REGULATION OF DENDRITIC SPINES BY 5-HT2A RECEPTOR SIGNALING PATHWAYS

By

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ABSTRACT

Dendritic spines are small membranous protrusions from the dendrites of neuron, which are thought to serve as basic units of synaptic transmission, learning and memory. Disruptions in dendritic spine shape, size or number are associated with many brain diseases. Mounting evidence suggests that serotonin 2A (5-HT2A) receptors, the most abundant serotonin receptors in the prefrontal cortex, are involved in the regulation of dendritic spines. It has been suggested that both agonists (such as DOI) and antagonists (such as atypical antipsychotics) of 5-HT2A receptors can modulate different aspects of dendritic spines, however, the underlying mechanisms still remains unknown. In this dissertation, mechanisms underlying regulation of dendritic spines by both agonists and antagonists of 5-HT2A receptors are extensively studied and presented. I hypothesize that 5-HT2A receptor agonist regulate dendritic spines via transglutaminase- (TGase) catalyzed serotonylation of small G protein of the Rho family, whereas atypical antipsychotics change dendritic spines via activation of the Janus Kinase 2 (JAK2) signaling pathway.

In the first study, the mechanisms and the functional consequences of 5-HT2A receptor-induced serotonylation of small G proteins of the Rho family were investigated in primary rat cortical neurons. Stimulation of 5-HT2A/2C receptors caused TGase-mediated transamidation and activation of Rac1 and Cdc42, but not RhoA, in both A1A1v cells and rat primary cortical culture. DOI-induced Rac1 transamidation occurs at Q61 in A1A1v cells, as demonstrated by site-directed mutagenesis at Q61 of Rac1. Furthermore, our findings were extended from 5-HT2A/2C receptors to another Gαq/11-coupled receptor, muscarinic acetylcholine receptors. In addition, stimulation of 5-

HT2A/2C receptors by DOI leads to a transient dendritic spine enlargement, which was blocked by TGase inhibitor cystamine, suggesting 5-HT2A/2C receptors-induced transamidation of Rac1 and Cdc42 is involved in the regulation of dendritic spines by 5-HT2A/2C receptors.

In the second study, to study the role of JAK2/ STAT pathway in the regulation of dendritic spines, Sprague-Dawley rats were pretreated with the JAK2 inhibitor AG490 or vehicle, followed by administration with olanzapine or vehicle daily for seven days. Microarray analysis of prefrontal cortices showed that 205 genes were significantly changed by AG490, olanzapine or the combination of both drugs compared to the controls. 92 of the 205 genes are changed by olanzapine via JAK2 signaling pathway. These genes are involved in the etiology of schizophrenia, neuronal signal transduction, neuronal growth factor, metabolism and energy, and synaptic plasticity. mRNA and protein levels of these genes were verified using real-time qPCR, western blot and the enzyme-linked immunosorbent assay (ELISA). Investigation on dendritic morphology shows that treatment with olanzapine induced a maturation in dendritic spines via both JAK2 dependent and independent pathways.

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LIST OF ABBREVIATIONS

5-HT	serotonin
AD	Alzheimer's disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
Arc	activity-regulated cytoskeleton-associated protein
ARF1	ADP-ribosylation 1
ASD	autism spectrum disorder
CaMK	Ca2+/calmodulin-dependent protein kinase
Cartpt	CART prepropeptide
Cbln1	cerebellin 1 precursor
Cbp	CREB binding protein
Cdc42	cell division cycle 42
Cdc42se2	CDC42 small effector 2
CDD	conserved domain database
CNS	central nervous system
Crh	corticotropin releasing hormone
DA	dopamine
DAG	1,2-diacylglycerol
Dcx	doublecortin
DIV	day in vitro

DOI	1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl
Drd1a	dopamine receptor D1A
ELISA	enzyme-linked immunosorbent assay
EPS	extrapyramidal symptoms
ER	endoplasmic reticulum
Fabp7	fatty acid binding protein 7, brain
Filip1	filamin A interacting protein 1
G protein	guanine nucleotide-binding protein
Gabrg1	GABA-A receptor, gamma 1
GAP	GTPase activating proteins
GDI	GDP dissociation inhibitors
GEF	guanine nucleotide exchange factor
GO	gene ontology
GPCR	G protein-coupled receptor
Grik4	glutamate receptor, ionotropic, kainate 4
Grp	gastrin releasing peptide
GST	glutathione transferase
HA	histamine
Homer1	homer homolog 1
Hspa1a/1b	heat shock 70kD protein 1A/1B
Htr2c	serotonin receptor 2C
IOD	integrated optical density
IP3	inositol-1, 4, 5-triphosphate

JAK2	Janus kinase 2
LTD	long-term depression
LTP	long-term potentiation
mDia	mammalian Diaphanous formin
NA	noradrenaline
Nav2	neuron navigator 2
Nfasc	neurofascin
NMDA	N-methyl-D-aspartic acid
Nr4a1	nuclear receptor subfamily 4, group A, member 1
Nrg1	neuregulin 1
Ntrk2	neurotrophic tyrosine kinase receptor type 2
Nts	neurotensin
PAK	p21-activated kinase
Pak1ip1	PAK1 interacting protein 1
Pde10a	phosphodiesterase 10A
PIP2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLA2	phospholipase A2
PLC	phospholipase C
PLD	phospholipase D
PNS	the peripheral nervous system
PSD	postsynaptic density
PVN	hypothalamic paraventricular nucleus

Rac1	Ras-related C3 botulinum toxin substrate 1
Rasal1	RAS protein activator like 1 (GAP1 like)
Rasgef1c	RasGEF domain family, member 1C
Reln	reelin
RGS	regulator of G protein signaling
RhoA	Ras homolog A
Rich2	Cdc-42-interacting protein 4 homolog 2
ROCK	Rho-associated coiled-coil kinase1/2
SA	spine apparatus
SEM	standard error of the mean
SERCA	sarco/endoplasmic reticulum Ca2+ ATPase
SERT	serotonin transporter
siRNA	small interfering RNA
SSRI	selective serotonin reuptake inhibitor
STAT	signal transducer and activator of transcription
TGase	transglutaminase
WT	wild type

CHAPTER ONE: INTRODUCTION

DENDRITIC SPINES

Structure

Dendritic spines are small membranous protrusions from dendrites that receive input from presynaptic axons. Human brain contains more than 10^{13} dendritic spines (Nimchinsky *et al*, 2002). More than 90% of excitatory synapses are located on spines, which contain neurotransmitter receptors, organelles, scaffolding proteins and other signaling systems mediating synaptic morphology and functional plasticity, and ultimately learning and memory. Spines are thought to serve as basic units of learning and memory, disruption of which is associated with many brain diseases. It has been a century since dendritic spines were described for the first time on Purkinje cell dendrites, however, we have just started to appreciate their complexity and functions.

As a structure specialized for synaptic transmission, dendritic spines typically consist of a head (volume from 0.001–1 μm^3) connected to dendritic shaft by a thin neck (length from 0.5-2 μm), which restrains the diffusion of cytoplasmic and membrane molecules in and out of the spine head. Dendritic spines are rich in actin but not microtubules or intermediate filaments. The change in spine morphology is driven by the remodeling of β- and γ- actin, the main isoforms of actin present in neurons (Matus *et al*, 1982).

Spine organelles

A subpopulation of spines (half of spines on hippocampal CA1 cells and almost all spines on Purkinje cells) contains a specialized form of smooth endoplasmic reticulum

(SER), referred as the spine apparatus (SA) (Gray and Guillery, 1963; Spacek and Harris, 1997). Large spines with a perforated synapse are more likely to contain SA than small spines. Classical SA consists of two or more stacked flat saccules derived from the SER in the dendritic shaft. The larger the synapse, the more saccules present. SA contains a variety of substances that are essential for synaptic functions, including Ca2+, actin, MAP2 kinase, sarco/endoplasmic reticulum Ca2+ ATPase (SERCA pumps) and inositol 1,4,5-triphospate 3 (IP3) kinase (Sabatini *et al*, 2002). Even though the function of the SA is not understood completely, several lines of evidence suggest that the SA has distinct functions. Notably, SA plays a role in Ca2+ buffering (sequestering and releasing) within the spine compartment (Sabatini *et al*, 2002). SA contains IP3 receptors and ryanodine receptors. Neurotransmitters causing increased IP3 levels inside the spines therefore can trigger Ca2+ release from the SER of SA (Andrews *et al*, 1988). Rapid large-amplitude of Ca2+ signals have been reported to underlie the dendritic spine plasticity (Nimchinsky *et al*, 2002). Consequently, the ability of SA to release Ca2+ into the cytosol is thought to be involved in the development of spine plasticity. In addition, SA is also involved in spine protein synthesis and post-translational protein processing.

Another very important organelle in spines is the postsynaptic density (PSD), which is a collection of membrane-attached signal processing molecules including receptors, cytoskeletal proteins and associated signaling molecules (Peters and Palay, 1991). The PSD was originally identified by electron microscopy as an electron-dense region in close apposition to the active zone of the synapse. The sizes of PSDs vary from 250nm to 500nm in diameter and from 25nm to 50nm in thickness(Meyer *et al*, 2014). Recent studies suggest that area of PSDs is proportional to the volume of spines and may

change with alterations of synaptic activity during long term potentiation. Hundreds of proteins have been identified in PSDs, including postsynaptic density-95 (PSD95), cellular adhesion proteins (Hirao *et al*, 1998), glutamine receptors (N-methyl-D-aspartic acid (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors) (Kornau *et al*, 1995; Xia *et al*, 2003), 5-HT2A receptors and Ca2+/calmodulin-dependent protein kinase (CaMK) II (Peters and Palay, 1991). Number of AMPA and NMDA receptors per synapse is also proportional to the area of PSDs and size of spines. Those proteins play essential roles in the regulation synaptic signaling and development of plasticity.

Spine morphology

Dendritic spines are highly heterogeneous in shape and size, which is one of the most remarkable characteristics of dendritic spines, especially on pyramidal neurons. The most classical nomenclature segregates dendritic spines into three categories, thin spines, which have a small head and a narrow neck; stubby spines, which have no obvious neck between the head and dendritic shaft; and mushroom spines, which have a large head and a narrow neck. Other authors also add another category, thin and hairlike dendritic filopodia, which having a length that is at least twice the diameter. Besides those four most common spines, there are some irregular shaped spines, such as branched spines that may contain more than one PSD. In adult cortex and hippocampus, >65% of spines are thin spines and around 25% of spines are mushroom spines (Bourne and Harris, 2007). The remaining 10% of spines are immature spines, including filopodia, stubby and branched spines (Bourne and Harris, 2007). Criteria for categorizing spine morphology

are mostly qualitative, therefore, making it difficult to compare data from different laboratories.

Dendritic spines stabilize with maturation. During the first postnatal week in rats, nascent synapses form resulting from the interaction between filopodia and presynaptic axon. Then filopodia may contract and form stubby spines. During the following postnatal week, thin and mushroom spines emerge. Thins spines are transient and emerge and eliminate over days, whereas mushroom spines that can last for months (Chen *et al*, 2014). Mushroom spines have larger PSDs, therefore they are more likely than thin spines to contain SA and thereby regulate Ca2+ handling and local protein synthesis (Bourne and Harris, 2007). Mushroom spines contain more AMPA receptors, which are a major indicator of synaptic efficacy and strength (Nimchinsky *et al*, 2004). On the contrary, thin spines have more NMDA receptors but few AMPA receptor, allowing for strengthening by insertion of AMPA receptors (Ganeshina *et al*, 2004; Matsuzaki *et al*, 2001). Long-term potentiation (LTP) tends to converts thin spines into mushroom spines by increasing spine head size and accumulating AMPA receptors. Long-term depression (LTD), on the other hand, results in depolymerization of actin and is accompanied by shrinkage and retraction of dendritic spines (Bourne and Harris, 2007). These facts suggest that mushroom spines are more stable "memory spines", while thin spines are more flexible candidate "learning spines" (Holtmaat *et al*, 2005).

Signaling

Modulation of spine morphology is dependent on actin cytoskeleton dynamics, which requires rapid treadmilling of actin filaments by adding monomers at one end and depolymerization of actin at the other end. Actin polymerization may produce the

mechanical force to push out the spine membrane, resulting in spine enlargement. The reorganization of actin also leads to morphological changes of PSD. These processes are regulated by a large number of signaling effectors.

Ca2+ and CaMKII

During LTP, stimulation of glutamate receptors results in a Ca2+ elevation in stimulated dendritic spines but not in adjacent spines, and this elevation lasts for only 0.1 s (Murakoshi and Yasuda, 2012). Following the Ca2+ elevation, Ca2+ binds to calmodulin, and Ca2+-bound calmodulin binds and phosphorylates CaMKII, thereby activating CaMKII. This process occurs within 10s in the stimulated spines.

The activity of CaMKII could persist for more than one hour (Fukunaga *et al*, 1993), and has been thought to act as a mechanism to maintain LTP (Lee *et al*, 2009; Malinow *et al*, 1989; Mayford *et al*, 1995). CaMKII may in turn activate PSD95 and stargazin by phosphorylation, resulting in PSD disassembly and AMPA receptor anchoring within the PSD (Steiner *et al*, 2008). In addition, CaMKII can directly regulate actin organization based on its ability to bind to actin filaments and stabilize spine structure. When activated, CaMKII dissociates from actin, thereby rendering actin filaments destabilized (Okamoto *et al*, 2009).

Small G proteins of Rho family

In neurons, small G proteins of the Rho family are well-known regulators of dendritic spine morphology. Specifically, activation of RhoA causes spine loss and shrinkage by preventing actin polymerization, whereas Rac1 and Ccd42 activation increases spine density and size by promoting actin polymerization (Martino *et al*, 2013). Glutamate uncaging can transiently activate RhoA and Cdc42 rapidly within 1 min and

decay over 3-5 min in the simulated spines, followed by a prolonged activation lasting more than 30 min (Murakoshi *et al*, 2011). Even though the motility of RhoA and Cdc42 are similar, they have different spatial patterns (Murakoshi and Yasuda, 2012). Active RhoA is able to diffuse out of spine and spread to its dendritic shafts, while activity of Cdc42 is restrained to the spines (Murakoshi and Yasuda, 2012). Inhibiting CaMKII activity leads to partial inhibition of activity of GTPases, suggesting Rho family are downstream of CaMKII (Lee *et al*, 2009). Taken together, we can now integrate numerous signals on multiple time scales. The initial Ca2+ signal, which last for 0.1s, is followed by CaMKII activation lasting for 1min. Subsequently, activation of small GTPase by CaMKII expands the signal to 30min, similar to the time scale of spine enlargement (Nimchinsky *et al*, 2002).

Pathology

Given the essential roles of dendritic spines in synaptic transmission, learning and memory, it is not surprising that disruptions in spine shape, size or number are associated with brain disorders, especially those are characterized by dysfunction in information processing and cognition, including Autism spectrum disorder (ASD), schizophrenia and Alzheimer's disease (AD) (Penzes *et al*, 2013; Penzes *et al*, 2011). ASD is characterized by persistent deficits in social communication and interaction, and repetitive behavior patterns. One of the emerging hypotheses for ASD is hyperconnectivity in local circuits and hypoconnectivity between brain regions, which may be caused by spine dysmorphology (Geschwind and Levitt, 2007). Recent post-mortem studies showed an increase in spine density in cortical pyramidal neurons, and spine density was negatively correlated with cognitive function(Hutsler and Zhang, 2010). Fragile X syndrome, which

is comorbid with ASD, also exhibits elevated spine density (Irwin *et al*, 2001). Schizophrenia is a heterogeneous disorder characterized by abnormal social behavior, failure to interpret reality, disrupted affect and cognition, with a typical age of onset in early adulthood. Studies have shown reductions in spine density in forebrain regions in schizophrenic patients (Glantz and Lewis, 2000; Silva-Gomez *et al*, 2003). AD, the most common type of dementia, is an age-related disorder. One of the most consistent findings in AD patients is dendritic spine loss in the hippocampus and cortex, the principal areas affected by AD (Selkoe, 2002). Together, these findings describe the remarkable spine pathology associated with ASD, schizophrenia and AD. Notably, symptoms of each of these disorders coincide with malfunction of dendritic spines at distinct stages of life (Penzes *et al*, 2011), suggesting spine pathology may contribute to the development of these disorders across the lifespan.

SEROTONIN

Serotonin (5-HT) signaling system has been shown to regulate dendritic spines (Feria-Velasco *et al*, 2002; Hajszan *et al*, 2005). The 5-HT2A receptor is one of the 5-HT receptor subtypes that mediate the effects of 5-HT on dendritic spines (Cornea-Hebert *et al*, 2002; Peddie *et al*, 2008). Both agonists (such as DOI) and antagonists (such as atypical antipsychotics) of 5-HT2A receptors can modulate different aspects of dendritic spines (Critchlow *et al*, 2006; Jones *et al*, 2009; Roppongi *et al*, 2013; Wang and Deutch, 2008; Yoshida *et al*, 2011). In this dissertation, mechanisms underlying regulation of dendritic spines by both agonists and antagonists of 5-HT2A receptors are extensively studied and presented.

Serotonin receptors

As one of the most complex families of monoamine neurotransmitter receptors, serotonin acts on the central nervous system (CNS), the peripheral nervous system (PNS), and non-neuronal tissues such as platelets, gastrointestinal tract and the cardiovascular system (Hannon and Hoyer, 2008; Pytliak *et al*, 2011). In the brain, serotonin is synthesized in serotonergic neurons concentrated in the rostral and caudal raphe nuclei of the midbrain (Hornung, 2003). Projections from serotonergic neurons link the raphe nuclei to hypothalamus, hippocampus, amygdala, basal ganglia and cortex (Hoyer *et al*, 2002; Nolte and Sundsten, 2002). These brain regions play major roles in numerous physiological functions, including mood, sleep, feeding, thermoregulation and sexual behavior (Hoyer *et al*, 2002; Nolte and Sundsten, 2002). Disruptions of these brain regions are also involved in pathological states, such as depression, anxiety and psychosis (Andreasen, 1997). The effects of 5-HT are mediated through 7 subfamilies (5-HT1 receptor - 5-HT7 receptor) and at least 14 subtypes of 5-HT receptors, according to their ligand recognition profiles, molecular structures, and intracellular transduction mechanisms (Hoyer *et al*, 1994). Except for the 5-HT3 receptors, which are ion channel receptors, 5-HT receptors are G protein-coupled receptors (GPCRs) (Niesler *et al*, 2007).

5-HT1 receptors are expressed on both pre-synaptic and post-synaptic terminals. This subfamily contains 5 members: 5-HT1A, 5-HT1B, 5-HT1D, 5-ht1E and 5-ht1F (Hoyer *et al*, 1994; Polter and Li, 2010). This subfamily preferentially couples to the Gαi/o family of Gα proteins, which negatively regulate adenylyl cyclase and thereby inhibit cAMP production (Polter and Li, 2010). Gαi/o proteins also stimulate protein kinase A activity, as well as induce opening of K+ channels (Raymond *et al*, 1999). 5-

HT2 receptor subfamily includes 3 members: 5-HT2A, 5-HT2B, and 5-HT2C, which activate phospholipase C (PLC) via coupling to Gαq/11 proteins (Hoyer *et al*, 1994).

5-HT3 receptor is unique among all the 5-HT receptor subtypes since it's the only ionotropic receptor subtype while the rest subfamilies belong to metabotropic GPCRs (Kilpatrick *et al*, 1990). 5-HT3 receptor is permeable to Na+, K+ and Ca2+ (Costall and Naylor, 2004; Niesler *et al*, 2007). Binding of 5-HT to the 5-HT3 receptor triggers a rapid depolarization because of a transient inward current subsequent to the opening of nonselective cation channels (Costall and Naylor, 2004; Niesler *et al*, 2007).

5-HT4, 5-HT6, and 5-HT7 receptors couple to Gαs proteins that stimulate adenylyl cyclase to increase cAMP, and activate protein kinase A. 5-HT4 receptors have a wide distribution including alimentary tract, urinary bladder, heart and adrenal gland as well as the CNS (Berger *et al*, 2009; Ford and Clarke, 1993). Emerging evidence indicates that 5-HT6 receptors are implicated in neuronal circuit formation, neuronal connectivity, and psychiatric relevant behaviors (Dayer *et al*, 2015; Woolley *et al*, 2004). 5-HT7 receptors are expressed in the hippocampus, amygdala, hypothalamus and cerebral cortex, as well as in the gastrointestinal tract (Hoyer *et al*, 1994; Ruat *et al*, 1993). 5-HT7 receptors play important roles in thermoregulation, circadian rhythm, learning and memory, mood regulation, and smooth muscle relaxation in the gastrointestinal tract (Lovenberg *et al*, 1993; Mnie-Filali *et al*, 2009).

The function and signal transduction of 5-HT5 receptors (5-HT5A and 5-HT5B) have not been fully characterized. However, current evidence indicates that this subfamily displays a pharmacological profile similar to 5-HT1 receptors. 5-HT5 receptors have been found in locus coeruleus, nucleus of the solitary tract, arcuate and

suprachiasmatic nuclei of the hypothalamus, regions with a wide range of physiological effects (Hoyer *et al*, 1994; Matthes *et al*, 1993). Therefore, ligands of 5-HT5 receptors can serve as potential drug candidates for various disorders such as anxiety, sleep disorder and pain (Roth, 1994; Volk *et al*, 2010).

5-HT2A receptor

Distribution and physiology roles

5-HT2A receptors are widely distributed throughout CNS and peripheral tissues. In brain, 5-HT2A receptors have been found mainly in the cortex, claustrum, limbic system, olfactory nuclei and basal ganglia (Hannon and Hoyer, 2008). In the cortex, high concentrations of 5-HT2A receptors have been found on GABAergic interneurons, as well as on apical dendrites of pyramidal cells, which are known to be glutamatergic and implicated in cognitive function, working memory and attention (Aghajanian and Marek, 1997; Brady *et al*, 2005). In the periphery, 5-HT2A receptors are highly expressed in platelets, vascular smooth muscle, uterine smooth muscle, and in neurons of the peripheral nervous system (PNS) (Brady *et al*, 2005; Nagatomo *et al*, 2004).

The 5-HT2A receptor is implicated in a wide variety of physiological processes. 5-HT2A receptor mediates the effects of 5-HT on peripheral sensitization and hyperalgesia in inflammation and nerve injury (Sommer, 2004). 5-HT potentiates adenosine diphosphate or thrombin-induced platelet aggregation, which is mediated by 5-HT2A receptors (Li *et al*, 1997). Direct activation of 5-HT2A receptor in hypothalamic paraventricular nucleus (PVN) increases the release of oxytocin, prolactin, ACTH, corticosterone and renin (Van de Kar *et al*, 2001; Zhang *et al*, 2002). In addition, 5-HT2A receptor signal transduction is also involved in many pathological states including

stress, anxiety, depression, psychotic disorder (Graeff *et al*, 1996), hypertension, and atherosclerosis.

Given the distribution pattern and physiological functions of 5-HT2A receptors in the CNS, it's not surprising that 5-HT2A receptors are implicated in pathophysiology of a variety of psychiatric disorders. Postmortem studies have shown that 5-HT2A receptor binding sites are increased in the brain of depressed patients and suicide victims (Dwivedi *et al*, 2005; Pandey *et al*, 2003), while 5-HT2A receptors are decreased in the cortex and the superior temporal gyrus of schizophrenia subjects (Arora and Meltzer, 1991, Dean and Hayes, 1996) (Hernandez and Sokolov, 2000). Chronic treatment of rodents with different types of antidepressants reduces the density of 5-HT2A receptors in the frontal cortex. Global CNS genetic inactivation of 5-HT2A receptors produces an anxiolytic phenotype that is reversed by expression of cortical 5-HT2A receptors, which illustrates the necessity of cortical 5-HT2A receptor in the serotonergic regulation of anxiety (Weisstaub *et al*, 2006). Furthermore, polymorphism of 5-HT2A receptor gene is involved in schizophrenia and various affective disorders such as bipolar disorder, major depressive disorder and obsessive-compulsive disorder (Ghadirivasfi *et al*, 2011; Noskova *et al*, 2009).

5-HT2A receptors also serve as targets of therapeutic agents for a number of psychiatric disorders. The 5-HT2A receptor agonists are involved in the behavioral effects of many hallucinogens. 5-HT2A/C receptor agonist DOI also exerts anxiolytic-like effects, which were only blocked by a 5-HT2A antagonist but not by a 5-HT2B/C antagonist, thereby indicating that these anxiolytic-like responses are mediated by 5-HT2A receptors (Nic Dhonnchadha et al, 2003). Selective 5-HT2A receptor antagonists

have been reported to have antidepressant-like effects in different rodent models. They are also used together with SSRIs to augment the clinical efficacy of SSRIs (Nic Dhonnchadha et al, 2003; Werneck et al, 2009). Atypical antipsychotic drugs used clinically for treating schizophrenia and major depressive disorder have actions at multiple receptors including antagonism at 5-HT2A receptors. In conclusion, 5-HT2A receptors are a major target for treatment of anxiety, depression and psychiatric disorders.

Receptor structure and signal transduction

The human 5-HT2A receptor gene HTR2A is located on chromosome 13q14-q21 and codes for a protein composed of 471 amino acids (Hoyer *et al*, 2002). This gene consists of three exons and two introns and the coding region is 1.4kb (Sanders-Bush *et al*, 2003). As a pleiotropic GPCR, 5-HT2A receptor protein has a seven trans-membrane structure and couples to different G proteins, resulting in broad range of responses. Canonical 5-HT2A receptor signaling is mediated via Gαq/11 which couples to the phospholipase C (PLC) signaling cascade. Activation of PLC results in release of inositol-1, 4, 5-triphosphate (IP3) and 1,2-diacylglycerol (DAG) through phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolysis. IP3 binds to its receptors on endoplasmic reticulum (ER) and mobilizes Ca2+ into cytosol, whereas DAG activates protein kinase C (PKC) (Roth *et al*, 1991).

5-HT2A receptor signaling can activate a variety of signal transduction cascades beyond the canonical pathways. 5-HT2A receptor has been shown to activate phospholipase A2 (PLA2) and subsequent release of the second messenger arachidonic acid through two signaling cascades. One is Ras-Raf-MEK-ERK pathway, mediated via coupling to Gαi/o; the other is Gα12/13-coupled RhoA-p38-MAPK pathway. 5-HT2A

receptor is also able to activate phospholipase D (PLD) through interaction with ADP-ribosylation 1 (ARF1) (Blaazer *et al*, 2008).

5-HT2A receptor has also been shown to mediate transamidation reaction of small G proteins RhoA and Rab4 in platelet cells (Walther *et al*, 2003), as well as transamidation of serotonin to Rac1 in A1A1v cells (Dai *et al*, 2011; Dai *et al*, 2008). In addition, Janus Kinase 2 / Signal Transducer and Activator of Transcription (JAK/STAT) pathways can also be activated by 5-HT2A receptors (Guillet-Deniau *et al*, 1997; Singh *et al*, 2009b). These two pathways are presented in detail in the following sections.

SEROTONYLATION AND MONOAMINYLATION

Besides via its canonical receptor-mediated signaling cascades introduced above, serotonin has appreciable effects through the covalent modification of protein substrates, a reaction termed serotonylation. Protein serotonylation and monoaminylation (such as histaminylation, dopaminylation and norepinephrinylation) are newly emerging post-translational modifications described during the last decade. The reactions are catalyzed by transglutaminases (TGases), and alter structures and functions of proteins, for example, activation of small G proteins. Serotonylation and monoaminylation are implicated in multiple physiological functions. In this section, the functions and regulatory system of serotonylation and monoaminylation are presented.

Transglutaminase, enzymes catalyzing serotonylation and monoaminylation

TGase (classified as EC 2.3.2.13) are a family of Ca2+-dependent enzymes that catalyze the formation of an isopeptide bond between the γ-carboxamide group of a peptide- or protein-bound glutamine residue and a free amine (such as 5-HT) or protein-

or peptide-bound lysine (Esposito and Caputo, 2005). Such covalent bonds formed by this reaction are highly resistant to proteolytic degradation.

Human TGases comprise nine isoforms, including TGase1 (keratinocyte TGase), TGase2 (tissue TGase), TGase3 (epidermal TGase), TGase 4 (prostate TGase), TGase5 (TGaseX), TGase6 (TGaseY), TGase7 (TGaseZ), FXIIIA (plasma TGase), and Band 4.2 (erythrocyte membrane protein 4.2) (Lorand and Graham, 2003; Ricotta *et al*, 2010). Among the nine isoforms, TGase2 is the most abundant and ubiquitously distributed member (Lorand and Graham, 2003). TGase1, 2, 3 and 7 have been shown to be expressed in human brain (Eckert *et al*, 2014; Zainelli *et al*, 2005).

TGases alter the function of protein substrates by adding new properties to the substrates. Given the wide distribution of TGases, TGases are involved in a variety of physiological functions.

For example, TGase1, 2, 3 and 5 have been found in mammalian epidermis and cross-link proteins of keratinocytes during terminal differentiation, thereby playing roles in the formation of cornified cell envelope (Esposito and Caputo, 2005; Hitomi, 2005; Kim *et al*, 1995; Lee *et al*, 1996). Dysfunction of these TGases is implicated in lamellar ichthyosis, psoriasis and dermatitis (Hitomi, 2005).

TGases2 has been found to catalyze the transamidation of both intracellular and extracellular proteins. Cell surface TGase2 has been involved in the stabilization of the extracellular matrix by crosslinking matrix proteins, such as fibronection, von Willebrand factor, vitronection, laminin and nidogen, thereby forming large polymeric structures that are resistant to proteolytic degradation (Esposito and Caputo, 2005; Fesus and Piacentini,

2002). Perturbation of extracellular matrix regulated by TGases2 can lead to liver diseases, renal diseases, pulmonary fibrosis, and atherosclerosis (Collighan and Griffin, 2009; Esposito and Caputo, 2005).

FXIIIA is a plasma TGase that consists of two subunits, catalytic subunit FXIIIA and carrier subunit FXIIIB (Suzuki *et al*, 1988). In the presence of Ca2+, FXIIIB dissociates from FXIIIA and FXIIIA in turn catalyzes the cross links between the side-chains of fibrin molecules, thus stabilizing blood clots (Lorand, 2001). FXIII is well known regulators of hemostasis, wound healing and the maintenance of pregnancy (Esposito and Caputo, 2005).

Evidence indicates that TGase2 and possibly TGase1 and TGase4 are implicated in monoaminylation (Dai *et al*, 2008; Hummerich *et al*, 2012; Johnson *et al*, 2012; Vowinckel *et al*, 2012).

Regulatory mechanisms of TGases

Enzymatic activities of TGases are tightly regulated by multiple physiological regulators. TGase activation is dependent on the presence of Ca2+ ion (Hand *et al*, 1985). Binding of Ca2+ unmasks the cysteine in the active site of TGases (Hand *et al*, 1985), and is further regulated by GTP (Monsonego *et al*, 1998), phospholipids (Ando *et al*, 1991), tumor necrosis factor alpha (Chen *et al*, 2000), nitric oxide (Jandu *et al*, 2013) and CaM (Dai *et al*, 2011; Zainelli *et al*, 2004). Increase in intracellular Ca2+ concentration may result from release of intracellular Ca2+ storage, extracellular influx, or release from Ca2+ binding proteins. GTP binding to TGase stabilizes its conformation in an inactive state thereby inhibiting the transamidation catalytic activity of the enzyme (Monsonego *et*

al, 1998). Phospholipids can interact with TGase and inhibit its activity by providing a hydrophobic environment (Ando *et al*, 1991). Nitric oxide also has an inhibitory effect on TGase activity through phospholipid S-nitrosylation of thiol groups (Bernassola *et al*, 1999). On the other hand, both tumor necrosis factor alpha and CaM have been show to increase TGase activity (Chen *et al*, 2000; Zainelli *et al*, 2004).

TGase-catalyzed serotonylation and monoaminylation

The transamidation of a monoamine, such as 5-HT, histamine (HA), dopamine (DA), and noradrenaline (NA), to a protein-bound glutamine residue catalyzed by TGases is termed as monoaminylation, or serotonylation when the particular monoamine is 5-HT (Muma and Mi, 2015; Walther *et al*, 2011). A wide range of proteins serve as targets for monoaminylation, including small G proteins such as Rac1, Rab3a, Rab4, Rab27a and RhoA, heterotrimeric Gα proteins, fibronectin, and cytoskeleton proteins such as actin, myosin heavy chain and filamin A (Muma and Mi, 2015). The serotonylation and monoaminylation of those proteins are implicated in a variety of physiological processes and pathological conditions, including platelet activation, serotonin transporter (SERT) regulation, insulin release, smooth muscle contraction, pulmonary hypertension, and bone extracellular matrix stabilization (Muma and Mi, 2015; Walther *et al*, 2011).

Serotonylation of RhoA and Rab3 in platelet cells activate RhoA and Rab3, in turn increasing α-granule exocytosis (Walther *et al*, 2003). High plasma 5-HT levels lead to activation of Rab4, rendering Rab4 active and causing the sequestration of SERT from membrane into the cytoplasm (Mercado and Kilic, 2010). Serotonylation of small G proteins Rab3a and Rab27a has been also involved in the regulation of insulin secretion (Paulmann *et al*, 2009). Monoaminylation of cytoskeleton proteins in smooth muscle

cells is involved in the enhancement of muscle contraction (Johnson *et al*, 2010; Watts *et al*, 2009). Extracellular matrix proteins such as fibronectin are transamidated by 5-HT, DA and NA, however, the functions of these reactions are not well understood (Hummerich and Schloss, 2010). Serotonylation of intracellular fibronectin, on the other hand, has been reported to be involved in the development of primary pulmonary hypertension (Penumatsa *et al*, 2014). Stimulation of 5-HT2A receptors causes serotonylation and activation of small G protein Rac1 in neuronal cells (Dai *et al*, 2011; Dai *et al*, 2008). The function and regulation of serotonylation of small G protein of Rho family in neuronal cells are extensively studied and presented in this dissertation.

SMALL G PROTEINS OF THE RHO FAMILY

As the one of the major substrates of serotonylation and monoaminylation, small guanine nucleotide-binding proteins (known as small G-proteins or small GTPases) are small monomeric G proteins, which are homologous to the alpha subunit of heterotrimeric G-proteins. The small G proteins of the Rho family, including Ras-related C3 botulinum toxin substrate 1 (Rac1), cell division cycle 42 (Cdc42), and Ras homolog A (RhoA), belong to the Ras superfamily, and are molecular switches that cycle between a GDP-bound inactive form and GTP-bound active form. The GTP-GDP cycle is regulated by three groups of proteins: guanine nucleotide exchange factors (GEFs), which activate small G proteins by stimulating dissociation of the GDP from GTPases; GTPase activating proteins (GAPs), which inactivate small G proteins by accelerating the intrinsically low GTP hydrolysis rate; and GDP dissociation inhibitors (GDIs), which maintain GTPases in their inactive form by preventing the dissociation of GDP and interaction with GEFs and GAPs (Bustelo *et al*, 2007).

Biological functions of small G proteins of Rho family

Once small G proteins of the Rho family are activated, they can interact with more than 70 downstream effectors to participate in a wide array of signaling cascades, thus regulating a variety of crucial cellular activities, such as cytoskeletal reorganization, activation of protein kinases, vesicular trafficking, cell growth, cell-cell adhesion, cell motility, neuronal and epithelial differentiation, cell death and enzymatic activities (Bustelo *et al*, 2007; Ridley, 2006).

Small G proteins of Rho family are important regulators of actin cytoskeleton, neurite outgrowth and neuronal differentiation, processes mediated by Rho family's interaction with downstream effectors such as Rho-associated coiled-coil kinase1/2 (ROCK), the p21-activated kinase (PAK), the mammalian Diaphanous formin (mDia) and proteins of the WASp family including WASp, N-WASp and WAVE (Spiering and Hodgson, 2011). Rac1, Cdc42 and RhoA play different roles in regulating actin cytoskeleton and neurite formation. Over expression of constitutively active Rac1 or Cdc42 results in neurite growth and filopodia production (Kozma *et al*, 1997; Van Leeuwen *et al*, 1997), whereas expression of dominant negative Rac1 or Ccd42 inhibits neurite formation (Kozma *et al*, 1997; Pan *et al*, 2005). Neurite formation induced by Rac1 or Cdc42 can be blocked by RhoA expression and be promoted by inactivating RhoA (Pan *et al*, 2005; Van Leeuwen *et al*, 1997). Rho GEFs and GAPs are also involved in the regulation of dendritic spines. Lfc, a Rho GEF, interacts with spinophilin selectively to regulate Rho-dependent organization of F-actin in dendritic spines (Ryan *et al*, 2005). Stimulation of 5-HT2A receptors induces a transient spine enlargement through a Rac1 GEF kalirin-7/ PAK1 dependent pathway (Jones *et al*, 2009). Another

Rac GEF Asef2 promotes dendritic spine formation via spinophilin-dependent targeting (Evans *et al*, 2015). Rho-GTPase-activating protein interacting with Cdc-42-interacting protein 4 homolog 2 (Rich2), is a Rac1 specific GAP protein. Overexpression of Rich2 increases size and reduces the density of dendritic spines, while knock-down of Rich2 induces a reduction in both dendritic size and density (Raynaud *et al*, 2014). Rho GTPases are involved in the pathological condition-induced dendritic spine alterations. For example, chronic social defeat stress decreases Rac1 expression in mouse nucleus accumbens and increases the density of stubby excitatory spines in a Rac1-dependent manner, while overexpression of constitutively active Rac1 reverses the social defeat-induced depression-related behaviors and increase in stubby spines (Golden *et al*, 2013).

Structure of Rac1

Like other members of the Rho family, Rac1 is molecular switch cycling between the active form and inactive form, processes tightly regulated by GEF, GAP and GDI. Most Rho proteins consist of short N- and C-terminals, and a GTPase domain, which is composed of a six-stranded β-sheet surrounded by α-helices (Vetter and Wittinghofer, 2001). The formation of the GTP-bound structural form is confined to a conformational change in two regions, referred to as switch I and II, which provide interacting platforms for GEF/GAP regulators and downstream effectors, thus initiating a network of signaling cascades (Dvorsky and Ahmadian, 2004). Binding of GEF to switch I/II displaces Mg2+ and releases GDP in exchange for GTP, while binding of GAP to switch I/II inserts a H_2O molecule into the catalytic pocket of the GTPases of the Rho family, thereby facilitating the hydrolysis of GTP into GDP(Spiering and Hodgson, 2011). In order to study small GTPases of the Rho family, multiple molecular tools have been created by

mutating some crucial amino acids among switch I/II region, including constrictively active mutants Q61L or G12V Rac1 and Cdc42 or Q63L or G14V RhoA, and dominant negative mutants T17N Rac1 or Cdc42 or T19N RhoA (Spiering and Hodgson, 2011). Likewise, post-translational modification, such as transamidation and phosphorylation of Rho family at those sites may also affect their ability to interact with regulating or downstream proteins, thereby altering the activity of GTPases.

Rac1 bears five glutamine residues (Q2, Q61, Q74, Q141, and Q162) that could sever as substrates for TGase-catalyzed transamidation (Dai et al, 2008). Using the NCBI Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi? db=cdd), we searched for GTP/Mg2, GAP, GEF, and GDI binding sites in the Rac1 sequence, and identified two glutamine residues, Q61 and Q74, and three lysine residues (K5, K16, and K116), within these functional domains (Dai *et al*, 2008). Previous studies found that transamidation or deamidation of Rac1 and Cdc42 at Q61 and RhoA at Q63 could inhibit both intrinsic and GAP-catalyzed hydrolysis of those GTPases, thereby rendering them constitutively active (Flatau *et al*, 2000; Lerm *et al*, 1999; Schmidt *et al*, 1999). Therefore, those residues may be potential targets of TGase-catalyzed modification, to inhibit GTP hydrolysis of Rho GTPases, thus prolonging their activity.

JAK/STAT SIGNALING PATHWAY AND 5-HT2A RECEPTORS

The JAK/STAT signaling system is composed of three main components, a receptor, Janus Kinase (JAK) which is a small family of cytoplasmic tyrosine kinase, and Signal Transducer and Activator of Transcription (STAT). The JAK family consists of

four members, JAK1, JAK2, JAK3 and ubiquitously expressed TYK2 (Aaronson and Horvath, 2002). Mammalian STATs contain seven members, including STAT1, STAT2, STAT3, STAT4, STAT5 (STAT5A and STAT5B), and STAT6 (Aaronson and Horvath, 2002). JAK-STAT signaling pathway is activated by receptors that respond to numerous signals including interferon, interleukin, growth factors and other chemical messengers (Aaronson and Horvath, 2002). On the other hand, a number of G protein coupled receptors such as 5-HT2A receptors (Guillet-Deniau *et al*, 1997), 2-adenoreceptors and angiotensin II receptors (Pan *et al*, 1997) also activate the JAK/STAT pathway. Activation of these receptors is followed by recruitment of JAKs and results in their autophosphorylation, which in turn phosphorylate receptor cytoplasmic tails and create docking sites for STAT. STATs are recruited to the receptors via their SH2 domain and get phosphorylated. Phosphorylated STAT dimerizes and translocates into the nucleus, where the dimer binds to DNA promoters and promote gene transcription, thereby altering cells function. Knockout studies have shown that the JAK-STAT pathways play important roles in regulation of the immune system. While knockout of JAK1 and JAK2 is lethal, JAK3 knockout animals show murine severe combined immune deficiency, which is associated with dysregulation of both B- and T-cells (Igaz *et al*, 2001). TYK2 knockout animals suffer from hypersensitivity to pathogens resulting from defects in interferon and IL-12 signaling (Shuai and Liu, 2003). Knockout of STAT1 and STAT2 leads to impairment in interferon signaling and susceptibility to viral infections, whereas knockout of STAT3 is embryonically lethal (O'Shea, 1997). STAT4, STAT5 and STAT6 knockouts also show various defects in the immune system.

Activation of 5-HT2A receptors in rat skeletal muscle myoblasts has been reported to cause a rapid and transient activation of JAK2-STAT3 (Guillet-Deniau *et al*, 1997). Acute treatment with 5-HT also activates JAK1, JAK2, and STAT1 via activation of 5-HT2A receptors but not 5-HT2C or 5-HT2B receptors in vascular smooth muscle (Banes *et al*, 2005). Chronic treatment with atypical antipsychotics olanzapine and clozapine, and MDL100907 [(R)-(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperinemethanol], which are well known antagonists/ inverse agonists of 5-HT2A receptors, activate the JAK2-STAT3 pathway in both A1A1v cells and in rat frontal cortex and hypothalamus (Muma *et al*, 2007b; Singh *et al*, 2009b; Singh *et al*, 2007a). And the activation of JAK2/STAT3 has been show to mediate desensitization of 5-HT2A receptors (Singh *et al*, 2009b). A recent study demonstrates that interleukin-6 receptor stimulation attenuates 5-HT2A receptor agonist-induced IP production by activating the JAK-STAT pathway (Donegan *et al*, 2015).

ATYPICAL ANTIPSYCHOTICS

Medical uses and adverse effects

The current first-line drug treatment for schizophrenia are antipsychotics, which fall into two major categories, first generation antipsychotics, known as typical antipsychotics, and second generation antipsychotics, known as atypical antipsychotics. Atypical antipsychotics are the most frequently prescribed medications for schizophrenia, and can ameliorate positive symptoms within 8-15 days (Benjamin, 2012). Compared to typical antipsychotics, atypical antipsychotics exhibit greater efficacy in treating psychotic conditions and have a lower risk of extrapyramidal symptoms (EPS) (Leucht *et al*, 1999). However, both typical and atypical antipsychotics fail to improve negative and

cognitive symptoms of schizophrenia. Although atypical antipsychotics have shown many benefits, they cause numerous metabolic adverse effects, such as weight gain, dyslipidemia, diabetes, and cardiovascular disease (Nasrallah, 2008). The atypical antipsychotic clozapine can also lead to a serious side effect called agranulocytosis, which is an acute blood disorder characterized by a severe reduction in granulocytes (Nasrallah, 2008). Atypical antipsychotics are also used to treat bipolar disorder, agitation associated with dementia, anxiety disorder, and autism spectrum disorder (Nasrallah, 2008).

Receptor binding profile

Different members of atypical antipsychotics exhibit unique receptor binding profiles by interacting with serotonergic, dopaminergic, histaminergic, adrenergic and muscarinic acetylcholine receptors with various affinity (Nasrallah, 2008). The unique receptor binding profile of atypical antipsychotics may underlie the particular adverse effects associated with each drug. For instance, binding to histamine H1 receptor is associated with weight gain (Nasrallah, 2008).

The common feature shared by typical and atypical antipsychotic agents is affinity for dopamine receptors, especially D2 receptors, antagonism of which is thought to mediate the effects of these drugs on positive symptoms as well as the induction of EPS. In contrast to typical antipsychotics, atypical antipsychotics are characterized with enhanced 5-HT2A/D2 affinity ratio, which may underlie the improved pharmacologic features of atypical antipsychotic drugs (Nasrallah, 2008). For example, treatment with the atypical antipsychotics risperidone and olanzapine, which have a relatively high 5-

HT2A/D2 affinity ratio, causes less severe EPS compare with haloperidol, which has high D2 affinity.

Atypical antipsychotics are potent 5-HT2A receptor antagonists, suggesting that 5-HT2A receptors play a role in the action of atypical antipsychotics (Marek *et al*, 2003). Chronic treatment with atypical antipsychotic drugs has been shown to desensitize the 5-HT2A receptor signaling transduction. For example, rats treated with clozapine for 1 year exhibit a 63% reduction in 5-HT2A binding sites in the frontal cortex. Burnet *et al* found a reduction in [3H] ketanserin binding sites and 5-HT2A receptor mRNA levels in the cingulate and frontal cortex following chronic clozapine treatment (Burnet *et al*, 1996). The ability of antipsychotic drugs to down regulate 5-HT2A receptors is correlated with their affinities for the 5-HT2A receptor (Gray and Roth, 2001). The role of transcriptional regulation has been studied for antipsychotic-induced 5-HT2A receptor downregulation. It is likely that atypical antipsychotics-induced 5-HT2A receptor down-regulation is primarily mediated by posttranslational mechanisms (e.g., endocytosis, proteolysis) instead of transcriptional regulation (Gray and Roth, 2001). In addition, our laboratory has previously reported that chronic treatment with olanzapine and clozapine-induced desensitization of 5-HT2A receptor associated with increases in regulator of G protein signaling (RGS) 7 protein levels in vitro and in vivo. The activation of the JAK-STAT pathway by olanzapine and clozapine is responsible for the increases in RGS7 protein level (Muma *et al*, 2007b; Singh *et al*, 2009b; Singh *et al*, 2007a).

STATEMENT OF PURPOSE

A growing body of evidence implicates dendritic spines as important targets of pathogenesis in many neurodevelopmental and neurodegenerative diseases, such as ASD, schizophrenia and AD, which are characterized by impairment in cognitive functions (Penzes *et al*, 2013; Penzes *et al*, 2011). Serotonergic transmission, particularly 5-HT2A receptor signaling, plays an important role in cognitive processing by regulating prefrontal cortical circuitries (Leiser *et al*, 2015). The 5-HT2A receptor is implicated in the action of several hallucinogens and serve as a target for several therapeutic interventions, including antipsychotics, anxiolytics and antidepressants (Amodeo *et al*, 2014; Gonz áez-Maeso *et al*, 2007; Mestre *et al*, 2013. Dysfunctions of 5-HT2A receptors are associated with many psychiatric disorders, such as depression, anxiety and schizophrenia (Berg *et al*, 2008; Gray and Roth, 2007; Herv ás *et al*, 2014). Emerging evidence indicates that 5-HT2A receptors play roles in regulating dendritic spine morphogenesis in prefrontal cortex, and may link synaptic pathologies with etiologies of psychiatric disorders. It has been reported that both agonists and antagonists of 5-HT2A receptors regulate different aspects of dendritic spines, however, the underlying mechanisms still remain unclear. Discovering the molecular mechanisms underlying the regulation of dendritic spine morphology by 5-HT2A receptors may provide us insight into the etiologies of these disorders and shed light on the development of new therapeutic molecules. Therefore, the purpose of this study is to identify the mechanisms underlying the regulation of dendritic spines by 5-HT2A receptor agonists and antagonists.

The previous studies in our laboratory showed that stimulation of 5-HT2A receptors induces TGase-catalyzed serotonylation of a small G protein of the Rho family

Rac1, resulting in Rac1 activation in A1A1v cells, a rat cortical cell line (Dai *et al*, 2011; Dai *et al*, 2008). However, the functional consequences of this Rac1 serotonylation in neuronal cells was still unknown. It has been reported that acute stimulation of 5-HT2A receptors alters dendritic spine area transiently via a kalirin-7/ PAK1 dependent pathway (Jones *et al*, 2009). Activation of 5-HT2A receptors also affects the actin cytoskeleton by decreasing drebrin clusters at dendritic spines (Roppongi *et al*, 2013). Small G proteins of the Rho family are important regulators of actin cytoskeleton dynamics and dendritic spine morphogenesis. Together, these findings lead us to hypothesize that 5-HT2A receptor-induced Rac1 transamidation and activation leads to changes in spine morphology.

To test this hypothesis, I first verified whether Rho family members, Rac1, Cdc42 and RhoA are modified by DOI-activated TGases in rat primary cortical neurons, which enable us to examine the morphology of dendritic spines. 5-HT2A receptor-coupled PLC activation and subsequent Ca^{2+} signaling are necessary for TGase-catalyzed Rac1 transamidation and the increase in intracellular Ca^{2+} is sufficient to cause Rac1 transamidation (Dai *et al*, 2011). Therefore I also further explored whether other Gαq/11-coupled receptors, including 5-HT2C receptors and muscarinic receptors, could induce Rac1 transamidation. Lastly, the effects of 5-HT2A/2C receptor-mediated TGase activity on dendritic spines in rat primary cortical cultures were studied.

On the other hand, treatment with atypical antipsychotics has been shown to induce changes in dendritic spines in multiple studies (Critchlow *et al*, 2006; Elsworth *et al*, 2011; Vincent *et al*, 1991; Wang and Deutch, 2008). Although inconsistency exists among these studies, these changes in dendritic spines were induced by chronic but not

acute treatment of atypical antipsychotics. We previously reported that a sub-chronic treatment with atypical antipsychotics olanzapine and clozapine activate the JAK2/STAT3 pathway in both A1A1v cells and in rat frontal cortex (Muma *et al*, 2007a; Singh *et al*, 2009a; Singh *et al*, 2007b). STAT3 is a transcription factor for RGS7 in A1A1v cells and increases expression of RGS7 mRNA and protein (Singh *et al*, 2009a). JAK2/STAT3 pathway has been shown to mediate desensitization of 5-HT2A receptors, probably via increasing RGS7 expression (Singh *et al*, 2009a). Therefore, I asked the question, what other genes are regulated by olanzapine via JAK2/STAT3 pathways, and whether these genes are involved in the regulation of dendritic spines.

To address these two questions, we pretreated Sprague-Dawley rats with the JAK2 inhibitor AG490 or vehicle, followed by administration with olanzapine or vehicle daily for seven days. A microarray study was then performed using mRNA extracted from these rat frontal cortices. Real-time qPCR, western blot and ELISA assay were used to verify the changes in genes. Then I examined the effects of olanzapine and AG490 on the dendritic spines in rat primary cortical cultures. Finally, we proposed a mechanism by which olanzapine treatment induces maturation of dendritic spines in both JAK2 dependent and independent pathways.

CHAPTER TWO

5-HT2A/2C RECEPTOR-MEDIATED TRANSAMIDATION OF RAC1 AND CDC42 IS INVOLVED IN THE REGULATION OF DENDRITIC SPINES

ABSTRACT

We previously reported a novel 5-HT2A receptor downstream effector, transglutaminase (TGase) -catalyzed serotonylation of the small G protein Rac1 in A1A1v cells, a rat embryonic cortical cell line. Here we further explore the mechanisms involved and demonstrate the functional consequences of 5-HT2A receptor-induced serotonylation of small G proteins of the Rho family in primary neuronal cells. We found that stimulation of 5-HT2A/2C receptors caused transamidation of Rac1 and Cdc42, but not RhoA, in both A1A1v cells and rat primary cortical culture. The transamidation is mediated by TGases, possibly TGase2 and TGase1. Transamidation of Rac1 and Cdc42 lead to their activation, since inhibition of TGases significantly decreased transamidation and activation. Stimulation of both 5-HT2A and 5-HT2C receptors contributed to DOI-induced Rac1 transamidation, since inhibition of 5-HT2A receptors by MDL100907 and 5-HT2C receptors by SB242084 blocked the DOI-stimulated Rac1 transamidation. DOI-induced TGase-catalyzed transamidation of Rac1 occurs at Q61 in A1A1v cells, as demonstrated by site-directed mutagenesis at Q61 of Rac1. Furthermore, we extended our findings from 5-HT2A/2C receptors to another Gαq/11-coupled receptor, muscarinic acetylcholine receptors. In SH-SY5Y cells, stimulation of muscarinic receptors by carbachol increased TGase-catalyzed Rac1 transamidation, thus resulting in activation of Rac1. In addition, stimulation of 5-HT2A/2C receptors by DOI leads to a transient dendritic spine enlargement, which was blocked by TGase inhibitor cystamine,

suggesting 5-HT2A/2C receptors-mediated transamidation of Rac1 and Cdc42 is involved in the regulation of dendritic spines by 5-HT2A/2C receptors. These studies suggest a novel serotonin signaling pathway in the regulation of dendritic spine in cortical neurons.

INTRODUCTION

In the central nervous system, the majority of the excitatory postsynaptic terminals are located on dendritic spines, which are small membranous protrusions from dendrites (Phillips and Pozzo-Miller, 2015). Changes in size, number and morphology of dendritic spines are tightly coordinated with synaptic function and plasticity, underlying the establishment and remodeling of neuronal circuits, learning and memory, and behavior (Penzes *et al*, 2011). Notably, malfunction of dendritic spines accompanies a large number of brain disorders, including bipolar disorder, autism spectrum disorder, schizophrenia and Alzheimer's disease, suggesting that dendritic spine can serve as a common target for those complex brain diseases (Konopaske *et al*, 2014; Penzes *et al*, 2013; Penzes *et al*, 2011). Understanding the molecular underpinnings of dendritic spine dynamics may provide us essential insight into the etiologies of those disorders and may reveal new drug targets.

Morphological changes of spines are driven by actin dynamics, which are regulated by small G proteins of the Rho-family. Small G proteins of the Rho-family, including Rac1, Cdc42 and RhoA, belong to the Ras superfamily of small (±21 kDa) GTPases, which are guanine nucleotide-binding proteins cycling between an inactive GDP-bound form and an active GTP-bound form(Tolias *et al*, 2011). The GDP/GTP

cycling is under the tight regulation of many molecular regulators including GTPase activating proteins (GAPs), guanine nucleotide exchange factors (GEFs), and GDP dissociation inhibitors (GDIs) (Tolias *et al*, 2011). At the synapse, Rac1, Cdc42 and RhoA play a pivotal role in spine formation and morphogenesis, and synaptic plasticity (Martino *et al*, 2013). Activation of Rac1 and Cdc42 has been shown to promote spine formation, growth and stabilization; conversely, RhoA activation leads to spine pruning. Perturbations in Rho family signaling are implicated in various brain disorders, particularly those associated with cognitive deficits, such as mental retardation, schizophrenia and Alzheimer's diseases (Ba *et al*, 2013; Bolognin *et al*, 2014; Datta *et al*, 2015). Given the significance of the Rho family in multiple pathophysiological processes, it is important to understand all of the mechanisms regulating activity of Rho family proteins in neuronal cells.

Numerous studies have demonstrated that the activity of small G proteins including those of the Rho family is regulated by monoaminylation (Muma and Mi, 2015). Monoaminylation is a post-translational modification of proteins in which transglutaminases (TGases) catalyze the transamidation of a primary amine molecule such as serotonin (5-HT) or dopamine to a protein-bound glutamine residue (Muma and Mi, 2015). Serotonylation is a term coined to describe the specific transamidation of 5-HT to a protein (Walther *et al*, 2003). We previously demonstrated that stimulation of serotonin 2A (5-HT2A) receptors induces serotonylation of Rac1, resulting in Rac1 activation (Dai *et al*, 2008). Together, these findings lead us to hypothesize that 5-HT2A receptor-induced Rac1 transamidation and activation leads to changes in spine morphology.

5-HT2A receptors are widely distributed in most forebrain regions. Disrupted function of 5-HT2A receptors has been identified in various neurological and psychiatric disorders such as schizophrenia, Alzheimer's disease (Fehér *et al*, 2013), autism, depression and anxiety (Berg *et al*, 2008; Gray and Roth, 2007; Hervás *et al*, 2014). 5-HT2A receptors are also the target for several antidepressants, anxiolytics, atypical antipsychotics and hallucinogens (Amodeo *et al*, 2014; González-Maeso *et al*, 2007; Mestre *et al*, 2013). 5-HT2A receptors localize to dendrites, dendritic shafts, and dendritic spines (Cornea-Hebert *et al*, 2002; Peddie *et al*, 2008). Initiation of 5-HT2A receptor expression coincides with the period of synaptogenesis (Roth *et al*, 1991). Jones and the colleagues reported that 5-HT2A receptor activation alters dendritic spine area via a kalirin-7 dependent pathway (Jones *et al*, 2009). Yoshida and colleagues have found that stimulation of 5-HT2A receptors also changes the density of specific subtypes of dendritic spines (Yoshida *et al*, 2011). And recently, Roppongi et al demonstrated that activation of 5-HT2A receptors affects the actin cytoskeleton by decreasing drebrin clusters at dendritic spines (Roppongi *et al*, 2013). Those studies suggest that 5-HT2A receptors play a role in the regulation of dendritic spine architecture and actin cytoskeleton. However, the underlying molecular mechanisms by which the 5-HT2A receptor signaling regulates dendritic spines and the role of serotonylation of Rac1 and possibly other members of the Rho family in the process are not clear.

In the present study, we use A1A1v cells, a rat cortical cell line, and rat primary cortical culture to test whether activation of TGase via stimulation of 5-HT2A receptors induces transamidation and activation of Rac1, Cdc42 and RhoA, and whether the transamidation results in changes of dendritic spine architecture. Overall, our results

indicate that 5-HT2A/2C receptor-induced transamidation and activation of Rac1 and Cdc42 are involved in the regulation of dendritic spine architecture.

METHODS

Reagents

The following drugs were used in this study: 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl (DOI) (Sigma-Aldrich, St. Louis, MO), (2-hydroxyethyl)trimethylammonium chloride carbamate (carbachol) (Sigma-Aldrich, St. Louis, MO) and 2-aminoethyl disulfide dihydrochloride (cystamine) (MP Biomedicals, Solon, OH) were dissolved in saline and further diluted before applied to cell cultures. SB 242084 (Sigma-Aldrich, St. Louis, MO) was dissolved in Ethanol. MDL100907 was kindly provided by Sanofi Aventis (Bridgewater, NJ) and dissolved in DMSO.

Cell culture and transfection

A1A1v cells are a rat cortical cell line, which endogenously express the 5-HT2A receptor signaling system but not the 5-HT2C receptor system (Dai *et al*, 2008). A1A1v cells were grown in Dulbecco's modified Eagle medium (Fisher Scientific, Pittsburgh, PA) containing 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) at 33 ℃ in 5% CO_2. Before each experiment, A1A1v cells were maintained for 24h in Dulbecco's modified Eagle medium containing 10% fetal bovine serum treated with charcoal to remove > 99% of endogenous 5-HT (Unsworth and Molinoff, 1992). SH-SY5Y cells, a human neuroblastoma cell line, were cultured in 50% Dulbecco's modified Eagle medium (Fisher Scientific, Pittsburgh, PA) and 50% Opti-MEM medium (Life Technologies, Grand Island, NY) containing 10% fetal bovine serum at 37 ℃ in 5% CO_2. Cells were transiently transfected for 5h with 8-12ug plasmid using Lipofectamine 2000

(Life Technologies, Grand Island, NY) 12-16h after plating on 100-mm2 plates. Cells were harvested using lysis buffer (25 mM Tris, pH 7.5, 250 mM NaCl, 5 mM EDTA and 1% Triton X-100, protease inhibitor cocktail 1:1000) 48h after transfection.

Primary cortical culture

Animal use was performed in accordance with the National Institute for Health Guide for the Care and Use of Laboratory Animals as approved by the University of Kansas Institutional Animal Care and Use Committee. Primary cortical neurons were isolated from E18 Sprague-Dawley rat embryos as described previously with minor modifications (Beaudoin III *et al*, 2012; Srivastava *et al*, 2011). Pups were decapitated and cortical tissues were dissected out, followed by 0.01% trypsin (Sigma-Aldrich, St. Louis, MO) digestion for 20min and gentle pipetting. Neurons were plated at a density of 5×10^5 cells/ml on 22mm diameter round cover glass (Neuvitro Corporation, Vancouver, WA) or at a density of 2.7×10^6 cells/ml on T25 cell culture flasks (Fisher Scientific, Lenexa, KS) coated with Poly-L-lysine (Sigma-Aldrich, St. Louis, MO). Cells were maintained in Neurobasal media (Life Technologies, Grand Island, NY) supplemented with 2% B27 (Life Technologies, Grand Island, NY), 0.5 nM glutamine (Sigma-Aldrich, St. Louis, MO) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO) at 37 ℃ in 5% CO_2, half of the medium was changed twice a week. Neurons were used for experiments at 21 days in vitro (DIV), when the dendritic spines display a relatively mature morphology.

Immunocytochemistry

Primary neurons were double-labeled with Alexa Fluor® 568 Phalloidin (Life Technologies, Grand Island, NY) for labeling actin, antibodies against microtubule-

associated protein 2 (Map2) clone HM-2 (Sigma-Aldrich, St. Louis, MO) for labeling dendrites, antibodies against 5-HT2A receptors (Singh *et al*, 2007b), and antibodies against PSD95 (6G6-1C9) (Life Technologies, Grand Island, NY). Neurons were fixed in 4% paraformaldehyde for 20min followed by three washes. Fixed neurons then were permeabilized for 15min in phosphate buffered saline (PBS) buffer containing 0.1% Triton X-100, following by incubation with PBS containing 1% bovine serum albumin for 30 minutes, to reduce the non-specific binding of phalloidin. 10μL methanol stock solution of Alexa Fluor® 568 Phalloidin in 200μL PBS was added to each coverslip, incubated for 20min, followed by three washes with PBS. Then phalloidin-labeled neurons were incubated in 1.5 ml 10% normal donkey serum (NDS) (Jackson Immunoresearch Labs Inc, West Grove, PA) containing 0.3% Triton X-100 at room temperature for 15min, to block the nonspecific binding of antibodies. After three washes with PBS, primary antibodies were added in PBS with 10% NDS overnight at 4 ℃. After three washes with PBS the next day, Alexa Fluor® 488 donkey anti-rabbit IgG (H+L) antibody and Alexa Fluor® 647 conjugated goat anti-mouse IgG (H+L) secondary antibody (Life Technologies, Grand Island, NY) in 10% NDS in PBS were added to coverslips and incubated for 2 hours at room temperature. Three more washes were performed before coverslips were mounted onto slides using ProLong anti-fade reagent (Invitrogen, Grand Island, NY).

Quantitative Analysis of Spine Morphology

Neurons were visualized with an Olympus/3I Spinning Disk Confocal Microscopy using a 100X TIRF oil immersion objective. Z-series of twenty to thirty images were taken at 0.2μm intervals, with 1024 x 1024 or 500 x 500 pixel resolution.

Three-dimensional maximum projection reconstructions and deconvolution were performed using Slidebook 5.5 or 6 (Intelligent Imaging Innovations, Inc). To examine the morphology of dendritic spines, dendrites and individual spines on dendrites were traced, and dendritic area was measured using CellProfiler (Broad Institute, Cambridge, MA). Length of dendrites was measuring using Image J (National Institutes of Health, Bethesda, Maryland). Spine density were measured by using Neuron Studio (Icahn School of Medicine at Mount Sinai, New York, NY). Spines on one or two primary dendrites of each neuron, and 5-10 neurons for each condition were analyzed from three separate experiments.

Site-directed Mutagenesis

Using the an interactive tool to identify conserved domains present in protein sequences in the Conserved Domain Database (available on http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), we searched for GTP/Mg2+ binding sites, and GAP, GEF, and GDI interaction sites in the Rac1 sequence (accession number: NP_008839) since TGase-catalyzed modifications at these sites would more likely lead to functional changes that would impact Rac1 activity. Four glutamine residues (Q61, Q74, Q161, and Q174) were located in the activity-related domains and were targeted for site-directed mutagenesis. Site-directed mutagenesis was performed with Rac1 DNA in TOPO® PCR Cloning vector using the Quikchange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). Oligonucleotides containing amino acid substitutions for glutamine 61, 74,161 and 174 of Rac1 were designed using QuikChange® Primer Design Program (Agilent Technologies, Santa Clara, CA) and synthesized by Life Technologies. Briefly, oligonucleotides

containing desired mutations were annealed to denatured DNA templates and extended with Pfu-based DNA Polymerase Blend. Then the parental methylated and hemimethylated DNA templates were digested with Dpn I enzyme. Mutated molecules were transformed into competent cells to replicate. Plasmids with desired mutations were purified from bacterial culture and confirmed by sequencing. Then wild type and Rac1 mutants were cloned into pcDNA™4/HisMax C Mammalian Expression Vectors (Life Technologies, Grand Island, NY) and transfected into A1A1v cells as described above.

Small Interfering RNA (siRNA)

To reduce TGase2 protein expression, siRNA duplex targeting the coding sequence of rat TGase2 mRNA was used as previously reported with minor modification(Dai *et al*, 2008). The target sequence is 5-AAGAGCGAGATGATCTGGAAT-3 and synthesized by QIAGEN (Germantown, MD). At DIV19, primary neurons were transfected with siRNA at a final concentration of 90 nM using Lipofectamine 3000 without P3000 reagent according to the manufacture's instruction. 72 hours after transfection, cells were treated with DOI or saline as a control. Cells incubated with Lipofectamine 3000 alone were used as a non-transfected control.

Immunoblot

Protein samples were separated on 12% or 14% SDS-polyacrylamide gels as previously described (Dai *et al*, 2008). Proteins were transferred into PDVF membranes followed by incubation for 1h in buffer (5% nonfat dry milk, 0.1% Tween20, 1X TBS buffer) to block non-specific binding. Primary antibodies were diluted in antibody buffer (2% nonfat dry milk, 0.1% Tween20, 1X TBS buffer) and incubated with membranes overnight at 4℃ on a shaker. The primary antibodies used are as follows: anti-Rac1

antibody, clone 23A8, mouse IgG, 1:700 (Millipore Corporation, Billerica, MA); anti-Cdc42 antibody, clone 44, mouse IgG, 1:500 (BD Biosciences, BD Biosciences); anti-actin, mouse IgG, 1:50,000 (MP Biomedicals, Solon, OH). The membranes were washed three times with TBS/Tween 20 buffer the next day, followed by incubation for 1h at room temperature in goat anti-rabbit IgG or donkey anti-mouse IgG diluted in antibody buffer. After the membranes were washed, the Amersham ECL Western Blotting Detection Reagents (GE Healthcare) were applied to the membranes, and the signals were detected using ChemiDoc™ XRS+ System (Bio-Rad, Hercules, CA) and quantified by calculating the integrated optical density (IOD) of each protein band using Image Lab™ Software (Bio-Rad, Hercules, CA).

Immunoprecipitation of TGase-Modified Protein

Immunoprecipitation of TGase-modified protein was performed as described previously with minor modifications (Dai *et al*, 2008). A1A1v cells, SH-SY5Y cells or rat primary cortical neurons were lysed using lysis buffer A (25 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 1:1000, protease inhibitor cocktail) and protein concentration was determined. 200 µg and 600 µg protein were used for detecting Rac1 and Cdc42 transamidation respectively. Cell protein was added to 20 µl of washed Sepharose-81D4 antibody beads directed against the TGase-modified proteins and buffer B (10mM Tris-HCI pH 7.5, 0.14M NaCl, 0.1% Tween 20) was added to achieve a 1 µg/µl final protein concentration. After 2h incubation at 37 ℃, the beads were spun down at 10,000xg and washed 4 times with buffer B. 25 µl of loading buffer (50mM Tris-HCI, pH6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol) was added to the final pellet and incubated at 95 ℃ for 5min to elute the TGase-modified protein.

Activity Assay for Small G Proteins

The activity of Rac1 and Cdc42 was measured using Glutathione Transferase (GST) -PAK1 bound Sepharose 4B beads as described previously (Dai *et al*, 2008). A1A1v cells, SH-SY5Y cells or primary cortical neurons were lysed using lysis buffer C (50mM Hepes, pH7.6, 100mM NaCl, 5mM MgCl2, 10% glycerol and 1:1000 protease inhibitor cocktail). 200µl lysed cells was added to 40µl of beads and incubate for 40min at 4 ℃ followed by three washes. 20µl 2 X PAGE sample buffer (0.187M Tris pH 6.8, 4.5% SDS, 22.3% glycerol, with 1:1000 protease inhibitor cocktail) was added to pellets and incubated at 90℃ for 5min followed by centrifuging at 9,000xg for 2min. Equivalent amounts of protein were loaded on 14% SDS PAGE and detected on immunoblots as described above.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). All data are analyzed by Student's t-test, one, or two-way ANOVA, or Kruskal-Wallis one-way ANOVA on Ranks. Post hoc tests were conducted using Newman-Keuls multiple comparison test or Dunn's method. Sigmaplot 12 (Systat Software, Inc., San Jose, CA) and Origin 8.0 (OriginLab Corporation, Northampton, MA) were used for statistical analysis. Significant differences are defined as those with a p-value< 0.05.

RESULTS

5-HT2A/2C receptor-stimulated Rac1 activation is dependent on TGase-catalyzed transamidation in rat primary cortical neurons in culture

To determine whether Rac1 activation by 5-HT2A/2C receptors stimulation alters dendritic spine morphology, we used primary cortical neurons from E18 rat pups cultured for DIV21. At this stage, dendritic spines display a relatively mature morphology and form connections with presynaptic partners.

The first step was to determine whether Rac1 transamidation is increased in rat primary culture, we treated the neurons at DIV21 with 3 μM DOI for 15min, the time point at which we observed the most abundant Rac1 transamidation in A1A1v cells (Dai *et al*, 2008). TGase-modified proteins were immunoprecipitated using the 81D4 antibody and examined on immunoblots using a Rac1 antibody. The ratio of TGase-modified Rac1 to total Rac1 is significantly increased following 15min of DOI treatment (Figure 2.1A), suggesting Rac1 is transamidated after stimulation of 5-HT2A/2C receptors in rat primary cortical cultures.

Next, to determine whether Rac1 activity is increased following 5-HT2A receptor stimulation in this primary culture model as previously demonstrated in A1A1v cells, we used a PAK1 pull down assay, since only activated GTP-bound Rac1 can bind to PAK1 (Parrini *et al*, 2002). We treated the primary neurons with 3 μM DOI, a 5-HT2A/2C receptor-selective agonist, for 5min, the period previously used to detect Rac1 activation in A1A1v cells (Dai *et al*, 2008). As shown in Figure 2.1B, the ratio of GTP-bound Rac1 to the total Rac1 is significantly increased by 100% after 5 min of DOI treatment, indicating that Rac1 becomes activated following DOI treatment in rat primary cortical cultures as in A1A1v cells.

To exam whether the DOI-stimulated increase of active Rac1 is due to TGase-catalyzed transamidation, we inhibited the activity or expression of TGase by treating the

primary neurons with 1mM cystamine, a TGase inhibitor, for 1 hour, or transfecting a siRNA targeting TGase2 into the primary neurons for 72 hours, followed by DOI treatment for 5min or 15min. As shown in Figure 2.1C, siRNA transfection successfully resulted in 45% decrease in TGase2 protein levels accompanied by an 85% increase in TGase1 protein levels. TGase3 was not detected on Western blot using a TGase3 specific antibody. DOI-induced Rac1 transamidation is significantly reduced by cystamine treatment and knock down of TGase2 expression with siRNA, suggesting 5-HT2A receptor-mediated Rac1 transamidation is dependent on the activity of TGases in primary culture. Compared with DOI-stimulated mock-transfected cells, cystamine treatment caused a 70% decrease and siRNA transfection caused 40% decrease in TGase-modified Rac1 following DOI treatment (Figure 2.1D). The reduction in DOI-induced Rac1 transamidation caused by cystamine is significantly higher than TGase2 siRNA, suggesting not only TGase2, but perhaps also TGase1, mediates DOI-induced Rac1 transamidation in rat primary cortical neurons. We also found that treatment with cystamine and siRNA transfection decreases the amount of DOI-induced Rac1 activation by 56% and 38%, respectively, compared with mock-transfected cells. These results indicate that TGase-catalyzed transamidation is necessary for the increase in Rac1 activity upon 5-HT2A/2C receptor stimulation in rat primary cortical neurons (Figure 2.1E).

DOI-induced Rac1 transamidation is mediated by both 5-HT2A and 5-HT2C receptors in rat primary cortical neurons

DOI is a selective 5-HT2A/2C receptor agonist. In order to test whether 5-HT2A or 5-HT2C, or both receptors mediate the DOI-stimulated Rac1 transamidation in rat

primary cortical cells, we pretreated the rat primary neurons at DIV21 with 3.6 nM MDL100907, a selective 5-HT2A receptor antagonist, 10 nM SB242084, a selective antagonist for the 5-HT2C receptors, or DMSO, for one hour. We then stimulated cells with DOI or saline for 15min and examined Rac1 transamidation as described above. As shown in Figure 2.2, the ratio of TGase-modified Rac1 to total Rac1 is significantly increased following 15min of DOI treatment, and both MDL100907 and SB24084 suppressed DOI-induced Rac1 transamidation. The mean of Rac1 transamidation levels in MDL100907 treated samples is lower than the one in SB24084 treated samples, however, the difference is not statistically significant. These results suggest that both 5-HT2A and 5-HT2C receptors contribute to DOI-stimulated Rac1 transamidation mediated by TGases.

Stimulation of 5-HT2A/2C receptors increase activation of Cdc42 in a TGase-dependent manner in A1A1v cells and rat primary cortical neurons

To explore whether another member of the Rho family, Cdc42, also becomes activated following stimulation of 5-HT2A receptors, we treated A1A1v cells with 3 μM DOI for 5 min, 15 min or 30min, and separated active Cdc42 using GST-PAK1 bound glutathione-sepharose beads. Since A1A1v cells do not express 5-HT2C receptors, the DOI-induced effects are mediated by 5-HT2A receptors (Dai *et al*, 2008). We observed an increase in active Cdc42 at these three time points. To further determine the duration of DOI-induced Cdc42 activation, we treated A1A1v cells with DOI again for longer time periods, including 30 min, 1 hour, 2 hours or 3 hours. As shown in Figure 2.3A, the activity of Cdc42 is significantly increased following 30 min, 1 hour and 2 hour DOI treatment, respectively. However, there is no significant change in the amount of GTP-

bound Cdc42 in cells treated with DOI for 3 hours compared with vehicle-treated cells, suggesting that DOI-induced Cdc42 activation lasts up to 2 hour in A1A1v cells.

We also examined TGase-modified Cdc42 after treatment with 3 μM DOI for 30 min, 1 hour, 2 hours or 3 hours. The Cdc42 transamidation is significantly elevated after 2 hour and 3 hour DOI treatment (Figure 2.3B). To examine whether the DOI-stimulated increase in Cdc42 activity is dependent on Cdc42 transamidation, A1A1v cells were treated with 1mM cystamine for 1 h, followed by treatment with 3 μM DOI for 30min, 1 hour or 3 hours. We found that the pretreatment with cystamine decreases the amount of activated Cdc42 at all three time points, suggesting the DOI-induced Cdc42 activation is due to the TGase-catalyzed transamidation of Cdc42 in A1A1v cells (Figure 2.3C).

Next, we determined whether Cdc42 is activated and transamidated after DOI treatment in primary cortical neurons. A significant reduction in TGase-modified Cdc42 by cystamine pre-treatment and TG2 siRNA transfection is shown in Figure 2.3D suggesting 5-HT2A receptor-mediated Cdc42 transamidation is also dependent on the activity of TGases in primary culture. Compared with DOI-stimulated mock-transfected cells, cystamine treatment caused an 84% decrease and siRNA transfection caused a 34% decrease in TGase-modified Cdc42 following DOI. As shown in Figure 2.3E, DOI treatment increases the ratio of GTP-bound Cdc42 to the total Cdc42. Pretreatment with 1mM cystamine decreased active Cdc42 by 75% and TGase2 siRNA transfection decreased active Cdc42 by 54% compared with DOI-stimulated mock-transfected cells. These results suggest that DOI-stimulated, TGase-catalyzed Cdc42 transamidation leads to Cdc42 activation in rat primary cortical neurons.

RhoA is not transamidated following DOI treatment in primary cortical neurons

We also tested whether RhoA is modified by TGases following DOI treatment. We were not able to detect TGase-catalyzed modification of RhoA in rat primary cortical cells (Figure 2.4). These results suggest that stimulation of 5HT2A/2C receptors does not induce significant levels of TGase-catalyzed RhoA transamidation in rat primary cortical cells.

TGase-catalyzed transamidation of Rac1 occurs at Q61 in neuronal cells

Rac1 bears five glutamine residues (Q2, Q61, Q74, Q141, and Q162) that could serve as substrates for TGase-catalyzed transamidation (Dai *et al*, 2008). Using the NCBI Conserved Domain Database, we searched for GTP/Mg2, GAP, GEF, and GDI binding sites in the Rac1 sequence, and identified two glutamine residues, Q61 and Q74 within these functional domains. To test which glutamine residue or residues are modified by TGases, we generated two plasmid constructs containing double mutations, one with a Q61/74N and the other with Q141/162N. We transfected wild type (WT) Rac1 and Rac1 mutants into A1A1v cells, and 48 hours later stimulated 5-HT2A receptors with DOI treatment. As shown in Figure 2.5A, DOI significantly increases TGase-modified WT Rac1. Transamidation of Q61/74N Rac1 was not increased following DOI treatment compared with vehicle-treated control. DOI-induced transamidation of Q61/74N Rac1 is significantly lower than DOI-induced Rac1 transamidation in both WT Rac1 and Q141/161N Rac1 transfected cells. This result suggests that 5-HT2A-induced TGase-catalyzed modification occurs at Q61 or/and Q74 of Rac1. To determine whether Q61 or Q74 or both are modified by TGase, we generated another two Rac1 mutants bearing a single mutation at Q61 or Q74, and transfected them into A1A1v cells. Forty eight hours after transfection, cells were treated with DOI for 15min. As shown in Figure 2.5B, DOI

stimulation significantly increased TGase-modified Q74N Rac1 and WT Rac1, but not Q61N Rac1, suggesting that Q61 is the site that is transamidated following stimulation of 5-HT2A receptors.

Muscarinic receptor stimulation in SH-SY5Y cells causes TGase-dependent Rac1 transamidation and activation

As previously reported, 5-HT2A receptor-induced PLC activation is necessary for TGase-catalyzed Rac1 transamidation, and an increase in intracellular Ca2+ is sufficient to cause Rac1 transamidation in A1A1 cells (Dai *et al*, 2011). Therefore, we hypothesized that other Gαq/11 linked-receptors that activate PLC and increase in intracellular Ca2+ also result in Rac1 transamidation. To test this hypothesis, we used a human neuroblastoma cell line SH-SY5Y cells, which express M1, M2 and M3 receptors (Kukkonen *et al*, 1992). In this cell line, 2-[(Aminocarbonyl) oxy]-N, N, N-trimethylethanaminium chloride (carbachol) treatment can activate muscarinic receptors and induce PLC activation and subsequently an increase in cytosolic Ca2+ (Wojcikiewicz *et al*, 1994). We first treated SH-SY5Y cells with vehicle or 1mM carbachol for 5min and 10min, and examined Rac1 activation as described above. As shown in Figure 2.6A, carbachol increases Rac1 activity significantly at 10min but not at 5min. To further test whether carbachol-increased Rac1 activity is due to transamidation, we pretreated SH-SY5Y cells with 1mM cystamine for 1 hour to inhibit TGase and then stimulated muscarinic receptors with carbachol for 10min. As shown in Figure 2.6B, carbachol increased Rac1 transamidation by 150% in vehicle-pretreated cells. Cystamine reduced the carbachol-stimulated Rac1 transamidation by 78% compared to vehicle-pretreated cells. As shown in Figure 2.6C, cystamine also decreased carbachol-induced Rac1

activity by 78% compared to vehicle-pretreated controls. These results suggest that other GPCRs that couple to Gαq/11 are able to increase TGase-catalyzed Rac1 transamidation and activation.

DOI-induced dendritic spine enlargement is dependent on TGase activity

Previous ultrastructural studies have shown that 5-HT2A receptors localize to dendrites, dendritic shafts, and dendritic spines of cortical and hippocampal neurons and co-localize with synaptic proteins in rat and in primate brains (Cornea-Hebert *et al*, 2002; Jones *et al*, 2009). Small G proteins of the Rho family are major regulators of actin cytoskeleton and dendritic spine morphology. In our cultured rat primary neurons, endogenous 5-HT2A receptors localize to the soma, dendritic shafts and dendritic spines, and co-localized with postsynaptic marker PSD95 (Figure 2.7A-C). To test whether stimulation of 5-HT2A receptors influences dendritic morphology, we performed a time course study in which DIV21 primary neurons were treated with 3 μM DOI for a maximum of 60 min. The actin cytoskeleton was labeled by Alexa Fluor® 568 Phalloidin (Life technologies) to visualize dendritic spines and immunofluorescent labeling was used to select spines that contain 5-HT2A receptors. The area of dendritic spine increased by DOI treatment at 30 min, and recovered to vehicle-treated control levels at 60min (Figure 2.7D). We hypothesized that TGase-catalyzed transamidation of small G proteins including Rac1 and Cdc42 may mediate the effect of DOI on dendritic spine morphology. To test this hypothesis, we inhibited transglutaminase by pretreatment of primary cortical neurons with 1mM cystamine for 1 hour, prior to DOI treatment for 30min. DOI-induced spine enlargement is prevented by cystamine pretreatment (Figure 2.7E), suggesting the 5-HT2A and/or 5-HT2C receptor stimulation induced increases in

spine area is dependent on TGase activity, likely transamidation of small G proteins Rac1 and Cdc42. Neither DOI nor cystamine had effects on dendritic spine density (Figure 2.7F).

DISCUSSION

We previously reported that the stimulation of 5-HT2A receptors induced a TGase-catalyzed Rac1 transamidation and activation, both of which can be blocked by inhibiting TGase (Dai *et al*, 2008). However, as a novel effector and second messenger of 5-HT2A receptor signaling pathway, the functional impact of Rac1 transamidation in neuronal cells was unknown. Small G proteins of Rho family are well known regulators of the actin cytoskeleton, neurite outgrowth and dendritic spine formation, morphogenesis and plasticity (Ba *et al*, 2013; Martino *et al*, 2013). Specifically, activation of Rac1 and Cdc42 leads to spine formation and enlargement. Stimulation of 5-HT2A receptors produces a transient increase in dendritic spine size that is dependent on Pak1 activation, a downstream effect of Rac1 and Cdc42 activation(Jones *et al*, 2009). Therefore, we hypothesized that the transient spine enlargement caused by stimulation of 5-HT2A receptors is dependent on TGase-catalyzed transamidation of Rac1 and Cdc42. The lack of dendritic spines in A1A1v cells limits their use for further study on the roles of small G proteins in the regulation of spines. Therefore, a rat primary cortical culture was used to allow for the visualizing the effects of 5-HT2A receptor stimulated transamidation on dendritic spines. Rat primary cortical neurons were obtained from E18 rat pups and cultured to DIV21. At this time, neurons express 5-HT2A receptors, 5-HT2C receptors, TGase1, TGase2 and small G proteins Rac1, Cdc42 and RhoA. Moreover, at

DIV21, dendritic spines of the primary neurons display a relatively mature morphology and form connections with presynaptic partners.

In present study, we first discovered that stimulation of 5-HT2A receptors causes TGase-mediated transamidation and activation of small G proteins Rac1 and Cdc42 in primary culture as well as A1A1v cells. We used two approaches to suppress TGase activity in primary culture, the competitive TGase inhibitor cystamine and TGase2 siRNA. Compared to cystamine, TGase2 siRNA produced less reduction in DOI-induced Rac1 and Cdc42 transamidation and activity. As a potential substrate for TGase, cystamine acts as a competitive inhibitor for all TGase subtypes, while the siRNA only targets TGase2. Forty-eight hours post-transfection with siRNA resulted in a 95% reduction of TGases2 protein level in A1A1v cells (Dai *et al*, 2008), however, only achieved a 45% reduction of TGase2 in rat primary cortical culture. We found that the TGase2 siRNA also increased TGase1 protein expression, which may be a compensatory effect of TGase2 reduction. TGase 2 is ubiquitously expressed in neuronal tissues, in contrast to TGase1 and 3 which are differentially expressed in various brain regions (Kim *et al*, 1999; Wilhelmus *et al*, 2009; Zainelli *et al*, 2005). TGase3 was not detectable in the primary neuronal cultures by Western blots. Both the limited reduction in TGase2 protein and increase in TGase1 protein may have contributed to the lower decrease in DOI-induced Rac1 and Cdc42 transamidation and activity with the TGases siRNA approach compared to the treatment with the pan-transglutaminase inhibitor cystamine.

Small G proteins of the Rho family are monomeric globular proteins functioning as molecular switches by cycling between GDP-bound inactive forms and GTP-bound active forms (Wennerberg and Der, 2004). Their activity is controlled by three groups of

regulatory proteins: GEFs, which stimulate dissociation of the GDP from Rho GTPase; GAPs, which accelerate the intrinsically low GTP hydrolysis rate; and GDIs, which prevent the dissociation of GDP and interaction with GEFs and GAPs (Tolias *et al*, 2011). The formation of the GTP-bound structural form is confined to a conformational change in two regions, referred to as switch I and II, which provide interacting platforms for regulators and downstream effectors, thus initiating a network of signaling cascades (Dvorsky and Ahmadian, 2004). Post-translational modifications of Rho GTPases at those regions are most likely to affect the activity of small G proteins. Among five glutamine residues in the Rac1 amino acid sequence, two of them (Q61, Q74) are located within activity-related domains and can serve as potential targets of TGase-catalyzed modification (Dai *et al*, 2008). In present study, we found that the Rac1 Q61N mutant cannot be transamidated following 5-HT2A/2C receptor stimulation and is constitutively active. Previous studies found that transamidation or deamidation of Rac1 and Cdc42 at Q61 and RhoA at Q63 could inhibit both intrinsic and GAP-catalyzed hydrolysis of those GTPases, thereby rendering them constitutively active (Flatau *et al*, 2000; Lerm *et al*, 1999; Schmidt *et al*, 1999). Together, these results suggest that transamidation of Rac1 at Q61 inhibits GTP hydrolysis of Rac1, thereby inhibiting inactivation of Rac1.

In this study, we found differences in the time course for Rac1 and Cdc42 transamidation compared to the increase in activity. As shown in Figure 1 and 3, transamidation of Cdc42 and Rac1 last longer than their activation. This discordant time course for transamidation and activation may result from that transamidation of glutamine residues other than Q61 which are not involved in the regulation of Rac1 and Cdc42 activity. Moreover, we hypothesize that transamidation of Rac1 and Cdc42

increases their activity by inhibiting inactivation via GAP-catalyzed hydrolysis. The inhibitory effects of transamidation may be overcome by increases in GAPs. Prolonged transamidation may also affect the function of GEFs, thereby counteracting the inhibition of GAPs.

Several studies indicate that stimulation of Gαq/11-protein-coupled receptors, such as bradykinin or endothelin-1 receptor, can cause small G protein activation (Clerk *et al*, 2001; van Leeuwen *et al*, 1999). However, the underlying mechanisms still remain unclear. Our previous study indicated that 5-HT2A receptor-coupled PLC activation and the subsequent increase in intracellular Ca2+ are necessary for TGase-catalyzed Rac1 transamidation and activation. Furthermore, an increase in intracellular Ca2+ is sufficient to cause Rac1 transamidation and activation in A1A1 cells, suggesting that receptor systems that increase in intracellular calcium may activate TGs to induce monoaminylation (Dai *et al*, 2011). In present study, we extended these findings to 5-HT2C receptors and another family of Gαq/11-protein-coupled receptors, muscarinic acetylcholine receptors. We found that stimulation of muscarinic receptors with carbachol increased the transamidation and activity of Rac1, both of which were suppressed by the TGase inhibitor cystamine. The muscarinic receptor family contains five subtypes, M1-M5. M1, M3 and M5 receptors are coupled to Gαq proteins, while M2 and M4 receptors are coupled to Gi/o proteins. SH-SY5Y cells predominantly express M3 muscarinic receptor subtype (74% of total), however, they also express significant amounts of M1 and M2 receptors (Kukkonen *et al*, 1992). In smooth muscle, M2 receptors are coupled via Gβγ3 to PLCβ3, activation of which leads to an increase in Cdc42 and Rac1 activity (Murthy *et al*, 2003). Therefore, the carbachol-induced Rac1

activation could be mediated by M1 and M3 receptor-induced TGase-mediated transamidation of Rac1 and Cdc42 and M2 activation of Rac1 and Cdc42 via Gβγ3/PLCβ3. However, the transglutaminase inhibitor completely blocked carbachol-stimulated Rac1 activation suggesting the role of M2 receptors in Rac1 activation is minimal in our primary neuronal cell culture model. Cholinergic neurons in the central nervous system are implicated in synaptic plasticity and cognition. Knockout of muscarinic receptors leads to disruption of cognition, learning, and memory (Wess, 2004). Muscarinic M1 receptors are located on dendritic shaft and dendritic spines of cortical pyramidal cells (Yamasaki *et al*, 2010). Brief activation of muscarinic receptors induces a rapid formation of fine filopodia from spine heads in hippocampal pyramidal neurons (Schätzle *et al*, 2011). Our results suggest that monoaminylation of Rac1 and Cdc42 may provide a novel mechanism underlying the regulation of dendritic spines by muscarinic receptors.

We found that cystamine is able to inhibit the DOI-induced transient spine enlargement, suggesting that TGase-catalyzed Rac1 and/or Cdc42 transamidation plays a vital role in mediating the regulation of dendritic spines via 5-HT2A/2C receptor signaling pathway. However, whether the transamidation of Rac1 and Cdc42 both play a role still need to be studied in the future. Mutation at the transamidation site of Rac1 leads to its constitutive activation, consequently, simply transfecting the mutant into primary neurons would not provide an answer to the question. In addition, even though 5-HT2A receptors are the most abundantly expressed subtype of serotonin receptors in neocortical neurons, DOI is also able to activate 5-HT2C receptors. 5-HT2C receptors are expressed in rat cortical primary cultures (Tohda, 2014; Tohda *et al*, 2009). We found

that DOI-induced Rac1 transamidation could be partially reduced by inhibition of 5-HT2A receptor signaling by MDL100907 or by blocking 5-HT2C receptor signaling cascade by SB242084, suggesting both 5-HT2A and 5-HT2C play roles in the TGase-catalyzed Rac1 transamidation. Therefore, 5-HT2C receptors may also contribute to the DOI-induced spine enlargement.

Numerous studies have shown that Gαq/11 coupled receptors, such as the 5-HT2A receptor, metabotropic glutamate receptor 5 and muscarinic receptors, as well as PLC, Ca2+ and calmodulin are upstream regulators of synaptogenesis and dendritic spines (Horne and Dell'Acqua, 2007; Spires *et al*, 2005; Wijetunge *et al*, 2008). However, little was known about the signaling molecules coordinating these mechanisms. Here we report that 5-HT2A/2C receptor signaling pathway regulates dendritic spine morphology in rat cortical neurons via TGase-mediated mechanism, likely monoaminylation of small G proteins. This study provides a link between Gαq/11 coupled receptors and the regulation of dendritic spines, thus providing further insight into neuropsychiatric disorders in which those receptors and dendritic spines are involved.

FIGURES AND FIGURE LEGENDS

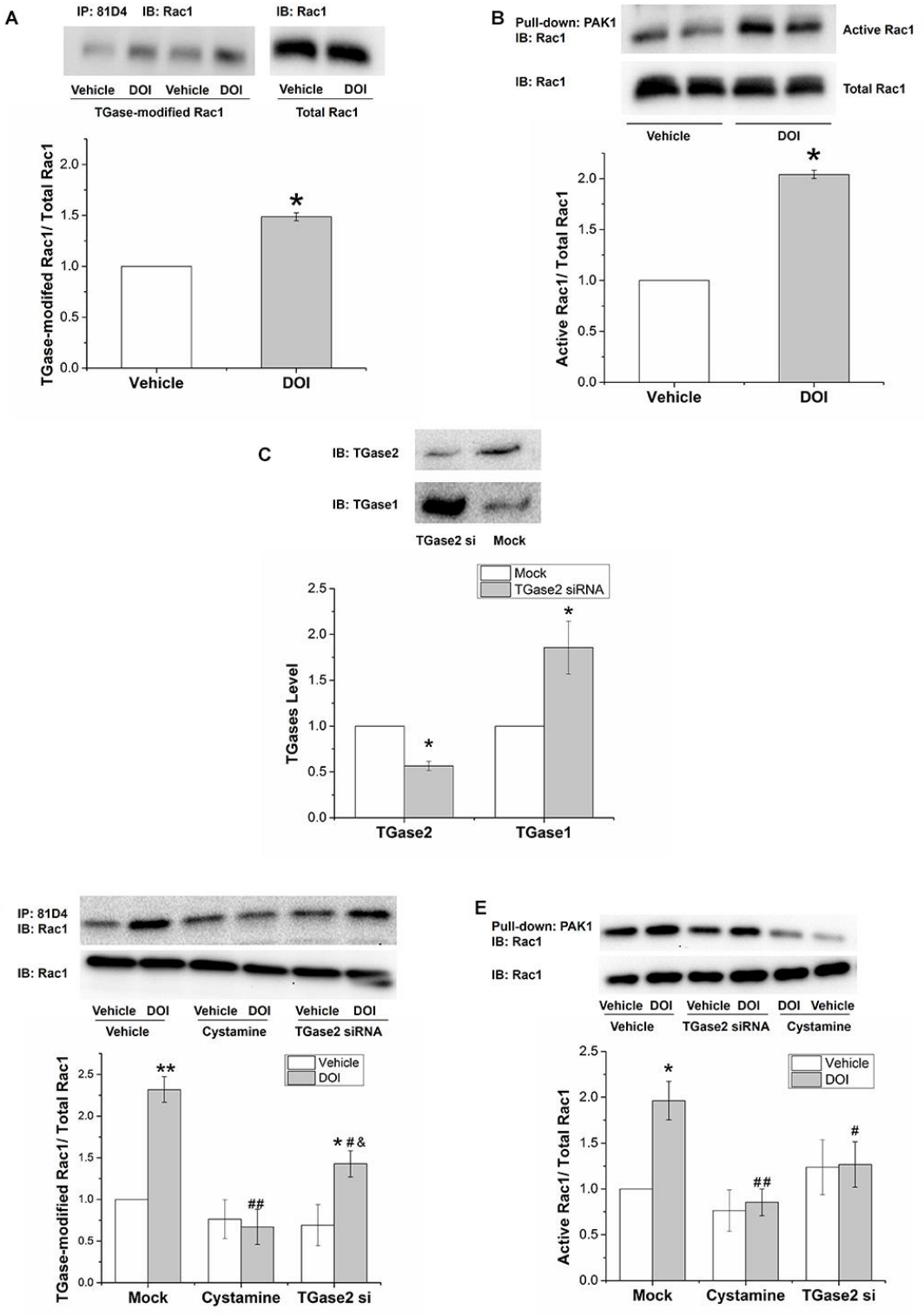
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Figure 2. 1

Figure 2.1 5-HT2 receptor-stimulated Rac1 activation is dependent on TGase-catalyzed transamidation in rat primary cortical neurons in culture.

A: DOI increased Rac1 transamidation in primary cortical neurons. Primary cortical neurons were treated with 3μM DOI treatment for 15 min, the TGase-modified proteins were immunoprecipitated with 81D4 antibody bound Sepharose beads and detected on immunoblots. Total amount of Rac1 in cell lysates was also detected on western blots using an anti-Rac1 antibody. Data are quantified by calculating the IOD of each protein band and the IOD ratio between TGase-modified Rac1 and total Rac1 was normalized to vehicle-treated control levels. Rac1 transamidation was significantly increased in DOI treated neurons. Student's t-test indicates * $p<0.001$, n=3.

B: DOI increased of the activity of Rac1 in primary cortical neurons. After 5 min of 3μM DOI treatment, active Rac1 was pulled down using GST-PAK1 coupled to glutathione-Sepharose beads and measured on western blots. IOD ratio between active Rac1 and total Rac1 was normalized to vehicle-treated control levels. DOI significantly increased activated Rac1 in DOI treated neurons. Student's t-test indicates * $p\leq0.001$, n=3.

C. TGase2 protein levels were significantly reduced 72 hours after transfection of primary neurons with 90 nM TGase2-specific siRNA (Student's t-test indicates * $p=0.001$, n=3). The reduction in TGase2 is accompanied by an increase in TGase1 protein levels. Student's t-test indicates * $p<0.05$, n=3.

D. Transamidation of Rac1 is reduced by inhibition of TGase activity by cystamine or knockdown of TGase2 by siRNA. Cells were incubated with TGase2-specific siRNA for 72 h or with 1mM cystamine for 1 h, and then they were treated with 3μM DOI or vehicle for 15 min, and TGase-modified Rac1 was detected on immunoblots. Two-way

ANOVA analysis of three separate experiments shows significant effects of transfection or pretreatment [$F(2,12)=13.011$, $p<0.001$], significant effect of DOI stimulation [$F(1,12)=18.343$, $p=0.001$], and a significant interaction between transfection or cystamine with DOI treatment on TGase-modified Rac1 [$F(2,12)=7.198$, $p<0.01$]., Newman-Keuls multiple comparison test indicates ** $p<0.001$, n=3 compared with vehicle treatment in mock-transfected cells; * $p< 0.05$, n=3 compared with vehicle treatment in TGase2 siRNA transfected cells; # $p< 0.01$, ## $p<0.001$, n=3 compared with DOI treatment in mock-transfected cells; & $p<0.05$, n=3 compared with DOI treatment in cystamine-pretreated cells.

E. Rac1 activation is abolished by inhibition of TGase activity by cystamine or knockdown of TGase2 by siRNA. Two-way ANOVA analysis of three separate experiments shows significant effects of transfection or cystamine pretreatment on Rac1 activation [$F(2,12)=5.223$, $p<0.05$]. Newman-Keuls multiple comparison test indicates * $p<0.05$, n=3 compared with vehicle treatment in mock-transfected cells; # $p< 0.05$, ## $p<0.01$, n=3 compared with DOI treatment in mock-transfected cells.

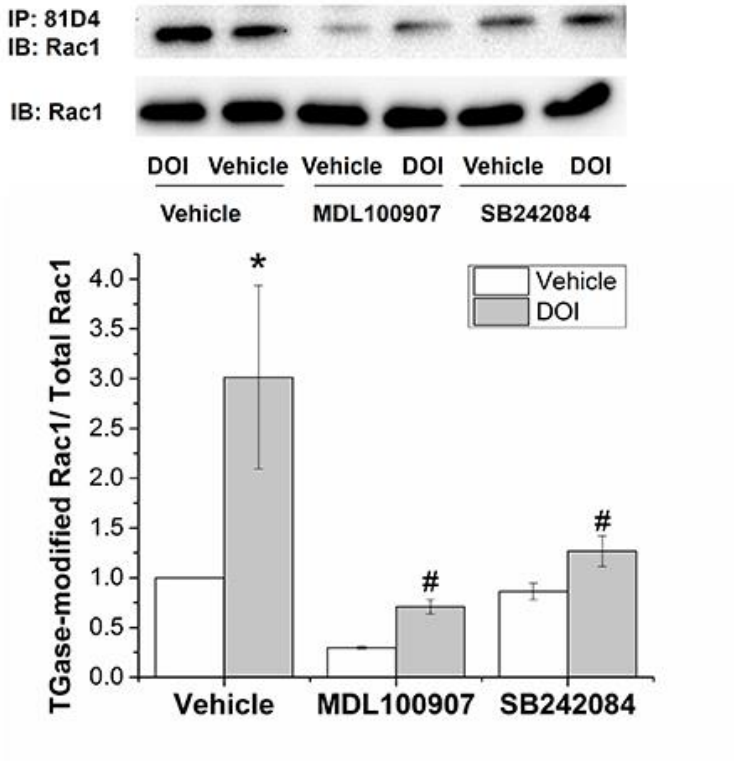


Figure 2. 2

Figure 2.2 DOI-induced Rac1 transamidation is mediated by both 5-HT2A and 5-HT2C receptors in rat primary cortical neurons. Cells were treated with MDL 100907, SB24084 or vehicle followed by DOI stimulation. TGase-modified Rac1 was detected on immunoblots. Two-way ANOVA analysis of three separate experiments shows significant effects of pretreatment [$F(2,12)=7.827$, $p<0.01$], significant effect of DOI stimulation [$F(1,12)=9.051$, $p<0.05$], Newman-Keuls multiple comparison test indicates * $p<0.01$, $n=3$ compared with vehicle treatment in vehicle-pretreated cells; # $p< 0.01$, $n=3$ compared with DOI treatment in vehicle-pretreated cells.

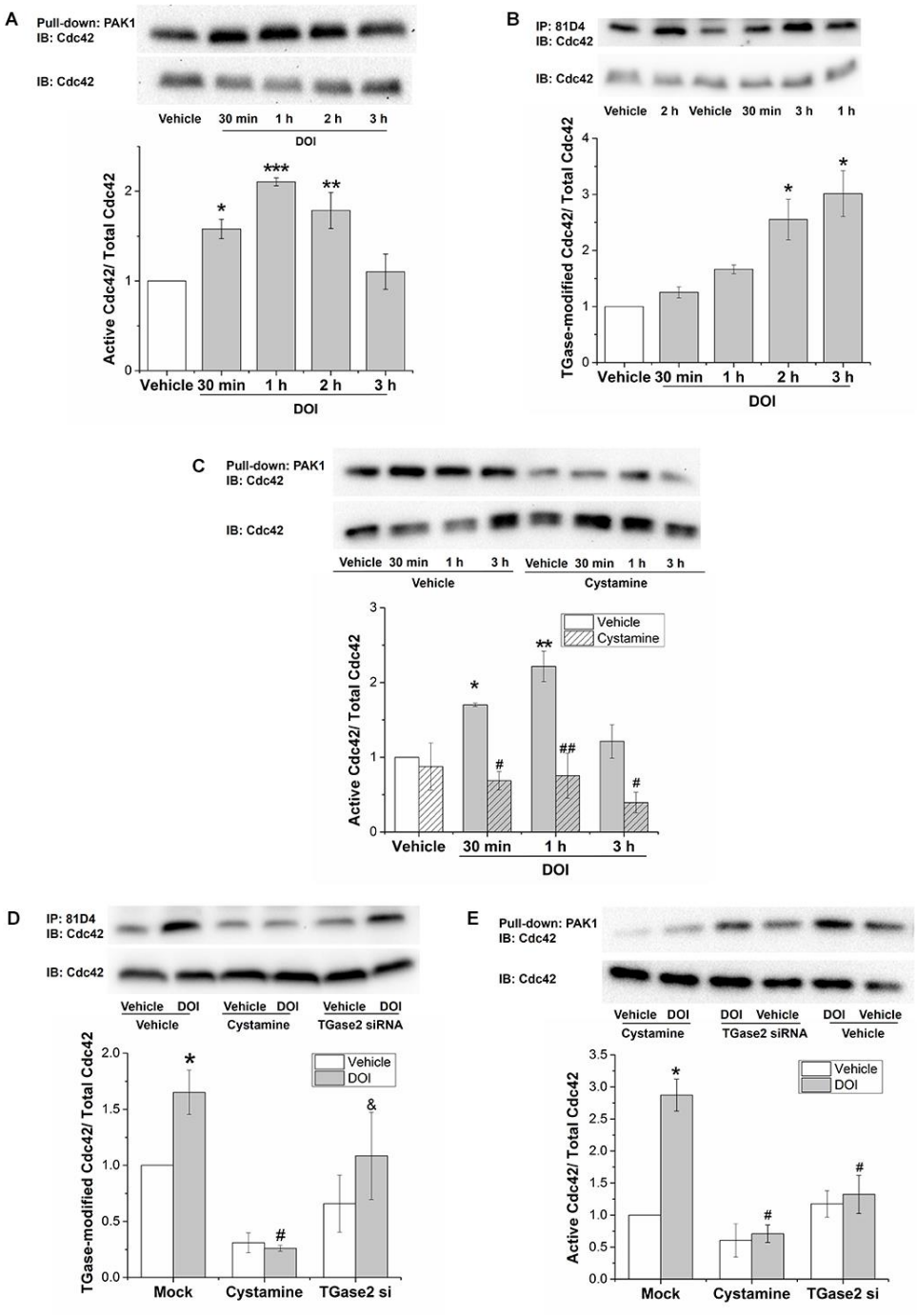


Figure 2. 3

Figure 2.3 Stimulation of 5-HT2 receptors increases activation of Cdc42 in a TGase-dependent manner in A1A1v cells and rat primary cortical neurons.

A. DOI increased the activity of Cdc42 in A1A1v cells. Cdc42 activity was transiently increased at 30min, 1hr and 2hr after DOI treatment and was reduced back to control levels at 3hr after DOI treatment. One-way ANOVA indicates a significant difference among groups [$F(4,10)=11.631$, $p<0.001$]. Newman-Keuls multiple comparison test indicates * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, n=3.

B. DOI increased transamidation of Cdc42 in A1A1v cells. One-way ANOVA indicates a significant difference among groups [$F(4,10)=11.828$, $p<0.001$]. Newman-Keuls multiple comparison test indicates * $p<0.01$, n=3.

C. Inhibition of TGase activity by cystamine prevents Cdc42 activation in A1A1v cells. Two-way ANOVA analysis of three separate experiments shows a significant effect of cystamine pretreatment [$F(1,16)=37.072$, $p<0.001$], a significant effect of DOI stimulation [$F(3,16)=4.595$, $p<0.05$], and a significant interaction between cystamine pretreatment with DOI treatment on Cdc42 activity [$F(3,16)=3.924$, $p<0.05$]. Newman-Keuls multiple comparison test indicates *, $p< 0.05$, **, $p<0.01$, n=3 compared with non-stimulated in vehicle pretreated cells; #, $p\leq 0.01$, ##, $p<0.001$, n=3 compared with vehicle-pretreated control cells stimulated with DOI for the same length of time.

D. Inhibition of TGase activity by cystamine or knockdown of TGase2 by siRNA prevents Cdc42 transamidation in rat primary cells. Two-way ANOVA analysis of three separate experiments shows significant effects of transfection or cystamine pretreatment [$F(2,12)=12.374$, $p<0.001$]. Newman-Keuls multiple comparison test indicates * $p<0.05$, n=3 compared with vehicle treatment in mock-transfected cells; # $p< 0.01$, n=3 compared with DOI treatment in mock-transfected cells; & $p<0.05$, n=3 compared with DOI treatment in cystamine-pretreated cells.

E. Inhibition of TGase activity by cystamine or knockdown of TGase2 by siRNA prevents Cdc42 activation. Two-way ANOVA analysis shows significant effects of transfection or cystamine pretreatment [$F(2,12)=17.503$, $p<0.001$], significant effect of DOI stimulation [$F(1,12)=16.065$, $p=0.002$], and a significant interaction between transfection or cystamine with DOI treatment on Cdc42 activation [$F(2,12)=10.895$, $p=0.002$]. Newman-Keuls multiple comparison test indicates * $p<0.001$, n=3 compared with vehicle treatment in mock-transfected cells; # $p<0.01$, n=3 compared with DOI treatment in mock-transfected cells.

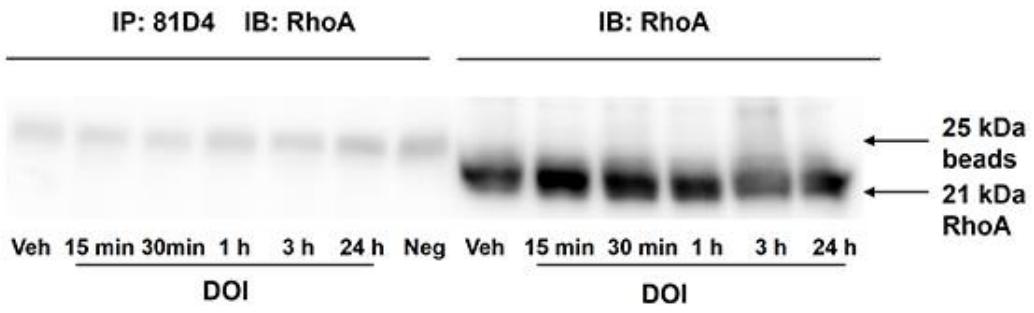
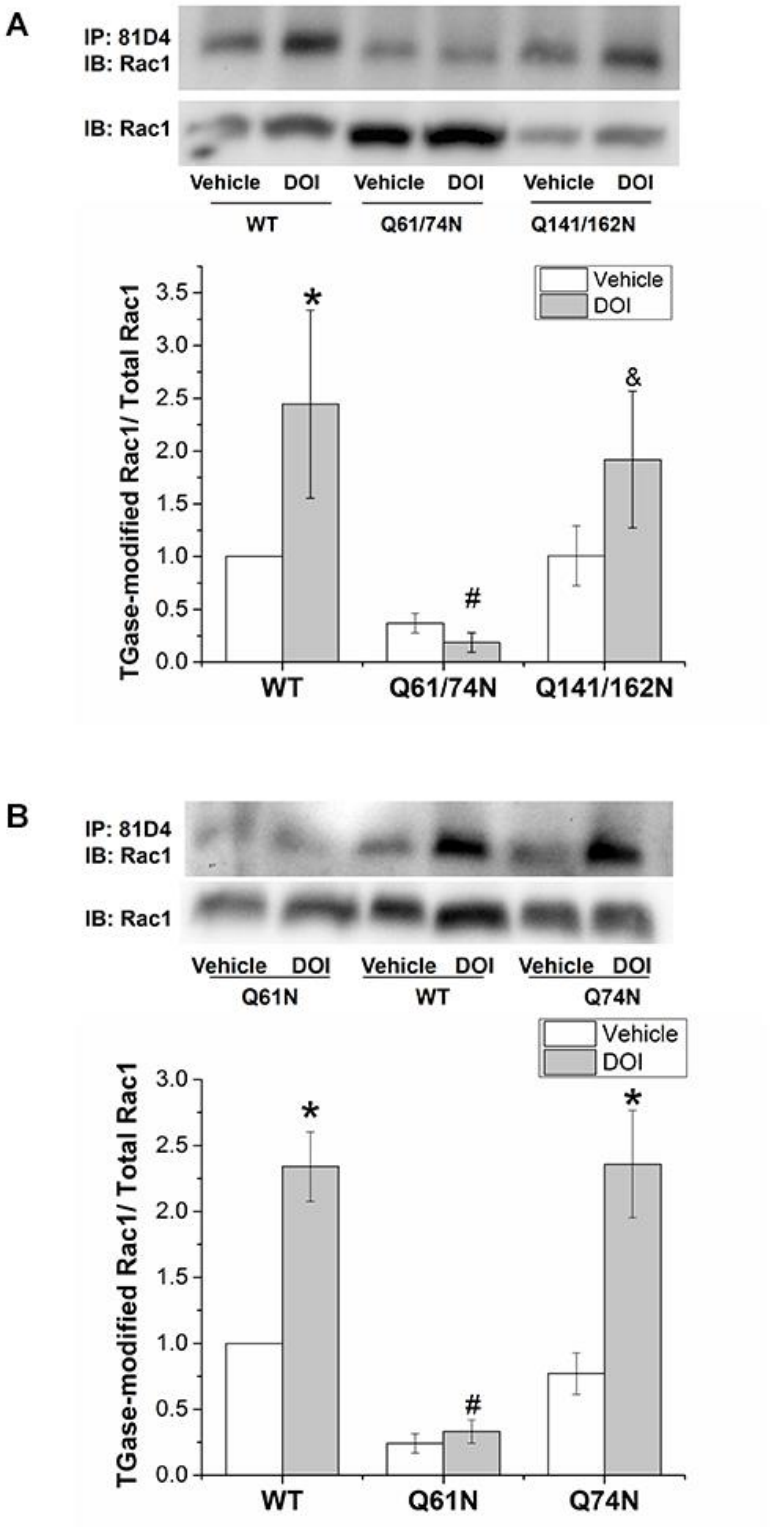


Figure 2. 4

Figure 2.4 RhoA is not transamidated following DOI treatment in primary cortical neurons. Primary cortical neurons were treated with 3μM DOI treatment for 15 min, 30 min, 1 hour, 3 hour and 24 hour. Then the TGase-modified proteins were immunoprecipitated with 81D4 antibody bound Sepharose beads and detected on immunoblots using anti-RhoA antibody. Total amount of RhoA in cell lysates was also detected on western blots using an anti-RhoA antibody. "Neg" indicates indicates 81D4 beads were only incubated with IP buffer but not protein lysate.


A
IP: 81D4
IB: Rac1
IB: Rac1
Vehicle DOI Vehicle DOI Vehicle DOI
WT
Q61/74N
Q141/162N
TGase-modified Rac1/ Total Rac1
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Q141/162N
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IP: 81D4
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Q61N
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TGase-modified Rac1/ Total Rac1
Vehicle
DOI
*
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WT
Q61N
Q74N

Figure 2. 5

Figure 2.5 TGase-catalyzed transamidation of Rac1 occurs at Q61 in neuronal cells.

A. DOI-stimulated transamidation was prevented in Q61/74N Rac1 mutants. A1A1v cells were transfected with Rac1 constructs containing amino acid substitutions for glutamine 61& 74, 141&162 and wild type (WT) Rac1 and 48 hours later, stimulated with DOI for 15min. Two-way ANOVA analysis shows a significant effects of transfection [$F(2, 12)= 5.442$, $p<0.05$] on Rac1 transamidation. Newman-Keuls multiple comparison test indicates * $p<0.05$ compared with vehicle treatment in WT Rac1-transfected cells; # $p<0.05$ compared with DOI treatment in WT Rac1-transfected cells, n=3; & $p<0.05$ compared with DOI treatment in Q61/74N Rac1-transfected cells.

B. DOI-stimulated transamidation was prevented in Q61N Rac1 mutants. Two-way ANOVA analysis of three separate experiments shows significant effects of transfection [$F(2,12)=26.132$, $p<0.001$], significant effects of DOI stimulation [$F(1,12)=33.442$, $p<0.001$], and a significant interaction between transfection with DOI treatment on TGase-modified Rac1 [$F(2,12)=7.107$, $p<0.01$]. Newman-Keuls post-hoc test indicates * $p<0.001$ compared with vehicle treatment in the same transfection groups; # $p< 0.001$ compared with DOI treatment in WT Rac1-transfected cells, n=3.

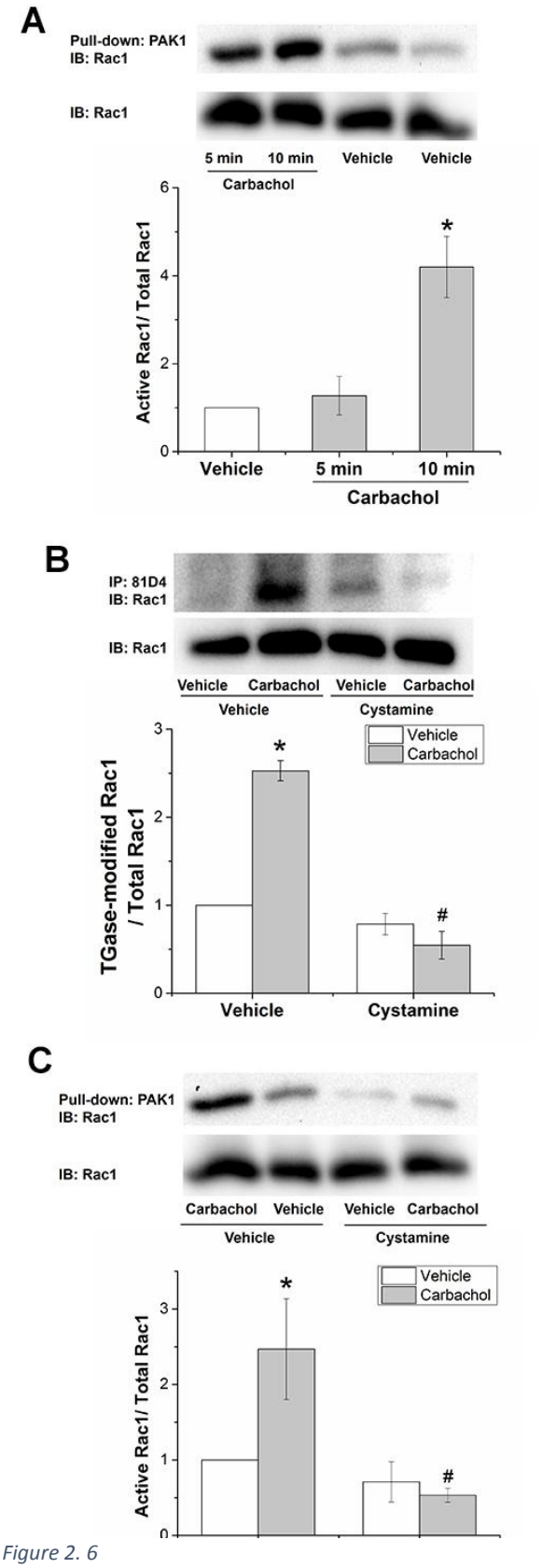

A
Pull-down: PAK1
IB: Rac1
IB: Rac1
5 min
10 min
Vehicle
Vehicle
Carbachol
Active Rac1/ Total Rac1
Vehicle
5 min
10 min
Carbachol
B
IP: 81D4
IB: Rac1
IB: Rac1
Vehicle
Carbachol
Vehicle
Carbachol
Vehicle
Cystamine
Vehicle
Carbachol
TGase-modified Rac1
/ Total Rac1
Vehicle
Cystamine
C
Pull-down: PAK1
IB: Rac1
IB: Rac1
Carbachol
Vehicle
Vehicle
Carbachol
Vehicle
Cystamine
Vehicle
Carbachol
Active Rac1/ Total Rac1

Figure 2. 6

Figure 2.6 Muscarinic receptor stimulation in SH-SY5Y cells causes TGase-dependent Rac1 transamidation and activation.

A. Carbachol treatment increased Rac1 activity in SH-SY5Y cells. SH-SY5Y cells were treated with vehicle or 1mM carbachol, a muscarinic receptor agonist, for 5min and 10min, and Rac1 activity was detected. One-way ANOVA shows a significant difference among groups [$F(2,6)=13.85$, $p<0.01$]. Newman-Keuls multiple comparison test indicates * $p<0.01$, n=3.

B. Carbachol-stimulated Rac1 transamidation is TGase dependent. Pretreatment with cystamine prevents carbachol-stimulated Rac1 transamidation. Two-way ANOVA analysis shows a significant effect of cystamine pretreatment, $F(1,8)=90.978$, $p<0.001$], significant effect of carbachol stimulation [$F(1,8)=31.28$, $p<0.001$], and a significant interaction between transfection or cystamine with carbachol treatment on Rac1 transamidation [$F(1,8)=59.003$, $p<0.001$]. Newman-Keuls multiple comparison test indicates * $p< 0.001$, n=3 compared with non-stimulated in vehicle pretreated cells; # $p<0.001$, n=3 compared with carbachol-stimulated controls in vehicle-pretreated cells.

C. Carbachol-stimulated Rac1 activation is TGase dependent. Cystamine pretreatment inhibited carbachol-stimulated activity of Rac1. Two-way ANOVA analysis shows a significant effect of cystamine pretreatment, [$F(1,8)=90.978$, $p<0.05$] on Rac1 activation. Newman-Keuls multiple comparison test indicates * $p< 0.05$, n=3 compared with non-stimulated in vehicle pretreated cells; # $p<0.01$, n=3 compared with carbachol-stimulated controls in vehicle-pretreated cells.

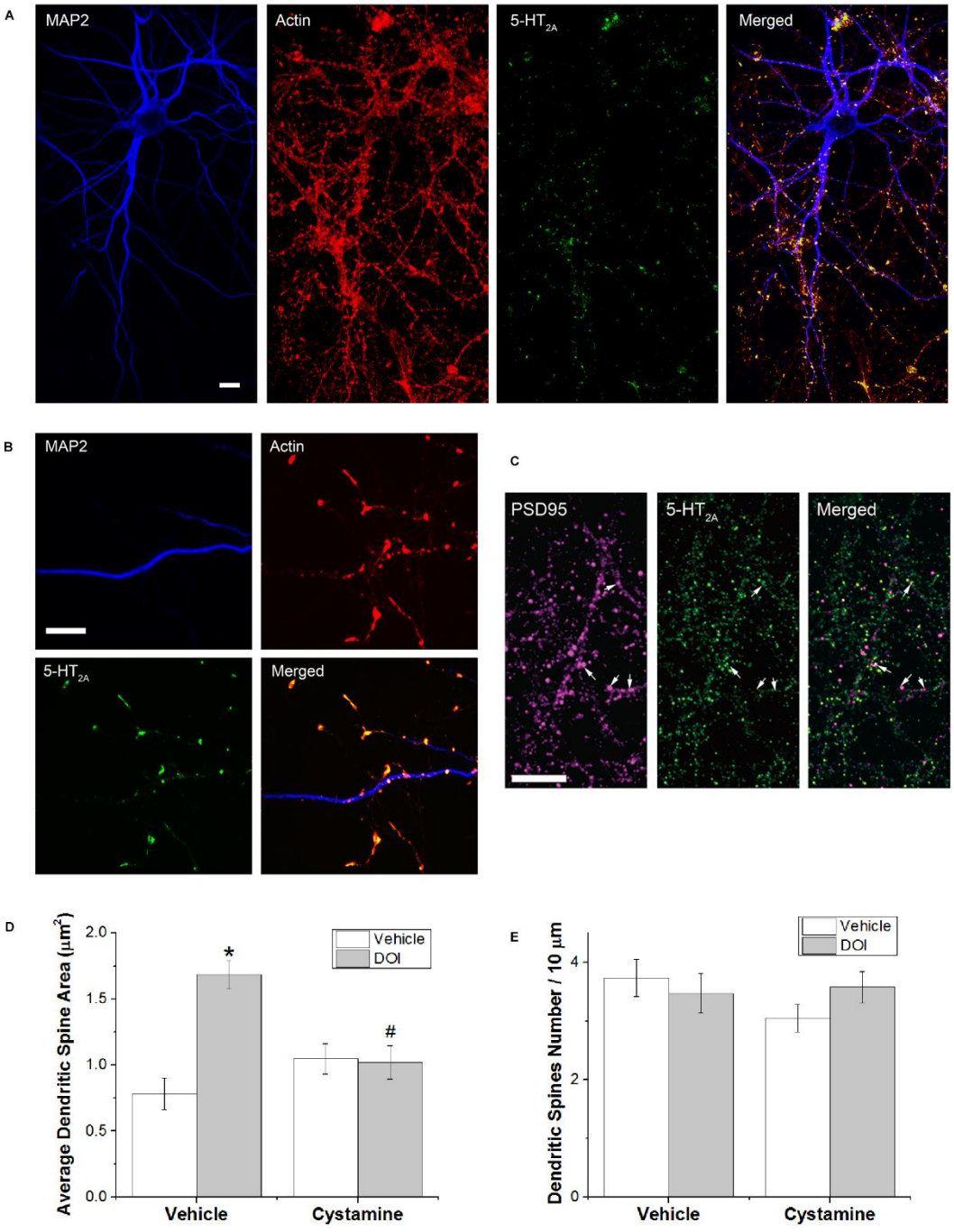


Figure 2. 7

Figure 2.7 DOI-induced dendritic spine enlargement is dependent on TGase activity.

A. 5-HT2A receptors (green) co-localize with actin (red) predominantly in the soma,

dendritic shafts and dendritic spines. Scale bar represents 10 μm.

B. Higher magnification of dendrites and dendritic spines. Scale bar represents 10 μm.

C. PSD-95 (magenta) colocalizes with 5-HT2A receptors (green) in the dendrites of cultured rat primary cortical neurons at DIV21 (arrowheads). Scale bar represents 10 μm.

D. DOI causes an increase in dendritic spine area after 30min treatment. Bars represent absolute value of dendritic spine area. Log transformation was performed on original data to achieved normality. One-way repeated measures ANOVA suggests a significant difference between five treatment groups [$F(4,34)= 9.342$, $p<0.001$]. Newman-Keuls multiple comparison test indicates * $p\leq 0.001$ compared with vehicle treatment group, and groups treated with DOI for 5min, 15min and 60min.

E. Cystamine inhibits the DOI-induced dendritic spine enlargement. Bars represent absolute value of dendritic spine area. Since log transformation does not enable the data to distribute normally, analysis was still performed on the original data using a non-parametric test. Kruskal-Wallis one-way ANOVA on Ranks indicates a significant difference between four treatment groups ($p<0.001$). Post hoc Dunn's test suggests * $p<0.05$ compared with vehicle-vehicle treatment; # $p<0.05$ compared with vehicle-DOI treatment.

F. DOI and cystamine have no significant effects on dendritic spine density. Bars represent absolute value of dendritic spine density. Log transformation was performed on original data to achieved normality. Two-way ANOVA test indicates that neither cystamine nor DOI has a significant effect on dendritic spine density.

CHAPTER THREE

OLANZAPINE TREATMENT ALTERS EXPRESSION OF GENES AND DENDRITIC SPINE MATURATION IN BOTH A JAK2-DEPENDENT AND INDEPENDENT PATHWAY

ABSTRACT

Recent studies show that atypical antipsychotics change the levels of genes that may play a role in the etiology and treatment of schizophrenia and mood disorders. We hypothesized that sub-chronic administration of olanzapine to rats would alter expression of various genes via Janus Kinase 2 (JAK2)/ signal transducer and activator of transcription 3 (STAT3) signaling pathway, thus exerting therapeutic or adverse effects. Sprague-Dawley rats were pretreated with the JAK2 inhibitor AG490 or vehicle, followed by administration with olanzapine or vehicle daily for seven days. Microarray analysis of prefrontal cortices showed that 205 genes were significantly changed by AG490, olanzapine or the combination of both drugs compared to the controls. These genes are involved in the etiology of schizophrenia, neuronal signal transduction, neuronal growth factor, metabolism and energy, and synaptic plasticity. 92 of the 205 genes are changed by olanzapine via JAK2 signaling pathway. Real-time qPCR verified that Crf, Cbln1, Nrg1, Hsp70, and Fabp7 were upregulated by olanzapine in a JAK2 dependent manner. Filip1, Homer1, Pak1ip1 and Arc were significantly changed by olanzapine, but not via the JAK2 pathway. Protein levels of neuregulin-1 (Nrg1) and heat shock protein 70 (Hsp70) were significantly increased by olanzapine via JAK2 pathway, while kalirin7 and spinophilin protein levels were changed by olanzapine independently of JAK2 signaling. Given the changes in numerous gene related to the cytoskeleton, post-

synaptic density and synaptic plasticity, dendritic spines in rat cortical primary culture were also examined. We found that treatment with olanzapine induced a maturation in dendritic spines. AG490 blocks olanzapine-induced changes in stubby spines and thin spines, but not the changes in dendritic filopodia and mushroom spines, suggesting the olanzapine-induced maturation of dendritic spines is only partially dependent on JAK2 pathway. Our results show for the first time that olanzapine causes changes in various important genes that may be involved in the etiology of schizophrenia and therapeutic effects of atypical antipsychotics in a JAK2 dependent manner.

INTRODUCTION

In the United States in 2014, drugs for the treatment of mental health rank second only to antihypertensives in the number of prescriptions dispensed and the atypical antipsychotic aripiprazole is ranked second drug in sales (Lindsley, 2012). Aripiprazole and other atypical antipsychotics are approved for the treatment of schizophrenia as well as for adjunctive therapy for bipolar disorder, major depressive disorder and autism disorders. Despite the extensive use of atypical antipsychotic drug therapy, new approaches for the treatment of schizophrenia are needed to combat the full range of disabling symptoms. The symptoms of schizophrenia cluster into positive symptoms (hallucinations and delusions), negative symptoms (social withdrawal and apathy), and cognitive deficits (impairments in attention, learning and memory, reasoning, and processing speed) (Andreasen, 1995). While positive symptoms can be ameliorated by atypical antipsychotics to various degrees, negative symptoms and cognitive deficits do not respond sufficiently to atypical antipsychotics and side effects, including metabolic

syndrome and weight gain, can limit the usefulness of atypical antipsychotics and patient compliance (Jones *et al*, 2011).

Atypical antipsychotic drugs have a broad binding profile with high affinity for both dopamine 2 receptors and serotonin 2A receptors thought to be important in therapeutic effects (Nasrallah, 2008). 5-HT2A receptor antagonism is the best known mechanism for the effects of atypical antipsychotics on the negative and cognitive symptoms (Marek *et al*, 2003). We previously reported that treatment with atypical antipsychotics olanzapine and clozapine activate the JAK2/STAT3 pathway in both A1A1v cells and in rat frontal cortex (Muma *et al*, 2007a; Singh *et al*, 2009a; Singh *et al*, 2007b). This effect is likely mediated by 5-HT2A receptors, because MDL-100,907 [(R)-(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperinemethanol], a selective 5-HT2A receptor antagonist, also activated the JAK2/STAT3 pathway (Singh *et al*, 2009a). Upon activation of the JAK2/STAT3 pathway, JAK2 becomes phosphorylated and dimerizes, then activates STAT3 via phosphorylation. STAT3 dimerizes and translocates to the nucleus, where it acts as a transcription factor. STAT3 is a transcription factor for RGS7 in A1A1v cells and increases expression of RGS7 mRNA and protein (Singh *et al*, 2009a). JAK2/STAT3 pathway has been shown to mediate desensitization of 5-HT2A receptors, probably via increasing RGS7 expression (Singh *et al*, 2009a). Interleukin-6 receptor stimulation also attenuates 5-HT2A receptor agonist-induced IP production by activating the JAK-STAT pathway (Donegan *et al*, 2015). Moreover, there is accumulating evidence that JAK-STAT pathway plays an important role in the regulation of neuronal functions in the central nervous system (CNS). For example, JAK/STAT regulates expression of a number of neurotransmitters, such as γ-

amino-butyric acid (GABA) (Lund *et al*, 2008), N-methyl-D-aspartate (NMDA) (Mahmoud and Grover, 2006), and muscarinic acetylcholine (Chiba *et al*, 2009). Inhibition of JAK/STAT pathway with JAK2 inhibitor AG490 in mice leads to impairment of spatial working memory (Chiba *et al*, 2009). JAK/STAT pathway also plays a key role in the synaptic plasticity. A recent study demonstrated that induction of NMDA-receptor dependent long-term depression (LTD) is dependent on the JAK/STAT pathway in the hippocampus (Nicolas *et al*, 2012).

Given the links of JAK/STAT pathway to atypical antipsychotics and neuronal functions, a better understanding of the 5-HT2A receptor/JAK2/STAT3 pathway could provide insight into the mechanisms of actin of atypical antipsychotics and shed light on novel medical interventions for schizophrenia. In the present study, we use microarray technology to screen for the expression of genes that are changed by chronic olanzapine treatment via the JAK/STAT pathway in the rat frontal cortex, and verified the mRNA and protein levels of select genes. We identified a number of schizophrenia susceptibility genes, as well as gene associated with regulation of cytoskeleton and synaptic function, which are changed by olanzapine or AG490 treatment. Changes in those genes may mediate the therapeutic effects of atypical antipsychotics. It is also worth noting that treatment with olanzapine induced the dendritic spine maturation in rat cortical primary culture via JAK/STAT pathway. Dendritic spine loss in forebrain regions is a consistent feature of schizophrenia (Glantz and Lewis, 2000; Konopaske *et al*, 2014; Penzes *et al*, 2011). Atypical antipsychotics may exert therapeutic effects on schizophrenia by reversing the dendritic spine pathologies in a JAK/STAT dependent manner.

METHODS

Reagents

The following drugs were used in this study: AG490 (Toronto Research Chemicals Inc., ON, Canada), olanzapine (Sandoz, Holzkirchen, Germany), 1-(2, 5-dimethoxy-4-iodophenyl)-2-aminopropane HCl (DOI) (Sigma-Aldrich, St. Louis, MO). MDL100907 was kindly provided by Sanofi Aventis (Bridgewater, NJ) and dissolved in DMSO. AG490 was reconstituted in 50% DMSO. DOI was dissolved in saline and further diluted before applied to cell cultures.

Animal

Twenty four male Sprague-Dawley rats (225-250 g) were obtained from Harlan Laboratories (Indianapolis, IN) and housed 2 per cage on a 12 h light/dark cycle (lights on 7:00 AM- 7:00 PM) in an environment controlled for temperature and humidity. All rats were provided with food and water ad libitum. Rats were randomly assigned to four experimental groups and given daily injections of vehicle (50% DMSO s.c.) or 10 mg/kg AG490 first (s.c.) for seven consecutive days. One hour later, these rats were injected with saline (s.c.) or 10mg/kg olanzapine. The AG490 and olanzapine doses were chosen based on previous results (Singh *et al*, 2009a). Rats were handled for several days before treatment to minimize stress and weighed every alternate day during the treatment period. 24 hours after the last injection, the rats were euthanized and whole brains were removed, frozen and stored at -80 ℃ for the following experiments. All procedures were performed in accordance with the National Institute for Health Guide for the Care and Use of

Laboratory Animals as approved by the University of Kansas Institutional Animal Care and Use Committee.

RNA Isolation

Total RNA was isolated from half of each frontal cortex preserved in RNA*later*® solution (Life Technologies, Carlsbad, CA), using the RNeasy Mini Kit (Qiagen Sciences, Valencia, CA) according to the manufacture's protocol. Total RNA was quantitated using a spectrophotometer and optical density ratios at 260/280 nm were determined.

Microarray Analysis

The Rat Genome GeneChip 230 2.0 Arrays were employed for the microarray study, which allowed us to probe the expression of over 30,000 transcripts and variants from the rat genome. To carry out the GeneChip analysis, the GeneChip 3' IVT Express Kit Labeling Assay was conducted at the University of Kansas Genomics Facility. In order to minimize experimental variability, standardized GeneChip operation was performed by an experienced investigator throughout the study. For each sample, 100 ng total RNA was used for cRNA target preparation, array hybridization, washing, staining and image scanning. The washing and staining steps were performed on a GeneChip Fluidics Station 450 and the scanning of hybridized arrays was conducted on a GeneChip Scanner 3000. After being generated from the Affymetrix GeneChip Operating Software (GCOS), the microarray data was first examined for quality, and the microarray data generated from all chips met quality control criteria set by Affymetrix.

Data Analysis. To facilitate direct comparison of gene expression data between different samples, the GeneChip data were first subjected to preprocessing. This step involved scaling (in GCOS) data from all chips to a target intensity value of 500, and further normalizations steps in GeneSpring GX11 (Agilent Technologies, Santa Clara, CA). Prior to identifying differentially expressed genes, genes that were detected as non-expressed in all samples, i.e., those with absence calls, were filtered out. To identify genes whose expression was changed by our compounds, a volcano-plot based approach was used. Based on this approach, in order to be called significantly altered, the expression value of a gene must pass two criteria, i.e. fold change ≥ 1.3 and $P \leq 0.05$. Multi-test correction was conducted using the Benjamini-Hochberg method. After the identification of differentially expressed genes, further bioinformatic data analyses were carried out at the levels of Gene Ontology (GO) and biological pathway using DAVID (http://david.abcc.ncifcrf.gov/).

Reverse transcription and quantitative real-time PCR (qPCR)

First strand cDNA was synthesized using Superscript III Reverse Transcriptase (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Real-time PCR amplification was performed using 7500 Real-Time PCR System using SYBR green PCR master mix (Life Technologies, Carlsbad, CA). Comparative Ct (ΔΔ Ct) was and used for analysis of real-time PCR data. Results were expressed as fold change in target mRNA levels for AG490-vehicle, vehicle-olanzapine, and AG490-olanzapine treated animals compared to vehicle-vehicle treated animals. The primers were synthesized by Life Technologies (Carlsbad, CA) as listed in Table 3.1.

Table 3.1 List of primers

Gene Name	Primer Sequence	Product Length	NCBI Reference Sequence
Arc	F:CCGTCCCCTCCTCTCTTGA R: AAGGCACCTCCTCTTTGTAATCCTAT	89	NM_019361.1
Reelin	F:GTACTCCCTGAACAACGGCA R:TCTGGAACCTTTCCGACGTG	116	NM_080394.2
Kalirin	F:TGGTATCTTTGGTATCTCCGCT R:ACGAGTTTCCGTAGGTCTTCCT	209	NM_032062.2
Hsp70	F:AACACGCTGGCTGAGAAAGAG R:TGATACAGCCCGCTGATGATC	86	NM_031971.2
Nr4a1	F:TGTCTGCTCTGGTCCTCATCAC R:CCACGGCGGCCATGT	111	NM_024388.2
Fabp7	F:GGACACAATGCACATTCAAGAAC R:CCGAATCACAGACTTACAGTTC	101	NM_030832.2
Pde10a	F:TGACCTGGACCACAGGGGC R:TGATGGCTTTGCGGATGATC	203	NM_022236.1
Nrg1	F:GGCAGTCAGCCCCTTTGTG R:TGCAGGGTTGTGATGAAAGGA	121	NM_001271130.1
Homer1	F:ACACTGTTTATGGACTGGGATTCTC R:TCCTGCGACTTCTCCTTTGC	109	NM_031707.1
Filip1	F:AACGGCTTACAGCTCAACTCG R:ACGGCTAGTTTACCCTGTGTTT	88	XM_008768653.1
Dcx	F:CTCCTATCTCTACACCCACAAGCC R:GAATCGCCAAGTGAATCAGAGTC	97	NM_053379.3
Cbln1	F:CACCGCGCCAGTTAAGCT R:GGTGAGTCTTTCGTCCGCTAA	62	NM_001109127.1

Pak1ip1	F:CGGCACCCTCACAAATGG R:TGCAAGGACAGAGTCTGAGAGAAA	64	NM_001037356.1
Nfasc	F:TGACCCAACCCCCAACGATCA R:CACTGCGGAAGTCGATCACC	207	NM_001160314.1
Spn	F:AAGGCCAAGCGTCTCATCAA R:TGGGCGGTCTCTTTTTTCAG	68	NM_053474.1
Cartpt	F:CTCAAGAGTAAACGCATTCC R:ACAAGCACTTCAAGAGGAAA	160	NM_017110.1
Nts	F:GTGATGACGACCTTGTTGCG R:AGCAGCGCTTCCAAGCTAAA	70	NM_001102381.1
Cdhr1	F:ATTCTCAGCCACCACGACTG R:GACGGAGATGGCTCCAGATG	238	NM_053572.1
Ntrk2	F:ACCAAACCAATCGGGAGCAT R:CCCCAGCCTTGTCTTTCCTT	192	NM_001163168.2
Crh	F:CTGATCCGCATGGGTGAAGA R:CAGCAACACGCGGAAAAAGT	156	NM_031019.1
Cbp	F:CGAGAACTTGCTGGACGGAC R:ATCTGTGCTGTCATTCGCGG	82	NM_133381.3

Table 3. 1

Tissue Preparation and Western Blot

Frontal cortex tissue was homogenized in ice-cold homogenization buffer, which contains 25 mM HEPES-Tris, pH 7.4, 1 mM EGTA, 1:1000 protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), by using a BulletBlender (Next Advance, Averill Park, NY). The homogenate was centrifuged at 14,000 rpm for 15 sec at 4℃. After centrifugation, the supernatant was collected as whole tissue homogenate. Protein concentration was determined using Pierce™ BCA Protein Assay Kit (Life Technologies,

Carlsbad, CA). Equal amounts of protein from different groups were separated on 12% SDS-polyacrylamide gels as described before (Dai *et al*, 2008). Non-specific binding was blocked in blotto (5% nonfat dry milk, 0.1% Tween20, 1X TBS buffer) for 1 hour. Primary and secondary antibodies were diluted in antibody buffer (2% nonfat dry milk, 0.1% Tween20, 1X TBS buffer). The following primary antibodies were used: anti-kalirin antibody, anti-JAK2 antibody, anti-phospho-Y1007-JAK2 antibody (Millipore Corporation, Billerica, MA), anti-spinophilin/neurabin 2 antibody, anti-neuregulin1 Type I antibody (Abcam, Cambridge, MA). Protein bands were detected using ChemiDoc™ XRS+ System (Bio-rad, Hercules, CA). The integrated optical density (IOD) of each protein band was quantified using Image Lab™ Software (Bio-rad, Hercules, CA).

Hsp70 ELISA Assay

Hsp70 protein levels were measured using rat Hsp70 ELISA Development Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, samples and standards were added to 96 well plates coated with capture antibody and incubated for 2 hours at room temperature. After 3 washes, detection antibody was added to each well followed by a 2 hour incubation. Wells were washed again and diluted Streptavidin-HRP was added to each well and incubated for 20 min at room temperature. Substrate solution was added followed by 20 min incubation, and then stop solution was added to each well. Optical density of each well was determined immediately, using a microplate reader (Bio-Tek, Winooski, VT) set to 450 nm.

Isolation of Rat Primary Cortical Neurons

Primary cortical neurons were isolated from Sprague-Dawley rat embryos at E18 as described previously with minor modifications (Beaudoin III et al, 2012; Srivastava et al, 2011). Pups were decapitated and cortical tissue was removed, followed by 0.01% trypsin (Sigma-Aldrich, St. Louis, MO) digestion for 20min. After gentle trituration, neurons were passed through a 40 μm cell strainer. Yield of viable cells were counted using a hemocytometer. Cells were plated at a density of 5 x 10^5 cells/ml on 22mm diameter round cover glass (Neuvitro Corporation, Vancouver, WA) or at a density of 2.7 x 10^6 cells/ml on T25 cell culture flasks (Fisher Scientific, Lenexa, KS) coated with Poly-L-lysine (Sigma-Aldrich, St. Louis, MO). Primary neurons were maintained in Neurobasal media (Life Technologies, Grand Island, NY) supplemented with 2% B27 (Life Technologies, Grand Island, NY), 0.5 nM glutamine (Sigma-Aldrich, St. Louis, MO) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO) at 37 ℃ in 5% CO_2. Half of the medium was changed twice a week until 21 days in vitro.

Immunocytochemistry

Primary neurons were labeled with Alexa Fluor® 568 Phalloidin (Life Technologies, Grand Island, NY), antibodies against 5-HT2A receptors (Singh *et al*, 2007b), and anti-MAP2 antibody (HM-2) (Abcam, Cambridge, MA). Neurons were fixed in 4% paraformaldehyde (pre-warmed, 37 ℃) for 20min followed by three washes. Fixed neurons then were permeabilized in phosphate buffered saline (PBS) buffer containing 0.1% Triton X-100 for 10 min, followed by incubation with PBS containing 10% serum for one hour. Then neurons were incubated with primary antibodies in 10% serum overnight in a humid chamber at 4 ℃. After three washes with PBS the next day, Alexa Fluor® 488 donkey anti-rabbit IgG (H+L) antibody, or Alexa Fluor® 647 goat anti-

mouse IgG (H+L) antibody (Life Technologies, Grand Island, NY) in 10% serum were added to coverslips and incubated for 1 hours at room temperature. 10μL methanol stock solution of Alexa Fluor® 568 Phalloidin in 200μL PBS was added to each coverslip. After a 20 min incubation, three more washes were performed before coverslips were mounted onto slides using ProLong anti-fade reagent (Invitrogen, Grand Island, NY).

Quantitative Analysis of Spine Morphology

Labeled neurons were visualized with an Olympus/3I Spinning Disk Confocal Microscopy using a 100X or 150X TIRF oil immersion objective. Z-series of twenty to thirty images were taken at 0.1μm intervals, with 1024 x 1024 and 1000 x 1000 pixel resolution. Three-dimensional maximum projection reconstructions and deconvolution were performed using Slidebook 5.5 or 6 (Intelligent Imaging Innovations, Inc). Length of dendrites was measuring using Image J (National Institutes of Health, Bethesda, Maryland). Density of different types of dendritic spines were measured by using Neuron Studio (Icahn School of Medicine at Mount Sinai, New York, NY). Spines on one or two primary dendrites of each neuron, and 5-10 neurons for each condition were analyzed from three separate experiments.

RESULTS

Identification of differential gene expression in the frontal cortex of rats treated with AG490 and olanzapine.

To identify the changes in gene expression induced by olanzapine via JAK2/STAT3 signaling pathway, we performed a microarray assay to compare gene expression in the frontal cortex from rats pretreated with vehicle or AG490 followed by

treatment with vehicle or olanzapine daily for seven days. We used multiple types of statistically analytical approaches (gene expression levels of 1.3-fold or more versus control rat, volcano-plot analysis, and two-way ANOVA) to analyze microarray data, which allows optimal gene mining. As indicated in the heat map of the cluster analysis (Figure 3.1A), 205 genes are significantly changed by the treatment with AG490, olanzapine or the combination of both drugs compared to the vehicle-treated controls. Among these genes, we noted that 92 genes were altered by vehicle/olanzapine but not by the combined treatment of AG490 and olanzapine, indicating that these 92 genes are regulated by olanzapine via JAK2 signaling pathway (Figure 3.1B). Of the 12 genes, which showed changes in gene expression in both the vehicle/olanzapine and AG490/olanzapine groups, the expression of several of these gene may not be regulated independently of JAK2/STAT signaling. A couple of genes in this group were changed less by AG490/olanzapine compared with vehicle/olanzapine, suggesting that JAK2/STAT pathway may be also involved in the olanzapine-induced changes of these genes. The Gene Ontology (GO) enrichment analysis revealed the functions which are significantly changed by AG490, olanzapine and the combined treatment in the identified genes collectively. As shown in Table 3.2, functions related to schizophrenia, adverse effects of atypical antipsychotics, as well as postsynaptic functions, cytoskeleton, and dendritic spine plasticity, are highly enriched in these genes.

Validation of the microarray results using qPCR

To validate the differential gene expression results obtained from microarray, we performed qPCR to verify the changes of a number of genes. In Figure 3.2A, post hoc Newman–Keuls method suggests that Crh, Cbln1, Nrg1, Hsp70 and Fabp7 are

significantly increased by vehicle-olanzapine when compared to vehicle-vehicle. The olanzapine-induced changes are significantly reduced by AG490, suggesting that olanzapine-induced increases in Crh, Cbln1, Nrg1, Hsp70 and Fabp7 are mediated by the JAK2 signaling pathway. In Figure 3.2B, two-way ANOVA tests indicate that olanzapine has a significant effect on Filip1, Homer1, Pak1ip1 and Arc, however, the impact of AG490 is not significant. Therefore, olanzapine-induced changes in expression of these genes may be not dependent on JAK2/STAT pathway. Two-way ANOVA analysis also shows that AG490 has a significant influence on Pde10, Ntrk2 and Cdrh1 (Figure 3.2C). Even though the microarray results suggest that Carpt, Nts, Nr4a1, Cbp and Reelin are significantly regulated by olanzapine in a JAK/STAT signaling pathway dependent manner, none of these genes is significantly affected by either AG490 or olanzapine based on the qPCR assay (Figure 3.2D). Given that GO biology processes related to cytoskeleton, and dendritic spine plasticity are enriched, we also measured the gene expression of kalirin7 and spinophilin (Spn), which are dendritic spine-enriched proteins and play important roles in the regulation of postsynaptic plasticity. Although we noted a tendency of increase in kalirin and Spn by olanzapine treatment, neither of these two genes are altered significantly by AG490 or olanzapine.

Validation of the qPCR results by measuring the protein levels

To follow up on the changes observed in qPCR assays, we also examined the protein levels of some genes of interest. We first measured the protein level of regulator of G-protein signaling 7 (RGS7). Treatment with olanzapine for 7 days significantly increased RGS7 in the whole tissue homogenate as shown in Figure 3.3A. The olanzapine-induced upregulation of RGS7 was reduced back to control level by

pretreatment with AG490. This result indicates that olanzapine increases RGS7 via JAK2/STAT3 signaling pathway in rat frontal cortex as previously demonstrate (Muma *et al*, 2007a). Next, we examined the protein level of Hsp70 and Nrg1 using ELISA Assay and Western blot respectively. We found that treatment with olanzapine significantly increased the protein level of Hsp70 by 30%, and AG490 pretreatment decreased it to control level (Figure 3.3B). Similarly, protein level of Nrg1 was significantly enhanced by olanzapine by 35%, and reduced to control level by AG490 (Figure 3.3C). These results suggest that olanzapine regulates both Hsp70 and Nrg1 in a JAK2/STAT dependent manner, consistent with the microarray and qPCR results. The protein levels of kalirin7 and spinophilin were also measured using SDS-PAGE. The protein levels of kalirin7 and spinophilin were significantly increased by olanzapine by 26% (Figure 3.3D). As shown in Figure 3E, the anti-spinophilin antibody recognizes the rat spinophilin at 120kD, and a band at ~95kDa which is suspected to be a cleavage product of spinophilin. Olanzapine significantly increases the protein levels of both bands. However, pretreatment with AG490 did not block the olanzapine-induced changes in kalirin7 and spinophilin, suggesting chronic treatment with olanzapine is able to increase protein levels of kalirin7 and spinophilin, but not via JAK2/STAT pathway.

JAK2/STAT pathway is activated by olanzapine in rat cortical primary culture

To determine whether olanzapine also activated JAK2/STAT3 signaling pathway in rat primary cortical culture, we pretreated the primary neurons at DIV14 with 15 μM AG490 or 50% DMSO as vehicle. One hour later, the neurons were treated with 300nM olanzapine, or 1 μM MDL100907, a selective 5-HT2A receptor antagonist, or drug vehicle. The doses of drugs were selected according to the previous studies (Singh *et al*,

2009a). We treated the neurons daily for seven days and harvested the whole cell lysate at DIV21. The protein level of phosphor-JAK2 (pJAK2) and total JAK2 were analyzed by western blot. Tyrosine phosphorylation of JAK2 was significantly increased by both olanzapine and MDL100907 treatment. As shown in Figure 3.4A, pretreatment with AG490 significantly decreased olanzapine- and MDL100907-induced increases in phosphorylation of JAK2. This result indicates that both olanzapine and MDL100907 treatment activate JAK2 in rat cortical neurons. Since MDL100907 is a selective 5-HT2A receptor antagonist, the activation of JAK2 by olanzapine is likely mediated by 5-HT2A receptors.

Moreover, to verify whether olanzapine causes same changes in protein expression in rat primary cortical neurons as in rat frontal cortex, we also examine the protein level of Nrg1. As shown in Figure 3.4B, both olanzapine and MDL100907 increased Nrg1 protein levels, and AG490 pretreatment reduced the protein back to control levels. This result suggests that, olanzapine upregulates Nrg1 via JAK2/STAT pathway in rat primary culture as well as in rat frontal cortex.

Olanzapine-induced dendritic spine maturation is partially dependent on JAK2/STAT pathway

To examine the influence of olanzapine on the morphology and function of dendritic spines, we treated the primary cortical neurons with 300nM olanzapine or vehicle for seven days, and fixed and stained the neurons at DIV21. As shown in Figure 3.5A, endogenous 5-HT2A receptors localize to the soma, dendritic shafts and dendritic spines in our cultured rat primary neurons. Treatment with olanzapine for seven days significantly decreased the density of immature dendritic filopodia while increasing the

mature mushroom spines, suggesting a maturation of dendritic spines by olanzapine treatment (Figure 3.5B left panel). There is a tendency for the density of total dendritic spines to increase, but it is not significant (Figure 3.5B right panel). To study the role of the JAK2 signaling pathway in the olanzapine-induced dendritic spine maturation, we treated the primary cortical culture with AG490 and olanzapine as described above, and examine the morphology of dendritic spines.

We found that olanzapine showed a tendency to decrease immature dendritic filopodia (Figure 3.5C), however it is not significant. AG490 treatment alone also significantly decreased the dendritic filopodia density. However, when combined with olanzapine, the effects of AG490 of olanzapine were inhibited. As shown in Figure 5D, olanzapine significantly reduced the density of stubby spines, another type of immature dendritic spines. AG490 pretreatment blocked the olanzapine-induced changes stubby spines (Figure 3.5D). We also found that olanzapine increased the density of thin spine, a relatively mature dendritic spine type, via JAK2 pathway (Figure 3.5E). The variability in the number of mushrooms spines was large resulting in not significant differences in either the AG490 or olanzapine effects (Figure 3.5F). As shown in Figure 3.5G, both olanzapine and AG490 treatment alone resulted in a small but significant increase in the total dendritic spine density. The olanzapine-induced increased was not blocked by AG490 pretreatment. These results suggest that the olanzapine-induced dendritic spine maturation is, at least, partially dependent on the JAK2 pathway.

DISCUSSION

Atypical antipsychotics are inverse agonists of 5-HT2A receptors/Gαq/11 protein signaling but are agonists when considering the 5-HT2A/JAK2/STAT3 pathway. We previously reported that the atypical antipsychotics olanzapine and clozapine induce desensitization of 5-HT2A receptor signaling at least partially via the JAK2/STAT3 signaling pathway, which is likely to contribute to therapeutic effects of atypical antipsychotics. Although we found that activation of JAK2/STAT3 pathways is necessary for full desensitization of 5-HT2A receptors by atypical antipsychotics, it is unclear whether activation of the JAK2/STAT3 pathway contributes to the beneficial effects of these antipsychotic drugs. The JAK2/STAT3 pathway regulates the expression of a number of transcription factors such as c-Fos, c-Jun and c-Myc, which can stimulate expression of select genes. To explore the genes regulated by atypical antipsychotics via JAK2/STAT3 pathway, we performed a microarray study and found a large number of genes are changed by olanzapine treatment and do so via the JAK2/STAT pathway. The expression of multiple genes was altered that are involved in the etiology of schizophrenia, the side effects of atypical antipsychotics, as well as cytoskeletal, post-synaptic density and synaptic functions, suggesting that the effects of olanzapine on dendritic spines may be mediated by synergistic functions of multiple genes via JAK2/STAT3 pathways.

We found that olanzapine increased both mRNA and protein levels of Nrg1 via JAK2/STAT3 pathway. Nrg1 is a widely accepted candidate susceptibility gene for schizophrenia (Bennett, 2011), but the question arises as to how Nrg1 contributes to the pathologies of schizophrenia. Nrg1 is an important regulator of GABAergic, dopaminergic and glutamatergic neurotransmission, and plays critical roles in neuronal

migration, myelination and synaptic plasticity (Agarwal *et al*, 2014; Chohan *et al*, 2014). It is suggested that acute and chronic Nrg1/ErbB4 activity exerts discriminative influences on dendritic spine plasticity. Acute Nrg1/ErbB4 activity inhibits synaptic plasticity in pyramidal neurons. Nrg1 suppresses long-term potentiation (LTP) by reducing AMPA receptor EPSCs in CA1 hippocampal neurons (Chen *et al*, 2010; Huang *et al*, 2000; Kwon *et al*, 2005). On the other hand, prolonged treatment with Nrg1 potentiates synaptic plasticity. Nrg1 restored amyloid β-induced impairment of LTP in mouse hippocampal slices in an ErbB4 dependent manner (Min *et al*, 2011). Knocking down ErbB2/B4 inhibited dendritic spine maturation while treatment with clozapine reversed the behavioral and spine abnormalities in this mice model (Barros *et al*, 2009). Treatment with Nrg1 for 21 days also significantly promoted spine maturation in the cultured neurons (Barros *et al*, 2009). Cahill and colleagues found that long-term incubation with Nrg1 increased both size and density of dendritic spines, which kalirin7-dependent manner. Kalirin7 is a Rac1 guanine nucleotide exchange factor (GEF) and a well-known regulator of dendritic spines (Cahill *et al*, 2013). Interestingly, we found kalirin7 protein levels were also increased by olanzapine. Total kalirin mRNA levels were not significantly changed by olanzapine, however, we did not measure kalirin7 mRNA, an isoform produced through alternative splicing.

We also found that olanzapine significantly increased the protein levels of spinophilin, a dendritic spine-enriched protein. The mRNA levels of spinophilin are also increased by olanzapine treatment, but not at a statistically significant level. Enhanced spinophilin expression may be a sign of increased dendritic activity or plasticity by olanzapine treatment. AG490 failed to block the enhanced spinophilin expression

induced by olanzapine, suggesting that the effects of olanzapine on dendritic spines may mediated by both JAK2/STAT3 and other pathways. A previous study found that fourteen-day treatment with olanzapine caused no changes in the mRNA level of spinophilin in rat hippocampus and cortex. (Law et al, 2004). Critchlow *et al* reported that clozapine increased spinophilin protein level by 70% (Critchlow *et al*, 2006). The inconsistent results on spinophilin among these studies may reflect species difference, brain region difference, different doses or differing durations of treatment.

Although the etiology of schizophrenia is not fully understood, mounting evidence suggests that schizophrenia is associated with malfunctions of brain circuitry, in particular, dendritic spines, in multiple brain regions. It has been reported that subjects with schizophrenia show a decrease in dendritic spine density on dorsolateral prefrontal cortex (DLPFC) layer III pyramidal neurons (Glantz and Lewis, 2000), auditory cortex on the superior temporal gyrus (Sweet et al, 2009), and subicular and CA3 region in schizophrenia brain (Kolomeets et al, 2005), brain regions exhibiting severe malfunction in schizophrenia. These studies demonstrate a strong association between brain region-specific loss of dendritic spines and brain dysfunction in schizophrenia. We examined the impact of olanzapine and the JAK2/STAT3 pathway on dendritic spines based on the importance of dendritic spines to functions that are abnormal in schizophrenia, the documented differences in dendritic spines in schizophrenia and our data demonstrating changes in the expression of genes involved in the regulation of dendritic spines.

Effects of antipsychotics on dendritic spines have been studied by various groups but the results are complex. One-year administration of typical antipsychotic haloperidol or atypical antipsychotic clozapine induced changes in axodendritic synapses of rat

medial prefrontal cortex layer VI (Vincent et al, 1991). Clozapine increased dendritic spine density, in particular, filopodia and mushroom spines in rat hippocampal neurons, while haloperidol reduced the number of filopodia (Critchlow et al, 2006). Olanzapine but not haloperidol reversed the dopamine depletion-induced loss of spine in layer V pyramidal cells in the prelimbic cortex (Wang and Deutch, 2008). In animal phencyclidine (PCP) models of schizophrenia, acute and chronic treatment with olanzapine also reversed the dendritic spine loss (Elsworth et al, 2011). However, in another recent study, early exposure of both haloperidol and olanzapine induced an reduction in spine density in layer III mouse pyramidal cells (Frost et al, 2010). The inconsistency of results between the studies may be due to various models, drugs and doses used. Moreover, criteria of spine morphology are mostly qualitative, therefore, it is difficult to compare data from different laboratories. Adult cortex and hippocampus contains >65% thin spines and around 25% mushroom spines (Bourne and Harris, 2007). The remaining 10% of spines are immature spines, including filopodia, stubby and branched spines (Bourne and Harris, 2007). Dendritic spines stabilize with maturation. Mushroom spines are stable memory spines containing more AMPA receptors, which are a major indicator of synaptic efficacy and strength (Nimchinsky *et al*, 2004). In contrast, thin spines contain more NMDA receptors but few AMPA receptor, making them ready to be strengthened by insertion of AMPA receptors (Ganeshina *et al*, 2004; Matsuzaki *et al*, 2001). Therefore they are learning spines. In the present study, we found that treatment with olanzapine for a week decreased immature dendritic filopodia and stubby spines, while increasing mature thin and mushroom spines. Olanzapine also caused a small increase in total dendritic spine density. These results suggest that olanzapine

induced an improvement of dendritic spine function in rat primary cortical culture. Only the changes in stubby spines and thin spines induced by olanzapine were inhibited by pretreatment of AG490, indicating that the olanzapine-induced maturation of dendritic spines is partially dependent on JAK2 signaling pathway. This result is consistent with our microarray and qPCR data. JAK2-dependent changes in genes such as Nrg1 may contribute to the JAK2-dependent changes in dendritic spines induced by olanzapine. Changes in the expression of other genes including Homer1, Arc, kalirin7 and spinophilin, which are not dependent on JAK2 pathways, may be responsible for the JAK2-independent portion of dendritic spine maturation and increase in total dendritic spine density. We also found AG490 alone has an effect on the total spine density, which may be due to the AG490-induced changes in gene expression.

In addition, we also measured the protein level of RGS7 and found a 30% increase in RGS7 by olanzapine, which is significantly attenuated to basal levels by AG490. We previously reported that olanzapine induced a 100% increase in RGS7 in rat frontal cortex (Singh *et al*, 2009a). The lower increase in RGS7 in the current study may be because we measured the protein level of RGS7 in the whole tissue homogenate in the current study instead of in the membrane fraction as reported by Singh *et al*.

Despite the beneficial neuropsychiatric effects, treatment with atypical antipsychotics is also accompanied by some adverse effects such as serious weight gain and other metabolic syndromes including dyslipidemia and glucose intolerance (Gareri *et al*, 2006). The molecular mechanisms leading to these side effects are not fully understood. One hypothesis is that atypical antipsychotics-induced weight gain is attributed to the changes of balance between neuropeptides and hormones that controls

food intake and energy homeostasis, thus resulting in an imbalance between energy intake and energy expenditure (de Kloet and Woods, 2010; Gonçalves *et al*, 2015). In current study, we identified several genes that may be involved in the adverse metabolic effects induced by olanzapine. For example, our microarray data shows that olanzapine treatment increases Cartpt, a prepropeptide for cocaine- and amphetamine-regulated transcript (CART), which is a hypothalamic neuropeptide that regulates food intake and energy expenditure(Lau and Herzog, 2014). Overexpression of CART in the CNS could lead to an anorexigenic effect (Kristensen *et al*, 1998; Larsen *et al*, 2000; Nakhate *et al*, 2013; Thim *et al*, 1998), while administration of CART into several specific brain regions results in appetite-promoting effects (Abbott *et al*, 2001; Hou *et al*, 2010; Kong *et al*, 2003; Smith *et al*, 2008). Effects of atypical antipsychotics on CART are also puzzling. Kursungoz et al reported that a four week treatment with risperidone elevated appetite and body weight gain, and the mRNA level of CART was significantly increased (Kursungoz *et al*, 2015). However, in another study, risperidone increased food intake and body weight in female juvenile rats, but failed to change CART mRNA levels (Lian *et al*, 2015). It is also reported that a four week risperidone treatment didn't change plasma CART levels in first-attack psychotic patients (Yanik *et al*, 2013). Consistent with our microarray data, the real-time PCR result showed a tendency for increased Cartpt mRNA level by olanzapine treatment, however, the change is not significant due to the large variation between animals. Larger sample size or longer treatment may be required to verify whether olanzapine increases Cartpt in a JAK2/STAT3 dependent manner. On the other hand, an increase in Cartpt does not indicate an increase in CART. CART is

produced by processing Cartpt by prohormone/ proprotein convertase, dysregulation of which may also result in accumulation of Cartpt.

In current microarray study, we observed that olanzapine significantly decreased Grp mRNA levels via JAK2/STAT3 pathway, which may play a role in the olanzapine-induced weight gain and metabolic side effects. As a mammalian counterpart of amphibian peptide bombesin, Grp in the CNS plays an important role in regulating synaptic plasticity, memory, emotions, social interaction and feeding behavior (Moody and Merali, 2004; Roesler and Schwartsmann, 2012). Both peripheral and central administration of Grp or bombesin have negative effects on the intake of food in numerous animal models and humans (Fekete *et al*, 2002; Gibbs, 1985; Gibbs *et al*, 1981; Himick and Peter, 1994; Rushing *et al*, 1996; Thaw *et al*, 1998). Knockout or blockade of Grp receptors in the brain increases food consumption significantly (Ladenheim *et al*, 2002; Ladenheim *et al*, 1996). Further verification of mRNA and protein levels of Grp in AG490 and olanzapine treated animals is needed to confirm the role of Grp in the atypical antipsychotics-induced weight gain.

A previous microarray study has also investigated the effects of chronic administration of olanzapine (2mg/kg per day for 21days) on differential expression of genes in the prefrontal cortex of rats (Fatemi *et al*, 2006). In this study, 38 genes were upregulated and 31 genes were down-regulated by olanzapine based on student t-test. Similar to our results, these differentially expressed genes are also involved in signal transduction, metabolism pathways, and synaptic plasticity. Their microarray results showed that olanzapine increased Homer1, Reelin and Cart, instead of Cartpt. They observed a reduction in ionotropic glutamate receptor NMDA3B, while we found a

reduction in another ionotropic glutamate receptor Grik4. They found a significant decrease in gene expression of insulin-like growth factor binding protein 2 (Igfbp2). We observed a 30% reduction in Igfbp6 but a 76% increase in Igfbp1 and 33% increase in Igfbp5. Compared to this previous microarray study, we tested a higher dose of olanzapine but a shorter treatment interval based on our previous results (Muma *et al*, 2007a). Moreover, we examined the contribution of the JAK2/STAT3 signaling pathway to the changes in gene expression using AG490.

Overall, our study suggests that chronic olanzapine treatment result in changes of a large number of genes in a JAK2/STAT3 signaling pathway dependent manner. Those genes may play important roles in mediating the therapeutic or side effects of olanzapine, such as improvement of synaptic functions. Our findings provide an insight into the molecular mechanisms underlying antipsychotic drug actions and adverse effects.

FIGURES AND FIGURE LEGENDS

Table 3.2 Genes listed in this table are selected based on (1), fold change ≥ 1.3 after log transformation and normalization; (2) volcano-plot analysis, p values are used to compare vehicle-olanzapine with vehicle-vehicle treatment; (3), two-way ANOVA analysis, p values are corrected using Benjamini-Hochberg method, and used to compare olanzapine treatment with vehicle treatment. *, genes also changed by AG490-olanzapine to a lesser extent. The fold change for Ntrk2 by AG490-olanzapine is +1.54, for Cartpt is +1.74, for Nts is +1.67, for Arc is +1.54.

Probe Set ID	Gene Symbol	Gene Title	GO Biological Process	Array Fold Change by Vehicle-Olanzapine	P
Genes related to schizophrenia					
1369093_at	**Reln** [2]	Reelin	regulation of synapse maturation, small GTPase mediated signal transduction, NMDA receptor activity, long-term synaptic potentiation, CREB transcription factor activity, cell adhesion, locomotory behavior, memory, brain development	+1.45	0.049
1370607_a_at	**Nrg1** [1]	neuregulin 1	MAPKKK cascade, nervous system development, synapse assembly, myelination, neurotransmitter receptor metabolic process, positive regulation of Ras protein signal transduction	+1.53	
1370652_at	**Ntrk2** [1]*	neurotrophic tyrosine kinase receptor type 2	brain-derived neurotrophic factor (BDNF) signaling pathway, feeding behavior, glutamate secretion, regulation of metabolic process, positive regulation of synaptic glutamatergic transmission	+1.87	
1376980_at	**Htr2c** [1]	serotonin receptor 2C	G-protein signaling, coupled to cyclic nucleotide second messenger, positive regulation of GABA secretion and acetylcholine secretion , behavioral response to nicotine negative regulation of locomotion, negative regulation of dopamine metabolic, regulation of sensory perception of pain	+1.51	
1368478_at	**Drd1a** [1]	dopamine receptor D1A	synaptic dopaminergic transmission, conditioned taste aversion, behavioral fear response, startle response, response to amphetamine and cocaine, synapse assembly, learning and memory, locomotory behavior, regulation of long-term neuronal synaptic plasticity, LTP and LTD	+1.49	
1369163_at	**Grik4** [3]	glutamate receptor, ionotropic, kainate 4	synaptic glutamatergic transmission	-1.11	0.055
1387706_at	**Gabrg1** [1]	GABA-A receptor, gamma 1	GABA-A receptor activity, neuron projection, inhibitory extracellular ligand-gated ion channel activity, synaptic transmission	-1.38	
1370024_at	**Fabp7** [2]	fatty acid binding protein 7, brain	startle response, cell proliferation in forebrain, neurogenesis, prepulse inhibition	+1.40	0.037
1380027_at	**Cbp** [1]	CREB binding protein	DNA-dependent regulation of transcription, histone acetylation	+1.30	
1392973_at	**Nav2** [2]	neuron navigator 2	nervous system development	+1.36	0.029
1368438_at	**Pde10a** [2]	Phosphodiester-ase 10A	cAMP catabolic process, cGMP catabolic	+1.31	0.002
1368247_at	**Hspa1a/1b** [2]	heat shock 70kD protein 1A/1B	DNA repair, anti-apoptosis, response to stress, negative regulation of cell growth, protein refolding, negative regulation of caspase activity, negative regulation of inclusion body assembly	+1.34	0.007
1369303_at	**Crh** [2]	corticotropin releasing hormone	glucocorticoid biosynthetic process, response to stress, inflammatory response, long-term memory, feeding behavior, positive regulation of cortisol secretion	+1.44	0.003
Genes related to side effects of atypical antipsychotics					
1368585_at	**Cartpt** [1]*	CART prepropeptide	activation of MAPKK activity, cellular glucose homeostasis, neuropeptide signaling pathway, synaptic transmission, adult feeding behavior, cellular response to starvation, positive regulation of epinephrine secretion, regulation of insulin secretion, negative regulation of glucagon secretion, somatostatin secretion	+2.39	
1380967_at	**Nts** [1]*	Neurotensin	regulation of blood vessel size, smooth muscle contraction, fat metabolism	+2.35	
1368611_at	**Grp** [2]	gastrin releasing peptide	neuropeptide signaling pathway, release of gastrointestinal hormones	-1.67	0.007
1386935_at	**Nr4a1** [2]	nuclear receptor subfamily 4, group A, member 1	regulation of transcription, DNA-dependent, induction of apoptosis negative regulation of caspase activity, regulation of energy homeostasis	+1.79	0.045
Genes related to cytoskeletal, post-synaptic density and synaptic plasticity					
1387068_at	**Arc** [2] *	activity-regulated cytoskeleton-associated protein	regulation of neuronal synaptic plasticity, long-term potentiation, and memory consolidation, AMPA glutamate receptor activity, actin cytoskeleton organization, dendritic spines	+2.76	0.0196
1370997_at	**Homer1** [2]	homer homolog 1	response to stress, metabotropic glutamate receptor signaling pathway, positive regulation of calcium ion transport via store-operated calcium channel activity, response to nicotine and cocaine	+1.74	0.0001
1379463_at	**Filip1** [1]	filamin A interacting protein 1	cytoskeleton	+1.46	
1374966_at	**Dcx** [1]	doublecortin	regulation of microtubule polymerization, neuron migration, nervous system development , neuron axonogenesis, dendrite morphogenesis	+1.45	
1393181_at	**Nfasc** [2]	neurofascin	cell adhesion, axon guidance, peripheral nervous system development, myelination, synapse organization	+1.32	0.035
1376873_at	**Cbln1** [1]	cerebellin 1 precursor	positive regulation of synapse assembly, cell adhesion, postsynaptic membrane	+1.31	
1382275_at	**Pak1ip1** [3]	PAK1 interacting protein 1	negative regulation of PAK1 kinase	-1.18	0.067
1388448_at	**Cdc42se2** [3]	CDC42 small effector 2	regulation of signal transduction, cytoskeleton, signal transduction	-1.08	0.037
1393416_at	**Rasal1** [3]	RAS protein activator like 1 (GAP1 like)	regulation of Ras protein signal transduction	-1.07	
1389811_at	**Rasgef1c** [3]	RasGEF domain family, member 1C	regulation of small GTPase mediated signal transduction	-1.20	0.028

Table 3. 2

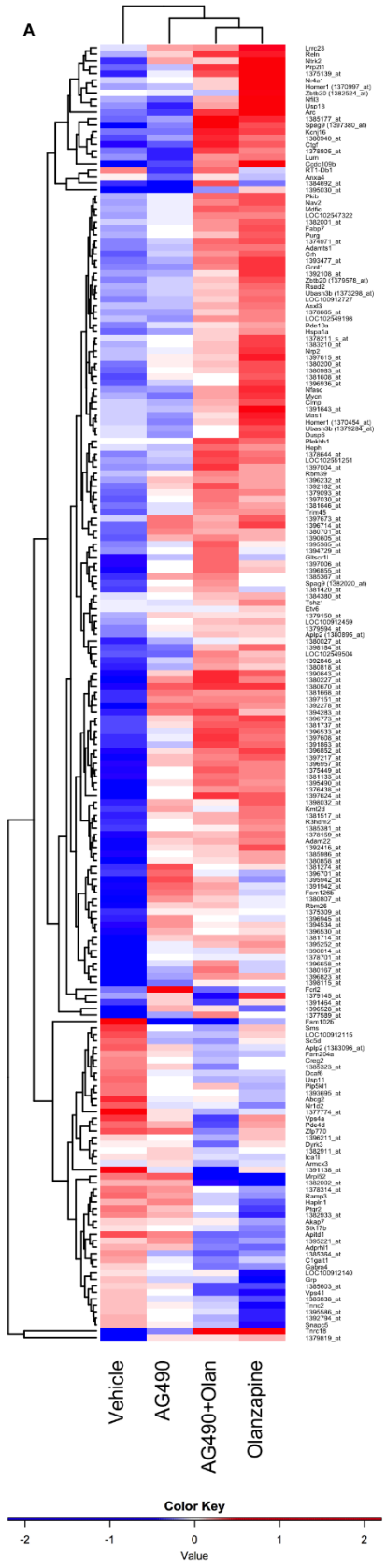

A
Vehicle
AG490
AG490+Olan
Olanzapine
Color Key
-2
-1
0
1
2
Value

Figure 3. 1

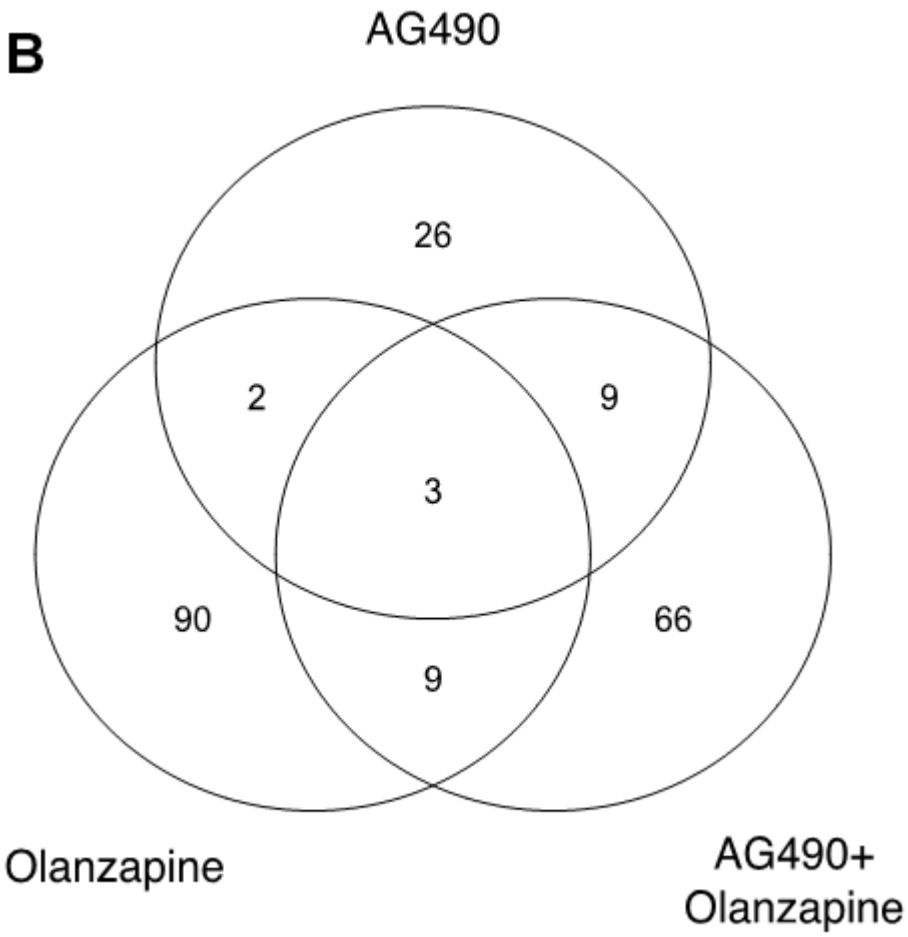


Figure 3.1. A, hierarchical clustering of 205 genes identified to be significantly affected by AG490, olanzapine, or the combination of both. The four columns represent treatments by these agents and the vehicle as marked at the bottom, respectively. The gene represented by each row is labeled on the right. The color bar at the bottom shows color representation of normalized gene expression values. Upregulated genes are shown in red and downregulated genes are in blue. The criteria for selection of genes in the differentially expressed gene set were fold change ≥ 1.3 and $P \leq 0.05$. B, Venn diagram shows the overlap of genes that are significantly altered by the treatments with AG490, olanzapine, or the combination of both.

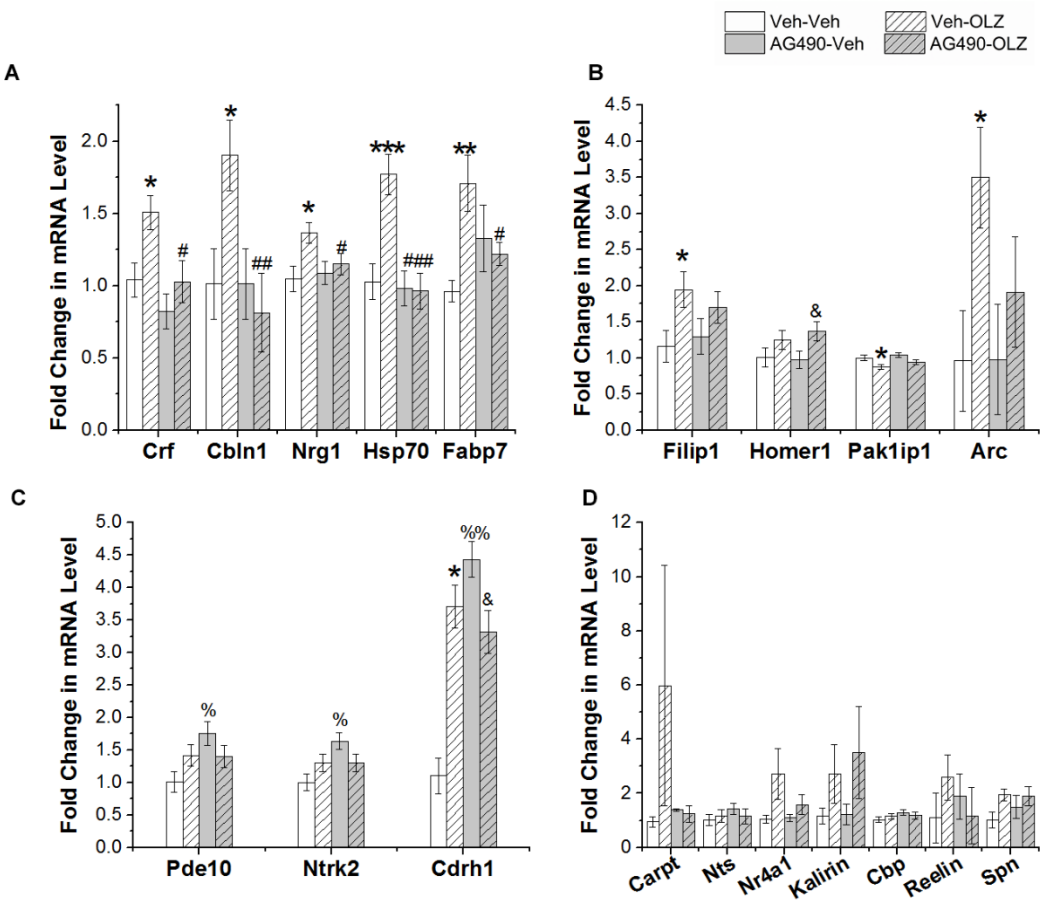
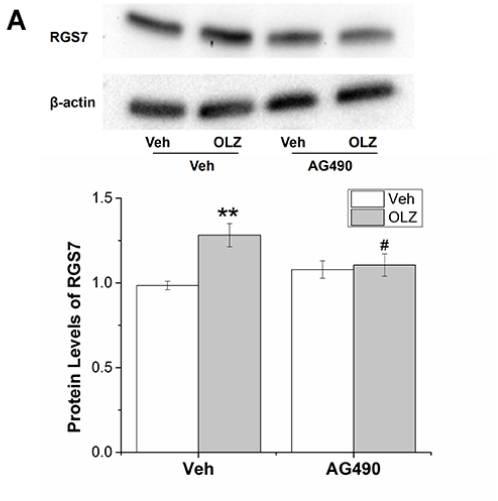


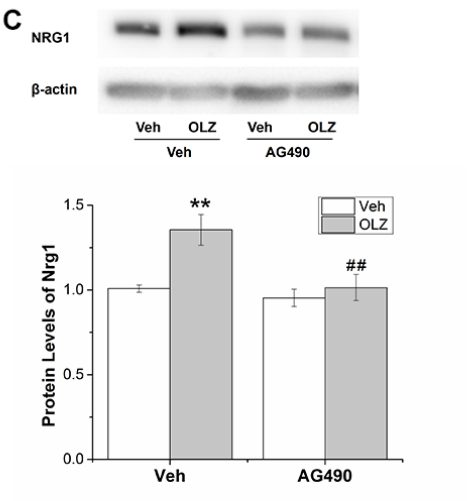
Figure 3. 2

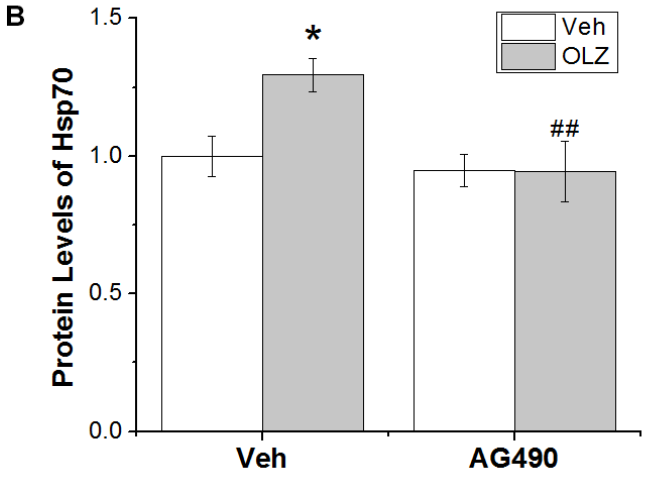
Figure 3.2. Genes of interests were validated by qPCR. A, Two-way ANOVA analysis shows significant effects of AG490 pretreatment [$F(1,18)= 7.677$, $p<0.05$], and significant effect of olanzapine treatment [$F(1,18)= 7.089$, $p<0.05$] on Crh expression; significant effects of pretreatment [$F(1,15)= 4.659$, $p<0.05$] on Cbln1 expression; significant effects of olanzapine treatment [$F(1,17)= 5.924$, $p<0.05$] on Nrg1 expression; significant effects of pretreatment [$F(1,15)= 11.277$, $p<0.01$], significant effect of treatment [$F(1,15)= 8.099$, $p<0.05$], and a significant interaction between AG490 pretreatment and olanzapine treatment on Hsp70 expression [$F(1,15)= 8.938$, $p<0.01$]; a significant interaction between AG490 pretreatment and olanzapine treatment on Fabp7

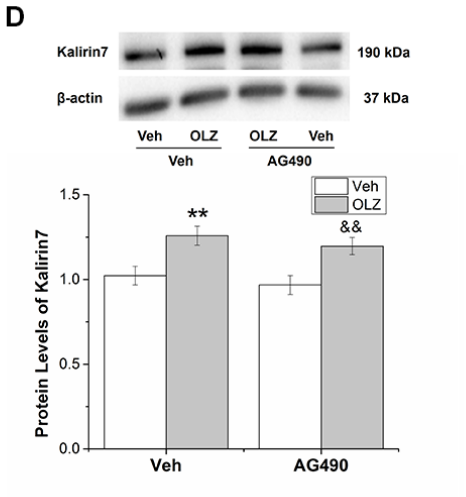
expression [$F(1,16)= 7.058$, $p<0.05$]. B, Two-way ANOVA analysis shows significant effects of olanzapine treatment on the expression of Filip1 [$F(1,18)= 6.436$, $p<0.05$], Homer1 [$F(1,17)= 5.917$, $p<0.05$], Pak1ip1 [$F(1,19)= 10.418$, $p<0.01$] and Arc [$F(1,18)= 5.65$, $p<0.05$]. C, Two-way ANOVA analysis shows significant effects of AG490 pretreatment on the expression of Pde10 [$F(1,15)= 4.587$, $p<0.05$], Ntrk2 [$F(1,18)= 5.764$, $p<0.05$], and Cdrh1 [$F(1,16)= 23.576$, $p<0.001$]. There is also a significant effect of olanzapine treatment [$F(1,16)= 6.045$, $p<0.05$], and a significant interaction between AG490 pretreatment and olanzapine treatment on Cdrh1 expression [$F(1,16)=38.012$, $p<0.001$]. D, Two-way ANOVA analysis shows no significant effects of either AG490 pretreatment or olanzapine treatment on the gene expression of Cartpt, Nts, Nr4a, Kalirin, Cbp, Reelin, and Spn., Newman-Keuls multiple comparison test indicates *** $p<0.001$, ** $p<0.01$, * $p<0.05$, compared with vehicle-vehicle treatment; ### $p<0.001$, ## $p<0.01$, # $p<0.05$, compared with vehicle-olanzapine treatment; &, $p<0.05$, compared with AG490-vehicle treatment; %%,

$p<0.001$, % $p<0.05$, compared with vehicle-vehicle treatment.


A
RGS7
β-actin
Veh
OLZ
Veh
OLZ
Veh
AG490
Protein Levels of RGS7
Veh
OLZ
**
#
Veh
AG490


C
NRG1
β-actin
Veh
OLZ
Veh
OLZ
Veh
AG490
Protein Levels of Nrg1
Veh
OLZ
**
##
Veh
AG490


B
Protein Levels of Hsp70
Veh
OLZ
*
##
Veh
AG490


D
Kalirin7
190 kDa
β-actin
37 kDa
Veh
OLZ
OLZ
Veh
Veh
AG490
Protein Levels of Kalirin7
Veh
OLZ
**
&&
Veh
AG490

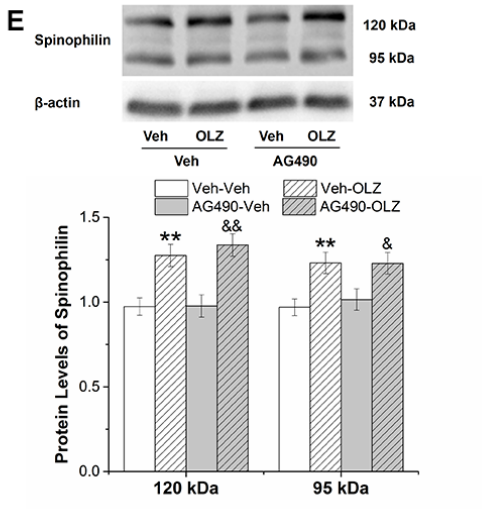

E
Spinophilin
120 kDa
95 kDa
β-actin
37 kDa
Veh
OLZ
Veh
OLZ
Veh
AG490
Veh-Veh
Veh-OLZ
AG490-Veh
AG490-OLZ
Protein Levels of Spinophilin
**
&&
**
&
120 kDa
95 kDa

Figure 3. 3

Figure 3.3. Protein levels of genes were verified by Western blot and ELISA assay. A, two-way ANOVA indicates a significant effect of olanzapine treatment [$F(1,16)= 9.652$, $p<0.01$], and a significant interaction between AG490 pretreatment and olanzapine treatment [$F(1,16)= 6.808$, $p<0.05$] on RGS7 protein levels. B, two-way ANOVA analysis of the ELISA assay shows a significant impact of AG490 treatment [$F(1,16)= 6.533$, $p<0.05$]. C, two-way ANOVA analysis shows a significant impact of AG490 treatment [$F(1,16)= 9.013$, $p<0.01$], a significant effect of olanzapine treatment [$F(1,16)= 9.59$, $p<0.01$], and a significant interaction between AG490 pretreatment and olanzapine treatment [$F(1,16)= 4.723$, $p<0.05$] on Nrg1 protein level. D, two-way ANOVA indicates a significant effect of olanzapine treatment [$F(1,17)= 18.009$, $p<0.001$] on kalirin7 protein level. E, two-way ANOVA indicates a significant effect of olanzapine treatment on the protein level of spinophilin at 120kDa [$F(1,10)= 27.906$, $p<0.001$], and on the band at 95kDa [$F(1,10)= 15.62$, $p<0.01$]. Newman-Keuls multiple comparison test indicates ** $p<0.01$, * $p<0.05$, compared with vehicle-vehicle treatment; ## $p<0.01$, # $p<0.05$, compared with vehicle-olanzapine treatment; && $p<0.01$, & $p<0.05$, compared with AG490-vehicle treatment.

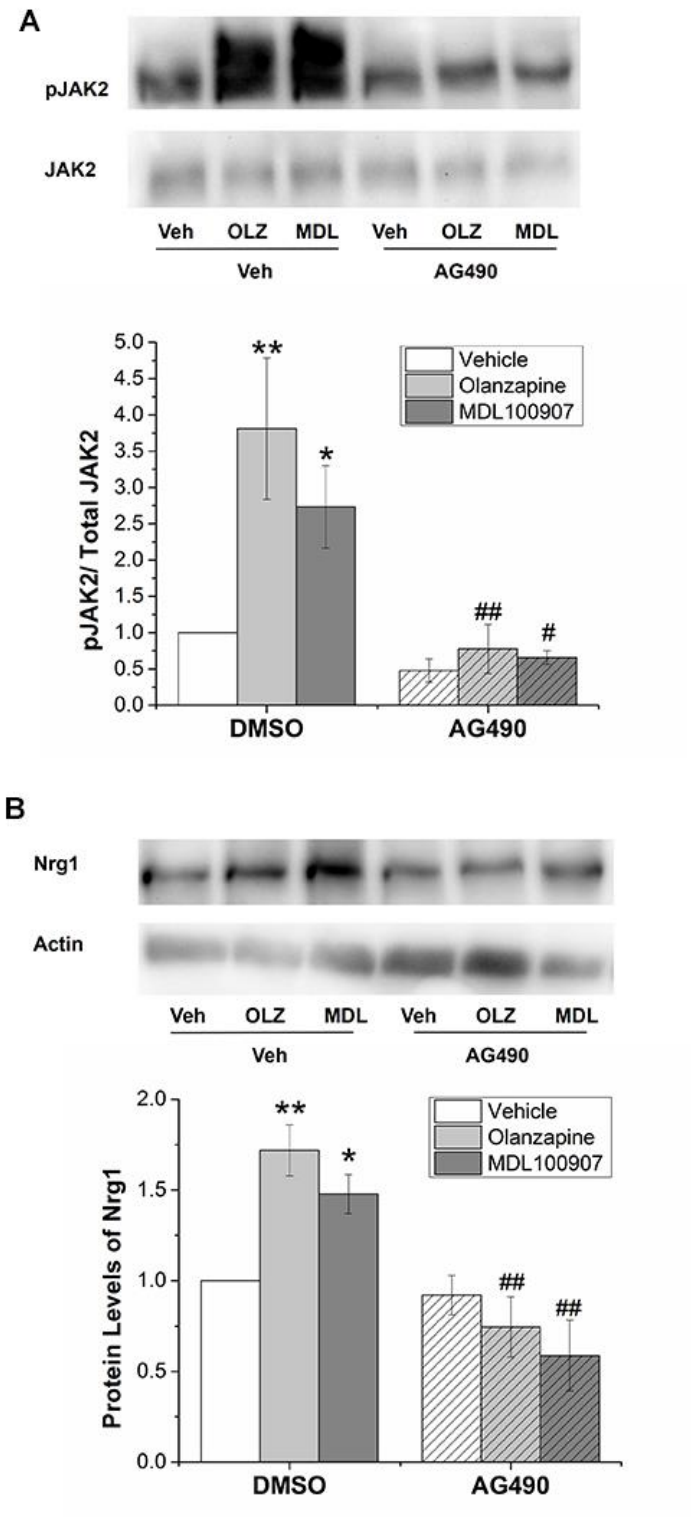

A
pJAK2
JAK2
Veh
OLZ
MDL
Veh
OLZ
MDL
Veh
AG490
pJAK2/ Total JAK2
Vehicle
Olanzapine
MDL100907
**
*
##
#
DMSO
AG490
B
Nrg1
Actin
Veh
OLZ
MDL
Veh
OLZ
MDL
Veh
AG490
Protein Levels of Nrg1
Vehicle
Olanzapine
MDL100907
**
*
##
##
DMSO
AG490

Figure 3. 4

Figure 3.4. Olanzapine and MDL100907 activate JAK2/STAT pathway in rat cortical primary culture. Primary cells at DIV14 were pretreated with AG490 (15μM) for 1 h before treatment with either olanzapine or MDL100907 daily for 7 days. Whole cell lysates were analyzed by western blot. A, The bar graph represents quantification of phosphor-JAK2 protein levels divided by JAK2 protein levels from three independent experiments. Two-way ANOVA analysis shows a significant impact of AG490 pretreatment [$F(1,12)= 22.301$, $p<0.001$], and a significant effect of olanzapine or MDL100907 treatment [$F(2,12)= 5.185$, $p<0.05$] on phosphorylation of JAK2. B, The bar graph represents quantification of Nrg1 protein levels normalized by β-actin protein levels. Two-way ANOVA analysis shows a significant impact of AG490 pretreatment [$F(1,12)= 34.699$, $p<0.001$], and a significant interaction between AG490 pretreatment and olanzapine or MDL100907 treatment [$F(2,12)= 6.729$, $p<0.05$] on Nrg1 protein level. Newman-Keuls multiple comparison test indicates ** $p<0.01$, * $p<0.05$, compared with vehicle-vehicle treatment; ## $p<0.001$, # $p<0.05$, compared with vehicle-olanzapine or vehicle-MDL100907 treatment.

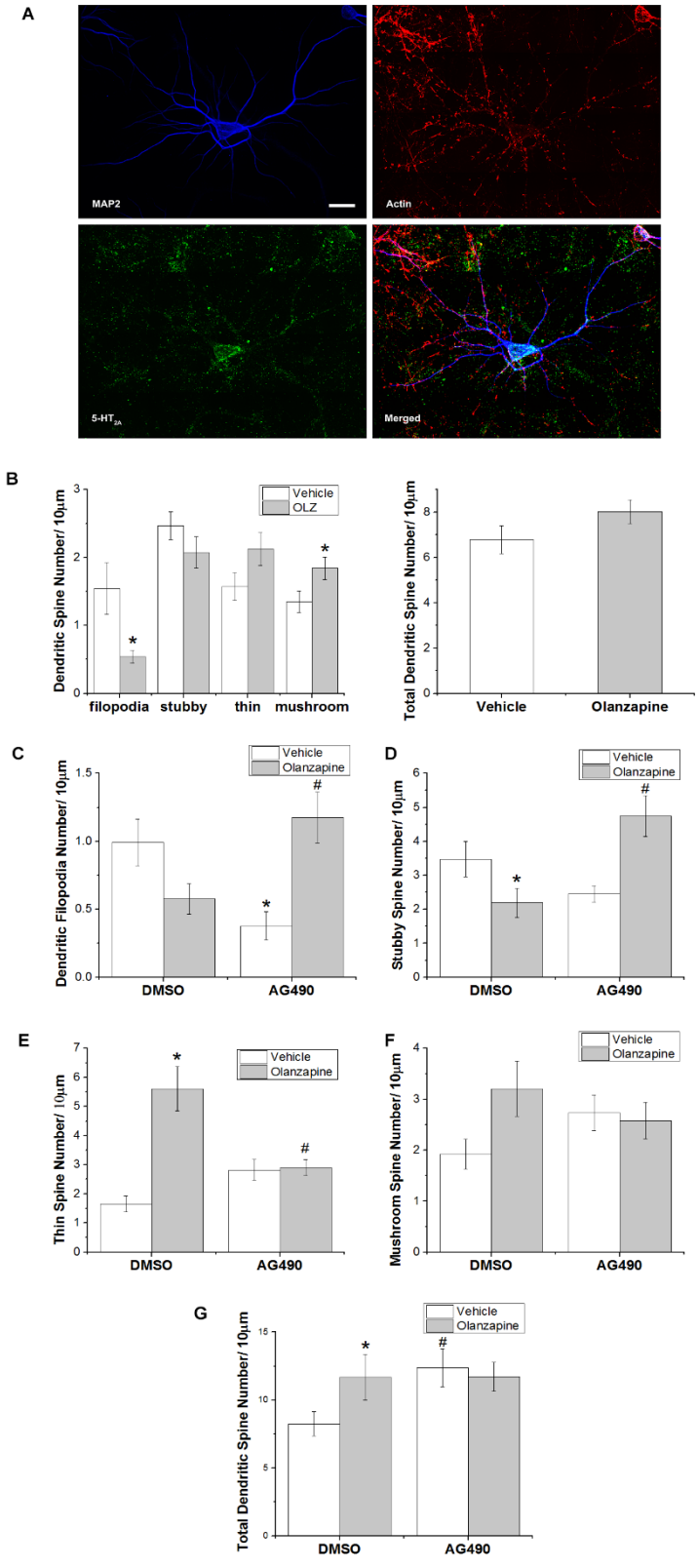

A
MAP2
Actin
5-HT2A
Merged
B
Dendritic Spine Number/ 10μm
Vehicle
OLZ
filopodia
stubby
thin
mushroom
Total Dendritic Spine Number/ 10μm
Vehicle
Olanzapine
C
Dendritic Filopodia Number/ 10μm
DMSO
AG490
D
Stubby Spine Number/ 10μm
E
Thin Spine Number/ 10μm
F
Mushroom Spine Number/ 10μm
G
Total Dendritic Spine Number/ 10μm

Figure 3. 5

Figure 3.5. Olanzapine-induced dendritic spine maturation is partially dependent on JAK2/STAT pathways. A, 5-HT2A receptors (green) co-localize with actin (red) predominantly in the soma, dendritic shafts and dendritic spines. Scale bar represents 20μm. B-G. Log transformation was performed on original data to achieve normality. B, left panel, olanzapine treatment for seven days induced dendritic spine maturation. Bars represent absolute value of dendritic spine numbers. Log transformation was performed on the original data of dendritic filopodia, stubby spines and thin spines to achieve normality. Student's t-test indicates * $p<0.05$ compared with vehicle treatment. Right panel, student's t-test suggests that olanzapine has no significant effect on the total dendritic spine density. C. Effects of olanzapine and AG490 on dendritic filopodia density. Log transformation does not enable the data to distribute normally, therefore analysis was performed on the original data using a non-parametric test. Kruskal-Wallis one-way ANOVA on Ranks indicates a significant difference between four treatment groups ($p<0.01$). Post hoc Dunn's test suggests * $p<0.05$ compared with vehicle-vehicle treatment; # $p<0.05$ compared with AG490-vehicle treatment. D. Olanzapine reduced the density of stubby spines via JAK2 pathway. Two-way ANOVA analysis shows a significant impact of AG490 pretreatment [$F(1,61)= 4.294$, $p<0.05$], and a significant interaction between AG490 pretreatment and olanzapine treatment [$F(1,61)=14.086$, $p<0.001$] on the density of stubby spines. Newman-Keuls multiple comparison test indicates * $p<0.05$ compared with vehicle-vehicle treatment; # $p<0.01$ compared with vehicle-olanzapine treatment and AG490-vehicle treatment. E. Olanzapine increased density of thin spines via JAK2 pathway. Two-way ANOVA analysis shows a significant impact of olanzapine treatment [$F(1,61)= 27.554$, $p<0.001$], and a significant interaction

between AG490 pretreatment and olanzapine treatment [$F(1,61)=16.867$, $p<0.001$] on the density of stubby spines. Newman-Keuls multiple comparison test indicates * $p<0.001$ compared with vehicle-vehicle treatment; # $p<0.01$ compared with vehicle-olanzapine treatment. F. Two-way ANOVA analysis suggests that olanzapine did not significantly increase mushroom spine density. G. Olanzapine increased the density of dendritic spines. Two-way ANOVA analysis shows a significant impact of olanzapine [$F(1,61)= 9.789$, $p<0.01$] on the density of stubby spines. Newman-Keuls multiple comparison test indicates * $p<0.05$ compared with vehicle-vehicle treatment; # $p<0.01$ compared with vehicle-olanzapine treatment.

CHAPTER FOUR: GENERAL CONCLUSION

SUMMARY OF RESULTS AND SIGNIFICANCE

As one of the most abundant serotonin receptors in the prefrontal cortex, 5-HT2A receptors are a major regulator of higher-order executive tasks such as learning, working memory, and behavioral flexibility, dysregulation of which is involved in many psychiatric disorders (Leiser *et al*, 2015; Williams *et al*, 2002). Mounting evidence suggests that 5-HT2A receptors regulate prefrontal cortical circuitries by controlling dendritic spines (Jones *et al*, 2009; Xia *et al*, 2003; Yoshida *et al*, 2011). However, the mechanisms underlying the regulation of dendritic spines by 5-HT2A receptors were unknown. The results presented here suggest two ways in which 5-HT2A receptor signaling pathways are involved in the regulation of dendritic spines: 1) serotonylation of small G protein of the Rho family, and 2) gene regulation via activation of the JAK2/STAT3 signaling pathway.

In chapter two, we demonstrated the functional consequences of 5-HT2A receptor-induced serotonylation of small G proteins of the Rho family in primary neuronal cells. We found that stimulation of 5-HT2A/2C receptors caused transamidation of Rac1 and Cdc42, but not RhoA, in both A1A1v cells and rat primary cortical culture. The transamidation is mediated by TGases, possibly TGase2 and TGase1, as suggested by TGase inhibitor cystamine or knock down of TGase2 with siRNA in rat primary cortical neurons. Transamidation of Rac1 and Cdc42 led to their activation, since inhibition of TGases significantly decreased their transamidation and activation. Moreover, we demonstrated that stimulation of both 5-HT2A and 5-HT2C receptors contributed to DOI-induced Rac1 transamidation, since inhibition of 5-HT2A receptors

by MDL100907 and 5-HT2C receptors by SB242084 blocked the DOI-stimulated Rac1 transamidation. We also found that DOI-induced TGase-catalyzed transamidation of Rac1 occurs at Q61 in A1A1v cells, as demonstrated by site-directed mutagenesis at Q61 of Rac1. Furthermore, we extended our findings from 5-HT2A/2C receptors to another Gαq/11-coupled receptor, muscarinic acetylcholine receptors. In SH-SY5Y cells, stimulation of muscarinic receptors by carbachol increased TGase-catalyzed Rac1 transamidation, thus resulting in activation of Rac1. In addition, stimulation of 5-HT2A/2C receptors by DOI leads to a transient dendritic spine enlargement, which was blocked by TGase inhibitor cystamine, suggesting 5-HT2A/2C receptors-induced transamidation of Rac1 and Cdc42 is involved in the regulation of dendritic spines by 5-HT2A/2C receptors.

Small G proteins are emerging as major targets for serotonylation and other monoaminylation, which are involved in a wide range of physiological functions, including platelet activation, SERT translocation and insulin secretion(Muma and Mi, 2015; Paulmann *et al*, 2009; Walther *et al*, 2003). We previously reported Rac1 serotonylation in A1A1v cells, derived from rat embryonic cortex (Dai *et al*, 2008). However, the function of Rac1 serotonylation in neuronal cells remained poorly understood. The Rho family, including Rac1, Cdc42 and RhoA, are associated with various cellular functions, particularly regulation of actin cytoskeleton, neurite outgrowth and neuronal differentiation(Tolias *et al*, 2011). Our study suggests that TGase activity plays a role in the 5-HT2A/2C receptor-stimulated dendritic spine enlargement. However, whether serotonylation of Rac1 and Cdc42 has a direct impact on dendritic spines needs to be further explored. Moreover, it has been suggested that Gαq/11 coupled receptors

and downstream PLC and Ca2+ signaling are involved with regulation of dendritic spines (Horne and Dell'Acqua, 2007; Spires et al, 2005; Wijetunge et al, 2008). However, little is known about the signaling molecules coordinating the mechanisms. Stimulation of Gαq/11-coupled receptors, such as bradykinin or endothelin-1 receptor, activates small G proteins (Clerk et al, 2001; van Leeuwen et al, 1999). The present study demonstrated that stimulation of muscarinic receptors activate Rac1 via TGase-modified Rac1 transamidation. The results indicate that besides 5-HT2A/2C receptors, other Gαq/11-coupled receptors may also regulate dendritic spines through the TGase-catalyzed monoaminylation of small G proteins of the Rho family.

In the studies presented in chapter three, we shifted our focus from acute stimulation of 5-HT2A receptors to chronic treatment with inverse agonists of 5-HT2A receptor. Our previous studies found that chronic treatment with atypical antipsychotics olanzapine and clozapine, and the selective 5-HT2A receptor antagonist MDL100907 activated JAK2/STAT3 pathway in both A1A1v cells and in rat frontal cortex. Activation of JAK2/STAT3 increased expression of RGS7 mRNA and protein levels, which may contribute to the olanzapine-induced desensitization of 5-HT2A receptors. In present study, a microarray assay was performed to explore other possible changes in gene expression caused by 5-HT2A receptor antagonist-induced JAK2/STAT signaling. The results showed that 205 genes were significantly changed by the JAK2 inhibitor AG490, olanzapine or the combination of both drugs compared to the vehicle treated controls. At least 92 of the 205 genes are changed by olanzapine via JAK/STAT signaling pathway. These genes are involved in the etiology of schizophrenia, neuronal signal transduction, neuronal growth factor, metabolism and energy, and synaptic plasticity. Real-time qPCR

validated that Crf, Cbln1, Nrg1, Hsp70, and Fabp7 were upregulated by olanzapine in a JAK2/STAT3 dependent manner. Filip1, Homer1, Pak1ip1 and Arc were significantly changed by olanzapine, but the effects of AG490 were not statistically significant. Western blotting and ELISA verified that the protein levels of Nrg1 and Hsp70 were significantly increased by olanzapine via JAK2/STAT3, while kalirin7 and spinophilin protein levels were changed by olanzapine independently of JAK2/STAT3. Given the changes in numerous genes related to cytoskeletal, post-synaptic density and synaptic plasticity, the impact of olanzapine administration on dendritic spines in rat cortical primary culture was also examined. Our results show for the first time that olanzapine causes changes in the expression of various important genes that may be involved in the etiology of schizophrenia and therapeutic effects of atypical antipsychotics, via JAK2/STAT3 signaling pathway.

LIMITATIONS OF THE PRESENT STUDIES

There are certain limitations that should be noted when interpreting the data of the present studies. One major limitation of present studies is that we did not directly establish a causal relationship between Rac1 transamidation and activation. Based on the effect of cystamine on Rac1 transamidation and activity, we only could conclude that 5-HT2A/2C receptor stimulation increased TGase-catalyzed transamidation of Rac1, which may contribute to the activation of Rac1. To solve this problem, we prepared Rac1 mutants and hypothesized that the mutation at the transamidation site of Rac1 would lead to deactivation of Rac1. However, we found that although the Rac1 Q61N mutant cannot be transamidated following 5-HT2A/2C receptor stimulation, but the mutant is constitutively active. It is possible that both transamidation and mutation at Q61 inhibit

GTP hydrolysis of Rac1, thereby inhibiting inactivation of Rac1. Due to this limitation, we were unable to prove that transamidation of Rac1 directly results in activation of Rac1. This difficulty also compromised our ability to build a causal relationship between serotonylation of Rho family members and changes in dendritic spines. Previous studies have demonstrated that overexpressing constitutively active Rac1 in hippocampal cultured neurons disrupted dendritic spine morphology (Nakayama *et al*, 2000). Transgenic mice expressing constitutively active Rac1 showed increased spine density in cerebellar Purkinje cells (Luo *et al*, 1996). Therefore, transfection Rac1 Q61N mutant in the rat primary cortical neurons will not enable us to elucidate this problem. Further experiments could be carried out to explore the role of Rac1 and Cdc42 transamidation in the 5-HT2A/2C receptor-induced dendritic spine enlargement.

Another limitation of present studies is that we did not confirm whether serotonin is associated with Rac1 and Cdc42 via a TGase-catalyzed covalent bond in rat primary cortical culture. A previous study showed that cystamine reduced the serotonin-associated Rac1 in A1A1v cells in immunoprecipitation experiments (Dai *et al*, 2008), suggesting that serotonin is the binding partner of Rac1. In the present study, the TGase-modified Rac1 did not show a significant upward shift on immunoblots compared with native Rac1 in cell lysates, suggesting that the binding partner of Rac1, is a small molecule likely an amine, such as serotonin. SHSY-5Y human neuroblastoma cell line has many features of dopaminergic neurons, such as expressing tyrosine hydroxylase and dopamine-beta-hydroxylase and dopamine transporter (Xie *et al*, 2010). Therefore, dopamine is likely to serve as the binding partner of Rac1 in SH-SY5Y cells.

There is a gap between the in vivo rat microarray studies and primary cortical culture studies in chapter three. The in vivo rat studies suggest that chronic treatment with olanzapine altered the expression of a number of genes at the mRNA and protein level, which are associated the regulation of the actin cytoskeleton, postsynaptic density, dendritic spine morphogenesis and synaptic plasticity. Therefore, we hypothesized that the olanzapine-induced changes in the expression of these genes, possibly via JAK2/STAT3 pathway, contributes to alterations in dendritic spines. However, we did not test this hypothesis in the rat frontal cortex in vivo, but examined the influences of olanzapine on dendritic spines in rat primary culture instead. Although a maturation of dendritic spines by olanzapine treatment was observed in primary culture, whether this effect could be replicated in rat frontal cortex remains unclear. Moreover, we verified that JAK2 was activated by chronic olanzapine and MDL100907 treatment, and observed an increase in Nrg1 protein level in rat primary cortical culture, but it is not known whether the expression pattern of other genes which were differentially altered by olanzapine in the rat frontal cortex is also affected in primary cortical neurons.

FUTURE STUDIES

First, the effects of selective serotonin reuptake inhibitors (SSRIs) and other monoamine reuptake inhibitors (MRIs) on serotonylation and other monoaminylation of small G proteins and DOI-induced changes in dendritic spines could be studied in the future. Reduction in hippocampal dendritic spine density in an olfactory bulbectomy model of depression was observed (Norrholm and Ouimet, 2001). Long-term depression is associated with dendritic spine shrinkage in neonatal rat hippocampus (Zhou *et al*, 2004). Zheng and colleagues showed that eighteen-day administration of fluoxetine and

fluvoxamine caused a reduction in dendritic spine density in neonatal mice, while increases dendritic spine density in adult mice (Zheng *et al*, 2011). Acute but not chronic SSRI treatment increased total dendritic length and dendritic spine density in CA1 region of juvenile rat brains (Norrholm and Ouimet, 2000). However, the mechanisms underlying the effects of SSRIs and other MRIs on dendritic spines still remain unknown. As a novel potential mechanism contributing to morphological regulation of dendritic spines by serotonin signaling, serotonylation and monoaminylation of small G protein could serve as a drug target for treating depression and other psychiatric disorders.

A primary amine, such as 5-HT, is required to be present in the cells as a substrate for serotonylation and other monoaminylation. Therefore, transporters for these monoamines, such as SERT, could contribute to the availability of the monoamines. In adult brains, SERT is expressed in serotonergic neurons and the distribution is limited to presynaptic axons in the cortex (Tao-Cheng and Zhou, 1999). In contrast, during development, SERT is broadly expressed in nonserotonergic neurons throughout the brain and distributed throughout the entire plasma membrane, including the postsynaptic dendritic spines (Zhou *et al*, 2000). Therefore, SERT is more likely to be involved in the serotonylation during the development, but not the adulthood. This may also explain why SSRIs exert differential effects on dendritic spines between neonatal and adult animals as introduced above. On the other hand, other monoamine transporters, such as norepinephrine transporter (NET), and dopamine transporter (DAT), which share a high level of structural and sequence homology and functional overlap with SERT, can transport 5-HT as well (Carboni *et al*, 1990; Larsen *et al*, 2011; Morón *et al*, 2002). Therefore, NET and DAT could also contribute to serotonylation and serotonylation-

regulated dendritic spines (Miner *et al*, 2003). Moreover, DA could be a substrate for Rac1 monoaminylation mediated Gαq/11-coupled receptor (Muma and Mi, 2015). Therefore, NET and DAT could also be involved in other monoaminylation, such as dopaminylation.

To study the role of serotonylation of small G proteins of the Rho family in the regulation of dendritic spines, further work should be done to test whether serotonin is the direct binding partner of Rac1 and Cdc42 in rat primary culture. Once serotonylation of small G proteins is validated in rat primary culture, studies could be performed to investigate whether treatment with SSRIs is able to block the DOI-induced serotonylation of Rac1 and Cdc42. Our preliminary immunoblot result showed that SERT is expressed in A1A1v cells and rat primary cortical culture at DIV21, therefore they could serve as cell models to study the effects of SSRIs. If serotonin is required for Rac1 transamidation, blocking the reuptake of serotonin into the cell could inhibit the DOI-stimulated Rac1 transamidation and activation. On the other hand, the extracellular accumulation of serotonin following acute SSRI treatment may enhance the stimulation of 5-HT2A/2C receptors, thus increasing Rac1 serotonylation and activation. The consequences of SSRI treatment on the DOI-induced dendritic spine enlargement need to be studied as well.

Disrupted-in-Schizophrenia-1 (DISC1) has been known to be involved in the etiology of schizophrenia, depression and other psychiatric diseases. Prolonged knockdown of DISC1 causes schizophrenia-like behaviors in animals. A recent study demonstrated that novel inhibitors to PAKs, downstream effectors of Rac1 and Cdc42, significantly ameliorated synaptic deterioration caused by DISC1 knockdown in vitro and

mitigated synaptic deterioration and ameliorated schizophrenia-like behavior in a DISC1 knockdown mouse model (Hayashi-Takagi *et al*, 2014). Inhibition of Rac1 and Cdc42 transamidation by TGase inhibitor cystamine or siRNA targeting TGase2 leads to decrease in Rac1 and Cdc42 activity and downstream PAK1 phosphorylation. Therefore, I hypothesize that inhibition of Rac1 and Cdc42 transamidation by cystamine or siRNA could also ameliorate dendritic spine deterioration and schizophrenia-like behavior induced by DISC1 knockdown. To test this hypothesis, we need to replicate 5-HT2A/2C receptor-mediated Rac1 and Cdc42 transamidation in vivo. The previous attempts to replicate DOI-induced Rac1 transamidation in rat frontal cortex failed. This could be attributed to the dosage, route, and time course used in our preliminary experiment did not mimic the conditions in our in vitro study.

We found that olanzapine induced the changes in the expression of a number of genes via the JAK2/STAT3 pathways. Whether these genes play roles in the regulation of dendritic spines, therapeutic and adverse effects of atypical antipsychotics, needs to be investigated. For instance, our results showed that Nrg1 was increased by olanzapine treatment via JAK2/STAT3 pathway. Prolonged treatment with Nrg1 promotes spine maturation and potentiates synaptic plasticity (Barros *et al*, 2009). I hypothesize that knockdown of Nrg1 would block the chronic olanzapine treatment-induced dendritic spine maturation. Furthermore, the microarray assay indicated a number of genes related to adverse metabolic effects of olanzapine, such as Cartpt, Grp, Nts and Nr4a1. All these genes exhibit a tendency to be regulated by olanzapine via JAK2/STAT3 signaling pathway. However, real-time qPCR indicated that the changes in these genes showed a relatively large variance between animals. Therefore, a larger sample size may be

required to further study these genes associated to olanzapine-caused adverse effects. Once the role of JAK2/STAT3 pathway in the regulation of these genes is validated, further studies could be performed to test whether inhibition of JAK2/STAT3 signaling by AG490 ameliorates olanzapine-caused metabolic side effects in animals. The results of these studies will improve our understanding of adverse effects of atypical antipsychotics, and shed light on the development of adjunctive therapy with atypical antipsychotics to reduce the side effects.

CONCLUSIONS

The data presented here indicates that both agonists and antagonists of 5-HT2A receptors are able to alter dendritic spines, however, in different aspects and via distinct mechanisms. The differences of these two aspects of the studies are compared in Table 4.1 and Figure 4.1. Our results suggest that acute stimulation of 5-HT2A/2C receptors and other Gαq/11-coupled receptors, which activates PLC and induces downstream Ca2+ release, increase TGase-catalyzed monoaminylation of small G proteins of Rho family, including Cdc42 and Rac1. Transamidation of Rac1 and Cdc42 leads to their activation, which in turn plays a role in the short-term regulation of actin cytoskeleton dynamic. Chronic treatment with 5-HT2A receptor antagonist olanzapine activates the JAK2/STAT3 pathway, which in turn regulate transcription of a number of genes, which play essential roles in the long-term regulation of dendritic spines.

Table 4.1 Comparison of studies on effects of 5-HT2A receptor agonist and antagonist on dendritic spines

Treatment	*Agonist: DOI*	*Antagonist: olanzapine*
Effect of drug	Increase in dendritic spine area	Type-specific change of dendritic spine number
Change of spine feature	Dendritic spine plasticity	Dendritic spine maturation
Time course	Acute	Chronic
Hypothetical mechanism	5-$HT_{2A/2C}$ receptor-induced serotonylation of Rho family	Transcriptional changes via JAK2/STAT3 pathway

Table 4. 1

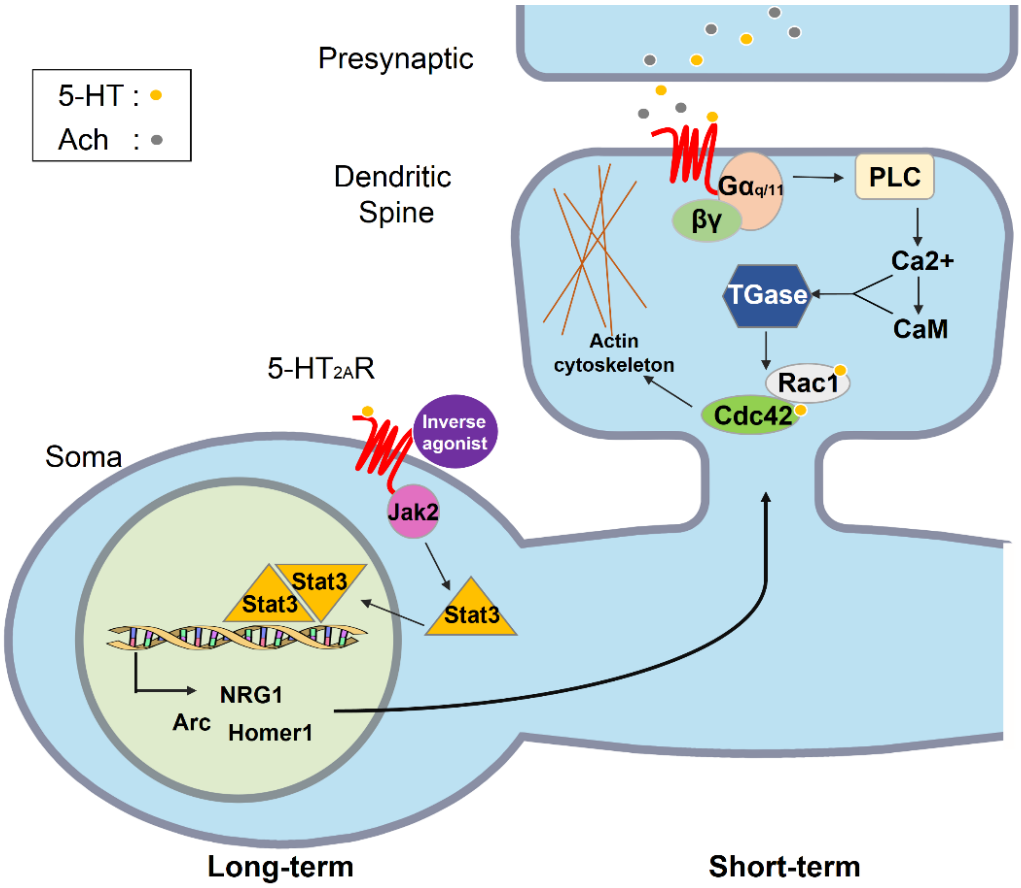


Figure 4. 1

Figure 4.1. Molecular mechanisms for 5-HT2A receptor-mediated regulation of dendritic spines. Short-term regulation of dendritic spines is produced by acute stimulation of 5-HT2A/2C receptors and other Gαq/11-coupled receptors, which activates PLC and induces downstream Ca2+-dependent TGase activation. TGases catalyze monoaminylation of small G proteins of Rho family, including Cdc42 and Rac1, thus causing Rac1 and Cdc42 activation, which in turn regulates actin cytoskeleton dynamics and causes a transient dendritic spine enlargement. Long-term regulation of dendritic spines is mediated via JAK2/STAT3 pathway activated by chronic treatment with 5-HT2A receptor agonists or inverse agonists including atypical antipsychotics.

JAK2/STAT3 pathway in turn controls transcription of a number of genes, which play essential roles in the regulation of dendritic spines.

LITERATURE CITED

Aaronson DS, Horvath CM (2002). A road map for those who don't know JAK-STAT. *Science* **296**(5573): 1653-1655.

Abbott C, Rossi M, Wren A, Murphy K, Kennedy A, Stanley S*, et al* (2001). Evidence of an orexigenic role for cocaine-and amphetamine-regulated transcript after administration into discrete hypothalamic nuclei. *Endocrinology* **142**(8): 3457-3463.

Agarwal A, Zhang M, Trembak-Duff I, Unterbarnscheidt T, Radyushkin K, Dibaj P*, et al* (2014). Dysregulated expression of neuregulin-1 by cortical pyramidal neurons disrupts synaptic plasticity. *Cell reports* **8**(4): 1130-1145.

Aghajanian G, Marek G (1997). Serotonin induces excitatory postsynaptic potentials in apical dendrites of neocortical pyramidal cells. *Neuropharmacology* **36**(4): 589-599.

Amodeo DA, Jones JH, Sweeney JA, Ragozzino ME (2014). Risperidone and the 5‐HT2A Receptor Antagonist M100907 Improve Probabilistic Reversal Learning in BTBR T+ tf/J Mice. *Autism Research* **7**(5): 555-567.

Ando Y, Imamura S, Owada MK, Kannagi R (1991). Calcium-induced intracellular cross-linking of lipocortin I by tissue transglutaminase in A431 cells. Augmentation by membrane phospholipids. *Journal of Biological Chemistry* **266**(2): 1101-1108.

Andreasen N (1995). Symptoms, signs, and diagnosis of schizophrenia. *The Lancet* **346**(8973): 477-481.

Andreasen NC (1997). Linking mind and brain in the study of mental illnesses: a project for a scientific psychopathology. *Science* **275**(5306): 1586-1593.

Andrews SB, Leapman RD, Landis D, Reese TS (1988). Activity-dependent accumulation of calcium in Purkinje cell dendritic spines. *Proceedings of the National Academy of Sciences* **85**(5): 1682-1685.

Ba W, van der Raadt J, Kasri NN (2013). Rho GTPase signaling at the synapse: implications for intellectual disability. *Experimental cell research* **319**(15): 2368-2374.

Banes AK, Shaw SM, Tawfik A, Patel BP, Ogbi S, Fulton D*, et al* (2005). Activation of the JAK/STAT pathway in vascular smooth muscle by serotonin. *American Journal of Physiology-Cell Physiology* **288**(4): C805-C812.

Barros CS, Calabrese B, Chamero P, Roberts AJ, Korzus E, Lloyd K*, et al* (2009). Impaired maturation of dendritic spines without disorganization of cortical cell layers in mice lacking NRG1/ErbB signaling in the central nervous system. *Proceedings of the National Academy of Sciences* **106**(11): 4507-4512.

Beaudoin III GM, Lee S-H, Singh D, Yuan Y, Ng Y-G, Reichardt LF*, et al* (2012). Culturing pyramidal neurons from the early postnatal mouse hippocampus and cortex. *Nature protocols* **7**(9): 1741-1754.

Benjamin J (2012). *Bipolar disorder-Unabridged Guide* Emereo Publishing.

Bennett M (2011). Schizophrenia: susceptibility genes, dendritic-spine pathology and gray matter loss. *Progress in neurobiology* **95**(3): 275-300.

Berg KA, Harvey JA, Spampinato U, Clarke WP (2008). Physiological and therapeutic relevance of constitutive activity of 5-HT 2A and 5-HT 2C receptors for the treatment of depression. *Progress in brain research* **172**: 287-305.

Berger M, Gray JA, Roth BL (2009). The expanded biology of serotonin. *Annual review of medicine* **60**: 355-366.

Bernassola F, Rossi A, Melino G (1999). Regulation of transglutaminases by nitric oxide. *Annals of the New York Academy of Sciences* **887**(1): 83-91.

Blaazer AR, Smid P, Kruse CG (2008). Structure–Activity Relationships of Phenylalkylamines as Agonist Ligands for 5‐HT2A Receptors. *ChemMedChem* **3**(9): 1299-1309.

Bolognin S, Lorenzetto E, Diana G, Buffelli M (2014). The Potential Role of Rho GTPases in Alzheimer's Disease Pathogenesis. *Molecular neurobiology* **50**(2): 406-422.

Bourne J, Harris KM (2007). Do thin spines learn to be mushroom spines that remember? *Current opinion in neurobiology* **17**(3): 381-386.

Brady S, Siegel G, Albers RW, Price D (2005). *Basic neurochemistry: molecular, cellular and medical aspects* Academic Press.

Burnet P, Chen C-H, McGowan S, Franklin M, Harrison P (1996). The effects of clozapine and haloperidol on serotonin-1A,-2A and-2C receptor gene expression and serotonin metabolism in the rat forebrain. *Neuroscience* **73**(2): 531-540.

Bustelo XR, Sauzeau V, Berenjeno IM (2007). GTP‐binding proteins of the Rho/Rac family: regulation, effectors and functions in vivo. *Bioessays* **29**(4): 356-370.

Cahill ME, Remmers C, Jones KA, Xie Z, Sweet RA, Penzes P (2013). Neuregulin1 signaling promotes dendritic spine growth through kalirin. *Journal of neurochemistry* **126**(5): 625-635.

Carboni E, Tanda G, Frau R, Di Chiara G (1990). Blockade of the noradrenaline carrier increases extracellular dopamine concentrations in the prefrontal cortex: evidence that dopamine is taken up in vivo by noradrenergic terminals. *J Neurochem* **55**(3): 1067-1070.

Chen C-C, Lu J, Zuo Y (2014). Spatiotemporal dynamics of dendritic spines in the living brain. *Frontiers in neuroanatomy* **8**.

Chen R, Gao B, Huang C, Olsen B, Rotundo RF, Blumenstock F*, et al* (2000). Transglutaminase-mediated fibronectin multimerization in lung endothelial matrix in response to TNF-α. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **279**(1): L161-L174.

Chen Y-J, Zhang M, Yin D-M, Wen L, Ting A, Wang P*, et al* (2010). ErbB4 in parvalbumin-positive interneurons is critical for neuregulin 1 regulation of long-term potentiation. *Proceedings of the National Academy of Sciences* **107**(50): 21818-21823.

Chiba T, Yamada M, Sasabe J, Terashita K, Shimoda M, Matsuoka M*, et al* (2009). Amyloid-β causes memory impairment by disturbing the JAK2/STAT3 axis in hippocampal neurons. *Molecular psychiatry* **14**(2): 206-222.

Chohan TW, Nguyen A, Todd SM, Bennett MR, Callaghan P, Arnold JC (2014). Partial genetic deletion of neuregulin 1 and adolescent stress interact to alter NMDA receptor binding in the medial prefrontal cortex. *Frontiers in behavioral neuroscience* **8**.

Clerk A, Pham FH, Fuller SJ, Sahai E, Aktories K, Marais R*, et al* (2001). Regulation of mitogen-activated protein kinases in cardiac myocytes through the small G protein Rac1. *Molecular and Cellular Biology* **21**(4): 1173-1184.

Collighan R, Griffin M (2009). Transglutaminase 2 cross-linking of matrix proteins: biological significance and medical applications. *Amino acids* **36**(4): 659-670.

Cornea-Hebert V, Watkins K, Roth B, Kroeze W, Gaudreau P, Leclerc N*, et al* (2002). Similar ultrastructural distribution of the 5-HT 2A serotonin receptor and microtubule-associated protein MAP1A in cortical dendrites of adult rat. *Neuroscience* **113**(1): 23-35.

Costall B, Naylor RJ (2004). 5-HT3 receptors. *Current Drug Targets-CNS & Neurological Disorders* **3**(1): 27-37.

Critchlow H, Maycox P, Skepper J, Krylova O (2006). Clozapine and haloperidol differentially regulate dendritic spine formation and synaptogenesis in rat hippocampal neurons. *Molecular and Cellular Neuroscience* **32**(4): 356-365.

Dai Y, Dudek NL, Li Q, Muma NA (2011). Phospholipase C, Ca2+, and calmodulin signaling are required for 5-HT2A receptor-mediated transamidation of Rac1 by transglutaminase. *Psychopharmacology* **213**(2-3): 403-412.

Dai Y, Dudek NL, Patel TB, Muma NA (2008). Transglutaminase-catalyzed transamidation: a novel mechanism for Rac1 activation by 5-hydroxytryptamine2A receptor stimulation. *Journal of Pharmacology and Experimental Therapeutics* **326**(1): 153-162.

Datta D, Arion D, Corradi JP, Lewis DA (2015). Altered expression of CDC42 signaling pathway components in cortical layer 3 pyramidal cells in schizophrenia. *Biological psychiatry*.

Dayer AG, Jacobshagen M, Chaumont-Dubel S, Marin P (2015). The 5-HT6 receptor: a new player controlling the development of neural circuits. *ACS chemical neuroscience*.

de Kloet AD, Woods SC (2010). Molecular neuroendocrine targets for obesity therapy. *Current opinion in endocrinology, diabetes, and obesity* **17**(5): 441.

Donegan JJ, Patton MS, Chavera TS, Berg KA, Morilak DA, Girotti M (2015). Interleukin-6 Attenuates Serotonin 2A Receptor Signaling by Activating the JAK-STAT Pathway. *Molecular pharmacology* **87**(3): 492-500.

Dvorsky R, Ahmadian MR (2004). Always look on the bright site of Rho: structural implications for a conserved intermolecular interface. *EMBO reports* **5**(12): 1130-1136.

Dwivedi Y, Mondal AC, Payappagoudar GV, Rizavi HS (2005). Differential regulation of serotonin (5HT)2A receptor mRNA and protein levels after single and repeated stress in rat brain: role in learned helplessness behavior. *Neuropharmacology* **48**(2): 204-214.

Eckert RL, Kaartinen MT, Nurminskaya M, Belkin AM, Colak G, Johnson GV*, et al* (2014). Transglutaminase regulation of cell function. *Physiological reviews* **94**(2): 383-417.

Elsworth JD, Morrow BA, Hajszan T, Leranth C, Roth RH (2011). Phencyclidine-induced loss of asymmetric spine synapses in rodent prefrontal cortex is reversed by acute and chronic treatment with olanzapine. *Neuropsychopharmacology* **36**(10): 2054-2061.

Esposito C, Caputo I (2005). Mammalian transglutaminases. *FEBS Journal* **272**(3): 615-631.

Evans JC, Robinson CM, Shi M, Webb DJ (2015). The Guanine Nucleotide Exchange Factor (GEF) Asef2 Promotes Dendritic Spine Formation via Rac Activation and Spinophilin-dependent Targeting. *Journal of Biological Chemistry* **290**(16): 10295-10308.

Fatemi SH, Reutiman TJ, Folsom TD, Bell C, Nos L, Fried P*, et al* (2006). Chronic olanzapine treatment causes differential expression of genes in frontal cortex of rats as revealed by DNA microarray technique. *Neuropsychopharmacology* **31**(9): 1888-1899.

Fehér Á, Juhász A, László A, Pákáski M, Kálmán J, Janka Z (2013). Serotonin transporter and serotonin receptor 2A gene polymorphisms in Alzheimer's disease. *Neuroscience letters* **534**: 233-236.

Fekete É, Vígh J, Bagi ÉE, Lénárd L (2002). Gastrin-releasing peptide microinjected into the amygdala inhibits feeding. *Brain research* **955**(1): 55-63.

Feria-Velasco A, del Angel AR, Gonzalez-Burgos I (2002). Modification of dendritic development. *Progress in brain research* **136**: 135-143.

Fesus L, Piacentini M (2002). Transglutaminase 2: an enigmatic enzyme with diverse functions. *Trends in biochemical sciences* **27**(10): 534-539.

Flatau G, Landraud L, Boquet P, Bruzzone M, Munro P (2000). Deamidation of RhoA glutamine 63 by the Escherichia coli CNF1 toxin requires a short sequence of the GTPase switch 2 domain. *Biochemical and biophysical research communications* **267**(2): 588-592.

Ford AP, Clarke DE (1993). The 5‐HT4 Receptor. *Medicinal research reviews* **13**(6): 633-662.

Frost DO, Page SC, Carroll C, Kolb B (2010). Early exposure to haloperidol or olanzapine induces long-term alterations of dendritic form. *Synapse (New York, NY)* **64**(3): 191.

Fukunaga K, Stoppini L, Miyamoto E, Muller D (1993). Long-term potentiation is associated with an increased activity of Ca2+/calmodulin-dependent protein kinase II. *Journal of Biological Chemistry* **268**(11): 7863-7867.

Ganeshina O, Berry R, Petralia R, Nicholson D, Geinisman Y (2004). Synapses with a segmented, completely partitioned postsynaptic density express more AMPA receptors than other axospinous synaptic junctions. *Neuroscience* **125**(3): 615-623.

Gareri P, De Fazio P, De Fazio S, Marigliano N, Ibbadu GF, De Sarro G (2006). Adverse Effects of atypical antipsychotics in the elderly. *Drugs & aging* **23**(12): 937-956.

Geschwind DH, Levitt P (2007). Autism spectrum disorders: developmental disconnection syndromes. *Current opinion in neurobiology* **17**(1): 103-111.

Ghadirivasfi M, Nohesara S, Ahmadkhaniha HR, Eskandari MR, Mostafavi S, Thiagalingam S*, et al* (2011). Hypomethylation of the serotonin receptor type‐2A Gene (HTR2A) at T102C polymorphic site in DNA derived from the saliva of patients with schizophrenia and bipolar disorder. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics* **156**(5): 536-545.

Gibbs J (1985). V. Effect of bombesin on feeding behavior peptides. *Life sciences* **37**(2): 147-153.

Gibbs J, Kulkosky P, Smith G (1981). Effects of peripheral and central bombesin on feeding behavior of rats. *Peptides* **2**: 179-183.

Glantz LA, Lewis DA (2000). Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. *Archives of general psychiatry* **57**(1): 65-73.

Golden SA, Christoffel DJ, Heshmati M, Hodes GE, Magida J, Davis K*, et al* (2013). Epigenetic regulation of RAC1 induces synaptic remodeling in stress disorders and depression. *Nature medicine* **19**(3): 337-344.

Gonçalves P, Araújo JR, Martel F (2015). Antipsychotics-induced metabolic alterations: focus on adipose tissue and molecular mechanisms. *European Neuropsychopharmacology* **25**(1): 1-16.

González-Maeso J, Weisstaub NV, Zhou M, Chan P, Ivic L, Ang R*, et al* (2007). Hallucinogens recruit specific cortical 5-HT 2A receptor-mediated signaling pathways to affect behavior. *Neuron* **53**(3): 439-452.

Graeff FG, Guimarães FS, De Andrade TG, Deakin JF (1996). Role of 5-HT in stress, anxiety, and depression. *Pharmacology Biochemistry and Behavior* **54**(1): 129-141.

Gray E, Guillery R (1963). A note on the dendritic spine apparatus. *Journal of anatomy* **97**(Pt 3): 389.

Gray JA, Roth BL (2001). Paradoxical trafficking and regulation of 5-HT 2A receptors by agonists and antagonists. *Brain research bulletin* **56**(5): 441-451.

Gray JA, Roth BL (2007). Molecular targets for treating cognitive dysfunction in schizophrenia. *Schizophrenia Bulletin* **33**(5): 1100-1119.

Guillet-Deniau I, Burnol A-F, Girard J (1997). Identification and localization of a skeletal muscle secrotonin 5-HT2A receptor coupled to the Jak/STAT pathway. *Journal of Biological Chemistry* **272**(23): 14825-14829.

Hajszan T, MacLusky NJ, Leranth C (2005). Short‐term treatment with the antidepressant fluoxetine triggers pyramidal dendritic spine synapse formation in rat hippocampus. *European Journal of Neuroscience* **21**(5): 1299-1303.

Hand D, Bungay P, Elliott B, Griffin M (1985). Activation of transglutaminase at calcium levels consistent with a role for this enzyme as a calcium receptor protein. *Bioscience reports* **5**: 1079-1086.

Hannon J, Hoyer D (2008). Molecular biology of 5-HT receptors. *Behavioural brain research* **195**(1): 198-213.

Hayashi-Takagi A, Araki Y, Nakamura M, Vollrath B, Duron SG, Yan Z*, et al* (2014). PAKs inhibitors ameliorate schizophrenia-associated dendritic spine deterioration in vitro and in vivo during late adolescence. *Proceedings of the National Academy of Sciences* **111**(17): 6461-6466.

Hervás A, Toma C, Romarís P, Ribasés M, Salgado M, Bayes M*, et al* (2014). The involvement of serotonin polymorphisms in autistic spectrum symptomatology. *Psychiatric genetics* **24**(4): 158-163.

Himick BA, Peter RE (1994). Bombesin acts to suppress feeding behavior and alter serum growth hormone in goldfish. *Physiology & behavior* **55**(1): 65-72.

Hirao K, Hata Y, Ide N, Takeuchi M, Irie M, Yao I*, et al* (1998). A Novel Multiple PDZ Domain-containing Molecule Interacting withN-Methyl-D-aspartateReceptors and Neuronal Cell Adhesion Proteins. *Journal of Biological Chemistry* **273**(33): 21105-21110.

Hitomi K (2005). Transglutaminases in skin epidermis. *European Journal of Dermatology* **15**(5): 313-319.

Holtmaat AJ, Trachtenberg JT, Wilbrecht L, Shepherd GM, Zhang X, Knott GW*, et al* (2005). Transient and persistent dendritic spines in the neocortex in vivo. *Neuron* **45**(2): 279-291.

Horne EA, Dell'Acqua ML (2007). Phospholipase C is required for changes in postsynaptic structure and function associated with NMDA receptor-dependent long-term depression. *The Journal of neuroscience* **27**(13): 3523-3534.

Hornung J-P (2003). The human raphe nuclei and the serotonergic system. *Journal of chemical neuroanatomy* **26**(4): 331-343.

Hou J, Zheng DZ, Zhou JY, Zhou SW (2010). Orexigenic effect of cocaine‐and amphetamine‐regulated transcript (CART) after injection into hypothalamic nuclei in streptozotocin‐diabetic rats. *Clinical and Experimental Pharmacology and Physiology* **37**(10): 989-995.

Hoyer D, Clarke DE, Fozard JR, Hartig P, Martin GR, Mylecharane EJ*, et al* (1994). International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin). *Pharmacological reviews* **46**(2): 157-203.

Hoyer D, Hannon JP, Martin GR (2002). Molecular, pharmacological and functional diversity of 5-HT receptors. *Pharmacology Biochemistry and Behavior* **71**(4): 533-554.

Huang YZ, Won S, Ali DW, Wang Q, Tanowitz M, Du QS*, et al* (2000). Regulation of neuregulin signaling by PSD-95 interacting with ErbB4 at CNS synapses. *Neuron* **26**(2): 443-455.

Hummerich R, Schloss P (2010). Serotonin—more than a neurotransmitter: transglutaminase-mediated serotonylation of C6 glioma cells and fibronectin. *Neurochemistry international* **57**(1): 67-75.

Hummerich R, Thumfart J-O, Findeisen P, Bartsch D, Schloss P (2012). Transglutaminase-mediated transamidation of serotonin, dopamine and noradrenaline to fibronectin: evidence for a general mechanism of monoaminylation. *FEBS letters* **586**(19): 3421-3428.

Hutsler JJ, Zhang H (2010). Increased dendritic spine densities on cortical projection neurons in autism spectrum disorders. *Brain research* **1309**: 83-94.

Igaz P, Toth S, Falus A (2001). Biological and clinical significance of the JAK-STAT pathway; lessons from knockout mice. *Inflammation Research* **50**(9): 435-441.

Irwin SA, Patel B, Idupulapati M, Harris JB, Crisostomo RA, Larsen BP*, et al* (2001). Abnormal dendritic spine characteristics in the temporal and visual cortices of patients with fragile‐X syndrome: a quantitative examination. *American journal of medical genetics* **98**(2): 161-167.

Jandu SK, Webb AK, Pak A, Sevinc B, Nyhan D, Belkin AM*, et al* (2013). Nitric oxide regulates tissue transglutaminase localization and function in the vasculature. *Amino acids* **44**(1): 261-269.

Johnson KB, Petersen-Jones H, Thompson JM, Hitomi K, Itoh M, Bakker EN*, et al* (2012). Vena cava and aortic smooth muscle cells express transglutaminases 1 and 4 in addition to transglutaminase 2. *American Journal of Physiology-Heart and Circulatory Physiology* **302**(7): H1355-H1366.

Johnson KB, Thompson JM, Watts SW (2010). Modification of proteins by norepinephrine is important for vascular contraction. *Frontiers in physiology* **1**.

Jones C, Watson D, Fone K (2011). Animal models of schizophrenia. *British journal of pharmacology* **164**(4): 1162-1194.

Jones KA, Srivastava DP, Allen JA, Strachan RT, Roth BL, Penzes P (2009). Rapid modulation of spine morphology by the 5-HT2A serotonin receptor through kalirin-7 signaling. *Proceedings of the National Academy of Sciences* **106**(46): 19575-19580.

Kilpatrick GJ, Bunce KT, Tyers MB (1990). 5‐HT3 receptors. *Medicinal research reviews* **10**(4): 441-475.

Kim S-Y, Chung S-I, Yoneda K, Steinert PM (1995). Expression of transglutaminase 1 in human epidermis. *Journal of investigative dermatology* **104**(2): 211-217.

Kim S-Y, Grant P, Lee J-H, Pant HC, Steinert PM (1999). Differential expression of multiple transglutaminases in human brain Increased expression and cross-linking by transglutaminases 1 and 2 in Alzheimer's disease. *Journal of Biological Chemistry* **274**(43): 30715-30721.

Kolomeets NS, Orlovskaya DD, Rachmanova VI, Uranova NA (2005). Ultrastructural alterations in hippocampal mossy fiber synapses in schizophrenia: a postmortem morphometric study. *Synapse* **57**(1): 47-55.

Kong WM, Stanley S, Gardiner J, Abbott C, Murphy K, Seth A*, et al* (2003). A role for arcuate cocaine and amphetamine-regulated transcript in hyperphagia, thermogenesis, and cold adaptation. *The FASEB Journal* **17**(12): 1688-1690.

Konopaske GT, Lange N, Coyle JT, Benes FM (2014). Prefrontal cortical dendritic spine pathology in schizophrenia and bipolar disorder. *JAMA psychiatry* **71**(12): 1323-1331.

Kornau H-C, Schenker LT, Kennedy MB, Seeburg PH (1995). Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science* **269**(5231): 1737-1740.

Kozma R, Sarner S, Ahmed S, Lim L (1997). Rho family GTPases and neuronal growth cone remodelling: relationship between increased complexity induced by Cdc42Hs, Rac1, and acetylcholine and collapse induced by RhoA and lysophosphatidic acid. *Molecular and cellular biology* **17**(3): 1201-1211.

Kristensen P, Judge ME, Thim L, Ribel U, Christjansen KN, Wulff BS*, et al* (1998). Hypothalamic CART is a new anorectic peptide regulated by leptin. *Nature* **393**(6680): 72-76.

Kukkonen J, Ojala P, Näsman J, Hämäläinen H, Heikkilä J, Akerman K (1992). Muscarinic receptor subtypes in human neuroblastoma cell lines SH-SY5Y and IMR-32 as determined by receptor binding, Ca++ mobilization and northern blotting. *Journal of Pharmacology and Experimental Therapeutics* **263**(3): 1487-1493.

Kursungoz C, Ak M, Yanik T (2015). Effects of risperidone treatment on the expression of hypothalamic neuropeptide in appetite regulation in Wistar rats. *Brain research* **1596**: 146-155.

Kwon O-B, Longart M, Vullhorst D, Hoffman DA, Buonanno A (2005). Neuregulin-1 reverses long-term potentiation at CA1 hippocampal synapses. *The Journal of neuroscience* **25**(41): 9378-9383.

Ladenheim E, Hampton L, Whitney A, White W, Battey J, Moran T (2002). Disruptions in feeding and body weight control in gastrin-releasing peptide receptor deficient mice. *Journal of endocrinology* **174**(2): 273-281.

Ladenheim E, Taylor J, Coy D, Moore K, Moran T (1996). Hindbrain GRP receptor blockade antagonizes feeding suppression by peripherally administered GRP. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **271**(1): R180-R184.

Larsen MB, Sonders MS, Mortensen OV, Larson GA, Zahniser NR, Amara SG (2011). Dopamine transport by the serotonin transporter: a mechanistically distinct mode of substrate translocation. *The Journal of Neuroscience* **31**(17): 6605-6615.

Larsen PJ, Vrang N, Petersen PC, Kristensen P (2000). Chronic intracerebroventricular administration of recombinant CART (42–89) peptide inhibits food intake and causes weight loss in lean and obese Zucker (fa/fa) rats. *Obesity research* **8**(8): 590-596.

Lau J, Herzog H (2014). CART in the regulation of appetite and energy homeostasis. *Frontiers in neuroscience* **8**.

Law AJ, Hutchinson LJ, Burnet PW, Harrison PJ (2004). Antipsychotics increase microtubule‐associated protein 2 mRNA but not spinophilin mRNA in rat hippocampus and cortex. *Journal of neuroscience research* **76**(3): 376-382.

Lee J-H, Jang S-I, Yang J-M, Markova NG, Steinert PM (1996). The proximal promoter of the human transglutaminase 3 gene stratified squamous epithelial-specific expression in cultured cells is mediated by binding of sp1 and ets transcription factors to a proximal promoter element. *Journal of Biological Chemistry* **271**(8): 4561-4568.

Lee S-JR, Escobedo-Lozoya Y, Szatmari EM, Yasuda R (2009). Activation of CaMKII in single dendritic spines during long-term potentiation. *Nature* **458**(7236): 299-304.

Leiser SC, Li Y, Pehrson AL, Dale E, Smagin G, Sanchez C (2015). Serotonergic Regulation of Prefrontal Cortical Circuitries Involved in Cognitive Processing: A Review of Individual 5-HT Receptor Mechanisms and Concerted Effects of 5-HT Receptors Exemplified by the Multimodal Antidepressant Vortioxetine. *ACS chemical neuroscience*.

Lerm M, Selzer J, Hoffmeyer A, Rapp U, Aktories K, Schmidt G (1999). Deamidation of Cdc42 and Rac by Escherichia colicytotoxic necrotizing factor 1: activation of c-Jun N-terminal kinase in HeLa cells. *Infection and immunity* **67**(2): 496-503.

Leucht S, Pitschel-Walz G, Abraham D, Kissling W (1999). Efficacy and extrapyramidal side-effects of the new antipsychotics olanzapine, quetiapine, risperidone, and sertindole compared to conventional antipsychotics and placebo. A meta-analysis of randomized controlled trials. *Schizophrenia research* **35**(1): 51-68.

Li N, Wallen N, Ladjevardi M, Hjemdahl P (1997). Effects of serotonin on platelet activation in whole blood. *Blood coagulation & fibrinolysis* **8**(8): 517-524.

Lian J, De Santis M, He M, Deng C (2015). Risperidone-induced weight gain and reduced locomotor activity in juvenile female rats: The role of histaminergic and NPY pathways. *Pharmacological Research* **95**: 20-26.

Lindsley CW (2012). The top prescription drugs of 2011 in the United States: antipsychotics and antidepressants once again lead CNS therapeutics. *ACS chemical neuroscience* **3**(8): 630-631.

Lorand L (2001). Factor XIII: structure, activation, and interactions with fibrinogen and fibrin. *Annals of the New York Academy of Sciences* **936**(1): 291-311.

Lorand L, Graham RM (2003). Transglutaminases: crosslinking enzymes with pleiotropic functions. *Nature Reviews Molecular Cell Biology* **4**(2): 140-156.

Lovenberg TW, Baron BM, de Lecea L, Miller JD, Prosser RA, Rea MA*, et al* (1993). A novel adenylyl cyclase-activating serotonin receptor (5-HT7) implicated in the regulation of mammalian circadian rhythms. *Neuron* **11**(3): 449-458.

Lund IV, Hu Y, Raol YH, Benham RS, Faris R, Russek SJ*, et al* (2008). BDNF selectively regulates GABAA receptor transcription by activation of the JAK/STAT pathway. *Science signaling* **1**(41): ra9.

Luo L, Hensch TK, Ackerman L, Barbel S, Jan LY, Jan YN (1996). Differential effects of the Rac GTPase on Purkinje cell axons and dendritic trunks and spines. *Nature* **379**(6568): 837-840.

Mahmoud GS, Grover LM (2006). Growth hormone enhances excitatory synaptic transmission in area CA1 of rat hippocampus. *Journal of Neurophysiology* **95**(5): 2962-2974.

Malinow R, Schulman H, Tsien RW (1989). Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. *Science* **245**(4920): 862-866.

Marek GJ, Carpenter LL, McDougle CJ, Price LH (2003). Synergistic action of 5-HT2A antagonists and selective serotonin reuptake inhibitors in neuropsychiatric disorders. *Neuropsychopharmacology* **28**(2): 402-412.

Martino A, Ettorre M, Musilli M, Lorenzetto E, Buffelli M, Diana G (2013). Rho GTPase-dependent plasticity of dendritic spines in the adult brain. *Frontiers in cellular neuroscience* **7**.

Matsuzaki M, Ellis-Davies GC, Nemoto T, Miyashita Y, Iino M, Kasai H (2001). Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons. *Nature neuroscience* **4**(11): 1086-1092.

Matthes H, Boschert U, Amlaiky N, Grailhe R, Plassat J, Muscatelli F*, et al* (1993). Mouse 5-hydroxytryptamine5A and 5-hydroxytryptamine5B receptors define a new family of serotonin receptors: cloning, functional expression, and chromosomal localization. *Molecular pharmacology* **43**(3): 313-319.

Matus A, Ackermann M, Pehling G, Byers HR, Fujiwara K (1982). High actin concentrations in brain dendritic spines and postsynaptic densities. *Proceedings of the National Academy of Sciences* **79**(23): 7590-7594.

Mayford M, Wang J, Kandel ER, O'Dell TJ (1995). CaMKII regulates the frequency-response function of hippocampal synapses for the production of both LTD and LTP. *Cell* **81**(6): 891-904.

Mercado CP, Kilic F (2010). Molecular mechanisms of SERT in platelets: regulation of plasma serotonin levels. *Molecular interventions* **10**(4): 231.

Mestre TA, Zurowski M, Fox SH (2013). 5-Hydroxytryptamine 2A receptor antagonists as potential treatment for psychiatric disorders. *Expert opinion on investigational drugs* **22**(4): 411-421.

Meyer D, Bonhoeffer T, Scheuss V (2014). Balance and stability of synaptic structures during synaptic plasticity. *Neuron* **82**(2): 430-443.

Min SS, An J, Lee J-H, Seol GH, Im JH, Kim H-S*, et al* (2011). Neuregulin-1 prevents amyloid β-induced impairment of long-term potentiation in hippocampal slices via ErbB4. *Neuroscience letters* **505**(1): 6-9.

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. *Journal of Comparative Neurology* **466**(4): 478-494.

Mnie-Filali O, Lambas-Senas L, Scarna H, Haddjeri N (2009). Therapeutic potential of 5-HT7 receptors in mood disorders. *Current drug targets* **10**(11): 1109-1117.

Monsonego A, Friedmann I, Shani Y, Eisenstein M, Schwartz M (1998). GTP-dependent conformational changes associated with the functional switch between G α and cross-linking activities in brain-derived tissue transglutaminase. *Journal of molecular biology* **282**(4): 713-720.

Moody TW, Merali Z (2004). Bombesin-like peptides and associated receptors within the brain: distribution and behavioral implications. *Peptides* **25**(3): 511-520.

Morón JA, Brockington A, Wise RA, Rocha BA, Hope BT (2002). Dopamine uptake through the norepinephrine transporter in brain regions with low levels of the dopamine transporter: evidence from knock-out mouse lines. *The Journal of neuroscience* **22**(2): 389-395.

Muma N, Singh R, Vercillo M, D'Souza D, Zemaitaitis B, Garcia F*, et al* (2007a). Chronic olanzapine activates the Stat3 signal transduction pathway and alters expression of components of the 5-HT 2A receptor signaling system in rat frontal cortex. *Neuropharmacology* **53**(4): 552-562.

Muma NA, Mi Z (2015). Serotonylation and transamidation of other monoamines. *ACS Chemical Neuroscience*.

Muma NA, Singh RK, Vercillo MS, D'Souza DN, Zemaitaitis B, Garcia F*, et al* (2007b). Chronic olanzapine activates the Stat3 signal transduction pathway and alters expression of components of the 5-HT2A receptor signaling system in rat frontal cortex. *Neuropharmacology* **53**(4): 552-562.

Murakoshi H, Wang H, Yasuda R (2011). Local, persistent activation of Rho GTPases during plasticity of single dendritic spines. *Nature* **472**(7341): 100-104.

Murakoshi H, Yasuda R (2012). Postsynaptic signaling during plasticity of dendritic spines. *Trends in neurosciences* **35**(2): 135-143.

Murthy K, Zhou H, Grider J, Brautigan D, Eto M, Makhlouf G (2003). Differential signalling by muscarinic receptors in smooth muscle: m2-mediated inactivation of myosin light chain kinase via Gi3, Cdc42/Rac1 and p21-activated kinase 1 pathway, and m3-mediated MLC20 (20 kDa regulatory light chain of myosin II) phosphorylation via Rho-associated kinase/myosin phosphatase targeting subunit 1 and protein kinase C/CPI-17 pathway. *Biochem J* **374**: 145-155.

Nagatomo T, Rashid M, Muntasir HA, Komiyama T (2004). Functions of 5-HT 2A receptor and its antagonists in the cardiovascular system. *Pharmacology & therapeutics* **104**(1): 59-81.

Nakayama AY, Harms MB, Luo L (2000). Small GTPases Rac and Rho in the maintenance of dendritic spines and branches in hippocampal pyramidal neurons. *The Journal of Neuroscience* **20**(14): 5329-5338.

Nakhate KT, Subhedar NK, Bharne AP, Singru PS, Kokare DM (2013). Involvement of cocaine-and amphetamine-regulated transcript peptide in the hyperphagic and body weight promoting effects of allopregnanolone in rats. *Brain research* **1532**: 44-55.

Nasrallah H (2008). Atypical antipsychotic-induced metabolic side effects: insights from receptor-binding profiles. *Molecular psychiatry* **13**(1): 27-35.

Nic Dhonnchadha BA, Hascoet M, Jolliet P, Bourin M (2003). Evidence for a 5-HT2A receptor mode of action in the anxiolytic-like properties of DOI in mice. *Behav Brain Res* **147**(1-2): 175-184.

Nicolas CS, Peineau S, Amici M, Csaba Z, Fafouri A, Javalet C*, et al* (2012). The Jak/STAT pathway is involved in synaptic plasticity. *Neuron* **73**(2): 374-390.

Niesler B, Walstab J, Combrink S, Möller D, Kapeller J, Rietdorf J*, et al* (2007). Characterization of the novel human serotonin receptor subunits 5-HT3C, 5-HT3D, and 5-HT3E. *Molecular pharmacology* **72**(1): 8-17.

Nimchinsky EA, Sabatini BL, Svoboda K (2002). Structure and function of dendritic spines. *Annual review of physiology* **64**(1): 313-353.

Nimchinsky EA, Yasuda R, Oertner TG, Svoboda K (2004). The number of glutamate receptors opened by synaptic stimulation in single hippocampal spines. *The Journal of neuroscience* **24**(8): 2054-2064.

Nolte J, Sundsten J (2002). *The human brain: an introduction to its functional anatomy* Mosby St. Louis. Vol 5.

Norrholm SD, Ouimet CC (2000). Chronic fluoxetine administration to juvenile rats prevents age-associated dendritic spine proliferation in hippocampus. *Brain research* **883**(2): 205-215.

Norrholm SD, Ouimet CC (2001). Altered dendritic spine density in animal models of depression and in response to antidepressant treatment. *Synapse* **42**(3): 151-163.

Noskova TG, Kazantseva AV, Gareeva AE, Gaisina DA, Tuktarova SU, Khusnutdinova EK (2009). [Association of several polymorphic loci of serotoninergic genes with unipolar depression]. *Genetika* **45**(6): 842-848.

O'Shea JJ (1997). Jaks, STATs, cytokine signal transduction, and immunoregulation: are we there yet? *Immunity* **7**(1): 1-11.

Okamoto K, Bosch M, Hayashi Y (2009). The roles of CaMKII and F-actin in the structural plasticity of dendritic spines: a potential molecular identity of a synaptic tag? *Physiology* **24**(6): 357-366.

Pan J, Fukuda K, Kodama H, Makino S, Takahashi T, Sano M*, et al* (1997). Role of angiotensin II in activation of the JAK/STAT pathway induced by acute pressure overload in the rat heart. *Circulation Research* **81**(4): 611-617.

Pan J, Kao YL, Joshi S, Jeetendran S, DiPette D, Singh US (2005). Activation of Rac1 by phosphatidylinositol 3‐kinase in vivo: role in activation of mitogen‐activated protein kinase (MAPK) pathways and retinoic acid‐induced neuronal differentiation of SH‐SY5Y cells. *Journal of neurochemistry* **93**(3): 571-583.

Pandey GN, Pandey SC, Ren X, Dwivedi Y, Janicak PG (2003). Serotonin receptors in platelets of bipolar and schizoaffective patients: effect of lithium treatment. *Psychopharmacology (Berl)* **170**(2): 115-123.

Parrini MC, Lei M, Harrison SC, Mayer BJ (2002). Pak1 kinase homodimers are autoinhibited in trans and dissociated upon activation by Cdc42 and Rac1. *Molecular cell* **9**(1): 73-83.

Paulmann N, Grohmann M, Voigt J-P, Bert B, Vowinckel J, Bader M*, et al* (2009). Intracellular serotonin modulates insulin secretion from pancreatic β-cells by protein serotonylation. *PLoS biology* **7**(10): e1000229.

Peddie C, Davies H, Colyer F, Stewart M, Rodriguez J (2008). Colocalisation of serotonin 2A receptors with the glutamate receptor subunits NR1 and GluR2 in the dentate gyrus: an ultrastructural study of a modulatory role. *Experimental neurology* **211**(2): 561-573.

Penumatsa K, Abualkhair S, Wei L, Warburton R, Preston I, Hill NS*, et al* (2014). Tissue transglutaminase promotes serotonin-induced AKT signaling and mitogenesis in pulmonary vascular smooth muscle cells. *Cellular signalling* **26**(12): 2818-2825.

Penzes P, Buonanno A, Passafaro M, Sala C, Sweet RA (2013). Developmental vulnerability of synapses and circuits associated with neuropsychiatric disorders. *Journal of neurochemistry* **126**(2): 165-182.

Penzes P, Cahill ME, Jones KA, VanLeeuwen J-E, Woolfrey KM (2011). Dendritic spine pathology in neuropsychiatric disorders. *Nature neuroscience* **14**(3): 285-293.

Peters A, Palay SL (1991). *The fine structure of the nervous system: neurons and their supporting cells* Oxford University Press, USA.

Phillips M, Pozzo-Miller L (2015). Dendritic spine dysgenesis in autism related disorders. *Neuroscience letters*.

Polter AM, Li X (2010). 5-HT1A receptor-regulated signal transduction pathways in brain. *Cellular signalling* **22**(10): 1406-1412.

Pytliak M, Vargova V, Mechírová V, Felsoci M (2011). Serotonin receptors-from molecular biology to clinical applications. *Physiol Res* **60**(1): 15-25.

Raymond JR, Mukhin YV, Gettys TW, Garnovskaya MN (1999). The recombinant 5‐HT1A receptor: G protein coupling and signalling pathways. *British journal of pharmacology* **127**(8): 1751-1764.

Raynaud F, Moutin E, Schmidt S, Dahl J, Bertaso F, Boeckers TM*, et al* (2014). Rho-GTPase-activating Protein Interacting with Cdc-42-interacting Protein 4 Homolog 2 (Rich2) A NEW Ras-RELATED C3 BOTULINUM TOXIN SUBSTRATE 1 (Rac1) GTPase-ACTIVATING PROTEIN THAT CONTROLS DENDRITIC SPINE MORPHOGENESIS. *Journal of Biological Chemistry* **289**(5): 2600-2609.

Ricotta M, Iannuzzi M, De Vivo G, Gentile V (2010). Physio-pathological roles of transglutaminase-catalyzed reactions. *World journal of biological chemistry* **1**(5): 181.

Ridley AJ (2006). Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends in cell biology* **16**(10): 522-529.

Roesler R, Schwartsmann G (2012). Gastrin-releasing peptide receptors in the central nervous system: role in brain function and as a drug target. *Frontiers in endocrinology* **3**.

Roppongi RT, Kojima N, Hanamura K, Yamazaki H, Shirao T (2013). Selective reduction of drebrin and actin in dendritic spines of hippocampal neurons by activation of 5-HT 2A receptors. *Neuroscience letters* **547**: 76-81.

Roth BL (1994). Multiple serotonin receptors: clinical and experimental aspects. *Annals of Clinical Psychiatry* **6**(2): 67-78.

Roth BL, Hamblin M, Ciaranello R (1991). Developmental regulation of 5-HT 2 and 5-HT 1C mRNA and receptor levels. *Developmental brain research* **58**(1): 51-58.

Ruat M, Traiffort E, Leurs R, Tardivel-Lacombe J, Diaz J, Arrang J-M*, et al* (1993). Molecular cloning, characterization, and localization of a high-affinity serotonin receptor (5-HT7) activating cAMP formation. *Proceedings of the National Academy of Sciences* **90**(18): 8547-8551.

Rushing PA, Gibbs J, Geary N (1996). Brief, meal-contingent infusions of gastrin-releasing peptide 1–27 and neuromedin B-10 inhibit spontaneous feeding in rats. *Physiology & behavior* **60**(6): 1501-1504.

Ryan XP, Alldritt J, Svenningsson P, Allen PB, Wu G-Y, Nairn AC*, et al* (2005). The Rho-specific GEF Lfc interacts with neurabin and spinophilin to regulate dendritic spine morphology. *Neuron* **47**(1): 85-100.

Sabatini BL, Oertner TG, Svoboda K (2002). The life cycle of Ca 2+ ions in dendritic spines. *Neuron* **33**(3): 439-452.

Sanders-Bush E, Fentress H, Hazelwood L (2003). Serotonin 5-ht2 receptors: molecular and genomic diversity. *Molecular interventions* **3**(6): 319.

Schätzle P, Ster J, Verbich D, McKinney RA, Gerber U, Sonderegger P*, et al* (2011). Rapid and reversible formation of spine head filopodia in response to muscarinic receptor activation in CA1 pyramidal cells. *The Journal of physiology* **589**(17): 4353-4364.

Schmidt G, Goehring U-M, Schirmer J, Lerm M, Aktories K (1999). Identification of the C-terminal Part of BordetellaDermonecrotic Toxin as a Transglutaminase for Rho GTPases. *Journal of Biological Chemistry* **274**(45): 31875-31881.

Selkoe DJ (2002). Alzheimer's disease is a synaptic failure. *Science* **298**(5594): 789-791.

Shuai K, Liu B (2003). Regulation of JAK–STAT signalling in the immune system. *Nature Reviews Immunology* **3**(11): 900-911.

Silva-Gomez AB, Juárez I, Flores G (2003). Decreased dendritic spine density on prefrontal cortical and hippocampal pyramidal neurons in postweaning social isolation rats. *Brain research* **983**(1): 128-136.

Singh RK, Dai Y, Staudinger JL, Muma NA (2009a). Activation of the JAK-STAT pathway is necessary for desensitization of 5-HT2A receptor-stimulated phospholipase C signalling by olanzapine, clozapine and MDL 100907. *International Journal of Neuropsychopharmacology* **12**(5): 651-665.

Singh RK, Dai Y, Staudinger JL, Muma NA (2009b). Activation of the JAK-STAT pathway is necessary for desensitization of 5-HT2A receptor-stimulated phospholipase C signalling by olanzapine, clozapine and MDL 100907. *Int J Neuropsychopharmacol* **12**(5): 651-665.

Singh RK, Shi J, Zemaitaitis BW, Muma NA (2007a). Olanzapine increases RGS7 protein expression via stimulation of the Janus tyrosine kinase-signal transducer and activator of transcription signaling cascade. *J Pharmacol Exp Ther* **322**(1): 133-140.

Singh RK, Shi J, Zemaitaitis BW, Muma NA (2007b). Olanzapine increases RGS7 protein expression via stimulation of the Janus tyrosine kinase-signal transducer and activator of transcription signaling cascade. *Journal of Pharmacology and Experimental Therapeutics* **322**(1): 133-140.

Smith KL, Gardiner JV, Ward HL, Kong WM, Murphy KG, Martin NM*, et al* (2008). Overexpression of CART in the PVN increases food intake and weight gain in rats. *Obesity* **16**(10): 2239-2244.

Sommer C (2004). Serotonin in pain and analgesia. *Molecular neurobiology* **30**(2): 117-125.

Spacek J, Harris KM (1997). Three-dimensional organization of smooth endoplasmic reticulum in hippocampal CA1 dendrites and dendritic spines of the immature and mature rat. *The Journal of neuroscience* **17**(1): 190-203.

Spiering D, Hodgson L (2011). Dynamics of the Rho-family small GTPases in actin regulation and motility. *Cell adhesion & migration* **5**(2): 170-180.

Spires TL, Molnár Z, Kind PC, Cordery PM, Upton AL, Blakemore C*, et al* (2005). Activity-dependent regulation of synapse and dendritic spine morphology in developing barrel cortex requires phospholipase C-β1 signalling. *Cerebral Cortex* **15**(4): 385-393.

Srivastava DP, Woolfrey KM, Penzes P (2011). Analysis of dendritic spine morphology in cultured CNS neurons. *Journal of Visualized Experiments*(53).

Steiner P, Higley MJ, Xu W, Czervionke BL, Malenka RC, Sabatini BL (2008). Destabilization of the postsynaptic density by PSD-95 serine 73 phosphorylation inhibits spine growth and synaptic plasticity. *Neuron* **60**(5): 788-802.

Suzuki K, Matsui K, Ito S, Fujita K, Matsumoto H (1988). Polymorphism of the A subunit of coagulation factor XIII: evidence for subtypes of the FXIIIA* 1 and FXIIIA* 2 alleles. *American journal of human genetics* **43**(2): 170.

Sweet RA, Henteleff RA, Zhang W, Sampson AR, Lewis DA (2009). Reduced dendritic spine density in auditory cortex of subjects with schizophrenia. *Neuropsychopharmacology* **34**(2): 374-389.

Tao-Cheng J-H, Zhou F (1999). Differential polarization of serotonin transporters in axons versus soma–dendrites: an immunogold electron microscopy study. *Neuroscience* **94**(3): 821-830.

Thaw AK, Smith JC, Gibbs J (1998). Mammalian bombesin-like peptides extend the intermeal interval in freely feeding rats. *Physiology & behavior* **64**(3): 425-428.

Thim L, Nielsen PF, Judge ME, Andersen AS, Diers I, Egel-Mitani M*, et al* (1998). Purification and characterisation of a new hypothalamic satiety peptide, cocaine and amphetamine regulated transcript (CART), produced in yeast. *FEBS letters* **428**(3): 263-268.

Tohda M (2014). Changes in the expression of BNIP-3 and other neuronal factors during the cultivation period of primary cultured rat cerebral cortical neurons and an assessment of each factor's functions. *Cell signalling and Trafficking* **2**(1): 1.

Tohda M, Hang PNT, Matsumoto K (2009). Developmental changes in serotonin 2C receptor mRNA editing in the rat cerebral cortex and primary cultured cortical neurons. *Biological and Pharmaceutical Bulletin* **32**(2): 289-292.

Tolias KF, Duman JG, Um K (2011). Control of synapse development and plasticity by Rho GTPase regulatory proteins. *Progress in neurobiology* **94**(2): 133-148.

Van de Kar LD, Javed A, Zhang Y, Serres F, Raap DK, Gray TS (2001). 5-HT2A receptors stimulate ACTH, corticosterone, oxytocin, renin, and prolactin release and activate hypothalamic CRF and oxytocin-expressing cells. *The Journal of Neuroscience* **21**(10): 3572-3579.

Van Leeuwen FN, Kain HE, Van Der Kammen RA, Michiels F, Kranenburg OW, Collard JG (1997). The guanine nucleotide exchange factor Tiam1 affects neuronal morphology; opposing roles for the small GTPases Rac and Rho. *The Journal of cell biology* **139**(3): 797-807.

van Leeuwen FN, van Delft S, Kain HE, van der Kammen RA, Collard JG (1999). Rac regulates phosphorylation of the myosin-II heavy chain, actinomyosin disassembly and cell spreading. *Nature Cell Biology* **1**(4): 242-248.

Vetter IR, Wittinghofer A (2001). The guanine nucleotide-binding switch in three dimensions. *Science* **294**(5545): 1299-1304.

Vincent S, McSparren J, Wang R, Benes F (1991). Evidence for ultrastructural changes in cortical axodendritic synapses following long-term treatment with haloperidol or clozapine. *Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology* **5**(3): 147-155.

Volk B, Nagy BJ, Vas S, Kostyalik D, Simig G, Bagdy G (2010). Medicinal chemistry of 5-HT5A receptor ligands: a receptor subtype with unique therapeutical potential. *Current topics in medicinal chemistry* **10**(5): 554-578.

Vowinckel J, Stahlberg S, Paulmann N, Bluemlein K, Grohmann M, Ralser M*, et al* (2012). Histaminylation of glutamine residues is a novel posttranslational modification implicated in G-protein signaling. *FEBS letters* **586**(21): 3819-3824.

Walther DJ, Peter J-U, Winter S, Höltje M, Paulmann N, Grohmann M*, et al* (2003). Serotonylation of small GTPases is a signal transduction pathway that triggers platelet α-granule release. *Cell* **115**(7): 851-862.

Walther DJ, Stahlberg S, Vowinckel J (2011). Novel roles for biogenic monoamines: from monoamines in transglutaminase‐mediated post‐translational protein modification to monoaminylation deregulation diseases. *FEBS Journal* **278**(24): 4740-4755.

Wang H-D, Deutch AY (2008). Dopamine depletion of the prefrontal cortex induces dendritic spine loss: reversal by atypical antipsychotic drug treatment. *Neuropsychopharmacology* **33**(6): 1276-1286.

Watts SW, Priestley JR, Thompson JM (2009). Serotonylation of vascular proteins important to contraction. *PLoS One* **4**(5): e5682.

Weisstaub NV, Zhou M, Lira A, Lambe E, Gonzalez-Maeso J, Hornung JP*, et al* (2006). Cortical 5-HT2A receptor signaling modulates anxiety-like behaviors in mice. *Science* **313**(5786): 536-540.

Werneck AL, Rosso AL, Vincent MB (2009). The use of an antagonist 5-HT2a/c for depression and motor function in Parkinson' disease. *Arq Neuropsiquiatr* **67**(2B): 407-412.

Wess J (2004). Muscarinic Acetylcholine Receptor Knockout Mice: Novel Phenotypes and Clinical Implications*. *Annu Rev Pharmacol Toxicol* **44**: 423-450.

Wijetunge LS, Till SM, Gillingwater TH, Ingham CA, Kind PC (2008). mGluR5 regulates glutamate-dependent development of the mouse somatosensory cortex. *The Journal of Neuroscience* **28**(49): 13028-13037.

Wilhelmus MM, Grunberg S, Bol JG, Van Dam AM, Hoozemans JJ, Rozemuller AJ*, et al* (2009). Transglutaminases and Transglutaminase‐Catalyzed Cross‐Links Colocalize with the Pathological Lesions in Alzheimer's Disease Brain. *Brain Pathology* **19**(4): 612-622.

Williams GV, Rao SG, Goldman-Rakic PS (2002). The physiological role of 5-HT2A receptors in working memory. *The Journal of neuroscience* **22**(7): 2843-2854.

Wojcikiewicz RJ, Tobin AB, Nahorski SR (1994). Muscarinic Receptor‐Mediated Inositol 1, 4, 5‐Trisphosphate Formation in SH‐SY5Y Neuroblastoma Cells Is Regulated Acutely by Cytosolic Ca2+ and by Rapid Desensitization. *Journal of neurochemistry* **63**(1): 177-185.

Woolley ML, Marsden CA, Fone KC (2004). 5-ht6 receptors. *Current Drug Targets-CNS & Neurological Disorders* **3**(1): 59-79.

Xia Z, Gray JA, Compton-Toth BA, Roth BL (2003). A direct interaction of PSD-95 with 5-HT2A serotonin receptors regulates receptor trafficking and signal transduction. *Journal of Biological Chemistry* **278**(24): 21901-21908.

Xie H-r, Hu L-S, Li G-Y (2010). SH-SY5Y human neuroblastoma cell line: in vitro cell model of dopaminergic neurons in Parkinson's disease. *Chinese medical journal* **123**(8): 1086-1092.

Yamasaki M, Matsui M, Watanabe M (2010). Preferential localization of muscarinic M1 receptor on dendritic shaft and spine of cortical pyramidal cells and its anatomical evidence for volume transmission. *The Journal of neuroscience* **30**(12): 4408-4418.

Yanik T, Kursungoz C, Sutcigil L, Ak M (2013). Weight gain in risperidone therapy: investigation of peripheral hypothalamic neurohormone levels in psychotic patients. *Journal of clinical psychopharmacology* **33**(5): 608-613.

Yoshida H, Kanamaru C, Ohtani A, Li F, Senzaki K, Shiga T (2011). Subtype specific roles of serotonin receptors in the spine formation of cortical neurons in vitro. *Neuroscience research* **71**(3): 311-314.

Zainelli GM, Dudek NL, Ross CA, Kim S-Y, Muma NA (2005). Mutant huntingtin protein: a substrate for transglutaminase 1, 2, and 3. *Journal of Neuropathology & Experimental Neurology* **64**(1): 58-65.

Zainelli GM, Ross CA, Troncoso JC, Fitzgerald JK, Muma NA (2004). Calmodulin regulates transglutaminase 2 cross-linking of huntingtin. *The Journal of neuroscience* **24**(8): 1954-1961.

Zhang Y, Damjanoska KJ, Carrasco GA, Dudas B, D'Souza DN, Tetzlaff J*, et al* (2002). Evidence that 5-HT2A receptors in the hypothalamic paraventricular nucleus mediate neuroendocrine responses to (−) DOI. *The Journal of neuroscience* **22**(21): 9635-9642.

Zheng J, Xu D-F, Li K, Wang H-T, Shen P-C, Lin M*, et al* (2011). Neonatal exposure to fluoxetine and fluvoxamine alteres spine density in mouse hippocampal CA1 pyramidal neurons. *International journal of clinical and experimental pathology* **4**(2): 162.

Zhou FC, Sari Y, Zhang JK (2000). Expression of serotonin transporter protein in developing rat brain. *Developmental Brain Research* **119**(1): 33-45.

Zhou Q, Homma KJ, Poo M-m (2004). Shrinkage of dendritic spines associated with long-term depression of hippocampal synapses. *Neuron* **44**(5): 749-757.