STUDIES OF SYNTHETIC PARTICLES AND NERVE ENDINGS ON MASS
TRANSPORT AND KINETICS AND INHIBITION OF THE
DEGLYCOSYLATED DOPAMINE TRANSPORTER

By

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And last but not least, I am so thankful to have endless support and encouragement from my family: mom, dad, my two lovely sisters (Anita and Bonnie), and my brothers-in-law (Jason and Martin). I love you all!
Unstirred layer (USL) is present at the interface of solids with solutions, and particles pass through these layers by diffusion. Rotating disk electrode voltammetry (RDEV) has been used in different biological applications. However, it is unknown whether brain tissue preparation in solution affects the mass transport at the electrode, and whether the USL in real biological samples affects the kinetic measurements. In Chapter Two, liposomes, silica, and Sephadex™ were used to model the tissue preparation particles and examined the effect on mass transport at the RDE. Both diffusion and hydrodynamic boundary layers formed between the solution and the electrode surface. In the presence of inert particles, the sensitivity, limiting current, apparent diffusion coefficient, boundary layers thicknesses, and the mixing time decreased with no change on the detection limit (6 ± 2 nM). In addition, the thicknesses of USL’s within the neuronal homogenates do not appear to affect the apparent kinetics of biological mass transport. Results also show that RDEV kinetically resolves transmembrane transport with a timing of approximately 30 ms.
It is known that the glycosylation on the dopamine transporter (DAT) is important for cocaine binding and the function of the DAT. However, the connection between carbohydrate moieties and the function of the DAT is unknown. In Chapter Three, effects of carbohydrate component modification on DA transport velocity were examined by using different glycosidases. DA transport activities decreased after different glycosidase treatments in rats’ striata. Then, α-mannosidase was used for the rest of the studies due to the rapid access to its substrate. After α-mannosidase treatment, $V_{\text{max}}$ of the DA transport decreased while the $K_m$ was unchanged. Moreover, removal of α-mannose abolished the effect of cocaine on the kinetic state of the DAT. Finally, cocaine, bupropion, mazindol, and β-phenylethylamine but not methamphetamine and tyramine block the effects of α-mannosidase on the DAT. Results lead to a conclusion that a derivative of mazindol may be a possible antagonist for cocaine.

Methamphetamine is a very addictive drug that will damage the brain. Chapter Four provides a summary on how methamphetamine affects different neurotransmitter contents and its transporter kinetics in different brain regions.
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Chapter Three is in the format of the journal *Synapse*, where it will be submitted. I performed all the experiment and manuscript preparation in consultation with my advisor, Dr. James O. Schenk, who is a coauthor.

Chapter Four is in the format of the *Current Drug Abuse Reviews*, where it will be submitted. I performed all the literature search and manuscript preparation in consultation with my advisor, Dr. James O. Schenk, who is a coauthor.
CHAPTER 1
INTRODUCTION

1. General Analytical Methods for Neurotransmitter Transport Kinetics

Different in vitro techniques including flow injection analysis (FIA), rapid filtration, and rotating disk electrode voltammetry (RDEV) have been applied to monitor the kinetics of neurotransmitter transport in different anatomical regions of the rat brain in the presence or absence of different drug treatments (Earles et al., 1998). In order to examine the kinetics of neurotransmitter transport, it is necessary to develop methods that can kinetically resolve transport activity. Neurotransmitter release occurs on a subsecond to second time scale, while reuptake occurs over a few seconds (Eccles and Jaeger, 1956; Jones et al., 1999; Schenk et al., 1990; Volz et al., 2006b). Samples can be tissue slices, homogenates, synaptosomes, or free cells (Earles et al., 1998).

1.1 Flow Injection Analysis

Continuous flow is one of the FIA methods. In continuous flow, a transporter-containing preparation and neurotransmitters are rapidly mixed and combined. Then, the combined solutions are passed through a flow tube in a fixed flow rate and are detected by a spectrophotometer, which is in a fixed position along the flow tube. The initial rate is calculated by set values for product formation or changes in the substrate concentration at different times (Earles et al., 1998).
1.2 Rapid Filtration

The rapid filtration technique can be performed by adding homogenized rat brain tissue in physiological buffer. $^3$[H]neurotransmitter is then added to the suspension, and neurotransmitter transport is measured by rapidly filtering the suspension at different time points. The filters are then rinsed and dissolved. A liquid scintillation counter is used to measure how much neurotransmitter is taken up. Finally, the experiments are repeated at different neurotransmitter concentrations (Earles et al., 1998).

1.3 Rotating Disk Electrode Voltammetry

RDEV is a faradic detection technique that can kinetically resolve transporter activity because the instrument response time (ca. 40 ms) is faster than the neurotransmitter release and reuptake. The first mass transport theory describing this technique was given by Levich in 1942 (Adams, 1969; Bard and Faulkner, 1980). However, RDEV did not get much attention until the 1950s, when researchers from England and the United States began to use this technique for their research on electrochemical reaction mechanisms. RDEV is a sensitive technique that is capable of making steady-state measurements (Bruckenstein and Miller, 1977). RDEV has been used to study different biological applications such as enzymology (Arechederra and Minteer, 2010; Bartlett and Pratt, 1995; Schubert et al., 2009; Van Stroe-Biezen et al., 1994). In neuroscience research, RDEV is used to measure neurotransmitters transporter kinetics, including the uptake for dopamine transporter (DAT) (Bjorklund et al., 2008; Chen et al., 2003; Chen et al., 1999; Earles and Schenk, 1999; McElvain and Schenk, 1992a; McElvain and Schenk, 1992b; Meiergerd and Schenk, 1994; Meiergerd et al., 1994a; Meiergerd et al., 1994b; Robinson et al., 2005; Sleipness et al., 2008; Volz and Schenk, 2004), serotonin transporter (SERT) (Hagan et al.,
norepinephrine transporter (NET) (Burnette et al., 1996; Chen and Justice, 1998; Chen et al., 1998; Reed et al., 2003), and the vesicular monoamine transporter 2 (VMAT-2) (Volz et al., 2009a; Volz et al., 2007; Volz et al., 2008; Volz et al., 2009b; Volz et al., 2006a; Volz et al., 2006b). RDEV can also measure the kinetics of DA depolarization by $K^+$ stimulation and then reuptake by the DAT (McElvain and Schenk, 1992). Besides neurotransmitters, RDEV can also detected uric acid (Ernst and Knoll, 2001; Gao et al., 1997), ascorbic acid (Earles and Schenk, 1998; Guo et al., 2012; Vasantha and Chen, 2005), and nitric oxide (Chen et al., 2006; Ciszewski et al., 1998).

When the electrode is rotating in a solution, the mixing of solution produces convection paths that are rapid and reproducible. An analyte, which must be electroactive, in the bulk solution is brought to the electrode surface by laminar flow from a field perpendicular to the electrode surface and then spun away in radial paths parallel to the electrode surface. Then, a sufficient potential is applied to oxidize or reduce the analyte at the electrode surface, and maximal oxidative or reductive limiting current is detected. The limiting current that is detected at the electrode surface is directly proportional to the concentrations of the electroactive molecules that are capable to undergo electron transfer processes. Dopamine (DA) is the analyte that my work is focused on for the next three chapters, and Scheme 1 shows the reaction of DA oxidized at the electrode surface. When DA is oxidized at the electrode surface, it becomes DA o-quinone with two protons and two electrons.

Adjacent to the electrode surface, two different layers are formed that may affect the temporal resolution. The diffusion boundary layer is the concentration gradient of the electroactive analyte adjacent to the electrode, while the hydrodynamic boundary layer is a velocity gradient from the electrode surface where the closest layer is unstirred to the bulk.
solution (Adams, 1969). Besides the unstirred layers at the electrode, biological membranes also contain unstirred layers, which are the region of stationary fluid where convection is not significant, and solute can only move through this region by diffusion (Barry and Diamond, 1984). This diffusion time represents the limit for resolution of the transport kinetics.

Studies have shown that the rate of mass transport can be affected by inert particles or red blood cells in the solution and thus affect the limiting current at the RDEs (Andersen et al., 1989; Caprani et al., 1985; Ficquelmont-Loizos et al., 1988; Sonneveld et al., 1990). However, it is not known whether brain tissue and the nerve endings in the solution will affect the mass transport at the RDE. Also, it is unknown if the unstirred layers at the membrane surface of nerve endings influence the kinetic measurements. In Chapter Two, inert particles were used to model the brain tissue preparations and the nerve endings in solution. Mass transport, sensitivity, temporal resolution, and detection limit of the RDEV were validated in the presence of inert particles. In addition, the thicknesses of the unstirred layers at the membrane surface of nerve endings were estimated and the effect on the kinetics measurements was evaluated.

2. Modification of the Dopamine Transporter

2.1 Synthesis of Dopamine in the Brain

Once the RDEV method was validated, my second part of the research was to examine DA transport kinetics under the modification of the DAT. Neurotransmitters are responsible for communication in the brain. DA is a catecholamine neurotransmitter, and its concentration in the synaptic cleft can be affected by psychostimulant drugs such as cocaine and methamphetamine (METH). DA is synthesized in the presynaptic terminal and present in several areas of the brain with a high density in the corpus striatum, which plays important roles in reward, reinforcement,
and motivation pathways (Purves et al., 2008). DA is generated from the amino acid precursor tyrosine. L-tyrosine is then converted to L-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase, and finally DA is formed from L-DOPA by L-aromatic amino acid decarboxylase. Upon generation, DA is loaded into synaptic vesicles through a vesicular monoamine transporter 2 (VMAT-2) (Cooper et al., 1996).

2.2 Effects of Cocaine and Methamphetamine on Dopamine Transport

The transmembrane protein called dopamine transporter (DAT) plays an important role in dopaminergic neurotransmission by removing DA from the synaptic space, thus regulating DA binding to DA receptors (Nestler, 2001). Under regular neurotransmission processes, DA is found in vesicles inside the presynaptic neuron, and a change in the membrane potential of the presynaptic cell activates vesicles to diffuse toward the terminal membrane. Then, DA is released from the presynaptic neuron into the synaptic cleft and binds to the DA receptor in the postsynaptic neuron to complete the transmission. The DAT will then transport DA from the postsynaptic neuron back to the presynaptic neuron for re-use (Cooper et al., 1996; Kandel et al., 2000). However, cocaine binds to the DAT and inhibits DA reuptake into the presynaptic neuron from the post-synapse resulting in a high concentration of DA remaining in the synaptic cleft (Gold, 1993). This increases the activation of the DA receptors and causes euphoria. METH has a different mechanism to inhibit DA transport. METH is a DA substrate analog, which competes with DA and gets transported into the presynaptic neuron by the DAT to the cytosol. Then, DA releases from the vesicle into the synaptic cleft via DAT. High concentrations of DA increase stimulation of DA receptors and causes euphoria.
2.3 The Dopamine Transporter

The DAT is a single gene product membrane protein with an approximate mass of 80 kDa (60 kDa for 619 amino acids and 20 kDa associated with glycosylation) with transport dependence on Na\(^+\) and Cl\(^-\) concentrations (Giros and Caron, 1993). The DAT contains 12 transmembrane regions connected by alternating intracellular and extracellular loops with N and C terminals facing the cytoplasm (Kilty et al., 1991; Shimada et al., 1991; Volz and Schenk, 2005). The common simulated and constitutive phosphorylation sites in the DAT have been focused on the first 21 residues at the N-terminus of the protein (Cervinski et al., 2005; Foster et al., 2003; Granas et al., 2003; Parnas and Vaughan, 2008). DAT phosphorylation is mediated by protein kinase C (Chen and Reith, 2000; Huff et al., 1997; Vaughan et al., 1997), which results in down-regulation of the transport activity (Chen and Reith, 2000; Huff et al., 1997; Kitayama et al., 1994; Vaughan et al., 1997; Zhang et al., 1997) and sequestration of the transport protein (Chen and Reith, 2000; Melikian and Buckley, 1999; Pristupa et al., 1998; Zhu et al., 1997). Figure 1 shows the schematic representation of the DAT (Giros and Caron, 1993; Smith et al., 2008). The first five transmembrane regions from the N-terminus are involved in the concentration gradient of the Na\(^+\)- and Cl\(^-\) ions as an energy source. Transmembrane regions six to eight are the target sites for inhibitors, and regions nine through the C-terminus are involved in substrate affinity and stereoselectivity (Schenk, 2002).

2.4 Glycosylation Sites on the Dopamine Transporter

The DAT is a glycoprotein, in which carbohydrates covalently linked to the peptide portion (Parnas and Vaughan, 2008; Spiro, 1969). Glycoproteins contain a characteristic group of sugars including D-mannose, D-glucose, D-xylose, D-galactose, L-fucose, N-acetyl-D-
glucosamine, N-acetyl-D-galactosamine, and sialic acids (Spiro, 1969). It has been known that the glycosylation sites on the DAT are N-linked oligosaccharides (Lew et al., 1991a). This is the common feature for Na⁺/Cl⁻-dependent neurotransmitter transporters and classic for transmembrane proteins (Giros et al., 1992). Giros et al. (1992) cloned cDNA coding for the rat, bovine, and human DAT, and Figure 1 shows the amino acid sequences of the human DAT. In addition, results show three potential glycosylation sites for human DAT at Asn¹⁸¹, Asn¹⁸⁸, and Asn²⁰⁵ (Vandenbergh et al., 1992; Volz and Schenk, 2005), and four potential glycosylation sites for the rat DAT at Asn¹⁸¹, Asn¹⁸⁸, Asn¹⁹⁶, and Asn²⁰⁴ (Surratt et al., 1993; Volz and Schenk, 2005) in the large second extracellular loop. DATs in the striatum and nucleus accumbens have molecular weights of 72 and 76 kD, respectively, and the difference is due to microheterogeneity (Lew et al., 1992; Lew et al., 1991b). In order to study the structure and function of a glycoprotein, exo- and endoglycosidase are usually used as a tool to remove a particular carbohydrate moiety or the entire glycosylation on the glycoprotein. SDS-PAGE and western blot analysis of the DAT after exoglycosidase treatments have suggested that sialic acid is present but not α-mannose in the glycosylation sites of the DAT (Bjorklund et al., 2008; Lew et al., 1991a). Also, it has been shown that by using neuraminidase to remove sialic acids, DA uptake velocity is dramatically decreased with no effect on the K_m (Meiergerd and Schenk, 1994; Zaleska and Erecinska, 1987). Therefore, DA cannot be transported back into the presynaptic cell if the DAT is not glycosylated (Meiergerd and Schenk, 1994). Glycosylation also affects cocaine binding to the DAT (Cao et al., 1989). When lectin was added to the striatal tissue, the lectin-DAT complex had a higher affinity for cocaine to the DAT while the number of binding sites was unchanged (Cao et al., 1989). When rats were treated with METH for five days and withdrawn for 24-hr before exo or endoglycosidase was added to the METH or saline pretreated
tissues, the results have no difference between the saline and METH pretreated samples (Bjorklund et al., 2008). Although it is known that modification in glycosylation affects molecular weights of the DAT and the uptake function of the DAT, the relationship between carbohydrate moieties and the DAT function is unknown. In Chapter Three, exo- and endoglycosidase were used to remove the oligosaccharides on the DAT in different experiments in order to examine the effects on the DA transport kinetics. α-Mannosidase was then used to investigate whether structurally dissimilar and similar DAT inhibitors inhibit at a glycosylated site.

3. Brain Circuitry

The brain is divided into several different regions that mediate different functions such as movement, judgment, sensation, vision, coordination, pain, memory, and reward (Kandel et al., 2000). A human brain contains at least 100 billion neurons, which play an important role in the communication in the brain (Kandel et al., 2000). An average neuron forms and receives approximately one thousand synaptic connections (Kandel et al., 2000). In the mesocorticollimbic dopamine system, dopaminergic neurons in the A10 cell body are grouped in the ventral tegmental area with projections to the nucleus accumbens, olfactory tubercle, amygdala, prefrontal cortex, and ventral striatal domains of the caudate putamen (the striatum in the rat) (Koob and Moal, 2006; Nestler, 2001). This dopaminergic system is responsible for drug and natural reward circuitry. Natural rewards such as food, water, sex and drug rewards like cocaine and METH provide positive reinforcement so that the behavior is repeated. Drugs such as cocaine and METH are hypothesized to cause changes in the mesocorticollimbic dopamine system, which lead to drug addiction (Koob and Moal, 2006). For instance, researchers
suggested that synaptic plasticity in the ventral striatum plays an important role in early stage of drug use, whereas synaptic plasticity in the dorsal striatum is likely to be involved in the acquisition of behavioral habits, which causes the advanced stages of drug addiction (Berke and Hyman, 2000; Everitt and Robbins, 2005; Everitt and Wolf, 2002; Gerdeman et al., 2003; Hyman and Melenka, 2001; Koob and Le Moal, 2001; Packard and Knowlton, 2002).

4. General Information on Cocaine and Methamphetamine

In 2010, there were approximately 22.6 million illicit drug users in the United States who were age 12 or older. Out of these populations, 1.5 million and 353,000 were cocaine and METH users, respectively (SAMHSA, 2011). Pure cocaine is extracted from coca leaves, which can be found mostly in Peru and Bolivia (Volkow, 2009). Cocaine can be used for legitimate medical uses of local anesthesia for eye, ear, and throat surgeries, but cocaine abuse can cause cardiovascular (heart attack), gastrointestinal (abdominal pain or nausea), and neurological (seizures) problems (Volkow, 2009). The half-life of cocaine in the human body is an hour (Volkow, 2009).

METH was synthesized from ephedrine by pharmacologist Nagayoshi Nagai and the chemist Akira Ogata in 1893 and 1919, respectively (Anglin et al., 2000; Weisheit and White, 2009). METH is a psychostimulant of the amphetamine class of psychoactive drugs. During World War II, American, German, and Japanese soldiers used METH to suppress their fatigue (Weisheit and White, 2009). METH can be used to treat sleeping disorders and attention deficit hyperactivity disorder (ADHD) at the dosage much lower than the abused level (Volkow, 2006). METH abuse can cause cardiovascular problems and hyperthermia (Volkow, 2006). The half-life of METH in the human body is 12 hours, which causes a prolonged stimulating effect resulting
in an extended duration of euphoria (Volkow, 2006). Both cocaine and METH are considered as powerful addictive stimulant drugs that cause changes in the brain structure. Chapter Four provides an overview on different effects observed in different brain regions following acute and chronic dosing of METH and withdrawal in rats.

The text for each chapter is in the format of the journal to which it will be submitted or published. Chapter Two is a research paper that describes how inert particles and nerves endings affect the mass transport on the rotating disk electrode and has been published in the journal Analytical Biochemistry. Chapter Three is a research paper that will be submitted to the journal Synapse, which explains how deglycosylation affects DAT kinetics. Chapter Four is a review paper that will be submitted to the journal Current Drug Abuse Reviews, which summarizes the history and statistics of METH usage and the effect of METH on the dopaminergic system of the rat brain.
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Fig. 1. Primary amino acid sequence of the human DAT. Amino acid residues that differ between the rat and human sequence are shown in black. Y-shaped symbols represent potential N-glycosylation sites. (Smith et al., 2008)
Scheme 1. Reaction scheme of DA oxidized at the RDE surface.
CHAPTER 2

MASS TRANSPORT AT ROTATING DISK ELECTRODES: EFFECTS OF SYNTHETIC PARTICLES AND NERVE ENDINGS

ABSTRACT

An unstirred layer (USL) exists at the interface of solids with solutions. Thus, the particles in brain tissue preparations possess an USL, as well as at the surface of a rotating disk electrode (RDE) used to measure chemical fluxes. Time constraints for observing biological kinetics based on estimated thicknesses of USL’s at the membrane surface in real samples of nerve endings were estimated. Liposomes, silica, and Sephadex™ were used separately to model the tissue preparation particles. Within a solution stirred by the RDE, both diffusion and hydrodynamic boundary layers are formed. It was observed that the number and size of particles decreased the following: the apparent diffusion coefficient excluding Sephadex™, boundary layers thicknesses excluding silica, the sensitivity excluding diluted liposomes which agreed with the results from other laboratory, limiting current potentially due to an increase in the path distance, and the mixing time. They have no effect on the detection limit (6 ± 2 nM). The RDE kinetically resolves transmembrane transport with a timing of ca. 30 ms.

INTRODUCTION

Rotating disk electrode (RDE) voltammetry has been used in a variety of biological applications such as enzymology [1 - 4], neurochemistry, and neuropharmacology of neurotransmitters [5, 6]. In our laboratory, the RDE has been used for the study of the kinetic mechanism(s) of the dopamine transporter (DAT) [7, 8], the kinetics of dopamine (DA)
depolarization stimulated release by K⁺ and coupled reuptake by the DAT [9], the study of the binding chemistry of DA [10], structure activity studies of the transport of DA [10], kinetic mechanisms of DA transport in human DAT expressed in human embryonic kidney cells (HEK hDAT) [11], and the function of the DAT in the presence of cocaine and after withdrawal from the repeated administration of cocaine or methamphetamine [7, 12, 13, 14].

RDE voltammetric experiments in these studies involve measurements of membrane fluxes in homogenized brain tissue or synaptosomes in physiological buffer [6, 7]. Tissue homogenates contain synaptosomes, other particles and inclusions with diameters ranging from 0.2 µm for nerve endings to 100 µm for some observed inclusions [15]. The interface between a particle and buffer solution has an unstirred layer (USL), a region of stationary fluid where convection is not significant. Thus solute(s) encounter the USL and move through this region by diffusion [16], a process slower than convection. This “barrier” produces errors in estimation of transport rates thereby affecting apparent values of $V_{\text{max}}$ and $K_m$ [16]. A number of techniques have been used to evaluate USL’s at biological membranes [17, 18] and the results have been reviewed [16, 19, 20]. Planar membranes have USL thicknesses from ca. 300 µm without convection [16] to ca. 100 µm with stirring. Other USL’s thicknesses range from 0.6 µm to 800 µm depending on the nature of the material and stirring rate [16]. The diffusion time through the USL’s represents a limit for resolution of kinetic measurements.

Electrolysis at the surface of still, solid electrodes in quiescent solutions produces a diffusion controlled concentration gradient [21]. In contrast, at an RDE, the signal is controlled by both convection and diffusion [21]. Here analyte in bulk solution is brought by laminar flow to the electrode from a field perpendicular to the electrode surface and spun away horizontally in a radial pattern across the electrode surface (Fig. 1A). Under these conditions and with the
application of a sufficient potential, a maximal oxidative or reductive current (limiting current, $i_L$) is observed and both diffusion boundary ($\delta$) and hydrodynamic boundary ($\delta_0$) layers are formed (Fig. 1B). Thus, there are unstirred layers at the electrode itself perhaps affecting temporal resolution. The $\delta_0$ is the thickness of the velocity gradient from the electrode surface where the closest layers are unstirred to the bulk solution [21], and the $\delta$ represents the concentration gradient of the electroactive analyte adjacent to the electrode [21]. The velocity of the solution and the concentration of the electroactive substance in the region closest to the electrode surface is zero. The thickness of $\delta$ is several times smaller than the thickness of $\delta_0$, and the thickness of $\delta_0$ can be reduced by increasing the rotation rate which enhances the $i_L$ [21].

Particles in the solution can affect the $i_L$ at RDE’s depending on their size and number [22 - 25]. Particles will rotate while the solution is stirring (Fig. 1B), increasing the rate of mass transport. Previous work studied the effect of different types of inert particles, ranging from 0.3 – 40 µm in diameter, on the $i_L$ in a large volumes (100’s to 800’s of mL) of solution, including soda-lime glass beads [24], SiC [23, 25], Al₂O₃ [23], B₄C [23], and red blood cells [22]. Ficquelmont-Loizos et al. [23] found that the $i_L$ is reduced when the particle size is below the $\delta$. Sonneveld et al. [25] found that the $i_L$ is increased when the number and the rotation rate are increased in the presence of SiC particles at the RDE.

Our laboratory uses 200 to 1,600 fold smaller volumes in containers where the walls are within 1.0 mm of the shaft and disk surface of the RDE. Convective flow as described above for an RDE requires “infinite” distance between the RDE and the walls of the solution container. It has been shown that at a fixed value of analyte concentration the $i_L$ is independent of the volume of the solution and the size of containers greater than 3.5 cm$^3$ [26, 27] and depends on the rotation rate. Volumes less than 3.5 cm$^3$ have not been fully tested.
Since it is unknown whether the preparation of the brain tissue and the nerve endings in the physiological buffer will affect the mass transport, it is important to model the effect of inert particles to mimic the nerve endings. Also, it is important to determine whether the USL in real samples of nerve endings affect the kinetic measurements.

The goals of this work were to (a) determine how $i_L$ is affected by numbers and sizes of particles in the low volume chamber; (b) determine whether the sensitivity and detection limit are affected by the numbers and sizes of particles; (c) determine how temporal resolution is affected by particles; (d) estimate the thickness of the USL at membrane surfaces in real samples of nerve endings; and (e) estimate a temporal limit for measurements of kinetics in preparations of nerve tissue.

MATERIALS AND METHODS

Instrumental set up

The setup for the RDE experiments has been described previously [5]. The 3-mm diameter glassy carbon electrode and MSRX precision rotator were from Pine Instrument, Inc. (Grove City, PA). The reference electrode was AgCl coated Ag, and the auxiliary electrode was a Pt wire. LC-4A or LC-4C potentiostats (Bioanalytical System, Inc., West Lafayette, IN) were used to apply a potential of +450 mV and to measure the current. Data were recorded onto a Nicolet 310 digital oscilloscope (Nicolet Instrument Corp., Madison, WI) set at 20 ms resolution. The custom-made (details upon request) Pyrex glass incubation chamber has an inner diameter of 1 cm and a depth of 1.5 cm, containing a volume of 500 µL of physiological buffer.

Chemicals and Materials
The reagents and composition of the pH 7.4 bicarbonate-based physiological buffer have been described previously [5]. Solutions were made in purified water from a Barnstead Nanopure water purification system (Dubuque, IA). The particles and their diameters were: 3 µm and 10 µm silica (Rainin, Woburn, MA) and 40 µm to 300 µm Sephadex™ (Sigma, St. Louis, MO). Dopamine hydrochloride and (-)-cocaine hydrochloride were also from Sigma.

**Liposomes**

Liposomes (0.03 to 0.1 µm diameter) were made according to the methods of Davis et al. [28] by first making a 15 mM solution of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) lipids (Avanti Polar Lipids, Inc., Alabaster, AL) in CHCl₃ (Fisher Scientific, Pittsburgh, PA). Then 4 µmoles DMPC from the solution above was transferred to 2.1 mL of CHCl₃ in a round bottom tube and dried under N₂ stream for 2 hours. This produced a lipid cake on the walls of the round bottom tube. After the lipid cake was dried, it was fully dissolved in 1.0 mL of physiological buffer, and sonicated (VWR Scientific Model 50 Aquasonic) at 60 ºC until the solution was translucent. The liposomes were transferred to an Eppendorf tube and stored at 4 ºC for up to a week.

**Animals**

Male Sprague-Dawley rats (350 to 450 g) were obtained from the Washington State University internal breeding colony, housed 2 to 3 per cage in a University vivarium with a 12 hour light/dark cycle, maintained at 22 to 24 ºC, and had ad libitum access to food and water. The rat procedures were in strict accordance with the NIH *Guide for the Care and Use of*
Laboratory Animals and were approved by the University Laboratory Animal Care and Use Committee.

Tissue Suspensions

Tissue suspensions were prepared as described previously [7]. An unanesthetized drug free rat was rapidly decapitated, and the brain was divided into two hemispheres midsagittally. The striata (sample weight 35 – 40 mg each) were dissected at stereotaxic coordinates of about 3.6 mm anteriorly to 2.0 mm posteriorly according to Pellegrino et al. [29]. Each striatum was chopped with a razor blade on an ice-cold watch glass and transferred into the incubation chamber into 500 µL of physiological buffer and incubated for 20 min at 37°C with a stream of a gas (mixture of 95% O₂/5% CO₂) directed across the surface. Then, the tissue suspensions were washed eight times with fresh physiological buffer. Finally, the RDE was lowered into the suspensions and monitored to obtain a baseline.

This preparation possesses the expected properties of DA neurotransmitter release and uptake [7, 9] including: 1) Ca²⁺ and K⁺ dependent release [9], 2) DA release and transport regulation by presynaptic autoreceptors [9, 30], 3) Na⁺ and Cl⁻ dependent DA transport [7], and transport inhibited by 100 µM cocaine [7] with other inhibitors exhibiting proper rank order of IC₅₀’s [31].

Levich Behavior and the Calculation of δ and δ₀

Levich behavior was examined by varying the RDE rotation rate from 1000 rpm to 9000 rpm with approximately 10 s holding time at each increment, measuring the iₓ, and plotting its versus ω¹/² (Eq.1). The experiment was repeated four times with 1.0 µM DA added to the
physiological with no particles present (open solution). The same procedure was used for inert particles in physiological buffer. The results were analyzed by GraphPad Prism 5 (GraphPad Software, San Diego, CA).

The $i_L$ (mA) is described by the Levich equation [21],

$$ i_L = k n F A D^{2/3} \omega^{1/2} \nu^{-1/6} C \quad \text{Eq. 1} $$

where $k$ is the corrected constant and calculated to be 0.605 according to Adams [21], $n$ is the number of electrons transferred per mole of DA ($n = 2$), $D$ is the diffusion coefficient in cm$^2$/s, $F$ is the Faraday’s constant, $A$ is the electrochemical area of the electrode in cm$^2$, $C$ is concentration in M, $\nu$ is the kinematic viscosity equal to 0.01 cm$^2$/s, and $\omega$ is the angular velocity of the RDE in radians/s where $\omega = 2\pi N$ and $N$ is in rps.

The $\delta_0$ thickness (cm) under laminar flow conditions at the RDE is given by [21],

$$ \delta_0 = 3\left(\frac{\nu}{\omega}\right)^{1/2} \quad \text{Eq. 2}, $$

and the $\delta$ thickness (cm) by [21],

$$ \delta = 1.61D^{1/3} \omega^{-1/2} \nu^{1/6} \quad \text{Eq. 3}. $$

Finally, $i_L$ and $\delta$ are related by [32, 33],

$$ \delta = \frac{D A n F C}{i_L} \quad \text{Eq. 4} $$

where the symbols were defined previously.

**Evaluation of Apparent $D$ in the Presence of Inert Particles**

Chronoamperometric measurements in 25.0 µM DA were used to determine the apparent value of $D$ of DA in the presence of silica and Sephadex™. First, a chronoamperogram was run for DA in the open solution. Then, 3 µm silica or 40 – 120 µm Sephadex™ was added so that the
electrode surface was covered by particles while the electrode was still in the solution with no potential applied. After the particles settled, a potential was applied again. The values of D and apparent D for DA in the physiological buffer and particles was calculated using the Cottrell equation [21],

$$i_t = \frac{nFAD^{1/2}C}{\pi^{1/2}t^{1/2}}$$  \hspace{1cm} \text{Eq. 5}

where $i_t$ is the current in amps, time is in s, $n = 2$, $A$ was independently measured and $C$ is [DA] in mol/cm$^3$.

_calibration_curves_for_da_with_and_without_particles_

_Calibration Curves for DA with and without Particles_

Calibration curves for DA were plotted over the concentration range of 0.25 to 1.18 µM, with the electrode rotation at 2000 rpm, but in a few cases at 6000 rpm. Sequential additions of DA were added approximately every 25 s. Three different conditions were chosen for this experiment. First, a control calibration curve of DA in the open solution was made. Second, different numbers and sizes of silica, Sephadex™, and liposomes were added to the physiological buffer. Finally, striatal suspensions in the physiological buffer with no inert particles present were used. In order to have a constant [DA] for each added increment, cocaine at 100 µM was added to the suspensions and incubated for 30 s before DA was added so the DAT activity was $\geq 98\%$ inhibited [7].

_instrument_response_time_and_effects_of_particles_on_temporal_resolution_

_Instrument Response Time and Effects of Particles on Temporal Resolution_

In this study, the RDE was rotated at 2000 rpm. The mixing time was measured by adding 0.1 µM DA in the open solution and the $i_L$ was measured from the time DA was added to the time at which the signal was at maximum. While the RDE was in the solution and rotating,
1.0 mg 3 µm silica or 10.0 mg 100 – 300 µm Sephadex™ was transferred to the open solution. Then, another 0.1 µM DA was added to the suspensions, and the mixing time was measured from the baseline before the second DA addition to the maximum signal.

**Unstirred Layers at Neuronal Membranes**

To estimate the thickness of the USL at the surface of neuronal membranes two types of experiments were performed in striatal suspensions. The first was to measure the time to observe the threshold of a DA signal (three times signal-to-noise ratio) following a depolarization stimulus and the second (in separate experiments) was to measure the timing between the addition of DA into a suspension and the initiation of inwardly directed transport by the DAT. These two experiments measure the sum of the timing of biological machinery and that for DA to traverse the USL. Thus, the USL thickness ($\delta^*$) in cm, evaluated by the Einstein relationship equation in three dimensions [34],

$$\delta^* = \sqrt{Dt} \quad \text{Eq. 6,}$$

represents a limit to the thickness of the USL. In the depolarization experiment, following the acquisition of a baseline, 15 mM K⁺ was added to initiate release as reviewed by Earles et al. [5]. In the transport experiment 1.0 µM of DA was added to the suspension and the time from its addition to initiation of uptake was recorded also as previously described by Earles et al. [5]. Results are expressed in µm.

**RESULTS**

*Calculated $\delta$ and $\delta_0$ Thicknesses*
As a benchmark for analyses, Table 1 lists calculated values for $\delta_0$ and $\delta$ which are in the range 97.7 to 293 $\mu$m, and 4.5 to 13.6 $\mu$m, respectively. Note that both the $\delta_0$ and $\delta$ thicknesses decrease by 67% as the rotation rate increases. In addition, the $\delta$ thickness is about 5% of the $\delta_0$ thickness.

**Levich Behavior**

Fig. 2 shows slopes of Levich plots for DA in the absence and presence of particles. The slopes of liposomes and 10 $\mu$m silica are significantly decreased when the number of particles is increased. The y-intercept for the Levich behavior is not recorded or shown because the baseline for each curve has a different offset value, and at $\omega = 0$, the $i_L$ cannot be predicted because the boundary condition for mass transport to the electrode is different than during rotation [21].

**Evaluation of $\delta$ Thickness in the Presence of Inert Particles**

Fig. 3 shows the results of how the $\delta$ thickness varies at different rotation rates in the presence of different numbers of inert particles. In general, the $\delta$ thickness decreases as the rotation rate increases. The results of the experimental values are lower than results of the theoretical values at low rotation rates up to 4000 rpm ($\omega^{1/2} = 20.5$). After 4000 rpm, the experimental values and the theoretical values have no statistical differences. In the presence of 1.0 and 10.0 mg of 3 $\mu$m silica, the $\delta$ is thicker than in the open solution. The $\delta$ in the presence of 40 – 120 $\mu$m Sephadex™ shows no statistical difference from the $\delta$ in the open solution (data not shown). The $\delta$ thickness in the presence of 100 – 300 $\mu$m Sephadex™ is less than that open solution. In the presence of liposomes, the $\delta$ thickness decreases when the concentration of the liposomes is diluted but increases with a higher concentration of liposomes.


**Evaluation of Apparent D in the Presence of Inert Particles**

Table 2 lists the apparent values of the D of DA in physiological buffer and in the presence of inert particles. Since inert particles affect the apparent D, a weighing constant \((x)\) of the D of DA in the presence of inert particles relative to its open solution value was calculated. Table 2 shows the \(x\) value of the D of DA in the presence of different sizes and numbers of inert particles. The \(x\) value was calculated from the ratio of D in the presence of inert particles and the physiological buffer only. The values of \(x\) were between 0 and 1, except 1.0 mg 40 – 120 \(\mu\)m Sephadex™ in RDE measurements.

**Calibration Curves for DA with and without Particles**

Fig. 4 shows the slopes of calibration curves (i.e. sensitivity) measured in physiological buffer, in the presence of liposomes, striatal suspensions, or various numbers and sizes of inert particles. Compared with the same size of the particles, increasing the number of particles in the physiological buffer significantly decreased the sensitivity except Sephadex™. The sensitivity in the presence of striatal suspensions is statistically the same as in the liposomes but lower when the liposomes are diluted. The detection limits in the absence and presence of inert particles as well as in striatal suspensions were statistically indistinguishable at 6 ± 2 nM.

Calibration curves were also measured at rotation rates up at 6000 rpm. In these experiments, 10.0 mg of 3 \(\mu\)m silica and 10.0 mg of 40 to 120 \(\mu\)m Sephadex™ were evaluated. Table 3 compares the slopes of the calibration curves and shows that the slopes significantly increase as the rotation rate increases. The theoretical calibration curve from the Levich Equation (Eq.1) at 2000 and 6000 rpm predicts the slope should increase by 1.7. Although the numerical values of the slopes were increased by the presence of particles, the increases as a function of
rotation rate was 1.7. Values of y-intercepts for the calibration curves are not recorded due to the fact that each baseline for the calibration curve has a different offset value.

Temporal Resolution

Table 4 shows that the mixing time represented by the DA signal reaching a maximum value is longer than the instrument response time. The addition of inert particles shortens the average mixing time by 55 ms but is not affected by the number and types of inert particles.

USL of the Neuronal Membrane

Fig. 5 shows raw data oscilloscope signals for the two experiments to estimate USL thicknesses at the surface of membranes and/or inclusions in striatal suspensions. The threshold time for observing release and initiation of uptake were 0.24 ± 0.03 s (n = 4) and 0.75 ± 0.05 s (n = 4), respectively. These values are statistically different, and using Eq. 6 the USL thicknesses were found to be in the range 12 ± 2 to 21 ± 2 µm.

DISCUSSION

The boundary conditions for Levich behavior at an RDE require the walls and bottom of the container to be at an “infinite” distance away from the shaft and surface of the rotating electrode. Previous studies of the effects of particles on RDE performance [22 - 25] were conducted in large volumes. Here we have investigated the effects of particles on the responses of an RDE in an incubation chamber where the walls and bottom were quite close to the RDE (within 1 – 2 mm). Temporal resolution and apparent D dependence were estimated as well as USL thicknesses both at the RDE surface and those associated with inert and biological particles.
in solution. The results show that inert particles affect the δ thickness, the apparent D, have variable effects on the sensitivity depending on particle number and size, enhance temporal resolution, and finally detection limits are unaffected. The thickness of USL’s within the neuronal homogenates agree with previous estimates by other analyses in the literature, are at the thinner limits reported, and do not appear to affect the apparent kinetics of biological mass transport.

Comments on the Effects of Particles on Detection at the RDE

When the numbers and sizes of the inert particles are increased, the slopes of Levich plots decrease over the range of $\omega^{1/2}$ from 10.2 to 30.7 (1000 – 9000 rpm) because particles occupy volume thus the solution volume fraction is reduced relative to open solution. Further tortuosity, the path length traversed by detected species to reach the electrode surface, is increased.

The δ thickness is inversely proportional to the $i_L$ and decreases as the rotation rate increases. This phenomenon has been experimentally validated by Pratt [36] and Mridha and Kibria [33]. Mridha and Kibria show that the experimental curve of the δ thickness vs. $\omega^{1/2}$ is lower than the theoretically predicted value [33], and the same trend is observed here. Several reasons could cause the experimental value of the δ thickness to be lower than its predicted value. DA undergoes an ECE (electron transfer-chemical-electron transfer) reaction [37] that occurs at low rotation rates. In addition, according to Mridha and Kibria, other electrochemical reactions may occur also and affect the $i_L$ [33]. The rotation rates are calibrated and thus, cannot be one of the factors causing lower experimental values. Further, the slope of $i_L$ vs. $\omega^{1/2}$ is not statistically different between the experimental and theoretical values [38]. The δ thickness at different rotation rates in our experiments agreed with the results by Pratt [36].
In biological samples, such as brain homogenates, tortuosity produced by the space occupying tissue preparation may affect apparent diffusion of small molecules or ions [39]. The presence of the inert particles in the physiological buffer here was intended to model the preparation of nerve endings and inclusions. The apparent D of DA decreased in the presence of particles (Table 2), suggesting that the inert particles extend the time it takes for the DA to diffuse from the bulk solution to the electrode surface because of increased path lengths and decreased volume fraction. Therefore, the steady-state mass transport (represented by $i_L$) at the RDE is reduced but the time to reach the steady-state is decreased as the mixing time is shorter in the presence of inert particles. The ratio of currents between those in particle beds and open solution (less than unity) in chronoamperometric measurements implies that the particles have an effect on the diffusion process. For 3 µm silica, the value of $x$ is 0.25, implying a lower flux of DA to the electrode surface as compared to open solution. For 40 – 120 µm Sephadex™, the ratio is closer to unity. This may be because Sephadex™, as a hollow sphere, is less resistant to diffusion of DA to the electrode surface. The ratio for different numbers of silica and Sephadex™ particles in the RDE measurements are higher than the chronoamperometric measurements, because in the stirred solution, mass transport is dominated by convection.

Fig. 4 shows that $i_L$ decreases when various numbers and sizes of particles are present at 2000 rpm, a condition where the particle sizes are smaller than the $\delta_0$ thickness (207 µm). The result follows the same trend as we have seen in the Levich plots, and the reasons have been discussed above. When the numbers of liposomes in the physiological buffer are reduced, the mass transport of DA is enhanced compared with that observed in striatal suspensions. The diameter of liposomes is much smaller compared to the size of nerve endings. We hypothesize that the liposomes can rotate freely within the $\delta$ and $\delta_0$. This may be the reason $i_L$ is larger in the
presence of diluted liposomes. In addition, sensitivity observed in striatal suspensions which include nerve endings and other inclusions is statistically identical to that observed in 40 – 300 µm Sephadex™. This may be because the size of the inclusions in the biological preparations may define the paths taken by analyte species to the electrode surface. When we compare the calibration curves measured at rotation rates at 2000 rpm or 6000 rpm (as listed in Table 3), inert particles do not affect the \(i_L\) dependence on angular velocity (\(\omega\)) of the RDE.

In kinetic studies in biological samples, it is important to know what effects particles have on the mixing time in the small incubation chamber. In the stirred physiological buffer any particles present will rotate as the solution stirs, hence the detected species can be transported to the electrode surface faster by enhanced convection mediated by particle movements. This effect leads to shorter mixing times relative to open solution and is observed here (Table 4).

**Comments on the Effects of USL’s in Brain Homogenates**

Several factors that affect the thickness of the USL include the method of mixing and the viscosity of the solution [40, 41]. The range of the estimated USL thickness at the neuronal membrane was from \(12 \pm 2 \mu m\) to \(21 \pm 2 \mu m\), a dimension larger than nerve endings and smaller than some observed inclusions. DA has been shown to be impermeant to lipid layers (pure lipid bilayers and dioleoyl phosphatidylcholine/cholesterol bilayers [42, 43]). Because the catecholamine neurotransmitters are not soluble in lipid, their path during uptake or release requires traversing an USL and then association with transmembrane biological machinery to mediate movement across the membrane. With DA as an example, it has been shown that DA transport in the striatal suspensions is completely blocked by 100 µM cocaine [44] suggesting that catecholamine neurotransmitters cannot permeate membranes without the aid of its
transporter. The timing of diffusion of DA across our observed range of USL thicknesses from 4.5 µm to 21 µm is 30 ms to 750 ms. In using the rate of uptake of DA, mediated by its transporter, it was found that the measured initial rate of transporter activity is unaffected by variation of USL thickness and mass transport across it [38]. Thus, the RDE kinetically resolves events of transmembrane transport, the USL thickness is not rate limiting, and membrane permeability is not in evidence. Comparisons of the RDE technique to other methods [45 - 56] to kinetically resolve DA transport were made and are listed in Table 5. Thus, it appears that the RDE microcell technique makes measurements $10^3$ to $10^4$ times more rapidly than the other techniques found in the literature and the limit of the timing of chemical fluxes at membranes, that could be measured by the RDE, is ca. 30 ms.

ACKNOWLEDGEMENTS

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REFERENCES


Fig. 1. A schematic diagram of the solution path and different boundary layers at the surface of a RDE. Panel A illustrates conditions of laminar flow. It shows fluid from a field perpendicular to the electrode surface and the radial paths of fluid flow across the disk surface. Panel B shows the boundary layers at the electrode surface, and the present particles may spin within the layers. Key: δ is the thickness of the diffusion boundary layer. δ₀ is the thickness of the hydrodynamic boundary layer.
**Fig. 2.** Slopes of Levich plots ($i_L$ vs. $\omega^{1/2}$) between $\omega^{1/2} = 10.2 - 30.7$ for 1.0 µM DA in the presence and absence of particles. The Levich plots (not shown) were linear with linear regression $p$ values $\geq 99.9\%$. The bar heights represent the average value ± standard error of regression (SER), $n = 4$. The asterisks represent slopes statistically different from those in open solution (plain physiological buffer) at $p \geq 96\%$ via a $z$ test. The following number and sizes of inert particles had no statistical differences from the open solution: 1.0 and 10.0 mg of 40-300 µm of Sephadex™ (data not shown), and 2X diluted liposomes.
Fig. 3. Plots of the δ thickness at the RDE at different rotation rates in the presence of different numbers of inert particles. The δ thickness from Eq.4 at the RDE in the presence of: A) 1.0 and 10.0 mg of 3 µm silica, B) 1.0 mg and 10.0 mg of 10 µm silica, C) 1.0 mg and 10.0 mg of 100 - 300 µm Sephadex™, and D) 2X diluted and concentrated liposomes. The results of A through D are statistically different from the results in open solution. Data points represent average values ± SD, n = 4.
Fig. 4. Slopes of the calibration curves (sensitivity) for DA in the range from 0.25 to 1.18 µM in the presence and absence of particles. The striatal suspension contains no inert particles. The calibration curves were linear with linear regression p values ≥ 99.9%. Bar heights represent average values ± SER, n = 4. Asterisks represent slopes significantly different from open solution with p values ≥ 96% via z test. The following number and sizes of inert particles had no statistical differences from open solution: 1.0 mg 10 µm silica, 1.0 mg 100 – 300 µm Sephadex™ (data not shown), and 2X diluted liposomes.
**Fig. 5.** Oscilloscope traces for depolarization (15mM $K^+$) stimulated and uptake of DA by the DAT. Panel A: 15 mM KCl was added to a striatal suspension. The inset shows the time between $K^+$ addition and the time of the initial observation of release. Panel B: In separate experiments 1.0 µM DA was added to a striatal suspension. The addition of 1.0 µM DA was at 7.06 s and the first initial observation of uptake was at 7.90 s.
Table 1
Calculated $\delta_0$ and $\delta$ thickness at different rotation rates

<table>
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<th>$\omega^{1/2}$</th>
<th>$\delta_0$ (µm)</th>
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<tr>
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<td>97.7</td>
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</tr>
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Note: The results were calculated from Eqs. 2 and 3 where $D = 0.61 \pm 0.03 \times 10^{-5}$ cm$^2$/s [35], $\nu$ = 0.01 cm$^2$/s and values of $\omega$ ranged from 1000 to 9000 rpm.
Table 2

Apparent D of DA and the ζ in the presence of inert particles

<table>
<thead>
<tr>
<th>Chromamperometric Measurement</th>
<th>Physiological Buffer</th>
<th>3 μm Silica</th>
<th>40-120 μm Sephadex™</th>
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<td>Avg slope (nA/μM/s)²</td>
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<td>D (cm²/g × 10⁻⁸)</td>
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<td>ζ</td>
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<th>RDE Measurement</th>
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<th>10.0 mg 3 μm Silica</th>
<th>1.0 mg 40-120 μm Sephadex™</th>
<th>10.0 mg 40-120 μm Sephadex™</th>
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<tbody>
<tr>
<td>Avg slope (nA/μM/s/mg/μM/s)²</td>
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<td>D (cm²/g × 10⁻⁸)</td>
<td>0.37 ± 0.04</td>
<td>0.31 ± 0.03 *</td>
<td>0.19 ± 0.01 *</td>
<td>0.40 ± 0.06 *</td>
<td>0.54 ± 0.02 *</td>
</tr>
<tr>
<td>ζ</td>
<td>0.55 ± 0.03</td>
<td>0.52 ± 0.10</td>
<td>1.08 ± 0.09</td>
<td>0.91 ± 0.05</td>
<td></td>
</tr>
</tbody>
</table>

Note: The results for the slope and ζ are presented as average value ± SER with n = 4. The D is presented as average value ± SE with n=4, except for the physiological buffer with n = 3. The asterisks (*) represent statistical differences from the physiological buffer at a p ≤ 0.05 via a t-test. The electrochemical area is A = 0.090 ± 0.004 cm² via chronamperometry in 25.0 μM K₃Fe(CN)₆.

* The slope of the plot of i vs. t is \( \frac{d[i]}{dt} \)

* The slope of the plot of i vs. \( \alpha \) is \( \frac{d[i]}{d[\alpha]} \),
<table>
<thead>
<tr>
<th></th>
<th>Rotation Rate</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>2000 rpm</td>
<td>6000 rpm</td>
</tr>
<tr>
<td></td>
<td>Slope</td>
<td>Slope</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(nA/µM)</td>
<td>(nA/µM)</td>
<td></td>
</tr>
<tr>
<td>Theoretical</td>
<td>114 ± 8</td>
<td>197 ± 13</td>
<td></td>
</tr>
<tr>
<td>Physiological Buffer</td>
<td>138 ± 9</td>
<td>222 ± 11</td>
<td></td>
</tr>
<tr>
<td>10.0 mg 3 µm Silica</td>
<td>72.8 ± 6.3 *</td>
<td>128 ± 7 *</td>
<td></td>
</tr>
<tr>
<td>10.0 mg 40 - 120 µm Sephadex™</td>
<td>100 ± 4.6 *</td>
<td>203 ± 16</td>
<td></td>
</tr>
</tbody>
</table>

Note: The result in each category is presented as average value ± SER, n = 4. The asterisks represent statistical differences from the physiological buffer at a p ≥ 96% via a z test. 10.0 mg 40 – 120 µm Sephadex™ has no statistical difference from the physiological buffer at the rotation rate of 6000 rpm.
### Table 4
Experimental response times for DA detection with different types and sizes of suspended particles

<table>
<thead>
<tr>
<th></th>
<th>Time (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instrument response time</td>
<td>40</td>
</tr>
<tr>
<td>Mixing time</td>
<td></td>
</tr>
<tr>
<td>Physiological buffer</td>
<td>130 ± 9</td>
</tr>
<tr>
<td>1.0 mg 3 µm silica</td>
<td>80 ± 3 *</td>
</tr>
<tr>
<td>10.0 mg 100 – 300 µm Sephadex™</td>
<td>70 ± 7 *</td>
</tr>
</tbody>
</table>

Note: The result in each category is presented as average value ± SD with n = 6. The asterisks represent statistical differences from the physiological buffer at a p ≥ 96 % via a z test.
### Table 5
Comparison of literature values of initial rates of DA release from rat striatal preparations to those estimated by the RDE microcell technique

<table>
<thead>
<tr>
<th>Striatal Preparation</th>
<th>Temp (°C)</th>
<th>Method of Kinetic Measurement</th>
<th>Stimulus (mM KCl)</th>
<th>Initial Time Point</th>
<th>Measurement Intervals</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slices</td>
<td>37</td>
<td>DA Superfusion</td>
<td>20</td>
<td>3 min</td>
<td>3 min</td>
<td>46</td>
</tr>
<tr>
<td>Slices</td>
<td>37</td>
<td>DA Superfusion</td>
<td>60</td>
<td>5 min</td>
<td>5 min</td>
<td>47</td>
</tr>
<tr>
<td>Minces</td>
<td>37</td>
<td>DA Superfusion</td>
<td>60</td>
<td>30 sec</td>
<td>1 min</td>
<td>48</td>
</tr>
<tr>
<td>Minces</td>
<td>37</td>
<td>DA Superfusion</td>
<td>50</td>
<td>2 min</td>
<td>2 min</td>
<td>49</td>
</tr>
<tr>
<td>Minces</td>
<td>37</td>
<td>³H-DA Superfusion</td>
<td>50</td>
<td>2 min</td>
<td>2 min</td>
<td>49</td>
</tr>
<tr>
<td>Minces</td>
<td>37</td>
<td>DA Static Incubation</td>
<td>22</td>
<td>20 min</td>
<td>20 min</td>
<td>50</td>
</tr>
<tr>
<td>Minces</td>
<td>37</td>
<td>DA Static Incubation</td>
<td>25</td>
<td>20 min</td>
<td>-</td>
<td>51</td>
</tr>
<tr>
<td>Minces</td>
<td>37</td>
<td>DA Vacuum Filtration</td>
<td>40</td>
<td>1 min</td>
<td>1 min</td>
<td>52</td>
</tr>
<tr>
<td>Minces</td>
<td>37</td>
<td>DA Vacuum Filtration</td>
<td>30</td>
<td>2 sec</td>
<td>2 sec</td>
<td>53</td>
</tr>
<tr>
<td>Minces</td>
<td>37</td>
<td>DA Filtration Tank</td>
<td>75</td>
<td>1.2 sec</td>
<td>1.2 sec</td>
<td>54</td>
</tr>
<tr>
<td>Minces</td>
<td>30</td>
<td>DA Superfusion</td>
<td>75</td>
<td>6 sec</td>
<td>6 sec</td>
<td>54</td>
</tr>
<tr>
<td>Minces</td>
<td>23</td>
<td>DA Superfusion</td>
<td>40</td>
<td>1 sec</td>
<td>-</td>
<td>55</td>
</tr>
<tr>
<td>Minces</td>
<td>37</td>
<td>DA Superfusion</td>
<td>30</td>
<td>1 sec</td>
<td>1 sec</td>
<td>56</td>
</tr>
<tr>
<td>Synaptosomes</td>
<td>30</td>
<td>DA Superfusion</td>
<td>30</td>
<td>1 sec</td>
<td>1 sec</td>
<td>Present Study</td>
</tr>
<tr>
<td>Synaptosomes</td>
<td>30</td>
<td>DA Filtration Tank</td>
<td>30</td>
<td>1 sec</td>
<td>1 sec</td>
<td>Present Study</td>
</tr>
<tr>
<td>Synaptosomes</td>
<td>37</td>
<td>DA Superfusion</td>
<td>15</td>
<td>0.18 sec</td>
<td>0.18 sec</td>
<td>Present Study</td>
</tr>
<tr>
<td>Synaptosomes</td>
<td>37</td>
<td>RDE Microcell</td>
<td>15</td>
<td>0.18 sec</td>
<td>0.18 sec</td>
<td>Present Study</td>
</tr>
</tbody>
</table>

Note: This is not intended to be a complete list of studies of DA release rate since the raw data used for the comparisons were not available in some literature source. (Adapted and modified from Dr. James McElvain’s dissertation [45]).
CHAPTER 3

KINETICS AND INHIBITION OF THE DEGLYCOSYLATED DOPAMINE TRANSPORTER

ABSTRACT

Dopamine (DA) is a catecholamine neurotransmitter that plays a role in reward, reinforcement, motivation pathways, and addiction. The dopamine transporter (DAT) is a single gene product exhibiting different glycosylation states in different areas of the brain, and the binding of cocaine to the DAT is glycosylation-dependent. Although it is known that glycosylation affects the apparent molecular weight of the DAT, the connection between carbohydrate moieties and function of the DAT is unknown. Herein we investigate the effects of carbohydrate component modification on DA transport velocity by using different glycosidases. Results show that DA transport activities decrease after neuraminidase, α-mannosidase, or N-glycanase treatments in the striatum. α-Mannosidase, which has the rapid access to its substrate, was chosen to further investigate the effects of deglycosylation of the DAT on the DA kinetics and examined whether cocaine, mazindol, bupropion, methamphetamine, tyramine, and β-phenylethylamine inhibit at a glycosylated site. Results show the following: 1) the $V_{\text{max}}$ of DAT decreased while the $K_{\text{m}}$ was unchanged after α-mannosidase treatment in both control and cocaine-treated samples, 2) both the $V_{\text{max}}$ and $K_{\text{m}}$ of the DAT increased after cocaine treatment, 3) α-mannosidase treatment abolished the cocaine effect on the DAT, 4) cocaine, bupropion, mazindol, and β-phenylethylamine block the effects of α-mannosidase, while tyramine and methamphetamine have no effects on blocking the α-mannosidase effect. Biochemically, it
seems that mazindol can be a possible candidate for the antagonist for cocaine on the DAT. However, mazindol treatment causes high cardiovascular risk in cocaine-dependent human studies by other researchers.

INTRODUCTION

Dopamine (DA) is a catecholamine neurotransmitter synthesized in the presynaptic terminal. DA plays an important role in fine motor skills, reward, reinforcement, and motivation pathways along with the dopaminergic affects of many drugs of abuse within the central nervous system (Purves et al., 2008). DA is presents in several areas of the brain with particularly high density in the corpus striatum (Purves et al., 2008). Researchers found that synaptic plasticity in the ventral striatum plays an important role in early stage of drug use, whereas synaptic plasticity in the dorsal striatum is likely to be involved in the acquisition of behavioral habits, which is associated with the advanced stages of drug addiction (Berke and Hyman, 2000; Everitt and Robbins, 2005; Everitt and Wolf, 2002; Gerdeman et al., 2003; Hyman and Melenka, 2001; Koob and Le Moal, 2001). Dopaminergic neurotransmission is regulated by the transmembrane protein called the dopamine transporter (DAT) (Nestler, 2001).

The Na\(^{+}\) and Cl\(^{-}\) dependent neuronal transmembrane DAT is a 60 kDa protein, a single gene product, made up of 619 amino acids, with an additional 20 kDa associated with glycosylation (Giros et al., 1992). The DAT contains 12 transmembrane regions connected by alternating intracellular and extracellular loops with N and COOH terminals within the cytoplasm (Kilty et al., 1991; Shimada et al., 1991; Volz and Schenk, 2005). In addition, the DAT is a heavily glycosylated protein at extracellular loop number two, E2. Human DAT contains three potential glycosylation sites at Asn\(^{181}\), Asn\(^{188}\), and Asn\(^{205}\) (Vandenbergh et al.,
1992; Volz and Schenk, 2005), whereas the DAT in rats contains four potential glycosylation sites at Asn\(^{181}\), Asn\(^{188}\), Asn\(^{196}\), and Asn\(^{204}\) (Surratt et al., 1993; Volz and Schenk, 2005). The apparent molecular weights of the DAT are different between anatomical areas due to different carbohydrate structures (Lew et al., 1991b, 1992).

Glycosylation links oligosaccharides to proteins by either a nitrogen atom or an oxygen atom to form a set of glycoforms which share an identical polypeptide chain but differ in oligosaccharide structures. Glycosylation stabilizes protein conformation, regulates intracellular and surface trafficking, controls protein folding, and protects against proteolysis (Li et al., 2004; Lis and Sharon, 1993). In addition, glycosylation also plays an important role on the DAT because DA cannot be transported by deglycosylated DAT (Meiergerd and Schenk, 1994a; Zaleska and Erecinska, 1987). It has also been shown that cocaine binding to the DAT is glycosylation-dependent (Cao et al., 1989; Li et al., 2004).

Glycosylation affects the apparent molecular weight of the DAT, and modifications in glycosylation are proposed to abolish the uptake function of the DAT (Bjorklund et al., 2008; Li et al., 2004; Meiergerd and Schenk, 1994a; Patel et al., 1993) as well as cocaine binding (Cao et al., 1989; Li et al., 2004). However, relationships between carbohydrate moieties and DAT function are unknown (Lew et al., 1992).

The DA uptake process can be inhibited by DAT inhibitors. These DAT inhibitors can be classified as either structurally similar substrate analogs or those strongly structurally dissimilar from DA. The structure of cocaine, bupropion, and mazindol are dissimilar from DA, in which they only inhibit the DAT but are not transported by the DAT. Tyramine, methamphetamine (METH), and β-phenylethylamine (β-PEA) are DA substrate analogs and competitive inhibitors that compete with DA to be transported by the DAT. Cocaine reduces the kinetic turnover of the
DAT by inhibiting the translocation step, not the binding, of DA into the presynaptic neuron from the post-synapse, resulting in a high concentration of DA remaining in the synaptic cleft (Gold, 1993; Meiergerd and Schenk, 1994a; Xu et al., 1995). This inhibition increases the activation of the DA receptors and causes euphoria. Bupropion, an antidepressant, is a DA reuptake inhibitor (Dhillon et al., 2008). It weakly inhibits DA uptake in order to extend the concentration and time DA remains in the synaptic cleft (Damaj et al., 2004; Stahl et al., 2004). Mazindol has been considered as an appetite suppressant and a DA reuptake inhibitor (Eshleman et al., 1994). It binds at a different site from cocaine on the DAT, but it competitively inhibits the inward transport of DA (Eshleman et al., 1994; Meiergerd and Schenk, 1994a; Wayment et al., 1998). Mazindol has been considered as a potential therapeutic drug for cocaine treatment (Chait et al., 1987; Li et al., 2006; Lima et al., 2002; Stine et al., 1995). Tyramine is a cosubstrate inhibitor of DA and competitively inhibits DA transport (Appell et al., 2004; Horn, 1979; Meiergerd and Schenk, 1994b). The amphetamines, of which METH is an example, are also DA substrate analogs. They compete with DA for binding sites and get transported into the presynaptic neuron by the DAT (Bjorklund et al., 2008; Jones et al., 1999; Wayment et al., 1998). Once METH enters into the cytosol, it then releases DA from vesicles, and DA transports into the synaptic cleft via DAT. High concentrations of DA in the synaptic cleft will increase the activation of DA receptors (Volkow, 2006). β-PEA is structurally similar to DA as well as the amphetamine (Janssen et al., 1999; Paterson et al., 1990; Saavedra, 1989; Sotnikova et al., 2004). In addition, β-PEA is an endogenous amine that is found in the nigrostriatal and mesolimbic regions of the mammalian brain (Paterson et al., 1990; Sotnikova et al., 2004). It has been known that β-PEA can stimulate the efflux of DA and inhibits DA uptake, similar to the stimulating
action of amphetamine (Bailey et al., 1987; Dyck, 1983; Horn and Snyder, 1972; Raiteri et al., 1977; Sotnikova et al., 2004).

In order to investigate the function of the glycosylation and the blocking of the inhibitors on the DAT after deglycosylation in the striatum, several studies were performed here. In the first study, four glycosidases were used to remove the carbohydrate components in order to examine the changes in the DA uptake velocities after each glycosidase treatment. Neuraminidase was used to remove the sialic acid. α-Mannosidase was used to remove mannose sugar residues which are α(1→2,3,6). N-glycanase was used to remove carbohydrate structure from the polypeptide at nitrogen groups on Asn residues and O-glycosidase was used to remove the oxygen groups on Ser/Thr residues. For the rest of the studies, only α-mannosidase was used because as an exoglycosidase, it has rapid access to its substrate, α-mannose, thereby leaving some glycosylation on the DAT. In the second study, the effects of carbohydrate components modification on DA kinetic activity (both uptake and affinity) at the DAT were determined after deglycosylation. In addition, low cocaine concentrations, which partially inhibited the DAT, were used to examine the effects of the inhibition of cocaine to the DAT after the modifications of carbohydrate components. In the third study, high concentrations of inhibitors that are structurally dissimilar from DA (cocaine, bupropion, and mazindol) and DA substrate analogs (tyramine, METH, and β-PEA) are used to completely saturated the DAT before the deglycosylation of the DAT to determine whether these inhibitors block the effect of α-mannosidase on the glycosylation site. These studies enabled us to further understand the influence of the glycosylation on the function of the DAT, the cocaine-DAT interactions at a glycosylated site, and examined whether inhibitor-DAT interacted at a glycosylated site.
MATERIALS AND METHODS

Measurement of DA Uptake

The uptake of DA was measured using rotating disk electrode (RDE) voltammetry, set at 2000 rpm, as described previously (Chiu et al., 2011; Earles et al., 1998). Briefly, the 3-mm diameter glassy carbon electrode and MSRX precision rotator were from Pine Instrument Inc. (Grove City, PA). An LC-4C potentiostat from Bioanalytical System, Inc. (West Lafayette, IN) was used to apply a potential vs. a AgCl coated Ag wire reference electrode sufficient to detect the oxidation of DA but not the inhibitors such as tyramine. A Pt wire was used as an auxiliary electrode. Currents were recorded with a Nicolet 310 digital oscilloscope (Nicolet Instrument Corp., Madison, WI) at 50 ms resolution. The custom-made (details upon request) Pyrex glass incubation chamber, which holds a volume of 500 µL physiological buffer, was maintained at 37°C by a VWR Scientific 1136 water circulator (Radnor, PA). A stream of 95% O₂/5% CO₂ gas mixture was gently directed across the surface of the physiological buffer at pH 7.4 [containing in (mM): 2.50 CaCl₂, 1.80 KCl, 1.24 KH₂PO₄, 1.40 MgSO₄, 124 NaCl, 26.0 NaHCO₃, and 10.0 glucose] in the incubation chamber.

General Procedure for Tissue Preparation

The tissue preparation was described previously (McElvain and Schenk, 1992) and Fig. 1 summarizes the experimental sequences for the studies described below. A drug free and nonanesthetized rat was rapidly decapitated, and the brain was divided into two hemispheres midsagittally. The striatum (sample weight 35 – 40 mg) was dissected at stereotaxic coordinates of about 3.6 mm anteriorly according to Pellegrino et al. (1990) and was weighed. The striatal sample was then chopped with a razor blade on an ice-cold watch glass and transferred into
physiological buffer in the incubation chamber. The striatum was then disrupted by pipetting and incubated for 20 min. Subsequently, the striatal suspension was washed eight times with fresh physiological buffer (McElvain and Schenk, 1992).

Kinetics of DA transport after glycosidase and cocaine treatments

In these studies, a glycosidase was added to the washed striatal suspension (see Sequence 1 in Fig.1). The amount of glycosidase added and the incubation time were optimized so that some of the DAT was still able to transport DA, and the kinetics of DA transport can be measured. This was done in a series of experiments where glycosidase activity was altered and/or the incubation time varied. The incubation time of an experimentally convenient time range of 5 – 15 min at 37°C with each glycosidase activity varied over 0.05 – 0.1 U. The RDE was then lowered into the incubation chamber. A final concentration of 1.0 µM of DA was added to the striatal suspension, and DA uptake velocity was measured. Control samples were homogenates with no glycosidase added.

Studies have shown that cocaine binding to the DAT is glycosylation-dependent (Cao et al., 1989; Li et al., 2004). Therefore, in order to determine if the DA transport kinetics would be altered after both cocaine and glycosidase treatments, an experiment was done with 0.05 U of α-mannosidase (see results section on the decision on using particular glycosidase) added to the striatal suspension and incubated for 5 min. Final concentrations of 0.5, 1.0, and 1.5 µM cocaine, respectively, were added to the striatal suspension and incubated for 30 s. After the incubation, sequential additions of DA were added every 20 s to the striatal suspension with concentrations of 0.25 – 11.6 µM. Control samples were homogenates with no glycosidase or cocaine added.
**Enzyme Assay**

In initial control studies, α-mannosidase activities in the presence and absence of the inhibitors were measured to verify that the presence of inhibitors did not affect enzyme activities directly. The α-mannosidase assay was performed according to the manufacture’s protocol (Sigma-Aldrich, St. Louis, MO). Briefly, a volume of 125 µL of 10 mM p-nitrophenyl-α-D-mannoside enzyme substrate and 125 µL of 0.1 M citrate buffer, pH 4.5 at 37°C were added to an Eppendorf tube and equilibrated to 37°C. Then, 50 µL of 0.05 U of α-mannosidase was transferred to the contents of the tube and incubated for 10 min at 37°C. Finally, 500 µL of 200 mM borate buffer, pH 9.8 at 37°C, was added to the solution and absorbance was measured using a Thermo Scientific Evolution 300 spectrophotometer (Waltham, MA) at 405 nm. When the test sample contained the inhibitor, the inhibitor was added to the solution 30 s before the α-mannosidase was added. Samples containing everything above except the inhibitor and the 0.05 U of α-mannosidase were the blanks. The enzyme activity was expressed in U/mL by,

\[
\frac{U}{mL} = \frac{(\Delta A_{test} - \Delta A_{blank})(0.8)(df)}{(10)(18.5)(0.050)}
\]

Eq. 1

where \(\Delta A_{test}\) is the absorbance of the sample, \(\Delta A_{blank}\) is the absorbance of the blank, 0.8 mL is the total volume of solution, df is the dilution factor, which is 2632 in this study, 10 min is the time of assay, 18.5 M\(^{-1}\)cm\(^{-1}\) is the \(\varepsilon\) of p-nitrophenol at 405 nm, and 0.050 mL is the volume of enzyme added. The data with and without inhibitors were compared using a paired t-test. Results in Fig. S1 show that the inhibitors have no direct effect on α-mannosidase activity.

**Study of the effects on the inhibitors at the glycosylated regions on the DAT**

To determine if the blocking of cocaine, bupropion, mazindol, tyramine, METH, or β-PEA, which are defined as inhibitors, are involved at the glycosylated regions on the DAT in the
striatum, an experiment was done so that a fixed high concentration of inhibitor was added before the addition of α-mannosidase (see Sequence 2 in Fig. 1). The following concentrations were used for each inhibitor and are based on 30- or 60- fold of the individual $K_m$ or $IC_{50}$ value: 30 µM cocaine, 33 µM bupropion, 10.2 µM mazindol, 1.02 mM tyramine, 17.5 µM METH, and 0.66 mM β-PEA. A concentration of 100 µM citalopram, which is a serotonin reuptake inhibitor, was used as a negative control. If the inhibitor blocks at the same site as a glycosidase, it may provide protection of DAT activity. In this study, after the striatal suspension was washed eight times with a physiological buffer (*vide ante*), the inhibitor was added and incubated for 30 s. Then, 0.05 U of α-mannosidase was added and incubated for 5 min. After the incubation, the striatal suspension was washed again eight times with a fresh physiological buffer, to remove the inhibitor and α-mannosidase, before 1.0 µM DA was added and transport activity was recorded. Control samples were homogenates with no inhibitor added.

**Animals**

Male Sprague-Dawley rats (350 to 450 g) were obtained from the Washington State University internal breeding colony and were housed 2 to 3 per cage in a University vivarium with a 12 hour light/dark cycle, maintained at 22 to 24 °C, and with access *ad libitum* to food and water. The animal use procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional University Animal Care and Use Committee.

**Data Analyses**

Initial DA uptake velocities were obtained by measuring the slope of a tangent line ([DA] vs. t) in which less than 10% of the DA concentration was transported (Meiergerd and Schenk,
The slope of the tangent line was obtained from linear regression using Graphpad Prism 5 (GraphPad Software, San Diego, CA), with units expressed in ρmol/s/ g wet weight tissue. The results were further analyzed to obtain values of $K_m$ and $V_{max}$ by fitting velocity vs. [DA] using the Michaelis-Menten equation,

$$v = \frac{V_{max}[DA]}{K_m + [DA]} \quad \text{Eq. 2}$$

where $V_{max}$ is the maximum velocity of DA uptake in ρmol/s/ g wet weight tissue, and $K_m$ is the Michaelis constant in µM. The values of $K_m$ and $V_{max}$ of DA transport after the α-mannosidase treatments at different cocaine concentrations were compared with the control using a z-test, with difference set at $p \geq 96\%$.

**Chemicals and Enzymes**

The buffer salts and glucose were purchased from Fisher Scientific (Fair Lawn, NJ) and/or JT Baker (Phillipsburg, NJ). DA hydrochloride, (-)-cocaine hydrochloride, (+)-METH hydrochloride, tyramine hydrochloride, mazindol, β-PEA hydrochloride, citalopram, and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO), and bupropion was purchased from Research Biochemicals Inc. (Natick, MA). Stock solutions were made in purified water from a Barnstead Nanopure water purification system (Dubuque, IA). Mazindol was made in DMSO, and the control samples have no statistical differences between the presence and absence of DMSO (data not shown). The glycosidases were purchased from Sigma-Aldrich (St. Louis, MO): neuraminidase (E.C. 3.2.1.18) type II from *Vibrio cholerae*, α-mannosidase (E.C. 3.2.1.24) from *Canavalia ensiformis* (Jack bean), PNGase F (E.C. 3.5.1.52) from *Chryseobacterium (Flavobacterium) meningoseptica*, and O-glycosidase (E.C. 3.2.1.97) from *Streptococcus pneumoniae*. 
RESULTS

Effects of Glycosidases on the Activity of DAT

In order to remove oligosaccharide moieties from DAT, the striatal suspensions in separate studies were treated with neuraminidase, α-mannosidase, N-glycanase, or O-glycosidase. Fig. 2 shows the results after the striatal suspensions were treated with different glycosidases. The DA uptake velocities are decreased by 47%, 32%, and 35%, respectively, after neuraminidase, α-mannosidase, and N-glycanase treatments in separate experiments. However, the DA uptake velocity is not statistically different from the control after O-glycosidase treatment.

Kinetics of the DA Transport after Deglycosylation

Our results in Fig. 2 show the presence of α-mannose in the DAT, which differs from SDS PAGE and western blot results (Bjorklund et al., 2008; Lew et al., 1991a). Our results also show that DA uptake velocities significantly decrease after the α-mannosidase treatment. The activity of α-mannosidase used in the study was only half of the activity of neuraminidase or N-glycanase. Based on this observation, α-mannosidase may have rapid access to the substrate on the glycosylation site. Therefore, further investigation on how the mannose in the DAT will affect the kinetics of DA transport and cocaine inhibition of DAT would be necessary. Results of $K_m$ and $V_{max}$ values of the DA transport in Fig. 3 were obtained from the nonlinear best-fit of the Michaelis-Menten equation (Eq. 2). The results of Fig. 3A show that the $K_m$ values of DA transport are not altered by α-mannosidase treatment. The $K_m$ values of DA transport in the presence of cocaine increased as a function of cocaine concentrations. However, there are no statistical differences between the treatments of cocaine only and cocaine with α-mannosidase.
Fig. 3B shows that α-mannosidase treatment reduces $V_{\text{max}}$. As cocaine concentrations increase, the $V_{\text{max}}$ values of DA transport are increased. Compared within the same cocaine concentration, $V_{\text{max}}$ values of DA transport decreased after α-mannosidase treatment.

Fig. 4 shows the results of further analysis of the data in Fig. 3. In the absence of α-mannosidase treatments, the $V_{\text{max}}/K_m$ ratio decreases as the cocaine concentration increases, and the slope is statistically different from zero. After the striatal suspension was treated with α-mannosidase, α-mannosidase blocks this effect.

**Shielding of DAT Deglycosylation by DAT Inhibitors**

Fig. 5 shows that cocaine, bupropion, and mazindol, which are structurally dissimilar from DA, block the effects of α-mannosidase. The effect of α-mannosidase on the DAT is decreased by more than 2-fold in the presence of cocaine, bupropion, or mazindol, with mazindol being the most effective to block the removal of α-mannose on the DAT by α-mannosidase. Tyramine and METH, which are DA substrate analogs, have no effects on deglycosylation of DAT by α-mannosidase. Although β-PEA is a DA substrate analog, the effect of α-mannosidase is attenuated by about 1.5-fold in the presence of β-PEA. Citalopram has no effects on deglycosylation of DAT by α-mannosidase.

**DISCUSSION**

This study determined the function of the glycosylation on the DAT and the interaction between the DAT inhibitors and the glycosylated site on the DAT. Results show 1) DA uptake velocities decrease after different glycosidase treatments, except for O-glycosidase, 2) the $V_{\text{max}}$ of DA uptake decreases, while $K_m$ undergoes no change after α-mannosidase treatment in both
control and cocaine-treated samples, 3) the effect of cocaine on the DAT is eliminated after the α-mannosidase treatment, and 4) high concentrations of cocaine, mazindol, bupropion, and β-PEA decrease the effect of α-mannosidase on the DAT, while METH and tyramine did not affect the deglycosylation on the DAT.

Deglycosylating DAT abolishes its transport activity (Cao et al., 1989; Meiergerd and Schenk, 1994a). In this study, exoglycosidase and endoglycosidase were used to verify that the function of the DAT is glycosylation-dependent. In endoglycosidase treatments, although both N-glycanase and O-glycosidase incubated with the same amount of activity and time in separate experiments, DA transport velocity decreases after N-glycanase treatment but undergoes no change after the O-glycosidase treatment, indicating that the DAT is N-linked. Although some of the glycoproteins in a striatum may be O-linked, results show that these deglycosylated glycoproteins do not affect DA transport velocity. Lew et al. (1991a) showed that the glycosylation of the DAT is N-linked, based on the study of N-glycanase treatment on $[^{125}\text{I}]1$-[2-(diphenylmethoxy)ethyl]-4-[2-(4-azido-3-iodophenyl)ethyl]piperazine ($[^{125}\text{I}]$DEEP) photolabeled DAT. In addition, based on the amino acid sequence of DAT, the possible glycosylation sites are located at either Asn-X-Ser or Asn-X-Thr (X is any amino acid except Pro), which also illustrates that it is an N-linked oligosaccharide.

In the exoglycosidase treatments, DA transport velocity has been shown to decrease after the neuraminidase treatment (Meiergerd and Schenk, 1994a; Zaleska and Erecinska, 1987). Lew et al. (1991a), along with our group (Bjorklund et al., 2008), have used the SDS PAGE and western blot, respectively, to study the effect of α-mannosidase on the apparent molecular weight of the DAT in striatum. Both SDS PAGE and western blot results show that the molecular weight of the DAT does not change after the α-mannosidase treatment, which indicates that no α-
mannose is present on the DAT. However, there is a possibility that SDS PAGE and western blot have insufficient resolution to define small changes on the DAT after α-mannosidase treatment. In this study, our results show that DA uptake velocities significantly decrease after the α-mannosidase treatment. It is possible that although a small amount of mannose sugar residues, which are in α(1→2,3,6) linkages, are present at the glycosylation sites on the DAT, they are located at the outer part of the glycosylation motif and play an important role in the DA transport. In addition, RDE seems to be a more sensitive method to detect the changes of carbohydrate moieties in the DAT than SDS PAGE and western blot.

After the α-mannosidase treatment, the DAT reduces the ability to transport DA but has no effect on the $K_m$. The same trend was shown in the result from Zaleska and Erečińska (1987), with neuraminidase as a glycosidase to remove sialic acid. In addition, cocaine loses its ability to block DAT after α-mannosidase treatment. Cao et al. (1989) had a similar result that when lectins were incubated with the striatal synaptosomes, both $[^3H]c$ocaine binding and the $[^3H]DA$ uptake increased. Their results showed that the lectin-DAT complex had a larger $V_{max}$ while the $K_m$ for DA to DAT was unchanged. This shows that both DA uptake and cocaine binding depend on the carbohydrates on the proteins. Another interesting result is that both the $V_{max}$ and the $K_m$ increase as the cocaine concentration increases with sequential addition of DA. This seems to contradict a statement that cocaine reduces the kinetic turnover of the DAT by inhibiting the translocation step and thus blocking DA transport (Carroll et al, 1992; McElvain and Schenk, 1992; Meiergerd and Schenk, 1994b; Meiergerd et al., 1994c; Riddle, 2005). Protein kinase A (Batchelor and Schenk, 1998) and membrane trafficking may be the cause of the increase of both $V_{max}$ and $K_m$ as the cocaine concentration increases with sequential addition of DA. Further investigation of this phenomenon is needed. Devés and Krupka (1987) have shown how
imidazole as a choline transport inhibitor affects choline transport. In that study, imidazole either inhibits, activates, or has no effect on the choline transporter depending on the choline concentration. Results show that at low concentrations of imidazole, choline transport is activated and the uptake velocity increases. At sufficiently high concentrations of imidazole, inhibition dominates. Zahniser et al. (1999) performed an experiment on DA clearance rates in rat striatum \textit{in vivo} with sequential addition of DA. Results show that the $V_{\text{max}}$ increases as the cocaine concentration increases and is due to the high uptake rates of the uninhibited DAT in response to the high DA concentrations.

Further analyses from the results in Fig. 3 show that cocaine affects the kinetic state of the DAT but the deglycosylation of the DAT removes the effect of cocaine on the DAT. Therefore the results suggest that cocaine binding to the DAT is glycosylation-dependent.

Our results show that mazindol is more effective than cocaine in blocking the removal of $\alpha$-mannose on the glycosylation site of the DAT by $\alpha$-mannosidase. Different studies have shown that mazindol has higher inhibition potency than cocaine on the DAT (Andersen, 1989; Javitch et al., 1984; Meiergerd and Schenk, 1994b; Parker and Cubeddu, 1988), which shows the same trend as our results. Since cocaine binding to the DAT is glycosylation-dependent and both mazindol and cocaine block at the same site with mazindol being more effective on the inhibition, mazindol can be a potential therapeutic drug for cocaine treatment. Biochemically, mazindol and its analogs have been found to be the potential inhibitor for the binding of cocaine at the DAT (Houlihan et al., 1998; Houlihan et al., 2002; Rothman, 1990). In addition, several researchers have tried to use mazindol to treat cocaine-dependent patients in their clinical studies (Chait et al., 1987; Malison et al., 1998; Preston et al., 1993; Stine et al., 1995). However, in human studies, low doses ($\leq 2$ mg/day) of mazindol do not attenuate the magnitude of the effect.
of cocaine (Malison et al., 1998; Preston et al., 1993; Stine et al., 1995). Malison et al. (1998) suggested that ≥30 mg/day is required to reduce about 50% of the euphoric effect of cocaine in humans. Yet, even a low dose of mazindol has high cardiovascular risk in cocaine-dependent patients (Preston et al., 1993). Bupropion is a DA reuptake inhibitor with a lower inhibition potency on DA uptake than mazindol or cocaine (Javitch et al., 1984), which matches our results that bupropion inhibits less than mazindol and cocaine at the glycosylation site of the DAT. METH, β-PEA, and tyramine are DA substrate analogs, so one may predict that these analogs would not inhibit the glycosidase binding since they would be transported by the DAT. Our previous work has shown that glycosylation of the DAT is not affected by METH exposure in either the striatum or the nucleus accumbens (Bjorklund et al., 2008). Our results here show METH has no effect on inhibition, which matches with our previous result. Tyramine is a co-substrate of DA, and the results match our prediction that it does not inhibit the glycosidase binding. β-PEA weakly inhibits the glycosidase cleavage, and it is unknown why it inhibits the cleavage, further investigation is required. Citalopram was chosen as a negative control due to the fact that it is three-orders of magnitude more selective than fluoxetine at inhibiting serotonin uptake than DA uptake (Rothman et al., 2001). As we predicted, inhibiting the serotonin transporter does not affect the deglycosylation of the DAT by α-mannosidase.

This study allows us to further understand the function of the glycosylation on the DAT and the inhibitors interactions at the glycosylated site on the DAT.

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Fig. 1. Flowchart of the experimental sequences used to determine the effects of deglycosylation on DA transport.
Fig. 2. DA transport velocities after different enzyme treatments of striatal homogenates. 0.1 U of neuraminidase, N-glycanase, or O-glycosidase were used and incubated for 15 min. For α-mannosidase, 0.05 U was used and incubated for 5 min. The error bars represent average value ± standard error of regression (SER), n = 4. The asterisks represent statistical differences from the controls at ≥ 96 % confidence level via a z test.
Fig. 3. $K_m$ and $V_{max}$ values of DA transport after the 0.05 U $\alpha$-mannosidase treatment at different concentrations of cocaine. The $K_m$ (Panel A) and the $V_{max}$ (Panel B) values of DA transport in the presence of different concentrations of cocaine and 0.05 U $\alpha$-mannosidase. The bar heights and error bars represent average values ± SER, $n = 8$. The asterisks represent statistical differences from the controls at ≥ 96 % confident level via a z test. The symbol (#) represents statistical differences from the cocaine treatment at ≥ 96 % confidence level via a z test.
Fig. 4. Plots of $\frac{V_{\text{max}}}{K_m}$ vs. [cocaine]. The points and error bars represent average values ± SER, n = 8. The slope of DA transport vs. [cocaine] in striatal suspensions without the α-mannosidase treatment is statistically different from zero by an F-test. The slope of the α-mannosidase treated striatal suspensions is not statistically different from zero. The asterisks represent statistical differences from the samples without α-mannosidase treatment at ≥ 96 % confidence level via a z test.
Fig. 5. Effects of each inhibitor on deglycosylation of the DAT by α-mannosidase. Bar heights and error bars represent average values ± SER, n = 4. The asterisks indicate statistical differences from the α-mannosidase treatment only at ≥ 96 % confidence level via a z test.
Fig. S1. Results of enzyme assays of α-mannosidase in the presence and absence of each inhibitor. The bar heights and error bars represent average value ± standard deviation, n = 3. The results show no statistical differences from each other at ≥ 96 % confidence level via a t-test. α-Mannosidase was used as the glycosidase, and p-nitrophenyl-α-D-mannoside was the substrate.
CHAPTER 4
MECHANISM OF ACTION OF METHAMPHETAMINE WITHIN THE CATECHOLAMINE AND SEROTONIN AREAS OF THE CENTRAL NERVOUS SYSTEM

ABSTRACT

Addiction to methamphetamine (METH) is thought to be mediated by dopaminergic effects in the reward pathway in the brain via the A10 dopaminergic pathway. Herein we describe an overview of the results of the basic preclinical science undertaken to provide mechanistic insights into the action of amphetamines in general and METH in particular. A brief history and demographic survey of amphetamine and METH use and abuse is given, and an overview of the relevant chemical aspects of amphetamine as they relate to neurotransmitters in general is made. A review of the methods used to study the biochemical effects of METH is outlined. Finally, a focused analysis of the kinetic mechanisms of action of the amphetamines in general and METH in particular at the transmembrane transporters and at the intracellular vesicular storage sites is made. A description of how catecholaminergic and serotonergic nerve signaling may be altered by METH is proposed. Overall, the emphasis here is on differences in effects observed between the striatal (the A9 substantia nigral dopamine pathway) and nucleus accumbens (the A10, ventral tegmental pathway) areas of the brain following acute as well as repeated dosing and withdrawal.
1. **INTRODUCTION**

METH is a highly addictive stimulant and is a derivative of amphetamine (1). It has a prolonged stimulating effect compared to other stimulants resulting in an extended duration of euphoria and an increased neurotoxicity. METH is known to harm the central nervous, cardiovascular, and pulmonary system (2). In addition, METH affects oral health and causes dermatological problems (2). This review provides an overview of the history of amphetamine and METH and recent statistics on the abuse of amphetamine and METH among 8th to 12th graders. Then, we will focus on the techniques of measuring the effects of METH on kinetics of the catecholamine and serotonin transporters (SERT). In addition, a summary of literature reports on how single or repeated administration(s) of METH affects the animal behavior, the concentrations of neurotransmitters, the levels and kinetics of the neurotransmitter transporters will be discussed. The history of amphetamine and METH from 1887 and literature reports for the last ten years will be covered here.

2. **HISTORY OF AMPHETAMINE AND METH**

In 1887, amphetamine was synthesized by the German chemist Lazar Edeleano (3). Later in 1893 and 1919, METH was synthesized from ephedrine by the Japanese pharmacologist Nagayoshi Nagai and the chemist Akira Ogata (3, 4). Fig. 1 shows both amphetamine and METH have similar chemical structures as ephedrine.
Although there has been increased use of amphetamine and METH in different countries such as the Philippines, Korea, England and Sweden (3, 5, 6), Japan and the United States have the greatest number of documented users. Before amphetamine was regulated as a controlled drug in 1950s, it was used to relieve the nasal congestion related to asthma (3, 7) and neurotic depression in the late 1930’s (8). In addition, it was used to treat other conditions such as fatigue, obesity, and hyperactivity in children (3, 9). Before amphetamine and METH were regulated, the drugs were thought to be safe and nonaddictive. Therefore, there was an increased usage of this drug by American and Japanese citizens, especially German, Japanese, and American soldiers during World War II to suppress their fatigue (3). Although the drug’s use began with people exposed through medical and military applications, it quickly spread to the mainstream population (3). In 1951, physicians prescriptions for amphetamine inhalers were required by the Food and Drug Administration’s 1959 use of the Drug Control Amendments of 1951 (3).

3. STATISTICS AND EFFECTS OF AMPHETAMINE AND METH USAGE

Amphetamine is usually used to treat attention-deficit hyperactivity disorder (ADHD). Prescribed medication to treat ADHD contains amphetamine (Adderall) or methylphenidate (Ritalin and Concerta). ADHD patients have low risk of amphetamine addiction if the prescription is followed. A survey conducted by the University of Michigan’s Institute for Social
Research, which tracked the illicit drug use among 8th, 10th, and 12th graders, shows the lifetime use of amphetamine has decreased from 15.5% in 1999 to 8.9% in 2010 (10). Amphetamine can cause health problems such as increased body temperature, blood pressure, and heart rate. In addition, it can cause serious cardiovascular complication in high dosages (11).

METH can be used to treat ADHD and sleeping disorders. However, the dosage is much lower than the abused level. The same survey previously mentioned shows that the lifetime use of METH among 8th, 10th and 12th graders in the United States has decreased from 6.5% in 1999 to 2.2% in 2010 (10). In addition, a research study shows that people who have abused METH use 3.3 g of the drug per week for an average of 12.5 years (12). Chronic METH abuse causes dental problems (known as “meth mouth”), visual and auditory hallucinations, paranoia, delusions (13), and dermatological problems such as cellulitis and abscess formation at the injection sites (2). In addition, it can cause hyperthermia and cardiovascular problems such as high blood pressure and rapid heart rate (13).

4. WHY IS IT SO EASY TO MAKE METH?

Based on the chemical structures in Fig. 1, the only difference between ephedrine and METH is an extra hydroxyl group on ephedrine. Therefore, by using the ephedrine/pseudoephedrine reduction method to remove the hydroxyl group, one can make METH from ephedrine (14). The phenylacetone (P2P) method used to be one of the methods for manufacturing METH (14). P2P and methyamine are the two main precursor chemicals used to make METH. However, the process requires a trained chemist, is time consuming, complex, and produces a low quality of product (14). P2P was classified as a Schedule II controlled substance in 1980. Since then, the ephedrine/pseudoephedrine reduction method has become more popular to make METH because
of the higher quality product and the fact that most of the chemicals needed to make METH can be found from household products such as the striking pads of matchbooks, anhydrous ammonia, lithium from lithium batteries, sodium hydroxide, and toluene from paint thinner or camping fuel (14). METH can exist as two different enantiomers (d- and l- METH) and both have similar stimulant efficacies (15).

5. DRUG REWARD PATHWAYS IN THE BRAIN

The reward pathway is responsible for making an individual feel pleasure when he or she is engaged in behaviors that are necessary for survival (16). Natural rewards such as food, water, and sex, and drug rewards like cocaine, amphetamine and METH, provide positive reinforcement so that the behavior is repeated (16). All drugs of abuse and other natural addictions (pathological overeating, pathological gambling, and sexual addiction) share a common brain reward circuitry in the brain’s limbic system (17-23). Fig. 2 shows the anatomical areas that are involved in the reward pathway.

Fig. 2. Schematic diagram of anatomical regions (in midseggital section) of the reward pathway in a rat brain. VTA stands for ventral tegmental area. (35)
This reward pathway, known as the mesocorticolimbic dopamine system, includes the ventral tegmental area (VTA) of the midbrain (a site of A10 dopaminergic cell bodies), the basal forebrain (includes the nucleus accumbens, frontal and limbic cortices, amygdale, and olfactory tubercle) (24). Other regions such as the hippocampus and hypothalamus, which have interactions with VTA and nucleus accumbens are also sometimes included (18, 19, 23-28). Recent research has found that ventral striatum plays an important role in the early stage of drug addiction, while the dorsal striatum is known to contribute to the later stages of drug addiction (19, 26, 29-33). Drugs such as cocaine, amphetamine and METH are hypothesized to cause changes in the dopaminergic drug reward pathway that lead to drug addiction (16). These psychostimulant drugs increase dopamine (DA), serotonin (5HT), and norepinephrine (NE) concentrations in the synaptic cleft.

5.1 Dopamine

Dopamine, which is a catecholamine neurotransmitter synthesized in the presynaptic terminal, plays an important role in fine motor skills, reward, reinforcement, and motivation pathways (34). There are four major dopaminergic pathways responsible for different functions: nigrostriatal, mesolimbic, mesocortical, and tuberoinfundibular (35).

Dopamine is present in several areas of the rat brain but has a highest density in the corpus striatum. Dopamine is synthesized by converting the amino acid precursor, L-tyrosine, to L-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH) (35). Then L-DOPA is converted to DA by L-aromatic amino acid decarboxylase (35). Dopamine is then loaded into synaptic vesicles via a vesicular monoamine transporter (VMAT-2) (34). Under normal conditions, DA is vesicularly released from the presynaptic neuron and then binds to
postsynaptic receptors to effect signaling. Subsequently, DA is cleared from the postsynaptic neuron and the synaptic cleft back to the presynaptic neuron for re-use by the transmembrane transporter protein, the dopamine transporter (DAT) (35). The DAT plays an important role in dopaminergic neurotransmission by removing DA from the synaptic space, thus regulating DA binding to the DA receptor (20, 36). Other catecholamine and 5HT have the same release and reuptake mechanisms as DA, but have their own transporters and vesicles transporters.

Addictive drugs such as cocaine and METH have different inhibition mechanisms on the DAT. Cocaine blocks the DAT and inhibits the DA reuptake into the presynaptic neurons from the post-synaptic neurons resulting a high concentration of DA remaining in the synaptic cleft (37). This increases the activation of the DA receptors and causes euphoria (38). Systematic treatment of METH increases the mesolimbic VTA cell body neurons response (1, 39) and decreases TH activity (40, 41). In addition, METH is a substrate analog of DA which competes with DA for binding sites and gets transported to the presynaptic neuron by the DAT (36, 42, 43). Once the METH enters into the cytosol, METH releases DA from the DA vesicle to the synaptic cleft through the DAT. In addition, METH reduces overall VMAT-2 protein which causes a further increase in concentration of DA in the cytosol, and DA forms a reactive oxygen species by auto-oxidation or monoamine oxidase (1, 44). High concentrations of DA in the synaptic cleft cause over-stimulation of the DA receptors. High concentrations of DA in the synaptic cleft cause by METH are contributed to have a greater neurotoxicity (45). Compared to the half-life of cocaine (60 - 90 min) (1, 45), the half-life of METH in humans is 12 hours, which causes a prolonged stimulating effect at the DA receptor resulting in an extended duration of euphoria (1).
5.2 Serotonin

Serotonin is a monoamine neurotransmitter synthesized in the presynaptic terminal by tryptophan hydroxylase (TPH) with the amino acid, tryptophan, as a precursor to form 5-hydroxytryptophan, and 5HT is then formed by decarboxylation (35). Serotonin is mainly found in the raphe nuclei of the pons and upper brainstem with projections to the limbic system, neostriatum, thalamus, cerebral and cerebellar cortices (35). The 5HT release and reuptake mechanisms are summarized in the DA section. The serotonin transporter (SERT) is responsible for regulating 5HT binding to the 5HT receptors by transporting the 5HT back to the presynaptic neuron from the synaptic cleft (34). METH affects the concentration of 5HT in the synaptic cleft similarly to that of DA (1). METH decreases the activity of TPH in nucleus accumbens and striatum when a single dose or multiple doses of METH is/are consumed (1, 40, 41). METH also competes with 5HT for a SERT-binding site and is transported to the presynaptic neuron. Then, METH releases the 5HT from the 5HT vesicles into the synaptic cleft (46, 47). The areas of brain affected include parietal cortex, amygdale, and hippocampus (40).

5.3 Norepinephrine

Norepinephrine is also a catecholamine neurotransmitter and is the metabolic product of DA by dopamine-β hydroxylase. Norepinephrine is present in the locus coeruleus with projections to hypothalamus and other area in the forebrain (34, 35). The NE release and uptake mechanisms are summarized in the DA section. Norepinephrine is removed from the synaptic cleft after it binds to the postsynaptic neuron by the norepinephrine transporter (NET) (34). METH is the inhibitor of NET, and increases extracellular NE in the striatum and hippocampus.
(1). METH competes with NE and gets transported into the presynaptic neuron by the NET. Then, METH forces the NE in the vesicles to be released into the synaptic cleft (46).

6. METHODS OF DETECTION

A number of analytical techniques have been used to measure the concentrations of neurotransmitters, densities of the neurotransmitter transporters, and the kinetics of neurotransmitter transport in different anatomical regions of a rat brain after amphetamine or METH treatments. High-performance liquid chromatography with electrochemical detection (HPLC-ED) has been used to measure the concentrations of neurotransmitters released after the amphetamine treatment (48). In general, a liquid chromatograph with an electrochemical detector is connected to a reverse phase column. General tissue preparations involve washing, centrifugation, and incubation steps (43, 48-50). Amphetamine or METH is then added to the tissue suspension and incubated. After the incubation, the tissue suspension is centrifuged at 4°C, and the supernatant is separated and saved to measure the amount of neurotransmitter released. Perchloric acid is then added to both the supernatant and the tissue pellets. Subsequently, homogenization and centrifugation are performed (48). The tissue extract is separated from the pellet and used to analyze the neurotransmitters content in the tissue homogenate (48). Neurotransmitter is then eluted with perchloric acid and injected into the HPLC-ED (43, 48-50). The amount of neurotransmitter of interest released and its concentration in the tissue homogenates is normalized by the original tissue weight.

HPLC-ED can also be used to measure concentrations of neurotransmitters during an in vivo microdialysis experiment (51, 52). An in vivo microdialysis experiment is used to examine the extracellular levels of neurotransmitters. After amphetamine/METH treatments, rats are implanted with guide cannule on the dural surface above the stereotaxic coordinates of striatum.
and nucleus accumbens (53). Before the experiment, each rat is anesthetized and a concentric dialysis probe is lowered through the guide cannula into the striatum or nucleus accumbens and secured by cyanoacrylic adhesive (53). The perfusion medium is flowed continuously through the probe at a constant rate (53). Timed dialysate samples are collected after the equilibration period and the samples are analyzed by HPLC-ED (51-53).

To study the effects of amphetamine or METH on the kinetics of the neurotransmitters transport, it is necessary to develop methods that can kinetically resolve transport activity. Neurotransmitter release occurs on a subsecond to second time scale, while reuptake occurs over a few seconds (42, 54-56). Investigators have used different methods to study the kinetics of the neurotransmitters transport such as \[^{3}\text{H}]\text{neurotransmitters}\) (40, 44, 57-67), and rotating disk electrode (RDE) voltammetry (36, 43, 49, 50, 68-74). \[^{3}\text{H}]\text{ labeled technique can be performed by incubating the tissue synaptosomes in assay buffer and pargyline (58). The nonspecific value of the neurotransmitter transport is determined by adding a high concentration of its inhibitor (58). In the experiment in which METH is directly added to the synaptosomes, the sample is incubated for 30 min in the presence of METH (67). Then, \[^{3}\text{H}]\text{neurotransmitter is added to initiate the assay and incubated at 37\textdegree\text{C}. A polyethylenimine soaked filter is used to filter the sample. The filter is then rapidly washed with ice-cold sucrose. Radioactivity collected on the filter is counted by liquid scintillation counter (58, 63, 67).

RDE voltammetry is another technique that can kinetically resolve transporter activity because the instrument response time (\textit{ca.} 40 ms) is faster than the neurotransmitter release and reuptake. RDE voltammetry is suitable to measure the velocity of plasmalemmal DA release (49) and uptake (74) as well as vesicular DA efflux and uptake (55) when DA is added directly to the tissue suspension. A glassy carbon electrode, AgCl coated Ag reference electrode and a Pt wire
auxillary electrode are used. A custom-made Pyrex glass incubation chamber with a volume of 500 µL of bicarbonate-based physiological buffer is thermostated at 37°C, and a stream of gas (mixture of 95% O₂/5% CO₂) is directed across the surface of the contents. A potentiostat is used to apply sufficient potential to oxidize neurotransmitter at the electrode surface, and the current is measured. Currents are recorded using a digital oscilloscope.

Tissue suspension is prepared by chopping the brain tissue on the ice-cold watch glass with a razor blade and transferred to the incubation chamber contained physiological buffer. Then, the tissue is disrupted and incubated, and the tissue suspension is washed with fresh physiological buffer. After the RDE is equilibrated to obtain a baseline, a neurotransmitter of interest is added to the suspension, and the transport data is recorded as extracellular neurotransmitter concentration vs. time ([A]₀ vs. t). For an inhibition experiment, an inhibitor is added and incubated for 30 s before the neurotransmitter is added.

7. METH INHIBITION OF CATECHOLAMINE AND SEROTONIN TRANSPORTERS

Our laboratory and other laboratories have studied the relationship between the catechol substrate binding site and cocaine, amphetamine and methylphenidate binding sites at the DAT. Cocaine is an inhibitor generally thought to block DA transport but is not a transported substrate analog (43, 71, 75, 76), whereas amphetamine and its derivatives are substrate analogs which compete with DA and get transported (77-84). Wayment et al. (43) performed an in vitro experiment on reserpine pretreated rats to study the amphetamine binding site in the striatal DAT. Results show amphetamine and DA compete at the same binding site, at the kinetically active DAT and are transported. Once amphetamine is transported, the preloaded intercellular DA is released. Therefore, amphetamine is a substrate analog for the plasmalemmal DAT. In
addition, the results show that amphetamine interacts with but does not bind to the cocaine inhibitory site on the DAT (43). Rutledge et al. (79, 81, 82, 85-87) performed different in vitro experiments to study the effects of amphetamine on NE in cerebral cortex as well as DA in striatum. Results show that the amphetamines increase DA and NE release and inhibit NE and DA uptake.

Methylphenidate, which is not a substrate analog, competitively inhibits the striatal DAT, and its inhibition on the DAT does not depend on time, whereas amphetamine exhibits time-dependent inhibition because it is taken up (73). Methylphenidate binds at both substrate and non-substrate binding sites (73). Wayment et al. (73) used methylphenidate and its analogs (threo-3,4-dichloromethylphenidate hydrochloride, threo-N-methyl-4-methylphenidate hydrochloride, and threo-N-benzylmethylphenidate hydrochloride) to study whether these analogs have effects on the kinetics of the DAT. Results show that methylphenidate analogs noncompetitively inhibit DA transport (73). Further, inhibition of the effects of amphetamine on DA transport is attenuated by the threo-N-methyl-4-methylphenidate hydrochloride and threo-N-benzylmethylphenidate hydrochloride, suggesting that these two methylphenidate analogs can be used as candidates for antagonism of amphetamine and METH (73).

In vitro experiments have been conducted to study the METH-induced changes on neurotransmitter transport. Results from Sandoval et al. (67) show that the uptake velocity of [3H]DA decreases after striatal synaptosomes is treated with METH but has no effect on [3H]NE in hippocampal synaptosomes (67). These results are consistent with the ex vivo experiment in which a single dose of 15 mg/kg of METH is subcutaneously delivered to a rat prior to in vitro experimentation (67). In addition, Westphalen and Stadlin (88) found that after 10 µM METH
was directly added to the striatal synaptosomes and was incubated for 60 min, the $[^{3}]$HDA $V_{\text{max}}$ significantly decreases.

*In vitro* experiments also involve the study of DA efflux using cells. Intracellular Ca$^{2+}$ as well as protein kinase C (PKC) play an important role in the amphetamine-stimulated release of DA (89). Down-regulated PKC causes a decrease of the amphetamine-stimulated release of DA but does not affect DA uptake through the NET in PC12 cells (90). When PKC$\beta_{II}$ is transfected into the hDAT-HEK 293 cells, amphetamine-stimulated release of DA is increased compared with the cells without the PKC$\beta_{II}$ (89). When intracellular Ca$^{2+}$ is chelated with the PC12 cells, the reduction of intracellular Ca$^{2+}$ causes the amphetamine-stimulated release of DA decrease and has no effect on the DA uptake through the NET (90).

The effect of amphetamine and its derivatives on the kinetics of the neurotransmitter transporters can be further investigated by either single or repeated administration(s) of the drug to a rat as experienced by a drug addict. The route of administration is either by subcutaneous (s.c.), intraperitoneal (i.p.), or self-administration (s.a.) to the rat. Rats were then withdrawn from drugs for 1-h or 24-h for tolerance studies or 1 week or more for sensitization studies. Tables 1 - 4 summarize literature results on the effect of METH on the kinetic constants (velocity, $V_{\text{max}}$, and $K_{m}$) of the DAT, VMAT-2, SERT, and NET in striatum, nucleus accumbens and hippocampus. The kinetic constants of these neurotransmitter transporters are measured by either $[^{3}]$H labeled (57-60, 63-66, 91-95) or RDE voltammetry technique (36). Tables 5 - 13 summarize literature results on the concentrations of catecholamine and 5HT in each anatomical region of interest and the levels of their transporters as well as the TH and TPH after METH treatments. Catecholamine and 5HT concentrations in the tissue are measured by HPLC-ED (52, 58, 64, 95-101), and the level of its transporters are measured by western blot (36, 52, 58, 97-
100, 102) or Lowry assay (96). The subjects were male Sprague-Dawley rats unless otherwise specified.
Table 1
Summary of how different METH doses, route of administration, treatment protocol and withdrawal time affects the kinetics of the DAT in striatum and nucleus accumbens.

### Striatum

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>Velocity</th>
<th>$V_{max}$</th>
<th>$K_m$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>i.p.</td>
<td>1X 5 days</td>
<td>24-hr</td>
<td>↑</td>
<td></td>
<td></td>
<td>(36)</td>
</tr>
<tr>
<td>6*</td>
<td>i.p.</td>
<td>1X 9 days</td>
<td>9 days</td>
<td>↑</td>
<td></td>
<td></td>
<td>(93)</td>
</tr>
<tr>
<td>5 or 15</td>
<td>s.c.</td>
<td>1X</td>
<td>1-hr</td>
<td>↓</td>
<td></td>
<td></td>
<td>(59)</td>
</tr>
<tr>
<td>15</td>
<td>s.c.</td>
<td>1X</td>
<td>0.5-, 1-, or 3-hr</td>
<td>↓</td>
<td></td>
<td></td>
<td>(59, 60)</td>
</tr>
<tr>
<td>10</td>
<td>s.c.</td>
<td>4X 2-hr intervals</td>
<td>1-hr or 9 days</td>
<td>↓</td>
<td></td>
<td></td>
<td>(60)</td>
</tr>
<tr>
<td>10</td>
<td>s.c.</td>
<td>4X 2-hr intervals</td>
<td>1-hr or 7 days</td>
<td>↓</td>
<td>↓</td>
<td>―</td>
<td>(65)</td>
</tr>
<tr>
<td>15</td>
<td>s.c.</td>
<td>1X</td>
<td>1-hr</td>
<td>↓</td>
<td>↓</td>
<td>―</td>
<td>(65)</td>
</tr>
<tr>
<td>10</td>
<td>s.c.</td>
<td>4X 2-hr intervals</td>
<td>1-hr, 24-hr or 7 days</td>
<td>↓</td>
<td>↓</td>
<td>―</td>
<td>(66)</td>
</tr>
<tr>
<td>15</td>
<td>s.c.</td>
<td>1X</td>
<td>1 hr</td>
<td>↓</td>
<td></td>
<td></td>
<td>(63)</td>
</tr>
<tr>
<td>7.5</td>
<td>s.c.</td>
<td>4X 2-hr intervals</td>
<td>1-hr, 24-hr or 48-hr</td>
<td>↓</td>
<td></td>
<td></td>
<td>(58)</td>
</tr>
<tr>
<td>50</td>
<td>s.c.</td>
<td>2X for 4 days</td>
<td>2-3 weeks</td>
<td>↓</td>
<td>↓</td>
<td>―</td>
<td>(92)</td>
</tr>
<tr>
<td>7.5</td>
<td>s.c.</td>
<td>4X 2-hr intervals</td>
<td>1-hr or 7 days</td>
<td>↓</td>
<td></td>
<td></td>
<td>(95)</td>
</tr>
<tr>
<td>0.6*</td>
<td>s.a.</td>
<td>8-hr/day for 7 days</td>
<td>8 or 30 days</td>
<td>↓</td>
<td></td>
<td></td>
<td>(103)</td>
</tr>
<tr>
<td>0.12*</td>
<td>s.a.</td>
<td>8-hr/day for 7 days</td>
<td>7 days</td>
<td>↓</td>
<td></td>
<td></td>
<td>(103)</td>
</tr>
<tr>
<td>0.12*</td>
<td>s.a.</td>
<td>METH pretreatment for 7 days, then 4X 7.5 mg/kg s.c., 2-hr interval</td>
<td>7 days</td>
<td>↓</td>
<td></td>
<td>(103)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>s.c.</td>
<td>1X</td>
<td>24-hr</td>
<td>―</td>
<td></td>
<td></td>
<td>(59, 60)</td>
</tr>
<tr>
<td>10</td>
<td>s.c.</td>
<td>4X 2-hr intervals</td>
<td>24-hr</td>
<td>―</td>
<td></td>
<td></td>
<td>(60)</td>
</tr>
<tr>
<td>0.5 or 1</td>
<td>s.c.</td>
<td>1X</td>
<td>1-hr</td>
<td>―</td>
<td></td>
<td></td>
<td>(60)</td>
</tr>
</tbody>
</table>

### Nucleus Accumbens

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration</th>
<th>Withdrawal Time</th>
<th>Velocity</th>
<th>$V_{max}$</th>
<th>$K_m$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>i.p.</td>
<td>1X for 5 days</td>
<td>24-hr</td>
<td>↓</td>
<td></td>
<td></td>
<td>(36)</td>
</tr>
<tr>
<td>7.5</td>
<td>s.c.</td>
<td>4X 2-hr</td>
<td>1-hr or 48-hr</td>
<td>↓</td>
<td></td>
<td></td>
<td>(58)</td>
</tr>
<tr>
<td>10</td>
<td>s.c.</td>
<td>4X 2-hr</td>
<td>1-hr</td>
<td>↓</td>
<td></td>
<td></td>
<td>(66)</td>
</tr>
<tr>
<td>7.5</td>
<td>s.c.</td>
<td>4X 2-hr</td>
<td>24-hr</td>
<td>↓</td>
<td></td>
<td></td>
<td>(58)</td>
</tr>
</tbody>
</table>

Abbreviations: no change (-), increase (↑), decrease (↓)
* Male Wister rats were used.
# indicates mg/infusion was dosed.
## Table 2

Summary of how different METH doses, route of administration, treatment protocol and withdrawal time affects the kinetics of the VMAT-2 in striatum and nucleus accumbens.

| Striatum | | | | | | | | |
|----------|----------|-----------|----------------|----------------|----------------|----------------|----------------|
| Dose (mg/kg) | Route | Duration of Treatment | Withdrawal Time | Velocity | V\text{max} | K\text{m} | Reference |
| 7.5 | s.c. | 4X 2-hr intervals | 1-, 24- or 48-hr | ↓ | | | (58) |
| 7.5 | s.c. | 4X 2-hr intervals | 1-hr or 7 days | ↓ | | | (95) |
| 10 | s.c. | 4X 2-hr intervals | 1- or 24-hr | ↓ | | | (57) |
| 15 | s.c. | 1X | 1-hr | ↓ | ↓ | — | (91, 94) |
| 15 | s.c. | 1X | 24-hr | — | | | (94) |
| 5 or 10 | s.c. | 1X | 1-hr | — | | | (94) |
| 0.6\textsuperscript{#} | s.a. | 8-hr/day for 7 days | 8 days | — | | | (103) |

| Nucleus Accumbens | | | | | | | | |
|-------------------|----------|-----------|----------------|----------------|----------------|----------------|----------------|
| Dose (mg/kg) | Route | Duration of Treatment | Withdrawal Time | Velocity | V\text{max} | K\text{m} | Reference |
| 7.5 | s.c | 4X 2-hr intervals | 1-hr or 24-hr | ↓ | | | (58) |
| 7.5 | s.c | 4X 2-hr | 48-hr | — | | | (58) |

Abbreviations: no change (-), decrease (↓)  
# indicates mg/infusion was dosed.
Table 3
Summary of how different METH doses, route of administration, treatment protocol and withdrawal time affects the kinetics of the SERT in striatum.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>Velocity</th>
<th>( V_{\text{max}} )</th>
<th>( K_m )</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>s.c.</td>
<td>4X 2-hr intervals</td>
<td>1-hr</td>
<td>↓</td>
<td></td>
<td></td>
<td>(60)</td>
</tr>
<tr>
<td>10</td>
<td>s.c.</td>
<td>4X 2-hr intervals</td>
<td>1-hr or 7 days</td>
<td>↓</td>
<td>↓</td>
<td>—</td>
<td>(65)</td>
</tr>
<tr>
<td>10</td>
<td>s.c.</td>
<td>4X 2-hr intervals</td>
<td>1-hr, 24-hr or 7 days</td>
<td>↓</td>
<td></td>
<td></td>
<td>(64)</td>
</tr>
<tr>
<td>15</td>
<td>s.c.</td>
<td>1X</td>
<td>1-hr</td>
<td>↓</td>
<td></td>
<td></td>
<td>(64)</td>
</tr>
<tr>
<td>10</td>
<td>s.c.</td>
<td>4X 2-hr intervals</td>
<td>18-hr or 24-hr</td>
<td>—</td>
<td></td>
<td></td>
<td>(65)</td>
</tr>
</tbody>
</table>

Abbreviations: no change (-), decrease (↓)
Table 4
Summary of how different METH doses, route of administration, treatment protocol and withdrawal time affects the kinetics of the NET in hippocampus.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>Velocity</th>
<th>V\textsubscript{max}</th>
<th>K\textsubscript{m}</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>s.c.</td>
<td>1X</td>
<td>1-hr</td>
<td>↓</td>
<td></td>
<td></td>
<td>(63)</td>
</tr>
<tr>
<td>10</td>
<td>s.c.</td>
<td>4X 2-hr</td>
<td>1-hr</td>
<td>↓</td>
<td>—</td>
<td>↑</td>
<td>(63)</td>
</tr>
</tbody>
</table>

Abbreviations: no change (-), increase (↑), decrease (↓)
Table 5
Summary of how different METH doses, route of administration, treatment protocol and withdrawal time affects the DA contents in different brain regions.

<table>
<thead>
<tr>
<th>Striatum</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg)</td>
<td>Route</td>
<td>Duration of Treatment</td>
<td>Withdrawal Time</td>
<td>DA Contents in Tissue</td>
<td>Reference</td>
</tr>
<tr>
<td>10</td>
<td>i.p.</td>
<td>4X 2-hr intervals</td>
<td>8 days</td>
<td>↓</td>
<td>(52)</td>
</tr>
<tr>
<td>15</td>
<td>s.c.</td>
<td>2X 12-hr for 4 days</td>
<td>18-hr</td>
<td>↓</td>
<td>(96)</td>
</tr>
<tr>
<td>14</td>
<td>s.a.</td>
<td>15-hr for 8 days</td>
<td>24-hr</td>
<td>↓</td>
<td>(99)</td>
</tr>
<tr>
<td>14</td>
<td>s.a.</td>
<td>15-hr for 8 days</td>
<td>7 days</td>
<td>↓</td>
<td>(99)</td>
</tr>
<tr>
<td>7.5</td>
<td>s.c.</td>
<td>4X 2-hr intervals</td>
<td>1-hr or 7 days</td>
<td>↓</td>
<td>(95)</td>
</tr>
<tr>
<td>4</td>
<td>i.p.</td>
<td>3X 3-hr intervals</td>
<td>24-hr or 7 days</td>
<td>↓</td>
<td>(101)</td>
</tr>
<tr>
<td>7.5</td>
<td>s.c.</td>
<td>4X 2-hr intervals</td>
<td>1-hr</td>
<td>↓</td>
<td>(58)</td>
</tr>
<tr>
<td>-</td>
<td>i.p.</td>
<td>Saline pretreatment for 14 days then METH challenge dose of 6X 5mg/kg 1-hr interval for 1 day or 2 days</td>
<td>14 days</td>
<td>↓</td>
<td>(104)</td>
</tr>
<tr>
<td>0.5 – 1.5</td>
<td>i.p.</td>
<td>METH pretreatment for 14 days then METH challenge dose of 6X 5mg/kg 1-hr interval</td>
<td>14 days</td>
<td>—</td>
<td>(104)</td>
</tr>
<tr>
<td>5</td>
<td>s.c.</td>
<td>2X 12-hr for 4 days</td>
<td>18-hr</td>
<td>—</td>
<td>(96)</td>
</tr>
<tr>
<td>0.6*</td>
<td>s.a.</td>
<td>8-hr/day for 7 days</td>
<td>8 or 30 days</td>
<td>—</td>
<td>(103)</td>
</tr>
<tr>
<td>0.12*</td>
<td>s.a.</td>
<td>8-hr/day for 7 days</td>
<td>7 days</td>
<td>—</td>
<td>(103)</td>
</tr>
<tr>
<td>0.24*</td>
<td>s.a.</td>
<td>8-hr/day for 7 days</td>
<td>7 days</td>
<td>—</td>
<td>(103)</td>
</tr>
<tr>
<td>0.12*</td>
<td>s.a.</td>
<td>METH pretreatment for 7 days, then 4X 7.5 mg/kg s.c., 2-hr interval</td>
<td>7 days</td>
<td>—</td>
<td>(103)</td>
</tr>
<tr>
<td>7 §</td>
<td>s.a.</td>
<td>1-hr 7-10 days followed by 1-hr s.a. for 12-14 days</td>
<td>24-hr</td>
<td>—</td>
<td>(97)</td>
</tr>
<tr>
<td>7 §</td>
<td>s.a.</td>
<td>1-hr 7-10 days followed by 6-hr s.a. for 12-14 days</td>
<td>24-hr</td>
<td>—</td>
<td>(97)</td>
</tr>
<tr>
<td>0.1-4.0 *</td>
<td>i.p.</td>
<td>3X for 14 days</td>
<td>48-hr</td>
<td>—</td>
<td>(100)</td>
</tr>
<tr>
<td>0.1-4.0 *</td>
<td>i.p.</td>
<td>3X for 14 days</td>
<td>14 days</td>
<td>—</td>
<td>(100)</td>
</tr>
<tr>
<td>4 *</td>
<td>i.p.</td>
<td>3X for 1 day</td>
<td>48-hr</td>
<td>—</td>
<td>(100)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nucleus Accumbens</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg)</td>
<td>Route</td>
<td>Duration of Treatment</td>
<td>Withdrawal Time</td>
<td>DA Contents in Tissue</td>
<td>Reference</td>
</tr>
<tr>
<td>102</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td>Route</td>
<td>Duration of Treatment</td>
<td>Withdrawal Time</td>
<td>DA Contents in Tissue</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------</td>
<td>-------</td>
<td>-----------------------</td>
<td>----------------</td>
<td>---------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>0.1-4.0 *</td>
<td>i.p</td>
<td>3X 14 days</td>
<td>14 days</td>
<td>↑</td>
<td>(100)</td>
</tr>
<tr>
<td>10</td>
<td>i.p</td>
<td>4X 2-hr</td>
<td>8 days</td>
<td>↓</td>
<td>(52)</td>
</tr>
<tr>
<td>0.1-4.0 *</td>
<td>i.p</td>
<td>3X 14 days</td>
<td>48-hr</td>
<td>↓</td>
<td>(100)</td>
</tr>
<tr>
<td>4</td>
<td>i.p</td>
<td>3X 14 days</td>
<td>48-hr</td>
<td>↓</td>
<td>(100)</td>
</tr>
<tr>
<td>7 §</td>
<td>s.a.</td>
<td>1-hr 7-10 days followed by 1-hr s.a. for 12–14 days</td>
<td>24-hr</td>
<td>—</td>
<td>(97)</td>
</tr>
<tr>
<td>7 §</td>
<td>s.a.</td>
<td>1-hr 7-10 days followed by 6-hr s.a. for 12-14 days</td>
<td>24-hr</td>
<td>—</td>
<td>(97)</td>
</tr>
<tr>
<td>4 *</td>
<td>i.p</td>
<td>3X for 1 day</td>
<td>14 days</td>
<td>—</td>
<td>(100)</td>
</tr>
<tr>
<td>7.5</td>
<td>s.c.</td>
<td>4X 2-hr</td>
<td>7 days</td>
<td>—</td>
<td>(58)</td>
</tr>
</tbody>
</table>

**Frontal Cortex**

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>DA Contents in Tissue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 §</td>
<td>s.a.</td>
<td>1-hr 7-10 days followed by 1-hr s.a. for 12–14 days</td>
<td>24-hr</td>
<td>—</td>
<td>(97)</td>
</tr>
<tr>
<td>7 §</td>
<td>s.a.</td>
<td>1-hr 7-10 days followed by 6-hr s.a. for 12-14 days</td>
<td>24-hr</td>
<td>—</td>
<td>(97)</td>
</tr>
<tr>
<td>14</td>
<td>s.a.</td>
<td>15-hr for 8 days</td>
<td>24-hr</td>
<td>—</td>
<td>(99)</td>
</tr>
<tr>
<td>14</td>
<td>s.a.</td>
<td>15-hr for 8 days</td>
<td>14 days</td>
<td>↓</td>
<td>(99)</td>
</tr>
</tbody>
</table>

Abbreviations: no change (-), increase (↑), decrease (↓)

§ Long-Evan rats were used.

* Wistar rats were used.

# indicates mg/infusion was dosed.
Table 6
Summary of how different METH doses, route of administration, treatment protocol and withdrawal time affects the 5HT contents in different brain regions.

<table>
<thead>
<tr>
<th>Striatum</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>5HT Contents in Tissue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>i.p.</td>
<td>4X 2-hr intervals</td>
<td>8 days</td>
<td>↓</td>
<td>(52)</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>s.a.</td>
<td>15-hr for 8 days</td>
<td>24-hr</td>
<td>↓</td>
<td>(99)</td>
<td></td>
</tr>
<tr>
<td>7 §</td>
<td>s.a.</td>
<td>1-hr 7-10 days followed by 1-hr s.a. for 12-14 days</td>
<td>24-hr</td>
<td>—</td>
<td>(97)</td>
<td></td>
</tr>
<tr>
<td>7 §</td>
<td>s.a.</td>
<td>1-hr 7-10 days followed by 6-hr s.a. for 12-14 days</td>
<td>24-hr</td>
<td>—</td>
<td>(97)</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>s.a.</td>
<td>15-hr for 8 days</td>
<td>7 days</td>
<td>—</td>
<td>(99)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nucleus Accumbens</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>5HT Contents in Tissue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>i.p.</td>
<td>4X 2-hr intervals</td>
<td>8 days</td>
<td>↓</td>
<td>(52)</td>
<td></td>
</tr>
<tr>
<td>7 §</td>
<td>s.a.</td>
<td>1-hr 7-10 days followed by 1-hr s.a. for 12-14 days</td>
<td>24-hr</td>
<td>—</td>
<td>(97)</td>
<td></td>
</tr>
<tr>
<td>7 §</td>
<td>s.a.</td>
<td>1-hr 7-10 days followed by 6-hr s.a. for 12-14 days</td>
<td>24-hr</td>
<td>—</td>
<td>(97)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Frontal Cortex</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>5HT Contents in Tissue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>s.a.</td>
<td>15-hr for 8 days</td>
<td>24-hr</td>
<td>↓</td>
<td>(99)</td>
<td></td>
</tr>
<tr>
<td>7 §</td>
<td>s.a.</td>
<td>1-hr 7-10 days followed by 1-hr s.a. for 12-14 days</td>
<td>24-hr</td>
<td>—</td>
<td>(97)</td>
<td></td>
</tr>
<tr>
<td>7 §</td>
<td>s.a.</td>
<td>1-hr 7-10 days followed by 6-hr s.a. for 12-14 days</td>
<td>24-hr</td>
<td>—</td>
<td>(97)</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>s.a.</td>
<td>15-hr for 8 days</td>
<td>7 days</td>
<td>—</td>
<td>(99)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: no change (-), decrease (↓)
§ Long-Evan rats were used.
Table 7
Summary of how different METH doses, route of administration, treatment protocol and withdrawal time affects the NE contents in different brain regions.

<table>
<thead>
<tr>
<th>Striatum</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>NE Contents in Tissue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>s.a.</td>
<td>15 hr for 8 days</td>
<td>24-hr</td>
<td>↓</td>
<td>(99)</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>s.a.</td>
<td>15 hr for 8 days</td>
<td>7 days</td>
<td>—</td>
<td>(99)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Frontal Cortex</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>NE Contents in Tissue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>s.a.</td>
<td>15 hr for 8 days</td>
<td>24-hr</td>
<td>↓</td>
<td>(99)</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>s.a.</td>
<td>15 hr for 8 days</td>
<td>7 days</td>
<td>—</td>
<td>(99)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: no change (-), decrease (↓)
Table 8
Summary of how different METH doses, route of administration, treatment protocol and withdrawal time affects the TH levels in different brain regions.

### Striatum

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>TH Levels</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 i.p.</td>
<td>4X 6-hr intervals</td>
<td>2-hr</td>
<td>↓</td>
<td>(41)</td>
<td></td>
</tr>
<tr>
<td>14 s.a.</td>
<td>15-hr for 8 days</td>
<td>14 days</td>
<td>↓</td>
<td>(99)</td>
<td></td>
</tr>
<tr>
<td>15 s.c.</td>
<td>1X</td>
<td>7 days</td>
<td>↓</td>
<td>(40)</td>
<td></td>
</tr>
<tr>
<td>- i.p. Saline pretreatment for 14 days then METH challenge dose of 6X 5mg/kg 1-hr interval for 1 day or 2 days</td>
<td></td>
<td>14 days</td>
<td>↓</td>
<td>(104)</td>
<td></td>
</tr>
<tr>
<td>0.5 – 1.5 i.p. METH pretreatment for 14 days then METH challenge dose of 6X 5mg/kg 1-hr interval</td>
<td></td>
<td>14 days</td>
<td>—</td>
<td>(104)</td>
<td></td>
</tr>
<tr>
<td>0.1-4.0 * i.p. 3X for 14 days</td>
<td>48-hr</td>
<td>—</td>
<td>(100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1-4.0 * i.p. 3X for 14 days</td>
<td>14 days</td>
<td>—</td>
<td>(100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 * i.p. 3X for 1 day</td>
<td>48-hr</td>
<td>—</td>
<td>(100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 * i.p. 3X for 1 day</td>
<td>14 days</td>
<td>—</td>
<td>(100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.6# s.a.</td>
<td>8-hr/day for 7 days</td>
<td>8 or 50 days</td>
<td>—</td>
<td>(103)</td>
<td></td>
</tr>
<tr>
<td>0.12# s.a.</td>
<td>8-hr/day for 7 days</td>
<td>7 days</td>
<td>—</td>
<td>(103)</td>
<td></td>
</tr>
<tr>
<td>0.24# s.a.</td>
<td>8-hr/day for 7 days</td>
<td>7 days</td>
<td>—</td>
<td>(103)</td>
<td></td>
</tr>
<tr>
<td>0.12# s.a. METH pretreatment for 7 days, then 4X 7.5 mg/kg s.c., 2-hr interval</td>
<td></td>
<td>7 days</td>
<td>—</td>
<td>(103)</td>
<td></td>
</tr>
<tr>
<td>15 s.c.</td>
<td>1X</td>
<td>1-hr</td>
<td>—</td>
<td>(40)</td>
<td></td>
</tr>
</tbody>
</table>

### Nucleus Accumbens

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>TH levels</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1-4.0 * i.p. 3X for 14 days</td>
<td>14 days</td>
<td>↑</td>
<td>(100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1-4.0 * i.p. 3X for 14 days</td>
<td>48-hr</td>
<td>↓</td>
<td>(100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 * i.p. 3X for 1 day</td>
<td>48-hr</td>
<td>↓</td>
<td>(100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 * i.p. 3X for 1 day</td>
<td>14 days</td>
<td>—</td>
<td>(100)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Frontal Cortex

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>TH levels</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>i.p.</td>
<td>4X 6-hr intervals</td>
<td>2-hr</td>
<td>↓</td>
<td>(41)</td>
</tr>
<tr>
<td>14</td>
<td>s.a.</td>
<td>8 days</td>
<td>14 days</td>
<td>↓</td>
<td>(99)</td>
</tr>
</tbody>
</table>

Abbreviations: no change (-), increase (↑), decrease (↓)

* Wistar rats were used.

# indicates mg/perfusion was dosed.
Summary of how different METH doses, route of administration, treatment protocol and withdrawal time affects the TPH levels in different brain regions.

### Striatum

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>TPH Levels</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>i.p.</td>
<td>4X 6-h intervals</td>
<td>2-hr</td>
<td>↓</td>
<td>(41)</td>
</tr>
<tr>
<td>15</td>
<td>s.c.</td>
<td>1X</td>
<td>1-hr</td>
<td>↓</td>
<td>(40)</td>
</tr>
<tr>
<td>15</td>
<td>s.c.</td>
<td>1X</td>
<td>7 days</td>
<td>↓</td>
<td>(40)</td>
</tr>
</tbody>
</table>

### Nucleus Accumbens

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>TPH levels</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>s.c.</td>
<td>1X</td>
<td>1-hr</td>
<td>↓</td>
<td>(40)</td>
</tr>
<tr>
<td>15</td>
<td>s.c.</td>
<td>1X</td>
<td>7 days</td>
<td>—</td>
<td>(40)</td>
</tr>
</tbody>
</table>

Abbreviations: no change (-), decrease (↓)
Table 10
Summary of how different METH doses, route of administration, treatment protocol and withdrawal time affects the DAT levels in different brain regions.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>DAT levels</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>s.c.</td>
<td>2X 12-hr for 4 days</td>
<td>18-hr</td>
<td>↓</td>
<td>(96)</td>
</tr>
<tr>
<td>7 §</td>
<td>s.a.</td>
<td>1-hr 7-10 days followed by 6-hr s.a. for 12-14 days</td>
<td>24-hr</td>
<td>↓</td>
<td>(97)</td>
</tr>
<tr>
<td>14</td>
<td>s.a.</td>
<td>15-hr for 8 days</td>
<td>24-hr</td>
<td>↓</td>
<td>(99)</td>
</tr>
<tr>
<td>14</td>
<td>s.a.</td>
<td>15-hr for 8 days</td>
<td>14 days</td>
<td>↓</td>
<td>(99)</td>
</tr>
<tr>
<td>4 ^</td>
<td>i.p.</td>
<td>3X 3-hr intervals</td>
<td>24-hr or 7 days</td>
<td>↓</td>
<td>(101)</td>
</tr>
<tr>
<td>5</td>
<td>i.p.</td>
<td>1X 5 days</td>
<td>24-hr</td>
<td>↓</td>
<td>(36)</td>
</tr>
<tr>
<td>10</td>
<td>i.p.</td>
<td>4X 2-hr intervals</td>
<td>1-hr or 7 days</td>
<td>↓</td>
<td>(98)</td>
</tr>
<tr>
<td></td>
<td>i.p.</td>
<td>Saline pretreatment for 14 days then METH challenge dose of 6X 5mg/kg 1-hr interval for 1 day or 2 days</td>
<td>14 days</td>
<td>↓</td>
<td>(104)</td>
</tr>
<tr>
<td>0.5 – 1.5</td>
<td>i.p.</td>
<td>METH pretreatment for 14 days then METH challenge dose of 6X 5mg/kg 1-hr interval</td>
<td>14 days</td>
<td>↓</td>
<td>(104)</td>
</tr>
<tr>
<td>0.6 §</td>
<td>s.a.</td>
<td>8-hr/day for 7 days</td>
<td>8 or 30 days</td>
<td>↓</td>
<td>(103)</td>
</tr>
<tr>
<td>0.12 §</td>
<td>s.a.</td>
<td>8-hr/day for 7 days</td>
<td>7 days</td>
<td>↓</td>
<td>(103)</td>
</tr>
<tr>
<td>0.24 §</td>
<td>s.a.</td>
<td>8-hr/day for 7 days</td>
<td>7 days</td>
<td>↓</td>
<td>(103)</td>
</tr>
<tr>
<td>0.12 §</td>
<td>s.a.</td>
<td>METH pretreatment for 7 days, then 4X 7.5 mg/kg s.c., 2-hr interval</td>
<td>7 days</td>
<td>↓</td>
<td>(103)</td>
</tr>
<tr>
<td>5</td>
<td>s.c.</td>
<td>2X 12-hr for 4days</td>
<td>18-hr</td>
<td>—</td>
<td>(96)</td>
</tr>
<tr>
<td>7 §</td>
<td>s.a.</td>
<td>1-hr 7-10 days followed by 1-hr s.a. for 12–14 days</td>
<td>24-hr</td>
<td>—</td>
<td>(97)</td>
</tr>
<tr>
<td>0.1-4.0 *</td>
<td>i.p.</td>
<td>3X for 14 days</td>
<td>48-hr</td>
<td>—</td>
<td>(100)</td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td>Route</td>
<td>Duration of Treatment</td>
<td>Withdrawal Time</td>
<td>DAT levels</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------</td>
<td>-------</td>
<td>-----------------------</td>
<td>-----------------</td>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td>0.1-4.0 *</td>
<td>i.p.</td>
<td>3X for 14 days</td>
<td>14 days</td>
<td>—</td>
<td>(100)</td>
</tr>
<tr>
<td>4 *</td>
<td>i.p.</td>
<td>3X for 1 day</td>
<td>48-hr</td>
<td>—</td>
<td>(100)</td>
</tr>
<tr>
<td>4 *</td>
<td>i.p.</td>
<td>3X for 1 day</td>
<td>14 days</td>
<td>—</td>
<td>(100)</td>
</tr>
<tr>
<td>7.5</td>
<td>s.c.</td>
<td>4X 2hr intervals</td>
<td>1-hr</td>
<td>—</td>
<td>(58)</td>
</tr>
<tr>
<td>2</td>
<td>i.p.</td>
<td>1X for 10 days</td>
<td>24-hr</td>
<td>—</td>
<td>(102)</td>
</tr>
</tbody>
</table>

### Nucleus Accumbens

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>DAT levels</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>i.p.</td>
<td>1X for 10 days</td>
<td>24-hr</td>
<td>↑</td>
<td>(102)</td>
</tr>
<tr>
<td>7 §</td>
<td>s.a.</td>
<td>1-hr 7-10 days followed by 6-hr s.a. for 12-14 days</td>
<td>24-hr</td>
<td>—</td>
<td>(97)</td>
</tr>
<tr>
<td>7 §</td>
<td>s.a.</td>
<td>1-hr 7-10 days followed by 1-hr s.a. for 12–14 days</td>
<td>24-hr</td>
<td>—</td>
<td>(97)</td>
</tr>
<tr>
<td>0.1-4.0 *</td>
<td>i.p.</td>
<td>3X for 14 days</td>
<td>48-hr</td>
<td>—</td>
<td>(100)</td>
</tr>
<tr>
<td>0.1-4.0 *</td>
<td>i.p.</td>
<td>3X for 14 days</td>
<td>14 days</td>
<td>—</td>
<td>(100)</td>
</tr>
<tr>
<td>4 *</td>
<td>i.p.</td>
<td>3X for 1 day</td>
<td>48-hr</td>
<td>—</td>
<td>(100)</td>
</tr>
<tr>
<td>4 *</td>
<td>i.p.</td>
<td>3X for 1 day</td>
<td>14 days</td>
<td>—</td>
<td>(100)</td>
</tr>
<tr>
<td>7.5</td>
<td>s.c.</td>
<td>4X 2hr intervals</td>
<td>1-hr</td>
<td>—</td>
<td>(58)</td>
</tr>
<tr>
<td>5</td>
<td>i.p.</td>
<td>1X 5 days</td>
<td>24-hr</td>
<td>—</td>
<td>(36)</td>
</tr>
</tbody>
</table>

### Frontal Cortex

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>DAT levels</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 §</td>
<td>s.a.</td>
<td>1-hr 7-10 days followed by 6-hr s.a. for 12-14 days</td>
<td>24-hr</td>
<td>↓</td>
<td>(97)</td>
</tr>
<tr>
<td>14</td>
<td>s.a.</td>
<td>15-hr for 8 days</td>
<td>24-hr</td>
<td>↓</td>
<td>(99)</td>
</tr>
<tr>
<td>7.5</td>
<td>s.c.</td>
<td>4X 2hr intervals</td>
<td>1-hr</td>
<td>↓</td>
<td>(58)</td>
</tr>
<tr>
<td>7 §</td>
<td>s.a.</td>
<td>1-hr 7-10 days followed by 1-hr s.a. for 12–14 days</td>
<td>24-hr</td>
<td>↓</td>
<td>(97)</td>
</tr>
<tr>
<td>2</td>
<td>i.p.</td>
<td>1X for 10 days</td>
<td>24-hr</td>
<td>—</td>
<td>(102)</td>
</tr>
</tbody>
</table>

Abbreviations: no change (-), increase (↑), decrease (↓)

§ Long-Evan rats were used.

* Wistar rats were used.

^ Female wild-type mice were used.

# indicates mg/infusion was dosed
Summary of how different METH doses, route of administration, treatment protocol and withdrawal time affects the VMAT-2 levels in different brain regions.

### Striatum

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatments</th>
<th>Withdrawal Time</th>
<th>VMAT-2 Levels</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>s.c.</td>
<td>4X 2-hr intervals</td>
<td>1-hr</td>
<td>↓</td>
<td>(58)</td>
</tr>
<tr>
<td>10</td>
<td>i.p.</td>
<td>4X 2-hr intervals</td>
<td>1-hr or 7 days</td>
<td>↓</td>
<td>(98)</td>
</tr>
<tr>
<td>0.1-4.0 *</td>
<td>i.p.</td>
<td>3X for 14 days</td>
<td>48-hr</td>
<td>―</td>
<td>(100)</td>
</tr>
<tr>
<td>0.1-4.0 *</td>
<td>i.p.</td>
<td>3X for 14 days</td>
<td>14 days</td>
<td>―</td>
<td>(100)</td>
</tr>
<tr>
<td>4 *</td>
<td>i.p.</td>
<td>3X for 1 day</td>
<td>48-hr</td>
<td>―</td>
<td>(100)</td>
</tr>
<tr>
<td>4 *</td>
<td>i.p.</td>
<td>3X for 1 day</td>
<td>14 days</td>
<td>―</td>
<td>(100)</td>
</tr>
</tbody>
</table>

### Nucleus Accumbens

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>VMAT-2 Levels</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1-4.0 *</td>
<td>i.p.</td>
<td>3X for 14 days</td>
<td>48-hr</td>
<td>―</td>
<td>(100)</td>
</tr>
<tr>
<td>0.1-4.0 *</td>
<td>i.p.</td>
<td>3X for 14 days</td>
<td>14 days</td>
<td>―</td>
<td>(100)</td>
</tr>
<tr>
<td>4 *</td>
<td>i.p.</td>
<td>3X for 1 day</td>
<td>48-hr</td>
<td>―</td>
<td>(100)</td>
</tr>
<tr>
<td>4 *</td>
<td>i.p.</td>
<td>3X for 1 day</td>
<td>14 days</td>
<td>―</td>
<td>(100)</td>
</tr>
<tr>
<td>7.5</td>
<td>s.c.</td>
<td>4X 2-hr intervals</td>
<td>1-hr</td>
<td>―</td>
<td>(58)</td>
</tr>
</tbody>
</table>

Abbreviations: no change (-), decrease (↓)

* Wistar rats were used
Table 12
Summary of how different METH doses, route of administration, treatment protocol and withdrawal time affects the SERT levels in different brain regions.

<table>
<thead>
<tr>
<th>Striatum</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>SERT Levels</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 § s.a.</td>
<td>1-hr 7-10 days followed by 1-hr s.a. for 12-14 days</td>
<td>24-hr</td>
<td>—</td>
<td>(97)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7 § s.a.</td>
<td>1-hr 7-10 days followed by 6-hr s.a. for 12-14 days</td>
<td>24-hr</td>
<td>—</td>
<td>(97)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nucleus Accumbens</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>SERT Levels</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 § s.a.</td>
<td>1-hr 7-10 days followed by 1-hr s.a. for 12-14 days</td>
<td>24-hr</td>
<td>—</td>
<td>(97)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7 § s.a.</td>
<td>1-hr 7-10 days followed by 6-hr s.a. for 12-14 days</td>
<td>24-hr</td>
<td>—</td>
<td>(97)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14 s.a.</td>
<td>15-hr for 8 days</td>
<td>14 days</td>
<td>—</td>
<td>(99)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
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<th>Frontal Cortex</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>SERT Levels</th>
<th>Reference</th>
</tr>
</thead>
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<td></td>
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<td>1-hr 7-10 days followed by 1-hr s.a. for 12-14 days</td>
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</tr>
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</tr>
</tbody>
</table>

Abbreviations: no change (-)
§ Long-Evan rats were used.
Table 13
Summary of how different METH doses, route of administration, treatment protocol and withdrawal time affects the NET levels in different brain regions.

<table>
<thead>
<tr>
<th>Striatum</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>NET Levels</th>
<th>Reference</th>
</tr>
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<td>—</td>
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</tr>
</tbody>
</table>

<table>
<thead>
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<th>Nucleus Accumbens</th>
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<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
<th>NET Levels</th>
<th>Reference</th>
</tr>
</thead>
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<td>1-hr 7-10 days followed by 1-hr s.a. for 12–14 days</td>
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<td>24-hr</td>
<td>—</td>
<td>(97)</td>
</tr>
<tr>
<td></td>
<td>2 i.p.</td>
<td>1X for 10 days</td>
<td>24-hr</td>
<td>—</td>
<td>(102)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Frontal Cortex</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Duration of Treatment</th>
<th>Withdrawal Time</th>
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<td>(102)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: no change (-)
§ Long-Evan rats were used.
Based on the doses, route of administration, duration of the treatments, and withdrawal time of METH, DA uptake velocity by the DAT tends to increase when METH is delivered via i.p. with long durations (5 – 9 days) of administration. Acute low doses (0.5 – 1 mg/kg) of METH with short withdrawal time (1-hr) do not change the DA uptake velocity. Compared among those acute administrations and short withdrawal time, DA velocities decrease when the doses are 10 times higher than 0.5 mg/kg. Dopamine velocity also decreases when the doses are 100 times higher than 0.5 mg/kg with multiple administrations and long withdrawal time (2 – 3 weeks). Fleckenstein et al. (60) found that both single and multiple administrations of METH cause DA uptake velocities to decrease after 1-hr withdrawal but partially (58, 66) or completely (60) recover to the control level after 24-hr withdrawal and decrease again 8 days later (60). The t1/2 of the DAT turnover is about 6.3 days (61). Therefore, the replacement of the DAT by synthesis is not the reason the DA uptake velocity is recovered after 24-hr withdrawal. Reactive oxygen species formed after repeated METH administrations cause oxidative inactivation of the DAT (60). Therefore, it is possible that the reducing agent, such as high concentrations of glutathione or ascorbate (60, 105), restores the activity of the DAT (60). In addition, METH self-administration pretreatment followed by a binge METH treatment attenuates the decrease in DA uptake, which may also cause the decrease of METH-induced hyperthermia (103).

The effects of METH treatments on DA uptake velocities by the DAT in nucleus accumbens do not depend on any parameters in Table 1. When comparing these parameters between striatum and nucleus accumbens, DA uptake velocities decrease in both of these brain regions after 48-hr withdrawal, but the results are different for 1-hr and 24-hr withdrawal between striatum and nucleus accumbens. Kokoshka et al. (66) found that the DA uptake velocity does not change after multiple METH treatments in nucleus accumbens, which shows
the METH-induced decrease in DA uptake is selectively occurs in striatum or has less susceptibility to a dopaminergic deficit in nucleus accumbens. Chu et al. (58) found that the DA uptake velocity recovered back to the level of the control after a 24-hr withdrawal and decreased again after another 24-hr withdrawal, which is similar to the repeated METH treatments in striatum.

Similarly, the effects of METH on DA uptake by the VMAT-2 in both striatum and nucleus accumbens do not depend on any parameters in Table 2. For a single dose of METH treatment, DA uptake velocity by VMAT-2 decreases after 1-hr but recovers back to the same level as the control after 24-hr withdrawal, which is the same trend found in the DAT transport in striatum. Dopamine transport velocities decrease after multiple METH treatments. Activation of the D2 receptor (D2R) may play an important role on the METH-induced decrease uptake by the VMAT-2 (94). When the rat was pretreated with D2 antagonist, eticlopride, prior to receive multiple METH regimens, 5HT uptake velocity in striatum increases when compared without the blockage (64). This seems the D2 blockage attenuated the effect of the METH on the 5HT uptake (64). Another result from the D2R knockout mice experiment shows that inactivation of D2R prevents the loss of DA content, the density of the DAT and TH in striatum, while the striatal DA overflow is reduced after METH treatments (101).

The effects of the METH treatments on 5HT uptake velocities by the SERT in striatum and NE uptake by the NET in the hippocampus do not depend on any parameters in Table 3. As we have seen before, 5HT uptake velocity decreases after repeated treatments with 1-hr withdrawal, then recovers back to control level after 24-hr withdrawal and finally decreases again after a week of withdrawal. Based on the summary in Table 4, the NE uptake velocity decreases after single or multiple METH administration(s).
The $V_{\text{max}}$ and $K_m$ of the DAT, VMAT-2, and SERT in striatum have the same trends after single or multiple METH administration(s). Results show that after METH treatments, $V_{\text{max}}$ of the DAT, VMAT-2, and SERT in striatum decrease with no effects on $K_m$. However, repeated METH treatments increase the $K_m$ but have no effect on the $V_{\text{max}}$ of the NET in the hippocampus.

Other studies (52, 53) have found that basal concentrations of DA increase in striatum and nucleus accumbens when a challenge dose of amphetamine or METH is given to a METH pretreated rat. The same result is found when amphetamine is added to the brain slice of striatum of an amphetamine pretreated rat (48). This phenomenon shows that amphetamine and METH are substrate analogs and stimulate the release of the catecholamine and 5HT neurotransmitters.

Repeated METH treatments also altered the concentrations of DA in tissues and the density of its transporters in striatum, nucleus accumbens, and frontal cortex. The effects of METH on the concentrations of DA in striatum and nucleus accumbens do not depend on any parameters in Table 5. When comparing the same METH treatment and withdrawal time between striatum and nucleus accumbens, drugs that are delivered via self-administration have the same results between these two brain regions. Also, results are the same in these regions when 10 mg/kg of METH is delivered via i.p. four times with 2-hr intervals and 8 days of withdrawal. After repeated METH treatments, the concentrations of DA in frontal cortex do not change with short withdrawal time (24-hr), but decrease with long withdrawal time (14 days).

The effects of METH on the concentrations of 5HT in striatum, nucleus accumbens, and frontal cortex do not depend on any of the parameters in Table 6. When compared results are among the same METH treatments and withdrawal time, the results show the same effects between striatum, nucleus accumbens, and frontal cortex. Therefore, METH may have the same
impact on the concentrations of 5HT in these three regions. Based on the summary in Table 7, the concentrations of NE in the striatum and frontal cortex decrease after repeated METH treatments with short withdrawal time (24-hr) but have no change with 7 days withdrawal.

Besides the catecholamine and 5HT neurotransmitters, METH also affects the activities of TH and TPH. The effects of METH on the TH and TPH activity in striatum, nucleus accumbens, and frontal cortex do not depend on any parameters in Table 8 and 9. Long duration of treatment with escalating doses and long withdrawal time tend to increase TH activity in nucleus accumbens but have no effect in striatum. Compared between the same METH treatments, the effects of METH on the TH and the TPH activities are different in striatum and nucleus accumbens. After 7 days of withdrawal, TH activity decreases in striatum but does not change in nucleus accumbens. The loss of TH and TPH during the long-term treatments may due to the neuronal degeneration (40). In addition, as we mentioned before, activation of the D2 receptor plays an important role on the densities of the TH and TPH during METH treatments (40).

METH affects the densities of the DAT and VMAT-2 but not SERT and NET in striatum, nucleus accumbens, and the frontal cortex. The effects of METH on the densities of the DAT in striatum, nucleus accumbens, and the frontal cortex do not depend on any parameters in Tables 10 - 13. Compared between striatum and nucleus accumbens, repeated METH treatments with 48-hr withdrawal time do not affect the density of the DAT in striatum and nucleus accumbens. However, when METH is delivered via i.p., the density of the DAT in nucleus accumbens increases but has no effect in striatum after 24-hr withdrawal. Repeated METH treatments do not affect the density of the VMAT-2 in nucleus accumbens but the VMAT-2 density decreases at
high doses in striatum. The decrease of the VMAT-2 density may due to the rapid nitrosylation (98).

When the DA transport activity is normalized by the DAT density in nucleus accumbens and striatum, the DAT is kinetically downregulated in nucleus accumbens and upregulated in striatum after the METH-sensitizing regime (36). Changes in the DAT kinetics can be caused by the changes in the glycosylation of the DAT, phosphorylation, or effector proteins binding to the DAT (36). The DAT is a glycoprotein with glycosylation sites on the second extracellular loop (106). It is known that carbohydrate structures are different between striatum and nucleus accumbens, which cause different responses to drugs of abuse (36, 107, 108). By using endoglycosidase (O-glycosidase and N-glycanase) and exoglycosidase (α-mannosidase and neuraminidase) to remove specific carbohydrate moieties on the DAT in striatum and nucleus accumbens of the METH treated rats as wells as the non-treated rats, western blot results show that the apparent molecular weights of the DAT in both striatum and nucleus accumbens are the same between the control and the one with METH treatment (36). Therefore, METH does not alter the glycosylation of the DAT in both the striatum and nucleus accumbens.

Amphetamine and its analogs such as METH are associated with the long-term deficits in dopaminergic and serotonergic functions. Toxic effects from amphetamine and its analogs are caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS) (109). Administrations of METH cause an increase accumulation of DA within the intracellular of dopaminergic neuron, in which DA can form reactive quinones and ROS by antioxidation (109, 110). In addition, monoamine oxidase metabolizes DA, which causes an increase in ROS formation (109). Once DA quinones are formed, it binds to the cysteine residues on proteins, which is toxic to the DA terminals (109, 111, 112). However, antioxidant such as ascorbic acid
and glutathione attenuated the DA quinone formation and the toxicity on DA terminals (112, 113). Repeated administrations of METH show a decrease in VMAT-2 and DAT proteins as well as DA content and an increase in nitrosylation of VMAT-2 protein. However, neuronal nitric oxide synthase inhibitor, S-methyl-L-thiocitrulline (SMTC), attenuated the decrease in the VMAT-2 and DAT proteins (98, 114). Therefore, METH leads to the rapid oxidation of VMAT-2, and the decrease in VMAT-2 mediates the continuing damage to DA terminals (98, 114).

Besides oxidative stress, chronic environmental stress enhances METH-induced toxicity in the brain. Rats experienced in ten days of unpredictable stress prior to METH administration result in an increase in hyperthermia, which may cause by the up-regulated 5HT2A/C receptor system (115). Rats exposed to stress prior to METH caused further decrease in VMAT-2 protein and the DA and 5HT contents when compared with those rats without stress prior administrated with METH. Prevention of hyperthermia attenuated the loss of VMAT-2, DA and 5HT contents (115). Therefore, through the increase in hyperthermia and the decrease in VMAT-2, chronic unpredictable stress enhances the long-term METH toxicity (115).

METH has a stronger impact on the DAT function than amphetamine because it causes strong stimulation, releases more DA, inhibits DA uptake more efficiently, and releases more Ca\(^{2+}\) from the intracellular store. All these impact may explain the increase and constant use of drug of abuse and the euphoria with which they are associated (116). In addition, METH is more lipophilic than amphetamine making it easier to penetrate and accumulate in cell membranes (117).

Amphetamine and METH also affect the rat stereotyped behavioral response. Stereotyped behavior of amphetamine- or METH-pretreated rats is more intense than the saline-pretreated rats (36, 48, 52), and the intensity increases when a challenge dose was injected after the
amphetamine withdrawal. Bjorklund et al. (36) observed that rats treated with METH show a significant sensitized response after four days of treatment. METH-treated rats show a continuous sniffing pattern with an increase in motion. However, the locomotor activity decreases after METH treatments (52). The locomotor activity also decreases after a challenge dose of amphetamine (48) was injected after the withdrawal. Paulson and Robinson (53) observed a large increase of basal activity when a challenge dose of amphetamine was injected to the amphetamine-withdrawal rat.

Several therapeutic agents were suggested as candidates to treat METH addiction. Lobeline (nicotinic receptor antagonist and neurotransmitter transporter inhibitor) and meso-transdiene, which is a lobeline analog, decrease METH self-administration on rats (118-120). meso-Transdiene inhibits METH-evoked DA release (119, 121) but has a high affinity for the DAT (119, 122). Structurally modified meso-transdiene molecules potentially improve the selectivity of the VMAT-2 and decrease DA release cause by METH stimulation (119). Bupropion is an antidepressant as well as DA and NE uptake inhibitor (123, 124). Rats pretreated with bupropion show a reduction on METH self-administration (124, 125). In clinical studies, bupropion attenuated METH effects such as euphoria on outpatients after METH administration and cue-induced craving (126).

8. CONCLUSION

This review has provided general information on the history, survey, and health issues of amphetamine and METH. The velocity of DA uptake increases when METH is delivered via i.p. with long withdrawal time. The $V_{\text{max}}$ of the DAT, VMAT-2, and SERT in striatum decrease while the $K_{\text{m}}$ has no effect after METH treatments. However, the $V_{\text{max}}$ of the NET in striatum
has no effect while the $K_m$ increases after METH treatments. The effects of METH on the catecholamine and 5HT contents and their transporters do not depend on doses, route of administrations, duration of treatments, or withdrawal time. The literature reviews may provide useful information on the prediction of the animal behavior, concentrations of the neurotransmitters, densities of the neurotransmitter transporters, and the kinetics of the neurotransmitter transporters under the effects of drugs of abuse, which may help for future experimental design of drugs of abuse-related research.

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