THE ROLE OF INSULIN SIGNALING ON DOPAMINE TRANSPORTER TRAFFICKING

By

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To my amazing, supportive parents,
for always keeping me smiling

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LIST OF ABBREVIATIONS

5-HT	**Serotonin**
ADHD	**Attention-deficit hyperactivity disorder**
Akt	**Serine/threonine protein kinase B**
AMPH	**Amphetamine**
ANOVA	**Analysis of variance**
BBB	**Blood-brain barrier**
BMI	**Body Mass Index**
CaMKII	**Calcium/calmodulin-dependent kinase II**
cAMP	**Cyclic adenosine monophosphate**
COMT	**Catechol-O-methyl transferase**
DA	**Dopamine**
DAT	**Dopamine transporter**
DIO	**Diet induced obesity**
DOPA	**3,4-dihydroxyphenylalanine**
DR	**Dopamine receptor**
ECL	**Extracellular loop**
ERK	**Extracellular signal-regulated kinase**
fMRI	**Functional magnetic resonance imaging**
GABA	**γ-aminobutyric acid**
GFP	**Green fluorescent protein**
GSK3	**Glycogen synthase kinase 3**

hDAT	**Human dopamine transporter**
HEK	**Human embryonic kidney**
HSCA	**High speed chronoamperometry**
HSV	**Herpes simplex virus**
IGF	**Insulin-like growth factor**
IR	**Insulin receptor**
IRS-2	**Insulin receptor substrate 2**
LeuT	**Leucine transporter**
MAOB	**Monoamine oxidase B**
MAPK	**Mitogen-activated protein kinase**
MPP^+	**1-methyl-4-phenylpyridium**
NE	**Norepinephrine**
NET	**Norepinephrine transporter**
PBS	**Phosphate-buffered saline**
PDK	**Phosphoinositol dependent kinase**
PET	**Positron emission tomography**
PI3K	**Phosphatidylinositol 3-kinase**
PICK1	**Protein interacting with C kinase**
PKA	**Protein kinase A**
PKC	**Protein kinase C**
PMA	**Phorbol 12-myristate 13-acetate**
PP2A	**Protein Phosphotase 2A**
RACK1	**Receptor for activated C-kinase**

RFP	**Red fluorescent protein**
RTK	**Receptor tyrosine kinase**
SERT	**Serotonin transporter**
STZ	**Streptozotocin**
SYN-1A	**Syntaxin 1a**
TH	**Tyrosine hydroxylase**
TMD	**Transmembrane domain**
VMAT	**Vesicular monoamine transporter**
VTA	**Ventral tegmental area**

CHAPTER I

INTRODUCTION

The Neurotransmitter Dopamine and Dopaminergic Pathways

Dopamine (3-hydroxytyramine; DA) is a catecholamine neurotransmitter that is a precursor to the synthesis of the neurotransmitter norepinephrine (NE). DA in synthesized from tyrosine by a two step process, where tyrosine hydroxylase (TH) is the rate-limiting enzyme in the reaction. Being that DA is a precursor to NE synthesis, it was originally thought that it did not have signaling properties on its own, but instead was only an intermediate to NE production. However, in 1958, Carlsson and colleagues demonstrated that DA had signaling properties on its own. Using 3,4-dihydroxyphenylalanine (DOPA), the precursor to DA, they showed that in rabbits depleted of catecholamines by reserpine, DOPA treatment could reverse the reserpine-mediated effects. Importantly, this reversal corresponded to an increase in DA, not NE (Carlsson, Lindqvist et al. 1958). Later work pointed to enrichments of DA in certain brain regions, namely the basal ganglia (Bertler and Rosengren 1959). Soon DA brain regions were mapped, displaying several distinct pathways of DA signaling (Fuxe 1965; Ungerstedt 1971). There are four main dopaminergic pathways: the tuberoinfundibular pathway, the nigrostriatal pathway, the mesocortical pathway, and the mesolimbic pathway (Figure 1). The tuberoinfundibular pathway, which

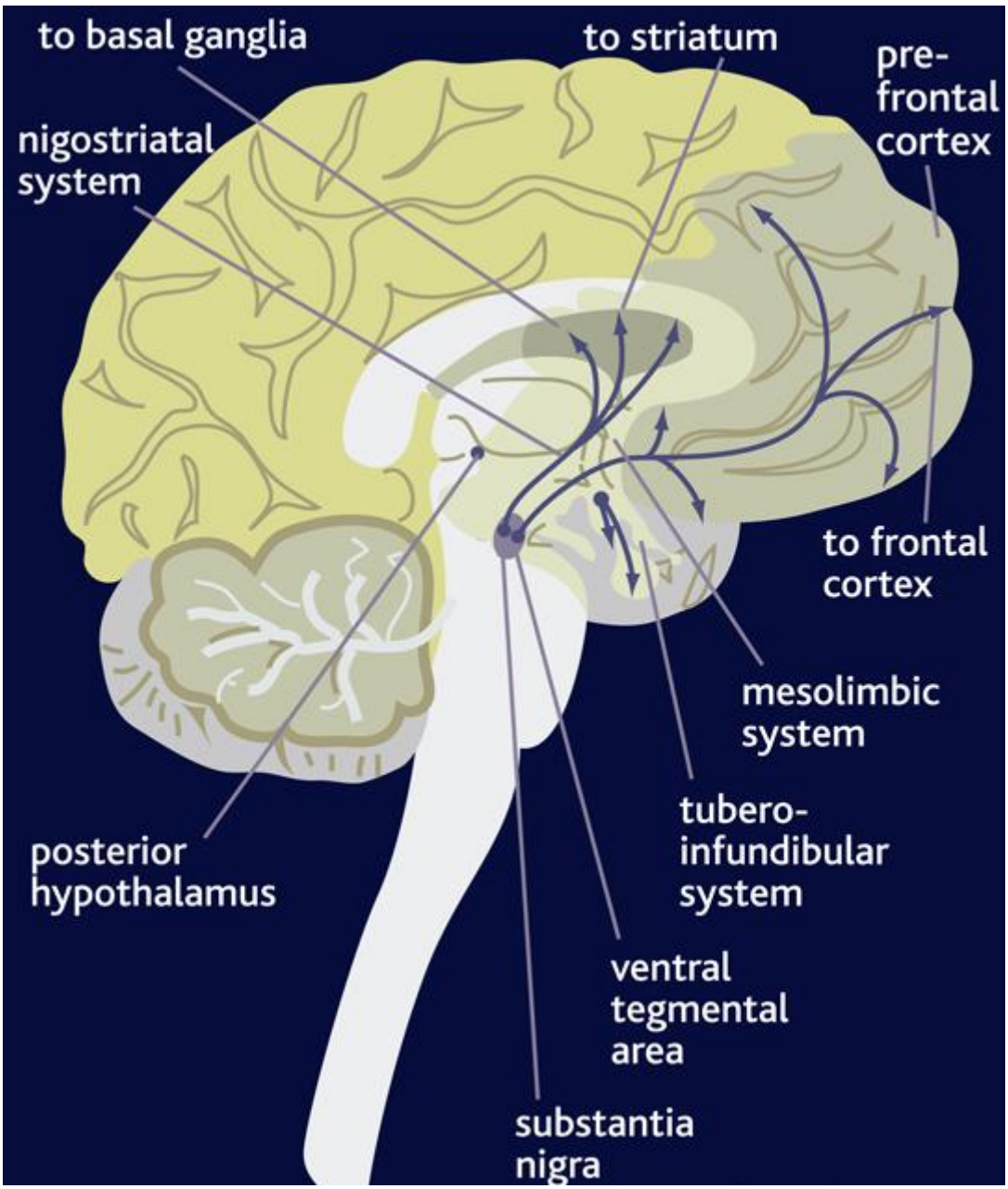


Figure 1. Pathways of Dopamine Signaling in the Brain. Illustration of major DA projections in the central nervous system. The nigrostriatal pathway originates in the substantia nigra and projects to the dorsal striatum. The mesolimbic and mesocortical projections originate in the ventral tegmental area and project both to ventral striatum and areas in the prefrontal cortex, respectively. The final system is the tuberoinfundibular system which projects from the hypothalamus to the pituitary. This image was obtained from cnsforum.com.

refers to a group of DA neurons in the arcuate nucleus of the hypothalamus that project to the median eminence, controls prolactin secretion from the anterior pituitary gland (Weiner and Ganong 1978). The nigrostriatal pathway consists of neurons whose cell bodies originate in the substantia nigra and terminate in the dorsal striatum. This area is implicated in movement since degeneration of these projections has been shown to cause Parkinson's Disease; characterized by tremors, rigidity, and overall improper movement (Barbeau 1962). Recently it has been demonstrated that this region is also important in feeding behavior (Volkow, Wang et al. 2002; Sotak, Hnasko et al. 2005; Robinson, Rainwater et al. 2007). Next, dopaminergic neurons in the mesocortical pathway project from the ventral tegmental area (VTA) to the frontal lobes of the cerebrum, particularly the prefrontal cortex, and are involved in cognition and emotion. Lastly, neurons of the mesolimbic pathway also originate in the VTA but instead innervate the ventral striatum, also known as the nucleus accumbens. This pathway is implicated in reward and pleasure.

DA is involved in a number of physiological and behavioral processes including cognition, locomotion, mood, motivation, and reward. Abnormalities in the central dopaminergic systems contribute to several neuropsychiatric diseases, including Parkinson's disease, attention-deficit hyperactivity disorder (ADHD), schizophrenia, bipolar disorder, binge eating disorder, and addiction (Wise 1998; Horschitz, Hummerich et al. 2005; Kienast and Heinz 2006; Volkow, Wang et al. 2007; Davis, Levitan et al. 2008; Koob and Le Moal 2008). In particular, the nigrostriatal system is thought to give us the motivation to seek

basic needs, such as food, while the mesolimbic pathway enables us to feel pleasure from them (Palmiter 2007). Our eating behavior and our desire for food is tied in closely with these systems, which receives input from areas of the brain that monitor our nutritional need for food, such as the hypothalamus (Obici, Feng et al. 2002; Obici, Zhang et al. 2002; Niswender and Schwartz 2003; Schwartz and Porte 2005). Therefore, it is not surprising that dysfunction in DA signaling has been linked to eating disorders and obesity (Wang, Volkow et al. 2001; Shinohara, Mizushima et al. 2004; Chen, Yang et al. 2008).

As such, DA dysregulation looks to be the basis of several neurological disorders. In order to develop effective pharmacotherapeutic approaches, it is critical to understand dopaminergic neurotransmission, the regulatory factors governing it, and how dysreguation of these factors can contribute to disease.

Dopaminergic Neurotransmission and the Dopamine Transporter

Several factors influence dopaminergic neurotransmission, such as the amount of DA synthesized and released, the number of DA receptors (DRs) at the synapse, and the length of time DA spends in the synaptic space. As noted above, DA is synthesized through a series of enzymatic reactions, beginning with the hydration of amino acid tyrosine to DOPA via TH. DOPA is decarboxylated by aromatic amino acid decarboxylase to produce DA (Figure 2). The transmitter is then packaged into synaptic vesicles by a vesicular monoamine transporter (VMAT) and released at nerve terminals into the synapse upon stimulation. Released DA then binds to DRs to elicit a response in the

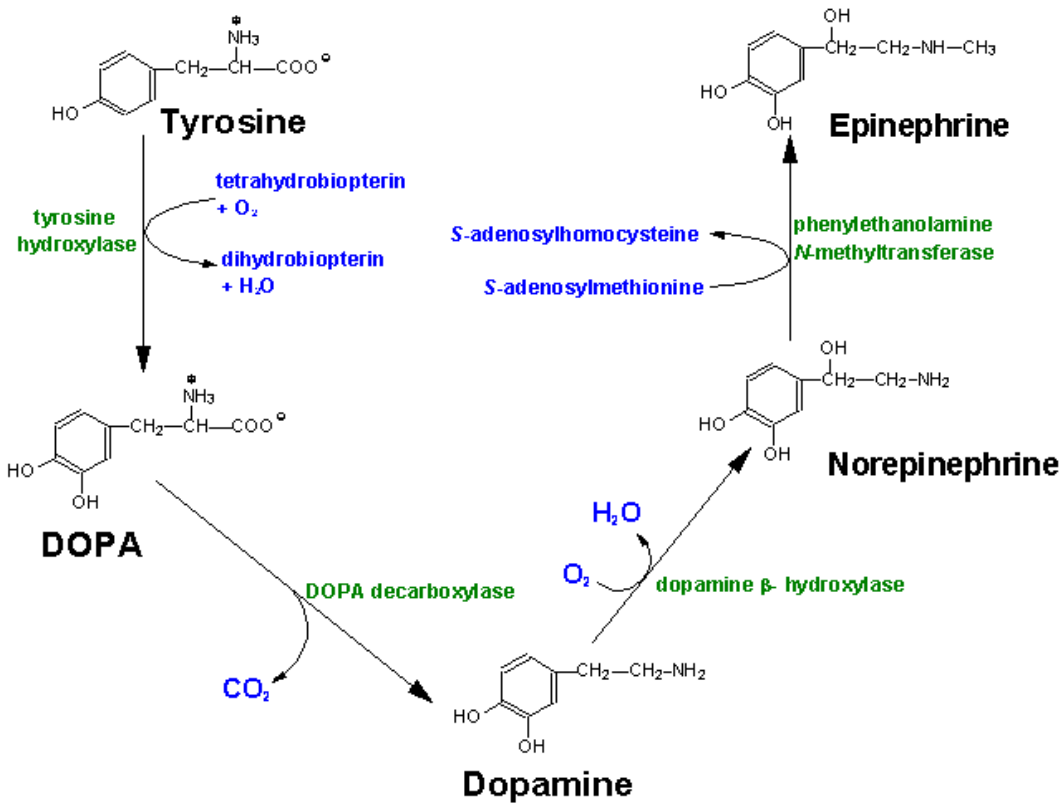


Figure 2. Biosynthesis of dopamine. Synthetic enzymes and their changes to each product are labeled in green and blue, respectively. This image was obtained from *www.neurosci.pharm.utoledo.edu.*

postsynaptic cell. The released DA is then cleared from the synapse primarily by the dopamine transporter (DAT), where it re-enters the presynaptic neuron to be recycled and repackaged into vesicles (Figure 3).

An important component in DA signaling is the receptor itself. DRs are a family of G protein-coupled receptors. There are five subtypes, which are divided into two groups. D1-like receptors, comprising the D1 and D5 receptors, are coupled to G proteins which stimulate adenylyl cyclase and cyclic adenosine monophosphate (cAMP) production, whereas D2-like receptors, comprising the D2 (D2R), D3 (D3R), and D4 (D4R) receptors, couple to Gi/o proteins and result in the inhibition of adenylyl cyclase and suppression of cAMP production (Kebabian and Calne 1979; Stoof and Kebabian 1981). D1-like receptors, by stimulating cAMP production, are excitatory, whereas activation of D2-like receptors is inhibitory. There are two isoforms of the D2R, a short form found presynaptically, and a long form found postsynaptically(Usiello, Baik et al. 2000). In fact, D2R is the main presynaptic autoreceptor of the dopaminergic system (Mercuri, Saiardi et al. 1997). D2Rs are expressed throughout DA regions of the brain. D3Rs, another member of the D2-like receptor family, also inhibit cAMP production. They are found postsynaptically, and have a higher density in limbic areas of the brain, such as the nucleus accumbens (Bouthenet, Souil et al. 1991). The diversity of DRs expressed at a given synapse help to define the response elicited when DA is released. Furthermore, this response is not only dependent on the receptor

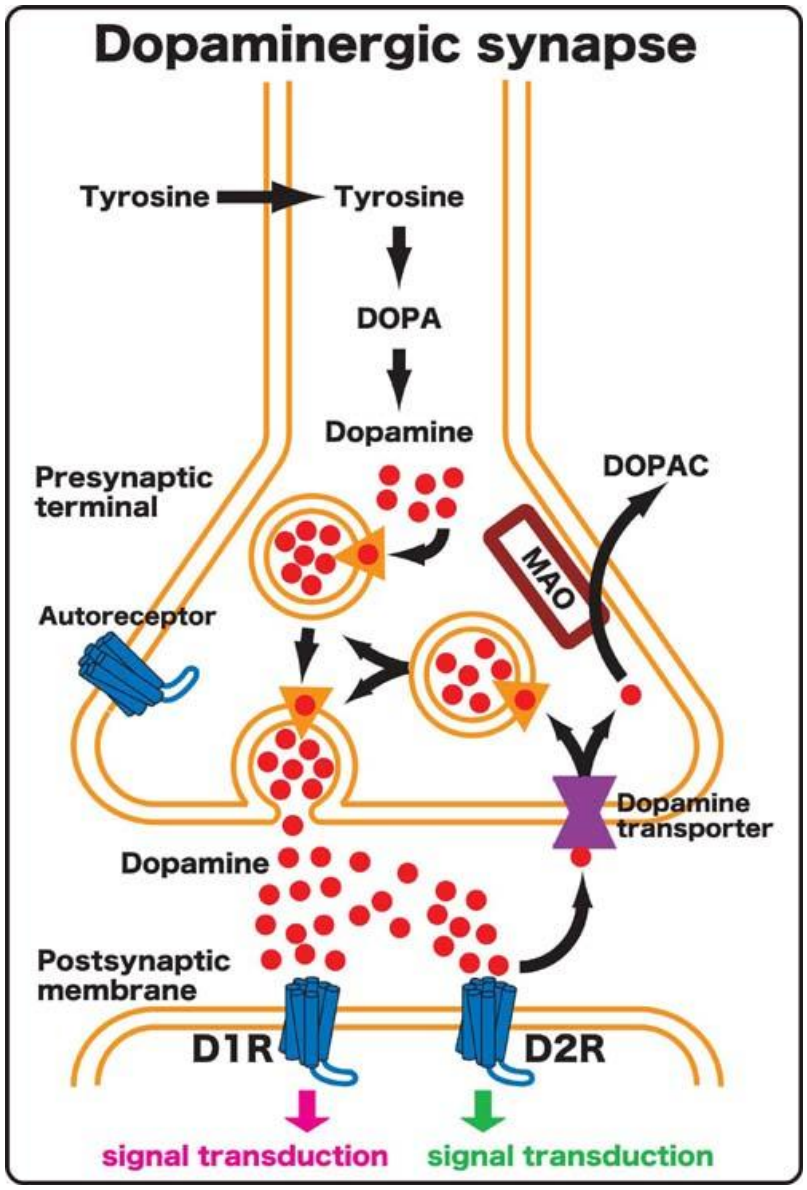


Figure 3. Diagram of a dopaminergic synapse. Enlarged view of a typical dopaminergic synapse. The presynaptic terminal is located at the top and the postsynaptic neuron is on the bottom. DOPA: 3,4-dihydroxyphenylalanine, DOPAC: 3,4-dihydroxyphenylacetic acid, D1R: type 1 dopamine receptor, D2R: type 2 dopamine receptor, MAO: monoamine oxidase. The dopamine transporter is shown on the presynaptic terminal in purple. The D2R subtype represents the main presynaptic autoreceptor of the dopaminergic system. The source for the image is http://www.nibb.ac.jp/annual_report/2004/img/240-01.jpg

type(s) at the synapse, but also the number of receptors present, lending another element to regulation of DA neurotransmission (Figure 3).

Termination of DA neurotransmission is another important component for maintaining proper dopaminergic tone. DA is degraded enzymatically by monoamine oxidase B (MAOB) and catechol-o-methyl transferase (COMT) (Figure 4), but enzymatic degradation does not account for inactivation of DA in the synapse. Instead, termination of DA neurotransmission is regulated by DAT. DAT allows DA to be cleared out of the synapse and taken up into the presynaptic bouton (Giros and Caron 1993). The importance of this transport system was demonstrated by the creation of DAT knockout mice, where DA clearance is significantly slower than in wild type mice. DA remained in the synapse at least 100 times longer than the control animals (Giros, Jaber et al. 1996; Gainetdinov, Jones et al. 1999), leading these animals to be hyperactive. In addition to its main role, DAT also provides a supply of DA for repackaging into vesicles for future release. This DAT-mediated recycling is the main source of DA for vesicular release in the neuron, thus decreasing the amount of synthesis needed to replenish vesicular stores of DA (Giros, Jaber et al. 1996). Indeed, when stimulated, the DA neurons of DAT knockout mice release significantly less DA than control animals (Giros, Jaber et al. 1996; Gainetdinov, Jones et al. 1999). It is thought that in DAT knockouts, there is a lack of DA available for packaging into vesicles, and consequently a reduction in the amount of DA available for vesicular release.

Furthermore, D2R expression and activity are reduced in mice lacking the

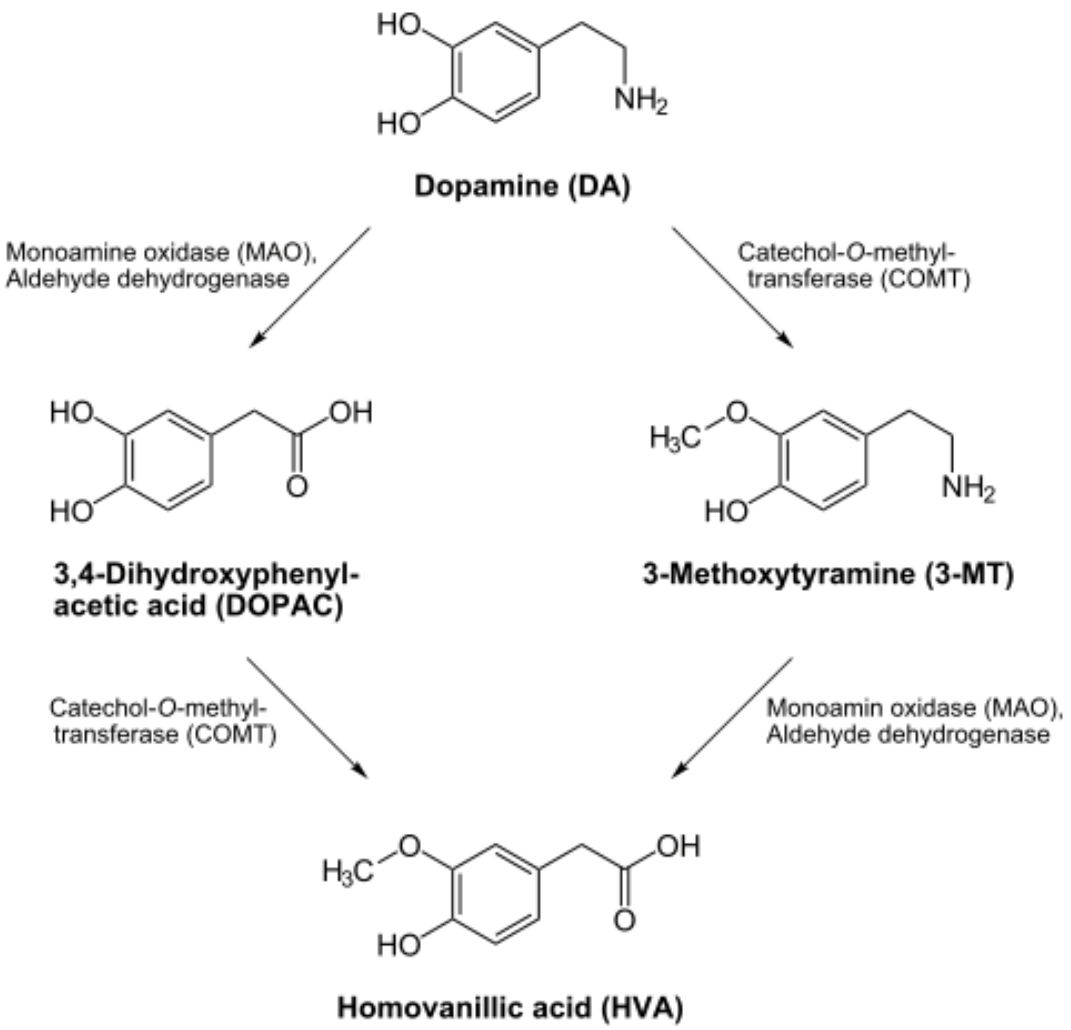


Figure 4. Enzymatic degradation of dopamine. Metabolic enzymes, COMT and MAO, that degrade unpackaged DA in synaptic areas.

DAT (Jones, Gainetdinov et al. 1999). These changes demonstrate that while DAT plays an important role in directly regulating DA signaling, it also influences several components of the dopaminergic synapse. Therefore changes in the activity and functioning of DAT can markedly disrupt DA neurotransmission.

Due to the important role of DAT in DA homeostasis, loss of proper function and regulation of the transporter has been implicated in several DA-related diseases. Decreased striatal DAT binding has been reported in first-episode schizophrenic patients (Mateos, Lomena et al. 2005), clinical depression (Laasonen-Balk, Kuikka et al. 1999), and obese individuals (Chen et al. 2008). Differences in the genomic variable number tandem repeat have been identified as risk factors for bipolar disorder (Greenwood, Alexander et al. 2001). Furthermore, genetic polymorphisms in the DAT coding region have been also associated with ADHD (Mazei-Robison, Couch et al. 2005; Yang, Chan et al. 2007; Binda, Dipace et al. 2008), as well as eating disorders (Shen, Hagino et al. 2004). DAT is also well-known for its role in addiction, including substance abuse of psychostimulants such as amphetamine (AMPH) (Giros, Jaber et al. 1996), which act on the transporter to elicit their behavioral effects. Due to its role in several neurological disorders and addiction, many researchers have focused on DAT structure, function, and regulation.

The Dopamine Transporter Structure and Function

The presence of a transport mechanism for biogenic amines was initially reported in the 1960s by Julius Axelrod. In 1961, Hertting and Axelrod showed

that that NE could be accumulated in nerve endings, and released upon stimulation (Axelrod, Whitby et al. 1961). Further characterization of catecholamine uptake regions in the brain revealed that both NE and DA could be accumulated by distinct regions of the brain, and that this accumulation could be inhibited by co-application of either tricyclic antidepressants or drugs of abuse, including cocaine and amphetamine (AMPH) (Glowinski and Axelrod 1964; Ross and Renyi 1967).

DAT is a member of the Na^+/Cl^--dependent neurotransmitter transporter family (SLC6) that contains high affinity transporters for NE, serotonin (5-HT), γ-aminobutyric acid (GABA), glutamate, and glycine (Kilty and Amara 1992; Torres, Gainetdinov et al. 2003). In 1991, DAT was cloned (Giros, el Mestikawy et al. 1991; Kilty, Lorang et al. 1991; Shimada, Kitayama et al. 1991; Usdin, Mezey et al. 1991), and analysis of the human DAT (hDAT) primary sequences revealed that the DAT cDNA encodes a protein of 620 amino acids. Hydropathy analysis predicts the presence of twelve transmembrane domains (TMDs) with intracellular amino and carboxyl termini (Figure 5). DAT is closely related to other catecholamine transporters, namely the NE transporter (NET), with which it shares 66% sequence identity (Blakely, Defelice et al. 2005). The structure of DAT also supports the notion that it is regulated by several signaling molecules. The termini of the transporter contain several serine, threonine, and tyrosine residues, allowing for regulation *via* phosphorylation. In fact, some of these residues are found in the consensus sequences for kinases

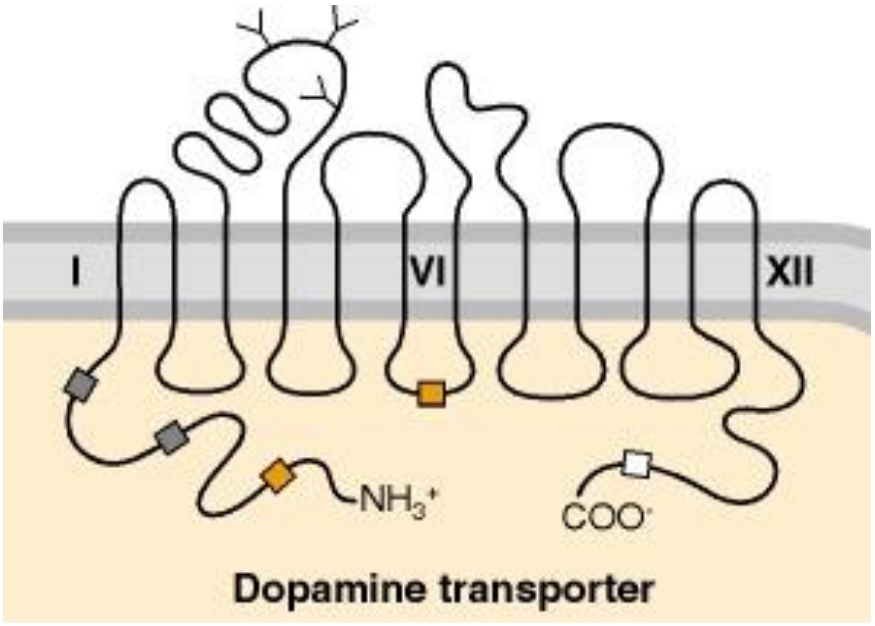


Figure 5. Illustration of the dopamine transporter. The human dopamine transporter is an integral membrane protein that contains twelve transmembrane domains (TMDs) with intracellular N- and C-termini, a large extracellular loop between TMDs 3 and 4 with three N-linked glycosylation sites, and multiple phosphorylation sites located on intracellular termini and loops. Putative glycosylation sites are indicated with *Y-shaped symbols* on extracellular sequences. Possible phosphorylation sites are indicated with boxes for various protein kinases: gray boxes, protein kinase A; orange boxes, protein kinase C; white boxes, calcium-calmodulin protein kinase. This image was obtained from (Siegal 1999).

such as protein kinase C (PKC), protein kinase A (PKA), and calcium/calmodulin-dependent kinase II (CaMKII) (Giros and Caron 1993). Additionally, there are three glycosylation sites in the large second extracellular loop (ECL) between TMDs 3 and 4.

Chimeric studies conducted on DAT and NET in heterologous expression systems initially defined DAT function as it related to structure. These studies led to the hypothesis that TMDs 1-3 and 9-12 were important for the affinity of substrates and sodium/chloride dependence, where TMDs 4-8 were involved in transporting the substrate and inhibitor binding (Buck and Amara 1994; Giros, Wang et al. 1994; Syringas, Janin et al. 2000). Mutagenesis studies have also helped to reveal the structure/function relationship of DAT. For example, mutation of glycosylation sites on ECL 2 results in a transporter that is expressed at the plasma membrane, but has a reduction in DA uptake, as well as an increase in sensitivity to inhibitors (Li, Chen et al. 2004). This predicts that glycosylation on ECL2 is important to the transport process.

A high resolution crystal structure of the leucine transporter (LeuT), a prokaryotic sodium-dependent transporter with approximately 25% homology to DAT and related neurotransmitter transporters, is reported (Yamashita, Singh et al. 2005). The LeuT is a fellow member of the SLC6 gene family and contains all 12 TMDs. Therefore, its structure has served as a point of reference for the structure/function relationship of DAT, allowing for the initial hypotheses of DAT topology to be confirmed (Yamashita, Singh et al. 2005). In fact, a recent study used LeuT as a reference for determination of the binding sites of cocaine and

DA in DAT. Beuming and collegues developed models of DAT based on the LeuT structure to predict a binding site for cocaine and dopamine between TMD 1, TMD 3, TMD 6, and TMD 8 (Beuming, Kniazeff et al. 2008) They then confirmed these predictions experimentally using site directed mutagenesis and chemical cross-linking methods (Beuming, Kniazeff et al. 2008). This study, which was possible only with the hi-resolution LeuT structure, demonstrates that cocaine utilizes the same binding site as DA. This work, which was aided with the LeuT structure, is important in helping develop therapies for cocaine abuse and addiction.

These structural studies have helped to further establish to a model of transporter action that was first proposed in 1966, known as the *alternating access model* (Jardetzky 1966), where external and internal gates alternately allow access to the transporter channel (Figure 6). First the gate to the extracellular space opens, allowing in the substrate and cotransported ions. This gate then closes, allowing the gate facing the intracellular space to open and release its cargo, thus "alternating" the access of the transporter channel between the outside and inside of the cell. This event is powered by the electrochemical gradient generated by the plasma membrane Na^+/K^+-ATPase (Torres, Gainetdinov et al. 2003). DA uptake by DAT, therefore, is reliant on both sodium and chloride, where two sodium ions and one chloride ion are co-transported with each DA molecule. It is important to note that stoichiometry

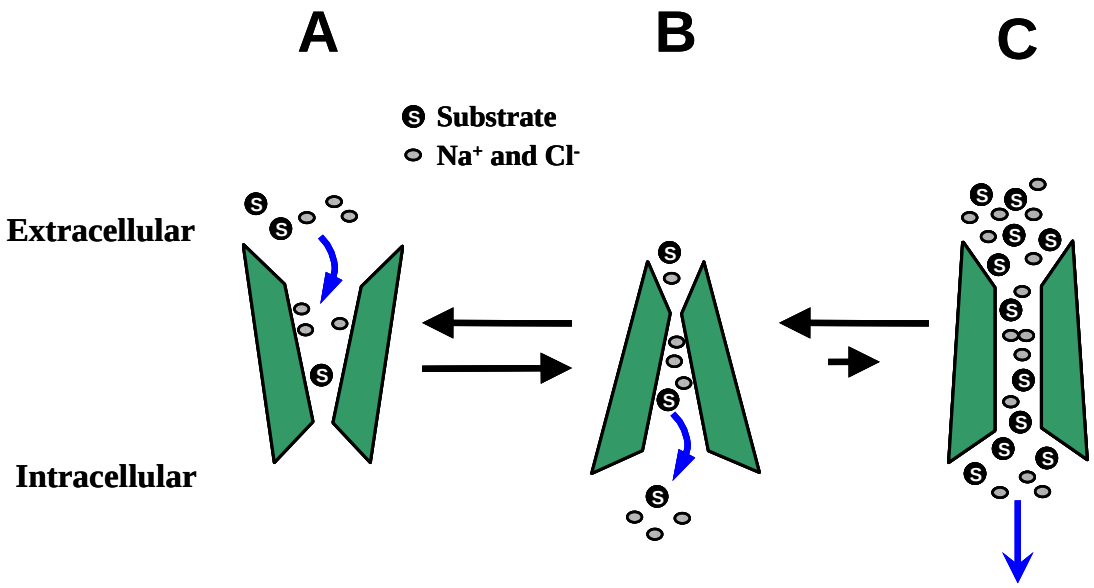


Figure 6. Dopamine transporter uptake action. The model for how DA uptake occurs is *via* the alternating access model. (A) The extracellular facing conformation of the transporter binds substrate and driving ions located in the extracellular space. (B) Following a conformational change, the now intracellular facing conformation of the transporter releases the cargo into the cytosol. The transporter is also thought to act as a channel, as demonstrated in (C). Courtesy of Dr. Kris Kahlig, Vanderbilt University.

predicts that this transport process should be electrogenic, with a net translocation of two positive charges per transport cycle. Due to the flux in electrical charge, this phenomenon can be measured by electrophysiology in a single cell expressing DAT, providing a useful tool in studying the transporter (Sonders, Zhu et al. 1997; Sitte, Huck et al. 1998; Khoshbouei, Wang et al. 2003; Pifl, Rebernik et al. 2004). Interestingly, these studies have found that the inward current is larger than the current predicted by the charge-to-flux ratio of the proposed stoichiometry (Lester, Cao et al. 1996; Sonders and Amara 1996). This suggests an uncoupled conductance. Therefore, the alternating access model cannot fully account for transporter actions.

Interestingly, some recordings taken of DAT currents have exhibited a large flux of DA crossing the membrane for a very brief amount of time, providing support for an alternative mode of action (Galli, Blakely et al. 1996; Galli, Blakely et al. 1998; Kahlig, Binda et al. 2005). This has led to one hypothesis to account for the uncoupled conductance of DAT; that it can act, albeit very briefly, as a channel. This channel-like mode is similar to the open conformation of a ligand-gated ion channel, consisting of large fluxes of substrate molecules and ions crossing the membrane (Figure 6). Due to the large number of molecules that move across the membrane very quickly, this state is of interest, even if it only occurs infrequently. Other functions have also been discovered by studying the effect of psychostimulants on the transporter. AMPH, for example, is shown to promote a channel-like state of DAT that contributes to DA efflux (Kahlig, Binda et al.

2005). The ability of DAT to function in several modes demonstrates the dynamic nature of this protein.

Psychostimulants and the Dopamine Transporter

DA has a well established role in pleasure and reward. It is not surprising then that this system is the target of several psychostimulant drugs, many of which elicit their effects through DAT. These drugs are mainly viewed as abuse liabilities, they produce rewarding effects, alter DA tone, and lead to drug addiction. DAT is a target for these drugs, which include cocaine, methamphetamine, and AMPH (Kuhar, Ritz et al. 1991; Zaczek, Culp et al. 1991; Giros and Caron 1993). They act to disrupt DAT's ability to properly function, causing increases in extracellular DA that stimulate postsynaptic receptors, thereby enhancing neurotransmission. These drugs can broadly be characterized through their mode of action. For example, one mechanism is to block DAT directly. This is the mode used by the drug cocaine. By directly blocking uptake of DA, cocaine allows for an increase in synaptic levels of DA and enhanced DA neurotransmission (Ross and Renyi 1967; Giros, el Mestikawy et al. 1991). Several lines of evidence have also demonstrated that cocaine alters the surface expression of DAT. Repeated cocaine administration was shown to increase DA uptake in rats (Ng, Hubert et al. 1991; Parsons, Schad et al. 1993), and acute exposure to 10 µM cocaine for 30 minutes increased DAT transport activity and DAT cell surface expression (Daws, Callaghan et al. 2002). Studies have also examined the impact of chronic cocaine treatment on DAT by looking at postmortem

tissue from cocaine addicts. Striatal synaptosomes obtained from such tissue display a significant increase in both the B_{max} of binding for the cocaine analog, WIN 35428, as well as the V_{max} for DA uptake (Mash, Pablo et al. 2002). Furthermore, reports have shown increased binding of WIN 35428 to DAT in postmortem brains of addicts (Little, Kirkman et al. 1993; Staley, Hearn et al. 1994; Little, Zhang et al. 1999). These studies indicate a functional upregulation of DAT following chronic cocaine abuse.

Another important method of action by psychostimulant drugs is to cause effux of DA *via* DAT into the synapse. These drugs act as a substrate of DAT, competing with DA for uptake. AMPH is perhaps the most well-known drug to utilize this mechanism. Once inside the cell, AMPH acts to reverse the conformation of the transporter, causing efflux of DA into the synapse (Fischer and Cho 1979; Pierce and Kalivas 1997). This allows for the drug's effects by acting as an indirect agonist of DRs, thus stimulating the postsynaptic cell and increasing DA neurotransmission.

Increases in DA signaling by either blocking DAT or by DAT-mediated DA efflux in limbic areas is thought to mediate the rewarding properties of psychostimulant drugs (Koob and Bloom 1988). Furthermore, AMPH-evoked DA efflux reveals another functional aspect of DAT beyond uptake of DA. Modifications to DAT function can be examined by changes to AMPH-induced DA efflux. For example, in order for AMPH to elicit its effects, DAT must be on the cell surface. Therefore alterations in the surface level of DAT will also change the amount of AMPH-induced DA efflux, which allows for another method

to examine alterations in DAT function. As such, beyond its effects as a psychostimulant drug, AMPH can be a useful tool to examine changes to DAT function.

AMPH effects include several behavioral changes, such as restlessness, reduced appetite, and hyperlocomotion. These drug actions are believed to occur due to AMPH-induced increase in extracellular DA. Consistent with this notion, DAT knockout mice do not show an increase in locomotion when administered AMPH (Giros, Jaber et al. 1996). These findings demonstrate that DAT plays a crucial role in the hyperlocomotor effects of AMPH, and indicate that alterations in DAT function or expression can affect the ability of AMPH to elicit its behavioral effects.

In addition to causing efflux and inducing behavioral changes such as hyperlocomotion, AMPH alters the surface expression of DAT, a phenomenon that has been shown by several groups (Fleckenstein, Haughey et al. 1999; Saunders, Ferrer et al. 2000; Carvelli, Moron et al. 2002; Gulley, Doolen et al. 2002; Kahlig, Javitch et al. 2004; Garcia, Wei et al. 2005; Johnson, Furman et al. 2005; Boudanova, Navaroli et al. 2008). Application of AMPH to DAT transfected cells for one hour reduced the rate of DA uptake and also decreased the surface expression of DAT as measured by biotinylation (Saunders, Ferrer et al. 2000). Similar results were obtained for the endogenous substrate, DA, as well (Saunders, Ferrer et al. 2000). Furthermore, measurements of a single transporter current after AMPH application demonstrated that the current itself was unaltered, confirming that changes to DA uptake were due to a redistribution of DAT away from the plasma

membrane (Kahlig, Javitch et al. 2004). Recently, live cell imaging was used to examine changes to DAT surface expression after rapid treatment (up to one minute) of AMPH in neuroblastoma N2A cells expressing DAT. These studies indicated that intially, AMPH acts to increase the expression of DAT on the cell surface, as does the endogenous substrate DA (Furman, Chen et al. 2009). Prolonged exposure, however, led to a decrease of DAT cell surface expression. Importantly, these results demonstrate that endogenous, as well as exogenous, substrates of DAT regulate its expression on the plasma membrane in a biphasic manner.

Due to the changes that occur to DA systems with chronic use, these drugs are often thought of in a negative manner and as an abuse liability. However, at times they have proved useful. AMPH is used frequently to treat ADHD, under the tradename Adderall. Also, the unique properties and functions they convey on DAT and DA transmission provide useful tools for studying the transporter. Still, most exogenous compounds that act on the transporter are, in fact, addictive and can have many negative consequences with frequent use.

DAT Regulation by Interacting Proteins

Being that DAT has an important role in DA neurotransmission, it is not surprising that it is tightly regulated. Several proteins that regulate DAT also physically interact with it, forming protein complexes, and much work continues to further define these. To date, some identified DAT interacting proteins are, but not limited to, protein interacting with C kinase (PICK1) (Torres, Yao et al. 2001),

Hic5 (Carneiro, Ingram et al. 2002), the catalytic subunit of protein phosphatase 2A (PP2A) (Bauman, Apparsundaram et al. 2000), the PKC isoforms β_1 and β_2 (Johnson, Guptaroy et al. 2005), the receptor for activated C-kinases (RACK1) (Lee, Kim et al. 2004), SYN-1a (Lee, Kim et al. 2004; Binda, Dipace et al. 2008), CaMKII (Fog, Khoshbouei et al. 2006; Wei, Williams et al. 2006), and D2R (Lee, Pei et al. 2007; Binda, Dipace et al. 2008).

PICK1 was found to interact with DAT via the PDZ recognition motif found on the C-terminal tail on the transporter. PKC, a well known modulator of DAT surface expression, also interacts with PICK1, leading to the possibility that PICK1 serves at an adaptor protein for these two molecules (Torres, Yao et al. 2001). Interestingly, RACK1 have been shown to interact with the DAT's N-terminus by a yeast-two hybrid assay (Lee, Kim et al. 2004). RACK1 also interacts with PKC, as well as several other kinases, and therefore may serve to facilitate kinase regulation of DAT by PKC and other kinases on the N-terminus (Ron and Mochly-Rosen 1994; Rodriguez, Ron et al. 1999). Therefore, both of these interacting proteins, PICK1 and RACK1, may help to facilitate PKC modulation of DAT at two distinct regions on the transporter. This indicates that PKC may modulate DAT in several ways, depending on the area of DAT available for binding and the interacting protein available to it.

SYN-1a is another protein identified to interact with DAT (Lee, Kim et al. 2004). This is of interest due to work on SYN-1a and DAT family members, including the GABA transporter, GAT1, and NET. SYN-1a interacts with the N-terminus of GAT1 and modulates the translocation of the transporter (Wang, Deken

et al. 2003), and is also shown to interact with NET (Sung, Apparsundaram et al. 2003), suggesting that it might serve a similar function in regulation of DAT. Recent studies have shown an increase in association between SYN-1a and DAT with AMPH treatment (Binda, Dipace et al. 2008), allowing for a possible role for SYN-1a in AMPH-mediated DAT trafficking.

In addition to the proteins mentioned above, recent work has also identified D2R to associate with DAT. Disruption of this interaction has been shown to decrease DA uptake (Lee, Pei et al. 2007). The short isoform of D2R functions as an inhibitory autoreceptor on the presynaptic cell, with a localization similar to DAT (Centonze, Usiello et al. 2002). Previous work has demonstrated that D2R stimulation may be regulating DAT through downstream activation of second messenger signaling cascades (Mayfield and Zahniser 2001). For example, Bolan and colleagues demonstrated that D2R stimulation causes enhanced substrate clearance through an increase in DAT cell surface expression, which was dependent upon extracellular signal-regulated kinases 1 and 2 (ERK 1/2), but independent of phosphatidylinositol 3-kinase (PI3K) (Bolan, Kivell et al. 2007). Lee and colleagues reported the first evidence of a direct association between DAT and D2R, and that disruption of this interaction affects DAT function (Lee, Pei et al. 2007). These studies suggest that there is a definite interplay, direct and indirect, between presynaptic D2Rs and DAT to help maintain DA homeostasis.

It is also quite interesting to note that DAT is thought to interact with itself. Cross linking and mutagenesis studies, as well as studies using fluorescence

resonance energy transfer (FRET) imaging, demonstrated that DAT forms an oligomer (Hastrup, Karlin et al. 2001; Hastrup, Sen et al. 2003; Sorkina, Doolen et al. 2003; Torres, Carneiro et al. 2003). Recently, the substrates DA and AMPH have been shown to reduce DAT oligomerization (Chen and Reith 2008), lending yet another opportunity for regulation of DAT function. Further work is needed to examine how second messager systems may also play a role in altering DA uptake by changing the ability of DAT to form oligomers.

Regulation of Dopamine Transporter Surface Expression

DA uptake capacity depends on the turnover rate of an individual transporter as well as on the number of functional transporters expressed at the plasma membrane. DAT function is dependent upon expression on the plasma membrane, and as such the surface expression of the transporter is a tightly regulated mechanism for controlling DA neurotransmission. Several signaling molecules have been identified as regulators of DAT cell surface expression, including PKC, PKA, ERK1/2, and members of the insulin signaling family (i.e. phosphoinositol 3 kinase (PI3K) and protein kinase B (PKB, or Akt)) (Batchelor and Schenk 1998; Carvelli, Moron et al. 2002; Page, Barc-Pain et al. 2004; Garcia, Wei et al. 2005; Bolan, Kivell et al. 2007).

Possibly the most well characterized of these pathways is PKC modulation of DAT. PKC activation by phorbol esters has been shown to cause trafficking of the transporter away from the plasma membrane, resulting in reduced DA uptake (Huff et al. 1997; Vaughan et al. 1997; Zhang et al. 1997; Zhu et al. 1997; Pristupa et

al. 1998; Daniels and Amara 1999; Melikian and Buckley 1999; Granas et al. 2003; Loder and Melikian 2003; Kahlig et al. 2004; Foster et al. 2008). This PKC-induced trafficking of DAT from the plasma membrane to intracellular compartments is a clathrin-mediated and dynamin-dependent endocytic mechanism (Daniels and Amara 1999; Sorkina, Hoover et al. 2005; Foster, Adkins et al. 2008). Beyond this work, PKC has been also shown to modulate the functionality of DAT by shifting it to a state that is more likely to result in DA efflux (Kantor and Gnegy 1998; Kantor, Hewlett et al. 2001; Johnson, Guptaroy et al. 2005). This work displays another aspect of the role of PKC in altering DAT function by supporting the reversal of DA transport.

Although it was originally believed that phosphorylation of DAT by PKC was required for internalization (Huff, Vaughan et al. 1997; Vaughan, Huff et al. 1997; Cowell, Kantor et al. 2000; Chang, Lee et al. 2001; Foster, Pananusorn et al. 2002; Granas, Ferrer et al. 2003; Foster, Adkins et al. 2008), recent lines of evidence have shown that this is not the case. Truncation of the PKC consensus sequence in the DAT N-terminus abolishes PKC-induced phosphorylation of DAT, yet this form of the transporter is still able to traffic in response to phorbol ester treatment (Granas, Ferrer et al. 2003). These data suggest that PKC regulation of DAT may involve separate phosphorylation and trafficking components. In fact, recent work by Foster and collegues demonstrated that, with PKC activation, the loss of surface DAT occurred only in concanavalin A-sensitive, non-raft membranes (Foster, Adkins et al. 2008). However, treatment with methyl-β-cyclodextrin, which destabilizes lipid rafts by depleting cholesterol, inhibited PKC-

induced downregulation of DAT activity but still allowed for internalization of DAT (Foster, Adkins et al. 2008). In addition, DAT phosphorylation was found to be at a significantly greater level in cholesterol-rich lipid raft microdomains. These findings suggest that regulation by PKC in the non-raft DAT population occurs through trafficking-dependent processes. Conversely, in lipid rafts DAT regulation by PKC is achieved through trafficking-independent processes (Foster, Adkins et al. 2008). Importantly, this study suggests that the localization of DAT is important in determining how it is regulated. Further work is needed to look at the importance of DAT localization and how regulation of these distinct populations affects the transporter.

Notably, recent work has revealed another interesting aspect of regulation of DAT trafficking. Furman and collegues explored the changes to DAT cell surface expression over time after exposure to a substrate, either DA or AMPH, using live cell confocal imaging and biotinylation assays in neuroblastoma N2A cells transfected with DAT. These investigations defined a biphasic regulation of DAT cell surface expression in response to substrate binding. Initially, within seconds, there is a rapid increase of DAT on the cell surface, beginning at 10 seconds and going to 2 minutes. This is followed by a decrease of cell surface DAT upon continued exposure to the substrate (Furman, Chen et al. 2009). Importantly, further work by this group also showed that inhibition of PKCβ blocked the initial rapid increase of cell surface DAT after exposure to DA or AMPH (Chen, Furman et al. 2009; Furman, Chen et al. 2009). Furthermore, PKCβ knockout mice were found to have a reduction in cell surface DAT

expression compared to wild type mice, while there was no change to the total levels of DAT protein between the two genotypes (Chen, Furman et al. 2009). In addition, the rapid increase in DAT cell surface expression upon substrate treatment seen in wild type mice was not observed in PKCβ knockout mice (Chen, Furman et al. 2009). Overall, these studies indicate that there are two phases of DAT trafficking in response to substrate binding, and that the initial rapid phase is dependent upon PKCβ. This work further displays the complexity to the regulation of DAT function *via* transporter trafficking.

PI3K signaling in regulating DAT Surface Expression. Much work has shown that DAT activity is modulated by multiple signal transduction pathways, and often this regulation involves modifying DAT trafficking and expression at the plasma membrane. The PI3K signaling pathway has been extensively studied for its role in modulating the surface expression of DAT. PI3K phosphorylates the D-3 position of phosphoinositol-2 phosphate ($PI(4,5)P_2$)to yield phosphoinositol-3 phosphate ($PI(3,4,5)P_3$). The generation of $PI(3,4,5)P_3$ at the plasma membrane upon the activation of PI3K allows for translocation of Akt (Taha and Klip 1999; Bondy and Cheng 2004). This localization of Akt to the plasma membrane is critical for its activation (Figure 7). Once bound to $PI(3,4,5)P_3$, Akt is phosphoylated by phosphoinositide dependent kinase 1 (PDK1), and may go on to signal to several downstream effectors (Yang, Tschopp et al. 2004).

In 2002, work by Carvelli and collegues showed that in hDAT expressing heterologous cells, inhibition of PI3K by LY294002 led to a decrease in surface

expression of hDAT. The investigators also reported a decrease in DA uptake with LY294002 treatment, not only in cells but also in rat striatal

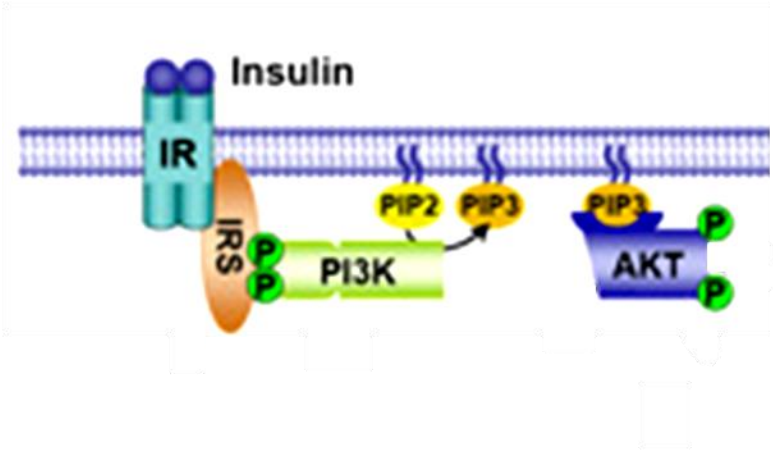


Figure 7. Activation of Akt by Insulin. Upon activation of the insulin receptor (IR), insulin receptor substrate (IRS) acivates phosphoinositol-3-kinase (PI3K). PI3K goes on to phosphorylate phosphoinositol-2 phosphate (PIP_2), converting it to phosphoinositol-3 phosphate (PIP_3) and allowing translocation of Akt to the plasma membrane, leading to its subsequent activation. Akt is now an active kinase and can phosphorylate downstream targets. This figure was obtained from http://www.hsph.harvard.edu/faculty/brendan-manning/images/Insulin_Signaling.jpg

synaptosomes. These effects are dynamin dependent as DA uptake and DAT cell surface expression after LY294002 treatment was inhibited by expression of a dominant negative mutant of dynamin I.

PI3K is activated by receptor tyrosine kinase stimulation. Importantly, RTK inhibitors have been shown to downregulate DAT activity and plasma membrane expression (Zahniser and Doolen 2001), thus fitting the model that PI3K activation through RTKs causes an increase in surface expression of DAT. Carvelli and coworkers also demonstrated that incubation with a RTK activator, insulin, showed increases in DAT transport capacity and surface levels (Carvelli, Moron et al. 2002).

As mentioned earlier, further downstream effects of PI3K signaling include the activation of Akt (Taha and Klip 1999). Akt is an important component in insulin and growth factor signaling pathways, and is thought to regulate several cellular functions including cell growth, glucose uptake and metabolism, as well as apoptosis (Hanada, Feng et al. 2004). Three isoforms of Akt have been identified (Hanada, Feng et al. 2004), all of which contain a pleckstrin homology domain that interacts with $PI(3,4,5)P_3$. This interaction allows for phosphorylation of Akt at residues threonine-308 and serine-473, that are required for full Akt activation (Hanada, Feng et al. 2004). Akt1 and Akt2 are ubiquitously expressed throughout the body, but Akt3 is only found to be expressed in the brain and testes. Insights

into the specificity of these isoforms in Akt-related functions such as glucose homeostasis and cell growth, have been provided by genetic studies where each isoform, or combinations of them, have been genetically deleted. Akt1 null mice knocked out show impaired growth, but similar glucose metabolism and maintenance of insulin levels as their wild type counterparts (Cho, Thorvaldsen et al. 2001). The opposite is true, however, for Akt2 knockout mice. These animals are normal in size, but are insulin resistant and hyperglycemic (Cho, Mu et al. 2001). These studies suggest that Akt1 is important in proper growth factor and cell survival signaling, whereas Akt2 is critical in insulin signaling and glucose metabolism. Clues to the specificity of Akt3 were also discovered using knock out animals. Knockouts of Akt3 show a reduced brain size (Tschopp, Yang et al. 2005). Interestingly, dual knockouts of isoforms 2 and 3 have both reduced brain size and impaired glucose metabolism, but a knockout of Akt1/Akt3 is embryonic lethal (Dummler, Tschopp et al. 2006). These data suggest that Akt2 and Akt3 serve distinct functions, because a dual knockout is not lethal. However, with both Akt1 and Akt3 knocked out, the mice do not survive, suggesting that one of these isoforms is needed for proper development, and Akt2 cannot serve that function. Therefore, it is thought that Akt1 and Akt3 are critical for proper cell growth, particularly Akt3 in brain, whereas the primary role of Akt2 is maintaining insulin signaling and keeping glucose at an appropriate level in the blood.

Being that Akt is downstream of PI3K activation, it is not surprising that the role of Akt in regulating DAT has been explored (Garcia, Wei et al. 2005; Wei, Williams et al. 2006). These studies provided further evidence that PI3K signaling

controls DAT plasma membrane expression by demonstrating that basal Akt activity sustains DAT on the surface. Using both pharmacological means (ML9, an Akt inhibitor) and genetic manipulations (dominant negative Akt mutant), Garcia and collegues demonstrated in heterologous expressing cells that inhibiting active Akt results in a decrease of DAT on the surface. Akt can be altered to remain active as well through myristylation (myr-Akt), which keeps the protein anchored at the plasma membrane in an active state. Importantly, transfection of this constitutively active, myr-Akt increased the surface expression of DAT (Garcia, Wei et al. 2005; Wei, Williams et al. 2006). The physiological relevance of these results was emphasized by demonstrating that DA uptake, measured in heterologous expressing cells and striatal synaptosomes, was also impaired with ML9 treatment (Garcia, Wei et al. 2005; Wei, Williams et al. 2006). These data provide compelling evidence to support the hypothesis that basal PI3K/Akt signaling is important in regulating DAT function. Furthermore, this work implies insulin regulates DAT trafficking, consistent with hormonal regulation of dopaminergic signaling. The suggestion that insulin signaling can regulate DA neurotransmission *via* DAT is compelling given that insulin levels fluctuate with food consumption (Niswender, Morrison et al. 2003), and DA is an important neurotransmitter in regulation of reward and motivation for feeding.

Insulin and the Dopamine Transporter

DAT is an important component in regulation of DA signaling, and therefore it is of interest that several lines of evidence suggest that insulin can

regulate the expression and activity of DAT. For example, chronic intracranial administration of insulin enhanced DAT mRNA in the substantia nigra (Figlewicz, Szot et al. 1994). Moreover, hyperinsulinemic rats showed increased DAT mRNA in the substantia nigra (Figlewicz, Patterson et al. 1998). Food restriction, which causes low circulating levels of insulin, results in a 32% decrease in the V_{max} of DA uptake in the striatum of rats, whereas the K_m remains unaltered, pointing to a reduction in the surface expression of DAT (Zhen, Reith et al. 2006). Furthermore, food deprivation also reduces DA uptake in striatal synaptosomes (Patterson, Brot et al. 1998). Incubation of these synaptosomes with a physiological concentration of insulin restored DA uptake to control levels (Patterson, Brot et al. 1998), suggesting that changes in circulating insulin can modulate DAT activity. As discussed previously, important components of the insulin signaling pathway, PI3K and Akt, have been shown to regulate the transporter's surface expression and function. These studies also show that activating PI3K and Akt by stimulating heterologous cells expressing DAT with insulin causes an increase of the transporter on the cell surface, as well as an increase in DA uptake.

Perhaps the most telling studies thus far related to insuilin regulation of DAT examined the effects of streptozotocin (STZ)-induced insulin depletion on DAT surface expression and its function. STZ enters the pancreatic β-cell via the glucose transporter GLUT2 and causes DNA damage, resulting in free radical production and subsequent β-cell necrosis (Szkudelski 2001). Without β-cells, animals suffer from hypoinsulinemia and hyperglycemia (Carr 1996). Rats made

hypoinsulinemic by streptozotocin treatment show decreased DAT mRNA in the substantia nigra (Figlewicz, Brot et al. 1996; Patterson, Brot et al. 1998), as well as decreased DA clearance as measured by high speed chronoamperometry (HSCA) (Owens, Sevak et al. 2005). Furthermore, rats treated with STZ were found to have a reduction AMPH-induced efflux, a phenomen that requires DAT on the cell surface. Here the authors used functional magnetic resonance imaging (fMRI), a technique that displays an image that correlates to the oxygenation of the tissue, which is a representation of the activity in that brain region. Upon receiving I.P. injection of AMPH, the rats treated with STZ had significantly less activity in the striatum compared to the control animals (Williams, Owens et al. 2007). The data from the fMRI scans were confirmed by using high speed chronoamperometry (HSCA). This technique measures DA efflux *in vivo* by carbon fiber amperometry in the brain of anesthetized animals after microinjection of AMPH in the striatum. Using HSCA, the investigators found that STZ-treated rats had a reduction in AMPH-induced efflux. Importantly, the authors also showed STZ to reduce active Akt, linking insulin signaling and Akt to DAT cell surface expression and function.

Although these studies highlight insulin regulation of DAT, they do use pharmacological manipulations to alter insulin levels. Further work has begun to examine DAT and the effect of high fat diets, which result in insulin resistance in the brain (De Souza, Araujo et al. 2005; Posey, Clegg et al. 2009). One study found that, after 20 days on a high fat diet, DAT binding density in the ventral and dorsal striatum was reduced (South and Huang 2008). Furthermore, an inverse

relationship between Body Mass Index (BMI) and striatal DAT availability has been shown (Chen, Yang et al. 2008). These studies begin to point to changes in DAT with a high fat diet, but they do not provide a mechanism for such changes. However, these data are of interest for they demonstrate alterations in a component of DA signaling in obesity. Still, it is not understood whether this dysfunction is a cause, or a consequence of a high fat diet. Additionally, co-morbidity between obesity and several DA-related disorders points to a need for a better understanding of the interactions between insulin signaling and DA systems.

Dopamine and Feeding Behaviors

DA is important in modulating several behaviors, ranging from movement to cognition to motivation and pleasure, including our motivation to eat and the pleasure we receive from eating. The role DA has in feeding behavior has been demonstrated by studies that illustrate improper DA signaling in obesity. For example, upon eating a palatable meal, dopaminergic areas of the brain, such as the dorsal striatum, increase in activity (Stice et al., 2008). Interestingly, in subjects with a BMI in the obese range, this activity is dampened, suggesting a dysregulation of DA neurotransmission in obese individuals (Stice et al., 2008). In studies on obese rats on high fat diets for up to 16 weeks, striatal DA turnover is impaired (Davis, Tracy et al. 2008), and mRNA levels of DAT, D4R, D2R, and TH are reduced (Huang, Yu et al. 2005; Huang, Zavitsanou et al. 2006). In humans, D2R occupancy as measured by positron emission tomography (PET)

is reduced in a BMI-dependent manner (Wang, Volkow et al. 2001), and similar results for DAT were also shown (Chen, Yang et al. 2008). Therefore, it is becoming clear that DA systems are very important in our consumption of food, as they are altered in states of excessive food consumption. The exact mechanism of this phenomenon, however, is still being explored.

DA has several roles in our consumption of food, namely motivation for seeking food and food consumption (Volkow, Wang et al. 2002; Palmiter 2007), and the reward and satiety we feel when we eat (Volkow and Wise 2005). The latter of these has been studied extensively. The DA reward system originates in the VTA, with the projections ending in the ventral striatum, also known as the nucleus accumbens. Several inputs feed into this system, including canabanoid and opiate systems, as well as signaling molecules that are regulated by our food consumption; which include insulin and leptin (Palmiter 2007). DA is well known for the role it plays as the signaling molecule for our endogenous reward system. Activation of DRs in the reward system is thought to give us a pleasant feeling for several tasks, such as consuming food or having sex. Drugs are known to hijack this system, leading to highs when consuming the drug and cravings when not. In fact, comparisons between drug addiction and obesity have been made (Volkow and Wise 2005). In fMRI scans, drug-addicted subjects had a reduction of activity in the striatum, a dopamine rich area, upon receiving the drug compared to non-addicted subjects. With constant drug use, their DA systems were altered, as shown by a reduction in striatal activity. This is thought to be the biological basis for addiction, causing them to crave the drug more, and also

need more to create a similar “high”. Some groups have presented a similar hypothesis for food consumption and obesity. In fact, fMRI scans of obese subjects also show a reduction in activity in the striatum when consuming a highly palatable food compared to normal subjects (Stice, Spoor et al. 2008; Stice, Spoor et al. 2008). It is hypothesized that this disparity will override one’s metabolic signals for when food is actually needed.

As researchers continue to explore DA signaling in feeding behavior, this view becomes overly simplistic. One study examined the effects of DA on feeding by creating mice that are DA deficient in the brain (Sotak, Hnasko et al. 2005). This was accomplished by knocking out expression of TH, the enzyme needed to synthesize DA, in neurons. Interestingly, these DA-deficient mice would not eat and die shortly after birth, having no motivation to seek and consume food. To attempt to rescue the animal’s motivation to eat, the researchers rescued DA signaling by injection of a virus expressing TH in different DA regions in the brain to determine exactly which region was responsible for feeding behavior. Only restoration to the dorsal striatum caused the animals to eat and survive. This study highlights the dorsal striatum, and therefore the nigrostriatal system, in feeding behavior. The authors suggest that the dorsal striatum may be the basis for our motivation for feeding; defining our basic need for food. Without DA signaling in this region, the animals did not eat. Therefore, alterations to DA signaling in the dorsal striatum and nigrostriatal system may have implications for our basic need for consuming food, thus altering feeding behavior. Importantly, restoration of signaling to the ventral

striatum had no effect, leading to the hypothesis that the dorsal striatum is necessary for food intake, and the ventral striatum may act just to fine tune feeding behavior (Sotak, Hnasko et al. 2005; Palmiter 2008).

Further work has supported the hypothesis of the importance of the dorsal striatum in feeding behavior. A study conducted by Volkow and colleagues examined extracellular DA levels using PET imaging, specifically a ratio of B_{max}/K_d of D2R ligand raclopride, in hungry subjects. These subjects were presented with food they could see and smell, but not consume. They found, that the B_{max}/K_d ratio of raclopride was decreased in the dorsal striatum (Volkow, Wang et al. 2002). They concluded these changes to be from an increase in extracellular DA as the hungry subjects were presented with food, which bound to the D2Rs and decreased the D2Rs available for raclopride binding. Interestingly, they saw no change in D2R availability in the ventral striatum (Volkow, Wang et al. 2002), further pointing to the role of the dorsal striatum in desire and motivation for food.

These studies highlight the importance of DA systems, in particular the dorsal striatum, to feeding behaviors. Notably, the effects of high fat diets on DA systems, including the dorsal striatum, have also begun to be explored. Geiger and colleagues examined the effects of obesity from a “cafeteria style” diet in rats, consisting of access to several different highly palatable, highly caloric foods such as meats, cheeses, cookies, sweetened condensed milk, etc., on DA systems. After 15 weeks on this diet, the rats became obese (Geiger, Haburcak et al. 2009). They then measured the amount of DA released after electrical

stimulation ex vivo and found that the obese rats displayed a reduction in the evoked DA potentials in slices from the ventral striatum, as well as from the dorsal striatum (Geiger, Haburcak et al. 2009). This study suggests that obese subjects have a reduction in DA neurotransmission in both the ventral and dorsal striatum. Although it is still uncertain the exact role each system plays in feeding behavior, it is clear that both affect feeding behavior, and therefore both areas need to be explored in order to gain an understanding of the role of DA systems in obesity.

Insulin Regulation of Feeding Behavior

Insulin is an important metabolic signal in our control of food consumption. Insulin is produced by pancreatic β-cells, and is well-known for its control of blood glucose levels. Insulin has long been recognized as a major endocrine regulator of the uptake, cellular transport, and metabolism of small nutrient molecules such as amino acids, fatty acids, and glucose. Insulin's classical role in the peripheral system in maintaining blood glucose levels has been well characterized, and its involvement in the central nervous system was originally thought to be minimal. However, evidence shows that insulin contributes to energy homeostasis through input to the hypothalamus (Baskin, Figlewicz Lattemann et al. 1999; Niswender, Morrison et al. 2003; Niswender, Baskin et al. 2004). Furthermore, additional roles of insulin signaling in the CNS have also begun to emerge (Figlewicz, Evans et al. 2003; Sevak, Koek et al. 2006; Russo, Bolanos et al. 2007; Williams, Owens et al. 2007).

The detection of insulin and insulin-related molecules in the brain provides plausible support for a regulatory role in the CNS (Kar, Chabot et al. 1993). At first it was believed that insulin was impermeable to the blood-brain barrier (BBB), but evidence since has demonstrated otherwise. Insulin is shown to rapidly enter the cerebrospinal fluid (Woods, Porte et al. 1985; Wallum, Taborsky et al. 1987). In addition, animals with high levels of plasma insulin, such as genetically obese rats, are found to have high insulin levels in the cerebrospinal fluid (Stein, Dorsa et al. 1983), showing the blood plasma levels and cerebrospinal fluid levels move in parallel. It is thought that movement across the BBB *via* a transport system is responsible for the parallel shifts between the two components (Woods, Porte et al. 1985). Such a system has since been identified, allowing for the transfer of insulin from the blood plasma to brain microvessels in the CNS (Frank and Pardridge 1983; Pardridge, Eisenberg et al. 1985). Further work confirmed such transport by showing the transfer of insulin into the CNS using radiolabeled insulin injected into the blood that was later observed in the brain. This transfer occured *via* a saturable transporter (Banks, Jaspan et al. 1997; Banks, Jaspan et al. 1997). The transporter for insulin in the BBB is now well characterized and is found to be widely distributed throughout the central nervous system (Mateo, Budygin et al. 2004). Insulin crosses the BBB with varying permeability, allowing for regulation of brain insulin levels, as well as dysregulation in disease states. The transport system can be altered by a number of physiological and pathological events including fasting, obesity, and diabetes mellitus (Baskin, Stein et al. 1985; Banks, Jaspan et al. 1997; Banks

and Kastin 1998). The existence of such a system suggests that insulin serves a physiological role in the brain.

Insulin receptors (IRs) are abundant in CNS, including striatum and hypothalamus (Havrankova, Roth et al. 1978; Havrankova, Brownstein et al. 1981; Hill, Lesniak et al. 1986; Figlewicz, Evans et al. 2003), however CNS glucose utilization is not insulin dependent. Instead, insulin serves functions in the brain beyond regulating glucose homeostasis, showing other important functions for this signaling peptide, including regulation of food intake. This was first demonstrated in a primate study, where researchers administered insulin directly to the brain by a intracerebroventricular (i.c.v.) infusion and found that food intake was decreased (Woods, Lotter et al. 1979). Furthermore, using mice that lacked IRs in the CNS, Bruning and collegues confirmed the importance of insulin signaling in the brain. These mice were overweight, insulin-resistant, and glucose intolerant (Bruning, Gautam et al. 2000). Taken together, these studies indicate the importance of insulin signaling in the brain in regulation of food intake, and body weight.

Understanding insulin signaling in all areas of the brain that contribute to feeding behavior is important to help understand improper food intake and obesity. As discussed earlier, studies have demonstrated the importance of DA systems on feeding behavior, and IRs have been identified in the substantia nigra and striatum by anatomical studies using receptor autoradiography and receptor immunochemistry (Hill, Lesniak et al. 1986; Werther, Hogg et al. 1987; Unger, Livingston et al. 1991; Schulingkamp, Pagano et al. 2000). Extensive

mRNA coexpression of the DA precursor TH and IRs was observed in the substantia nigra (Figlewicz, Evans et al. 2003). With expression of IRs on DA neurons, it is not surprising that a role for insulin in regulating components of DA neurotransmission, including DAT, has begun to emerge.

Insulin, Diabetes, and DA-related Diseases

Several lines of evidence exist to link insulin to DAT and DA neurotransmission. Interestingly, dysfunction in these systems overlaps as well. Insulin resistance and type II diabetes have been linked to several disorders involving improper DA signaling, such as Parkinson's Disease (Sandyk 1993; Morris, Zhang et al. 2008), schizophrenia (Mukherjee, Decina et al. 1996), bipolar disorder (Fiedorowicz, Palagummi et al. 2008), and depression (Golden, Lazo et al. 2008). A recent study found that, among diabetic patients, there was a significant increase in the risk for Parkinson's Disease (Driver, Smith et al. 2008). Several studies have also shown a predisposition to the precursor to diabetes, metabolic syndrome, as well as diabetes itself, and obesity, in patients with bipolar disorder (Fiedorowicz, Palagummi et al. 2008; van Winkel, De Hert et al. 2008).

Perhaps the most well studied interplay of diabetes and DA-related disease is schizophrenia, a disorder that is well known to involve DA dysfunction (Kapur and Mamo 2003; Howes and Kapur 2009). Diabetes and insulin resistance are known to be prominent in schizophrenic patients, where 18-19% of patients have a family history of diabetes mellitus (Mukherjee, Schnur et al.

1989), compared to approximately 1.2-6.3% of the general population (Adams and Marano 1995). Studies have linked this increase to second generation, atypical antipsychotics, specifically clozapine and olanzapine, which have been shown to increase insulin resistance and improper glucose metabolism in a matter of a few months after beginning treatment (Newcomer 2001; van Winkel, De Hert et al. 2008). This is of interest considering these drugs target dopamine receptors. However, recent studies have demonstrated that use of these drugs does not fully explain the increase of diabetes in schizophrenic patients. Ryan and colleagues found in drug naïve patients that there was already insulin resistance and impaired glucose tolerance (Ryan, Collins et al. 2003). Furthermore, another group found that the prevalence of diabetes in the schizophrenic population was increased before the use of new, atypical antipsychotics (Dixon, Weiden et al. 2000). Lastly, Zhao and colleagues examined insulin signaling in the prefrontal cortex of postmortem tissue from schizophrenic patients. These subjects had been treated with first generation antipsychotics, not the second generation, atypical drugs thought that were previously hypothesized to be responsible for this correlation. They found a significant decrease in several aspects of insulin signaling, including insulin receptor phosphorylation and Akt phosphorylation (Zhao, Ksiezak-Reding et al. 2006), again suggesting improper insulin signaling in schizophrenic patients regardless of the drugs used to treat them. These studies certainly demonstrate a link between schizophrenia and diabetes, but the cause is still not clear.

Studies have not yet determined whether the dysfunction in insulin signaling seen in these patients is a cause, or a consequence, of schizophrenia.

Although the exact relationship between insulin resistance and diseases involving DA dysfunction are still not fully understood, these studies demonstrate an important link between the two systems.

Specific Aims

DA signaling influences a wide range of behaviors, including movement, motivation and cognition, and desire and reward. A growing body of literature points to DA regulation of feeding behavior *via* signaling in the dorsal striatum. DAT is an important component of DA neurotransmission, that functions to clear away extracellular DA and terminate transmission. Therefore, changes to DAT function have important implications in altering DA signaling and DA-mediated behaviors, such as feeding behavior.

DAT function is regulated by changes in transporter expression on the plasma membrane. The insulin signaling pathway, including PI3K and Akt, is known to alter DAT function by regulating the transporter's cell surface expression (Figure 8). Therefore, alterations in insulin signaling could lead to changes in DAT function and DA neurotransmission. Interestingly, dysregulated insulin signaling and diabetes is seen in patients with DA-related disorders. Furthermore, the occurrence of insulin resistance and obesity is increasing in our nation (Mokdad, Ford et al. 2003; Flegal, Carroll et al. 2010), yet the effects of this on DA systems and DA-related behaviors is not well understood. As a role

for insulin in regulating DA-related behaviors begins to be revealed, understanding on a molecular level how changes to insulin signaling alter DAT function is needed.

Therefore the aims of this dissertation are:

1) To further define components of the insulin signaling pathway that alter DAT cell surface expression.
2) Examine diet-induced molecular changes to insulin signaling and DAT function in the nigrostriatal pathway.

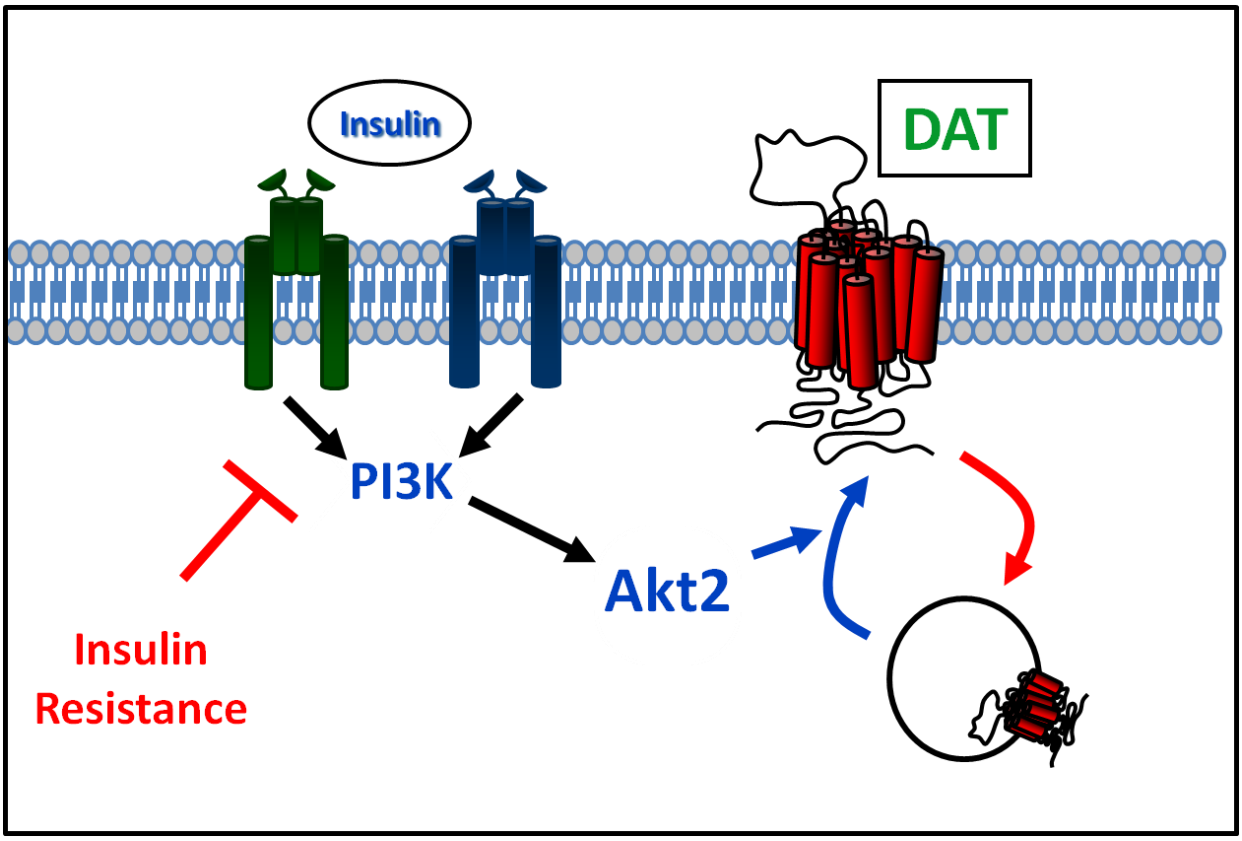


Figure 8. Schematic of insulin regulation of the dopamine transporter. Upon binding insulin to its receptor, the insulin signaling pathway, which consists of phosphoinositol-3-kinase (PI3K) and Akt, is activated. This acts to support the dopamine transporter (DAT) on the plasma membrane (blue). A high fat diet causes insulin resistance, inhibiting the insulin signaling pathway and therefore reducing DAT cell surface expression (red).

CHAPTER II

ISOFORM SPECIFIC REGULATION OF DAT CELL SURFACE EXPRESSION BY AKT2

Introduction

DA is a neurotransmitter that plays an important role in movement, motivation, and cognition. DA is also a key regulator of reward (Wise 1998). An essential element in fine tuning DA neurotransmission is the DA transporter (DAT) (Giros, el Mestikawy et al. 1992; Borowsky, Adham et al. 2001). DAT function is required to clear released DA by active reuptake into the presynaptic bouton (Giros, Jaber et al. 1996; Jones, Gainetdinov et al. 1998), thereby terminating DA signaling. Therefore, changes in DAT function have profound implications in DA homeostasis and signaling (Gelernter, Kranzler et al. 1994; Spencer, Biederman et al. 2005).

Both function and trafficking of the DAT are tightly regulated by several signaling pathways, including protein kinase C (PKC), mitogen activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and importantly, protein kinase B (Akt) (Gonzalez and Robinson 2004; Torres 2006). It is well documented that inhibition of PI3K decreases surface levels of DAT and reduces DAT function, including DA clearance measured *in vivo* (Carvelli, Moron et al. 2002; Owens, Sevak et al. 2005; Williams, Owens et al. 2007; Lute, Khoshbouei

et al. 2008). Similarly, inhibition of Akt results in a decrease in DAT surface expression and function (Garcia, Wei et al. 2005).

While it is known that Akt regulates DAT cell surface expression, it is unclear which Akt isoform is involved in this regulation. Importantly, different Akt isoforms appear to serve distinct functions in brain (Tschopp, Yang et al. 2005; Dummler, Tschopp et al. 2006). Akt exists as three isoforms; Akt1 and Akt2 are ubiquitously expressed, whereas Akt3 is found only in the brain and testes (Dummler, Tschopp et al. 2006). Knockout studies reveal that Akt1 is primarily associated with cell survival and growth (Cho, Thorvaldsen et al. 2001), and Akt3 appears to have similar functions in the brain (Tschopp, Yang et al. 2005). In contrast, Akt2 is associated with insulin modulation of glucose homeostasis, including regulation of glucose transporter (GLUT4) trafficking (Cho, Mu et al. 2001; Bae, Cho et al. 2003).

Many studies have linked dysfunctions in Akt signaling to the underlying mechanisms of disorders such as schizophrenia, which also involves dysregulation of DA signaling (Emamian, Hall et al. 2004; Schwab, Hoefgen et al. 2005; Bajestan, Sabouri et al. 2006). Therefore, defining the isoform of Akt that is responsible for altering DAT cell surface expression will further advance our knowledge of how aberrant Akt signaling leads to abnormal DA neurotransmission and may be clinically relevant to central dopaminergic disorders.

Methods

Cell surface protein biotinylation. Biotinylation experiments were performed on intact cells as described previously (Sung, Apparsundaram et al. 2003; Garcia, Wei et al. 2005; Dipace, Sung et al. 2007). Briefly, HEK-293 stably transfected with hDAT (hDATcells) were plated at a density of 1 x 10^6 per well in a six-well poly-(D-lysine) coated plate. Cells were washed with cold PBS containing Ca^{2+}/Mg^{2+} and treated for the indicated times. Then, cells were incubated with 1.0 mg/mL sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate [NHS-SS-biotin] (Pierce/Thermo Scientific, Rockford, IL) for 30 minutes, washed, quenched with 100 mM glycine, and extracted in lysis buffer (PBS Ca2+/Mg2+, 1% Triton 100-X, and 0.5 mM PMSF at 4°C). Lysates were centrifuged, total fractions reserved, and supernatants incubated with immobilized streptavidin beads (Pierce/ThermoScientific) for 1 hr at room temperature. Beads were washed three times in lysis buffer, and bound proteins eluted with 2X sample buffer containing 2-mercaptoethanol. Proteins were separated by SDS-PAGE and immunoblotted. For estimation of relative amounts of proteins, the exposed films of the immunoblots were scanned, and band intensities were measured with Scion Image (Scion Corporation, Frederick, MD).

Brain slice preparation. Methods are as described (Grueter and Winder 2005). All procedures were conducted in accordance with the Vanderbilt Institutional Animal Care and Use Committee. Briefly, Sprague Dawley rats (approx. 300 g) were decapitated. The brains were quickly removed and placed in an ice-cold, low-sodium/high-sucrose dissecting solution. Hemisected (300 µm)

coronal brain slices containing the striatum were prepared on a vibratome. Slices were allowed to recover in a submerged holding chamber (37°C) containing oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (aCSF) that contained the following (in mM): 124 NaCl, 4.4 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1 NaH_2PO_4, 10 glucose, and 26 $NaHCO_3$ for a recovery period of 60 min before beginning experiments. Slices were then incubated with Akt inhibitors: 12 µM (I-Akt1 and I-Akt2), 5 µM (I-Akt ½) in aCSF, or aCSF containing the vehicle DMSO for one hour at 37 degrees. Biotinylation assays were then performed.

Biotinylation assays. For slice assays, hemisected coronal slices (300 µm) were transferred to multiwell submerged chambers containing oxygenated aCSF with NHS-SS-Biotin (1 mg/ml) on ice at 4°C and incubated for 45 minutes, then washed twice for 10 min each in aCSF, and finally incubated in aCSF containing glycine (100mM) for two 20 min periods. Slices were then placed onto dishes on dry ice and the striatum was removed and placed into eppendorf tubes. Tissue punches of the striatum were homogenized in ice-cold homogenization buffer (1% Triton, 2 mM sodium orthovanadate, 2 mM sodium fluoride, 25 mM HEPES, 150 mM NaCl, 10 µg/ml aprotinin, and 10 µg/ml leupeptin, and 100 µM phenylmethylsulfonyl fluoride) and centrifuged for 30 min at 1000 x g at 4°C. Protein levels were obtained, and equal amounts were added to strepavidin beads with pulldown buffer (0.1 % Triton, 25 mM HEPES, 150 mM NaCl, 2 mM sodium orthovanadate, 2 mM sodium fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin, and 100 µM phenylmethylsulfonyl fluoride) and incubated overnight at 4°C. Samples were washed, eluted, and immunoblot analysis was carried out.

Total slice lysates and the biotinylated (slice surface) fraction underwent immunodetection for rDAT.

Immunoprecipitation. After treatment with either vehicle or each inhibitor as described, hDAT expressing HEK cells were washed with ice-cold PBS/Ca^{2+}/Mg^{2+} and incubated in 400 µl/well of lysis buffer containing 50 mM NaH_2PO_4, 10 mM Tris, 100 mM NaCl, 0.5 mM PMSF, pH 8.0, plus 1% Triton X-100 for 1 h at 4°C. Cell lysates recovered by centrifugation at 20,000*g* for 30 min were incubated overnight at 4°C either with Akt1 (1:250; Cell Signaling Technology; Danvers, MA) or Akt2 (1:800; Santa Cruz Biotechnology, Sana Cruz, CA) antibodies. Complexes were retrieved by the addition of 20 µl of protein G-Sepharose (GE Healthcare, Little Chalfont, Buckinghamshire, UK), washed three times with lysis buffer. Bound proteins were then eluted and processed for immunoblot analysis as described.

Immunoblotting. Determination of immunoreactivity was conducted according to previously described methods (Garcia, Wei et al. 2005; Williams, Owens et al. 2007). Briefly, tissue samples were separated by SDS-PAGE, and resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes (BioRad), which were incubated for 1 hr in blocking buffer (5% BSA and 0.1% Tween20 in Tris-buffered saline). The blots were incubated with primary antibody overnight at 4°C. Primary antibodies used for immunostaining were CamKII (1:2000; Affinity BioReagents, Rockford, IL), p-Akt (Thr308; 1:1000; Millipore, Billerica, MA) and hDAT (1:1000, Cell Signaling Technology, Danvers, MA). For rat DAT (rDAT) immunostaining, mouse monoclonal primary

antibodies were used (antibody 16, 1:1000; (Gaffaney and Vaughan 2004)). All proteins were detected using HRP-conjugated secondary antibodies (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA). After chemiluminescent visualization (ECL-Plus; Amersham; Piscataway, NJ) on Hyper-film ECL film (Amersham), protein band densities were quantified (Scion Image; Frederick, MD) and normalized to control.

Immunohistochemistry. For tissue staining, slices were prepared as described above and subsequently fixed with PBS Ca^{2+}/Mg^{2+},4% paraformaldehyde, washed three times with PBS, permeabilized and blocked with PBS 4% bovine serum albumin (BSA)/0.15% Tween-20, and immunostained with the appropriate antibody dissolved in PBS 4% BSA/0.05% Tween-20. Primary antibodies used for immunostaining were used: Akt2 (1:200; Santa Cruz Technology; Santa Cruz, CA) and rDAT (1:400; antibody 16, (Gaffaney and Vaughan 2004)) overnight at 4°C. After incubation with secondary fluorophores, immunofluorescence was imaged using a Perkin Elmer UltraView confocal with a Nikon Eclipse 2000-U microscope equipped with a 60X lens with an N.A. of 1.49. Image processing was performed using Image J and Adobe Photoshop.

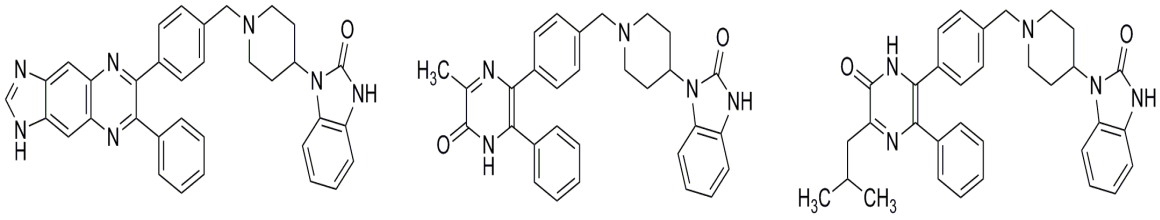
HSCA. HSCA was conducted using the FAST-12 system (Quanteon; http://www.quanteon.cc) as previously described with some modification (Owens et al., 2005; Williams et al., 2007). Recording electrode/micropipette assemblies were constructed using a single carbon-fiber (30 lm diameter; Specialty Materials; Tulsa, OK), which was sealed inside fused silica tubing (Schott, North America; Elmsford, NY). The exposed tip of the carbon fiber (150 µm in length)

was coated with 5% Nafion (Aldrich Chemical Co., St. Louis, MO; 3–4 coats baked at 200 °C for 5 min per coat) to provide a 1000-fold selectivity of DA over its metabolite dihydroxyphenylacetic acid (DOPAC). Under these conditions, microelectrodes displayed linear amperometric responses to 0.5–10 μM DA during in vitro calibration in 100 mM phosphate-buffered saline (pH 7.4). Animals were anesthetized with injections of urethane (850 mg/kg, i.p.) and α-chloralose (85 mg/kg, i.p.), fitted with an endotracheal tube to facilitate breathing, and placed into a stereotaxic frame (David Kopf Instruments; Tujunga, CA). To locally deliver test compounds (see below) close to the recording site, a glass single or multi-barrel micropipette (FHC; Bowdion, ME) was positioned adjacent to the microelectrode using sticky wax (Moyco; Montgomeryville, AL). The center-to-center distance between the microelectrode and the micropipette ejector was 300 μm. The study used a multibarrel configuration in which barrels contained AMPH (400 μM) or vehicle (aCSF) and additional barrels contained the Akt inhibitors (1mM). The electrode/micropipette assembly was lowered into the striatum at the following coordinates (in mm from bregma [68]): A/P +1.5; M/L, +/- 2.2; D/V, -3.5 to -5.5. The application of drug solutions was accomplished using a Picospritzer II (General Valve Corporation; Fairfield, NJ) in an ejection volume of 100–150 nl (5–25 psi for 0.25–3 s). After ejection of test agents, there is an estimated 10–200-fold dilution caused by diffusion through the extracellular matrix. To record the efflux of DA at the active electrode, oxidation potentials—consisting of 100-ms pulses of 550 mV, each separated by a 1-s interval during which the resting potential was maintained at 0 mV—were applied with respect to

an Ag/AgCl reference electrode implanted into the contralateral superficial cortex. Oxidation and reduction currents were digitally integrated during the last 80 ms of each 100-ms voltage pulse. For each recording session, DA was identified by its reduction/oxidation current ratio: 0.55–0.80. At the conclusion of each experiment, an electrolytic lesion was made to mark the placement of the recording electrode tip. Rats were then decapitated while still anesthetized, and their brains were removed, frozen on dry ice, and stored at -80˚C until sectioned (20 μm) for histological verification of electrode location within the striatum. HSCA data were analyzed with GraphPad Prism.

Results

Pharmacological inhibition of Akt has been shown to reduce DAT cell surface expression (Garcia, Wei et al. 2005). However, the isoform of Akt involved is unknown. Therefore, we utilized allosteric, isoform-specific inhibitors of Akt1 (I-Akt1), Akt2 (I-Akt2), as well as a dual Akt1 and Akt2 inhibitor (I-Akt1/2), to identify the Akt isoform that regulates DAT trafficking (Figure 9). These selective inhibitors were developed and characterized in cell lines and primary tissue (DeFeo-Jones, Barnett et al. 2005; Lindsley, Zhao et al. 2005; Zhao, Robinson et al. 2008). HEK-293 cells stably expressing the human DAT (hDAT cells) were plated at the same density and treated for one hour with either I-Akt1 (12 uM), I-Akt2 (12 uM), or I-Akt1/2 (5 uM). These concentrations have previously been shown to be isoform specific (DeFeo-Jones, Barnett et al. 2005; Lindsley, Zhao et al. 2005). Using biotinylation assays



1, Dual Akt1/Akt2 Selective

Akt 1 IC_{50} = 58 nM
Akt 2 IC_{50} = 210 nM
Akt 3 IC_{50} = 2,200 nM

2, Akt1 Selective

Akt 1 IC_{50} = 760 nM
Akt 2 IC_{50} >20,000 nM
Akt 3 IC_{50} >20,000 nM

3, Akt2 Selective

Akt 1 IC_{50} >20,000 nM
Akt 2 IC_{50} = 325 nM
Akt 3 IC_{50} >20,000 nM

Figure 9. Structures of isoform selective, allosteric Akt Inhibitors. The structures of the potent dual Akt1/Akt2 selective (**1**), Akt1 selective (**2**), and Akt2 (**3**) selective inhibitors are shown. IC_{50} for each Akt isoform is shown for each compound.

(Saunders, Ferrer et al. 2000; Garcia, Wei et al. 2005), we examined surface expression of hDAT after both vehicle treatment and drug treatment. Figure 10A (inset) shows representative immunoblots for hDAT obtained from hDAT cells treated with vehicle (Control), I-Akt1, I-Akt2, or I-Akt1/2. Densitometric analysis of the immunoblots was performed and the level of surface hDAT was normalized to total hDAT. This data demonstrated that only inhibition of Akt2 significantly reduced hDAT cell surface expression (Fig. 10A, surface hDAT was normalized to total hDAT and expressed as percent of control; *$p<0.01$ by one-way ANOVA followed by Bonferroni post hoc test). Consistently, dual inhibition of both isoforms significantly decreased surface hDAT as well (Fig. 10A). Importantly, inhibition of Akt1 had no significant effect on hDAT surface expression.

Active Akt is phosphorylated at Thr 308 and Ser 473, and therefore application of isoform specific inhibitors should reveal reduction of the phosphorylation of the appropriate isoform. Therefore, to confirm the isoform specificity of these inhibitors in our assay, hDAT cells were treated as described above and specific Akt isoforms were immunoprecipitated and analyzed by immunoblot analysis using phospho-specific antibodies. We probed for phosphorylated Akt (Thr-308) to gauge the active, phosphorylated state of each Akt isoform after drug treatment. Fig. 10B demonstrates that the each inhibitor dramatically decreased basal phosphorylation of the relevant isoform(s), confirming that these inhibitors are isoform specific at the concentrations and treatment time used in our assay. Thus, these data suggest that Akt2, not Akt1,

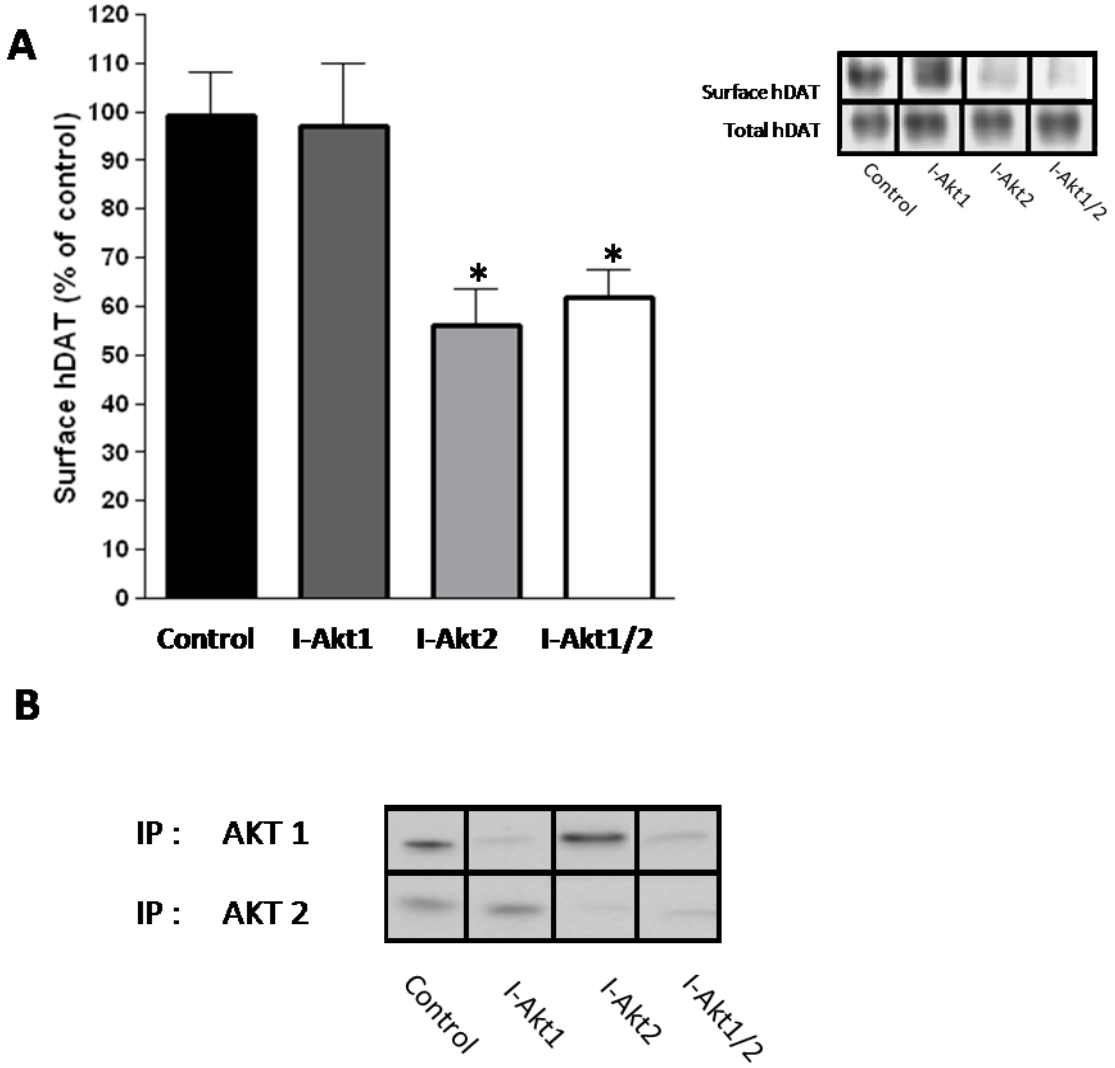


Figure 10. Inhibition of Akt2 reduces hDAT cell surface expression in hDAT cells. (A) Representative immunoblots obtained from HEK cells expressing hDAT after treatment for one hour with isoform-specific inhibitors of Akt1 (I-Akt1, 12 µM), Akt2 (I-Akt2, 12 µM) or a dual inhibitor of Akt1 and Akt2 (I-Akt1/2, 5 µM). For quantification, the density of each biotinylated hDAT band was normalized to the density of its corresponding total hDAT band and expressed as a percentage of control (* = $p<0.01$; One Way ANOVA, followed by Bonferroni post-hoc test; n=4). All data are represented as mean ± S.E.M. (B) Immunoprecipitation of Akt1 and Akt2 was performed from hDAT cells after treatment with each inhibitor as described in panel A. Immunoblots were probed for p-Akt (Thr308) to assess the phosphorylation state of each isoform after drug treatment.

is responsible for regulating hDAT cell surface expression in this heterologous expression system.

Next we confirmed our findings *ex vivo* by biotinylation of striatal slices (300 µm), which are enriched in DAT positive nerve termini (Giros and Caron 1993). Slices were treated with either I-Akt1, I-Akt2, or I-Akt1/2 as shown in Fig. 3. The samples from each tissue punch were normalized to total level of proteins. Similar to the results obtained from hDAT cells, treatment of striatal slices with I-Akt2 reduced rat DAT (rDAT) cell surface expression, as did treatment with the dual inhibitor I-Akt1/2 (Fig. 11A, inset). The cytosolic protein CaMKII was found only in the cytosolic fraction, confirming the plasma membrane nature of the biotinylated fraction of the striatal preparation. Importantly, CaMKII levels (loading control) were not affected by Akt inhibitor treatment (Fig. 11A, inset). Densitometric analysis of the immunoblots showed that inhibition of Akt2 significantly decreased DAT cell surface expression with respect to vehicle treated control (Fig. 11A). Similarly, dual inhibition of both Akt1 and Akt2 significantly decreased DAT cell surface expression (Fig. 11A). Importantly, inhibition of Akt1 alone had no effect on DAT trafficking in our striatal preparation (Fig. 11A).

To demonstrate further the role of Akt2 in the regulation of DAT, we determined whether Akt2 is expressed in striatum in DAT positive projections. Figure 11B shows that in striatal slices, Akt2 is heavily enriched in dopaminergic terminals marked by DAT immunoreactivity. These data further support our

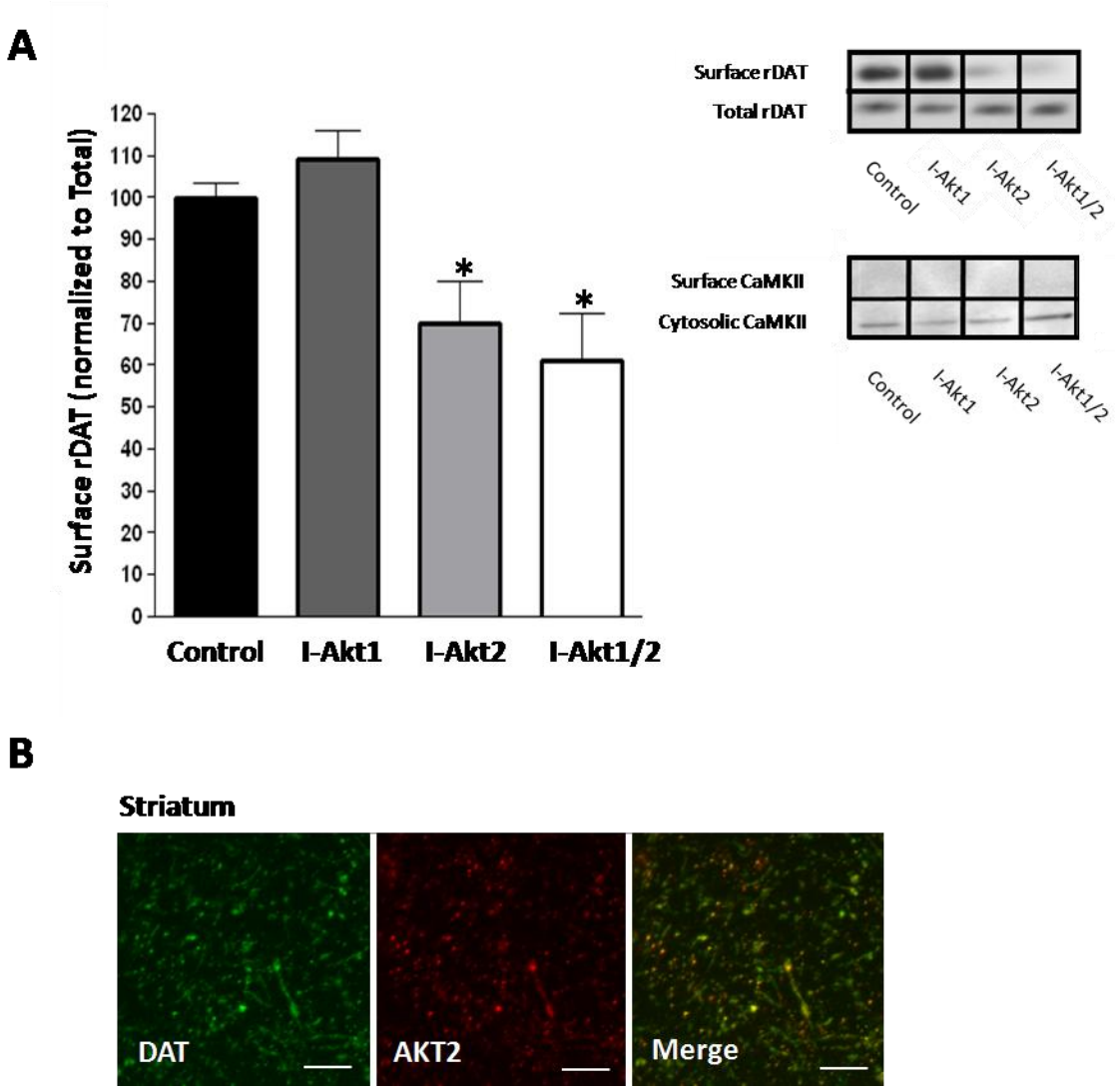


Figure 11. Inhibition of Akt2 reduces rDAT cell surface expression in rat striatal tissue. (A) Representative immunoblots obtained from striatal slices of biotinylated and total rat DAT (rDAT) after treatment for one hour with isoform-specific inhibitors of Akt1 (I-Akt1; 12 µM), Akt2 (I-Akt2; 12 µM), or dual inhibitor of Akt1 and Akt2 (I-Akt1/2; 5 µM). Immunoblots of CaMKII were used to determine the plasma membrane identity of the biotinylated fraction and control for loading. For quantification, the density of each biotinylated rDAT band was normalized to that of its corresponding total rDAT band and expressed as a percentage of control (* = $p<0.05$; One Way ANOVA followed by Bonferroni post-hoc test; n=11). All data are represented as mean ± S.E.M. (B) Confocal imaging of rat striatal slices, where green indicates DAT positive regions and red indicates Akt2 positive regions (scale bar, 12 µm). The merged image depicts yellow regions indicating high levels of expression of both Akt2 and DAT in dopaminergic projections (n=3).

finding that Akt2 is involved in regulating DAT trafficking, demonstrating for the first time isoform specificity of Akt regulation of DAT trafficking in native tissue.

DAT function is regulated both by the number of transporters at the plasma membrane and by the rate of their transport cycle. Therefore, we determined *in vivo* whether inhibition of Akt decreases DAT function. For this, we utilized amphetamine (AMPH), a substrate of DAT that reverses its transport cycle to cause DA efflux. We monitored AMPH-induced DA efflux in striatum of anesthetized rats, as a measure of DAT activity, by high speed chronoamperometry (HSCA). Since Akt2 inhibition leads to a decrease in DAT cell surface expression, we hypothesized that it would also lead to a reduction in AMPH-induced DA efflux. Figure 12A shows striatal AMPH-induced DA efflux recorded 45 min after injecting the Akt inhibitors I-Akt1, I-Akt2, or vehicle (artificial cerebrospinal fluid [aCSF] in DMSO). The inhibitors, AMPH, and aCSF were intrastriatally applied by way of a calibrated micropipette positioned adjacent to the recording electrode. First AMPH was applied to obtain a baseline measure for DA efflux. Inhibitors or aCSF were then applied and AMPH pressure-ejected again 45 minutes later. Inhibition of Akt2 led to a significant reduction in the ability of AMPH to cause DA efflux (Fig. 12B) further supporting our hypothesis that Akt2 is the isoform that regulates DAT surface expression and function.

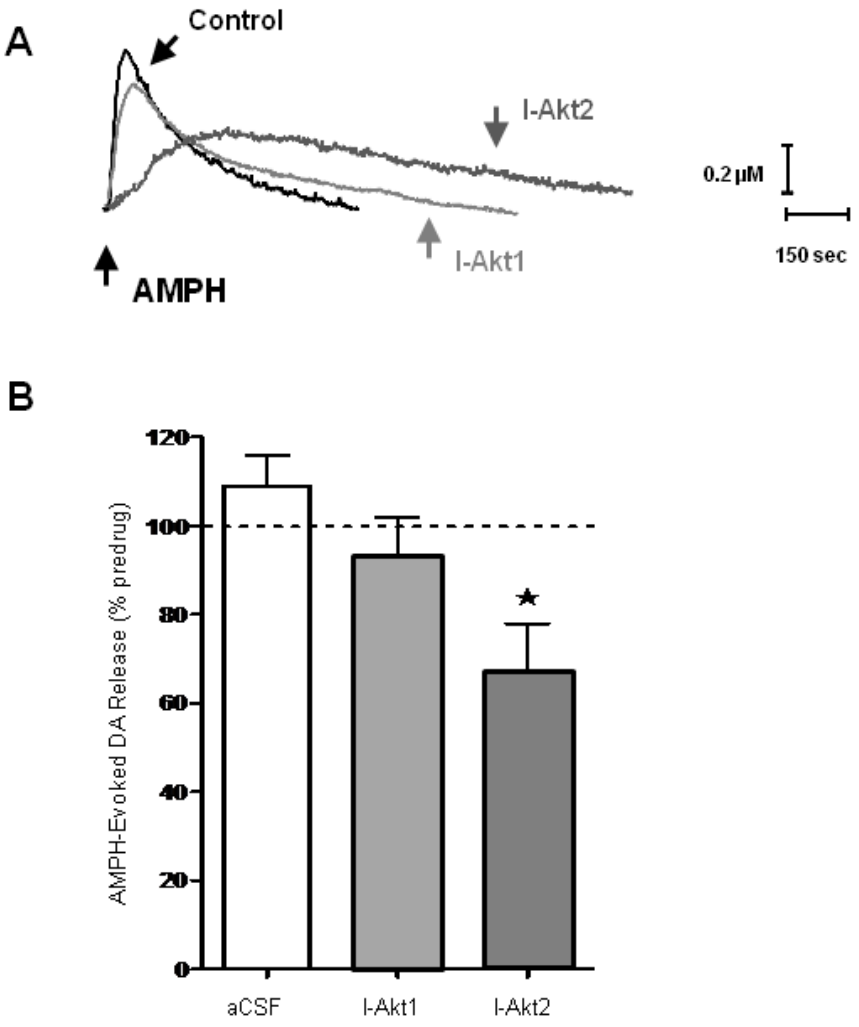


Figure 12. Inhibition of Akt2 reduces DAT-mediated reverse transport of DA. (A) Representative recordings of striatal extracellular DA after microinjection of AMPH (400 µM /125 nl), as measured by HSCA. Traces were obtained 45 minutes after microinjection of I-Akt1, I-Akt2 (1 mM/125 nl), or vehicle control. (B) Quantification of the total DA released after microinjection of AMPH. Inhibition of Akt2 significantly reduced AMPH-induced DA efflux. One way ANOVA followed by Bonferroni post hoc test (*=$p<0.05$; control, n=9; I-Akt1, n=8; I-Akt2, n=6). All data are represented as mean ± S.E.M.

Discussion

DA clearance is regulated both by DAT turnover rate and by the number of active transporters at the plasma membrane (Giros, el Mestikawy et al. 1991). As a consequence, DAT membrane expression is thought to fine tune DA homeostasis and dopaminergic signaling (Amara 1996; Blakely, Defelice et al. 2005; Spencer, Biederman et al. 2005). Previously, we have shown that insulin signaling through Akt regulates DAT cell surface expression, DA clearance, and the ability of psychostimulants such as amphetamine to target the DAT and thereby increase extracellular DA levels (Garcia, Wei et al. 2005; Owens, Sevak et al. 2005; Williams and Galli 2006; Lute, Khoshbouei et al. 2008). Here, we demonstrate by using selective inhibitors of Akt1 and Akt2 that, in striatum, Akt2 activity regulates DAT trafficking, whereas Akt1 does not.

Akt activation has diverse functions ranging from cell survival and growth to glucose homeostasis (Cho, Mu et al. 2001; Cho, Thorvaldsen et al. 2001; Somwar, Kim et al. 2001; Bae, Cho et al. 2003; Yang, Tschopp et al. 2004; Krizman-Genda, Gonzalez et al. 2005). In order to contribute to such diverse physiological processes, it is thought that each Akt isoform serves a distinct role. This hypothesis is postulated from the phenotypes observed in the Akt isoform-specific knockout mouse models (Cho, Mu et al. 2001; Cho, Thorvaldsen et al. 2001). In particular, Akt2 knockout mice are hyperglycemic and insulin resistant (Cho, Mu et al. 2001). These observations obtained from knockout models are consistent with data supporting a pivotal role of Akt2 in the increase of glucose uptake (Bae, Cho et al. 2003). This insulin-mediated increase in glucose uptake

is supported by trafficking of the glucose transporter GLUT4 to the plasma membrane, an effect shown to be mediated through Akt2, not Akt1 (Bae, Cho et al. 2003). Although insulin crosses the blood-brain barrier (Banks, Jaspan et al. 1997), neurons use insulin-independent mechanisms to transport glucose. Insulin receptors (IRs) are found throughout the brain, including DA-rich areas such as the striatum (Schulingkamp, Pagano et al. 2000). Importantly, abnormal insulin status has been shown to alter DAT cell surface expression and function (Patterson, Brot et al. 1998; Owens, Sevak et al. 2005; Williams, Owens et al. 2007; Lute, Khoshbouei et al. 2008). Therefore, it is conceivable that insulin signaling regulates DAT surface expression by modulating Akt2 activity. Furthermore, disease states with dysfunctional insulin signaling, such as Type II Diabetes, could alter Akt2 activity in brain and affect DAT function and DA homeostasis. Further work is needed to determine the extent that improper insulin tone is affecting DAT function and DA homeostasis.

Notably, these isoform-specific, allosteric Akt inhibitors exert their effects by blocking the phosphorylation of Akt itself (Lindsley, Zhao et al. 2005), making Akt unable to activate downstream targets. Importantly, they do not have inhibitory activity on other cellular kinases, such as protein kinase A (PKA) or protein kinase C (PKC) (DeFeo-Jones, Barnett et al. 2005). Previously, these inhibitors have been used to examine the isoform specificity of increased Akt activity in tumor cell lines and tissues (DeFeo-Jones, Barnett et al. 2005; Zhao, Robinson et al. 2008). While each drug stimulates apoptosis, inhibition of both isoforms by the dual inhibitor I-Akt1/2 was most effective, providing evidence that

tumor cell growth is somehow not isoform specific (DeFeo-Jones, Barnett et al. 2005). Conversely, in striatal preparations, we found isoform specificity to Akt regulation of DAT trafficking, despite the fact that Akt1 is also expressed in dopaminergic terminals (data not shown).

While the data shown here firmly point to Akt2 as an important regulator of DAT, only Akt1 and Akt2 were examined. A third isoform, Akt3, is expressed in the brain and testes (Gonzalez and McGraw 2009). Importantly, the inhibitors used in this study do not alter Akt3. Previous work demonstrates that Akt3 plays a role similar to Akt1 in the brain in regulating cell growth and survival, whereas Akt2 contributes to mediating insulin receptor signaling (Tschopp, Yang et al. 2005; Dummler, Tschopp et al. 2006). The possibility that Akt3 regulates monoamine transporter trafficking is intriguing and warrants further evaluation in future studies when a specific inhibitor becomes available.

In summary, our data indicate that basal Akt2 activity is responsible for maintaining DAT cell surface expression, implicating Akt2 as a key regulator of DAT function and DA homeostasis. Akt2 is known to be coupled to insulin receptor activation, further confirming insulin signaling as an important modulator DAT function and dopaminergic tone.

CHAPTER III

DIET-INDUCED CHANGES IN INSULIN SIGNALING REGULATES THE TRAFFICKING AND FUNCTION OF THE DOPAMINE TRANSPORTER

Introduction

High fat diets and the resulting obesity are known to cause several changes in the periphery, including insulin resistance, which can lead to Type 2 Diabetes (Kahn, Hull et al. 2006), heart conditions (Hubert, Feinleib et al. 1983), and depression (Golden, Lazo et al. 2008). Still, the impact of obesity on brain function is not fully understood. Clinical evidence has shown a correlation between disorders involving DA dysfunction to dysregulation in insulin signaling and food intake. Binge eating disorder, for example, is associated with impairments in DA signaling (Davis, Levitan et al. 2008; Davis, Levitan et al. 2009; Frieling, Romer et al. 2009). Furthermore, patients with Parkinson's Disease have been shown to have increased Type 2 Diabetes rates (Sandyk 1993; Morris, Zhang et al. 2008), as do patients with schizophrenia (Mukherjee, Decina et al. 1996).

Physiological homeostasis of DA signaling in striatum has been linked to motivation for feeding (Palmiter 2007). Such studies highlight a tight relationship between insulin and DA signaling in the brain, such as that dysregulation of the insulin pathway will cause impairment in DA signaling and *vice versa*. A tight

relationship is further supported by the comorbid nature of feeding behaviors abnormalities with DA signaling dysfunction.

A key element fine tuning DA signaling is DAT (Giros, el Mestikawy et al. 1992; Borowsky, Adham et al.). DAT function is required to clear vesicular released DA by active reuptake into the presynaptic bouton (Giros, Jaber et al. 1996; Jones, Gainetdinov et al. 1998), thus terminating DA signaling. DA is then re-packaged into vesicles for release. Thus, changes in DAT function have profound implications in DA signaling (Gelernter, Kranzler et al. 1994; Spencer, Biederman et al. 2005). Therefore, is not surprising that both function and trafficking of the DAT are tightly regulated by several signaling pathways including PKC, mitogen activated protein kinase (MAPK), and importantly PI3K (Gonzalez and Robinson 2004; Torres 2006).

It is well documented that a high density of IRs are expressed in DA regions, including the striatum (Hill, Lesniak et al. 1986; Manzanares, Canton et al. 1988; Bergstedt and Wieloch 1993; Kar, Chabot et al. 1993; Schulingkamp, Pagano et al. 2000; Figlewicz, Evans et al. 2003), a brain region enriched in DA projections and DATs (Pilotte, Sharpe et al. 1994; Ciliax, Heilman et al. 1995; Ciliax, Drash et al. 1999). IRs signal through PI3K to activate Akt (Taha and Klip 1999; Bondy and Cheng 2004). Inhibition of PI3K decreases surface levels of DAT, reduces DA function, as well as DA clearance *in vivo* (Carvelli, Moron et al. 2002; Owens, Sevak et al. 2005; Williams, Owens et al. 2007; Lute, Khoshbouei et al. 2008). Consistently, inhibition of Akt results in a decrease in both DAT surface expression and function (Garcia, Wei et al. 2005). AMPH is a well-

characterized psychostimulant that elicits its effects by its ability to reverse DAT-mediated transport of DA, inducing efflux and therefore increasing extracellular DA levels. Due to the requirement of DAT on the cell surface for this phenomenon to occur, inhibition of Akt and the consequent reduction in DAT cell surface expression results in a decrease of AMPH-induced DA efflux (Garcia, Wei et al. 2005).

Interestingly, the isoforms of Akt appear to serve distinct functions. Akt has three isoforms; Akt1 and Akt2 are ubiquitously expressed where as Akt3 is found only in the brain and testes (Dummler, Tschopp et al. 2006). Many studies have linked dysfunctions in Akt1 signaling to the underlying mechanisms of schizophrenia (Emamian, Hall et al. 2004; Schwab, Hoefgen et al. 2005; Bajestan, Sabouri et al. 2006). As shown in Chapter II, Akt2 is the isoform responsible for regulating DAT cell surface expression. Importantly, several DA related disorders have been linked to insulin dysfunction, including schizophrenia (Mukherjee, Decina et al. 1996). Therefore, defining the isoform involved in regulation of DAT in a state of insulin signaling dysfunction may help to further define the role of insulin signaling in brain diseases such as schizophrenia. Knockout studies have shown that Akt1 is primarily associated with cell survival and growth (Cho, Thorvaldsen et al. 2001). Consistently, Akt3 appears to function similarly for survival and growth in the brain (Tschopp, Yang et al. 2005). In contrast, Akt2 has been associated with glucose homeostasis and insulin signaling, including regulation of the glucose transporter (Cho, Mu et al. 2001; Bae, Cho et al. 2003).

In vitro insulin treatment causes an increase in DAT on the surface and an increase in DA uptake (Carvelli et al., 2002). *In vivo*, studies have tried to address the functional regulation of DAT by depleting circulating insulin with administration of STZ, a drug that is toxic to the insulin producing pancreatic β cells. These animals have reduced striatal levels of p-Akt, and decreased DAT surface expression and AMPH-induced efflux (Williams, Owens et al. 2007). Consistently, food restriction, a manipulation that also results in hypoinsulinemia, causes a reduction in DA uptake in rat striatal synaptosomes (Patterson et al., 1998). These studies suggest that changes in insulin status such as diabetes or obesity could affect DAT membrane expression by impairing Akt function.

Obesity and insulin resistance are highly prevalent in the United States (Mokdad, Bowman et al. 2001). In western nations, it is estimated that as much as 25% of the population is considered obese, and high fat diets are known to result in insulin resistance (Wisse, Kim et al. 2007). In this pre-diabetic state, the body maintains proper glucose levels by increasing insulin production as cells become resistant due to increasing adiposity, resulting in euglycemia and hyperinsulinemia (Schwartz and Porte 2005). This state is also known to result in reduced p-Akt (active) levels (De Souza, Araujo et al. 2005; Posey, Clegg et al. 2009). Therefore, we sought to explore whether a high fat diet could affect DAT trafficking and function. This is particularly relevant, considering that DA homeostasis supports food intake (Palmiter 2007) and possibly regulates feeding behaviors including motivation for food seeking.

Considering that DA is involved in behaviors from cognition to movement to motivation for food, this work may shed light on the comorbid aspects of obesity and DA related diseases.

Methods

Diet induced obesity (DIO) model. Male Sprague-Dawley rats were ordered from Charles River (Indianapolis, Indiana) at a body weight range of 275-300 g. Upon arrival to the vivarium rats were individually housed in a facility kept on a 12-hour light cycle and were given standard rodent chow and water *ad libidum*. In the first phase of DIO, rats were given a control diet consisting of 10% fat (Research Diets, New Brunswick, NJ) for 7 days. After this lead-in period, half of the rats were switched to an isocaloric, nutrient matched high-fat (HF) diet of 60% fat for 28 more days; the remaining control rats were kept on the control, low fat (LF) diet for the same amount of time. All experiments were performed in the morning.

Tissue preparation. Tissue punches from specific brain regions were collected (dorsal striatum and substantia nigra) and homogenized on ice in buffer containing 20mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM b-glycerolphosphate, 1 mM Na_3VO_4, 1 lg/ml leupeptin and 1 mM PMSF, then spun at 13000xg for 30 minutes at 4°C. The supernatant was taken, the protein content was assessed, and analysis was performed.

Brain slice preparation. Methods are as described by (Grueter and Winder 2005). Briefly, rats were decapitated. The brains were quickly removed and placed in an ice-cold, low-sodium/high-sucrose dissecting solution. Hemisected (300 µm) coronal brain slices containing the striatum were prepared on a vibratome. Slices were allowed to recover in a submerged holding chamber (37°C) containing oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (aCSF) that contained the following (in mM): 124 NaCl, 4.4 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1 NaH_2PO_4, 10 glucose, and 26 $NaHCO_3$ for a recovery period of 60 min before beginning experiments. If treated with Akt inhibitors, slices were incubated with 12 µM (I-Akt1 and I-Akt2), 5 µM (I-Akt ½) in aCSF, or aCSF containing the vehicle DMSO for one hour at 37 degrees. Biotinylation assays were then performed.

Biotinylation assays. For slice assays, hemisected slices (300 µm) were made as described above and transferred to multiwell submerged chambers containing oxygenated aCSF with NHS-SS-Biotin (1 mg/ml) on ice at 4°C and incubated for 45 minutes, then washed twice for 10 min in aCSF, and finally incubated in aCSF containing glycine (100mM) for two 20 min periods. Slices were then placed onto dishes on dry ice and the striatum was removed and placed into eppendorf tubes. Tissue punches were homogenized in ice-cold homogenization buffer (1% Triton, 2 mM sodium orthovanadate, 2 mM sodium fluoride, 25 mM HEPES, 150 mM NaCl, 10 µg/ml aprotinin, and 10 µg/ml leupeptin, and 100 µM phenylmethylsulfonyl fluoride) and centrifuged for 30 min at 1000 x g at 4°C. Protein levels were obtained, and equal amounts were taken

and added to strepavidin beads with pulldown buffer (0.1 % Triton, 25 mM HEPES, 150 mM NaCl, 2 mM sodium orthovanadate, 2 mM sodium fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin, and 100 µM phenylmethylsulfonyl fluoride) and incubated overnight at 4°C. Samples were then washed, eluted, and western analysis was carried out.

Immunostaining. For western blotting, determination of immunoreactivity was conducted according to previously described methods (Garcia, Wei et al. 2005; Williams, Owens et al. 2007). Briefly, tissue samples were separated by SDS-PAGE, and resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes (BioRad), which were incubated for 1 hr in blocking buffer (5% BSA and 0.1% Tween20 in Tris-buffered saline). The blots were then incubated with primary antibody overnight at 4°C. The primary antibodies used are as follows: Akt (1:1000; Cell Signaling Technology; Danvers, MA), Akt1 (1:1000; Cell Signaling Technology; Danvers, MA), phospho-Akt (Thr308) (1:1000; Cell Signaling Technology; Danvers, MA), Akt2 (1:1000; Santa Cruz Biotechnology; Santa Cruz, CA), Na/K ATPase (1:450; Dr. Fambrough, Johns Hopkins University; Baltimore, MD), and CamKII (1:2000; Affinity BioReagents; Rockford, IL), IRS2 (1:1000; Upstate Technologies; Billerica, MA). For rat DAT (rDAT) immunostaining, mouse monoclonal primary antibodies were used (antibody 16, 1:1000; (Gaffaney and Vaughan 2004). All proteins were detected using HRP conjugated secondary antibodies (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA). After chemiluminescent visualization (ECL-Plus; Amersham;

Piscataway, NJ) on Hyper-film ECL film (Amersham), protein band densities were quantified (Scion Image; Frederick, MD) and normalized to control.

Assay of Akt Activity. Akt activity assays were performed as described previously. Tissue was lysed for 45 min at 4 degrees in a buffer containing 20mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM b-glycerolphosphate, 1 mM Na_3VO_4, 1 lg/ml leupeptin and 1 mM PMSF. Lysed proteins (400 ug) underwent immunoprecipitation with an Akt-specific monoclonal antibody as part of a commercially available Akt activity assay kit (BioVision, Mountain View, CA). Activity of the immunoprecipitated Akt was determined in vitro with the addition of recombinant GSK3α a the kinase substrate; the resulting phosphorylated was determined by immunoblotting using phosphospecific antibodies to GSK3α (Ser21, diluted 1:1000), provided in the kit.

Immunohistochemistry. For tissue staining, slices were prepared as described above and subsequently fixed with PBS Ca^{2+}/Mg^{2+},/4% paraformaldehyde, washed three times with PBS Ca^{2+}/Mg^{2+}, permeabilized and blocked with PBS Ca^{2+}/Mg^{2+}/4% bovine serum albumin (BSA)/0.15% Tween-20, and immunostained with the appropriate antibody dissolved in PBS 4%, BSA/0.05%, Tween-20/0.05%. Primary antibodies used for immunoblotting were used here at the following concentrations: Akt2 at 1:200 and DAT at 1:400 overnight at 4°C. For immunohistochemistry experiments to confirm viral injections, rats were perfused with 4% paraformaldehyde in PBS and the intact brains were removed, postfixed for 24 hours, then put into PBS with 20% sucrose

overnight, and then sectioned and processed according to previously published protocols (Russo, Bolanos et al. 2007). Briefly, sections were incubated in blocking buffer (containing BSA) and then with rabbit antibody to GFP (1:5000; Abcam Inc.; Cambridge, MA) and rat tyrosine hydroxylase (1:500; Chemicon; Billerica, MA). After incubation with secondary fluorophores, immunofluorescence was imaged using a Perkin Elmer UltraView confocal with a Nikon Eclipse 2000-U microscope equipped with a 60X lens with an N.A. of 1.49. Image processing was performed using Image J and Adobe Photoshop.

Locomotor activity. Locomotor activity was assessed by placing the rat in a 26 × 61 × 23 cm high plexiglass chamber located within sound-attenuating cubicles. Horizontal activity was measured with four pairs of infrared photobeams positioned 4 cm above the floor of the chamber. Each beam was placed 15 cm away from the next immediate photobeam and the two extreme photobeams were located 8 cm away from the floor sides. An hour baseline was recorded, animals were given amphetamine (1.78 mg/kg) by I.P. injection, and placed immediately back into the chambers to continue recording for 60 minutes. The data was collected in 5 minute periods over each 60 minute test.

HSCA. HSCA was conducted using the FAST-12 system (Quanteon; http://www.quanteon.cc) as previously described with some modification (Owens et al., 2005; Williams et al., 2007). Recording electrode/micropipette assemblies were constructed using a single carbon-fiber (30 lm diameter; Specialty Materials; Tulsa, OK), which was sealed inside fused silica tubing (Schott, North America; Elmsford, NY). The exposed tip of the carbon fiber (150 μm in length)

was coated with 5% Nafion (Aldrich Chemical Co., St. Louis, MO; 3–4 coats baked at 200 °C for 5 min per coat) to provide a 1000-fold selectivity of DA over its metabolite dihydroxyphenylacetic acid (DOPAC). Under these conditions, microelectrodes displayed linear amperometric responses to 0.5–10 μM DA during in vitro calibration in 100 mM phosphate-buffered saline (pH 7.4). Animals were anesthetized with injections of urethane (850 mg/kg, i.p.) and α-chloralose (85 mg/kg, i.p.), fitted with an endotracheal tube to facilitate breathing, and placed into a stereotaxic frame (David Kopf Instruments; Tujunga, CA). To locally deliver test compounds (see below) close to the recording site, a glass single or multi-barrel micropipette (FHC; Bowdion, ME) was positioned adjacent to the microelectrode using sticky wax (Moyco; Montgomeryville, AL). The center-to-center distance between the microelectrode and the micropipette ejector was 300 μm. The electrode/micropipette assembly was lowered into the striatum at the following coordinates (in mm from bregma [68]): A/P +1.5; M/L, +/- 2.2; D/V, -3.5 to -5.5. The application of drug solutions was accomplished using a Picospritzer II (General Valve Corporation; Fairfield, NJ) in an ejection volume of 100–150 nl (5–25 psi for 0.25–3 s). After ejection of test agents, there is an estimated 10–200-fold dilution caused by diffusion through the extracellular matrix. To record the efflux and clearance of DA at the active electrode, oxidation potentials—consisting of 100-ms pulses of 550 mV, each separated by a 1-s interval during which the resting potential was maintained at 0 mV—were applied with respect to an Ag/AgCl reference electrode implanted into the contralateral superficial cortex. Oxidation and reduction currents were digitally integrated during the last 80 ms of

each 100-ms voltage pulse. For each recording session, DA was identified by its reduction/oxidation current ratio: 0.55–0.80. At the conclusion of each experiment, an electrolytic lesion was made to mark the placement of the recording electrode tip. Rats were then decapitated while still anesthetized, and their brains were removed, frozen on dry ice, and stored at -80˚C until sectioned (20 µm) for histological verification of electrode location within the striatum. HSCA data were analyzed with GraphPad Prism.

Viral Injections. At day 25 of the diet, rats were anesthetized with isoflurane inhalation and given 0.5 ul bilateral microinjections of HSV vectors encoding GFP (as a control), or wild-type IRS2 over 5 min into the substantia nigra (A/P -5.3, M/L +/- 2.0, D/L -7.8, measured from bregma). After a 5 minute pause, the needle was slowly withdrawn over 5 minutes. Biochemical and behavioral assays were performed as described above 3 days after surgery.

Results

Diet-induced obesity results in reduced Akt activity in striatum and substantia nigra. We hypothesized that insulin resistance would also lead to decreased DAT surface expression. To test this hypothesis we induced obesity and insulin resistance by high-fat feeding in rats (diet-induced obesity (DIO) (De Souza, Araujo et al. 2005; Posey, Clegg et al. 2009). Rats were fed a 60% lard-based high-fat diet (HF) for 28 days and controls fed a micro-nutrient matched 10% low-fat diet (LF). Throughout the 28 day period, HF animals consumed significantly more calories than LF animals (Fig. 13A; 3001 ± 134.3 cal. vs. 2633

± 95.65 cal, *=p<0.05 by Student's t-test). Also, HF animals gained significantly more weight than LF animals (Fig. 13B; 187.0 ± 9.63 g vs. 160.8 ± 5.38 g, *=p<0.05 by Student's t-test). Importantly, while blood glucose levels were not significantly different in the HF versus LF animals (Fig. 14A), plasma insulin levels were significantly elevated in the HF animals, indicating the presence of insulin resistance (Fig. 14B). Furthermore, levels of tyrosine hydroxylase (TH) and dopamine were found to be similar between LF and HF groups (Fig. 15 and Fig. 16).

Akt is activated by phosphorylation at threonine 308 in response to insulin. Therefore, Akt phosphorylation is commonly utilized as a marker of insulin action (Luque et al., 2006). To confirm that the DIO model resulted in impaired Akt phosphorylation, we assessed phosphorylated Akt (p-Akt) at position 308 in striatal extracts by western blot. HF feeding resulted in reduction of p-Akt to 57 ± 9% of LF animals (Fig. 17A; *=p<0.01 by Student's t-test). Similar results were obtained in the substantia nigra, where the HF feeding decreased Akt phosphorylation to 69 ± 5% of LF animals (Fig. 17B; *=p<0.05 by Student's t-test). Importantly, total Akt levels were unaffected by DIO. To further confirm a decrease in active Akt, we performed Akt activity assays. For this, Akt was immunoprecipitated from striatal tissue of LF and HF animals. A substrate of Akt, glycogen synthase kinase 3 (Gsk-3) was then added and phosphorylation of the substrate was assessed as a measurement of Akt activity in each preparation. DIO led to a decrease in Akt activity, as measured by p-Gsk-3 (Fig. 18, *=p<0.05 by Student's t-test, n=4), further confirming a reduction in active Akt in HF rats.

To assess the isoform specificity of the observed decreases in active Akt in the HF animals, immunoprecipitations were performed to pull down specific Akt isoforms, and then probed for phosphorylation. In the HF animals, there was a significant reduction in p-Akt2 with respect to LF animals (Fig. 19A; *=$p<0.05$, by Student's t-test), while no significant changes in the level of p-Akt1 were observed (Fig. 19B). As a control, each isoform was immunoprecipitated and then probed for Akt1 or Akt2. Figure 19C demonstrates the specificity of the immunoprecipitations.

HF feeding reduces DAT cell surface expression. Because HF feeding impairs Akt2 activation in the striatum, we next determined whether DIO induces a decrease in DAT cell surface expression using biotinylation assays on striatal slices. Fig. 18A shows representative immunoblots of both biotinylated (surface) and total fraction obtained from either HF or LF animals. As a control, for non-specific membrane protein trafficking, we also determined surface levels of the Na/K ATPase, a protein found predominantly at the plasma membrane. We observed a lack of immunoreactivity for Na/K ATPase in the cytosolic fraction, confirming the specificity of our assay. Quantification by western blot analysis shows that levels of surface DAT in HF animals were reduced to 73 ± 9% of LF animals (Fig. 20B, *=$p<0.05$ by Student's t-test). This HF feeding-induced phenomenon was likely due to impaired trafficking since the total levels of DAT are unchanged (Fig. 20A). Furthermore, since Na/K ATPase surface levels are unchanged, our data suggest that this DIO-induced trafficking does not target membrane proteins indiscriminately.

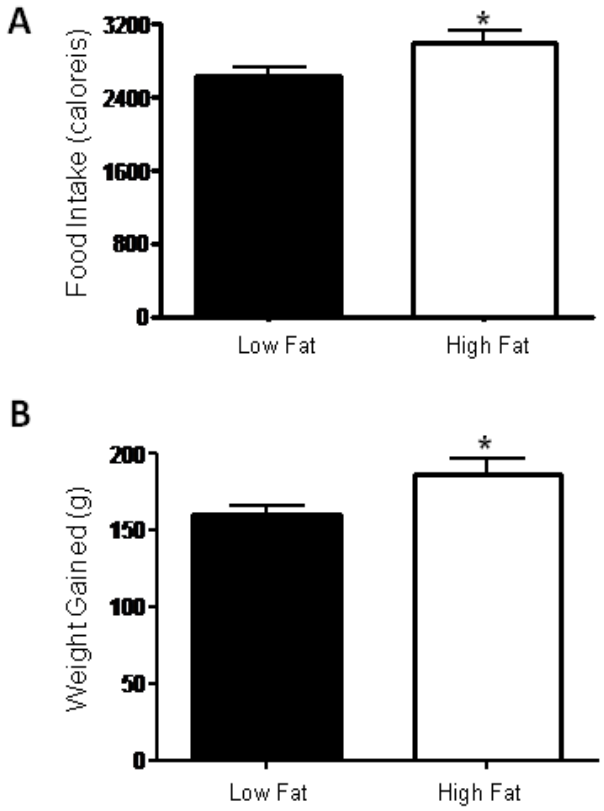


Figure 13. High fat feeding results in increased food intake and weight gain. Rats were fed a diet of either 10 % fat (LF) or 60% fat (HF) for 28 days. The HF-fed rats have a significant increase in food intake (A, n=13; *=$p<0.05$ by Student's t-test) and weight gain (B, n=13; *=$p<0.05$ by Student's t-test). (*=$p<0.05$ by Student t-test; LF, n=13; HF, n=16). All data are represented as mean ± S.E.M.

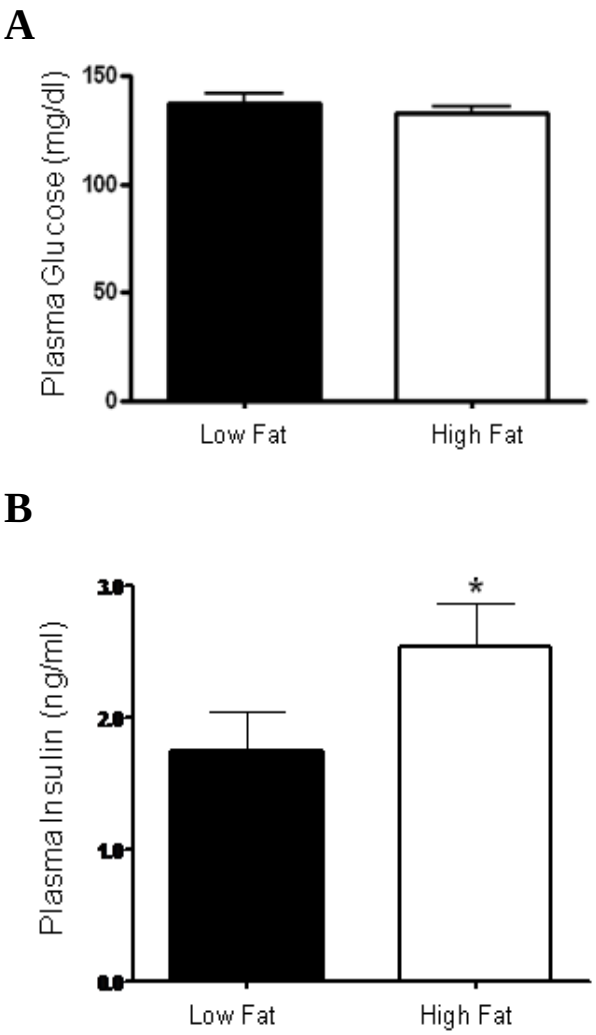


Figure 14. High fat feeding results in insulin resistance. Rats were fed a diet of either 10 % fat (LF) or 60% fat (HF) for 28 days. On day 28, blood was collected and plasma glucose and insulin levels were measured. (A) Plasma glucose levels were not significantly different between the two groups. (B) Insulin levels in the HF rats were significantly higher as compared to the LF group (*=$p<0.05$ by Student t-test; LF, n=13; HF, n=16). All data are represented as mean ± S.E.M.

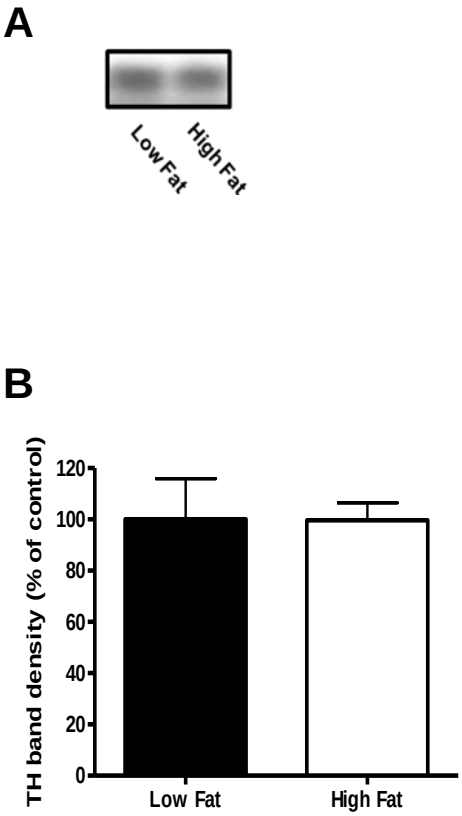


Figure 15. Tyrosine hydroxylase levels are unchanged by high fat feeding. Rats were fed a diet of either 10 % fat (LF) or 60% fat (HF) for 28 days. (A) Representative immunoblot of tyrosine hydroxylae (TH) from the striatum of LF and HF animals. (B) Band densities of TH were quantified and are shown as a percentage of control. There is not a significant difference between the two groups (n=3, $p>0.05$ by Student's t-test). All data are represented as mean ± S.E.M.

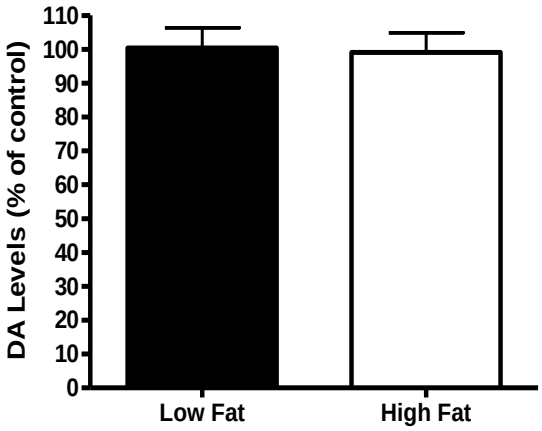


Figure 16. Dopamine (DA) levels in striatum are unchanged by high fat feeding. (B) Dopamine levels were assessed in LF and HF rats on day 28 and no significant difference is seen between the two groups (shown as a percentage of control, n=5, $p>0.05$ by Student's t-test). All data are represented as mean ± S.E.M.

Importantly, levels of DA were found to be unchanged between HF and LF animals (Fig. 20A), as well as protein levels of tyrosine hydroxylase, the rate-limiting enzyme in DA synthesis (Fig. 20B).

HF feeding reduces DA clearance in vivo. DAT activity is affected by the number of transporters at the cell surface and by their catalytic function. Therefore, the DIO-induced reduction in DAT surface expression was predicted to lead to a consequent reduction in DAT activity. We assessed this hypothesis by determining DA clearance *in vivo* using HSCA. A calibrated micropipette positioned adjacent to the recording electrode was lowered into the striatum of anesthetized rats. DA was applied into the striatum and its clearance measured over time. Figure 21 shows the kinetic profile for DA clearance. There was no effect on the signal amplitude or on the rise time (not shown) of the signal attained for a given amount of exogenously applied DA, which suggests that there is no change in the rate of diffusion of DA through the extracellular matrix between the two groups. In contrast, compared to LF rats, HF animals showed a marked and significant reduction in the rate at which DA was cleared from extracellular fluid (Fig. 21, $p<0.0001$ by two-way repeated measures ANOVA, $F_{4,24}=60.83$; *=$p<0.01$, with Bonferroni post-hoc analysis). These data demonstrate that the decrease observed in the surface expression of DAT results in a functional decrease in DAT activity *in vivo*.

HF feeding impairs AMPH-induced locomotion. Alterations in Akt phosphorylation, thus, lead to changes in surface levels of DAT, as well as DAT

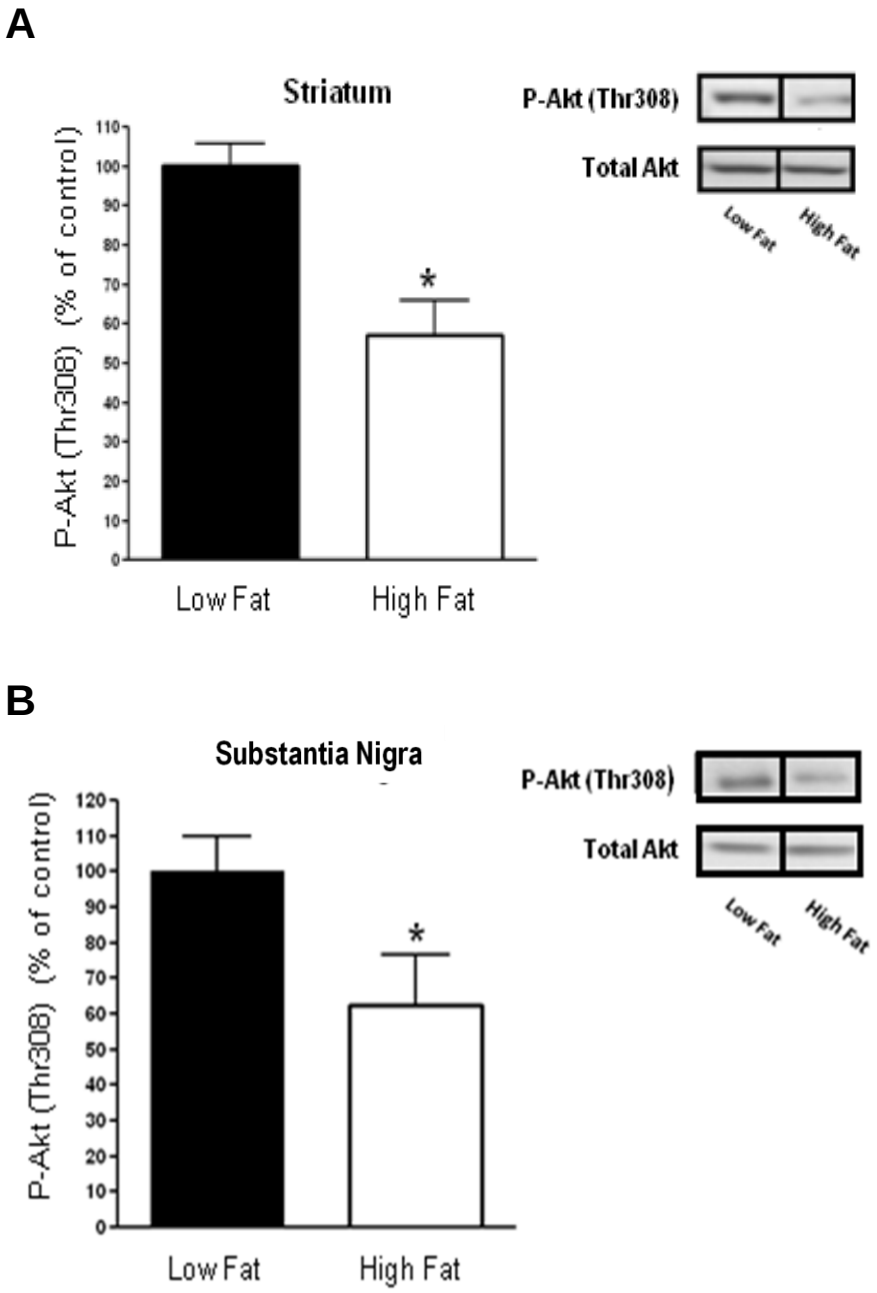


Figure 17. DIO induces a decrease in p-Akt in striatum and substantia nigra. (A,B) Tissue from the striatum (A) and substania nigra (B) was analyzed by western blot for levels of p-Akt (Thr308) and total Akt (insets). Quantification of immunoreactivity for p-Akt shows a significant reduction in HF rats compared to LF rats for both brain regions. Data is expressed as a percentage of LF control (*=$p<0.05$ by Student t-test; LF, n=6; HF, n=7). All data are represented as mean ± S.E.M.

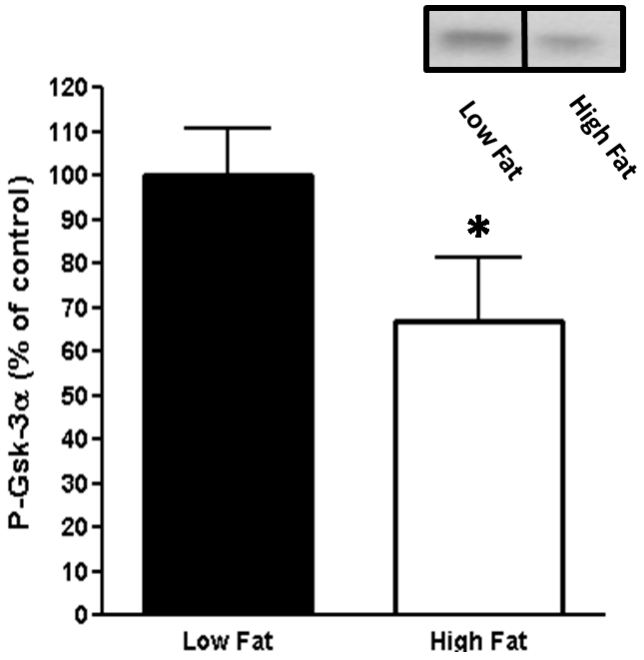


Figure 18. DIO induces a decrease in Akt activity in striatum. Akt activity was measured by the ability of immunoprecpitated Akt from striatal tissue of LF and HF fed animals to phosphorylate the substrate glycogen synthase kinase 3 (Gsk-3) in vitro. Levels of phosphorylated Gsk-3 were analyzed by western blot (insets). Quantification of immunoreactivity for p-Gsk-3 shows a significant reduction in HF rats compared to LF rats. Data is expressed as a percentage of LF control (*=$p<0.05$ by Student t-test;n=4). All data are represented as mean ± S.E.M.

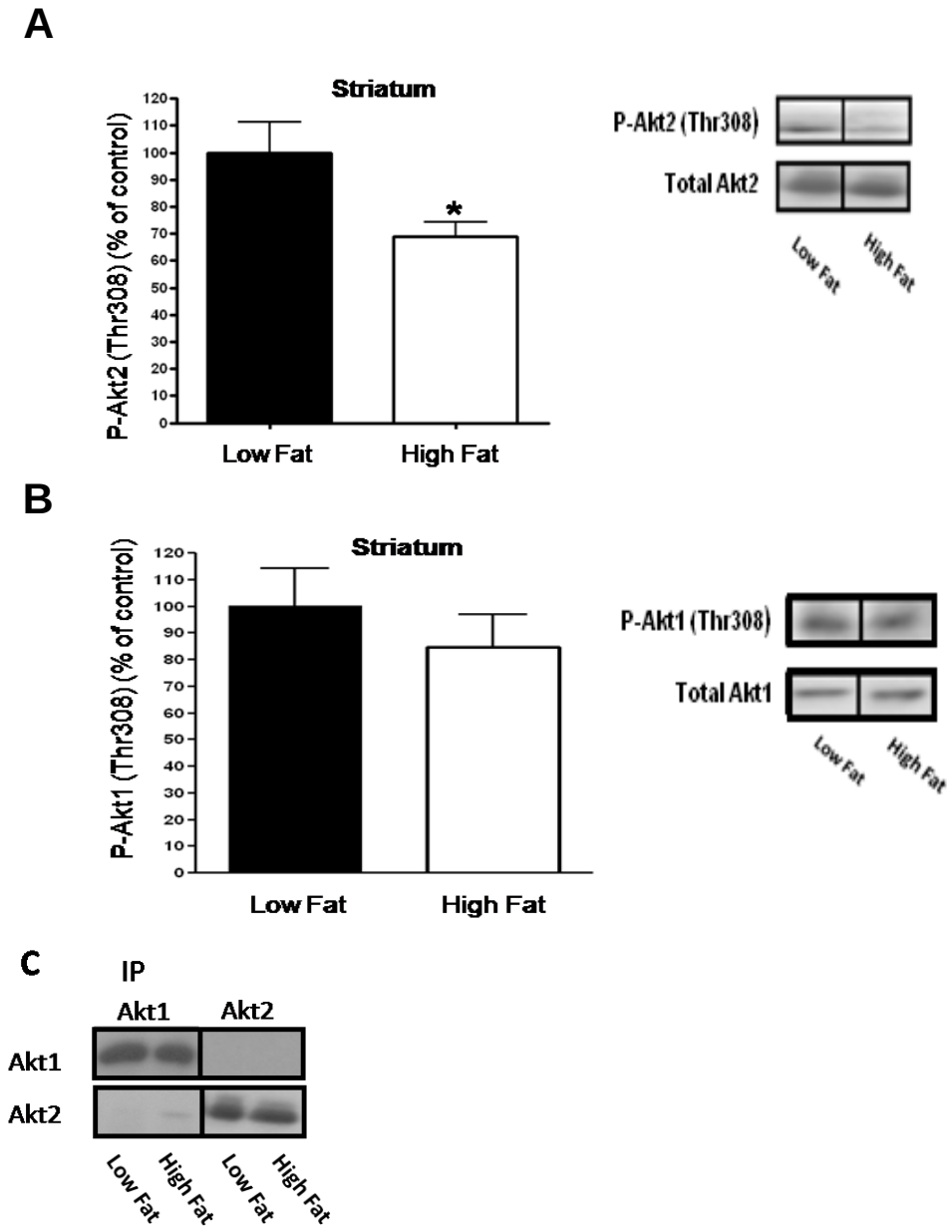


Figure 19. DIO induces a decrease in p-Akt2. Immunoprecipitation of Akt2 (A) and Akt1 ()b was performed on striatal tissue of LF and HF rats. Immunoblots were probed for p-Akt (Thr308) to assess the phosphorylation state of each isoform (insets). Quantification of the immunoreactivity shows that p-Akt2 levels were significantly decreased (A). In contrast p-Akt1 levels were not significantly changed (B) (*=$p<0.05$, Students t-test; LF, n=4; HF, n=5). (C) Each isoforms was pulled down and probed, demonstrating specificity to the immunoprecipitation for each isoform. All data are represented as mean ± S.E.M.

activity. AMPH elicits increased locomotion by causing DA efflux through reverse transport of DA, mediated by DAT. Therefore, we used AMPH-induced changes in locomotion to probe functional changes in DAT mediated behavior between LF and HF rats. Movement was assessed by the number of beam breaks (activity counts) in a 5 minute period. Figure 21A shows rat locomotor activity over a 90 min time period, wherein AMPH (1.78 mg/kg) was administered by I.P. injection at time = 60 minutes (arrow). Baseline locomotor activity (between 0 and 60 min) was unchanged by HF feeding (Fig. 21B). Importantly, after injection of AMPH, locomotion was increased in both groups, but the total distance traveled was reduced in HF animals (Fig. 21C, 8618 ± 835.7 vs. 6345 ± 573.0 mean activity counts, *=$p<0.05$ by Student's t-test). These data indicate that DIO reduces AMPH-induced locomotion, providing additional behavioral evidence that a high-fat diet impairs DA signaling in striatum. Furthermore, levels of AMPH in the striatum were found to be the same between LF and HF animals. Animals were given an I.P. injection of AMPH (1.78 mg/kg) and then tissue was taken at three different time points, 5, 10, and 20 minutes. The amount of AMPH in the tissue was similar over time, demonstrating that the ability of AMPH to reach the striatum between the two groups is not significantly different (Figure 23).

Virally mediated IRS2 expression restores Akt activity in the substantia nigra. Our data demonstrate that DIO leads to a reduction in DAT surface expression as a consequence of decreased Akt phosphorylation. To cement this hypothesis, we employed a viral expression system to increase expression of

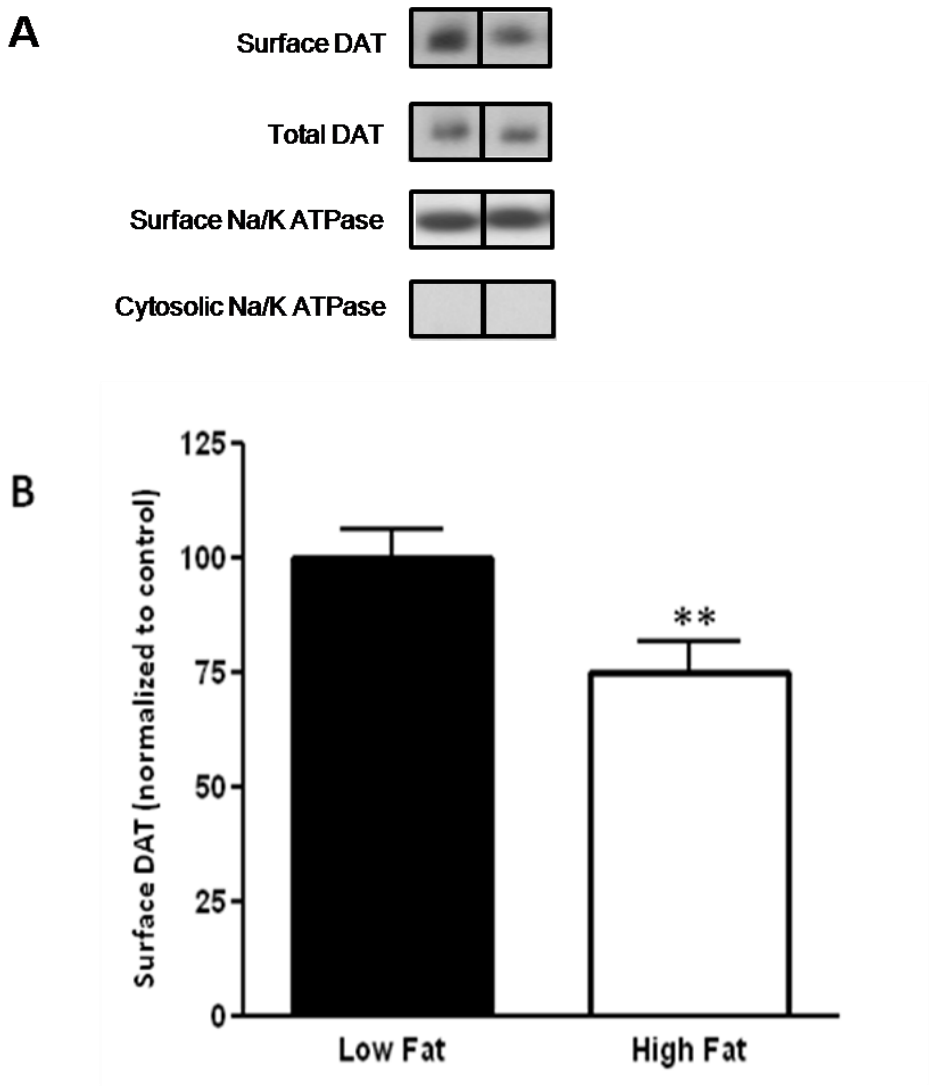


Figure 20. Dopamine transporter cell surface expression is reduced in striatal slices of HF rat. (A) Representative immunoblots of biotinylated (surface) and total proteins for the dopamine transporter (DAT) obtained from LF and HF rats. As a control we used both surface and cytosolic levels for the Na/K ATPase. Na/K ATPase is found only at the surface and is unchanged across groups. (B) Quantification of rDAT immunoreactivity. rDAT surface levels were normalized to the total amount of rDAT and expressed as a percent of control. The levels of surface rDAT were significantly reduced in HF animals as compared to LF animals (**=$p<0.01$ by Students t-test, LF, n=4; HF, n=5). Data are represented as mean ± S.E.M.

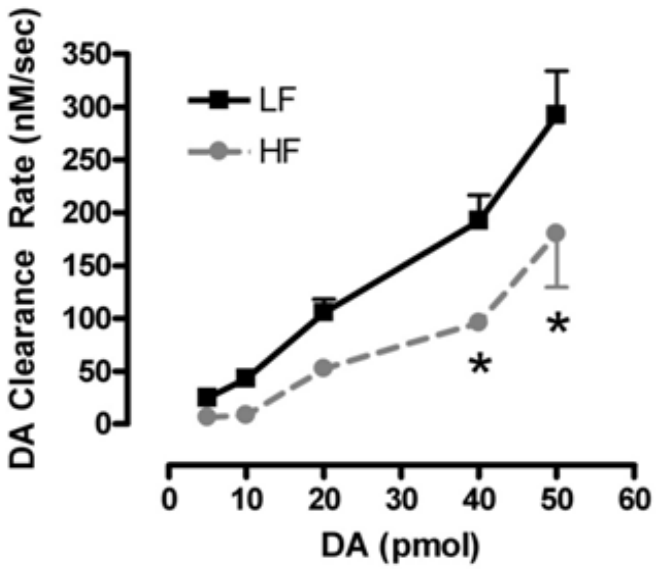


Figure 21. DA clearance rate is reduced in DIO animals. Dopamine (DA) clearance rates obtained by pressure ejecting different concentrations of DA in striatum of anesthetized LF and HF rats. HF rats show a significant reduction in DA clearance as compared to LF rats (*=$p < 0.05$ by two way repeated measures ANOVA followed by Bonferroni post hoc test, n=4). All data are represented as mean ± S.E.M.

IRS2, a cytosolic protein upstream of Akt whose activation increases Akt function (Gelling, Morton et al. 2006). We injected a herpes simplex virus (HSV) encoding IRS2 (HSV-IRS2) into the substantia nigra of HF and LF animals, as well as HSV expressing GFP (HSV-GFP) as a control. This virus has been characterized previously in dopaminergic regions of the rodent brain (Russo, Bolanos et al. 2007). Virally mediated GFP expression in the cell bodies of the nigral dopaminergic neurons and in their terminal projection to the striatum was confirmed by immunohistochemistry (Fig. 24A). Neurons were labeled after injection of HSV-GFP GFP with antibody against TH, a marker for dopaminergic neurons. Figure 24 shows expression of GFP in TH-positive neuronal cell bodies (top) and nerve endings (bottom). Next we confirmed that injection of HSV-IRS2 restores basal Akt phosphorylation in HF animals. Since IRS2 is upstream of Akt, we expected that overexpression of IRS2 would restore p-Akt levels in HF animals, as observed by others (Gelling, Morton et al. 2006). In the HF animals injected with HSV-IRS2, a significant increase in IRS2 protein level was observed in the substantia nigra compared to HF animals injected with HSV-GFP (Fig. 25A, *=$p<0.05$; one way ANOVA followed by Bonferroni post hoc test). Importantly, IRS2 overexpression reversed the impairment in basal Akt phosphorylation seen between HF and LF rats (Fig. 25B). Although IRS2 expression did not increase p-Akt levels in the LF animals, it was able to significantly increase p-Akt level in the HF animals as compared to HF animals injected with HSV-GFP (*=$p<0.05$, one way Anova followed by Bonferroni post hoc test).

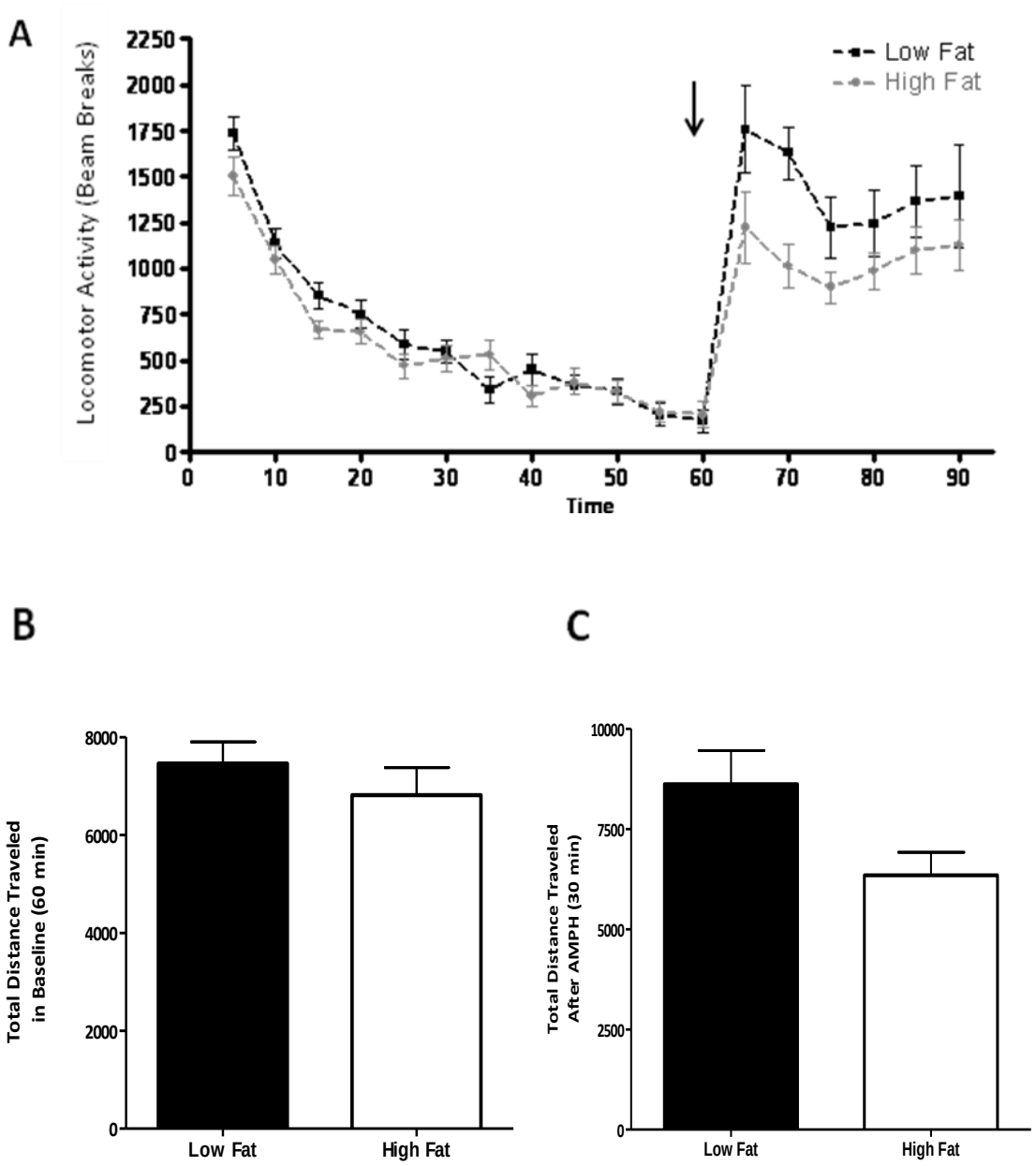


Figure 22. AMPH-induced locomotor activity is reduced in DIO animals. Locomotor activity was assessed in HF and LF rats before and after an I.P. injection of AMPH (1.78 mg/kg). (A) Locomotor activity measured by beam breaks over time. Each data point represents 5 minutes of recording expressed as a mean ± SEM. The arrow indicates administration of AMPH. (B) Total distance traveled by HF and LF rats measured during the first 60 minutes (before AMPH; $p \geq 0.05$ by Student's t-test, n=12). (C) Total distance traveled measured in HF and LF rats throughout a 30 min time period after AMPH injection (*=$p < 0.05$ by Students t-test, n=12). All data are represented as mean ± S.E.M.

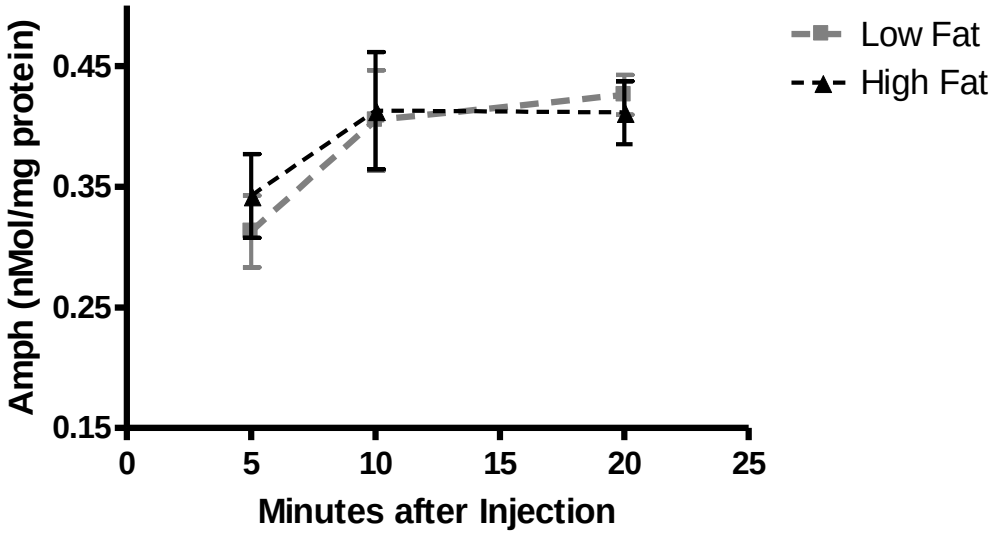


Figure 23. AMPH levels in striatum after I.P. injection of AMPH remain the same between LF and HF fed rats. AMPH levels were assessed at different time point in the striatum of LF and HF rats after an I.P. injection of AMPH (1.78 mg/kg). There was not a significant difference between the two groups (n=3 for each time point). All data are represented as mean ± S.E.M.

Overexpression of IRS2 in the substantia nigra restores DAT cell surface expression in striatum as well as AMPH-induced locomotion. We next determined whether the increase in p-Akt in the nigra due to injection of HSV-IRS2 restores normal surface expression of DAT (Fig. 26). Both LF and HF animals were injected in the substantia nigra with either HSV-GFP or HSV-IRS2. Consistent with its inability to increase p-Akt, injection of HSV-IRS2 in the nigra did not increase DAT cell surface expression in striatum of LF animals compared to GFP injected controls. Conversely, IRS2 overexpression in HF animals restored DAT plasma membrane expression to the level detected in LF animal injected with HSV-GFP (Fig. 26). Importantly, injection of either virus did not affect the levels of the Na/K ATPase, showing this result is not due to changes to all surface proteins (Figure 27).

We next determined whether restoration of DAT plasma membrane expression reversed the reduction in locomotor response seen after administering AMPH in the HF animals. We again injected either HSV-GFP or HSV-IRS2 into the substantia nigra of LF and HF animals and then, three days later, measured locomotor responses after an IP injection of AMPH (1.78 mg/kg). HF HSV-GFP injected rats traveled less distance 30 minutes after AMPH injection compared to LF HSV-GFP injected animals (Fig. 28B, 5184 ± 281.5 vs. 3835 ± 335.4, *=$p<0.05$ by one way ANOVA followed by Bonferroni post hoc test) as previously observed in uninjected animals (Fig. 23). Importantly, IRS2 is able to restore the deficit in cell surface expression of DAT and AMPH-induced locomotor activity in HF animals (Figure 28).

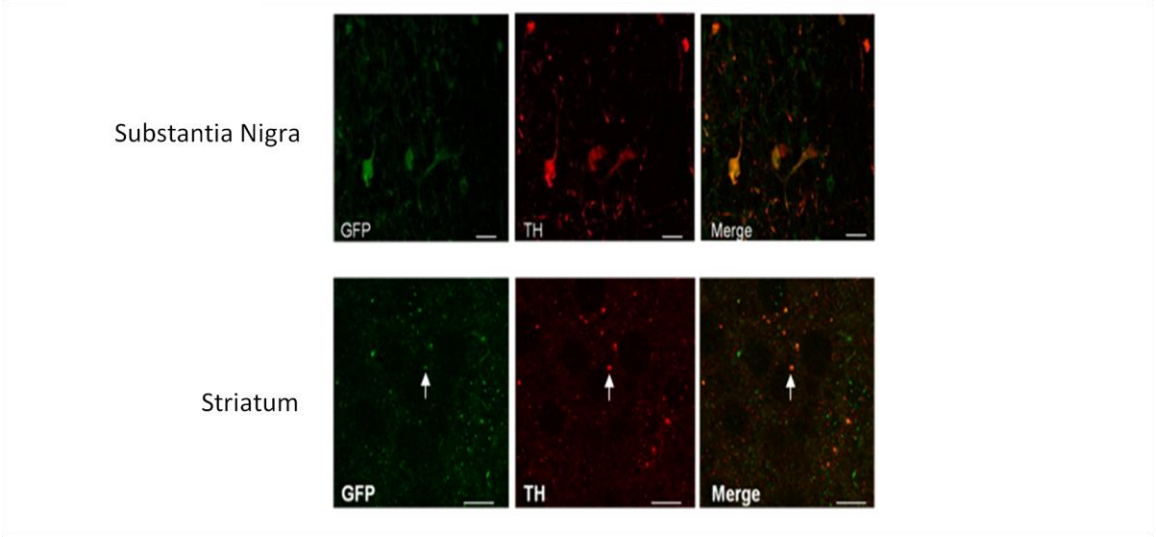


Figure 24. Viral-mediated expression of HSV-GFP. (A) DA neurons from the substantia nigra and projection terminals in the striatum were stained for TH immunoreactivity after injection of HSV-GFP into the nigra. (scale bars, 20 uM, top panel; 12 uM, bottom panel). Injection coordinates were A/P +5.3, M/L+/- 2.0, D/V -7.8. All coordinated measured from the top of the skull at bregma.

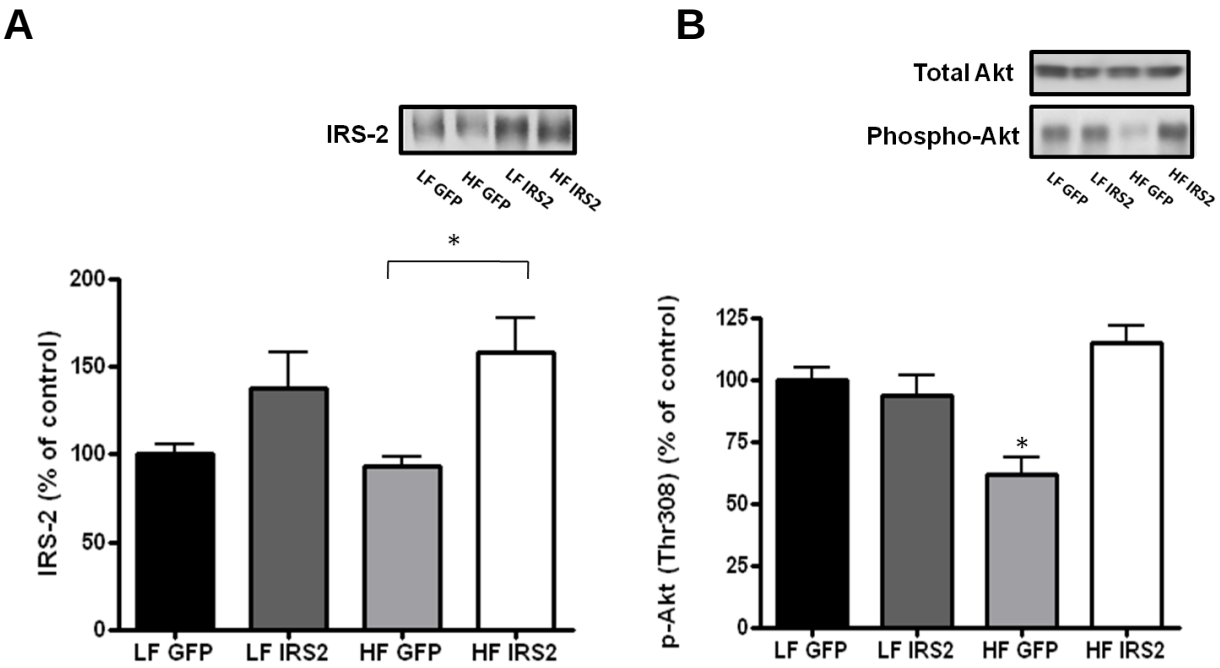


Figure 25. Viral-mediated expression of IRS2 in DIO rats restores p-Akt levels. (A) Representative immunoblot (inset) and quantification of IRS2 levels in the substania nigra after injection of HSV-IRS2 (*=p<0.05 by one way ANOVA followed by Bonferroni post hoc test; n=4). (B) Representative immunoblot (inset) and quantification of p-Akt (Thr308) levels in the substania nigra after injection of HSV-IRS2. Injection of HSV-IRS2 significantly increases p-Akt levels in HF rats (*=p < 0.05 by one way ANOVA followed by Bonferroni post hoc test; LF, n=3; HF, n=4). All data are represented as mean ± S.E.M.

Discussion

Food ingestion is not only controlled by hypothalamic function (Niswender, Baskin et al. 2004), but is also regulated by nigrostriatal DA pathways that are required for motivation to engage in feeding behaviors (Palmiter 2007). Excessive stimulation of DA signaling inhibits feeding; for example, elevating intrasynaptic DA concentration with either AMPH or cocaine, or stimulating DA receptors with apomorphine reduces food intake (Ladurelle, Duterte-Boucher et al. 1991). Still, how pathological feeding affects DA signaling is poorly understood. We sought here to determine how high-fat feeding leads to defects in DA homeostasis. Initial results from both animal and human studies support the concept that obese subjects suffer "hypodopaminergic, reward-deficiency syndrome (HRDS)", based upon evidence of derangements in the striatal dopaminergic system (Volkow and Wise 2005; Stice, Spoor et al. 2008). Importantly, in rodents, dietary-induced obesity leads to deficits in mesolimbic DA neurotransmission and decreases DAT density in striatum (South and Huang 2008; Geiger, Haburcak et al. 2009).

To date there is not a clear understanding of how HRDS develops and whether obesity-driven brain insulin resistance plays a significant role in this process. Based upon evidence for insulin regulation of DA homeostasis (Williams, Owens et al. 2007; Geiger, Behr et al. 2008; South and Huang 2008), it is imperative to clarify whether insulin resistance in striatum triggers striatal hypodopaminergia and whether rescuing the insulin signaling in obese animals also rescues normal DA homeostasis.

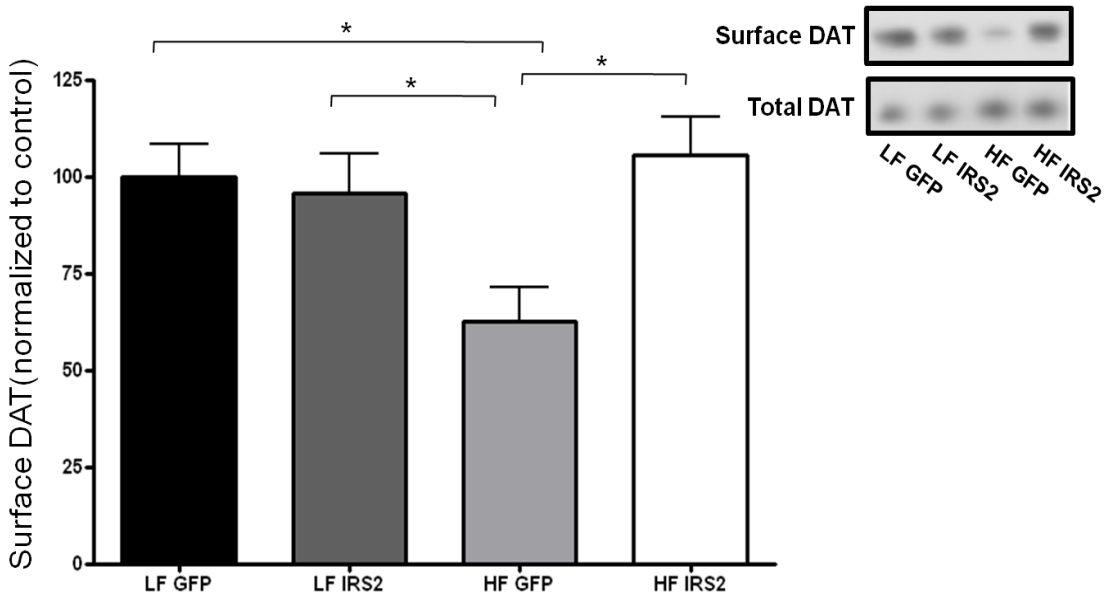


Figure 26. Viral-mediated expression of IRS2 restores surface expression of DAT. (A) Representative immunoblots (inset) of biotinylated (surface) and total rDAT obtained from LF and HF rats injected either with HSV-GFP or HSV-IRS2. Quantification of surface levels, normalized to total and expressed as a percentage of control (LF, HSV-GFP injected rats) are shown (*=$p < 0.05$ by one way ANOVA followed by Bonferroni post hoc test; n=6). All data are represented as mean ± S.E.M.

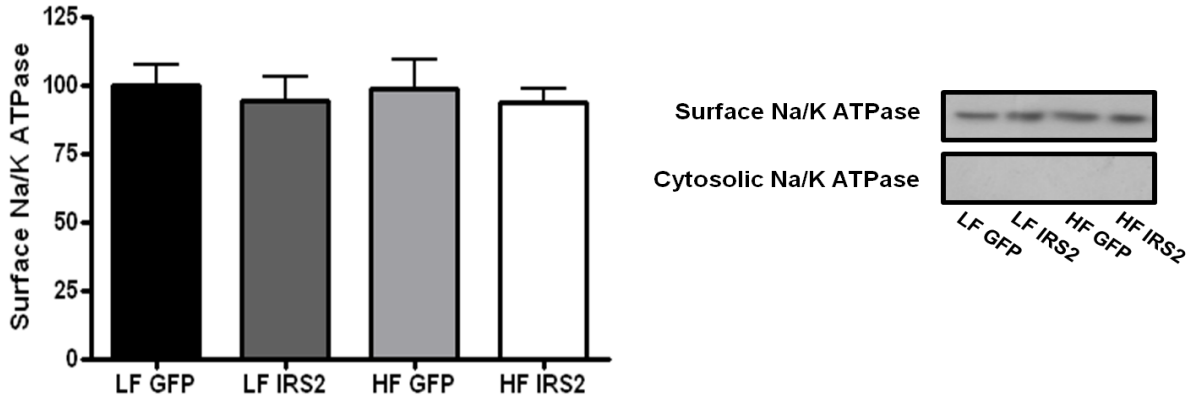


Figure 27. Viral-mediated expression of IRS2 does not alter the surface expression of Na/K ATPase. Representative immunoblots (inset) of biotinylated (surface) and total Na/K ATPase levels obtained from LF and HF rats injected either with HSV-GFP or HSV-IRS2. Quantification of surface levels are shown, expressed as a percentage of control. ($p>0.05$ by one way ANOVA; n=6)

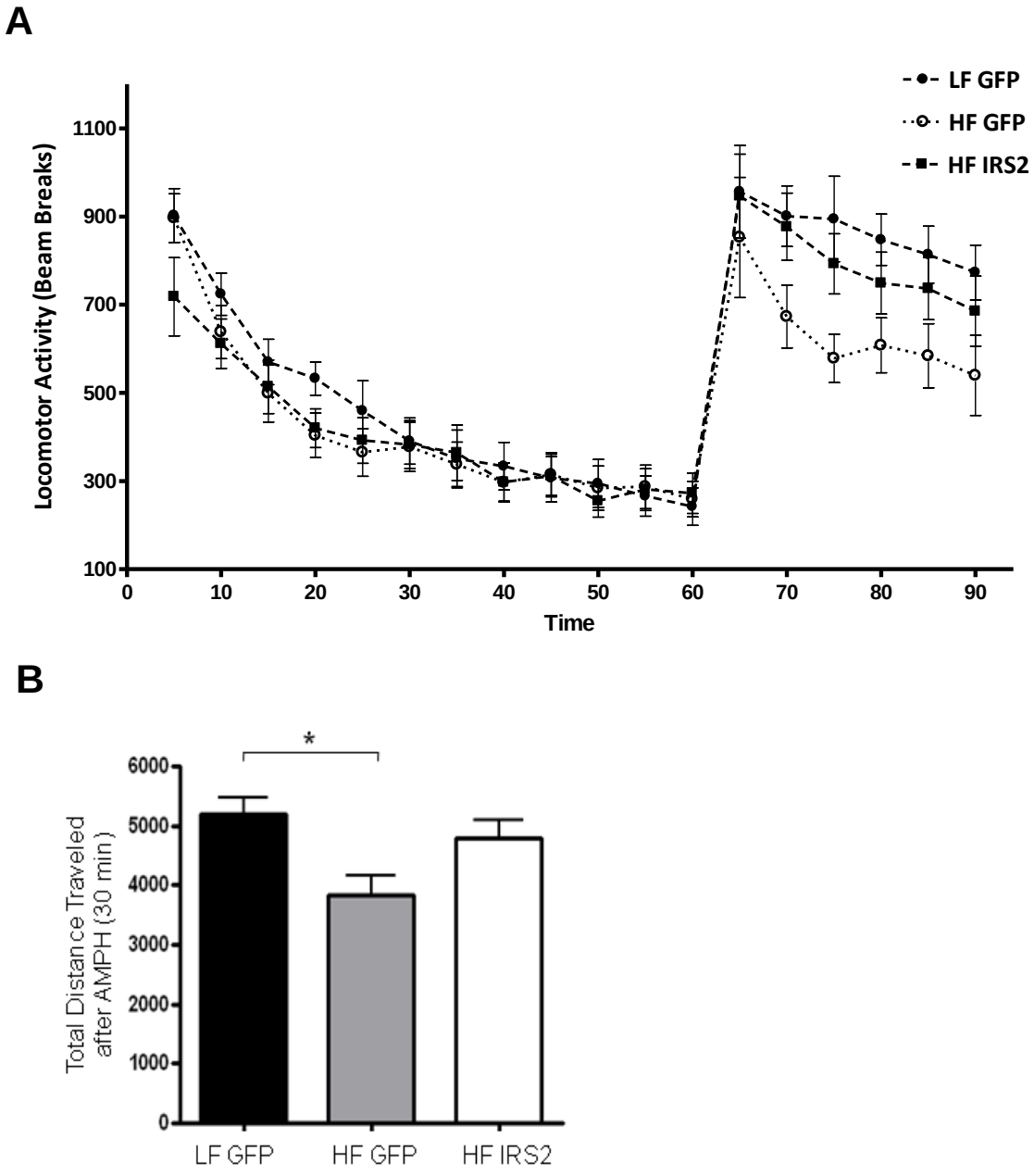


Figure 28. Viral-mediated expression of IRS2 restores surface expression of DAT and AMPH-induced locomotor activity in DIO rats. (A) Locomotor activity measured by beam breaks over time, with AMPH (arrow) given at 60 minutes (I.P., 1.78 mg/kg) to HF rats injected either with HSV-IRS2 (HF IRS2) or HSV-GFP (HF GFP) and LF rats injected with HSV-GFP (LF GFP). (B) Total distance traveled throughout a 30 min time period after injection of AMPH in HF IRS2, HF GFP and LF GFP (*=$p<0.05$ by one way ANOVA followed by Bonferroni post hoc test; LF GFP, n=13; HF GFP, n=12; HF IRS2, n=13). All data are represented as mean ± S.E.M.

The two main DA projections to the striatum originate from either the substantia nigra to the dorsal striatum, or from the ventral tegmental area to the ventral striatum. The ventral striatum does not underlie the primary motivation for feeding, since disruption of DA signaling in the nucleus accumbens does not impair food intake (Salamone, Correa et al. 2005; Palmiter 2008). This notion is further support by studies demonstrating that DA synthesis in dorsal striatum, in contrast to the ventral striatum, is essential for feeding (Cannon, Abdallah et al. 2004; Palmiter 2008). Thus, we focused our attention to whether the development of obesity impairs DA signaling in the nigrostriatal system and whether this dysregulation can be triggered by acquired dietary-induced brain insulin resistance. Here, we show that high-fat feeding decreases cell surface expression of a key element of DA homeostasis, namely, DAT. This decrease in DAT cell surface expression could therefore lead to a reduction in striatal DA clearance and mechanistically contribute to HRDS. Further work into changes in DA signaling in the nigrostriatal system during the development of obesity will be required uncover the role this signaling pathway plays in feeding and identify possible drug targets for pharmacotherapies of obesity.

The insulin signaling pathway, via Akt, regulates DAT trafficking to and from the plasma membrane as well as DAT-mediated DA clearance (Carvelli, Moron et al. 2002; Owens, Sevak et al. 2005; Williams, Owens et al. 2007). High-fat feeding and/or obesity leads to changes in insulin signaling (Edelman 1998; Clegg, Benoit et al. 2005; Posey, Clegg et al. 2009), including defects in Akt activation. After 28 days of high-fat feeding we found a reduction in

phosphorylation of Akt, at position Thr308, concomitant to the reduction in DAT plasma membrane expression. Importantly, we extended this observation by examining whether DIO impairs Akt phosphorylation in an isoform specific manner and observed specific defects in striatal Akt2 phosphorylation.

It is compelling then, given past work linking insulin signaling to DAT surface expression (Carvelli, Moron et al. 2002; Garcia, Wei et al. 2005; Williams, Owens et al. 2007), that inhibition of Akt2, not Akt1, affects levels of DAT at the plasma membrane. Since inhibition of Akt2 reduces the ability of AMPH to cause DA efflux *in vivo*, as measured by chronoamperometry, Akt2-mediated DAT trafficking, therefore, supports changes in DAT function and possibly DA homeostasis. Because AMPH relies on DAT-mediated reverse transport of DA in order to cause its behavioral effects, we sought to extend these findings to determine whether DIO affects AMPH-induced hyperlocomotion: indeed DIO leads to a significant reduction in AMPH-induced locomotion, further supporting the notion that DIO impairs DAT function.

In order to solidify the hypothesis that high-fat feeding causes decreases in striatal DAT cell surface expression by impairing Akt phosphorylation/activity we sought next to determine whether genetic rescue of insulin signaling also rescues DAT expression/trafficking in DIO animals. Overexpression of a viral vector expressing IRS-2, a kinase upstream of Akt, in substantia nigra increased phosphorylation of Akt in the DIO animals to a level comparable to that of low-fat fed control animals. The substantia nigra has DA projections to the dorsal striatum. Concomitant with rescue of Akt phosphorylation, IRS-2 gene therapy

also restored DAT cell surface expression in the dorsal striatum of DIO animals to the level observed on LF controls. Consistent with our functional model, normalization of DAT cell surface expression normalized the ability of AMPH to induce locomotion.

Thus, our work demonstrates that 4 weeks of a high-fat diet is sufficient to impair striatal DA clearance as well as the ability of AMPH to cause DA efflux and locomotion and this functional alteration is mediated by impaired activation of Akt isoform 2. Our results further suggest that high-fat feeding decreases DAT cell surface expression by inducing brain insulin resistance. As the role of DA signaling in feeding behavior begins to be revealed, a higher resolution understanding of alterations in DA systems by high-fat diets may yield insight into comorbid neuropsychiatric disorders and/or novel obesity therapeutic targets.

CHAPTER IV

GENERAL DISCUSSION, IMPLICATIONS, AND FUTURE DIRECTIONS

DA signaling plays an important role in mood, reward, behavior, and motor function. Imbalances in dopaminergic signaling are thought to underlie various psychiatric disorders, including Parkinson's disease, bipolar disorder, schizophrenia, drug abuse and ADHD (Wise 1998; Horschitz, Hummerich et al. 2005; Kienast and Heinz 2006; Volkow, Wang et al. 2007; Davis, Levitan et al. 2008; Koob and Le Moal 2008). DAT plays a critical role in dopaminergic signaling by taking up DA released into the synapse back into the presynaptic terminal (Borowsky, Adham et al. 2001). Importantly, DA signaling is influenced by several signals, including peptides that are well known for their role in regulating food intake and metabolism, such as insulin. Dysregulated insulin signaling and diabetes is seen in patients with DA-related disorders (Mukherjee, Decina et al. 1996; Dixon, Weiden et al. 2000; van Winkel, De Hert et al. 2008). Therefore, it is not surprising that researchers are discovering that insulin plays a role in regulating DA homeostasis. As a role for insulin in regulating DA-related behaviors begins to be revealed, understanding on a molecular level how peptides related to food intake, such as insulin, affect DA systems is needed. Therefore this dissertation: 1) further defines components of the insulin signaling pathway that alter DAT cell surface expression and 2) examines diet-induced molecular changes to insulin signaling and DAT function in the nigrostriatal

pathway. With obesity and diabetes rising in occurrence, further studies into how changes in insulin tone affect dopaminergic systems are warranted.

Obesity and Diabetes

According to the Center for Disease Control, obesity is quickly becoming an epidemic in our nation, with rates of obesity increasing markedly over the past 20 years (Flegal, Carroll et al. 2010). More than half of Americans are either overweight or obese, as defined by a BMI above 24 or 30, respectively (Mokdad, Bowman et al. 2001). Perhaps even more startling is the increase of obesity in children and adolescents. Approximately 31% of children, age 2-18, have a BMI above the 85th percentile for their age, sex, and height (Ogden, Carroll et al. 2010). Furthermore, these increases are leading to a financial burden to the health care system. It is estimated that there has been a 36% increase in spending on obesity and obesity related disorders (Finkelstein, Fiebelkorn et al. 2003). This trend is quite alarming, given the association between obesity and many chronic diseases, including type 2 diabetes, cardiovascular disease, several types of cancer, musculoskeletal disorders, sleep apnea, and gallbladder disease (Must, Spadano et al. 1999; Visscher and Seidell 2001).

Whereas obesity and diabetes mellitus continue to increase in occurrence, the understanding of how these conditions affect the brain is only beginning to be uncovered. Americans over consume foods, and often these are high fat foods. Several ideas were put forth that the signaling of insulin, and other hormones relating to food consumption such as leptin, were not just important to the

periphery, but also in the brain. It has been well established that these peptides signal to the hypothalamus, which helps balance our body's rate of energy expenditure to the amount of food we have consumed. Dysfunction here has been hypothesized to account for obesity, and surely does play a role. However, this view alone is too simplistic. Often individuals will eat when full, and the motivation for food and the pleasure of eating is believed to be an important component in feeding behavior. In fact, fMRI studies have shown activity in the areas of our brain known to be involved in pleasure and reward, which involve DA as a neurotransmitter, are activated as we eat something palatable (Stice, Spoor et al. 2008). Increased DA turnover and release occur in the nucleus accumbens and the dorsal striatum in response to feeding (Yoshida, Yokoo et al. 1992). As discussed, DA has been shown to have a role in motivation for food and the pleasure we feel from eating, but this role is not yet well understood. Interestingly, diabetes and obesity occur in patients with diseases involving DA dysfunction (Sandyk 1993; Morris, Zhang et al. 2008). This overlap implies an important interplay between the two systems. Insulin receptors are expressed in DA rich areas, including the substantia nigra, the VTA, and the striatum (Figlewicz, Evans et al. 2003). Therefore, it is plausible that insulin signaling in DA regions helps to regulate our feeding behavior. In order to understand overconsumption of food and obesity, it is critical to understand at a molecular level how metabolic signaling molecules such as insulin regulate DA homeostasis, and how this, as a whole, regulates food intake.

DA signaling comprises several components, all which provide an opportunity for regulation. Proper dopaminergic tone is dependent upon several factors, including the amount of DA synthesized, vesicular release of DA, DRs on the post synaptic cell, and termination of the signal by uptake of DA through DAT. PET imaging studies have demonstrated an inverse relationship between BMI and DAT binding in human subjects (Chen, Yang et al. 2008), pointing to alterations in the surface expression of DAT in obese subjects. The work in this dissertation focuses on the alterations in DAT cell surface expression after a 4 week period on a 60% high fat diet. Prior work has demonstrated that improper insulin signaling can modify DAT function (Owens, Sevak et al. 2005; Williams, Owens et al. 2007). These studies used an animal model of insulin depletion by injecting STZ, a drug that is toxic to the insulin producing β-cells in the pancreas. After treatment, these animals had a reduction in DAT function, shown by a reduction in DA clearance and AMPH-induced DA efflux as well as striatal Akt activity. Therefore, we hypothesize that insulin signals through Akt to maintain DAT on the cell surface, thereby regulating DAT function and DA transmission.

In this dissertation, this hypothesis was tested using a Diet Induced Obesity (DIO) model where rats were given a 60% high fat diet for 28 days. Control animals were kept on a 10% fat diet, which contains the same amount of fat as rat laboratory chow, but is also nutrient matched with the high fat diet. The data collected demonstrate that consuming a high fat diet alters insulin signaling, namely phosphorylation of Akt, in the nigrostriatal system. It also results in a reduction in DAT cell surface expression, DA clearance, and AMPH-induced

hyperlocomotion. Importantly, restoration of Akt signaling by overexpression of IRS-2 by viral injection restores DAT to the cell surface. This work indicates that insulin is signaling through Akt to regulate DAT function, demonstrating insulin regulation of DA neurotransmission.

To help identify how insulin modulates DAT function, it is important to define the pathway insulin signals through to regulate DAT cell surface expression. Studies examining PI3K and Akt, two important components of this pathway, show that they regulate the cell surface expression of DAT (Carvelli, Moron et al. 2002; Garcia, Wei et al. 2005; Lute, Khoshbouei et al. 2008). Here, the isoform specificity of Akt in regulating DAT was explored, which was possible due to the development of isoform specific inhibitors of Akt. Prior to this work, it was unknown if Akt regulation of DAT was primarily due to activation of insulin, being that Akt is stimulated by a variety of signals and controls several cellular processes in addition to serving as a mediator of insulin signaling, Akt signaling supports survival and cell growth (Hanada, Feng et al. 2004). Interestingly, the isoforms of Akt appear to serve distinct roles. It is known that Akt2 is the isoform responsible for mediating insulin signaling (Cho, Mu et al. 2001). In contrast, Akt1 is thought to govern signaling for growth and cell survival (Cho, Thorvaldsen et al. 2001). Therefore, establishing that Akt2 is the isoform of Akt regulating DAT helps to further define that insulin itself is mediating DAT function. In fact, Akt2 inhibition alone was sufficient to reduce DAT cell surface expression in the striatum, as well as inhibit AMPH-induced efflux. Therefore, this work supports

the hypothesis that insulin is an important mediator of DAT cell surface expression *via* PI3K and Akt2.

Previously, studies used these inhibitors to try and identify an isoform-specific target for tumor cells (DeFeo-Jones, Barnett et al. 2005). Rapid cellular growth is often characterized with high levels of active Akt, and researchers looked to define the isoform of Akt responsible in hopes to be able to define a pharmacological target for halting tumor growth without affecting the other important roles of Akt. However, they found that neither isoform was solely responsible, but only inhibition of both forms reduced tumor growth (DeFeo-Jones, Barnett et al. 2005). In contrast, here we did identify isoform specificity to a biological process. We found inhibition of Akt2 alone, not Akt1, can alter a component of DA signaling, specifically by reducing DAT cell surface expression. Alterations in DAT cell surface expression affect its function, allowing DA to remain in the synapse and enhance DA neurotransmission, thereby affecting dopaminergic tone. As such, this data displaying isoform specificity to DAT trafficking is particularly interesting for it is the first work using these inhibitors to demonstrate Akt isoform specificity. Furthermore, it allows for an isoform-specific pharmacological target for altering DAT function, which may be useful in treating diseases of DA dysfunction. For example, DA neurotransmission could be modulated by inhibiting Akt2, resulting in a decrease of DAT function. Knowing the specific isoform to target would prevent potential unwanted outcomes of inhibiting all isoforms of Akt. Perhaps defining a specific Akt isoform responsible in tumor growth was not possible because several processes are altered in this

state of cellular dysfunction, and therefore biological functions such as cell growth are greatly skewed. As such, extreme situations of DA dysfunction may also lead to differential regulation of DAT by Akt, and therefore further study is warranted before treatment of a disease.

Alternative Interpretations

High fat feeding and DIO. Here we examine alterations to DAT, a critical component in DA homeostasis, after 28 days on a high fat diet. We report that high fat feeding causes alterations to Akt phosphorylation and DAT cell surface expression. It is important to note that prior work using high fat diets demonstrates several changes throughout the body (Montague 2003; De Souza, Araujo et al. 2005; Posey, Clegg et al. 2009). Furthermore, alterations from high fat diets continue to be defined. Several studies have examined changes to components of DA neurotransmission in response to alterations in diet. For example, Geiger and colleagues utilized animals with either obesity-prone or obesity-resistant phenotypes to examine differences in dopamine release between the two groups. In this model, obesity-prone rats were hyperphagic and had a 20% higher body weight than the obesity-resistant rats. Using slice preparations from these animals, they found that electrically evoked DA release was significantly reduced in obesity-prone rats in the dorsal striatum, along with other DA regions such as the ventral striatum and prefrontal cortex, suggesting that there may be a widespread dysfunction in mechanisms regulating dopamine release (Geiger, Behr et al. 2008). Unlike the work in this dissertation, the model

of obesity used by the investigators in this study did not alter the type of diet. However, their data demonstrates alterations in stimulated release in an obese state. Therefore, it is possible that there are alterations to DA release upon stimulation in the DIO model as well. If so, such changes could influence the modifications we show in DAT cell surface expression and are an important consideration for future studies.

Another important consideration is the role of diet changes and obesity on DRs. For example, it is possible that the reduction in active Akt is affecting the presynaptic D2R, which has been shown to associate with DAT and affect its function (Lee, Pei et al. 2007). Therefore, Akt may be affecting the receptor first, and the effects seen on the transporter may be indirect. A recent study examined changes to D2R in the striatum in another model of obesity, which they refer to as a "cafeteria-style" diet. Johnson and Kenny fed animals several forms of "junk food" to model a typical poor diet that results in obesity. They found that striatal levels of the D2R were signficantly reduced in the animals fed a "cafeteria-style" diet compared to control animals (Johnson and Kenny 2010). However, D2Rs are found both pre- and postsynaptically, and the authors did not examine which population was altered. Still, modulation of either population could affect DAT cell surface expression and function, whether directly or indirectly. Therefore, changes to D2R are another important consideration for interpretation.

Pharmacological manipulations. The data in this dissertation indicate that Akt2 is responsible for sustaining DAT cell surface expression. These experiments made use of pharmacological inhibitors of Akt1 and Akt2. It is

important to note that treatment with these drugs affects all cells in the striatum, not only DA neurons. Therefore, it is possible that isoform-specific inhibition of Akt in other neuronal populations in the striatum could be the primary change, and changes to DAT cell surface expression may be secondary. Experiments utilizing a preparation that enriches for DA terminals would be useful to confirm that it is indeed alterations to active Akt2 in DA neurons responsible for changes in DAT cell surface expression.

Viral intervention with wild type IRS2. We clearly show that expression of wild type IRS2 in the substantia nigra leads to an increase in phosphorylated Akt and DAT cell surface expression, as well as changes to AMPH-induced locomotion. However, it is important to note that these data could have alternative interpretations. Viral expression in the substantia nigra could directly alter presynaptic components of DA neurotransmission, such as vesicular release, DA production, or changes to presynaptic DRs. In addition to modulation of DA signaling components, viral expression of IRS2 could alter receptors important to the excitability of the neuron itself. For example, it is possible that the surface expression of other proteins is modulated as well. To test this possibility, experiments looking for changes in other membrane bound receptors important to excitability, for example AMPA receptors, would be useful to determine if IRS2 injection is altering important components to neurotransmission beyond DAT.

Future Directions

Downstream effectors of Akt regulating DAT cell surface expression. With Akt2 identified as an important component in regulation of DAT cell surface expression, further work is now needed in determining what is downstream of Akt2 activation in order to alter DAT trafficking. This exact process is still unknown. Several kinases downstream of Akt2 have been identified (Frame and Cohen 2001; Kramer, Witczak et al. 2006; Thong, Bilan et al. 2007; Wieman, Wofford et al. 2007), allowing for several candidates for an intermediate between Akt2 and DAT. However, one model of transporter trafficking by insulin activation leads to a hypothesis for a similar model for regulation of DAT trafficking. The glucose transporter 4 (GLUT4) cell surface expression is mediated by insulin signaling *via* Akt2 (Sano, Kane et al. 2003). Prior studies demonstrate that adipocytes from Akt2 null animals had a decrease of GLUT4 at the cell surface, as well as decreased insulin-stimulated glucose uptake (Bae, Cho et al. 2003). Rescue of Akt2 by viral expression restored GLUT4 surface expression, while viral expression of Akt1 had no effect (Bae, Cho et al. 2003). Therefore, GLUT4 cell surface expression is mediated through Akt2. Importantly, this specificity is also demonstrated in this dissertation with Akt regulation of DAT. This allows for a potential model to identify molecules downstream of Akt2 that are responsible for DAT trafficking, particularly because one such molecule has been identified in GLUT4 trafficking. AS160 is a GTPase activating protein that catalyzes the inactivation of several Rab proteins (Kane, Sano et al. 2002). These proteins are known to be important in the translocation of molecules throughout the cell,

including inter-membrane trafficking (Gonzalez and McGraw 2009). Interestingly, AS160 is known to regulate GLUT4 cell surface expression, leading to the possibility that it may function in a similar manner to regulate the trafficking of DAT. For example, knockdown of AS160 by RNAi expression increases the basal levels of GLUT4 on the cell surface in adipocytes (Eguez, Lee et al. 2005). Importantly, inhibition of AS160 is dependent on phosphorylation by Akt (Bae, Cho et al. 2003; Thong, Bilan et al. 2007). In fact, GLUT4 increases in cell surface expression by activation of Akt2 have been shown to be reliant on the subsequent inhibition of AS160 (Eguez, Lee et al. 2005). This indicates that Akt2 activation leads to AS160 inhibition, supporting GLUT4 on the cell surface. DAT cell surface expression has been shown to be regulated in a similar manner, via insulin and Akt2. Therefore, AS160 should be targeted as a possible downstream effector of insulin receptor and Akt2 activation, resulting in modulation of DAT cell surface expression. Experiments testing whether inhibition or overexpression of AS160 alters DAT cell surface expression and DA uptake could examine this hypothesis. Furthermore, it would be interesting to determine whether the effects seen by inhibition of Akt2 on DAT cell surface expression require AS160.

This work defining Akt2 in the regulation of DAT cell surface expression helped to further define the role of insulin signaling in DA systems. The work in this dissertation also examined the effects of improper insulin signaling by using a model of obesity and insulin resistance; the DIO model. Importantly, the levels of active Akt2 in the striatum of DIO animals were reduced, whereas active Akt1

was unchanged. Further work examining changes to targets downstream of Akt2 in DIO animals would help to define the effecter proteins contributing to the changes seen with improper insulin signaling to DAT. If AS160 is involved in DAT trafficking, it would be expected that its phosphorylation is increased in HF fed animals compared to LF fed control animals, allowing it to remain active. Using viral techniques similar to those used with IRS-2, expression of dominant negative AS160 could reduce active AS160 and maintain DAT on the cell surface in HF rats. Conversely, overexpression of AS160 in LF animals should produce an effect similar to that seen in HF animals, with DAT reduced on the cell surface.

Although AS160 is an interesting candidate for regulation of DAT trafficking, it is important to note that there are many downstream effectors of Akt2 that could be regulating DAT trafficking. For instance, glycogen synthase kinase 3 (GSK3) is a kinase targeted by Akt. GSK3 is probably most well known for its role in activating glycogen synthase, which occurs when GSK3 is inhibited by Akt, allowing for the formation of glycogen upon insulin stimulation (Cohen, Alessi et al. 1997). Phosphorylation by Akt of either GSK3α at serine-21 or GSK3β at serine-9 promotes inhibition of GSK3 activity (Cohen, Alessi et al. 1997; Frame and Cohen 2001). GSK3 signaling has been shown to regulate the plasma membrane expression of the glucose transporter-1 (GLUT1) (Wieman, Wofford et al. 2007), integrins (Roberts, Woods et al. 2004), and the megalin receptor (Yuseff, Farfan et al. 2007). Therefore, as with AS160, it would be interesting to test whether GSK3 modulates DAT cell surface expression and

function. Also similar to AS160, activation of Akt inhibits GSK3 activity (Cohen, Alessi et al. 1997). Therefore, experiments similar to those described above for AS160 could test for the involvement of GSK3 in the regulation of the transporter. Alterations to GSK3 have been noted in schizophrenia (Emamian, Hall et al. 2004), a disease of DA dysfunction that has been linked to polymorphisms to Akt1 (Emamian, Hall et al. 2004; Bajestan, Sabouri et al. 2006; Ikeda, Iwata et al. 2006). In fact, GSK3 is the target of the drug lithium, a well-known treatment for another DA-related disease, Bipolar disorder. The relation of GSK3 signaling to diseases of DA dysfunction makes a possible interaction between DAT and GSK3 very intriguing. Evidence collected so far support regulation of GSK3 by Akt1, due to the changes seen in schizophrenic patients (Emamian, Hall et al. 2004), however this is far from conclusive and only an observation of a disease state. Much work remains to be done to establish the isoform specificity to Akt regulation of GSK3 in brain, and therefore the potential regulation of DAT function. AS160 and GSK3, as well as all effector proteins of Akt, should be explored as possible candidates for insulin regulation of DAT cell surface expression. Identification of the exact components of the pathway that insulin acts through to modify DAT function would provide a better understanding of how Akt regulates DA systems.

Alterations to DAT and interacting proteins. Another important component in understanding how insulin signaling affects DAT cell surface expression is to examine if the post-translational modifications and interacting proteins of the transporter is itself, and how they are altered with inhibition of Akt2 or in the DIO

model. For example, much work has been done examining the phosphorylation of DAT in response to activation of PKC, a well known regulator of DAT cell surface expression. PKC activators such as phorbol 12-myristate 13-acetate (PMA) and protein phosphatase inhibitors such as okadaic acid (OA) have been shown to induce DAT endocytosis and reduce transporter V_{max} (Daniels and Amara 1999; Melikian and Buckley 1999; Doolen and Zahniser 2001). Initial models suggested that this PKC-dependent downregulation of DAT activity is primarily due to the intracellular accumulation of the transporter (Zhang, Coffey et al. 1997; Zhu, Kavanaugh et al. 1997; Pristupa, McConkey et al. 1998; Daniels and Amara 1999; Melikian and Buckley 1999; Chang, Lee et al. 2001; Granas, Ferrer et al. 2003; Loder and Melikian 2003). It has been postulated that PKC downregulation of DAT is mediated by the phosphorylation of the transporter, therefore modifications such as phosphorylation could also be possible for insulin regulation of DAT cell surface expression. When examining the effects of PKC activation on DAT, direct phosphorylation of N-terminal serines was observed using $^{32}PO_4$-labeled DAT (Vaughan, Huff et al. 1997; Foster, Pananusorn et al. 2002). Such experiments looking at the phosphorylation of DAT after Akt2 activation by insulin, or Akt2 inhibition by inhibitors used in this dissertation, could help to define if DAT itself is phosphorylated in response insulin. Another possibility is to examine DAT mutants with truncations of the N-terminus or the C-terminus that are transfected into cell lines, or mutations with alterations to the amino acids that can be phosphorylated on these termini. One could treat these cells with the Akt2 inhibitor, I-Akt2, and look to see if DAT cell surface expression is reduced, as with

wild-type DAT, or not. If there is no change, then the region mutated is important to Akt2-mediated DAT trafficking. Overall, it is important to determine if post-translational modifications, such as phosphorylation, to DAT by insulin occur and determine what region of the transporter is important for insulin-mediated DAT trafficking. This would help to further define how insulin regulates DAT cell surface expression, which is critical to understand the interaction between insulin and DA systems.

*Mechanism of DAT trafficking in response to insulin signaling. D*ecreases to the surface expression of a protein can occur either by removal from the plasma membrane, or by prevention of insertion onto the cell surface, or both. For the former, experiments examining whether certain proteins known to be involved in internalization, such as clathrin or dynamin, are required for DAT surface expression changes would be useful. For example, the redistribution of DAT from the plasma membrane by a member of the insulin signaling pathway, PI3K, is known to be dynamin dependent (Carvelli, Moron et al. 2002). Therefore, one would hypothesize that modulation of DAT cell surface expression by other insulin signaling members, such as Akt2, is also dynamin dependent, but this remains to be tested. Defining if internalization of DAT is responsible for DAT surface expression changes, and then understanding how that internalization is accomplished is important to understand how insulin signaling regulates DAT cell surface expression. In fact, internalization of DAT is an extensive area of study, and recent work has indicated it could be dependent upon the location of DAT on the plasma membrane. This was examined by

again looking at PKC-dependent internalization. PKC stimulated trafficking of DAT is also believed to be dynamin-dependent, as with PI3K, as well as clathrin mediated (Daniels and Amara 1999; Sorkina, Hoover et al. 2005; Foster, Adkins et al. 2008). Foster and coworkers demonstrated that a combination of trafficking and trafficking-independent processes are involved in PKC-dependent downregulation of DAT activity based upon the location of DAT (Foster, Adkins et al. 2008). They found that PKC induced loss of cell surface DAT occurs only from non-raft populations. Conversely, in cholesterol-rich lipid raft microdomains, PKC activation led to an increase in DAT phosphorylation compared to non-raft populations, as well as a loss of DAT activity, but there was not a decrease in the cell surface expression (Foster, Adkins et al. 2008). This study indicates that non-raft DATs are regulated by trafficking events and that cholesterol-dependent nontrafficking regulatory mechanisms occur in lipid rafts. Due to the changes seen in this study in cell surface expression, one would hypothesize that only the non-raft populations of DAT are regulated by insulin signaling. Experiments looking at the localization of DATs regulated by insulin similar to those conducted looking at PKC and DAT would be needed to determine if this is the case. Understanding the population of DATs that are susceptible to regulation by insulin would help to further our knowledge on insulin regulation of DAT and DA systems. By determining the population of DATs on the plasma membrane that are affected by insulin signaling, therapies that are specific to that population can be developed. This allows for a more specific target for treatment of diseases with improper insulin signaling, such as insulin resistance and obesity.

With the emerging data indicating a role for insulin signaling in modulation of DA systems, the list of the detrimental effects of a high fat diet and insulin resistance grows beyond dysregulation of blood glucose levels. DA signaling is important for a variety of behaviors and implicated in several diseases, from schizophrenia, depression, addiction, ADHD, binge eating disorder, and several others (Wise 1998; Horschitz, Hummerich et al. 2005; Kienast and Heinz 2006; Volkow, Wang et al. 2007; Davis, Levitan et al. 2008; Koob and Le Moal 2008). Alterations to DA homeostasis by improper insulin tone, therefore, have important implications for DA-based behaviors. Here it is shown that a high fat diet results in a decrease in DAT cell surface expression, DAT function, and AMPH-induced DA efflux. These changes are rescued with overexpression of a kinase directly downstream of the insulin receptor, IRS2. Importantly, this trafficking event is attributed to Akt2 activity, the isoform of Akt known to be involved in insulin signaling. Overall, the work in this dissertation demonstrates that insulin signaling *via* Akt2 is regulating DAT function, and that a high fat diet leads to improper insulin signaling thereby altering DAT function. However, it is not fully understood how DA signaling alters feeding behavior. Future studies aimed at revealing the role of DA systems in feeding behavior, as well as how alterations in insulin affect this will help to elucidate the co-morbitity of obesity and DA-related disorders, and hopefully begin to identify treatments.

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