Catecholamine Transporters: Differential Regulation by Insulin

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Abstract

Accumulating evidence supports growing appreciation for the prevalence of comorbidity of metabolic disorders and mental illness. Historically, pancreatic hormone insulin is considered to be one of the most important metabolic regulators in the body. Recently, it has also been shown that insulin signaling pathway is implicated in brain catecholamine homeostasis, perturbations of which manifest in many psychiatric disorders. Synaptic control of catecholamine neurotransmission is accomplished by an intricately regulated system of catecholamine reuptake, facilitated by dopamine (DA) and norepinephrine (NE) transporters (DAT and NET, respectively). Despite structural homology and functional similarity of DAT and NET, their dynamic regulation is transporter specific and cell context dependent. Thus, metabolic insulin signaling has been demonstrated to differentially regulate DAT and NET trafficking to control brain catecholamine neurotransmission.

Introduction

A sophisticated system of chemical neurotransmission between neurons enables the brain to control our physiology and behavior. Complex dynamics of the fast neuronal communication is modulated by slow-acting monoaminergic system[1]. Of particular interest here is catecholamine neurotransmission, which is essential for many brain functions such as learning, memory, attention, reward, mood, and stress[2,3]. Catecholamine signaling fidelity is maintained by transporter proteins, DAT and NET, which govern duration and magnitude of dopamine and norepinephrine neurotransmission by actively translocating catecholamines from the extracellular space into presynaptic neurons[4–6]. The essential role of DAT and NET is demonstrated by the adverse health consequences resulting from the polymorphisms in the human DAT and NET genes[7,8]. Also, transgenic mouse models lacking DAT or NET reveal phenotypes of aberrant brain physiology and severe behavioral alterations[9,10].

DAT and NET are expressed in their respective catecholaminergic neurons, which project throughout the brain from a few midbrain nuclei. The four major DA projections include the nigrostriatal, mesocortical, mesolimbic, and tuberoinfundibular pathways, while the locus coeruleus (LC) NE neurons innervate all brain regions[11]. DAT and NET belong to the solute carrier 6 (SLC6) gene family, which constitutes Na^+/Cl^- -dependent neurotransmitter-sodium symporters. These transporters utilize secondary active transport by coupling neurotransmitter reuptake with sodium gradient across the cellular plasma membrane. Cloning of NET[4] and DAT[6,12] revealed a high level of amino acid sequence homology between transmembrane domains and intracellular loops of the two transporter proteins. The predicted topological model of catecholamine transporters was later confirmed by high-resolution X-ray crystallographic structure of the bacterial leucine transporter (LeuT), a prokaryotic homolog of the SLC6 family that is structurally and functionally related to monoamine transporters[13]. Structural similarity between DAT and NET proteins may explain why the transporters are "promiscuous" for each other's neurotransmitters[3,5]. However, despite the fact that DAT and NET may substitute for each other in fulfilling their function[14], regulation of the two proteins is transporter-specific and depends on regional and cellular contexts. While highlighting general principles that control transporters' function, this review will specifically focus on how insulin signaling pathway exerts differential regulation of DAT and NET.

Potential mechanisms of transporter regulation

Transporter activity can be regulated by two distinct mechanisms: modulation of the intrinsic molecular properties and control of protein expression on the plasma membrane[2]. We will briefly discuss both potential mechanisms.

1) Transcription, translation, and anterograde trafficking to the plasma membrane are the fundamental processes that modulate transporter function[15]. However, regulatory checkpoints guiding these processes for transporters are not well understood. Only two NET and DAT transcription factors have been discovered: Phox2 and Nurr1. Overexpression of Phox2 and Nurr1 have been shown to elevate

mRNA and protein levels of NET[16] and DAT[17], respectively. Nonetheless, neither the mechanism, nor the upstream molecular regulators of Phox2 and Nurr1 have been identified.

Moreover, translation and anterograde trafficking of the transporters are also not fully understood. During protein synthesis, transporters are co-translationally translocated through the endoplasmic reticulum (ER) membrane[18]. Upon formation of oligomers in the ER, they are transported to the *cis*-Golgi network by COP (coatomer) II vesicles[19]. Oligomer formation was found to be essential for the ability of the transporters to exit the ER[20]. In order to move from the Golgi to the cell surface, both DAT and NET require N-glycosylation in the second extracellular loop[15]. Therefore, the mechanisms guiding transporters oligomerization and glycosylation indirectly regulate DAT and NET cell surface expression.

Thus, *de novo* protein synthesis, its half-life, as well as the rate of initial insertion of the transporters into the plasma membrane are essential processes controlling transporter function. Unfortunately, molecular machinery responsible for quality control of NET and DAT production as well as the mechanisms that guide sorting of the transporter proteins at the ER/Golgi interface and that govern anterograde transporter trafficking are not completely understood yet.

2) Immediate control over transporter function is maintained within the neuronal bouton *via* intraterminal trafficking and intrinsic protein modifications of NET and DAT. Initially, the transporters were thought to be the stagnant monitors of synaptic neurotransmitter concentration. Transporter conformation was thought to be the only determinant of the transient reuptake rate[2]. Understanding of the transporter regulation mechanism was propelled to a new level when cortical NET membrane expression was shown to be dependent on the extracellular norepinephrine concentration. This result suggested that neurons could control the rate of neurotransmitter reuptake by regulating the concentration of transporter proteins on the plasma membrane[21]. This regulatory method is slower than the rapid "on-site" modification of the intrinsic protein structure. However, transporters exhibit the slow kinetics of substrate translocation (approximately one substrate molecule per second per transporter)[3]. Thus, speedy intraterminal transporter trafficking to and from the plasma membrane in response to immediate external stimuli (such as changes in extracellular neurotransmitter concentration) is a plausible method to control the rate of catecholamine reuptake.

Endocytosis as a means of transporter function regulation

The transporter membrane availability is supported by local protein trafficking to and from the plasma membrane *via* exocytic and endocytic processes[20,22–24]. The process of endocytic recycling is guided by a number of different molecular mediators that maintain specificity of endosomal compartments and control the process of endosomal maturation. Endosomal regulators define the fate of the cargo – whether the endocytosed proteins recycle back to the membrane or undergo lysosomal degradation. Endosomal differentiation, mediated in large by Rab GTPases, allows for temporal and spatial segregation of the recycled cargo[25]. Rab GTPases provide organelle identity markers and serve as multifaceted organizers of nearly all membrane trafficking processes in eukaryotic cells. The array of proteins associated with Rab GTPases (such as guanine-nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), GDP dissociation inhibitors (GDIs), and GDI displacement factors (GDFs)) help to maintain the multi-level regulation system that allows precise control over the movement and longevity of endocytosed proteins[26].

Recent efforts have identified a few Rab GTPases involved in NET and DAT trafficking[23,24,27,28]. As excellent endosomal identity markers, Rab GTPases can reveal which endosomal pathway is utilized during transporters intraterminal redistribution. Various Rab GTPases are differentially involved in early and late recycling endosomes, in mature endosomes, and in lysosomal compartments[25,26]. Unraveling the sequence in which Rab proteins co-localize with translocating transporters will help understanding the timeline of trafficking events, as well as the fate of the transporter proteins during those events. Thus, analysis of NET anterograde transport allowed to exclude the possibility of NET segregation to either small or large dense core vesicles[23]. This suggested that endosomes could be involved in NET trafficking. Indeed, studies conducted in the superior cervical ganglion (SCG) nerve terminals showed co-localization of NET with Rab4 and Rab11 (recycling endosome markers)[24]. Based on the special case of amphetamine (AMPH)[a] induced NET trafficking, this seminal research provides grounds for further investigations aiming to understand the mechanisms behind intraterminal transporter trafficking.

SCG neurons elaborate profuse noradrenergic fibers in culture and present large terminals extending laterally from axonal membranes[23]. This makes SCG a convenient endogenous experimental model to study NET function.

a. **Amphetamine (AMPH)** - sympathomimetic drug inducing monoamine release from the nerve terminals into the extracellular milieu.

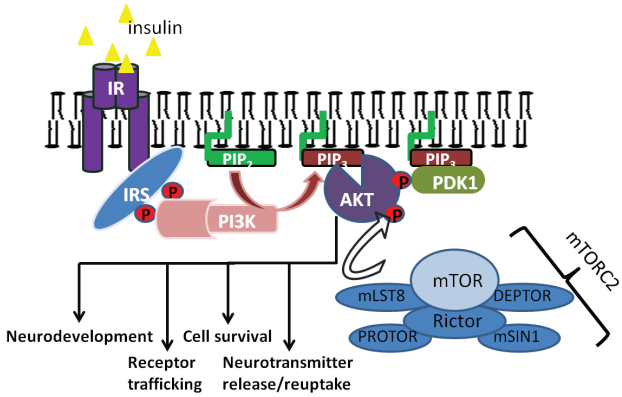

Figure 1: Insulin signaling pathway in the brain

Absence of similar experiment-friendly natural dopaminergic model system forces researchers to study DAT trafficking in heterologous expression systems. Studying constitutive and PKC-induced DAT internalization revealed co-localization of DAT with Rab11 and Rab5, respectively[28]. Interestingly, Rab5 may be substituted for Rab7 during the course of endosome maturation. This switch is a well-known trigger for endosome fusion with a lysosome, signifying a degrading pathway[25]. However, it has not been determined whether DAT trafficking undertakes this molecular route.

In vitro evidence: insulin enhances DAT function and reduces NET function

Metabolic hormone insulin was shown to influence a broad spectrum of cellular function in the nervous system *via* PI3K (phosphatidylinositol 3-kinase) / Akt signaling pathway[29] (Figure 1). Importantly, catecholamine transporters function was also found to be dependent on the integrity of the PI3K-Akt pathway, the main molecular players of which are briefly described here. Upon ligand binding, insulin receptor (IR) is autophosphorylated on its intracellular tyrosine residues, an essential step in the activation cascade. Activated IR is a tyrosine kinase (RTK), which binds and phosphorylates scaffold protein insulin receptor substrate (IRS). The downstream cascade is generated through signaling complexes that are assembled around the tyrosine-phosphorylated IRS[30]. PI3K is a lipid kinase that gets recruited to the activated IRS and converts phosphatidyl-inositol into phosphoinositide phosphates PIP_2 and PIP_3. PIP_2 and PIP_3 are "docking" lipids that trigger activation of serine/threonine kinases including 3-phosphoinoitide-dependent protein kinase-1 (PDK1) and Akt (also known as protein kinase B (PKB)) by recruiting them to the plasma membrane. Membrane-localized Akt is subsequently activated by phosphorylation at two key residues – Thr308 (by PDK1)[29], and Ser473 (by mammalian target of rapamycin complex 2 (mTORC2))[31]. Phosphorylated Akt is involved in multiple cellular functions, including metabolism, cell stress, cell-cycle, apoptosis, as well as regulation of protein synthesis and trafficking[32].

In vitro studies demonstrate that PI3K-Akt signaling differentially influences trafficking of catecholamine transporters. In case of the DAT, inhibition of the insulin signaling pathway was shown to rapidly decrease DAT function. Particularly, broad-spectrum tyrosine kinase inhibitors reduced DAT transport-associated currents, decreased DAT surface expression, and diminished DA uptake into DAT expressing *Xenopus* oocytes[33]. Brief application of PI3K inhibitors resulted in clathrin-mediated dynamin-dependent DAT endocytosis[34]. The effect was reversed with acute insulin treatment. Utilizing DAT-mediated DA releasing properties of AMPH[35,36], researchers were able to show that insulin signaling is required to maintain DAT cell surface expression, since application of PI3K inhibitors resulted in dramatic reduction of AMPH-induced DAT-mediated DA efflux in heterologous cells and dopaminergic neurons[37]. Later, *in vivo* studies confirmed these results[38].

Continuing to unravel the mechanism, researchers turned their attention to Akt, a serine/threonine protein kinase at the center of metabolic insulin signaling[32]. Expression of the dominant-negative Akt mutant or application of pharmacological Akt inhibitors induced a decrease in cell-surface expression of DAT, whereas a constitutively active form of Akt inhibited AMPH-induced DAT internalization[39]. Importantly, DAT trafficking effect was observed within minutes after stimulus application[34,37,39]. These data do not eliminate the possibility of intrinsic transporter modifications, which could occur prior to trafficking events. Further research is needed to understand whether insulin signaling has a direct role in the mechanism of maintaining DAT on the plasma membrane. Another important question is whether cytosol-redistributed DAT is capable of returning to the surface, i.e., which endosomal pathway – recycling or degrading - is employed during inhibition of insulin signaling.

In contrast with the DAT phenotype, decrease of the NET function was caused by activation of the insulin signaling pathway. Insulin application inhibited tritiated NE uptake in dissociated NET-expressing brain cells, whole brain synaptosomes, and in acute brain slices[40–43]. The mechanism behind such NET downregulation remains unknown. Interestingly, a later study conducted in the SK-N-SH cells (a human neuroblastoma cell line) demonstrated a contradicting result of elevated NET function upon insulin

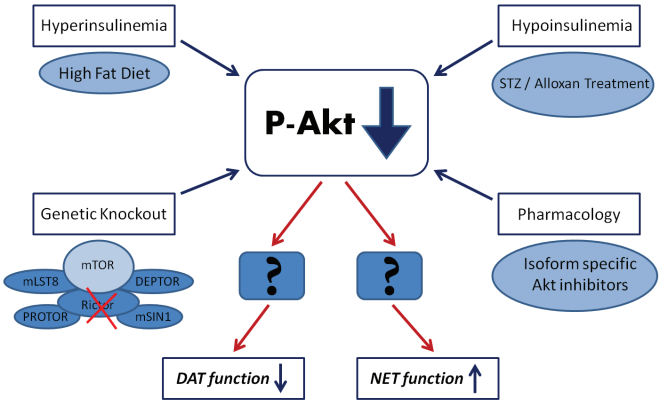

Figure 2: Multiple ways lead to disrupted insulin signaling and aberrant Akt activity

treatment[44]. Perhaps, a detailed analysis of the differences within the molecular machineries of the systems used will help deducing the occurrence of opposing results described above. This may bring us closer to understanding how insulin causes the opposite dynamics of two structurally and functionally similar transporters: DAT and NET.

Insulin signaling regulates the transporter function *in vivo*

a) Insulin in the brain: direct dependence on the peripheral insulin tone. The notion of insulin presence in the brain was controversial until 1967, when the use of sensitive radioimmunoassay techniques demonstrated not only that insulin is present in the cerebro-spinal fluid (CSF), but also that CSF levels are increased with peripheral insulin infusion[45]. Furthermore, IR is abundantly expressed in the brain, including dopaminergic and noradrenergic neurons[46]. Despite the ongoing debate on the source of brain insulin, the majority of evidence demonstrates that CNS insulin concentration depends on the fidelity of the active saturable transport of pancreatic insulin past the blood brain barrier[47–49]. Indeed, alterations in the plasma insulin concentration are mirrored by the changes in the CSF insulin level[50]. Human positron emission tomography (PET) studies showed attenuated neuronal activity evoked by a peripheral insulin injection in non-diabetic subjects with insulin resistance[51]. Such tight correlation between peripheral and central insulin tone supports the fact that alteration in plasma insulin level is capable of disrupting insulin signaling in the brain, which will consequently cause disturbance in catecholamine transporter function.

b) Pathophysiological alterations in the insulin signaling pathway and the animal models mimicking these alterations. Disruption in insulin signaling is commonly caused by persistent pathological alterations in the plasma insulin level known as hypo- or hyperinsulinemia. Both conditions result in inhibition of Akt phosphorylation[52,53] (Figure 2). In case of hypoinsulinemia, the lack of IR ligand shuts down the PI3K-Akt signaling cascade. In response to chronic hyperinsulinemia, cells develop insulin resistance by increasing degradation of IRS proteins[30,52] (Figure 1). Consequently, both hypo- and hyperinsulinemia disrupt Akt activity, leading to alteration of multiple intracellular functions, including transcription, protein synthesis and trafficking.

How can we induce alterations in peripheral insulin level in order to study its influence on the brain? In humans, hypoinsulinemia – a feature of type I diabetes mellitus – results from disrupted production of insulin by the pancreatic β cells[54]. To mimic this disease in an animal model, rodents are injected with streptozotocin (STZ) or alloxan – drugs that selectively destroy the pancreatic β cells. Hyperinsulinemia is a hallmark of an array of metabolic disturbances in humans, such as metabolic syndrome, obesity, and type II diabetes mellitus, all of which feature various levels of insulin resistance. An animal model of hyperinsulinemia may be created by feeding rodents with high fat diet. Both hypo- and hyperinsulinemic animal models are used to study how the disturbance in the insulin signaling pathway influences cellular physiology. Molecularly, both models converge on the downregulation of the Akt phosphorylation and activity (Figure 2). As discussed above, peripheral and central insulin tone are tightly interconnected, making hypo- and hyperinsulinemic animal models a good platform to study how disrupted insulin signaling is implicated in neurophysiology.

Perturbed insulin signaling *in vivo* causes aberrant DAT and NET cell surface expression

a) Disrupted insulin signaling causes DAT function downregulation. The evidence that insulin signaling may regulate catecholamine homeostasis was initially obtained from the STZ or alloxan-treated hypoinsulinemic rodents. AMPH exerts its psychostimulant action via DAT-mediated DA efflux; thus, the effect of AMPH is highly dependent on the DAT plasma membrane availability. Alloxan-treated rats demonstrated diminished locomotor activity and stereotyped behavior following AMPH administration. Importantly, the attenuated behavior was reversed by subsequent insulin treatment[55]. Such reduced response to AMPH in hypoinsulinemic animals suggested that basal insulin signaling is critical for appropriate DAT cell surface expression. Subsequent research demonstrated the ability of insulin to specifically regulate DAT plasma membrane availability. The direct assessment of striatal DAT plasma membrane expression in STZ-pretreated hypoinsulinemic rats showed reduced surface DAT[38]. *In vivo* chronoamperometric recordings in hypoinsulinemic animals demonstrated decreased striatal DA clearance, which signified of the reduced DAT cell surface

expression[56,57]. Importantly, acute insulin application rescued this phenotype. Moreover, high fat-fed insulin resistant rats were found to exhibit downregulation of the striatal DAT function that was rescued *via* restoration of the nigrostriatal Akt phosphorylation by the recombinant viral vector expression technology[58]. These findings demonstrated the plasticity of the system and showed that insulin acts rapidly *via* the PI3K/Akt pathway to regulate DAT function. However, it was also demonstrated that DAT mRNA in the ventral tegmental area (VTA) and substantia nigra (SN) regions was reduced in STZ-treated rats compared to control animals[59]. Such multifaceted evidence underlines the level of complexity, as well as the diversity of the mechanisms involved in insulin regulation of DAT. Further studies will allow deducing what external factors lead to the divergence in regulation: whether it occurs at the level of transcription or at the level of transient intraterminal protein trafficking.

*b) Disrupted insulin signaling causes NET function upregulation***.** As mentioned before, alterations in the insulin signaling pathway cause opposing dynamics for NET and DAT function. Using *in vivo* microdialysis, Shimizu et al showed reduced hypothalamic extracellular NE content in freely moving hypoinsulinemic rats[60]. In line with this finding, an increase in NE tissue content (an assessment of the intracellular neurotransmitter concentration) in the hypothalamus was also found in hypoinsulinemic animals[61]. With no significant changes in NE metabolites, these data supports the fact that altered NE reuptake could be the driving force of such an imbalance between intra- and intercellular concentration of the neurotransmitter. Recently published evidence demonstrated that the hypoinsulinemic condition induced NET trafficking *via* the Akt signaling pathway. In particular, STZ-treated mice showed enhanced NE brain tissue content levels, increased NE clearance, and elevated NET cell surface expression, a phenotype that was recapitulated by pharmacological Akt inhibition[53]. An excellent illustration of the insulin signaling influence on NET function *via* the PI3K/Akt pathway was provided in a study which analyzed cortical NE homeostasis in a genetic mouse model with attenuated ability to phosphorylate Akt in neurons. Mice with aberrant neuronal Akt function exhibited increase in total and cell surface NET expression[14]. Earlier investigations demonstrated an increase in NET mRNA in the LC of STZ-treated rats[59]. Similarly to the DAT story, NET regulation by insulin may depend on other factors and, thus, occurs at different stages of the protein life time. Further research is needed in order to pinpoint influencing factors and understand the mechanism.

Concluding remarks

Catecholamine neurotransmission is essential for normal brain physiology. Given the importance of transporters in maintaining brain catecholamine homeostasis, substantial effort must be invested to enhance our knowledge of NET and DAT regulation. The studies described above provide strong evidence that metabolic dysfunction, induced by impaired insulin signaling, impacts brain catecholamine neurotransmission by altering transporter function. Insulin was shown to influence brain NE and DA homeostasis by dynamic regulation of DAT and NET *via* PI3K/Akt signaling. Importantly, activation of insulin signaling causes downregulation of NET and upregulation of DAT function. Thus, two structurally and functionally homologous transporters with affinity for each other's neurotransmitters are regulated differently by the insulin signaling pathway. This could be the consequence of the divergent amino acid sequence within transporters intracellular domains. Another plausible explanation for such difference in transporter regulation may be the unique regional and cellular contexts of DAT and NET. Initial studies show that Akt, a kinase in the center of metabolic insulin signaling pathway, plays the key role in transporter function regulation. Further studies are warranted in order for us to understand the mechanisms underlying the comorbidity of metabolic disorders and mental illness. Identification of the molecular players will lead to new therapeutic approaches and, hopefully, to prevention of mental illnesses manifested by aberrant catecholamine homeostasis.

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