# BRAIN WITHOUT RICTOR:

# mTORC2 SIGNALING REGULATES CENTRAL DOPAMINE HOMEOSTASIS

By

Olga Igorevna Dadalko

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

December, 2015

Nashville, Tennessee

**Approved:**

Aurelio Galli, Ph.D.
Danny Winder, Ph.D.
Eugenia Gurevich, Ph.D.
Todd Graham, Ph.D.
Ariel Deutch, Ph.D.

## DEDICATION

Dedicated to my parents, Igor and Natalia Dadalko,

whose love and encouragement is felt despite long distances.

## ACKNOWLEDGMENTS

I would like to acknowledge the funding without which this work would not have been possible. Vanderbilt Interdisciplinary Graduate Program training grant and the Vanderbilt Brain Institute Scholars Award provided excellent support during the initial years of my predoctoral career. Further financial support was contributed by National Institutes of Health Grants awarded to my mentor, Dr. Aurelio Galli (DK085712).

I thank my advisor, Dr. Aurelio Galli, for his mentoring and support. Graduate training is a challenging endeavor in a young person's life. I highly value and appreciate Dr. Galli's advice to help me navigate both scientific and life challenges. It is with his guidance that these challenges were transformed into great opportunities for professional and personal growth. I am also forever grateful to the members of my thesis committee: Drs. Danny Winder, Eugenia Gurevich, Ariel Deutch, and Todd Graham, for their suggestions and guidance throughout my graduate career. I thank my former committee member and our collaborator Dr. Gregg Stanwood for all his work, support and encouragement.

I am indebted to many people at Vanderbilt, which collaborative and friendly environment is stimulating and nurturing for a young scientist. I would like to acknowledge Drs. Eugenia Gurevich, Kevin Niswender, David Wasserman, and Gregg Stanwood for all their time and effort in equipping me with knowledge, reagents, and encouragement where I needed it to master various techniques and to allow me to thoroughly explore my interdisciplinary project. Such

collaborative spirit is now rooted in the essence of my scientific quest, which I think is an invaluable quality for a scientist in the modern world.

I would like to recognize the Clinical Neuroscience Scholars Program and Dr. Neil Woodward, my clinical neuroscience mentor. His role in my understanding the neuroimaging research is invaluable. I greatly appreciate his insight and guidance as I plan my future career with neuroimaging in mind. It has also been a real pleasure and a great advantage to be a part of NPISA (Neuroscience Program in Substance Abuse) club, founded by Drs. Galli and Winder. The topics discussed during the NPISA meetings elevated my understanding of molecular neuroscience and provided an opportunity to meet and talk to the ones of the most inspirational scientists in the field.

The members of the Galli laboratory have truly become my second family. I cannot imagine working in a more friendly, encouraging, and productive environment. I thank Drs. Kevin Erreger, Christine Saunders, and Heiner Matthies for providing their expertise on scientific matters, as well as offering guidance throughout my tenure as a graduate student. I would specifically like to recognize the help and support of Amanda Poe and Nicole Bibus Christianson, who are the glue of the Galli lab and are indispensible for every single person working in the laboratory.

Finally, I would like to thank my family and friends for their support. Even from overseas, my parents' love and encouragement are palpable. I would not be where I am today without their example of hard work, perseverance and devotion.

## TABLE OF CONTENTS

**Page**

## LIST OF FIGURES

**Figure** **Page**

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AADC | Aromatic-L-amino acid decarboxylase |
| ADHD | Attention deficit hyperactivity disorder |
| AMPH | Amphetamine |
| C | Celsius |
| COMT | Catechol-O-methyl transferase |
| CNS | Central nervous system |
| $D_2R$ | D2 dopamine receptor |
| DA | Dopamine |
| DAT | Dopamine transporter |
| g | Gram |
| IR | Insulin receptor |
| IRS | Insulin receptor substrate |
| kDa | Kilo Dalton |
| KO | Knockout |
| L | Liter |
| M | Molar |
| µg | Microgram |
| µM | Micromolar |
| mg | Milligram |
| min | Minute |
| mL | Milliliter |

| | |
|---|---|
| mM | Millimolar |
| mTORC2 | Mammalian target of rapamycin complex 2 |
| NAc | Nucleus accumbens |
| NE | Norepinephrine |
| NET | Norepinephrine transporter |
| ng | Nanogram |
| PET | Positron emission tomography |
| PDK1 | Phosphoinositide-dependent kinase-1 |
| PFC | Pre-frontal cortex |
| PI3K | Phosphatidylinositol 3-kinase |
| PIP2 | Phosphatidylinositol-4,5-bisphosphate |
| PIP3 | Phosphatidylinositol-3,4,5-trisphosphate |
| sec | Second |
| TH | Tyrosine hydroxylase |
| VMAT | Vesicular monoamine transporter |
| wt/WT | Wild-type |

# CHAPTER I

# INTRODUCTION

## Overview of Dopamine Neurotransmission

*Dopamine as a neurotransmitter: Discovery*

The sophisticated ability of the nervous system to rapidly convert electrical signals into chemical, appropriately integrating excitatory, inhibitory and modulatory inputs, enables the brain to control our physiology and behavior. Neurotransmitters are chemical transducers propagating intraneuronal electrical signals of an action potential interneuronally, thereby forming neurocircuitry that enables the brain to function. Modulatory neurotransmitters, such as catecholamines, are essential to shaping both the amplitude as well as the duration of excitatory and inhibitory neuronal signaling. Dopamine (DA, 3,4-dihydroxyphenethylamine) is a monoamine neurotransmitter, which contains a catechol group connected to an aromatic ring by a two-carbon chain. DA shares the common biosynthetic pathway with another catecholamine neurotransmitter, norepinephrine (NE) (Figure 1), and initially was considered only as an intermediate in its production. The importance of the brain DA system was recognized once the studies on Parkinson disease revealed the dependence of movement on central DA: the loss of normal motor function was shown to stem from the degeneration of DA neurons in substantia nigra pars compacta (SNc) in

the midbrain. DA received its neurotransmitter status after seminal work of Avid Carlsson and coworkers showing that it is indeed DA, and not NE, which was essential for the DOPA-mediated restoration of reserpine-induced movement abnormalities (Carlsson et al., 1957). Further research revealed the essential role of DA for many brain functions, including motor control, salience, reward, learning, memory, attention, and mood.

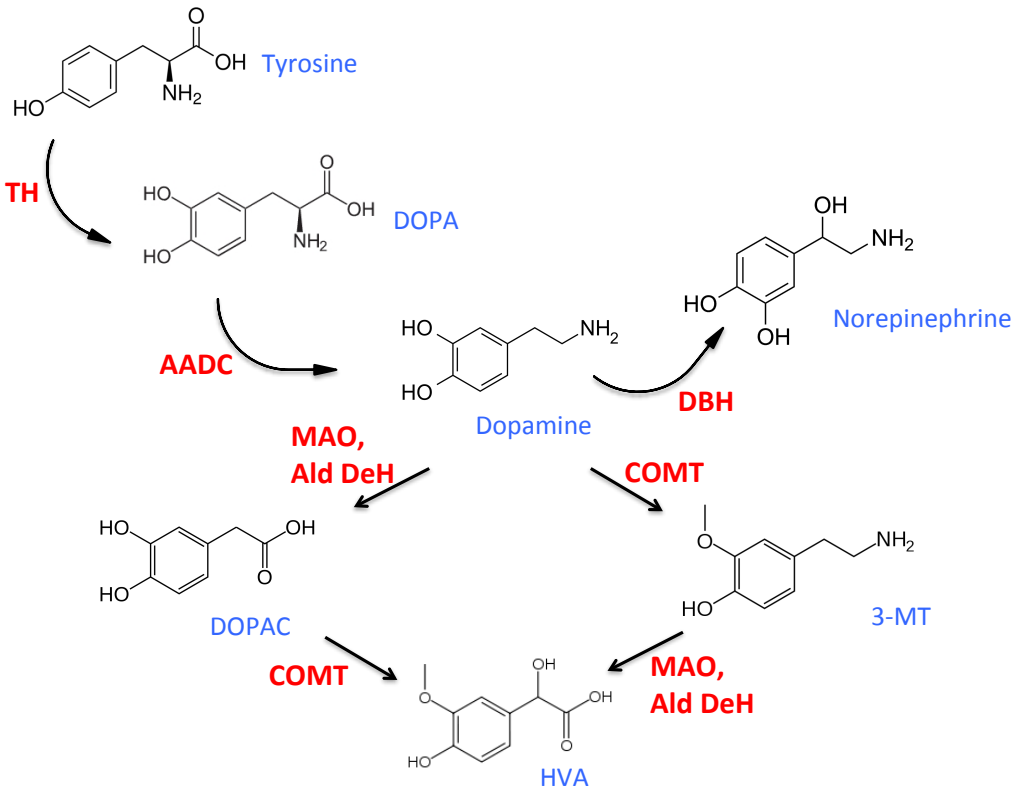



**Figure 1. Dopamine biosynthesis and metabolism.** Enzymes are labeled in red, and reactants/products are labeled in blue.

Early studies on brain DA demonstrated that it was enriched in the basal ganglia (Bertler and Rosengren, 1959), highlighting the importance of the brain region specificity in DA signaling. In addition, Parkinsonian patients showed reduced DA levels in the basal ganglia, linking striatal DA neurotransmission to movement (Ehringer and Hornykiewicz, 1960, 1998). Finally, the important role of DA in motor control was established when treatment with L-DOPA (L-3,4-dihydroxyphenylalanine, the immediate DA precursor, allowing to bypass the rate limiting step in DA biosynthesis) provided therapeutic effect in patients with Parkinson disease (Barbeau, 1962; Birkmayer and Hornykiewicz, 1962; Carlsson, 1987). The specificity of the brain region involved suggested that unique dopaminergic pathways may play a fundamental role in disease pathophysiology.

Accumulating evidence linking striatal DA tone and motor function served a pivotal role in the DA hypothesis of schizophrenia. A pioneering idea of Henri Laborit to use chlorpromazine to treat psychotic patients was tested in 1951 by John Delay and Pierre Deniker, who confirmed that high doses of this drug calm highly agitated schizophrenic or manic depressive patients. Chlorpromazine-induced extrapyramidal side effects targeted DA-dependent motor system, which allowed for the hypothesis that DA neurotransmission may be altered in schizophrenia. Indeed, Carlsson and Lindqvist analyzed the action of chlorpromazine and haloperidol, another typical antipsychotic with similar properties, in the mouse brain. By demonstrating an increased rate of DA turnover, they suggested that the drugs acted by blocking DA receptors (1963)

(Carlsson and Lindqvist, 1963). This theory was later confirmed by radioactive binding (Burt et al., 1975; Seeman et al., 1975).

*Overview of the DA neuroanatomy*

Development of fluorescent DA allowed for mapping of the dopaminergic pathways in the brain (Dahlstroem and Fuxe, 1964; Fuxe et al., 1965; Ungerstedt, 1971). Combining pharmacological approaches with lesion techniques, scientists started to tease apart the DA pathways and their unique disease implications (Anden, 1970; Anden et al., 1970b; Anden et al., 1970a; Anden, 1972). The three most extensive DA pathways include mesolimbic (projecting from the VTA to the nucleus accumbens, septum, amygdala, olfactory tubercle, and piriform cortex), mesocortical (from the VTA to the prefrontal, cingulate, and entorhinal cortices), and nigrostriatal pathway (supplying DA from the SNc to the dorsal striatum: nuclei of caudate and putamen) (Figure 2). These DA circuits are implicated in a variety of physiological and behavioral processes. They are extensively studied to determine each pathway's individual contribution, as well as their intricate interplay in health and disease. Recently, techniques such as optogenetics, DREADDs (designer receptors exclusively activated by designer drugs), and CLARITY (Clear, Lipid-exchanged, Anatomically Rigid, Imaging/immunostaining compatible, Tissue hYdrogel) has tremendously advanced our understanding of DA pathways in health and disease (Baik, 2013; Lerner et al., 2015; Saunders et al., 2015; Zheng and Rinaman, 2015).

DA biosynthesis is a two-step process limited to the DA neurons, the majority of which originate in mesencephalon (particularly in the SNc and the VTA) and diencephalon (predominantly in the hypothalamus), as well as in the dopaminergic cells within so-called ultra-short DA systems in the retina and olfactory bulb.

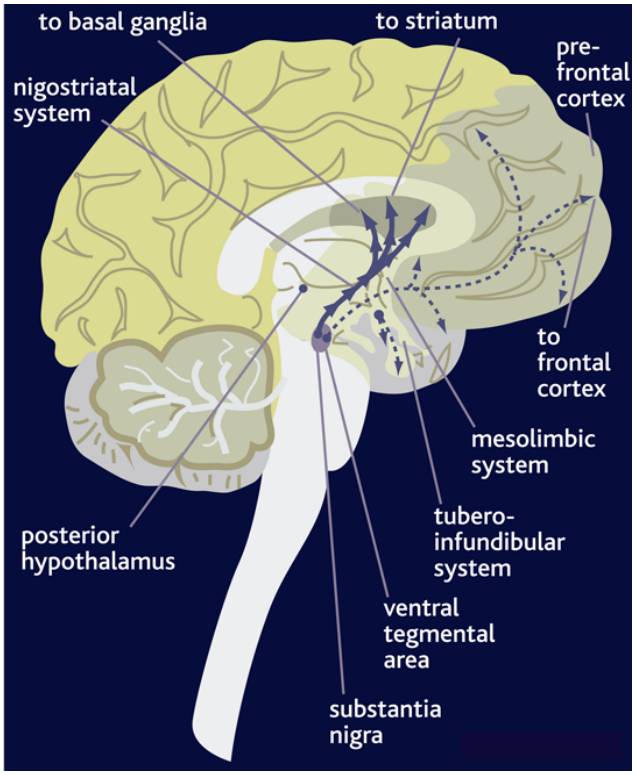



**Figure 2. Dopamine pathways in the brain.** Shown are the four major dopaminergic tracts in the brain: nigrostriatal and mesocorticolimbic systems originate in the midbrain in the substantia nigra and the VTA, respectively. The nigrostriatal pathway supplies DA to the dorsal striatum (implicated in movement disorders). Mesocorticolimbic system projects to the cortical structures (important for affect, emotion, and motivation; implicated in schizophrenia). The smaller, tuberoinfundibular system originates in the arcuate nucleus of the hypothalamus and projects to the pituitary gland (important for the endocrine system). Image obtained from *cnsforum.com*

Tyrosine hydroxylase (TH), the rate-limiting enzyme in DA biosynthesis, converts amino acid tyrosine into L-DOPA. Tyrosine penetrates the blood brain barrier by means of an active transport process, which, considering normal dietary consumption of this amino acid, fully saturates TH activity. Therefore, in a case of decreased brain dopamine content, administration of supplementary tyrosine does not produce a therapeutic effect, in contrast to L-DOPA, which increases catecholamine synthesis by penetrating the blood brain barrier and bypassing this rate-limiting enzymatic step. L-DOPA is then further synthesized into DA by aromatic amino acid decarboxylase (AADC) (Figure 1). Utilizing a steep electrical gradient created by ATP-powered proton pumps, DA is then packaged into vesicles against its chemical gradient by the vesicular monoamine transporter (VMAT) and prepared for synaptic release.

The integrity of DA neurotransmission depends on the rates of DA synthesis and release as well as on the efficiency of extracellular DA clearance. For signal termination, many neurotransmitter systems rely on the system of enzyme-mediated catabolism. Similarly, both monoamine oxidase (MAO) and catecholomethyl transpherase (COMT) aid in the process of signal termination by mediating the enzymatic DA catabolism into its metabolites 3-MT, DOPAC, and HVA (Figure 1). However, for the catecholaminergic systems, the main method of neurotransmitter clearance and, therefore, signal termination, is transporter-mediated. The first evidence for a transport mechanism for biogenic amines dates to the early 1960's, when Axelrod and colleagues showed accumulation of norepinephrine in nerve endings, followed by its stimulated release (Axelrod et

al., 1961). Further characterization of the newly discovered mechanism revealed that DA undergoes similar process in distinct brain regions (Ross and Renyi, 1967). It took two decades to clone and categorize the monoamine transporters(Kilty et al., 1991; Pacholczyk et al., 1991; Shimada et al., 1991). Availability of the cDNAs allowed for extensive investigations of the transport mechanism.

*Dopamine Transporter*

At the dopaminergic synapse and perisynaptic space, the DA transporter (DAT) is an integral membrane protein consisting of 12 transmembrane domains, with intracellular N- and C-termini. DAT actively translocates DA from the extracellular milieu back into the dopaminergic neurons, efficiently terminating DA signaling, and thereby preventing DA spillover to other synapses. Once recycled, DA is then repackaged into the vesicles (Giros and Caron, 1993), or degraded by MAO. DA recycling is essential for the integrity of synaptic DA release: the recycled pool is the main source of DA for the vesicular packaging (Giros et al., 1996). For fulfilling its vital functions, DAT is deemed to be the key regulator of DA neurotransmission in the brain. Studies in transgenic animal models as well as clinical research show that altered expression or function of the DAT has significant impact on DA-dependent behaviors and disease. Thus, DAT knockout (KO) mice exhibit at least a 100 times slower DA clearance compared to control animals, consequentially showing hyperdopaminergic-like behaviors, such as extreme hyperactivity, impulsivity, and anxiety in the novel

environment. In addition, DAT KO mice demonstrate a substantial reduction in the stimulated vesicular DA release, supporting the importance of DAT-mediated DA recycling for the intact DA signaling (Giros et al., 1996; Gainetdinov et al., 1999a; Jones et al., 1999).

An important contribution to the understanding of DAT function in regulating central DA neurotransmission and DA-dependent behaviors is provided by the research exploring DAT overexpression. These studies utilized amphetamine (AMPH) and cocaine, two psychostimulants adopted for studying DAT physiology and proven to be excellent readouts for DA-dependent endophenotypes. Cocaine acts by blocking DAT and preventing DAT-mediated DA reuptake, which increases extracellular DA concentration (Sulzer et al., 2005; Williams and Galli, 2006). AMPH acts to reverse DAT and causes DAT-mediated DA efflux (Figure 3). Mouse model of elevated DAT protein expression (DAT-tg), as well as mice with an increased DAT surface expression (Gpr37-/-), exhibit exaggerated AMPH-induced hyperlocomotion, with no change in basal locomotion (Marazziti et al., 2004; Marazziti et al., 2007; Salahpour et al., 2008). In addition, cocaine-induced locomotion is unaltered in the DAT-tg mice, but is reduced in the Gpr37-/- mice compared to control animals. In contrast to DAT KO mice, animals overexpressing DAT have reduced extracellular DA. Thus, DAT expression and function dictate synaptic DA bioavailability as observed in the DAT KO and DAT overexpressing mice.

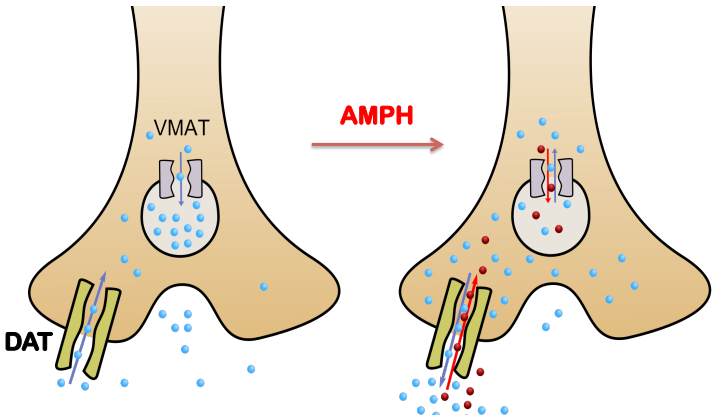



**Figure 3. Amphetamine-induced DA efflux.** Amphetamine (AMPH, red dots) is a DAT substrate, which reverses the transport of DA (blue dots), resulting in DAT-mediated DA efflux. AMPH also acts as a competitive inhibitor, preventing high-affinity DAT-mediated DA reuptake. Once in cytosol, AMPH increases the intracellular DA concentration (providing additional substrate for the escalating efflux) *via* activating TH, inhibiting MAO, and depleting DA-containing synaptic vesicles by collapsing vesicular pH gradient and disrupting function of the VMAT-2 (vesicular monoamine transporter-2, which sequesters DA from the cytosol creating highly concentrated vesicles, utilizing energy from an ATP-driven inward vesicular proton pump). Reviewed by (Sulzer, 2011).

Of interest, the Palmiter group showed that expression of DAT is not altered after DA depletion in the neonatal brain (Zhou and Palmiter, 1995), suggesting that DAT availability is not dependent on DA levels. Importantly, such DA-dependent phenotypes are produced despite multiple compensatory developmental adaptations that occur in the transgenic mice in an effort to reestablish normal DA neurotransmission. Despite accumulating data, the complex dynamic interactions of the molecular players maintaining the integrity of DA homeostasis remain incompletely understood. However, available evidence

suggests that aberrant DA-dependent physiology and behaviors may be caused by impaired DAT function.

Highlighting the importance of DAT in the central DA neurotransmission is the fact that DAT is implicated in many DA-related psychiatric disorders, as reviewed by (McHugh and Buckley, 2015). DAT polymorphisms were associated with treatment-resistant schizophrenia (Bilic et al., 2014) as well as attention deficit hyperactivity disorder (ADHD) (Kovtun et al., 2015). *De novo* genetic variants were identified in the DAT of patients with autism spectrum disorder (Neale et al., 2012), a pivotal discovery implicating DAT in the pathophysiology of autism. This finding provided a great opportunity to study how changes in transporter's structure impact its function and, therefore, alters DA-dependent physiology (Hamilton et al., 2013; Bowton et al., 2014; Cartier et al., 2015; Hamilton et al., 2015). Despite technical difficulties surrounding the method of crystallization of integral membrane proteins, the rapid progress in the field of crystallography in the last decade rendered a high-resolution X-ray crystallographic structure of the bacterial leucine transporter (LeuT), a prokaryotic homolog of the solute carrier 6 (SLC6) gene family that is structurally and functionally related to DAT (Yamashita et al., 2005). Based on the crystal structure, and utilizing homology modeling to engineer simulation systems that include genetic mutations in question, structural biologists employ electron spin resonance technique to measure the distances between amino acids and protein moieties with high precision. This allows for construction of electronic protein models and simulations of DAT and its genetic variants, which are used to

understand how intrinsic molecular biomechanics of the transporter effect its reuptake function (Hamilton et al., 2013).

*Dopamine Transporter Function and Regulation*

As mentioned before, monoamine transporters are homologous, and belong to the SLC6 gene family, which constitutes $Na^+/Cl^-$ -dependent neurotransmitter-sodium symporters (Pacholczyk et al., 1991). These transporters utilize secondary active transport by coupling neurotransmitter reuptake with a sodium gradient across the cellular plasma membrane. Logically, cloning of the DAT stemmed immediately from already known sequences of norepinephrine and serotonin transporters, revealing a high level of homology between the transmembrane domains and the intracellular loops of these transporter proteins (Kilty et al., 1991; Shimada et al., 1991). Currently, discovery of a genetic variation that changes one of the transporters physiology permits scientists to extrapolate the outcomes and assess similar questions for the other SLC6 family members, allowing for fruitful findings that advance the transporter field as a whole.

Availability of DAT cDNA allowed for detailed studying of this protein in heterologous expression systems, advancing our knowledge of transporter biomechanics, pharmacological properties, and means of regulation. Performing high affinity DA reuptake, the DAT plays a crucial role in brain DA neurotransmission. It is not surprising, therefore, that expression and function of DAT are tightly regulated. Two mechanisms can be specified: the long-term one

is executed *via* control over protein biosynthesis and anterograde trafficking, and the transient one, which controls the intrinsic properties of the transporter function (Blakely and Bauman, 2000). Despite accumulating data regarding DAT function, the control of transcription, translation, and anterograde trafficking of the transporter remain poorly understood. For example, the most extensively studied DAT transcription factor, Nurr1, was shown to be influenced by many kinases and phosphatases, but regulatory checkpoints of the DAT gene *SLC6A3* transcription have not yet been elucidated. Similarly, neither the mechanism nor the upstream molecular regulators of coordinating Nurr1 function in *SLC6A3* transcription have been identified.

In contrast to DAT expression and anterograde trafficking, regulation of the transporter reuptake properties and intrinsic biomechanics has been extensively studied in the last three decades. Many DAT posttranslational modifications were shown to regulate transporter function. Naturally occurring DAT gene variants coupled with site-directed mutagenesis techniques have provided scientists with material to study structure/function relationships. This allowed for the identification of important motifs for post-translational modifications of DAT, as well as paradigms of both biomechanics of transporters' function and its physiology. It was shown that the DAT requires N-glycosylation in the second extracellular loop for trafficking from the Golgi to the cell surface (Zahniser and Sorkin, 2009). In addition, utilizing cross-linking reagents and FRET imaging techniques in heterologous expression systems, DAT was found to form oligomers in the ER, an essential step for the DAT to get shuttled to the

plasma membrane (Hastrup et al., 2001; Hastrup et al., 2003; Sorkina et al., 2003), suggesting a possible mechanism for the DA neuron to control DAT surface expression. The integrity of the DAT homodimer was found to be sensitive to psychostimulants binding (Hastrup et al., 2003), suggesting that DAT oligomerization may play a role in the transporter function. Indeed, a recent study showed that the transporter function and its relationship with psychostimulants depend on the composition of multimeric proteins: some mutant protomers were able to change the function of the wild-type DAT components, as well as the entire multimer function (Zhen et al., 2015). Considering the abundance of DAT genetic variants in the human population, as well as epidemiologic data linking mutant DAT alleles to DA-related mental illnesses, further studies are warranted to unravel the detailed mechanism of DAT oligomerization and its physiological relevance *in vivo*.

Furthermore, DAT phosphorylation was found to regulate transporter reuptake and efflux functions, as well as DAT cell surface expression. Cloning of the DAT allowed for the proposal of its putative structure, which permitted identification of multiple consensus sequences for protein kinase C (PKC) protein kinase A (PKA), and calcium/calmodulin-dependent kinase II (CaMKII) in the N- and C-terminus and intracellular loops of the transporter (Giros and Caron, 1993). *In vitro* studies utilizing chimeras identified discrete DAT domains responsible for substrate recognition, translocation, and affinity (Buck and Amara, 1994; Giros et al., 1994; Syringas et al., 2000). Further research showed that such vital DAT functions as high affinity DA reuptake, psychostimulant-

mediated DA efflux, transporter cell surface expression, and interaction of DAT with other proteins depend on the phosphorylation state of the transporter termini (Bauman et al., 2000; Torres et al., 2001; Carneiro et al., 2002; Lee et al., 2004). For example, it was shown that CaMKII binding to the DAT C-terminus facilitates phosphorylation of the N-terminus, mediating AMPH-induced DA efflux (Fog et al., 2006). Of interest, many studies now focus on the importance of electrostatic interactions of the DAT N-terminus with the negatively charged $PIP_2$ lipids of the plasma membrane, suggesting that the preferred cell surface location of the transporter is within the lipid rafts (Adkins et al., 2007; Jones et al., 2012; Gabriel et al., 2013; Kovtun et al., 2015). Therefore, both intrinsic transporter function and trafficking are tightly regulated by multiple signaling pathways, including PKC, mitogen-activated protein kinase (MAPK), and, importantly, insulin-PI3K signaling pathway (Torres, 2006). Considering how critical DAT is in regulating central DA tone, fine-tuning of the abovementioned signaling pathways is expected to have high importance in the integrity of the brain DA neurotransmission.

## Insulin in the Central Nervous System

In the periphery, insulin signaling is indispensable for the regulation of plasma glucose levels. Brain glucose utilization, however, is not insulin dependent. Rather, functions of insulin in the brain range from signaling peripheral metabolic status to the regulation of reward, development, cognition

and others (Schulingkamp et al., 2000; Daws et al., 2011). The notion of insulin presence in the brain was controversial until 1967, when the use of sensitive radioimmunoassay techniques demonstrated not only that insulin is present in the cerebro-spinal fluid (CSF), but also that its CSF levels are increased with peripheral insulin infusion (Margolis and Altszuler, 1967). Furthermore, the insulin receptor (IR) is widely expressed throughout the brain, including in dopaminergic and noradrenergic neurons (Schulingkamp et al., 2000). Despite the ongoing debate over the source of brain insulin, the majority of evidence demonstrates that the CNS insulin concentration depends on the fidelity of the active and, saturable transport of pancreatic insulin at the blood brain barrier (Schwartz et al., 1992; Baura et al., 1993; Banks, 2004). Indeed, alterations in the plasma insulin concentration are mirrored by the changes in the CSF insulin level (Schwartz et al., 1990). Human positron emission tomography (PET) studies showed attenuated neuronal activity evoked by a peripheral insulin injection in non-diabetic subjects with insulin resistance (Anthony et al., 2006). Such a tight correlation between peripheral and central insulin tone supports the fact that altered peripheral insulin levels are capable of disrupting insulin signaling in the brain.

*Insulin regulates brain DA tone via altering DAT function*

Due to the escalating obesity epidemic in the United States, an important public health concern is the comorbidity of metabolic disorders and mental illness. Mounting evidence suggests that metabolic insulin-PI3K signaling is

implicated in the pathogenesis of DA-associated brain dysfunctions, which manifest in many mental diseases such as schizophrenia (Emamian et al., 2004; Siuta et al., 2010), drug addiction (Mazei-Robison et al., 2011; Neasta et al., 2011; Chen et al., 2012; Collo et al., 2012), and bipolar disorder (Beaulieu and Caron, 2008). One of the crucial regulators of DA tone in the brain, the DAT, is shown to heavily rely on the intact insulin-PI3K signaling (Garcia et al., 2005; Williams et al., 2007; Lute et al., 2008).

The pioneering evidence that insulin signaling regulates brain catecholamine homeostasis was obtained from rodents pharmacologically rendered hypoinsulinemic. These animals demonstrated diminished AMPH-induced locomotor activity and stereotypy, both of which were reversed by insulin treatment (Marshall, 1978). As mentioned earlier (Figure 3), AMPH exerts its psychostimulant action *via* DAT-mediated DA efflux (Fischer and Cho, 1979; Sulzer et al., 2005; Sulzer, 2011), therefore its effect is highly dependent on the DAT cell surface expression. Alleviation of the decreased AMPH-induced behaviors with insulin treatment showed that insulin signaling may affect DAT trafficking *in vivo*. Later studies demonstrate that insulin signaling is required to maintain DAT cell surface expression. Thus, pharmacological or diet-induced insulin-PI3K pathway inhibition causes clathrin-mediated dynamin-dependent DAT endocytosis (Carvelli et al., 2002), leading to a dramatic reduction of DAT surface expression and function *in vitro* (Doolen and Zahniser, 2001; Carvelli et al., 2002; Lute et al., 2008), and *in vivo* (Owens et al., 2005; Williams et al., 2007; Sevak et al., 2008b; Sevak et al., 2008a; Speed et al., 2011). Thus, a strong link

between metabolic insulin-PI3K signaling and brain DA homeostasis was established, and studies began to explore the mechanisms supporting this connection.

*Insulin Signaling Pathway in the Brain*

In addition to DAT trafficking, insulin has been shown to be the key mediator of essential neuronal functions in the brain, including neurotransmitter release and reuptake, receptor function, neuronal development and survival (van der Heide et al., 2006). PI3K (phosphatidylinositol 3-kinase) lipid kinase and protein kinase B (Akt) were found to be the essential molecules regulating insulin function in the CNS (Banks et al., 2012). Upon ligand binding and intracellular tyrosine autophosphorylation, the insulin receptor activates the scaffold protein IRS (insulin receptor substrate) (White, 2002). PI3K, a lipid kinase activated by IRS, converts phosphatidyl-inositol into the phosphoinositide phosphates $PIP_2$ and $PIP_3$, which recruit 3-phosphoinoitide-dependent protein kinase-1 (PDK-1) and Akt (protein kinase B) to the plasma membrane (Figure 4). Membrane-localized Akt is subsequently activated through phosphorylation at two key residues – Thr308 by PDK1 (van der Heide et al., 2006) and Ser473 by mTORC2 (mammalian target of rapamycin complex 2) (Sarbassov et al., 2005). Phosphorylated Akt is involved in multiple cellular functions including metabolism, cell stress, cell-cycle, apoptosis, and the regulation of protein synthesis and trafficking (Franke, 2008b, a).

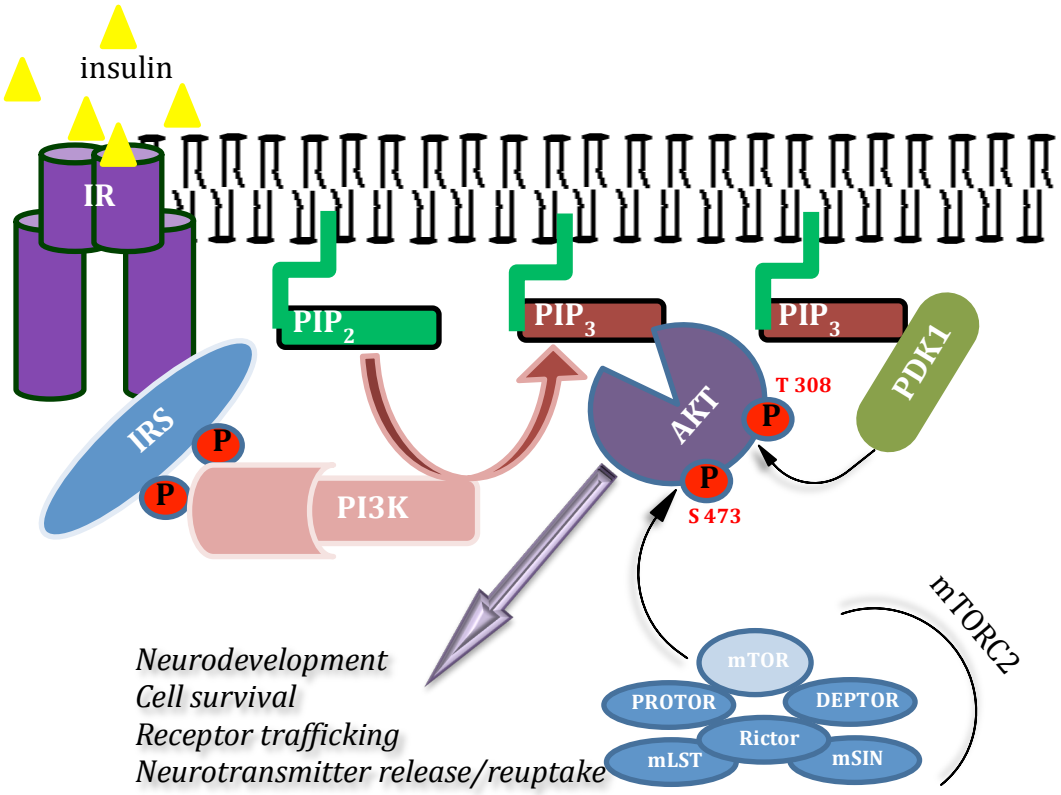



**Figure 4. Insulin signaling in the brain.**

mTORC2, rapamycin-insensitive complex containing mammalian target of rapamycin (mTOR), is ubiquitously expressed and is involved in cell survival, growth, and metabolism (Weber and Gutmann, 2012). Rictor (rapamycin-insensitive companion of TOR) is a component of the mTORC2 complex, the scaffolding protein that holds the multiprotein mTORC2 together, allowing for its kinase activity. mTORC2 is required for phosphorylation and activation of AGC kinase family members (Oh and Jacinto, 2011), including serum/glucocorticoid regulated kinase 1 (SGK1), protein kinase C α (PKCα), and Akt (Weber and

Gutmann, 2012). In the periphery, Akt has been shown to be the key kinase that mediates insulin's glucoregulatory effects (Bae et al., 2003; Kumar et al., 2008).

Akt is a serine/threonine protein kinase at the center of metabolic insulin signaling (Franke, 2008b). In the CNS, Akt activity is indispensible for both homeostatic neuroendocrine responses involved in energy balance, peripheral fat metabolism, and reproduction (Bruning et al., 2000; Obici et al., 2002; Niswender et al., 2003; Koch et al., 2008; Kim et al., 2012), and for higher brain functions involved in memory acquisition and storage, reward and salience processing, and mood and affect states (Kalkman, 2006; Spencer, 2009; Kitagishi et al., 2012; Kanoski et al., 2013; Shi et al., 2014; Machado-Vieira et al., 2015). Akt activity and substrate selectivity depends on the phosphorylation state of its residues Thr308 and Ser473 (Franke, 2008b, a). Despite accumulating evidence, molecular and cellular conditions that influence Akt phosphorylation are not fully understood. Available data shows that both the extracellular environment as well as intracellular context may lead to differential phosphorylation of Akt, predicting appropriate substrate selection by this kinase. Phosphorylation of Akt at Ser473 requires the rapamycin-insensitive mTORC2 (Zoncu et al., 2011). This suggests that mTORC2 is essential in controlling the activity as well as the substrate selectivity of the kinase, makingmTORC2/Akt signaling one of the main regulators of downstream insulin signaling in the brain.

### *mTORC2/Akt in DA-Associated Neuropsychiatric Disorders*

As discussed above, accumulating data support that metabolic kinase Akt is implicated in the pathogenesis of DA-associated brain dysfunctions, which manifest in many neuropsychiatric diseases. Data from our laboratory and others show that Akt signaling regulates brain monoamine homeostasis (Robertson et al., 2010; Siuta et al., 2010; Saunders et al., 2014), particularly DA neurotransmission (Speed et al., 2011; Dadalko et al., 2015), providing a possible molecular link between aberrant Akt signaling and disrupted central DA neurotransmission. Important in this context are findings demonstrating that fine tuning of Akt activity by its phosphorylation at residues Thr308 and Ser473 plays essential role in regulating central DA homeostasis.

One of the key regulators of Akt function is mTORC2 that renders Akt active by phosphorylating its Ser473 residue. Rictor, the rapamycin insensitive component of mTORC2, is essential for the complex formation, making mTORC2/Rictor necessary for Akt Ser473 phosphorylation (Sarbassov et al., 2005). Clinical evidence supports a role for mTORC2/Rictor - Akt signaling in mental health. Lithium, used to treat bipolar disorder, stimulates phosphorylation of Akt at Ser473 (Chalecka-Franaszek and Chuang, 1999). Certain antidepressants (Krishnan et al., 2008), along with both typical (Emamian et al., 2004) and atypical antipsychotics (Lu and Dwyer, 2005), also increase Akt Ser473 phosphorylation. Furthermore, findings of diminished Akt Ser473 phosphorylation and/or its activity in post-mortem brains of patients with schizophrenia (Zhao et al., 2006) and depression (Karege et al., 2007)

strengthen the association between dysregulation of mTORC2/Akt signaling and development of DA-associated psychiatric illnesses. Nonetheless, the molecular mechanisms linking mTORC2/Rictor - Akt signaling to impairments in central DA neurotransmission and behaviors have remained elusive.

*mTORC2/Rictor/Akt Signaling Regulates DA Tone: DAT*

Studies conducted in the last decade have unearthed many details in the mechanism of DA neurotransmission regulation by the mTORC2/Rictor - Akt signaling. As discussed above, one of the key regulators of presynaptic DA is DAT. *In vitro* studies show that expression of the dominant-negative Akt mutant or application of pharmacological Akt inhibitors decreased the cell-surface expression of DAT, whereas a constitutively active form of Akt inhibited AMPH-induced DAT internalization in the human embryonic kidney 293 cells (Garcia et al., 2005). Importantly, these DAT trafficking effects were observed within minutes after the applied stimulus (Carvelli et al., 2002; Garcia et al., 2005; Lute et al., 2008). In addition, it was found that mTORC2/Rictor - Akt signaling plays a crucial role in regulating trafficking of catecholamine transporters in the brain: norepinephrine transporter (NET) in the cortex (Siuta et al., 2010) and hippocampus (Robertson et al., 2010), and DAT in the striatum (Speed et al., 2011). Both DAT and NET cell surface expression were shown to depend on the altered phosphorylation state of the key Akt residues, Thr308 and Ser473. *In vitro* studies using isoform-specific, allosteric Akt inhibitors that block the phosphorylation of Akt (Lindsley et al., 2005), suggest that the molecular

machinery linking mTORC2/Rictor - Akt signaling and brain DA homeostasis relies on Akt phosphorylation and function (Speed et al., 2010). Using AMPH-induced DA efflux as a biochemical and behavioral readout, our published *in vivo* data reveal that Akt function supports DAT plasma membrane expression (Williams et al., 2007; Speed et al., 2011). In contrast with the precise pharmacologic or genetic Akt targeting used in the *in vitro* studies, more physiologically relevant approaches were utilized to disrupt Akt activity in these *in vivo* investigations (induction of diabetes type I-like and diabetes type II-like states). In the study of Williams and collaborators, streptozotocin-treated and therefore insulin-depleted rats showed the reduction of pGSK$\alpha$, an Akt target, in the dorsal striatum (Williams et al., 2007). Speed et al used diet-induced obesity in rats to cause brain insulin resistance, thereby reducing pAkt-308 (Speed et al., 2011). Of interest, most studies show that the reduction in pAkt-308 or the complete oblation of Akt activity (pharmacologically or *via* dominant negative mutation) is the underlying cause of reduced surface DAT expression. Limited data is available, to date, to tease apart differential Akt phosphorylation and its role in the DAT expression and trafficking. In this context, mTORC2/Rictor signaling may play a determining factor controlling Akt activity and its substrate selectivity.

*mTORC2/Rictor/Akt Signaling Regulates DA Tone: Tyrosine Hydroxylase*

Another crucial regulator of presynaptic DA tone is tyrosine hydroxylase (TH), the rate-limiting enzyme in DA biosynthesis. TH activity is essential for the

integrity of the DA system, as shown by the Parkinson's disease research (Haavik and Toska, 1998; Tabrez et al., 2012), and in studies employing TH inhibitors (Moore et al., 1987; Goldstein and Lieberman, 1992). Missense mutations in the TH gene are associated with severe infantile Parkinsonism (Bademci et al., 2012). Mice that lack TH in the DA neurons (deemed DA deficient mice) are not able to sustain feeding behavior and die early after birth (Zhou and Palmiter, 1995). Furthermore, TH KO mice are embryonic lethal (Thomas et al., 1995). These data highlight the importance of the TH in general and within DA neurons in particular. In this context, intriguing is the data showing that mTORC2/Akt pathway is implicated in growth and survival of TH-expressing DA neurons. *In vitro* data shows that stimulation of the upstream kinases, RTK and PI3K, promotes DA neuron growth and survival, and conversely, inhibition of RTK/PI3K interferes with these processes (Kim et al., 2004; Terada et al., 2014). Furthermore, *in vivo* studies which inhibit or enhance Akt signaling in SNc and VTA show that the DA neuron soma size, the integrity of DA innervation, and the levels of DA and its metabolites in the midbrain and target areas are dependent on the fidelity of mTORC2/Rictor signaling and correlate with Akt phosphorylation (Ries et al., 2006; Russo et al., 2007; Ries et al., 2009).

In addition to influencing the morphological properties of TH-expressing neurons, mTORC2/Rictor - Akt signaling was proposed to modulate TH function. The function of TH is regulated by transcriptional and post-translational mechanisms, including protein phosphorylation. Seminal finding by Morgenroth et al showed that cAMP-dependent protein kinase activated TH launched a

search for TH phosphorylation sites (Morgenroth et al., 1975). Four major TH phosphorylation sites were later characterized: Ser-8, Ser-19, Ser-31, and Ser-40 (Haycock, 1990). *In vitro* and *ex vivo* studies found a few kinases and phosphatases regulating TH activity, including PKA, PKG, PKC, PP2A (Ser-40); ERK1/2, PP2A (Ser-31); CaMKII, PP2A (Ser-19) (Dunkley et al., 2004). Ser-31 phosphorylation is shown to be co-variant with DA tissue content *in vivo* (Salvatore et al., 2009). Furthermore, Ser-31 phosphorylation is implicated in DA biosynthesis, especially in somatodendritic dopaminergic regions such as the SNc and VTA (Salvatore and Pruett, 2012).

To date, no evidence suggests that mTORC2/Akt activity directly affects TH function at either the transcriptional or post-translational level. However, upstream activators of mTORC2/Akt signaling were shown to enhance DA release in PC12 cells (Amino et al., 2002), and in striatal brain slices (Goggi et al., 2003). It is possible that the decreased AMPH-induced DAT-mediated DA efflux observed in rodent models with perturbed mTORC2/rictor-Akt signaling (Dadalko et al., 2015) stems from the impaired TH function since inhibition of TH leads to a reduction in psychostimulant-induced DA release (Butcher et al., 1988; Thomas et al., 2008). This may be because the newly synthesized pool of DA is essential for the AMPH-induced increase in extracellular DA (Butcher et al., 1988; Thomas et al., 2008). In light of recent evidence showing that DA biosynthesis may be autonomously regulated between somatodendritic and terminal field compartments (Salvatore and Pruett, 2012), further investigations

carefully discriminating between brain regions and evaluating if mTORC2/Akt signaling impacts TH activity in the brain region-specific manner are necessary.

*mTORC2/Rictor/Akt Signaling Regulates DA Tone: DA Receptors*

Dopamine Receptors Neurophysiology: anatomy and overview

The multitude of physiological functions controlled by DA in the central nervous system is mediated by the DA receptors. DA receptors belong to the G protein-coupled receptor (GPCR) superfamily, and have canonical seven-transmembrane structure. Binding of DA initiates a conformational change in the receptor so that it activates a G protein or follows a non-canonical G-protein independent mechanism. This in turn generates second-messenger signaling cascades, participating in regulation of ion channels, cell excitability, posttranslational protein modifications, control of translational mechanisms, and many others, as reviewed by (Beaulieu et al., 2015). The signals downstream DA receptors are often found overlapping and converging; however, each receptor has its unique pharmacologic properties and expression. Specificity of action is dependent on the cell types in which a particular receptor is expressed, the location of the receptors on these cells (soma, proximal or distal dendrites, dendritic spines or shafts, or presynaptic terminals), and the interactions with other receptors and signaling systems. DA receptors are ubiquitous throughout the brain, modulating control over such functions as movement, reward, affect, attention, feeding, olfaction, vision, cognitive function, hormonal and sleep regulation.

Dopamine receptors are divided into two groups. The $D_1$ family consists of $D_1$ and $D_5$ receptors, which are coupled to $G_s$ or $G_{olf}$, which stimulate the production of cAMP turning on the PKA signaling cascade. $D_2$, $D_3$, and $D_4$ receptors belong to $D_2$ family, and signal through $G_i/G_o$ by inhibiting cAMP production. As discussed above, the rates of DA synthesis, release, and recycling determine the presynaptic component of DA signaling fidelity. Interestingly, seminal work by Carlsson and Lindqvist allowed for the postulation of a receptor-mediated feedback mechanism to control the rate of DA synthesis and turnover (Carlsson and Lindqvist, 1963). Further investigations discovered DA autoreceptors, located on the DA neurons and orchestrating the presynaptic DA tone by responding to the DA concentration in the extracellular milieu. DA autoreceptors ($D_2$-short) were found to be alternatively spliced $D_2$ DA receptor variants, which differ from the postsynaptic ones by lacking 29 amino acids in their third intracellular loop. Postsynaptic $D_2$ receptors, or $D_2$-long receptors, were found to have distinct physiological, signaling, and pharmacological properties from their $D_2$-short counterparts.

Dopamine Receptors Neurophysiology: Signaling

Until a decade ago, the prevailing dogma was that DA receptors signal exclusively through G-protein dependent mechanisms. By regulating cAMP levels, DA receptors were shown to control DARPP-32, DA and cAMP-regulated neuronal phosphoprotein (also known as protein phosphatase 1 regulatory subunit 1B, PPP1R1B), which is a negative regulator of protein phosphatase 1 (PP1). DARPP-32 plays an essential role in integrating signals from a number of

behaviorally important neurotransmitters and neuromodulators that target the striatum (Yger and Girault, 2011). DA neurotransmission and DA-dependent behaviors rely on DARPP-32 signaling, which is highly specific to the neuronal population where it is expressed. For example, ablation of DARPP-32 in $D_1$ receptor expressing striatonigral medium spiny neurons (MSNs) decreased basal and cocaine-induced locomotion and abolished dyskinetic behaviors in response to the Parkinson's disease drug L-DOPA, whereas the loss of DARPP-32 in striatopallidal $D_2$ expressing MSNs produced a robust increase in locomotor activity and a strongly reduced cataleptic response to the antipsychotic drug haloperidol (Bateup et al., 2010). DARPP-32 is also shown to regulate DA receptor-mediated control over transcriptional process: a signaling cascade downstream of PP1, where phosphorylation of mTORC1 and rpS6 influences CAP-dependent mRNA translation (Santini et al., 2009; Santini et al., 2010; Valjent et al., 2011; Santini et al., 2012; Bonito-Oliva et al., 2013).

In the last decade, our knowledge of the repertoire of DA receptor signaling has grown exponentially. Thus, it was shown that DA receptors biological effects could be accomplished *via* cAMP-independent, non-canonical signaling pathways. Such alternative signaling includes, but is not limited to, transactivation of BDNF receptors (Swift et al., 2011), internalization of $Ca^{2+}$ channels (Kisilevsky et al., 2008; Kisilevsky and Zamponi, 2008), and heteromer formation and alternative coupling to $G\alpha_q$ to mediate $IP_3$ signaling (Hazelwood et al., 2008; Blom et al., 2012; Medvedev et al., 2013; Perreault et al., 2014).

## $D_2$ Dopamine Receptors: Neurophysiology and Function

Of special interest here is the $D_2R$. The diversity of downstream mechanisms activated by $D_2R$ supports its key role in the regulation of the DA neurotransmission, both pre- and post-synaptically. Presynaptically, $D_2R$ plays a pivotal role in the regulation of the DAT trafficking and function through phosphorylation of ERK1/2 (Meiergerd et al., 1993; Batchelor and Schenk, 1998; Dickinson et al., 1999; Bolan et al., 2007; Lee et al., 2009). In addition, $D_2R$ regulates *de novo* DA synthesis (Wolf and Roth, 1990; De Mei et al., 2009). Postsynaptically, $D_2R$ is indispensable for DA signal transduction, making it a key target in pharmacological treatment of DA-dependent neuropsychiatric disorders. To date, $D_2R$ antagonism is one of the unequivocally efficacious therapeutic strategies for schizophrenia, indicating a critical role of $D_2R$ signaling in this devastating disease (Howes and Kapur, 2009). Accumulating data support the importance of brain region segregation when studying multiple pathways activated by the $D_2R$, which have been shown to depend on various molecular contexts.

In addition to the well-known $G\alpha_{i/o}$-mediated cAMP/PKA/DARPP-32 pathway, $D_2R$ has been shown to activate a multitude of signaling cascades. Thus, $D_2R$ couple to the $G\beta\gamma$ subunits to regulate the PLC signaling pathway (Hernandez-Lopez et al., 2000), the activity of L- and N-type $Ca^{2+}$ channels (Yan et al., 1997), as well as G-protein coupled inwardly rectifying potassium channels, or GIRKs (Kuzhikandathil et al., 1998).

*mTORC2/Rictor/Akt signaling through $D_2$ Dopamine Receptors*

Particularly interesting is the accumulating data supporting G-protein independent signaling of the $D_2R$ by complex formation with β-arrestin2 (βArr2), PP2A, and Akt (Beaulieu et al., 2004), reviewed in (Beaulieu et al., 2011). βArr2 is a well-known regulator of GPCR function. βArr2 plays a crucial role in GPCR signal termination through receptor desensitization and G-protein uncoupling, followed by βArr2-mediated clathrin-dependent endocytosis (Lohse et al., 1990; Ferguson et al., 1996). Furthermore, βArr2 was found to act as a scaffold for kinases and phosphatases, forming multiprotein complexes that influence many intracellular signaling cascades (Luttrell et al., 1999; Luttrell and Gesty-Palmer, 2010).

The formation of βArr2 - Akt - PP2A complex in response to $D_2R$ activation was shown to inactivate PI3K/Akt signaling and cause an increase in GSK3 activity. GSK3 dysfunction has been suggested as a potential pathway for the development of DA-related neuropsychiatric diseases, as reviewed in (Beaulieu et al., 2011). Lithium, which is used to alleviate manic symptoms of bipolar disorder as well as schizophrenia and schizoaffective psychoses, has been shown to activate Akt and enhance inhibitory phosphorylation of GSK3 in the rodent models of aforementioned DA-related disorders (Beaulieu et al., 2004; Emamian et al., 2004; Li et al., 2007). The key finding demonstrating the direct contribution of DA in the regulation of Akt/GSK3 signaling is the study in DAT-KO mice utilizing inhibition of *de novo* DA synthesis by the irreversible TH inhibitor, α-methyl-*para*-tyrosine (Beaulieu et al., 2004). The virtual absence of striatal DA

upon TH inhibition enhanced Akt activity and increased phosphorylation of GSK3 in the DAT-KO mice. Similar biochemical changes were observed with application of $D_2R$ blockade or administration of lithium salts, suggesting a major role for $D_2R$ – Akt – GSK3 signaling in DA-related neuropsychiatric disorders.

### *Introduction to the neuronal Rictor KO mouse model*

Out of the three Akt isoforms identified, *Akt1* genetic variants have been associated with the etiology of schizophrenia (Emamian et al., 2004). For example, Akt1 null mice demonstrate enhanced sensitivity to psychostimulant effects of dopaminergic and noradrenergic agents (Emamian et al., 2004; Lai et al., 2006). Decreases in Akt1 mRNA, protein, and activity levels were found in the brains of schizophrenia patients (Thiselton et al., 2008). It is important to remember that Akt activity depends on its two key phosphorylation sites: Thr308 and Ser743, both of which control Akt conformation, interaction with other proteins, as well as its substrate selectivity (Franke, 2008a, b). Clinical evidence supports a role for mTORC2/Akt signaling in DA-dependent mental illnesses. Lithium, used to treat bipolar disorder, stimulates phosphorylation of Akt at Ser473 (Chalecka-Franaszek and Chuang, 1999). Certain antidepressants (Krishnan et al., 2008), along with both typical (Emamian et al., 2004) and atypical antipsychotics (Lu and Dwyer, 2005), also increase Akt Ser473 phosphorylation. Furthermore, findings of diminished Akt Ser473 phosphorylation and/or its activity in post-mortem brains of patients with schizophrenia (Zhao et

al., 2006) and depression (Karege et al., 2007) strengthen the association between dysregulation of mTORC2/Akt signaling and development of DA-associated psychiatric illnesses. Nonetheless, the molecular mechanisms linking mTORC2/Akt to impairments in DA neurotransmission and behaviors have been elusive.

To engineer a mouse model with aberrant mTORC2/Akt signaling, we used the Cre-LoxP mediated genetic recombination technology, which allows for the selective deletion of genes along tissue specific promoters. The Nestin-Cre promoter was chosen for its ability to delete the gene of interest in the entire brain. Deletion of a gene along a Nestin-Cre promoter generates viable mice for the Rictor gene, but not for the PDK1 gene (Oishi et al., 2009). As a result of this genetic strategy, the Rictor/Nestin-Cre transgenic cross, or nRictor KO mice, have severely depleted brain Akt Ser473 phosphorylation. This provides a great model in which to study how aberrant mTORC2/Akt signaling in the brain impacts central DA homeostasis.

A previously published study in the nRictor KO mouse model demonstrated severe alterations in cortical DA tone (Siuta et al., 2010). Disrupted mTORC2/Akt signaling and decreased brain Akt Ser473 phosphorylation led to cortical hypodopaminergia and pre-pulse inhibition deficits. These characteristic schizophrenia-associated endophenotypes are thought to stem from altered cortical and striatal DA neurotransmission (Braff et al., 2001; Emamian et al., 2004; Howes and Kapur, 2009; Keri et al., 2009). Striatal DA bioavailability is fundamental in the development of schizophrenia (Davis et al., 1991b; Howes

and Kapur, 2009), and is increased during prodromal and psychotic states (Howes and Kapur, 2009). Such elevated DA levels correlate with the positive symptoms of the disease, and antidopaminergic drugs, such as a $D_2R$ blocker typical antipsychotic haloperidol, are effective in reducing these symptoms in most patients (Abi-Dargham et al., 2000).

The specific aims of this thesis will focus on exploration of impairments in nigro-striatal DA neurotransmission as they occur under the influence of aberrant mTOR2/Akt signaling. Specific attention will be given to such DA homeostasis regulators as DAT, TH, and $D_2R$, as well as DA-regulated stiatum-dependent behaviors. In addition to the nRictor KO mouse, a TH-Rictor KO transgenic line will be introduced. This mouse model, with disrupted mTORC2/Akt signaling in the TH expressing cells, will allow to hone in on the mTORC2/Akt function within pre-synaptic monoaminergic pathways. Finally, using a viral intervention technique, we will disrupt mTORC2/Akt function in the dorsal striatum, targeting the brain region of interest to assess DA-related behaviors, as well as eliminate possible confounding developmental compensations of transgenic mouse models.

Thus, our Specific Aims are:

I) *To test the hypothesis that impairment in brain mTORC2/Rictor signaling disrupts central DA tone and to assess the key elements responsible for aberrant striatal DA homeostasis in the nRictor KO mice.*

II) *To determine whether aberrant brain mTORC2/Rictor signaling alters DA (subcortical) related behaviors.*

III) *To assess which neuronal populations or subcortical loci are vulnerable to disruption of mTORC2/Rictor signaling in producing biochemical and behavioral DA-dependent phenotypes.*

# CHAPTER II

## *mTORC2/RICTOR SIGNALING DISRUPTS DOPAMINE-DEPENDENT BEHAVIORS VIA DEFECTS IN STRIATAL DOPAMINE NEUROTRANSMISSION**

### *Abstract*

Disrupted neuronal protein kinase B (Akt) signaling has been associated with dopamine (DA) related neuropsychiatric disorders, including schizophrenia, a devastating mental illness. We hypothesize that proper DA neurotransmission is, therefore, dependent upon intact neuronal Akt function. Akt is activated by phosphorylation of two key residues, Thr308 and Ser473. Blunted Akt phosphorylation at Ser473 (pAkt-473) has been observed in lymphocytes and post-mortem brains of schizophrenia patients, and psychosis-prone normal individuals. Mammalian target of rapamycin (mTOR) complex 2 (mTORC2) is a multiprotein complex which is responsible for phosphorylation of Akt at Ser473 (pAkt-473). We demonstrate that mice with disrupted mTORC2 signaling in brain exhibit altered striatal DA-dependent behaviors, such as increased basal locomotion, stereotypic counts and exaggerated response to the psychomotor effects of amphetamine (AMPH). Combining *in vivo* and *ex vivo* pharmacological, electrophysiological, and biochemical techniques we demonstrate that the

* The work presented in this chapter is published as Olga I. Dadalko, Michael Siuta, Amanda Poe, Kevin Erreger, Heinrich J.G. Matthies, Kevin Niswender, Aurelio Galli (*J. Neuroscience;* 2015 Jun 10;35(23):8843-54)

changes in striatal DA neurotransmission and associated behaviors are caused, at least in part, by elevated D2 DA receptor (D2R) expression and upregulated ERK1/2 activation. Haloperidol, a typical antipsychotic and D2R blocker, reduced AMPH hypersensitivity and elevated pERK1/2 to the levels of control animals. By viral gene delivery, we downregulated mTORC2 solely in the dorsal striatum of adult wild-type mice, demonstrating that striatal mTORC2 regulates AMPH stimulated behaviors. Our findings implicate mTORC2 signaling as a novel pathway regulating striatal DA tone and D2R signaling.

## Introduction

Impaired brain dopamine (DA) homeostasis is strongly implicated in neuropsychiatric disorders such as schizophrenia and psychostimulant abuse (Narendran and Martinez, 2008; Howes and Kapur, 2009; Espana and Jones, 2013; Nestler, 2013). Evidence from studies in animal models supports the key role of insulin resistance in aberrant striatal DA signaling (Wang et al., 2001; Wang et al., 2002; Johnson and Kenny, 2010; Daws et al., 2011; Niswender et al., 2011; Speed et al., 2011)

In the CNS, insulin signaling regulates reward, development, and cognition (Schulingkamp et al., 2000; Daws et al., 2011). Importantly, insulin activates intracellular kinases including Akt (van der Heide et al., 2006). Three isoforms of Akt have been identified and their brain expression characterized. Seminal

findings revealed a strong correlation between genetic variants of *Akt1* and schizophrenia (Emamian et al., 2004; Nicodemus et al., 2008; Nicodemus et al., 2010; Tan et al., 2012), a DA-associated neuropsychiatric disorder (Howes and Kapur, 2009). Thus, it has been proposed that brain DA dysfunction could stem from altered Akt signaling (Niswender et al., 2011). Recently, we and others have shown that aberrant brain Akt function stemming from either an obesogenic diet or diabetes results in impaired striatal DA homeostasis contributing to DA-dependent behaviors (Williams et al., 2007; Speed et al., 2010). However, the molecular mechanisms linking Akt dysfunction with altered DA neurotransmission have yet to be established.

Mammalian target of rapamycin (mTOR) complex 2 (mTORC2) is a multiprotein complex that is a critical regulator of cell growth and metabolism. mTORC2 contains Rictor, mSIN1, mLST8, and mTOR. Akt, along with other kinases, is a primary substrate of mTORC2 which is responsible for phosphorylation of Akt at Ser473 (pAkt-473), one of two key phosphorylation sites. To study how aberrant mTORC2 signaling influences central DA neurotransmission, we used Cre-LoxP technology to disrupt the mTORC2 complex by neuronal ablation of the Rictor protein (nRictor KO mouse model; (Shiota et al., 2006; Siuta et al., 2010)). We show that impaired mTORC2/Akt signaling alters striatal DA tone, increases basal and AMPH-induced locomotion, and stereotypic counts. These behaviors, traditionally associated with elevated striatal DA signaling (Sharp et al., 1987; Rebec, 2006; Kreitzer and Malenka, 2008), were associated instead with diminished striatal DA bioavailability.

Furthermore, viral-mediated recombination specifically in dorsal striatum supports the hypothesis that this brain region has a major role in mediating DA-driven behavioral dysfunction in response to aberrant mTORC2 signaling and possibly Akt Ser473 phosphorylation.

## Results

*Neuronal deletion of Rictor results in impaired Akt Ser473 phosphorylation and increased DA transporter expression and function in the dorsal striatum:* Akt is a serine/threonine protein kinase, whose activity and substrate selectivity is regulated by phosphorylation at two key residues: Thr308 by phosphoinositide-dependent kinase-1, PDK1, and Ser473 by mTORC2. Rictor – rapamycin insensitive companion of mTOR – is the scaffolding protein that maintains mTORC2 integrity, allowing for its kinase activity. nRictor KO mice were generated by crossing floxed *Rictor* animals with nestin-Cre transgenic mice, as previously described (Siuta et al., 2010). We have shown that nRictor KO mice lack both rictor mRNA and protein expression in a gene-dosage dependent manner within the brain (Siuta et al., 2010). Figure 5*A (inset)* reveals a lack of Akt Ser473 phosphorylation in the dorsal striatum of the nRictor KO animals, whereas total Akt is unchanged. Our laboratory and others have previously shown that Akt function regulates DA transporter (DAT) trafficking (Doolen and Zahniser, 2001; Carvelli et al., 2002; Garcia et al., 2005; Franke, 2008b; Lute et al., 2008; Speed et al., 2010) and, thereby, DA-related behaviors (Williams et al.,

2007; Speed et al., 2011; Owens et al., 2012). Thus, to understand the role of mTORC2/Akt signaling in DA neurotransmission, we first evaluated DAT surface and total expression in the dorsal striatum of nRictor KO and control (CTR)

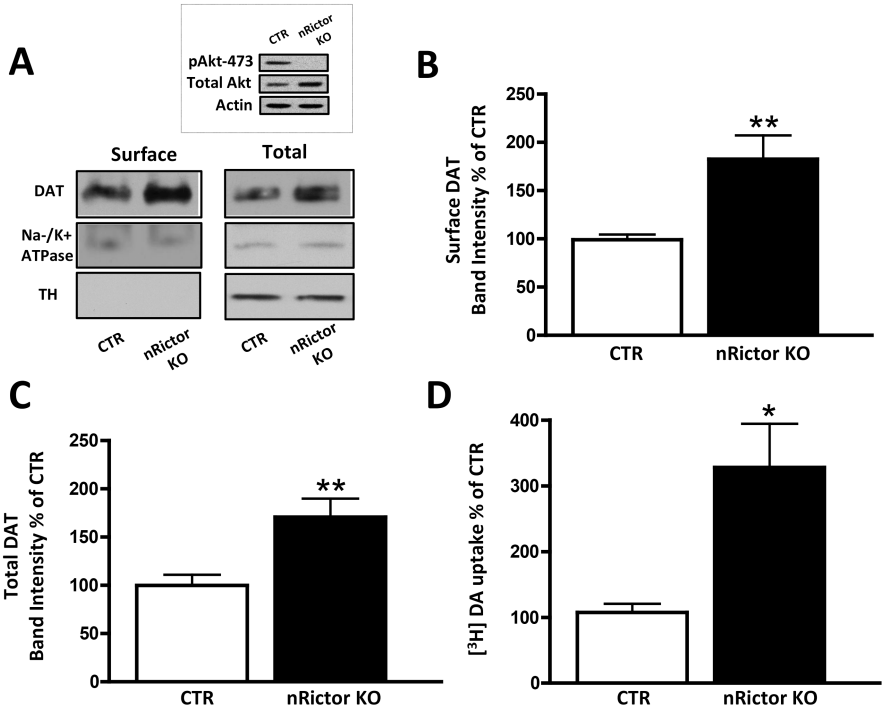



**Figure 5. DAT expression and function is increased in the dorsal striatum of the nRictor KO mouse.** A) Representative immunoblots of surface and total protein fractions probed with a selective anti-DAT antibody or with an anti-Na-K ATPase antibody to serve as a loading control. TH, a cytosolic protein was detected in the total fraction but not in the biotinylated fraction. *Inset:* representative immunoblot of pAkt-473 in the dorsal striatum of nRictor KO mice and CTR animals. B) Quantitation of the optical density of DAT surface fraction normalized to CTR. C) Quantitation of the optical density of the total protein fraction normalized to CTR. D) [$^3$H]-DA uptake in anatomically paired striatal slices obtained from CTR and nRictor KO mice expressed as a percent of control.

animals. Using biotinylation (representative immunoblots are shown in Fig. 5*A*), we observed that defects in mTORC2 signaling lead to increased DAT plasma membrane expression (Fig.5*B*; $t(12)=3.3$, $p\leq0.01$, paired t-test) and elevated total expression of DAT (Fig. 5*C;* $t(15)=3.1$, $p\leq0.01$, paired t-test). The absence of the

cytosolic protein tyrosine hydroxylase (TH) in the surface fraction indicates the integrity of the experimental preparation while Na/K ATPase serves as a loading control (Fig. 5*A*). We next sought to determine whether augmented total and membrane DAT expression in nRictor KO mice leads to an increase in DAT function. To quantify DAT function, we assessed [$^3$H]-DA uptake in acute striatal slices. Consistent with the strong increase in DAT protein expression, nRictor KO mice exhibited significantly higher [$^3$H]-DA uptake (Fig. 5*D; t*(16)=2.9, $p \leq 0.01$, paired t-test).

*nRictor KO mice exhibit increased novelty-induced locomotion, stereotypic counts, and exaggerated response to AMPH:* DA reuptake by the DAT is the primary mechanism of terminating DA transmission in the dorsal striatum, shaping the duration and amplitude of DA signaling (Kristensen et al., 2011). DA neurotransmission is essential for initiation and organization of motor function (Birkmayer and Hornykiewicz, 1961; Hornykiewicz, 1966; Cotzias et al., 1967; Fischer and Heller, 1967; Ungerstedt, 1968; Ungerstedt and Pycock, 1974; Goldstein et al., 1975; Langston and Ballard, 1983), and therefore DAT function is critical in DA-dependent behaviors, such as locomotor activity. Thus, we determined whether changes in DAT expression and function translate to altered DA dependent behaviors in nRictor KO mice.

First, we examined basal locomotion. After five days of handling (see methods), animals were placed in open field chambers and allowed to freely explore. Locomotor activity was measured as distance travelled in five-minute bins. Compared to CTR, nRictor KO mice exhibit a dramatic and stable increase

in horizontal locomotion (n=12, two-way ANOVA, effect of genotype $F_{(1,528)}$=1492, $p$<0.0001; followed by Bonferroni post-hoc test ***$p$<0.001 (Fig. 6*A*)). Furthermore, stereotypic counts (stereotypic episodes counted in beam breaks within a small 4-beam box) in nRictor KO mice are significantly elevated with respect to CTR animals (n=10, two-way ANOVA, effect of genotype $F_{(1,648)}$=523.8, $p$<0.0001; followed by Bonferroni post-hoc test **$p$<0.01, ***$p$<0.001 (Fig. 6*B*)). These data are consistent with the notion that impaired striatal DA neurotransmission contributes to aberrant "stereotypic behaviors" (Kelly et al., 1975; Andrews et al., 1982; Carr and White, 1984; Porrino et al., 1984; Sharp et al., 1987). Mice heterozygous for rictor deletion did not show alterations in locomotor activity or stereotypic counts (data not shown), demonstrating that nestin-Cre transgene has no effect on these DA associated behaviors. Importantly, this also strongly suggests that the full blockade of mTORC2 signaling is necessary to induce the phenotype observed in the nRictor KO.

AMPH exerts its action mainly *via* DAT, causing non-vesicular DAT-mediated DA efflux (Kahlig et al., 2005; Sulzer et al., 2005). The observed increase in DAT expression in the dorsal striatum of the nRictor KO mice allowed us to hypothesize that AMPH would cause exaggerated behavioral responses in nRictor KO animals. Consistent with this hypothesis, mice that overexpress DAT (DAT-tg), as well as animals with an increased DAT surface expression (Gpr37-/-) exhibit exaggerated AMPH-induced hyperlocomotion (Marazziti et al., 2004; Salahpour et al., 2008). In order to evaluate changes in the locomotor response

caused by AMPH in nRictor KO mice, a 6-day long habituation protocol (see methods) was empirically used to decrease the basal locomotion of the nRictor KO to the level of CTR mice. On the test day (day 7), an initial saline injection

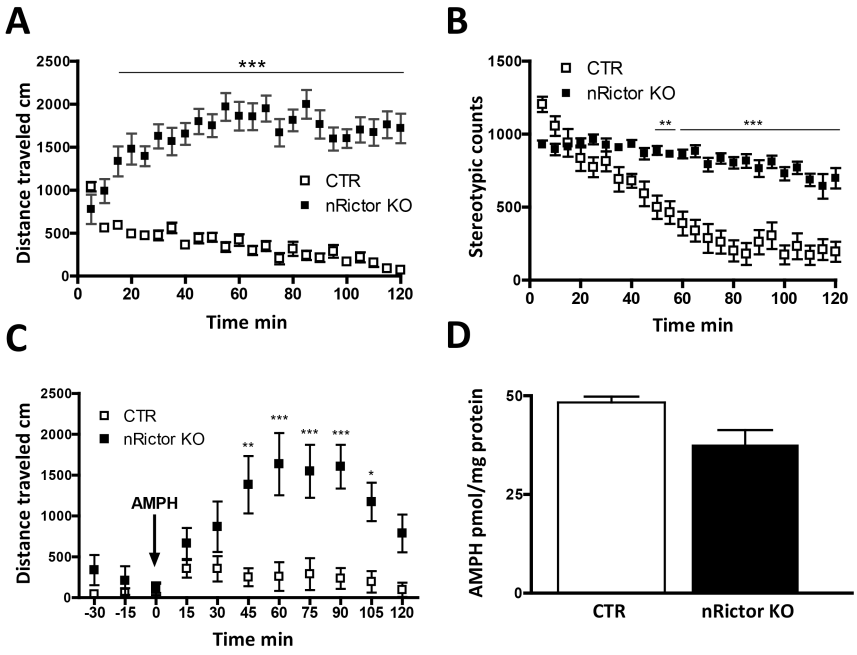



**Figure 6. Neuronal deletion of Rictor results in novelty-induced hyperactivity, increased stereotypic counts, and exaggerated locomotor response to AMPH.** A) Horizontal locomotion measured in open field chambers in 5-min intervals. Shown is the distance traveled in cm. B) Stereotypic counts binned in 5-min intervals. C) AMPH (i.p., 2mg/kg) stimulated locomotor activity binned in 15-min intervals. Both CTR and KO mice were habituated to achieve non-significant differences in basal locomotor activity before AMPH injection. Data represent distance traveled in cm. D) 30 minutes after 2mg/kg AMPH i.p., animals were sacrificed, and tissue content of AMPH was measured by HPLC in striatal homogenates.

was followed by a 2h period in the chamber, a sufficient time for all animals to reach comparable baseline locomotion. AMPH (2mg/kg) was then administered i.p. (time 0) and horizontal locomotion was recorded in five-minute bins for the next two hours. nRictor KO animals demonstrate exaggerated psychomotor response to AMPH, with locomotor activity markedly higher than that of CTR

animals (n=10, two-way ANOVA, effect of genotype $F_{(1,198)}$=71.15, $p$<0.0001; followed by Bonferroni post-hoc test *$p$<0.05, **$p$<0.01, ***$p$<0.001 (Fig. 6*C*)). The AMPH tissue content in the dorsal striatum of the nRictor KO mouse is comparable to the CTR animals (Fig. 6*D; t*(4)=2.7, $p$>0.05, unpaired t-test), and, importantly, is clearly not elevated relative to CTR, indicating that AMPH transport across the blood brain barrier is not altered by Rictor deletion.

DAT expression and cellular distribution is precisely regulated, and alterations in DAT availability could contribute to DA dysfunction and to the pathophysiology of neuropsychiatric disorders. However, clinical studies have not been consistent on this issue, showing increased, decreased, or no change in striatal DAT availability in patients with schizophrenia (Brunelin et al., 2013). We have shown that decreased brain mTORC2 signaling also results in deficits in pre-pulse inhibition (Siuta et al., 2010). Here, we demonstrate that elevated striatal surface and total DAT support AMPH hypersensitivity. Psychostimulants, including AMPH, cause changes in behavior by elevating striatal DA bioavailability (Rebec, 2006). Thus, our data suggest elevated DA tone in the dorsal striatum of nRictor KO mice. Therefore, our next goal was to determine the neuronal adaptation caused by mTORC2 signaling within the DA network.

*nRictor KO mice exhibit reduced AMPH-induced DA release, tissue DA content, and TH phosphorylation:* Basal hyperlocomotion and exaggerated locomotor response to AMPH are phenotypes suggestive of striatal DA hyperfunction (Bardo et al., 1990; Rebec, 2006). To further test this, we evaluated AMPH-induced DA release in the dorsal striatum of nRictor KO mice by *in vivo*

microdialysis and *ex vivo* chronoamperometry. The nRictor KO mouse has reduced brain size (Siuta et al., 2010) consistent with the phenotype of the Akt3 KO mouse (Easton et al., 2005). Thus, we empirically defined the stereotactic coordinates to ensure accurate and parallel placement of the probe in the nRictor KO and CTR animals. 18-24 hours after mice underwent guide cannula placement, microdialysis probes were inserted into the dorsal striatum of nRictor KO and CTR mice. Dialysate samples were collected in 20-minute intervals, and analyzed by HPLC for monoamines and metabolites content. After establishing a stable baseline, AMPH was administered (2mg/kg i.p.). AMPH-induced DA release was significantly reduced in the dorsal striatum of nRictor KO mice relative to CTR (n=2, two-way ANOVA, effect of genotype $F_{(1,12)}$=13.8, $p$<0.01; followed by Bonferroni post-hoc test *$p$<0.05 (Fig. 7*A*)).

To confirm this reduction in AMPH-induced DA efflux, we employed *ex vivo* chronoamperometry to measure AMPH-induced DA release directly from striatal slices. Coronal slices with dorsal striatum were recovered in 28°C oxygenated aCSF for a minimum of one hour prior to recording. The carbon fiber electrode was angularly deepened into the dorso-lateral portion of the striatum (same *locale* as microdialysis). AMPH (10 μM) was bath applied after 30 minutes of baseline recordings. In agreement with the microdialysis data, AMPH-induced DA release was significantly reduced in the striatal tissue of the nRictor KO compared to CTR animals (n=6, two-way ANOVA, effect of genotype $F_{(1,410)}$=106.4, $p$<0.0001; followed by Bonferroni post-hoc test *$p$<0.05, **$p$<0.01, ***$p$<0.001 (Fig. 7*B*)).

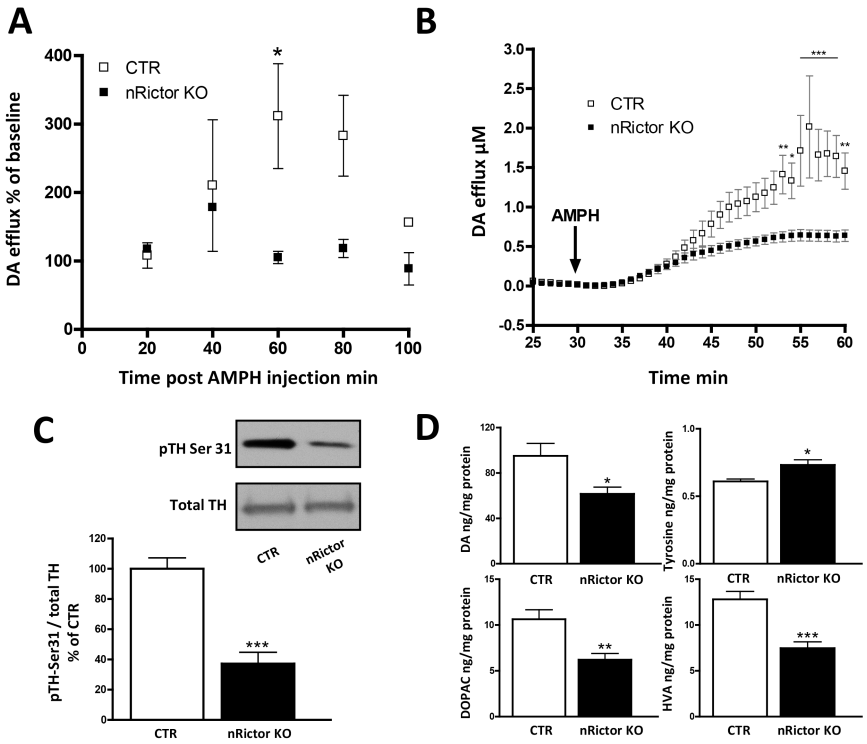



**Figure 7. nRictor KO exhibit reduced AMPH-induced DA release, tissue DA content, and TH phosphorylation.** A) Time course of extracellular DA concentration as measured by microdialysis coupled with HPLC from the dorsal striatum of CTR and nRictor KO mice after i.p. administration of AMPH (2mg/kg). Results are shown as percent of baseline in 20-min intervals. B) AMPH-induced (10 µM) DA release measured by oxidation currents from striatal slices reported as µM DA release. C) Top, pTH-Ser31 and TH representative immunoblots obtained from striatal tissue. Bottom, quantification of the ratio of pTH-Ser31 to total TH expressed as a percent of CTR. D) DA tissue content, its precursor tyrosine, and its metabolites DOPAC and HVA as measured by HPLC in striatal homogenates.

It is possible that the observed decrease in AMPH-induced DA efflux stems from impaired function of TH, the rate limiting enzyme for DA synthesis. Indeed, it has been shown that TH inhibition leads to a reduction in psychostimulant-induced DA release, confirming that the newly synthesized pool of DA is essential for AMPH induced DA efflux (Butcher et al., 1988; Thomas et al., 2008). TH conformation and activity is regulated by four phosphorylation sites. Ser31 is

the key residue whose phosphorylation correlates with TH activity *in vivo* (Salvatore et al., 2009; Damanhuri et al., 2012; Salvatore and Pruett, 2012). Further, it has been shown that DA levels also correlate with the level of TH phosphorylation at Ser31 (Salvatore, 2014). Thus, we quantified pTH-Ser31 in nRictor KO mice relative to CTR animals. Indeed, phosphorylation of TH at Ser31 is reduced in nRictor KO mice with respect to CTR animals (Fig. 7*C;* $t(12)=6.0$, $p<0.001$, unpaired t-test).These data reveal a strong association between mTORC2 signaling, TH phosphorylation and the ability of AMPH to cause DA efflux. In line with the down regulation of pTH-Ser31, we found a reduction in DA and its metabolites in the dorsal striatum tissue of nRictor KO animals, as well as a consistent increase in tyrosine, the DA precursor (Fig. 7*D;* DA: $t(21)=2.6$; tyrosine: $t(8)=2.9$; DOPAC: $t(22)=3.6$; HVA: $t(21)=4.7$; $^{*}p<0.05$; $^{**}p<0.01$, $^{***}p<0.001$, unpaired t-test).

*Elevated striatal expression of D2R and pERK1/2 in nRictor KO mice supports exaggerated AMPH-induced locomotion:* Basal hyperlocomotion, elevated stereotypic counts, and the ability of AMPH to induce exaggerated hyperactivity, are behaviors that rely, at least in part, on increased DA tone in the dorsal striatum (Bardo et al., 1990; Rebec, 2006). However, nRictor KO animals exhibit reduced AMPH-induced DA release (Figure 3). This is consistent with lowered DA tissue content and attenuated phosphorylation of TH at Ser31. Thus, it is possible that altered expression and/or function of DA receptors (e.g., D1R and D2R) are involved. In support of this hypothesis are data demonstrating that mice with elevated striatal D2R expression display increased locomotion in a novel

environment (Kramer et al., 2011). Further, toxin ablation and functional disruption of D2R-expressing striatopallidal medium spiny neurons (MSN) inhibits locomotor activity (Durieux et al., 2009; Bateup et al., 2010). Consistently, we found total protein levels of D2R in the dorsal striatum of nRictor KO mice to be significantly elevated relative to CTR animals, (Fig. 8*A;* $t(26)=3.3$, $p<0.01$, unpaired t-test). Surface expression of D2R determined by slice-surface biotinylation was also significantly increased (CTR 100±12.7; nRictor KO 245±49.0; $t(8)=3.8$, $p<0.01$, paired t-test). To corroborate these findings, we isolated plasma membranes from the dorsal striatum tissue, and observed elevated [$^3$H]-nemonapride (a potent D2R antagonist) binding in the nRictor KO mice, further evidence of increased D2R (Fig. 8*B;* $t(17)=1.9$, $p<0.05$, unpaired t-test). Importantly, the levels of D1R in the nRictor KO mice were not significantly different from those of CTR (Fig. 8*C;* $t(23)=1.19$, $p>0.05$, unpaired t-test).

Activation of D2R results in enhanced phosphorylation of extracellular signal-related kinase (ERK1/2) (Luo et al., 1998; Cai et al., 2000; Wang et al., 2005; Bolan et al., 2007). The relationship between D2R signaling, ERK1/2 phosphorylation, and locomotion has been defined by Cai and collaborators, who demonstrated that activation of ERK1/2 in DA-deficient dorsal striatum is required for D2R signaling to drive locomotor hyperactivity (Cai et al., 2000). Here, we observe that the increase in D2R expression in nRictor KO mice leads to a marked basal increase in striatal pERK1/2 (Fig. 8*D;* $t(12)=3.0$, $p<0.01$, unpaired t-test).

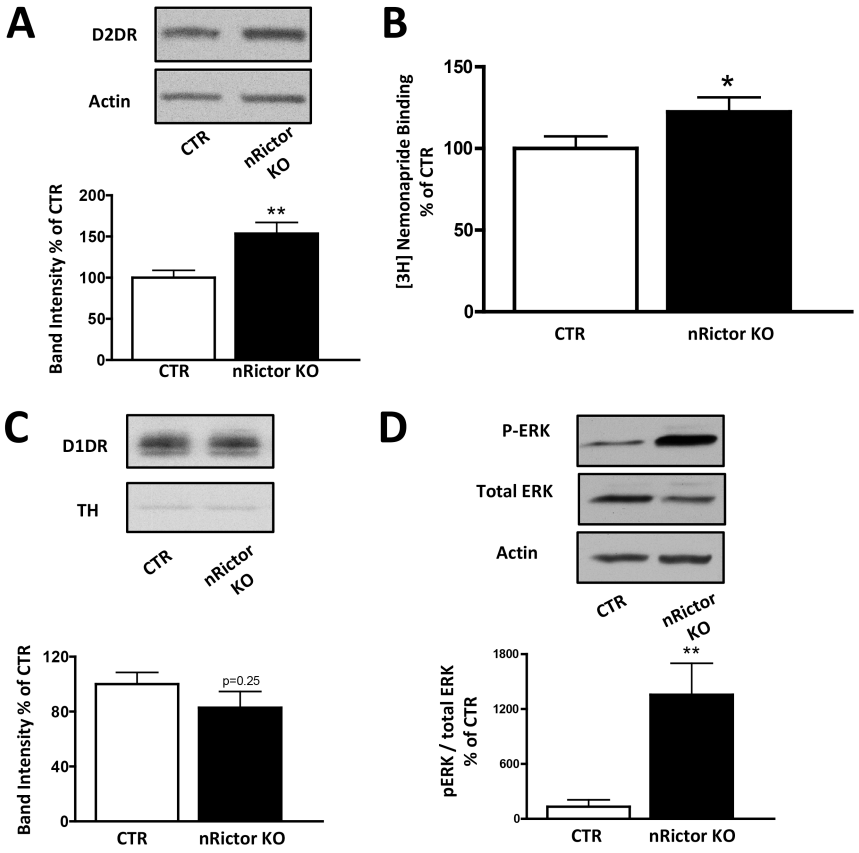



**Figure 8. Elevated expression of D2R and not D1R increases pERK1/2 in the nRictor KO mice.** A) Representative immunoblots (*top*) and optical density quantitation (*bottom*) of D2R expression in the dorsal striatum of nRictor KO and CTR animals. Actin was probed as a loading control. Data are expressed as percent of CTR B) [$^3$H]nemonapride binding to striatal plasma membranes of CTR and nRictor KO mice: nonspecific binding (in the presence of 10μM sulpiride) was subtracted from the specific binding which was counted in duplicates in DPM and averaged within a sample. Data normalized to CTR levels C) Representative immunoblots (*top*) from striatal homogenates obtained from nRictor and CTR mice probed with D1R antibody, and quantitation of the respective optical densities (*bottom*). Data are shown as a percent of control. Actin immunoblots served as a loading control D) Representative immunoblot and quantification of pERK1/2 (shown as a percent of control) and total ERK in the dorsal striatum of CTR and nRictor KO. Actin is the loading control.

In order to determine the role of D2R in the hyperlocomotion observed in nRictor KO mice, we first tested the effect of haloperidol, a typical antipsychotic and D2R antagonist, on ERK1/2 phosphorylation in the dorsal striatum. Haloperidol was

administered (0.8 mg/kg i.p., a dose of therapeutic relevance) to efficiently block the elevated D2R of nRictor KO mice (Clapcote et al., 2007; Lipina et al., 2010). One hour after injection, mice were euthanized and dorsal striatal homogenates probed for pERK1/2. In nRictor KO mice, haloperidol reduced pERK1/2 to CTR levels (Fig. 9*A; t*(16)=1.7, $p<0.05$, unpaired t-test), indicating that the enhanced D2R signaling causes increased ERK1/2 phosphorylation in the KO animals. D2R are involved in the symptomology of patients with neuropsychiatric disorders, and human imaging studies show elevated efficacy of DA to stimulate D2R in both drug naïve and treated schizophrenia patients (Abi-Dargham et al., 2000). Next, we determined whether blockade of D2R with haloperidol (administered as above) alters levels of pTH-Ser31 in nRictor KO animals. In nRictor KO mice, haloperidol significantly increased levels of pTH-Ser31 with respect to saline injected animals (Fig. 9*B; t*(16)=3.9, $p<0.01$, unpaired t-test).This data indicates that enhanced D2R signaling contributes, a least in part, to the decreased pTH-Ser31 in the KO animals. To further define the role of D2R in the phenotypes of the nRictor KO mice, we tested whether haloperidol would block the exaggerated response to AMPH in nRictor KO mice. Co-administration of haloperidol with AMPH reversed the exaggerated AMPH-induced hyperactivity of nRictor KO mice, reducing their locomotor activity to the level of CTR mice (Fig. 9*C*: locomotor activity represented as area under the curve from the time of injection to 60 min, AMPH treatment: *t*(9)=2.4, $*p<0.05$, unpaired t-test; AMPH + Haloperidol (AMPH Haldol) treatment: *t*(9)=1.2, $p>0.5$, unpaired t-test).

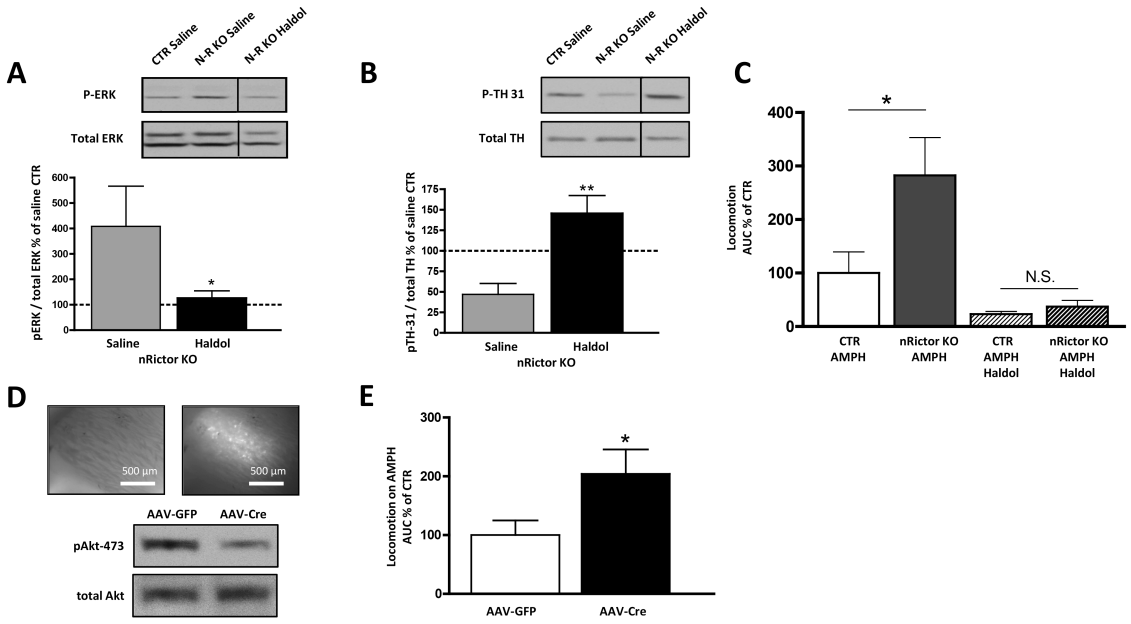



**Figure 9**. **Treatment with haloperidol supports the role of D2R in exaggerated AMPH-induced hyperactivity in nRictor KO mice, while viral intervention reveals that Akt signaling specifically in the dorsal striatum supports this phenotype.** A) Acute administration of haloperidol reduced expression of pERK1/2 in the dorsal striatum of nRictor mice. (*top*) Representative immunoblots for pERK1/2 from striatal tissue of CTR and nRictor mice injected either vehicle or haloperidol (Haldol). (*bottom*) Data were normalized to saline injected CTR. B) Acute administration of haloperidol (i.p., 0.8mg/kg) decreased expression of pTH-Ser31 in the dorsal striatum of nRictor mice. (*top*) Representative immunoblots for pTH-Ser31 and total TH obtained from striatal tissue of CTR and nRictor mice injected either vehicle or haloperidol (Haldol). (*bottom*) Data were normalized to the correspondent total TH and expressed as a percent of saline injected CTR. C) CTR and nRictor KO mice were habituated to saline injections and open field chambers for six days. On day seven drugs were administered and horizontal locomotor activity recorded in 5-min intervals. Data are represented as area under the curve from time of injection to 60 min. D) Viral downregulation of striatal Rictor results in decreased pAkt-473: AAV-Cre-GFP and AAV-eGFP viral vectors were injected into the dorsal striatum of floxed animals. Shown are representative immunoblots of AAV-Cre injected animals vs. AAV-eGFP CTRs. *Inset:* representative images (4X) demonstrating GFP in the targeted brain region: white light on the left; FITC filter on the right. E) AMPH (i.p. 2mg/kg) induced locomotion measured following the same handling/habituation protocol as was used for the genetic KO. Data are represented as area under the curve from the time of injection to 60 min.

*Reduced mTORC2 signaling in the dorsal striatum supports exaggerated AMPH-induced hyperactivity:* Recombinant adeno-associated viral vectors (rAAV) are widely used for temporally and spatially controlled gene delivery. Out of many naturally available serotypes, rAAV-5 was shown to be the most efficient for genetic transduction in the dorsal striatum of mice (Aschauer et al., 2013) and non-human primates (Markakis et al., 2010). In order to strengthen our hypothesis that AMPH-induced hypersensitivity of nRictor KO animals stems, at least in part, from impaired mTORC2 signaling specifically in the dorsal striatum, we selectively deleted Rictor by injecting rAAV-Cre into the dorsal striatum of floxed *Rictor* mice. For biochemical assessment, floxed *Rictor* animals were injected unilaterally with rAAV-Cre, while the other hemisphere was injected with rAAV-eGFP as a control. Light and fluorescence microscopy confirmed localization of injections to dorsal striatum (Fig. 9*D* top). Dorsal striata injected with rAAV-Cre showed a reduction in pAkt-473 compared to rAAV-eGFP injected striata (Fig. 9*D* bottom). Four weeks after bilateral rAAV-Cre injection, animals exhibited exaggerated AMPH-induced hyperlocomotion relative to rAAV-eGFP animals (Fig. 9*E:* locomotor activity represented as area under the curve from the time of injection to 60 min, $t(12)=2.2$, $p<0.05$, unpaired t-test).

## Discussion

The mTORC2 complex is responsible for phosphorylation of AGC kinases to promote their maturation, stability and allosteric activation (Oh and Jacinto,

2011). These kinases include Akt, serum/glucocorticoid regulated kinase 1 (SGK1) and protein kinase Cα (PKCα). Clinical evidence supports a role for Akt in DA-dependent mental illness (Emamian et al., 2004; Lu and Dwyer, 2005; Krishnan et al., 2008). Nonetheless, the molecular mechanisms linking altered mTORC2/Akt signaling to impaired DA neurotransmission and corresponding behaviors have been elusive. Here, we demonstrate how genetic deletion of Rictor protein and the parallel reduction of pAkt-473 in the brain results in altered striatal DA tone.

Our laboratory has extensively studied how Akt signaling influences DA tone in the brain (Williams et al., 2007; Speed et al., 2010; Speed et al., 2011). These published data reveal that Akt function supports DAT surface expression . Here we expand upon these findings, demonstrating that genetic deletion of the protein Rictor causes a reduction in Akt Ser473 phosphorylation and an increase in DAT expression. These data are consistent with a differential role of Akt Ser473 phosphorylation and Akt function in DA homeostasis. However, in the current study, we cannot rule out the possibility that other AGC kinases regulated by mTORC2 participate in this process. These data warrant further mechanistic investigation to understand the differential effects that perturbations of the mTORC2 pathway might have on central DA neurotransmission. In this context, it is important to point out seminal work by Mazei et al. (Mazei-Robison et al., 2011).

Here we show that genetic disruption of mTOR/Rictor signaling in the brain in nRictor KO mice behaviorally manifests in novelty-induced hyperactivity and

AMPH hypersensitivity, two phenotypes that have been associated in animal models with schizophrenia (Gainetdinov et al., 2001). These behaviors were mechanistically supported by both pre- and post-synaptic changes in the DA system. These include diminished DA bioavailability and elevation in D2R and its downstream signaling. Further, we demonstrate that mTORC2 signaling specifically in the dorsal striatum regulates AMPH-induced locomotion.

Striatum is a basal ganglia nucleus critically involved in integrating motor control, reward, and motivation. It is also implicated in brain disorders such as Parkinson's and Huntington's disease, drug addiction, and schizophrenia (Nestler, 2005; Kellendonk et al., 2006; Kreitzer and Malenka, 2008; Simpson et al., 2010; Durieux et al., 2012). Studies in animal models link stereotypy to aberrant DA signaling in the dorsal striatum (Kelly et al., 1975; Andrews et al., 1982; Carr and White, 1984; Porrino et al., 1984; Sharp et al., 1987). Our results demonstrate that global brain impairment of mTORC2 signaling results in hyperactivity, increased stereotypy, and exaggerated response to AMPH. A limitation to the genetic approach undertaken here is that nRictor KO mice have reduced brain size (Siuta et al., 2010). Therefore, we cannot exclude the possibility that the observed phenotype was influenced by developmental impairment. However, virally mediated deletion of Rictor specifically in the dorsal striatum of adult mice significantly increased the ability of AMPH to stimulate locomotion, recapitulating, at least in part, nRictor KO mouse behavior. Overall, our data strongly support a primary role for mTORC2 signaling in modulating striatal DA neurotransmission.

AMPH-induced changes in behavior are caused by elevated intra-synaptic DA promoted by non-vesicular DAT-mediated DA release (Fischer and Cho, 1979; Sulzer et al., 2005; Rebec, 2006; Hamilton et al., 2014). Surprisingly, we found a decrease in DA bioavailability in response to AMPH in the dorsal striatum of nRictor KO mice. This finding is further supported by the observed reduction of tissue DA levels, as well as lowered pTH-Ser31. This key phosphorylation site correlates with TH activity *in vivo,* and is a reliable predictor of DA tissue content (Salvatore et al., 2009; Damanhuri et al., 2012; Salvatore and Pruett, 2012). All together these data suggest the involvement of compensatory mechanisms that employ DA receptors for the observed behavior. In this respect, the nRictor KO mouse represents a unique model of aberrant brain mTORC2 signaling causing a plethora of changes in central DA neurotransmission including altered D2R signaling. The finding that nRictor KO mice have elevated D2R expression, with no change observed in D1R levels, supports this hypothesis.

nRictor KO mice have reduced striatal DA content in the context of elevated D2R. These data are in line with previous studies suggesting that striatal DA depletion leads to an increase in D2R, and not D1R expression (Dewar et al., 1990; Radja et al., 1993). However, we cannot exclude the possibility that overexpression of D2R drives the reduction in DA content. In support of this mechanistic hypothesis, acute administration of haloperidol increased TH phosphorylation at Ser31 in nRictor KO mice. We also hypothesize that increased D2R expression drives the nRictor KO behavioral phenotype. Consistent with this hypothesis, the AMPH locomotor hypersensitivity of these

mice was blocked by the D2R inhibitor, haloperidol. The involvement of D2R in the regulation of locomotion is further supported by D2R-eGFP mice, which overexpress D2R in the medium spiny neurons and exhibit higher basal activity (Kramer et al., 2011). Therefore, elevated D2R in this model appears to drive both reduced DA synthesis and hypersensitivity to AMPH.

In nRictor KO animals with impaired mTORC2 signaling phosphorylation of ERK1/2 is markedly elevated, a known downstream target of D2R activation (Luo et al., 1998; Cai et al., 2000; Wang et al., 2005; Bolan et al., 2007). Therefore, the observed increase in D2R expression in nRictor KO mice likely accounts for the increase striatal ERK1/2 phosphorylation. This possibility is strengthened by the finding that haloperidol treatment lowered ERK1/2 phosphorylation in nRictor KO mice. Prior studies have shown that in DA-depleted striatum, pERK1/2 was increased in response to D2R, but not D1R stimulation, which led to a characteristic rotating phenotype that could be blocked by the inhibition of MAPK/ERK pathway (Cai et al., 2000). In addition, a recent study revealed that disruption of upstream activators of the Akt signaling pathway in D2R expressing striatopallidal MSNs, results in spontaneous and drug-induced hyperlocomotion along with an increase in ERK1/2 phosphorylation (Besusso et al., 2013). Taken together, our data suggest a “cross-talk” between mTORC2 function and D2R-ERK1/2 signaling that drives specific DA-dependent dorsal striatum-associated behaviors. Indeed, the significant increase in striatal pERK1/2 was restored to control levels with an acute treatment of haloperidol, a typical antipsychotic and D2R antagonist. Furthermore, our behavior experiments demonstrate that

haloperidol blocks the exaggerated AMPH-induced hyperlocomotion in mice with reduced brain mTORC2 signaling.

In this study we functionally isolate the dorsal striatum as a brain region where mTORC2 signaling regulates DA-dependent behaviors by altering D2R signaling. D2R are clearly involved in schizophrenia, supported by the fact that typical antipsychotics that target D2R alleviate schizophrenic symptoms in humans (Chouinard et al., 1993; Min et al., 1993; Odou et al., 1996; Kasper et al., 1997). Furthermore, D2R antagonists attenuate schizophrenia-like phenotypes associated with striatal DA dysfunction in animal models (Cazorla et al., 2014). Here, we show that aberrant mTORC2 signaling in the dorsal striatum is sufficient to alter fundamental DA-dependent behaviors in mice. Furthermore, we demonstrate that reduction of mTORC2 function influences DA levels in the dorsal striatum, and causes abnormal D2R signaling that leads to aberrant phosphorylation of ERK1/2, ultimately supporting the observed basal and AMPH-induced hyperactivity in the KO animals.

*Translational relevance:* Our prior findings reveal that nRictor KO mice have altered cortical DA neurotransmission associated with impaired pre-pulse inhibition (Siuta et al., 2010). Besides cortical circuits, dysregulated striatal DA neurotransmission is also thought to be fundamental to the etiology of schizophrenia (Davis et al., 1991a; Abi-Dargham et al., 2000; Howes and Kapur, 2009). Pivotal evidence supporting the DA hypothesis of schizophrenia stemmed from studies investigating psychotogenic effects of psychostimulants (Lieberman et al., 1987). Psychostimulants, including AMPH, disrupt striatal DA homeostasis

(Rebec, 2006). Importantly, they were shown to cause paranoia and psychosis in healthy individuals, and further exacerbate psychoses in schizophrenic patients (Lieberman et al., 1987). Imaging studies implicate the dorsal striatum in AMPH responses in both healthy humans and patients with schizophrenia (Weinberger and Laruelle, 2002). This study enhances our understanding of how metabolic signaling via mTORC2 can influence DA neurotransmission in brain and reveals that mTORC2 is a pivotal regulator of striatal DA neurotransmission and AMPH action. Our data also support dysfunction of mTORC2 signaling as a possible mechanism underlying in the etiology of schizophrenia.

## Materials and Methods

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

### Generation of mice

Mice were engineered as described previously (Siuta et al., 2010). Briefly, mice with floxed *Rictor* alleles were crossed to Nestin-Cre transgenic animals to produce neuron specific Rictor knockout mice (rictor f/f Nes+/+ or +/-; nRictor KO). Control mice (CTR) were littermates that lacked Cre recombinase. All mice were backcrossed to C57Bl6 background for at least 10 generations. To genotype the animals, DNA from tail clippings was analyzed by PCR with primers for the floxed, nestin, and recombined alleles as previously described (Shiota et

al., 2006).

**Brain slice preparation and biotinylation**

For brain slice preparation and biotinylation, all procedures were performed as previously described (Robertson et al., 2010). Briefly, 8-30 week old mice were sacrificed by rapid decapitation, and corticostriatal (300 $\mu$m) slices were prepared with a vibratome (Leica VT1000S) in ice cold oxygenated (95% O2 / 5% CO2) sucrose solution (sucrose 210 mM; NaCl 20 mM; KCl 2.5 mM; MgCl2 1 mM; NaH2PO4•H2O 1.2 mM). Slices were then collected in oxygenated artificial cerebral spinal fluid (ACSF) (NaCl 125 mM, KCl 2.5 mM, NaH2PO4•H2O 1.2 mM, MgCl2 1 mM, CaCl2•2H2O 2 mM), washed twice with oxygenated 4°C ACSF, and then incubated with 4°C ACSF solution containing 1 mg/mL of EZ-Link Sulfo-NHS-SS-Biotin (Pierce/ThermoScientific; Rockford, IL) for 45 min. After biotin incubation, the slices were rinsed, washed, and the reaction terminated with ACSF containing glycine. Dorsal striatum slices from CTR and nRictor KO mice were anatomically paired, dissected on ice, and frozen at −80°C until use. After homogenization, biotinylated (surface proteins) fraction was isolated with ImmunoPure immobilized streptavidin beads (Pierce). Total slice lysates and biotinylated fractions underwent immunodetection for dopamine (DA) transporter (DAT) and DA D2 receptor (D2R).

**Tissue harvest. Monoamine, metabolites, and amphetamine (AMPH) content**

Mice (8-30 weeks old) were sacrificed by rapid decapitation under volatile

isoflurane anesthesia, brains were removed and chilled on ice. Dorsal striatum was dissected out from two hemispheres to create comparable samples for both monoamine content and immunoblotting. After dissection, tissue was frozen on dry ice and stored in -80°C until use. Monoamine content was determined at the Vanderbilt University Neurochemistry Core *via* high performance liquid chromatography (HPLC) with amperometric detection as described previously (Robertson et al., 2010). To evaluate AMPH concentration in the dorsal striatum, procedures as above were followed in mice injected with 2 mg/kg AMPH i.p. 30 min prior to decapitation.

**Immunoblotting**

Tissue was lysed in 1% Triton lysis buffer (25 mM Hepes, 150 mM NaCl, 2 mM sodium orthovanadate, 2 mM NaF, plus a cocktail of protease inhibitors and phosphatase inhibitors) and centrifuged at 17,000g for 30 min at 4°C. Supernatant was collected into 0.1% Triton pulldown buffer (25 mM HEPES, 150 mM NaCl, 2 mM sodium orthovanadate, 2 mM NaF, plus a cocktail of protease inhibitors and phosphatase inhibitors). Protein concentration was determined using Bio-Rad protein concentration kit and all samples were equalized for total protein amount. Proteins were denatured with SDS-PAGE sample loading buffer at 95°C for 5 min, cooled to room temperature, and separated by 10% SDS-PAGE. Resolved proteins were then transferred to polyvinylidene difluoride (PVDF) membrane and blocked in either 5% milk or 2.5% BSA in 0.1% Tween20 Tris-buffered saline. Blots were then incubated in primary antibody rocking either

at room temperature for 1 hour or overnight at 4°C. The primary antibodies used in this study included Akt (1:1000, Cell Signaling Technology), phospho-Akt serine 473 (pAkt-Ser473) (1:800, Cell Signaling Technology), D2R (1:100; Santa Cruz Biotechnology), D1 DA receptor (1:500; Sigma-Aldrich, US), tyrosine hydroxylase (1:1000; Cell Signaling Technology; Danvers, MA), phospho-tyrosine hydroxylase serine 31 (1:800; Cell Signaling Technology; Danvers, MA), DA transporter (1:1000; Dr. Roxanne Vaughan, University of North Dakota School of Medicine), ERK 1/2 (1:1000; Promega; Madison, WI), phospho-ERK 1/2 (1:800; Promega; Madison, WI), β-actin (used as a loading control; 1:1000; Sigma-Aldrich; St. Louis, MO), Na-K ATPase (used as a control for biotinylation; 1:1000; DSHB, Department of Biology, The John Hopkins University). All proteins were detected using HRP conjugated secondary antibodies (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA). After chemiluminescent visualization (PerkinElmer, Inc., Waltham, MA) on Hyblot CL film (Denville Scientific, South Plainfield, NJ), protein band densities were quantified using ImageJ software (ImageJ, National Institutes of Health, Bethesda, MD).

### *Ex-vivo* DA uptake

Corticostriatal slices were prepared as described above. After collection, slices were allowed to recover in 28°C oxygenated ACSF for one hour. The slices were then placed into 37°C ACSF uptake buffer for 10 min (NaCl 125 mM, KCl 2.5 mM, NaH2PO4•H2O 1.2 mM, MgCl2 1 mM, CaCl2•2H2O 2 mM, pH 7.4) containing [$^3$H]-DA (50 nM). Immediately after uptake, slices were washed in ice-

cold ACSF three times, homogenized in 1% Triton lysis buffer (see Immunoblotting), and spun down at 17,000g for 30 min at 4°C. Supernatant was collected into 0.1% Triton pulldown buffer, and samples equalized for total protein concentration. Ecoscint H scintillation solution (National Diagnostics, Atlanta, GA) was added to samples and they were quenched overnight, after which radioactivity was measured. Specific uptake was defined as total uptake minus uptake obtained in the presence of 10 μM cocaine (non-specific uptake).

**Locomotor behavior**

Male mice (8–12 weeks old), were housed in temperature and humidity controlled rooms and kept on a 12-h light/dark cycle. Food and water were available *ad libitum*. Experiments were conducted in accordance with the NIH guidelines for the care and use of animals and were approved by the Vanderbilt University Institutional Animal Care and Use Committee. Initial handling lasted 5 days, with daily i.p. saline injections. On day 6 mice were tested for open field locomotor activity. Four hour long sessions were performed using automated experimental chambers (27.9×27.9 cm; MED-OFA-510; MED Associates, Georgia, VT) under constant illumination in a sound-attenuated room. During days 7-12 mice were habituated to the chambers and saline injections: four hour sessions with two saline injections daily (at times -120 min and 0 min). On day 13, mice were injected with saline at time -120 min and allowed to explore the chambers for two hours to settle to comparable baseline. At time 0 min, drugs or vehicle were administered i.p. (AMPH 2mg/kg; haloperidol 0.8mg/kg in DMSO)

and locomotion recorded for the next two hours. Analysis of open field activity, as well as stereotypic counts was performed using Activity Monitor (MED Associates).

### *In-vivo* microdialysis

Mice under anesthesia with isoflurane were placed in a stereotaxic frame (Kopf Instruments) with a mouse adapter. A guide cannula (CMA7 microdialysis, USA) was placed above the dorsal striatum (+1.4 AP, ±1.4 ML from Bregma and −0.9 DV from dura for CTR mice, and +1.0 AP, ±1.4 ML from Bregma and −0.3 DV from dura for nRictor KO mice) and secured to the skull with epoxy adhesive (Plastics One). Animals were allowed 24 hours to recover from the surgery. One day before the experiment, animals were placed in individual dialysis chambers and the microdialysis probe (CMA7 microdialysis, USA) with the active length of 2mm was inserted into the guide cannula. One end of a tether (Plastics One) was attached to a harness and the other end attached to a swivel (Instech) mounted on a counterbalanced arm above the dialysis chamber. The probe was perfused overnight at a flow rate of 0.5 µL/min with artificial cerebral spinal fluid containing 149 mM NaCl, 2.8 mM KCl, 1.2 mM CaCl2, 1.2 mM MgCl2, 5.4 mM d-glucose, pH 7.2. On the day of the experiment the flow rate was changed to 1.0 µL/min and after equilibration, dialysis fractions (20 min each) were collected to establish baseline concentrations of neurotransmitter efflux. Dialysate samples were stored at −80°C and analyzed by HPLC-EC for DA levels. Probe placement was verified after collection of slices.

### *Ex vivo* high speed chronoamperometry

Corticostriatal slices were prepared and allowed to recover as specified above for DA uptake. DA concentration was measured by chronoamperometry in the dorsal striatum as previously described (Hoffman and Gerhardt, 1999; Gerhardt and Hoffman, 2001). Briefly, carbon fiber electrodes (100 µm length × 30 µm O.D.) coated with nafion for dopamine selectivity were lowered into the dorsolateral portion of the striatum so that the tip of the recording electrode was positioned at a depth of 75-100 µm. The voltage was stepped from 0 mV to 550 mV for 100 ms and then back to 0 mV and the charging current of the carbon fiber electrode was allowed to decay for 20 ms before the signals were integrated. Data were collected at a frequency of 1 Hz with an Axopatch 200B amplifier. The integrated charge was converted to dopamine concentration based on *in vitro* calibration with dopamine.

### Radioactive nemonapride binding

Dorsal striatum tissue was harvested as described, and stored at -80°C until processed. To quantitatively assess D2R expression, plasma membranes were obtained by tissue homogenization in 10 volumes of ice-cold 50mM Tris-HCl buffer (containing also 1mM EDTA, 5mM $MgCl_2$, 1.5mM $CaCl_2$, 120mM NaCl, 5mM KCl, pH 7.4), and then centrifuged at 40,000 × g for 30 min. The pellet was re-suspended in ice-cold 50mM Tris-HCl buffer (containing 1mM EDTA, 5mM $MgCl_2$, 1.5mM $CaCl_2$, 120mM NaCl, 5mM KCl, 12µM pargyline, 0.1% ascorbic

acid, pH 7.4), after which protein content was determined by Bradford method. Samples were diluted to the same final protein concentration.

D2R binding assay was performed on ice, using 1.5nM [$^3$H]nemonapride to determine $V_{max}$. Nonspecific binding was determined using 10μM sulpiride. For the ligand-binding assays, the membrane preparations were incubated on a shaker (to prevent precipitation of the membranes) for 60 min in a final volume of 200μL. The reaction was stopped by rapid filtration through Whatman GF/A glass fiber filters. Filters were washed twice with 5 mL of ice-cold 50mM Tris-HCl buffer, and, after an overnight incubation, assayed for radioactivity on a liquid scintillation counter. Ligand binding was determined by average of duplicates with subtraction of non-specific binding, which was observed in the presence of 10μM sulpiride (30-35%).

**Stereotactic surgeries and viral injections**

Mice were anesthetized with isoflurane and placed in a stereotaxic frame (Kopf Instruments) with subsequent injection into two striatal locations. The tip of a 5.0μl Hamilton microsyringe needle (30-gauge) was inserted to stereotaxic coordinates relative to bregma: anterior-posterior (AP) +0.75cm; medial-lateral (ML) +/-1.50cm; and dorsal-ventral (DV) –3.57cm, and second DV coordinate, -3.00cm. Viral vector suspension in a volume of 1.5μl per hemisphere (0.750μl into each DV site) was injected at 0.15μl/min. After microinjection, the needle was left for 3 min before withdrawal to reduce the efflux of injectate along the

injection tract. Mice were excluded from injection failures. For the biochemical assessment, the mice were injected with rAAV-Cre into the right hemisphere, and with rAAV-eGFP into the left as control. Animals were sacrificed 3 weeks after microinjection, and analyses of the dorsal striatum tissue was performed as described above. For the behavioral experiments, mice were injected bilaterally with either rAAV-Cre or rAAV-eGFP, and locomotor behavior and AMPH-induced hyperactivity were assessed as described above.

**Statistical analysis**

All data are expressed as the mean ± SEM. Mean differences between groups were determined using Student's *t* test or two-way ANOVAs followed by *post hoc* testing when the main effect or interaction was significant at $p < 0.05$. Statistical analyses were conducted using GraphPad Prism. The number of animals and specific statistical analyses used in each experiment are indicated in the text.

## CHAPTER III

# *IMPAIRED mTORC2 SIGNALING IN CATECHOLAMINERGIC NEURONS EXAGGERATES HIGH FAT DIET-INDUCED HYPERPHAGIA**


## Abstract

*Objective:* Food intake is highly regulated by central homeostatic and hedonic mechanisms in response to peripheral and environmental cues. Neutral energy balance stems from proper integration of homeostatic signals with those "sensing" the rewarding properties of food. Impairments in brain insulin signaling causes dysregulation of feeding behaviors and, as a consequence, hyperphagia. Here, we sought to determine how the mammalian target of rapamycin complex 2 (**mTORC2),** a complex involved in insulin signaling, influences high fat feeding.

*Methods:* Rictor is a subunit of mTORC2, and its genetic deletion impairs mTORC2 activity. We used Cre-LoxP technology to delete *Rictor* in tyrosine hydroxylase (TH) expressing neurons (TH Rictor KO). We assessed food intake, body weight, body composition and DA dependent behaviors.


* The work presented in this chapter is *in press* as Olga I. Dadalko, Kevin D. Niswender, Aurelio Galli (*Heliyon*) 2015.

*Results:* TH Rictor KO mice display a high-fat diet specific hyperphagia, yet, when on low-fat diet, their food intake is indistinguishable from controls. Consistently, TH Rictor KO become obese only while consuming high-fat diet. This is paralleled by reduced brain DA content, and disruption of DA dependent behaviors including increased novelty-induced hyperactivity and exaggerated response to the psychostimulant amphetamine (AMPH).

*Conclusions:* Our data support a model in which mTORC2 signaling within catecholaminergic neurons constrains consumption of a high-fat diet, while disruption causes high-fat diet-specific exaggerated hyperphagia. In parallel, impaired mTORC2 signaling leads to aberrant striatal DA neurotransmission, which has been associated with obesity in human and animal models, as well as with escalating substance abuse. These data suggest that defects localized to the catecholaminergic pathways are capable of overriding homeostatic circuits, leading to obesity, metabolic impairment, and aberrant DA-dependent behaviors.

## Introduction

A range of factors contributes to obesity, including nutritional trends, availability of highly palatable foods, changes to the built environment, economic stresses, and others (Mokdad et al., 2003). Feeding is a centrally controlled complex biological behavior fine-tuned by both homeostatic metabolic drive (hunger or satiety) and hedonic motivational drive (reward and salience).

Therefore, identifying the neurobiological circuits in which deficits lead to the development of both positive energy balance (i.e. “homeostatic” or “metabolic” obesity) and/or exaggerated responses to palatable food (i.e. “hedonic” obesity) is essential for defining mechanisms involved in the central regulation of food intake.

Akt is a key insulin dependent kinase that influences both peripheral endocrine responses and higher brain functions such as learning and memory, reward, and salience (Bae et al., 2003; Beaulieu et al., 2004; Russo et al., 2007; Kumar et al., 2008; Miller et al., 2014; Wong et al., 2015). Akt is activated by phosphorylation of two key residues, Thr308 and Ser473. Mammalian target of rapamycin (mTOR) complex 2 (mTORC2) is a multiprotein complex responsible for phosphorylation of Akt at Ser473 (pAkt-473). Within hypothalamic neurons, mTORC2 activity is implicated in mechanisms that control homeostatic neuroendocrine responses (Kocalis et al., 2014). In the catecholaminergic system, mTORC2 function regulates monoamine turnover, DA neurotransmission, as well as psychostimulant action (Siuta et al., 2010; Speed et al., 2011; Dadalko et al., 2015). These processes are implicated in reward and salience (Daws et al., 2011). This concept is also supported by data demonstrating that impairment in mTORC2 signaling leads to escalating morphine self-administration (Mazei-Robison et al., 2011).

The mTORC2 complex consists of Rictor, mSIN1, mLST8, and mTOR. Recent studies show that conditional deletion of *Rictor* in hypothalamic centers leads to hyperphagia, impaired peripheral glucose homeostasis, and obesity

(Kocalis et al., 2014). In order to determine whether Rictor function specifically within catecholaminergic neurons plays a role in feeding behaviors and metabolism, we crossed *Rictor-Flox* mice with mice expressing *Cre recombinase* under control of the tyrosine hydroxylase (TH) promoter (TH Rictor KO). We show that disruption of mTORC2 signaling within catecholaminergic circuits supports exaggerated hyperphagia in response to palatable high-fat diets.

## Results

*Rictor deletion* within *catecholaminergic circuits results in increased lean mass.* We first assessed body weight and composition, as well as feeding in animals consuming a low fat diet for 8 weeks (LF, 10% fat). TH Rictor KO mice weigh more than control (CTR) mice (Fig. 10A; CTR mice were littermates of TH Rictor KO mice that lacked *Cre recombinase*). The increase in body weight is due to elevated lean mass as determined by NMR (Fig. 10A). DA neurons in the hypothalamus have been shown to regulate plasma growth hormone (GH) concentration (Bosse et al., 1997). The increase in lean mass, therefore, could stem from elevated GH levels. There were no differences in GH between genotypes (data not shown). Importantly, cumulative food intake was not different for TH Rictor KO mice relative to CTR animals (Fig. 10B) while on the LF diet. Feed efficiency, the change in total body weight, fat, or lean mass divided by the cumulative calories consumed over a period of time was not different (Fig. 10C).

These data strongly suggest that TH Rictor KO mice with disrupted mTORC2 signaling within catecholaminergic neurons have intact homeostatic energy regulation. However, since catecholamines are implicated in salience and reward, we next determined the role of mTORC2 signaling on the intake of a high fat (HF, 60% fat), palatable diet.

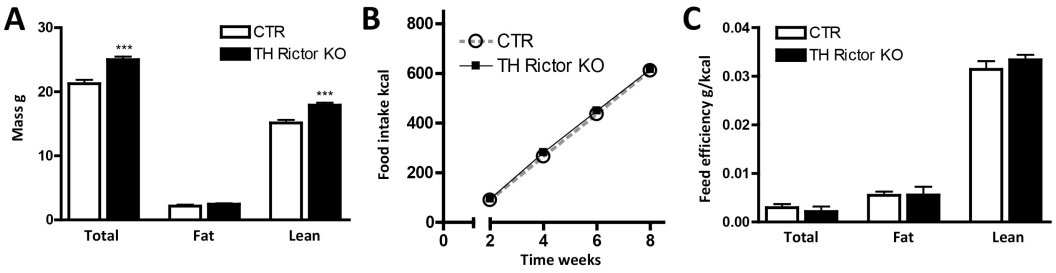



**Figure 10. Energy balance and body composition of TH Rictor KO mice on low-fat diet (LF).** (A) Body composition of TH Rictor KO and CTR mice; n=8 per genotype (B) Cumulative food intake (LF diet) was measured over the course of eight weeks and was found to be indistinguishable from the LF food intake of CTR animals; n=6-7 per genotype. (C) Feed efficiency over the corresponding eight week period was calculated as change in total body weight, fat, or lean mass in grams divided by total kcal consumed. Values represent mean±SEM; *** $p<0.001$.

*Conditional deletion of Rictor in catecholaminergic neurons results in escalating hyperphagia on high fat (HF) diet.* The mesolimbic system is an essential component of the circuitry that evaluates saliency of natural rewards, including food (Castro et al., 2015; Cone et al., 2015; McCutcheon, 2015; Perello and Dickson, 2015). Catecholaminergic neurons are an essential component of this system. Given their role in food reward, we hypothesized that mTORC2 signaling in TH expressing neurons might play a pivotal role in regulating food consumption in animals exposed to palatable high fat diet (HF, 60% fat). Unlike

on LF diet, TH Rictor KO mice consume significantly more calories than CTR animals on HF diet (Fig. 11A, *inset*). When excess HF diet intake is visualized

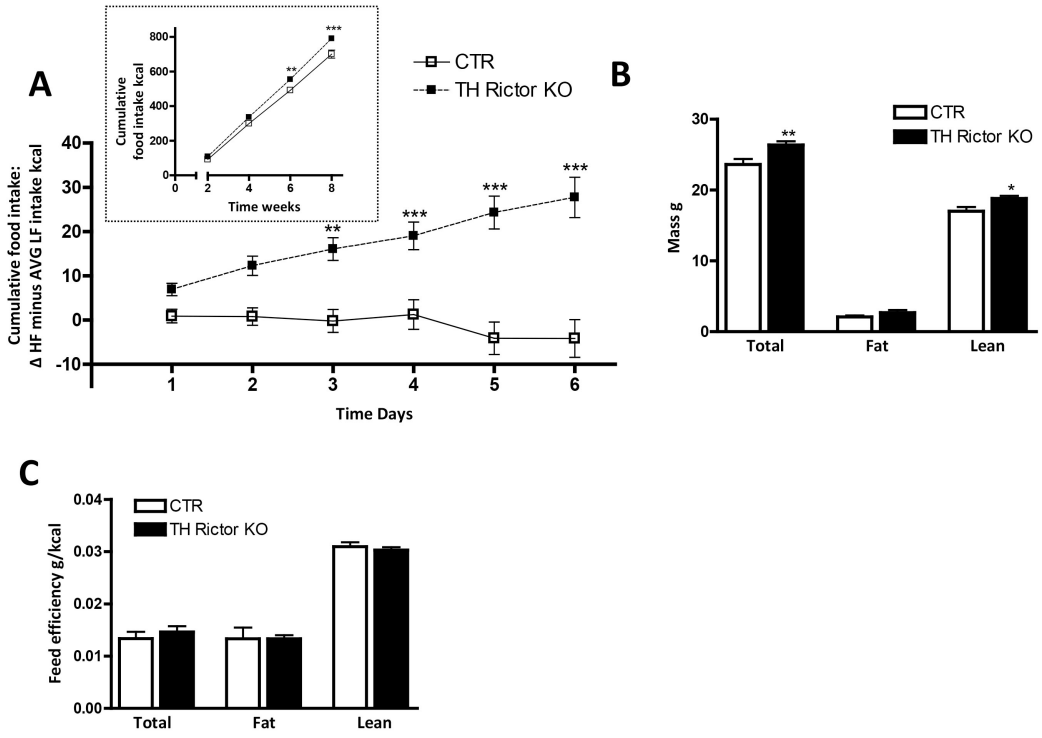



**Figure 11. Energy balance of TH Rictor KO mice on high-fat diet (HF).** (A) Genotype-specific difference in cumulative consumption of HF food relative to LF food consumption (average low-fat food intake was subtracted from high-fat food intake in kcal); n=7-9 per treatment per genotype. Inset: Cumulative food intake (high-fat diet) was measured over the course of eight weeks. In both graphs, high-fat food-induced escalating hyperphagia is shown for TH Rictor KO mice compared to CTR animals; n=6-7 per genotype. (B) Body composition over the corresponding eight week period was measured and graphed to compare with CTR mice; n=8 per genotype. (C) Feed efficiency over the corresponding eight week period was calculated as change in total body weight, fat, or lean mass in grams divided by total kcal consumed); n=4-5 per genotype. Values represent mean±SEM; *** p<0.001, ** p<0.01, * p<0.05.

cumulatively by subtracting average LF diet consumption from daily HF diet consumption, continuously escalating HF diet consumption is observed only in TH Rictor KO mice (Fig. 11A). This was calculated by subtracting the average

caloric consumption on a low fat diet from the caloric consumption on the high fat diet of the correspondent genotype each day. Thus, CTR animals modulate their high-fat food intake, while TH Rictor animals continuously increase consumption of the high-fat diet over time (Fig. 11A). Concomitantly, TH Rictor KO mice show a rapid increase in body weight and lean mass after only six days on a high fat diet (Fig. 11B) without changes in feed efficiency (Fig. 11C).

*Conditional deletion of Rictor in TH expressing neurons results in impaired DA neurotransmission and aberrant DA-dependent behaviors.* The catecholaminergic system modulates motivated behaviors such as feeding, drinking, and locomotion as well as reward. TH Rictor KO mice exhibit normal food intake when exposed to a LF diet. However, they display exaggerated hyperphagia to a HF diet. This hyperphagia could stem from disrupted DA neurotransmission in midbrain. To explore this possibility, we first analyzed DA tissue content in the ventral striatum. TH Rictor KO mice have reduced DA tissue content in the Nucleus Accumbens (NAc) relative to CTR animals (Fig. 12A), suggesting impaired mesolimbic DA tone in the conditional KO mice. Importantly, DA metabolites DOPAC, 3-MT, and HVA were not significantly changed in the TH Rictor KO mice (data not shown), suggesting faster DA metabolism. This hypothesis awaits further exploration.

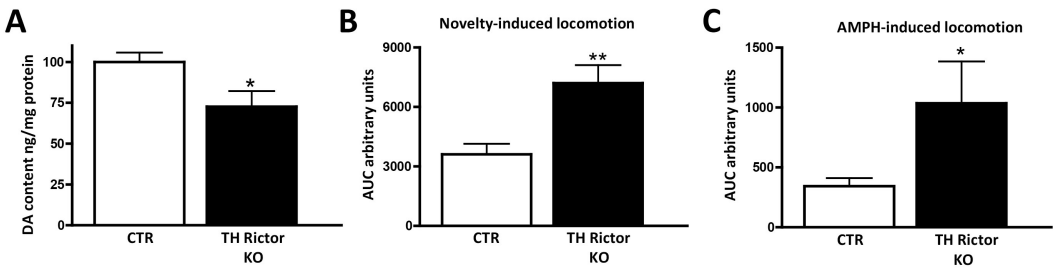



**Figure 12. Aberrant NAc DA tone and disrupted DA-dependent behaviors in TH Rictor KO mice.** (A) DA tissue content as measured by HPLC in NAc homogenates; n=6-8 per genotype. (B) Novelty-induced locomotion: horizontal movement measured in open field chambers in 5-min intervals; n=8 per genotype. Data are represented as area under the curve (AUC) for the first 30 minutes in the chamber. (C) AMPH-induced locomotion; n=7-8 per treatment per genotype. CTR and TH Rictor KO mice were habituated to saline injections and open field chambers for six days. On day seven AMPH (2mg/kg) was administered i.p. and horizontal locomotor activity recorded in 5-min intervals. Data are represented as area under the curve (AUC) from time of injection to 30 minutes. Values represent mean±SEM; ** $p<0.01$, * $p<0.05$.

Analysis of locomotor activity is an efficient method to evaluate the integrity of the DA neurotransmission in rodents. Novelty-induced hyperactivity is modulated by the mesolimbic DA pathway (Bardo et al., 1990). TH Rictor KO mice display exaggerated hyperlocomotion in a novel environment relative to CTR animals (Fig. 12B). Importantly, rodents that are prone to elevated novelty-induced hyperactivity, also exhibit heightened sensitivity to psychostimulants, such as amphetamine (AMPH) (Siuta et al., 2010; Kramer et al., 2011; Dadalko et al., 2015). Therefore, we challenged habituated (see methods) TH Rictor KO mice with a single dose of AMPH. TH Rictor KO animals show increased AMPH-induced hyperactivity compared to their wild type counterparts (Fig. 12C). These data support the notion that genetic deletion of *Rictor* in the TH expressing cells results in altered mesolimbic DA tone associated with exaggerated high fat hyperphagia and AMPH-induced hyperlocomotion.

## Discussion

Regulation of energy balance is intricately regulated by the central nervous system. Food intake is controlled by both homeostatic and hedonic circuits, which rely not only on objective physiological cues supported by peripheral systems, but also on subjective experiences such as memory, motivation, and pleasure, all supported by environmental cues (Porte et al., 2002; Berthoud, 2006; Daws et al., 2011; Castro et al., 2015; Perello and Dickson, 2015). One of the key regulators of energy balance, the adiposity negative feedback hormone insulin, signals *via* mTORC2/Akt pathway in the brain, and modulates both homeostatic and hedonic neural circuits (Daws et al., 2011; Niswender et al., 2011; Kocalis et al., 2014). Proper integration of homeostatic signals with those "sensing" the saliency of food is necessary for appropriate energy balance regulation. Aberrant brain insulin signaling causes abnormal feeding behaviors, including hyperphagia (Speed et al., 2011) and has been shown to regulate catecholaminergic neurotransmission through Akt (Robertson et al., 2010; Siuta et al., 2010).

Dopamine is essential in modulation of many vital behaviors including movement, cognition, motivation, and salience. Motivation to obtain natural rewards such as food is vital for the organismal survival, and was shown to depend on central DA neurotransmission. Indeed, motivation for seeking food as well as the reward and satiety we feel when we eat have been extensively studied in humans using imaging techniques (Volkow et al., 2003; Volkow and

Wise, 2005). Mere consumption of food is a motivated behavior that is controlled by the DA signaling (Palmiter, 2007).

Our laboratory and others have previously shown *in vitro* data that aberrant Akt signaling, and its upstream regulators such as insulin, PI3K, and IRS-2 are capable of disrupting central DA neurotransmission by altering expression and/or function of the DA homeostasis molecular players (Carvelli et al., 2002; Garcia et al., 2005; Wei et al., 2007; Lute et al., 2008). In addition, our *in vivo* studies demonstrated that aberrant peripheral insulin signaling caused by either high-fat diet or pharmacological interventions leads to altered central DA signaling (Williams et al., 2007; Speed et al., 2011). Therefore, in this study we sought to look if the disrupted mTORC2/rictor/Akt signaling in the TH expressing neurons, which include DA cells, lead to abnormal feeding behaviors and altered metabolism.

*Conclusion:*

Here, we demonstrate that mTORC2 signaling in catecholaminergic neurons modulates brain DA homeostasis, and is implicated in DA-related behaviors such as novelty-induced hyperlocomotion, hypersensitivity to the psychostimulant AMPH, and, importantly, HF diet-specific hyperphagia. Importantly, studies in the metabolic chambers (conducted with the support of the Mouse Metabolic Phenotyping Center, Vanderbilt) allowed to compare energy expenditure, level of activity, oxygen consumption, heat production, and circadian rhythms in the TH Rictor KO and control animals (data not shown). This did not reveal any differences in basal activity or locomotion between genotypes,

suggesting that the increased high-fat food intake was not caused by the elevated activity of the TH Rictor mice. Our data support a model in which disrupted mTORC2 signaling within catecholaminergic neurons creates aberrant striatal DA neurotransmission, and causes HF diet-specific exaggerated hyperphagia. These data suggest that mTORC2 signaling defects localized to the catecholaminergic pathways are capable of overriding homeostatic circuits, and drives aberrant DA-dependent behaviors.

## Materials and methods

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

### Experimental animals: generation and care

Mice were engineered as previously described (Siuta et al., 2010; Saunders et al., 2014; Dadalko et al., 2015). Briefly, C57Bl6 mice with *floxed Rictor* alleles were crossed to *TH-Cre* transgenic animals to produce TH-cell specific *Rictor* knockout (TH Rictor KO) mice. Control mice (CTR) were littermates that lacked the floxed *Rictor* allele. To genotype the animals, DNA from tail clippings was analyzed by PCR with primers for the *floxed*, *Cre recombinase*, and recombined alleles as previously described (Shiota et al., 2006). Male mice were studied from 8 to 18 weeks of age. Mice were housed in a temperature (22 ° C) and light (12 h light/dark cycle) controlled room with free

access to standard laboratory rodent chow diet (#5001, LabDiet; St. Louis, MO) and water except where indicated.

**Food intake and body composition analysis**

Mice (male, 8-12 weeks old) were housed individually; diets (Research Diets, Inc., NJ, USA: high fat (HF) product number D12492; low fat (LF) product number D12450) were randomly assigned. Body weight was determined once per week, on a standard balance. Body composition was measured by nuclear magnetic resonance (NMR) in a Bruker Body Composition Analyzer (Bruker Optics; Billerica, MA). Adiposity (% body fat) was calculated as ((fat mass/lean mass)$\times$100). Caloric intake (kcal) was determined daily for the first seven days of diet administration and weekly thereafter. Feed efficiency is the ratio of weight gained (total body weight, fat and lean), divided by calories consumed (kcal) over the indicated period.

**Tissue harvest. Monoamine and their metabolites tissue content**

Mice (male, 8-12 weeks old) were sacrificed by rapid decapitation under volatile isoflurane anesthesia, brains were removed and chilled on ice. Dorsal striatum was dissected out from two hemispheres to create comparable samples for both monoamine content and immunoblotting. After dissection, tissue was frozen on dry ice and stored in -80°C until use. Monoamine content was determined at the Vanderbilt University Neurochemistry Core *via* high

performance liquid chromatography (HPLC) with amperometric detection as described previously (Robertson et al., 2010).

**Locomotor behavior**

Male mice (8–12 weeks old), were housed in temperature and humidity controlled rooms and kept on a 12-h light/dark cycle. Food and water were available *ad libitum*. Experiments were conducted in accordance with the NIH guidelines for the care and use of animals and were approved by the Vanderbilt University Institutional Animal Care and Use Committee. Initial handling lasted 5 days, with daily i.p. saline injections. On day 6 mice were tested for open field locomotor activity. Four-hour long sessions were performed using automated experimental chambers (27.9×27.9 cm; MED-OFA-510; MED Associates, Georgia, VT) under constant illumination in a sound-attenuated room. During days 7-12 mice were habituated to the chambers and saline injections: four-hour sessions with two saline injections daily (at times -120 min and 0 min). On day 13, mice were injected with saline at time -120 min and allowed to explore the chambers for two hours to settle to comparable baseline. At time 0 min, drugs or vehicle (saline) were administered i.p. (AMPH 2mg/kg) and locomotion recorded for the next two hours. Analysis of open field activity, as well as stereotypic counts was performed using Activity Monitor (MED Associates).

**Statistical analysis**

All data are expressed as the mean ± SEM. Mean differences between groups were determined using Student's *t* test or one- or two-way ANOVAs followed by *post hoc* testing when the main effect or interaction was significant at $p < 0.05$. Statistical analyses were conducted using GraphPad Prism. The number of animals and specific statistical analyses used in each experiment are indicated in the figure legends and/or text.

# CHAPTER IV

# DISCUSSION

Diseases stemming from aberrant peripheral insulin signaling, such as obesity and metabolic impairment, are becoming alarmingly common in the United States today. According to NIH health surveys, 68.8% of US adults are overweight, and 35.7% are obese with body mass index (BMI) above 30. The statistics are even more disconcerting for children and adolescents – one third of all young people aged 2 to 19 are overweight or obese. Therefore, the comorbidity of obesity and mental illness is an important public health concern. The first observations of such comorbidity became apparent two centuries ago. Sir Henry Maudsley, a pioneering British psychiatrist, wrote, "*Diabetes is a disease which often shows itself in families in which insanity prevails." (1868, The Physiology and Pathology of Mind").* While it might not seem surprising to understand that obesity and/or diabetes may trigger CNS disorders due to inflammation and altered vasculature permeability (Gavard et al., 1993; Anderson et al., 2001; Lustman et al., 2005; Anderson et al., 2007), emerging literature supports peripheral metabolic disorders often preceding psychiatric diagnoses by a few years (Mukherjee et al., 1989; Mukherjee et al., 1996; Ryan et al., 2003).

Disorders caused by insulin signaling impairment have been extensively studied in the periphery, where they lead to obesity and diabetes, which, in turn, are linked to the wide-spread life-threatening diseases such as heart disease,

stroke, and certain types of breast, kidney, colon, and endometrial cancers (niddk.nih.gov). In the last decade, impaired peripheral insulin signaling and, stemming from it, altered Act activity, were also found to result in aberrant brain monoamine neurotransmission (Sevak et al., 2007; Williams et al., 2007; Robertson et al., 2010; Owens et al., 2012). Important to note is that brain insulin levels depend on the active saturated transport of peripheral insulin across the blood brain barrier. This enables peripheral insulin to control central insulin/Akt signaling (Banks, 2004). Such vital brain functions as neurodevelopment, neurotransmitter release and reuptake, and neuronal receptor trafficking were shown to depend on central insulin/Akt signaling (van der Heide et al., 2006). Particularly important for this work is that brain DA neurotransmission was found to rely on central insulin/Akt signaling (Siuta et al., 2010; Daws et al., 2011; Speed et al., 2011; Dadalko et al., 2015). Human epidemiologic data as well as studies in animal models showed that aberrant Akt activity is linked to monoamine related neuropsychiatric disorders, particularly DA-associated brain dysfunctions, which manifest in many mental diseases including schizophrenia (Emamian et al., 2004; Siuta et al., 2010), drug addiction (Mazei-Robison et al., 2011; Neasta et al., 2011; Chen et al., 2012; Collo et al., 2012), and bipolar disorder (Beaulieu et al., 2007; Beaulieu et al., 2008; Beaulieu et al., 2009). Of utmost interest and relevance to this thesis was to elucidate how fine-tuning of Akt activity, particularly its upstream mTORC2/rictor signaling, regulates striatal DA homeostasis and subcortical DA-dependent behaviors. Therefore, the specific aims of this project were to:

I) *To test the hypothesis that impairment in brain mTORC2/rictor signaling disrupts central DA tone and assess the key elements responsible for aberrant striatal DA homeostasis in the nRictor KO mice.*

II) *To determine whether aberrant brain mTORC2/rictor signaling alters DA (subcortical) related behaviors.*

III) *To assess which neuronal populations or subcortical loci are vulnerable to disruption of mTORC2/rictor signaling in producing biochemical and behavioral DA-dependent phenotypes.*

Collectively, our studies reveal that disrupted central mTORC2/Akt signaling results in aberrant subcortical DA neurotransmission and disrupted DA-dependent behaviors. We utilized transgenic mouse models and viral intervention techniques to induce whole brain or region-specific deletion of protein rictor, an essential mTORC2 component. As discussed in Chapter I, mTORC2 is an AGC kinase, necessary to phosphorylate Akt at Ser-473. Here, we demonstrate how deletion of rictor and the consequential reduction of pAkt-473 in the brain result in altered striatal DA tone. Rictor deletion in the *nestin*-expressing cells as it occurs in the nRictor KO mouse was mechanistically supported by both pre- and post-synaptic changes in the DA system. Thus, nRictor KO mice exhibit diminished striatal DA bioavailability and increased DAT expression along with an elevation in D2R and its downstream signaling.

Striatal DA neurotransmission is tightly linked to the open field locomotor behavior and to the psychomotor response to AMPH (Fahn et al., 1971; Davis et al., 1991b; Lotharius and Brundin, 2002; Howes and Kapur, 2009). In line with these data, we have found that the biochemical and neurochemical phenotype of nRictor KO mice manifests in exaggerated novelty- and AMPH-induced locomotor activity. These two hyperactive behaviors have previously been associated with increased striatal DA tone (Giros et al., 1996; Gainetdinov et al., 1999b), which is contrary to our observations in our mouse model with reduced striatal DA content and decreased TH Ser-31 phosphorylation (Chapter II). Importantly, we have also shown that conditional deletion of rictor in the dorsal striatum (*via* viral intervention) or in the TH-expressing cells (TH Rictor KO transgenic mouse model) caused exaggerated AMPH-induced hyperactivity in mice. Of interest, TH Rictor KO mice show decreased DA tissue levels in the dorsal striatum, exhibiting a similar but less pronounced phenotype to the nRictor KO animals. This suggests that low DA tone may result from the disrupted mTORC2/rictor signaling in the presynaptic circuitry. This hypothesis is supported by the data from virally treated mice, with conditional deletion of rictor in the dorsal striatum: these animals do not show significant changes in the striatal DA content (data not shown). In addition to the total brain rictor knockout and the TH-specific rictor KO, we engineered Dlx5.6 and Nkx2.1 rictor KO mice, where deletion of rictor was targeted mostly to the projection neurons of the dorsal striatum (Dlx5.6), or mostly to the striatal interneurons (Nkx2.1). These animals exhibited no change in biochemical, neurochemical or behavioral

phenotypes as pertinent to striatal DA neurotransmission. This suggests that the behavioral phenotype of brain rictor deletion may result from a combination of altered mTORC2/rictor signaling in the presynaptic and projection striatal systems. Our data support the idea that the hyperactive behavior, usually ascribed to a hyperdopaminergic state, can be produced with low subcortical DA content.

Recent studies from our laboratory as well as others show that central DA tone depends on intact mTORC2/rictor signaling (Daws et al., 2011). Indeed, inhibition of the mTORC2 upstream activator IRS-2 in the VTA caused a decrease in the electrically-evoked DA release in the NAc. Furthermore, conditional knockout of rictor in the VTA significantly alters the size and excitability of the DA neurons, and reduces rewarding responses to morphine (Mazei-Robison et al., 2011). In addition, genetic deletion of rictor in the brain results in cortical hypodopaminergia, supporting pre-pulse inhibition (PPI) deficits in the transgenic mice (Siuta et al., 2010). As discussed in Chapter I, mTORC2/rictor/Akt signaling is implicated in the TH neuron survival. Importantly, the number of the TH-positive cells in the VTA and SNc was not altered in mice that lack neuronal rictor (Siuta et al., 2010). However, we found that DA neuron soma size was decreased in the VTA and unaltered in the SNc in the nRictor KO mice (Dr. Stanwood, personal communication). With these data in mind, it was especially interesting to find significant alterations in the *striatal* DA tone of the nRictor KO mice (Dadalko et al., 2015). These data suggest that mTORC2/rictor signaling engages different mechanisms to influence central DA

neurotransmission in a region-specific manner. Future investigations are warranted to analyze and compare the electrophysiological properties of the VTA and SNc DA neurons with aberrant mTORC2/rictor signaling. Additionally, rescue experiments employing viral interventions could be employed to tease apart which distinct pathways of the dopaminergic neurons in the VTA and SNc drive the phenotype.

DA homeostasis in the brain is supported by the availability and function of monoamine transporters. Compared to the cortical brain regions, where DA tone is regulated by the NET (Gresch et al., 1995; Yamamoto and Novotney, 1998; Miner et al., 2003; Siuta et al., 2010), in the dorsal striatum, it is the DAT that controls DA bioavailability (Giros et al., 1996; Jones et al., 1998). In the nRictor mice, striatal tissue DA content is low, whereas DAT expression and function are elevated compared to their wild-type counterparts. The Palmiter group showed that neonatal DA depletion from DA neurons did not change the expression of DAT (Zhou and Palmiter, 1995), suggesting that scarcity of DA would not change the levels of the DAT. Conversely, DAT expression and function determine DA synaptic bioavailability, as shown in studies of DAT-KO and DAT overexpression mice (Giros et al., 1996; Jones et al., 1998; Marazziti et al., 2004; Marazziti et al., 2007; Salahpour et al., 2008). DAT trafficking and expression is regulated by the insulin signaling, particularly by its downstream kinase Akt (Garcia et al., 2005; Speed et al., 2010; Speed et al., 2011). Since mTORC2/rictor signaling regulates Akt activity (Sarbassov et al., 2005; Franke, 2008b, a), these data suggest a

possible pathway that may be implicated in driving DAT overexpression in low tissue DA content that we observed in the nRictor KO mice.

DAT expression and function are significantly elevated in the dorsal striatum of the nRictor KO mice, with disrupted mTORC2/rictor/Akt signaling (Dadalko et al., 2015). Previous studies in our laboratory show that downregulation of Akt phosphorylation at Thr308, as well as full pharmacologic blockade of Akt by allosteric inhibitors (Lindsley et al., 2005; Speed et al., 2010; Speed et al., 2011) causes a reduction of DAT surface expression in the dorsal striatum. As discussed, nRictor KO mice show the opposite phenotype for DAT. This may be supported, at least in part, by an increase in pAkt308 (Appendix Figure 1), which exists simultaneously with the reduction of pAkt473 (Dadalko et al., 2015) in the dorsal striatum of the KO mice. Supporting an increase in pAkt308 phosphorylation, we show elevated pGSK3β in the dorsal striatum of nRictor KO mice (Appendix Figure 1). It is also possible that another AGC family kinase, downstream of mTORC2/rictor signaling, is involved in driving the nRictor KO increased DAT phenotype. For example, the AGC family contains 60 members (reviewed in (Pearce et al., 2010)), including, PKC and PKA, two kinases heavily involved in DAT trafficking, biomechanics, and function regulation (Pristupa et al., 1998; Blakely and Bauman, 2000; Chang et al., 2001; Granas et al., 2003; Khoshbouei et al., 2004; Page et al., 2004). Further studies are needed to tease apart how various brain regions employ mTORC2/rictor signaling and differential Akt phosphorylation to orchestrate DA homeostasis.

In addition to regulating DAT trafficking and function, Akt activity plays an important role in DA receptor signaling. For example, prolonged stimulation of D2R causes specific dephosphorylation of Akt at Thr308 (Beaulieu et al., 2004). Interestingly, phosphorylation of Akt at its second key residue Ser473 is not affected (Beaulieu et al., 2004). Furthermore, depletion of striatal DA in the hyperdopaminergic DAT-KO mice or haloperidol induced D2R blockade resulted in enhanced phosphorylation of Akt at Thr308, indicating that D2R are responsible for the regulation of Akt by DA (Beaulieu et al., 2004; Emamian et al., 2004). Importantly, Akt Thr308 residue is not directly dependent on mTORC2/rictor activity (Sarbassov et al., 2005; Franke, 2008b). This underscores the significance and novelty of our finding that D2R, as well as their downstream signaling pathways, are increased in the dorsal striatum of nRictor KO mice with aberrant mTORC2/rictor signaling and depleted Akt Ser473 phosphorylation.

As discussed above, DA levels do not control the expression of DAT, however, DA availability dictates the sensitivity and responsiveness of DA receptors. Thus, DA deficient mice reveal oversensitivity of DA receptors without exhibiting a change in receptor expression, manifested by an exaggerated psychomotor response and c-fos immunoreactivity to DA agonists (Kim et al., 2000). Importantly, nRictor KO mice demonstrate behaviors (i.e., increased novelty-induced hyperlocomotion, AMPH hypersensitivity) traditionally associated with elevated DA tone, which is contrary to what we find in nRictor KO mice. Hence, we hypothesized that postsynaptic DA receptor signaling is altered in the

nRictor KO. In the nRictor KO mice, we show elevated D2R in the dorsal striatum. Haloperidol administration confirmed our hypothesis by alleviating AMPH-induced hyperactivity and reducing pERK (Chapeter II) and pAkt308 to control levels (Appendix Figure 2) in nRictor KO mice. Similarly, in DA deficient mice, a mixture of D1-D2 receptor antagonists inhibited the hyperactivity caused by the DA agonists (Szczypka et al., 1999).

D2R may signal *via* a G-protein coupled pathway, or employ non-conventional signaling by forming a complex with β-arrestin, Akt, and PP2A (Beaulieu et al., 2011). Another mechanism that may be utilized to control D2R signaling is MDM2/β-arrestin mediated D2R internalization and its subsequent lysosomal degradation. Similar pathway has been previously shown in trafficking and degradation of beta2-adrenergic receptors (Shenoy et al., 2001). MDM2, E3 ubiquitin ligase for β-arrestin, requires phosphorylation by Akt for its activity (Milne et al., 2004; Fenouille et al., 2011). In the nRictor KO mice with aberrant mTORC2/rictor signaling, the activity of MDM2 may be decreased due to the impaired Akt function. Thus, a decrease in β-arrestin mediated D2R internalization may be expected. We have shown an increase in D2R expression and function in nRictor KO mice. Recently, we have also found that β-arrestin is elevated in the dorsal striatum of nRictor KO mice (Appendix Figure 3). Assessing the expression of MDM2 and its phosphorylation state is complicated by the fact that MDM2 is rapidly cleaved at the DVPD site by Caspase-2, which inhibits MDM2 E3 ubiquitin ligase activity (Oliver et al., 2011). Our preliminary data demonstrates downregulation of MDM2 in the dorsal striatum of nRictor KO

mice (Appendix Figure 4). Further immunoprecipitation experiments, along with biochemical assessment of MDM2 phosphorylation, will eventually help elucidate this paradigm.

Our findings with regard to aberrant central DA signaling in the nRictor KO mice are intriguing for several reasons. Besides offering many insights about the mechanisms of DA-dependent neuropsychiatric phenotypes, this mouse model provides a broad platform for studying central regulation of peripheral neuroendocrinology and metabolism. As discussed above, brain DA signaling and mTORC2/rictor/Akt pathway are underlying the interdependent mechanism implicated in the well-established comorbid nature of metabolic impairments and DA-related neuropsychiatric disorders. nRictor KO is a great model to explore the specific pathways that drive such comorbidity. Indeed, nRictor KO mice combine neuropsychiatric-like endophenotypes as well as metabolic abnormalities: cortical hypodopaminergia, aberrant subcortical DA neurotransmission, and altered DA-dependent behaviors (Siuta et al., 2010; Dadalko et al., 2015), as well as insulin resistance, impaired glucose utilization, hyperphagia and obesity (Kocalis et al., 2014). These data emphasize that aberrant central mTORC2/rictor signaling heavily impacts peripheral metabolic regulation.

In the last decade, significant attention has been devoted to isolating homeostatic and hedonic regulators that control energy balance and metabolism (Blundell et al., 2000; Castro et al., 2015). Hypothalamic homeostatic mechanisms are in place to match energy expenditure with appropriate calorie consumption. However, brain circuitry responsible for sensing and predicting

reward and salience for palatable diet may override homeostatic mechanisms, resulting in overconsumption and metabolic impairment. Accumulating evidence from clinical studies and animal models shows that brain circuits supporting substance abuse behaviors are implicated in “food addiction” (Johnson and Kenny, 2010; Kenny, 2011, 2013; Volkow et al., 2013; Tomasi et al., 2015). After seeing the robust metabolic phenotype in the nRictor KO mice, we became interested to explore how extensive the reward/salience neurocircuitry is in producing such severe metabolic imbalance. We began by engineering a mouse model with aberrant mTORC2/rictor singling in the TH expressing neurons: TH Rictor KO mice.

TH Rictor KO mice exhibit exaggerated overconsumption of high-fat (60% fat) diet. Our data strongly support a link between disrupted catecholaminergic mTORC2/rictor signaling and high-fat induced hyperphagia (Dadalko et al, *in press*). Hence, for the first time, we show that the mice with a deletion of rictor in the catecholaminergic neurons are extremely hyperphagic only on a palatable, high-fat diet, which makes them obese at much faster rates compared to their wild-type counterparts fed the same diet. It should be noted that the TH Rictor KO mice maintain normal food consumption and exhibit no obesity phenotype while on regular or low-fat chow (Dadalko et al, *in press*). Of interest, we also assessed the food intake of mice that lack rictor in the Dlx and Nkx expressing neurons, finding no difference in their consumption of high-fat food relative to the control wild-type mice (data not shown). Moreover, viral transgene-mediated deletion of rictor in the dorsal striatum has not produced either similar feeding

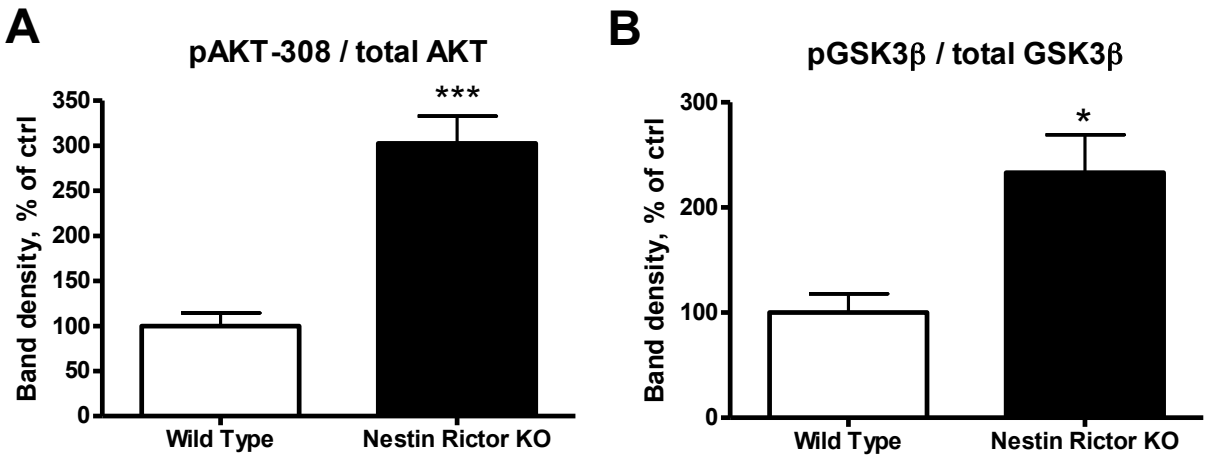
phenotype or metabolic abnormalities, despite causing AMPH-induced hypersensitivity. These data highlight the importance of intact mTORC2/rictor signaling within catecholaminergic neurons to maintain the integrity of high-fat food feeding. Future studies employing viral intervention techniques and metabolic chambers will help to pinpoint the neurocircuits responsible for obscuring the homeostatic energy balance. Specifically, condition place preference tests with highly palatable food and drugs of abuse will also help elucidate the mechanisms involved in the convergence of neuroendocrinology with the neurobiology of substance abuse disorders.

Similar to the nRictor KO, the VTA DA neurons of the TH Rictor KO animals have decreased soma size (Dr. Horvath, personal communication). This phenotype has been implicated in abnormal reward for morphine (Mazei-Robison et al., 2011), and is predicted not to impact the rewarding properties of ethanol, cocaine or nicotine (Mazei-Robison et al., 2014). These data suggest a pathway linking the soma size of the VTA DA neurons to the mechanism that drives higher tolerance for the rewarding properties of morphine, a μ-opioid receptor-specific drug. Importantly, high-fat food has been shown to induce a quick spike in CSF β-endorphin, an endogenous opioid acting on μ-opioid receptor (Mizushige et al., 2006; Mizushige et al., 2009). Additionally, stimulation of μ-opioid receptors in the NAc has been shown to stimulate feeding through reward-related mechanisms (Baldo et al., 2013; Richard et al., 2013). Further exploration of this hypothesis will allow to understand if the aberrant mTORC2/rictor within the VTA DA neurons disrupts the ability of DA to properly

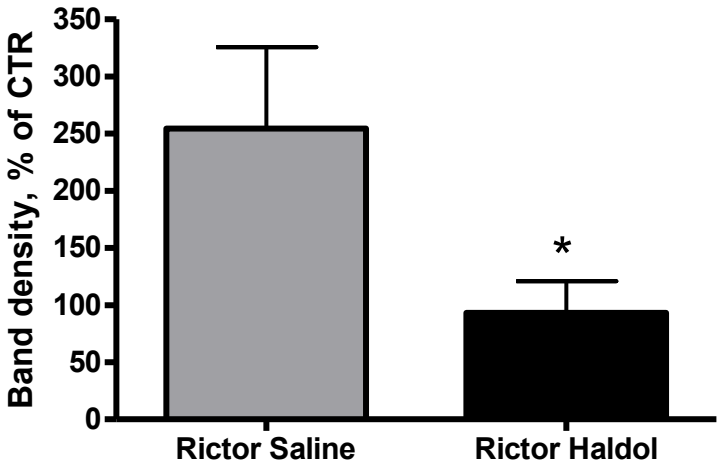
regulate central β-endorphin levels (Deutch and Martin, 1983), leading to the increased tolerance for the rewarding properties of the high-fat food and consequentially to the exaggerated escalating consumption of high-fat diet as it occurs in the TH Rictor KO mice.

In conclusion, the data presented here enhances the understanding of how metabolic signaling *via* mTORC2/rictor can influence central DA homeostasis and reveals that mTORC2/rictor pathway is a pivotal regulator of subcortical DA neurotransmission, AMPH action, and high-fat food intake. Our data also support the fact that dysfunction of mTORC2/rictor signaling in the brain may be a possible mechanism underlying the etiology of schizophrenia and the comorbidity of DA-related neuropsychiatric disorders and peripheral metabolic impairments.

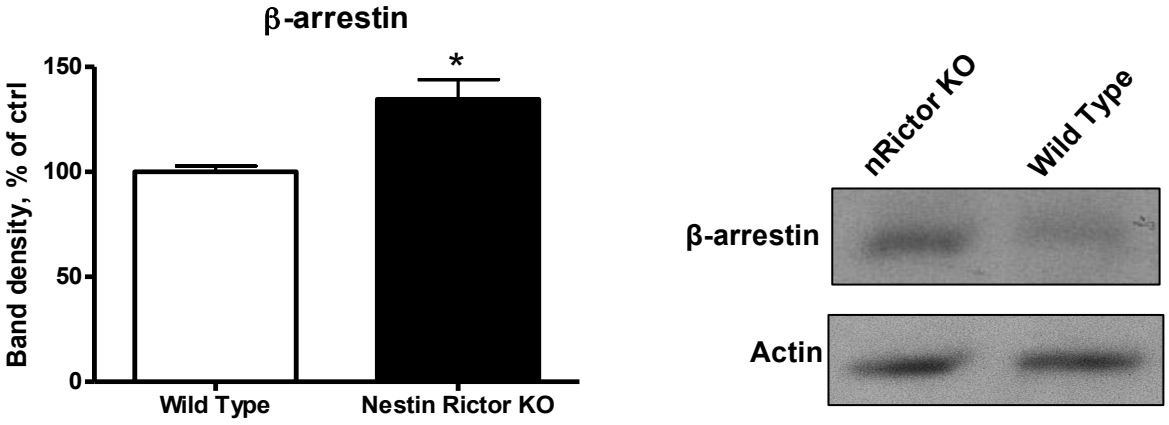
## APPEXDIX



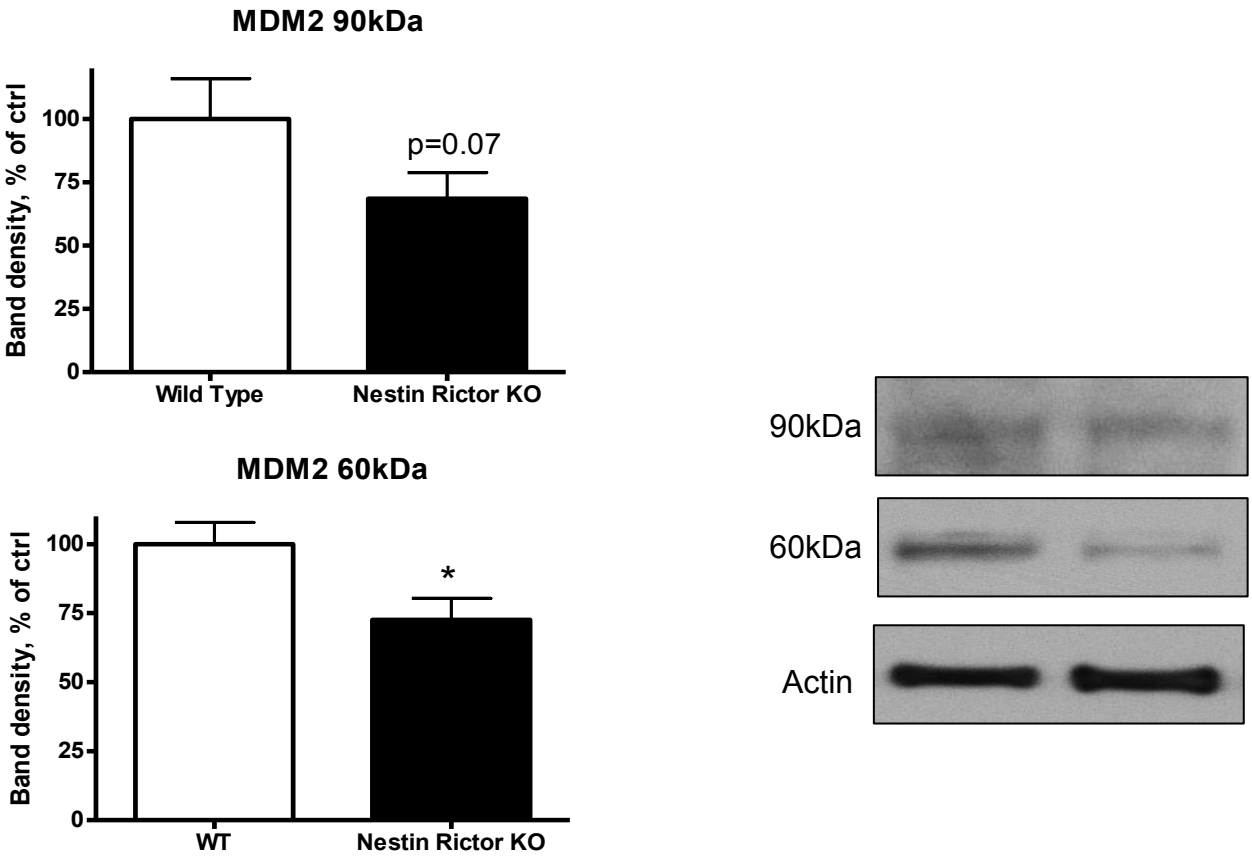

**Appendix Figure 1. Akt-308 and GSK3β phosphorylation in the nRictor KO mouse dorsal striatum.** (A) Quantification of pAkt308 normalized to total Akt (expressed as a percent of control) in the dorsal striatum of control wild type and nRictor KO mice. (Student's t-test, p=0.0002, df=13) (B) Quantification of pGSK3β normalized to total GSK3 (shown as a percent of control) dorsal striatum of control wild type and nRictor KO mice. (Student's t-test, p=0.01, df=13)




**Appendix Figure 2. Haloperidol reduces pAkt-308 in the dorsal striatum of nRictor KO mice.** Acute administration of haloperidol (i.p., 0.8mg/kg) reduced expression of pAkt308 in the dorsal striatum of nRictor mice. Shown is quantification of immunoblot optical densities. Data obtained from striatal tissue of CTR and nRictor mice injected with either vehicle or haloperidol (Haldol). Data were normalized to saline injected CTR. (Student's t-test, p=0.03, df=10)




**Appendix Figure 3. β-arrestin is elevated in the dorsal striatum of nRictor KO mice.** Optical density quantitation (*left*) and representative immunoblots (*right*) of β-arrestin expression in the dorsal striatum of nRictor KO (Nestin Rictor KO) and control wild type (Wild Type) animals. Actin was probed as a loading control. Data are expressed as percent of control. (Student's t-test, p=0.01, df=13[1])




**Appendix Figure 4. MDM2 is decreased in the dorsal striatum of nRictor KO mice.** Optical density quantitation (*left*) and representative immunoblots (*right*) of MDM2 expression in the dorsal striatum of nRictor KO (Nestin Rictor KO) and control wild type (WT) animals. Actin was probed as a loading control. Data are expressed as percent of control. (Student's t-test, p=0.02, df=12[2])

## References


Abi-Dargham A, Rodenhiser J, Printz D, Zea-Ponce Y, Gil R, Kegeles LS, Weiss R, Cooper TB, Mann JJ, Van Heertum RL, Gorman JM, Laruelle M (2000) Increased baseline occupancy of D2 receptors by dopamine in schizophrenia. Proceedings of the National Academy of Sciences of the United States of America 97:8104-8109.

Adkins EM, Samuvel DJ, Fog JU, Eriksen J, Jayanthi LD, Vaegter CB, Ramamoorthy S, Gether U (2007) Membrane mobility and microdomain association of the dopamine transporter studied with fluorescence correlation spectroscopy and fluorescence recovery after photobleaching. Biochemistry 46:10484-10497.

Amino S, Itakura M, Ohnishi H, Tsujimura J, Koizumi S, Takei N, Takahashi M (2002) Nerve growth factor enhances neurotransmitter release from PC12 cells by increasing Ca(2+)-responsible secretory vesicles through the activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase. Journal of biochemistry 131:887-894.

Anden NE (1970) [Parkinson's syndrome. 2. Nuclei and extrapyramidal tracts]. Lakartidningen 67:4807-4812 passim.

Anden NE (1972) Dopamine turnover in the corpus striatum and the lumbic system after treatment with neuroleptic and anti-acetylcholine drugs. The Journal of pharmacy and pharmacology 24:905-906.

Anden NE, Butcher SG, Engel J (1970a) Central receptor activity of amines formed from L-dopa and some dopa analogs. Acta pharmacologica et toxicologica 28:30.

Anden NE, Butcher SG, Corrodi H, Fuxe K, Ungerstedt U (1970b) Receptor activity and turnover of dopamine and noradrenaline after neuroleptics. European journal of pharmacology 11:303-314.

Anderson RJ, Freedland KE, Clouse RE, Lustman PJ (2001) The prevalence of comorbid depression in adults with diabetes: a meta-analysis. Diabetes care 24:1069-1078.

Anderson SE, Cohen P, Naumova EN, Jacques PF, Must A (2007) Adolescent obesity and risk for subsequent major depressive disorder and anxiety disorder: prospective evidence. Psychosomatic medicine 69:740-747.

Andrews CD, Fernando JC, Curzon G (1982) Differential involvement of dopamine-containing tracts in 5-hydroxytryptamine-dependent behaviours caused by amphetamine in large doses. Neuropharmacology 21:63-68.

Anthony K, Reed LJ, Dunn JT, Bingham E, Hopkins D, Marsden PK, Amiel SA (2006) Attenuation of insulin-evoked responses in brain networks controlling appetite and reward in insulin resistance: the cerebral basis for impaired control of food intake in metabolic syndrome? Diabetes 55:2986-2992.

Aschauer DF, Kreuz S, Rumpel S (2013) Analysis of transduction efficiency, tropism and axonal transport of AAV serotypes 1, 2, 5, 6, 8 and 9 in the mouse brain. PloS one 8:e76310.

Axelrod J, Whitby LG, Hertting G (1961) Effect of psychotropic drugs on the uptake of H3-norepinephrine by tissues. Science 133:383-384.
Bademci G, Vance JM, Wang L (2012) Tyrosine hydroxylase gene: another piece of the genetic puzzle of Parkinson's disease. CNS & neurological disorders drug targets 11:469-481.
Bae SS, Cho H, Mu J, Birnbaum MJ (2003) Isoform-specific regulation of insulin-dependent glucose uptake by Akt/protein kinase B. The Journal of biological chemistry 278:49530-49536.
Baik JH (2013) Dopamine signaling in reward-related behaviors. Frontiers in neural circuits 7:152.
Baldo BA, Pratt WE, Will MJ, Hanlon EC, Bakshi VP, Cador M (2013) Principles of motivation revealed by the diverse functions of neuropharmacological and neuroanatomical substrates underlying feeding behavior. Neuroscience and biobehavioral reviews 37:1985-1998.
Banks WA (2004) The source of cerebral insulin. European journal of pharmacology 490:5-12.
Banks WA, Owen JB, Erickson MA (2012) Insulin in the brain: there and back again. Pharmacology & therapeutics 136:82-93.
Barbeau A (1962) The pathogenesis of Parkinson's disease: a new hypothesis. Canadian Medical Association journal 87:802-807.
Bardo MT, Bowling SL, Pierce RC (1990) Changes in locomotion and dopamine neurotransmission following amphetamine, haloperidol, and exposure to novel environmental stimuli. Psychopharmacology 101:338-343.
Batchelor M, Schenk JO (1998) Protein kinase A activity may kinetically upregulate the striatal transporter for dopamine. Journal of Neuroscience 18:10304-10309.
Bateup HS, Santini E, Shen W, Birnbaum S, Valjent E, Surmeier DJ, Fisone G, Nestler EJ, Greengard P (2010) Distinct subclasses of medium spiny neurons differentially regulate striatal motor behaviors. Proceedings of the National Academy of Sciences of the United States of America 107:14845-14850.
Bauman AL, Apparsundaram S, Ramamoorthy S, Wadzinski BE, Vaughan RA, Blakely RD (2000) Cocaine and antidepressant-sensitive biogenic amine transporters exist in regulated complexes with protein phosphatase 2A. The Journal of neuroscience : the official journal of the Society for Neuroscience 20:7571-7578.
Baura GD, Foster DM, Porte D, Jr., Kahn SE, Bergman RN, Cobelli C, Schwartz MW (1993) Saturable transport of insulin from plasma into the central nervous system of dogs in vivo. A mechanism for regulated insulin delivery to the brain. The Journal of clinical investigation 92:1824-1830.
Beaulieu JM, Caron MG (2008) Looking at lithium: molecular moods and complex behaviour. Mol Interv 8:230-241.
Beaulieu JM, Gainetdinov RR, Caron MG (2009) Akt/GSK3 signaling in the action of psychotropic drugs. Annual review of pharmacology and toxicology 49:327-347.
Beaulieu JM, Espinoza S, Gainetdinov RR (2015) Dopamine receptors - IUPHAR Review 13. British journal of pharmacology 172:1-23.

Beaulieu JM, Del'guidice T, Sotnikova TD, Lemasson M, Gainetdinov RR (2011) Beyond cAMP: The Regulation of Akt and GSK3 by Dopamine Receptors. Frontiers in molecular neuroscience 4:38.

Beaulieu JM, Sotnikova TD, Yao WD, Kockeritz L, Woodgett JR, Gainetdinov RR, Caron MG (2004) Lithium antagonizes dopamine-dependent behaviors mediated by an AKT/glycogen synthase kinase 3 signaling cascade. Proceedings of the National Academy of Sciences of the United States of America 101:5099-5104.

Beaulieu JM, Tirotta E, Sotnikova TD, Masri B, Salahpour A, Gainetdinov RR, Borrelli E, Caron MG (2007) Regulation of Akt signaling by D2 and D3 dopamine receptors in vivo. The Journal of neuroscience : the official journal of the Society for Neuroscience 27:881-885.

Beaulieu JM, Marion S, Rodriguiz RM, Medvedev IO, Sotnikova TD, Ghisi V, Wetsel WC, Lefkowitz RJ, Gainetdinov RR, Caron MG (2008) A beta-arrestin 2 signaling complex mediates lithium action on behavior. Cell 132:125-136.

Berthoud HR (2006) Homeostatic and non-homeostatic pathways involved in the control of food intake and energy balance. Obesity 14 Suppl 5:197S-200S.

Bertler A, Rosengren E (1959) Occurrence and distribution of dopamine in brain and other tissues. Experientia 15:10-11.

Besusso D, Geibel M, Kramer D, Schneider T, Pendolino V, Picconi B, Calabresi P, Bannerman DM, Minichiello L (2013) BDNF-TrkB signaling in striatopallidal neurons controls inhibition of locomotor behavior. Nature communications 4:2031.

Bilic P, Jukic V, Vilibic M, Savic A, Bozina N (2014) Treatment-resistant schizophrenia and DAT and SERT polymorphisms. Gene 543:125-132.

Birkmayer W, Hornykiewicz O (1961) [The L-3,4-dioxyphenylalanine (DOPA)-effect in Parkinson-akinesia]. Wiener klinische Wochenschrift 73:787-788.

Birkmayer W, Hornykiewicz O (1962) [The L-dihydroxyphenylalanine (L-DOPA) effect in Parkinson's syndrome in man: On the pathogenesis and treatment of Parkinson akinesis]. Archiv fur Psychiatrie und Nervenkrankheiten, vereinigt mit Zeitschrift fur die gesamte Neurologie und Psychiatrie 203:560-574.

Blakely RD, Bauman AL (2000) Biogenic amine transporters: regulation in flux. Current Opinion in Neurobiology 10:328-336.

Blom H, Ronnlund D, Scott L, Spicarova Z, Rantanen V, Widengren J, Aperia A, Brismar H (2012) Nearest neighbor analysis of dopamine D1 receptors and Na(+)-K(+)-ATPases in dendritic spines dissected by STED microscopy. Microscopy research and technique 75:220-228.

Blundell J, Finlayson G, Halford J, King N (2000) The Regulation of Food Intake in Humans. In: Endotext (De Groot LJ, Beck-Peccoz P, Chrousos G, Dungan K, Grossman A, Hershman JM, Koch C, McLachlan R, New M, Rebar R, Singer F, Vinik A, Weickert MO, eds). South Dartmouth (MA).

Bolan EA, Kivell B, Jaligam V, Oz M, Jayanthi LD, Han Y, Sen N, Urizar E, Gomes I, Devi LA, Ramamoorthy S, Javitch JA, Zapata A, Shippenberg TS (2007) D2 receptors regulate dopamine transporter function via an extracellular signal-regulated kinases 1 and 2-dependent and phosphoinositide 3 kinase-independent mechanism. Molecular pharmacology 71:1222-1232.

Bonito-Oliva A, Pallottino S, Bertran-Gonzalez J, Girault JA, Valjent E, Fisone G (2013) Haloperidol promotes mTORC1-dependent phosphorylation of ribosomal protein S6 via dopamine- and cAMP-regulated phosphoprotein of 32 kDa and inhibition of protein phosphatase-1. Neuropharmacology 72:197-203.

Bosse R, Fumagalli F, Jaber M, Giros B, Gainetdinov RR, Wetsel WC, Missale C, Caron MG (1997) Anterior pituitary hypoplasia and dwarfism in mice lacking the dopamine transporter. Neuron 19:127-138.

Bowton E, Saunders C, Reddy IA, Campbell NG, Hamilton PJ, Henry LK, Coon H, Sakrikar D, Veenstra-VanderWeele JM, Blakely RD, Sutcliffe J, Matthies HJ, Erreger K, Galli A (2014) SLC6A3 coding variant Ala559Val found in two autism probands alters dopamine transporter function and trafficking. Translational psychiatry 4:e464.

Braff DL, Geyer MA, Swerdlow NR (2001) Human studies of prepulse inhibition of startle: normal subjects, patient groups, and pharmacological studies. Psychopharmacology (Berl) 156:234-258.

Brunelin J, Fecteau S, Suaud-Chagny MF (2013) Abnormal striatal dopamine transmission in schizophrenia. Current medicinal chemistry 20:397-404.

Bruning JC, Gautam D, Burks DJ, Gillette J, Schubert M, Orban PC, Klein R, Krone W, Muller-Wieland D, Kahn CR (2000) Role of brain insulin receptor in control of body weight and reproduction. Science 289:2122-2125.

Buck KJ, Amara SG (1994) Chimeric dopamine-norepinephrine transporters delineate structural domains influencing selectivity for catecholamines and 1-methyl-4-phenylpyridinium. Proceedings of the National Academy of Sciences of the United States of America 91:12584-12588.

Burt DR, Enna SJ, Creese I, Snyder SH (1975) Dopamine receptor binding in the corpus striatum of mammalian brain. Proceedings of the National Academy of Sciences of the United States of America 72:4655-4659.

Butcher SP, Fairbrother IS, Kelly JS, Arbuthnott GW (1988) Amphetamine-induced dopamine release in the rat striatum: an in vivo microdialysis study. Journal of neurochemistry 50:346-355.

Cai G, Zhen X, Uryu K, Friedman E (2000) Activation of extracellular signal-regulated protein kinases is associated with a sensitized locomotor response to D(2) dopamine receptor stimulation in unilateral 6-hydroxydopamine-lesioned rats. The Journal of neuroscience : the official journal of the Society for Neuroscience 20:1849-1857.

Carlsson A (1987) Perspectives on the discovery of central monoaminergic neurotransmission. Annual review of neuroscience 10:19-40.

Carlsson A, Lindqvist M (1963) Effect of Chlorpromazine or Haloperidol on Formation of 3methoxytyramine and Normetanephrine in Mouse Brain. Acta pharmacologica et toxicologica 20:140-144.

Carlsson A, Lindqvist M, Magnusson T (1957) 3,4-Dihydroxyphenylalanine and 5-hydroxytryptophan as reserpine antagonists. Nature 180:1200.

Carneiro AM, Ingram SL, Beaulieu JM, Sweeney A, Amara SG, Thomas SM, Caron MG, Torres GE (2002) The multiple LIM domain-containing adaptor protein Hic-5 synaptically colocalizes and interacts with the dopamine transporter. The

Journal of neuroscience : the official journal of the Society for Neuroscience 22:7045-7054.
Carr GD, White NM (1984) The relationship between stereotypy and memory improvement produced by amphetamine. Psychopharmacology 82:203-209.
Cartier E, Hamilton PJ, Belovich AN, Shekar A, Campbell NG, Saunders C, Andreassen TF, Gether U, Veenstra-Vanderweele J, Sutcliffe JS, Ulery-Reynolds PG, Erreger K, Matthies HJ, Galli A (2015) Rare autism-associated variants implicate syntaxin 1 (STX1 R26Q) phosphorylation and the dopamine transporter (hDAT R51W) in dopamine neurotransmission and behaviors. EBioMedicine 2:135-146.
Carvelli L, Moron JA, Kahlig KM, Ferrer JV, Sen N, Lechleiter JD, Leeb-Lundberg LM, Merrill G, Lafer EM, Ballou LM, Shippenberg TS, Javitch JA, Lin RZ, Galli A (2002) PI 3-kinase regulation of dopamine uptake. Journal of neurochemistry 81:859-869.
Castro DC, Cole SL, Berridge KC (2015) Lateral hypothalamus, nucleus accumbens, and ventral pallidum roles in eating and hunger: interactions between homeostatic and reward circuitry. Frontiers in systems neuroscience 9:90.
Cazorla M, de Carvalho FD, Chohan MO, Shegda M, Chuhma N, Rayport S, Ahmari SE, Moore H, Kellendonk C (2014) Dopamine D2 receptors regulate the anatomical and functional balance of basal ganglia circuitry. Neuron 81:153-164.
Chalecka-Franaszek E, Chuang DM (1999) Lithium activates the serine/threonine kinase Akt-1 and suppresses glutamate-induced inhibition of Akt-1 activity in neurons. Proceedings of the National Academy of Sciences of the United States of America 96:8745-8750.
Chang MY, Lee SH, Kim JH, Lee KH, Kim YS, Son H, Lee YS (2001) Protein kinase C-mediated functional regulation of dopamine transporter is not achieved by direct phosphorylation of the dopamine transporter protein. Journal of neurochemistry 77:754-761.
Chen YC, Chen YW, Hsu YF, Chang WT, Hsiao CK, Min MY, Lai WS (2012) Akt1 deficiency modulates reward learning and reward prediction error in mice. Genes, brain, and behavior 11:157-169.
Chouinard G, Jones B, Remington G, Bloom D, Addington D, MacEwan GW, Labelle A, Beauclair L, Arnott W (1993) A Canadian multicenter placebo-controlled study of fixed doses of risperidone and haloperidol in the treatment of chronic schizophrenic patients. Journal of clinical psychopharmacology 13:25-40.
Clapcote SJ, Lipina TV, Millar JK, Mackie S, Christie S, Ogawa F, Lerch JP, Trimble K, Uchiyama M, Sakuraba Y, Kaneda H, Shiroishi T, Houslay MD, Henkelman RM, Sled JG, Gondo Y, Porteous DJ, Roder JC (2007) Behavioral phenotypes of Disc1 missense mutations in mice. Neuron 54:387-402.
Collo G, Bono F, Cavalleri L, Plebani L, Merlo Pich E, Millan MJ, Spano PF, Missale C (2012) Pre-synaptic dopamine D(3) receptor mediates cocaine-induced structural plasticity in mesencephalic dopaminergic neurons via ERK and Akt pathways. Journal of neurochemistry 120:765-778.

Cone JJ, Roitman JD, Roitman MF (2015) Ghrelin regulates phasic dopamine and nucleus accumbens signaling evoked by food-predictive stimuli. Journal of neurochemistry 133:844-856.
Cotzias GC, Van Woert MH, Schiffer LM (1967) Aromatic amino acids and modification of parkinsonism. The New England journal of medicine 276:374-379.
Dadalko OI, Siuta M, Poe A, Erreger K, Matthies HJ, Niswender K, Galli A (2015) mTORC2/Rictor Signaling Disrupts Dopamine-Dependent Behaviors via Defects in Striatal Dopamine Neurotransmission. The Journal of neuroscience : the official journal of the Society for Neuroscience 35:8843-8854.
Dahlstroem A, Fuxe K (1964) A Method for the Demonstration of Monoamine-Containing Nerve Fibres in the Central Nervous System. Acta physiologica Scandinavica 60:293-294.
Damanhuri HA, Burke PG, Ong LK, Bobrovskaya L, Dickson PW, Dunkley PR, Goodchild AK (2012) Tyrosine hydroxylase phosphorylation in catecholaminergic brain regions: a marker of activation following acute hypotension and glucoprivation. PloS one 7:e50535.
Davis KL, Klar H, Coyle JT (1991a) Foundations of psychiatry. Philadelphia: Saunders.
Davis KL, Kahn RS, Ko G, Davidson M (1991b) Dopamine in schizophrenia: a review and reconceptualization. The American journal of psychiatry 148:1474-1486.
Daws LC, Avison MJ, Robertson SD, Niswender KD, Galli A, Saunders C (2011) Insulin signaling and addiction. Neuropharmacology 61:1123-1128.
De Mei C, Ramos M, Iitaka C, Borrelli E (2009) Getting specialized: presynaptic and postsynaptic dopamine D2 receptors. Current opinion in pharmacology 9:53-58.
Deutch AY, Martin RJ (1983) Mesencephalic dopamine modulation of pituitary and central beta-endorphin: relation to food intake regulation. Life sciences 33:281-287.
Dewar KM, Soghomonian JJ, Bruno JP, Descarries L, Reader TA (1990) Elevation of dopamine D2 but not D1 receptors in adult rat neostriatum after neonatal 6-hydroxydopamine denervation. Brain research 536:287-296.
Dickinson SD, Sabeti J, Larson GA, Giardina K, Rubinstein M, Kelly MA, Grandy DK, Low MJ, Gerhardt GA, Zahniser NR (1999) Dopamine D2 receptor-deficient mice exhibit decreased dopamine transporter function but no changes in dopamine release in dorsal striatum. Journal of neurochemistry 72:148-156.
Doolen S, Zahniser NR (2001) Protein tyrosine kinase inhibitors alter human dopamine transporter activity in Xenopus oocytes. Journal of Pharmacology & Experimental Therapeutics 296:931-938.
Dunkley PR, Bobrovskaya L, Graham ME, von Nagy-Felsobuki EI, Dickson PW (2004) Tyrosine hydroxylase phosphorylation: regulation and consequences. Journal of neurochemistry 91:1025-1043.
Durieux PF, Schiffmann SN, de Kerchove d'Exaerde A (2012) Differential regulation of motor control and response to dopaminergic drugs by D1R and D2R neurons in distinct dorsal striatum subregions. The EMBO journal 31:640-653.

Durieux PF, Bearzatto B, Guiducci S, Buch T, Waisman A, Zoli M, Schiffmann SN, de Kerchove d'Exaerde A (2009) D2R striatopallidal neurons inhibit both locomotor and drug reward processes. Nature neuroscience 12:393-395.

Easton RM, Cho H, Roovers K, Shineman DW, Mizrahi M, Forman MS, Lee VM, Szabolcs M, de Jong R, Oltersdorf T, Ludwig T, Efstratiadis A, Birnbaum MJ (2005) Role for Akt3/protein kinase Bgamma in attainment of normal brain size. Molecular and cellular biology 25:1869-1878.

Ehringer H, Hornykiewicz O (1960) [Distribution of noradrenaline and dopamine (3-hydroxytyramine) in the human brain and their behavior in diseases of the extrapyramidal system]. Klinische Wochenschrift 38:1236-1239.

Ehringer H, Hornykiewicz O (1998) Distribution of noradrenaline and dopamine (3-hydroxytyramine) in the human brain and their behavior in diseases of the extrapyramidal system. Parkinsonism & related disorders 4:53-57.

Emamian ES, Hall D, Birnbaum MJ, Karayiorgou M, Gogos JA (2004) Convergent evidence for impaired AKT1-GSK3beta signaling in schizophrenia. Nature genetics 36:131-137.

Espana RA, Jones SR (2013) Presynaptic dopamine modulation by stimulant self-administration. Frontiers in bioscience 5:261-276.

Fahn S, Libsch LR, Cutler RW (1971) Monoamines in the human neostriatum: topographic distribution in normals and in Parkinson's disease and their role in akinesia, rigidity, chorea, and tremor. Journal of the neurological sciences 14:427-455.

Fenouille N, Puissant A, Tichet M, Zimniak G, Abbe P, Mallavialle A, Rocchi S, Ortonne JP, Deckert M, Ballotti R, Tartare-Deckert S (2011) SPARC functions as an anti-stress factor by inactivating p53 through Akt-mediated MDM2 phosphorylation to promote melanoma cell survival. Oncogene 30:4887-4900.

Ferguson SS, Downey WE, 3rd, Colapietro AM, Barak LS, Menard L, Caron MG (1996) Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. Science 271:363-366.

Fischer E, Heller B (1967) Antagonic effects of cocaine and adrenaline on the psychomotor activity of mice. Acta physiologica latino americana 17:118-119.

Fischer JF, Cho AK (1979) Chemical release of dopamine from striatal homogenates: evidence for an exchange diffusion model. Journal of Pharmacology & Experimental Therapeutics 208:203-209.

Fog JU, Khoshbouei H, Holy M, Owens WA, Vaegter CB, Sen N, Nikandrova Y, Bowton E, McMahon DG, Colbran RJ, Daws LC, Sitte HH, Javitch JA, Galli A, Gether U (2006) Calmodulin kinase II interacts with the dopamine transporter C terminus to regulate amphetamine-induced reverse transport. Neuron 51:417-429.

Franke TF (2008a) Intracellular signaling by Akt: bound to be specific. Science signaling 1:pe29.

Franke TF (2008b) PI3K/Akt: getting it right matters. Oncogene 27:6473-6488.

Fuxe K, Hoekfelt T, Nilsson O (1965) A Fluorescence and Electronmicroscopic Study on Certain Brain Regions Rich in Monoamine Terminals. The American journal of anatomy 117:33-45.

Gabriel LR, Wu S, Kearney P, Bellve KD, Standley C, Fogarty KE, Melikian HE (2013) Dopamine transporter endocytic trafficking in striatal dopaminergic neurons: differential dependence on dynamin and the actin cytoskeleton. The Journal of neuroscience : the official journal of the Society for Neuroscience 33:17836-17846.

Gainetdinov RR, Jones SR, Caron MG (1999a) Functional hyperdopaminergia in dopamine transporter knock-out mice. Biological psychiatry 46:303-311.

Gainetdinov RR, Mohn AR, Caron MG (2001) Genetic animal models: focus on schizophrenia. Trends in neurosciences 24:527-533.

Gainetdinov RR, Wetsel WC, Jones SR, Levin ED, Jaber M, Caron MG (1999b) Role of serotonin in the paradoxical calming effect of psychostimulants on hyperactivity. [see comments]. Science 283:397-401.

Garcia BG, Wei Y, Moron JA, Lin RZ, Javitch JA, Galli A (2005) Akt is essential for insulin modulation of amphetamine-induced human dopamine transporter cell-surface redistribution. Molecular pharmacology 68:102-109.

Gavard JA, Lustman PJ, Clouse RE (1993) Prevalence of depression in adults with diabetes. An epidemiological evaluation. Diabetes care 16:1167-1178.

Gerhardt GA, Hoffman AF (2001) Effects of recording media composition on the responses of Nafion-coated carbon fiber microelectrodes measured using high-speed chronoamperometry. Journal of neuroscience methods 109:13-21.

Giros B, Caron MG (1993) Molecular characterization of the dopamine transporter. Trends in pharmacological sciences 14:43-49.

Giros B, Jaber M, Jones SR, Wightman RM, Caron MG (1996) Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. Nature 379:606-612.

Giros B, Wang YM, Suter S, McLeskey SB, Pifl C, Caron MG (1994) Delineation of discrete domains for substrate, cocaine, and tricyclic antidepressant interactions using chimeric dopamine-norepinephrine transporters. The Journal of biological chemistry 269:15985-15988.

Goggi J, Pullar IA, Carney SL, Bradford HF (2003) Signalling pathways involved in the short-term potentiation of dopamine release by BDNF. Brain research 968:156-161.

Goldstein M, Lieberman A (1992) The role of the regulatory enzymes of catecholamine synthesis in Parkinson's disease. Neurology 42:8-12; discussion 41-18.

Goldstein M, Battista AF, Miyamoto T (1975) Modification of involuntary movements by centrally acting drugs. Advances in neurology 9:299-305.

Granas C, Ferrer J, Loland CJ, Javitch JA, Gether U (2003) N-terminal truncation of the dopamine transporter abolishes phorbol ester- and substance P receptor-stimulated phosphorylation without impairing transporter internalization. The Journal of biological chemistry 278:4990-5000.

Gresch PJ, Sved AF, Zigmond MJ, Finlay JM (1995) Local influence of endogenous norepinephrine on extracellular dopamine in rat medial prefrontal cortex. Journal of neurochemistry 65:111-116.
Haavik J, Toska K (1998) Tyrosine hydroxylase and Parkinson's disease. Molecular neurobiology 16:285-309.
Hamilton PJ, Shekar A, Belovich AN, Christianson NB, Campbell NG, Sutcliffe JS, Galli A, Matthies HJ, Erreger K (2015) Zn(2+) reverses functional deficits in a de novo dopamine transporter variant associated with autism spectrum disorder. Molecular autism 6:8.
Hamilton PJ, Belovich AN, Khelashvili G, Saunders C, Erreger K, Javitch JA, Sitte HH, Weinstein H, Matthies HJ, Galli A (2014) PIP2 regulates psychostimulant behaviors through its interaction with a membrane protein. Nature chemical biology 10:582-589.
Hamilton PJ, Campbell NG, Sharma S, Erreger K, Herborg Hansen F, Saunders C, Belovich AN, Consortium NAAS, Sahai MA, Cook EH, Gether U, McHaourab HS, Matthies HJ, Sutcliffe JS, Galli A (2013) De novo mutation in the dopamine transporter gene associates dopamine dysfunction with autism spectrum disorder. Molecular psychiatry 18:1315-1323.
Hastrup H, Karlin A, Javitch JA (2001) Symmetrical dimer of the human dopamine transporter revealed by cross-linking Cys-306 at the extracellular end of the sixth transmembrane segment. Proceedings of the National Academy of Sciences of the United States of America 98:10055-10060.
Hastrup H, Sen N, Javitch JA (2003) The human dopamine transporter forms a tetramer in the plasma membrane: cross-linking of a cysteine in the fourth transmembrane segment is sensitive to cocaine analogs. The Journal of biological chemistry 278:45045-45048.
Haycock JW (1990) Phosphorylation of tyrosine hydroxylase in situ at serine 8, 19, 31, and 40. The Journal of biological chemistry 265:11682-11691.
Hazelwood LA, Free RB, Cabrera DM, Skinbjerg M, Sibley DR (2008) Reciprocal modulation of function between the D1 and D2 dopamine receptors and the Na+,K+-ATPase. The Journal of biological chemistry 283:36441-36453.
Hernandez-Lopez S, Tkatch T, Perez-Garci E, Galarraga E, Bargas J, Hamm H, Surmeier DJ (2000) D2 dopamine receptors in striatal medium spiny neurons reduce L-type Ca2+ currents and excitability via a novel PLC[beta]1-IP3-calcineurin-signaling cascade. The Journal of neuroscience : the official journal of the Society for Neuroscience 20:8987-8995.
Hoffman AF, Gerhardt GA (1999) Differences in pharmacological properties of dopamine release between the substantia nigra and striatum: an in vivo electrochemical study. The Journal of pharmacology and experimental therapeutics 289:455-463.
Hornykiewicz O (1966) Dopamine (3-hydroxytyramine) and brain function. Pharmacological reviews 18:925-964.
Howes OD, Kapur S (2009) The dopamine hypothesis of schizophrenia: version III--the final common pathway. Schizophrenia bulletin 35:549-562.

Johnson PM, Kenny PJ (2010) Dopamine D2 receptors in addiction-like reward dysfunction and compulsive eating in obese rats. Nature neuroscience 13:635-641.

Jones KT, Zhen J, Reith ME (2012) Importance of cholesterol in dopamine transporter function. Journal of neurochemistry 123:700-715.

Jones SR, Gainetdinov RR, Wightman RM, Caron MG (1998) Mechanisms of amphetamine action revealed in mice lacking the dopamine transporter. The Journal of neuroscience : the official journal of the Society for Neuroscience 18:1979-1986.

Jones SR, Gainetdinov RR, Hu XT, Cooper DC, Wightman RM, White FJ, Caron MG (1999) Loss of autoreceptor functions in mice lacking the dopamine transporter. Nature neuroscience 2:649-655.

Kahlig KM, Binda F, Khoshbouei H, Blakely RD, McMahon DG, Javitch JA, Galli A (2005) Amphetamine induces dopamine efflux through a dopamine transporter channel. Proceedings of the National Academy of Sciences of the United States of America 102:3495-3500.

Kalkman HO (2006) The role of the phosphatidylinositide 3-kinase-protein kinase B pathway in schizophrenia. Pharmacology & therapeutics 110:117-134.

Kanoski SE, Fortin SM, Ricks KM, Grill HJ (2013) Ghrelin signaling in the ventral hippocampus stimulates learned and motivational aspects of feeding via PI3K-Akt signaling. Biological psychiatry 73:915-923.

Karege F, Perroud N, Burkhardt S, Schwald M, Ballmann E, La Harpe R, Malafosse A (2007) Alteration in Kinase Activity But Not in Protein Levels of Protein Kinase B and Glycogen Synthase Kinase-3[beta] in Ventral Prefrontal Cortex of Depressed Suicide Victims. Biological psychiatry 61:240-245.

Kasper S, Barnas C, Heiden A, Volz HP, Laakmann G, Zeit H, Pfolz H (1997) Pramipexole as adjunct to haloperidol in schizophrenia. Safety and efficacy. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology 7:65-70.

Kellendonk C, Simpson EH, Polan HJ, Malleret G, Vronskaya S, Winiger V, Moore H, Kandel ER (2006) Transient and selective overexpression of dopamine D2 receptors in the striatum causes persistent abnormalities in prefrontal cortex functioning. Neuron 49:603-615.

Kelly PH, Seviour PW, Iversen SD (1975) Amphetamine and apomorphine responses in the rat following 6-OHDA lesions of the nucleus accumbens septi and corpus striatum. Brain research 94:507-522.

Kenny PJ (2011) Reward mechanisms in obesity: new insights and future directions. Neuron 69:664-679.

Kenny PJ (2013) The food addiction. Scientific American 309:44-49.

Keri S, Seres I, Kelemen O, Benedek G (2009) Neuregulin 1-stimulated phosphorylation of AKT in psychotic disorders and its relationship with neurocognitive functions. Neurochemistry international 55:606-609.

Khoshbouei H, Sen N, Guptaroy B, Johnson L, Lund D, Gnegy ME, Galli A, Javitch JA (2004) N-terminal phosphorylation of the dopamine transporter is required for amphetamine-induced efflux. PLoS biology 2:E78.

Kilty JE, Lorang D, Amara SG (1991) Cloning and expression of a cocaine-sensitive rat dopamine transporter. Science 254:578-579.
Kim DH, Woods SC, Seeley RJ (2012) Hypothalamic Akt/PKB signaling in regulation of food intake. Frontiers in bioscience 4:953-966.
Kim SJ, Chun JY, Kim MS (2000) Insulin stimulates production of nitric oxide via ERK in osteoblast cells. Biochemical and biophysical research communications 278:712-718.
Kim Y, Seger R, Suresh Babu CV, Hwang SY, Yoo YS (2004) A positive role of the PI3-K/Akt signaling pathway in PC12 cell differentiation. Mol Cells 18:353-359.
Kisilevsky AE, Zamponi GW (2008) D2 dopamine receptors interact directly with N-type calcium channels and regulate channel surface expression levels. Channels 2:269-277.
Kisilevsky AE, Mulligan SJ, Altier C, Iftinca MC, Varela D, Tai C, Chen L, Hameed S, Hamid J, Macvicar BA, Zamponi GW (2008) D1 receptors physically interact with N-type calcium channels to regulate channel distribution and dendritic calcium entry. Neuron 58:557-570.
Kitagishi Y, Kobayashi M, Kikuta K, Matsuda S (2012) Roles of PI3K/AKT/GSK3/mTOR Pathway in Cell Signaling of Mental Illnesses. Depression research and treatment 2012:752563.
Kocalis HE, Hagan SL, George L, Turney MK, Siuta MA, Laryea GN, Morris LC, Muglia LJ, Printz RL, Stanwood GD, Niswender KD (2014) Rictor/mTORC2 facilitates central regulation of energy and glucose homeostasis. Molecular metabolism 3:394-407.
Koch L, Wunderlich FT, Seibler J, Konner AC, Hampel B, Irlenbusch S, Brabant G, Kahn CR, Schwenk F, Bruning JC (2008) Central insulin action regulates peripheral glucose and fat metabolism in mice. The Journal of clinical investigation 118:2132-2147.
Kovtun O, Sakrikar D, Tomlinson ID, Chang JC, Arzeta-Ferrer X, Blakely RD, Rosenthal SJ (2015) Single-quantum-dot tracking reveals altered membrane dynamics of an attention-deficit/hyperactivity-disorder-derived dopamine transporter coding variant. ACS chemical neuroscience 6:526-534.
Kramer PF, Christensen CH, Hazelwood LA, Dobi A, Bock R, Sibley DR, Mateo Y, Alvarez VA (2011) Dopamine D2 receptor overexpression alters behavior and physiology in Drd2-EGFP mice. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:126-132.
Kreitzer AC, Malenka RC (2008) Striatal plasticity and basal ganglia circuit function. Neuron 60:543-554.
Krishnan V, Han M-H, Mazei-Robison M, Iñiguez SD, Ables JL, Vialou V, Berton O, Ghose S, Covington Iii HE, Wiley MD, Henderson RP, Neve RL, Eisch AJ, Tamminga CA, Russo SJ, Bolaños CA, Nestler EJ (2008) AKT Signaling within the Ventral Tegmental Area Regulates Cellular and Behavioral Responses to Stressful Stimuli. Biological psychiatry 64:691-700.
Kristensen AS, Andersen J, Jorgensen TN, Sorensen L, Eriksen J, Loland CJ, Stromgaard K, Gether U (2011) SLC6 neurotransmitter transporters: structure, function, and regulation. Pharmacological reviews 63:585-640.

Kumar A, Harris TE, Keller SR, Choi KM, Magnuson MA, Lawrence JC, Jr. (2008) Muscle-specific deletion of rictor impairs insulin-stimulated glucose transport and enhances Basal glycogen synthase activity. Molecular and cellular biology 28:61-70.

Kuzhikandathil EV, Yu W, Oxford GS (1998) Human dopamine D3 and D2L receptors couple to inward rectifier potassium channels in mammalian cell lines. Molecular and cellular neurosciences 12:390-402.

Lai WS, Xu B, Westphal KG, Paterlini M, Olivier B, Pavlidis P, Karayiorgou M, Gogos JA (2006) Akt1 deficiency affects neuronal morphology and predisposes to abnormalities in prefrontal cortex functioning. Proceedings of the National Academy of Sciences of the United States of America 103:16906-16911.

Langston JW, Ballard PA, Jr. (1983) Parkinson's disease in a chemist working with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. The New England journal of medicine 309:310.

Lee FJ, Pei L, Liu F (2009) Disruption of the dopamine transporter-dopamine D2 receptor interaction in schizophrenia. Synapse 63:710-712.

Lee KH, Kim MY, Kim DH, Lee YS (2004) Syntaxin 1A and receptor for activated C kinase interact with the N-terminal region of human dopamine transporter. Neurochemical research 29:1405-1409.

Lerner TN, Shilyansky C, Davidson TJ, Evans KE, Beier KT, Zalocusky KA, Crow AK, Malenka RC, Luo L, Tomer R, Deisseroth K (2015) Intact-Brain Analyses Reveal Distinct Information Carried by SNc Dopamine Subcircuits. Cell 162:635-647.

Li Z, Tan F, Thiele CJ (2007) Inactivation of glycogen synthase kinase-3beta contributes to brain-derived neutrophic factor/TrkB-induced resistance to chemotherapy in neuroblastoma cells. Molecular cancer therapeutics 6:3113-3121.

Lieberman JA, Kane JM, Alvir J (1987) Provocative tests with psychostimulant drugs in schizophrenia. Psychopharmacology 91:415-433.

Lindsley CW, Zhao Z, Leister WH, Robinson RG, Barnett SF, Defeo-Jones D, Jones RE, Hartman GD, Huff JR, Huber HE, Duggan ME (2005) Allosteric Akt (PKB) inhibitors: discovery and SAR of isozyme selective inhibitors. Bioorganic & medicinal chemistry letters 15:761-764.

Lipina TV, Niwa M, Jaaro-Peled H, Fletcher PJ, Seeman P, Sawa A, Roder JC (2010) Enhanced dopamine function in DISC1-L100P mutant mice: implications for schizophrenia. Genes, brain, and behavior 9:777-789.

Lohse MJ, Benovic JL, Codina J, Caron MG, Lefkowitz RJ (1990) beta-Arrestin: a protein that regulates beta-adrenergic receptor function. Science 248:1547-1550.

Lotharius J, Brundin P (2002) Pathogenesis of Parkinson's disease: dopamine, vesicles and alpha-synuclein. Nature reviews Neuroscience 3:932-942.

Lu XH, Dwyer DS (2005) Second-generation antipsychotic drugs, olanzapine, quetiapine, and clozapine enhance neurite outgrowth in PC12 cells via PI3K/AKT, ERK, and pertussis toxin-sensitive pathways. Journal of molecular neuroscience : MN 27:43-64.

Luo Y, Kokkonen GC, Wang X, Neve KA, Roth GS (1998) D2 dopamine receptors stimulate mitogenesis through pertussis toxin-sensitive G proteins and Ras-involved ERK and SAP/JNK pathways in rat C6-D2L glioma cells. Journal of neurochemistry 71:980-990.

Lustman PJ, Clouse RE, Ciechanowski PS, Hirsch IB, Freedland KE (2005) Depression-related hyperglycemia in type 1 diabetes: a mediational approach. Psychosomatic medicine 67:195-199.

Lute BJ, Khoshbouei H, Saunders C, Sen N, Lin RZ, Javitch JA, Galli A (2008) PI3K signaling supports amphetamine-induced dopamine efflux. Biochemical and biophysical research communications 372:656-661.

Luttrell LM, Gesty-Palmer D (2010) Beyond desensitization: physiological relevance of arrestin-dependent signaling. Pharmacological reviews 62:305-330.

Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG, Lefkowitz RJ (1999) Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. Science 283:655-661.

Machado-Vieira R, Zanetti MV, Teixeira AL, Uno M, Valiengo LL, Soeiro-de-Souza MG, Oba-Shinjo SM, de Sousa RT, Zarate CA, Jr., Gattaz WF, Marie SK (2015) Decreased AKT1/mTOR pathway mRNA expression in short-term bipolar disorder. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology 25:468-473.

Marazziti D, Mandillo S, Di Pietro C, Golini E, Matteoni R, Tocchini-Valentini GP (2007) GPR37 associates with the dopamine transporter to modulate dopamine uptake and behavioral responses to dopaminergic drugs. Proceedings of the National Academy of Sciences of the United States of America 104:9846-9851.

Marazziti D, Golini E, Mandillo S, Magrelli A, Witke W, Matteoni R, Tocchini-Valentini GP (2004) Altered dopamine signaling and MPTP resistance in mice lacking the Parkinson's disease-associated GPR37/parkin-associated endothelin-like receptor. Proceedings of the National Academy of Sciences of the United States of America 101:10189-10194.

Margolis RU, Altszuler N (1967) Insulin in the cerebrospinal fluid. Nature 215:1375-1376.

Markakis EA, Vives KP, Bober J, Leichtle S, Leranth C, Beecham J, Elsworth JD, Roth RH, Samulski RJ, Redmond DE, Jr. (2010) Comparative transduction efficiency of AAV vector serotypes 1-6 in the substantia nigra and striatum of the primate brain. Molecular therapy : the journal of the American Society of Gene Therapy 18:588-593.

Marshall JF (1978) Further analysis of the resistance of the diabetic rat to d-amphetamine. Pharmacology, Biochemistry & Behavior 8:281-286.

Mazei-Robison MS, Appasani R, Edwards S, Wee S, Taylor SR, Picciotto MR, Koob GF, Nestler EJ (2014) Self-administration of ethanol, cocaine, or nicotine does not decrease the soma size of ventral tegmental area dopamine neurons. PloS one 9:e95962.

Mazei-Robison MS et al. (2011) Role for mTOR signaling and neuronal activity in morphine-induced adaptations in ventral tegmental area dopamine neurons. Neuron 72:977-990.
McCutcheon JE (2015) The role of dopamine in the pursuit of nutritional value. Physiology & behavior.
McHugh PC, Buckley DA (2015) The structure and function of the dopamine transporter and its role in CNS diseases. Vitamins and hormones 98:339-369.
Medvedev IO, Ramsey AJ, Masoud ST, Bermejo MK, Urs N, Sotnikova TD, Beaulieu JM, Gainetdinov RR, Salahpour A (2013) D1 dopamine receptor coupling to PLCbeta regulates forward locomotion in mice. The Journal of neuroscience : the official journal of the Society for Neuroscience 33:18125-18133.
Meiergerd SM, Patterson TA, Schenk JO (1993) D2 receptors may modulate the function of the striatal transporter for dopamine: kinetic evidence from studies in vitro and in vivo. Journal of neurochemistry 61:764-767.
Miller JS, Barr JL, Harper LJ, Poole RL, Gould TJ, Unterwald EM (2014) The GSK3 signaling pathway is activated by cocaine and is critical for cocaine conditioned reward in mice. PloS one 9:e88026.
Milne D, Kampanis P, Nicol S, Dias S, Campbell DG, Fuller-Pace F, Meek D (2004) A novel site of AKT-mediated phosphorylation in the human MDM2 onco-protein. FEBS letters 577:270-276.
Min SK, Rhee CS, Kim CE, Kang DY (1993) Risperidone versus haloperidol in the treatment of chronic schizophrenic patients: a parallel group double-blind comparative trial. Yonsei medical journal 34:179-190.
Miner LH, Schroeter S, Blakely RD, Sesack SR (2003) Ultrastructural localization of the norepinephrine transporter in superficial and deep layers of the rat prelimbic prefrontal cortex and its spatial relationship to probable dopamine terminals. The Journal of comparative neurology 466:478-494.
Mizushige T, Matsumura S, Yoneda T, Tsuzuki S, Inoue K, Fushiki T (2006) Daily increase of fat ingestion mediated via mu-opioid receptor signaling pathway. Biomedical research 27:259-263.
Mizushige T, Saitoh K, Manabe Y, Nishizuka T, Taka Y, Eguchi A, Yoneda T, Matsumura S, Tsuzuki S, Inoue K, Fushiki T (2009) Preference for dietary fat induced by release of beta-endorphin in rats. Life sciences 84:760-765.
Mokdad AH, Ford ES, Bowman BA, Dietz WH, Vinicor F, Bales VS, Marks JS (2003) Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. Jama 289:76-79.
Moore KE, Demarest KT, Lookingland KJ (1987) Stress, prolactin and hypothalamic dopaminergic neurons. Neuropharmacology 26:801-808.
Morgenroth VH, 3rd, Hegstrand LR, Roth RH, Greengard P (1975) Evidence for involvement of protein kinase in the activation by adenosine 3':5'-monophosphate of brain tyrosine 3-monooxygenase. The Journal of biological chemistry 250:1946-1948.
Mukherjee S, Schnur DB, Reddy R (1989) Family history of type 2 diabetes in schizophrenic patients. Lancet 1:495.
Mukherjee S, Decina P, Bocola V, Saraceni F, Scapicchio PL (1996) Diabetes mellitus in schizophrenic patients. Comprehensive psychiatry 37:68-73.

Narendran R, Martinez D (2008) Cocaine abuse and sensitization of striatal dopamine transmission: a critical review of the preclinical and clinical imaging literature. Synapse 62:851-869.
Neale BM et al. (2012) Patterns and rates of exonic de novo mutations in autism spectrum disorders. Nature 485:242-245.
Neasta J, Ben Hamida S, Yowell QV, Carnicella S, Ron D (2011) AKT signaling pathway in the nucleus accumbens mediates excessive alcohol drinking behaviors. Biological psychiatry 70:575-582.
Nestler EJ (2005) Is there a common molecular pathway for addiction? Nature neuroscience 8:1445-1449.
Nestler EJ (2013) Cellular basis of memory for addiction. Dialogues in clinical neuroscience 15:431-443.
Nicodemus KK, Marenco S, Batten AJ, Vakkalanka R, Egan MF, Straub RE, Weinberger DR (2008) Serious obstetric complications interact with hypoxia-regulated/vascular-expression genes to influence schizophrenia risk. Molecular psychiatry 13:873-877.
Nicodemus KK, Law AJ, Radulescu E, Luna A, Kolachana B, Vakkalanka R, Rujescu D, Giegling I, Straub RE, McGee K, Gold B, Dean M, Muglia P, Callicott JH, Tan HY, Weinberger DR (2010) Biological validation of increased schizophrenia risk with NRG1, ERBB4, and AKT1 epistasis via functional neuroimaging in healthy controls. Archives of general psychiatry 67:991-1001.
Niswender KD, Daws LC, Avison MJ, Galli A (2011) Insulin regulation of monoamine signaling: pathway to obesity. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 36:359-360.
Niswender KD, Morrison CD, Clegg DJ, Olson R, Baskin DG, Myers MG, Jr., Seeley RJ, Schwartz MW (2003) Insulin activation of phosphatidylinositol 3-kinase in the hypothalamic arcuate nucleus: a key mediator of insulin-induced anorexia. Diabetes 52:227-231.
Obici S, Zhang BB, Karkanias G, Rossetti L (2002) Hypothalamic insulin signaling is required for inhibition of glucose production. Nature medicine 8:1376-1382.
Odou P, Vaiva G, Luyckx M, Brunet C, Dine T, Gressier B, Cazin M, Cazin JC (1996) Neuroleptic monitoring: relation between antipsychotic efficiency and radioreceptor assay of serum haloperidol. European journal of clinical pharmacology 50:357-363.
Oh WJ, Jacinto E (2011) mTOR complex 2 signaling and functions. Cell cycle 10:2305-2316.
Oishi K, Watatani K, Itoh Y, Okano H, Guillemot Fo, Nakajima K, Gotoh Y (2009) Selective induction of neocortical GABAergic neurons by the PDK1-Akt pathway through activation of Mash1. Proceedings of the National Academy of Sciences:-.
Oliver TG, Meylan E, Chang GP, Xue W, Burke JR, Humpton TJ, Hubbard D, Bhutkar A, Jacks T (2011) Caspase-2-mediated cleavage of Mdm2 creates a p53-induced positive feedback loop. Molecular cell 43:57-71.
Owens WA, Williams JM, Saunders C, Avison MJ, Galli A, Daws LC (2012) Rescue of dopamine transporter function in hypoinsulinemic rats by a D2 receptor-

ERK-dependent mechanism. The Journal of neuroscience : the official journal of the Society for Neuroscience 32:2637-2647.
Owens WA, Sevak RJ, Galici R, Chang X, Javors MA, Galli A, France CP, Daws LC (2005) Deficits in dopamine clearance and locomotion in hypoinsulinemic rats unmask novel modulation of dopamine transporters by amphetamine. Journal of neurochemistry 94:1402-1410.
Pacholczyk T, Blakely RD, Amara SG (1991) Expression cloning of a cocaine- and antidepressant-sensitive human noradrenaline transporter. Nature 350:350-354.
Page G, Barc-Pain S, Pontcharraud R, Cante A, Piriou A, Barrier L (2004) The up-regulation of the striatal dopamine transporter's activity by cAMP is PKA-, CaMK II- and phosphatase-dependent. Neurochemistry international 45:627-632.
Palmiter RD (2007) Is dopamine a physiologically relevant mediator of feeding behavior? Trends in neurosciences 30:375-381.
Pearce LR, Komander D, Alessi DR (2010) The nuts and bolts of AGC protein kinases. Nature reviews Molecular cell biology 11:9-22.
Perello M, Dickson SL (2015) Ghrelin signalling on food reward: a salient link between the gut and the mesolimbic system. Journal of neuroendocrinology 27:424-434.
Perreault ML, O'Dowd BF, George SR (2014) Dopamine D(1)-D(2) receptor heteromer regulates signaling cascades involved in addiction: potential relevance to adolescent drug susceptibility. Developmental neuroscience 36:287-296.
Porrino LJ, Lucignani G, Dow-Edwards D, Sokoloff L (1984) Correlation of dose-dependent effects of acute amphetamine administration on behavior and local cerebral metabolism in rats. Brain research 307:311-320.
Porte D, Jr., Baskin DG, Schwartz MW (2002) Leptin and insulin action in the central nervous system. Nutrition reviews 60:S20-29; discussion S68-84, 85-27.
Pristupa ZB, McConkey F, Liu F, Man HY, Lee FJ, Wang YT, Niznik HB (1998) Protein kinase-mediated bidirectional trafficking and functional regulation of the human dopamine transporter. Synapse 30:79-87.
Radja F, el Mansari M, Soghomonian JJ, Dewar KM, Ferron A, Reader TA, Descarries L (1993) Changes of D1 and D2 receptors in adult rat neostriatum after neonatal dopamine denervation: quantitative data from ligand binding, in situ hybridization and iontophoresis. Neuroscience 57:635-648.
Rebec GV (2006) Behavioral electrophysiology of psychostimulants. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 31:2341-2348.
Richard JM, Castro DC, Difeliceantonio AG, Robinson MJ, Berridge KC (2013) Mapping brain circuits of reward and motivation: in the footsteps of Ann Kelley. Neuroscience and biobehavioral reviews 37:1919-1931.
Ries V, Henchcliffe C, Kareva T, Rzhetskaya M, Bland R, During MJ, Kholodilov N, Burke RE (2006) Oncoprotein Akt/PKB induces trophic effects in murine models of Parkinson's disease. Proceedings of the National Academy of Sciences of the United States of America 103:18757-18762.

Ries V, Cheng HC, Baohan A, Kareva T, Oo TF, Rzhetskaya M, Bland RJ, During MJ, Kholodilov N, Burke RE (2009) Regulation of the postnatal development of dopamine neurons of the substantia nigra in vivo by Akt/protein kinase B. Journal of neurochemistry 110:23-33.

Robertson SD, Matthies HJ, Owens WA, Sathananthan V, Christianson NS, Kennedy JP, Lindsley CW, Daws LC, Galli A (2010) Insulin reveals Akt signaling as a novel regulator of norepinephrine transporter trafficking and norepinephrine homeostasis. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:11305-11316.

Ross SB, Renyi AL (1967) Inhibition of the uptake of tritiated catecholamines by antidepressant and related agents. European journal of pharmacology 2:181-186.

Russo SJ, Bolanos CA, Theobald DE, DeCarolis NA, Renthal W, Kumar A, Winstanley CA, Renthal NE, Wiley MD, Self DW, Russell DS, Neve RL, Eisch AJ, Nestler EJ (2007) IRS2-Akt pathway in midbrain dopamine neurons regulates behavioral and cellular responses to opiates. Nature neuroscience 10:93-99.

Ryan MC, Collins P, Thakore JH (2003) Impaired fasting glucose tolerance in first-episode, drug-naive patients with schizophrenia. The American journal of psychiatry 160:284-289.

Salahpour A, Ramsey AJ, Medvedev IO, Kile B, Sotnikova TD, Holmstrand E, Ghisi V, Nicholls PJ, Wong L, Murphy K, Sesack SR, Wightman RM, Gainetdinov RR, Caron MG (2008) Increased amphetamine-induced hyperactivity and reward in mice overexpressing the dopamine transporter. Proceedings of the National Academy of Sciences of the United States of America 105:4405-4410.

Salvatore MF (2014) ser31 Tyrosine hydroxylase phosphorylation parallels differences in dopamine recovery in nigrostriatal pathway following 6-OHDA lesion. Journal of neurochemistry 129:548-558.

Salvatore MF, Pruett BS (2012) Dichotomy of tyrosine hydroxylase and dopamine regulation between somatodendritic and terminal field areas of nigrostriatal and mesoaccumbens pathways. PloS one 7:e29867.

Salvatore MF, Pruett BS, Spann SL, Dempsey C (2009) Aging reveals a role for nigral tyrosine hydroxylase ser31 phosphorylation in locomotor activity generation. PloS one 4:e8466.

Santini E, Valjent E, Fisone G (2010) mTORC1 signaling in Parkinson's disease and L-DOPA-induced dyskinesia: A sensitized matter. Cell cycle 9:2713-2718.

Santini E, Heiman M, Greengard P, Valjent E, Fisone G (2009) Inhibition of mTOR signaling in Parkinson's disease prevents L-DOPA-induced dyskinesia. Science signaling 2:ra36.

Santini E, Feyder M, Gangarossa G, Bateup HS, Greengard P, Fisone G (2012) Dopamine- and cAMP-regulated phosphoprotein of 32-kDa (DARPP-32)-dependent activation of extracellular signal-regulated kinase (ERK) and mammalian target of rapamycin complex 1 (mTORC1) signaling in experimental parkinsonism. The Journal of biological chemistry 287:27806-27812.

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307:1098-1101.

Saunders BT, Richard JM, Janak PH (2015) Contemporary approaches to neural circuit manipulation and mapping: focus on reward and addiction. Philosophical transactions of the Royal Society of London Series B, Biological sciences 370.

Saunders C, Siuta M, Robertson SD, Davis AR, Sauer J, Matthies HJ, Gresch PJ, Airey DC, Lindsley CW, Schetz JA, Niswender KD, Veenstra-Vanderweele JM, Galli A (2014) Neuronal ablation of p-Akt at Ser473 leads to altered 5-HT1A/2A receptor function. Neurochemistry international 73:113-121.

Schulingkamp RJ, Pagano TC, Hung D, Raffa RB (2000) Insulin receptors and insulin action in the brain: review and clinical implications. Neuroscience & Biobehavioral Reviews 24:855-872.

Schwartz MW, Figlewicz DP, Baskin DG, Woods SC, Porte D, Jr. (1992) Insulin in the brain: a hormonal regulator of energy balance. Endocr Rev 13:387-414.

Schwartz MW, Sipols A, Kahn SE, Lattemann DF, Taborsky GJ, Jr., Bergman RN, Woods SC, Porte D, Jr. (1990) Kinetics and specificity of insulin uptake from plasma into cerebrospinal fluid. The American journal of physiology 259:E378-383.

Seeman P, Chau-Wong M, Tedesco J, Wong K (1975) Brain receptors for antipsychotic drugs and dopamine: direct binding assays. Proceedings of the National Academy of Sciences of the United States of America 72:4376-4380.

Sevak RJ, Owens WA, Koek W, Galli A, Daws LC, France CP (2007) Evidence for D2 receptor mediation of amphetamine-induced normalization of locomotion and dopamine transporter function in hypoinsulinemic rats. Journal of neurochemistry 101:151-159.

Sevak RJ, Koek W, Owens WA, Galli A, Daws LC, France CP (2008a) Feeding conditions differentially affect the neurochemical and behavioral effects of dopaminergic drugs in male rats. European journal of pharmacology 592:109-115.

Sevak RJ, Koek W, Daws LC, Owens WA, Galli A, France CP (2008b) Behavioral effects of amphetamine in streptozotocin-treated rats. European journal of pharmacology 581:105-112.

Sharp T, Zetterstrom T, Ljungberg T, Ungerstedt U (1987) A direct comparison of amphetamine-induced behaviours and regional brain dopamine release in the rat using intracerebral dialysis. Brain research 401:322-330.

Shenoy SK, McDonald PH, Kohout TA, Lefkowitz RJ (2001) Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin. Science 294:1307-1313.

Shi X, Miller JS, Harper LJ, Poole RL, Gould TJ, Unterwald EM (2014) Reactivation of cocaine reward memory engages the Akt/GSK3/mTOR signaling pathway and can be disrupted by GSK3 inhibition. Psychopharmacology 231:3109-3118.

Shimada S, Kitayama S, Lin CL, Patel A, Nanthakumar E, Gregor P, Kuhar M, Uhl G (1991) Cloning and expression of a cocaine-sensitive dopamine transporter complementary DNA. Science 254:576-578.

Shiota C, Woo JT, Lindner J, Shelton KD, Magnuson MA (2006) Multiallelic disruption of the rictor gene in mice reveals that mTOR complex 2 is essential for fetal growth and viability. Developmental cell 11:583-589.

Simpson EH, Kellendonk C, Kandel E (2010) A possible role for the striatum in the pathogenesis of the cognitive symptoms of schizophrenia. Neuron 65:585-596.

Siuta MA, Robertson SD, Kocalis H, Saunders C, Gresch PJ, Khatri V, Shiota C, Kennedy JP, Lindsley CW, Daws LC, Polley DB, Veenstra-Vanderweele J, Stanwood GD, Magnuson MA, Niswender KD, Galli A (2010) Dysregulation of the norepinephrine transporter sustains cortical hypodopaminergia and schizophrenia-like behaviors in neuronal rictor null mice. PLoS biology 8:e1000393.

Sorkina T, Doolen S, Galperin E, Zahniser NR, Sorkin A (2003) Oligomerization of dopamine transporters visualized in living cells by fluorescence resonance energy transfer microscopy. The Journal of biological chemistry 278:28274-28283.

Speed N, Saunders C, Davis AR, Owens WA, Matthies HJ, Saadat S, Kennedy JP, Vaughan RA, Neve RL, Lindsley CW, Russo SJ, Daws LC, Niswender KD, Galli A (2011) Impaired striatal Akt signaling disrupts dopamine homeostasis and increases feeding. PloS one 6:e25169.

Speed NK, Matthies HJ, Kennedy JP, Vaughan RA, Javitch JA, Russo SJ, Lindsley CW, Niswender K, Galli A (2010) Akt-dependent and isoform-specific regulation of dopamine transporter cell surface expression. ACS chemical neuroscience 1:476-481.

Spencer JP (2009) The impact of flavonoids on memory: physiological and molecular considerations. Chemical Society reviews 38:1152-1161.

Sulzer D (2011) How addictive drugs disrupt presynaptic dopamine neurotransmission. Neuron 69:628-649.

Sulzer D, Sonders MS, Poulsen NW, Galli A (2005) Mechanisms of neurotransmitter release by amphetamines: a review. Progress in neurobiology 75:406-433.

Swift JL, Godin AG, Dore K, Freland L, Bouchard N, Nimmo C, Sergeev M, De Koninck Y, Wiseman PW, Beaulieu JM (2011) Quantification of receptor tyrosine kinase transactivation through direct dimerization and surface density measurements in single cells. Proceedings of the National Academy of Sciences of the United States of America 108:7016-7021.

Syringas M, Janin F, Mezghanni S, Giros B, Costentin J, Bonnet JJ (2000) Structural domains of chimeric dopamine-noradrenaline human transporters involved in the Na(+)- and Cl(-)-dependence of dopamine transport. Molecular pharmacology 58:1404-1411.

Szczypka MS, Rainey MA, Kim DS, Alaynick WA, Marck BT, Matsumoto AM, Palmiter RD (1999) Feeding behavior in dopamine-deficient mice. Proceedings of the National Academy of Sciences of the United States of America 96:12138-12143.

Tabrez S, Jabir NR, Shakil S, Greig NH, Alam Q, Abuzenadah AM, Damanhouri GA, Kamal MA (2012) A synopsis on the role of tyrosine hydroxylase in Parkinson's disease. CNS & neurological disorders drug targets 11:395-409.

Tan HY, Chen AG, Chen Q, Browne LB, Verchinski B, Kolachana B, Zhang F, Apud J, Callicott JH, Mattay VS, Weinberger DR (2012) Epistatic interactions of AKT1 on human medial temporal lobe biology and pharmacogenetic implications. Molecular psychiatry 17:1007-1016.

Terada K, Kojima Y, Watanabe T, Izumo N, Chiba K, Karube Y (2014) Inhibition of nerve growth factor-induced neurite outgrowth from PC12 cells by dexamethasone: signaling pathways through the glucocorticoid receptor and phosphorylated Akt and ERK1/2. PloS one 9:e93223.

Thiselton DL, Vladimirov VI, Kuo PH, McClay J, Wormley B, Fanous A, O'Neill FA, Walsh D, Van den Oord EJ, Kendler KS, Riley BP (2008) AKT1 is associated with schizophrenia across multiple symptom dimensions in the Irish study of high density schizophrenia families. Biological psychiatry 63:449-457.

Thomas DM, Francescutti-Verbeem DM, Kuhn DM (2008) The newly synthesized pool of dopamine determines the severity of methamphetamine-induced neurotoxicity. Journal of neurochemistry 105:605-616.

Thomas SA, Matsumoto AM, Palmiter RD (1995) Noradrenaline is essential for mouse fetal development. Nature 374:643-646.

Tomasi D, Wang GJ, Wang R, Caparelli EC, Logan J, Volkow ND (2015) Overlapping patterns of brain activation to food and cocaine cues in cocaine abusers: association to striatal D2/D3 receptors. Human brain mapping 36:120-136.

Torres GE (2006) The dopamine transporter proteome. Journal of neurochemistry 97 Suppl 1:3-10.

Torres GE, Yao WD, Mohn AR, Quan H, Kim KM, Levey AI, Staudinger J, Caron MG (2001) Functional interaction between monoamine plasma membrane transporters and the synaptic PDZ domain-containing protein PICK1. Neuron 30:121-134.

Ungerstedt U (1968) 6-Hydroxy-dopamine induced degeneration of central monoamine neurons. European journal of pharmacology 5:107-110.

Ungerstedt U (1971) Stereotaxic mapping of the monoamine pathways in the rat brain. Acta physiologica Scandinavica Supplementum 367:1-48.

Ungerstedt U, Pycock C (1974) Functional correlates of dopamine neurotransmission. Bulletin der Schweizerischen Akademie der Medizinischen Wissenschaften 30:44-55.

Valjent E, Bertran-Gonzalez J, Bowling H, Lopez S, Santini E, Matamales M, Bonito-Oliva A, Herve D, Hoeffer C, Klann E, Girault JA, Fisone G (2011) Haloperidol regulates the state of phosphorylation of ribosomal protein S6 via activation of PKA and phosphorylation of DARPP-32. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 36:2561-2570.

van der Heide LP, Ramakers GM, Smidt MP (2006) Insulin signaling in the central nervous system: learning to survive. Progress in neurobiology 79:205-221.

Volkow ND, Wise RA (2005) How can drug addiction help us understand obesity? Nature neuroscience 8:555-560.

Volkow ND, Wang GJ, Tomasi D, Baler RD (2013) The addictive dimensionality of obesity. Biological psychiatry 73:811-818.

Volkow ND, Wang GJ, Maynard L, Jayne M, Fowler JS, Zhu W, Logan J, Gatley SJ, Ding YS, Wong C, Pappas N (2003) Brain dopamine is associated with eating behaviors in humans. Int J Eat Disord 33:136-142.

Wang C, Buck DC, Yang R, Macey TA, Neve KA (2005) Dopamine D2 receptor stimulation of mitogen-activated protein kinases mediated by cell type-dependent transactivation of receptor tyrosine kinases. Journal of neurochemistry 93:899-909.

Wang GJ, Volkow ND, Fowler JS (2002) The role of dopamine in motivation for food in humans: implications for obesity. Expert Opin Ther Targets 6:601-609.

Wang GJ, Volkow ND, Logan J, Pappas NR, Wong CT, Zhu W, Netusil N, Fowler JS (2001) Brain dopamine and obesity. Lancet 357:354-357.

Weber JD, Gutmann DH (2012) Deconvoluting mTOR biology. Cell cycle 11:236-248.

Wei Y, Williams JM, Dipace C, Sung U, Javitch JA, Galli A, Saunders C (2007) Dopamine transporter activity mediates amphetamine-induced inhibition of Akt through a Ca2+/calmodulin-dependent kinase II-dependent mechanism. Molecular pharmacology 71:835-842.

Weinberger D, Laruelle M (2002) Neurochemical and neurophramacological imaging in schizophrenia. In: Neuropsychopharmacology: The Fifth Generation of Progress (Davis K, Charney D, Coyle J, Nemeroff C, eds).

White MF (2002) IRS proteins and the common path to diabetes. American journal of physiology Endocrinology and metabolism 283:E413-422.

Williams JM, Galli A (2006) The dopamine transporter: a vigilant border control for psychostimulant action. Handbook of experimental pharmacology:215-232.

Williams JM, Owens WA, Turner GH, Saunders C, Dipace C, Blakely RD, France CP, Gore JC, Daws LC, Avison MJ, Galli A (2007) Hypoinsulinemia regulates amphetamine-induced reverse transport of dopamine. PLoS biology 5:e274.

Wolf ME, Roth RH (1990) Autoreceptor regulation of dopamine synthesis. Annals of the New York Academy of Sciences 604:323-343.

Wong P, Sze Y, Chang CC, Lee J, Zhang X (2015) Pregnenolone sulfate normalizes schizophrenia-like behaviors in dopamine transporter knockout mice through the AKT/GSK3beta pathway. Translational psychiatry 5:e528.

Yamamoto BK, Novotney S (1998) Regulation of extracellular dopamine by the norepinephrine transporter. Journal of neurochemistry 71:274-280.

Yamashita A, Singh SK, Kawate T, Jin Y, Gouaux E (2005) Crystal structure of a bacterial homologue of Na+/Cl--dependent neurotransmitter transporters. Nature 437:215-223.

Yan Z, Song WJ, Surmeier J (1997) D2 dopamine receptors reduce N-type Ca2+ currents in rat neostriatal cholinergic interneurons through a membrane-delimited, protein-kinase-C-insensitive pathway. Journal of neurophysiology 77:1003-1015.

Yger M, Girault JA (2011) DARPP-32, Jack of All Trades... Master of Which? Frontiers in behavioral neuroscience 5:56.

Zahniser NR, Sorkin A (2009) Trafficking of dopamine transporters in psychostimulant actions. Seminars in cell & developmental biology 20:411-417.

Zhao Z, Ksiezak-Reding H, Riggio S, Haroutunian V, Pasinetti GM (2006) Insulin receptor deficits in schizophrenia and in cellular and animal models of insulin receptor dysfunction. Schizophrenia research 84:1-14.
Zhen J, Antonio T, Cheng SY, Ali S, Jones KT, Reith ME (2015) Dopamine transporter oligomerization: impact of combining protomers with differential cocaine analog binding affinities. Journal of neurochemistry 133:167-173.
Zheng H, Rinaman L (2015) Simplified CLARITY for visualizing immunofluorescence labeling in the developing rat brain. Brain structure & function.
Zhou QY, Palmiter RD (1995) Dopamine-deficient mice are severely hypoactive, adipsic, and aphagic. Cell 83:1197-1209.
Zoncu R, Efeyan A, Sabatini DM (2011) mTOR: from growth signal integration to cancer, diabetes and ageing. Nature reviews Molecular cell biology 12:21-35.