Heterogeneous dopamine neurochemistry in the striatum:

the fountain-drain matrix

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Abbreviations:
BG: basal ganglia
DA: dopamine
DAT: dopamine transporter
MFB: medial forebrain bundle
FCV: fast cyclic voltammetry
AMPT: α-methyl-l-tyrosine
Type-I_R: amperometric signal increase
Type-II_R: amperometric signal decrease
GBL: gamma-butyrolactone GBL
Abstract

In contrast to the relatively high attention paid to the structural heterogeneity of striatal dopamine (DA) innervation, little attention has been focused on the possible striatal heterogeneity for release and uptake of DA. By using amperometric methods, we found striatal regions showing a DA-decrease during the medial forebrain bundle stimulation (drain areas) near to other zones that showed an increase in DA-concentration (fountain areas). Both areas were intermixed to form a tri-dimensional matrix to regulate DA-concentration throughout the striatum (fountain-drain matrix). The response to electrical stimuli of different amplitudes and durations and to different drugs (α-methyl-l-tyrosine, cocaine, gamma-butyrolactone and haloperidol) suggests that regional differences for both DA-release/DA-uptake and DA-cell firing autoregulation are behind the striatal fountain-drain matrix. The high-diversity of DA-activity observed in the striatum is a new framework for analyzing experimental and clinical phenomena.
Introduction

The striatum is the largest structure of the basal ganglia (BG) where its medium-sized spiny neurons process BG-inputs coming from all cortical areas and return the processed information to the cortex via the “direct” (striatum-substantia nigra/internal pallidum-thalamus-cortex) and “indirect” (striatum-external pallidum-subthalamus-substantia nigra/internal pallidum-thalamus-cortex) pathways. Therefore, it is involved in forming a closed loop which connects the subcortical projections from each cortical area with the thalamic projections to the same cortical area (Alexander et al., 1986; Albin et al., 1989; DeLong, 1990; Smith et al., 1998; Obeso et al., 2000b). The striatum is massively innervated by meso-striatal dopamine (DA) cells, which, by modulating the cortical action on medium-sized spiny neurons (Dahlstroem and Fuxe, 1964; Ungerstedt, 1971; Fallon and Moore, 1978; Gerfen et al., 1987b; Joel and Weiner, 2000), influence motor and non-motor functions of the cortex (Gerfen, 2000). At a subcellular level, DA-actions are induced at both the synaptic cleft (where DA behaves as a short-lasting neurotransmitter), and the extracellular non-synaptic space (where it behaves as a long-lasting volume transmitter) (Gonon, 1988; Kawagoe et al., 1992; Sesack et al., 1994; Descarries et al., 1996; Schultz, 1998). The role of each DA-pool and the interaction between them is still being researched.

Based on the distribution of different neurochemical markers, the striatum has been segregated into two intricately related compartments, the striosoma (i.e. patch) which has a high density for substance P, dynorphin and neurotensin markers, and the matrix which has a high density for cholinergic enzymes and calbindin (Goldman-Rakic, 1982; Groves et al., 1988; Graybiel, 1990). There is also a non-homogeneous DA-distribution...
across the striatum, with local heterogeneity for DA-innervation (Voorn et al., 1986; Gerfen et al., 1987a; Gerfen et al., 1987b; Graybiel et al., 1987; Langer et al., 1991; Gerfen, 1992a; Gerfen, 1992b; Joel and Weiner, 2000), DA-concentration (Voorn et al., 1986), tyrosine hydroxylase level (Gerfen et al., 1987b; Gerfen, 1992a), DA-receptor (Gerfen et al., 1990) and DA-transporter (DAT) (Graybiel et al., 1993; Gonzalez-Hernandez et al., 2004) density. A part of this heterogeneity has been linked to the striosomes/matrix dichotomy (Gerfen et al., 1987b; Graybiel and Moratalla, 1989; Halpain et al., 1990; Hanley and Bolam, 1997). There are electrochemical works suggesting regional diversity for striatal DA-transmission (May and Wightman, 1989; Peters and Michael, 2000), but these differences have not been systematically evaluated. In the present work, the regional diversity for DA-activity was studied in the striatum by quantifying extrasynaptic DA with a selective and sensitive amperometric method which made it possible to analyze the local heterogeneity of DA-response to medial forebrain bundle (MFB) stimulation and its modulation by DA-receptor and DAT-activity.

Methods

Experiments were carried out on male Sprague-Dawley rats weighing 300-350 g. Animals were housed at 22° C, two per cage, under normal laboratory conditions on a standard light-dark schedule (12:12 with 3.00-15.00 light on) and free access to food and water. Experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) regarding the care and use of animals for experimental procedures and adequate measures were taken to minimise pain and discomfort.
Electrodes and electrochemical methods

DA concentration was quantified here with two high temporal resolution (<1s) electrochemical methods, amperometry (Dugast et al., 1994; Gonon, 1997; Benoit-Marand et al., 2001; Puopolo et al., 2001) and fast cyclic voltammetry (FCV) (May et al., 1988; Wightman et al., 1988; Wightman and Zimmerman, 1990; Garris and Wightman, 1994; Wu et al., 2001). The recording electrode was a carbon fiber microelectrode (8 µm diameter and 100 µm long), whose tip was pulled to ≈1 µm (Fu and Lorden, 1996) in order to minimize tissue damage (Fig. 1A). As an additional precaution, the electrode was very slowly introduced in the striatal tissue (20 µm / min) with a micro-manipulator (MO-8 by Narishige, Tokyo, Japan) modified in our laboratory to include a stepper motor and a digital-controller for vertical displacement (1 µm resolution; Sylvac, Crissier, Switzerland). The response to MFB stimulation was tested every 50 µm (half the length of the carbon fiber electrodes) and 5 min after the last movement. Electrodes were pretreated for 20 sec with a 70 Hz triangular wave (0-2.3V vs. Ag/AgCl) to optimize their in vivo electrochemical behavior. This treatment causes a very small decrease in the time-resolution of electrode (compare 2.5V-treated electrodes in Fig. 1B with that obtained in untreated electrodes and in 2.9V-treated electrodes). The sensitivity of the electrodes was about 50 times higher (> 1nM) than that obtained with untreated electrodes. The reference electrode (silver wire in vitro coated with a layer of silver chloride by applying +5V for 3 min) was placed inside a pulled glass capillary tube (filled with a 3 M potassium chloride solution saturated with AgCl; Fluka) with a 300 µm diameter tip.
Electrode calibration and testing

Selectivity and sensitivity of electrochemical methods and electrodes were tested before their implantation. The active part of the carbon-fiber electrode was placed 5 mm into a Teflon capillary tubing (125 μm i.d. and 5 cm in length), which was connected to the exit of a liquid switch (CMA110; CMA Microdialysis, Stockholm, Sweden) which was in turn connected to a dual syringe perfusion pump (CMA102; CMA Microdialysis, Stockholm, Sweden). The perfusion fluid was a phosphate-buffered (PBS) saline solution (NaCl 148 mM, KCl 2.7 mM, CaCl₂ 1.2 mM and MgCl₂ 0.8 mM) and the flow rate was 40 μl/min. Oxidizable compounds were dissolved in this solution and loaded in one of the two perfusion syringes (the other was loaded with the solution used to dissolve the test compound). The internal volume of the whole system was 40 μl.

In vivo preparation

Following previously reported procedures (Rodriguez and Gonzalez-Hernandez, 1999), in vivo studies were performed under chloral hydrate anesthesia (400 mg/kg i.p.) in a sound-proofed dark room (5.00-10.00 hours after turning the light on) of the and with the body temperature maintained between 36.5 and 37.0°C. According to previous studies (Chergui et al., 1994; Gonon, 1997), DA released during the electrical stimulation of the MFB was monitored with a carbon fiber microelectrode introduced in the striatum. The electrode was connected to an amperometry detector (BECA Instruments, Tenerife, Spain) which controlled the electrode potential (versus a reference electrode whose open tip was immersed in a saline solution in contact with the meninges through a previously made hole in the skull) by means of a potentiostat. The location of the striatal recording electrode was 0.0-1.2 mm anterior to bregma, 2.6-3.4
mm lateral to the midline and 3.8-5.0 mm below the cortical surface (Paxinos and Watson, 1986). Activation of DA-inputs to the striatum was performed by the electrical stimulation of the MFB (electrode placed in a position 4 mm anterior to lambda, 2.2 mm lateral to the midline and 8.0 mm below the cortical surface). Electrical stimuli were provided by a S-8800 Grass model and a bipolar electrode made with two tungsten rods (100µm diameter; A-M Systems INC.) etched with an oxyacetylene torch flame (Braga et al., 1977), insulated with glass (fused silica capillary, Composite Metal Service, Hallow, UK) and placed with the tips 500µ apart. The exposed surface of the tip was about 0.5 mm. Stimuli were given in 0.2-0.7 mA and 1 msec square biphasic pulses. The striatal DA concentration change induced by the MFB stimulation was estimated on the basis of the calibration of the carbon fiber electrodes before their implantation.

In the last experiments, the effect of DA depletion on striatal response to MFB-stimulation was tested. Thus, α-methyl-l-tyrosine (AMPT) was ip. injected (250 mg/kg) during the periodic stimulation of MFB (20-30 Hz - 0.3 mA / 1.0-1.5 sec stimulus duration each 20-60 sec). Sixty min after AMPT administration extracellular DA was replenished by injecting exogenous dopamine (5 mM) throughout a fused silica capillary tube (75 µm inner diameter) located 400 µm from the recording-electrode tip (Figure 8A). DA was dissolved in a ringer solution (148 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl$_2$ and 0.8 mM MgCl$_2$) supplemented with L-glutathione reduced (1mM) to avoid DA oxidation and whose osmolarity (auto-osmometer Osmostat OM-6020; CagaK Co. LTD, Kyoto, Japan) was between 280-285 mOsm. Two DA-fluxes were used (CMA-102 Microdialysis pump; CMA, Stockholm, Sweden), one which induced a fast and transitory increase of extracellular DA (3 µl/min during 3 sec; Figure 8D) and
the other which induced a slow and progressive increase of DA (continuous perfusion of 0.2 µl/min; Figure 8E).

**Amperometry method**

Amperometry uses a continuous oxidation potential (0.4 V for DA) that, although less selective than FCV, provides a very high sensitivity (nA) and time resolution (µs). The amperometry method used here was similar to that previously used by others (Chergui et al., 1994; Gonon, 1997; Staal et al., 2004), with some modifications aimed at optimizing its selectivity for DA and its time and spatial resolution. Amperometric current through the electrode was continuously measured with a time constant of 1 ms and the detector output was digitized and recorded at 1 kHz (PowerLab system connected to a PC computer, ADInstruments, Castle Hill, Australia).

It has been shown that the rapid increase in the current evoked in the striatum by MFB electrical stimulation is caused entirely by the evoked DA-overflow (Chergui et al., 1994; Dugast et al., 1994; Gonon, 1997). As an additional precaution, and in order to rule out the possible interference of serotonin, we used a potential of 200 mV instead of the 0.4 V oxidation potential that is generally used for DA (Fig. 1D). In some experiments, the amperometric response was tested at different oxidation potentials. Thus, the oxidation potential was slowly modified between 800 and –800 mV and the response to MFB stimulation was tested every 50 mV. A three-dimension map of data (response amplitude x post-stimulus time x oxidation potential) was computed and visualized with a color plot similar to that previously used for FCV data (Peters and Michael, 2000).

The electrical artifacts induced by the stimulation pulses on the amperometric signal were initially withdrawn using two methods, one previously used in other
laboratories (Chergui et al., 1994; Dugast et al., 1994; Gonon, 1997) and the other reported here. With the previously reported method a number of stimuli (12 in Fig. 1E) are averaged and the mean response obtained at 0 V oxidation potential (Fig. 1F) are subtracted (electrical artifacts) from that obtained at 0.4 V (DA oxidation + electrical artifacts). Thus, the artifact can be reduced without disturbing the amplitude of the amperometric response (Fig. 1G). We also tested a new method which noticeably decreased the stimuli artifact by: 1. decreasing the high-frequency components of the stimuli (square pulses were modified by shaving the pulse corners in order to obtain a stimulus wave-form with the initial and final portions of the rising and falling phases similar to those of a sigmoid waveform), and 2. fast bi-phasic stimulating pulses were used (the artifact induced by each pulse was very symmetric and lasted 1-2 msec and was withdrawn by using a moving average which computed each signal value as the average of the data obtained a few milliseconds before and after it). This new procedure was used in most studies because, as it is as efficient at rejecting artifacts as the previously reported method, it does not need the averaging of a number of amperometric responses which increases the time-resolution of the study. As shown in Fig. 1H, all amperometric responses to MFB-stimulation (and not only the mean recording; Fig. 1G) can be used to quantify the experimental response with this method.

**Fast cyclic voltammetry method.**

FCV quantifies DA-concentration by studying currents recorded when the oxidation potential is moved quickly between two boundaries (generally between 1.0 to -1.0 V). Under these conditions most DA is oxidized in a range between 0.5-0.7 V. The FCV method used here was similar to that previously used by others (May et al., 1988; Wightman and Zimmerman, 1990; Garris and Wightman, 1994; Wu et al., 2002). The electrode was held at 0V, scanned to -1V, ramped to 1.0V, back to -1V and returned to
0V. The duration of this cycle was 15 msec and the cycle was repeated every 100 msec. Current through the electrode was continuously digitized at 20 kHz (PowerLab system connected to a PC computer, ADInstruments, Castle Hill, Australia).

Drug and chemicals

Haloperidol, gamma-butyrolactone (GBL), α-methyl-l-tyrosine (AMPT), L-glutathione reduced and acetazolamide were obtained from Sigma Chemical Corp. (St. Louis, USA). Cocaine chlorhydrate was obtained from Avello (Barcelona, Spain).

Histology

In order to verify the location of the recording microelectrode, a lesion was made by passing DC current through the carbon fiber electrode (15 µA of cathodal DC current for 50 sec). At the end of each experiment, the rats were transcardially perfused with 200 ml of 0.9% saline solution followed by 400 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH=7.4). Brains were removed and stored in the same fixative at 4°C for 12-24 hours and then the midbrain was cut at 50 µm with a vibratome in the coronal plane and stained with the formal thionine procedure.

Statistics

Mathematical analyses were performed using the one way ANOVA followed by the LSD test for post hoc comparisons. Analysis was performed using the Statistica program (Statsoft; Tulsa, U.S.A.). A level of p<0.05 was considered as critical for assigning statistical significance.
Results

In most striatal regions the amperometric current increased during the MFB stimulation, a response that will be referred to as type-I response (type-I, Fig. 2A). This response was similar to that previously reported by others, showing a response-amplitude and a response-duration that rose when the stimulation rate increased (Fig. 2A). This response has been previously considered as an indication of striatal DA-release from stimulated DA-inputs.

In some striatal regions a current decrease was observed during MFB stimulation, a response that will be referred to as type-II response (type-II, Fig. 2B). The amplitude and duration of type-II also rose when the rate (Fig. 2B) and duration (Fig. 2D) of stimuli increased. Type-II often began after the stimulus switch-off when a low-rate stimulation was used (10 Hz in Fig. 2E). In some recordings type-II began during stimulation and lasted for 1-2 sec after stimulus switch-off (40 Hz in Fig. 2E). In this case the stimulus-rate needed to induce a within-stimulus response was often higher than that needed to induce only the post-stimulus response (Fig. 2F). Type-II was selective for the oxidation potential of DA (Fig. 2G), and was not observed for potentials lower than 50 V. The finding of type-II suggested that MFB stimulation decreases extracellular DA in certain striatal regions.

Finally, a mixture of type-I and type-II was also observed in some striatal regions (type-I/II). In this case type-I generally began before type-II and often delayed the beginning of type-II. In some striatal loci, type-I and type-II were equally vigorous. In this case the response-amplitude of both responses progressively increased with the stimulation-rate (Fig. 2C).
The response type was different and specific in each striatal position and changed when the electrode was moved to neighboring striatal regions, with type-I\textsubscript{R} being more frequently observed in the dorso-lateral edges of the striatum. Striatal loci with type-I\textsubscript{R} will be referred to as \textit{fountain areas} and those with type-II\textsubscript{R} as \textit{drain areas}. Figure 3 shows response examples recorded across a striatal tract. As can be observed, the response was very stable within each area (the response to two consecutive stimuli are shown in each region) but can be very different when the recording electrode was moved 200 µm. The frequency for each response type clearly depends on the region of the striatum that is being recorded (e.g. it is more frequent in the dorsal regions of the striatum just under the corpus callosum than in the middle striatal zones). We did not perform a systematic screening of all striatal regions and no information about the relative frequency for each response type is provided.

\textbf{Cocaine action}

The possible involvement of dopamine transporter on the different response types was tested by administering cocaine, a transporter activity inhibitor. Cocaine increased the type-I\textsubscript{R} (Fig. 4A and 4C), an effect observed for a wide stimulus-range (200-1200 ms in the Fig. 4B example). Cocaine induced a complex modification of the type-II\textsubscript{R}. In most cases, cocaine changed the type-II\textsubscript{R} responses into a type-I/II\textsubscript{R}. This change was observed in striatal regions showing a marked type-II\textsubscript{R} preceded by a small type-I\textsubscript{R} (Fig. 4D), but also in regions showing a pure type-II\textsubscript{R} (Fig. 4E). In striatal areas showing a type I/II\textsubscript{R}, the cocaine effect (Fig. 4F) was much more marked on type-I\textsubscript{R} (600% increase) than on type-II\textsubscript{R} (25% decrease).
**GBL action**

The possible influence of DA nigro-striatal cell impulse flow on the different response types was tested by administering GBL, a drug that at high doses (injected here in a single dose of 750 mg/kg ip.) inhibits the impulse flow of these neurons (Walters and Roth, 1972; Walters et al., 1973; Diana et al., 1991). GBL induced a slight increase (30%) of type-I_R (Figs. 5A and 5B) and a marked decrease (70%) of type-II_R (Figs. 5C, 5D and 5E). In regions showing a type I/II_R, the initial type-I_R increased (to 300% of the basal response) and the posterior type-II_R decreased (to 25% of the basal response) after GBL administration (Fig. 5F and 5G).

**Haloperidol action**

The possible involvement of DA-cell receptors on the different response types was tested by administering haloperidol, a DA-receptor antagonist which is partially selective for D2-like receptors. Haloperidol increased the type-I_R (Fig. 6A). Type-II_R showed a marked attenuation (see an example in Fig. 6B) after haloperidol administration to about 40% of its basal response (Fig. 6C). In regions showing a type I/II_R, the marked increase of the initial type-I_R together with the decrease of type-II_R made type-II_R undetectable (see 30 Hz in Fig. 6D).

**pH and type-II_R**

A recent FCV study has reported pH changes which could interfere with the DA signal after MFB stimulation (Venton et al., 2003). Thus, we performed different studies to evaluate the possible involvement of pH on type-II_R with amperometry and FCV methods.
Initially we tested *in vitro* the electrode sensitivity for DA and pH with amperometry methods. In this technique, electrodes showed a more marked sensitivity for DA-concentration than for pH modification (Fig. 1C). In addition, no DA-pH overlapping was observed at the oxidation potential typically used for DA quantification with amperometry methods. Thus, for positive oxidation potentials the DA wave increased with the oxidation potential (Figs. 7D and 7G), whereas no response was observed for pH-pulses (Figs. 7E and 7H). When negative oxidation potentials were used, a small response to DA was observed for potentials lower than -300 mV (Figs. 7D and 7G) whereas pH-pulses induced a more marked effect, especially at -500 mV (Figs. 7E and 7H). These data show that, when an oxidation potential of +200 mV is used (i.e. *in vivo* studies), the pH change does not affect the amperometric signal (Fig. 7F), at least when pH oscillates within the boundaries previously observed in the striatum (<0.1) after the MFB stimulation (Venton et al., 2003).

The DA-pH segregation was not as evident for FCV, even when using the same *in vitro* preparation and the same electrodes as those previously used with amperometry. With this technique, DA induced an oxidation wave around 600 mV and a reduction wave around -200 mV (Figs. 7I and 7L). On the other hand, the pH-change (7.4 → 7.6) increased at oxidation potentials around -400 mV and decreased it at +600 mV (Figs. 7J). Therefore, the positive peak induced by DA at +600 mV could be reduced by a negative peak induced by a simultaneous increase of pH (Fig. 7K). In agreement with a previous study, present data suggest that care should be taken not to misinterpret pH shifts accompanying terminal activity as DA changes when FCV is used (Venton et al., 2003). In this respect, the amperometric evaluation of DA at +200 mV did not have the same pH-interference problem as FCV.
Finally, acetazolamide (a carbonic anhydrase inhibitor that selectively decreases the pH response to MFB stimulation without modifying the DA-response) (Venton et al., 2003) was used for in vivo testing the possible influence of pH on type-II$_R$ with amperometric methods. This amperometric study showed that after drug administration no type-II$_R$ modifications were observed (Figs. 7A) either in the response amplitude (Figs. 7B) or in the response latency (time taken by the oxidation current to reach its minimum point) (Figs. 7C). Taken together present data show that pH modifications cannot explain type-II$_R$.

*Tyrosine hydroxylase inhibition and dopamine action.*

This experiment was aimed at obtaining direct evidence of DA involvement on type-II$_R$. Thus, the effect of $\alpha$-methyl-p-tyrosine (an inhibitor of the rate-limiting enzyme tyrosine hydroxylase) (Michael et al., 1987; Watanabe et al., 2005) on type-II$_R$ was evaluated before and 50-120 min after AMPT administration, and then (120-200 min after AMPT) DA was directly perfused in the striatum with phasic (3 $\mu$l/min x 3 sec) or tonic (0.2 $\mu$l/min x 1 min) perfusion rates. AMPT dramatically reduced type-II$_R$ in three of the six rats studied, decreasing both the response amplitude (Figure 8C - left side) and the response duration (Figure 8C - right side). In the other three rats a complete disappearance of type-II$_R$ was observed after AMPT (Figure 8B). In rats with complete type-II$_R$ disappearance, DA-perfusion increased the amperometric current, inducing a fast and transitory increase (phasic perfusion; Figure 8D) or a slow and progressive increase (tonic perfusion; Figure 8E). A recovery of the type-II$_R$ which disappeared after AMPT was observed after both the phasic DA-administration (Figure 8D) and the tonic DA-administration (Figure 8E). In the second case the type-II$_R$
recovery was progressive and followed the slow increase of the amperometric current induced by DA-perfusion.

**Discussion**

The present study reports a marked diversity for DAergic activity in the striatum, where some regions showed an extracellular DA-increase after MFB stimulation (type-IR in fountain areas), whereas other regions showed a DA-decrease (type-II_R in drain areas). In addition, a mixed type-IR / type-II_R response was observed in the same striatal region (type-I/II_R in mixed areas). Both the disappearance of type-II_R after AMPT and its recovery by local DA-perfusion in the striatum show that type-II_R is the consequence of a transitory decrease of extracellular DA. The cocaine, GBL and haloperidol effects suggest that DA-receptors and DAT modulate type-II_R. The finding of fountain-drain areas intermixed across the striatum is a new framework for analyzing experimental (e.g. the high DA-diffusion observed in the striatum after the partial degeneration of DA-cells) and clinical (e.g. dyskinesias and other motor complications often observed in Parkinson’s disease after chronic DA-drug administration or DA-cell striatal implants) phenomena.

**Type-IR**

Type-IR showed the typical DA-response to MFB-stimulation reported in previous voltammetry (Wightman et al., 1988; Garris et al., 1994; Garris and Wightman, 1994; Wu et al., 2001) and amperometry (Chergui et al., 1994; Dugast et al., 1994) studies. The extracellular DA-concentration is generally the result of two opposing mechanisms: DA-release and DA-uptake. It has been shown that the increase
of the electrochemical-signal observed after MFB-stimulation is induced by an excess of DA-release which, after saturating DAT, diffuses until the recording electrode (DA-overflow) (Wightman and Zimmerman, 1990; Gonon, 1997; Wu et al., 2001). From this perspective: 1. the rapid decrease of the electrochemical-signal observed after the MFB-stimulation switch off (which corresponds to a fast DA-concentration decrease) is induced by an efficient activity of DAT (although DA-diffusion may play a minor role); 2. the type-I increase induced by cocaine is secondary to a DA-uptake inhibition (thus facilitating DA-overflow) (Greco and Garris, 2003); and 3. the type-I increase induced by haloperidol is secondary to a blockade of the receptor-mediated pre-synaptic self-inhibition (thus facilitating DA-release and inhibiting DA-uptake) (Benoit-Marand et al., 2001; Wu et al., 2002). The mechanisms for the GBL actions here reported are less evident. Bearing in mind the above mentioned model, the type-I increase observed after GBL administration could be induced by the persistent reduction of extracellular DA secondary to the GBL-induced inhibition of DA-cell firing rate (Walters et al., 1973; Bannon et al., 1981; Bannon et al., 1982). This hypothesis explains the similar effect observed for GBL (which decreases the presynaptic DA-receptor stimulation as a consequence of an extracellular DA-concentration decrease) and haloperidol (which decreases the presynaptic DA-receptor stimulation as a consequence of a direct blockade) administration.

**Type-II**

Some previous electrochemical studies have suggested a regional diversity for striatal DA-transmission (May and Wightman, 1989; Peters and Michael, 2000) but, as far as we know, this is the first paper reporting striatal regions which respond to the MFB stimulation with a decrease of extracellular DA (type-II). The finding of type-II
increases in some experimental conditions (use of bipolar short-lasting electrical stimuli, highly-sensitive pre-treated electrodes and electrochemical methods, and short carbon fibers which reduce the probability of recording adjacent fountain and drain regions simultaneously) and decreases in others (use of long-lasting high-frequency stimuli which increase the type-I component of type-I$_R$/II$_R$, recording in non-peripheral regions of the striatum where the finding of type-II$_R$ is more common, and administration at the beginning of experiments of repetitive stimuli with short inter-stimulus). Although a mixture of all these factors can markedly reduce the probability of finding drain regions, we cannot explain why type-II$_R$ has never been previously reported. We believe that this response has probably been observed in other laboratories but it was never systematically studied because its biological explanation and significance is less evident than for type-I$_R$.

At the beginning, we found evidence suggesting that type-II$_R$ was not produced by collateral non-dopaminergic associated phenomena such as electrical artifact, modifications in other transmitters or modifications in pH or oxygen. Type-II$_R$ vanished after decreasing the oxidation potential (Fig 2G), which shows that it is not a non-specific artifact induced by MFB stimulation. The amperometric conditions used also suggest that type-II$_R$ is not the consequence of changes in other neurotransmitters such as serotonin (the oxidation potential used does not oxidate serotonin -Fig. 1D- and the position of MFB-electrodes made the stimulation of serotonergic ascending fibers very improbable) and GABA (GABA is not directly detectable with present methods) released from non-dopaminergic meso-striatal neurons. Another two possible confusing variables are oxygen and pH, which can be detected by carbon-fiber electrodes and which change in the striatum after MFB-stimulation (Zimmerman and Wightman, 1991; Zimmerman et al., 1992; Cramer et al., 1997; Venton et al., 2003). DA-oxidation and
oxygen-reduction occur at different potentials (+0.3 v and -1.4 v respectively) (Zimmerman et al., 1992; Venton et al., 2003) and show a different kinetic response (Venton et al., 2003), ruling out the oxygen involvement on type-II. Previous (Venton et al., 2003) and present data show a partial overlapping between pH and DA signals when studied with FCV. However, this was not the case for amperometry. Thus, in vitro studies showed DA and pH amperometric waves which did not overlap at the oxidation potential used in in vivo studies (+200 mV). In addition, acetazolamide, a drug that decreases the pH response to MFB-stimulation but not the DA-response (Venton et al., 2003), did not change type-II R. The pH changes previously observed with FCV after MFB stimulation are slower (began 2 sec after stimulation) and longer-lasting (> 30 sec) than type-II R studied here (which generally began during stimulation and persisted for only a few seconds). Thus, in vitro (no pH wave at +200mV oxidation potential) and in vivo (no type-II R modification after acetazolamide administration and a different kinetic for type-II R to that reported for pH-response) studies do not support pH modifications as the basis for type-II R.

On the other hand, we found evidence showing that, similarly to that previously reported for type-I R, type-II R is produced by a short-lasting modification of the DA concentration. Thus, type-II R changed after administration of drugs that modify DA-synapse (cocaine, haloperidol and AMPT) and DA-cell firing activity (GBL). Data obtained after DA-synthesis inhibition were particularly significant. Type-II R markedly decreased or completely vanished after inhibiting tyrosine hydroxilase-activity with AMPT, showing that this response needs endogenous catecholamines. Under these circumstances, type-II R was quickly restored by the local perfusion of DA in the striatum, showing that this is the catecholamine involved in type-II R. Contrary to the DA-increase associated with type-I R (Chergui et al., 1994; Dugast et al., 1994; Gonon,
1997), type-II$_R$ is caused by a transitory decrease in the extracellular DA-concentration. Thus, the DA-cell firing rate activation can increase (Wightman and Zimmerman, 1990; Gonon, 1997; Wu et al., 2001) or decrease extracellular DA, with the striatal region under study being the factor determining one response or the other.

**Mechanisms to explain type-II$_R$**

Two factors could be involved in the type-I$_R$/type-II$_R$ dichotomy observed in different regions of the striatum: 1. anatomical differences in the DA-innervation vs. DAT-density proportion (Ciliax et al., 1995; Gonzalez-Hernandez et al., 2004), and 2. functional differences of mechanisms involved in the DAT-activation induced by DA-cell spike firing (Falkenburger et al., 2001; Khoshbouei et al., 2003)(Meiergerd et al., 1993; Mortensen and Amara, 2003). It has been suggested that the electrochemical heterogeneity of the striatum could be related to the striosoma/matrix architecture of this center (May and Wightman, 1989). However, the functional organization of the striatum (Alexander and DeLong, 1985b; Alexander and DeLong, 1985a; Graybiel, 1990), and the heterogeneity for DA-innervation (Voorn et al., 1986; Gerfen et al., 1987a; Gerfen et al., 1987b; Graybiel et al., 1987; Langer et al., 1991; Gerfen, 1992a; Gerfen, 1992b; Joel and Weiner, 2000), DA-concentration (Voorn et al., 1986), DA-receptor (Gerfen et al., 1990) and DAT-density (Graybiel et al., 1993; Gonzalez-Hernandez et al., 2004) do not always fit in with the striosoma/matrix architecture. We are presently trying to establish an electrochemical-morphological relationship between type-II$_R$ and local density for DAT and D2-receptors. However, the electrochemical heterogeneity could be more qualitative (functional status) than quantitative (number of molecules). This might be the case for DAT, whose activity can quickly increase during cell hyperpolarization (Sonders et al., 1997; Falkenburger et al., 2001; Khoshbouei et al.,...
2003) or D2-autoreceptor stimulation (Meiergerd et al., 1993; Cass and Gerhardt, 1994; Mortensen and Amara, 2003). The acute modification of type-II_{R} observed after the spike firing blockade with GBL or the D2-receptor inhibition with haloperidol supports this possibility. The explanation for type-I_{R}/II_{R} could be a mixture of those proposed here for type-I_{R} and type-II_{R}. When DA-release (more efficient in type-I_{R} regions) and DA-uptake (more efficient in type-I_{R} regions) are balanced in one striatal region, a type-I_{R}/II_{R} could be observed.

**Possible functional meaning of fountain-drain striatal matrix**

Bearing in mind the physical dimension of the electrode (8 x 100 µm) and the response modifications observed after small changes (200-500 µm) in the brain position of the electrode, present data suggest the existence of an intricate 3-D arrangement of fountain-drain regions within the striatum (fountain-drain striatal matrix). Besides other proposed regulatory mechanisms such as synaptic regulation of DA-release (Benoit-Marand et al., 2001; Wu et al., 2002) or somatic regulation of the firing rate (Rodriguez et al., 2003a; Rodriguez et al., 2003b), this fountain-drain matrix could provide another way to modulate the striatal action of DA. Considering the ability of DA to diffuse from the synaptic cleft and to cover long-distances across the extracellular space (Kawagoe et al., 1992; Agnati et al., 1995; Descaries et al., 1996; Schultz, 1998; Hoistad et al., 2000), the existence of highly effective striatal regions for sequestering DA-excesses could prevent the deterioration of striatal activity induced by an excessive DA-diffusion (Doucet et al., 1986; Schneider et al., 1994). An early FCV-study of the electrochemical heretogeneity of the striatum (May and Wightman, 1989) found some characteristics in the voltammetric response to MFB-stimulation (type-I responses because no type-II_{R} was reported in this study) suggesting the existence of striatal
regions particularly suitable for preventing an excessive DA-diffusion (“mass transfer barrier”). Type-II\textsubscript{R} of drain areas are a suitable substrate for this task. In this regard, disturbances in the fountain-drain matrix distribution may contribute to the deterioration of the therapeutic response generally observed in advanced Parkinson’s disease. This is the case of dyskinesias, a motor complication often observed in Parkinson’s disease after chronic DA-drug administration (Cotzias et al., 1969; Fahn, 2000; Obeso et al., 2000a), and after DA-cell implants (Lindvall and Hagell, 2000; Dunnett et al., 2001; Hagell and Brundin, 2001; Winkler et al., 2005). Dyskinesias has been associated with a transitory increase of DA-activity in specific striatal regions. Because DAT-expression down-regulates in surviving DA-neurons (Chinaglia et al., 1992; Miller et al., 1999; Pirker, 2003; Poewe and Scherfler, 2003; Gonzalez-Hernandez et al., 2004), a functional mitigation of drain regions (perhaps together with other factors such as a loss of firing-rate autoregulation) (Rodriguez et al., 2003a), probably facilitates the diffusion (Doucet et al., 1986; Schneider et al., 1994) and accumulation of DA, therefore triggering motor complications.

In summary, we report here evidence showing a marked heterogeneity for the DA-release and DA-uptake in the striatum, where some regions show DA-uptake activation during DA-cell firing which is more intense than the corresponding DA-release. As these regions probably mitigate DA-diffusion throughout the striatum, their disturbance could facilitate motor complications in basal ganglia disorders. Now we are analyzing the possible morphological basis for the fountain-drain matrix, and identifying the synaptic mechanisms involved in type-II\textsubscript{R}. 
References


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Footnotes

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Figure Legends

Figure 1. Amperometric method. A: tip of the electrode pulled to about 1 µm. B: Time resolution of electrodes with different treatments (electrodes used were pretreated with a triangular wave 0-2.3v x 70 Hz during 20 sec). C1: increase of sensitivity after electrode treatment - maximum response in 10 electrodes (amperometric response to DA was evaluated in ascorbic acid -AA- or glutation -glt- solutions) (data are shown as mean ± standard error). C2: increase of sensitivity after electrode treatment – an example of the amperometric current at different oxidation potentials (amperometric response to DA was evaluated in ascorbic acid -AA- or glutation -glt- solutions), response of PBS is shown by dashed line. C3: differential sensitivity for DA and pH in treated electrodes (mean ± standard error of 20 measures). D: in vitro selectivity of the electrode shown as amperometric current at different oxidation potential (BKG: background; DA: dopamine; 5HT: 5-hydroxytryptamine). An example of twelve striatal response-waves to MFB stimulation recorded at 200 mV and 0 mV oxidation potential are shown in E and F respectively. Electrical artifacts induced by the stimulation pulses on the amperometric signal were withdrawn using two different methods: 1. subtraction of the mean response obtained at 0 V (electrical artifacts) from that obtained at 250 mV (DA oxidation + electrical artifacts) (Fig. G), and 2. a moving average of the response wave (Fig. H).

Figure 2. Type of amperometric responses observed in the striatum. This figure shows examples (individual stimulus traces) of the different striatal responses to MFB stimulation. Depending on the striatal region under study the oxidation current increased (type-I_R in A), decreased (type-II_R in B) or showed a mixed modification (type-I/I_R in C), responses that were directly related to the stimulus frequency (stimuli
of 400 msec with frequencies between 5 and 30Hz). Duration and amplitude of type-II_R also increased with the duration of the stimulus (from 200 ms to 2000 ms in D where 20 Hz stimuli were used). Depending on the stimuli frequency the type-II_R began during stimulation or after stimulation switch off (E; 1 sec stimulus). Occasionally the type-II_R began during stimulation but increased after the stimulation switch off (F; 1 sec stimuli). Type-II_R was observed at the oxidation potentials which correspond to DA and completely vanished when the oxidation potential was lower than 0 mv (G; 20 Hz 1 sec stimuli). The stimulus duration is shown in all pictures by a black column.

**Figure 3. Modification of the amperometric response to MFB-stimulation in adjacent striatal regions.** Recordings were performed in a position 0 mm anterior to bregma, 3.2 mm lateral to the midline and 3.5-5.1 mm below the cortical surface. The stimulus (30 Hz / 500 msec) presentation is shown in all pictures by a black column.

**Figure 4. Cocaine action on type-I_R, type-II_R and type-I/II_R.** A shows 10 pre-drug and 20 post-drug (20-35 min after 20 mg/kg ip. in saline solution) type-I_R responses to MFB stimuli (40Hz, 600 ms, 0.5 mA each 3 min). B shows pre-drug and post-drug type-I_R response to MFB stimuli of different duration (50Hz, 0.5 mA). C shows the increase (response area shown as a percentage of basal response) of type-I response-area (data obtained from 4 rats) for MFB stimuli of different frequencies (600 ms, 30 Hz, 0.5 mA). D shows pre-drug and post-drug response for MFB stimuli of different frequencies (500 ms, 0.5 mA). E shows the time-course of the cocaine effect (500 ms, 30 Hz, 0.5 mA). F shows the cocaine effect (response area) on type-I_R and type-II_R (data were obtained from 5 rats (500 ms, 30 Hz, 0.5 mA) and are shown as a percentage of the mean value of pre-drug response to MFB-stimulation).
Figure 5. GBL action on type-Iₐ, type-IIₐ and type-I/Iₐ. A shows pre-drug and post-drug type-Iₐ response to MFB stimuli of different durations (40Hz, 0.5 mA). B shows (as a percentage of basal response) the increase of type-Iₐ area (n=6 rats) to MFB stimuli (40 Hz, 1000 ms, 0.5 mA) 20-40 min after GBL administration (750 mg/kg ip). C shows the decrease of type-Iₐ to MFB stimuli of different duration (20Hz, 0.5 mA). D shows (as a percentage of basal response area) the increase of type-Iₐ area (n=5) to MFB stimuli (40 Hz, 1000 ms, 0.5 mA). E shows the time-course of the GBL effect on type-IIₐ (1000 ms, 40 Hz, 0.5 mA). F shows (as a percentage of basal response area) the modifications of the initial (type-Iₐ) and late (type-IIₐ) components of the type-I/Iₐ (n=6) to MFB stimulation (40 Hz, 1000 ms, 0.5 mA) 20-40 min after GBL administration (750 mg/kg ip.). G shows the time-course of the GBL effect on the type-I/Iₐ (1000 ms, 40 Hz, 0.5 mA).

Figure 6. Haloperidol action on type-Iₐ, type-IIₐ and type-I/Iₐ. A shows pre-drug and post-drug (50 min after 0.5 mg/kg ip. in saline solution) type-Iₐ response to MFB stimuli of different frequencies (2000 ms, 0.5 mA). B shows an example of the drug-induced decrease of type-IIₐ response. C shows (as a percentage of basal response average) the decrease of the response area (n=5 rats) of type-IIₐ to MFB stimuli (30 Hz, 1000 ms, 0.5 mA). D shows an example of the type-I/Iₐ response increase to MFB stimuli at different stimulus rates (500 ms, 0.5 mA).

Figure 7. Type-IIₐ and pH: in vivo and in vitro studies. An example of acetazolamide in vivo action on type-IIₐ is shown in A. Acetazolamine (25 mg/kg ip. in saline solution) did not induce any modification in the response amplitude (B) and duration of the
oxidation current decrease during the first portion of type-II\textsubscript{R} (C). Data in B and C were recorded 25-50 min after drug-administration (n=30 stimuli in 3 rats), and are shown as a percentage of the mean pre-drug value (± standard error). The sensitivity of amperometry (D-H) and FCV (I-L) methods to DA and pH changes was also tested \textit{in vitro}. The amperometric response to DA and pH modifications was tested at different oxidation potentials with a voltage interval of 50 mV. Two precautions were taken to facilitate the stabilization of the baseline before a new oxidation potential was studied: 1. oxidation potentials were moved from positive to negative values (which facilitates the rapid stabilization of the baseline), and 2. at least ten minutes elapsed between the voltage modification and the next amperometric recording. An example of these recordings, computed as a three-dimensional map (response amplitude x post-stimulus time x oxidation potential), is visualized in a color plot at D (DA) and E (pH). DA-pH overlapping was not observed at the oxidation potential (200 mV) typically used for \textit{in vivo} studies (an example is shown in F with blue-line representing DA and red-line representing pH). The maximum amplitude of FCV response to DA and pH change is shown in G and H respectively (mean of type-II\textsubscript{R} maximum amplitude ± standard error). An example of the FCV voltamogram (Faradic current for oxidation and reduction plotted against the input voltage to the potentiostat to form a cyclic representation) recorded in basal (red line) and in a 2 µM DA-solution (blue line) is shown in L. Similar to the amperimetric data computation, the FCV was also visualized as a three-dimensional map (response amplitude x post-stimulus time x oxidation potential). A FCV example (recorded with the same electrode used in the amperometry study shown in D and E) is shown in I (DA) and J (pH). DA-pH overlapping was observed at the oxidation potential (600 mV) generally used in \textit{in vivo} studies (an example in shown in K with blue-line representing DA and red-line representing pH).
Figure 8. Inhibition of type-II_{R} with AMPT: response recovery after perfusing DA in the striatum. A: tip of the electrode pulled to about 1 µm (left-side) and micro-canula showing a drop of the solution used to perfuse DA in the striatum (right-side). B: examples of the response disappearance to MFB-stimulation before and after AMPT peripheral injection (250 mg/kg ip). C: decrease of amplitude and duration of type-II_{R} (n=4) to MFB-stimulation after AMPT administration (data are shown as mean ± standard error). D: two examples of amperometric response to DA phasic-perfusion (3 µl/min during 3 sec). Type-II_{R} disappeared after AMPT administration (left side of figure) and was recovered after the local DA-perfusion (right-side of figure). D: example of amperometric response to DA tonic-administration (perfusion of 0.2 µl/min for 20 min). Type-II_{R} disappeared after AMPT administration showed a progressive recovery during the progressive arrival (as shown by the increase of the amperometric signal) of injected-DA.
Striatal distribution of responses

3.5 mm  3.7 mm  3.9 mm

4.1 mm  4.3 mm  4.5 mm

4.7 mm  4.9 mm  5.1 mm

50 pA  1.5 s
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**Type-I**

- **A**
  - 200 ms
  - 400 ms
  - 600 ms
  - 800 ms
  - 1000 ms
  - 40 Hz

- **B**
  - % pre-GBL average
  - 160
  - 140
  - 120
  - 100
  - 80
  - 60
  - 40
  - 20
  - 0
  - 40 Hz / 1000 ms
  - * p<0.001 vs. basal response
  - basal
  - GBL

**Type-II**

- **C**
  - 400 ms
  - 1000 ms
  - 1600 ms
  - 50pA

- **D**
  - % pre-GBL average
  - 120
  - 100
  - 80
  - 60
  - 40
  - 20
  - 0

- **E**
  - 40 Hz / 400 ms
  - 50pA
  - 0
  - 0.5
  - 1.0
  - 1.5
  - 2.0
  - 2.5
  - sec
  - 0
  - 20 min

**Type-I/II**

- **F**
  - % pre-GBL average
  - 350
  - 300
  - 250
  - 200
  - 150
  - 100
  - 50
  - 0

- **G**
  - 40 Hz / 1000 ms
  - 60pA
  - 0
  - 0.5
  - 1.0
  - 1.5
  - 2.0
  - sec
  - 0
  - 20 min
"In vitro" amperometry

Time Response: 200 mV

Response Amplitude: 1400-1600 msec

"In vitro" fast cyclic voltammetry

Time Response: 600 mV
α-methyl-p-tyrosine decreases Type-II_R

DA perfusion after α-methyl-p-tyrosine restores Type-II_R