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The Long-term Effects of Chronic Antipsychotic Drug Treatment in Juvenile Rats on Adult Behavioural Attributes, Dopamine and Serotonin Neurotransmitter Systems

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The Long-term Effects of Chronic Antipsychotic Drug Treatment in Juvenile Rats on Adult Behavioural Attributes, Dopamine and Serotonin Neurotransmitter Systems

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MICHAEL DE SANTIS (BMEDSCI, MSc-Res)

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ABSTRACT

The prescription and use of antipsychotic drugs (APDs) to treat various mental illnesses and behavioural disorders in children and adolescents has exponentially increased in recent years on a global scale. This significant increase in rates of use across both male and female sexes is despite a limited knowledge of what the long-term effects of early APD treatment are. With critical neurodevelopmental phases well-known to extend through this juvenile time period, there is the potential that exposure to the potent actions of APDs on numerous multifunctional neurotransmitter (NT) systems may alter brain topography on a long-term and/or permanent basis. Furthermore, alterations to NT systems including dopamine (DA) and serotonin (5-HT) have previously been heavily implicated in both the pathogenesis of mental illness, and known to play significant roles in behavioural attributes such as activity levels and anxiety. With the second generation APDs aripiprazole, olanzapine and risperidone three of the most commonly prescribed to the juvenile population, and the therapeutic effects of APDs predominantly based on a partial agonist and/or antagonist mechanism of action on DA and 5-HT receptors and hence modulation of NT signal, this thesis investigated the long-term effects of juvenile APD treatment during this critical neurodevelopmental time period on adult behavioural attributes, and subsequently uncovered potential long-term alterations to the DA and 5-HT NT system in the adult brain across both male and female cohorts.

The first study, an investigation into potential long-term changes to behavioural attributes, demonstrated that juvenile APD treatment resulted in changes to locomotor

activity, anxiety and depressive-like behaviours when compared to the control. Differing effects upon comparison of sexes were also uncovered. In the male cohort, juvenile treatment with olanzapine and risperidone resulted in long-term hyper-locomotor effects, and a decrease in depressive-like behaviour in those treated with olanzapine. Furthermore, an anxiolytic-like effect was observed across all 3 APD treatment groups in comparison to controls. Lesser effects were observed in the female cohort, with a long-term increase to depressive-like behaviour and hypo-locomotor activity exhibited following juvenile treatment with risperidone and olanzapine. Such observations clearly indicated the potential for juvenile APD treatment to cause long-term changes to behavioural attributes into adulthood, long after drug treatment had concluded.

With alterations to the DA and 5-HT NT systems well known to play a significant role in both APD treatment efficacy, along with the aforementioned behavioural attributes and the pathogenesis of various mental illnesses, I further investigated the effects of juvenile APD treatment on long-term alterations to both the DA and 5-HT NT systems across both male and female cohorts.

Investigations into the effects of juvenile APD treatment on the DA NT system uncovered long-term alterations to DA synthesis (TH & p-TH), receptor (D_1 and D_2 receptors) and transporter (DAT) levels in mesocortical, mesolimbic, nigrostriatal and hippocampal brain regions. In the male cohort, early treatment with risperidone increased p-TH levels in both the PFC and hippocampus, and DAT levels in the VTA,

whilst decreases to D_1 receptor expression in the NAc and DAT levels in the CPu were found. Decreases to TH levels in the PFC were also uncovered following early aripiprazole treatment. Alterations across all three drug treatment groups were revealed in the female cohort, with early aripiprazole treatment decreasing TH and D_1 receptor levels in the VTA and p-TH levels in the PFC, olanzapine treatment decreasing p-TH levels in the PFC and increasing the D_2 receptor expression in the PFC and NAc and early risperidone treatment increasing D_1 receptor levels in the hippocampus. These results clearly indicated the capability of APD treatment during critical phases of neurodevelopment to elicit long-lasting and potentially permanent changes to the way the DA NT system functions. Furthermore, differing effects across sexes and investigated drug treatment groups were also uncovered.

Along with investigations into the long-term effects on the DA NT system, I also investigated alterations to 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors in the cortical, hippocampal and striatal brain regions, previously found to play significant roles in both the therapeutic efficacy of APDs and furthermore the pathogenesis of various mental illnesses. Juvenile treatment with APDs in the male cohort was found to elicit long-term changes across all three receptor subtypes. Early risperidone treatment resulted in decreases to 5-HT_{1A} receptors in the PFC and NAc and 5-HT_{2A} receptors in the PFC and hippocampus, whilst increases to 5-HT_{2C} receptors were observed in the hippocampus. Olanzapine treatment was found to decrease 5-HT_{2A} and 5-HT_{2C} receptor expression in the hippocampus and PFC respectively, whilst juvenile aripiprazole treatment was found to decrease 5-HT_{2A} and 5-HT_{2C} levels in the PFC, and increase 5-HT_{2C} expression in the hippocampus. Lesser alterations to the 5-HT receptors were found in

the female cohort, with early risperidone and olanzapine treatment resulting in a decreased 5-HT$_{2A}$ receptor density levels in the hippocampus, and aripiprazole decreasing 5-HT$_{1A}$ receptor levels in the NAc. It is therefore evident that juvenile APD treatment also has the potential to result in long-lasting changes to the density of 5-HT receptors, well-known to play significant roles in both the mechanism of action and therapeutic efficacy of APD treatment.

In summary, this thesis provides foundational evidence that juvenile treatment with the commonly prescribed APDs aripiprazole, olanzapine and risperidone has the potential to elicit long-term changes to various behaviours, and furthermore result in long-lasting, permanent alterations to the functioning of both the DA and 5-HT NT systems in adulthood.

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STATEMENT FOR THE STYLE OF THE THESIS

In accordance with the University of Wollongong thesis committee "Guidelines for Preparation and Submission of Higher Degree Research (HDR) theses" (2018) and "HDR Thesis by Compilation Rules" (2018), this PhD thesis is presented in "Journal Article Compilation Style Format". This comprises a series of three original studies published in peer-reviewed journals, including *Journal of Psychopharmacology*, *International Journal of Molecular Sciences,* and *Neuropsychiatric Disease and Treatment*. I am the first author of the three publications. I hereby declare that I am the primary designer of these studies, and have carried out all experiments, data analysis and manuscript preparation.

Mr Michael De Santis

March 2018

I consent to the presentation of this PhD in 'Journal Article Style' and I acknowledge the above statement pertaining to student contribution to be correct.

Professor Chao Deng, Principal Supervisor

2018

LIST OF PUBLICATIONS INCLUDED AS PART OF THE THESIS

The following three refereed journal papers are included as part of the thesis:

De Santis, M., Lian, J., Huang, X-F, and Deng, C. (2015). Early antipsychotic treatment in childhood/ adolescent period has long-term effects on depressive-like, anxiety-like and locomotor behaviours in adult rats. *Journal of Psychopharmacology.* 30(2):204-214.

De Santis, M., Lian, J., Huang, X. F. and Deng, C. (2016). Early Antipsychotic Treatment in Juvenile Rats Elicits Long-Term Alterations to the Dopamine Neurotransmitter System. *International Journal of Molecular Sciences.* 17(11): 1944.

De Santis, M., Huang, X. F. and Deng, C. (2017). Early Antipsychotic Treatment in Juvenile Rats Elicits Long-term Alterations to the Adult Serotonin Receptors. *Neuropsychiatric Disease and Treatment*. (Accepted 9th March 2018)

Other publications and presentations related to the thesis:

Published Abstract

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Congress, Seoul, Republic of Korea, 3-5th July 2016. *International Journal of Neuropsychopharmacology*. 19 (S1): 23.

Conference Proceedings

De Santis M., Huang X-F., Deng C. (2014). Long-term Behavioural Effects of Childhood/Adolescent Antipsychotic Drug Treatment in Adult Rats. *Biological Psychiatry Australia 4th Annual Scientific Meeting 2014,* Melbourne, Australia, 13-14 October 2014.

De Santis M., Huang X-F., Deng C. (2014). Long-term Effects of Childhood/Adolescent Antipsychotic Drug Treatment on Binding of Dopamine Receptor and Transporter in Adult Rats. *25th International Society for Neurochemistry/13th Asian Pacific Society for Neurochemistry/35th Australasian Neuroscience Society Meeting,* Cairns, Australia, 23-27 August 2015.

De Santis M., Huang X-F., Deng C. (2015). Long-term Effects of Childhood/Adolescent Antipsychotic Drug Treatment on 5-HT Receptor and 5-HTT Binding in Adult Rats. *Biological Psychiatry Australia 5th Annual Scientific Meeting 2015,* Coogee, Australia, 20-22 September 2015.

De Santis M., Huang X-F., Deng C. (2017). Early antipsychotic treatment in juvenile rats elicits long-term alterations to the adult serotonin receptors. *Biological Psychiatry*

Australia 7th Annual Scientific Meeting 2017, Wollongong, Australia, 29-31st October 2017.

Additional publications from other projects I have been involved in during my doctoral studies:

Publications in Referred Journals

De Santis, M., Pan B., Lian J., Huang X-F., Deng C. (2014) Different effects of bifeprunox, aripiprazole, and haloperidol on body weight gain, food and water intake, and locomotor activity in rats. *Pharmacology Biochemistry and Behavior.* 124:167-173.

Lian, J., **De Santis, M.**, He, M. and Deng, C. (2015). "Risperidone-induced weight gain and reduced locomotor activity in juvenile female rats: The role of histaminergic and NPY pathways." *Pharmacological Research* 95–96(0): 20-26.

STATEMENT OF CONTRIBUTION OF OTHERS

I, Michael De Santis, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Medicine, University of Wollongong, is entirely my own work unless otherwise referenced or acknowledged. Two co-authors (Prof. Chao Deng, Snr Prof. Xu-Feng Huang) of the 3 journal articles included in the thesis are my PhD supervisors, and another co-author of 2 journal articles (Dr Jiamei Lian) has provided comments on experimental design, data analysis, results interpretation, and revision of manuscripts.

Mr Michael De Santis

March 2018

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Chao Deng   Xu-Feng Huang

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Associate Professor Kelly Newell

Michael De Santis

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

5-HT – Serotonin (5-hydroxytryptamine)

5-HTP - 5-hydroxytryptophan

5-HTT – Serotonin (5-hydroxytryptamine) Transporter

AADC - Aromatic L-amino Acid Decarboxylase

APD – Antipsychotic Drug

cAMP - cyclic Adenosine Monophosphate

CNS – Central Nervous System

CPu – Caudate Putamen

DA – Dopamine

DAT – Dopamine Active Transporter

DOPA - L-3,4-dihydroxyphenylalanine

EPM – Elevated Plus Maze

EPS – Extra-pyramidal Symptoms

FS – Forced Swim

GABA - gamma-aminobutyric acid

NAc – Nucleus Accumbens

NT - Neurotransmitter

p-TH – phosphorylated-Tyrosine Hydroxylase

PD – Postnatal Day

PFC – Prefrontal Cortex

RN – Raphe Nucleus

SN – Substantia Nigra

TH – Tyrosine Hydroxylase

t.i.d. – Three Times Daily

TPH – Tryptophan Hydroxylase

VMAT2 - Vesicular Monoamine Transporter 2

VTA – Ventral Tegmental Area

CHAPTER 1 - GENERAL INTRODUCTION

The prescription and use of antipsychotic drugs (APDs) in children and adolescents has exponentially increased on a worldwide scale in recent times, despite serious limitations in the safety and efficacy of APD use in these population groups (Rani et al. 2008; Varley and McClellan 2009; Olfson et al. 2010; Alexander et al. 2011; Olfson et al. 2012; Seida et al. 2012; Hoekstra 2014; Sharma et al. 2016). In particular, second generation APDs (e.g. aripiprazole, olanzapine and risperidone) are commonly prescribed (mostly off-label) for treating various childhood disorders; from mental illnesses including anxiety and child-onset schizophrenia (Olfson et al. 2010; Memarzia et al. 2014; Schneider et al. 2014), to various behavioural disorders (Haw and Stubbs 2007; Vitiello et al. 2009; Sharma and Shaw 2012). Whilst APDs are known to elicit their therapeutic efficacy mostly through antagonist or partial agonist actions on the dopamine (DA) and serotonin (5-HT) neurotransmitter (NT) systems, the dopaminergic and serotonergic systems are also heavily involved in and undergo multiple critical neurodevelopmental processes during this juvenile time period (Levitt et al. 1997; Frost and Cadet 2000; Andersen 2003; Andersen and Navalta 2004; Yildirim et al. 2008; Klomp et al. 2012; Milstein et al. 2013; Cousins and Goodyer 2015). There is therefore the potential for APD use in such a critical neurodevelopmental time period to carry a substantial risk of long-term alterations to the functioning (from NT synthesis to transport and binding to receptors) of the DA and 5-HT NT systems in the adult brain, with significant implications to the pathogenesis of mental illness (Andersen and Navalta 2004; Marco et al. 2011; Bottelier et al. 2014; De Santis et al. 2016). Therefore, this thesis investigated the long-term effects of childhood/adolescent APD

treatment with aripiprazole, olanzapine and risperidone on adult behavioural attributes, and subsequent changes to the DA and 5-HT NT systems in relevant brain regions.

The investigation in Chapter 3 showed evidence that early treatment (Postnatal Day (PD) 22-50) with aripiprazole (1 mg/kg, three times daily (t.i.d.)), olanzapine (1 mg/kg, t.i.d.) and risperidone (0.3 mg/kg, t.i.d.) resulted in long-term changes to behavioural attributes in the adult rat across both male and female sexes. Following an early drug treatment period during a human equivalent time of childhood/adolescence in humans (PD 22-50) (Andersen 2003) APD treatment was ceased, and a brain maturation period of no drug treatment (PD 51-71) ensued. Following this time period mature, adult rats were put through a series of behavioural tests (from PD 72-94) including open-field/holeboard, elevated plus maze (EPM), social interaction and forced swim (FS) tests to investigate potential changes to behavioural attributes including locomotor activity, anxiety-like, depressive-like and social behaviours. The study found that in the male cohort, early treatment with risperidone, and olanzapine resulted in long-term hyper-locomotor effects (demonstrated in the open-field/holeboard and FS tests), whilst a decrease in depressive-like behaviour was also revealed in the olanzapine treatment group following analysis of FS test results. Lesser effects were uncovered to the female cohort, with alterations to FS test results demonstrating an increase in depressive-like behaviour following juvenile drug treatment with risperidone and olanzapine. These results demonstrated that juvenile APD treatment causes long-term changes to adult behavioural attributes, including locomotor activity, anxiety and depressive-like behaviours, with clear differences in effects across sexes.

As mentioned previously, with changes to the aforementioned behavioural attributes previously linked to alterations to both the DA and 5-HT NT systems (Karl et al. 2006; Seo et al. 2008; de Oliveira et al. 2009; Beaulieu and Gainetdinov 2011; Biojone et al. 2011), and APDs known to elicit their therapeutic effects through actions predominantly on DA and 5-HT receptors (Meltzer 2002; Meltzer et al. 2003; Grace et al. 2007; Kegeles et al. 2010; Purves-Tyson et al. 2012; Milstein et al. 2013), this supported the case for further research into potential long-term alterations to the DA and 5-HT NT systems which may have resulted in the observed effects. The focus for the investigations of Chapters 4 and 5 therefore delved into potential long-term alterations to the adult DA (Chapter 4) and 5-HT (Chapter 5) NT systems following the juvenile APD treatment period, utilising the brain tissue from the animal cohort detailed in Chapter 3. Following the period of behavioural testing described above (PD 72-94), animals were then allowed to rest with access to food and water until PD 106, when they were sacrificed and brain tissue was collected for use in western blot and receptor autoradiography experiments.

Chapter 4 subsequently details the results of the long-term effects of early APD treatment on the DA NT system. Specifically, we investigated the effects of aripiprazole, olanzapine and risperidone on DA synthesis (tyrosine hydroxylase (TH) and phosphorylated-tyrosine hydroxylase (p-TH)), dopamine active transporter (DAT) and D_1 and D_2 receptor levels in cortical and striatal brain regions, along with the hippocampus, substantia nigra and ventral tegmental area, regions known to play key

roles in the synthesis and regulation of the DA signal. Clear long-term alterations to the DA NT system were uncovered, and furthermore the observed results demonstrated clear differences in the effects of early APD treatment upon comparison of sexes. Alterations in the female cohort included decreases to synthesis markers (TH and p-TH) and increases in D_2 receptor density levels in mesocortical brain regions following early aripiprazole and olanzapine treatment, whilst no alterations were uncovered in risperidone drug treatment groups. In the male cohort however, alterations were almost exclusively restricted to the risperidone drug treatment group in comparison to control, with increases to DA synthesis and re-uptake markers were observed across mesocortical, hippocampal and striatal brain regions. Additionally, changes in DA D_1 receptor levels only were observed, with no changes to D_2 receptor detected. These results clearly demonstrated that whilst early treatment with APDs results in long-term, potentially permanent alterations to the functioning of the DA NT system, there are clearly also dissimilarities in their effects on the male and female sexes.

Investigations into potential changes to the 5-HT NT system following early APD treatment are described in Chapter 5. In particular, we investigated the long-term effects of juvenile APD treatment with aripiprazole, olanzapine and risperidone on 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor subtypes, in cortical, striatal and hippocampal brain regions, all known to play key roles in the therapeutic mechanisms of APD action. Once again significant changes to 5-HT receptor subtypes were uncovered in the adult brain following the juvenile APD treatment period, in particular the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor subtypes in cortical and hippocampal brain regions. Furthermore, clear variances between sexes were once again uncovered, with the majority of alterations

found in the male cohort. Specifically, decreases to $5\text{-}HT_{2A}$ receptors were uncovered in cortical and hippocampal brain regions across all 3 drug treatment groups in the male cohort, and $5\text{-}HT_{2C}$ density levels in cortical regions following early aripiprazole treatment. Increases to hippocampal $5\text{-}HT_{2C}$ receptor density levels were also found following early aripiprazole and risperidone treatment. Decreases to $5\text{-}HT_{1A}$ receptors were also found in cortical and striatal regions following early risperidone treatment. As mentioned previously, lesser effects were found in the female cohort, with decreases to $5\text{-}HT_{2A}$ receptor density levels in hippocampal brain regions following early olanzapine and risperidone drug treatment, whilst early aripiprazole treatment was found to decrease $5\text{-}HT_{1A}$ receptor levels in the NAc. These results clearly demonstrated that APD treatment during the critical neurodevelopmental time period of childhood/adolescence results in permanent alterations to 5-HT receptor density levels. The results of this investigation once again clearly indicated that early APD treatment with aripiprazole, olanzapine and risperidone has the potential to cause long-term, permanent alterations to 5-HT receptors, in particular across cortical and hippocampal brain regions in the male cohort.

In conclusion, this thesis has uncovered that APD treatment during the critical neurodevelopmental time period of childhood/adolescence results in long-term changes to various behavioural attributes, and permanent alterations to various receptor subtypes, synthesis and re-uptake markers in the DA and 5-HT NT systems. Additionally, sex and drug specific differences in treatment effects were also evident. With APDs known to have high affinities for both the DA and 5-HT NT systems to elicit their therapeutic effects, and both DA and 5-HT also known to play key roles in

the modulation of various behavioural attributes and in the pathophysiology of numerous mental illnesses, treatment during such a critical period of neurodevelopment has clearly resulted in permanent changes to the functioning of both NT systems in the mature, adult brain. Increasing the awareness of the potential long-term, permanent effects of the prescription and use of APDs in children and adolescents on adult behaviours and the DA and 5-HT NT systems will allow clinicians to weigh up the potential risks vs benefits of prescribing APD treatment during such a critical time of neurodevelopment. Furthermore, the results of the current thesis has shed light on the fact that careful consideration needs to be cast over both the pharmacology of the APD being prescribed, and also the sex of the child being treated.

CHAPTER 2 – LITERATURE REVIEW

2.1 Introduction

Long-term mental illnesses have been found to affect 13-20% of children globally (Younger 2017) and is an important public health problem in Australia, seen to impact approximately 14% of Australian children and adolescents (Sawyer et al. 2001). Whilst the exact processes involved in the pathogenesis of mental illness are still mostly unknown, evidence suggests that both the dopamine (DA) and serotonin (5-HT) neurotransmitter (NT) systems play crucial roles in the aetiology of various mental disorders such as childhood-onset schizophrenia, bipolar disorder and psychosis. Alterations to the functioning of both the DA and 5-HT NT systems, including changes to various pre- and post-synaptic receptor subtypes, especially the DA D_1, D_2 and $5\text{-}HT_{1A}$ and $5\text{-}HT_{2A/2C}$ receptors along specific pathways/areas of the brain have been implicated in the pathogenesis of numerous mental illnesses. The aforementioned changes and subsequent dysregulation of the neurotransmission of both DA and 5-HT has hence associated both the DA and 5-HT system in the mechanisms underlying the disease state (Grace 2000; Lewis and Lieberman 2000; Feenstra et al. 2001; DeLeon et al. 2004; Conley and Kelly 2005; Grace et al. 2007; Nasrallah 2008; Correll 2010).

Antipsychotic drugs (APDs) have been used as a basis of treatment of various mental illnesses for over 50 years (Gray and Roth 2007; Correll 2010), however recent investigations into the trends of APD prescription and use have exposed significant increases in the prescription and use in the juvenile population globally (Olfson et al. 2006; Danielyan et al. 2007; Rani et al. 2008; Varley and McClellan 2009; Egger 2010; Olfson et al. 2010; Alexander et al. 2011; Olfson et al. 2012; Seida et al. 2012; Hoekstra

2014; Karanges et al. 2014). Alarmingly, significant levels of off-label prescription and use within the juvenile population was also highlighted (Haw and Stubbs 2007; Alexander et al. 2011). Whilst second generation APDs (including aripiprazole, olanzapine and risperidone) are known to produce varying levels of therapeutic benefits to patients with a higher safety profile than their first generation counterparts (Findling et al. 2005; Vitiello et al. 2009; Zuddas et al. 2011), their potent affinity and mechanisms of action on DA and 5-HT receptors during what is a crucial period of brain development has highlighted the potential for long-term, potentially permanent alterations to brain functioning, in particular changes to the DA and 5-HT NT systems.

Therefore, investigating the potential long-term effects of juvenile treatment with the commonly prescribed APDs aripiprazole, olanzapine and risperidone on adult behavioural attributes, and furthermore potential permanent changes to the DA and 5-HT NT systems, will provide an important insight for physicians, allowing them to weight up the risk vs benefit ratio of prescribing APDs to the juvenile population.

2.2 Literature Review

2.2.1 Antipsychotic Drugs

2.2.1.1   Overview of APDs

APDs have been used since the 1950s to provide relief to sufferers of various mental illnesses. Decades of research into the pathology of mental illness have led to the continual development of new APDs with a greater therapeutic efficacy and lesser

detrimental side effects, used as a treatment for a wide range of mental disorders (Correll 2008; Almandil et al. 2013; Deng 2013). Two 'generations' of APDs have subsequently been developed, categorised according to their clinical action and/or mechanism of action. Pharmacological analysis of all APDs has found that whilst the majority of APDs display a high affinity of the DA NT system (in particular the DA D_2 receptor) (Feenstra et al. 2001; DeLeon et al. 2004; Conley and Kelly 2005; Nasrallah 2008; Correll 2010), corresponding affinities/actions on other NT systems including the 5-HT NT system and receptors in order to regulate neurotransmission is also correlated with and critical to recent advances in treatment and an increased therapeutic efficacy (Kapur and Remington 1996; Moore et al. 1999; Meltzer and Huang 2008).

In particular, the actions of APDs on DA D_2 receptors and 5-HT$_{2A}$ receptors in regions encompassing the mesolimbic, mesocortical and nigrostriatal pathways have been highlighted as essential in the alleviation of psychotic symptomology, and increased cognitive abilities (Grace et al. 2007; Kegeles et al. 2010; Purves-Tyson et al. 2012). Whilst their affinities for, and mechanism of action on certain NT groups (including both the DA and 5-HT NT systems) are key to their ability to reduce psychosis (Kane and Freeman 1994), severe detrimental side effects are seen to correlate with first and second generation APD use. Moreover, therapeutic benefits have also been found with prescription and use of the aripiprazole, an example of an APD with a partial agonist mechanism of action across multiple DA and 5-HT receptors, with far fewer side effects (DeLeon et al. 2004; Han et al. 2009).

2.2.1.2   First Generation APDs

First generation APDs (also known as 'typical' or 'conventional' antipsychotics) were the first effective form of treatment of some symptomology of numerous mental illnesses, including schizophrenia. First generation APDs have a high affinity for dopaminergic receptors (e.g. Haloperidol; Ki value 1.4nM for D_2 receptors, 21nM for D_3 receptors and 11nM for D_4 receptors) (Table 1) (DeLeon et al. 2004; Conley and Kelly 2005; Kusumi et al. 2015), with its potent non-selective antagonism of these receptor subtypes causing alleviation of some symptomology of mental illness (Seeman et al. 1976; Creese et al. 1996; Ginovart and Kapur 2012).

Although very effective in the treatment of various facets of the symptomology of mental illness, particularly the positive symptoms of schizophrenia, 1st generation APDs have also been found to have very limited benefits in treating other symptomology, including negative symptoms and cognitive deficits (Agid et al. 2008). Furthermore, while providing their therapeutic benefits, the potent, non-selective antagonism of DA receptors (DA D_2 receptor inclusive) by first generation APDs has been strongly linked with inducing the observed Extrapyramidal Symptoms (EPS; e.g. tardive dyskinesia and Parkinsonism) at a very high rate (Tarsy and Baldessarini 2006). The therapeutic efficacy and side effects (e.g. EPS) induced by first generation APDs have been linked to the potent antagonism of D_2 receptors in the mesolimbic and nigrostriatal dopaminergic pathways (Seeman et al. 1976; Creese et al. 1996; Kapur and Mamo 2003; Tarsy and Baldessarini 2006; Ginovart and Kapur 2012), of which are some of the neuronal areas of interest in the present study.

2.2.1.3 Second Generation APDs

In the 1960s, the first of the second generation of APDs (also known as atypical antipsychotics) - clozapine (Clozaril) was developed, and was first used clinically in the 1970s. Further second generation APD development soon followed, including olanzapine (Zyprexia), ziprasidone (Geodon) and risperidone (Risperdal), all of which were used clinically in the treatment of various mental illnesses including schizophrenia (Kane and Freeman 1994). In comparison to their first generation counterparts, the newly developed second generation APDs have an increased therapeutic efficacy in the treatment of the multi-faceted symptomology of various mental illnesses (Cleghorn et al. 1990; Danion et al. 1999; Ho et al. 1999; Conley and Kelly 2005).

Whilst the exact pharmacological mechanisms of action behind second generation APD efficacy are complicated, investigations have uncovered a lower affinity and less potent antagonistic actions on dopaminergic receptor groups than their first generation counterparts. As demonstrated in Table 1, second generation APDs have a more extensive affinity for multiple neuroreceptor groups (Scatton and Sanger 2000; Feenstra et al. 2001; DeLeon et al. 2004; Nasrallah 2008; Correll 2010; Kusumi et al. 2015), encompassing 5-HT (e.g. risperidone Ki value: 0.16 for $5\text{-}HT_{2A}$ receptors), muscarinic (e.g. clozapine Ki value: 1.4 for M_1 receptors), histaminergic (e.g. olanzapine Ki value: 3.5 for H_1 receptors) and α adrenergic receptors (e.g. clozapine Ki value: 6.8 for α_1 receptors) (Mathews and Muzina 2007; Correll 2010). Whilst the lower antagonistic potency of second generation APDs at the DA D_2 receptors has been correlated to fewer EPS than were found with first generation APD use, second generation drug treatment has been found to potentially induce weight gain and other metabolic disorders (e.g.

cardiovascular disease and type II diabetes) (Lieberman et al. 2005; Zipursky et al. 2005; Ujike et al. 2008; Weston-Green et al. 2008; Patel et al. 2009), with antagonism of histamine H_1 receptors in particular previously correlated with such side effects (Deng et al. 2010; He et al. 2013).

Although second generation APDs are known to elicit widespread effects across multiple neuroreceptor groups, a common pharmacological profile on DA and 5-HT receptors (located on dopaminergic neurons in mesolimbic and mesocortical brain regions) is known to play a key role in achieving their increased therapeutic effects (Duncan et al. 1999; Farah 2005; Meltzer and Huang 2008; Meltzer and Massey 2011). Of particular importance is APD occupation of DA receptors (in particular the DA D_2 receptor), with an optimal occupancy rate of between 65-80% found crucial to achieving the greatest therapeutic benefits and least EPS side effects (Seeman 2011; Ginovart and Kapur 2012). Furthermore, the increased antagonistic affinity for 5-HT_{2A} receptors in comparison to DA D_2, and higher antagonistic actions for 5-HT_{2A} and 5-HT_{2C} receptors is thought to be the basis of difference between the vast majority of first and second generation APDs (Meltzer and Massey 2011). Previous studies have found that the antagonism of second generation APDs (e.g. olanzapine) on 5-HT_{2A} and 5-HT_{2C} receptors facilitates the release of DA in cortical, striatal and hippocampal brain regions, which is correlated to the alleviation of mental illness symptomology (Di Matteo et al. 2001; Horacek et al. 2006; Kuroki et al. 2008).

Receptor	HAL (Ki)	OLAN (Ki)	RISP (Ki)	ARIP (Ki)
D_1	270	**250**	**620**	**1960**
D_2	1.4	**17**	**3.3**	**0.74**
D_3	21	54	13	1.0
D_4	11	28	16	510
$5\text{-}HT_{1A}$	3081	**2720**	**250**	**5.6**
$5\text{-}HT_{2A}$	25	**1.9**	**0.16**	**8.7**
$5\text{-}HT_{2C}$	>5000	**7.1**	**63**	**76**
α_1	19	60	2.3	26
H_1	727	3.5	2.6	25
M_1	>1500	1.9	>10 000	>10 000

Table 1 – Receptor binding affinities for 1st and 2nd Generation APDs.

Abbreviations: HAL- haloperidol; RISP- risperidone; OLAN- olanzapine; ARIP- aripiprazole; D_1 = dopamine$_1$; D_2 = dopamine$_2$; D_3 = dopamine$_3$; D_4 = dopamine$_4$; $5\text{-}HT_{1A}$ = serotonin$_{1A}$; $5\text{-}HT_{2A}$ = serotonin$_{2A}$; $5\text{-}HT_{2C}$ = serotonin$_{2C}$; H_1 = histamine$_1$; M_1 = muscarinic$_1$. Note: All values documented as Ki values (nM).

(Feenstra et al. 2001; DeLeon et al. 2004; Nasrallah 2008; Correll 2010; Kusumi et al. 2015)

2.2.1.4 Further Development of Second Generation APDs

Aripiprazole is one of the newest APDs developed, and has been observed to have a more favourable safety and tolerability profile than its predecessors. Whilst it can be categorized as a second generation APD, it has been found to display a unique mechanism of action, different from that of other second generation APDs (Mailman and Murthy 2010). This enhanced therapeutic efficacy with low incidence of detrimental side-effects has been attributed mostly to its partial agonist mechanism of action at DA D_2 receptor (Burris et al. 2002; Shapiro et al. 2003; DeLeon et al. 2004; Wood and Reavill 2007; Etievant et al. 2009; Natesan et al. 2011). It has been found to exhibit a very high affinity (Ki value: 0.45 nM) (Table 1) (DeLeon et al. 2004; Correll 2010; de Bartolomeis et al. 2015) and high occupancy rate for D_2 receptors (more than 90%) at the regular clinical dosage rate of 15-30mg (Yokoi et al. 2002; DeLeon et al. 2004; Hamamura and Harada 2007). Based on these differences in pharmacology, aripiprazole has also been classified as a third generation APD in some literature (Newman-Tancredi et al. 2007; Mailman and Murthy 2010). This thesis however will follow the literature classification of aripiprazole that defines it as being a second generation APD.

As evident in Table 1, aripiprazole also has a high affinity for $5\text{-}HT_{1A}$ and $5\text{-}HT_{2A}$ receptors. Additionally, studies into the pharmacology of aripiprazole have discovered a partial agonist mechanism of action at $5\text{-}HT_{1A}$ and a partial antagonist mechanism of action for $5\text{-}HT_{2A}$ receptors (Shapiro et al. 2003; DeLeon et al. 2004; Newman-Tancredi et al. 2005; Mamo et al. 2007; Correll 2010; Mailman and Murthy 2010; de Bartolomeis

et al. 2015). Furthermore, there is evidence that at relevant therapeutic doses, aripiprazole has a low occupancy and activity at 5-HT_{1A} and 5-HT_{2A} receptors, rather acting predominantly on DA D_2 receptors (Mamo et al. 2007; Wood and Reavill 2007). Our laboratory has also found evidence that long-term aripiprazole treatment did not affect 5-HT_{1A} receptors (Han et al. 2009). Despite these high affinity and occupancy rates for DA D_2 receptors (Yokoi et al. 2002; DeLeon et al. 2004; Hamamura and Harada 2007; Correll 2010), the therapeutic benefits of aripiprazole have been correlated with very low rates of the EPS discussed earlier compared with first generation APDs (e.g. Haloperidol). Furthermore, a very low rate of metabolic side-effects (seen with other second generation APDs such as clozapine and olanzapine) has been found with aripiprazole use (DeLeon et al. 2004; Han et al. 2009; Snyder et al. 2015).

2.2.1.5   Prescription and Use of APDs in Childhood/Adolescence

The prescription and use of APDs in children and adolescents is exponentially increasing worldwide (Olfson et al. 2006; Danielyan et al. 2007; Rani et al. 2008; Varley and McClellan 2009; Egger 2010; Olfson et al. 2010; Alexander et al. 2011; Olfson et al. 2012; Seida et al. 2012; Hoekstra 2014; Karanges et al. 2014), reported to have doubled in the UK (Haw and Stubbs 2007; Rani et al. 2008) and US (Alexander et al. 2011; Olfson et al. 2012; Hoekstra 2014; Karanges et al. 2014; Memarzia et al. 2014) over 10 year periods up to 2005 and 2010 respectively. In Australia, childhood/adolescent APD prescriptions (< 14 years) were found to be relatively stable in the 4 year period to 2007 (around approx. 0.25 dispensed drug utilisation/1000

population/day), however strong trends towards increases is becoming more apparent in recent years (Hollingworth et al. 2013; Karanges et al. 2014).

Whilst first generation APDs (e.g. haloperidol) have been used in the treatment of various childhood mental illnesses since the 1970s, they were seen to exhibit a moderate treatment efficacy (Zuddas et al. 2011) whilst eliciting serious EPS. Second generation APDs (including aripiprazole, olanzapine and risperidone) however were perceived to have a higher safety profile and treatment efficacy in the adolescent population, leading to substantial increases in its prescription and use (Findling et al. 2005; Vitiello et al. 2009; Zuddas et al. 2011) in the treatment of various childhood disorders; from mental illnesses including anxiety, depression and child-onset schizophrenia (Olfson et al. 2010; Memarzia et al. 2014; Schneider et al. 2014), to various behavioural disorders (Findling et al. 2005; Haw and Stubbs 2007; Vitiello et al. 2009; Loy et al. 2012; Sharma and Shaw 2012).

Furthermore, investigations into this growing trend of APD use in childhood/adolescent populations has found it to be mostly through off-label prescriptions (Cheng-Shannon et al. 2004; Haw and Stubbs 2007; Alexander et al. 2011; Olfson et al. 2012; Milstein et al. 2013; Hoekstra 2014; Larkin et al. 2014; Lòpez-De Fede et al. 2014; Schneider et al. 2014). With governing bodies only approving the use of drugs over a specific age and for the treatment of a specific illness (e.g. risperidone, >5yrs for autism spectrum disorder)(Lòpez-De Fede et al. 2014), all childhood/adolescent prescriptions for APDs

outside this licenced age group and treatment purpose is deemed to be off-label use (Cote and E. 1996; Patel et al. 2005; Cooper et al. 2006; Haw and Stubbs 2007).

Although APD use has some benefits in the treatment of mental and behavioural disorders in the childhood/adolescent period (Loy et al. 2012), there is a lack of substantial scientific evidence on the safety and efficacy during this critical neurodevelopmental phase over both a short and long-term period (Andersen and Navalta 2004; Cheng-Shannon et al. 2004; Loy et al. 2012; Memarzia et al. 2014; Xu et al. 2015). Detrimental side-effects including weight gain and EPS have currently been reported, and furthermore, there is the potential for long-term alterations to NT pathways including DA and 5-HT, critical to both the therapeutic efficacy of APDs, along with brain development and functioning (Crews et al. 2007; Correll 2008; Kumra et al. 2008; Almandil et al. 2013; Memarzia et al. 2014; Schneider et al. 2014).

2.2.2 The Dopamine Neurotransmitter System: Its Role in Behaviour, Mental Illness & Potential Long-term Effects of Childhood Adolescent Antipsychotic Drug Use

2.2.2.1 DA: Synthesis, Release and Re-uptake

The DA NT is produced in several areas of the brain, including the substantia nigra (SN) and ventral tegmental area (VTA), with projections to various cortical (e.g. prefrontal cortex (PFC)) and sub-cortical (e.g. caudate putamen (CPu), nucleus

accumbens (NAc), hippocampus) regions of the brain (Figure 1). The synthesis, release and re-uptake of DA is demonstrated in Figure 2. DA biosynthesis begins by the hydroxylation of the amino acid L-tyrosine to L-3,4-dihydroxyphenylalanine (DOPA) via the enzyme tyrosine hydroxylase (TH) (Cooper et al. 1996). This initial phase is the rate limiting step in the synthesis of DA, with the activation of the TH enzyme the results of phosphorylation by a variety of protein kinases at multiple serine residues on the regulatory domain of TH. This phosphorylation of TH produces the phosphorylated-tyrosine hydroxylase (p-TH) enzyme (Haycock 1990; Fitzpatrick 1999), with both the TH and p-TH enzymes well-known indicators of the synthesis levels of DA. The produced DOPA then undergoes decarboxylation by aromatic L-amino acid decarboxylase (AADC) (Cooper et al. 1996). Following synthesis, DA is then transported via the vesicular monoamine transporter 2 (VMAT2) into vesicles, which are then released into the synapse in response to a presynaptic action potential (Cooper et al. 1996; Eiden et al. 2004). Following transmission of the signal, the remaining DA in the synaptic cleft is re-absorbed via the dopamine active transporter (DAT) for recycling (Cooper et al. 1996; Masson et al. 1999).

Nigrostriatal Pathway

Substantia Nigra → Caudate Putamen
. Motor control

Mesocortical Pathway

Ventral Tegmental Area →
Prefrontal Cortex
. Memory
. Motivation and emotional response
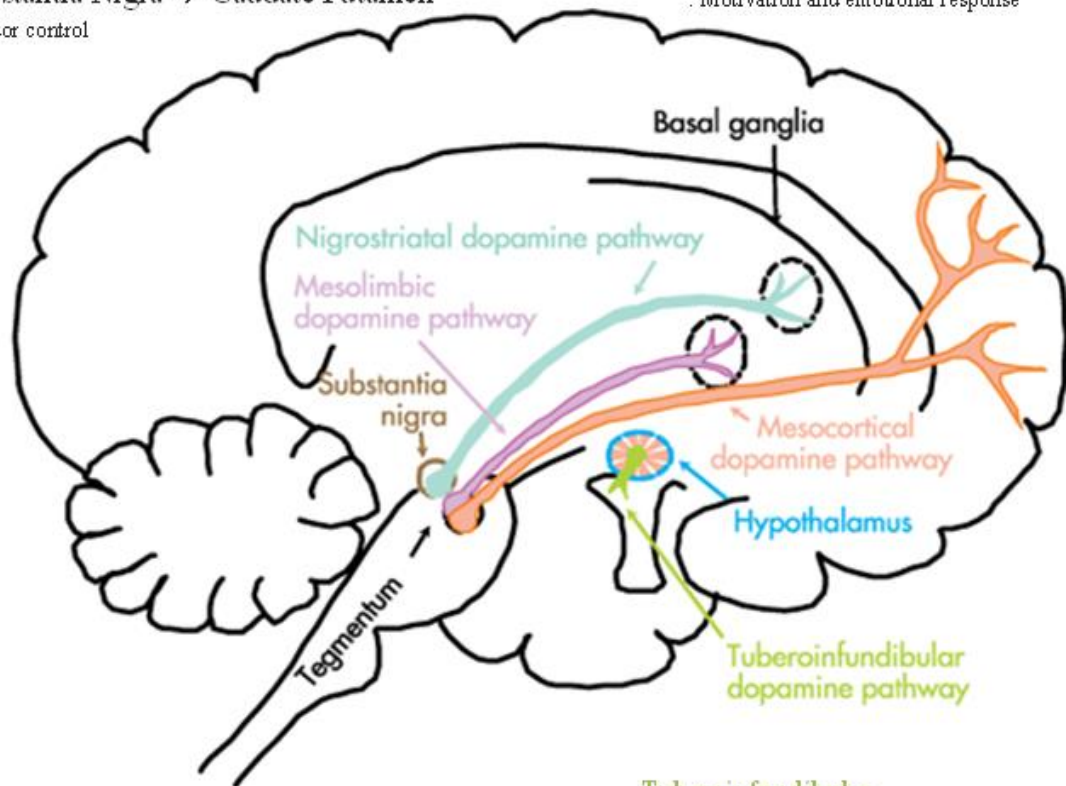
Mesolimbic Pathway

Ventral Tegmental Area → Nucleus
Accumbens, Hippocampus
. Reward and desire
. Addiction

Tuberoinfundibular Pathway

Hypothalamus to Pituitary Gland
. Hormonal regulation

Figure 1 - The four major Dopaminergic Pathways of the Brain & Associated Functions.

(1) Nigrostriatal Pathway, (2) Mesolimbic Pathway, (3) Mesocortical Pathway, (4) Tuberoinfundibular Pathway.

Adapted from Kobayashi 2001; Nasrallah 2009

The transmitted DA is the primary endogenous ligand for DA receptors, which are seven transmembrane domain G-protein coupled receptors (Missale et al. 1998; Beaulieu and Gainetdinov 2011). There are 5 DA receptor subtypes (D_1 receptor to D_5 receptor), of which are divided into 2 groups; the D_1-like family and the D_2-like family. The D_1-like family if of DA receptors consists of the D_1 and D_5 receptor subtypes, and

are coupled to the $G\alpha_{s/olf}$, which is linked to adenylate cyclise activation and subsequent increased intracellular concentration levels of the important second messenger cyclic adenosine monophosphate (cAMP). The D_2-like family consists of the D_2, D_3 and D_4 DA receptor subtypes, which are coupled to the G-protein $G_{\alpha i}$, which is linked to the inhibition of adenylate cyclise, and hence decreased intracellular concentration levels of cAMP (Missale et al. 1998; Neves et al. 2002; Girault and Greengard 2004; Beaulieu and Gainetdinov 2011). Furthermore, the DA D_2 receptor subtype has subsequently been found to be expressed in 2 separate forms (D_2-short chain and D_2-long chain), with differing functions. D_2-short chain receptors have been found pre-synaptically with modulatory functions, whilst D_2-long chain receptors have been found to act and function as classical post-synaptic receptors (Khan et al. 1998). Both DA D_1 and D_2 receptor subtypes have been found in abundance in the striatal and NAc regions of the brain, and are up to 100 times more abundant than the D_3 to D_5 receptor subtypes (Sokoloff and Schwartz 1995; Missale et al. 1998; Hurley and Jenner 2006). Whilst the DA D_2 receptor is of particular interest as it is a high affinity target in the mechanism of APD action, both the DA D_1 and D_2 receptors have a clear role in shaping the dopaminergic signal.
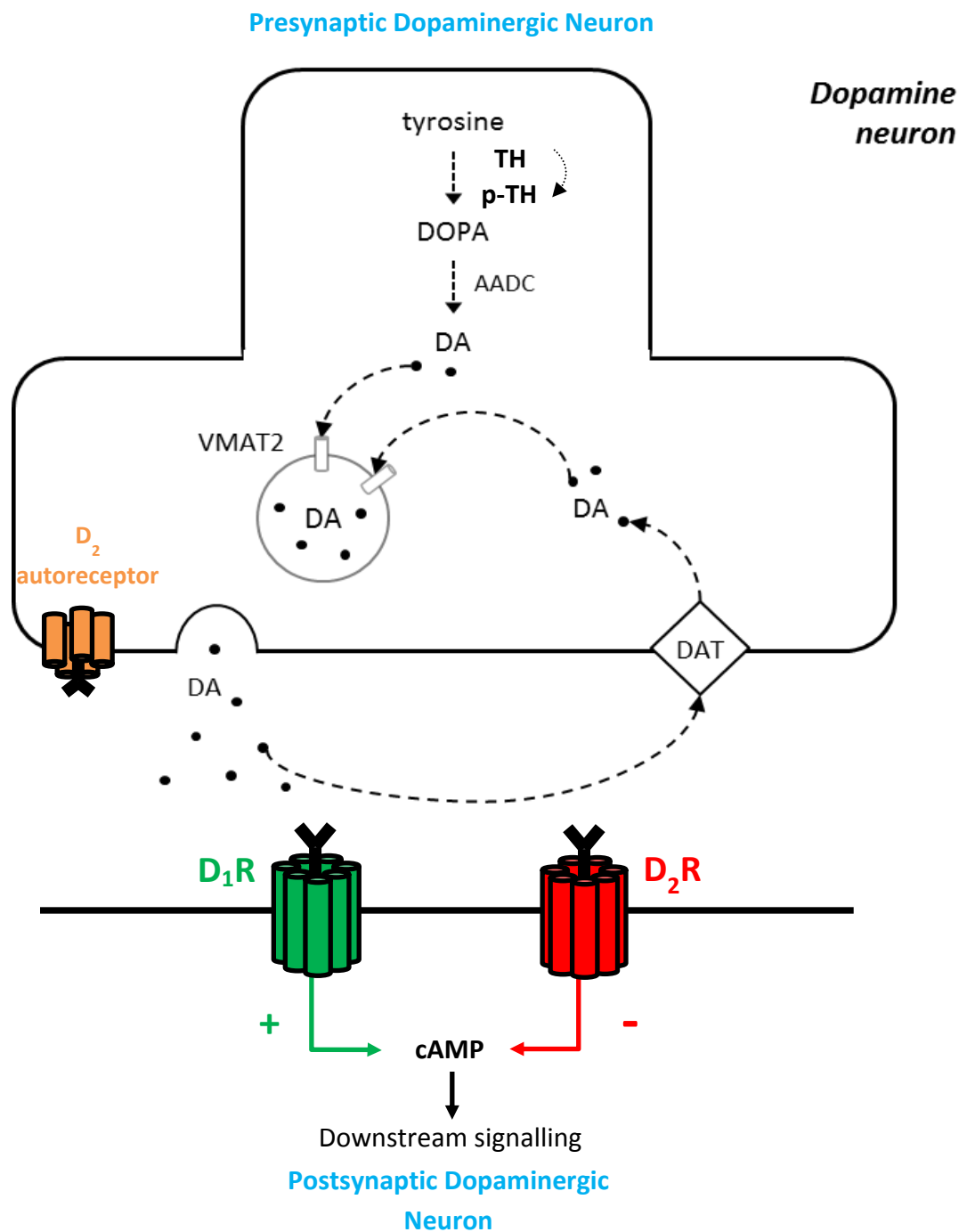
Figure 2 – The synthesis, release and reuptake of Dopamine (DA)

Abbreviations: AADC - Aromatic L-amino acid decarboxylase; D_1R - Dopamine 1 receptor; D_2R - Dopamine 2 receptor; D_2 autoreceptor - Dopamine 2 autoreceptor; DA - Dopamine; DAT - Dopamine Active Transporter; DOPA - L-3,4-dihydroxyphenylalanine; p-TH - Phosphorylated Tyrosine Hydroxylase; TH - Tyrosine Hydroxylase; VMAT2 - Vesicular Monoamine Transporter 2.

2.2.2.2 Regulation of DA Synthesis and Release

As mentioned in Section 2.2.2.1, following the synthesis and release of the DA NT into the synapse for binding with DA receptors, the re-uptake of any excess DA in the synaptic cleft occurs (Schmitz et al. 2003; Lyon et al. 2011; Miyake et al. 2011). This re-uptake process is part of the complex and highly regulated dopaminergic system, where the DAT and DA D_2 autoreceptors interact in order to modulate the synthesis and production of DA, and hence in effect helping to shape DA signalling (Schmitz et al. 2003).

Whilst the system that regulates the production and transmission of DA is highly complex, studies have found a clear role of both the DAT and DA D_2 autoreceptor in the modulation of dopaminergic neurotransmission. While DAT performs the action of DA re-uptake/reabsorption, the DA D_2 autoreceptor has been found to regulate the dopaminergic NT via complex feedback systems at the soma and axon terminal (Schmitz et al. 2003). Activation of the D_2 autoreceptor has been linked to numerous 'regulatory steps' in the synthesis and release of DA, including eliciting hyperpolarization of the membrane, which subsequently decreases NT firing rate via the activation of potassium (K^+) ion channels, and thus the modulation of TH synthesis (the rate-limiting step in DA NT production; Section 2.2.3.1) (O'Hara et al. 1996; Cathala and Paupardin-Tritsch 1999; Schmitz et al. 2003). Finally, a role of 5-HT receptors (including the 5-HT_1 and 5-HT_2 receptor classes) on the regulation/modulation of the DA signal has also been uncovered, with a presence of 5-HT receptors in nigrostriatal, mesolimbic and mesocortical pathways found to act in a either a facilitative (e.g. 5-

HT$_{1A}$ and 5-HT$_{2A}$ receptors) and inhibitory (5-HT$_{2C}$ receptor) manner, shaping the overall DA signal (Alex and Pehek 2007) (see Section 2.2.3.2).

2.2.2.3 The DA Pathways and its Role in the Pathogenesis of Mental Illness

There are 4 major dopaminergic pathways in the brain, demonstrated in Figure 1. They are (1) the nigrostriatal pathway – extending from the SN to the CPu of the striatum, and found to play critical roles in processes including motor control; (2) the mesolimbic pathway – projecting from the VTA to the NAc, amygdala and hippocampus, and involved in the feeling of reward and desire; (3) the mesocortical pathways – from the VTA to the PFC, and involved in memory and emotional responses; and (4) the tuberoinfundibular pathway – extending from the arcuate nucleus of the hypothalamus to the median eminence of the pituitary gland, and is involved in hormonal regulation and sensory processes (Kobayashi 2001). Furthermore, studies have indicated a further role for the nigrostriatal pathway (extending from the SN to the CPu) in the motor control based side-effects of non-selective DA D$_2$ receptor antagonist APDs (e.g. EPS) (Stephen and Stahl 2003).

Neurotransmission along the mesolimbic, mesocortical and nigrostriatal pathways are of particular interest in regard to their involvement in the pathophysiology of mental illness (Carlsson 1988; Kapur and Mamo 2003; Kegeles et al. 2010; Howes et al. 2012; Purves-Tyson et al. 2012). Projections along the mesolimbic and mesocortical pathways especially have been found essential to the mediation of behaviour, anxiety and working memory (Grace 2000; Goto and Grace 2005; Grace et al. 2007). In particular, the NAc

has been found to be somewhat of a convergence centre of neurotransmission projections from the VTA, hippocampus and PFC. Furthermore, an alteration to these dopaminergic projections via DA D_1 receptor and D_2 receptor (from the hippocampus and PFC respectively) to the NAc results in an instability of the DA system, disrupting the tonic (facilitating D_2 receptor activation from the PFC) and phasic (facilitating D_1 receptor activation from the hippocampus) DA neurotransmission (Grace 2000; Goto and Grace 2005; Grace et al. 2007). Any disruptions to DA neurotransmission, such as through APD use, has been found to be fundamental to the pathophysiology of major mental illnesses such as schizophrenia (Csernansky and Bardgett 1998; Meyer-Lindenberg et al. 2002; Goto and Grace 2005; Grace et al. 2007). Therefore, an undisrupted and highly regulated synthesis of DA, along with a balanced transmission from DA neurons in the VTA, PFC and hippocampus to the NAc may assist in the alleviation of some behavioural, anxiety and working memory issues affecting those with various mental illnesses.

2.2.2.4   Potential Long-term Effects of Early APD Use on the DA NT System

As mentioned in Section 2.2.1.1, APDs are known to predominantly produce their therapeutic effects through a mechanism of action concentrated around DA receptors, specifically the D_2 receptor subtype, to modulate the DA signal in mesolimbic, mesocortical and nigrostriatal pathways (Grace et al. 2007; Kegeles et al. 2010; Purves-Tyson et al. 2012). Although relatively unknown in the clinical setting, there is growing evidence through animal studies that juvenile treatment with APDs for a period of up to 4 weeks has the potential to elicit long-term permanent changes to the DA NT system,

including alterations to synthesis markers (TH & p-TH), transporter (DAT) and DA D_1 and D_2 receptor distribution and density levels (Moran-Gates et al. 2006; Der-Ghazarian et al. 2010; Vinish et al. 2012; Xu et al. 2012; Milstein et al. 2013; Klomp et al. 2014; Varela et al. 2014; Lian et al. 2016; Moe et al. 2016). Whilst numerous short-term investigations have found that early APD treatment for 1-4 weeks can result in various immediate changes to the aforementioned features of the DA system (Moran-Gates et al. 2006; Varela et al. 2014; Lian et al. 2016), studies into the longer term effects into adulthood (where a 4 week treatment period in adolescence is utilised) are lesser in number (Vinish et al. 2012; Milstein et al. 2013). Although investigations have uncovered changes to the density and distribution of DA receptors (including D_1 and D_2 receptors), there is limited evidence on the long term effects on the production and regulation of the DA signal along dopaminergic pathways.

2.2.2.4.1 The DA D_1 receptor, mental illness and the therapeutic efficacy of APDs

The DA D_1 receptor is expressed throughout the brain, however the highest receptor density levels are found in nigrostriatal, mesolimbic and mesocortical areas (Missale et al. 1998; Beaulieu and Gainetdinov 2011). In particular, brain regions including the PFC, CPu, NAc, SN and amygdala have been found to express the highest D_1 receptor levels, whilst lower levels have been found in hippocampal and hypothalamic brain regions. As mentioned in Section 2.2.2.1, the D_1 receptor is a G-protein coupled receptor found exclusively on the post-synaptic cleft of DA receptive cells, with its activation following the phasic release of the DA signal known to play a critical role in working memory, particularly in cortical brain regions (Aultman and Moghaddam 2001;

Milstein et al. 2013), and the regulation of DA transmission along DA pathways (Grace 2000; Goto and Grace 2005; Grace et al. 2007).

Although second generation APDs including aripiprazole, olanzapine and risperidone are known to elicit their therapeutic effects predominantly through high affinity actions on the DA D_2 receptor (with a low affinity for the D_1 receptor), investigations have uncovered decreases to D_1 receptor density levels in the NAc and cortical regions following APD treatment in young animals. Specifically, APD treatment with olanzapine and clozapine in young animals over a short-term period have resulted in immediate decreases to D_1 receptor density levels (Moran-Gates et al. 2006; Lian et al. 2016), whilst long-term treatment has resulted in long-term decreases (Vinish et al. 2012; Milstein et al. 2013) in male rats. With the D_1 receptor known to play a key role in the regulation of the DA signal, APDs targeting the D_1 receptor may play a key role in the therapeutic effects of APDs and the alleviation of the symptomology of mental illness.

2.2.2.4.2 The DA D_2 receptor, mental illness and the therapeutic efficacy of APDs

The DA D_2 receptor is known to be located throughout the brain, with varying receptor density levels. Investigations have found the highest levels in the NAc, striatum and olfactory tubercle, whilst they are also expressed at significant densities throughout cortical, hippocampal, SN, VTA, hypothalamic, amygdala and septal brain regions (Missale et al. 1998; Seeman 2006; Beaulieu and Gainetdinov 2011). As specified in Section 2.2.2.1, the DA D_2 receptor is a G-protein coupled receptor known to be found

in both short-form (as a pre-synaptic autoreceptor), and long-form (as a traditional post-synaptic receptor)(Khan et al. 1998). Its activation is linked to the $G\alpha_{i/o}$ class of G-proteins, known to induce inhibition of adenylate cyclase and decreased secondary messenger signalling (Neves et al. 2002; Girault and Greengard 2004; Beaulieu and Gainetdinov 2011).

Previous studies have accumulated undoubted evidence of the critical role the DA D_2 receptor plays in the pharmacological efficacy of first and second generation APD treatment (Meltzer 2002; Nasrallah 2008; Kegeles et al. 2010). As mentioned previously, APDs including risperidone and olanzapine are known to have a high affinity, antagonist mechanism of action on the DA D_2 receptor, with their therapeutic benefits seen around a 60-85% receptor occupancy rate, however detrimental movement and metabolic side effects were also clearly apparent (Lieberman 2004; Seeman 2011; Ginovart and Kapur 2012). The introduction of the APD aripiprazole however saw an enhanced therapeutic efficacy in patients with a decrease in detrimental side effects. Studies into the pharmacology of aripiprazole revealed a critical role of its high affinity (see Table 1), high receptor occupancy (above 90%), partial agonist mechanism of action on the DA D_2 receptor in the regulation of the DA signal, and subsequent alleviation of some symptomology of mental illness (Burris et al. 2002; Shapiro et al. 2003; DeLeon et al. 2004; Wood and Reavill 2007; Etievant et al. 2009; Natesan et al. 2011). A combined vital role of the partial agonist actions of aripiprazole on the 5-HT$_{1A}$ and 5-HT$_{2C}$ receptors and antagonist actions on the 5-HT$_{2A}$ receptor and correlation to therapeutic efficacy was also found (Kapur and Remington 1996; Kusljic et al. 2003; Seo et al. 2008).

2.2.2.5 The Role of the DA System in Behavioural Attributes & Potential Effects of Early APD Treatment

The DA NT system has also been found to play a role in multiple behavioural attributes, with alterations to the production/synthesis, transport and binding of DA in cortical, striatal and ventral tegmental brain regions directly linked to changes to various behavioural attributes (Kelly et al. 1975; Karl et al. 2006; de Oliveira et al. 2009; Beaulieu and Gainetdinov 2011; Biojone et al. 2011). Changes to behaviours including locomotor activity levels, social interaction, depressive- and anxiety-like behaviours have all previously been linked to alterations in the DA NT system, with increases to DA signalling and receptor density levels in areas of the brain including the PFC, NAc and VTA correlated with decreases to anxiety and depressive-like behaviours (Karl et al. 2006; de Oliveira et al. 2009; Adriani et al. 2010; Biojone et al. 2011), and increased locomotor levels (Seo et al. 2008; Beaulieu and Gainetdinov 2011).

With short and long-term APD treatment in juvenile rodents known to have the potential to elicit immediate and long-term changes to DA receptor density levels and DA neurotransmission (Moran-Gates et al. 2006; Vinish et al. 2012; Milstein et al. 2013; Varela et al. 2014), there is the subsequent potential for early treatment with APDs to significantly influence numerous behavioural attributes.

2.2.3 The Serotonin Neurotransmitter System: Its Role in Behaviour, Mental Illness & Potential Long-term Effects of Childhood Adolescent Antipsychotic Drug Use

2.2.3.1 5-HT: Synthesis, Release and Re-uptake

The 5-HT NT is one of the major monoaminergic NTs in the brain, responsible for signal transduction between 5-HT neurons (Silber and Schmitt 2010). Predominantly produced in neurons located in the Raphe Nucleus (RN) of the midbrain (where the majority of 5-HT neurons are located), 5-HT projects throughout the brain including the cortex, striatum and hippocampus, with brain regions including the SN and NAc innervated by projections from the RN (Dawson 1999; Kusljic et al. 2003; Müller and Jacobs 2010; Beliveau et al. 2017; Wong-Lin et al. 2017).

The process of 5-HT synthesis, release and re-uptake is shown in Figure 3, and begins with the conversion of the amino acid tryptophan to 5-hydroxytryptophan (5-HTP) via tryptophan hydroxylase (Dawson 1999), an enzyme that is synthesized in 5-HT neurons in the RN and is the sole precursor and rate limiting step in the production of the 5-HT NT (Dawson 1999; Richard et al. 2009). The conversion of 5-HTP to 5-HT then occurs via the decarboxylation of 5-HTP by the enzyme AADC, the same enzyme present in the synthesis of DA from DOPA (see Section 2.2.2.1) and found to be located in both serotonergic cells in the RN and catecholaminergic cells in relevant brain regions (Dawson 1999). The synthesised 5-HT is then transported via vesicular monoamine transporters and released into the synapse, triggered by 5-HT cell firing rate from the RN (Dawson 1999). Following transmission of the 5-HT signal, termination of potential

synaptic effects are controlled through the binding of 5-HT to the 5-HT transporter (5-HTT), located on pre-synaptic neurons and enables recycling of the 5-HT signal and hence concentration in the synaptic cleft (Dawson 1999).
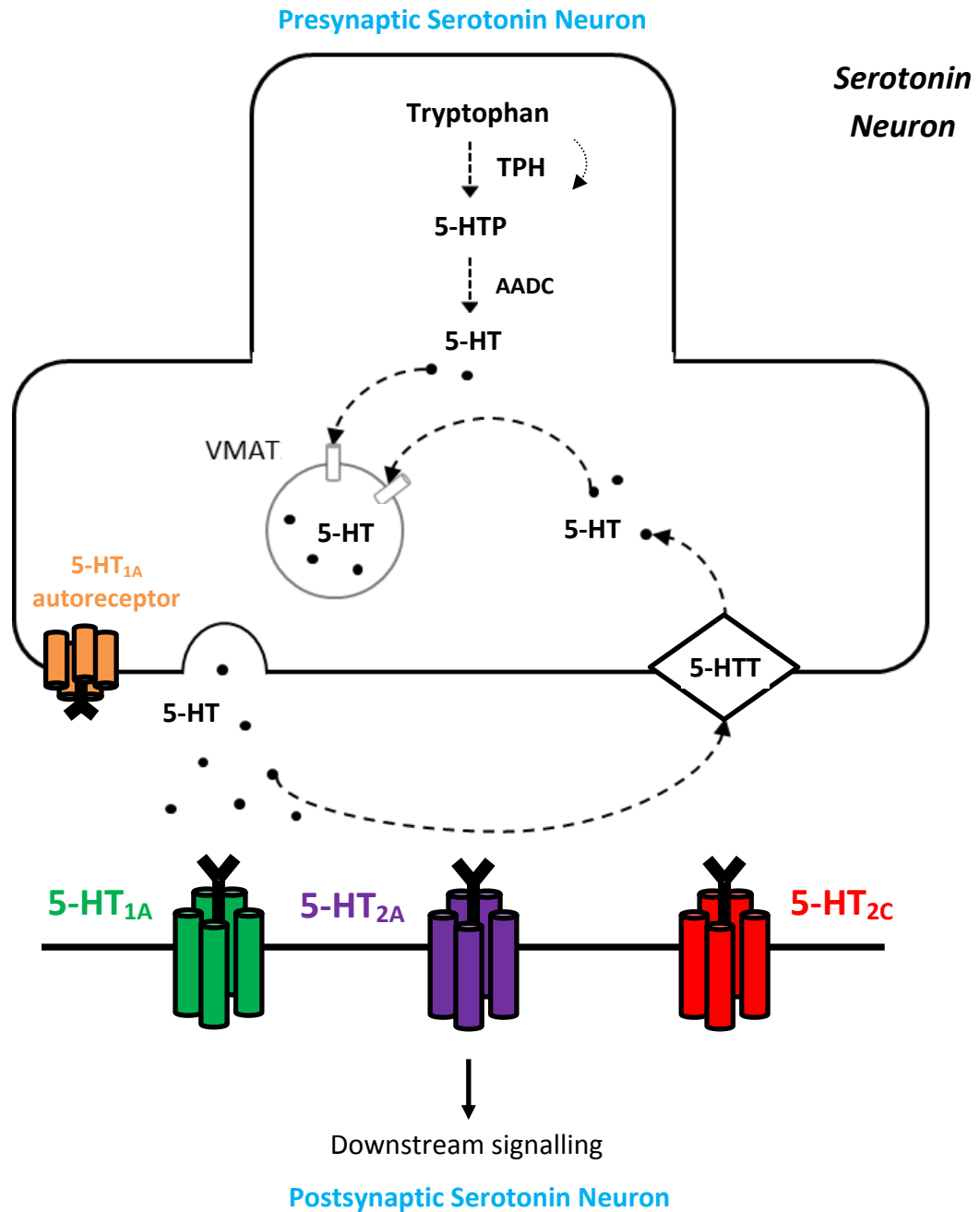
Figure 3 - The synthesis, release and reuptake of Serotonin (5-HT)

Abbreviations: $5\text{-}HT_{1A}$ - Serotonin 1A receptor; $5\text{-}HT_{1A}$ autoreceptor - Serotonin 1A autoreceptor; $5\text{-}HT_{2A}$ - Serotonin 2A receptor; $5\text{-}HT_{2C}$ - Serotonin 2C receptor; 5-HT - Serotonin; 5-HTP - 5-hydroxy-L-tryptophan; 5-HTT - Serotonin transporter; AADC - Aromatic L-amino acid decarboxylase; TPH - Tryptophan Hydroxylase; VMAT - vesicular monoamine transporter.

A monoamine NT, the effects of 5-HT are known to be mediated by approximately fourteen distinctively dissimilar 5-HT receptor subtypes, categorised into seven different classes based on their structural and operational characteristics (5-HT$_1$-5-HT$_7$) (Barnes and Sharp 1999; Fink and Göthert 2007; Hannon and Hoyer 2008; Meltzer and Huang 2008; Beliveau et al. 2017). Whilst the vast majority of 5-HT receptor classes are G-protein coupled receptors (5-HT$_{1-2}$, 5-HT$_{4-7}$) and linked to secondary messenger systems & subsequent modulatory effects (Araneda and Andrade 1991; Wong-Lin et al. 2017), the 5-HT$_3$ class has been identified as a ligand-gated ion channel and subsequent excitatory postsynaptic potential (see Table 2) (Barnes and Sharp 1999; Wong-Lin et al. 2017). Of particular interest are the 5-HT$_{1A}$ and 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor subtypes that are known to be widespread throughout the brain and play opposing modulatory functions (see Sections 2.2.3.4.1, 2.2.3.4.2 and 2.2.3.4.3), and furthermore have been heavily implicated in both the pathology and treatment of various mental illnesses (Araneda and Andrade 1991; Meltzer and Massey 2011; Beliveau et al. 2017; Carhart-Harris and Nutt 2017; Wong-Lin et al. 2017).

Table 2 – Profile of 5-HT receptor classes & associated G-protein & secondary messenger system			
Receptor class	**Class Subtypes**	**Associated G-protein**	**Secondary Messenger/Signal Transduction**
5-HT$_1$	5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{1E}$, 5-HT$_{1F}$	Gα_i/Gα_o	cAMP, PKA / ↓
5-HT$_2$	5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_{2C}$	Gα_q	PLC, IP3, DAG, PKC / ↑
5-HT$_3$	5-HT$_3$	Ion Channel	Ligand-gated Ion Channel
5-HT$_4$	5-HT$_4$	Gα_s	cAMP, PKA / ↑
5-HT$_5$	5-HT$_{5A}$, 5-HT$_{5B}$	Gα_i/Gα_o	cAMP / ↓
5-HT$_6$	5-HT$_6$	Gα_s	cAMP, PKA
5-HT$_7$	5-HT$_7$	Gα_s	cAMP, PKA

Abbreviations: 5-HT- 5-hydroxytryptamine (serotonin); cAMP- cyclic adenosine monophosphate; DAG- diacylglycerol; IP3- inositol 1,4,5-triphosphate; PKA- protein kinase A; PKC- protein kinase C; PLC- phospholipase C

(Barnes and Sharp 1999; Fink and Göthert 2007; Hannon and Hoyer 2008; Wong-Lin et al. 2017)

2.2.3.2   Regulation of 5-HT Synthesis and Re-uptake

As indicated in Section 2.2.3.1, the regulation of the synthesis and release of 5-HT into the synaptic cleft for binding with 5-HT receptors is regulated through both presynaptic autoreceptors and the 5-HTT (Dawson 1999; Alex and Pehek 2007; Fink and Göthert 2007). Both the 5-HT_{1A} and $5\text{-HT}_{1B/D}$ receptor subtypes are well-known to also be located presynaptically in the RN where they function as autoreceptors, with the 5-HT_{1A} autoreceptor located on the cell body of the 5-HT neuron, and $5\text{-HT}_{1B/D}$ autoreceptors located on nerve terminals. Upon the release of 5-HT somatodendritically, 5-HT_{1A} autoreceptor activation inhibits further 5-HT cell firing (Alex and Pehek 2007), and combined with the 5-HTT, which facilitates the re-uptake of excess 5-HT in the synaptic cleft, they act as feedback systems to regulate and modulate the production and transmission of the 5-HT signal (Blier and Ward 2003; Kish et al. 2005; Alex and Pehek 2007; Fink and Göthert 2007).

Furthermore, in addition to the regulation of the 5-HT signal itself, previous studies have demonstrated that the highly interconnected networks of 5-HT projections may also play a critical role in the autoregulation of other NT systems, including glutamate, gamma-aminobutyric acid (GABA) and DA (Lemos et al. 2006; Bang et al. 2012; Wong-Lin et al. 2017). In particular, a significant involvement of 5-HT receptors in the modulation of DA in mesolimbic, mesocortical and nigrostriatal pathways has been highlighted (Kapur and Remington 1996; Alex and Pehek 2007). With 5-HT neurons known to project from the RN to multiple dopaminergic regions of the brain, different 5-HT receptor subtypes have subsequently been found to affect dopaminergic function in different ways.

2.2.3.3 Serotonergic Projections and its Role in the Pathogenesis of Mental Illness

As mentioned previously, 5-HT neurons are known to be predominantly located in the RN of the midbrain, and project through the majority of the brain including the cortex, striatum, hippocampus and SN (Wilson and Molliver 1991; Dawson 1999; Kusljic et al. 2003; Müller and Jacobs 2010; Beliveau et al. 2017; Wong-Lin et al. 2017). Whilst investigations have found that the 5-HT NT plays a key role in various sensory and motor processes in the central nervous system (CNS) (Carlsson 1987; Tecott et al. 1995; Sawa and Snyder 2002; Carlsson et al. 2004; Dolžan et al. 2008), there is growing evidence of its involvement in the pathophysiology of various mental illnesses and behavioural attributes, including schizophrenia, anxiety and depression (Kusljic et al. 2003; Carlsson et al. 2004; Matsumoto et al. 2005; Dolžan et al. 2008; Seo et al. 2008; Meltzer 2012; Teissier et al. 2015).

The link between the 5-HT NT system and the physiology of numerous mental illnesses has also been found to be multi-faceted, with investigations also uncovering a clear negative correlation between the pharmacological actions on and alterations to 5-HT receptors and signal (including 5-HT_{1A} and 5-HT_{2A} receptors), and the modulation of the DA signal and NT system functioning (Kapur and Remington 1996; Abi-Dargham et al. 1997; Horacek et al. 2006; Alex and Pehek 2007; Seo et al. 2008) as indicated in Section 2.2.3.2. Specifically, actions on 5-HT receptors in cortical, striatal and hippocampal brain regions has been found critical to the ability of APDs to modulate the DA signal (Meltzer and Huang 2008; Kusumi et al. 2015), with 5-HT receptor subtypes including the 5-HT_{1A} and 5-HT_{2A} receptor found to facilitate the release of DA

along mesocortical, mesolimbic and nigrostriatal pathways upon agonist and antagonist actions on them respectively. The 5-HT$_{2C}$ receptor subtype has also demonstrated the capability to regulate the DA signal, with inhibitory effects on DA release upon both agonist actions on the receptor and also in phasic periods due to high levels of constitutive activity. Whilst the 5-HT$_{2C}$ receptor has been found to modulate DA release through tonic inhibition from terminal regions of the mesolimbic and nigrostriatal pathways, studies have uncovered an ability of the PFC to produce similar effects in the mesocortical DA pathway (Kapur and Remington 1996; Abi-Dargham et al. 1997; Kusljic et al. 2003; Alex and Pehek 2007). With alterations to the DA signal/DA NT system in mesocortical, mesolimbic and nigrostriatal pathways correlated to the pathophysiology and thus treatment of various mental illnesses (see Section 2.2.2.3) (Carlsson 1988; Kapur and Mamo 2003; Kegeles et al. 2010; Howes et al. 2012; Purves-Tyson et al. 2012), the 5-HT NT system clearly has a significant role to play as a secondary/dual pharmacological target of APDs in modulating the DA signal and enhancing the therapeutic effects of treatment with decreased side effects.

2.2.3.4   Potential Long-term Effects of Early APD Use on the 5-HT NT System

With the enhanced therapeutic efficacy of second generation APDs, attention turned to the importance of the 5-HT system in the pharmacological treatment of mental illnesses (Meltzer et al. 2003; Meltzer and Massey 2011; Amato 2015). Investigations into the pharmacology of second generation APDs quickly uncovered a relatively lower antagonistic effect on the DA D$_2$ receptor and 5-HT$_{1A}$ receptor, and higher affinity for

the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor subtypes, with the functional interaction between the 5-HT and DA systems resulting in the modulation of the DA signal and hence an alleviation of symptomology with decreased side effects (Kapur and Remington 1996; Abi-Dargham et al. 1997).

As indicated in Section 2.2.2.4, with the aforementioned exponential increases in the prescription and use of APDs in the juvenile population, there is growing evidence that treatment during such a critical time of neurodevelopment may cause long-term alterations to the functioning of the 5-HT NT system. Studies have uncovered that early APD treatment may result in immediate alterations to both the density of 5-HT receptors (in particular increases to 5-HT$_{1A}$, and decreases to 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors following treatment with APDs including olanzapine and risperidone), and also the production and transmission of the 5-HT NT signal (Maciag et al. 2006; Choi et al. 2010; Choi et al. 2012; Klomp et al. 2012; Klomp et al. 2014; Lian et al. 2016; Choi et al. 2017). Although current investigations into the effects of short-term APD treatment on the 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors have uncovered various immediate alterations to the density/distribution of 5-HT receptors (Moran-Gates et al. 2006; Varela et al. 2014; Lian et al. 2016; Choi et al. 2017), the long-term effects following an early treatment period is currently unknown.

2.2.3.4.1 The 5-HT$_{1A}$ receptor, mental illness and the therapeutic efficacy of APDs

The 5-HT$_{1A}$ receptor is a G-protein coupled receptor, found to be most highly expressed in the RN and limbic brain regions, including the hippocampus, as well as cortical areas

(Barnes and Sharp 1999; Hannon and Hoyer 2008; Amato 2015). As mentioned in Sections 2.2.3.1 and 2.2.3.2, 5-HT$_{1A}$ receptors are well-known to be located both post-synaptically to 5-HT neurons acting as a normal post-synaptic receptor, and also pre-synaptically on the cell body of 5-HT neurons in the RN where they act as an autoreceptor, modulating the 5-HT signal via inhibition of 5-HT cell firing (Kapur and Remington 1996; Barnes and Sharp 1999; Blier and Ward 2003; Alex and Pehek 2007; Hannon and Hoyer 2008). Investigations into the functional processes involved in the modulatory role of 5-HT$_{1A}$ on the 5-HT NT system have found that 5-HT$_{1A}$ receptor agonism/activation has an antagonist effect on 5-HT$_{2A}$ receptors (Meltzer et al. 2003), which subsequently decreasing 5-HT function (Alex and Pehek 2007). In addition to its modulatory effects on the 5-HT NT system, 5-HT$_{1A}$ receptors have also been found to play an important role in the regulation of DA release in areas of the brain including the PFC, CPu, NAc, hippocampus, SN and VTA (see Section 2.2.3.2) (Kapur and Remington 1996; Meltzer et al. 2003; Alex and Pehek 2007).

With alterations to DA signalling in mesocortical and mesolimbic pathways correlated to the appearance of multiple features of mental illness symptomology, this has resulted in the 5-HT$_{1A}$ receptor being identified as a pivotal target for APD treatment and the alleviation of mental illness symptomology (Rollema et al. 1997; Ichikawa et al. 2001; Li et al. 2004). Correlating with advancements in second generation APD treatment has been an increased affinity and partial agonist mechanism of action to the 5-HT$_{1A}$ receptor (Shapiro et al. 2003; DeLeon et al. 2004; Newman-Tancredi et al. 2005; Mamo et al. 2007; Correll 2010; Mailman and Murthy 2010; de Bartolomeis et al. 2015), as one part of a combined pharmacology for both DA (in particular the D$_2$ receptor) and 5-

HT (in particular the 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors) receptor subtypes (Mamo et al. 2007; Wood and Reavill 2007).

Whilst the majority of previous investigations into the effects of APD treatment on 5-HT$_{1A}$ receptors have shown no alterations, some studies into the immediate effects of treatment of up to 3 weeks in juvenile rats have found that APDs including clozapine, olanzapine and risperidone has the potential to increase 5-HT$_{1A}$ receptor levels in areas of the brain including the PFC and hippocampus (Choi et al. 2010; Choi et al. 2017). Investigations into the adult cohort have found even lesser effects of APD treatment, with a small number of studies over identical treatment periods finding immediate increases to 5-HT$_{1A}$ receptor binding in the PFC of tested animals (Tarazi et al. 2002; Choi et al. 2017).

2.2.3.4.2 The 5-HT$_{2A}$ receptor, mental illness and the therapeutic efficacy of APDs

The 5-HT$_{2A}$ receptor is a G-protein coupled receptor found to be most highly expressed in 5-HT terminal regions of the brain including the CPu, NAc, hippocampus and PFC (Barnes and Sharp 1999; Alex and Pehek 2007; Amato 2015), with lower density levels also uncovered on the cell bodies of DA neurons in the SN and VTA (Jakab and Goldman-Rakic 1998; Doherty and Pickel 2000; Alex and Pehek 2007) and cortical GABAergic and cortico-striatal glutamatergic neurons (Choi et al. 2017). Primarily known as a post-synaptic receptor, stimulation of the receptor has been linked to an enhanced intracellular molecular signal-transduction cascade and thus 5-HT signal, with conversely, antagonism of the 5-HT$_{2A}$ receptor seen to result in decreased 5-HT

function (Alex and Pehek 2007; Fink and Göthert 2007). Furthermore, as described in Section 2.2.3.3, actions on the 5-HT$_{2A}$ receptor have also been linked to changes in the DA signal across mesocortical, mesolimbic and nigrostriatal pathways, with activation of the 5-HT$_{2A}$ receptor resulting in an increase to DA activity (Alex and Pehek 2007). Furthermore, small density levels of the 5-HT$_{2A}$ receptor have also been found pre-synaptically in cortical brain regions, and thus a potential regulatory role is possible yet still to be confirmed (Miner et al. 2003; Alex and Pehek 2007).

With the enhanced therapeutic efficacy and lowered side effects of second generation APD use, the change in pharmacology to a higher affinity for the 5-HT$_{2A}$ receptor and lesser affinity for the DA D$_2$ receptor was highlighted as having a key role in these advancements in treatment (Kusumi et al. 2000; Horacek et al. 2006; Meltzer and Massey 2011). Although there are no current investigations into the long-term effects of early APD treatment on the density of 5-HT$_{2A}$ receptors in the adult brain, some recent studies into the immediate effects of treatment with second generation APDs including olanzapine and risperidone on both young and adult rodents have all demonstrated a capability to decrease 5-HT$_{2A}$ density levels across cortical and brain regions (Tarazi et al. 2002; Choi et al. 2010; Lian et al. 2016; Choi et al. 2017)

2.2.3.4.3 The 5-HT$_{2C}$ receptor, mental illness and the therapeutic efficacy of APDs

The 5-HT$_{2C}$ receptor is known to have a similar pharmacological profile, structure and secondary messenger systems to 5-HT$_{2A}$ receptors. Investigations into their distribution however have found them to be only located in the CNS, with highest density

populations found post-synaptically in the cortex, CPu, limbic system (including the NAc and hippocampus) and the SN, along with the striatum and VTA (Radja et al. 1991; Rick et al. 1995; Alex and Pehek 2007; Amato 2015). As indicated previously, stimulation of the 5-HT$_{2C}$ receptor has a similar effect to the 5-HT$_{2A}$ receptor, with increases to secondary messenger signalling ultimately resulting in an enhanced 5-HT signal (Alex and Pehek 2007; Fink and Göthert 2007).

Along with the 5-HT$_{2A}$ receptor, the 5-HT$_{2C}$ receptor has also been similarly linked to changes in the DA signal, and hence involved in the pathophysiology of various mental illnesses (as described in Section 2.2.3.3) (Meltzer and Huang 2008; Meltzer and Massey 2011). Unlike the 5-HT$_{2A}$ receptor, the 5-HT$_{2C}$ receptor has also been found to have an inhibitory effect on DA release both upon agonist actions on the receptor, and furthermore in phasic periods due to its high levels of constitutive activity(Alex and Pehek 2007). Unique to the 5-HT$_{2C}$ receptor in comparison to its 5-HT$_{2A}$ counterpart is this aforementioned higher level of constitutive activity (Berg et al. 2005), an attribute that may be a key factor in its relationship to both the pathophysiology of mental illness and as a pharmacological target of APD treatment.

Although no previous studies have investigated the long-term effects of early APD on 5-HT$_{2C}$ receptors in the adult brain, a small number of investigations into the immediate effects of treatment in both young (Lian et al. 2016) and adult (Tarazi et al. 2002; Huang et al. 2006; Lian et al. 2015) rodent models have been completed. Whilst immediate decreases to 5-HT$_{2C}$ receptor were found in brain regions including the PFC, NAc, CPu and hippocampus following treatment with olanzapine, a potentially role of

the 5-HT$_{2C}$ receptor in APD induced weight gain was also evident (Hannon and Hoyer 2008; Sicard et al. 2010).

2.2.3.5 The Role of the 5-HT System in Behavioural Attributes & Potential Effects of Early APD Treatment

Along with the DA NT system, changes to the production, transmission and binding of 5-HT from the dorsal RN and median RN to cortical and striatal brain regions has also been correlated to alterations in various behavioural attributes (Tanaka et al. 2007; Dalley et al. 2008; Choi et al. 2010; Young 2013; Teissier et al. 2015; Wong-Lin et al. 2017). Alterations to the 5-HT NT system have uncovered associations to a range of mental illnesses and subsequent behavioural attributes, including reward, depression and anxiety levels (Millan 2004; Joca et al. 2007; Teissier et al. 2015; Wong-Lin et al. 2017). Furthermore, whilst APDs such as aripiprazole, olanzapine and risperidone known to elicit antagonistic effects on 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors and decrease 5-HT projections, the negative correlation and regulatory role of the 5-HT NT system on the DA NT system may therefore also be indirectly resulting in an effect on DA-linked behavioural attributes, including locomotor activity levels (see Section 2.2.2.5) (Kusljic et al. 2003; Seo et al. 2008). Specifically, previous investigations have linked decreases in 5-HT NT signalling and projections from the RN with decreased anxiety and depressive-like behaviours, and increases to activity levels (Kusljic et al. 2003; Seo et al. 2008; Biojone et al. 2011).

With previous investigations clearly highlighting the potential of short and long-term APD treatment on 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors to result in immediate and

long-term changes to both the density of receptors and the 5-HT signal (Mora et al. 1997; Choi et al. 2017), there is the potential that this may result in long-term alterations to behavioural attributes linked to alterations to the 5-HT (and DA) NT systems, including anxiety and depressive behaviours, later in life.

2.2.4 Brain Development, the Dopamine and Serotonin Neurotransmitter Systems & Potential Impact of APD Exposure

2.2.4.1 Mammalian Neurodevelopmental Processes

Investigations into the development of the mammalian brain have identified seven phases of neural development (see Table 3), with numerous critical maturational changes highlighted during the early postnatal phases all the way through to early adulthood (Andersen 2003; Andersen and Navalta 2004; Kolb et al. 2013). Comparative studies of the human and rodent brain have identified the time periods over which each of these neurodevelopmental phases occur, from early embryonic development and initial postnatal days through childhood/adolescence into the adult brain, with the adolescent period defined as the time encompassing the initial postnatal days through the most rapid phases of development (Stiles and Jernigan 2010; Kolb et al. 2013). A comparative overview of the neurodevelopmental process of the DA and 5-HT NT systems in rats and humans, from birth to adulthood, is outlined in Figure 4.

| Table 3 – Seven Stages of Brain Development ||
Stage	Process
1	Neurogenesis & Gliogenesis
2	Cell Migration
3	Cell Differentiation
4	Cell Maturation
5	Synaptogenesis
6	Cell Death & Synaptic Pruning
7	Myelogenesis
	Adapted from (Stiles and Jernigan 2010; Kolb et al. 2013)

During the embryonic time period until birth, neural progenitor cells are known to develop, migrate and differentiate, before elimination of up to 50% occurs in the period immediately before birth (Andersen 2003). From birth and through the childhood/adolescent period, significant increases to synapse and receptor density levels to adult levels occurs, followed by a process of regressive elimination into adulthood until the brain reaches its mature topography (Andersen 2003) (see Figure 4).

Monoamine NTs including DA and 5-HT have been identified as playing critical roles in the aforementioned development of NT producing neurons and target tissues

throughout the brain (Mazer et al. 1997; Andersen and Navalta 2004). Inclusive in this is the development of neurons that produce DA and 5-HT respectively, along with relevant receptor subtypes across both NT systems and in respective brain regions (Andersen and Navalta 2004). Previous investigations have highlighted these numerous maturational changes during this early post-natal period (McCutcheon and Marinelli 2009), with the various phases of development within each NT typified by age-dependent responses to the stimulation of various receptor subtypes and firing of respective neurons (Marinelli and White 2000; Tseng and O'Donnell 2007; Tseng and O'Donnell 2007; McCutcheon and Marinelli 2009). Specifically, investigations have uncovered significant increases to synapse and receptor density levels across both the DA and 5-HT NT systems into early stages of adulthood, followed by a period of regressive elimination (Dinopoulos et al. 1997; Andersen 2003; Andersen and Navalta 2004).
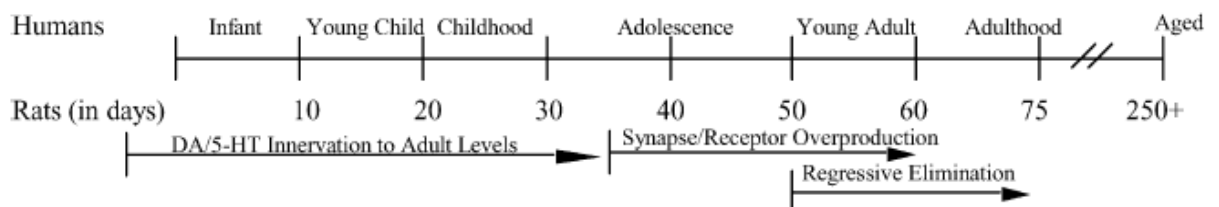
Figure 4 – Comparative timeline of human (below) and rat (above) brain development.

Adapted from (Andersen and Navalta 2004)

It is during the neurodevelopmental phase that differences between male and female sexes have also been identified (Andersen and Teicher 2000; De Bellis et al. 2001; Andersen 2003). Specifically, differences in the rate and age of myelination upon

comparison of sexes have been found, along with the differences between the rate and age of synapse and receptor production and elimination, including within both the DA and 5-HT NT systems (Andersen and Teicher 2000; Andersen 2003).

2.2.4.2 The Trophic Role of the DA & 5-HT NT Systems in Neurodevelopment, and Potential Effects of Early APD Treatment

Monoamine NT systems, including DA and 5-HT, have also been found to play a critical trophic role in the neurodevelopmental processes that shape the final functionality of the adult brain (Levitt et al. 1997; Mazer et al. 1997; Frost and Cadet 2000; Whitaker-Azmitia 2001; Andersen 2003; Andersen and Navalta 2004; Marco et al. 2011; Klomp et al. 2012; Piontkewitz et al. 2012; Milstein et al. 2013; Cousins and Goodyer 2015). Studies have uncovered both NT's significant, concentration-dependent, trophic effect on the various phases of brain development; including the development of receptors, sprouting and formation of synapses and overall axonal growth (Kalsbeek et al. 1988; Lankford et al. 1988; Gelbard et al. 1990; Andersen and Navalta 2004; Lauder 2016). Specifically, decreases to the availability of endogenous DA and 5-HT NT levels in the early development phases following birth, and the subsequent lack of stimulation of receptor subtypes has been found to impact the development of receptors within each NT system, and the overall development and growth of neurites (Diefenbach et al. 1995; Dinopoulos et al. 1997; Whitaker-Azmitia 2001; Andersen 2003; Andersen and Navalta 2004; Song et al. 2017).

The childhood/adolescent period in particular that has been highlighted as a critical phase for the aforementioned neurodevelopment, with numerous processes in which shape the final adult topography of the brain underway during this time of extreme flux (Andersen 2003; Andersen and Navalta 2004; Piontkewitz et al. 2012). During this period until final adult brain topography is reached, several factors have been identified as capable of influencing the functionality of the brain long-term (Levitt et al. 1997; Frost and Cadet 2000; Andersen 2003; Marco et al. 2011; Klomp et al. 2012; Piontkewitz et al. 2012; Milstein et al. 2013; Cousins and Goodyer 2015). Prescription and use of drugs such as APDs have been identified as having the potential to elicit long-term, permanent alterations to NT pathways throughout the brain during this developmental period (Andersen and Navalta 2004; Andersen 2005; Bock 2010; Andersen and Navalta 2011; Alcantara et al. 2014). With APDs known to have a high affinity for and produce their therapeutic effects through potent actions on receptors within the DA and 5-HT NT system, and with both NT systems known to undergo numerous critical neurodevelopmental processes and play a significant role in overall brain development during the childhood/adolescent period (see Section 2.2.4.1 above), there is the potential that drugs with pharmacological actions targeting these NT systems will impact their maturation in a process known as neuronal imprinting (Andersen 2003; Frost et al. 2010; Kolb et al. 2013; Bottelier et al. 2014). Limited studies however have thus far been completed to identify this capability for drug treatment during this critical neurodevelopmental phase to cause long-term alterations to the functionality of the DA and 5-HT NT systems across both sexes, (Moran-Gates et al. 2006; Vinish et al. 2012; Milstein et al. 2013), and forms the premise for this thesis.

2.3 Rationales, Aims and Hypotheses

2.3.1 Rationales of this thesis

As reviewed above, although there may be some short-term benefits of early APD prescription and use, there is the potential for widespread alterations to numerous NT systems, including the DA and 5-HT NT systems. APDs including aripiprazole, olanzapine and risperidone have a high affinity for, and produce their therapeutic effects through potent mechanisms of action on DA and 5-HT receptors, including DA D_2 and 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor subtypes. With both the DA and 5-HT NT systems also known to undergo, and be involved in, numerous critical neurodevelopmental processes, early use of APDs has the potential to cause long-term alterations to normal brain functioning.

2.3.2 Aims

As discussed above, the **general aim** of this study was to investigate whether juvenile APD treatment, at a time where critical neurodevelopmental phases are occurring, would cause long-lasting, potentially permanent changes to behavioural attributes and the DA and 5-HT NT systems.

The **specific aims** of this research study were:

1. Investigate the effects of early APD treatment with aripiprazole, olanzapine and risperidone on long-lasting behavioural attributes, including social interaction, locomotor activity, anxiety-like and depressive-like behaviours.

2. Examine whether early treatment with the APDs aripiprazole, olanzapine and risperidone results in long-lasting alterations to DA NT receptors, synthesis and re-uptake markers in key brain areas including the prefrontal cortex (PFC), caudate putamen (CPu), nucleus accumbens (NAc), hippocampus, substantia nigra (SN) and ventral tegmental area (VTA).

3. Reveal whether early treatment with the APDs aripiprazole, olanzapine and risperidone causes long-term alterations to the key 5-HT receptors 5-HT$_{1A}$, 5-HT$_{2A}$ and 5-HT$_{2C}$, involved in both the efficacy of APD action and the pathogenesis of mental illness, in key brain areas including the PFC, CPu, NAc and hippocampus.

2.3.3 Hypotheses

The **hypotheses** of this research studies were:

1. Early treatment with the APDs aripiprazole, olanzapine and risperidone will result in long-lasting changes to locomotor activity, anxiety-like, depressive-like and social behaviours in across both male and female sexes, as these behavioural attributes have previously been associated to actions on DA and 5-HT receptor subtypes and subsequent modulation of the NT signal. (Chapter 3).

2. Treatment with the APDs aripiprazole, olanzapine and risperidone during the critical neurodevelopmental time period will cause long-term changes to the density of DA D$_2$ receptors in both the male and female cohort, with little effect

on the D_1 receptor subtype. Subsequent alterations to TH and p-TH synthesis markers and DAT will also be uncovered. (Chapter 4).

3. Juvenile treatment with aripiprazole, olanzapine and risperidone will result in long-lasting, permanent changes to the density of $5\text{-}HT_{2A}$ receptor subtypes through multiple brain regions across both the male and female cohorts. Alterations to the $5\text{-}HT_{1A}$ and $5\text{-}HT_{2C}$ receptor subtypes will also be present, however to a lesser extent. (Chapter 5).

CHAPTER 3 - EARLY ANTIPSYCHOTIC TREATMENT IN CHILDHOOD/ADOLESCENT PERIOD HAS LONG-TERM EFFECTS ON DEPRESSIVE-LIKE, ANXIETY-LIKE AND LOCOMOTOR BEHAVIOURS IN ADULT RATS

Early antipsychotic treatment in childhood/adolescent period has long-term effects on depressive-like, anxiety-like and locomotor behaviours in adult rats

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Abstract

Childhood/adolescent antipsychotic drug (APD) use is exponentially increasing worldwide, despite limited knowledge of the long-term effects of early APD treatment. Whilst investigations have found that early treatment has resulted in some alterations to dopamine and serotonin neurotransmission systems (essential to APD efficacy), there have only been limited studies into potential long-term behavioural changes. This study, using an animal model for childhood/adolescent APD treatment, investigated the long-term effects of aripiprazole, olanzapine and risperidone on adult behaviours of male and female rats. Open-field/holeboard, elevated plus maze (EPM), social interaction and forced swim (FS) tests were then conducted in adult rats. Our results indicated that in the male cohort, early risperidone and olanzapine treatment elicited long-term hyper-locomotor effects (open-field/holeboard and FS tests), whilst a decrease in depressive-like behaviour (in FS test) was observed in response to olanzapine treatment. Furthermore, anxiolytic-like behaviours were found following testing in the open-field/holeboard and EPM in response to all three drug treatments. Effects in the female cohort, however, were to a far lesser extent, with behavioural attributes indicative of an increased depressive-like behaviour and hypo-locomotor activity exhibited in the FS test following early risperidone and olanzapine treatment. These results suggest that various APDs have different long-term effects on the behaviours of adult rats.

Keywords

Antipsychotic drug, risperidone, olanzapine, motor activity, anxiety, depression, development

Final manuscript can be accessed from Research Online

CHAPTER 4 - EARLY ANTIPSYCHOTIC TREATMENT IN CHILDHOOD/ADOLESCENT PERIOD HAS LONG-TERM EFFECTS ON THE DOPAMINE NEUROTRANSMITTER SYSTEM IN ADULT RATS

Article

Early Antipsychotic Treatment in Juvenile Rats Elicits Long-Term Alterations to the Dopamine Neurotransmitter System

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Abstract: Prescription of antipsychotic drugs (APDs) to children has substantially increased in recent years. Whilst current investigations into potential long-term effects have uncovered some alterations to adult behaviours, further investigations into potential changes to neurotransmitter systems are required. The current study investigated potential long-term changes to the adult dopamine (DA) system following aripiprazole, olanzapine and risperidone treatment in female and male juvenile rats. Levels of tyrosine hydroxylase (TH), phosphorylated-TH (p-TH), dopamine active transporter (DAT), and D_1 and D_2 receptors were measured via Western blot and/or receptor autoradiography. Aripiprazole decreased TH and D_1 receptor levels in the ventral tegmental area (VTA) and p-TH levels in the prefrontal cortex (PFC) of females, whilst TH levels decreased in the PFC of males. Olanzapine decreased PFC p-TH levels and increased D_2 receptor expression in the PFC and nucleus accumbens (NAc) in females only. Additionally, risperidone treatment increased D_1 receptor levels in the hippocampus of females, whilst, in males, p-TH levels increased in the PFC and hippocampus, D_1 receptor expression decreased in the NAc, and DAT levels decreased in the caudate putamen (CPu), and elevated in the VTA. These results suggest that early treatment with various APDs can cause different long-term alterations in the adult brain, across both treatment groups and genders.

Keywords: antipsychotic; dopamine; risperidone; olanzapine; aripiprazole; development; juvenile

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1. Introduction

Prescription and use of antipsychotic drugs (APDs) in children and adolescents is increasing rapidly worldwide, despite a lack of knowledge on the safety and efficacy of APD use on the developing brain [1–9]. Second-generation APDs including aripiprazole, olanzapine and risperidone are commonly being prescribed (mostly off-label) for the treatment of a variety of childhood disorders, from mental illnesses including anxiety, depression and child-onset schizophrenia [5,10], to various behavioural disorders [11–13]. Risperidone especially has been found to be highly effective in the treatment of male childhood behavioural disorders, and subsequently approved for use by regulatory and governing bodies, and prescribed at a higher rate [12]. Furthermore, recent investigations have also highlighted the potential capability of APD treatment in reducing suicidal risk factors, commonly associated with the aforementioned mental illnesses [14].

Although APDs are known to produce their therapeutic effects predominantly through potent antagonistic and/or partial agonist mechanisms of action on the dopamine (DA) D_2 and serotonin (5-HT) 5-HT_{1A} and 5-$HT_{2A/2C}$ receptors [15–21], the dopaminergic and serotonergic neurotransmitter (NT) systems also undergo, and are heavily involved in, multiple critical neurodevelopmental processes during the childhood/adolescent period [18,22–27].

There is therefore the potential that use of potent APDs at this critical time period of neurodevelopment has the ability to cause long-term alterations to NT systems, including DA signalling pathways, in a manner preceding normal brain functioning [8,26,28]. With alterations to mesocortical, mesolimbic and nigrostriatal DA NT pathways previously implicated in the pathophysiology of the mental illness state [15,21,29,30], prescription and use of APDs in the childhood/adolescent period may be potentially leading to long-term deficits in brain functioning [31].

Whilst current clinical investigations into the effects of APD use in the adolescent population has found some benefits in the control of various mental illness symptomology short term (1–2 months) and over a longer period (up to six months) [32–34], whether or not childhood/adolescent APD use is causing long-lasting alterations to adult brain functioning is mostly unknown [18,35,36].

Previously, several animal studies investigating the effects of early APD use on the developing brain, including previous investigations completed in our laboratory, has found that early treatment of up to 4 weeks can result in various significant alterations to both behavioural attributes [31], and numerous NT systems, including the DAergic NT system [18,36–40]. Whilst numerous investigations have found that early treatment with various APDs in juvenile rats has resulted in various short-term alterations immediately after treatment [36,38,40], studies into potential long-term alterations are fewer in number [18,37]. Changes to both the distribution/density of various NT receptor subtypes, including DA D_1 and D_2 receptors have been uncovered in cortical and striatal brain regions, along with alterations to dendritic architecture [41], with limited evidence on potential long-lasting effects to the synthesis, production and regulation of DA.

The aim of the current study was therefore to further investigate the long-lasting effects of early APD exposure in juveniles with aripiprazole, olanzapine and risperidone on the DA NT system in both male and female adult rats. In particular, adult brain levels of dopamine active transporter (DAT), and the DA synthesis markers tyrosine hydroxylase (TH) and phosphorylated-tyrosine hydroxylase (p-TH), as well as D_1 and D_2 receptors, were investigated in both cortical and striatal brain regions, along with regions involved in the synthesis and regulation of the DA signal, including the hippocampus, substantia nigra (SN) and ventral tegmental area (VTA), via Western blot and/or receptor autoradiography experiments.

2. Results

2.1. Long-Term Effects of Adolescent APD Treatment on Tyrosine Hydroxylase (TH) and Phosphorylated-Tyrosine Hydroxylase (p-TH) Levels

2.1.1. Tyrosine Hydroxylase

There was a significant effect of Gender on TH expression in the nucleus accumbens (NAc) ($F_{1,46} = 10.487$, $p < 0.01$), SN ($F_{1,44} = 38.456$, $p < 0.001$), Hippocampus ($F_{1,45} = 5.416$, $p < 0.05$) and VTA ($F_{1,46} = 15.486$, $p < 0.001$) following analysis via two-way ANOVA (Analysis of Variance). Post hoc analysis revealed that whilst males expressed higher TH density levels in the SN, Hippocampus and VTA, females were found to have higher levels in the NAc. Furthermore, a significant effect of treatment on TH expression was found in the VTA ($F_{3,46} = 4.851$, $p < 0.01$) (Figure 1E′,E″), whilst a trend to significant effect of Treatment was observed in the caudate putamen (CPu) ($F_{3,46} = 2.352$, $p = 0.087$). Analysis via one-way ANOVA of the female cohort uncovered a significant effect of early APD treatment on TH expression in the VTA ($F_{3,22} = 6.098$, $p < 0.01$). Further post hoc tests found a significant decrease in TH expression following early treatment with the APD aripiprazole (-59.2%, $p < 0.01$). In the male cohort, a significant effect of early APD treatment on TH expression was found in the prefrontal cortex (PFC) ($F_{3,22} = 3.201$, $p < 0.05$) of adult rats. Post hoc analysis discovered that early aripiprazole treatment significantly decreased TH expression (-11.2%, $p < 0.05$), whilst a trend to significant decrease was also found in the olanzapine drug treatment group (-9.0%, $p = 0.096$) (Figure 1A′,A″).
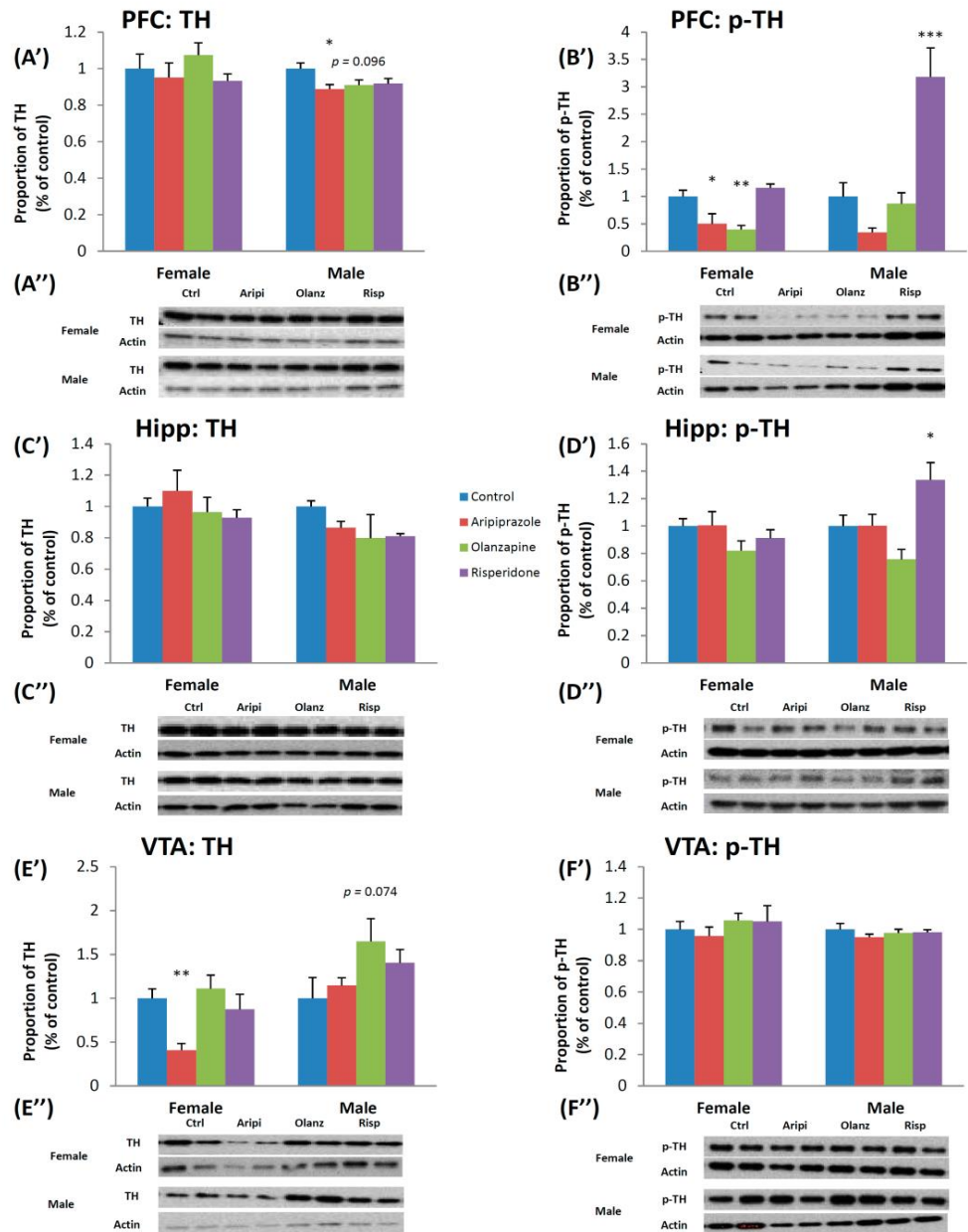
Figure 1. Effects of three APDs on TH and p-TH expression levels in the PFC (TH: (**A′**,**A″**); p-TH: (**B′**,**B″**)), Hipp (TH: (**C′**,**C″**); p-TH: (**D′**,**D″**)) and VTA (TH: (**E′**,**E″**); p-TH: (**F′**,**F″**)) of female and male rats. Sprague-Dawley rats were treated chronically with Aripiprazole (1.0 mg/kg, t.i.d), Olanzapine (1.0 mg/kg, t.i.d), Risperidone (0.3 mg/kg, t.i.d) or control (vehicle). Data expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control. The representative bands of Western Blot are shown. APD: Antipsychotic drug; Hipp: Hippocampus; p-TH: Phosphorylated-tyrosine hydroxylase; PFC: Prefrontal cortex; t.i.d.: Three times daily; TH: Tyrosine hydroxylase; VTA: Ventral Tegmental Area.

2.1.2. Phosphorylated-Tyrosine Hydroxylase

Analysis via two-way ANOVAs (Gender $\times$ Treatment) of p-TH expression levels revealed a significant effect of Gender ($F_{1,46} = 11.602$, $p < 0.01$), Treatment ($F_{3,46} = 6.759$, $p < 0.001$) and a significant interaction between the two factors ($F_{3,46} = 8.272$, $p < 0.001$) in the PFC of animals, with a higher expression in the male cohort. In the SN, a significant effect of Gender was also observed ($F_{1,44} = 6.884$, $p < 0.02$), with higher p-TH expression in the female cohort, whilst in the Hippocampus, a significant effect of Treatment ($F_{3,45} = 5.301$, $p < 0.01$) and a significant interaction between the two factors ($F_{3,45} = 3.430$, $p < 0.05$) was uncovered. In the female cohort, analysis via one-way ANOVA uncovered a significant effect of early APD treatment on p-TH expression in the PFC ($F_{3,22} = 9.070$, $p < 0.01$), whilst post hoc analysis revealed p-TH expression in the PFC was found to significantly decrease in both the aripiprazole (-49.4%, $p < 0.05$) and olanzapine (-60.2%, $p < 0.01$) treatment groups (Figure 1B',B''). In the male cohort, analysis via one-way ANOVA found a significant effect of treatment on p-TH expression in the PFC ($F_{3,22} = 15.887$, $p < 0.001$) and Hippocampus ($F_{3,22} = 6.103$, $p < 0.01$). Post hoc analysis revealed that early risperidone treatment significantly increased p-TH expression in both the PFC (218.3%, $p < 0.001$) (Figure 1B',B'') and Hippocampus (33.7%, $p < 0.05$) (Figure 1D',D'') of male rats.

2.2. Long-Term Effects of Adolescent APD Treatment on Dopamine Active Transporter (DAT) Levels

Two-way ANOVA analysis revealed a significant effect of Gender found in the CPu ($F_{1,46} = 6.509$, $p < 0.02$), NAc ($F_{1,44} = 6.300$, $p < 0.02$) and VTA ($F_{1,46} = 15.977$, $p < 0.001$) of adult rats following early APD treatment, with a higher protein expression of DAT in the male cohort uncovered. Furthermore, a trend to significant interaction between the Gender and Treatment factors was found in the VTA ($F_{3,46} = 2.554$, $p = 0.07$). One-way ANOVA analysis of the male cohort found that early APD treatment had a significant effect on DAT protein expression in the VTA ($F_{3,22} = 3.170$, $p < 0.05$). Furthermore, post hoc analysis revealed that risperidone treatment significantly increased DAT protein expression in the VTA ($+107.3\%$, $p < 0.05$). No significant differences in DAT protein expression were found following analysis of the female cohort.

Examples of [^{3}H]WIN35428 binding to DAT are presented in Figure 2A'–D'. Non-specific binding was observed to be less than 20% in the CPu, and less than 25% in the SN and VTA due to relatively lower DAT binding densities. There was a significant effect of Gender on DAT bindings in the SN ($F_{1,44} = 11.803$, $p < 0.01$) and VTA ($F_{1,46} = 13.941$, $p < 0.01$) of rats following two-way ANOVA, with significantly higher levels of DAT bindings in the male cohort. Furthermore, a significant effect of Treatment was found in the CPu ($F_{3,46} = 2.976$, $p < 0.05$). Post hoc analysis revealed that early risperidone treatment significantly decreased DAT binding by -35.9% ($p < 0.02$). Arranged by gender, one-way ANOVA revealed a significant effect of early APD treatment on DAT bindings was observed in the CPu of the male cohort ($F_{3,21} = 4.065$, $p < 0.05$). Post hoc analysis revealed that early risperidone treatment significantly decreased DAT bindings in the CPu (-37.1%, Controls 176.9 ± 8.4 fmoles/mg vs. risperidone 111.3 ± 15.7 fmoles/mg, $p < 0.02$). Due to very weak DAT binding present in the PFC, NAc and Hippocampus of both females and males, DAT bindings in these areas were not quantified. No significant effects were found in the female cohort between APD treatment group and controls.
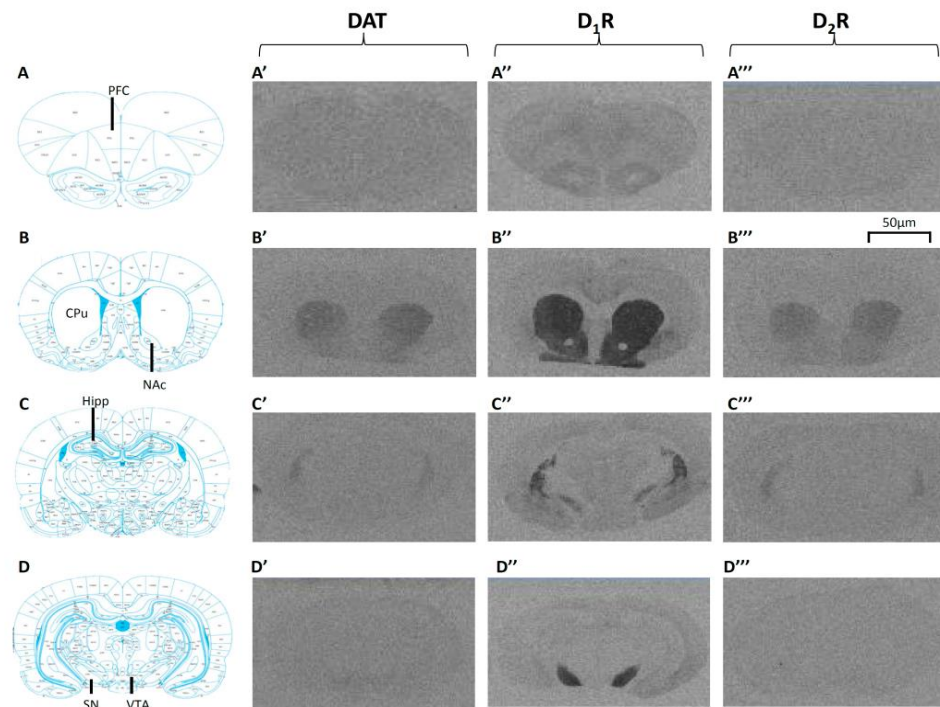
Figure 2. Examples of DAT, D_1 and D_2 receptors binding in the adult rat brain following childhood/adolescent APD treatment. (**A**–**D**) The schematic diagram adapted from a rat brain atlas [42] showing the level of Bregma for each investigated region ((**A**) PFC: 4.68 mm; (**B**) CPu & NAc: 1.08 mm; (**C**) Hippocampus: −2.76 mm; (**D**) SN & VTA: −4.92 mm). (**A′**–**D′**) Examples of autoradiograms to demonstrate [^{3}H]WIN35428 binding to DAT; (**A″**–**D″**) Examples of autoradiograms to demonstrate [^{3}H]SCH23390 binding to DA D_1 receptors (D_1R); (**A‴**–**D‴**) Examples of autoradiograms to demonstrate [^{3}H]Raclopride to DA D_2 receptors (D_2R). APD: Antipsychotic drug; CPu: Caudate Putamen; DA: Dopamine; DAT: Dopamine Active Transporter; Hipp: Hippocampus; NAc: Nucleus Accumbens; PFC: Prefrontal cortex; SN: Substantia Nigra; VTA: Ventral Tegmental Area.

2.3. Long-Term Effects of Adolescent APD Treatment on Dopamine D_1 Receptor (D_1R) Levels

Two-way ANOVA found a significant effect of Gender on the protein levels of D_1R in the NAc ($F_{1,45} = 11.529$, $p < 0.01$), with a higher D_1 receptor protein level in the female cohort, whilst male protein levels of D_1R was found to be higher overall in the VTA ($F_{1,46} = 1.720$, $p < 0.01$). A significant interaction between Gender and Treatment factors in the VTA ($F_{3,46} = 3.273$, $p < 0.05$) was also uncovered. In the female cohort, one-way ANOVA uncovered a significant long-lasting effect of treatment observed in the VTA ($F_{3,22} = 5.551$, $p < 0.01$). Post hoc analysis revealed that early aripiprazole treatment significantly decreased D_1R protein levels in the VTA by 73.7% ($p < 0.02$) (Figure 3C′,C″). Analysis of the male cohort revealed early APD treatment had significant long-term effects on the D_1R protein levels in the NAc ($F_{3,21} = 3.749$, $p < 0.05$). Post hoc tests demonstrated that early treatment with risperidone was found to decrease D_1R protein levels by 21.0% ($p < 0.02$) (Figure 4C′,C″).

Examples of [^{3}H]SCH23390 binding to D_1R are presented in Figure 2A″–D″. DA D_1 receptor binding was observed in the CPu, NAc and SN, with non-specific binding was observed to be less than 10%. However, there was a weak binding in the PFC, Hippocampus and VTA, and was therefore discounted from analysis. There was a significant effect of Gender in the SN ($F_{1,46} = 20.093$, $p < 0.001$) of rats following two-way ANOVA, with higher levels uncovered in the female cohort. Additionally,

a trend to a significantly higher D_1R binding was found in the CPu of female rats ($F_{1,46} = 2.966$, $p = 0.093$).
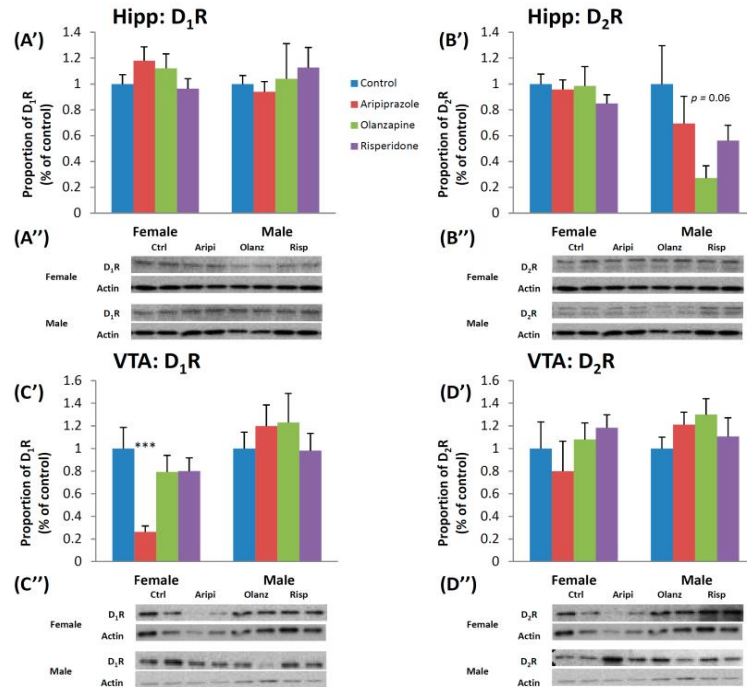
Figure 3. Effects of three APDs on D_1 and D_2 receptor expression levels in the Hippocampus (D_1R: (**A′**,**A″**); D_2R: (**B′**,**B″**)) and VTA (D_1: (**C′**,**C″**); D_2: (**D′**,**D″**)) of female and male rats. Sprague-Dawley rats were treated chronically with Aripiprazole (1.0 mg/kg, t.i.d), Olanzapine (1.0 mg/kg, t.i.d), Risperidone (0.3 mg/kg, t.i.d) or control (vehicle). Data expressed as mean $\pm$ SEM. *** $p < 0.001$ vs. control. The representative bands of Western blot are shown. APD: Antipsychotic drug; Hipp: Hippocampus; t.i.d.: Three times daily; VTA: Ventral Tegmental Area.

2.4. Long-Term Effects of Adolescent APD Treatment on Dopamine D_2 Receptor (D_2R) Levels

There was a significant effect of Gender on DA D_2R protein levels in the SN ($F_{1,45} = 35.633$, $p < 0.001$) and hippocampus ($F_{1,45} = 7.418$, $p < 0.02$) following two-way ANOVA, with higher D_2R protein levels found in the female cohort in both brain regions. In the female cohort, one-way ANOVA analysis revealed that APD treatment had a significant effect on D_2R protein in the PFC ($F_{3,21} = 4.228$, $p < 0.02$). Early treatment with olanzapine significantly increased D_2R protein in the PFC by 55.1% ($p < 0.02$) (Figure 4B′,B″). Whilst no significant alterations to D_2R protein levels were found between drug treatment groups in the male cohort, a trend to significant effect of treatment was found in the SN ($F_{3,21} = 2.897$, $p = 0.064$) and in the hippocampus between olanzapine and control groups (-72.8%, $p = 0.06$) (Figure 3B′,B″).

Examples of [^{3}H]Raclopride binding to D_2R are presented in Figure 2A‴–D‴. Non-specific binding in the CPu and NAc were observed to be below 10%. Weak binding, however, was found in the PFC, Hippocampus, SN and VTA, and were thus discounted from analysis. Analysis via two-way ANOVAs (Gender × Treatment) uncovered a trend towards a significant interaction between the two factors on D_2R binding density in the NAc ($F_{1,46} = 3.790$, $p = 0.059$). Analysis of the female cohort via one-way ANOVA, a significant effect of treatment on D_2R expression was found in the NAc ($F_{3,24} = 3.362$, $p < 0.05$). Post hoc analysis demonstrated that early olanzapine treatment significantly

increased D_2R binding in the NAc of female rats (+35.0%, Control 79.8 ± 7.5 fmoles/mg vs. olanzapine 107.6 ± 6.8 fmoles/mg, $p < 0.05$), whilst trends to significant increases were found following early treatment of olanzapine (+11.8%, Control 161.2 ± 1.5 fmoles/mg vs. olanzapine 180.2 ± 9.0 fmoles/mg, $p = 0.083$) in the CPu of adult female rat brains. No significant alterations were found following analysis in the male cohort.
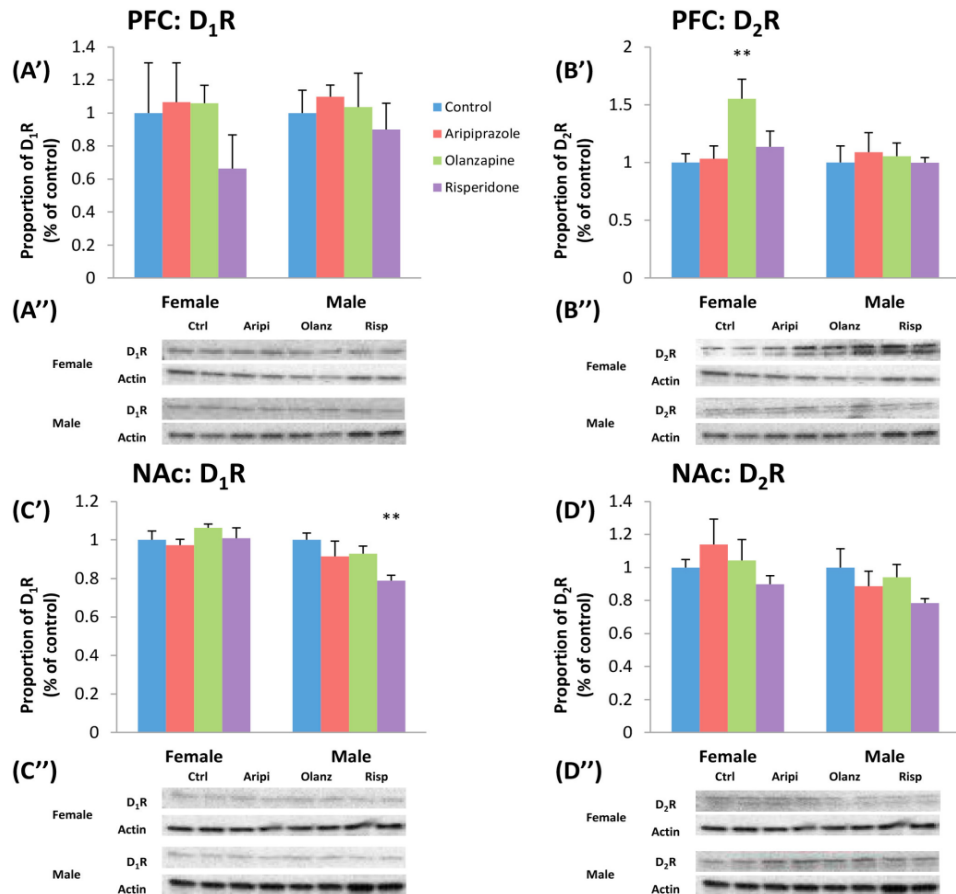
Figure 4. Effects of three APDs on D_1 and D_2 receptor (D_1R and D_2R) expression levels in the PFC (D_1R: (**A′,A″**); D_2R: (**B′,B″**)) and NAc (D_1R: (**C′,C″**); D_2R: (**D′,D″**)) of female and male rats. Sprague-Dawley rats were treated chronically with Aripiprazole (1.0 mg/kg, t.i.d), Olanzapine (1.0 mg/kg, t.i.d), Risperidone (0.3 mg/kg, t.i.d) or control (vehicle). Data expressed as mean $\pm$ SEM. ** $p < 0.01$ vs. control. The representative bands of Western blot are shown. APD: Antipsychotic drug; NAc: Nucleus Accumbens; PFC: Prefrontal cortex; t.i.d.: Three times daily.

3. Discussion

The present study investigated for the first time the long-term effects of early treatment (in juvenile rats) with the commonly used APDs aripiprazole, olanzapine and risperidone, on DA neurotransmission in the hippocampus, SN and VTA, and provides further evidence of potential alterations in the PFC, CPu and NAc in adult male and female rats. Our findings provide evidence that early APD treatment during the critical neurodevelopmental period of youth causes long-lasting alterations to DA synthesis and re-uptake markers in the mesocortical DA NT pathway, CPu and

Hippocampus in the adult brain. Additionally, alterations to DA D_1 and D_2 receptors were also uncovered across the mesolimbic and nigrostriatal brain regions. Furthermore, different effects between gender cohorts were also uncovered.

Whilst previous studies investigating the long-term effect of juvenile APD use on DA synthesis markers in the adult brain have to our knowledge not been completed, several studies investigating the immediate and long-lasting effects of APD treatment with aripiprazole, olanzapine and risperidone on DA synthesis markers, precursors, and NT levels in young [37,43] and adult [43–50] rodent models have demonstrated differing effects.

In this study, long-term alterations in DA synthesis markers were uncovered in the present study. In the female cohort, significant decreases in the DA synthesis markers TH and p-TH were found following early treatment with aripiprazole and olanzapine in comparison to the control; specifically in the PFC and VTA brain regions of the DA NT system. Rats that received early treatment with aripiprazole showed decreased levels of TH and p-TH in the VTA and PFC respectively, whilst early olanzapine treatment resulted in decreased p-TH levels in the PFC upon comparison to the control. Additionally, decreased TH levels in the PFC were also uncovered in the male cohort following early aripiprazole and olanzapine treatment. Previous investigations into the effect of the partial D_2 agonist aripiprazole over acute and short-term durations in the adult rodent have uncovered increases to DA markers indicative of an increased DA synthesis and/or production in the PFC of male rats [43–47]. However, studies into the effects of short-term aripiprazole treatment on DA production in the VTA have revealed an opposite effect, with reductions in DA firing observed [48,50]. Furthermore, whilst investigations into the effects of olanzapine over short-term time periods in adult rats have also found increases to DA and its precursor 3,4-dihydroxyphenylalanine (DOPA) in the PFC of treated animals [44], an investigation into the long-term effects of adolescent treatment with olanzapine on the stimulated release of the DA NT in the adult brain revealed decreases to DA transmission in the NAc following electrical stimulation of the VTA [37].

Interestingly, our study revealed that early treatment with risperidone, however, was found to increase p-TH levels in both the PFC and hippocampus of male rats. Previous acute and short-term studies into the effects of the potent D_2 receptor antagonist risperidone revealed increased levels of DA production and transmission in the PFC and NAc of the adult rats [49,51]. This indicates that whilst the actions of all APDs in the juvenile neurodevelopmental phases resulted in long-term alterations to DA synthesis levels, the more potent D_2 antagonist pharmacology of risperidone resulted in an increase to the level of DA synthesis markers in the long-term, compared with the decreased levels observed in the other APDs investigated. Furthermore, a clear difference between genders was observed with the change elicited by risperidone only observed in the male rodent model.

Whilst similar effects of aripiprazole treatment on DA production in the VTA, and risperidone treatment effects on DA levels in the PFC was observed in the present study, opposing effects to those previously described in the literature were also revealed. Specifically, decreases in the production of TH and p-TH were found in the PFC of both female and male animals following treatment with both aripiprazole and olanzapine in our study. It is possible that these opposing results to that reported in the literature may be due to factors including; the difference in age of the animals treated, the treatment duration of the study, and the duration of time between cessation of treatment and detection of DA markers, with the current investigation specifically investigating what the effects of early APD treatment is on DA NT synthesis in the adult brain. The observed alterations to the measured variables may have therefore subsequently occurred following the withdrawal period following the cessation of APD treatment. Furthermore, the altered levels of TH and p-TH observed following aripiprazole, olanzapine and risperidone treatment may have been the result of its pharmacological actions on DA receptors undergoing a critical neurodevelopmental period, which has previously been found to result in long-term alterations to NT functioning [18,37].

Although investigations into the long-term effects of early APD treatment on DAT levels have not been completed in either young or adult treatment models to our knowledge, adult animal studies

demonstrating the acute and short-term effects of drugs with a pharmacology based around the DA D_2 receptor on DAT availability and subsequent DA re-uptake have shown region specific results [52–54]. Along with alterations in DA synthesis markers, our study uncovered region specific alterations to DAT levels in the male cohort. Whilst early APD treatment with risperidone was found to increase DAT expression in the VTA (in correlation to the effect seen on TH and p-TH levels in the PFC and hippocampus), a decrease in DAT level was observed in the CPu in comparison to the control. Previous studies into the effects of D_2 receptor antagonists have found decreases to DAT re-uptake and availability in striatal regions, whilst opposite effects have been observed in the NAc [52,54]. Additionally, investigations into the effects of D_2 receptor agonists have indeed uncovered an increase in DAT availability in the striatum [52,54].

As previously mentioned, whilst the observed alterations to DAT levels were parallel to the majority of changes in DA synthesis markers, the decrease in DAT expression in the CPu of the male cohort following risperidone treatment was not expected. This potentially indicates that whilst APD treatment may result in an up/down-regulation of DA synthesis, and concurrent alteration in DAT in mesocortical/mesolimbic DA pathways, different alterations may be caused along other DAergic projections. With alterations to specific DAergic regions/projections previously implicated in numerous facets of mental illness symptomology, including alterations to activity levels [17,55] and cognitive functioning [56], such alterations following early APD treatment may be indicative of changes to such symptomology.

With the pharmacology of the APDs investigated in our study based around either partial agonist or antagonist mechanism of action of the DA D_2 receptor, it can be said that strong similarities can be drawn between results. Whilst it is clear that the levels of DAT present in the investigated DA pathways are heavily influenced by the pharmacological actions of drugs on the DA D_2 receptor, it is also clear that different effects of APD treatment may also be observed between brain regions [53]. Similar region specific alterations were also observed in our investigation, and it has been heavily postulated that this may be due to the differences in the density and availability of DA receptors (both D_1 and D_2) between brain regions, and the subsequent ability of agonist/antagonist actions on these receptors to influence levels of DAT.

Regional differences in adult DA D_1 receptor densities were also uncovered following juvenile APD treatment in both the female and male cohorts. In the female cohort, a decreased D_1 receptor density was found in the VTA of female animals treated with the partial agonist aripiprazole, whilst a similar decrease in D_1 receptor levels were found in the male cohort, with early risperidone treatment found to cause long-term decreases to D_1 receptor density in the NAc. Whilst similar investigations into the adult animal model has found no alterations to DA D_1 receptor density levels in the PFC, CPu, NAc and hippocampus following long-term olanzapine and risperidone drug treatment [57], similar decreases in DA D_1 receptor levels have been uncovered in previous investigations in young animals. Studies investigating the immediate effects of juvenile APD treatment following a short-term treatment period [38,40], and long-lasting effects following long-term treatment period [18,37], has found that both olanzapine and clozapine resulted in a decreased D_1 receptor density level in the PFC and NAc of young male rats.

Physiological differences between genders, variances in the density and availability of DA D_1 receptors across the investigated brain regions, along with the pharmacological actions of the APDs used may account for the region and treatment factor differences in DA D_1 receptor density levels observed. More specifically, with the D_1 receptors playing a critical role in the regulation of DA transmission along DAergic projections via facilitation from hippocampal regions [15,58,59], the partial agonist actions of aripiprazole on the D_2 receptor pre-synaptically will subsequently be heavily influenced by the concentration of extracellular DA NT linked to D_1 receptor density. This potentially may result in the observed alterations in the present study, with decreased D_1 receptor density uncovered in the VTA of female rats following aripiprazole treatment, whilst risperidone was found to increase D_1 receptor density in hippocampal regions.

The present study found increases in the expression of the DA D_2 receptor in numerous brain regions in the female rodent model between drug treatment group and control. Female rats that received early olanzapine drug treatment exhibited increased D_2 receptor binding density in the PFC, NAc and CPu when compared to the control group. Previous investigations into the effects of APD treatment on DA D_2 receptor levels in an animal model have also uncovered either similar significant increases to D_2 receptor expression following treatment, or no change to D_2 receptor expression across both male and female cohorts [36–38,40,43,57,60–62]. Whilst studies into the effects of both short and long-term APD treatment with olanzapine and risperidone on D_2 receptor densities have found increases in regions including the PFC, CPu, NAc and hippocampus, both immediately following cessation of treatment [38,57] and after long-term time periods [18,37,38,57], the effects of treatment with the partial agonist drug aripiprazole, over short and long-term treatment periods, have found conflicting results. APD treatment in both young and adult rat models have been found to result in no immediate alterations to D_2 receptor density in striatal brain regions [43,60,61], whilst a more recent investigation in young rats uncovered an increased expression of D_2 receptor levels in the CPu following a short-term treatment period [36]. Surprisingly, despite the well-document high affinity and mechanism of action of APDs through DA D_2 receptors, no alterations to D_2 receptor levels were observed in the male cohort in any of the investigated regions in the present study.

Whilst the current study investigated the effects on the D_1 and D_2 receptors specifically due to their high expression levels in investigated regions, there is also the potential for juvenile APD treatment to cause long-term alterations to DA D_3 receptor levels in the adult brain. With the D_3 receptor linked to both the therapeutic effects of APD actions, and correlated to the expression of D_2 receptors [63], further investigations into potential impacts of juvenile APD expression will shed further light on the depth of the effects of early APD treatment on the DA NT system. Additionally, it is well documented that in the majority of cases, a multi-drug approach is implemented in the clinic [64], and there is subsequently the potential for a multi-drug treatment approach to result in similar or even more widespread alterations. Therefore, further studies into the effects of APD co-treatment with other drugs (such as the antidepressant bupropion) have the potential to uncover further detrimental effects of these drugs on the developing brain.

The current study has uncovered numerous gender-specific effects across multiple measured variables as a result of juvenile APD treatment. Most profoundly however, were the observed opposing effects of APD treatment on p-TH and DA D_2 receptor levels, upon comparison of the male and female cohorts. As mentioned in a previous article, there are numerous potential factors that may be resulting in the observed variances between genders [31]. Firstly, the well-documented gender difference in neurodevelopmental phases of the DA system has the potential to have an obvious significant influence on the observed differences in the present study. In particular, previous investigations have uncovered both a regional and gender-based difference in the development of the DA NT system, including differences in; striatal DA D_1 and D_2 receptor overproduction and elimination, DA D_1 receptor densities in the NAc, and rates of myelination [25,55]. Together, these gender-based differences in the neurodevelopmental phases of the DA NT system in particular, combined with the highly potent pharmacological mechanism of action of APDs in the critical neurodevelopmental window, may explain the observed differences in DA-related measured variables. Furthermore, with such long-term differences in APD treatment response to measured DA variables across genders observed in this study, there is also subsequently the potential gender differences in clinical response to prescribed APD treatments later in life.

Additionally, the sex hormones testosterone and oestrogen may also have played a significant role in the observed gender differences of the present study, in particular the dissimilar alterations to p-TH expression following APD treatment. It is well documented that during adolescence, both testosterone and oestrogen strongly influence the development and maturation of the brain in DAergic regions, including the PFC and striatum [17,25,55]. Similarly, they are also known to play a significant role in shaping the DA signal, with previous animal studies that augmented both testosterone and oestrogen levels found correlative alterations to DA neurotransmission, including DA synthesis, receptor mRNA

and transporter levels [17,55,65]. Furthermore, studies have also uncovered some "neuro-protective" effects of oestrogen on the DA NT system, with the hormone exhibiting an ability to inhibit DA D_2 receptor, along with 5-HT_{1A} receptor-induced mediated behavioural changes in sensorimotor gating/information processing, of which has previously been found deficient in people with mental illnesses [66,67].

4. Materials and Methods

4.1. Animals and Housing

Timed pregnant Sprague-Dawley rats were obtained at gestation day 16 from the Animal Resource Centre (Perth, Australia). They were housed in individual cages under environmentally controlled conditions (22 °C, light cycle from 07:00 to 19:00 and dark cycle from 19:00 to 07:00), and allowed ad libitum access to water and standard laboratory chow diet (3.9 kcal/g: 10% fat, 74% carbohydrate, 16% protein). Day of birth was considered postnatal day (PD) 0. Pups were sexed on PD14, and then 96 Sprague-Dawley rats (48 males and 48 females) were weaned on PD20 and housed in individual cages.

4.2. Drug Treatment Groups

Before the drug treatment commenced, the rats were trained for self-administration by feeding them cookie dough (0.3 g) without drugs 2 times per day for PD 18–21. Animals were then assigned randomly to one of four experimental groups per gender on PD21 (n = 12/group): (1) aripiprazole (Otsuka, Tokyo, Japan); (2) olanzapine (Eli Lilly, Indianapolis, IN, USA); (3) risperidone (Apotex, Toronto, ON, Canada); or (4) control (vehicle). The drug treatment period from PD 22–50 in juvenile rats was carried out at the equivalent time of the childhood/adolescent phase in humans [25]. A staggered drug treatment pattern, where lower APD dosages are slowly increased to a final dosage amount, was used to mimic a clinical setting [68]. The APD doses were initiated on PD22 at 0.2 mg/kg for aripiprazole; 0.25 mg/kg, 3 times per day for olanzapine; and 0.05 mg/kg, 3 times per day for risperidone, and then increased in 3 steps over the first 7 days of the 4 week treatment period to achieve a final dose on PD28 of 1 mg/kg, 3 times per day for aripiprazole; 1 mg/kg, 3 times per day for olanzapine; and 0.3 mg/kg, 3 times per day for risperidone. Rats were observed throughout the treatment to ensure that they completely consumed the cookie dough pellet. The rats in the control group received an equivalent pellet without the drug. In consideration of a shorter half-life of APDs in rats [69], APDs were administered 3 times per day (at 07:00, 14:00 and 22:00; with 8 $\pm$ 1 h intervals) in order to ensure consistently high concentrations and mirror the clinical scenario of oral administration once per day. The proposed dosages are within the recommended dosage ranges for the psychiatric treatment of paediatric patients, based on the body surface area formula for dosage translation between humans and rats in the FDA guideline for clinical trials [34,68,70,71]. The relevant human equivalent dose (HED) is therefore calculated by the formula: Animal dose (mg/kg) $\times$ Animal Km (6)/Human Child Km (25) $\times$ Body Weight (Km factor, body weight (kg) divided by body surface area (m^2), is used to convert the mg/kg dose to a mg/m^2 dose). Therefore, for an adolescent with an average weight of 40 kg, the utilized dosages for aripiprazole (1 mg/kg) and olanzapine (1 mg/kg) equals 9.6 mg, whilst risperidone (0.3 mg/kg) equates to a dosage of 2.88 mg, all within a clinically relevant range. It has been previously reported that, at these used dosages, aripiprazole treatment reaches above 90% DA D_2 receptor occupancy rates in the rat brain [72], while olanzapine and risperidone reach 65%–80% DA D_2 receptor occupancy [73,74]. These dosages have also been shown to be physiologically and behaviourally effective in our laboratory, with similar dosages seen to induce weight gain and changes in hypothalamic neuropeptide Y expression in adolescent rats [75], whilst alterations to both DA and 5-HT receptor binding has been reported in juvenile rats [40]. All experimental procedures were approved by the Animal Ethics Committee, University of Wollongong, Wollongong, Australia (AE 12/20), and complied with Australian Code of Practice for the Care and Use of Animals for Scientific Purpose (2004).

4.3. Histological Procedures

All rats were sacrificed via carbon dioxide asphyxiation on PD106 between 09:00 and 11:30 to minimise the potential circadian-induced variation of protein expression. Following euthanasia, the brain tissue was immediately removed, frozen in liquid nitrogen, and stored at -80 °C until analysis. Six brains from each drug treatment group ($n = 12$) were then randomly selected for Western blot analyses (outlined in Sections 4.3.1 and 4.4), and the remaining six brains from each treatment group were then used for receptor autoradiography experiments (outlined in Sections 4.3.2 and 4.5). The brain regions involved in both dopaminergic signalling and the therapeutic actions of APDs, including the PFC, CPu, NAc, hippocampus, SN and VTA, were dissected in order to detect DA receptor, transporter, and synthesis levels.

4.3.1. Histology—Microdissection (Western Blot Analyses)

Brain microdissection puncture techniques were used to collect aforementioned selected brain regions following a standard procedure in our laboratory [76–79]. Briefly, 500 μm thick sections were cut at -14 °C using a cryostat (Leica CM1850, Leica Microsystems, Wetzler, Germany) and collected bilaterally using a microdissection puncher on glass slides.

4.3.2. Histology—Receptor Autoradiography

Brains selected for receptor autoradiography were sectioned coronally at -18 °C into 14 μm using a cryostat (Leica CM1850, Leica Microsystems, Wetzler, Germany). Sections were then thaw mounted onto Poly-L-Lysine (Sigma-Aldrich, Castle Hill, NSW, Australia) coated glass slides and stored at -20 °C. A set of sections from each animal was stained with 0.5% cresyl violet solution (Nissl staining) and used to confirm identification of anatomical structures.

4.4. Western Blot Analyses

All brain tissue dissected from individual rats (outlined in Section 4.3.1) were homogenised in ice-cold homogenising buffer (9.8 mL NP-40 cell lysis buffer (Invitrogen, Camarillo, CA, USA), 100 μL β-Glycerophosphate (50 mM; Invitrogen), 33.3 μL PMSF (0.3 M; Sigma-Aldrich, St. Louis, MO, USA), and 100 μL Protease Inhibitor Cocktail (Sigma-Aldrich)). The samples were then centrifuged, and the supernatant solution was then collected and stored at -80 °C until required.

Total protein concentration was quantified spectrophotometrically via Bio-Rad DC Protein Assay (#500-0114, Bio-Rad, Hercules, CA, USA) at A750 nm. A range of sample protein concentrations was pre-tested in each region (2, 2.5, 4, 5, 6, 7.5, 8, and 10 μg). Ten micrograms of protein was selected for PFC, CPu and NAc regions, whilst 8 μg of protein was selected for Hippocampus, SN and VTA regions as it best fitted the linear range of signal detection for all tested antibodies. Homogenised brain samples containing the aforementioned μg concentration of protein were then firstly heated at 95 °C in the loading buffer (950 μL Laemmli buffer (Bio-Rad) and 50 μL β-mercaptoethanol (Sigma-Aldrich)) for 5 min to denature the protein, then placed on ice and centrifuged for 2 min at 4 °C. The samples were then loaded into Criterion™ TGX™ 4%–20% Precast Gels (Bio-Rad), and underwent electrophoresis in SDS-PAGE running buffer (100 mL 10× SDS-PAGE running buffer (Bio-Rad) and 900 mL distilled water) at 140 V for 70 min. Proteins on the gels were then transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) electrophoretically using the Bio-Rad Midi Format 1-D Electrophoresis Systems for 1 h at 100 V.; in ice-cold transfer buffer (150 mL 10× Tris/Glycine Buffer (Bio-Rad), 300 mL cold methanol and 1050 mL distilled water). In order to detect the proteins of interest, PVDF membranes were incubated in Tris-Buffered Saline-Tween (TBST) (Sigma-Aldrich) solution containing 5% Blotting Grade Blocker (Non-Fat Dry Milk Powder) (Bio-Rad) for 1 h at room temperature. Membranes were then incubated overnight with the primary antibody, including; D_1R (1:5000; #ab20066, Abcam, Cambridge, United Kingdom), D_2R (1:5000; #ab21218, Abcam, Cambridge, United Kingdom), DAT (1:1000; #SC-14002, Santa Cruz, Dallas, TX, USA), TH (1:2000; #AB9983,

Millipore, Toronto, ON, Canada) and p-TH (1:10,000; #AB5935, Millipore, Toronto, ON, Canada), diluted in TBST buffer containing either 1% Non-Fat Dry Milk Powder (D_1R, D_2R, DAT) or 1% Bovine Serum Albumin (BSA) (TH, p-TH). Following primary antibody incubation, TBST was used to wash membranes (3 × 5 min), followed by a 1 h incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (D_1R and D_2R—1:5000, DAT—1:4000, TH—1:3333, p-TH—1:3333; Millipore, Temecula, CA, USA) at room temperature. Secondary antibodies were diluted in TBST buffer containing either 1% Non-Fat Dry Milk Powder (D_1R, D_2R and DAT) or 1% BSA (TH and p-TH). Three TBST washes then followed secondary antibody incubation, and proteins of interest were visualised using Classico Western horseradish peroxidase (HRP) Substrates (Millipore) and AmershamHyperfilm ECL (GE Healthcare, Life Science, Wauwatosa, WI, USA). Membranes were then re-probed with mouse anti-actin polyclonal antibody (1:10,000; Millipore, #MAB1501) and HRP-conjugated rabbit anti-mouse secondary antibody (1:3000; Cell Signalling, #7076).

Immunoreactive signals were quantified using GS-800 image densitometry and Quantity One software (Bio-Rad, Version 4.6.7), and the values were corrected based on their corresponding actin levels. For D_1R, the band at ~48 kDa corresponding with amino acids 9–21 of DA D_1R was detected and quantified [80,81]. For the D_2R, a pair of bands detected at ~48 and ~51 kDa representing the short and long-forms respectively and corresponding to amino acids 272–282 of DA D_2R were quantified [82]. For DAT, a band at ~50 kDa was detected and quantified, corresponding to amino acids 541–620 at the C-terminus of the dopamine transporter (DAT) [83]. For TH, a single band at ~58 kDa recognising the C-terminus of TH was detected and quantified [84], whilst for p-TH, a single band at ~60 kDa was detected and quantified [84]. Quantification of the β-actin protein was at 46 kDa. Normalization of results was accomplished by taking the value of the vehicle group as 100%. Each sample for all groups (n = 6 per group) has been performed in duplicate to confirm reliability of results.

4.5. Receptor Autoradiography and Quantification

Experimental procedures for DAT, D_1R, and D_2R binding autoradiography were based on those reported previously [40,85–89].

4.5.1. Dopamine Active Transporter (DAT) Binding Procedures

Brain sections for DAT binding were pre-incubated in 50 mM Tris-HCl buffer containing 120 mM NaCl and 0.1% bovine serum albumin (pH 7.4) for 20 min at 4 °C. Sections were then incubated for 2 h in 15 nM [^{3}H]WIN35428 (specific activity, 85.9 Ci/mmol; PerkinElmer, Waltham, MA, USA). Non-specific binding was determined by the addition of 10 μM GBR12909 (Sigma-Aldrich, Castle Hill, NSW, Australia) to subsequent sections. Sections were then washed twice for 1 min in ice-cold buffer, dipped in ice-cold distilled water and then air dried [40,85,88].

4.5.2. Dopamine D_1 Receptor Binding Procedures

Briefly, brain sections containing PFC, CPu, NAc, Hippocampus, SN and VTA were thawed at room temperature (RT) and pre-incubated in 50 mM Tris-HCl buffer (pH 7.4), containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ for 20 min at RT. Sections were then incubated for 1.5 h at RT in 50 mM Tris-HCl buffer with 4 nM [^{3}H]SCH23390 (specific activity: 85 Ci/mmol; PerkinElmer, Waltham, MA, USA) and 30 nM Spiperone (Sigma-Aldrich, Castle Hill, NSW, Australia) to prevent non-specific binding to DA D_2 receptor. Non-specific binding was determined with the addition of 100 μM (+)-Butaclamol (Sigma-Aldrich, Castle Hill, NSW, Australia) to subsequent sections. Slides with sections were then washed twice for 10 min in ice-cold buffer, dipped in ice-cold distilled water and then dried under a stream of cool air to remove excess buffer salts [40,85].

4.5.3. Dopamine D_2 Receptor Binding Procedures

D_2R binding procedures began with pre-incubation for 30 min at RT in 50 mM TrisHCl buffer, containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.001% ascorbic acid (pH 7.4).

Incubation for 1 h in 5 nM [^{3}H]Raclopride (specific activity 76 Ci/mmol; PerkinElmer, Waltham, MA, USA) at RT was then used for total D_2 receptor binding. Non-specific binding was determined with addition of 10 μM (+)-Butaclamol (Sigma-Aldrich, Castle Hill, NSW, Australia) to subsequent sections. Following incubation, sections were washed twice for 5 min in ice-cold buffer, dipped in ice-cold distilled water and then air dried [40,85,88,89].

4.5.4. Quantification

All receptor binding slides were exposed to Amersham Hyperfilm ECL (GE Healthcare, Life Science, Wauwatosa, WI, USA) for 2–3 months, along with autoradiographic standards ([^{3}H]microscales from Amersham), in X-ray film cassettes. Following exposure time, quantitative analysis of binding images was conducted using the Multi-Analyst image analysis system (Bio-Rad, Hercules, CA, USA), connected to a GS-800 Imaging Densitometer (Bio-Rad, Hercules, CA, USA). Optical density measurement was then converted into fmoles [3H] ligand per mg TE (tissue equivalent) by comparing to the standard. Density of specific binding was calculated through subtraction of non-specific binding from total binding. Confirmation of anatomical structures and specific brain regions was accomplished through reference to a set of Nissl stained (0.5% cresyl violet solution) from each animal, along with a standard rat brain atlas [42].

4.6. Statistical Analysis

SPSS software (Windows version 19.0, SPSS Inc., Chicago, IL, USA) was then used to analyse all collected data. Normal distribution of data from all experiments was examined through the use of the Kolmogorov–Smirnov test. All normally distributed data from male and female rats were analysed by two-way analysis of variances (ANOVAs) (Gender × Treatment). Data from males or females were then analysed separately by one-way ANOVA, followed by post hoc Dunnett (correction) tests for multiple comparisons between the treatment groups. Data that were not normally distributed were analysed via the non-parametric Mann–Whitney U-test. The data were expressed as mean $\pm$ standard error of the mean (SEM). Statistical significance was accepted when $p < 0.05$.

5. Conclusions

In summary, this study has revealed that early APD treatment during the critical neurodevelopmental time period has the potential to cause long-lasting alterations to the DA NT system, including changes to DA synthesis markers, transporter and receptor density levels. The observed changes provide potential reasoning for previously documented alterations to behavioural attributes including locomotor activity, cognition and anxiety, previously reported both in our laboratory [31], and other investigations [18,35,37,90], following early treatment with olanzapine, clozapine and risperidone. Although these observed changes to the DA system do provide some insight into and evidence of long-term physiological alterations following early APD use, further investigations into potential effects on other NT groups however may uncover further confounding and more widespread effects related to the previous behavioural alterations observed. Other NT systems including the 5-HT and adrenergic systems are known targets of APD pharmacology, and, furthermore, are well-documented to have significant interplay and thus strong influence on shaping the DAergic signal [56,91,92], as well as a strong correlation to the modulation of anxiety, depressive-like and locomotor behaviours observed in the previously mentioned studies. The modifications uncovered in the present study provides evidence to clinicians of the potential of juvenile treatment with commonly prescribed APDs such as risperidone to result in long-term alterations to the DA NT system in the animal model. The full extent of the potential effects of juvenile APD treatment across multiple brain regions and genders in a clinical setting is currently not well understood, with further investigations (including imaging studies) having the potential to provide further insight into the potential effects of early APD use in children/adolescents, and subsequently allowing clinicians to weigh up the potential risks vs. benefits of APD prescription and use. In particular, the significant alterations to the

DA synthesis markers as a result of risperidone treatment in the male cohort should be highlighted, with risperidone currently approved for use in children as young as five in the US, in particular widely used in males.

Author Contributions: Chao Deng and Michael De Santis designed the study. Michael De Santis performed the animal treatment, conducted the experiments and analysed data. Jiamei Lian contributed to the receptor binding experiments. Michael De Santis prepared the initial draft of the manuscript. Michael De Santis, Chao Deng, Jiamei Lian and Xu-Feng Huang revised the manuscript and interpreted the data. All of the authors approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

5-HT	Serotonin
ANOVA	Analysis of Variance
APD	Antipsychotic Drug
CPu	Caudate Putamen
DA	Dopamine
DAT	Dopamine Active Transporter
DOPA	3,4-Dihydroxyphenylalanine
HED	Human Equivalent Dose
HRP	Horseradish Peroxidase
NAc	Nucleus Accumbens
NT	Neurotransmitter
p-TH	Phosphorylated-Tyrosine Hydroxylase
PD	Postnatal Day
PFC	Prefrontal Cortex
PVDF	Polyvinylidene Difluoride
RT	Room Temperature
SEM	Standard Error of the Mean
SN	Substantia Nigra
TBST	Tris-Buffered Saline-Tween
TH	Tyrosine Hydroxylase
VTA	Ventral Tegmental Area

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CHAPTER 5 - EARLY ANTIPSYCHOTIC TREATMENT IN JUVENILE RATS ELICITS LONG-TERM ALTERATIONS TO THE ADULT SEROTONIN RECEPTORS

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ORIGINAL RESEARCH

Early antipsychotic treatment in juvenile rats elicits long-term alterations to the adult serotonin receptors

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Background: Antipsychotic drug (APD) prescription/use in children has increased significantly worldwide, despite limited insight into potential long-term effects of treatment on adult brain functioning. While initial long-term studies have uncovered alterations to behaviors following early APD treatment, further investigations into potential changes to receptor density levels of related neurotransmitter (NT) systems are required.

Methods: The current investigation utilized an animal model for early APD treatment with aripiprazole, olanzapine, and risperidone in male and female juvenile rats to investigate potential long-term changes to the adult serotonin (5-HT) NT system. Levels of 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} receptors were measured in the prefrontal cortex (PFC), caudate putamen (CPu), nucleus accumbens (NAc), and hippocampus via Western Blot and receptor autoradiography.

Results: In the male cohort, long-term changes to 5-HT_{2A} and 5-HT_{2C} receptors were found mostly across hippocampal and cortical brain regions following early aripiprazole and olanzapine treatment, while early risperidone treatment changed 5-HT_{1A} receptor levels in the NAc and PFC. Lesser effects were uncovered in the female cohort with aripiprazole, olanzapine and risperidone to alter 5-HT_{1A} and 5-HT_{2A} receptors in NAc and hippocampal brain regions, respectively.

Conclusion: The results of this study suggest that early treatment of various APDs in juvenile rats may cause gender and brain regional specific changes in 5-HT_{2A} and 5-HT_{2C} receptors in the adult brain.

Keywords: antipsychotic drug, serotonin, risperidone, olanzapine, aripiprazole, development, juvenile

Introduction

Antipsychotic drug (APD) prescription and use is rapidly increasing globally, despite a lack of knowledge on the safety and efficacy of APD use on the developing brain.[1–10] Second-generation APDs including aripiprazole, olanzapine, and risperidone are currently commonly being prescribed (mostly off-label) for the treatment of a variety of childhood disorders from mental illnesses, including depression and child-onset schizophrenia,[5,11,12] to various behavioral disorders, including autism spectrum disorder.[13–15]

While APDs are known to elicit their therapeutic effects predominantly through a strong affinity and subsequent antagonistic mechanism of action on both the dopamine (DA) D_2 and serotonin (5-HT) 5-HT_{1A} and $5\text{-HT}_{2A/2C}$ receptors,[16–22] both the dopaminergic and serotonergic neurotransmitter (NT) systems have been proven to undergo, and be heavily involved in, numerous critical neurodevelopmental processes during

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1569

Michael De Santis

84

the childhood/adolescent period.[19,23–29] Specifically, 5-HT is known to play an early significant and concentration-dependent trophic role in neural development and neurite growth[8,26,30–33] and then also undergo specific phases of development as a NT system (eg, synaptogenesis and regressive elimination).[8,31]

Subsequently, there is the potential that early insult/use of potent APDs at this critical time of neurodevelopment may have the ability to cause long-term alterations to the functionality of the NT systems, including that of 5-HT, in a manner that precedes normal brain functioning.[8,27,34,35] With alterations to the 5-HT NT system previously linked to changes in both behavioral attributes (including locomotor, anxiety, and depressive-like behaviors) and furthermore negatively correlated to dopamine NT functioning,[36–38] prescription and use of APDs during the critical neurodevelopmental time period have the potential to lead to long-term deficits in brain functioning.[10,35,39]

Although current clinical investigations have found some benefits to childhood/adolescent APD use in the treatment of the symptomology of various mental illnesses over a short-term time period (1–2 months) and a time period of up to 6 months,[40–42] the potential for the use of potent APDs to cause long-term alterations to adult brain functioning, especially in a clinical setting, is still mostly unknown.[19,43,44]

Previously completed animal studies investigating the effects of juvenile APD use on the developing brain, including previous studies completed in our laboratory, have found that early treatment of up to 4 weeks can result in various significant long-term changes to behavioral attributes[39] and immediate alterations to NT pathways including the 5-HT NT system.[45,46] While investigations into the distribution/density of various NT receptor subtypes, including 5-HT$_{1A}$, 5-HT$_{2A}$, and 5-HT$_{2C}$ receptors, have found various immediate alterations following short-term APD treatment,[44,45,47] studies investigating the long-term effects of early APD treatment have been found to be limited to the DA NT system.[19,35,48]

The present study was subsequently conducted in order to investigate the long-term effects of juvenile APD use with aripiprazole, olanzapine, and risperidone on the adult 5-HT NT system in both male and female rats. Specifically, investigations into adult brain levels of 5-HT$_{1A}$, 5-HT$_{2A}$, and 5-HT$_{2C}$ receptors were investigated in cortical, striatal, and hippocampal brain regions via Western Blot and/or receptor autoradiography experiments.

Materials and methods
Animals and housing
Timed pregnant Sprague Dawley rats were obtained at gestation day 16 from the Animal Resource Centre (Perth, WA,

Australia) and housed in individual cages under environmentally controlled conditions (22°C, light cycle from 7 am to 7 pm and dark cycle from 7 pm to 7 am). Each was allowed ad libitum access to standard laboratory chow diet (3.9 kcal/g: 10% fat, 74% carbohydrate, and 16% protein) and water. The day of birth was considered postnatal day (PD) 0. Pups were sexed on PD14, and then, 96 rats (48 males and 48 females) were weaned on PD20. Rats were housed in individual rat cages with top wire lids, in which they were able to smell and see each other through the lids.

Drug treatment groups
After weaning and prior to the commencement of drug treatment, all animals were trained for self-administration by feeding them cookie dough (0.3 g) without drugs two times per day for PD18–21. Rats were then randomly assigned to one of the four experimental groups per gender on PD21 (n=12/group): 1) aripiprazole (Otsuka, Tokyo, Japan), 2) olanzapine (Eli Lilly, Indianapolis, IN, USA), 3) risperidone (Apotex, Toronto, ON, Canada), and 4) control (vehicle). Drug treatment was carried out in juvenile rats from PD22–50, a time period equivalent to the childhood/adolescent phase in humans.[26] In order to replicate a clinical setting, a staggered drug treatment pattern was used, where lower APD dosages are slowly increased to a final dosage amount.[49] Specifically, APD doses were initiated on PD22 at 0.2 mg/kg for aripiprazole, 0.25 mg/kg three times per day for olanzapine, and 0.05 mg/kg three times per day for risperidone and were increased in three steps over the first 7 days of the 4-week treatment period to achieve a final dose on PD28 of 1 mg/kg three times per day for aripiprazole, 1 mg/kg three times per day for olanzapine, and 0.3 mg/kg three times per day for risperidone. Drug treatment was administered orally to each drug treatment group via mixing cookie dough powder (containing cornstarch 37%, sucrose 37%, gelatine 17%, and casein 9%) with a small amount of distilled water until even in consistency. All animals were individually observed for the duration of each treatment to ensure that they completely consumed the cookie dough pellet and thus received a full dosage. Animals in the control group also received an equivalent pellet without the drug. In consideration of a shorter half-life of APDs in rats, and to ensure a consistently high drug concentration in replication of the clinical scenario of oral administration once per day,[50] APDs were administered three times per day (at 7, 2, and 10 h) with 8±1 hour intervals. The proposed dosages are translated from a clinical setting and within the recommended dosage ranges for the psychiatric treatment of pediatric patients. Dosage calculations are based on the body surface area formula for dosage translation between humans and

rats in the US Food and Drug Administration guideline for clinical trials.[42,49,51,52] The relevant human equivalent dose (HED) is therefore calculated by the following formula: animal dose (mg/kg) × animal Km (6)/human child Km (25) × body weight (Km factor, body weight (kg) divided by body surface area (m²), is used to convert the mg/kg dose to a mg/m² dose). Therefore, for an adolescent with an average weight of 40 kg, the utilized dosages for aripiprazole (1 mg/kg in rats) and olanzapine (1 mg/kg in rats) equals to a clinical dosage of 9.6 mg, while risperidone (0.3 mg/kg) equals to a clinical dosage of 2.88 mg, all within a clinically relevant range for adolescent patients. Previous reports have demonstrated that at this dosage amount, aripiprazole drug treatment reaches >90% DA D_2 receptor occupancy rates in the rat brain,[53] while olanzapine and risperidone reach 65%–80% DA D_2 receptor occupancy rates.[54,55] These dosage amounts have also been shown to be physiologically and behaviorally effective in our laboratory, with similar dosages seen to induce weight gain and changes in hypothalamic neuropeptide Y expression in adolescent rats,[56] while immediate alterations to both DA receptor and 5-HT receptor binding have been reported in juvenile rats.[45] All experimental procedures were approved by the Animal Ethics Committee, University of Wollongong, Wollongong, NSW, Australia (AE 12/20) and complied with Australian Code of Practice for the Care and Use of Animals for Scientific Purpose (2004).

Histological procedures

After a maturation period where all animals were monitored regularly and allowed to mature (PD51–105), all rats were sacrificed on PD106 via carbon dioxide asphyxiation. Euthanasia was completed between 9 am and 11.30 am to minimize the potential circadian-induced variation of protein expression. Brain tissue was removed immediately following euthanasia, frozen in liquid nitrogen, and stored at −80°C until analysis. Six brains from each drug treatment group (n=12) were then randomly assigned for Western Blot analyses, and the remaining six brains from each treatment group were then used for receptor autoradiography experiments. Brain regions involved in both serotonergic signaling and the therapeutic actions of APDs, including the prefrontal cortex (PFC), caudate putamen (CPu), nucleus accumbens (NAc), and hippocampus, were dissected in order to detect 5-HT receptor levels.

Microdissection (Western Blot analyses)

Tissue from aforementioned brain regions to be used for Western Blot analysis was collected using microdissection puncture techniques, following a standard procedure in our laboratory.[57–60] Briefly, 500 μm thick sections were cut at −14°C using a cryostat (Leica CM1850; Leica Microsystems, Wetzler, Germany) and collected bilaterally using a microdissection puncher on glass slides.

Receptor autoradiography

Tissue from brains selected for receptor autoradiography were collected via coronally dissected sections at −18°C into 14 μm using a cryostat (Leica CM1850). Once dissected, sections were thaw-mounted onto poly-L-lysine (Sigma-Aldrich Co., St Louis, MO, USA)-coated glass slides and stored at −20°C. A set of sections from each animal were stained with the 0.5% cresyl violet solution (Nissl staining) and used to confirm the identification of anatomical structures.

Western Blot analyses

Tissues obtained from individual rats were homogenized in ice-cold homogenizing buffer (9.8 mL of NP-40 cell lysis buffer; Thermo Fisher Scientific, Waltham, MA, USA; 100 μL of β-glycerophosphate; 50 mM; Thermo Fisher Scientific; 33.3 μL of phenylmethane sulfonyl fluoride; 0.3 M; Sigma-Aldrich Co.; and 100 μL of Protease Inhibitor Cocktail; Sigma-Aldrich Co.). All samples were then centrifuged, with the supernatant solution collected and stored at −80°C until required.

DC™ Protein Assays (#500-0114; Bio-Rad Laboratories Inc., Hercules, CA, USA) were completed at $A_{750\,nm}$ to spectrophotometrically quantify total protein concentrations. A range of sample protein concentrations were pretested in each region (2, 2.5, 4, 5, 6, 7.5, 8, and 10 μg). A total of 10 μg of protein was selected for PFC, CPu, and NAc regions, while 8 μg of protein was selected for the hippocampus as it best fitted the linear range of signal detection for all tested antibodies. Homogenized brain samples containing the aforementioned microgram concentration of protein were then first heated at 95°C in the loading buffer (950 μL of Laemmli buffer; Bio-Rad Laboratories Inc.; and 50 μL of β-mercaptoethanol; Sigma-Aldrich Co.) for 5 minutes to denature the protein, then placed on ice, and centrifuged for 2 minutes at 4°C. The samples were then loaded into 4%–20% Criterion™ TGX™ Precast Gels (Bio-Rad Laboratories Inc.) and underwent electrophoresis in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) running buffer (100 mL of 10× SDS-PAGE running buffer; Bio-Rad Laboratories Inc.; and 900 mL of distilled water) at 140 V for 70 minutes. Proteins on the gels were then transferred electrophoretically using the Bio-Rad Midi Format 1-D Electrophoresis Systems onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories Inc.) in ice-cold transfer buffer (150 mL of 10× tris/glycine buffer; Bio-Rad Laboratories Inc.; 300 mL of cold methanol, and 1,050 mL

Michael De Santis

86

of distilled water) at 100 V for 1 hour. In order to detect the proteins of interest, PVDF membranes were incubated in tris-buffered saline–Tween (TBST) (Sigma-Aldrich Co.) solution containing 5% Blotting-Grade Blocker (nonfat dry milk powder) (Bio-Rad Laboratories Inc.) for 1 hour at room temperature (RT). Membranes were then incubated overnight with the primary antibody, including 5-HT$_{1A}$ (1:2,000; #ab85615; Abcam, Cambridge, UK), 5-HT$_{2A}$ (1:1,000; #sc-50397; Santa Cruz Biotechnology Inc., Dallas, TX, USA), and 5-HT$_{2C}$ (1:1,000; #sc-15081; Santa Cruz Biotechnology Inc.), diluted in TBST buffer containing either 1% bovine serum albumin (BSA) (5-HT$_{1A}$) or 1% nonfat dry milk powder (5-HT$_{2A}$ and 5-HT$_{2C}$). Membranes were either washed three times with TBST for 5 minutes (5-HT$_{1A}$ and 5-HT$_{2A}$) and then incubated with horseradish peroxidase (HRP)-conjugated goat antirabbit secondary antibody for 1 hour at RT (5-HT$_{1A}$ – 1:5,000 and 5-HT$_{2A}$ – 1:3,000; EMD Millipore, Billerica, MA, USA) or washed three times for 20 minutes (5-HT$_{2C}$) and then incubated with HRP-conjugated donkey antigoat secondary antibody for 45 minutes at RT (5-HT$_{2C}$ – 1:2,000; Abcam). Secondary antibodies were diluted in TBST buffer containing either 1% BSA (5-HT$_{1A}$) or 1% nonfat dry milk powder (5-HT$_{2C}$ and 5-HT$_{2A}$). Three TBST washes then followed secondary antibody incubation, and proteins of interest were visualized using the Classico Western horseradish peroxidase (HRP) substrates (EMD Millipore) and Amersham Hyperfilm ECL (GE Healthcare Life Sciences, Wauwatosa, WI, USA). Membranes were then re-probed with mouse antiactin polyclonal antibody (1:10,000, #MAB1501; EMD Millipore) and HRP-conjugated rabbit antimouse secondary antibody (1:3,000, #7076; Cell Signaling Technology, Danvers, MA, USA).

Immunoreactive signals were quantified using the GS-800 image densitometry and Quantity One software (Bio-Rad Laboratories Inc.), and the values were corrected based on their corresponding actin levels. For 5-HT$_{1A}$, the band at ~62 kDa was detected and quantified,[61] while for 5-HT$_{2A}$, the band at ~55 kDa was detected and quantified.[62,63] Furthermore, for 5-HT$_{2C}$, a band at ~55 kDa was detected and quantified.[64] The β-actin protein was quantified at 46 kDa. Western Blot gels were arranged by gender, in which each gel contained 24 samples (six rats/group × four treatments [ie, three APDs and one vehicle] × one gender). In order to control for variability, all samples were run in duplicate at second gels at the same sample arrangement and the values of each drug treatment group and control corrected based on their corresponding actin levels. Samples from male and female rats were run in different gels. All results were normalized by taking the value of the vehicle group of each gender as 100% to obtain a comparative value.

Receptor autoradiography and quantification

Experimental procedures for 5-HT$_{2A}$ binding autoradiography were based on those completed and reported previously.[45,65–67] 5-HT$_{1A}$ and 5-HT$_{2C}$ binding autoradiography was also completed; however, binding results were too low and thus discounted from further analysis.

5-HT$_{2A}$ receptor binding procedures

Brain sections for 5-HT$_{2A}$ receptor binding were thawed at RT and then preincubated in 170 mM tris buffer (pH 7.4) for 15 minutes. Slides with sections were then incubated for 2 hours with 2 nM [^{3}H]Ketanserin (specific activity: 47.3 Ci/mmol; PerkinElmer Inc., Waltham, MA, USA) in 170 mM tris buffer at RT to determine total binding. Nonspecific binding was determined with the addition of 2 μM Spiperone (Sigma-Aldrich Co.) to subsequent sections. Following incubation, sections were washed four times for 2 minutes in ice-cold buffer, dipped in ice-cold distilled water, and then air dried.[66,67]

Quantification

Following the completion of receptor binding experiments, all slides were exposed to Amersham Hyperfilm ECL for 2–3 months, along with autoradiographic standards ([^{3}H] microscales from Amersham), in X-ray film cassettes. Quantitative analysis of binding images was conducted following the relevant exposure time, using the Multi-Analyst image analysis system (Bio-Rad Laboratories Inc.), connected to a GS-800 Imaging Densitometer (Bio-Rad Laboratories Inc.). Optical density measurement was then converted into femtomoles of [^{3}H] ligand per milligram of tissue equivalent (TE) by comparing to the standard. Specific binding was calculated by subtracting nonspecific binding from total binding. A set of sections from each animal were stained with the 0.5% cresyl violet solution (Nissl staining), for the purpose of confirmation of anatomical structures. Specific brain regions in this project were identified by reference to the Nissl-stained sections, along with a standard rat brain atlas.[68]

Statistical analysis

Statistical analysis of collected data was completed with the use of SPSS software (Windows Version 19.0; IBM Corporation, Armonk, NY, USA). Distribution of data was examined through the Kolmogorov–Smirnov test. All normally distributed data were also analyzed by two-way analysis of variance (ANOVA) (gender × treatment). Male and female data sets were then also analyzed separately by one-way ANOVA, followed by post hoc Dunnett's tests for

Michael De Santis

87

multiple comparisons between the treatment groups. Data that were not distributed normally were analyzed via the non-parametric Mann–Whitney U test. All data were analyzed per investigated brain region. The data were expressed as mean ± standard error of the mean (SEM). Statistical significance was accepted when $P<0.05$.

Results

Long-term effects of adolescent APD treatment on 5-HT$_{1A}$ receptor levels

A significant effect of treatment on 5-HT$_{1A}$ receptor protein expression was found in the PFC ($F_{3,45}$=4.973, $P<0.01$) and NAc ($F_{3,44}$=3.791, $P<0.02$), while a significant effect of gender was also observed in the NAc ($F_{1,44}$=13.584, $P<0.01$). Furthermore, a significant interaction between the two factors was found in the CPu ($F_{3,46}$=2.860, $P=0.050$) and a trend toward a significant interaction was found in the NAc ($F_{3,44}$=2.559, $P=0.070$). Post hoc analysis uncovered that early risperidone treatment decreased 5-HT$_{1A}$ expression in the PFC (−23.8%, $P<0.02$) when compared with the control (Figures 1A′ and A″ and S1). In the male cohort, early APD treatment had a significant effect on the expression of 5-HT$_{1A}$ receptors in the NAc ($F_{3,22}$=5.091, $P<0.01$) of adult rats. Further analysis revealed that early risperidone treatment trended to significantly decrease 5-HT$_{1A}$ receptor expression in the NAc (−7.0%, $P=0.081$) (Figures 1 and S1). Analysis of the female cohort found trends toward significant effects of early APD treatment in both CPu ($F_{3,22}$=2.853, $P=0.065$) and NAc ($F_{3,20}$=2.695, $P=0.079$). Post hoc analysis revealed that early aripiprazole treatment decreased 5-HT$_{1A}$ receptor expression in the NAc (−16.6%, $P=0.054$) when compared with the control group. No significant alterations were uncovered in the CPu (Figures 1B′ and B″ and S1) or hippocampus (Figures 1D′ and D″ and S1) of treated animals in comparison to the control, across either gender.

Long-term effects of adolescent APD treatment on 5-HT$_{2A}$ receptor levels

Two-way ANOVA tests (gender × treatment) revealed a significant effect of treatment on 5-HT$_{2A}$ receptor protein expression levels in the hippocampus ($F_{3,45}$=4.913, $P<0.01$), while a significant effect of gender was found in the hippocampus ($F_{1,45}$=17.745, $P<0.001$) and CPu ($F_{1,46}$=4.541, $P<0.05$). Additionally, a significant interaction between the factors was uncovered in the hippocampus ($F_{3,45}$=3.340, $P<0.05$) and PFC ($F_{3,46}$=3.972, $P<0.02$). Analysis of the male cohort via one-way ANOVA (treatment) uncovered a significant effect of early APD treatment on 5-HT$_{2A}$ receptor expression in the NAc ($F_{3,22}$=3.378, $P<0.05$), and hippocampus

($F_{3,22}$=4.054, $P<0.05$). Furthermore, a trend to significant effect was also discovered in the PFC ($F_{3,22}$=3.035, $P=0.054$). Post hoc analysis discovered that aripiprazole treatment was found to decrease 5-HT$_{2A}$ receptor levels in the PFC (−78.0%, $P=0.081$) upon comparison to the control group. In the female cohort, one-way ANOVA found a significant effect of early APD treatment on 5-HT$_{2A}$ expression in the PFC ($F_{3,22}$=3.233, $P<0.05$) (Figures 2A′ and A″ and S2) and hippocampus ($F_{3,21}$=4.738, $P<0.02$) (Figures 2D′ and D″ and S2). Further analysis via post hoc tests discovered decreases to 5-HT$_{2A}$ receptor expression in the hippocampus following early olanzapine (−62.4%, $P<0.01$) treatment. No significant changes in the expression of 5-HT$_{2A}$ receptors were uncovered in the CPu (Figures 2B′ and B″ and S2) or NAc (Figures 2C′ and C″ and S2) of APD animals in comparison to the control, across both male and female cohorts.

Examples of [^{3}H]Ketanserin binding to 5-HT$_{2A}$ are presented in Figure 3. Detected levels of 5-HT$_{2A}$ in the CPu of females and males, however, were discounted, as expression was too low for accurate quantification. Analysis via two-way ANOVA (gender × treatment) found a significant effect of early APD treatment on the expression of 5-HT$_{2A}$ receptors in the hippocampus ($F_{3,41}$=2.106, $P<0.01$), along with a significant interaction between the two factors ($F_{3,41}$=1.228, $P<0.05$). A trend to significant effect of treatment was also uncovered in the PFC of rats ($F_{3,43}$=4.004, $P=0.079$). Post hoc analysis revealed that early treatment with both aripiprazole (−49.0%, $P<0.02$) and risperidone (−51.1%, $P<0.01$) significantly decreased 5-HT$_{2A}$ expression in the hippocampus in comparison to the control. When subsequently divided by gender, analysis of the male cohort demonstrated a significant effect of early APD treatment on 5-HT$_{2A}$ receptor expression in the PFC ($F_{3,21}$=4.010, $P<0.05$) and hippocampus ($F_{3,21}$=6.274, $P<0.01$). Further analysis via post hoc revealed that early treatment with aripiprazole decreased 5-HT$_{2A}$ binding in the PFC (−44.3%, $P=0.064$) and hippocampus (−48.8%, $P<0.05$). Similar decreases were also observed following risperidone treatment in the PFC (−60.2%, $P<0.02$) and hippocampus (−69.5%, $P<0.01$) and olanzapine treatment in the hippocampus (−44.7%, $P=0.063$). No significant effects were found in the female cohort between the APD treatment group and control.

Long-term effects of adolescent APD treatment on 5-HT$_{2C}$ receptor levels

Analysis of 5-HT$_{2C}$ expression via two-way ANOVA (gender × treatment) uncovered a significant effect of treatment on 5-HT$_{2C}$ receptor protein expression in the PFC ($F_{3,44}$=4.286, $P<0.02$) and hippocampus ($F_{3,45}$=10.791,
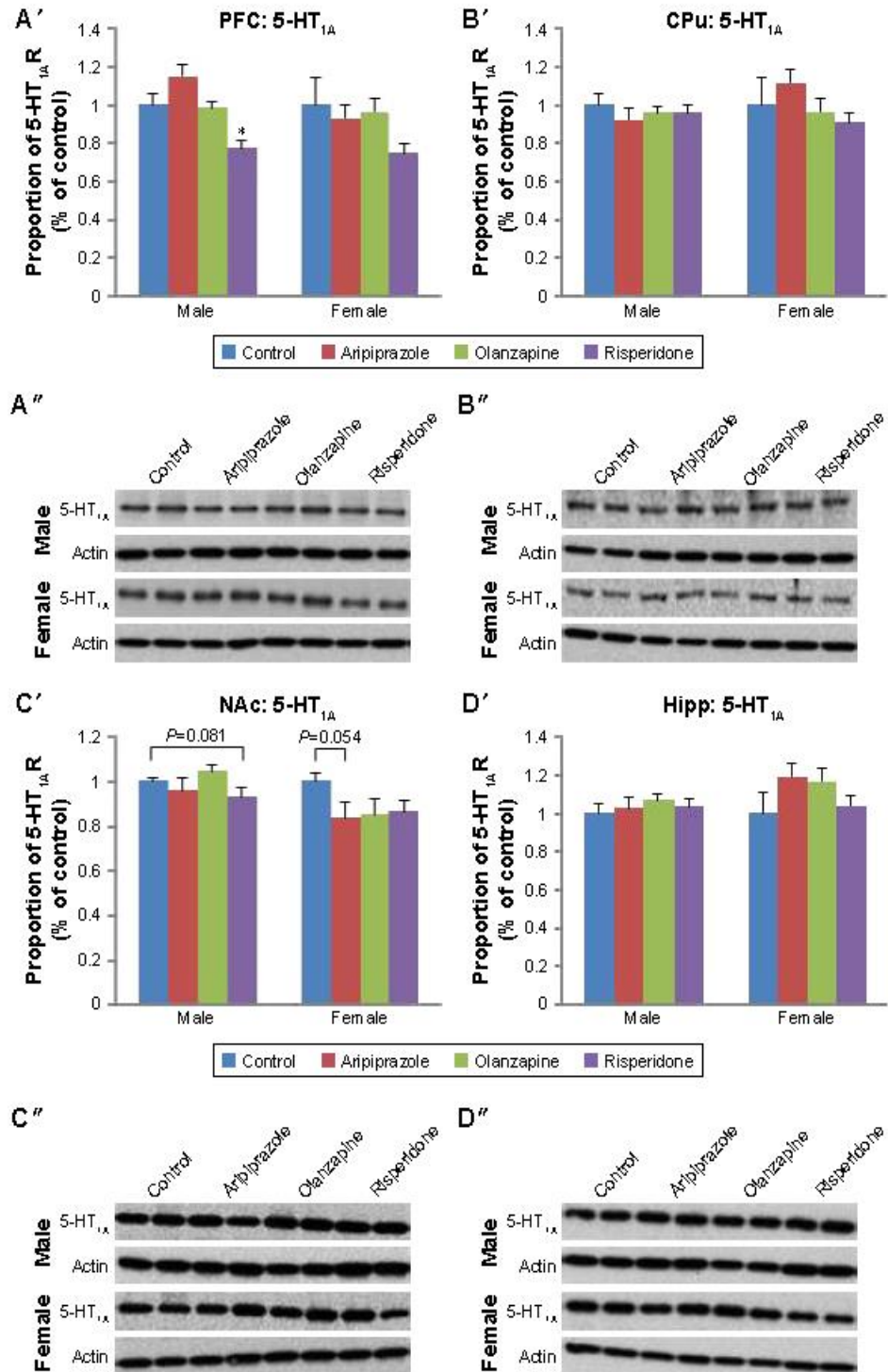
Figure 1 Effects of three APDs on 5-HT$_{1A}$ expression levels in the PFC (A', A''), CPu (B', B''), NAc (C', C''), and Hipp (D', D'') of female and male rats.

Notes: Sprague Dawley rats were treated chronically with aripiprazole (1.0 mg/kg, tid), olanzapine (1.0 mg/kg, tid), risperidone (0.3 mg/kg, tid), or control (vehicle). The number of samples per gender per group is 6. Data were expressed as mean ± SEM. *$P<0.05$ vs control. The representative bands of Western Blot are shown.

Abbreviations: APDs, antipsychotic drugs; CPu, caudate putamen; Hipp, hippocampus; 5-HT, serotonin; NAc, nucleus accumbens; PFC, prefrontal cortex; SEM, standard error of the mean; tid, three times daily.

Michael De Santis   89
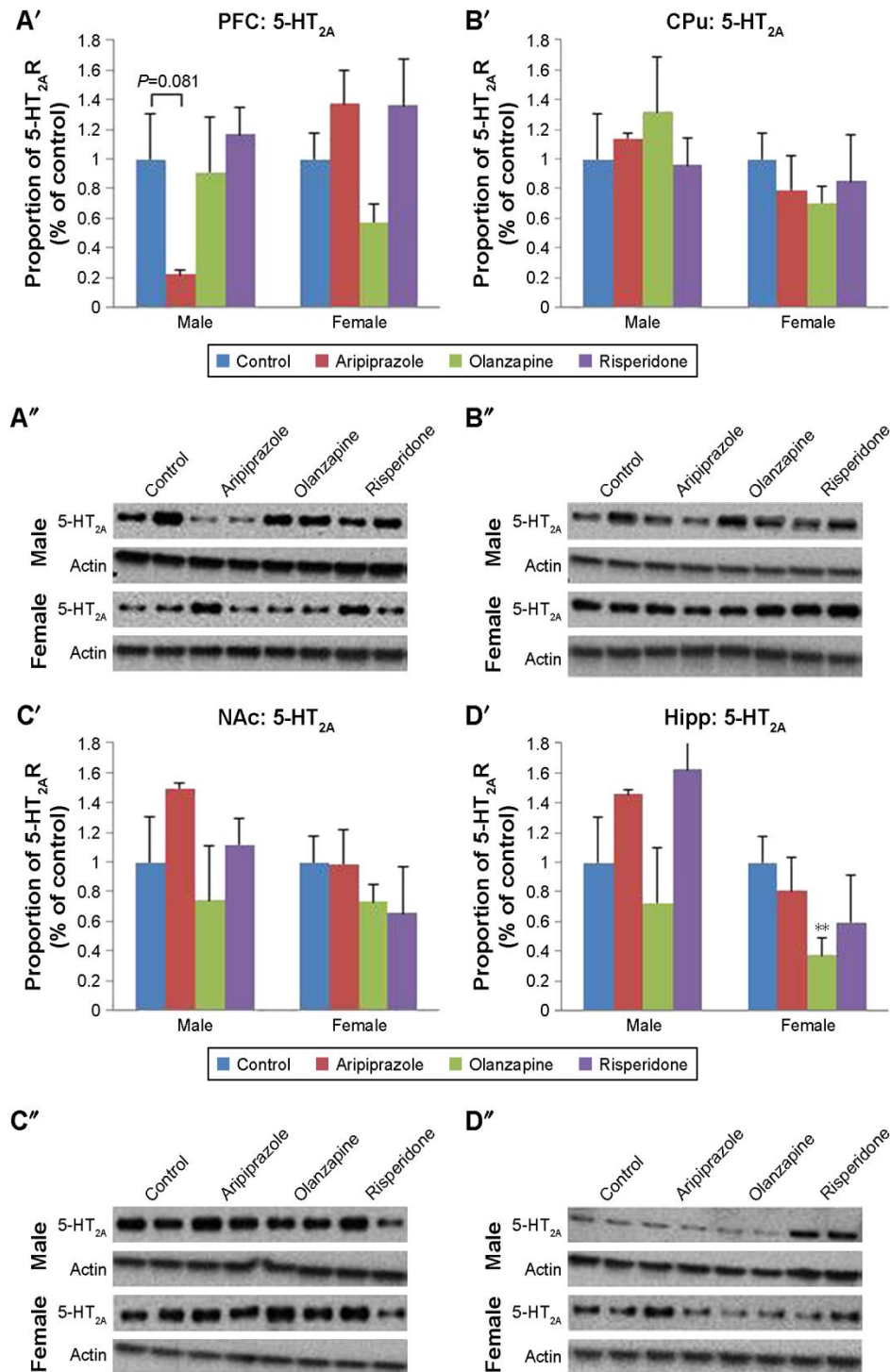
Figure 2 Effects of three APDs on 5-HT$_{2A}$ expression levels in the PFC (**A'**, **A''**), CPu (**B'**, **B''**), NAc (**C'**, **C''**), and Hipp (**D'**, **D''**) of female and male rats.
Notes: Sprague-Dawley rats were treated chronically with aripiprazole (1.0 mg/kg, tid), olanzapine (1.0 mg/kg, tid), risperidone (0.3 mg/kg, tid), or control (vehicle). The number of samples per gender per group is 6. Data expressed as mean ± SEM. **P<0.01 vs control. The representative bands of Western Blot are shown.
Abbreviations: APDs, antipsychotic drugs; CPu, caudate putamen; Hipp, hippocampus; NAc, nucleus accumbens; 5-HT, serotonin; PFC, prefrontal cortex; SEM, standard error of the mean; tid, three times daily.

Michael De Santis   90
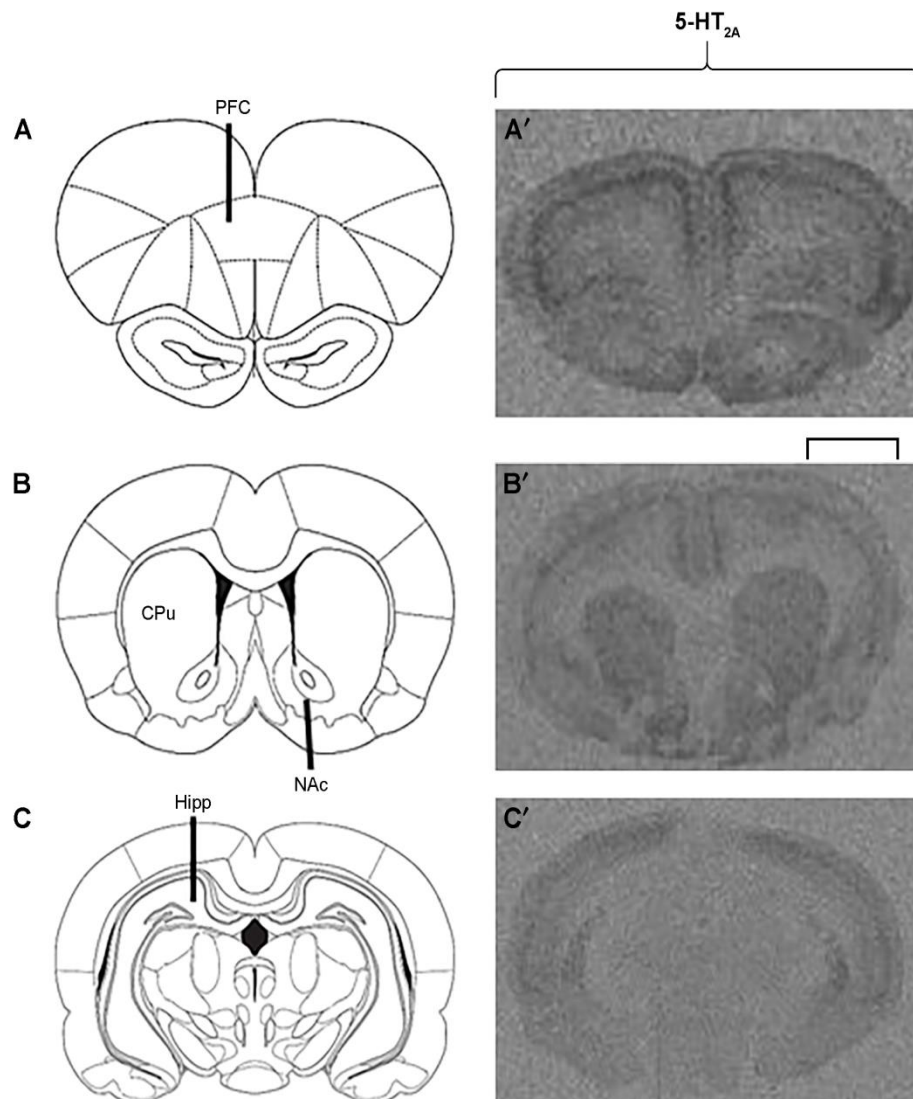
5-HT$_{2A}$

Figure 3 Examples of 5-HT$_{2A}$ receptor binding in the adult rat brain following childhood/adolescent APD treatment.
Notes: The schematic diagram is adapted from Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates*. 6th edition. London: Elsevier; 2007. © Academic Press 2007.[68] Showing the level of Bregma for each investigated region (**A**: PFC, 4.68 mm; **B**: CPu and NAc, 1.08 mm; **C**: Hipp, −2.76 mm). (**A'–C'**) Examples of autoradiograms to demonstrate [³H]Ketanserin binding to 5-HT$_{2A}$ receptors. The scale bar applies to all autoradiograms and is 2.0 mm for A', 2.8 mm for B', and 3.2 mm for C'.
Abbreviations: APD, antipsychotic drug; CPu, caudate putamen; Hipp, hippocampus; 5-HT, serotonin; NAc, nucleus accumbens; PFC, prefrontal cortex.

$P<0.001$) of rats. Additionally, a significant effect of gender was found in the hippocampus ($F_{1,45}=16.265$, $P<0.001$), while a significant interaction between the factors was also found in the hippocampus ($F_{3,45}=7.511$, $P<0.001$). Post hoc analysis uncovered that early treatment with both aripiprazole (−50.2%, $P<0.05$) and olanzapine (−42.5%, $P=0.078$) significantly decreased 5-HT$_{2C}$ expression in the PFC in comparison to the control. Following analysis of the male cohort via one-way ANOVA, a significant effect of early

APD treatment was discovered in the PFC ($F_{3,20}=8.004$, $P<0.01$) and hippocampus ($F_{3,22}=15.474$, $P<0.001$), while a trend to significant effect was found in the CPu ($F_{3,22}=2.946$, $P=0.059$). Post hoc analysis found decreases in 5-HT$_{2C}$ receptor expression in the PFC following early APD treatment with aripiprazole (−45.1%, $P<0.05$) and olanzapine (−50.1%, $P<0.01$) (Figures 4A′ and A″ and S3). Additionally, increases in 5-HT$_{2C}$ receptor expression were uncovered in the hippocampus following early treatment

Michael De Santis   91

Figure 4 Effects of three APDs on 5-HT$_{2C}$ expression levels in the PFC (**A′**, **A″**), CPu (**B′**, **B″**), NAc (**C′**, **C″**), and Hipp (**D′**, **D″**) of female and male rats.
Notes: Sprague Dawley rats were treated chronically with aripiprazole (1.0 mg/kg, tid), olanzapine (1.0 mg/kg, tid), risperidone (0.3 mg/kg, tid), or control (vehicle). The number of samples per gender per group is 6. Data expressed as mean ± SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control. The representative bands of Western Blot are shown.
Abbreviations: APDs, antipsychotic drugs; CPu, caudate putamen; Hipp, hippocampus; 5-HT, serotonin; NAc, nucleus accumbens; PFC, prefrontal cortex; SEM, standard error of the mean; tid, three times daily.
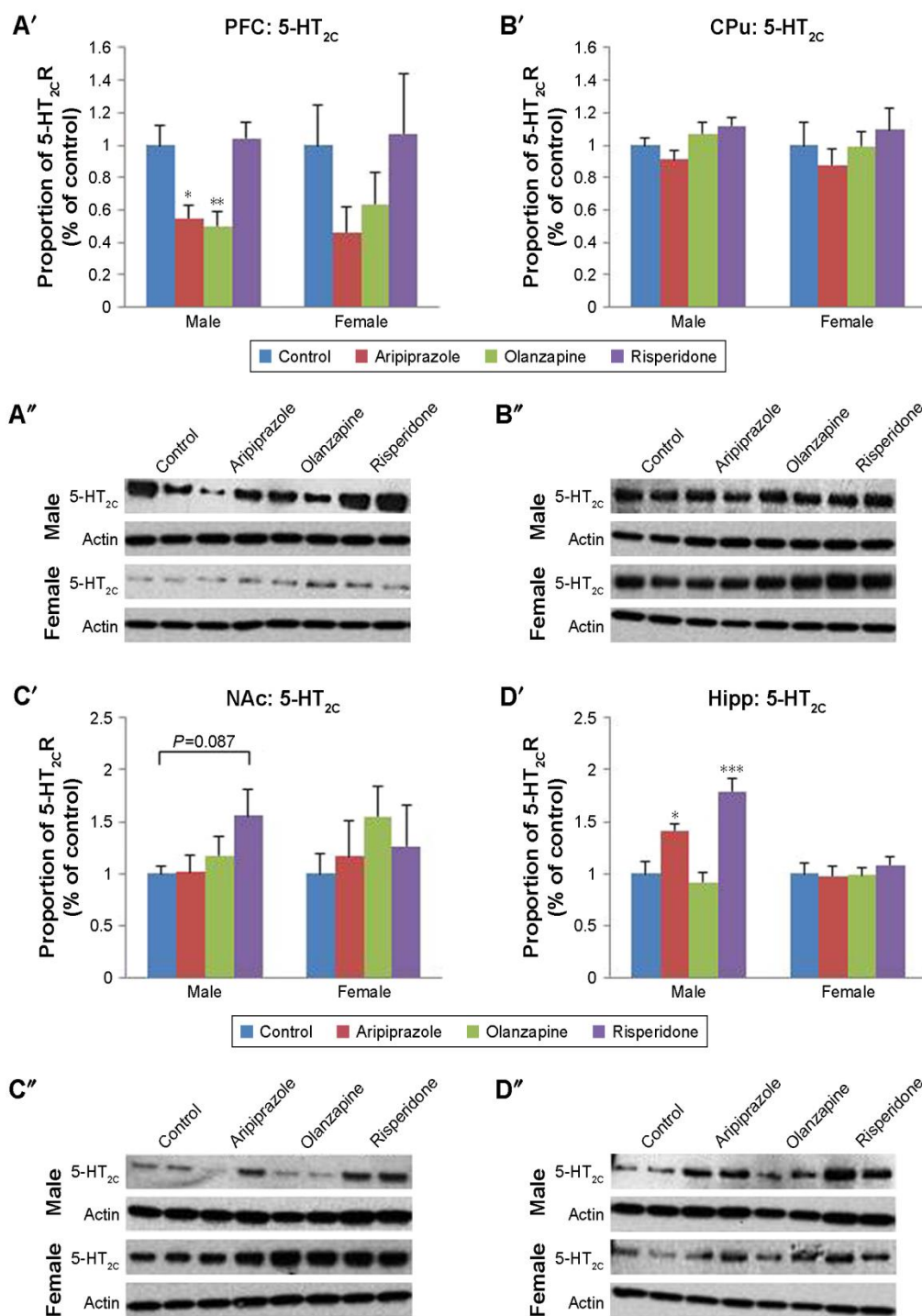
Michael De Santis   92

with both aripiprazole (+41.5%, $P<0.05$) and risperidone (+79.6%, $P<0.001$) (Figures 4D′ and D″ and S3), while no alterations were uncovered in either the CPu (Figures 4B′ and B″ and S3) or the NAc (Figures 4C′ and C″ and S3). No significant alterations to $5\text{-}HT_{2C}$ receptor expression, however, were found in the female cohort.

Discussion

The present study has, for the first time, provided insight into the long-term effects of early (juvenile) treatment with the APDs aripiprazole, olanzapine, and risperidone on the density of 5-HT receptors in the adult brain. Our investigation has revealed that juvenile APD treatment during the critical neurodevelopmental time period resulted in significant long-term alterations to $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ receptors, predominantly in hippocampal and cortical brain regions. Furthermore, we have uncovered more widespread alterations to the density of male 5-HT receptors in comparison to female 5-HT receptors, with changes in $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ receptors uncovered across multiple drug treatment groups.

While previous investigations into the long-term effects of juvenile APD use on $5\text{-}HT_{1A}$, $5\text{-}HT_{2A}$, and $5\text{-}HT_{2C}$ receptors have, to our knowledge, not been completed, numerous studies have examined the immediate effects of aripiprazole, olanzapine, or risperidone treatment on the density of 5-HT receptors in young[45,46,69] and adult[65,67,70,71] rodents, over both short- and long-term treatment periods. Such investigations have uncovered a trend for APD treatment to result in immediate decreases to both $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ receptor subtypes following a cessation of treatment. We believe that our investigation is the first to identify that if treated in a juvenile animal, this alteration to 5-HT receptor density is still prominent in adulthood.

Long-term alterations to $5\text{-}HT_{2A}$ receptors after early APD exposure were uncovered in hippocampal and cortical brain regions in the current study. In the male cohort, significant decreases to the density of $5\text{-}HT_{2A}$ receptors were found in the hippocampus of adult brains following juvenile treatment with the APDs aripiprazole, olanzapine, and risperidone. Furthermore, similar decreases were also found in the PFC of those that underwent early treatment with aripiprazole and risperidone in comparison to the control. Decreases in $5\text{-}HT_{2A}$ receptor densities were also found in the hippocampus of females following treatment with the APDs olanzapine and risperidone.

Although previous investigations into the immediate effects of short- and long-term treatments with olanzapine have also found region-specific alterations to the density

levels of $5\text{-}HT_{2A}$ receptors of both young[45,69] and adult[67,70,71] rats, results from the current study extended that of previous findings. Specifically, while decreases in $5\text{-}HT_{2A}$ levels have been observed in the hippocampus of the present study, previous investigations over multiple time periods, and across both genders, have found no changes to hippocampal $5\text{-}HT_{2A}$ receptor levels in the adult brain following olanzapine treatment.[70,71] Furthermore, while no changes in $5\text{-}HT_{2A}$ levels were uncovered in the PFC of our investigation, olanzapine has been found to immediately decrease PFC $5\text{-}HT_{2A}$ receptor levels in young rats following short-term treatment periods[45,69] and adult rats following both short-term[70,71] and long-term treatment periods.[67] While studies on the short- and long-term effects of risperidone treatment in both young and adult rats have also uncovered similar significant decreases in $5\text{-}HT_{2A}$ receptor levels in the PFC,[45,46,70] no changes in $5\text{-}HT_{2A}$ levels in brain regions including the CPu, NAc, and hippocampus have been previously uncovered.[45,69,70] In addition, although significant decreases in $5\text{-}HT_{2A}$ receptors were observed in both the hippocampus and PFC of animals treated with aripiprazole in the present study, limited investigations have previously been completed investigating the potential for aripiprazole's antagonistic actions on $5\text{-}HT_{2A}$ receptors to cause short- or long-term changes. While one investigation into the immediate effects of short-term treatment with aripiprazole has uncovered decreased levels of $5\text{-}HT_{2A}$ receptors in the PFC of young male rats,[45] further investigations into the immediate and long-term effects of short- and long-term treatments will shed further light and allow further comparisons to be drawn to the current investigation findings.

In addition to the $5\text{-}HT_{2A}$ receptor changes, alterations to the $5\text{-}HT_{2C}$ receptor were found in the PFC and hippocampus of males only in the present study. Contrasting results were revealed between the aforementioned regions, with decreases in $5\text{-}HT_{2C}$ receptor levels uncovered in the PFC of adult male rodents following juvenile treatment with aripiprazole and olanzapine, while increases were found in the hippocampus of aripiprazole- and risperidone-treated groups in comparison to control.

Previous investigations into the immediate effects of APD treatment on $5\text{-}HT_{2C}$ receptor density levels have found differing results to that observed by the current study, where long-lasting effects were revealed. First, contrary to our study, alterations to $5\text{-}HT_{2C}$ receptors have been found across both male and female rodents in studies of varying treatment durations, utilizing both young and adult models, and through multiple brain regions. In addition, while olanzapine treatment has been found to decrease $5\text{-}HT_{2C}$ levels in

Michael De Santis   93

the PFC of studies, investigations into the immediate effects of short- and long-term APD treatment on 5-HT_{2C} receptors in the hippocampus of young and adult rodents have found treatment with olanzapine resulted in either a decrease or no change in the density of receptors.[45,67,70,71] In particular, while short-term olanzapine treatment of both young male and female rodents resulted in immediate decreases across cortical and striatal brain regions,[45] variations in results have been found in the immediate effects of adult olanzapine treatment models over both short-term[70,71] and long-term periods,[67] with either decreases or no change in receptor densities found in investigated brain regions including PFC, CPu, NAc, and hippocampus. Investigations into the effects of aripiprazole and risperidone on 5-HT2C receptor have been concentrated on the immediate effects of short-term treatment periods, with no alterations to any brain regions found following short-term aripiprazole treatment across both male and female young animals[45] or short-term risperidone treatment in adult male rodents.[70]

Minimal changes in adult 5-HT_{1A} receptors were uncovered following juvenile APD treatment in the present study. Decreases in 5-HT_{1A} receptor density levels in the adult brain were uncovered in the NAc following juvenile risperidone APD treatment in the male cohort, and in the NAc of female rodents treated with aripiprazole in comparison to the control. Previous investigations once again centered upon uncovering the immediate effect of treatment on 5-HT_{1A} receptor density levels, with the majority of studies revealing no changes in receptor levels following APD treatment with aripiprazole, olanzapine, or risperidone in investigated regions. Some short-term studies did however reveal that acute and short-term treatment in both young and adult rats increased 5-HT_{1A} receptor density levels.[46,70] Specifically, while increases in 5-HT_{1A} receptor density levels have been found previously across both the PFC and hippocampus of both young and adult male rodents following short-term treatment with risperidone and olanzapine,[46,70] acute treatment of female rats with aripiprazole has also uncovered increases in 5-HT_{1A} levels in the hippocampus. No alterations, however, were found following acute and long-term olanzapine or haloperidol treatments and across cortical and hypothalamic brain regions.[65]

The minimal observed alterations to the 5-HT_{1A} receptor in comparison to 5-HT_{2A} and 5-HT_{2C} may be due to a number of factors. First, of the three APDs investigated in the current study, only aripiprazole has been found to have a significant affinity for the 5-HT_{1A} receptor,[65,72] while the similar antagonistic pharmacological profile of olanzapine and risperidone

on 5-HT_{2A} and 5-HT_{2C} receptors may be resulting in the comparable decreases in adult brain receptor density levels between the two receptor subtypes.[22] The 5-HT_{1A} is known to be located both pre- and postsynaptically and have autoreceptor functions.[38,73,74] Investigations have found that the 5-HT_{1A} receptor located presynaptically in the dorsal raphe nucleus, performing regulatory functions for the 5-HT NT, and also located postsynaptically in limbic structures including the hippocampus, performing traditional postsynaptic receptor functions.[38,73,74]

The repeated antagonism of the 5-HT_{2A} and 5-HT_{2C} receptors, along with the presynaptic 5-HT_{1A} receptor, has the potential to result in a downregulation in number and sensitivity and subsequently a long-term deficiency in 5-HT NT signaling.[38,75,76] With the 5-HT_{2A} receptor in particular well known to play critical roles in both APD treatment efficacy[19–22] and the regulation and functioning of the 5-HT NT system,[36–38,77,78] and with previous investigations demonstrating a correlative functions of the 5-HT NT in the pathophysiology of multiple mental illnesses,[16,22,29,38,79,80] any disturbances to the regulation of the 5-HT NT system, such as through early APD treatment targeting 5-HT receptors, have the potential to alter 5-HT transmission and, thus, elicit related changes to multiple facets of mental illness over long term. Furthermore, the subsequent deficiency in projections of the 5-HT NT has been found to result in the disinhibition and therefore enhancement of the DA signal[36–38] and correlated to changes in behaviors, as demonstrated in our previous investigations.[35,39] Changes in behaviors including enhanced locomotor activity[36,37] and anxiolytic and decreased depressive-like behaviors[8,36,75,81–84] have previously been uncovered and correlated to the repeated antagonism of the 5-HT_2 receptor, potentially the negatively correlated alterations to the DA signal.

APDs such as aripiprazole have also been found to elicit partial agonist effects on presynaptic 5-HT_{1A} receptors in the dorsal raphe nuclei of previously investigated brains,[76,85] Investigations into the effects of APD treatment on 5-HT receptors in the dorsal raphe nuclei, however, will need to be the focus of future studies, as the focus of the current investigations only shifted to the 5-HT NT system following previous results, and thus, no relevant tissue is available for analysis.

With previous investigations into the effects of APD treatment on 5-HT receptors centered upon revealing any immediate changes in receptor density levels, and the current study looking into long-lasting effects, the contrasting results between the current and previous investigations may be the

Michael De Santis

94

product of a myriad of influencing factors. Factors including the treatment duration of the study, differences in age of the animals treated, and duration of time between the cessation of APD treatment and detection of 5-HT receptors have previously been highlighted as having the potential to influence the observed results, with the current study specifically investigating the long-term effects of juvenile APD treatment on 5-HT receptors in the adult brain. There is the potential that the alterations to measured variables uncovered in the present study may have occurred following the cessation of APD treatment and during the drug withdrawal period. During such time, the antagonistic action of APDs on 5-HT receptors in an adult brain may result in a short-term over-compensatory increase in receptor numbers (as observed in previous investigations[65]), followed by a regulation of density over long term. Drug treatment during the critical neurodevelopmental time period may subsequently result in a long-term decrease in morphology and/or density over the large time duration, in a process previously labeled as neuronal imprinting.[25] Previous studies have demonstrated similar age-dependent effects of drug treatment, with psychotropic drugs such as fluoxetine (a selective serotonin re-uptake inhibitor [SSRI]) previously proven to elicit different effects on a juvenile compared to an adult, mature brain.[25,34,86,87]

Chronologically, and as outlined briefly earlier, 5-HT is also known to play a significant role first as a trophic factor in overall brain development and then undergo significant neurodevelopmental phases itself as the NT system develops from birth through to adulthood.[8,31] Specifically, the 5-HT ligand, along with the 5-HT$_{1A}$ receptor, has been found to play key roles in overall axonal growth and synapse formation throughout the brain.[88] Alterations to baseline levels of 5-HT during these early critical phases of neurodevelopment, through either intrinsic or extrinsic factors (eg, early APD treatment), have been found to alter the developmental trajectory of the adult brain and subsequently impact the adult brain functioning.[8,26,30,32,88] Therefore, there is the potential that the juvenile APD treatment utilized in the present study, with a high affinity and potent actions on the 5-HT NT system, has impacted not only the observed long-lasting changes in 5-HT receptors but also widespread long-lasting alterations to overall axonal growth, neurite, and dendrite formations.

Additionally, and as indicated previously, gender-specific alterations to adult 5-HT receptor density levels were also observed following juvenile APD treatment in the present study. In particular, more widespread alterations to the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors were uncovered in the PFC and hippocampus of males, while minimal alterations were observed in the female cohort across all investigated 5-HT receptor subtypes and across all four brain regions and APD treatment groups. Potential influencing factors on the observed gender differences in results have been outlined extensively in previous publications.[35,39] Specifically, the well-known differences in the development and expression of 5-HT receptors between genders[89] and with the influence of the sex hormones testosterone and estrogen[26,82,89–91] have the potential to play a role in the observed gender differences. Previously demonstrated gender variations to 5-HT-mediated functions have an obvious potential to influence the observed results,[89,92] with the sex hormones testosterone and estrogen found to play a critical role.[89,91,93–96] Changes in the levels of the sex hormone estrogen have been found to influence the levels of 5-HT ligand in brain regions including the cortex and raphe nucleus[94–96] and, furthermore, alter the density levels of 5-HT$_{1A}$ and 5-HT$_2$ receptors in brain regions including the cortex, raphe nucleus, and hippocampus.[89] Additionally, estrogen has previously been found to play a neuro-protective effect on 5-HT NT system, with studies uncovering its ability to inhibit behavioral changes in information processing mediated by both the 5-HT$_{1A}$ and DA D$_2$ receptors, an attribute that found deficient in people suffering from mental illness.[90]

Conclusion

The current study has uncovered the potential for treatment with the APDs aripiprazole, olanzapine, and risperidone during the critical neurodevelopmental period to cause long-lasting alterations to the density of 5-HT receptors in the adult brain. In particular, significant alterations to 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors in cortical and hippocampal brain regions were observed in the male cohort across aripiprazole, olanzapine, and risperidone APD treatment groups in comparison to controls. These observed changes are in addition to the alterations to various behavioral attributes (including anxiety and depressive-like behaviors) and the dopamine NT system (including receptors, transporters, and synthesis markers) previously reported with the same treatment model in our laboratory.[35,39] Although the observed alterations to the 5-HT NT system in the investigated regions provide some evidence of the potential for early APD treatment to elicit long-term alterations to a NT system functioning, further investigations have the potential to uncover both the scope of changes elicited on the 5-HT NT system and potential alterations to other NT groups. Other NT systems including the adrenergic and muscarinic NT systems are also known to be a part of the pharmacological mechanisms of action of

Michael De Santis

95

APDs and interplay with other NT systems,[37,97–99] and thus, antagonist actions during the critical neurodevelopmental time period have the potential to elicit long-lasting changes that may be exhibited clinically. Furthermore, investigations into the effects of juvenile APD in a disease animal model would also provide invaluable insight into the potential long-lasting effects of treatment during such a critical neurodevelopmental time period. The alterations observed in the present study provide some of the first evidence of the potential of juvenile APD treatment with aripiprazole, olanzapine, and risperidone to elicit long-term alterations to the 5-HT NT system in the adult brain. With all three APDs approved for use in adolescents with various medical conditions and also known to be prescribed off-label, the potential long-term effects of early use should be highlighted before they are prescribed clinically, especially in the male cohort where the vast majority of alterations have been found.

Acknowledgments

Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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Michael De Santis

96

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Michael De Santis

97

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Michael De Santis

98

CHAPTER 6 - GENERAL DISCUSSION

6.1 Overall Discussion

As previously outlined, significant increases in the prescription and use of APDs in the juvenile population has occurred in recent years, despite a serious lack of understanding of the long-term effects of treatment. APDs such as aripiprazole, olanzapine and risperidone are known to produce their therapeutic effects through a potent affinity for and mechanism of action on DA and 5-HT receptors. The DA and 5-HT NT systems are also known to undergo and have a significant role in brain development during the critical neurodevelopmental period of childhood and adolescence (Levitt et al. 1997; Frost and Cadet 2000; Andersen 2003; Klomp et al. 2012; Milstein et al. 2013; Cousins and Goodyer 2015), and previous investigations have correlated alterations to the DA and 5-HT NT systems with changes in various behavioural attributes, and the pathogenesis of various mental illnesses (Meltzer and Huang 2008; Seo et al. 2008; Kegeles et al. 2010; Beaulieu and Gainetdinov 2011; Howes et al. 2012; Meltzer 2012; Ellenbroek and Cesura 2015; Teissier et al. 2015). There is therefore the potential that use of APDs during this phase of neurodevelopment may result in permanent or long-lasting alterations to NT systems including that of DA and 5-HT as well as behaviours.

The studies presented in Chapters 3 to 5 of this thesis therefore investigated the effects of early use of the commonly prescribed APDs aripiprazole, olanzapine and risperidone on potential long-term alterations to behavioural attributes, and changes to the DA and 5-HT NT systems in adulthood. With in-depth discussions for each study included within Chapters 3 to 5 of this thesis, this chapter will provide a general discussion

around the key outcomes that have been achieved, and potential mechanisms for observed alterations based on interactions between the examined behaviours and the DA and 5-HT NT systems. Finally, limitations and recommendations of avenues for further research will be addressed.

6.1.1 Provided Evidence that Juvenile Antipsychotic Drug Treatment Elicits Long-term Changes to Adult Behavioural Attributes and the DA and 5-HT NT Systems

In Chapter 3 of this thesis (De Santis et al. 2016), the long-term effects of juvenile APD treatment on adult behavioural attributes in rodents was investigated. Evidence has subsequently demonstrated that early APD treatment with aripiprazole, olanzapine and risperidone results in long-term alterations to locomotor activity, anxiety and depressive-like behaviours in both the male and female animal models. Based on the premise of the uncovered behavioural changes and knowledge of the established basis of physiology from the literature, investigations into both the DA (Chapter 4) and 5-HT (Chapter 5) NT systems were also conducted, and alterations across both NT systems was also found.

As described in Chapter 3, this thesis has demonstrated that locomotor activity levels were altered in both the male and female cohort following early APD treatment with both olanzapine and risperidone. Although contrasting effects were uncovered across sexes (discussed in Section 6.1.3 below), the physiology behind changes to locomotor activity behaviours can been directly linked to alterations to the production and

transmission of the NT and density of receptors in the DA and 5-HT NT systems (Seo et al. 2008; Choi et al. 2010; Beaulieu and Gainetdinov 2011; Teissier et al. 2015; Wong-Lin et al. 2017), as discussed in Chapters 1 and 3. Given the findings in Chapter 3 that early treatment with risperidone and olanzapine (to a lesser extent) significantly increased numerous behavioural parameters indicative of locomotor activity in the male cohort, it is interesting to find that corresponding increases to p-TH and DAT levels were detected in the PFC, hippocampus and VTA respectively, along with reduced levels of 5-HT$_{2A}$ receptor density levels in similar brain regions. The findings discussed in Chapters 4 and 5 across both the male and female cohorts are in support of the results of current studies that theorise that early exposure to the potent DA receptor antagonist mechanisms of risperidone and olanzapine may have resulted in long-term changes to the DA NT system, including an enhancement/up-regulation of DA synthesis and re-uptake markers in brain regions previously linked to hyper-locomotor activity in males, with converse correlative effects in females once again in cortical and hippocampal brain regions (Beaulieu and Gainetdinov 2011; Shu et al. 2014). Furthermore, although limited changes to the D$_1$ or D$_2$ receptors were observed in Chapter 4, they were in line with previous results in a similar long-term APD treatment model (Vinish et al. 2012; Milstein et al. 2013), and may be due to the ongoing maturational/developmental phases of DA receptors as discussed in Chapter 1. Specifically, the receptor density levels of monoamine NT systems including DA are known increase significantly before a period of regressive elimination into adulthood (Dinopoulos et al. 1997; Andersen 2003; Andersen and Navalta 2004). It is during this period of regressive elimination that APD treatment was ceased in the current animal treatment model and hence a return to 'normal' receptor density levels may have occurred, whilst the production/synthesis rate

of the DA NT and re-uptake marker (DAT) may still be left elevated. This may potentially be due to the antagonist actions of APDs on DA receptors including the D_2 autoreceptor (known to play a critical role in the regulation of DA synthesis) during the early neurodevelopmental phase. Along with increases to synthesis and re-uptake markers in the DA NT system found in Chapter 4, the reductions to the density of the 5-HT_{2A} and 5-HT_{2C} receptors in the PFC and hippocampus discussed in Chapter 5 are also in line with the observed increases to locomotor activity levels of Chapter 3. With previous studies linking a deficient neurotransmission of 5-HT to a disinhibited and thus elevated DA signal (Kusljic et al. 2003; Seo et al. 2008), and 5-HT_{2A} and 5-HT_{2C} receptor activation known to have opposing effects on the DA release (Alex and Pehek 2007; Howell and Cunningham 2015), there is collectively a clear potential association between the observed locomotor behaviours and contrasting alterations to the physiology in both the DA and 5-HT NT systems.

Further to the observed changes to locomotor activity behaviours, Chapter 3 also details the uncovered alterations to anxiety- and depressive-like behaviours found across 3 APD treatment groups, with contrasting effects across sexes again observed. Correlations between the observed alterations to anxiety-like and depressive-like behavioural parameters in both sexes, and the previously described changes to the 5-HT and DA NT systems (discussed in Chapters 4 and 5) can again be drawn. Collectively, the observed decreases to 5-HT_{2A} and 5-HT_{2C} receptor subtypes in cortical and hippocampal brain regions described in Chapter 5, together with the uncovered increases to DA synthesis and re-uptake markers detailed in Chapter 4, are in line with the findings of previous studies which have correlated reductions to the density of 5-

HT$_{2A}$ and 5-HT$_{2C}$ receptors and decreased serotonergic signalling (Mora et al. 1997; Andersen and Navalta 2004; Karl et al. 2006; Seo et al. 2008), and associated alterations to DA signalling (Dunlop and Nemeroff 2007; Seo et al. 2008; de Oliveira et al. 2009) with anxiolytic and decreased depressive-like behaviours, as observed in Chapter 3 of this thesis.

6.1.2   Provided Evidence that Juvenile Antipsychotic Drug Treatment with Aripiprazole, Olanzapine and Risperidone Causes Differing Long-term Effects

As discussed in Chapters 3-5, early treatment with aripiprazole, olanzapine and risperidone was found to result in differing effects on long-term behaviours and the DA and 5-HT NT systems in the adult brain. Whilst early treatment with olanzapine and risperidone was found to result in long-term widespread, sex-specific effects across the 3 investigations, early aripiprazole treatment was found to have minimal effects on adult behavioural attributes (as discussed in Chapter 3), yet still result in sex-dependent changes to the DA (Chapter 4) and 5-HT (Chapter 5) NT systems.

Specifically, the high occupancy, partial agonist effects of aripiprazole on the DA D$_2$ receptor was seen to result in long-term decreases to DA synthesis markers in mesocortical regions of both the male and female cohorts, whilst its antagonist actions on 5-HT$_{2A}$ receptors was seen to result in similar correlative decreases to the 5-HT$_{2A}$ receptor in the hippocampus. Increases to 5-HT$_{2C}$ receptor density levels in the hippocampus of males were also uncovered following early aripiprazole treatment,

together with decreases in the PFC. The observed contrasting changes may be due to both the varied regional capabilities of the 5-HT_{2C} receptor to tonically regulate/modulate DA signalling from the terminal regions (e.g. hippocampus) of mesolimbic and nigrostriatal pathways, something that is yet to have been proven in the mesocortical pathway as discussed in Chapter 1 (Section 2.2.3.3) (Kapur and Remington 1996; Abi-Dargham et al. 1997; Kusljic et al. 2003; Alex and Pehek 2007). Together with the observed changes to DA synthesis markers TH and p-TH uncovered in the PFC in Chapter 4, these regional variations in the relationship between the 5-HT_{2C} receptor and DA signal provides a foundational basis for the regional differences in aripiprazole treatment effects.

In contrast, early treatment with the high affinity, antagonist actions of olanzapine and risperidone on 5-HT_{2A}, 5-HT_{2C} and DA D_2 receptors was found to have more widespread, sex specific effects across all 3 investigations. As discussed in Chapter 3 and Section 6.1.1 above, sex based and somewhat contrasting changes to locomotor activity, anxiety- and depressive-like behaviours were found across both the olanzapine and risperidone drug treatment groups, whilst the effects of early treatment with olanzapine and risperidone on the DA and 5-TH NT systems respectively, and potential reasons for the differences in effects are detailed and discussed in Chapters 4 and 5. Specifically, the antagonist actions of olanzapine on both the pre- and post-synaptic DA D_2 receptor (inclusive of the D_2 autoreceptor, with its regulatory functions) was found to affect the DA NT system of the female cohort only, decreasing the DA synthesis marker p-TH by 60% in the PFC, and increasing the adult density levels of the D_2 receptor in both the PFC and NAc of the mesocorticolimbic DA pathway. Furthermore,

olanzapine's high affinity, inverse agonist actions on 5-HT_{2A} and 5-HT_{2C} receptors was found to decrease 5-HT_{2A} receptor density levels of females and the 5-HT_{2C} receptor of males only. In risperidone treated animals, increases to p-TH levels were observed in the PFC and hippocampus and decreases to D_1 receptors were observed in contrast to the effects of olanzapine, whilst increases to 5-HT_{2C} receptors were observed.

The higher affinity, more potent antagonist actions of risperidone on the DA NT system receptors in comparison to olanzapine may be resulting in the different long-term effects of the APDs on the DA NT system. Furthermore, with a similar inverse agonist mechanism of action of olanzapine and risperidone on the 5-HT_{2A} and 5-HT_{2C} receptor subtypes (Meltzer and Massey 2011), it is interesting that whilst early treatment with olanzapine decreased 5-HT_{2A} and 5-HT_{2C} receptor levels in the female and male cohorts respectively, and risperidone was similarly found to decrease male 5-HT_{2A} density levels in the PFC and hippocampus, increases to 5-HT_{2C} receptors were found in the hippocampus of risperidone treated animals. Whilst both the higher affinity of olanzapine for the 5-HT_{2C} receptor (Meltzer 2002; Meltzer and Massey 2011) and the aforementioned different regional capabilities of the 5-HT_{2C} receptor to regulate the DA signal may play a role in this contrasting change (Kapur and Remington 1996; Abi-Dargham et al. 1997; Kusljic et al. 2003; Alex and Pehek 2007), the higher level of constitutive activity of 5-HT_{2C} receptors in comparison to its counterpart may also provide some basis of understanding for the observed differences in long-term effects (Berg et al. 2005), although further investigations into this may provide further clarity. The findings of this thesis has provided further evidence for, and highlighted the potentially fundamental role and intricate regional differences of the relationship that

the 5-HT NT system plays in the regulation of the DA signal, in particular the 5-HT$_{2C}$ receptor.

6.1.3 Revealed that Juvenile Antipsychotic Drug Treatment Elicited Different Long-term Changes Across Sexes to Adult Behavioural Attributes, and the Dopamine and Serotonin Neurotransmitter Systems

As discussed in Chapters 3-5 and briefly in Sections 6.1.1 and 6.1.2 above, early APD treatment with aripiprazole, olanzapine and risperidone was surprisingly found to result in sex-specific effects across all 3of the investigations detailed in this thesis.

In Chapter 3, contrasting alterations were uncovered across locomotor activity, anxiety- and depressive-like behavioural attributes upon comparison of the male and female cohorts. Specifically, whilst early APD treatment in the male cohort resulted in behavioural attributes indicative of increased locomotor activity levels and decreased anxiety-like and depressive-like behaviours, on the contrary, treatment in the female cohort was found to result in changes to behaviours indicative of decreased locomotor activity and increased depressive-like behaviour, with no change in anxiety-like behaviour in comparison to the control group. As detailed in Chapter 4 above, the contrasting and sex-specific effects observed in Chapter 3 were again uncovered in the DA NT system upon comparison of male and female cohorts. Specifically, whilst early treatment with aripiprazole and olanzapine was found to result in decreases to DA

synthesis markers, and increases to D_2 receptor density levels were uncovered following olanzapine treatment in the female cohort, both increases to DA synthesis and re-uptake markers were found in the male cohort following risperidone drug treatment, and furthermore minimal effects were observed in the olanzapine and aripiprazole drug treatment groups. Finally, sex-specific alterations were similarly observed in investigations into the 5-HT NT system. As detailed in Chapter 5, whilst alterations to 5-HT_{2A} and 5-HT_{2C} receptors were uncovered in cortical and hippocampal brain regions of male animals across all 3 drug treatment groups, a lone decrease to the 5-HT_{2A} receptor was uncovered in the hippocampus of olanzapine treated female rats.

Overall, there are numerous potential factors that need to be deliberated when considering the reasons for sex-specific variances observed in the results across all 3 studies (Chapters 3-5). Firstly, as detailed in Chapter 1, it is well known that there are significant differences in the timeline of neurodevelopmental phases between the male and female brain, including that of the monoamine NT systems DA and 5-HT investigated in this thesis (Andersen and Teicher 2000; De Bellis et al. 2001; Andersen 2003). Specifically, sex differences have been found of the age and rate that myelination, synapse and receptor production and elimination occurs within both the DA and 5-HT NT systems (Andersen and Teicher 2000; Andersen 2003), as well as the individual neurodevelopmental differences between specific receptor subtypes and brain regions (Andersen 2003; Sinclair et al. 2014). Furthermore, previous results have also highlighted that basic differences between males and females on a biological level (Bolea-Alamanac et al. 2018) also provides a potential reasoning for the observed sex differences in affects. Additionally, the well documented influence of the sex hormones

estrogen and testosterone on the development of the DA and 5-HT NT systems, including the synthesis of each ligand and density of numerous receptor subtypes within each NT system also highlights their capability to impact the observed results (Sumner and Fink 1993; Sumner and Fink 1995; Zhang et al. 1999; Andersen 2003; Gogos et al. 2010; Gogos et al. 2012; Purves-Tyson et al. 2012; Purves-Tyson et al. 2014; Sinclair et al. 2014; Bolea-Alamanac et al. 2018). Furthermore, a potential 'neuro-protective' role of estrogen on the DA and 5-HT NT systems has also been uncovered in some investigations (Dunlop and Nemeroff 2007; Gogos et al. 2012), and once carries the potential to influence results. Further studies into the role of the sex hormones testosterone and estrogen on shaping the DA and 5-HT NT systems, and its thus a potential neuroprotective role through the period of neurodevelopment will shed further light on its exact capabilities to influence the potential long-term effects of early APD treatment that were observed in the current thesis.

6.2 Recommendations for Further Research

In reflecting on the findings of this thesis, the following are recommendations of areas in which further research will provide greater insight into the potential long-term effects of juvenile APD treatment on the adult brain:

a) The DA NT system is well known to play a critical role in both the pathogenesis of mental illness, and as a pharmacological target of APD treatment. This thesis uncovered that juvenile treatment with the APDs including aripiprazole, olanzapine and risperidone caused alterations to DA synthesis markers, transporter and D_1 and

D_2 receptor density levels across mesocortical, mesolimbic and nigrostriatal pathways (Chapter 4). Further investigations however into potential long-term effects on the DA D_3 receptor, also known to play a role in the therapeutic benefits of APD actions, would provide further insight into the scope of long-term effects on the adult brain.

b) The investigations in Chapter 5 revealed that $5-HT_{2A}$ and $5-HT_{2C}$ receptor density levels were altered in cortical and hippocampal brain regions long-term, following early treatment with the APDs aripiprazole, olanzapine and risperidone. With the scope of the completed study into the 5-HT system only spanning the PFC, NAc, CPu and hippocampus (as tissue had already been collected), further investigations conducted into brain regions including the RN will reveal to a greater extent potential long-term changes to the 5-HT system. Known to play a key role in the synthesis/production of the 5-HT NT (Choi et al. 2012), investigations into the effects of early treatment on 5-HT synthesis markers will also provide into the effects of early APD treatment on the production and transmission of a NT known to play numerous critical functions within the brain. Furthermore, additional studies into potential changes to other 5-HT receptor subtypes in the hippocampus including $5-HT_4$, $5-HT_6$ and $5-HT_7$ will provide greater insight into the full array of potential effects of early APD treatment, with the region and receptors known to play a critical role in cognitive and memory capabilities (Berumen et al. 2012).

c) The current thesis aimed to establish whether APD treatment had the capability to result in any long-term behavioural changes, and subsequently behavioural tests

investigating numerous behavioural attributes were selected. The results of the investigations have subsequently thrown to light the need for further investigations into both the potential effects of early APD use on other behaviours (e.g. object recognition, pre-pulse inhibition etc.), and also the benefits that extensive testing into the uncovered behavioural changes would have, including the light-dark transition test to further investigate anxiety-like behaviours, which we now recommend be carried out.

d) Investigations into the potential for long-term physiological alterations on other NT systems known to play a critical role in the physiology of the behavioural attributes investigated will also provide insight into potential further, more extensive effects across numerous NT systems. The noradrenergic, glutamate and GABA NT systems are all well-known to play critical roles in the regulation of behavioural attributes observed in the current thesis, and furthermore are well-known to interact significantly with the DA & 5-HT NT systems in order to shape the NT signal.

e) Whilst the current juvenile animal treatment model was utilised to investigate the effects of APD treatment in healthy rats, further studies across the numerous developmental animal models of mental illness (e.g. hippocampal lesion model for schizophrenia) will provide further knowledge on the potential long-term effects during the critical neurodevelopmental period. Thus far, investigations into the effects of adolescent treatment with APDs in animal models of mental illness have uncovered that early intervention can alter structural brain abnormalities long-term in a manner that precedes normal brain functioning (Piontkewitz et al. 2009;

Piontkewitz et al. 2012). With no single disease or genetic model capable of covering the wide-range of antipsychotic treatment conditions in children/adolescents, investigations across numerous animal models of mental illness will need to be completed to correctly investigate the effects of treatment across the numerous mental illnesses that APDs are currently used to treat, which fell outside the scope of the current thesis.

f) With sex comparative differences in effects observed across all 3 studies (Chapter 3-5), further investigations into the potential impact of the presence and absence of the sex hormones testosterone and estrogen will shed further light on the role of each in the observed changes. With estrogen and testosterone both known to play influential roles in the development and function of both the DA and 5-HT NT systems, investigations into the effects of both sex hormones on the long-term effects of juvenile APD in the DA and 5-HT systems will provide further insight into the potential role of each in the observed alterations.

g) Although aripiprazole, olanzapine and risperidone were selected and utilised in the current investigations as they are three of the most commonly prescribed/used APDs in the juvenile population (Haw and Stubbs 2007; Vitiello et al. 2009; Olfson et al. 2010; Sharma and Shaw 2012; Memarzia et al. 2014; Schneider et al. 2014), consideration also needs to be given to the various other APDs currently being prescribed to children and adolescents. Therefore, further investigations into the potential long-term effects of other APDs widely used in the treatment of psychosis

related disorders (e.g. clozapine) will also provide a greater insight into the effects of early APD use on the adult brain.

h) Whilst the current studies have demonstrated that early APD treatment with aripiprazole, olanzapine and risperidone caused long-term changes to both the DA and 5-HT NT systems in an in vivo animal model, the potential effects in a clinical setting is currently not well understood. With APD use in the child and adolescent population still currently increasing globally, extending the premise of the current investigation into the clinical setting and across multiple brain regions will provide further, more clinically relevant information to clinicians on the long-term implications of early APD prescription and use.

6.3   Conclusions and Implications

In conclusion, the series of investigations that is encompassed in this thesis has demonstrated that APD treatment in juvenile rodents has the capability to cause sex and drug specific long-term alterations to various behavioural attributes in adulthood. With the knowledge that both the DA and 5-HT NT systems play a vital role in the physiology behind investigated behavioural attributes, this thesis has also provided evidence that early treatment with the high affinity, potent agonist/antagonist actions of aripiprazole, olanzapine and risperidone on the DA and 5-HT NT systems has the capability to cause sex, drug and region specific long-term alterations to DA receptors, synthesis and re-uptake markers, and furthermore 5-HT receptors in the adult brain. These results hence confirm that treatment with the APDs aripiprazole, olanzapine and

risperidone during a time that critical neurodevelopmental phases are occurring has the potential to cause long-lasting and potentially permanent alterations to both the DA and 5-HT NT systems, and furthermore implicate a potential correlation with the observed changes to various physiologically related behavioural attributes. Whilst detected alterations to locomotor activity, anxiety- and depressive-like behaviours were uncovered following the investigations within the scope of the current thesis, further behavioural and cognitive tests may reveal a more extensive array of long-term changes caused by this early insult of APDs, with both the DA and 5-HT NT systems known to play critical roles in numerous cognitive abilities from working memory to sensory and motor processes (Carlsson et al. 2004; Dolžan et al. 2008; Teissier et al. 2015).

The findings from the present investigations provide an initial examination into the potential long-term effects of 3 APDs widely and increasingly prescribed in the juvenile population (Vitiello et al. 2009; Zuddas et al. 2011), in an area where there is a need for further information around the safety of its use long-term. Further and more extensive investigations over short and long-term time periods to uncover the full array of potentially permanent effects that the potent actions of APD prescription and use may have on the juvenile brain will provide further evidence of the alterations to both the neural topography and functioning in adulthood. A well-established evidence base will allow psychologists/psychiatrists to weigh up the risk/benefit ratio of prescribing APDs to the juvenile population, with alternative treatment options potentially addressed first (Haw and Stubbs 2007; Vitiello et al. 2009; Memarzia et al. 2014). Furthermore, clinical tools/assessments to monitor the risks and benefits associated with juvenile

APD prescription and use clinically will also provide an additional tool for valuation of the risk/benefit ratio of use.
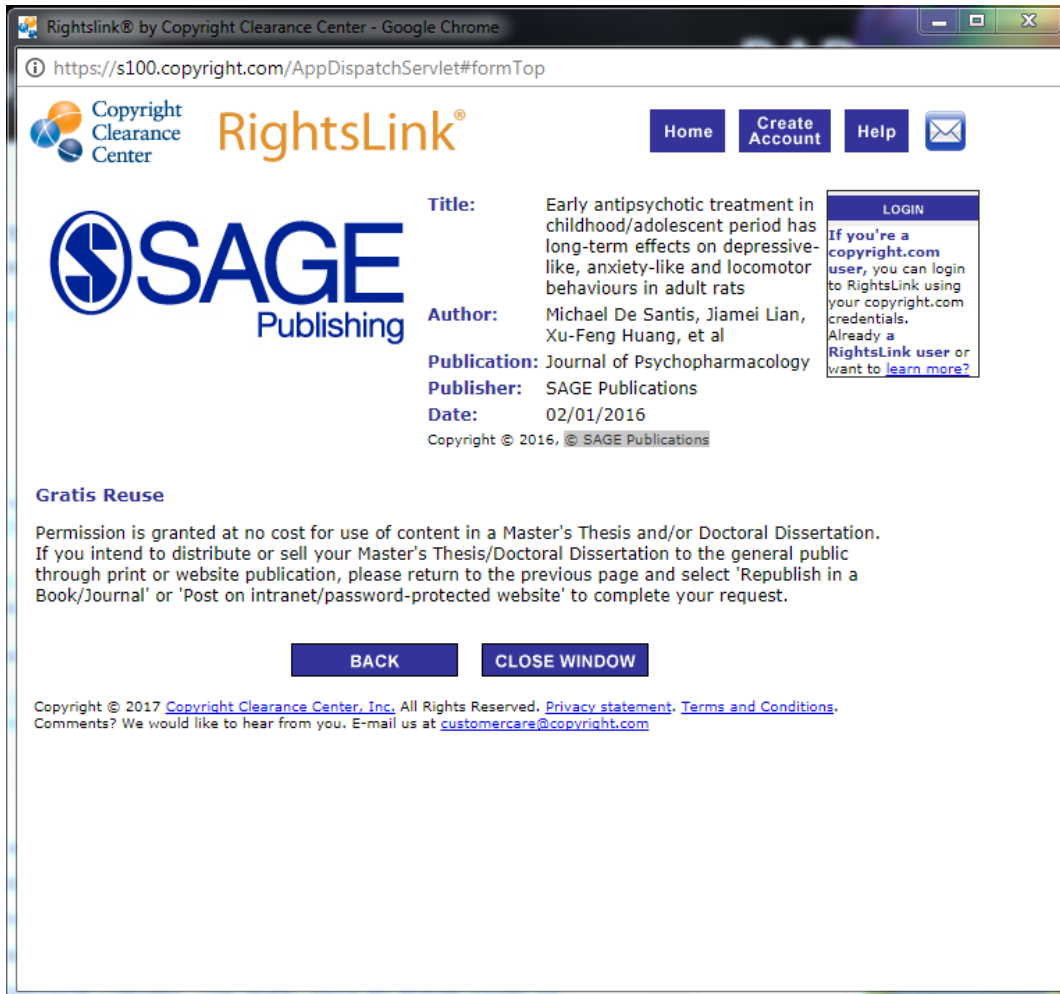
APPENDICES

Appendix A – Chapter 3 Supplementary

Supplement A1 – Statement from co-authors

This is to attest that the PhD candidate, Michael De Santis, contributed significantly to the investigation (De Santis, M., Lian, J., Huang, X-F. and Deng, C. (2015)). Early antipsychotic treatment in childhood/adolescent period has long-term effects on depressive-like, anxiety-like and locomotor behaviours in adult rats. *Journal of Psychopharmacology*, 30 (2) 204-214: designed and performed the experimental work, analysed the data, interpreted results, and wrote the manuscript. Two of the co-authors (C.D and X-F.H) are my PhD supervisors, who have provided comments on experimental design, data analysis, results interpretation, and revision of manuscripts. The other (J.L) assisted with experimental procedures and provided comments on revision of manuscripts.

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Xu-Feng Huang	
Chao Deng	

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Appendix B – Chapter 4 Supplementary

Supplement B1 – Statement from co-authors

This is to attest that the PhD candidate, Michael De Santis, contributed significantly to the investigation (De Santis, M., Lian, J., Huang, X-F. and Deng, C. (2016). Early Antipsychotic Treatment in Juvenile Rats Elicits Long-term Alterations to the Dopamine Neurotransmitter System. *International Journal of Molecular Sciences*, 17(11): 1944: designed and performed the experimental work, analysed the data, interpreted results, and wrote the manuscript. Two of the co-authors (C.D and X-F.H) are my PhD supervisors, who have provided comments on experimental design, data analysis, results interpretation, and revision of manuscripts. The other (J.L) assisted with experimental procedures and provided comments on revision of manuscripts.

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Jiamei Lian	
Xu-Feng Huang	
Chao Deng	

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Appendix C – Chapter 5 Supplementary

Supplement C1 – Statement from co-authors

This is to attest that the PhD candidate, Michael De Santis, contributed significantly to the investigation (De Santis, M., Huang, X-F. and Deng, C. (2018). Early Antipsychotic Treatment in Juvenile Rats Elicits Long-term Alterations to the Adult Serotonin Receptors. *Neuropsychiatric Disease and Treatment*, (accepted 9th March 2018) designed and performed the experimental work, analysed the data, interpreted results, and wrote the manuscript. Two of the co-authors (C.D and X-F.H) are my PhD supervisors, who have provided comments on experimental design, data analysis, results interpretation, and revision of manuscripts.

Author	Signature
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Xu-Feng Huang	
Chao Deng	

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Supplement C2 – Supplementary Figures from Chapter 5 Investigation

<u>Title:</u> **Early Antipsychotic Treatment in Juvenile Rats Elicits Long-term Alterations to the Adult Serotonin Receptors**

Running title – *Juvenile APD affects 5-HT Receptors in adult rats*

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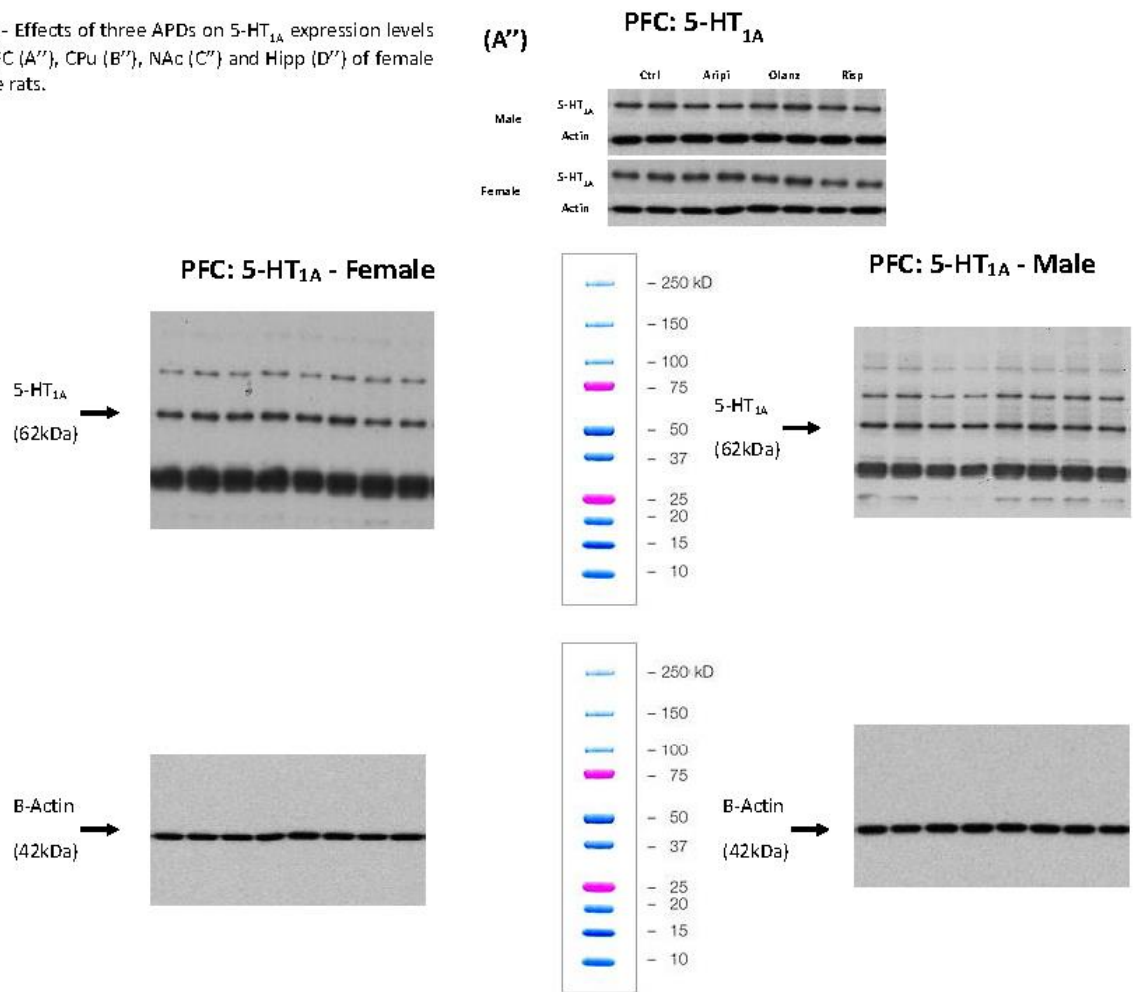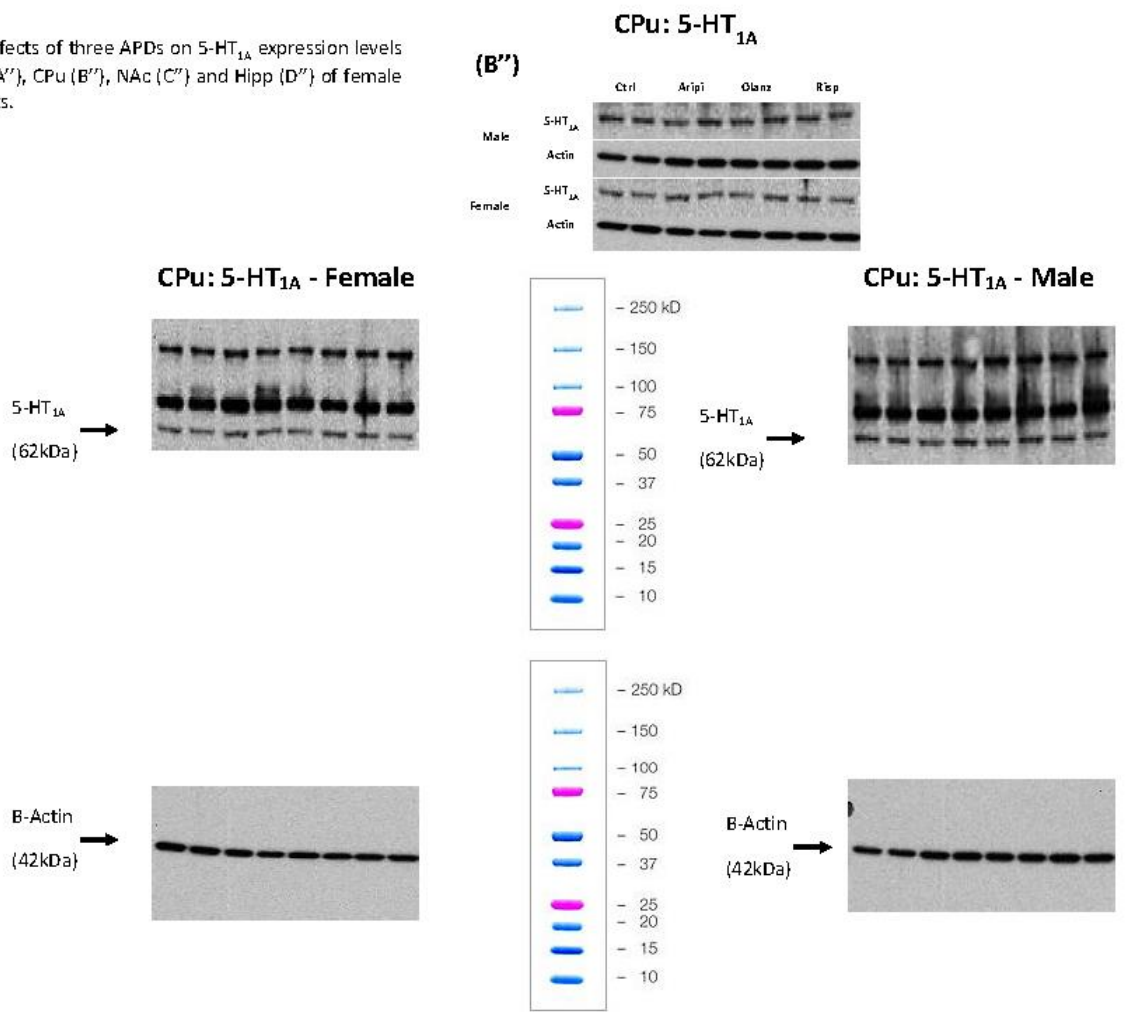
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Figure 1 - Effects of three APDs on 5-HT$_{1A}$ expression levels in the PFC (A''), CPu (B''), NAc (C'') and Hipp (D'') of female and male rats.

(A'')

PFC: 5-HT$_{1A}$

PFC: 5-HT$_{1A}$ - Female

5-HT$_{1A}$
(62kDa)

B-Actin
(42kDa)

PFC: 5-HT$_{1A}$ - Male

5-HT$_{1A}$
(62kDa)

B-Actin
(42kDa)

Figure 1 - Effects of three APDs on 5-HT$_{1A}$ expression levels in the PFC (A''), CPu (B''), NAc (C'') and Hipp (D'') of female and male rats.

CPu: 5-HT$_{1A}$

(B'')

CPu: 5-HT$_{1A}$ - Female

5-HT$_{1A}$
(62kDa)

CPu: 5-HT$_{1A}$ - Male

5-HT$_{1A}$
(62kDa)
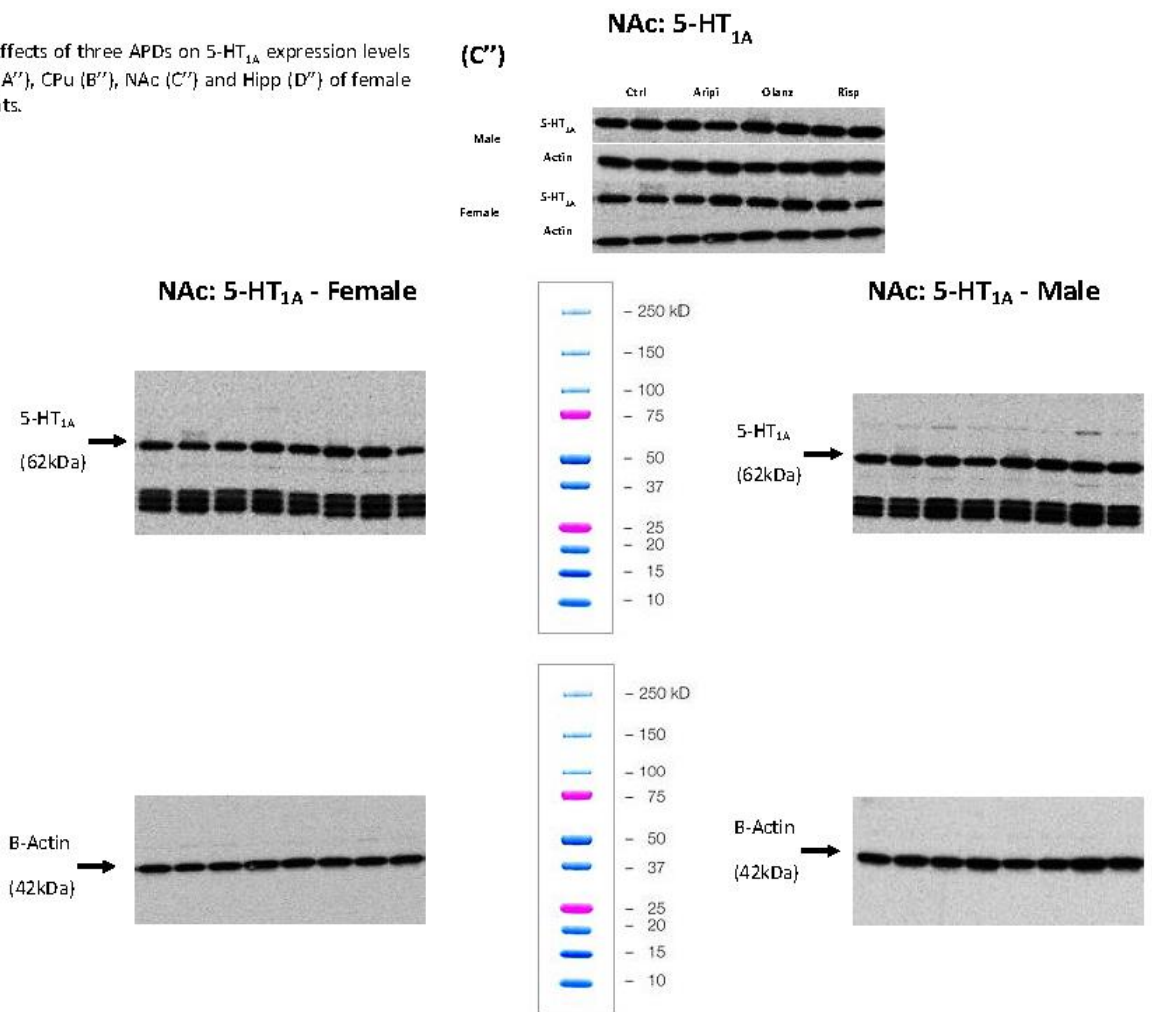
B-Actin
(42kDa)

B-Actin
(42kDa)

Figure 1 – Effects of three APDs on 5-HT$_{1A}$ expression levels in the PFC (A''), CPu (B''), NAc (C'') and Hipp (D'') of female and male rats.

(C'')

NAc: 5-HT$_{1A}$

NAc: 5-HT$_{1A}$ - Female

NAc: 5-HT$_{1A}$ - Male

Figure 1 - Effects of three APDs on 5-HT$_{1A}$ expression levels in the PFC (A''), CPu (B''), NAc (C'') and Hipp (D'') of female and male rats.
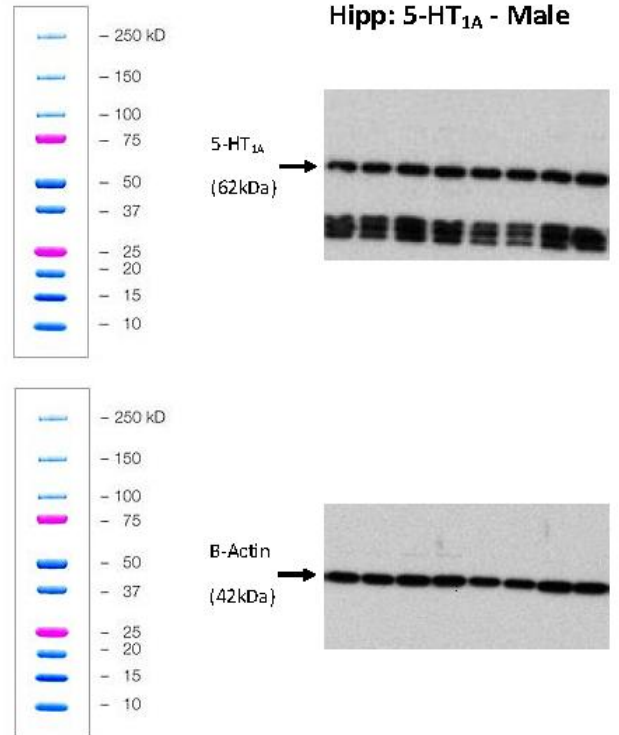
Figure 3 - Effects of three APDs on 5-HT$_{2A}$ expression levels in the PFC (A''), CPu (B''), NAc (C'') and Hipp (D'') of female and male rats.
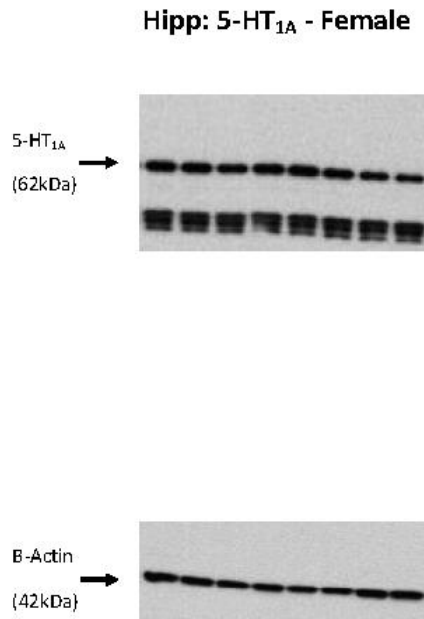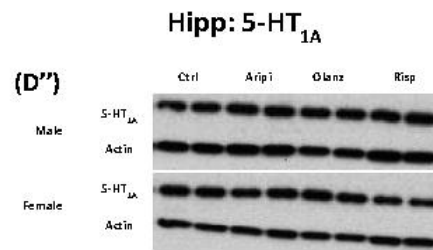
Figure 3 - Effects of three APDs on 5-HT$_{2A}$ expression levels in the PFC (A''), CPu (B''), NAc (C'') and Hipp (D'') of female and male rats.

Figure 3 - Effects of three APDs on 5-HT$_{2A}$ expression levels in the PFC (A''), CPu (B''), NAc (C'') and Hipp (D'') of female and male rats.

(C'')

NAc: 5-HT$_{2A}$

NAc: 5-HT$_{2A}$ - Female

5-HT$_{2A}$
(55kDa)

B-Actin
(42kDa)

NAc: 5-HT$_{2A}$ - Male

5-HT$_{2A}$
(55kDa)

B-Actin
(42kDa)

Hipp: 5-HT$_{2A}$

(D'')

Figure 3 - Effects of three APDs on 5-HT$_{2A}$ expression levels in the PFC (A''), CPu (B''), NAc (C'') and Hipp (D'') of female and male rats.
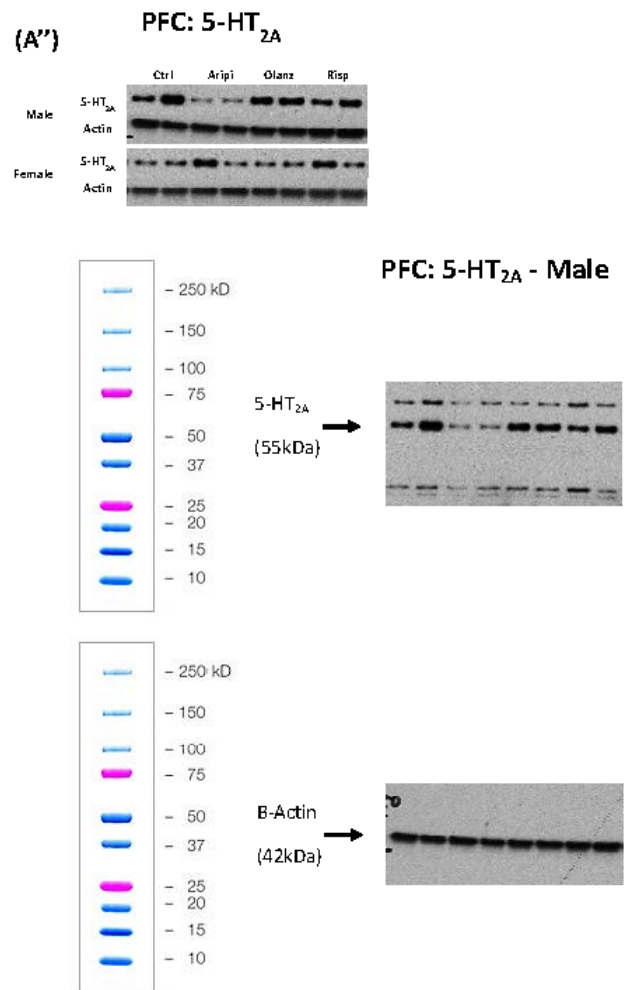
Hipp: 5-HT$_{2A}$ - Female

Hipp: 5-HT$_{2A}$ - Male

Figure 4 - Effects of three APDs on 5-HT$_{2c}$ expression levels in the PFC (A''), CPu (B''), NAc (C'') and Hipp (D'') of female and male rats.

Figure 4 - Effects of three APDs on 5-HT$_{2C}$ expression levels in the PFC (A''), CPu (B''), NAc (C'') and Hipp (D'') of female and male rats.
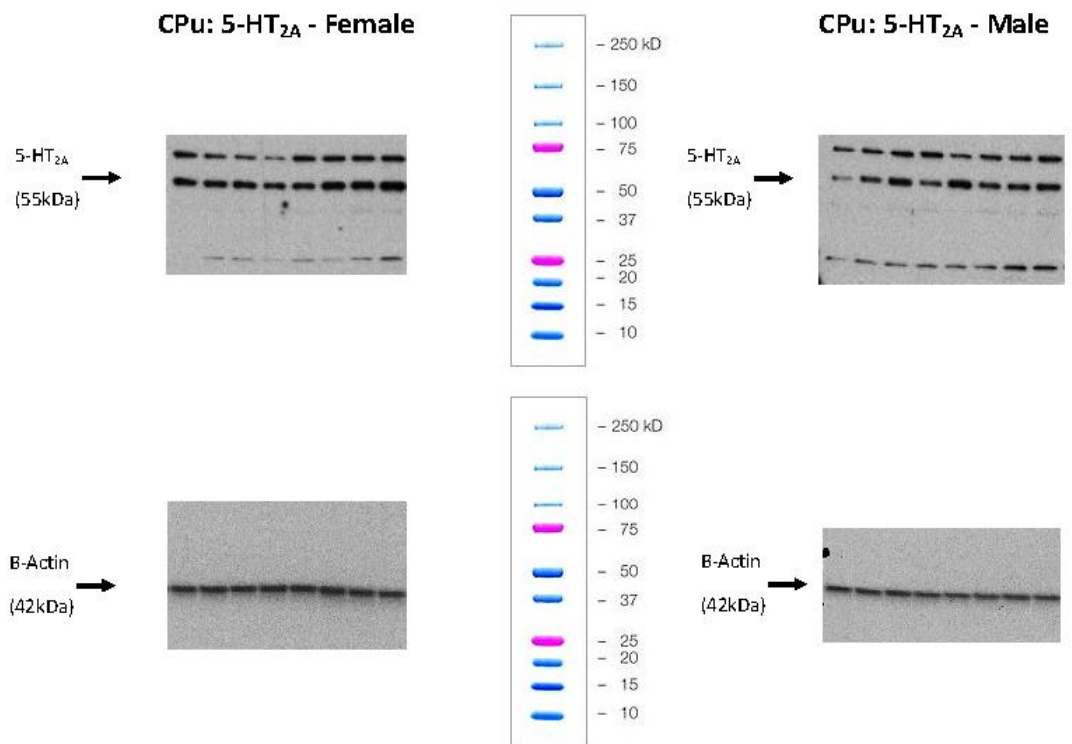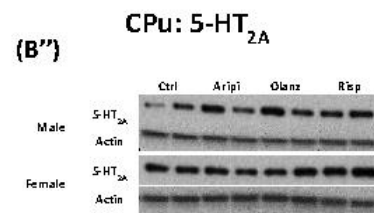
CPu: 5-HT$_{2C}$

(B'')

CPu: 5-HT$_{2C}$ - Female

CPu: 5-HT$_{2C}$ - Male
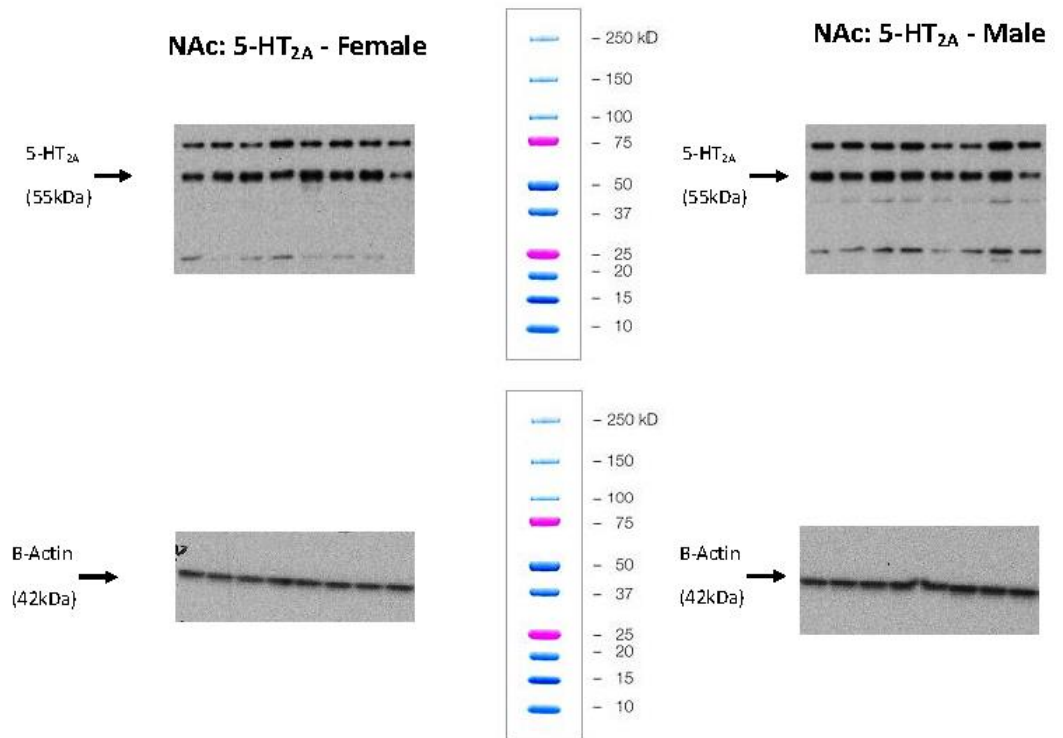
5-HT$_{2C}$ (55kDa)

B-Actin (42kDa)

Figure 4 - Effects of three APDs on 5-HT$_{2C}$ expression levels in the PFC (A''), CPu (B''), NAc (C'') and Hipp (D'') of female and male rats.

NAc: 5-HT$_{2C}$

(C'')

NAc: 5-HT$_{2C}$ - Female

NAc: 5-HT$_{2C}$ - Male
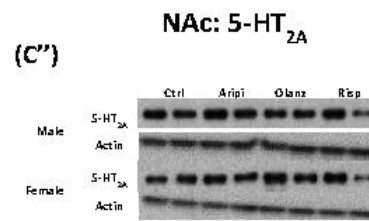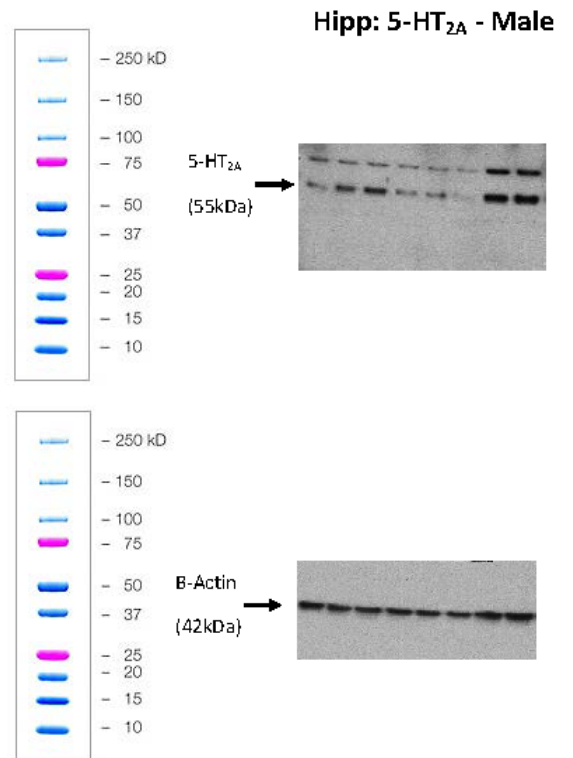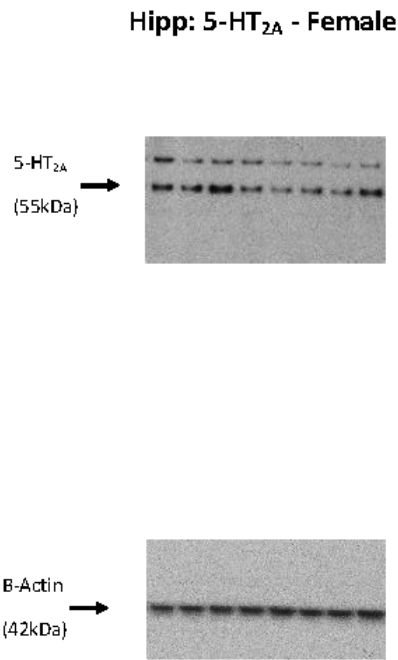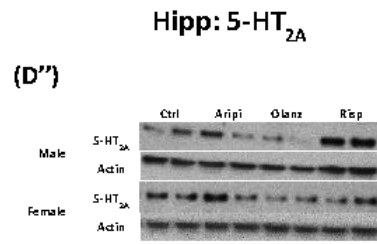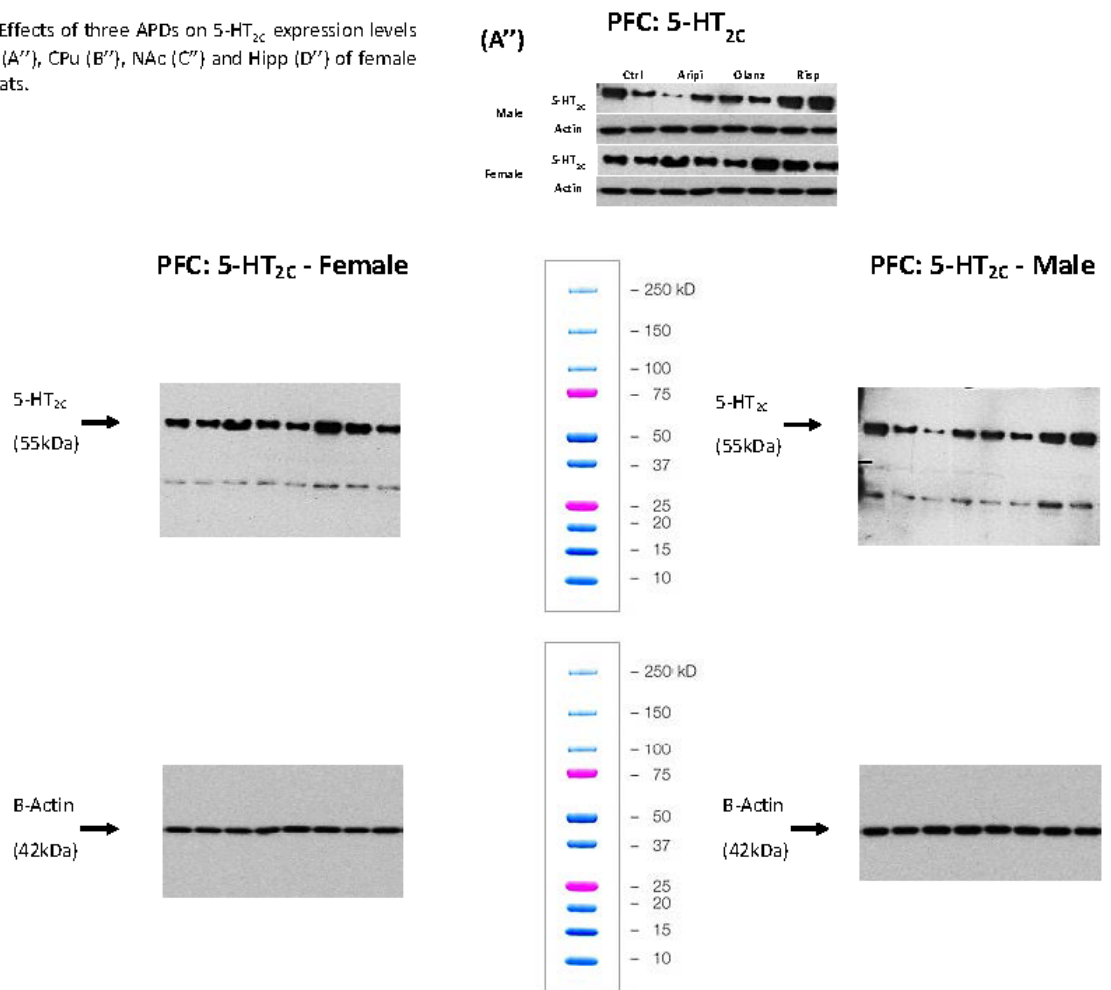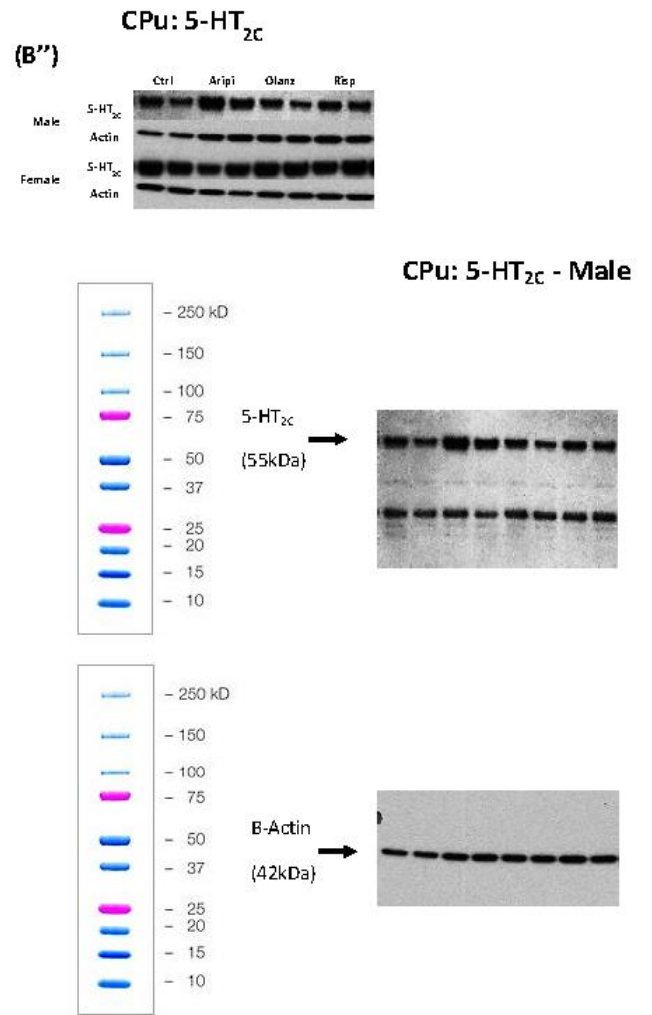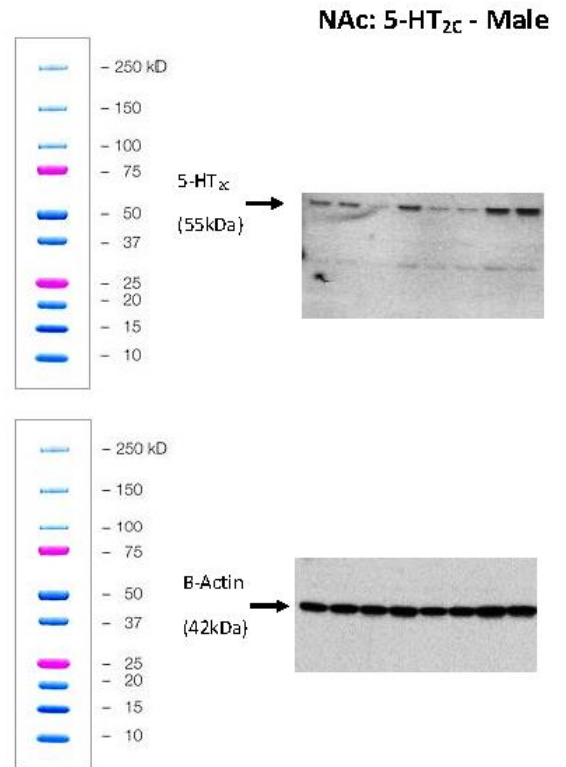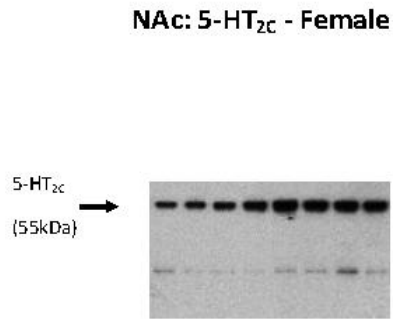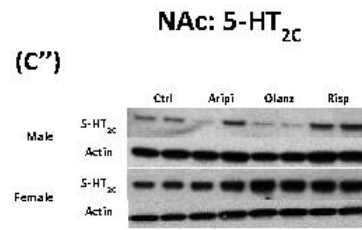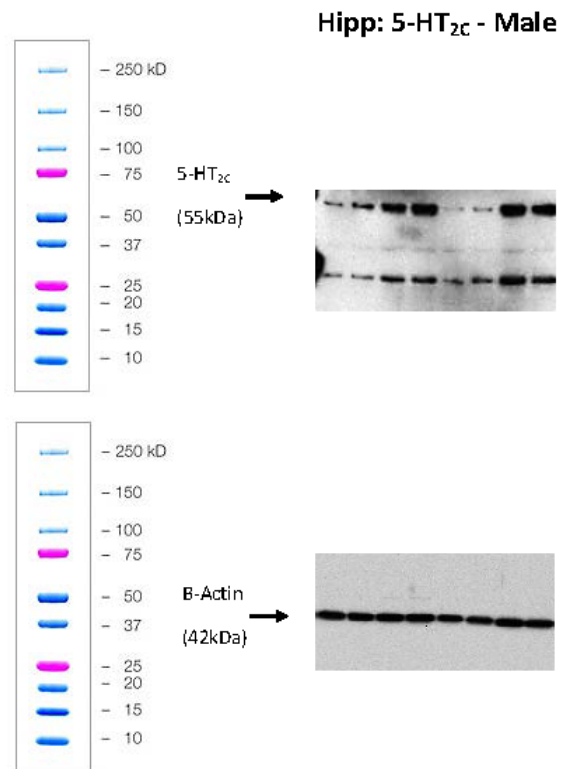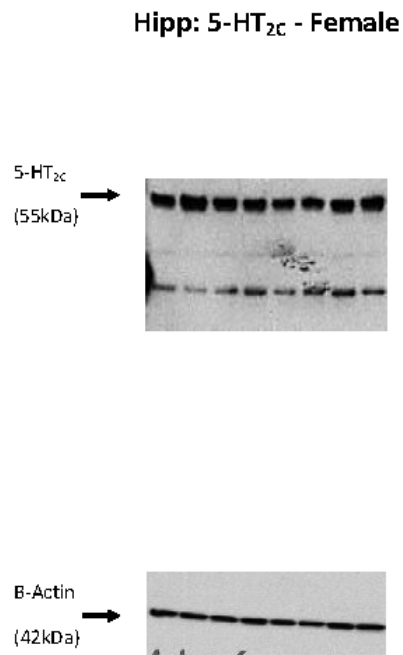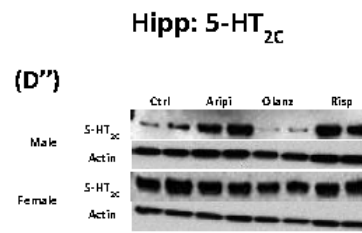
Hipp: 5-HT$_{2C}$

Figure 4 - Effects of three APDs on 5-HT$_{2C}$ expression levels in the PFC (A''), CPu (B''), NAc (C'') and Hipp (D'') of female and male rats.

(D'')

Hipp: 5-HT$_{2C}$ - Female

Hipp: 5-HT$_{2C}$ - Male

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