Amphetamine-fueled insights into dopaminergic diseases: the protein kinase Akt drives responses to psychostimulants

Michael Siuta

Amphetamine (AMPH) is a psychostimulant that exerts its behavioral effects, in part, through release of pre-synaptic dopamine (DA) via reversal of the dopamine transporter (DAT) at mesostriatal synapses. Due to the characteristic and robust release of DA in response to AMPH, this drug is often used to study animal models of diseases where DA dysfunction at mesostriatal synapses is implicated, namely schizophrenia, Parkinson's disease, and drug addiction. Interestingly, the function of the protein kinase Akt (also known as protein kinase B) has recently been associated, in both human and animal studies, with both the pathogenesis and treatment of these DA-related diseases. Akt is stimulated by phosphotidylinositol 3-kinase (PI3K) signaling, which itself is activated by growth factors (such as brain derived neurotrophic factor) and hormones (such as insulin) through receptor tyrosine kinases (RTKs). Many of these growth factors and hormones also influence the actions of psychostimulants through cellular and molecular mechanisms that include promotion of DAT trafficking, increased axonal innervation of the striatum, and enhanced synthesis of pre-synaptic dopamine. Recent evidence suggests that many of these mechanisms may be profoundly regulated by Akt. Collectively, studies of the activation and inhibition of PI3K/Akt signaling, through pharmacologic, genetic, or viral manipulations, suggest a prominent role for Akt signaling in neuronal growth, neuronal migration, and regulation of DA neurotransmission. These findings hold promise for development of future strategies aimed at more directly influencing Akt signaling in the brain in order to treat dopaminergic diseases.

Neuroscience Graduate Program, Vanderbilt University School of Medicine, U1205 Medical Center North, Nashville, TN 37232, USA.
Correspondence e-mail: michael.siuta@vanderbilt.edu.

Psychostimulants like amphetamine (AMPH) are used to study behavior and physiology in animal models of Parkinson's disease, schizophrenia, and addiction [1, 2, 3]. While the symptoms of these diseases are quite disparate in humans, they are all, to some degree, linked to the function of dopaminergic systems in brain. Recent evidence suggests that common intracellular signaling pathways may be important in the treatment and pathogenesis of these diseases. One such pathway involves the serine/threonine protein kinase Akt. Human studies demonstrate that genetic variation in the isoform Akt1 influences dopamine-associated structures and functions in humans[4], and, potentially, the risk for schizophrenia, methamphetamine abuse[5], and Parkinson's disease[6]. Human studies have also discovered defects in phosphorylation of Akt related to mental illness diagnoses[7, 8, 9], suggesting that activators of Akt, like the phosphotidylinositol 3-kinase (PI3K) proteins, also modulate dopamine (DA) in brain. PI3K is activated by receptor tyrosine kinases (RTKs), which, in turn, are activated by a diverse set of hormones, including insulin[10], and growth factors, including brain-derived neurotrophic factor[11] (BDNF). Intriguingly, many RTK ligands, along with PI3K/Akt itself, influence the actions of AMPH and other psychostimulants[12-17].

One of the most studied functions of AMPH is its ability to increase synaptic DA. AMPH accomplishes this by multiple mechanisms, including DA-efflux through reversal of the dopamine transporter (DAT), the major protein involved in synaptic clearance of DA. AMPH is also capable of entering the cell to trigger release of DA from pre-synaptic vesicle stores, again by reversal of transporter function. Trafficking of the DAT to the cell surface has recently shown to be dependent on RTKs[18], PI3K[19], and Akt[20], providing a molecular mechanism to explain the potential for hormones and growth factors to modulate DA systems and responses to stimulants.

In addition to surface levels of DAT, the magnitude of DA release elicited by AMPH, and the effects on consequent behaviors, are also governed by the amount of pre-synaptic DA available. Pre-synaptic DA can be influenced by several factors, including DA synthesis, the health of DA neurons, and the density of DA terminals, processes where PI3K/Akt also plays a role[13]. Thus, the goals of the present review are to (1) model the regulation of the DAT and responses to psychostimulants by PI3K/Akt, (2) review the activators of PI3K/Akt in brain, and analyze their PI3K/Akt dependent functions, and (3)

integrate evidence from animal and culture studies to assess mechanisms underlying the relationship between RTKs, PI3K/Akt signaling, and responses to psychostimulants. As activation or inhibition of PI3K/Akt signaling profoundly influences DA-related behaviors, understanding the different levels (cellular and molecular) at which Akt modulates AMPH actions provides insights into how this pathway regulates both pre-synaptic DA and the DAT, an important pharmacological target. Understanding AMPH responses may help to inform ways to target Akt for the treatment of psychiatric and neurologic diseases.

PI3K/AKT SIGNALING, DAT SURFACE EXPRESSION, AND RESPONSES TO PSYCHOSTIMULANTS

The PI3K/Akt signaling cascade can be activated following stimulation of RTKs[21]. The tyrosine-phosphorylated protein products of receptor stimulation interact with the SH2 domain on growth factor sensitive isoforms of PI3K, stimulating its lipid kinase activity. PI3K then catalyzes phosphorylation of phosphoinositides at the 3-position in the inositol ring, causing an increase in the generation of PIP2 and PIP3. The Pleckstrin homology (PH) domain of Akt interacts with these phosphorylated phosphoinositide byproducts, which causes membrane translocation of Akt. This translocation allows Akt to be phosphoryated itself at the Threonine-308 and Serine-473 residues by phosphoinositide-dependent kinase 1 (PDK1) and the mammalian target of rapamycin (mTOR) complex 2 (mTORC2). Phosphorylation of Akt at the 308 and 473 residues is necessary for full activation of the enzyme's kinase function.[21]

Inhibition of PI3K pharmacologically with LY294002 decreases cell surface expression of the DAT both *in vitro*, in heterologous cell culture lines, and *ex vivo*, in striatal synaptosomes[22]. Stimulation of PI3K activity with either insulin pretreatment or constitutively active PI3K results in an enhancement of DA uptake[22]. A direct role for Akt in these effects is suggested by studies *in vitro* where AMPH-induced internalization of the DAT, and consequent reductions in DA uptake, are blocked by a virus expressing constitutively active Akt or insulin stimulation, in a PI3K- and Akt-dependent manner[20]. Compelling *in vivo* evidence to support the relationship between PI3K/Akt signaling and the DAT comes from studies in hypoinsulinemic animals, which show reduced Akt activity in brain along with reduced DAT cell surface expression, DA clearance, and amphetamine-induced efflux of DA[12]. Pharmacologic inhibition of PI3K in the rodent striatum causes a parallel reduction in AMPH-induced DA efflux, and local pretreatment with insulin restores the effects of DA clearance and AMPH-induced efflux in hypoinsulinemic mice[12]. Together, this evidence suggests that local activitation of RTK/PI3K/Akt signaling is the mediator of these effects in hypoinsulinemic animals.

The decreased DAT cell surface expression and AMPH-induced DA efflux with PI3K inhibition provides a potential mechanism to explain how Akt activation and inhibition affects psychostimulant- and reward-related behaviors observed in other studies. Hypoinsulinemic animals show diminished self-administration of AMPH[3], consistent with the diminished availability of surface DAT to promote DA release with drug use. In a similar fashion, administration of the PI3K inhibitor LY294002 reduces the sensitizing effects of cocaine[16]. In addition to addiction models, Parkinson's disease models also often rely on AMPH-induced behavioral endpoints to track functional effects of various lesions and treatments. Usually, these models involve AMPH-induced locomotor rotations following unilateral lesions or treatments to DA cell bodies in the substantia nigra. A unilateral 6-hydroxydopamine lesion (6-OHDA) to the substantia nigra, for example, results in differential AMPH-induced release of DA between the lesioned and unlesioned sides of brain, and this functional asymmetry is reflected in increased turning behavior toward (ipsiversive) the lesioned side. Unilateral injections of associated adenovirus vectors (AAVs) expressing myristolated Akt (myr-Akt), a constitutively active form of Akt, results in contraversive turning behaviors. This suggests a relative increase in AMPH-induced DA in the myr-Akt expressing side. This enhanced AMPH response is likely due at least in part to elevated nigral DA associated with myr-Akt expression, which supports the overall ability of Akt signaling to promote the actions of AMPH[13].

Characteristic cellular changes associated with Akt signaling also reflect differences in reward sensitivity and responses to stimulants observed with Akt modulation. Withdrawal periods following chronic opiate administration, for example, cause diminished sensitivity to opiate reward (as measured by conditioned place preference (CPP)), reductions in Akt phosphorylation, and decreased midbrain DA neuron size [23]. The cellular basis of the effects on sensitivity to reward are emphasized in this particular study, as viral inhibition of PI3K/Akt signaling in the midbrain itself reduces cell body size and CPP, suggesting the Akt downregulation is sufficient to cause the observed cellular and behavioral responses to chronic opiates. Viral enhancement of the pathway, conversely, reverses the effects of chronic opiates on cell size and reward-related behaviors[23]. Similarly, myr-Akt injections, which increase responses to AMPH[13], as stated above, also enlarge tyrosine hydroxylase (TH) neuron cell bodies in

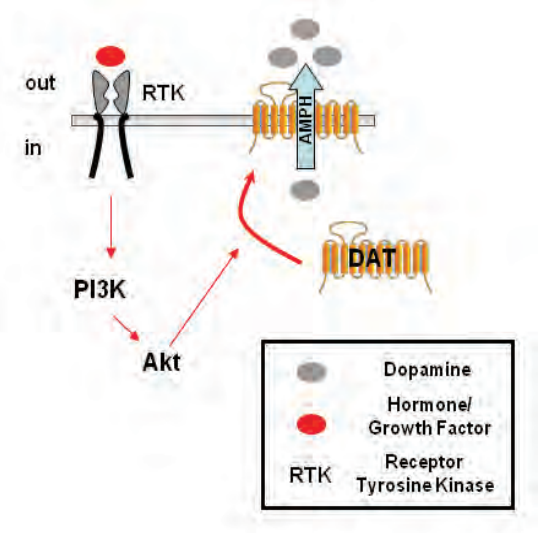

Figure 1 | **Model of PI3K/AKT influence on the dopamine transporter.**

midbrain[13, 24] and increase the density of striatal TH terminals[13]. Indeed, oftentimes it is difficult to disentangle the potential cellular versus molecular influences of Akt on responses to psychostimulants, unless the effects evaluated are compared on an acute time scale (where molecular effects like trafficking presumably predominate) versus a chronic time scale, when the trophic influence of Akt become prominent. RTK activators, which have a growing number of documented PI3K-dependent effects, have long been studied as modulators of responses to psychostimulants in different contexts. Thus, findings from these studies provide insight into the mechanisms whereby Akt signaling in brain can promote DA release in response to psychostimulants (See model in **Figure 1**).

PI3K/AKT-DEPENDENT INFLUENCES OF RTKS ON DOPAMINE SYSTEMS

RTKs that stimulate Akt signaling in brain: Insulin stimulates PI3K/Akt signaling through activation of a receptor tyrosine kinase (RTK) and promotes DAT trafficking to the plasma membrane[20]. While the insulin receptor is widely distributed in brain[25], there are many other RTKs in brain which affect DA systems that also have PI3K-dependent effects. Among the RTK ligands also capable of inducing Akt phosphorylation are nerve growth factor[26](NGF), brain-derived neurotrophic factor[11](BDNF), glial-derived neurotrophic factor[27](GDNF), fibroblast growth factor [28](FGF), and the epidermal growth factor (EGF) family of proteins, which includes neuregulin-1[8](NRG-1). A role for many of these RTKs has been postulated in either the pathogenesis or treatment of schizophrenia[29], psychostimulant addiction[30] and Parkinson's[31], suggesting that RTKs influence dopaminergic systems in a similar fashion to PI3K/Akt signaling.

PI3K-dependent cellular influences of RTKs: An increasing number of PI3K-dependent effects of RTK ligands have recently been uncovered, largely focused on the trophic effects of Akt. For example, the promotion of neurite outgrowth in dopaminergic cell lines by NGF is partly inhibited by the PI3K inhibitor LY290042[32]. In addition, the ability of NRG-1 to induce chemotactic migration is blocked by inhibition of PI3K and the NRG-1-associated RTK, erbB2[33]. IGF-1 stimulation of growth cone expansion in cultured neurons is also attenuated by treatment with LY294002[34]. Intriguingly, myr-Akt expression in the substantia nigra, described above, results in increased tyrosine hydroxylase positive terminals in the striatum without changing cell density in the nigra itself. This suggests that the increased terminal density is not due to changes in cell number but changes in target innervation[13]. These findings suggest that one potential mechanism for the influence of Akt on DA systems is through promotion of axonal outgrowth from DA cell bodies, resulting in increased DA terminal density. Together with evidence supporting the influence of Akt on cell size, mentioned above, and the PI3K-dependence of BDNF, IGF-1, and estrogen on neuroprotection *in vitro*[27] and *in vivo*[10], Akt seems to be a powerful positive modulator of DA systems[13].

RTKs, PI3K, and DA synthesis and release: In addition to cellular events, which occur over a longer time course, RTKs also promote short-term modulation of DA systems through PI3K/Akt signaling. In PC12 cells, NGF, EGF, and IGF-1 enhance stimulated release of DA release in a manner subject to inhibition of PI3K[35, 36]. Recent evidence implicates that this effect is true in brain also, as treatment with BDNF in striatal slice preparations also enhances stimulated release of DA, and this effect is blocked by LY249002 administration[37]. The mechanisms underlying the enhanced release of DA by RTKs is unknown, but they are believed to be presynaptic[37], and could potentially involve a combination of factors including stimulation of DA synthesis by TH[13], enhancement of calcium-responsible secretory vesicles[35], and promotion of DA recycling via DAT trafficking to the cell surface[20]. These mechanisms are all consistent with the overall effect of myr-Akt viruses in the dopaminergic midbrain- a promotion of pre-synaptic DA function reflected by increased cell size, terminal density, total nigrostriatal dopamine content, and AMPH-induced behaviors[13, 24]. These mechanisms, in conjunction with promotion of cell surface DAT, contribute to the ability of Akt to promote DA release in response to AMPH.

ACTIVATORS OF PI3K/AKT SIGNALING AND RESPONSES TO PSYCHOSTIMULANTS

RTKs in DAT trafficking: According to the model provided in **Figure 1**, RTK activators will promote DAT cell surface expression, DA uptake, and responses to stimulants, and inhibitors, such as LY249002, will diminish these effects. One study supporting this model showed that, in rat striatal synaptosomes, both RTK inhibition (with genistein and tyrphostin) and PI3K inhibition led to a rapid downregulation of DA clearance and DAT cell surface expression[18]. Conversely, acute growth factor (BDNF) treatment increased DA uptake, and this increase is prevented upon co-treatment with the PI3K inhibitor LY294002[18], paralleling previous findings on the effects of insulin. In addition, the effect of RTKs on DA uptake in this study are primarily dependent on the Vmax for uptake, as opposed to the Km. Thus, this effect of RTKs on DA clearance is attributable to the total number of available DAT, rather than a change in affinity[18].

BDNF and responses to stimulants: Thus, the regulation of the DAT by RTKs directly parallels the modulation of DAT by insulin[12], and which is dependent in part on PI3K. This is significant for the established role of BDNF in the regulation of DA release and related behaviors in response to psychostimulants[15, 38]. Both intra-NAc or intra-VTA infusions of BDNF enhance locomotor responses to cocaine[15], consistent with the model in **Figure 1** of increased DAT availability and overall promotion of pre-synaptic DA by Akt. Several studies support this relationship between BDNF and psychostimulant behaviors, with anti-BDNF antibodies decreasing and viral enhancement of BDNF increasing locomotor activity in response to methamphetamines[38,39]. In line with these findings, antibodies directed against either BDNF or its RTK also diminish DA release in response to methamphetamine[38], suggesting BDNF promotes mechanisms related to increasing stores of pre-synaptic dopamine.

GDNF-related responses to psychostimulants: Interestingly, BDNF and GDNF seem to have opposite effects on reward-related behaviors, as studies show that GDNF *decreases* cocaine and opiate conditioned place preference[40], while BDNF *increases* drug reward and promotes self-administration of stimulants[30]. While the effects of GDNF seem contrary to our model, studies that measure GDNF effects on AMPH-induced release of D, support our model, with GDNF stimulation increasing and GDNF inhibition decreasing AMPH-induced DA efflux[41, 42]. This is in direct parallel to the proposed influence of BDNF on methamphetamine-induced efflux[38], suggesting that BDNF and GDNF may not ultimately have entirely opposite effects on responses to AMPH. Other findings on GDNF in support of our model include pronounced enhancements of DA uptake in GDNF-treated midbrain neuron cultures[43] and enhanced AMPH-induced locomotion with single nigral injections of GDNF [44]. Studies in animals with nigrostriatal lesions show that GDNF treatment enhances striatal DA content[45] and increases cell surface labeling of the DAT by radioligands[46, 47], suggesting an overall support of pre-synaptic DA function by GDNF. GDNF thus appears to enhance locomotor effects of stimulants, although conditioned place preference is diminished in treated animals.

Potential role of BDNF in cocaine sensitization: BDNF, in contrast to GDNF, is theorized to have an important role in the initiation of drug addiction[30]. The role of BDNF in models of psychostimulant addiction is particularly intriguing, as cocaine self-administration has been shown to increase midbrain BDNF levels[30]. In mice trained to self-administer cocaine, local deletion of BDNF in the nucleus accumbens, through conditional knockout strategies, diminishes cocaine self-administration[30]. The dynamics of BDNF signaling in the acquisition of cocaine addiction are therefore in line with our model. In normal animals, upregulation of BDNF with cocaine administration[30], according to our model, would lead to net activation of PI3K/Akt signaling. This, in turn, would stimulate DAT trafficking, providing an increased numbers of substrate for cocaine to bind to with repeated drug administration and also promoting replenishment of pre-synaptic DA. Intact Akt signaling, we hypothesize, is required for appropriate reuptake and recycling of DA into pre-synaptic terminals with DA release. Future biochemical and physiological studies are needed to determine the validity of this model.

Other RTKs and modulation of DAT: There are many other RTKs that may influence DA function similarly, including IGF-1, estrogen, FGF, and EGF. Some evidence already exists for modulation of DA function by these RTK ligands. Both FGF and epidermal growth factor (EGF) increase DA uptake in cultured cells[48], and FGF acutely enhances DAT cell surface expression[28]. However, the Akt dependence of these effects have yet to be determined.

CONCLUSIONS

The consensus in the literature on overall effects of Akt on DA systems is toward a promotion of DA release and DA-related behaviors in response to AMPH. Growth factor and hormonal signaling through RTKs is an increasingly well understood mechanism for regulation of nigrostriatal DA with therapeutic implications. The multiple mechanisms whereby RTKs and Akt potentially enhance AMPH actions converge at the promotion of are pre-synaptic DA function, causing increases in cell size, axonal

density, DAT trafficking and, potentially, upregulation of tyrosine hydroxylase. Animal models that focus on the temporal relationship between RTK signaling and DAT dynamics are warranted in order to separate contributions of DAT trafficking (on an acute time course) and cellular trophism (on a chronic time course) to AMPH actions; the Akt-dependence of any observed effects should also be established. Future studies in humans will bear out the potential of these mechanisms to translate into treatments of dopaminergic diseases.

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FURTHER INFORMATION

Aurelio Galli's Lab:
https://medschool.mc.vanderbilt.edu/facultydata/php_files/part_dept/show_part.php?id3=4070