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Voltammetric characterization of the effect of monoamine uptake inhibitors and releasers on dopamine and serotonin uptake in mouse caudate-putamen and substantia nigra slices

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Summary

Fast scan cyclic voltammetry is an electrochemical technique used to measure dynamics of transporter-mediated monoamine uptake in real time and provides a tool to evaluate the detailed effects of monoamine uptake inhibitors and releasers on dopamine and serotonin transporter function. We measured the effects of cocaine, methylphenidate, 2 β -propanoyl-3 β -(4-tolyl) tropane (PTT), fluoxetine, amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), phentermine and fenfluramine on dopamine and serotonin uptake following electrically stimulated release in mouse caudate-putamen and substantia nigra pars reticulata slices. We determined rank orders of uptake inhibition effects based on two variables; increases in apparent K_m for dopamine and serotonin uptake and inhibition constant (K_i) values. For example, the rank order of uptake inhibition based on apparent K_m values at the dopamine transporter was amphetamine $\geq$ PTT $\geq$ methylphenidate $\gg$ methamphetamine = phentermine = MDMA $>$ cocaine $\gg$ fluoxetine = fenfluramine, and at the serotonin transporter was fluoxetine = methamphetamine = fenfluramine = MDMA $>$ amphetamine = cocaine = PTT $\geq$ methylphenidate $>$ phentermine. Additionally, changes in electrically stimulated release were documented. This is the first study using voltammetry to measure the effects of a wide range of monoamine uptake inhibitors and releasers on dopamine and serotonin uptake in mouse brain slices. These studies also highlight methodological considerations for comparison of effects between heterogeneous brain regions.

Keywords

dopamine; serotonin; voltammetry; mouse; caudate-putamen; substantia nigra

Introduction

The behavioral effects of psychostimulants and antidepressants result primarily from their interactions with the brain biogenic amine transporters, the dopamine (DA) transporter (DAT), the serotonin (5-HT) transporter (SERT) and the norepinephrine transporter. These monoamine transporter proteins share genetic, structural and functional similarities, and all belong to a superfamily of 12 transmembrane domain, Na⁺/Cl⁻-dependent transporters (Amara and Arriza,

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1993). Monoamine reuptake is a multi-step process involving extracellular ligand binding, transport across the plasma membrane, intracellular ligand release and transporter reorientation. It is well appreciated that these transporters are vital for the termination of monoaminergic neurotransmission and a greater role for monoamine transporters in regulating presynaptic homeostasis and monoaminergic tone is emerging (Bengel et al., 1998; Jones et al., 1998; Xu et al., 2000).

Pharmacological agents that interact with monoamine transporters come in two varieties: pure uptake inhibitors and releasers (Blakely and Bauman, 2000). Uptake inhibitors (e.g., cocaine) bind to transporter proteins and inhibit uptake, slowing the clearance of monoamines from the extrasynaptic space, thereby increasing extracellular monoamine concentrations. Releasers (e.g., amphetamines) increase extracellular monoamine levels both by competitively inhibiting neurotransmitter reuptake and promoting reverse transport. Amphetamines also disrupt vesicular storage of monoamines and prevent intracellular monoamine degradation, further increasing the likelihood of transporter mediated monoamine efflux. There is a growing literature that DA efflux through the DAT may also occur by a fast, channel-like mechanism, which may be important in amphetamine actions (Kahlig et al., 2005). The mechanistic differences between uptake inhibitors and releasers is due in part to the fact that binding sites on monoamine transporters differ for uptake inhibitors and substrates/releasers (Kitayama et al., 1992). Releasers promote efflux in a non-exocytotic process that is dependent upon transport into the nerve terminal as a substrate; therefore, uptake inhibitors can block releaser effects. Unlike uptake inhibitors, many releasers can be neurotoxic, which is related to their ability to cause reverse transport as well as induce hyperthermia and oxidative stress. Like reverse transport, neurotoxicity induced by amphetamines can be blocked by administration of an uptake inhibitor (McCann and Ricaurte, 2004).

Monoamine uptake inhibitors and releasers are used in a wide variety of ways; some have no therapeutic utility and high abuse liability while others are effective in the treatment of disorders such as obesity, attention deficit hyperactivity disorder, narcolepsy, depression, panic disorder and obsessive-compulsive disorder. While many drugs are specific to a particular transporter, others are non-selective and affect uptake in multiple monoaminergic systems. It is the interaction of pharmacological agents at each of these transporters that results in unique behavioral profiles (Ginsburg et al., 2005; Izenwasser et al., 1999). It has been suggested that stimulant dependence, withdrawal, and pharmacotherapeutic strategies targeted for treating addiction involve both the dopaminergic and serotonergic systems (Baumann et al., 1995; Hitzig, 1993; Parsons et al., 1995; Rocha et al., 1998; Rothman et al., 1998; Rothman and Baumann, 2003; Sora et al., 2001; Walsh and Cunningham, 1997). For example, Davies et al. (1994) created tropane analogs that bind DA and 5-HT transporters with greater affinity and slower dissociation rates than cocaine. While originally designed to characterize the cocaine pharmacophore, these analogs have been found to alter drug-taking and depression-like behavior in rats and non-human primates (Hemby et al., 1997; Lile et al., 2000; Nader et al., 1997; Roberts et al., 2003; Sizemore et al., 2004). Because of the great focus of the psychostimulant literature on DA and 5-HT systems, we chose to evaluate the effects of a wide variety of uptake inhibitors and releasers on DA and 5-HT uptake using fast scan cyclic voltammetry (FSCV).

The ability of uptake inhibitors and releasers to bind to monoamine transporters and inhibit monoamine uptake has been measured most often with exogenously applied, radiolabeled neurotransmitters in synaptosomal preparations, membrane binding experiments and transporter expression systems (Baumann et al., 2000; Piffl et al., 1995). FSCV is a technique that allows the kinetic effects of pharmacological manipulation of endogenous monoamine uptake to be monitored in real time in discrete brain regions. To date, no voltammetric studies have comprehensively determined drug-transporter effect relationships using a variety of

uptake inhibitors and releasers in two neurotransmitter systems, which was the goal of this study. We characterized the uptake inhibition profiles of cocaine, methylphenidate, 2 β -propanoyl-3 β -(4tolyl) tropane (PTT) (Davies et al., 1993), fluoxetine, amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), phentermine and fenfluramine in DA and 5-HT terminal field regions, the caudate-putamen (CPu) and substantia nigra pars reticulata (SNr), respectively.

Preliminary reports of these data have appeared in abstract form (John and Jones, 2005; John et al., 2003).

Methods

Animals

Male and female C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine) were used at 2–4 months of age for the experiments described. They were housed with approximately three other littermates of the same sex in an animal care facility at 23 °C with a 12-hour light/dark cycle and given food and water *ad libitum*. Animal care and experimental protocols were in accordance with national and institutional guidelines.

Brain slices

Mice were anesthetized, decapitated and their brains rapidly removed and cooled in ice-cold, pre-oxygenated (95% O₂/5% CO₂), artificial cerebrospinal fluid (aCSF) consisting of (in mM) NaCl (126), HEPES acid (20), NaHCO₃ (25), glucose (11), KCl (2.5), CaCl₂ (2.4), MgCl₂ (1.2), NaH₂PO₄ (1.2), L-ascorbic acid (0.4), pH adjusted to 7.4. Coronal slices (400 μ m thick) containing the CPu or SNr were prepared using a vibrating tissue slicer (Leica VT1000S, Vashaw Scientific Inc., Norcross, GA). Slices were kept in a reservoir of oxygenated aCSF at room temperature until required. Thirty minutes before each experiment, a brain slice was transferred to a locally constructed submersion recording chamber, perfused at 1 ml/min with 32°C oxygenated aCSF and allowed to equilibrate.

Cyclic Voltammetry

Carbon-fiber microelectrodes were prepared as previously described (Cahill et al., 1996) and reference electrodes were Ag/AgCl wires. A bipotentiostat (EI400, Cypress Systems, Lawrence, KS) was used for FSCV. Monoamine detection with FSCV is based on relative innervation levels; while many electroactive neurochemicals are present in the brain slice, we predominantly detect DA in the dorsal CPu and 5-HT in the SNr of mice (Giros et al., 1996; John et al., 2006). For CPu DA recording, the electrode potential was linearly scanned from –400 mV to 1200 mV and back to –400 mV vs Ag/AgCl, at 300 V/sec, repeated every 100 ms (Mateo et al., 2004), while for 5-HT recording in the SNr, the electrode was scanned from 0 mV to 1200 mV to –600 mV and back to 0 mV vs. Ag/AgCl, at 300 V/sec, also repeated every 100 ms (John et al., 2006). Stimulation parameters used to evoke DA and 5-HT release produced consistent, measurable signals, and these parameters were chosen based on previous work in mice (John et al., 2006; Mateo et al., 2004). DA release was evoked every 5 minutes by a 1 pulse stimulation (monophasic, 350 μ A, 4 ms pulse width) and 5-HT release was evoked every 10 minutes by 30 pulse, 30 Hz stimulations (monophasic, 350 μ A, 1 ms pulse width) from an adjacent bipolar stimulating electrode (Plastics One, Roanoke, VA) placed on the surface of the slice, 100–200 μ m away from the carbon-fiber electrode. The carbon-fiber microelectrode was positioned 75 μ m below the surface of the slice. Each slice served as its own precondition control. Background subtracted cyclic voltammograms were constructed by subtracting the background current obtained before release from the current measured after release. In each case, the substance detected was identified by its characteristic cyclic

voltammogram. The oxidation current for DA or 5-HT was converted to concentration by electrode calibration with 1 μ M DA or 5-HT, respectively, at the end of the experiment.

Drugs were applied to brain slices by superfusion for 30 minutes per concentration, generally in a cumulative concentration paradigm (Jones et al., 1995b). We have determined that cumulative concentrations of pharmacological agents applied to brain slices do not differ in their ability to alter release or uptake when compared to the same pharmacological agents applied at single concentrations. Drugs were applied at increasing concentrations until voltammetric signals were no longer detectable and this generally occurred at concentrations ≤ 1 mM. An exception to this was PTT, which appeared to have maximal pharmacological effects at a concentration of 2 μ M and only one concentration higher than this (10 μ M) was tested.

Data Analysis

For this work, a Michaelis-Menten based kinetic model was used to evaluate release and uptake kinetics of extracellularly measured monoamine concentrations (Wightman et al., 1988; Wightman and Zimmerman, 1990). Temporal changes in extracellular monoamine concentrations evoked by transient electrical stimulation, which are a balance between the opposing processes of release and uptake during the rising phase, and uptake alone during the declining phase, were characterized by the following formula (with DA as an example).

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

Here, [DA] is the instantaneous extracellular concentration of DA released, f is the stimulation frequency, [DA] p is the release rate constant, expressed as concentration of DA released per stimulus pulse, and $V_{\max}$ and K_m are Michaelis-Menten uptake rate constants. The equation assumes that (1) a fixed concentration of DA ([DA] p) is released into the extracellular space with each stimulus pulse (but see Limberger et al., 1991; Kennedy et al., 1992) (2) uptake is a saturable process and (3) uptake via the neuronal DAT is the primary mechanism for clearing DA, which can occur between each stimulus pulse and in the time interval after the stimulation. Furthermore, a control K_m value (inversely related to the affinity of the transporter for its monoamine) of approximately 0.2 μ M for DA and 5-HT at their respective transporters was used and $V_{\max}$, which is proportional to the number of monoamine transporters, was also determined. These assumptions are best suited for evaluating release and uptake in striatal regions where one-pulse stimulations are used and that have rapid uptake, but can also be used with multiple pulse stimulations in areas with low uptake rates such as amygdala or midbrain (Bunin et al., 1998; John et al., 2006; Jones et al., 1995a). The curve fitting algorithm, based on simplex minimization and goodness of fit, was described by a nonlinear regression coefficient (r) (Jones et al., 1995b).

The concentration of DA or 5-HT released and $V_{\max}$ values for uptake were determined from current vs. time curves before and after drug application. The K_m values of 0.16 μ M DA at DAT and 0.17 μ M 5-HT at SERT were used for CPu and SNr measurements, respectively (Bunin et al., 1998; Near et al., 1988; Shaskan and Snyder, 1970; Wu et al., 2001b). When pharmacological agents were used, the change in the current vs. time profile was evaluated as a change in apparent K_m ; inhibition of DA or 5-HT uptake was reflected as an increase in apparent K_m (John et al., 2006; Jones et al., 1995a). The drugs studied have previously been described to competitively inhibit monoamine transport (Chen and Justice, 1998; Davies et al., 1993; Jones et al., 1995b; Krueger, 1990; Missale et al., 1985; Richelson and Pfenning, 1984; Schuldiner et al., 1993; Wu et al., 2001a; but see, e.g., Coyle and Snyder, 1969; Eshleman et al., 1999; McElvain and Schenk, 1992).

As described by Jones et al. (1995b), inhibition constants (K_i values) were determined by plotting the linear concentration-effect profiles and determining the slope of the linear regression; a straight line is expected for competitive uptake inhibition (Figure 1, representative example). To properly fit the linear regression, in some cases, the extreme concentrations (i.e. low concentrations that produced little effect and high concentrations that reproduced maximal effects that had previously been measured at a lower concentration) were not calculated in the linear regression. The r^2 for the linear regression was ≥ 0.90 in each case, indicating competitive inhibition. The slope of the linear regression is equal to K_m/K_i where the K_m values 0.16 μM and 0.17 μM for DA at the DAT and 5-HT at the SERT, respectively, were used.

Drugs

All drugs and all components of the aCSF were obtained from Sigma (St. Louis, MO) except for PTT and MDMA. PTT, a novel, high affinity tropane monoamine uptake inhibitor developed for characterization of cocaine binding sites (Davies et al., 1993), was a gift from Dr. Huw Davies. MDMA was a gift from the National Institute on Drug Abuse (Rockville, Maryland). (-)-Cocaine, (-)-PTT fumerate, (+)-amphetamine sulfate and (+)-methamphetamine were tested with the indicated formulation, otherwise, racemic mixtures of hydrochloride salts of the tested compounds were used.

Statistics

All statistical analyses were carried out using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). All drugs were tested in at least 4 brain slices, which were obtained from at least 4 animals. Data are shown as mean $\pm$ SEM. Data were evaluated with a one-way ANOVA. A p value of < 0.05 was considered significant.

Results

Figure 2 and Table I show the effects of cocaine (COC), methylphenidate (MPH), PTT, fluoxetine (FLU), amphetamine (AMPH), methamphetamine (METH), MDMA, phentermine (PHEN) and fenfluramine (FEN) on DA system function. Note that not all drugs were tested in the same concentration range; uptake inhibitors and releasers have effects on electrically stimulated monoamine release such that signals are abolished at high concentrations of drug, preventing uptake measurements. Therefore, several of the curves are not carried out to maximal, plateau concentrations.

Two parameters can be used to quantitatively describe uptake inhibition, apparent K_m and the inhibition constant, K_i . The Michaelis-Menten constant, K_m , describes the affinity of the monoamine for its transporter. In the presence of an uptake inhibitor, the “apparent” affinity of the monoamine substrate for its transporter is decreased. In this relationship, the increase in apparent K_m after pharmacological uptake inhibition reflects the magnitude of uptake inhibition achieved by these compounds; higher apparent K_m values represent greater uptake inhibition than lower apparent K_m values. In our studies we were able to determine an apparent K_m for DA at the DAT for each concentration of drug tested. Figure 2 and Table I describe the concentration-effect relationship of monoamine uptake inhibitors and releasers on apparent K_m for the DA system. All of the drugs tested, except fluoxetine and fenfluramine, significantly and dose-dependently inhibited DA uptake.

The inhibition constant (K_i) is related to the concentration of the drug that produces a half-maximal effect. This is an inverse relationship such that a high K_i value represents low drug potency while a low K_i value represents high drug potency. Although the values for apparent K_m and K_i are related, they measure two different aspects of uptake inhibition. K_i values were determined using voltammetric apparent K_m data, as described by Jones et al. (1995; see

methods and Figure 1). The calculated K_i values for all compounds are presented in Table I. Table I also shows average $V_{\max}$ values for all experiments with a given drug; average values are given, as $V_{\max}$ did not vary significantly ($p>0.05$) with drug concentration for any compound tested.

Figure 3 and Table I show the effects of monoamine uptake inhibitors and on 5-HT system function. Figure 3 shows the concentration-effect relationships of the drugs on apparent K_m for the 5-HT system, of which cocaine, PTT, fluoxetine, methamphetamine, MDMA, and fenfluramine had significant, concentration-dependent effects. Table I shows the K_i values and average $V_{\max}$ values; $V_{\max}$ was not significantly changed ($p>0.05$) by application of any of the drugs.

Peak release values and changes in peak release (Figure 4) followed predictable patterns (John and Jones, 2006b). Uptake inhibitors typically caused a small, but non-significant, increase in monoamine signal peak height at low concentrations (Garris and Rebec, 2002; Schmitz et al., 2003) while at higher concentrations there was a decrease in peak release resulting from the increased extracellular monoamine concentrations activating presynaptic autoreceptors (Wieczorek and Kruk, 1994a); this latter effect could be prevented with an autoreceptor antagonist (data not shown). In contrast to uptake inhibitors, the decrease in peak, evoked transmitter release produced by releaser drugs is only partially attributable to autoreceptor activation. Releaser-induced vesicular depletion may also contribute to this decrease in peak, evoked release by reducing the available transmitter pool (Iravani and Kruk, 1995; Schmitz et al., 2001; Wieczorek and Kruk, 1994b).

Discussion

The goal of this study was to assess the activity of various monoamine uptake inhibitors and releasers at DA and 5-HT transporters with FSCV. We evaluated the effects of cocaine, methylphenidate, PTT, fluoxetine, amphetamine, methamphetamine, MDMA, phentermine and fenfluramine on DA and 5-HT terminal dynamics in the CPu and SNr, respectively. Based on the effect of these compounds to maximally increase apparent K_m , we found the rank order for maximal DA uptake inhibition in the CPu to be amphetamine $\geq$ PTT $\geq$ methylphenidate $\gg$ methamphetamine = phentermine = MDMA $>$ cocaine $\gg$ fluoxetine = fenfluramine. Based on the K_i values obtained, the rank order of inhibitory potency (where a smaller K_i value reflects greater inhibitory potency) for the DA system was PTT $\gg$ methylphenidate $>$ cocaine $>$ amphetamine = methamphetamine $>$ phentermine = MDMA $\gg$ fluoxetine $\gg$ fenfluramine. In the 5-HT system, the rank order of uptake inhibition, based on maximal effects on apparent K_m , was fluoxetine = methamphetamine = fenfluramine = MDMA $>$ amphetamine = cocaine = PTT $\geq$ methylphenidate $>$ phentermine, while based on K_i values was PTT $>$ phentermine $\gg$ cocaine $\geq$ MDMA $\geq$ fenfluramine $>$ methylphenidate $>$ amphetamine $\gg$ fluoxetine $\gg$ methamphetamine. These rank orders of DA and 5-HT uptake inhibition are quite similar to previously published rank orders using synaptosomal preparations (Crespi et al., 1997; Fleckenstein et al., 1999) and stably transfected cell lines (Wall et al., 1995). It is important to remember that K_i and apparent K_m are constants that reflect different aspects of ligand-transporter interactions. K_i reflects the potency of the drug to inhibit substrate uptake by the transporter which can operate independently of the efficacy of the drug to inhibit uptake (which is reflected by the maximal apparent K_m). Functionally, the effect of these pharmacological agents is to increase the extracellular lifetime of monoamines which also increases the time available for DA or 5-HT to interact with their respective receptors and impact behavioral processes. To our knowledge, this is the first report of voltammetric characterization of the uptake-inhibiting properties of various monoamine uptake inhibitors and releasers in both the DA and 5-HT systems.

Binding and uptake inhibition studies have provided a great deal of information regarding the actions of monoamine uptake inhibitors and releasers at the monoamine transporters. In intact tissue, such as brain slices, potency and efficacy are often different than in membrane binding or synaptosomal preparations, indicating a need to measure uptake in brain slices with voltammetry. Table II shows a survey of published K_i and EC_{50} values for uptake for the compounds studied in this report. Nicolaysen et al. (1988) found that a 30 mg/kg i.p. dose of cocaine produced brain concentrations of 10 μ M in the rat striatum. Voltammetric studies have shown that 10 μ M cocaine increases apparent K_m for DA uptake to 2.5–11 μ M in rat CPu slices (Jones et al., 1995a; Jones et al., 1995b); consistent with those reports, we determined that 10 μ M cocaine increased the apparent K_m for DA uptake to approximately 6 μ M. Methylphenidate is generally thought to be relatively DAT selective and have twice the potency for DA uptake compared to cocaine (see Table II). Our results also show that methylphenidate is twice as potent as cocaine at inhibiting DA uptake. PTT, a tropane analog developed for characterization of cocaine binding sites (Davies et al., 1993), was reported to be relatively selective for the DAT compared to the SERT (Davies et al., 1994). We find potent actions of PTT at the DAT and this compound maximally inhibits DA uptake; PTT also inhibited 5-HT uptake but only submaximally and at relatively high concentrations. Fluoxetine is a selective 5-HT uptake inhibitor (Table II) and our results show that fluoxetine concentration-dependently inhibits the SERT but has no effect on DA uptake.

Amphetamine is thought to be a relatively DAT selective monoamine releaser (Table II). Voltammetric methods have previously shown that 10 μ M amphetamine increased apparent K_m for DA to 30 μ M (Schmitz et al., 2001), nearly identical to the results shown here. Phentermine is also thought to be a DA selective releaser, but less potent than amphetamine (Table II). *In vivo* microdialysis studies have, however, shown that at high doses/concentrations, phentermine increases both DA and 5-HT, but DA to a greater extent (Baumann et al., 2000). We found that phentermine did not inhibit DA uptake to the same magnitude and is less potent than amphetamine at the DAT, and that phentermine did not have significant effects at the SERT. The methylated amphetamine derivative, methamphetamine, is thought to have greater action at SERT than amphetamine itself (Table II) and our results are consistent with this. MDMA is often thought of as 5-HT selective, based on its neurotoxicity profile in rats and primates (Morton, 2005); however, MDMA is also known to interact with DA transporters (Table II). We show that MDMA can maximally inhibit 5-HT uptake, but only partially inhibits DA uptake compared to the efficacy of the other compounds studied. Fenfluramine is a selective 5-HT releaser (Table II); we find that fenfluramine concentration-dependently inhibited 5-HT uptake, while having no significant actions at the DAT.

In some cases, the K_i values we obtained (Table I) differ greatly from those reported in other studies (Table II). While we speculate that these differences are methodological/analytical (see below), it must also be considered that experimental conditions differ between voltammetric recordings in intact tissue and other uptake experiments. Unlike many other uptake inhibition studies utilizing exogenously added monoamines, our voltammetric approach used endogenously released monoamine to measure pharmacologically induced changes in uptake in brain slices. Differences found in our study compared to the reported literature may also result from species differences, or other methodological differences that would affect the ligand-transporter interactions including assay temperature, interactions with binding proteins, transporter internalization, drug sequestration or post-translational modifications, such as glycosylation or phosphorylation of the transporter.

One explanation for our high K_i values relates to the fact that they are directly based on apparent K_m values, leading to vast differences in the magnitude of our reported K_i values between the DA and 5-HT systems. By comparing Figures 2 and 3 it can be seen that the maximal apparent K_m in the DA system is two orders of magnitude greater than for the 5-HT system; as shown

in the present results, fully efficacious 5-HT uptake inhibition reveals a maximal apparent K_m value of approximately 1 μM while fully efficacious DA uptake inhibition produces a maximal apparent K_m value of approximately 100 μM . However, these maximal apparent K_m values each represent maximal uptake inhibition within the given system, i.e., uptake rates consistent with a complete transporter blockade (a pharmacological transporter knockout). This discrepancy in maximal K_m values appears to be due to the fact that baseline DA uptake rates in the CPu are greater than for 5-HT in the SNr. It seems that the maximal magnitude of apparent K_m , as determined by Michaelis-Menten modeling, is affected by transporter density and baseline uptake rates before application of pharmacological agents. Baseline DA uptake rates in the CPu (approximately 4 $\mu\text{M}/\text{sec}$) are much greater than 5-HT uptake rates in the SNr (≤ 0.1 $\mu\text{M}/\text{sec}$); uptake rates are related to the density of DA and 5-HT transporters in the CPu and SNr, respectively. Faster uptake rates allow for a greater range of change by uptake inhibition compared to already slow uptake rates. Therefore, we advise the reader that direct comparisons of apparent K_m values or K_i values between the DA and 5-HT systems may not be valid. It appears that the method we used to determine K_i values may be most appropriately used when baseline uptake rates are rapid; however, our data (i.e. rank orders) are qualitatively consistent with other studies. Additionally, we wished to directly compare the uptake inhibiting properties of the monoamine uptake inhibitors and releasers on the DA and 5-HT systems. However, this proved difficult due to the difference in magnitude of baseline uptake rate and therefore of potential maximal uptake inhibition between the DA and 5-HT systems; different analysis techniques will be required to make this comparison.

Differences in baseline uptake rates prove relevant when evaluating the *in vivo* effect of transporter-interacting drugs. It is thought that the reinforcing efficacy drugs of abuse is related to their ability to elevate DA, although actions at other monoamine transporters is also important (Ginsburg et al., 2005). Microdialysis studies show that drugs of abuse, like cocaine, preferentially increase DA levels in the nucleus accumbens (NAc) shell, compared to NAc core or CPu (Carboni et al., 1989; Wu et al., 2001a), even though cocaine appears to have similar efficacy in the CPu and NAc both *in vitro* and *in vivo* (Boja and Kuhar, 1989; Izenwasser et al., 1990; Jones et al., 1995a). This suggests that the preferential elevation of NAc DA by cocaine does not occur at the level of drug-transporter interaction. It was previously thought that lower DA uptake rates in the NAc were responsible for this preferential increase, however, it appears that rates for uptake *and* release, as well as diffusion and extracellular monoamine levels all influence the functional impact of monoamine uptake inhibitors and releasers in the brain (Daws et al., 2005; Michael et al., 2005; Wu et al., 2001a).

There are other drug-transporter interactions that may be reflected in our assay. For example, amphetamines not only competitively inhibit extracellular monoamine uptake but also concurrently cause transporter reversal. Although we are currently unable to distinguish these effects voltammetrically, this later action of releasers may also account for some aspects of uptake inhibition in our measurements and both are potentially reflected in our apparent K_m values. Furthermore, this reverse operation of the transporter often causes an apparent new equilibrium, whereby voltammetric signals do not return to baseline (John and Jones, 2006b). Unfortunately, we can not appropriately model signals which do not return to their original baseline with the Michaelis-Menten method and these signals were eliminated from our analysis. Another effect of transporter-interacting drugs on DAT and SERT function is PKC-dependent trafficking of transporters. Overall, it appears that substrates cause the trafficking of DAT molecules away from the cell surface, and uptake inhibitors have an opposite effect; in contrast, SERT density is increased by substrates and decreased by pure uptake inhibitors (reviewed in Elliott and Beveridge, 2005). While we do not directly measure monoamine transporter trafficking in our voltammetric measurements, it seems reasonable that these effects of monoamine transporter inhibitors and releasers may be reflected in our uptake measurements. *In vitro*, uptake inhibitors and releasers have consistently been found to traffic

transporters within minutes to one hour (Chi and Reith, 2003; Daws et al., 2002; Kahlig et al., 2004; Ramamoorthy and Blakely, 1999; Sandoval et al., 2001; Saunders et al., 2000), which is well within the time frame of our recordings. However, since our concentration-effect relationships exhibit a linear relationship (see methods), and $V_{\max}$ is unchanged, competitive inhibition appears to be the predominant mode of action for the acute effect of the compounds tested. While amphetamines are known to induce neurotoxicity, characterized by reduced transporter number that would be consistent with a reduced $V_{\max}$, we do not find acute neurotoxic effects of amphetamines in our superfused slice preparation.

As described in the results, there were effects of many of these drugs on our measures of monoamine release, although none of these were statistically significant for 5-HT release. Most surprisingly, there were significant effects of the selective serotonergic uptake inhibitor and releaser, fluoxetine and fenfluramine, on impulse-dependent DA release. We have not previously found effects of 10 μM fluoxetine on any aspect of DA dynamics (Budygin et al., 2002; John and Jones, 2006a; John et al., 2006), however, the results shown here only found significant effects of selective serotonergic drugs on DA release at concentrations of drug ≥ 100 μM . Previous studies, both *in vitro* and *in vivo*, concerning the control of DA release by 5-HT have provided conflicting results (Benloucif and Galloway, 1991; Clark et al., 1996; Dewey et al., 1995; Lucas and Spampinato, 2000; Westfall and Tittermary, 1982; Yi et al., 1991). Therefore, the precise mechanism resulting in reduced electrically-stimulated DA release by serotonergic drugs is unclear without further pharmacological characterization. Although this effect may be mediated through 5-HT heteroreceptors on DA terminals, there is also evidence that this may be an indirect effect (see Barnes and Sharp, 1999), especially given the high concentrations of drug needed to produce this effect.

In conclusion, this work describes the effects of monoamine uptake inhibitors and releasers on DAT and SERT function. Uptake inhibition studies are often accomplished using synaptosomal preparations or cell expression systems. Here, we used an electrochemical technique, FSCV, to monitor endogenously released DA and 5-HT and pharmacological effects on reuptake processes in brain slices. In addition, these studies document methodological considerations for comparison of monoamine uptake inhibitors between heterogeneous brain regions.

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Figure 1.

Effect of MDMA on apparent K_m values for DA uptake in CPu slices as used to determine K_i values (see methods). The concentration effect relationship was plotted and linear regression was used to determine the slope of the line, which is equal to K_m/K_i .

Figure 2.

Effect of monoamine uptake inhibitors and releasers on apparent K_m for DA uptake. (A) Representative voltammetric signals (plotted every 100 ms) measured by FSCV in response to a 1 pulse (350 μ s pulse width) electrical stimulation in a single mouse CPu slice. Note the changes in DA release and uptake in response to increasing concentrations of the catecholamine uptake inhibitor, methylphenidate (MPH). (B) Concentration-effect curves for uptake inhibitors and (C) releasers. Each concentration-effect curve was analyzed with a one-way ANOVA (***) $p < 0.001$.

Figure 3.

Effect of monoamine uptake inhibitors and releasers on apparent K_m for 5-HT uptake. (A) Representative voltammetric signals (plotted every 100 ms) measured by FSCV in response to a 30 pulse, 30 Hz (350 μ A, 1 ms pulse width) electrical stimulation in a single mouse SNr slice. Note the changes in 5-HT release and uptake in response to increasing concentrations of the monoamine releaser, MDMA. (B) Concentration-effect curves for uptake inhibitors and (C) releasers. Each concentration-effect curve was analyzed with a one-way ANOVA (* p <0.05; ** p <0.01; *** p <0.001).

Figure 4. Concentration-effect curves for monoamine uptake inhibitors (left) and releasers (right) on DA (top) and 5-HT (bottom) release. Each concentration effect-curve was analyzed with a one-way ANOVA (* $p < 0.05$, ** $p < 0.01$).

Table I

K_i and average V_{max} values for the monoamine uptake inhibitors and releasers used in this study. Please see text for interpretation of these values.

Drug	K_i (μ M)		Average V_{max} (μ M /sec) across the drug concentration range	
	DA	5-HT	DA	5-HT
Control			3.1 $\pm$ 0.20	0.080 $\pm$ 0.006
Cocaine	0.35	10	3.1 $\pm$ 0.29	0.054 $\pm$ 0.005
Methylphenidate	0.21	19	3.9 $\pm$ 0.30	0.062 $\pm$ 0.005
PTT	0.0054	0.88	2.8 $\pm$ 0.26	0.100 $\pm$ 0.011
Fluoxetine	270	59	4.5 $\pm$ 0.21	0.080 $\pm$ 0.006
Amphetamine	0.42	23	3.1 $\pm$ 0.25	0.078 $\pm$ 0.005
Methamphetamine	0.47	84	2.7 $\pm$ 0.16	0.11 $\pm$ 0.005
MDMA	1.7	13	3.2 $\pm$ 0.26	0.083 $\pm$ 0.010
Phentermine	1.6	1.5	3.8 $\pm$ 0.50	0.076 $\pm$ 0.008
Fenfluramine	580	15	4.3 $\pm$ 0.21	0.080 $\pm$ 0.008

Table IIPublished K_i and IC_{50} values for transporter-interacting compounds on DA and 5-HT uptake.

Drug	K_i (μ M)		IC_{50} (μ M)	
	DA	5-HT	DA	5-HT
Cocaine	0.058–0.79 (^{5, 6, 14, 16, 21, 22})	0.11–0.18 (^{5, 21, 22})	0.095–5.0 (^{1, 4, 10, 11, 12, 13, 15, 17, 18, 28})	0.30–0.85 (^{2, 11, 12, 17, 18})
Methylphenidate	0.10–0.39 (^{7, 21, 22})	15–22 (^{21, 22})	0.09–0.28 (^{1, 10, 12, 13})	>1.0–26 (^{2, 12})
PTT			0.0016 (¹⁵)	
Fluoxetine	1.6–5.4 (^{21, 24})	0.0096–0.017 (^{21, 24, 26})	>5–20 (^{11, 23})	0.0073–0.16 (^{11, 19, 23, 27})
Amphetamine	0.034–3.6 (^{16, 14, 21, 22, 26})	1.8–8 (^{21, 22, 26})	0.033–0.40 (^{1, 3, 9, 10, 11, 12})	>1–11 (^{2, 3, 9, 11, 12})
Methamphetamine	0.11–0.33 (^{16, 25})	2.1 (²⁵)	0.082–0.47 (^{1, 8, 10, 11})	>1–21 (^{2, 8, 11})
MDMA	1.6 (²⁵)	0.24 (²⁵)	1.1–14 (^{8, 9, 12, 20, 27})	0.20–8.3 (^{8, 9, 12, 19, 20, 27})
Phentermine	1.58 (²⁶)	14 (²⁶)	1.4 (³)	13 (³)
Fenfluramine	24 (²⁶)	0.27 (²⁶)	11–20 (^{3, 9, 12})	0.33–5.0 (^{3, 9, 12})

¹ Numbers in parentheses refer to references: (Andersen, 1987);² (Andersen, 1989);³ (Baumann et al., 2000);⁴ (Boja et al., 1990);⁵ (Carroll et al., 1995);⁶ (Chen and Justice, 1998);⁷ (Chen et al., 1999);⁸ (Cozzi et al., 1999);⁹ (Crespi et al., 1997);¹⁰ (Deutsch and Schweri, 1994);¹¹ (Eshleman et al., 1999);¹² (Fleckenstein et al., 1999);¹³ (Gatley et al., 1996);¹⁴ (Giros et al., 1992);¹⁵ (Letchworth et al., 2000);¹⁶ (Lin and Uhl, 2002);¹⁷ (Matecka et al., 1996);¹⁸ (Miller et al., 2001);¹⁹ (Mortensen et al., 1999);²⁰ (Pifl et al., 2005);²¹ (Richelson and Pfenning, 1984);²² (Ritz et al., 1987);²³ (Rothman et al., 1993);²⁴ (Rothman et al., 1994);²⁵ (Rothman et al., 2000);

²⁶(Rothman et al., 2001);

²⁷(Verrico et al., 2007);

²⁸(Zhang et al., 1998).