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In vivo analysis of a salt bridge at the external gate of the Drosophila melanogaster serotonin transporter in response to amphetamines

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IN VIVO ANALYSIS OF A SALT BRIDGE AT THE EXTERNAL GATE OF THE *DROSOPHILA MELANOGASTER* SEROTONIN TRANSPORTER IN RESPONSE TO AMPHETAMINES

A Thesis

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For my family.

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ABSTRACT

Beenen, Timothy J. M.S., Purdue University, May 2015. *In Vivo* Analysis of a Salt Bridge at the External Gate of the *Drosophila Melanogaster* Serotonin Transporter in Response to Amphetamines. Major Professor: Eric Barker.

Monoamine neurotransmitter transporters are membrane proteins responsible for the clearing of biogenic amines from a synapse. These transporters are targets for many important pharmaceuticals including antidepressants, as well as psychostimulant drugs such as cocaine and amphetamines. Amphetamines are believed to elicit their psychostimulant activity primarily by inducing a reversal of the transport cycle and increasing neurotransmitter release into the synapse, though the mechanism of this activity is incompletely understood. Previous *in vitro* research has suggested functional significance of a conserved salt bridge in the serotonin transporter (SERT) in amphetamine-induced 5-HT efflux. This salt bridge is disrupted in the *Drosophila melanogaster* SERT. Here, a mutant line of *D. melanogaster* expressing a SERT with a restored salt bridge (dSERT N484D) was studied. Changes in neurochemistry induced by methamphetamine (METH) or 3, 4-methylenedioxymethamphetamine (MDMA) were examined *in vivo*. HPLC/Mass spectrometry was used to quantify brain concentrations of neurotransmitters in fly tissue after drug treatment. N484D flies were found to have significantly depleted

5-HT in response to 0.05% MDMA relative to wild-type. This depletion of 5-HT was not observed after treatment with 0.6% MDMA or 0.6% METH. No significant drug-induced changes were observed in concentrations of other neurotransmitters examined. The results show that the presence of the salt bridge at the external gate of SERT may be important for amphetamine-induced 5-HT efflux, and helps explain pharmacological differences observed between hSERT and dSERT.

CHAPTER 1. INTRODUCTION

1.1 History of MDMA and methamphetamine

MDMA

MDMA (N-methyl-1-(3, 4-methylenedioxyphenyl)-propan-2-amine), also known as ecstasy, is a psychostimulant drug that induces powerful psychoactive experiences in humans (figure 1.1). MDMA was first synthesized in 1912 by the Merck Corporation as an intermediary product intended for the synthesis of 3-methyl-hydrastinine, an anti-hemorrhagic drug. Merck patented MDMA as a part of this reaction, though no application of the patent was ever utilized by the company.[1] Years after its discovery the psychoactive properties of MDMA became better known. MDMA shares both structural and psychoactive commonalities with psychostimulant drugs such as methamphetamine, as well as psychedelics such as mescaline and LSD.[2] The subjective experience of MDMA use includes feelings of euphoria, stimulation, a sense of wellbeing, communicativeness, and visual hallucinations.[3] Drugs that produce subjective experiences of this sort are considered to be members of a unique group, and the special term “entactogen” has been conceived to describe and classify them.[4]

Recreational use of MDMA first rose in popularity during the 1960s and 1970s amongst members of the western counterculture. By the 1980s MDMA

had become a staple drug used at night clubs and rave parties. The stimulant effects of MDMA suppress a person's perception of the need to consume food or water. Consequently, MDMA use—particularly in the context of all-night dance parties—earned repute for risk of injury or death by dehydration.[5]

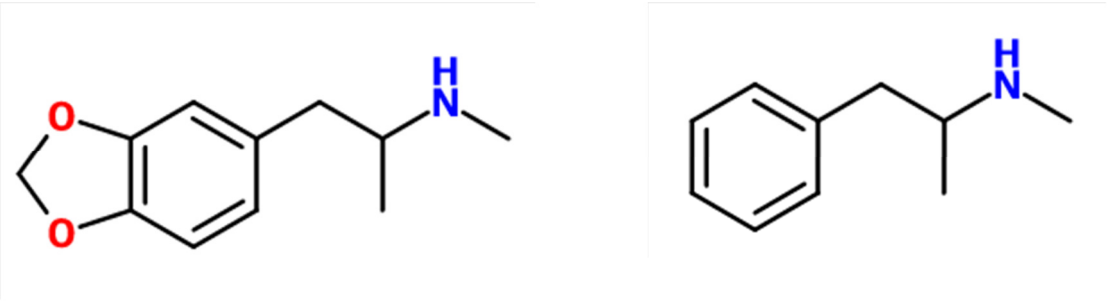
During the 1970s and early 1980s, MDMA found medical application as an adjunct to psychotherapy in individuals suffering from depression and post-traumatic stress disorder.[1, 4] More recently, interest in use for such applications has reemerged.[6] However, earlier research had suggested that the MDMA metabolite MDA is toxic to humans, and other investigations pointed to the high likelihood that the MDMA being consumed as a street drug was often dangerously impure. Based on this, in 1984, the Drug Enforcement Agency of the United States classified it as a schedule 1 controlled substance. Schedule 1 classification indicates that a drug has a high abuse potential, without any accepted medical value and, as a result, medical use of MDMA was promptly halted. This scheduling came as a consequence of the DEA being given emergency drug scheduling authority. Despite this classification being contested as inappropriate for MDMA by psychiatrists, researchers, and courts,[7, 8] this regulatory status was ultimately upheld in the United States in 1988, after a four year legal battle.[9] Subsequent studies have shown that MDMA does cause an acute neurodegeneration of serotonergic neurons.[4, 10] However, debate still persists in the medical community regarding the mechanisms by, and, extent to which MDMA may induce chronic neurotoxicity or neurodegeneration. [4, 11, 12, 13, 14]

METH

Methamphetamine (N-methyl-1-phenylpropan-2-amine) is a powerful stimulant drug with great potential for abuse and addiction (figure 1.1).[15] Methamphetamine (METH) use induces feelings of euphoria, intense arousal, increased libido, and reduced appetite and fatigue. [5, 16, 17] A structural derivative of amphetamine, METH was first synthesized from ephedrine in 1919 by Japanese pharmacologist Nagai Nagayoshi.[16] In subsequent years the drug was used medically for its appetite-suppressing and wakefulness-promoting effects. As the potential for addiction became better known, METH was classified as a schedule 2 controlled substance in the United States in 1970. Today, legal efforts have reduced the availability of chemical precursors commonly used for METH production (most notably pseudoephedrine), yet the drug is still extensively produced and abused illegally. The quality and purity of illicitly-produced METH varies widely, and the side effects of potential contaminants are unknown.[16] Despite its notoriety for addiction, METH has accepted medical indications for the treatment of exogenous obesity and attention deficit disorder with hyperactivity. When used for these purposes it is marketed under the brand name Desoxyn.[16, 18]

METH use, and particularly chronic use, is associated with numerous health deficits, including cardiomyopathy, psychosis, degradation of teeth and enamel, depression, sleep disturbances, reproductive dysfunction, oxidative stress and abnormal metabolism.[11, 15, 17, 19] Amphetamines are used around the world, and their abuse is quite prevalent. Use and abuse of amphetamines is a

global phenomenon, with approximately 17.2 million people dependent as of 2010. Worldwide amphetamine dependence is also increasing, especially in Asia.[20]



A. MDMA B. METH

Figure 1.1 Structure of 3,4-methylenedioxymethamphetamine (MDMA) and methamphetamine (METH)

1.2 The serotonin transporter

Serotonin (5-hydroxytryptamine; 5-HT) is a neurotransmitter responsible for many biological functions including regulation of sleep, libido, appetite, and memory. 5-HT signaling is governed by several systems including ion channels, pre and post-synaptic receptors, biosynthetic enzymes, and transporters.[21]

The serotonin transporter (SERT) is a pre-synaptic membrane protein. SERT is a member of the solute carrier 6 (SLC6) family of transporters, and is responsible for the clearing of 5-HT from the synaptic cleft, thereby terminating 5-HT neurotransmission.[22,23, 24] SLC6 transporters are 12-transmembrane alpha-helical membrane proteins. They are neurotransmitter-sodium symporters (NSSs), which couple the “downhill” transport of sodium to the “uphill” transport

of their substrate. This family includes the monoamine transporters SERT, the dopamine transporter (DAT), the norepinephrine transporter (NET), three GABA transporter (GATs), 2 glycine transporters (GLYs), and a subset of amino acid transporters.[24]

During normal neurotransmission, neurotransmitters such as dopamine (DA), norepinephrine (NE), and 5-HT are released from pre-synaptic neurons into the synapses via vesicles. These particular neurotransmitters are monoamines, and are known to play an important role in normal rewarding and reinforcing behaviors. However, drugs of abuse utilize these same neurotransmitter systems, leading to their addictive potential.[25]

Upon being released, these neurotransmitters can bind to their target receptors on post-synaptic neurons, thereby propagating a chemical signal. After a neurotransmitter has been released into a synapse and has transmitted its chemical signal, it is brought back into a pre-synaptic cell via a transporter. Once inside a cell, a monoamine neurotransmitter is either broken down by a monoamine oxidase (MAO) or repackaged into a vesicle by the vesicular monoamine transporter (VMAT) for subsequent reuse.[23] (Figure 2A)

SERT is an important target for many pharmaceuticals, including most antidepressants, as well as notable drugs of abuse such as cocaine, and amphetamines.[26] As serotonin is critically involved in the regulation of mood, arousal, appetite, and libido, it naturally became the subject of research in the treatment of depression and anxiety disorders.[27] During the 1960s the antihistamine drug diphenhydramine was discovered to have serotonin reuptake

inhibiting effects. This observation led to a search for structural derivatives of diphenhydramine that would have more specific effects on SERT. This search culminated in a class of drugs that would come to be known as selective serotonin reuptake inhibitors (SSRIs). SSRIs include antidepressants such as sertraline and fluoxetine, which are now some of the most prescribed pharmaceuticals.[28] Eli Lilly developed the drug fluoxetine during the 1970s, which was brought to market in the United States in 1987, and marketed under the brand name Prozac.[27] Much progress has been made in understanding the mechanisms of antidepressant pharmacology in the decades following their initial discovery.[29] Unipolar depression is an extremely debilitating mental illness, and is currently increasing in prevalence globally. It has been projected that by 2020 unipolar depression will be the second most burdensome disease behind ischemic heart attack.[20, 30] It is likely that as rates of mental illnesses diagnosed increase, use of SSRIs will increase as well. Thus, it is important to continue to study the targets of these drugs to increase understanding of their pharmacology, and improve available medication therapies.

1.3 Amphetamine action on monoamine systems

Molecular pharmacology of psychostimulants

Amphetamines exert their action by being transported into presynaptic neurons and inducing a reversal of the transport cycle of the biogenic amine transporters. Primary targets are SERT, DAT, NET, and VMAT. By doing so, amphetamines increase synaptic concentrations of 5-HT, DA, and NE.[31] As they

are weak bases, amphetamines also disrupt the proton gradient required for normal vesicular uptake, further contributing to altered transport. This mechanism of action is unique among psychostimulants. In contrast, the action of cocaine is mediated by its binding to the substrate-binding site of monoamine transporters, competitively inhibiting the reuptake of monoamines.[32, 33] This unique mechanism of amphetamine action is incompletely understood, and this research aims, in part, to further elucidate this process.

Monoamine transporters are principal targets for amphetamines. SERT is the primary molecular target for MDMA, although MDMA also acts on DAT, likely contributing to the subjective experience of the drug.[34] In addition to inducing neurotransmitter release through effects on transporters, MDMA may also interact with biogenic amine receptors, further contributing to its mechanism of action.[19, 31] METH acts on monoamine amine transporters as well, but is much less selective for SERT than MDMA, and has considerably greater affinity for DAT and NET. METH induces the release of NE twice as much as it does DA, and sixty times more than it does 5-HT.[19, 35] Additionally, MDMA and METH are both agonists of the trace amine associated receptor (TAAR1). Activation of TAAR1 by its native ligands is associated with excitation of the sympathetic nervous system. [36] Agonism of TAAR1 by amphetamines has similar effects, and also increases DAT efflux and transporter internalization. [37] Amphetamines are able to induce their effects on numerous pathways, primarily through action on monoamine systems, and especially catecholamine and 5-HT transporters. Amphetamine action on catecholamine and 5-HT receptors is important as well,

and activity on trace amine systems also contributes to their effects.[37] The structures of monoamine neurotransmitters pertinent to this research, as well as a brief overview of their principal functions can be found in table A.1.

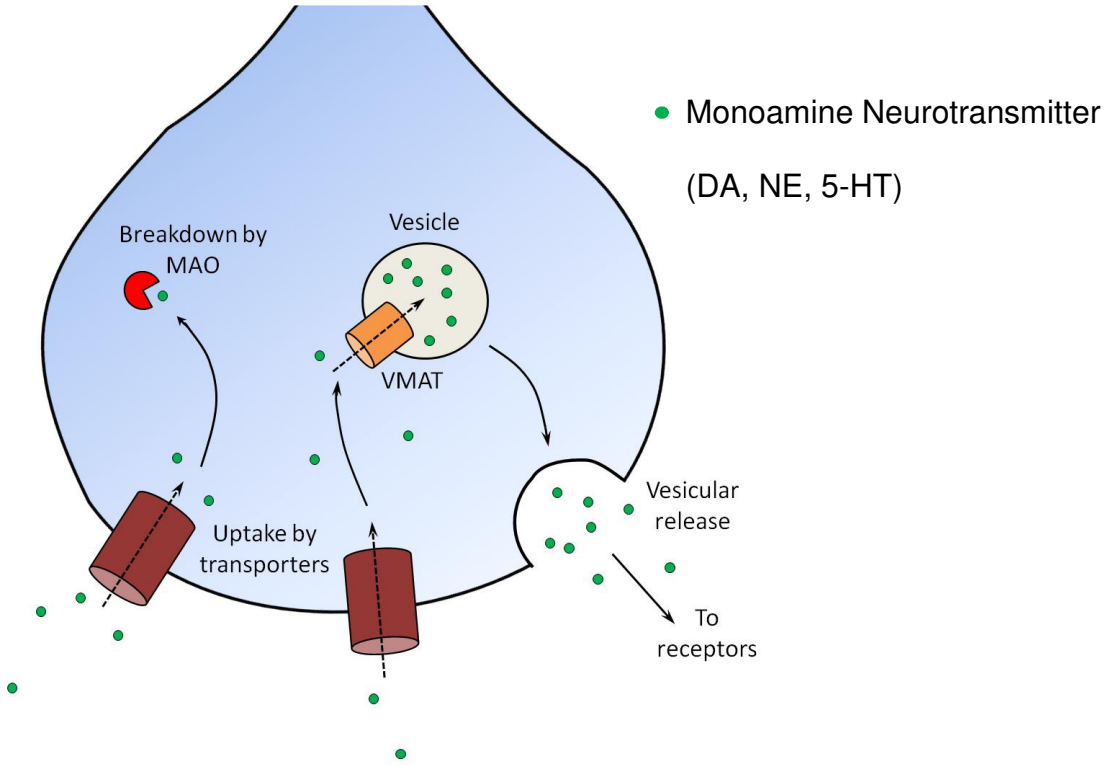


Figure 1.2 Normal transporter function. Neurotransmitters are released from presynaptic neurons via vesicles into the synaptic space to transmit a chemical signal by activating receptors on post-synaptic neurons and glia. Transporters clear neurotransmitters from the synapse to terminate signaling. Neurotransmitters can then be repackaged into vesicles by VMAT for subsequent reuse, or broken down by MAO.

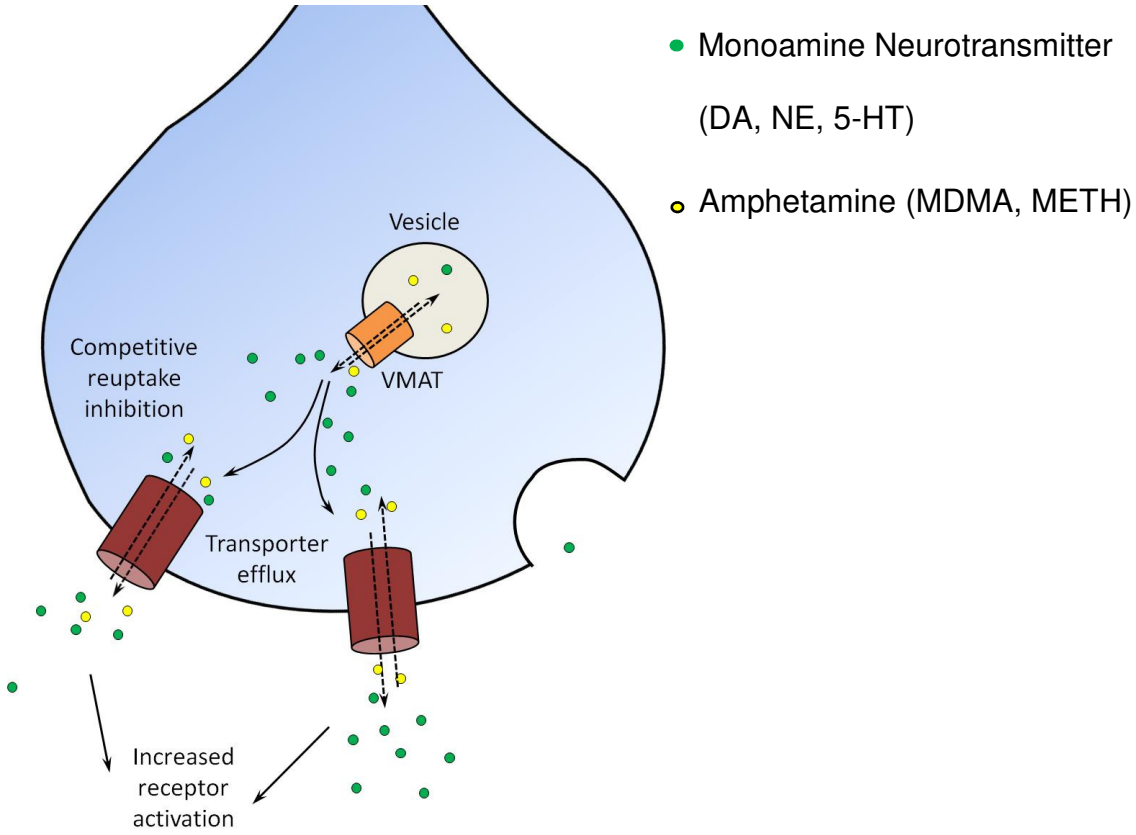


Figure 1.3 Amphetamine action on monoamine transporters. Amphetamines reverse the flow of neurotransmitters through transporters and inhibit neurotransmitter uptake. Neurotransmitters are pumped out of vesicles into the cytoplasm where transporters then mediate efflux, thereby increasing synaptic concentration of neurotransmitters and receptor activation.

1.4 Current problem

SERT and the external gate

A crystal structure of SERT has not been resolved, but inferences made from the crystal structure of the leucine transporter (LeuT), a bacterial homologue of Na/Cl-dependent neurotransmitter transporters (including SERT) from *Aquifex aeolicus* revealed a number of important characteristics of transporter architecture.[38] Of note, it revealed the presence of internal and external gates in the transporter that help to govern the transport cycle by regulating access to the substrate binding site. This allows for regulation of transport from either side of the membrane. More recently, a structure of the *D. melanogaster* dopamine transporter (dDAT) has been reported,[39] as well as mutational variants of LeuT, in which residues involved in ligand recognition and transporter function have been altered to their corresponding residues in mammalian biogenic amine transporters.[40, 41, 42] This structural data has illuminated much about the mechanics of transport, and factors determining substrate binding. Additionally, it has been instrumental in developing a homology model of hSERT.[43]

The external gate of NSSs, based on the crystal structure of LeuT, contains a pair of amino acids that are able to form a salt bridge between two transmembrane helices.[38] Amino acid sequence alignment of transporters from several species revealed the absence of one of these critical external gating residues in dSERT. (Figure 2.3) Additionally, dSERT shows greatly reduced 5-HT efflux in response to amphetamine exposure.[44] These observations lead to

the hypothesis that the lack of this conserved residue contributes to the variation in amphetamine-induced efflux between hSERT and dSERT.

As previously stated, the primary target for amphetamines are biogenic amine transporters. MDMA and other ring-substituted amphetamines exhibit a particularly selective affinity for SERT due to the substitutions at the 3 and 4 positions of the phenyl ring.[45] The selectivity of MDMA for the serotonin transporter, and the resulting increasing in synaptic concentrations of serotonin, is considered to be a significant factor in the unique “entactogenic” quality of the drug.[46] Additionally, this selectivity for SERT is thought to be partially responsible for the reduced addictive potential of MDMA relative to other stimulant drugs. For example, METH and cocaine are considered much more addictive than MDMA owing to their capacity to increase synaptic DA concentrations by acting on DAT. This activation of the DA system is an important factor in the addictive potential of these drugs. However, the role of DA in the action of MDMA should not be overlooked, as it also plays some role in the effects of MDMA.[34]

The exact mechanism for how amphetamines are able to induce reverse transport is unclear, although mechanisms relating to phosphorylation of the N-terminus, as well as structural alterations induced by changes in pH gradient have been proposed.[47, 48, 49] Understanding the role of this salt bridge may help to explain how amphetamines are able to induce reverse transport. Additionally, by studying systems-scale drug effects, this work could lead to development of novel treatments for amphetamine abusers through improved understanding of their mechanisms of addiction and toxicity.

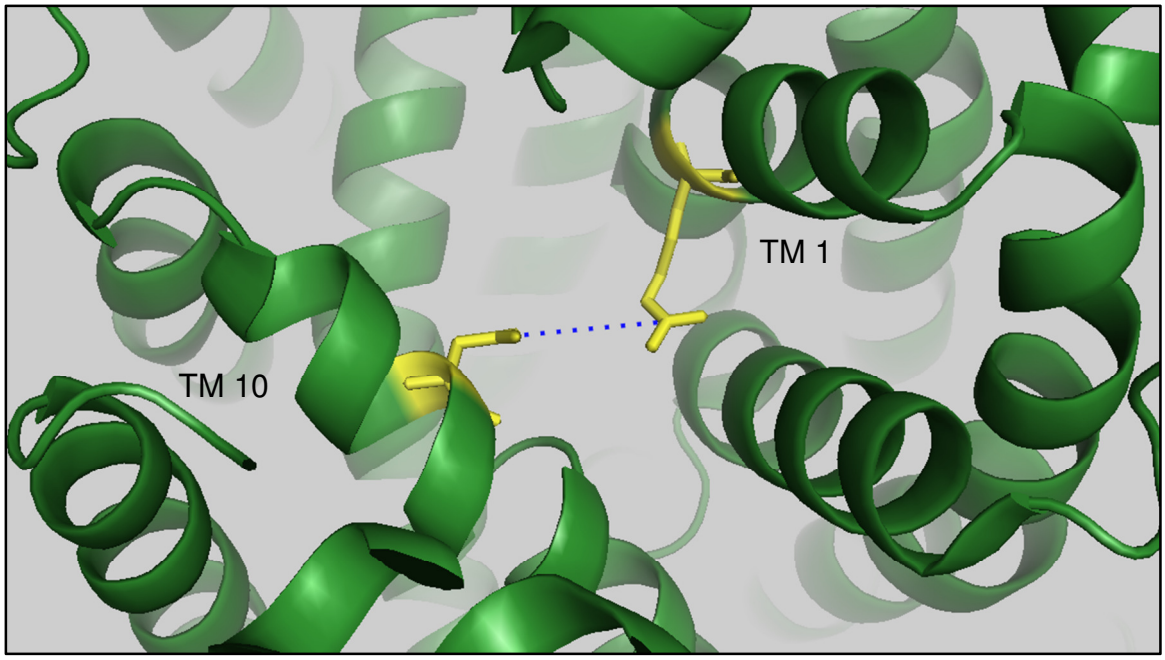


Figure 1.4 Model of the interaction of the residues forming the salt bridge in monoamine transporters. This shows the amino acids forming the salt bridge in the external gate of LeuT, consisting of R30 and D404. In hSERT the homologous residues are R104 and D484, and in dSERT they are R99 and N484. Adapted from [38]; PDB code 2A65.

dSERT and hSERT

The amino acid sequences of the human serotonin transporter (hSERT) and *D. melanogaster* serotonin transporter (dSERT) are approximately 51% identical.[50] This sequence similarity corresponds to similarities in 5-HT recognition and uptake of 5-HT between the *D. melanogaster* and mammalian transporters. However, differences in transport of amphetamines and other substrates between the hSERT and dSERT have been observed.[51, 44] Previous research in the Barker lab has identified a key region of SERT that appears to be important in the molecular mechanisms of amphetamine activity. Sequence alignment of NSSs shows a highly conserved salt bridge in the extracellular vestibule of transporters across numerous species that is lacking in dSERT (figure 4). dSERT is unique amongst transporters of other species examined in that this acidic residue is highly conserved, but is a basic residue in *D. melanogaster*. The salt bridge in hSERT is composed of Arginine 104 and Glutamate 493. The homologous residues in the dSERT are Arginine 99 and Asparagine 484. Generally, a salt bridge has a stabilizing effect on protein structure, reducing conformational flexibility.[52] The salt bridge found in monoamine transporters is hypothesized to have such an effect, aiding in the tight closing of the transporter and preventing the leak of neurotransmitters out of the cell.

Additional *in vitro* assays were previously performed in the Barker lab utilizing HEK-293 cells transfected with SERT salt bridge mutants. These mutants restored the acidic residue in dSERT to the hSERT equivalent (N484D),

	Transmembrane Domain I											Transmembrane Domain X							
hSERT	101	N	V	W	R	F	P	Y	107	…	489	K	L	L	E	E	Y	A	496
dSERT	96	N	V	W	R	F	P	Y	102	…	481	N	F	L	**N**	V	Y	G	487
LeuT	27	N	F	L	R	F	P	V	33	…	401	D	E	M	D	F	W	A	407
mSERT	101	N	I	W	R	F	P	Y	107	…	489	T	L	L	E	E	Y	A	495
rSERT	101	N	I	W	R	F	P	Y	107	…	489	T	L	L	E	E	Y	A	495
bSERT	101	N	V	W	R	F	P	Y	107	…	489	K	L	L	E	E	F	A	495
gpSERT	101	N	I	W	R	F	P	Y	107	…	489	K	L	L	E	E	Y	A	495
hDAT	82	N	V	W	R	F	P	Y	88	…	473	T	L	L	D	H	F	A	479
dDAT	49	N	V	W	R	F	P	Y	55	…	472	H	L	L	D	R	Y	A	478
mDAT	82	N	V	W	R	F	P	Y	88	…	472	T	L	L	D	H	F	A	478
rDAT	82	N	V	W	R	F	P	Y	88	…	472	T	L	L	D	H	F	A	478
hNET	79	N	V	W	R	F	P	Y	85	…	469	T	L	L	D	T	F	A	475
mNET	78	N	V	W	R	F	P	Y	106	…	470	T	L	L	D	T	F	A	476
rNET	78	N	V	W	R	F	P	Y	84	…	470	T	L	L	D	T	F	A	476
mGAT	54	N	V	W	R	F	P	Y	60	…	444	Q	L	F	D	Y	Y	A	450

Figure 1.5 Transporter sequence alignment. An acidic residue (aspartate or glutamate, E or D) is highly conserved across monoamine transporters from several species, but is replaced by an asparagine (N) in dSERT (bold). All sequences obtained from www.Uniprot.org.

and changed hSERT to the dSERT equivalent (E493N). These experiments revealed numerous differences between hSERT and dSERT: dSERT exhibits basal efflux of 5-HT that does not occur in hSERT. This is hypothesized to be a consequence of the absence of the salt bridge, which likely helps to keep hSERT closed more tightly, preventing 5-HT leak.

Amphetamine-induced efflux is greatly reduced in dSERT relative to hSERT. Electrophysiology experiments showed that amphetamines are substrates for hSERT, but not dSERT.[51] The N484D mutation in dSERT was able to restore amphetamine-induced currents to levels comparable to hSERT in response to MDMA

The lack of a salt bridge likely accounts for some differences in pharmacology observed between hSERT and dSERT.[51] Altering amino acid residues in dSERT to the corresponding residues in hSERT can make dSERT pharmacology more like that of hSERT.[53] A mutant line of *D. melanogaster* (N484D) containing this salt bridge is currently being investigated *in vivo*. This line of flies is not a knockout mutant, and contains both wild-type dSERT and dSERT N484D.

Current Problem

The goal of this project was to investigate the role of a salt bridge at the external gate of the SERT in MDMA/METH-induced neurotransmitter efflux *in vivo*. 5-HT efflux is believed to be a major component underlying the effects of these drugs. Previous in-vitro studies in our lab identified reduced amphetamine-induced efflux in dSERT relative to hSERT. These differences have been

hypothesized to be due to the lack of this highly conserved salt bridge in *D. melanogaster*.

To further investigate this problem *in vivo* a mutant SERT has been generated and inserted into *D. melanogaster* which contains a mutation (N484D) that restores this salt bridge. It is hypothesized that this dSERT mutation will facilitate tighter closing of the external gate, and thus restore the amphetamine-induced efflux found in hSERT.

Significance of the Problem

The mechanisms underlying amphetamine-induced efflux are poorly understood.[54] Previous work has demonstrated that single amino acid changes in SERT in different species are able to alter ligand binding and pharmacological activity.[53, 55, 56]

In vivo experiments may reveal how this residue is able to influence MDMA activity and amphetamine-induced changes in neurochemistry. Previous cell based assays demonstrated the importance of this residue in *in vitro* drug-activity, and this work corroborates those assays using an *in vivo* model. Additionally, *D. melanogaster* has previously been used to study genomic, proteomic, and transcriptomic changes after exposure to METH.[15] This model could also be extended to MDMA to observe systems-level changes resulting from MDMA-induced 5-HT depletion in N484D flies in efforts to discover new therapeutic techniques to fight addiction and toxicity.

CHAPTER 2. AMPHETAMINE PHARMACOLOGY IN *D. MELANOGASTER*

2.1 Use of *D. melanogaster* as a model system

Rationale for the use of *D. melanogaster*

Previous research in the Barker lab utilizing *in vitro* assays demonstrated important pharmacological differences between dSERT and hSERT. Specifically, it was observed that hSERT will readily efflux when exposed to amphetamines, however dSERT has minimal response to amphetamines.[51] As the conserved acidic residue present in most monoamine transporters is an asparagine in dSERT, an important role may exist for this residue in mediating MDMA/METH action on SERT. Indeed, previous research in the lab, as described above, confirmed that this gating residue plays a key role in the differences between hSERT and dSERT in amphetamine-induced efflux.

To further understand the role of this residue, the fruit fly, *D. melanogaster* was used for the study of drug action *in vivo*. *D. melanogaster* is a very suitable and ubiquitous model organism in part because of the relative ease of husbandry, and its well-characterized genome. Additionally, useful inferences about other animals can be made from evaluating genetic manipulations in the fly, owing to the high degree of homology between the two genomes.[57] Capitalizing on this genetic similarity can be useful for research, as over 75% of genes associated

with human diseases have been found to be shared between humans and *D. melanogaster*.[58] Thus, using *the fly* for *in vivo* studies such as those performed here is worthwhile and convenient.

Previous research has shown that METH produces behavioral changes in *D. melanogaster* that are very similar to those observed in humans (e.g. increased locomotion, reduced food intake, altered metabolism), making *D. melanogaster* an appropriate organism for use in studying amphetamine pharmacology.[59, 60] Inferences made from the impact of amphetamines in *D. melanogaster* can be extended to humans as well, and are likely to assist in the development of therapeutics to fight addiction and reduce amphetamine toxicity. For example, METH has been found to alter carbohydrate metabolism in *D. melanogaster* and this is believed to contribute to its toxicity.[15] Experiments already performed have revealed that increased dietary trehalose was able to significantly reduce METH toxicity by attenuating a METH-induced Warburg-like effect in *D. melanogaster*.[15, 59] Thus, further systems-scale studies hold promise to reveal additional pathways contributing to amphetamine toxicity and its treatment.

D. melanogaster husbandry and drug administration

Wild-type (w^{1118} strain) and dSERT N484D mutant flies were received from collaborator Dr. Barry Pittendrigh (University of Illinois at Urbana-Champaign). Flies were reared on the Formula 4-24® Drosophila diet (Carolina Biological Supply, Burlington, NC) at 22-23 C and 60-70% humidity. A targeted gene expression system under the control of TrH-GAL4 (tryptophan hydroxylase) was used for insertion of the N484D gene. Flies were contained in capped vials

containing holes for gas exchange and a single cotton swab by which food and drugs were administered. Immediately upon arrival a fresh solution of 10% sucrose was prepared as food for the flies. Control flies were given 150 µL of the sucrose solution, and the experimental group was given 150 µL of sucrose containing either MDMA or METH. A red dye was added to provide a means of confirming consumption of food and drugs by visual observation of the presence of a red pigment in the abdomen. Drug concentrations were 0.6% for METH, and either 0.05% or 0.6% for MDMA. Both wild-type w^{1118}, and transgenic flies containing the N484D mutant serotonin transporter were used. Each group of flies was then exposed to sucrose alone or sucrose with drug for different duration of time, ranging from 2 hours to 48 hours. After the appropriate time had passed, the experiment was terminated by promptly placing the vials of flies in a -80˚C freezer.

2.2 Isolation of *D. melanogaster* brain tissue

To examine changes in neurochemistry, flies were kept cold on dry ice and were decapitated under a microscope. Males were separated from females in order to observe potential sex-specific drug effects. Only the fly heads were used for further analysis, and all other tissue was discarded, so as to measure only changes in brain neurochemistry specifically.

Flies from each group were kept separately and prepared for analysis by high-performance liquid chromatography coupled to tandem mass spectroscopy (HPLC-MS/MS). 90µL of 0.1M formic acid and10µL of deuterated serotonin (d_4-5-

HT) internal standard was added to a 1.5mL Eppendorf tube containing approximately 15 heads for each sample. Samples were kept cold and homogenized using a Polytron homogenizer. After homogenization each sample was sonicated for 60 seconds, and then stored in a -80˚C freezer until analysis by HPLC-MS/MS.

2.3 Mass spectrometry

HPLC-MS/MS was used to identify and quantify biogenic amine concentrations in each sample. This technique is useful because it is highly sensitive and is able to detect very small concentrations of molecules of interest. The technique has previously been used with success by the Barker lab.[61] HPLC separates molecules by size by forcing a sample through a column under high pressure.

In these experiments, an internal standard of deuterated 5-HT was used for calibration of the HPLC-MS/MS instrument, serving as a constant to quantify the concentrations of the monoamines being analyzed in each sample. This is an appropriate internal standard because it is similar, but not identical, to each of the other biogenic amines being studied.

Samples were analyzed at Bindley Biosciences Center, Purdue University. The instrument employed was an Agilent 1200 HPLC pump and an Agilent 6460 triple quadrupole mass spectrometer. The chromatography utilized a Waters Atlantis T3 polar amide C18 HPLC column with a mobile phase of a water/methanol gradient, buffered with 0.1% formic acid. The total run time was

22 minutes with an elution time of 3.8 minutes for 5-HT. The mass spectrometer source used a positive polarity electrospray ionization process. The MRM transitions were 177.1 to 160.1 for 5-HT and 181.1 to 164.1 for d4-5-HT.

2.4 Changes in neurochemistry

Using an *in vivo* model allows for the assessment of changes in many systems, as opposed to previous *in vitro* work, which only examined changes in SERT activity, specifically. Concentrations of five biogenic amine neurotransmitters, 5-HT, DA, octopamine (OCT), NE, and tyramine (TYR) were measured to assess for MDMA or METH-induced changes in neurochemistry in wild-type and N484D mutant flies *in vivo*. Changes in concentration were analyzed for each neurotransmitter in wild-type vs. dSERT N484D flies, drug-treated or untreated, and male vs. female.

2.4.1 5-HT results

5-HT is a neurotransmitter synthesized from the amino acid tryptophan. It is involved in numerous core biological functions, including regulation of sleep and wakefulness, appetite, and mood.[62, 63] As it is the native substrate for SERT, as well as a principal target for amphetamines, it was crucial to assess for amphetamine-induced changes in levels of 5-HT. Previous *in vitro* data suggest that dSERT is insensitive to MDMA.[51] The N484D point mutation is proposed to restore sensitivity to MDMA. Flies expressing dSERT N484D were therefore expected to have time-dependent depletion of 5-HT after MDMA exposure, and

indeed this was observed. The concentration of 5-HT was significantly reduced in N484D flies relative to wild type when flies were treated with 0.05% MDMA (figure 2.1 A, B, 2.2 A). Post-hoc analysis by multiple t-test using the Holm-Sidak method revealed that 0.05% MDMA significantly reduced 5-HT in N484D flies at 6 and 24 hours. The effects were not yet observable after 2 hours, and were no longer observed after 30 hours. Treatment with 0.6% MDMA produced direct drug effects in both wild-type and N484D flies (figure 2.1 C, D), but did not result in significant differences between the two (figure 2.2, B). METH exposure produced direct drug effects in WT flies on 5-HT levels only at the 6 hour time point (figure 2.1 E), and no differences were observed between wild-type and N484D flies (figure 2.2 C).

Figure 2.1 Direct Drug Effects on 5-HT concentrations in wild type and N484D mutant flies. One sample t-test with a theoretical mean of 1.0 was performed to assess for changes in neurotransmitter concentrations between drug-treated and untreated flies to assess for drug response. No significant effects were observed in Control Flies treated with 0.05% MDMA (Panel A). Significance was reached at the 2 hour and 6 hour time points, P=0.0047 and 0.0479, respectively, for N484D flies. Effects were present, but did not reach statistical significance by the 24 hour point, P=0.0640 (Panel B). Significant drug effects were also observed at all times measured in wild-type flies treated with 0.6% MDMA, $P < 0.01$, and at the 2 hour and 6 hour time points in N484D flies, P=0.0007 and 0.0262, respectively, though they were also not observed by the 24 time point (Panels C and D). In flies treated with 0.6% METH significant direct drug effects were reached only at the 6 hour point in wild-type flies, P=0.0319 (Panels E and F). One sample was measured as approximately 30 flies for each drug treatment at each time point. Error bars represent the mean ± SEM of independent experiments for each time point measured.

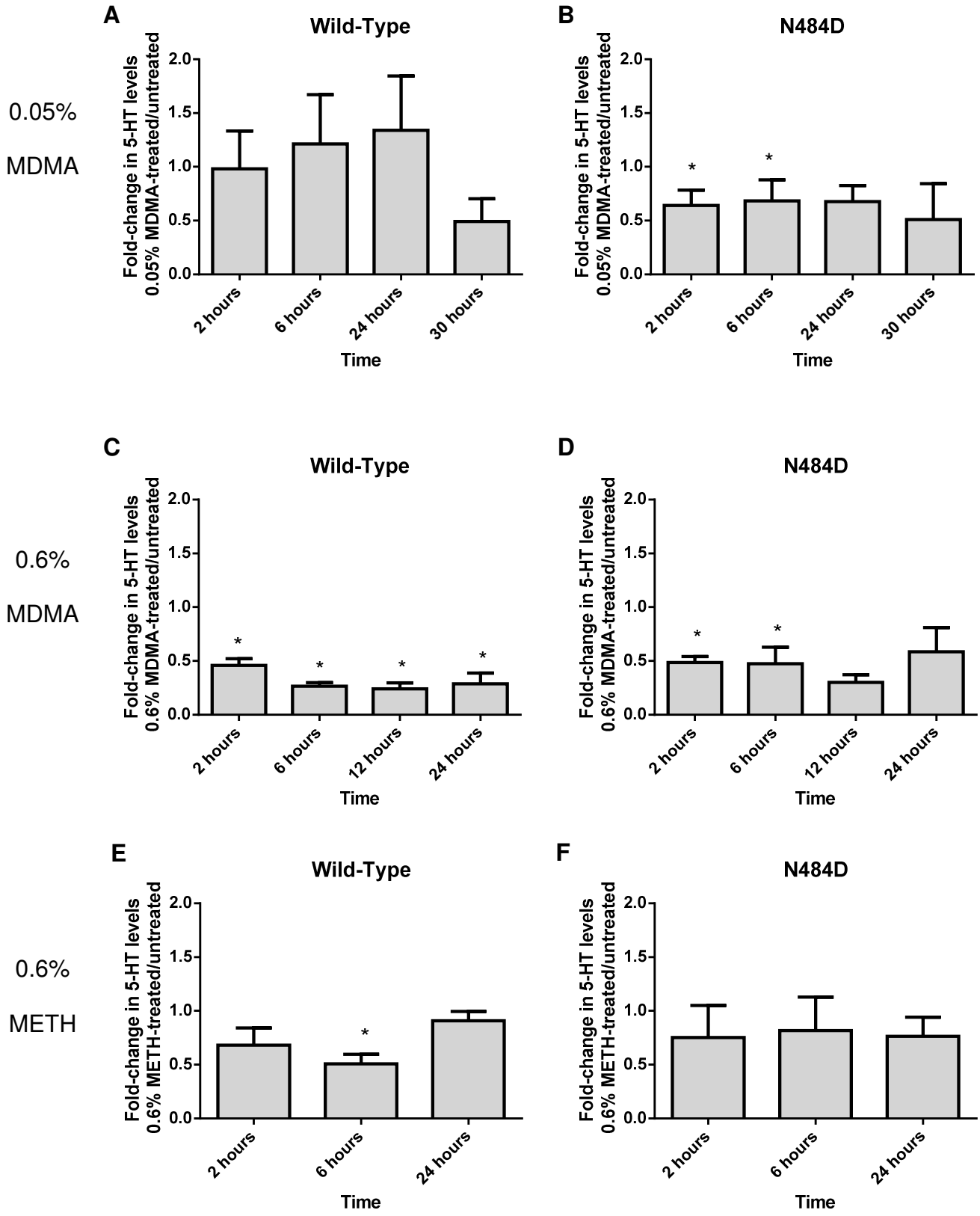
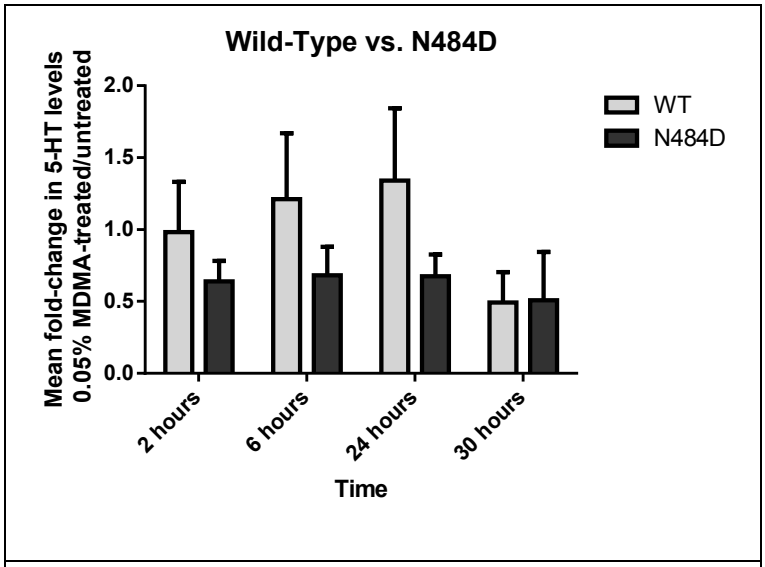

A
Wild-Type
B
N484D
0.05%
MDMA
Fold-change in 5-HT levels
0.05% MDMA-treated/untreated
2.0
1.5
1.0
0.5
0.0
2 hours
6 hours
24 hours
30 hours
Time
C
Wild-Type
D
N484D
0.6%
MDMA
Fold-change in 5-HT levels
0.6% MDMA-treated/untreated
2 hours
6 hours
12 hours
24 hours
Time
E
Wild-Type
F
N484D
0.6%
METH
Fold-change in 5-HT levels
0.6% METH-treated/untreated
2 hours
6 hours
24 hours
Time

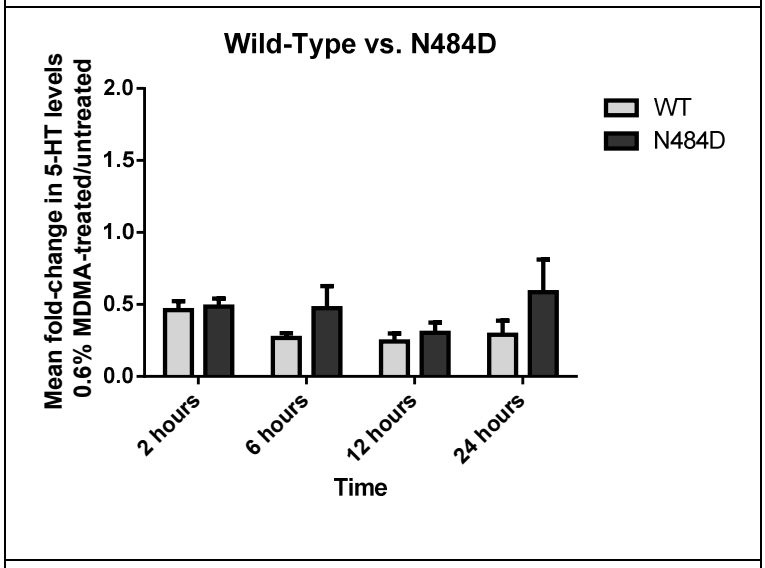
Figure 2.1

Figure 2.2 Comparison of 5-HT concentrations in wild-type and N484D flies. Two-Way ANOVA showed significantly reduced 5-HT levels in N484D flies relative to wild-type after treatment with 0.05% MDMA, P= 0.0039 Post-hoc analysis by multiple t-test using the Holm-Sidak method revealed that 5-HT was reduced in N484D flies after 6 and 24 hours, P=0.02181 and 0.02536, respectively (Panel A). Significant differences were not observed with 0.6% MDMA treatment. (Panel B) Two-Way ANOVA failed to show a significant reduction in 5-HT levels in N484D flies relative to wild-type after 0.6% METH treatment:, P=0.6853 (Panel C).

A



B



C

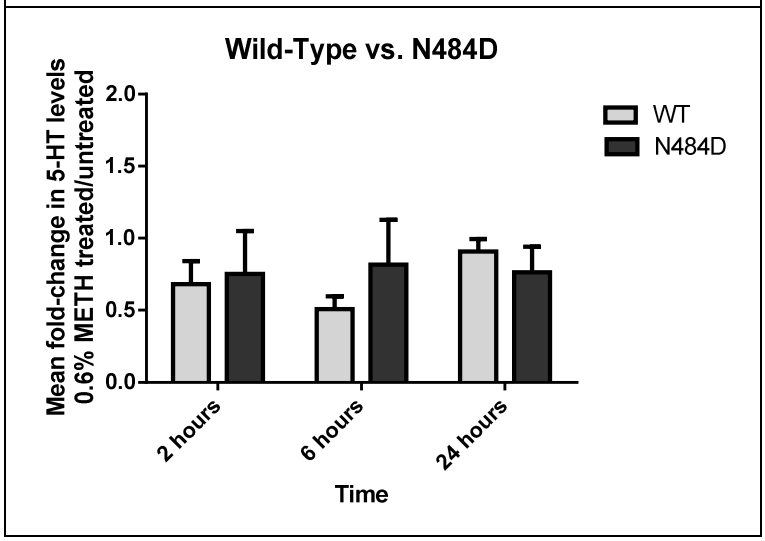


Figure 2.2

2.4.2 Results for Dopamine

DA is a neurotransmitter derived from the amino acid tyrosine. DA is the neurotransmitter most commonly associated with the reinforcing properties of addictive psychoactive substances, and is also involved in the coordination of movement, reward, attention, memory, and motivation.[64]

Based on existing dating that amphetamines induce DA release, amphetamine exposure was predicted to reduce concentrations of DA in fly tissue samples. Drug-effects were observed in flies treated with 0.05% MDMA at two time points, and at one time point in METH-treated flies (figure 2.3). Two-Way ANOVA showed significantly reduced DA levels in N484D flies relative to wild-type, P=0.0262 (figure 2.4 A). However, no differences were observed in flies treated with 0.6% MDMA (P = 0.7548) or with METH (P = 0.5431) (figure 2.4 B, C).

Figure 2.3 Direct Drug Effects on DA concentrations in wild type and N484D mutant flies. One sample t-test with a theoretical mean of 1.0 was performed to assess for neurotransmitter concentrations between drug-treated and untreated flies. No significant effects were observed in Control or N484D flies treated with 0.05% MDMA (Panels A and B). In wild-type flies treated with 0.6% MDMA, significant drug effects were observed at the 6 and 24 hour time points P=0.0198 and 0.030, respectively. No effects were observed in N484D flies (Panels C and D). In flies treated with 0.6% METH significant direct drug effects were reached only at the 6 hour point in wild-type flies, P=0.0134, but not in N484D flies (Panels E and F). One sample was measured as approximately 30 flies for each drug treatment at each time point. Error bars represent the mean ± SEM of independent experiments for each time point measured.

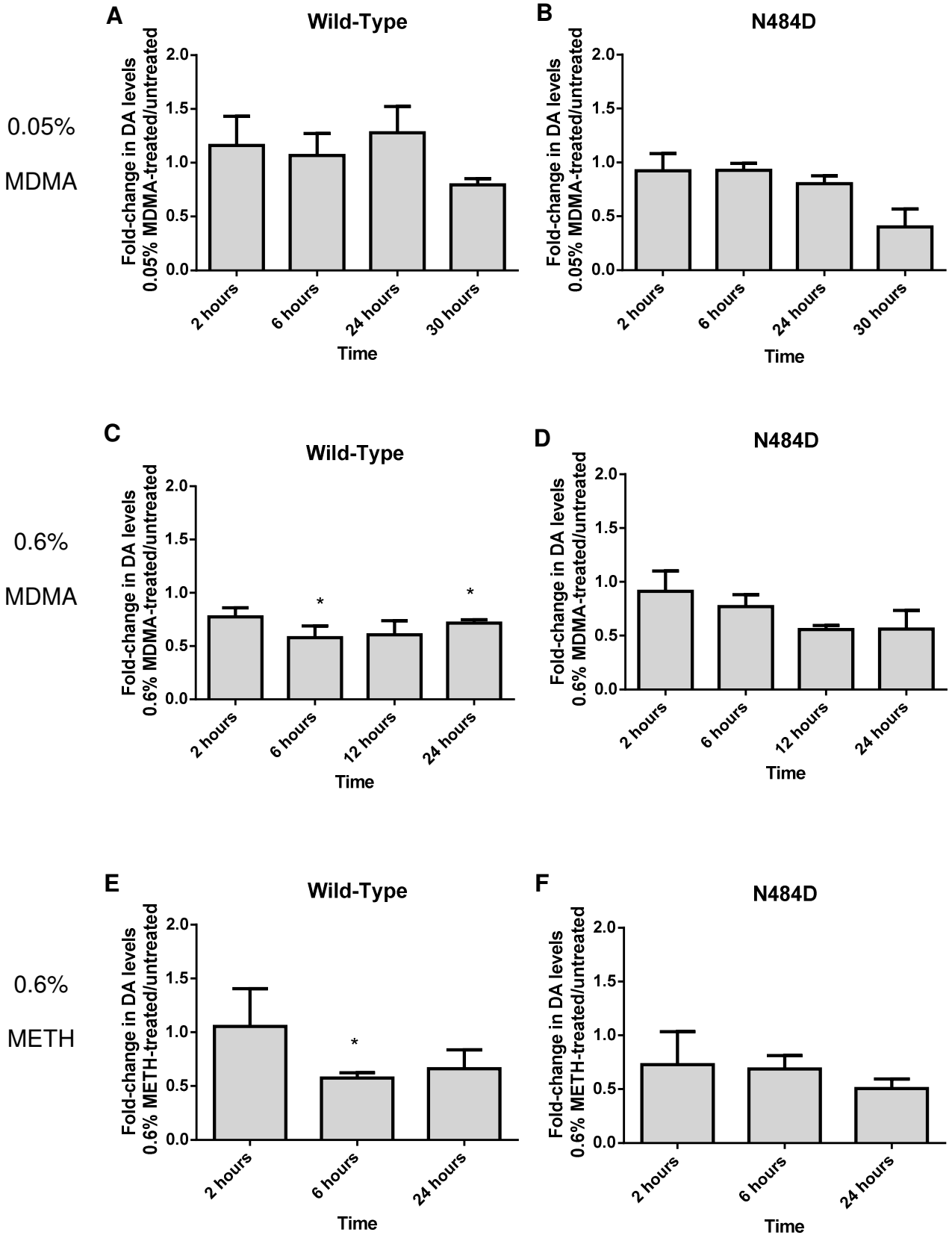

A
Wild-Type
B
N484D
0.05%
MDMA
Fold-change in DA levels
0.05% MDMA-treated/untreated
2.0
1.5
1.0
0.5
0.0
2 hours
6 hours
24 hours
30 hours
Time
C
Wild-Type
D
N484D
0.6%
MDMA
Fold-change in DA levels
0.6% MDMA-treated/untreated
*
*
2 hours
6 hours
12 hours
24 hours
Time
E
Wild-Type
F
N484D
0.6%
METH
Fold-change in DA levels
0.6% METH-treated/untreated
*
2 hours
6 hours
24 hours
Time

Figure 2.3

Figure 2.4 Comparison of DA concentrations in wild-type and N484D flies. Two-Way ANOVA showed significantly reduced DA levels in N484D flies relative to wild-type after 0.05%MDMA treatment, P=0.0262 (Panel A). However, no differences were observed in flies treated with 0.6% MDMA (P = 0.7548) or with METH (P = 0.5431) (Panels B and C).

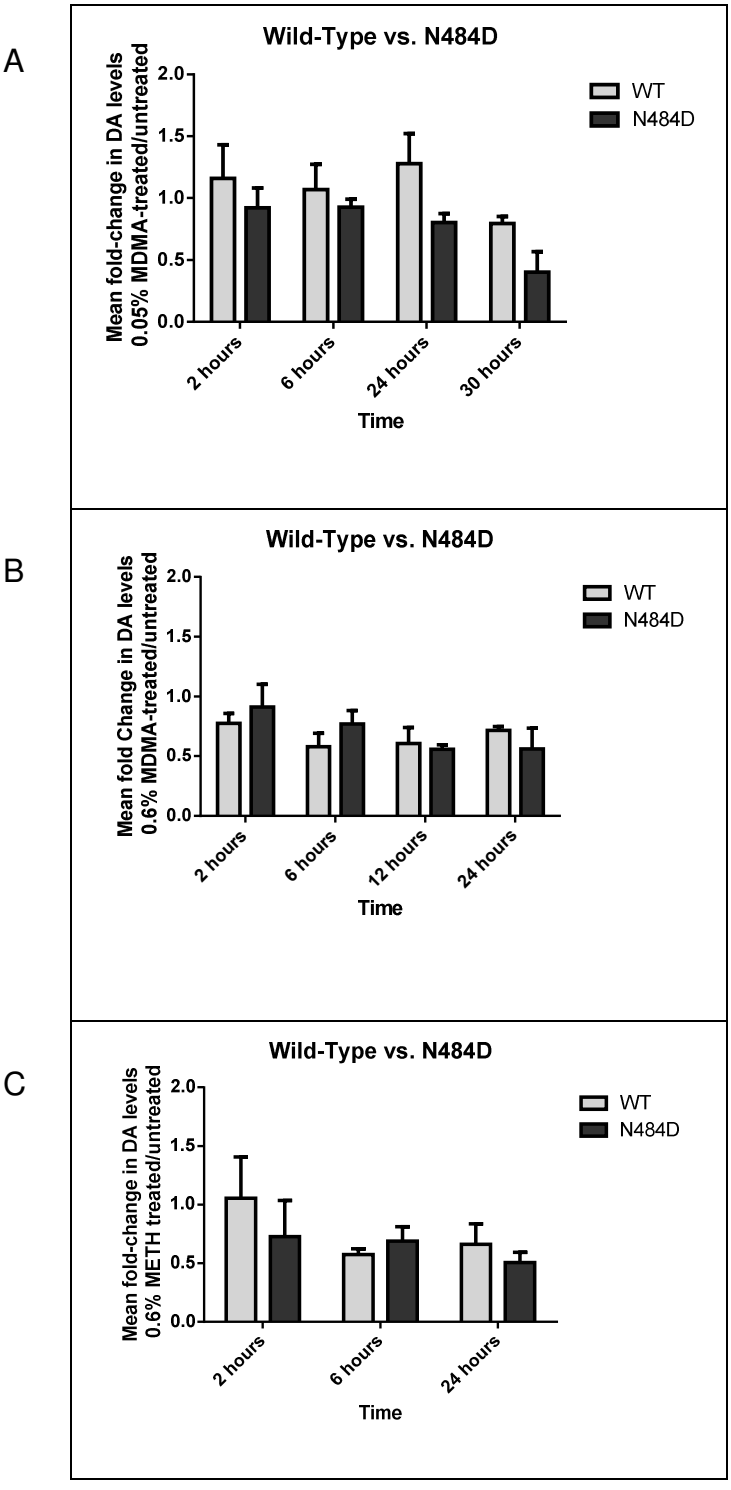

A
Wild-Type vs. N484D
Mean fold-change in DA levels
0.05% MDMA-treated/untreated
2.0
1.5
1.0
0.5
0.0
WT
N484D
2 hours
6 hours
24 hours
30 hours
Time
B
Wild-Type vs. N484D
Mean fold Change in DA levels
0.6% MDMA-treated/untreated
2.0
1.5
1.0
0.5
0.0
WT
N484D
2 hours
6 hours
12 hours
24 hours
Time
C
Wild-Type vs. N484D
Mean fold-change in DA levels
0.6% METH treated/untreated
2.0
1.5
1.0
0.5
0.0
WT
N484D
2 hours
6 hours
24 hours
Time

Figure 2.4

2.4.3 Norepinephrine, tyramine, octopamine, and gender results

It is well established that MDMA and METH have effects on the 5-HT, DA, and NE systems in humans. As such, it was natural to study these neurotransmitters. In mammals, NE Influences sleep, wakefulness, attention, and feeding behavior.[65] Although it is the key neurotransmitter in the sympathetic nervous system in humans, NE is not present in *D. melanogaster*. In invertebrate species, NE is functionally replaced by OCT. NE concentration was still analyzed by HPLC/mass spectroscopy as a control assessing for contamination. In mammals, NE is the principal neurotransmitter governing the sympathetic nervous system. Found only in trace amounts, little is known about the function of OCT in humans.[66] In invertebrates, OCT is present in very high concentrations, and is believed to be the principal invertebrate neurotransmitter[67], making it important to study changes in OCT levels in *D. melanogaster*. However, results from this work found OCT in only very low concentrations, sometimes undetectable by HPLC-MS/MS. Consequently, no drug effects, or differences between wild-type or N484D flies were observed.

Additionally, interest in the trace amine tyramine (TYR) as a catecholamine-releasing agent contributing to the effects of drugs of abuse is increasing. TYR is a trace amine derived from the amino acid tyrosine. It may induce vasoconstriction and regulate blood pressure, and is essential for female sperm storage, and for larval locomotion in *D. melanogaster*.[66] It is known for having moderate psychoactive effects associated with the consumption fermented foods, particularly strong cheeses (e.g. blue cheese). As cheese is high in tyramine,

excess consumption, especially in persons concurrently taking an MAOI, can have psychoactive sympathomimetic effects.[68] Much like OCT, TYR also has effects on the adrenergic and dopaminergic systems.[69] . It has been suggested that TYR may act as a direct agonist of brain DA receptors.[69] This makes the role of tyramine in amphetamine-induced changes in neurochemistry important to examine. No significant differences were observed between WT *D. melanogaster* and N484D mutants in respect to TYR or OCT concentrations in this study.

Lastly, it was unknown if gender-specific differences would be observed amongst drug-treated flies. No differences were observed between male and female flies in the drug-treated or untreated groups.

CHAPTER 3. DISCUSSION

3.1 Discussion of results

Amphetamines are a class of potent psychoactive drugs that are used for both therapeutic and recreational purposes. The pharmacological activity of amphetamines is mainly caused by their action on monoamine neurotransmitter systems. In particular, a reversal of the transport cycle of the monoamine transporters SERT, DAT, and NET leads to increased synaptic concentrations of neurotransmitters, and contributes to their psychoactivity.[68]

Previous research has shown that dSERT is insensitive to MDMA.[51, 70] However, a dSERT point mutation (N484D) has been identified that restores sensitivity to MDMA *in vitro*. Here, an *in vivo* analysis of the effects of MDMA and METH exposure on biogenic amine levels in wild-type flies and flies expressing dSERT N484D was performed. Results showed that treatment with 0.05% MDMA significantly reduced 5-HT levels in dSERT N484D flies relative to wild-type flies. As significant drug-induced changes were not observed in the levels of other neurotransmitters investigated, it may be that the effects of this mutation in dSERT exclusively influence 5-HT levels. This was particularly clear in experiments with MDMA, which has greater affinity for SERT relative to other amphetamines.[2, 45] Although lower DA content was observed in N484D flies than

wild-type flies treated with 0.05% MDMA (figure 2.4 A), this was not the result of drug effects (figure 2.3 A, B).

Previous *in vitro* work performed in the Barker laboratory show that the N484D mutation restores amphetamine-induced efflux to dSERT. Without the presence of an acidic residue to create a functional salt bridge between the two transmembrane helices, wild-type dSERT exhibits minimal responsiveness to amphetamines. When the salt bridge is restored by the mutation of asparagine 484 to an aspartate this functionality is also reintroduced. The results for 0.05% MDMA-treated flies complement this *in vitro* data from the Barker lab suggesting an essential role for this salt bridge in mediating the ability of amphetamines to be substrates for SERT, and underline the importance of this specific residue in the native function of SERT. However, the lack of a response to 0.6% MDMA and 0.6% METH was unexpected.

Though a large number of flies were used (approximately 30 flies per data point), no more than 6 data points were generated for any time point, and most had only 2 or 3. It may be that significant changes were not observed due to this relatively small sample size, potentially skewing the results. Much of the data did not show the predicted changes in neurochemistry. However, it is noteworthy that where the predicted changes were observed, the trend was for N484D flies to have lower neurotransmitter levels than wild-type flies (e.g. figure 2.1 A, B). It may be that with a greater number of replicates at each time point more conclusive results could be generated.

Additionally, this *in vivo* model used a transgenic targeted expression system that inserted dSERT N484D into *D. melanogaster* without altering native dSERT. This may pose a potential limitation to this study, as the presence of dSERT may confound the interpretation of results (i.e. a lack of detectable effects may be due to native dSERT mitigating drug effects mediated by dSERT N484D). The relative abundance of either protein was not assessed, so the extent of dSERT involvement was not determined. However, it is important to note that although dSERT was present it would still be expected that amphetamines would inhibit uptake by dSERT, allowing for neurotransmitter efflux through dSERT N484D to lead to drug-induced neurochemical changes.

3.2 Future directions

The present study examined the role of a conserved acidic residue in SERT *in vivo* by analyzing changes in neurochemistry in *D. melanogaster* expressing the N484D mutant transporter after MDMA or METH exposure. After completing these studies, experiments using *D. melanogaster* tissue for the generation of synaptosomes were attempted. Synaptosomes are a useful way to study the activity of proteins involved in neurotransmission.[71] This could be used to provide an alternative means of assessing drug-induced changes in *D. melanogaster* neurochemistry. Synaptosomes are routinely prepared from mammalian tissue for laboratory studies. To our knowledge, only one report exists wherein *D. melanogaster* tissue was used for synaptosome generation. [72] Using invertebrates rather than mammals for synaptosome preparation requires

considerably more samples to yield sufficient quantities of tissue, and is more difficult to complete.[73] Efforts to make synaptosomes from *D. melanogaster* have thus far been unsuccessful.

Additionally, it was observed in these experiments that all flies treated with MDMA or METH seemed to respond to treatment in terms of locomotor activity, irrespective of whether they were wild-type or N484D. However, this behavior was not measured quantitatively. Future behavioral studies would also be useful to further understand amphetamine pharmacology and the role of this salt bridge in restoring MDMA sensitivity in *D. melanogaster*. Experiments previously performed in the Pittendrigh laboratory using 0.6% METH showed increased locomotor activity. It would be predicted that while wild-type flies would not respond after MDMA treatment, owing to dSERT insensitivity to MDMA, N484D flies would have increased locomotion.

Lastly, *D. melanogaster* has previously been used to study genomic, proteomic, and transcriptomic changes after exposure to METH.[15] This model could also be extended to MDMA to observe systems level changes resulting from MDMA-induced 5-HT depletion in N484D flies. Future research using this model is likely to be useful fighting amphetamine addiction and toxicity.

REFERENCES

REFERENCES

1. Úxler, F. *et al.* The origin of MDMA (‘ Ecstasy ’) – separating the facts from the myth. *Pharmazie* **61,** (2006).

2. Steele, T., Nichols, D. & Yim, G. Stereochemical effects of 3,4-methylenedioxymethamphetamine (MDMA) and related amphetamine derivatives on inhibition of uptake of [3H]monoamines into synaptosomes from different regions of rat brain. *Biochem. Pharmacol.* **36,** 2297–2303 (1987).

3. Nelson, a J. D., Thur, K. E., Marsden, C. a & Cassaday, H. J. Paradoxical effects of low dose MDMA on latent inhibition in the rat. *Neuropharmacology* **67,** 331–6 (2013).

4. Biezonski, D. K. & Meyer, J. S. The Nature of 3 , 4-Methylenedioxymethamphetamine (MDMA) -Induced Serotonergic Dysfunction : Evidence for and Against the Neurodegenera- tion Hypothesis. *Curr. Neuropharmacol.* 84–90 (2011).

5. Hornfeldt, C. S. Rave drugs: pharmacological considerations. *AANA J.* **72,** 334–5; author reply 335 (2004).

6. Kupferschmidt, K. High hopes. *Science* **345,** 18–23 (2014).

7. Nichols, D. MDMA represents a new type of pharmacologic agent and cannot be considered to be either a hallucinogenic agent or an amphetamine-type stimulant. *Doc. from DEA Sched. Hear. MDMA* (1985). at <http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:MDMA+represents+a+new+type+of+pharmacological+agent+and+cannot+be+considered+either+an+hallucinogenic+agent+or+an+amphetamine-type+stimulant#3>

8. Justice, department of. Deletion of MDMA from Schedule I following appeals court decision. *Fed. Regist.* 17 (1988).

9. Justice, D. of. DEA scheduling of MDMA as Schedule I. *Fed. Regist.* **53,** (1988).

10. Molliver, M. E. & Street, W. Distinct morphologic classes of axons in primates exhibit differential vulnerability. *Neuroscience* **28,** 121–137 (1989).

11. Quinton, M. S. & Yamamoto, B. K. Causes and consequences of methamphetamine and MDMA toxicity. *AAPS J.* **8,** E337–47 (2006).

12. Parrott, A. C. MDMA and 5-HT neurotoxicity: the empirical evidence for its adverse effects in humans - no need for translation. *Br. J. Pharmacol.* **166,** 1518–20; discussion 1521–2 (2012).

13. Urban, N. B. *et al.* Sustained recreational use of ecstasy is associated with altered pre and postsynaptic markers of serotonin transmission in neocortical areas: a PET study with [^{11}C]DASB and [^{11}C]MDL 100907. *Neuropsychopharmacology* **37,** 1465–73 (2012).

14. Verrico, C. D., Miller, G. M. & Madras, B. K. MDMA (Ecstasy) and human dopamine, norepinephrine, and serotonin transporters: implications for MDMA-induced neurotoxicity and treatment. *Psychopharmacology (Berl).* **189,** 489–503 (2007).

15. Sun, L. *et al.* Systems-scale analysis reveals pathways involved in cellular response to methamphetamine. *PLoS One* **6,** e18215 (2011).

16. Grobler, S. R., Chikte, U. & Westraat, J. The pH Levels of Different Methamphetamine Drug Samples on the Street Market in Cape Town. *ISRN Dent.* **2011,** 974768 (2011).

17. Hsieh, J. H., Stein, D. J. & Howells, F. M. The neurobiology of methamphetamine induced psychosis. *Front. Hum. Neurosci.* **8,** 537 (2014).

18. Recordati Rare Diseases. Desoxyn prescribing information. (2013).

19. Cruickshank, C. C. & Dyer, K. R. A review of the clinical pharmacology of methamphetamine. *Addiction* **104,** 1085–99 (2009).

20. Degenhardt, L. *et al.* The global epidemiology and burden of psychostimulant dependence: Findings from the Global Burden of Disease Study 2010. *Drug Alcohol Depend.* **137,** 36–47 (2014).

21. Blakely, R. D. *et al.* Regulated phosphorylation and trafficking of antidepressant-sensitive serotonin transporter proteins. *Biol. Psychiatry* **44,** 169–78 (1998).

22. Rudnick, G. Bioenergetics of neurotransmitter transport. *J. Bioenerg. Biomembr.* **30,** 173–85 (1998).

23. Rudnick, G. & Clark, J. From synapse to vesicle: the reuptake and storage of biogenic amine neurotransmitters. *Biochim. Biophys. Acta* **1144,** 249–63 (1993).

24. Pramod, A. B., Foster, J., Carvelli, L. & Henry, L. K. SLC6 transporters: structure, function, regulation, disease association and therapeutics. *Mol. Aspects Med.* **34,** 197–219 (2013).

25. Celik, L. *et al.* Binding of serotonin to the human serotonin transporter. Molecular modeling and experimental validation. *J. Am. Chem. Soc.* **130,** 3853–65 (2008).

26. Gabrielsen, M., Ravna, A. W., Kristiansen, K. & Sylte, I. Substrate binding and translocation of the serotonin transporter studied by docking and molecular dynamics simulations. *J. Mol. Model.* **18,** 1073–85 (2012).

27. Domino, E. F. History of Modern Psychopharmacology : A Personal View With an Emphasis on Antidepressants. *Psychosom. Med.* **598,** 591–598 (1999).

28. Jayanthi, L. D. & Ramamoorthy, S. Regulation of monoamine transporters: influence of psychostimulants and therapeutic antidepressants. *AAPS J.* **7,** E728–38 (2005).

29. Andersen, J., Kristensen, A. S., Bang-Andersen, B. & Strømgaard, K. Recent advances in the understanding of the interaction of antidepressant drugs with serotonin and norepinephrine transporters. *Chem. Commun. (Camb).* 3677–92 (2009). doi:10.1039/b903035m

30. Flint, J. & Kendler, K. S. The Genetics of Major Depression. *Neuron* **81,** 484–503 (2014).

31. Amara, S. G. & Sonders, M. S. Neurotransmitter transporters as molecular targets for addictive drugs. *Drug Alcohol Depend.* **51,** 87–96 (1998).

32. Moritz, A. E. *et al.* Phosphorylation of dopamine transporter serine 7 modulates cocaine analog binding. *J. Biol. Chem.* **288,** 20–32 (2013).

33. Chen, R. *et al.* Abolished cocaine reward in mice with a cocaine-insensitive dopamine transporter. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 9333–8 (2006).

34. Saldaña, S. N. & Barker, E. L. Temperature and 3,4-methylenedioxymethamphetamine alter human serotonin transporter-mediated dopamine uptake. *Neurosci. Lett.* **354,** 209–212 (2004).

35. Rothman, R. B. *et al.* Amphetamine-Type Central Nervous Norepinephrine More Potently Than They Release Dopamine and Serotonin. *Synapse* **41,** 32–41 (2001).

36. Bunzow, J. R. *et al.* Amphetamine, 3,4-Methylenedioxymethamphetamine, Lysergic Acid Diethylamide, and Metabolites of the Catecholamine Neurotransmitters Are Agonists of a Rat Trace Amine Receptor. *Mol. Pharmacol.* **60,** 1181–1188 (2001).

37. Xie, Z. & Miller, G. M. A receptor mechanism for methamphetamine action in dopamine transporter regulation in brain. *J. Pharmacol. Exp. Ther.* **330,** 316–25 (2009).

38. Yamashita, A., Singh, S. K., Kawate, T., Jin, Y. & Gouaux, E. Crystal structure of a bacterial homologue of Na+/Cl--dependent neurotransmitter transporters. *Nature* **437,** 215–23 (2005).

39. Aravind Penmatsa, Kevin H. Wang, and E. G. X-ray structure of the dopamine transporter in complex with tricyclic antidepressant. *Nature* **503,** 85–90 (2014).

40. Wang, H. *et al.* Structural basis for action by diverse antidepressants on biogenic amine transporters. *Nature* **503,** 141–5 (2013).

41. Madura, J. D. & Surratt, C. K. Dopamine transporter comparative molecular modeling and binding site prediction using the LeuT. *Proteins* 1033–1046 (2007). doi:10.1002/prot

42. Zhou, Z. *et al.* Antidepressant specificity of serotonin transporter suggested by three LeuT-SSRI structures. *Nat. Struct. Mol. Biol.* **16,** 652–7 (2009).

43. Jørgensen, A. M. *et al.* Homology modeling of the serotonin transporter: insights into the primary escitalopram-binding site. *ChemMedChem* **2,** 815–26 (2007).

44. Roman, D. L. *et al.* Distinct Molecular recognition of Psychostimulants by Human and Drosophila Serotonin Transporters. *J. Pharmacol. Exp. Ther.* **308,** 679–687 (2004).

45. Cozzi, N. V, Sievert, M. K., Shulgin, A. T., Jacob, P. & Ruoho, A. E. Inhibition of plasma membrane monoamine transporters by b - ketoamphetamines. *Eur. J. Pharmacol.* (1999).

46. Ravna, A. W., Sylte, I. & Dahl, S. G. Structure and localisation of drug binding sites on neurotransmitter transporters. *J. Mol. Model.* **15,** 1155–64 (2009).

47. Sucic, S. *et al.* The N terminus of monoamine transporters is a lever required for the action of amphetamines. *J. Biol. Chem.* **285,** 10924–38 (2010).

48. Cervinski, M. a, Foster, J. D. & Vaughan, R. a. Psychoactive substrates stimulate dopamine transporter phosphorylation and down-regulation by cocaine-sensitive and protein kinase C-dependent mechanisms. *J. Biol. Chem.* **280,** 40442–9 (2005).

49. Khoshbouei, H. *et al.* N-terminal phosphorylation of the dopamine transporter is required for amphetamine-induced efflux. *PLoS Biol.* **2,** E78 (2004).

50. Demchyshyn, L. L. *et al.* Cloning, expression, and localization of a chloride-facilitated, cocaine-sensitive serotonin transporter from Drosophila melanogaster. *Proc. Natl. Acad. Sci. U. S. A.* **91,** 5158–62 (1994).

51. Rodriguez, G., Roman, D. & White, K. Distinct recognition of substrates by the human and Drosophila serotonin transporters. *J. Pharmacol. Exp. Ther.* **306,** 338–346 (2003).

52. Kumar, S. & Nussinov, R. Close Range Electrostatic Interactions in Proteins Close-Range Electrostatic Interactions in Proteins. 604–617 (2002).

53. Barker, E. L. *et al.* High Affinity Recognition of Serotonin Transporter Antagonists Defined by Species-scanning Mutagenesis. *J. Biol. Chem.* **273,** 19459–19468 (1998).

54. Wall, C. Non-neurotoxic Amphetamine Derivatives Release Serotonin through Serotonin Transporters. *Mol. Pharmacol.* 271–276

55. Barker, E. L. & Kimmel, L. Chimeric Human and Rat Serotonin Transporters Reveal Domains Involved in Recognition of Transporter Ligands. *Mol. Pharmacol.* (1994).

56. Torres-Altoro, M. I., Kuntz, C. P., Nichols, D. E. & Barker, E. L. Structural analysis of the extracellular entrance to the serotonin transporter permeation pathway. *J. Biol. Chem.* **285,** 15369–79 (2010).

57. Pandey, U. B. & Nichols, C. D. Human disease models in Drosophila melanogaster and the role of the fly in therapeutic drug discovery. *Pharmacol. Rev.* **63,** 411–36 (2011).

58. Reiter, L. T., Potocki, L., Chien, S., Gribskov, M. & Bier, E. A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster. *Genome Res.* **11,** 1114–25 (2001).

59. Walters, Jr., K. R. *et al.* Methamphetamine causes anorexia in Drosophila melanogaster, exhausting metabolic reserves and contributing to mortality. *J. Toxicol. Sci.* **37,** 773–790 (2012).

60. Cadet, J. L., Jayanthi, S. & Deng, X. Speed kills: cellular and molecular bases of methamphetamine-induced nerve terminal degeneration and neuronal apoptosis. *FASEB J.* **17,** 1775–88 (2003).

61. Han, B. *et al.* Quantitative LC-MS/MS analysis of arachidonoyl amino acids in mouse brain with treatment of FAAH inhibitor. *Anal. Biochem.* **432,** 74–81 (2013).

62. Ressler, K. J. & Nemeroff, C. B. Role of serotonergic and noradrenergic systems in the pathophysiology of depression and anxiety disorders. *Depress. Anxiety* 2–19 (2000).

63. Cowen, P. J. Serotonin and depression: pathophysiological mechanism or marketing myth? *Trends Pharmacol. Sci.* **29,** 433–6 (2008).

64. Beaulieu, J. & Gainetdinov, R. R. The Physiology , Signaling , and Pharmacology of Dopamine Receptors. *Pharmacol. Rev.* **63,** 182–217 (2011).

65. Tanaka, M., Yoshida, M., Emoto, H. & Ishii, H. Noradrenaline systems in the hypothalamus, amygdala and locus coeruleus are involved in the provocation of anxiety: basic studies. *Eur. J. Pharmacol.* **405,** 397–406 (2000).

66. Roeder, T. Tyramine and octopamine: ruling behavior and metabolism. *Annu. Rev. Entomol.* **50,** 447–77 (2005).

67. Roeder, T. Octopamine in invertebrates. *Prog. Neurobiol.* **59,** 533–561 (1999).

68. Sulzer, D., Sonders, M. S., Poulsen, N. W. & Galli, A. Mechanisms of neurotransmitter release by amphetamines: a review. *Prog. Neurobiol.* **75,** 406–33 (2005).

69. Lillian E. Dyck, C. W. K. and C. T. D. The role of catecholamines, 5-hydroxytryptamine and m-tyramine in the behavioural effects of m-tyrosine in the rat. *Eur. J. Pharmacol.* **84,** 139–149 (1982).

70. Dasari, S., Viele, K., Turner, a C. & Cooper, R. L. Influence of PCPA and MDMA (ecstasy) on physiology, development and behavior in Drosophila melanogaster. *Eur. J. Neurosci.* **26,** 424–38 (2007).

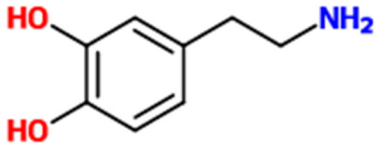
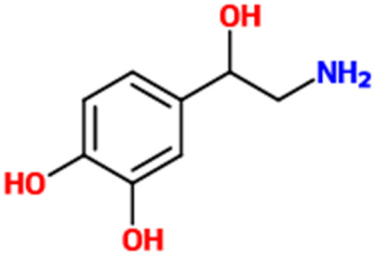
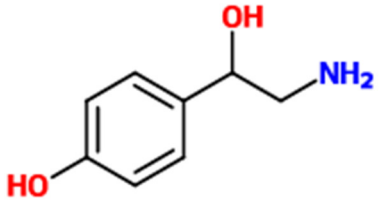
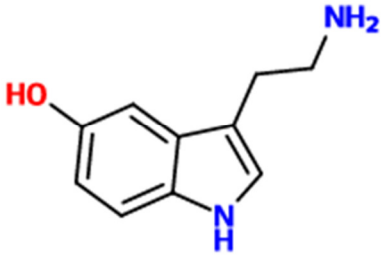
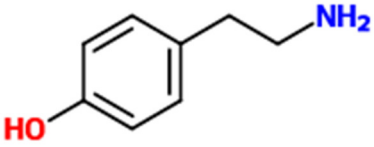
71. Bai, F. & Witzmann, F. A. Synaptosome Proteomics. *Subcell. Biochem.* (2010).

72. Ramarao, C. S., Acharya, S. R., Krishnan, K. S. & Kenkare, U. W. High affinity uptake of L-glutamate and γ-aminobutyric acid in Drosophila melanogaster. *J. Biosci.* **11,** 119–135 (1987).

73. Btochem, I. A microscale flotation technique for the isolation of synaptosomes from nervous tissue. *Insect Biochem.* **10,** (1980).

APPENDIX

Table A1.Biogenic amines structures and functions

Biogenic amine	Structure	Function
Dopamine		Involved in coordination of movement, reward and motivation. Many addictive drugs stimulate dopamine release, and this is believed to be a crucially involved in addiction.[64]
Norepinephrine		Influences sleep, wakefulness, attention, and feeding behavior. Although it is the key neurotransmitter in the sympathetic nervous system in humans it is not present in *Drosophila*.[65]
Octopamine		Found only in trace amounts in humans. Little is known about its function. The main neurotransmitter in invertebrates. Replaces norepinephrine in the sympathetic nervous system.[66] Essential for female sperm storage, and larval locomotion.[67]
Serotonin		Involved in numerous core biological functions, including regulation of sleep and wakefulness, appetite, and mood.[62]
Tyramine		A trace amine which acts as a catecholamine-releasing agent. May induce vasoconstriction and regulate blood pressure. Essential for female sperm storage, and for larval locomotion in *D. melanogaster*.[66]

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