

PREFRONTAL AND STRIATAL CATECHOLAMINE DYSFUNCTION IN THE NEURONAL

RICTOR NULL

By

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For God, family, country.

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LIST OF ABBREVIATIONS

Akt	Serine/threonine protein kinase B
AMPH	Amphetamine
AMPT	Alpha-methyl
ANOVA	Analysis of variance
COMT	Catechol-o-methyltransferase
DA	DA
DAT	Dopamine transporter
DAT-KO	Dopamine transporter knockout mouse
dnAKT	Dominant-negative Akt
dnIRS	Dominant-negative insulin receptor substrate
DOPAC	Dihydroxyphenoacetic acid
DR	Dopamine receptor
HVA	Homovanillic acid
IGF	Insulin growth factor
IR	Insulin resistance
IRS	Insulin receptor substrate
LC	Locus coeruleus
mTOR	Mammalian target of rapamycin
mTORC2	mammalian target of rapamycin complex 2
NE	Norepinephrine
NET	Norepinephrine transporter
NET-KO	Norepinephrine transporter knockout mouse
NGF	Nerve growth factor
NRG-1	Neuregulin-1

NX	Nisoxetine
PDK1	Phosphoinositide-dependent kinase 1
PH	Pleckstrin homology domain
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol (4,5)-biphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PPI	Prepulse inhibition (of the acoustic startle response)
Rictor	Rapamycin-insensitive companion of TOR signaling
RTK	Receptor tyrosine kinase
TH	Tyrosine hydroxylase
PI3K	Phosphatidylinositol 3-kinase
VMAT	Vesicular monoamine transporter
VTA	Ventral tegmental area
6OHDA	6-hydroxydopamine

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Insulin resistance in brain disorders

The relationship between metabolic and neuropsychiatric diseases has been a focus of intensive investigation in recent years. Indeed, significant overlap exists, in terms of both disease comorbidity and regulatory mechanisms, between cardiovascular, glucose control, and nervous system disorders. Impaired glucose tolerance, for example, is frequently encountered in patients with schizophrenia (Bushe and Holt 2004; Scheen and De Hert 2007; van Winkel, van Os et al. 2008; Ferentinos and Dikeos 2012). While this association is generally attributed to the side effects of antipsychotic medications, recent findings demonstrate that even first-episode, medication-naïve patients have higher rates of glucose intolerance (Ryan, Collins et al. 2003; Venkatasubramanian, Chittiprol et al. 2007; Verma, Subramaniam et al. 2009). Additional findings suggest the interrelatedness of metabolic disease and mental illness extends beyond schizophrenia. For example, cardiovascular disease, a key component of metabolic syndrome, is more frequently encountered in the bipolar population, and patients with Type 1 diabetes commonly suffer from comorbid depression (Anderson, Freedland et al. 2001; Grey, Margaret et al. 2002; Barnard, Skinner et al. 2006; Swartz and Fagiolini 2012; Vancampfort, Vansteelandt et al. 2013; Westman, Hallgren et al. 2013). The association between metabolic disease and brain disorders additionally extends beyond psychiatry, as recent evidence indicates that insulin resistance (IR) may in itself be a risk factor for both Alzheimer's and Parkinson's disease (Hu,

Jousilahti et al. 2007; Li and Hölscher 2007). Imaging studies provide insight into neuroanatomic foci underlying the relationship between metabolic and neuropsychiatric disorders, as patients with IR demonstrate global cerebral atrophy, particularly in frontal cortical and hippocampal regions (Bruehl, Sweat et al. 2011; Yates, Sweat et al. 2012).

Given historical treatments of schizophrenia, the recent clinical and neuroimaging associations between metabolic and neuropsychiatric illness are particularly intriguing. In the pre-phenothiazine era, induction of coma with high doses of insulin was a common treatment for patients with severe cases of psychosis. While the safety and efficacy of such approaches were attributed to low powered studies and anecdotal evidence at the time, the paucity of alternatives allowed insulin shock therapy to become an important part of schizophrenia treatment before the advent of antidopaminergic medications (Doroshov 2007). While insulin coma naturally became extinct as newer, safer medications arose, the emerging relationship between metabolic and neuropsychiatric disease suggests that understanding insulin signaling in the brain may yield insights into the mechanisms of nervous system disorders.

Mechanisms of insulin resistance

IR, as it is clinically defined, refers to an impaired ability of a known amount of exogenous or endogenous insulin to trigger glucose uptake and utilization compared to normal individuals (Lebovitz 2001). At a molecular level, insulin affects glucose metabolism by increasing the cell surface expression of the glucose transporter, enhancing the net function of glycogen synthase, and other mechanisms (Lebovitz 2001). IR is hypothesized to occur when there is dysfunction in

the components of insulin signaling that are responsible for communication between insulin and downstream glucose-disposal mechanisms (Shulman 2000; Kahn, Hull et al. 2006).

IR, as it refers to the central nervous system, has different implications than in the periphery, as glucose uptake in the CNS is not insulin-dependent. Rather, IR in the CNS is more accurately conceptualized as impairment in the ability of insulin to activate its intracellular effectors. There are many potential signaling components, therefore, that can serve as a molecular basis for IR.

The initiating component in the insulin signaling cascade occurs when insulin itself binds to its receptor, a surface bound-protein that is part of the receptor tyrosine kinase (RTK) family. Receptor stimulation leads to phosphorylation of tyrosine moieties on downstream proteins, including both the receptors themselves and scaffolding proteins like the insulin receptor substrate (IRS). The tyrosine-phosphorylated protein products of receptor stimulation interact with the SH2 domain on growth factor sensitive isoforms of protein phosphoinositide 3-kinase (PI3K) (Rameh and Cantley 1999). PI3K then catalyzes phosphorylation of phosphoinositides at the 3-position in the inositol ring, leading to additional phosphorylation steps that generate phosphatidylinositol (3,4,5)-triphosphate (PIP3) from phosphatidylinositol (4,5)-biphosphate (PIP2). These phosphorylated phosphoinositide byproducts interact with the Pleckstrin homology (PH) domain of the protein kinase Akt, causing membrane translocation of Akt through the formation of salt bridges. Once at the membrane, the upstream kinases phosphoinositide-dependent kinase 1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2) act directly on Akt to phosphorylate this kinase at the Threonine-308 (T308) and Serine-473 (S473) residues. These phosphorylation steps increase the ability of Akt to

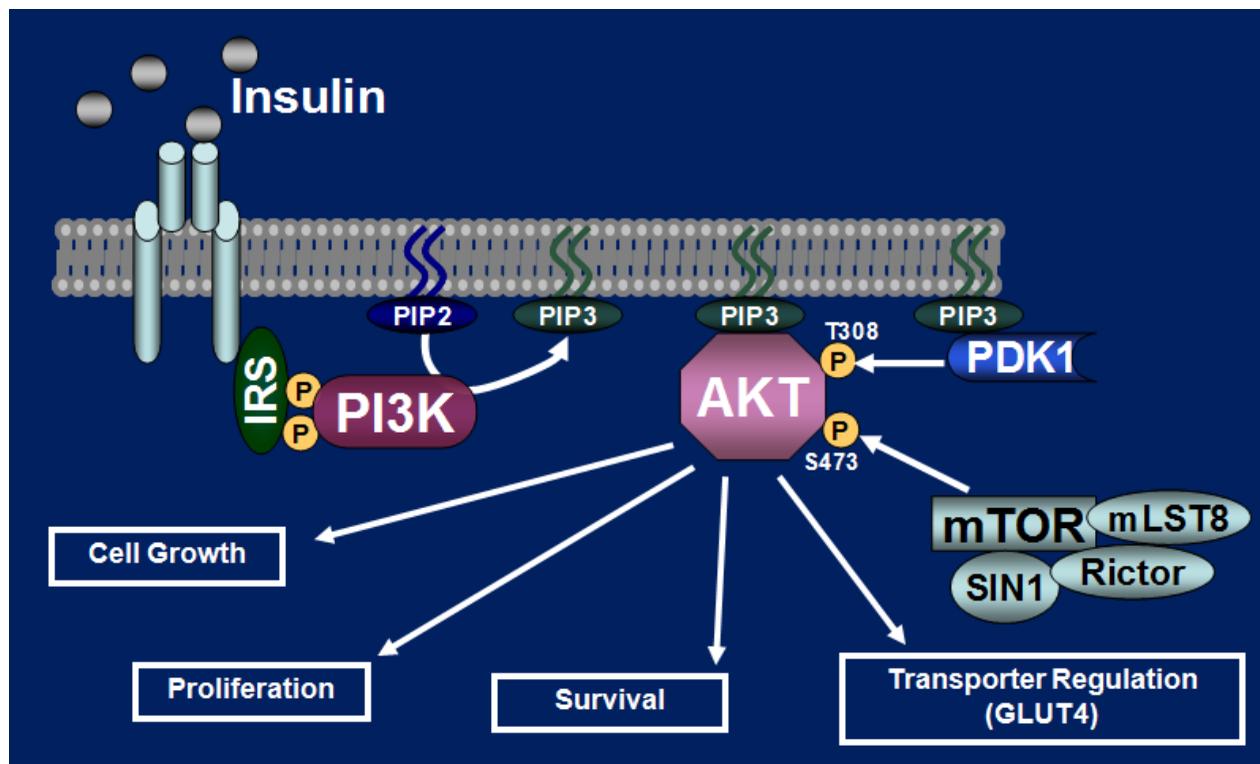
phosphorylate downstream substrates (Rameh and Cantley 1999). A schematic depicting the insulin/Akt signaling pathway is depicted in Fig. 1.

Given the multitude of insulin signaling effectors, the exact mechanisms underlying IR at a physiological level are likely to be multifactorial at a molecular level. Indeed, there is some support for IR at levels including IRS, PI3K, and Akt itself (Pessin and Saltiel 2000; Cho, Mu et al. 2001; Vosseller, Wells et al. 2002). In addition to the Akt pathway, parallel signaling pathways stimulated in response to insulin are also potential mechanisms of IR, like mitogen-activated protein kinase (MAPK) and extracellular regulated kinase (ERK) (Sesti 2006; Asano, Fujishiro et al. 2007). In terms of isolating a molecular link between IR and neuropsychiatric disease, however, Akt is a promising mechanism for further investigation, as Akt dysfunction, in itself, is tied to the development of neurologic and psychiatric disorders (Beaulieu, Gainetdinov et al. 2009).

The evidence linking an Akt loss of function to development of both IR and neuropsychiatric disease stems from a constellation of findings at genetic, animal model, neuroimaging, and post-mortem levels. Genetic findings, regarding loss of function mutations in the isoform Akt2, supports that monogenic dysfunction in Akt signaling is sufficient to cause IR in both animal models and even rare patients (Cho, Mu et al. 2001; George, Rochford et al. 2004). Intriguingly, post-mortem studies in neuropsychiatric patients suggests that an Akt signaling loss of function, at the level of decreased expression and/or phosphorylation, is associated with depression, schizophrenia, and Parkinson's disease (Emamian, Hall et al. 2004; Karege, Perroud et al. 2007; Timmons, Coakley et al. 2009). Indeed, while a loss of function in Akt signaling is therefore a potential causative factor for both IR and neuropsychiatric disease, common treatments for both classes of disorders, including insulin, mood stabilizers, and

antipsychotics, are also potent Akt stimulators (De Sarno, Li et al. 2002; Beaulieu, Sotnikova et al. 2004; Krishnan, Han et al. 2008; Asano, Fujishiro et al. 2007). Thus, a preliminary framework suggests that a loss of function of Akt leads to the development of brain disorders, and stimulation of Akt leads to its treatment. Therefore, Akt signaling dysfunction has high yield in terms of dissecting the relationships between IR and neuropsychiatric disease.

Figure 1. The insulin/Akt signaling pathway.



Schematic of the insulin/Akt signaling pathway. Insulin activates Akt signaling by triggering its phosphorylation at the Threonine-308 and Serine-473 residues. The surface receptor for insulin is an RTK that stimulates PI3K signaling after binding of its respective ligand. Once activated, PI3K catalyzes the conversion of PIP2 to PIP3, which causes the translocation of Akt from the cytosol to the cell surface. At the cell surface, Akt can be activated through phosphorylation by the kinases PDK1 and mTORC2, a multi-subunit signaling complex that depends on multiple proteins, including SIN1 and Rictor, to function. Once activated, Akt exerts a diverse array of effects, including regulation of growth, proliferation, and transporter function. Akt is regulated by numerous upstream factors in addition to insulin, such as other RTKs and neurotransmitter receptors, and in turn regulates diverse downstream signals. Image courtesy of Dr. Aurelio Galli.

Insulin resistance, Akt signaling, and schizophrenia

The disorder with the strongest evidence to support links between IR, Akt signaling, and dysfunctional neurotransmission is schizophrenia. Schizophrenia and Type II diabetes, as addressed earlier, are frequently comorbid conditions, a finding generally attributed to the side effects of antipsychotic medications (Lebovitz 2001). While the effects of the atypical antipsychotics on weight gain and diabetes risk are undeniable, compelling evidence suggests that impairments in glucose metabolism may be associated with the underlying disease process itself (Ryan, Collins et al. 2003; Venkatasubramanian, Chittiprol et al. 2007; Verma, Subramaniam et al. 2009). A recent age- and sex-matched study, for example, demonstrates impaired fasting glucose tolerance in 15% of patients with new-onset, first episode schizophrenia, compared with 0% of controls (Ryan, Collins et al. 2003). Later studies built on these findings, not only reproducing the observation of glucose intolerance in first episode patients, but building on them to demonstrate increased IR rates in the first degree relatives of probands compared to individuals with no psychiatric history (Spelman, Walsh et al. 2007). This evidence suggests not only that IR is present in first episode patients with schizophrenia, but that this IR observed in patient populations has a genetic basis.

While there therefore appears to be strong evidence for IR even in first episode psychotic patients, the body of work linking Akt to schizophrenia evolved separately from Akt's role as an insulin effector. In terms of the study of schizophrenia, Akt has value as a candidate gene, a biomarker, a therapeutic target, and a molecular component for building pathogenic models. The breakthrough study regarding the role of Akt in schizophrenia was first published by Emamian and colleagues. Guided by a hypothesis regarding the role of Akt as a synaptic plasticity protein,

Emamian *et al.* analyzed lymphocytes derived from control and patient populations at a biochemical level, identifying decreased expression of the isoform Akt1 in lymphocyte cell lines derived from patient populations. This effect was additionally true in post-mortem brain, as they found decreased expression of Akt1 in cortical and hippocampal brain regions of schizophrenia patients (Emamian, Hall et al. 2004). Emamian *et al.* then validated the biologic plausibility of their findings, using a murine model to directly probe how a loss of Akt signaling influences experimental phenotypes of schizophrenia. To do so, they examined a reflex called sensorimotor gating in Akt1 null mice. Sensorimotor gating is considered a cross-species endophenotype of schizophrenia that has a strong genetic influence. Sensorimotor gating deficits are observed in putative animal models of schizophrenia, patients with schizophrenia, and their first degree relatives (Braff, Geyer et al. 2001; Perry, Minassian et al. 2001; Swerdlow, Geyer et al. 2001; Powell, Zhou et al. 2009). In murine models, sensorimotor gating is assessed through a task called prepulse inhibition (PPI) of the acoustic startle response. Intriguingly, PPI assessments of Akt1 nulls demonstrate normal sensorimotor gating at baseline, but increased sensitivity to disruption with the indirect dopamine agonist amphetamine (AMPH), as opposed to the PCP-derivative phencyclidine (Emamian, Hall et al. 2004). This suggests that dopaminergic systems, as opposed to glutamatergic systems, are particularly susceptible to disruption in an Akt-deficient state.

Lastly, Emamian and colleagues looked at how treatment of schizophrenia affects Akt signaling. Utilizing animal models, they demonstrated that the antipsychotic medication haloperidol increases the T308 and S473 phosphorylation of Akt (Emamian, Hall et al. 2004). From this study, Emamian therefore developed the initial framework to understand the links between Akt and schizophrenia. They suggested, using convergent evidence across species, that

loss of function in Akt is associated with development of schizophrenia, and gains of function in Akt signaling are associated with its treatment.

Serine-473 phosphorylation of Akt and schizophrenia

In the years since publication of the study by Emamian et al, several studies expanded on the role of Akt, and the S473 phosphorylation site, in particular, as a potential biomarker for schizophrenia (Kéri, Seres et al. 2009; Keri, Beniczky et al. 2010; Kéri, Seres et al. 2011). Due to its intracellular nature, and the difficulties with assessment of S473 in post-mortem tissue (Zhao, Ksiezak-Reding et al. 2006), phosphorylation studies of Akt are frequently performed using cultured lymphoblasts. While there are many differences between lymphoblasts and neurons, they are a system that allows for assessment of intracellular signaling pathways directly in patient populations without significant confounds. Indeed, in terms of a cellular migration phenotype, lymphoblasts are similar to cultured neurons in that both cell types will migrate toward the source of a neurotrophic ligand like neuregulin (NRG) (Sei, Ren-Patterson et al. 2007).

In the study of schizophrenia, both baseline and stimulated studies of lymphoblasts are performed, generally using an agonist like neuregulin, and biochemical findings are frequently correlated to additional experimental results. For example, one such study from lymphoblasts analyzed how NRG affects S473 phosphorylation in normal volunteers and unmedicated, first-episode patients. In patients with schizophrenia, Sei *et al.* demonstrated impaired NRG-stimulated S473 phosphorylation and cell migration compared to controls (Sei, Ren-Patterson et al. 2007). Intriguingly, NRG-1 is itself a schizophrenia candidate gene (Harrison and

Weinberger 2004), and activates Akt in an RTK/PI3K-dependent manner, similar to the RTK/PI3K-dependent effects of insulin (Kanakry, Li et al. 2007). The blunted Akt activation and cell migration observed in the study by Sei *et al.*, therefore, suggests that defective machinery underlying Akt activation is present in patients with schizophrenia.

Subsequent studies strengthen the relationship between a blunted activation of Akt, schizophrenia itself or schizophrenia-associated phenotypes. For example, Keri *et al.* expand on the link between Akt signaling and brain function through correlation of Akt signaling in lymphocyte cell lines to personality assessments of the normal population. Intriguingly, Keri *et al.* demonstrated that NRG-1 dependent stimulation of S473 inversely correlates with measures of delusional personality. The inverse relationship between Akt signaling and measures of delusional personality therefore suggests that Akt signaling impairments, in individuals with no psychiatric history, leads to a psychosis-prone personality and, potentially, sensitivity to mental illness (Kéri, Seres et al. 2011).

While the link between an Akt signaling deficit and a delusional personality is the first finding of its kind, Keri et al expanded on their findings to demonstrate how defects in Akt signaling affect psychophysical variables in schizophrenia patients. To do so, Keri *et al.* repeated their biochemical analysis of Akt signaling in lymphocytes derived from patient populations, but this time coupled their findings to analysis of the P50 evoked sensory potential assay (Keri, Beniczky et al. 2010). This test is performed by measuring electroencephalographic and electrooculographic responses to a pair of auditory stimuli presented in quick succession. In healthy populations, the brainwave responses to the second stimuli are less than the brainwave responses to the first, a phenomenon called sensory gating (Keri, Beniczky et al. 2010). Sensory gating phenotypes are very similar to sensorimotor gating phenotypes, in that individuals with

schizophrenia, and their first degree relatives, have impairments in sensory gating, suggesting it is a genetically-influenced reflex and potential endophenotype of schizophrenia (Siegel, Waldo et al. 1984). Keri *et al.*'s analysis revealed not only that schizophrenia patients have impaired sensory gating compared to controls, but that these deficits also correlate with impairments in activation of Akt at the S473 residue (Keri, Beniczky et al. 2010). Intriguingly, given the AMPH-induced impairments in sensorimotor gating observed in Akt1 null mice (Emamian, Hall et al. 2004; Keri, Beniczky et al. 2010), the association between Akt and psychophysical phenotypes of schizophrenia appears to extend across species.

Despite the strength of the association between Akt and schizophrenia across species, the mechanisms underlying this association remain incompletely understood. Indeed, Akt is a major cellular regulatory pathway that has diverse molecular effects. The promiscuous regulatory potential of Akt makes it difficult to precisely identify how Akt influences the risk for schizophrenia. However, understanding such mechanisms has the potential to yield therapeutic insights, as our current understandings of the mechanisms underlying schizophrenia itself are somewhat limited. Indeed, our understanding of schizophrenia is based, to a significant extent, off our understanding of the neurocircuitry and pharmacology that influences its symptomatology. Therefore, to help elucidate the underlying molecular mechanisms, the neurocircuits that appear to be particularly sensitive to disruption by both IR and Akt will be addressed in the following section.

Akt, the prefrontal cortex, and dopamine

In addition to evidence supporting Akt as a biomarker of schizophrenia, emerging evidence suggests that the function of prefrontal cortical circuits, specifically prefrontal dopamine (DA), are particularly susceptible to disruptions in insulin/Akt signaling. Neuroimaging studies help to identify, at a structural and functional level, the effects of metabolic signaling disruptions on brain function. For example, children with IR demonstrate reductions in prefrontal and hippocampal brain volumes (Bruehl, Sweat et al. 2011; Yates, Sweat et al. 2012). In a similar fashion, genetic variation in Akt influences not only the size of prefrontal cortical structures, but also verbal learning and memory performance (Pietiläinen, Paunio et al. 2009). Post-mortem studies of both depressed and schizophrenia patients augments the importance of insulin/Akt signaling pathways in prefrontal cortical regions, with separate cohorts demonstrating deficits in either phosphorylated Akt or Akt expression in patients with both major depression and schizophrenia (Karege, Perroud et al. 2007; Dwivedi, Rizavi et al. 2010).

In addition to morphologic and post-mortem findings, recent investigations continue to develop the link between Akt and prefrontal cortex at genetic, biochemical, and functional levels. In one recent study, Tan and colleagues collected lymphocytes from normal volunteers, finding decreased expression of the Akt1 protein in individuals with Akt risk haplotypes for schizophrenia. Additionally, Tan and colleagues used functional magnetic resonance imaging (fMRI) to assess the effects of Akt genetic variation on cortical networks during a working memory task. They found that individuals with these “hypoactive” Akt1 alleles demonstrate impaired efficiency in the function of prefrontal networks, a phenotype also observed in the schizophrenia population (Tan, Nicodemus et al. 2008). Separate neuroimaging cohorts recapitulate not only the association between Akt signaling and prefrontal cortical function, but

also suggest the penetrance of Akt risk alleles on PFC function increases in the presence of other schizophrenia candidate genes (Nicodemus, Law et al. 2010; Tan, Chen et al. 2012).

Given that working memory tasks in prefrontal cortex are linked to the function of dopamine systems (Weinberger and Berman 1988), a “hypofrontality” of prefrontal DA systems is therefore hypothesized to result from impairments in Akt signaling. The sensitivity of both working memory function and DA systems to disruptions in Akt-signaling is reflected by findings from animal models. Behavioral analysis of mice lacking the isoform Akt1, for example, demonstrate that, under baseline conditions, Akt1 nulls are capable of normal acquisition of a working memory task called the delayed T-maze task. However, Akt1 nulls, compared to their wild type counterparts, are more sensitive to working memory disruption in response to quinpirole and guanfacine, agents that challenge dopaminergic and noradrenergic systems, respectively (Emamian, Hall et al. 2004; Lai, Xu et al. 2006). The NE disruption observed in Akt1 nulls is particularly intriguing, given the role of NE in PFC function, and CSF findings of NE alterations in patients with schizophrenia (Finlay, Zigmond et al. 1995; Friedman, Adler et al. 1999; Aston-Jones and Cohen 2005).

Therefore, although Akt is a kinase whose dysfunction is likely to affect multiple tissue types, brain regions, and molecular systems, neuroimaging findings support that PFC networks are particularly prone to Akt signaling disruptions. Additionally, while a loss of function in Akt is likely to impact multiple neurotransmitter systems, many of the functions that are impaired in these neuroimaging studies are particularly dependent on prefrontal DA networks. , Animal models corroborate neuroimaging findings, as pharmacologic studies in animals lacking the isoform Akt1 suggests that working memory performance is sensitive to disruption in response to both DA and NE challenge. Collectively, therefore, evidence across species suggests that

catecholamine systems, particularly DA systems in the prefrontal cortex, are critical components of the association between Akt and the central nervous system.

Overview of dopamine and norepinephrine systems in brain

In order to understand the links between IR, Akt signaling, and the catecholamines, it is helpful to first review relevant catecholamine neuroanatomy. DA and norepinephrine (NE) are the two major catecholamines in brain. There are four major DA projections in brain- the mesolimbic, mesofrontal, mesostriatal, and tuberoinfundibular. The mesolimbic, mesofrontal, and mesostriatal projections are those most frequently studied in the context of neuropsychiatric disease. The cell bodies for these projections lie in the midbrain, specifically the ventral tegmental area (VTA) and substantia nigra (SN) (Dunnett, Björklund et al. 2005). The distal axonal projections of these circuits lie in the prefrontal cortex, nucleus accumbens, and dorsal striatum, respectively. The three separate functions of reward, cognition, and locomotion are frequently ascribed to DA projections in brain. While an oversimplification of a complex system, the mesofrontal circuit is most tightly linked to cognition, the mesolimbic circuit to reward, and the mesostriatal system to locomotion (Dunnett, Björklund et al. 2005).

Unlike the relatively restricted projections of the dopaminergic system, NE neurons innervate virtually all areas of the brain including the spinal cord. Indeed, a single noradrenergic neuron can innervate regions as disparate as the cortex and spinal cord (Room, Postema et al. 1981). The noradrenergic system is subdivided into two major divisions, the lateral tegmental (LT) system and the locus coeruleus (LC). The LT system, which is substantially less well studied than the LC, projects primarily to the spinal cord, brainstem, hypothalamus, and basal

forebrain (Moore and Bloom 1979). The LC, in contrast, both receives information from numerous brain regions and subsequently projects to a wide array of regions, providing a potential point of convergence between disparate streams of information (Simpson, Altman et al. 1997). As reflected in these projections, the LC noradrenergic system plays important roles in the regulation of food intake, stress, anxiety, and sympathetic nervous system activation. Disruption in both DA and NE systems, therefore, and pharmacological targeting of these same systems, are key components of the pathogenesis and treatment of many neuropsychiatric disorders. A summary of DA and NE projections in the brain are depicted in Fig. 2.

Figure 2. Dopaminergic and noradrenergic pathways in brain.

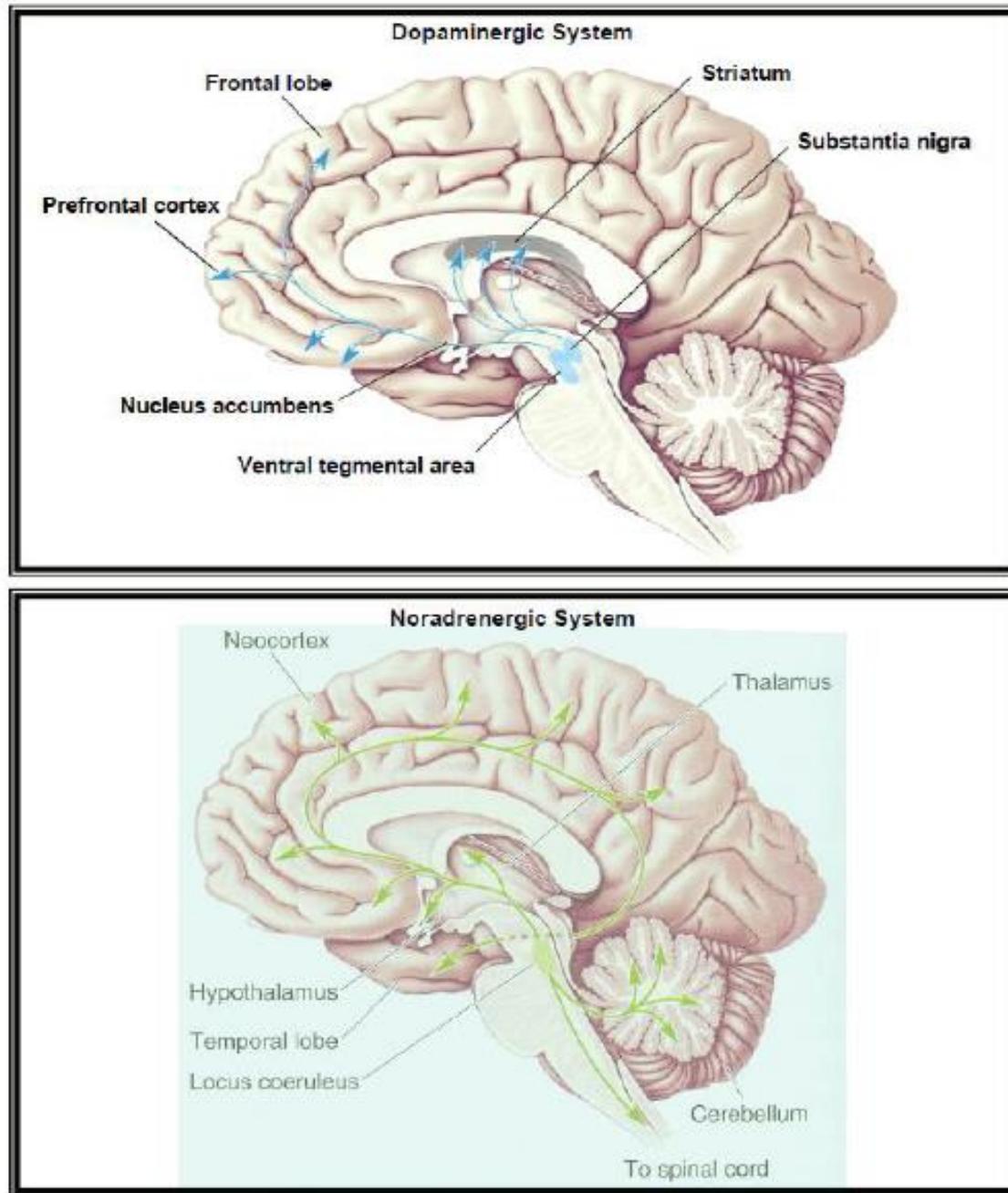


Diagram of dopaminergic (top) and noradrenergic projections (bottom) in brain. The mesocortical DA neurons, which originate in the VTA and project to the medial/frontal lobes of cortex, and mesostriatal DA neurons, which originate in SN and project to the dorsal striatum, are depicted. The more diffuse pattern of NE innervation can be appreciated, in contrast. Co-innervation of DA and NE fibers of cortical regions can be appreciated in this image. Image adapted from Neuroscience Exploring the brain Third Edition by Mark F. Barry, Barry Connor, and Mark. F. Paradiso.

Dopaminergic and noradrenergic networks in schizophrenia

Of the four dopaminergic pathways in brain, the mesostriatal and mesocortical systems are the best characterized in terms of neuropsychiatric disease. Mesostriatal DA circuits are hypothesized to underlie the movement symptoms of Parkinson's disease and the positive symptoms of schizophrenia. In the context of Parkinson's disease, post-mortem findings suggest that a loss of mesostriatal DA precipitates the onset of movement symptoms in this disorder (Kish, Shannak et al. 1992). Conversely, enhanced release of DA from mesostriatal DA neurons are associated with the positive symptoms of schizophrenia (Howes and Kapur 2009). The evidence supporting an increased DA release in schizophrenia is based off of separate, indirect lines of evidence. One key clinical finding supporting an enhanced release of striatal DA in schizophrenia depends on the fact that the effective dose of antipsychotic medications correlates with their ability to antagonize the DA D2 receptor (Creese, Burt et al. 1976; Snyder 1976). A second piece of evidence centers on historical reports describing how high doses of AMPHs – drugs that trigger the extracellular release of catecholamines like DA – produce a psychotic state clinically indistinguishable from schizophrenia (Snyder 1973). Another critical piece of evidence is derived from data produced by the advent of positron emission tomography (PET) in the 1990's. This data demonstrates that, in patients with schizophrenia compared to controls, there is increased displacement of a DA-receptor ligand in the striatum after AMPH administration. The authors use this increased displacement of DA receptors as an indirect indicator of increased striatal dopaminergic transmission. This triad of evidence, demonstrating AMPH-induced psychosis, ability of antipsychotics to antagonize the D2 receptor, and increased striatal DA release after AMPH administration, formed a cornerstone for understanding the

treatment and pathogenesis of schizophrenia for decades (Laruelle, Abi-Dargham et al. 1999; Howes and Kapur 2009).

While increased release of mesostriatal DA is hypothesized to underlie the positive symptoms of schizophrenia, additional evidence suggests that mesofrontal DA circuitry is differentially impaired. Mesofrontal circuits are hypothesized to underlie the negative and cognitive symptoms of schizophrenia (Howes and Kapur 2009). At a neurochemical level, the basis between DA and the function of prefrontal cortical networks is best established in a landmark study by Weinberger et al. Utilizing functional imaging techniques coupled with neurochemical assessment of the cerebrospinal fluid (CSF), Weinberger et al. demonstrated not only that prefrontal activation during cognitive tasks was impaired in patients with schizophrenia, but that this impairment correlates with reductions of the DA metabolite homovanillic acid (HVA) in the CSF (Weinberger et al. 1988). This led to the reconceptualization of the DA hypothesis of schizophrenia, to suggest that, while a DA exaggeration in striatal brain regions underlies the positive symptoms of the disease, a DA deficit, present in prefrontal cortex, underlies the negative and cognitive symptoms of disease (Weinberger et al. 1988). As these symptoms are a significant cause of the disability associated with the diagnosis (Hafner, Löffler et al. 1999; Niendam, Bearden et al. 2006; Leifker, Bowie et al. 2009), much attention, in recent years, focuses on cortical DA dysfunction as a means to treat cognitive dysfunction.

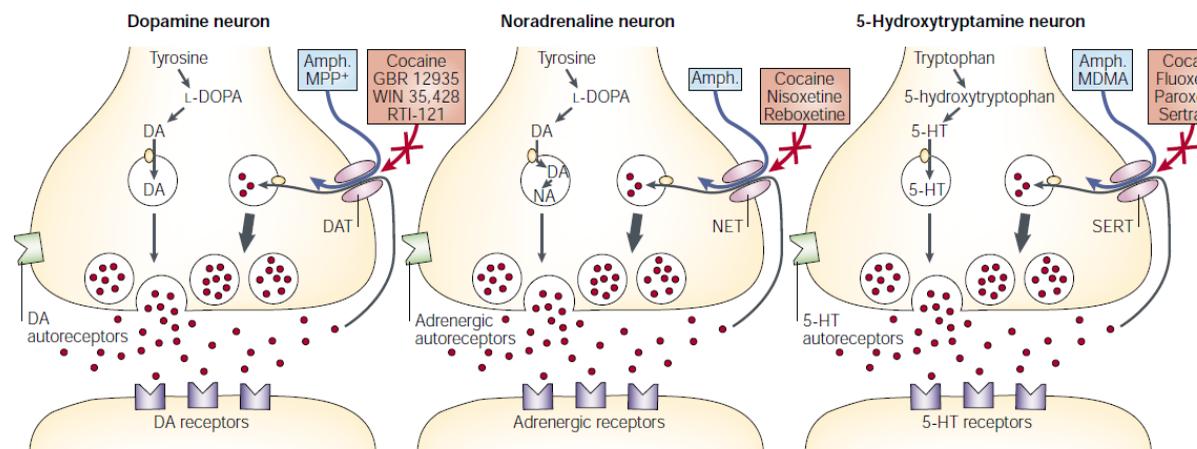
It is important to note that complex neurotransmitter systems, in addition to DA, are found to be abnormally regulated in schizophrenia. Indeed, strong evidence supports a role for NE dysfunction. In the 1980's, for example, CSF studies of patients with schizophrenia demonstrate consistent and reproducible elevations of NE in patients compared to controls.

Cerebrospinal elevations in NE levels led to the evolution of hypotheses regarding a basis for noradrenaline in schizophrenia (Hornykiewicz 1982).

In addition to a potential role in manifestation of schizophrenia, NE is also a critical regulator of prefrontal cortex function. For example, animal models demonstrate that NE release in the PFC is important for the maintenance of attention and ultimately memory (Abercrombie and Jacobs 1988; Aston-Jones, Rajkowski et al. 1999; Usher, Cohen et al. 1999; Aston-Jones and Cohen 2005). Additional evidence suggests that NE release, particularly in the PFC, correlates with the amount of arousal of an organism experiences and its response to stress (Aston-Jones and Bloom 1981; Nakane, Shimizu et al. 1994; Finlay, Zigmond et al. 1995). Given that attention and the stress response are two symptom dimensions altered in schizophrenia, noradrenergic agents remain an attractive pathway for development of neuropsychiatric therapeutics (Arnsten 2004).

Therefore, clinical and animal model evidence, to this point, suggests that disruptions in Akt signaling profoundly affects the function of prefrontal cortex systems, potentially due to its effects on DA and NE systems. Intriguingly, Akt, DA, and NE systems are all disrupted in schizophrenia, and promising targets for the treatment of neuropsychiatric disease in general. However, the mechanistic links underlying the relationships between Akt, DA, and NE systems remain only partially understood. Therefore, the impact of Akt signaling on DA and NE systems, at cellular and molecular levels, will be addressed in subsequent sections.

Figure 3. Regulation of synaptic monoamines.



Schematic depicting the mechanisms underlying regulation of the major monoamine neurotransmitters in brain, namely DA, NE, and 5-hydroxytryptamine (5HT, commonly known as serotonin). The monoamines share in common aromatic amino acid precursors, namely tyrosine for the DA and NE systems, and tryptophan for 5HT. 5HT neurons are included as a reference, given similarities between the regulation and pharmacology of all monoamines, but DA and NE neurons are the focus of this thesis. TH catalyzes the formation of DA from tyrosine, which is in turn metabolized into NE by dopamine beta hydroxylase (DBH) in NE neurons. The packaging of transmitter into vesicles, release into the synaptic cleft following presynaptic stimulation, diffusion into the synaptic cleft, stimulation of pre- and post-synaptic receptors, and uptake by pre-synaptic transporters are all depicted for the respective neurons. Transport mechanisms can be targeted by diverse pharmacological agents, depicted above. Adapted from Torres et al., 2003.

Dopamine neuron survival and Akt

Given the classical role of Akt as an oncoprotein, and its associated role in determining cellular growth and survival (Testa and Bellacosa 1997; Manning and Cantley 2007; Cicenas 2008; Kim, Duan et al. 2009; Mairet-Coello, Tury et al. 2009), one important mechanism to consider when evaluating the role of Akt on the function of DA systems is the survival of DA neurons. The effects of DA neuron cell death can be modeled through administration of the neurotoxic DA analogue 6-hydroxydopamine (6-OHDA), an agent capable of destroying both dopaminergic and

noradrenergic nuclei in the brain (Jeste and Smith 1980; Bing, Zhang et al. 1994). In line with causing reductions in DA neuron cell number after administration of neurotoxic agents, there are neurochemical reductions in total DA levels following administration of 6-OHDA (Fink and Smith 1979). DA neurotoxic agents, in turn, cause characteristic behavioral effects. For example, in the assessment of open field behavior, 6-OHDA-treated animals show diminished exploratory locomotion (Fink and Smith 1979). As these open field exploratory deficits are ameliorated by DA repletion (Fink and Smith 1979), this suggests that DA depletion is sufficient to cause reductions in locomotor activity.

In addition to the effects of DA depletion on striatal DA systems and dependent behaviors, DA neurotoxins like 6-OHDA also affect the function of cortical catecholamine systems. For example, 6-OHDA lesions directed specifically at the medial forebrain bundle, which connects ventral tegmental DA neurons to their projections in the prefrontal cortex, cause deficits in sensorimotor gating behaviors that correlate with reductions in cortical DA levels (Bubser and Koch 1994; Koch and Bubser 1994; Swerdlow, Shoemaker et al. 2006).

Therefore, the potential effect of Akt on neuronal survival is an important mechanism to consider when analyzing the impact of insulin/Akt signaling on neuropsychiatric disease. Indeed, the role of insulin/Akt pathways in neuronal growth and survival dates back, to a certain extent, to classic studies by Stanley Cohen and Rita Levi-Montalcini in the 1950s. Cohen and Levi-Montalcini demonstrated, in chick embryos, that isolation and administration of the ligand nerve growth factor (NGF) promotes neurite outgrowth (Cohen, Levi-Montalcini et al. 1954; Cohen and Levi-Montalcini 1956; Cohen and Levi-Montalcini 1957). Subsequent evidence determines that NGF exerts its effects, in part, through an RTK/PI3K dependent manner in dopaminergic cell lines (Kim, Seger et al. 2004). Indeed, other stimulators of RTK signaling

cascades, like insulin-like growth factor (IGF), affect neuronal migration, cellular growth, and survival of dopaminergic neurons in an analogous manner to NGF (Bogush, Pedrini et al. 2007; Sei, Ren-Patterson et al. 2007; Altar, Hunt et al. 2008). The pro-survival effect of IGF appears to be PI3K dependent, as the PI3K inhibitor LY294002 attenuates the ability of IGF-1 to stimulate growth cone expansion in cultured neurons (Laurino, Wang et al. 2005).

Therefore, stimulation of RTK/PI3K signaling networks promotes DA neuron growth and survival, and inhibition of RTK/PI3K signaling interferes with these processes. In line with the ability of RTK/PI3K signaling to regulate Akt, transgenic mice with gains and loss of function in Akt signaling, respectively, recapitulate the effects of RTK agonism and PI3K inhibition on cell growth, survival, and neuronal migration. For example, a loss of the isoform Akt3 leads to prominent (~25%), symmetric reductions in rodent brain size, an effect driven by reductions in both neuron size and number. This reduction in brain size in Akt3 nulls is consistent with effects of PI3K inhibition on neuronal outgrowth and survival observed in cellular models (Yang, Tschopp et al. 2004; Easton, Cho et al. 2005). Additionally, Parkinson mimetics like 6-OHDA upregulate proteins that are potent inhibitors of Akt signaling, a finding which suggests that Akt signaling impairments underlie cell death in Parkinson's disease (Malagelada, Jin et al. 2008).

In contrast to a loss of Akt signaling impairing growth and survival of DA neurons, opposing cellular phenotypes are observed with gains of function in Akt. One such model is a transgenic mutant with neuronal loss of function in PTEN (phosphatase and tensin homolog). PTEN is a tumor suppressor protein that acts as a negative regulator of Akt, interfering with its function. PTEN-deficient mice, therefore, leads to gains of function in Akt signaling, and subsequent enhancements in brain size at 10 weeks of age (Backman, Stambolic et al. 2001). Similar effects are observed when deletion of PTEN is restricted to dopaminergic cells, as

animals lacking PTEN in DAT neurons show increases in midbrain DA neurons and striatal markers of DA synapses (Diaz-Ruiz, Zapata et al. 2009), suggesting that gains of function in Akt serve to promote the cellular growth of dopaminergic systems.

The development of viral vectors encoding specific components of Akt signaling cascades reinforce the overall role of Akt on neuronal growth and survival. The effects of genetic Akt manipulation on neuronal survival can be best studied following injection of viral vectors into either the SN or VTA, where DA neuron cell bodies reside. Inhibition of Akt signaling in DA neurons, for example, as accomplished via injection of vectors encoding dominant negative insulin receptor substrates (dnIRS), reduces both DA neuron cell size and Akt phosphorylation (Russo, Bolanos et al. 2007). Viruses encoding dominant negative forms of Akt (dnAkt) have similar effects to dnIRS, as dnAkt viruses injected directly into the midbrain reduces DA neurons cell number and size. Additionally, dnAkt viruses also limit the extent of dorsal striatum innervation, where DA neurons project (Ries, Cheng et al. 2009).

In contrast to the effects of viral interference of Akt signaling, viral enhancement of Akt signaling can also be achieved. One strategy to enhance Akt signaling involves the engineering of viruses that express Akt proteins with myristoylated moieties (myrAkt), resulting in constitutive activation of this protein by promoting its trafficking to the cell surface. MyrAkt treatments, in stark contrast to dnAKT treatments, increase TH neuron density in the SN, target innervations of the striatum, and, importantly, total DA levels in the midbrain (Ries, Henchcliffe et al. 2006; Ries, Cheng et al. 2009). In addition to its role in the promotion of neuronal survival and target innervation, animals receiving injections of the DA neurotoxin 6-hydroxydopamine (6-OHDA), following myrAkt pretreatment, show reductions in the extent of DA lesioning.

Together, this evidence suggests that a loss of Akt function interferes with DA neuron survival, and gains of function in Akt exerts prominent protective effect on DA neurons.

Akt and dopamine synthesis

Tyrosine hydroxylase is the rate-limiting protein responsible for catecholamine biosynthesis. It is expressed in the central and peripheral nervous system, including adrenal chromaffin cells. The function of TH is tightly regulated through transcriptional and post-translational mechanisms, including protein phosphorylation. The effects of TH function at a behavioral level can be understood through pharmacologic and genetic interference with protein function. TH can be inhibited with alpha-methyl-tyrosine (AMPT), an agent classically used as an antipsychotic and antihypertensive agent. AMPT administration causes reductions in CNS catecholamines and locomotor activity in mice (Jeste and Smith 1980), analogous to the effects of DA neurotoxins on locomotor behavior. Therefore, while it is clear from the previous section that Akt has an effect on the growth and survival of DA neurons, mechanisms like tyrosine hydroxylase function may also explain the influence of Akt on the function of DA systems.

In the 1990's Richard Palmiter and colleagues generated a series of knockout (KO) mice, targeting the catecholamine synthetic pathway through TH deletion, in order to better evaluate the effects of TH (TH-KO) (Thomas, Matsumoto et al. 1995; Zhou and Palmiter 1995; Zhou, Quaipe et al. 1995). Interestingly, TH-KO proved to be embryonic lethal, emphasizing the importance of the catecholamines (Thomas, Matsumoto et al. 1995), as a group, for embryogenesis. In later years, Dr. Palmiter was eventually able to construct a TH-KO model where TH was restored in cells co-expressing DBH, leading to the generation of what were

termed DA-deficient mice (Zhou and Palmiter 1995). These mice show normal levels of NE and epinephrine (EPI), and survive past birth (Zhou and Palmiter 1995). However they also show marked reductions in brain DA content, similar to that observed in mice with 6-OHDA lesions. Remarkably, these mice show similar behavioral phenotypes as 6-OHDA models, and are profoundly hypokinetic in the open field (Zhou and Palmiter 1995). In fact, their hypokinesia is so profound that it is described as a total amotivational state. Their hypokinesia is so severe that it leads to a near complete lack of self-care, and without intervention, DA-deficient mice die within seven days of birth (Zhou and Palmiter 1995). This amotivational state is attributed to a complete lack of extracellular DA in DA-deficient mice, leading to a subsequent lack of exploratory activity and locomotor response to AMPH. These behaviors, however, are restored when DA levels are restored through pharmacologic (Szczyepka, Rainey et al. 1999) or genetic methods (Heusner, Hnasko et al. 2003; Palmiter 2008).

While there is no evidence, to date, to suggest that Akt directly affects the function of TH at either a transcriptional or post-translational level, there is evidence to suggest that signals upstream of Akt enhance pre-synaptic function of DA neurons, potentially via a TH-dependent mechanism. For example, many growth factors, including NGF, EGF, and IGF-1, enhance the release of DA in pheochromocytoma (PC12) cells, a cultured cell line that expresses all the necessary machinery for synthesis and release of DA. Intriguingly, the ability of these growth factors to enhance the release of DA from PC12 cells is subject to inhibition of PI3K (Amino, Itakura et al. 2002; Itakura, Yamamori et al. 2005). Recent evidence implicates that this effect is true in brain also, as treatment with BDNF in striatal slice preparations enhances DA release in a manner blocked by the PI3K inhibitor LY249002 (Goggi, Pullar et al. 2003).

While the effects of RTK/PI3K on the enhancement of presynaptic DA release, therefore, appears to be reproducible by different groups, a dependence of this effect on TH has yet to be revealed. Indeed, while some RTKs appear able to modulate TH activity, this effect may be dependent on RTK effectors that are activated in parallel to Akt, and not Akt itself. For example, one study, focused on the effects of fibroblast growth factor (FGF), an RTK ligand linked to Parkinson's disease, demonstrates that FGF administration does indeed increase TH activity (Murase and McKay 2006). However, the downstream mediators of the FGF effect appear to be driven by the MAPK cascade, and not signaling through PI3K/Akt (Murase and McKay 2006). Thus, current findings do not support a direct effect of Akt on TH, although a broad body of evidence, addressed in the previous section, supports a role for Akt in the growth and survival of TH neurons.

Akt and the dopamine transporter

In addition to deficiencies in the total number or survival of DA neurons, and beyond control of DA synthesis, another critical mechanism that regulates the function of DA systems is the DA transporter (DAT). DAT allows DA to be cleared out of the synapse and taken up into the presynaptic bouton. Therefore, DAT is a critical protein for regulation of synaptic DA levels and the recycling of DA from the extracellular space. Interference with the DAT interferes with both of these functions (S.P 1995). Consistent with genetic interference in the ability to recycle released DA, mice lacking the DAT (DAT-KO) mice show marked reductions in total DA levels. In fact, the striata of DAT-KO mice contain only 5% the tissue content of their wild type counterparts (Giros, Jaber et al. 1996; Gainetdinov, Jones et al. 1999). This reduction in tissue

DA levels is of sufficient magnitude to resemble the DA-deficiencies observed in DA neurotoxin models. However, despite this marked reduction in tissue DA levels, DAT-KO mice are markedly hyperactive in the open field, showing increases in exploratory behavior that are an order of magnitude greater than wild type controls. Indeed, the increase in locomotor activity observed in DAT-KO mice is linked to adaptations, at the level of DA synthesis and autoreceptor downregulation, that creates fivefold elevations in extracellular DA, despite marked overall reduction in tissue DA (Giros, Jaber et al. 1996; Gainetdinov, Jones et al. 1999). Inhibition of TH, however, at a dose that causes minimal impairment in control animals, markedly interferes with both extracellular DA levels and locomotor activity in DAT-KO (Cyr, Beaulieu et al. 2003; Sotnikova, Caron et al. 2006).

Based off the parallels between elevated synaptic DA levels in DAT-KO mice and elevations in striatal DA transmission observed in schizophrenia patients, DAT-KO mice are considered putative schizophrenia models (Howes and Kapur 2009). In line with their role as a schizophrenia model, DAT-KO mice also demonstrate sensorimotor gating impairments. As described earlier, these sensorimotor gating impairments are additionally found in patients with schizophrenia and their first degree relatives. Intriguingly, sensorimotor gating deficits in DAT-KO mice are corrected toward baseline with antipsychotic medications (Gainetdinov, Mohn et al. 2001; Ralph, Paulus et al. 2001; Powell, Young et al. 2008; Powell, Zhou et al. 2009).

Indeed, as an emerging body of evidence suggests that both IR and Akt signaling have profound effects on DAT function, DAT dysregulation is a potential mechanism to explain the role of IR in neuropsychiatric disease. There is a broad base of knowledge, in fact, supporting RTK/PI3K signaling, in general, as a mechanism of DAT regulation. For example, in rat striatal synaptosomes, both RTK inhibition and PI3K inhibition lead to decreases in DAT cell surface

expression, causing subsequent reductions in DA clearance rates (Hoover, Everett et al. 2007). Conversely, acute growth factor (BDNF) treatment increases DA uptake, in a manner that is prevented by co-treatment with the PI3K inhibitor LY294002 (Hoover, Everett et al. 2007). Additionally, FGF treatment of cultured dissociated DA neurons, which increases TH function, as described previously, also promotes DAT surface expression (Murase and McKay 2006).

These *in vitro* and *ex vivo* findings, which suggest that that RTKs act via PI3K to increase DAT surface expression and function, are corroborated by *in vivo* models of insulin function and dysfunction. The first model to demonstrate a link between insulin signaling cascades and DAT function is the streptozotocin (STZ) model, which demonstrates hypoinsulinemia secondary to beta cell destruction. Consistent with the effects of insulin on Akt signaling, the STZ model is S473 deficient in striatal brain regions (Robertson, Matthies et al. 2010). STZ-treated mice additionally show reductions in midbrain DAT mRNA (Figlewicz, Brot et al. 1996), and have reductions in striatal DAT surface expression (Williams, Owens et al. 2007). This effect appears to be dependent on impairments in PI3K/Akt signaling, and not an effect of a confound of the model like inflammation, as direct treatment of striatal slices with the PI3K inhibitor LY294002, along with direct inhibition of Akt itself, additionally decrease cell surface expression of the DAT (Williams, Owens et al. 2007; Speed, Matthies et al. 2010). *In vivo* evidence validates that Akt deficient states affect DA systems at a functional level. Reductions in both DA clearance rates, and the amount of DA released following AMPH stimulation, are observed in both the STZ model and following PI3K inhibition. Therefore, an Akt-deficient state, according to these models, impairs the ability of the DAT to both clear synaptic DA or release DA in response to AMPH stimulation (Williams, Owens et al. 2007).

In contrast to insulin/Akt signaling deficiencies causing an apparent impairment in DAT function, stimulation of Akt signaling appears to increase DAT function. For example, chronic injections of insulin directly into the brain increase DAT mRNA expression in the VTA (Figlewicz, Szot et al. 1994). Additionally, direct injections of insulin into the CNS, acutely, increases striatal DA clearance rates (Williams, Owens et al. 2007). Intriguingly, DA release and DA clearance rates are rescued in the STZ model following direct injections of insulin into striatal brain regions, further suggesting that DAT deficits observed in this model are due to local neuronal signaling impairments (Williams, Owens et al. 2007),

Akt and the norepinephrine transporter

While it is therefore clear that Akt dysfunction is likely to impact DAT function, this effect may not be able to explain the effects of Akt on DA signaling in cortical brain regions. Indeed, while DAT expression is high in striatal brain regions, its expression is low in the PFC. Microdialysis studies confirm this suspicion, as prefrontal cortex DA clearance is insensitive to selective DAT-inhibitors (Gresch, Sved et al. 1995; Yamamoto and Novotney 1998; Moron, Brockington et al. 2002; Miner, Schroeter et al. 2003; Liprando, Miner et al. 2004).

One mechanism to consider, therefore, when evaluating the effects of Akt on prefrontal regulation of DA, is via its influence on the NET. Evidence supporting that Akt acts as a negative regulator of the NET stems from observations regarding the ability of insulin to decrease NE uptake in neuronal cultures, dissociated brain cells, and both hypothalamic and hippocampal slices. Treatment of hippocampal slices and cultured sympathetic neurons reveals that the ability of insulin to decrease NE uptake depends on its ability to internalize the NET.

Additionally, co-treatment of hippocampal slices with both insulin and Akt inhibitors blocks the ability of insulin to decrease NET expression. Lastly, the STZ-model has increased surface expression of the NET, increased total NE levels in the hippocampus, and increased NE clearance rates, all of which are normalized with insulin treatments (Robertson, Matthies et al. 2010). This evidence, suggests, collectively, that a loss of Akt signaling would, theoretically, lead to an upregulation of the NET.

NET dysfunction, hypothetically, can have dramatic influence on the homeostasis of other monoamines, particularly DA in the cortical brain regions. In fact, the NET has a higher affinity for DA than its nominal substrate *in vitro* (Xu, Gainetdinov et al. 2000). Indeed, microdialysis studies suggest that NET reuptake of DA, in regions of low DAT expression such as the cortex, or even in regions with substantial DAT expression such as the shell of the nucleus accumbens, can significantly influence dopaminergic tone in the brain (Gresch, Sved et al. 1995; Yamamoto and Novotney 1998; Moron, Brockington et al. 2002; Miner, Schroeter et al. 2003; Liprando, Miner et al. 2004). Mice lacking the NET (NET-KO), confirm the effects of NET disruption on DA systems, as NET-KO mice display disrupted DA clearance in the cortex and DA receptor supersensitivity, a phenomenon hypothesized to underlie their increased responsiveness to psychostimulants like AMPH and cocaine (Xu, Gainetdinov et al. 2000; Moron, Brockington et al. 2002; Keller, Diedrich et al. 2006).

Introduction to the Rictor-nKO model

Therefore, while a broad body of evidence suggests that dysfunction in Akt signaling influences the function of prefrontal cortical networks, and subsequent risk for neuropsychiatric

disease, the mechanisms underlying this association remain partly understood. Pharmacological evidence in Akt1 null mice demonstrate normal sensorimotor gating and working memory function, two endophenotypes of schizophrenia, at baseline, but enhanced sensitivity to disruption with dopaminergic and noradrenergic agents (Emamian, Hall et al. 2004; Lai, Xu et al. 2006). While these single transgene studies are particularly striking, and provide biologic validity to the clinical evidence linking Akt to mental illness, advances in transgenic technology allow greater molecular dissection into the mechanisms underlying mental illness.

To date, is not yet known what the effects of the upstream kinases that activate Akt signaling, namely PDK1 and mTORC2, have on dopaminergic neurotransmission. Part of that reason is genetic interference with these kinases, through constitutive deletion of the PDK1 enzyme, and Rictor, an mTORC2 subunit protein critical for the S473 phosphorylation of Akt, yield nonviable organisms (Shiota, Woo et al. 2006). However, as these enzymes catalyze the T308 and S473 phosphorylation of Akt, and these steps are altered either in association with schizophrenia or by antipsychotic medications (Emamian, Hall et al. 2004; Kéri, Seres et al. 2009; Keri, Beniczky et al. 2010; Kéri, Seres et al. 2011), a further understanding of these proteins in brain function may yield novel insights into neuropsychiatric disorders.

The development of Cre-lox mediated genetic recombination technology allows the selective deletion of genes along tissue specific promoters, increasing our ability to study the effects of genes whose constitutive deletions are embryonically lethal. Indeed, the Nestin-Cre promoter is an intriguing neuronal promoter to consider for initial studies, due to its ability to delete this gene in the entire brain. This genetic strategy, driving the deletion of a gene of interest along a Nestin-Cre promoter, generates viable mice for the Rictor gene, but not for the PDK1 gene (Oishi, Watatani et al. 2009).

The Rictor/Nestin-Cre transgenic cross, therefore, will be the subject of the initial investigations of this model. The specific aims of the thesis will focus on elaboration of pre-synaptic mechanisms, including TH, DAT, and NET regulation, for the initial inquiry. Evaluation of post-synaptic hypothesis will be considered throughout, and specific experiments to evaluate post-synaptic mechanisms will be considered in the Future directions section.

The specific aims, therefore, of this thesis are as follows:

- 1.) Assess the effects of neuronal Rictor deletion behaviors which depend on the appropriate function of DA systems, including sensorimotor gating, open field locomotion, and the locomotor response to AMPH.
- 2.) Assess the effects of neuronal Rictor deletion on cortical catecholamine systems, with particular emphasis of the effects of Rictor deletion on tissue catecholamine levels and transporter function.
- 3.) Assess the effects of neuronal Rictor deletion on striatal DA catecholamine systems, and discuss preliminary data and potential post-synaptic mechanism underlying the effects of Rictor on striatal physiology.

CHAPTER II

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 S473 site has been linked to schizophrenia. While 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 for how aberrant Akt signaling leads 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 S473 phosphorylation. Rictor-null (Rictor-nKO) mice show prepulse inhibition (PPI) deficits, a schizophrenia-associated behavior. In addition, they also 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. Importantly, in Rictor-nKO mice NET blockade reversed cortical deficits in DA and PPI deficits, 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 defects 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.

Introduction

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 while 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, while protein kinase B, also known as Akt, is the primary substrate of mTORC2 (Sarbasov, Guertin et al. 2005). Specifically, mTORC2 is the kinase responsible for phosphorylation of Akt at serine residue 473, one of two key phosphorylation sites (Sarbasov, Guertin 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, Gainetdinov et al. 2009).

Post-mortem, imaging and genetic association studies in humans (Emamian, Hall et al. 2004; Dawn, Vladimir et al. 2008; Tan, Nicodemus 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, Hall et al. 2004; Lai, Xu 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 S473, the mTORC2 phosphorylation site (Chalecka-Franaszek and Chuang 1999). The link between mTORC2 signaling deficits and mental illness has been strengthened by seminal work demonstrating that certain antidepressants (Krishnan, Han et al. 2008), along with both typical (Emamian, Hall et al. 2004) and atypical antipsychotics (Lu and Dwyer 2005) increase Akt S473 phosphorylation. Furthermore, findings of diminished S473 phosphorylation and/or activity in post-mortem brains of patients with schizophrenia (Zhao, Ksiezak-Reding et al. 2006) and depression (Karege, Perroud 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, Ohnishi et al. 2006). Recent observations that blunted S473 phosphorylation occurs in lymphocytes derived from patients with schizophrenia and psychosis-prone normal individuals (Kéri, Seres 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, Xu et al. 2006; Tan, Nicodemus

et al. 2008). Importantly, models of schizophrenia suggest that cortical deficits in DA neurotransmission and content defined here as cortical hypodopaminergia contributes to both the cognitive deficits and the negative symptoms characteristic of this disorder (Davis, Kahn 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, Nicodemus et al. 2008) by decreasing DA availability at prefrontal synapses (Lotta, Vidgren 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, Sved et al. 1995; Moron, Brockington et al. 2002; Miner, Schroeter et al. 2003), which takes up both major brain catecholamines, DA and NE (Yamamoto and Novotney 1998; Moron, Brockington et al. 2002). Interestingly, insulin administration, which stimulates mTORC2/Akt signaling, decreases NET transcription in brain while, hypoinsulinemia, and decreased mTORC2/Akt signaling, increases NET transcription (Figlewicz, Brot 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 prefrontal hypodopaminergia.

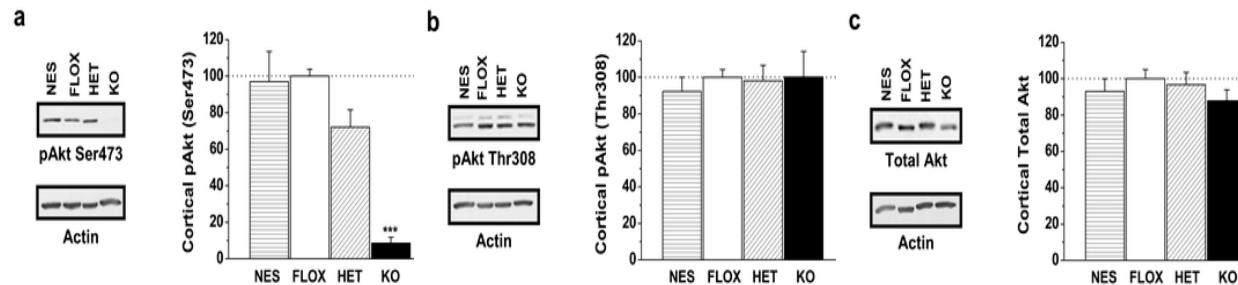
To test this hypothesis, we have generated an animal model in which mTORC2/Akt signaling down-regulation is achieved by neuronal deletion of a key mTORC2 regulatory subunit, rictor. We used a Cre-lox strategy to restrict the genetic deletion to neurons and bypass the embryonic lethality associated with whole body deletion (Shiota, Woo 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 S473 phosphorylation

Akt deficiency is mechanistically linked to prefrontal cortex abnormalities and schizophrenia-linked phenotypes in several mouse models (Lai, Xu et al. 2006), although a clear

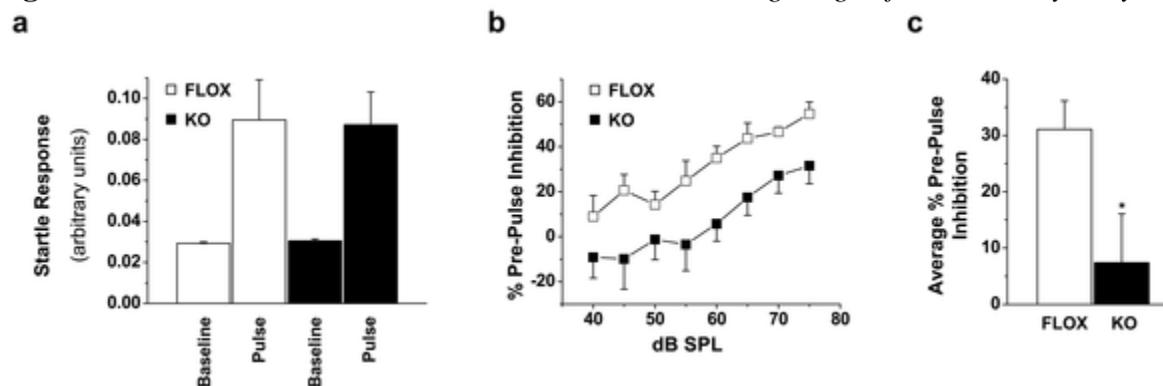
Figure 4. Akt phosphorylation in cortex of neuronal Rictor null.



(A) Phosphorylation of Akt on residue S473 in the cortex. (B) Phosphorylation of Akt at T308 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 knockout 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 S473 (A) T308 (B), total Akt (C), and actin to serve as a loading control. Samples $n = 9-16$. *** $p < 0.001$ one-way ANOVA. Representative immunoblots are shown, as probed with antibodies to phosphorylated Akt at S473 (a) T308 (b), total Akt (c), and actin to serve as a loading control. Samples $n=9-16$. *** $P < 0.001$ One-way ANOVA.

molecular mechanism for how Akt regulates cortical function remains elusive. Here, we investigate the dopaminergic consequences of abolishing Akt phosphorylation at S473 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, Woo 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 (Fig. S1; $P < 0.01$ 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 S473 within the cortex of rictor-nKO mice (Fig. 4a; $***P < 0.001$ 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 T308 (Fig. 4b) and total levels of Akt (Fig. 4c) within the cortex are not different among the genotypes, allowing a direct evaluation of the effects of S473 phosphorylation. Similarly, Rictor deletion also abolishes Akt phosphorylation at S473 in other brain regions, such as the substantia nigra (SN)/ventral tegmental area (VTA) of Rictor-nKO mice, while T308 phosphorylation and total levels of Akt are unaltered (data were normalized to control (FLOX) and reported as mean \pm s.e.m., P values by Student's t-test; pAkt S473 FLOX=100 \pm 13%, KO=6 \pm 1%, $P < 0.001$; pAkt T308 FLOX=100 \pm 13%, KO=89 \pm 8%, $P = 0.49$; total Akt FLOX=100 \pm 13%, KO=101 \pm 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 \pm s.e.m.; FLOX=100 \pm 9%, NES=130 \pm 12%, HET=118 \pm 12%, KO=116 \pm 12%; $P = 0.32$ by one-way ANOVA followed by Dunnett's test).

Figure 5. 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 KO and littermate FLOX control mice. (b) The percentage of PPI was reduced significantly across the entire dB range in KO mice. (c) The average PPI across the entire dB range reveals a significant deficit in PPI in KO mice; n=6-7 animals. *P<0.05 Student's t-test

Neuronal Rictor-nKO mice display sensorimotor gating deficits

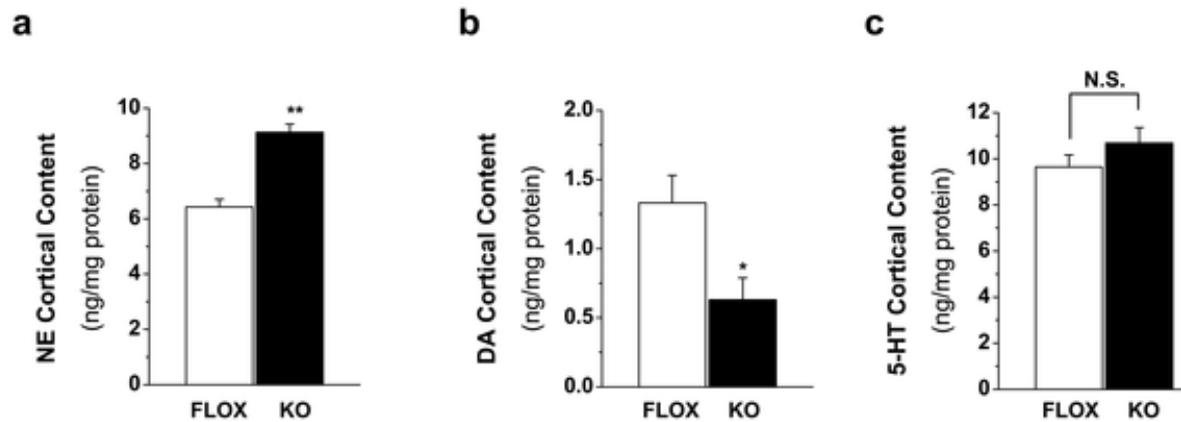
Pre-pulse inhibition (PPI) behavior has long been identified as a promising phenotype for translational studies of schizophrenia owing to the direct parallels in expression between rodent and human subjects. While PPI deficits are present in psychiatric disorders other than schizophrenia, they have a 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 which is characteristic of schizophrenia (Powell, Zhou et al. 2009). The PPI behavioral assay measures the degree to which the startle response elicited by a loud “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

ric1or-nKO mice demonstrate profound deficits in Akt phosphorylation, we tested whether ric1or-nKO 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. 5a; 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. 5b). In ric1or-nKO mice, prepulse sound levels between 40-55 dB did not appreciably attenuate the startle reflex. PPI was not observed in ric1or-nKO 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. 5b; $*P < 0.05$ by ANOVA). This PPI deficit can also be expressed as a significant decrease in average PPI across all sound levels in ric1or-nKO mice (Fig. 5c; Student's t-test, $*P < 0.05$). Thus, neuronal Ric1or deletion with loss of Akt S473 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.

Ric1or-nKO 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, Deniker et al. 1952; Carlsson and Lindqvist 1963; Seeman and Lee 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, Berman et al. 1988; Davis, Kahn et al. 1991; Howes and Kapur 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-nKO mice could be partially explained by alterations in cortical DA content (Koch and Bubser 1994; Swerdlow, Shoemaker 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 infralimbic, prelimbic, and anterior cingulate cortex. Interestingly, HPLC with electrochemical detection of DA, NE, and 5-HT in the PFC of rictor-nKO mice revealed striking alterations in the DA and NE tissue content of these animals, while 5-HT levels remained unchanged (Fig. 6). While both DA and NE levels are significantly different in rictor-nKO mice they change in opposite directions; NE tissue content is significantly increased (Fig. 6a; $**P < 0.01$ by one-way ANOVA followed by Dunnett's test) while DA levels are significantly decreased (Fig. 6b; 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-nKO 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. 6c; 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

Figure 6. Monoamine content in the rostral cortex is significantly altered in *ric1-1* mice.



Tissue content of (a) NE, (b) DA, and (c) serotonin (5-HT) in rostral cortical homogenates. Results are presented as mean \pm s.e.m ng/mg of protein, n=4-10. *P<0.05; **P<0.01 Student's t-test.

noradrenergic systems. In addition, extracellular levels of DA and NE were determined in the PFC of *Rictor-nKO* mice by microdialysis. Under basal conditions, extracellular DA is not significantly different in *Rictor-nKO* mice compared to FLOX controls (data are reported as pg of DA/ μ L, mean \pm s.e.m.; FLOX=0.54 \pm 0.15, KO=0.76 \pm 0.18, P = 0.35 by Student's t-test). However, basal levels of extracellular NE, are significantly decreased in *Rictor-nKO* mice (data are reported as pg of NE/ μ L, mean \pm s.e.m.; FLOX=14.8 \pm 3.4, KO=6.2 \pm 1.5, P < 0.05 by Student's t-test). While basal extracellular levels of DA are unaltered in *ric1-1* mice, these animals do display significant deficits in DA tissue content suggesting that maintenance of DA homeostasis is perturbed.

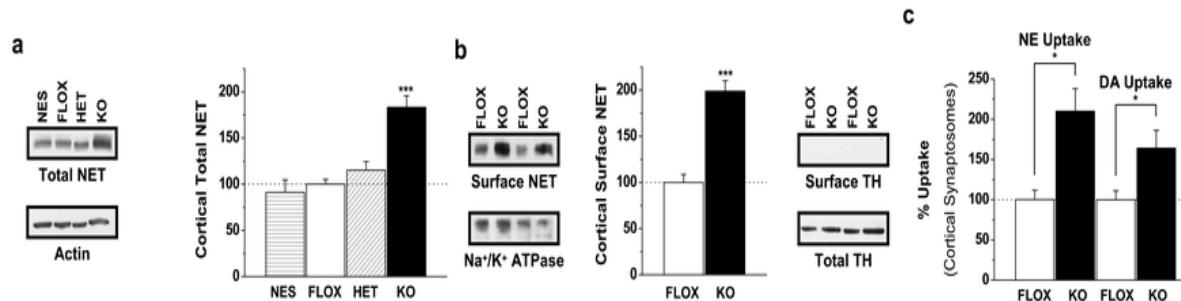
While prefrontal hypodopaminergia has been linked to PPI deficits, other studies clearly demonstrate a link between striatal hyperdopaminergia and PPI deficits (Ralph, Varty et al. 1999; Ralph 2001). Thus, we sought to determine if *Rictor* deletion increases tissue levels of DA in projecting DA neurons from the SN and VTA. DA levels in the SN/VTA are not altered in

ric1-*nKO* mice (data are reported as ng of DA/mg protein, mean \pm s.e.m.; FLOX=4.4 \pm 0.5, KO=4.8 \pm 0.4, P = 0.60 by Student's t-test). Thus, our data indicates that the PPI deficits observed in ric1-*nKO* 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 Ric1-*nKO* mice. Importantly, decreases in mTORC2/Akt signaling induced by hypoinsulinemia have been shown to increase NET transcription (Figlewicz, Brot 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, Clarke et al. 1986; Figlewicz, Szot et al. 1993; Figlewicz, Brot et al. 1996). Thus, we predict that altered DA homeostasis in Ric1-*nKO* 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, Brockington et al. 2002; Miner, Schroeter et al. 2003). Given the pivotal role of Ric1 in Akt regulation and the role of Akt signaling in determining NET availability, we hypothesize that Ric1-*nKO* mice will display aberrant NET regulation that sustains the alterations in NE and DA levels seen in the rostral cortex.

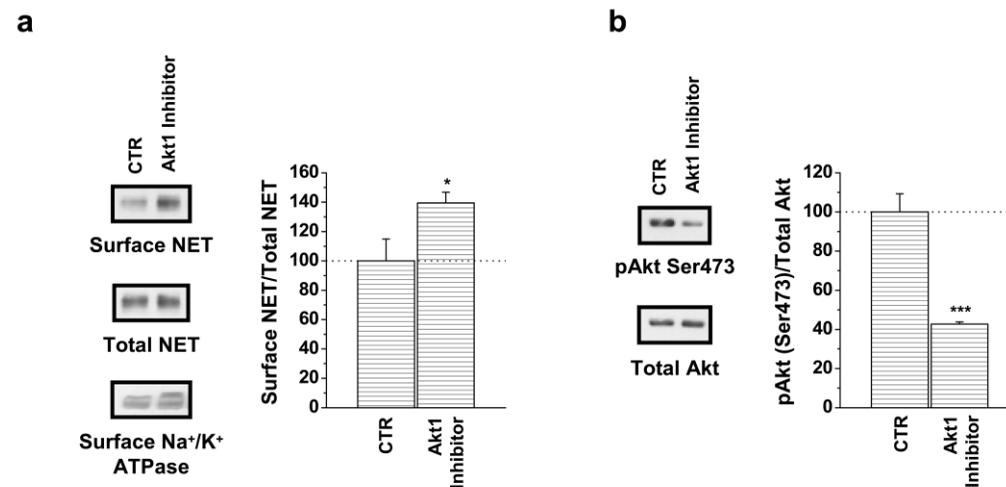
Figure 7. Neuronal Rictor deletion results in increased NET expression and function.



(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) [³H]NE and [³H]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.

As hypothesized, total cortical NET protein is increased approximately two-fold in rictor-nKO mice compared to all other genotypes (Fig. 7a; *** $P < 0.0001$ by one-way ANOVA followed by Dunnett's test). Furthermore, biotinylation assays reveal that cell surface levels of NET are also significantly increased (Fig. 7b; 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-nKO mice results in a significant increase in NET function as assayed by cortical synaptosomal NE uptake (Fig. 7c; Student's t-test, * $P<0.05$). The nearly two-fold increase in cortical synaptosomal NE uptake was also observed for DA (Fig. 7c; Student's t-test, * $P<0.05$) indicating that rictor deletion increases DA clearance by NET in noradrenergic neurons and as a consequence reduces

Figure 8. *Akt1* inhibition enhances NET surface availability in cortical slices.

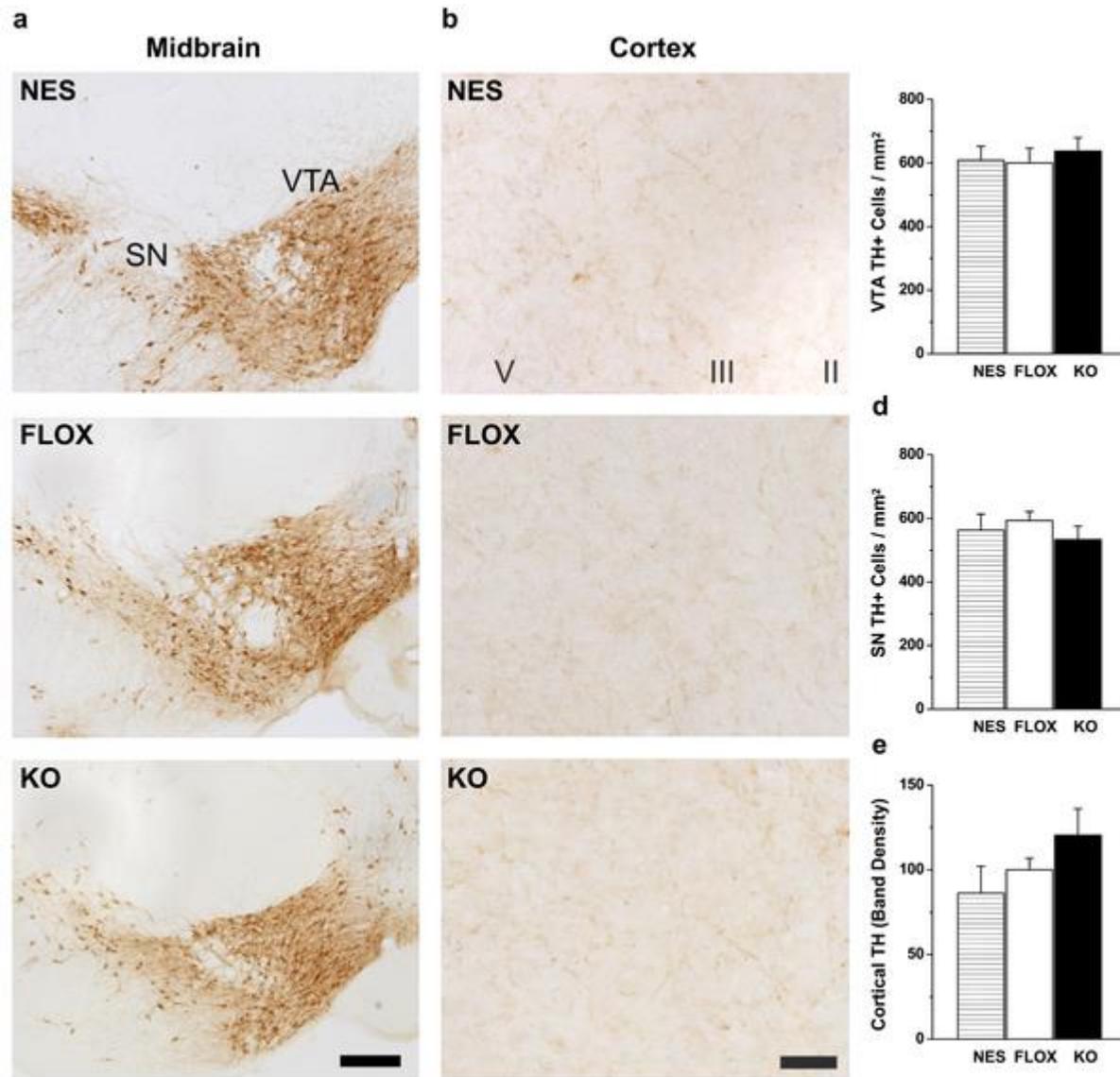


(a) Levels of NET as measured from the different fractions (surface, total) of cortical slices from NES control mice. Slices were treated with either vehicle-DMSO (CTR) or 12 μ M of the Akt1 inhibitor. Mean \pm s.e.m optical density were normalized to total NET and are shown as a percentage of CTR. Representative 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 \pm 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.

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 Rictor-nKO mice has the potential to mechanistically explain both the increased NE tissue content and decreased cortical DA tissue content described earlier (Fig. 6). 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,

Barnett et al. 2005; Lindsley, Zhao et al. 2005; She, Chandarlapaty et al. 2008) in cortical slices to show that Akt inhibition, rather than rictor manipulation, is capable of determining NET surface availability (Fig. 8). Surface levels of NET are significantly enhanced in biotinylated cortical slices treated with the Akt1 inhibitor (Fig. 8a; *P < 0.05 by Student's t-test). Importantly, the levels of Akt S473 phosphorylation are substantially diminished in samples of these inhibitor treated slices (Fig. 8b; ***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-nKO 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 most likely does not require additional afferent connections or neural circuitry.

Figure 9. 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 μ m. 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 \pm s.e.m TH⁺ cells/mm² are shown; n=6. (e) TH protein levels in the cortex. Mean \pm 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.

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

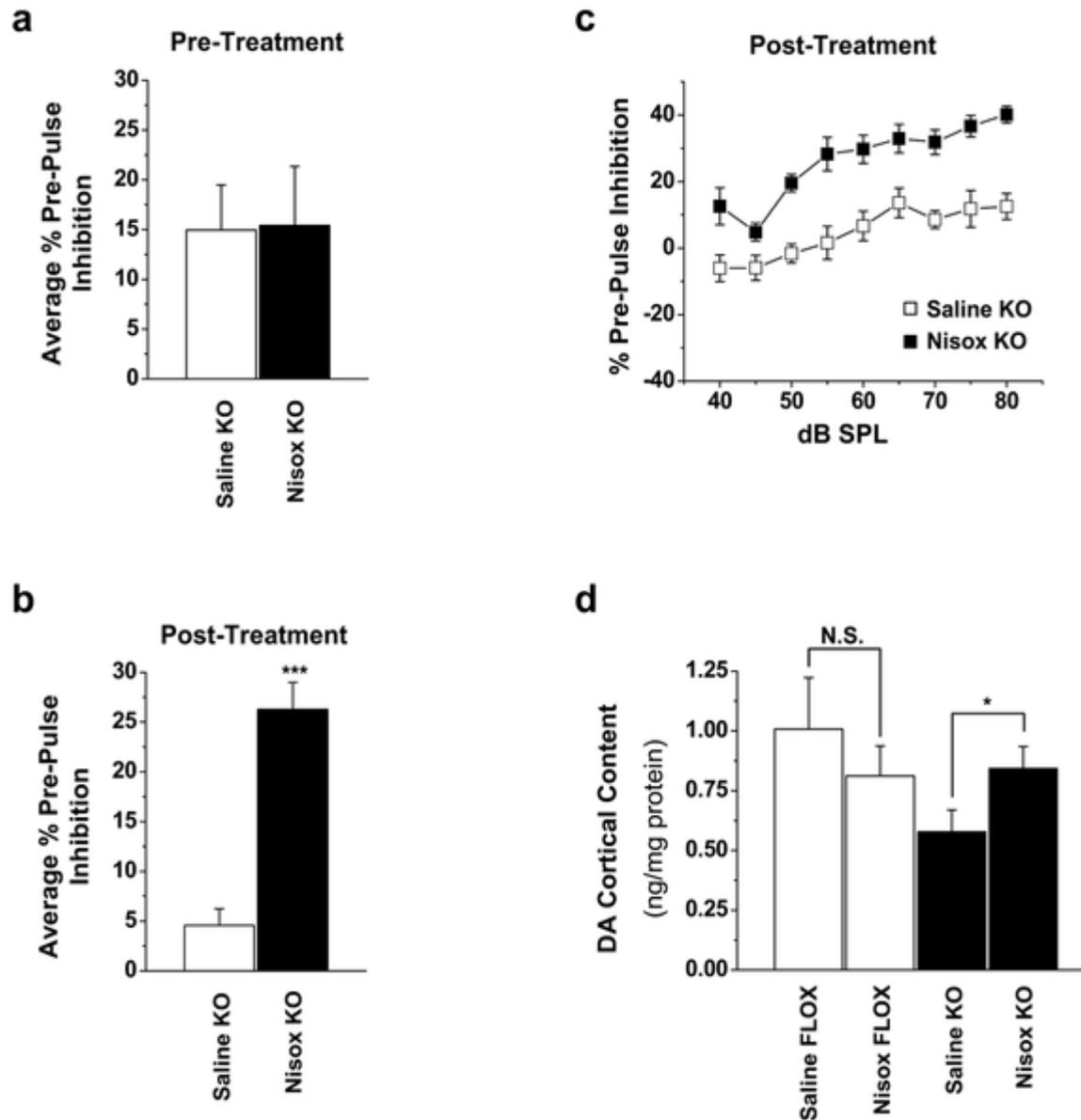
to NE and ultimately supporting both increased NE tissue content and a state of hypodopaminergia. Such a mechanism within the prefrontal cortex provides an elegant molecular mechanism linking Akt hypophosphorylation to both cortical hypodopaminergia and 8

Midbrain dopaminergic neurons and cortical monoaminergic projections are unaltered in Rictor-nKO 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. While Rictor-nKO 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 9a and 9c-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. 9b). The immunostaining was confirmed with western blot analysis of total cortical TH protein levels (Fig. 9e; $P = 0.20$ by one-way ANOVA). Other markers of dopaminergic neurons in the cortex were not significantly altered in the Rictor-nKO 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= $96 \pm 9\%$, KO= $108 \pm 18\%$; $P = 0.85$ by one-way ANOVA followed by Dunnett's test). These data demonstrate that the DA

and noradrenergic systems in the Rictor-nKO mice are not globally altered. Therefore, we propose that the changes in cortical NE and DA tissue content can be primarily accounted for the specific enhancement of NET expression in noradrenergic neurons.

Figure 10. Nisoxetine restores PPI deficits and DA levels in the rostral cortex of Rictor-nKO mice.



(a) panel b and d are cutNo 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-nKO mice; n=4-6. *P<0.05 Student's t-test.

NET blockade reverses the sensorimotor gating deficits and hypodopaminergia in rictor-nKO mice

We hypothesize that aberrant Akt phosphorylation in Rictor-nKO 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 mechanism, we treated Rictor-nKO mice with nisoxetine (NET specific blocker) or saline. Prior to treatment, both groups of Rictor-nKO mice demonstrated comparable startle reflex amplitude similar to Fig. 5b (data not shown). The average PPI across all sound levels also were not different between the two Rictor-nKO groups prior to treatment (Fig. 10a; Student's t-test, $P = 0.95$). Following the initial PPI trial, the Rictor-nKO 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-nKO PPI deficits compared to saline treated animals with a ~5 fold increase of average PPI (Fig. 10b Student's t-test, $***P < 0.0001$). Similar to FLOX control mice (Fig. 10b), nisoxetine treated Rictor-nKO 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. 10c). Importantly, saline treated animals exhibited PPI at prepulse levels > 55 dB, albeit at a weaker level as compared to nisoxetine treated animals, consistent with Fig. 5b. Thus, nisoxetine treatment significantly reverses the PPI deficits observed in Rictor-nKO mice (Fig. 10c; $P < 0.0001$ 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-nKO mice. Unlike nisoxetine,

acute clozapine treatment (i.p. 3 mg/kg for 30 minutes prior to PPI) did not rescue PPI deficits in Rictor-nKO mice (data are reported as average percent PPI mean \pm s.e.m. as in Fig. 5c; saline-KO $24.3 \pm 8.6\%$, clozapine-KO $20.5 \pm 6.4\%$; Student's t-test, $P = 0.73$). Interestingly, previous studies have shown that antipsychotic treatment enhances activity and phosphorylation of Akt at S473, and this increase is hypothesized to be important for the efficacy of such drugs (Emamian, Hall et al. 2004; Kang, Seo et al. 2004; Roh, Seo et al. 2007). Consistently, FLOX mice subjected to the same clozapine treatment as described above show significantly enhanced cortical phosphorylation of Akt at S473 (data are normalized to total levels of Akt and expressed as percent of control mean \pm s.e.m.; saline-FLOX $100 \pm 20\%$, clozapine-FLOX $215 \pm 50\%$; $*P < 0.05$ by one way ANOVA). Importantly, the same treatment does not alter S473 phosphorylation of Akt in rictor-nKO mice (data are normalized to total levels of Akt and expressed as percent of control (saline treated FLOX) mean \pm s.e.m.; saline-KO $5 \pm 1\%$, clozapine-KO $5 \pm 2\%$; $P > 0.05$ by one way ANOVA). Thus, the inability of clozapine to rescue PPI deficits in Rictor-nKO mice may be partially due to the genetic neuronal deletion of rictor, which abolishes the ability of clozapine to enhance Akt S473 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-nKO 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-nKO 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. 6, saline treated Rictor-

nKO mice had reduced DA tissue content levels in comparison to saline treated controls (Fig. 10d; Student's t-test, *P<0.04) and 8 days of nisoxetine treatment significantly enhanced DA content (Fig. 10d; 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. 9d; 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-nKO mice (data are reported as ng of NE/mg protein, mean \pm s.e.m.; FLOX-saline=5.7 \pm 0.4, FLOX-nisoxetine=6.5 \pm 0.5, Student's t-test, P = 0.29; KO-saline=8.3 \pm 0.7, KO-nisoxetine=8.6 \pm 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-nKO 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-nKO 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, Fukushima 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, Buchanan 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 with increasing evidence for a role of Akt in 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.

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, Moron et al. 2002; Moron, Brockington et al. 2002; Miner, Schroeter et al. 2003), Akt-linked changes in NET expression may thereby translate to cognitive deficits and negative symptoms (Davis, Kahn 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, Zhou 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, Hall et al. 2004; Keri, Beniczky et al. 2010). For example, studies show diminished Akt1 protein content in lymphocytes of patients with schizophrenia (Emamian, Hall et al. 2004; Kéri, Seres 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, Facchinetti et al. 2006) may yield new insights into the genetic basis of mental illness.

While 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 and Kapur 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, Sternberg et al. 1980), support our model by which increased cortical NET expression leads to increased NE content but decreased DA content. While 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, Cabib 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 and Kapur 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, Collins 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, Li et al. 2007; Tan, Nicodemus et al. 2008) as well as environmental risk factors for schizophrenia including obstetric complications and early life stressors (Krishnan, Han et al. 2008; Nicodemus, Marenco 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 and Kapur 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, Nicodemus et al. 2008) and proneness to 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.

Methods

All procedures were performed according to Vanderbilt University Institutional Animal Care and Use Committee approved procedures.

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) obtained from Jackson Laboratories to create double heterozygous (rictorf/w Nes^{Cre+/-}; 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, Woo 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, Woo 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 using a scope 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, $\approx 30\mu\text{g}$ 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-nKO 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 °C. 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-nKO 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 $\mu\text{L}/\text{min}$ 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 $\mu\text{L}/\text{min}$ 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 monoamine levels.

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 $\approx 4^\circ\text{C}$ sucrose solution (sucrose 210mM; NaCl 20mM; KCl 2.5mM; MgCl₂ 1mM; NaH₂PO₄•H₂O 1.2mM), 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 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 (MabTechnologies 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 \pm 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 legend sections.

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CHAPTER 3

HYPERLOCOMOTION AND DISRUPTIONS IN STRIATAL CATECHOLAMINE CONTENT IN THE NEURONAL RICTOR NULL

Abstract

Background/methods: Based on evidence outlined in the preceding chapter, a compelling argument can be made that NET overexpression serves to precipitate cortical DA dysfunction in the neuronal Rictor null. However, abnormal striatal DA function is also a possibility in this model, given the link between Akt and disorders of striatal DA signaling, including schizophrenia and Parkinson's disease. Evidence from prior studies in animal models support a link between Akt signaling and the function of mesolimbic/mesostriatal DA networks. Therefore open field locomotion and psychomotor responses to the stimulant AMPH, two behavioral phenotypes classically linked to the function of striatal DA networks, were analyzed in the Rictor-nKO, along with neurochemical and biochemical assessments of striatal DA function.

Results/Conclusions: A DA-deficiency characterizes striatal DA systems of the Rictor null, similar to the phenotypes observed in prefrontal cortical regions. Behavioral phenotypes observed in the Rictor null, however, including open field hyperactivity, and hypersensitivity to AMPH, are more typically associated with those observed in functionally hyperdopaminergic and hypernoradrenergic models. Preliminary biochemical data is discussed, although it is non-conclusive. The potential role of noradrenergic dysfunction in both basal and AMPH-stimulated

locomotion in the Rictor-nKO is also discussed, given the findings of elevated NE and NET observed in many of the brain regions studied in the Rictor-nKO. Post-synaptic mechanisms are considered in addition to the pre-synaptic mechanisms that are the basis of this thesis, and technical considerations regarding how to make inferences between biochemical and functional findings are proposed. Prospective follow up studies, utilizing pharmacologic and genetic methods are described as priorities in order to analyze separate contributions of DA synthesis, DA reuptake, post-synaptic signaling and noradrenergic systems to the observed phenotypes. A noradrenergic focus for follow up experiments is particularly encouraged. Such an approach would serve both to validate whether the DA-NE interactions proposed in Chapter 2 additionally extends to the regulation of striatal systems that are the focus of this chapter. Additional study of noradrenergic influences on the phenotypes described in this chapter may also help further development of noradrenergic agents in clinical practice, including NET inhibitors for cognitive enhancement and depression, alpha-1 antagonists for post-traumatic stress disorder, alpha-2 agonists for hypertension and anesthesia, and DBH-selective inhibitors for cocaine addiction.

Introduction

Aberrant signaling through the protein kinase Akt is an increasingly well-studied mechanism underlying neuropsychiatric disease. While the mechanisms linking Akt signaling to neuropsychiatric diseases are manifold, cellular and animal models suggest that dysfunctional Akt signaling profoundly influences the machinery underlying DA neurotransmission (Emamian, Hall et al. 2004; Lai, Xu et al. 2006). The preponderance of the evidence suggests that deficits in Akt signaling lead to alterations in DA signaling, through a combination of mechanisms

including an influence on cell survival and transporter regulation. The initial characterization of a novel model of Akt dysfunction, the neuronal Rictor null, recapitulates the influence of Akt signaling on DA neurotransmission, at least in cortical brain regions. Intriguingly, levels of tissue NE are markedly elevated in cortical brain regions. Additionally, Rictor-nKO mice show reduced levels of prefrontal DA compared to their wild type counterparts. Histological assessments suggest that this effect is not due to survival, as TH projections to cortical regions are equivalent between genotypes, and density of TH cell bodies in SN and VTA are similarly analogous. However, NET levels are markedly different between genotypes, an intriguing finding given elevations in NET observed in hypoinsulinemic mice (Robertson, Matthies et al. 2010).

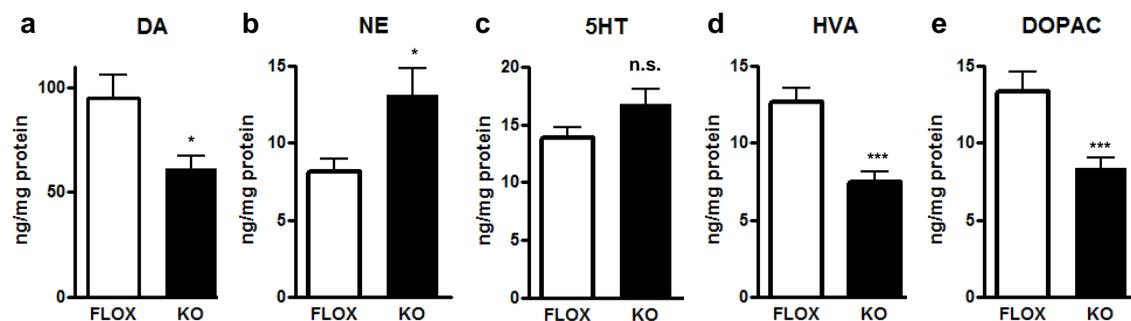
While cortical DA dysfunction seems dependent on NET dysfunction in the Rictor-nKO, striatal DA neurotransmission remains relatively uncharacterized. Given recent evidence implicating dysfunctional Akt signaling in brain disorders like schizophrenia and Parkinson's disease (Emamian, Hall et al. 2004; Timmons, Coakley et al. 2009), we sought to investigate alterations in subcortical DA systems in the Rictor-nKO. We therefore analyzed two behaviors tightly linked to the function of striatal DA systems, namely open field behavior and the locomotor activating effects of AMPH (Fahn, Libsch et al. 1971; Davis, Kahn et al. 1991; Lotharius and Brundin 2002; Howes and Kapur 2009). Additionally, we analyzed monoamine content in striata of the neuronal Rictor null, and performed biochemical assessments of striatal DA systems. The significance of these findings and prospective experiments to disentangle the underlying mechanisms are outlined in the following chapter.

Results

Reductions in tissue dopamine content in dorsal striatum of Rictor-nKO

We dissected and evaluated the neurochemical content of striata from several cohorts of mice. Rictor-nKO mice show a 30-40% reduction in both striatal DA content (Fig. 11a, data demonstrated Rictor-FLOX versus Rictor-nKO reported as mean \pm s.e.m., in ng/mg protein; FLOX=95.06 \pm 10.97, KO=61.53 \pm 5.904; P=0.016 by Student's t-test) and its major metabolites, namely 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), compared to wild type controls (Fig. 11d-e, for DOPAC, FLOX=10.64 \pm 1.028, KO=6.7 \pm 0.508; P=0.0032 by Student's t-test, for HVA, FLOX=12.68 \pm 0.8413, KO=7.47 \pm 0.6993; P<0.001 by Student's t-test). Given that the decreases in DA and its metabolites DOPAC and HVA were of similar magnitude, DA turnover rates are not significantly different between genotypes. Importantly, 5HT levels did not differ significantly between groups (Fig. 11c, FLOX=14.2 \pm 0.9002, KO=16.72 \pm 1.32; P=0.1189 by Student's t-test). This suggests that, although signaling through

Figure 11. Neurochemical assessment of dorsal striata in Rictor-nKO mice.



Tissue content of (a) DA, (b) NE, and (c) 5-HT, (d) HVA, and (e) DOPAC in dorsal striatum homogenates. Results are presented as mean \pm s.e.m ng/mg of protein, n=11-10. *P<0.05; ***P<0.01 Student's t-test.

mTORC2 is likely to interfere with a range of physiological functions, cortical catecholamine systems are particularly disrupted in the Rictor-nKO.

The finding of reduced DA in a putative loss of function Akt model is consistent with findings from previous studies. For example, flies lacking the Parkinson's-related gene DJ-1 show reductions in both p-Akt and tissue DA content (Yang, Gehrke et al. 2005), dominant negative Akt viruses inhibit DA neuron growth and survival (Ries, Cheng et al. 2009), and prefrontal regions previously examined in the Rictor-nKO show reductions in tissue DA content (Siuta, Robertson et al. 2010).

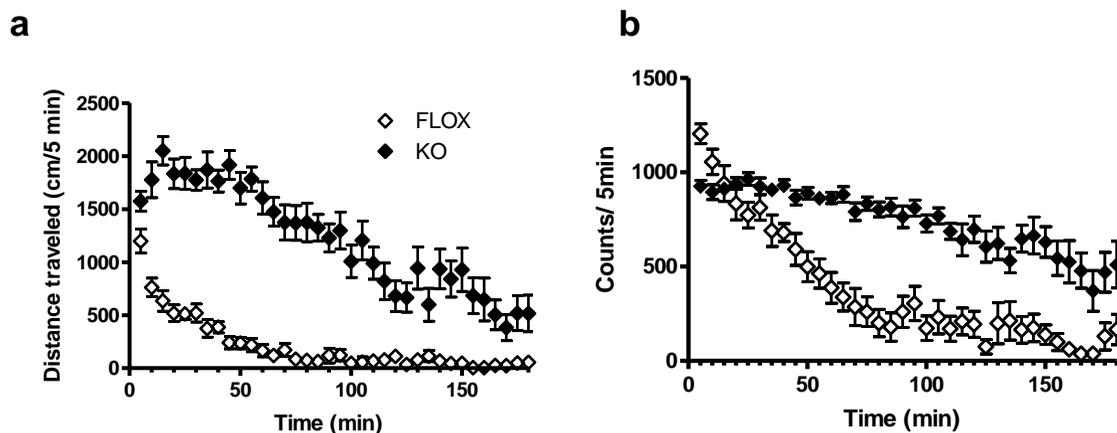
Unexpectedly, elevations in striatal NE levels were also observed in Rictor-nKO mice (Fig. 11b, FLOX=6.49 ± 0.69, KO=10.49± 1.36; P=0.014 by Student's t-test). Elevations in tissue NE levels are also observed in cortical regions of the Rictor-nKO (see Fig. 6) and in additional brain regions like the hippocampus, hypothalamus, and cerebellum (data not published). However, the significance of elevated NE levels must be interpreted cautiously in the Rictor-nKO, as reviews of the literature reveal controversies regarding whether significant noradrenergic innervation of the striatum exists. One side of the argument holds that while there may be NE projections to ventral regions of striatum, the projections to the dorsal regions of striatum characterized in our study are relatively sparse (Rommelfanger and Weinshenker 2007). However, while NE projections to the striatum may be limited compared to NE innervation of other brain regions, there is some evidence to suggest a physiological and pathophysiological role of NE in striatal function. For example, studies of cultured striatal neurons reveals that adrenergic receptors modulate DA signaling pathways (Hara, Fukui et al. 2010), and elevated striatal NE levels are observed in other pathological mutant models (Raber, Mehta et al. 1997). Additional information demonstrating a role of NE in striatal neurotransmission stems from studies demonstrating both endogenous NE levels and the accumulation of radiolabeled NE into striatal brain regions following intraventricular injection (Glowinski and Iversen 1966).

Furthermore, striatal brain homogenates from canines are capable of synthesizing NE from DA, suggesting the presence of the noradrenergic synthetic enzyme DBH in this brain region (Udenfriend and Creveling 1959). Therefore, the potential significance of striatal NE in the Rictor-nKO, warrants further investigation.

Pronounced open field hyperactivity in neuronal Rictor null (Rictor-nKO) mice.

The implications of a tissue DA deficiency, at the levels of physiology and behavior, are

Figure 12. Locomotor assessment of Rictor-nKO mice in a novel, open field environment.



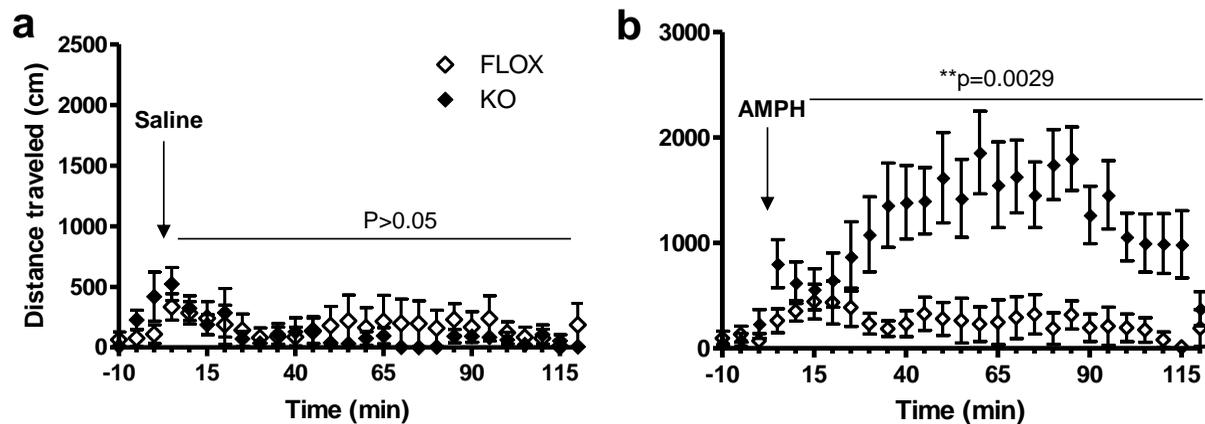
Markedly enhanced horizontal locomotion (12a) and stereotypy (12b) characterize the Rictor-nKO, which persists on the first day for a 180 minutes period. Rictor-nHET mice show comparative locomotor activity to wild type animals, suggesting that complete loss of signaling through Rictor is required the hyperactivity phenotype that characterizes the mutant(data not shown).

markedly different depending on the context of the animal model in which it is observed. For example, both animals with neurotoxic (6-OHDA) lesion of nigrostriatal DA pathways, and mice lacking the DA transporter are characterized by reductions in total tissue DA content (Kelly and Iversen 1976; Jones, Gainetdinov et al. 1998). However, these models differ markedly in terms

of their exploratory behavior in the open field, with 6-OHDA-lesioned mice showing profound hypokinesia and DAT-KO mice demonstrating profound hyperactivity (Kelly and Iversen 1976; Jones, Gainetdinov et al. 1998).

Therefore, to gain insight into the relationship between tissue DA concentration and locomotor activity in this model, we evaluated the behavior of Rictor-nKO mice in the open field. Compared to control conditions, Rictor-nKO mice demonstrate dramatic elevations in locomotor activity (Fig. 12a, $P < 0.001$ for effect of genotype by two way ANOVA), with mutants demonstrating an order of magnitude increase in horizontal locomotion that persists for over a three hour period. Additionally, stereotypy shows a similar degree of enhancement in the mutants compared to control animals, demonstrating that overall psychomotor activity of the Rictor-nKO is dramatically elevated (Fig. 12b, $P < 0.001$ for effect of genotype by two way ANOVA). Mice with conditional genetic deletion of one functioning Rictor allele (Rictor-nHET)

Figure 13. Locomotor response of Rictor-nKO mice to saline and AMPH.



Rictor-nKO mice demonstrate enhanced sensitivity to the locomotor activating effects of AMPH. All animals were habituated to the chamber for 6 days before AMPH challenge exposure to ensure the locomotor response to saline were equivalent between groups (a). Significant activation occurred in Rictor-nKO mice on in response to 2mg/kg of AMPH challenge day (b, arrow denotes when AMPH is administered, $**p < 0.01$ by two way ANOVA for genotype effects on drug response versus rictor-FLOX animals).

show comparative locomotor activity to Rictor-FLOX mice (data not shown, $P > 0.05$ by two way ANOVA), serving as a control for the effects of the Nestin-Cre transgene and demonstrating that complete blockade of Rictor-mediated signaling is required for the phenotypes observed in the mutants.

Rictor-nKO mutants are hypersensitive to the locomotor activating effects of AMPH.

The exaggerated locomotor activity in the Rictor-nKO is intriguing, given that similar enhancements in locomotion occur in hyperdopaminergic models like the DAT-KO (Giros, Jaber et al. 1996). DAT-KO mice resemble the Rictor-nKO according to several other key phenotypes, beyond this elevation in locomotion. DAT-KO mice also display PPI deficits at baseline that are corrected with nisoxetine (Yamashita, Fukushima et al. 2006). Intriguingly, DAT-KO display paradoxical effects to the psychostimulant AMPH. As opposed to wild type animals, DAT-KO mice demonstrate a reduction in psychomotor activity in response to AMPH (Beaulieu, Sotnikova et al. 2006). Indeed, the response to AMPH is a well-studied phenomenon in many models of insulin, PI3K, and Akt dysfunction. However, there are important discrepancies in the literature regarding the influence of Akt on the response to AMPH. One group of findings suggests, collectively, that Akt deficits reduce the locomotor response to AMPH, while increases in Akt signaling enhance the locomotor response to AMPH. Hypoinsulinemic mice, for example, show diminished self-administration of AMPH (Galici, Galli et al. 2003) at particular doses. Similarly, administration of the PI3K inhibitor LY294002 reduces the locomotor sensitizing effects of the psychostimulant cocaine (Izzo, Martin-Fardon et al. 2002). Additionally, *in vivo* voltammetric studies of hypoinsulinemic mice reveal that

AMPH-induced striatal DA release is markedly diminished in this model, which is restored with local insulin pretreatment (Williams, Owens et al. 2007). PI3K inhibition causes similar reductions in DA release to STZ treatment, suggesting that reductions in AMPH-induced DA release are Akt-dependent (Williams, Owens et al. 2007). In addition to these findings, injections of associated adenovirus vectors (AAVs) expressing myr-Akt, a constitutively active form of Akt, corrects the AMPH-induced locomotor deficit in 6-OHDA models (Ries, Henchcliffe et al. 2006). This line of evidence suggests, collectively, that a loss of function in Akt signaling leads to a diminished sensitivity to AMPH, while gains of function in Akt enhance sensitivity to AMPH.

However, the correlations between Akt signaling deficits and reduced sensitivities to AMPH are challenged by separate lines of evidence. For example, in an analysis of the ability of AMPH to affect sensorimotor gating responses, Akt1-deficient mice show a greater sensitivity to AMPH-induced disruptions than wild type animals (Emamian, Hall et al. 2004). Additionally, the inhibition of Akt signaling observed in hyperactive mutants and animals given psychostimulants is hypothesized to underlie locomotor activity in general. For example, studies of DAT-KO mice suggest that Akt signaling deficits, at least in post-synaptic compartments, stimulates locomotor activity, and activation of Akt, in response to medications like lithium and the antipsychotics, reduces locomotion (Beaulieu, Sotnikova et al. 2004; Beaulieu, Sotnikova et al. 2005).

Given these discrepant findings in the literature, we sought to test the ability of AMPH to modulate open field activity in Rictor-nKO mice. We first assessed the qualitative effects of AMPH administration in a novel, open field environment. We found, unexpectedly, that administration of AMPH in the open field markedly increased the basal hyperactivity already

present in the Rictor-nKO (data not shown). On initial exposure to the open field, however, significant differences in locomotor response to AMPH was difficult to elicit, as baseline locomotion is already significantly elevated in this model (Fig. 12). Therefore, we developed an experimental design for AMPH-stimulated locomotion involving a paradigm where each animal served as its own internal control in habituated conditions. This involved analysis of the open field activity of the Rictor-nKO every day for 6 days. Upon reintroduction to the open field environment, the hyperactivity of Rictor-nKO mutants persisted, but, on subsequent days, eventually habituated to wild type levels after two hours of open field re-exposure (data not shown). By day 6, their locomotor response to saline approached wild type levels, with Rictor-nKO mice displaying habituated responses to saline injections (Fig. 13A, $P > 0.05$ for effect of genotype by two way ANOVA). On open field testing on the subsequent day (Day 7) we found that locomotor activation in response to AMPH, in terms of both local exploration and stereotypic behaviors, was greater in the Rictor-nKO compared to the wild type (Fig. 13B, $P < 0.01$ by two-way ANOVA). This data suggests that, despite what would be predicted by an overall reduction in DA content, Rictor-nKO mice are hypersensitive to the locomotor activating effects of AMPH.

Discussion/Future directions

The mechanisms underlying the behavioral responses to AMPH are complex. AMPH is a drug that exerts its behavioral influence through elevation of extracellular levels of monoamines. A complex array of mechanisms underlie the ability of AMPH to stimulate open field locomotion in other models, including TH, the DAT, DA receptors, adrenergic receptors, and

even the NET (Tyler and Tessel 1979; Giros, Jaber et al. 1996; Ventura, Cabib et al. 2003; Beaulieu, Sotnikova et al. 2004; Beaulieu, Sotnikova et al. 2006; Salahpour, Ramsey et al. 2008). Additionally, AMPH is a psychostimulant that, depending on the context of its use, can either stimulate or inhibit locomotor activity (Beaulieu, Sotnikova et al. 2006; Alttoa, Eller et al. 2007). For example, in DAT-KO mice, which are considered functionally hyperdopaminergic, AMPH exerts an inhibitory influence on exploratory activity (Beaulieu, Sotnikova et al. 2006). In contrast, in NET-KO mice, which are considered functionally hypernoradrenergic, AMPH elicits a hyperactive locomotor response compared to controls, an effect attributed to development of D2 hypersensitivity in this model (Xu, Gainetdinov et al. 2000).

While AMPH therefore has complex effects on locomotion, the exact relationship between Akt signaling and the mechanisms underlying locomotor is unclear, as there are discrepant findings in the literature regarding how gains and losses of function in Akt, respectively, influence the locomotor response to AMPH. For example, reduced responsiveness to AMPH in an Akt-deficient state is suggested by models of hypoinsulinemia and PI3K/Akt inhibition (Izzo, Martin-Fardon et al. 2002; Ries, Henchcliffe et al. 2006; Williams, Owens et al. 2007). In contrast, an Akt-deficient state underlying either enhanced locomotor activity or sensitivity to AMPH is suggested by DAT-KO mice (Beaulieu, Sotnikova et al. 2004), Akt1 nulls (Emamian, Hall et al. 2004) and neuronal Rictor nulls on the other (Fig. 13B).

One potential explanation for these discrepancies is that the source of the Akt signaling defect occurs in different cellular populations in these disparate models. Indeed, in the STZ model, which demonstrates reduced AMPH-stimulated DA efflux (Williams, Owens et al. 2007), Akt deficiencies are believed to be particularly in pre-synaptic DA neurons, as prior studies demonstrate that these neurons express the insulin receptor (MARKS, PORTE et al. 1990). In

DAT-KO mice, however, Akt signaling defects are hypothesized to be in post-synaptic striatal neurons (Beaulieu, Sotnikova et al. 2004). In Akt1 null mice, the signaling deficit involves a germline mutation, and thus is present in all cells where Akt1 is expressed (Emamian, Hall et al. 2004). Intriguingly, both dopaminergic and noradrenergic systems are particularly prone to disruption in Akt1 nulls (Lai, Xu et al. 2006), perhaps due to the fact that the Akt signaling defect in this mutant line is systemic, and therefore impacts all neurons expressing Akt1.

While the exact mechanisms are still unknown, similar disruptions in DA/NE signaling are present in the Rictor-nKO, which possibly underlies the altered response to AMPH observed in this model. Indeed, neurochemical findings in both cortical (Fig. 6) and striatal (Fig. 11) brain regions suggest that alterations in both systems, namely a DA deficit and a NE excess, characterize this model. *A priori* hypotheses regarding increased NET expression in the cortex of Rictor-nKO mice were confirmed, based off prior evidence in hypoinsulinemic mice. Conversely, similar *a priori* hypotheses regarding decreased DAT expression in the Rictor-nKO were also expected, based off findings from hypoinsulinemic mice. Indeed, while initial biochemical assessments suggested that a decreased DAT phenotype was observed in the striata of the first Rictor-nKO cohort examined (data not shown), this phenotype was observed in an aged cohort, and was not consistently replicated in the younger mice used in the studies described in this Chapter (data not shown).

While a reduction in tissue DAT levels was expected, based on prior studies of Akt-inhibited/hypoinsulinemic animals, this phenotype, in and of itself, lacks explanatory power for the AMPH hyperactivity phenotype in Rictor-nKO mice. Indeed, prior studies suggest that a loss of DAT expression leads to reduced sensitivity to AMPH, rather than enhanced (Giros, Jaber et al. 1996; Zhuang, Oosting et al. 2001; Beaulieu, Sotnikova et al. 2006; Williams, Owens et al.

2007). Therefore, *in vivo* electrochemical assessment of the striatal responses to AMPH was attempted in Rictor-nKO nulls, to assert whether particular components of AMPH release, such as total amount of DA release, versus DA clearance rates were disrupted in this model. While this approach would provide insight into the phenotypes of Rictor-nKO mice at a physiological level, these experiments were discontinued due to difficulties of the Rictor-nKO to survive the anesthesia involved in study protocol.

Thus, *in vivo* microdialysis investigations in the future may be warranted, if carefully planned *a priori* hypotheses are available and proper internal and external controls can be established. Future chronoamperometric investigations into the function of striatal systems in Rictor-nKO mice are also warranted, although this model is perhaps better suited to an *ex vivo* assessment utilizing striatal slices as opposed to an *in vivo* assessment. Such an approach would allow for investigations of DA clearance but free of confounds of genotype by anesthesia interactions on the measurements.

Additional biochemical assessments of TH and striatal DA receptors function in the Rictor-nKO were similarly inconclusive. TH and DA receptors were not different in either striatal or cortical regions of this model (data not shown). However, further pharmacological challenge of TH and DA receptor in Rictor-nKO may yield functional insights that are not apparent at the biochemical level. For example, in mice lacking the DAT, TH levels biochemically are reduced 95%, but TH at a functional level is markedly upregulated in these mutants (Jones, Gainetdinov et al. 1998). Indeed, many phenotypes characteristic of the DAT-KO, such as pronounced locomotor activity and elevations in extracellular DA, are dependent on these upregulations in TH activity. Similar findings are also observed at the level of the DA receptor in DAT-KO mice. Biochemically, there are marked reductions of DA receptor at a

total level in DAT-KO mice, but aberrant behavioral responses to DA receptor stimulation are additionally noted, as, following apomorphine administration, the DAT-KO becomes markedly rigid with strong tremor (Raul Gainetdinov, personal communication).

Therefore, particularly for the synthetic and receptor proteins involved in the response to AMPH, functional assessments of the behavioral response to these agents are warranted. The contribution of TH activity to hyperactivity in the Rictor-nKO, for example, can be determined by measuring the ability of the mutant to generate locomotor activity when challenged alpha-methyl-tyrosine, an agent that inhibits TH activity. Additionally, the sensitivity of the phenotypes in the Rictor-nKO to reagents like apomorphine can provide insight into receptor dysfunction in this model. Observations regarding the behavioral response to these agents, therefore, can then guide further lines of experimental inquiry.

Apart from the classical dopaminergic mechanisms underlying the locomotor response to AMPH, an important future direction for the Rictor-nKO involves disentangling the role of noradrenergic systems in locomotor and psychostimulant-induced phenotypes. Indeed, one potential explanation for the observed hypersensitivity to AMPH in the Rictor-nKO is the noradrenergic phenotype observed in this model. Rictor-nKO mice demonstrate increased NE tissue content, observed in cortex (Fig. 6), hippocampus, hypothalamus, cerebellum (data not shown), and striatum (Fig. 11). Additionally, NET expression is also markedly elevated in this model, in cortical regions (Fig. 7), hippocampus, and potentially the striatum (data not shown). While the effects of AMPH are traditionally attributed to the DAT, the NET is also a target of AMPH action, and both NE and the NET play important roles in behavioral response to AMPH in wild type animals. Prior studies, for example, involving microdialysis assessment of the locomotor response to AMPH, demonstrate that cortical NE is a critical mediator of locomotor

sensitization to AMPH (Darracq, Blanc et al. 1998). Additionally, prior studies also demonstrate that pretreatment with selective NET inhibitors like nisoxetine, in wild type animals, blocks the locomotor response to AMPH (Tyler and Tessel 1979).

It is clear that NE in the brain is a significant modulator of the response to AMPH, though it is currently unclear whether elevations in striatal NE in the Rictor-nKO (Fig. 11) contributes to either hyperactivity (Fig. 12) or enhanced locomotor response to AMPH (Fig. 13) in the Rictor-nKO. While controversies regarding the significance of NET innervation of the striatum are addressed previously, there is some precedent for the potential physiological significance of striatal NE, as recent evidence suggests NE-DA cross-talk occurs in the striatum, where adrenergic receptors serve to modulate DA signaling pathways in post-synaptic neurons (Hara, Fukui et al. 2010).

Therefore, one additional direction to consider for future study of the Rictor-nKO would be to use appropriately controlled studies with immunohistochemical or *in vivo* microdialysis methods to thoroughly validate whether there is any striatal NE in the Rictor-nKO, as suggested by the neurochemical data outlined in Fig. 11. There is, indeed, some data to suggest there is a role for NE in striatal behaviors. Elevations in striatal NE, for example, have been linked to phenotypes of hyperactivity in other mutant models (Jones and Hess 2003). Additionally, microdialysis studies support the existence of NE in striatal brain regions, as previous studies suggest that extracellular NE levels are $\frac{1}{4}$ those of DA levels in this brain region (Cenci, Kalén et al. 1992; McKittrick and Abercrombie 2007). Lastly, while immunohistochemical and biochemical data lack in terms of demonstrating NET expression in striatum (Miner, Schroeter et al. 2003), uptake studies, utilizing transporter knockouts as important controls, demonstrate that selective NET inhibitors affect 20% of DA uptake in this brain region (Moron, Brockington et al.

2002). Indeed, as disruption of both NE and DA systems are observed in other mouse models of Akt deficiency, validation of striatal NE transmission in the Rictor-nKO may yield novel insights into the pathogenesis of neuropsychiatric disease (Lai, Xu et al. 2006).

While the pharmacologic methods outlined above provide one methods to disentangle the contributions of various targets of AMPH and neurotransmitter systems, alternative genetic methods are also possible. A possible approach to genetically dissect out the contributors to the locomotor response to AMPH, would be to generate Rictor deletions driven by TH-cre, DAT-cre, and NET-cre, leading to deletion of Rictor in DA and NE neurons, DA neurons, and NE neurons, respectively. Correlation of locomotor, biochemical, and neurochemical phenotypes across genetic models would help to dissect out not only the molecular aspects controlling these phenotypes, but also more precisely contributions of both neurodevelopmental processes and neuroanatomy to the observed phenotypes.

It is important to note that, as with other genetic mouse models, diverse neurotransmitter systems are likely to modulate the dysfunctional phenotypes observed in Rictor-nKO mice. Conversely, the same transgenic animal can be developed to model the phenotypes of seemingly disparate conditions. Indeed, a genetic model like the DAT-KO, when challenged appropriately, can model both a hyperkinetic disorder like ADHD (Beaulieu, Sotnikova et al. 2006) or a hypokinetic disorder like Parkinson's (Sotnikova, Beaulieu et al. 2005). Thus, future evaluation of the Rictor-nKO is warranted over time, and in the context of different environmental and pharmacological challenges, to fully understand the value of the Rictor-nKO for modeling complex human disorders.

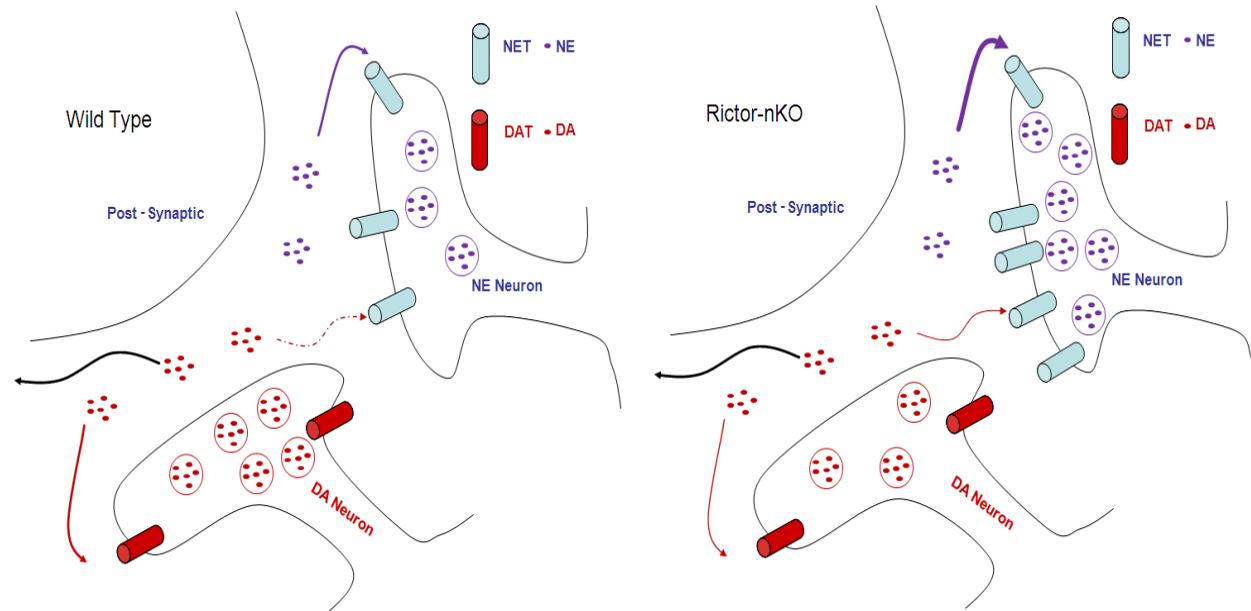
Methods

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 °C. 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).

CHAPTER 4

CONCLUSIONS, FUTURE DIRECTIONS, AND SIGNIFICANCE

Figure 14. Model of postulated changes at catecholamine synapses occurring in Rictor-nKO mice.



Model for the dysfunction observed in the Rictor-nKO, which is hypothesized to occur as a brain adaptation to an Akt-deficient or insulin resistant state. The figure above depicts a synapse where a NE and DA terminal innervate a common post-synaptic target. Extracellular levels of transmitter are assumed to be equivalent between genotypes at this time, although future studies are warranted to validate if this is true. Changes in intracellular content of transmitter between genotypes is depicted, in particular less DA (red neuron) and more NE (blue neuron) in the Rictor-nKO. Evidence from biochemical, uptake, and pharmacological studies suggest that a gain of function of the NET occurs in the Rictor-nKO (blue arrow), and this gain of function particularly impacts DA neurochemistry. A decrease in function of DAT is predicted in the Rictor-nKO (red arrow), based off evidence of prior models of PI3K/AKT inhibition, but this pathway warrants further investigation in this model. One novel component of these findings is that a minor pathway in DA clearance in wild type animals (dotted red arrow in wild type) takes on pathophysiological significance in the Rictor-nKO (red arrow in Rictor-nKO). Diffusion in the extracellular space is not yet understood in the mutant versus wild type animals (black arrow), although it can be modeled in future studies. Functional consequences of loss of mTORC2 signaling on specific neurotransmitter systems require further investigation in order to fully understand the translational potential of this model.

Implications: Insights into the mechanism of action of the NET

Emerging evidence suggests that DA and NE systems are exquisitely interrelated, with disruptions in the regulation of one of these systems causing concomitant dysfunction in the other. DA and NE are the two major catecholamines in brain, and modulate many analogous functions, including reward, locomotion, and cognition. In addition to their shared control of brain function, DA and NE derive from a common biosynthetic pathway, with DA serving as the precursor for NE. Synaptic control of both of these transmitters is accomplished by an intricately regulated system that includes reuptake by their respective transporters, namely the DAT and NET.

There is significant potential for interaction between DA and NE systems in brain, at the level of both transporter and receptor function (Rommelfanger and Weinshenker 2007; Hara, Fukui et al. 2010). At the level of transporter interactions, in prefrontal cortical regions, where DAT expression is low (Miner, Schroeter et al. 2003; Liprando, Miner et al. 2004), the NET is the major transporter responsible for DA uptake, as inhibition of the NET increases extracellular DA levels, as measured by *in vivo* microdialysis (Yamamoto and Novotney 1998; Moron, Brockington et al. 2002). In the Rictor-nKO model, the evidence outlined in Chapter 2 suggests that the relationship between the NET and cortical DA takes on a disruptive role. Pharmacological, neurochemical, and biochemical data collectively suggest that a gain of function of the NET leads to neurobehavioral and neurochemical defects in this mutant. These findings have significance for the treatment of neuropsychiatric disease, given that disorders like depression, schizophrenia, Parkinson's disease, and drug addiction are prominently linked to dysfunction of catecholamine systems and the Akt signaling pathway (Emamian, Hall et al.

2004; Arnold, Talbot et al. 2005; Bajestan, Sabouri et al. 2006; Ries, Henchcliffe et al. 2006; Cookson, Dauer et al. 2007; Russo, Bolanos et al. 2007; Krishnan, Han et al. 2008; Aubry, Schwald et al. 2009; Bitanhirwe, Weber et al. 2010).

In line with this evidence, and the findings presented in Figures 4-10, we developed the model in Fig. 14 to explain the change in the dynamics at cortical synapses that results from loss of signaling through mTORC2/Akt, as is hypothesized to occur in numerous disorders of the central nervous system. Fig. 14 depicts a hypothetical synapse where a DAT neuron and a NET neuron innervate a common post-synaptic target. In addition to a DA and NE neuron, arrows demonstrating the motion of substrate in the synaptic space are depicted in Fig. 14. The reuptake of DA and NE into their respective presynaptic terminals is depicted by red and blue arrows, respectively. The weighting of the arrows in Fig. 14 represents qualitative differences in uptake of DA and NE between genotypes. Enhanced NET function in the Rictor-nKO, in terms of an enhanced ability to take up both DA and NE, as demonstrated in Fig. 7, is represented by increased weighting of the blue arrows depicting NE uptake. In contrast to the gain of function of the NET, reductions in tissue DA content (red neuron) are depicted in the dopaminergic neurons. This reduction in DA content is reflected from findings in both striatal (Fig. 11) and cortical (Fig. 6) brain regions, and consistent with prior studies showing that loss of Akt function impairs presynaptic DA functions (Williams, Owens et al. 2007; Iniguez, Warren et al. 2008; Ries, Cheng et al. 2009).

In addition to the changes in catecholamine content that occurs in the Rictor-nKO, Fig. 14 also depicts another component of DA homeostasis that is hypothesized to occur, namely uptake of DA by the NET (dotted arrow in Fig. 14, wild type). Based off of our findings, this pathway of DA homeostasis takes on pathophysiological significance in the Rictor-nKO. This

finding is based off of evidence demonstrating increased desipramine-sensitive uptake of both DA and NE into cortical synaptosomes, and the findings that inhibition of the NET both rescues behavior phenotypes in the Rictor-nKO and increases total DA levels (Fig. 10). This evidence fits with the ability of the NET to alter DA clearance that is suggested by previous *in vivo* microdialysis and *ex vivo* synaptosomal uptake studies (Yamamoto and Novotney 1998; Moron, Brockington et al. 2002; Miner, Schroeter et al. 2003).

This potential function of the NET is intriguing given the history of NET inhibitors in treatment of neuropsychiatric disease. The tricyclic class of medications (TCA) are potent blockers of monoamine transporters in general, and used to be first line treatments for depression. However, despite the advent of selective serotonin uptake inhibitor classes of medications for the treatment of depression, TCAs remain effective alternatives for depressed patients that fail current first-line treatments (Zhou 2004). The combination DAT/NET inhibitor bupropion has efficacy as monotherapy for treatment of both depression and cigarette smoking (Zhou 2004). Other recent studies suggest that selective NET inhibitors like reboxetine have efficacy for the treatment of depression in patients with Parkinson's disease (Pintor, Baillès et al. 2006). Additionally, selective NET inhibitors like atomoxetine are important components of treatment for attention deficit hyperactivity disorder, (ADHD) (Zhou 2004), and NET inhibition has neuroprotective effects in neurotoxin models of Parkinson's disease (Rommelfanger, Edwards et al. 2007).

Most studies of NET inhibition in schizophrenia demonstrate modest effects. No benefits on cognitive symptoms have been demonstrated, although indices of cerebral blood flow are increased in prefrontal cortex of atomoxetine-treated patients (Bangs, Emslie et al. 2007; Rao, Venkatasubramanian et al. 2007; Friedman, Carpenter et al. 2008; Kelly, Buchanan et al. 2009;

Poyurovsky, Faragian et al. 2009; Sacco, Creedon et al. 2009; Ball, Warren et al. 2011). However, none of these studies employed a design where NET inhibitors were given as monotherapy. Intriguingly, while there has not yet been a demonstrated effect of NET inhibitors on primary cognitive outcomes in schizophrenia yet, there is some evidence to suggest that NET inhibition prevents the manifestation of extrapyramidal motor defects in patients treated with antipsychotic medications (Kelly, Buchanan et al. 2009), an intriguing findings given the neuroprotective effects of NE in animal models of parkinsonism (Rommelfanger, Weinshenker et al. 2004; Rommelfanger, Edwards et al. 2007). Indeed, given that depressive and cognitive symptoms are part of the prodrome for both schizophrenia and Parkinson's disease, future prospective studies of normal individuals treated with NET inhibitors has value for determining the value of the NET in the manifestation of neuropsychiatric symptoms over time.

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