THE ROLE OF INSULIN AND AKT IN THE REGULATION OF THE

NOREPINEPHRINE TRANSPORTER AND MONOAMINE HOMEOSTASIS

By

Sabrina Elizabeth Robertson

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Approved:

Randy Blakely

Roger Colbran

Aurelio Galli

Anne Kenworthy

For Charles and Jean Doughty

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| | |
|---|---|
| 5-HT | Serotonin |
| AADC | Aromatic-L-amino acid decarboxylase |
| ADHD | Attention deficit hyperactivity disorder |
| AMPH | Amphetamine |
| C | Celsius |
| COMT | Catechol-O-methyl transferase |
| CNS | Central nervous system |
| D2DR | D2 dopamine receptor |
| DA | Dopamine |
| DAT | Dopamine transporter |
| DBH | Dopamine $\beta$ hydroxylase |
| g | Gram |
| h | Hour |
| IP | Immunoprecipitation |
| IR | Insulin receptor |
| IRS | Insulin receptor substrate |
| kDa | Kilo Dalton |
| KO | Knockout |
| L | Liter |
| LC | Locus Coerleus |
| LeuT | Leucine transporter |

| | |
|---|---|
| LT | Lateral Tegmental |
| M | Molar |
| μg | Mirogram |
| μM | Miromolar |
| mg | Milligram |
| min | Minute |
| mL | Milliliter |
| mM | Millimolar |
| mTORC2 | Mammalian target of rapamycin complex 2 |
| NAcc | Nucleus accumbens |
| NE | Norepinephrine |
| NET | Norepinephrine transporter |
| ng | Nanogram |
| OI | Orthostatic intolerance |
| PET | Positron emission tomography |
| PDK1 | Phosphoinositide-dependent kinase-1 |
| PFC | Pre-frontal cortex |
| PI3K | Phosphatidylinositol 3-kinase |
| PIP2 | Phosphatidylinositol-4,5-bisphosphate |
| PIP3 | Phosphatidylinositol-3,4,5-trisphosphate |
| PNS | Peripheral nervous system |
| s | Second |
| SERT | Serotonin transporter |

| | |
|---|---|
| SNP | Single nucleotide polymorphism |
| SNRI | Selective norepinephrine reuptake inhibitor |
| SSRI | Selective serotonin reuptake inhibitor |
| TH | Tyrosine hydroxylase |
| TMD | Transmembrane domain |
| VMAT2 | Vesicular monoamine transporter type 2 |
| WT | Wild-type |

# CHAPTER I

## INTRODUCTION

### Overview of Monoamine Systems

*Catecholamine synthesis and discovery*

As the brain's endogenous chemical messengers, neurotransmitters provide the foundation for the sophisticated system of communication in the nervous system that enables us not only to eat and breathe, but also to feel and think. The first neurotransmitter discovered was acetylcholine in 1921 by the German Otto Loewi (Bennett 2000). Since this original discovery, the list of neurotransmitters has expanded tremendously and subsequent studies designed to understand the intricacies of their function in the brain grow exponentially by the day. Neurotransmitters that contain a single amino group connected by a two carbon chain to an aromatic ring are classified as monoamine neurotransmitters. Of particular interest here are those monoamines that contain a catechol group, the catecholamines such as norepinephrine (NE), also known as noradrenaline, and dopamine (DA). NE and DA are generated from the same synthetic pathway in the brain, NE by a three step process and DA by a simple two step process (Figure 1). This pathway begins with tyrosine which is converted in the initial rate limiting step by the enzyme tyrosine hydroxylase (TH) into 3,4-dihydroxyphenylalanine. 3,4-dihydroxyphenylalanine is then utilized to generate DA by aromatic amino acid decarboxylase (AADC), which is then accumulated within the vesicle and ready for synaptic signaling in dopaminergic neurons or, in noradrenergic neurons, where DA β
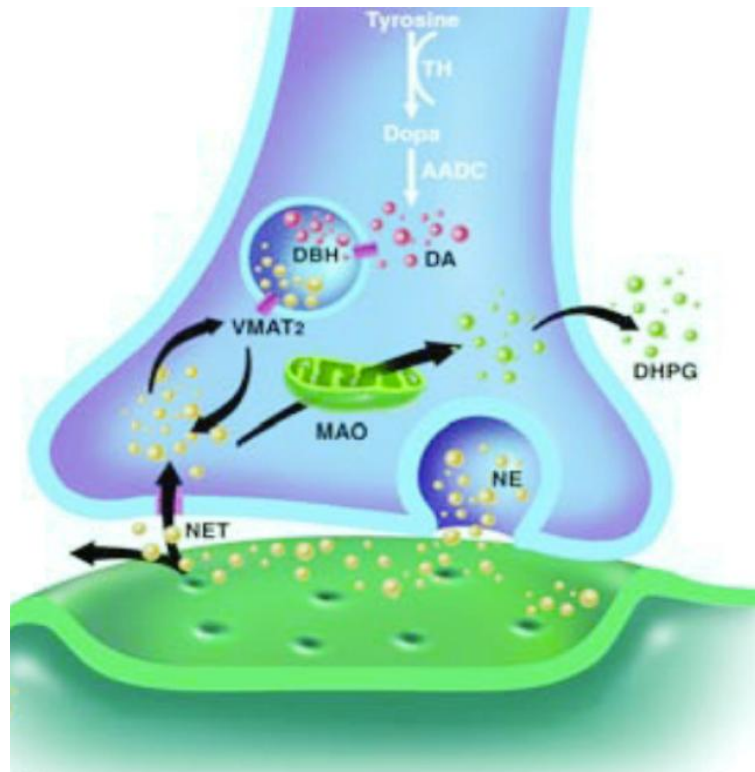
**Figure 1**. Noradrenergic synapse. In the presynaptic terminal of a noradrenergic neuron, conversion of tyrosine into Dopa via tyrosine hydroxylase, the rate limiting enzyme in catecholamine synthesis, initiates the production of NE. Following Dopa production, aromatic-L-amino acid decarboxylase is responsible for the production of DA which is then loaded via the vesicular monoamine transporter type 2 (VMAT2) into vesicles. At this point, in dopaminergic neurons, vesicles are ready for neurotransmitter release. In the noradrenergic neuron, however, once inside the vesicle, DA is converted by dopamine β hydroxylase into NE for subsequent release. Importantly, after the arrival of an action potential and corresponding calcium influx, fusion of the NE-loaded vesicles allows for NE to fill the synaptic space. In the synapse, the NE transporter is absolutely critical for clearing synaptic NE and thereby determining the duration and intensity of noradrenergic signaling. Following reuptake through the transporter, NE is then either reloaded into synaptic vesicles or is broken down by monoamine oxidase within the mitochondria. Adapted from Dr. Randy Blakely at Vanderbilt University.

hydroxylase (DBH) is responsible for the conversion of this DA into NE.

Today, while the importance of these two monoamine neurotransmitters as chemical messengers within the brain is widely accepted, years of research provided the foundation for this easily accepted notion. Indeed it was not until almost 30 years after

the initial discovery of the first neurotransmitter that the Swedish scientist Ulf Svante von Euler revealed NE as the neurotransmitter utilized by neurons in the sympathetic nervous system (Ordway et al. 2007). Soon to follow, Peter Wilhelm Joseph Holtz expanded the role of NE from the peripheral nervous system (PNS) to the central nervous system (CNS) by his discovery of its presence within the brain. Unlike NE, the discovery of DA in the brain predates its recognition as a neurotransmitter by some time. As an intermediary in the synthesis of NE, initially researchers believed DA was simply this and not a neurotransmitter in its own right. It was not until 1958 that seminal work, by Avid Carlsson and colleagues, recognized that DA itself was a vital neurotransmitter for movement (Carlsson et al. 1958). Since these initial discoveries years of research have been dedicated to understanding the role these monoamines play in the brain and ultimately behavior. In this thesis, through my studies and discussion of the NE transporter (NET) trafficking and function, I hope to provide new insights about the role and regulation of these monoamine neurotransmitters in the brain. As represented schematically in Figure 1, NET is a protein critical for clearing NE and sometimes DA from the synapse, and thus NET is essential for controlling both the duration and intensity of noradrenergic and at times dopaminergic signaling in the brain. Given this significant contribution to monoamine homeostasis, discussion and study of the transporter will afford not only perspective of how aberrant regulation of monoamine neurotransmitters may ultimately contribute to mental health disorders but also promises to reveal novel therapeutics for treating such disorders.

*Monoamine function*

Groundbreaking research over the last several decades has illuminated fundamental insight into the precise role NE and DA play in brain function. In particular, the development of sophisticated transgenic mouse models has afforded us the unique opportunity to study the physiological and behavioral consequences of preventing catecholamine production *in vivo*. In the 1990's Richard Palmiter and colleagues generated a series of knockout (KO) mice targeting key enzymes in the catecholamine synthetic pathway such as TH and DBH (Thomas et al. 1995; Zhou et al. 1995; Zhou et al. 1995). KO of TH, which catalyzes the initial rate limiting step in the synthesis of both DA and NE, enabled researchers to examine *in vivo* the physiological effects of abolishing catecholamine production altogether. Interestingly, TH knockout proved to be embryonic lethal further emphasizing the importance of these catecholamines (Thomas et al. 1995). Additional studies from the same group demonstrate that DBH, the enzyme required for the conversion of DA into NE, KO also disrupts appropriate embryonic development in mice, and in fact 88% of DBH KO mice do not survive (Thomas et al. 1995; Thomas et al. 1997; Thomas et al. 1997). Those DBH KO mice that do survive are believed to do so only through the maternal transfer of the minimal levels of NE required, and of these survivors 60% die within 5 weeks of life. In addition, DBH KO mice have significantly reduced body mass, experience hypothermia more easily than wild type controls, and fail to piloerect or constrict peripheral vasculature (Thomas et al. 1995; Thomas et al. 1997; Thomas et al. 1997). While these studies firmly establish a vital role for NE in fetal development, study of DBH KO mice supplemented with NE to bypass fetal requirements allowed for more sophisticated investigation into the role of NE in

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behavior. Indeed, such DBH KO mice show decreased maternal care, normal learning, but decreased memory, and disturbed motor function (Thomas et al. 1997; Thomas et al. 1997; Thomas et al. 1998; Weinshenker et al. 2002). Interestingly, although the condition is incredibly rare, humans with severe DBH deficiency have been found (Man in 't Veld et al. 1987; Usera et al. 2004; Senard et al. 2006). Despite the existence of such a condition, detailed study of these individuals is still lacking, and thus it remains to be seen if phenotypes of the DBH KO mice are consistent with the human condition. Although, consistent with the notion that these catecholamines are critical for normal fetal development, mothers of DBH deficient patients have a history of spontaneous abortions and still births (Vincent et al. 2002).

*Monoamine projections*

Whereas KO animal studies emphasize the importance of catecholamines, years of research on the neurons that synthesize, store, and release NE and DA provide an excellent foundation for understanding the role of these neurotransmitters in mediating specific behaviors. Figure 2 provides a schematic representation of the most highly studied and well characterized noradrenergic and dopaminergic nuclei in the brain as well as representations of their neuronal projections throughout the brain. The widespread projections of these monoaminergic neurons in the CNS insinuate an essential role for these neuromodulators in numerous brain functions.

In comparison to the noradrenergic system, the DA system is significantly more restricted with respect to its distribution within the brain. The four major dopaminergic projections include the tuberoinfundibular pathway (not pictured in Figure 2), the

**Figure 2**. Monoamine projections in the central nervous system. Schematic illustration of dopaminergic (top) and noradrenergic (bottom) projections. DA neurons in the nigrostriatal pathway project to the striatum from the substantia nigra. DA neurons from the ventral tegmental area that project to the nucleus accumbens and the prefrontal cortex, constitute the mesolimbic and mesocortical pathways, respectively. The tuberoinfundibular pathway is not depicted. NE neurons in the locus coeruleus project to a vast array of regions within the CNS. Given the widespread distribution of these projections, noradrenergic function modulates a variety of brain functions ranging from memory and mood to feeding behavior and attention. Other noradrenergic nuclei are not depicted such as the lateral tegmental nucleus which has more limited projections to regions such as the amygdala and spinal cord. Adapted from Neuroscience Exploring the Brain Third Edition by Mark F. Bear, Barry W. Connors, and Michael A. Paradiso.

**Dopaminergic System**

Frontal lobe

Striatum

Substantia nigra

Prefrontal cortex

Nucleus accumbens

Ventral tegmental area



**Noradrenergic System**

Neocortex

Thalamus

Hypothalamus

Temporal lobe

Locus coeruleus

Cerebellum

To spinal cord
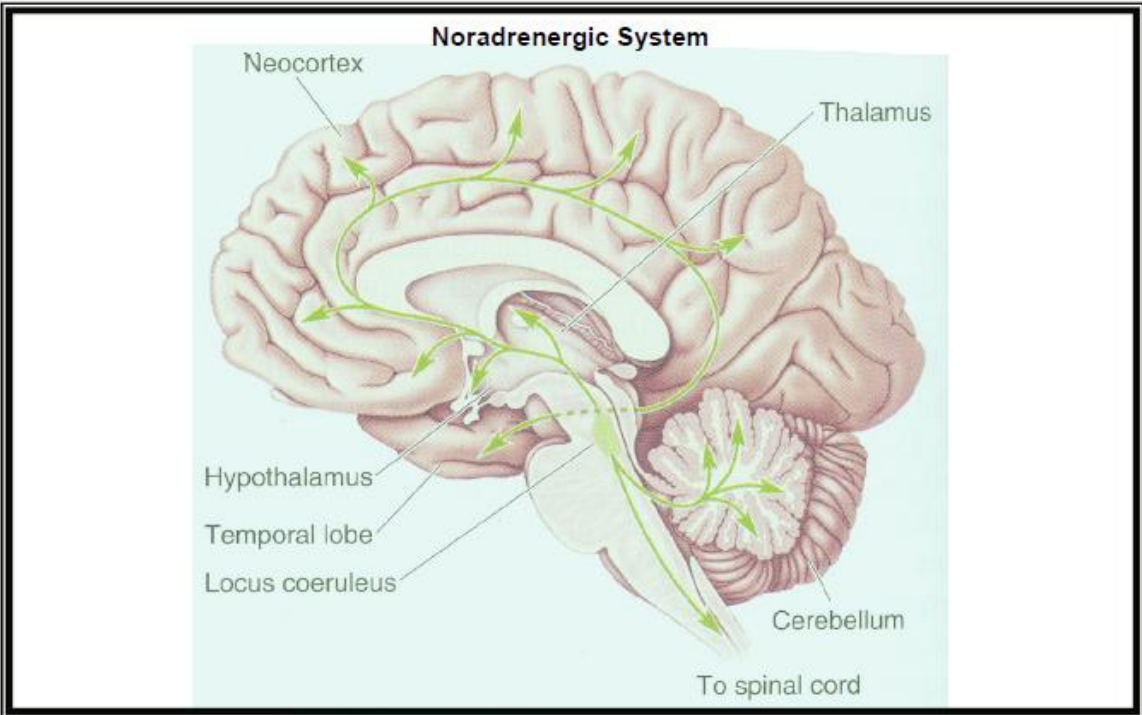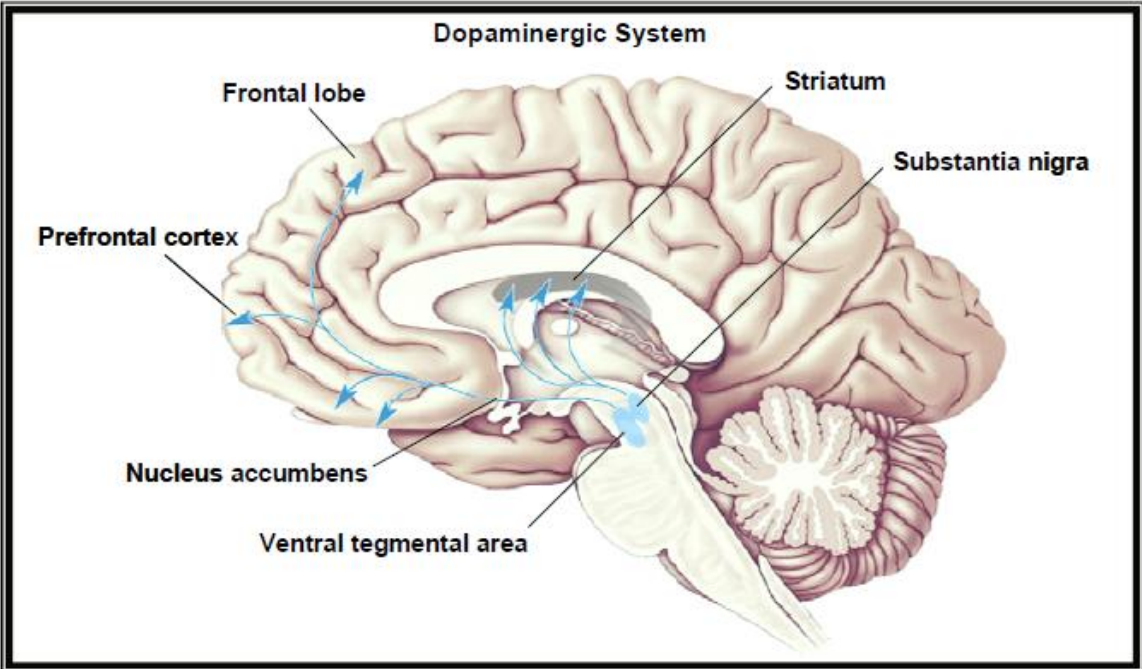
nigrostriatal pathway, the mesocortical pathway, and the mesolimbic pathway. Globally, proper dopaminergic tone in the brain is important for motor function, cognition, mood, motivation, and reward. Given the focus of this thesis on NET, however, and its capacity to dramatically influence noradrenergic and at times dopaminergic activity in the brain, our discussion of DA and dopaminergic projections will be limited to those regions of the brain which can be influenced by NET activity. Two particular regions of DA release impacted by NET function are the prefrontal cortex (PFC), which is part of the mesocortical pathway, and the nucleus accumbens (NAcc) shell, a component of the mesolimbic DA pathway (Gresch et al. 1995; Yamamoto et al. 1998; Moron et al. 2002; Miner et al. 2003). Significantly low levels of the dopamine transporter (DAT) within the cortex, necessitate the need for other means of DA clearance (Sesack et al. 1998). Indeed, the primary mechanisms for clearing synaptic DA in the PFC is through enzymatic degradation by catechol-O-methyl transferase (COMT) and reuptake by NET (Axelrod et al. 1958; Moron et al. 2002). Thus, NET function can significantly impact DA levels in this mesocortical pathway that modulates brain functions such as cognition, motivation, and reward. Although reuptake of DA through NET is less substantial in the NAcc shell, NET can play a minor role in DA clearance here and thus has the potential to impact mesolimbic pathway functions such as reward and pleasure. Importantly, deficits in the mesocortical pathway are associated with cognitive disorders such as schizophrenia and attention-deficit hyperactivity disorder (ADHD), whereas deficits in both the mesocortical and mesolimbic pathways have been linked to substance abuse disorders. Thus, abnormal NET function or regulation in these DA rich areas of the brain has the potential to contribute to such DA related disorders.

Unlike like the relatively restricted projections of the dopaminergic system, NE neurons innervate virtually all areas of the brain including the spinal cord. Indeed, single noradrenergic neurons have been shown to innervate regions as disparate as the cortex and the spinal cord (Room et al. 1981). The noradrenergic system is subdivided into two major divisions, the lateral tegmental (LT) system (not pictured in Figure 2) and the locus coeruleus (LC). The LT system contains cell groups, defined by Moore and Card, A1-A3, A5, A7 whereas the LC is comprised of cell groups A4 and A6 (Moore et al. 1979). The LT system, which is substantially less well studied than the LC, projects primarily to the spinal cord, brainstem, hypothalamus, and the basal forebrain. As reflected in these projections, the LT noradrenergic system plays an important role in the regulation of food intake, stress, anxiety, and mediating the body's fight or flight system through modulation of the PNS.

*The locus coeruleus*

Although the LT system remains relatively uncharacterized, the LC is one of the most heavily studied neuronal groups to date. Indeed, the first documented description of the LC by Reil in 1809 significantly predates the discovery of NE itself (Reil 1809). The early identification of this noradrenergic nucleus in large part is due to its unique blue black color in postmortem brain tissue. In fact, the distinct color of this nucleus prompted Wenzel in 1812 to coin the term 'locus coeruleus' which means 'the blue spot'(Wenzel 1812). Approximately, 55,000 to 65,000 cells comprise the LC in humans which can be divided into four distinct sub-zones according to the region of brain (i.e. hippocampus, cortex, hypothalamus, or spinal cord/cerebellum) innervated by projecting

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neurons along the dorsal-ventral and anterior-posterior axes (Iversen et al. 1983; Pearson et al. 1983; Baker et al. 1989; Chan-Palay et al. 1989). Whereas NE neurons from the LC can be somewhat subdivided based on projections to these four different regions, this is certainly not an inclusive list of all the diverse regions of the brain that LC activity may influence. Indeed, the projections of LC neurons innervate essentially all of the regions of the brain, and even single neurons within the LC can project to areas as distinct as the PFC and brainstem.

Given the expansive reach of LC projections, this noradrenergic nucleus has been implicated in a variety of brain functions such as sleep, arousal, stress, attention, memory, etc. Indeed, extensive research of LC activity has yielded significant insight into the role of NE in the nervous system. Current conceptualizations of LC function indicate that the LC, which receives information from numerous brain regions and subsequently projects to a wide array of regions, may provide a site where converging streams of information are prioritized (Simpson 2007). Consistent with this hypothesis, LC activity is responsive to both alterations in organism homeostasis as well as changes in environmental stimuli. For example, LC firing correlates extremely well with the sleep wake cycle, and thus this nucleus and NE play a critical role in regulating sleep (Aston-Jones et al. 1981). Years of research on this aspect of noradrenergic function is summarized elegantly in a 2007 review by Gary Aston-Jones (Aston-Jones 2007). In addition to evidence for the involvement of LC activity in the regulation of sleep, increases in LC activity, NE release, and NE turnover are observed in response to stress. Indeed, the amount of NE release correlates well with the degree of stress or arousal experienced, particularly within the PFC (Nakane et al. 1994; Finlay et al. 1995; Ramos et al. 2007). Similarly,

NE release in the PFC and hippocampus is important for the maintenance of attention and ultimately for memory (Abercrombie et al. 1988; Aston-Jones et al. 1999; Usher et al. 1999; Southwick et al. 2002; Aston-Jones et al. 2005). Interestingly, although NE release is important for attention, arousal, and memory, conditions of too little or excessive stress and subsequent too little or excessive NE release are believed to disrupt these PFC related functions. Indeed, current models of NE function in the brain support an inverted-U relationship between LC activity/NE release/stress and performance on tasks that require
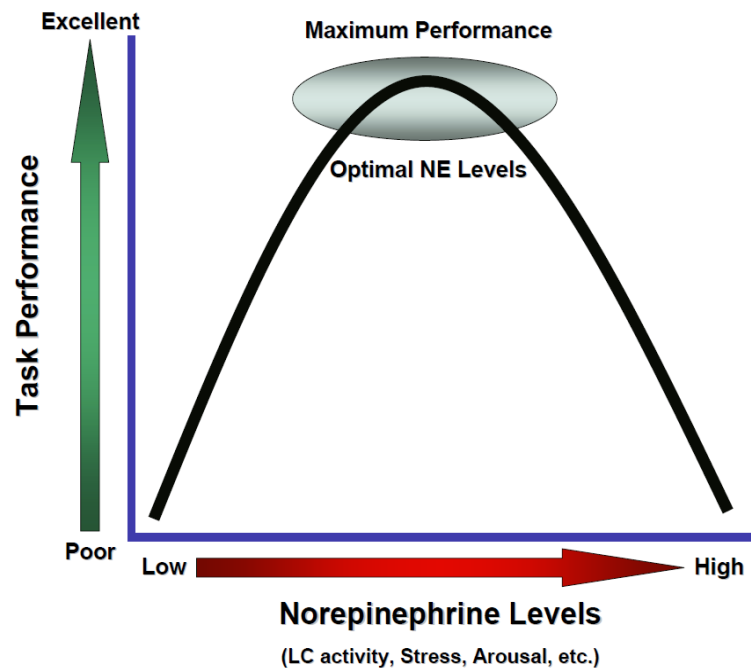


**Figure 3**. Optimal NE release and performance on cognitive tasks. Schematic illustration of inverted-U relationship between NE release and performance on cognitive tasks. LC activity and subsequent NE release in the PFC correlates well with arousal state and stress levels in an individual. Importantly, too much or too little stress/arousal negatively impacts performance on PFC related tasks. Correspondingly, excessive or too little NE release in the prefrontal cortex is also associated with poor performance on these cognitive tasks. Thus, an inverted-U relationship is said to exist between NE release in the PFC and cognitive function, where moderate levels of NE release result in optimal performance.

attention and memory (Aston-Jones et al. 2005; Arnsten 2007; Aston-Jones 2007; Ramos et al. 2007). For example, hypoactive LC firing is associated with little NE release, drowsiness, and inattention which ultimately reflects in poor performance on attention related tasks. On the other hand, moderate levels of LC activity and NE release are believed to support optimal attention and performance, while excessive LC firing is associated with distractibility and poor performance. Interestingly, such hyperactive modes of LC function are hypothesized to play a role in disorders like ADHD, whereas hypoactive modes have been implicated in the negative symptoms associated with schizophrenia such as poor attention and deficits in working memory. Research to date, suggests that this inverted-U relationship between NE release and performance is partially mediated by the differential affinity of α1 versus α2 adrenergic receptors for NE and the differential effects of their distinct signaling pathways on PFC function. Detailed studies supporting this theory are summarized nicely in two reviews by Amy Arnsten (Arnsten 2007; Ramos et al. 2007).

Cumulatively, noradrenergic and dopaminergic signaling, in both the CNS and PNS, impacts a vast array of brain and body functions. As exemplified by TH and DBH KO studies, these neurotransmitters are not only important for appropriate adult brain function they are also crucial for fetal development. In addition, studies of the neurons that synthesize and release these monoamines reveal unique insight about their role in a plethora of brain functions ranging from motivation and reward to cognition, food intake, attention, stress, arousal, motor control, etc. Indeed, imbalances in monoamine homeostasis are believed to underlie diseases related to abnormalities in these functions such as substance abuse disorders, depression, schizophrenia, ADHD, Parkinson's

disease, anorexia, etc. Thus, proper maintenance of monoaminergic tone within the brain is absolutely imperative for appropriate DA and NE signaling and subsequent brain function. Here, monoaminergic tone is defined by the signaling of these monoamines at the synapse and thus is determined by three important signaling components: the amount of neurotransmitter released into the synapse, the time neurotransmitter is available for signaling within the synapse, and finally the affinity/sensitivity of neurotransmitter receptors in the synapse. Monoamine transporters, such as NET, can impact all three aspects of monoaminergic tone defined here. First, transporters directly determine, through their reuptake of monoamines in the synapse, the time these neurotransmitters remain available within the synapse for signaling. In addition, reuptake of monoamines through transporters is crucial for neurotransmitter recycling and thus transporter function can significantly impact the amount of neurotransmitter released from the synapse. Finally, synaptic receptor availability and sensitivity is altered by the duration and concentration of neurotransmitter at the synapse, and thus this aspect of synaptic signaling may be influenced indirectly by transporter function also. The remainder of this thesis will focus on understanding the intricacies of monoamine transporter function, with particular focus on NET, and its capacity to impact brain monoamine homeostasis and thus brain function and behavior.

**Monoamine Transporter Structure and Function**

The first descriptions of neurotransmitter re-uptake were provided by Julius Axelrod in 1959 and 1961 (Axelrod et al. 1969; Axelrod 1971). His studies demonstrated the selective accumulation of radiolabeled epinephrine and NE in organs innervated by

the sympathetic nervous system such as the spleen and heart. These studies were extended by Iverson in 1963 who further demonstrated the capacity of [$^3$H] NE uptake in the heart, and finally by Coyle and Snyder in 1969 who showed, for the first time, re-uptake of neurotransmitter in brain tissue (Iversen 1963; Coyle et al. 1969). Since these original descriptions of neurotransmitter reuptake, the identification of specific transporters responsible for the reuptake of particular neurotransmitters has opened an entire field of fruitful research on these proteins and their role in synaptic signaling.

The monoamine transporters—DAT, NET, and the serotonin (5-HT) transporter (SERT)—belong to the SLC6 gene family of Na$^+$/Cl$^-$ dependent transporters and are critical for regulating extracellular levels of neurotransmitters. These transporters rely mainly on the co-transport of Na$^+$ down its electrochemical gradient to facilitate the uptake of biogenic amines from the inter- and extrasynaptic space. This transporter mediated re-uptake controls both the duration and the intensity of monoamine signaling at the synapse and is hypothesized to occur via an alternating access mechanism (Axelrod 1965; Jardetzky 1966; Forrest et al. 2008). This model of transporter function suggests that substrate and Na$^+$ binding trigger conformational changes that shift the transporter from an "outward-facing" conformation, in which the substrate is exposed extracellularly, to an "inward-facing" conformation where the substrate is exposed to the intracellular milieu (Jardetzky 1966; Erreger et al. 2008; Forrest et al. 2008; Shi et al. 2008). In addition to the classical alternating access model of transporter function, studies on monoamine transporters also revealed the existence of channels in transporters as an important aspect of transporter function (Galli et al. 1995; Galli et al. 1996; Galli et al. 1998; DeFelice et al. 2007). Indeed, bursts of NE release have been demonstrated to be

associated with discrete channel opening events (Galli et al. 1995; Galli et al. 1996; Galli et al. 1998). Collectively, both alternating access and channel like activity enables monoamine transporters to accumulate neurotransmitters back into the intracellular compartment after vesicular release in order to ensure both appropriate regulation and maintenance of synaptic signaling.

*Monoamine transporter structure*

Topological predictions and experimental data to date indicate that the monoamine transporters have 12 transmembrane domains (TMD) with intracellular amino and carboxy termini (Figure 4), and these predictions have been confirmed by the crystal structure of the bacterial leucine transporter (LeuT), a bacterial homolog of the neurotransmitter transporters (Pacholczyk et al. 1991; Bruss et al. 1995; Hersch et al. 1997; Chen et al. 1998; Androutsellis-Theotokis et al. 2002; Yamashita et al. 2005). Subsequent structural studies of the LeuT structure have yielded additional insight into important structural domains of the monoamine transporters. For example, sites critical for substrate binding,
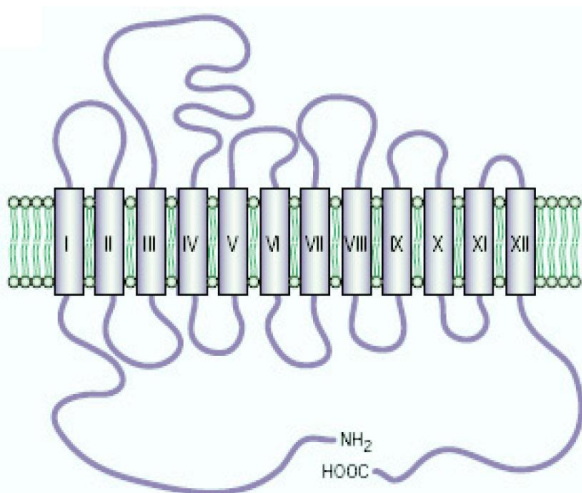


**Figure 4**. Monoamine transporter topology. Illustration of monoamine transporter topology. The monoamine transporters are predicted to have twelve transmembrane segments with cytoplasmic N and C termini. These predictions have been experimentally confirmed by various studies and in particular via the crystal structure of the close leucine transporter homolog. Adapted from (Torres et al., 2003).

ion dependence, and even antidepressant binding have now been identified (Henry et al. 2007; Torres et al. 2007). In addition, support for the existence of these transporters as functional dimers or multimers has also come from these types of structural studies. Another important structural domain of the transporters is the large extracellular domain, located between TMD3 and TMD4, that is post-translationally modified in order to ensure appropriate targeting of the transporters to the surface (Li et al. 2004). Finally, numerous putative phosphorylation sites and binding domains have also been identified within the intracellular domains of the various monoamine transporters, and these domains are considered vital for transporter regulation (Blakely et al. 1998; Granas et al. 2003; Sung et al. 2003; Khoshbouei et al. 2004; Fog et al. 2006; Blakely et al. 2007).

Initially, research on monoamine transporters focused primarily on understanding the intricacies of transporter function. A milestone in monoamine transporter research, the cloning of the human NET, propelled the field further by allowing for not only more detailed studies of transporter function but also for studies on transporter regulation (Pacholczyk et al. 1991). Today, evidence clearly supports the notion that NET and the other monoamine transporters are not merely stagnant regulators of synaptic monoamine levels, but rather are dynamically modulated proteins that contribute to synaptic plasticity. This dynamic regulation of NET will be the subject of the remainder of the thesis. First, however, to provide a foundation for these discussions, it is important to take an in-depth look at the transporter and its role in maintaining monoamine homeostasis in the brain.

*NET localization*

As described previously, NET is the primary mechanism by which NE is cleared from the synapse. As such, the transporter is specifically localized to noradrenergic neurons within the CNS. In the periphery, however, NET expression is not limited to sympathetic neurons, but it is also found in the lungs, placenta, and adrenal medulla (Ramamoorthy et al. 1994; Westwood et al. 1996; Schroeter et al. 2000). At the level of a single neuron, NET expression is observed as punctuate not only at the periphery of the synapse (as pictured in Figure 1), but also as punctuate along the axons, somata, and dendrites (Lorang et al. 1994; Miner et al. 2003; Matthies et al. 2009). In addition, intracellular compartments contain NET and thus suggest the potential for redistribution of NET from intracellular pools to the surface or vice versa in response to stimuli (Matthies et al. 2009). Importantly, while NET expression is limited to noradrenergic neurons in the CNS, the ability of this transporter to reuptake other neurotransmitters besides NE, adds additional weight to its importance in the maintenance of monoamine homeostasis. In particular, NET reuptake of DA, in regions of low DAT expression such as the cortex or even in regions with substantial DAT expression such as NAcc shell, can significantly influence dopaminergic tone in the brain (Gresch et al. 1995; Yamamoto et al. 1998; Moron et al. 2002; Miner et al. 2003; Liprando et al. 2004).

*NET KO mice*

The significance of NET function for maintenance of brain monoamine homeostasis cannot be fully appreciated without discussion of NET KO models. In 2000, Xu et al. published the first description of NET KO mice (Xu et al. 2000). As

anticipated, clearance of NE in these KO mice was at least 6-fold slower in comparison to WT controls. Indeed, defective clearance is seen in the other monoamine transporter KO mice, thus these deficits further substantiate the claim that transporters are critical for both temporal and spatial control of synaptic signaling (Jones et al. 1998). Interestingly, in addition to abnormal NE clearance and elevated extracellular levels of NE, NET KO mice also display a 55% to 70% reduction in NE tissue content in NE enriched regions of the brain. Once NE is taken up by the transporter it can either be degraded enzymatically or be taken up into vesicles by the vesicular monoamine transporter 2 (VMAT2). In fact, 70% of NE recaptured by NET is recycled rather than degraded (Schomig et al. 1989; Xu et al. 2000). Thus, the significant reduction in NE tissue content observed in NET KO mice, despite compensatory increases in TH, is reflected in the inability of neurons to recycle NE into vesicles for subsequent release. Not surprisingly then, even synaptic NE release is diminished by 60% in these KO mice. In addition to disruptions in noradrenergic homeostasis, NET KO mice display a plethora of interesting phenotypes. For example, NET KO mice are smaller, exhibit reduced body temperatures, and behave similarly to normal mice treated with antidepressants in various behavioral paradigms. Interestingly, perhaps in part due to the ability of NET to reuptake DA, DA related functions are also disrupted in NET KO mice. For instance, NET KO mice display disrupted DA clearance in the cortex and DA receptor 'supersensitivity' which is believed to underlie their increased responsiveness to drugs of abuse such as amphetamine (AMPH) and cocaine (Xu et al. 2000; Moron et al. 2002; Keller et al. 2006). Altogether, the striking behavioral and physiological phenotypes of NET KO mice further emphasize the vital role NET plays in maintaining monoaminergic tone

within the brain, and in addition lend gravity to the notion that disruptions in NET function may contribute to abnormalities in brain function and behavior.

*Monoamine transporters as pharmacological targets*

Not only is NET of substantial interest given its ability to significantly influence monoaminergic signaling, but the transporter is also the target of therapeutics and drugs of abuse. A vast array of drugs utilized to treat a number of different brain-related disorders target the monoamine transporters. Among the first to be identified as therapeutically useful were the tricyclic antidepressants which typically block both SERT and NET (Owens et al. 1997; Richelson 2003). Since then, both 5-HT selective and NE selective reuptake inhibitors (SSRI and SNRI respectively) have been developed for the treatment of depression. Interestingly, while the majority of treatments for ADHD have focused on manipulation of the dopaminergic system and DAT itself, atomoxetine, a NET specific inhibitor, has become recognized as another effective strategy for treating this disorder in some individuals (Corman et al. 2004). Not surprisingly, perhaps, given both the ability of NET to impact cortical DA levels, and the importance of cortical NE itself in the regulation of attention. Along with this rationale, clinical trials with NET specific inhibitors are ongoing in populations of schizophrenic patients with the hopes of alleviating negative symptoms of the disorder such as deficits in the attention and working memory (clinicaltrials.gov). Although pharmacological targeting of NET and other monoamine transporters can be useful in the treatment of various disorders, these transporters are also the targets of psychostimulant drugs of abuse such as cocaine and AMPH. Indeed, drugs such as AMPH not only inhibit monoamine reuptake and thereby

disrupt brain monoamine homeostasis, but they can also hijack the sophisticated signaling pathways that dynamically regulate these transporters to exacerbate their effects.

In summary, as exemplified by monoamine transporter knockout studies and by study of the drugs that target them, appropriate transporter function is absolutely critical for maintaining monoamine homeostasis in the brain. Furthermore, this maintenance ensures proper brain function, and thus disruptions in NET function ultimately have the potential to impact behavior. In addition, years of research on these transporters has revealed that they are more than mere stagnant monitors of synaptic monoamine levels, but rather through their own dynamic regulation they are important components of synaptic plasticity. This dynamic regulation will be the focus of the remainder of the thesis.

## Monoamine Transporter Regulation

*Amphetamine regulation of monoamine transporters*

Although early research on monoamine transporters laid an excellent foundation for a detailed understanding of how transporters facilitate the reuptake of neurotransmitter, recent studies over the last decade have focused more intensely on the dynamic regulation of these transporters. Importantly, transporter regulation manifests through two distinct mechanisms. First, uptake or the activity of the transporter can be manipulated (i.e. slowed, accelerated, reversed, or inhibited entirely), or secondly, the availability of the transporter on the surface can be altered. An excellent example of monoamine transporter regulation that encompasses both mechanisms is the regulation of the transporters by AMPH. Indeed, the AMPH regulation of monoamine transporters has

been studied in depth and thus serves as an excellent case study for monoamine transporter regulation.

AMPH and its derivatives are regularly used in the treatment of a wide array of disorders such as ADHD, obesity, traumatic brain injury, and narcolepsy (Prinzmetal 1935; Olfson et al. 2002; Knutson et al. 2004; Martinsson et al. 2004; Sulzer et al. 2005; Fleckenstein et al. 2007). Despite the important medicinal role for AMPH, it is more widely known for its psychostimulant and addictive properties as a drug of abuse. The primary molecular targets of AMPH are both the VMATs and plasma membrane monoamine transporters, DAT, NET, and SERT. The rewarding and addicting properties of AMPH rely on its ability to act as a substrate for these transporters and ultimately increase extracellular levels of monoamines. AMPH achieves this elevation in extracellular levels of neurotransmitter by inducing synaptic vesicle depletion, which increases intracellular monoamine levels, and also by promoting reverse transport of monoamines (efflux) through plasma membrane monoamine transporters (Burn et al. 1958; Carlsson et al. 1962; Kirshner 1962; Ritz et al. 1987; Sulzer et al. 1995; Fon et al. 1997; Jones et al. 1998). Here, the focus will remain on two important aspects of AMPH-induced regulation of the plasma membrane monoamine transporters—transporter mediated monoamine efflux (reverse transporter activity) and transporter trafficking (changes in transporter surface availability).

The molecular mechanism underlying AMPH action remained a mystery until the late 1950's when the work of Burn and Rand revealed that AMPH acts by "releasing a noradrenaline-like substance" (Burn et al. 1958). Thus the foundation of the field was established, and since then numerous studies have focused intently on discovering the

detailed mechanism behind AMPH's ability to induce monoamine release into the extracellular milieu. Investigations following the work of Burn and Rand implicated both vesicular and plasma membrane monoamine transporters as important conduits for monoamine release. This discussion, however, will focus on efforts surrounding the plasma membrane monoamine transporters. The reader is directed to a substantial review for a thorough discussion of VMAT contribution to AMPH-mediated monoamine release (Sulzer et al. 2005).

Early evidence demonstrated that AMPH-like drugs act as substrates for monoamine transporters and that AMPH-induced monoamine release could be blocked by uptake inhibitors such as cocaine and nomifensine (Heikkila et al. 1975; Raiteri et al. 1979; Parker et al. 1988; Seiden et al. 1993; Sulzer et al. 1995; Jones et al. 1998; Schenk 2002). In tandem with these studies, Fischer and Cho proposed the facilitated exchange diffusion model as a model for AMPH-induced monoamine release via DAT (Fischer et al. 1979; Burnette et al. 1996). Fischer and Cho hypothesized that AMPH is transported as a substrate into the cell via DAT which subsequently results in the counter transport of DA extracellularly. Given that AMPH serves as a substrate for DAT, its transport into the cell increases the number of transporters in the inward facing conformation, and thus increases the probability that intracellular DA will bind to DAT and induce reverse transport. Evidence in support of this model of AMPH-induced efflux demonstrates that AMPH accumulation in rat synaptosomes is saturable, temperature-dependent, and ouabain-sensitive, implicating an active transport mechanism for AMPH (Zaczek et al. 1991). Additional evidence for an active transport mechanism has been supported by several electrophysiology studies illustrating AMPH's ability to generate DA-like

transporter associated currents (Sonders et al. 1997; Sitte et al. 1998; Khoshbouei et al. 2003; Kahlig et al. 2004).

Since the introduction of facilitated exchange diffusion in 1979, new experimental results have emerged that challenge this model.  For example, direct intracellular injections of AMPH into the giant DA neuron of *Planorbis corneus*, the pond snail, induce reverse transport despite the fact that AMPH has not been taken up via the transporter (Sulzer et al. 1995).  Furthermore, AMPH increases intracellular $Na^+$ concentration and an increase in intracellular $Na^+$ is sufficient to drive DA efflux even in the absence of extracellular AMPH (Khoshbouei et al. 2003).  In fact, numerous studies to date on AMPH-induced changes in uptake, charge transfer, and efflux all support the notion that the influx of extracellular sodium ions via the transporter triggers reverse transport (Pifl et al. 1995; Pifl et al. 1997; Sitte et al. 1998; Pifl et al. 1999; Schenk 2002; Pifl et al. 2004).  Other experiments, utilizing concatemers of SERT (AMPH sensitive) and GAT (AMPH insensitive) reveal that AMPH-induced efflux also depends upon the oligomeric nature of the monoamine transporters (Seidel et al. 2005).  Finally, additional studies have revealed that AMPH can cause DAT-mediated DA efflux by a process that results in rapid bursts of DA efflux through a channel-like mode of DAT, a process that is independent from the slow exchange-like mechanism (Kahlig et al. 2005).  This was demonstrated in outside-out patches from both heterologous cells stably expressing DAT and from dopaminergic neurons.  Interestingly, this channel-like mode of DA release is approximately equivalent to a quantum of DA release from synaptic vesicle fusion.  Therefore, this channel-like burst mode may actually influence the synaptic action and psychostimulant properties of AMPH.  These studies suggest that although facilitated

exchange diffusion may contribute to AMPH-mediated monoamine release, it cannot

account for all experimental observations to date (Figure 5).
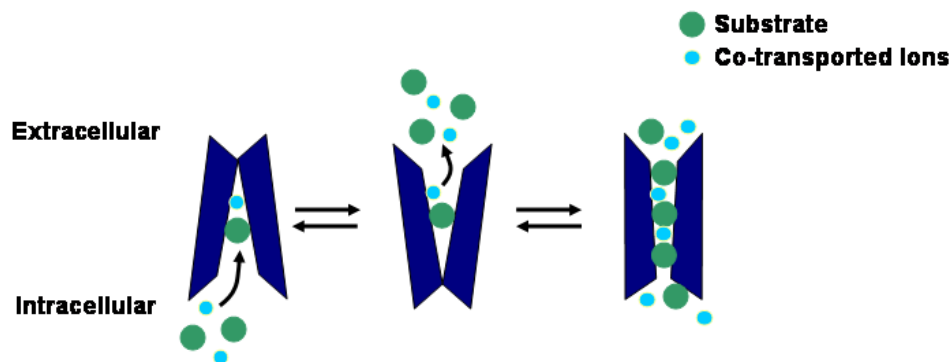


**Figure 5**. Transporter-mediated monoamine efflux. Schematic representation of transporter mediated monoamine efflux. Transporters in the inward facing conformation are capable of mediating monoamine efflux by binding substrate and co-transported ions. Transporter reversal can be enhanced under certain conditions such as in the presence of AMPH or increased intracellular Na+. In addition to the slow exchange-like mechanism of efflux, AMPH can also induce efflux through a channel-like mode of the transporter.

While the model of AMPH-induced efflux evolved to include not only facilitated

exchange diffusion but also channel-like modes of the transporter, its development is

incomplete without considering regulation by second messenger systems. As mentioned

previously, numerous putative phosphorylation sites for various protein kinases have

been identified within the intracellular regions of the monoamine transporters. In fact,

several studies have demonstrated that DAT function is heavily regulated by a plethora of

protein kinases (Melikian et al. 1999; Carvelli et al. 2002; Granas et al. 2003; Loder et al.

2003). As evidence emerged that AMPH is capable of increasing protein kinase C (PKC)

activity *in vivo*, the possibility that kinase regulation may impact AMPH's ability to

induce transporter-mediated monoamine efflux became enticing (Giambalvo 1992). Soon thereafter, strong evidence for the involvement of PKC in AMPH-induced DAT-mediated DA efflux appeared in experiments that utilized specific PKC inhibitors to prevent AMPH-stimulated DA release altogether (Kantor et al. 1998; Cowell et al. 2000). A similar role for PKC was also established for AMPH regulation of NET efflux in undifferentiated PC12 cells (Kantor et al. 2001). Not surprisingly, these experiments also revealed a requirement for intracellular $Ca^{2+}$ in AMPH-induced NET efflux, along with the necessity for PKC activity. In addition to a role for intracellular $Ca^{2+}$, the effects of repeated AMPH exposure on NET were shown to depend on N-type and L-type $Ca^{2+}$ channel activity (Kantor et al. 2004). These studies were further extended by research that demonstrated the ability of intracellular $Ca^{2+}$ to regulate both AMPH-induced DAT currents and efflux (Gnegy et al. 2004). Finally, recent studies investigating PKC's involvement in AMPH mediated DA release have provided evidence for the importance of an actual physical association of PKC$\beta_{II}$ with DAT in the rat striatum (Johnson et al. 2005).

Considering the wealth of data supporting a role for PKC in AMPH stimulated neurotransmitter release, the finding that PKC activation leads to N-terminal phosphorylation of DAT in the rat striatum is not surprising (Foster et al. 2002). Nevertheless, a DAT mutant lacking the first 22 amino acids of the N-terminus, which eliminates $^{32}$P incorporation in response to PKC, shows no significant deficits in uptake, inhibitor binding, internalization, or oligomerization (Granas et al. 2003). Given the substantial evidence for PKC involvement in AMPH-induced transporter efflux, Galli and coworkers chose to investigate the role of the N-terminus in regulating DAT-

mediated DA efflux by using the 22 amino acid deletion mutant. The study revealed an 80% reduction in AMPH-induced DA efflux in the mutant DAT (Khoshbouei et al. 2004). Additionally, they demonstrated that mutation of 5 N-terminal serine residues to alanine residues (S/A) produced an identical phenotype to that of the 22 amino acid deletion DAT mutant. Furthermore, mutation of the same five residues to aspartate (S/D) restored AMPH-induced DA efflux to normal levels. From this study, the authors proposed a novel model for AMPH-induced regulation of DAT efflux that implies a role for N-terminal phosphorylation in shifting DAT from a "reluctant" to a more "willing" state for efflux.

Although an abundance of evidence insinuates a role for phosphorylation via PKC in AMPH-induced DAT efflux, additional data implicates the involvement of other kinases in this complex process (Pierce et al. 1997; Kantor et al. 1999). In fact, recent data indicates that $Ca^{2+}$/calmodulin-dependent protein kinase α (CaMKIIα) is a key component of AMPH-induced regulation of DAT efflux (Fog et al. 2006). In this study, the researchers clearly demonstrated that inactivation or inhibition of CaMKIIα dramatically reduced AMPH-induced DA efflux. Furthermore, a physical association between CaMKIIα and the DAT C-terminus was observed, and subsequent disruption of this association was sufficient to diminish AMPH-stimulated DA release. Thus, research clearly indicates a role for both PKC and CaMKIIα signaling in AMPH-induced DAT mediated DA efflux. Whether or not these two signaling pathways contribute to efflux in parallel or sequentially, however, has yet to be determined. Interestingly, recent research focused on the role of CaMKII in AMPH-mediated DA efflux has identified syntaxin1A (SYN1A) as an important link between CaMKII signaling and transporter reversal (Binda

et al. 2008).  SYN1A is a SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) that is critical for synaptic vesicle release.  In addition to its role in vesicular fusion, SYN1A also interacts with and regulates numerous transmembrane proteins including ion channels and importantly, neurotransmitter transporters.  In fact, DAT/SYN1A and NET/SYN1A associations have been shown to increase in response to AMPH, and evidence now indicates that this enhancement in SYN1A association is vital for AMPH-mediated DA efflux (Dipace et al. 2007; Binda et al. 2008).  Interestingly, SYN1A interaction with NET and DAT has also been shown to influence transporter channel-like activity, a mode of the transporter that is important for AMPH-induced efflux (Sung et al. 2003; Carvelli et al. 2008).  By utilizing pharmacological and peptide inhibitors of CaMKII, experiments have clearly established that the increase in SYN1A/DAT association in response to AMPH requires CaMKII activity.  From these findings, a model for AMPH-induced DA efflux can be formulated, whereby the binding

**Figure 6**. Regulation of AMPH-induced DAT efflux by second messengers. AMPH-induced DAT efflux is regulated by second messengers. A model of AMPH-induced regulation of DAT mediated DA efflux via intracellular signaling pathways. AMPH transport via DAT into the intracellular milieu results in an increase in both intracellular Na+ and Ca2+ levels. As a consequence, PKC and CaMKII activation initiates both phosphorylation of the N terminus of DAT and enhancement of DAT/SYN1A association, shifting the transporter from a "reluctant" to a "willing" state for efflux.
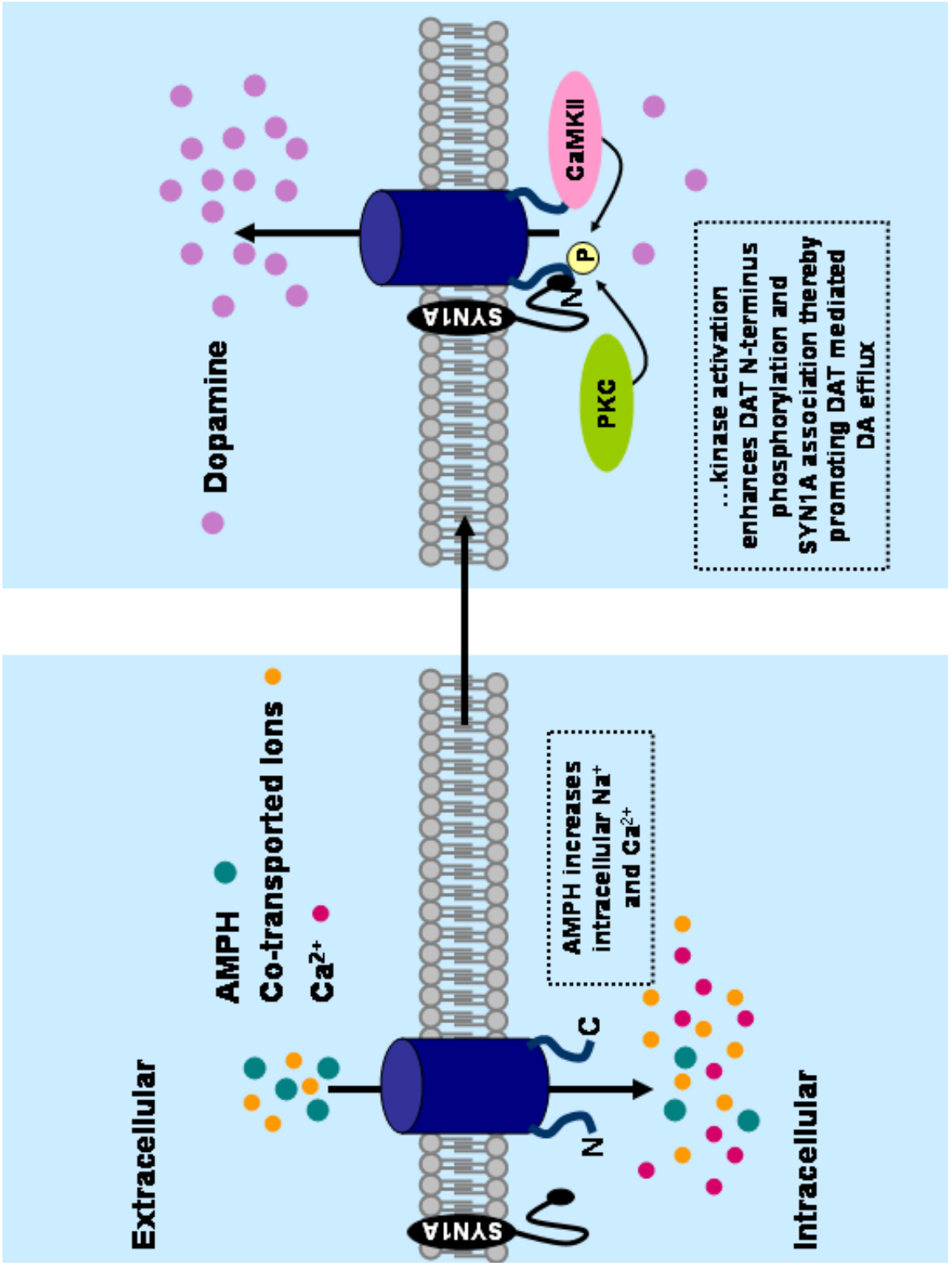
of CaMKIIα to the C-terminus of DAT facilitates the phosphorylation of the N-terminus and the binding of SYN1A, promoting the shift of DAT towards a 'willing' state for AMPH-induced DA efflux (Figure 6).

Unlike AMPH-induced neurotransmitter efflux, a phenomenon recognized in the late 1950's, another aspect of AMPH regulation of monoamine transporters did not emerge from the literature until the late 1990's. Fleckenstein and coworkers hypothesized that AMPH not only induces transporter efflux, but also may regulate transporter surface expression levels based on their observation that in rats a single high dose injection of AMPH results in a decrease in DAT function one hour later (Fleckenstein et al. 1997). Since then, AMPH-induced regulation of DAT trafficking has been confirmed by numerous studies, and the investigation into the mechanisms underlying this phenomenon has become an area of intense research (Saunders et al. 2000; Kahlig et al. 2003; Kahlig et al. 2004; Kahlig et al. 2006).

The first comprehensive demonstration of AMPH-induced trafficking of DAT in heterologous systems surfaced a few years after Fleckenstein's original proposal. In this study, acute treatment with the DAT substrates AMPH and DA not only reduced [$^3$H]DA uptake and AMPH-induced currents, but also clearly decreased DAT cell surface expression (Saunders et al. 2000). By utilizing a dominant negative mutant of dynamin I (K44A) to prevent substrate-induced trafficking, the authors also provided evidence to suggest that AMPH-stimulated DAT endocytosis occurs via a dynamin dependent pathway. Following these experiments, a series of investigations verified these results in other heterologous systems (*Xenopus* oocytes), rat synaptosomal preparations, and finally, indirectly, *in vivo* via high speed chronoamperometry (Gulley et al. 2002; Chi et

al. 2003; Kahlig et al. 2004; Owens et al. 2005; Williams et al. 2007). Interestingly, application of DAT inhibitors such as cocaine, mazindol, and nomifensine is sufficient to prevent the AMPH-induced DAT trafficking, implying that transport of AMPH into the cell may be an important component of this regulation. To address this hypothesis, a mutant DAT (Y335A) capable of substrate binding but impaired in substrate transport, was exposed to AMPH and analyzed for redistribution from the cell surface to the cytosol. Interestingly, extracellular AMPH application did not induce internalization of the uptake-impaired DAT, but when applied directly into the intracellular milieu, AMPH was sufficient for inducing trafficking of the mutant (Kahlig et al. 2006). From this, the researchers concluded that whereas the DAT transport cycle is unnecessary for AMPH-induced DAT trafficking, an increase in intracellular AMPH is an essential component of this regulation. An important caveat to consider in these studies is the timing of transporter cell surface redistribution in response to AMPH application. Indeed, recent studies point out that AMPH-induced trafficking of DAT is dependent upon the time of AMPH exposure. For example, a rapid enhancement of DAT surface expression occurs within seconds of AMPH application and diminishes by 2.5 minutes implying that AMPH's regulation of transporter trafficking differs with respect to acute versus long lasting effects (Johnson et al. 2005).

As AMPH-induced DAT trafficking became well established, researchers shifted their focus towards identifying underlying key components of the phenomenon. Considering the wealth of evidence supporting a role for AMPH-induced PKC activation in transporter efflux, the idea that PKC activity may also be involved with transporter trafficking seemed plausible. In fact, numerous studies had already demonstrated that

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PKC activation leads to the rapid redistribution of DAT from the cell surface in both heterologous and neuronal systems (Pristupa et al. 1998; Daniels et al. 1999; Granas et al. 2003; Loder et al. 2003; Sorkina et al. 2003)  However, recent results show that while AMPH application induces N-terminal phosphorylation of DAT via PKC, prevention of this phosphorylation by mutation does not deter AMPH-induced DAT trafficking (Granas et al. 2003; Cervinski et al. 2005).  Furthermore, more recent studies have demonstrated that residues required for PKC-induced internalization are not critical for AMPH-triggered DAT sequestration, and PKC inhibition also failed to inhibit AMPH-induced DAT redistribution (Boudanova et al. 2008; Boudanova et al. 2008).  Therefore, while experiments to date clearly implicate a role for PKC in AMPH-induced regulation of DAT efflux, at this time it does not appear to be involved in AMPH-induced transporter trafficking from the surface.  Interestingly, new work from Vaughan and co-workers indicates that PKC induced DAT regulation may differ depending on the membrane localization of the transporter (Foster et al. 2008).  In this study the authors, demonstrate that PKC-stimulated phosphorylation of DAT occurs to a significantly higher level in lipid raft populations of DAT compared to non-lipid raft populations.  In fact, the PKC-triggered DAT internalization happens primarily from non-raft population.  Thus, PKC regulation of DAT depends heavily upon the discrete membrane localization of the transporter.  Given AMPH's dual effect on transporters, one can imagine a scenario where non-raft populations are primarily responsible for AMPH-induced DAT internalization whereas raft populations may primarily constitute transporters-mediating monoamine efflux.  Whether membrane localization is important or not for AMPH-induced efflux and trafficking has yet to be determined, but it will be an interesting

question for future studies. In addition to PKC, other kinases have also been implicated in AMPH-induced DAT trafficking. For example, AMPH application results in a time-dependent increase in CaMKII activity which is required for DAT trafficking (Wei et al. 2007). Importantly, this CaMKII activation inhibits an important kinase in the insulin signaling pathway, Akt. This study suggests that insulin signaling is involved in DAT trafficking and as a consequence may impact DA homeostasis. Thus, the current model of AMPH-induced DAT internalization proposes an intersection of AMPH-stimulated signaling and the insulin signaling pathway.

Whereas evidence for AMPH-induced internalization of DAT has accumulated since the 1990's, experiments supporting a similar phenomenon with respect to NET did not appear until the 2000's (Zhu et al. 2000; Dipace et al. 2007). The first studies, from Ordway and colleagues, which investigated the effects of chronic AMPH exposure on NET revealed that long term AMPH exposure reduces NET expression in both a time and concentration dependent manner (Zhu et al. 2000). Furthermore, a study from 2007 demonstrated that, like DAT, acute AMPH application stimulated a slow, significant reduction in surface levels of NET in a catecholaminergic cell line (Dipace et al. 2007). Although these results come as no surprise, the study also revealed novel aspects of AMPH-induced regulation of the transporter. First, the authors clearly demonstrated that this process is $Ca^{2+}$ dependent by utilizing BAPTA-AM and $Cd^{2+}$ to diminish intracellular $Ca^{2+}$ levels and prevent AMPH-stimulated NET internalization. Furthermore, inhibition of CaMKII via KN93 also prevented AMPH-induced NET trafficking, implying that this process for NET is both $Ca^{2+}$ and CaMKII dependent just as it is for DAT. Finally, the study of an N-terminal deletion mutant (hNET$\Delta_{28-47}$) also

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indicated that AMPH-stimulated regulation of NET internalization may be mediated through the N-terminus (Dipace et al. 2007).  Interestingly, whether a similar region is critical for AMPH-induced DAT internalization has yet to be determined. In comparison to the wealth of research dedicated to determining how AMPH regulates both trafficking and efflux of DAT, knowledge related to AMPH-induced regulation of NET is rather limited.  Despite this gap in the field, much research has been done on β-PMA induced downregulation of NET and evidence supports a role for lipid rafts in NET internalization (Jayanthi et al. 2004; Jayanthi et al. 2006).  Perhaps, similar mechanisms will be elucidated for AMPH-mediated NET trafficking which would suggest a divergence in the regulation of NET compared to DAT given the clathrin dependence of AMPH-induced DAT trafficking.  Thus, much research is still needed in the field of AMPH-induced NET internalization and efflux as many important questions remain unanswered, such as which compartment does the transporter travel through during AMPH-stimulated trafficking and what intracellular signals and modifications of NET are required for this trafficking phenomenon? Furthermore, does the insulin signaling pathway play any role in the ability of AMPH to induce NET mediated NE efflux and NET trafficking?

Since the first clues behind AMPH's mechanism of action began to emerge as early as the late 1950's, this field of research has seen an exponential amount of growth. After almost 50 years of investigation, the original model for AMPH-induced monoamine efflux has evolved from its simplest form of facilitated exchange diffusion to a multifaceted mechanism that requires not only exchange diffusion and channel-like modes of release but also regulation by second messenger systems.  Perhaps, even more remarkable than the transformation of the efflux field, is the discovery of a second

mechanism of action for AMPH altogether—transporter trafficking. Despite the advancements over the last few years, both the broad implications and the intricate details of AMPH's actions continue to elude us. For instance, how does AMPH's ability to dynamically regulate monoamine transporter membrane expression contribute to its psychostimulant and addictive properties? Which intracellular signaling pathways are critical for AMPH-induced regulation of transporter efflux and trafficking, and how do they differ? Research aimed at addressing these types of questions promises to bring us one step closer to comprehending the basis of not only AMPH abuse and addiction but also its role as a treatment for various pathological conditions. As our understanding of AMPH action continues to progress, so too will our comprehension of monoaminergic regulation in general. For example, recent work involving a DAT coding variant associated with ADHD that effluxes DA, as if it were exposed continuously to AMPH, has been shown to have aberrant regulation under normal conditions that closely parallels regulation of wild-type DAT by AMPH (Mazei-Robison et al. 2008). Thus, uncovering the secrets of AMPH-mediated monoamine transporter regulation promises to enhance our capacity to generate novel therapeutic strategies for treating drug abuse as well as disorders associated with monoaminergic dysfunction in general such as depression and ADHD.

*Insulin/Akt regulation of monoamine transporters*

The complexity with which AMPH regulates both monoamine transporter function and transporter surface expression offers critical insight into the dynamic nature of transporter regulation. Here, however, a less well characterized mechanism of

monoamine transporter regulation is of particular interest, the regulation of these transporters by the insulin signaling pathway. Unlike in the periphery where insulin signaling is primarily important for the regulation of glucose levels, within the CNS it has been demonstrated to influence a broad array of functions (Figure 7) such as neurodevelopment, cell survival, neurogenesis, receptor trafficking, neurotransmitter release, and neurotransmitter reuptake (Woods et al. 1996; Bruning et al. 2000; Schulingkamp et al. 2000; van der Heide et al. 2006). Given its diversity in function, it is not surprising then that insulin signaling is hypothesized to impact numerous brain functions such as learning, memory, regulation of food intake etc. and that disruptions in insulin signaling perhaps contribute to brain related disorders. Importantly, while the source of CNS insulin is still debated, the majority of evidence supports the active transport of insulin past the blood brain barrier from the periphery (Schwartz et al. 1992; Banks 2004). Indeed, peripheral manipulations of insulin are mirrored by alterations in CNS insulin (Schwartz et al. 1990). Thus, the exponential growth in the number of individuals afflicted with peripheral insulin related disorders such as obesity and diabetes further necessitates a full appreciation of the role of insulin signaling pathways in the brain

The notion that insulin signaling may be involved in the regulation of monoamine homeostasis began with studies that revealed striking alterations in the dopaminergic system of rodents rendered diabetic through streptozotocin (STZ) treatment, which results in necrosis of insulin producing pancreatic $\beta$ cells (Chu et al. 1986; Karkanias et al. 1997; Saitoh et al. 1998). Although these initial studies provided preliminary

**Figure 7**. Insulin signaling pathway in the brain. Representation of simplified insulin signaling pathway. Insulin binding to the insulin receptor (IR) triggers autophosphorylation of the tyrosine kinase receptor and subsequent recruitment of the scaffolding adaptor, insulin receptor substrate (IRS). IRS then binds and activates phosphatidylinositol 3-kinase (PI3K) which is responsible for the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). Protein kinase B, also known as Akt, then binds to PIP3 at the membrane where phosphoinositide-dependent kinase-1 (PDK1) and the mammalian target of rapamycin complex 2 (mTORC2) are responsible for the phosphorylation and full activation of Akt. Downstream of Akt, numerous intracellular pathways are activated and impact a variety of cell functions such as cell growth, survival, and proliferation. In particular, for the brain, insulin signaling has been shown to be important for the regulation of food intake, neurogenesis, learning and memory, neurodevelopment, neurotransmitter release and importantly neurotransmitter reuptake.

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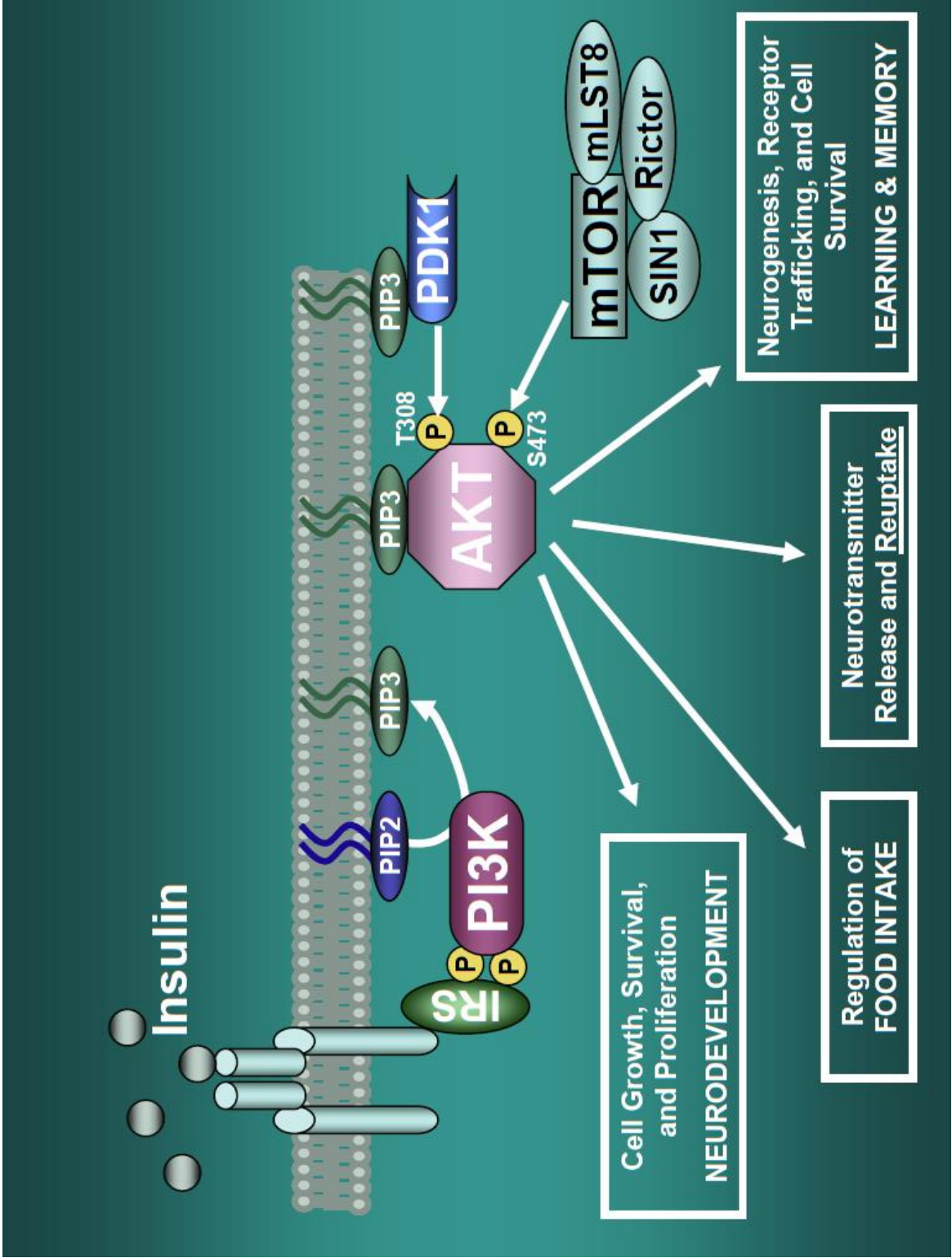
evidence that insulin is capable of altering monoaminergic signaling in the brain, subsequent studies demonstrated specifically the ability of insulin to regulate DAT and thus dopaminergic signaling. For example, tyrosine kinase inhibitors, which block the kinase activity of the insulin and insulin-like growth factor receptors were shown to reduce DA clearance due to a decrease in the surface expression levels of DAT (Doolen et al. 2001). Concurrently, other investigators expanded these results by illustrating that inhibition of downstream components of the insulin signaling pathway, such as phosphatidylinositol 3- kinase (PI3K) and Akt, also dramatically reduce DA clearance and surface expression of DAT (Carvelli et al. 2002; Garcia et al. 2005). Furthermore, surface levels and function of DAT are significantly diminished in both STZ models of diabetes and diet-induced obese states of insulin resistant rats ((Williams et al. 2007) and unpublished data). Thus, basal insulin signaling is critical for appropriate dopaminergic tone via its maintenance of DAT at the cell surface. Given the importance of insulin signaling for maintaining DAT at the plasma membrane, researchers quickly realized the ability of insulin signaling to impact AMPH action in the brain. For example, the reinforcing properties of AMPH are tremendously diminished in the STZ model of diabetes, as demonstrated by the AMPH self-administration paradigm (Galici et al. 2003). Additionally, selective inhibition of PI3K via LY294002 results in a dramatic reduction in AMPH's ability to elicit DAT-mediated DA efflux in heterologous cells, dopaminergic neurons, and *in vivo* within the striatum of rats as measured by both *in vivo* voltammetry and functional magnetic resonance imaging (Williams et al. 2007; Lute et al. 2008). These data suggest that kinases linked to both glucose homeostasis and food intake regulation are also capable of regulating the reward pathways in the brain that are

targeted by psychostimulants such as AMPH. Although studies continue to elucidate new aspects of the insulin regulation of DAT, similar in-depth research on the role of insulin in the regulation of noradrenergic homeostasis is clearly lacking.

In addition to disruptions in the dopaminergic system, early studies on diabetic rodents also revealed abnormalities in noradrenergic tone (Ganguly et al. 1986; Lucas et al. 1989; Lackovic et al. 1990; Shimizu 1991). Differences in the types of animals studied and the regions of the nervous system examined, however, failed to offer a consensus on whether deficits in insulin signaling stimulate or depress noradrenergic function. Despite these discrepancies in diabetic models, initial studies on insulin regulation of NET function were more consistent. For example, insulin inhibits NE uptake in whole brain neuronal cultures, dissociated brain cells, and whole brain synaptosomes (Boyd et al. 1985; Boyd et al. 1986; Masters et al. 1987; Raizada et al. 1988). Furthermore, Figlewicz et al. demonstrated the ability of nanomolar concentrations of acute insulin to decrease NE uptake from both hypothalamic and hippocampal slices (Figlewicz et al. 1993). Subsequently, these studies were extended to demonstrate that insulin also inhibits NE uptake in PC12 cells which endogenously synthesize NE and express NET (Figlewicz et al. 1993). Since these original studies in the late 1980's and early 1990's insulin regulation of noradrenergic signaling and NET function has remained a relatively untouched area of research. Indeed, only a single additional study on the topic was published in 2001, which in SK-N-SH cells demonstrated that insulin increases NE uptake in contrast to earlier studies in PC12 cells (Apparsundaram et al. 2001). Thus, in this thesis, I explore the nature of insulin's regulation of NET with particular interest in how disruptions in this regulation have the

potential to impact monoamine homeostasis, behavior, and perhaps ultimately mental health.

## Monoamine Dysfunction and Disease

The notion that monoamine dysfunction in the nervous system may contribute to various neurological and psychiatric disorders has persisted nearly since their discovery as neurotransmitters in the brain. Indeed, drugs that alter the monoaminergic systems have been utilized for the treatment of mental health disorders for over 50 years now. In particular, the ability of the NE and DA systems to modulate attention, arousal, mood, food intake, reward, and memory has implicated them in mood disorders associated with deficits in these realms such as ADHD, schizophrenia, depression, anorexia, and substance abuse disorders. In addition to mood disorders, these monoamines are also implicated in different neurological disorders such as Parkinson's disease and Alzheimer's disease. Indeed, for the noradrenergic system in particular, a substantial loss of LC neurons is well documented in both of these devastating diseases (Chan-Palay et al. 1989). For now, however, the focus will remain on evidence supporting a link between noradrenergic dysfunction and mood disorders with specific attention paid to those disorders in which disrupted insulin/Akt signaling has been preliminarily implicated, depression and schizophrenia. Whereas the NE system received significant attention in early studies of mood disorders, in the last decade the plausibility of noradrenergic signaling involvement in such disorders has largely been ignored while hypotheses abound for the other monoamines such as DA and 5-HT. Thus, it is absolutely imperative to remind ourselves of the plentiful evidence supporting a link between anomalous NE signaling and disease.

*NE and Depression*

First and foremost, in the case of depression, LC activity and thus NE release in the brain is heavily affiliated with behaviors that are disturbed in depression such as sleep, attention, memory, and stress. Furthermore, antidepressants that target NET and SERT, and thereby increase synaptic levels of NE and 5-HT in the brain, are still the primary treatment for the disorder. Although SSRIs are more heavily used and researched in comparison to NSRIs, studies evaluating SSRIs relative to NSRIs suggest that the two classes of drugs are of similar efficacy (i.e. individuals treated with either of these on average show approximately a 50% improvement in symptoms) (Anderson 1998; Anderson 2000). In addition to the effectiveness of targeting the noradrenergic system in depressed individuals, numerous studies investigating the levels of NE and its metabolites in depressed patients have revealed striking alterations in the system (Roy et al. 1985; Roy et al. 1988; Kelly et al. 1998; Ressler et al. 1999). For example, a recent positron emission tomography (PET) study, revealed alterations in NE specifically within the PFC of patients with major depression (Fu et al. 2001). However, a consensus from these types of studies on whether depressed patients are afflicted with excessive NE or too little NE is lacking. Indeed depending on the individual study and the type of depressed individuals chosen for the study, the results can vary significantly. This, perhaps, is not surprising considering the studies of LC activity, which demonstrate that non-moderate NE release regardless of whether it is too little or too much can have similar adverse consequences for behavior. Thus, it is easy to imagine that individuals with either too little or excessive NE may manifest similar depression related symptoms. Or, perhaps, diminished NE levels may be characteristic of a particular subtype of

depression in comparison to another subtype defined by excessive levels of the monoamine, or even subtypes may exist where abnormalities in NE fail to contribute at all. In addition to these issues, measurements of NE levels from humans are also particularly susceptible to changes in movement, exercise, stress, methodological differences, etc. Thus additional research utilizing more advanced and reliable measures of NE as well as enhancements in research design (i.e. careful selection of research subjects for specific symptoms or subtypes of depression) is necessary to understand more clearly the abnormalities seen in the noradrenergic system during depression. In addition to exploring alterations in NE itself, other studies have focused on other noradrenergic signaling components for connections to depression, such as α2 adrenergic receptors and TH. Indeed, higher densities of α2 receptors have been demonstrated in the frontal cortex, hippocampus, temporal cortex, hypothalamus, and LC of depressed suicide victims in several reports (Meana et al. 1987; Meana et al. 1992; Gonzalez et al. 1994; Ordway et al. 1994; Callado et al. 1998). Some studies have even shown evidence for altered α2 affinity and density on the platelets of depressed patients (Piletz et al. 1990). In the case of TH, studies are less consistent and some have shown increases in TH levels in the LC of depressed individuals while others have shown support for decreased TH (Biegon et al. 1992; Zhu et al. 1999). Finally, catecholamine depletion studies in humans have also been preformed to provide evidence for a link between NE and depression. In unaffected individuals catecholamine depletion will not precipitate depressive symptoms; however, similar treatment in subjects with a history of depression instigates expression of depressive symptoms (Salomon et al. 1997; Charney 1998; Berman et al. 1999). Altogether, while the precise nature of the noradrenergic system's contribution to

depression remains elusive divergent human studies of individuals with depression consistently supports the idea that noradrenergic dysfunction may contribute to aspects of this disorder.


*NE and Schizophrenia*

In the schizophrenia research field predominant hypotheses classically focus on dopaminergic dysfunction as a key component of the disorder, primarily due to the efficacy of D2 dopamine receptor (D2DR) antagonists in the treatment of the disorder. While other neurotransmitters, such as glutamate and 5-HT, are just beginning to receive attention in the field, investigation of noradrenergic contribution to the disorder is minimal to non-existent. The role of NE, however, should not be overlooked, given early findings in the 1980's of elevated NE in the CSF and altered DBH activity in patients with schizophrenia (Farley et al. 1978; Breier et al. 1990; Maas et al. 1993; van Kammen et al. 1994). Indeed, numerous studies have examined NE and its metabolites in the CSF of patients, and most often elevations in NE are seen in association with both negative and positive symptoms of the disorder. Since these original observations, however, the reliability of such measurements has been brought into question, and consequently interest in NE as part of schizophrenia pathology has remained relatively shelved. In addition to the well known positive symptoms of schizophrenia, the disorder is also characterized by a subset of negative symptoms which include deficits in cognition (i.e. attention and working memory). As described previously, noradrenergic activity within the PFC is an essential component of cognitive function. Thus more recent research has focused on the role of NE in the manifestation of negative symptoms associated with the

disorder. For example, significant reductions in PFC NE have been seen in cognitively impaired schizophrenics relative to schizophrenics with low levels of cognitive deficits (Bridge et al. 1985; Bridge et al. 1987). Consistently, enhanced NE turnover (i.e. reduced levels of NE and increased MHPG) in the NAcc has been associated with cognitive impairments in schizophrenics. Despite growing interest, especially within the realm of the disorder's cognitive deficits, research on noradrenergic function in schizophrenia is still insufficient. This point is further emphasized by the little recognized fact that all effective antipsychotics, typical or atypical, have been shown to block the noradrenergic α1 receptor as well as D2DRs (Cohen et al. 1986; Baldessarini et al. 1992). It remains to be determined, however, whether this additional α1 receptor blockade is important for the efficacy of these drugs. In summary, substantial research is needed to fully appreciate and understand the contribution of the noradrenergic system to schizophrenia in general and more specifically to particular symptoms of the disorder.

*NET Dysfunction and Disease*

Through its re-uptake of synaptic NE and other monoamines such as DA, NET is pivotal for maintaining the integrity of monoaminergic signaling in the brain and periphery (Iversen 1971; Pacholczyk et al. 1991; Xu et al. 2000; Moron et al. 2002). Thus, disruption of NET function has the potential to impact both autonomic function and mental health. Indeed, studies supporting a direct link between transporter dysfunction and disease continue to accumulate. The first of such studies revealed a pivotal role for aberrant NET function in the autonomic nervous system disorder, orthostatic intolerance (OI), a disease characterized by the inability of an individual's body to withstand the

physiological stressors associated with postural changes (i.e. moving from a sitting position to standing upright) (Shannon et al. 2000; Robertson et al. 2001; Hahn et al. 2003). From this work, a single nucleotide polymorphism (SNP), alanine457proline (A457P), was identified in the transporter from a family of patients with OI. Interestingly, A457P NET cannot appropriately express at the surface, and in addition the mutant transporter acts in a dominant negative manner to further prevent the surface expression of wild-type NET resulting in severely deficient NE reuptake. Thus, individuals with this particular allele experience dizziness, tachycardia, and fainting upon standing because of an inability to control synaptic levels of NE in the periphery.

Since this original description of a direct role for anomalous NET function in disease, substantial effort has been invested in identifying other NET SNPs which may contribute to both autonomic and central nervous system disorders. For example, as emphasized by the role of NET in OI, NE plays a critical role in the peripheral nervous system in the regulation of heart rate and blood pressure. Not surprisingly then, several NET SNPs have been identified and associated with hypertension, and researchers continue to search for additional associations with other peripheral disorders such as myocardial ischemia, cardiomyopathy, etc. (Halushka et al. 1999; Rumantir et al. 2000; Ono et al. 2003; Hahn et al. 2005; Nonen et al. 2008). As for monoamine related mood disorders, NET SNPs have not only been identified but they have also been functionally characterized (Hahn et al. 2005; Kim et al. 2006; Haenisch et al. 2008; Hahn et al. 2009). In the case of ADHD, for example, a SNP associated with the disorder was found within the promoter of the NET gene where it creates a novel repressor site and thus results in altered transcription of the transporter (Kim et al. 2006). NET SNPs not associated with

particular disorders have also been identified and functionally characterized (Hahn et al. 2005). For some of these, subsequent to their functional investigations, association of the mutant transporters with particular disorders was soon to follow. For example, the NET SNP F528C was first shown to have increased plasma membrane expression and function as well as deficits in antidepressant binding and PKC induced trafficking, before its association with major depression was described (Hahn et al. 2005; Haenisch et al. 2008). On the other hand, the list of newly identified, non-functionally characterized NET SNPs, that are associated with disorders such as depression, ADHD, OI, and hypertension continues to grow exponentially (Hahn et al. 2008; Kim et al. 2008; Nonen et al. 2008). Whereas the identification of NET SNPs offers new insight into the connection between monoamines and nervous system related disorders, it is important to remind ourselves that transporter function is sophistically regulated by a battery of other proteins and complex signaling pathways. Thus, expanding research beyond the transporter itself, in an effort to encompass important regulators of these dynamically modulated proteins, promises to offer new and exciting insight into how aberrant monoamine transporter regulation may also contribute to disease states.

## Specific Aims

Monoamine homeostasis in the nervous system supports a variety of vital functions ranging from mood, memory, and reward to sleep, heart rate, and food intake. As an integral component of this delicate system, transporters play the key role of monitoring monoamine synaptic availability. Therefore, understanding the intricacies of how these transporters are regulated and function is absolutely imperative. Indeed,

examples of aberrant transporter function linked to specific diseases emphasize this need. Here, of particular interest, is the role of insulin signaling in the regulation of NET. Today, as sedentary lifestyles and access to high fat, high sugar, food abounds, the number of individuals afflicted with disorders characterized by aberrant insulin signaling grow exponentially. Thus, it is critical to understand how these changes impact transporter function and ultimately the brain and behavior. As such, the specific aims of this project were to:

I.   Determine the role of insulin signaling in the regulation of NET in both the central nervous system (CNS) and the peripheral nervous system (PNS).

II.  Identify components of the insulin signaling pathway that are required for the regulation of NET.

III. Study the physiological and behavioral consequences of manipulating this regulation *in vivo*.

# CHAPTER II

INSULIN REVEALS AKT SIGNALING AS A NOVEL REGULATOR OF

NOREPINEPHRINE TRANSPORTER TRAFFICKING AND NOREPINEPHRINE

HOMEOSTASIS*

**Abstract**

Noradrenergic signaling in the central nervous system plays an essential role in circuits involving attention, mood, memory, and stress as well as providing pivotal support for autonomic function in the peripheral nervous system. The high affinity norepinephrine (NE) transporter (NET) is the primary mechanism by which noradrenergic synaptic transmission is terminated. Data indicates that NET function is regulated by insulin, a hormone critical for the regulation of metabolism. Given the high co-morbidity of metabolic disorders such as diabetes and obesity with mental disorders such as depression and schizophrenia we sought to determine how insulin signaling regulates NET function and thus noradrenergic homeostasis. Here, we show that acute insulin treatment, through the downstream kinase protein kinase B (Akt), significantly decreases NET surface expression in mouse hippocampal slices and superior cervical ganglion neuron (SCGN) boutons (sites of synaptic NE release). In vivo manipulation of insulin/Akt signaling, with streptozotocin (STZ), a drug that induces a Type 1-like diabetic state in mice, also results in aberrant NET function and NE homeostasis. Notably, we also demonstrate that Akt inhibition or stimulation, independent of insulin, is

capable of altering NET surface availability. These data suggest that aberrant states of Akt signaling such as in diabetes and obesity have the potential to alter NET function and noradrenergic tone in the brain. Furthermore, they provide one potential molecular mechanism by which Akt, a candidate gene for mood disorders such as schizophrenia and depression, can impact brain monoamine homeostasis.

## Introduction

Appropriate regulation of critical brain functions such as learning, memory, attention, sleep, mood, and stress depend on the fidelity of noradrenergic signaling in the nervous system. The norepinephrine transporter (NET) is fundamental for maintaining this fidelity by controlling both the duration and strength of NE signaling through its reuptake of synaptic NE (Iversen 1971; Pacholczyk et al. 1991; Bonisch et al. 2006). Indeed, disruption of NET function has been shown to directly impact both autonomic function and mental health (Ganguly et al. 1986; Klimek et al. 1997; Rumantir et al. 2000; Shannon et al. 2000; Hahn et al. 2003; Hahn et al. 2005; Kim et al. 2006; Haenisch et al. 2008; Hahn et al. 2008; Hahn et al. 2009).

Prior studies illustrate that NET function is dynamically regulated both by changes in transporter turnover rate and by trafficking of the transporter to and from the plasma membrane (Apparsundaram et al. 1998; Apparsundaram et al. 1998; Uchida et al. 1998; Apparsundaram et al. 2001; Miner et al. 2003; Sung et al. 2003; Jayanthi et al. 2004; Dipace et al. 2007). Importantly, previous studies implicate a clear role for insulin, a metabolic hormone, in the regulation of NET function. Indeed, insulin inhibits NE uptake in whole brain neuronal cultures, dissociated brain cells, and whole brain

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synaptosomes (Boyd et al. 1985; Boyd et al. 1986; Masters et al. 1987; Raizada et al. 1988). Furthermore, Figlewicz et al. demonstrated the ability of nanomolar concentrations of acute insulin to decrease NE uptake from both hypothalamic and hippocampal slices (Figlewicz et al. 1993). These studies were elegantly extended to demonstrate that insulin also inhibits NE uptake in PC12 cells which endogenously synthesize NE and express NET (Figlewicz et al. 1993). Conversely, more recent studies have shown in different preparations that insulin increases NE uptake (Apparsundaram et al. 2001). Our studies, both in vitro and in vivo, indicate that insulin plays an inhibitory role in the regulation of NET function by controlling its surface availability. Moreover, we reveal that protein kinase B (Akt), a multifunctional kinase downstream of numerous signaling pathways including insulin, is required for this insulin regulation. Importantly, we also show that Akt activity potently regulates NET surface levels independently of changes in insulin status.

Diseases characterized by aberrant insulin and Akt signaling such as diabetes and obesity have a high co-morbidity with monoamine related mental disorders such as schizophrenia and depression (Mukherjee et al. 1989; Mukherjee et al. 1996; Lustman et al. 2005; Zhao et al. 2006). Our data reveal for the first time, Akt function as a potent regulator of NET activity/trafficking and thus provide an interesting and plausible link between metabolic dysfunction and mood disorders. Considering the identification of Akt as a candidate susceptibility gene in schizophrenia and perhaps depression (Hsiung et al. 2003; Emamian et al. 2004; Karege et al. 2007; Arguello et al. 2008), these data also provide a plausible molecular mechanism linking anomalous Akt function to altered monoamine homeostasis which is characteristic of these disorders.

## Results

*Acute insulin treatment decreases NET surface levels in the central and peripheral nervous systems as well as in heterologous preparations*

NET plays a pivotal role in controlling global noradrenergic tone and its aberrant regulation has the capacity to impact mental health (Ganguly et al. 1986; Klimek et al. 1997; Rumantir et al. 2000; Shannon et al. 2000; Hahn et al. 2003; Hahn et al. 2005; Kim et al. 2006; Haenisch et al. 2008; Hahn et al. 2008; Hahn et al. 2009). Evidence indicates that insulin signaling regulates NE homeostasis (Shimizu 1991; Figlewicz et al. 1993; Figlewicz et al. 1996; Barber et al. 2003). Thus, understanding how insulin/Akt fine tunes NET trafficking and/or function may provide an interesting and plausible link between metabolic dysfunction and mental disorders such as depression and schizophrenia (Mukherjee et al. 1989; Mukherjee et al. 1996; Lustman et al. 2005; Zhao et al. 2006). Our goal is to determine the nature of insulin's regulation of NET in the central and peripheral nervous systems. Here, we provide evidence for the role of insulin in the down regulation of the transporter from the surface. Utilizing a new assay, slice biotinylation, we show for the first time that acute *in vitro* treatment of mouse hippocampal slices with 1 nM insulin significantly diminishes surface levels of NET (Fig. 8A). Tyrosine hydroxylase (TH), a cytosolic protein, is detected primarily in the total fraction and comprises less than 1% of the surface fraction. Therefore, these data demonstrate that the biotinylated fraction represents cell surface proteins and speaks to the health of noradrenergic TH positive neurons in the assay. These results provide an ANOVA followed by Dunnett's test; N=3-8). molecular mechanism to describe the decreased NE uptake described by Figlewicz and collaborators (Figlewicz et al. 1993). In addition to

**Figure 8.** Insulin stimulation decreases NET surface levels in mouse hippocampal slices, SCGN boutons, and hNET cells. **A,** (Left) Mouse hippocampal slices treated with either 1 nM insulin or vehicle (CTR) for 20 minutes. Shown are representative Western blots of NET surface and total levels, as well as surface $Na^+/K^+$ ATPase levels to serve as a loading control. (Center) Quantification of the insulin-induced decrease in NET surface expression is also shown. Data are normalized to total NET and expressed as a percent of control (mean ± s.e.m, **$P<0.01$ by Student's t-test; N=7-6). (Right) Representative Western blot of TH levels in the biotinylated and total fractions. **B**, (Left) Confocal images of NET distribution in SCGN boutons treated with vehicle (CTR), 1 nM, or 100 nM insulin for 20 minutes. Insulin treatments induce an increase in intracellular NET accumulation. (Right) NET fluorescence intensity plots (see Materials and Methods) across SCGN boutons treated with vehicle (open squares) and 100 nM insulin (solid squares). The normalized NET intensity was plotted against the normalized distance as described in Material and Methods (mean ± s.e.m, $P<0.0001$ by Two-way ANOVA; N=18-8 boutons). Panel B special thank you to Heinrich Matthies. **C**, (Left) Acute 100 nM insulin treatment for 20 minutes significantly increases NET and Rab11a colocalization in SCGN boutons with respect to vehicle (CTR). (Right) Quantification of NET and Rab11a colocalization using the ICQ analysis (see Materials and Methods). Insulin significantly increases NET colocalization with the strictly intracellular protein Rab11a (mean ± s.e.m, ***$P<0.001$ by Student's t-test; N=23). Panel C special thank you to Heinrich Matthies **D,** Insulin decreases NET surface levels in a time dependent manner in hNET cells. (Left) Shown are representative Western blots for surface and total NET in cells treated with vehicle (CTR) or insulin at varying time points. (Center) Western blot analysis showed that 1 µM of insulin exposure significantly decreases surface NET. Data are normalized to total NET and expressed as a percent of control (mean ± s.e.m, *$P<0.05$ by one-way ANOVA followed by Dunnett's test; N=4). (Right) The cytosolic kinases ERK1/2 are found in the total fraction but not in the surface fraction. **E,** Quantitation of Western blots from hNET cells exposed to 5 minutes of insulin at varying concentrations (mean ± s.e.m, *$P<0.05$ and **$P<0.01$ by one-way ANOVA followed by Dunnett's test; N=3-8).

**A**

Insulin (1 nM)

Surface NET

Total NET

Surface Na⁺/K⁺ ATPase

Insulin (1 nM)

Surface TH

Total TH

**B**

Insulin (20 min)

NET

CTR

Insulin (1 nM)

Insulin (100 nM)

1 μm

□ CTR
■ Insulin (100nM)
P < 0.0001

Normalized NET Intensity

Normalized Distance

**C**

NET and Rab11a

CTR          20 min

Insulin (100 nM)

1 μm

NET and Rab11a ICQ

CTR    20 min

Insulin (100 nM)

***

**D**

Insulin (1 μM)

Surface NET

Total NET

Insulin (1 μM)

Surface ERK1/2

Total ERK1/2

Surface NET/Total NET (% of CTR)

CTR    1 min    5 min

Insulin (1 μM)

*

**E**

Surface NET/Total NET (% of CTR)

CTR    1 nM    10 nM    100 nM    1 μM

Insulin (5 min)

*    *    **

revealing a role for insulin in the regulation of NET trafficking in slices from the central nervous system, we also sought to demonstrate the ability of insulin to regulate NET in the peripheral nervous system.

Superior cervical ganglion neurons (SCGN) enable us to investigate the role of insulin in the peripheral nervous system. Importantly, due to the large diameter (2-4 micron) of SCGN boutons, these cultures also provide us with the unique opportunity to study NET regulation at presynaptic sites of NE release (Matthies et al. 2009). Before exploring the role of insulin in the regulation of these noradrenergic neuronal cultures, we sought to demonstrate that these cells express the insulin receptor (IR). Not only is IRβ staining abundant throughout the SCGN (data not shown), the receptor is also specifically localized to single boutons (Appendix Fig. A1). After demonstrating the presence of IRs on noradrenergic SCGNs, we serum-starved the SCGN cultures for 1 hour and then treated them with vehicle, 1 nM, or 100 nM insulin for 20 minutes and discovered a dramatic increase in intra-bouton NET immunoreactivity (Fig. 8B). We quantified these observations using pixel intensity plots of a single confocal section (see Materials and Methods). Pixel intensity plots were obtained from a straight line drawn across the widest region of the approximately spherical bouton, starting at the brightest spot of fluorescence on one side of the bouton. The subsequent line spanned the entire diameter of the bouton, and was extended beyond the limits of the bouton to provide a background value. This line was then divided arbitrarily into 20 bins, and the NET fluorescence intensity of each bin was normalized to the fluorescence intensity of the brightest spot on the perimeter of the bouton (100%). The mean ± s.e.m. pixel intensity (normalized NET intensity) is plotted against each bin (normalized distance). Figure 8B shows that in

insulin-treated neurons, the NET intra-bouton fluorescence signal is significantly higher than in vehicle-treated neurons as shown by a shift in the curve upward. This demonstrates that NET is accumulated intracellularly upon 100 nM insulin treatment. Our result that 100 nM insulin treatment significantly increases intracellular NET accumulation is further substantiated by co-staining for the recycling endosome marker Rab11a. Rab11a is a GTPase that plays an important role in the trafficking of numerous proteins including NET via "slow" recycling endosomes (Matthies et al. 2010). Importantly, we show that 100 nM insulin exposure significantly enhances NET colocalization with the strictly intracellular protein Rab11a (Fig. 8C). This increase in colocalization was quantified by utilizing the intensity correlation analysis/intensity correlation quotient (ICA/ICQ) method (Li et al. 2004). We calculated the ICQ of NET and Rab11a in boutons and found a significant increase upon 100 nM insulin treatment (Fig. 8C).

Our results, for the first time, demonstrate the ability of insulin to induce NET trafficking, in both the central and peripheral nervous systems. Furthermore, this regulation is seen not only at the level of hippocampal slices but also at the level of a single bouton. In order to dissect this insulin-induced regulation further, we chose to study it in a more malleable system, Chinese hamster ovary (CHO) cells with stably transfected HA-tagged human NET (hNET cells). Here we validate this model by demonstrating that insulin-induced trafficking of NET away from the cell surface is both time and dose-dependent in hNET cells. Incubation of hNET cells for 5 minutes with 1 µM insulin results in a significant reduction of NET in the biotinylated fraction (Fig. 8D). Additionally, 5 minutes of insulin exposure at varying doses results in significant

decreases in surface NET (Fig. 8E). In addition, to insulin's ability to modulate NET surface availability in these cells, uptake assays also support an insulin mediated decrease in NET function. Consistent with previous results, our data reveal a significant decrease in [$^3$H] DA uptake after exposure to 10 nM insulin (Vehicle $1.162 \pm 0.020$ and Insulin $0.986 \pm 0.020$ pmol*well$^{-1}$*min$^{-1}$; mean $\pm$ s.e.m., **P<0.01 by Student's t-test; N=4-3). The time and dose dependent changes in NET surface levels and function in response to insulin in hNET cells illustrates the utility of this system for studying the intricacies of regulation of this transporter by insulin. In particular, we sought to uncover specifically which components of the insulin signaling pathway are essential for insulin-induced NET regulation.

*Insulin requires Akt to induce changes in NET surface expression*

Insulin receptors are tyrosine kinase receptors which upon activation recruit both phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) to the membrane for subsequent activation. Akt, in particular, is a multifunctional kinase that is involved with diverse pathways in cell growth, survival, metabolism etc. Indeed aberrant Akt function has been implicated in a vast array of disorders such as diabetes, obesity, cancer, autoimmune disease, as well as mental disorders such as depression and schizophrenia (Hsiung et al. 2003; Emamian et al. 2004; Dummler et al. 2007; Karege et al. 2007; Manning et al. 2007; Krishnan et al. 2008). While genetic evidence for the involvement of Akt in mental disorders grows, how Akt dysregulation impacts these diseases at the molecular level is still unclear. Importantly, it has been shown that Akt is critical for the regulation of other transporters such as the glucose and dopamine transporter by insulin

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(Carvelli et al. 2002; Garcia et al. 2005; He et al. 2007; Zaid et al. 2008). Here, we investigate whether Akt plays a critical role in the insulin regulation of NET by utilizing the isoform specific Akt1, Akt2, or Akt1/2 inhibitors (DeFeo-Jones et al. 2005; Lindsley et al. 2005; She et al. 2008). These inhibitors are allosteric inhibitors that require the pleckstrin homology (PH) domain of Akt to inhibit phosphorylation and activation of the kinase. Although they require this domain for inhibition, binding of the inhibitors to Akt requires the whole protein since *in vitro* assays show the PH domain alone is insufficient for binding. Furthermore, the inhibitors reversibly inhibit both the activation and activity of Akt and are highly specific for Akt compared to other similar kinases such as PKA, PKC, and SGK. In our studies, 5 μM application of the dual Akt1/2 inhibitor to hNET cells 30 minutes prior to and during insulin exposure prevents insulin stimulated trafficking of NET away from the surface (Fig. 9A). In addition to pharmacological blockade, we also utilize a "kinase-dead" dominant negative mutant (K179R) construct of Akt (Akt-KD) to show that Akt activity is required for insulin induced regulation of the transporter (Garcia et al. 2005). Consistent with our previous results, transient transfection of hNET cells with the Akt- KD construct 48 hours prior to insulin application abolishes insulin stimulated trafficking of NET away from the plasma membrane (Fig. 9B). While both genetic and pharmacological inhibition of Akt prevents insulin induced regulation of the transporter in hNET cells, we sought to extend these observations to a more physiologically system.

Given the abundance of NET expression in mouse hippocampal slices, we chose this experimental system to investigate the role of Akt in the insulin regulation of NET. While all three isoforms of Akt are expressed within the brain, the distribution and

**Figure 9**. In hNET cells, Akt inhibition and Akt-KD overexpression prevents insulin induced NET trafficking. **A**, (Left) Representative Western blot of NET from hNET cells incubated in medium containing 0.1% DMSO or the dual Akt 1/2 inhibitor (5 μM) dissolved in DMSO 30 min prior to and during either vehicle (CTR) or insulin treatment (5 min). The absence of cytosolic ERK1/2 bands in the biotinylated fraction supports the integrity of the biotinylation assay. (Right) Quantitation of Western blots represented by the left panel. Data are normalized to total NET and expressed as a percent of control (mean ± s.e.m, **$P<0.01$ by one-way ANOVA followed by Dunnett's test; N=4-5). **B**, 48 hours prior to treatment with vehicle (CTR) or insulin (5 min), hNET cells are transfected with either Akt-KD, a kinase dead dominant negative form of Akt or pcDNA3.1 (vector). (Left) A representative Western blot shows that overexpression of Akt-KD inhibits the insulin induced reduction of surface NET compared to vector transfected control. Overexpression of Akt-KD was confirmed by probing for total levels of Akt. The cytosolic kinases ERK1/2 are found in the total fraction but not in the surface fraction. (Right) Quantitation of multiple Western blots represented by the left panel. Surface NET is normalized to total NET levels and expressed as a percent of control (mean ± s.e.m, **$P<0.01$ by one-way ANOVA followed by Dunnett's test; N=4-5).

**A**



**B**



60
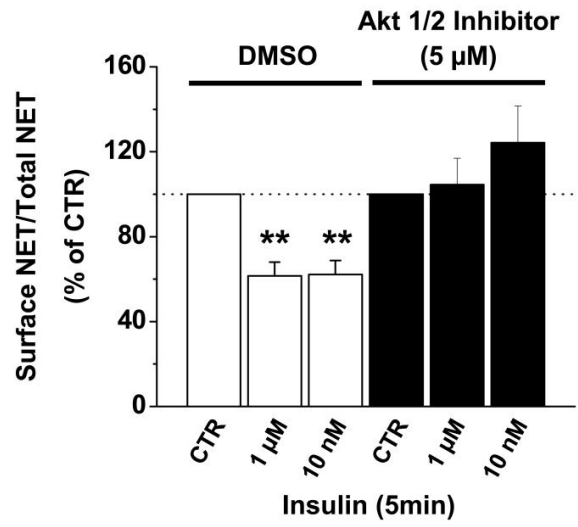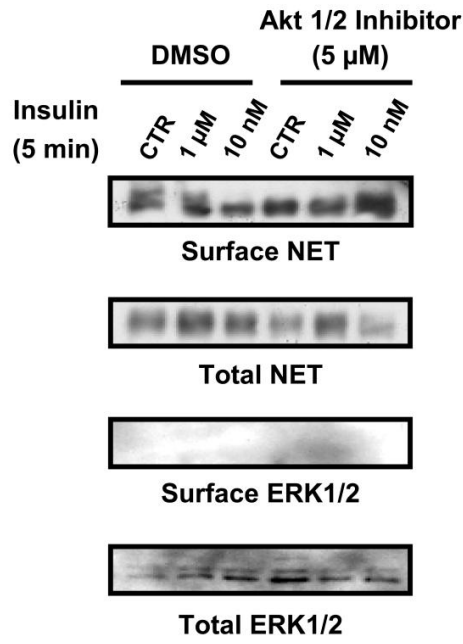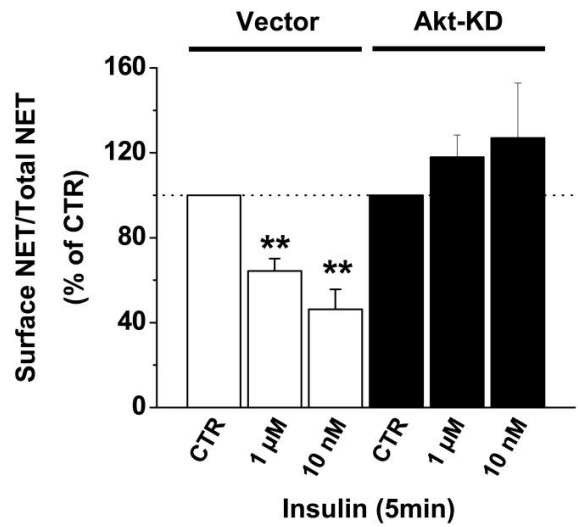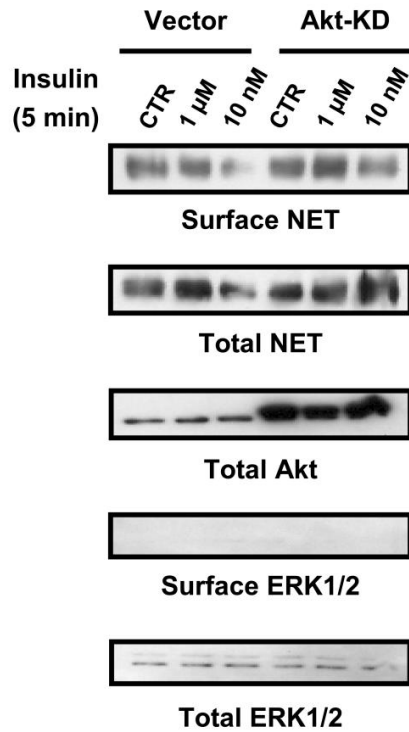
differences in their expression across various regions of the brain is relatively uncharacterized (Easton et al. 2005). We anticipate that for Akt to regulate the transporter it should be localized in the same neuronal domain. Indeed, NET, Akt1, and Akt2 co-staining of slices reveals that Akt1 and Akt2 are heavily expressed in NET positive hippocampal terminals (Fig. 10A). Thus, Akt is poised for regulating the transporter in hippocampal slices. Consistent with these findings, insulin-stimulated decreases in NET are abolished by pre-treatment with either the Akt1 or Akt2 inhibitor for 1 hour prior to and during insulin exposure (Fig. 10B). These data demonstrate that insulin-induced down-regulation of NET requires Akt activity. Interestingly, changes in the phosphorylation status of Akt at serine residue 473, which relates to Akt activation, correlate well with changes in NET surface levels (Fig. 10C). This correlation is seen consistently throughout our studies and led us to hypothesize that Akt alone, which can be stimulated by numerous pathways, is a pivotal regulator of the transporter. Before investigating the effects of altering Akt activity independent of insulin signaling, however, we sought to determine if *in vivo* manipulation of insulin and Akt signaling results in significant changes in NET regulation and consequential monoamine homeostasis.

*Hypoinsulinemic mice have reduced Akt phosphorylation, enhanced NE levels, and elevated NET surface expression/function*

To induce a hypoinsulinemic state, mice were injected with streptozotocin (STZ), a selectively toxic compound targeting the insulin producing pancreatic beta cells (Lenzen 2008). These mice display hallmarks of hypoinsulinemia such as aberrant

**Figure 10**. Akt1 and Akt2 colocalize with NET at terminals in mouse hippocampal slices, and their inhibition precludes insulin stimulated decreases in surface NET. **A,** Confocal images of NET (green), Akt1 (red top panel), and Akt2 (red bottom panel) immunoreactivity in mouse hippocampal slices. The merge panels provide evidence of colocalization between NET and both isoforms of Akt. **B**, Mouse hippocampal slices were treated with vehicle or the specific isoform inhibitor for Akt1 or Akt2 (12 μM) for 40 minutes prior to and during 1 nM insulin treatment for 20 minutes. (Left) Shown is a representative Western blot of surface and total levels of NET while surface $Na^+/K^+$ ATPase levels serve as a loading control. TH is detected in the total fraction but absent in the surface fraction. (Right) Quantification of multiple Western blots represented by the left panel. Surface NET is normalized to total NET and expressed as a percent of control (mean ± s.e.m, **$P<0.01$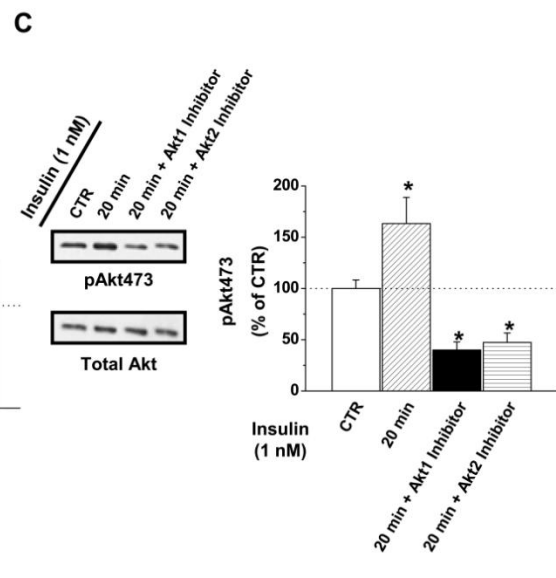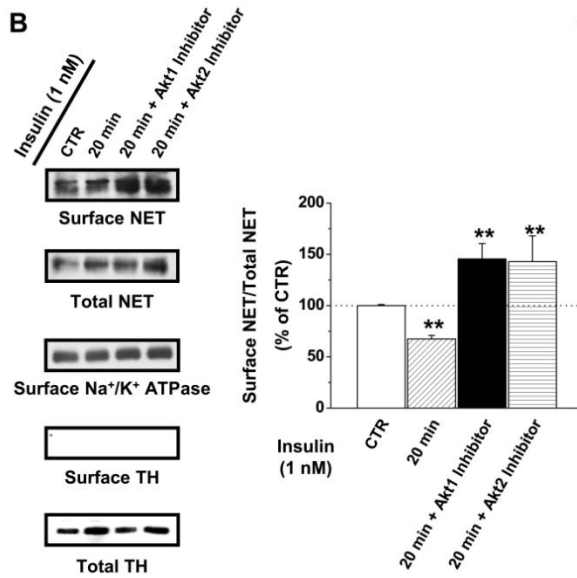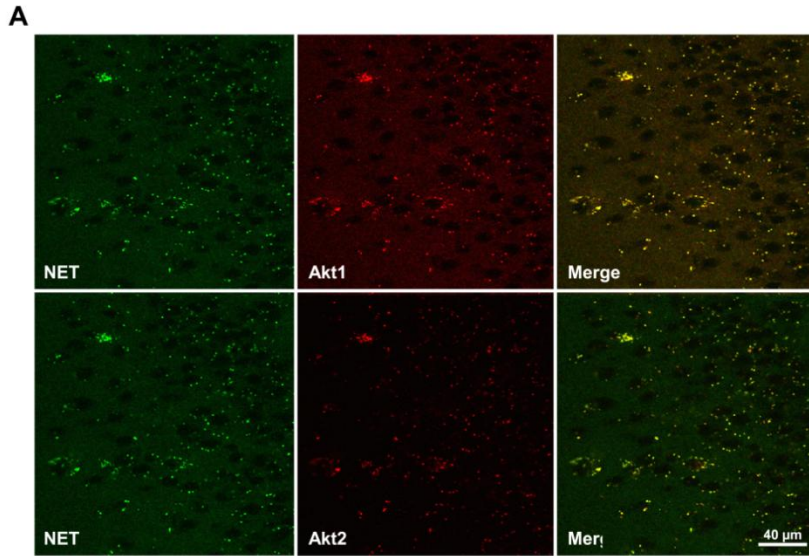 by one-way ANOVA followed by Dunnett's Test; N=10-7). **C**, (Left) Representative Western blot from the same mouse hippocampal slice samples used for quantitation in panel B probed for phosphorylated Akt at serine residue 473 (pAkt473) and total levels of Akt. (Right) Quantification of pAkt473 levels in slices treated as in panel B. pAkt473 levels are expressed as a percent of control (mean ± s.e.m, *$P<0.05$, by one-way ANOVA followed by Dunnett's Test; N=8-9).

**A**

NET · Akt1 · Merge

NET · Akt2 · Merge

40 μm

**B**

Insulin (1 nM)

CTR · 20 min · 20 min + Akt1 Inhibitor · 20 min + Akt2 Inhibitor

Surface NET

Total NET

Surface Na⁺/K⁺ ATPase

Surface TH

Total TH

Surface NET/Total NET (% of CTR)

Insulin (1 nM)

CTR · 20 min · 20 min + Akt1 Inhibitor · 20 min + Akt2 Inhibitor

**C**

Insulin (1 nM)

CTR · 20 min · 20 min + Akt1 Inhibitor · 20 min + Akt2 Inhibitor

pAkt473

Total Akt

pAkt473 (% of CTR)

Insulin (1 nM)

CTR · 20 min · 20 min + Akt1 Inhibitor · 20 min + Akt2 Inhibitor

glucose regulation, hyperphagia, hypouria, etc (Hernandez et al. 1972; Bell et al. 1983). In our studies, mice received one 200 mg/kg i.p. injection of STZ, and 48 hours later blood glucose levels were measured to confirm drug efficacy. Control mice, which received vehicle injections, maintained normal glucose levels while STZ injected mice showed aberrantly elevated levels of blood glucose (Appendix Fig. A2). After measurement of blood glucose levels, mice were left untreated in the STZ-induced diabetic state for 7 to 10 days and were then sacrificed for biochemical studies. STZ mice display deficits in Akt phosphorylation at ser473 in the hippocampus, which is accompanied by an increase in NE tissue content in the region (Fig. 11A, B). Importantly, studies show that appropriate NET function is critical for regulating NE tissue content (Xu et al. 2000). Thus, given the deficits in Akt phosphorylation and the significant elevation of NE tissue content, we hypothesize that these mice will display significantly enhanced NET surface levels. Moreover, evidence from previous studies show that STZ rats have elevated steady state levels of NET mRNA in the locus coeruleus (Figlewicz et al. 1996). Importantly, hippocampal slice biotinylation reveals a significant increase in surface NET in mice with STZ-induced hypoinsulinemia (Fig. 11C). To determine if enhanced surface expression of NET in hippocampal slice preparations from STZ-treated mice results in increased NE clearance in hippocampus *in vivo*, we utilized high speed chronoamperometry (HSCA). Clearance rate of NE locally injected into the dentate gyrus was significantly increased in STZ treated mice, as indicated by the decreased time required for the NE signal to diminish in STZ mice as compared with saline treated mice (Fig. 11D, left panel). Consistently, the average clearance rate of NE over a range of concentrations, measured as described in Material and Methods, was significantly

**Figure 11**. STZ-induced hypoinsulinemia decreases hippocampal Akt phosphorylation while significantly increasing NET surface expression and function. Mice were injected with either vehicle (CTR) or 200mg/kg i.p. STZ to mimic a type I diabetic state as described in Materials and Methods. **A,** (Left) Representative Western blot of pAkt473 and total Akt levels in mouse hippocampal slices from CTR or STZ mice. (Right) Quantitation of pAkt473 levels from CTR and STZ mouse hippocampal slices. Data are represented as percent of CTR (mean ± s.e.m, *$P<0.05$ by Student's t-test; N=14-11). **B,** Tissue content of norepinephrine as measured by HPLC from hippocampal homogenates in CTR versus STZ mice (mean ± s.e.m, *$P<0.05$ by Student's t-test; N=9-8). **C,** (Left) Shown is a representative Western blot of surface and total levels of NET from hippocampal slices of either CTR or STZ mice. Surface $Na^+/K^+$ ATPase levels serve as a loading control. (Center) Quantitation of Western blots represented by the left panel. Surface NET is normalized to total levels of NET and expressed as percent of CTR (mean ± s.e.m, *$P<0.05$ by Student's t-test; N=14-11). (Right) The absence of cytosolic TH bands in the biotinylated fraction supports the integrity of the hippocampal slice biotinylation assay. **D,** (Left) Oxidation currents (converted to µM concentration using a calibration factor determined *in vitro*) produced by pressure ejection of 0.8 to 60 pmol of NE into the dentate gyrus region of the hippocampus of anaesthetized CTR and STZ mice. Corresponding summary data for the rates (nM/s) (Right) and time (s) (Bottom) of NE clearance plotted as a function of increasing concentrations of NE (CTR n=8; STZ n=7; *Rate*: main effect treatment, p=0.0453; main effect NE concentration, p<0.0001; *Time*: main effect treatment, p=0.0255; main effect NE concentration, p<0.0001; by Two-way ANOVA with Bonferroni post-hoc comparisons, *p<0.05) Panel D courtesy of Anthony Owens and Lynette Daws.
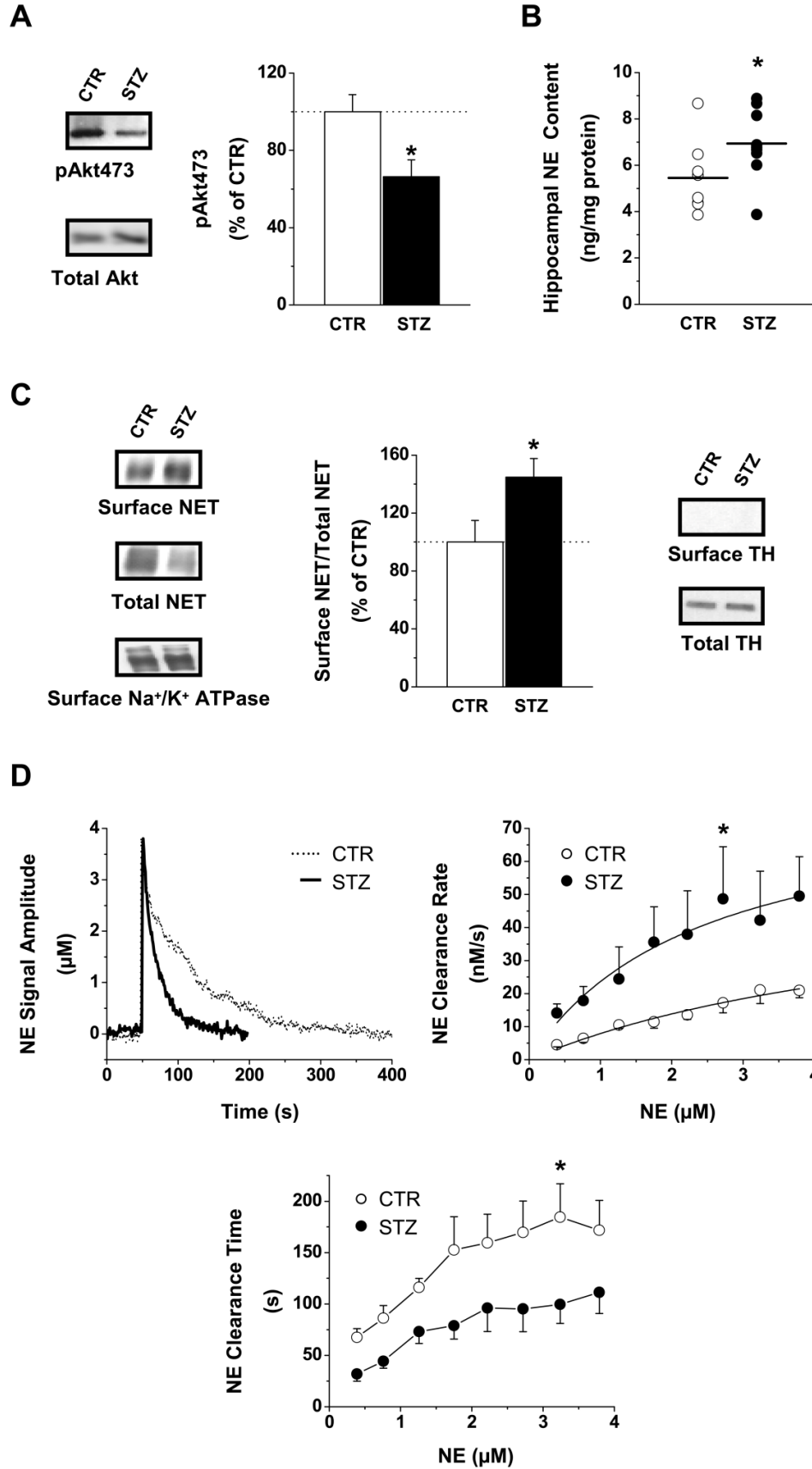
enhanced in STZ mice as compared to control animals, such that the apparent maximal velocity for NE clearance was increased approximately 2-fold compared to control mice (Fig. 11D, right panel). Similarly, the average time required for NE clearance (NE clearance time) over the same range of concentrations was significantly reduced in STZ treated mice (Fig. 11D, bottom panel). Thus, STZ mice display aberrant Akt regulation and increased NE tissue content in the hippocampus which corresponds with increased NET surface expression and function. Importantly, the STZ-induced diabetic mice display normal protein levels of other noradrenergic markers in the hippocampus such as TH (CTR N=9 100.0 ± 5.5; STZ N=11 94.5 ± 7.2; unpaired T-Test p=0.57) and dopamine beta hydroxylase (CTR N=7 100.0 ± 8.5; STZ N=9 101.7 ± 8.2; unpaired T-Test p=0.89). These data indicate that changes in NE tissue content correlates with altered NET surface expression and function and are not due to changes in NE synthesis. In addition, to support our hypothesis that hypoinsulinemia induced by STZ underlies the alterations observed in NET expression and function, we sought to reverse these deficits *in vitro* and *in vivo* with acute insulin treatment. Again, STZ treated mice had significantly reduced levels of Akt ser473 phosphorylation (Fig. 12A) and enhanced surface expression of NET (Fig. 12B) in biotinylated hippocampal slices relative to control mice. However, 1nM insulin treatment for 20 minutes restores Akt phosphorylation levels and surface expression of the transporter to control levels (Fig. 12A, B). Thus, acute *in vitro* insulin treatment is sufficient to rectify STZ-induced alterations in hippocampal preparations. To determine if a similar rescue is possible *in vivo* we again utilized HSCA. As before, the average time required for NE clearance was significantly reduced, indicating enhanced NET function, in STZ treated,

hypoinsulinemic mice, relative to saline treated control mice (Fig. 12C, left panel). Similar to our *in vitro* paradigm, local infusion of insulin (10 μM / 100 nL to deliver 1 pmol) was then utilized in the dentate gyrus of STZ mice in an attempt to rectify the changes observed in NET function. First, exogenous NE was locally applied (200 μM / 10 nL to deliver 2 pmol) to achieve reproducible signals with amplitudes recorded at the carbon fiber electrode in the range of 1 μM. Insulin was then infused and two minutes later NE was applied again. Importantly, insulin itself did not produce any electrochemical signal. Exogenous insulin application 5 minutes before NE infusion, however, significantly increased the time required for NE to clear from the extracellular fluid in the dentate gyrus thus implying a significant reduction in NET function (Fig. 12C, center and right panel). The ability of *in vitro* and *in vivo* insulin application to restore STZ-induced deficits further supports the notion that peripheral hypoinsulinemia underlies NET dysfunction in these mice. Thus, our data from biotinylated hippocampal slices and *in vivo* HSCA in STZ-induced diabetic mice show for the first time, that *in vivo* manipulation of insulin levels in the periphery impacts both Akt signaling/phosphorylation and NET function in the brain with consequences for monoamine homeostasis.

*Akt signaling regulates NET surface availability in hNET cells, SCG neurons, and in cortical slices following in vivo antipsychotic treatment*

We hypothesize that manipulation of Akt signaling either by pharmacological means or receptor stimulation, independently of insulin, alters the surface availability of NET. To investigate if Akt signaling is capable of regulating NET trafficking, we

**Figure 12.** Acute insulin treatment *in vitro* and *in vivo* restores Akt deficits, NET surface expression, and function in the hippocampus of STZ-treated mice. Mice were injected with either vehicle (CTR) or 200mg/kg i.p. STZ to mimic a type I diabetic state as described in Materials and Methods. **A,** (Left) Representative Western blot of pAkt473 and total Akt levels in hippocampal slices from control mice (CTR) or STZ mice. Slices from STZ mice were then subdivided and treated either with 1 nM insulin for 20 minutes (STZ + Insulin) or with vehicle (STZ). (Right) Quantitation of pAkt473 levels from CTR, STZ, or STZ + Insulin mouse hippocampal slices. Data are represented as percent of CTR (mean ± s.e.m, *P<0.05 by one-way ANOVA followed by Dunnett's Test; N=9). **B,** (Left) Shown is a representative Western blot of surface and total levels of NET from hippocampal slices of either CTR or STZ mice, with slices from STZ mice subdivided and treated either with 1 nM insulin for 20 minutes (STZ + Insulin) or with vehicle (STZ). Surface Na$^+$/K$^+$ ATPase levels serve as a loading control. (Center) Quantitation of Western blots represented by the left panel. Surface NET is normalized to total levels of NET and expressed as percent of CTR (mean ± s.e.m, *P<0.05 by one-way ANOVA followed by Dunnett's Test; N=8). (Right) The absence of cytosolic TH bands in the biotinylated fraction supports the integrity of the hippocampal slice biotinylation assay. **C,** (Left) Summary data from HCSA experiments comparing clearance time of exogenous NE in the dentate gyrus of STZ treated versus saline treated control mice (see Figure 4D and materials and methods for details; mean ± s.e.m, *P<0.05 by Student's t-test; N=6). Local application of exogenous NE to the dentate gyrus of anesthetized mice was utilized to produce signal amplitudes of approximately 1 μM. After three reproducible NE signals were obtained, exogenous insulin (10 μM / 100 nl to deliver 1 pmol) was locally applied to the dentate gyrus and 2 minutes later, NE was applied again, and then at 5 minute intervals, until the NE signal returned to its pre-insulin baseline. (Center) Representative oxidation currents (converted to μM concentration using a calibration factor determined *in vitro*) produced by pressure ejection of 1 pmol of NE into the dentate gyrus region of the hippocampus of anaesthetized STZ mice prior to (STZ) and 5 minutes post insulin (STZ + insulin) application. (Right) Average NE clearance time in STZ treated mice following acute pressure injection of insulin. Data are expressed as percent of pre-insulin (baseline) clearance time. Insulin significantly increased NE clearance time in STZ treated mice (mean ± s.e.m, *P<0.05 and **P<0.01 by Friedman test followed by Dunn's Multiple Comparison Test on data pictured. Identical results were obtained by one-way ANOVA followed by Dunnett's Test on raw data; N=6). Panel C courtesy of Anthony Owens and Lynette Daws.

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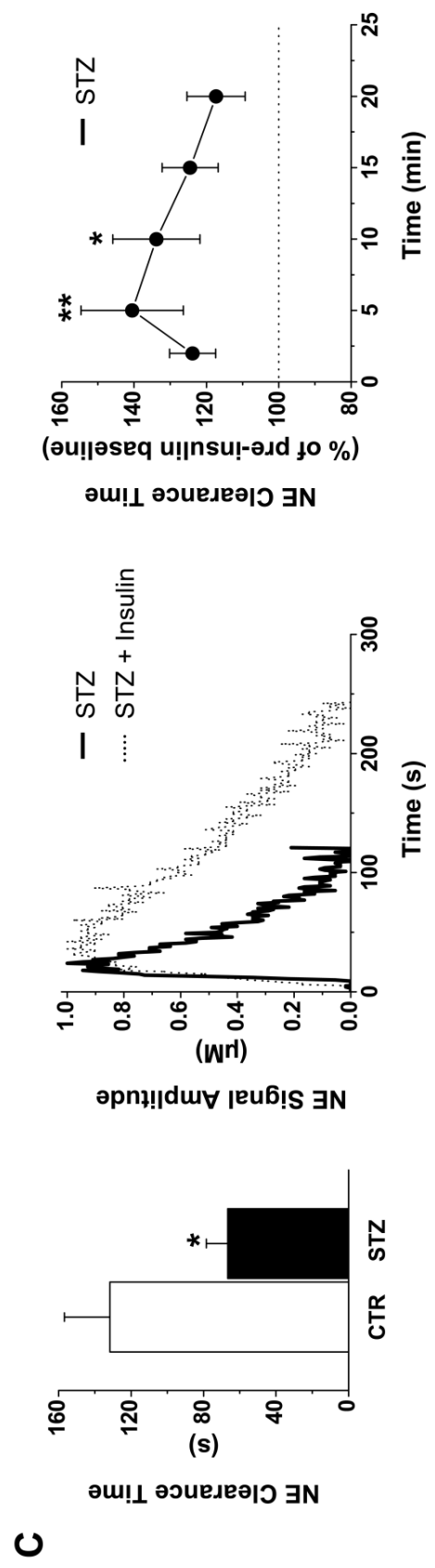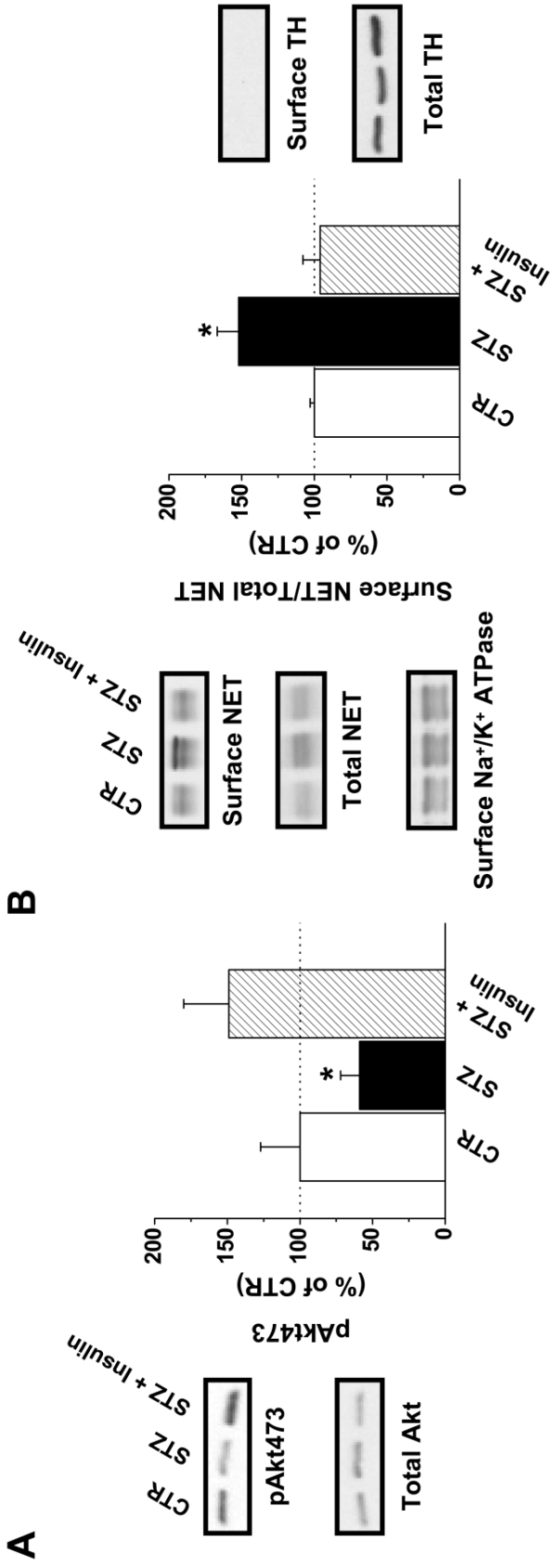
exposed hNET cells to the Akt1/2 inhibitor (5 μM) for varying time periods (Fig. 13A). Inhibition of Akt significantly increased NET surface expression in a time-dependent manner. Importantly, this pharmacological manipulation of Akt is independent of insulin status. To determine if Akt regulates NET at sites of NE release, we extended these studies to boutons of SCGN cultures. Co-staining of SCGN cultures for NET, Akt1, and Akt2 indicates that Akt is indeed present in the bouton and poised for regulation of the transporter (Appendix Fig. A3). Given the close proximity of both Akt isoforms to NET in the SCGN boutons, we exposed SCGN cultures to vehicle, Akt1 inhibitor, or Akt2 inhibitor for 1 hour to determine if both isoforms are capable of driving NET to the surface. In order to allow for Akt signaling inhibition by the Akt inhibitors the neurons were not serum starved. Here we show, at sites of synaptic NE release, that incubation of cultures with either the Akt1 or Akt2 isoform specific inhibitors results in a significant increase in NET immunoreactivity on the perimeter of the bouton (Fig. 13B). Thus, both Akt1 and Akt2 are capable of regulating NET surface availability in SCGN. Again, we quantified these observations using pixel intensity plots of a single confocal section (see Materials and Methods). Upon Akt inhibition, the Pixel intensity plots reveal a significant downward shift, indicating an increase in NET surface levels in response to Akt inhibition. Furthermore, the average NET intensity across inner bins (bins 3-17) reveals a significant decrease in NET immunoreactivity within the bouton in the presence of either Akt inhibitor (Fig. 13B, inset). These data demonstrate, for the first time, that Akt activity is a potent regulator of NET.

Next, we determined if activation of Akt by signaling pathways distinct from insulin are capable of altering NET surface expression. Numerous studies have shown

**Figure 13**. Basal Akt inhibition significantly increases NET surface expression. **A**, hNET cells are treated with vehicle (CTR) or the dual Akt1/2 inhibitor (5 μM) for varying amounts of time. (Left) Shown is a representative Western blot of surface and total levels of NET after vehicle or Akt1/2 inhibitor treatment. Cytosolic ERK1/2 bands are not present in the surface fraction. (Right) Quantitation of the increase in NET surface expression induced by Akt inhibition is also shown. Surface NET is normalized to total levels of NET and expressed as percent of CTR (mean ± s.e.m, *P<0.05 by one-way ANOVA followed by Dunnett's Test; N=6-4). **B**, Mouse SCGNs were cultured and processed for immunocytochemistry as described in Material and Methods. (Top Left) Confocal images of NET immunoreactivity in SCGN cultures treated with vehicle or 12 μM of either the Akt1 or Akt2 inhibitor for 1 hour. (Bottom) Analysis of intensity plots spanning SCGN boutons. The normalized NET intensity is plotted against the normalized distance as described in Material and Methods (mean ± s.e.m, P<0.0001 by Two-way ANOVA; N=14). (Dotted Inset) The average NET intensity across bins 3-18, which represent bins that are within the interior of the bouton, in CTR, Akt1 inhibition, or Akt2 inhibition conditions (mean ± s.e.m, **P<0.01 by one-way ANOVA followed by Dunnett's Test; N=14).

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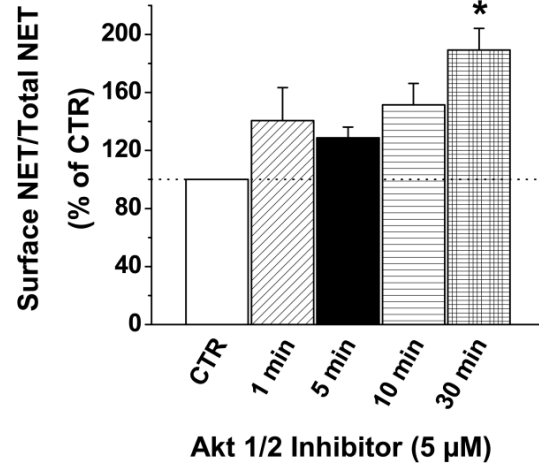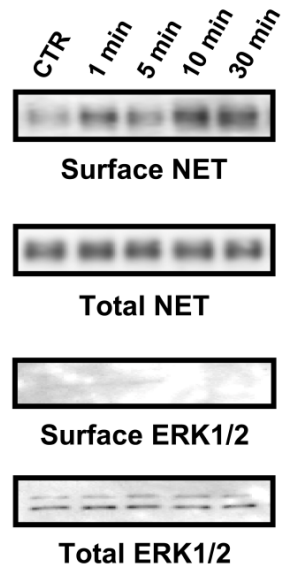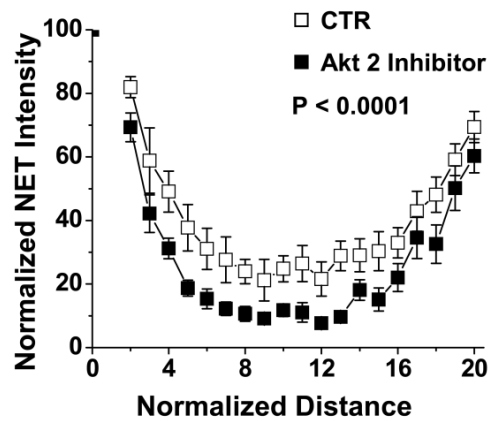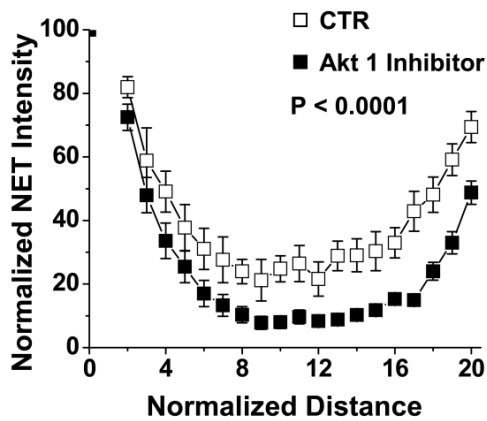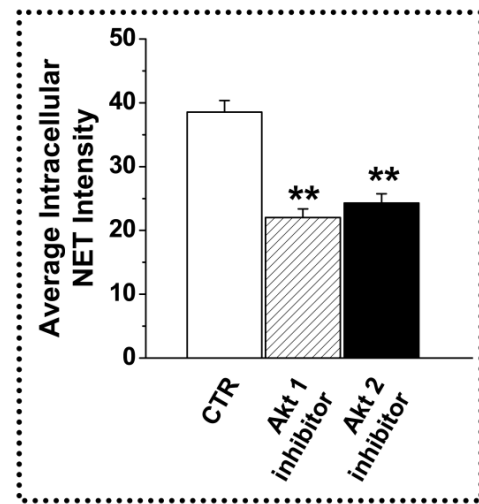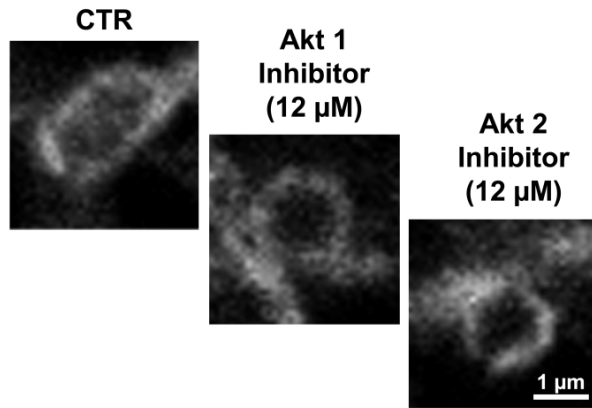
that antipsychotics, such as clozapine, influence the phosphorylation status and activity of Akt in the cortex (Emamian et al. 2004; Kang et al. 2004; Roh et al. 2007). Our evidence suggests that alterations in the phosphorylation status of Akt correlate well with changes in NET surface expression. Thus, we determined if *in vivo* i.p. injections of clozapine are capable of altering both the phosphorylation state of Akt at ser473 as well as surface levels of NET. Mice received 30 mg/kg i.p. single injections of clozapine and one hour after injection the mice were sacrificed and cortical slices were taken and biotinylated to examine both Akt phosphorylation and surface levels of NET. As expected, clozapine treatment significantly enhanced phosphorylation of Akt at ser473 in the cortex while total levels remained unaltered (Fig. 14A). Importantly, this increase in Akt phosphorylation is accompanied by a significant decrease in the levels of surface NET (Fig. 14B). Thus, activation of Akt through receptor signaling distinct from that of insulin alters the dynamics of NET surface expression.

## Discussion

Through its re-uptake of synaptic NE and other monoamines such as DA, NET is pivotal for maintaining the integrity of monoaminergic signaling in the brain and periphery (Iversen 1971; Pacholczyk et al. 1991; Xu et al. 2000; Moron et al. 2002). NET function is intricately controlled not only through regulation of transporter activity but also through the dynamic trafficking of the transporter to and from the plasma membrane (Apparsundaram et al. 1998) (Apparsundaram et al. 2001; Sung et al. 2003; Wersinger et al. 2006; Dipace et al. 2007). Importantly, single nucleotide polymorphisms in NET that
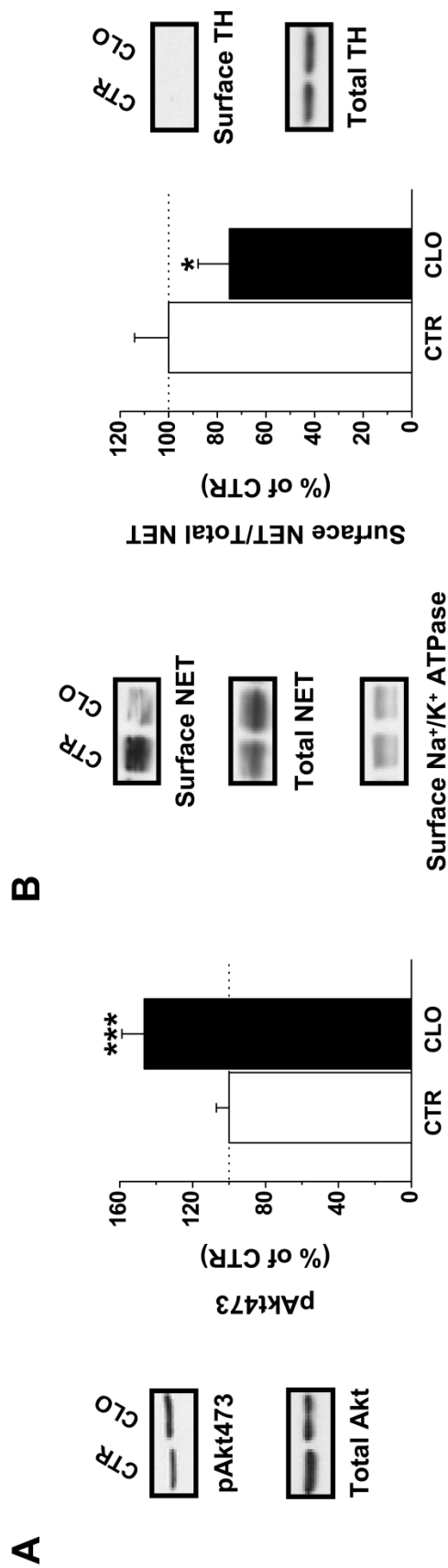
**Figure 14.** *In vivo* treatment with clozapine, an atypical antipsychotic, activates Akt and significantly reduces NET surface levels in mouse cortical slices. **A**, Mice are injected *in vivo* with either vehicle (CTR) or clozapine (i.p. 10 mg/kg for 1 hour) (CLO). (Left) Representative Western blot of pAkt473 and total Akt levels from cortical slices of either CTR or CLO mice. (Right) Quantification of pAkt473 levels from cortical slices of multiple animals (mean ± s.e.m, ***P<0.001 by Student's t-test; N=21). **B,** Following *in vivo* clozapine injection and cortical slice preparation, slices are immediately biotinylated. (Left) Shown is a representative Western blot of NET surface and total levels from CTR and CLO mice. Surface Na$^+$/K$^+$ ATPase levels serve as a loading control. (Center) A quantification of NET surface levels normalized to total NET levels and represented as percent of control (mean ± s.e.m, *P<0.05 by Student's t-test; N=21). (Right) Cytosolic TH is absent in the biotinylated fraction.

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impact surface availability have been shown to be directly linked to both central and peripheral nervous system disorders, such as depression, ADHD, orthostatic intolerance, and blood pressure abnormalities (Halushka et al. 1999; Hahn et al. 2003; Hahn et al. 2005; Haenisch et al. 2008; Hahn et al. 2009). Thus, the ability of abnormal NET surface expression to impact mental health and autonomic function makes it imperative that we understand how dysregulation of signaling pathways linked to these disorders impacts NET function. Notably, aberrant Akt signaling has been implicated in diseases such as diabetes and obesity, which have a high co-morbidity with monoamine related mental disorders, as well as with the pathology of schizophrenia and depression (Mukherjee et al. 1989; Mukherjee et al. 1996; Hsiung et al. 2003; Emamian et al. 2004; Lustman et al. 2005; Zhao et al. 2006; Karege et al. 2007; Krishnan et al. 2008). Here, our goal was to characterize how changes in Akt signaling, regulates NET function in the nervous system.

We demonstrate for the first time, that insulin and Akt signaling are capable of fine tuning NET surface levels and function in both the central and peripheral nervous systems. We show that acute insulin treatment significantly decreases NET surface availability in mouse hippocampal slices, at sites of synaptic NE release (SCGN boutons), and in heterologous cells. Moreover, our studies demonstrate that Akt is required for insulin regulation of NET. Consistently, *in vivo* manipulation of insulin signaling in the periphery via STZ treatment, results in aberrant Akt signaling in the hippocampus, and this deficit correlates with enhanced NE levels, NET surface expression, and NE clearance. The aberrant NE levels and NET regulation in STZ-induced diabetic mice provides a strong proof of principle, that metabolic dysfunction in

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the periphery has the potential to impact NET function and monoamine homeostasis in the brain. These data provide one plausible avenue to explain the depressive-like behavioral deficits observed in diabetic rodents (Hilakivi-Clarke et al. 1990). STZ-induced diabetic mice undergo physiological changes beyond hypoinsulinemia, and therefore we cannot exclude the possibility that these changes in NET expression are exclusively due to insulin deficits. Still, our *in vitro* data strongly indicate a direct role for insulin in regulating NET surface levels. Furthermore, the ability of acute insulin treatment in STZ hippocampal slices to restore NET surface levels and phosphorylation of Akt to control levels, as well as its ability to restore NET function in STZ animals *in vivo* supports our notion that hypoinsulinemia underlies these alterations. Altogether, these data provide convincing support for an inhibitory role of insulin/Akt signaling in the regulation of NET function, and importantly, this regulation of NET in the brain can be altered by abnormal insulin signaling in the periphery.

Interestingly, this down regulation of NET in response to insulin is opposite to the effect of insulin signaling in the regulation of the dopamine transporter (DAT). For example, early studies showed that *in vivo* manipulation of insulin levels via STZ treatment and food deprivation results in decreased DAT mRNA and DAT-mediated DA uptake respectively (Figlewicz et al. 1996; Patterson et al. 1998). More recent studies confirm these original observations and substantial evidence supports the notion that insulin/PI3K/Akt signaling is critical for maintaining basal levels of DAT at the surface (Carvelli et al. 2002; Galici et al. 2003; Garcia et al. 2005; Owens et al. 2005; Zhen et al. 2006; Wei et al. 2007; Williams et al. 2007; Lute et al. 2008; South et al. 2008). This ability of insulin to differentially regulate DAT and NET surface expression is intriguing

given the high homology of the two transporters, and thus speaks to the specificity of insulin signaling in the regulation of monoamine homeostasis.

In addition, to describing the nature of insulin's regulation of NET we also establish a novel role for Akt in controlling NET surface availability. We show that Akt inhibition, independent of insulin, in heterologous cells and SCGN boutons results in enhanced NET surface expression. Indeed, overall, our studies show that NET surface expression correlates well with the phosphorylation status of Akt at ser473. Therefore, Akt is not only required for insulin regulation of the transporter, but manipulation of Akt alone impacts NET surface availability. Distinct signaling pathways such as receptor tyrosine kinase (RTK), integrin, and G-protein coupled receptor (GPCR) signaling converge on Akt. Thus, divergent signaling pathways have the potential to similarly impact NET function via Akt signaling as a final common pathway. Indeed, here we demonstrate that similar changes in NET surface availability can be induced by either stimulation of insulin signaling or manipulation of GPCR signaling with an antipsychotic. The ability of the atypical antipsychotic clozapine, which can target α2-adrenergic receptors present on presynaptic noradrenergic terminals, to enhance Akt phosphorylation at ser473 is well documented in the cortex (Ashby et al. 1996; Emamian et al. 2004; Kang et al. 2004; Karkoulias et al. 2006; Roh et al. 2007). We show that i.p. injection of clozapine not only increases Akt ser473 phosphorylation, but also, similar to acute insulin treatment, it stimulates a subsequent decrease in NET surface expression. These data substantiate the hypothesis that completely distinct signaling pathways which converge on Akt have the potential to similarly influence NET availability and function. In addition, the ability of clozapine to manipulate the surface availability of NET could be

important for its enhanced efficacy in the treatment of cognitive symptoms associated with schizophrenia (Woodward et al. 2005). Indeed, a number of clinical trials utilizing NET specific inhibitors are ongoing and promise to reveal if NET specific inhibition will prove effective in treating schizophrenia cognitive symptoms such as poor concentration and memory deficits (clinicaltrials.gov).

The importance of the discovery of Akt as a potent regulator of NET is further emphasized when one considers the wealth of information supporting a role for Akt dysfunction in the development of mental disorders such as schizophrenia and depression. A link between Akt and schizophrenia was first described in (Emamian et al. 2004). The authors revealed decreased levels of Akt protein in lymphocytes and post-mortem brain tissue from schizophrenic patients along with evidence for a genetic association between schizophrenia and an Akt haplotype. Since these original studies numerous reports substantiate an association between Akt dysfunction and schizophrenia (Norton et al. 2007; Arguello et al. 2008; Dawn et al. 2008). In particular, human genetic and imaging studies corroborate a relationship between an Akt1 variant associated with schizophrenia and disruptions in DA-associated behaviors linked to the disorder (Tan et al. 2008). These studies indicate a possible link between Akt dysregulation, monoamine function, and predisposition to schizophrenia. Correspondingly, our studies provide one molecular mechanism by which Akt dysfunction has the potential to impact monoamine homeostasis, via its regulation of NET, a protein critical for controlling both global NE homeostasis and cortical DA homeostasis (Yamamoto et al. 1998; Moron et al. 2002). Thus, future studies investigating the impact of schizophrenia-linked Akt1 variants on the noradrenergic system (i.e. NET expression, NE homeostasis) and ultimately NE-related

behaviors will strengthen the growing link between aberrant Akt function, monoamine homeostasis, and schizophrenia. Interestingly, related studies from our laboratory in mice show that ablation of phosphorylation of Akt at Ser473 in neurons results in dramatically enhanced NET surface expression as well as behavioral and biochemical phenotypes that are hallmark characteristics of schizophrenia (Siuta and Robertson et al. *in press* at *PLoS Biology*).

Whereas a role for abnormal Akt signaling in schizophrenia is well supported, studies supporting a role for Akt in depression are just beginning to appear. For example recent studies reveal deficits in Akt activity in the PFC post-mortem tissue of depressed individuals (Hsiung et al. 2003; Karege et al. 2007). Interestingly, social defeat induced depression in mice has also been shown to depend on a decrease in Akt activity (Krishnan et al. 2008). Thus impairments in Akt activity may sustain depression. In addition, chronic antidepressant treatment in both mice and humans results in enhanced levels of phosphorylated Akt (Krishnan et al. 2008) which our studies correlate with decreased surface NET. Interestingly, chronic antidepressant treatment has also been shown to result in a down regulation of NET (Kitayama et al. 2006; Song et al. 2008; Jeannotte et al. 2009; Jeannotte et al. 2009). Whether this antidepressant-stimulated decrease in surface NET is Akt dependent, however remains to be determined. Thus, increases in NET cell surface expression could underlie the etiology of depression. Indeed, one NET SNP F528C that is associated with major depression has enhanced membrane expression of the transporter along with deficits in trafficking of the transporter away from the plasma membrane (Hahn et al. 2005; Haenisch et al. 2008).

Given the widespread influence of NET on monoamine homeostasis in both the CNS and PNS, and the evidence supporting a link between NET dysfunction and disease, substantial effort must be invested into enhancing our understanding of NET trafficking and regulation. Here, we show that Akt signaling is key for fine tuning NET surface availability. As our picture of NET regulation becomes more complete, so too will our ability to create unique approaches for treating disorders such as, depression, schizophrenia, drug addiction, and ADHD.

## Materials and Methods

*Cell culture and transfection*

We gratefully acknowledge Dr. Randy Blakely for the gift of CHO cells stably transfected with HA tagged human NET (hNET cells). The cells were maintained in Ham's F12 Media/10% FBS/L-Glu/pen/strep. Cells were plated on poly-D-lysine (Chemicon/Millipore, Billerica, MA)-coated plates for each experiment and incubated for 24 to 48 h before each experiment. Transient transfections with Akt-KD, a kinase dead (catalytically inactive) construct of Akt with the lysine at residue 179 replaced with an arginine (AKT-K179R) (Garcia et al., 2005), was graciously provided by Dr. R. Roth Stanford University (Stanford, CA). Transfections were performed with Fugene6 according to manufacturer's instructions (Roche), and experiments on transfected cultures were performed 48 hours following transfection. Mouse superior cervical ganglion neurons (SCGNs) were cultured according to Savchenko *et al*. (Savchenko et al. 2003; Matthies et al. 2009). Briefly, superior cervical ganglions were dissected from 1-3 day old C57BL/6J mice and dissociated for 30 minutes in 3 mg/mL collagenase/0.5

mg/mL trypsin, followed by 10% FBS in DMEM. Cells were plated and incubated with 3% fetal bovine serum in UltraCulture medium containing NGF for 2 hrs at 37° C to allow fibroblasts to adhere. Supernatant medium (containing SCG cells) was centrifuged, resuspended in medium supplemented with FBS, and cultured on poly-D-lysine- and collagen-coated glass-bottom plates for 14 days before experiments (treating with 1 μM 5-fluoro-5-deoxyuridine after 24 hrs).

*Antibodies and other reagents*

Monoclonal anti-mNET (NET-05) and monoclonal anti-hNET (NET 17-1) from MAb Technologies, Stone Mountain, GA (Matthies et al. 2009)), monoclonal anti-Na+/K+ ATPase α subunit (Developmental Studies Hybridoma Bank, Iowa City, IA), anti-Tyrosine Hydroxylase (MAB5280, Millipore/Chemicon, Billerica, MA), anti-ERK ½ (V114A, Promega, Madison, WI), anti-Akt (9272, Cell Signaling, Danvers, MA), anti-phospho-Akt (ser473) (4060, Cell Signaling) were used at dilutions of 1:200, 1:1000, 1:50, 1:1000, 1:5000, 1:1000 and 1:1000, respectively, for immunoblotting. Detection was obtained with secondary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) and by enhanced chemiluminescence reaction (ECL Plus Western Blotting Detection System, RPN2133, GE Healthcare). Monoclonal anti-mNET (NET-05 MAb Technologies, Stone Mountain, GA (Matthies et al. 2009)), anti-Rab11a (Lapierre et al. 2001), anti-Akt1 (2938, Cell Signaling), anti-Akt2 (D-17, sc-7127, Santa Cruz Biotechnology), anti-insulin Rβ (C-19, sc-711, Santa Cruz Biotechnology) were used at dilutions of 1:500, 1:110, 1:400, 1:100, and 1:50 for immunocytochemistry. Fluorescent secondary antibodies included highly cross-absorbed anti-mouse, goat, or rabbit IgG

(Molecular Probes/invitrogen, Carlsbad, CA). Insulin from bovine pancreas (I6634), clozapine (C6305), NE, and STZ (S0130) were obtained from Sigma (I6634 St. Louis, MO). Akt1/2, Akt1, and Akt2 inhibitors (Lindsley et al. 2005) were generously provided by Dr. C. Lindsley (Vanderbilt University, Nashville, TN).

*Cell and slice surface protein biotinylation*

Biotinylation experiments were performed on intact cells as described previously (Sung et al. 2003; Garcia et al. 2005; Dipace et al. 2007). Briefly, hNET cells were plated at a density of 1 x 106 per well in a six-well poly-(D-lysine) coated plate. Cells were serum starved for 30 minutes to 1 hour prior to treatment, and washed with cold PBS containing $Ca^{2+}/Mg^{2+}$. Then, cells were incubated with 1.0 mg/mL sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate [NHS-SS-biotin] (Peirce/ThermoScientific, Rockford, IL) for 30 minutes, washed, quenched with 100 mM glycine, and extracted in lysis buffer (PBS Ca2+/Mg2+, 1% Triton 100-X, and 0.5 mM PMSF at 4°C). Lysates were centrifuged, total fractions reserved, and supernatants incubated with immobilized streptavidin beads (Pierce/ThermoScientific) for 1 hr at room temperature. Beads were washed three times in lysis buffer, and bound proteins eluted with 2X sample buffer containing 2-mercaptoethanol. Proteins were separated by SDS-PAGE and immunoblotted. For estimation of relative amounts of proteins, the exposed films of the immunoblots were scanned, and band intensities were measured with Scion Image (Scion Corporation, Frederick, MD). For brain slice preparation and biotinylation all procedures were performed according to Vanderbilt University Institutional Animal Care and Use Committee approved procedures. Brain slices were prepared from C57BL/6J 15 to 20-

week-old male mice from Jackson Laboratories that were anesthetized with isoflurane and rapidly decapitated. Following, removal the brain was chilled in oxygenated 4°C sucrose solution (sucrose 210 mM; NaCl 20 mM; KCl 2.5 mM; MgCl2 1 mM; NaH2PO4•H2O 1.2 mM), and then while in sucrose solution 300 μm coronal slices were made using a vibratome. Slices were then collected in oxygenated artificial cerebral spinal fluid (ACSF) (NaCl 125 mM, KCl 2.5 mM, NaH2PO4•H2O 1.2 mM, MgCl2 1 mM, CaCl2•2H2O 2 mM). For *in vitro* drug treatments slices were then allowed to recover for 1 hour at 37°C in oxygenated ACSF and were then followed by drug treatment (i.e. insulin) and subsequent biotinylation. For *in vivo* treated animals (STZ or Clozapine experiments), slices were immediately washed twice with oxygenated 4°C ACSF following collection, and then incubated with 4°C ACSF solution containing 1mg/mL of EZ-Link Sulfo-NHS-SS-Biotin (Pierce/ThermoScientific; Rockford, IL.) for 45 min. After biotin incubation, the slices were rinsed twice quickly and for two 10min washes in oxygenated 4°C ACSF. The reaction was quenched by washing twice for 20min each with oxygenated 4°C ACSF containing glycine. Following quenching, slices were frozen on dry ice and the hippocampus was cut out and frozen at -80°C until used. For each experiment, a minimum of 4 animals were used for the collection of slices per treatment group, and a single slice was utilized for each sample (represented in the text as "N"). Single slices were lysed in 1% Triton buffer (25 mM Hepes, 150 mM NaCl, 2 mM Sodium orthovanadate, 2 mM NaF, plus a cocktail of protease inhibitors). Lysates were then centrifuged at 17,000g for 30 min at 4°C. After isolation of supernatant 0.1% Triton pulldown buffer (25 mM HEPES, 150 mM NaCl, 2 mM Sodium orthovanadate, 2 mM NaF, plus a cocktail of protease inhibitors) was added. Total protein was taken and the

samples were processed for protein concentration determination using Bio-Rad's protein assay and spectrometry at 595 nm Biotinylated proteins were then isolated using ImmunoPure immobilized streptavidin beads (Pierce/ThermoScientific) overnight at 4°C with agitation. Beads were washed three times with 0.1% Triton pulldown buffer and biotinylated proteins were then eluted in 50 μL of 2X SDS-PAGE sample loading buffer at 95°C and then room temperature. Total slice lysates and the biotinylated (slice surface) fraction underwent immunodetection for NET, pAkt473, total Akt, $Na^+/K^+$ ATPase, and TH as described previously.

*Immunostaining*

SCG neurons were either serum starved for one hour (insulin treatment) or non-starved (Akt inhibitor treatment) in DMEM:F12 and treated with vehicle, insulin, Akt1, or Akt2 inhibitor for 20 or 60 minutes respectively. Slices were obtained as previously described (cell and slice biotinylation methods). Slices and neurons were subsequently fixed with PBS $Ca^{2+}/Mg^{2+}/4\%$ paraformaldehyde, washed three times with PBS $Ca^{2+}/Mg^{2+}$, permeabilized and blocked with PBS/4% bovine serum albumin (BSA)/0.15% Tween-20, and immunostained with the appropriate antibody dissolved in PBS plus 4% BSA and 0.05% Tween-20. Primary antibodies were visualized with the appropriate covalently Alexa-labeled secondary antibody from Molecular Probes. Immunofluorescence was imaged using a Perkin Elmer UltraView confocal with a Nikon Eclipse 2000-U microscope equipped with a 60X lens with N.A.=1.49, or an Olympus FV 1000 using a 60X lens of N.A.=1.45 (VUMC Cell Imaging Shared Resource). Image processing was performed using Image J and Adobe Photoshop.

*Analysis of NET internalization*

The quantitation of NET intracellular accumulation was achieved using pixel intensity plots of a single confocal section along a line through the center of each bouton using ImageJ. The line intersects the brightest spot on one side of the widest portion of the bouton perimeter. The line was extended beyond the limits of the bouton to generate a background value, and then divided into 20 bins (bin # 10 is approximately at the center of the bouton) for the pixels spanning the bouton. NET fluorescence intensity of each bin was normalized to the fluorescence intensity of the brightest spot on the bouton perimeter (100%) and the mean ± SEM pixel intensity of each bin plotted.

*Colocalization analysis*

Only images in which there was no pixel saturation were analyzed. Background fluorescence was first subtracted in ImageJ by selecting an unstained area of each image and running the background subtraction plugin available at UHN research facilities (CA). The intensity correlation quotient (ICQ) was then determined by running the intensity correlation analysis (ICA) plugin for ImageJ developed by Tony Collins and Elise Stanley (Toronto Western Research Institute), also available at the above link. The ICQ indicates whether the intensity of staining for two proteins varies in synchrony over space. An ICQ value of +0.5 means that in any pixel with a certain intensity of staining for one protein, the intensity of staining for the other protein studied will be exactly the same, while an ICQ value of 0 signifies no relation between the two staining patterns. Consistently, an ICQ value of –0.5 indicates an inverse relationship for colocalization.

This method is based on the synchrony around which two signals vary in space (Li et al. 2004).

*Uptake assay*

hNET cells were seeded into 6-well plates 24-48 h prior to the experiment and grown to confluency. After 30 minutes of serum starvation, the cells were incubated with 10 nM insulin for 1 min (Sigma, St Louis, MO, USA) in KRH/glucose buffer at 37°C. The cells were then placed into 37°C KRH/glucose uptake buffer for 10 min (130 mM NaCl, 1.3 mM KCl, 10 mM Hepes, 1.2 mM MgSO4, 1.2 mM KH2PO4, 2.2 mM CaCl2, 10mM glucose, pH 7.4) containing, 100 μM ascorbic acid, 100 μm pargyline, and [$^3$H]DA (50 nM) for uptake and depending on the treatment group the uptake buffer also had vehicle, 10 nM insulin, or cocaine to measure non-specific uptake. Immediately following uptake, cells were washed in cold 4°C KRH/glucose buffer 3 times. All solution was removed and plates were allowed to dry on the 37°C plate. Once dry, cells were lysed with 1 mL of 0.01% SDS. Radioactivity was measured in a Beckman scintillation counter with UniverSol cocktail. Specific uptake was defined as total uptake minus non-specific uptake in the presence of 10 μM cocaine.

*STZ treatment*

All procedures were approved by the Vanderbilt University Medical Center and the University of Texas Health Science Center at San Antonio Institutional Animal Care and Use Committees and were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For all experiments, male C57BL/6J

15 to 20-week-old mice from Jackson Laboratories served as experimental subjects. STZ is an antibiotic that destroys the insulin-secreting beta cells of the pancreas and has previously been used to induce chronic hypoinsulinemia in rats by our laboratories (Galici et al. 2003; Owens et al. 2005). STZ (Sigma- Aldrich;) was freshly dissolved in ice-cold 100 mM citrate saline (pH 4.5) for all studies. Mice received STZ (200 mg/kg, i.p. for biochemical and high-speed chronoamperometry (HSCA) studies) and were returned to their home cages for 7-10 days. Weight and blood glucose were measured prior to injection and post injection. Blood glucose was measured with a glucometer (Advantage Accu-Chek, Roche Diagnostics;) before STZ, at 48 hours after injection and at animal sacrifice. Animals were considered hypoinsulinemic when their blood glucose levels exceeded 300 mg/dl.

*Tissue extraction for neurochemistry*

The brain sections were homogenized in 100-750 ul of 0.1M TCA, containing 10-2 M sodium acetate, 10-4 M EDTA, 5ng/ml isoproterenol (as internal standard) and 10.5 % methanol (pH 3.8). Samples were spun in a microcentrifuge at 10000 g for 20 minutes. The supernatant was removed and stored at −80 degrees (3). The pellet was saved for protein analysis. Supernatant was then thawed and spun for 20 minutes. Samples of the supernatant were then analyzed for biogenic monoamines and/or amino acids. Biogenic amines were determined by a specific HPLC assay utilizing an Antec Decade II (oxidation: 0.5) electrochemical detector operated at 33° C. Samples of the supernatant were injected using a Water 717+ autosampler, twenty onto a Phenomenex Nucleosil (5u, 100A) C18 HPLC column (150 x 4.60 mm). Biogenic amines were eluted with a mobile

phase consisting of 89.5% 0.1M TCA, 10-2 M sodium acetate, 10-4 M EDTA and 10.5 %

methanol (pH 3.8). Solvent was delivered at 0.6 ml/min using a Waters 515 HPLC pump.

Using this HPLC solvent the following biogenic amines elute in the following order:

noradrenaline, MHPG, adrenaline, DOPAC, dopamine, 5-HIAA, HVA, 5-HT, and 3-MT

(2). HPLC control and data acquisition are managed by Millennium 32 software.

*High-speed chronoamperometry (HSCA)*

HSCA is an electrochemical recording technique, which affords the kinetics of

NE clearance to be measured *in vivo*. Detailed methods are published elsewhere (Daws &

Toney, 2007; Daws et al. 2005). Mice were anaesthetized by intraperitoneal injection (2

ml/kg body weight) of a mixture of chloralose (25 mg/kg) and urethane (250 mg/kg)

followed by tracheal intubation and placement into a stereotaxic frame. Body temperature

was maintained at 36-37°C and blood oxygen levels monitored (MouseOximeter,

StarrLifeSciences, USA) and maintained above 90%. A Nafion-coated carbon fiber

electrode was attached to a glass micropipette containing NE. The assembly was lowered

into the brain region of interest (stereotaxic coordinates in mm: DG region of

hippocampus AP -1.6 to -1.7; ML 0.5; DV -1.8 to -2.0) and NE pressure ejected to

achieve concentrations at the recording electrode ranging from about 0.2–4.0 μM. High-

speed chronoamperometric recordings were made using the FAST-12 and FAST-16

systems (Quanteon, USA). Oxidation potentials consisted of 100-ms pulses of +0.55 V.

Each pulse was separated by a 900-ms interval during which the electrode potential was

maintained at 0.0 V. Voltage at the active electrode was applied with respect to a

Ag/AgCl reference electrode positioned in the extracellular fluid of the ipsilateral

superficial cortex. Electrode placement was confirmed by making an electrolytic lesion at the recording site at the conclusion of the experiment.

*Statistical analysis*

All data are expressed as the mean ± s.e.m. Mean differences between groups were determined using t-tests or one and two-way ANOVAs followed by post hoc tests when the main effect or interaction was significant at $P < 0.05$. Statistical analyses were conducted using software from Graph-Pad Prism. The number of animals and specific statistical analyses used in each experiment are indicated in the figure legends and/or text.

DYSREGULATION OF THE NOREPINEPHRINE TRANSPORTER SUSTAINS

CORTICAL HYPODOPAMINERGIA AND SCHIZOPHRENIA-LIKE BEHAVIORS

IN NEURONAL RICTOR NULL MICE**

**Abstract**

**Background**: The mammalian target of rapamycin (mTOR) complex 2 (mTORC2) is a multimeric signaling unit that phosphorylates protein kinase B/Akt following hormonal and growth factor stimulation. Defective Akt phosphorylation at the mTORC2-catalyzed Ser473 site has been linked to schizophrenia. Although human imaging and animal studies implicate a fundamental role for Akt signaling in prefrontal dopaminergic networks, the molecular mechanisms linking Akt phosphorylation to specific schizophrenia-related neurotransmission abnormalities have not yet been described. Importantly, current understanding of schizophrenia suggests that cortical decreases in DA neurotransmission and content, defined here as cortical hypodopaminergia, contribute to both the cognitive deficits and the negative symptoms characteristic of this disorder. We sought to identify a mechanism linking aberrant Akt signaling to these hallmarks of schizophrenia.

**Methodology/Findings:** We used conditional gene targeting in mice to eliminate the mTORC2 regulatory protein rictor in neurons, leading to impairments in neuronal Akt

Ser473 phosphorylation. Rictor-null (KO) mice exhibit prepulse inhibition (PPI) deficits,a schizophrenia-associated behavior. In addition, they show reduced prefrontal dopamine (DA) content, elevated cortical norepinephrine (NE), unaltered cortical serotonin (5-HT), and enhanced expression of the NE transporter (NET). In the cortex, NET takes up both extracellular NE and DA. Thus, we propose that amplified NET function in rictor KO mice enhances accumulation of both NE and DA within the noradrenergic neuron. This phenomenon leads to conversion of DA to NE and ultimately supports both increased NE tissue content as well as a decrease in DA. In support of this hypothesis, NET blockade in rictor KO mice reversed cortical deficits in DA content and PPI, suggesting that dysregulation of DA homeostasis is driven by alteration in NET expression which we show is ultimately influenced by Akt phosphorylation status.

**Conclusions/Significance:** These data illuminate a molecular link, Akt regulation of NET, between the recognized association of Akt signaling deficits in schizophrenia with a specific mechanism for cortical hypodopaminergia and hypofunction. Additionally, our findings identify Akt as a novel modulator of monoamine homeostasis in the cortex.

<div align="center">

**Introduction**

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Mammalian target of rapamycin (mTOR) complex 2 (mTORC2) is one of two highly conserved protein kinases that are critical regulators of cell growth and metabolism. mTOR complex 1 (mTORC1) and mTORC2 are functionally distinct multiprotein complexes that are defined by their subunit composition, rapamycin sensitivity, and substrate selectivity. Raptor, mLST8, PRAS40, and mTOR comprise the rapamycin sensitive mTORC1 whereas the rapamycin insensitive mTORC2 contains

rictor, mSIN1, mLST8, and mTOR. Two key substrates of mTORC1 are S6K and 4E-BP, which are important regulators of translation, whereas protein kinase B, also known as Akt, is the primary substrate of mTORC2 (Sarbassov et al. 2005). Specifically, mTORC2 is the kinase responsible for phosphorylation of Akt at serine residue 473, one of two key phosphorylation sites (Sarbassov et al. 2005). Akt is an extensively studied kinase that has been implicated in numerous disorders such as diabetes, obesity, cancer, and mental disorders such as schizophrenia(Beaulieu et al. 2009). Post-mortem, imaging and genetic association studies in humans (Emamian et al. 2004; Dawn et al. 2008; Tan et al. 2008) reveal that Akt deficiencies are associated with schizophrenia. Genetic studies in rodents further corroborate the relationship between dysregulation in Akt signaling and disruptions in dopamine (DA)-associated behaviors linked to schizophrenia (Emamian et al. 2004; Lai et al. 2006).

Putative evidence for a role of defects in mTORC2 signaling in mental illnesses preceded the discovery of the mTORC2 complex itself. Indeed, lithium, used to treat bipolar disorder, stimulates phosphorylation of Akt at Ser473, the mTORC2 phosphorylation site (Chalecka-Franaszek et al. 1999). The link between mTORC2 signaling deficits and mental illness has been strengthened by work demonstrating that certain antidepressants (Krishnan et al. 2008), along with both typical (Emamian et al. 2004) and atypical antipsychotics (Lu et al. 2005) increase Akt Ser473 phosphorylation. Furthermore, findings of diminished Ser473 phosphorylation and/or activity in post-mortem brains of patients with schizophrenia (Zhao et al. 2006) and depression (Karege et al. 2007) potentially fortify the association between dysregulation of mTORC2-Akt signaling and development of psychiatric illnesses, although these findings may be

confounded by perimortem artifacts (Ide et al. 2006). Recent observations that blunted Ser473 phosphorylation occurs in lymphocytes derived from patients with schizophrenia and psychosis-prone normal individuals (Keri et al. 2009) also support the plausibility that mTORC2-Akt deficits are involved in schizophrenia.

While human imaging and animal studies implicate a fundamental role for Akt signaling in prefrontal DA networks, the molecular mechanisms linking mTORC2/Akt to schizophrenia-related neurotransmission abnormalities have been elusive (Lai et al. 2006; Tan et al. 2008). Importantly, models of schizophrenia suggest that cortical deficits in DA neurotransmission and content, defined here as cortical hypodopaminergia, contribute to both the cognitive deficits and the negative symptoms characteristic of this disorder (Davis et al. 1991). Consistent with this hypothesis, imaging studies reveal that genetic variation associated with low activity Akt alleles interact epistatically with catechol-O-methyltransferase (COMT), a gene responsible for degradation of prefrontal synaptic DA. Together, these interactions ultimately affect the fidelity of prefrontal networks in humans (Tan et al. 2008) by decreasing DA availability at prefrontal synapses (Lotta et al. 2002). Thus, a compelling hypothesis in schizophrenia is that impaired mTORC2/Akt signaling triggers aberrant regulation of DA homeostasis.

Termination of DA signaling at prefrontal synapses involves two mechanisms: degradation via enzymes including COMT, and clearance via the norepinephrine (NE) transporter (NET) (Gresch et al. 1995; Moron et al. 2002; Miner et al. 2003), which takes up both major brain catecholamines, DA and NE (Yamamoto et al. 1998; Moron et al. 2002). Interestingly, insulin administration, which stimulates mTORC2/Akt signaling, decreases NET transcription in brain, while hypoinsulinemia and decreased

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mTORC2/Akt signaling increases NET transcription (Figlewicz et al. 1996). Therefore, we hypothesized that dysregulation of mTORC2/Akt signaling may provide a mechanistic link to cortical hypodopaminergia. Specifically, we propose that reduced Akt activity mediates increased NET expression and increased DA clearance by noradrenergic neurons in cortex; a novel molecular mechanism that explains how Akt dysfunction contributes to a reduction in prefrontal dopamine.

To test this hypothesis, we have generated an animal model in which mTORC2/Akt signaling down-regulation is achieved by neuronal deletion of a key mTORC2 regulatory subunit, rictor. We used a Cre-lox strategy to restrict the genetic deletion to neurons and bypass embryonic lethality associated with whole body deletion (Shiota et al. 2006). The goal of the present study is to test how alteration in Akt signaling affects dopamine homeostasis in the prefrontal cortex.

## Results

*Rictor deletion attenuates Akt Ser473 phosphorylation*

Akt deficiency is mechanistically linked to prefrontal cortex abnormalities and schizophrenia-linked phenotypes in several mouse models (Lai et al. 2006), although a clear molecular mechanism for how Akt regulates cortical function remains elusive. Here, we investigate the dopaminergic consequences of abolishing Akt phosphorylation at Ser473 in neurons by utilizing the Cre/LoxP system to delete rictor specifically in neurons. Mice were engineered with a floxed rictor allele, as previously described (Shiota et al. 2006), and crossed with neuron-specific nestin gene (NES mice) Cre driver line. Validating our approach, rictor knockout mice (KO) lack rictor mRNA expression and

rictor protein expression in a gene-dosage dependent manner within the brain and cortex (Appendix Fig. B1; $P < 0.01$ and $P < 0.05$ respectively, by one-way ANOVA followed by Dunnett's test). Importantly for the current hypothesis, neuronal rictor deletion abolishes Akt phosphorylation at Ser473 within the cortex of rictor KO mice (Fig. 15a; ***$P <$ 0.001 by one-way ANOVA Dunnett's test) compared to FLOX (floxed allele(s) in the absence of Cre), NES (Cre allele in the absence of a FLOX allele), and heterozygous rictor neuronal knockout mice (HET). Phosphorylation of Akt at Thr308 (Fig. 15b) and total levels of Akt (Fig. 15c) within the cortex are not different among the genotypes, allowing a direct evaluation of the effects of Ser473 phosphorylation. Similarly, rictor deletion also abolishes Akt phosphorylation at Ser473 in other brain regions, such as the substantia nigra (SN)/ventral tegmental area (VTA) of rictor KO mice, while Thr308 phosphorylation and total levels of Akt are unaltered (data were normalized to control (FLOX) and reported as mean ± s.e.m., P values by Student's t-test; pAkt Ser473 FLOX=100 ± 13%, KO=6 ± 1%, $P < 0.001$; pAkt Thr308 FLOX=100 ± 13%, KO=89 ± 8%, $P = 0.49$; total Akt FLOX=100 ± 13%, KO=101 ± 10%, $P = 0.96$). Furthermore, total protein levels of mTOR in the cortex are not altered by neuron-specific rictor knockout (data were normalized to control (FLOX) and reported as mean ± s.e.m.; FLOX=100 ± 9%, NES=130 ± 12%, HET=118 ±12%, KO=116 ± 12%; $P = 0.32$ by one-way ANOVA followed by Dunnett's test).

*Neuronal rictor KO mice display sensorimotor gating deficits*

Pre-pulse inhibition (PPI) behavior has long been identified as a promising phenotype for translational studies of schizophrenia owing to the direct parallels in expression between rodent and human subjects. While PPI deficits are present in

**Figure 15**. Neuronal rictor deletion specifically abolishes Akt phosphorylation at Ser473 in the cortex. (**a**) Phosphorylation of Akt on residue Ser473 in the cortex. (**b**) Phosphorylation of Akt at Thr308 and (**c**) total Akt in the cortex are similar for all genotypes. Shown are mean ± s.e.m of optical densities as a percentage of FLOX control mice. Genotypes shown include animals expressing only nestin-CRE (NES), mice expressing two copies of the 'floxed' rictor allele (FLOX) only, heterozygous mice which express nestin-CRE and a single copy of the 'floxed' rictor allele (HET), and knock-out mice expressing both nestin-CRE and two copies of the 'floxed' rictor allele (KO). Total cortical protein extract was loaded in each lane. Representative immunoblots are shown, as probed with antibodies to phosphorylated Akt at Ser473 (a) Thr308 (b), total Akt (c), and actin to serve as a loading control. Samples n=9-16. ***$P<0.001$ One-way ANOVA.
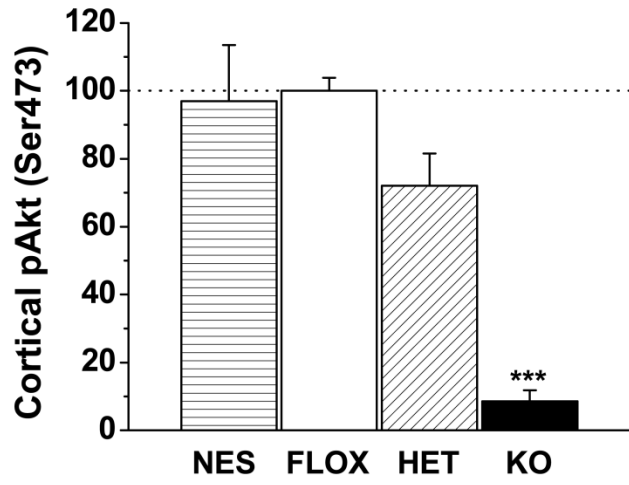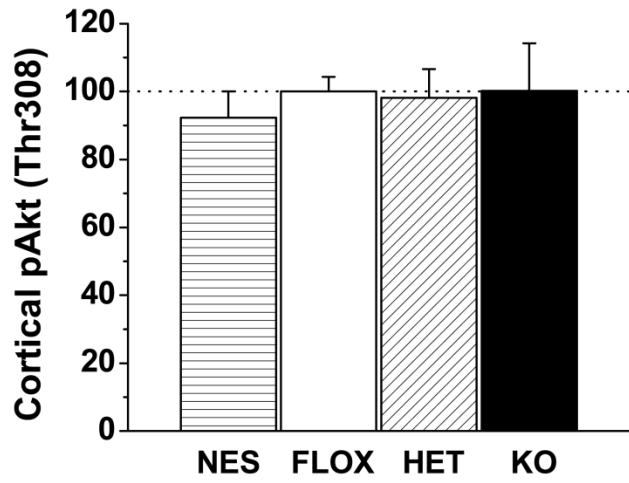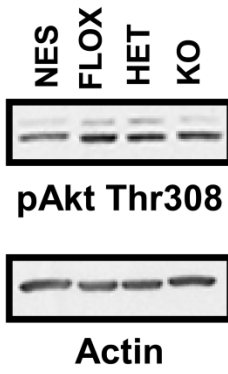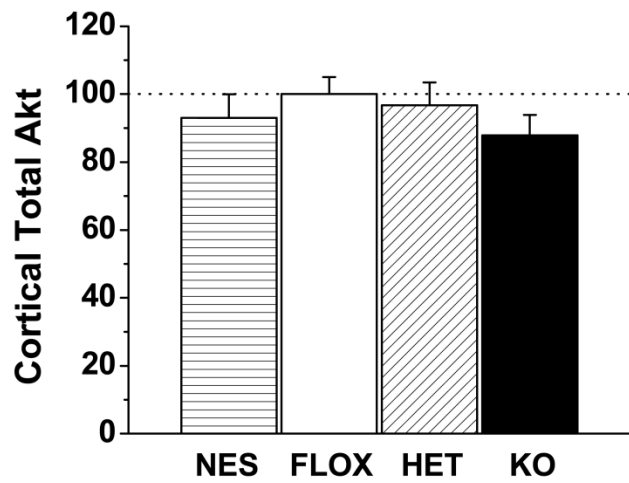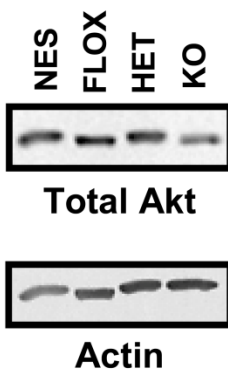
psychiatric disorders other than schizophrenia, they have a clear heritable component in schizophrenic families and these deficits can be attenuated by antipsychotic drug administration. Furthermore, PPI deficits are also linked to the hypofunction of corticostriatal forebrain circuits that is characteristic of schizophrenia (Powell et al. 2009). The PPI behavioral assay measures the degree to which the startle response elicited by a loud sound "pulse" sound is attenuated when immediately preceded by a non-startling "prepulse" sound. As such, it assays the degree to which a brief sensory trace can rapidly modify a subsequent motoric response, thereby representing a straightforward approach towards quantifying sensorimotor dysregulation, which is generally regarded as an endophenotype of schizophrenia. Since evidence suggests a role for Akt in schizophrenia, and rictor KO mice demonstrate profound deficits in Akt phosphorylation, we tested whether rictor KO mice display impaired PPI relative to FLOX control mice. No differences in startle responses elicited by a 94 dB sound pressure level (SPL) noise burst were observed, suggesting that hearing and gross motor function were similar between groups (Fig. 16a; Student's t-test; P = 0.44). By contrast, analysis of PPI behavior revealed clear differences between genotypes; startle reflex amplitude was inhibited at all prepulse intensities in FLOX control mice, with the amount of PPI increasing monotonically from 40 to 75 dB SPL (Fig. 16b). In rictor KO mice, prepulse sound levels between 40-55 dB did not appreciably attenuate the startle reflex. PPI was not observed in rictor KO mice until prepulse levels > 55 dB, albeit at a weaker level in comparison to FLOX mice. These differences gave rise to a significant reduction of PPI across prepulse sound levels (Fig. 16b; *P < 0.05 by ANOVA). This PPI deficit can also be expressed as a significant decrease in average PPI across all sound levels in
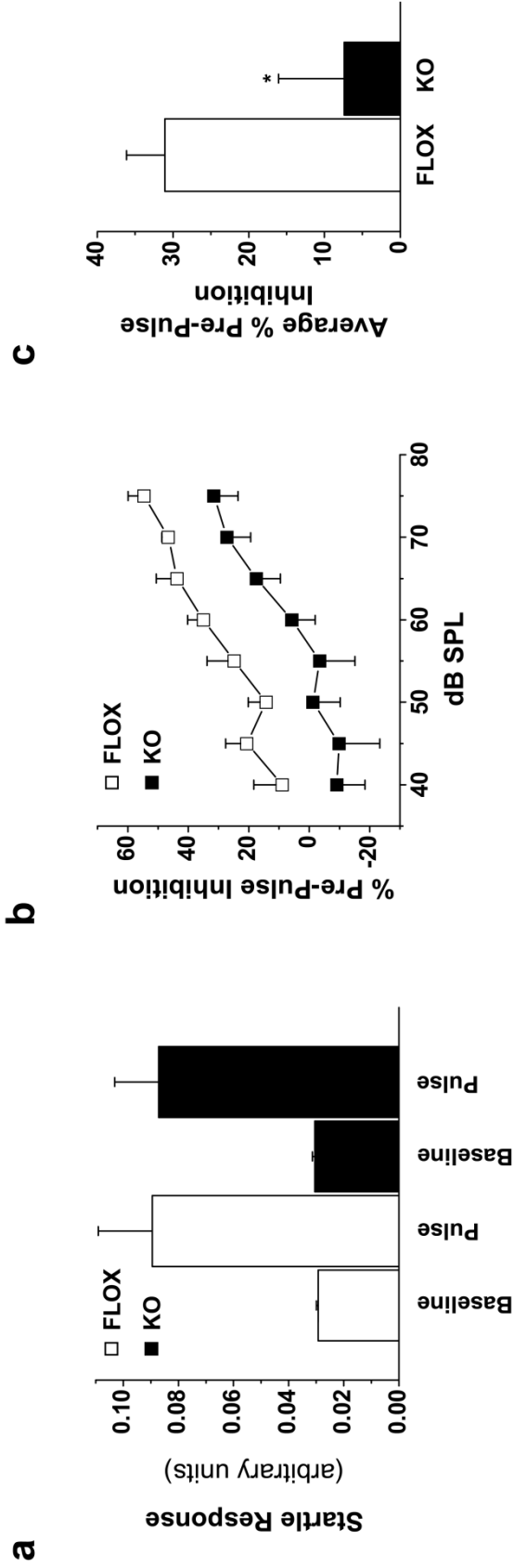
**Figure 16.** Neuronal rictor deletion results in sensorimotor gating deficits as assayed by PPI. (**a**) No difference in startle reflex elicited by a 94 decibel (dB) sound pressure level (SPL) noise was observed between rictor KO and littermate FLOX control mice. (**b**) The percentage of PPI was reduced significantly across the entire dB range in rictor KO mice. (**c**) The average PPI across the entire dB range reveals a significant deficit in PPI in rictor KO mice; n=6-7 animals. *P<0.05 Student's t-test. Special thank you to Daniel Polley for assistance.

rictor KO mice (Fig. 16c; Student's t-test, *P < 0.05). Thus neuronal rictor deletion, with loss of Akt Ser473 phosphorylation, impairs the forebrain circuits critically involved with sensorimotor integration. Given the strong evidence for PPI deficits in schizophrenic patients, we hypothesize that this mouse model has the potential to lend novel insight into the molecular mechanisms by which Akt deficits contribute to the schizophrenic phenotype.

*Rictor KO mice display hypodopaminergia in rostral cortex*

For almost fifty years, schizophrenia research has centered on dopaminergic signaling as a crucial component of the etiology of the disease (Delay et al. 1952; Carlsson et al. 1963; Seeman et al. 1975). In particular, the original "DA hypothesis" heavily supported the notion of excessive DA neurotransmission and DA content, defined here as hyperdopaminergia, within the brain (Matthysse 1973; Snyder 1976). Subsequent revision of the hypothesis, however, has transformed thinking from a global hyperdopaminergia to a regional hyperdopaminergia within the striatum and dopaminergic hypofunction within the cortex (Weinberger et al. 1988; Davis et al. 1991; Howes et al. 2009). While this conceptualization is an oversimplification of a highly complex disorder, we utilized this hypothesis to hone in on molecular mechanisms that contribute to cortical hypodopaminergia. Furthermore, previous studies have linked pre-frontal dopamine deficits with PPI deficits in animal models, and perhaps the PPI deficits observed in the rictor KO mice could be partially explained by alterations in cortical DA content (Koch et al. 1994; Swerdlow et al. 2006). Thus, we investigated steady state levels of dopamine (DA), serotonin (5-HT), and norepinephrine (NE) in a region of

mouse brain that is roughly analogous to the human prefrontal cortex and contains areas such as the intralimbic, prelimbic, and anterior cingulate cortex. Interestingly, HPLC with electrochemical detection of DA, NE, and 5-HT in the PFC of rictor KO mice revealed striking alterations in the DA and NE tissue content of these animals, while 5-HT levels remained unchanged (Fig. 17). Although both DA and NE levels are significantly different in rictor KO mice they change in opposite directions; NE tissue content is significantly increased (Fig. 17a; **$P < 0.01$ by one-way ANOVA followed by Dunnett's test) while DA levels are significantly decreased (Fig. 17b; Student's t-test, *$P < 0.05$). In addition, NES mice show similar PFC DA content levels (6.4±0.2 ng/mg protein) as FLOX mice. Thus, rictor KO mice display a key feature of the "dopamine hypothesis" of schizophrenia, namely hypodopaminergia in the rostral cortex, which may explain the sensorimotor gating deficits described earlier. Importantly, 5-HT levels are unaltered in the cortex (Fig. 17c; Student's t-test, $P=0.26$) indicating that rictor deletion does not simply result in global monoaminergic alterations but rather specific changes in the dopaminergic and noradrenergic systems. In addition, extracellular levels of DA were determined in the PFC of rictor KO mice by microdialysis. Under basal conditions, extracellular DA is not significantly different in rictor KO mice compared to FLOX controls (data are reported as pg of DA/µL, mean ± s.e.m.; FLOX=0.54 ± 0.15, KO=0.76 ± 0.18, n=4-5; $P = 0.35$ by Student's t-test). Whereas basal extracellular levels of DA are unaltered in rictor KO mice, these animals do display significant deficits in DA tissue content suggesting that maintenance of DA homeostasis is perturbed.

Although prefrontal hypodopaminergia has been linked to PPI deficits, other studies
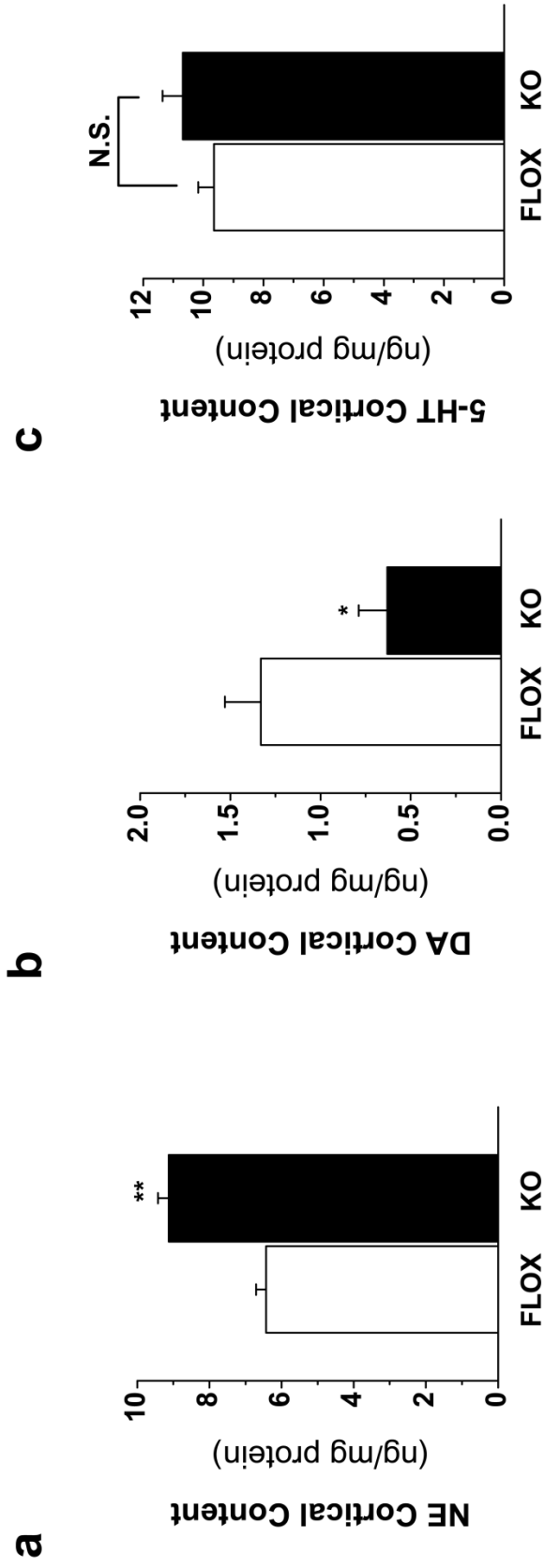
**Figure 17**. Monoamine content in the rostral cortex is significantly altered in rictor KO mice. Tissue content of (**a**) NE, (**b**) DA, and (**c**) serotonin (5-HT) in rostral cortical homogenates. Results are presented as mean ± s.e.m ng/mg of protein, n=4-10. *P<0.05; **P<0.01 Student's t-test.

clearly demonstrate a link between striatal hyperdopaminergia and PPI deficits (Ralph et al. 1999; Ralph et al. 2001). Thus, we sought to determine if rictor deletion increases tissue levels of DA in the striatum or in projecting DA neurons from the SN and VTA. DA levels in the SN/VTA are not altered in rictor KO mice (data are reported as ng of DA/mg protein, mean ± s.e.m.; FLOX=4.4 ± 0.5, KO=4.8 ± 0.4, P = 0.60 by Student's t-test). Importantly, similar to the cortex, DA tissue content in the striatum of rictor KO mice is significantly decreased (data are reported as ng of DA/mg protein, mean ± s.e.m.; FLOX=101.2 ± 7.7, KO=80.1 ± 3.4, *P < 0.05 by Student's t-test). Thus, our data indicate that the PPI deficits observed in rictor KO mice are likely to arise from impairments in DA neurotransmission.

*Rictor deletion increases NET expression and function*

It is intriguing that DA content is decreased while NE content is increased in rictor KO mice. Importantly, decreases in mTORC2/Akt signaling induced by hypoinsulinemia have been shown to increase NET transcription (Figlewicz et al. 1996). Moreover, early studies and unpublished data from our laboratory implicate deficits in Akt signaling with not only increases in NET transcription, but also with acute increases in NET cell surface expression (i.e. intact Akt signaling decreases NET availability at the plasma membrane) (Boyd et al. 1986; Figlewicz et al. 1993; Figlewicz et al. 1993; Figlewicz et al. 1996). Thus, we predict that altered DA homeostasis in rictor KO mice is due to changes in NET cell surface expression mediated by impaired Akt phosphorylation. Importantly, unlike other brain regions where DAT is the primary mechanism for removing DA from the synapse, in cortex DAT contributes relatively little

and NET performs the majority of DA clearance. Indeed NET has a higher affinity for DA than NE itself, but DA can also be degraded in the synapse by catechol-o-methyltransferase (COMT) (Moron et al. 2002; Miner et al. 2003). Given the pivotal role of rictor in Akt regulation and the role of Akt signaling in determining NET availability, we hypothesize that rictor KO mice will display aberrant NET regulation that sustains the alterations in NE and DA levels seen in the rostral cortex.

As hypothesized, total cortical NET protein is increased approximately two-fold in rictor KO mice compared to all other genotypes (Fig. 18a; ***$P < 0.0001$ by one-way ANOVA followed by Dunnett's test). Furthermore, biotinylation assays reveal that cell surface levels of NET are also significantly increased (Fig. 18b; Student's t-test, ***$P<0.0001$). Tyrosine hydroxylase (TH), a cytosolic protein, was detected exclusively in the total protein fraction but not in the surface fraction, indicating that the biotinylated fraction represents exclusively cell surface proteins. Finally, the striking enhancement in surface NET detected in rictor KO mice results in a significant increase in NET function as assayed by cortical synaptosomal NE uptake (Fig. 18c; Student's t-test, *$P<0.05$). The nearly two-fold increase in cortical synaptosomal NE uptake was also observed for DA (Fig. 18c; Student's t-test, *$P<0.05$) indicating that rictor deletion increases DA clearance by NET in noradrenergic neurons and as a consequence reduces cortical DA content. Furthermore, DA content is not decreased due to increased degradation since COMT levels were not different in cortex compared to FLOX control mice (data were normalized to control (FLOX) and reported as mean $\pm$ s.e.m.; FLOX=$100 \pm 8\%$, NES=$78 \pm 11\%$, HET=$130 \pm 24\%$, KO=$80 \pm 11\%$; $P = 0.10$ by one-way ANOVA followed by Dunnett's test). Thus, the increase in NET expression and function within the cortex of

**Figure 18**. Neuronal rictor deletion results in increased NET expression and function. (**a**) NET protein levels in the cortex. Mean $\pm$ s.e.m optical densities are shown as a percentage of FLOX control mice. Representative immunoblots are shown, as probed with antibodies to NET, and actin (loading control); n=10. (**b**) Levels of surface NET as measured from the biotinylated fraction of cortical slices. Mean $\pm$ s.e.m optical density is shown as a percentage of FLOX control mice. Representative immunoblots are shown, as probed with antibodies to NET, $Na^+/K^+$ ATPase to serve as plasma membrane/loading control (n=3-5), and TH which is absent in the biotinylated fractions since it is a cytosolic protein. (**c**) [$^3$H]NE and [$^3$H]DA uptake into cortical synaptosomes of FLOX and KO mice. Mean $\pm$ s.e.m uptake is shown as a percentage of uptake in FLOX control mice; n=12-18 *P<0.05; ***P<0.001 Student's t-test.
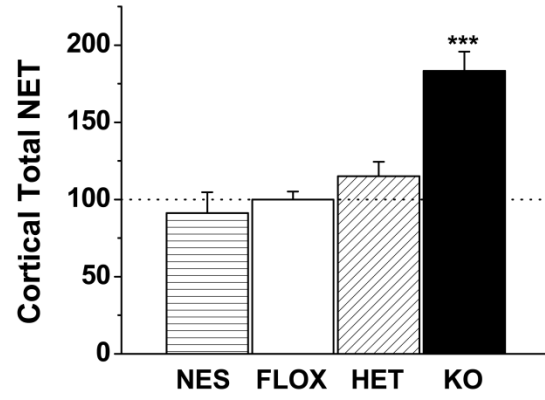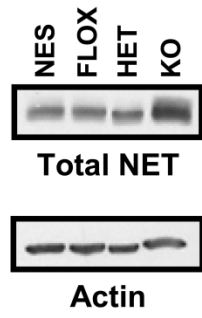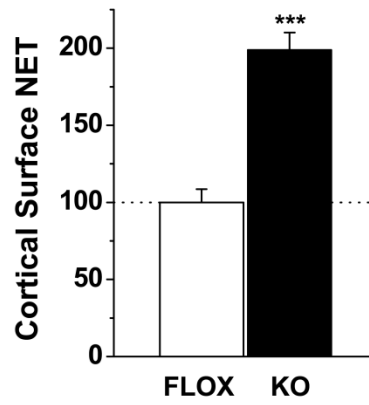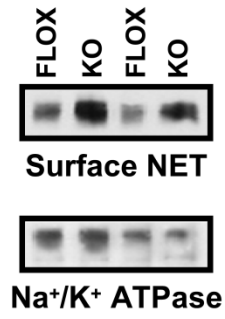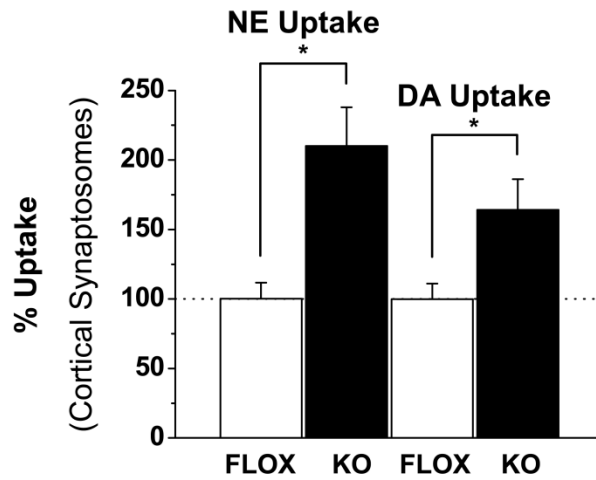
rictor KO mice has the potential to mechanistically explain both the increased NE tissue content and decreased cortical DA tissue content described earlier (Fig. 17). Interestingly, we did not find a significant difference in serotonin transporter expression within the PFC of the rictor KO mice, as measured by citalopram binding (data not shown).

While our data indicates that global neuronal mTORC2 dysfunction enhances NET function and induces cortical hypodopaminergia, we sought to demonstrate more specifically that these alterations could arise from downregulation of cortical Akt activity. Thus, we utilized the isoform specific Akt1 inhibitor (DeFeo-Jones et al. 2005; Lindsley et al. 2005; She et al. 2008) in cortical slices to show that Akt inhibition is capable of directly determining NET surface availability (Appendix Fig. B2). Surface levels of NET are significantly enhanced in biotinylated cortical slices treated with the Akt1 inhibitor (Appendix Fig. B2a; *P < 0.05 by Student's t-test). Importantly, the levels of Akt Ser473 phosphorylation are substantially diminished in samples of these inhibitor treated slices (Appendix Fig. B2b; ***P < 0.0001). Together, these data support the notion that Akt stimulated regulation of the transporter occurs not only at the level of transcription, as is seen in rictor KO mice, but also at the level of transporter trafficking. Furthermore, the ability of Akt inhibition to enhance NET surface expression in cortical slices indicates that all the molecular machinery necessary for this rictor/Akt regulation of NET is intact within the PFC, and thus is consistent with our hypothesis that altered cortical monoamine homeostasis via aberrant NET regulation underlies PPI deficits in rictor KO mice.

We hypothesize that amplified NET function in rictor KO mice enhances the accumulation of both NE and DA within the noradrenergic neuron leading to conversion

of DA to NE and ultimately supporting both increased NE tissue content and a state of hypodopaminergia. Such a mechanism within the prefrontal cortex provides an elegant molecular mechanism linking Akt hypophosphorylation to both cortical hypodopaminergia and PPI deficits, two key hallmarks of schizophrenia.

*Midbrain dopaminergic neurons and cortical monoaminergic projections are unaltered in rictor KO mice*

Considering the widespread function of Akt, and its role in cell growth and proliferation, we next sought to demonstrate that the changes in DA and NE levels within the cortex were specifically due to increased NET expression rather than global changes in the number or projections of dopaminergic and noradrenergic neurons. Although rictor KO mice do display a gross reduction in brain size, similar to what is seen for Akt3 deficient mice (brain weight normalized to body weight; FLOX $2.49 \pm 0.18\%$ compared to KO $1.63 \pm 0.10\%$; $P < 0.0005$ by one-way ANOVA followed by Dunnett's test), coronal brain sections stained for TH revealed no significant alterations in dopaminergic cell number within the VTA or SN (Fig 19a and 19c-d; VTA $P = 0.82$ by one-way ANOVA, SN $P = 0.53$ by one-way ANOVA). Furthermore, TH staining of dopaminergic and noradrenergic projections within the cortex do not reveal any gross alterations among the groups (Fig. 19b). The immunostaining was confirmed with western blot analysis of total cortical TH protein levels (Fig. 19e; $P = 0.20$ by one-way ANOVA). Other markers of dopaminergic neurons in the cortex were not significantly altered in the rictor KO mice such as total levels of D2 dopamine receptors (data were normalized to control (FLOX) and reported as mean $\pm$ s.e.m.; FLOX=$100 \pm 5\%$, NES=$95 \pm 13\%$, HET=$96 \pm 9\%$,
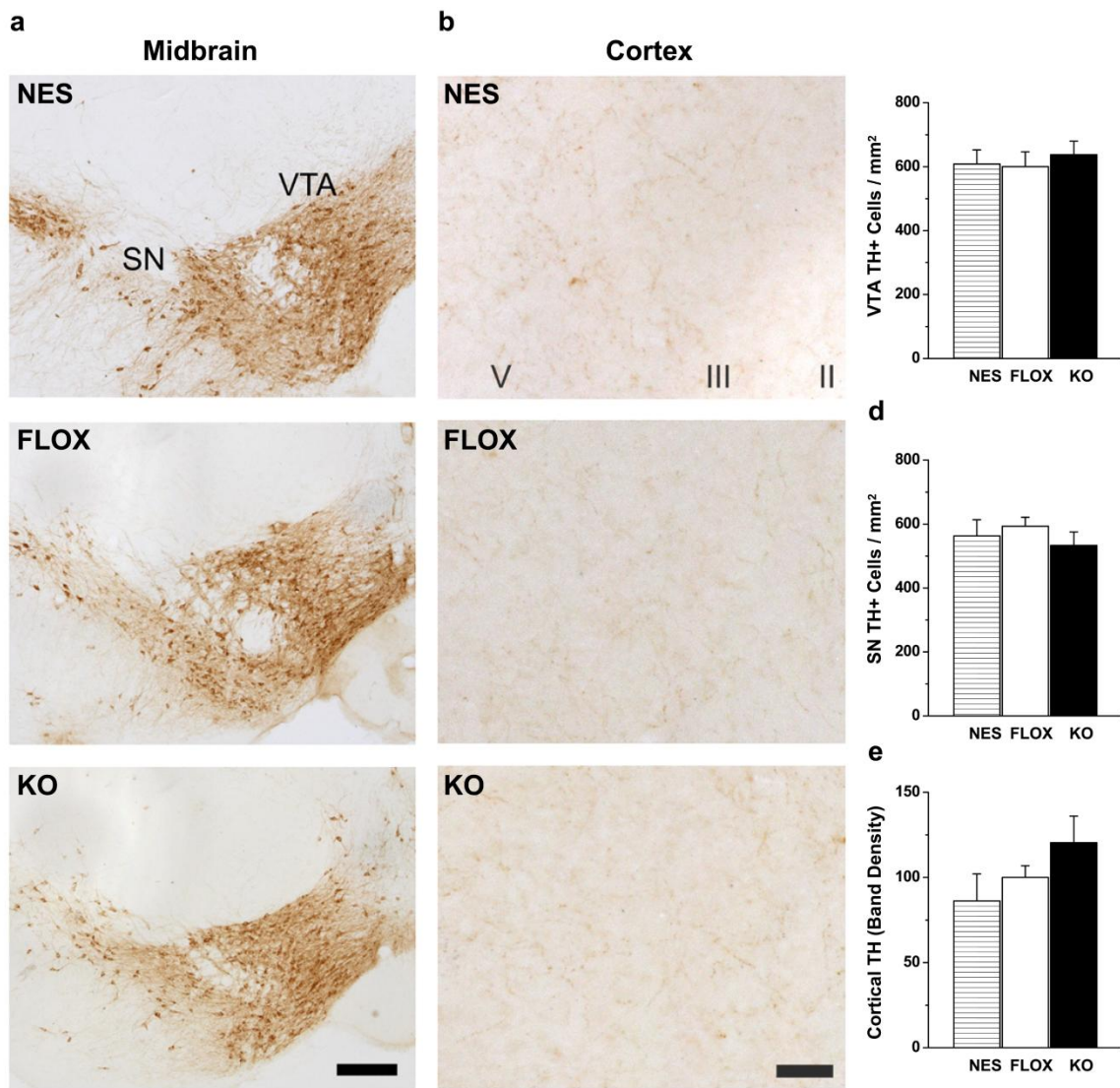
**Figure 19**. TH staining and expression in the midbrain and cortex is similar in NES, FLOX, and KO mice. TH immunoreactivity in the (**a**) midbrain substantia nigra (SN) and ventral tegmental area (VTA) and the (**b**) cortex. Scale bars = 50 µm. Coronal brain sections were stained with TH antibody and cell counts of TH$^+$ cells were taken. Cell counts are similar in NES, FLOX, and KO matched mice in both the (**c**) VTA and the (**d**) SN. Mean ± s.e.m TH$^+$ cells/mm$^2$ are shown; n=6. (**e**) TH protein levels in the cortex. Mean ± s.e.m optical densities are shown as a percentage of FLOX control mice; n=19-22. One-way ANOVA analysis reveals no significant difference between genotypes. Panels a-d courtesy of Gregg Stanwood.

KO=108 ± 18%; P = 0.85 by one-way ANOVA followed by Dunnett's test). These data demonstrate that the DA and NE systems in the rictor KO mice are not globally altered. Therefore, we propose that the changes in cortical NE and DA tissue content can be primarily accounted for by the specific enhancement of NET expression in noradrenergic neurons.

*NET blockade reverses the sensorimotor gating deficits and hypodopaminergia in rictor*

*KO mice*

We hypothesize that aberrant Akt phosphorylation in rictor KO mice results in enhancement of NET function within the cortex, resulting in changes in both DA and NE homeostasis. If this model is valid, then NET inhibition should reverse both PPI behavioral deficits and cortical deficits in DA tissue content. In order to test this hypothesis, we treated rictor KO mice with nisoxetine (NET specific blocker) or saline. Prior to treatment, both groups of rictor KO mice demonstrated comparable startle reflex amplitude similar to Fig. 16b (data not shown). The average PPI across all sound levels also were not different between the two rictor KO groups prior to treatment (Fig. 20a; Student's t-test, P = 0.95). Following the initial PPI trial, the rictor KO mice received i.p. injections of either saline or nisoxetine (30 mg/kg) and 30 minutes later began a second PPI trial. Nisoxetine reversed rictor KO PPI deficits compared to saline treated animals with a ~5 fold increase of average PPI (Fig. 20b Student's t-test, ***P<0.0001). Similar to FLOX control mice (Fig. 16b), nisoxetine treated rictor KO mice display startle reflex amplitudes that are inhibited by all prepulse intensities, with the amount of PPI increasing monotonically from 40 − 75 dB SPL (Fig. 20c). Importantly, saline treated animals

**Figure 20**. Nisoxetine restores PPI deficits and DA levels in the rostral cortex of rictor KO mice. (**a**) No differences are observed in the average %PPI across the experimental range of dB in KO mice prior to treatment with saline or nisoxetine. (**b**) Average %PPI 30 min after i.p. injection of either saline or nisoxetine (30mg/kg) in KO mice reveals a significant increase in %PPI in nisoxetine treated KO mice. (**c**) The mean PPI across all sound levels in KO mice treated with either saline or nisoxetine for 30 min; n=5-6, ***$P<0.001$ Student's t-test. (**d**) 8 days of nisoxetine treatment restores DA levels in the rostral cortex of rictor KO mice; n=4-6. *$P<0.05$ Student's t-test.
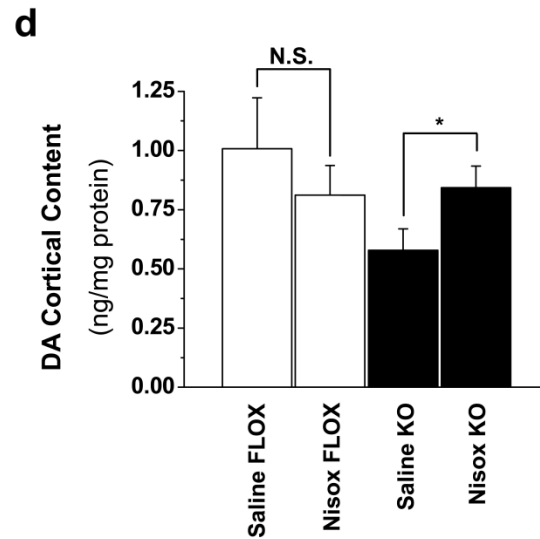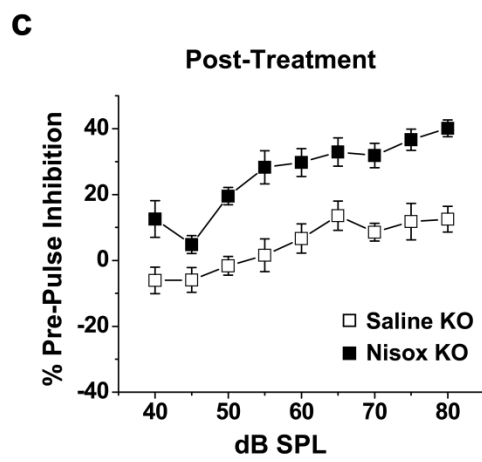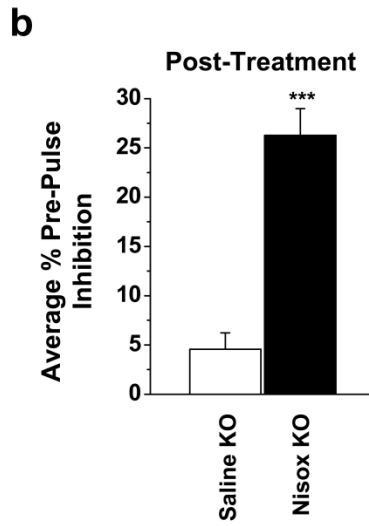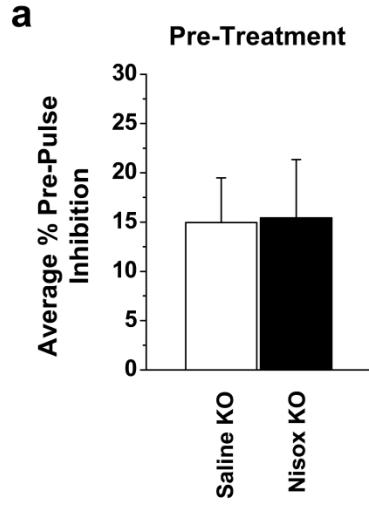
exhibited PPI at prepulse levels > 55 dB, albeit at a weaker level as compared to nisoxetine treated animals, consistent with Fig. 16b. Thus, nisoxetine treatment significantly reverses the PPI deficits observed in rictor KO mice (Fig. 20c; P < 0.0001 by two way ANOVA) providing support for our model of mechanistic linkage between Akt, NET, and cortical DA and NE homeostasis.

In addition to nisoxetine treatment, we sought to determine if traditional antipsychotics were capable of reversing the PPI deficits displayed in rictor KO mice. Unlike nisoxetine, acute clozapine treatment (i.p. 3 mg/kg for 30 minutes prior to PPI) did not rescue PPI deficits in rictor KO mice (data are reported as average percent PPI mean ± s.e.m. as in Fig. 16c; saline-KO 24.3 ± 8.6%, clozapine-KO 20.5 ± 6.4%; Student's t-test, P = 0.73). Interestingly, previous studies have shown that antipsychotic treatment enhances activity and phosphorylation of Akt at Ser473, and this increase is hypothesized to be important for the efficacy of such drugs (Emamian et al. 2004; Kang et al. 2004; Roh et al. 2007). Consistently, FLOX mice subjected to the same clozapine treatment as described above show significantly enhanced cortical phosphorylation of Akt at Ser473 (data are normalized to total levels of Akt and expressed as percent of control mean ± s.e.m.; saline-FLOX 100 ± 20%, clozapine-FLOX 215 ± 50%; *P < 0.05 by one way ANOVA). Importantly, the same treatment does not alter Ser473 phosphorylation of Akt in rictor KO mice (data are normalized to total levels of Akt and expressed as percent of control (saline treated FLOX) mean ± s.e.m.; saline-KO 5 ± 1%, clozapine-KO 5 ± 2%; P > 0.05 by one way ANOVA). Thus, the inability of clozapine to rescue PPI deficits in rictor KO mice may be partially due to the genetic neuronal deletion of rictor, which abolishes the ability of clozapine to enhance Akt Ser473

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phosphorylation in these animals. These data are consistent with the hypothesis that Akt deficits play an important role in the etiology of schizophrenia, and that perhaps some degree of antipsychotic efficacy is due to their ability to enhance Akt function.

The success of nisoxetine treatment in rescuing the PPI deficits in rictor KO mice was consistent with our model and led us next to determine if NET inhibition could normalize prefrontal cortex DA tissue content. In this experiment, rictor KO and FLOX control mice received either nisoxetine (30 mg/kg) or saline injections i.p. daily for eight consecutive days and were euthanized 30 min following their last i.p. injection. DA levels were measured in the rostral cortex by electrochemical detection. Similar to the results seen in Fig. 17, saline treated rictor KO mice had reduced DA tissue content levels in comparison to saline treated controls (Fig. 20d; Student's t-test, *$P<0.04$) and 8 days of nisoxetine treatment significantly enhanced DA content (Fig. 20d; Student's t-test, *$P<0.03$) while the same treatment in FLOX control mice did not have a significant impact on DA levels (Fig. 20d; Student's t-test, $P = 0.21$). Moreover, chronic nisoxetine treatment does not significantly alter cortical NE tissue content in either FLOX control or rictor KO mice (data are reported as ng of NE/mg protein, mean ± s.e.m.; FLOX-saline=5.7 ± 0.4, FLOX-nisoxetine=6.5 ± 0.5, Student's t-test, $P = 0.29$; KO-saline=8.3 ± 0.7, KO-nisoxetine=8.6 ± 0.4, Student's t-test, $P = 0.74$). These data further support the model that enhanced NET expression alters DA content in the rictor KO mice, and indicate that hypodopaminergia (and PPI deficits) can be rectified with NET specific inhibition *via* nisoxetine. The observations that chronic nisoxetine administration rescues DA content and acute treatment reverses impaired PPI are consistent with a model whereby impairment of PPI is due to decreased intra-synaptic DA in rictor KO mice. Our

data suggest that reduced intra-synaptic DA content could arise from enhanced clearance of DA via NET.

NET specific inhibition with nisoxetine has been utilized to rectify PPI deficits in some animal models of schizophrenia (Yamashita et al. 2006). Only a small number of studies, however, have investigated the utility of NET specific inhibition in schizophrenic patients with a particular focus on symptoms related to cortical hypofunction, and the efficacy of such treatment is still controversial (Kelly et al. 2009). Currently, a number of additional clinical trials with substantial sample sizes are ongoing and promise to reveal if NET specific inhibition is therapeutic in schizophrenia, especially for cognitive symptoms such as poor concentration and memory (clinicaltrials.gov).

## Discussion

The "dopamine hypothesis" for schizophrenia has enjoyed a resurgence of interest, and increasing evidence links Akt to DA related behaviors, yet key molecular mechanisms linking Akt to DA homeostasis have been elusive. Given evidence for a role of Akt in the regulation of NET expression and function in cortex and evidence that NET transports DA in this brain area, it has been our priority to develop a compelling experimental model to uncover a molecular link between Akt and cortical DA homeostasis. Here, we conducted the first studies, to our knowledge, in an animal model where a genetic deletion that disrupts Akt phosphorylation enhances expression of NET and leads to a cortical hypodopaminergic phenotype with schizophrenia-linked behavioral consequences.

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Our data are consistent with pathophysiological models of schizophrenia that emphasize "hypofrontality" of DA systems. Given the role of NET in DA clearance in prefrontal synapses (Carvelli et al. 2002; Moron et al. 2002; Miner et al. 2003), Akt-linked changes in NET expression may thereby translate to cognitive deficits and negative symptoms (Davis et al. 1991). These data, as well as our proof-of-principle results in mice, lead to the compelling hypothesis that NET inhibition would have therapeutic potential to selectively enhance DA tone in the prefrontal cortex and perhaps alleviate negative symptoms. Consistent with this reasoning, several clinical trials are currently investigating NET blockers for cognitive deficits in patients with schizophrenia.

Our data demonstrate that neuronal mTORC2 dysfunction is sufficient to generate cortical hypodopaminergia and schizophrenia-linked behaviors. In particular, we show that genetic mTORC2 disruption impairs PPI, a schizophrenia-linked phenotype, which has been validated in genetic mouse models of the disorder (Powell et al. 2009). An emerging body of evidence associates Akt phosphorylation deficits with mental illness in humans, giving our findings of impaired Akt phosphorylation in mice more translational viability (Emamian et al. 2004; Keri et al. 2009). For example, studies show diminished Akt1 protein content in lymphocytes of patients with schizophrenia (Emamian et al. 2004; Keri et al. 2009). In concert with our findings, candidate gene approaches aimed at identifying genetic variation in proteins associated with mTORC2/Akt signaling pathways, including rictor and other mTORC2 subunit proteins like mSin1 (Jacinto et al. 2006) may yield new insights into the genetic basis of mental illness.

Although DA is classically implicated in the pathogenesis of schizophrenia, concepts of neurotransmitter dysfunction in this disease process are constantly evolving.

This is reflected by popular glutamatergic and recently proposed revisions to dopaminergic hypotheses of schizophrenia (Howes et al. 2009). However, the role of elevated cortical NE, while not typically emphasized, should not be overlooked. Indeed, findings of elevated NE in CSF of patients with schizophrenia, which led to noradrenergic hypotheses of schizophrenia in the early 1980's (Lake et al. 1980), support our model by which increased cortical NET expression leads to increased NE content but decreased DA content. Although the sensitivity of these measurements to acute stressors made the reproducibility and reliability of these methods in the aforementioned studies questionable, a role for elevated NE in the development of schizophrenia-like phenotypes in humans cannot be completely ruled out. Indeed, our current findings, as well as others, intimately and mechanistically link DA alterations together with NE changes (Ventura et al. 2003). As a recent revision to the DA hypothesis of schizophrenia suggests, schizophrenia could be conceptualized as a disease where DA dysfunction is a "final common pathway" that can be elicited by a number of more proximal causes, including both genetic and epigenetic factors and disruption in other neurotransmitter systems (Howes et al. 2009), and including, as we propose here, increased NET function.

Schizophrenia is thought to arise from rather complex gene-environment interactions, and, therefore, acquired (rather than monogenetic) dysfunction in mTORC2/Akt signaling is a particularly intriguing mechanism. For example, acquired Akt defects are associated with impaired regulation of blood glucose and diabetes, which is over-represented in first episode, medication-naive patients with schizophrenia (Ryan et al. 2003). mTORC2/Akt signaling also provides a promising portal into "multiple hit" models of schizophrenia, as Akt is positioned to interact with other candidate genes such

as neuregulin-1 and COMT (Kanakry et al. 2007; Tan et al. 2008) as well as environmental risk factors for schizophrenia including obstetric complications and early life stressors (Krishnan et al. 2008; Nicodemus et al. 2008). The effects of neuronal mTORC2 dysfunction on NET expression observed in our study ultimately illustrates a potential molecular mechanism to link disparate genetic and environmental factors (i.e. obesity/diabetes/insulin resistance) to dysfunction in a putative "final common pathway" of schizophrenia, namely alterations in DA signaling (Howes et al. 2009). Indeed, while more remains to be learned about mTORC2, deficiencies in Akt signaling even in "healthy" individuals are associated with impaired prefrontal cortex activation on working memory tasks (Tan et al. 2008) and risk for psychosis, suggesting a subtle influence on brain function and behavior that may require other genetic and environmental hits to result in clinical disease. Thus, our data provide one molecular mechanism, NET regulation, towards a framework linking environmental stressors and/or lifestyle factors to mTORC2/Akt signaling and ultimately to DA dependent behaviors. Our studies provide a potential molecular mechanism linking Akt dysfunction to a schizophrenia-like phenotype and suggest the viability of targeting both Akt phosphorylation and NET as pharmacotherapies for schizophrenia.

## Materials and Methods

*Generation of mice*

      All mice were fully backcrossed to C57Bl6 background. Mice homozygous for an allele containing LoxP sites flanking exon 3 of the rictor gene (rictor f/f Nes-/-; FLOX) were crossed with neuron specific Nestin cre transgenic mice (rictorw/w Nes+/+; NES)

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obtained from Jackson Laboratories to create double heterozygous (rictorf/w NesCre+/-; HET) offspring (w is the wildtype allele). HET mice were then crossed to produce neuron specific rictor knockout mice (rictor f/f Nes+/+ or +/-; KO) and HET KO mice. Control animals were of the following genotypes (rictorf/f Nes-/- FLOX, rictorf/w Nes-/- and rictorw/w Nes+/+, Nes+/- or Nes-/-). Subsequent crossings between FLOX and HET mice were used to generate additional study animals. Genotyping was performed by PCR using DNA obtained from tail clippings with primers for the floxed, nestin and recombined alleles as described (Shiota et al. 2006).

*mRNA expression*

Total brain RNA was extracted from with Trizol reagent (Invitrogen). cDNA was synthesized with a High Capacity cDNA reverse transcription kit (Applied Biosystems). rictor mRNA was quantified with real time RT PCR on a Bio-Rad iCycler using iQ SYBR green Supermix reagent (Bio-Rad) and primer pairs as reported in (Shiota et al. 2006). Expression was normalized to levels of the housekeeping gene RPL13.

*Immunoblot analysis*

Mice were anesthetized with volatile isoflurane after which rapid decapitation allowed brain removal. Brains were chilled on ice and dissected under a microscope for either total cortex, rostral cortex, or caudal cortex. Tissue was homogenized and lysed in a Triton based buffer that contained a cocktail of protease inhibitors plus NaF (2mM) and sodium orthovanadate (2mM). After homogenization samples were centrifuged at 17,000Xg for 30 min, and the supernatant was collected and processed for protein

concentration determination. For western blotting, ≈30μg of protein per sample was run on a 10-12% acrylamide gel, transferred to a PVDF membrane blocked with 5% milk and incubated with primary antibodies to a variety of proteins. Akt, rictor, and mTOR antibodies were obtained from Cell Signaling. We also used actin (Sigma), Na+/K+ ATPase (DBH), NET (Mab Technologies Inc. NET05-2), and tyrosine hydroxylase (Chemicon/Millipore) antibodies. Secondary antibodies were obtained from Santa Cruz Biotechnology. After chemiluminescent visualization on Hyper-film ECL film, protein band densities were quantified and analyzed (Scion Image; http://www.scioncorp.com).

*Pre-pulse inhibition*

Startle responses were elicited with a 94 dB SPL broadband noise burst (2-75 kHz, 50 ms duration, 0 ms rise/fall time) and measured through a floor plate mounted on piezo force transducers (PCB Piezotronics). In two-thirds of the trials, the noise burst (pulse) was preceded by a prepulse (2.4-78.2 kHz frequency-modulated sweep, 40 octaves/s, 0.14 ms stimulus onset asynchrony) ranging from 40-75 dB SPL. Startle responses were measured across 297 trials (20 ± 6.4 sec intertrial interval, initial 27 trials discarded to minimize within-session habituation), as max − min of the force signal within 400 ms following onset of the noise burst. Presence of a normal startle response (significant difference between force plate amplitude -600 to -200 ms (baseline) vs. 0-400 ms (startle) from noise burst onset) was a prerequisite for subsequent analysis to minimize possible contributions of gross sensorimotor deficits in either genotype. This criterion excluded 2/8 WT and 0/7 rictor KO mice from further analysis. Inhibition of the

startle reflex was quantified for each prepulse intensity as PPI = 100 x ((pulse-alone) – (prepulse + pulse) / pulse alone).

*Tissue extraction for neurochemistry*

Brain sections were homogenized in 100-750 ul of 0.1M TCA, which contains 10-2 M sodium acetate, 10-4 M EDTA, 5ng/ml isoproterenol (as internal standard) and 10.5 % methanol (pH 3.8). Samples were spun in a microcentrifuge at 10000 g for 20 minutes, the supernatant removed and stored at –80 ºC. The pellet was saved for protein analysis. Supernatant was then thawed and spun for 20 minutes and then analyzed for biogenic monoamines and/or amino acids by a specific HPLC assay (Vanderbilt Neurochemistry Core). Biogenic amines were eluted in the following order: NE, MHPG, epinephrine, DOPAC, DA, 5-HIAA, HVA, 5-HT, and 3-MT (2).

*In vivo microdialysis*

Mice were anesthetized with isoflurane and placed in a stereotaxic frame using mouse-specific ear bars (Kopf Instruments, Tujunga, CA). A guide cannula (CMA7 microdialysis, USA) was placed above the medial prefrontal cortex (+2.0 AP, ± 0.7 ML from Bregma and -1.0 DV from skull for FLOX or NES mice and (+1.9 AP, ± 0.6 ML from Bregma and -1.0 DV from skull for rictor KO mice and secured to the skull with epoxy adhesive (Plastics one). Animals were allowed to recover from the surgery (1-3 days). The day before the experiment, animals were placed in individual dialysis chambers and the microdialysis probe (CMA7 microdialysis, USA) with the active length of 2 mm was inserted into the guide cannula. One end of a tether (Plastics One) is

attached to a harness and the other end attached to a swivel (Instech) that is mounted on a counterbalanced arm above the dialysis chamber. The probe was perfused overnight at a flow rate of 0.5 μL/min with artificial cerebral spinal fluid containing 149 mM NaCl, 2.8 mM KCl, 1.2 mM CaCl2, 1.2 mM MgCl2, 5.4 mM d-glucose, pH 7.2. On the day of the experiment the flow rate was changed to 1.0 μL/min and after equilibration dialysis fractions (20 min each) were collected to establish baseline concentrations of neurotransmitter efflux. Dialysate samples were stored at -80°C and analyzed by HPLC-EC for DA levels. Probe placement was verified after collection of slices by Nissl staining of coronal slices.

*Brain slice preparation and biotinylation*

Brain slices were prepared from 6- to 10-week-old mice that were anesthetized with isoflurane and rapidly decapitated. Following, brain removal the brain was chilled in oxygenated ≈4°C sucrose solution (sucrose 210mM; NaCl 20mM; KCl 2.5mM; MgCl2 1mM; NaH2PO4•H2O 1.2mM), and then while in sucrose solution 300μm coronal slices were made using a vibratome. Slices were then collected in oxygenated artificial cerebral spinal fluid (ACSF) (NaCl 125mM, KCl 2.5mM, NaH2PO4•H2O 1.2mM, MgCl2 1mM, CaCl2•2H2O 2mM). For *in vitro* drug treatments slices were then allowed to recover for 1 hour at 37°C in oxygenated ACSF (NaCl 125mM, KCl 2.5mM, 1nM insulin, NaH2PO4•H2O 1.2mM, MgCl2 1mM, CaCl2•2H2O 2mM)with either vehicle or Akt1 inhibitor and following recovery slices were then biotinylated. For non-treated slices, slices were immediately washed twice with oxygenated 4°C ACSF following collection, and then incubated with 4°C ACSF solution containing 1mg/mL of EZ-Link Sulfo-NHS-

SS-Biotin (Pierce/ThermoScientific; Rockford, IL.) for 45 min. Slices were then washed twice with oxygenated 4°C ACSF, and then incubated with 4°C ACSF solution containing 1mg/mL of EZ-Link Sulfo-NHS-SS-Biotin (Pierce Chemical; Rockford, IL) for 45min. After biotin incubation, the slices were rinsed twice quickly and for two 10 min washes in oxygenated 4°C ACSF. The reaction was quenched by washing twice for 20 min each with oxygenated 4°C ACSF containing glycine. Following quenching, slices were frozen on dry ice and the cortex was cut out and frozen at -80°C until used. Slices were lysed in 1% Triton buffer (25mM Hepes, 150mM NaCl, 2mM sodium orthovanadate, 2mM NaF, plus a cocktail of protease inhibitors) and centrifuged at 17,000g for 30min at 4°C. After isolation of supernatant 0.1% Triton pulldown buffer (25mM HEPES, 150mM NaCl, 2mM sodium orthovanadate, 2mM NaF, plus a cocktail of protease inhibitors) was added. Protein concentration was determined using Bio-Rad's protein concentration kit. Biotinylated proteins were then isolated using ImmunoPure immobilized streptavidin beads (Pierce) overnight at 4°C with agitation. Beads were washed three times with 0.1% Triton pulldown buffer and biotinylated proteins were then eluted in 50μL of 2X SDS-PAGE sample loading buffer at 95°C and then cooled to room temperature. Total slice lysates and the biotinylated (slice surface) fraction underwent immunodetection for NET (MabTechnoligies Inc. Stone Mountain, GA) as described previously.

*Synaptosomal uptake*

Mice were sacrificed by rapid decapitation and cortex dissected using an ice cold metal block and then homogenized at 400 rpm in at least 10 volumes (w/v) of ice-cold

0.32 M glucose with a Teflon-glass homogenizer (Wheaton Science Products, Millville, NJ). After centrifugation of the homogenate at 800g for 10 min at 4°C, the supernatant was again centrifuged at 10,000g for 15 min. The final pellet was gently resuspended in Krebs-Ringer HEPES (KRH) medium containing 118 mM NaCl, 4.8 mM KCl, 25 mM NaHCO3, 1 mM NaH2PO4, 1.3 mM CaCl2, 1.4 mM MgSO4, 10 mM glucose, 1 mM tropolone, 0.1 mM pargyline, and 0.1 mM ascorbic acid, pH 7.4 and protein concentration assayed. Aliquots (0.2 ml) of synaptosomal preparations (50 ng of protein) were prepared on ice. DA and NE transport assays (5 min at 37°C) were initiated by the addition of [3H]DA or [3H]NE (~100 Ci/mmol, Amersham) and were terminated by immediate filtration over 0.3% polyethylenimine-coated glass fiber filters using a cell harvester (Brandel Inc., Gaithersburg, MD). The filters were washed three times with 1.5 ml of ice-cold phosphate-buffered saline (PBS) and incubated overnight in Ecoscint H (National Diagnostics, Atlanta, GA). Radioactivity bound to filters was counted using a Beckman LS 6000 liquid scintillation counter. Nonspecific uptake, defined as the accumulation of [3H]DA or [3H]NE in the presence of 1 μM desipramine, was subtracted from total uptake values to obtain specific uptake values.

*TH immunohistochemistry*

Mice (n = 6/genotype) were anesthetized and transcardially perfused with 4% paraformaldehyde. Brains were removed and cryopreserved, and coronal sections were cut on a microtome at 40 μm and stained using minor modifications of published protocols. Sections were blocked in 4% Blotto (Nestlé Carnation dried milk), 0.2% Triton-X 100 in PBS and incubated at 4°C for 3 days with a monoclonal anti-tyrosine

hydroxylase antibody (Sigma, 1:4000). Sections were thoroughly washed and then incubated in biotinylated anti-mouse IgG (Jackson Immunoresearch, 1:1000) for 60 min. Avidin-biotin amplification (Vectastain ABC Standard, Vector Labs) and 3-3'-diaminobenzidine reactions were used to visualize labeled proteins. Sections from all genotypes were processed in parallel to minimize variability between groups. Negative controls in which primary antibody was omitted revealed no specific labeling.  Slides were coded so that the investigator was blinded to the genotype and sections were imaged using a Zeiss Axioskop microscope and Axiocam HR. TH-immunoreactive (IR) cells were counted in images obtained with a 40X objective in two fields per hemisphere derived from 2-3 sections per animal. Values were corrected for cell diameter, but no differences in cell diameter were found across genotypes.

*Statistical analysis*

All data are expressed as the mean ± s.e.m. Statistical significance between groups was determined using t-tests or one and two-way ANOVAs followed by post hoc tests when the main effect or interaction was significant at $P < 0.05$. Statistical analyses were conducted using software from Graph-Pad Prism. The number of animals and specific statistical analyses used in each experiment are indicated in the results and figure legends.

**CHAPTER IV**

DISCUSSION

Diseases characterized by peripheral insulin and Akt dysfunction such as diabetes and obesity are reaching epic proportions in the United States. Indeed, recent national health surveys reveal that approximately two-thirds of adults in the United States are overweight, and almost one-third of the adult population is obese which translates into 63.6 million people (www.niddk.nih.gov). This alarming increase in the waistline of the average American is reflected by similarly increasing numbers of obesity related disorders such as diabetes which is currently estimated to affect 7.8 percent of the population. On a more global level, these same trends are spreading rampantly as developing countries continue to adopt the 'Western lifestyle' of diminished exercise and overconsumption of high calorie, high fat food. As such, the already staggering 171 million people afflicted worldwide with diabetes in 2000 is projected to increase to 366 million by 2030 (Wild et al. 2004). This barrage of data on a growing global problem highlights the necessity for understanding how insulin related disorders impact our health.

In particular, a largely overlooked statistic is the substantial number of diabetic patients that suffer from monoamine related mental health disorders such as depression and schizophrenia. In fact, depression affects more than one quarter of the diabetic population, a level that is nearly 5 times higher than that in the general population (Gavard et al. 1993; Anderson et al. 2001; Lustman et al. 2005). While affliction with a life altering disorder such as diabetes may be a trigger for brain disorders, it is interesting

that a significant proportion of these individuals actually present with depressive symptoms several years prior to the onset of diabetes. The high co-morbidity between schizophrenia and diabetes is equally surprising. For example, a family history of type II diabetes is three times more likely in schizophrenic patients (Mukherjee et al. 1989; Mukherjee et al. 1996). Consistently, studies of glucose tolerance in first episode, drug naive (these individuals have never received antipsychotics) schizophrenics, have revealed significant impaired fasting glucose tolerance implying diabetes-linked metabolic disruptions prior to the onset of mental illness (Ryan et al. 2003). Thus, the increased prevalence of monoamine related mood disorders in the diabetic and obese populations insinuates a link between peripheral insulin signaling and brain function. In addition, studies revealing altered levels of monoamines in various animal models of diabetes and in humans with diabetes also support this notion (Chu et al. 1986; Lucas et al. 1989; Lackovic et al. 1990; Shimizu 1991; Barber et al. 2003).

Importantly, the primary source of brain insulin is from actively transported peripheral insulin across the blood brain barrier, thus aberrant alterations in peripheral insulin have the potential to significantly impact brain insulin signaling (Banks 2004). Insulin signaling in the brain has been shown to influence a variety of brain functions such as neurodevelopment, neurogenesis, neurotransmitter release, and importantly neurotransmitter reuptake (van der Heide et al. 2006). Of particular interest here, was to understand the role of insulin signaling in the regulation of monoamine reuptake, specifically *via* its regulation of NET. As such, the specific aims of this project were to:

I. Determine the role of insulin signaling in the regulation of NET in both the central nervous system (CNS) and the peripheral nervous system (PNS).

II. Identify components of the insulin signaling pathway that are required for the regulation of NET.

III. Study the physiological and behavioral consequences of manipulating this regulation *in vivo*.

Collectively, our studies from heterologous systems, the CNS, and the PNS demonstrate that acute insulin exposure diminishes NET mediated NE uptake by decreasing the surface availability of the transporter. These results were further extended by illustrations that severe chronic manipulations of peripheral insulin, as in the case of STZ-induced diabetes, significantly disrupt NET function and noradrenergic homeostasis. Support for functional consequences of these changes in monoamine homeostasis, comes from studies demonstrating behavioral deficits in STZ-induced hypoinsulinemic mice (Massol et al. 1989; Hilakivi-Clarke et al. 1990; Hirano et al. 2007; Alvarez et al. 2009). For example, these diabetic mice exhibit behavioral deficits particularly within the realm of monoamine related behaviors such as Porsolt's swim test, which is also held as an indicator of depressive symptoms in mice. Thus, our studies provide proof of principle that aberrant peripheral insulin can directly impact brain insulin signaling, subsequent monoamine homeostasis, and perhaps ultimately behavior. Correspondingly, this work provides implications for the high co-morbidity of insulin related disorders such as diabetes and obesity with noradrenergic related mood disorders such as depression.

While the data reported here supports a role for peripheral insulin in the regulation of brain monoamine homeostasis, STZ initiated depletion of insulin is a manipulation of body insulin that most closely resembles the untreated type I diabetic state in humans. Today, as noted previously, the staggering worldwide increases in diabetes is primarily of the type II state which is almost always secondary to substantial weight gain or obesity and is characterized by a state of excessive insulin and insulin resistance rather than insulin depletion. Thus, the consequences of this more subtle and slower in onset change in peripheral insulin on NET function and subsequent noradrenergic homeostasis remains to be determined. Indeed, studies in the lab are currently underway to determine if a model of high fat, diet-induced obesity in rats, that more closely resembles the growing obesity epidemic, can contribute to changes in monoaminergic signaling and behavior.

In addition to exploring the nature of insulin's regulation of NET, we also sought to identify specific components of the insulin signaling pathway that are critical for this regulation. Our data, utilizing both pharmacological inhibitors and overexpression of a dominant negative form of Akt, supports a requirement for Akt in the insulin regulation of NET. In addition, our studies demonstrate that Akt stimulation or inhibition, independent of insulin, also dynamically modulates NET surface levels thereby identifying Akt as a key regulator of the transporter. Indeed, the phosphorylation status of Akt at residue ser473 correlates remarkably well with the surface availability of the transporter. This identification of Akt as a potent regulator of NET function is particularly interesting when one considers the accumulating evidence for Akt dysfunction in various psychiatric disorders such as depression and schizophrenia. The

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newfound interest in Akt as a potential candidate gene for these monoamine related mood disorders is a rather recent development. In fact, the first study that provided substantial converging evidence for Akt dysfunction in schizophrenia did not appear until 2004 (Emamian et al. 2004). Here, the authors demonstrated a significant decrease in Akt1 protein levels in the peripheral lymphocytes and post-mortem brain tissue from individuals with schizophrenia. In addition, they also illustrated the ability of antipsychotics to increase Akt phosphorylation as well as provided evidence for a significant association between schizophrenia and an Akt haplotype associated with diminished protein levels. Since these original observations, numerous studies have followed suit and provided additional evidence for an association of Akt gene variants with schizophrenia (Ikeda et al. 2004; Schwab et al. 2005; Bajestan et al. 2006; Norton et al. 2007; Xu et al. 2007; Dawn et al. 2008). In addition to these reports, other patient studies have investigated the phosphorylation of Akt in response to activation of upstream signaling pathways. Indeed, Neuregulin 1-stimulated phosphorylation of Akt has been shown to be deficient in schizophrenics and other patients with psychosis (Keri et al. 2009; Keri et al. 2009). Similarly, substantial deficits in the insulin receptor (IR) signaling pathway have also been reported (Zhao et al. 2006). In this particular study, 12 schizophrenic patients had significant deficits in IR levels, IR phosphorylation, Akt levels, and phosphorylation of Akt at ser473 in the dorsolateral PFC. These findings are of particular interest when one considers our demonstration of correlations between Akt ser473 phosphorylation and NET surface levels. Perhaps, these individuals, with substantial IR signaling deficits may also have significantly enhanced NET function.

In conjunction with the substantial number of post-mortem human studies supporting a link between Akt dysfunction and schizophrenia, Akt deficient animal models as well as functional imaging studies on humans with low expressing Akt variants also support a role for this pathway in the pathology of the disorder. Indeed, work from the Weinberger lab connects Akt variation with disruption of schizophrenia-linked prefrontal-striatal monoamine related brain functions (Tan et al. 2008). While studies on Akt1 deficient mice also reveal deficits in PFC development and structure which the authors propose predisposes these mice to deficits in PFC related tasks (Lai et al. 2006). Importantly, while these studies link Akt dysfunction with specific aspects of schizophrenia pathology they fail to provide plausible molecular mechanisms by which this dysfunction may actually contribute to the disorder.

Given the role of Akt in the regulation of NET and the importance of the transporter in maintaining the fidelity of cortical dopaminergic and global noradrenergic signaling, we hypothesized that disruptions in Akt signaling *in vivo* may disrupt monoamine homeostasis and lead to subsequent behavioral deficits. Indeed, neuronal deletion of rictor, a vital component of the mTORC2 complex responsible for Akt ser473 phosphorylation, results in schizophrenia-linked behavioral deficits (i.e. abnormal PPI) as well as alterations in cortical monoamine homeostasis. In particular, these mice exhibit a pronounced increase in cortical NE tissue content as well as a significant diminishment of cortical DA tissue content. Interestingly, current conceptualizations of schizophrenia support a role for such cortical hypofunction and hypodopaminergia as is seen in these rictor KO mice. In addition, we provide evidence that these deficits arise due to aberrant increases in NET surface expression and function, given pharmacological inhibition of

the transporter restores both deficits. Thus, these data provide one plausible molecular mechanism, the anomalous Akt regulation of NET, by which Akt dysfunction may lead to specific impairments (i.e. PPI deficits and cortical hypodopaminergia) associated with schizophrenia.

Indications of Akt dysfunction in neuropsychiatric disorders, however, are not limited to the pathology of schizophrenia. In fact, a few, rather recent studies have implicated Akt dysfunction in depression, a disorder that is already heavily associated with noradrenergic dysfunction and in some instances specifically with abnormally functioning NET variants (Hahn et al. 2005; Haenisch et al. 2008; Hahn et al. 2008). For instance, significant reductions in Akt activity were found in the ventral PFC of both depressed non-suicide and depressed suicide victims but not in non-depressed suicide victims (Karege et al. 2007). Similarly, reductions in cortical PI3K and Akt activity were also reported by an independent group (Hsiung et al. 2003). Furthermore, the disrupted-in-schizophrenia 1 (DISC1) gene has been associated with major depressive disorder, and signaling through this pathway ultimately converges on and stimulates Akt activity (Hashimoto et al. 2006). Finally, studies in stress induced animal models of depression also reveal significant reductions in the levels of Akt activity and phosphorylation (Krishnan et al. 2008). Also, the authors demonstrate that these reductions in Akt activity are both necessary and sufficient to initiate depressive behaviors. To extend their studies further, the authors show that, like antipsychotics, chronic antidepressant treatment significantly increases Akt phosphorylation not only in their animal model but also in postmortem brain tissue from chronically treated depressed patients. Altogether, these studies supporting a link between Akt dysregulation and depression encourage further

investigation into the subject. For instance, are Akt1 variants associated with lower protein levels which have been linked to schizophrenia also associated with depressive disorders? Or, perhaps, depressive or anxiety related behaviors stand out in individuals possessing such Akt alleles. Similarly, do depressive/anxiety phenotypes express in Akt defective animal models such as the rictor KO mice? Answers to questions such as these, promise to offer new insight into the role Akt may play in depression.

Cumulatively, our data indicates that deficits in Akt signaling sustain abnormally elevated levels of NET surface expression and function. Given the recent identification of schizophrenic patients with Akt variants associated with low levels of protein, it would be interesting to determine if these specific individuals have altered NET levels *via* post mortem and PET imaging studies. Considering the heterogeneous nature of schizophrenia, such studies in general schizophrenic populations may yield little insight. However, narrowing the criterion for inclusion (i.e. only Akt1 variant possessing schizophrenics) in studies searching for changes in transporter levels/function may increase the chances of seeing through correlates from rictor KO mice to human studies. Similarly, examining these individuals for deficits in specific NE related functions such as attention, working memory, and anxiety may be advantageous. Another important consideration for Akt1 variant possessing individuals is the use of NET specific inhibitors for treatment especially for cognitive related symptoms. Although studies of NET specific inhibition for the alleviation of negative symptoms in schizophrenia are currently underway (www.clinicaltrials.gov), such studies may be aided by again narrowing the criterion for inclusion to only those individuals with Akt1 variation. Perhaps, this alternative treatment will prove more efficacious in individuals whom we would

hypothesize to have aberrant transporter regulation. A similar rationale could be applied for the antipsychotic clozapine, which targets numerous receptors beyond the D2DR and in particular impacts noradrenergic receptor signaling, and as we show here actually increases Akt activity and subsequently reduces NET surface levels. Interestingly, some, still debated, reports indicate that clozapine is especially effective at treating the hard to alleviate cognitive symptoms of schizophrenia. Perhaps, this increased efficacy may in part be due to this drug's ability to manipulate NET levels and thus presumably alter NE within the cortex which is crucial for optimal attention/cognition. Clozapine however, is not the only antipsychotic that increases Akt phosphorylation and activity (Kang et al. 2004; Lu et al. 2004; Lu et al. 2005; Roh et al. 2007), although whether these other antipsychotics are capable of impacting NET function/availability remains to be determined. Despite our focus on NET here, given the general implication of Akt dysfunction in schizophrenia and the fact that antipsychotics appear to combat these deficits, we ought not to forget evidence for Akt regulation of the DAT. Perhaps antipsychotics further impact the dopaminergic system beyond D2DR signaling by stimulating Akt regulation of the DAT.

As mentioned previously, our studies reveal a significant divergence in the Akt regulation of two fairly homologous transporters, the DAT and the NET. Indeed, for the DAT insulin appears to be crucial for transporter function by maintaining DAT levels at the surface while insulin/Akt stimulation decreases NET function and surface expression. The obvious question then becomes, how does this divergent regulation occur for two very similar transporters. Interestingly, similar paradoxical scenarios exist for the insulin regulation of other important CNS proteins, such as AMPA receptors. Indeed, for AMPA

receptors, depending on the developmental context, insulin will stimulate AMPA receptor internalization under one condition and exocytosis in another (Man et al. 2000; Plitzko et al. 2001; Zhao et al. 2004). As in our case, however, the reason for such divergent regulation is unknown. One plausible explanation for the differential regulation of NET versus DAT lies in the structural differences between the two transporters. Whereas the majority of NET and DAT is fairly homologous, the intracellular N- and C- termini of these transporters are not very well conserved (Gu et al. 2001). In addition, these regions are also the most heavily targeted areas of the transporter by intracellular signaling pathways for trafficking regulation. Thus, creating and examining insulin induced trafficking in a NET construct with the DAT's N-terminus rather than its own (or vice versa) may offer unique insight about the structural determinants which dictate the direction of insulin regulation. Another plausible explanation for the divergent Akt regulation of the monoamine transporters is the cellular context in which the signaling is initiated. In other words, insulin/Akt signaling in noradrenergic neurons may impact differential downstream targets in comparison to the Akt initiated signaling in dopaminergic neurons. Exogenous expression of NET in DAT KO dopaminergic neurons (or vice versa) could determine if indeed neuronal environment dictates the direction of transporter regulation. Along a similar rationale, insulin/Akt-induced NET trafficking correlates very well with phosphorylation status of Akt at ser473. Alternatively, ongoing studies in the lab indicate that insulin induced changes in DAT function correspond more so with the phosphorylation of Akt at residue thr308. Importantly, studies support the notion that Akt phosphorylation status dictates differential substrate specificity and thus activation of unique signaling pathways

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(Guertin et al. 2006; Jacinto et al. 2006). Consistent with this notion, ser473 phosphorylation of Akt is absolutely required for phosphorylation of the Akt substrate FoxO1/3a but not for GSK3 targeting. Interestingly, in support of this hypothesis, so far, changes in DAT expression in Akt ser473 deficient rictor KO mice are not nearly as striking as changes in NET expression and function in these animals. Thus, perhaps phosphorylated ser473 Akt initiated signaling pathways impact NET function more significantly than DAT. This hypothesis raises the question, of whether PDK1 KO mice, which would be deficient in thr308 Akt phosphorylation, would show substantial changes in NET expression. Such studies promise to uncover how seemingly similar monoamine transporters are so differentially regulated by insulin and Akt signaling.

Another interesting, yet overlooked aspect of this research is the evidence for a role of Akt in the regulation of NET transcription. Indeed, several early investigations into the insulin regulation of the transporter supported its ability to downregulate NET mRNA (Figlewicz et al. 1993; Figlewicz et al. 1996). In addition, our data involving chronic manipulation of insulin/Akt signaling also supports this notion. Both STZ induced hypoinsulinemic mice and Akt signaling deficient rictor KO mice exhibit significantly increased levels of total NET protein. This ability of Akt to initiate both trafficking and transcription renders the question, what downstream components instigate these two distinct aspects of transporter regulation? Although Akt is known to target several transcription factors, none of these known factors have been shown to impact NET transcription. Indeed, relatively few transcription factors for NET have been identified (i.e. Phox2, MeCP2, Slug) (Kim et al. 2006; Fan et al. 2009; Harikrishnan et al. 2010). Thus, investigations of Akt related changes in NET transcription would be a very

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new and exciting avenue of research. Similarly, the number of potential downstream targets for the Akt regulation of NET trafficking is staggering. Although insulin signaling regulates the availability of other membrane targeted proteins beyond the monoamine transporters, such as the glucose transporter, the epithelial Na+ channel (ENaC), the insulin receptor itself, and AMPA receptors, the majority of these examples are instances of insulin stimulated increases in protein surface levels. Those of which are examples of insulin initiated decreases in surface expression offer few parallels from our current knowledge of NET trafficking regulation. Thus, here to, an entirely new avenue of research is open ground to develop a complete understanding of how Akt initiates decreases in NET surface expression. As noted before, one potential starting point for both of these avenues of research is the striking correlation between Akt ser473 phosphorylation and NET regulation. Since phosphorylation status dictates differential substrate specificity, starting the search off with Akt ser473 specific substrates may narrow the vast pool of potential downstream components.

In conclusion, the data presented here demonstrates that peripheral changes in insulin and Akt signaling are capable of impacting transporter function, subsequent monoamine homeostasis, and ultimately behavior. Given the unprecedented rise in insulin related disorders worldwide, these data further necessitate our continued study of how disruptions in brain insulin signaling may impact mental health. Furthermore, independent of peripheral insulin disorders, Akt dysfunction in its own right has been implicated in a number of neuropsychiatric disorders associated with monoamine dysfunction. Here, we provide evidence that Akt dysfunction directly impacts monoamine homeostasis via its aberrant regulation of NET. Thus, providing one small

molecular insight into how Akt dysfunction may contribute to the pathology of mood disorders. More importantly, when trying to understand the pathological underpinnings of these monoamine related mental diseases, these studies remind us not to forget the neglected noradrenergic system and in particular the beloved NET. Indeed, prior experience has demonstrated that single mutations in the transporter are capable of instigating complex and devastating disease states (i.e. orthostatic intolerance). Thus, understanding transporter function and importantly transporter regulation at an intricate level promises only new insight and unique therapeutic opportunities in the realm of mental health research.
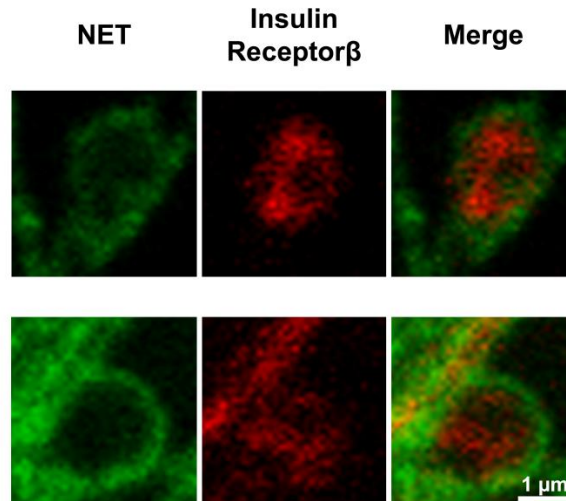
**Appendix Figure A1**. SCGN express the insulin receptor at NET enriched boutons. Mouse SCGNs were cultured and processed for immunocytochemistry as described in Material and Methods. Shown is a confocal image of a SCGN bouton co-stained for NET (green) and the insulin receptor $\beta$ subunit ($IR_\beta$) (red). The images depict a single bouton with associated axons (linear structures). The bouton is marked by NET immunoreactivity as well as $IR_\beta$ immunoreactivity.
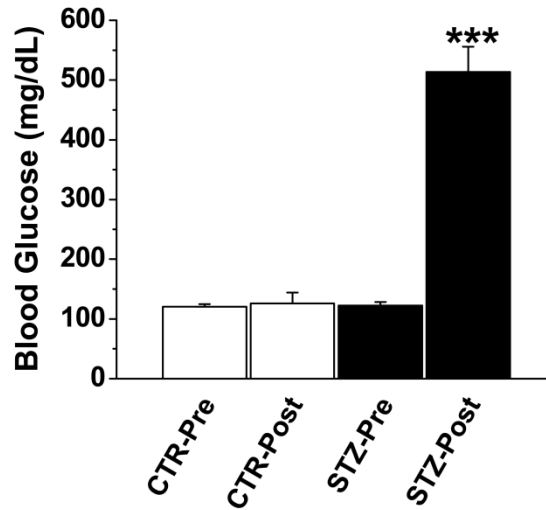
**Appendix Figure A2**. STZ treatment induces a significant increase in blood glucose levels. Male C57BL/6J 15 to 20-week-old mice from Jackson Laboratories receive either vehicle (CTR) or STZ (200 mg/kg, i.p.) injections and then are returned to their home cages for 7-10 days. Blood glucose levels are measured prior to injection and post injection. Blood glucose measurements of CTR versus STZ mice pre and post injection are represented. (mean ± s.e.m, ***P<0.001 by Student's t-test; N=8-7).
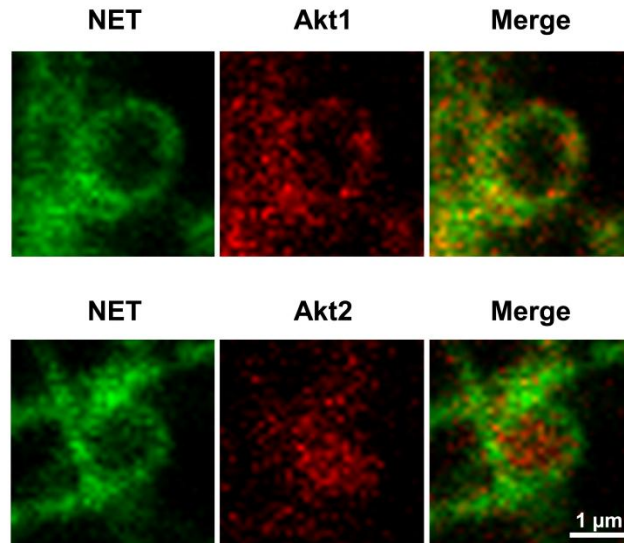
**Appendix Figure A3**.  Both Akt1 and Akt2 isoforms are found at boutons of SCGN cultures.   Mouse SCGNs were cultured and processed for immunocytochemistry as described in Material and Methods.  SCGN boutons co-stained for NET (green) and Akt1 (red top panel) or Akt2 (red bottom panel).  Images depict single boutons with associated axons (linear structures).    The   perimeter   of   the   boutons   is   marked   by   NET immunoreactivity as well as intra-bouton Akt1 and Akt2 immunoreactivity.
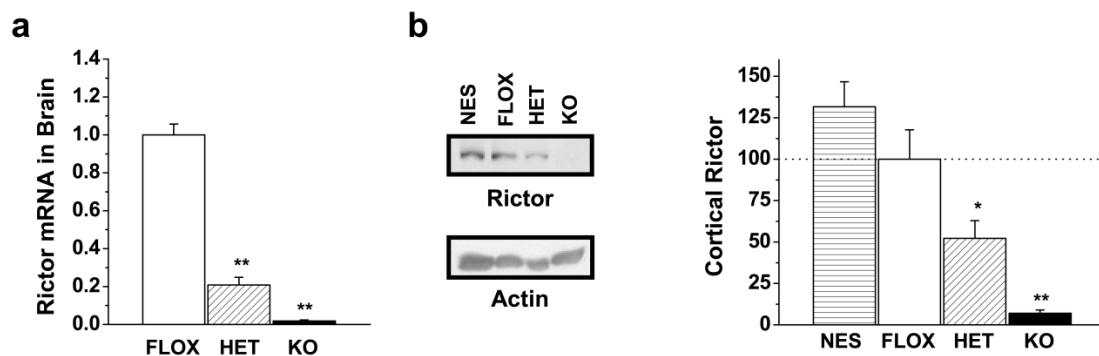
**Appendix Figure B1**.  Rictor mRNA levels and protein expression in the brain are reduced in a gene-dosage dependent manner.  (**a**) qRT-PCR confirms down regulation of the rictor gene in HET and KO mice.  Mean ± s.e.m relative expression shown as a percentage of FLOX control mice; n=4-5 animals.  Panel A courtesy of Kevin Niswender. (**b**) Rictor protein levels in the cerebral cortex.  Mean ± s.e.m optical densities are shown as a percentage of FLOX control mice; n=5, *P<0.05; **P<0.01 One-way ANOVA.
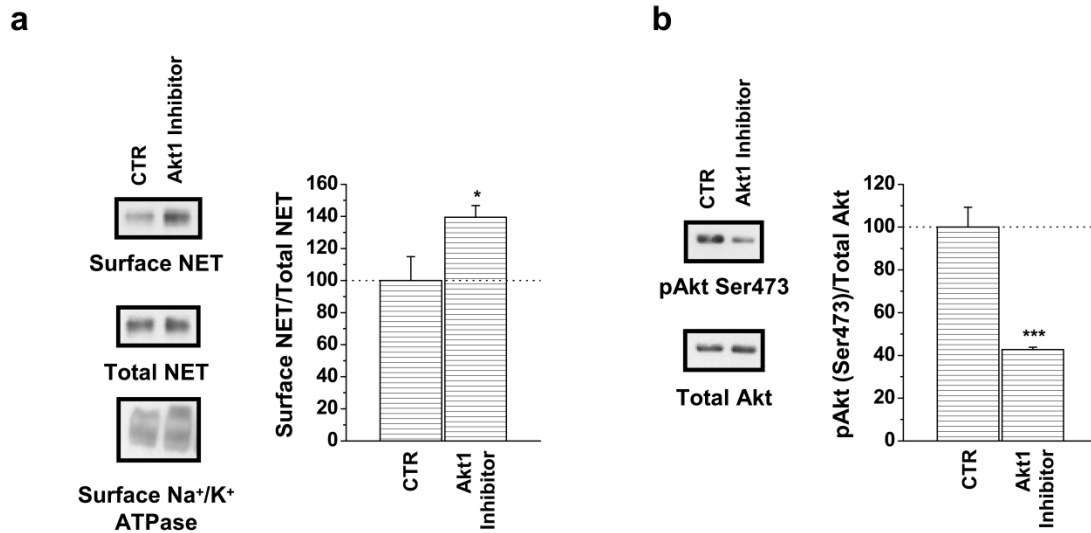
**Appendix Figure B2**. Akt1 inhibition enhances NET surface availability in cortical slices. (**a**) Levels of NET as measured from the different fractions (surface, total) of cortical slices from NES control mice. Slices were treated with either vehicle-DMSO (CTR) or 12 µM of the Akt1 inhibitor. Mean ± s.e.m optical density were normalized to total NET and are shown as a percentage of CTR. Representative immunoblots are shown, as probed with antibodies to NET, Na$^+$/K$^+$ ATPase to serve as plasma membrane/loading control; n=4, *P<0.05 Student's t-test. (**b**) Phosphorylation of Akt on residue Ser473 measured from the same samples as panel A. Mean ± s.e.m of optical densities normalized to total Akt and shown as a percentage of CTR; n=4, ***P<0.001 Student's t-test.

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