

EXPLORING EXTRACELLULAR DOPAMINE CONCENTRATION AND ITS
REGULATION ON DOPAMINE RELEASE BY VOLTAMMETRY

by

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Extracellular dopamine (DA) is critical in regulating DA release as well as interacting with other neurotransmission systems. Microdialysis and voltammetry are the major techniques for extracellular DA measurement *in vivo*. These two techniques provide distinct results due to their different detection volumes. Carbon fiber microelectrode is 10,000 times smaller than that of a microdialysis probe. With such a small size, carbon fiber microelectrode provides a high spatial resolution and causes unobservable damage to the brain which paints a completely different picture of DA release in the brain as compared to the knowledge obtained by microdialysis.

In Chapter I, with the high temporal and spatial resolution provided by carbon fiber microelectrode in conjunction with fast scan cyclic voltammetry (FSCV), we are able to detect DA terminal populations with different autoinhibition levels in rat striatum. We revealed a coupling between resting DA and local autoinhibition level. The recording sites with high resting DA concentration (μM) exhibit a high autoinhibition on evoked DA release induced by medial forebrain bundle (MFB) stimulation, and *vice versa*. These different types of DA release will never be observed by microdialysis due to its large dimension. On the contrary, microdialysis result is an average of all the DA release sites (high and low) that the microdialysis probe goes through. This averaging method could contribute to the low measurement of the DA concentration by microdialysis.

In Chapter II, we examined the resting DA by a carbon fiber microelectrode at ~200 μm away from a microdialysis probe. We found TTX-insensitive DA was decreased by microdialysis probe implantation. This reduction contributes to the low DA measurement by microdialysis.

In Chapter III, we monitored evoked DA induced by MFB stimulation in the tissue near microdialysis probe. We found DA terminals near a microdialysis probe are hyper-sensitive to D2 receptor antagonist and DA transporter inhibitor. This suggests that the DA terminals in the tissue near microdialysis probe are under an altered neurochemical state with a loss of DA homeostasis.

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INTRODUCTION

Dopamine (DA) is an important neurotransmitter highly involved in many brain functions. Dysfunction of DA system is related to many neurological and psychiatric diseases, such as Parkinson's disease (Booij et al. 1999; Dagher and Robbins 2009) attention deficit hyperactivity disorder (Levy and Swanson 2001), and substance abuse (Koob and Bloom 1988). Over the past several decades, central DA systems have been the subject of intense investigation utilizing numerous methodologies such as fluorescence, voltammetry, electrophysiology, microdialysis, positron emission tomography and etc. (Aghajanian and Roth 1970; Kawagoe and Wightman 1994; Calabresi et al. 2000; Chefer et al. 2006; Nader et al. 2008). Many studies focus on measuring extracellular DA concentration and identification of the factors responsible for regulation and maintenance of extracellular DA.

Among these techniques, microdialysis and voltammetry evolved to be the major techniques for measuring extracellular DA *in vivo*, many studies focused on the rat striatum as a model system (Church et al. 1987; Hurd and Ungerstedt 1989; Garris et al. 1997; Borland and Michael 2004; Robinson and Wightman 2004; Katagiri et al. 2009; Wang et al. 2010). Despite both being applied widely, these two techniques utilized distinct methodologies.

Microdialysis utilized a semi-permeable membrane to form a cylindrical shape at the microdialysis probe tip with an active detection volume of 220 μm diameter and 4 mm long. The inlet solution of artificial cerebral spinal fluid (aCSF) was pumped into the microdialysis probe to exchange with brain fluid through the semi-permeable membrane and then pumped out of the outlet tubing. Outlet dialysate was analyzed on HPLC or capillary electrophoresis. These coupled analyzing equipment made microdialysis technique highly sensitive and selective and capable of monitoring multiple chemical species simultaneously. While on the other hand, the disadvantage of this technique is that it is incapable of dynamic DA (sub-second events) measurement due to the low perfusion rate to facilitate a substantial exchange between inlet solution and brain fluid and hence result in a long collection time (20 min in most

studies) to obtain enough sample for detection. With its temporal resolution limitation, the major applications of microdialysis are the slow events lasting for minutes, such as drug effects on basal DA and behavior related DA fluctuations (see examples of Church et al. 1987, Westerink et al. 1987, Mitala et al. 2008). Another limitation of microdialysis lies in its large dimension, the inlet solution exchanges with all the brain tissue around the semi-permeable membrane. Hence the microdialysis result is an average from all the brain tissue surrounding the probe membrane, which masks the heterogeneity of the sub-populations of DA terminals around microdialysis probe.

Fast scan cyclic voltammetry (FSCV) performed on a carbon fiber microelectrode is the other major technique for measuring extracellular DA *in vivo*. The carbon fiber is 7-10 μm of diameter with a length of 100 or 400 μm which is approximately 1/10,000th of the volume of microdialysis probe. This small detection volume makes the carbon fiber microelectrode an excellent approach for locating small population of terminals to monitor local DAergic activities. Additionally, the high potential scan rate (300 V/s) utilized in FSCV facilitates detection of sub-second events on carbon fiber microelectrode which makes this technique capable of monitoring dynamic DA release. While on the flip side, the high potential scan rate generates a high background current which contains a large portion of non-faradic current. DA detection is achieved by current subtraction performed on two states with different DA concentrations. The subtracted cyclic voltammogram is used to identify DA by recognizing its signature oxidation and reduction peaks. With the high temporal resolution, voltammetric measurements have revealed a wealth of information on dynamic DA release (Wightman and Zimmerman 1990; Garriss et al. 1994; Benoit-Marand et al. 2001; Heien et al. 2005), which includes DA transients associated with the electrophysiological activity of midbrain DA neurons (Robinson et al. 2001; Wightman and Robinson 2002; Phillips et al. 2003; Roitman et al. 2004; Stuber et al. 2005; Sombers et al. 2009).

Although microdialysis and voltammetry monitor DA activities at different timescales, they both focus on measuring extracellular DA concentration and identifying the factors regulating DA. Extracellular DA concentration reflects a balance between DA release from terminals and clearance from

the extracellular space via DA transporter (DAT) (Horn 1990; Wightman and Zimmerman 1990; Smith and Justice 1994; Booij et al. 1999; Chefer et al. 2006), a transmembrane protein that locates on the plasma membrane of DA cells (Reith et al. 1997). However, both DA release and clearance are themselves subject to multiple forms of regulation, including the factors from DA terminal itself (i.e. autoinhibition) and from other molecules as well (i.e. nicotine, H₂O₂, etc.) (Horn 1990; Wiczorek and Kruk 1995; Zahniser et al. 1999; Cowell et al. 2000; Chen et al. 2001; Avshalumov and Rice 2003; Rice and Cragg 2004; Zhang and Sulzer 2004).

DA release measured by means of voltammetry and microdialysis clearly show that DA release involves synaptic vesicle fusion, a Tetrodotoxin (TTX)-sensitive event (Millar et al. 1985; Westerink et al. 1987; Westerink and De Vries 1988; Santiago et al. 1992). On the other hand, voltammetric studies from our laboratory have begun to suggest that tonic DA release also occurs by a non-vesicular mechanism (Kulagina et al. 2001; Borland and Michael 2004; Mitala et al. 2008). In our hands, intrastriatal infusions of kynurenate (KYN, a broad spectrum antagonist of ionotropic glutamate receptors (Stone 1993), an inhibitor of the glycineB site of the N-methyl-D-aspartate receptor (Kessler et al. 1989), and an antagonist of the α 7 nicotinic acetylcholine receptor (Pereira et al. 2002)) elicit a decrease in extracellular DA from the basal level (Kulagina et al. 2001; Borland and Michael 2004; Mitala et al. 2008). The ability of KYN to decrease DA from the basal level is insensitive to TTX and sensitive to nomifensine, a DAT inhibitor, characteristics which suggest that the basal extracellular DA in the striatum includes a tonic pool derived from DA reverse transport (Borland and Michael 2004), i.e. DA release via the DAT. While our findings are consistent with other studies of DA reverse transport (Eshleman et al. 1994; Leviel 2001), they are surprising in light of the evidence from in vivo microdialysis that basal DA release is exclusively vesicular (Westerink and De Vries 1988; Santiago et al. 1992).

Indeed the TTX-sensitive basal DA concentration measured by microdialysis is in low nanomolar, which could be undetectable by voltammetry (detection limit: 20-50 nM). But the non-vesicular resting

DA concentration observed by voltammetry is in micromolar range which far exceeds the detection limit of microdialysis. This motivates us to investigate into this issue.

The heterogeneity of evoked DA within striatum has been well known for decades, which has been documented as early as in 90s (Garris et al. 1994). Both slow and fast evoked DA release phenomena have been observed in rat striatum. Our previous study found that the slow and fast DA release is due to the autoinhibition level of the recording sites (Moquin and Michael 2009). Autoinhibition is a self-regulation of DA release from DAergic terminal triggered by extracellular DA binding to presynaptic D2 receptors. We hypothesize that the different autoinhibition level is due to the different activation of D2 receptor by their local resting DA. By monitoring the static and dynamic DA in the rat striatum by FSCV, we demonstrated a coupling relationship between the autoinhibition and the local resting DA levels (Wang et al. 2010, also see Chapter I). We found both high and low resting DA sites could exist in the rat striatum. Considering the large area that microdialysis probe goes through, averaging these sites with different DAergic tones, including both high and low resting DA could contribute to the low DA measurement by microdialysis.

Despite the contributions due to the methodology difference described above, we believe the discrepancy is also the result of tissue disruption caused by microdialysis probe implantation due to its large dimension (220 μm diameter). Microscopic studies revealed extensive tissue disruption around microdialysis probe, which includes cell death, blood system disruption and gliosis response (Clapp-Lilly et al. 1999; Zhou et al. 2001; Jaquins-Gerstl and Michael 2009; Jaquins-Gerstl et al. 2011). Considering the dialysate DA is exclusively TTX-sensitive (Westerink and De Vries 1988; Santiago et al. 1992), we hypothesize that the TTX-insensitive DA is disrupted by microdialysis probe implantation. We tested this hypothesis by infusing kynurenate in rat striatum and measuring static DA response by a carbon fiber microelectrode in the affected tissue (~ 200 μm away from probe) before and after microdialysis probe implantation (Chapter II). We observed a decrease of the response of resting DA to kynurenate infusion in the tissue near microdialysis probe. This suggests that microdialysis probe implantation would lead to a

decrease or even disappearance of TTX-insensitive DA in the nearby tissue which would result in low nanomolar and purely TTX-sensitive DA measurement in dialysate.

In Chapter III, we investigate the sources that cause DA suppression in the brain tissue near a microdialysis probe. In rat striatum, evoked DA was decreased and slowed down by microdialysis probe implantation (Borland et al. 2005). This suppression of DA release resembles the effect of quinpirole, a D2 agonist (Moquin and Michael 2009; Wang et al. 2010). Hence we hypothesize that the DA terminals near microdialysis probe were under high autoinhibition regulations. We tested this hypothesis by monitoring the effect of raclopride, a D2 receptor antagonist on the evoked DA in the affected tissue by a carbon fiber microelectrode implanted near a microdialysis probe (~200 μm away). We found that the terminals near microdialysis probe were experiencing a high autoinhibition level than non-probe implanted tissue. Further raclopride treatment alone cannot remove the suppression in the disrupted tissue completely. A following dose of nomifensine, a DA uptake inhibitor still exhibited a dramatic effect on the evoked DA release while in the non-probe implanted tissue, nomifensine effect was minimal after raclopride treatment. These results suggest that DA terminals in the disrupted tissue near a microdialysis probe are hyper-autoinhibited and hyper-sensitive to DAergic drugs.

Chapter I. The Coupling between Resting and Dynamic Extracellular DA in Rat Striatum

(This chapter is adapted from Wang et al. 2010 with modifications)

1.1 INTRODUCTION

Central DA systems attract intense interest because they participate in multiple aspects of normal brain function and because DA dysfunctions are implicated in multiple CNS disorders, including Parkinson's disease (Booij et al. 1999; Hornykiewicz 2002; Dagher and Robbins 2009), schizophrenia (Stone et al. 2007), attention deficit hyperactivity disorder (Levy and Swanson 2001), and substance abuse (Koob and Bloom 1988). Current theories on the multiple functions of DA invoke the concept that extracellular DA operates on multiple timescales. In a recent review, Schultz pointed out that DA functions can be divided approximately between those involving rapid changes in extracellular DA concentrations (sub-seconds to seconds) and slow changes (minutes to tens-of-minutes) (Schultz 2007). Numerous studies have employed microdialysis (e.g. Hernandez et al. 2007) and voltammetry (e.g. Robinson and Wightman 2007) to elucidate DA events on different time scales. Extracellular DA concentrations generally reflect a balance between the rate of DA release and clearance (Wightman and Zimmerman 1990; Smith and Justice 1994). As DA release and clearance are each tightly regulated, extracellular DA concentrations appear to be precisely and intricately controlled (Wieczorek and Kruk 1995; Zahniser et al. 1999; Cowell et al. 2000; Chen et al. 2001; Rice and Cragg 2004; Zhang and Sulzer 2004). Voltammetry is effective for monitoring rapid DA dynamics (Wightman and Robinson 2002) whereas microdialysis is effective on the longer timescales (Watson et al. 2006; Nandi and Lunte 2009).

However, the objective of the present study was to test the hypothesis that a coupling between the multiple DA timescales contributes to the control of extracellular DA concentrations. Thus, we used a single technique, voltammetry, to monitor DA on multiple timescales.

Fast-scan cyclic voltammetry in conjunction with carbon fiber microelectrodes facilitates the monitoring of extracellular DA dynamics in the rat striatum during electrical stimulation of the medial forebrain bundle (MFB) (Kuhr et al. 1987; Wightman et al. 1988). The dynamics of evoked release are both heterogeneous and sensitive to the manipulation of pre-synaptic D2 autoreceptors (Benoit-Marand et al. 2001; Kita et al. 2007; Moquin and Michael 2009). The D2 antagonist, raclopride, increases the rate of evoked release whereas the D2 agonist, quinpirole, has the opposite effect. For this reason, herein we hypothesize that the inherent local heterogeneity of DA dynamics stems from different levels of occupation of D2 autoreceptors. Such a hypothesis implies local variations in the basal extracellular DA concentration, although direct observations of such a phenomenon have not been described to date because microdialysis, the preferred approach to assessing basal DA levels, averages the DA concentration over both distance (i.e. the length of the probe) and time (i.e. the sampling duration). Thus, in the present study, we employed voltammetry, which offers higher spatial resolution, to investigate local variations in basal DA.

Previously (Kulagina et al. 2001; Borland and Michael 2004; Mitala et al. 2008), we reported that voltammetry, under specific circumstances, provides a measure of long-timescale DA. Intra-striatal infusions of the glutamate antagonist, kynurenatate (KYN), elicit a decrease in basal (i.e. non-evoked) DA as measured with a voltammetric electrode placed near the infusion site. We demonstrate herein that the striatum is heterogeneous in its response to KYN infusions. Thus, in regards to the basal DA concentration, the striatum exhibits both KYN-sensitive and KYN-insensitive sites. Moreover, sites where basal DA is KYN-sensitive and KYN-insensitive yield substantially and significantly different evoked DA responses during MFB stimulation. Collectively, our findings show that basal DA (i.e. long-timescale DA) contributes to the regulation of evoked DA (i.e. sub-seconds to seconds timescale DA), supporting the conclusion that a coupling exists between the multiple timescales of DA function.

1.2 METHODS AND MATERIALS

1.2.1 Carbon fiber microelectrodes and Fast scan cyclic voltammetry

Carbon fiber microelectrodes were constructed by inserting a single carbon fiber (7 μm diameter, Thornell Carbon Fiber, T300, Amoco Performance Products, Inc., Greenville, SC, USA) into a borosilicate glass capillary (0.75 mm I.D., 1 mm O.D.; Sutter Instruments, Co., Novato, CA, USA). The capillary was pulled to a fine tip around the fiber with a vertical micropipette puller (Narishige, Tokyo, Japan). The resulting tip was sealed with Spurr Epoxy (Polysciences, Inc., Warrington, PA, USA) and the exposed fiber was trimmed to a length of 100 μm . This comparatively short carbon fiber length is used for selectively locate the areas with different DAergic kinetics. Each electrode was pre-treated with a triangular potential waveform (0–2 V vs. Ag/AgCl reference at 200 V/s for 1 s) in artificial cerebrospinal fluid (145 mM Na^+ , 1.2 mM Ca^{2+} , 2.7 mM K^+ , 1.0 mM Mg^{2+} , 152 mM Cl^- and 2.0 mM phosphate, adjusted to pH 7.4). For fast scan cyclic voltammetry, the resting potential was 0 V vs. Ag/AgCl and the voltage was swept linearly to 1.0 V, then to -0.5 V and back to 0 V at 300 V/s. During experiments involving MFB stimulation, scans were performed at 10 Hz. During longer duration experiments involving KYN infusion, scans were performed at 2.5 Hz. The DA voltammetric signal was obtained in the 500–700 mV potential range of the first potential sweep, which gives the maximum DA oxidation current. Voltammetric currents were converted to DA concentrations by post-calibration of the microelectrodes after they were extracted from the rat brain.

Data collected during KYN infusions were corrected for signal drift by the method described by Borland and Michael (2004). Current signals collected in the range of 500-700 mV were subtracted by the current values collected in the range of 200-300 mV, to correct the baseline drift. Accordingly, the post-calibration data were corrected by the same method and in vivo current recording during KYN infusions were converted by the corrected post-calibration results.

Calibration was performed in artificial cerebrospinal fluid. DA identification was based on the appearance of background subtracted voltammograms (Borland and Michael 2004).

1.2.2 Animals and surgical procedures

All procedures involving animals were carried out with the approval of the Institutional Animal Care and Use Committee of the University of Pittsburgh. Male Sprague–Dawley rats (Hilltop, Scottsdale, PA, USA) (250–375 g) were anesthetized with isoflurane (5% initially, 2.5% for maintenance) carried by oxygen throughout all experiments. While anesthetized, rats were wrapped in a homeothermic blanket at 37 °C (EKEG Electronics, Vancouver, BC, USA) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) with the incisor bar 5 mm above the interaural line (Pellegrino et al. 1979). Holes were drilled through the skull and dura mater was dissected to permit electrode implantations. Electrical contact between brain tissue and a Ag/ AgCl reference electrode was established with a salt bridge made with a plastic pipette tip plugged with a paper wick and filled with artificial cerebrospinal fluid.

Carbon fiber microelectrodes were placed in the striatum 2.5 mm anterior to bregma, 2.5 mm lateral from midline, and 4.5 mm below dura (Pellegrino et al. 1979). Stainless steel bipolar stimulating electrodes (MS303/a; Plastics One, Roanoke, VA, USA) were lowered towards the ipsilateral MFB (2.2 mm posterior to bregma, 1.6 mm from midline, and initially 7.5 mm below dura). From its initial position, the stimulating electrode was lowered slowly until evoked DA release was detected in the striatum: this is a well established procedure for locating nigrostriatal DA fibers (Ewing et al. 1983; Kuhr et al. 1984; Heien et al. 2005). The optically isolated stimulus was a biphasic, constant-current square wave (2 ms pulse duration, 280 μ A pulse height). Stimulation was delivered as either a single train (10 s) or four 1-s trains separated by 2-s intervals. The stimulation frequency is 60 Hz.

In some instances, as explained in the Results section, the placement of the recording electrode was optimized to facilitate the recording of fast-type or hybrid stimulus responses. This optimization involved lowering the electrode until a site yielding fast-type responses was located. In addition, in some of the experiments described below, we expressly wished to avoid fast-type and hybrid sites. In these instances, we employed the shorter (100 μm) electrodes as preliminary experiments showed that these shorter electrodes were more routinely located into slow-type sites without any procedure to optimize the placement.

1.2.3 Micropipettes and kynurenate infusion

Micropipets for the KYN infusions were constructed from fused silica capillary tubing (350 μm O.D., 25 μm I.D., Polymicro Technologies, Phoenix, AZ, USA). The capillary was pulled to a fine tip with a horizontal laser-powered puller (Sutter instruments, Novato, CA, USA) and trimmed to make the outer tip diameter 30 μm . The inlet end of the capillary was attached to a 50 μL gastight syringe (Hamilton, Reno, NV, USA) driven by a microprocessor-controlled driver (NA-1; Sutter Instruments). The syringe and pipette were pre-filled with 1 mM KYN in artificial cerebrospinal fluid. The micropipette was mounted in a stereotaxic carrier at 10° from vertical and aligned with the microelectrode before either was lowered into the brain. Stereotaxic procedures were used to position the tip of the pipet 150 μm laterally from the striatal microelectrode in the same coronal plane. The infusion volume was 200 nL (2×10^{-10} mol KYN) and the infusion duration was 2 min.

1.2.4 Statistics

Three-way analysis of variance (ANOVA) was performed on the evoked DA concentration results in Figure 1.5 and 1.6. Two-way ANOVA was performed on the non-evoked DA concentration

results in Figure 1.3, evoked DA concentration results in Figure 1.4, and normalized post-KYN evoked DA results in the inset of Figure 1.6. Post hoc comparison was performed using Tukey's criterion.

1.2.5 Chemicals and drugs

Kynurenic acid (kynurenate) and DA hydrochloride were used as received from Sigma (St Louis, MO, USA) and dissolved in artificial cerebrospinal fluid. Raclopride tartrate (2 mg/kg i.p.) (Sigma Aldrich) were dissolved in phosphate buffered saline (100 mM phosphate, pH 7.4). Isoflurane was obtained from Halocarbon (River Edge, NJ, USA). All solutions were prepared with ultrapure water (NANOPure; Barnstead, Dubuque, IA, USA).

1.3 RESULTS

1.3.1 Kynurenate infusions affect evoked DA release

Intrastriatal infusion of KYN suppresses evoked DA release during both the continuous and multiple-train stimuli used in this study (Figure 1.1). Figure 1.1A is an example response to continuous stimulation with the onset of evoked release delayed for about 2 s after start of the stimulus. KYN infusion both extended the delay in the onset of release and decreased the overall response amplitude. Figure 1.1B is an example response to consecutive 1-s stimulus trains separated by 2-s intervals. KYN infusion suppressed the response to all four stimulus trains. Throughout this study, KYN infusions suppressed all evoked responses within the striatum, regardless of the style of the stimulus (continuous or multiple trains) and regardless of the dynamic features of the pre-infusion response.

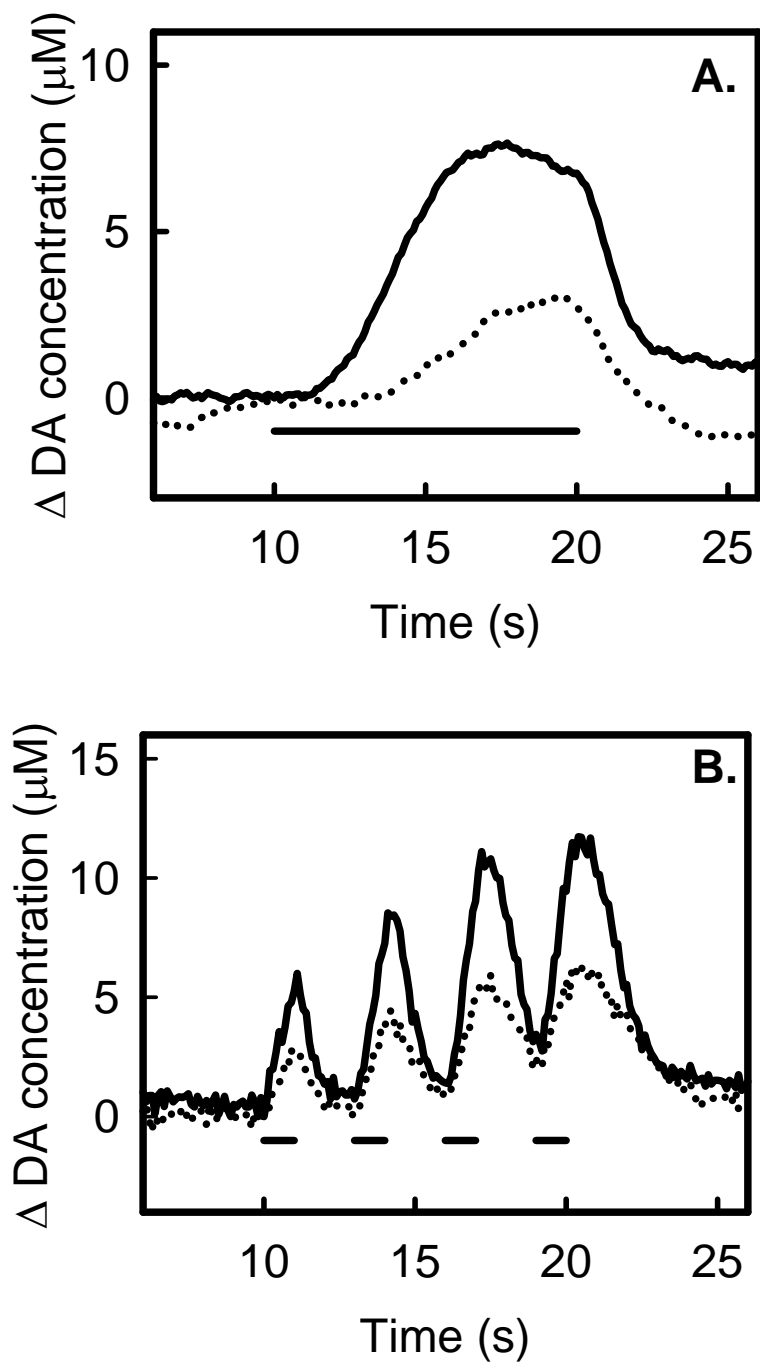


Figure 1.1 Intraatrial KYN infusion diminishes the amplitude of evoked DA release in response to continuous (A) and multiple-train (B) stimulation of the medial forebrain bundle (solid line: pre-KYN, dotted lines: post-KYN). Horizontal bars show the stimulation time.

1.3.2 The effect of KYN infusion on the tonic basal DA pool

Rats were implanted with a stimulating electrode in the MFB and a voltammetric recording electrode in the ipsilateral striatum. No optimization of the placement of the recording electrode to locate fast-type or hybrid sites was performed; however, sites exhibiting long onset delays in the response (e.g. Figure 1.1A) were excluded from the remainder of this study. Once the stimulating and recording electrodes were in position, a pre-infusion stimulus response was recorded. Then, the voltammetric signal (non-evoked) was recorded for 3 min before and 15 min after the start of KYN infusion. Finally, a post-infusion stimulus response was recorded: the post-infusion response was recorded 25 min after the pre-infusion response. Intrastratial KYN infusion decreased extracellular DA from its resting level at some striatal recording sites but not at others (Figure 1.2): these sites will henceforth be called KYN-sensitive and KYN-insensitive sites, respectively. KYN sensitive sites are identified by a decrease in the basal voltammetric signal and by the appearance of characteristic DA oxidation and reduction peaks in the background subtracted voltammograms: this identification scheme was explained in detail by Borland and Michael (2004). In some cases, the infusion causes an artifact, which is responsible for the spike in the averaged response near 400 s in the KYN insensitive group in Fig. 1.2. Such spikes are infrequent (one of 20 in this study) but have been observed before (Kulagina et al. 2001; Borland and Michael 2004). Inspection of the background-subtracted voltammograms associated with these spikes confirms that they are not DA-related. Hence, the striatum exhibits sites where the basal DA concentration is either KYN-sensitive or KYN-insensitive.

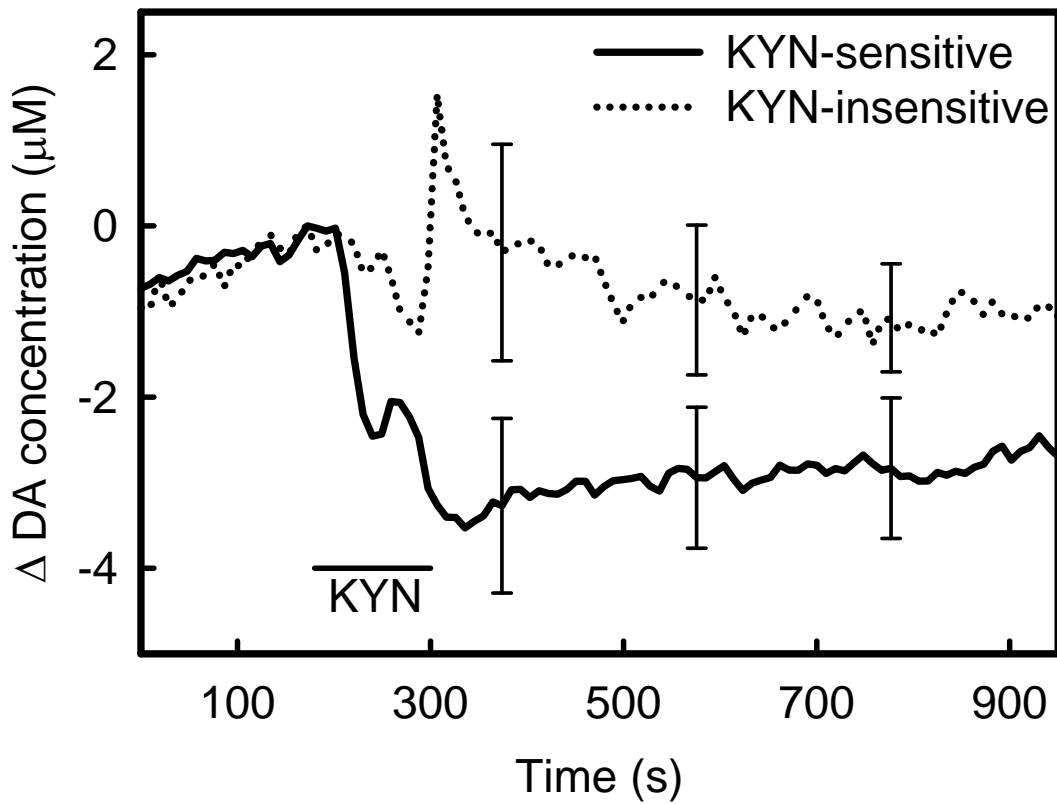


Figure 1.2 The striatum is heterogeneous with respect to the response to KYN-infusion. The horizontal bar indicates the KYN infusion (2×10^{-10} mol in 200 nL aCSF). Some sites are KYN-insensitive (dotted line) while others are KYN-sensitive (solid line). Each response is the average (\pm SEM) of responses from 10 different animals. The data points indicated by error bars were subjected to two-way ANOVA: the KYN-insensitive and KYN-sensitive groups are significantly different [$F(df = 1, 72) = 8.93, p < 0.004$]: time was not a significant factor.

1.3.3 Heterogeneity of evoked DA responses

The remainder of this study examines the differences between evoked responses recorded at KYN-insensitive and KYN-sensitive sites (all evoked responses in this study were affected by the KYN infusions: the terms ‘KYN insensitive’ and ‘KYN-sensitive’ refer specifically to the basal DA response, as in Figure 1.2).

Electrical stimulation of the MFB with a continuous 10-s train (60 Hz) evokes substantially and significantly different responses at KYN-insensitive and KYN-sensitive sites (Figure 1.3). The responses in Figure 1.3 were recorded prior to KYN-infusion. The identification of the recording site as KYN-insensitive or KYN-sensitive was based on the voltammetric response to a subsequent KYN infusion. The infusion responses obtained at the sites in Figure 1.3 ($n = 4$ each) are included in the data set used to construct Figure 1.2. Pre-KYN infusion evoked responses were also recorded using four 1-s trains separated by 2-s intervals (Figure 1.4). The responses evoked by individual stimulus trains were re-zeroed to account for the fact that the voltammetric signal usually does not return to the pre-stimulus baseline during the 2-s interval between trains (Moquin and Michael 2009). As with Figure 1.4, the recording sites were identified as being KYN-insensitive or KYN-sensitive during a subsequent KYN-infusion. The infusion responses obtained at the sites in Figure 1.4 ($n = 6$ each) are included in the data set used to construct Figure 1.2. The rising phase of the evoked responses from all 12 sites (Figure 1.4) were subjected to three-way ANOVA: the three factors were the experimental group [KYN insensitive (triangles) and KYN-sensitive (circles)], the stimulus train (first, second, third, and fourth), and the recording time within each train (100, 200, 300 ms, etc.).

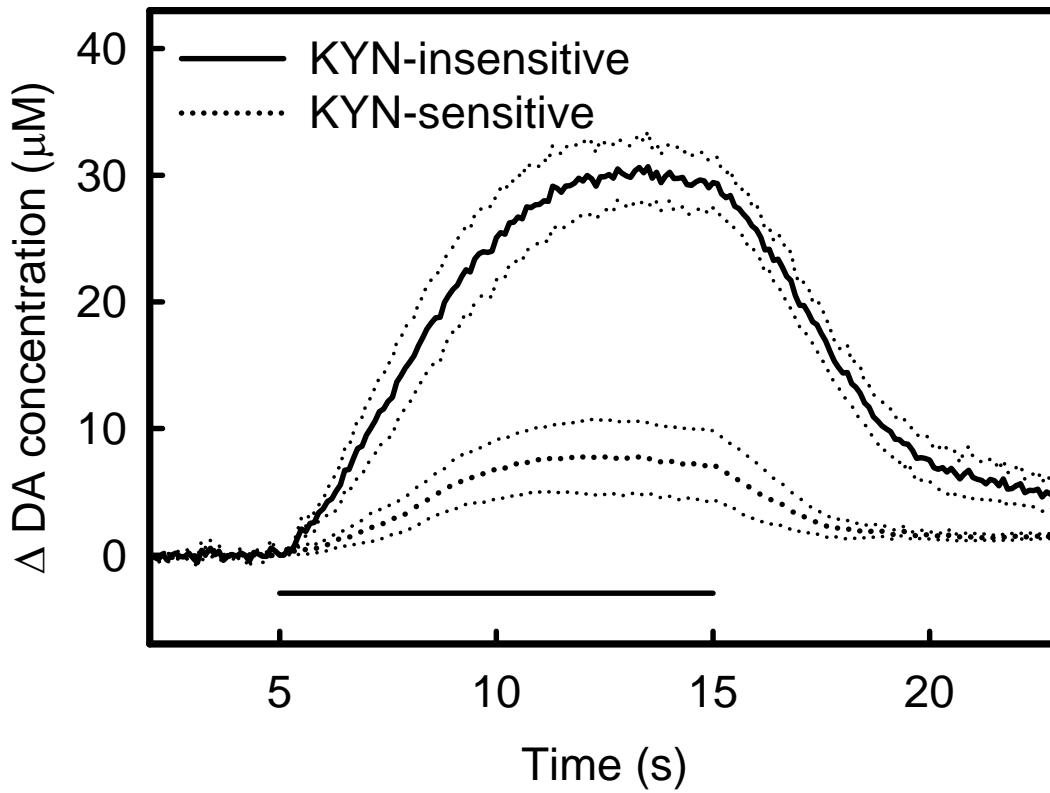


Figure 1.3 KYN-insensitive and KYN-sensitive recording sites yield substantially and significantly different evoked DA responses during a continuous 10 s stimulus train at 60 Hz (horizontal bar). Each trace is the mean \pm standard error of the responses recorded in four animals each. These data were subjected to two-way ANOVA with group (KYN-insensitive and KYN-sensitive) and time as the classifications: evoked responses at the KYN-insensitive and KYN-sensitive sites are significantly different [$F(df = 1, 606) = 1984, p < 0.000000000000001$].

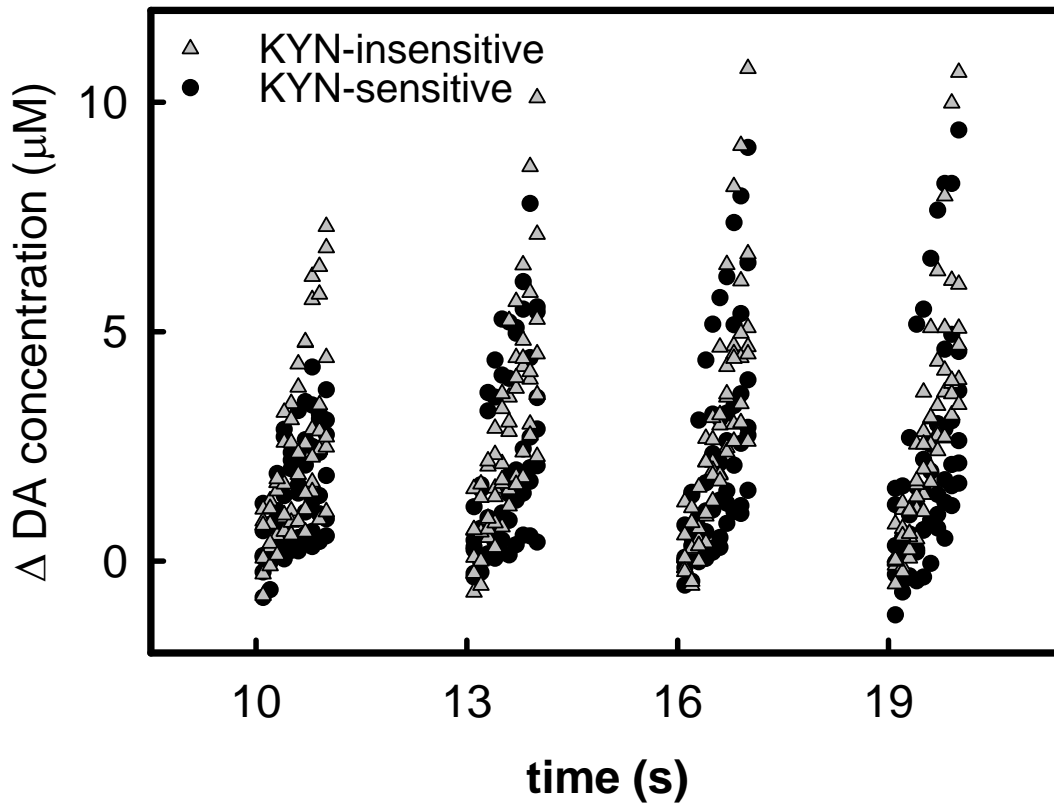


Figure 1.4 The rising phase of evoked DA responses during four consecutive stimulus trains (1-s trains with 2-s intervals) at KYN-insensitive (gray solid triangles) and KYN-sensitive (black solid circles) sites are significantly different. The response to each train was re-zeroed in this plot. These responses were subjected to three-way ANOVA: the responses at the KYN-insensitive and KYN-sensitive sites are significantly different [$F(df = 1, 400) = 20.6, p < 0.00001$].

The group factor is a highly significant contributor to the variance [$F(df = 1, 400) = 20.6, p < 0.00001$]: thus, evoked responses at KYN-insensitive and KYN-sensitive sites are significantly different. The maximum amplitudes of the responses to each stimulus train before and after KYN infusion (Figure 1.5) were also subjected to three-way ANOVA (factors: experimental group, before and after KYN infusion, and the stimulus train number). All three factors are significant and there is a significant interaction between the experimental groups and the stimulus train. The experimental group is a significant factor [$F(df = 1, 80) = 6.1, p < 0.02$] and, according to Tukey's pair-wise comparisons, the pre-infusion evoked responses in the KYN-insensitive and KYN-sensitive groups (Figure 1.5 top black bars v. Figure 1.5 bottom black bars) are significantly different ($p < 0.01$). In both groups, the pre-and post-infusion amplitudes (Figure 1.5 top black bars v. white bars; Figure 1.5 bottom black v. grey bars) are significantly different (Tukey's pair-wise comparisons: KYN-insensitive $p < 0.0000001$; KYN-sensitive $p < 0.005$). Interestingly, there is no significant difference between the absolute post-infusion amplitudes (Figure 1.5 top white bars v. bottom gray bars). However, when the post-KYN responses are normalized to the amplitude of the first stimulus train (Figure 3.5, inset), the KYN-sensitive and KYN-insensitive responses are significantly different [two-ANOVA, $F(df = 1, 40) = 5.19, p < 0.03$].

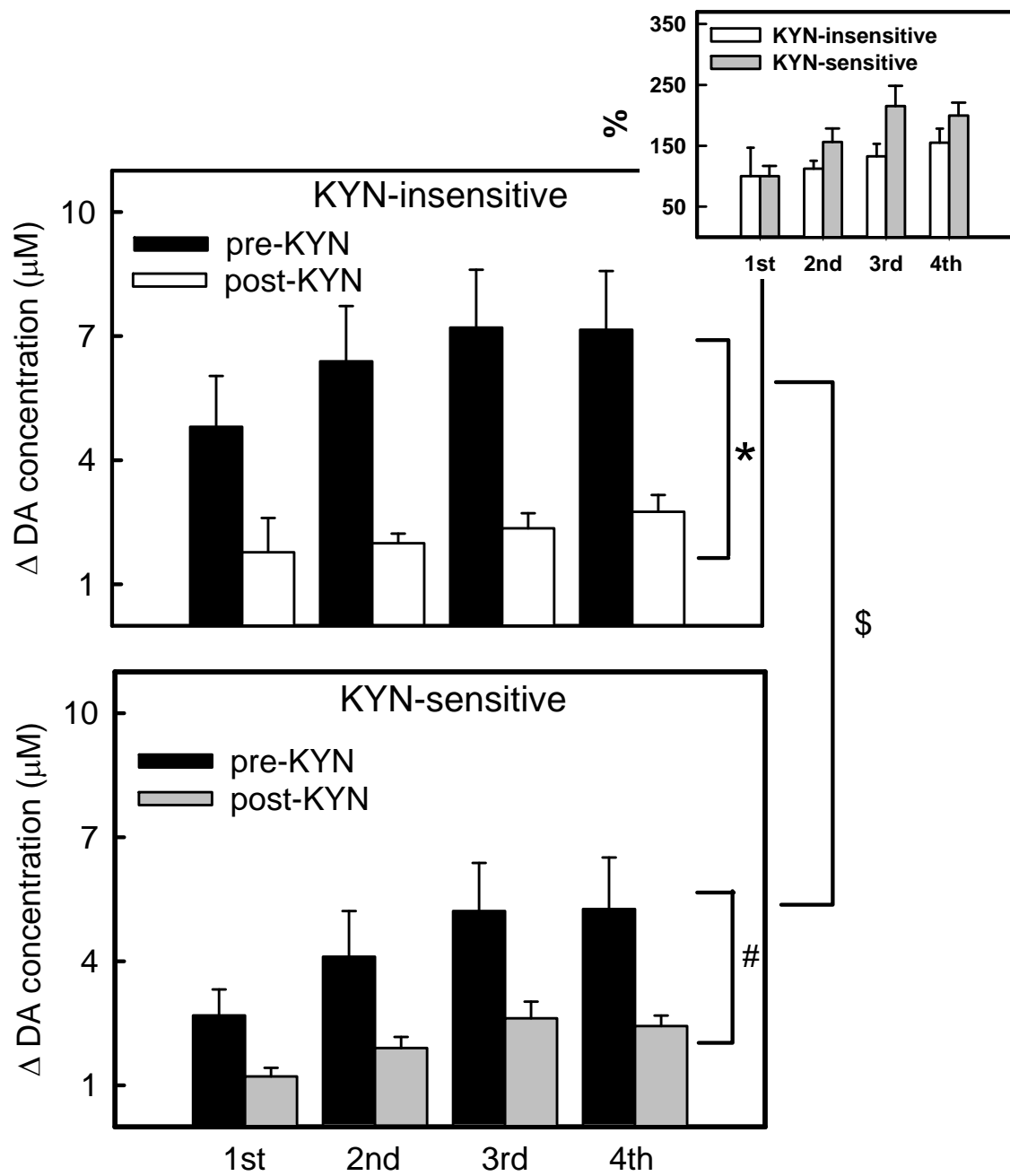


Figure 1.5 Histograms of the peak amplitude of the response to four consecutive stimulus trains (1-s trains with 2-s intervals) at KYN-insensitive (top panel) and KYN-sensitive (bottom panel) striatal sites before (black bars) and after (white and gray bars) KYN-infusion. The inset panel shows the post-infusion amplitudes normalized with respect to the amplitude of the response to the first train. Data in the main panels were subjected to three-way ANOVA: the KYN-insensitive and KYN-sensitive groups are significantly different [$F(df = 1, 80) = 6.11, *, p < 0.02$]. The Tukey's test confirmed that the pre-infusion responses at the KYN-insensitive and KYN-sensitive sites are different ($p < 0.01$) but not the post-infusion responses. The pre- and post-infusion groups are significantly different [$F(df = 1, 80) = 50.5, p < 0.00000001$]. The effect of infusion was significant in both the KYN-insensitive (*, $p < 0.0000001$) and KYN-sensitive sites (#, $p < 0.005$). Inset panel: these normalized responses were also subjected to two-way ANOVA: The KYN-insensitive and KYN-sensitive sites are significantly different [$F(df = 1, 40) = 5.19, p < 0.03$].

1.4 DISCUSSION

Evoked DA responses in the rat striatum are heterogeneous in both their amplitude and dynamic profiles. Originally, this heterogeneity was attributed to an anatomical variation in the local density of DA terminals (May and Wightman 1989a; May and Wightman 1989b) and sites within the striatum were broadly classified as DAergic or non-DAergic. However, reexamination of this issue leads to the alternative conclusion that the heterogeneity of evoked release is instead caused by local variations in the degree to which DA terminals are autoinhibited (Moquin and Michael 2009). The present study is focused on the underlying source of the heterogeneity in autoinhibition. We examined the hypothesis that heterogeneity in the basal DA content of the striatal extracellular space is a contributing factor. Local heterogeneity in the basal DA concentration is not described in the existing literature because basal DA is typically measured by microdialysis, which is an averaging technique (Watson et al. 2006; Nandi and Lunte 2009). In the present study, we find that the rat striatum, in addition to exhibiting heterogeneity in evoked responses, is also heterogeneous in the manner in which basal (non-evoked) DA responds to intrastriatal KYN infusion (Figure 1.2). At some striatal sites (KYN-sensitive sites), the infusions cause the basal DA concentration to decrease from its resting level. At other sites (KYN-insensitive sites), there is no such effect.

Furthermore, these distinct striatal sites yield substantially and significantly different evoked responses (Figures 1.3-1.5), supporting the overall conclusion that the heterogeneity in evoked release is coupled to the heterogeneity in the KYN sensitivity of the basal DA concentration. This coupling further supports the concept that DA terminals within the striatum are subdivided into fundamentally, and thus presumably functionally, distinct subpopulations in distinct dynamical states. It is interesting to note that the sites yielding the largest amplitude evoked responses (Figures 1.3-1.5) yielded the lowest amplitude infusion responses (Figure 1.2), and vice-versa, so the distinction between sites is not related in any simple manner to the density of local DA innervations (i.e. DA sites vs. non-DA sites). Thus, this study provides two findings that we regard as highly novel. First, that the striatum is spatially heterogeneous

with respect to the steady-state concentration of extracellular DA. Second, that the heterogeneity of the steady-state extracellular DA contributes to the heterogeneity of the dynamical properties of DA terminals.

1.4.1 Autoreceptors contribute to the heterogeneity of evoked release

Evoked release in the rat striatum exhibits a range of dynamic behaviors, which we broadly classified as fast-type and slow-type (Moquin and Michael 2009). Hybrid responses (Moquin and Michael 2009; Fig. 1) indicate that DA terminals in the fast and slow states exist side-by-side within the striatum. A following study from our lab (Wang et al. 2010; Fig.2) adds to the characterization of these sites by confirming that activation of D2 receptors with quinpirole abolishes the fast-type component of release. Quinpirole slows, but does not abolish, slow type release (Moquin and Michael 2009; Fig. 2). These effects of quinpirole support the explanation that fast-type release is observed when the basal DA concentration is too low to activate D2 autoreceptors. On the other hand, slow type release is observed when the basal DA concentration rises to a level sufficient to activate D2 autoreceptors.

The effects of the D2 antagonist, raclopride, on the dynamics of evoked release are complementary to those of quinpirole. Raclopride abolishes the delay in the onset of evoked release (Moquin and Michael 2009), even in cases where that delay is substantial (i.e. several seconds), as in Figure 3A of Wang et al. (2010). This is consistent with the explanation that the slow release behavior is a consequence of autoinhibition. These observations also show that sites yielding slow-type behavior cannot be considered non-DAergic sites (Venton et al. 2003) as DA's basal activity is clearly responsible for the on-going autoinhibition. Moreover, the non-DA sites defined anatomically by Venton et al. (2003) are much smaller than the dimensions of the cylindrical (100 μm long) voltammetric electrodes used for this study. Finally, previous studies have established that D2 antagonists minimally affect fast-type release. When evoked release is sufficiently fast to be detected during so-called pseudo-1-pulse stimuli (1-to-4 pulses), D2 antagonists have little to no effect on the response (Garris et al. 1994; Garris and

Wightman 1995; Benoit-Marand et al. 2001). This supports the conclusion that fast evoked responses occur when DA terminals are free of autoinhibition. At first glance, it might appear counter-intuitive that the amplitude of fast-type release should be consistently smaller than the amplitude of slow-type release. However, it is necessary to consider the dynamics of release and the amplitude of release separately. This situation arises for two reasons. First, the fast component of release is short lived because, although autoinhibition is absent prior to the onset of the stimulus, the stimulus itself raises the extracellular DA concentration, leading to a rapid onset of autoinhibition (Garris et al. 1994; Garris and Wightman 1995; Benoit-Marand et al. 2001) that results in the short-term depression of further release (Montague et al. 2004; Kita et al. 2007). This phenomenon is also observed in tissue slice preparations (Limberger et al. 1991; Kennedy et al. 1992; Phillips et al. 2002; Avshalumov and Rice 2003). On the other hand, slow-type release exhibits short-term facilitation rather than depression, so, whereas the initial rate of release is slow, the rate of release increases as the stimulation proceeds (Moquin and Michael 2009; Figs 3 and 4). Thus, slow-type release eventually exceeds fast-type release in amplitude.

As discussed in our previous study (Moquin and Michael 2009), we remain uncertain as to the mechanism underlying the short term facilitation of slow-type release.

1.4.2 Origin of the variations in autoinhibition

Voltammetric responses to intrastriatal infusion of KYN (Figure 1.2) reveal the existence within the striatum of sites wherein the basal DA concentration is KYN-insensitive or KYN-sensitive. These distinct sites yield substantially and significantly different evoked responses (pre-infusion) (Figures 1.3-1.5). The amplitude of evoked release, regardless of the details of the stimulus, was significantly higher at sites where basal DA was KYN-insensitive, consistent with the interpretation that these sites contain lower basal DA concentration, making the basal response more challenging to detect but also causing less autoinhibition of evoked release. On the other hand, sites where basal DA appears KYN-sensitive

exhibited significantly lower evoked release, consistent with the interpretation that these sites contain higher basal DA concentrations, making the basal response more detectable and resulting in greater autoinhibition of evoked release.

It is important to emphasize again that the sites yielding larger amplitude evoked responses yielded lower amplitude basal responses, and vice-versa. In our opinion, this eliminates the possibility that differences between the sites can be attributed to the properties of the recording or stimulating electrodes. Overall, these findings support the conclusion that the presence within the striatum of subpopulations of DA terminals in different dynamical states is a direct consequence of variable autoinhibition stemming from local variations in the basal extracellular DA concentration. The ability of steady-state DA levels to affect the dynamical state of DA terminals may be described as a form of coupling between the various timescales of DA activity (Schultz 2007).

1.4.3 Autoinhibition is not the sole determinate of DA dynamics

However, our results also suggest that the tonic basal extracellular DA concentration is not the sole factor that determines the dynamics of evoked release. KYN infusion suppresses evoked release at both KYN-insensitive and KYN-sensitive locations (Figure 1.1 and 1.5), i.e. at all striatal locations in this study. This is not the expected result if the KYN infusion affects evoked release solely by adjusting the DAergic tone on autoreceptors. If that were the case, the effects of KYN infusions should more closely resemble those of a D2 antagonist and increase evoked release. In fact, the effects of KYN infusions (decreasing evoked release) more closely resemble those of an agonist, such as quinpirole. We attribute these findings to KYN's well-known DA-independent properties, which have been the subject of several prior studies of DA release (Lonart and Zigmond 1991; Kulagina et al. 2001; Borland and Michael 2004). These properties include KYN's ability to block both ionotropic glutamate receptors and the $\alpha 7$ nicotinic receptor (Hilmas et al. 2001; Rassoulpour et al. 2005; Poeggeler et al. 2007; Mitala et al. 2008; Amori et

al. 2009). KYN's DA-independent actions, however, do not appear to call into question the conclusion that the heterogeneity in evoked release prior to any KYN infusion is largely driven by local variations in autoinhibition.

It is interesting to note, however, that KYN infusions eliminated the significant difference in the absolute amplitude of evoked responses at KYN-insensitive and KYN sensitive locations (Figure 1.5 top white bars vs. bottom gray bars), although a slight difference remains between the normalized responses (Figure 1.5 inset). This observation shows that KYN almost abolishes the heterogeneity of evoked release. Thus, KYN's combined DA-dependent and DA-independent actions homogenize evoked release, supporting the conclusion that subpopulations of DA terminals within the striatum exist in different dynamical states as a consequence of the pre-synaptic inputs they receive, which include but are not necessarily limited to the autoinhibition caused by the basal extracellular DA concentration. Although we show that the basal DA concentration is not the only source of the well-known heterogeneity in evoked DA release, it clearly plays a major role. For this reason, we conclude that a coupling exists between the slow-acting basal DA and the fast-acting DA release associated with impulse flow in nigrostriatal DA fibers.

1.4.4 Kynurenic acid

In this study, we employed KYN as a pharmacological tool to manipulate both the basal DA concentration and evoked DA release. KYN is an endogenous compound in the brain formed as a result of tryptophan metabolism by glia (Amori et al. 2009). Disruptions in endogenous KYN levels are associated with schizophrenic symptoms in human patients (Schwarcz et al. 2001), so there is intense interest in KYN itself. At micromolar concentrations, KYN acts as a broad spectrum antagonist of the ionotropic glutamate receptors. However, at endogenous nanomolar concentrations, KYN also blocks the $\alpha 7$ nicotinic receptor (Hilmas et al. 2001), which has led to the suggestion that KYN's actions on glutamate

may be an indirect consequence of its anti-cholinergic actions. Delivery of exogenous KYN to the striatum by reverse dialysis decreases dialysate DA levels (Rassoulpour et al. 2005) and inhibition of endogenous KYN synthesis enhances DA levels (Amori et al. 2009). This study was not specifically designed to address the actions of endogenous KYN as we used exogenous KYN at high concentration (1 mM). Nevertheless, our observations that intrastriatal KYN infusions decrease DA, both basal and evoked, are consistent with the actions ascribed to endogenous KYN (Rassoulpour et al. 2005; Amori et al. 2009). Possible functional implications DA depletion studies provide evidence of the functional significance of steady-state extracellular DA concentrations (Abercrombie et al. 1990; Kirchoff et al. 2009). The deficits associated with experimental or clinical DA depletions are alleviated by DA agonists (Maneuf et al. 1997; Schwarz 2003; Antonini and Barone 2008) and DA replacement with L-DOPA (Abercrombie et al. 1990; Birkmayer and Hornykiewicz 1998; Nagatsua and Sawadab 2009), therapies that in the absence of functional DA terminals are presumed to restore steady-state rather than dynamical DAergic transmission. Thus, our direct observation of steady-state basal DA functionally impacting the dynamics of DA release complements the insights derived from the effectiveness of these pharmacotherapeutic strategies for addressing the symptoms associated with DA depletion. Moreover, other studies have identified pathological consequences of higher-than-normal steady-state DA concentrations (Murphy et al. 1996; Miyazaki and Asanuma 2008), giving rise to the concept that steady-state DA concentrations must be carefully regulated, i.e. that normal brain function depends upon steady-state DA concentrations that are neither too low nor too high. However, this study substantially extends this concept by suggesting that point-to-point variations in steady-state DA concentrations are functionally significant as well, i.e. our findings lead us to suggest that there should also be control over the size of DA terminal subpopulations exhibiting fast (non-autoinhibited) and slow (autoinhibited) dynamical behaviors.

1.5 CONCLUSIONS

In this study, we have demonstrated a coupling between static DA regulated by kynurenate and autoinhibition level in rat striatum. We have found that recording sites with high resting DA exhibit high autoinhibition than sites with low resting DA concentration. This provide valuable platform on studying the heterogeneity of DAergic release within striatum. Our study also suggests the possible regulation factors on the autoinhibition heterogeneity, such as resting DA, glutamate and acetylcholine inputs.

Chapter II. Microdialysis Probe Implantation Disturbs Resting DA Level in the nearby Brain Tissue

2.1 INTRODUCTION

Microdialysis and voltammetry are the major techniques for measuring DA concentrations in the brain. These two techniques have distinct dimensions (microdialysis probe: 220 μm diameter, 4 mm long; carbon fiber microelectrode: 7-10 μm diameter, 100 or 400 μm long) and provide disparate results on DA measurements in striatum. Microdialysis detects resting DA concentration in low nanomolar (e.g. Smith and Justice 1994; Qian et al. 1999; Borland et al. 2005) and is purely TTX-sensitive (Millar et al. 1985; Westerink et al. 1987; Westerink and De Vries 1988; Santiago et al. 1992). While on the other hand, voltammetry observed micromolar TTX-insensitive DA in rat striatum (Borland and Michael 2004).

In Chapter I, we have demonstrated the heterogeneity of static DA in rat striatum, which would contribute to the low DA measurement by microdialysis due to the averaging property of this technique. But still the absence of TTX-insensitive DA remains to be the disparity.

Microscopic studies had shown that tissue around microdialysis probe implantation site is disturbed and showing tissue response (Clapp-Lilly et al. 1999; Zhou et al. 2001; Mitala et al. 2008; Jaquins-Gerstl and Michael 2009). In the disrupted tissue, axonal damage, neuron degeneration (Clapp-Lilly et al. 1999), blood brain barrier opening (Mitala et al. 2008), blood vessel disruptions, blood flow deficiency and gliosis (Jaquins-Gerstl and Michael 2009) were observed. These results suggest an abnormal neurochemical environment and possibly a DAergic disruption in the brain tissue around microdialysis probe.

To monitor the DA activities in tissue near microdialysis probe, in our previous studies (Borland et al. 2005; Yang and Michael 2007) we implanted a carbon fiber microelectrode near the microdialysis probe (~ 200 μm from a microdialysis probe's outer wall) to monitor the DA release in rat striatum evoked by medial forebrain bundle (MFB) stimulation. Carbon fiber microelectrode has a small diameter (7-10

µm) which causes minimal damage to the brain tissue (Peters et al. 2004; Jaquins-Gerstl and Michael 2009). At the same time, the high temporal resolution provided by fast scan cyclic voltammetry (FSCV) makes this technique capable of monitoring dynamic DA activities. We observed a 10-fold decrease of evoked DA release in the tissue near microdialysis probe. These results suggest a disruption of DAergic release near microdialysis probe. Hence we hypothesize that the static DA concentration in the tissue near microdialysis probe is disrupted.

To test this hypothesis, we monitored the effect of kynurenate (KYNA) on resting DA concentration by a carbon fiber microelectrode in the tissue near a microdialysis probe. Carbon fiber microelectrode was placed ~200 µm away from microdialysis probe implantation site. The placement of carbon fiber microelectrode is determined by our previous observations (Borland et al. 2005; Yang and Michael 2007) to be located within the disrupted brain tissue area by microdialysis probe implantation.

(The results in this chapter were published in Mitala et al. (2008), the following part of this chapter is adapted from Mitala et al. (2008) with modifications)

Kynurenate was an endogenous compound in the brain formed as a result of tryptophan metabolism by glia (Amori et al. 2009), which is a broad-spectrum antagonist of the ionotropic glutamate receptors (e.g. Ivanova et al. 2003; Wu et al. 2007). Our previous results showed that microinfusion of kynurenate directly into the striatum decreases micromolar extracellular DA from the basal level (Kulagina et al. 2001; Borland and Michael 2004). The effect of kynurenate was insensitive to tetrodotoxin and sensitive to nomifensine, consistent with the classical profile of reverse DA transport (Leviel 2001). On the other hand, our conclusion that the striatal ECS might contain a pool of DA derived from glutamate-triggered reverse transport is consistent with the finding of the in vitro study of Lonart and Zigmond (1991). During the present study we found that the voltammetric response to kynurenate infusion is significantly diminished near microdialysis probes, suggesting the possibility that alterations of basal reverse DA transport might also be a component of the tissue response to the probes.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals and solutions

Chloral hydrate, kynurenic acid (kynurenate) and dopamine were used as received from Sigma (St. Louis, MO). Chloral hydrate was dissolved in phosphate buffered saline (PBS: 155 mM NaCl, 100 mM phosphate, pH 7.4). Microdialysis probes were perfused with artificial cerebrospinal fluid (aCSF: 144 mM Na⁺, 1.2 mM Ca²⁺, 2.7 mM K⁺, 152 mM Cl⁻, 1.0 mM Mg²⁺, and 2.0 mM PO₄³⁻, adjusted to pH 7.4). Kynurenate was dissolved in aCSF. All solutions were prepared with ultrapure water (NANOPure; Barnstead, Dubuque, IA).

2.2.2 Microdialysis probes

Vertical, concentric microdialysis probes (220 μm o.d., 4 mm long) were constructed with hollow fiber dialysis membrane (Spectra-Por RC Hollow Fiber, MWCO: 6,000, 160 μm i.d., Spectrum Laboratories, Inc., Rancho Dominguez, CA) and fused silica outlet lines (150 μm o.d., 75 μm i.d., Polymicro Technologies, Phoenix, AZ). The probes were perfused with aCSF at 0.586 μl/min during the whole experiment.

2.2.3 Microinfusion pipettes

Microinfusion pipettes were constructed using fused silica capillary tubing (350 μm o.d., 25 μm i.d., Polymicro Technologies, Phoenix, AZ). The outlet tip of the capillary was pulled by a horizontal laser powered capillary puller (Sutter instruments, Novato, CA, USA) and trimmed to make the tip diameter of ~30 μm. The inlet end of the capillary was attached to a 50 μl gastight syringe (Hamilton,

Reno, NV) driven by a microprocessor-controlled driver (Sutter Instruments, Novato, CA) and pre-filled with kynurenate solution (1 mM in aCSF). The infusion rate was 100 nl/min. The infusion duration was 2 min.

2.2.4 Carbon fiber microelectrodes and fast scan cyclic voltammetry

Carbon fiber microelectrodes were constructed by inserting a single carbon fiber (7-10 μm diameter, Thornell Carbon Fiber, T300, Amoco Performance Products, Inc., Greenville, SC, USA) into a borosilicate glass capillary (0.75 mm I.D., 1 mm O.D.; Sutter Instruments, Co., Novato, CA, USA). The capillary was pulled to a fine tip around the carbon fiber with a vertical micropipette puller (Narishige, Tokyo, Japan). The resulting tip was sealed with Spurr Epoxy (Polysciences, Inc., Warrington, PA, USA) and the exposed fiber was trimmed to a length of 400 μm . Each electrode was pre-treated with a triangular potential waveform (0–2 V vs. Ag/AgCl reference at 200 V/s for 1 s) in aCSF. For fast scan cyclic voltammetry, the resting potential was 0 V vs. Ag/AgCl and the voltage was swept linearly to 1.0 V, then to -0.5 V and back to 0 V at 300 V/s. Scans were performed at 400 ms intervals. The DA voltammetric signal was obtained in the 500–700 mV potential range of the first potential sweep, which gives the maximum DA oxidation current. Voltammetric currents were converted to DA concentrations by post-calibration of the microelectrodes after they were extracted from the rat brain. Data collected during kynurenate infusions were corrected for signal drift by subtracting the current collected in the range of 200-300 mV from the current collected between 500-700 mV, as described by Borland and Michael (2004). Post-calibration was performed in aCSF. Post-calibration signals were subjected to the same baseline subtraction method. DA identification was based on the appearance of background subtracted voltammograms (Borland and Michael 2004).

2.2.5 Animals and surgical procedures

All procedures involving animals were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Male Sprague–Dawley rats (Hilltop, Scottsdale, PA) (250–375 g) were initially anesthetized with chloral hydrate (400 mg/kg i.p., dissolved in PBS) and kept anesthetized with additional injections of chloral hydrate (50 mg/kg) when needed. The rats were wrapped in a homoeothermic blanket (EKEG Electronics, Vancouver, BC, Canada) set to 37 °C. The rats were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) with the incisor bar 5 mm above the interaural line (Pellegrino et al. 1979). Holes were drilled through the skull in the appropriate positions to expose the underlying dura and brain tissue. The dura was removed with a scalpel to allow for placement of the microelectrode and microdialysis probe into the brain tissue with minimal disruption to the surrounding blood vessels. The connection between the Ag/AgCl reference electrode and the brain was accomplished through a salt bridge by filling aCSF to a plastic pipette tip with paper tissue sticking out from the tip.

2.2.6 Device placements and experiment procedures

For the group of animals with a microdialysis probe involved, all the devices (a microdialysis probe, a carbon fiber microelectrode and a microinfusion pipette) were implanted into rat striatum eventually (Figure 2.1). Before implantation, all the devices were lined up on the brain surface, and their distances were calculated to facilitate their final positions as shown in Figure 2.1 once they were dialed to their expected depths in the brain. The microdialysis probe was vertically placed at 2.5 mm anterior to bregma, 2.5 mm lateral from midline. The carbon fiber microelectrode and microinfusion pipette were held 10° from vertical, but at opposite coronal sides from microdialysis probe. On the brain surface, the tips of microelectrode and micropipette were placed on the same coronal plane ~200 µm posterior from the microdialysis probe outer wall. The distance between microelectrode and pipette were calculated to

make their tips 150 μm apart from each other, when both dialed down to their expected depths, 4.5 mm and 4.3 mm below dura. The carbon fiber depth was 200 μm deeper to allow the pipette tip to be placed at the mid-point of the carbon fiber. The first recording of DA signal in response to kynurenate infusion on carbon fiber microelectrode was performed before microdialysis probe was implanted into the brain. After this recording, the microdialysis probe was dialed down slowly in 30 min to the expected depth of 7 mm below dura. After microdialysis probe implantation, a 2 hr equilibration was allowed before another kynurenate infusion to be conducted.

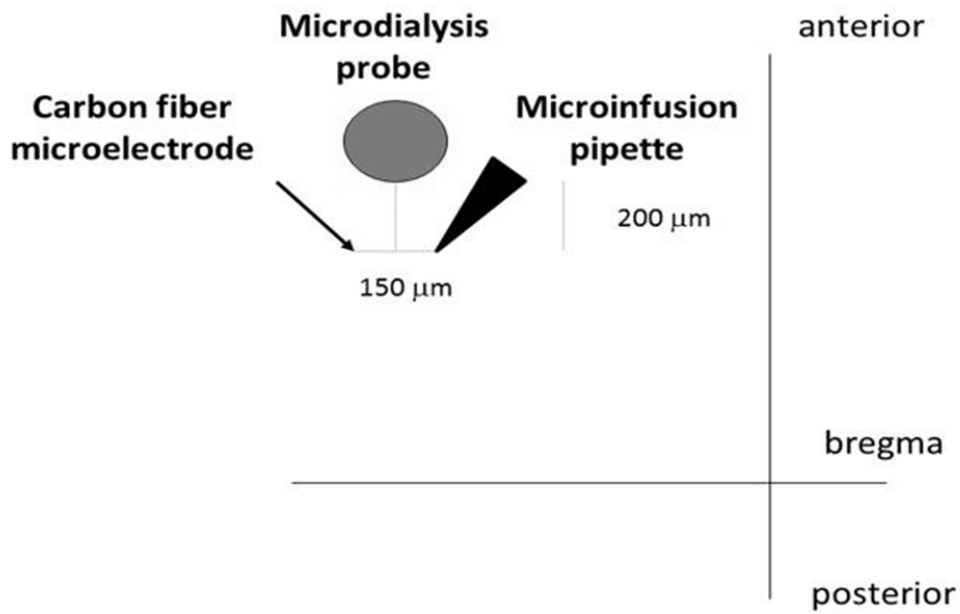


Figure 2.1 Placements of devices in the experiments with a microdialysis probe involved. Figure shows the positions and relative distances between devices (a microdialysis probe, a microinfusion pipette and a carbon fiber microelectrode) in the horizontal view (not to the scale).

For the control group, without a microdialysis probe implanted, devices were implanted in rat striatum as demonstrated in Figure 2.2. The carbon fiber microelectrode was placed at 2.5 mm anterior to bregma, 2.5 mm lateral from midline. The microinfusion pipette was held at 10° from vertical, and lined up with carbon fiber microelectrode on the brain surface to allow their placements in the same coronal plane and their distance to be 150 µm apart from each other when both dialed down to their expected depths, 4.3 mm and 4.5 mm below dura. In this group of animals, no microdialysis probe was implanted. The second kynurenate infusion was performed 3.2 hr after the first infusion to keep a same time interval between two kynurenate infusions as that of the group involving a microdialysis probe.

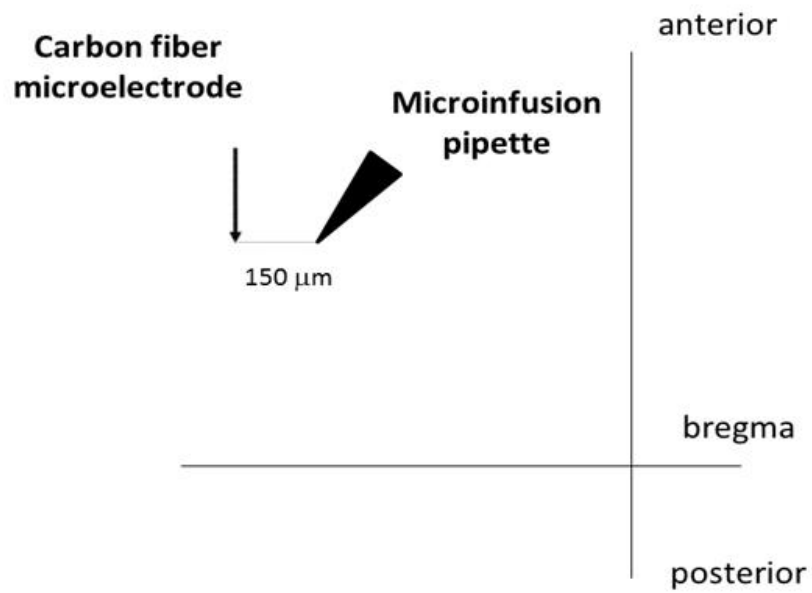


Figure 2.2 Placements of devices in the experiments without a microdialysis probe involved. Figure shows the positions and relative distances between devices (a carbon fiber microelectrode and a microinfusion pipette) in the horizontal view (not to the scale).

2.3 RESULTS

2.3.1 Background subtraction and DA signal recognition

Fast potential scan rate (300 V/s) caused large background currents (Figure 2.3A). This background current is due to both faradic and non-faradic current contributions. By subtracting the background voltammograms between two treatment stages—before and after kynurenate infusion in this experiment (in Figure 2.3A: solid vs. dotted), the voltammograms was confirmed as DA (Figure 2.3B) by identifying its signature oxidation and reduction peaks.

2.3.2 Kynurenate infusion caused DA decrease

Intrastriatal infusion of kynurenate via a pipette placed 150 μm from the carbon fiber microelectrode caused a decrease in extracellular DA levels as measured by fast scan cyclic voltammetry (Figure 2.4A and 2.4B are examples from two different animals). The cyclic voltammograms were obtained and compared to that from post-calibration to confirm DA. For example the cyclic voltammogram obtained from 1900-2100 s of Figure 2.4A (Figure 2.5A) was confirmed as DA by comparing to that of post-calibration (Figure 2.5C). Occasionally, kynurenate infusion caused current spike during infusion as shown in Figure 2.4A at around 300 s. The background subtraction voltammogram showed this was not DA related signal (Figure 2.5B). In other cases, kynurenate infusion caused DA decrease without generating current spike (Figure 2.4B).

Currents were corrected for baseline drift in long period recordings (~40 min) by subtracting the current values collected in the range of 500-700 mV of DA oxidation potential by the current values collected from a non-DA related potential range: 200-300 mV, as described in Borland and Michael (2004). The subtracted current values were converted to DA concentrations according to post-calibration curve of the carbon fiber microelectrode. The post-calibration results were subjected to baseline correction with the same method.

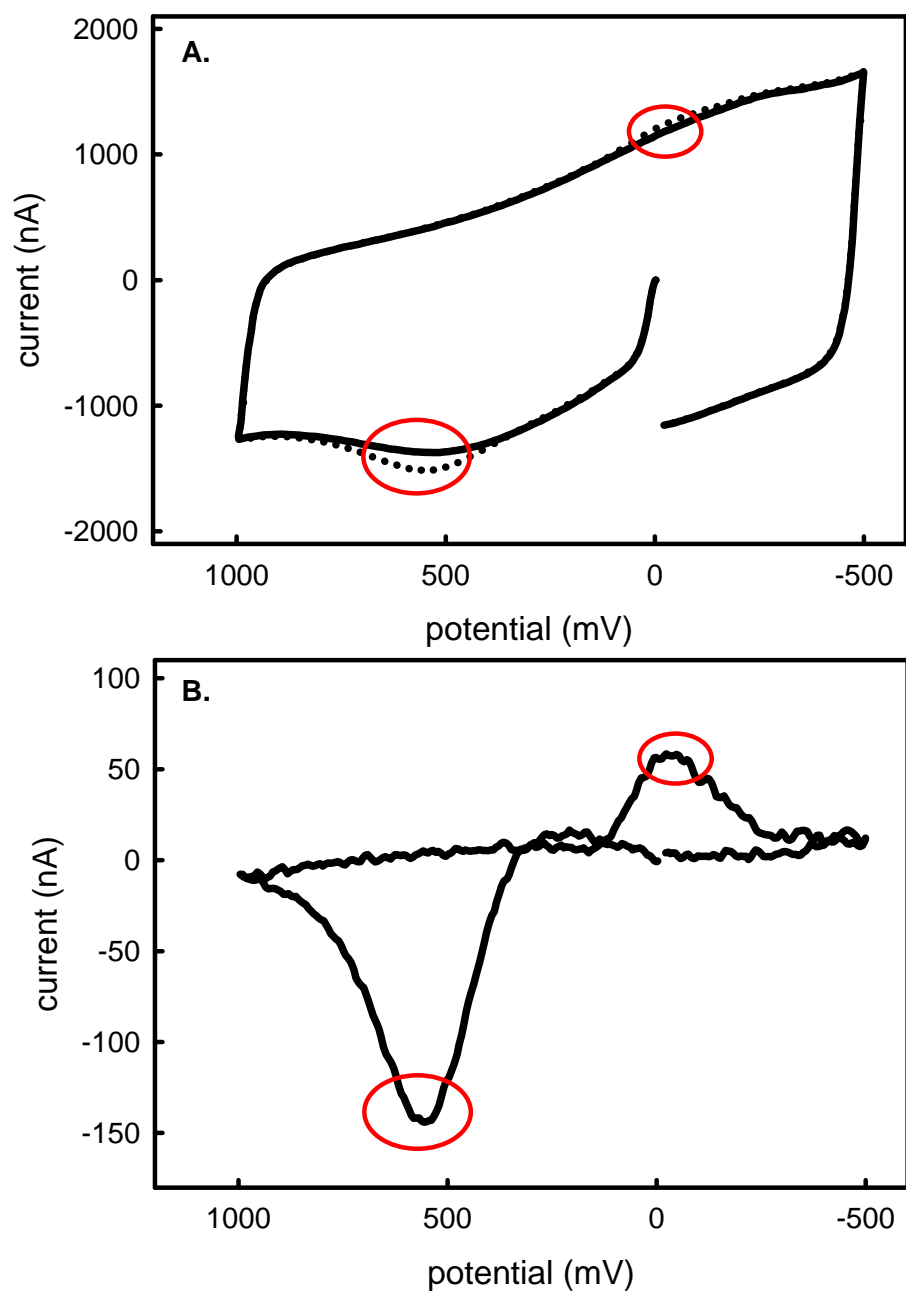


Figure 2.3 Background subtraction and DA identification. A. shows two background currents obtained at different DA concentrations (solid vs. dotted). B. shows the subtracted voltammogram from A. The signature oxidation and reduction peaks identify DA.

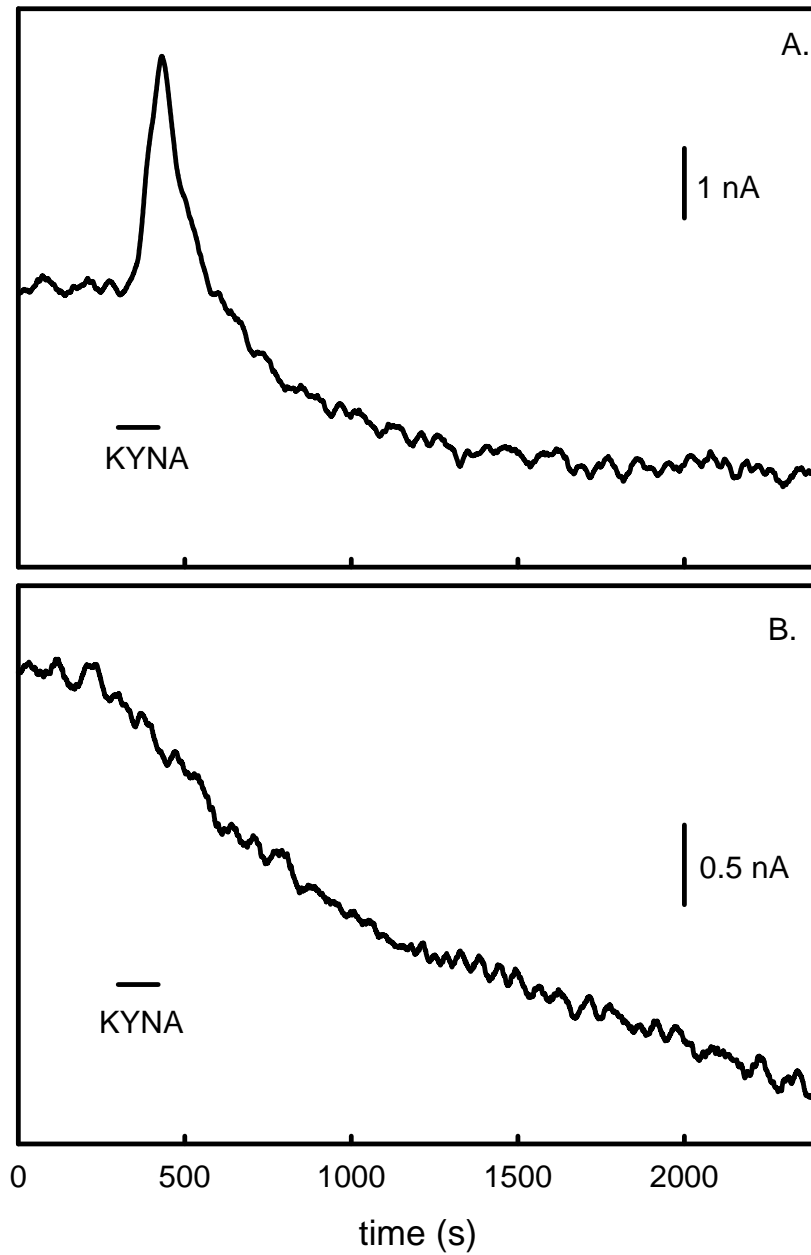


Figure 2.4 Kynurenate infusion caused DA decrease in rat striatum. A: an example of current recording in response to kynurenate infusion, in this rat kynurenate infusion caused a current spike starting around 300 s; B: current recording in response to kynurenate infusion in another rat, in this example kynurenate infusion caused DA decrease without a current spike. Kynurenate was perfused for 2 min (horizontal bar).

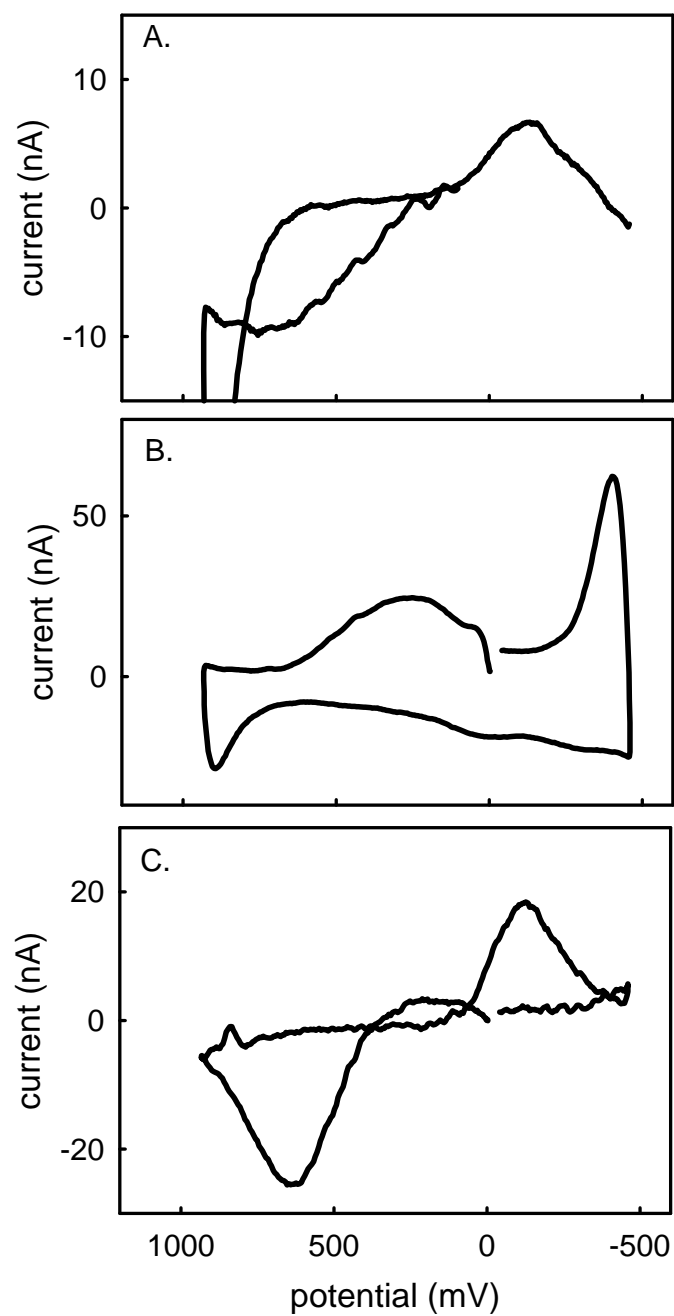


Figure 2.5 Cyclic voltammograms identification. A: cyclic voltammogram obtained at the time range of 1900-2100 s from the recording of Figure 2.4A. B: cyclic voltammogram obtained at 300-500 s from Figure 2.4A. C: cyclic voltammogram of 5 μM DA obtained in the post-calibration from the same carbon fiber microelectrode.

2.3.3 Voltammetric Response to Intrastratial Infusion of Kynurenate in the Tissue before and after Microdialysis Probe Implantation

Two consecutive infusions performed 3.2 h apart in striatum without a microdialysis probe implanted induced similar responses (Figure 2.6 A). However, implantation of a microdialysis probe ~200 μm away from the tip of the carbon fiber microelectrode significantly decreased the amplitude of the response to the second infusion (Figure 2.6 B). The histogram in Figure 2.6 C shows the normalized amplitude of the response to the second infusion with respect to the first infusion at the time points indicated by symbols and error bars in Figure 2.6 A and B. The pre- and post-probe data are significantly different (ANOVA, $F = 37.6$, $p < 0.001$). Hence, probe implantation significantly decreased the impact of intrastratial infusion of kynurenate on extracellular DA concentrations as measured by voltammetry near the probe.

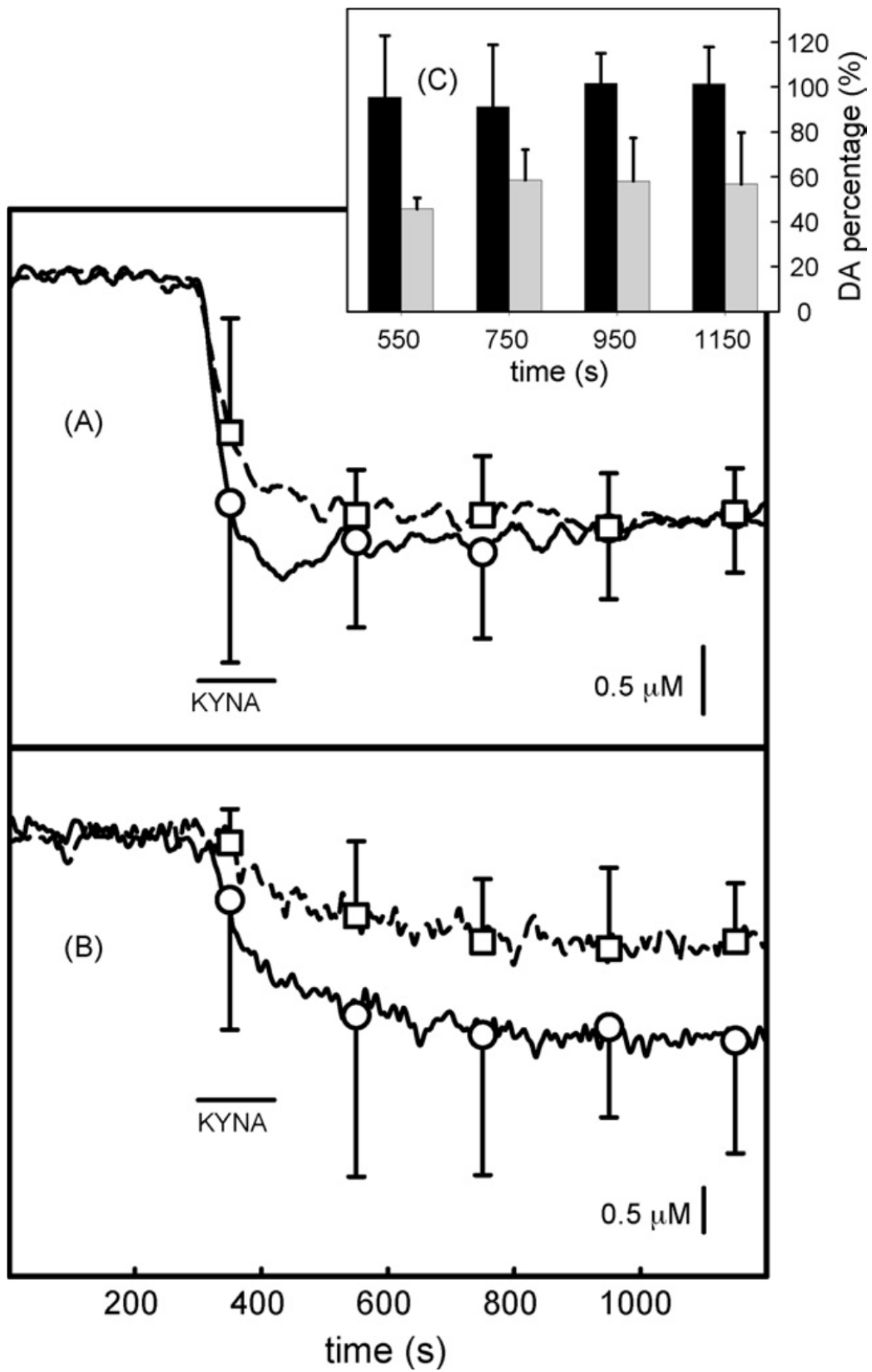


Figure 2.6 Intrastriatal infusions of kynurenate decrease extracellular DA as measured by voltammetry. The traces in this figure are the average of voltammetric signals from four individual animals. Two infusions were performed in each animal. The solid trace with round symbols is the response to the first infusion and the broken trace with square symbols is the response to the second infusion. The symbols indicate the mean and standard deviation of the responses at 200 s intervals. In panel A the two infusions were performed 3.2 h apart. In panel B the two infusions were performed 3.2 h apart and a microdialysis probe was implanted after the first infusion. Panel C is a histogram that reports the amplitude of the second response normalized with respect to the amplitude of the first infusion at the time points indicated by the symbols in panels A and B. The effect of probe implantation is significant (two-way ANOVA, $p < 0.001$).

2.4 DISCUSSION

Previously, we reported that intrastriatal infusions of kynurenate cause a TTX-insensitive, nomifensine-sensitive decrease in extracellular DA levels as measured by voltammetry (Borland and Michael 2004). This observation supports the conclusion that a TTX-insensitive mode of DA release, presumably reverse DA transport, contributes to basal extracellular DA in the striatum. This conclusion, however, is unexpected in light of reports that basal dialysate DA levels are completely TTX-sensitive (Westerink and De Vries 1988; Westerink et al. 1989). However, the implantation of a microdialysis probe significantly diminished the effect of kynurenate infusions on extracellular DA as measured by voltammetry near microdialysis probes (Figure 2.6). This observation indicates that a suppression of reverse DA transport might also be a component of the DAergic alterations associated with the tissue response to microdialysis probes. Reverse transport is a well-established mechanism of DA release (Leviel 2001; Sulzer et al. 2005; Fleckenstein et al. 2007) and has long been accepted as the mechanism by which amphetamine elevates dialysate DA levels (Westerink et al. 1987). Nevertheless, except in cases such as amphetamine treatment, the TTX-insensitive dialysate DA is attributed to the tissue damaging effects of implanting the probe. For this reason, the majority of microdialysis protocols include a wait-time of roughly 24 h after probe implantation before samples are collected for analysis. The wait-time is understood to be necessary to washout any DA released due to physical injury of DA terminals near the probe site. While injury-derived DA release is likely to be TTX-insensitive, it has not been established that all TTX-insensitive DA release is injury-related. Indeed, several studies have suggested that reverse DA transport might be a physiologically relevant phenomenon independent of amphetamine (Lonart and Zigmond 1991; Cowell et al. 2000; Leviel 2001). Hence, the findings of the present study suggest that the contribution of reverse DA transport to basal DA release deserves further investigation and consideration.

2.5 CONCLUSION

The present study provides complimentary evidence for probe-induced changes in DAergic neurochemistry. In previous studies we noticed alterations in depolarization-dependent DA release (Yang et al., 1998; Borland et al., 2005; Yang and Michael, 2007), whereas herein we also present evidence for probe-induced disruption of a DA release process that fits the profile of reverse DA transport. The present study was not designed to establish any specific cause-and-effect relationship between probe-induced tissue damage and neurochemical alterations but rather to establish that two coexist and are therefore correlated. In doing so, this study adds important insights into the nature and extent of the differences between measures of ECS DA based on microdialysis and voltammetry.

Chapter III. Microdialysis Probe Implantation Disturbs Evoked DA Release in Rat Striatum

(This chapter is adopted from the manuscript of Wang and Michael to be submitted to J Neurosci Meth with modifications)

3.1 INTRODUCTION

Microdialysis and voltammetry have evolved to become two widely adopted methodologies for measuring extracellular DA concentrations in the brain and many studies have focused on the rat striatum as a model system (Church et al. 1987; Hurd and Ungerstedt 1989; Garris et al. 1997; Borland and Michael 2004; Robinson and Wightman 2004; Katagiri et al. 2009; Wang et al. 2010). Both methods are thoroughly described in the literature and numerous laboratories are expert in the procedures of one or the other or both. Nevertheless, a concern arises over the matter of the interpretation of microdialysis and voltammetry data, since experimental conditions have been documented under which the methods produce disparate results (Lu et al. 1998; Yang et al. 1998; Borland and Michael 2004). In some respects, contributions to these disparities are obvious because the two methods have different attributes of sensitivity, selectivity, and temporal response (Wightman and Robinson 2002; Watson et al. 2006; Nandi and Lunte 2009). A less obvious but, in our opinion, even more important additional contribution stems from the tissue responses to implantation of microdialysis probes and voltammetric microelectrodes. The latter is the subject of this report.

In the previous two chapters, we have demonstrated from two aspects on the sources that could contribute to the low measurement of resting DA by microdialysis: the heterogeneity of resting DA within striatum and the disruption of TTX-insensitive DA caused by microdialysis probe implantation. In this study, we further investigate into the factors that cause DA suppression in the tissue around microdialysis probe.

Two factors have come to light as key regulators of extracellular DA concentrations in the rat striatum, DA uptake and autoinhibition, each of which can be manipulated by pharmacological means

(Wieczorek and Kruk 1995; Zahniser et al. 1999; Benoit-Marand et al. 2001; Kita et al. 2007; Moquin and Michael 2009). In previous studies (Yang et al. 1998; Borland et al. 2005; Yang and Michael 2007), we found that the presence of a microdialysis probe alters voltammetric responses to nomifensine, a DA uptake inhibitor. Specifically, we found greater sensitivity of the DA system to nomifensine, as measured by voltammetry in the presence versus absence of a microdialysis probe. Similarly, in the present study we found greater sensitivity of the DA system to systemic administration of raclopride, a D2 receptor (D2R) antagonist, as measured by voltammetry in the presence compared to the absence of a microdialysis probe. Collectively, our findings suggest that the tissue response to probe implantation disrupts DA regulation, rendering the DA system hypersensitive to pharmacological manipulations.

3.2 MATERIALS AND METHODS

3.2.1 Voltammetric electrode and techniques

Carbon fiber microelectrodes were constructed by inserting a single carbon fiber (7 μm diameter, Thornell Carbon Fiber, T300 3K, Amoco Performance Products, Inc., Greenville, SC) into a borosilicate glass capillary (0.75 mm I.D., 1 mm O.D., Sutter Instruments, Co., Novato, CA). The glass capillary was pulled to a fine tip using a vertical micropipette puller (Narishige, Tokyo, Japan). The carbon fiber was sealed into the pulled capillary tip with Spurr Epoxy (Polysciences, Inc., Warrington, PA). After curing the epoxy at 70° C for at least 8 hours, the exposed carbon fiber was trimmed to a length of 400 μm with a scalpel blade with the aid of a stereo microscope. Electrical contact between the fiber and a nichrome hook-up wire was achieved with a small droplet of mercury (conductive paint or carbon paste are suitable alternatives). The microelectrodes were electrically pretreated in room temperature, N₂-purged artificial cerebrospinal fluid (aCSF: 145 mM Na⁺, 1.2 mM Ca²⁺, 2.7 mM K⁺, 1.0 mM Mg²⁺, 152 mM Cl⁻ and 2.0 mM phosphate adjusted to pH 7.4) with a triangular potential waveform (0-2 V vs. Ag/AgCl at 200V/s for 1 s). This pretreatment method increased the sensitivity of carbon fiber microelectrodes for by oxidizing the carbon surface to enhance DA adsorption (Feng et al. 1987).

Fast scan cyclic voltammetry (FSCV) (Baur et al. 1988) was performed with an EI400 potentiostat (originally obtained from Ensmann Instrumentation, Bloomington, IN: this company is no longer in business) and a software control program developed in-house. The applied potential was held at 0 V vs. Ag/AgCl between potential scans: each scan involved a linear sweep to 1 V, then to -0.5 V, and back to 0 V v. Ag/AgCl at a rate of 300 V/s. The scan frequency was 7.5 Hz (in these experiments the scan frequency and stimulus frequency, which was 45 Hz, must be integer multiples of one another to assure that the voltammetric and stimulus waveforms are properly synchronized). DA oxidation signals were monitored by integrating the current between 500 and 700 mV on the first potential sweep of each voltammetric scan: this potential range produced the maximum DA oxidation current. Oxidation current values were converted to DA concentrations by post-calibration of the microelectrodes after they were removed from brain tissue.

3.2.2 Microdialysis probes

Concentric-style microdialysis probes with an outer diameter of 220 μm and an active length of 4 mm were constructed with hollow fiber membrane (Spectra/Por RC hollow fiber, 200 μm I.D., Spectrum, Rancho Dominguez) with a molecular weight cutoff of 6000 Da. The inlet line was PE-20 tubing (0.38 mm I.D., 1.09 mm O.D., \sim 40 cm long, Becton Dickinson, Franklin Lakes, NJ) and the outlet line was a fused silica capillary (70 μm I.D., 145 μm O.D. and \sim 20 cm long, Polymicro Technologies, Phoenix). All the connections and the tip of the microdialysis probe were sealed with epoxy (Devcon, Danvers, MA). The inlet tubing was connected to a 250 μL gas-tight syringe (Hamilton) filled with aCSF driven by a microliter syringe pump (Harvard, Holliston, MA) at a perfusion rate of 0.586 $\mu\text{L}/\text{min}$.

3.2.3 Animal and surgical procedures

All procedures involving animals were carried out with the approval of the Institutional Animal Care and Use Committee of the University of Pittsburgh. Male Sprague-Dawley rats (Hilltop, Scottsdale, PA) (250-375 g) were anesthetized with chloral hydrate (400 mg/kg i.p.) and were kept unconscious with additional doses of chloral hydrate (~50 mg/kg i.p.). While anesthetized, rats were wrapped in a homoeothermic blanket (EKEG Electronics, Vancouver, BC) with their body temperature monitored and maintained at 37°C. The rats were placed in a stereotaxic frame (David Kopf, Tujunga, CA) with the incisor bar 5 mm above the interaural line. The scalp was shaved and cleaned before a midline incision exposed the skull. Holes were drilled through the skull at the appropriate positions and the dura was dissected to allow for placements of devices with minimal disruption to any surrounding blood vessels.

3.2.4 Stereotaxic procedures

In experiments involving microdialysis, the tip of the microdialysis probe was initially placed on the brain surface at a position 2.5 mm anterior from bregma and 2.5 mm lateral from midline (Pellegrino et al. 1979). The microelectrode was mounted 10° from vertical on a second stereotaxic carrier and positioned so as to be placed ~200 µm posterior with respect to the probe's outer wall once each device was lowered to their final experimental depth within the striatum.

The microelectrode was dialed down first to a depth of 4.5 mm below dura while the microdialysis probe remained at the brain surface. Electrical contact between brain tissue and Ag/AgCl reference electrode was accomplished via a salt bridge constructed of a plastic pipette tip plugged with tissue paper and filled with aCSF. The ipsilateral medial forebrain bundle (MFB) was stimulated with a stainless steel bipolar stimulating electrode (MS303/a; Plastics One, Roanoke, VA, USA) placed 2.2 mm posterior from bregma, 1.6 mm lateral from midline and 7.5 mm below dura initially: the stimulating

electrode was lowered until evoked DA release was observed in the striatum. The stimulus was an optically isolated, biphasic, constant-current square wave with pulse duration of 2 ms, pulse height of 280 μ A, frequency of 45 Hz, and a train length of 10 s.

Once evoked DA release was detected in the striatum, the stimulating electrode was not moved for the duration of the experiment. Experiments proceeded after a stable evoked DA release of at least 30 nA was detected.

In experiments involving microdialysis, the probe was slowly lowered over a 30-min period until the tip reached 7 mm below dura. Thereafter, stimulus trains were delivered at 20 min intervals.

The positions of devices of the experiments involving a microdialysis probe were shown in Figure 3.1. For the other group without a microdialysis probe involved, only a carbon fiber microelectrode was implanted in striatum, and a stimulating electrode in medial forebrain bundle.

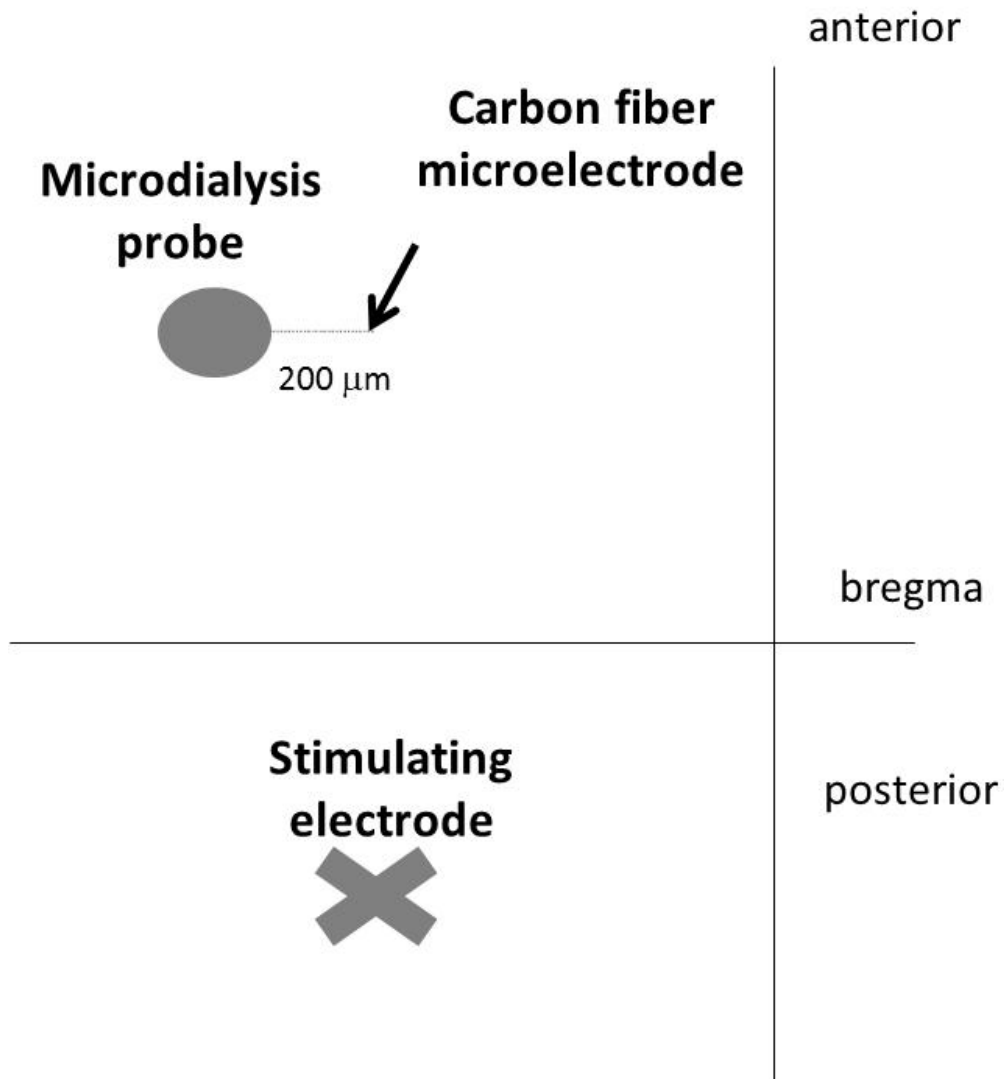


Figure 3.1 Devices placements in rat brain for experiments involving a microdialysis probe. The picture shows a horizontal view of rat brain. Microdialysis probe and carbon fiber microelectrode were implanted in rat striatum. Stimulating electrode was implanted in MFB. In the experiments without a microdialysis probe involved, only carbon fiber microelectrode was implanted in striatum. Figure shows the relative positions and distance between devices (not to scale).

3.2.5 Pharmacological agents and procedures

After placement of the microelectrode and microdialysis probe a 2-hr equilibration period was used prior to drug administration: the choice of a 2 hr equilibration time is explained in the Discussion section, below. Raclopride (2 mg/kg, i.p.) was administered 5 min after recording the last pre-drug evoked response and 15 min before the next. Nomifensine (20 mg/kg, i.p.) was administered 40 min after raclopride injection.

3.2.6 Drugs and solutions

Chloral hydrate, raclopride tartrate, and nomifensine were used as received from Sigma (St. Louis, MO, USA). Chloral hydrate was dissolved to 0.2 g/mL in phosphate buffered saline (PBS: 155 mM NaCl, 100 mM phosphate, pH 7.4). Raclopride and nomifensine were dissolved in 1 mL of PBS. All drugs were administered by intraperitoneal injections (i.p.). All solutions were prepared with ultrapure water (NANOPure; Barnstead, Dubuque, IA, USA).

3.2.7 Statistics

Two-way repeated measures ANOVA was performed on evoked DA in Figures 3.4. Three-way repeated measures ANOVA was performed on normalized evoked DA overflow concentration in Figure 3.5.

3.3 RESULTS

3.3.1 Voltammetry in the striatum: no microdialysis probe

In this study, the voltammetric microelectrodes were lowered into the striatum to the coordinates given in the Materials and Methods section: thereafter, no further adjustment or optimization of the recording electrode's position was performed, as is often done to locate 'hot spots' of evoked DA release (Kawagoe et al. 1992; Robinson et al. 2003; Venton et al. 2003). Without such optimization of the placement of the recording electrode, evoked responses usually exhibit the slow-type response characteristics evident in Figure 3.2 (solid circle labeled line). These characteristics include a slow initial rate of evoked overflow and an increase in the rate of evoked overflow (short-term facilitation) as the stimulus continues. Combined, these features impart a complex, almost sigmoidal, shape to the rising phase of the stimulus response. After the stimulus ends, the DA signals return towards the baseline with no noticeable delay. The initial segment of the descending phase of the response is approximately linear, a sign of zeroth-order clearance kinetics and saturation of the DA reuptake transporter. The prolonged delay in the onset of DA release after the start of the stimulus is attributable to autoinhibition of DA terminals at the recording site: consistent with our recent reports (Moquin and Michael 2009; Wang et al. 2010), administration of raclopride, a D2R antagonist, abolishes both the initial delay in evoked release and the short-term facilitation (Fig 3.2, solid triangle labeled line). In the present study, we followed the initial dose of raclopride with a subsequent dose of the DA uptake inhibitor, nomifensine. Interestingly, when administered after raclopride, nomifensine had only minor additional impact on the evoked response (Fig 3.2, open triangle labeled line). The initial rise in the signal was slightly faster but by the end of the stimulus the signal was actually less, but not significantly so, than before nomifensine administration.

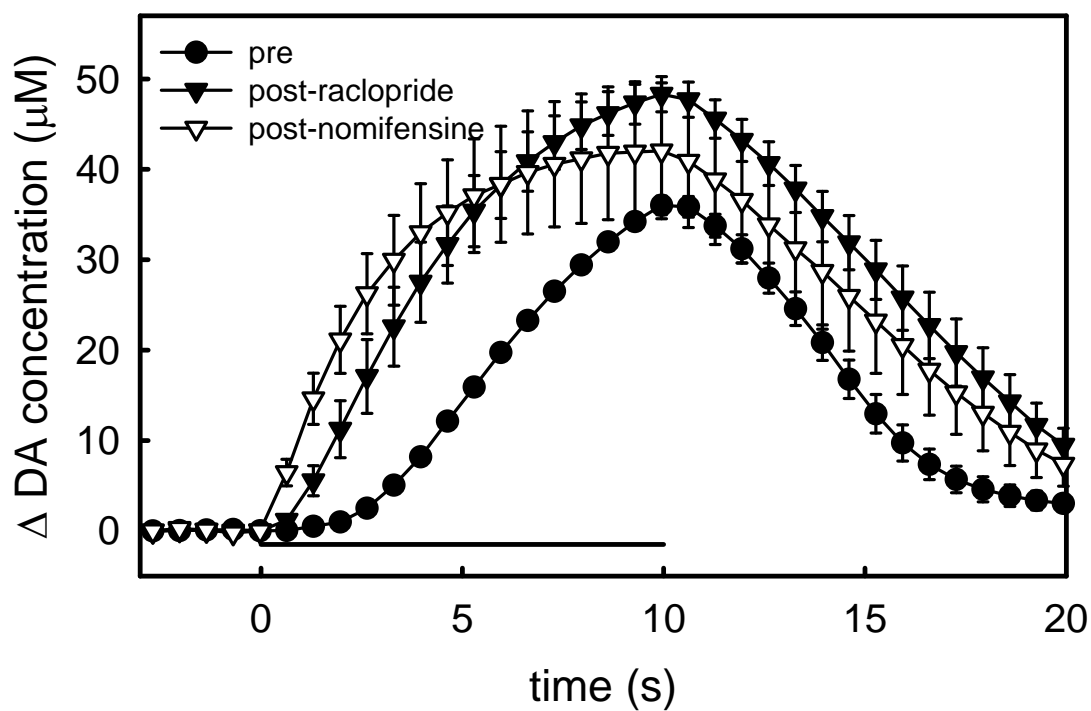


Figure 3.2 Voltammetric recordings of evoked DA release in the rat striatum in the absence of microdialysis probes. The solid circle labeled line is the mean of the pre-drug responses, the solid triangle labeled line is the mean of the responses after raclopride, and the open triangle labeled line is the response after the final dose of nomifensine (each trace is the average responses recorded in a group of 4 rats). The error bars represent standard errors. The horizontal bar represents the timing of the 10-s stimulus. Note the delay after the beginning of the stimulus before the onset of the pre-drug response: this delay is a hallmark feature of the slow-type evoked responses in the striatum (see text).

3.3.2 Voltammetry near a microdialysis probe in the striatum

In a second group of rats, voltammetric microelectrodes were implanted into the striatum and, since placements were not optimized to identify evoked release ‘hot spots,’ slow-type evoked responses were again observed (Fig 3.3, solid circle labeled line). After recording these ‘pre-probe’ responses, a microdialysis probe was lowered into the striatum. The stereotaxic carriers were arranged to set the spacing between the microelectrode’s tip and the outer surface of the microdialysis probe to 200 μm : however, the precise spacing was not confirmed histologically because the microelectrodes tracks are too small to visualize by light microscopy (Peters et al. 2004). The implantation of a microdialysis probe in this manner substantially and significantly reduced the amplitude of a subsequent evoked response (Fig 3.3, open circle labeled line) recorded without any other manipulation of the voltammetric microelectrode, the stimulating electrode, or the stimulation parameters, i.e. we attribute the change in the evoked response to the tissue response to implantation of the probe. However, an interesting finding is that probe implantation did not simply change the amplitude of the evoked response but also changed its temporal profile: specifically, probe implantation further delayed the onset of evoked overflow with respect to the start of the stimulus. This is very similar to the manner in which administration of quinpirole, a D2R agonist, affected the response (Moquin and Michael 2009) and raises the possibility that some of the impact of probe implantation on the evoked response is associated with an increased autoinhibitory tone on DA terminals in the vicinity of the probe. Consistent with this idea, the effect of raclopride on the evoked responses recorded near the probe (Fig 3.3, solid triangle labeled line) was clearly exaggerated in comparison to the effect of raclopride in the absence of the probe (quantitative discussion of the magnitude of the raclopride effect follows in subsequent sections of this chapter). As in the case of the experiment reported in Fig 3.2, we administered a final dose of nomifensine to the rats and recorded a final evoked response (Fig 3.3, open triangle labeled line). Again, the effect of this final dose of nomifensine was exaggerated in comparison to what we observed in the absence of microdialysis probes.

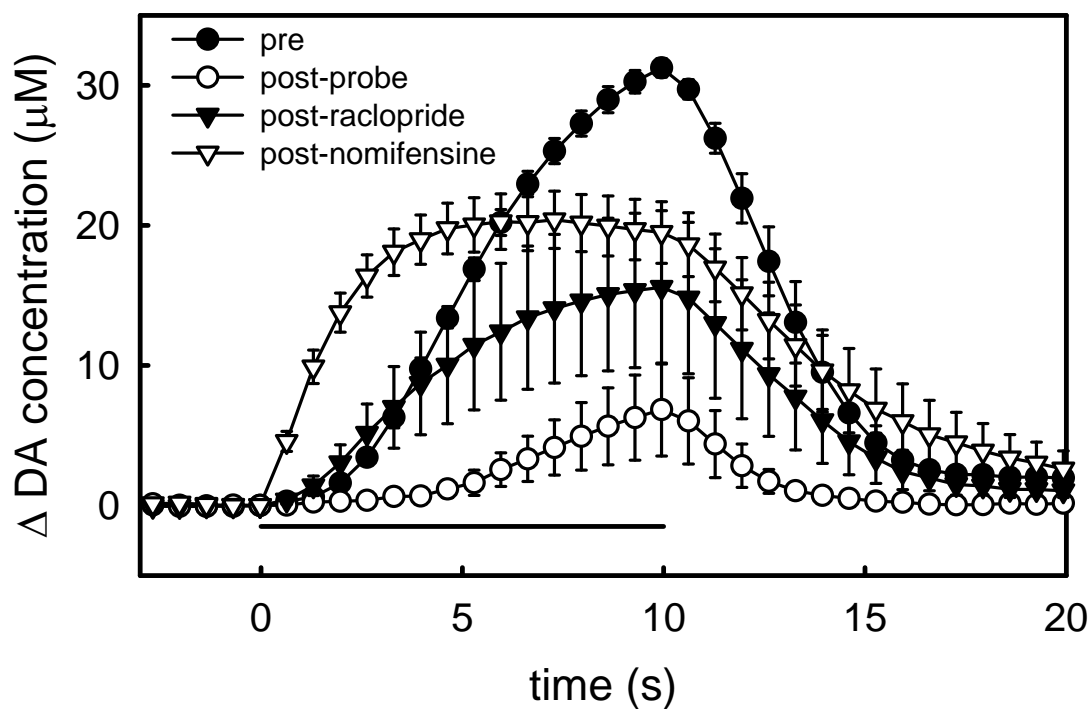


Figure 3.3 The impact of microdialysis probes on evoked DA release in the rat striatum. The traces in this figure show the average evoked responses recorded before (solid circle labeled) and after (open circle labeled) probe implantation, and after a sequential doses of raclopride (solid triangle labeled) and nomifensine (open triangle labeled). Each trace is the average of recordings in 4 rats: the error bars represent standard errors of the mean.

3.3.3 Absolute effects of drugs on evoked DA responses

Fig 3.4 compares the evoked responses recorded with (open circle labeled lines) and without (solid circle labeled lines) the implantation of a microdialysis probe before drug administration (Fig 3.4a), after systemic raclopride administration (Fig 3.4b), and after a final systemic dose of nomifensine (Fig 3.4c). Even though this experiment involved two separate groups of rats, the initial evoked responses in the two groups (pre-drug and pre-probe) were similar to each other (Fig 3.4a, inset). According to 2-way repeated measures ANOVA, there is no significant difference between these initial responses ($F(1,6)=0.305$, $p=0.6$). In each experimental condition (pre-drug, after raclopride, and after the final dose of nomifensine), evoked responses exhibited a significantly smaller absolute amplitude in the presence of the microdialysis probe. Each data set in Fig 3.4 was subjected to a 2-way repeated ANOVA: the factors were the probe (presence and absence) and time (repeated). In each case, the probe factor was significant (Fig 3.4a $F(1,6)=119.016$, $p<0.00004$; Fig 3b $F(1,6)=18.542$, $p=0.005$; Fig 3c $F(1,6)=6.592$, $p<0.05$). Thus, consistent with prior reports (Lu et al. 1998; Yang et al. 1998; Borland et al. 2005; Yang and Michael 2007), the presence of the probe significantly suppresses DAergic activity as measured by evoked responses recorded in the surrounding tissues.

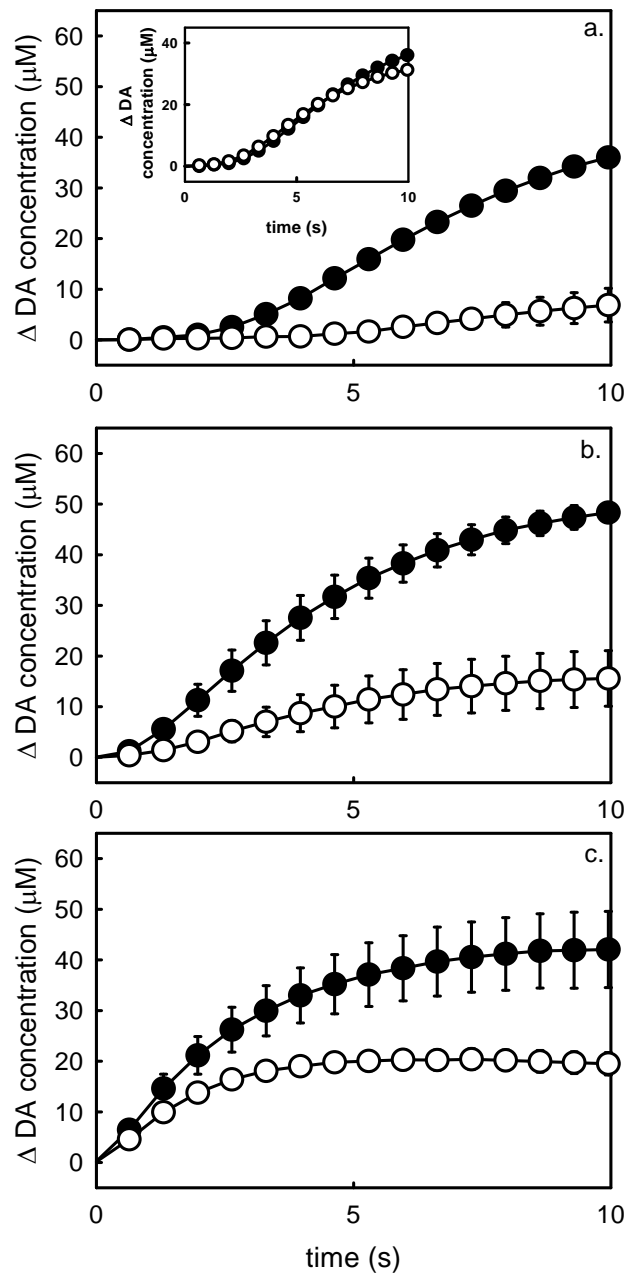


Figure 3.4 Comparison of absolute evoked DA responses recorded in the rat striatum in the absence (solid circle labeled lines) and presence (open circle labeled lines) of microdialysis probes before drug administration (a), after raclopride (b), and after a final dose of nomifensine (c). The inset in panel a compares the initial pre-drug/pre-probe responses from the two groups.

3.3.4 Proportional effects of drugs evoked DA responses

As implicated by the qualitative description of Figs 3.2 and 3.3, above, the proportional effects of raclopride and nomifensine (after raclopride) on evoked DA release are different in the presence and absence of a microdialysis probe. To demonstrate this quantitatively, Fig 3.5 re-plots the results of Figs 3.2 and 3.3 after normalizing the data with respect to the amplitude of the initial evoked response (pre-drug and post-probe-implantation) in each group. This normalized format makes it immediately obvious that, on a proportional basis, the effect of raclopride and nomifensine (after raclopride) are vastly exaggerated in the presence of the microdialysis probe compared to the absence of the probe.

The data in Fig 3.5 were subjected to a 3-way repeated ANOVA: the factors were the probe (presence and absence), the drug treatment (pre-drug, after raclopride, and after nomifensine), and time (repeated). The probe factor was significant ($F(1,18)=4.657$, $p<0.05$). Thus, overall, there is a significant difference between these normalized data sets in the presence and absence of the microdialysis probes. The drug treatment was significant as well ($F(2,18)=3.687$, $p<0.05$). Thus, on a normalized basis, both raclopride and nomifensine (after raclopride) significantly increased the normalized response amplitude. These normalized results demonstrate that the DAergic elements in the tissue impacted by probe implantation are hypersensitive to the pharmacological manipulations investigated in this study.

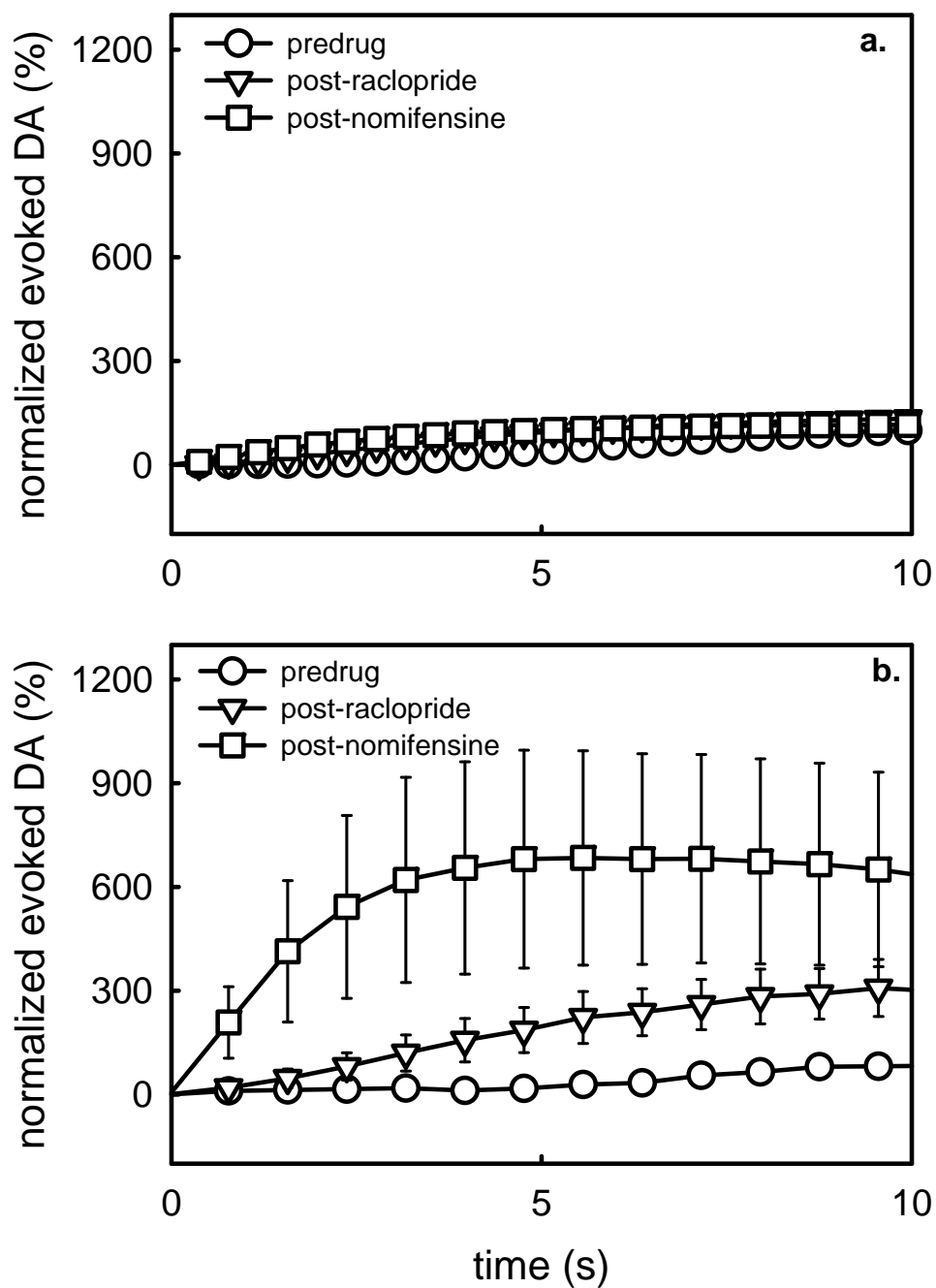


Figure 3.5 Normalized evoked responses recorded in the absence (a) and presence (b) of microdialysis probes. The responses were normalized with respect to the maximum DA concentration observed during the initial (pre-drug and pre-probe) stimulus.

3.4 DISCUSSION

The implantation of microdialysis probes into brain tissue results in injury and triggers a tissue response (Clapp-Lilly et al. 1999; Zhou et al. 2001; Mitala et al. 2008; Jaquins-Gerstl and Michael 2009). This is not unique to microdialysis probes and the subject of the tissue response to brain implants has become a central issue in the brain machine interface field as well (Szarowski et al. 2003; Winslow and Tresco 2010). So, it is important to understand if and how the brain tissue response affects the function and properties of neurochemical systems. In the case of DA, voltammetry offers a secondary methodology for *in vivo* measurements, so comparative studies are possible. The implantation of voltammetric microelectrodes into brain tissue is also injurious, but evidence suggests that microelectrodes are less injurious than larger devices, such as microdialysis probes and neuroprosthetic devices (Peters et al. 2004; Jaquins-Gerstl and Michael 2009). For example, in a recent immunocytochemical study we were unable to visualize any astrocytic activation by voltammetric electrodes, whereas astrocytes were clearly activated by microdialysis probes (Jaquins-Gerstl and Michael 2009). In our opinion, this justifies the experimental approach of ‘voltammetry near microdialysis probes’, which is based on the idea that the injury associated with microelectrode does not add substantially to that associated with the probes.

In the present study, which is consistent with previous ones using this same approach, we noticed a reduction in the amplitude of 10 s, 45 Hz stimulus responses when a microdialysis probe was implanted at a site approximately 200 μm from the voltammetric microelectrode. This supports the conclusion that the tissue response to the probe alters DA function as measured by voltammetry in the tissue surrounding the implantation site. In the short run, it is interesting to understand the consequences of the altered DA function as a way to improve the interpretation of *in vivo* results, especially in the context of resolving the perplexing differences between the outcome of microdialysis and voltammetry measurements. In the longer run, understanding the mechanism(s) of these alterations might provide guidance towards mitigating some of the disruptive aspects of probe implantation.

The voltammetric recordings described in this report were initiated 2 hr after probe implantation. There has been vituperative discussion and debate over the matter of what is the correct amount of time to wait after probe implantation before initiating experiments. Many protocols use a wait time of 24 hr after probe implantation (Enrico et al. 1998; Westerink et al. 1998; Vidal et al. 2005; Chefer et al. 2006), although various times have been used (see for example Young and Rees (1998), Yoshimoto et al. (2006)). However, we simply no longer agree that there is a preferred wait time or that 24 hr is more valid or appropriate than, say, 2 hr. According to our findings, the disruption of DA function by the probe begins at the time of implantation. Evoked DA responses remain suppressed at 16 hr after probe implantation and actually are more severely depressed than at 2-4 hr after implantation (Yang and Michael 2007). Consistent with this, we found intense glial activation surrounding microdialysis probe tracks after 24 hr implants (Jaquins-Gerstl and Michael 2009), which histologically confirms that the tissues remain in an abnormal state after 24 hrs. Furthermore, one of the main justifications for waiting 24 hr after probe implantation is based on the observation that basal DA levels exhibit complete sensitivity to tetrodotoxin (TTx) at that time, i.e. are purely derived from vesicle fusion events and are free of contributions from non vesicular routes of DA release, such as reversal of the DA transporter (Westerink et al. 1987; Westerink and De Vries 1988). On the other hand, recent studies from our laboratory (Borland and Michael 2004; Wang et al. 2010) provide evidence that the release of DA via reverse transport is functionally significant in the rat striatum, so we not agree that ‘complete TTx sensitivity’ is an appropriate or necessary criterion for valid measurements of DA in this brain region.

The findings of the present study support two interrelated points. First, on an absolute basis, probe implantation leads to an absolute suppression of evoked DA signals (Figure 3.4). Second, on a proportional basis, probe implantation leads to an enhancement in the effects of pharmacological agents, in this study raclopride and nomifensine after raclopride, on the evoked responses (Figure 3.5). Together, these findings paint a complex picture of the nature of the probe-induced DAergic disruption. Some loss in DA function can be attributed to a loss of DA terminals, which is consistent with histological findings. However, it appears that the DA terminals surviving 2 hr after probe implantation are in an abnormal

neurochemical state. As mentioned above, probe implantation does not simply decrease the amplitude of the evoked response (Figure 3.3) but also alters its temporal features. After probe implantation, evoked release is further delayed after the onset of the stimulus, which might be a sign that the terminals are subject to greater autoinhibition than is normally the case, since the D2R agonist, quinpirole, had the same delaying effect (Moquin and Michael 2009; Wang et al. 2010). This explanation is further supported by the extremely large proportional effect of raclopride after probe implantation (Fig 3.5b). This might be a sign that the extracellular space in the tissue surrounding the probe contains a higher than usual basal DA concentration, perhaps due to an injury-related DA release. While this is a possible explanation, our voltammetric recordings, which continued throughout the probe implantation procedure, did not provide any evidence that the probe implantation elevated the basal DA concentration. On the contrary, in Chapter II we observed a reduced basal dopamine detected by a carbon fiber microelectrode within 200 μm away from a microdialysis probe. In our first impression this contradicts with the hyper-autoinhibition we have observed in this study. Because a low basal dopamine is supposed to correlate with a fast evoked dopamine release, in another word a low autoinhibition level (Wang et al. 2010, also see Chapter I). But when think one step further, in the same study (Wang et al. 2010), after basal dopamine was decreased by kynurenate, the evoked dopamine was slowed down instead of being speeded up. This is because kynurenate, the drug we use to manipulate basal dopamine is a complicated drug. It does not only decrease basal dopamine but also affect glutamate and acetylcholine neurotransmission. This provides us a clue to explain the paradox of the low basal dopamine and high autoinhibition in the injured tissue. Dopamine release is not purely self-regulated. Other neurotransmitters, such as glutamate and acetylcholine affect dopamine release by acting on the hetero-receptors. When a microdialysis probe is implanted, other neurotransmission systems are possibly affected as well and their input on dopamine terminals could be altered.

There are interesting parallels between the raclopride results of this study and the nomifensine results of our prior studies (Borland et al. 2005; Yang and Michael 2007). In both cases, we found that absolute measures of DA were decreased after probe implantation but that on a proportional basis

pharmacological effects were exaggerated in the presence of the probe. Collectively, these findings suggest a situation in the tissue surrounding the probe wherein the DA system exhibits a hypersensitivity to certain pharmacological manipulations. This might begin to explain why reports of pharmacologically induced changes in dialysate DA concentrations are widely reported in the literature, whereas this is not the case in the voltammetry literature. For example, DA uptake inhibitors are well known for their ability to raise dialysate DA levels, although a similar phenomenon has been difficult to detect by voltammetry. But, we observed an increase in DA, i.e. basal or non-evoked DA, by voltammetry near a microdialysis probe (Borland et al. 2005) after nomifensine administration, consistent with our idea that the DA system surrounding the microdialysis probe is hypersensitive to pharmacological manipulation. Our present findings reinforce this point of view: the impact of our final dose of nomifensine on the evoked DA response was negligible in the absence of a microdialysis probe (Fig 3.2, open triangle labeled line) but substantial in the presence of a probe (Fig 3.3, open triangle labeled line).

An interesting aspect of these findings is the manner in which they suggest that DA terminals surrounding the probe are ‘suppressed’ rather than just ‘killed’ 2 hr after probe implantation. While in the brain 16 hr after microdialysis implantation, there is no evoked dopamine can be detected on the carbon fiber microelectrode in the disrupted tissue, even after uptake inhibition (Yang and Michael 2007). After raclopride administration, the maximal evoked DA in injured tissue recovers to 50% of the pre-probe level (probe implantation decreased it to 23% of pre-probe). A following nomifensine brings it back even more, to 62%. In the slow sites we usually observed without optimization, raclopride alone cannot remove the suppression completely, especially in the initial stage of the stimulation (Fig 3.2 and 3.4a). A following administration of nomifensine removes the suppression completely and evoked DA is immediately obvious upon stimulation. This is still true in the injured tissue near a microdialysis probe. After raclopride, evoked DA is still suppressed in the initial stage but to a higher degree as compared to non-probe implanted tissue. Interestingly, after the following nomifensine treatment, although the maximal evoked DA in the injured tissue is still much lower than non-probe implanted animals, the immediate DA release upon stimulation of the two groups are almost identical during the first two

seconds of stimulation. It will be interesting in the future to ascertain whether steps can be taken to stabilize those terminals that survive the initial stages of the tissue response to the probe, and thereby mitigate some of the disruptive impact of the probes.

3.5 CONCLUSION

This study lends additional evidence in support of the idea that implantation of a microdialysis probe into the rat striatum triggers a tissue response that, among other things, alters DAergic function at the probe's implantation site. A typical evoked response measured by voltammetry during a 10-s, 45-Hz stimulus decreases upon probe implantation at a site 200 μm away. The extent of this signal loss is surprising, however, in light of an ultrastructural analysis (Clapp-Lilly et al. 1999) that found only a partial loss of neuronal terminals at similar distances from the probe (that study, furthermore, utilized larger diameter probes that might be expected to cause greater damage than the probes we used). The present study sheds valuable light on this matter by showing that the terminals near the probe are in a suppressed neurochemical state. Thus, the new insight provided here is that the probe-induced disruption is partially caused by terminal loss and partially caused by an altered regulation of the surviving terminals. That the tissue near the probe contains a greater than previously realized complement of DA terminals is certainly an intriguing finding that gives rise to the possibility of taking measures to promote their longer term survival and perhaps so mitigate the disruptive consequences of probe implantation.

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