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EFFECTS OF REPEATED ARIPIPRAZOLE TREATMENT ON THE cAMP AND AKT PATHWAYS IN THE DORSAL STRIATUM OF PREADOLESCENT AND ADULT RATS

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A Thesis

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ABSTRACT

The positive symptoms of schizophrenia primarily result from an excess of high affinity D2-like receptors (i.e. $D2^{High}$ receptors). First-generation antipsychotics, such as haloperidol, are D2-like antagonists that can cause severe extrapyramidal effects. Aripiprazole, a dopamine and serotonin partial agonist, has fewer side effects, making it tolerable for adults and children. Extrapyramidal effects (e.g. Parkinsonism, dystonia, and akathisia) are among the most problematic side effects produced by antipsychotic compounds, which likely result from an excess of D2-like receptors in the dorsal striatum. In order to examine the effects of repeated antipsychotic treatment on dopamine system functioning, this thesis compared the molecular effects of repeated haloperidol and aripiprazole administration on $D2^{High}$ receptors, as well as various indices of dopamine second messenger system functioning. Preadolescent and adult rats were pretreated with haloperidol or aripiprazole for 11 consecutive days. After either a 4- or 8-day drug abstinence period dorsal striatal tissue was extracted. [^{35}S]GTPγS binding assays were conducted to assess the effects of repeated haloperidol and aripiprazole treatment on the efficacy and potency of D2-like receptors. PKA subunits and components of the Akt pathway were measured using Western Blots. Results showed that repeated treatment with haloperidol or aripiprazole did not significantly affect D2-like receptor efficacy or potency in young or adult rats. In both age groups, haloperidol significantly increased the

expression of PKA-Cα, PKA-Cβ, and PKA-RII, but not p-PKA. Haloperidol also significantly increased PKA-Cβ and PKA-RII levels relative to aripiprazole. Repeated administration of haloperidol significantly increased p-GSK-3β levels in young and adult rats, but neither haloperidol nor aripiprazole significantly affected GSK-3β, Akt, or p-Akt levels. Overall, the results of this thesis indicate that repeated aripiprazole and haloperidol treatment differentially affects D2 signaling pathways in the dorsal striatum. Aripiprazole has less extreme or prolonged effects on D2 receptor signaling pathways than haloperidol, as evidenced by the lack of post-treatment upregulation in the cAMP and Akt pathways. Upregulation of D2-like receptors and, in turn, upregulation of proteins in the cAMP and Akt pathways may be partially responsible for the side effects induced by long-term antipsychotic treatment.

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CHAPTER ONE

INTRODUCTION

Approximately 4 out of 1,000 people around the world live with schizophrenia: a disorder that strains relationships and makes appropriate social interaction difficult (Stip & Tourjman, 2010). The DSM-V no longer classifies schizophrenia in categories (paranoid, disorganized, catatonic, undifferentiated, and residual), but rather it is based on a spectrum of symptoms (Bhati, 2013). According to the *Diagnostic and Statistical Manual of Mental Disorders* (5th ed.; *DSM-5*; American Psychiatric Association, 2013) diagnosis consists of "two or more psychotic symptoms for six or more months" that can be acute, ongoing, or in either partial or full remission (Bhati, 2013). Diagnostic criteria for schizophrenia include the following symptoms: "delusions (e.g., beliefs not rooted in reality), hallucinations (e.g., sensory experiences not rooted in reality), disorganized speech (e.g., incoherent verbal communication), disorganized behavior or catatonia (e.g., inappropriate or unusual actions or movements), and negative symptoms (e.g., diminished motivation or behavior)" (American Psychiatric Association, 2013; Elis, Caponigro, & Kring, 2013, p. 915).

Neural changes underlying these symptoms, as indicated by enlarged lateral ventricles and lower gray matter volumes in the frontal and medial temporal lobes, are generally consistent across adult patients (Fusar-Poli et al., 2013; Shenton, Dickey, Frumin, & McCarley, 2001; Shepard, Laurens, Matheson,

Carr, & Green, 2012). MRI studies show that neural changes progress as the brain matures; however, the brains of adults with advanced symptoms of schizophrenia have similar amounts of damage, suggesting that at some point during maturation this progression stabilizes (Andreasen et al., 2011; Olabi et al., 2011; Pantelis et al., 2005). Recent clinical studies have focused on the prodromal symptoms of schizophrenia in children and adolescents in an attempt to understand the development of the disorder (Woodberry et al., 2014). Evidence suggests that there is a strong genetic predisposition for schizophrenia, and these individuals may be vulnerable to the development of the disorder at various points during maturation: prenatal, perinatal, childhood, and post-adolescence (Cannon, 2005; Pantelis et al., 2005; Walder, Faraone, Glatt, Tsuang, & Seidman, 2014). Environmental factors, such as prenatal exposure to infection, malnutrition, toxins, obstetric complications, stress due to social isolation or familial conflict, brain injury, and drug use, contribute to the onset of schizophrenia at these vulnerable periods (Brown, 2011; McDonald & Murray, 2000; Seeman & Seeman, 2014).

Although probably involving a complex interplay between various neurotransmitter systems, the positive symptoms of schizophrenia primarily result from dopamine system dysfunction. Three brain areas likely to be involved are the prefrontal cortex, nucleus accumbens, and dorsal striatum (Krug et al., 2014; Rausch et al., 2014). Clinical efforts focus on reducing dopamine transmission through the use of dopamine receptor antagonists or partial

agonists. Specifically, the D2-like receptors, which include the D_2, D_3, and D_4 subtypes, are linked to schizophrenia (Glatt, Faraone, & Tsuang, 2003; Shafer & LeVant, 1998; Wang, Zhong, Gu, & Yan, 2003). D2-like receptors can function in two different states: a state of high affinity and a state of low affinity (i.e. $D2^{High}$ and $D2_{Low}$ receptors; Seeman, 2011). Indeed, Seeman (2011) has proposed that an excess of $D2^{High}$ receptors is responsible for the positive symptoms associated with schizophrenia.

First-generation antipsychotics or “typical antipsychotics” emerged as a treatment for schizophrenia in the 1950’s, followed by second-generation anti-psychotics or “atypical antipsychotics” in the 1980’s (Abou-Setta et al., 2012). First-generation antipsychotics are potent dopamine D2-like receptor antagonists, which exhibit a dose-dependent correlation between clinical effectiveness and affinity for D2-like receptors (Mailman & Murthy, 2010). Despite their clinical efficacy, first-generation antipsychotics have many undesirable side effects, including sedation, autonomic and cardiovascular changes, weight gain, extrapyramidal side effects (i.e. Parkinsonism, dystonia, and akathisia), neuroendocrine effects, and tardive dyskinesia (Mailman & Murthy, 2010). Extrapyramidal side effects can become permanent, and the acetylcholinergic drugs used to treat these motor deficits have their own set of side effects (dry mouth, constipation, delirium, and memory problems) (Mailman & Murthy, 2010). As a consequence, many patients are reluctant to take first-generation antipsychotics, as evidenced by the number of patients who stop long-term

treatment (Warikoo, Chakrabarti, & Grover, 2014); thus making the search for an effective antipsychotic with few complications both necessary and complex.

Second-generation antipsychotics (such as amisulpride, clozapine, olanzapine, and risperidone) cause a reduction of positive symptoms, and produce fewer side effects than first-generation antipsychotics (Croxtall, 2012; Leucht et al., 2009). Even so, second-generation antipsychotics do have some side effects, such as weight gain, myocarditis, sexual dysfunction, and acute extrapyramidal symptoms (Gardner, Baldessarini, & Waraich, 2005; Shirzadi & Ghaemi, 2006; Uçok & Gaebel, 2008). Patients may be just as likely to discontinue taking second-generation antipsychotics as first-generation antipsychotics, since long-term preference for either first- or second-generation drugs remains inconclusive (Warikoo et al., 2014). Overall, first- and second-generation antipsychotics appear about equally effective at mitigating positive symptoms, but neither are particularly effective at reducing negative symptoms (Leucht et al., 2009; McEvoy, Zygman, & Margolese, 2010; White et al., 2006). Today, 20 first-generation and second-generation antipsychotics are approved by the Food and Drug Administration for the treatment of schizophrenia, and some drugs are used off-label for the treatment of other disorders (Abou-Setta et al., 2012).

Despite these advances, there is a continuing desire to develop drugs with a superior side effect profile and a greater efficacy for treating negative symptoms. Aripiprazole is a dopamine and serotonin partial agonist with a high

affinity for D2-like receptors, thus placing it in a class of its own as a “third-generation” antipsychotic. Aripiprazole is recognized throughout the literature for its efficacy, tolerability among patients, and its ability to reduce symptoms of other mental disorders, including off-label use for severe agitation in geriatric dementia patients (Leslie, Mohamed, & Rosenheck, 2009; Maglione et al., 2009; Shekelle et al., 2007). Additionally, aripiprazole may be more effective than other drugs at treating negative symptoms (DeLeon, Patel, & Crismon, 2004; Naber & Lambert, 2004; Stip & Tourjman, 2010), although some studies suggest that aripiprazole and second-generation antipsychotics do not differ according to negative symptom reduction (Bianchini et al., 2014). According to Kirino (2012) aripiprazole has fewer side effects than other antipsychotics, although some aversive symptoms have been reported. Interestingly, actions at serotonin receptors may be involved in the muted extrapyramidal effects experienced after repeated aripiprazole treatment, since serotonin receptor antagonism increases dopaminergic transmission and, thus, could soften dopamine receptor upregulation in the dorsal striatum (Kapur & Mamo, 2003; Kuroki, Nagao, & Nakahara, 2008; Meltzer, Li, Kaneda, & Ichikawa, 2003). Aripiprazole was approved by the Food and Drug Administration in 2002 for the treatment of schizophrenia (Kirino, 2012).

Due to the high tolerability of aripiprazole, it is also administered to children and adolescents diagnosed with schizophrenia (ages 13-17) and bipolar disorder I (ages 10-17), either alone or in conjunction with lithium or valproate

(Kirino, 2012). Aripiprazole has also been administered to children for tic disorders, autism, aggression or conduct disorder, and depression (Masi et al., 2009; Stachnik & Nunn-Thompson, 2007; Yoo et al., 2011). The few studies assessing the long-term effects of aripiprazole in children are promising. Yoo et al. (2011) compared the effects of the first-generation antipsychotic haloperidol to aripiprazole, and found that the two drugs were equally efficacious at reducing tic symptoms, but aripiprazole had fewer side effects.

In summary, haloperidol and aripiprazole are equally effective at reducing positive symptoms in patients with schizophrenia, with aripiprazole being more efficacious when treating negative symptoms. Haloperidol is associated with pronounced side effects, most notably hyperprolactinemia and extrapyramidal symptoms; whereas, the development of hyperprolactinemia, weight gain, and sedation is generally much lower with aripiprazole (Kane et al., 2002; Marder et al., 2003; McEvoy, Daniel, Carson, McQuade, & Marcus, 2007). With this information as a backdrop, the purpose of this thesis is to compare the effects of haloperidol and aripiprazole at a molecular level across ontogeny, in order to determine the basis for these different treatment outcomes in humans. To this end, I assessed the effects of haloperidol and aripiprazole on $D2^{High}$ receptors and various indices of second messenger system functioning (e.g. protein kinase A subunits and Akt levels). Understanding the neuronal effects of these compounds in preadolescent and adult rats could help explain the unique treatment and side effect profiles of first-, second-, and third-generation

antipsychotics and expose potential ontogenetic differences in receptor functioning.

CHAPTER TWO

DOPAMINE SYSTEMS IN ADULT RODENTS

Overview of Dopamine Pathways and Schizophrenia

The mammalian brain contains four prominent dopamine pathways: nigrostriatal, mesolimbic, mesocortical, and tuberoinfundibular. Dysregulation of dopamine systems within two of these pathways is thought to underlie the symptoms of schizophrenia (Davis, Kahn, Ko, & Davidson, 1991). The positive symptoms of schizophrenia are attributed to hyperdopaminergia in the mesolimbic pathway, while negative symptoms are attributed to hypodopaminergia in the mesocortical pathway (Davis et al., 1991). Additionally, dopamine antagonism in the nigrostriatal dopamine pathway is responsible for extrapyramidal side effects (Nguyen, Pradel, Micallef, Montastruc, & Blin, 2004; Ossowska, 2002).

Dahlström and Fuxe (1964) categorized dopaminergic cells into nine clusters within the rat brain, with higher concentrations of dopamine in the rostral mesencephalon and lower concentrations of dopamine in the diencephalon. This patterning suggests that dopamine fibers project from caudal to rostral areas (Dahlström & Fuxe, 1964). Further research showed that clusters of cells containing high levels of dopamine originate in the ventral tegmental area and the substantia nigra pars compacta (Beckstead, Domesick, & Nauta, 1979; Moore & Bloom, 1978; Smith & Kieval, 2000). The axons of cells located in the

ventral tegmental area extend to the ventral striatum (nucleus accumbens), and are referred to as the mesolimbic pathway (Björklund & Dunnett, 2007). Some neurons project directly from the ventral tegmental area to the prefrontal cortex, and this pathway is known as the mesocortical pathway (Emson & Koob, 1978; Fuxe et al., 1974). The two pathways projecting to the nucleus accumbens are sometimes jointly referred to as the mesocorticolimbic pathway (White, 1996). The axons of cells located in the substantia nigra terminate in the dorsal striatum (caudate nucleus and putamen), which is known as the nigrostriatal pathway (Hanley & Bolam, 1997; Matsuda et al., 2009; Prensa & Parent, 2001). A fourth pathway, labeled the tuberoinfundibular pathway, projects from the hypothalamus to the anterior pituitary (Ben-Jonathan, Arbogast, & Hyde, 1989; Gudelsky, 1981; McCann et al., 1986).

Mesolimbic Pathway

Ventral Tegmental Area

Dorsal to the substantia nigra, the ventral tegmental area is involved in reward, motivation, and cognition (Lewis & O'Donnell, 2000). The ventral tegmental area is divided into four sections: the paranigral nucleus, the parabrachial pigmented area, the parafasciculus retroflexus area, and the ventral tegmental tail (Oades & Halliday, 1987). Both the paranigral and parabrachial pigmented areas contain high concentrations of dopaminergic cells, whereas the parafasciculus retroflexus area and the ventral tegmental tail contain much lower

concentrations (Ikemoto, 2007). The ventral tegmental tail contains GABAergic neurons that inhibit the firing of dopaminergic neurons within other portions of the ventral tegmental area. The prominent tract projecting from the ventral tegmental area innervates the nucleus accumbens, olfactory tubercle, amygdala, septal area, and the anterior limbic area (Ikemoto, 2007; Swanson, 1982).

Nucleus Accumbens

The nucleus accumbens, also known as the ventral striatum, is composed of a core and a shell (Heimer, Zahm, Churchill, Kalivas, & Wohltmann, 1991). Dopaminergic cells are robustly located in the shell, with few dopaminergic neurons found in the core (Deuscht & Cameron, 1992; Zahm, 1992). Dopaminergic afferents innervate the shell from the ventral tegmental tail in addition to limbic structures such as the hippocampus, lateral hypothalamus, entorhinal cortex, and amygdala (Deuscht & Cameron, 1992). Neurons located in the shell exhibit two main structural distinctions from neurons in the core. Namely, neurons in the shell have fewer dendritic spines and fewer terminals than do neurons located in the core (Meredith, Agolia, Arts, Groenewegen, & Zahm, 1992). Evidence suggests that dopaminergic release in the nucleus accumbens is modulated by glutamatergic afferents from the prefrontal cortex and the basal lateral amygdala (Jackson & Moghaddam, 2001).

Mesocortical Pathway

Neurons that comprise the mesocortical pathway project from the ventral tegmental area to limbic and medial prefrontal cortices (Björklund & Dunnett, 2007; Voorn, Jorritsma-Byham, Van Dijk, & Buijs, 1986). Most notably, neurons in the paranigral and parabrachial nuclei of the ventral tegmental area project to the medial prefrontal cortex (Lammel et al., 2008; Oades & Halliday, 1987; Ranganath & Jacob, 2015). The medial prefrontal cortex is located in the most rostral area of the brain near the midline. In rats, it is functionally involved in working memory, attention, and inhibitory response control, although cognitive abilities associated with the prefrontal cortex vary among species (Dalley, Cardinal, & Robbins, 2004; Heidbreder & Groenwegen, 2003; Ranganath & Jacob, 2015). In addition to these more prominent limbic and cortical projections, neurons originating in the ventral tegmental area also terminate in the thalamus, periaqueductal gray, parabrachial nucleus, locus coeruleus, and the median raphe nucleus (Swanson, 1982).

Nigrostriatal Pathway

Substantia Nigra

The substantia nigra contains a large concentration of dopaminergic neurons. It is subdivided into two nuclei: the substantia nigra pars compacta and the nondopaminergic substantia nigra pars reticulata. The pars compacta and pars reticulata are associated with the control of voluntary movement.

Dopaminergic neurons located in the substantia nigra pars compacta project to the dorsal striatum (caudate nucleus and putamen), globus pallidus, and entopeduncular nucleus (Gauthier, Parent, Lévesque, & Parent, 1999; Gerfen, Staines, Fibiger, & Arbuthnott, 1982). A higher concentration of these neuronal cell bodies reside in the rostral portion of the substantia nigra pars compacta in comparison to the caudal region (Tieu, 2011).

Dorsal Striatum

The caudate nucleus, which is a component of the dorsal striatum, is essential for the control of voluntary movement. The caudate nucleus lies adjacent to the thalamus and the anterior horn of the lateral ventricle. Dopaminergic efferents from the substantia nigra terminate in the caudate nucleus (Gauthier, Parent, Lévesque, & Parent, 1999; Matsuda et al., 2009; Nelson & Kreitzer, 2014), with outputs extending to the subthalamic nucleus and globus pallidus. The putamen, a second component of the dorsal striatum, also plays a critical role in the regulation of voluntary movement, and it is structurally and functionally associated with the caudate nucleus in rodents.

Subthalamic Nucleus

The subthalamic nucleus consists mainly of glutamatergic neurons that have a modulatory effect on dopamine pathways. Located posteromedially to the substantia nigra, the subthalamic nucleus modulates voluntary movement, as lesions to the subthalamic nucleus result in contralateral hemiballismus (Hamani, Saint-Cyr, Fraser, Kaplitt, & Lozano, 2004). The subthalamic nucleus receives

dopaminergic input from the dorsal striatum, and maintains a feedback loop with the globus pallidus.

Globus Pallidus

The globus pallidus is an integrated structure in rats, whereas in primates the globus pallidus has distinct external and internal components. The globus pallidus is located caudally to the dorsal striatum and is involved in controlling voluntary movement. The globus pallidus receives afferents from the substantia nigra, caudate nucleus, putamen, and subthalamic nucleus (Kita & Kita, 2001). GABAergic neurons in the globus pallidus project to the lateral and anterior nuclei of the thalamus and the subthalamic nucleus (Bevan, Booth, Eaton, & Bolam, 1998).

Entopeduncular Nucleus

The entopeduncular nucleus can be found in non-primate mammals and is located lateral to the hypothalamus. This area corresponds to the medial pallidum in primates (Benhamou & Cohen, 2014), and is functionally similar to the globus pallidus. The entopeduncular nucleus receives dopaminergic afferents from the substantia nigra pars compacta and projects GABAeric fibers to the thalamus.

Thalamus

The thalamus is comprised of nuclei that synthesize information about sensory stimuli and voluntary movement. Among the many nuclei, the ventromedial nucleus of the thalamus is involved in motor control in rats (Clavier,

Atmadja, & Fibiger, 1976). Efferents from the putamen and entopeduncular nucleus converge in the ventromedial thalamus, while outputs from the thalamus project to the supplementary motor area, the cerebellum, and the spinal cord.

Tuberoinfundibular Pathway

Hypothalamus

The hypothalamus is located below the thalamus and is a highly interconnected region comprised of many small nuclei involved in the regulation of the endocrine system and other homeostatic mechanisms (DeGroot, 1959; Reichlin, Saperstein, Jackson, Boyd, & Patel, 1976). As a consequence, the hypothalamus regulates the sympathetic nervous system and modulates stress, sleep, body temperature, physical growth, metabolism, sexual maturation, ovulation and spermatogenesis, lactation, hunger, and thirst (Besser & Mortimer, 1974; Schally, Kastin, & Arimura, 1977). The tuberoinfundibular pathway, which projects from the arcuate and paraventricular nuclei of the hypothalamus to the anterior pituitary, releases dopamine at the target site.

Anterior Pituitary

The pituitary gland contains an anterior lobe (adenohypophysis) and a posterior lobe (neurohypophysis). The neurosecretory cells in the anterior lobe release various hormones into the blood (e.g. growth hormone, luteinizing hormone, follicle-stimulating hormone, thyroid-stimulating hormone, ACTH, and prolactin) (Ben-Jonathan, Arbogast, & Hyde, 1989; Besser & Mortimer, 1974;

Buckingham, 1981; Schally et al., 1977). Dopaminergic efferents from the tuberoinfundibular pathway inhibit the release of prolactin (Enjalbert et al., 1986; Macleod & Lehmeyer, 1974; Maurer, 1980). Prolactin is primarily known for inducing lactation in mammals, but also regulates water and electrolyte balance, growth, metabolism, parental behavior, reproduction, and immunoregulation (Bole-Feysot et al., 1998).

CHAPTER THREE

DOPAMINE RECEPTORS IN ADULT RODENTS

Dopamine Receptor Subtypes and the cAMP Signaling Pathway

Dopamine receptors are relatively homologous, but they are classified functionally based on their modulation of adenylyl cyclase via G protein coupled receptors (Beaulieu & Gainetdinov, 2011; Missale, Nash, Robinson, Jaber, & Caron, 1998). D1-like receptors include both D_1 receptors (D_{1A} receptors in rats) and D_5 receptors (D_{1B} receptors in rats) (Emilien, Maloteaux, Geurts, Hoogenberg, & Cragg, 1999). When an agonist binds to D_1 or D_5 receptors, $G_{s\alpha}$ and $G_{olf\alpha}$ subunits detach from the β and γ subunits through the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP). This triggers an increase in adenylyl cyclase activity, which causes adenosine triphosphate (ATP) to lose two phosphate groups and become adenosine monophosphate, or cyclic AMP (cAMP). Cyclic AMP stimulates protein kinase A (PKA), which consequently regulates protein transcription and gene expression.

Receptors in the D2-like subclass, which include D_2, D_3, and D_4 receptors, activate the G protein subunit $G_{i\alpha}$. As the $G_{i\alpha}$ subunit dissociates from the βγ complex, through the exchange of GDP for GTP, adenylyl cyclase is inhibited. Inhibition of adenylyl cyclase results in decreased cAMP levels and PKA activity which, in turn, suppresses protein transcription and gene expression (Beaulieu & Gainetdinov, 2011; Missale et al., 1998). Thus, PKA is active when cAMP binds

to the PKA regulatory subunit, causing the alpha and beta catalytic subunits to separate (Dell'Acqua & Scott, 1997; Hubbard & Cohen, 1993).

D2 Receptors and the Akt Signaling Pathway

Akt, also referred to as Protein Kinase B, is a serine/threonine kinase that is part of a D2 receptor second messenger cascade involved in the gene regulation of cell proliferation and apoptosis (Brunet, Datta, & Greenberg, 2001; Kennedy et al., 1997; Song, Ouyang, & Bao, 2005). Thus, this pathway plays an important role in normal functioning of dopamine-regulated behaviors and is distinct from the cAMP pathway (Arguello & Gogos, 2008; Li & Gao, 2011). Activation of the Akt signaling pathway appears to be unique to D_2 receptors, but may be augmented by D_3 receptors (Beaulieu et al., 2007). When dopamine binds to D_2 receptors, the phosphorylation of $G_{i\alpha}$ by G protein receptor kinases signals β-arrestin2 to activate protein phosphatase 2A (PP2A). PP2A inhibits Akt, which inhibits glycogen synthase kinase 3 (GSK-3β) (Beaulieu, Del'Guidice, Sotnikova, Lemasson, & Gainetdinov, 2011; Li & Gao, 2011). GSK-3β, in turn, inhibits β-catenin. Evidence suggests that the Akt signaling pathway is active under hyperdopaminergic conditions, and β-catenin may mediate synaptic plasticity via glutamatergic AMPA and NMDA receptors (Li & Gao, 2011). When D2 receptors are stimulated by an agonist, the inhibitory effects of GSK-3β on β-catenin are disinhibited due to the inhibition of Akt by PP2A, thus attenuating the modulatory effects of β-catenin (Beaulieu et al., 2011).

D1-like Receptor Localization

In terms of the D1-like receptor family, D_1 receptors are the most prolific dopamine receptor subtype and are found densely in the striatum, nucleus accumbens, substantia nigra pars reticulata, olfactory bulb, amygdala, frontal cortex, hippocampus, cerebellum, thalamus, and hypothalamus (Beaulieu & Gainetdinov, 2011; Dawson, Ghelert, Yamamura, Barnett, & Wamsley, 1985). High concentrations of D_1 receptors are found in the entopenduncular nucleus and the islands of Calleja, with moderate to low concentrations in the frontal cortex and subthalamic nucleus (Dawson et al., 1985; Emilien et al., 1999). Additionally, high levels of D_1 receptor mRNA are located in the striatum, nucleus accumbens, olfactory tubercle, amygdala, and anterior cingulate cortex (Meador-Woodruff, 1991, 1994). Low levels of D_1 mRNA are found in the septum, hippocampus, hypothalamus, thalamus, cerebellum, and cortex (Meador-Woodruff, 1991, 1994).

D_5 receptors are less abundant than other dopamine receptor subtypes and are found primarily on pyramidal neurons of the prefrontal cortex, premotor cortex, cingulate cortex, entorhinal cortex, olfactory tubercle, substantia nigra pars compacta, hypothalamus, hippocampus, and dentate gyrus (Beaulieu & Gainetdinov, 2011). Relatively low levels of D_5 receptors are also located on the medium spiny neurons of the caudate nucleus and the nucleus accumbens (Beaulieu & Gainetdinov, 2011; Khan et al., 2000). Moderate levels of D_5 receptor mRNA are detected in the parafascicular nucleus of the thalamus and

hippocampus (Meador-Woodruff, 1992, 1994). Additionally, high levels of D_5 receptor mRNA are found in the endoperiform, reuniens and perifascicular nuclei of the thalamus (Apostolakis et al., 1996).

D2-like Receptor Localization

D_2 receptors are the most abundant of the D2-like receptors and are found in many of the same locations as D_1 receptors (Boyson, McGonigle, & Molinoff, 1986). Higher concentrations of D_2 receptors are evident in the striatum, nucleus accumbens, olfactory tubercle, substantia nigra, ventral tegmental area, hypothalamus, striate cortex, entorhinal cortex, choroid plexus, islands of Calleja, septum, amygdala, hippocampus, pituitary gland, and retina (Boyson et al., 1986; Charuchinda, Supavilai, Karobath, & Palacios, 1987; Gehlert & Wamsley, 1986). D_2 receptor mRNA is abundant in the substantia nigra, ventral tegmental area, and zona incerta (Meador-Woodruff, 1991, 1994). Additionally, high levels of D_2 mRNA are located in the striatum, nucleus accumbens, olfactory tubercle, and entorhinal cortex, with moderate levels in the anterior cingulate, orbital and insular cortices. In contrast, low levels of D_2 receptor mRNA are found in the septum, amygdala, hippocampus, cortex, hypothalamus, thalamus, and cerebellum (Meador-Woodruff, 1991, 1994). D_2 receptors have two distinct isoforms: D_2 short (D_2S) and D_2 long (D_2L). D_2S receptors are located presynaptically and function as dopamine autoreceptors, whereas D_2L receptors

are located postsynaptically (Jomphe, Tiberi, & Trudeau, 2005; Lindgren et al., 2003; Usiello et al., 2000).

The highest densities of D_3 receptors are predominately found in the shell of the nucleus accumbens, olfactory tubercle, islands of Calleja, ventral pallidum, substantia nigra, and cerebellum (Lévesque et al.,1992; Stanwood et al., 2000). Lower numbers of D_3 receptors are found in the striatum, ventral tegmental area, hippocampus, septum, and prefrontal cortex (Beaulieu & Gainetdinov, 2011; Lévesque et al.,1992). Not surprisingly, D_3 receptor mRNA is highly expressed in the nucleus accumbens and islands of Calleja, with low levels in the substantia nigra, ventral tegmental area, striatum, septum, olfactory tubercle, amygdala, hippocampus, cortex, hypothalamus, thalamus, and cerebellum (Lévesque et al.,1992; Meador-Woodruff, 1991, 1994).

D_4 receptors are the least common of the D2-like receptors and are expressed in relatively low levels in the hippocampus, striatum, olfactory tubercle, amygdala, hypothalamus, globus pallidus, substantia nigra, nucleus accumbens core, thalamus, frontal and entorhinal cortices, and cerebellum (Defagot & Antonelli, 1996; Defagot, Malchiodi, Villar, & Antonelli, 1997; Primus et al., 1997; Tarazi, Kula, & Baldessarini, 1997). D_4 receptor mRNA is located in the substantia nigra and ventral tegmental area (Meador-Woodruff, 1994), while low levels of D_4 receptor mRNA are expressed in the nucleus accumbens, amygdala, hippocampus, cortex, hypothalamus, and thalamus (Meador-Woodruff, 1994).

$D2^{High}$ Receptor Affinity and Functionality

As mentioned in Chapter 1, D2-like receptor systems are intimately associated with psychosis. D2-like receptors exist in two different functional states: a state of high affinity and a state of low affinity (Seeman, 2011). The high-affinity state of the D2-like receptor, known as $D2^{High}$, is thought to be responsible for dopamine supersensitivity, which is most commonly observed as an exaggerated behavioral response to agonist drugs. Various manipulations increase $D2^{High}$ receptors and produce dopamine supersensitivity, including ethanol treatment and withdrawal, hippocampal lesion, amphetamine- or quinpirole-induced sensitization, Cesarean section, cocaine self-administration, and excitotoxic lesions of the entorhinal cortex (Briand, Flagel, Seeman, Robinson, 2008; Seeman et al., 2005; Sumiyoshi et al., 2005). Among humans, dopamine agonists can trigger psychotic symptoms in normal populations and exacerbate psychosis in schizophrenics (Angrist, Rotrosen, & Gershon, 1980; Bell, 1973; Ellison, 1994). Thus, the positive symptoms characteristic of schizophrenia may not result from a simple increase in D2-like receptor numbers in particular dopaminergic pathways, but rather a change in the functionality of the D2-like receptors. Specifically, Seeman (2011) has proposed that an increased proportion of $D2^{High}$ receptors relative to $D2_{Low}$ receptors is responsible for schizophrenia. Therefore, an increased percentage of $D2^{High}$ receptors may underlie the hyperdopaminergia of the mesolimbic dopamine pathway (Davis et al., 1991).

CHAPTER FOUR

DOPAMINE SYSTEMS AND RECEPTORS IN YOUNG RODENTS

Overview

Dopamine neurons increase steadily in number from the prenatal period through adolescence (Tarazi & Baldessarini, 2000). Around PD40, dopamine receptor levels peak and a period of pruning occurs in which receptors decline to adult levels at PD60 through PD120 (Andersen, Thompson, Rutstein, Hostetter, & Teicher, 2000; Teicher, Andersen, & Hostetter, 1995). Dopamine neurons are apparent at embryonic day (E)13 in the prosencephalon; and by E14 two prominent clusters of dopamine neurons extend from the mesencephalon to the ganglionic eminence (Voorn, Kalsbeek, Jorritsma-Byham, & Groenewegen, 1988). By E17, these trajectories innervate the dorsal striatum, and dopamine neurons further differentiate into distinct clusters that form the substantia nigra, VTA, dorsal striatum, nucleus accumbens, and olfactory tubercles (Fishell & van der Kooy, 1987; Voorn et al., 1988). Rostral dopaminergic afferents also spread to the area of the future prefrontal cortex (Kalsbeek, Voorn, Buijs, Pool, & Uylings, 1988). Dopamine neurotransmitter levels during early development (E18 to PD5) are about 8-17% of adult levels (Noisin & Thomas, 1988).

By the first postnatal day (PD1), dopamine neurons in the substantia nigra exist in small distinct clusters. These clusters more closely resemble adult distributions by PD4 and continue to increase in size through PD11 (Fishell & van

der Kooy, 1987; Murrin & Ferrer, 1984). Around PD4, dopamine neurons innervate the nascent prefrontal cortex (Kalsbeek et al., 1988). Dopamine neurons throughout the dorsal striatum continue to develop from PD8 through PD13 (Voorn et al., 1988). A shift in development occurs in the dorsal striatum during adolescence, as dopamine receptor levels peak at PD40 and then decline to adult levels by PD60 (Teicher et al., 1995). This developmental pattern is apparent in the dorsal striatum, but similar changes in receptor density are also observed in the nucleus accumbens (Teicher et al., 1995). Overall, dopamine neurotransmitter levels fluctuate during development and are heterogeneous across brain areas. Dopamine levels eventually stabilize at adult levels at different time points depending on brain region, but adult dopaminergic systems are established by PD120 (Andersen et al., 2000; Rao, Molinoff, & Joyce, 1991).

Overview of Dopamine Receptor Subtype Development

In the dorsal striatum, D1-like and D2-like receptors increase at similar rates, thus suggesting that development of the two receptor subtypes are controlled by similar mechanisms (Zeng, Hyttel, & Murrin, 1988). Despite a general increase in receptors with age, the emergence of D1-like and D2-like receptors is non-linear, since the dorsal striatum, substantia nigra, and globus pallidus contain heterogeneous levels of receptor subtypes at any given age (Rao et al., 1991). Within the dorsal striatum and nucleus accumbens, D_1, D_2, and D_4 receptors reach peak levels at PD28 and decline to adult levels between

PD35 and PD60 (Tarazi & Baldessarini, 2000). Limbic and cortical structures also demonstrate a similar developmental pattern, with a relative absence of pruning during adolescence (Tarazi & Baldessarini, 2000). D1 and D2 receptor mRNA is apparent before dopaminergic innervation, however postnatal dopamine receptor binding correlates with dopamine innervation (Jung & Bennett, 1996). At birth, D1-like and D2-like receptor mRNA is at about 60% of adult levels, with mRNA levels peaking at PD30, and then declining to adult levels (Creese, Sibley, & Xu, 1992).

D1-like Receptor Localization

Embryonic development of D1-like receptors increases slowly from E14 through birth (Jung & Bennett, 1996). Early evidence suggested that the number of D1-like receptors stabilizes between birth and seven postnatal weeks (Broaddus & Bennett, 1989); however, it is more generally reported that D1-like receptors double in number every 7 to 10 days for the first 3 weeks (Murrin & Zeng, 1990; Zeng et al., 1988). This pattern of development is clearly evident in areas such as the dorsal striatum, nucleus accumbens, and olfactory tubercle, but the same pattern is also apparent in brain regions with more moderate levels of D1-like receptors such as the frontal cortex, claustrum, and endoperiform nucleus (Murrin & Zeng, 1990). The function of the D1-like receptor is unaltered during maturation, since juvenile D1-like receptors exhibit the same pharmacological properties as adult D1-like receptors (Zeng et al., 1988). For

example, D1-like receptors are coupled to their G-proteins by PD5 (Jung & Bennett, 1996). D1-like receptor levels increase steadily throughout the developing brain until adolescence. At PD40, D1-like receptor densities in the dorsal striatum peak and then decline to adult levels where they remain relatively stable from PD60 through PD140 (Teicher et al., 1995). D1-like receptor mRNA is apparent by E14 in the dorsal striatum, olfactory tubercle, frontal, cingulate, parietal, and insular cortices, epithalamus, thalamus, hypothalamus, and pons. By E18, mRNA localization parallels that of adults (Schambra et al., 1994). In the nigrostriatal pathway, D1-like receptor mRNA increases linearly until birth, peaks at PD5, and then decreases by 20% until adult levels are maintained (Jung & Bennett, 1996).

D2-like Receptor Localization

D_2 receptors are the most abundant and diffuse of the D2 receptor family (Stanwood, McElligot, Lu, & McGonigle, 1997). Even so, most studies examining the ontogeny of D2-like receptors use displacing ligands that do not differentiate among the D2-like receptor subtypes. Despite this issue, D2-like receptor autoradiography is a good estimate of D_2 receptor distribution due to the relatively large number of D_2 receptors compared to D_3 and D_4 receptors (Stanwood et al., 1997). At E12, D2-like receptors are found in the caudal part of the neural tube and extend outward to regions that will comprise the forebrain. This pattern of development leads to a dramatic increase in the number of dorsal

striatal receptors between E15 through E17 (Sales, Martres, Bouthenet, & Schwartz, 1989). Large increases in D2-like receptor levels occur from E18 through E22 across the forebrain, and this late prenatal period is also characterized by the emergence of D2-like receptors in the olfactory tubercles (Sales et al., 1989). Generally, D2-like receptors increase linearly in number from PD2, and approach adult levels by PD21 (Murrin & Zeng, 1986; Stanwood et al., 1997). D2-like receptor numbers and G-protein-induced inhibition of adenylyl cyclase increases between PD21 through PD28, which coincides with a substantial increase in dopamine terminals in the dorsal striatum (Broaddus & Bennett, 1989). In terms of D2-like receptor subtypes, expression of D_2 receptors antecedes expression of D_3 receptors, since D_3 receptors emerge at a slower rate in the dorsal striatum and nucleus accumbens than D_2 receptors (Stanwood et al., 1997). D_3 receptors are detectable by PD14 in the islands of Calleja and by PD21 in the nucleus accumbens. D_3 receptors show a steady increase in numbers until PD60 (Stanwood et al., 1997). In terms of receptor affinity, the D2-like receptors of young and adult rats show a similar binding affinity (i.e. K_d) by PD7 (Murrin & Zeng, 1986).

D2-like receptor mRNA is present at E14 in the dorsal striatum, olfactory tubercle, frontal, cingulate, parietal, and insular cortices, epithalamus, thalamus, hypothalamus, and pons (Schambra et al., 1994; Srivastava, Morency, & Mishra, 1992). At E18, D2-like receptor mRNA dispersion is similar to what is observed in adult rat brains, although D2-like receptor mRNA levels show a different pattern

of development depending on brain region (Schambra et al., 1994). For example, in most brain areas D2-like receptor mRNA increases steadily until adulthood, although some studies report a slight decline in the dorsal striatum between PD16 and PD28 (Chen & Weiss, 1991; Jung & Bennett, 1996; Srivastava et al., 1992; Weiss, Chen, Zhang, & Zhou, 1992). In contrast, D2-like receptor mRNA in the midbrain peaks by PD14 and then declines to adult levels (Creese et al., 1992).

$D2^{High}$ Receptors and Measures of D2 Functionality

Evidence suggests that there are ontogenetic changes in the functional state of D2-like receptors. A study by McDougall et al. (2015) assessed the number of $D2^{High}$ receptors in preadolescent, adolescent, and adult rats. Their results indicated that the percentage of $D2^{High}$ receptors in the dorsal striatum of preadolescent rats was twice that of adolescent (PD40) and adult (PD80) rats, although there were no differences in the percentage of $D2^{High}$ receptors between the various preadolescent ages (PD5, PD10, PD15, PD20). In an experiment particularly important to this thesis, we examined the effects of haloperidol and aripiprazole on the dorsal striatal $D2^{High}$ receptors of young rats. Haloperidol and aripiprazole was administered via intraperitoneal injection from PD10 through PD20. Following either a 4 or 8 day drug abstinence period, dorsal striatal tissue was extracted and analyzed via dopamine competition assays using [^{3}H]domperidone. Regardless of drug abstinence period, our results indicated

that both haloperidol and aripiprazole significantly increased the percentage of $D2^{High}$ receptors in the dorsal striatum of preadolescent rats. Thus, these results demonstrate that antipsychotic drugs can alter $D2^{High}$ receptors during early development as well as in adulthood (Seeman, 2008).

CHAPTER FIVE

EFFECTS OF REPEATED TREATMENT WITH DOPAMINE COMPOUNDS ON D2 RECEPTORS

Overview

Evidence suggests that as many as 70% of people with schizophrenia are supersensitive to dopamine agonists, as evidenced by agonist-induced exacerbation of psychotic symptomology (Lieberman, Kane, & Alvir, 1987). Supersensitivity is not simply due to an increase in the quantity of D2-like receptors, but it can be caused by changes in the functional state of the receptor (Seeman et al., 2005). Repeated treatment with dopamine antagonists, especially first-generation antipsychotics, can cause dopamine receptor upregulation (Burt, Creese, & Snyder, 1977; Laruelle et al., 1992; O'Dell et al., 1990; Wilmot & Szczepanik, 1989). Upregulation is manifested as an increase in the number of dopamine receptors, which results from dopamine denervation or prolonged pharmacological blockade of dopamine receptors (Ginovart, Wilson, Hussey, Houle, & Kapur, 2008; Seeman, Lee, Chau-Wong, & Wong, 1976; Staunton, Magistretti, Koob, Shoemaker, & Bloom, 1982). The behavioral impact of dopamine receptor upregulation often takes the form of extrapyramidal side effects or tardive dyskinesia (Creese & Snyder, 1980; Klawans & Rubovitz, 1972; Tarsy & Baldessarini, 1977). Repeated treatment with second-generation antipsychotics results in an upregulation of D2-like receptors, but long-term

extrapyramidal side effects do not appear as severe. Kapur and Seeman (2014) proposed that the lack of side effects may be due to the fast dissociation of these compounds at D2-like receptors, although the more favorable treatment outcome of second-generation antipsychotics has yet to be fully explained (Dwivedi, Rizavi, & Pandey, 2002). The third-generation antipsychotic aripiprazole is thought to be more of a "stabilizer" of receptor activity, thus resulting in fewer side effects in people with schizophrenia (Kirino, 2012). There is conflicting evidence as to whether aripiprazole causes D2-like receptor upregulation in the dorsal striatum, since either no changes or slight increases in D2 receptor numbers have been reported (Inoue et al., 1997; Tadokoro et al., 2012).

Effects of Repeated Haloperidol or Aripiprazole Treatment on D2-like Receptors in the Dorsal Striatum of Adult Rodents

Upregulation of D2-like Receptors

It is well established that repeated treatment with haloperidol causes an upregulation of D2-like receptors in the dorsal striatum. Following 21 days of haloperidol treatment, D2-like receptors in the dorsal striatum increase by approximately 50% (O'Dell et al., 1990; Wilmot & Szczepanik, 1989). When combined with denervation of dopamine neurons, repeated treatment with haloperidol leads to even greater increases in D2-like receptor numbers (Reches, Wagner, Jackson, Yablonskaya-Alter, & Fahn, 1983; Shaunton, Magistretti, Koob, Shoemaker, & Bloom, 1982). In general, studies comparing the effects of repeated haloperidol and aripiprazole treatment conclude that the first-generation

antipsychotic is much more likely to induce D2-like receptor upregulation. Following repeated administration of haloperidol (1, 2, or 4 mg/kg) or aripiprazole (12 or 100 mg/kg) for 21 days, haloperidol caused a 40% increase in dorsal striatal D2-like receptors (i.e. B_{max} values). In contrast, aripiprazole produces only a nonsignificant upregulation of D2 receptors when compared to control values, suggesting that aripiprazole acts as an antagonist at dorsal striatal D2 receptors regardless of its potency (12 or 100 mg/kg) (Inoue et al., 1997). In a similar study, Tadokoro et al. (2012) showed that a 14-day regimen of haloperidol caused an upregulation of dorsal striatal D2-like receptors (B_{max} = 153% increase), while the same regimen of aripiprazole produced only a slight, nonsignificant increase in the density of D2-like receptors (B_{max} = 126% increase). As with the Inoue et al. (1997) study, the B_{max} values of aripiprazole and vehicle control groups did not differ. In conclusion, repeated treatment with haloperidol increases the number of dorsal striatal D2-like receptors, but the impact of repeated aripiprazole treatment on these receptors remains elusive.

Alterations in the Percentage of $D2^{High}$ Receptors

A separate issue is whether repeated treatment with dopamine antagonists alters the affinity state of D2-like receptors. This issue is particularly important because aripiprazole appears to exert antagonistic effects when D2-like receptors are in a state of high affinity. In a critical study for this thesis, Seeman (2008) reported that repeated treatment with aripiprazole caused a significant increase in the percentage of dorsal striatal $D2^{High}$ receptors. Not

surprisingly, chronic treatment with haloperidol also results in an elevated percentage of $D2^{High}$ receptors (Seeman et al., 2005).

In summary, repeated treatment with haloperidol causes a dramatic upregulation of D2-like receptors, whereas the effect of repeated aripiprazole treatment on D2-like receptor numbers is ambiguous (Inoue et al., 1997; Tadokoro et al., 2012). In contrast, repeated treatment with both haloperidol and aripiprazole increases the percentage of D2-like receptors in a high affinity state. If anything, aripiprazole may increase $D2^{High}$ receptors to a greater extent than haloperidol (Seeman et al., 2005); this suggests that repeated aripiprazole treatment may cause long-term modifications in behavior by altering D2 affinity as opposed to upregulating D2-like receptors.

Changes in D2 Receptor cAMP and Akt Signaling Pathways

Repeated treatment with haloperidol and aripiprazole affects the cAMP and Akt signaling pathways. As mentioned in a previous chapter, D2-like receptor stimulation activates a G_i protein which decreases adenylyl cyclase activity and, as a consequence, reduces cAMP levels and PKA activity (Beaulieu & Gainetdinov, 2011; Kebabian & Cote, 1981; Missale et al., 1998). The Akt pathway is also modulated by D2-like receptor stimulation. Specifically, D2-like receptor activation decreases Akt levels and inhibits GSK-3β activity which, in turn, inhibits β-catenin (Freyberg, Ferrando, & Javitch, 2010; Li & Gao, 2011). Importantly, GSK-3β is a critical component regulating hyperdopaminergic behaviors, since decreased activity in this pathway is associated with

hyperdopaminergic conditions (Li & Gao, 2011; Li, Xi, Roman, Huang, & Gao, 2009).

D2-like receptor blockade increases activity within the cAMP pathway. For example, chronic treatment with haloperidol increases adenylyl cyclase activity (Iwatsubo & Clouet, 1975). *In vitro* studies show a pronounced increase in cAMP following exposure to haloperidol, and lesser increases in cAMP levels after treatment with second-generation antipsychotics (Masri et al., 2008). Curiously, aripiprazole decreases cAMP levels *in vitro* (Masri et al., 2008). Chronic treatment with haloperidol increases PKA activity in the dorsal striatum, while other evidence suggests that acute haloperidol treatment may also result in phosphorylation of PKA (Pan, Chen, Lian, Huang, & Deng, 2015; Turalba, Leite-Morris, & Kaplan, 2004). Acute treatment with aripiprazole did not produce a significant change in the amount of phosphorylated PKA (Pan et al., 2015).

The Akt pathway is also affected by repeated treatment with dopaminergic compounds (Alimohamad, Rajakumar, Seah, & Rushlow, 2005). For example, a long-term regimen of haloperidol significantly increased phosphorylated Akt levels in the dorsal striatum of both rats and mice (Emamian, Hall, Birnbaum, Karayiorgou, & Gogos, 2004; Sutton & Rushlow, 2011); however, the acute effects of haloperidol treatment on phosphorylated Akt remain unclear, since there are conflicting reports that haloperidol either increases phosphorylated Akt levels (Emamian et al., 2004) or has no effect (Pan et al., 2015). Acute aripiprazole treatment had somewhat different actions, as acute treatment with

aripiprazole produced only a nonsignificant increase in phosphorylated Akt levels (Pan et al., 2015).

Other proteins within the Akt pathway are also altered after treatment with antipsychotics. Repeated treatment with a moderate dose of haloperidol increased both GSK-3β and β-catenin levels in dorsal striatal tissue (Alimohamad et al., 2005). Within the dorsal striatum, increased GSK-3β labeling was observed in cell bodies and nearby dendrites, whereas increased β-catenin labeling occurred more densely in nuclear or neuropil areas. Elevated levels of GSK-3β and β-catenin were also found in deeper layers of the prefrontal cortex, which coincides with the location of D2-like receptors (Alimohamad et al., 2005; Berendse, Galis-de Graaf, & Groenewegen, 1992; Larson & Ariano, 1995). Acute haloperidol treatment did not alter GSK-3β or β-catenin levels (Pan et al., 2015); however, a single injection of aripiprazole did increase phosphorylated GSK-3β (Pan et al., 2015).

In summary, these results indicate that repeated treatment with haloperidol or aripiprazole affects the cAMP and Akt signaling pathways of adult rats, although the effects of haloperidol are generally more pronounced. Regarding the cAMP pathway, both acute and repeated treatment with haloperidol increased PKA catalytic activity (Dwivedi et al., 2002; Pan et al., 2015). There is a lack of evidence regarding the effects of repeated aripiprazole treatment on phosphorylated PKA, but acute aripiprazole did increase the alpha PKA catalytic subunit with no significant increase in the ratio of p-PKA to PKA

(Pan et al., 2015). Within the Akt pathway, repeated haloperidol treatment lead to increases in phosphorylated Akt, GSK-3β, and β-catenin (Alimohamad et al., 2005; Emamian et al., 2004; Sutton & Rushlow, 2011). In acute models, there is conflicting evidence regarding the effects of haloperidol on phosphorylated Akt levels, but studies consistently find that haloperidol does not increase the amount of phosphorylated GSK-3β (Emamian et al., 2004; Pan et al., 2015; Sutton & Rushlow, 2011). On the other hand, the effects of repeated aripiprazole treatment on the Akt pathway are unknown. That being said, acute aripiprazole administration did increase the amount of phosphorylated GSK-3β and, perhaps, phosphorylated Akt (Pan et al., 2015).

Effects of Repeated Treatment of Haloperidol and Aripiprazole on D2-like Receptors in the Dorsal Striatum of Young Rodents

Repeated treatment with haloperidol and aripiprazole in young rats causes upregulation of D2-like receptors in the dorsal striatum (Der-Ghazarian, Charntikov, Varela, Crawford, McDougall, 2010). Similar to adult rodents, haloperidol appears to produce greater D2-like receptor upregulation than aripiprazole (Der-Ghazarian et al., 2010). To date, no studies have investigated the acute or chronic effects of haloperidol or aripiprazole on the cAMP or Akt second messenger systems of young rats.

CHAPTER SIX

THESIS STATEMENT

Conclusion

Despite the effectiveness of first-generation antipsychotics in reducing positive symptoms, extrapyramidal side effects remain a major problem when treating schizophrenia. Therefore, there is a need for an effective pharmacotherapy with fewer side effects, such as aripiprazole. The ability of repeated aripiprazole treatment to mitigate psychosis has been demonstrated, although relatively little is known about its mechanism of action. Aripiprazole is frequently prescribed to children and adolescents because of its low side effect profile (Kirino, 2012; Masi et al., 2009; Stachnik & Nunn-Thompson, 2007; Yoo et al., 2011); however, few preclinical studies have examined the neural effects of aripiprazole in young populations. For this reason, more information is needed regarding the effects of repeated aripiprazole treatment across ontogeny.

Alterations in D2-like receptor functioning are responsible for many of the symptoms associated with schizophrenia as well as the extrapyramidal side effects that result from dopamine antagonist treatment. For example, D2-like agonists induce psychosis and exacerbate psychotic symptoms, whereas D2-like receptor antagonists reduce positive symptoms (Li & Gao, 2011; Lieberman et al., 1987). In terms of side effects, D2-like agonists alleviate Parkinsonism and related motoric disturbances (Li & Gao, 2011). Many years ago, Davis et al.

(1991) proposed that schizophrenia is primarily due to dysregulation of dopamine systems in the nucleus accumbens and prefrontal cortex. More recently, Seeman (2011) has extended this model by proposing that an excess of $D2^{High}$ receptors is responsible for psychosis. Although research examining the effects of antipsychotic drugs on the $D2^{High}$ receptors of adult rats is well along (Seeman, 2008), it is important to determine the effects of repeated haloperidol and aripiprazole treatment on the percentage of $D2^{High}$ receptors in young rats.

Traditionally, it is understood that repeated treatment with first-generation antipsychotics lessens psychosis through antagonism of D2-like receptors, which has the unwanted side effect of upregulating these receptors. Despite symptom reduction, first-generation antipsychotics cause more extrapyramidal side effects than second- or third-generation antipsychotics (Croxtall, 2012; Leucht et al., 2009). As an example, repeated treatment with haloperidol results in an upregulation of D2-like receptors, a larger percentage of $D2^{High}$ receptors, and behavioral supersensitivity (Inoue et al., 1997; Seeman et al., 2005; Tadokoro et al., 2012; Varela et al., 2014). In contrast, treatment with the third-generation antipsychotic aripiprazole results in a slight, nonsignificant upregulation of D2-like receptors, an increased percentage of $D2^{High}$ receptors, and relatively less supersensitivity (Seeman, 2008; Tadokoro et al., 2012; Varela et al., 2014). The lack of pronounced D2-like receptor upregulation suggests that aripiprazole does not operate under the traditionally understood mechanisms of first-generation

antipsychotics, although both haloperidol and aripiprazole do increase the percentage of $D2^{High}$ receptors (Seeman, 2008).

Since dopamine system dysfunction is a hallmark of schizophrenia, it is not surprising that second messenger systems associated with D2-like receptors are often dysregulated in schizophrenia. Indeed, some researchers speculate that dysfunction involving the cAMP and Akt pathways may be a critical neural mechanism underlying schizophrenia (Beaulieu, Del'Guidice, Sotnikova, Lemasson, & Gainetdinov, 2011; Emamian et al., 2004; Karam et al., 2010; Lovestone, Killick, Di Forti, & Murray, 2007). For example, Li et al. (2009) have hypothesized that GSK-3β, a component of the Akt pathway, may modulate dopamine-dependent behaviors by inhibiting NMDA receptors in the prefrontal cortex. Thus, hyperdopaminergic behaviors may be a consequence of GSK-3β attenuating glutamatergic functioning (Li et al., 2009). Regardless of the exact explanation, it is clear that PKA, Akt, and GSK-3β are dysregulated in the frontal cortex of schizophrenics (Emamian et al., 2004).

Consistent with the latter findings, repeated and acute treatment with antipsychotic drugs alters the functioning of the cAMP and Akt pathways. Within the dorsal striatum of adult rats, repeated haloperidol treatment increases phosphorylated PKA, Akt, GSK-3β, and β-catenin (Alimohamad et al., 2005; Dwivedi et al., 2002; Emamian et al., 2004; Sutton & Rushlow, 2011). In contrast, there is a general lack of information regarding the effects of repeated aripiprazole treatment on the cAMP and Akt pathways. The only available

evidence suggests that acute treatment with aripiprazole slightly enhances the phosphorylation of GSK-3β and possibly Akt (Pan et al., 2015).

In addition to possibly being involved in the manifestation of schizophrenia, alterations of the cAMP and Akt pathways may be responsible for some of the side effects (e.g. extrapyramidal effects) associated with antipsychotic drugs. In other words, D2-like receptor antagonists may produce side effects by causing persistent change in the cAMP and/or Akt second messenger systems through actions at D2-like receptors. Based on the evidence presented above, it is possible that aripiprazole has a lesser impact on these second messenger systems, thereby resulting in fewer side effects. Consistent with this idea, repeated aripiprazole treatment causes relatively less behavioral supersensitivity in young rats than does haloperidol (Varela et al., 2014).

This thesis examined the effects of repeated aripiprazole and haloperidol treatment on dopamine systems in the dorsal striatum of preadolescent and adult rats. We previously reported that repeated haloperidol and aripiprazole treatment increased the percentage of $D2^{High}$ receptors in young rats; however, we did not assess whether these changes had any functional consequence. Therefore, I examined the functionality of D2-like receptors by conducting GTPγS assays and measuring the cAMP and Akt second messenger systems, after repeated exposure to haloperidol or aripiprazole. My expectation was that aripiprazole and haloperidol would increase the efficacy of D2-like receptors as

measured by the GTPγS assay. These D2-like receptor changes, in turn, were predicted to cause enhanced responsiveness in the cAMP and Akt pathways.

Proposed Hypotheses

In my first experiment, [^{35}S]GTPγS binding assays were conducted to assess the effects of repeated haloperidol and aripiprazole treatment on the efficacy of D2-like receptors (i.e. the coupling of $G_{i\alpha}$ to D2 receptors). Preadolescent and adult rats were pretreated with haloperidol or aripiprazole for 11 consecutive days. After either a 4 or 8 day drug abstinence period, rats were euthanized and dorsal striatal tissue dissected out and stored until assay. Based on the previously gathered $D2^{High}$ data, I hypothesized that the D2-like receptors of younger rats would show a greater efficacy than adult rats. I did not expect that the different drug abstinence periods would affect GTPγS binding. I also hypothesized that the D2-like receptors of haloperidol-treated rats would display greater efficacy than aripiprazole-treated groups, and that D2-like receptors of both drug groups would demonstrate greater efficacy than those treated with saline.

In my second experiment, I assessed the cAMP second messenger system. More specifically, I measured PKA subunits (i.e. alpha and beta catalytic subunits, regulatory subunits, and phosphorylated PKA) in order to determine if upregulation of any of these subunits occurred after repeated haloperidol or aripiprazole treatment. Preadolescent and adult rats were repeatedly

administered haloperidol, aripiprazole, or vehicle for 11 days, and dorsal striatal tissue was extracted after a 4 or 8 day drug abstinence period. Levels of PKA subunits in the dorsal striatum was measured using Western Blot analysis. I hypothesized that there would be greater increases in the amount of phosphorylated PKA in younger rats compared to adult rats, but no differences due to the 4 or 8 day drug abstinence periods. Additionally, I hypothesized that there would be increased phosphorylated PKA in haloperidol-treated rats in comparison to aripiprazole-treated rats, and that both haloperidol and aripiprazole would induce elevated levels of phosphorylated PKA when compared to rats that receive vehicle.

I also assessed components of the Akt pathway, specifically, the amount of phosphorylated Akt relative to nonphosphorylated Akt, and the amount of phosphorylated GSK-3β relative to nonphosphorylated GSK-3β. As mentioned previously, preadolescent and adult rats received repeated injections of haloperidol, aripiprazole, or vehicle for 11 days. Dorsal striatal tissue was extracted after a 4 or 8 day drug abstinence period and phosphorylated Akt and GSK-3β was measured using Western Blot analysis. I hypothesized that younger rats would demonstrate larger increases in phosphorylated Akt and GSK-3β levels than adult rats, and that there would be no differences between the 4 or 8 day drug abstinence periods. I also hypothesized that rats treated with haloperidol and aripiprazole would display elevated amounts of phosphorylated Akt and GSK-3β relative to rats that receive vehicle.

CHAPTER SEVEN

METHODS

Subjects

Subjects were 164 male and female Sprague-Dawley rats (Charles River Laboratories, Hollister, CA, USA). Eighty subjects were preadolescent rats born and raised at California State University, San Bernardino (CSUSB). Preadolescent rats were culled to 10 pups per litter by PD3 and housed with the dam in the same cage. Eighty-four adult subjects were purchased from Charles River Laboratories and arrived on PD51-59. Approximately equal numbers of male and female rats were assigned to all conditions.

The colony room was maintained at a temperature of 22-24°C and was kept under a 12 hour light/dark cycle. Drug administration was conducted during the light cycle. Food and water were available at all times. All subjects were treated according to the “Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research” (National Research Council, 2003) and the research protocol was approved by the Institutional Animal Care and Use Committee at CSUSB.

Drugs

Haloperidol was dissolved in saline, whereas aripiprazole was dissolved in (2-hydropropyl)-β-cyclodextrin (HBC) solution. Saline served as the control

vehicle for haloperidol, and HBC served as the control vehicle for aripiprazole. Drugs were injected at a volume of 2.5 ml/kg for preadolescent rats and 2 ml/kg for adults.

Design

These experiments used 2 x 2 x 3 between-subjects experimental designs (Subject Age: young vs. adult; Drug Type: haloperidol vs. aripiprazole vs. saline/HBC; Drug Abstinence Period: 4 or 8 days). A drug abstinence period of either 4 or 8 days was chosen because these durations are commonly used for adult rats, although there is a dearth of evidence regarding an ideal drug abstinence period for young rats. Similar drug abstinence periods were used to assess the effects of repeated aripiprazole and haloperidol treatment on the dorsal striatum of early adolescent rats (Varela et al., 2014).

D2 receptor function was measured using GTPγS assays and Western Blots. Specifically, the GTPγS assay measured the coupling of $G_{i\alpha}$ to D2 receptors and the Western Blots assessed Akt, GSK-3β, and PKA subunit levels. Male and female rats were included in the study, with sex differences analyzed separately from the previously mentioned statistical analyses.

Procedures

Pretreatment Phase

Young rats began injections at PD10, while adult rats began injections at PD80. Haloperidol (1 mg/kg), aripiprazole (10 mg/kg), and vehicle (saline/HBC), were administered for 11 days: postnatal day PD10 through PD20 for young rats and PD70 through PD80 for adult rats. Injections were followed by a drug abstinence period of either 4 or 8 days. Subjects were then sacrificed by rapid decapitation (PD24 or PD28 for juveniles and PD84 or PD88 for adults). Dorsal striatal tissue was dissected out and kept at -80 °C until assay.

[S^{35}]GTPγS Binding Assay

On the day of assay, tissue was thawed and homogenized in 100 volumes of 50 mM Tris–HCl buffer (pH 7.4) for approximately 20 s using a Brinkman Polytron. Homogenates were centrifuged at 20 000 × g for 30 min. The pellet was then resuspended in 100 volumes of the same buffer and centrifuged again at 20 000 × g for 30 min. The final pellet was suspended in approximately 20 volumes of buffer (pH 7.4) and incubated for 30 min at 37 °C to remove endogenous transmitter.

[^{35}S]GTPγS binding was conducted in duplicate tubes in assay buffer (50 mM Tris–HCl, 120 mM NaCl) containing 30 μM GDP, 10–20 μg protein, and dopamine in various concentrations. Nonspecific binding was determined in the presence of 30 μM cold GTPγS. The tubes were preincubated for 15 min at 37 °C, after which 0.1 nM [^{35}S]GTPγS were added. Following the addition of

[^{35}S]GTPγS, tubes were incubated for an additional 45 min at 37 °C. The incubation period was terminated by filtering the contents of the tubes using glass fiber filters. Net agonist-stimulated [^{35}S]GTPγS binding values were calculated by subtracting basal binding values (without agonist) from agonist-stimulated values (with agonist) and dividing by basal values. Agonist potency (pEC_{50}) and agonist efficacy (E_{max}) were determined by iterative nonlinear regression fitting using Prism (Graph Pad Software).

Western Blot Analyses for PKA and Akt

The levels of Akt/p-Akt, GSK-3β/p-GSK-3β, and PKA/p-PKA were determined using Western Blotting. Four different proteins were used to assess PKA: the alpha catalytic subunit (PKA-Cα), the beta catalytic subunit (PKA-Cβ), the regulatory subunit (PKA-RII), and p-PKA. Briefly, dorsal striatal samples (20 µg protein) were mixed with lamemmeli buffer, boiled for 5 min at 95 °C, and loaded on 12% polyacrylamide gels. Homogenates were then transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were rinsed in TBST buffer (20 mM Tris-HCl, 150 mM NaCl, and 0.5% Tween20) and then blocked with 5% non-fat milk in TBST (diluted in blotto) for 2 h. Membranes were then incubated with the primary rabbit antibody for Akt [1:20,000 (diluted in blotto); #C67E7], phospho-Akt [Thr308; 1:10,0000 (diluted in blotto); Cell Signaling Technology, #D25E6], PKA-Cβ [1:10,000 (diluted in blotto); Santa Cruz Biotechnology, #SC-904], or p-PKA [Thr197; 1:50,000 (diluted in blotto); Cell Signaling Technology, #5661] overnight at 4 °C. The next day, the blots were

washed five times with TBST for 5 min each and then incubated in anti-rabbit IgG-HRP secondary antibody [1:50,000 (diluted in blotto); Santa Cruz Biotechnology] 2 h at room temperature. After membranes were washed five times in TBST, immunoreactive proteins were revealed with enhanced chemiluminescence (Thermo Fisher SuperSignal West Dura Extended Duration Substrate, Thermo Fisher Scientific). Immunoreactive bands were then visualized and quantified using a computer-assisted digital imagining program (Chemi-Doc, Bio-Rad). Following quantification, blots were mildly striped with a commercially available buffer (Restore Western Blot Stripping Buffer, Thermo Fisher Scientific), and the blotting procedure was redone using primary rabbit antibodies for GSK-3 α/β [1:20,000 (diluted in blotto); Cell Signaling, #D75D3], phospho-GSK-3β [Ser9; 1:20,000 (diluted in blotto); Cell Signaling Technology, #D3A4], PKA-RII [1:10,000 (diluted in blotto); Santa Cruz Biotechnology, #SC-908], and PKA-Cα [1:75,000 (diluted in blotto); Cell Signaling Technology, #5842].

Data Analyses

Data were initially analyzed using four-way ANOVAs (Age x Drug x Abstinence Period x Sex) for the GTPγS and Western Blot experiments. Sex was excluded as a factor in the final statistical analyses if the main effect and interactions involving the sex independent variable were nonsignificant. Tukey tests ($p<.05$) were used for making planned and post hoc comparisons. Litter

effects were controlled by assigning no more than one subject from each litter to a particular group (for a fuller discussion of litter effects, see Zorrilla, 1997).

CHAPTER EIGHT

RESULTS

Experiment 1

GTPγS Binding

Repeated treatment with haloperidol or aripiprazole did not significantly affect D2-like receptor efficacy (i.e. E_{max} values) in young or adult rats (see Table 1) [Drug x Age interaction, $F(2,60) = .68$, $p = .51$]. Likewise, neither haloperidol or aripiprazole altered the potency (i.e. pEC_{50} values) of NPA-stimulated [^{35}S]GTPγS specific binding in either age group [Drug x Age interaction, $F(2,60) = 1.43$, $p = .25$]. D2 receptor efficacy and potency were not differentially affected by the drug abstinence period [$F(1,60) = .34$, $p = .56$; $F(1,60) = .044$, $p = .835$].

Table 1. Mean D2 Receptor Efficacy and Potency (E_{max} and pEC_{50}, respectively) in the Dorsal Striatum of Young (PD24 and PD28) and Adult (PD84 and PD88) Rats.

Drug	E_{max}		pEC_{50}	
	Young	Adult	Young	Adult
Vehicle	27.08 (+3.89)	32.96 (+4.27)	6.35 (+0.26)	6.23 (+0.31)
Aripiprazole	20.61 (+2.66)	30.15 (+3.02)	7.14 (+0.30)	6.47 (+0.32)
Haloperidol	27.17 (+2.47)	28.92 (+3.75)	6.46 (+0.29)	6.78 (+0.23)

Note: E_{max} (mean, +SEM) is expressed as fmol/mg weight wet tissue; pEC_{50} (mean, +SEM) is the log of EC_{50} (nm)

Experiment 2

Western Blots

In both age groups, haloperidol significantly increased the expression of dorsal striatal PKA-Cα (122.82% ± 7.2), PKA-Cβ (115.98% ± 5.6), and PKA-RII (122.30% ± 6.3), but not p-PKA (see Tables 2 and 3) [Drug main effect, $F(2,84) = 5.30$, $p = 0.007$; $F(2,84) = 5.04$, $p = 0.009$; and $F(2,84) = 6.08$, $p = 0.003$, respectively]. Haloperidol also significantly increased PKA-Cβ and PKA-RII levels relative to aripiprazole (96.87% ± 5.1 and 105.26% ± 5.4, respectively). After 4 abstinence days, PKA-Cα and PKA-RII levels were higher in young rats than adult rats (see Figures 1 and 2) [Age x Drug Abstinence Period interaction, $F(1,84) = 6.15$, $p = .015$; and $F(1,84) = 7.25$, $p = .009$, respectively]. Adult rats had higher levels of both proteins when assessed after 8 rather than 4 abstinence days. No significant main effects or interactions involving the sex independent variable were apparent.

Table 2. Mean Dorsal Striatal PKA-Cα and PKA-Cβ Values of Young (PD24 and PD28) and Adult (PD84 and PD88) Rats.

Drug	PKA-Cα		PKA-Cβ	
	Young	Adult	Young	Adult
Vehicle	100	100	100	100
Aripiprazole	120.26 (+8.9)	110.33 (+6.1)	99.98 (+8.0)	93.75 (+6.6)
Haloperidol	127.52 (+11.9)*	118.12 (+8.4)*	119.07 (+8.1)*†	112.89 (+8.0)*†

Note: PKA-Cα and PKA-Cβ values (mean, +SEM) are expressed as percent of same-aged vehicle controls.
*Significantly different from rats administered vehicle (saline/HBC)
†Significantly different from rats administered aripiprazole

Table 3. Mean Dorsal Striatal PKA-RII and p-PKA values of Young (PD24 and PD28) and Adult (PD84 and PD88) Rats.

Drug	PKA-RII		p-PKA	
	Young	Adult	Young	Adult
Vehicle	100	100	100	100
Aripiprazole	107.99 (+7.2)	102.52 (+8.4)	121.06 (+11.5)	106.75 (+8.2)
Haloperidol	122.64 (+8.6)* †	121.97 (+9.4)* †	116.13 (+9.1)	116.78 (+11.6)

Note: PKA-RII and p-PKA values (mean, +SEM) are expressed as percent of same-aged vehicle controls.

*Significantly different from rats administered vehicle (saline/HBC)

†Significantly different from rats administered aripiprazole

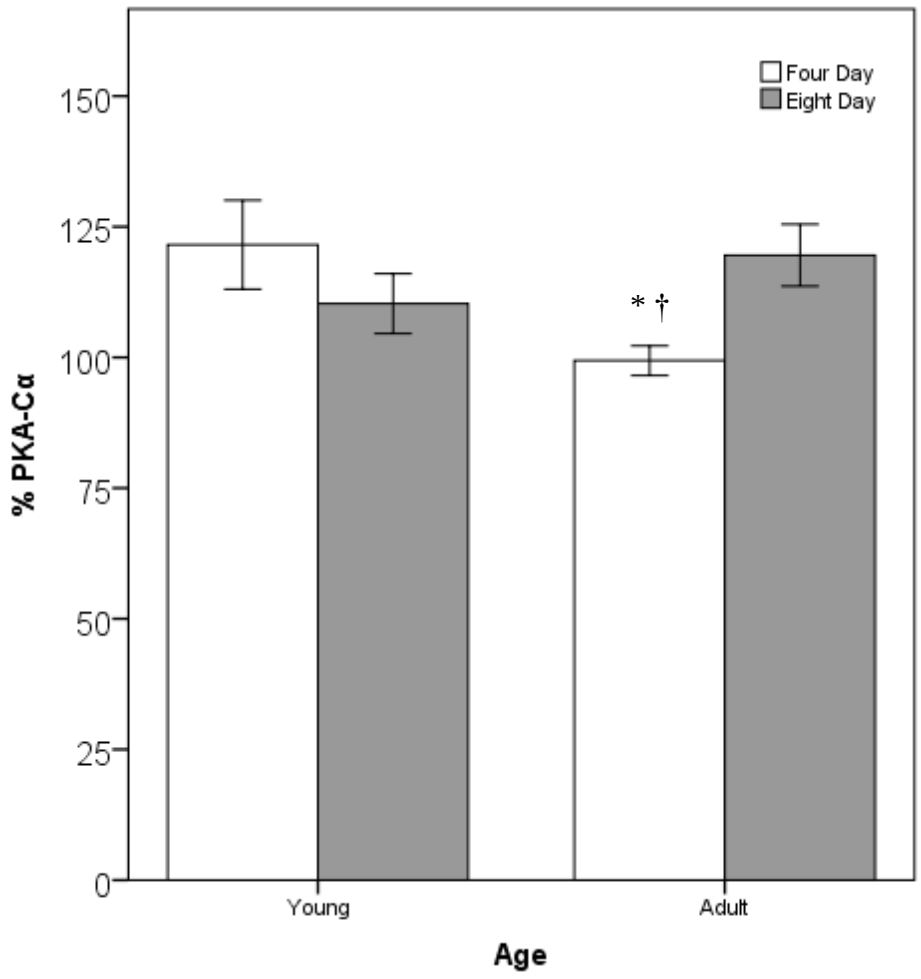

Figure 1. Mean percent (+SEM) PKA-Cα levels of young and adult rats after four or eight drug abstinence days.
*Significantly different from adult rats tested after 8 abstinence days
†Significantly different from young rats tested after 4 abstinence days

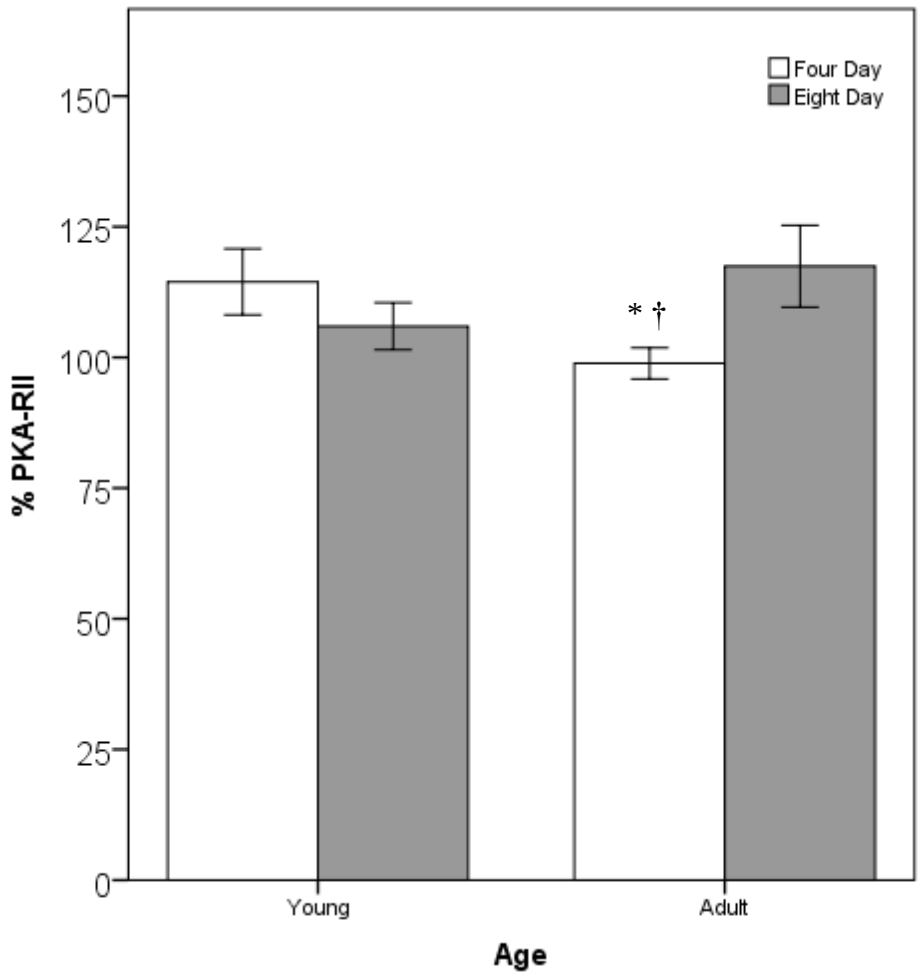

Figure 2. Mean percent (+SEM) PKA-RII levels of young and adult rats after four or eight drug abstinence days.
*Significantly different from adult rats tested after 8 abstinence days
†Significantly different from young rats tested after 4 abstinence days

Repeated administration of haloperidol significantly increased p-GSK-3β levels in the dorsal striatum of young and adult rats (see Table 4) [Drug main effect, $F(2,84) = 4.96$, $p = .009$], but neither haloperidol nor aripiprazole significantly affected GSK-3β, Akt, or p-Akt levels (see Tables 4 and 5). A significant Age x Drug Abstinence Period interaction was found [$F(1,84) = 6.90$, $p = .01$], as Akt levels were elevated in PD24 rats when compared to PD84 rats (see Figure 3). Among adult rats, Akt levels were greater after 8 abstinence days than at 4 days. No significant main effects or interactions involving the sex independent variable were apparent.

Table 4. Mean Dorsal Striatal GSK-3β and p-GSK-3β Values of Young (PD24 and PD28) and Adult (PD84 and PD88) Rats.

Drug	GSK-3β		p-GSK-3β	
	Young	Adult	Young	Adult
Vehicle	100	100	100	100
Aripiprazole	102.09 (+11.0)	119.86 (+19.0)	90.35 (+10.6)	134.19 (+15.4)
Haloperidol	128.11 (+11.1)	109.61 (+11.0)	148.12 (+25.6)*	140.00 (+16.8)*

Note: GSK-3β and p-GSK-3β values (mean, +SEM) are expressed as percent of same-aged vehicle controls.
*Significantly different from rats administered vehicle (saline/HBC)

Table 5. Mean Dorsal Striatal Akt and p-Akt Values of Young (PD24 and PD28) and Adult (PD84 and PD88) Rats.

Drug	Akt		p-Akt	
	Young	Adult	Young	Adult
Vehicle	100	100	100	100
Aripiprazole	105.04 (+5.8)	108.97 (+8.7)	119.2 (+14.2)	135.21 (+19.6)
Haloperidol	108.74 (+4.9)	100.88 (+8.6)	140.69 (+19.8)	123.65 (+15.9)

Note: Akt and p-Akt values (mean, +SEM) are expressed as percent of same-aged vehicle controls.

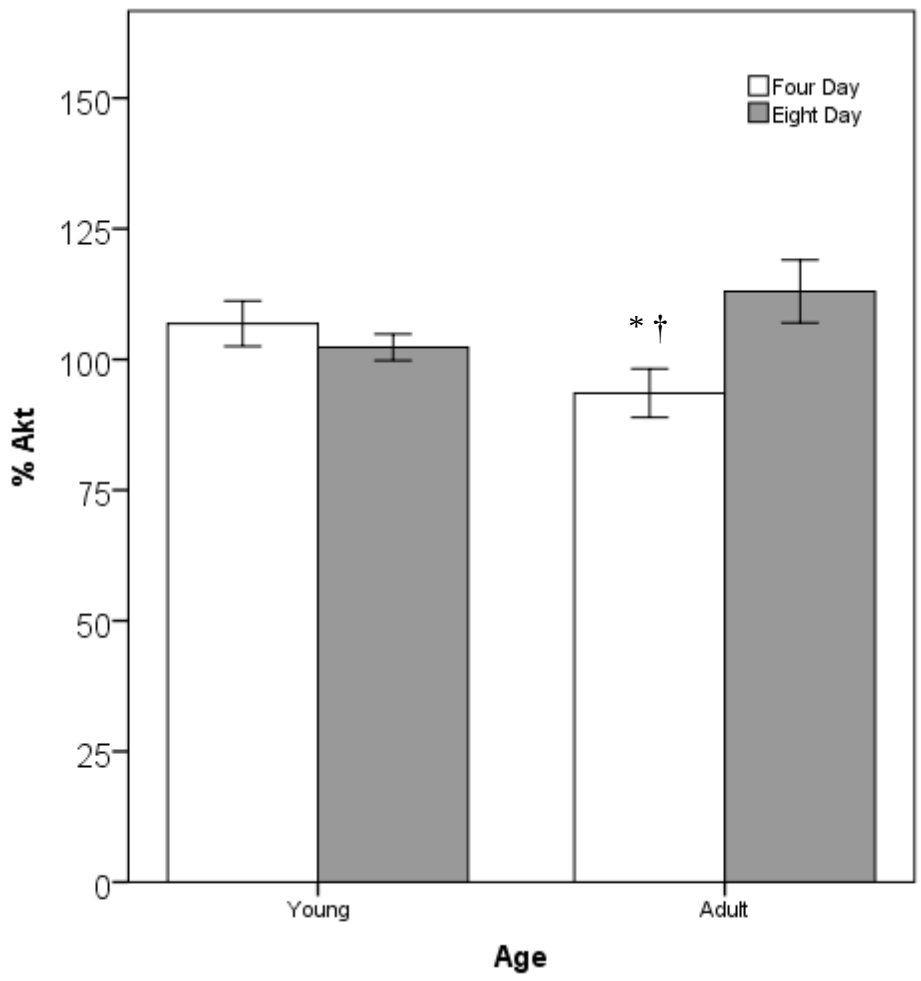

Figure 3. Mean percent (+SEM) Akt levels of young and adult rats after four or eight drug abstinence days.
*Significantly different from adult rats tested after 8 abstinence days
†Significantly different from young rats tested after 4 abstinence days

CHAPTER NINE

DISCUSSION

Schizophrenia is a disorder that can affect humans across the life-span, and a variety of factors are thought to initiate its onset throughout maturation. Although first-, second-, and third-generation antipsychotics reduce positive symptoms, this is often at the cost of persisting extrapyramidal effects (Croxtall, 2012; Leucht et al., 2009; Mailman & Murthy, 2010). These undesirable effects are likely the result of an excessive number of D2 receptors in the dorsal striatum (Inoue et al., 1997; Schröder, Bubeck, & Sauer, 2000, Silvestri et al., 2000; Varela et al., 2014). Consistent with this explanation, repeated treatment with haloperidol and aripiprazole causes long-term increases in dorsal striatal $D2^{High}$ receptors in young rats, as well as adults (Seeman, 2008). Importantly, the ramifications of these receptor changes on D2 second messenger systems have not previously been determined. Specifically, it is unclear whether drug-induced changes in the percentage of $D2^{High}$ receptors affect the functioning of downstream signaling pathways. In an effort to examine this idea, I sought to determine whether repeated haloperidol or aripiprazole treatment would alter the efficacy of D2 receptors and/or impact the cAMP and Akt signaling pathways. Both young and adult rats were included in this study since (1) haloperidol and aripiprazole are commonly prescribed to humans across ontogeny, and (2) young rats possess a higher percentage of $D2^{High}$ receptors than adults.

Development has emerged as an important factor when assessing D2 receptor function. Dorsal striatal D2 receptors appear to function differently across development, as young rats possess a greater percentage of $D2^{High}$ receptors than adolescent or adult rats (McDougall et al., 2015). Because of this finding, the current study focused on the effects of chronic aripiprazole treatment on the dorsal striatum during development. For example, we previously demonstrated an increase in the percentage of $D2^{High}$ receptors in the dorsal striatum of young rats following repeated haloperidol and aripiprazole treatment. Whether these receptor changes have functional implications is unknown. To assess functionality, I measured the long-term effects of haloperidol and aripiprazole on GTPγS binding, as well as D2 signaling mechanisms.

Effects of Repeated Haloperidol and Aripiprazole Treatment on D2 Receptor Efficacy and Potency

The GTPγS assay is used to determine the efficacy or functionality of D2 receptors (Harrison & Traynor, 2003; Milligan, 2003). Because young rats have a greater percentage of $D2^{High}$ receptors than adults (McDougall et al., 2015), I hypothesized that the D2 receptors of young rats, when compared to adults, would have greater efficacy. Following either a 4- or 8-day drug abstinence period, haloperidol-treated rats were predicted to display more efficacious receptors than aripiprazole-treated rats, while rats in both treatment groups were predicted to show enhanced D2 receptor efficacy relative to control rats. In contrast to original expectations, D2 receptor efficacy did not significantly change

following repeated haloperidol or aripiprazole treatment. In fact, D2 receptor efficacy did not differ between young and adult rats, drug groups, drug abstinence periods, nor sexes. Likewise, potency (pEC_{50}) did not differ according to age, drug, abstinence period, or sex. These results are contrary to original expectations. One possibility is that drug-induced increases in the percentage of $D2^{High}$ receptors are independent of changes in D2 receptor efficacy. Alternatively, D2 receptors in the high affinity state may not initiate changes in the cAMP system through traditionally understood mechanisms (i.e. the dissociation of $G_{i\alpha}$ from the βγ complex). This possibility suggests that the effects of increased $D2^{High}$ receptors are independent of the activation of the cAMP signaling cascade. Thus, PKA subunits may upregulate independently of changes in D2/G_{ia} coupling, and alternate D2 receptor signaling pathways may be responsible for changes in the cAMP system.

Effects of Repeated Haloperidol and Aripiprazole Treatment on the cAMP Pathway

Although haloperidol and aripiprazole did not alter the dissociation of G_{ia} from the βy complex, it remains possible that alterations in the percentage of $D2^{High}$ receptors might affect downstream proteins within the cAMP pathway. I previously hypothesized that young rats would display greater increases in p-PKA relative to adult rats, and that haloperidol treatment would lead to greater elevations of p-PKA than aripiprazole. Interestingly, repeated haloperidol treatment did not alter p-PKA, but it did increase the amount of PKA-Cα, PKA-

Cβ, and PKA-RII relative to vehicle controls. The PKA-Cβ and PKA-RII levels of haloperidol-treated rats were also significantly greater than the aripiprazole-treated groups. No differences were found between age groups, sex, nor drug abstinence period. The increases in PKA subunits may be attributed to the general upregulation of D2 receptors after repeated haloperidol treatment. Interestingly, since haloperidol and aripiprazole both increase the percentage of $D2^{High}$ receptors, it is likely that this factor cannot solely account for the changes in the cAMP pathway after haloperidol treatment. On the contrary, since D2 receptors do not cause long-term changes in the quantity of PKA subunits after aripiprazole treatment, perhaps the unique actions of aripiprazole as a partial agonist allow for an increase in $D2^{High}$ receptors without lasting effects on the cAMP pathway. This pattern of results preserves the possibility that increased levels of PKA are linked to some of the more lasting actions of haloperidol, such as extrapyramidal effects.

Using a different model, Pan and colleagues have attempted to determine the short-term effects of haloperidol and aripiprazole treatment on PKA subunits. However, a critical distinction must be made in comparing the methods of Pan et al. (2015, 2016a, 2016b) to the present study. Notably, the work of Pan et al. (2015, 2016a, 2016b) assessed the short-term effects of haloperidol and aripiprazole treatment on second messenger components, while this thesis focused on the long-term effects of these drugs on D2 second messenger systems after 4- or 8-day drug abstinence period. Thereby, the interpretation of

Pan et al. (2015, 2016a, 2016b) is centered on the effects of haloperidol and aripiprazole shortly after the conclusion of drug treatment; whereas, the current study is concerned with long-term, potentially permanent changes in receptor function after repeated drug treatment. Pan et al. (2015, 2016b) reported increased levels of PKA-Cα after both a 1- and 7-day haloperidol *and* aripiprazole treatment regimen. p-PKA was also increased after a 7-day regimen of haloperidol but not aripiprazole (Pan et al., 2016b). Though this evidence suggests that aripiprazole exerts less intense effects on the cAMP system when compared to haloperidol, the present results also highlight the importance of the drug abstinence period when considering the effects of drug-induced receptor changes on the cAMP system.

The current study demonstrated increases in PKA subunits after repeated haloperidol treatment, which was unlike effects occurring after aripiprazole treatment. Specifically, upregulation of PKA subunits (i.e., PKA-Cα, PKA-Cβ, and PKA-RII) was present 4 and 8 days after cessation of repeated haloperidol treatment, whereas upregulation of the PKA system was not apparent following repeated aripiprazole treatment. In terms of haloperidol, it is likely that the increase in regulatory subunits (PKA-RII) was in direct response to increases in catalytic activity. This effect was not observed when using an acute treatment model, as Pan et al. (2015) reported decreased PKA-RII levels after a single injection of haloperidol. It is clear that both haloperidol and aripiprazole cause acute, short-term activation of the cAMP system (Pan et al., 2015); whereas, the

present thesis indicates that haloperidol has more long-lasting effects on the cAMP system than aripiprazole. One possibility is that the antagonistic actions of haloperidol are more pronounced than those of aripiprazole, thus causing more long-term effects. It is also possible that the partial agonist properties of aripiprazole "soften" its upregulation of the cAMP system following the conclusion of treatment. The lack of phosphorylation observed in the current study may also provide insight about the long-term changes in the cAMP system after haloperidol and aripiprazole treatment. Interestingly, the lack of p-PKA observed in our chronic model could actually indicate increased PKA activity, since phosphorylation of PKA can mute some activity of the kinase (Yu et al., 2013). Regardless, the present results are consistent with the idea that some of the behavioral differences observed after repeated haloperidol and aripiprazole treatment may be due to upregulation in the cAMP system.

Effects of Repeated Haloperidol and Aripiprazole Treatment on the Akt Pathway

As previously mentioned, the Akt pathway may also play a critical role in the therapeutic effects of aripiprazole, and the activity of proteins in this pathway may even explain its low side effect profile (Pan et al., 2015). Since increases in p-GSK-3β have been demonstrated in a short-term model, in which dorsal striatal tissue was assessed 24 hrs following repeated haloperidol treatment (Pan et al., 2016a), I hypothesized that haloperidol would also increase the p-GSK-3β and p-Akt levels of adult rats when measured 4 and 8 days after drug discontinuation.

Young rats were hypothesized to exhibit an even greater increase in p-GSK-3β and p-Akt. The present results showed that haloperidol increased the amount of p-GSK-3β, but not p-Akt, in both age groups. Aripiprazole caused a somewhat different pattern of effects, as the partial agonist did not alter p-GSK-3β nor p-Akt in either young or adult rats. Similar to the cAMP pathway, haloperidol has a lasting impact on the Akt pathway, whereas aripiprazole does not. The differential actions of these two compounds on p-GSK-3β levels may be due to the partial agonist actions of aripiprazole. It is noteworthy that haloperidol and aripiprazole affect other neurotransmitter systems (e.g., serotonergic, adrenergic, GABAergic, glutamatergic, etc.) to differing extents, so it is also possible that the generally more muted effects of aripiprazole may be a consequence of complex interplay of many neurotransmitter systems.

The present results show that long-term models, in which neural effects are measured 4 or 8 days after drug discontinuation, produce a very different pattern of effects than short-term models. For example, we found that p-GSK-3β signaling was elevated both 4 and 8 days after cessation of a 10-day haloperidol regimen. In contrast, after a 24 hr drug abstinence period, Pan et al. (2015) reported increased p-GSK-3β levels after a single injection of aripiprazole but not haloperidol. Additionally, there were no short-term increases in p-GSK-3β after a 7 day regimen of haloperidol or aripiprazole, but haloperidol did increase Akt levels (Pan et al., 2016a). When considered together with the cAMP data, the results of this thesis and Pan et al. (2015, 2016a, 2016b) indicate the importance

of treatment length and drug abstinence period when assessing the effects of repeated haloperidol and aripiprazole treatment. Perhaps phosphorylation of GSK-3β after aripiprazole treatment provides some beneficial effects, such as reduced extrapyramidal effects, but post-treatment phosphorylation of GSK-3β may lead to less desirable side effects. Regardless, the consequences of haloperidol treatment on p-GSK-3β leave open the possibility that aripiprazole's more favorable side effect profile may be partly due to the differential actions of these drugs on the Akt pathway.

Development and the cAMP and Akt Pathways

Given its favorable treatment outcomes among humans of various ages, it was critical to assess the actions of aripiprazole on D2 receptor systems in both young and adult rats. Somewhat surprisingly, the effects of repeated haloperidol and aripiprazole treatment did not differ between age groups. This finding has interesting implications, for it supports the idea that D2 receptors function similarly regardless of age and, therefore, the potential consequences of drug action at these receptors is more or less the same. Previous research has demonstrated a progressive linear increase in dopamine receptor content from prenatal ages through preadolescence, followed by an overabundance of receptors during adolescence, and then a decline to adult levels (Andersen, Thompson, Rutstein, Hostetter, & Teicher, 2000; Tarazi & Baldessarini, 2000; Teicher, Andersen, & Hostetter, 1995. $D2^{High}$ receptors also vary according to

age, with young rats having a greater percentage of the high affinity D2 receptors than adolescent or adult rats (McDougall et al., 2015). Due to changes observed in D2 receptor content and affinity state throughout development, it was plausible to hypothesize that the effects of haloperidol and aripiprazole would differ according to age. In terms of the Akt and cAMP pathways, at least, our hypotheses concerning age-dependent differences were not supported by the data.

Drug Abstinence Period

Drug abstinence period is an important factor to take into account when assessing the long-term effects of dopaminergic drugs. If behavioral or neural effects are measured shortly after DA antagonist treatment then acute drug effects are being examined; however, if assessment occurs days or weeks after drug discontinuation then the effects of receptor upregulation are often at issue. Haloperidol causes profound D2 receptor upregulation in preweanling and adult rats (Der-Ghazarian, Charntikov, Varela, Crawford, & McDougall, 2010; O'Dell et al., 1990; Wilmot & Szczepanik, 1989); whereas, repeated aripiprazole treatment causes upregulation of D2 receptors in young rats, and may cause slight, non-significant D2 receptor upregulation in adult rats (Inoue et al., 1997; Tadokoro et al., 2012). Thus, differences in upregulation could be responsible for the more pronounced actions of haloperidol, relative to aripiprazole, on the cAMP and Akt pathways across both age groups. Such an explanation is consistent with the

idea that upregulation of D2 receptors is responsible for the extrapyramidal effects caused by first-generation antipsychotics (Creese & Snyder, 1980; Klawans & Rubovitz, 1972; Tarsy & Baldessarini, 1977).

In the present study, there were relatively few differences observed between the 4- and 8-day drug abstinence periods, but that is probably because upregulation was apparent at both time points. Among the differences that were observed, young rats, when compared to adult rats, had elevated levels of PKA-Cα and PKA-RII than adults after 4 abstinence days. In terms of the Akt pathway, PD24 rats had higher Akt levels when compared to PD84 rats. In adult rats, Akt levels were greater after 8 abstinence days than at 4 days. These findings suggest the possibility that adult cAMP and Akt systems display upregulation of various proteins well after termination of antipsychotic treatment, and that the extent of these changes may be somewhat more pronounced 8 days after drug cessation. In this regard, it would be interesting to assess the effects of haloperidol and aripiprazole on Akt and cAMP system functioning weeks and months after drug discontinuation.

Clinical Relevance

Considering the clinical aspects of these findings, it has long been known that first-generation antipsychotics cause more extrapyramidal effects than second-generation antipsychotics (Croxtall, 2012; Leucht et al., 2009). Aripiprazole, which is often referred to as a “third-generation” antipsychotic, also

has a favorable side effect profile with few extrapyramidal effects (DeLeon et al., 2003; Keck et al., 2004; Marder et al., 2003). The results of this thesis are consistent with the idea that the long-term effects of haloperidol on the cAMP and Akt pathways may underlie its adverse effects; whereas, the lack of persistent change in cAMP and Akt pathways after aripiprazole treatment may be responsible for its superior side effect profile. In regard to development, haloperidol and aripiprazole likely exert their unique long-term effects consistently across young and adult ages. With this in mind, these results reflect the minimal side effects reported after aripiprazole treatment in adults as well as children. With that being said, some studies have reported that aripiprazole causes increased extrapyramidal effects in younger ages when compared to second-generation antipsychotics (Amor, 2012; Doey, 2012; McKinney & Renk, 2011). Perhaps children display elevated extrapyramidal effects after aripiprazole treatment because of increased D2 receptor upregulation that is independent of actions on the cAMP and Akt systems.

In terms of limitations, one concern regarding the translational relevance of this work is the dosage of aripiprazole (10 mg/kg/day) administered to rats, which is much higher than typical human doses (10-30 mg/kg/day). However, the different dosages were actually used to control elimination rate, which is much faster in rats than humans (Greenaway & Elbe, 2009; Mallikaarjun, Salazar, & Bramer, 2004; Shimokawa, Akiyama, Kashiyama, Koga, & Miyamoto, 2005). Another concern is the length of the drug abstinence period used in rats when

compared to the much longer abstinence periods experienced by humans. Although these time factors are difficult to compare, the drug abstinence periods used in this study likely map to human brain development and aging over months or years (Varela et al., 2014).

Conclusion

Overall, the results of this thesis indicate that aripiprazole has different long-term effects on D2 receptor signaling pathways than haloperidol. Aripiprazole has less extreme or prolonged effects on D2 receptor signaling pathways, as evidenced by the lack of post-treatment upregulation in cAMP and Akt pathways. These results are consistent with the idea that the superior side effect profile of aripiprazole may be due to reduced D2 receptor upregulation in the dorsal striatum (Inoue et al., 1997). This thesis expands on this hypothesis, suggesting that D2 receptor functioning is important for determining how aripiprazole reduces psychosis and has fewer extrapyramidal effects. Specifically, haloperidol and aripiprazole both act to alter the affinity of D2 receptors, which may explain their therapeutic effects in reducing the positive symptoms of schizophrenia. Additionally, upregulation of D2 receptors and, in turn, upregulation of proteins in the cAMP and Akt pathways may be partially responsible for side effects. However, making comparisons between animal studies and humans with schizophrenia is complicated, because the cellular environment differs substantially between rats and clinical populations (Pan et

al., 2016a). The cellular environment is important because aripiprazole is thought to have either agonist or antagonist actions depending on the level of dopaminergic activity which, of course, differs according to disease state and the brain areas being assessed (Pan et al., 2016b). Despite this caution, the results of this thesis indicate that aripiprazole does not affect the cAMP and Akt systems as intensely as haloperidol.

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