The Role of the Dopamine Transporter in Attention Deficit Hyperactivity Disorder

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Research focused on neuropsychiatric disorders has shown time and time again that these diseases are extremely complex. As advances in our understanding of disease mechanisms are made, it seems that more questions arise than are answered. This review will introduce the many complexities of attention deficit hyperactivity disorder (ADHD) and focus on the role of the dopamine transporter (DAT) in the disease, specifically addressing various mechanisms of transporter regulation and the primary methods of studying DAT in ADHD.

ADHD PRIMER

Attention deficit hyperactivity disorder is a relatively common condition characterized by impulsive behavior, hyperactivity, distractibility, and impairments in sustained attention. There are currently no biological markers for ADHD so there is no test for the disorder; diagnosis is based solely on clinical observations and interviews with parents and teachers1. The DSM-IV outlines three subtypes of ADHD: predominantly inattentive, predominantly hyperactive and impulsive, and a combined subtype that possesses aspects of both of the other classifications. A positive diagnosis is made when a subject has 6 of 9 inattentive symptoms and/or 6 of 9 hyperactive/impulsive symptoms (Table 1)2. The DSM-IV-TR diagnostic criteria also require that symptoms are present before age 7, but some would argue that this requirement is too restrictive2,3.

ADHD is estimated to affect 3-7% of school age children1 in the general population, a measure in line with the findings of a broad review of more than 100 ADHD surveys that reported a worldwide prevalence rate of 5.29%4. However, some researchers contend that ADHD is drastically over- or underestimated on a population level. A recent review of ADHD surveys performed between 1997 and 2007 found ADHD prevalence rates as low as 0.2% and as high as 27%5. It is also notable that ADHD exhibits a distinct male-to-female bias, with estimates ranging from 2:1 to as high as 9:1 depending on the subtype1. The reasons for this bias are unclear but might include differences in cultural reinforcement of certain gender roles or merely sex differences in biological factors contributing to the disorder itself6.

Although studies have shown that ADHD symptoms tend to decline as subjects grow older, research suggests that 4% of adults (age 18-44) retain ADHD symptoms6,9 though some recent studies contend that adult ADHD rates may be as high as 15% for full ADHD diagnosis and as high as 60% for ADHD in partial remission3,5. Adults with ADHD have an increased risk for substance abuse6 and comorbid psychiatric disorders, especially anxiety disorders, eating disorders, anti-social personality disorders, depressive syndromes, tics, and learning (usually reading and spelling) disabilities10.

Treatment for ADHD typically involves administration of psychostimulants such as methylphenidate (MPH; Ritalin; Novartis Pharmaceuticals, Basel, Switzerland) or amphetamine (AMPH; Adderall; Shire Pharmaceuticals, Basingstoke, England). Both of these pharmacological agents primarily target the dopamine system immediately implicate dopaminergic signaling as a major player in ADHD symptoms and suggest that dopaminergic dysfunction may underlie ADHD pathology.
Table 1 | DSM-IV symptom criteria for ADHD.

<table>
<thead>
<tr>
<th>Inattention (6 or more)</th>
<th>Hyperactivity/Impulsivity (6 or more)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fails to attend to details</td>
<td>Blurs out answers</td>
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<tr>
<td>Has difficulty sustaining attention</td>
<td>Difficulty awaiting turn</td>
</tr>
<tr>
<td>Does not seem to listen</td>
<td>Interrupts or intrudes</td>
</tr>
<tr>
<td>Fails to finish</td>
<td>Talks excessively</td>
</tr>
<tr>
<td>Has Difficulty organizing tasks</td>
<td>Fidgets with hands or feel</td>
</tr>
<tr>
<td>Avoids sustained effort</td>
<td>Leaves seat in classroom</td>
</tr>
<tr>
<td>Loses things</td>
<td>Runs about or climbs</td>
</tr>
<tr>
<td>Is distracted by extraneous stimuli</td>
<td>Difficulty laying quietly</td>
</tr>
<tr>
<td>Is forgetful</td>
<td>Motor Excess</td>
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Taken from Mazei-Robison and Blakely, 2006. Used with permission.

DOPAMINE CIRCUITRY

Neurotransmitters are typically categorized as excitatory or inhibitory depending on how the transmitter affects its target neuron. Dopamine, however, can be excitatory or inhibitory depending on the type of DA receptor it binds—excitatory D1-like receptors (D1 and D2) or inhibitory D2-like receptors (D2, D3, and D4)13. When D1-like receptors are activated, adenylate cyclase (the enzyme that generates cyclic AMP (cAMP)) is stimulated, but D2-like receptor activation results in inhibition of adenylate cyclase. The activation or inhibition of adenylate cyclase and resulting changes in cAMP levels lead to depolarization (D1-like) or hyperpolarization (D2-like) of the cell membrane. Clearly, the dual excitatory and inhibitory roles of DA make the system quite complicated, especially when trying to understand how dopaminergic signaling fits into ADHD pathology14.

There are four major dopaminergic circuits in the brain (reviewed in 14)—nigrostriatal, hypothalamic-tubero infundibular (HTI), mesocortical, and mesolimbic—and the latter two are most closely linked to ADHD. The nigrostriatal system begins in the substantia nigra pars compacta and projects to the striatum. This circuit primarily regulates motor function and it is the loss of these dopaminergic neurons that leads to the development of Parkinson’s disease15-17. The HTI pathway starts in the arcuate nucleus of the hypothalamus and projects mostly to the pituitary gland. In this case, dopamine operates under the alias “prolactin inhibiting factor (PIF)” and regulates the secretion of prolactin and luteinising hormone18.

ADHD, however, is linked to dopaminergic dysfunction in fronto-limbic brain areas19, specifically the prefrontal cortex20, nucleus accumbens, and striatum21 (brain areas involved in ADHD reviewed in 14,22). These areas are parts of the mesocortical and mesolimbic circuits. Both pathways originate in the ventral tegmental area (VTA), a nucleus medial to the substantia nigra and ventral to the red nucleus in the midbrain23. Mesocortical projections go to prefrontal and frontal cortical areas where it regulates information processing, attention, working memory, language, and planning24. Mesolimbic projections are directed primarily to the nucleus accumbens (NAc) where they are involved in reward processing and addiction25, psychosis26, and major depression27. The mesolimbic pathway also makes connections with several other brain regions including the hypothalamus, ventral pallidum, and amygdala28.

In all of the dopamine circuits, dopamine signaling is terminated by the actions of the dopamine transporter. This plasma membrane protein is located in perisynaptic regions29,30 and works to recover DA from the synaptic cleft then transport it back into the presynaptic neuron where it is re-packaged into synaptic vesicles for re-release30. Reuptake of neurotransmitter is one of the main mechanisms utilized in the brain to limit signaling and is seen in several neurotransmitter systems including the other biogenic amines, norepinephrine and serotonin. With such an important role in regulating neurotransmission, it is easy to speculate how transporter dysfunction could contribute to a disease phenotype.

DAT GENE AND PROTEIN BASICS

The human DAT gene was originally cloned in 1992 by screening a cDNA library derived from the substantia nigra31. Further work used phage library screening and restriction site mapping to determine that the fifteen exons and fourteen introns33 of the DAT gene span over 64 kb of chromosome 531. This work also confirmed that the gene codes for a 620-amino acid protein. To date, there are no reports of alternative splicing in the DAT gene. In addition to the protein-coding sequence, the DAT gene contains a 40-base pair repeat (commonly referred to as a variable number tandem repeat (VNTR) in the 3'-untranslated region of the gene, with individuals
carrying anywhere from three to eleven copies of the repeat sequence. The precise function of the VNTR is unclear, but it has been shown that the 10-repeat VNTR allele is associated with ADHD.

The DAT protein was originally predicted to have twelve transmembrane (TM) domains and intracellularly oriented amino and carboxy termini, a structure that was ultimately confirmed when a homologous bacterial leucine transporter (LeuT) was crystallized. Early work on DAT focused on uptake kinetics, inhibitor sensitivity, and ion dependence, finding that one Cl⁻ and two Na⁺ ions are co-transported with each DA molecule. Work using chimeric DAT-NET fusion proteins later uncovered the structural determinants for the observed Na⁺ and Cl⁻ ion dependence of DAT-mediated transport, specifically involving the C- and N-terminal regions (DAT and other related transporters reviewed in ref. 41).

DAT REGULATION AND INTERACTING PROTEINS

At the most basic level, DAT function seems relatively simple—it merely recovers dopamine as it diffuses out of the synapse. However, DAT is a highly regulated protein; its function is finely tuned by phosphorylation, ubiquitination, and several interacting proteins. The most frequently studied DAT-regulator is protein kinase C (PKC). Direct activation of PKC by phorbol esters or indirect PKC activation via Gq-coupled G-protein coupled receptor stimulation leads to decreases in DAT activity, primarily by internalization of DAT to intracellular compartments via a clathrin- and dynamin-dependent process. There is evidence, however, that DAT phosphorylation is not required for internalization, it seems that phosphorylation regulates reverse transport through DAT. Collaborative work from the Galli, Javitch, and Gnegy labs showed that alanine substitution for five serines in the DAT N-terminal abolished phosphorylation, but did not affect PKC-induced endocytosis. Rather, the loss of phosphorylation inhibited AMPH-induced DA efflux. Conversely, substitution of aspartates for the N-terminal serines (mimicking phosphorylation) rescued AMPH-induced efflux. It has been suggested that PKC primarily regulates DAT via internalization, but that DAT phosphorylation by PKC or other kinases stabilizes at least some DAT in an “efflux-willing” conformation.

DAT regulation by kinases, however, is not as simple as PKC-induced down-regulation. In fact, DAT is a substrate for several other kinases. Carvelli and coworkers (2002) found that insulin stimulates DAT activity in a phosphatidylinositol 3-kinase (PI3K) dependent manner that causes a redistribution of DAT to the cell surface. Members of the mitogen activated protein kinase (MAPK) family have also been shown to regulate DAT activity; p42 and p44 MAPK inhibitors lead to decreased DAT activity and plasma membrane expression. Last of all, calcium/calmodulin-dependent protein kinase II (CaMKII) has also been shown to facilitate DAT reversal in response to amphetamine. The precise details of how all of these kinase pathways interact and converge on DAT remain unclear and are being actively researched.

Since it was shown the DAT internalization occurred independent of phosphorylation, researchers began looking for other mechanisms to explain DAT trafficking. Work in yeast has shown that plasma membrane trafficking of various transport proteins is regulated by ubiquitination, specifically mono-ubiquitination. Since DAT is trafficked independent of phosphorylation and lacks protein sequence motifs that often serve as sorting signals, researchers in the Sorkin lab examined DAT’s ubiquitination state using mass spectrometry. These studies showed that upon PKC activation with the phorbol ester PMA, DAT is ubiquitinated in both the N- and C-terminal domains, specifically on lysines 19, 27, 35, and 599. There is some redundancy in the ubiquitination signal, as DATs harboring mutations at single lysines (ubiquitin conjugation sites) have normal trafficking, but endocytosis is disrupted when more than one lysine is eliminated. Sorkin’s group went on to utilize RNAi methods to identify Nedd4-2 (neural precursor cell expressed, developmentally downregulated 4-2) as DAT’s ubiquitin E3 ligase. This raises an obvious question—if DAT endocytosis occurs independent of PKC-mediated phosphorylation, then why does PKC activation still result in DAT endocytosis? It is known that Nedd4-2 is regulated by phosphorylation and, although it has not been demonstrated directly, it is reasonable to hypothesize that Nedd4-2 activity is regulated by PKC. Thus, PKC may be increasing Nedd4-2 activity or somehow allowing Nedd4-2 access to ubiquitination sites on the DAT molecule, and it is the ubiquitination that ultimately causes endocytosis.

The structure of DAT lends itself to many protein-protein interactions, as both termini are oriented towards the intracellular compartment. It comes as no surprise, then, that proteins interacting with DAT are responsible for regulating transport function. For example, several of the kinases that regulate DAT have direct protein-protein interactions with the transporter. It has been shown that both PKCβ-II and CaMKII interact with DAT (PKC on the N-terminal and CaMKII on the C-terminal) and facilitate AMPH-induced DA efflux. DAT phosphorylation is also regulated via DAT’s direct interaction with protein phosphatase 2A (PP2A); in a
Table 2 | DAT-interacting proteins.

<table>
<thead>
<tr>
<th>N-terminal Interactions</th>
<th>Function</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA D2 Receptor</td>
<td>targets DAT to active synapses; anchors DAT into membrane</td>
<td>69</td>
</tr>
<tr>
<td>Receptor of Activated C Kinase (RACK1)</td>
<td>localizes DAT to synapses; may serve as a scaffold for signaling complexes</td>
<td>70</td>
</tr>
<tr>
<td>Syntaxin 1A</td>
<td>unknown; inhibits transport in GABA transporter (GAT1) system</td>
<td>71</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C-terminal Interactions</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Synuclein</td>
<td>clustering DAT in membrane; DA-induced apoptosis</td>
<td>72</td>
</tr>
<tr>
<td>Hic-5</td>
<td>focal adhesion adaptor; likely a scaffold for signaling complexes</td>
<td>73</td>
</tr>
<tr>
<td>Piccolo</td>
<td>presynaptic scaffold; role in assembling synaptic active zones</td>
<td>74</td>
</tr>
<tr>
<td>Protein Interacting with C Kinase (PICK1)</td>
<td>targets DAT to the plasma membrane; tether PKC to DAT</td>
<td>75</td>
</tr>
</tbody>
</table>

role opposing the kinases, PP2A de-phosphorylates DAT and promotes surface expression\(^66\).

Besides the kinases and phosphatase, several other proteins interact with DAT. It is beyond the scope of this review, however, to address the function of them all in detail (interacting proteins are reviewed in ref. 67). The identity of interacting proteins and a brief description of the proposed role of each interaction can be found in Table 2. In nearly all cases, the impact of the protein-protein interaction is not fully understood and is still being actively investigated.

STUDYING DAT IN ADHD

Several studies have been able to make significant links of the dopamine transporter to ADHD. Twin studies have suggested that ADHD is highly heritable—approximately 80% of cases have some significant and identifiable genetic component\(^25,75\) (twin, family, and adoption studies in ADHD reviewed in \(^76\)). A plethora of genome-wide linkage studies have been conducted using various cohorts of ADHD subjects that resulted in linkage at several chromosomal locations including 5p12, 10q26, 12q23, 16p13.7,77,78; 17p11\(^2\); 15q and 7p (although failure to replicate linkage at 16p13 and 17p11\(^8\); and 6q12 and 5p13\(^37\). As the resolution of linkage mapping methods improved, studies identified smaller regions linked to ADHD including 4q13.2, 5q33.3, 11q22, and 17p11\(^8\), as well as 2q21.1 and 13q12.1\(^1\);\(^79\); and 2q35, 5q13.1, 6q22-23, 7q21.11, 9q22, 14q12, and 16q24.1\(^8\). To summarize, chromosome 5 is most frequently linked to ADHD. Interestingly, the specifically linked region at 5p13 is near the DAT gene locus\(^8\). The overall lack of consistency among linkage studies may be accounted for by several factors including differences in ADHD diagnosis or the identity of ADHD study populations\(^7\). It is also possible that ADHD is a complex disorder caused by several common polymorphisms in only a few genes. In this case, it is most likely that several variants in a localized pathway or a functionally related set of genes are contributing to the disorder.

Since genome-wide linkage studies yielded only limited data, many groups opted to study ADHD using a candidate gene approach. In such a method, researchers choose genes that are likely involved in the disorder and look for association of specific alleles to that disorder. In ADHD candidate gene studies, the catecholaminergic neurotransmitter systems are the most common candidates examined (ADHD associated genes reviewed in refs. 86 and 87). Studies of smaller populations as well as larger meta-analyses\(^8\)\(^8\)\(^9\)\(^8\);\(^9\)\(^8\)\(^9\)\(^8\)\(^9\)\(^8\)\(^9\)\(^8\) have found association of several genes with ADHD including dopamine \(\beta\)-hydroxylase (DBH)\(^88-90\); dopamine \(D_2\)\(^90,91\), \(D_4\)\(^88-90,92\), and \(D_5\) receptors\(^88-90,92\); the serotonin transporter (SERT)\(^78,90,92\); and various serotonin receptors\(^8,89,92\); acetylcholine receptors\(^8,89,92\); monoamine oxidases A\(^92\) and B\(^94\); synaptosomal associated protein of size 25 kDa (SNAP25)\(^88,90,92\); and, most importantly, DAT and the DAT 3′-VNTR\(^85,88-90,92,95\). The linkage data clearly point to a complex genetic basis for ADHD, and the most consistent findings invariably point to DAT.

Perhaps the most direct link of DAT function to ADHD comes from studying the function of rare coding variants of the DAT protein. Several studies have looked for single nucleotide polymorphisms (SNPs) in the dopamine transporter gene and identified only seven low-frequency coding variants—V24M, V55A, R237Q, V382A, A559V, E602G, and R615C\(^34,96-100\). However, only the work of Mazei-Robison and coworkers examined subjects diagnosed with strictly ADHD (i.e. without comorbid psychiatric disorders); the A559V variant was identified in two brothers from this population\(^34\). Later functional
characterization of this mutant transporter revealed a basal DA leak. DA efflux that typically only occurs upon stimulation (i.e. AMPH treatment) is happening without any pharmacological manipulation\textsuperscript{103}. The only other DAT variant with a phenotype of interest thus far is V382A, a transporter that does not properly traffic to the plasma membrane and can exist in the plasma membrane in a transport-inactive state\textsuperscript{102}.

Research on nearly all aspects of DAT regulation and function are still being actively studied. Many open questions remain regarding DAT regulation, trafficking, and involvement in signaling networks, as well as molecular characterization of rare coding variants. It is noteworthy that there are several useful animal models of ADHD (animal models of ADHD reviewed in ref. 14) including the DAT knockout mouse that displays hyperactivity and learning impairments\textsuperscript{100,101,104} and a DAT knockdown mouse that displays hyperactive behavior and allows for pharmacological manipulation since some DAT remains\textsuperscript{105-107}. These models allow for in vivo studies of DAT mutations as well as DAT mutant function in the context of other genetic manipulations.

CONCLUSIONS

It should be abundantly clear that ADHD is an incredibly complex disorder. The etiology is not fully understood, but it is obvious that several genes and proteins are somehow connected in a diffuse web of interactions, regulations, and cross-communications. The dopamine transporter, however, stands out as a key player in ADHD. Research continues to investigate the function and regulation of DAT. Ultimately, a further understanding of DAT is essential for understanding the role of altered dopamine signaling in ADHD and guiding future therapeutic strategies.

REFERENCES


This work provides in vivo evidence that DAT surface expression is dynamically regulated by its phosphorylation state. Although our current thinking about the mechanism of DAT trafficking has changed, this paper demonstrated the key role of PKC in DAT endocytosis and addressed the functional impacts of such regulation on neural signaling.


49. Holton KL, Loder MK, Melkian HE (2005). Nonclassical, distinct endocytic signals dictate constitutive and PKC-regulated neurotransmitter...


61. Endocytosis is a major mechanism used to regulate DAT function. This paper is the most current published information regarding DAT ubiquitination and trafficking and reflects our current understanding of the mechanisms of DAT trafficking.


101. Mazei-Robison MS, Bowton E, Holy M, Schmudermayer M, Freimuth M, Sitte HH, Galli A, Blakely RD (2008). Anomalous dopamine release associated with a human dopamine transporter coding variant. J. Neurosci. 28 (28): 7040-7046. This paper is a fine example of the type of functional studies that are used to characterize novel coding variants of DAT. In addition, it supports the hypothesis that identifying low-frequency coding variants does provide new insight regarding possible mechanisms to explain ADHD.


FURTHER INFORMATION
Randy Blakely’s Lab: http://www.blakelylab.org