface). Syringe was left in place for additional 10 minutes to let diffusion and maximize tissue retention.

Turning behavior

The success of 6-OHDA lesions was tested evoking turning behavior 7 days after the surgery at PD22 (Fig. 1A). The protocol used was identical to that described before (Flores-Barrera et al. 2010; Hudson et al. 1993; Schwarting and Huston, 1996). Rats were first placed in automated rotometer bowls during 10 minutes to acclimatize them. Then, amphetamine (4mg/kg, i.p.) was administered. Left and right full body turns were recorded for 90 minutes by a home-made computerized activity monitor system. Animals showing >500 turns ipsilateral toward the lesioned side were considered for further experiments. After behavioral test of Parkinsonism, additional 3-8 days passed before in vitro slice
electrophysiological experiments were performed and thus avoid indirect effects caused by amphetamine (see time line in Fig 1A).

Probably, it is worth mentioning that while neonate-lesioned rats (e.g., PD0-5) are proposed to serve as a model of the destruction of dopaminergic neurons to mimic Lesch–Nyhan disease as well as other disorders such as hyperactivity and schizophrenia, it has been shown that young animals at later ages (>PD10) have already approached signs of striatal circuit maturity (Dehorter et al. 2011), and therefore, can reproduce the signs of Parkinsonism associated with the loss of dopamine (rev. in: Breese et al, 2005; Reader and Dewar, 1999). Nevertheless, a standard locomotor test for rodent Parkinsonism was performed at PD22 (turning behavior; Ungerstedt, 1968) and electrophysiological experiments at PD>25 (decrease in feedback inhibition; Taverna et al. 2008), corroborating Parkinsonian signs in our samples of dopamine deprived animals and comparing with the controls at a time when dopamine innervation has almost reached full development (Antonopoulos et al. 2002).

Brain Slices

Animals were deeply anaesthetized with isoflurane and transcardially perfused with 5-7 ml ice-cold perfusion solution containing (in mM): 225 sucrose, 2.5 KCl, 7 MgCl₂, 0.5 CaCl₂, 28 NaHCO₃, and 7 glucose, 1 ascorbic acid, and 3 pyruvate (pH 7.4 with NaOH; saturated with 95% O₂ & 5% CO₂). Rat brains were removed into ice-cold saline. Sagittal slices (250 µm) were prepared from 25-30 day-old Wistar rats and stored for recuperation 30 minutes in normal saline solution containing (in mM): 123 NaCl, 3 KCl, 1 MgCl₂, 2.0 CaCl₂, 25 NaHCO₃, and
glucose (pH 7.4 with NaOH, 298 mOsm/l with glucose; saturated with 95% CO₂ & 5% O₂) (Guzmán et al. 2003; Pérez-Rosello et al. 2005; Tecuapetla et al. 2007). Slices were transferred to a custom plexiglas recording chamber and were continuously superfused with oxygenated saline (3 ml/min). Individual neurons were visualized (40X water immersion objective) under differential interference contrast (DIC) enhanced visual guidance using infrared video microscopy.

**Electrophysiology**

Whole-cell patch-clamp recordings were performed with micropipettes made with borosilicate glass and fire polished for D.C. resistances of about 3-6 MΩ. Internal solution had a high Cl⁻ concentration so that inhibitory postsynaptic currents (IPSCs) are seen as inward currents (in mM): 72 KH₂PO₄, 36 KCl, 2 MgCl₂, 10 HEPES, 1.1 EGTA, 0.2 Na₂ATP, 0.2 Na₃GTP, 5 QX-314 and 0.5% biocytin (pH = 7.2, 275 MOsm/l). QX-314 prevented antidromic action potentials from occurring and allowed for stable voltage-clamp recordings.

Synaptic events were evoked with field stimulation with the use of a pencil shaped bipolar concentric tungsten electrode (12.5 or 50 µm at the tip; 50 ± 8 kΩ). Paired shock stimulation (interstimulus interval = 50 ms; 0.2-0.4 ms duration; 50-200 µA) delivered through the stimulating electrode at a frequency of 0.1 Hz was controlled with a computer interface (see below) and isolation units (Digitimer LTD, Hertfordshire UK) between the computer and the stimulating electrodes to quickly adjust stimulus parameters during the experiment. The field electrode was positioned in the external globus pallidus (GPe). Distance between recording and stimulating electrode in all configurations was about 1000 µm. All experiments
were realized in presence of 6-cyano-2, 3-dihydroxy-7-nitro-quinoxaline disodium salt (10 µM CNQX) and D-(-)-2-amino-5-phosphonovaleric acid (50 µM APV). Traces shown are the average of near 5 minutes recordings (25-30 traces) taken once the amplitude had been stabilized in a given condition. A small hyperpolarizing voltage command (10 mV) was constantly given during the experiment to monitor input conductance. The morphological identity of stained biocytin-filled neostriatal neurons was examined as described previously (Guzmán et al. 2003, Tecuapetla et al. 2007). In addition, BAC mice with GFP associated to either the D1 or D2 receptors promoters (e.g., dSPNs or iSPNs) were used to observe whether in control conditions antidromic stimulation from the GP preferentially activates dSPNs or iSPNs. This was not the case: both kinds of SPNs were activated equally with this stimulation (unpublished data). Whole-cell access resistances were in the range 5–20 MΩ. Access resistance was continuously monitored and experiments abandoned if changes > 25% were encountered. No cell capacitance, series resistance or liquid junction potential (2 mV) compensations were made. All recordings were filtered at 1-3 KHz and digitized with an AT-MIO-16E10 (National Ins., Austin TX) DAQ (NI-DAQ) board in a PC clone. On-line data acquisition used custom programs made in the Labview environment (National Ins). The NI-DAQ board was used to save the data on binary files in the computer hard disk for further off-line analysis. Digitized data saved on disk was imported for analysis and graphing into commercial graphing software (Origin v. 6. Microcal, Northampton MA). IPSCs amplitudes were
measured from basal line to peak from the first response. The paired pulse ratio was $\text{PPR} = \frac{2\text{nd IPSC}}{1\text{st IPSC}}$.

**Immunocytochemistry**

Recorded neurons were injected with biocytin and sub-samples of rat neurons were immunoreacted to either substance P (SP) or enkephalin (ENK) antibodies. Slices containing injected neurons were fixed overnight in 4% paraformaldehyde and 1% picric acid in 0.1 M PBS, pH 7.4. The slices were then infiltrated with 30% sucrose and cut on a vibratome into 40 µm sections. The sections were incubated 4–6 hr in PBS solution containing 0.2 Triton X-100 and streptavidin conjugated to Cy3 (6 µl/ml; Zymed, Invitrogen Carlsbad, CA) to label the recorded neuron. Sections were then processed for conventional immunocytochemistry and fluorescence to demonstrate either SP or ENK using commercially available antisera (Millipore, Temecula, CA) conjugated to fluorescein isothiocyanate. Immunocytochemical preparations were visualized on a confocal microscope (see Guzmán et al. 2003).

**RNA quantification by real time PCR**

Extraction of RNA was made by the conventional TRIzol protocol (Invitrogen, Carlsbad, CA). Dorsal striatum dissected from naïve or control animals and either 6-OHDA injected or vehicle injected (control) hemispheres were used in a sterile surface cleaned with RNAzap (Invitrogen, Carlsbad, CA). RNA was measured for 260/280 ratio and concentration with a NanoDrop2000C (ThermoScientific, Wilmington, DE) to confirm its quality. Only RNA with $260/280 \geq 2$ ratio values.
was considered and stored at -70°C. RNA (1-2 µg) was reverse-transcribed with *SuperScript*® II kit (Invitrogen, Carlsbad, CA) following manufacturer instructions. cDNA was precipitated and re-suspended in nuclease-free water and stored at -20°C. Gene-specific primers were designed and tested for efficiency and specificity (Primer-BLAST, NCBI). Other subset of primers was purchased from PrimerDesign Ltd (Southampton, UK). Sequences were: primers for SST were GCT CTG CAT CGT CCT GGC TTT G and CTT GGC CAG TTC CTG TTT CCC G which gave a PCR product of 115 bp. Primers for SSTR1 were TGG TAT GCT TGG TGG GAC TG and GCC AGG TTT AGA ATG TAG ATG TTG which gave a PCR product of 103 bp. Primers for SSTR2 were AAG AGG AAA AAG TCA GAG AAA AAG G and CAG ACA CGG ACG AGA CAT TG which gave a PCR product of 109 bp. Primers for SSTR3 were TGC CAC GGC TTA GGA CGC AA and AGG CCC AAT GCT CAG ACG CT which gave a PCR product of 144 bp. Primers for SSTR4 were AGG GCA TGC GGT GAT GCA GG and GTT GCG TAG ACG TCT CAT TTA GC which gave a PCR product of 100 bp. Primers for SSTR5 were TTG TGG TGG TCC TAT CTT ATG C and ATC CTC CAT ACC GTA TCC TCT AC which gave a PCR product of 125 bp and primers for GAPDH were AAT GGT GAA GGT CGG TGT GAA C and AGG TCA ATG AAG GGG TCG TTG which gave a PCR product of 103 bp. Eva qPCR Super mix kit (BioChain, Hayward, CA) was used for real time PCR with the manufacturer’s specifications. Thermal cycling included 40 cycles of three steps 95°C for 30 s, 60°C for 1 min and 72°C for 30 s. The PCR cycle threshold (C_t) was measured at exponential phase of the amplification curve with
AbiPrism 7000 detection system (Applied Biosystems, Foster City, CA). mRNA quantification was made by means of ΔΔCt method, using GAPDH as housekeeping gene for reference (Chan et al. 2011; Coulson et al. 2008). Experiments for each gene of interest were run for triplicate. Results were presented as fold difference relative to control side or relative to receptor SSTR2.

Statistics

Distribution-free statistical procedures were used to find data significance at the level P<0.05 or less with commercial software: Wilcoxon T test in the case of paired samples, Mann-Whitney U test in the case of unpaired samples and Friedman analysis in the case of more than two treatments on the same sample with post-hoc Dunn test (Systat v.7. SPSS Inc., Chicago IL). To separate inhibitory and facilitatory presynaptic actions of SST into two samples a reasonable hypothesis, supported by immunocytochemistry, posited that each action (sample) comes from a different cell class.

Drugs Used

Somatostatin-14 (SST) (Peninsula Laboratory, Inc., San Carlos, CA, USA), SST antagonist cyclo (7-aminoheptanoyl-phe-D-try-lys-O-benzyl-thr) acetate (CycosST) (ICN Pharmaceuticals, Mexico), [Ac-4-NO2-Phe-c (DCys-Tyr-DTrp-Lys-Thr-Cys)-D-Tyr-NH2] or CYN 154806 (CYN), N2-[4-(5,7-Difluoro-2-phenyl-1H-indol-3-yl)-1-oxobutyl]-L-arginine methyl ester trifluoroacetate or L-803 087 Trifluoroacetate (TFA) and SMS201-995 also called octreotide (OCT) (Tocris Bioscience, Ellisville, Missouri), 6-cyano-2,3-dihydroxy-7-nitro-quinoxaline
disodium salt (CNQX), D-(_)-2-amino-5-phosphonovaleric acid (APV), QX-314 and bicuculline (Sigma-Aldrich-RBI, St Louis, MO, USA) were kept in stock solutions freshly prepared and added to the superfusion during the experiment to give the required final concentration. SST was prepared with dimethylsulfoxide (0.01%).

RESULTS

Isolation of IPSCs between SPNs indicates less connections after 6-OHDA

165 SPNs from neostriatal sagittal slices from naïve (rats with no treatment) or control side (non injected), and 96 from 6-hydroxydopamine (6-OHDA) unilaterally injected side, were recorded (Fig. 1A; see Materials and Methods; Ungersted, 1968). Young animals were used for 6-OHDA lesions and slice recordings, however, these animals already surpassed postnatal days (PD) in which DA deprivation produces Parkinsonism and not other disorders (Breese et al, 2005; Dehorter et al. 2011; Reader and Dewar, 1999): PD15. In addition, a standard locomotor test for rodent Parkinsonism was performed at PD22 (turning behavior; Ungerstedt, 1968): animals showed more than 95% turning behavior unbalance (Fig. 1B; Carman et al. 1991) and SPNs recorded from DA depleted striata frequently showed spontaneous activity with burst firing (Fig. 1C; Galarraga et al. 1987; Jaidar et al. 2010; Liang et al. 2008; Tseng et al. 2001); a behavior rarely observed in control conditions (Tseng et al. 2001; Vergara et al. 2003).

Moreover, electrophysiological experiments at PD>25 showed a decrease in feedback inhibition (Taverna et al. 2008; see below), thus corroborating several reported signs of Parkinsonism in our dopamine deprived animals while comparing
them with control animals that did not show these signs at a time when dopamine innervation has reached almost full development (Antonopoulos et al. 2002). In addition, inward and outward rectifying currents, inactivating outward currents and latency for firing filled the electrophysiological profile of spontaneously activated SPNs (Figs. 1D-G; Surmeier et al. 2011).

Scheme in Figure 2A shows how antidromic stimulation of striatofugal axons, from the GPe, isolates IPSCs from synapses between SPNs (Guzman et al., 2003; Tecuapetla et al. 2007): stimulation of SPNs axons activates inhibitory postsynaptic currents (IPSCs) on neighboring SPNs (QX-314 is used inside the recorded cell to avoid antidromic action currents; see Material and Methods). Evoked IPSCs (Figs. 2B, C) in the presence of 10 µM CNQX and 50 µM APV were completely but reversibly blocked by 10 µM of the GABA_A-receptor antagonists gabazine (Fig. 2B; López-Huerta et al. 2008; Tecuapetla et al. 2007) or bicuculline (not shown).

Electrophysiological profiles of biocytin filled neurons matched immunoreactivity to either substance P or enkephalines (see above: SP; Figs. 2D-F; ENK; Figs. 2G-I) with similar probability. Moreover, antidromic stimulation from the GP in BAC mice with GFP associated to either the D_1 or D_2 receptors promoters (e.g., dSPNs or iSPNs) were used to observe whether antidromic stimulation preferentially activates dSPNs or iSPNs. The result was that both kinds of SPNs were activated equally with this stimulation (unpublished). Mean whole cell input resistance was 205 ± 86 MΩ in neurons from the injured side (n = 14; NS with respect to controls; Flores-Barrera et al. 2009).

In control-naïve animals, an IPSC could be evoked by antidromic stimulation in 97% of trials (Figs. 1, 3A; n = 160/165), that is, failures to evoke an IPSC < 5%.
In contrast, in 6-OHDA injured animals successful evocation of an IPSC using antidromic stimulation was reduced to 68% (n = 65/96) with failures about 30% (Fig. 3A; P < 0.005). Using minimal stimulation strengths (Tecuapetla et al. 2005) to activate less axons, the number of failures to evoke an IPSC increased five-fold with respect to controls (not shown). In addition, maximal IPSC response in control-naïve preparations was (mean ± SEM): 157 ± 13 pA (Fig. 3B left, black traces) while it was 66 ± 11 pA in DA deprived preparations (Fig. 3B right, black traces). The reduction amounts to about 57 ± 12% (n = 6; P < 0.05), suggesting less number of release sites for feedback inhibition after DA deprivation (Taverna et al. 2008). In contrast, other types of inhibition such as those coming from the GP or interneurons are preserved or augmented during DA deprivation (e.g., Aceves et al. 2011; Dehorter et al. 2009; Magill et al. 2001).

The amount of SST induced reduction of IPSCs is denoted by gray traces and compared in Figure 3B. Note that this modulation appears to be enhanced in a sample of 6-OHDA injured animals when compared to the controls (Fig. 3C, asterisk) as it is explained below.

**Modulation of feedback inhibition by SST in the DA deprived striatum**

Presynaptic SST-mediated modulation of IPSCs generated by connections between SPNs has been reported (Lopez-Huerta et al. 2008). In the present work, these findings have been corroborated in a sample of experiments in control-naïve animals: IPSCs obtained with half maximal stimulation were reduced by 1 µM SST from 140 ± 19 pA to 92 ± 16 pA (n = 6; P<0.05; cf., black and gray traces in Fig. 3B left; see Lopez-Huerta et al. 2008). When striatal slices were taken from DA
deprived striata, IPSCs were not always reduced by SST, on the contrary, some IPSCs were enhanced. However, synaptic events that were reduced were significantly more depressed than the controls, from 49 ± 9 pA to 20 ± 3 pA (n = 13; P < 0.01; cf., black and gray traces in Fig. 3B right). Thus, SST induced depression of IPSC amplitude in a sample of experiments is significantly larger (gray traces) in DA deprived animals: 36 ± 6% in control/naïve vs. 69 ± 6% in DA deprived subjects (Fig. 3C; n = 6; P < 0.04). A representative time course of SST-induced IPSC depression is illustrated in Figure 4A (bar indicates SST application).

Representative traces before and during SST are illustrated in Figures 4B1, 2. A summary of SST depressing actions in a sample of neurons from DA depleted striata is seen in Figure 4C (n = 7; P < 0.01). When recordings are normalized to the first IPSC and superimposed, a concomitant increase in the paired pulse ratio (PPR) is disclosed (Fig. 4G; n = 7; P < 0.01), suggesting that SST actions are presynaptic in origin in this experimental sample. Because after DA deprivation a sample of IPSCs was depressed and another sample (see below) was enhanced, we then hypothesized that each one of these samples should correspond to different classes of SPNs (Bolam, 2010). Accordingly, neurons recorded after SST-induced IPSC reduction were immunoreactive for substance-P (SP) antibodies (n = 6; P < 0.05; Figs. 2D-F; see Material and Methods), thus supporting the preceding hypothesis. Coincidently, the most affected connections after DA depletion are between direct pathway neurons (dSPNs), which preferentially connect with each other (Taverna et al. 2008).

In another sample of IPSCs recorded after DA depletion, 43% of IPSCs, were the subject of an increase in amplitude during SST: from 61 ± 11 pA to 111 ±
24 pA; or about 82 % enhancement. A time course of a representative experiment is illustrated Figure 4D, and representative traces before and during SST are shown in Figures 4E1, 2. A summary of this action in a sample of cells is shown in Figure 4F (n = 6; P < 0.05). SST-induced enhancement of IPSCs was accompanied by a PPR decrease (Fig. 4H; P < 0.05), suggesting again a presynaptic action. Following our above stated hypothesis, SST-induced enhancement of IPSCs should belong to the other class of SPNs; and supporting such hypothesis, neurons in which SST-induced enhancement of IPSCs was recorded were immunoreactive to enkephalin (ENK) antibodies (n = 6; P < 0.05). Interconnected indirect pathway neurons (iSPNs) are less affected after DA depletion and iSPNs also connect with dSPNs (Taverna et al. 2008; Tecuapetla et al. 2009). Changes in PPR were from: 0.83 ± 0.1 in controls to 1.37 ± 0.2 during SST-induced decrease in IPSC amplitude and from 0.93 ± 0.1 in the controls to 0.7 ± 0.15 during SST-induced increase in IPSC amplitude (Figs. 4G, H).

In addition, a mean-variance analysis on both responses was carried out (Clements and Silver, 2000; Salgado et al. 2005): the SST-induced decrease in IPSC amplitude produced a decrease in the weighted probability of release, P_{rw}, from 0.22 ± 0.03 in the controls to 0.14 ± 0.02 during SST (n = 6; P < 0.05; Fig. 5A), and a decrease in the number of release sites from N = 14 ± 2 in the controls to 10 ± 1 during SST (P < 0.05; Fig. 5A). Traces from a representative experiment are shown in Figure 5B. In addition, the SST-induced increase in IPSC amplitude was accompanied by an increase in the weighted probability of release, P_{rw}, to 0.42 ± 0.02 during SST (n = 6; P < 0.05; Fig. 5A) as well as an increase in the number of release sites to N = 49 ± 8 (P < 0.05; Fig. 5A). Traces from a representative
experiment are illustrated in Figure 5C. None of these actions produced a change in weighted quantal amplitude: $Q_W = 16 \pm 2 \text{ pA}$. This analysis further supported presynaptic actions of SST.

The presence of functional SST receptors (sstrs) in SPNs has been reported (Schulz et al. 2000; Vasilaki et al. 2004; Vilchis et al. 2002). In the present work, application of the non selective antagonist 1µM cyclo-SST (not shown), prevented all SST actions and agonists actions, see below, in both control and DA deprived animals (Lopez-Huerta et al, 2008).

Receptors involved in SST actions

Modulation by SST in mammals is exerted via a family of six G protein coupled receptors (sstr1 to sstr5 including two splice variants for sstr2: A and B; Schulz et al. 2000). In addition, their pharmacological and molecular similarities allow a division into two groups (Hoyer et al. 1995; Olias et al. 2004): one set including sstr2, sstr3 and sstr5, other group containing sstr1 and sstr4. sstrs are expressed in the striatum (Galarraga et al. 2007; Ramirez et al. 2002; Vilchis et al. 2002) and, although still under debate, there are few selective analogues available for these receptors (Olias et al. 2004). In this initial exploratory study, we assayed two widely used sstr-agonists, one for each group.

1 µM octreotide (OCT) was used to selectively activate sstr2 and/or sstr3 at these concentrations since there is little expression of sstr5 (Schulz et al. 2000). This agonist decreased IPSC amplitude by $36 \pm 4 \%$ ($n = 6$; $P < 0.05$; Fig. 6A). The reduction in amplitude was accompanied by an increase in PPR of $31 \pm 11 \%$ ($P < 0.05$; see superimposed and normalized representative traces in Fig. 5A as well as
a summary box plot of the same sample in Fig. 6C for IPSC amplitude and Fig. 6D for PPR change). In two experiments, the selective sstr2 antagonist CYN 154806 (CYN; see Olias et al. 2004 for analogues affinities), was added to the superfusion. The results were basically the same as those without the antagonist, suggesting that the receptor implied in presynaptic inhibition of feedback connections is sstr3.

In addition, we applied 1 µM L-803 087 trifluoroacetate (TFA), a more selective agonist for sstr1 and/or sstr4 at these concentrations, although with more affinity for sstr4. TFA also reduced IPSC amplitude by 48 ± 6% (n = 7; P < 0.05; Figs. 6B-D) while increasing PPR by 45 ± 22 % (P < 0.05). This result eliminated the simple possibility that each receptor group had a different function. Actions of both agonists were blocked by the unselective sstr antagonist 1µM cyclo-SST in both control and DA deprived striata (not shown).

Next, striatal PCR exploration of the expression of SST and its receptors was performed (Fig. 7). Relative quantification of mRNA was accomplished with respect to sstr2 because this abundant receptor was the only one that did not change in 6-OHDA injured animals as compared to the controls (Galarraga et al. 2007; Piwko et al. 1996; Ramirez et al. 2002; Tatsuoka et al. 1987). We found that sstr1, sstr4 and sstr5 decrease their expression after DA depletion by 48 ± 8 %, 46 ± 25% and 67 ± 29% respectively (cf., Figs. 1 A and B; n = 4; P < 0.05). But surprisingly, sstr3 expression suffered a considerable relative augmentation in DA depleted striata: to 225 ± 46 % as compared to the controls (Fig. 1C). This result coincides with the actions of one tested agonist: OCT (see above), reinforcing the suggestion that sstr3 needs further attention (out of the scope of the present work).
Further research is necessary to have a hint on the receptor responsible for
the SST-induced increase in feedback inhibition (Hathway et al. 1999). It is known
that these receptors can form oligomers that change their properties and actions
(Olias et al. 2004).

In addition, SST mRNA was decreased in DA depleted striata by about 57 ±
15 % as compared to the controls (n = 4; P < 0.05; Asanuma et al. 1990; Augood
et al. 1991; Espino et al. 1995; Soghomonian and Chesselet, 1991; but see:

DISCUSSION

Presynaptic modulation of feedback inhibition by somatostatin is altered after
dopamine deprivation

Pair recordings demonstrated that synaptic connectivity between SPNs, or
feedback inhibition, is decreased in the striatum of DA deprived animals (Taverna
et al. 2008). This finding was confirmed here with a population recording approach:
it was harder to evoke feedback IPSCs with antidromic stimulation (cf., Guzman et
al. 2003; Tecuapetla et al. 2007). In addition, we show that remaining feedback
inhibition becomes differentially modulated by SST in distinct subsets of SPNs.

SST inhibits the release of various hormones and transmitters (Gillies, 1997;
Lanneau et al. 2000). It induces presynaptic modulation in excitatory and inhibitory
synapses in the hippocampus (Boehm and Betz 1997; Tallent and Siggins 1997),
thalamus (Leresche et al. 2000; Sun et al. 2002), striatum (Lopez-Huerta et al.
2008) and hypothalamus (Gillies, 1997; Lanneau et al. 2000), where it commonly
decreases transmitter release. SST has also been reported to increase GABA
release (Gillies, 1997). But to our knowledge, this is the first time that a differential modulation is reported in different subsets of the same type of projection neuron: the direct and indirect spiny projection neurons of the basal ganglia. DA depletion induced SST to depress feedback inhibition in 57% of SPNs, and to enhance feedback inhibition in 43% of SPNs. In naïve animals, only depression is observed (Lopez-Huerta et al. 2008), although relatively less potently. Therefore, it was not only showed that SST actions are altered in Parkinsonian animals, but it was also hypothesized that these differential actions become opposite and segregated into the two projection cell classes: dSPNs and iSPNs. This hypothesis was supported by performing immunocytochemical experiments in samples of recorded neurons. Probably, a sampling increase would tend to divide SST actions in between two halves of SPNs, since there is 50% of each: dSPNs and iSPNs. This is forecasted since SST induced depression of feedback inhibition was found in SP immunoreactive neurons (dSPNs) while augmentation was found in ENK immunoreactive cells (iSPNs). These findings are perhaps predicted by the preferential connections that these neurons exhibit (Taverna et al. 2008): dSPNs preferentially connect with dSPNs and iSPNs mainly, but not only, connect with iSPNs. Thus, the connections between dSPNs and iSPNs became differentially affected. dSPNs connections are the most affected after DA deprivation, explaining much of the loss in feedback inhibition. Connections among iSPNs are only partially affected (Taverna et al. 2008), and perhaps, the enhancing action of SST in some iSPNs connections contributes to their preservation.
Possible somatostatin receptors involved in presynaptic modulation of feedback inhibition

Available tools to begin exploring the receptors involved may not be very specific (Hoyer et al. 1995; Momiyama and Zsaborsky 2006; Olias et al. 2004). In spite of this fact, we chose to test two widely used drugs, each one belonging to each receptor group (Hoyer et al. 1995; Olias et al. 2004): OCT for sstr2, sstr3 and sstr5 and TFA for sstr1 and sstr4.

In addition, relative PCR quantification has indicated that sstr2 is abundant, is mostly postsynaptic, but it is not modified by DA deprivation (the present work and: Galarraga et al. 2007; Piwko et al. 1996; Ramirez et al. 2002; Schulz et al. 2000; Tatsuoka et al. 1987). Besides, sstr5 expression is scant in the neostriatum (e.g., Schulz et al. 2000).

On the other hand, sstr3 was the only receptor that had a large relative enhancement after DA deprivation while SST mRNA was decreased (Asanuma et al. 1990; Augood et al. 1991; Espino et al. 1995; Soghomonian and Chesselet, 1991), suggesting a sstr3 hypersensitive response. Moreover, the agonist octreotide (OCT), mimicked SST induced depression of feedback inhibition even when sstr2s were blocked with the selective antagonist CYN. Taken together, the data suggests that one possible receptor affected by OCT to induce presynaptic inhibition of feedback connections is sstr3. Nonetheless, a clear pre or postsynaptic location for sstr3 has not been completely defined (Schulz et al. 2000).

In addition, TFA, the agonist used to test the other group of receptors: sstr1 and sstr4, also depressed inhibition. In this second group of receptors, sstr1 is probably the autoreceptor present in SST interneurons (Schulz et al. 2000; Vasilaki
et al. 2004). This receptor has lower affinity for TFA (Olias et al. 2004), it is 
decreased after DA depletion and it is hard to imagine how antidromic activation of 
SPNs axons (feedback inhibition) will activate interneurons (feedforward inhibition). 
On the other hand, sstr4 has larger affinity for TFA, it is localized with sstr2 
receptors in postsynaptic dendrites (Schulz et al. 2000), and it cannot be ruled out 
that may depress inhibition in feedback connections. In conclusion, both sstr3 and 
sstr4 become suspects to explain the enhanced SST induced presynaptic inhibition 
among SPNs.

In any case, we found possible pharmacological explanations for the 
depression but failed to find agonists that mimicked the facilitation. Among the 
many possible explanations are that sstrs may work as oligomers, they may 
produce different actions depending on location (Olias et al. 2004), or some type of 
indirect effects. All these hypotheses are out of the scope of the present work.

Possible functional consequences of differential SST modulation for Parkinsonism

A main pathophysiological model of basal ganglia is based on segregated 
“direct” and “indirect” pathways, each with particular projection cells: SP containing 
dSPNs and ENK containing iSPNs, respectively (Albin et al. 1989; DeLong and 
Wichmann, 2007; Surmeier et al. 2011). dSPNs tend to decrease their excitability 
after DA depletion, while iSPNs tend to enhance their excitability in the same 
condition, thus causing an imbalance between both pathways (Albin et al. 1989; 
Day et al. 2006, Flores-Barrera et al, 2010). This imbalance is the underlying 
change originating the signs of the disease (DeLong and Wichmann, 2007).
We found that connections among dSPNs and among iSPNs are differentially affected by SST after DA deprivation: the first are depressed and the second are enhanced. Feedback inhibition among dSPNs appears to be more depressed by SST than in the controls.

Notably, these actions of SST are in opposition to the changes in excitability that the two classes of projection neurons exhibit after DA depletion, that is: a reduction of inhibition among dSPNs would go in a direction opposite to the reduction of dSPNs excitability during DA depletion. An increase in inhibition among iSPNs would act in opposition to the increase in excitability that these neurons exhibit during DA depletion, and in addition, would compensate the partial loss of connections among iSPNs seen after DA depletion (Taverna et al. 2008). It is then suggested that SST modulation may counteract the unbalance seen among BG direct and indirect pathways after DA deprivation.

Unfortunately, SST mRNA appears to decrease under these conditions (Asanuma et al. 1990; Augood et al. 1991; Espino et al. 1995; Soghomonian and Chesselet, 1991; but see: Nilsson et al. 2009) as confirmed in this work, thus hampering the potential homeostatic correction mechanism given by SST interneurons. In fact, during Parkinsonism, some local interneurons modify their output supporting the activation of homeostatic mechanisms (Dehorter et al. 2009; Sánchez et al. 2011), and apparently, SST interneurons are among these (Dehorter et al. 2009; Tepper et al. 2010), thus confirming that DA deprivation not only affects SPNs but also interneurons and microcircuit dynamics (Jaidar et al., 2010). Therefore, the decrease in SST expression may be a part of the changes accompanying Parkinsonism that we still do not understand. Understanding them
is important given the continuous development of novel sstrs agonists for other reasons (e.g., cancer therapy). Perhaps, some new agonists being made disposable to the living brain may work as alternative or adjuvant tools for the therapeutics of Parkinsonism.

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REFERENCES


Flores-Barrera E, Vizcarra-Chacón BJ, Bargas J, Tapia D, Galarraga E. Dopaminergic modulation of corticostriatal responses in medium spiny
projection neurons from direct and indirect pathways. *Front Syst Neurosci.* 5:15, 2011.


Soghomonian JJ, Chesselet MF. Lesions of the dopaminergic nigrostriatal pathway alter preprosomatostatin messenger RNA levels in the striatum, the


Surmeier DJ, Carrillo-Reid L, Bargas J. Dopaminergic modulation of striatal neurons, circuits, and assemblies. *Neurosci* 198: 3-18, 2011


Figure Legends

**FIG 1.** The 6-OHDA model of rodent Parkinsonism. A. Top: A time line showing the data of 6-OHDA lesion (PD15), next the time of observing turning behavior after amphetamine administration (PD22), and finally, the days of electrophysiological experiments (PD25-30). Middle: Some animals were sacrificed after the lesion to check for Tyrosine Hydroxylase (TH) immunohistochemistry in sagittal striatal slices similar to those used for electrophysiology. A virtual absence of TH immunoreactivity is appreciated in the side ipsilateral to the 6-OHDA injection. TH immunoreactivity is preserved in the non injected side (contralateral). Bottom: coronal sections showing the substantia nigra TH immunoreactivity in
contralateral and ipsilateral (dashed border) sides to the injection. B. Histogram shows the number of complete body turns towards the ipsilateral or contralateral sides after 6-OHDA injection; amphetamine (i.p.) was used. Inset shows net unbalance: up to 95% ($P < 0.001$). C. Electrophysiological recordings of representative neurons. Top: a neuron recorded in the control side (contralateral to 6-OHDA injection): resting potential = -80 mV, with spontaneous synaptic activity. Bottom: two traces showing recordings from a more depolarized neuron with spontaneous spike and burst discharges in the DA-depleted side (ipsilateral to 6-OHDA injections). D. Recordings of voltage responses to hyperpolarizing and depolarizing current injections (top) in a spiny projection neuron (SPN) in current-clamp mode. Notice inward rectification and time to first action potential. E. Current-voltage relationship (I-V plot) from records at left. F. Recordings of inward and outward rectifying currents to hyperpolarizing and depolarizing voltage commands (bottom) in the same neuron in voltage-clamp mode. Holding potential = –85 mV. G. I-V plot in voltage-clamp mode shows the amount of inward rectification.

**FIG 2.** Feedback inhibition evoked by antidromic stimulation. A. Scheme of the stimulation protocol: a field stimulus in the GPe activates striatofugal axons from SPNs which in turn make inhibitory connections with neighboring SPNs. B. Time course of representative inhibitory postsynaptic current (IPSCs) amplitudes evoked with this protocol. 10 µM CNQX plus 50 µM APV block AMPA/KA/NMDA excitatory synaptic currents. Addition of 10 µM gabazine into the bath saline blocked the IPSCs. C. Representative IPSCs recorded at different times - indicated by numbers.
Superimposed traces at the bottom. D. Overlay of immunofluorescence against substance P (green) and red biocytin-signal (CY3) after whole cell recording during antidromic stimulation. E, F. Biocytin-filled neuron and SP immunofluorescence. G. Overlay of immunofluorescence against enkephalins (ENK, green) and red biocytin-signal (CY3). SP and ENK neurons had the same probability in being obtained. H, I. Recorded biocytin-filled neuron and enkephalin immunofluorescence. Scale bars 10 µm.

**FIG 3.** Antidromically evoked IPSCs and their decrease induced by SST in control and 6-OHDA injured animals. A. Evoking an IPSC in SPNs with antidromic stimulation is relatively robust in control conditions: failures < 5%. In contrast, evoking antidromic IPSCs after DA depletion is more difficult: failures = 32% (P < 0.005). This supports the loss of feedback inhibition after DA deprivation. B. Comparing feedback inhibition and its SST modulation in neurons from either the control non injured side or naïve animals (left), or neurons from DA deprived animals (right). It was found that: maximal amplitude of evoked IPSC is larger (black traces) in control/naïve animals than in DA deprived animals (P < 0.05). Second, SST induced depression of IPSC amplitude is significantly larger (gray traces) in DA deprived animals: 36% in control/naïve vs. 69% in DA deprived. C. Box plots summarize the results in a sample of experiments (n = 6, 7; *P < 0.04).

**FIG 4.** Presynaptic modulation of feedback inhibition by somatostatin in dopamine deprived striatum. A. As in control cases, the time course shows that 1 µM SST (bar) depresses absolute (@) IPSCs amplitude. B. Representative average IPSCs
(n = 25) in control (1) and during SST application (2). Superimposed traces at the bottom: note transition from paired pulse depression to facilitation. C. Box plots show results from a sample of experiments (P < 0.01). D. Opposite cases are seen in this time course: 1 µM SST (bar) enhance @IPSCs amplitudes. This response is never seen in control animals. E. Representative average IPSCs (n = 25) in control (1) and during SST application (2). Superimposed traces at the bottom: note transition from paired pulse facilitation to depression. F. Box plots show results from a sample of experiments (n = 6; P < 0.05). G, H. Changes in the paired pulse ratio (PPR) before and during SST.

**FIG 5.** Mean-variance analysis. A. Mean-variance analysis was performed in control, depressed and enhanced antidromically evoked IPSCs. Different experiments with different stimulus strengths were used. Parabolas (continuous lines) of the form $y = Ax - Bx^2$ could be fitted to the data. Mean weighted probability of release (Prw) and weighted quantal content (Qw) were obtained from fitted data: Qw was about 16 in all cases and Prw changed from 0.14 in depressed synapses to 0.42 in enhanced ones. Changes in Prw were accompanied with parallel changes in the number of release sites (N). B. Sample of traces in which SST depressed the IPSC. C. Sample of traces in which SST enhanced the IPSC.

**FIG 6.** Role of sstr3 and sstr4 mediating SST depressing actions. A. Actions of the sstr agonist, octreotide (OCT 1 µM), on the feedback IPSC (each trace is the average of 30 recordings): IPSC is reduced while PPR is enhanced (n = 6 experiments; P < 0.05; see superimposition). In these conditions OCT should be
selected to sstr2 and sstr3 but the same result (not shown) was observed in the presence of the sstr2 selective antagonist CYN 154806. B. Actions of the sstr agonist, trifluoroacetate (TFA 1 µM), on the feedback IPSC: IPSC is reduced while PPR is enhanced (n = 6 experiments; P < 0.05; see superimposition). At this concentration TFA is reported to be more selective to sstr4. C. Box plots summarize IPSC depression by both drugs as compared to SST. D. Box plots confirm, in a sample of experiments, that activation of sstrs changes PPR, suggesting presynaptic actions of both drugs.

**FIG 7.** DA deprivation modifies relative expression of somatostatin receptors. A. Relative quantification of sst and its receptors by qPCR in control striatal tissue. Expression of each receptor is plotted relative to sstr2 expression. B. After DA deprivation there is a different profile of sstrs mRNA expression. C. Relative quantification of sstrs mRNA expression in DA deprived tissue with respect to the expression found in the controls: note a large increase in the relative expression of sstr3. D. Agarose gel electrophoresis of final product qPCR of sst and receptors.
Figure 1

A. Diagram of the experimental timeline showing PD15, PD22, and PD25-30 stages with 6-OHDA, behavior, and slice sections.

B. Bar graph showing the unbalance percentage between ipsilateral and contralateral groups.

C. Graphs illustrating voltage and current measurements with labeled voltages (-80 mV, -70 mV, -200 mV, etc.) and currents (200 pA, 100 pA).

D. Graph showing the relationship between voltage and current, with a resistance of RN = 154 MΩ.

E. Graph depicting the voltage-current relationship with horizontal and vertical dashed lines for reference.

F. Graph illustrating current and voltage measurements with time (100 ms) and current (100 pA, 200 pA).

G. Graph showing the current-voltage relationship with voltage and current axes labeled.
Figure 4

A. @IPSC Amplitude (pA) in CNQX + APV

B. 25 pA superimposed & normalized

C. P<0.01

D. @IPSC Amplitude (pA) in CNQX + APV

E. 50 pA superimposed & normalized

F. P<0.05

G. P<0.01

H. P<0.05

I. Increase 43%
Decrease 57%
López-Huerta et al Figure 5

A

Mean IPSCs amplitude (pA)

IPSCs amplitude variance (pA$^2$)

$Q_w = 16$

- $Pr_w = 0.14$, $N = 10$, SST depression
- $Pr_w = 0.22$, $N = 15$, control
- $Pr_w = 0.42$, $N = 49$, SST facilitation

B

C

$25$ pA, $50$ ms

$50$ pA
A. Control

B. Control

C. P<0.05

D. P<0.05

IPSC amplitude decrease (%)

PPR change (%)

SST OCT TFA

SST OCT TFA