Quantitative Voltammetric Analysis in the Brain: Functional Significance of Tonic Extracellular Dopamine Levels

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Extracellular (EC) dopamine (DA) levels in several regions of the brain are implicated in the rewarding effects of psychostimulants, including cocaine. These DA levels can be referred to as either tonic (long term) or phasic (short term). Microdialysis and voltammetry are currently the most popular methods to determine EC DA levels *in vivo*. Recently voltammetric work has demonstrated increases in phasic DA following uptake inhibition. Microdialysis studies have monitored a longer term increase in dialysate DA following uptake inhibition, but it has yet to be confirmed whether this is a phasic or tonic increase. The voltammetric procedures in our laboratory are designed to monitor tonic DA levels. In our lab, cocaine and nomifensine only increase tonic DA levels when preceded with a D2 antagonist. In this dissertation, I will discuss my work in determining the role of tonic DA levels following uptake inhibition and the functional significance of tonic DA.

Initially I investigated the effect of cocaine and nomifensine on tonic EC DA levels in both the striatum and the nucleus accumbens brain regions of anesthetized animals. Cocaine and nomifensine only increased tonic DA levels when preceded with the D2 antagonist, raclopride. Similar results were found in unanaesthetized animals, leading to the conclusion that the lack of response following uptake inhibition was not an anesthetic effect. These findings introduce the novel concept that tonic DA levels in the striatum and nucleus accumbens are stabilized following uptake inhibition, and this stabilization involves the D2 receptors.

Additionally, voltammetric recordings were conducted in the nucleus accumbens of an unanesthetized rat that received cocaine after pretreatment with raclopride in order to evaluate cocaine-induced hyperactivity. The combination of raclopride and cocaine produced two treatment outcomes, a tonic increase in EC DA levels and locomotor activation. However, these treatment outcomes were mutually exclusive in that individual animals exhibited one outcome or the other but, with one exception, not both. This data suggests that tonic elevations of EC DA suppress cocaine-induced hyperactivity. By quantitatively measuring DA levels via voltammetry, we observe an entirely new phenomenon: an excess of DA in the EC space decreases activity.

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PREFACE

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1.0 EVIDENCE FOR A TONIC/ PHASIC DOPAMINE SYSTEM

1.1 INTRODUCTION

Dopamine has been characterized as an important neurotransmitter that is implicated in various diseases, including Parkinson's, schizophrenia, and drug abuse (Koob and Bloom 1988; Carlsson and Carlsson 1990; Carlsson *et al*. 1991). My research has focused on drug abuse, specifically cocaine, and its effects on DA levels in the extracellular space (ECS) of the brain. Cocaine inhibits the DA transporter which is responsible for removing DA from the ECS and is also suggested to play a role in releasing DA into the ECS by means of reverse transport (Zahniser *et al.* 1999; Leviel 2001; Borland and Michael 2004)

DA concentrations in the ECS are maintained by a balance between the rates of dopamine release and uptake (Wightman and Zimmerman 1990). It is well established that DA uptake occurs through the transporter, but the mechanisms of release are not as clearly understood. Over the past 50 years, there have been multiple studies regarding the release of DA (Giorguieff *et al.* 1977; Raiteri *et al.* 1979; Grace 1991; Desce *et al.* 1992; Leviel 2001). Many of these studies suggest the possibility of two modes of DA release. These modes of release include a tonic (long term) and phasic (short-term) type.

1.2 HISTORICAL SUPPORT

Historically the terms tonic and phasic referred to the type of release, where tonic release was attributed to release from the DA transporter (DAT) and phasic release was viewed to be an exocytotic process that was initiated by neuronal firing. Since phasic release was dependent upon an action potential, it was sensitive to tetrodotoxin (TTX), a neurotoxin which blocks action potentials in nerves. Tonic DA release was therefore termed TTX-insensitive.

The idea of two modes of release was strengthened by experiments performed in both synaptosomes and brain slices which exhibited both TTX-sensitive and TTX-insensitive DA release (Giorguieff *et al.* 1977; Raiteri et al. 1979; Desce *et al.* 1992). Based upon multiple studies, Grace proposed a theory for schizophrenia where he described how both tonic and phasic DA function together to affect DAergic neurotransmission (1991).

Grace's theory involved both tonic (impulse independent) and phasic (impulse dependent) DA release. He suggested, based on previous experiments that tonic release was mediated by corticostriatal glutamate projections (Roberts and Sharif 1978; Roberts and Anderson 1979; Clow and Jhamandas 1989) and was responsible for the steady state (basal) levels of DA in the ECS. On the other hand, DA released in the phasic mode is most likely removed rapidly by uptake and therefore would not be expected to contribute to ECS DA levels (Holz and Coyle 1974; Tassin et al. 1974). Grace's theory concludes that there is a gating mechanism between tonic and phasic DA. When tonic levels are too high, the phasic DA responses are filtered. Contrarily, a prolonged low level of tonic DA, as is believed to occur in schizophrenia, causes the phasic release to be hyper-responsive.

Although this theory has received a great deal of attention, there is conflicting evidence from microdialysis studies. These studies indicated that all DA release is TTX-sensitive or

impulse dependent (Westerink et al. 1987; Westerink and de Vries 1988). Conversely, Grace's theory suggested that tonic release was TTX-insensitive. However, microdialysis studies suggested that all DA was TTX-sensitive and therefore, the term tonic no longer characterized long term release. Following this discovery, the term tonic began to characterize pacemaker firing. We believe this is a misnomer because pacemaker firing involves a lone action potential firing at a regular interval. This type of firing involves rapid release and clearance of DA. Due to the swift reuptake of DA released by pacemaker firing, the DA spends a minimal amount of time in the ECS space. Nonetheless, pacemaker firing can not account for the gating mechanism described by Grace. In the gating mechanism proposed by Grace, tonic DA provides a homeostatic regulation and underlies the background level of DA in the ECS. Only a large steady level of DA would be able to play this role; whereas pacemaker firing involves DA rapidly released and taken back up into the terminal.

For our purposes, any reference to tonic DA will refer to DA that remains in the ECS for a significant amount of time. It is most likely that this DA enters the extra-synaptic space through reverse transport. DA transporters are located extra-synaptically, thereby releasing DA directly into the extra-synaptic space and not into the synaptic cleft. Phasic DA will refer to DA that is quickly released and then quickly taken back up into the synapse. This process is most likely due to a neuronal impulse causing vesicular DA to be released into the synaptic cleft. It is possible for non-impulse dependent DA to be taken up quickly and therefore be categorized as phasic. Similarly, DA released by a neuronal impulse may escape the synaptic cleft before it is taken up by a transporter and therefore be referred to as tonic DA. To summarize, I will use the terms tonic and phasic to refer to a type of DA present in the ECS not as a type of release. Tonic

DA will represent DA that is long-lasting in the ECS, while phasic DA is transient or DA that spends a short time in the ECS because it is rapidly taken back up.

1.3 VOLTAMMETRIC EVIDENCE

Voltammetry provides compelling evidence for the existence of a tonic/phasic DA system. In 2002, Wightman and colleagues presented the first voltammetric data that displayed "subsecond fluctuations in DA concentrations" or phasic DA (Robinson *et al.* 2001). In our laboratory, experiments have been conducted to determine the concentration of tonic DA levels (Kulagina *et al.* 2001; Peters *et al*. 2002; Khan and Michael 2002; Borland *et al.* 2004; Borland and Michael 2005; Mahon *et al* 2005). In order to investigate phasic and tonic DA via voltammetry there are specific procedures involved in selectively monitoring either tonic or phasic DA levels.

In order to detect phasic DA, the electrode must be optimally placed at a location in the brain that has a large amount of DA ("hotspot") present. This "hotspot" is determined by the DA released following an electrical stimulation. The 50-160 μm carbon fiber microelectrode is lowered at 75 μm increments in order to find a "hotspot." Only when there is a detectable response at the electrode is the placement optimal (Robinson *et al.* 2003). It is speculated that the "hotspot" may correspond to a rapidly firing DA neuron. Once the location of the carbon fiber microelectrode is optimized, voltammetry is able to detect phasic DA. The voltammetric parameters that are used with the detection of phasic release (transients) are extremely sensitive and have a DA detection limit if 13 nM in awake rats (Cheer et al. 2004). This system is suitable for the detection of small transient changes, but can not be used for long term detection. If data

collection exceeds two minutes, there is too large of a drift in the baseline and transients can not be determined, even with the use of a high-throughput algorithm (Heien *et al.* 2005).

Studies have shown that DA transients can be naturally occurring without an overt sensory input or any specific behavior. Both the presence and frequency of transients increased following uptake inhibition or an exposure to a salient stimulus (i.e. food, noise) in an awake rat (Robinson and Wightman 2004). For example, following the administration of nomifensine, the number of transients increased 570% in the nucleus accumbens (Robinson and Wightman 2004). Additionally, evidence suggests that fluctuations in phasic DA release corresponded to DA signaling during behavior (Robinson et al. 2001, 2002; Phillips et al. 2003; Cheer et al. 2004).

In our laboratory we are detecting sustained DA levels or tonic DA. We utilize microelectrodes that are between 300-400 μm in length and we do not optimize the electrode's placement. The data was subjected to a 41-point averaging to eliminate non-DA spikes caused by movements of the animal. However this averaging may also eliminate any transients present in the recorded signal.

Our voltammetric method has allowed us to investigate various aspects of the DA system, including the basal level of TTX-insensitive DA and the response to uptake inhibition at various distances from a microdialysis probe (Kulagina *et al.* 2001; Borland and Michael 2004; Borland *et al.* 2005). Additionally, preliminary studies have investigated the effect of uptake inhibition on the tonic level of DA (Peters *et al.* 2002; Khan and Michael 2003; Mahon *et al.* 2005). The studies involving uptake inhibition are further detailed in the following two chapters.

1.4 VOLTAMMETRIC AND MICRODIALYSIS DISCREPANCIES

Although microdialysis does not detect TTX-insensitive DA under basal conditions, voltammetry is able to detect a large pool (~2.0 µM) of basal TTX-insensitive DA (Borland and Michael 2004). Microdialysis only detects TTX-insensitive DA when the probe is initially implanted, and it is suggested that this TTX-insensitive DA is due to damage that is caused by the initial implantation. For this reason, when conducting microdialysis experiments, researchers wait 24 hours after probe implantation to begin collecting data (Benveniste *et al.* 1987). Although it is possible that damage causes TTX-insensitive DA, this does not mean that all TTX-insensitive DA is caused by damage.

Discrepancies also exist over the concentration of basal DA levels in the brain. Microdialysis indicates that the basal level of DA in the brain is ~5 nm (Smith and Justice 1994), but recent voltammetric data puts the level at ~2.0 µM (Borland and Michael 2004). It is unknown why these discrepancies exist between microdialysis and voltammetry, but previous experiments performed in our laboratory suggest a gradient of DA release in the tissue surrounding a microdialysis probe (Borland *et al.* 2005). When a microelectrode was placed 200 µm from a microdialysis probe, both devices had a similar responses to uptake inhibition. This response was not detected by a microelectrode that was placed 1 mm away from the microdialysis probe (Borland *et al.* 2005). We believe these discrepancies may be due to tissue disruption surrounding the microdialysis probe (Clapp-Lilly *et al*. 1999; Zhou *et al.* 2001).

1.5 KINETIC EVIDENCE

Extensive work has been conducted on the uptake and release kinetics of the DA system, but two distinct results have been seen regarding the rate of uptake. Wightman and colleagues report rapid uptake in both the nucleus accumbens and the striatum with studies utilizing voltammetry to monitor the disappearance of electrically evoked DA release (Jones et al. 1995). Their specific kinetic parameters of DA uptake as determined by non-linear regression of the Michaelis-Menten equation were a K_T of 0.22 μM and V_{max} of 3.8 μM/sec. Zahniser and colleagues employed pressure injection of exogenous DA to investigate the release and uptake kinetics of the DA system and they reported a much slower rate of uptake, K_T of 2.5 μM and V_{max} 0.14 μM/sec. (1999). In Wightman's calculation of the K_T, he assumes the basal level of DA to be zero. According to previous studies conducted in our laboratory, the basal level of DA is 2.0 μM (Borland and Michael 2004). If Wightman had calculated the K_T based on a basal level of 2.0 μM DA, the resulting K_T would have been 2.2 μM, which is similar to Zanhiser's calculation.

1.6 CENTRAL HYPOTHESIS

Due to the various lines of evidence indicating the existence of tonic DA, my central hypothesis is that tonic DA levels are controlled by the D2 receptors following uptake inhibition. I investigate how this control is established and the functional significance of tonic DA. In Chapter 2, I discuss the effect of uptake inhibition on tonic DA levels in both anesthetized and unanaesthetized rats. I also examine the function of D2 receptors in the stabilization of tonic

DA. In Chapter 3, the behavioral consequences of tonic DA are explored as well as the roles of the D1 and D2 receptors.

2.0 CONTROL OF TONIC EXRACELLULAR DOPAMINE CONCENTRATIONS BY D2 AUTORECEPTORS FOLLOWING UPTAKE INHIBITION

2.1 ABSTRACT

The psychostimulant properties of dopamine uptake inhibitors are attributed to their impact on extracellular dopamine levels in several regions of the brain. Dopamine uptake inhibitors increase the frequency and amplitude of rapid, phasic dopamine-related voltammetric transients. However, the literature is not clear on the effects of these drugs on slower, tonic dopamine-related voltammetric signals. In this study, extracellular dopamine was monitored in the rat striatum and nucleus accumbens using voltammetric procedures adapted to monitoring tonic dopaminergic processes. We tested the hypothesis that tonic dopamine levels are stabilized after uptake inhibition by a decrease in the velocity of dopamine release by a mechanism that involves dopamine D2 autoreceptors. The present study examines the effect of cocaine and nomifensine on tonic dopamine levels in anesthetized and unanesthetized rats pretreated with the D2 antagonist raclopride. Cocaine and nomifensine concentration increased tonic dopamine levels in rats pretreated with D2 antagonists. These findings are consistent with the concept that tonic dopamine levels in the striatum and nucleus accumbens are controlled following inhibition of the dopamine transporter and that the mechanism of that stabilization involves D2 autoreceptors.

2.2 INTRODUCTION

Cocaine and nomifensine are two of several drugs that inhibit the dopamine transporter (DAT), a transmembrane protein that deactivates the neurotransmitter dopamine (DA) by transporting it from the brain extracellular space (ECS) to the cytoplasm of DAergic cells. The transporter plays a critical role in DAergic neurotransmission by contributing to the regulation the amount of DA in the ECS to interact with pre- and postsynaptic DA receptors. Drugs that inhibit the transporter exhibit psychostimulant properties and cocaine in particular is a widely abused substance. Hence, it is significant to understand how DAT inhibition (DAT-I) affects brain ECS DA concentrations and DAergic neurotransmission. Microdialysis and voltammetry are techniques that have been applied to the determination of ECS DA levels. DAT-I increases the microdialysis efflux of DA from the striatum and nucleus accumbens (Church et al. 1987; DiChiara and Imperato 1988; Nakachi et al. 1995; Pontieri et al. 1995; Bradberry et al. 2000), two forebrain structures that receive dense DAergic projections from the midbrain. Furthermore, DAT-I increases the amplitude and duration of electrically evoked (Chergui et al. 1994; Suaud-Chagny et al. 1995; Wu et al. 2001; Greco and Garris 2003) and spontaneous (Phillips et al. 2003; Robinson and Wightman, 2004) DA transients recorded with high-speed in vivo voltammetry, suggesting that DAT-I alters phasic DAergic signaling in the brain. When suitably time-averaged, the high-speed in vivo voltammetry data agree qualitatively and quantitatively with the microdialysis results, supporting the conclusion that the microdialysis and high-speed voltammetry techniques provide information about phasic DA release after DAT-I (Heien et al. 2005).

However, considerable attention has been paid to the concept that DAergic neurotransmission might involve both phasic and tonic processes that occur on relatively fast

(second to second) and relatively slow (minute to minute) time scales, respectively (Grace 1991; Nicholson 1995; Cragg et al. 2001; Schmidtz et al. 2001; Goto and Grace 2005). In previous studies, we demonstrated the use of voltammetry to monitor a sustained decrease in ECS DA after the local infusion of kynurenate, a broadly-acting antagonist of ionotropic glutamate receptors, via pipets placed adjacent to voltammetric microelectrodes in the striatum of anesthetized rats (Kulagina et al. 2001; Borland and Michael, 2004). Since kynurenate caused a decrease in ECS DA from the basal level, and since this effect of kynurenate was insensitive to tetrodotoxin (TTX), our findings appear to suggest the presence of a tonic basal DA level in the striatal ECS. Thus, we hypothesize that this basal level of DA might be involved in a tonic component of DAergic transmission in the striatum.

In preliminary studies (Peters et al. 2002; Khan and Michael, 2003; Mahon et al. 2005), however, we were unable to obtain voltammetric evidence that DAT-I increases the level of ECS DA associated with the tonic DA component in the rat striatum, except in the case of animals pretreated with D2 antagonists, including sulpiride, haloperidol, and raclopride. In animals pretreated with a D2 antagonist, DAT-I induced robust and long-lasting, i.e. tonic, increases in DA (preliminary studies cited above; further results presented below). Thus, the tonic component of ECS DA in the striatum appears to respond differently to DAT-I compared to the phasic DA probed by the microdialysis and high-speed voltammetry techniques discussed above. The objective of the present investigation is to further understand the role of D2Rs in controlling the tonic component of ECS DA.

D2 receptors, including terminal autoreceptors (Usiello et al. 2000), modulate the kinetics of both DA release (Kawagoe et al. 1992; Kennedy et al. 1992; Benoit-Marand et al. 2001; Rougé-Pont et al. 2002; Wu et al. 2002) and uptake (Meiergerd et al. 1993; Cass and Gerhardt

1994; Mayfield and Zahniser 2001; Wu et al. 2002; Schmitz et al. 2003). Hence, the ability of the D2R to stabilize tonic DA near basal levels after DAT-I potentially could involve adaptations of both DA release and DA uptake. However, there is ample evidence in the literature to demonstrate that DAT inhibitors, including cocaine and nomifensine, slow the kinetics of DA uptake under in vivo conditions (Suaud-Chagny et al. 1995; Zahniser et al. 1999; Budygin et al. 2000; Kiyatkin et al. 2000; Wu et al. 2001; Garris et al. 2003; Sabeti et al. 2002), which would not lead to stabilization of DA levels after DAT-I. For this reason, an adaptive decrease in the velocity of DA release appears to be the most likely mechanism underlying the ability of D2Rs to control tonic ECS DA levels after DAT-I.

The present investigation was designed to address several aspects of the D2R-mediated control of tonic ECS DA after DAT-I in rats. First, we were interested to know if this mechanism is active in the NAc as well as the striatum, since the NAc is centrally involved in substance abuse (Koob and Nestler 1997; Wise 1998). Then, we were interested in confirming that the D2R control of tonic DA occurs in both unanesthetized as well as anesthetized animals, since several reports show that anesthetics affect DA systems (Sabeti et al. 2003). In conjunction with other experiments conducted in our laboratory, our findings are consistent with the idea that DA autoreceptors stabilize a tonic component of ECS DA concentrations in the striatum and NAc after DAT-I.

2.3 METHODS AND MATERIALS

2.3.1 Voltammetric electrodes and techniques

Voltammetric electrodes (7 µm in diameter, 400 µm in length) were constructed by sealing individual carbon fibers (Thornell, T-300, Amoco Performance Products, Inc., Greenville, SC, USA) into pulled borosilicate glass capillaries with a low-viscosity epoxy (Spurr embedding kit, Polysciences, Inc., Warrington, PA, USA). The reference electrode for experiments in unanesthetized rats was a 5-mm section of 500 µm diameter silver wire anodized at 1V vs. a platinum electrode for 25 s in 1 M HCl. The Ag/AgCl reference electrodes for calibration and for experiments in anesthetized rats were purchased from Bioanalytical Systems (West Lafayette, IN, USA). The electrodes were mounted in a flow system for pretreatment, testing, and calibration in artificial cerebrospinal fluid (aCSF: 144 mM Na^+, 1.2 mM Ca^{2+}, 2.7 mM K_+, 1.0 mM Mg^{2+}, 152 mM Cl^-, 1 mM Mg^{2+} and 2.0 mM PO_4^{3-} adjusted to pH 7.4 with NaOH). Electrodes were pretreated with a triangular potential waveform (0-2 V vs. Ag/AgCl, 200 V/s, 3 s), allowed to requilibrate to a stable voltammetric baseline, and stored in air until needed for in vivo measurements. Fast-scan cyclic voltammetry was performed with a high-speed potentiostat (EI-400, Ensman Instruments, Bloomington, IN). The applied potential was held at 0 mV vs Ag/AgCl between voltammetric scans. Scans comprised sequential linear voltage sweeps to 1000 mV, -500 mV, and back to 0 mV at 300 V/s. The voltammetric current was digitized at 40 kHz.

2.3.2 Identification of dopamine

The time course of responses was obtained from the current measured during successive scans in the potential window corresponding to the maximum DA oxidation current (typically near 700 mV vs Ag/AgCl). During experiments in unanesthetized animals, the time course traces were post-processed with a 41-point moving average to eliminate nonDA-related noise spikes associated with the movements of the animals. These noise features were most prominent after DAT-I induced hyperactivity. When drug treatment induced a voltammetric signal in vivo, background subtracted voltammograms were used to identify whether the response was DA-related (Borland and Michael, 2004). Responses were identified as DA-related when voltammograms exhibited DA-like oxidation and reduction peaks, near 700 mV and -100 mV, respectively for anesthetized rats. For unanesthetized rats, oxidation and reduction peaks were 800 mV and 0 mV, respectively. This difference is due to the differences in reference electrodes. DA-related voltammetric responses were converted to units of DA concentration by postcalibration of each electrode after its removal from the rat. Postcalibration was performed in room-temperature, nitrogen-purged aCSF: no corrections for the volume fraction or tortuosity of the ECS were attempted. Postcalibration was not performed in cases where postmortem histology was used to confirm the placement of the microelectrodes.

2.3.3 Drugs and Solutions

Chloral hydrate, atropine, raclopride, nomifensine maleate, dopamine, and cocaine hydrochloride were purchased from Sigma (St. Louis, MO, USA). All drugs were dissolved in phosphate

buffered saline (PBS: 354 mM Na^+, 154 mM Cl^-, and 100 mM HPO_4^{2-} adjusted to pH 7.4). Isoflurane was purchased from Easerling Vetrinary Supply (West Columbia, SC). Low odor 10% Formalin (Buffered Formalde Fresh®) was obtained from Fisher Scientific (Pittsburgh, PA). All solutions were prepared with ultrapure water (Nanopure, Barnstead, Dubuque, IA, USA).

2.3.4 Animals and procedures for voltammetry in vivo

All experiments involved male Sprague-Dawley rats (Hilltop, Scottsdale, PA, USA) (250-375g) and were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

2.3.4.1 Voltammetry in unanaesthetized rats

Aseptic surgery was performed 2 to 5 days prior to voltammetric recording. Rats were anesthetized with isoflurane as a vapor with oxygen through a trachea tube. Initial induction involved a 2% isoflurane vapor and maintenance was conducted at 0.5% isoflurane vapor. A small hole was drilled through the skull to permit a guide cannula with a locking ring (MD 2251, Bioanalytical Systems, Inc.) to be mounted over the nucleus accumbens. The tip of the guide cannula was lowered 1 mm below the brain surface. A Ag/AgCl reference electrode was placed in contact with the contralateral cortex. The cannula and reference electrode were anchored to the skull with jeweler's screws and acrylic cement. Experiments only involved those animals that appeared healthy after surgery and had recovered to at least 80% of their pre-surgical weight.

On the day of recording, a voltammetric electrode was mounted in a micromanipulator (UNC Chemistry Department, Chapel Hill, NC) that locked into the guide cannula (Rebec et al.

1993; Garris et al. 1997). The microelectrode and Ag/AgCl reference electrode were connected to a head-mounted preamplifier. All of the unanaesthetized rat experiments were conducted in the nucleus accumbens, therefore the microelectrode was lowered to a point 1.2 mm anterior to bregma, 1.4 mm lateral from midline, and 7.4 mm below dura (Paxinos and Watson 1998). The animals were tethered into a Raturn chamber (Bioanalytical Systems, West Lafayette, IN). Since the Raturn chamber was a novel environment for these animals, each recording session began with a habituation period lasting at least 1 1/2 hours. Typically, the animals briefly explored their new surroundings and resumed sleeping before the end of the habituation period.

2.3.4.2 Voltammetry in anesthetized rats

Rats were anesthetized with an initial dose of chloral hydrate (400 mg/kg i.p.) and received a single dose of atropine (0.1 mg/kg i.p.). Additional chloral hydrate was delivered as needed to maintain anesthesia. The rats were then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) and wrapped in a 37°C homoeothermic blanket (EKEG Electronics, Vancouver, BC, Canada). Holes were drilled through the skull in appropriate positions to permit placement of electrodes. A salt bridge provided the electrical contact between the brain and reference electrode. The voltammetric microelectrodes were positioned in the striatum (1.2 mm anterior to bregma, 2.5 mm from midline, and 6.0 mm below dura) or the core of the nucleus accumbens (1.2 mm anterior to bregma, 1.4 mm from midline, and 7.4 mm below dura) (Paxinos and Watson 1998). Recording began not less than 1 1/2 hr after electrode placement once a flat voltammetric baseline signal was established.

2.3.5 Experiments in unanaesthetized rats

2.3.5.1 Drug administration

Unanesthetized rats received either cocaine (30 mg/kg i.p.) or nomifensine (20 mg/kg i.p.) during voltammetric recording in the nucleus accumbens. Groups of rats were pretreated 30 min prior to inhibitor administration with vehicle solution (PBS) or raclopride (2 mg/kg i.p.).

2.3.5.2 Histology

After voltammetric recording, the rats were deeply anesthetized with chloral hydrate and the microelectrodes were used to make an electrolytic lesion to mark the microelectrode implantation site. The lesions were formed by connecting the electrodes to an 11-V power supply for 1 s. The animals were perfused transcardially with saline followed by 10% formalin and the brain was removed from the skull and stored in formalin for 2 days. The tissue was sliced into 40-µm sections, stained with cresyl violet, and inspected with a microscope. Lesioning destroyed the electrodes, so postcalibration could not be performed after histology.

2.3.6 Experiments in anesthetized rats

Voltammetric recording was performed in the striatum and nucleus accumbens. The rats were pretreated with vehicle solution (PBS) or raclopride (2 mg/kg i.p.) 30 min prior to receiving either cocaine (30 mg/kg i.p.) or nomifensine (20 mg/kg i.p.).

2.3.7 Data Analysis

Voltammetric responses were expressed as the change from baseline observed 200 sec prior to administration of the DAT inhibitors subtracted from those recorded over 200 sec near the maximal voltammetric response. The background-subtracted time course signals obtained in anesthetized animals were converted to units of DA concentration for background subtracted voltammograams that resembled the DA signal obtained in the post calibration. In unanesthetized animals, the time dependent signal was smoothed with a 41-point (16 sec) moving average to reduce occasional movement artifacts. Post calibrations were not performed in unanaesthetized animals in order to verify electrode placement. The presence of DA in both anesthetized and unanaesthetized animals was determined by the presence of the DA oxidation and reduction peaks in the background subtracted voltammogram. For statistical analysis, the average signal for groups of similarly treated rats was calculated at 10-min intervals from the time of administration of the DAT inhibitor. Statistical analysis was based on one-way or two-way ANOVA followed by Duncan's multiple range test.

2.4 RESULTS

2.4.1 Unanaesthetized rats

Neither cocaine (30 mg/kg i.p.) nor nomifensine (20 mg/kg i.p.) affected voltammetric signals in the nucleus accumbens of rats pretreated with vehicle (Figure 1; traces 2, 4). Background-subtracted voltammograms did not exhibit DA-like features (Figure 1, right panel). Both

inhibitors induced immediate and significant DA-related voltammetric responses in the nucleus accumbens of rats systemically pretreated with raclopride (2 mg/kg i.p.) (Figure 1; traces 1, 3). Nomifensine induced DA-related responses in six out of six raclopride-pretreated rats. However, cocaine induced DA-related responses in 5 out of 8 raclopride-pretreated rats. Background subtracted voltammograms exhibited DA-like oxidation and reduction peaks near 800 mV and 0 mV, respectively. Throughout this work, these oxidation and reduction peaks serve to identify DA-related voltammetric responses (see section on Validation, below). Results from the remaining 3 rats are not included in Figure 1. Histology confirmed that all the electrodes used to generate Figure 1 were correctly placed in the nucleus accumbens (Figure 2).

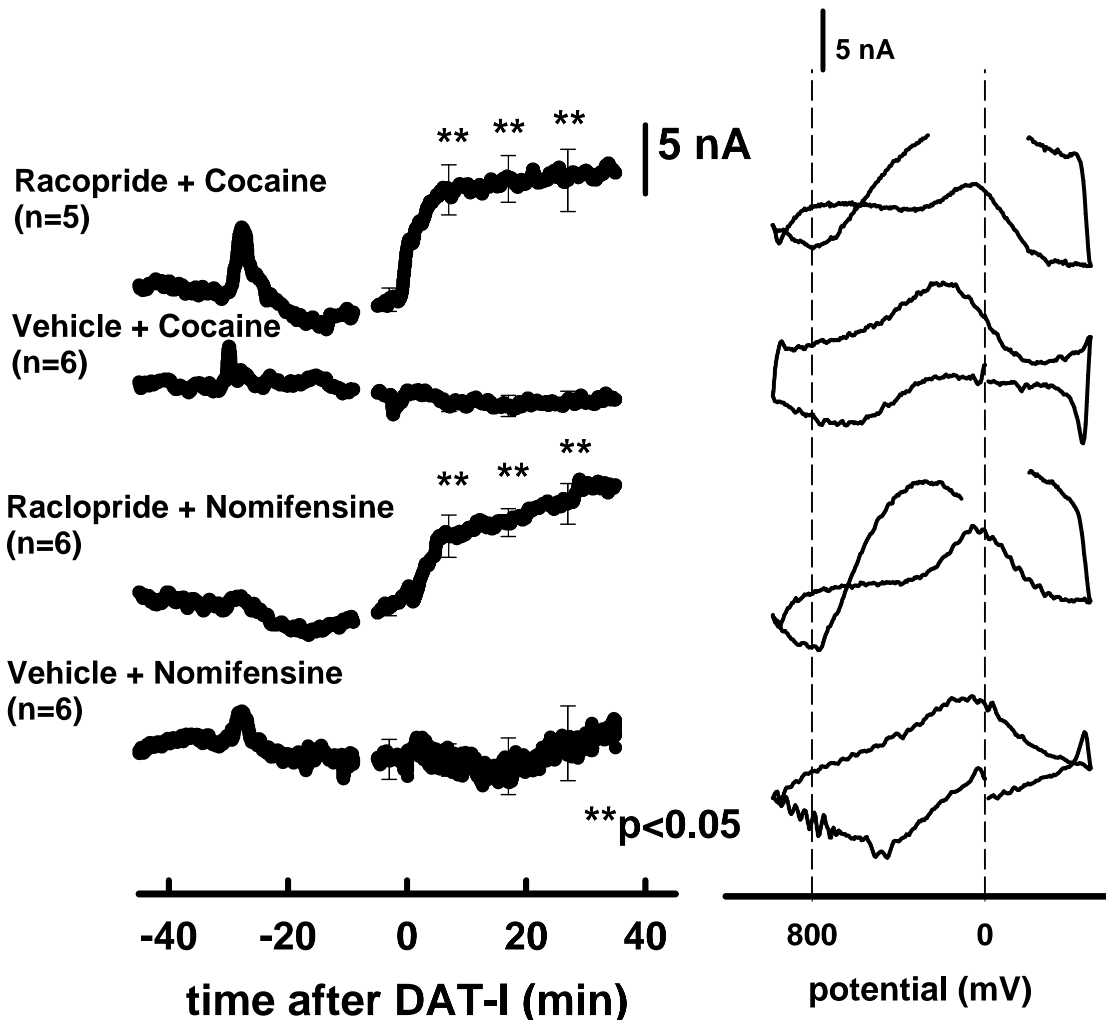

Figure 1: The effect of cocaine (30 mg/kg i.p.) and nomifensine (20 mg/kg i.p.) on voltammetric recordings from the nucleus accumbens of an awake rat pretreated with vehicle or raclopride (2.0 mg/kg i.p.). Left panel: Traces obtained by averaging the voltammetric time course signals recorded from groups of similarly treated rats. Right panel: Representative background subtracted voltammograms. Note the oxidation and reduction features near 800 mV and 0 mV, respectively, from rats pretreated with raclopride. Note the absence of these features in rats pretreated with vehicle. The vertical dashed lines are guides to clarify the alignment of the voltammogram. The asterisk indicates that points are significantly different (=$p<0.05$, ANOVA followed by Duncan's test) from the signal at the time of DAT-I administration (t=0 min).**

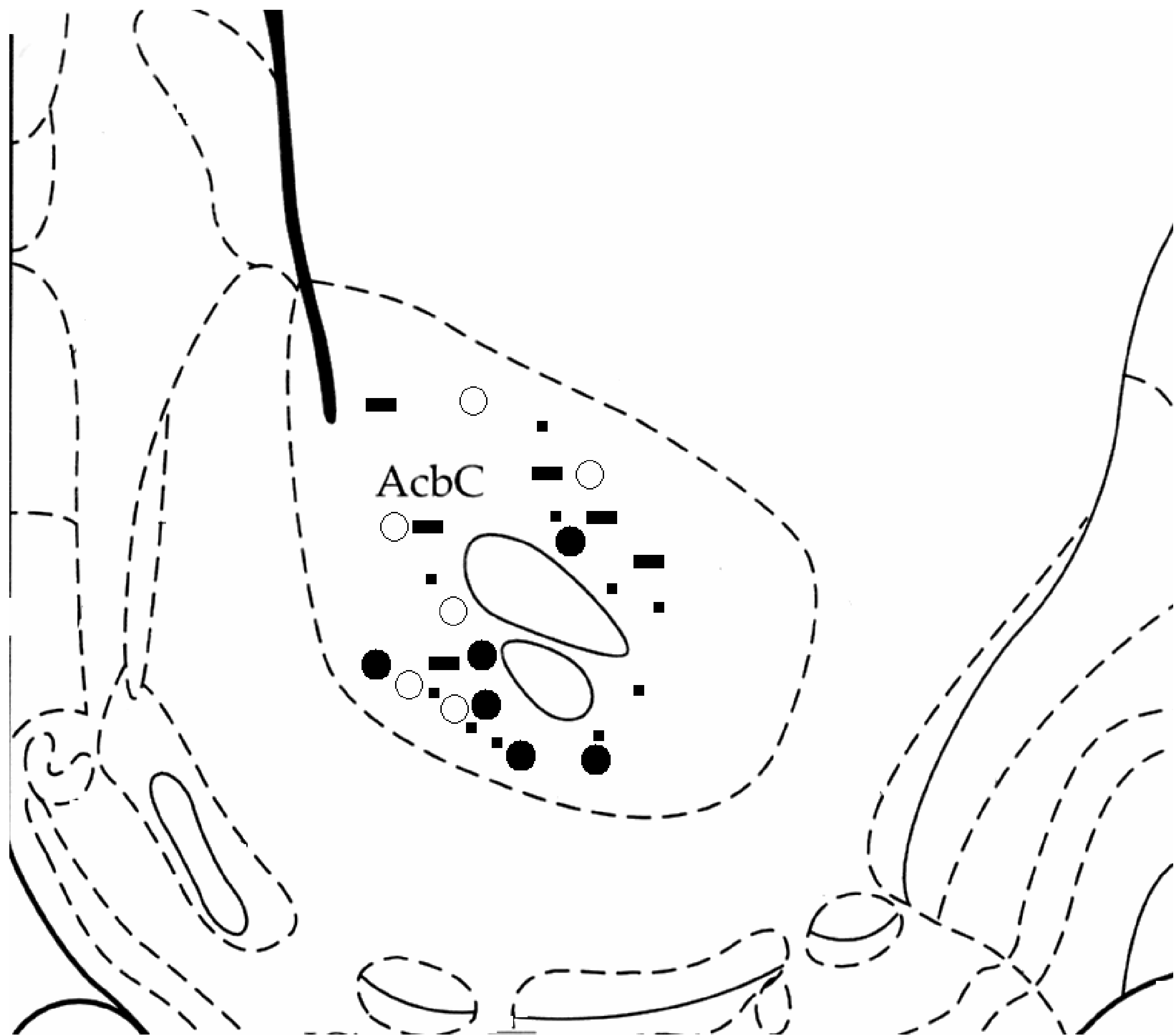

Figure 2: A drawing from the atlas of Paxinos and Watson (1998) showing the location of microelectrodes in the nucleus accumbens: Squares- raclopride followed by cocaine, rectangles- vehicle followed by cocaine, ovals- vehicle followed by nomifensine, circles- raclopride followed by nomifensine.

2.4.2 Anesthetized rats

Cocaine (30 mg/kg i.p.) and nomifensine (20 mg/kg i.p.) induced only nonDA-related, non-significant voltammetric responses in the striatum and nucleus accumbens of vehicle-pretreated anesthetized rats (Figure 3). Cocaine and nomifensine induced immediate DA-related voltammetric responses signals in the striatum and nucleus accumbens of rats pretreated with raclopride (2 mg/kg i.p.) (Figure 4). The amplitude of the responses in the striatum reached statistical significance. The amplitude of the responses in the nucleus accumbens did not reach significance due to intra-animal variability. The voltammogram from the nucleus accumbens of one raclopride-pretreated rat that received cocaine did not exhibit DA-like features: data from this rat is not included in Figure 4.

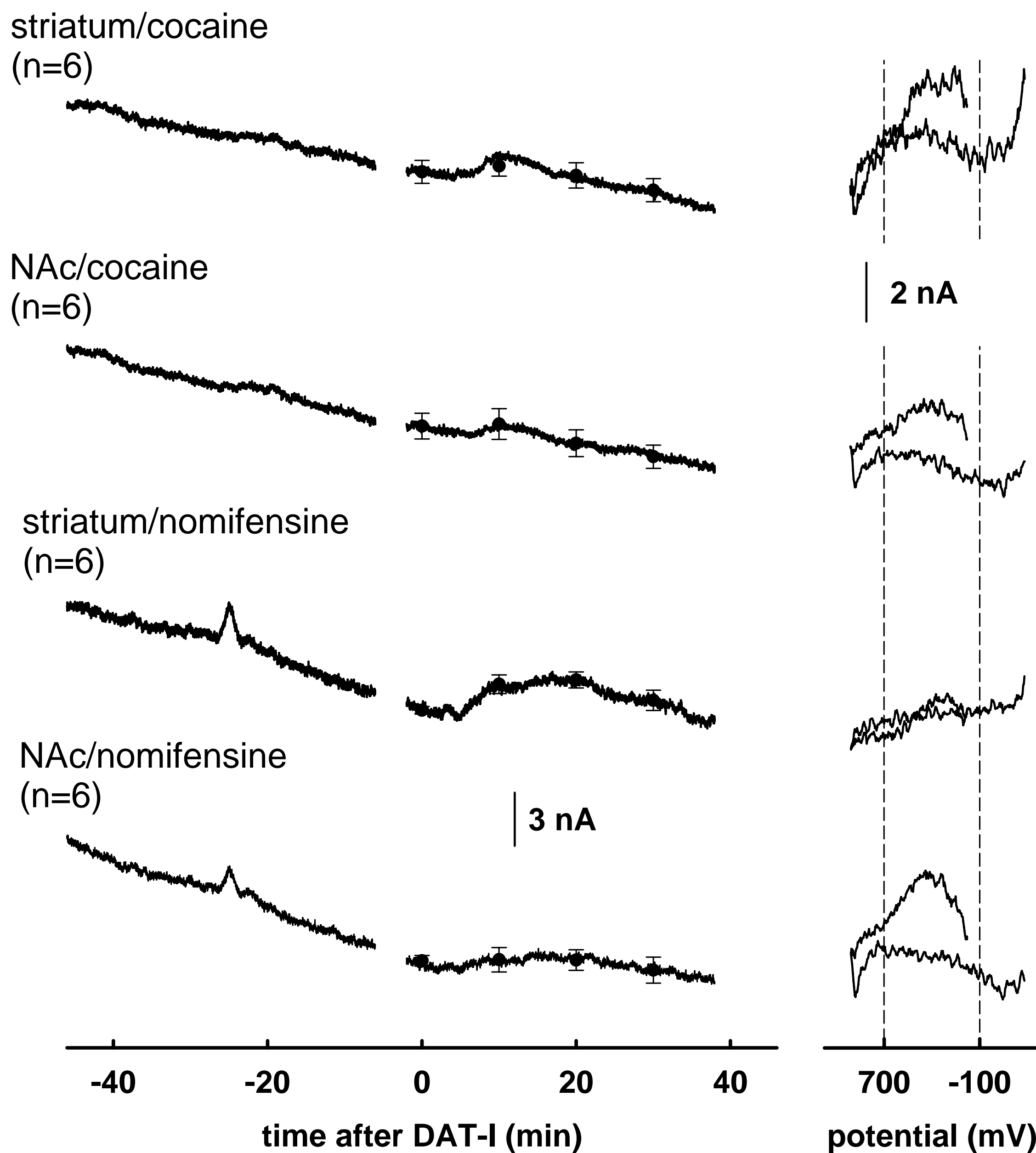

Figure 3: The effect of cocaine (30 mg/kg i.p.) and nomifensine (20 mg/kg i.p.) on the voltammetric signal recorded from the striatum and nucleus accumbens of anesthetized rats pretreated with vehicle solution (PBS). Main panel: Traces of the averaged time course signals. Right panel: Representative background subtracted voltammograms.

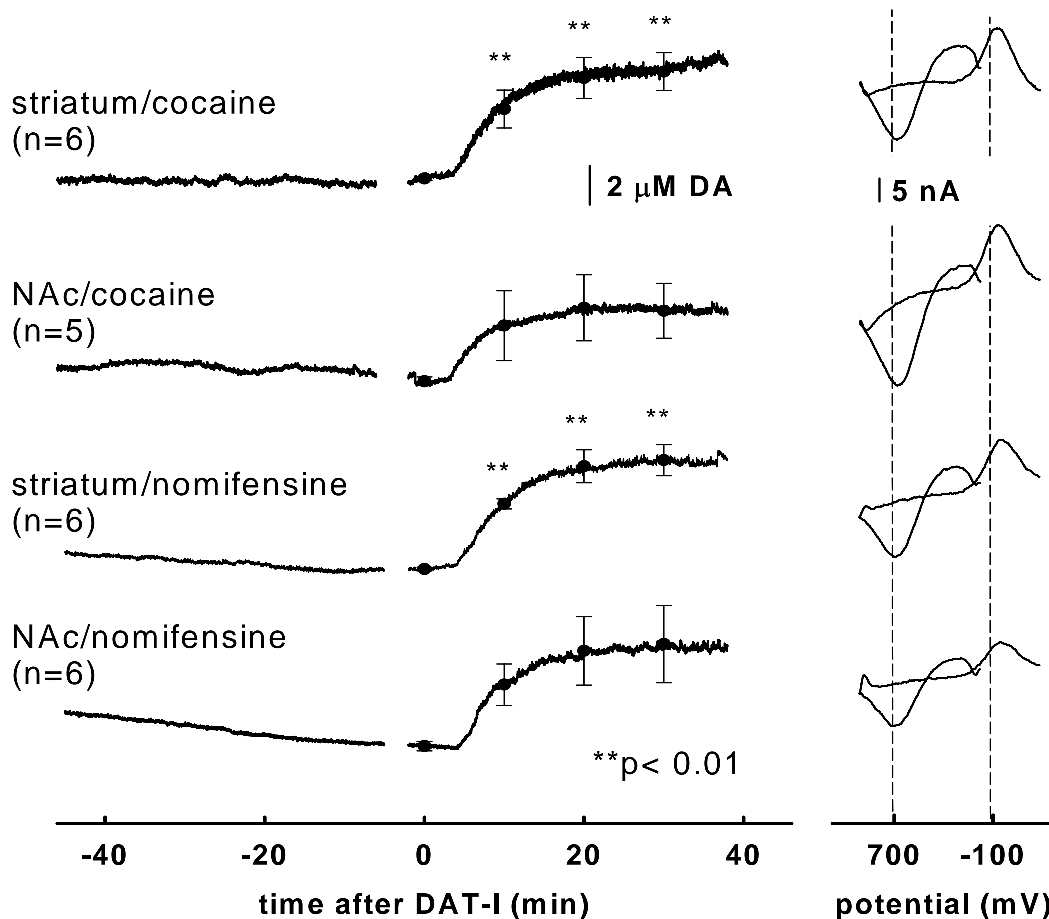

Figure 4: The effect of cocaine (30 mg/kg i.p.) and nomifensine (20 mg/kg i.p.) on the voltammetric signal recorded from the striatum and nucleus accumbens of anesthetized rats pretreated with raclopride (2 mg/kg i.p.). Main panel: Traces of the averaged time course signals. Asterisks indicate data points that are significantly different from the data point at t=0 (=p<.01). Right panel: Representative background subtracted voltammograms. Note the DA-like oxidation peak near 700 mV vs Ag/AgCl and reduction peak near -100 mV vs Ag/AgCl.**

2.4.3 Validation of the identification of DA-related voltammetric responses

During this study, the identification of DA-related voltammetric responses was based on the appearance of background subtracted voltammograms exhibiting DA-related features, including an oxidation peak near 700 mV vs. Ag/AgCl and a reduction peak near -100 mV vs. Ag/AgCl for anesthetized animals. The oxidation peak was near 800 mV and the reduction peak was near 0 mV for unanaesthetized animals. However, some of the voltammograms observed during this study also exhibit a feature near 250 mV, which needs to be identified. Here, we show that this feature is derived from the voltammetric background signal. Voltammetric recording was performed in the striatum of anesthetized rats. These rats first received a combined dose of pargyline (75 mg/kg i.p.) and raclopride (2.0 mg/kg) followed 30 min later by cocaine (30 mg/kg). DA-related voltammetric responses were observed in these animals (Figure 5; panel A) despite the use of pargyline, a monoamine oxidase inhibitor, which confirms that DOPAC is not a major contributor to the voltammetric responses reported in this study. Background subtracted voltammograms obtained prior to the administration of cocaine exhibit the feature near 250 (Figure 5; panel B), which arises from a drift in the voltammetric background signal. This feature is routinely observed during the early stages of in vivo recording sessions and does not depend on the administration of drugs (data not included). The magnitude of this drift-related feature depends on the time after electrode implantation but also exhibits variations between individual electrodes. Background subtracted voltammograms obtained after cocaine administration clearly shows that the DA-related oxidation and reduction peaks are superimposed on the drift response (Figure 5; panel C). This point is further supported by the background subtracted voltammogram obtained during electrical stimulation of the medial forebrain bundle in Figure 5; panel D, middle and bottom voltammogram. The middle

voltammogram was obtained with background scans recorded 30 min prior to the stimulus: this procedure results in voltammograms that exhibit both the DA-related features and the drift-related feature. The bottom voltammogram is from the same stimulation, but the background scan was recorded 20 sec before the stimulus in order to obtain the voltammogram. Finally, the top voltammogram in Figure 5 was collected during the postcalibration of a microelectrode in 250 μM DOPAC: this voltammogram shows that our voltammetric recording procedures are essentially insensitive to DOPAC.

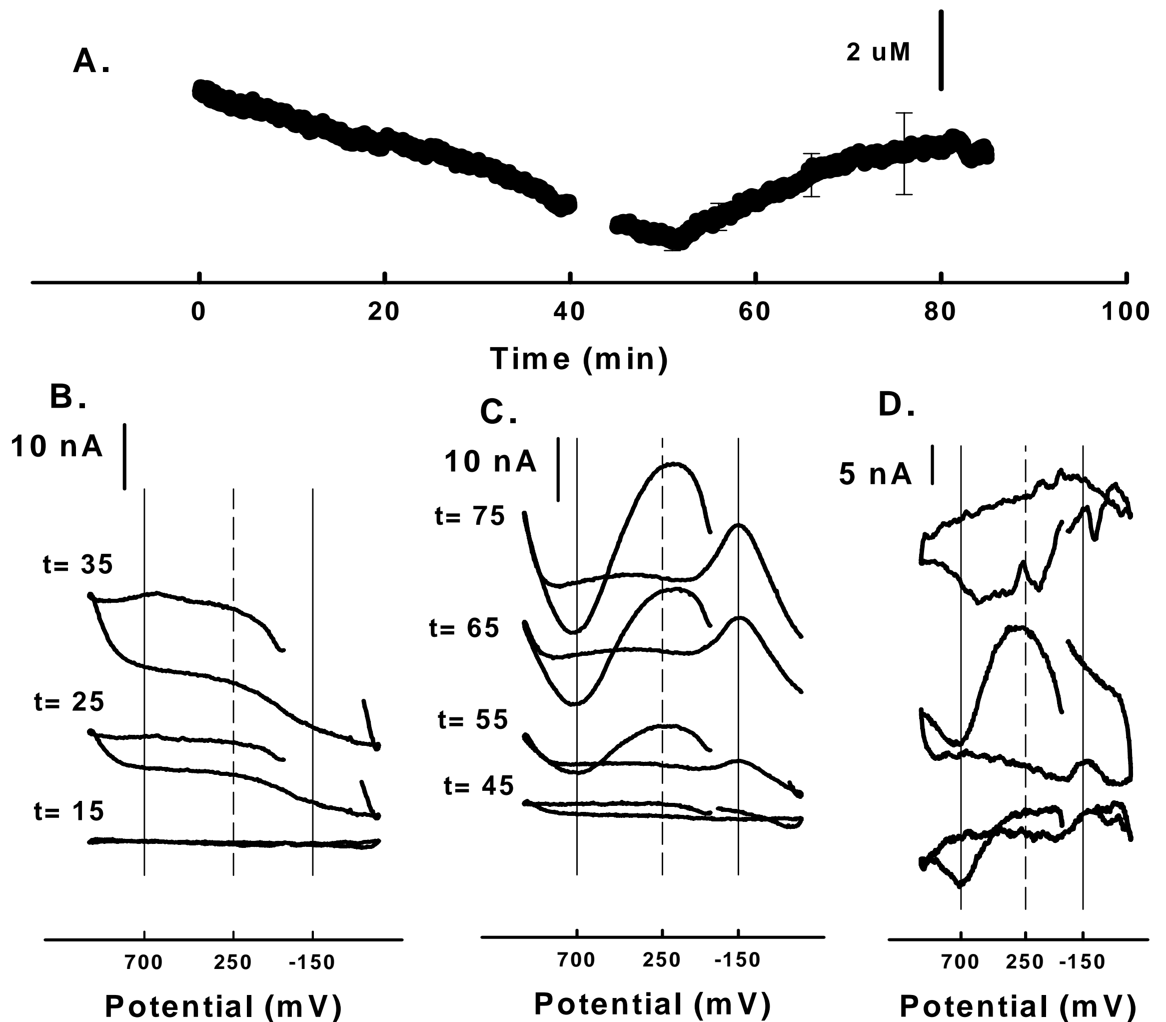

Figure 5: The effect of cocaine (30 mg/kg i.p.) on voltammetric recordings from the striatum of rats pretreated by systemic administration of pargyline (75 mg/kg i.p.) and raclopride (2.0 mg/kg i.p.). Panel A: Averaged time course signals. Panel B and C: Representative background subtracted voltammograms at various time points in the voltammetric recording. Panel D: Top trace is post calibration of 250 uM DOPAC. The middle and bottom trace is background subtraction of a MFB stimulation with a 30 minute foreground. The middle trace was background subtracted at time= 200 sec subtracted from the stimulation increase at 2100 sec. The bottom trace was background subtracted from time= 2080 sec from t= 2100 sec. Note the large increase at 250 in the middle trace and its absence in the bottom trace. Note the DA-like oxidation and reduction features near 700 mV and -100 mV, respectively, in the DA sample (Trace C and D middle and

bottom). This feature is not present in the DOPAC trace (Trace D, top trace). Feature at 250 mV is derived from the voltammetric background signal (Trace B, C, and D).

2.5 DISCUSSION

The finding of the present study are consistent with the hypothesis that D2 receptors control this tonic component of ECS DA in the striatum of anesthetized rats and the NAc of unanaesthetized rats following the systemic administration of cocaine and nomifensine. Neither cocaine alone nor nomifensine alone induced tonic DA increases in the striatum or NAc (Figures 1; trace 2, 4 and Figure 3). However, both DAT inhibitors induced DA-related responses in rats pretreated systemically with the D2 antagonists raclopride (Figures 1; trace 1, 3 and Figure 4). Presumably, the D2 antagonists interrupt a feedback mechanism that normally autoinhibits DA release in order to stabilize tonic ECS DA levels after DAT-I.

2.5.1 Methodological considerations

The findings of the present study are different from those obtained by the techniques of microdialysis and high speed voltammetry where DAT-I is concerned. As discussed in the introduction, DAT-I increases the microdialysis efflux of DA (Church et al. 1987; DiChiara and Imperato 1988; Nakachi et al. 1995; Pontieri et al. 1995; Bradberry et al. 2000). However, it is also well established that the microdialysis efflux of DA both under basal conditions and after

DAT-I is sensitive to TTX. TTX decreases microdialysis DA efflux to below detectable levels and prevents the ability of DAT inhibitors to increase DA efflux (Westerink et al. 1987). In contrast, the basal DA level that we describe herein as being stabilized after DAT-I appears to be insensitive to TTX (Borland and Michael, 2004). The ability of kynurenate infusions to decrease tonic DA from its basal level is unaffected by infusions of TTX into either the striatum or the medial forebrain bundle. Hence, microdialysis and the voltammetric technique used for the present study appear to have measured fundamentally different DAergic phenomena. This is consistent with the idea of both a phasic and tonic pool of DA.

Wightman's laboratory has demonstrated that DAT inhibitors increase both the frequency and amplitude of nonevoked DA voltammetric transients in the nucleus accumbens (Robinson and Wightman 2004; Stuber et al. 2005). Like the microdialysis results, the high-speed voltammetric recordings appear to report on phasic DA-related events that occur on a much faster time scale than the tonic events we report. The phasic events described by Wightman's laboratory comprise sub-second DA transients whereas the events we have reported are much longer duration, lasting for several tens of minutes. Thus, the high speed events reported by Wightman's laboratory and the tonic events that we describe are fundamentally different phenomena.

This contrast between our findings and those of Wightman's are due to the different voltammetric procedures that are employed. We use electrodes between 300-400 µm in length that have been subjected to electrochemical pretreatment, which increases their response time, but make them more sensitive to DA detection. Wightman and coworkers use shorter electrode approximately 50-150 µm and optimize the placement of the electrode in order to detect sub-second or transient DA signals. In our experiments, the voltammetric signal from

unanaesthetized animals were post-processed with a 41-point moving average to eliminate nonDA-related noise spikes, but this also would filter out any DA transients that may have been present. Due to these difference, our procedures are better adapted to monitoring tonic changes in ECS DA concentrations, as our prior studies confirm (Kulagina et al. 2001; Borland and Michael 2004; Borland et al. 2005). Since the DA responses observed during the present study were long-lasting, out focus is on tonic DA.

Early on in this study, we noticed that cocaine and nomifensine induce nonDA-related voltammetric responses in anesthetized rats (Figure 3), similar to those reported by Venton et al. (2003). However, these nonDA-related responses are much less evident in unanesthetized rats, especially in the case of cocaine (Figures 1). When nomifensine was administered to unanesthetized rats, a nonDA-related response in the striatum was delayed by nearly 1 hr after drug administration and did not reach significant levels (Figure 1; trace 4). However, especially in the unanesthetized animals, the nonDA-related responses did not interfere with the detection of DA, because the nonDA-related responses were small in amplitude, delayed in time, and did not involve the potential window corresponding to the reduction of dopamine-o-quinone (near -100 mV). Some studies have reported the effects of DAT inhibitors on tonic voltammetric signals, including decreases in the signals (Gratton and Wise 1994; Kiyatkin and Stein 1996). However, these studies were based on a different voltammetric procedure and the possibility that this procedure produced nonDA-related responses has not been excluded (see discussion in Venton et al. 2003). One voltammetry study reported a sustained increase of DA after DAT-I, but the electrodes had a very slow response time possibly indicating that the increase was due to an accumulation of phasic DA (Gonon and Buda 1985).

One implication of the absence of an increase in tonic ECS DA after the administration of cocaine or nomifensine is that the autoinhibition of DA release must be able to rapidly adapt to inhibition of the DAT. In fact, the work of Benoit-Marand et al. (2001) confirms that autoinhibition is a rapid process that can occur within 200 ms of DA release and persist for nearly 1 s thereafter. Hence, autoinhibition can occur faster than the temporal response of our voltammetric procedures.

As mentioned above, our voltammetric findings are different from those based on microdialysis, which suggest that DAT-I increases phasic ECS DA. On the other hand, our voltammetric techniques agrees with the microdialysis literature showing that D2 antagonists enhance the increase in microdialysis DA efflux induced by DAT inhibitors (Westerink et al. 1990). Our voltammetric findings further agree with the microdialysis literature that DAT-I-induced increases in ECS DA are TTX-sensitive, since intracranial infusions of TTX abolished the effect of nomifensine in sulpiride-pretreated rats (Borland 2005). A perplexing question that remains incompletely answered is why the basal microdialysis efflux of DA does not appear to include a TTX-insensitive component, consistent with our voltammetric findings. To address this issue, we placed carbon fiber microelectrodes along side of microdialysis probes in the striatum of anesthetized rats and found that DAT-I does increase ECS DA as measured by voltammetry under these circumstances (Borland et al. 2005). Hence, when voltammetry and microdialysis are performed side-by-side, the two techniques agree that DAT-I elevates ECS DA. What remains unclear is why it is necessary to place the two devices side-by-side in order to achieve this agreement. This is a matter still under investigation in our laboratory.

2.5.2 Tonic DA after DAT-I in animals pretreated with D2 antagonists

The DA increase following the combined administration of a D2 antagonist and a DAT inhibitor may have more than one origin. The D2 antagonist might act to prevent the autoinhibition of DA release or might act synergistically with DAT inhibitors to produce an overall greater inhibition of DA uptake than the DAT inhibitors alone. However, several considerations point to suppression of autoinhibition as the likely predominant mechanism at work. For example, the kinetics of the DA clearance are similar in wild-type and D2R-deficient mice (Benoit-Marand et al. 2001; Rougé-Pont et al. 2002; however, see Dickinson et al. 1999), which suggests that DAT kinetics are subject to multiple forms of regulation in addition to the D2R (Hoffman et al. 1999; Gulley and Zahniser 2003; Schmitz et al. 2003; Zahniser and Sorkin 2004). Furthermore, early analyses of electrically evoked DA release as recorded by voltammetry concluded that D2 antagonists mainly affect DA release compared to DA uptake, suggesting a more prominent effect on DA release (Wightman and Zimmerman 1990; Kawagoe et al. 1992). A more recent study concluded that haloperidol decreases DA clearance after MFB stimulation while concurrently suppressing the autoinhibition of DA release (Wu et al. 2002). However, synergistic effects of haloperidol and the noncompetitive DAT inhibitor, RTI-76, on uptake kinetics were not observed. Thus, although the ability of D2 antagonists to decrease DA uptake might have contributed to the magnitude of the DA responses observed during our work, suppression of autoinhibition of DA release appears to stand as the main factor preventing the stabilization of tonic ECS DA after DAT-I. We conclude, therefore, that autoinhibition of DA release is the main contributor to the stabilization of tonic DA levels after DAT-I. Our conclusion is strengthened by the fact that DAT inhibitors are well known to decrease the effective velocity of DA uptake in vivo (Suaud-Chagny et al. 1995; Budygin et al. 2000;

Kiyatkin et al. 2000; Wu et al. 2001; Sabeti et al. 2002; Garris et al. 2003), which confirms autoreceptors do not prevent pharmacological inhibition of the DAT. Thus, stabilization of tonic ECS DA following DAT-I cannot be attributed solely to adaptations of DA uptake kinetics.

DAT-I decreases the firing of midbrain DA neurons (Studer and Schultz 1987; Einhorn et al. 1988; Henry et al. 1989; Mercuri et al. 1991; Mercuri, et al. 1992; Mercuri et al. 1997), an effect that is blocked also by D2 antagonists (Studer and Schultz 1987; Einhorn et al. 1988). Hence, a decrease in the firing of midbrain DAergic neurons after DAT-I might contribute to the ECS DA-stabilizing decrease in DA release. However, the regulation of firing rate is generally attributed to D2Rs located in the midbrain, whereas the role of presynaptic D2Rs has been demonstrated in our laboratory because the D2 antagonist, sulpiride, applied directly to the striatum by microinfusion followed by DAT-I will still result in an increase in DA (Borland 2005).

2.5.3 Tonic DA is stabilized after DAT inhibition

Based on the findings of this study and previous studies in our laboratory, we have developed the working hypothesis that the ECS in the striatum and in the NAc contains a tonic DA component that is stabilized by D2 autoreceptors after the administration of DAT inhibitors. Thus, compared to the phasic DA component probed by microdialysis and high-speed voltammetric techniques, the tonic DA component is less impacted by DAT-I due a compensatory ability of D2 autoreceptors to autoinhibit DA release. In the next chapter, I will address the potential functional significance of the tonic DA component described herein.

2.6 ACKNOWLEDGEMENTS

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3.0 SUPRESSION OF COCAINE INDUCED HYPERACTIVITY BY TONIC EXTRACELLULAR DOPAMINE

3.1 ABSTRACT

Voltammetric recording was conducted in the extracellular space of the nucleus accumbens in unanesthetized rats in order to correlate extracellular dopamine levels with cocaine-induced hyperactivity. The voltammetric recording procedure employed was one adapted to monitoring tonic, i.e. sustained and long-lasting, dopamine changes. The rats were housed in a chamber that counter rotated during bouts of locomotor activity and the number of chamber rotations was used as a quantitative index of activation. Observations were performed in rats that received cocaine (10, 20, or 30 mg/kg i.p.) after pretreatment with the D2 receptor antagonist, raclopride (0, 0.2, 0.5, or 2.0 mg/kg i.p.). These drug combinations produced two treatment outcomes, a tonic increase in extracellular dopamine levels in the nucleus accumbens and locomotor activation. However, these treatment outcomes were mutually exclusive in that individual animals exhibited one outcome or the other but, with one exception, not both. The mutually exclusive relationship between these treatment outcomes supports the conclusion that tonic elevations of extracellular dopamine suppress cocaine-induced hyperactivity.

3.2 INTRODUCTION

The mesolimbic dopamine (DA) system terminates in the nucleus accumbens (NAc), a brain region centrally involved in the actions of psychostimulants, including cocaine, that inhibit the dopamine transporter (DAT) (Koob and Bloom 1988; Wise, 2002). The DAT is responsible for removing DA from the extracellular space, thereby terminating its actions on both pre- and post-synaptic DA receptors (Zahniser et al. 1999). Hence, much attention has been paid to understanding how DAT inhibition (DAT-I) affects DA concentrations in the extracellular space (ECS) of NAc and other DAergic brain regions. Furthermore, the expression in the NAc of several types of DA receptor, broadly classified as D1-like and D2-like, has generated interest in identifying their respective roles in DAT-I-induced hyperactivity. Several lines of neurochemical evidence from microdialysis, PET, and voltammetry show that DAT-I increases ECS DA levels in the NAc (e.g. Di Chiara and Imperato, 1988; Le Moal and Simon, 1991; Wise, 2004; Volkow et al. 1996; Gonon and Buda 1985). Furthermore, the ability of both D1 and D2 antagonists to diminish hyperactivity following DAT-I suggests that both receptor types are involved in cocaine-induced behaviors (Cabib et al. 1991; Tella 1994).

However, recent studies from our laboratory suggest that the striatum and NAc also contain a pool of ECS DA that remains relatively constant after DAT-I (Peters et al. 2002; Khan and Michael 2003; Mahon et al. 2005; also see Chapter 1). Our in vivo experiments involved a voltammetric technique adapted to monitoring tonic, i.e. minute-to-minute, changes in ECS DA levels. According to measurements based on this technique, microinfusions of kynurenate via pipets positioned adjacent to voltammetric microelectrodes induce sustained decreases in ECS DA (Kulagina et al. 2001; Borland and Michael 2004). The effect of kynurenate is insensitive to tetrodotoxin (TTX) but sensitive to the DAT inhibitor, nomifensine, leading us to ascribe our

observations to a tonic pool of ECS DA. However, our voltammetric technique has produced no evidence of an increase in the amount of DA associated with this tonic ECS pool following DAT-I in anesthetized and unanesthetized rats, even with relatively high doses of cocaine (30 mg/kg i.p.) and nomifensine (20 mg/kg i.p.) (see Chapter 1).

Obviously, the suggestion that there exists a pool of ECS DA that does not increase in response to DAT-I stands in contrast to other findings. For example, DAT-I increases the efflux of DA as measured by microdialysis (DiChiara and Imperato 1988; Le Moal and Simon 1991). However, it is well established that the basal DA efflux and the DAT-I-induced increase in DA efflux as measured by microdialysis are TTX-sensitive (Westerink et al. 1987), which suggests that microdialysis and tonic voltammetric recording might be observing distinct DA-related phenomena. To further illustrate this point, we conducted in vivo voltammetric recordings with microelectrodes placed adjacent to microdialysis probes (Borland et al. 2005). We clearly detected a nomifensine-induced increase in ECS DA at microelectrodes placed about 200 μm from microdialysis probes, which lends support to the idea that the microdialysis result reflects a DAT-I-induced response that occurs near the probes. Thus, we conclude that tonic DA levels as measured by voltammetry in tissues far removed from microdialysis probes fluctuate less in response to DAT-I than might be expected based on the magnitude of DAT-I-induced changes in DA efflux as measured by microdialysis.

Our suggestion that there exists a pool of ECS DA that does not increase after DAT-I also raises the question as to why this pool is unaffected by DAT-I. In Chapert 1, I suggest that the tonic ECS DA pool is maintained near its basal value after DAT-I by an autoreceptor-mediated decrease in DA release that compensates for the decrease in DA clearance. DAT-I produced a clear increase in voltammetrically detected ECS DA in rats pretreated with a D2, but not a D1,

antagonist (Kahn and Michael 2003). In this regard, our voltammetric findings concur with the microdialysis literature, which reports that D2 antagonists increase DAT-I-induced DA efflux (Westerink et al. 1990).

Despite the fact that ECS DA levels increase in the NAc after DAT-I in rats pretreated with the D2 antagonists, raclopride (Chapter 1), these animals were not hyperactive. They did not exhibit locomotor activity nor did they exhibit obvious stereotyped behaviors. The absence of stereotyped behaviors is consistent with the concept that these behaviors require simultaneous activation of both D1 and D2 receptors. The absence of locomotor activity is also not altogether surprising, as both D1 and D2 antagonists are known to prevent cocaine-induced locomotor hyperactivity (Cabib et al. 1991; Tella 1994). However, the ability of D2 antagonists to prevent cocaine-induced hyperactivity is surprising in light of studies involving knockout mice, which suggest that the D1 receptor, but not the D2 receptor, is both necessary and sufficient for cocaine-induced hyperactivity. For example, cocaine-induced hyperactivity is preserved in D2 knockout mice, whereas cocaine induces hypoactivity in D1 knockout mice (Chausmer et al. 2002; Xu et al. 2002; Xu et al. 1994). Furthermore, the full D1 agonist, SKF 81297, induces near-normal hyperactivity in D2 knockout mice, which further supports the concept that the D1 receptor function is preserved in the absence of the D2 receptor (Usiello et al. 2000). Although it is often mentioned that comparing the effects of antagonists with receptor knockout studies is challenging (Xu et al. 2000; Gringrich and Hen 2000; Boulay et al. 1999), studies in knockout mice provide a basis of expecting cocaine-induced hyperactivity to be preserved under the conditions of our experiments involving rats pretreated with D2 antagonists. Under these conditions, both voltammetry and microdialysis confirm large increases in ECS DA after DAT-I.

Thus, the question arises as to why this elevated ECS DA does not induce a D1-mediated state of hyperactivity.

In order to address this question in a direct manner, we set out to assess more thoroughly how tonic ECS DA in the NAc correlates with locomotor activity. For this purpose, we conducted voltammetric recording in the NAc of unanesthetized rats that received raclopride (0, 0.2, 0.5, or 2 mg/kg i.p.) followed by cocaine (10, 20, or 30 mg/kg i.p.). During voltammetric recording, the rats were housed in a chamber that counter-rotated during bouts of locomotor activity. We used the number of chamber rotations as a quantitative index of locomotor activity.

3.3 METHODS AND MATERIALS

3.3.1 Animals, surgical procedures, and voltammetric procedures.

All procedures involving animals were carried out with the approval of the Intuitional Animal Care and Use Committee of the University of Pittsburgh. With only one exception, all procedures for surgery and voltammetric recording used in this study were identical to those described in Chapter 1. The only difference is that during the present study we removed the electrodes from the rat for postcalibration. So, histological verification of the in vivo placement, which involves destroying the electrode, was not performed. However, as Figure 2 of Chapter 1 confirms, stereotaxic placement of electrodes in the NAc is a reliable procedure.

3.3.2 Data analysis

Voltammetric signals over time were obtained by averaging the current in the potential range giving the maximum DA oxidation signal. The time-dependent signal was smoothed with a 41-point (16 s) moving average to reduce occasional movement artifacts. A change in the current after the administration of a drug was analyzed for the presence of dopamine by examining background subtracted voltammograms for the presence of DA oxidation and reduction features. Voltammograms recorded over 200 s prior to drug administration (time= 45 minutes; t1, Figure 6) were subtracted from those recorded over 200 s (time=48 minutes; t2, Figure 6) near the maximal voltammetric response. Background subtracted voltammograms obtained during the in vivo recording sessions were compared to those obtained during post-calibration of the electrode. The current response was converted to units of dopamine concentration by means of the post-calibration. The data from multiple experiments was pooled and averaged. The pooled data at specific time points was analyzed by one-way ANOVA followed by Duncan's multiple range test.

3.3.3 Drugs

Animals received either vehicle (phosphate buffer solution: 155 mM NaCl, 100 mM phosphate, pH 7.4) or raclopride (0.2, 0.5, or 2.0 mg/kg i.p.) followed by cocaine (10, 20, or 30 mg/kg i.p.). Voltammetric recording began at 0 minutes, with vehicle or raclopride administered at 20 minutes and cocaine administered at 50 minutes. Both raclopride and cocaine hydrochloride salt were dissolved in phosphate buffer solution and were purchased from Sigma (St. Louis, MO).

Isoflurane was purchased from Easterling Veterinary Supply (West Columbia, SC). All solutions were prepared in ultrapure water (Nanopure, Barnstead Inc., Dubuque, IA).

3.4 RESULTS

3.4.1 Voltammetry after raclopride and cocaine in unanaesthetized rats

Consistent with our previous observations in Chapter 1, cocaine (10, 20, or 30 mg/kg i.p.) by itself did not induce tonic DA-related voltammetric responses in the NAc (data not shown). However, when administered 30 min after raclopride (2 mg/kg i.p.), cocaine (10, 20, or 30 mg/kg i.p.) induced sustained increases in DA-related voltammetric signals in the NAc (Figure 6). Transient voltammetric responses associated with the injections were nonDA-related. At a dose of 20 or 30 mg/kg, cocaine increased ECS DA levels in all rats pretreated with 2 mg/kg raclopride. At a dose of 10 mg/kg, cocaine increased DA in three of the six rats (Figure 6; panel A, only includes data from the three animals in which DA-related responses were obtained). Representative voltammograms are shown in Panel B. The DA-related voltammetric currents were converted to units of DA concentration by postcalibration after the electrodes were recovered from the rats. There were no statistically significant differences between the DA responses observed at the three doses of cocaine. The DA responses were therefore pooled (Figure 6; panel C). The increase in the pooled response 8 min after cocaine administration corresponded to 1.32 ± 0.23 μM (mean ± standard error, n=15): analysis of variance of the DA concentration observed at 10-min intervals revealed a significant effect ($F=8.76$; $df=3,56$; $p<0.05$).

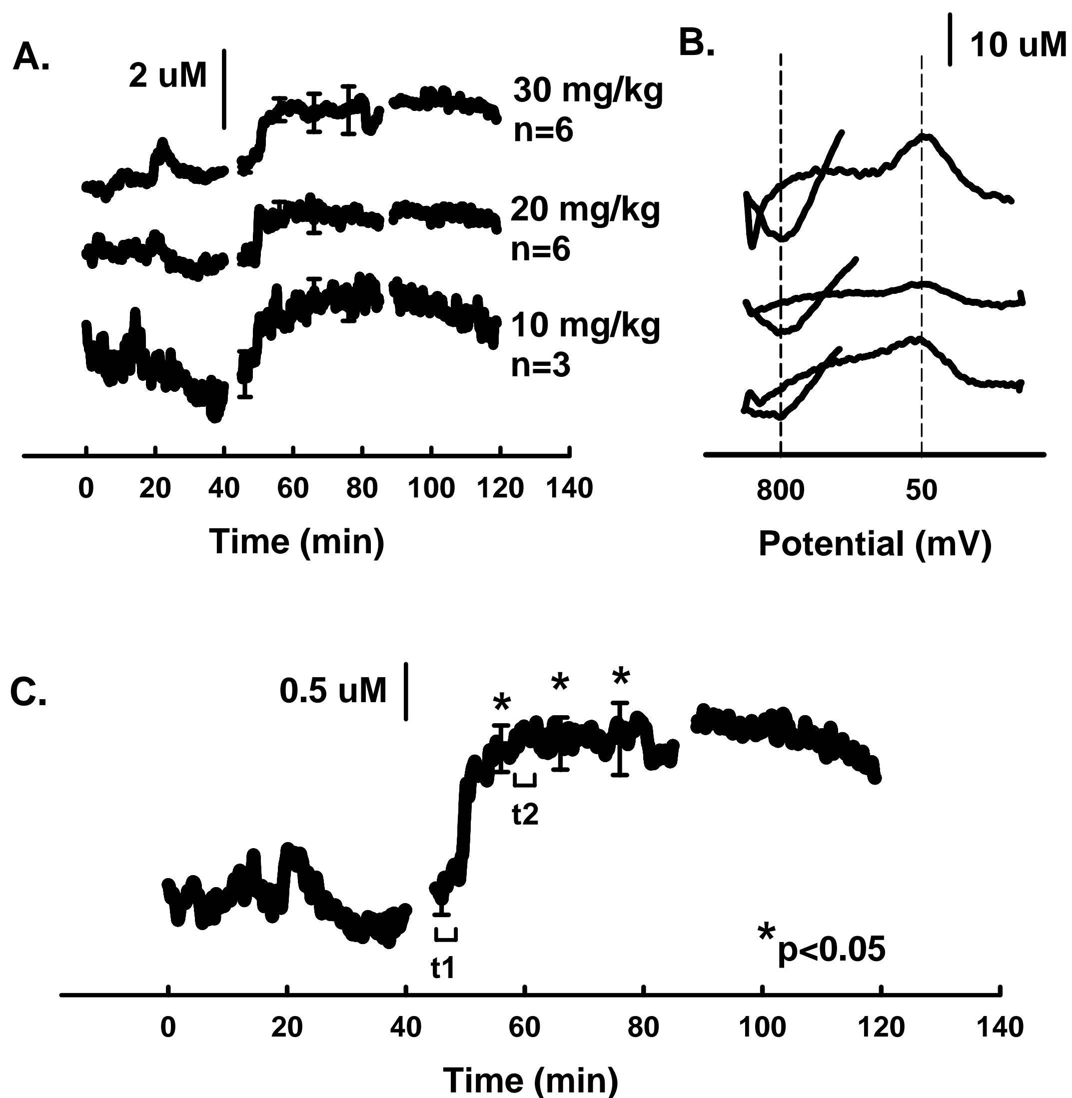

Figure 6: The effect of cocaine (10, 20, and 30 mg/kg i.p.) on voltammetric recordings from the nucleus accumbens of an awake rats pretreated with raclopride (2.0 mg/kg i.p.). Panel A: Traces obtained by averaging the voltammetric time course signals recorded from groups of similarly treated rats. Panel B: Representative background subtracted voltammograms. Note the oxidation and reduction features near 800 mV and 50 mV, respectively. The vertical dashed lines are guides to clarify the alignment of the

voltammogram. Panel C: Trace obtained by averaging the voltammetric time course signals recorded from all doses of cocaine. The asterisks indicate points that are significantly difference (*=p<0.05, ANOVA followed by Duncan's test) from the signal at the time of cocaine administration (t=50 min).

3.4.2 Locomotor activity after raclopride and cocaine

Without raclopride, each dose of cocaine (10, 20, and 30 mg/kg i.p) induced consistent locomotor activity throughout the test interval (Figure 7). Although the rats that received 30 mg/kg cocaine exhibited the greatest number of rotations, there were no significant differences between doses. Raclopride (2 mg/kg i.p.) administered 30 min prior to cocaine abolished the cocaine-induced locomotor activity in all animals. The locomotor counts accumulated during the test interval in these animals were due only to occasional, intermittent shifts in body position and posture rather than sustained locomotor activity. Within a few minutes of drug administration, these rats typically returned to sitting or lying in the chamber: they exhibited no unusual or atypical body postures, they were not catatonic, and no animals in this study exhibited stereotypic behaviors.

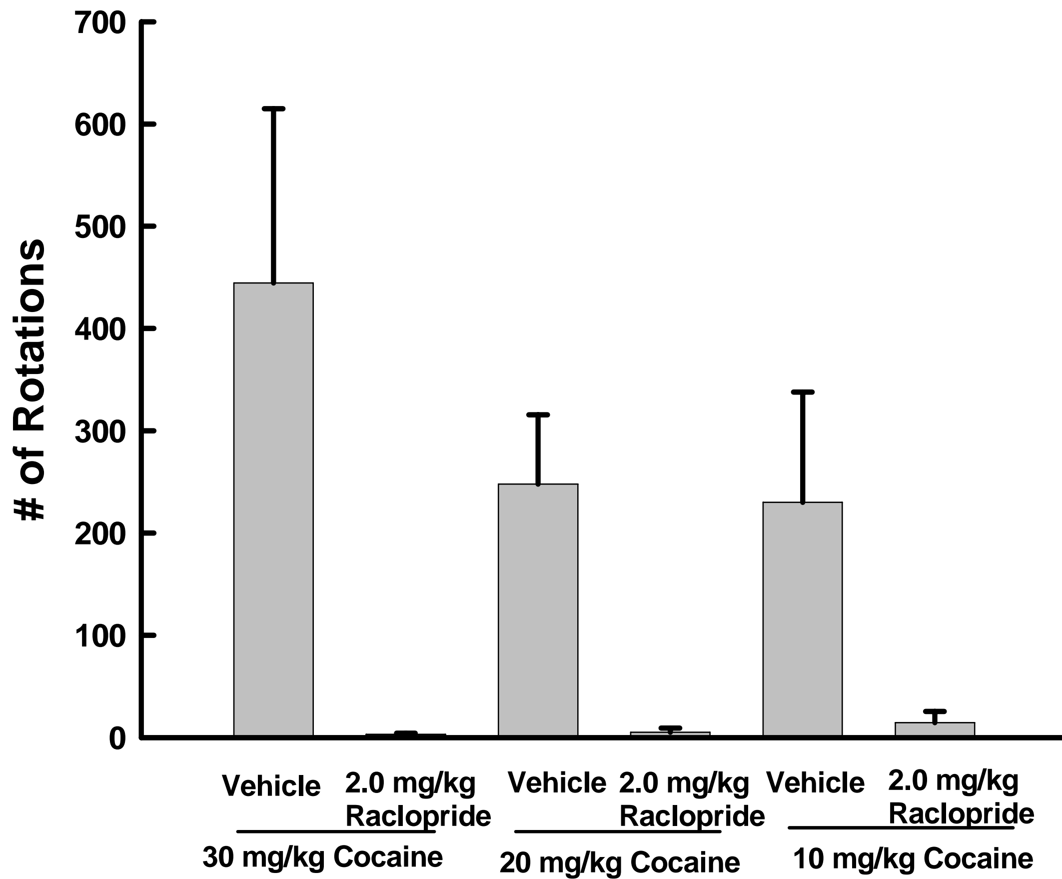

Figure 7: The effect of cocaine (10, 20, and 30 mg/kg i.p.) on locomotor activity of rats pretreated with either raclopride (2.0 mg/kg i.p.) or vehicle (1 mL PBS). Locomotor activity was analyzed from rats for 65 minutes following the administration of cocaine. The data is the average of n=6 for all doses.

3.4.3 Raclopride dose effects

Voltammetric recording was performed in two additional groups of rats pretreated with 0.2 or 0.5 mg/kg raclopride 30 min prior to receiving 30 mg/kg cocaine (Figure 8). Each drug combination was administered to a total of 6 rats. Cocaine (30 mg/kg) induced a significant increase in extracellular DA in all six rats pretreated with 2.0 mg/kg raclopride (one-way ANOVA: $F=3.23$; $df = 3,20$; $p<0.05$). However, a sustained increase in extracellular DA was obtained in only 2 of the 6 rats that received 0.5 mg/kg raclopride and in only 1 of the six rats that received 0.2 mg/kg raclopride. The lower doses of raclopride partially reduced the locomotor counts in the animals that received 30 mg/kg cocaine (Figure 9). There was a significant difference between the effect of 0.2 mg/kg and 2.0 mg/kg raclopride on the locomotor activity counts (one-way ANOVA: $F=16.29$; $df=1,10$; $p<0.05$).

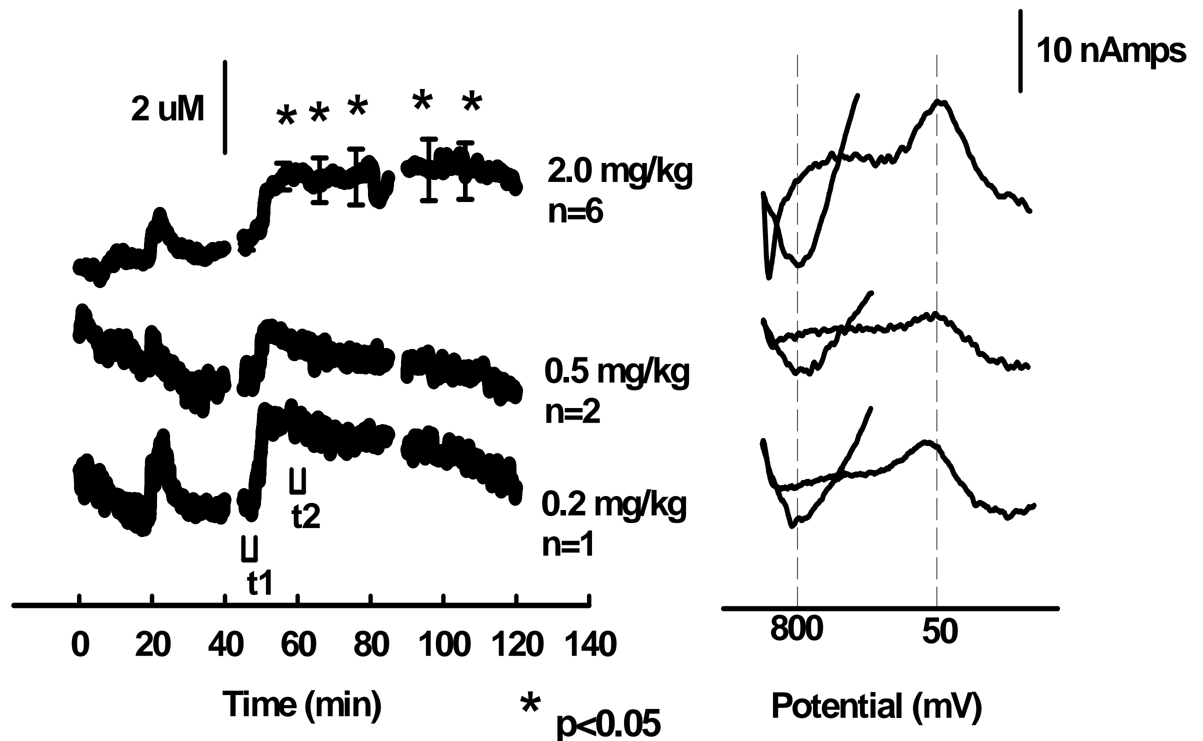

Figure 8: The effect of cocaine (30 mg/kg i.p.) on voltammetric recordings from the nucleus accumbens of awake rats pretreated with raclopride (0.2, 0.5, and 2.0 mg/kg i.p.). Left Panel: Traces obtained by averaging the voltammetric time course signals recorded from groups of similarly treated rats. Right Panel: Representative background subtracted voltammograms. Note the oxidation and reduction features near 800 mV and 50 mV, respectively. Background subtracted voltammograms were obtained from the subtraction of data recorded over 200 s prior to drug administration (time= 45 minutes; t1) from those recorded over 200 s (time=48 minutes; t2) near the maximal voltammetric response. The vertical dashed lines are guides to clarify the alignment of the voltammogram. The asterisks indicate points that are significantly difference (*=$p<0.05$, ANOVA followed by Duncan's test) from the signal at the time of cocaine administration (t=50 min).

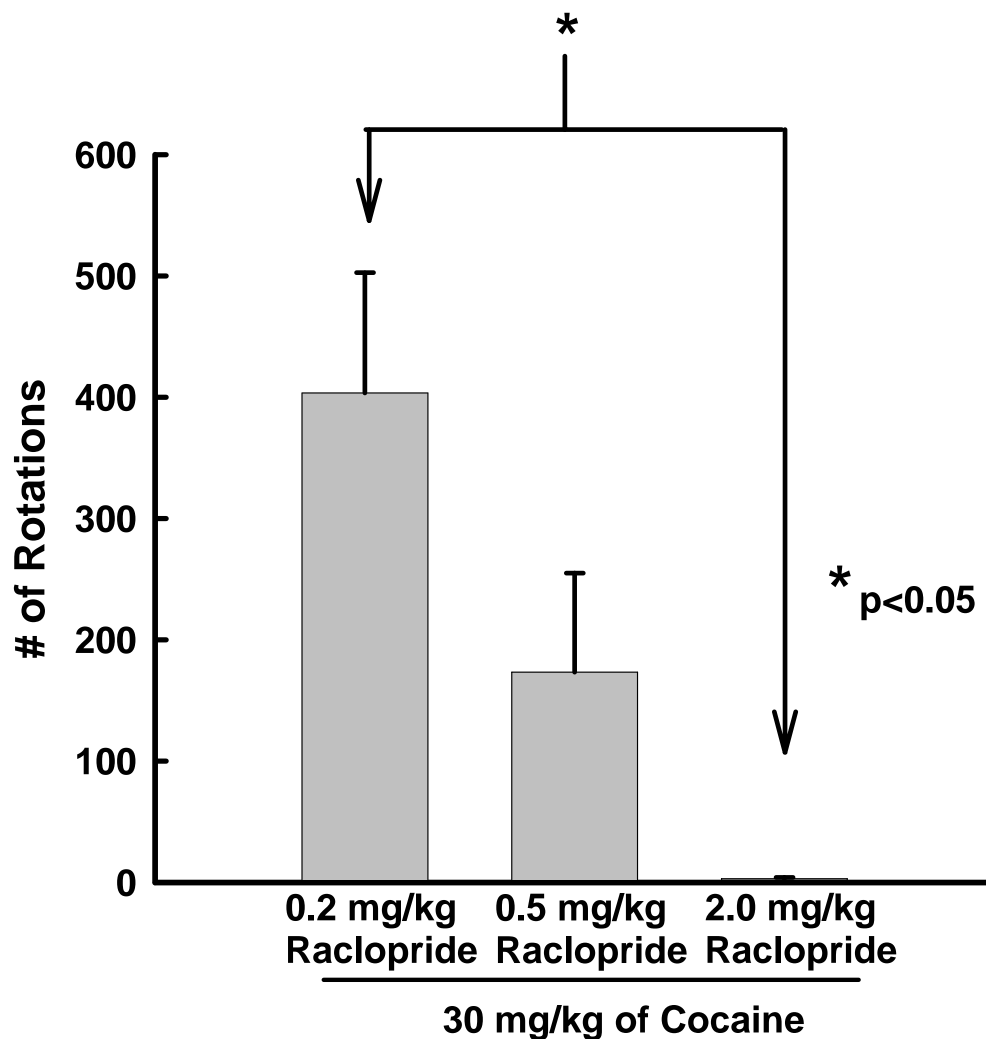

Figure 9: The effect of cocaine (30 mg/kg i.p.) on locomotor activity of rats pretreated with raclopride (0.2, 0.5, and 2.0 mg/kg i.p.). Locomotor activity was analyzed from rats for 65 minutes following the administration of cocaine. The data is the average of n=6 for all doses. The asterisks indicate raclopride doses that are significantly difference (*=p<0.05, ANOVA followed by Duncan's test).

3.4.4 Correlation of extracellular DA with locomotor activity

By correlating the concentration of the drug-induced increase in ECS DA with the locomotor activity counts for individual animals, 47 of the 48 rats in this study were clearly associated with one of the two drug-treatment outcomes we observed (Figure 10, main panel). The drug combinations induced either sustained elevations of ECS DA in the NAc or locomotor activity, but not both. Only one rat in our study exhibited both a sustained DA elevation and locomotor activity (Figure 10, asterisk). Six drug combinations were uniquely associated with one of the two treatment outcomes (Table 1). Cocaine after 0 or 0.2 mg/kg raclopride induced locomotor activity but did not elevate tonic DA. The higher doses of cocaine (20 and 30 mg/kg) after 2 mg/kg raclopride elevated tonic DA but did not induce locomotor activity. The remaining drug combinations (0.5 or 2 mg/kg raclopride and 10 or 30 mg/kg cocaine) produced mixed outcomes. However, in the individual rats, these drug combinations produced, on a mutually exclusive basis, either elevated tonic DA or locomotor activity but not both (see inset panel in Figure 10 for details). A summary of locomotor activity and increases in DA concentrations for all rats are included in the Appendix.

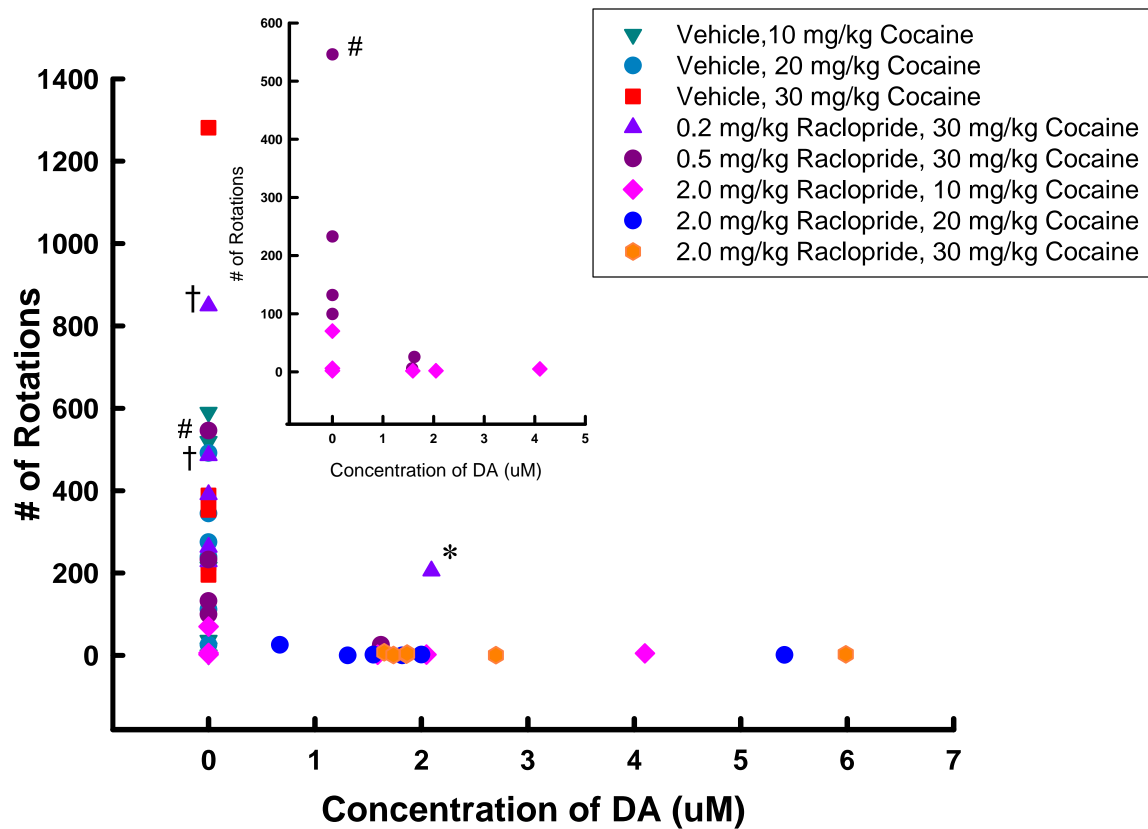

Figure 10: The effect of cocaine (10, 20, and 30 mg/kg i.p.) on locomotor activity of rats pretreated with either raclopride (0.2, 0.5, and 2.0 mg/kg i.p.) or vehicle (1 mL PBS i.p.). Locomotor activity was analyzed from rats for 65 minutes following the administration of cocaine.

Experimental Outcome		Drug Treatment	
Locomotor Activity	Sustained increase in ECS DA	Raclopride (mg/kg)	Cocaine (mg/kg)
Yes	No	0	10
		0	20
		0	30
		0.2*	30*
No	Yes	2	30
		2	20
Yes/No	No/Yes	2	10
		0.5	30

Table 1: Table summarizing the experimental outcomes, including locomotor activity and increase in ECS DA vs. the drug treatment administered. *The one animal that exhibited both locomotor activity and a sustained increase in extracellular DA received 0.2 mg/kg raclopride and 30 mg/kg cocaine.

3.5 DISCUSSION

The concept that DAergic neurotransmission occurs on multiple time scales is frequently discussed in the literature (Grace 1991; Nicholson 1995; Cragg *et al.* 2001; Schmidtz *et al.* 2001; Goto and Grace 2005). This concept invokes the idea that relatively slow, minute-to-minute changes in extracellular DA concentrations are associated with tonic DAergic transmission, whereas rapid, subsecond DA transients are associated with phasic DAergic transmission (Grace 1991; Grace 1995; Floresco *et al.* 2003). Since the DA responses observed during this study were sustained and long-lasting, tonic DAergic transmission is the focus of our attention. Our findings suggest that tonic elevations of ECS DA in the NAc abolish cocaine-induced hyperactivity. In 47 of the 48 rats in our study, drug administration induced, on a mutually exclusive basis, either a tonic elevation of DA or hyperactivity but not both (Figure 10). This study involved 8 different drug combinations, six of which induced either a tonic elevation of DA or hyperactivity (Figure 10, Table 1). Two drug combinations produced both outcomes, but those outcomes remained mutually exclusive in individual rats (Figure 10 (inset), Table 1). These findings suggest that tonic DA elevations abolish cocaine-induced hyperactivity and therefore tonic DA is functionally significant.

At first glance, the results of this study appear irreproducible. However, this apparent irreproducibility is not derived from the techniques or procedures employed for this study. Rather, it derives from a variation in the response of individual rats to the drug treatments. For example, the combination of 0.5 mg/kg raclopride with 30 mg/kg cocaine induced a tonic DA elevation in only 2 of six animals, neither of which exhibited hyperactivity (Figure 10). The other four rats exhibited hyperactivity but not elevated DA. Hence, the irreproducible effect of

the drug treatment on ECS DA levels is fully consistent with, and complementary to, the irreproducible effect on hyperactivity.

Consistent with previous reports (Cabib *et al.* 1991; Ushijima *et al.* 1995; Chausmer and Katz 2001), we observed a dose-dependent decrease in cocaine-induced hyperactivity in animals pretreated with raclopride (Figure 9). Such results have been used to support the conclusion that D2R activity is a necessary component of cocaine-induced hyperactivity. However, this conclusion, while parsimonious, does not account for the observation that cocaine induces hyperactivity in D2R knockout animals (Chausmer et al. 2002). Our findings show instead that the effect of raclopride on cocaine-induced hyperactivity is a sensitive function of the effect of the drug treatment on extracellular DA levels. All rats in Figure 9 received 30 mg/kg cocaine: of the six rats that received 0.2 mg/kg raclopride, six exhibited hyperactivity but only one exhibited elevated DA; of the six rats that received 0.5 mg/kg raclopride, four exhibited hyperactivity and the other 2 exhibited elevated DA; of the six rats that received 2.0 mg/kg raclopride, none exhibited hyperactivity but all exhibited elevated DA. Thus, the intermediate average number of rotations recorded in animals that received 0.5 mg/kg raclopride (Figure 9) derives from the fact that some animals in this group rotated normally, whereas some did not rotate at all. For example, one of these rats accumulated more activity counts than 5 of the rats that received the same dose of cocaine but no raclopride (Figure 10, # symbol). Likewise, two rats that received 0.2 mg/kg raclopride and 30 mg/kg cocaine accumulated more activity counts than 5 of the rats that received only 30 mg/kg cocaine (Figure 10, † symbol). Thus, we conclude that large increases in ECS DA are responsible for suppressing activity, indicating that hyperactivity is inversely related to tonic ESC DA levels in the NA.

3.5.1 Methodological considerations

Microdialysis is frequently chosen for slow DA measurements in vivo whereas voltammetry is often chosen for rapid DA measurements. However, voltammetric recording protocols vary widely in the style, dimension, and pretreatment of the microelectrode, the details of the voltammetric waveform (speed, voltage range, and interval between scans), surgical techniques (e.g. optimization of electrode placement), and data post-processing (background subtraction, signal integration, low-pass filtering, etc.). In several respects, our procedures are well-adapted to monitoring tonic DA. We employ electrochemically pretreated microcylinder voltammetric electrodes, which are sensitive to DA but have relatively slow response times, i.e. the time to reach 90% of the new steady-state signal following a change in DA concentration, of more than a hundred milliseconds. Furthermore, our electrodes are 300 μm in length, which provides a large electrode surface area for DA detection at the expense of spatial resolution. Since this study was aimed at monitoring tonic DA, we post-processed our data with a low-pass filter based on a 41-point (16-s) moving average to reduce nonDA-related noise spikes encountered during experiments in hyperactive animals. This low-pass filter may however remove DA transients from our signals but enabled the monitoring of tonic DA responses.

Substantial evidence now supports the idea that our voltammetric procedure is highly effective for monitoring tonic DAergic events. For example, we have recorded tonic decreases in ECS DA following the intrastriatal infusion of kynurenic acid (Kulagina et al. 2001; Borland and Michael 2004), we have recorded tonic DA increases after DAT-I in rats pretreated with D2 antagonists in both anesthetized and unanesthetized animals (Mahon et al. 2005; Willoughby Chapter 1; this study), and we have recorded sustained increases in ECS DA after DAT-I in tissue adjacent to microdialysis probes (Borland et al. 2005). Furthermore, in Chapter 1, I

provided a detailed validation of our approach to identifying voltammetric responses related to tonic ECS DA changes.

3.5.2 Effect of tonic DA on hyperactivity involves the D1R

According to our findings (Peters et al. 2002; Chapter 1; the present study), DAT-I alone does not induced tonic elevations of ECS DA in NAc even when relatively high inhibitor doses are used. It appears instead that tonic DA levels are stabilized near basal levels after DAT-I via a mechanism that involves the autoinhibition of DA release by D2 autoreceptors. The autoinhibition of DA release compensates for the decrease in the velocity of DA uptake. Interference with the autoinhibition by means of a D2R antagonist thus leads to sustained increases of ECS DA after DAT-I (Peters et al. 2002; Mahon et al. 2005; Chapter 1, and this study) and presents the opportunity to address the involvement of tonic DA acting at postsynaptic D1 receptors, which are not blocked by raclopride, in cocaine-induced hyperactivity.

The mutually exclusive relationship between tonic DA elevations and hyperactivity suggests that the tonic elevations of DA themselves were responsible for abolishing locomotor activity in these animals. This raises the question as to why a tonic ECS DA elevation inhibits cocaine-induced hyperactivity, considering that the elimination of cocaine-induced hyperactivity in the D1R knockout demonstrates that D1R activity is necessary for behavioral activation after DAT-I (Xu et al. 1994; Xu et al. 2000). Since activation of the D1R by DA is viewed as the underlying cause of cocaine-induced hyperactivity, our finding that tonic elevations of DA abolish locomotor activity is unexpected.

One point to consider is that the drug combinations used during this study elevated DA to levels sufficient to saturate the D1R. In combination with 2 mg/kg raclopride, cocaine increased ECS DA concentrations in the NAc, on average, by 1.3 μM, according to the postcalibration procedures used during this study. This concentration, which is presumably superimposed on pre-drug basal DA, exceeds the EC_{50} (ca. 0.5 μM) of DA in assays of D1R activity based on the rate of cAMP production (Kim et al., 2004). However, saturation of the D1R does not by itself appear to abolish its function. For example, in assays of D1R activity, maximal production of cAMP is achieved at saturating DA concentrations, suggesting that the D1R remains positively coupled to adenylate cyclase in the presence of high DA concentrations (Ng et al. 1995).

Another point to consider is the possibility that the sustained elevations of ECS DA during these studies triggered agonist-induced desensitization of D1Rs. The D1R is one of many G protein-coupled receptors that is known to exhibit agonist-induced desensitization. Agonist binding triggers phosphorylation of the cytoplasmic loops of the receptor that results in decoupling of the receptor from G proteins and targets the receptor for internalization (Kim et al. 2004). However, in transfected cells expressing the D1R, exposure to 10 μM DA for 90 min only doubled the DA EC_{50} and only decreased the maximal rate of cAMP production by 50%. Thus, agonist-induced desensitization appears to decrease, but not abolish, D1R activity (Kim et al. 2004).

Although it appears likely that the conditions leading to the inhibition of locomotor activity involve saturation and desensitization of the D1R in the NAc, these phenomena appear insufficient to abolish D1R activity and so only partially explain the abolition of cocaine-induced hyperactivity by elevated ECS DA levels. Another point to consider is that several recent reports from Wightman's laboratory show that cocaine increases the amplitude and frequency of high-

speed DA transients in the NAc (Phillips et al. 2003; Robinson and Wightman 2004). This leads us to hypothesize that, specifically, transient activation of D1Rs is necessary for cocaine-induced hyperactivity. In such a case, a tonic elevation of ECS DA might abolish cocaine-induced hyperactivity by preventing the D1R from recognizing phasic DA events. In this respect, tonic DA levels would appear to be acting as a gate upon phasic DAergic transmission in the manner proposed by Grace sometime ago (Grace 1991).

3.5.3 Tonic DA release

The origins of tonic ECS DA remain an open question. DA reaches the extracellular space via two release mechanisms, vesicular release and reverse transport. Microdialysis studies suggest that basal extracellular DA levels derive entirely from the vesicular route, since basal DA efflux is sensitive to tetrodotoxin (Westerink et al. 1987). This has lead to the attribution of tonic DA to the pacemaker-like, slow firing mode of midbrain DA neurons. On the other hand, using voltammetry, we observed a tonic decrease of ECS DA from basal levels upon the intrastriatal infusion of kynurenic acid (Borland and Michael 2004). The effect was insensitive to tetrodotoxin and sensitive to nomifensine, indicating that reverse transport also contributes to basal DA. In the anesthetized rat, however, the sustained increase of ECS DA observed after the administration of both a D2 antagonist and a DAT inhibitor was tetrodotoxin-sensitive (Borland 2005), confirming that vesicular DA release contributes to the tonic DA elevations reported herein.

This study suggests a mechanism whereby tonic elevations of ECS DA levels in the NAc suppress behavioral activity in animals. This runs counter the general view that elevated DA levels are associated with behavioral activation. However, in at least one well-known case, a

link between elevated ECS DA and a suppression of hyperactivity appears to exist. The most prevalent pharmacotherapy for attention deficit hyperactivity disorder is the DAT inhibitor, methylphenidate (Rev. Elia *et al.* 1999). The efficacy of methylphenidate is often described as paradoxical, given the general expectation that DAT-I should increase ECS DA levels and promote hyperactivity. This study provides new insight into the relationship between tonic ECS DA and activity which corresponds with the effectiveness of methylphenidate.

3.6 ACKNOWLEDGMENTS

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APPENDIX

Table 2: Summary of locomotor activity and concentration of dopamine at various raclopride and cocaine dose.

Dose of Raclopride (mg/kg)	Dose of Cocaine (mg/kg)	Number of Rotations	Concentration of DA (uM)
0	10	214.50	0.00
0	10	36.50	0.00
0	10	18.00	0.00
0	10	590.25	0.00
0	10	1.25	0.00
0	10	519.50	0.00
0	20	239.25	0.00
0	20	275.25	0.00
0	20	491.25	0.00
0	20	344.50	0.00
0	20	25.50	0.00
0	20	111.50	0.00
0	30	224.25	0.00
0	30	388.50	0.00
0	30	1281.25	0.00
0	30	195.75	0.00
0	30	224.25	0.00
0	30	353.50	0.00
2	10	2.00	2.05
2	10	70.00	0.00
2	10	2.00	0.00
2	10	6.00	0.00
2	10	5.00	4.10
2	10	1.75	1.59
2	20	1.75	1.55
2	20	2.00	2.00
2	20	0.00	1.82
2	20	0.00	1.31
2	20	25.50	0.67
2	20	1.25	5.41
2	30	7.75	1.65
2	30	1.00	1.74
2	30	2.00	1.86
2	30	2.75	5.99
2	30	0.50	2.70
2	30	4.50	1.87
0.2	30	263.00	0.00
0.2	30	390.75	0.00

0.2	30	849.00	0.00
0.2	30	485.50	0.00
0.2	30	227.75	0.00
0.2	30	205.25	2.10
0.5	30	99.25	0.00
0.5	30	545.75	0.00
0.5	30	232.75	0.00
0.5	30	132.00	0.00
0.5	30	25.50	1.62
0.5	30	5.50	1.58

Summary of locomotor activity and dopamine levels for all animals involved in experiments in Chapter 3.

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