KINETICS, ZINC MODULATION, AND GLYCOSYLATION STATES OF THE DOPAMINE TRANSPORTER: EFFECTS OF METHAMPHETAMINE AND COCAINE

By

NICOLE LUCIA BJORKLUND

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of Nicole Lucia Bjorklund find it satisfactory and recommend that it be accepted.

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Chair

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KINETICS, ZINC MODULATION, AND GLYCOSYLATION STATES OF THE DOPAMINE TRANSPORTER: EFFECTS OF METHAMPHETAMINE AND COCAINE

Abstract

by Nicole Lucia Bjorklund, Ph.D.
Washington State University
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Chair: James O. Schenk

This research focuses on various molecular aspects of the dopamine transporter (DAT): In the third chapter Zn^{2+} modulates dopamine (DA) transport by the human DAT (hDAT) but not the rat DAT (rDAT) expressed in human embryonic kidney cells. Zn^{2+} reduced and increased the inhibition by cocaine in hDAT and rDAT, respectively. Zn^{2+} and cocaine were not competitive with each other. Zn^{2+} increased the second order binding rate constant for DA to the DAT for hDAT and rDAT. In striatal homogenates Zn^{2+} increased dopamine transport velocity and decreased cocaine inhibition providing evidence for differences in sensitivity to Zn^{2+} between the different preparations and also between the hDAT and rDAT.

In the fourth and fifth chapters, DAT was investigated in animals sensitized to either methamphetamine (METH) or cocaine. Effects induced by a sensitizing dose of METH on behavior, dopamine (DA) transport by the DAT, DAT density, and inhibition of DAT by METH in the nucleus accumbens (NAcc) and striatum (Str) were

investigated. Changes in glycosylation of the DAT after METH sensitization were examined. Sensitization caused increased stereotypic responses in rats. Animals treated with METH, had lower and higher DAT activity in the NAcc and Str, respectively. Western blots demonstrated that DAT density was unchanged in the NAcc of METH-treated animals, but striatal DAT density decreased. The IC_{50} of METH increased in the NAcc and decreased in the Str. Studies using glycosidase treatments and western blots revealed that glycosylation was not affected by METH exposure.

Studies examining cocaine sensitization 24 hours post treatment found that dopamine transport was decreased in the NAcc, but increased in the Str, persisting for 3 weeks after treatment. DAT density remained unchanged in both areas 24 hours post treatment, but decreased in the NAcc at 3 weeks. DAT was kinetically up-regulated in the NAcc at 3 weeks post treatment, thus the NAcc and Str may have different, but long-term alterations following treatment with cocaine.

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ATTRIBUTIONS

Chapter Two is in the format of the *Journal of Neuroscience Methods* as this is where it was published. Courtney Wright did some preliminary experiments to validate the pre-steady state theory in this manuscript. Dr. James O. Schenk wrote the parts concerning steady state kinetics. I wrote the section on pre-steady state kinetics and along with Dr. Schenk produced the final manuscript.

Chapter Three is in the format of *European Journal of Pharmacology* where it was published. Dr. Trent J. Volz provided assistance in the preparation of the manuscript and his name appears as a co-author. I performed all the work and manuscript preparation in consultation with my advisor, Dr. James O. Schenk, who also shares authorship.

Chapter Four is in the format of the journal *Synapse*, where it was submitted. Dr Barbara A. Sorg provided guidance and material for the electrophoresis and behavioral studies and therefore shares authorship. Experiments and preparation of the manuscript was done by myself in consultation with my mentor, Dr. James O. Schenk, who is a co-author.

Chapter Five is on the format of the journal *Neuropharmacolgy* as this is where the manuscript will be submitted. Dr Barbara A. Sorg provided guidance and material for the electrophoresis and is a co-author. Experiments and preparation of the manuscript

was performed by me in consultation with my mentor, Dr. James O. Schenk, who is a co-author.

CHAPTER ONE

INTRODUCTION

General Introduction

The dopaminergic neurons in the A8 – A10 cell groups in the brain include the substantia pars compacta and areas of the midbrain tegmentum. These neurons innervate the striatum, frontal and temporal cortices, and the limbic structures of the basal forebrain. These pathways are involved in the movement, emotion, thought, and memory storage (E.R. Kandel et al., 2000).

In addition, specific areas of the brain located within these cell groups have been found to be essential for drug seeking and abuse, specifically stimulants. The ventral tegmental area, important for reward, sends axons to the nucleus accumbens, striatum, and prefrontal cortex which are all involved in motivation (E.R. Kandel et al., 2000; R.A. Wise, 1998). Figure 1 shows the location of some of these brain areas important for stimulant abuse.

Stimulants such as cocaine and methamphetamine (METH) cause an increase of the neurotransmitter dopamine in the brain. Increased dopamine levels produce pleasure and reinforce drug seeking (J.R. Cooper et al., 2003; E.R. Kandel et al., 2000). This is achieved by inhibition of the dopamine transporter, which is described fully in the next section

Molecular Properties of the Dopamine Transporter

The DAT is a plasma membrane protein and a member of the Na^+/Cl^- dependent transporter family with the gene name SLC6A3 (B. Giros et al., 1992). This family

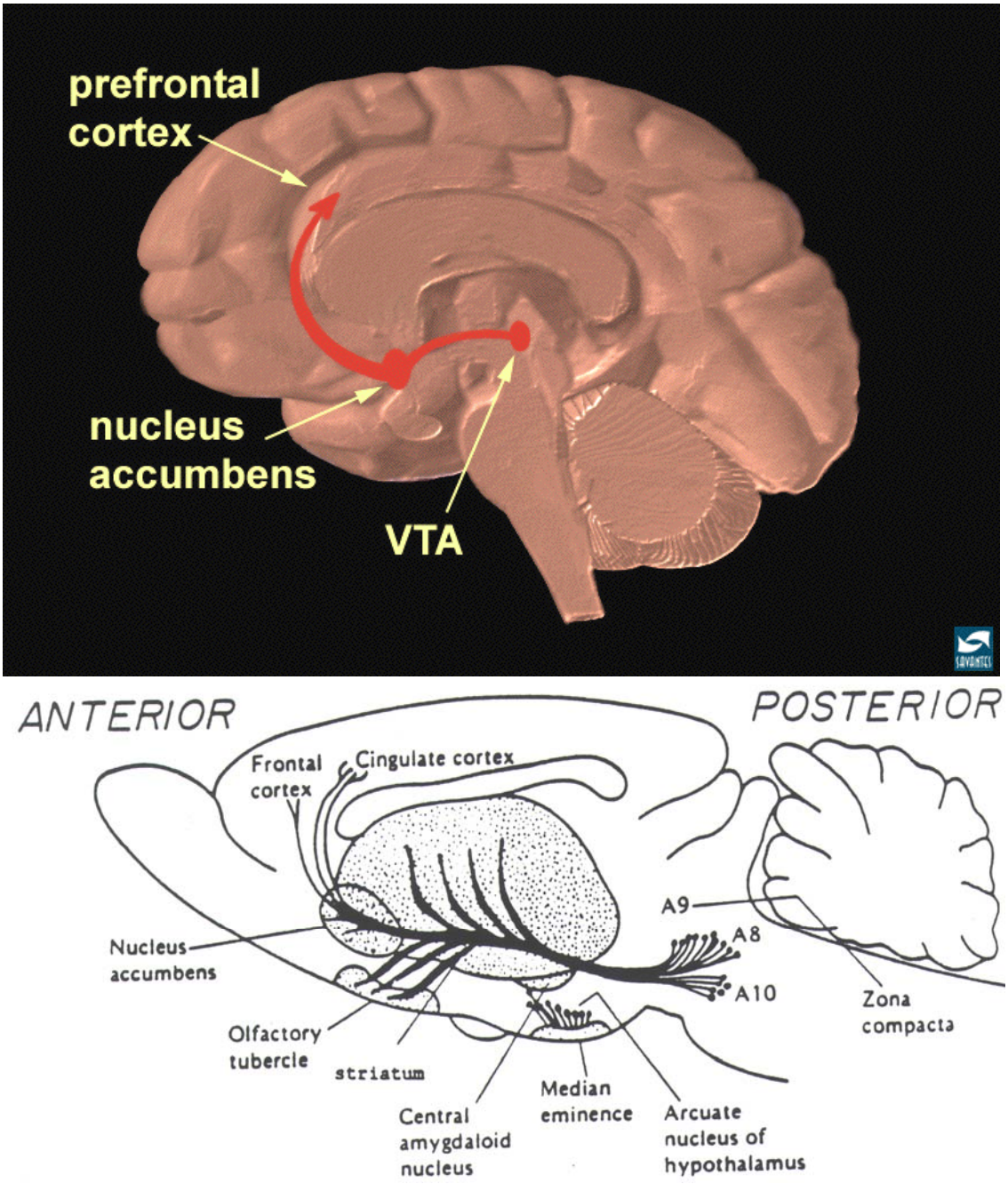

Figure 1. Areas of the human and rat brain important for drug abuse and addiction. VTA stands for ventral tegmental area. The top picture is from the National Institute of Drug Abuse web site; www.nida.nih.gov/pubs/Teaching/. The bottom picture of dopaminergic pathways in the rat is modified from Cooper, Bloom, and Roth 1982.

includes the serotonin and norepinephrine transporters as well. The gene contains 15 exons that spread across almost 60 kilobases (See Figure 2) (C. Guindalini et al., 2006). The DAT gene has a proximal strong core promoter (C. Guindalini et al., 2006). The restricted pattern of gene expression suggests silencing elements are also present. One silencing element has been found in intron 1 and is neural-specific (T.A. Greenwood and J.R. Kelsoe, 2003). Enhancer elements have been located in introns 9, 12, and 14 which also regulate expression (T.A. Greenwood and J.R. Kelsoe, 2003). There are different haplotypes of introns in the DAT gene sequence which alter expression. Different haplotypes have been implicated in a variety of neuropsychiatric disorders; including attention deficit hyperactivity disorder, alcoholism, schizophrenia, and Parkinson's disease (C. Guindalini et al., 2006).

The processed gene products of the rat DAT and human DAT are 68,746 D (619 amino acids) and 68,495 D (620 amino acids) respectively (B. Giros et al., 1992; S. Shimada et al., 1991). Hydrophobicity analysis of the DAT predicts 12 membrane spanning domains with the N and C termini located in the cytoplasm as shown in Figure 3 (B. Giros et al., 1992; S. Shimada et al., 1991).

The DAT has multiple types of post-translational modifications. The second extracellular loop is predicted by sequence to contain 3 to 4 glycosylation sites, depending on the species (B. Giros et al., 1992; S. Shimada et al., 1991). The extent of glycosylation on the DAT can vary greatly depending upon the stage of development, species, and area of the brain (R. Lew et al., 1992; A.P. Patel et al., 1994; R.A. Vaughan et al., 1996). Using exo and endoglycosidases, it was determined that the glycosylation

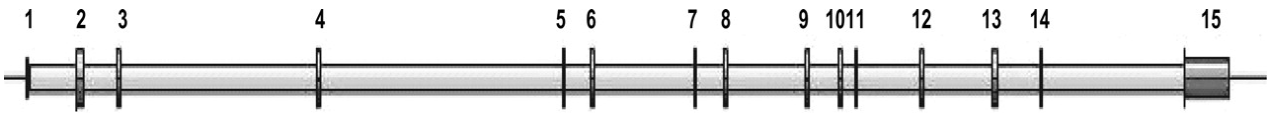

Figure 2. Gene structure of the dopamine transporter. Numbered segments are exons and introns are the segments in between. Modified from Guindalini et al. (2006) Proc. Natl. Acad. Sci. USA 103, 4552-4557.

was made of complex carbohydrates and not high in mannose (C.J. Cao et al., 1989; R. Lew et al., 1991). The western blot analysis of the DAT in the nucleus accumbens displays a higher weight species (76 kD) than in the striatum (72 kD) (R. Lew et al., 1992). Removal of sialic acids by neuraminidase causes a 5 kD decrease in molecular weight for both areas (R. Lew et al., 1991). It has been shown that removal of sialic acid residues severely inhibits transport of dopamine by the DAT (S.M. Meiergerd and J.O. Schenk, 1994; M. Zalewska and M. Erecinska, 1987). The extent and type of glycosylation, however, do not appear to alter inhibitor binding ([^{3} H]WIN 35,428) nor are they altered by treatment of various drugs of abuse (Chapters Four and Five) (R. Lew et al., 1992). The extent of glycosylation appears to be mainly important for the trafficking and stability of the DAT in the membrane (L.-B. Li et al., 2004).

The DAT is also phosphorylated (J.D. Foster et al., 2002). The N terminus has multiple phosphorylation sites which are important for signaling in terms of trafficking and internalization (M.A. Cervinski et al., 2005; R.A. Vaughan et al., 1997). METH increases protein kinase C activity and DAT phosphorylation which leads to internalization of the DAT (R.A. Vaughan et al., 1997). Inhibition of protein kinase A reduces dopamine transport by the DAT, suggesting another regulatory mechanism (M. Batchelor and J.O. Schenk, 1998). Additionally, active ubiquitination sites have recently been found on the amino terminus (M. Miranda et al., 2006; M. Miranda et al., 2005). Therefore, the DAT is an active signaling partner and the activity of this protein depends on multiple proteins and substrates.

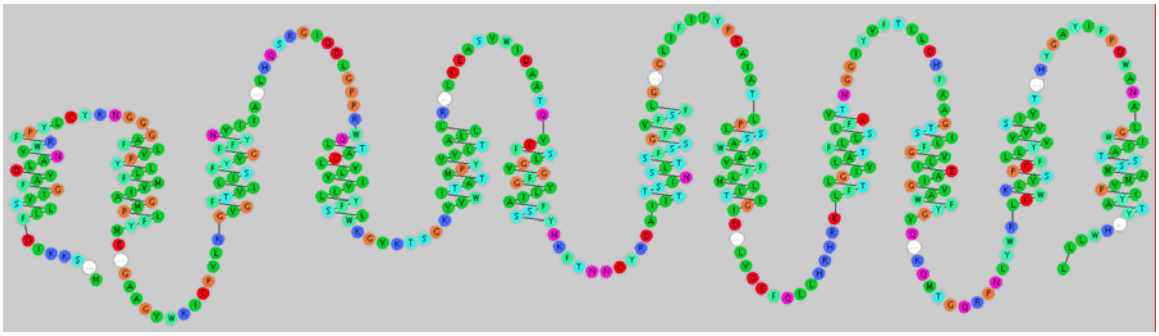

Figure 3. Rat dopamine transporter secondary structure diagram. The extracellular loops are on top and the intracellular loops are on the bottom connected by alpha helices spanning the plasma membrane. The N and C-termini are located intracellularly. Residues are colored according to residue types (the color scheme colors residues of similar physicochemical properties in a similar way). The white circles represent contiguous stretches of residues which are omitted. This diagram was created by exporting the rat DAT sequence from the EXPASY database and using the Residue-based diagram of proteins (RbDe) program provided by the Institute for Computational Biomedicine at the Weill Medical College at Cornell University. (F. Campagne and H. Weinstein, 1999)

The DAT is also hypothesized to contain a Zn^{2+} binding site (L. Norregaard et al., 1998). Residues His^{193}, His^{375}, and Glu^{396} make up the binding site (See Figure 4) (C.J. Loland et al., 1999). Zn^{2+} usually has four coordination sites in proteins, therefore the last coordinate would be occupied by a water molecule (I.L. Alberts et al., 1998). The presence of Zn^{2+} inhibits dopamine transport by a noncompetitive mechanism, suggesting a sort of regulatory mechanism if Zn^{2+} is available in the synapse to the DAT (L. Norregaard et al., 1998 and Chapter Three). The presence of Zn^{2+} also affects cocaine inhibition, offering insight into possible cocaine antagonists (E.K. Richfield, 1993). Please see Chapter Three for a complete report.

The DAT and Drugs of Abuse

Methamphetamine

The DAT is the primary target for various drugs of abuse including METH and cocaine. METH is a substrate of the DAT and acts as a competitive inhibitor (H.K. Wayment et al., 1999). METH competes with dopamine in binding to the DAT and can be transported into the cell by the DAT (E.A. Anderzhanova et al., 2005). METH has a half life of up to 70 hrs in humans and the effects of the drugs last from 6-12 hours (J. Caldwell et al., 1972; S.B. Karch, 1996). The toxicological longevity of this molecule enhances the toxicity and damage it can do to cells (E.A. Anderzhanova et al., 2005).

Once the METH is inside the cell, it can be transported into synaptic vesicles and cause release of dopamine from the vesicles into the cytoplasm of the presynaptic neuron (F.J. White and P.W. Kalivas, 1998). The increase in dopamine concentration causes the DAT to reverse direction and transport dopamine into the synapse, instead of taking it up

into the pre-synaptic neuron (E.A. Anderzhanova et al., 2005). This process is termed efflux and once the dopamine is transported into the synapse it can bind to dopamine receptors on both the pre and post-synaptic neurons, thus altering neurotransmission. Along with efflux, the release of dopamine from the synaptic vesicles in the presynaptic neuron can cause other disruptions to the cell. The oxidizing environment of the cytosol (versus the synaptic vesicle) causes dopamine to oxidize forming semi-quinone radicals which are very reactive and can, upon further reaction, form reactive oxygen species (E.A. Anderzhanova et al., 2005; F.J. White and P.W. Kalivas, 1998) Reactive oxygen species can cause many different forms of cell damage, ranging from DNA damage to lipid peroxidation (E.A. Anderzhanova et al., 2005). There are repair systems in the cell to fix this damage, but these systems can be overloaded and may not be able to repair all the damage.

METH exposure alters the trafficking of the DAT as well. Immediately after exposure, the DAT density of the plasma membrane is increased (L.A.A. Johnson et al., 2005). After 30 min the density is decreased by internalization of the DAT (C. Saunders et al., 2000). It has been found in an injection paradigm which models a METH binge situation that the DAT levels return to normal after 24 hours (A.E. Fleckenstein et al., 1997). The results of METH exposure can also vary on the injection schedule/protocol (R. Stefanski et al., 1999; R. Stefanski et al., 2002). It is also known that rats are not as sensitive to METH as

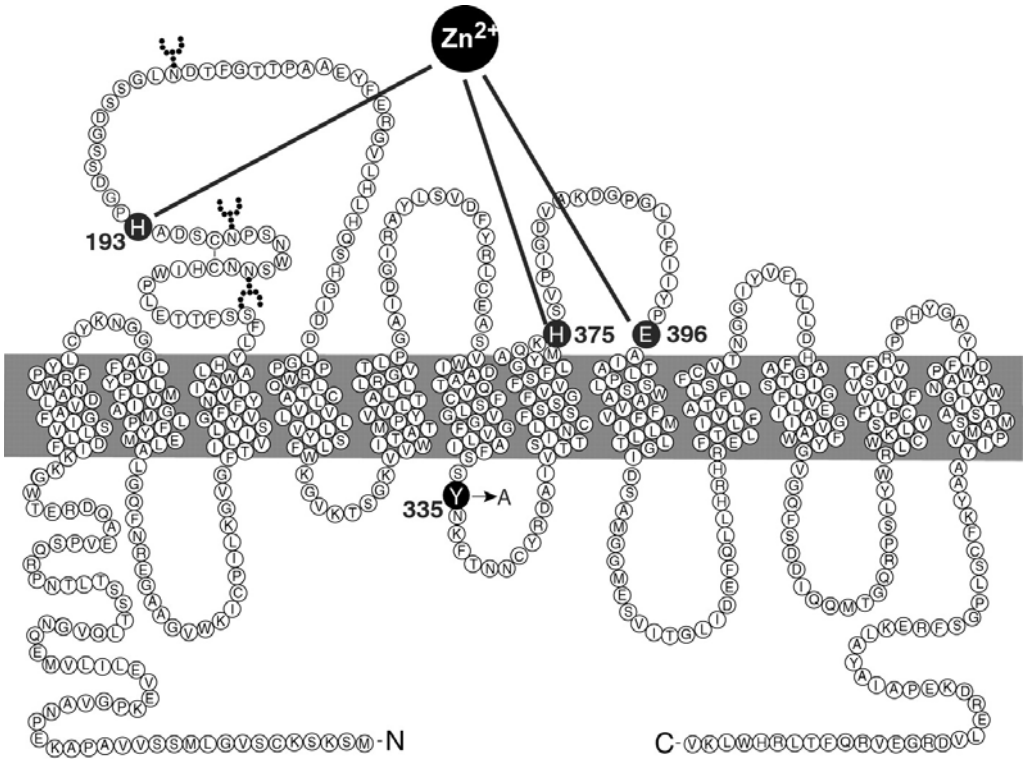

Figure 4. Zn^{2+} binding site on the dopamine transporter. Norregaard, L. et al (1998) EMBO J. 17(15), 4266-4273.

humans. For instance, the effects don't last as long and METH can be metabolized more efficiently (J. Caldwell et al., 1972). Therefore, if METH results in minimal damage in rats this may not directly correlate to humans.

Despite differences in experimental methods and conditions, it can be concluded that METH alters DAT levels at the plasma membrane. One very important study looked at abstinent METH abusers 4 years later and found their DAT levels were lower than in people who had never used METH (N.D. Volkow et al., 2001). This suggests that METH abuse may have very long lasting effects in human. Chapter Four is a detailed molecular study of DAT alterations in rats sensitized to METH.

Cocaine

Cocaine also inhibits dopamine transport causing an increase of dopamine in the synapse; however, the mechanism of inhibition is different than that of METH. Cocaine can bind to the DAT and inhibit transport in a competitive, noncompetitive, and uncompetitive mechanism depending upon experimental parameters (J.S. McElvain and J.O. Schenk, 1992; S.L. Povlock et al., 1996; S.L. Povlock and J.O. Schenk, 1997). However, the results are similar in that DAT is blocked from taking dopamine back up into the synapse and dopamine is able to continue to bind and stimulate receptors. Cocaine is rapidly broken down in the cellular milieu by esterases and the physiological effects of cocaine only last around 20 min in humans (S.B. Karch, 1996; D.J. Stewart et al., 1979). The metabolized molecules of cocaine are readily excreted by the body (F. Fish and W.D.C. Wilson, 1969; D.J. Stewart et al., 1979).

Despite the relatively quick high and degradation of cocaine, the compound is extremely addictive (E.J. Nestler, 2006). Researchers at NIDA are actively searching for

a therapeutic capable of combating cocaine abuse and addiction (J.L. Flippen-Anderson et al., 2003). Chapter Five documents a study in which rats were sensitized to cocaine, thus modeling a specific stage in the addiction process; craving. After 24 hours and 3 weeks the dopamine transport velocity and DAT density were measured to assess how they had been altered. The study of how specific areas and proteins in the brain are altered due to exposure to drugs of abuse can aid in the understanding of how to develop therapeutics. This kind of study is important for both the study of drug addiction and brain disease.

The following dissertation documents research concerning two different aspects concerning the DAT; 1) examining the effects of Zn^{2+} on DAT activity, cocaine inhibition, and DAT preparation and 2) investigating how drugs of abuse alter DAT activity, density, and glycosylation.

The text for each chapter is in the format of the journal to which it was submitted, published. Chapter Two is a detailed essay on the theory of studying kinetics using rotating disk electrode voltammetry and is in the format of *Journal of Neuroscience Methods*. Chapter Three is a kinetic study of how Zn^{2+} affects the DAT and cocaine binding and is in the format for *European Journal of Pharmacology*. Chapter Four is a molecular and activity study of how methamphetamine treatment affects DAT activity, METH inhibition, and glycosylation and is formatted for *Synapse*. Chapter Five is study of how sensitizing cocaine treatment alters DAT activity, density, and glycosylation and is in the format of *Neuropharmacology*.

REFERENCES

Alberts, I.L., Nadassy, K. Wodak, S.J., 1998. Analysis of zinc binding sites in protein crystal structures. Protein Science. 7, 1700-1716.

Anderzhanova, E.A., Oja, S.S., Saransaari, P. Rayevsky, K.S., 2005. 'non-specific' mechanisms of neuronal damage in amphetamine neurotoxicity in the rodent striatum. Biogenic Amines. 19, 1-23.

Batchelor, M. Schenk, J.O., 1998. Protein kinase a activity may kinetically upregulate the striatal transporter for dopamine. Journal of Neuroscience. 18, 10304-10309.

Caldwell, J., Dring, L.G. Williams, R.T., 1972. Metabolism of [14c]methamphetamine in man, the guinea pig, and the rat. Biochemical Journal. 129, 11-22.

Campagne, F. Weinstein, H., 1999. Schematic representation of residue-based protein context-dependent data: An application to transmembrane proteins. J Mol Graph Model FIELD Full Journal Title:Journal of molecular graphics & modelling. 17, 207-213.

Cao, C.J., Young, M.M., Wong, J.B., Mahran, L.G. Eldefrawi, M.E., 1989. Putative cocaine receptor in striatum is a glycoprotein with active thiol function. Membr Biochem FIELD Full Journal Title:Membrane biochemistry. 8, 207-220.

Cervinski, M.A., Foster, J.D. Vaughan, R.A., 2005. Psychoactive substrates stimulate dopamine transporter phosphorylation and down-regulation by cocaine-sensitive and protein kinase c-dependent mechanisms. Journal of Biological Chemistry. 280, 40442-40449.

Cooper, J.R., Bloom, F.E. Roth, R.H., 2003, The biochemical basis of neuropharmacology (Oxford Press, Oxford) p. 405.

Fish, F. Wilson, W.D.C., 1969. Excretion of cocaine and its metabolites in man. Journal of Pharmacy and Pharmacology. 21, 135-138.

Fleckenstein, A.E., Metzger, R.R., Gibb, J.W. Hanson, G.R., 1997. A rapid and reversible change in dopamine transporters induced by methamphetamine. European Journal of Pharmacology. 323, R9-R10.

Flippen-Anderson, J.L., George, C. Deschamps, J.R., 2003. Molecular approaches to treatments for cocaine abuse. Journal of Molecular Structure. 647, 259-267.

Foster, J.D., Pananusorn, B. Vaughan, R.A., 2002. Dopamine transporters are phosphorylated on n-terminal serines in rat striatum. Journal of Biological Chemistry. 277, 25178-25186.

Giros, B., el Mestikawy, S., Godinot, N., Zheng, K., Han, H., Yang-Feng, T. Caron, M.G., 1992. Cloning, pharmacological characterization, and chromosome assignment of the human dopamine transporter. Mol Pharmacol. 42, 383-390.

Greenwood, T.A. Kelsoe, J.R., 2003. Promoter and intronic variants affect the transcriptional regulation of the human dopamine transporter gene. Genomics. 82, 511-520.

Guindalini, C., Howard, M., Haddley, K., Laranjeira, R., Collier, D., Ammar, N., Craig, I., O'Gara, C., Bubb, V.J., Greenwood, T., Kelsoe, J., Asherson, P., Murray, R.M.,

Castelo, A., Quinn, J.P., Vallada, H. Breen, G., 2006. A dopamine transporter gene functional variant associated with cocaine abuse in a brazilian sample. Proceedings of the National Academy of Sciences of the United States of America. 103, 4552-4557.

Johnson, L.A.A., Furman, C.A., Zhang, M., Guptaroy, B. Gnegy, M.E., 2005. Rapid delivery of the dopamine transporter to the plasmalemmal membrane upon amphetamine stimulation. Neuropharmacology. 49, 750-758.

Kandel, E.R., Schwartz, J.H. Jessell, T.M., 2000, Principles in neural science (McGraw-Hill, New York) p. 1414.

Karch, S.B., 1996, The pathology of drug abuse (CRC Press, Boca Raton) p. 462.

Lew, R., Grigoriadis, D., Wilson, A., Boja, J.W., Simantov, R. Kuhar, M.J., 1991. Dopamine transporter: Deglycosylation with exo- and endoglycosidases. Brain Research. 539, 239-246.

Lew, R., Patel, A., Vaughan, R.A., Wilson, A. Kuhar, M.J., 1992. Microheterogeneity of dopamine transporters in rat striatum and nucleus accumbens. Brain Research. 584, 266-271.

Lew, R., Vaughan, R., Simantov, R., Wilson, A. Kuhar, M.J., 1991. Dopamine transporters in the nucleus accumbens and the striatum have different apparent molecular weights. Synapse (New York, NY, United States). 8, 152-153.

Li, L.-B., Chen, N., Ramamoorthy, S., Chi, L., Cui, X.-N., Wang, L.C. Reith, M.E.A., 2004. The role of n-glycosylation in function and surface trafficking of the human dopamine transporter. Journal of Biological Chemistry. 279, 21012-21020.

Loland, C.J., Norregaard, L. Gether, U., 1999. Defining proximity relationships in the tertiary structure of the dopamine transporter. Identification of a conserved glutamic acid as a third coordinate in the endogenous zn(2+)-binding site. J Biol Chem. 274, 36928-36934.

McElvain, J.S. Schenk, J.O., 1992. A multisubstrate mechanism of striatal dopamine uptake and its inhibition by cocaine. Biochemical Pharmacology. 43, 2189-2199.

Meiergerd, S.M. Schenk, J.O., 1994. Striatal transporter for dopamine: Catechol structure-activity studies and susceptibility to chemical modifications. Journal of Neurochemistry. 62, 998-1008.

Miranda, M., Dionne, K.R., Sorkina, T. Sorkin, A., 2006. Three ubiquitin conjugation sites in the amino terminus of the dopamine transporter mediate protein kinase c-dependent endocytosis of the transporter. Molecular Biology of the Cell. 18, 313-323.

Miranda, M., Wu, C.C., Sorkina, T., Korstjens, D.R. Sorkin, A., 2005. Enhanced ubiquitylation and accelerated degradation of the dopamine transporter mediated by protein kinase c. Journal of Biological Chemistry. 280, 35617-35624.

Nestler, E.J., 2006. Molecular mechanisms of drug addiction. Cell Biology of Addiction. 393-412.

Norregaard, L., Frederiksen, D., Nielsen, E.O. Gether, U., 1998. Delineation of an endogenous zinc-binding site in the human dopamine transporter. Embo J. 17, 4266-4273.

Patel, A.P., Cerruti, C., Vaughan, R.A. Kuhar, M.J., 1994. Developmentally regulated glycosylation of dopamine transporter. Developmental Brain Research. 83, 53-58.

Povlock, S.L., Meiergerd, S.M. Schenk, J.O., 1996, Kinetic mechanism of the dopamine transporter: A comparison with other biogenic amine transporters, in: Cns neurotransmitters and neuromodulators: Dopamine, ed. T.W. Stone (CRC Press, Boca Raton) p. 21-39.

Povlock, S.L. Schenk, J.O., 1997. A multisubstrate kinetic mechanism of dopamine transport in the nucleus accumbens and its inhibition by cocaine. Journal of Neurochemistry. 69, 1093-1105.

Richfield, E.K., 1993. Zinc modulation of drug binding, cocaine affinity states, and dopamine uptake on the dopamine uptake complex. Molecular Pharmacology. 43, 100-108.

Saunders, C., Ferrer, J.V., Shi, L., Chen, J., Merrill, G., Lamb, M.E., Leeb-Lundberg, L.M.F., Carvelli, L., Javitch, J.A. Galli, A., 2000. Amphetamine-induced loss of human dopamine transporter activity: An internalization-dependent and cocaine-sensitive mechanism. Proceedings of the National Academy of Sciences of the United States of America. 97, 6850-6855.

Shimada, S., Kitayama, S., Lin, C.L., Patel, A., Nanthakumar, E., Gregor, P., Kuhar, M. Uhl, G., 1991. Cloning and expression of a cocaine-sensitive dopamine transporter complementary dna. Science (Washington, DC, United States). 254, 576-578.

Stefanski, R., Ladenheim, B., Lee, S.H., Cadet, J.L. Goldberg, S.R., 1999. Neuroadaptations in the dopaminergic system after active self-administration but not after passive administration of methamphetamine. European Journal of Pharmacology. 371, 123-135.

Stefanski, R., Lee, S.-H., Yasar, S., Cadet, J.L. Goldberg, S.R., 2002. Lack of persistent changes in the dopaminergic system of rats withdrawn from methamphetamine self-administration. European Journal of Pharmacology. 439, 59-68.

Stewart, D.J., Inaba, T., Lucassen, M. Kalow, W., 1979. Cocaine metabolism: Cocaine and norcocaine hydrolysis by liver and serum esterases. Clinical Pharmacology & Therapeutics (St. Louis, MO, United States). 25, 464-468.

Vaughan, R.A., Brown, V.L., McCoy, M.T. Kuhar, M.J., 1996. Species- and brain region-specific dopamine transporters: Immunological and glycosylation characteristics. Journal of Neurochemistry. 66, 2146-2152.

Vaughan, R.A., Huff, R.A., Uhl, G.R. Kuhar, M.J., 1997. Protein kinase c-mediated phosphorylation and functional regulation of dopamine transporters in striatal synaptosomes. Journal of Biological Chemistry. 272, 15541-15546.

Volkow, N.D., Chang, L., Wang, G.-J., Fowler, J.S., Franceschi, D., Sedler, M., Gatley, S.J., Miller, E., Hitzemann, R., Ding, Y.-S. Logan, J., 2001. Loss of dopamine transporters in methamphetamine abusers recovers with protracted abstinence. Journal of Neuroscience. 21, 9414-9418.

Wayment, H.K., Deutsch, H., Schweri, M.M. Schenk, J.O., 1999. Effects of methylphenidate analogs on phenethylamine substrates for the striatal dopamine transporter: Potential as amphetamine antagonists? Journal of Neurochemistry. 72, 1266-1274.

White, F.J. Kalivas, P.W., 1998. Neuroadaptations involved in amphetamine and cocaine addiction. Drug and Alcohol Dependence. 51, 141-153.

Wise, R.A., 1998. Drug-activation of brain reward pathways. Drug and Alcohol Dependence. 51, 13-22.
Zalewska, M. Erecinska, M., 1987. Involvement of sialic acid in high-affinity uptake of dopamine by synaptosomes from rat brain. neuroscience letters. 82, 107-112.

Unraveling neuronal dopamine transporter mechanisms with rotating disk electrode voltammetry

James O. Schenk[a,b,c*], Cortney Wright[a], and Nicole Bjorklund[b]

[a]Department of Chemistry,[b] School of Molecular Biosciences, and [c]Programs in Pharmacology/Toxicology and Neuroscience, Washington State University, Pullman, WA 99164

*Corresponding author. Department of Chemistry, Washington State University, Pullman, WA 99164-4630

E-mail address: geni@wsu.edu

Abstract

Herein we describe how the rotating disk electrode voltammetric technique can be used to examine the mechanism(s) of the inward transport of dopamine by the neuronal transporter for dopamine (DAT). The usefulness of making measurements kinetically resolving dopamine transport, interpretations of changes in K_m and V_{max}, approaches to defining pre-steady state binding of dopamine to DAT, interactions between competing inhibitors, chemical modification of functional groups within DAT, and a presentation of a hypothetical multi-state model of dopamine transport are presented and discussed.

Key words: cocaine, amphetamine, transporter mechanism

1. Introduction

In dopaminergic chemical neurotransmission dopamine is released from a presynaptic cell and a local extracellular concentration-time (C-T) profile results. At first the extracellular concentration of dopamine increases until release modulating autoreceptors begin to slow the process. Extracellular dopamine is then cleared from synaptic areas by diffusion and reuptake via the dopamine transporter, DAT (Schenk and Bunney, 1987; Amara and Kuhar, 1991; Garris and Wightman, 1994; Torres et al., 2003). The extracellular dopamine is the communicating signal that is ultimately transduced into physiological function. Alterations in this signal manifested by a change in the dopamine C-T profile via changes in regulation of DAT or by pharmacological effects on DAT will influence how dopamine modulates its own release and the functioning of dopamine sensitive postsynaptic neurons.

Very briefly, the DAT is a Na^+ and Cl^- dependent, 80 kDa transporter comprised of 619 amino acids with 12 transmembrane regions which have varying functional roles. The first five transmembrane regions are thought to be involved in functions common to all members of the family including ion dependence of transport. Transmembrane spanning regions six through eight appear to be the target sites for inhibitors while regions nine through the C-terminus contain the determinants for substrate affinity and stereoselectivity (Kitayama et al., 1992; Giros et al.,1993; Buck and Amara, 1994). A recent review of DAT and its structure and function can be consulted for more molecular details of the DAT (Torres et al., 2003).

Understanding the function, regulation, and pharmacology of DAT is very important in defining dopaminergic signaling. Alteration of DAT function can cause three types of modulation to chemical neurotransmission: (a) postsynaptic sites could "see" only changes in the magnitude of the dopamine signal with no changes in timing (amplitude modulated signaling), (b) a prolongation of the C-T profile with minimal changes in the magnitude of $[DA]_o$ (frequency modulated signaling) and (c), a combination of both (a) and (b). These alterations in signal affect downstream pathways that can ultimately lead to altered physiological function. Thus, it is important to be able to assess kinetic changes in DAT activity.

Our laboratory has spent a number of years developing a technique, rotating disk electrode voltammetry (RDEV), to resolve the kinetic activity of DAT in various neuronal preparations and under various experimental conditions. Reviews and other presentations of the experimental approach have been made (Meiergerd and Schenk, 1997; Earles et al., 1998; Earles and Schenk, 1998). Further a recent review of the results of studies of DAT kinetic mechanisms has appeared (Schenk, 2002; Schenk et al., 2003). Thus, this presentation is focused on how data from work with RDEV or other methods to measure the kinetics of DAT may be analyzed and interpreted.

2. Kinetic resolution of DAT activity

Cell membranes are essentially impermeable to water soluble, polar and charged molecules (Stein, 1986, 1989; 1990; Weiss; 1996). The DAT functions to catalyze the movement of dopamine across the neuronal membrane. Thus, the DAT can be viewed as

analogous to an enzyme (Meiergerd and Schenk, 1994a) – it chemically recognizes dopamine based on chemical structure, binds dopamine, and undergoes a conformational change to lower the energy barrier for the movement of dopamine across the membrane. After the movement of dopamine across the membrane the DAT reorients or "relaxes" to undergo another cycle. This process is very rapid; therefore a system to kinetically resolve DAT activity is required to study it. It is known that the dopamine transported as described begins outside the cell and ends up inside. These are the beginning and ending conditions for dopamine transported by DAT. This is a thermodynamic statement. Dopamine and other players in this scheme could act to move between these two conditions in perhaps an infinite number of ways. Thus, measurements of DAT in the kinetic domain, continuously monitoring dopamine concentration changes, are required to begin to develop hypotheses about the mechanism of dopamine transport by DAT.

Fig. 1 is an illustration of the set-up we developed to measure the kinetics of DAT. The references cited above can be consulted for the details of construction and use. In brief, the system consists of a thermostatted, small volume (< 500 μL) incubation chamber with reference and auxiliary electrodes sealed into the glass walls. A preparation of dopamine nerve terminals or cells expressing DAT is placed into the incubation chamber in physiological buffer and then a glassy carbon rotating disk electrode is immersed into the preparation. Then a potential sufficient to oxidize and detect dopamine is applied. This results in a detection current signal which is offset and monitored in a way completely analogous to high pressure liquid chromatography with electrochemical detection except that the potentiostat in this application responds very rapidly. The instrument response time plus the incubation chamber mixing time is

around 20 milliseconds. The experiment is initiated by addition of agents to cause release of dopamine from the preparation or by addition of dopamine itself. The signal is then monitored. If the signal has timing that is longer than the instrumental response plus the mixing time of the system then it is kinetically resolved. We have shown that RDEV kinetically resolves DAT function in synaptosomes (McElvain and Schenk, 1992), tissue homogenates (McElvain and Schenk, 1992; Povlock and Schenk, 1997), and in suspensions of cells in an expression system (Earles and Schenk, 1999).

3. General kinetic studies of DAT and interpretation of kinetic parameters

The scheme for dopamine binding and transport can be simply represented as,

$$DA + T \underset{k_2}{\overset{k_1}{\rightleftharpoons}} DA\text{-}T \xrightarrow{k_3} DA + T$$

where DA is dopamine, T is the transporter, DA-T is dopamine bound to the transporter, k_1 is the binding constant, k_2 is the dissociation constant, and k_3 is the translocation constant. This model should be referred to for a more complete understanding of mathematical models presented below.

In general studies of the DAT the experimenter measures the initial velocity of transport of dopamine as a function of dopamine concentration and fits the result to the Michaelis-Menten expression preferably by non-linear curve fitting (Ratkowski, 1990),

$$\upsilon = \frac{V_{\max}[DA]_0}{[DA]_0 + K_m} \qquad (1)$$

where formally K_m is the concentration of dopamine at half-maximal velocity and $V_{\max} = k_3[DAT]$, the product of the kinetic constant for the movement of dopamine

across the cell membrane and the density or concentration of DAT in the preparation. This value is dependent on tissue or cell concentrations and has to be normalized for tissue weight, cell count, or protein. Frequently this measurement is made to determine whether a drug or a change in state of the animal (Meiergerd et al., 1993; 1994a; Batchelor and Schenk, 1998) or a cell preparation affects the transport of dopamine. Values for K_m and V_{max} and changes in these values induced by an experimental manipulation are obtained. How are the results interpreted? First the experimenter needs to know the physical definition of the K_m, for it is not necessarily a dissociation constant as popularly viewed. There are guidelines to determine the form of the constant (see Fersht, 1985; Segel, 1993). For example, if k_3 is comparable to k_2 then the K_m = the dissociation constant of dopamine from DAT. Values of these constants for DAT from estimations in the literature (Meiergerd and Schenk, 1994a; Earles and Schenk, 1999; Rudnick and Clark, 1993) suggest that k_1 is very fast and probably under diffusion control, k_2 is difficult to measure because it is slow and k_3 is in the range 1 to 10 s^{-1}. Thus, the form of the K_m is likely to be,

$$K_m = \frac{k_3 + k_2}{k_1} \qquad (2)$$

where the kinetic constant, k_3, within V_{max} is also present in the value of K_m. Thus, if V_{max} were found to change with no change in K_m then one may assume that the value for [DAT] was the likely change and if K_m and V_{max} proportionally changed then it may be assumed that k_3 may have changed, and finally if K_m changes without a change in V_{max} then the binding of dopamine at DAT could be assumed to have changed. These findings should then be used to guide further investigations such as studies of membrane trafficking, separate ligand binding studies, and so forth.

Based on results of velocity changes in dopamine transport at different dopamine and drug concentrations Eq. 1 can be modified to fit the effects of competitive, noncompetitive and uncompetitive inhibitions. These forms of Eq 1 are commonly encountered (see Fersht, 1985; Segel, 1993). For the purposes of this discussion, however, it should be pointed out that dopamine signaling would be altered differently between the three possibilities. Competitive inhibition would produce the most change in the timing of extracellular dopamine clearance at low dopamine concentrations, where in noncompetitive inhibition the effect is independent of dopamine concentration, and finally in uncompetitive inhibition the inhibition observed increases with the extracellular dopamine concentration. From a physiological point of view these differing effects would alter the degree and timing of postsynaptic receptor occupation by dopamine (Meiergerd and Schenk, 1994b) and be a basis for the different types of signal modifications suggested in the Introduction.

4. Pre-Steady State Kinetics

The study of steady state kinetics is key to understanding enzymatic mechanisms as shown in the previous section. However, this method is only able to reveal effects on the slowest step in the reaction sequence. In order to investigate the steps before the rate-limiting step an investigator must look at the pre-steady state kinetics (Meiergerd et al., 1994b). In the study of dopamine transport by the DAT it is the binding step of dopamine to the transporter that is studied by pre-steady state kinetics. The value of pre-steady state measurements is that binding and translocation steps can be differentiated.

This allows an investigator to study how certain substrates and inhibitors affect the binding of dopamine to the DAT.

There is now an arsenal of techniques for studying pre-steady state kinetics. These include stopped-flow, rapid quench, and relaxation techniques just to name a few. These are all acceptable for certain experiments and conditions. Our lab has found RDEV to be a good tool for measuring the binding of dopamine in the kinetic time scale (Meiergerd et al. 1994b). It allows the direct measurement of dopamine binding to the transporter in real time. The binding of dopamine to the DAT is under diffusion control, therefore requiring rapid measurement. RDE voltammetry has been measured to have a resolution of $\leq$ 20ms and can routinely detect dopamine concentrations of at levels $< K_m$, thereby making it more than capable of measuring this phenomenon.

There are two different methods using RDEV to delineate the pre-steady state kinetics of dopamine binding that have been explored in this lab. The first is by the use of the pseudo-first order rate equation and the second is using the second order binding equation. These require a few important assumptions to be made. These assumptions are 1) that [DA]>>[DAT], 2) the binding rate, k_1>> the translocation rate, k_3 and 3) that the chemistry (translocation) is irreversible. There are limitations to each of these methods; however they both have value.

Use of the pseudo-first order rate equation takes advantage of the fact that a drop in temperature will alter translocation kinetics and lengthen the time window in which translocation occurs. Lowering the dopamine concentration to less than the value of K_m also facilitates observation of the binding of dopamine (Wayment et al. 1998; Earles and Schenk 1999) and RDEV has detection and quantification limits sufficient to make this

measurement (Meiergerd et al., 1994b; Earles and Schenk, 1998). By using these conditions, the integrated first order rate expression can be used to calculate the rate constants (Capello and Bielski, 1972). The integrated first order rate equation is:

$$k_1' = \frac{1}{t} \ln \frac{[DA]_0}{([DA]_0 - [DA])} \quad (3)$$

In this equation, k_1' is the binding rate constant, t is the time elapsed, $[DA]_0$ is the initial dopamine concentration and [DA] is the dopamine concentration at time t. The values obtained from non-linear curve fitting are fit to this expression. This approach does not require a value for [DAT]. This method is useful when screening for substrates or inhibitors that affect dopamine binding.

Lowering substrate concentration to pre-steady state levels ($<<K_m$) and measuring the velocity of dopamine transport under physiological conditions (37°C) can also be used to study dopamine binding (Meiergerd et al., 1994b). However, this requires the use of the second order binding equation. The rate of the reaction is given by: (Capellos and Bielski, 1972)

$$\frac{dx}{dt} = k([DA]_0 - x)([DAT]_0 - x) \quad (4)$$

At time (t) = 0, the dopamine (DA) and DAT concentrations are symbolized as $[DA]_0$ and $[DAT]_0$, respectively. When time has elapsed, these concentrations are designated as $[DA]_0 - x$ and $[DAT]_0 - x$, and the concentration of DA bound to DAT is x. The second order binding constant is k.

The integration of this equation leads to:

$$kt = \frac{1}{([DA]_0 - [DAT]_0)} \ln \left[\frac{[DAT]_0([DA]_0 - x)}{[DA]_0([DAT]_0 - x)} \right] \quad (5)$$

This integration is used only when $[DA]_o \neq [DAT]_0$. The concentration of DAT must be estimated or measured in order to use this expression. By fitting the dopamine transport velocities to this rate equation an investigator can obtain the second order binding constant for dopamine binding to the DAT. This method compliments and helps complete the steady state mechanistic picture of dopamine transport in the DAT.

5. Multisubstrate kinetic mechanism(s) of DAT functioning

The kinetic mechanism of dopamine uptake (Fig. 2) involves the binding of dopamine and Na^+ to the dopamine transporter followed by Cl^- binding (see McElvain and Schenk, 1992, Povlock and Schenk, 1997, and Povlock et al. 1996). Upon completion of binding events the transporter reorients in the membrane and releases dopamine into the cytosol (McElvain and Schenk, 1992) where it can be sequestered in storage vesicles or degraded by monoamine oxidase (MAO) to dihydroxy phenyl acetate (DOPAC). Under basal conditions dopamine concentrations are ~10^4 higher intracellularly as compared to that in the extracellular medium (McElvain and Schenk, 1992; Povlock and Schenk, 1997). Therefore the asymmetric nature of the dopamine transporter enables the clearance of dopamine from the extracellular milieu into dopaminergic neurons. In addition, the rat DAT exhibits apparent transacceleration (Meiergerd et al, 1994*a*; Povlock and Schenk, 1997), a characteristic of transporters as opposed to pores or channels (Weiss, 1996). This characteristic suggests that the rate-limiting step in the transport cycle is the reorientation of the inward facing conformation of the transporter to its outward facing form.

Some drugs of abuse such as amphetamine alter the asymmetry of the dopaminergic transporter by causing release of cytosolic dopamine via the DAT whereas cocaine does not appear to do so. The kinetic events related to the alteration in functioning are not known and likely are mediated by changes in the kinetics of transitions between the inward and outward conformations of the transporter (Meiergerd et al., 1994b; Wayment et al., 1995; Schenk, 2002).

The four state model of Fig 3 may represent the action of transporters where the accompanying rate constants are depicted by the lower case letters (Stein, 1986; Weiss, 1996):

A three state model for transporters has also been proposed and a justification of the four state model is given in Schenk (2002). Both of these models assume the transporter is a protein undergoing enzyme-like changes rather than opening like a channel or employing ferry boat mechanisms (Stein, 1986; 1990; Weiss, 1996). The equation describing unidirectional flux is identical in both versions (Stein, 1986) and is given by

$$\nu_{12} = \frac{KS_1 + S_1S_2}{K^2R_{00} + KR_{12}S_1 + KR_{21}S_2 + R_{ee} + S_1S_2} \qquad (6)$$

where the subscripts "1" and "2" refer to different sides of the membrane, the velocity is a function of substrate concentrations S_1 and S_2 at the two faces of the membrane, the R's are resistance (equal to $1/V_{max}$ and the rate constants relevant to the direction of transport), K is a constant (defined as $K = \frac{h_1}{b_2} + \frac{h_2}{k_1}$ obtained by collecting the rate constants (Stein, 1986; Weiss, 1996), and the labels on the R values indicate direction of transport except for ee and 00 signifying equilibrium exchange and kinetic activity of the unloaded transporter, respectively. This expression indicates that an increase in

concentration of substrate on the opposite side of the membrane from which the transport experiment is initiated positively influences the rate of transport toward that side. It is considered to be a principle test to distinguish transporters from pores or channels (Stein, 1986; Weiss, 1996) and in virtually all of the transport systems tested in the literature have observed this qualitative characteristic (Stein, 1986; Weiss, 1996). Additionally, using Michaelis-Menten kinetics and the constant, K, for the four state model, it is possible to determine or set limits on the values of the rate constants for the turnover of the transporter between inward and outward conformations. With knowledge of the kinetics of transporter reorientation in the membrane it will be possible to study the effects of drugs of abuse on these processes and gain more insight into mechanistic function of drugs such as amphetamine, methamphetamine, and cocaine. Further because a drug could bind to the intracellular side, the extracellular side or both sides, models such as that of Fig. 3 need to be taken into consideration. The basis for considering these more complex possibilities is given by Deves and Krupka (1989), Krupka and Deves (1983) and Deves (1991).

6. Studies of competing inhibitors of membrane transport by DAT

An area of intense interest with regard to DAT pharmacology is whether an antagonist to drugs of abuse (cocaine or amphetamine) can be developed. Thus a means of determining the relationships between inhibitor binding sites needs to be developed. The RDEV approach has provided one such approach by measuring the effects of DAT inhibitors, alone or in combination with a second inhibitor, on the velocities of kinetically

resolved dopamine transport. There are three possibilities: two inhibitors can react at a common site, two completely independent sites, or two sites that reciprocally influence binding. The expressions for these possibilities were developed for membrane transporters by Deves and Krupka (1980) and have been shown to be useful in examining the pharmacology of DAT. The expressions are relatively simple and have the forms,

$$\left(\frac{v}{v_b}-1\right)=\left(\frac{v}{v_{test1}}-1\right)+\left(\frac{v}{v_{test2}}-1\right) \qquad (7)$$

for simple, same site competition. The expression,

$$\left(\frac{v}{v_b}-1\right)=\left(\frac{v}{v_{test1}}-1\right)+\left(\frac{v}{v_{test2}}-1\right)+\left(\frac{v}{v_{test1}}-1\right)\left(\frac{v}{v_{test2}}-1\right)A \qquad (8)$$

for independent sites where A is an expression unique to the type of transport experiment being conducted. Here $A = (1 + \frac{f_1}{f_{-1}})\,(1 + \frac{[DA]_o}{K_m})$ where the ratio $\frac{f_1}{f_{-1}}$ is the equilibrium distribution of carrier between inward-facing and outward-facing forms. Finally, the expression,

$$\left(\frac{v}{v_b}-1\right)=\left(\frac{v}{v_{test1}}-1\right)+\left(\frac{v}{v_{test2}}-1\right)\left(1+\frac{[I]_{test1}}{K_{\text{int}}}\right) \qquad (9)$$

where K_{int} is the half saturation constant for test inhibitor 1 in the presence of saturating concentrations of test inhibitor 2.

In all of these expressions the v represents a measured transport velocity in the absence of an inhibitor (no subscript), in the presence of one or the other test inhibitor (subscripts *test1* and *test 2*), or in the presence of both inhibitors (subscript *b*). Experimentally, these values are all measured and the value observed in the presence of both inhibitors is compared to the predicted values. Making a distinction between binding models for inhibitors of DAT with agreement between predicted and

experimental values of v_b to within 5% have been demonstrated (Meiergerd and Schenk, 1994b, Wayment et al., 1998; 1999). A review of these studies has been published recently (Schenk, 2003).

7. Studies of the role of functional groups in membrane transport

A final set of experiments in which the RDEV approach has been useful is the examination of the effects of modification of functional groups on DAT activity. Most work to date has been performed by site directed mutagenesis (Giros and Caron, 1993; Kitayama et al., 1992). The modifications used here were covalent and noncovalent modification of existing functional groups within DAT. In early studies we showed that N-ethylmaleimide, a sulfhydryl alkylating agent, differentially inhibited dopamine transport at striatal DAT at low versus physiological temperatures suggesting that DAT can undergo conformational changes (Meiergerd and Schenk, 1994). More recently (Volz et al., 2004) we have shown that the RDEV can be used to detect changes in DAT activity following covalent and noncovalent chemical modification of arginine residues at DAT. Dopamine and some hydroxyl containing substrate analogs attenuated the effects of the arginine agents (phenylglyoxal, 2,3-butandione, 1,2-cyclohexanedione, and arginine cork) whereas non-hydoxyl containing substrate analogs were without effect. Further the inhibitor, cocaine, and some tropanes but not benztropine or inhibitors of the GBR family were able to attenuate the effects of arginine modification. These results suggest that the DAT related functional groups involved in substrate and inhibitor binding as well as structural features of substrates and inhibitors can be mapped.

In summary RDEV and the proper mathematical analysis are useful tools for examining the mechanism of dopamine transport by the DAT. This methodology can make direct measurement of the kinetics of DAT in real time, delineate binding from translocation steps in DAT activity in single experiments, and evaluate the mechanisms pharmacological agents on DAT activity. This approach can also be extended to studies of the mechanisms of the serotonin and norepinephrine transporters.

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References

Amara SG, Kuhar MJ. Neurotransmitter transporters: recent progress. Ann. Rev. Neurosci., 1993; 16: 73-93.

Bard AJ, Faulkner LR. Electrochemical Methods: Fundamentals and Applications. John Wiley and Sons: New York, 1980: 28.

Batchelor M, Schenk. JO. Protein kinase A activity may kinetically upregulate the striatal transporter for dopamine. J. Neurosci., 1998; 18: 10304-10309.

Buck KJ, Amara SG. Chimeric Dopamine-Norepinephrine Transporters Delineate Structural Domains Influencing Selectivity for Catecholamines and 1-Methyl-4-Phenylpyridinium. PNAS, 1994; 91:12584-12588.

Capellos C, Bielski BMJ. Kinetic Systems: Mathematical Description of Chemical Kinetics in Solution. Wiley- Interscience: New York, 1972: 14-18.

Devés R, Krupka RM. Inhibition kinetics of carrier systems. Meth. Enzymol., 1989; 171: 113-132.

Devés R, Krupka RM. Testing transport systems for competition between pairs of reversible inhibitors, inhibition of erythrocyte glucose transport by cytochalasin B and steroids. J. Biol. Chem, 1980; 255: 11870-11874.

Devés R. Kinetics of transport: characterizing the interaction of substrates and inhibitors with carrier systems. In Yudilevich DL, Deves R, Peran S, Cabantchik, editors. Cell Membrane Transport Experimental Approaches and Methodologies. Plenum Press: New York, 1991: 3.

Earles C, Schenk JO. Multisubstrate mechanism for the inward transport of dopamine by the human dopamine transporter expressed in HEK cells and its inhibition by

cocaine. Synapse, 1999; 33: 230-238.

Earles C, Schenk JO. Rotating disk electrode voltammetric measurements of dopamine transporter activity: an analytical evaluation. Analyt. Biochem., 1998; 264: 191-198.

Earles C, Wayment H, Green M, Schenk JO. Resolution of biogenic amine transporter kinetics by rotating disk electrode voltammetry: methodology and mechanistic interpretations. Meth. Enzymol., 1998; 296: 660-675..

Fersht A. Enzyme Structure and Mechanism. W. H. Freeman Co.: New York, 1985: 133.

Fromm HJ. Initial Rate Enzyme Kinetics. Springer-Verlag: New York, 1975.

Garris PA, Wightman RM. Different kinetics govern dopaminergic transmission in the amygdala, prefrontal cortex, and striatum: an in vivo voltammetric study. J. Neurosci., 1994; 14: 442-450.

Giros B, Caron MG. Molecular characterization of the dopamine transporter. Trends Pharmacol., 1993; 14: 43-49.

Kitayama S, Shimada S, Xu H, Markham L, Donovan DM, Uhl G. Dopamine transporter site-directed mutations differentially alter substrate transport and cocaine binding. Proc. Nat. Acad. Sci. USA, 1992; 89: 7782-7785.

Krupka RM, Devés R. Kinetics of inhibition of transport systems. Int. Rev. Cytol., 1983; 84: 303-352.

McElvain JS. Schenk JO. A multisubstrate mechanism for uptake of striatal dopamine uptake and its inhibition by cocaine. Biochem. Pharmacol., 1992a; 43: 2189-2199.

McElvain JS. Schenk JO. Dopamine autoreceptor blockade by haloperidol and the apparent dynamics of endogenous dopamine release from and reuptake into striatal suspensions. Neuropharmacology, 1992b; 31: 649-659.

Meiergerd SM, Hooks S, Schenk JO. The striatal transport of dopamine in the rat may be kinetically up-regulated following three weeks of withdrawal from induction of self-administration of cocaine. J. Neurochem., 1994a; 65: 127-128.

Meiergerd SM, McElvain JS, Schenk JO. The effects of cocaine and repeated cocaine followed by withdrawal: alteration of dopaminergic transporter turnover with no changes in the kinetics of substrate recognition. Biochem. Pharmacol., 1994b; 47: 1627-1634.

Meiergerd SM, Patterson TA, Schenk JO. D2 receptors may modulate the function of the striatal transporter for dopamine: direct evidence from studies of transporter function in vitro and in vivo. J. Neurochem., 1993; 61: 764-767.

Meiergerd SM, Schenk JO. Striatal transport for dopamine: catechol structure activity studies and susceptibility to chemical modification. J. Neurochem., 1994a; 62: 998-1008.

Meiergerd SM, Schenk JO. Kinetic evaluation of the commonality between the site of action of cocaine and some other inhibitors of the striatal transporter for dopamine. J. Neurochem., 1994b; 63: 1683-1692.

Meiergerd SM, Schenk JO. The measurement of the neuronal transport of biogenic amine in the kinetic domain. In Boulton AA, Baker G, Adams RN, editors. Neuromethods. Raven Press: New York, 1995; 27.

Povlock SL, Meiergerd SM, Schenk JO. Kinetic mechanism of the neuronal transport of dopamine. In Snow TW, editor. CNS Neurotransmitters and Neuromodulators: Dopamine. CRC Press: Boca Raton, 1996;. 21.

Povlock SL, Schenk JO. A multisubstrate kinetic mechanism of dopamine transport in the

nucleus accumbens and its inhibition by cocaine. J. Neurochem., 1997; 69: 1093-1105.

Ratkowski DA. Handbook of non-linear regression models. In Owen DB, editor. Statistics: Textbooks and Monographs. Marcel Dekker: New York, 1990; 107.

Rudnick G, Clark J. From synapse to vesicle: the reuptake and storage of biogenic amine neurotransmitters. Biochim. Biophys. Acta, 1993; 249.

Schenk JO. The functioning neuronal transporter for dopamine: kinetic mechanisms and effects of amphetamine, cocaine, and methylphenidate. Prog. Drug Res. 2002; 59:111-131.

Schenk JO, Bunney BS. In vivo chronoamperometry: clues toward the spatial and temporal influences of dopamine. In Justice JB, editor. Voltammetry in the Neurosciences. Humana Press: Clifton, 1987; 139.

Schenk JO, George SE, and Schumacher PD. What can be learned from studies of multisubstrate mechanisms of neuronal dopamine transport? Eur. J. Phamacol., 2003; 479: 223-228.

Segel IH. Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems. John Wiley & Sons: New York, 1993.

Stein WD . Transport and Diffusion Across Cell Membrane. Academic Press: San Diego, 1986: 231-274.

Stein WD. Channels, Carriers, and Pumps: An Introduction to Membrane Transport. Academic Press: San Diego, 1990: 127.

Stein WD. Kinetics of transport: analyzing, testing, and characterizing models using kinetic approaches. Adv. Enzymol., 1989; 171: 23-62.

Torres G, Gainetdinov RR, Caron MG. Plasma membrane monoamine transporters: structure, regulation, and function. Nature Rev., 2003; 4: 13-25.

Volz TJ, Kim M, Schenk JO. Covalent and noncovalent chemical modifications of arginine residues decrease dopamine transporter activity. Synapse, 2004;52:272-282.

Wayment HK, Deutsch H, Schweri MM, Schenk JO. Effects of methylphenidate analogues on phenethylamine substrates for the striatal dopamine transporter: potential as amphetamine antagonists? J. Neurochem., 1999; 72: 1266-1274.

Wayment HK, Meiergerd SM, Schenk JO. Relationships between the catechol substrate binding site and amphetamine, cocaine, and mazindol binding sites in a kinetic model of the striatal transporter for dopamine in vitro. J. Neurochem., 1998; 70: 1941-1949.

Weiss TF. Carrier-mediated transport. In Cellular Biophysics: Transport. The MIT Press: Cambridge, 1996; 1: 333

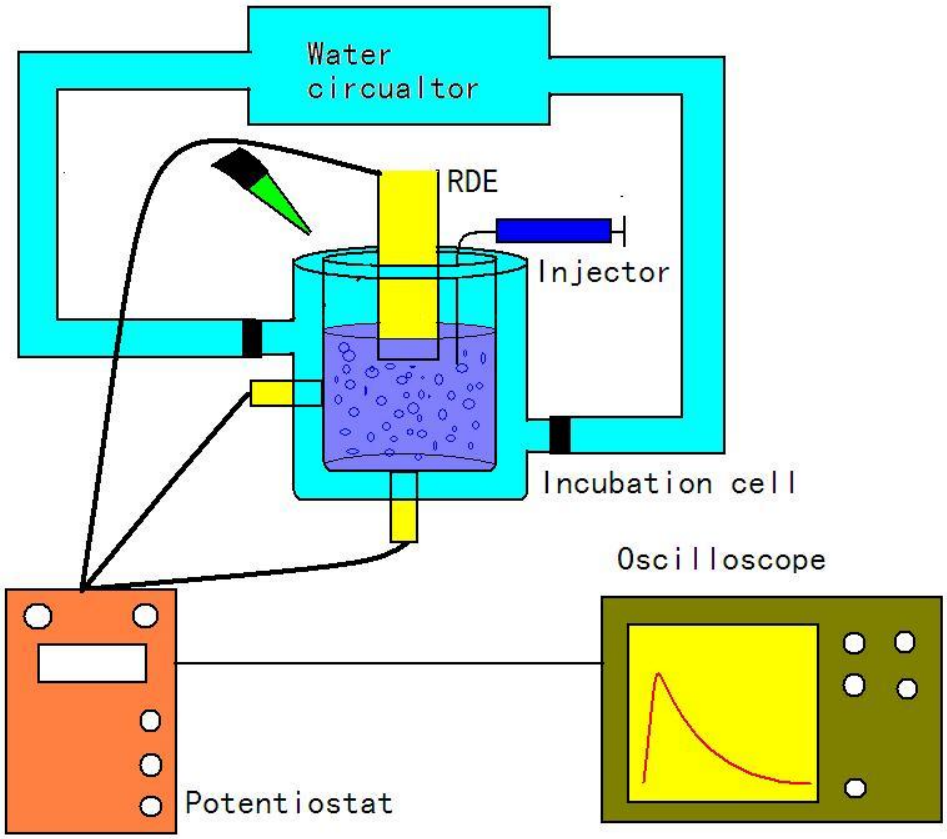

Figure 1 A schematic diagram of the experimental set up for the RDEV. The rotating disk electrode (RDE) is placed into the glass, water-jacketed incubation chamber containing a preparation of the DAT in physiological buffer saturated with a gas mixture of 95%O_2/5%CO_2. The reference and auxiliary electrodes are sealed into the walls of the glass incubation chamber, the potentiostat applies a potential sufficient to oxidize dopamine, and the resulting current is monitored by a digital oscilloscope. The cartoon trace on the oscilloscope depicts the signal for uptake of dopamine added from and exogenous source. Details of construction of the set up and treatment of raw data can be found in Meiergerd and Schenk (1995), Earles et al., 1998; and Earles and Schenk, 1998. The theory of RDEV is fully described in Bard and Faulkner (1980).

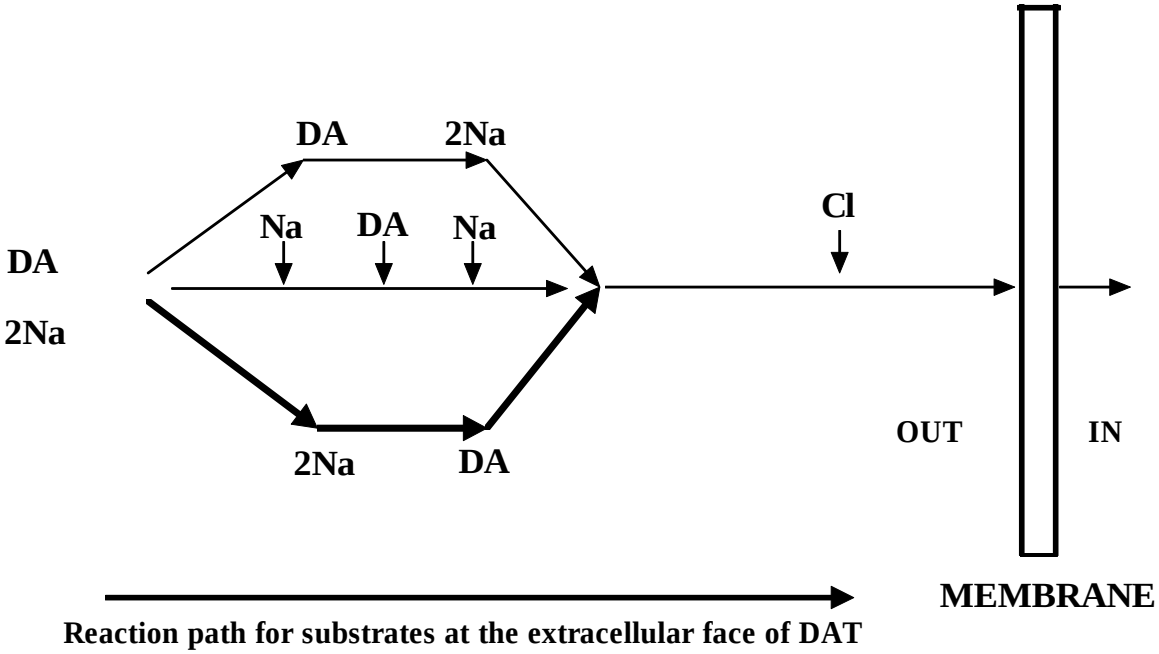

Figure 2. Illustration of the multisubstrate mechanisms of dopamine transport. The striatum (McElvain and Schenk, 1992) and nucleus accumbens Povlock and Schenk, 1997) exhibit partially random sequential mechanisms where dopamine and two Na^+'s find randomly before the binding of Cl^-. Human DAT expressed in human embryonic kidney cells exhibits an ordered binding sequence (Earles and Schenk, 1999). The fundamental kinetic basis for the techniques used can be found in Fromm (1975).

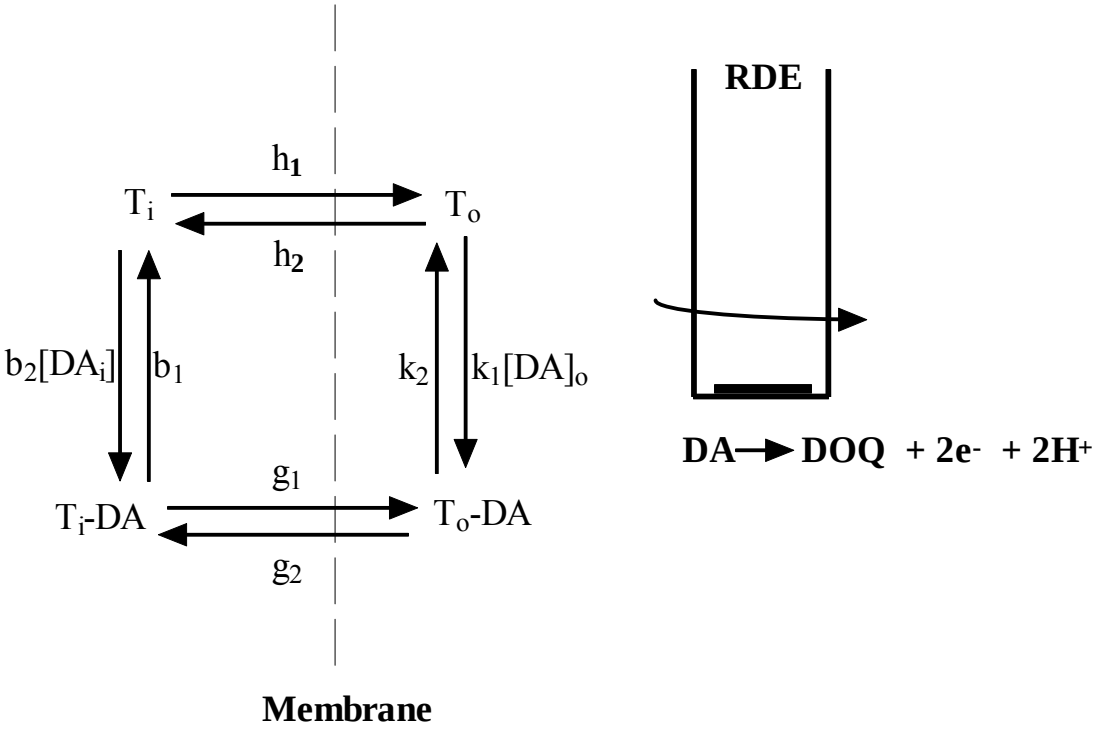

Figure 3. Illustration of the four state transport model that may apply to the function of DAT. The various transitions of the DAT molecule empty or loaded with dopamine (T and T-DA) are illustrated. To emphasize the challenge of studying this system the RDE and its detection reaction are shown only on the outside of the membrane. Other methods may have to be developed to make measurements inside the membrane. The subscripts *i* and *o* are labels to indicate *i*nside and *o*utside, respectively. Key: DA, dopamine; DOQ, dopamine-*o*-quinone.

Differential effects of Zn^{2+} on the kinetics and cocaine inhibition of dopamine transport by the human and rat dopamine transporters

Nicole L. Bjorklund[a] , Trent J. Volz [b], and James O. Schenk[a,b,c*]

[a]School of Molecular Biosciences, Washington State University, Pullman, Washington 99164, USA

[b]Department of Chemistry, Washington State University, Pullman, Washington 99164, USA

[c]Programs in Pharmacology/Toxicology and Neuroscience, Washington State University, Pullman, Washington 99164, USA

Running Title: Effects of Zinc on the Kinetics of the Dopamine Transporter

[*]Corresponding Author. Washington State University, Department of Chemistry, Pullman, WA 99164-4630, Tel. 509-335-4300; Fax. 509-335-8867.

E-mail address: geni@wsu.edu

Abstract

Zn^{2+} may play a major role in the modulation of neurotransmission because it modulates membrane receptors and channels. Recent literature has shown Zn^{2+} inhibits dopamine transport by the dopamine transporter (DAT), the main target of cocaine and some other drugs of abuse. Cocaine inhibits DAT and modulation of the DAT by Zn^{2+} may alter effects of cocaine on dopamine neurotransmission. This study investigates how Zn^{2+} changes DAT kinetics and its inhibition by cocaine. Steady state and pre-steady state kinetics of DAT activity were investigated using rotating disk electrode voltammetry. Values of K_M and V_{max} in human DAT (hDAT) and effects of cocaine match those in the literature. Zn^{2+} allosterically inhibited transport in the hDAT with a $K_I = 7.9 \pm 0.42$ μM. Removal of endogenous Zn^{2+} with penicillamine in hDAT increased transport values. In contrast, Zn^{2+} did not alter transport by rat DAT (rDAT), with K_M and V_{max} values of 1.2 ± 0.49 μM and 15.7 ± 2.57 pmol/(s x 10^6 cells), respectively, and removal of Zn^{2+} did not increase dopamine transport values. Zn^{2+} allosterically reduced the inhibition by cocaine in hDAT. Results of pre-steady state studies demonstrated that Zn^{2+} increases the second order binding rate constant for dopamine to hDAT (3.5 fold to 19.2 x 10^6 $M^{-1}s^{-1}$ for hDAT). In rat striatal homogenates Zn^{2+} increased initial dopamine transport velocity and decreased cocaine inhibition providing evidence for differences in sensitivity to Zn^{2+} between the three different preparations. Modulation of the DAT by Zn^{2+} needs to be assessed further in development of cocaine antagonists.

Keywords: dopamine transporter, Zn^{2+} modulation, pre-steady state kinetics, cocaine, rotating disk electrode voltammetry, inductively-coupled plasma ion emission spectroscopy

1. Introduction

Zn^{2+} plays a crucial role in brain chemistry and function (A. Takeda, 2000). It is a vital component in many proteins and possesses structural and/or catalytic functions. This transition metal is differentially sequestered within cells and is tightly regulated by the actions of metallothioneins and cell partitioning (J. Hidalgo et al., 2001). Disruption of Zn^{2+} regulation leads to dysfunction and disease. For example, Zn^{2+} deficiency in the diet increases seizure susceptibility, causes learning impairment, and leads to olfactory dysfunction (for review see (A. Takeda, 2001; A. Takeda et al., 2003)). Zn^{2+} can also act as a neuromodulator. For example, Zn^{2+} has been found to modulate various receptors and ion channels (S.Y. Assaf and S.H. Chung, 1984; G.L. Westbrook and M.L. Mayer, 1987). Westbrook and Mayer found that Zn^{2+} acts as an NMDA and GABA receptor antagonist in hippocampal neurons and modulates both excitory and inhibitory neurotransmission (G.L. Westbrook and M.L. Mayer, 1987). Zn^{2+} may also be a modulator of the dopaminergic system because it inhibits dopamine transport by the neuronal dopamine transporter (DAT) (L. Norregaard et al., 1998). Subsequently, a binding site for Zn^{2+} on the DAT was proposed based on results from site-directed mutagenesis studies (C.J. Loland et al., 1999; L. Norregaard et al., 1998).

The binding site for Zn^{2+} on the DAT includes the functional groups from His^{193}, His^{375}, and Glu^{396} as ligands, where the fourth coordinate position is occupied by a water molecule (C.J. Loland et al., 1999; L. Norregaard et al., 1998). This four coordination geometry (versus five or six coordination sites) is favored for both catalytic and structural Zn^{2+} binding sites in proteins (I.L. Alberts et al., 1998). In transformed African green

monkey kidney fibroblast (COS) cells and human embryonic kidney (HEK293) cells expressing the DAT, 10 μM Zn^{2+} causes inhibition of dopamine transport by decreasing the V_{max} (C.J. Loland et al., 1999). Further studies demonstrated that dopamine could compete with [^{3}H]WIN 35,428 binding in the presence of Zn^{2+}, thus suggesting that only the translocation step was inhibited (L. Norregaard et al., 1998). These findings suggest that Zn^{2+} is acting allosterically relative to the dopamine binding site on the DAT (L. Norregaard et al., 1998).

This report describes the results of a detailed kinetic study that was carried out to further investigate the effects of Zn^{2+} on DAT function using steady state and pre-steady state dopamine transport experiments. Specifically, to determine 1) if added Zn^{2+} modulates binding or translocation steps in the dopamine transport processes, 2) whether Zn^{2+} modulates the DAT differently depending on the preparation (tissue or cell line) or species of origin (human (hDAT) or rat (rDAT)) of the transporter, and 3) the effect of Zn^{2+} on cocaine inhibition of the DAT. In steady-state kinetics the concentration of the dopamine-bound DAT complex is unchanging with time thereby allowing the measurement of dopamine-DAT binding and translocation. Pre-steady-state kinetics describe initial binding step or steps of dopamine binding to DAT (for review, see (J.O. Schenk et al., 2005) and references therein). Penicillamine, a specific Zn^{2+} chelator, was used in separate sets of experiments to remove endogenous Zn^{2+} and to determine if transport is altered in the absence of Zn^{2+}. The relationship between cocaine and Zn^{2+} was investigated using steady-state kinetics and evaluation of a number of binding models.

2. Materials and Methods

2.1. Chemicals and solutions

Solutions were prepared with deionized water purified further with a Barnstead (Dubuque, IA) Nanopure water purification system. Chemicals were reagent grade and were used as received. The common buffer salts were purchased from JT Baker Chemical Co. (Philipsburg, NJ). Dopamine hydrochloride, (-)cocaine hydrochloride, D-(-)-penicillamine hydrochloride, Dulbecco's modified eagle media (DMEM), bovine calf serum (BCS), fetal clone serum (FCS), G418 sulfate, penicillin-streptomycin, and amphotericin B were purchased from Sigma (St. Louis, MO). $ZnCl_2$ was purchased from Fisher Scientific (Pittsburgh, PA). The composition of the pH 7.4 physiological buffer was 124 mM NaCl, 1.8 mM KCl, 1.3 mM $MgSO_4$, 1.24 mM KH_2PO_4, 2.5 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose saturated with 95 % O_2/ 5 % CO_2. The pH 7.3 Ca^{2+} and Mg^{2+} free phosphate buffered saline (CMF-PBS) had a composition of 139 mM NaCl, 4.1 mM KCl, 5.1 mM Na_2HPO_4, 5.0 mM KH_2PO_4, and 2 % (wt/vol) glucose.

2.2. Cell culture and preparation

HEK293 cells expressing hDAT and rDAT were cultured in the laboratory and maintained as previously described (C. Earles and J.O. Schenk, 1999). HEK293 cells expressing hDAT were a gift from Dr. Amy Eshleman at the VA Hospital at the Oregon Health Sciences University (Portland, OR) and rDAT from Dr. Marc Caron at the

Howard Hughes Medical Institute Laboratories at Duke University (Durham, NC).

DMEM was supplemented with 5 % (v/v) FCS, 5 % (v/v) BCS, 0.05 % (v/v) penicillin-streptomycin, 0.001 % (v/v) amphotericin B, and 0.001 % (v/v) G418 antibiotic. Once at 90 % confluence, the cells were harvested by first rinsing gently with CMF-PBS. Physiological buffer was then used to gently remove the cells from the bottom of the plate and cells were collected into a centrifuge tube (for Zn^{2+} and penicillamine experiments, 10 μM $ZnCl_2$ or 300 nM penicillamine was added to the physiological buffer prior to dislodging cells). The cell suspension were centrifuged at 17 x *g* for 8 min and the supernatant was removed and 1 ml of fresh, 37 ºC physiological buffer was added for each plate of cells used. The average cell counts, estimated using a hemocytometer (Haussen Scientific, Harsham, PA), ranged from 1.5-2.5 x 10^6 cells for each 500 μl experiment. The cells were kept in a 37 ºC water bath and used for experimentation within three hours.

2.3. Animals

Male Sprague-Dawley rats (275-325 g) from Animal Technologies Ltd. (Kent, WA) were maintained on a 12 hour light/dark cycle with food and water provided *ad libitum*. Animal procedures were conducted in accordance to the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* and were approved by the Washington State University Institutional Animal Care and Use Committee.

2.4. Tissue preparation

Rat striatal suspensions were prepared as previously described (T.J. Volz et al., 2004) in 500 µl of physiological buffer. Briefly, the striatal tissue from unanesthetized rats was obtained by rapid decapitation and dissection of the brain. The striatal tissue was weighed (35-40 mg) and kept on ice until experimentation took place. Before voltammetric studies, the tissue was chopped using an ice-cold razor blade on an ice-cold watch glass and placed in 500 µl physiological buffer (for Zn^{2+} experiments, 10 µM $ZnCl_2$ was first added to the buffer, justification below) in a glass incubation chamber with a circulating 37 ºC water jacket. The tissue was homogenized using repetitive pipetting and settled in 18 min. Then, tissue was washed six times with fresh physiological buffer, leaving a final volume of 500 µl.

2.5. Steady-state measurement of dopamine transport velocity

The inwardly-directed velocity of dopamine transport in 500 µl of rat striatal suspensions or 500 µl HEK293 cell suspensions were performed using rotating disk electrode voltammetry as described previously (C. Earles and J.O. Schenk, 1999; T.J. Volz et al., 2004). The electrode was a glassy carbon rotating disk electrode (Part No. AFM DO3GC) rotated at 2000 rpm with a MSRX precision rotator from Pine Instruments (Grove City, PA). A LC-4C potentiostat with a 200 ms time constant from Bioanalytical Systems (West Lafayette, IN) was used to apply +450 mV relative to a Ag/AgCl reference electrode and the resulting detection current was recorded onto a Nicolet model 310 digital oscilloscope (Madison, WI). Data were sampled every 20 ms

for 1.5 min. The initial velocity was calculated by measuring the slope of the tangent line of the clearance profile over the time range of 0 to 3 s using 6 data points at 500 ms intervals after dopamine injection. Cocaine was introduced to a suspension 30 s before dopamine injection as described previously (C. Earles and J.O. Schenk, 1999; S.L. Povlock and J.O. Schenk, 1997). Initial rates of dopamine clearance for initial concentrations of dopamine in the range of 0.5-2.5 μM were fit to the Michaelis-Menten expression by nonlinear regression analysis using GraphPad Prism Version 2.0 (San Diego, CA). Precision of kinetic parameters from the curve fits are given as standard errors of regression (S.E.R.) and statistical testing between kinetic parameters used the z-test (L.L. Havlicek and R.D. Crain, 1988). In experiments in which inhibition by cocaine or Zn^{2+} was measured, the curves were first fit to the Michaelis-Menten expression to obtain values for K_M and V_{max}. Changes in these parameters relative to control were used to choose the appropriate modified expression for competitive, noncompetitive, or uncompetitive inhibition (I.H. Segel, 1975). Curve fits were then run again and K_I was determined using the appropriate model.

To determine the relationship between Zn^{2+} and cocaine binding sites, data were fit to the multiple inhibitor binding models where there is a single-site, two independent sites, and two interacting but separate sites (R. Devés and R.M. Krupka, 1980; H. Wayment et al., 1998).

For the single site model,

$$(\nu / \nu_{IL} - 1) = (\nu / \nu_L - 1) + (\nu / \nu_I - 1) \qquad (1)$$

ν is the initial velocity of dopamine transport in the absence of inhibitor, ν_I is the initial velocity in the presence of inhibitor I at a given concentration; ν_L is the initial velocity of

transport in the presence of a given concentration of inhibitor L, and v_{IL} is the initial velocity of transport in the presence of both inhibitors, I and L, at the same concentrations previously used to obtain v_L and v_I.

For the model of independent sites,

$$(v/v_{IL} - 1) = (v/v_I - 1) + (v/v_L - 1) + (v/v_I - 1)(v/v_L - 1)A \qquad (2)$$

A is a constant unique to the type of experiment (R. Devés and R.M. Krupka, 1980) and the form for zero-trans entry experiment was chosen as before (S.M. Meiergerd and J.O. Schenk, 1994; H.K. Wayment et al., 1999). The rest of the variables have the same meaning as in Eq. 1.

In the interacting sites equation,

$$(v/v_{IL} - 1) = (v/v_I - 1) + (v/v_L - 1)(1 + [I]/K_{IL}) \qquad (3)$$

K_{IL} is the half-saturation constant for the test inhibitor (Zn^{2+}) under saturating concentrations of cocaine. [I] is the concentration of Zn^{2+} and the other values are as in Eq. 1.

The experimental velocities for v, v_I, and v_L were used to calculate what the initial velocity (v_{IL}) would be in each particular model where the two inhibitors were present simultaneously. The calculated v_{IL} was then compared to the experimentally-determined v_{IL} as described previously (S.M. Meiergerd and J.O. Schenk, 1994; H. Wayment et al., 1998). A fit was determined to be adequate if the model and experimental initial velocities were numerically within ± 5 % of predictions and statistically indistinguishable at $p > 0.05$.

2.6. Pre-steady-state binding experiments and analysis

Pre-steady-state experiments were carried out to determine the apparent second order rate constant (k_1) for the binding of dopamine to the DAT. These experiments were conducted under the same conditions as the steady-state experiments except the temperature of the incubation chamber was reduced to 22 º C and a non-saturating concentration of 10 nM dopamine was injected and its concentration - time profile was measured as previously described (J.O. Schenk et al., 2005). These changes in experimental condition were made to ensure the measurement was of dopamine binding and not transport. The translocation step is the rate limiting step under steady-state conditions and in the time window measured and therefore lowering the temperature inhibits this step. For these measurements the use of a non-saturating concentration of dopamine (100 fold below the K_M) was used. The resulting profile, as dopamine concentration vs. time, was then fit to the integrated second order binding equation,

$$k_1 t = \frac{1}{([DA]_0 - [DAT]_0)} \ln\left[\frac{[DAT]_0([DA]_0 - x)}{[DA]_0([DAT]_0 - x)}\right] \qquad (4).$$

In this equation DAT and dopamine concentrations at time = 0 are $[DAT]_0$ and $[DA]_0$ respectively. At some elapsed time, the concentrations are defined as $[DAT]_0 - x$ and $[DA]_0 - x$, where the dopamine-bound DAT concentration is x. The rate of the reaction is k_1. The $[DAT]_0$ was calculated using previously published estimates of DAT expression in HEK293 cells (C. Earles and J.O. Schenk, 1998). Graphing the right side of the equation (termed "kinetic parameter") versus time yields a slope which is the second order binding rate constant in $M^{-1}s^{-1}$. Further detailed discussion of pre-steady-state experiments for DAT can be found elsewhere (S.M. Meiergerd et al., 1994; J.O. Schenk et al., 2005).

2.7. Inductively Coupled Plasma Optical Emission Spectroscopy

After selected voltammetric studies were complete, samples were placed into Eppendorf tubes and centrifuged for 8 min at 17 g. The supernatant was removed and the cell samples were kept at 0º C until analysis. On the day of Zn^{2+} measurement the cell samples were digested in a 1:1 (v/v) ratio with concentrated HNO_3 for 2 hours at 120º C. A standard curve was made using 10,000, 5000, 1000, 100, and 10 ppb Zn^{2+} in 4 % HNO_3. A Perkin Elmer Optima 3200RL inductively coupled plasma optical emission spectrometer with peristaltic pump and A5 90plus autosampler was used to detect Zn^{2+} at 213.9 nm. The sample was introduced into the plasma at a flow rate of 1.5 ml/min. Three replicates were taken for each sample and there were three samples for each condition. The system was flushed with deionized water between each sample.

3. Results

3.1. Steady-state dopamine transport by HEK293 cells expressing hDAT

Zn^{2+} has been shown to inhibit the transport of dopamine by hDAT expressed in HEK293 cells (C.J. Loland et al., 2002). In preliminary experiments it was found that 10 μM Zn^{2+} containing buffer without tissue or cells had no effect on the dopamine signal (data not shown). Fig. 1 shows the result of steady-state experiments of dopamine transport in controls and experiments in which Zn^{2+} and cocaine were added individually

and in combination. An initial velocity was measured for each dopamine concentration and each data set was fit using nonlinear regression to the Michaelis-Menten expression. Values of K_M and V_{max} for the various conditions are listed in Table 1. The addition of 10 μM Zn^{2+} lowered the V_{max} of dopamine transport by hDAT, but did not affect K_M, consistent with previously-reported results (J.J. Bonnet et al., 1994; L. Norregaard et al., 1998), suggesting a noncompetitive mechanism. The K_I for Zn^{2+} inhibition of hDAT dopamine transport was found to be 18 μM from fitting the experimental data to the noncompetitive Michaelis-Menten model.

Zn^{2+} has been shown to increase the binding of the cocaine analog [^{3}H] WIN 35,428 to DAT by increasing the B_{max} (L. Norregaard et al., 1998). Experiments were carried out to determine the effects of Zn^{2+} and cocaine alone and in combination on dopamine transport to further investigate this phenomenon in hDAT. Steady-state experiments were first conducted with cocaine alone to determine its mechanism of inhibition. Cocaine inhibition of dopamine transport by hDAT was found to be competitive with a K_I of 0.3 μM (Table 1). This mechanism and K_I value match a previous report from this laboratory (C. Earles and J.O. Schenk, 1999). The dopamine transport curve in the presence of both Zn^{2+} and cocaine has less inhibition than with cocaine alone, suggesting possible antagonism of one inhibitor by the other.

3.2. Steady-state experiments in HEK293 cells expressing rDAT

Zn^{2+} is able to modulate dopamine transport by hDAT. Historically, rDAT is typically used for *in vivo* pharmacological experimentation, preclinical development of

dopamine treatments and/or behavioral correlations. In ^{3}[H] dopamine uptake assays using rat synaptosomes, it was found that 10 μM Zn^{2+} inhibited dopamine transport (E.K. Richfield, 1993). Therefore, the effect of Zn^{2+} on dopamine transport activity was investigated in HEK293 cells expressing rDAT. 10 μM Zn^{2+} statistically had no effect on the translocation of dopamine across the membrane by rDAT compared with control (Fig. 2).

3.3. Penicillamine effects on dopamine transport in hDAT and rDAT

To further examine the effect of Zn^{2+} on DAT activity, penicillamine, a Zn^{2+} chelator, was used to remove endogenous Zn^{2+}. In panel A of Figure 3, ICP-OES analysis of Zn^{2+} concentration of untreated and cells treated with 300 nM penicillamine is shown. Over 50 % endogenous Zn^{2+} was removed in both cell lines after penicillamine treatment. Panel B demonstrates how removing endogenous Zn^{2+} affects dopamine transport. In cells expressing hDAT, dopamine transport velocity increased in a penicillamine-concentration dependent manner. However, activity of rDAT remained unchanged. The linear regression fit to the rDAT data demonstrated that the slope of the line does not deviate from zero. This demonstrates the lack of effect of Zn^{2+} on rDAT.

3.4. Pre-steady-state kinetics of dopamine transport by HEK293 cells expressing hDAT

It has been shown that Zn^{2+} noncompetitively inhibits the translocation step of dopamine transport (L. Norregaard et al., 1998), which was corroborated by the data

above. The effect of Zn^{2+} on dopamine binding to the DAT has not yet been characterized therefore, to further elucidate the inhibitory role of Zn^{2+}, pre-steady-state experiments were conducted with hDAT. It was found (Fig. 4) that the addition of 10 μM Zn^{2+} to the physiological buffer produced an increased k_1 compared with control.

3.5. Zn^{2+} effects on cocaine inhibition of hDAT activity expressed in HEK293 cells

Because of the results in Figure 1, experiments were conducted to investigate possible antagonism between Zn^{2+} and cocaine because the inhibition was not additive when the two were combined. At 1 and 10 μM cocaine, the inhibition was decreased in the presence of Zn^{2+} relative to cocaine alone (Fig. 5). At 100 μM cocaine the inhibition was not altered by Zn^{2+}. These data suggest that perhaps Zn^{2+} and cocaine may compete for the same binding site or each allosterically alters the binding of the other inhibitor as has been suggested previously (J.J. Bonnet et al., 1994; L. Norregaard et al., 2003; L. Norregaard et al., 2000).

3.6. Comparison of binding models to experimental data in hDAT in the presence of Zn^{2+} and cocaine

To test for the possibility that Zn^{2+} and cocaine bind to the same binding site or independent binding sites on the DAT, data were fit to the binding models (Eqs.1-3).

Experimentally, the combination of 10 μM Zn^{2+} and 1 μM cocaine yielded less inhibition than the simple same site model (Fig. 6) with a + 40 % difference from

predicted values. This result suggests there is antagonism between Zn^{2+} and cocaine. The same site model yields the least amount of inhibition among the models, therefore the other models do not need to be considered. Cocaine competitively inhibits hDAT (C. Earles and J.O. Schenk, 1999), whereas the relationship between binding sites for cocaine and Zn^{2+} here did not fit simple models of identical or allosteric sites.

3.7. Comparison of the effects of Zn^{2+} and cocaine on steady-state initial velocity measurements in HEK293 cells expressing rDAT and in striatal homogenate preparations

As shown above, results of experiments on HEK293 cells expressing rDAT conflict with hDAT results. Studies in the literature, carried out in rat striatal synaptosomes, found that Zn^{2+} inhibits dopamine transport (E.K. Richfield, 1993). To probe the question of whether the differences arise from the preparation or are species related, initial velocity experiments were carried out in rat striatal tissue homogenates to compare how Zn^{2+} affects dopamine transport and cocaine inhibition (Fig. 7). Unlike the absence of an effect by Zn^{2+} on transport in rDAT transfected into HEK293 cells (Fig. 2), Zn^{2+} produced a slight but significant *increase* in the initial velocity of dopamine transport in rat striatal homogenates. The addition of Zn^{2+} and cocaine in combination decrease inhibition as seen in hDAT (Fig. 5).

4. Discussion

In this study, we report that Zn^{2+} disrupts the transport of dopamine across the membrane by hDAT expressed in HEK293 cells as measured by rotating disk electrode voltammetry. Inhibition by Zn^{2+} resulted in a statistically significant decrease in the V_{max} while K_M remained statistically indistinguishable from the control. These results suggest, using a different experimental method, an allosteric mechanism of Zn^{2+} relative to dopamine as has been previously proposed (L. Norregaard et al., 1998) and independently confirms previous results showing Zn^{2+} effects on hDAT dopamine transport (L. Norregaard et al., 1998; C. Pifl et al., 2004).

Zn^{2+} was hypothesized to disrupt the translocation of dopamine and not the binding of dopamine to the DAT (C.J. Loland et al., 1999; L. Norregaard et al., 1998; E.K. Richfield, 1993). In hDAT-expressing HEK293 cells, the addition of 10 μM Zn^{2+} causes the second order binding rate constant to increase, suggesting an increase in the binding of dopamine to the DAT. These data fit with the hypothesis that binding of dopamine is not disrupted (L. Norregaard et al., 1998), but enhanced by Zn^{2+}. In addition to increasing dopamine binding, Zn^{2+} may also alter the dissociation rate of dopamine from hDAT. Using measured values of K_M, V_{max}, and k_1, the dissociation constant was estimated assuming that DAT follows Briggs-Haldane kinetics as found previously (C. Earles and J.O. Schenk, 1999; S.R. Jones et al., 1999). In control experiments, the dissociation constant was estimated to be 6 s^{-1} and in experiments with 10 μM Zn^{2+} added, the calculated dissociation rate constant could not be determined as it was a negative value. These results suggest that Zn^{2+} may stabilize the dopamine – DAT complex. This stabilization leads to increased dopamine binding, decreased dopamine dissociation and slows translocation of dopamine across the membrane.

The combined Zn^{2+}/cocaine curve from Fig. 1 warranted more examination because it was not additive compared to the two inhibitors alone. Studies were conducted to investigate Zn^{2+} effects of dopamine transport velocities under increasing cocaine concentrations (Fig. 5). In order to explore the binding relationship of Zn^{2+} and cocaine data were fit to various binding models. Previously, this laboratory has used these binding models to describe the binding sites of multiple inhibitors on the DAT (S.M. Meiergerd and J.O. Schenk, 1994; H.K. Wayment et al., 1999). This laboratory has calibrated the system and found the models to work well in predicting the same binding site relationship between two inhibitor structural analogs (benztropine and cocaethylene) whereas interactive allosteric interactions were found with structurally dissimilar compounds (mazindol and nomifensine). In the present study, when the experimental data was compared to predictions from the same site binding model the inhibition observed was less than expected for this model. This suggests negative binding influences on cocaine by Zn^{2+}. Modulation of cocaine binding by Zn^{2+} is similar to other findings that found Zn^{2+} alters the binding of ligands and drugs to receptors (C. Acuña-Castillo et al., 2000; A.M. Hosie et al., 2003; J.A. Schetz et al., 1999).

Literature binding studies found that 10 μM Zn^{2+} increased the binding of WIN 35,428, a cocaine structural analog inhibitor, to the DAT by increasing the B_{max}, but without altering the K_d (L. Norregaard et al., 1998). However, other binding studies found this increased affinity of dopamine uptake blockers at submicromolar concentrations of Zn^{2+} but not at 5-10 μM Zn^{2+}(J.J. Bonnet et al., 1994). Gether et al. hypothesized that Zn^{2+} binding to the DAT may cause a conformational change or perhaps stabilize the conformation required of DAT for binding certain inhibitors or

transporting substrates (U. Gether et al., 2001). The binding site for cocaine is separate from that of Zn^{2+} (L. Norregaard et al., 2000). This is corroborated in this study by the difference between inhibition of DAT by Zn^{2+} (noncompetitive) and DAT inhibition by cocaine (competitive). The lack of fit to the same site binding model reinforces this notion. Thus the binding of Zn^{2+} may induce a conformational change in the DAT reducing the affinity of cocaine for its binding site.

Surprisingly, we found that rDAT and hDAT react differently in the presence of 10 μM Zn^{2+} when expressed in HEK293 cells. Steady-state experiments in hDAT demonstrate that Zn^{2+} decreased V_{max}, but in Zn^{2+} treated rDAT the K_M and V_{max} are unaffected (Fig. 2). Moreover, treatment with the Zn^{2+} chelator, penicillamine, increased dopamine transport in hDAT but had no effect in rDAT. In this study both hDAT and rDAT are expressed in HEK293 cells and are thus in the same membrane lipid environments and subject to the same regulatory machinery. These identical environments suggest that these observed functional differences are due to differences between the transporters themselves.

The proposed Zn^{2+} binding site in hDAT includes His^{193}, His^{375}, and Glu^{396} (C.J. Loland et al., 1999; L. Norregaard et al., 1998). These residues are conserved in the rDAT; however the overall sequence for hDAT and rDAT are not identical (92% homology, for review see (T.J. Volz and J.O. Schenk, 2005)). Additionally, rDAT has 4 possible glycosylation sites (B. Giros et al., 1991; J.E. Kilty et al., 1991; S. Shimada et al., 1991), while hDAT has only 3 (B. Giros et al., 1992; D.J. Vandenbergh et al., 1992). The glycosylation sites are located on the large second extracellular loop. The additional glycosylation site in rDAT is located at Asp^{196} and glycosylation here would likely

interfere with the Zn^{2+} binding coordinate at His^{193} either by blocking coordination by Zn^{2+} or altering the conformation of the second extracellular loop.

There have been many published studies describing functional and physical differences between rDAT and hDAT. Along with differences in glycosylation, some pharmacological properties (F.A. Paczkowski et al., 1999) and substrate affinities (M.J. Kuhar et al., 1999) are also different between hDAT and rDAT. These differences, along with the difference in amino acid sequence between hDAT and rDAT, may contribute to a different rDAT conformation and thus prevent Zn^{2+} coordination and subsequent regulation of the rDAT.

In order to compare cell culture studies with the rat model of the dopaminergic system, dopamine transport in rat striatal homogenates was studied. In striatal homogenates, 1 μM dopamine in the presence of 10 μM Zn^{2+} increased the initial dopamine transport velocity. This result differs from previous studies (E.K. Richfield, 1993); which used different experimental methods and conditions, Richfield used 10 nM dopamine to measure initial transport velocity. In our experiments, this concentration is suitable for pre-steady-state studies only. In addition, values of K_M and V_{max} were not assessed. In striatal homogenates, Zn^{2+} decreased the inhibition by cocaine, which differed from previous findings where Zn^{2+} increased cocaine inhibition of the DAT (E.K. Richfield, 1993). This difference is most likely due to the Zn^{2+} and cocaine concentrations used and also how the tissue was prepared. We chose to use 100 μM cocaine for tissue and 10 μM cocaine for cells because in this and other laboratories these concentrations are able to produce up to 100 % inhibition of dopamine transport and/or binding ((L.T. Kennedy and I. Hanbauer, 1983; J.M. Masserano et al., 1994; J.S.

McElvain and J.O. Schenk, 1992) for example). Further, this is the lower limit of cocaine concentrations used in some uptake and binding assays which have used up to 1 mM cold cocaine (L.T. Kennedy and I. Hanbauer, 1983; R.R. Metzger et al., 2000; V. Sandoval et al., 2001). A modulation of this complete cocaine-induced transport inhibition by Zn^{2+} is more easily detected and thus reduces the risk of a false-negative result when testing for Zn^{2+} effects. Inhibition by cocaine was decreased by the presence of Zn^{2+} in hDAT as well (Fig. 5).

Rat striatal preparations were chosen because they have been used extensively to study native rDAT function and have previously been used to study the effects of Zn^{2+} on rDAT (E.K. Richfield, 1993). Brain homogenates and HEK293 cells are very different in composition, including but not limited to the membrane lipid environment in which DAT is inserted. When investigating kinetics using brain tissue, it must be kept in mind other neuronal signaling systems and neuronal intracellular biochemical mechanisms important for dopamine metabolism and sequesterization may complicate interpretation of the data. HEK293 cells are kidney cells with different intracellular biochemistry with regard to dopamine and modify the DAT differently when it is processed in the endoplasmic reticulum and Golgi apparatus (A. Patel et al., 1993). It is therefore not surprising that results may shift when comparing the two. As was mentioned above, rDAT has an extra glycosylation site that may interfere with Zn^{2+} binding. Glycosylation of the DAT has been shown to be altered when expressed in cell culture. This difference in glycosylation between rDAT expressed in HEK293 cells and rat striatal tissue may cause the different effects of Zn^{2+} on dopamine transport. The above data suggest that both species of DAT and preparation (cells versus tissue) may produce differences in regard to the inhibition

of dopamine transport by Zn^{2+}. The bulk of studies have used rat striatal tissue or hDAT in a cell line, and these two preparations seem to follow the same trends in terms of Zn^{2+} and cocaine inhibition when used alone and in combination.

Many processes are modulated by Zn^{2+} and a significant number are important for neurotransmission. Many ion channels, receptors and various signaling systems are influenced by Zn^{2+} (for review see (A. Mathie et al., 2006)). Zn^{2+} levels have been found to be high in the striatum, an area rich in DAT (E. Mengual et al., 1995). Therefore, along with observed modulation and anatomical proximity it is possible that DAT may be modulated by Zn^{2+}. The present data suggest that Zn^{2+} is a modulator of DAT, and affects the binding of dopamine and cocaine to DAT. If Zn^{2+} modulation of dopamine transport is functionally relevant, it must be taken into account in investigations of neurodegenerative disease and addiction. Additionally, the ability of Zn^{2+} to reduce inhibition of DAT by cocaine furthers the notion that cocaine antagonists can be designed.

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References

Acuña-Castillo, C., Morales, B. Huidobro-Toro, J.P., 2000. Zinc and copper modulate differentially the p2x4 receptor. J. Neurochem. 74, 1529-1537.

Alberts, I.L., Nadassy, K. Wodak, S.J., 1998. Analysis of zinc binding sites in protein crystal structures. Protein Sci. 7, 1700-1716.

Assaf, S.Y. Chung, S.H., 1984. Release of endogenous Zn^{2+} from brain tissue during activity. Nature. 308, 734-736.

Bonnet, J.J., Benmansour, S., Amejdki-Chab, N. Costentin, J., 1994. Effect of CH3HgCl and several transition metals on the dopamine neuronal carrier; peculiar behaviour of Zn^{2+}. Eur. J. Pharmacol. 266, 87-97.

Devés, R. Krupka, R.M., 1980. Testing transport systems for competition between pairs of reversible inhibitors. Inhibition of erythrocyte glucose transport by cytochalasin b and steroids. J. Biol. Chem. 255, 11870-11874.

Earles, C. Schenk, J.O., 1998. Rotating disk electrode voltammetric measurements of dopamine transporter activity: An analytical evaluation. Anal. Biochem. 264, 191-198.

Earles, C. Schenk, J.O., 1999. Multisubtrate mechanism for the inward transport of dopamine by the human dopamine transporter expressed in hek cells and its inhibition by cocaine. Synapse. 33, 230-238.

Gether, U., Norregaard, L. Loland, C.J., 2001. Delineating structure-function relationships in the dopamine transporter from natural and engineered Zn^{2+} binding sites. Life Sci. 68, 2187-2198.

Giros, B., El Mestikawy, S., Bertrand, L. Caron, M.G., 1991. Cloning and functional characterization of a cocaine-sensitive dopamine transporter. FEBS Lett. 295, 149-154.

Giros, B., el Mestikawy, S., Godinot, N., Zheng, K., Han, H., Yang-Feng, T. Caron, M.G., 1992. Cloning, pharmacological characterization, and chromosome assignment of the human dopamine transporter. Mol. Pharmacol. 42, 383-390.

Havlicek, L.L. Crain, R.D., 1988, Practical statistics for the physical sciences (American Chemical Society, Washington D.C.).

Hidalgo, J., Aschner, M., Zatta, P. Vašák, M., 2001. Roles of the metallothionein family of proteins in the central nervous system. Brain Res. Bull. 55, 133-145.

Hosie, A.M., Dunne, E.L., Harvey, R.J. Smart, T.G., 2003. Zinc-mediated inhibition of GABAa receptors: Discrete binding sites underlie subtype specificity. Nature Neurosci. 6, 362-369.

Jones, S.R., Joseph, J.D., Barak, L.S., Caron, M.G. Wightman, R.M., 1999. Dopamine neuronal transport kinetics and effects of amphetamine. J. Neurochem. 73, 2406-2414.

Kennedy, L.T. Hanbauer, I., 1983. Sodium-sensitive cocaine binding to rat striatal membrane: Possible relationship to dopamine uptake sites. J. Neurochem. 41, 172-178.

Kilty, J.E., Lorang, D. Amara, S.G., 1991. Cloning and expression of a cocaine-sensitive rat dopamine transporter. Science. 254, 578-579.

Kuhar, M.J., McGirr, K.M., Hunter, R.G., Lambert, P.D., Garrett, B.E. Carroll, F.I., 1999. Studies of selected phenyltropanes at monoamine transporters. Drug Alc. Dep. 56, 9-15.

Loland, C.J., Norregaard, L. Gether, U., 1999. Defining proximity relationships in the tertiary structure of the dopamine transporter. Identification of a conserved glutamic acid as a third coordinate in the endogenous $Zn^{(2+)}$-binding site. J. Biol. Chem. 274, 36928-36934.

Loland, C.J., Norregaard, L., Litman, T. Gether, U., 2002. Generation of an activating $Zn^{(2+)}$ switch in the dopamine transporter: Mutation of an intracellular tyrosine constitutively alters the conformational equilibrium of the transport cycle. Proc. Natl. Acad. Sci. U S A. 99, 1683-1688.

Masserano, J.M., Venable, D. Wyatt, R.J., 1994. Effects of chronic cocaine administration on [^{3}H]dopamine uptake in the nucleus accumbens, striatum and frontal cortex of rats. J. Pharmacol. Exp. Ther. 270, 133-141.

Mathie, A., Sutton, G.L., Clarke, C.E. Veale, E.L., 2006. Zinc and copper: Pharmacological probes and endogenous modulators of neuronal excitability. Pharmacol. Ther. 111, 567-583.

McElvain, J.S. Schenk, J.O., 1992. A multisubstrate mechanism of striatal dopamine uptake and its inhibition by cocaine. Biochem. Pharmacol. 43, 2189-2199.

Meiergerd, S.M., McElvain, J.S. Schenk, J.O., 1994. Effects of cocaine and repeated cocaine followed by withdrawal. Alterations of dopaminergic transporter turnover with no changes in kinetics of substrate recognition. Biochem. Pharmacol. 47, 1627-1634.

Meiergerd, S.M. Schenk, J.O., 1994. Kinetic evaluation of the commonality between the site(s) of action of cocaine and some other structurally similar and dissimilar inhibitors of the striatal transporter for dopamine. J. Neurochem. 63, 1683-1692.

Mengual, E., Casanovas-Aguilar, C., Pérez-Clausell, J. Giménez-Amaya, J.M., 1995. Heterogeneous and compartmental distribution of zinc in the striatum and globus pallidus of the rat. Neurosci. 66, 523-537.

Metzger, R.R., Haughey, H.M., Wilkins, D.G., Gibb, J.W., Hanson, G.R. Fleckenstein, A.E., 2000. Methamphetamine-induced rapid decrease in dopamine transporter function: Role of dopamine and hyperthermia. J. Pharmacol. Exp. Ther. 295, 1077-1085.

Norregaard, L., Frederiksen, D., Nielsen, E.Ø. Gether, U., 1998. Delineation of an endogenous zinc-binding site in the human dopamine transporter. EMBO J. 17, 4266-4273.

Norregaard, L., Loland, C.J. Gether, U., 2003. Evidence for distinct sodium-, dopamine-, and cocaine-dependent conformational changes in transmembrane segments 7 and 8 of the dopamine transporter. J. Biol. Chem. 278, 30587-30596.

Norregaard, L., Visiers, I., Loland, C.J., Ballesteros, J., Weinstein, H. Gether, U., 2000. Structural probing of a microdomain in the dopamine transporter by engineering of artificial Zn^{2+} binding sites. Biochem. 39, 15836-15846.

Paczkowski, F.A., Bryan-Lluka, L.J., Porzgen, P., Bruss, M. Bonisch, H., 1999. Comparison of the pharmacological properties of cloned rat, human, and bovine norepinephrine transporters. J. Pharmacol. Exp. Ther. 290, 761-767.

Patel, A., Uhl, G. Kuhar, M.J., 1993. Species differences in dopamine transporters: Postmortem changes and glycosylation differences. J. Neurochem. 61, 496-500.
Pifl, C., Rebernik, P., Kattinger, A. Reither, H., 2004. Zn^{2+} modulates currents generated by the dopamine transporter: Parallel effects on amphetamine-induced charge transfer and release. Neuropharmacol. 46, 223-231.
Povlock, S.L. Schenk, J.O., 1997. A multisubstrate kinetic mechanism of dopamine transport in the nucleus accumbens and its inhibition by cocaine. J. Neurochem. 69, 1093-1105.
Richfield, E.K., 1993. Zinc modulation of drug binding, cocaine affinity states, and dopamine uptake on the dopamine uptake complex. Mol. Pharmacol. 43, 100-108.
Sandoval, V., Riddle, E.L., Ugarte, Y.V., Hanson, G.R. Fleckenstein, A.E., 2001. Methamphetamine-induced rapid and reversible changes in dopamine transporter function: An in vitro model. J. Neurosci. 21, 1413-1419.
Schenk, J.O., Wright, C. Bjorklund, N., 2005. Unraveling neuronal dopamine transporter mechanisms with rotating disk electrode voltammetry. J. Neurosci. Meth. 143, 41-47.
Schetz, J.A., Chu, A. Sibley, D.R., 1999. Zinc modulates antagonist interactions with D2-like dopamine receptors through distinct molecular mechanisms. J. Pharmacol. Exp. Ther. 289, 956-964.
Segel, I.H., 1975, Enzyme kinetics; behavior and analysis of rapid equilibrium and steady-state enzyme systems (Wiley-Interscience, New York) p. 957.
Shimada, S., Kitayama, S., Lin, C.L., Patel, A., Nanthakumar, E., Gregor, P., Kuhar, M. Uhl, G., 1991. Cloning and expression of a cocaine-sensitive dopamine transporter complementary DNA. Science. 254, 576-578.
Takeda, A., 2000. Movement of zinc and its functional significance in the brain. Brain Res. Brain Res. Rev. 34, 137-148.
Takeda, A., 2001. Zinc homeostasis and functions of zinc in the brain. Biometals. 14, 343-351.
Takeda, A., Hirate, M., Tamano, H., Nisibaba, D. Oku, N., 2003. Susceptibility to kainate-induced seizures under dietary zinc deficiency. J. Neurochem. 85, 1575-1580.
Vandenbergh, D.J., Persico, A.M. Uhl, G.R., 1992. A human dopamine transporter cDNA predicts reduced glycosylation, displays a novel repetitive element and provides racially-dimorphic taqi rflps. Mol. Brain Res. 15, 161-166.
Volz, T.J., Kim, M. Schenk, J.O., 2004. Covalent and noncovalent chemical modifications of arginine residues decrease dopamine transporter activity. Synapse. 52, 272-282.
Volz, T.J. Schenk, J.O., 2005. A comprehensive atlas of the topography of functional groups of the dopamine transporter. Synapse. 58, 72-94.
Wayment, H., Meiergerd, S.M. Schenk, J.O., 1998. Relationships between the catechol substrate binding site and amphetamine, cocaine, and mazindol binding sites in a kinetic model of the striatal transporter of dopamine in vitro. J. Neurochem. 70, 1941-1949.
Wayment, H.K., Deutsch, H., Schweri, M.M. Schenk, J.O., 1999. Effects of methylphenidate analogs on phenethylamine substrates for the striatal dopamine transporter: Potential as amphetamine antagonists? J. Neurochem. 72, 1266-1274.

Westbrook, G.L. Mayer, M.L., 1987. Micromolar concentrations of zinc antagonize NMDA and GABA responses of hippocampal neurons. Nature. 328, 640-643.

Table 1
Effect of Zn^{2+} and cocaine alone and in combination on kinetic constants of dopamine transport by hDAT

Table 1:	K_M	V_{max}	K_I
	(μM)±S.E.	(pmol/(s x 10^6cells))±S.E.	(μM)±S.E.
Control	2.01±1.15	22.9±7.86	-
10 μM Zn^{2+}	0.880±0.640	8.49±2.65	17.9±0.420[a]
1 μM Cocaine	8.26±3.71	31.1±11.7	0.320±0.010[b]
1 μM Cocaine + 10 μM Zn^{2+}	2.40±0.870	15.1±3.42	-

[a] data were fit to the noncompetitive model, [b]data were fit to the competitive model.

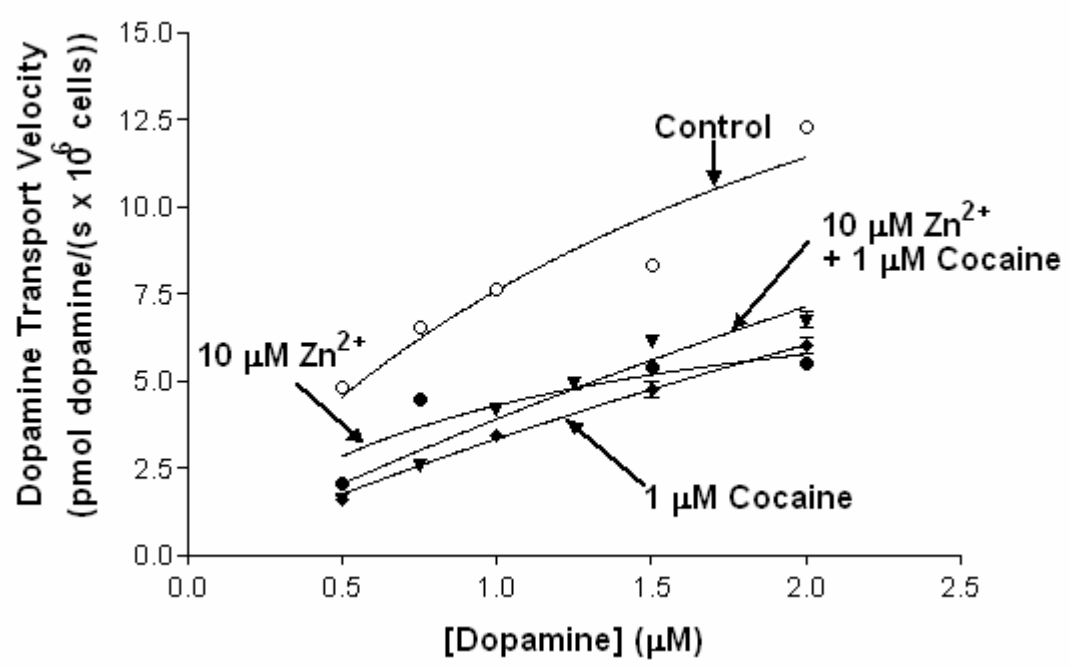

Figure 1. - The effect of 10 μM Zn^{2+} alone and in combination with 1 μM cocaine on steady state DA transport in HEK293 cells expressing hDAT. The solid lines are fits to the Michaelis-Menten equation. Each datum point is the mean ± S.E.M. of 3 or 4 replicates.

The K_M and V_{max} values are listed in Table 1. Values of K_I for inhibition by either Zn^{2+} or cocaine were obtained from fits of the data to the inhibition model suggested by the changes in K_M and V_{max} observed.

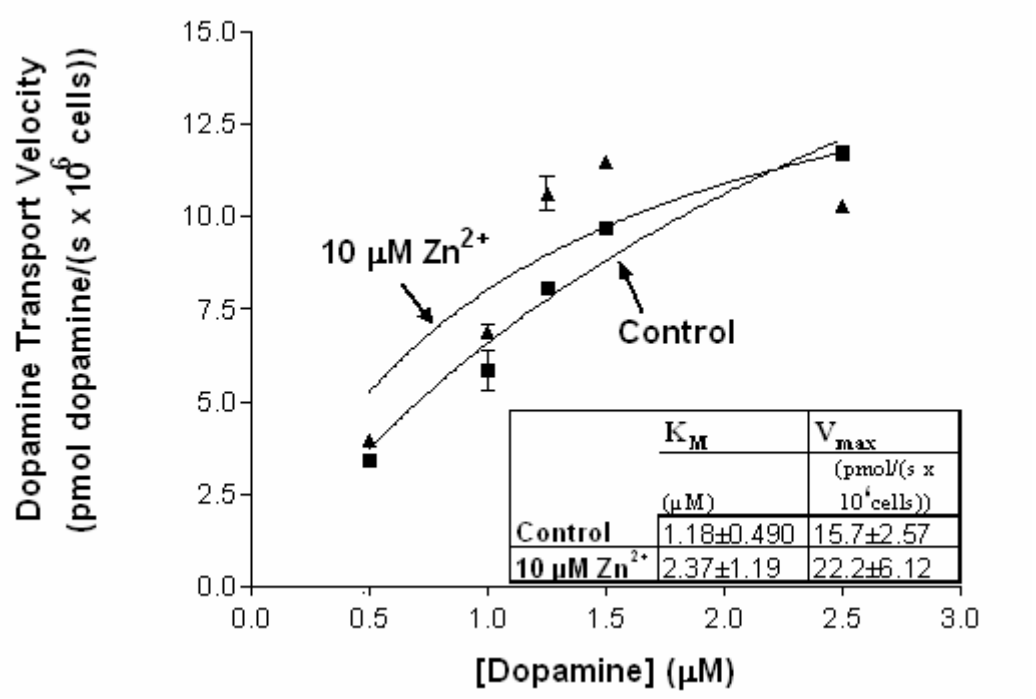

Figure 2. - Fits of DA transport velocities to the Michaelis-Menten expression in rDAT expressed in HEK293 cells in the presence and absence of 10 μM Zn^{2+}. The solid lines are fits to the Michaelis-Menton equation. The K_M and V_{max} values are listed in the inset. Each datum point is the mean ± S.E.M. of 3 or 4 replicates.

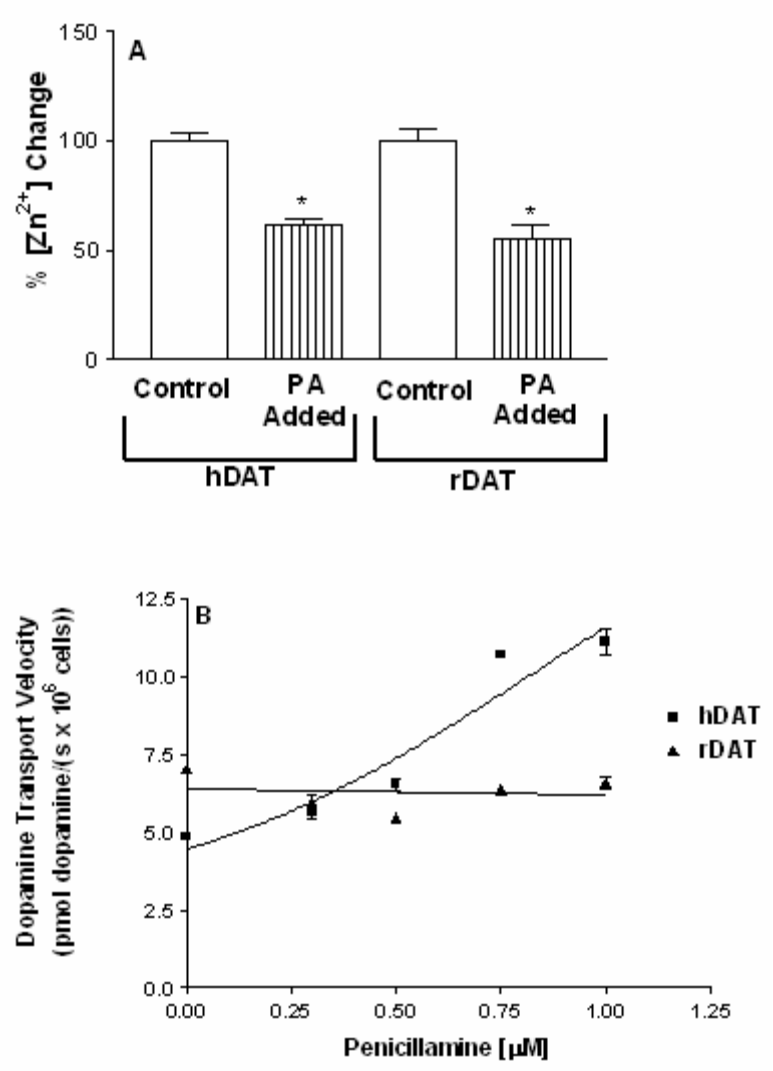

Figure 3. – Chelation of endogenous Zn^{2+} by penicillamine (PA) increases the activity of hDAT but not rDAT. Panel A shows ICP-OES analysis of $[Zn^{2+}]$ in cells treated with penicillamine versus non-treated cells for each cell line. The asterisks represent a value that is statistically different from control at $p \leq 0.0001$ via t-test. Panel B is a graph showing the initial velocity of dopamine transport in the presence of increasing concentrations of penicillamine for hDAT and rDAT. Each datum point is the average velocity ± S.E.M. of 4 replicates. The hDAT data was fit to a curve using nonlinear regression, while rDAT data was fit using linear regression and the slope is not significantly different from zero.

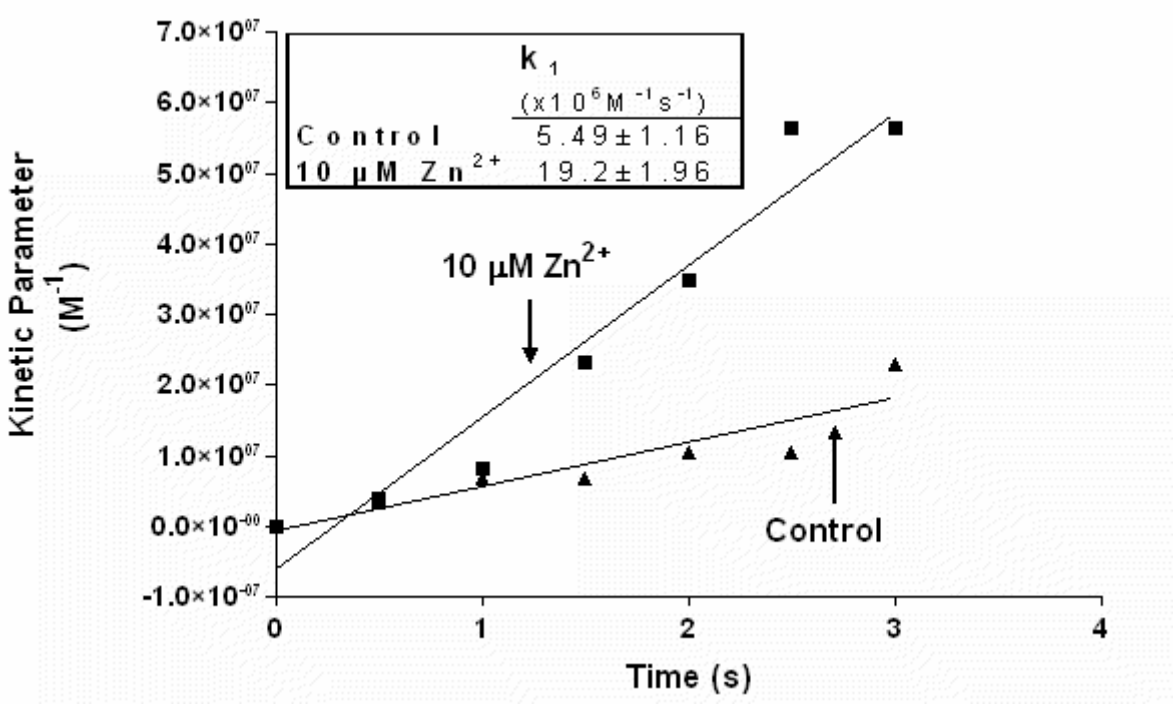

Figure 4. - Pre-steady state binding to hDAT in the presence and absence of 10 µM Zn^{2+}. The data were fit to Equation 4 using linear regression analysis and the resulting slopes are equal to k_1 and are listed ± S.E.R. in the inset. The *p*-values for linear regression fits for control and 10 µM Zn^{2+} were 0.002 and 0.0036, respectively.

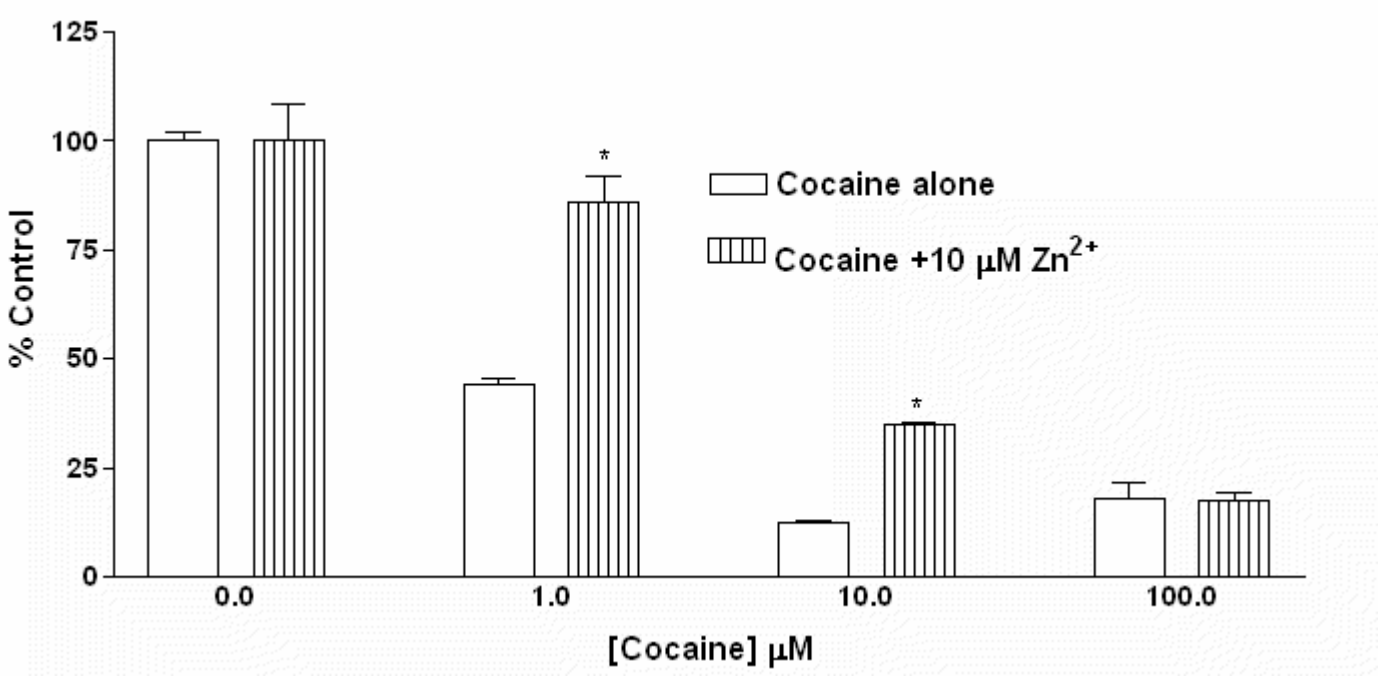

Figure 5. - Zn^{2+} attenuates the effects on cocaine inhibition in hDAT. [Zn^{2+}] was at 10 µM and cocaine at 1 µM, 10 µM, and 100 µM was added 30 s prior to addition of dopamine. Each datum point is the mean ± S.E.M. of 4 replicates. The asterisks represent values that are statistically different from cocaine alone at $p < 0.01$ via t-test.

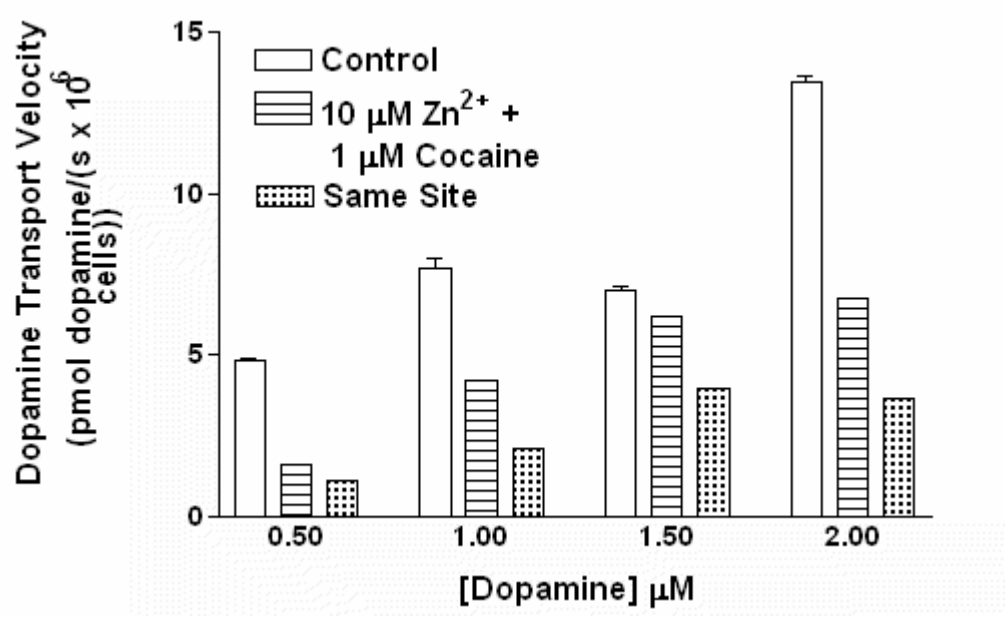

Figure 6. - Comparison of binding models to experimental kinetic data of hDAT in the simultaneous presence of Zn^{2+} and cocaine. The solid bars represent experimental data of initial transport velocities at varying [dopamine] values. The striped bars are measured initial velocities in the presence of 10 μM Zn^{2+} and 1 μM cocaine. Dotted bars are predicted initial velocities where Zn^{2+} and cocaine bind to the same site. Initial velocity values are in the form of mean ± S.E.M. S.E.M. values not visible are within the dimensions of the bar. Model velocities were at least 25 % different from experimental velocities, thereby demonstrating a lack of fit to the model.

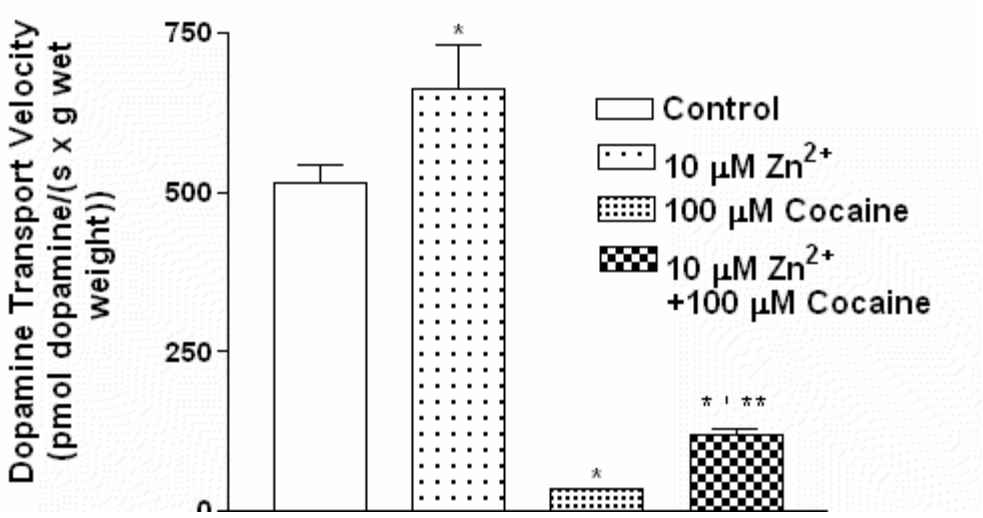

Figure 7. - Zn^{2+} effects on initial velocity measurements of DA transport in striatal tissue homogenates from rats. Each bar is the mean ± S.E.M. of 4 replicates. One asterisk represents a statistical difference between control velocities and velocities with $Zn^{2+}$and cocaine alone or combined, where $p \leq 0.003$. Double asterisks represent a statistical difference between dopamine transport in the presence of 100 μM cocaine alone and transport in the presence of Zn^{2+} and cocaine in combination, where $p = 0.0101$.

CONTEMPORANEOUS EFFECTS OF BEHAVIORALLY SENSITIZING DOSING OF METHAMPHETAMINE ON DOPAMINE TRANSPORTER ACTIVITY AND METHAMPHETAMINE INHIBITION IN RAT NUCLEUS ACCUMBENS AND STRIATUM

Nicole L. Bjorklund[1], Barbara A. Sorg[2], and James O. Schenk[1,2,3]

School of Molecular Biosciences[1], Programs in Pharmacology/Toxicology and Neuroscience[2], and the Department of Chemistry[3], Washington State University, Pullman, Washington 99164

Running Title: Methamphetamine and dopamine transporter

Correspondence should be addressed to Dr. James O. Schenk, Department of Chemistry, Washington State University, Pullman, WA 99164, geni@wsu.edu

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ABSTRACT

Animals sensitized to methamphetamine (METH) have altered dopaminergic systems, including dopamine transporter (DAT) activity. We investigated the effects induced by a sensitizing dose (5 mg/kg, i.p. per day for 5 days) of METH on behavior, dopamine (DA) transport by the DAT, DAT density, and inhibition of DA transport by METH in both the nucleus accumbens and striatum. We further investigated possible changes to glycosylation of the DAT after METH sensitization. The dosing paradigm caused an increased stereotyped response in rats treated with METH compared to saline controls. In animals treated with METH, DA transport velocities were lower in the nucleus accumbens and increased in the striatum. Western blots demonstrated that DAT density was unchanged in the nucleus accumbens of METH-treated animals, but striatal DAT density was decreased. Further studies investigating METH inhibition of DA transport found that in the nucleus accumbens of METH-treated animals, the IC_{50} was shifted to the right from 0.81 to 1.45 μM. In the striatum of METH-treated animals, the IC_{50} decreased compared to saline-treated controls. Studies using glycosidase treatments and western blots revealed that glycosylation was effectively removed by N-glycanase and neuraminidase, but not O-glycosidase or α-mannosidase. These studies also suggest that glycosylation was not altered in METH-treated animals. This study demonstrates that in animals sensitized to METH, the DAT is differentially regulated in different areas of the brain important for drug abuse and that DA transport changes induced by METH are not due to DAT density, but changes in kinetic regulation. Additionally, this study suggests that glycosylation may not play a role in DAT activity changes after METH exposure.

Introduction

Repeated subchronic exposure to methamphetamine (METH) causes increased locomotor activity in rodents. This specific behavioral manifestation is termed sensitization and is believed to model certain aspects of drug craving (Robinson and Berridge, 1993). Sensitization is apparent after a few days of exposure and can last for several weeks. Sensitization is caused by specific neuroadaptations in the brain (White and Kalivas, 1998).

An area of the brain affected by METH sensitization and is involved in the sensitized motor response is the nucleus accumbens (Pierce and Kalivas, 1997). Long-term sensitization produces neuroadaptations and structural changes in this area lasting several weeks or months after discontinuing drug exposure (Nestler, 2001). Neuroadaptation after METH treatment has also been found in the striatum (Fleckenstein et al., 1997; Genova et al., 1997). This area is rich in dopaminergic inputs, but is not suspected to be involved in drug seeking/craving behavior (Freed et al., 1995; Letchworth et al., 2001; McBride et al., 1999; Porrino et al., 2004). However, striatal areas are sensitive to neurotoxic doses of METH (For review see (Volz et al., 2007)).

The dopamine transporter (DAT) is one of the targets of METH-induced changes in the brain (Fumagalli et al., 1998; Itzhak et al., 2002; Raiteri et al., 1975; Raiteri et al., 1977). The DAT controls the actions of monoamines on receptor desensitization and postsynaptic responses by transporting dopamine (DA) from the synapse into the presynaptic neuron after its release in chemical neurotransmission. Amphetamines, of which METH is an example, competitively inhibit DA transport and can be transported

into the presynaptic neuron by the DAT (Jones et al., 1999; Wayment et al., 1998). Both the nucleus accumbens and striatum have high DAT densities compared to the rest of the central nervous system (Freed et al., 1995). The DAT protein, which is the product of a single gene, located in the nucleus accumbens has a higher level of glycosylation than the striatum (Lew et al., 1992; Lew et al., 1991; Povlock and Schenk, 1997; Vaughan et al., 1996). Conversely, striatal DAT has a lower molecular weight, suggesting that there is less glycosylation in this brain region (Lew et al., 1992; Lew et al., 1991; Meiergerd and Schenk, 1994). The general kinetic mechanism of the DAT is similar in both brain areas (McElvain and Schenk, 1992; Povlock and Schenk, 1997). However, in the nucleus accumbens, DA transport is more tightly regulated by extracellular Na^+, has a higher turnover rate, and is more sensitive to cocaine (Povlock and Schenk, 1997). A comparative study between the nucleus accumbens and the striatum on the inhibition of DA transport by METH treatment causing behavioral sensitization has not yet been undertaken.

The literature has described various effects of METH treatment on DAT activity (Bennett et al., 1998; Fleckenstein et al., 1997; Kokoshka et al., 1998; Nakayama et al., 1993). The outcome relies heavily on the dosing protocol and the withdrawal time (Stefanski et al., 1999; Stefanski et al., 2002). One study found striatal DA uptake was decreased by 36 % (Nakayama et al., 1993). Conversely, in another study, DA uptake was increased in striatal synaptosomes with an increase in METH-induced DA release (Yamada et al., 1988). Basal DA release is unchanged after METH sensitization (Robinson and Becker, 1982), however amphetamine, cocaine, nomifensine, and phenylethylamine stimulate a higher concentration of DA during release in METH-

treated animals (Kazahaya et al., 1989; Robinson and Berridge, 1993; Yamada et al., 1988). DAT density, as determined by binding experiments, also varies between reports. In one study, the DAT density decreased by 29 % (Nakayama et al., 1993), while in other studies there was no change in the nucleus accumbens or striatum after METH treatment (Belcher et al., 2006; Frey et al., 1997).

DAT density alone cannot account for the changes in DA transport after METH sensitization (Bennett et al., 1998; Kokoshka et al., 1998). It has been suggested that changes in glycosylation of the DAT may account for changes in DA transport kinetics ((Kokoshka et al., 1998; Povlock et al., 1996; Povlock and Schenk, 1997; Volz et al., 2007). The removal of sialic acids from the DAT alters substrate affinity and transport (Meiergerd and Schenk, 1994; Zalewska and Erecinska, 1987). Glycosylation changes may also explain differences between brain regions in sensitivity to METH.

The present study investigates behavioral sensitization due to METH treatment and the subsequent effects on DAT kinetics and METH inhibition in the nucleus accumbens and striatum.

MATERIALS AND METHODS

Chemicals and Solutions

Solutions were prepared with deionized water purified further with a Barnstead Nanopure (Dubuque, IA) water purification system. Chemicals were reagent grade and used as received. The common buffer salts were purchased from JT Baker Chemical Co. (Philipsburg, NJ). Dopamine hydrochloride (DA), (+)-methamphetamine hydrochloride, and TWEEN 20 were purchased from Sigma-Aldrich (St. Louis, MO). The composition

of the pH 7.4 physiological buffer was 124 mM NaCl, 1.8 mM KCl, 1.3 mM $MgSO_4$, 1.24 mM KH_2PO_4, 2.5 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose saturated with 95 % O_2/ 5 % CO_2. Tris-buffered saline with TWEEN 20 (TTBS) contained 10 mM Tris base, 150 mM NaCl and 0.05% TWEEN 20, pH 7.6.

Animal Protocol

Male Sprague-Dawley rats weighing 350-500 g were obtained from the WSU internal breeding colony. Animals were housed 2 per cage in the vivarium with a 12 h light/ dark cycle (lights on at 6:00 a.m.) and had access *ad libitum* to food and water. After four days of brief handling, the injection protocol was initiated. The METH group received intraperotineal (i.p.) injections of 5 mg/kg METH each day for 5 days. It has been shown previously that this dosing protocol produces sensitization in rats (Brady et al., 2005). The METH dose is the weight of salt/ kg rat weight and was dissolved in saline (0.9 % NaCl, pH 7.4). The saline control group received 1 ml/kg (i.p.) saline each day for 5 days. Injections were administered at the same time each day. Rats were sacrificed 24 hrs after the last injection.

Procedures were approved by the Institutional Animal Care and Use Committee and followed the guidelines in accordance to the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals.*

Behavioral Assessment

Rats were observed on the first and fifth (last) day of injection of either saline or METH. Observation took place for 30 s every 2 min from 2 min before the injection to

10 min after injection. Rats were then observed for 30 s every 5 min from 10 to 45 min after injection and then for 30 s every 10 min from 45 to 65 min. Rats were given a single numerical score for each 30 s interval on a scale of 1 to 10 as outlined previously (Kalivas et al., 1988). The scoring protocol is defined as follows; 1) asleep or still 2) inactive, grooming, mild licking 3) locomotion (all four feet move in 10 s), rearing or sniffing (≤ 3 s duration) 4) any combination of two: locomotion, rearing or sniffing 5) continuous sniffing for 10 s without locomotion or rearing 6) continuous sniffing for 10 s with locomotion or rearing 7) patterned sniffing for 5 s 8) patterned sniffing for 10 s 9) continuous gnawing 10) bizarre diskinetic movements or seizures. The values for each time point were averaged for each treatment group. Statistical testing between treatment groups was done using a two-way ANOVA.

Tissue Collection

Twenty four hours after the last injection, animals were rapidly decapitated and the brain was removed and placed on a watch glass over ice for ~ 2 min. To dissect the nucleus accumbens, using a razor blade, a slice of tissue was cut at a 45 º angle between the anterior limits of the olfactory tubercles and ~ 1 mm rostral to the optic chiasma. The slice was positioned with the caudal aspect uppermost and punches (1.5 mm diameter) were taken including the anterior commisure (Sleipness et al., 2007). Samples from each brain were pooled. The average weight of the pooled nucleus accumbens tissue was ~13 mg.

The striatum was dissected out of the rest of the brain caudal to the second cut for the slice described above. The average weight of the striatum was ~30 mg. Striata were

not pooled. All tissue samples were kept on ice after dissection until their further preparation for rotating disk electrode (RDE) voltammetry or western blotting.

Measurements of DA Transport Velocity

Inward velocity measurements were performed using RDE voltammetry as described previously (Volz et al., 2004). Both the glassy carbon RDE (Part No. AFM DO3GC) and the precision rotator, set at 2000 rpm, were from Pine Instruments (Grove City, PA). An LC-4C potentiostat from Bioanalytical Systems (West Lafayette, IN) was used to apply +450 mV to the RDE and the resulting detection current was recorded onto a model 310 digital oscilloscope from Nicolet (Madison, WI). Data were sampled every 20 ms for 1.5 minutes. The initial velocity was calculated by measuring the slope of a tangent line to the clearance profile and expressing the result in pmol/(s x g wet weight tissue). Indicators of precision are given as standard errors of regression (SER) and statistical testing between data sets was done using the z- test (Havlicek and Crain, 1988).

Tissue Preparation and Western Blotting

The brain was rapidly removed and the striatum and nucleus accumbens were dissected. The samples were weighed and kept on ice at all times. For each mg of tissue, 10 µl of a buffer containing 10 mM Tris and 1 mM EDTA (Tris-EDTA) at pH 7.5 with protease inhibitors (Roche Diagnostics, Indianapolis, IN) was added and then samples were homogenized. The homogenized samples were then centrifuged at 2000 x g for 10 min at 4 ºC and 10 % Triton X-100 was added to each sample supernatant to a final concentration of 1.5 %. The protein concentration was determined using the BCA™ kit

from Pierce (Rockford, IL) and adjusted with Tris-EDTA buffer so an equal concentration and amount of protein was loaded in each lane. Laemmli sample buffer (Bio-rad, Hercules, CA) with 5% 2-mercaptoethanol (Fisher Scientific, Fair Lawn, NJ) was then added in a 1:1 ratio to each sample. The samples were then heated in a 95º C water bath for 5 min and centrifuged for ~ 1 min. The samples were then loaded alongside Dual Color molecular weight markers (Bio-rad) onto a Criterion™ precast 7.5 % Tris-HCl polyacrylamide gel (Bio-rad) and separated by electrophoresis. Following electrophoresis, the proteins were transferred to a polyvinylidene fluoride membrane in 4º C transfer buffer (192 mM glycine, 25 mM Tris-base, and 15 % methanol in distilled H_2O). The membranes were then rinsed with TTBS and blocked for 1 h in TTBS with 10 % (w/v) powdered milk (TTBS-M). The membranes were rinsed again and probed overnight with a monoclonal mouse anti-DAT antibody (1:1000 in TTBS-M), a kind gift from Dr. Roxanne Vaughan (University of North Dakota, Department of Biochemistry and Molecular Biology, Grand Forks, ND). After another rinse, the membranes were probed with a donkey anti-mouse horseradish peroxidase secondary antibody from Affinity Bioreagents (Golden, CO) at a dilution of 1:2500 in TTBS-M for 2 h. Following the secondary antibody incubation, the membranes were rinsed repeatedly with TTBS and DAT was detected by chemiluminescence using the ECL Plus™ kit from Amersham Biosciences (Piscataway, NJ) and exposing to Kodak (Rochester, NY) Biomax MR™ film. The blots were then stripped and re-probed as described above with anti-actin (Sigma-Aldrich) to use as a loading control.

Film from the western blots was scanned on a Molecular Dynamics (Sunnyvale, CA) Personal Densitometer SI version 4.0 and quantified using the Quantity One (Bio-

Rad) program. Data from blots were obtained as background-subtracted optical densities and were normalized to levels of actin expression in the samples (actin levels do not change with treatment, $p = 0.26$).

Glycosidase Treatments

Samples were treated with α-mannosidase according to the manufacturer's information (Sigma-Aldrich) to remove terminal mannose residues. 12 U/ml of enzyme (EC 3.2.1.24) was added to 30 µg of protein in a total reaction volume of 60 µl of 100 mM, pH 4.5 citrate buffer and the mixture was incubated for 24 h at 25 º C. The reaction was stopped with the addition of Laemmli sample buffer and heating to 95 º C for 5 min.

Neuraminidase treatment to remove sialic acid residues was carried out as described by (Vaughan et al., 1996). Three U/ml of neuraminidase, EC 3.2.1.18 (Roche Diagnostics) in 50 mM sodium acetate, pH 5.5 was added 1:1 to the sample for a final protein concentration of 0.5 mg/ml and incubated at 25 º C for 16 hours.

N-Glycanase treatment was carried out according to the manufacturer's instructions (Prozyme, San Leandro, CA) to remove all N-linked glycans. Using 45 µl of sample (2.4 mg/ml) with 0.1 % SDS, 50 mM 2-mercaptoethanol, and 0.75% Nonidet P-40, 5 mU of N-glycanase (EC 3.5.1.52) was added. The mixture was incubated at 37 º C for 24 hours.

O-glycosidase treatment was carried out according to the manufacturer's instructions (Sigma-Aldrich) to remove o-linked glycosylation. 30 µg of sample was first incubated with neuraminidase for 1 hour at 37 º C to remove sialic acids. Two µl O-

glycosidase (EC 3.2.1.97) was then added to the sample and the mixture was incubated for 24 hours at 37 º C.

RESULTS

Behavioral characterization of repeated METH treatment

Figure 1 shows the behavioral response of 5 mg/kg METH or saline injection on treatment days one and five. Daily treatment with METH produced higher behavioral ratings than saline-treated controls, and by the fifth day, rats treated with METH displayed patterned sniffing earlier in the observation period. Saline treated animals produced consistently low behavioral scores that did not change significantly.

DA transport in the nucleus accumbens and striatum after METH treatment

The activity of the DAT 24 hours after the last METH or saline injection was assessed to determine if this parameter was altered. Figure 2A shows an oscilloscope trace of typical raw data observed in transport experiments. The signal decreases over time as DA is transported into the cell where it is not detected by the electrode. Figure 2B, shows the effects of saline and METH treatments on DA transport velocity in striatal and nucleus accumbens tissues. In nucleus accumbens tissue, METH treatment reduced DA transport by 21 %. In contrast, in the striatum, METH treatment increased transport velocity by a small but significant amount (5.9 % higher than saline-treated animals).

Measurement of DAT density after METH treatment

Different areas of the brain have different DAT densities, and METH treatment can alter the density of DAT (Bennett et al., 1998; Freed et al., 1995). Additionally, as shown in Figure 2, the DA transport by DAT is altered differently after repeated METH treatment in the nucleus accumbens and striatum. In Figure 3, representative western blots for each brain area from animals treated with either METH or saline are shown along with densitometric analysis of these blots. Normalization of DA transport activity by DAT density is shown to the right of the densitometric analyses. Figure 3A is of the nucleus accumbens. The density of both lower and higher molecular weight DAT forms tended toward a decrease following METH treatment but differences between the treatments were not statistically significant. However, when DA transport (from Fig 2A) was normalized by DAT density, the METH-treated samples are statistically lower than the saline-treated controls. In the striatum, shown in Figure 3B, both the lower molecular weight DAT and total DAT density were statistically lower in METH-treated compared with saline-treated animals. When DA transport velocity (from Fig 2B) was normalized by DAT density, the METH-treated samples were significantly higher than controls.

Measurement of METH inhibition of DA transport

After it was determined that METH altered DA transport by the DAT in both the nucleus accumbens and the striatum, we assessed the extent to which METH inhibited DA transport after repeated METH treatment. The inset bar graph in Figure 4A, shows the results of DAT function in the nucleus accumbens. Inhibition was significantly decreased in the METH-treated samples compared to saline-treated controls, with the IC_{50} increasing from 0.81 ± 0.07 to 1.45 ± 0.08 μM METH. Figure 4B shows that

striatal samples from METH-treated animals show increased inhibition to METH. The IC_{50} has decreased from 1.00 ± 0.09 to 0.81 ± 0.08 μM METH. The METH concentrations used ranged from above and below the K_I of METH at the DAT (Povlock et al., 1996).

Endo and exoglycosidase treatment

As shown in Figure 3, the DAT density cannot account for altered activity in the two brain areas. Glycosylation of the DAT has been suggested to possibly play an important role in the differences in DAT activity between the nucleus accumbens and striatum (Bennett et al., 1998; Schenk, 2002). To take a closer look at whether the glycosylation state of DAT in these brain areas was affected by repeated METH treatment, we treated the dissected tissue samples with exo and endoglycosidases. If the glycosylation of DAT is altered due to METH treatment, the reduction in molecular weight may be different between the saline-treated and METH-treated samples. Results from the experiment using endo and exoglycosidase on METH and saline-treated samples are shown in Figure 5. The top two rows of panels are western blots of endoglycosidase treated samples. O-glycosidase (OG) (first row of panels) did not alter the molecular weight of the DAT under either condition or brain area. N-glycanase (NG) treatment reduced the molecular weight of DAT to just above 37 kD, consistent with previous studies (second row of panels) (Lew et al., 1991; Lew et al., 1992). The reduction appears to be the same for both treatment groups (METH versus saline). The bottom two rows of panels show western blots of samples treated with an exoglycosidase. The samples treated with α- mannosidase (αM) did not have a lowered molecular weight for

either treatment or brain area (third row of panels). Neuraminidase (NA) treatment reduced the molecular weight of the DAT to approximately the same degree under both treatment regimes (fourth row of panels). The reduction in molecular weight is similar to that found in previous studies, around 5 kD (Lew et al., 1991; Lew et al., 1992).

DISSCUSSION

The present study was carried out to determine what changes in DAT kinetics, inhibition by METH, and glycosylation occur in animals behaviorally sensitized by METH. We show that in rats behaviorally sensitized by METH, there are 1) decreases in DA transport in the nucleus accumbens and increases in the striatum 2) decreased density of DAT in the striatum 3) changes in kinetic regulation of the DAT in both the nucleus accumbens and striatum 4) shifting of the IC_{50} of METH to larger values in the nucleus accumbens, and 5) no alteration of glycosylation of the DAT in either area.

In Figure 2B, the initial transport velocities of DA by the DAT are shown for samples from METH and saline-treated rats from the nucleus accumbens and striatum. Rats sensitized by METH have a reduction in DA transport in the nucleus accumbens and an increase in the striatum. The increase of DA transport in the striatum has been observed previously (Yamada et al., 1988), with no change in total DA concentration.

Changes in transport velocity have been linked to DAT density (Gulley and Zahniser, 2003; Zahniser and Sorkin, 2004). In the present study, western blotting techniques were utilized to measure DAT density after treatment with either METH or saline (Fig 3). Two areas of the immunoreactive bands were measured to account for both high and lower molecular weight species of this protein. Figure 3A shows that in the

nucleus accumbens the DAT levels decreased somewhat, but were not statistically different. Therefore the decrease in DA transport velocity shown in Figure 2 may not be due to decreased DAT density, but kinetic down-regulation. This is shown by the normalized DA transport velocity. Western blots and densitometric analysis of the striatal samples show that DAT levels are reduced in sensitized animals, at least after 24 hours post injection. A decrease in striatal DAT has been noted previously in the literature when a METH-sensitizing regime was followed (Nakayama et al., 1993). In the present study, the protein levels are reduced in the lower molecular weight DAT species but not in the higher molecular weight ones. This observation may suggest that more highly glycosylated DATs are not altered by METH treatment. The decreased DAT density in the striatum suggests that DAT activity may be decreased, but as shown in Figure 2, DA transport has increased. Normalized DA transport values show a statistically significant increase in METH-treated samples. Therefore, in the striatum, the DAT is kinetically up-regulated by METH exposure.

Further kinetic studies were carried out to determine if METH-sensitized animals had altered DAT inhibition to METH (Fig. 4). The different effects of METH treatment on brain area continue when looking at the inhibition of DA transport by METH. The DAT in the nucleus accumbens of METH-treated animals seems to have an altered mechanism as the inhibition is less than saline-treated controls. In fact the IC_{50} for METH has shifted to the right by 35.4 %. This increase makes sense as the initial DA transport velocity is already lower, therefore it should take a higher METH concentration to inhibit the DAT in the nucleus accumbens. The IC_{50} of striatal samples (Fig. 4B) from METH-treated animals remain the same as saline-treated controls. The lack of effect on

the IC_{50} in the striatum suggests that the DAT located in this brain area may be upregulated and transporting DA more efficiently (Fig. 2) despite the decrease in DAT density. These data further suggest that DAT kinetics have been altered in both brain areas.

In past METH studies, the cause of alteration to the DAT was unknown (Bennett et al., 1998; Kokoshka et al., 1998). Kokoshka et al. (1998) and Bennett et al (1997) both found the decrease in DA transport after METH treatment was not due to a decrease in DAT density. It has been suggested that glycosylation may be the key to the differences in brain areas to drugs of abuse (Lew et al., 1992; Patel et al., 1994). Glycosylation can also affect substrate binding and transport (Meiergerd and Schenk, 1994; Zalewska and Erecinska, 1987). Additional experiments were done to analyze the types and levels of glycosylation on the DAT to see if glycosylation was altered by METH treatment, thus causing changes in DAT function. The reduction of DAT molecular weight after N-glycanase and neuraminidase treatments was similar to previous studies (Lew et al., 1991). O-glycosidase and α-mannosidase did not seem to have any effect on DAT molecular weight, as previously described (Lew et al., 1992; Lew et al., 1991). Analysis of glycosidase-treated samples by western blot suggests that glycosylation of DAT is not altered by METH treatment in either the striatum or nucleus accumbens. Additionally, O-glycosidase and α-mannosidase treatment demonstrated that the DAT most likely does not contain the substrates for these enzymes and these types of glycosylation are not added after METH treatment.

It has recently been found that alcohol increases the whole cell level of glycosylation of neuronal cells in culture (Braza-Boils et al., 2006). In different disease

states, the glycosylation of certain proteins is altered (Natarajan et al., 2006; Saez-Valero and Small, 2001). It appears, however, the presence of METH does not alter the glycosylation state or density of DAT in the studies presented here. A caveat to the interpretation of these results is that the changes may be undetectable using western blotting techniques.

The differences in activity but not glycosylation state due to METH in the striatum versus the nucleus accumbens are significant. The nucleus accumbens has been repeatedly shown to be important for reward and motivation of drug-seeking behavior (Vanderschuren and Everitt, 2005; Wise, 1998). This area is hypothesized to be involved in the initial stages of the addiction process (Vanderschuren and Everitt, 2005). The striatum, on the other hand, is seen to be important for habituation (Vanderschuren and Everitt, 2005). This area is involved in the long-term reinforcement of drug use. Therefore these two areas are believed to have different roles in the process of drug abuse and also, as shown here, have different DAT activities and possibly molecular or regulatory changes accompanying METH sensitization.

This study demonstrates brain region specific effects of METH sensitization on DAT kinetics and inhibition with no changes in glycosylation. Future studies regarding METH effects on the DAT should therefore remain focused on other post translational modifications, such as phosphorylation and should include interacting proteins that may alter signaling systems kinetically regulating the DAT.

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REFERENCES

Belcher AM, O'Dell SJ, Marshall JF. 2006. A sensitizing regimen of methamphetamine causes impairments in a novelty preference task of object recognition. Behavioural Brain Res 170(1):167-172.

Bennett BA, Hollingsworth CK, Martin RS, Harp JJ. 1998. Methamphetamine-induced alterations in dopamine transporter function. Brain Research 782(1,2):219-227.

Brady AM, Glick SD, O'Donnell P. 2005. Selective disruption of nucleus accumbens gating mechanisms in rats behaviorally sensitized to methamphetamine. J Neurosci 25(28):6687-6695.

Braza-Boïls A, Tomás M, Marín MP, Megías L, Sancho-Tello M, Fornas E, Renau-Piqueras J. 2006. Glycosylation is altered by ethanol in rat hippocampal cultured neurons. Alcohol and Alcoholism (Oxford, United Kingdom) 41(5):494-504.

Fleckenstein AE, Metzger RR, Gibb JW, Hanson GR. 1997. A rapid and reversible change in dopamine transporters induced by methamphetamine. Eur J Pharmacol 323(2/3):R9-R10.

Freed C, Revay R, Vaughan RA, Kriek E, Grant S, Uhl GR, Kuhar MJ. 1995. Dopamine transporter immunoreactivity in rat brain. JComp Neurol 359(2):340-349.

Frey K, Kilbourn M, Robinson T. 1997. Reduced striatal vesicular monoamine transporters after neurotoxic but not after behaviorally-sensitizing doses of methamphetamine. Eur J Pharmacol 334(2/3):273-279.

Fumagalli F, Gainetdinov RR, Valenzano KJ, Caron MG. 1998. Role of dopamine transporter in methamphetamine-induced neurotoxicity: evidence from mice lacking the transporter. J Neurosci 18(13):4861-4869.

Genova L, Berke J, Hyman SE. 1997. Molecular adaptations to psychostimulants in striatal neurons: toward a pathophysiology of addiction. Neurobiol Disease 4(3 & 4):239-246.

Gulley JM, Zahniser NR. 2003. Rapid regulation of dopamine transporter function by substrates, blockers and presynaptic receptor ligands. Eur J Pharmacol 479(1-3):139-152.

Havlicek LL, Crain RD. 1988. Practical Statistics for the Physical Sciences. Washington D.C.: American Chemical Society.

Itzhak Y, Martin JL, Ali SF. 2002. Methamphetamine-induced dopaminergic neurotoxicity in mice. Long-lasting sensitization to the locomotor stimulation and desensitization to the rewarding effects of methamphetamine. Progress in Neuro-PsychopharmacolBiol Psych 26(6):1177-1183.

Jones SR, Joseph JD, Barak LS, Caron MG, Wightman RM. 1999. Dopamine neuronal transport kinetics and effects of amphetamine. J Neurochem 73(6):2406-2414.

Kalivas PW, Duffy P, DuMars LA, Skinner C. 1988. Behavioral and neurochemical effects of acute and daily cocaine administration in rats. J Pharmacol Exp Ther 245(2):485-492.

Kazahaya Y, Akimoto K, Otsuki S. 1989. Subchronic methamphetamine treatment enhances methamphetamine- or cocaine-induced dopamine efflux in vivo. Biol Psych 25(7):903-912.

Kokoshka JM, Vaughan RA, Hanson GR, Fleckenstein AE. 1998. Nature of methamphetamine-induced rapid and reversible changes in dopamine transporters. Eur J Pharmacol 361(2/3):269-275.
Letchworth SR, Nader MA, Smith HR, Friedman DP, Porrino LJ. 2001. Progression of changes in dopamine transporter binding site density as a result of cocaine self-administration in rhesus monkeys. J Neurosci 21(8):2799-2807.
Lew R, Grigoriadis D, Wilson A, Boja JW, Simantov R, Kuhar MJ. 1991. Dopamine transporter: deglycosylation with exo- and endoglycosidases. Brain Res 539(2):239-246.
Lew R, Patel A, Vaughan RA, Wilson A, Kuhar MJ. 1992. Microheterogeneity of dopamine transporters in rat striatum and nucleus accumbens. Brain Res 584(1-2):266-271.
Lew R, Vaughan R, Simantov R, Wilson A, Kuhar MJ. 1991. Dopamine transporters in the nucleus accumbens and the striatum have different apparent molecular weights. Synapse (New York, NY, United States) 8(2):152-153.
McBride WJ, Murphy JM, Ikemoto S. 1999. Localization of brain reinforcement mechanisms: intracranial self-administration and intracranial place-conditioning studies. Behav Brain Res 101(2):129-152.
McElvain JS, Schenk JO. 1992. A multisubstrate mechanism of striatal dopamine uptake and its inhibition by cocaine. Biochem Pharmacol 43(10):2189-2199.
Meiergerd SM, Schenk JO. 1994. Striatal transporter for dopamine: catechol structure-activity studies and susceptibility to chemical modifications. J Neurochem 62(3):998-1008.
Nakayama M, Koyama T, Yamashita I. 1993. Long-lasting decrease in dopamine uptake sites following repeated administration of methamphetamine in the rat striatum. Brain Res 601(1-2):209-212.
Natarajan SK, Ramamoorthy P, Thomas S, Basivireddy J, Kang G, Ramachandran A, Pulimood AB, Balasubramanian KA. 2006. Intestinal mucosal alterations in rats with carbon tetrachloride-induced cirrhosis: changes in glycosylation and luminal bacteria. Hepatol (Hoboken, NJ, United States) 43(4):837-846.
Nestler EJ. 2001. Molecular basis of long-term plasticity underlying addiction. Nat Rev Neurosci 2(2):119-128.
Patel AP, Cerruti C, Vaughan RA, Kuhar MJ. 1994. Developmentally regulated glycosylation of dopamine transporter. Dev Brain Res 83(1):53-58.
Pierce RC, Kalivas PW. 1997. A circuitry model of the expression of behavioral sensitization to amphetamine-like psychostimulants. Brain Res Rev 25(2):192-216.
Porrino LJ, Lyons D, Smith HR, Daunais JB, Nader MA. 2004. Cocaine self-administration produces a progressive involvement of limbic, association, and sensorimotor striatal domains. J Neurosci 24(14):3554-3562.
Povlock SL, Meiergerd SM, Schenk JO. 1996. Kinetic mechanisms of the dopamine transporter: A comparison with other biogenic amine transporters. CNS Neurotransmitters and Neuromodulators: Dopamine:21-39.
Povlock SL, Schenk JO. 1997. A multisubstrate kinetic mechanism of dopamine transport in the nucleus accumbens and its inhibition by cocaine. J Neurochem 69(3):1093-1105.

Raiteri M, Bertollini A, Angelini F, Levi G. 1975. d-Amphetamine as a releaser or reuptake inhibitor of biogenic amines in synaptosomes. Eur J Pharmacol 34(1):189-195.
Raiteri M, Del Carmine R, Bertollini A, Levi G. 1977. Effect of sympathomimetic amines on the synaptosomal transport of noradrenaline, dopamine and 5-hydroxytryptamine. Eur J Pharmacol 41(2):133-143.
Robinson TE, Becker JB. 1982. Behavioral sensitization is accompanied by an enhancement in amphetamine-stimulated dopamine release from striatal tissue in vitro. Eur J Pharmacol 85(2):253-254.
Robinson TE, Berridge KC. 1993. The neural basis of drug craving: an incentive-sensitization theory of addiction. Brain Res Rev 18(3):247-291.
Sáez-Valero J, Small DH. 2001. Altered glycosylation of cerebrospinal fluid butyrylcholinesterase in Alzheimer's disease. Brain Res 889(1,2):247-250.
Schenk JO. 2002. The functioning neuronal transporter for dopamine: kinetic mechanisms and effects of amphetamines, cocaine and methylphenidate. Prog Drug Res 59:111-131.
Sleipness EP, Sorg BA, Jansen HT. 2007. Diurnal differences in dopamine transporter and tyrosine hydroxylase levels in rat brain: Dependence on the suprachiasmatic nucleus. Brain Res 1129(1):34-42.
Stefanski R, Ladenheim B, Lee SH, Cadet JL, Goldberg SR. 1999. Neuroadaptations in the dopaminergic system after active self-administration but not after passive administration of methamphetamine. Eur J Pharmacol 371(2/3):123-135.
Stefanski R, Lee S-H, Yasar S, Cadet JL, Goldberg SR. 2002. Lack of persistent changes in the dopaminergic system of rats withdrawn from methamphetamine self-administration. Eur J Pharmacol 439(1-3):59-68.
Vanderschuren LJMJ, Everitt BJ. 2005. Behavioral and neural mechanisms of compulsive drug seeking. Eur J Pharmacol 526(1-3):77-88.
Vaughan RA, Brown VL, McCoy MT, Kuhar MJ. 1996. Species- and brain region-specific dopamine transporters: immunological and glycosylation characteristics. J Neurochem 66(5):2146-2152.
Volz TJ, Fleckenstein AE, Hanson GR. 2007. Methamphetamine-induced alterations in monoamine transport: implications for neurotoxicity, neuroprotection and treatment. Addiction 102(s1):44-48.
Volz TJ, Kim M, Schenk JO. 2004. Covalent and noncovalent chemical modifications of arginine residues decrease dopamine transporter activity. Synapse (New York, NY, United States) 52(4):272-282.
Wayment H, Meiergerd SM, Schenk JO. 1998. Relationships between the catechol substrate binding site and amphetamine, cocaine, and mazindol binding sites in a kinetic model of the striatal transporter of dopamine in vitro. J Neurochem 70(5):1941-1949.
White FJ, Kalivas PW. 1998. Neuroadaptations involved in amphetamine and cocaine addiction. Drug and Alcohol Dependence 51(1,2):141-153.
Wise RA. 1998. Drug-activation of brain reward pathways. Drug Alc Depend 51(1,2):13-22.

Yamada S, Kojima H, Yokoo H, Tsutsumi T, Takamuki K, Anraku S, Nishi S, Inanaga K. 1988. Enhancement of dopamine release from striatal slices of rats that were subchronically treated with methamphetamine. Biol Psych 24(4):399-408.
Zahniser NR, Sorkin A. 2004. Rapid regulation of the dopamine transporter: role in stimulant addiction? Neuropharmacol 47(Suppl. 1):80-91.
Zalewska M, Erecinska M. 1987. Involvement of sialic acid in high-affinity uptake of dopamine by synaptosomes from rat brain. Neurosci Lett 82:107-112.

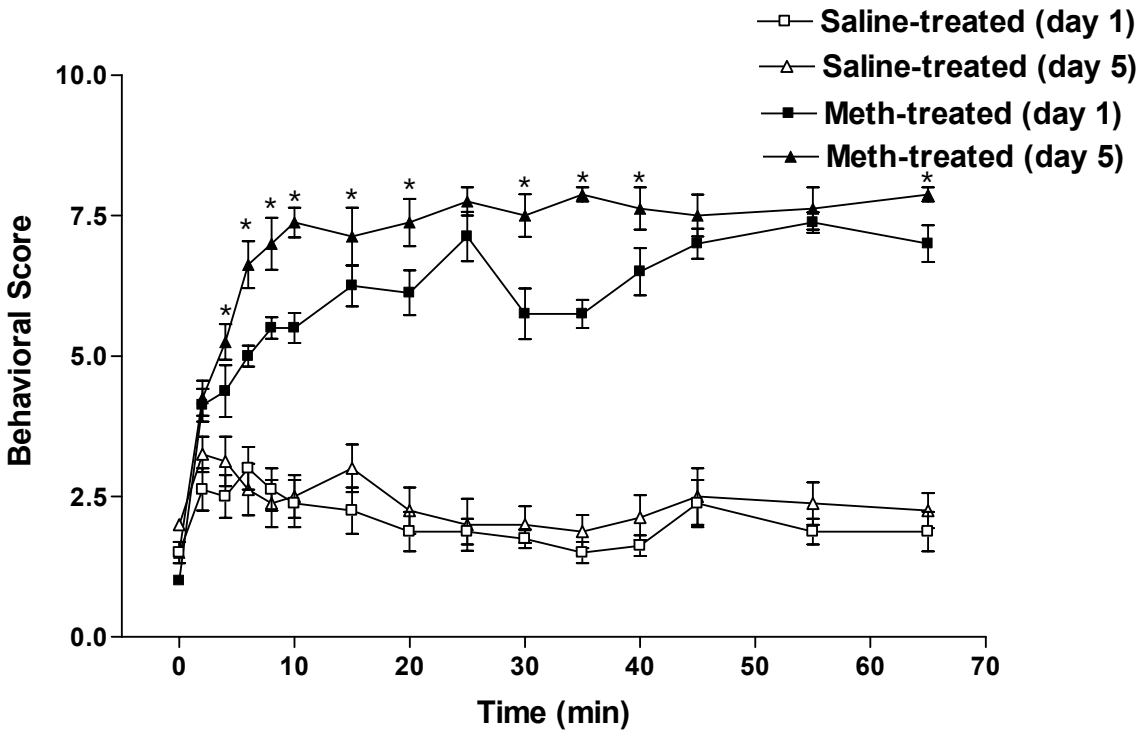


Figure 1. Effects of METH treatment on behavioral scores versus time. Open squares and open triangles represent the mean behavioral score ± SEM of saline-treated animals on days 1 and 5 respectively (n = 8). The closed squares and closed triangles represent the mean behavioral score ± SEM of METH-treated animals on days 1 and 5 respectively, (n = 8). Time = 0 corresponds to behavioral score 5 min before injection of either saline or METH. Differences of the responses between 1) saline and METH treated animals and 2) the days of METH injection were analyzed by ANOVA and were 1) found to be significantly different with $P < 0.0001$ and 2) METH day 5 was statistically higher than day 1 beginning at 6 min after treatment. Doses: 5 mg/kg METH or 1.0 ml/kg saline per day for 5 days.

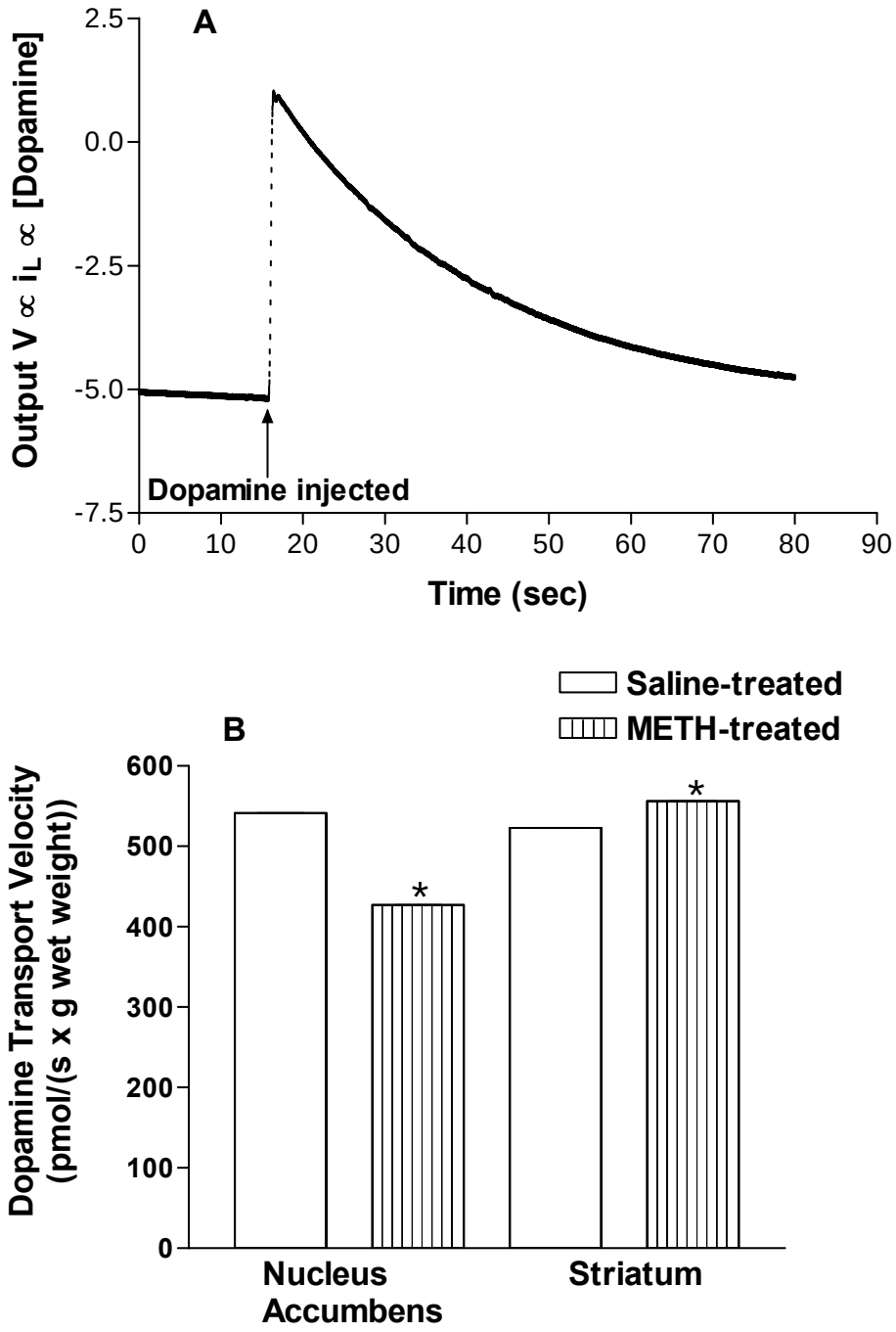
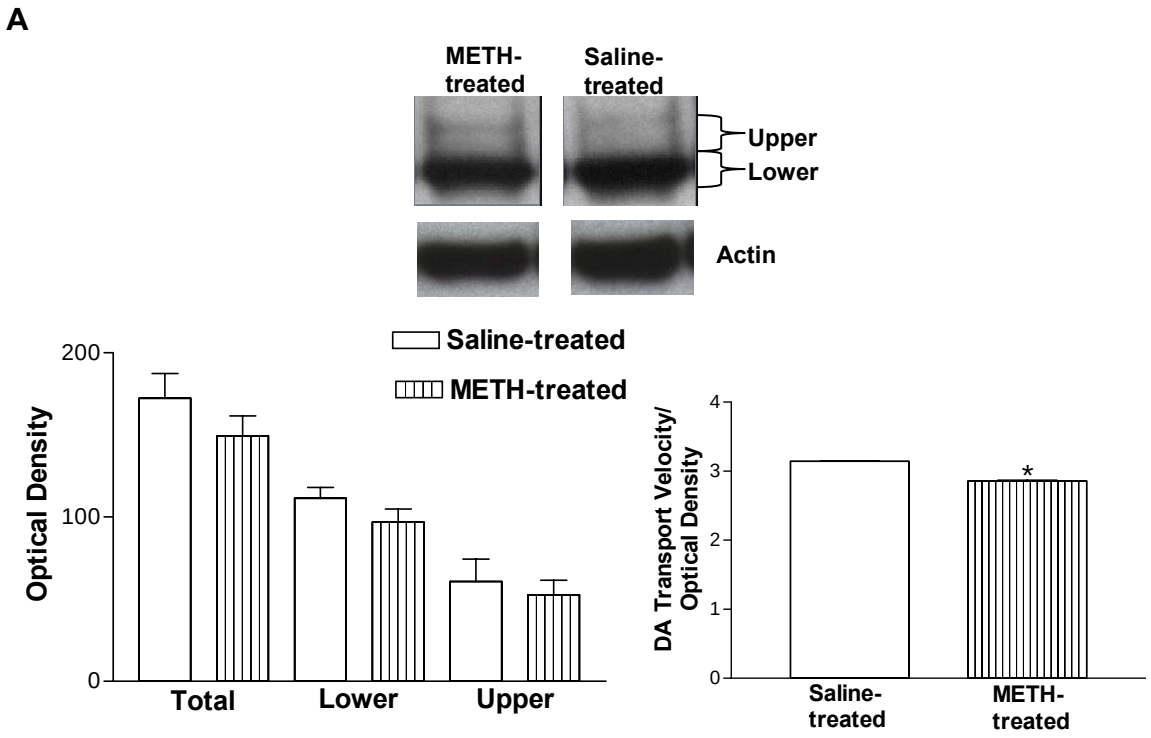


Figure 2. Effects of METH treatment on initial DA transport velocity. Panel **A** shows an oscilloscope reading before and after injection with 1.0 μM DA. Panel **B** is a bar graph representing initial DA transport velocity by the DAT in the nucleus accumbens and striatum. Open bars represent saline-treated controls (n = 4) and striped bars represent velocities for METH-treated animals (n = 4). Asterisks denote a statistically significant difference in transport velocity for the METH-treated samples versus saline-treated ($P <$ 0.001 via the *z*-test). Standard errors of regression (SER) are within the dimensions of the bars.


A
METH-treated
Saline-treated
Upper
Lower
Actin
Saline-treated
METH-treated
Optical Density
200
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Total
Lower
Upper
DA Transport Velocity/ Optical Density
4
3
2
1
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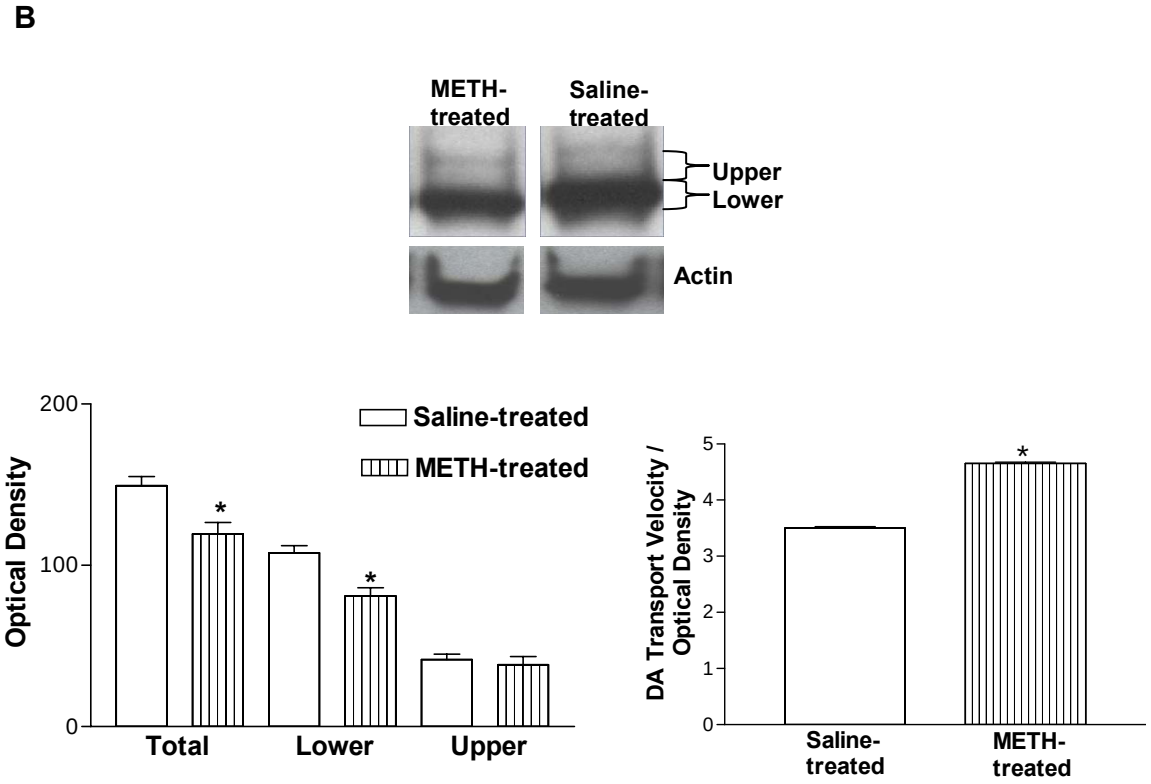
Saline-treated
METH-treated


B
METH-treated
Saline-treated
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Actin
Saline-treated
METH-treated
Optical Density
200
100
0
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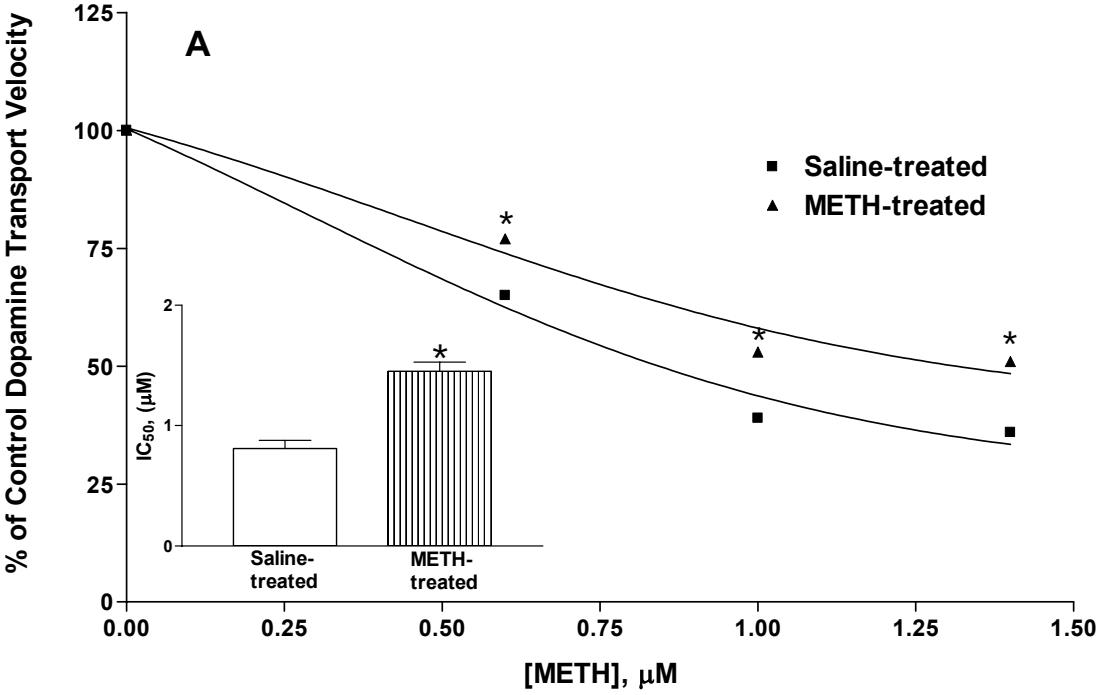
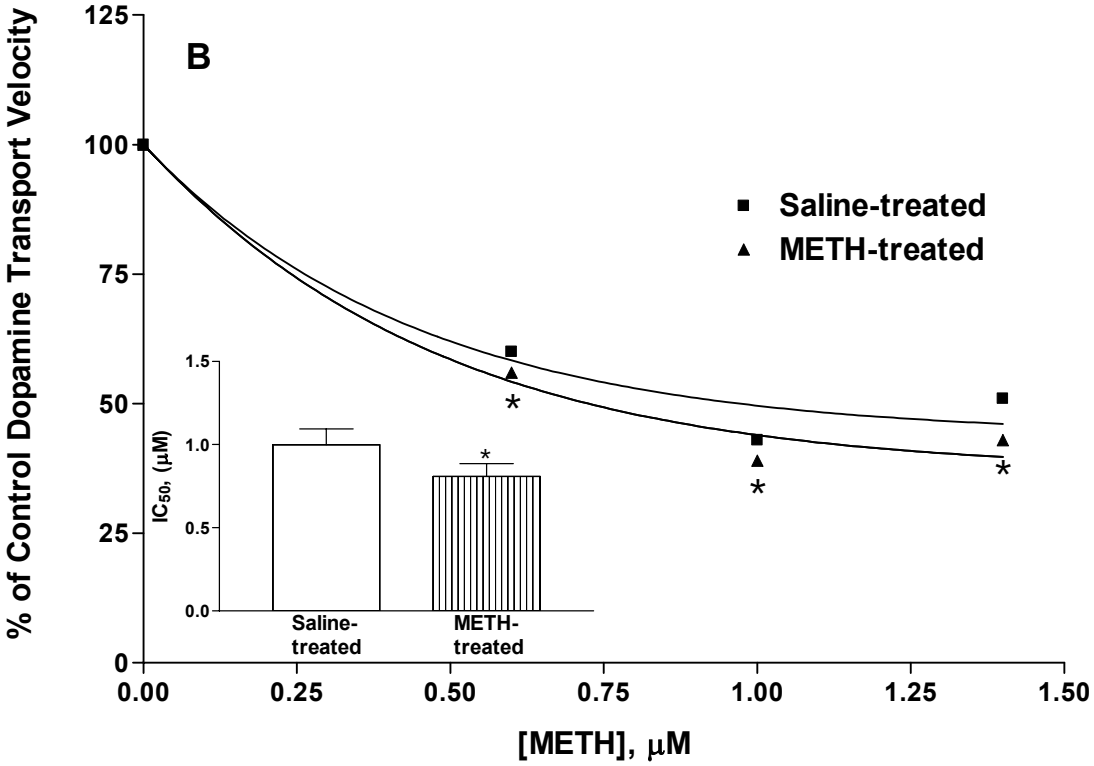
*
Total
Lower
Upper
DA Transport Velocity / Optical Density
5
4
3
2
1
0
*
Saline-treated
METH-treated

Figure 3. Effects of METH treatments on DAT density in the nucleus accumbens and striatum. Rats treated with METH show similar levels of DAT in the nucleus accumbens (Panel A) but lower levels in the striatum (Panel B). Representative western blots are shown above the densitometric analyses and normalized DA transport velocities in each panel. Error bars represent the mean ± S.E.M. with an n = 7 or 8 from different groups of animals. Asterisks represent density values significantly different from saline-treated controls at $P = 0.0086$ for lower MW DAT and $P = 0.0151$ for total DAT via a *t*-test. The density values for DAT were normalized to β-actin expression. Average DA transport velocity values from Figure 2 were normalized by the corresponding DAT density ± SE ($P < 0.0001$).


A
% of Control Dopamine Transport Velocity
125
100
75
50
25
0
Saline-treated
METH-treated
*
IC50, (μM)
2
1
0
Saline-treated
METH-treated
0.00
0.25
0.50
0.75
1.00
1.25
1.50
[METH], μM


B
% of Control Dopamine Transport Velocity
125
100
75
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0
Saline-treated
METH-treated
*
IC50, (μM)
1.5
1.0
0.5
0.0
Saline-treated
METH-treated
0.00
0.25
0.50
0.75
1.00
1.25
1.50
[METH], μM

Figure 4. Rats sensitized to METH show decreased METH inhibition at the DAT in the nucleus accumbens but not the striatum. METH was injected along with 1 μM DA to measure DA transport velocity and examine METH inhibition. Panels **A** and **B** represent values measured in the nucleus accumbens and striatum, respectively. Each datum point represents the percent ± % SE of DA transport velocity at various METH concentrations versus control (no METH) where n = 4. Asterisks represent values statistically different from saline-treated controls ($P < 0.0001$). The inset in each graph is the average ± SER IC_{50} values for each treatment group. An asterisk represents an IC_{50} value statistically different from the saline-treated animal value via a *z*-test at $P < 0.0001$.

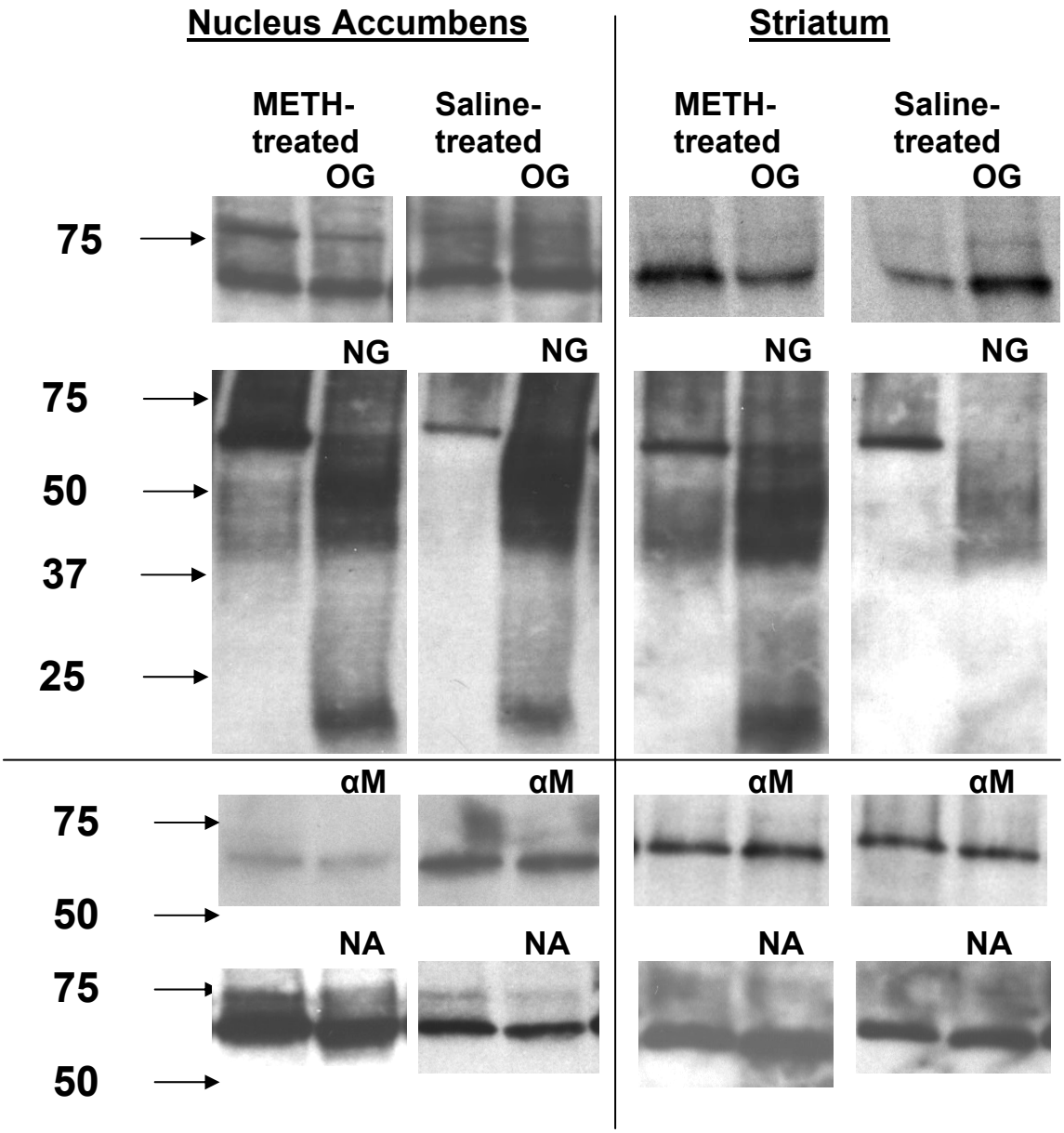

Figure 5. Endo and exoglycosidase treatments of DAT show no difference in glycosylation states after METH treatment. Representative western blots of O-glycosidase (OG) treated samples are shown in the top panels where samples in lanes on the left were controls and those on the right were treated with OG. The second row of panels show representative western blots of N-glycanse (NG) treated samples alongside control samples. Representative western blots of α-mannosidase (αM)-treated samples are shown in the third row of panels where samples in lanes on the left were controls and those on the right were treated with αM. The bottom row of panels show representative western blots of neuraminidase-treated (NA) samples alongside control samples. Nucleus accumbens samples are pooled from 4 animals, while striatal samples were from individual animals (n = 4).

Withdrawal from cocaine treatments: Differences in neuroadaptations of the dopamine transporter in the nucleus accumbens versus striatum

Nicole L. Bjorklund [a], Barbara A. Sorg [b], and James O. Schenk [a,b,c,*]

[a]*School of Molecular Biosciences,* [b]*Programs in Neuroscience/Toxicology, and*
[c]*Department of Chemistry, Washington State University*
Pullman, Washington 99164, USA

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[*]Corresponding author: Tel.:+1-509-335-4300; fax: 1- 509-335-8867.
E-mail address: geni@wsu.edu (J.O. Schenk)

Abstract

Cocaine is an extremely addictive substance. Small doses are able to permanently sensitize rats and alter dopaminergic neurotransmission. Here the effects following withdrawal from a sensitizing dosing of cocaine (15 mg/kg, i.p. per day for 3 days) were measured on dopamine transport by the DAT and DAT density in the nucleus accumbens and striatum. Dopamine transport velocities were lower in the nucleus accumbens and increased in the striatum at 24 hours withdrawal. Western blots and densitometric analyses demonstrated that at 24 hours DAT density was unchanged in both brain areas. Thus, DAT appears to be kinetically down-regulated in the nucleus accumbens. After 3 weeks withdrawal, dopamine transport returned to control values in the nucleus accumbens but was still increased in the striatum. DAT density was decreased in the nucleus accumbens, specifically the lower molecular weight form of DAT. Normalization of transport by DAT density showed that the DAT is kinetically up-regulated in the nucleus accumbens after 3 weeks withdrawal. However, dopamine transport normalized by DAT density from cocaine-sensitized striatal samples was similar to saline-treated controls. This study demonstrates that areas important for sensitization have long-term neuroadaptations. The striatum is involved in later stages of the addiction process, but as shown in the present study, dopamine transport remains increased in this area.

1. Introduction

Behavioral sensitization represents the craving aspect of drug abuse of cocaine (White and Kalivas, 1998). Repeated treatment with cocaine in rats causes behavioral sensitization (Kalivas et al., 1988). The increased locomotor response to a challenge dose of cocaine in rats previously treated with cocaine demonstrates how sensitive they are to the stimulant. This sensitivity to cocaine is permanent and has been found to increase over withdrawal time (Peris et al., 1990).

The main target of cocaine regulating sensitization is the dopamine transporter (DAT). The DAT plays an important role in dopaminergic neurotransmission by removing dopamine from the synaptic space and therefore regulating the availability of dopamine to react with dopamine receptors. Cocaine binds to the DAT and inhibits dopamine from binding and being transported into the pre-synaptic neuron. The increase of dopamine by cocaine in the synapse causes a "high" and increases the activation of dopamine receptors (Nestler, 2001). The inhibition of the DAT by cocaine causes immediate effects but it also causes long lasting molecular effects (Nestler, 2001).

The molecular effects in sensitized animals occur in brain areas in which cocaine acts and also where the cell bodies project (Koob, 1992). The reward pathway for stimulants includes the ventral tegmental area, nucleus accumbens, and the prefrontal cortex. The striatum (caudate and putamen in humans), which has high DAT density compared to other areas of the central nervous system, is also affected by cocaine

exposure (Little et al., 1999). The striatum plays a later role in the drug addiction process. It converts an action into a habit, thus abuse into addiction (Weiss, 2005).

The reported effects of cocaine on DAT have varied, most likely due to different dosing protocols, withdrawal times, and experimental procedures. The research presented here specifically is investigating how sensitizing doses of cocaine affect DAT. In previous studies it was found after a three day withdrawal, sensitized rats have a decrease in dopamine transport in the nucleus accumbens and increased stimulated dopamine release (Cass et al., 1993; Chefer and Shippenberg, 2002). Decreased transport was also found in another study which also found the striatum was not affected by sensitizing doses of cocaine (Cass et al., 1993). The DAT density does not change in the medial prefrontal cortex in response to sensitizing doses of cocaine (Williams and Steketee, 2005). DAT density results in nucleus accumbens and striatum after cocaine treatment have been measured and found not to change (Cass et al., 1993), however the dosing protocol and withdrawal times are different than presented here. Previous studies in this laboratory have found the V_{max} and turnover number of striatal dopamine transport to be increased in cocaine-treated rats at both 24 hours and 2 weeks withdrawal (Meiergerd et al., 1994). The present work is an extension of this research.

The different areas of the brain play different roles in the drug abuse process and therefore are affected in different ways by exposure to drugs of abuse. The study of how areas are altered in specific models of drug addiction aid in the understanding of 1) how exposure to drugs of abuse alter the brain, 2) how the brain is altered in addiction states, 3) how the alteration of neurotransmission can disrupt brain function, and 4) possible strategies to circumvent alterations due to drugs of abuse or disease.

This research examines alterations to the DAT in animals treated with sensitizing dosing of cocaine. The activity, density, and normalized dopamine transport velocities were assessed at both 24 hours and 3 weeks withdrawal. Therefore this investigation takes into account both short and long-term changes in cocaine-treated animals.

2. Materials and Methods

2.1 *Chemicals and Solutions*

Solutions were prepared with deionized water purified further with a Barnstead Nanopure (Dubuque, IA) water purification system. Chemicals were reagent grade and used as received. The common buffer salts were purchased from JT Baker Chemical Co. (Philipsburg, NJ). Dopamine hydrochloride (DA), (-)-cocaine hydrochloride, and TWEEN 20 were purchased from Sigma-Aldrich (St. Louis, MO). The composition of the pH 7.4 physiological buffer was 124 mM NaCl, 1.8 mM KCl, 1.3 mM $MgSO_4$, 1.24 mM KH_2PO_4, 2.5 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose saturated with 95 % O_2/ 5 % CO_2. Tris-buffered saline with TWEEN 20 (TTBS) contained 10 mM Tris base, 150 mM NaCl and 0.05% TWEEN 20, pH 7.6.

2.2 *Animal Protocol*

Animal procedures were approved by the Institutional Animal Care and Use Committee.

Male Sprague-Dawley rats from the Washington State Univerisyt breeding colony weighing 350-500 g were used for the present studies. Animals were housed in the vivarium with a 12 h light/ dark cycle (lights on at 6:00 a.m.) and had access to food and water *ad libitum*. After brief handling once a day for four days, an injection protocol was initiated. The cocaine group received 15 mg/kg interperitonily per day for 3 days (Kalivas et al., 1988). The saline control group received 1 ml/kg saline each day for 3 days. Injections were administered at the same time each day.

At 24 hours or 3 weeks after the last injection, animals were rapidly decapitated and the brain removed and placed on a watchglass over ice for ~ 2 min. To dissect out the nucleus accumbens, using a razor blade, a slice of tissue was cut at a 45º between the anterior limits of the olfactory tubercles and ~ 1 mm rostral to the optic chiasma. The slice was positioned with the caudal aspect uppermost and punches were taken including the anterior commisure. The nucleus accumbens from each brain were pooled. The average weight of the nucleus accumbens sample was ~13.0 mg.

The striatum was dissected out of the rest of the brain caudal to the second cut for the slice described above. The average weight of striatum was 30 mg. Striata were not pooled. All tissue samples were kept on ice until used for voltammetry experiments or preparation for western blotting.

2.3 *Dopamine Transport Velocity Measurements*

Inward velocity measurements were performed using rotating disk electrode voltammetry as described previously (Volz et al., 2005). The working electrode was a

glassy carbon rotating disk electrode (Part No. AFM DO3GC) with a precision rotator at 2000 rpm from Pine Instruments (Grove City, PA). A potentiostat from Bioanalytical systems was used to apply +450 mV to the rotating disk electrode and the resulting detection current was recorded onto a digital oscilloscope from Nicolet (Madison, WI). Data was sampled every 20 ms for 1.5 minutes. The initial velocity was calculated by measuring the slope of a tangent line of the clearance profile between 0 and 15 s. Statistical analysis of the kinetic parameters including indicators of precision are given by standard errors of regression (SER) and statistical testing between sets of data used the z-test (Havlicek and Crain, 1988).

2.4 *Purification and Western Blotting*

The brain was rapidly removed and the striatum and nucleus accumbens were dissected. The samples were weighed and kept on ice at all times. For each mg of tissue, 10 µl of a buffer containing 10 mM Tris and 1 mM EDTA (Tris-EDTA) at pH 7.5 with protease inhibitors (Roche Diagnostics, Indianapolis, IN) was added and then samples were homogenized. The homogenized samples were then centrifuged at 2000 x g for 10 min at 4 ºC and 10 % Triton X-100 was added to each sample supernatant to a final concentration of 1.5 %. The protein concentration was determined using the BCA™ kit from Pierce (Rockford, IL) and adjusted with Tris-EDTA buffer so an equal concentration and amount of protein was loaded in each lane. Laemmli sample buffer (Bio-rad, Hercules, CA) with 5% 2-mercaptoethanol (Fisher Scientific, Fair Lawn, NJ) was then added in a 1:1 ratio to each sample. The samples were then heated in a 95º C

water bath for 5 min and centrifuged for ~ 1 min. The samples were then loaded alongside Dual Color molecular weight markers (Bio-rad) onto a Criterion™ precast 7.5 % Tris-HCl polyacrylamide gel (Bio-rad) and separated by electrophoresis. Following electrophoresis, the proteins were transferred to a polyvinylidene fluoride membrane in 4º C transfer buffer (192 mM glycine, 25 mM Tris-base, and 15 % methanol in distilled H_2O). The membranes were then rinsed with TTBS and blocked for 1 h in TTBS with 10 % (w/v) powdered milk (TTBS-M). The membranes were rinsed again and probed overnight with a monoclonal mouse anti-DAT antibody (1:1000 in TTBS-M), a kind gift from Dr. Roxanne Vaughan (University of North Dakota, Department of Biochemistry and Molecular Biology, Grand Forks, ND). After another rinse, the membranes were probed with a donkey anti-mouse horseradish peroxidase secondary antibody from Affinity Bioreagents (Golden, CO) at a dilution of 1:2500 in TTBS-M for 2 h. Following the secondary antibody incubation, the membranes were rinsed repeatedly with TTBS and DAT was detected by chemiluminescence using the ECL Plus™ kit from Amersham Biosciences (Piscataway, NJ) and exposing to Kodak (Rochester, NY) Biomax MR™ film. The blots were then stripped and re-probed as described above with anti-actin (Sigma-Aldrich) to use as a loading control.

Film from the western blots was scanned on a Molecular Dynamics (Sunnyvale, CA) Personal Densitometer SI version 4.0 and quantified using the Quantity One (Bio-Rad) program. Data from blots were obtained as background-subtracted optical densities and were normalized to levels of actin expression in the samples (actin levels do not change with treatment, $p = 0.26$).

3. Results

3.1 *Dopamine transport velocities in the nucleus accumbens of cocaine-sensitized versus saline-treated rats at 24 hours and 3 weeks withdrawal*

Dopamine transport was measured in the nucleus accumbens of animals treated with either a sensitizing dose of cocaine or saline. Transport of 1 μM dopamine was measured at either 24 hours or 3 weeks post-treatment. As shown in Fig. 1, the transport in the nucleus accumbens was decreased 24 hours after cocaine treatment by 25 % but returns to saline-treated levels 3 weeks later.

3.2 *Dopamine transport velocities in the striatum of cocaine-sensitized versus saline-treated rats at 24 hours and 3 weeks withdrawal*

In the striatum of animals given sensitizing dosing of cocaine, the dopamine transport velocity is increased 7.3 % 24 hours after the last cocaine dose compared to saline-treated animals (Fig. 2). Dopamine transport increases up to 15 % of saline-treated controls even after 3 weeks of withdrawal in this brain area.

3.3 *DAT density in the nucleus accumbens at 24 hours withdrawal in cocaine-sensitized or saline-treated rats; normalization of dopamine transport*

Cocaine exposure can cause changes in DAT density (Little et al., 2002). This may affect dopamine transport velocities (Zahniser and Sorkin, 2004). Fig. 3 shows on the left a representative western blot of nucleus accumbens samples taken 24 hours after treatment of either cocaine or saline. The densitometric analysis is shown on the upper right hand side. Both the lower and upper molecular weight bands were analyzed to account for possible changes in both lower and higher molecular weight DAT forms. DAT density was unchanged in cocaine-treated samples compared to saline-treated with neither the lower or higher molecular weight band density changing. Dopamine transport measured in Fig. 1 was normalized by the measured DAT density and is shown on the lower right hand side. Cocaine-treated samples tended toward a decrease but were statistically the same as control.

3.4 *DAT density in the nucleus accumbens at 3 weeks withdrawal in cocaine-sensitized or saline-treated rats; normalization of dopamine transport*

Fig. 4 shows a representative western blot of nucleus accumbens samples taken after 3 weeks withdrawal from either cocaine or saline. Densitometric analysis of the blot is on the upper right hand side and shows a decrease in DAT density in cocaine-treated samples. The lower molecular weight DAT glycosylation form was decreased, but the more highly glycosylated DAT forms remained unchanged. Normalization of

dopamine transport after 3 weeks withdrawal with corresponding DAT density found the DAT to be kinetically up-regulated in the nucleus accumbens after treatment with cocaine.

3.5 *DAT density in the striatum at 24 hours withdrawal in cocaine-sensitized or saline-treated rats; normalization of dopamine transport*

The DAT in the striatum has been shown to be sensitive to stimulants such as amphetamine and cocaine (Volz et al., 2007). A change in DAT density may alter dopamine transport kinetics (Zahniser and Sorkin, 2004). Fig. 5 shows a representative western blot of striatal samples taken 24 hours after either cocaine or saline treatment. The densitometric analysis on the upper right hand side shows no significant change in DAT density in either the lower or higher molecular weight forms of DAT. Further, normalization of dopamine transport in cocaine-treated samples tends toward an increase but is not statistically different from saline controls.

3.6 *DAT density in the striatum 3 weeks post-treatment in cocaine-sensitized or saline-treated rats; normalization of dopamine transport*

A representative western blot of samples taken after 3 weeks withdrawal from either cocaine or saline is shown on the left hand side of Fig. 6 and the densitometric analysis is on the right hand side. This figure shows that in the striatum of cocaine-treated animals the DAT density tends toward an increase, but is not statistically

significant. This increase was seen in the lower molecular weight form of DAT, but not the more highly glycosylated form. Normalization of dopamine transport by DAT density shows no difference between cocaine and saline-treated samples after 3 weeks withdrawal in the striatum.

4. Discussion

The main findings in animals receiving sensitizing dosing of cocaine are 1) in the nucleus accumbens, dopamine transport by the DAT was decreased after 24 hours withdrawal, but returned to control levels 3 weeks later, 2) in the striatum, dopamine transport was increased after 24 hours and remained increased at 3 weeks withdrawal 3) DAT density in the nucleus accumbens was unchanged at 24 hours withdrawal, but decreased at 3 weeks suggesting the DAT was kinetically up-regulated, and 4) DAT density in the striatum was unaffected with no change in kinetic regulation at either time point of withdrawal.

Previous studies examining dopaminergic changes in animals sensitized to cocaine have used various dosing protocols and withdrawal times. Therefore direct comparisons may not be entirely valid. This study used a dosing protocol validated by (Kalivas et al., 1988) in which extensive behavioral testing was done and long-term changes in dopamine neurochemistry was established in sensitized animals. The present study incorporates changes in DAT activity and expression information to the characterization of this addiction model.

The nucleus accumbens had short and long-term changes associated with sensitizing cocaine dosing. After 24 hours post-treatment, there is a decrease in dopamine transport (Fig. 1). A similar decrease has been found previously (Cass et al., 1993; Chefer and Shippenberg, 2002). When DAT density is used to normalize dopamine transport the numbers tend to be lower in cocaine-treated animals, but are statistically the same as control (Fig. 3). This suggests the DAT may be somewhat down-regulated but not significantly so. In addition, both the lower and higher molecular weight glycosylated forms of DAT were measured in the western blots and no change was detected.

The nucleus accumbens in cocaine-sensitized animals was found to have long-term changes. After 3 weeks withdrawal, the activity of the DAT in cocaine-treated animals is the same as saline-treated, but the DAT density is lower (Fig. 4), specifically the lower molecular weight form of DAT. The higher molecular weight species is unchanged after sensitizing doses of cocaine. Perhaps the more highly glycosylated DAT is not as sensitive to cocaine exposure or more stable. When dopamine transport is normalized, we find the DAT in cocaine-treated animals is kinetically up-regulated. The reduction of the lower molecular weight DAT and kinetic up-regulation shows long-term neuroadaptation in the nucleus accumbens to sensitizing cocaine dosing.

The striatum was affected differently than the nucleus accumbens in cocaine-treated animals. Dopamine transport was increased in cocaine-treated animals after 24 hours post-treatment (Fig. 1), as predicted by analysis of previous studies performed in this laboratory (Meiergerd et al., 1994). However, the density of both low and high molecular weight DAT forms was the same as saline-treated samples. When dopamine

transport was normalized, the cocaine-treated samples tended toward and increase, but were statistically the same as control. The DAT in the striatum may be slightly up-regulated after cocaine sensitizing doses, but changes in stability of certain glycosylation states of the DAT do not seem to be affected.

Striatal samples from cocaine-treated animals taken 3 weeks after treatment had increased transport velocities. The increase in transport velocity at both 24 hours and 3 weeks was similar to that predicted using earlier studies from this laboratory (Meiergerd et al., 1994) and has also been reported by other laboratories using slightly different dosing protocols (Yi and Johnson, 1990).The DAT density remained unchanged and normalization showed no up or down-regulation. The sustained increase of dopamine transport is the major finding in the striatum.

This study highlights the changes that occur to the DAT in cocaine-sensitized animals. Importantly, the differences shown concern the adaptations that occur in different brain areas are involved in distinct roles of the addiction process. The nucleus accumbens plays an active role in sensitization. The striatum is rich in DAT density but is only affected differently by exposure to cocaine.

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References

Cass, W. A., Gerhardt, G. A., Gillespie, K., Curella, P., Mayfield, R. D., Zahniser, N. R., 1993. Reduced clearance of exogenous dopamine in rat nucleus accumbens, but not in dorsal striatum, following cocaine challenge in rats withdrawn from repeated cocaine administration. Journal of Neurochemistry 61, 273-283.
Chefer, V. I., Shippenberg, T. S., 2002. Changes in basal and cocaine-evoked extracellular dopamine uptake and release in the rat nucleus accumbens during early abstinence from cocaine: quantitative determination under transient conditions. Neuroscience (Oxford, United Kingdom) 112, 907-919.
Havlicek, L. L., Crain, R. D., 1988. Practical Statistics for the Physical Sciences. American Chemical Society, Washington D.C.
Kalivas, P. W., Duffy, P., DuMars, L. A., Skinner, C., 1988. Behavioral and neurochemical effects of acute and daily cocaine administration in rats. Journal of Pharmacology and Experimental Therapeutics 245, 485-492.
Koob, G. F., 1992. Drugs of abuse: anatomy, pharmacology and function of reward pathways. Trends in Pharmacological Sciences 13, 177-184.
Little, K. Y., Elmer, L. W., Zhong, H., Scheys, J. O., Zhang, L., 2002. Cocaine induction of dopamine transporter trafficking to the plasma membrane. Molecular Pharmacology 61, 436-445.
Little, K. Y., Zhang, L., Desmond, T., Frey, K. A., Dalack, G. W., Cassin, B. J., 1999. Striatal dopaminergic abnormalities in human cocaine users. Am J Psychiatry FIELD Full Journal Title:The American journal of psychiatry 156, 238-245.
Meiergerd, S. M., McElvain, J. S., Schenk, J. O., 1994. Effects of cocaine and repeated cocaine followed by withdrawal. Alterations of dopaminergic transporter turnover with no changes in kinetics of substrate recognition. Biochemical Pharmacology 47, 1627-1634.
Nestler, E. J., 2001. Molecular basis of long-term plasticity underlying addiction. Nature Reviews Neuroscience 2, 119-128.
Peris, J., Boyson, S. J., Cass, W. A., Curella, P., Dwoskin, L. P., Larson, G., Lin, L. H., Yasuda, R. P., Zahniser, N. R., 1990. Persistence of neurochemical changes in dopamine systems after repeated cocaine administration. Journal of Pharmacology and Experimental Therapeutics 253, 38-44.
Volz, T. J., Bjorklund, N. L., Schenk, J. O., 2005. Methylphenidate analogs with behavioral differences interact differently with arginine residues on the dopamine transporter in rat striatum. Synapse (Hoboken, NJ, United States) 57, 175-178.
Volz, T. J., Fleckenstein, A. E., Hanson, G. R., 2007. Methamphetamine-induced alterations in monoamine transport: implications for neurotoxicity, neuroprotection and treatment. Addiction 102, 44-48.
Weiss, F., 2005. Neurobiology of craving, conditioned reward and relapse. Current Opinion in Pharmacology 5, 9-19.
White, F. J., Kalivas, P. W., 1998. Neuroadaptations involved in amphetamine and cocaine addiction. Drug and Alcohol Dependence 51, 141-153.

Williams Jason, M., Steketee Jeffery, D., 2005. Effects of repeated cocaine on the release and clearance of dopamine within the rat medial prefrontal cortex. Synapse FIELD Full Journal Title:Synapse (New York, N.Y.) 55, 98-109.
Yi, S. J., Johnson, K. M., 1990. Effects of acute and chronic administration of cocaine on striatal uptake, compartmentalization and release of [3H]dopamine. Neuropharmacology 29, 475-486.
Zahniser, N. R., Sorkin, A., 2004. Rapid regulation of the dopamine transporter: role in stimulant addiction? Neuropharmacology 47, 80-91.

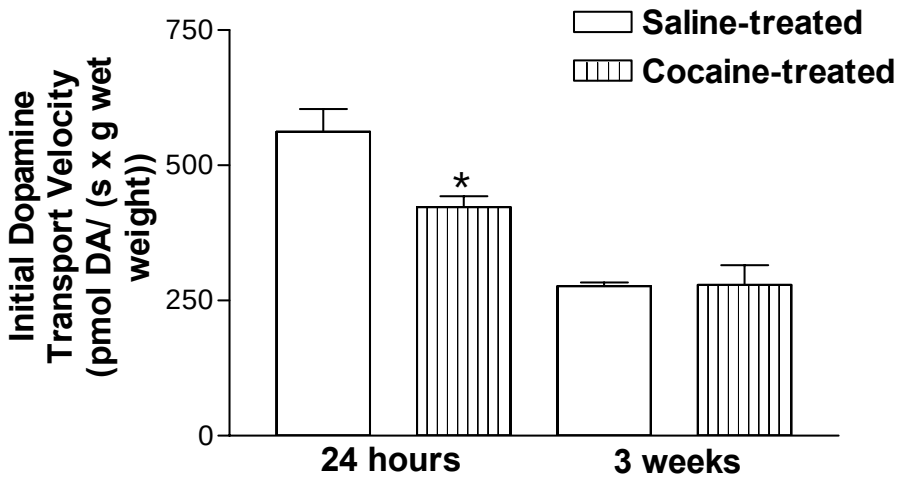

Fig. 1. Dopamine transport velocity in the nucleus accumbens of cocaine or saline-treated rats 24 hours and 3 weeks after withdrawal. Bars represent the mean initial transport velocity at 1 μM dopamine ± SER (n = 4). The asterisk denotes a value statistically different than saline-treated sample value with a $p < 0.0001$ as determined by a z-test.

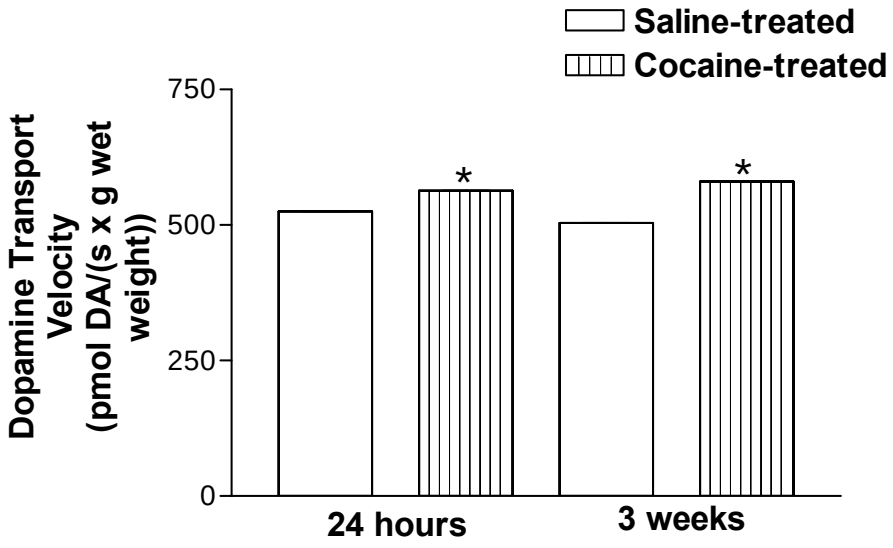

Fig. 2. Dopamine transport velocity in the striatum of cocaine or saline-treated rats 24 hours and 3 weeks after withdrawal. Bars represent the mean initial transport velocity at 1 μM dopamine ± SER (n = 4). The asterisks denote a value statistically different than saline-treated sample value with a $p < 0.0001$ as determined by a z-test.

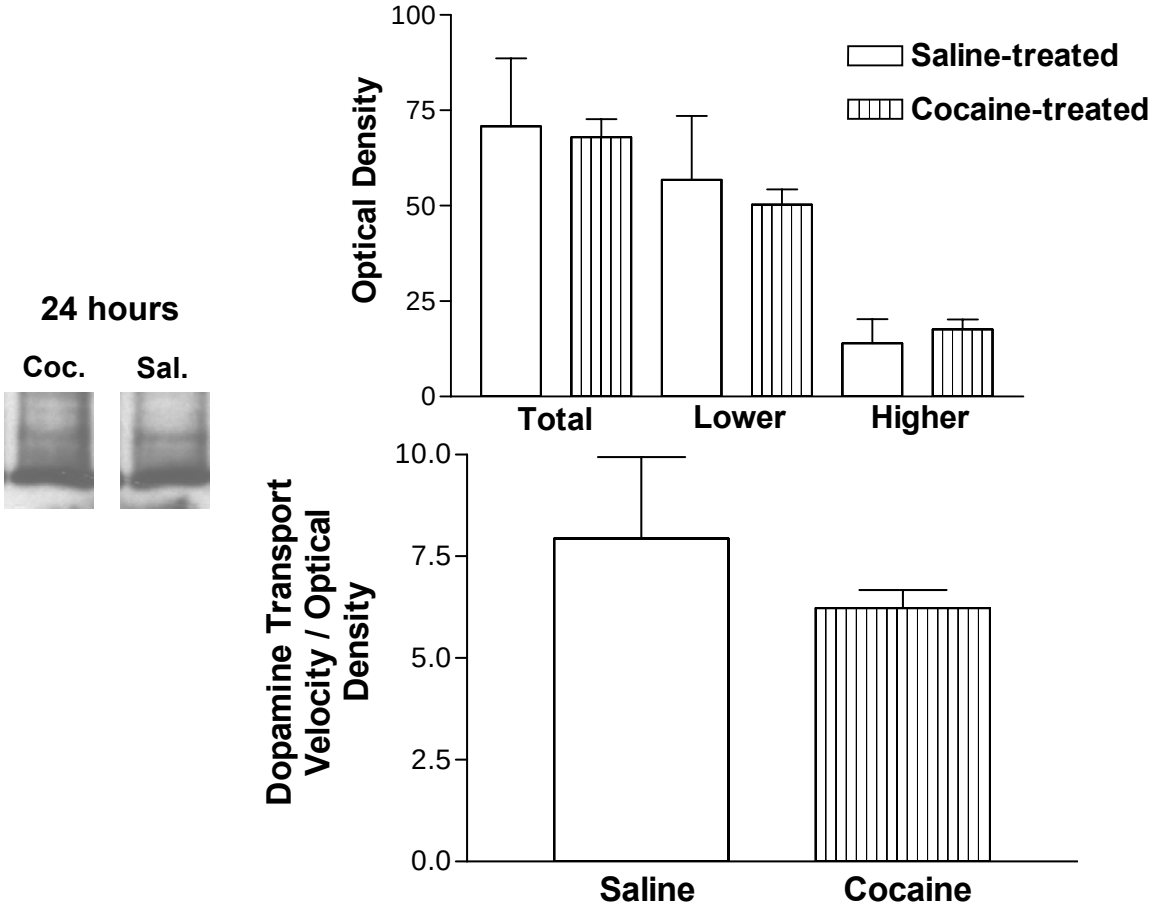

Fig. 3. Effects of cocaine treatments on DAT density in the nucleus accumbens after 24 hours withdrawal. A representative western blot is shown to the left of the densitometric analyses and normalized dopamine transport velocities. Error bars represent the mean ± SEM with an n = 7 or 8. The density values for DAT were normalized to β-actin expression. The mean dopamine transport velocity values from Figure 1 were normalized by the corresponding DAT density. The error bars represent the propagation of error by division.

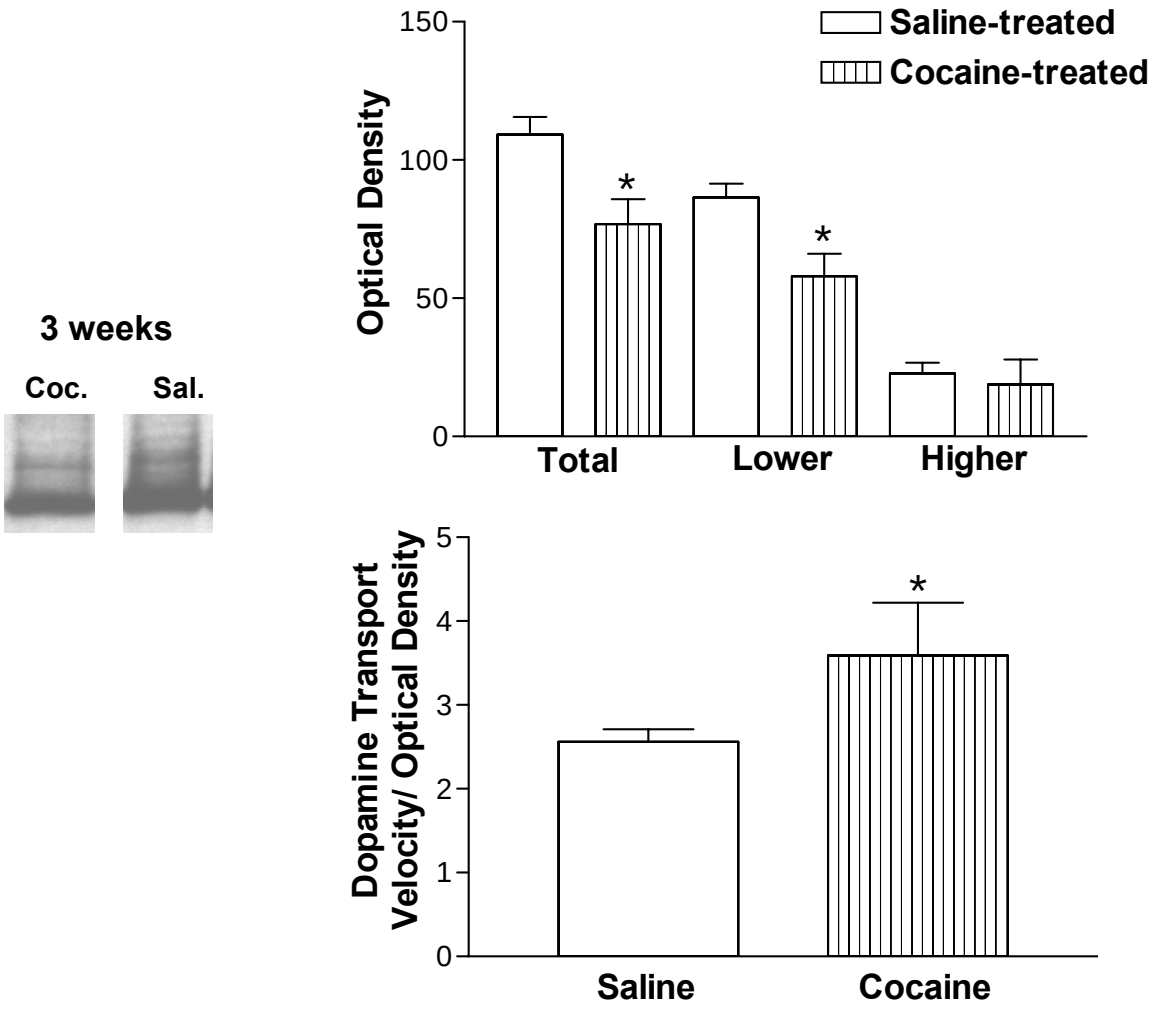

Fig. 4. Effects of cocaine treatments on DAT density in the nucleus accumbens at 3 weeks. A representative western blot is shown to the left of the densitometric analyses and normalized dopamine transport velocities. Error bars represent the mean ± SEM with an n = 7 or 8 from different groups of animals. Asterisks represent density values significantly different from saline-treated controls at $p \leq 0.002$ as calculated by a t-test. The density values for DAT were normalized to β-actin expression. The mean dopamine transport velocity values from Figure 1 were normalized by the corresponding DAT density. The error bars represent the propagation of error by division ($p < 0.011$).

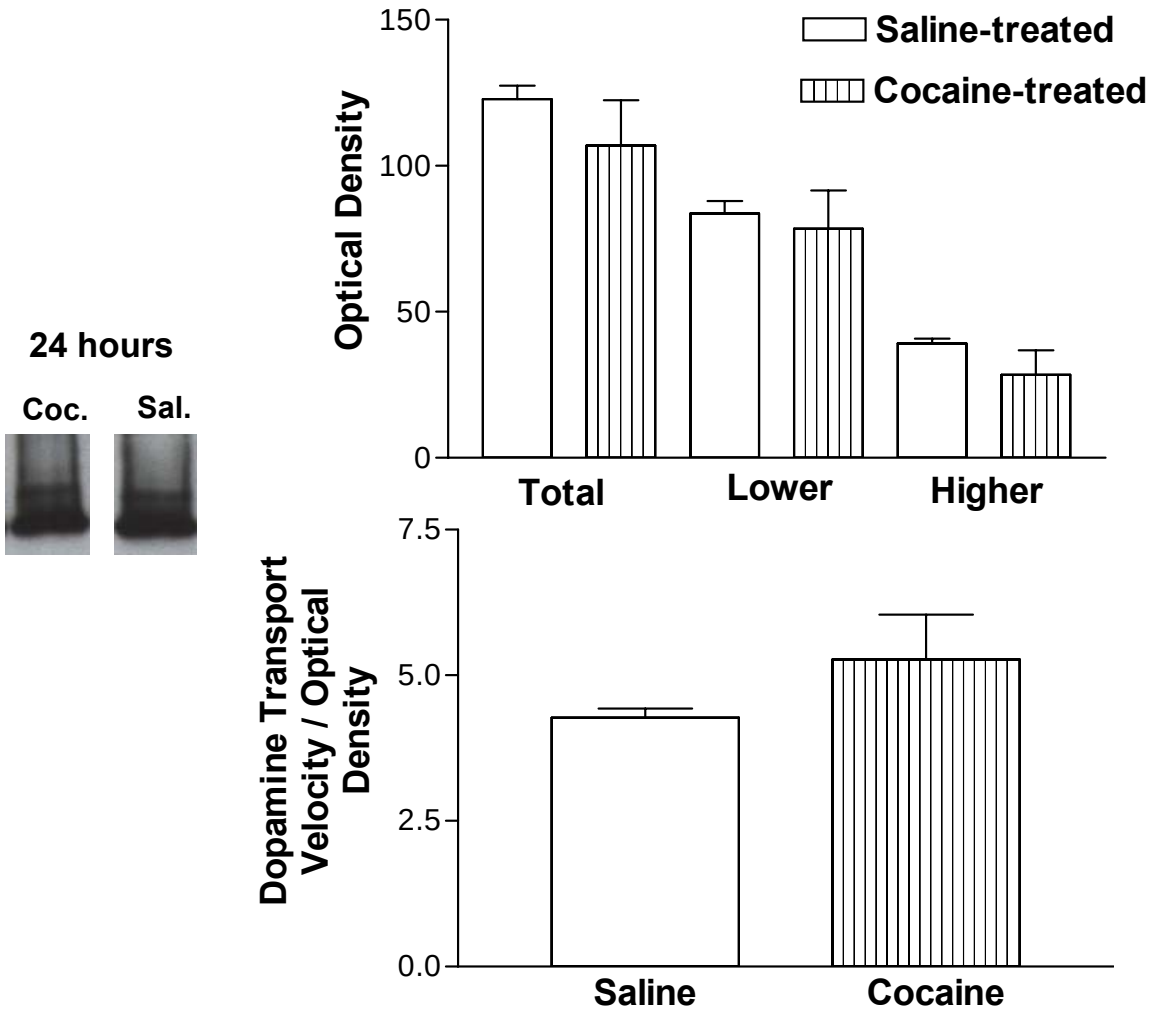

Fig. 5. Effects of cocaine treatments on DAT density in the striatum 24 hours after withdrawal. A representative western blot is shown to the left of the densitometric analyses and normalized dopamine transport velocities in each panel. Error bars represent the mean ± SEM with an n = 7 or 8. The density values for DAT were normalized to β-actin expression. Average dopamine transport velocity values from Figure 2 were normalized by the corresponding DAT density. The error bars represent the propagation of error by division.

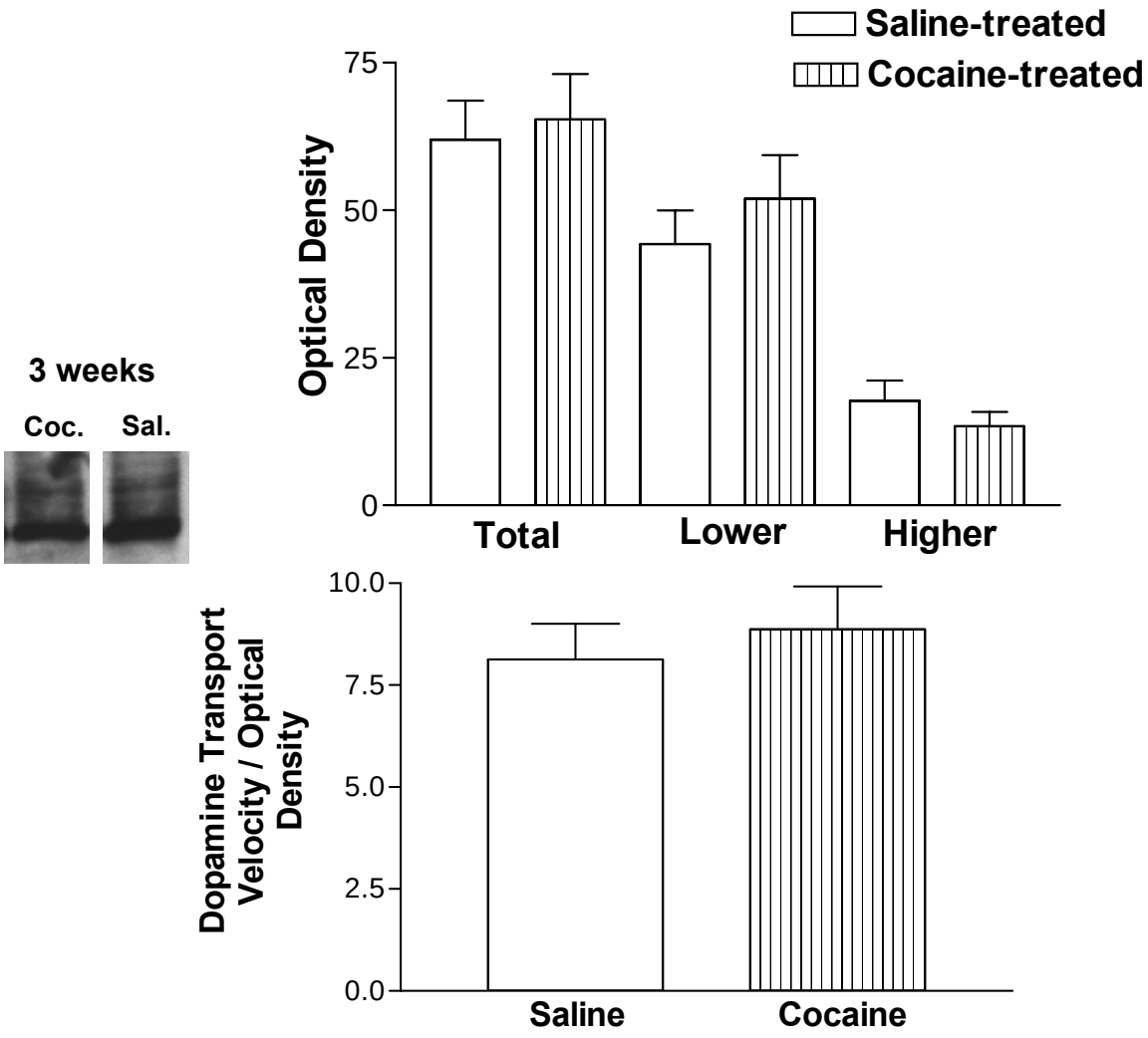

Fig. 6. Effects of cocaine treatments on DAT density in the striatum 3 weeks after treatment. A representative western blot is shown to the left of the densitometric analyses and normalized dopamine transport velocities in each panel. Error bars represent the mean ± SEM with an n = 7 or 8. The density values for DAT were normalized to β-actin expression. Average dopamine transport velocity values from Figure 2 were normalized by the corresponding DAT density. The error bars represent the propagation of error by division.

CHAPTER SIX

CONCLUSION

The preceding chapters document the experiments done to learn more about the molecular nature of the dopamine transporter (DAT). Specifically, the topics rotating disk electrode voltammetry, kinetics, Zn^{2+} modulation, and methamphetamine (METH) and cocaine effects are covered.

The DAT is the primary mechanism regulating dopamine (DA) concentration in the synapse and therefore the activation of postsynaptic DA receptors and autoreceptors. The modulation of this protein may enhance or depress dopaminergic neurotransmission. DAT activity and expression have been found to be very important in various disorders of the brain, including attention deficit hyperactivity disorder, alcoholism, Parkinson's disease, and stimulant abuse (J.O. Schenk, 2002). The study of how the DAT is regulated and altered due to metal ions and stimulants can help shed light on how the activity of DAT is changed and how this can be prevented or reversed if necessary.

The second chapter outlines the main technique used in the experiments, rotating disk electrode voltammetry (RDEV). Using this technique allows the rate of DA transport to be measured under many conditions. Detailed kinetics can be measured using both pre-steady state and steady state transport rates. RDEV is a very sensitive method that can be used to measure how the activity of the DAT has changed after exposure to various agents (metal ions, inhibitors, activators, etc.). The second chapter goes through how an investigator would measure the DA transport rate, giving all the mathematics necessary for performing the experiment and interpreting the results. It can

be used as a guide to the technique itself and when reading the subsequent chapters. This study was published in the *Journal of Neuroscience Methods* (J.O. Schenk et al., 2005).

The third chapter discusses experiments done concerning the role of Zn^{2+} on DAT activity. It was previously found the DAT was inhibited by the presence of Zn^{2+} (L. Norregaard et al., 1998; E.K. Richfield, 1993). A Zn^{2+} binding site is located on the extracellular face of the DAT (L. Norregaard et al., 1998). The study discussed in Chapter Three goes further to investigate Zn^{2+} modulation using both steady state and pre-steady state kinetics. In addition, the study looked at both human and rat DATs and the effects of cocaine on Zn^{2+} modulation. The results of this investigation led to many interesting conclusions on how Zn^{2+} can modulate the DAT. It was found that Zn^{2+} was increasing the binding of DA to the human DAT but inhibiting the translocation. Rat DAT translocation was unaffected by Zn^{2+}, but binding was increased. The lack of effect on the rat DAT is most likely due to the extra glycosylation site. Zn^{2+} reduced and increased the inhibition by cocaine in human DAT and rat DAT, respectively. These effects were not the result of competition between cocaine and Zn^{2+}. In striatal homogenates Zn^{2+} increased initial dopamine transport velocity and decreased cocaine inhibition providing evidence for differences in sensitivity to Zn^{2+} between the different preparations and also between the human DAT and rat DAT. This study was important in elucidating the details of Zn^{2+} modulation of the DAT and how it differs between species and preparation types. This research was published in the *European Journal of Pharmacology* (N.L. Bjorklund et al., 2007).

Along with Zn^{2+} modulation, the effects of METH exposure were studied and the results of this study are summarized in Chapter 4. In this study, rats were sensitized to

METH and then the activity of the DAT, inhibition by METH, DAT density, and changes to glycosylation were assessed. The initial experiment examined the behavior of rats given 5 mg/kg of METH per day for 5 days. It was found that rats treated with METH had higher locomotor scores than those treated with saline. In addition, on day 5 of METH treatment animals began patterned sniffing significantly earlier than on day 1. Experiments measuring DAT transport rates in the nucleus accumbens found that METH treated samples had lower transport velocity than saline-treated. This effect was reversed in the striatum, where in METH-treated samples the velocity was increased. Experiments examining METH inhibition of DA transport found that in the nucleus accumbens METH-treated samples had an increased IC_{50} value, thus requiring higher concentrations of METH to inhibit transport. Conversely, in the striatum, the IC_{50} was decreased. When DAT density was measured in the two brain areas it was found that in METH-treated samples the density was decreased in the striatum but remained unchanged in the nucleus accumbens. The DAT density was used to normalize transport velocity in both areas. This normalization showed that the DAT was kinetically down-regulated in the nucleus accumbens after METH exposure while the striatum DAT was up-regulated. Glycosylation was analyzed to determine if modification of this post-translational modification was causing the differences between METH and saline-treated samples and differences in the brain areas. It was found that N-glycanase, which removes all N-glycosylation, and neuraminidase, which removes sialic acids, were able to decrease the molecular weight of the DAT. Alpha mannosidase, which removes terminal mannose residues, and O-glycosidase, which removes all O-glycosylation, did not affect DAT molecular weight. These results agree with previous studies examining DAT

glycosylation (R. Lew et al., 1991; R. Lew et al., 1992). The glycosylation did not appear to be changed after METH treatment or between the brain areas. In conclusion, this study found that DAT activity was significantly altered in animals that were behaviorally sensitized to METH. It was also found the changes in DAT activity, METH inhibition, and densities were different in the different brain areas. Lastly, the study demonstrated that glycosylation may not be affected by METH exposure. This study is important because it shows the effects of METH at a specific stage of the addiction process. By defining the changes in the DAT after METH exposure perhaps treatments to reverse these changes can be devised. The results of this study were submitted to the journal *Synapse*.

Chapter 5 summarizes a study about changes of the DAT in animals that are sensitized to cocaine. This study is different in that it examines the DAT both at 24 hours and 3 weeks after treatment. This study examined both the nucleus accumbens and striatum, both important areas in terms of drugs of abuse. The nucleus accumbens is important for the initial steps of drug seeking and reward (F.J. White and P.W. Kalivas, 1998). The striatum is activated later in the abuse process, converting abuse into a habit and essentially an addiction (F. Weiss, 2005). Sensitization studies highlight the initial stages of drug craving (F.J. White and P.W. Kalivas, 1998). This study found the nucleus accumbens was altered in terms of DA transport and DAT density. The DAT was kinetically up-regulated even after 3 weeks of withdrawal. The striatum, on the other hand was found to have increased transport velocities at both 24 hours and 3 weeks post cocaine treatment. However, when dopamine transport was normalized by DAT density it was found to be the same as saline-treated controls. Therefore the striatum was not

affected by this sensitization protocol as the nucleus accumbens was. This study highlights how the different areas of the brain react to a specific abuse model. In addition, the examination of how drugs of abuse can alter dopaminergic neurotransmission is informative on how to possibly reverse these changes in addiction states but also disease states.

In conclusion, these research projects were performed to investigate both basic modulation of the DAT (Zn^{2+} modulation) and also assess how exposure to drugs of abuse (METH and cocaine) can alter the DAT in a specific addiction model.

REFERENCES:

Bjorklund, N.L., Volz, T.J. Schenk, J.O., 2007. Differential effects of Zn2+ on the kinetics and cocaine inhibition of dopamine transport by the human and rat dopamine transporters. European Journal of Pharmacology. 565, 17-25.

Lew, R., Grigoriadis, D., Wilson, A., Boja, J.W., Simantov, R. Kuhar, M.J., 1991. Dopamine transporter: Deglycosylation with exo- and endoglycosidases. Brain Research. 539, 239-246.

Lew, R., Patel, A., Vaughan, R.A., Wilson, A. Kuhar, M.J., 1992. Microheterogeneity of dopamine transporters in rat striatum and nucleus accumbens. Brain Research. 584, 266-271.

Norregaard, L., Frederiksen, D., Nielsen, E.O. Gether, U., 1998. Delineation of an endogenous zinc-binding site in the human dopamine transporter. Embo J. 17, 4266-4273.

Richfield, E.K., 1993. Zinc modulation of drug binding, cocaine affinity states, and dopamine uptake on the dopamine uptake complex. Molecular Pharmacology. 43, 100-108.

Schenk, J.O., 2002. The functioning neuronal transporter for dopamine: Kinetic mechanisms and effects of amphetamines, cocaine and methylphenidate. Progress in Drug Research. 59, 111-131.

Schenk, J.O., Wright, C. Bjorklund, N., 2005. Unraveling neuronal dopamine transporter mechanisms with rotating disk electrode voltammetry. Journal of Neuroscience Methods. 143, 41-47.

Weiss, F., 2005. Neurobiology of craving, conditioned reward and relapse. Current Opinion in Pharmacology. 5, 9-19.

White, F.J. Kalivas, P.W., 1998. Neuroadaptations involved in amphetamine and cocaine addiction. Drug and Alcohol Dependence. 51, 141-153.