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A ROLE FOR PRESYNAPTIC MECHANISMS IN THE ACTIONS OF NOMIFENSINE AND HALOPERIDOL

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Abstract—Psychomotor stimulants and neuroleptics exert multiple effects on dopaminergic signaling and produce the dopamine (DA)-related behaviors of motor activation and catalepsy, respectively. However, a clear relationship between dopaminergic activity and behavior has been very difficult to demonstrate in the awake animal, thus challenging existing notions about the mechanism of these drugs. The present study examined whether the drug-induced behaviors are linked to a presynaptic site of action, the DA transporter (DAT) for psychomotor stimulants and the DA autoreceptor for neuroleptics. Doses of nomifensine (7 mg/kg i.p.), a DA uptake inhibitor, and haloperidol (0.5 mg/kg i.p.), a dopaminergic antagonist, were selected to examine characteristic behavioral patterns for each drug: stimulant-induced motor activation in the case of nomifensine and neuroleptic-induced catalepsy in the case of haloperidol. Presynaptic mechanisms were quantified *in situ* from extracellular DA dynamics evoked by electrical stimulation and recorded by voltammetry in the freely moving animal. In the first experiment, the maximal concentration of electrically evoked DA ($[DA]_{max}$) measured in the caudate-putamen was found to reflect the local, instantaneous change in presynaptic DAT or DA autoreceptor activity according to the ascribed action of the drug injected. A positive temporal association was found between $[DA]_{max}$ and motor activation following nomifensine ($r=0.99$) and a negative correlation was found between $[DA]_{max}$ and catalepsy following haloperidol ($r=-0.96$) in the second experiment.

Taken together, the results suggest that a dopaminergic presynaptic site is a target of systemically applied psychomotor stimulants and regulates the postsynaptic action of neuroleptics during behavior. This finding was made possible by a voltammetric microprobe with millisecond temporal resolution and its use in the awake animal to assess release and uptake, two key mechanisms of dopaminergic neurotransmission. Moreover, the results indicate that presynaptic mechanisms may play a more important role in DA-behavior relationships than is currently thought. **© 2003 IBRO. Published by Elsevier Science Ltd. All rights reserved.**

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Abbreviations: AP, anteroposterior; CP, caudate-putamen; DA, dopamine; DAT, dopamine transporter; ML, mediolateral; SSRI, serotonin reuptake inhibitor.

Key words: dopamine, transporter, autoreceptor, haloperidol, nomifensine, voltammetry.

Psychomotor stimulants and neuroleptics possess reinforcing properties and high therapeutic value, respectively (Julien, 1985). Both classes of drugs profoundly influence dopamine (DA)-related behavior and target dopaminergic signaling. Psychomotor stimulants enhance motor activity (Fog, 1972) and increase DA levels in brain extracellular fluid by inhibiting the DA transporter (DAT) (Boja and Kuhar, 1989; Hurd and Ungerstedt, 1989). The drug-induced behavioral activation is sensitive to lesions of midbrain dopaminergic neurons (Kelly et al., 1975; Roberts et al., 1975). Neuroleptics produce catalepsy (Fog, 1972) and bind to DA receptors (Seeman, 1977), leading to a complex neurochemical response. The antagonism of autoreceptors prevents negative feedback control, also elevating ambient DA concentrations (Moghaddam and Bunney, 1990), whereas blockade of postsynaptic receptors opposes the disinhibition. However, a clear relationship between the drug-induced changes in dopaminergic activity and behavior has been very difficult to establish in the awake animal. Indeed, the poor correlation between DA levels measured by microdialysis and behavior produced by either psychomotor stimulants (Sharp et al., 1987; Kuczenski et al., 1991; Nakachi et al., 1995; Badiani et al., 1998) or neuroleptics (Zetterstrom et al., 1984; Imperato and Di Chiara, 1985; Osborne et al., 1994) has led some to re-evaluate the dopaminergic basis of the drug-induced motor activation and catalepsy.

An alternative hypothesis for the reported discrepancies between drug assessment by microdialysis and behavior is that dialysate DA does not precisely characterize time-dependent changes in extracellular DA. This postulate is based on evidence that the microdialysis probe causes damage to adjacent tissue (Benveniste and Diemer, 1987; Clapp-Lilly et al., 1999), distorting extracellular DA dynamics in the sampled region relative to intact tissue (Yang et al., 1998; Lu et al., 1998). To test this hypothesis, we recently compared the effects of GBR 12909 on dialysis DA and electrically evoked DA measured by voltammetric microsensors, which cause minimal tissue damage when implanted (Allen et al., 2001). A close temporal association was observed between the increase of electrically evoked DA in the caudate-putamen (CP) and motor activation following systemic administration of the DA uptake inhibitor (Budygin et al., 2000). In contrast, dialysate DA correlated poorly, reaching a plateau approximately 60–80 min after that for the behavior. This finding is important for at least two reasons. First, it re-affirms a primary

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role for DA in stimulant-induced motor activation. Second, it suggests that the delay in dialysate DA is an artifact of sampling. Whether the observed discrepancies between neuroleptic-induced changes in dialysate DA and catalepsy have a similar origin is not known.

Given that the electrically evoked voltammetric signal does not directly reflect functional levels of the neurotransmitter, our result also raises other intriguing questions. For example, why does the indirect neurochemical measure parallel stimulant-induced behavior so faithfully? Additionally, is the measure generally applicable for the study of dopaminergic drugs? Multiple actions on dopaminergic neurons are expected in response to systemically applied GBR 12909. DAT blockade would increase extracellular DA levels in both dendritic and terminal regions (Santiago and Westerink, 1992) where the protein is localized (Nirenberg et al., 1996). Soma firing rate, in turn, could be indirectly inhibited by the elevated DA locally via autoreceptors (Mercuri et al., 1991) and in projection fields via long feedback loops such as the striatonigral tract (Engberg et al., 1997). Terminal autoreceptors governing release (Starke et al., 1989), synthesis (Wolf and Roth, 1990) and uptake (Hoffman et al., 1999) could also be activated. Voltammetric studies in the anesthetized animal demonstrate that increases of electrically evoked DA observed in the striatum following systemic administration of DAT inhibitors are well described by altered uptake kinetics in the terminal region (Wightman and Zimmerman, 1990). In contrast to the poor sampling rate of microdialysis, the rapid voltammetric measurements permit resolving drug-induced changes in the DA signal into the respective components governed by release and uptake. It is therefore tempting to speculate that altered presynaptic DAT activity links the GBR 12909-induced changes in evoked DA and motor activation (Budygin et al., 2000). However, the pharmacological control of dopaminergic presynaptic mechanisms has not been evaluated in the freely moving animal. A better understanding of the relationship between the voltammetric and functional indices could provide new insight into central drug action as well as DA-behavior relationships.

The goal of this study was to investigate the relationship between dopaminergic presynaptic mechanisms and behavior induced by a DA uptake inhibitor, nomifensine (7 mg/kg i.p.), and a mixed D1/D2 antagonist, haloperidol (0.5 mg/kg i.p.). Doses were selected to examine the characteristic behavioral patterns produced by each drug: stimulant-induced motor activation in the case for nomifensine (Costall et al., 1975) and neuroleptic-induced catalepsy for haloperidol (Rebec et al., 1985). The effects of both drugs were examined on electrically evoked DA levels measured in the CP of the freely moving rat by voltammetry. This region, innervated by nigrostriatal dopaminergic neurons (Bjorklund and Lindvall, 1984), was selected to examine nomifensine effects for three reasons. First, psychomotor stimulants robustly increase extracellular DA levels in the CP (Sharp et al., 1987; Kuczenski et al., 1991; Kawagoe et al., 1992; Nakachi et al., 1995; Badiani et al., 1998; Wu et al., 2001). Second, we had previous success linking GBR 12909-induced changes in electrically evoked DA in the CP with motor activation (Budygin et al., 2000). And third, nigrostriatal, along with mesolimbic, dopaminergic neurons, are believed to play a role in mediating the behavioral effects of psychomotor stimulants (Costall et al., 1972; Kuczenski et al., 1991; Whishaw et al., 1992). In the first experiment, presynaptic drug action was kinetically quantified *in situ* from the evoked DA dynamics in terms of parameters for DA release and uptake. The time-dependent effects of drugs on behavior were compared with the voltammetric signal in the second experiment. Thus, the present study is the first to evaluate central drug action, determined at the synaptic level in the awake animal, directly with altered neurotransmitter dynamics and behavioral status.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague–Dawley rats (weighing 250–350 g, 8–12 weeks old) were purchased from Charles Rivers (Raleigh, NC, USA) or Harlan (Indianapolis, IN, USA) and housed under controlled lighting and temperature. Food and water were available *ad libitum*. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 86-23) and was approved by the Institutional Animal Care and Use Committees of the University of North Carolina and Illinois State University. All efforts were made to minimize the number of animals used and their suffering.

Surgeries: general procedure

After anesthesia animals were immobilized in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA), skin and muscle layers were retracted and holes were drilled through the skull for placement of reference, auxiliary, stimulating and working electrodes. During surgery and postoperative care body temperature was maintained at approximately 36 °C using a Deltaphase Isothermal Pad (Braintree Scientific, Braintree, MA, USA). All stereotaxic coordinates described herein are given in millimeters according to the atlas of Paxinos and Watson (Paxinos and Watson, 1986). Anteroposterior (AP) and mediolateral (ML) coordinates are referenced from bregma; dorsoventral coordinates are referenced from dura. The stimulating electrode was positioned in the substantia nigra (-5.6 AP, $+1.0$ ML, -7.5 to -8.5 DV) and the working electrode was positioned in the CP ($+1.2$ AP, $+2.0$ ML, -4.5 to 5.5 DV). Reference (chloridized silver wire) and auxiliary (stainless steel wire) electrodes were implanted in the contralateral cortex. *Nonsurvival surgery*. These experiments were performed under urethane anesthesia (1.5 g/kg i.p.) as previously described (Wu et al., 2001). The location of ascending DA neurons was determined by incrementally lowering the stimulating electrode until a robust signal voltammetrically identified as DA was recorded in the CP. The position of the working electrode was then optimized to support a high rate of DA release. *Survival surgery*. Surgery to prepare animals for voltammetrically monitoring levels of electrically evoked DA during free behavior was performed under aseptic conditions with equithesin (1.0 ml/kg i.p.) or ketamine (80 mg/kg i.p.) and xylazine (12 mg/kg i.p.) as previously described (Garris et al., 1997; Budygin et al., 2000). After optimization of the evoked DA signal, the stimulating electrode was cemented to the skull with the aid of surgical screws.

Electrochemistry

Fast-scan cyclic voltammetry at carbon-fiber microelectrodes was used to monitor brain extracellular DA (Wiedemann et al., 1991; Michael et al., 1999). Electrochemistry was computer controlled using an EI 400 potentiostat (Ensman Instruments, Bloomington, IN, USA). The potential of the working electrode, which rested at a bias of −0.4 V versus Ag/AgCl, was linearly scanned to 1.0 V and back every 100 ms at a rate of 300 V/s. The output of the potentiostat was digitized and stored to computer file using locally written software. Extracellular DA concentrations were calculated from the current at the peak oxidation potential for DA (typically 500–700 mV) using a calibration factor determined *in vitro* for each working electrode post-experiment. Background-subtracted voltammograms were obtained by subtracting voltammograms collected during baseline from those collected during electrical stimulation. Although not shown, all recordings used in the present study exhibited a voltammogram consistent with DA. Cylindrical carbon-fiber microelectrodes were prepared as previously described (Cahill et al., 1996). Approximately 50–200 μm of the carbon fiber (r=2.5 μm) extended beyond the glass insulation.

Electrical stimulation

Constant current, biphasic stimulus pulses, 2 ms each phase, were computer generated and applied to a twisted, bipolar stimulating electrode using optical isolation (NL 800, Neurolog, Medical Systems, Great Neck, NY, USA). A current intensity of ±125 μA and train duration of 0.4 s were used. The frequency of the train varied and is indicated in the text for each experiment. Tips of the stimulating electrode (0.2 mm diameter; Plastics One, Roanoke, VA, USA) were separated by approximately 1 mm.

Voltammetry recording sessions

After a post-surgery recovery period of at least 1 week, electrically evoked levels of DA were monitored in freely behaving rats as previously described (Garris et al., 1997; Budygin et al., 2000). Rats were first habituated for approximately 30 min to a recording chamber consisting of a 60×60 cm flat-surface area bounded by 20 cm walls angled away at 45°. After this time the stimulating electrode connector, voltammetric head stage and micromanipulator, containing a fresh carbon-fiber microelectrode, were attached to the animal. Connections to instrumentation were made via a cable and electronic swivel, which permitted complete access in the recording chamber.

Two experimental protocols were used in this study. The first protocol examined the frequency dependence of evoked DA before and after drug administration. The effects of drugs were measured beginning 20 min after drug administration. Frequencies between 10 and 60 Hz were used with a duration of 0.4 s (trains ranging from 4 to 24 pulses), and the responses to these trains were analyzed to determine rate constants for DA release and uptake. In the second protocol, the time course of the effects of nomifensine and haloperidol was examined. A baseline response to a 30 Hz train of pulses was collected and then the drug was injected. The response to sequential 30 Hz trains applied at 10- and 30-min intervals was recorded for 90 min after drug administration.

Behavioral analysis

Drug-induced behaviors were evaluated together with the monitoring of DA by voltammetry. Motor activation was measured during the 1-min period prior to electrical stimulation using a 0–6-point scale (Murray and Waddington, 1990): 0, asleep or inactive; 1, episodes of normal activity; 2, discontinuous activity with bursts of prominent sniffing or rearing; 3, continuous stereotyped activity such as sniffing or rearing along a fixed path; 4, stereotyped sniffing or rearing that is fixated in one location; 5, focused stereotyped behavior with bursts of licking or gnawing; 6, continuous licking or gnawing. Catalepsy was measured during the 3-min period prior to electrical stimulation by placing forelimbs on a 10 cm-high bar and recording the time when both forepaws returned to the ground (Imperato and Di Chiara, 1985). The rating scale was as follows: 1, 0–25 s; 2, 26–60 s; 3, 61–110 s; 4, 111–180 s; 5, >180 s. The doses of nomifensine (7 mg/kg) and haloperidol (0.5 mg/kg) were selected to elicit the characteristic behavioral patterns produced by each drug (Costall et al., 1975; Rebec et al., 1985). Identical analysis of behavior was done in animals not surgically prepared for voltammetry to establish whether the electrical stimulation and measurement of DA affected the response.

Data analysis

Voltammetric recordings were analyzed using a kinetic model describing evoked levels of extracellular DA as a balance between the opposing processes of release and uptake (Wightman et al., 1988). During application of the stimulus train the change in DA concentration with respect to time is given by:

$$d[DA]/dt = [DA]_p * f - V_{max}/(K_m/[DA]+1) \quad (1)$$

where f is the frequency of stimulation, [DA] is the concentration of DA measured at the electrode, $[DA]_p$ is the concentration of DA released per stimulus pulse, and V_{max} and K_m are Michaelis-Menten rate constants for DA uptake. At the end of the stimulation the DA concentration reaches a maximum, $[DA]_{max}$, whose value is dependent on the balance between release and uptake. After stimulation only uptake is operable and the change in DA concentration with respect to time is described by:

$$d[DA]/dt = -V_{max}/(K_m/[DA]+1) \quad (2)$$

A nonlinear regression employing a simplex minimization algorithm was used to fit equations (1) and (2) to experimental data (Jones et al., 1995). Data from the entire frequency response (i.e. 10–60 Hz) were simultaneously analyzed. For kinetic analysis of baseline recordings, $[DA]_p$ and V_{max} were determined after fixing K_m at 0.2 μM (Garris et al., 1997). Limited time for signal averaging of the low-frequency responses, due to the desire to reduce the number of stimulus trains applied to the animal and the requirement for collecting two frequency series for evaluating drug effects, prevented the assessment of K_m under pre-drug conditions in the present study. The narrow time window for evaluating drug effects in ambulant animals (see Results) also limited the number of evoked responses collected. As a result, V_{max} was fixed at the calculated control value for fitting recordings collected after drug administration. For responses collected after nomifensine, $[DA]_p$ was also fixed at the baseline value and K_m was determined. $[DA]_p$ was determined after fixing K_m for evaluation of responses collected after haloperidol. This strategy produced results consistent with the drug mechanisms previously determined in the anesthetized animal using more robust kinetic analysis (Wightman et al., 1988; Kawagoe et al., 1992; Wiedemann et al., 1992; Wu et al., 2001). The time delay caused by DA adsorption to the electrode was removed by deconvolution (Bath et al., 2000).

Where applicable, data are expressed as the mean±S.E.M. where n is the number of animals. Significance was tested by repeated measures analysis of variance (Sokal and Rohlf, 1995), unless indicated otherwise in the text, using SPSS software (Chicago, IL, USA). Post hoc analysis of means, when appropriate, used the method of least squares with a Bonferoni correction. Differences were deemed significant when $P<0.05$.

Drugs and reagents

All chemicals and drugs were used as received and purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise

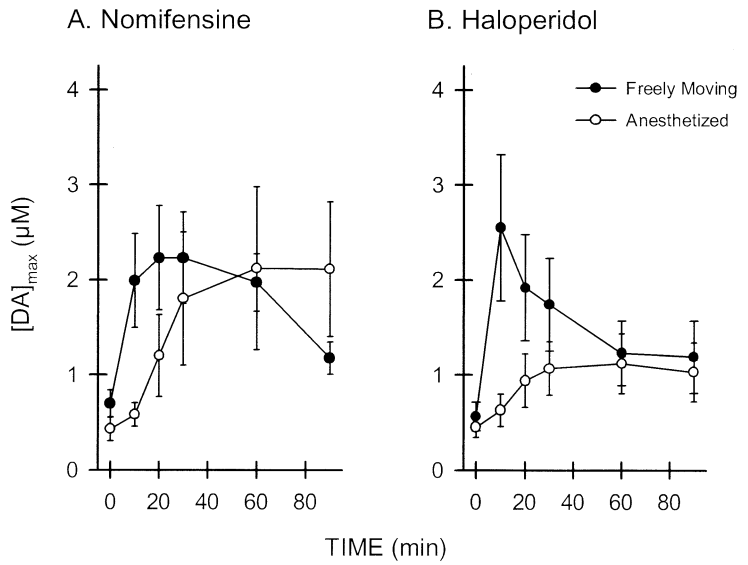

Fig. 1. Effects of anesthesia on the time course of extracellular DA increased by nomifensine and haloperidol. Changes in $[DA]_{max}$ obtained during a 0.4-s, 30- Hz train of pulses delivered at 10- and 30-min intervals following drug injection are shown. Drugs were injected immediately after collection of two baseline measurements that were averaged and plotted at time zero. Open and closed circles describe data in anesthetized and awake rats, respectively. Data are the mean±S.E.M. (n=6–8). (A) Time course for nomifensine. (B) Time course for haloperidol.

indicated. Nomifensine (Hoechst Roussel Pharmaceuticals, Somerville, NJ, USA), and haloperidol were dissolved in physiological saline. To aid in dissolution, minimal amounts of tartaric acid were added to the nomifensine solution and minimal amounts of acetic acid were added to the haloperidol solution. Aqueous solutions were prepared in doubly distilled, deionized water (Mega Pure System, Corning Glasswork, Corning, NY, USA).

RESULTS

Technical considerations for experiment 1

The pharmacological control of DAT and DA autoreceptor activity in the freely moving animal was evaluated in experiment 1. Because this goal has not been achieved previously, a design from related experiments performed under anesthesia was used. The design incorporated electrical stimulation, voltammetry and kinetic analysis, and has been successfully employed to characterize the effects of the DA uptake inhibitor, nomifensine (May et al., 1988; Kawagoe et al., 1992; Wu et al., 2001), and the dopaminergic antagonist, haloperidol (Kawagoe et al., 1992; Wiedemann et al., 1992), as well as other drugs (Wightman and Zimmerman, 1990), on DA presynaptic mechanisms. Because of potentially important differences between measuring evoked DA in anesthetized and unanesthetized animals, several issues must be considered before applying the design. These considerations are discussed in detail below.

Specificity of analyte measurement. Selectivity of the measured signal is a key consideration for all voltammetric studies, especially with new conditions and in a behavioral context (Marsden et al., 1988; Joseph, 1996). There is also the specific concern that the drugs used to alter dopaminergic presynaptic mechanisms, nomifensine and haloperidol, exhibit noradrenergic properties (Gianutsos et al., 1982; Helmeste and Seeman, 1983). Selectivity in the present study is provided by the electrochemical information in the voltammogram, collected with the technique of fast-scan cyclic voltammetry, and the anatomical specificity of the electrical stimulation. Throughout this work, voltammograms collected before and after drug administration were identical to those for DA determined during post-calibration (data not shown). While voltammograms for DA and norepinephrine are electrochemically indistinguishable (Baur et al., 1988), we conclude that drug-induced increases in the evoked response are largely if not completely due to DA, because the CP is nearly devoid of noradrenergic terminals (Moore and Card, 1984). Furthermore, noradrenergic neurons innervating the CP are unlikely to be activated by stimulation of the substantia nigra. A related concern is whether the administered drugs alter the recording characteristics of the microsensor. Diminished electrode sensitivity to DA after nomifensine as reported elsewhere (Davidson et al., 2000) was not observed. Similarly, haloperidol did not alter electrode post-calibration.

Time course for nomifensine and haloperidol effects. A stable response is necessary to acquire the frequency series used to assess kinetic effects of drugs on evoked DA dynamics (Wightman and Zimmerman, 1990). Because extrapolation from experiments in anesthetized to unanesthetized animals is uncertain, time courses were determined for the freely moving condition. Time-dependent effects of nomifensine (7 mg/kg i.p.) and haloperidol (0.5 mg/kg i.p.) on evoked DA are shown in Fig. 1A and B, respectively (open circles). A short (0.4 s), low-frequency (30 Hz) and low-intensity (125 μA) train of 12 pulses was used to evoke DA. Both drugs elicited a rapid but transient increase in $[DA]_{max}$, the maximum DA concentration elic-

ited by the stimulus train. In contrast to nomifensine, whose effects progressively decreased following the maximal response, haloperidol-induced increases in $[DA]_{max}$ fell to a constant level about two and a half times lower than maximal response. On the basis of the reasonably stable response between 20 and 40 min following administration of each drug, this time frame was selected to collect the post-drug frequency series.

For comparison, time courses for nomifensine and haloperidol were also determined under urethane anesthesia using identical drug doses and conditions for electrical stimulation (closed circles; Fig. 1A and B, respectively). Nomifensine increased $[DA]_{max}$ in both groups of animals to a similar extent. However, drug effects were delayed in the anesthetized animal, reaching a maximal value more slowly and remaining high during the course of the measurements. Statistical analysis showed a significant main effect of time ($F_{1,65}=2.67$, $P<0.05$) but not of animal group ($F_{5,65}=1.51$, $P>0.20$). In addition to delaying the haloperidol-induced increase in $[DA]_{max}$, anesthesia also decreased peak response. Thereafter, $[DA]_{max}$ remained high and at a similar level as that in the awake animal. Statistical analysis demonstrated a significant main effect of animal group ($F_{1,83}=8.63$, $P<0.01$) but not time ($F_{5,83}=1.96$, $P>0.10$). The time course for haloperidol in anesthetized animals is similar to one reported by us using more robust pulse trains applied at shorter intervals (Wiedemann et al., 1992), although somewhat slower in onset perhaps due to different stimulation parameters. These results suggest that urethane affects the time-dependent changes in evoked DA induced by both drugs and is consistent with anesthesia altering the dopaminergic properties of neuroleptics (Mereu et al., 1995) and psychomotor stimulants (Opacka-Juffry et al., 1991).

Quantifying dopaminergic presynaptic mechanisms in the freely moving animal. The general design developed in the anesthetized animal (Wightman and Zimmerman, 1990) is to collect recordings evoked by a range of frequencies between 10 and 60 Hz, administer the drug, and repeat the frequency series after stabilization of the response. Evoked signals are then resolved into respective rates for release and uptake by mathematical means (Wightman et al., 1988) and calculated parameters compared between pre- and post-drug conditions. The frequency series provides more dynamic information for curve fitting compared with a single response, thus increasing the accuracy of the calculations. A sample frequency series is shown in Fig. 2 (control, open circles) for the pre-drug condition in a freely moving animal. The striking increase in signal amplitude with frequency is even more clearly seen in the compiled results for $[DA]_{max}$ (Fig. 3A; control, open circles). There was also a tendency for the individual signal elicited by 10 Hz to reach steady state, whereas extracellular DA continued to increase during the stimulation train at higher frequencies. These dynamic changes are qualitatively similar to those observed under anesthesia. However, the use of a longer pulse train (≥2 versus 0.4 s) and higher-intensity current (≥300 versus 125 μA) produce a greater concentration range (up to 6 μM) and more clearly pronounced steady-state responses in anesthetized animals (Wightman et al., 1988).

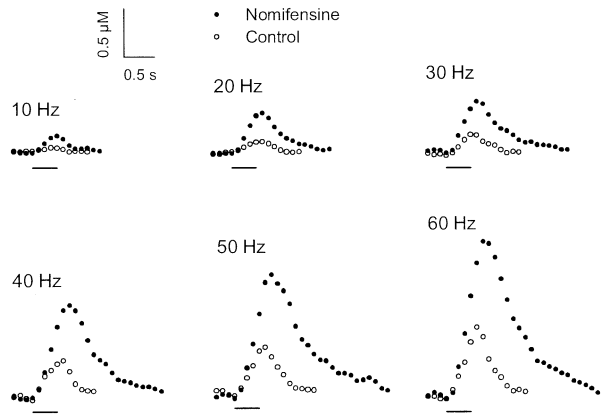

Fig. 2. Effects of nomifensine on the dynamics of electrically evoked DA in the caudate-putamen of the freely moving rat. The responses measured prior to drug administration (control) are shown as open circles. Data collected between 20 and 40 min after nomifensine (7 mg/kg i.p.) are shown as solid circles. All data were collected in the same rat during one recording session. Train frequency is denoted at the top left of each set. Each datum is the change of concentration of extracellular DA determined from a single voltammogram collected at intervals of 100 ms. Curves at frequencies below 40 Hz are the average of four to six replications. The solid line underneath each set of curves demarcates application of the 0.4-s stimulus train. Scale bars for both time and DA concentration apply to all curves.

Analysis of curves collected in all animals during pre-drug recording determined an averaged $[DA]_p$ and V_{max} of 65±18 nM and 2.73±0.51 μM/s, respectively (average $r=0.90$ for fit of data to model). These values are similar to those reported previously by us in the ambulant animal in a study that did not examine drug effects ($[DA]_p=40\pm6$ nM, $V_{max}=2.17\pm0.29$ μM/s, average $r=0.93$) (Garris et al., 1997). Using identical stimulation parameters, it was also shown in the same study that urethane does not alter the overall shape of the evoked response, the ability of the kinetic model to describe the response or DA uptake, but reduces DA release by a factor of about two. DAT activity, however, may be decreased by other anesthetics such as chloral hydrate (Sabeti et al., 2000) and equithesin (Kiyatkin et al., 2000).

Experiment 1: drug-induced changes in DAT and DA autoreceptor activity

The goal of experiment 1 was to quantify the pharmacological control of DAT by nomifensine and DA autoreceptors by haloperidol in the freely moving animal. Effects of the DA uptake inhibitor are shown in Fig. 2 for an individual frequency series describing evoked DA (nomifensine, closed circles). The dose, 7 mg/kg i.p., is moderate and much lower than that (25 mg/kg) used in an early study by us investigating this drug under urethane anesthesia (Kawagoe et al., 1992). The high dose (25 mg/kg) used previously in anesthetized animals was lethal in the present experiments with awake animals. The moderate dose (7 mg/kg) robustly increased evoked DA at all fre-

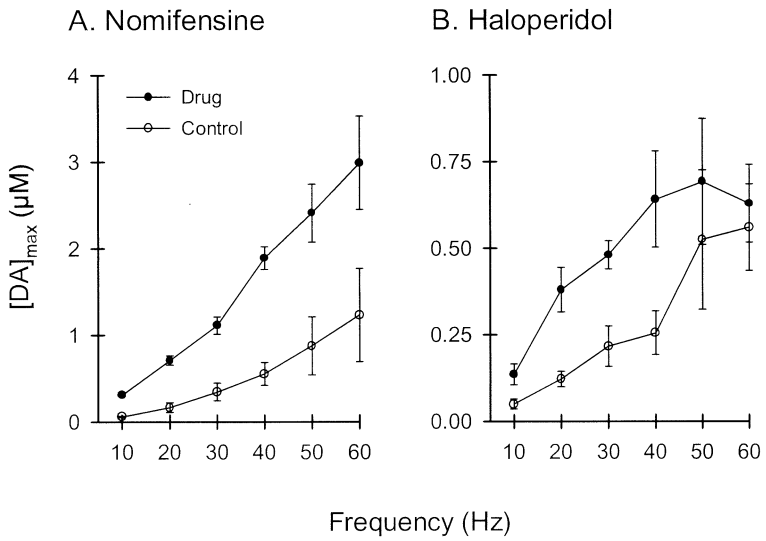

Fig. 3. Drug effects on the frequency dependence of evoked DA levels. $[DA]_{max}$ during each stimulation was determined and averaged for each frequency, and plotted for control (open circles) and drug (solid circles) groups. Data are the mean±S.E.M. (n=4). (A) Effects of nomifensine. (B) Effects of haloperidol.

quencies as demonstrated by individual responses and averaged values for $[DA]_{max}$ (Fig. 3A: drug, closed circles). Statistical analysis of compiled results yielded main effects of drug ($F_{1,18}$=41.32, P<0.001) and frequency ($F_{5,18}$=7.48, P<0.001) but no interaction ($F_{1,5}$=2.33, P>0.1). Kinetic analysis demonstrated that the increase in DA levels following nomifensine administration is suitably modeled by an 11.5-fold increase in K_m (2.2±0.49 μM average r=0.86), which is comparable to our recent work in the anesthetized animal at a dose of 10 mg/kg i.p. (Wu et al., 2001). Taken together, these results strongly suggest that nomifensine increases $[DA]_{max}$ in the freely moving animal by altering DAT activity.

Frequency-dependent effects of haloperidol on electrically evoked DA in freely moving animals are shown in Fig. 3B. Similar to nomifensine, these responses were averaged from individual recordings (data not shown). Administration of a moderate dose of the dopaminergic antagonist (0.5 mg/kg i.p.) elicited marked increases in $[DA]_{max}$ (closed circles). Statistical analysis demonstrated main effects of drug ($F_{1,24}$=51.12, P<0.0001) and frequency ($F_{5,24}$=3.28, P<0.05), with a significant interaction ($F_{1,5}$=2.69, P<0.05). Only the mid-range frequencies were statistically different from controls (P<0.01 for 20, 30 and 40 Hz). The significant interaction term for haloperidol but not for nomifensine also indicates different drug action. Consistent with this finding is that haloperidol effects on evoked DA could be suitably modeled by an increase in $[DA]_p$ (average r=0.735). Compared with the average value for pre-haloperidol baseline recordings (43±12 nM), the increase was 42% (62±13 nM) and significant (P<0.05; paired t-test), and similar to our previous work in the anesthetized animal at an identical dose (Wiedemann et al., 1992). Because autoreceptors governing DAT have also been identified (Hoffman et al., 1999), the increase in $[DA]_p$ must be considered a semi-quantitative measure of the negative feedback mechanism. Resolving a simultaneous change in more than one parameter is very difficult and beyond the scope of the present study. Nevertheless, these results suggest that haloperidol increases $[DA]_{max}$ in the freely moving animal by altering DA autoreceptor activity.

Technical considerations for experiment 2

The relationship between drug-induced changes in presynaptic mechanisms and behavior was evaluated in experiment 2. An important concern for this experiment is whether electrical stimulation, which must be applied to quantify altered DAT and autoreceptor function, affects the drug-induced behavior under study. The robust parameters typically used in anesthetized animals markedly alter behavior in awake animals, in large part because the stimulus train appears to be strongly aversive (Garris et al., 1997). Reducing stimulus parameters to levels used in the present study prevents the marked reaction but does not eliminate all evoked behavior. Immediately coincident with the pulse train is a fast head turn, which is followed by a brief (few seconds) episode of behavioral activation characterized by head bobbing, sniffing, grooming and rearing (Garris et al., 1999; Kilpatrick et al., 2000). To determine potential artifacts of the electrical stimulation, we compared drug-induced behavior with and without application of the pulse train.

Fig. 4A compares drug-induced behavior between animals that had not undergone surgery (i.e. unoperated) and those stimulated with a 30 Hz train of pulses (0.4 s, 125 μA) at intervals as in Fig. 1 (i.e. operated). Nomifensine-induced motor activation was almost identical in the two groups of animals (panel A). The activation score increased rapidly after drug administration, reached a plateau by 20 min and decreased at the last time point. Statistical analysis demonstrated significant changes in behavior over time ($F_{5,48}$=26.76, P<0.001) but no effect of

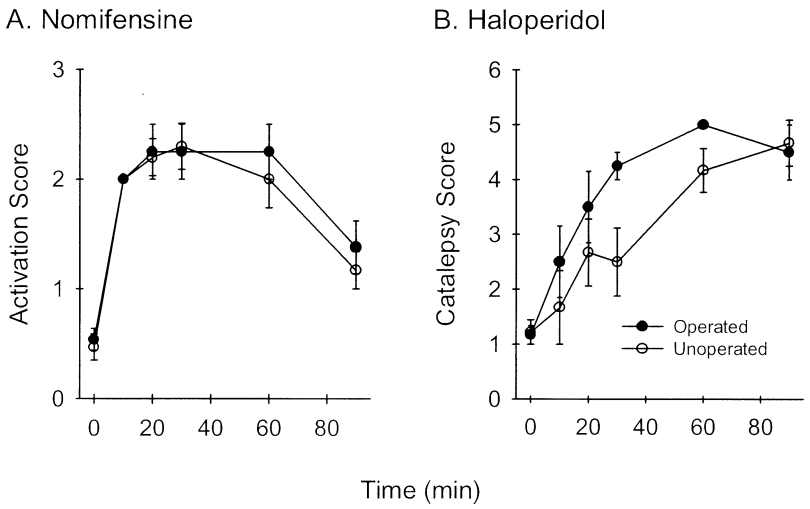

Fig. 4. Effects of the voltammetric measurements on behavior. Patterns of motor activation and catalepsy were measured in two groups of animals as described in Experimental Procedures. The first group was animals with simultaneous voltammetric measurements of DA (operated) and the second group was animals not surgically prepared for the voltammetric measurements (unoperated). Data are the mean±S.E.M. (n=4–6). (A) Nomifensine-induced motor activation. (B) Haloperidol-induced catalepsy.

animal group ($F_{1,48}$=0.52, P>0.95). The effects of haloperidol on catalepsy under identical experimental conditions are shown in Fig. 4B. The progression of behavior was similar in both electrically stimulated and unoperated rats, as catalepsy gradually increased during the course of the experiments (main effect of time: $F_{5,48}$=13.59, P<0.001). Although peak catalepsy score reached a similar level at the end of the time course, there was a significant effect of animal group ($F_{1,48}$=5.03, P<0.05), as development of catalepsy was initially faster in electrically stimulated rats. However, the interaction between time and electrical stimulation was not significant ($F_{1,5}$=0.92, P>0.50), preventing further analysis of the stimulation effect but supporting the conclusion that haloperidol induced a similar time course of behavior in both animal groups.

Thus, the results show that electrical stimulation only slightly alters the time course of drug-induced motor activation and catalepsy. The basis for the behavioral differences following haloperidol in stimulated and unoperated rats is unknown. The stimulating electrode may have destroyed some DA neurons, perhaps leading to receptor supersensitivity as seen in 6-hydroxydopamine-lesioned animals (Heikkila et al., 1981). However, supersensitivity requires substantial denervation, and the nigrostriatal tract appears largely intact given the robust evoked DA signals. Although the nomifensine response is unaffected by the stimulating electrode, fewer DAT sites to target in denervated tissue could be offset by a compensatory increase in postsynaptic receptors.

Experiment 2: relationship between $[DA]_{max}$ and behavior

The goal of experiment 2 was to evaluate the relationship between drug-induced changes in DAT or DA autoreceptor activity, and animal behavior. Changes in these presynaptic mechanisms were indexed by $[DA]_{max}$, which in the case of nomifensine reflects altered DAT function and in the case of haloperidol reflects altered DA autoreceptor function according to the analysis above. Qualitative inspection of the effects of nomifensine on $[DA]_{max}$ in awake animals (Fig. 3A) and activation score (Fig. 4A) indicated similar changes in both measures. This relationship is more clearly seen in Fig. 5 as both measures were characterized by a rapid increase followed by a somewhat slower decline. Data for this figure were obtained from Fig. 1A (solid circles), and behavioral data were the mean of values in Fig. 4A. Pooling of behavior data are supported by the lack of statistical difference between stimulated and unoperated animals. On the other hand, qualitative inspection of the effects of haloperidol on $[DA]_{max}$ in awake animals (Fig. 1B) and catalepsy score (Fig. 4B) indicated a relationship opposite to that for nomifensine. Indeed, as shown in Fig. 5B, the peak effect of the dopaminergic antagonist occurs early in the time course for $[DA]_{max}$ but later for behavior, with gradual changes in between. Similar to Panel A, data were obtained from Figs. 1B and 4B. However, because of statistical differences between stimulated and unoperated animals, only the behavioral data from the former group were included to illustrate the effects of haloperidol.

To evaluate the relationship between $[DA]_{max}$ and behavior quantitatively, the two measures were correlated. As shown in Fig. 6A, time courses for $[DA]_{max}$ and motor activation following nomifensine overlapped considerably. Indeed, statistical analysis demonstrated a strong, positive correlation between the two measures (r=0.99). A similar analysis is shown for haloperidol and behavior in Fig. 6B. Interestingly, there was a strong, inverse correlation between $[DA]_{max}$ and catalepsy (r=−0.96). Data for Fig. 6 were obtained from Fig. 5 except that the pre-drug (0 min) time point was not used in the analysis, because we were interested in drug effect, not baseline. These results demonstrate a tight temporal relationship between changes in

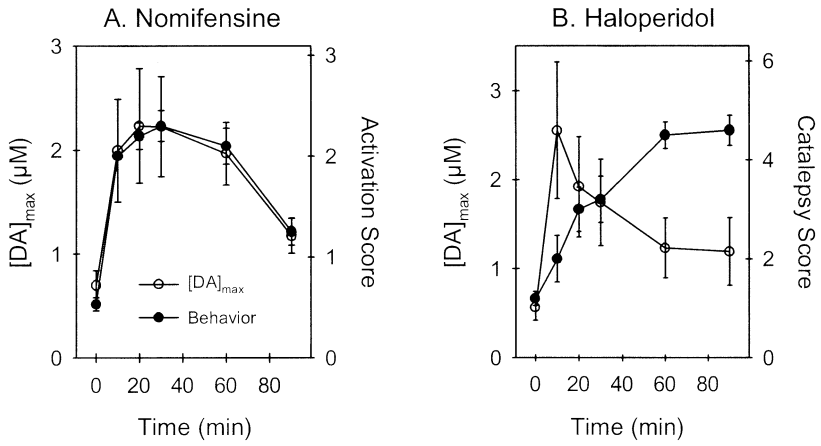

Fig. 5. Qualitative comparison of drug-induced increases in DA and behavior. Data are the mean±S.E.M. (n=4–10) and were compiled from Figs. 3 and 4. (A) Nomifensine-induced increases in $[DA]_{max}$ and motor activation. (B) Haloperidol-induced increases in $[DA]_{max}$ and catalepsy.

$[DA]_{max}$ and the DA-related behaviors elicited by both nomifensine and haloperidol.

DISCUSSION

The present study investigated the kinetic, neurochemical and behavioral effects of the DA uptake inhibitor, nomifensine, and the dopaminergic antagonist, haloperidol, in the freely moving animal. In the first experiment, both drugs were found to increase electrically evoked DA levels in the CP. Urethane anesthesia slowed these time-dependent changes in $[DA]_{max}$. Kinetic analysis demonstrated that nomifensine and haloperidol increased the evoked DA signal by inhibiting the presynaptic activity of DAT and DA autoreceptors, respectively. The voltammetric measurements were compared with behavior in the second experiment. The electrical stimulation, required to evaluate presynaptic drug effects, was shown to have minimal effects on behavior. A close temporal association was also observed between $[DA]_{max}$ and motor activation produced by nomifensine and catalepsy produced by haloperidol. Taken together, the results provide new insight into central drug action and DA-behavioral relationships.

Relationship of drug-induced changes in DA and behavior

The positive correlation between the pattern of motor activation following nomifensine and $[DA]_{max}$ is remarkable (Fig. 6A). Apparently, increased basal levels of extracellular DA produced by nomifensine-induced blockade of DAT (Nomikos et al., 1990; Nakachi et al., 1995) lead to DA receptor stimulation, and ultimately motor activation. The correlation between the evoked signal measured by voltammetry and behavior is therefore strong, because $[DA]_{max}$ reflects altered presynaptic DAT activity in real time. Although we measured evoked signals only in the CP, systemic administration of psychomotor stimulants also increases DA levels in projection fields of mesolimbic dopaminergic neurons (Kawagoe et al., 1992; Wu et al., 2001). Thus, the observed motor activation most likely reflects the combined responses of midbrain dopaminergic systems

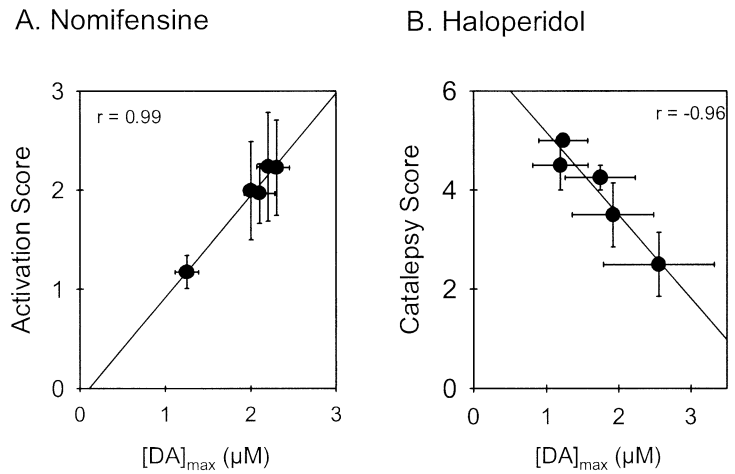

Fig. 6. Quantitative comparison of drug-induced increases in DA and behavior. Data are the mean±S.E.M. (n=4–10) and were obtained from Fig. 5 as described in the text. (A) Correlation of nomifensine-induced increases in $[DA]_{max}$ and motor activation. (B) Correlation of haloperidol-induced increases in $[DA]_{max}$ and catalepsy.

(Costall et al., 1972; Kuczenski et al., 1991; Whishaw et al., 1992).

The negative correlation between haloperidol-induced changes in catalepsy and $[DA]_{max}$, in this case an index of presynaptic autoreceptor activity, indicates a more complex mechanism of action than that for nomifensine. An interplay between DA autoreceptors and postsynaptic receptors, whose blockade would produce opposite behavioral effects, is anticipated to contribute to the observed catalepsy induced by haloperidol. The rapid increase in voltammetric signal indicates that haloperidol quickly enters the brain, binds to presynaptic autoreceptors and disinhibits DA synthesis and release (Starke et al., 1989; Wolf and Roth, 1990), which increases both levels of electrically evoked DA (Wiedemann et al., 1992) and basal DA levels in between the application of stimulus trains (Zetterstrom et al., 1984). The initial elevation of extracellular DA is apparently able to compete with haloperidol for postsynaptic receptor sites, because development of catalepsy is delayed even though the drug has reached the terminal region. As suggested from microdialysis studies, the response to autoreceptor blockade undergoes rapid tolerance (Di Chiara and Imperato, 1985; Imperato and Di Chiara, 1985). Progressively decreasing DA levels then appear to enable the neuroleptic to compete more favorably for postsynaptic receptors, eventually leading to catalepsy. We therefore propose that the close but inverse temporal correlation observed between $[DA]_{max}$ and catalepsy arises because voltammetrically measured DA reflects the competition at postsynaptic receptors between haloperidol and endogenous DA elevated by disinhibited presynaptic autoreceptors.

The observed link between the evoked voltammetric signal and behavior strongly supports the notion that dopaminergic mechanisms play a primary role in mediating the behavioral effects of both nomifensine and haloperidol. These results are contrasted with the reported discrepancies between dialysate DA and motor activation produced by psychomotor stimulants (Sharp et al., 1987; Kuczenski et al., 1991; Nakachi et al., 1995; Badiani et al., 1998) or catalepsy produced by neuroleptics (Zetterstrom et al., 1984; Imperato and Di Chiara, 1985; Osborne et al., 1994). One possible explanation for these discrepancies is that traumatized tissue surrounding the dialysis probe (Benveniste and Diemer, 1987; Clapp-Lilly et al., 1999) distorts apparent DA dynamics (Yang et al., 1998; Lu et al., 1998). Because of its smaller size, the voltammetric microsensor causes considerably less damage (Allen et al., 2001). Indeed, in a study directly comparing the two techniques, we showed that the evoked voltammetric signal correlated well with motor activation following GBR 12909 administration whereas DA levels measured by microdialysis did not, reaching a plateau over 1 h after that for the behavior (Budygin et al., 2000). Thus, taken together, the available evidence indicates that the origin of the reported discrepancies between psychomotor stimulant- and neuroleptic-induced changes in dialysate DA and behavior is a sampling artifact, and unrelated to a non-dopaminergic drug mechanism.

Central drug action and DA-behavior relationships

The present study suggests that the behavioral effects of systemically applied nomifensine and haloperidol are mediated at least in part by altered DA presynaptic control. Because all of the multiple effects exerted by the drugs on dopaminergic signaling potentially contribute to the behavioral status of the animal, the extraordinary association between a single mechanism and either motor activation or catalepsy is quite surprising. The correlation for nomifensine is especially interesting given that the drug elicits opposite changes at dopaminergic somatodendrites and terminals. Presynaptic DAT blockade apparently overcomes the inhibition of soma firing rate (Mercuri et al., 1991; Engberg et al., 1997) to elevate extracellular DA in the projection field. Although haloperidol acts in unison at both ends of the dopaminergic neuron to enhance neurotransmission (Chiodo and Bunney, 1983; Bunney et al., 1991; Wiedemann et al., 1992), it is not known whether soma electrical activity tracks catalepsy in the same manner as demonstrated here for evoked DA. In any event, altered presynaptic autoreceptor function favorably predicts catalepsy presumably by modifying the efficacy of haloperidol for blocking postsynaptic receptors through elevated extracellular DA. This result is quite unexpected in that a proximal site, presynaptic autoreceptors, drives the most immediate drug target underlying catalepsy, postsynaptic receptors.

Similar to catalepsy, it is thought that neuroleptics are effective in treating schizophrenia by interrupting dopaminergic neurotransmission at the postsynaptic level (Seeman et al., 1976). Consequently, our result may have important implications for the management of schizophrenics, because it also suggests that the onset of haloperidol's neuroleptic action in humans is delayed by the drug's presynaptic effect (Phillips et al., 2001). Although mechanisms are different, a parallel can thus be drawn between haloperidol and selective serotonin reuptake inhibitors (SSRIs), whose therapeutic effects are delayed by serotonin 1A autoreceptor stimulation (Artigas et al., 2001). Inhibition of serotonin uptake increases extracellular levels of the neurotransmitter in the vicinity of raphe cell bodies, which in turn inhibits cell firing via the serotonin 1A autoreceptor and ultimately decreases serotonergic control of target cells in project fields. For this reason, serotonin 1A receptor antagonists have been proposed to accelerate the action of SSRIs (Artigas et al., 2001). Similarly, it is possible that delayed neuroleptic action could be countered by developing and co-administering selective antagonists of the DA autoreceptor or developing neuroleptics with less DA autoreceptor activity.

While the correlation that we observe between the evoked DA signals and the animal's change in behavior is strong, it obviously does not indicate whether or not the dopaminergic neurons that are being measured are driving the behaviors. Rather, the evoked DA signal provides an index of the time scale of action of the drug on a group of presynaptic dopaminergic terminals in the brain. This time scale is a composite of the rate at which the drug reaches the terminal as well as the time required to exert its effect. For

nomifensine, the effect is simply inhibition of the transporter. For haloperidol the effect is more complex since it involves competition between pre- and postsynaptic effects. However, for each drug their rates and associated mechanisms of action are likely similar throughout the brain. Furthermore, in the case of nomifensine, similar actions are exerted at noradrenergic as well as dopaminergic terminals.

CONCLUSIONS

The major finding of the present study is that presynaptic DAT and DA autoreceptor activity appear to be behaviorally relevant targets of systemically applied psychomotor stimulants and neuroleptics, respectively. This finding was made possible by a voltammetric microprobe with millisecond temporal resolution, and its use in the awake animal to assess release and uptake, two key mechanisms of dopaminergic neurotransmission, in a behavioral context. In addition to identifying the central action of drugs prescribed for the treatment of a wide variety of debilitating neuropathologies such as schizophrenia, Tourette's syndrome, depression, attention deficit disorder and narcolepsy (Julien, 1985), the results also indicate that presynaptic mechanisms may play a more important role in DA-behavior relationships than is currently thought. Future studies should investigate other dopaminergic drugs, especially more selective D2 antagonists; dose dependency, although high doses of psychomotor stimulants may be precluded from evaluation; and a wider time window, both before and after the measurements described herein. The rapid tolerance of DA autoreceptor blockade should also be further characterized.

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