The Role of human Dopamine Transporter Threonine 261 and Serine 262 in Amphetamine Regulation of Transporter Function and Surface Expression

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The Role of human Dopamine Transporter Threonine 261 and Serine 262 in Amphetamine Regulation of Transporter Function and Surface Expression

A thesis submitted in partial fulfillment of the requirements for the degree of Master of science at Virginia Commonwealth University.

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List of Abbreviations

5-HT = Serotonin

ADHD = attention deficit hyperactivity disorder

Akt = Protein kinase B

AMPH = Amphetamine

CAMKII = Calcium-calmodulin dependent kinase II

Cdk5 = Cyclin dependent kinase

CNS = Central Nervous System

CPP = Conditioned Place preference

DA = Dopamine

DAT = Dopamine transporter

ERK = Extracellular signal-regulated protein kinase

ICL2 = Intracellular loop 2

IV = Intravenous

METH = Methamphetamine

NE = Norepinephrine

NET = Norepinephrine transporter

OA = Okadaic acid

PI-3-kinase: phosphoinositide 3 kinase

PKC = Protein kinase C

PMA = phorbol 12-myristate 13-acetate

SERT = Serotonin transporter

VMAT = Vesicular monoamine transporter
Abstract

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Amphetamine (AMPH) has been used clinically and recreationally around the world for years. A 2021 study reports a 67.5% rise in AMPH use from 2010-2017 across all fifty states. Study also shows that 30% of patients with AMPH-induced psychosis end up with primary psychosis over time. AMPH works by inhibiting monoamine (Norepinephrine (NE), Serotonin (5-HT), and Dopamine (DA) reuptake followed by triggering transporter mediated monoamine release and consequently increases monoamine concentrations in extracellular synaptic space. AMPH also works as a substrate for monoamine transporters, enters the neuron, and once its inside the neuron, it will prevent the storage of monoamines in vesicles by inhibiting vesicular monoamine transporter 2 (VMAT2). AMPH also regulates monoamine transporter surface expression by affecting transporters trafficking. Creating this neurochemical imbalance corrupts the normality of central nervous system function and results in reduced monoamines in vesicles. Because of the neurochemical imbalance AMPH causes, there has been no drug to fully treat and remain effective on abstinence. Recreational use of AMPH and Methamphetamine (METH) can cause addiction
which can eventually lead to death. Since monoamine transporter is a phosphoprotein, various kinases such as protein kinase C (PKC) downregulates the transporters. An in vitro study was done with mutated T258A and S259A motif in norepinephrine transporter (NET) sequence to see the double mutant (DM) transporter attenuating the effects of AMPH. The results showed low AMPH-mediated downregulation of DM as well as resistance to AMPH-induced endocytosis of NET. In rats, same motif was injected into intra-accumbal region by using membrane permeable TAT-NET peptide and the results indicate that TAT-peptide containing NET-T258/S259 motif blocked AMPH-mediated NET inhibition. Similarly, AMPH-induced conditioned place preference (CPP)/reinstatement and locomotor sensitization was attenuated by NET-T258/S259. Since human DAT and NET have similar sequence homology especially in intracellular loop 2 (ICL2), and AMPH affects all three transporters by the same mechanism, we explored the AMPH-mediated regulation of DAT by mutating the phosphorylation sites threonine 261 and serine 262 to non-phosphorylatable alanine in hDAT. We examined the mechanisms involved in AMPH-induced DAT regulation in human embryonic kidney cells (HEK-293) expressing wild-type (WT)-hDAT or double mutant (DM)-hDAT. Our uptake experiment reveals that treatment of AMPH on cells carrying WT-hDAT results in decrease in hDAT-mediated DA uptake while DM-hDAT carrying cells blunted the effects of AMPH. The kinetic analysis of DA uptake in HEK-293 cells transfected with hDAT showed that AMPH treatment resulted in a significant decrease in the maximal velocity ($V_{max}$) in WT-hDAT and a non-significant change in the case of DM, while not affecting the affinity ($K_m$) of DA for DAT. Our surface biotinylation studies showed decrease cell surface density of hDAT in AMPH-treated cells expressing WT-hDAT but not DM-hDAT. Therefore, our findings with previous results demonstrate that T261 and S262 are involved in AMPH-mediated DAT downregulation.
Introduction

History of Amphetamine (AMPH)

AMPH was “discovered” when Lazar Edeleano, Romanian chemist, synthesized the base, simply because he was interested in synthetic dyes but not its pharmacological activity (Edeleano, 1887). AMPH has been used clinically and recreationally around the world for years. AMPH (α-methylphenethylamine) was first discovered by Barger and Dale in 1910. However, later in 1927, for the purpose of creating a substitute for ephedrine, G. A. Alles synthesized AMPH. Alles and others performed experiments in human and animal subjects to conclude that AMPH has ability to produce arousal and insomnia and reverse drug-induced anesthesia (Bett, 1946; Guttmann and Sargent, 1937). In 1932, Gordon Alles got a patent on AMPH as to specify its medicinal use. In 1934, he assigned his patent to Smith, Kline, and French (SKF) drug firm of Philadelphia where around 1935, supervised AMPH’s additional clinical development and confirmed that AMPH had strong patent protection in Europe and elsewhere (Piness, Miller, & Alles, 1930; Rasmussen, 2008) and then tradename Benzedrine® was given. In 1935, Benzedrine was released to market by Smith, Kline, and French to be used as a treatment of mild depression, narcolepsy, post-encephalitic Parkinsonism, and a few other disorders. (Bett, 1946; Guttmann and Sargent, 1937; Tidy, 1938). But as more research was done, it was clear to them that AMPH existed in 2 active forms Dextro (d-) (Dexedrine®), which was more potent and Levo (l-) (Cydril ®). Dexedrine and Benzedrine had unrestricted sales until 1939, the drugs could then be purchased by a prescription or signing the Poison Register (Bett, 1936). Charles Bradley, psychiatrist, was first to report the benefits of Benzedrine in treatment of children with behavioral problems now known as ADHD. (Bradley, 1937; American Psychiatric Association, 1994). In 1970s, the clinical trials established that both
AMPH isomers, \(l\)-AMPH and \(d\)-AMPH were effective for treatment of ADHD (Arnold et al., 1972, 1973, 1976). Current medications have an enantiomeric mixture of both \(d\)- and \(l\)- with a 3:1 ratio. Some brand names include Adderall® (Immediate release), Adderall XR® (Extended release), and Vyvanse® (Lisdexamfetamine) which is L-lysine bond to d-amphetamine via amide linkage (Heal et al., 2013).

**Synthesis and precursor of AMPH and METH**

AMPH and METH are synthetic drugs and have many precursors as shown in figure 1. To manufacture AMPH and METH, the primary precursor chemicals are ephedrine, 1-phenyl-2-propanone (P-2-P) and pseudoephedrine. While ephedrine and pseudoephedrine can be found in manufacture of bronchodilators and decongestants, P-2-P has a primary use for production of AMPH and METH by pharmaceutical industry. On top of that, phenylacetic acid is used as a precursor for P-2-P. Another precursor used for AMPH synthesis is norephedrine (UNODC, 2014).
AMPH synthesis is based on reduction of P-2-P in presence of methylamine or ammonia. Leukart method, a non-metal reduction reaction, is one of the most popular reduction methods for AMPH synthesis. As presented in figure 2, for the first step for AMPH synthesis is heating a mixture of P-2-P and Formamide (or ammonium formate) until it results in production of intermediate product, N-formylamphetamine; which is hydrolysed in the next step using hydrochloric acid (HCl). The product is then precipitated out of the solution and AMPH base is an oily liquid with “fishy-amine” odor (United Nations Office on Drugs and Crime et al., 2006).
Figure 2. Leuckart reaction for AMPH synthesis. Pictural representation of AMPH synthesis as described. (Adapted from (United Nations Office on Drugs and Crime et al., 2006)).

Epidemiology

In 2015, AMPH-like stimulants had between 13.8 million and 53.8 million users (world drug report 2015; https://www.unodc.org). A 2018 paper shows that in 2016, about 6.1 million children in U.S. were diagnosed with ADHD. Out of the 5.4 million of them that currently had ADHD, about 62% of them take medications and about 46.7% of them received behavioral treatment (Danielson et al., 2018). ADHD is one of the most prevalent mental disorders among children in the United States. (O’Connell et al., 2009). In United States, ADHD has a prevalence rate of 5.5% to 9.3 in 4- to 17-year-old children and adolescents (CDC, 2010) and a prevalence rate of 4.4% in 18- to 44-year-old adults (Kessler et al., 2006). According to United Nations World Drug Report in 2019, it was projected that about 0.6% of global population between the ages of 15-64 (which is about 29 million people) used AMPH in 2017. About 5.1 million people in U.S. aged 12 or older misused prescription stimulations in 2020 according to National Survey of Drug Use and Health. Based on 2018 survey, about 1.9 million people in U.S. have been using AMPH steadily since 2015 and deaths caused by overdose have increased by 33% from 2015 to 2016; not
to mention around 10,333 overdose deaths in 2017 (Kariisa et al., 2019). The major demographic characteristics with increased risk of AMPH abuse are Rural areas, Caucasian, Hispanic and Asian ethnicities, lower socioeconomic status, male, preexisting mood disorder adverse childhood events, and prior substance use disorder (Callaghan et al., 2018; Dhein et al., 2018; Ma et al., 2018). About 59% of patients with AMPH use disorder are being referred by criminal justice system to enter substance abuse treatment centers compared to about 38% of patients with all other substance use disorders combined. AMPH users are more likely to receive long-term treatment compared to other drug abusers. Study also shows that 30% of patients with AMPH-induced psychosis end up with primary psychosis over time (Mullen et al., 2020). According to World Health Organization estimates, AMPH and METH are the second most common form of illicit substance abuse (world drug report 2015; https://www.unodc.org). Since COVID-19 pandemic started on March 2020, it had a huge impact on AMPH abusers. METH, developed from its parent drug, AMPH (NIDA), causes pulmonary hypertension and cardiomyopathy (Zhao et al., 2018) and there is a huge risk for hostile COVID-19 outcomes in METH users. COVID-19 compromises lung functions and it can put METH users at risk. Diseases such as chronic obstructive pulmonary disease, cardiovascular disease have been shown to exacerbate prognosis with coronaviruses including the ones causing Middle East respiratory syndrome and severe acute respiratory syndrome (NIH, 2020). When it comes to COVID-19 care, patients with history of substance abuse are deprioritized for care. Support groups have a huge influence on helping drug abusers but due to social distancing, the support groups are canceled or switched to online virtual meetings which are not as helpful as in person meetings. Pandemic stress urges abusers to turn to substance to alleviate their negative feelings (Volkow, 2020). A 2021 study reports a 67.5% rise in AMPH use from 2010-2017 across all fifty states (Vaddadi et al., 2021).
Pharmacology of AMPH

In humans, AMPH administration in form of ADDERALL®, with d-AMPH and l-AMPH in ratio of 3:1, reaches peak plasma concentration in 3 hours. The half-life for d-AMPH in adults is 10 hours compared to 13 hours half-life of l-AMPH in adults (FDA). One study showed that AMPH levels are not affected by food and maximum cardiovascular effects of AMPH occurred generally 1 hour after administration, while behavioral effects occurred after 2 hours (Angrist et al., 1987). Recreational use of AMPH in form of METH can cause addiction which can lead to neurochemical imbalance, tachyphylaxis, cognitive deficiency, psychosis, and eventually organ damage and death (Baig, 2018). For treatment, Benzodiazepines such as midazolam, diazepam, or lorazepam in form of IV has been used with acutely agitated psychotic patients as first-line agents; but for second-line agents, if necessary, antipsychotic medicines such as haloperidol, olanzapine, risperidone, and ziprasidone were found to be successful in managing AMPH-associated psychosis. Other signs of hyperadrenergic and agitation has been resolved by lipophilic beta-blockers like labetalol and metoprolol (Richards et al., 2015). However, METH-induced depression, excessive sleeping anxiety, psychosis and a few other related symptoms must be treated at the same time with combination of drugs since no drug has been effective when administered alone. No treatment regimens remained effective on abstinence (Baig, 2018). Clinically, AMPH has been used as in treatment for ADHD and other CNS disorders such as narcolepsy (Heal et al., 2013). AMPH has many routes of administration such as inhalation, IV, intramuscular, or transmucosal. AMPH is metabolized through cytochrome CYP2D6 pathway and are excreted renally and hepatically. Psychiatric disorders related to AMPH use are often linked with acute and chronic use of AMPH, and most studies have been done with METH for these disorders. AMPH is classified as phenethylamines, a class of CNS stimulants. AMPH is highly
distributed in body and stimulates CNS due to containing a methyl group to alpha position on its carbon chain which made AMPH lipophilic (Mullen et al., 2020). METH is a sympathomimetic drug just like other AMPHs. Small doses of IV administration leads to prominent central stimulant effects (Nurnberger et al., 1984; Hoffman & Lefkowitz, 1982; Schnidler et al., 1992). Absorption of METH (after consumption) occurs in the GI tract (Caldwell, 1976) and about 90% of METH is eliminated in urine (Shimosato et al., 1986). About 22% of METH is excreted unchanged in urine of pH 6-8, and 4-7% as AMPH (Schepers et al., 2003). Drugs of abuse (such as AMPH) elevate levels of DA in nucleus accumbens. This area is highly critical for rewarded behaviors. Figure 3 shows Pathways for DA signaling, and DAT is expressed in pathways. DAT plays important role in controlling DA neurotransmission by clearing synaptic DA (Brunton et al., 2018).
Figure 3. Dopaminergic projections in the brain. This is pictorial representation of DA projections in CNS. This shows nigrostriatal pathway (DA projection to dorsal striatum), mesocortico/mesolimbic pathway (DA projection to nucleus accumbens), and tuberoinfundibular pathway (DA projection to hypothalamus). (Adapted from Brunton et al., 2018)).

Role and synthesis of DA

DA has many functions such as motor activity, mood, and cognition. Imbalances in brain levels of DA can be associated with Parkinson’s disease, bipolar disorder, drug addiction, and ADHD (Kristensen et al., 2011; Pramod et al., 2013). As seen in Figure 4, Initial step in DA synthesis is conversion of dietary phenylalanine to tyrosine by phenylalanine hydroxylase. Tyrosine then crosses into brain via uptake and is converted to L-dopa by tyrosine hydroxylase (which is the rate limiting step of the process). Via the enzyme L-aromatic amino acid decarboxylase (AADC), L-dopa is converted to DA. As seen in Figure 5, DA is stored inside VMAT2 until it is released by exocytosis. Synaptic DA activates dopamine receptors in pre-
(autoreceptors) and postsynaptic (D1 and D2) neurons. DAT terminates this action by reuptake of DA from synapse. In CNS, the 3 major pathways for DA projection are mesocortico/mesolimbic (in ventral tegmental area), tuberoinfundibular (in hypothalamus), and nigrostriatal (in substantia nigra pars compacta). Besides the diseases mentioned previously, dysregulation of dopaminergic system can also cause Tourette syndrome and schizophrenia. An important pathway is mesolimbic which is associated majorly with reward and minorly learned behaviors. Dysfunction in mesolimbic pathway can cause psychoses, schizophrenia, addiction, and learning deficits (Brunton et al., 2018).
**Figure 4.** DA synthesis and inactivation. DA is synthesized from conversion of l-phenylalanine to l-tyrosine to l-DOPA to DA. DA is broken down by either COMT or MAO/ALDH to HVA. Enzymes are in blue and co-factors are in black. (Adapted from (Brunton et al., 2018)).

**How Monoamine Transporter function**

Although the exact mechanism of AMPH is unknown until now, a large number of research has clarified the partial mechanism of AMPH. AMPH targets the monoamine transporters DAT, NET and SERT. Generally, many pharmacological agents including antidepressants, neurotoxins, and psychostimulants target the monoamine transporter which can alter brain function (Barker and Blakely 1995; Amara and Sonders 1998; Miller et al. 1999). DAT, along with other monoamine transporters have similar mechanism of action that involved their corresponding monoamine. As a part of SLC6 gene family of Na+/Cl− dependent transporters, it is believed that the monoamine transporters consist of 12 transmembrane-spanning domains in which both the N- and C-terminals are placed in cytoplasm (Foster et al., 2006). Monoamine transporters are vital for regulation of extracellular levels of neurotransmitters; and they rely on co-transport of Na+ down its electrochemical gradient to accelerate uptake of amines from inter- and extra-synaptic space. This reuptake of monoamines by the transporter from the synapse controls the intensity and duration of monoamine signaling (Axelrod, 1965; Jardetzky, 1966; Forrest, 2008). Monoamine transporters uptake function is active when the substrate with Na+ and Cl− produce conformational change which shifts the “outward-facing” of the transporter to “inward-facing”. Substrate is exposed extracellularly in the “outward-facing” and exposed intracellularly in the “inward-facing” phases of the transporter (Jardetzky, 1966; Forrest, 2008; Erreger et al., 2008; Shi et al., 2008; Kniazeff et al., 2008).
Effect of AMPH on Monoamine transporters

Acting as a substrate for monoamine transporters that gets transported into cytosol, (Rothman and Baumann, 2003; Sulzer et al., 2005) AMPH works by inhibiting monoamine (NE, 5-HT, DA) reuptake and increases monoamine concentration in pre-synaptic neuron and extracellular synaptic space (Mullen et al., 2020). AMPH and METH are the most potent at NET, their potency decreases about 5-9 fold at DAT and 200-500 fold at SERT (Han and Gu, 2006). The substances that produce dependence function by increasing the extracellular concentrations of NE and DA (Koob and Bloom, 1988; Di Chiara and Imperato, 1988; Wozniak et al., 1991). AMPH has very similar structure to DA and NE, therefore, it is carried by DAT or NET inside the neurons (Ritz et al., 1988) and it forces DA and NE out of VMAT (Sulzer, 2010). Consequently, increased levels of DA/NE forces DAT/NET to perform efflux of DA/NE into synaptic cleft (Sulzer et al., 2005; Carvelli et al., 2010). AMPH effects are also accompanied by inhibition of monoamine oxidase and reuptake inhibition which can associate synergistically or additively to increase monoamine concentrations in synapse (Heal et al., 2013). Since AMPH acts as a substrate on monoamine transporters, then its action can be blocked by uptake inhibitors like nomifensine and cocaine (Jones et al., 1998; Sulzer et al., 1995; Schenk, 2002; Seiden et al., 1993; Raiteri et al., 1979; Heikkila et al., 1975; Parker et al., 1988). AMPH and METH are DAT substrates that compete with DA for transport while cocaine is a transport inhibitor that binds to DAT and block transmitter uptake without being transported (Foster et al., 2006).
Figure 5. Illustration of dopaminergic synapse. This figure shows the synthesis of DA from the initial step of phenylalanine converting to Tyrosine. Tyrosine then enters the presynaptic terminal and is converted to DA by TH and AADC. Synthesized DA is then stored in VMAT2 until it’s released out to the synapse where it will bind postsynaptic as well as autoreceptor DA receptors. It’s also seen that the neurotransmission is terminated by DAT when it uptakes DA back into the presynaptic neuron where it can be stored back into VMAT2 or it can be broken down by MAO or ALDH and COMT and final metabolic product is HVA. Cocaine is seen as an inhibitor of DAT where it inhibits the uptake by DAT. (Adapted from (Brunton et al., 2018)).

Downregulation of DAT via AMPH and Kinases

Besides the reverse transport mechanism of AMPH, research has proven that exposure to AMPH decreases surface expression of DAT in heterologous expression systems (Chi and Reith, 2003; Gulley et al., 2002; Kahlig et al., 2006; Saunders et al., 2000), in vivo (Sandoval et al., 2000, 2001), and in striatal synaptosomes (Johnson et al., 2005a, Mannangatti et al., 2021). Many studies over the years compared the effect of AMPH with kinases downregulating DAT. Reverse
transport, forward transport, and cell surface expression are properties of DAT that are regulated by psychostimulant drug exposure and signaling pathways. Alteration of these properties can influence total DA clearance capacity hence neurotransmission. Multiple kinases have been shown to regulate DAT function such as PKC, CAMKII, and ERK (German et al., 2015; Vaughan and Foster, 2013). However, most of the studies have been done specifically with PKC. Studies demonstrated that human, rat, and mouse isoform of DATs (hDAT, rDAT, and mDAT respectively) are all phosphoproteins through metabolic phosphorylation of DAT with $^{32}\text{PO}_4$. Increased phosphorylation of DAT using PKC activators, such as phorbol 12-myristate 13-acetate (PMA), and protein phosphatase inhibitors, okadaic acid (OA), has been confirmed in previous studies as well as AMPH and METH (Huff et al. 1997; Vaughan et al. 1997; Cowell et al. 2000; Granas et al. 2003; Lin et al. 2003; Cervinski et al. 2005). It’s been shown in many studies that PMA-induced activation of PKC decreases DA transport capacity in several cell lines transfected with DAT (M. Y. Chang et al., 2001; R. Chen et al., 2009; Daniels & Amara, 1999; Doolen & Zahniser, 2002; Eriksen et al., 2009; Melikian & Buckley, 1999; Reith et al., 1997; Sorkina et al., 2005; S. J. Zhu et al., 1997). There has been reports of similar downregulation in studies with synaptosomes (Copeland et al., 1996; Foster et al., 2008; Foster et al., 2002; Vaughan et al., 1997). Interestingly, many (Cervinski et al., 2005; Chen et al., 2009; Fog et al., 2006; Johnson et al., 2005; Richards and Zahniser, 2009) but not all studies (Boudanova et al., 2008) show that pretreatment of cells or striatal tissue with METH or AMPH causes numerous responses such as efflux, endocytosis, and DAT downregulation with dependence on PKC and/or CAMKII. PKC activation can lead to reduction of transport $V_{\text{max}}$, elevated efflux $V_{\text{max}}$ and enhanced DAT internalization (German et al., 2015; Vaughan and Foster, 2013) which can result in extracellularly increased DA levels indicating that PKC is a positive regulator for neurotransmission of DA (Foster & Vaughan,
Interestingly, AMPH and METH effects are blocked by PKC inhibitors (Cervinski et al., 2005). Similarities between PMA and substrate-induced effects are indications of common mechanism between the two (Foster et al., 2006).

A rationale for study

Because of the neurochemical imbalance caused and consequent neuroplasticity adaptations, there has been no drug to fully treat and remain effective on abstinence, most likely because the neurobiology underlying the disease is complex and not completely understood. Thus, there is an unmet need to gain a deeper knowledge of neurobiological targets with causal and mechanistic links to AMPH use disorder. Since monoamine transporters are phosphoprotein, various kinases regulate the transporters through post-translational modification. However, which phosphorylation site(s)/motif(s) in DAT involved in specific kinase mediated phosphorylation is largely unknown. One study identified Threonine 53 on DAT as a phosphosite responsible in regulation of DAT kinetic properties when phosphorylated in response to AMPH (Challasivakanaka et al., 2017). Mutation of that site to avoid phosphorylation led to decrease DAT $V_{\text{max}}$ and loss of AMPH-induced substrate efflux which indicates that Threonine 53 has a huge role in DAT kinetic mechanism (Foster et al., 2012). One study showed inhibition of AMPH-induced DA efflux by PKC inhibitor, Go6976, and by mutation of serine 4,7,13 residues (shown in Figure 6) to alanine. Their results indicate the PKC phosphorylation of the residues in N-terminal of DAT contributes to AMPH-induced DA efflux (Wang et al., 2016). However, phosphorylation of DAT can occur on other residues of DAT such as ICL2. Our lab has been successful targeting those residues to prevent phosphorylation. For example, Threonine 258 (T258) and Serine 259 (S259) phosphorylation of NET is involved in PKC-mediated NET trafficking and NE uptake. Mutation of T258 and S259 to non-phosphorylatable alanine attenuated
AMPH-induced NET endocytosis and NE uptake (Annamalai et al., 2010). As to translate in-vitro cell culture study to in-vivo model, membrane-permeable TAT-sequence conjugated NET-T258/S259 motif was injected into rat intra-accumbal region to intervene T258/S259 phosphorylation in intact NET protein. Consistently, TAT- NET-T258/S259 motif blocked AMPH-mediated NET inhibition. Behaviorally, TAT- NET-T258/S259 motif infusion also attenuated AMPH-induced CPP, reinstatement, and locomotor sensitization. (Mannangatti et al., 2018). One study done to measure the effects of AMPH on DA and DAT levels in vivo (using mice synaptosomes) demonstrated that treatment with AMPH significantly decreased DAT-mediated DA-uptake and also AMPH treatment decreased surface DAT while increasing intracellular DAT (Mannangatti et al., 2021) without altering total DAT expression. It is unknown how AMPH affects DAT activity and trafficking at the molecular level and what are the motif(s) engaged in AMPH-linked DAT regulations. Understanding the causal relationship of AMPH interactions with its target DAT will open a new opportunity to develop novel therapeutic agents to treat AMPH-use disorder.
Figure 6. Phosphorylation sites in DAT. Phosphorylation sites in DAT are shown in yellow, green, and cyan for serine, threonine, and tyrosine respectively. The target of our research are Threonine 261 and Serine 262 on ICL2. (Adapted from (Bermingham & Blakely, 2016)).

In 1992, Giros et al., cloned the first hDAT (Figure 7). Figure 7 shows the nucleic acid and amino acid sequences of hDAT with transmembrane domains separated in boxes. Comparing Figure 6 to Figure 7, the sequence for ICL2 amino acids can be found immediately after transmembrane domain 4 box where threonine 261 and serine 262 can be seen.
Figure 7. Amino acid sequences of cloned human DAT. This was done in 1992 by Giros et al., and it shows the nucleic acid and amino acid sequences of their cloned hDAT. All 12 transmembrane domains have been boxed. (Adapted from Giros et al., 1992).

More fascinating data from that paper is shown in Figure 8. In Figure 8, Giros et al., compared the protein sequences of hDAT, to hNET (as well as bovine and rDAT). It can be seen that hDAT...
and hNET has similar consecutive protein sequences especially between the transmembranes 4 and 5. Amino acid sequence alignment NET and DAT revealed similar sequences in ICL2.

Figure 8. Comparison of the protein sequences of human DAT and human NET. This figure shows how the similarity between hDAT and hNET (as well as rat and bovine DAT). All the conserved protein sequences are boxed. (Adapted from (Giros et al., 1992)).
Objectives

Therefore, due to similarities seen between DAT and NET, and our previous results from our work with AMPH and mutating NET sequences, we hypothesize Threonine 261 and Serine 262 in DAT (Figure 6) could modulate AMPH-mediated DAT regulation and DA-transport, and that mutating Threonine 261 and Serine 262 to Alanine will attenuate or block AMPH- effects on DAT function and regulation.

Methods

Site-directed mutagenesis and polymerase chain reaction

The following primer was used to confirm the mutations by automated sequencing of entire DNA sequences for both sense and anti-sense strands. (5’ to 3’):

DAT (S): GAC CAC ACC TGC CGA G
DAT (AS): AG GTA TGC TCT GAT GCC

The following primer was used to create DM hDAT (5’ to 3’):

T261A-S262A Sense: aag ggc gtg aag gcc gca ggg aag gtt gta tgg
T261A-S262A Anti-Sense: cca tac ctt ccc tgc ggc ctt cac gcc ctt

| **Table 1. Components for reaction mixture for PCR** |
| --- | --- |
| **Components** | **Volume (µL)** |
| Reaction Buffer | 5 µL |
| hDAT DNA (75 ng) | 7.5 µL (10 ng/µL from stock) |
| T261A/S262A Sense | 5 µL |
| T261A/S262A Antisense | 5 µL |
| 10 mM dNTPs | 1 µL |
Quick solution & 1.5 µL 
Hyclone water & 25 µL 

1 µL of QuickChange™ Lightning enzyme was added to above components.

Table 2. PCR conditions

<table>
<thead>
<tr>
<th>Step</th>
<th>Cycle step</th>
<th>Temperature</th>
<th>Time</th>
<th># of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial Denaturation</td>
<td>94°C to 98°C</td>
<td>3 minutes</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>94°C</td>
<td>30 seconds</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Annealing</td>
<td>55°C</td>
<td>30 seconds</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Extension</td>
<td>80°C</td>
<td>30 seconds</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Repeat step 2-4 for 35 cycles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Final Extension</td>
<td>70°C to 80°C</td>
<td>5 minutes</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>Hold</td>
<td>4°C</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>

After PCR termination, Dpn-1 digestion was done by adding 2 µL of Dpn-1 restriction enzyme directly to the PCR reaction mixture and incubated for 1 hour at 37°C.

Molecular transformation in E.Coli DH5-alpha

Two 14-mL BD Falcon polypropylene round-bottom tubes were pre-chilled on ice (one for WT-hDAT and one for DM-hDAT) and SOC medium was preheated to 42°C. Once the cells thawed on ice, cells were mixed gently and aliquoted 50 µL into the pre-chilled tubes. 50 ng of WT-hDAT or DM-hDAT was added to respective tubes and tubes were incubated in ice for 20 minutes. In 42°C water bath, tubes were heat-pulsed for 45 seconds, and then incubated in ice for
2 minutes. Then, 150 µL of the preheated SOC medium was added to the tubes and tubes were incubated for 1 hour at 37°C while shaking at 225-250 rpm. Next, about 200 µL of transformation mixture was plated on LB agar plates containing ampicillin. Plates were then incubated overnight at 37°C.

**Plasmid isolation**

After ~16-hour of post incubation of plates at 37°C, individual colony was picked from the LB agar plate, and then inoculated into 3 mL LB broth with ampicillin and kept at shaking for 6 hours at 37°C. Then, 300 µL of liquid culture was inoculated into 300 mL of LB broth with ampicillin and kept overnight shaking at 37°C. Next step was to Pellet 300 mL of overnight LB culture at 6000 x g for 15 minutes at 4°C And later homogenously resuspend the bacterial pellet in 6 mL buffer P1. 6 mL of buffer P2 was added and mixed, and then incubated at room temperature for 5 minutes. During incubation, cap was screwed onto the outlet nozzle of QIAfilter Cartridge and QIAfilter Cartridge was placed into a convenient tube. Then, 6 mL of prechilled buffer P3 was added and mixed. Lysate was then poured into the barrel of the QIAfilter Cartridge and was incubated at room temperature for 10 minutes. Next step was to equilibrate the HiSpeed Tip with 4 mL buffer QBT, to allow it to enter the resin. Then cap was removed from QIAfilter Cartridge outlet nozzle and plunger was inserted into the cartridge and filtered cell lysate into the HiSpeed Tip. HiSpeed Tip was washed with 20 mL Buffer QC after lysate has entered. DNA was eluted with 5 mL buffer QF. 3.5 mL isopropanol was added, mixed, and incubated for 5 minutes to precipitate DNA. While incubated, plunger was removed from 20 mL syringe and QIAprecipitator Module was attached to the outlet nozzle. QIAprecipitator was then placed over a waste bottle to filter the eluate-isopropanol mixture through. QIAprecipitator was removed from syringe and plunger was pulled out. And then QIAprecipitator was re-attached and 2 mL of 70% ethanol was
added to syringe. DNA was washed by inserting plunger and pressing ethanol through QIAprecipitator. QIAprecipitator was removed from syringe and plunger was pulled out. QIAprecipitator was re-attached and plunger was inserted and membrane was dried by forcefully pressing air through the QIAprecipitator repeatedly for several times. Then, outlet nozzle of QIAprecipitator was dried using adsorbent paper. Next, the plunger was removed from a new 5 mL syringe, QIAprecipitator was attached, and outlet was held over a 1.5 mL collection tube. 1 mL buffer TE was then added to the 5 mL syringe. Plunger was then inserted and eluted the DNA into the collection tube using constant pressure. QIAprecipitator was removed from 5 mL syringe, plunger was pulled out and re-attached the QIAprecipitator to 5 mL syringe. Lastly, eluate from previous steps was transferred to 5 mL syringe and eluted for a second time into the same 1.5 mL tube.

**Quantification of plasmid DNA**

Samples were removed from freezer and left to thaw in ice. Samples were then mixed by gently tapping side of the tube. 4 µL of respective DNA sample were added in 996 µL of deionized water. Deionized water was served as blank. Samples were mixed by vortexing and left for 10 minutes to ensure the suspension of DNA. Respective DNA samples were transferred into a cuvette and quantified using spectrophotometer. Concentration of DNA was calculated by using the following formula:

\[ \text{Optical density at } A_{260} \times \text{standard concentration of DNA} \times \text{dilution factor} = \text{concentration of testing DNA} \]

\( A_{260} \) represents the wavelength of light which DNA can absorb. This determines the concentration of our DNA sample according to conversion factor (50 µg/mL/1000). Concentration of DNA was calculated based on the conversion and dilution factor.
Restriction digestion & Agarose gel electrophoresis

The DAT cDNA was cloned in pcDNA3 plasmid vector using Ecor I and Kpn I sites. Therefore, to validate the DAT cDNA size, restriction digestion was done by the use of restriction enzymes: Ecor I and Kpn I with respective plasmid WT-hDAT or DM-hDAT.

**Table 3. Components for Restriction Digestion for WT-hDAT or DM-hDAT**

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x CUM Stat buffer</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>Ecor I</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>Kpn I</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>Water</td>
<td>21.5 µL</td>
</tr>
<tr>
<td>WT-hDAT or DM-hDAT cDNA</td>
<td>0.5 µg cDNA in 1 µL</td>
</tr>
</tbody>
</table>

Keep the reaction mixture for 1 hour at 37°C water bath.

In a microwaveable flask, 1 g of agarose was mixed with 100 mL 1xTAE (shown in table 4) and microwaved for 1-3 minutes until dissolved. Agarose solution was then cooled at 50°C for 5 minutes. Ethidium bromide (EtBr) was added in approximate concentration of 0.2-0.5 µg/mL. Agarose was then poured into a gel tray with well comb in place and allowed to solidify for 20-30 minutes at room temperature. Once solidified, it was placed into the gel box and 1x TAE was added until gel was covered.

**Table 4. Components for 1 Liter 50x Stock of TAE**

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-base</td>
<td>242 g</td>
</tr>
<tr>
<td>Acetate (100% acetic acid)</td>
<td>57.1 mL</td>
</tr>
<tr>
<td>EDTA</td>
<td>100 mL 0.5 sodium EDTA</td>
</tr>
</tbody>
</table>

Add dH₂O up to one Liter

To make 1x TAE from 50X TAE stock: Dilute 20 mL of Stock into 980 mL of DI water

Gel loading dye was added to the respective restricted and non-restricted DNA samples and the samples were loaded into the wells. A molecular weight ladder was loaded into the first
lane of the gel to determine the size of DNA fragments. Gel was ran at 80-150 V at room temperature and after dye line was about 75-80% of the way down through the gel. Gel was then removed from the tray and placed under UV transilluminator to visualize the DNA fragments, and photo documentations. As described in detail below under results section, the mutations were confirmed by sequencing.

Cell culture and transfections

HEK-293 (Human Embryonic Kidney) cells were cultured (T25 flasks) in DMEM medium supplemented with 10% (v/v) FBS (fetal bovine serum), 100 units/mL penicillin, 100 µg/mL of streptomycin, 2mM glutamine in a 5% CO2 at humidified atmosphere of 95% air. Cells were trypsonized and seeded in 24 well plates (100K cells/well). After ~16 hours seeding, cDNA 0.3 µg (WT-hDAT or DM-hDAT) or empty vector pcDNA3 control were transiently transfected by using lipofectamine according to manufacture instructions (Figure 9).
Figure 9. Schematic of Seeding and Transfection 24 well plate. 24 well plates were used to seed HEK-293 cells (100K cells/well). First and third row was transfected with WT-hDAT; second and forth wells were transfected with DM-hDAT. This seeding and transfection plan was used for uptake and kinetics experiments.

DA Uptake and Kinetics experiments

After 24-hour post transfection, [{\textsuperscript{3}H}]-DA uptake was performed. Transfected HEK-293 cells were washed with Krebs-Ringer assay buffer (KRH-AB) (25 mM Na\textsubscript{2}HCO\textsubscript{3},124 mM NaCl, 5 mM KCl, 5mM MgSO\textsubscript{4}, 1.5 mM CaCl\textsubscript{2}, 10 mM glucose, 0.1 mM pargyline, and 0.1 mM ascorbic acid pH 7.3) The KRH-AB contains an optimal level of ions (Na, K, CaCl\textsubscript{2}, MgSO\textsubscript{4}) and glucose to provide an optimal isotonic atmosphere with the nutrition of optimal cell viability. Moreover, DAT-mediated DA transport is Na and Cl-dependent mechanism. Thus, KRH buffer is highly desirable to assay DAT-mediated DA transport while maintaining cell viability. Cells were then treated with vehicle or AMPH (1x concentration mixed with 500 µL KRH AB) at 37°C with different concentrations [5 or 10 µM]. After treatment AMPH for 10 minutes. AMPH was removed and cells were washed twice with 2 mL of KRH AB. DA uptake was performed by incubating the
cells for 10 minutes at 37°C with 15 nM [3H]-DA. Uptake assay was terminated by removing the radiolabeled DA by rapid washing of cells twice with 2 mL of KRH AB. For saturation analysis, [3H]-DA and unlabeled DA was mixed ranging from 25 nM to 4000 nM. Non-specific [3H]-DA uptake is defined as the accumulation in presence of 10 mM cocaine and was subtracted from total DA uptake. We then compared non-specific background by using empty pcDNA3 vector-transfected cells. Cells were lysed with 400 µL of Optiphase Supermix scintillant and we measured the radioactivity using MicroBeta2 LumiJET liquid scintillation counter (Perkin Elmer Inc.). Different passages of HEK-293 cells were used and we performed uptake in duplicates as results are presented as mean ± SD. For DA uptake, Vmax and Km values were determined by non-linear curve fits and the values were plotted DA uptake against concentration of DA using Michaelis-Menten equation, 

\[ v = \frac{V_{\text{max}}[S]}{K_m + [S]} \]

Cell surface DAT measurement by Biotinylation and Immunoblot

HEK cells were trypsonized and seeded in 12 well plates (200K cells/well). HEK cells were then transfected as described earlier but done with twice the concentration since the wells in a 12 well plate are twice the size of the wells in a 24 well plate and seeded double the amount of cells. Cells were later treated with vehicle and AMPH on 37°C environment. Following treatment, cells were incubated at 4°C and washed with ice-cold phosphate-buffered saline (PBS/Ca-Mg)(138 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 9.6 mM NaHPO4, 1 mM MgCl2, 0.1 mM CaCl2, pH 7.3). Cells were then incubated with Sulfo-NHS-Biotin for 1 hour at 4°C in ice-cold PBS/Ca-Mg. Biotinylating agent was detached by washing twice and incubating the cells in 100 mM glycine for 20 minutes. Cells were washed with ice-cold PBS/Ca-Mg and then 600 µL RIPA buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium
deoxycholate) containing protease inhibitors and phosphatase inhibitors was used to lyse the cells at 4°C. Lysates were then centrifuged at 2500 g for 30 minutes at 4°C. 50 µL of supernatant was collected that serves as total protein. 500 µL of remaining supernatant were incubated with NeutrAvidin beads overnight at 4°C on nutator. Beads were washed 2 times with ice-cold PBS/Ca-Mg and 1 time with RIPA buffer. Samples were then centrifuged at 5000 rpm for 5 mins at 4°C and supernatant was collected that serves at unbound protein. Beads were washed twice with RIPA buffer, then added 45 µL of 2X Laemmli buffer (62.5 mM Tris–HCl, pH 6.8, 20% glycerol, 2% SDS, 5% b- mercaptoethanol and 0.01% bromophenol blue) and centrifuged at 5000 rpm, 5 mins at room temperature; the samples serve as biotinylated surface proteins. 42 µL of those samples were taken, and for total and unbound proteins, 30 µL of samples were mixed into 12 µL of 4X laemmli buffer, and the samples were analyzed by immunoblotting with Rabbit-DAT polyclonal antibody. To confirm identical loading and surface localization of biotinylated DAT protein, total, bound, and unbound blots were stripped and reprobed with anticalnexin antibody and were quantified using NIH ImageJ. To ensure quantitation within the linear range of the film, pre-calibration of exposures was done, and multiple exposures were taken as well to validate linearity of quantitation. Total, nonbiotinylated, and surface DAT protein values were normalized using levels of calnexin immunoreactivity in total cell extract and values averaged across all experiments. The biotinylated DAT and nonbiotinylated DAT were normalized with total DAT and the ratio are used to determine the surface DAT (biotinylated DAT) and intracellular DAT (nonbiotinylated DAT).

**Statistical analysis**

Statistical analyses were done by the use of GraphPad Prism software. All values are expressed as mean ± standard deviation (SD). Figures are presented in bar graphs showing every
individual value representing a single experiment. For the uptake experiment, two-way ANOVA was followed by post hoc Bonferroni multiple comparisons. Student’s t-test were used for kinetics and biotinylation studies. Statistical significance was considered at value of P < 0.05.

Results

Confirmation of Threonine 261 and Serine 262 mutations (DM-hDAT) in hDAT by Sequencing & gel electrophoresis

Site-directed mutagenesis of hDAT was performed and all mutations were verified by sequencing (Figure 10) and used this plasmid for our cell culture studies. WT and DM hDATs were subcloned into pcDNA3.1-hDAT vector. This chromatogram in Figure 10 showing the conversion of ACC (threonine) to GCC (alanine) and GCC (serine) to GCA (alanine) respectively in positions 261 and 262.

![Figure 10. Site directed mutagenesis for DM-hDAT. Verification of site directed mutagenesis of Threonine 261 and Serine 262 in hDAT. Threonine261Alanine + Serine262Alanine: ACC (Threonine) to GCC (Alanine) + TCA (Serine) to GCA (Alanine).](image)

For further confirmation, restriction digestion was done and restricted plasmid was done in agarose gel electrophoresis to visualize the DNA (Figure 11). Figure 11A shows similar uncut band sizes between WT and DM indicating similar plasmid structure. Because human DAT is cloned into EcoR I and Kpn I enzymes, we used these two enzymes to release the hDAT from the plasmid. As
expected, digestion of plasmid with these two enzymes released hDAT with similar size in both WT and DM (Figure 11B). The sizes of the uncut hDAT is 7229 bp, pcDNA3 alone is 5400 bp, and hDAT inserts are 1829 bp.

Figure 11. Agarose gel electrophoresis of both WT-hDAT and DM-hDAT. (A & B) Uncut (100 ng cDNA) and restricted digestion (500 ng cDNA) of both WT-hDAT and DM-hDAT were run in 0.6% agarose gel and visualized the DNA fragments under UV transilluminator. On top of the lanes, the name of the mutants are shown. Uncut plasmid: 7229 bp, pcDNA3 alone: 5400 bp, Insert: 1829 bp.

AMPH-mediated downregulation of DA uptake was blunted in DM-hDAT

We examined if the phosphosites T261 and S262 in hDAT are involved in AMPH-mediated hDAT regulation. We substituted threonine 261 and serine 262 in hDAT with non-phosphorylatable alanine to prevent phosphorylation. WT-hDAT or DM-hDAT are expressed in HEK-293 cells and studied the effect of AMPH on DA uptake. Exposure of different concentrations of 5 µM or 10 µM of AMPH, to HEK-293 cells expressing WT-hDAT significantly inhibited DA uptake compared to vehicle (5 µM $P = 0.0160$; 10 µM: $P = 0.0003$) (Figure 12). DA
uptake for vehicle treated cells was $0.0917 \pm 0.03069$ pmol/$10^6$ cells/min and for respective concentration of AMPH treatment was $0.05199 \pm 0.01818$ pmol/$10^6$ cells/min for 5 µM and $0.03954 \pm 0.01484$ pmol/$10^6$ cells/min for 10 µM of AMPH. Remarkably, AMPH-induced inhibition of DA uptake was nonsignificantly reduced in cells expressing the non-phosphorylatable form of T261/S262 (DM). DA uptake for vehicle treated cells was $0.08139 \pm 0.03237$ pmol/$10^6$ cells/min and for respective concentration of AMPH treatment was $0.06084 \pm 0.02528$ pmol/$10^6$ cells/min for 5 µM and $0.0482 \pm 0.02371$ pmol/$10^6$ cells/min for 10 µM of AMPH.

Figure 12. Effect of AMPH on DAT-mediated DA uptake. HEK-293 cells transfected with WT-hDAT or DM-hDAT were treated with vehicle or 5 or 10 µM of AMPH (shown in red and green respectively) for 10 minutes at 37°C. DAT-mediated [3H]DA uptake was measured using 15 nM of DA label. Uptake assays were executed in duplicates in 24 well plates for WT and DM. Each data point shows the average of duplicates. *P= 0.0213 for DAT-mediated DA uptake of 5 µM AMPH treated WT-hDAT compared with vehicle; ***P = 0.0004 for DAT-mediated DA uptake of 10 µM AMPH treated WT-hDAT compared with vehicle followed by 2-way ANOVA test. ns: non-significant.
AMPH-mediated alterations in DA kinetics are attenuated in DM-hDAT

The kinetic analysis of DA uptake in HEK-293 cells transfected with WT-hDAT showed that AMPH (10 µM, 30 min) treatment resulted in a significant decrease in the maximal velocity ($V_{\text{max}}$) ($t=3.460$, df=4; $P = 0.0258$) and non-significant changes in $K_m$ between vehicle and AMPH treatment ($t=0.5569$, df=4; $P = 0.6011$). $V_{\text{max}}$ for vehicle treated cells was $8.868 \pm 1.22$ pmol/10$^6$ cells/min and $5.938 \pm 0.812$ pmol/10$^6$ cells/min for AMPH treated cells. DA affinity ($K_m$) for vehicle treated cells was $677.46 \pm 348.83$ nM and AMPH treated cells was $557.53 \pm 112.22$ nM. (Figure 13B & C). Interestingly in mutating S261 and T262 into a non-phosphorylatable alanine in hDAT prevented AMPH mediated decrease in $V_{\text{max}}$. DM-hDAT transfected cells showed non-significant differences in $V_{\text{max}}$ between vehicle and AMPH ($t=0.6698$, df=4; $P = 0.5397$). However, expression of DM-hDAT in HEK cells did not alter $K_m$ values between vehicle and AMPH treatment and showed non-significant differences ($t=0.2988$, df=4; $P = 0.7800$). $V_{\text{max}}$ for vehicle treated cells was $8.495 \pm 1.49$ pmol/10$^6$ cells/min and $7.882 \pm 0.52$ pmol/10$^6$ cells/min for AMPH treated cells. DA affinity ($K_m$) for vehicle treated cells was $609.36 \pm 337.44$ nM and AMPH treated cells was $674.2 \pm 165.49$ nM. (Figure 13B & C).
Figure 13. Effect of AMPH on DAT kinetics. (A) HEK-293 cells transfected with WT-hDAT or DM-hDAT were treated with vehicle or AMPH (10 µM) for 30 minutes at 37°C. DAT specific [3H]DA uptake was measured using several concentrations of DA. Assays were executed in duplicates in two 24 well plates for WT and DM. Each data point shows the average of duplicates. Non-linear curve fits of uptake data for vehicle (N=3) and AMPH (N=3) were shown (mean ± SD). *P = 0.0258 for V_{max} of AMPH treated WT-hDAT compared with vehicle; *P = 0.0254 for V_{max} of AMPH treated WT-hDAT and DM-hDAT (paired t-test). Charts are aimed at the comparison for the effect of AMPH on WT-hDAT or DM-hDAT focused on hDAT V_{max} (DA transport velocity) (B) and K_{m} (DA affinity for DAT) (C). ns: non-significant.
AMPH downregulates cell-surface expression of WT-hDAT but not DM-hDAT

DAT activity is the representative of functional properties of DAT located on the surface of the plasma membrane. Changes in the DAT activity may occur due to altered expression of surface DAT or changes in catalytic transport cycle. Therefore, we sought to assess if decreased hDAT $V_{\text{max}}$ following AMPH treatment is attributable to decreased surface hDAT expression in WT-hDAT transfected cells. Surface hDAT were determined in HEK-293 cells transfected with WT-hDAT or DM-hDAT following treatment with vehicle or AMPH (10 µM, 30 min). The results from biotinylolation studies showed decreased cell surface DAT density in WT-hDAT following AMPH treatment compared to respective vehicle treatment (Figure 14B & E). Quantified band densities showed AMPH treatment in WT-hDAT significantly reduced surface expression than vehicle treated cells ($t=5.031, \text{df}=4; P = 0.0073$) (Figure 14B). However, in DM-hDAT AMPH treatment failed to alter surface DAT expression ($t=0.6617, \text{df}=4; P = 0.5444$) (Figure 14E). Total DAT protein expression (Figure 14A) and quantified band densities showed no significant differences between AMPH and vehicle treatments or between WT-hDAT and DM-hDAT (Figure 14D). Reflecting the changes in surface DAT band densities, there were concomitant increases in intracellular DAT and showed significant effect between vehicle and AMPH treatment in WT-hDAT ($t=4.618, \text{df}=4; P = 0.0438$) (Figure 14C & F) and whereas the increase in intracellular DAT was not evident in DM-hDAT ($t=0.0418, \text{df}=4; P = 0.9686$) (Figure 14C & F). While intracellular ER marker calnexin was present in total and nonbiotinylated fraction, it was completely undetectable in biotinylated fraction, establishing that the biotinylation of proteins occurred exclusively on the surface not intracellular proteins.
Figure 14. Effect of AMPH on surface DAT. 24 hours post transfection of WT-hDAT and DM-hDAT, cells were treated with vehicle or AMPH (10 µM) for 30 minutes at 37°C and cell surface biotinylation was executed. Representative immunoblots of three independent experiments show total (A), biotinylated surface hDAT (B), and non-biotinylated intracellular hDAT (C) as well as the intracellular marker calnexin immunoblot shown under respective DAT immunoblots. Total DAT was normalized to total calnexin, biotinylated surface DAT was normalized to total DAT, and nonbiotinylated DAT was normalized to nonbiotinylated calnexin and values are represented as mean ± SD (D & E & F). AMPH treated samples (N=3) are shown relative to vehicle treated samples (N=3) for WT-hDAT or DM-hDAT, and represent each independent cell culture preparations. Student’s t-test: **P= 0.0073 for biotinylated vehicle and AMPH treated WT-hDAT; *P= 0.0403 for biotinylated AMPH treated WT-hDAT and DM-hDAT; *P=0.0438 for non-biotinylated vehicle and AMPH treated WT-hDAT; *P=0.0197 for non-biotinylated AMPH treated WT-hDAT and DM-hDAT; ns: non-significant.

Discussion

As an integral membrane neuronal protein, DAT’s main function is termination of dopaminergic neurotransmission. DAT provides the primary mechanism of clearing the synapse of DA by reuptake of DA into presynaptic neurons; therefore, determining the duration and intensity of dopaminergic neurotransmission (Giros et al., 1996). DAT knockout studies in mice
have shown that AMPH is required for outward transport of large concentration of DA (from cytoplasm) through DAT after its depletion from the vesicles (Jones et al., 1998). DAT knockdown models have shown inhibition of locomotor activity triggered by AMPH administration (Zhuang et al., 2001). It’s been demonstrated that DAT downregulation occurs corresponding to DAT phosphorylation (Vaughan et al., 1997). Studies with expression of DAT in heterologous systems (Granas et al., 2003; Huff et al., 1997), and rat (Vaughan et al., 1997; Foster et al., 2002) striatal tissues demonstrated rapid elevation of $^{32}$P labeling incorporation, by PKC activators such as PMA, diacylglycerol analogs, or Gq receptor agonists (Granas et al., 2003; Huff et al., 1997; Vaughan et al., 1997). Such effects have been blocked by PKC inhibitors (Foster and Vaughan, 2017). It’s been studied that AMPH and METH increase $^{32}$P labeling in DAT and their actions are blocked by PKC inhibitors (Cervinski et al., 2005). AMPH and METH are substrates of DAT; they compete with DA for DAT transport and raise extracellular DA by inducing DA efflux through reversal of DAT (Eshlemen et al., 1994; Sulzer et al., 1995). A lot of literature describes the effects of AMPH or METH on DAT but in this study, AMPH was used for all experiments since AMPH has also been used in a lot of our recently published articles. AMPH and METH show no difference when it comes to changes in DA release in striatum, rates of elimination, and some more pharmacokinetic properties (Melega et al., 1995). Not only AMPH can release more DA in prefrontal cortex, but it elicits more locomotor activity in rodents when compared to METH (Shoblock et al., 2003). Many studies (Cervinski et al., 2005; Chen et al., 2009; Fog et al., 2006; Johnson et al., 2005; Richards and Zahniser, 2009) demonstrated that AMPH- and METH-mediated downregulation, efflux, and endocytosis of DAT (in cells or striatal tissues) depends on PKC or CAMKII. Reports demonstrated that in striatal and heterologously expressed rDAT, about 90% of $^{32}$P labeling occurs on serine while about 10% occurs on threonine
(Foster et al., 2002). Figure 6 shows numerous residues on DAT that serve as phosphorylation sites in N-terminal, C-terminal and ICLs. Many of these residues are conserved in hDAT and rDAT as well as hNET (Figure 8). This suggests numerous similarities in phosphorylation residues across species and monoamine transporters. Studies shown that mutation of serine to alanine at 7th position caused increase in DA uptake $V_{\text{max}}$ (Moritz et al., 2015), and decreased AMPH-induced efflux (Khoushbourie et al., 2004; Vaughan et al., 1997; Wang et al., 2016). A study from our laboratory shows that the threonine 258 and serine 259 residues of ICL2 in NET are involved in AMPH-induced NET endocytosis. However, mutation of those sites to alanine, resulted in resistant to AMPH-mediated NET downregulation (decreased uptake and reduced cell surface expression of NET) and blunted AMPH-induced NET endocytosis (Annamalai et al., 2010). The result from this study suggests that the residues in ICL2 are involved in transporter downregulation caused by AMPH as well as phosphorylation. Based on the fact that threonine and serine residues are conserved in both NET and DAT in ICL2 (Figure 8), we hypothesized that threonine 261 and serine 262 residues located in ICL2 of DAT may be involved in AMPH-mediated DAT regulation. In this study, AMPH was used to treat HEK-293 cells transfected with WT-hDAT or DM-hDAT and the effects of AMPH was seen in uptake, kinetics, and biotinylation experiments. For our studies, Lipofectamine was used to transfected the cells. Higher concentrations of Lipofectamine is toxic to cells, although we used optimum lesser concentration of Lipofectamine in our studies. However, using stable cell lines is safer and will help us to ensure the equivalent expression of DAT protein in cells. As mentioned in literature, downregulation of DAT by actions of AMPH can demonstrates a possible link between PKC and phosphosites of DAT. Reports of mutagenesis and peptide mapping of native and expressed rDATs recognized the distal N-terminus of DAT as major site of PKC- and AMPH-stimulated phosphorylation (Cervinski et al., 2005; Foster et al.,
2002). However, our experiments demonstrate a possible link between threonine 261/serine 262 sites of DAT and phosphorylation-mediated downregulation of DAT. As a result of our uptake, kinetics, and biotinylation experiments, AMPH downregulated WT-hDAT but not DM-hDAT. Mutation of threonine 261 and serine 262 sites to alanine attenuated the effects of AMPH on DAT indicating the importance of those sites in AMPH downregulation. Mutational analysis of phosphosites, threonine 261 and serine 262, in hDAT and their expression in heterologous cell system provides evidence that these sites are involved in AMPH-mediated DAT regulation. Our uptake experiment reveals that pharmacological treatment of AMPH on cells carrying WT-hDAT results in decrease in hDAT-mediated DA uptake. Remarkably, hDAT carrying mutation of threonine 261 and serine 262 results in a non-significant change in DA uptake in response to different concentrations of AMPH treatment. Our kinetics experiment demonstrates that treatment with AMPH resulted a significant decrease in $V_{\text{max}}$ of WT-hDAT but not DM-hDAT. To elaborate, AMPH significantly reduces the transport of DA in WT-hDAT. However, mutation of threonine 261 and serine 262 to alanine results in a non-significant change in transport of DA. However, there was no change in $K_m$ values in both WT-hDAT and DM-hDAT between vehicle and AMPH treatments, which indicates that AMPH does not alter the affinity of DA for DAT. Biotinylation approach was performed to determine the surface expression of DAT in response to AMPH treatment. Heterologously expressed WT-hDAT in hEK-293 cells showed reduced surface DAT expression in treatment with AMPH whereas DM-hDAT completely abolished this effect. Notably, the total expression of DAT was not altered in both WT-hDAT and DM-hDAT between treatments. Remarkably, the intracellular expression of DAT was increased in WT-hDAT with AMPH treatment, but not in DM-hDAT. Our present data matched with previous data reported with AMPH downregulation of WT-hNET and DM-hNET since that study targeted the same motif
in ICL2 of NET (Annamalai et al., 2010). In native tissue, it’s been reported that AMPH-mediated downregulation of both NET and DAT in striatal synaptosomes (Mannangatti et al., 2021). Phosphosites of DAT are regulated by various kinases. Activation of PKC reduces DAT V_max, reduce surface DAT expression, elevate DAT internalization, elevate phosphorylation of striatal DAT on serines located on N-terminus, and regulate AMPH-induced DA efflux without altering DA K_m. (Chang et al., 2001; Chen et al., 2009; Daniels and Amara, 1999; Melikian & Buckley, 1999; Vaughan et al., 1997; Zhu et al., 1997). Alternatively, inhibition of ERK can decrease DAT V_max and reduce surface expression of DAT without altering DA K_m (Morón et al., 2003). CaMKII assists with phosphorylation of DAT N-terminus and mediates AMPH-induced DA efflux through DAT (Fog et al., 2006). Akt is engaged in insulin and AMPH-mediated DA release (Garcia et al., 2005; Wei et al., 2007). Inhibition of PI-3-kinase reduces DAT V_max as well as decreasing DAT surface expression without altering DA K_m (Carvelli et al., 2002; Lute et al., 2008). Inhibition of Cdk5 reduces DAT V_max without changing DAT surface expression (Price et al., 2009). Inhibition of tyrosine kinase can reduce DAT V_max while decreasing surface expression of DAT (Doolen and Zahnisser, 2001). Based on previous studies, several kinases involved in DAT regulation and our studies aim to check which kinases are involved in these residues. There is no commercially available antibody for Threonine 261 and Serine 262 but it is important to examine total DAT phospho-status with vehicle or AMPH treated cells in both WT and DM. Notably, It is possible that phosphorylation of T261 and S262 by other kinases may play a major role in AMPH-induced DAT regulation. Further studies examine DAT phospho-status in DM following AMPH treatment will help us delineate the involvement of the T261A and S262A phosphorylation in AMPH-mediated DAT regulation. Our future plans are to screen different kinases either through pharmacological inhibition or genetic silencing in the same model of experiments and distinguish
the kinase-mediated modulation of DAT to determine which kinase phosphorylates the threonine 261 and serine 262 residues. Also, our future goal is to evaluate whether these residues are involved in AMPH-mediated DAT endocytosis or exocytosis. DA has a huge role on body’s everyday function, therefore, the more we understand about the processes of phosphorylation of DAT, the more it can guide to more potential strategies to apply these DAT modifications to be utilized as therapeutic targets in disorders related to DA.

Conclusion

In conclusion, present study confirms for the first time two important residues, threonine 261 and serine 262, located in ICL2 of hDAT and their significance in regulation of AMPH-mediated DAT. However, our previous findings showed AMPH-mediated regulations of NET depends on threonine 258 and serine 259 in heterologous system (Annamalai et al., 2010). Keeping in mind the fact that hDAT and hNET share similar amino acid sequence surrounding ICL2, we tested effect of AMPH after mutating threonine 261 and serine 262 into non-phosphorylatable form in hDAT. Notably, these two non-phosphorylatable sites in hDAT completely blunted the effect of AMPH-mediated regulation of DAT protein in heterologous cell system. However, it is essential to identify which receptor and downstream kinase signaling are involved in the phosphorylation of threonine 261 and serine 262 residues in hDAT and how AMPH interacts with these signaling cascade require further endeavor. However, non-phosphorylated state of threonine 261 and serine 262 can possibly block AMPH-triggered DAT down regulation which can further suggest that phosphorylation of threonine 261 and serine 262 sites regulate DAT response to AMPH. Potentially, threonine 261 and serine 262 are targets of cellular signaling molecules in response to binding of AMPH to DAT.
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Vita

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